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Dual compartment neurofluidic system for electrophysiological measurements in physically isolated neuronal cell cultures

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Abstract— **This work investigates an approach to record electrophysiological measurements of neuronal cell cultures in a dual compartment neurofluidic system. The two compartments are separated by 10-µm-wide and 3-µm-high microchannels and this provides a physical isolation of neurons allowing only neurites to grow between the compartments. We present long-term cell viability in closed compartment devices, neurite growth across the microchannels and a recording setup for the long-term recording of the network activity over 21 Days-in-Vitro (DIV). Structural and fluidic isolation between the compartments are demonstrated using transfection experiments and neurotoxin exposure, respectively.**

I. Introduction

Following the paradigm of cell culture analogs [1], the development of in-vitro models for specific neuronal pathways should provide insights into pathological neurodegenerative diseases and their treatment modalities that lack sufficient understanding. Further, using microsystem technologies to build and sustain such models offers easy accessibility and manipulation capabilities that are not available in traditional in-vivo studies [2]. In this work, we present our progress in the development of such a system for in-vitro neuronal network cultures.

Following Campenot's pioneering work [3], microsystem technologies have recently been used to initiate in-vitro studies of separated neuronal populations, either for chemical studies in both closed [4] and open [5, 6] compartments, or for extracellular recordings of electrical activity in open compartments [7].

Here, we present a closed-compartment neurofluidic device with microchannels connecting two compartments, the whole system being integrated on a planar micro-electrode array (MEA). We discuss long term neuronal cell cultures in our device, and demonstrate neurite growth through the microchannels connecting the compartments. Preliminary results of electrophysiological recording from the compartmented cultures are presented. Suppression of network activity within a compartment using neurotoxins and subsequent recovery of network activity through successive wash cycles are presented to demonstrate spontaneous network activity, and highlight the physical and fluidic separation between the compartments.

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II. Materials and Methods

A. Neurofluidic device fabrication

The 3-mm-thick polydimethylsiloxane (PDMS) devices used for this study have 2 microfluidic compartments ('B' & 'C' in Figure 1) of 100µm height and 8mm length interconnected with microchannels of 10µm height, 3µm width and 150µm length that are spaced at regular intervals of 60 µm [4]. The microchannels prevent the movement of cells between compartments. The devices were fabricated by conventional soft lithographic molding techniques pioneered by Whitesides and his collaborators (see for instance [8]), and that we described elsewhere [9]. Four 6-mm-diameter reservoir holes ('A' in Figure 1) were drilled in the fabricated devices using laser technique. These fabricated PDMS stamps were rinsed thoroughly in an ultrasonic bath, stored in Milli-Q water for 24h and decontaminated in a 70% ethanol bath. Each PDMS device was then sterilized in a dry oven at 120° C for 15 min and placed on cover-slips with the microchannels facing downwards for oxygen-plasma treatment. This treatment renders the microfluidic compartment and the microchannels hydrophilic, while preserving hydrophobicity of the contact surface, thereby preventing leakage. The PDMS devices were then aligned and reversibly deposited onto planar Microelectrode Arrays

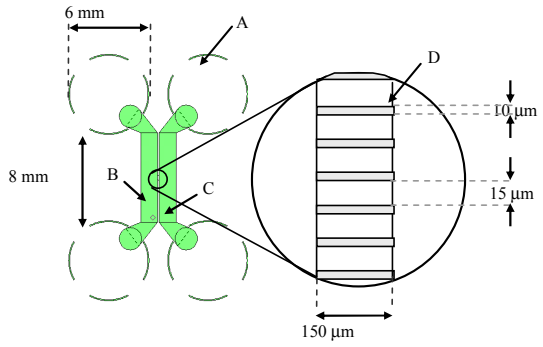


Fig. 1. Schematic layout of dual compartment device design used for neuronal cell culture; A- Reservoir (diameter = 6mm), B and C – Dual compartments (width = 1.5mm, height 0.1mm, length = 8mm), D – Microchannels (width = 10um, height = 3 um)

(MEAs). Prior to the placement of the PDMS devices, MEAs are sterilized in a vacuum oven, coated overnight with a solution of Polyethylenimine (PEI) (Sigma-Aldrich, stock solution at 50% w/v in water, Ca. No.P3143) at a concentration of 40 µg/ml and rinsed thoroughly in sterile water (tissue culture grade, GIBCO, Invitrogen, Cat. No. 15230).

