Dual-compartment microfluidic device for neuronal co-cultures: Design, implementation and validation
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Chapter 6

Selective Pharmacological Manipulation of Cortical-Thalamic Co-cultures

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ABSTRACT

In this study, we demonstrate capabilities to selectively manipulate dissociated co-cultures of neurons plated in dual-compartment devices while allowing simultaneous multi-site electrophysiological recordings. Synaptic receptor antagonists and tetrodotoxin solutions were used to selectively control and study the network-wide burst propagation and cell firing in Cortical-Cortical and Cortical-Thalamic co-culture systems. The results show that in cortical-thalamic dissociated co-cultures, burst events initiate in the cortical region and propagate to the thalamic region and the burst events in thalamic region can be controlled by blocking the synaptic receptors in the cortical region. Whereas, in Cortical-Cortical co-culture system, one of the region acts as a site of burst initiation and facilitate propagation of bursts in the entire network. Tetrodotoxin, a sodium channel blocker, when applied to either of the regions blocks the firing of neurons in that particular region with significant influence on the firing of neurons in the other region. The results demonstrate selective pharmacological manipulation capabilities of co-cultures in a dual compartment device and helps understand the effects of neuroactive compounds on networks derived from specific CNS tissues and the dynamic interaction between them.
6.1 Introduction

A microelectrode array (MEA) based recording system is useful for detecting and analyzing the network-wide dynamics of cultured neurons (Kamioka et al., 1996; Jimbo and Robinson, 2000; Wagenaar et al., 2006). Coupled with compartmentalization technique, the system can be used for studying neuronal sub-population and may provide an in vitro model system to study interactions between cell types (Davenport et al., 1996; Berdondini et al., 2006; Dworak and Wheeler, 2009; Gao et al., 2011; Hosmane et al., 2011; Majumdar et al., 2011). Compartmentalization and fluidic separation of dissociated cultures of neurons in a novel PDMS microfabricated device was demonstrated earlier (Taylor et al., 2003; Park et al., 2006). Recently, co-cultures of two different types of neuron have been reported (Takayama et al.; Majumdar et al., 2011). In our earlier work, we demonstrated co-culturing cortical and thalamic cells in a dual compartment system coupled to MEA and characterized interaction between the cell types (Kanagasabapathi et al., 2012). Interaction between cortex and thalamus are responsible for generating rhythmic neuronal network oscillations and plays a significant role in fundamental functions such as sleep modulation (Andolina et al., 2007; Iyengar et al., 2007; Crunelli et al., 2011). In vivo, interactions between cortical and thalamic regions have been widely studied (Miller, 1996; Nicolelis, 2005; Briggs and Usrey, 2008) and studies have shown that seizure like network-wide activities originate in the cortex and spreads to the thalamus (Pinault, 2003). In a recent work, Adams and co-workers (Adams et al., 2011) demonstrated co-culturing organotypic cortex and thalamus slices to characterize the initiation and spreading of oscillations within thalamus and cortex. However, both in organotypic slice studies and in vivo approaches it is often cumbersome to manipulate a specific region of interest pharmacologically to study the interaction dynamics of the cell types in isolated conditions. In vitro co-culture system utilizing dissociated cells in compartmented devices with microfluidic separation may provide sufficient flexibility in selectively manipulating an individual region.

In this study, we demonstrate a dual compartment system that permits selective pharmacological manipulation of co-cultures in vitro while recording the electrophysiological activity, simultaneously, from two cultures. The effect of neuroactive compounds such as Synaptic receptor antagonist (SRA) and Tetrodotoxin (TTX) on networks derived from specific CNS tissues and the dynamic interaction between the specific cell types was examined. A hierarchical behavior in network-wide burst response was observed both in cortical-cortical and cortical-thalamic co-cultures. We report that in cortical-cortical co-cultures, a particular compartment acts as a network-wide burst leading compartment, and plays a major role in regulating the
network bursting of both compartments. While in Cortical-Thalamic co-cultures, the compartment with cortical cells always acts as a network burst leading compartment, controlling the spread of network bursts to the thalamic compartment.

