# LIPID BILAYER CHAMBER ARRAY
# FOR FLUORESCENT AND ELECTROCHEMICAL MEASUREMENT
# OF MEMBRANE PROTEINS

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## ABSTRACT

We proposed a method to easily form a lipid bilayer chamber array for the simultaneous measurement of the fluorescent measurement and the electrochemical one. The simultaneous measurement has potential to reveal novel membrane protein properties owing to the complementary relationship between the two measurements. In order to realize the simultaneous measurement, a following feature is required to the lipid bilayer chambers. The feature is that each of the chambers has (i) a lipid bilayer which can be directly observed by microscopes and (ii) an electrode for electrochemical measurement. The previous methods to form such lipid bilayer chambers for the simultaneous measurement were not suitable for an array system. Here, we propose the method suitable for forming the array system to achieve the high-throughput measurement. Our method was realized by combining droplet interface bilayer technique and MEMS technology.

## KEYWORDS

Lipid bilayer, Membrane protein, MEMS, Simultaneous measurement, Chamber array.

## INTRODUCTION

An artificial lipid bilayer chamber is an attractive tool for membrane protein analysis because of its purity [1, 2]. The previous studies of the artificial lipid bilayer chamber for membrane protein analysis had been divided into two directions: one was for fluorescent measurement [3, 4], the other for electrochemical one [5, 6]. Basically, the fluorescent measurement is suitable to spatiotemporal analysis of molecule interaction, on the other hand, the electrochemical one to exam electrical properties of ion channels. Recently, the two measurements have been combined into one device. Such devices can execute a simultaneous measurement of fluorescent imaging and electrochemical recording [7, 8]. The simultaneous measurement allows us to combine information of channel conformational change and molecular interaction between channels and ligands [9, 10]. By using the measurement, therefore, we can directly analyze time transition from attaching effector molecules to passing currents. Thus, the simultaneous measurement has potential to demonstrate novel membrane protein characters. However, the throughput of the previous systems is still insufficient for the practical application because the previous systems are not suitable for forming the array. For example, the previous systems need to locate the electrode for electrochemical measurement into a small droplet one by one by using a micro-manipulator [7, 8]. Here, we propose a method to easily form the array system leading to the multi-channel simultaneous measurement as a next challenge for high-throughput membrane protein analysis.

## FABRICATION

Fig. 1 represents the concept of this study. The key feature is the lipid bilayer chamber array which can execute simultaneous measurement of membrane proteins. Our strategies to achieve a suitable technique for the array system are follows. (i) Preparing the transparent substrate on which hydrophilic/hydrophobic areas are patterned; (ii) Electrodes are patterned as well in advance onto the areas to be inside the chambers; (iii) Lipid bilayers are formed by applying the droplet interface bilayer technique using the substrate.

The device was fabricated by a standard photolithography and an electrochemical technique. The fabrication process is shown in Fig. 2. The process is divided into three steps. The first step is for patterning of the gold electrodes on the substrate (Fig. 2i-iv). The second one is for the hydrophilic/hydrophobic patterning of the substrate such that the gold electrodes are exposed only at the hydrophilic areas (Fig. 2v-ix). The third one is fabrication of the Ag/AgCl electrodes by chemically changing the patterned gold electrodes on the substrate (Fig. 2x, xi).

In the first step, the substrate was sonicated in acetone for 5 min and rinsed in IPA. ZPN (Zeon, Japan), a negative photoresist, was spincoated on the glass
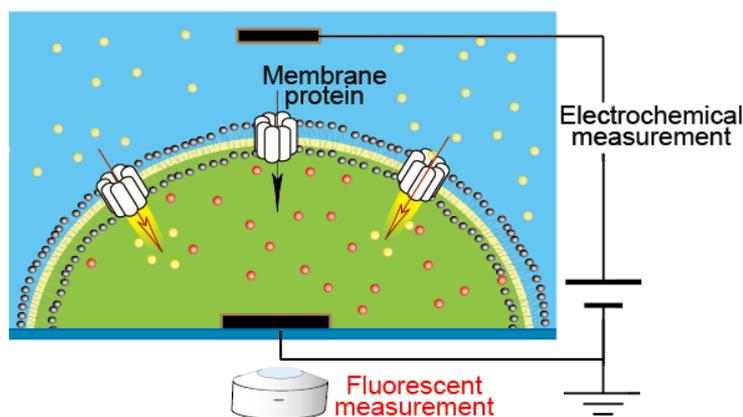


