Electrical stimulation of cultured neurons using a simply patterned indium-tin-oxide (ITO) glass electrode

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Running Title

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Abstract

-Background

Indium-tin-oxide (ITO) glass electrodes possess the properties of optical transparency and high electrical conductivity, which enables the electrical stimulation of cultured cells to be performed whilst also measuring the responses with fluorescent imaging techniques. However, the quantitative relationship between the intensity of the stimulating current and the cell response is unclear when using conventional methods that employ a separated configuration of counter and stimulation electrodes.

-New Method

A quantitative electrical current stimulation device without the use of a counter electrode was fabricated.

-Results

Nerve growth factor (NGF)-induced differentiated PC12 cells were cultured on an ITO single glass electrode, and the Ca^{2+} response to electrical stimuli was measured using fluorescent Ca^{2+} imaging. ITO electrode devices with a width less than 0.1 mm were found to evoke a Ca^{2+} response in the PC12 cells. Subsequent variation in the length of the device in the range of 2–10 mm was found to have little influence on the efficiency of the electric stimulus. We found that the stimulation of the cells was dependent on the electrical current, when greater than 60 μA , rather than on the Joule heat, regardless of the width and length of the conductive area.

-Comparison with Existing Method(s)

Because of the cells directly in contact with the electrode, our device enables to stimulate the cells specifically, comparing with previous devices with the counter electrode.

-Conclusions

The ITO device without the use of a counter electrode is a useful tool for evaluating the quantitative neural excitability of cultured neurons.

Introduction

The activity of excitable cells, including neurons, are regulated by their physico-chemical membrane properties (Catterall 2010). Intracellular recordings and patch clamp methods have been widely used to measure neuronal activity and to stimulate individual neurons, (Cohen et al. 2008; Hatakeyama et al. 2010; Wallach and Marom

2012). Expanding on this approach, multi-electrode arrays (MEAs) enable the stimulation of cells whilst simultaneously measuring their activity, allowing the network properties of multiple neurons dissociated in culture or tissue to be investigated (Wagenaar et al. 2005; Furukawa et al. 2013). Another effective method for simultaneously recording the neural activity of multiple neurons *in vitro* or *in vivo* is calcium imaging (Ikegaya et al. 2004; Ogawa et al. 2006). Calcium imaging enables both the observation of neuronal activity in regions of interest, and the subsequent analyses of the interactions and connectivity among them (Brustein et al. 2003; Bock et al. 2011). In recent years, devices employing indium-tin-oxide (ITO) glass electrodes have been developed to study the electrical stimulation of specific neurons via calcium imaging (Behrend et al. 2011; Weitz et al. 2013, 2014).

ITO glass electrodes possess both high optical transparency and electrical conductivity (Konry and Marks 2005), which enables the effective stimulation of cells on the electrode during calcium imaging without interference to the fluorescence signal, whilst also evaluating the activity of the directly stimulated neurons and their surrounding cells. Using an ITO electrode device, the excitability of stem cells and retinal neurons in response to electrical current stimuli has been measured (Behrend et al. 2011; Takayama et al. 2011). In these studies, cells in the experimental medium were stimulated by the electrical field between a stimulating ITO glass electrode and a counter electrode. However, uncertainty exists when determining the area where the current is passing between the stimulation and the counter electrode. Furthermore, the stimulus intensity may vary according to the distance between the ITO electrode and the counter electrode, as well as with the position of the cell on the electrode. As a consequence of the above, the current intensity required for neuronal excitation is difficult to determine.

In this study, a device was fabricated that could uniformly stimulate multiple cells without the use of a counter electrode. To achieve this, we first confirmed that the cells

were being directly activated by the current flowing through the electrode upon which the cells were attached. To develop the device, we designed and patterned ITO glass electrodes that were sufficient to excite Nerve Growth Factor (NGF)-differentiated PC12 cells. Although PC12 cells possess a neuron-like structure and excitability, they do not form functional neural connections. Therefore, these cells are suitable for evaluating both the efficacy of a device in evoking the Ca^{2+} response, and the spatial resolution of the input stimulation. Using the ITO device, we investigated the relationship between the strength of the stimulation current and cell excitability. Moreover, we were able to quantitatively evaluate the optimal configuration of the patterned electrodes by varying the length and width of the thin area, in order to stimulate the cells effectively. As a result, we are able to demonstrate that our system enables multiple cells to be stimulated on the ITO electrode. The device was then used to stimulate cultured hippocampal neurons within the neural circuit.

