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LONG-TERM BRAIN SLICE CULTURING IN A MICROFLUIDIC PLATFORM

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ABSTRACT

In this work, we present the development of a transparent poly(methyl methacrylate) (PMMA) based microfluidic culture system for handling long-term brain slice cultures independent of an incubator. The different stages of system development have been validated by culturing GFP producing brain slices from 8-day old (P8) mouse pups. Fluorescence microscopic monitoring of GFP was utilized as an indicator of tissue viability. The final format of the developed system, featuring "plug-and-play" technolgy with a reusable fluidic connection board and easily changeable microfluidic chips, facilitated brain slice culturing for 16 days.

KEYWORDS: Microfluidic Tissue Culture, Brain Slice, Long-Term Culture, Stem Cell Therapy

INTRODUCTION

In vitro brain slice cultures are extensively used in neuroscience and neuroengineering [1] to mimic many aspects of *in vivo* conditions in the study of complex neuronal networks. Organotypic brain slice cultures are useful to study e.g. short and long term plasticity, mechanism of memory, learning and neurochemical behaviour [2]. Another application of organotypic brain slice cultures is stem cell therapy as a treatment of Parkinson's disease, in which degenerated dopaminergic neurons are replaced by implanting neuronal stem cells into brain tissue. Initial studies have established the basic knowledge regarding the necessary procedures [3]. The conventional techniques, microscopy and electrophysiology, can show that the stem cells become a part of brain tissue and respond to stimulation; however, using these methods, it is difficult to acquire real-time recordings of responses from the entire neuronal network upon stimulation of the stem cells.

The most commonly used techniques to culture brain tissue slices are the roller tube method and the interface method [2]. In the roller tube method, a glass cover slip, on which brain slices are fixed with collagen or plasma clot, is placed in a plastic tube containing culture medium and undergoing slow rotation to ensure oxygenation due to continuous change of liquid-gas interface. Although the roller tube method is ideal for protocols requiring optimal optical conditions and access to individual neurons, it suffers from large experimental variability due to thinning of the tissue. In the interface method, brain slices are maintained in a stationary culture, where they are placed at the air-medium interface on a semi-porous membrane maintained stationary over the entire culture period. The tissue receives oxygen from above and medium from underneath by capillary suction. Although the method provides optimal *in vitro* conditions for long-term brain slice culture, it cannot facilitate real-time electrophysiological or electrochemical measurements.

To circumvent the limitations of the conventional culture methods, a recent development is to apply microfluidic platforms to perform tissue culture experiments. Various microfluidic systems are developed to achieve, e.g. local chemical stimulation and improved viability of brain slices as well as culturing of thick brain slices. Blake et al. introduced a multi layer perfusion chamber to provide nutrients in a controlled manner and promote fast exchange and localization of medium and cellular effectors [4]. This approach was applied to perform electrical recording for 60 min. Choi et al. fabricated hollow micro needles, which could penetrate tissue [5]. The whole microchip with the needles was then placed in the petri dish to supply nutrients to all the layers of the tissue. Although, they have showed the new idea, the experiment was performed only for 4 h. Rambani et al. developed a system for brain slice culturing by implementing a forced convection system [6] to handle thick brain slices (700 µm), which is very challenging. They suggested that the forced convection feeding is better for tissue survival.

We develop a microfluidic system for long-term brain tissue slice culturing to facilitate integration of neuronal stem cells as a tool in research on stem cell therapy. Ultimately, such a system can be integrated with 3D microelectrodes [7] for potential recordings in the tissue upon selective stimulation of the integrated stem cells. Successful implementation comprises two main requirements: i) Culturing of brain tissue for at least three weeks under proper conditions (nutrients, O_2, pH and temperature), and ii) monitoring of stem cell integration and differentiation. As an initial part of the research, we have developed a microfluidic tissue culture system for long-term culturing to be later adapted for integration of neuronal stem cells into brain tissue.

EXPERIMENTAL

PMMA substrates for fabricating the different components of the microfluidic systems were machined using either laser ablation or micromilling. To obtain a complete microfluidic system, the fabricated PMMA components were bonded to-

gether using a UV assisted thermal bonding process (1 min UV exposure, 88ºC, ca. 35 bar for 30 min). Fluidic connections to the systems were made using 19 gauge needles. O-rings for connecting microfluidic chips to fluidic connection board were made of polydimethylsiloxane (PDMS) using a micromilled mould.

