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Microfluidic compartmentalized co-culture platform for CNS axon myelination research

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Abstract This paper presents a circular microfluidic compartmentalized co-culture platform that can be used for central nervous system (CNS) axon myelination research. The microfluidic platform is composed of a soma compartment and an axon/glia compartment connected through arrays of axon-guiding microchannels. Myelin-producing glia, oligodendrocytes (OLs), placed in the axon/glia compartment, interact with only axons but not with neuronal somata confined to the soma compartment, reminiscent to *in vivo* situation where many axon fibres are myelinated by OLs at distance away from neuronal cell bodies. Primary forebrain neurons from embryonic day 16–18 rats were cultured inside the soma compartment for two weeks to allow them to mature and form extensive axon networks. OL progenitors, isolated from postnatal day 1-2 rat brains, were then added to the axon/glia compartment and co-cultured with neurons for an additional two weeks. The microdevice showed fluidic isolation between the two compartments and successfully isolated neuronal cell bodies and dendrites from axons growing through the arrays of axon-guiding microchannels into the axon/glia compartment. The circular co-culture device developed here showed excellent cell loading characteristics where significant numbers of cells were positioned near the axon-guiding microchannels. This significantly increased the probability of axons crossing these microchannels as demonstrated by the more than 51 % of the area of the axon/glia compartment covered with axons two weeks after cell seeding. OL progenitors co-cultured with axons inside the axon/glia compartment successfully differentiated into mature OLs. These results indicate that this device can be used as an excellent *in vitro* co-culture platform for studying localized axon-glia interaction and signalling.

Keywords Cell culture microsystem · Neuron co-culture · Axon-glia interaction

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1 Introduction

To develop effective treatments for diseases originated from neural damage, intensive research have been carried out to reveal how neuronal cells grow, differentiate, network and respond to various external stimuli (Knusel et al. 1991; James et al. 2004; Chao et al. 2005; Taylor et al. 2007). Conventional cell culture methods, typically done in plastic cell culture plates, are commonly used in studying various properties of cells, but these culture plates offer a vastly different environment compared to *in vivo* surroundings where both physical and biochemical environments are tightly controlled in a spatial and temporal manner. These make it extremely challenging to study neuron cell development *in vitro*, especially since neurons are highly polarized cells and various factors such as intercellular signalling molecules, extracellular matrix and growth factors all play a critical role (Mains and Patterson 1973).

In the vertebrate nervous system, formation of insulating myelin sheaths around axons is essential for rapid nerve impulse conduction. Myelination is a sequential, multi-step

process that requires reciprocal signals between axons and myelin-producing cells — oligodendrocytes (OLs) in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) (Baumann and Pham-Dinh 2001; Li et al. 2005; Sherman and Brophy 2005). Dysfunction of myelin-forming cells and/or loss of myelinating sheath underline many neurological disorders including multiple sclerosis, Alzheimer's disease and psychological disorders such as schizophrenia, but the signals that regulate myelination in the mammalian CNS remain largely unknown. This is mainly due not only to the complexity of the highly regulated myelination process in the CNS, but also to the lack of suitable *in vitro* models of CNS myelination to unravel the cellular and molecular basis of axon-glia signals. An *in vitro* myelination model system that can mimic physiological axon-glia interactions will allow detailed understanding into axon-glia communications.

An *in vitro* culture device allowing compartmented culture of neurons with localized fluidic environment control was first introduced by Campenot in 1977 (Campenot 1977). Since then, Campenot chambers have been widely used to study axon-glia interaction and axonal biology of dorsal root ganglion (DRG) (Bi et al. 2006; Ishibashi et al. 2006; Ng et al. 2007). This chamber utilizes a Teflon® divider attached to a substrate via a thin layer of silicone grease. The Teflon® divider separates the chamber into two compartments, confining cells into each compartment while the axons can grow through the silicone grease into the neighbouring compartment. Nerve growth factor (NGF) is typically used to promote axonal growth through the grease layer. Since cerebral neurons do not have the same dependency on NGF for axonal growth, they have not been successfully cultured in these Campenot chambers. Other drawbacks of Campenot chambers include their tendency to leak due to imperfect grease seal, difficulty in assembly, and limitation in adapting to sophisticated microscopy.

Recently, there have been numbers of microfluidic devices developed for culturing neurons (Heuschkel et al. 1998; Thiebaud et al. 2002; Morin et al. 2006; Millet et al. 2007). Microfluidic devices, when applied to neuron cell culture platforms, can provide a physically and biochemically controlled microenvironment by using guiding structures or microfluidic channels. Taylor et al. recently reported a microfluidic neuronal culture device that enables culturing neuronal cell bodies and axons in two different fluidic environments and successfully isolated axonal mRNA (Taylor et al. 2005). Ravula et al. developed a compartmentalized microfluidic culture system for electrophysiological signaling studies in cultured neurons (Ranula et al. 2006). These devices are typically built in poly (dimethylsiloxane) (PDMS) due to their good biocompatibility, ease of fabrication, mass production capability, and low cost.

