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One-photon and two-photon stimulation of neurons in a microfluidic culture system†

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In this study, we demonstrate a novel platform for optical stimulation of neural circuits combined with a microfluidic culture method and microelectrode array measurements. Neuron-on-a-chip was designed and fabricated to isolate axons without a soma or dendrite. Thus, it is readily able to manipulate the neuronal alignment and to investigate the neuronal activity at the locations we want to observe. We adapted the optical stimulation technique to the arranged neurons to generate the neuronal signals in a non-invasive fashion. A blue light-emitting diode and a femtosecond laser with 780 nm center wavelength were used for neuronal activation and the corresponding neuronal signals were measured by MEAs at the same time. We found that one-photon light *via* caged glutamate provoked periodic spiking. In contrast, the femtosecond pulse irradiation generated repetitive firing at constant rates. Response times of one-photon and two-photon stimulation were around 200 ms and 50 ms, respectively. We also quantified neural responses, by varying optical parameters such as exposure time and irradiation power.

Introduction

As microfluidic technology has advanced, the neuron-on-a-chip has matured enough to allow investigation of neuronal functionality and connectivity in neuroscience research. Neuron-on-a-chip offers many advantages in terms of experimental reproducibility, flexibility, as well as cost. One of the remarkable merits of neuronal culture in the device is that it allows arranging the neural network while guiding neurite outgrowth at the designated location and in the required direction.[1–5] Recently, integration of microfluidic devices with microelectrode arrays (MEAs) has led to investigate neuronal networks using electrophysiological approaches.[6–12] This platform offers non-invasive, long-term multi-site recording of neuronal networks. These advantages offer an alternative method to patch-clamp experiments that require experienced and sophisticated skills. Even though it enables biological comparability with electrophysiological readout, it has inherent limitations for the study of localized neural functionality. Because most MEAs have a discrete and fixed pattern of electrodes, the current device structure cannot afford selectively to stimulate neurons.

To overcome the above mentioned problems, optical stimulations, categorized as endogenous and exogenous, have generated interest.[13] Exogenous optical stimulation is associated with photochemical reactions originating outside the cell organism that induce neural activation. This method requires ion-channel mediators such as caged neurotransmitters,[14] photo-switches,[15] and genetic modification of neurons.[16] In contrast, endogenous optical stimulation does not need genetic manipulation or other interventions to evoke action potentials.[17–24]

In previous studies, various integrated concepts between optical stimulation and the electrical recording of neuronal signals based on the planar MEAs were introduced. The first introduction was the "PhotoMEA platform", which coupled MEAs recording and optical uncaging.[25] Several studies tried to apply an optogenetic technique into neural networks on the planar MEAs.[26,27] These previous studies showed clear feasibility of each technology in the *in vitro* environment. However, merging all the technologies, including optical stimulation, planar MEAs, and microfluidic culture platform, has not been carried out despite the clear need and potential. The PDMS-based microfluidic device is well known as a good optical device[28] because it enables focused light to be

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delivered to the neuron with less light attenuation and beam distortion. Thus, unified devices integrating the optical stimulation and the electrical detection in a microfluidic device would be a new research platform in neuroscience research and may expand a wide range of applications. In particular, there is also lack of electrophysiological characterization of neuronal response to each of optical stimulation. This was because of the restriction to monitor and analyze systemically both the neural morphology and response. Thus, unified devices integrating the optical stimulation and the electrical detection in a microfluidic device would be new research platform in neuroscience research and expand wide range of applications.

Experimental section

Fabrication

The microfluidic device was fabricated with the typical process of the PDMS moulding technique. The first layer on a clean wafer is spin-coated with SU-8 2002 (Microchem, USA) to 3 μm height for the micro-grooves (1000 rpm, 30 s). The wafer is then soft-baked on a hot plate for 1 minute at 65 °C and 2 minutes at 95 °C continuously. An FCG mask (Microtech, Korea) is then tightly put on the wafer. They are exposed with UV radiation for 105 mW using a MA6 mask aligner (SUSS MicroTech AG, Germany). After the exposure, the wafer is post-baked on a 95 °C hotplate for 2 minutes. The wafer is developed (SU8 developer, MicroChem, USA) leaving exquisite patterns on the wafer for 1 minute. A second layer for the cell culture part is processed. The 100 μm height for the cell culture channel is spin-coated with SU-8 2050 (1600 rpm, 30 s). The wafer is soft-baked on a hot plate for 45 minutes at 95 °C. The second FCG mask is precisely aligned over the first layer and tightly contacted. They are exposed with UV radiation for 240 mW to get a pattern. The wafer is post-baked on a hotplate for 30 minutes and the remaining photoresists are developed using SU8 developer for 10 minutes. Following development the wafer was rinsed with isopropyl alcohol.