The microfluidic systems were sterilized with 0.5 M NaOH for 15 min followed by rinsing with cell culture tested water and PBS. The culture chambers were coated with Poly-D-lysine (10 µg/ml) for 2hrs at room temperature and laminin (20 µg/ml) overnight at 37ºC. All the used chemicals and cell culture tested water were from Sigma-Aldrich. Solutions were prepared using cell culture tested water.

Vibratome was used to prepare 250 µm thick brain slices from genetically modified P8 Balb/c mice having GFP production linked to expression of the gene encoding for glutamate decarboxylase (GAD). The preparation of tissue slices was done based on standard procedures [8] according to local guidelines. In order to start microfluidic tissue culture, each utilized tissue slice was transferred into a dedicated culture chamber using a large-bore glass pipette. After placing the tissue slices into the chambers, each chamber was half-filled with culture medium [8]. Prior to closing the chambers, the culture systems were placed in a CO_2 incubator for 15-30 min to allow adhesion of the slices on the coated surface. The chambers were then closed with a 500-µm PMMA window using a double-sided adhesive silicon gasket to ensure fluid tightness. Air retained in the chambers upon closing the windows was removed through a dedicated channel by slowly perfusing culture medium. During microfluidic culturing, medium (presaturated with CO_2 in an incubator) to each culture chamber was delivered at the flow rate of 75 µl/h from a polypropylene syringe with Luer lock (B Braun AG, Melsungen, Germany) operated by a laboratory syringe pump. Interfacing to the microfluidic systems was done using BD Connecta tubing with 3-way valve (Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden). The culture medium in the syringes was changed every second day. Temperature during culturing on microscope stage was maintained by a custom made Peltier heater controlled by a TC-36-25 RS232 temperature controller (TE Technology, Inc., Traverse City, MI).

Microscopic imaging during tissue culturing was done using either inverted Olympus microscope or upright Zeiss microscope.

RESULTS AND DISCUSSION

The primary goal in developing a microfluidic tissue culture system has been to enable culturing of brain tissue slices for a sufficiently long period (up to 3 weeks) to facilitate studies on integration of neuronal stem cells into brain tissue and differentiation into dopaminergic neurons. To provide possibility for time-lapse microscopic imaging, the system has to possess optimal optical properties and function independent of a CO_2 incubator. To achieve this, the transparent and sufficiently gas impermeable PMMA was chosen as the substrate for fabrication.

