Multichannel recording and stimulation of neuronal cultures grown on microstamped poly-D-lysine

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Abstract—**We report progress toward designable, reproducible, patterned in vitro neuronal cultures. Cell adhesive proteins were directly microcontact printed on microelectrode arrays (MEAs) used as substrates for hippocampal neurons grown in a defined culture medium. The patterned neuronal network circuits were maintained up to one month. The recorded activity was comparable to that of cultures grown on unpatterned uniform surfaces. Time-locked evoked responses were recorded across the networks.**

Keywords—**Microelectrode array, hippocampal neurons, microstamping, spontaneous activity, evoked response**

I. INTRODUCTION

Using planar microelectrode arrays, we can study the network properties of neuronal cultures *in vitro* [1]. There are a few suggestive schemes available to implement patterned cultures on MEAs [1, 2]. In order to produce a large quantity of cultures for the characterization of network activities, it is desirable to have a simple scheme to create the protein pattern on MEA. Microstamping is one of many ways to create the micrometer resolution pattern without sophisticated clean room environment [3].

Here we report the creation of patterned cultures on poly-D-lysine (PDL) patterned MEAs using the microstamping technique. Even though no specific linking schemes were involved, there was sufficient PDL to promote neural growth and form patterned networks for up to a month; multichannel recording and stimulation was reproducibly achieved.

II. METHODOLOGY

A. Cell Culture

Hippocampal neurons (BrainBitsLLC.com) from 18-day gestation Sprague-Dawley rat embryos were dissected mechanically 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 [4]. Plating density was 75 – 100 cells/mm^2. Half of the medium was changed weekly, without glutamate. Before each recording session, the status of the culture was evaluated under the microscope and images were taken by phase contrast microscope.

B. Microelectrode array

MEAs were purchased from Multi Channel Systems (Reutlingen, Germany). Electrodes were 10 µm or 30 µm in diameter and the interelectrode distance was 200 µm. The insulator was PECVD silicon nitride (0.5 µm). In order to remove any organic contaminants on the surface, MEAs were cleaned in acetone ultra-sonication bath for 10 min and successively rinsed with isopropyl alcohol (IPA) and deionized water (DI water) before any use. Each MEA was reused more than twice.

C. Microstamping

PDMS stamps were created from microfabricated molds made of a thick photoresist (AZ4620, Clariant Corp., Sommerville, NJ) [5].

We followed the microstamping procedure introduced by [3]. The stamp surface was modified by soaking the stamp in aqueous SDS solution (10% w/v in DI water, sodium dodecyl sulfate) for 15 min. Excess SDS layers were removed by dipping the coated stamp into DI water once and drying under gentle nitrogen gas. Poly-D-lysine (PDL, MW 70,000 – 150,000, 0.1 mg/mL in DI, Sigma-Aldrich) was loaded for 30 min and dried gently using nitrogen. Each PDL-inked PDMS stamp and an MEA were aligned using a custom-built contact aligner and brought in contact for 3 min. Printed substrates were rinsed with DI water.

D. Data acquisition and analysis

An MEA was connected with MEA1060 amplifier (Gain 1200, 10 Hz – 3 kHz, Multi Channel Systems, Reutlingen, Germany) and amplified signals were fed to MC card (sampling rate 40 kHz, Multi Channel Systems, Reutlingen, Germany) or MAP (Multichannel Acquisition Processor, sampling rate 40 kHz, Plexon Inc, TX, USA). Dedicated software such as MC Rack (Multi Channel Systems) or RASPUTIN (Plexon) was used to control the hardware (MC card or MAP). Digitized data were further processed to estimate the RMS value of background noise for all channels. Spikes were detected by setting the threshold at 5 or 6 times the RMS value. Analog raw data or spike waveforms were stored for off-line analysis. Off-line Sorter (Plexon Inc., TX, USA) was used for spike sorting

