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Positioning of the sensor cell on the sensing area using cell trapping pattern in incubation type planar patch clamp biosensor

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ABSTRACT

Positioning the sensor cell on the micropore of the sensor chip and keeping it there during incubation are problematic tasks for incubation type planar patch clamp biosensors. To solve these problems, we formed on the Si sensor chip's surface a cell trapping pattern consisting of a lattice pattern with a round area 5 μm deep and with the micropore at the center of the round area. The surface of the sensor chip was coated with extra cellular matrix collagen IV, and HEK293 cells on which a chimera molecule of channel-rhodopsin-wide-receiver (ChR-WR) was expressed, were then seeded. We examined the effects of this cell trapping pattern on the biosensor's operation. In the case of a flat sensor chip without a cell trapping pattern, it took several days before the sensor cell covered the micropore and formed an almost confluent state. As a result, multi-cell layers easily formed and made channel current measurements impossible. On the other hand, the sensor chip with cell trapping pattern easily trapped cells in the round area, and formed the colony consisted of the cell monolayer covering the micropore. A laser (473 nm wavelength) induced channel current was observed from the whole cell arrangement formed using the nystatin perforation technique. The observed channel current characteristics matched measurements made by using a pipette patch clamp.

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1. Introduction

The pipette patch clamp technique has been used to investigate many electro-physiological subjects, including cell biological function, neural cell signal transduction characteristics, and the effects of chemical compounds on ion channels [1–7]. The theory and technique of this extremely powerful method are well established. However, its application to high throughput screening is hampered by its large size. The pipette patch clamp is also easily disturbed by mechanical vibrations, and a high level of skill is required to make measurements with it.

The planar patch clamp has been developed for high throughput screening [8–15], and it has certain advantages over the pipette patch clamp. It is relatively easy to perform and is insensitive to mechanical vibrations; it is thus suitable for long-term measurements. High-throughput screening devices based on the planar patch clamp are already commercially available [16]. The commercialized devices, however, cannot be used in a system that requires long incubation times.

We have developed an incubation type of planar patch clamp biosensor on which the whole-cell channel current of the transient receptor potential vanilloid type 1 (TRPV1) channel expressed on human embryonic kidney 293 (HEK293) cells can be observed using capsaicin as a ligand molecule [15,17]. To enable a long incubation, the surface of the Si biosensor chip was coated with extra cellular matrix (ECM) fibronectin (FN). Although the FN coating lowered the seal resistance (~7 MΩ versus the pipette patch clamp's giga-ohm seal), the whole cell channel current had a good signal-to-noise ratio [15,17]. A crucial issue in the planar patch clamp method is the positioning of the cell on the sensor chip's micropore, which electrically connects the bath solution side to the pipette solution side. This issue is especially serious in the incubation type, where the cell tends to move away from the micropore during incubation. To solve this problem, we examined the possibility of using micro contact printing of an ECM round pattern about 20 μm in diameter (about twice the size of the HEK293 cell) around the micropore as a way to keep the cell around it during incubation. We successfully measured the channel current of TRPV1 expressed on HEK293 using capsaicin as a ligand molecule (not reported). However, we noticed

