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Automatic positioning and sensing microelectrode array (APSMEA) for multi-site electrophysiological recordings

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Abstract

Technological improvement of measurements for the electrical recordings from individual neurons within network is essential in neuroscience today. Here, we present a novel automatic positioning and sensing microelectrode array (APSMEA), which simultaneously positioned desired number of neurons onto 48 recording microelectrodes automatically and scathelessly by use of negative dielectrophoretic (DEP) forces, and facilitated the measurement of the electrophysiological activities of neuronal populations after functional synaptic connections formed between neurons. The results of multi-site electrophysiological recordings during drug administration also demonstrated the application of APSMEA in bioassay with cultured rat cortical neurons. Therefore, this device should benefit the investigation of neuronal networks *in vitro* with more comprehensive electrophysiological experiments, and also promise the possibility of a modular device for both cell manipulation and cell-based biosensor on microchip.

Keywords: Microelectrode array; Dielectrophoresis; Cell positioning; Neuronal network; Electrophysiology

1. Introduction

The nervous system, due to its extraordinary complexity and plasticity, requires extensive primary analysis employing highly simplified model systems before the function of the actual neural circuits *in vivo* can be approached effectively (Marom and Shahaf, 2002). *In vitro* studies are therefore increasingly important in allowing research on simpler systems, such as small networks of neurons. Providing an interface between such cultured neuronal network and an external electrical circuit for multi-site electrophysiological measurement or for stimulation, microelectrode array (MEA) enabled the studies related to formation of neuronal network, synaptic modification, regulation of network activity patterns, pharmacological evaluation, biological rhythms, and so on (Arnold et al., 2005; Darbon et al., 2002; Eytan et al., 2003; Gross et al., 1977; Jimbo et al., 1999; Martinoia et al., 2005; Pine, 1980; Xiang et al., 2007a,b; Wagenaar et al., 2005). To achieve higher amplitude of electrophysiological signals and to improve the effectiveness of stimulation, however, neurons should be positioned near a recording/stimulation site. Thus, a simple and effective approach for defining neuronal networks is of great importance in improving the performance of MEA-based recording and stimulation.

Traditionally, two approaches have been used to control the locations of dissociated neurons on a certain region of the substrate, either employing containment such as micro-wells, micro-channels and micro-drops (Jimbo et al., 1993; Macis et al., 2007; Maher et al., 1999; Suzuki et al., 2005; Zeck and Fromherz, 2001), or adopting appropriate materials for surface patterning (Chang et al., 2003; Kleinfeld et al., 1988; Nam et al., 2004; Scholl et al., 2000). Indeed these approaches achieved a lot of successes, but it is still a challenge for precise manipulation of cells or treating the surface regions.

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The use of dielectrophoretic (DEP) forces has recently been explored to assist engineering of neuronal networks (Heida et al., 2001; Prasad et al., 2004; Yu et al., 2004). As earlier reports demonstrated, DEP forces, created on cells when a non-uniform electrical field interacts with the field-induced electrical polarization on the cells, are fast, flexible and easy to control (Becker et al., 1995; Cheng et al., 1998a,b; Pohl, 1978). Depending on the dielectric properties of the cells relative to the suspension medium, positive or negative forces can be achieved, which direct these cells toward strong or weak electrical fields, respectively. It should be thoughtfully noted that, during positive DEP forces, the strong electrical field and the working medium with low conductivity were both negative for the viability of neurons (Prasad et al., 2004; Yu et al., 2004). As demonstrated in our previous studies, negative DEP forces guaranteed the viability while neurons were positioned on a bioelectronic chip without recording electrodes, and these neurons maintained *in vitro* up to 2 weeks (Yu et al., 2004).

In this study, we developed a novel automatic positioning and sensing microelectrode array (APSMEA), which combined two sets of arrayed electrodes, with one set for electrophysiological measurements and the other set for generating negative DEP forces. Such device allowed the positioning of neurons accurately and scathelessly onto the recording/stimulation microelectrodes within a short period of time, and facilitated multi-site electrophysiological recordings upon functional synaptic connections formed between neurons. In addition, to illustrate the general use of such device, APSMEA with cortical neuronal network was applied as an example in pharmacological analysis.

