Dual-compartment microfluidic device for neuronal co-cultures: Design, Implementation and validation
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Chapter 7

General Discussion
In this thesis, a dual-compartment device for co-culturing cells from two different brain regions and selective pharmacological manipulation of an individual sub-population in the co-culture is designed, implemented and validated. In the preceding chapters, functional connectivity between sub-populations and an experimental approach to selectively manipulate individual cell types using neuro-active chemical substances and their influence on signal propagation between the regions was presented.

We chose cortical and thalamic dissociated cells for the preparation owing to the interactions between these cell types in generating rhythmic neuronal network oscillations in vivo which, from a physiological point of view, play a significant role in fundamental functions such as epilepsy, sleep modulation, and sensor-motor information integration (Andolina et al., 2007; Iyengar et al., 2007; Crunelli et al., 2011). In vivo, strong reciprocal connections between the thalamus and cortex are responsible for generating rhythmic oscillatory neuronal network activity (Adams et al., 2011). An ability to reproduce such configuration in vitro using dissociated cell cultures may provide sufficient control of temporal and spatial characteristics of synchronized network wide burst activities and this may provide useful insights in sustained rhythmic oscillatory activity.

For the co-culture studies used in this work, we custom built a dual-compartment PDMS device that can be integrated in a commercial MEA. MEAs provide non-invasive extracellular stimulation and recording of cell soma, along with stimulating axons and dendrites present at the recording sites. We optimized device design by several iterations of compartment design, microchannels of different length between the compartments, and medium change protocols to establish long-term viability (>DIV30).

Dissociated cell cultures on MEAs generate large quantities of electrophysiological network wide spike response data for long-term studies. However, maintaining viable cultures for an extended period of time continues to be a challenge. Stable environmental conditions during the developmental phase and electrophysiological recording, avoiding pH and osmolarity shocks, providing nutrients and oxygen to the cells by having a continuous perfusion system with supplement enriched media are critical for long-term cell culture studies. Recent advances in microfluidics technology enable design and fabrication of a variety of microvalves and micropumps on-chip thereby providing a controlled fluidic environment for cell culture (Jeon et al., 2002; Eddings and Gale, 2006; Hosokawa et al., 2007; Nakanishi et al., 2008).

For studies involving interaction between sub-population of neurons, the dual compartment system offers several advantages compared to the conventional one
component dissociated cell culture systems, namely:

1. Capability to segregate and to establish functional connectivity between sub-populations of neurons in vitro

2. The sub-population of neurons being studied is in isolation from other brain regions enabling us to rule out the influence of other neuronal populations. Although this does not represent the three dimensional in vivo conditions with inter-connected regions, this technique can be highly beneficial for studies exploring interaction between two specific regions of the brain

3. Capability to screen individual pharmacological compounds on cell bodies and neurites in isolation and their subsequent influence in synaptic connectivity can be verified

4. In case of cortical-thalamic co-cultures, the system provides sufficient spatial sampling to record activities from both the regions simultaneously.

5. High experiment throughput from fewer animals (low brain tissue requirement), low volume of cell culture medium and pharmacological compounds, makes it an ideal platform for drug screening applications in primary cell types. From a pharmacological screening standpoint, the system may be used for studying interaction between two different homogeneous cell lines, thereby minimizing primary cell requirement.

6. Potential to integrate the PDMS device with high density MEAs with 128 to 1028 recording / stimulation channels thereby providing very high spatial sampling of cells in culture. Custom built MEA substrates with electrode arrays segregated into several regions may assist in expanding the system into three or more compartment system with functional connectivity between several cell types.

7. In addition, optical transparency of PDMS provides capabilities to integrate with optogenetic techniques. Inactive caged glutamate can be converted to active glutamate using UV lights to mimic synaptic inputs of single cells.