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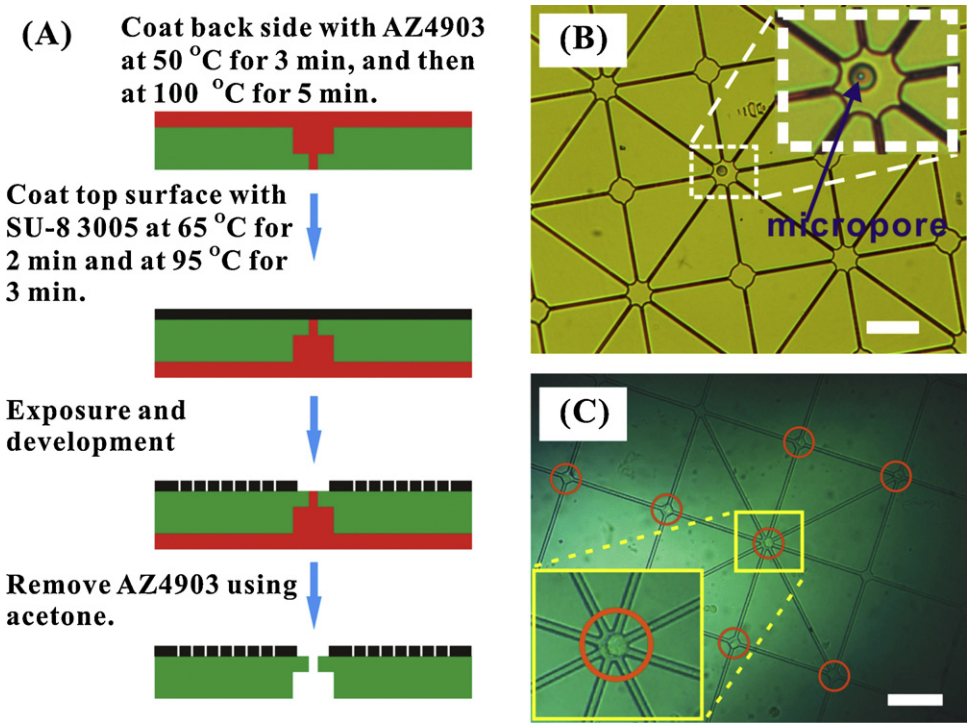

Fig. 1. (A) The formation procedure of the cell trapping structure on the surface of the biosensor chip. (B) Optical microscopy image of the cell trapping pattern formed on the chip surface. Micropore of about 2 μm diameter in the round area (20 μm diameter) at the crossing point of the lattice is indicated by the arrow. Here the circle pattern (~8 μm diameter) surrounding the micropore shows that the back side round hole formed by FIB is observed transparently. (C) A single cell is put in each round area at the crossing point through single cell manipulation using a micro injector. Scale bar is 50 μm.

that the success probability of getting a channel current was low for several reasons, such as misaligned printing of the round pattern and/or blurred printing.

Recent brain science studies have used light-gated ion channels such as channel rhodopsine-2 [18–22]. Thanks to their extremely high time and space resolution, such channels have performed extremely well in neural network analysis. We think that such an optogenetic method would also be very useful for ion channel biosensors, since we can easily examine the performance of the biosensor by using light-gated ion channel expressing cells as sensor cells. Concerning the light-gated ion channels, other than the wild type molecules such channelrhodopsine-1 (ChR1) and -2 (ChR2), several unique chimera molecules such as channelrhodopsine wide receiver (ChR-WR) and channel rhodopsine fast receiver (ChR-FR) have been developed [21,22].

In this study, we examined the use of a cell trapping pattern as an aid to position and keep the sensor cell in the micropore region of the chip. We also used a light-gated ion channel, ChR-WR, as a sensor cell to demonstrate the usefulness of this technique.

2. Materials and methods

2.1. Preparation of the Si sensor chip

The fabrication of the sensor chip is described in detail in the previous reports [14,15]. It is briefly shown in Fig. 1. A 1-μm-thick SiO_2 layer was formed on the Si on insulator (SOI) substrate surface by thermal oxidation at 900 °C with a water-saturated O_2 flow. The SOI substrate consisted of a 2.5-μm Si layer, 3-μm SiO_2 (box) layer, and 600-μm Si layer. A large well on the reverse side was made by diamond drilling. Subsequently, a pyramid-shaped hole was formed by etching with 8% (v/v) tetramethylammonium hydroxide (TMAH) at 90 °C for about 40 min, until the buried SiO_2 layer was reached. Finally, a micropore with 1.5–2.0 μm diameter through the residual silicon membrane was formed by focused ion beam (FIB) milling from the reverse side.