2. Experimental

2.1. Materials and reagents

Neurobasal medium, B27 supplement and glutamine were obtained from Invitrogen Corp. (Carlsbad, CA). Papain, glutamate, β-mercaptoethanol, tetrodotoxin (TTX), *N*-methyl-D-aspartate (NMDA) and D-2-amino-5-phosphonovaleric acid (APV) were purchased from Sigma Chemical Company (St. Louis, MO). Sylgard 184 was supplied by Dow Corning Corp. (Midland, MI). Capitalbio Corp. provided clean room facilities, chemicals and equipment for APSMEA fabrication.

2.2. APSMEA fabrication and encapsulation

APSMEAs were produced using a conventional semiconductor process and subsequently encapsulated, as modified from the protocols of Xiang et al. (2007a). Briefly, a layer of SiO_2 (3000 Å) was grown on the polished side by thermal oxidation, followed by a layer of Si_3N_4 (1500 Å) using low-pressure chemical vapor deposition (LPCVD) process. Then the conductive layer of Au/Ti film (Au 2000 Å and Ti 200 Å) was sputtered, allowing the recording electrode array structure made by lift-off technology. Afterwards, using a plasma-enhanced chemical vapor deposition (PECVD) process, the $SiO_2/Si_3N_4/SiO_2$ (4000 Å/5000 Å/1000 Å) triplex layers were formed as the passivation layer, on which the DEP electrode array structure and its passivation layer were produced in the same way. Finally, these passivation layers on the recording electrodes, DEP electrodes, and the bonding-pads were removed by reactive ion etching (RIE) (Fig. 1a–c). The wafers were diced and the chip (15 mm × 15 mm) was assembled on a printed circuit board (PCB) (60 mm × 60 mm) by wire bonding. A polystyrene ring (Ø9 mm) and a plastic petridish (Ø35 mm) with a hole (Ø11 mm) were positioned on and glued to the chip with Sylgard 184. Fig. 1d shows a photo of an assembled APSMEA device with a cell culture chamber. The recording electrodes were platinized in a solution containing 1% chloroplatinic acid and 0.01% lead acetate at a constant plating voltage of 0.8 V for 20 s.

2.3. Cell preparation

In accordance with institutional guidelines for care and use of animals, dissociated cortical neurons were prepared as previously described (Yu et al., 2004). Briefly, cortical tissue was taken from 18-day rat embryos and dissociated by trituration after digestion with papain (20 U/ml). The neurons were then resuspended in neurobasal medium with B27 supplement and 500 μM glutamine. The medium initially added to new cultures was also supplemented with 25 μM glutamate and 25 μM β-mercaptoethanol. With four different concentrations, i.e., 1×10^5 cells/ml, 2×10^5 cells/ml, 5×10^5 cells/ml and 1×10^6 cells/ml, neurons were seeded on the chips, which had been coated with poly-L-lysine (50 μg/ml) and laminin (10 μg/ml).

2.4. Cell manipulation

The method for cell manipulation was according to our previous studies (Yu et al., 2004). Briefly, the neurons in suspension were introduced onto the chips, and then manipulated by applying a pair of 5 MHz, 2 V sinusoidal signals with the phase-angle difference of 180° for 30 min. Using an upright microscope in the reflection mode equipped with differential interference contrast optic (DMR/HCS, Leica, Germany), the process of cell manipulation was monitored and the images from the microscope were acquired by a charge-coupled-device (CCD) video camera (WV-GP410, Panasonic, Japan). Controls were also prepared with no signals applied. These devices were then transferred into a humid, 37 °C and 5% CO_2 incubator. Feeding was performed with half of the culture medium changed twice a week.

2.5. Electrophysiology

The method for multi-site electrophysiological recordings was similar to that of Xiang et al. (2007a). Briefly, with the APSMEA connected with the commercial multi-channel amplifier and filtering stage (Cyberkinetics, Salt Lake City, UT), measurements were carried out in 14-day-old cortical cultures at 37 °C, allowing the signals from the recording electrodes amplified (5000×), band-pass filtered (250–7500 Hz), and sampled at 30 kHz/channel on 25 channels simultaneously. Using ±3 times of the standard deviation of the noise level as the thresh-

