Model of ‘implant-host’ neural circuits in a microfluidic chip in vitro

V N Kolpakov1, Y I Pigareva1, A A Gladkov1,2, A S Bukatin3,4, V B Kazantsev1,5,6, I V Mukhina1,2 and A S Pimashkin1

1 Neurotechnology Department, Lobachevsky State University of Nizhny Novgorod, 603950 Nizhny Novgorod, Russia
2 Cell Technology Department, Central Research Laboratory, Privolzhsky Research Medical University, 603005 Nizhny Novgorod, Russia
3 The Laboratory of Renewable Energy Sources, Alferov Saint-Petersburg National Research Academy University of the Russian Academy of Sciences, 194021 Saint-Petersburg, Russia
4 The Laboratory of Bio and Chemosensor Microsystems, Institute for Analytical Instrumentation of the RAS, 198095 Saint-Petersburg, Russia
5 Neuroscience and Cognitive Technology Laboratory, Center for Technologies in Robotics and Mechatronics Components, Innopolis University, 1 Universitetskaya Str., 420500 Innopolis, Russia
6 Center for Neurotechnology and Machine Learning, Immanuel Kant Baltic Federal University, 14 Nevsky Str., 236016 Kaliningrad, Russia

pimashkin@neuro.nnov.ru

Abstract. In this study, we developed a new model of neuronal cells plating into a developed neural network to study functional integration using microfluidic methods. The integration was modeled in a three-chamber microfluidic chip by growing two weakly coupled neuronal networks and enhancing its connectivity by plating new dissociated cells. The direction of connections was formed by the asymmetric design of the chip. Such technology can be used to develop a new type of scaffold to recover the modular structure of the network.

1. Introduction

Neural tissue regeneration in the brain injury area can be performed with the implantation of scaffold structures composed of various biocompatible and biodegradable materials (polymers, hydrogels, and hyaluronic acid) that carry neuronal cells to restore the cellular structure. Such recovery must also restore a heterogeneous or modular network architecture and its functional structure. A neurite outgrowth direction can be defined by scaffold structure to form a synaptic connection with the host brain tissue and restore signaling pathways that form functions.

However, a method to integrate neuronal cells in the network and localize in precise spatial structure in order to restore its functionality remains one of the main technical challenges. Recent advances in microfluidic methods combined with microelectrode arrays allow the development of an in vitro model of such network monitoring and spatial reconfiguration to study the formation of new
functional connections which enhances network functionality. Guidance of axon growth by microchannels between separate networks enabled modeling of the complex biologically inspired architecture of hippocampal regions using primary rodent cell cultures. In this study, we developed a microfluidic chip with three chambers connected by microchannels with the asymmetric design which allows integration of new cells between unidirectionally and synaptically weakly coupled networks in order to enhance these functional connections and provide spike pattern propagation in a predefined direction.

2. Materials and methods

2.1. Microfluidic Device Fabrication
Microfluidic chips were fabricated using a 10:1 mix of PDMS (polydimethylsiloxane) silicon elastomer and its curing agent (Sylgard 184, Dow Corning, UK). Liquid PDMS was poured onto a master mold and cured in an oven at 70°C at least for two hours. The wells for cell plating were cut with a puncher (2 mm in diameter) at two opposite sides of each chamber.

The chip consisted of three chambers: a Source, an Implant, and a Target (figure 1). The Source chamber and the Implant chamber were connected by 16 microchannels, each of which was 400 μm long and consisted of two triangular sections. The Implant chamber and the Target chamber were connected by 16 asymmetric microchannels 600 μm in length, each consisted of three sections. The second and the third sections contained two ‘traps’ that prevented axons growing in the Target - Implant direction.

2.2. Cell Culturing
Mouse hippocampal cells (E18) with a density of 5500 cells/mm² were cultured in the Source and the Target chambers of the chip. On 7th day of culture development in vitro (DIV) in the Source and the Target chambers, the new dissociated hippocampal cells were plated into the Implant chamber, modeling the cell integration of new cells into the host neuronal network (figure 1). Details of the culturing protocol can be found in a previous study [1].

![Figure 1](image-url)

**Figure 1.** Microfluidic chip design and scheme of the experiment. Dissociated hippocampal cells were plated in the Source and the Target chamber, the Implant chamber remained empty (DIV1). The axons of the Source network grew into the Implant chamber within 6 days after plating (DIV6). Axons from the Target mostly grew into the “trap” section. Dissociated hippocampal cells were plated into the Implant chamber 7 days after plating in the host chambers (DIV7).