*Figure 1: The concept of this study: The lipid bilayer chamber array for the simultaneous measurement.*

substrate (No. 3 glass, Matsunami, Japan) at 3000 rpm. The substrate was baked at 90 degrees C for 1.5 min. ZPN except for the area to be a gold electrode pattern was exposed to UV light for 5 sec with the glass mask. The substrate was baked again at 110 degrees C for 1 min. ZPN was etched by NMD for 30-60 sec. Gold was deposited by using a vacuum evaporator. The substrate was sonicated in acetone for 1-2 min and rinsed in IPA.
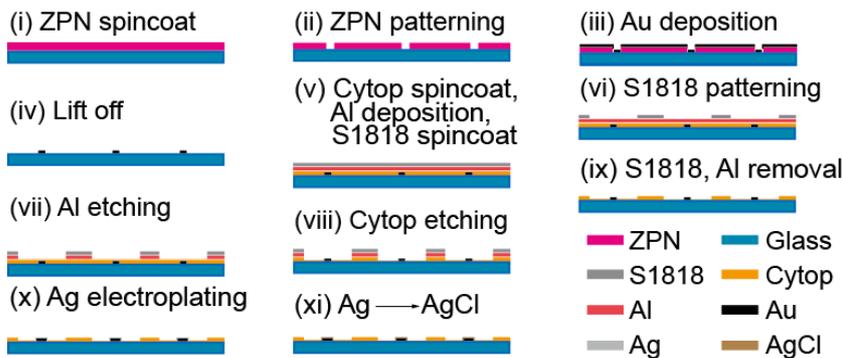


**(i) ZPN spincoat**

**(ii) ZPN patterning**

**(iii) Au deposition**

**(iv) Lift off**

**(v) Cytop spincoat, Al deposition, S1818 spincoat**

**(vi) S1818 patterning**

**(vii) Al etching**

**(viii) Cytop etching**

**(ix) S1818, Al removal**

**(x) Ag electroplating**

**(xi) Ag ⟶ AgCl**

| ZPN | Glass |
| S1818 | Cytop |
| Al | Au |
| Ag | AgCl |

*Figure 2: Fabrication of the device.*

In the second step, 9 wt% of Cytop solution (Asahi glass, Japan) was spincoated at 2000 rpm on the substrate on which the gold electrodes were patterned. Aluminum was deposited by using the vacuum evaporator. This aluminum layer was the mask during the Cytop etching process. Next, S1818 was spincoated on the substrate at 3000 rpm. The substrate was baked at 100 degrees C for 1min. S1818 was exposed to UV light for 7 sec through the glass mask. S1818 was etched by NMD for 45 sec. Aluminum was etched by aluminum etchant. Cytop was etched by applying oxygen plasma for 30 min under the condition of 50 W and 20 ml by using a plasma etching machine. Finally, S1818 and aluminum were removed by acetone and aluminum etchant respectively. A cross-sectional profile of the single hydrophilic area of the substrate was measured by a stylus profile meter (Dektak 6m, ULVAC, Japan) after the second step. In this substrate, the diameter of the hydrophilic circle area was 1 mm and the thickness of the Cytop layer was 1 μm and the gold layer 200 nm. The dimension of the gold pattern exposed at the hydrophilic area was about 200 μm by 800 μm.

In the third step, silver was electroplated on the gold electrodes exposed at the hydrophilic area by applying DC voltage (1 V, 2-3 min): at that time, the plating solution was 0.3 M $AgNO_3$ in 1 M $NH_3$ aqueous solution and the anode was the gold electrode on the substrate and the cathode the gold wire. Finally, the silver is replaced to Ag/AgCl by applying DC voltage (1 V, 1 min) in 0.1 M HCl aqueous solution: the cathode was the silver on the substrate and the anode the gold wire.

## METHOD OF LIPID BILAYER CHAMBER FORMATION

The process of lipid bilayer membrane formation is explained below. The process is composed of three steps: droplet pattern formation using the first buffer solution; addition of the lipid solution; and contact of the second buffer solution to the first buffer solution.

In the first step, the first buffer solution was patterned on the substrate by using the hydrophilic/hydrophobic patterned substrate. The first buffer solution was pipetted onto the hydrophilic circle areas one by one. The solution was easily patterned within the circle areas because the area was surrounded by the hydrophobic area.

In the second step, the lipid solution was added on the substrate on which the droplets were patterned. The lipid solution was organic solvent in which 20 mg/ml of phospholipid was dispersed. After the step, the lipid molecules were self-assembled to form the monolayer at the interface between the first buffer solution and the lipid solution.

In the third step, the substrate after the second step was immersed into the second buffer solution which is poured into a petri dish. After the step, the lipid molecules formed the monolayer at the interface between the second buffer solution and the lipid solution as well. The second buffer solution pushed the lipid solution, and the first buffer solution and the second buffer solution were brought into contact. After that, the layer of the lipid solution got thinner and, finally, the lipid bilayer was formed by contact of the monolayers (Fig. 3a). Fig. 3b is a microscopic image of the completed lipid bilayer chamber. Figure 3c shows the lipid bilayer chamber array (c-1: bright field image, c-2: fluorescent image). Fluorescent molecules contained in the first buffer solution properly remained in the chambers.