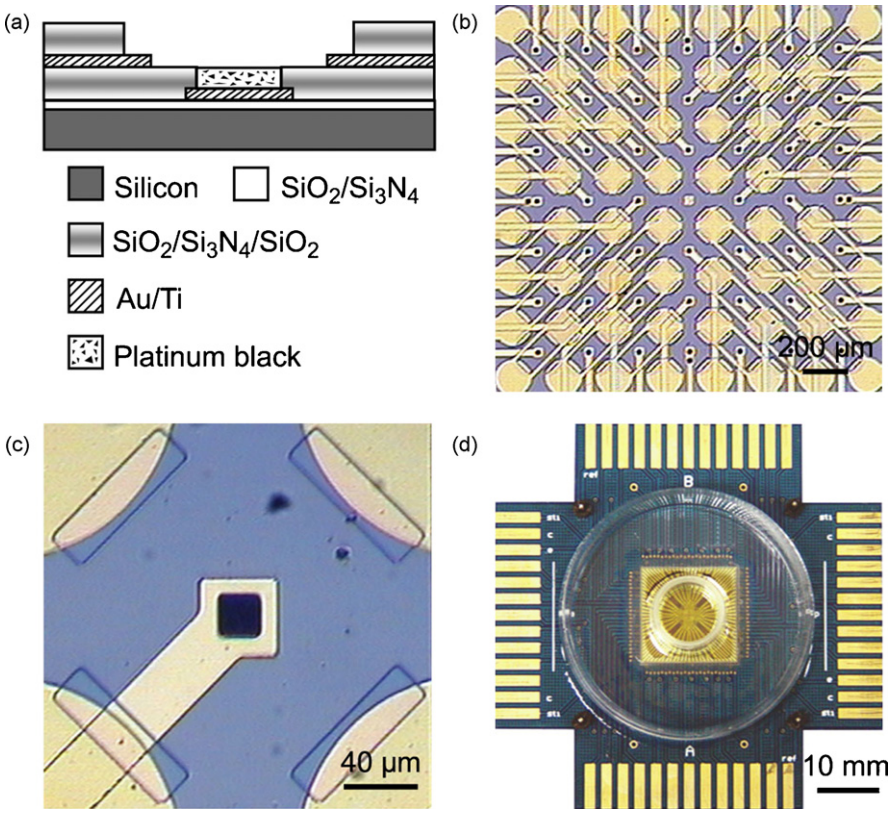

Fig. 1. Fabrication and encapsulation of APSMEAs. (a) Cross-sectional view of an APSMEA (not to scale). (b) Layout of the APSMEA with 49 groups of DEP electrodes arranged in a 7 × 7 square grid, at each center of which is the recording electrode (plated with platinum black). (c) Functional unit of the APSMEA with a recording electrode surrounded by four DEP electrodes. (d) A photo of an assembled APSMEA with a cell culture chamber.

old value for each channel, electrophysiological activities were detected on-line or off-line. Meanwhile, a spike-sorting algorithm (Shoham et al., 2003) was used to minimize the confusion from different neural signal sources picked up by the same electrode. NMDA (5 μM) and APV (5 μM) were also applied in the culture medium, and their related electrophysiological activities before and after drug administration were both recorded for 10 min. The excitatory or inhibitory effect of drugs on neurons was then simply defined as percentage changes in sum of spikes over all the active sites and the time course with drug treatment, compared with that of control.

3. Results and discussion

3.1. Convenient fabrication and flexible encapsulation of APSMEAs

Using standard microelectronic fabrication technologies, APSMEAs were conveniently fabricated, providing their high reproducibility of manufacture and consistent performance due to the tight dimensional control of photolithography. Within one microchip, there were 48 recording electrodes (20 μm^2 each) plus one pad located in the center, arranged in a matrix of 7 × 7, with 225 μm center-to-center spacing (Fig. 1b). To enable the establishment of dielectrophoretic traps, 49 groups of DEP electrodes were fabricated to surround the above electrodes (Fig. 1a–c). The DEP electrodes were also shaped according to the equipotential boundaries determined by the curves ($xy = \pm 6400$), which would facilitate the generation of negative DEP forces as previously described (Yu et al., 2004). Due to the complexity of the array with the combination of the two types of electrodes, the layout of the leads was carefully considered. The APSMEA was subsequently encapsulated with the PCB according to the specifications of the user, and the pads on the PCB were readily connected to the commercial amplifier with zebra strips (Fig. 1d).