6.2 Materials and Methods

Dual-compartment device and primary co-culture preparation

Dual compartment polydimethylsiloxane (PDMS) devices used for this study have 2 microfluidic compartments (Compartment A and compartment B as shown in figure 6.1A) of 100 μm height and 8 mm length interconnected with microchannels of 10 μm width, 3 μm height and 150 μm length that are spaced at regular intervals of 50 μm. The small cross-section of the microchannels prevent the movement of cells between compartments while providing sufficient access path for neurites to cross-over (Kanagasabapathi et al., 2011a). As per the approved protocol for the care and use of lab animals in The Netherlands, primary cultures of embryonic (E18) Wistar rat cortical-cortical co-cultures (i.e. with cortical cells in both compartment A and compartment B of the dual-compartment device) and cortical-thalamic co-cultures (i.e. with cortical cells in compartment A and thalamic cells in compartment B of the dual-compartment device) were prepared as reported in our earlier work (Kanagasabapathi et al., 2011b). Prior to plating the cells, the MEA substrates were coated overnight with a solution of Polyethylenimine (PEI) (Sigma-Aldrich, US) at a concentration of 40 μg/ml and rinsed thoroughly in sterile water (GIBCO, Invitrogen, US). PEI coating is used to promote stronger attachment of cells to the surface (Lelong et al., 1992; Vancha et al., 2004). PEI, a positively charged organic polymer, attracts negatively charged outer surface of cells to the coated surface. Experiments in the absence of coating agent showed a characteristic tendency of cells to form heterogeneous clusters in the compartment. The cells are firmly attached among themselves into many clusters, but very weakly attached to the MEA surface. This resulted in significant loss of cells during medium change and wash cycles. Further, during development phase, the strong force exerted by neurites growing between the clusters, tend to move the cell clusters towards the center of the compartment with very few cells remaining close to the microchannels. Pretreatment of MEAs with PEI resulted in a homogeneous distribution of cells in the compartment, with cells attaching firmly to the surface while showing very low tendency to clustering. Primary cortical and thalamic cells were plated at a density of ~ 2 x 10^5 cells/cm² and the cultures were stored in an incubator with 5% CO₂ to air mixture and ~ 100% relative humidity at 37 °C. Co-cultures are refreshed thrice a week starting from days-in-vitro (DIV) 4, the cells in cortical compartment were refreshed with
Lonza neurobasal medium (Lonza Ltd., Ch), while those in thalamic compartment were refreshed with the neurobasal medium supplemented with 3% fetal-calf serum (GIBCO, Invitrogen, US) and 1% horse serum (GIBCO, Invitrogen, US). The serum concentration in thalamic compartment is progressively reduced during medium change until DIV 9 and completely removed on DIV 11 to avoid glia overgrowth during long term culture (more than DIV 15). Medium was refreshed by completely removing the medium from all four reservoirs except the two compartments and by adding ~80 µl of freshly prepared medium to one reservoir per compartment. The osmotic pressure difference between the reservoirs connected to the compartment resulted in medium refreshing within the compartment due to capillary force. Once the level of medium in the two reservoirs connected to a compartment saturates, an additional ~40 µl of medium was added to compensate for the initial volume removed. The chosen medium refreshing frequency offered optimal cell survival thus far resulting in active cultures up to DIV 35.

**Transfection Imaging**

Cultures were transfected with a thy1-eGFP construct in one compartment, using lipofectamine 2000 (Invitrogen, CA, USA). 1 mg DNA was mixed with 100 ml neurobasal medium and mixed with 3 mg lipofectamine 2000 diluted in 100 ml neurobasal medium (without supplement), and left at room temperature for 20 minutes. After diluting the transfection mixture with four volumes of neurobasal medium with supplement, it was gently flushed into one of the compartments. Images of transfected neurons (typically only a few neurons were stained per compartment) were recorded after fixation of the cultures with 4% paraformaldehyde in phosphate buffered saline at DIV 18 to 21.

