Technological developments in studying neural information processing in patterned neuronal networks *in vitro*

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***Abstract*—Spatially defined biological neural networks were created from dissociated rat brain tissues and grown on culture dishes with embedded metal electrodes (planar multi-electrode array). Mature Neural networks matured in two weeks and were spontaneously active. Electrical stimulations evoked phase-locked responses. Multichannel recordings provided a chance to study the neural coding developed in live neural networks *in vitro*.**

***Index Terms*—cell culture, microelectrode array, multichannel recording, patterned neuronal network**

I. Introduction

PLANAR multielectrode arrays (MEAs) have been used to study the properties of electrogenic tissues including acute brain slices, brain cells (cortical neurons, spinal cord neurons, hippocampal neurons, etc.), and heart cells. One could either detect small extracellular potentials created by action potentials or deliver currents to generate electrical fields to stimulate nearby tissues through the electrodes (usually 60 electrodes) [1-4]. There have been efforts to develop cell-based biosensors for screening neurotoxins using this type of system [5, 6]. It has also been demonstrated that one can study learning related phenomena in cultured cortical neural networks [7, 8].

In our laboratory, we have been developing technologies to design neural circuits *in vitro* by controlling the growth of neurons from rat embryos [9-11]. Advanced lithography, surface chemistry, cell culture techniques and multichannel data processing capability made it possible to consider designing and characterizing the patterned neuronal network *in vitro*.

Here we overview the recent progress on the implementation of patterned neuronal circuits on MEAs. Dissociated hippocampal neurons derived from rat embryos were successfully grown in pattern for a month. Multichannel recordings allowed us to monitor the spatially distributed or correlated network activities. Time-locked evoked responses were induced by electrical stimulation through theses electrodes. Statistical analyses such as rate histogram, interspike interval histogram, auto-correlogram, cross-correlogram, perievent histogram were routinely used to characterize the neural data. Devising the proper measure to represent the multidimensional data was very difficult task in general.

II. Methods

A. Cell culture

Dissected hippocampi (18-day gestation Sprague-Dawley rat embryos) were purchased from Brain Bits™ (www.BrainBitsLLC.com). Tissues were mechanically dissociated and cultured at 37°C, 5% CO_2, 9% O_2, in serum free B27/Neurobasal medium (Invitrogen, Gaithersburg, MD) with 0.5 mM glutamine and 25 μM glutamate. Each dish gets about 4,000 ~ 16,000 cells depending on the area and plating density (50 ~ 200 cells/mm^2). Cultures were maintained by changing half of the medium weekly, without glutamate.

B. Multi electrode arrays

MEAs (Fig. 1(a)) serve as a culture dish as well as a metal electrode array. The metal layer pattern is photolithographically defined on the substrate (glass) and coated by an insulation layer. Then via holes are opened to be microelectrodes (size of 10 ~ 30 μm in diameter). Contact pads are formed at the edge of the substrate to make external connections.

There are a few commercially available MEAs. We purchased 60 channel MEAs from Multi Channel Systems (Fig. 1(a), Reutlingen, Gemany). They had 60 electrodes (8×8 configuration with four corners empty) and each electrode was made of TiN (titanium nitride, 10 μm in diameter) and AC impedance was 300 kΩ ~ 500 kΩ at 1 kHz. A metal layer is insulated by silicon nitride (0.5 μm in thickness). We also used custom designed MEAs from Elume Inc. (Semi Valley, CA). These MEAs were insulated with polyimide (4 ~ 5 μm in thickness) layer and electrodes were made of transparent indium-tin oxide conductor. The tip of the electrodes was platinized to reduce the AC impedance for low noise recording