We have developed a microfluidic co-culture platform where neurons and OLs can be co-cultured in two separate compartments connected by arrays of axon-guiding microchannels, providing physical isolation for cell bodies but not for axons, while maintaining fluidic isolation. Embryonic CNS neurons and postnatal OL progenitors were co-cultured inside the device for up to four weeks to study axon-glia interaction and myelination. The circle shaped platform with open access neuron compartment has been tested with different axon-guiding channel dimensions and cell loading protocols to achieve high density of axonal network growth inside the axon/glia compartment. We expect that these results can provide critical guidelines for designing various co-culture microfluidic devices to study interactions and signaling between axons and glia.

2 Materials and methods

2.1 Design and fabrication

The microfluidic co-culture platform is composed of two cell culture compartments, one for neuron cells and the other for OL cells. The two compartments are connected by arrays of 15–30 μm wide, 2.5 μm high microchannels (n= 140–180). Typical sizes of embryonic cortical neuron cells isolated from E16-18 rats and OL progenitor cells from P1–2 rat range from 5 to 15 μm. These shallow microchannel arrays that connect the two compartments (60 to 100 μm high) work as a barrier to keep neuron cell bodies in the soma compartment, while allowing axons to pass through and grow into the axon/glia compartment. The length of microchannels (200 to 800 μm long) restricts the growth of dendrites into the axon/glia compartment, leaving only axonal network layer formation in the axon/glia compartment. This enables OL progenitors loaded into the axon/glia compartment to interact only with axons, but not with neuronal somata or dendrites (Fig. 1 (a)).

This microfluidic co-culture platform also fluidically isolates the axonal microenvironment. Fluidic isolation of the microfluidic co-culture platform was established by creating a minute fluidic level difference of culture media between the two compartments. When the levels of culture media inside the two compartments are at equilibrium, molecules resolved in one compartment can diffuse to the other through the microchannel arrays. On the other hand, slight difference in fluidic levels between the two compartments produce small but sustained flow from the higher side to the lower side that counters diffusion, thus enabling fluidic isolation (Fig. 1 (b)). This fluidic isolation between the two compartments is essential for localized treatments

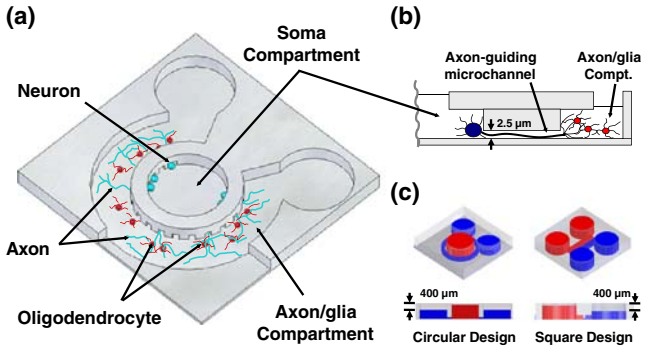

Fig. 1 Schematic illustration of the microfluidic compartmentalized CNS neuron co-culture platform. (**a**) 3D-view of the circular device, (**b**) cross-sectional view showing the physical isolation of axons and OLs from neuronal soma and dendrites, and (**c**) circular and square design culture platforms with cross-sectional views showing a minute difference in fluidic levels for fluidic isolation. The circular compartment in the center of the circular device is an open access compartment for neurons and is connected to the outer ring-shaped axon/glia compartment for OLs through arrays of axon-guiding microfluidic channels

of axons or OL cells with various growth factors, inhibition factors, and also potentially with drugs.

The platform is composed of one circular shape open compartment (soma compartment) located in the middle for neurons and one closed co-centric ring compartment (axon/glia compartment) for glia. The open circular soma compartment is 7 mm in diameter and the closed outer ring compartment is 100 µm high and 2 mm wide, and distanced away from the soma compartment by the length of the axon-guiding microchannels (200–800 µm long). Culture media for the neuron cells can be held in the soma compartment whereas culture media for the OLs are held in the two reservoirs connected to the axon/glia compartment. The soma compartment is an open compartment to provide better cell living conditions by facilitating CO_2 exchange. Although PDMS, the material this co-culture platform is made of, is known to be gas permeable, primary cultured neurons are often very sensitive to small environmental changes and an open access compartment design reduces the possible negative effects of an enclosed compartment. The open compartment design also reduces mechanical stress to cells during culture media exchange compared to a closed compartment design by reducing the shear stress during such processes.

To characterize our design and also to compare the design to other previously reported co-culture designs such as that from Taylor et al. (Taylor et al. 2005), we have also fabricated and tested a square shaped co-culture device (Fig. 1 (c)). This square design is composed of two rectangle shaped compartments connected through arrays of axon-guiding microchannels. The compartment width and height varied from 1.2 to 5 mm and 60 to 100 µm, and the channel length and width varied from 200 to 800 µm and 15 to 30 µm, respectively, to optimize the geometry of the design.