Preparation

To obtain complete mould piece from replicable moulds fabricated through photo-lithography, we use polydimethylsiloxane (PDMS, Sylgard 184, Dow Chemical, USA) which is biocompatible, non-flammable, optically transparent, gas permeable and very flexible. PDMS is degassed with vacuum to get a clear polymer before use. PDMS is poured about 2 mm thick on the patterned wafer. Replica moulds are cut out into proper pieces and holes are punched out to get a complete piece. The bonding side of the complete piece is cleaned with sticky tape and irreversibly bonded on a sterilized glass cover slip. Then, it was treated with plasma cleaning for 5 minutes to make the surface hydrophilic and coated by poly-D-lysine for cell plating. The microfluidic device used in this study has micro-channels and micro-grooves. The dimension of the micro-channel was 100 μm in height and 2 mm in width, which was designed so that the cells were located. The micro-groove performs the axonal growth and isolation, and its height and width were 3 μm and 10 μm, respectively. Because the microfluidic device is made from polydimethylsiloxane as a good optical material, it enables focused light to be delivered to the neuron with less light attenuation and beam distortion.

Neural recoding recording and signal analysis

For electrical recording, we utilized a 60-channel extracellular recording system (USB-ME64, Multi Channel Systems, Germany) mounted on the microscope. The neural signals were measured at a 10 kHz sampling rate using the MC_Rack software. The commercial 60-channel MEA (60MEA200/30, Multi Channel Systems, Germany), coupled with the microfluidic culture platform, was used to detect action potentials from the neurons. Time sequential signals acquired from the MEA were sorted by a thresholding value considered standard deviation to delineate the action potential. The average noise level of the measured signal was less than 50 μV peak-to-peak.

The measured neural signals were then transferred into a file type accessible to MATLAB (MathWorks, USA). Because the recorded signals in a channel came from in and around the electrode, it is required to distinguish the neurons affected by optical stimulation from any other neuron. Therefore, we used the spike sorting technique to extract the same form of neural spikes for the purpose of comparing the individual neuron's response to each optical stimulation. The spike sorting was executed with customized code, based on PCA analysis and the Kalman filter mixture model of clustering.[29]

The dot in the raster plot represents an action potential and each occurrence of an action potential was plotted in the time scale. In the PSTH, the total response time (2 seconds) was segmented by 25 milliseconds. The number of spikes in each unit was then divided by stimulation trial numbers and the corresponding results were plotted as PSTH. To quantify each neural response to two types of optical stimulation using our platform, we calculated the value of spike variation in the single neuron. We examined using different intensity (mW) and exposure time (ms). The number of spikes was counted for a second before stimulation and after stimulation. The value of spike variation was calculated by subtracting the number of spikes before stimulation from that after stimulation and taking an average.

Cell culture

To acquire cells, the 18 day-old embryos (E18) from pregnant Sprague-Dawley rats were prepared and their hippocampal neurons were dissected into HBSS (Gibco, USA). Hippocampal neurons were then separated from the cortex, dissociated in 0.25% trypsin–EDTA solution (Gibco, USA) and neutralized in DMEM (Gibco, USA) containing 10% horse serum. The cells were plated at a concentration of 6×10^6 cells per mL in neurobasal media (Gibco, USA) supplemented with 50× B27

(Gibco, USA), 100× GlutaMax (Gibco, USA), and 100 μg mL^{-1} primocin (InvivoGen, USA).