Materials and Methods

Electrode patterning design and fabrication of the photomasks

Patterned ITO electrodes were fabricated using photomasks of their respective patterns. These patterns were designed using a vector-based drawing program, Canvas Version 11, and printed on transparent OHP films with a laser printer (LBP9600C, Canon, Tokyo, Japan). The drawings were then reproduced on emulsion masks (HIGH RESOLUTION PLATE 3 inch×3 inch, Konica, Tokyo, Japan) with the size reduced vertically and horizontally by five times, and with the black and transparent areas inverted. The process was performed by exposing the emulsion masks through the printed OHP films with a stepper (MM605, Nanometric Technology Inc., Tokyo, Japan). The exposed emulsion masks were immersed in developing (CDH-100, Konica) and fixing fluid (CFL-X, Konica) in a darkroom to reproduce the designs on the photomasks.

Etching of conductive region of ITO electrode

ITO-coated glass slides with a resistance of 50 Ω and a size of 100×100 mm were purchased from Kinoene (Tokyo, Japan). ITO was patterned according to a photolithographic and wet-etching procedure (Zhan et al. 2002; Sun and Gillis 2006). A thick layer (0–10 μ m) of AZP4210 positive photoresist (AZ Electronic Materials, NC, USA) was spin-coated onto the ITO glass slide at 1500 rpm using a spin coater (SC200, Nanometric Technology Inc.) and was subsequently baked at 100°C for 2 min on a hot plate (ND-1, AS ONE, Osaka, Japan). The glass slide was then exposed to UV light from a mask aligner (MaskAligner LA310k, Nanometric Technology Inc.) for 1.5 sec through the fabricated photomasks for each respective pattern. The photoresist was then developed in 20% AZ400K developer solution (AZ Electronic Materials). An acidic solution composed of 5% HNO₃ and 20% HCl was used to etch the portion of the ITO that was not protected by the photoresist. The remaining photoresist was then removed in 100% AZ400K developer. The patterned ITO glass electrode was rinsed with acetone and dried.

Cell culture

PC12 cells were obtained from RIKEN Tsukuba Institute. Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% horse serum (HS, Invitrogen), 5% fetal bovine serum (FBS, Invitrogen), 25 U/mL penicillin, and 25 μ g/mL streptomycin (Nacalai Tesque, Kyoto, Japan). To culture cells on the patterned ITO electrode, four Press-to-Seal silicone isolators (Invitrogen) were reversibly sealed to the patterned ITO electrode slide in piles. For experimental use, the PC12 cells (passage number 5–9) were cultured on the patterned ITO glass electrode coated with Cellmatrix Type I-C (Nitta Gelatin Inc., Osaka, Japan), and differentiated by culturing for 3 days in 50 ng/mL nerve growth factor (NGF, Alomone Labs, Jerusalem, Israel) containing a serum free medium.

The primary culture of hippocampal neurons was prepared from day 19 embryonic Wistar rats (Charles River Laboratories Japan, Tokyo, Japan, with all animal procedures approved by the ethical committee of Keio University, permission number 09106-(1)). The hippocampal neurons were excised in ice-cold PBS and dissociated using a dissociation solution kit (Sumitomo Bakelite, Tokyo, Japan). The neurons were plated on poly-D-lysine (PDL, Sigma-Aldrich, St. Louis, MO, USA)-coated patterned ITO glass electrodes and cultured in neurobasal medium supplemented with B-27, 2 mM L-glutamine, 50 U/mL penicillin, and 10 µg/mL streptomycin (Invitrogen). The neurons were cultured for 4 weeks prior to experimental use.

Dye Loading

Fluo-4-AM (Invitrogen) was stored at -20°C as a 5 mM stock solution in DMSO (Nacalai Tesque). Fluo-4 is suitable to measure Ca^{2+} concentration changes in response to neuronal activity due to its dissociation constant ($K_d=345\text{nM}$). For optical imaging, the PC12 cells, or the hippocampal neurons located on the patterned ITO glass, were incubated with 10 µM fluo-4-AM and 0.02% pluronic F-127 (Invitrogen) in the culture medium for 30 min at 37°C. The cells were washed twice with Krebs-Ringer Henseleit solution (KRH) containing (in mM): NaCl, 125; KCl, 5; MgSO_4 , 1.2; CaCl_2 , 2; KH_2PO_4 , 1.2; glucose, 6; HEPES, 25; at a pH adjusted to 7.4 using NaOH and HCl. The medium was then replaced with fresh KRH and further incubation was carried out for 15 min for complete hydrolysis of the acetoxymethyl ester form of fluo-4-AM in the cells.