B. Cell culture

As per the approved protocols for the care and use of lab animals in the Netherlands, primary cultures of Wistar Rat embryonic cortical neurons were prepared by Trypsine (GIBCO, Invitrogen, Ca. No. 25050-014) digestion of day-18 embryonic rat whole cortices. The dissociated cells were cultured in Neurobasal medium (GIBCO, Invitrogen, Ca. No. 21103) supplemented with 1% fresh, stable L-Glutamine (GlutaMAX™ 100x, GIBCO, Invitrogen, Ca. No. 35050-038), 1% Penicillin-Streptomycin Solution (GIBCO, Invitrogen, Ca. No. 15140-122), 2% B27 Supplement™ (GIBCO, Invitrogen, 17504-044) and 10nM Triiodo thyronine. The cultured cells were plated on PEI coated MEAs substrates at a surface concentration of ~ 2 x 10^5 cells / cm^2 and ~ 1 x 10^5 cells / cm^2. The plating occurred by injecting the cell suspension from one reservoir into the inlet of each compartment. The devices were then incubated in a humidified incubator at 37° C supplied with 5% CO_2.

C. Transfection imaging

Cultures were transfected with a thy1-eGFP construct in one compartment and a CMV-DsRed construct in the other compartment, using lipofectamine 2000, as per the manufacturer's instructions (Invitrogen). Briefly, 1 mg DNA was mixed with 100 ml neurobasal medium and mixed with 3 mg lipofectamine 2000 diluted in 100 ml neurobasal medium (without supplement), and left at room temperature for 20 minutes. After diluting the transfection mixture with four volumes of neurobasal medium with supplement, it was gently flushed into one of the compartments. Images of transfected neurons (typically only a few neurons were stained per compartment) were recorded after fixation of the cultures with 4 % paraformaldehyde in phosphate buffered saline at 18 to 21 days.

D. Recording setup

Network activity in the devices was recorded twice per week using standard MEA1060 system [MEA 1060-Inv-Standard amplifier, Multichannel Systems, Germany]. The total medium volume of the reservoirs being 0.34 ml, particular care was taken to prevent excessive evaporation during the recording sessions. A sterile recording box was custom built with a 90% relative humidity and 5% CO_2 supply.

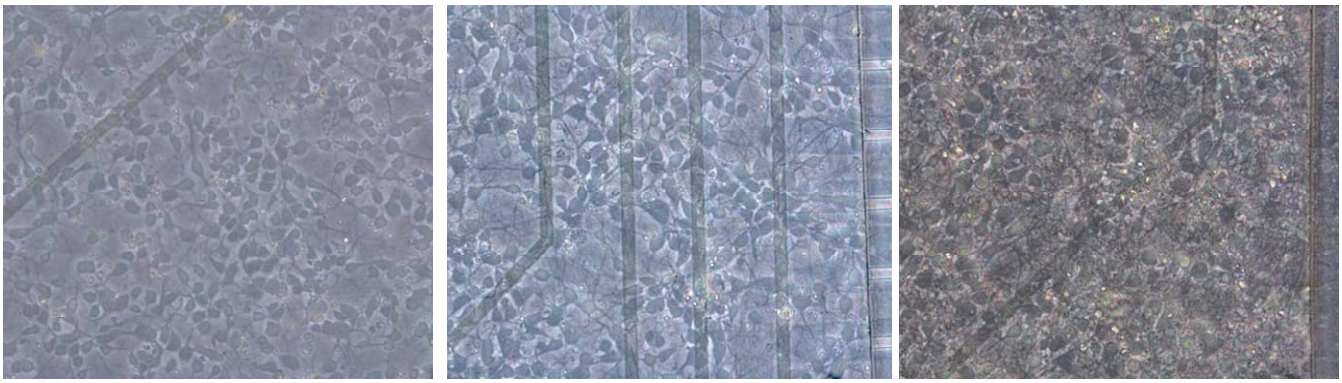
E. Device Cleaning and reuse

MEA devices were rinsed in DI water and placed in 1% Tergazyme [Alconox Inc., USA] solution at 37 °C overnight to enhance tissue break-down and removal of cell debris. The devices were then rinsed in DI water overnight and prepared as described earlier. Freshly prepared sets of PDMS devices were used for each experiment.

