Selective Stimulation of a Target Neuron in Micropatterned Neuronal Circuits Using a Pair of Needle Electrodes

Kouhei HATTORI,a,*, Hekiru KURAKAKE,a Junko IMAI,a Takuya HASHIMOTO,a Mihoko ISHIDA,a Koki SATO,a Honoka TAKAHASHI,a Soichiro OGUMA,b Hideaki YAMAMOTO,b Ayumi HIRANO-IWATA,b,c and Takashi TANIIa

a Faculty of Science and Engineering, Waseda University, Tokyo 169-8555, Japan
b Research Institute of Electrical Communication, Tohoku University, Sendai 980-8577, Japan
c WPI-Advanced Institute for Materials Research (WPI-AIMR), Tohoku University, Sendai 980-8577, Japan

* Corresponding author: k_hattori@aoni.waseda.jp

ABSTRACT

Neurostimulation is an essential technique to trigger and modulate the spatiotemporal activity of local neuronal circuits. Current stimulation methods have a trade-off relationship among aiming precision, temporal resolution, and noninvasiveness, making it difficult to stimulate and monitor a single target neuron for a long term. Here, we show that a method using two needle electrodes in combination of micropatterning techniques provides new possibilities for targeting and stimulating a single neuron selectively. Results of physiological experiments as well as analog circuit simulation reveal that two needle electrodes can stimulate a target neuron selectively by placing the two needle electrodes in proximity to and to straddle the target neuron, and that the steepness of voltage applied to two needle electrodes is important for the target neuron to fire at a low voltage. The proposed method enables a noninvasive stimulation suitable for measuring long-term activity of local neuronal circuits.

1. Introduction

Neurostimulation is an essential technique to trigger neuronal firing and clarify the spatiotemporal signal propagation.1,2 This technique is also used for modulating synaptic strength, hence neuronal firing patterns.3,4 Stimulating a target neuron in a neuronal network makes it possible to identify the most influential neuron projecting to multiple neurons with effective synaptic connection strength. The global network can be activated upon the firing of such influential neurons,5–7 and the active state is maintained for 30–40 min.8 Therefore, a noninvasive single-cell stimulation provides new possibilities for analyzing network connections and the time course of firing patterns.

The whole-cell patch clamp method enables the stimulation of a target neuron.9 The stimulation pattern can be controlled with a high time resolution.10 However, the whole-cell patch clamp damages the clamped neuron, limiting survival time. The application of voltage pulses using multielectrode arrays (MEAs) is a noninvasive method of neurostimulation, but multiple neurons around the electrode are stimulated simultaneously; hence, the spatial resolution is comparatively low.11 Moreover, cell culture on the electrodes fixed on a substrate is disadvantageous in terms of targeting a neuron after its growth. Recent optogenetics has the aiming precision at a single-neuron level, but it requires additional optical systems such as a laser and a digital mirror device.12,13 The side effects of photoactive chemicals may also be a concern. A simple and noninvasive method capable of targeting a single neuron after network formation is required for long-term analyses of a neuronal network.

Electrical stimulation with two needle electrodes is a noninvasive method that can stimulate neurons after network formation.14,15 Two needle electrodes have been used for stimulating local regions of brain slices to investigate the functional connectivity of neuronal circuits16,17 and temporal changes.18,19 A needle electrode has been used as a stimulation electrode to treat neurological disorders such as Parkinson’s disease.20,21 It has been theoretically predicted that the aiming precision can be improved by placing both the needle electrodes moving independently in the vicinity of the target.22,23 This suggests the possibility of stimulating a single neuron selectively in the network.

Micropatterning techniques are useful to predefine the position of neurons in primary culture systems.24–26 For example, arranging two neurons on a glass substrate at an intercellular distance with or without intercellular connection is made possible by micropatterning techniques. For investigating intercellular signaling, applying electrical stimulation to either of two neurons is required. However,
if two neurons with intercellular connection emit simultaneous firing, it is difficult to determine whether both the neurons fire depending upon electrical stimulation or signal propagation through the intercellular connection. In this sense, it is useful to arrange neurons on a glass substrate in isolation using micropatterning techniques because the possibility of signal propagation is screened out, and the effectiveness of electrical stimulation can be measured directly.

