

Agarose microwell based neuronal micro-circuit arrays on microelectrode arrays for high throughput drug testing

Gyumin Kang,^a Ji-Hye Lee,^b Chang-Soo Lee^b and Yoonkey Nam^{*a}

Received 2nd June 2009, Accepted 14th August 2009

First published as an Advance Article on the web 10th September 2009

DOI: 10.1039/b910738j

For cell-based biosensor applications, dissociated neurons have been cultured on planar microelectrode arrays (MEAs) to measure the network activity with substrate-embedded microelectrodes. There has been a need for a multi-well type platform to reduce the data collection time and increase the statistical power for data analysis. This study presents a novel method to convert a conventional MEA into a multi-well MEA with an array of micrometre-sized neuronal culture ('neuronal micro-circuit array'). An MEA was coated first with cell-adhesive layer (poly-D-lysine) which was subsequently patterned with a cell-repulsive layer (agarose hydrogel) to both pattern the cell adhesive region and isolate neuronal micro-circuits from each other. For a few weeks, primary hippocampal neurons were cultured on the agarose microwell MEA and the development of spontaneous electrical activities were characterized with extracellular action potentials. Using neurotransmission modulators, the simultaneous monitoring of drug responses from neuronal micro-circuit arrays was also demonstrated. The proposed approach will be powerful for neurobiological functional assay studies or neuron-based biosensor fields which require repeated trials to obtain a single data point due to biological variations.

Introduction

A microelectrode array (MEA) is a planar substrate embedded with an array of microelectrodes that are capable of measuring extracellular action potentials from electrogenic tissues.^{1–4} It has been used to study the electrophysiological properties of *in vitro* neural tissues such as brain slices and dissociated neuronal cultures. Cultured neurons form an interconnected network on an MEA and network dynamics including synaptic connections, signal propagations, and plasticity have been extensively studied.^{5–9} In addition, there have been advances in MEA platforms based on state-of-the-art nano- and micro-technology for the purpose of improving electrode quality,^{10,11} increasing the spatial resolution,¹² enhancing the selectivity of neural interfaces,^{13,14} and patterning cultured neuronal networks.^{15,16}

Researchers have tried to use the dissociated neuronal cultures as a cell-based biosensor using the signals detected by MEAs for past decades. Gross *et al.* first showed that *in vitro* non-sensory neuronal networks on MEAs can be used as biological sensors which can recognize specific chemical changes.¹⁷ In addition, dissociated neuronal networks out of various neural tissues—hippocampus, cortex, suprachiasmatic nucleus, dorsal root ganglion, and embryonic stem cell—were tested using MEAs to characterize network responses to pharmacological agents and toxic compounds.^{18–22} The most common and convenient practice to quantify the network response is to find the average spike rate or burst rate of all available channels and use either or both of them as an activity index of one neuronal network cultured on

an MEA.^{18,23,24} The spike rate stands for single neuron level activity in the network, while the burst rate represents the network-level activity.

Multiple MEA experiments under various conditions are often performed to collect sufficient data points that are necessary for the statistical analysis. Unlike common cell culture substrates such as coverslip or Petri-dish, the supply of an MEA is limited due to its cost and life cycle, thus the number of MEAs that can be used at a time is limited accordingly. Under these constraints, it is very difficult to gather large sets of data points readily from multiple MEA experiments. In addition, repeated MEA experiments may take days, weeks, or months depending on the experimental conditions such as cell culture techniques and culture ages. Thus, a multi-well approach for an MEA based biosensing applications can improve the statistical analysis power to estimate network responses more reliably and greatly enhance the throughput of a single MEA experiment. To this end, some commercial multi-well or multi-chamber type MEAs are available with a limited number of wells from MEA supplier, such as Multi Channel Systems (Reutlingen, Germany), Ayanda Biosystems (Lausanne, Switzerland) and Alpha MED Sciences (Osaka, Japan).

The goal of this work is to convert the conventional MEA platform into a multi-well MEA platform without redesigning either the MEA or the neural recording system. The main idea is to form an array of micrometre-sized neuronal networks on an MEA and record network responses from them simultaneously. To this end, we adopted the 'micro-island culture technique' which grows neurons in a micrometre-sized confined area by spotting poly-lysine or collagen on an agarose hydrogel layer.²⁵ The formed micrometre-sized neuronal networks have been proposed as a miniaturized neuronal circuit model due to dense synapse formation and bursting electrical activity.^{25–31} The

^aDepartment of Bio and Brain Engineering, KAIST, 335 Gwahangno, Yuseong-gu, Daejeon, 305-701, Korea. E-mail: ynam@kaist.ac.kr; Fax: +82 42 869 4310; Tel: +82 42 869 4322

^bDepartment of Chemical Engineering, Chungnam National University, 79 Daehangno, Yuseong-gu, Daejeon, 305-764, Korea

technical challenge is to fabricate an array of isolated neuronal micro-circuit whose size is in the order of 100~200 micrometres in diameter on an MEA so that they can be coupled to individual microelectrodes and multiple networks can be monitored simultaneously.