System configuration

The novel configuration for these experiments comprised a microfluidic device coupled to microelectrode arrays, an inverted microscope (IX70, Olympus, Japan) with a customized culture chamber (Live Cell Instruments, Korea), a CCD camera (Hamamatsu, Japan), a 60-channel extracellular recording system (USB-ME64, Multi Channel Systems, Germany), and two types of light sources for one- and two-photon stimulation, as shown in Fig. 1. Whenever any incident light passes the mechanical shutter (SH05, Thorlabs, USA), a trigger signal was delivered to all devices for time synchronization. In the experiment, both 140 fs, 780 nm center-wavelength pulsed light from an amplified Ti:sapphire laser (Chameleon, Coherent, USA) and continuous visible light ranging from 300 to 600 nm LED (SOLA light engine, Lumencor, USA) entered an inverted microscope *via* a dichroic mirror. They were focused by a 0.7-N.A. 60× objective lens to the center of the cultured neurons inside the microfluidic channel device and were coupled with 1 mm thick glass planar microelectrode arrays on an inverted microscope. Images were delivered to a CCD camera through a phase contrast microscope. To measure the neuronal activities accurately, all devices related to light irradiation and neuronal recording were synchronized by a command trigger signal. The experimental setup employs a customized chamber supported with a 37 °C temperature controller and a humidified 5% CO_2 gas inlet for the prolonged culturing of neurons on the inverted microscope (Fig. S1†). In addition, an XY-auto stage (Live Cell Instruments, Korea) and a Z-motorized stage (Applied Scientific Instruments, USA) are installed on the microscope. The precise position of stimulation on the specimen was adjusted by three axis motorized stages installed in the microscope. The PDMS device is minimally-sized to insert it into the glass ring of the microelectrode arrays. The space between the device and the ring of the microelectrode array is filled with media to prevent evaporation, which changes medium osmolality and causes cell death. Furthermore, the design permits simultaneous monitoring of cell morphology in the microfluidic device through the microscope and measurement of their electrical activity with the microelectrode arrays. We continuously acquired images of cultured neurons in a microfluidic device using the Micro-Manager software (NIH, USA) and their raw neural signals using the MC_Rack software (Multi Channel Systems, Germany) simultaneously on the same computer.

Results and discussion

Fig. 1(a) shows a novel neurophysiology platform for optical stimulation, combined with the microfluidic culture method and microelectrode arrays measurement. The microfluidic device was reversibly attached to MEAs as a single device (Fig. 1(b)–(d)). It was designed to separate the cell bodies in a micro-channel and the axons in a micro-groove as our