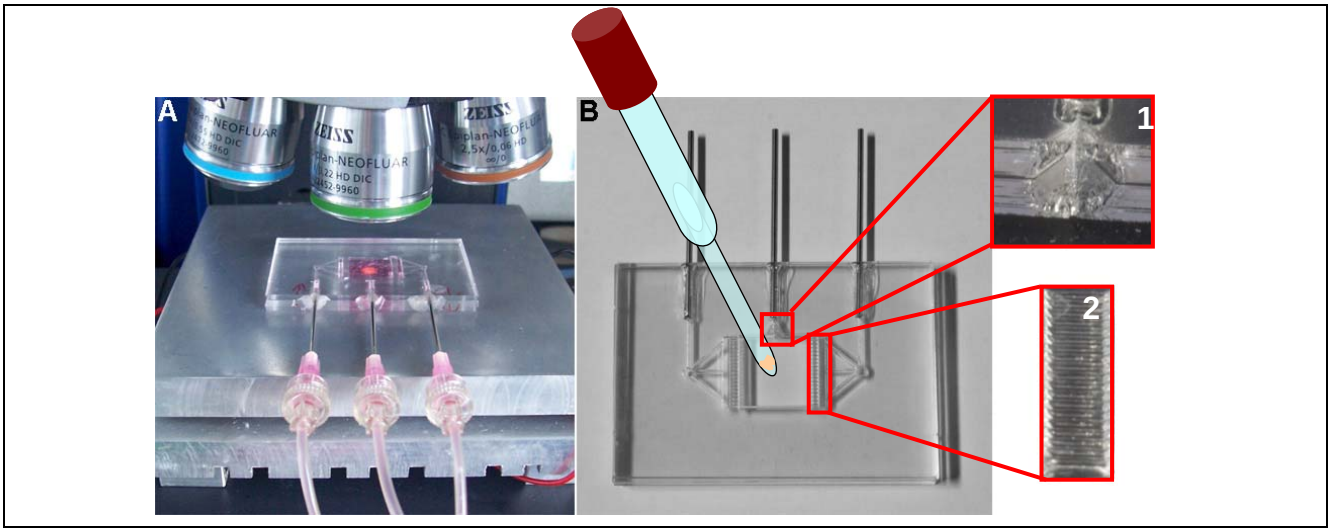

Figure 1: (A) A single-chamber brain slice culture system designed for culturing on a microscope stage having a Peltier heater. B) A schematic view of tissue loading into the culture chamber and magnification of the chamber design with a funnel-shaped channel (inset 1) and flow equalizers (inset 2).

Figure. 1A shows a single-chamber culture system on a Peltier heater installed on the stage of an upright Zeiss microscope to provide temperature control during culturing. Figure 1B shows a magnified view of the system consisting of 3 pieces of 1.5 mm thick PMMA sheets machined using laser ablation and having dedicated fluidic connections. The system is a modified version of a previously presented microfluidic cell culture system [9], having "flow equalizers" (inset 2, Figure

1B) to provide an equal lateral flow throughout the chamber and an optimized chamber height to eliminate shear stress on the growing tissue. Figure 1B also shows a schematic view of tissue loading using a large-bore pipette. Since a tissue culture chamber cannot be completely filled during tissue loading, air is occluded in the chamber when it is closed using a PMMA window. To ensure proper microfluidic performance without problems with bubbles, the occluded air has to be easily and effectively removed. This can be achieved through the funnel-shaped channel in the middle of the chamber (inset 1, Figure 1B), which after removal of air can be closed using the connected 3-way valve. Figure 2 shows microscopic images of a hemispherical brain slice cultured on microscope stage under perfusion for 20 h (A) and for 6 days (B). The shown images were acquired using an inverted microscope to obtain to obtain better optical quality. Although the system could successfully sustain tissue culture for 6 days, further development was necessary in order to improve imaging using an upright microscope which is necessary for real-time imaging on a Peltier heater which is not transparent from underneath.

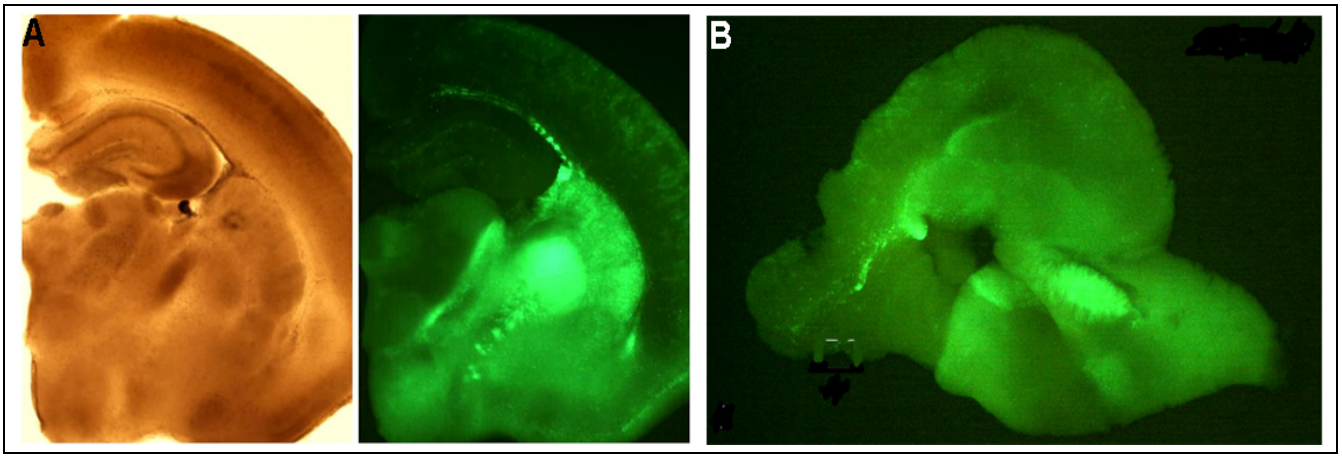

Figure 2. Microscopic images of a hemispherical brain slice from P8 transgenic GAD-GFP mouse cultured in the system shown in figure 1 for A) 20 h (left panel: bright field; right panel: GFP fluorescence) and B) 6 days (GFP fluorescence). Imaging was done using an inverted Olympus microscope.