All co-culture platforms were fabricated in PDMS using the soft lithography technique (Xia and Whitesides 1998) and were assembled on poly-D-lysine (PDL) or Matrigel™ coated glass or polystyrene substrates. First, the master mold was fabricated on a 3 inch diameter silicon substrate using a two-layer photolithography process by sequentially patterning two layers of photosensitive epoxy (SU-8™, Microchem, Inc., Newton, MA) with different thicknesses. The first layer forming the axon-guiding microchannels was 2.5 µm thick and the second layer forming the cell culture compartment was 60 to 100 µm thick. The SU-8™ master was coated with (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane (United Chemical Technologies, Inc., Bristol, PA) to facilitate PDMS release from the master after replication. Devices were replicated from the master by pouring PDMS pre-polymer (10:1 mixture, Sylgard® 184, Dow Corning, Inc., Midland, MI), followed by curing at 85°C for 40 min. The reservoirs to hold culture media were punched out using a 7 mm diameter punch bit mounted on a drill press. To improve the bonding of PDMS devices onto substrates and to make the device hydrophilic for easy cell and culture media loading, the PDMS culture devices were exposed to oxygen plasma for 90 s. For sterilization, the device was immersed in 70 % ethanol for 30 min prior to bonding on PDL or Matrigel™ coated glass coverslips or polystyrene culture plates. Each device was placed inside each well of a conventional 6-well polystyrene cell culture plate. Figure 2 shows the overall fabrication steps and assembly processes.

2.2 Tissue dissociation and cell culture

Primary CNS neurons were prepared from forebrains of embryonic day 16–18 Sprague-Dawley rats. Briefly, forebrains free of meninges were dissected in ice-cold dissection buffer (Ca^{2+}/Mg^{2+}–free Hank's Balanced Salt Solution containing 10 mM HEPES), dissociated with L-cysteine activated papain (10 units/ml) in dissection buffer for 5 min at 37°C, and resuspended in dissection media containing trypsin inhibitor (10 mg/ml) for 2–3 min. Following two more washes with the trypsin inhibitor solution, the tissue was resuspended in a plating media (Neurobasal media containing 2 % B27, 1 mM Glutamine, 25 µM glutamic acid and 100 units/ml of penicillin and 100 µg/ml streptomycin) and triturated with a fire-polished glass Pasteur pipette until all clumps disappeared. The cells were then passed through a 70 µm cell sieves, and live cells were counted using a hemocytometer and trypan blue exclusion assay. The viability of isolated cells was constantly greater than 90–95 %. Cells were loaded into the soma compartment of the device and cultured at 37°C in a humidified

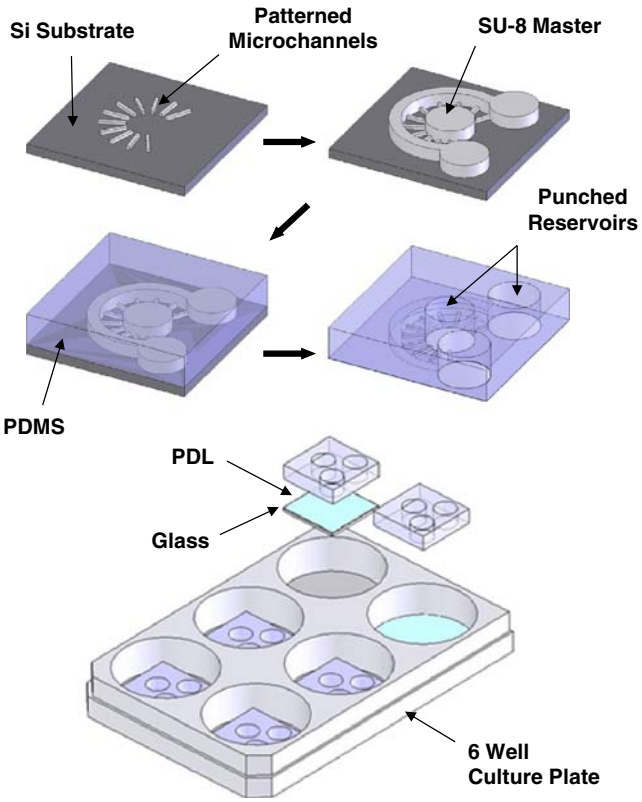

Fig. 2 Fabrication and assembly steps for the microfluidic co-culture device. Two SU-8™ layers with different thicknesses were patterned on top of a silicon substrate to form the axon-guiding microchannel array and the two cell culture compartments (soma, axon/glia). PDMS devices were replicated from the SU-8™ master using soft lithography process and 7 mm diameter reservoirs were punched out followed by sterilization in 70 % ethanol for 30 min and bonding onto PDL or Matrigel™ coated substrates. Each device fits into one well of a conventional 6-well polystyrene culture plate

5 % CO_2 incubator. Areal density, defined as the number of cells per culture area, was 500 cells/mm^2. At 4 days *in vitro* (DIV 4), culture medium was changed to plating medium without glutamic acid, and thereafter, cells were fed every 3–4 days by changing out half of the medium.

Primary rat OL progenitors were prepared from the cerebral hemispheres of Sprague-Dawley rats at postnatal day 1–2 as previously described (Li et al. 2005). Briefly, forebrains free of meninges were chopped into 1 mm^3 blocks and placed into HBSS containing 0.1 % trypsin and 10 µg/ml DNase I. After digestion, the tissue was collected by centrifugation and triturated with the plating media DMEM20S (DMEM, 20 % fetal bovine serum and 1 % penicillin-streptomycin). Cells were plated onto PDL-coated 75 cm^2 flasks and were fed with fresh DMEM20S medium every other day for 10–11 days at 37°C in a humid atmosphere of 5 % CO_2 and 95 % air. To isolate OL progenitors, the flasks were pre-shaken for 1 h at 200 rpm at 37°C to remove microglia. OL progenitors were then separated from the astrocyte layer by shaking at 200 rpm for 18–22 h. The suspension was plated onto uncoated petri-dishes and incubated for 1 h to further remove contaminating microglia and astrocytes. Purified OL progenitors were then collected by passing through a 15 µm sieve, centrifuged and loaded into the device at an areal density of 400 cells/mm^2 per device. Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution, Neurobasal media, B27, penicillin, streptomycin and goat serum were from Invitrogen (Carlsbad, CA). Poly-D-lysine, papain, trypsin inhibitor, glutamine, glutamic acid, paraformaldehyde and Triton X-100 were from Sigma (St. Louis, MO).