Here, we report the feasibility of using two needle electrodes to stimulate a single target neuron. First, we show experimental results of calcium imaging of single neurons cultured on a micropattern in isolation and stimulated electrically with two needle electrodes. Next, we analyze the effectiveness of stimulation with two needle electrodes by conducting simulations to combine a lumped parameter circuit with a Hodgkin–Huxley model. A two-compartment model is used to clarify the mechanism by which the target neuron fires upon electrical stimulation with two needle electrodes placed near this neuron. Finally, we discuss the key parameters that determine the spatiotemporal resolution of the stimulation.

2. Methods

2.1 Cell culture

All experiments were approved by the Office of Research Ethics, Waseda University (Approval Numbers: 2018-A065, 2019-A046, 2020-A058). Timed-pregnant Sprague Dawley rats were obtained from Oriental Yeast Co., Ltd., Japan. Primary neurons were obtained from cortices of embryonic day 18 rats and seeded on glass substrates (CS-15R15, Harvard Apparatus) with micropatterns. The process for micropatterning has been described elsewhere.30,31 In short, the cell-permissive area of the glass surface was modified with poly-D-lysine, whereas the nonpermissive area was modified with an octadecylsilane monolayer, which becomes cell-repellent upon the adsorption of albumin. The neurons were cultured in a culture medium (148-09671, FUJIFILM Wako Pure Chemical Corporation). A regular array of cell-permissive circles of 100 µm diameter at 200 µm intervals was used for culturing neurons in the circles in isolation.

2.2 Calcium imaging

Calcium imaging was performed on 12–14 days in vitro (DIV) to visualize neuronal activity. Cultured neurons were loaded with the fluorescence calcium indicator fluo-4 AM (Invitrogen) by first rinsing the cells in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (HBS) containing 128 mM NaCl, 4 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM D-glucose, 10 mM HEPES, and 45 mM sucrose and by subsequently incubating in HBS containing 2 mM CaSO4 AM and 0.01 % pluronic F-127 for 30 min at 37 °C. The cells were then rinsed in fresh HBS and incubated for an additional 10 min to complete the deesterification of the loaded AM dyes. The samples were imaged under an inverted microscope (TE300, Nikon) equipped with a 20× objective lens (numerical aperture, 0.45), filter cube (B-2A, Nikon), a home-built light source high-power blue LED (OSB5XNE3C1E, OptoSupply), and a scientific digital charge-coupled device camera (ORCA-R2, Hamamatsu Photonics). Fluorescence intensity was measured as a 12-bit (4096 steps) digital output. All recordings were performed at 37 °C. Fluorescence imaging was performed at 10 frames/s using HCImage software (Hamamatsu Photonics).

2.3 Electrical stimulation

Tungsten tips were used as the electrodes for stimulation after electropolishing a tungsten wire (W-461337, Nilaco) in 100 mM NaOH. Two tungsten needles except for their tip apexes were coated with an insulating polymer and mounted on manipulators. An optical micrograph of the tip is shown in Supporting Information Fig. S1. The exposed area of the hand-made tips without polymer coating was evaluated to be 5000 to 50000 µm². These two needles were immersed in the medium such that the target neuron was placed between them. The distance from the neuron to the needle tips was controlled to be in the range from 0 to 100 µm. The tip height was controlled to be 0.25 µm above the substrate surface. A direct digital synthesizer function generator (FY6600, Kuman) was used for applying a bipolar square pulse to neurons cultured on micropatterns.

2.4 Simulation

LTspice software32 was used for analyzing the subthreshold membrane potential of a neuron. The leaky integrate-and-fire (LIF) neuron model was used as an equivalent circuit model where a LIF neuron was connected electrically with two needle electrodes via the electric double layers and solution resistances. The key feature of circuit simulation is to divide the neuron into two compartments, each is stimulated with either of the two needle electrodes. It is conjectured that the neuron fires if the membrane potential of either of the two compartments exceeds the threshold potential upon electrical stimulation. Unless stated otherwise, the parameters in Table 1 were used.33–35 We used the pseudocapacitance model for the electric double layer at the needle electrodes to take the ionic current flow into account.36

