

# A NEUROSPHEROID CULTURED ON THE TIP OF A FLEXIBLE MICROELECTRODE FOR CORTICAL MICROSTIMULATION

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## ABSTRACT

This paper describes a flexible microelectrode combined with a neuronal spheroid (neurospheroid) cultured on the electrode tip. Prior works have found that neurons of a neurospheroid can extend their axons to form synaptic connections with neurons inside living neuronal tissues, that could enable such tissues to be stimulated via transplanted neurons (*i.e.*, instead of through the insertion of invasive probes). Here we present a parylene-based flexible microelectrode that enables activation of neurospheroid cultured on the electrode. Experiments revealed that our probe successfully activated neurons within neurospheroids, resulting in neurite extension onto the electrode. These results mark an important step toward achieving minimally-invasive cortical microstimulation via neurospheroid-based probing systems.

## KEYWORDS

Neurospheroid, neural probe, electrode, microstimulation, parylene

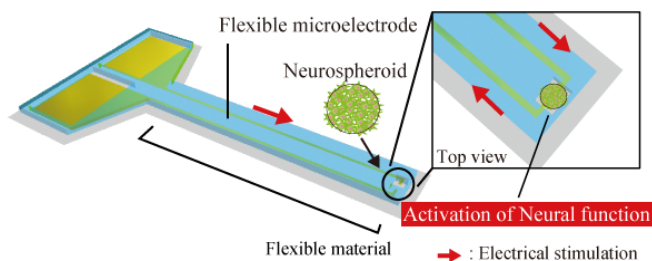
## INTRODUCTION

Electrical stimulation of the central nervous system offers a powerful method for treating diverse neurological disorders [1]. For example, stimulation of the basal ganglia can be remarkably effective in restoring motor function to Parkinson's patients [2, 3]. Additionally, cortical microstimulation via microampere pulses to different regions of the cochlea has enabled hearing restoration in deaf patients [4, 5]. Silicon-based solid electrodes connected with implantable pulse generators are commonly used for such brain stimulation treatments [6, 7]; however, these conventional electrode and brain stimulation systems are inflexible and rigid. Consequently, surgical implantation typically leads to damage of the complex neuronal network. Recently, we presented a glass-based neural probe equipped with a neurospheroid cultured on the electrode tip [8]. Micromotion is the relative movement between the implant electrode and the tissue. Brain pulsations can be attributed to changes in intracranial pressure due to breathing and cardiac pulse. For the neural probe that is tethered to the skull, micromotion can also result from relative movement between the skull and brain. Unfortunately, the large mismatch in stiffness between the glass-based neural probe and the brain tissue can contribute to shear-induced inflammation at the implant site.

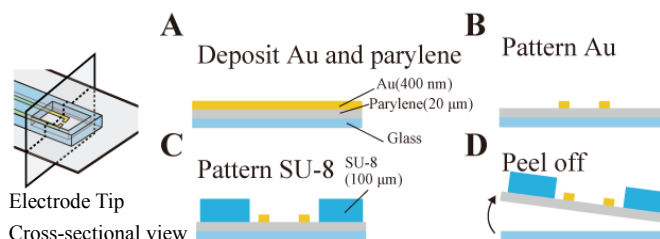
To minimize this issue, here we introduce a flexible, parylene-based biocompatible neural probe (**Figure 1**). In this paper, we demonstrated our microelectrode by activating a neurospheroid cultured on the electrode tip via electrical stimulation.

## EXPERIMENTAL

**Figure 2** shows the fabrication process for the flexible microelectrode. Initially, we evaporated a 20  $\mu\text{m}$  thick parylene film onto a glass substrate. A 400 nm thick layer of gold was deposited and patterned with aqua regia to form a pair of electrodes (**Figure 2(A) and (B)**). We spincoated a 100  $\mu\text{m}$  thick layer of



**Figure 1.** Conceptual illustration of the flexible microelectrode equipped with a neurospheroid cultured on the electrode tip. Our goal is stimulation of the neural tissue through a cultured neurospheroid on the electrode. In this work, we focus on the fabrication of the flexible microelectrode equipped with a cultured neurospheroid on the electrode tip. We can stimulate the tissue without inserting a solid needle through the neurospheroid cultured on the flexible electrode.



**Figure 2.** Schematic diagrams of the fabrication process (the reference cross-section is shown in the left-side illustration). (A)-(D) We fabricated the microelectrode via standard photolithography and microfabrication processes. Because the substrate of the microelectrode is a parylene film, the microelectrode can be peeled off from the glass plate.

(**Figure 2(A) and (B)**). We spincoated a 100  $\mu\text{m}$  thick layer of

SU-8 photoresist onto the surface of the electrode. The resist was exposed to UV through a photomask and developed using SU-8 developer to form an insulating layer and to make a microchamber at the tip (**Figure 2(C)**). Finally, we peeled off the microelectrode from the glass substrate (**Figure 2(D)**).