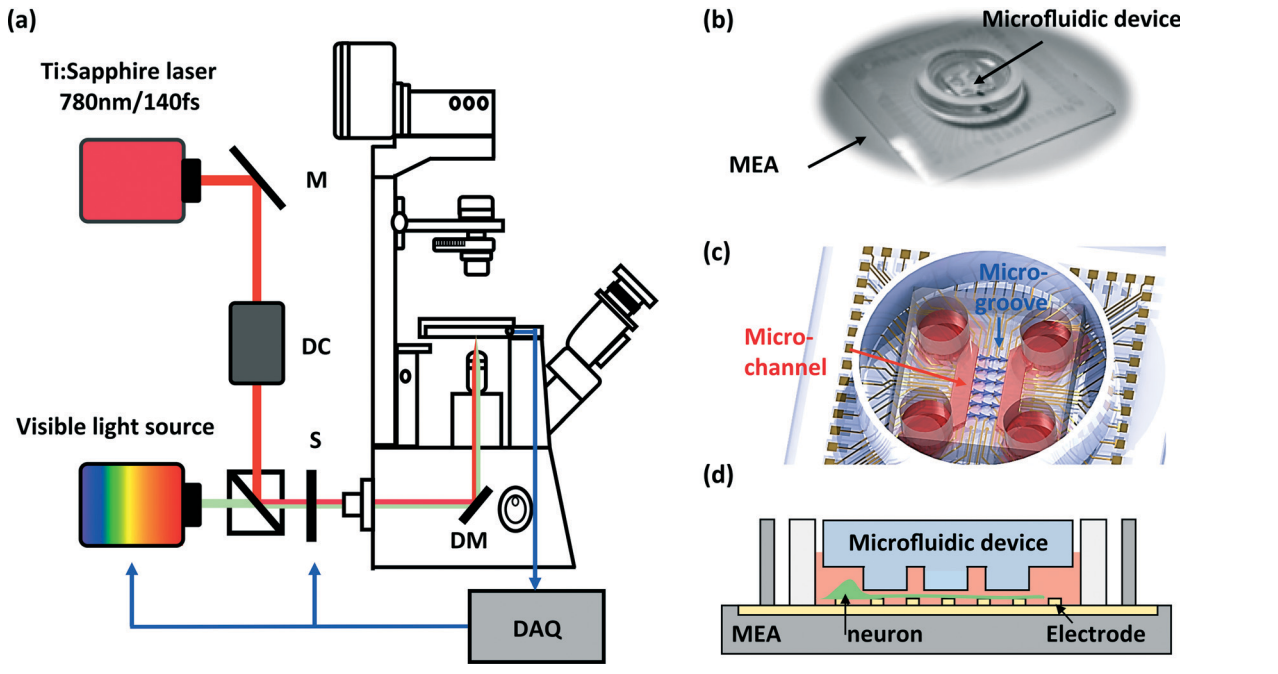

Fig. 1 Schematics of experiment setup (a) Ti:sapphire laser and visible light source for two-photon and one-photon optical stimulation were focused and delivered into neurons in the microfluidic culture system. Neural signals from multi-channels were recorded through the DAQ when neurons are optically stimulated in the customized live cell chamber on the microscope (DM: dichroic mirror, M: mirror, DC: dispersion compensator, S: shutter). (b) Actual image and (c, d) a diagram of a microfluidic device coupled with planar microelectrode array showed that the system was designed to separately measure both soma and axonal response to optical stimulation on the soma.

previous methods suggested (Fig. 2). Because it needs a small volume media (<25 μL) to grow the neurons of the same cell number as 24-well culture, it could efficiently monitor neural response in the experiments with a small amount of material such as exosome. The spontaneous activities of cultured hippocampal neurons in our culture platform were detected in both micro-channel and micro-groove region, but the signals obtained in each channel had different amplitudes (Fig. 2(c)). The amplitude signals of peak-to-peak voltage exceeding 250 μV are common in the micro-grooves, which are 20 times greater than that recorded from the micro-channels.[30] The large signal in the micro-groove is caused by an increase of total impedance. The culture platform within a live cell chamber on an inverted microscope provides reproducibility of optical stimulation out of an incubator for a long time, as well as precise position control over wherein the light is focused. For the one-photon and two-photon stimulation, continuous visible light from an LED source and a 140 fs light from a Ti:sapphire laser were utilized. Both lights entered into the microscope *via* a dichroic mirror and were focused by a 0.7 N.A. 60× objective lens to the soma of the cultured neurons.

To compare neuronal activities evoked by the two types of optical stimulation, we investigated each response of soma and axon in the neurons. Neurons cultured for more than 21 days *in vitro* were used for the one-photon and two-photon stimulation. Because unlike the case with one-photon stimulation, the soma's response to two-photon stimulation started in cultures older than 21 days. To derive the photolysis effect of caged glutamate as one-photon stimulation, the MNI-caged glutamate (2 mM) was bathed in the warmed media. 405 nm light with 174.9 mW was then exposed for 200 milliseconds. For two-photon stimulation, the focused ultra-short pulsed light from a Ti:sapphire laser with 70 mW was also exposed to a neuron for 5 milliseconds to generate spikes without exterior materials. Fig. 3 shows the representative neuronal response when two types of optical stimulation were applied to a soma near to the electrodes in the micro-channel. The evoked action potential by one-photon stimulation was detected at both the soma and axon and its response time was about 200 milliseconds after stimulation (Fig. 3(a) and (b)). Conversely, two-photon light generated repetitive spikes more than a second long (Fig. 3(c)). However, neuronal activation was measured in only the soma region, not in the axon (Fig. 3(d)).