**Electrophysiological Measurements and Signal Analysis**

Dual compartment PDMS device was bonded reversibly on planar Microelectrode Arrays (MEA) with 60 electrodes (i.e. 30 electrodes per compartment) of 30 µm diameter and 200 µm spacing placed in an 8 x 8 array structure (Multichannel Systems, Germany) (Kanagasabapathi et al., 2011a) and the spontaneous network activity of the cultures was recorded using a standard MEA1060 system (MEA 1060 Inv-Standard amplifier, Multichannel Systems, Germany). Spontaneous electrical activity was recorded twice per week starting from DIV 14 and the electrophysiological signals were sampled at a frequency of 25 kHz with an internal band pass filter of 10 Hz – 3 kHz. Raw data of single channel bursts from a sample electrode in cortical and thalamic compartment of a Cx-Th co-culture is shown in figure 6.1B.
Figure 6.1: Dual compartment device used for the co-culture studies. (A) – Schematic layout of a PDMS dual compartment device used for culturing Cx-Cx and Cx-Th co-cultures; (B) – Raw data from four sample electrodes of a cortical-thalamic co-culture system with synchronized single channel burst events; (C) – Transfection image of a single neuron and associated neurite crossover through the microchannels connecting the compartments.
6.2 Materials and Methods

Figure 6.2: Network burst detection and burst initiation in a dual compartment system.
(A) - Raster plot of a sample device with network bursts identified in both the compartments (X-axis represents the recording time and on y-axis, electrode index 1 through 30 belongs to compartment A and electrode index 31 through 60 belongs to compartment B); Line 61 (‘+’ in blue) represents the network bursts (NB) detected in compartment A and line 62 (‘*’ in red) represents the network bursts detected in compartment B; (B) – Burst initiation histogram of a sample Cx-Cx co-culture device with burst initiation in compartment B (n=9 devices); (C) – Burst initiation histogram of a sample Cx-Cx co-culture device with burst initiation in compartment A (n=6 devices); (D) – Burst initiation histogram of a sample Cx-Th co-culture device with burst initiation in cortical compartment (compartment A) (n=21 devices).
Spike and Burst detection

To detect spikes from individual electrode, a Precise Timing Spike Detection (PTSD) algorithm as demonstrated by Maccione et al. was used (Maccione et al., 2009). The algorithm depends on the differential threshold (DT), peak lifetime period (PLP) and the refractory period (RP) of a spike. The amplitude based threshold is set manually for each channel based on the standard deviation of the biological and thermal noise of the signal. The peak lifetime and the refractory period refer to the duration of a spike and the minimum interval between two consecutive spike events respectively.

PTSD computes the Relative Maximum/Minimum (RMM) of the raw data signal. When the RMM is Minimum, the algorithm looks for the nearest Maximum within the PLP window, and vice versa. If the difference between the two found RMM (differential value) is greater than DT, the spike is identified and its timestamp is stored.