Formation of cortical neurospheroids using a polydimethylsiloxane (PDMS) microchamber array has been reported previously [8,9]. Briefly, to form uniform-sized cortical neurospheroids, the cerebral cortices of Wistar rats (E16-19) were dissected and dissociated with 0.15 U/ml papain. The resultant cell suspension was seeded at a density of  $4 \times 10^6$  cells/ml on the PDMS microchamber array to form neurospheroids in the microchambers (**Figure 3**). The diameter of each neurospheroid was approximately 150  $\mu\text{m}$  (*i.e.*, due to the 150  $\mu\text{m}$  diameter of each PDMS microchamber).

## RESULTS AND DISCUSSION

The fabricated electrode had a microchamber for neurospheroid culture on the microelectrode tip (**Figure 4(A) and (B)**). Because the fabricated electrode is parylene-based, it can be easily bent due to its flexibility (**Figure 4(C)**). The impedance of the microelectrode was about 600 kohm at 1 kHz. The relative flexibility of the fabricated microelectrode is advantageous for practical applications, such as transplantation.

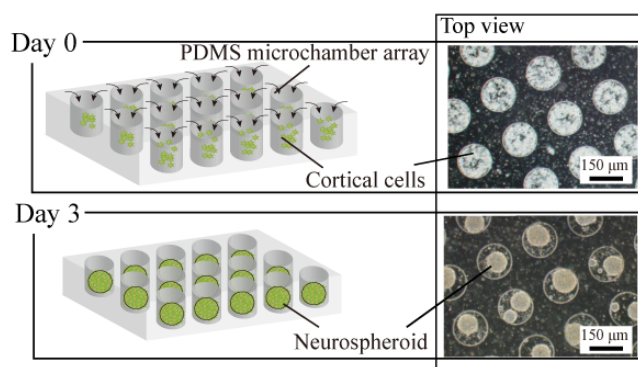
We performed calcium imaging analysis of the neurospheroid response to 100  $\mu\text{M}$  ATP stimulation to confirm the neuronal activity of the neurospheroid, (**Figure 5**). The neurospheroid was loaded with 3  $\mu\text{M}$  Fluo-8/AM for 30 min and observed. This experiment was conducted under the condition of physiological concentrations ( $\sim 1$  mM) of  $\text{Mg}^{2+}$ , which prevents neurons from producing spontaneous oscillations. The trace represents the activation of a neurospheroid due to the ATP stimulation. We found that the formed neurospheroid has neuronal activity.

Using the functional neurospheroid, we tested the biocompatibility of the electrode by culturing neurospheroid on the electrode tip. After 3 days of culture in the PDMS microchamber, a neurospheroid was taken from the chamber by gentle pipetting and then loaded into a microchamber on the electrode tip.

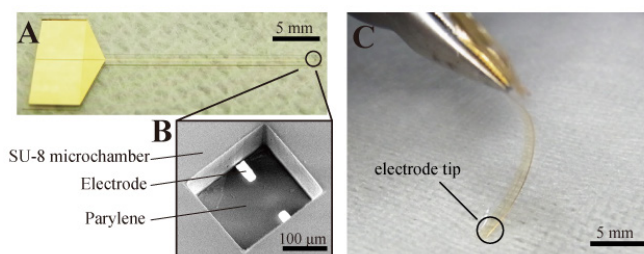
As culture periods increased, the neurospheroid was still confined to the microchamber on the electrode tip and extended their neurites on the surface of the electrode (**Figure 6(A-D)**).

After 7 days of culture, the neurons and glial cells were determined by immunocytochemical staining for microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP). We observed the cells using a confocal laser microscope (**Figure 6(E)**). The distributions of the two cell-type-specific proteins were indicated. We found that the neurites of the neurospheroid extended over the microchamber on the electrode tip.

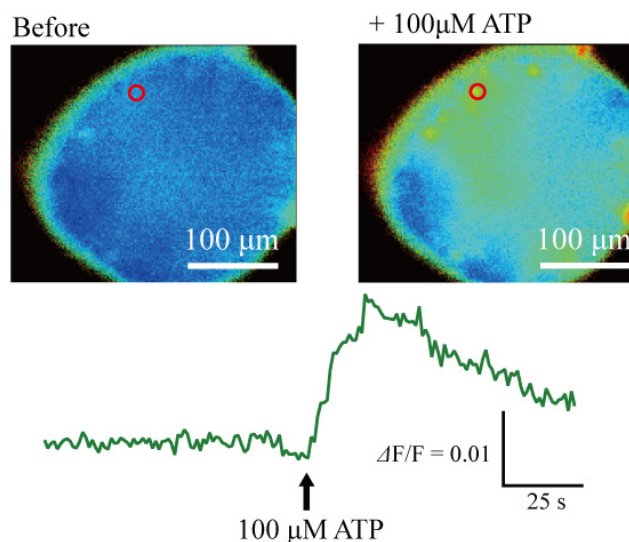
We finally checked the activation of the neurons in the neurospheroid on the electrode tip by electrical stimulation through the pair of gold electrodes (**Figure**