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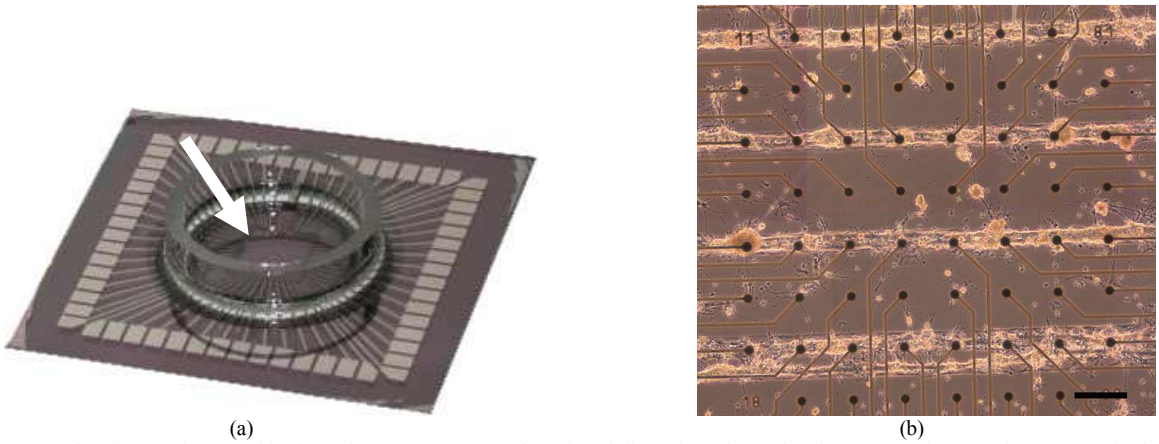

(a) (b)

Fig. 1. (a) Example of MEA from Multi Channel Systems. Center glass ring defines the culture chamber. External connections are made though edge contact pads. The arrowed area is magnified in part (b). Substrate size: 5 cm × 5 cm, (b) Center of MEA with live cultures at 24 days *in vitro*. This culture was linearly patterned (see text), scale bar: 200 μm.

and large charge storage for electrical stimulation.

C. Surface chemistry

The surface of the substrates (here, the insulator of MEAs) needs to be treated to permit attachment and growth of neurons for a long term (at least two to three weeks in culture). Usually, this is done by coating the whole surface with cell adhesive biomoelcules such as poly-D-lysine (PDL, applied 0.1 mg/ml in deionized water).

To create the surface pattern that would control the growth of neurons, we imprint PDL pattern in micrometer scale by inking the PDL solution onto silicone rubber microstamp and transferring to the surface by micro-contact printing (stamping) procedure [12]. The procedure was often assisted by the protein linking chemistry to form a strong covalent bond between the surface and biomolecules, which also proved to extend the longevity of the patterned cultures up to a month[9]. Detailed procedures could be found elsewhere [10, 11]

D. Neural recording and stimulation

During the experimental sessions, a culture containing MEA dish was pulled out of the incubator and transferred to the recording chamber, where the MEA was connected with the 60-channel amplifier unit (Gain 1200, bandwidth 10 – 3000 Hz, MEA 1060 system, Multi channel Systems). The temperature of the MEA was maintained at 37 °C with a heating stage and controlled gas (5% CO_2, 10% O_2, backfilled with nitrogen) was fed to the recording chamber to maintain the pH level of culture medium near 7.4. A silver-silver chloride wire or a large ITO electrode was used as a reference electrode.

The continuous stream of 60-channel data were digitized with a 64-channel AD board (MC Card, f_s = 40 kHz per channel, Multi channel Systems) and displayed and stored using dedicated software (MC Rack, Multi channel Systems). Post analyses were usually performed by replaying the recorded data and identifying the spikes with a thresholding method and classifying the spikes ('spike sorting') if there were multiples units in a same channel.

For real time experiments, the Multichannel Acquisition Processor developed by Plexon Inc. (Dallas, TX) was used to do tasks such as spike detection, spike sorting, spike rate histogram, and interspike interval histogram. Signals bigger than 5 times the standard deviation of the background noise were regarded as neural spikes. The spike detection window was 1.4 ms in total with 0.6 ms for prethreshold. When a certain number of spikes (usually more than 100 spikes) were collected, they were sorted by template matching or principal component analysis. Each channel could have four different units that were processed individually.