Based on the previously established methods (Chiappalone et al., 2005; van Pelt et al., 2005), an algorithm for burst and network burst detection used in this work was developed by Pasquale et al (Pasquale et al., 2010). A Single channel burst is defined as sequence of spikes with inter-spike interval smaller than 100 ms and containing at least 10 spikes within a burst (Chiappalone et al., 2005). However, this method doesn’t take into account the variation in spike firing within bursts. Hence, the algorithm developed by Pasquale is based on the computation of the logarithmic inter-spike interval histogram in order to detect automatically the best threshold between inter-burst (i.e., between bursts and/or outside bursts) and intra-burst (i.e., within burst) activity for each recording channel of the array. Once the burst detection is performed, a matrix containing the timestamps of the first spike in each burst, the timestamps of the last spike, the number of spikes and the burst duration is saved for each recording channel. A burst event train containing only the first spike of each burst is used to detect network bursts based on the algorithm proposed by van Pelt (van Pelt et al., 2005; Vajda et al., 2008). In brief, a network burst is detected when the product of the number of active channels and the total number of spikes at these channels in consecutive time bin of 25 ms duration exceeds a threshold value of 9 (van Pelt et al., 2005), with a minimum time period of 2 seconds between two consecutive network bursts (Pasquale et al., 2010). Time of initiation of network burst (from the first spike of each network burst) and its distribution in compartment A and compartment B is computed separately. For each network burst in compartment A, the burst in compartment B immediately before and after is located. Utilizing cross-correlation based techniques (Garofalo et al., 2009; Kanagasabapathi et al., 2011a), a correlation function was built from the two
burst trains (one per compartment containing the first spike of each network burst). It measures the probability of occurrence of spike in one burst train as a function of time with respect to the presence of spike in the other burst train (Knox, 1981). Based on the time of occurrence, the directionality in burst propagation can be deduced. Distribution of network burst initiation in Cx-Cx and Cx-Th co-culture devices based on this analysis is shown in figure 6.2.

**Pharmacological experiments**

Neuroactive compounds were used to selectively manipulate an individual region of the co-culture in dual-compartment devices. For the analysis used in this work, pharmacological experiments with both Cx-Cx and Cx-Th co-cultures were performed between DIV 21 and DIV 30. Experiments with pharmacological compounds consisted of three phases with an initial spontaneous activity (SA) recording of 5 minutes followed by electrophysiological recording with the addition of either synaptic receptor antagonist (SRA) or Tetrodotoxin (TTX) solution in one of the two compartments and a final phase of SA recording after three time (x3) wash out cycle. Between each phase, the cultures were allowed to equilibrate for 30 minutes to allow for diffusion and to stabilize the effects due to medium change (Wagenaar et al., 2004). Initial recording started at least 20 minutes after the device was moved from the incubator in order to soften the effects of mechanical perturbation on the electrophysiological activity (Biffi et al., 2011).

Synaptic receptor antagonist (SRA) solution consisting of a cocktail of 50 µM bicuculline methiodide (BMI) - GABA receptor antagonist, 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 in the experiments to block synaptically evoked network wide burst response (Bakkum et al., 2008). Tetrodotoxin (TTX) at a concentration of 100 nM in cell culture medium at 37˚C was used to block spontaneous firing of the neurons (Ramakers and Boer, 1991).

When replacing medium after the phase with pharmacological compound, the compartment with pharmacological solution was washed by completely removing the reagent from the reservoir and replacing it with freshly prepared neurobasal medium at 37˚C. The wash out cycle was repeated 3 times to ensure complete removal of the pharmacological solution. For each wash out cycle, the cell culture medium was filled from the inlet reservoir and allowed to flow through the entire compartment before being flushed through the outlet reservoir.
**Figure 6.3:** Effect of synaptic receptor antagonist (SRA) in Cortical-Cortical (Cx-Cx) co-culture systems. The box-plot represents the change in network burst rate on multiple Cx-Cx co-culture devices during three phases of experiments (phase 1: SA - spontaneous activity; phase 2: SRA in BL/BF - addition of SRA in either burst leading or burst following compartment and phase 3: SA - Recovery of spontaneous activity after three times wash cycle). (A) - SRA in Burst Leading compartment (n= 9 devices), (B) – SRA in burst following compartment (n= 6 devices). Addition of SRA in burst leading compartment blocks the network bursts significantly (P< 0.01) in that compartment with a marginal drop in bursting in the other compartment (A), while addition of SRA in burst following compartment does not have any significant impact on the network bursting of burst leading compartment (B).

