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Multichannel Simultaneous Measurements of Single-Molecule Translocation in α-Hemolysin Nanopore Array

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We present a microarray system that enables simultaneous monitoring of multiple ionic currents through transmembrane α-hemolysin nanopores arrayed at bilayer lipid membranes. We applied the self-assembling ability of lipid molecules interfaced between an aqueous solution and organic solvent to induce bilayer membrane formation at a microfluidic device; the device consists of a hydrophobic polymer film that serves to suspend the lipid-containing solvent at micrometer-sized apertures as well as to separate the aqueous solution into two chambers. In this study, we confirmed that expeditious and reproducible bilayer formation is realized by control of the composition of the solvent, a mixture of *n*-decane and 1-hexanol, which permits simultaneous incorporation of the α-hemolysin nanopores to the membrane array. Monitoring the eight wells on the array at once, we obtained a maximum of four relevant, synchronous signals of translocating ionic current through the nanopores. The system was also able to detect translocation events of nucleic acid molecules through the pore via the profile of a blocked current, promising its potential for high-throughput applications.

Biological membranes contain wide varieties of membrane proteins, carrying out essential roles such as signal transduction and neurotransmitter transport in the body, and are considered as a target for more than half of all pharmaceutical drugs.[1,2] Despite their importance, researchers must tackle a formidable problem that these proteins preserve their original structures and activities only within the membranes.[3,4] One of the platforms for membrane protein study is the use of artificial, planar bilayer lipid membranes (BLM). Among them, suspended BLM is applied especially for the study of ion-channel proteins due to its suitable electrical properties.[5] The suspended BLM was usually formed by a painting method; a researcher manually paints mineral oil solution containing lipid molecules at a small aperture on a hydrophobic plastic sheet, where the lipid−oil layer becomes thinner over time. However, the formation of BLM is often unpredictable and the formed membrane is fragile, and the large scale of the system is inadequate for using precious biological substances. Recently several research groups have innovated a well-controlled method rather than using manual procedures;[6−9] this method applies the principle of the Langmuir−Blodgett technique combined with microfluidic systems, where lipid solution and aqueous buffer are infused into microchannels or microchambers. In our previous works, we developed a microarray chip focused on the implementation of parallel BLM formation successful in mass production of BLM as well as incorporation of gramicidin-A peptide channels.[10,11]

As the next challenge, we are interested in the incorporation of α-hemolysin, a more complex protein forming a transmembrane nanopore. α-Hemolysin (αHL) nanopore from *Staphylococcus aureus* consists of homoheptametric subunits with the most narrow constriction at 1.5 nm in diameter, able to translocate single-stranded DNA (ssDNA), RNA, or linear polymers, but not double-stranded DNA (dsDNA) (see Materials and Methods). Numbers of studies have demonstrated that this specific αHL pore size is useful for biosensor applications by monitoring the shift of its electrical conductance, for example, DNA/RNA sequencing, to measure DNA−protein interaction and mass spectrometry of poly(ethylene glycol).[12−15] The nanopore DNA sequencer is at present especially remarked as a highly attractive approach since

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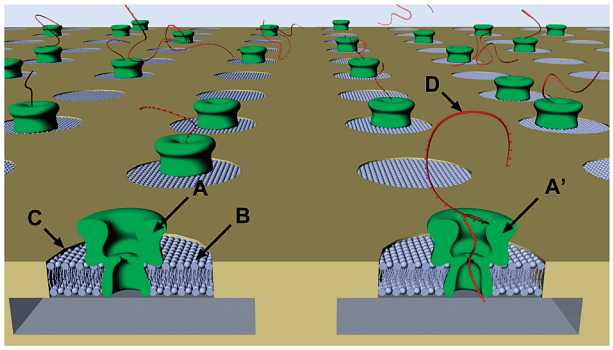

Figure 1. Schematic diagram of the system that enables detection of multichannel signals from translocations of single molecules at the αHL nanopores. Ionic current through a nanopore is significantly reduced with the translocation of a single linear polymer such as ssDNA/RNA. Multichannel monitoring will be achieved by the incorporation of the nanopores at electrically isolated BLM chambers (A, αHL nanopore; A′, αHL nanopore blocked by a translocating molecule; B, bilayer lipid membrane; C, micrometer-sized aperture; D, analyte polymer).

the method does not need any amplification or chemical modification of DNA.[16] However, there are still critical issues remaining toward practical use, such as insufficient signal-to-noise ratio and limited data-throughput. Although devices in recent studies have been adapted for sensitivity, handling robustness, and the reproducibility of BLM formation,[17−22] we find few attempts with the nanopore array system to gain higher throughput.

On these accounts, we target multichannel signal detection of the αHL nanopores, as shown in Figure 1, which demands refining our previous system. Most importantly the drawbacks with suspended BLM must be considered; organic-solvent molecules, intrinsically remaining between the two lipid layers by the suspension method, are considered to inhibit the incorporation of membrane proteins including αHL but not simple peptides due to a slight increase in membrane thickness.[3,5] In addition, rapid and reproducible formation of stable BLM will be required for the following αHL incorporation and the electrical measurements.