Fluorescent Measurements

Fluorescent imaging experiments were performed using a fluorescent microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) equipped with a 10× objective (S Fluor, Nikon), a 505 dichroic mirror, and a 535/55-nm barrier filter. Fluo-4 was excited at 488 nm using a Xe lamp (150 W) with a monochromator unit, and fluorescence was measured with a CCD camera (HiSCA, Hamamatsu Photonics, Shizuoka, Japan). The fluorescence was

calculated as the mean intensity over a defined region of interest (ROI) on the cell body of each cell using the software package, Aquacosmos (Hamamatsu Photonics).

Electrical Stimulation and Measurement of Current

The stimulation current was applied using an Electronic Stimulator (SEN-8203, NIHON KOHDEN, Tokyo, Japan), with the applied voltage varying from 10 V to 50 V. The duration of single stimulation was set to 70 or 90 μ sec, with intervals of 50 msec, and a train 200 times. The above settings produced square wave voltage with a frequency of 20 Hz, pulse-widths of 70 or 90 μ sec (Only 90 μ sec is illustrated in Fig. 1–3), and a period of 10 sec. Cells were stimulated every 30 sec whilst the applied voltage was gradually increased from 10 V to 50 V.

In order to measure the current flowing through the ITO electrode, a digital multimeter (CDM-2000D, CUSTOM, Tokyo, Japan) was connected to the circuit which comprised of the cell-stimulating ITO electrode and the stimulator connected in series. The digital multimeter was set to the ACA mode.

Detection of Ca^{2+} responses

In order to determine the response of Ca^{2+} to the electrical stimuli, the time differentials of the relative fluorescent intensities ($d(F/F_0)/dt$) in the respective cells were calculated, F_0 is the initial value of fluorescent intensity. The onset of the response timing was detected as the supra-threshold period ($2.58 \times \text{SD}$ of noise) using the value $d(F/F_0)/dt$, with the noise value being the calcium signal associated with the non-stimulated period (0–1 min) (Ikegaya et al. 2004).

Measurement of the temperature on the electrode

The temperature on the electrode was measured using a Visual IR Thermometer (VT04, FLUKE, WA, USA). Thermography images for the ITO device were captured at the end of every 10 sec electrical current application period.

Results

Excitation of PC12 cells induced by a patterned ITO glass electrode without a counter electrode

The conductive region of the ITO electrode device was initially designed and patterned in order to effectively evoke a Ca^{2+} response in NGF-differentiated PC12 cells without the use of a counter electrode. To generate enough intensity to excite cells on the electrode, whilst also ensuring that the conductive region was within the field of view across the width of the electrode, the middle section of the conductive region was thinly patterned (Fig. 1A).

We aimed to confirm whether the patterned ITO glass electrode could stimulate cells on the middle section of the conductive region, and if the responses of these cells could be simultaneously observed. Since ITO glass electrode has transmittance about or over 80% (Konry and Marks 2005; Chen et al. 2008), fluorescence of fluo-4 in the differentiated PC12 cells was successfully observed through the ITO glass electrode (Fig. 1B). The input voltage was gradually increased from 10 to 50 V (Fig. 1C lower), where it was observed that some cells responded with relatively weak stimulation at 35 V, with the number of responding cells then gradually increasing as a function of voltage (Fig. 1C upper). The timing of the observed increases in fluorescence by the responding cells was found to correspond with the application of the electrical stimulation, with fluorescence increasing during the stimulation, and then decreasing during the interval period. The amplitude of Ca^{2+} responses increased depending on the voltage (Fig. S1, $P < 0.0001$ in Jonckheere's trend test). Moreover, the amplitude of electrically evoked Ca^{2+} responses was larger than that of spontaneous activity (Fig. S1B, $P < 0.05$ in Dunnett's test). These results indicate that numerous PC12 cells can be electrically stimulated using a patterned ITO glass electrode device without the presence of a counter electrode.