**Figure 6.4:** Effect of synaptic receptor antagonist in Cortical-Thalamic (Cx-Th) co-culture systems. Statistical analysis of network burst rate on multiple Cx-Th devices with (A) - SRA in cortical compartment (n= 12 devices), (B) – SRA in Thalamic compartment (n= 9 devices). The box-plot represents the change in network burst rate on multiple Cx-Cx co-culture devices during three phases of experiments (phase 1: SA - spontaneous activity; phase 2: SRA in Cx/Th compartment - addition of SRA in either Cx or Th compartment and phase 3: SA - Recovery of spontaneous activity after three times wash cycle). As shown in (A), addition of SRA in Cx compartment completely suppresses the network burst in both Cx and Th compartments. While addition of SRA in Th compartment does not have any significant effect on the network wide bursts in Cx compartment.
Figure 6.5: Effect of TTX on the average firing rate (AFR) of Cx-Th co-culture system. Change in AFR with the addition of TTX in cortical compartment (A) – (B); and thalamic compartment (C) – (D); (A) – Change in AFR during three phases of an experiment (phase 1 - spontaneous activity; phase 2 - addition of TTX in cortical compartment and phase 3 - Recovery of spontaneous activity after three times wash cycle); (B) – Box-plot of AFR analysis on multiple Cx-Th devices (n= 6 devices) with the addition of TTX in cortical compartment; (C) – Change in the average firing rate during three phases of an experiment with the addition of TTX in thalamic compartment. (phase 1 - spontaneous activity; phase 2 - addition of TTX in thalamic compartment and phase 3 - Recovery of spontaneous activity after three times wash cycle); (D) – Box-plot of average firing rate analysis on multiple Cx-Th devices (n= 9 devices) with the addition of TTX in thalamic compartment.
6.3 Results

Neurite growth through microchannels

Transfection of individual neurons was performed on DIV 11. Cells in one compartment were transfected and a single transfected neuron with extensive neuritic arborization within the compartment of origin, as well as neurite crossing over to the other compartment through the microchannels is shown in figure 6.1C. The density of neurite branching within the compartment of origin is considerably higher than those neurites that pass through the microchannels. Figure 1C further shows the extent to which the neurites of a single neuron spread across both the compartments.

Network bursts and region of burst initiation

Co-cultures of both cortical-cortical and cortical-thalamic cells in dual compartment devices were observed to exhibit synchronized network bursts from third week in development (DIV 21). A sample raster plot of a Cx-Cx device on DIV 21 is shown in figure 6.2A. In figure 6.2A, timestamps of spikes recorded from all the recording channels with electrode index 1 through 60 along Y axis (electrode index 1 through 30 represents compartment A and electrode index 31 through 60 represents compartment B) over a period of 60 seconds is presented. The raster plot shows the presence of synchronized network bursts in both compartments, network bursts in compartment A are represented by ‘+’ (line 61) while network bursts in compartment B are identified by ‘*’ (line 62) respectively.

In cortical-thalamic co-cultures presented in this work, cortical cells were always plated in compartment A (electrode index 1 through 30) and thalamic cells were plated in compartment B (electrode index 31 through 60). For a sample of n = 21 Cx-Th devices, time of initiation of network bursts (TNBi) in cortical and thalamic compartments was computed as explained earlier (section 2.3.1) and based on TNBi, distribution of network burst initiation between the compartments is presented as shown in figure 6.2B. Similarly, TNBi was computed for two groups of Cx-Cx co-cultures of sample size n = 6 devices and n = 9 devices from 3 different cultures respectively. In the first group of n = 6 devices, distribution of bursts initiated in compartment B is considerably higher than those from compartment A (figure 6.2B). On the other hand, in the second group of n = 9 devices (figure 6.2C), compartment A acts as the region of burst initiation followed by compartment B. It may be observed that in Cx-Th co-cultures, network bursts were observed to originate in the Cx compartment in majority of the cases closely followed by thalamic bursts. However,
in case of cortical-cortical co-cultures, the choice of either of the compartment as the region of burst initiation and burst following was not predetermined.

