Dual-compartment microfluidic device for neuronal co-cultures: Design, implementation and validation
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Chapter 4

In Vitro Cortical-Thalamic Co-cultures

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In this paper, we propose an experimental approach to develop an in vitro dissociated cortical-thalamic co-culture model using a dual compartment neurofluidic device. The device has two compartments separated by 10 μm wide and 3 μm high microchannels. The microchannels provide a physical isolation of neurons allowing only neurites to grow between the compartments. Long-term viable co-culture was maintained in the compartmented device, neurite growth through the microchannels was verified using immunofluorescence staining, and electrophysiological recordings from the co-culture system was investigated. Preliminary analysis of spontaneous activities from the co-culture shows a distinctly different firing pattern associated with cultures of individual cell types and further analysis is proposed for a deeper understanding of the dynamics involved in the network connectivity in such a co-culture system.
4.1 Introduction

We aim to develop an in vitro neuronal network system to provide insights into the interactions between the cortex and the thalamus. We believe this will help understand the connectivity pathways involved in the pathological neurodegenerative conditions and their treatment modalities. Until recently, in vivo studies have provided the necessary tools to understand the thalamo-cortical interactions (Nicolelis, 2005). However, in vivo studies are often limited by the complexities in simultaneous multiple site recordings and by the influence of other regions of the brain in the cortical-thalamic circuitry. Developing an in vitro dissociated co-culture model with cortical and thalamic cells alone may provide tools to circumvent these issues. Microsystem technologies offer unique possibilities in developing such in vitro models; this technology allows easy accessibility and manipulation capabilities that are not available in traditional in vivo studies. However, developing dissociated cortical-thalamic co-culture systems poses serious challenges related to the maintenance of a long term thalamic cell culture in vitro. Survival of thalamic cells in isolation is not easily achievable and it is attributed to the absence of external influences like the absence of cortical signals with specific developmental properties (Magowan and Price, 1996; Asavaritikrai et al., 2003). The influence of cortical cues in the development of thalamo-cortical connectivity or in the remodelling of networks following environmental modifications was demonstrated by Coronas et al. (Coronas et al., 2000). Asavaritikrai et al. has shown that thalamic cells require trophic support from cortex for their survival (Asavaritikrai et al., 2003). Considering these challenges, we propose to develop an in vitro co-culture system that can support long-term dissociated thalamic cells in combination with cortical cells.

In this work, we discuss culturing cortical and thalamic cells in a dual compartment neurofluidic device with microchannels connecting the two compartments. The microchannels provide the necessary access paths for neurites to cross-over to the adjacent compartment. The neurofluidic device is integrated on a planar micro-electrode array (MEA) to facilitate electrophysiological measurements from the co-culture system. Neurite growth through the microchannels connecting the compartments is observed and the structural connectivity between the cell types was verified using immunofluorescence selective staining of cortical and thalamic compartments. Preliminary results of electrophysiological recordings (figure 4.1A) from the compartmented cultures are presented. Analysis of the spontaneous activities of the co-cultures shows a distinctively different firing pattern associated with cultures of individual cell types.
4.2 MATERIALS AND METHODS

NEUROFLUIDIC DEVICE

The 3-mm-thick polydimethylsiloxane (PDMS) devices used for this study have 2 microfluidic compartments (figure 4.1D) of 100 \( \mu \text{m} \) height and 8 mm length interconnected with microchannels of 10 \( \mu \text{m} \) height, 3 \( \mu \text{m} \) width and 150 \( \mu \text{m} \) length that are spaced at regular intervals of 50 \( \mu \text{m} \) (Taylor et al., 2003). The small cross-section of the microchannels prevent the movement of cells between compartments while allowing neurites to cross-over to the adjacent compartment and form functional network (Kanagasabapathi et al., 2010; Kanagasabapathi et al., in press). The compartmented PDMS device (figure 4.1D) is reversibly bonded to Multi electrode array (MEA) substrates (Multichannel Systems, Germany) with 60 electrodes in an 8 x 8 matrix (without four corner electrodes) and the compartments are aligned to include 30 electrodes each. 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, US) at a concentration of 40 \( \mu \text{g/ml} \) and rinsed thoroughly in sterile water (GIBCO, Invitrogen, US).