7.1 Dual-Compartment Device For Dissociated Neuron Cell Culture

Although, environmental conditions and dynamics of the nervous systems in an in vitro dissociated cell culture are significantly different from in vivo condition, in vitro systems allow viable cells that are electrophysiologically active, make synaptic connections and express their channels and receptors. Primary cultures of rat neurons express several aspects of nervous system function, including spontaneous
activity, plasticity, basic excitatory / inhibitory synaptic responses and comparable pharmacological sensitivity (Usher et al.; Dichter, 1978; Morefield et al., 2000; Marom and Shahaf, 2002; van Pelt et al., 2005; Pasquale et al., 2008).

Primary rat neuronal cultures are well characterized and dissociated neuronal cultures on MEA have been used as a preparation in many electrophysiological studies (Ramakers et al., 1991; Watanabe et al., 1996; DeMarse et al., 2001; Potter and DeMarse, 2001; Shahaf and Marom, 2001; Eytan et al., 2003; Novellino et al., 2003; Marom and Eytan, 2005; Ruaro et al., 2005; van Pelt et al., 2005; Gabay et al., 2007; Nakanishi et al., 2008). In addition, in vitro systems offer excellent accessibility in selectively manipulating single cells or group of cells. Recently, studies involving electrophysiological recording and stimulation of dissociated cells, pharmacological manipulation (Pancrazio et al., 1999; Morefield et al., 2000; Chiappalone et al., 2003; Stett et al., 2003; Beggs and Plenz, 2004; Wagenaar et al., 2005; Jun et al., 2007; Vajda et al., 2008) and effects of drugs such as ch-ABC in promoting axonal extensions in spinal injuries have been reported (Vahidi et al., 2008). Developing a compartmented system offers further possibilities in network electrophysiological studies and to assess the effects of pharmacological agents in signal propagation between different neuronal cell types. For instance, in co-culture studies involving cortex and thalamus, the role played by each of these two regions in initiation and propagation of oscillatory network activity is still not completely understood. Although in vivo electrophysiological studies offer an ideal brain condition to study the interaction dynamics, they are limited by relatively poor spatial sampling due to the limited number of simultaneous recording sites. In vitro slice studies offer a suitable alternative to in vivo approach – however, manipulating an individual region of the co-culture is often cumbersome. Optical stimulation with caged compounds or genetic methods to express photosensitive proteins in combination with Ca+ imaging may provide an alternative for electrophysiological studies. By gene expression of specific cell types, it is possible to limit optical stimulation to a selected sub-population of neurons in vivo and in vitro slice studies. It is possible to achieve an extremely sensitive temporal and spatial scale of a particular cell type without any indirect effects associated with the presence of other cell types. However, activating a whole circuit containing multiple cell types is cumbersome.

Developing a multi-compartment system with cells from different regions of the brain offers flexibility in manipulating individual cell types while simultaneously monitoring the circuitry. As a step towards that direction, we present a dual-compartment device for co-culturing dissociated cortical and thalamic cells. This technique offers high spatial resolution – providing electrodes to simultaneously record both cortical and
thalamic regions, while offering capabilities to selectively manipulate an individual region.

Dual compartment devices presented in this thesis is designed for co-culture studies of neuronal sub-populations. The device has two microfluidic compartments (width = 1.5 mm, height = 100 µm, length = 8 mm) connected by microchannels of very low aspect ratio (width = 10 µm and height = 3 µm, length = 150 µm). Low aspect ratio microchannels provide the necessary neuronal soma and fluidic isolation between the compartments while providing access path for neurites to cross-over. The small size of the microfluidic compartment is instrumental in creating capillary force between the inlet and outlet reservoirs. Cell culture is loaded in the compartment through capillary action and the laminar flow through the compartment allows for uniform distribution of cells in the compartment.

Compartmented systems similar to those presented here are often challenged by very low volume of medium available to the cell culture (Rhee et al., 2003; Dworak and Wheeler, 2009). Cells in the center of the compartment were observed to deteriorate from second week in culture (~DIV 10), by implementing a medium change protocol that helps in supplying cells with fresh oxygen and supplements over the developmental period, the viability of cells extended beyond DIV 30. Medium change of three times per week offered good culture stability and viable neurons with electrophysiological activities over DIV 30. However, medium change protocol reported in this work depends entirely on the volume of medium available in the reservoir. With larger reservoir it may be possible to have a lower medium change frequency or with a continuous perfusion system of very low volume flow rate, the cell viability in the compartment can be further increased. However, in our present work, since the PDMS devices were designed to suit the commercially available MEA setup, increasing the reservoir size to more than 6mm (in diameter) resulted in a low clearance space between the reservoir and device boundary and this often resulted in medium leakage to the recording electrode surface.