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Effect of SRA on Cx-Cx co-cultures

Raster plot of spontaneous electrophysiological activity of a sample cortical-cortical (Cx-Cx) co-culture with detected network bursts from both the compartments is shown in figure S6.1. Distribution of initiation time of network bursts in compartment A and compartment B of Cx-Cx co-cultures was used to determine the burst leading (BL) and burst following (BF) compartment (figure S6.1-C). Addition of synaptic receptor antagonist (SRA) in the compartment with leading network bursts resulted in the loss of network bursts in that compartment. When SRA is added to BL compartment, majority of network bursts (indicated by a * in the raster plot) in that compartment has disappeared with a small change in the bursting of the BF compartment. Raster plot of spontaneous activity of Cx-Cx co-culture device before the addition of SRA (figure S6.1-A) and with SRA in BL compartment (figure S6.1-B) shows a drop in network burst in that compartment. Experiment was repeated in 9 Cx-Cx devices and a box-plot of change in network burst rate during three phases of the experiment in these 9 devices is shown in figure 6.3A. By confirming with Lilliefors’ test1 that the data does not come from a normal distribution, Kruskal-Wallis Non-parametric test, was used to determine the statistical significance in the change in network burst rate. A significant drop in network bursts in BL compartment (P between the three phases in BL compartment is < 0.01) with only a marginal drop in network bursts in BF compartment (P between initial SA phase and the phase with SRA is 0.07) was observed.

Under similar conditions, change in network burst rate with the addition of SRA in burst following compartment of 6 Cx-Cx co-culture devices is shown in figure 6.3B. Addition of SRA in BF compartment resulted in the loss of network burst activity in that compartment with no change in the bursting behavior of the BL compartment. In the compartment with SRA, with Kruskal Wallis test, P between initial SA and the phase with SRA was estimated to be 0.87. P value between the two phases in the BL compartment is > 0.20 and it is attributed to the localized bursting of cortical cells.

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1 Lilliefors test was used to check if the population of the data was from a normal distribution. It is a variant of the Kolmogorov-Smirnov normality test and it is used when the variance of the distribution and the expected value is not known. In case of the normal distribution we would use a parametric test (that makes use of the parameters like std or expected value to assess if the two sets of data come from the same distribution). In our case, Lilliefors test always showed that the distribution was not normal and hence, we used Kruskal-Wallis non-parametric test to see if the sets of data came from the same distribution or not.
in the compartment without SRA. See supplementary figure S6.1-(D) and (E) for a sample raster plot of spontaneous activity of a Cx-Cx co-culture device before the addition of SRA in burst following compartment.

**Effect of SRA on Cx-Th co-cultures**

In Cx-Th co-cultures, experiments were performed with the addition of SRA in either cortical or thalamic compartment and the electrophysiological activities were recorded during the initial spontaneous activity, with the addition of SRA and after three times wash out cycle as explained earlier. 21 Cx-Th devices were used for the analysis and in all 21 devices used, NB was observed to initiate from the cortical compartment. For experiments used in this analysis, the devices were grouped into 2 groups based on the addition of SRA in either cortical or thalamic compartment.

**SRA in Cx compartment**

In 12 Cx-Th co-culture devices used in the analysis, with a predominant distribution of network burst initiation in Cx compartment (figure 6.2D), addition of SRA in cortical compartment resulted in a significant drop in network bursting in that compartment with subsequent drop in network bursting in the Thalamic compartment. Raster plot of a sample Cx-Th device before the addition of SRA and with SRA in cortical compartment (see supplementary figure S6.2 (A) and (B)), shows the influence of blocking the cortical bursting on the entire network. Box-plot of 12 devices with SRA in Cx compartment is shown in figure 6.4A and statistical test shows a significant drop in NB with p between any of the two phases in both compartments < 0.01.