Figure 3A shows the result of further development featuring a microfluidic system consisting of 3 pieces of micromilled 1 mm PMMA sheets to shorten the optical path through the culture medium. In order to simplify both fabrication and operations the system features a fluidic connection board to facilitate a "plug-and-play" assembly for experiments. In this format, the microfluidic chips can also accommodate several culture chambers to handle parallel tissue cultures. Fluid tight connection of the microfluidic system to the fluidic connection board is facilitated using PDMS O-rings (inset 1, Figure 3A). Since the O-rings are fully embedded in the PMMA structures, the gas permeability of PDMS does not influence the function of the system. Since the structure of the flow equalizers shown in figure 1 requires a sufficiently thick PMMA substrate, the system shown in figure 3A is equipped with a new type of flow equalizers (inset 2), which also simplify the fabrication process. Tissue loading in the chambers is still done as shown in figure 1B but the geometry of the channel used to remove the occluded air is slightly altered (inset 3). The structural features of this system, described above, require increased fabricational accuracy, which necessitates the usage of micromilling as the fabrication technique instead of laser ablation. This system was used to culture hippocampal tissue slices for 16 days. An upright fluorescence microscopic image of a tissue slice after 16-day culture period is shown in figure 3B. Despite the fact that, the system showed improved optical quality due to the shorter optical path and optimal microfluidic function for handling of tissue culture, onset of local necrosis was observed.

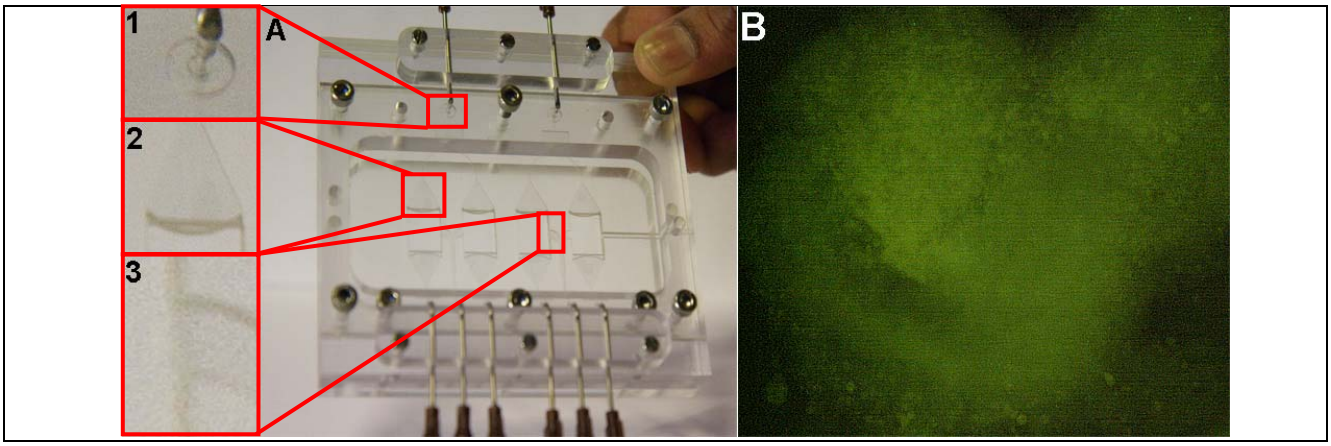

Figure 3. A) A multichamber brain slice culture system (magnification of a flow equalizer (inset 2) and funnel-shaped channel (inset 3)) with a fluidic connection board interfaced to the microfluidic chip via PDMS O-rings (inset 1). B) A GFP fluorescence image of a hippocampal brain slice from a P8 transgenic GAD-GFP mouse after 16 days of culture. Imaging was done using an upright Zeiss microscope.

CONCLUSION

We have developed a brain slice culture system for facilitating long-term culturing on a microscope stage to perform real-time microscopic imaging during studies on integration of neuronal stem cells into brain slices. The system has sustained culturing for 16 days; however, to further extend the culture time to 3 weeks and avoid the observed onset of local necrosis, further optimization of conditions, such as the flow rate and initial tissue handling, is being conducted. The versatility of the developed "plug-and-play" type system provides the basis for further work toward stem cell integration in brain tissue.

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