2.3 Immunocytochemistry

Neurons and OLs co-cultured in the platform were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBS) for 10 min, washed with PBS, and blocked with TBST (50 mM Tris•HCL, pH 7.4, 150 mM NaCl and 0.1 % Triton X-100) containing 5 % goat serum. The fixed cells were incubated overnight at 4°C with antibodies against neurofilament-H (NF) at 1:1000 dilution (Chemicon, Temecula, CA), microtubule-associated protein 2 (MAP-2) at 1:1000 dilution (Chemicon, Temecula, CA) or myelin basic protein (MBP) at 1:1000 (Covance, Berkeley, CA). After washing with TBST, secondary antibody conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (1:1000, Molecular Probes, Inc., Eugene, OR) was incubated with the cells for 1 hour at room temperature. The substrates were then washed 2-3 times and mounted onto glass coverslips with FluoroMount™ and kept in the dark at 4°C. Cell images were captured using a fluorescent microscope (IX71, Olympus America, Inc., Center Valley, PA) equipped with a digital camera (DP70, Olympus America, Inc.).

2.4 Image analysis

Fluorescent images were first converted into black and white at the threshold where no background fluorescent was detected using a commercial image editing software (Photoshop®, Adobe Systems, Inc., San Jose, CA). Then, the percentage of the white colored area on the images indicating the area covered with axons was measured with NIS-Element 2.30 (Nikon Instruments, Inc., Tokyo, Japan).

3 Results and discussions

3.1 Fabrication

Figure 3 shows images of the microfabricated PDMS co-culture device. The scanning electron micrographs show arrays of 15 µm wide axon-guiding microchannels separat-

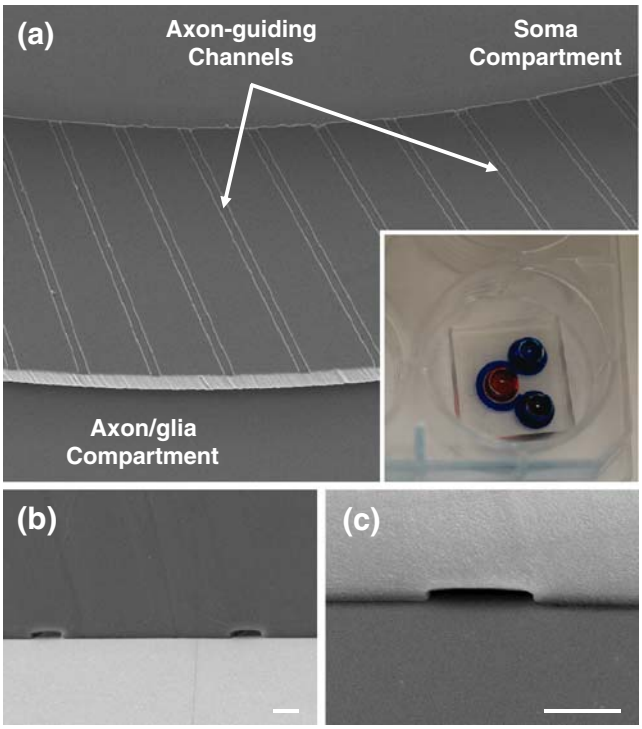

Fig. 3 Scanning electron micrographs (SEMs) and an optical photograph of the PDMS co-culture device showing (**a**) bottom side of the axon-guiding channel arrays before bonding (Inset: Device filled with color dye for visualization and placed inside a conventional 6-well polystyrene culture plate), (**b**) PDMS device bonded onto a glass coverslip showing two 15 μm wide axon-guiding microchannels with approximately 60 μm separation, and (**c**) close up view of one axon-guiding microchannel. Scale bars, 10 μm

ed from one another by approximately 60 μm distance. The inset in Figure 3(a) shows the overall device (22 mm by 22 mm) placed inside a conventional 6-well polystyrene culture plate. Axon-guiding channel having width of 30 μm with 30 μm distance between channels and 15 μm with 60–500 μm distances have been tested to find the optimal dimension that maximizes the channel area between the compartments (i.e. maximum channel width and minimum distance between channels) for high density axon growth into the axon/glia compartment while maintaining a stable fluidic seal. Too wide of a channel caused the PDMS channel to sag and blocked the channel. Too short distance between channels resulted in insufficient contact area between the PDMS structure and the substrate and prevented a tight fluidic seal. Experimental results showed that axon-guiding channels with 15 μm width and 60 μm distance provided maximum opening between the two compartments while maintaining a robust fluidic seal between the PDMS device and the substrate throughout the culture period of four weeks without leakage. Without the oxygen plasma treatment, the fluidic seal between the device and the PDL or Matrigel™ coated substrate broke after several hours, and the hydrophobic property of the PDMS caused bubbles to be trapped inside the compartment while loading cells or changing out the culture media. The 30 μm wide channels with 30 μm distances provided more than two folds of opening area between compartments, but the short distance between the channels deteriorated the adhesion of the PDMS device to the substrate, resulting in a fluidic seal failure after several hours. Forty to sixty PDMS microfluidic co-culture platform devices have been routinely fabricated and tested simultaneously in one cell culture run that typically last for 4–5 weeks.