The response of a neuron to extracellular stimulation was also analyzed using the two-compartment Hodgkin–Huxley model.37 The membrane potential of each compartment V_{\text{m1}}(t) or V_{\text{m2}}(t) was calculated as

\[ V_{\text{m1}} = V_{\text{m1, intra}} - V_{\text{m1, extra}} \ (x = 1,2), \]

\[ C_{\text{m1, intra}} \frac{dV_{\text{m1, intra}}}{dt} = -I_{\text{Na}}(V_{\text{m1, intra}}) - I_{\text{K}}(V_{\text{m1, intra}}) - I_{\text{L}}(V_{\text{m1, intra}}), \]

\[ C_{\text{m2, intra}} \frac{dV_{\text{m2, intra}}}{dt} = -I_{\text{Na}}(V_{\text{m2, intra}}) - I_{\text{K}}(V_{\text{m2, intra}}) - I_{\text{L}}(V_{\text{m2, intra}}) - R_{\text{m2, intra}}(V_{\text{m2, intra}} - V_{\text{m1, intra}}), \]

where the extracellular potentials V_{\text{m1, extra}} and V_{\text{m2, extra}} are changed in accordance with the results of the LTspice simulation. \( I_{\text{Na}}, I_{\text{K}}, \) and \( I_{\text{L}} \) are the voltage-dependent sodium current, voltage-dependent potassium current, and leakage current, respectively. Two compartments are connected via cytoplasmic resistance \( R_{\text{m1, link}} = R_{\text{m2, link}} = 100 \ \Omega/cm² \). The constant \( C = 1 \ \mu F/cm² \) is the membrane capacitance. The channel currents \( I_{\text{Na}}, I_{\text{K}}, \) and \( I_{\text{L}} \) represent the membrane potential dynamics in accordance with the following equations:

\[ I_{\text{Na}} = g_{\text{Na}} m h (V_{\text{m1, extr}} - E_{\text{Na}}) \ (x = 1,2), \]

\[ I_{\text{K}} = g_{\text{K}} n (V_{\text{m1, extr}} - E_{\text{K}}) \ (x = 1,2), \]

\[ I_{\text{L}} = g_{\text{L}} (V_{\text{m1, extr}} - E_{\text{L}}) \ (x = 1,2). \]

The functions m, h, and n are given by Table 1.

Table 1. Parameters used in the simulation.

| Name | Value and unit | Description |
|------|----------------|-------------|
| R₁ and R₂ | 400 MΩ | Membrane resistance |
| R₃ | 10 mΩ | Cytoplasmic resistance |
| R₄ | 30 Ω | Solution resistance |
| R₅ and R₆ | 500 Ω | Solution resistance |
| R₇ and R₈ | 50 kΩ | Interfacial resistance |
| C₁ and C₂ | 6 pF | Membrane capacitance |
| C₃ and C₄ | 500 nF | Electric double layer capacitance |
| E_L | −70 mV | Reversal potential |
dy/dt = a_x(V_m)(1 - y) - b_y(V_m)y \quad (x = 1, 2; \; y = m, h, n), \quad (7)

where the voltage-dependent functions \( a_x(V) \) and \( b_y(V) \) obey the following equations:

\[
\begin{align*}
\frac{dV}{dt} &= \frac{0.1(V + 40)}{1 - \exp \left( - \frac{V + 40}{10} \right)} \\
\frac{dV}{dt} &= 4 \exp \left( - \frac{V + 65}{18} \right), \\
\frac{dV}{dt} &= 0.07 \exp \left( - \frac{V + 65}{20} \right), \\
\frac{dV}{dt} &= 1 \\
\frac{dV}{dt} &= \frac{1}{1 + \exp \left( - \frac{V + 35}{10} \right)}, \\
\frac{dV}{dt} &= \frac{0.1(V + 55)}{1 - \exp \left( - \frac{V + 55}{80} \right)}. 
\end{align*}
\]

Here, \( g_{Na}, g_{K}, \) and \( g_L \) indicate the maximum sodium, maximum potassium, and maximum leakage conductances, which are assumed to be 56, 5, and 0.0205 mS/cm², respectively. \( E_{Na}, E_{K}, \) and \( E_L \) are the reversal potentials of the potassium channel, the sodium channel, and the leakage channel and are assumed to be 50 mV, -90 mV, and -70.3 mV, respectively. The above equations represent the ion channel dynamics of cerebral cortical neurons. The electrical stimulation using two needle electrodes was reproduced 500 ms after the start of the simulation by depolarizing or hyperpolarizing the extracellular potential of each compartment by 10 to 60 mV. This protocol is based on the finding that the transient change in membrane potential strongly depends on the change in not intracellular but extracellular membrane potential when the stimulation pulse is input. The time course of the membrane potential was calculated by incorporating the above procedure into Eqs. (2) and (3).