**SRA in Th compartment**

In the 9 Cx-Th co-culture devices, addition of SRA in the thalamic compartment had only marginal impact on the NB of both thalamic and cortical compartment. Raster plot of spontaneous activity of a sample Cx-Th co-culture before the addition of SRA and with SRA in Th compartment is shown in figure S6.1-(D) and S1-(E) respectively. Statistical test from the box-plot distribution of 9 devices (figure 6.4B) shows that P-values between initial SA phase and the phase with SRA (i) in Cx compartment = 0.94 and (ii) in Th compartment = 0.65. This non-significant drop in NB rate may be attributed to the influence of thalamic network bursting from the burst leading cortical compartment.
6.3 Results

Effect of TTX on the average firing rate (AFR)

TTX blocks sodium channels and completely suppresses spontaneous firing of neurons. In our experiments, addition of TTX to the co-culture system affected spontaneous firing of the entire culture and the synchronized bursting of the network didn’t immediately recover after the wash cycle with cell culture medium. Hence, to quantify the effects due to the addition of TTX in the co-culture system, we calculated the change in average firing rate induced by TTX in both Cx-Cx and Cx-Th co-cultures.

In Cx-Cx co-cultures, addition of TTX in either of the compartment had a significant impact on the average firing of the cells. The average firing rate of the cells in that compartment dropped significantly (P<0.01) with subsequent drop in the firing of the cortical cells in the other compartment (P<0.01) (Data not shown). In Cx-Th co-cultures, addition of TTX in either the cortical compartment or the thalamic compartment affected the firing rate of the cells in that compartment with subsequent drop in the spontaneous firing of cells in the adjacent compartment as shown in figure 6.5 (P<0.01 in both the compartments).

6.4 Discussion

The techniques described in this work to study selective chemical manipulation of an individual cell type in a Cx-Th co-culture system demonstrates how a two compartment system coupled to standard MEA can be useful in studying the influence of a particular cell type in a co-culture model. The two compartment systems described here may further provide tools for understanding the effects of neuroactive compounds on a particular neuronal cell type. The net effectiveness of synaptic connections between neurons influences the probability that the bursting of neurons in one region could spread to other region (Maeda et al., 1995). Synchronized bursting in the entire network starts around DIV21 signaling the maturation of the culture (Habets et al., 1987; Chiappalone et al., 2006). In synchronized network-wide bursts, bursts were observed to initiate from a particular region and played a dominant role in driving the entire network response (Maeda et al., 1995) and the same system of propagation of synchronized burst firing appears to occur in vivo (Steriade et al., 1993). Capabilities to identify and manipulate such regions may play an important role in understanding network dynamics.
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MASTER / SLAVE REGIONS IN Cx-Cx HOMOGENEOUS CO-CULTURES

In Cx-Cx co-cultures, synchronized network bursts were observed to initiate from a particular compartment, thus acting as master compartment with predominant effect on the burst pattern in the adjacent compartment, acting as slave. Our experimental observation has shown that controlling the cells in master compartment with synaptic receptor antagonist has an influence on the behavior of the network wide burst response. However, controlling the spontaneous bursting of slave region did not have any significant impact on the master compartment. Thus, the master region supersedes the activity of the slave region and it may also determine the frequency and duration of bursting in the network. However, in our experimental approach, selection of either of the compartments to behave as a master or a slave region is not predetermined. This, we believe, may depend on the development of the network, the extent of neuritic arborization within the individual compartments and to a large extent on the strength of synaptic connections within each region (Maeda et al., 1995).