2.2. Formation of the cell trapping pattern on the Si chip

The formation process of the cell trapping pattern is schematically shown in Fig. 1. The Si sensor chip was cleaned using a standard Piranha solution (H_2O_2:H_2SO_4 = 4:1 v/v) above 100 °C for 20 min; it was thoroughly rinsed with deionized water and dried with a nitrogen blow. The cell trapping pattern was formed by using SU8 negative photoresist. To protect the micropore from being clogged by SU8, which is difficult to remove, we filled it with positive photo resist AZ4903, which can be easily removed by acetone. That is, the backside of the Si chip was spin-coated with AZ4903 at 500 rpm for 10 s and then at 4000 rpm for 30 s. The chip was baked at 50 °C for 3 min and then at 100 °C for 5 min. After that, the top surface was spin-coated with SU-8 3005 (Microchem Co., Ltd.) at 500 rpm for 10 s and then at 3000 rpm for 30 s. The prebaking condition was 65 °C for 2 min followed by 95 °C for 3 min. We formed a lattice pattern with round patterns at the crossing points, as shown in Fig. 1(B). We designed this pattern not only for the present experiments but also for future applications such as multisensor point measurements and neural network formation. After precisely aligning the round pattern center of the photomask to the micropore point by using a mask aligner (Mikasa MA-10), exposure was carried out at 200 mJ/cm^2. The post-bake condition was 65 °C for 1 min followed by 95 °C for 2 min. After cooling to the room temperature, the chip was dipped into SU-8 developer (Microchem. Co., Ltd.) for 2 min and rinsed in isopropanol and deionized water. After drying the chip with an N_2 blow, it was hard-baked at 175 °C for 3 min. All of the back side AZ4903 positive photo-resist was removed by immersing the chip in acetone for 1 day. The fabricated pattern endured autoclave sterilization and repeated use. The

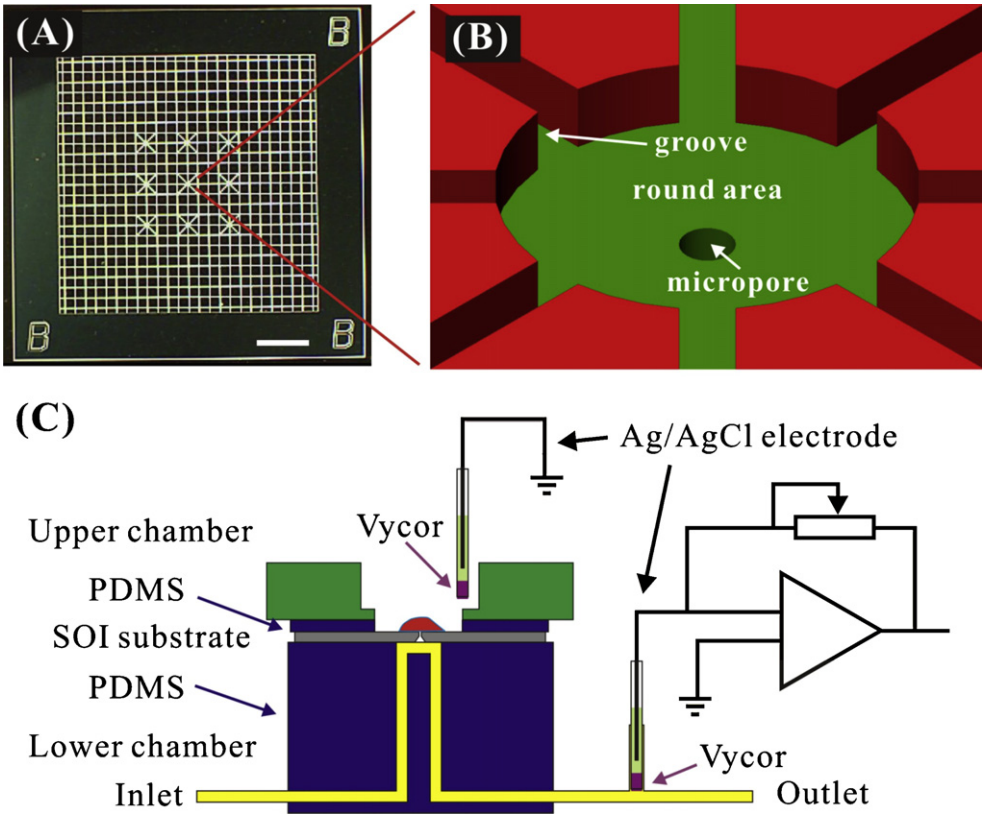

Fig. 2. The detailed structure of the cell trapping patterns (A) and (B) and schematic drawing of the biosensor having a sensor chip with a silicon on insulator (SOI) substrate. The chip is sandwiched between polydimethylsiloxane (PDMS) plates.

detailed structure of the cell trapping pattern and the schematic structure of the biosensor device are shown in Fig. 2.