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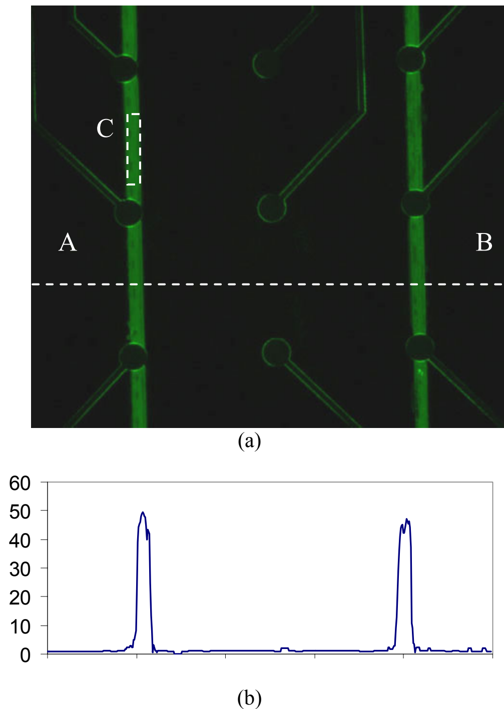

Fig.1. Direct protein printing on MEA. (a) PLL-FITC (0.1 mg/mL in 1x PBS) stamped on MEA surface. Line width: 20µm, spacing: 400µm. (b) Intensity profile from part (a) along AB.

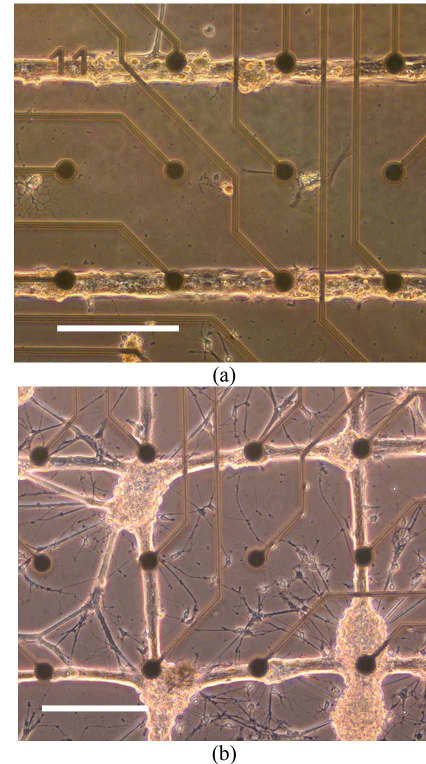

Fig.2. Hippocampal neurons grown on PDL stamped MEAs, (a) line pattern, 27 DIV (days *in vitro*), (b) square grid pattern, 8 DIV. Scale bar: 200µm.

and Neuroexplorer (NEX, Nex Technologies, MA, USA) was used for spike train analysis.

E. Electrical stimulation and analysis

A stimulus generator (STG-8, MCS) was used to deliver voltage pulses to the electrodes. The stimulating electrode was shunted to the system ground through a 33 kΩ resistor and the input of the corresponding amplifier was also grounded to the system ground (this is the fixed configuration of MEA 1060). Biphasic pulses (positive first, amplitude ± 0.4 V – ± 2.2 V, pulse width 200 µs) were used to stimulate the cells. Pulses were delivered every 1 or 2 sec.

If the stimulus level was low, the stimulation artifact did not affect spike detection. As the stimulus level increased stimulation artifacts significantly affected the baseline of the recorded traces; the artifacts were estimated using a 4th order polynomial and subtracted from the original trace. This was effective as long as the amplifier outputs were not saturated. This process was modified from [6].

III. Results

A. Microstamping PDL on MEA

According to our fluorescence study, PDL was readily printed on an MEA surface. Fig. 1(a) shows an example of printed pattern on an MEA. The intensity profile (Fig.1(b)) indicates the selective printing of protein on the surface. The intensity of the pixel values were uniform on pattern (47.8 ± 5.09, mean ± s. d., n = 1315, Fig. 1(a) C).

B. Cell growth on patterned MEA

Fig.2 (a) shows the growth of neurons in line patterns. Each of twelve MEAs was cultured with four 40 µm wide lines. For a few cultures, some lines were washed off during the media change and only one or two well-attached lines were available. When we estimated the outgrowth of neural processes on off-pattern areas, 7 cultures out of 12 had negligible cell growth in background areas. Three cultures had significant proliferation of neural processes or glial cells in the background. In terms of the fidelity or compliance of the cell growth to the pattern, 10 cultures had good compliance to the printed PDL lines and the rest of the cultures were evaluated as fair. When we counted the cultures that had a good compliance as well as the good suppression of cell growth in background, 7 cultures met this condition.