Biphasic or monophasic voltage pulses were delivered directed to one of the electrodes on the MEA. The magnitude of the stimulus varied from 0.1 to 2.2 V. The pulse duration was 0.2 ms for each phase. Each stimulus was delivered every 1 second (1 Hz) or 2 seconds (0.5 Hz). Both recording and stimulating channels suffered from large stimulation artifacts. It was not possible to access the stimulating channel for more than a few hundred milliseconds after the stimulation. For neighboring electrodes, early responses were readily accessible after digitally adjusting the baseline as long as the amplifier outputs are not saturated by the stimulation artifacts. In this way, spikes that appeared as early as 3 – 5 ms could be detected easily by simple threshold crossing method.

E. Data analysis

After extracting spikes from raw data, time stamp series (spike trains) were processed using Neuroexplorer (NEX, Nex Technologies, Littleton, MA). Rate histograms, auto correlograms, cross correlograms and perievent histograms were calculated. The mean frequency and confidence limits were calculated under the assumption that the spike train is a Poisson train. These limits were used to tell whether the calculated histogram shows any statistically significant event. More complicated analysis such as burst analysis or neural population vector analysis (principal component analysis) could be carried out by this software.

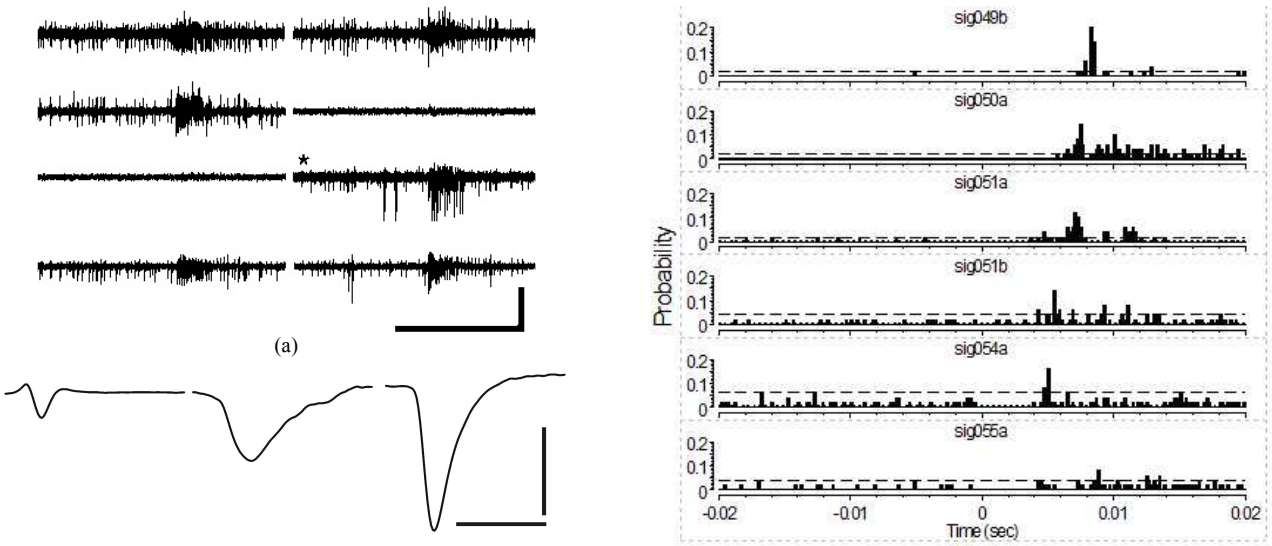

Fig. 2. (a) Spontaneous recording from patterned cultured in fig. 1(b), scale bar: 1 s, 200 μV. (b) Three different units sorted from the marked channel in part (a), scale bar: 1 ms, 200 μV. (c) Perievent histogram constructed after recording evoked responses from a line pattern similar to fig.1(b). Sig055a was stimulated by biphasic voltage pulses.