To evaluate the reproducibility of optical stimulation, we analyzed the time series activation patterns using spike raster plots and peri-stimulus time histograms (Fig. 4). The major response to photolysis of caged glutamate begins regularly from about 200 milliseconds in every trial. The evoked spikes activated by one-photon stimulation periodically emerged for both soma and axon at the same time. All responses to the

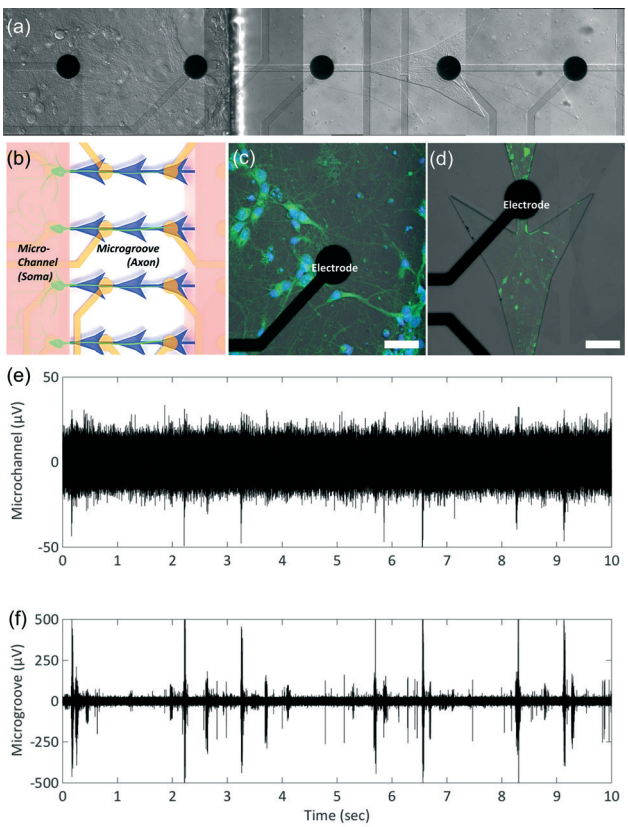

Fig. 2 (a) A DIC image, (b) diagram and (c, d) florescent images clearly show the separation between (c) cell bodies at a micro-channel and (d) axons at a microgroove in the microfluidic device on microelectrode arrays. The DIC images were stitched with ImageJ software (scale bar: 50 μm). The fluorescent images were acquired with fluorescent dyes – DAPI (blue) and Tau (green). Simultaneously, neural signals of spontaneous activity for 10 seconds were observed from each region corresponding to (e) the micro-channel and (f) the micro-groove (scale bar: 30 μm) on 14 days *in vitro*.

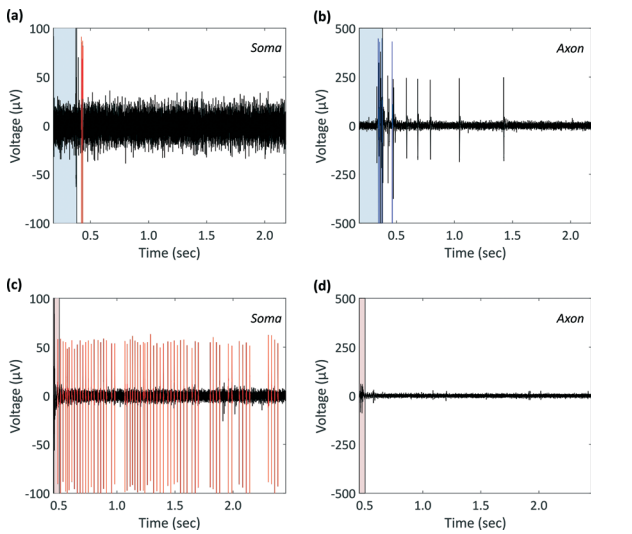

Fig. 3 Optically-evoked neuronal signals were measured in the region of soma and axon. One-photon exogenous stimulation on the area of soma (duration: 200 ms) was effective in both (a) soma and (b) axon. On the contrary, two-photon endogenous stimulation on the area of soma (duration: 5 ms) induced neural activities only in the region of (c) soma without any activities in (d) axon.