2.3. Neurite Analysis
The axon growth cone regions in microchannels were observed using bright-field microscopy with
Axio Observer.A1, Carl Zeiss, Germany. We analyzed neurite growing each day from 1 to 6 DIV (n = 4 chips, 64 microchannels). We estimated the percent of the microchannels, in which neurites grew from the Source to the Target chambers on 5 DIV. The axons from the Source and Target chambers were visually difficult to separate within the same microchannel on later days of culture development.

2.4. Extracellular Spike Recording and Stimulation protocol
Microfluidic chips were manually mounted onto the surface of a planar microelectrode array (MEA, Multichannel Systems, Germany). The electrophysiological activity was recorded from 59 (1 reference) TiN electrodes of the MEA system USB-MEA120-Inv-2-BC-System (Multichannel Systems, Germany) at a sample rate of 20 kHz. Electrode diameter was 30 μm with a 200 μm interelectrode distance.

Stimulation of the electrodes on the MEA was performed using the STG-4004 stimulator (Multichannel Systems, Reutlingen, Germany). Analysis of the recorded bioelectrical activity was performed with custom-made software in Matlab [1]. We used a low-frequency stimulation which consisted of biphasic voltage pulses ±800 mV, 260 μs per phase, positive first, with 3s interstimulus intervals. Series of 60 stimuli were applied separately on two electrodes in the Source and the Target chambers.

Figure 2. Axon growth in three-chamber microfluidic chip. (a) Microphotographs of the axons on hippocampal cells in the microchannels on 4, 5, 6 and 9 DIV. The maximum distances to which the neurites grew from the Source chamber are marked by green arrows, the neurites grew from the Target chamber are marked by blue arrows. The cells were plated into the Implant chamber on 7 DIV. Scale bar = 100 μm. (b) The number of the microchannels (%), where neurites grew to the corresponding section from the Source (white) and the Target (black) chambers at 5 DIV (64 microchannels, 4 chips).

2.5. Analysis of network activity
Spike and burst detection methods were described in detail in a previous study [2]. Spontaneous spiking activity was recorded on 10, 14, 18, 21, 27 DIV.

The probability of the burst propagation in the forward direction was estimated as a number of bursts propagated [4] from the Source to the Implant normalized to the burst number in the Source. The probability of the burst propagation in the opposite direction was estimated as the number of bursts propagated from the Implant to the Source normalized to the burst number in the Implant. The
characteristics were also estimated between the Implant and the Target. *Burst propagation delay* was estimated as a mean time delay between the maximums of array-wide spiking rate with a 1 ms time bin found for each pair of propagated bursts.

Electrical stimulation applied to the electrodes in the chambers evoked a response in the form of a network burst recorded by a group of electrodes. We used a *post-stimulus time histogram* (PSTH) to characterize the evoked network bursts. PSTH values on the electrodes of the Source, Implant, and Target chambers were analyzed separately and were normalized on the number of analyzed electrodes. The PSTH was estimated at 10–50 ms intervals after stimulation with a 2 ms time-bin and calculated by averaging the responses to 60 stimuli.

3. Results and discussion
Axons of cells from the Source chamber grew into the Implant chamber at a distance of 400-600 μm on 5 DIV in more than 95% of microchannels (n=64 microchannels from 4 chips, figure 2b). The microchannel length 400 μm between the Source and the Implant chambers did not allow cell dendrites from the Source chamber to grow into the Implant chamber and reduced the effect of the implanted cells on cells in the Source chamber. These axons of the Source neurons formed the basis for directed synaptic connections with the new dissociated implanted cells in the Implant chamber. In addition, the axons continued to grow further than the Implant chamber, entering the next channel connecting the Implant chamber and the Target chamber, where they met the neurites (usually dendrites) from the Target chamber culture at 5 DIV in 48% of microchannels. A weak unidirectional connection could be formed between the Source and the Target cultures before the new dissociated cells were plated into the Implant chamber. Axons of cells from the Target chamber grew at a distance of 600-800 μm on 5 DIV only in 1.6% of all microchannels.