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 and thalamic neurons are prepared by Trypsine (GIBCO, Invitrogen, USA) digestion of day-18 embryonic rat whole cortices and ventral basal thalamus. The dissociated cortical cells are cultured in Neurobasal medium (Lonza lifesciences, USA) and the dissociated thalamic cells are cultured in the same medium supplemented with an additional 3% Fetal bovine serum (FBS) and 1% Horse serum (HS). The cultured cells are platted on PEI coated MEAs substrates at a concentration of ~ 2 x 10^5 cells / cm^2. The plating occurs by injecting the cell suspension from one reservoir of each compartment. The devices are then incubated in a humidified incubator at 37 °C supplied with 5% CO\textsubscript{2}. Neurite growth in the co-culture is checked at regular intervals and the neurobasal medium is replaced by freshly prepared medium on days-in-vitro (DIV) 4, 7, 9, 11, etc., The presence of serum in the culture medium for thalamic cells is maintained for at least (DIV) 5 days in culture, then progressively reduced and finally completely eliminated after DIV 9 to avoid the glia overgrowth during long term culture (more than DIV 15).
IMMUNOFLOUORESCENCE IMAGING

Cultured neurons in the dual compartments are fixated for 20 min. at room temperature with 4%wt/vol paraformaldehyde in phosphate buffer solution (PBS) and permeabilized for 10 min. with 0.1% TritonX-100. Blocking reaction is performed with a buffer containing 3%BSA, 2%FBS, 0.05% Triton-X 100 for 90 min. at room temperature. Cultures are then washed two times with PBS and incubated with primary antibodies towards NeuN (Chemicon Millipore MAB377 1:300 dilution) and anti Neurofilaments-200Kd (Sigma aldrich N4142 1:300) followed by secondary antibodies of Goat antimouse conjugated to Alexafluor546 and Goat anti-rabbit conjugated to Alexafluor 488 (Invitrogen Molecular Probes). Staining of NeuN antibody is primarily found within the nucleus of post-mitotic neurons (Mullen et al. 1992) while Neurofilament-200Kd shows strong staining of neural processes.

RECORDING SETUP

The spontaneous activity from the co-culture was recorded using a standard MEA1060 system [MEA 1060-Inv-Standard amplifier, Multichannel Systems, Germany]. A sterile recording box is custom built with a 90% relative humidity and 5% CO\textsubscript{2} supply. Electrophysiological recordings are analyzed using the Spycode toolset (Bologna et al.).

SPIKE DETECTION

The spontaneous spiking within the culture is detected using a threshold based ‘Precise Timing Spike Detection’ (PTSD) algorithm (Maccione et al., 2009). In brief, the threshold is computed according to the standard deviation (i.e. n-times SD) of the biological and thermal noise of the signal and it is set independently for each channel (Chiappalone et al., 2003). The PTSD algorithm computes the Relative Maximum/Minimum (RMM) of the raw data signal and when the RMM is a Minimum, the algorithm looks for the nearest Maximum within the duration of a spike and vice versa. When the difference between the two peaks in RMM (differential value) exceeds the differential threshold (DT), the spike is identified and its timestamp is stored. To detect spikes, a differential threshold (DT) of 7 times the standard deviation of the noise, a peak lifetime period (PLP) of 2 ms and refractory period (RP) of 1 ms are used.
Figure 4.1: Electrophysiological measurements from Cortical-Thalamic co-culture in a dual compartment device. A: Spontaneous activity recordings from the 60 electrode MEA layout (red line shows the separation between the two compartments); B: Tonic firing pattern of thalamic cells; C: Typical bursting behavior from cortical cells; D: Schematic layout of dual compartment device design used for neuronal cell culture; I and II – compartments for cell culture (width = 1.5 mm, height = 100 μm, length = 8 mm), III – Microchannels (width = 10 μm, height = 3 μm), IV- Reservoirs for neurobasal medium (diameter = 6 mm).
4.2 Materials and Methods

Figure 4.2: A: Cells plated in a dual compartment device; B: Immunofluorescence image of a cortical-thalamic co-culture confirms the isolation of cells within their respective compartments and bidirectional cross-over of neurites to the adjacent compartment.