2.3. Collagen IV coating on the chip surface

The Collagen IV solution (Sigma, USA) was very slowly thawed at 4 °C overnight and subsequently stirred with a Vortex stir machine for 10–15 s. Then, it was diluted by 10 ml of 50 mM HCl, dispensed into a 1 ml micro tube, and kept under −80 °C until use. The stock solution was diluted to a final usage concentration of 100 μg/ml by using phosphate buffered saline (PBS, Wako Pure Chemical Industries Ltd.). We stored this diluted solution at 4 °C and used it up within 2 weeks. Collagen IV stock solution was dropped on the surface of the Si chip until it covered the whole surface, and the chip was kept in a clean bench for 1 h. After that, the chip was rinsed with PBS and deionized water.

2.4. Expression of ChR-WR on HEK293

ChR-WR is the name given to the chimeric molecule ChR(ABCDEfg) in Ref. [22]. Here, we used ChR-WR tagged with the green fluorescent protein derivatives, Venus [23]. Its plasmid construction is reported in detail in Ref. [22]. HEK293 cells, which were a generous gift from Mr. Minoru Wakamori at Tohoku University, were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Biological Industries Ltd.) and transfected using Effectene transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. After cloning twice together with G418 (Gibco) in a 10 cm dish, single colonies with bright Venus fluorescence were selected by using a cloning cylinder (IWAKITE-32, Asahi Glass Co., Ltd., Japan) and cultured in a medium containing G418. Laser evoked channel current of ChR-WR-expressing HEK293 sensor cell was measured. The wave form unique to ChR-WR and its dependence on the membrane potential almost agreed to those observed under the similar conditions using pipette patch clamp [22] as mentioned later.

2.5. Cell culture and placement on the Si chip

ChR-WR expressing HEK293 cells were cultured in a Petri dish until they were about 70% confluent. The culture medium was composed of DMEM, supplemented with 10% (v/v) FBS, 1% (v/v) GlutaMAX (Gibco) and 0.5% (v/v) penicillin streptomycin (Gibco). 500 μg/ml G418 was added to the dish in order to eliminate untransfected HEK293 normal cells. After washing the dish in PBS, 0.05% trypsin-EDTA (Gibco) was added to it, and the culture was continued for 1 min to detach the cells from the Petri dish. After centrifuging and aspiration to remove the upper solution, the cells were diluted to 5×10^4–2×10^5 cells/ml. They were then seeded at a density of 100–400 cells/mm^2 above the collagen IV coated Si chip in the biosensor (Fig. 3). The channel current was measured after confirming that the cells had covered the micropore.

2.6. Channel current measurement

The channel current was measured using a patch-clamp amplifier (Axonpatch 200B, Axon Instruments) and a data acquisition system (Digidata 1440A, Axon Instruments) with a whole cell mode at room temperature. All data were recorded using a 2-kHz low pass filter at an output gain of 1 mV/pA and analyzed using pClamp 10.2 software (Axon Instrument).

Just before the channel current measurement, the medium in the upper and lower chambers of the biosensor was replaced by a bath solution (BS) and pipette solution (PS), respectively. The PS was composed of (in mM): 100 L-glutamine, 120 CsOH,