**Diffusion through microchannels**

By maintaining hydrostatic pressure difference between the compartments, diffusion through microchannels connecting the compartments was estimated to be ~ 20 hrs (Park et al., 2006). For studies performed in this work, pharmacological compounds were used for a very short duration (~ 30 minutes) and 3x wash cycle was performed at the end of each experiment. Further, to ensure complete fluidic isolation during the course of the experiment, a pressure difference was maintained between the compartments by increasing the level of medium in the reservoirs of the compartment without pharmacological insults.
7.2 Connectivity In Homogeneous Co-Culture

In chapter 2 and 3, viability of neurons with recorded electrophysiological activities over DIV 30 was presented. Immunofluorescence labeling of neurites demonstrate that the microchannels provided an access path for the neurites to propagate to the adjacent compartment and form a functional neuronal network. Extracellular recording of electrophysiological activities of neurons in culture show spontaneous spikes and synchronized network-wide bursts activities.

Simultaneous recording of activity in both compartments showed strong network cross-correlation revealing functional connectivity between two neuronal populations in the compartments.

Functional connectivity is evaluated by considering spike trains from all the recording sites. Correlation in spike train determines the level of connectivity in the system whether they are connected by either direct or indirect synaptic connections (Garofalo et al., 2009).

Statistical analysis using correlation reveals that the two neuronal populations are not only functionally connected to cells in the same compartment but also to cells in the other compartment.

To conclude, presence of genuine functional connectivity between cortical cells cultured in both compartments of dual-compartment microfluidic device was demonstrated. Based on the cross-correlation analysis, neurons within the same compartment have stronger functional connections than with neurons in the adjacent compartment. Moreover, inter compartmental functional connectivity did not show a statistically significant dependency on distance from the physical barrier, suggesting that well connected networks are formed despite the presence of the physical barrier of microchannels.

Cortical cells in compartments express similar connectivity dynamics and plasticity to those reported in dissociated cultures of cortical neurons (Marom and Shahaf, 2002; Bonifazi et al., 2005; van Pelt et al., 2005; Wagenaar et al., 2006a) and the results were also in agreement to those reported from intact cortex (Silberberg et al., 2004). In vivo, bursts are reported to occur during developmental phase and play a key role in establishing connections (Meister et al., 1991; Ben-Ari, 2001; Zhang and Poo, 2001; Leinekugel et al., 2002; Wagenaar et al., 2006b). In vitro, similar response can be observed at a simplified level of organization (Fields and Nelson, 1992; O’Donovan et al., 1998), cortical cultures start to fire stochastic spontaneous spikes from DIV 7 gradually maturing into network wide spike and organized population bursts by DIV 21 (Chiappalone et al., 2006). Synchronization in network wide burst
events was observed from third week in culture similar to those reported in literature (Kamioka et al., 1996; Chiappalone et al., 2006). Presence of synchronized network wide burst events was considered a sign of maturity in network (Wagenaar et al., 2005; Chiappalone et al., 2006; Wagenaar et al., 2006b).

In dual compartment devices with cortical cells, presence of microchannel barrier does not restrict signal propagation between compartments. Electrophysiological response of our homogeneous cortical-cortical co-culture model expresses similar evolution of spontaneous, network-wide spike and synchronized burst events in the culture. Investigating this spontaneous electrophysiological activity during development phase is an important factor in understanding the formation of functional neuronal circuits and their implications in network plasticity.

7.3 Network Dynamics in Cx-Th Co-Culture System

Cortical and thalamic cells in co-culture may provide a suitable platform to exclusively study the signal propagation between cortex and thalamus in isolation without influence from other brain regions (Avoli and Gloor, 1982; Danober et al., 1998; Andolina et al., 2007; Iyengar et al., 2007; Crunelli et al., 2011).