CONTROLLING BURST PROPAGATION IN Cx-TH CO-CULTURES

In Cx-TH co-cultures, synchronized network bursts initiate in the cortical region and the strong cortical-thalamic connections were observed to initiate the propagation of network bursts into the thalamic region (Adams et al., 2011; Kanagasabapathi et al., 2012). In our earlier work with Cx-TH co-culture samples, the certainty of cortical origin of the burst was confirmed by analyzing burst initiation, that showed a high probability of burst initiation in cortical region compared to the thalamic region. In this current work, initiation of bursts in the cortical region was experimentally confirmed by suppressing network bursts at its origin and thereby subsequently controlling NBs in the thalamic region. Addition of SRA in thalamic compartment had a marginal effect on the NB of thalamic cells with no significant change in cortical compartment further confirming the origin of bursts in a Cx-TH co-culture. Here, the cortical region acts as a master region and controls the propagation of bursts to the thalamic region. The results also suggest that the propagation of spontaneous synchronized network bursts in the co-culture system is controlled by the region of burst initiation.
CHANGING SPONTANEOUS FIRING PATTERN OF THE CO-CULTURE SYSTEM

Although the use of synaptic receptor antagonist demonstrated capabilities to selectively manipulate a particular region of the co-culture system and thereby influence activity of the adjacent compartment, direct subtle influence of a particular region of the culture on the distribution and firing pattern of the other region is demonstrated using experiments with TTX.

Addition of TTX solution in either of the master or the slave compartment in a co-culture system caused considerable loss of spontaneous activity in that particular region. However, the drop in the firing rate resulted in subsequent change in the firing rate of the other compartment, which provides an indication of the strength of direct connections between both the regions. In addition, experiments with TTX suggest that although propagation and distribution of NB in the culture was determined by the region of burst initiation, spontaneous firing of the network does not show any hierarchy. Manipulating the firing pattern of a particular region induces a strong reciprocal change in the firing behavior of the other cell type in the co-culture system.

Based on the results presented, the dual compartment devices demonstrated in this work offers a valuable platform to approach questions systematically related to the study of neuronal sub-populations. Unlike in vivo conditions, where multiple neuronal pathways influence any recorded region, isolated networks can be studied in a controlled and isolated environment. The high susceptibility to manipulations obtained when working in vitro allows the construction of networks of heterogeneous co-cultures and possibilities for selective chemical manipulation of an individual region without directly affecting the adjacent region.

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6.5 Supplementary Information
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Figure S6.1: Raster plot of a sample Cx-Cx co-culture device with the addition of SRA in either the Burst Leading (A) – (C); or Burst Following compartments (D) – (F). (A) – Spontaneous activity of 30 s of a sample Cx-Cx co-culture system with identified network bursts in compartment A (detected network in line 61 of the raster plot) and compartment B (line 62); (B) – Spontaneous activity with the addition of SRA in burst leading compartment; (C) – Change in network burst rate during three phases of this experiment (phase 1 - spontaneous activity; phase 2 - addition of SRA in Burst Leading compartment and phase 3 - Recovery of spontaneous activity after three times wash cycle). Similarly, (D) through (F) shows the effect of SRA in Burst following compartment.
**Figure S6.2:** Raster plot of a sample Cx-Th co-culture device with the addition of SRA in either Cortical (A) – (C); or Thalamic compartments (D) – (F). (A) – Spontaneous activity of 30 s of a sample Cx-Th co-culture device with identified network bursts in compartment A (line 61 of the raster plot) and compartment B (line 62); (B) – Spontaneous activity with the addition of SRA in cortical compartment; (C) – Change in network burst rate during three phases of this experiment (phase 1: SA - spontaneous activity; phase 2: SRA in Cx - addition of SRA in cortical compartment and phase 3 - Recovery of spontaneous activity after three times wash cycle). Similarly, (D) through (F) shows the effect of SRA in Thalamic compartment. From (C) and (F), it may be observed that by controlling network-wide bursts in cortical region, the bursting in thalamic region can be controlled. However, controlling thalamic bursts did not have significant effect on the cortical network bursts.