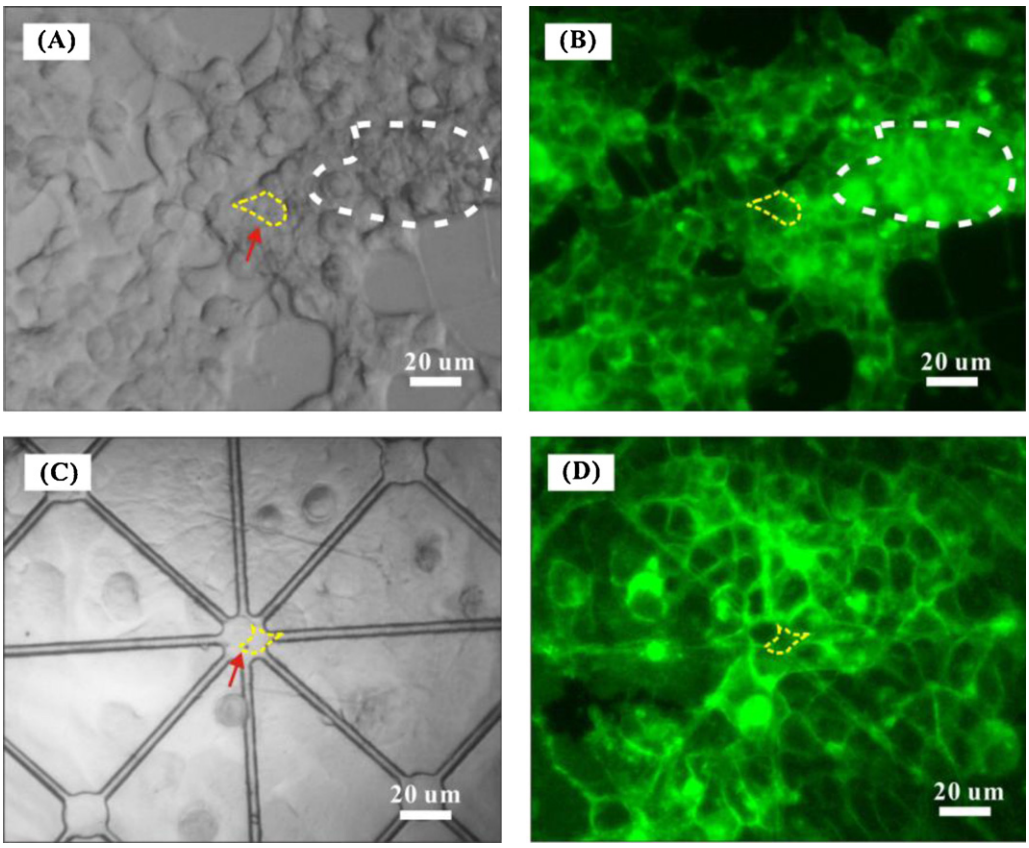

Fig. 3. Optical microscopy image of the chip surface in the biosensor after the culture of ChR-WR expressing HEK293. (A) and (B) Bright and fluorescence images of unpatterned surface, respectively. The cell on the micropore is outlined with a dotted line. Multilayer region is indicated by a bold dotted line. The fluorescent protein Venus [23] was tagged to the ChR-WR. (C) and (D) Bright and fluorescence microscopy images of patterned surface, respectively. Single monolayer colony is observed. The cell on the micropore is outlined with a dotted line. The micropores are indicated by the red arrow in (A) and (C).

50 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES), 2.5 $MgCl_2$ and 1.25 Na_2EGTA (pH 7.4). The BS was composed of (in mM): 2.5 $CaCl_2$, 1.25 $MgCl_2$, 10 D-glucose, 140 NaCl, 3 KCl and 10 HEPES (pH 7.4). After measuring the resistance and capacitance of the biosensor, the lower chamber's PS was replaced with nystatin (Sigma) containing PS (typically 100 μg/ml). After about 10 min, conductive pores formed through the cell membrane at the micropore and the cell inside and the PS side became electrically connected, forming the whole cell arrangement [24].

The capacitance of the Si chip was quite large, typically about 100 pF, so it was often difficult to observe the small (typically about 6 pF) increase in capacitance due to perforation of the cell membrane by nystatin. A laser beam with a 473 nm wavelength and 1.5 mW maximum output power was used (Sumitomo Osaka Cement Co., Ltd.) to irradiate the cell on the micropore. The focused spot size of the laser beam was about 10–50 μm.

3. Results and discussion

We made a lattice pattern with a round area at the crossing point as shown in Fig. 2(A) and (B), with the idea that this pattern would be good for not only HEK293 cells but also several other cells such as PC12 and neural cells. Furthermore, the round areas had four different diameters: 20, 25, 30 and 35 μm. The width and depth of the groove were 4 μm and 5 μm, respectively, when SU8 3005 was used. We can easily put a single cell into the round area by single cell manipulation using a micro injector (CellTram Air, Eppendorf, Japan), as shown in Fig. 1(C). This means that the usual planar patch clamp channel current measurements can be easily conducted by adding suitable negative pressure from the PS side. The aim of the present work is, however, to investigate the effect of the cell trapping pattern on the sensor chip on the cell culture in the biosensor. Hence, we seeded cells on the chip surface by using a conventional pipette (Nichipet 200 μl, Nichiryo Co., Ltd.) instead of a micro injector, if it is not mentioned. Cells were put in the round pattern area and also in the groove lines and terraces. Then we compared the cases with and without a pattern as shown in Fig. 3.