Here, we report the fabrication and verification of an agarose hydrogel based multi-well MEA. Micrometre-sized wells were fabricated on an MEA using micro-molding in capillary (MIMIC) method and dissociated hippocampal neurons were cultured on the MEA. An array of isolated neuronal micro-circuit was coupled to microelectrodes and the development of extracellular neural signals was investigated for a few weeks. The new MEA platform was further verified by collecting tens of independent drug responses from multiple neuronal micro-circuits simultaneously.

Experimental

Fabrication of PDMS mold

A PDMS (poly(dimethylsiloxane)) mold was fabricated by soft-lithography (Fig. 1). First, SU-8-2025 (MicroChem, MA) was spin-coated on a 4-inch silicon wafer at 500 rpm for 10 sec and 1600 rpm for 30 sec to obtain 50 μm thickness. Then the coated wafer was baked at 65 $^{\circ}\text{C}$ for 3 min and 95 $^{\circ}\text{C}$ for 7 min in a convection oven. The soft-baked SU-8-2025 was exposed to UV light for 20 sec at 20.3 mW/cm² under the photofilm mask and was baked at 65 $^{\circ}\text{C}$ for 1 min and at 95 $^{\circ}\text{C}$ for 3 min in a convection oven. After the hard baking, SU-8-2025 was developed in SU-8 developer for 3 min followed by thorough rinse with de-ionized (DI) water. The developed SU-8 master was blow-dried with nitrogen gas.

To cast a PDMS mold, the mixture of PDMS prepolymer and curing agent (10 : 1 (w/w), Sylgard 184 silicone elastomer kit, Dow Corning Corp., MI) was poured on the master and cured at 60 $^{\circ}\text{C}$ in a convection oven for 2–3 hours. Cured PDMS was peeled off from the master and cut into pieces of 7 mm $\times$ 7 mm square. The PDMS mold was consisted of 100 μm in diameter circular positive features with 200 μm pitch in a square array.

Substrate preparation

Planar-type microelectrode arrays (MEAs) purchased from MultiChannel Systems (Reutlingen, Germany) had 59 micro-electrodes (TiN, 30 μm in diameter, 200 μm spacing, Si₃N₄ insulation, electrode impedance of 30 k Ω at 1 kHz) for extracellular neural recording and one large TiN electrode for grounding.⁴

The surface of MEA was sonicated with acetone, isopropyl alcohol (IPA), and DI water for 5 min per each and blow-dried by air stream. MEAs were coated with poly-D-lysine (PDL, MW 70,000–150,000, Sigma-Aldrich, MO) by applying a few drops of PDL solution (0.1 mg/ml in borate buffer, pH 8.5) on the MEA surface at 37 $^{\circ}\text{C}$. After overnight PDL adsorption, PDL solution was aspirated and the MEAs were soaked in DI water for 6 hrs to remove PDL residues. The substrates were finally dried by air-stream and ready to be assembled with a PDMS mold.

Microwell array fabrication by agarose hydrogel

Microwell arrays were fabricated by patterning agarose hydrogel using a soft-lithographic technique known as MIMIC (micro-molding in capillary). MIMIC utilizes a capillary force to fill the microchannels with molding solutions. Both cured PDMS

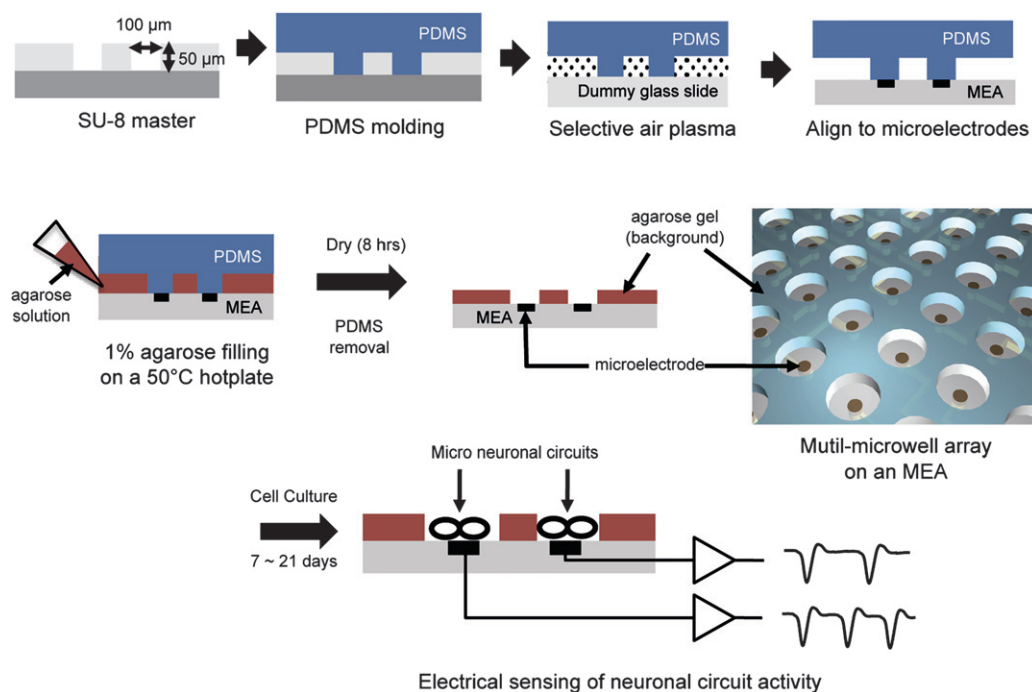


Fig. 1 Schematic illustration of multi-well MEA fabrication and neural signal recording process. A PDMS mold was replicated from a SU-8 master and its internal walls were selectively treated by air plasma. The PDMS mold was aligned and attached with an MEA and agarose hydrogel microwells were replicated by MIMIC process. Neuronal micro-circuits were formed only inside the wells.