All the recording electrodes within an APSMEA were platinized with a constant voltage simultaneously (0.8 V, 20 s), with the results shown in Fig. 1b and c. The platinum plated on electrodes served as an excellent polarizable interface in solution, which consequently decreased the noise and increased the signal/noise ratio (Gesteland et al., 1959).

3.2. Manipulation of neurons with arrayed negative DEP forces

In agreement with our earlier report (Yu et al., 2004), arrayed negative DEP forces were generated in the culture medium, by applying a pair of 5 MHz, 2 V sinusoidal signals with the phase-angle difference of 180° on the four DEP electrodes each group, and the cells, as shown in Fig. 2a–d, were manipulated within 30 min by means of being pushed and positioned over the recording electrodes where the electrical field was minimal and harmless to the cells. A very few of neurons were positioned on

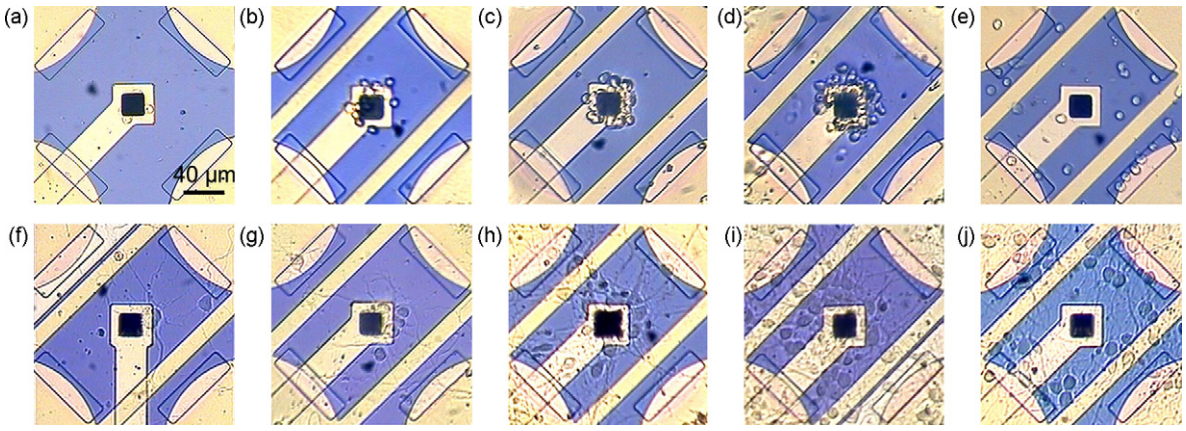

Fig. 2. Positioning of neurons with negative DEP forces at different cell concentrations. (a)–(d) Positioned neurons at the seeding concentrations of 1×10^5 cells/ml, 2×10^5 cells/ml, 5×10^5 cells/ml, and 1×10^6 cells/ml, respectively. (f)–(i) Outgrowth of neurites *in vitro* after 3-day culture corresponding to (a)–(d), respectively. (e) Neurons seeded without negative DEP forces at the concentration of 5×10^5 cells/ml and (j) growth of randomly positioning cell bodies after 3 days.

the recording electrodes at the concentration of 1×10^5 cells/ml, and they accumulated with the increase of cell concentrations, until a large number of them gathered around the recording electrodes at the concentration of 1×10^6 cells/ml. Obviously different from the negative DEP forces-treated patterns, neurons for the control randomly settled on the APSMEA (Fig. 2e). Supporting the cell growth, the culture medium used during the manipulation of neurons need not be changed (Yu et al., 2004).

Although it was reasonable that the proportion of the recording electrodes covered with neurons started relatively low and gained as time passed, cells were apt to be manipulated at the initial time of being seeded. At the lowest concentration of 1×10^5 cells/ml, the proportion arrived at $44.4 \pm 12.7\%$ after applying negative DEP forces just for 5 min, and finally $76.4 \pm 9.8\%$ for 30 min ($n = 4$, separate APSMEAs). At the concentration of 1×10^6 cells/ml, each of the electrodes possessed of at least one cell after 5 min without exception, and continuation of the DEP forces resulted in congregation of cells over the electrodes ($n = 4$, as above). At the concentrations of 2×10^5 cells/ml and 5×10^5 cells/ml, the proportions of $88.2 \pm 3.2\%$ and $99.5 \pm 1.0\%$ occurred after 30 min ($n = 4$, as above), respectively. Fig. 3a shows a panoramic view of all the recording electrodes with neurons positioned on at the concentration of 5×10^5 cells/ml. To facilitate the formation of networks after the outgrowth of neurites, neurons also settled down between the groups of DEP electrodes due to the passivation layer maintained there. Taken together, these results showed that APSMEAs could conveniently and effectively position desired numbers of neurons over the recording electrodes.