3.2 Fluidic isolation

The efficiency of fluidic isolation was tested on both the circular design and the square design by creating a minute fluidic level difference between the reservoirs that resulted in difference in hydrostatic pressure. Initially, color dye (red and blue) was mixed with PBS and loaded into each reservoir. Fluidic level differences of 130, 400, 650, and 1000 μm were achieved by creating volume differences of 5, 15, 25, 40 μl between reservoirs, respectively. Successful fluidic isolation between the compartments was achieved with as small as 400 μm fluidic level difference that was maintained for over 70 h in both the circular and the square design culture platform (Fig. 4). Fluidic pressure from the soma compartment (red color, higher fluidic level) to the axon/glia compartment (blue color, lower fluidic level) through the 2.5 μm high microchannel array counteracted diffusion. For more accurate test, the axon/glia compartment and the soma compartment were filled with fluorescent dye (FITC) and PBS respectively, with the soma compartment having a slightly higher fluidic level. With a fluidic level difference of 400 μm between the two compartments, FITC was confined to the axon/glia compartment as

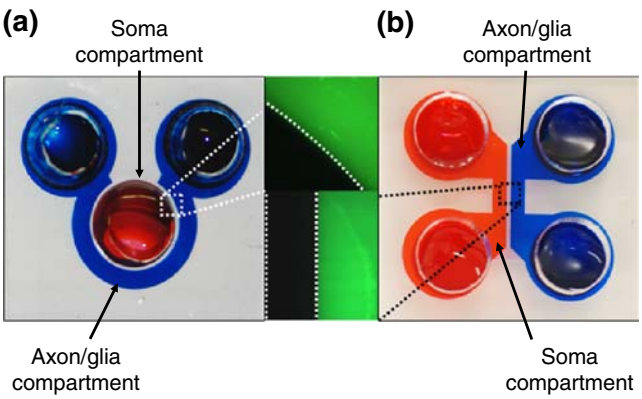

Fig. 4 Fluidic isolation in the (**a**) circular design and (**b**) square design neuron culture platform. Dotted white lines in the center fluorescence images delineate compartment boundaries. Green fluorescent part indicates the axon/glia compartment while the black part indicates the array of axon-guiding microchannels

shown in Fig. 4 where a sharp boundary can be seen between the compartment and the axon-guiding microchannel arrays.

3.3 Cell loading

To facilitate high density of axons to cross into the axon/glia compartment through the axon-guiding microchannels, neurons loaded into the soma compartment of the microfluidic co-culture platform have to be positioned close to the entrance of the microchannels during the initial cell loading process. Neuron cells were loaded into the soma compartment to characterize and compare the distances between the loaded cells and the entrance of the axon-guiding microchannels in the two different microdevice designs. The areal cell density was same in both designs (500 cells/mm^2). We first tested the square design neuron culture platform. In our initial experiments, 120 µl/reservoir of additional culture media was added to each reservoir immediately after loading the neurons to the soma compartment. This cell loading protocol, however, resulted in many of the cells inside the soma compartment washing out to the outlet reservoir due to the rather strong culture media flow from one reservoir to the other reservoir caused by the addition of culture media. To prevent such washing out of cells, a short incubation step was added before loading culture media since neurons loaded inside the compartment attach to the substrate after a while and their locations remain relatively stable. Incubation times of 10–60 min were tested to optimize the incubation step. Sufficient adhesion of the cells to the substrate was achieved after 30 min of incubation that prevented cell wash-out while maintaining good cell viability. Longer incubation times (more than 30 min) provided stronger adhesion of the cells but with a drop in cell viability. Neurons incubated for 60 min before adding culture media showed slight aggregation inside the soma compartment after 24 h, and most of them were observed dead at DIV 4. Short incubation times (less than 30 min) resulted in many cells still being washed out to the other side of the reservoir while adding the culture media.

Although cell adhesion to the substrate is an important factor and the loading method has been optimized for the square design, the chances of axons entering the axon-guiding microchannels and growing into the axon/glia compartment are low unless neurons are positioned close to the inlet of the axon-guiding microchannels during the initial loading process. The location of the cells during the initial cell loading step is heavily influenced by the microfluidic design of the culture compartments and cell loading inlets/outlets. The circular design co-culture platform has one open access compartment in the center for neuron cells. This design allows neuron cells loaded into the center compartment to flow radially toward the axon-guiding channel inlets. This small but sustained flow keeps cells close to the entrance of the axon-guiding channels.