surfaces and PDL coated MEA surfaces were not so hydrophilic to generate capillary force needed for the MIMIC. Thus, the PDMS mold was attached to a dummy glass slide and oxidized in an air plasma cleaner (6 min at 30 W, $\approx$ 200 mTorr, model: PDC-001, Harrick Plasma, NY) to render the exposed PDMS surface hydrophilic (Fig. 1). Through this process, only the side walls of the PDMS molds were selectively oxidized, while the bottom side that made a contact with the dummy glass slide remained hydrophobic, which eliminated any undesirable permanent bonding problem between the hydrophilic PDMS surface and silicon nitride insulator surface of an MEA.^{32,33} The plasma-treated PDMS mold was aligned with an MEA under an inverted transmitted-light microscope and brought in contact with the MEA surface to form a reversible bonding and it was transferred onto a hotplate at 55 °C.

Agarose (gel strength > 12 000 g/cm², gelling range: 36–39 °C, melting range: 87–89 °C, Amresco Inc., Ohio) was dissolved in 90 °C DI water and 2% (w/v) agarose solution was formed. After placing the PDMS-MEA assembly on the hotplate, the PDMS mold was filled with the agarose solution by applying the 20 μ l of agarose solution to two sides of the PDMS mold (Fig. 1). The filled PDMS-MEA assembly was placed at room temperature for 8 hrs. Once the agarose hydrogel was completely dried, the PDMS mold was detached manually with care and the agarose-microwell was completed (Fig. 1). The dried MEAs with agarose microwells were kept in a plastic container until future usage.

The fabricated thin agarose microwell structures were stable for over 3 weeks in culture medium without any detachment, which was reportedly a problem in long-term culture condition.³⁴ The fabricated multi-well MEAs could be stored in dried state for a week and still be used without the compromise of its functions, which would be advantageous for commercial development.

To confirm the formation of the microwell structure, we measured the surface profile of dried agarose microwell using White Light Interferometry (Pemtron, Korea). Next, we estimated the actual thickness of the microwell when it was swollen in solution. Since the White Light Interferometry apparatus was not operable in solution, the following procedure was used: The molded agarose microwell was soaked in poly-L-lysine-FITC (PLL-FITC; Sigma-Aldrich) solution and the laser-scanning confocal microscope (LSM510, Car Zeiss, Germany) was used to collect z-stacked images.

To use the substrates for cell culture experiments, they were sterilized by immersing them in 70% ethanol solution for 3 min and dried thoroughly in a sterile cell culture hood. A PDMS ring (1 cm inner radius, 5 cm outer radius, and 2 cm height) was attached on the MEA and the ring formed a cell culture chamber to hold approximately 1 ml of culture medium.

Cell culture

Primary hippocampal neurons were cultured in serum-free condition. Hippocampus dissected from E-18 Sprague-Dawley rat embryos were treated with 0.25% trypsin for 15 min at 37 °C. After rinsing residual trypsin with Hank's Balanced Salt Solution (HBSS), the hippocampus was triturated using a fire-polished Pasteur pipette in 1 ml of HBSS. The cell suspension was centrifuged at 1000 rpm for 3 min and cell pellet was extracted by aspirating supernatant. The cell pellet was suspended in

Neurobasal media (Invitrogen, MD) supplemented with B-27 (Invitrogen, MD), 2 mM Glutamax (Invitrogen, MD), 12.5 μ M L-glutamic acid (Sigma-Aldrich, MO) and Penicillin–Streptomycin (Invitrogen, MD). Dissociated cells were plated at the density of 800 cells/mm².

Cultures were maintained under 5% CO₂ in an incubator at 37 °C. After 1 DIV (days *in vitro*), the substrate surface was gently washed by exchanging media few times to remove floating cells from the non-adhesive agarose region, which covered the large portion of the surface. To supply fresh nutrient, the half of media was replaced with new maintenance media (plating media without L-glutamic acid) every 3–4 days till the termination of the culture.

At the completion of these experiments, the agarose hydrogel was removed by dipping the MEA in boiling water for 2–3 min. The MEA was further sonicated in 1% Terg-a-zyne® enzyme detergent (Sigma-Aldrich, MO) solution for 15 min, rinsed in running DI water, and dried under an air stream. We reused the MEAs for further experiments.

Neural signal recording and analysis

To record neural signals from the cultured neurons on multi-well MEAs, they were connected with an MEA 1060 amplifier (Gain: 1200, bandwidth: 10 Hz – 5 kHz, MultiChannel Systems, Germany) (Fig. 1). The recording was done under the following condition: the substrate temperature was set at 37 °C and the water-impermeable plastic membrane (ALA Scientific Instruments Inc., NY) was installed to prevent water evaporation. Humidified 5% CO₂ gas was continuously streamed to maintain the media pH.