3.3. Formation of neuronal networks in vitro

Compared with the control (Fig. 2j), the neurites of the positioned neurons (3 DIV) freely grew out to contact with

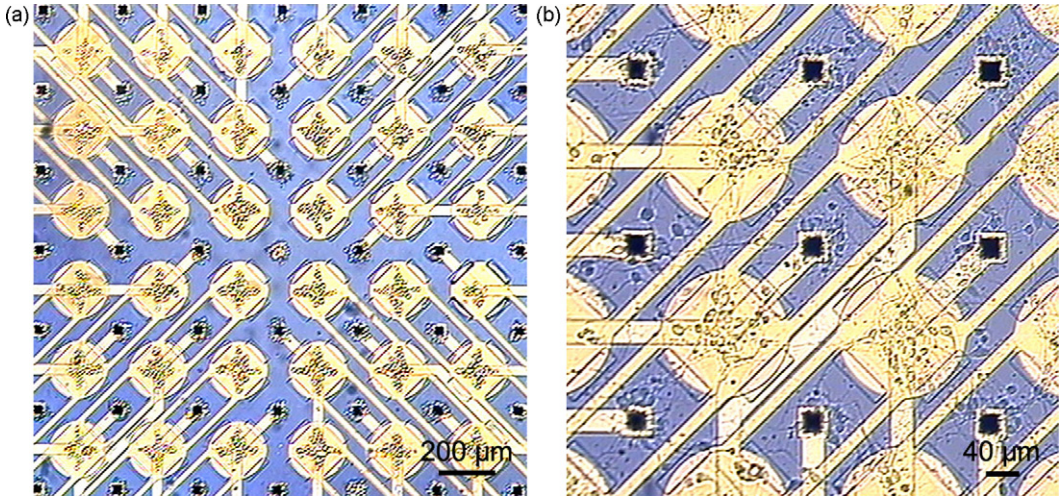

Fig. 3. Positioning of neurons with arrayed negative DEP forces on multiple recording electrodes. (a) An example of positioned neurons on an APSMEA at the seeding concentration of 5×10^5 cells/ml and (b) the outgrowth of their neurites *in vitro* after 3-day culture. Note that cells settled down between the groups of DEP electrodes due to the passivation layer maintained there, allowing neurons to form network easily.

other neurons while most of their somas retained in position (Figs. 2f–i and 3b), indicating that APSMEAs ensured the sufficient synapse formation between neurons and kept the functional activities of neuronal networks during development. Throughout 2-week culture period, the neurites continued to grew dense and the glial cells proliferated, leading the engineered structure of the neuronal networks to become slightly blurred. It is important to mention that glial cells provide necessary trophic factors for cultured neurons, and that direct contact between neurons and glia are also crucial for neuronal survival (Potter, 2001; Slezak and Pfrieger, 2003).

3.4. Detection of spontaneous electrophysiological activities with APSMEAs

To demonstrate the sensing properties of APSMEAs, we recorded spontaneous electrophysiological activities from cortical neuronal networks (14 DIV), at the seeding concentration of 5×10^5 cells/ml (corresponding to 1.56×10^5 cells/cm^2 in culture area), either engineered or not (referred to as DEP and Ctrl). It is should be noted that appropriate cell seeding concentration is of importance to neural signal detection. In this work, the seeding concentration of 5×10^5 cells/ml or less is appropriate for developing neuronal network *in vitro* and preventing overpopulation at a single electrode.

