Microfluidic device for unidirectional axon growth

E.Malishev[1*], A.Pimashkin[2], A.Gladkov[2,3], Y.Pigareva[2] A.Bukatin[1], V.Kazantsev[2], I.Mukhina[2,3], M.Dubina[1]

[1] Saint-Petersburg Academic University - Nanotechnology Research and Education Centre of the RAS, Saint-Petersburg, Russia
[2] N.I. Lobachevsky State University of Nizhny Novgorod - Neurocenter, Nizhny Novgorod, Russia
[3] Nizhny Novgorod State Medical Academy, Nizhny Novgorod, Russia
[*] corresponding author. E-mail: malishevg@gmail.com

Abstract. In order to better understand the communication and connectivity development of neuron networks, we designed microfluidic devices with several chambers for growing dissociated neuronal cultures from mice fetal hippocampus (E18). The chambers were connected with microchannels providing unidirectional axonal growth between “Source” and “Target” neural sub-networks. Experiments were performed in a hippocampal cultures plated in a poly-dimethylsiloxane (PDMS) microfluidic chip, aligned with a 60 microelectrode array (MEA). Axonal growth through microchannels was observed with brightfield, phase-contrast and fluorescence microscopy, and after 7 days *in vitro* electrical activity was recorded. Visual inspection and spike propagation analysis showed the predominant axonal growth in microchannels in a direction from “Source” to “Target”.

1. Introduction

Microfluidic chips combined with microelectrode arrays are used in a wide range of electrophysiological studies of neural networks at subcellular, cellular and network-wide scale. Such devices provide a method to control morphology of the grown culture of dissociated neurons in order to model a brain regions, study interaction or coupling of different cell types and formation of functional signalling pathways in the brain. Dissociated neuronal cultures can be grown in two separated chambers connected with microchannels which are long enough to provide a growth only for axons of the neurons [1]. Specific design of the microchannels can define axon outgrowth direction and organize functional connectivity in the neural culture. In such circuits presynaptic sub-population is connected with post-synaptic sub-population of the neurons [2]. Such dual compartment *in vitro* system, coupled with a microelectrode array and can provide unique approach to study network-wide synaptic plasticity as a basics of information coding, memory and learning. In this study we proposed a microfluidic device with different asymmetric microchannel designs to investigate unidirectional axonal growth during hippocampal culture development in order to localize presynaptic and postsynaptic subnetworks.

2. Materials and Methods

2.1 Chip design

Proposed design of microfluidic chip is based on the probabilistic nature of the direction of axon outgrowth. In order to maximize the probability of axon growth in desired area it is sufficient to place some "focusing" side walls, limiting available directions of axon growth (fig. 1.a). For solving the reverse problem of axon backward outgrowth minimization it is necessary to reduce the area from which axons may grow in an undesired direction. This can be done, for example, by creating a narrow "bottleneck" and false directions for growth (fig. 1.b). In our chip we have combined both these methods and proposed microchannels consisting of the sequence of triangular segments (fig. 1.c,d)

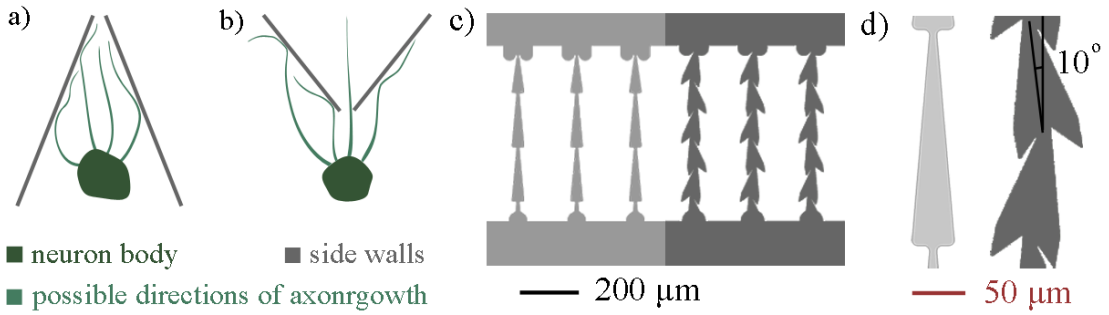

Figure 1. Microchannels' design: **a)** model of axon "focusing", **b)** model of a "bottleneck" for backward growing axons, **c)** proposed designs of microchannels: "Straight" type (left) and "Zig-zag" type (right), **d)** triangular segments' design, blue bar – 200 µm.