III. Results and Discussion

A. Viability of neurons in closed channel devices

Cell viability and neurite formation were observed at regular intervals over the developmental period. The development of a typical culture in a compartment is shown in Fig. 2. The distribution of cells along the inlet, the active electrode region and the outlet region of the compartment confirm the uniformity of cell plating. Each reservoir, with a volume of ~60µl contains enough nutrient supply for the culture. However, we observed that the cells in the center of the compartment started to degenerate after approximately 7 DIV, probably from oxygen and nutrients depletion and/or waste accumulation. This resulted in cell death in the center of the compartment propagating towards the periphery. To circumvent the issue of low medium availability to the cells and to provide cells with sufficient oxygen, we performed experiments with different medium change frequencies over the developmental period. Cultures with a medium change frequency of three times per week offered the most favorable results thus far resulting in active cultures until DIV 21.



DIV 2 DIV 4 DIV 9

Fig. 2. Cell culture in a compartment over the developmental stage with tri-weekly medium change schedule.

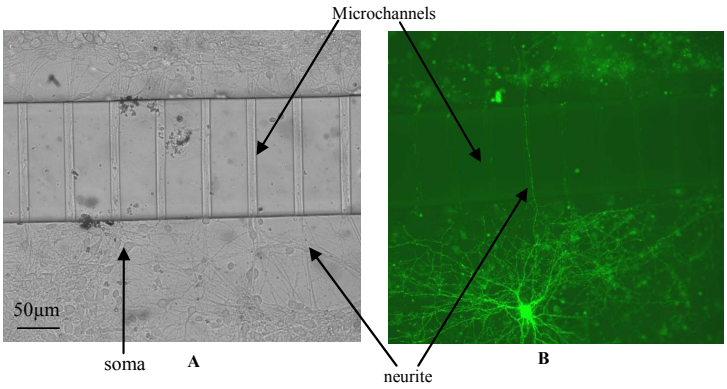


Fig. 3. Neurite growth through microchannels. A: Phase contrast image of neurites grown across the microchannels connecting the cell bodies in both compartments; B: Transfection imaging of a neurite grown across the microchannels connecting the compartments.

B. Neurite growth through Microchannels

Visual observation of the culture over the developmental period indicates neurite growth across the compartment from DIV 3. Neurites were observed to cross-over to the adjacent compartment through the microchannels along the whole length of the compartment. Phase contrast imaging of cell bodies isolated within a compartment and neurites crossing over to the adjacent compartment confirmed the physical containment effected by the microchannels (Figure 3a). To further substantiate structural trans-compartment connectivity by axonal crossover to the adjacent compartment, transfection of individual neurons with eGFP (green fluorescent protein) and DsRed (not displayed here) was performed in compartments B and C, respectively. Figure 3b shows extensive dendritic and axonal arborization within the compartment of origin, as well as individual axons crossing over to the other compartment through the channels.

C. Electrophysiological recordings

Electrophysiological recording of spontaneous activity within the culture is as shown in Fig 4. The raster plot shows the spontaneous activity of a culture in the closed compartmented device on DIV 14. In the figure, electrodes along the y-axis are number from 11 through 88 representing the corresponding rows and columns of the electrode layout and each dot represents an action potential recorded by one of the micro-electrode array channels.

D. Experiment with Tetrodotoxin (TTX)

To ensure the biological origin of the recorded activity within the compartments, experiments were performed using Tetrodotoxin (TTX), to silence the spontaneous activity within a compartment. Stock solutions of TTX were made in Neurobasal medium and warmed to 37 °C on the day of the experiment. As a control condition, the spiking activity was recorded for 2 minutes before adding TTX (Figure 5a). Next, 100 µl of medium in one of the compartment was removed by pipetting and replaced with an equivalent volume of TTX stock solution at a concentration of 1 nM. The addition of TTX at this concentration immediately suppressed all activity in the compartment. The spiking activity was recorded for 2 minutes with TTX in the compartment (Figure 5b). A rinsing cycle then followed with supplement enriched Neurobasal medium. The recovery of spikes in the compartment was recorded after successive rinses (Figures 5c and d) with very few electrodes regaining activity after the first rinse cycle, while the majority of the electrodes regain network activity after three rinse cycles as shown in Figure 5d.