The "cell loading efficiency" in the two different microfluidic co-culture platform designs was analyzed by measuring the average distance of the closest cells from the inlets of the axon-guiding microchannels. Cells loaded into the square design co-culture platform moved smoothly into the soma compartment; however, the average distance of the cells from channel inlets, measured from 263 cells in multiple devices, was 26.0±32.3 µm (means ± SD). In contrast, the circular design co-culture platform showed an average distance of 3.8±11.2 µm, measured from 225 cells in multiple devices, which indicates that the loaded cells are located almost exactly at the inlet of the axon-guiding microchannels (Fig. 5). To see the statistical significance, at a significance level of 1 %, we performed a two-sample pooled T-test showing $P<0.001$. This shows that the cells are located closer to the inlets of the axon-guiding microchannels when using the circular shape design compared to the square design, increasing the probability of axons growing into the microchannels. Cells loaded into the open soma compartment were naturally positioned close to the channel inlets due to the radial flow pressure resulting from the circular fluidic design, and the fluidic pressure from the added culture media of 120 µl moved the cells even closer to the channel inlets. Although neurons were located at the channel inlets, channel openings were not blocked by the neurons, and axons could successfully pass through the channels into the axon/glia compartment.

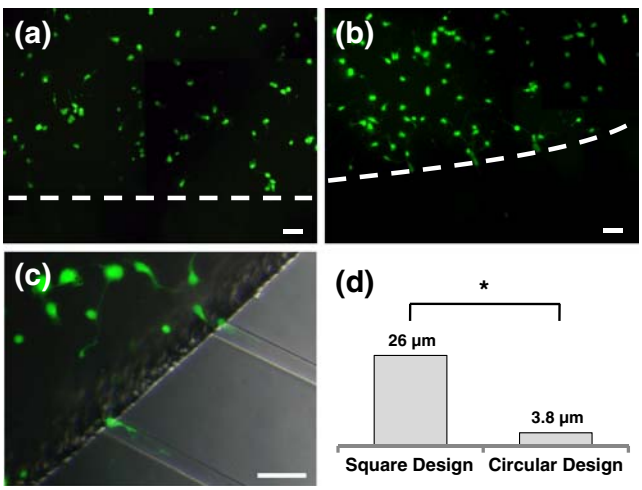

Fig. 5 Fluorescent images of neuron cells inside the soma compartment at DIV 1. (**a**) Cells inside the square design device; (**b**–**c**) cells inside the circular design device; (**d**) average distance of the closest cells from the axon-guiding channel inlets measured (square design, $n=263$; circular design, $n=225$). White dotted lines indicate where the axon-guiding microchannels start. Cells were stained with Calcein-AM at DIV 1 before fixing. Scale Bars, 50 µm. *$P<0.001$

The cell loading efficiency was not analyzed for the OLs since they have to attach uniformly on top of the axonal network layer inside the axon/glia compartment rather than being concentrated to the axon-guiding channel outlet area. OL progenitors were loaded to the axon/glia compartment at DIV 14 for the co-culture experiments with a final areal cell density of 400 cells/mm^2. A total culture media volume of 10 µl was added through the axon/glia compartment reservoir after aspirating out the excessive culture media inside the reservoir. It is important not to remove the culture media inside the axon/glia compartment during aspiration, since axons inside the axon/glia compartment can be aspirated out with the culture media, causing damages to the axonal network. OL progenitors were uniformly distributed over the axonal network inside the axon/glia compartment in both the circular and the square shaped designs.

3.4 Axon growth

Two different types of substrates, glass coverslips and 6-well polystyrene culture plates, were coated with either PDL or Matrigel^{TM} prior to assembly with the PDMS microfluidic co-culture devices. Substrates coated with Matrigel^{TM} resulted in all axon-guiding microchannels being blocked due to rehydration of the Matrigel^{TM}. Therefore, all following experiments were conducted on PDL coated substrates.

The microdevice was designed so that neuron cell bodies are isolated from axons by the height of the axon-guiding microchannels (2.5 µm) that block the cell bodies from moving into the axon/glia compartment. On the other hand, isolation of dendrites from axons is achieved by controlling the length of the axon-guiding channels because both axons and dendrites can grow through the axon-guiding microchannels but dendrites can grow only for a short distance. Microchannels with length ranging from 200 to 800 µm were tested to find the minimum length required for axon/dendrite isolation. Axons and dendrites successfully developed from neurons loaded inside the co-culture platform, and no toxicity or contamination issues were observed throughout four weeks of culture. Axons crossed and filled most of the axon-guiding microchannels by DIV 6 and started to spread out rapidly inside the axon/glia compartment. The microchannel arrays were not only efficient in guiding axons but also successful in physically isolating cell bodies and dendrites from axons. The 2.5 µm high shallow microchannels prevented cell bodies from moving into the axon/glia compartment, and the length of the microchannels kept dendrites from reaching the axon/glia compartment. Figure 6 clearly demonstrates the physical isolation of axons (stained for neurofilament (NF), red) from cell bodies and dendrites (stained for neuron marker, MAP2, green) via the axon-guiding microchannels. A