In this study, we therefore examined the composition of the solvent itself that enables control of the process of BLM formation. The strategy is to mix alcohol within the solvent that partly dissolves into aqueous phase. By the diffusion of alcohol into the buffer we expect that the solvent layer between the aqueous phases becomes thinner swiftly so that bilayer formation will be accelerated. Furthermore, the enriched lipid fraction may reduce the chance that solvent molecules remain at the lipid bilayer and may strengthen the electrical resistance of the membrane that is important for electrophysiological experiments. Consequently, the incorporation of αHL to the BLM will be induced and accelerated,

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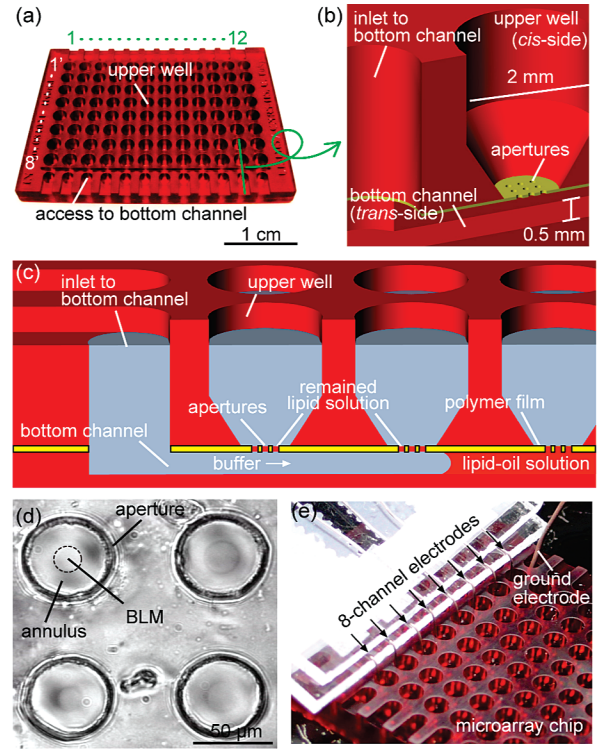

Figure 2. Multichannel nanopore biosensor system. (a) Overview and (b and c) cross-sectional images of the device. (d) Microscopic image of suspended BLM at four apertures on the well. One of the BLM edges is indicated with the dashed line. (e) Experimental setup of multichannel recording. The eight-channel Ag/AgCl electrodes are inserted into the upper wells.

affording the possibility of simultaneous nanopore formations at the BLM array. In the following sections, first we present the principles and the fabrication process of the microarray device for transmembrane αHL nanopores. We chose 1-hexanol as the alcohol with mineral oil *n*-decane[9] and screened the mixing ratio to obtain optimal solvent conditions for parallel BLM formation. Then we performed multichannel monitoring of the αHL nanopore signals with the array device, also using an RNA analogue of polyuridylic acid and a synthetic short ssDNA as translocating analytes of the pore to examine the applicability of our system.

MATERIALS AND METHODS

Principle of On-Chip BLM Formation and αHL Incorporation. The fundamental concept for developing a multichannel BLM microarray device is reported in our recent work.[11] Briefly, the device consists of three parts (Figure 2). The intermediate thin film fabricated by a hydrophobic polymer suspends BLM at micrometer-sized apertures. The upper component forms wells at the cis side of the liquid chambers, and the upper wells in a straight row share one microfluidic channel at the bottom component as the trans side. Here we adopt a 96-well format, eight wells in each of 12 rows, designed within the dimension of 40 × 30 × 3.5 mm^3. The detailed design is shown in Figure 2a−c. In our device we put nine apertures at each well to increase the chance of bilayer formation. If the number of the apertures increases at each well we may face the risk that one of the membranes ruptures and fail signal monitoring. On the other hand the success rate of bilayer formation will be improved by the

increase of the number of apertures. We determined the number of aperture by the balance of these conditions and controlled the concentration ratio of 1-hexanol to *n*-decane.

The following three-step protocol provides formation of suspended BLM on the device (see Figure 2c): initially the cis-chambers were filled with 5 μL of buffer solution. Next, 3 μL of 20 mg/mL lipid solution dissolved in the mixture of *n*-decane and 1-hexanol was injected into the bottom microfluidic channel, and 50 μL of buffer solution was introduced sequentially. Due to the hydrophobic property of the polymer thin film, the lipid solution remained at the apertures as a lipid-containing oil membrane. The amphiphilic lipid molecules autonomously assembled at the interface between the oil and buffer aqueous solution, and the membrane became thinner with the diffusion of 1-hexanol from the membrane to the buffer as well as the share stress by the buffer flow. When a particular thickness was attained, the assembled lipids came together to form a bilayer membrane.[5] A lipid concentration of 20 mg/mL, and 500 mM KCl with 10 mM MOPS (pH 7) for the buffer was used.