In the case of the unpatterned surface, it generally took a long time (more than 5 days) for the cells to reach the micropore's place and become almost confluent. Thus, multilayers often formed, as shown in Fig. 3(A) and (B). Such a multilayer formation on the micropore makes channel current measurements impossible. Thus, the multilayer formation significantly decreased (to ~20%) the success probability (= number of devices in which the reliable channel current profiles as shown in Fig. 4 were obtained/total number of fabricated devices) of the channel current measurement. On the other hand, in the case of the patterned surface, single or several cells were easily trapped in the round area by seeding cells using the pipette, and they did not move out but formed a monolayer colony covering the micropore around the round area by the cell division of several days incubation as shown in Fig. 3(C) and (D). The not so high (~5 μm) sidewall of the round area is considered to be suitable for the colony formation, although we did not investigate in detail about this point. The monolayer colony formation made the sensor cell's adsorption on the chip surface stronger than the single cell adsorption. If one or more of the cells become trapped in the round area during the initial culturing stage, a monolayer colony easily forms in a relatively short time; thus, the success probability of the channel current measurement significantly increased (~60%).

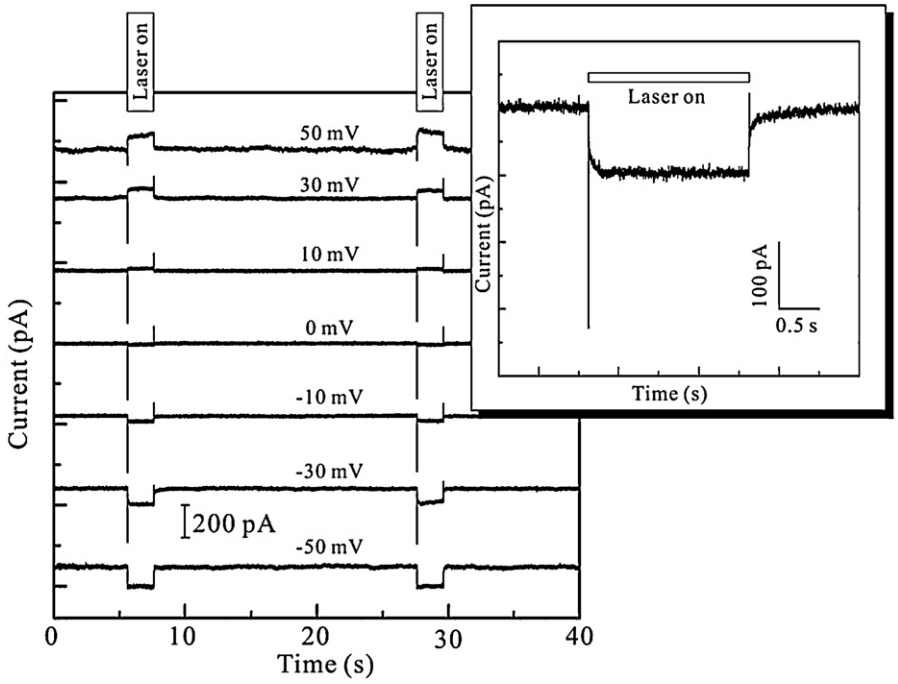

Fig. 4. Observed dependence of the laser-gated channel current recordings on the membrane potential. Inset is the expanded current profile measured at membrane potential of −30 mV.

However, the measurements tended to fail when there were very small cells or only edges of cells on the micropore. Thus, we think that the success probability can be increased a bit more by optimizing the diameter of the round area for HEK293 cells. We have compared the success probability among the four kinds of round areas with diameters, 20, 25, 30 and 35 μm. Relatively good results were obtained in the case of the diameter 20 or 25 μm (Supporting information S1), where the size of the HEK293 cells were 15 ± 5 μm.