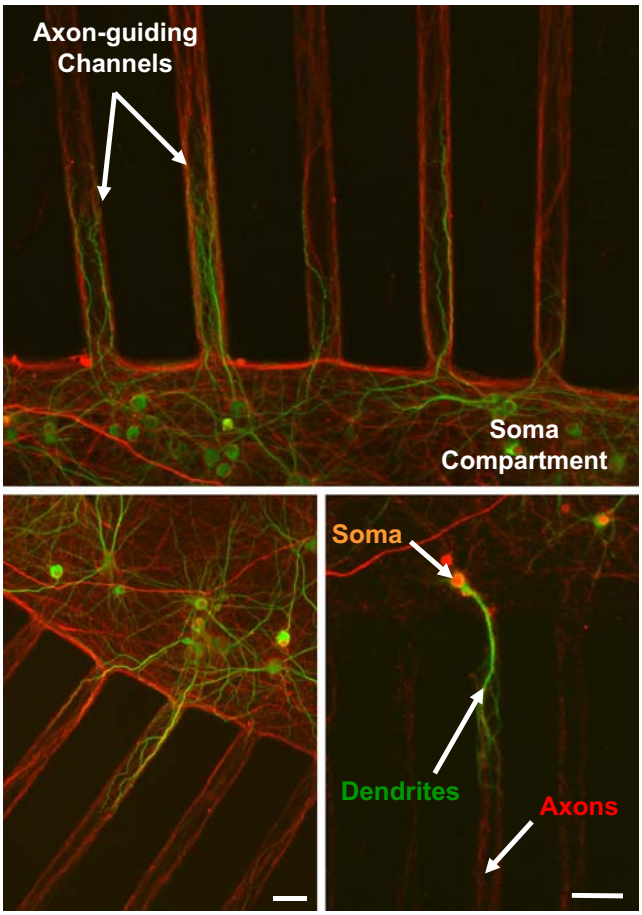

Fig. 6 Immunocytochemistry images of neurons at two weeks in culture demonstrate that axons grew from the soma compartment into the axon/glia compartment through the arrays of axon-guiding microchannels but dendrites and neuronal soma could not reach into the axon/glia compartment due to the length of the microchannels (200-800 µm long) and the height of the microchannels (2.5 µm) respectively. Axons were immunostained for NF (red) and dendrite for MAP2 (green). Scale bars, 20 µm

shorter axon-guiding channel has the advantage to form an extensive axonal network inside the axon/glia compartment much earlier compared to longer axon-guiding channels. Axon-guiding channel as short as 200 µm was sufficient to isolate dendrites from growing into the axon/glia compartment. Having a dense axonal network forming inside the axon-glia compartment as early as possible has the advantage of maximizing the co-culture period with OLs, since the overall culture becomes unhealthy after about five weeks of culture inside the co-culture platform.

To compare how the two microfluidic culture platform designs and the different substrates influence axon growth inside the co-culture platform, neurons were cultured in both the circular and the square design assembled on PDL coated polystyrene culture plates and PDL coated glass coverslips. The axon growth efficiency by different conditions was analyzed by the axon coverage ratio (ACR), defined as the percentage of area covered with axons inside

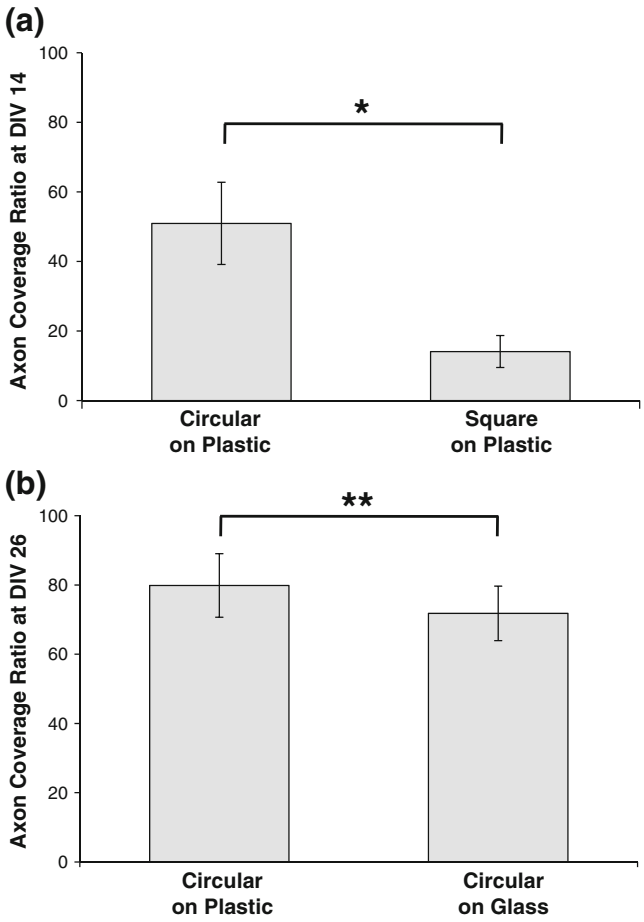

Fig. 7 Axon coverage ratio (ACR) of the axon/glia compartment by (**a**) different culture platform designs at DIV 14 and by (**b**) different substrates (polystyrene, glass) at DIV 26 analyzed from 79 images. Neurons cultured on the polystyrene culture plate using the circular design shows enhanced axon growth when compared to the square design on plastic or the circular design on glass coverslip. *$P<0.0001$, **$P<0.05$

the axon/glia compartment. To analyze the ACR by the different device designs, neuron cells were cultured at an areal density of 500 cells/mm^2 on top of PDL coated polystyrene culture plates and were fixed at DIV 14. After two weeks of neuron cell culture, the average ACR of the circular design co-culture platform attached on polystyrene culture plate was 51.0±11.8 % (means ± SD), which is statistically significantly higher than that of the square design on the same substrate showing 14.1±4.6 % (Fig. 7, $P<0.0001$). Therefore, we concluded that the novel circular co-culture design developed here enables the formation of a denser axonal network layer when compared with the square design.