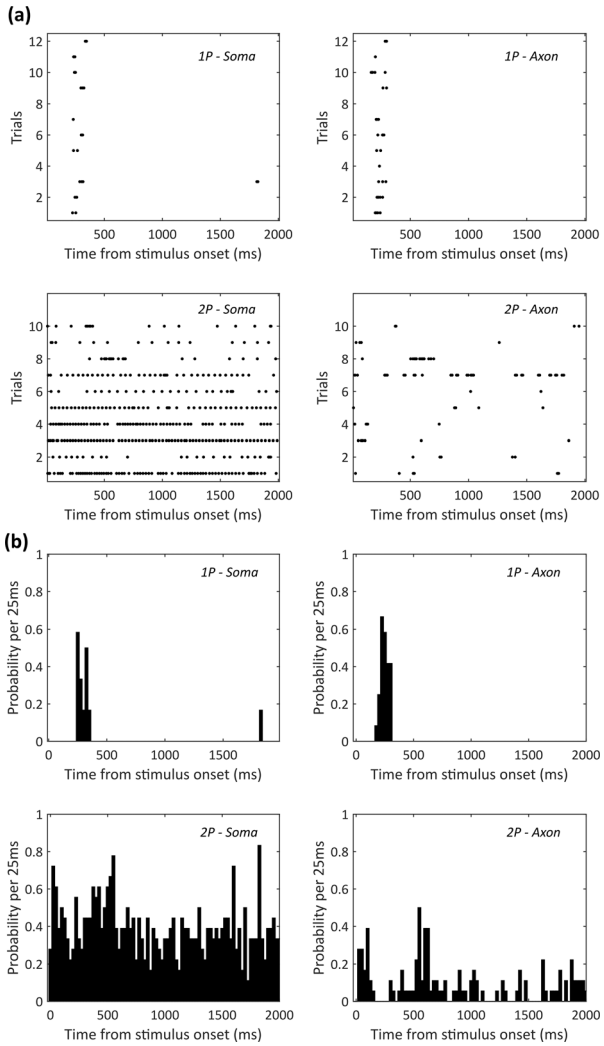

Fig. 4 Neural responses to two types of optical stimulation were reproduced. The results of one-photon exogenous stimulation showed the periodic response in the soma emerged repeatedly as well as in the axon at every trial. However, two-photon endogenous stimulation generated irregularly repetitive spiking on (a) the raster plot and (b) PSTH.

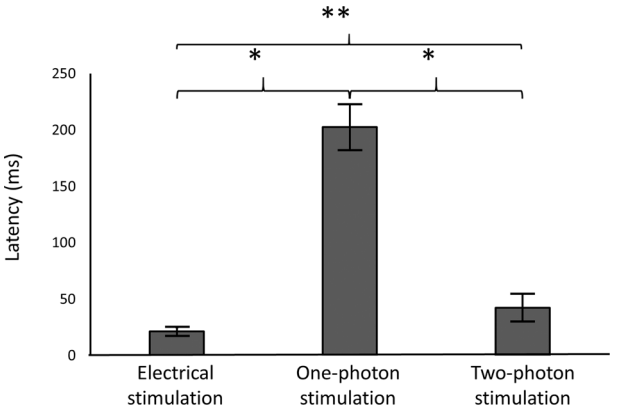

Fig. 5 Latency graph of the electrical stimulation, the one-photon stimulation with photolysis of caged glutamate, and the two-photon stimulation the time of the first neural response to each of optical stimulation was compared. The latency of the two-photon stimulation is 42.37 ± 12.19 milliseconds (n = 10), slower than that of the electrical stimulation (21.22 ± 3.80 milliseconds, n = 13) and faster than that of the one-photon stimulation using photolysis of MNI-caged glutamate (202.32 ± 20.49 milliseconds, n = 12). The electrical stimulation with 1 μA and 1 millisecond duration was applied into an electrode around soma in the micro-channel. The others were analyzed based on the data utilized in Fig. 4. Error bars, SEM (*$p < 0.001$, **$p < 0.5$; Wilcoxon test).

two-photon stimulation were approximately equal to all stimulation trials, showing repetitive spikes at rapid speed for every trial at only the soma. The spike pattern appears as an all-or-none discharge of 20–30 action potentials with each successive spike. This tendency thereafter has a long flat plateau in the PSTH. The response time for two-photon stimulation was about 50 milliseconds, which was a fast feedback compared to the one-photon response (Fig. 5). Such a delayed response in one-photon stimulation is because of the few biochemical processes involved in generating action potentials such as the uncaging process and neurotransmitter delivery.