Organotypic co-cultures of cortex and thalamus slices extracted from P0-P3 rats to study and characterize the initiation and spreading of rhythmic network oscillations within these regions has been reported (Adams et al., 2011). Manipulating an individual region of organotypic co-culture is often cumbersome; a dual compartment device with dissociated cells may provide better capabilities to selectively expose an individual cell type to pharmacological agents. However, preparation and survival of dissociated thalamic cells in vitro in isolation is challenging. Thalamic cells cultured from the early stages of development (~ E15) survived only up to 5 days in vitro (Lotto and Price, 1995; Asavaritikrai et al., 2003) and the survival of thalamic cells from older prenatal (> E15) and early postnatal brains in isolation was not successful due to the absence of external influences such as the absence of cortical signals with specific developmental properties (Magowan and Price, 1996; Asavaritikrai et al., 2003).

Simultaneous electrophysiological recordings from both the compartments with cortical and thalamic cells during the early stages of development (between DIV 14 and DIV 21) shows that compartments containing cortical cells are dominated by highly synchronized network burst events (Chapter 1: section 1.4) while the compartment with thalamic cells exhibit tonic spiking (Chapter 4). However, later in the developmental period, spontaneous firing pattern of both cortical and thalamic
cells in the compartments has changed considerably. Synchronised network bursts were observed in the compartment with thalamic cells and they appeared to be time-locked to the cortical bursting phenomenon. Based on cross-correlation analysis of synchronized network bursts and burst time histogram (Chapter 5: Figure 5.6), time of initiation of burst events in cortical and thalamic compartment was estimated. Burst events originate in the cortical region and the presence of strong cortical connections drives the network and in-turn, the thalamic cells discharge network bursts. The results were in agreement to the earlier reported studies on cortical-thalamic co-culture slice models (Avoli and Gloor, 1982; Adams et al., 2011). In the cortical compartment, instantaneous firing rate (IFR) distribution of cortical cells was significantly different from those cortical controls cultured in isolation. Further, burst patterns in the cortical compartment appear to be highly influenced by the presence of thalamic population. The duration of cortical bursts in the co-culture is elongated with an average burst duration of $389 \pm 24$ ms (mean ± standard error), when compared to the cortical bursts in isolation - with an average duration of $246 \pm 4$ ms, ($p < 0.01$, Kruskal-Wallis nonparametric test). Reciprocal connections between the cortical and thalamic region were observed to play a relevant role in cortical behavior by modulating the duration and shape of burst events. Prolonged synchronized bursting pattern was observed in the cortical culture and the duration of cortical bursts were elongated by up to 57%.

7.4 Selective Pharmacological Manipulation

Chemically induced changes in the network behavior are often a reflection of the effects on pre- and post-synaptic activity in the network (Foy et al.). Experiments presented here, demonstrate how controlling activity in one portion of a network through manipulating synaptic neurotransmission can influence the global activity of the entire network. Understanding the mechanism of change in such activity patterns may provide explanations to the transitions occurring during oscillatory rhythms underlying many physiological states (Blumenfeld and McCormick, 2000).

Preliminary analysis of spontaneous activities from the co-culture showed a distinctively different firing pattern associated with cortical and thalamic cell types. During early stages of development in vitro (i.e. DIV 14), burst events were observed in cortical region while distinct tonic firing patterns were observed from the thalamic compartment. The presence of distinct firing patterns of individual cell types in vitro demonstrates that the dissociated cells in culture retain their individual phenotype. Functional connectivity is re-established in a sub-population of thalamic and cortical dissociated cells indicating a natural tendency of cortex and thalamus to
form reciprocal interconnections.