## EXPERIMENT

By using the substrate and the method of membrane formation, we conducted the electrophysiological measurement of a membrane protein as follows. The protein measured by the experiment was α-hemolysin. α-hemolysin monomers can be dissolved in aqueous solution and inserted spontaneously into lipid bilayers: heptamers form pores and their diameters are 1.5 nm. 1M KCl aqueous solution was used as the first buffer solution and the second buffer solution. In the first buffer solution, 100 nM of α-hemolysin was included. The Ag/AgCl electrode on the substrate and the Ag/AgCl wire were connected to a patch-clamp amplifier (CEZ2400, Nihon Kohden, Japan). After lipid bilayer formation, the Ag/AgCl wire was located in the second buffer solution and voltage clamping (50 mV) was applied across the membrane. During the experiment, the substrate was observed by using an inverted optical microscope and a CCD camera.

The fluorescent measurement of membrane proteins is under way. So far, we have conducted $Ca^{2+}$ imaging by using $Ca^{2+}$ ion fluorescent indicator.

## RESULT AND DISCUSSION

The representative result of the electrophysiological measurements of α-hemolysin is as follows. The current signals were stepwise and each step was about 50 pA. The value is equivalent to the conductance of the pore formed by α-hemolysin (~1 nS). This result indicates that an electrical property of α-hemolysin was successfully measured by the device and the method. The steps of the current signals less than 50 pA (about 40 pA) were also measured. We inferred that those signals were from α-hemolysin hexamers. As for the fluorescent measurement, we have detected influx of $Ca^{2+}$ ions from the outside of the chamber to the inside.
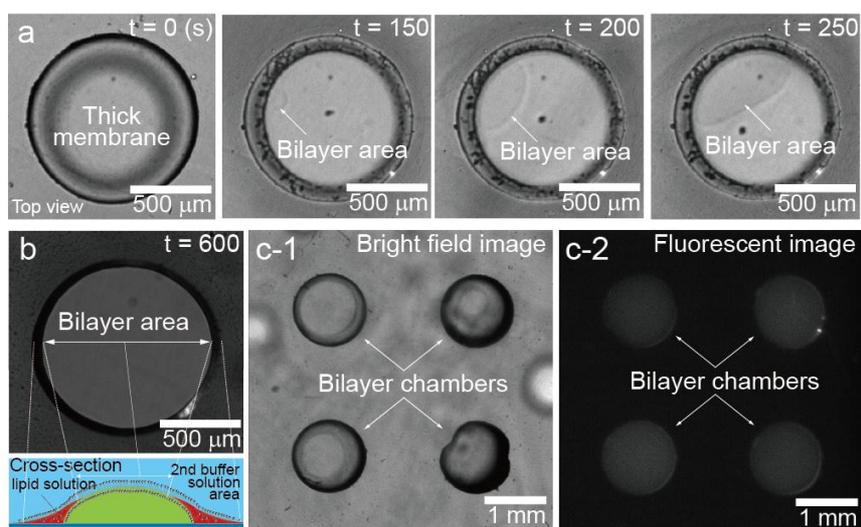


*Figure 3: (a) The lipid bilayer formation. (b) The complete lipid bilayer chamber. (c) The lipid bilayer chamber array (c-1: bright field image, c-2: fluorescent image).*

## CONCLUSION

We proposed a device that easily forms the lipid bilayer chamber array for the simultaneous measurement of membrane proteins. In contrast to the previous methods, our device integrated the electrode for the electrochemical measurement onto the chip by using MEMS technology, therefore suitable for the array system. We successfully measured the property of the nano-pore membrane protein by the lipid bilayer chamber array.

## ACKNOWLEDGEMENT

## REFERENCES

[1] H. Bayley , B. Cronin , A. Heron , M. A. Holden , W. L. Hwang , R. Syeda , J. Thompson, and M. Wallace, *Mol. BioSyst.*, 4, pp. 1191-1208, (2008).
[2] M. Zagnoni, *Lab Chip*, 12, pp. 1026-1039, (2012).
[3] S. Ota, H. Suzuki, and S. Takeuchi, *Lab Chip*, 11, pp. 2485-2487, (2011)
[4] A. Kleefen, D. Pedone, C. Grunwald, R. Wei, M. Firnkes, G. Abstreiter, U. Rant, and R. Tampe, *Nano Lett.*, 10, pp. 5080-5087, (2010).
[5] R. Kawano, T. Osaki, H. Sasaki, and S. Takeuchi, *Small*, 6, pp. 2100-2104, (2010).
[6] A. M. El-Arabi, C. S. Salazar, and J. J. Schmidt, *Lab Chip*, 12, pp. 2409-2413, (2012).
[7] A. J. Heron, J. R. Thompson, B. Cronin, H. Bayley, and M. I. Wallace, *J. Am. Chem. Soc.*, 131, pp. 1652-1653, (2009).
[8] O. K. Castell, J. Berridge, and M. I. Wallace, *Angew. Chem. Int. Ed.*, 51, pp. 3134-3138, (2012).
[9] T. Ide, *ChemPhysChem*, 11, pp. 3408-3411, (2010).
[10] R. Schmauder, D. Kosanic, R. Hovius, and H. Vogel, *ChemBioChem*, 12, pp. 2431-2434, (2011).

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