2.2 Microfluidic device fabrication

PDMS microfluidic chips containing an array of microchannels between two chambers were fabricated by two layer lithography and PDMS molding techniques (see fig. 2.a). Mold design contained: first 5 µm-thick layer, which formed microchannel structure and second 50 µm-thick layer, which formed chambers. Microchannels' structure was based on two types of segments, shown in figure 1.d. and their length varied from 200 µm to 1000 µm (2 - 10 segments).

For mold fabrication, the negative photoresist SU8 2025 and SU8 Thinner (MicroChem, USA) were used. Some portion of SU8 2025 was diluted by SU8 Thinner in a way to decrease the solids concentration from 68.55% (original concentration) to 45% (desired concentration, similar to SU8 2005) in order to achieve 5 µm layer thickness.

Silicon wafers were spin-coated with SU8 using a headway spinner at speeds of 4000 rpm. Softbake was done on a hotplate at 95 °C for 4 and 7 minutes for the first and second layer respectively. The lithography was performed with a MJB4 (SUSS Microtec, Germany) and an UV-filter PL-360-LP (Omega Optics, USA) with a cutoff wavelength 350 nm. Then wafers were post exposure-baked on a hotplate at 95 °C for 5 and 7 minutes respectively. Patterns were developed by using SU8 Developer. Finally, molds were hard baked at 200 °C for 7 minutes.

In order to create PDMS chip, PDMS (Dow Corning, USA) was mixed in a 10:1 ratio. Then, uncured PDMS was degassed in a desiccators and poured onto the master molds and cured in an oven at 70°C at least for two hours. After that PDMS chips were removed from molds and chamber structures were punched.

In order to investigate spike propagation inside the microchannels, each PDMS chip was positioned and mounted onto the surface of a planar microelectrode array (MEA), so as to locate several electrodes in the microchannels (fig 2.b). PDMS chips were adhered to MEA plate by heating them after mounting to 120°C for 20 minutes. Before cell plating the device was pre-treated with the adhesion promoting molecule polyethyleneimine (Sigma P3143).

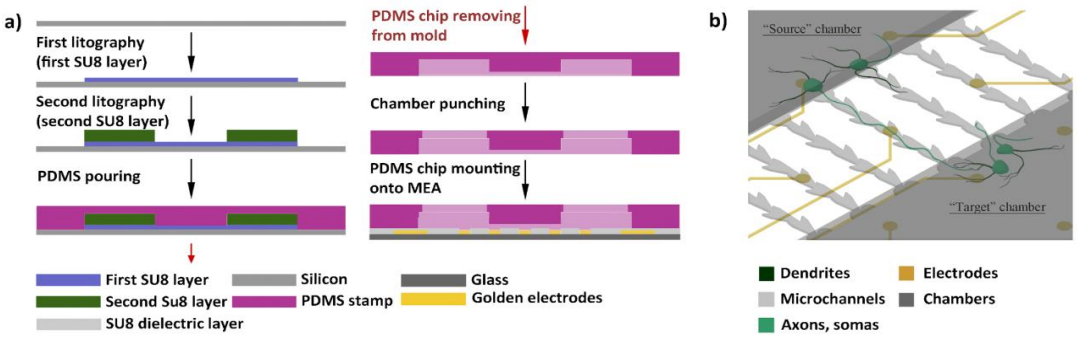

Figure 2. **a)** microfluidic device fabrication process **b)** Scheme of the microfluidic device with plated cells and branches.

2.3 Culture preparation

Hippocampal cells were dissociated from embryonic mice (E18) and plated into separate subcompartments on MEAs at a final density of approximately 15,000–20,000 cells/mm^2. C57Bl/6 mice were euthanized via cervical dislocation, according to the protocols approved by the Russian National Ministry of Public Health for the care and use of laboratory animals. The cultures were stored in incubator at 36-37°C and 5% CO_2 for 3-4 weeks.