Multichannel neural signals were digitized at 40 kHz and stored in PC using MC Rack (MultiChannel Systems, Germany). Recorded raw data stream were filtered with 200 Hz digital high pass filter and spikes were detected by setting the threshold level at –6 times the standard deviation of the background noise of each channel. Spike time stamps were imported from a spike-train analysis program NeuroExplorer (Nex Technologies, MA) and spike rate and burst rate was calculated. The spike bursting was analyzed using surprise based algorithm with a minimum surprise parameter of 2.

Spike rate (spikes/sec) and burst rate (bursts/min) were used as measures of neural activity. A channel was classified as an active channel if the average spike rate was greater than 0.1 spikes/sec from 10 min recording data.¹⁶ Spike-rate histogram was constructed by counting the number of spikes in specific time bin (10 sec). For a channel to be included in the burst rate count, the average burst rate should be greater than 0.5 bursts/min in 10 min recording data. Statistical data was presented in mean $\pm$ S.D. (standard deviation).

Drug response test

Drug response test was performed at 7 DIV. After 10 min of stabilization period ('baseline'), 20 μ L of fresh culture medium was directly applied to the existing medium and the activity was measured for 10 min ('control'). The control activity served as a negative control for the following drug responses. Then, the same volume of bicuculline (14340, Sigma-Aldrich, MO)

solution was applied to the medium twice every 10 min while recording the activity. This allowed us to test two different concentration level of bicuculline, which was calculated to be 20 μM and 40 μM , respectively. Using the same protocol, AP5 (A8054, Sigma-Aldrich, MO) with the final concentration of 25 μM and 50 μM and NMDA (N-methyl-D-aspartic acid, M3262, Sigma-Aldrich, MO) with 20 μM and 40 μM were also tested.

Results

Fabrication of agarose microwells

Fig. 2(a) shows a phase contrast image of agarose microwell fabricated on an MEA using the MIMC technique. Each microwell was centred over a single microelectrode of the MEA and the bottom of each microwell was a cell adhesive PDL surface. The remainder of the surface was covered with the non-adhesive agarose hydrogel. Measured electrode impedance was $73.9 \text{ k}\Omega \pm 8.2 \text{ k}\Omega$ (mean $\pm$ S.D., $n = 56$, $V = 100 \text{ mV}$, $f = 1 \text{ kHz}$). To minimize any structural defects while casting the microwells, it was necessary to wait for the agarose hydrogel to dry completely before removing the PDMS mold. The fabrication process was reliable and reproducible to integrate microwells up to 2500 wells per 1 cm^2 . The selective air plasma treatment inside the channel allowed a rapid filling of the boiled agarose solution without any other measures such as negative pressure³⁵ or ethanol solvent.^{28,34}

The surface profile of dried microwell is shown in figure 2(b). The measured diameter of the microwell was 100 μm and the average thickness was $796.6 \pm 75.5 \text{ nm}$ ($n = 4$) at

dried state. The estimated thickness of the hydrated agarose hydrogel was 13.3 μm . This was thinner than the height of the PDMS mold (50 μm) that was used to cast the microwell structure.

Neuronal micro-circuit formation in agarose microwells

Fig. 2(c) shows neuronal micro-circuit array formed on an MEA at 7 DIV. Neuronal growth was confined inside the agarose microwells and there was no neuron or neurite growing on the background agarose region. There were no neurites bridging the space between the wells.

Fig. 2(d) shows the distribution of the number of neurons in each well at 1 DIV with the cell plating density of 800 cells/ mm^2 . Nearly all of the microwells (over 99%) had at least one neuron at 1 DIV and the average number of neurons in each well was 11.4 ± 6.8 cells ($n = 354$ microwells from 6 MEAs). The average number was higher than the expected number (6.3 cells/well) of neurons calculated from the plating density and the microwell size. The discrepancy could be due to the migration of non-adhered neurons on the background non-adhesive area into nearby microwells.³⁶

Fig. 2(e) shows the development of a neuronal micro-circuit in a same microwell over three weeks. At 1 DIV, neurons were localized in the microwell and adhered to the MEA surface. At 3 DIV, neurite extensions were prominent and neurites were entangled in the well. The neurites could not escape from the well due to the background agarose layer. Thus, the neurite outgrowth was successfully confined to the 100 μm wide circular area. After 7 DIV, neurites started to unravel and somata seemed