With the single action potentials (AP) usually biphasic or triphasic in shape, such activities recorded by the recording electrodes were similar both from DEP and Ctrl (Fig. 4a). These activities were inhibited by 1.5 μM TTX (known to be a blocker of voltage-dependent Na^+ channels), verifying the physiological basis for the observed extracellular action potentials (data not shown). These results were consistent with the earlier reports (Eytan et al., 2003; Gross et al., 1977; Jimbo et al., 1999; Nam et al., 2004; Xiang et al., 2007a). Besides, the recording electrodes could be also used to stimulate neurons non-destructively (data not shown), providing another application of APSMEAs in neuroscience studies (Eytan et al., 2003; Jimbo et al., 1999; Wagenaar et al., 2005).

It is well known that the AP initiated from the axon hillock where the axon branches from the soma. With soma (~10 μm) positioned on the recording electrode (20 μm^2), the electrode is thus much easy to couple with the axon hillock and pick up AP. In this way, the proportion of electrodes with the electrophysiological signals detected within each APSMEA was taken into account as well as the maximal amplitude of the signals from each electrode. As shown in Fig. 4b, $93.8 \pm 7.4\%$ of the recording electrodes from DEP networks were active, significantly greater than $60.9 \pm 14.2\%$ from Ctrl networks ($P < 0.01$; Student's t-test; $n = 4$, separate APSMEAs). With $166.7 \pm 78.5\ \mu V_{peak-peak}$ of the maximal amplitude of the signals from DEP networks ($n = 180$, recording electrodes pooled from four separate APSMEAs in Fig. 4b) and $116.3 \pm 68.0\ \mu V_{peak-peak}$ from Ctrl networks ($n = 117$, as above), the cumulative percentage histograms also showed the significant differences between DEP and Ctrl networks ($P < 0.001$; two-sample Kolmogorov–Smirnov tests), as shown in Fig. 4c. Although it was unavailable to strictly constrain the positioned neurons in their original locations after 2-week culture, these results strongly suggested that APSMEAs achieved the improved performance in MEA-based recordings.