Figure 4.3: Raster plot of spontaneous activities recorded from a cortical-thalamic co-culture on DIV 18. The spontaneous activity in the cortical compartment predominantly exhibits synchronized network burst events while thalamic cells exhibit tonic spiking behavior during early stages of their development period (Horizontal dark blue lines in the thalamic compartment represent densely firing tonic spiking electrodes).
MEA devices are 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 are then rinsed in DI water overnight and prepared as described earlier. Freshly prepared sets of PDMS devices are used for each experiment.

4.3 RESULTS AND DISCUSSION

VIABLE CO-CULTURE IN DUAL COMPARTMENT DEVICES

Cell viability and neurite growth through the microchannels are observed at regular intervals over the developmental period. A sample cell culture in a dual compartment device is shown in figure 4.2A. Each reservoir, with a volume of ~60 μl contains enough nutrient supply for the culture. However, for a long term culture, the cell culture medium needs to be changed at regular interval to maintain a balanced supply of nutrients to the cell culture and at the same time, to avoid excess removal of neurotrophic growth factors secreted by the cells. We performed experiments with different medium change frequencies over the developmental period and found that a medium change frequency of three times per week offered the most favorable results thus far resulting in active co-cultures until DIV 35.

NEURITE GROWTH THROUGH MICROCHANNELS

Visual observation of the culture over the developmental period indicates neurite growth across the compartment from DIV 3. Neurites are 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 confirms the physical containment effected by the microchannels (data not shown). To further substantiate structural cross-compartment connectivity between the co-cultures by neuritic crossover, immunofluorescence staining of neurons in both compartments is performed. Neurites from the cortical compartment are stained with green fluorescent protein (GFP) and those from thalamic compartments are stained with DsRed. Figure 4.2B shows extensive neuritic arborization within the compartment of origin, as well as neurites crossing over to the other compartment through the microchannels.
**Electrophysiological recordings**

Electrophysiological recording of spontaneous activity from a co-culture on DIV 18 is shown in figure 4.1A. Compartments containing cortical cells (compartment I in figure 4.1A) are dominated by highly synchronized network burst events (figure 4.1C) while the compartment with thalamic cells (compartment II in figure 4.1A) exhibit tonic spiking (figure 4.1B). Spontaneous spikes in a co-culture are detected as explained earlier and the results are represented figure 4.3. The raster plot shows the activity recorded over 300 sec in a dual compartment, electrodes along the y-axis are numbered from 12 through 87 representing the corresponding rows and columns of the electrode layout (electrodes 12 through 48 are in cortical compartment, electrodes 51 through 87 are in thalamic compartment) and each dot represents the spikes (time bin = 1 sec) recorded by one of the MEA channels. Our preliminary observations indicate strong cortical burst events preceding much weaker thalamic bursts.

**4.4 Conclusion**

In this novel application, an experimental approach to develop an in vitro cortical-thalamic co-culture system utilizing a dual compartment neurofluidic system is demonstrated. Critical issues in maintaining a long term co-culture is discussed. An optimal medium change protocol is implemented that helps in supplying cells with fresh oxygen and supplements over the developmental period; The small cross-section of the microchannels prevents the migration of cells between compartments while allowing neurites to cross-over to the adjacent compartment and form functional network. With the microchannels offering the necessary physical isolation between the compartments, viable cell cultures are maintained for up to DIV 35. Electrophysiological recordings of the spontaneous activity confirm the viability of cells in the co-culture system. Good inter-compartment neurite growth and connectivity is observed, and bidirectional neurite cross-over to the adjacent compartment is verified using immunofluorescence imaging.

These results show that the system supports healthy neuronal cultures with good somatic separation. Although further analyses are required to compare with cultures of individual cell types and to understand the dynamics in signal propagation between cortical and thalamic regions, the preliminary results presented in this work may provide some fundamental blocks in understanding the interactions between the cortex and thalamus. Further, the dual compartment system demonstrated in this work offers the unique possibility that the neuronal circuitry being measured in the system is independent of external influences from other neuronal populations. This may provide a useful tool in studying the sub-circuitry of neuronal pathways in isolation.
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