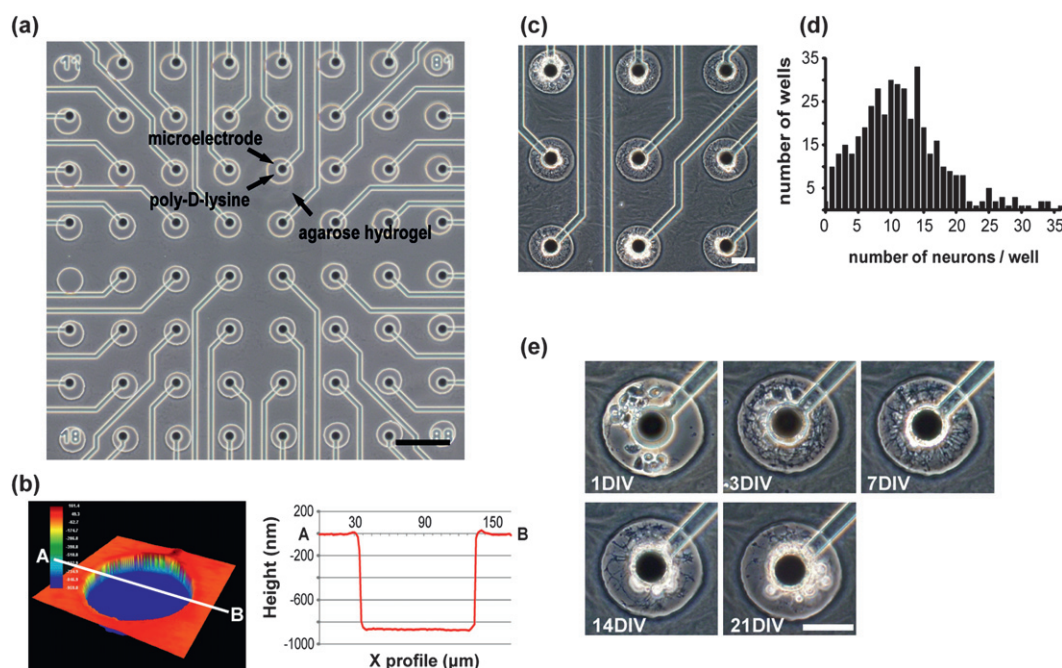


Fig. 2 Multi-well MEA. (a) A phase contrast microscopic image of agarose microwells on an MEA. One agarose microwell is composed of a microelectrode, poly-D-lysine coated surface, and agarose hydrogel wall. Scale bar: 200 μm . (b) Surface profile of dried agarose microwell measured using white light interferometry. Measured diameter: 100 μm , depth: 860 nm. (c) A phase contrast microscopic image of neuronal micro-circuits on a multi-well MEA at 7 DIV. Scale bar: 50 μm . (d) Distribution of the number of neurons in a well at 1 DIV. ($n = 354$ wells from 6 MEAs) (e) The growth of a same neuronal micro-circuit for three weeks. This well is from the 3rd row, 1st column in (c). Scale bar: 50 μm .

to form a small cluster on the electrode. When there were more than three neurons in a microwell, neurons frequently formed a neural cluster and its size increased by time.

Characterization of neural signals

Neural recordings were carried out from 6 different MEAs at 5, 7, and 14 DIV. Extracellular spikes generated by neuronal

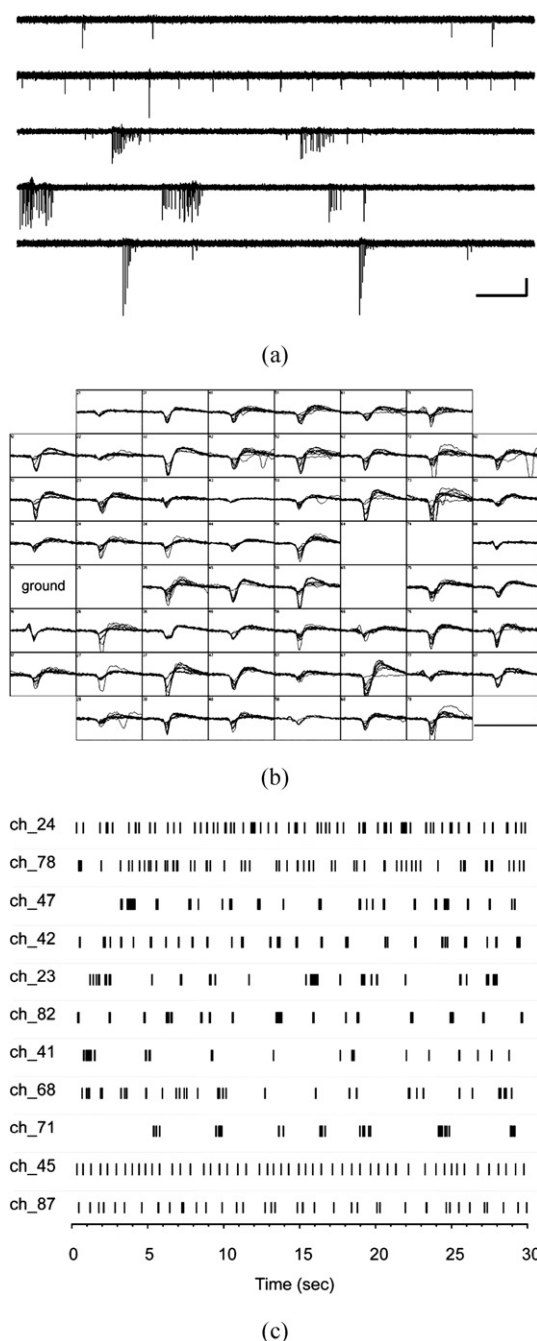


Fig. 3 (a) Extracellular neural signal traces recorded simultaneously from 5 different neuronal micro-circuits on a same MEA at 7 DIV. Scale bar: 500 ms, 100 μ V. (b) Overlay plot of 20 spike waveforms from 56 microelectrodes. Scale bar: 3 ms, 100 μ V. (c) Raster plot of spiking activity from an MEA.

micro-circuits in each microwell were measured by the 30 μ m TiN microelectrode located at the centre of the microwell.