To quantify neural response in accordance with stimulation parameters of optical activation, the value of spike variation was studied. We counted the number of spikes for one second before and one second after stimulation and then calculated when different optical parameters, such as intensity and exposure time, were induced. The value of spike variation was then analyzed by subtracting the number of spikes before stimulation from the number of spikes after stimulation and taking an average. All experimental procedures were repeated more than 10 times with 30 seconds period at each stimulation condition for 300 seconds. Fig. 6(a) shows that one-photon stimulation periodically evokes action potentials in the neurons of more than 30.5 mW power and 10 milliseconds light exposure time. When optical intensity was increased from 6.2 mW to 174.9 mW, the number of periodic spikes ranged from 0 to 3. Similarly, the number of the evoked spikes increased from 0 to 3 according to given exposure time from 10 to 500 milliseconds. This indicates that there is a threshold condition for generating action potential in one-photon stimulation. However, our results could be different, as well as the threshold level of one-photon

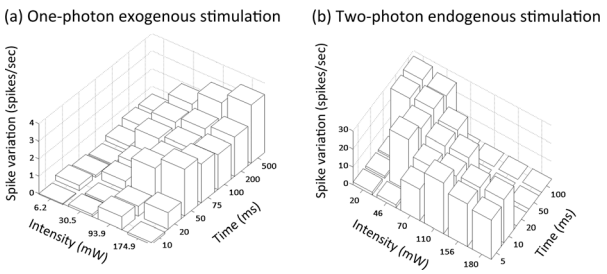

Fig. 6 3D diagram of spike variation generated by (a) one-photon exogenous stimulation and (b) by two-photon endogenous stimulation. The neural responses of two types of optical stimulation were quantified ($n \geq 10$) according to intensity and time. While photolysis of the commercial caged glutamate periodically generated spikes ranged from 0 to 3 for a second, two-photon endogenous stimulation evoked repetitive spikes with the 20–30 spikes for a second.

stimulation, if another variable was considered, the concentration of MNI-caged glutamate bathed in media.

Fig. 6(b) shows the response of two-photon endogenous stimulation. The characteristic spike pattern produced by two-photon stimulation is of repetitive spikes and rapid spike trains at a constant rate. The two-photon response was bounded on the intensity with more than 70 mW at the 5 milliseconds exposure time and with less than 46 mW at the 100 milliseconds exposure time. Over 110 mW intensity and 20 milliseconds exposure time, neurons became unable to respond to stimulation, causing a decreased number of spikes. In this experiment, we found that the number of action potentials induced by single two-photon stimulation ranged from 20 to 30 spikes for duration of one second. Our results show that both types of optical stimulation have threshold parameters in terms of optical intensity, as well as exposure time. As mentioned above, we are convinced that both optical stimulations are very reliable and reproducible techniques for generating action potential in neuronal research, as long as it is operated in the threshold region.

Conclusions

In conclusion, the cultured hippocampal neurons in a microfluidic device were activated using a customized neurophysiology setup that integrates optical stimulation and electrical recording. The system could be employed to measure neural response, with imaging its morphology at the same time when neurons are stimulated optically. Through the experimental results, we confirmed that both one-photon and two-photon stimulation produces reliable and repeatable activation of neurons, identifying their electrophysiological properties. The results imply that optical stimulation and microelectrode measurements in microfluidic culture systems may achieve good technical harmony. Our research platform has the potential to be applied where the conventional approaches such as the capillary glass electrode are difficult to access. Our approach is applicable to neurons and neural circuits cultured in both two- and three-dimensional environments and used to investigate spinogenesis and synaptogenesis involved in synaptic plasticity. Furthermore, it could extend into electrophysiological/electrochemical study for high content drug screening when combined with a disease model of neuronal circuits developed with iPSC-derived neurons. In addition, two-photon stimulation can offer an alternative new optical stimulation modality without genetic manipulation.

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