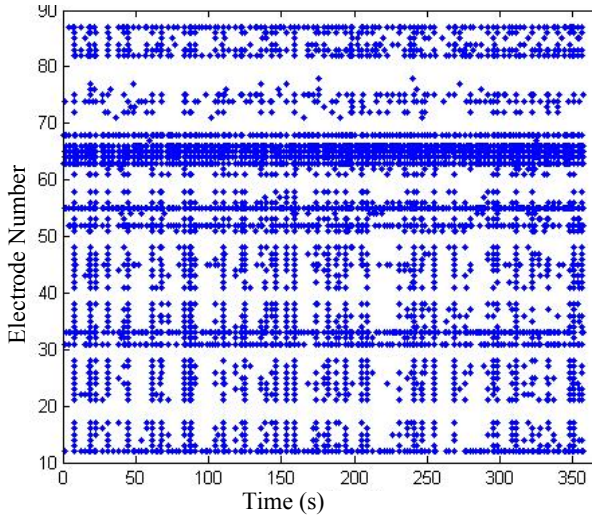


Fig. 4. Spontaneous activity of a culture on DIV 14 (350s time interval).

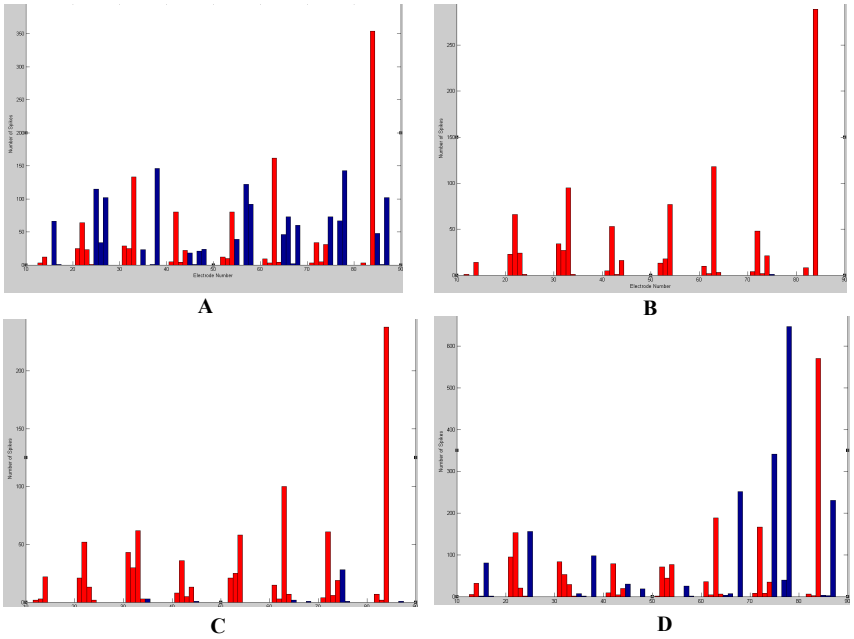


Fig. 5. Network activity in the dual-compartment device. A: Spike rate analysis in both compartments before addition of TTX (Compartment A in red and Compartment B in Blue); B: Suppression of network activity in compartment B with the addition of TTX; C: Recovery of spikes in compartment B after first washout cycle; D: Recovery of spikes in compartment B after three washout cycles.

IV. Conclusion

We presented neuron cultures in a closed dual compartment devices. Closed compartment systems similar to those presented in this work are often challenged by very low volume of medium available to the cell culture. We addressed this issue by implementing a medium change protocol that helps in supplying cells with fresh oxygen and supplements over the developmental period. We found that three times medium change per week offered good culture stability up to DIV 21.

With the microchannels offering the necessary physical isolation between the compartments, the cells were observed to be plated uniformly along the compartment length. In addition, the small feature size of the microchannels (width = 10 µm and height = 3 µm) also provided the necessary somatic and fluidic isolation between the compartments. The best evidence of the good quality of the neural network was the electrophysiological recording of spontaneous activity by means of a planar micro-electrodes array (MEA).

Good inter-compartment neurite growth and connectivity was observed, and supported by transfection imaging. Experiments with TTX injected in one compartment showed the suppression of spontaneous activity in that particular compartment, without observable influence on the spontaneous activity of the neighboring compartment. The suppressed network activity was regained after a few wash cycles.

These results show that the system supports healthy neuronal cultures with good fluidic and somatic separation. We now envisage extending its use to culture multi-compartment, differentiated neuronal subpopulations, thereby providing a tool to establish in-vitro network models of specific neuronal pathways.

Acknowledgments

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