2.4 Microscopy

Axon growth and culture development monitoring was performed using brightfield and phase-contrast mode microscopy under a fluorescent inverted microscope DMIL HC (Leica, Germany), with a 20x/1.0 objective. Also fluorescence micrographs were done. For that immunostained cultures were fixed for 15 min in 4% formaldehyde containing phosphate-buffered saline (PBS) (pH=7.4), washed in PBS and permeabilized for 30 min with 0.1% Triton X-100 (Sigma 93443-100ML) and 2% bovine serum albumin (BSA). Subsequently, the cells were incubated for 2 hours at room temperature in PBS containing 1% BSA and the primary antibodies: rabbit polyclonal Anti-Tau (314002, SySy, Germany) to stain axons. After washing in PBS, the cell cultures were incubated for 2 hours at room temperature with the following secondary antibodies: goat anti- rabbit conjugated Alexa Fluor 647 (A21245, Molecular Probes®, US).

2.5 Electrical recording and stimulation

Electrical activity was recorded after 7 days in vitro by USB-MEA system (Multichannel systems, Germany). Signals were recorded simultaneously from microelectrode array with 60 planar electrodes with 30 μm diameter and 200 μm interelectrode space. Electrical stimulation was applied using a STG-4004 stimulator (Multichannel Systems, Germany). Spikes were detected using threshold detection estimated from the noise statistics in each channel separately. The amplitudes of detected spikes were in the range of 10–40 μV. All signal analysis and statistics were performed using custom made software in Matlab® (see [3] for details).

3. Results and Conclusions

3.1 results

First we produced the microfluidic devices with different microchannel designs (see Materials and Methods) and plated hippocampal cells into interconnected chambers (fig. 3). In order to find optimal design of the microchannels for unidirectional connectivity between neuronal sub-populations we studied axon growth dynamics varying microchannels' length and shape. We examined a microsope images taken each day after plating and found that in most cases axons, growing in "forward" direction of microchannels (from Source to Target chamber) reached their ends, while only a few number of the axons, growing "backward" passed more, than 2 segments (see fig. 4.a,b). In summary we analyzed axon growth in 26 cultures with two shapes of microchannel segments and 5 types of microchannel length and found that in most cases efficiency of "Zig-zag"-shaped microchannels was less than in "Straight"-shaped. We confirmed that the branches in microchannels were axons by staining axon with fluorescent markers using immunostaining methods (fig. 4 b).

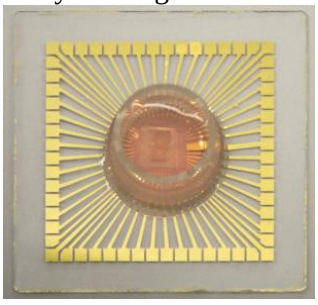

Figure 3. General view of a microfluidic device.

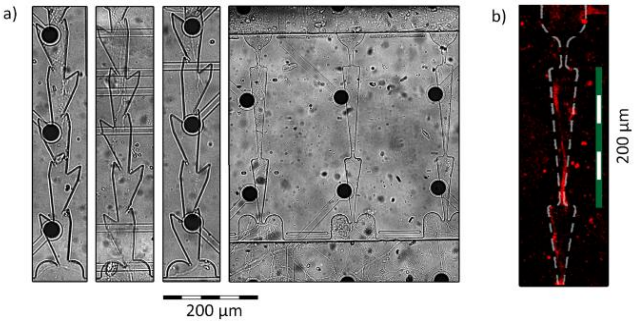

Figure 4. Axon growth microscopy **a)** brightfield micrographs of axons in different types of microchannels, **b)** fluorescence micrographs of Tau immunostained neurons.

We tested functional connections between neurons in microchannels using microelectrode array. Bioelectrical activity of the hippocampal culture starting form 7 DIV consists of network-wide synchronous spiking activity which referred as a bursts. Such bursts last for several hundreds of milliseconds with a few seconds of interburst interval. At the initiation phase of the burst spikes propagate through the network recruiting most of the neurons which induces reverberating activity for the rest of the burst.