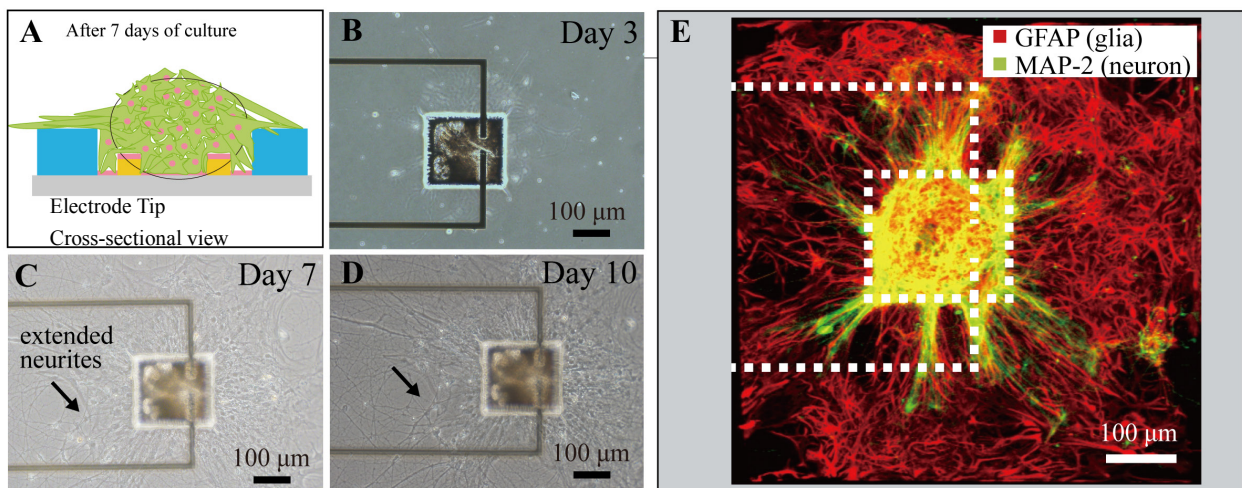
**Figure 3.** Conceptual illustrations and photographs of the method to form neurospheroids. Cortical cells were seeded on a microchamber array (top). The diameter of each microchamber was 150  $\mu\text{m}$ . After 3 days of culture, neurospheroids were formed in the chambers (bottom).



**Figure 4.** (A) Photograph of the microelectrode. (B) Scanning electron microscope (SEM) image of the microelectrode tip. (C) Photograph showing the flexibility of the fabricated electrode.



**Figure 5.**  $\text{Ca}^{2+}$  response of neurospheroid to ATP stimulation. Pseudocolor images of changes in fluo8 before and after 100  $\mu\text{M}$  ATP stimulation. The bottom trace demonstrates the  $\text{Ca}^{2+}$  change in a red circle. An arrow indicates the timing of 100  $\mu\text{M}$  ATP application.



**Figure 6.** (A) Illustration of the cross-sectional view of neurospheroid cultured on the electrode tip after 7 days of culture. (B-D) Growth of the neurospheroid cultured on the electrode tip. Neurite extension of the neurons increased over time. (E) Fluorescence image of the neurospheroid onto the electrode tip. Neurites were extended over the microchamber on the electrode tip.

7). The observed increase in calcium demonstrates that the neurons of the neurospheroid can be activated *via* electrical stimulation using the flexible parylene-based microelectrode.

## CONCLUSION

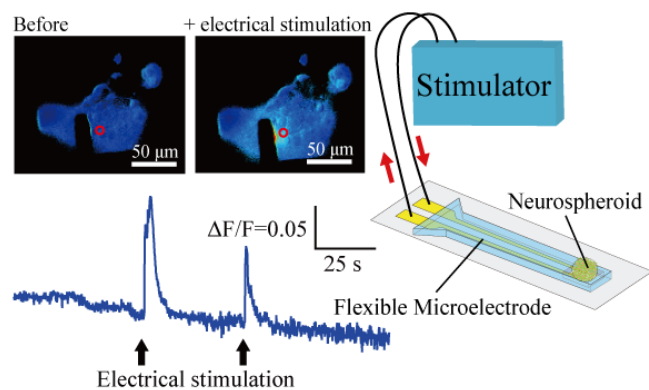
We have fabricated a flexible, parylene-based biocompatible neural probe, and observed neurites extension for a neurospheroid cultured on the microelectrode. Electrical stimulation experiment demonstrated the activation of the neurospheroid cultured on the microelectrode tip *via* electrical stimulation. These results suggest that the neurospheroid cultured on the microelectrode has the potential capability of enabling the neurites extension and synapse formation with neurons in the living neuronal tissue. Our electrode may also offer a platform for stimulating living neuronal tissues by activation of the neurospheroid.

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**Figure 7.** Activation of a neurospheroid by electrical stimulation using the parylene-based flexible microelectrode. Pseudocolor images indicate changes in fluo8 before and after electrical stimulation. The trace demonstrates the  $\text{Ca}^{2+}$  responses of cells in a red circle. Arrows indicate the timing of electrical stimulations (50Hz,  $\pm 2\text{V}$ , block wave).