Eight MEAs had a square grid pattern similar to Fig.2(b). Initially, cells preferentially attached to the pattern and grew in compliance with the pattern. After three weeks in culture, however, only one culture had negligible cell growth in the off-pattern area and a perfect compliance to the surface pattern. Most of the cultures suffered from the

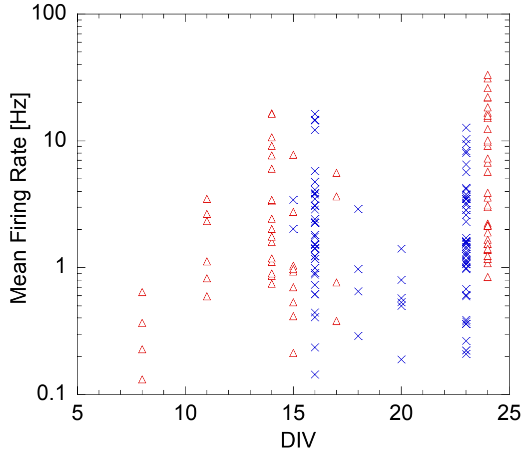

Fig. 3. Mean firing rate of spontaneously active units from line pattern ('△', N = 5) or square grid pattern ('×', N = 7). Two recordings from line pattern are sampled at two different stages (11 & 17 DIV and 14 & 24 DIV).

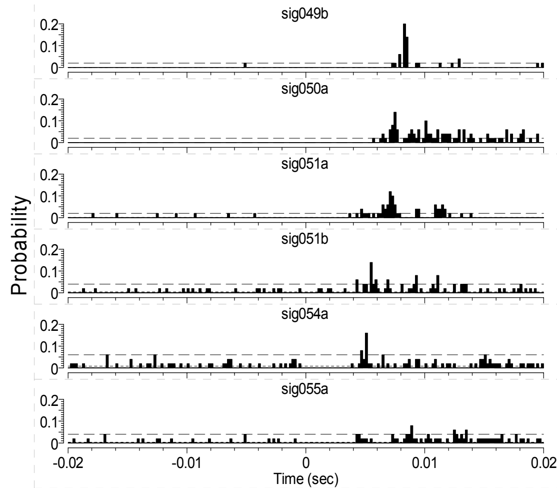

Fig.4. Perievent histogram showing the propagation of action potential. Network is grown to form a line pattern, covering recording electrodes labeled sig49 to sig55 and stimulation channel sig56 which was stimulated with biphasic voltage pulses. Each channel is 200μm apart. Dashed lines indicate 99% confidence level.

significant cell growth in the off-pattern and aggregation of cell bodies forming microspheres. These microspheres often pulled each other and degraded the compliance to the pattern.

C. *Spontaneous activity*

MEAs with line patterns were able to detect spikes as early as 8 DIV. Eleven cultures out of twelve had spontaneously active lines. On average, 2.56 ± 1.26 (s.d., n=16, 4 lines per pattern) lines were active. Among the electrodes that were aligned to the pattern, 33 % ± 18 % (s.d., n=16) detected extracellular spikes. Fig. 3 is a scatter plot of mean firing rates estimated from five cultures. The spike rates ranged from 0.1 Hz to 30 Hz as cultures matured. Average spike rate increased from 8 DIV to 14 DIV. After the second week, however, the frequency range remained same. When we compared the same cultures at different stages (11 DIV vs. 17 DIV or 14 DIV vs. 24 DIV), more firing units were discriminated after spike sorting.

Cultures on square grid patterns had active neurons as early as 6 DIV and displayed robust activity after 14 DIV. The number of active electrodes varied from 2 to 29. The spike rates varied from 0.14 Hz to 16. 28 Hz.

After 2 weeks in culture, most of the cultures had synchronized bursting activity, which was evident from crosscorrelograms of time-stamps across the channels. In case of line patterns, some neighboring lines had different bursting periods. Often there was also short-term (less than 2 ms) correlation between the channels, indicating axonal conduction.

D. *Evoked responses*

Stimulating electrodes were selected as those reporting the robust spontaneous activity. Stimulation of these electrodes was effective at inducing time-locked responses in other channels. Channels that had significant correlation in spontaneous activity also had similar correlation when one of the channels was stimulated. Time delays of the responses varied from 2 ms to 15 ms after the stimulation. When the stimulus level was increased from low (± 0.4) to high (± 2.2 V), a threshold level was found below which there was no response. Above the threshold, induced spikes were precisely aligned at fixed time delay and nearly every stimulus induced a response. By increasing the stimulus level, more channels responded to the stimulation.