Such bursts from 7 DIV to 10 DIV emerged spontaneously and independently in each of two chambers. We found that after 20 DIV the spontaneously emerged bursts in the Source chamber consequently evoked the bursts in the Target chamber through axons in microchannels (fig.5. a,b,c,d). A probability of such burst propagation was higher than the probability of evoking the bursts in opposite direction. The results for different 3 cultures are represented in fig. 5.e as a percentage of "Source to Target" bursts (blue bar) and "Target to Source" (green bar). We summarized the results and found that in average the probability of burst propagation through grown pathways was by 4 ± 1.5 times higher than in opposite direction.

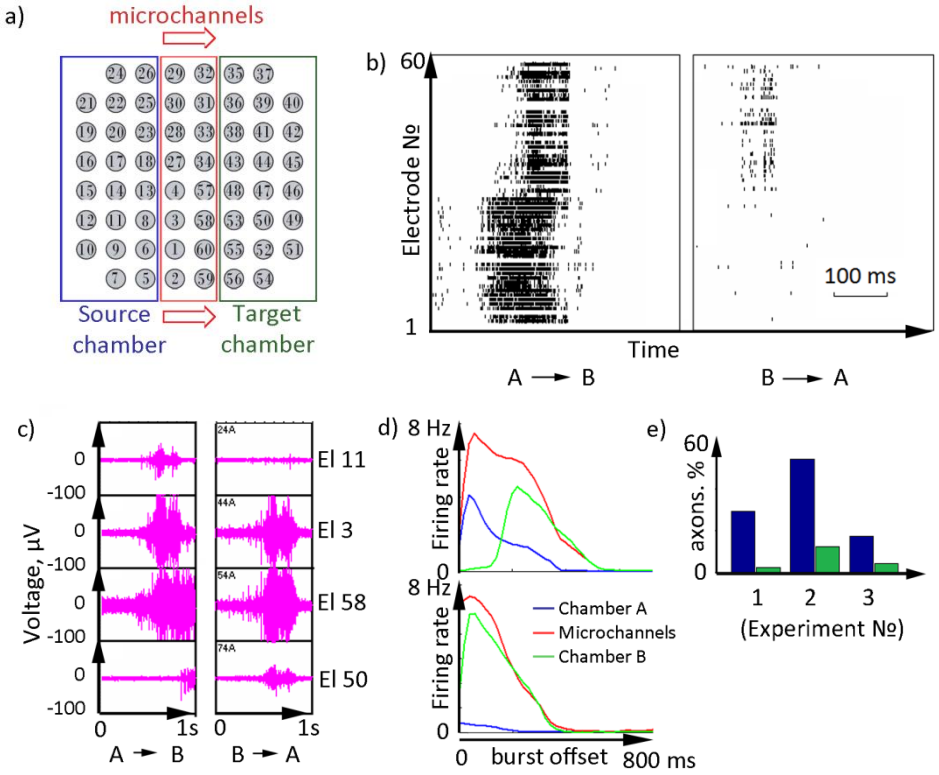

Figure 5. **a)** electrode numeration map, blue area depicts electrodes coupled with Source chamber, red - with microchannels, green - with Target chamber. **b)** raster plots of the bursts from Source chamber (A) evoked the burst in the Target (B) chamber (left). Burst in the Target chamber didn't propagate backward to Source chamber (right). **c)** Signals from 4 selected electrodes in Source chamber (top), two middle electrodes - coupled with microchannels, bottom electrode - electrode in Target chamber. Left column – burst spontaneously emerged in Source chamber (A), Right - in Target chamber (B). **d)** Top panel - Average spontaneous burst profile emerged in Source chamber. Each line represents spiking firing rate in each 5 ms time bin in Source chamber (A) (blue line), axons (red line) and Target chamber (B) (green line). Bottom panel – Average spontaneous burst profile emerged in Target chamber in opposite to axon grow direction. **e)** burst evoke probability from "Source to Target" chamber (blue bar) and "Target to Source" chamber (green bar). Each pair of bars represents on culture 20 min. recording on 20-25 DIV.

