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Microfluidic hydrogel layers with multiple gradients to stimulate and perfuse three-dimensional neuronal cell cultures

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Abstract

We present a simple and easy to handle PDMS microfluidic device for neuronal cell culture studies in three-dimensional hydrogel scaffolds. The hydrogel is structured in parallel layers to reconstruct cell layers close to the natural environment. Dissociated cortical neurons of embryonic rats have been cultured in 0.5% w/v agarose including 0.2% w/v alginate. The cells formed neurite networks through neighboring cell free hydrogel layers. The cell culture showed neurite outgrowth in the microfluidic channel over more than seven days *in vitro* without perfusion. Culturing neurons in hydrogel layers surrounded by a liquid phase containing culture medium resulted in denser neuronal networks.

Keywords: microfluidics; gradient; perfusion; neurons; hydrogel; 3D cell culture

1. Introduction

Cell cultures of neurons *in vitro* are extremely sensitive to environmental cues [1]. To analyze the function and structure of neuronal cultures in a proper way, cells should be entrapped in a three-dimensional micro-environment and arranged in multiple cell layers. For three-dimensional scaffolds, natural hydrogels are suitable to entrap different kinds of cells. Neuronal cells have already been cultured in agarose, collagen or matrigel [2, 3], but the arrangement of multiple cell layers has not yet been achieved.

Microfluidic devices can be applied to generate multiple cell layers by laminar flow. We fabricate two microfluidic devices with either four inlets or six inlets and one outlet in polydimethylsiloxane (PDMS). By injecting a pre-warmed hydrogel solution in the inlets, four or six parallel hydrogel layers can be generated in the main channel. We call them the four layer or six layer device. Cells can be entrapped in the hydrogel layers, which are functioning as a three-dimensional matrix. Hydrogel layers can also be enriched with supplemental growth factors to stimulate the cell culture layers. In the six layer device, the two boundary layers are filled with liquid and function as perfusion channels.

Dissociated cortical neurons from embryonic rats E17 have been cultured in 0.5% w/v agarose hydrogel layers containing 0.2% w/v alginate. In the four layer device cell free hydrogel layers have been enriched with 4% v/v B27 supplement generating a 2% v/v homogeneous concentration profile in steady state.

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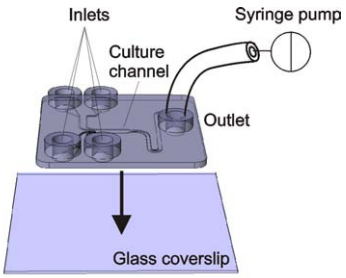

Fig. 1. Drawing of the four layer device. A microfluidic chip fabricated in polydimethylsiloxan (PDMS) and bonded to standard glass coverslip for cell culture studies.

The six layer device generated a pseudo perfusion condition, because the medium and the B27 supplement in the perfusion channel was renewed every 24h. Neuronal cell viability is related to their neurite network formation. The pseudo perfusion of cells, cultured in hydrogel layers, resulted in a denser neurite network and a higher cell viability compared to a homogeneous stimulation.

2. Microfluidic set up for three dimensional cell culture in hydrogel layers

To establish parallel hydrogel layers in a microfluidic device, a simple channel design was used including four inlet channels that led into a main channel. One outlet channel with a higher fluid resistance followed the main channel. Figure 1 shows the four inlet microfluidic device. The four layer design was enhanced by two additional inlet channels to perfuse the hydrogel layers in the middle of the main channel. Both designs were transferred into a silicon master by standard photolithography and deep reactive ion etching. Dow Corning PDMS 184 was molded on the silicon master. Oxygen bonding of PDMS to a glass coverslip closed the microchannels on the bottom. The surface of the PDMS channels was pre-treated with biomolecular coatings for cell culture test. Parallel hydrogel layers in the main channel are generated by injecting a pre-warmed agarose solution mixed with cells or nutriments and simultaneously sucking from the outlet. Hydrogel free layers were obtained by injecting water or medium. Laminar layers of hydrogel immediately gellified, through the thermo responsive behavior of agarose, by cooling the device down to 26°C for low gelling agarose.