Spontaneous neural activities developed from each neuronal micro-circuit were successfully measured after 5 DIV. Fig. 3(a) shows the representative traces recorded from MEAs at 7 DIV. Individual neuronal micro-circuits exhibited various patterns of activity including low frequency spike activity, high frequency spike activity, single spike activity, and burst activity, which were also reported in *in vitro* massive neuronal cultures and micro neuronal cultures.^{3,26,37} Fig. 3(b) shows the overlay of spike waveforms from each channel. The background noise level was about 10 μ V_{pp} and the average spike amplitude was 152 μ V_{pp} ($n = 21,638$) and it ranged from 30 to 697 μ V_{pp}. Occasionally, more than two different spike waveforms were detected from one channel. Fig. 3(c) shows the raster plot of spike trains from a single MEA. Cross correlation analysis of the spike activity in individual micro-circuits confirmed that the micro-circuits were functionally decoupled from each other (data not shown).

Development of spontaneous activity

To investigate the development of spontaneous network activity, neural recording data from 6 MEAs were analyzed.

Fig. 4(a) shows spike rate and burst rate at different developmental stages: 5 DIV, 7 DIV and 14 DIV. Both measures increased from 5 to 7 DIV as neurons matured. The number of

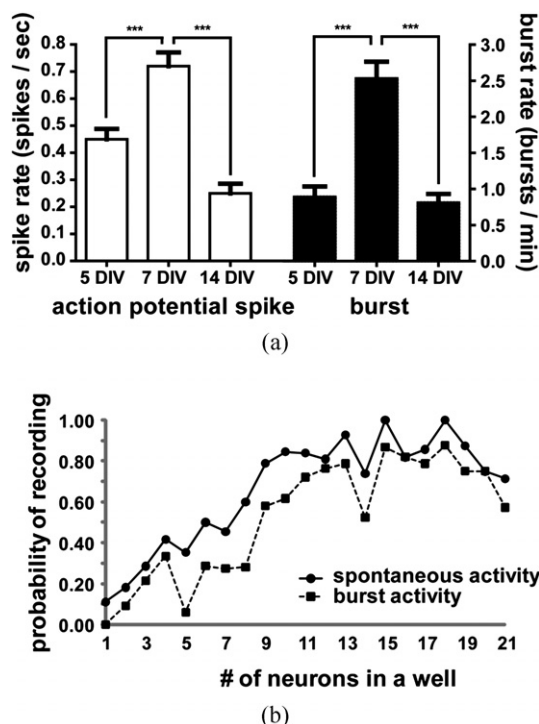


Fig. 4 (a) Spike rates and bursts rates of neuronal micro-circuits on multi-well MEAs at 5, 7 and 14 DIV. Both spike rate and burst rate were significantly high at 7 DIV compared to 5 DIV and 14 DIV. Mean $\pm$ S.E.M. $p < 0.0001$ (b) Percentage of active microwells according to number of neurons in an agarose microwell at 7 DIV. ($n = 350$ wells at 5 DIV, 348 wells at 7 DIV and 334 wells at 14 DIV from same multi-well MEAs. Among 354 wells, 4 wells at 5 DIV, 6 wells at 7 DIV and 20 wells at 14 DIV did not have neuronal micro-circuits.)

microwells that generated electrical activity ('active') also increased. The percentage of microwells per MEA that were active was as follows: $51.7 \pm 15.0\%$ (5 DIV), $66.4 \pm 20.6\%$ (7 DIV), and $31.9 \pm 18.5\%$ (14 DIV). The percentage of microwells that generated burst was $15.8 \pm 8.9\%$ (5 DIV), $51.1 \pm 21.6\%$ (7 DIV), and $24.9 \pm 12.3\%$ (14 DIV).

The number of neurons in microwells affected the activity of the microwells. Fig. 4(b) shows the percentage of microwells that were determined to be active from the detected spiking activity at

7 DIV. When there were more than 10 neurons in a microwell, it was very likely to be active. In contrast, microwells were mostly inactive when there were less than 3 neurons in a microwell. The lack of the activity with few neurons could be due to the insufficient coupling between neurons and the microelectrode. The microwells tend to generate bursting activity when there were more than 9 neurons seeded in the microwells.

Drug response tests

Fig. 5 shows individual multi-well MEA responses to bath application of NMDA (Fig. 5(a)), AP5 (Fig. 5(b)), and bicuculline (Fig. 5(c)) which activate or inhibit chemical synapses. The traces in Fig. 5 are the spike-rate histograms obtained from each well of the MEA sorted by the baseline activity level. NMDA, which activates excitatory glutamate receptors, increased the activity level and the increase of the dose of NMDA also increased the spike rate accordingly (Fig. 5(a)). Some of the microwells that were inactive at the baseline and control condition also responded to NMDA. Next, we tested with AP5 which blocks the NMDA receptors. At the concentrations used here, AP5 essentially abolished spike activity in all neuronal micro-circuits (Fig. 5(b)). This effect was reversible, as spike activity reinitiated upon AP5 washout. Moreover, some micro-circuits that were silent under baseline conditions exhibited spike activity after AP5 washout. Bicuculline, which blocks inhibitory GABA receptors, dramatically affected the temporal pattern of micro-circuit activity (Fig. 5(c)). All of the well activity changed into regular bursts with higher burst rates. The cross-correlation of microwell activities were nearly zero implying that the induced change of network activity was solely isolated from well to well. The similarity of resulting drug responses of each microwell to conventional MEA experiments implies that each microwell activity recorded by a single microelectrode could be a single trial that represents one MEA experiment with a massive cultured neuronal network.¹⁸