3. Results and Discussion

First, we investigated the feasibility of using two needle electrodes for neurostimulation. As shown in Fig. 1, the neuron on the circular micropattern in isolation was stimulated using two needle electrodes. We used a calcium indicator to examine if the neuron fires upon the stimulation. The target neuron emitted fluorescence signals when stimulation pulses with an amplitude of \( 5 V_{p-p} \) or greater were applied (Fig. 1b). This indicates that the target neuron fires if the amplitude is sufficiently large. Figure 1c shows that the voltage amplitude to trigger neuronal firing depends on the distance from the needle to the target neuron. The results suggest that a bipolar square pulse with an amplitude of approximately \( 5 V_{p-p} \) induces the firing of the target neuron placed between two needles, while other neurons at a distance remain quiescent.

Figure 2 shows the selectivity of the stimulation. Neurons were cultured on the circular micropatterns in isolation. Only the target neuron was placed between two needle electrodes. Neurons outside the circles adhered non-specifically on the substrate surface without any neuronal processes, hence no synaptic connection was established between neurons in the different circles, and no signals propagated from a neuron to others. Therefore, the direct response to the electrical stimulation was revealed by calcium imaging. As shown in Fig. 2, only the target neuron fired upon the stimulation,
while the nontarget neurons remained quiescent. These results indicate that the stimulation is selective if a nontarget neuron is at least 300 µm away from the target neuron. Although the minimum voltage required for firing is higher than that shown in Fig. 1, we conjecture that the difference is mainly due to the fluctuation of the electrical characteristics of the electrodes, as shown in Supporting Information.

Next, we performed computational simulation to calculate the membrane potential change upon the application of a bipolar square pulse. Figure 3a shows the circuit model used in the LTspice simulation. We used the two-compartment model with which two compartments were stimulated by two needle electrodes. The compartments were connected electrically to two needle electrodes. The compartments were connected electrically to two needle electrodes via the lumped solution resistances and the parallel connection of a resistance and a capacitance representing the electric double layers near the electrodes. We assumed that the neuron fires when the membrane potential of either of the two compartments exceeds the threshold. In Fig. 3b, the membrane potential \( V_1 \) appears as transient up-down-up pulses in the left-side compartment. At the same time, three corresponding pulses of \( V_2 \) appear in the opposite direction in the right-side compartment. Such transient pulses appear only at the rising and falling edges of the bipolar square pulse. This is because, while electric current flows mainly outside the neuron, the transient change in extracellular current becomes larger than that in intracellular current at the rising and falling edges, inducing the transient membrane potential change. Since the change in applied voltage is greatest at the center rising edge of the bipolar square pulse, the greatest change in membrane potential is induced. In Fig. 3c, the peak potential is \(-2.6 \text{ mV}\) when a bipolar square pulse of \( 5 V_{p-p} \) is applied. In Fig. 3d, the peak potential increases with the amplitude of the bipolar square pulse at a constant steepness of \(125 \text{ mV/µs}\). However, the peak potential also depends on the steepness. In Fig. 3e, the peak potential increases with the steepness when the amplitude of the bipolar square pulse is maintained at \( 5 V_{p-p} \). This result indicates that a voltage pulse with a steepness greater than \(50 \text{ mV/µs}\) is required for the neuron to fire when stimulating with a voltage pulse of \( 5 V_{p-p} \).

Figures 3f and 3g show the time course of the membrane potential of a neuron simulated with the two-compartment Hodgkin–Huxley model after a transient change in the membrane potential was applied only once. For convenience, the stimulation with two needle electrodes was realized by directly changing the extracellular potentials of the two compartments. In Fig. 3f, when the peak potential is \(-40 \text{ mV}\), the membrane potentials of the depolarized and hyperpolarized compartments return to the resting potential after the stimulation without generating an action potential. In Fig. 3g, when the peak potential is \(-10 \text{ mV}\), the membrane potentials of both compartments return to the resting membrane potential temporarily after the stimulation, but the sodium ion channels begin to open with a delay, causing the depolarized compartment to generate an action potential. As a result, electric current flows from the depolarized compartment to the hyperpolarized compartment via the cytoplasmic resistance \( R_{\text{link}} \), such that the hyperpolarized compartment also generates an action potential accordingly, and eventually, both compartments generate action potentials. The minimum peak potential required for both compartments to generate action potentials was evaluated to be \(-25 \text{ mV}\) by the simulation. In this Hodgkin–Huxley model, the cell was divided equally into two compartments; hence, the parameters for the two compartments were the same. However, if we assume that the axon hillock is
contained only in the depolarized compartment, the peak potential required to generate an action potential is lower than that evaluated by the simulation because the concentration of voltage-dependent sodium channels in the axon hillock is higher than that used in this simulation.40