3. Different gradients through hydrogel layers

Different hydrogel layers can function as a tissue-like three-dimension scaffold for *in vitro* studies. The hydrogel layers can immobilize cells or act as drug supplier to neighboring cell culture regions. Fig. 2 shows different concepts of either a fully filled main channel with two alternating hydrogel layers containing cells and drugs, or a main channel partially filled with hydrogel that is perfused by a liquid phase. Within the enhanced six layer device the perfusable hydrogel width can be enlarged compared to the four layer device, or the alternating cell layers of the four layer design can be perfused or a six layer cell tissue can be created.

As an example application for neuronal cell culture experiments, we studied the influence of two different culturing conditions on the neurite outgrowth. An alternating cell tissue was generated with entrapped neurons in the agarose in two non-neighboring layers. The cells have been mixed 1:1 with the pre-warmed agarose solution and injected in the first and third layer. The other two agarose layers function as a barrier, where neurites can grow through. In the four layer device the barriers are enriched with 4% v/v B27 supplement to stimulate the neurite outgrowth [4]. In the six layer devices, the 3rd and the 5th layer were filled with hydrogel containing culture medium. We used the boundary layers as perfusion channels. Here in one layer 8% v/v B27 supplement in neurobasal medium was injected, while the other medium layer was B27 free. Both concentrations have been renewed every 24h. Neuronal network formation have been observed with differential interference contrast microscopy and traced with NeuronJ, a software plugin for ImageJ [5].

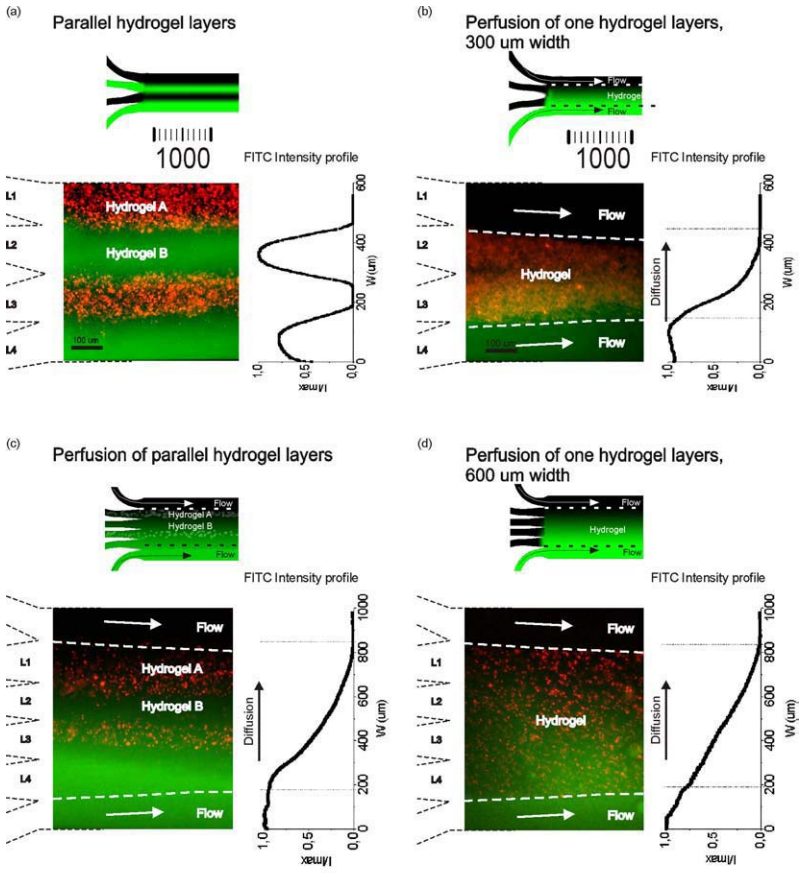

Fig. 2. Four different types of gradients and hydrogel layer formation in the four or six layer device. a) Alternate hydrogel layers of red fluorescent microspheres and green diffusible fluorescein molecules. b-d) Fluorescein perfusion of two or four hydrogel layers. The scale indicated 1000 µm

4. Results and Discussion

After the three-dimensional cell and hydrogel layers are established, the flow in the main channel was stopped and the B27 molecules diffused into the cell layers. If we consider that the rate of cell endocytosis of the molecules is lower than the diffusion rate, endocytosis can be neglected. Simulations for 150 kDa molecules showed a homogenous concentration of 2 % v/v in all hydrogel layers after 4h for both culturing conditions. Because we are renewing the liquid phase in the six layer device every day only, we call this condition as a pseudo-perfusion. We see no influence of the initial gradient on the neurite extension, but the fact of renewing consumed B27 molecules boosts the neurite density.