![Figure 3](image_url)

**Figure 3.** Spontaneous activity of neuronal culture in a three-chamber microfluidic chip. (a) The number of bursts per minute in the Source (green), Implant (red) and Target (blue) chambers. (b) Percentage of bursts propagation from chamber Source to chamber Implant (green), from Implant chamber to Target chamber (blue). (c) Burst propagation delay from the Source chamber to the Implant chamber (green), from the Implant chamber to the Target chamber.

We evaluated the characteristics of spontaneous network activity during culture development and after plating new cells (figure 3a). The *number of bursts per minute* was 264 in the Source chamber and 859 in the Target chamber on 10 DIV while the culture in the Implant chamber remained inactive (see Methods). The bursting activity appeared on 14 DIV (7 days after new cell plating). The *burst propagation* was not observed between the Source and the Implant chambers until 18 DIV and
between the Implant and the Target chambers until 21 DIV (figure 3b). The probability of the burst propagation in the forward direction was in the range of 5-16.6% between the Source and the Implant chambers as well as between the Implant and the Target chambers on 18-27 DIV. There was no propagation in the opposite direction.

The burst propagation delay (figure 3a) from the Source chamber to the Implant chamber on 18 DIV was equal to 9 ms, and 10 ms on 27 DIV. The burst propagation delay from the Implant chamber to the Target chamber was 19.7 ms on 21 DIV, and 72 ms on 27 DIV.

Figure 4. The responses induced by low-frequency stimulation applied to the electrode in the Source chamber. The number of spikes after stimuli in the Implant (a) and the Target (b) chambers on 3 DIV after cell plating in the Implant chamber (10 DIV from first culture plating). The number of spikes after stimuli in the Implant (c) and the Target (d) chambers on 11 DIV after cell plating in the Implant chamber (18 DIV from first plating). (e) PSTH of the responses in the Implant chamber induced by the Source and the Target chamber stimulation. (f) PSTH of the responses in the Source chamber induced by the Target chamber stimulation and the responses in the Target chamber induced by the Source chamber stimulation. Mann-Whitney Rank Sum Test, *p < 0.001.

The postsynaptic responses were estimated in the Implant and the Target chambers in response to electrical stimulation of the Source chamber electrodes. The stimulation evoked spikes in the Implant and the Target chambers after 3 days from cell plating in the Implant chamber (figure 4 a - d, 10 DIV from the plating of first cultures). The average number of stimulus-evoked spikes (PSTH) on the Implant chamber electrodes increased from 0.01 ± 0.08 on 3 DIV to 12.2 ± 6.5 on 20 DIV after cell plating in the Implant chamber (Kruskal-Wallis test, p < 0.001). The electrical stimulation of the
Target chamber electrodes evoked spikes in the Source and the Implant chambers. The PSTH in the Implant chamber was significantly less to stimulation of the Target than the Source chamber during days: 7, 11, 15, and 20 DIV (Mann-Whitney Rank Sum Test, p < 0.001). The PSTH in the Source chamber (stimulation in the Target) was significantly less than the PSTH in the Target chamber (stimulation in the Source) during days: 7, 11, 15, and 20 DIV (Mann-Whitney Rank Sum Test, p < 0.001).

4. Discussion
Many studies have demonstrated the convenience of platforms based on MEA and microfluidic chips for modeling cellular and neurites disorders. It was shown that neurons derived from stem (pluripotent) cells can functionally interact with primary cultures of neurons [3] and enhance information processing performance [4]. In this work, we investigated the integration of cells between unidirectionally and weakly coupled two subnetworks to enhance its connectivity. We applied electrical stimulation to evoke spiking activity and analyzed functional connectivity between subnetworks. Network bursts in the Implant and Target chambers, recorded with delays of 10-50 ms in response to the stimulus in the Source chamber, indicated the formation of synaptic connections with the cells in the corresponding chambers. Stimulation in the opposite direction to axon growth also demonstrated a spiking response which might be explained by back-propagation of action potentials through the axons and then could evoke a network response in the chamber. Nonetheless, the responses in integrated cell culture and further connected with it were significantly greater than to opposite suggesting activitypropagation dominantly in a predefined direction.

5. Conclusion
In this study, we investigated a three-chamber chip platform to study spiking activity changes after the integration of the cells into weakly coupled cultured neural networks. The method was based on a previously proposed experimental scheme. We showed that integrated cells formed synaptic connectivity in the form of a “bridge” to transfer spontaneous and stimulus-evoked spikes. Such an experimental platform allows to model the recovery of the functional structure of neural networks and can be further developed using IPS cells for recovery of the brain injury.

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