Figure 4 demonstrates the estimation of axonal conduction velocity in linearly patterned network. Action potentials were initiated from the stimulating electrode (sig 56) and propagated along the pattern. Measured conduction velocity was 0.3 m/s when we correlate the time point of significant peaks in perievent histogram and the distance between the electrodes. In case of square grid pattern, conduction velocity was estimated to be 0.15 m/s which were significantly slower than axonal conduction velocity measured in line pattern.

Figure 5 shows the example of reciprocal connection within the square grid pattern. When we stimulated CH 33, there were time-locked responses at 4.6 ms on CH 26 (Fig.5(a)), while stimulation CH 26 induced time-locked spikes at 6.0 ms on CH 33 (Fig.5(b)). Non-time-locked spikes occurred frequently between 10 ms and 15 ms after stimulation.

IV. Discussion

We have realized geometrically defined neuronal

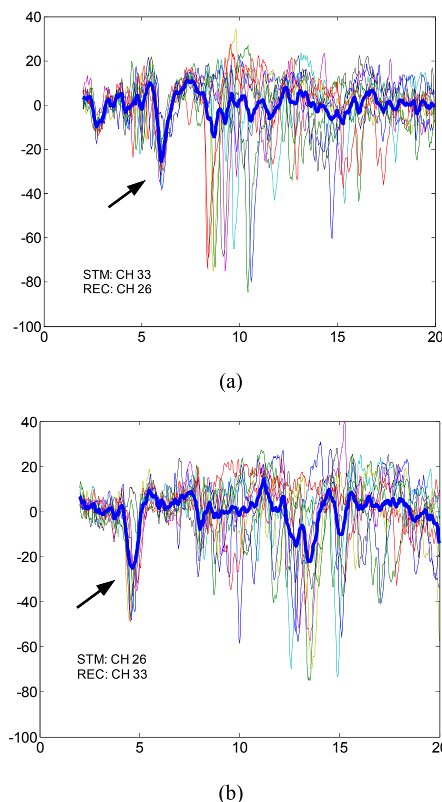

Fig. 5. Example of reciprocal connection within the network (square grid pattern). Arrows show time-locked single unit responses. (a) Stimulate CH 33 and record CH 26, (b) Stimulate CH 26 and record CH 33. Thick solid line is the average trace (N = 10). Biphasic voltage pulse (ten pulses, ± 1.0 V, 200 μs, 1 Hz). Stimulation artifact removed. x-axis: time in millisecond, y-axis: amplitude in μV.

networks *in vitro* by directly printing a cell adhesive biomolecule on a microelectrode array. Preferential cell growth on PDL pattern indicates that we have printed sufficient PDL to functionalize the surface without any chemical linking scheme. However, it was hard to prevent the growth of glia and neuroprogenitors from growing in the off-pattern area after two weeks in culture. This was the result of the lack of active control of the off-pattern area to prohibit the cell growth [2, 7]. Simple line patterns were more effective at maintaining the original morphology than more complex square grid patterns.

Mean firing rates of discriminated firing unit were distributed over a wide range and without a significant difference between line patterns and square grid patterns. Within the culture, there exist neurons firing at low frequency (< 1 Hz) and high frequency (> 10Hz) at the same time. Both patterned cultures show regular bursting patterns after two weeks. Comparison of bursting patterns may reveal the difference between patterns.

Electrical stimulation readily depolarized neuronal processes near the electrode. Cell and electrode coupling was sufficient to observe the threshold behavior reproducibly. Stimulated electrodes induced responses from multiple channels. Most of the responses consisted of time-locked action potentials; there were some examples suggesting reciprocal connections within the network. Unidirectional signal propagation was measured in a line network with axonal conduction velocity of 0.3 m/s. It was difficult to capture the pattern of the signal propagation in square grid pattern.

V. Conclusion

It is hoped that geometrically controlled neuronal network will be a useful model to study the behavior of small population of neuronal network for basic understating in neuroscience field. Here we applied multichannel recording and stimulation technique to study patterned neuronal network in culture grown on surface patterned planar microelectrode array.

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