Electrical stimulation was applied by controlling not the electrode potential with respect to the solution but the relative potential between electrodes, while membrane potential was measured as the intracellular potential with respect to the extracellular potential. Therefore, neuronal firing occurs if extracellular solution potential differs depending on the location. To calculate the variance of solution potential as well as that of membrane potential, we performed simple LTspice simulation with lumped parameters. However, the membrane potential calculated by the LTspice simulation is not affected by ionic current upon electrical stimulation. If we take the effect of potassium channels into account and calculate the membrane potential by charge accumulation,41 depolarization upon electrical stimulation to fire is conjectured to be retarded, as compared to the result shown in Fig. 3g, where the electrical stimulation was realized by directly changing the extracellular potential of both compartments by 60 mV, which corresponds to the peak potential of -10 mV.

Electrodes were placed into the nervous tissue so that they were targeted to a single neuron. To investigate this feasibility, we changed the lumped parameters of the LTspice simulation model.

Figure 3. Results of LTspice simulation (a–e) and the simulation using Hodgkin–Huxley model (f, g). (a) Two-compartment model used in the LTspice simulation. (b) Time course of the membrane potential calculated by LTspice simulation. The rising and falling times of the stimulation pulse are both 40 µs; namely, the voltage steepness of the first and last falling edges is 62.5 mV/µs and that of the center rising edge is 125 mV/µs. (c) Magnified view of (b) from 25.0 to 26.0 ms around the center pulse of the membrane potential. The peak potential is defined as the highest membrane potential of the depolarized component when the center edge of the bipolar square pulse is rising. (d) Relationship between the peak potential and the amplitude of the bipolar square pulse. (e) Relationship between the peak potential and the steepness of the bipolar square pulse. (f) Membrane potential obtained by the simulation using Hodgkin–Huxley model. The stimulation at 500 ms is realized by changing the extracellular potential of both compartments by 30 mV, which corresponds to the peak potential of -40 mV. (g) Membrane potential obtained by changing the extracellular potential of both compartments by 60 mV, which corresponds to the peak potential of -10 mV.
Micropatterning techniques with two needle electrodes is effective to stimulate a target neuron in neuronal networks. This means that combination of microcellular distance between the target and nontarget neurons using selectively stimulate only the target neuron by controlling the stimulation. Spatial resolution, it was demonstrated in Fig. 2 that it is possible to neuron can be placed on a glass substrate apart from other neurons, whereas that from Cell B is changed from 10 to 100 µm. We assume that the solution resistances can be converted to the cell–electrode distance with a conversion ratio of 50 Ω/µm.

Finally, we investigated the selectivity of the stimulation by LTspice simulation. The peak potential when solution resistance of only the Elec. #1 side is changed is also shown in (c). (d) Cell–electrode geometry for investigating the stimulation selectivity. (e) Relationship between the cell–electrode distance and the peak potential. We conducted LTspice simulation where two cells composed of two compartments are connected to two electrodes in parallel. The distance from Cell A to the electrodes is fixed at 10 µm, whereas that from Cell B is changed from 10 to 100 µm. We assume that the solution resistances can be converted to the cell–electrode distance with a conversion ratio of 50 Ω/µm.

4. Conclusion

The feasibility of the method using two needle electrodes was investigated. The physiological experiments performed by combining the micropatterning technique and the neurostimulation with two needle electrodes demonstrate that the method may be potentially used for stimulating the target neuron to fire selectively. The simulation performed using the two-compartment model revealed that the target neuron can be stimulated to fire selectively by placing the needles in proximity to and to straddle the target neuron. These findings indicate that the method has the aiming precision at the single-cell level. This noninvasive stimulation method can be applied to the long-term monitoring of neuronal network activity after stimulation.

Supporting Information

The Supporting Information is available on the website at DOI: https://doi.org/10.5796/electrochemistry.21-00032.

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Electrochemistry, (in press) 1–7

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