III. Results and Discussion

A. Formation of patterned neuronal network

We can form a patterned network on MEA and maintain it up to a month. So far, most of the patterned cultures were dense with clusters of cell bodies and bundles of neural fibers. Low density high resolution cultures were also possible with relative low yield due to the poor viability. We are actively pursing the techniques to improve the yield of the low density high resolution cultures.

The longevity and the compliance of the patterns were dependent upon the physical dimensions of the pattern. For example, 40 μm wide linear pattern produced high yield cultures. 83% of the linearly patterned cultures (10 out of 12 cultures) showed good compliance to the surface pattern. When grid patterns were tested, only 50 % (6 out of 12 cultures) had evaluated to be in a good compliance.

B. Spike detection and sorting

The background noise level was 3 ~ 4 $\mu V_{(rms)}$. Spikes larger than 15 ~ 20 $\mu V_{(0\text{-}peak)}$ were readily detected by the thresholding method. Often, when cells were tightly coupled to the electrode, we were able to record spikes as large as 500 ~ 700 $\mu V_{(peak\text{-}peak)}$. As an example, Fig.2(a) shows the spontaneously bursting activities recorded from linearly patterned network (Fig.1(b)). Multiple units were detected from a single channel (see Fig.2(b)). Various spike waveforms spikes were detected.

C. Spontaneous activity of patterned cultures

Neurons in patterned cultures showed spontaneous activities as early as 7 days in culture (Fig.2(a)). However, robust and reproducible recordings could be found only after 2 weeks in culture. Most of the electrodes were showed correlated and periodic bursting. Often there were a few channels displaying tonic firing behaviors. We constructed 64 lines from 16 cultures and 54 lines were intact and healthy. Each line was overlaid with 8 electrodes which could report activities. 41 lines were spontaneously active at the time of recording. Mean firing rate was distributed from 0.1 Hz to 30 Hz. There was no significant difference in mean firing rate when we compared different patterns of cultures.

These spontaneous activities could be modulated by applying drugs such as bicuculline or Mg^{2+}. Bicuculline increased the spike rate and made the bursting rate more regular. High level of Mg^{2+} could reversibly block the activities suggesting the involvement of NMDA receptors in observed activities.

D. Electrical stimulation and evoked responses

Voltage stimulation was used since this was easier to control the electrode voltage than current stimulation. Evoked responses showed all or none behaviors near the threshold level. Positive first biphasic voltage pulses were more effective than negative first biphasic pulses.

More electrodes recorded evoked responses as the stimulus intensity increased. Since we were stimulating through extracellular fields, a higher stimulation level depolarized more units near the electrode.

The latency of the evoked spikes was within 10 ms, which is likely to be synaptically or axonally conducted responses. Estimated conduction velocity ranged from 0.1 – 0.3 m/s. Fig.2(c) [13] is an example of axonally propagated direct response in linearly patterned cultures. Estimated conduction velocity was 0.3 m/s.

E. Connectivity of neuronal network

Patterned cultures had a fan-in and fan-out structure. We performed a detailed analysis of one culture with a grid pattern culture and found that each electrode connected with 5.0 ± 4.3 electrodes (N = 21, mean ± s.d.) and more than one electrode (3.9 ± 3.3 electrodes, N = 21, mean ± s.d.) evoked responses from the same electrode. There were some pairs of electrodes

that showed reciprocal connectivity with slightly different latencies.

More elaborate analysis paradigm is required to efficiently investigate multichannel recording and stimulation data set.

IV. Conclusion

Designing the neural circuit in dish is in general a very challenging task. Our works show that it would be possible to create a patterned network *in vitro* by combining the state-of-the-art technologies in various fields including micro-fabrication, surface chemistry, and cell cultures. Multichannel data analysis techniques showed that artificially created neural circuits had desired characteristics to be minimally functional. We are currently using these model networks to study the relationship between the form and the function of the network.

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