It was noticeable that the neuronal micro-circuits were responsive to neurotransmission modulators at relatively early stage of the culture (7 DIV). According to previous reports,^{38,39} the increase of neuron density accelerates maturation of neuron and increases synaptic protein level and glutamate level. A confined neuronal growth in microwells locally increased the neuron density (about 1400 cell/mm²) and it seemed to accelerate the maturation process. This could be advantageous for neuron-based sensor applications in a sense that one can obtain 'functional and responsive' neuronal cultures at an earlier period.

Summary and Conclusions

In this work, a novel agarose hydrogel based multi-well type MEA platform for a neuron-based biosensor has been proposed. It provided a high-density array of neuronal micro-circuits on a single MEA and simultaneous neural recordings from individual circuits. This can be used as a substitute for repeated MEA trials to obtain a single data point for statistical purposes. In combination with the state-of-the-art microfluidic platforms, the current platform can be a powerful tool for high-throughput neuron-based drug screening systems.

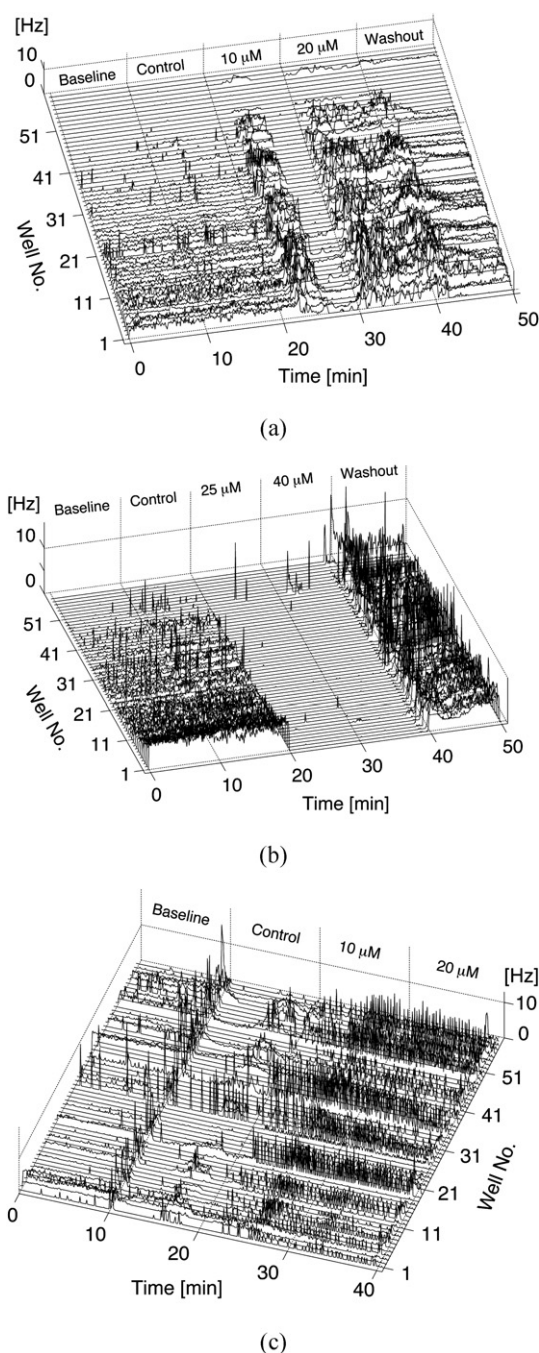


Fig. 5 Drug responses collected from neuronal micro-circuit arrays in a single MEA trial. (a) NMDA, (b) AP5, (c) bicuculline. Each trace represents a spike rate histogram (bin size: 10 sec).

Acknowledgements

We thank NanoBiotechlaboratory and Biophotonics laboratory in KAIST for the use of their facilities and Jisoon Lim and Minjee Jang for very helpful assistance. This work was supported by the System IC 2010 Program of Ministry of Knowledge Economy in Korea, KRF-2007-313-D00169, KRF-2008-313-D00614 grants from Ministry of Education, Science And Technology in Korea, and Chung Moon Soul Center for Bio-information And Electronics in KAIST.