Effect of ITO electrode design on the number of excited cells

To examine the relationship between the features of the patterned electrode and the effect of the electrical stimulus on the differentiated PC12 cells, the Ca^{2+} responses measured for each of various patterns imprinted on the ITO electrodes were compared. Varying the length and width of the middle section of the ITO glass electrode enabled further examination of the effect of the pattern changes on cell responses because it altered both the resistance of the narrow part of the electrode, and the total current flowing through the electrode (Fig. 1A). At first, the length was set to 2 mm and the width was varied at 0.05, 0.1, 0.25, 0.5, 1, and 3 mm, respectively. The device with the electrode width set to 3 mm was treated as the control device, as the middle section was then as wide as the other areas on the electrode (Fig. 1A). To determine the intensity of the stimulus necessary to evoke a Ca^{2+} response on the ITO glass electrode, the voltage of the electric stimulator was increased gradually from 10 to 50 V (Fig. 1C lower). The relationship between the ratio of responding cells and the input voltage is shown in Fig. 2A. The ratio of responding cells was calculated as the ratio of total number of cells that responded at least once at a given applied voltage to total number of cells located on the electrode. The ratio was found to gradually increase with increasing voltage for electrodes with widths of 0.05 and 0.1 mm (Fig. 2A).

The ratio of responding cells was then compared for electrodes of constant widths but with varying lengths. As shown in Fig. 2A, cells were stimulated the most effectively on the device with a 0.05 mm electrode width. The width of the electrode was therefore set to 0.05 mm, and the length was varied at 2, 5, 10, and 20 mm, respectively. Obvious increases in the ratio of the responding cells with increasing voltage was similarly found for the devices with a 2, 5, and 10 mm electrode length, but not for the device with a 20 mm electrode length (Fig. 2B). Our results demonstrate that devices with an electrode width less than 0.1 mm were able to stimulate cells at the conductive area, whilst varying

the length of these devices within the 2–10 mm range had little further effect on the stimulus efficiency.

Total electrical current determines the excitation efficiency of cells

The data presented in Fig. 2 was re-plotted as a function of the total current passing through the ITO electrode device (Fig. 3). Irrespective of the electrode pattern, the plots indicate a threshold and linear dependency relationship, whereby the cells seldom responded to stimuli below about 60 μA , with the ratio of responding cells then increasing linearly as the stimuli exceeded 60 μA ($R^2=0.8154$).

The data indicates that the ratio of responding cells can be explained by the electrical current value, and that the cells respond when the current value exceeds a certain stimulation threshold. However, there still remains a possibility that the Joule heat generated by the current passing through the ITO electrodes may influence the activation of the cells. To examine this possibility, the temperature of the electrode was measured during stimulation using infrared thermography. The maximum temperature change observed did not exceed one degree Celsius, indicating that Joule heat wasn't a factor influencing the cells' response to the stimuli (data not shown). In summary, it was concluded that when using a patterned ITO glass electrode, PC12 cells were directly activated by current flowing through the conductive area, with a stimulation threshold of about 60 μA .

Spatial resolution of the stimuli using the patterned ITO electrode

We examined whether the cells located on the outside of the conductive area were also excited by the electrical current. Whilst NGF-differentiated PC12 cells form synapse-like structures, prompting dopamine release and transmitter functionality, they do not function to promote an excitatory effect on proximate cells and therefore the Ca^{2+} responses shouldn't propagate among cells. Previous results have indicated that PC12 cells are suitable for evaluating the localization of stimulation (Greene and Tischler 1976; Cao et

al. 2007; Jeon et al. 2010). At the end of each experiment, KCl, with a final concentration of 40 mM, was applied directly into the recording chamber to identify living cells after the electrical stimulation experiment. The input voltage was increased gradually to maximum 50 V in the same way as experiment of Fig. 2, and the ratio of responding cells was calculated by dividing the total number of cells that responded at least once to the electrical stimulation by the total number of cells responding to either the electrical stimulation or KCl. The ratio of responding cells was calculated for cells located both directly on the electrode and those external to the electrode. For the areas external to the electrode, the ratio was calculated for each 20 μ m interval extending out from both sides of the electrode. The experiments were performed using electrodes with a width of 0.05 mm (Fig. 2). The length of the electrode was randomly assigned at either 2, 5, or 10 mm.