We also observed spike propagation through a grown synaptic pathway by single short pulse of electrical voltage stimulation of the neural network. We have randomly chosen the electrode for stimulation and applied a bipolar voltage pulse (500 μs, ±600 mV) in order to evoke bursting activity in both chambers. Stimulus responses propagated between chambers only from Source chamber into Target, but not vice versa (fig. 6).

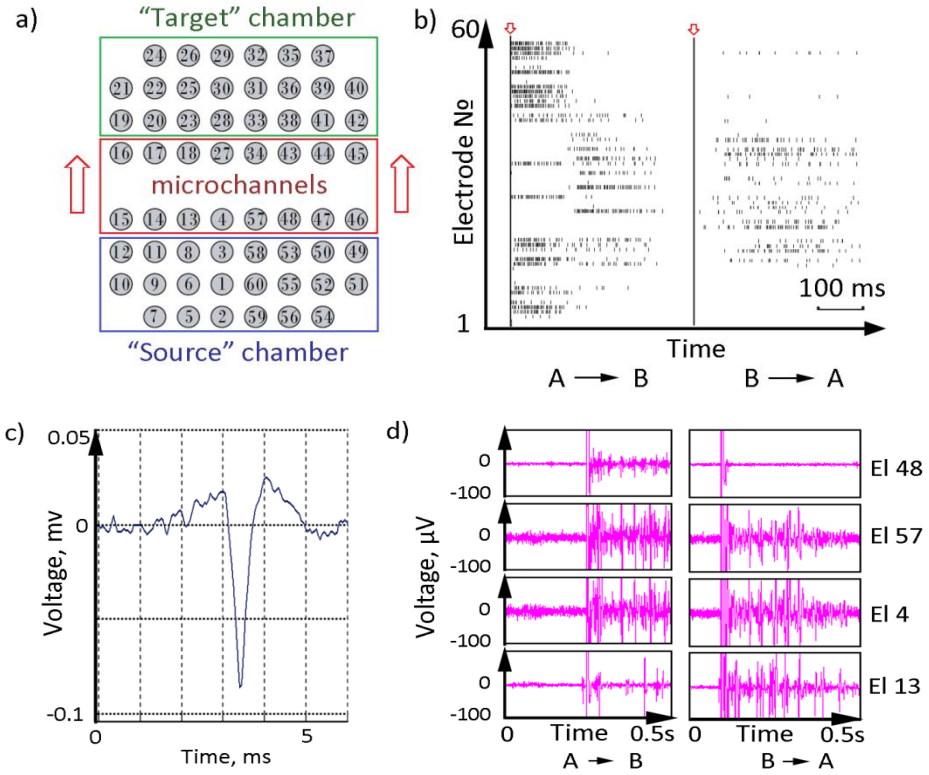

Figure 6. Electrical activity of hippocampal culture: **a)** electrode numeration map. **b)** raster plots of spiking activity in response to electrical stimulus (red arrow) applied to the electrode in Source chamber (A) (left) and Target chamber (B) (right). Each vertical line represents a spike timestamp, **c)** example of a spike timestamp, **d)** electrophysiological signals in response to electrical stimulus recorded from 4 selected electrodes: top - electrode from Source chamber (A), two middle electrodes - coupled with microchannels, bottom electrode - Target chamber (B). Left column (A->B) - burst emerged in Source chamber, Right (B->A) - burst emerged in Target chamber.

3.2 conclusions

In this study we developed microfluidic chips with dual chambers coupled by specifically designed microchannels for unidirectional neural axon outgrowth between sub-populations of dissociated hippocampal networks. We tested several designs varying length and inner design to find optimal design of microfluidic device. We found that the "Straight" type of the microchannel was optimal for unidirectional synaptic spike propagation during spontaneous and stimulus evoked bursts. The minimal length for microchannel to provide unidirectional coupling was found to be 400 μm, indicating that dendrites cannot overgrow such length from Source chamber to Target and axons are not able to grow backward from Target chamber to Source Source chamber in order to provide backward synaptic connections in Target chamber or Source chamber respectively. Such microfluidic chip coupled with MEA can be widely used in the research of network dynamics in a realistic morphology, network-wide synaptic plasticity and interaction between cell types which is the basics of the higher cognitive functions in the brain.

Acknowledgments

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4. References

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