Propagation of electrical activities between cortical and thalamic regions and their inter-dependency in connectivity during later stages in development (DIV 21) was observed. Burst events originated in the cortical region and drive the entire cortical-thalamic network bursting behavior while mutually weak thalamic connections played a vital role in sustaining longer burst events in cortical cells. By selectively blocking glutamatergic and gabaergic receptors in cortical regions, propagation of synchronous bursts can be blocked. Synaptic receptor antagonist (SRA) solutions, a cocktail of 50 µM bicuculline methiodide (BMI) to block GABA receptors, 100 µM of 2-amino-5-phosphonovaleric acid (APV) to block NMDA receptors and 100 µM of 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) to block AMPA receptors in cell culture medium at 37˚ C was used to block network wide burst propagation in the cortical region.

We demonstrated that by controlling the origin of network bursts in a co-culture system (i.e. the cortical region) it is possible to control the spread of bursting activity to the thalamic region.

**Dominant region in a homogeneous co-culture system**

In an in vitro culture of cortical cells, whether one or more pacemaker type cells trigger synchronized network spikes and bursts is of interest in understanding the dynamics in network firing. Attempts have been made to localize the region of burst initiation within a network of neurons (Eckmann et al., 2008). In a dissociated cortical culture, an intrinsic pacemaker type mechanism to account for the random spatial features of origin and propagation of population bursts (Maeda et al., 1995) and to explain correlations between collective activity and plasticity changes in synaptic efficacy (Canepari et al., 1997; Jimbo and Robinson, 2000) has been reported. An important question is whether the intrinsic spiking cells are indeed capable of recruiting enough cells to start network bursts. For the experiments reported in Chapter 6, SRA was used for blocking the majority of synaptic transmission. Applying SRA in the burst initiating compartment had a significant influence on the behavior of the network wide burst response in both compartments. However, applying SRA in burst following compartment did not have an impact on the other compartment. Controlling spontaneous synchronized network bursting of burst following compartment had only a marginal effect on both the compartments.

In both cases, synchronized network bursts continued to exist in certain clusters of the electrodes and this activity is attributed to the intrinsic spiking neurons.
In conclusion, the results demonstrate that several cells in a cortical cell culture in vitro are capable of intrinsic spiking. However, a particular compartment acts as the region of burst initiation and the choice of the specific compartment may depend on the development of the network, the extent of synaptic arborization within the individual compartments and to a large extent on the strength of functional connections within each region (Maeda et al., 1995).

**Synchronized network burst propagation in Cx-Th co-cultures**

In dissociated Cx-Th co-cultures, cortical origin of synchronized network bursts was experimentally verified with selective chemical manipulation of individual compartment as reported in the previous section. Although propagation and distribution of synchronized network bursts in the culture was determined by the region of burst initiation, spontaneous firing of the network does not show hierarchy (Adams et al., 2011).

Based on the results presented in the preceding chapters, dual compartment devices offer a valuable platform to systematically approach questions related to the study of neuronal sub-populations. In case of cortical-thalamic region in vivo, strong reciprocal connections between the thalamus and cortex were observed to be responsible for generating rhythmic oscillatory neuronal network activity (Adams et al., 2011). In organotypic co-cultures in vitro, seizure-like rhythms were observed to originate in the deep layers of cortex (Adams et al., 2011). However, specific role played by each of these regions in initiation and propagation of pathophysiological oscillatory network activity is still unclear. In vivo electrophysiological analysis suggests that network wide synchronized burst events are initiated in cortex region and secondarily engage the thalamus (Meeren et al., 2002; Pinault, 2003; Polack et al., 2007). In the simplified in vitro cortical-thalamic co-culture system presented in this work, the results support a functional model where cortical bursts recruit the thalamic region. The results from our studies demonstrate that sustained network burst events were initiated in the cortical region and secondarily spread to involve the thalamus region. This may provide insights into the evolution of thalamocortical network activity in vivo, and inter-relationship between the cortex and the thalamus.
7.5 Perspectives Of Microfluidic Compartment Technology And Implications For Neuroscience Research

MEA technology is slowly applied in pharmacological research to streamline drug screening experiments. Traditionally, neurobiological drug discovery programs involve an early compound screening to an advanced in vivo studies.

To compliment the early drug screening studies of in vitro organotypic slices, compartmented MEA systems can be used to study the drug effects on a network level and the results may be correlated to in vivo studies to better understand the neurophysiological effects.