The ratio of responding cells was found to reach a maximum for those located on the electrode, with the ratio of responding cells located external to the electrode decreasing depending on the distance from the electrode. The ratios calculated for the cells located 0–20 and 20–40 μ m away from the electrode were not significantly lower than those on the electrode, whereas the ratios for the cells located at a distance greater than 40 μ m away were significantly lower ($P < 0.05$ in Dunnett's test, Fig. 4). The maximum amplitudes of Ca^{2+} responses decreased as the function of the distance from conductive area, and Ca^{2+} responses from cells located at a distance greater than 20 μ m away were significantly lower than that on electrode (Fig. S2, $P < 0.05$ in Dunnett's test).

Application to a cultured hippocampal neuron network

The ITO glass electrode developed in this study was further utilized to simultaneously excite multiple neurons in a cultured neuron network. In a hippocampal neuron network cultured for 4 weeks *in vitro*, sparse spontaneous Ca^{2+} responses were observed in the neurons both on and external to the conductive region (ITO glass electrode). The application of the electrical current stimulation evoked synchronized responses in the

neurons located directly on the conductive region. External to the electrode, small numbers of neurons evoked Ca^{2+} responses, which were synchronized with the responses of the neurons located on the electrode (Fig. 5).

Discussion

In this report, an ITO glass electrode device was developed to stimulate cells without the use of a counter electrode. The device enabled the activation of excitable cells on the electrode whilst simultaneously observing the activity of the cells with fluorescent calcium imaging (Fig. 1). The ITO electrode device evoked Ca^{2+} response in PC12 cells depending on the current flowing in the electrode with a stimulation threshold of 60 μA , unrelated to the Joule heat, and regardless of the width and length of the conductive area (Fig. 2 and 3). Moreover, only the cells located on the electrode and near the electrode were excited in this device (Fig. 4). Because the cells were stimulated directly by the current flowing through the patterned ITO electrode, the system did not require a counter electrode, enabling the uniform and simultaneous stimulation of multiple cells regardless of the arrangement of a counter electrode. The device developed here was also applied to stimulate multiple neurons in a cultured hippocampal neuron network (Fig. 5).

As shown in Fig. 4, the ratio of responding cells outside of the electrode decreased gradually depending on the distance from the electrode. The ratio calculated for those cells located on the electrode was approximately 0.35, which was consistent with the results displayed in Fig. 3. A ratio up to about 0.1 is thought to correlate with a spontaneous response, which is supported by the observation that the ratios calculated for the devices with a 0.25–3 mm electrode width increased similarly up to about 0.1, regardless of the width (Fig. 2). Expanding on this theory, almost no response was observed in the cells located in the area greater than 40 μm away from the electrode because the rates were within the range of the spontaneous response (Fig. 4). Moreover,

the amplitudes of Ca^{2+} response of those cells were as small as those of spontaneous response shown in Fig. S1 ($P > 0.05$ in t-test). This result also indicates that the ratio of responding cells at a distance greater than $40\text{ }\mu\text{m}$ is due to spontaneous response rather than response to the current flowing ITO glass electrode. Conversely, the ratios calculated for the cell responses observed at $0\text{--}20$ and $20\text{--}40\text{ }\mu\text{m}$ from the electrode were not significantly lower than the ratio calculated for those cells located on the electrode ($P > 0.05$ in Dunnett's test).

After 3 days of NGF treatment, the PC12 cells used in this experiment were estimated to have neurites of about $50\text{ }\mu\text{m}$ in length (Das et al. 2004). Hence, some of the cells located in the areas that were $0\text{--}20$ and $20\text{--}40\text{ }\mu\text{m}$ away from the electrode likely extended their neurites to make contact with the electrode. Consequently, these cells may also be excited by the current flowing through the ITO electrode. It is presumed that the wider the contacting area between neurites and the conductive region, the higher the probability of evoking a response, and the results of this experiment are consistent with this presumption. Therefore, it can be concluded that our system excites only the cells touching the ITO electrode on the device.

The spatial resolution of an electrical stimulus applied to cells has previously been examined *via* the prosthesis of retinal cells on MEA (Behrend et al. 2011). In the study, it was concluded that MEA electrodes, as small as $10\text{ }\mu\text{m}$, excited neurons in the relatively large area about $150\text{ }\mu\text{m}$ in diameter. The findings from our study indicate that our system may be superior to the described MEA application concerning the spatial resolution of stimuli. Moreover, sinusoidal input of current has been reported to improve the spatial resolution of stimuli in retinal cells, when compared to short-duration pulses as employed in this study, as it effectively avoids the activation of axons (Freeman et al. 2010; Weitz et al. 2013). Using this method, it is anticipated that only the cells with their cell bodies located on the conductive area will be excited on our device. We have compared

characteristics of our method to that of other methods to measure electrical activity in neurons (Table S1). Our method mentioned in this report is superior to other methods in points of measuring the activities of large number of neurons simultaneously and stimulating specific neurons in high spatial resolution.