For both culture conditions, we detected neurite outgrowth already through hydrogel layers after one day *in vitro* (DIV). After 5 DIV the neurite length reached a maximum in the six layer device, see Fig.3. The cell culture, as shown in Fig.4, has established its fully neural network. The main differences between the two culture-conditions are the neurite density and the maximum neurite length. The four layer self-contained system can only be supplied by culture medium and supplements, from the reservoirs that are located at the end of the inlet and outlet channels. After the layer formation, a long diffusion way is imposed, that forces long neurite extensions. After 5 DIV the nutriment in the four layer device have also reached the cells in the main channels and induce continuous neurite growth. In the six layer device the four hydrogel layers are continuously supplied with new nutriments, through the direct contact of the hydrogel to the culture medium phase. This condition stimulates a denser neurite network compared to the homogenous condition.

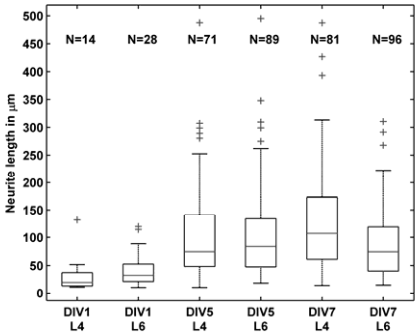

Fig. 3. Boxplot of neurite length after one, five and seven days *in vitro.* L4 indicates the four layer device with homogeneous culture condition and L6 indicates the six layer device with pseudo-perfusion conditions. N is the number of neurites in a 600 x 600 µm^2 area in one focus plane.

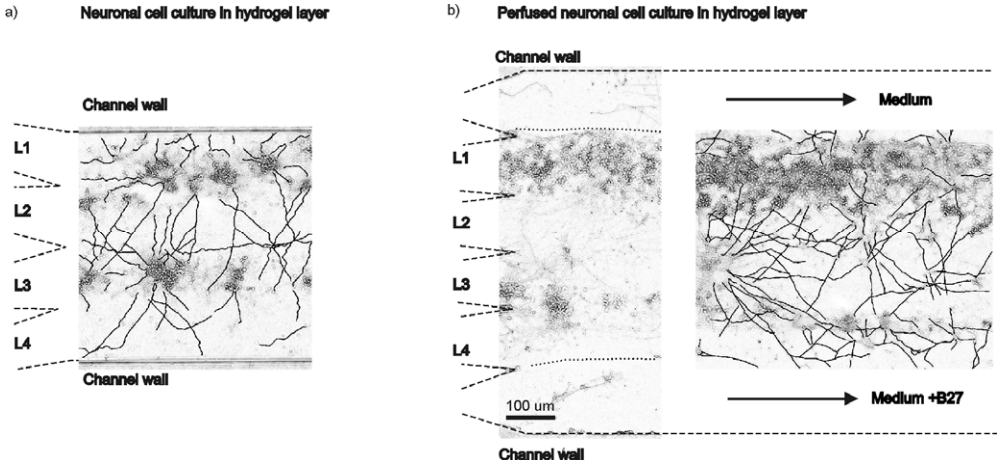

Fig. 4. Neuronal network formation after five days *in vitro* under a) homogenous and b) semi-perfusion conditions

5. Conclusion

A multi-functional microfluidic device based on PDMS has been fabricated and tested with neuronal cells cultured in different 3D cell layers under two different conditions. Our microfluidic device has the potential to help answering questions in neuronal network development. The PDMS chip can easily be bonded to other surfaces, e.g. multi electrode arrays for electrophysiological studies of neurite networks. Co-cultures of different cell types can also be implemented for developmental questions. The advantage of the six layer device are the two liquid channels that can be used for perfusion or even drug testing experiments.

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