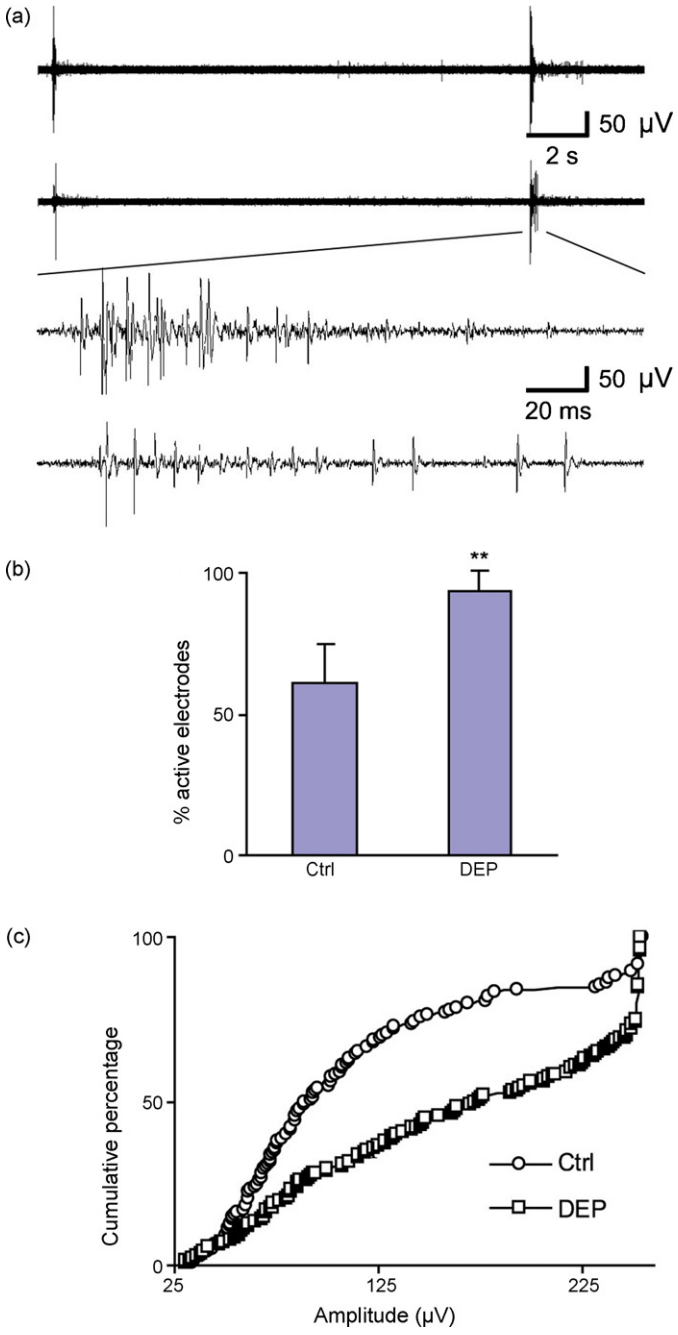

Fig. 4. Multi-site recordings of extracellular spontaneous electrophysiological activities from cortical neuronal networks (14 DIV) either engineered or not (referred to as DEP and Ctrl) at the seeding cell concentration of 5×10^5 cells/ml. (a) Representative 20-s long traces recorded with the two electrodes (upper panel) and typical bursts shown on the expanded time scale (lower panel, 0.2-s traces). (b) Comparison of the percentage of active electrodes within each APSMEA between Ctrl and DEP. $^{**}P < 0.01$, Student's t-test, $n = 4$ (separate APSMEAs). Error bars represent S.D. (c) Cumulative percentage histograms of the maximal amplitudes of the signals from Ctrl ($n = 117$, recording electrodes pooled from four separate APSMEAs in (b)) and DEP ($n = 180$, as above).

Based on the multi-site electrophysiological recordings of the APSMEA, the responses of neuronal populations *in vitro*

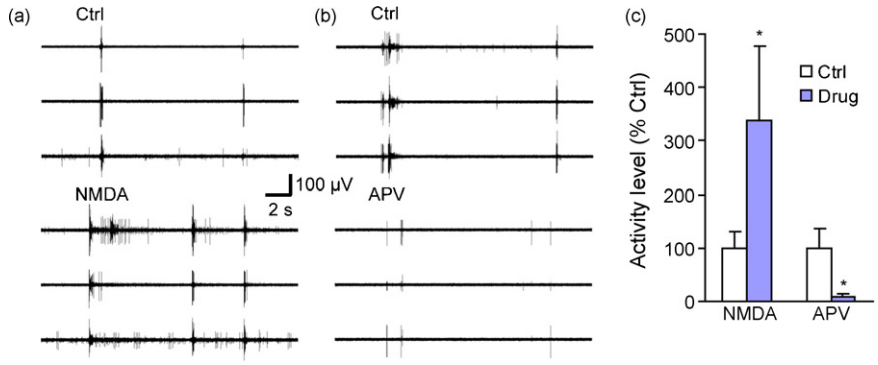

Fig. 5. Drug evaluation using APSMEAs with cortical neurons (14 DIV). (a) and (b) Typical examples of spontaneous activity (20-s plot of three channels) before and after neurons were treated with 5 μM NMDA and 5 μM APV, respectively. (c) Histograms of NMDA- and APV-induced changes in activity level compared with the control (100%). *$P < 0.05$, Student's t-test, $n = 4$ (separate APSMEAs). Error bars represent S.D.

(14 DIV) during drug administration, to illustrate its general use, were monitored. As shown in Fig. 5, the NMDA receptor agonist NMDA (5 μM) triggered the neurons to fire intensively, causing the induced activity level three times more than that of controls ($n = 4$, separate APSMEAs). On the contrary, 5 μM APV (NMDA receptor antagonist) drastically decreased the activity level ($7.8 \pm 7.5\%$ of control; $n = 4$, as above). Consistent with the earlier reports (Kemp and McKernan, 2002; Xiang et al., 2007a), NMDA and APV showed the excitatory and inhibitory effects on neuronal networks, respectively.

4. Conclusions

With the optimized electromotive parameters, the strategically designed DEP electrodes on the APSMEA effectively positioned desired numbers of neurons onto multiple recording electrodes simultaneously and without injury. The neurites then grew out freely to form functional synapses while the somas were retained in the defined areas of the APSMEA. The electrophysiological activities of neuronal networks were examined by the recording electrodes, demonstrating the marked improvement of the APSMEA in performance and its utility in bioassay.

The convenience and effectiveness of such system should stimulate the use of APSMEAs, facilitating a better understanding of the function of neuronal networks *in vitro*. The methods of DEP forces employed in this study were not restricted to neurons, which would satisfy many specific needs for the manipulation and positioning of different kinds of cells for a variety of desired evaluations.

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