Our device was found to activate cells when the stimulating current was greater than approximately 60 μA , regardless of the shape of the conductive areas (Fig. 2 and 3). When the range of the current was less than 60 μA , the ratio of responding cells increased in a range up to 0.1, which is thought to be attributed to a spontaneous response as previously mentioned (Fig. 3). The stimulation threshold seems to vary according to the physico-chemical characteristics of the cells, such as the shape of the cell, the expression levels of the voltage-gated channels, and with other properties of the cells. The stimulation threshold may be lower in more excitable cells, such as differentiated PC12 cells, and neurons (Homma et al. 2006; Simms and Zamponi 2014). Previous studies have reported the stimulation threshold changes depending on the input current pattern in cells cultured on MEAs (Shepherd and Javel 1999; Weitz et al. 2014). It might be possible that investigations into the influence of the input current may further optimize the cell responses observed in this study.

In this study, the propagation of the Ca^{2+} signal from neurons located on the electrode to neurons external to the electrode was shown (Fig. 5). The device developed in this study allows the application of any pattern on the conductive region of an ITO electrode. The ITO device is then capable of stimulating specific cells located in the viewing field, whilst also being able to alternatively stimulate multiple cells in contact with the device. Previous studies have demonstrated the perturbation effects that neurons contained within a network can have on the activities of other neurons in the network (Hanakawa et al. 2009). Studies into the effect of varying the subthreshold input amplitude of surrounding neurons on the activity of individual neurons and on neurons within the network have also

previously been reported (Stacey and Durand 2002; Stacey et al. 2009). The device developed in this study would be a useful tool in the field of neuronal networks when elucidating neural activities.

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Figure legends

Fig. 1 (A) The patterned ITO glass electrode used in this experiment. The width and length of the middle section of the conductive region were varied in this study. Responses of cells on this section of the electrode were observed in the following experiments. (B) Fluorescence of fluo-4 in NGF-differentiated PC12 cells on the patterned ITO glass electrode. The length of the middle section of the electrode is 0.2 mm and the width is 0.1 mm. The area between the two red dotted lines is on the ITO glass electrode. The other areas are non-conductive regions outside of the electrode. The scale bar indicates 100 μ m. (C) Time-course of Ca^{2+} response in PC12 cells indicated with the arrowheads in Fig. 1B (upper), and applied voltage from the electrical stimulation (lower).

Fig. 2 Ratio of responding cells on the ITO glass electrode associated with gradually increasing voltage at various widths (A) and lengths (B). The input voltage was changed from 10 V to 50 V. (A) The length of the middle section of the electrode was 2 mm and

the width was changed from 0.05 to 3 mm. (B) The width of the middle section of the electrode was 0.05 mm, and the length was changed from 2 to 20 mm. Error bars indicate the standard error of the mean (SEM).

Fig. 3 Relationship between the ratio of responding cells and mean current flowing through the ITO glass electrode, derived from the data plotted in Fig. 2. A linear approximation of the plots ranging above the stimulation threshold of 60 μ A was performed. The coefficient of determination of the inclined line above the stimulation threshold is $R^2=0.8154$.

Fig. 4 Ratio of responding cells located on the electrode and external to the electrode (N=8). “Electrode” indicates the area on the electrode and “X–Y μ m” indicates areas located X–Y μ m away from the electrode. Each area outside the electrode has a width of 20 μ m, respectively. Error bars indicate the SEM. * indicates $P < 0.05$ in Dunnett’s test.

Fig. 5 Application to cultured hippocampal neuron network. (A) Fluorescence image of cultured hippocampal neurons stained with fluo-4-AM on an ITO glass electrode. The area between the two red dotted lines is on the ITO glass electrode. The scale bar indicates 100 μ m. (B) Raster-plot indicates the onset of the Ca^{2+} response for neurons located on the electrode (shaded region) and neurons external to the electrode (upper). Histograms represent the rate of responding neurons located external to the electrode (upper) and located on the electrode (bottom) measured every 30 sec (middle). The time-course of applied voltage is also shown (bottom).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5