The ACR was also affected by the substrate type. Neurons with an areal cell density of 3100 cells/mm^2 were cultured inside the circular design culture platforms assembled on PDL coated glass coverslips and PDL coated polystyrene culture plates, respectively. After four weeks of culture, including two weeks of co-culture period, neurons cultured on glass coverslips showed an average ACR of 71.8±7.9 %, while the neurons cultured on polystyrene culture plates showed an ACR of 79.9±9.2 % (Fig. 7). Again, to see the statistical significance, at a significance level of 1 %, two-sample pooled T-test was performed showing P value of 0.016 and therefore, concluded that neurons form denser axonal network when cultured on polystyrene substrates compared to glass substrates.

In addition, the adhesion of cells to substrates was also different depending on the substrate types. Axons grown on top of polystyrene culture plates were firmly attached to the substrate while many axons cultured on glass coverslips peeled off when detaching the PDMS devices for fixing and staining at the end of the culture periods.

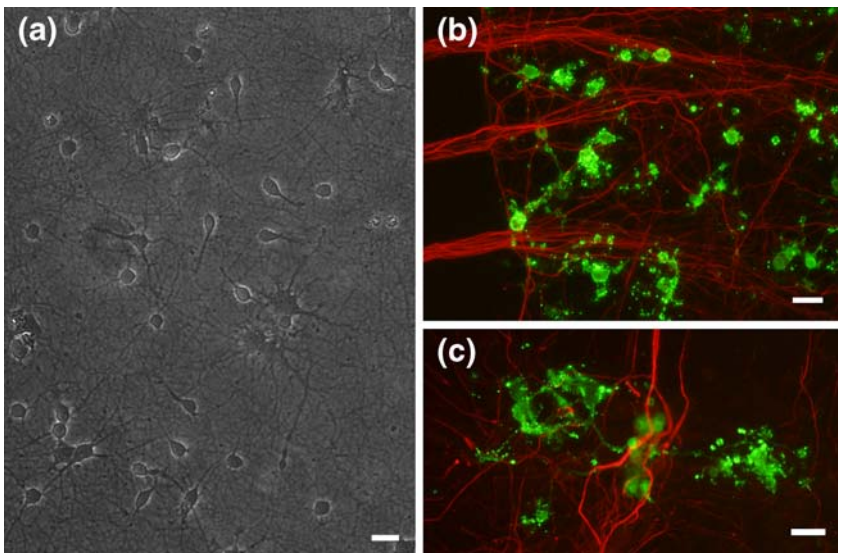

Fig. 8 A phase contrast image and immunocytochemistry images of axons and OLs co-cultured inside the axon/glia compartment for two weeks. (**a**) Phase contrast image of the axon/glia compartment; (**b**–**c**) immunostaining of mature OLs grown on top of axonal network layer inside the axon/glia compartment. Axons were stained for NF (red) and mature OLs were stained for MBP (green). Scale bars, 20 μm

3.5 Co-culture of CNS neurons and oligodendrocyte progenitors

The co-culture capability of the developed platform was tested by plating OL progenitors on top of the axonal network inside the axon/glia compartment that was already formed during the initial two-week culture period. OL progenitors with an areal cell density of 400 cells/mm^2 were loaded uniformly on top of the axonal layer inside the axon/glia compartment without any disturbance to the existing axonal network layer. The 2.5 μm high axon-guiding microchannels physically prevented OLs from crossing the axon-guiding channels, and no OLs were observed inside the soma compartment upon OL loading. After loading, neurons and OLs were co-cultured for up to two more weeks and fixed at DIV 26. Figure 8 shows axons (stained for NF, red) and OLs (stained for MBP, green) labelled with fluorescent dyes inside the axon/glia compartment. Myelin basic protein (MBP), stained with green fluorescence, expresses only in mature OLs, thus, the expression of MBP shown in Fig. 8 is a clear indication that OLs co-cultured on top of the axonal network successfully developed into mature OLs inside the PDMS microfluidic co-culture platform.

4 Conclusions

We have developed a circular design PDMS microfluidic compartmentalized co-culture platform composed of two compartments connected via arrays of shallow axon-guiding microfluidic channels. These microfluidic channels allowed both physical and fluidic isolation between the soma compartment and the axon/glia compartment. Fluidic isolation between the compartments was achieved with a 400 μm fluidic level difference between the compartments. Neuron cell bodies and dendrites inside the soma compartment were successfully isolated from the axons growing into the axon/glia compartment. This novel circular design allowed cells to be positioned right next to the inlets of the axon guiding channel as well as showed enhanced axonal growth characterized by the significantly increased axon coverage ratio inside the axon/glia compartment. The co-culture capability of the device was confirmed by successfully co-culturing OL progenitors with axons inside the axon/glia compartment that resulted in maturation of OLs. We expect that this novel CNS axon-glia co-culture microsystem platform will serve as a powerful tool for future mechanistic dissection of CNS axon-glia signaling networks *in vitro*.

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