At molecular level, the influence of specific growth factor on the response of cell signaling can be examined with compartmented system. Major drawback of current technologies available for compartmentalizing cells is that although cells are segregated into separate regions either through surface patterning (Ruardij et al., 2000; Sorkin et al., 2006; Massobrio and Martinoia, 2008) or through physical compartments (Berdondini et al., 2006), they still share the same cell culture medium. However, to understand the importance and the effect of cell level changes that causes this effect, it is also essential to study the influence of growth factors secreted by a particular cell types. Using compartmented system presented in this thesis, complete fluidic isolation between the compartments can be achieved and the culture medium from individual cell types can be collected for examining the changes in the secreted growth factors with or without external environmental cues.

In studies related to spinal injuries, when damaged and regenerating axons needs to be exposed to inhibitory environment in isolation from the cell bodies, direct effects of growth inhibitor molecules in neuronal development and regeneration can be investigated at the cellular and molecular level. Conventional systems with random plating of cells do not resemble the microenvironment of spinal injuries, where the cells are often located away from the region of injury (Neumann et al., 2002). Dual compartment devices, on the other hand, can be used for such studies by platting cells in one compartment and exposing the neurite projection that extends to the adjacent compartment to an inhibitory environment (Taylor et al., 2010). This technique of organizing cells in a dual compartment system is advantageous because it permits separation of cell bodies from the neurites, resembling the segmental structure of the spinal cord where the cell bodies are located away from the descending projections that are faced with the inhibitory environment.

Our approach to culturing cells in restricted compartments with complete fluidic isolation between the cell types while allowing neurites to extend synaptic
connections to the cells in adjacent compartment distinguishes this method from other methods of cell segregation reported in literature (Davenport et al., 1996; Berdondini et al., 2006; Mourzina et al., 2006). We showed that while cell bodies were uniformly platted in the compartment, the neurites extends to adjacent compartment in both directions and forms a bi-directional connectivity. We demonstrated the capability of this new in vitro cell culture system by culturing cortical and thalamic neurons in compartments and establishing functional connectivity between them. We demonstrated that functional connectivity is re-established in a sub-population of thalamic and cortical dissociated cells indicating a natural tendency of cortex and thalamus to form reciprocal interconnections. As a proof of concept to demonstrate fluidic isolation between compartments and to selectively manipulate an individual cell type, we used SRA to suppress electrophysiological burst activity of cells in one compartment.

Compartmented system for neuronal network studies

The compartment system can be further extended to a three compartment system (figure 7.1), with the possibility of inducing directionality in connectivity. Cells can be plated with time delay in each compartment thereby directing specificity in neurite growth. For instance, a Cx-Th-Cx culture can be studied in the three compartment schematic shown in Figure 6.1. With Cx cells in compartment 1 (C1), Th cells in compartment 2 (C2) can be plated with a time delay (i.e. 48 hrs) followed by

![Figure 7.1: Proposed three compartment system with cells in mutual communication with each other.](image)
7.5 Perspectives Of Microfluidic Compartment Technology

Cx cells in compartment 3 (C3) with a longer time delay (i.e. DIV 7). Considering the time required for axons to proliferate into other compartments, this system will provide a unique Cx -> Th -> Cx -> Cx connectivity pattern. The influence of cortical cues on thalamic action potential and the reciprocal weak and strong connectivity on the Cx cells on either of the other compartment can be studied. The system can be combined with electrical or optical stimulation modules to selectively manipulate signal propagation between compartments. Such unique combination of cell types in vitro and control of directionality in connectivity can be achieved with compartmented technology. Compartmented devices may also be used for culturing cells from different regions of the brain including cortical, thalamus and basal ganglia. Although the three dimensional in vivo brain conditions cannot be replicated in such an in vitro model, the system does represent the essential connectivity. Understanding communication pathways between different regions of the brain and their response to external cues is of significant interest for understanding the mechanism behind many pathophysiological conditions such as epilepsy, schizophrenia and Parkinson’s disease.
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