Fig. 4 shows a typical example of the measured channel current. A rectangular pulse of a laser beam was used to irradiate the sensor cell after the monolayer colony had formed around the sensor cell. Although sharp spike-like pulses with a μs order width can be seen around the rectangular wave form due to the photo current induced by the laser irradiation (Supporting information Fig. S2), these did not have any influence on the channel current wave forms. We also carried out control experiments using HEK293 cells without transfection of ChR-WR and measured no laser induced channel current.

The seal resistance of several hundreds MΩ is easily obtained in the usual planar patch clamp [16]. In the incubation type, however, it becomes impossible to "get giga-ohm seal", which is an important concept in patch clamp, since ECM molecules coated on the sensor chip surface made a several tens nm gap between the sensor cell membrane and the chip surface. In the present case, the seal resistance was 10–30 MΩ after suction from the PS side with a negative pressure about 10 kPa. We think that the present seal resistance is sufficiently large for the whole cell mode operation. Very stable and sufficiently good signal to noise ratios can be easily obtained as shown in Fig. 4 by using stable AgCl/Ag electrodes of the salt bridge type [25] and doing measurements inside an electrically sealed box. We think that a much larger seal resistance would be necessary to observe a single channel recording. We are now trying to increase the seal resistance by changing the shape of the micropore's entrance and by using other ECM materials. However, we have not yet succeeded in improving much on the results presented here. The observed channel current wave forms matched the reported form of ChR-WR (which correspond to the chimera molecule, ABCDEfg in Ref. [22]): a sharp rise with no clear desensitization, a rather rectangular wave form, and slightly slower fall [22]. We observed a pulse train with a variable pulse interval. Attenuation of the second pulse, which has been observed in ChR-2 in dark surroundings, was not observed over the range of the pulse intervals larger than 500 ms. These are unique characteristics of ChR-WR [22], and shows that reliable channel current reflecting the molecular structure of the ion channel can be observed by the incubation type planar patch clamp.

The observed dependence of the channel current on the membrane bias (*I–V* characteristics) (Fig. 5) shows characteristics of a cation channel, and it matches the reported *I–V* characteristics of ChR-WR reported in [22], where ChR-WR was expressed on HEK293, stimulated with a filtered Xe lamp light (477 ± 10 nm), and measured by pipette patch clamp under almost the same cation concentrations as with PS and BS. From the current profile and *I–V* relations, we conclude that the laser evoked channel current of ChR-WR expressed on HEK293 was correctly measured by our incubation type planar patch clamp device.

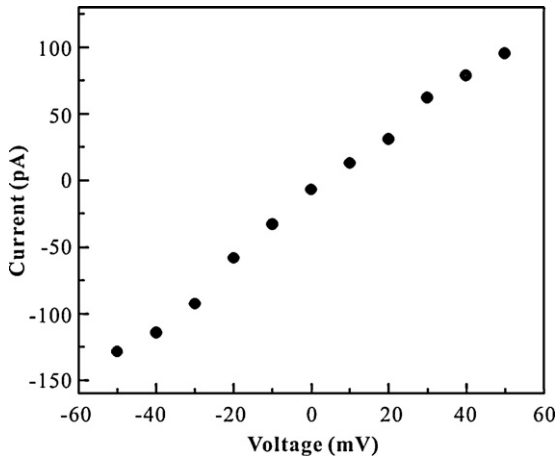

Fig. 5. Dependence of channel current on applied membrane potential.

4. Conclusions

How to put the sensor cell on the micropore and keep it there during the incubation are problematic aspects of incubation type planar patch clamp biosensors. To solve these problems, we formed a cell trpping pattern consisting of lattice pattern with a round area of about 5 μm in depth at the crossing point on the sensor Si chip and with a micropore at the center of the round area. We seeded the cell in this round area and incubated it there. In the case of a flat sensor chip with no cell trapping pattern, it took several days before the sensor cell covered the micropore and was almost confluent on the sensor chip. The multi-layers of cell often grew and made channel current measurements impossible. On the other hand, cells were easily trapped on the sensor chip surface with the cell trapping pattern and stayed in the round pattern during the cell culture. A stable cell monolayer colony easily formed, and a correct light induced channel current could be measured. We concluded that cell trapping patterns on their sensor chip's surface were extremely useful to increase the success probability of the incubation type planar patch clamp biosensors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2012.03.018.

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