Notes and references

- 1 C. A. Thomas, Jr., P. A. Springer, G. E. Loeb, Y. Berwald-Netter and L. M. Okun, *Exp. Cell Res.*, 1972, **74**, 61–66.
- 2 J. Pine, *J. Neurosci. Methods*, 1980, **2**, 19–31.
- 3 M. H. Droge, G. W. Gross, M. H. Hightower and L. E. Czisny, *J. Neurosci.*, 1986, **6**, 1583–1592.
- 4 U. Egert, B. Schlosshauer, S. Fennrich, W. Nisch, M. Fejt, T. Knott, T. Muller and H. Hammerle, *Brain Research Protocols*, 1998, **2**, 229–242.
- 5 Y. Jimbo, T. Tateno and H. P. Robinson, *Biophys. J.*, 1999, **76**, 670–678.
- 6 G. Shahaf and S. Marom, *J. Neurosci.*, 2001, **21**, 8782–8788.
- 7 Y. Nam, J. Chang, D. Khatami, G. J. A. B. G. J. Brewer and B. C. A. W. B. C. Wheeler, *IEE Proceedings - Nanobiotechnology*, 2004, **151**, 109–115.
- 8 D. A. Wagenaar, J. Pine and S. M. Potter, *Journal of Negative Results in BioMedicine*, 2006, **5**, 16.
- 9 J. D. Rolston, D. A. Wagenaar and S. M. Potter, *Neuroscience*, 2007, **148**, 294–303.
- 10 G. Gabriel, R. Gomez, M. Bongard, N. Benito, E. Fernandez and R. Villa, *Biosens. Bioelectron.*, 2009, **24**, 1942–1948.
- 11 T. Gabay, M. Ben-David, I. Kalifa, R. Sorkin, Z. e. R. Abrams, E. Ben-Jacob and Y. Hanein, *Nanotechnology*, 2007, **18**, 35201.
- 12 S. Hafizovic, F. Heer, T. Ugniwenko, U. Frey, A. Blau, C. Ziegler and A. Hierlemann, *J. Neurosci. Methods*, 2007, **164**, 93–106.
- 13 S. K. Ravula, M. S. Wang, S. A. Asress, J. D. Glass and A. Bruno Frazier, *J. Neurosci. Methods*, 2007, **159**, 78–85.
- 14 B. J. Dworak and B. C. Wheeler, *Lab Chip*, 2009, **9**, 404–410.
- 15 I. Suzuki, Y. Sugio, Y. Jimbo and K. Yasuda, *Lab Chip*, 2005, **5**, 241–247.
- 16 Y. Nam, D. W. Branch and B. C. Wheeler, *Biosens. Bioelectron.*, 2006, **22**, 589–597.
- 17 G. W. Gross, B. Rhoades and R. Jordan, *Sens. Actuators, B*, 1992, **6**, 1–8.
- 18 G. W. Gross, B. K. Rhoades, H. M. E. Azzazy and W. Ming-Chi, *Biosens. Bioelectron.*, 1995, **10**, 553–567.
- 19 M. Parviz and G. W. Gross, *NeuroToxicology*, 2007, **28**, 520–531.
- 20 W. Nakamura, S. Honma, T. Shirakawa and K. Honma, *Nutritional Neuroscience*, 2002, **5**, 399–400.
- 21 S. K. Ravula, M. S. Wang, M. A. McClain, S. A. Asress, B. Frazier and J. D. Glass, *Neurosci. Lett.*, 2007, **415**, 34–39.
- 22 S. Illes, W. Fleischer, M. Siebler, H. P. Hartung and M. Dihne, *Experimental Neurology*, 2007, **207**, 171–176.
- 23 E. W. Keefer, A. Gramowski, D. A. Stenger, J. J. Pancrazio and G. W. Gross, *Biosens. Bioelectron.*, 2001, **16**, 513–525.
- 24 S. I. Morefield, E. W. Keefer, K. D. Chapman and G. W. Gross, *Biosens. Bioelectron.*, 2000, **15**, 383–396.
- 25 G. Banker and K. Goslin, *Culturing nerve cells*, MIT Press, Cambridge, Mass., 1998.
- 26 M. M. Segal and E. J. Furshpan, *J. Neurophysiol.*, 1990, **64**, 1390–1399.
- 27 J. M. Bekkers and C. F. Stevens, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 7834–7838.
- 28 C. M. Nelson and C. S. Chen, *FEBS Lett.*, 2002, **514**, 238–242.
- 29 H. Moriguchi, Y. Takayama, K. kotani and Y. Jimbo, *MEA-Meeting 2008 Proceedings*, 2008, **1**, 270–273.
- 30 V. Parpura and P. G. Haydon, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 8629–8634.
- 31 S. Mennerick, J. Que, A. Benz and C. F. Zorumski, *J. Neurophysiol.*, 1995, **73**, 320–332.
- 32 S. W. Rhee, A. M. Taylor, C. H. Tu, D. H. Cribbs, C. W. Cotman and N. L. Jeon, *Lab Chip*, 2005, **5**, 102–107.
- 33 H. Hwang, G. Kang, J. H. Yeon, Y. Nam and J.-K. Park, *Lab Chip*, 2009, **9**, 167–170.
- 34 C. M. Nelson, S. Raghavan, J. L. Tan and C. S. Chen, *Langmuir*, 2003, **19**, 1493–1499.
- 35 H. Takano, J. Y. Sul, M. L. Mazzanti, R. T. Doyle, P. G. Haydon and M. D. Porter, *Anal. Chem.*, 2002, **74**, 4640–4646.
- 36 J. M. Corey, B. C. Wheeler and G. J. Brewer, *J. Neurosci. Res.*, 1991, **30**, 300–307.
- 37 I. Suzuki, J. Hayashi and K. Yasuda, *MEA-Meeting 2008 Proceedings*, 2008, **1**, 89–90.
- 38 D. Parnas and M. Linial, *Developmental Brain Research*, 1997, **101**, 115–124.
- 39 Y. Bogoch and M. Linial, *Exp. Cell Res.*, 2008, **314**, 677–690.