Subsequently the αHL molecules added at the cis-chamber formed nanopores at the BLM. The formation of BLM induced the incorporation of the αHL from the buffer to the bilayer. Once the incorporation occurred, the αHL formed homoheptamers that opened up nonselective open-state transmembrane nanopores (see Figure 1). The constriction of the nanopore defined pore conductance so that the electrical current increased stepwise depending on the number of the formed pores. Furthermore, the narrow pore size resulted in a steep drop of the current by translocation of analyte molecules such as RNA or ssDNA whose size is similar to that of the constriction, yet the current recovered when the translocation ended.[23−25] Here, the final concentration of 5−10 μg/mL of αHL was added to the cis-side wells, and the translocation events were monitored with a synthetic RNA analogue and a short ssDNA.

Fabrication Process of the Device. The aperture-patterned polymer film for the intermediate layer was fabricated with a common photolithography process: utilizing vapor deposition, poly(chloro-*p*-xylylene) (parylene) dimer was polymerized and coated on a silicon wafer with thickness of 20 μm (PDS2010, Specialty Coating Systems, Indiana). Then a thin aluminum layer was deposited and patterned by photolithography. We obtained the patterned parylene film by applying oxygen plasma. The actual diameter of the aperture was 55−57 μm.

Stereolithography allowed instant fabrication of a three-dimensional model designed with CAD software (Perfactory, Envisiontec, Germany). In principle, visible light polymerizes a thin layer of photocurable resin illuminated with the designed pattern so that the layer-by-layer projection of the light can materialize the designed structure. The resolution of 16 μm in the $x-y$ plane is sufficient for the developing device. The patterned parylene film was simply inserted at the middle of the process between the upper and bottom portions of the array device. The detailed process was previously reported.[11]

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Table 1. Electrical Properties of the Formed Bilayer Lipid Membranes[a]

solvent	capacitance (μF/cm^2)	estimated thickness (nm)[b]
n-decane	0.65 ± 0.05	6.8 ± 0.5
mixture with 1-hexanol[c]	0.85 ± 0.19	5.4 ± 1.1

[a] The standard deviations of the data were also included (n > 15). [b] The thickness of the membrane was estimated from the capacitance using an assumed dielectric constant of 5. [c] The ratio of 1-hexanol to *n*-decane is 2 to 1.

Multichannel Electrophysiological Measurement. The apparatus for electrophysiological experiments is shown in Figure 2e. We monitored eight wells in a row, i.e., on one channel, at a time. After BLM formation described above, the ground electrode was placed at the inlet of the bottom channel and a set of eight electrodes was immersed in the measuring wells. Since the upper wells are electrically isolated when membranes are formed, electrical monitoring is independently available at each well. The Ag/AgCl electrodes were connected to an eight-channel patch-clamp amplifier (Triton, Tecella, CA), acquiring data by a voltage-clamping mode. The sampling rate and the low-pass filter were 5 or 1 kHz, and 1 kHz, respectively.

Materials. Synthetic lipid 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids, Inc. Transmembrane nanopore, αHL from *S. aureus*, and an RNA analogue polymer, polyuridylic acid (poly(U)), were from Sigma-Aldrich Corp. Single-stranded DNA of 19 nt (GGGGGAGTATTGCGGAGGA, ssDNA) was synthesized at BEX Co., Ltd. Poly(chloro-*p*-xylylene) (parylene) for the polymer film of the device was provided by Parylene Japan, Inc. Other reagents and materials were purchased from Sigma-Aldrich, Kanto Chemical, Wako Pure Chemical Industries, Tokyo Ohka Kogyo, Rohm and Haas, and Ferrotec Silicon Corp. All compounds were used without further purification.

RESULTS AND DISCUSSION

Multiple Bilayer Formation Regulated with the Solvent of Lipids. We confirmed the formation of bilayer lipid membranes by evaluating the membrane thickness from microscopic images and electrical capacitance data (see details in the Supporting Information). As mentioned above we adopted a mixed solvent of 1-hexanol and *n*-decane and found that mixing 1-hexanol rapidly reduced the thickness of the lipid-containing solvent layer and effectively promoted BLM formation process. Mixing of 1-hexanol also changes the electrical properties of the formed bilayer membrane. As clearly presented in Table 1 the capacitance increases by mixing the cosolvent. This result agrees well with the assumption that the mixed hexanol makes the membrane thinner and reduces the chance of solvent molecules remaining within the bilayer. Here the optimal ratio of 1-hexanol to *n*-decane was determined at 80−20%. If 1-hexanol is used below 50% level, the process of thinning the solvent layer apparently slowed down. On the other hand, the formed BLM (or the half-formed lipid−oil membrane) easily broke at ratios higher than 90% since *n*-decane cannot reinforce the annulus of the membrane at such a low composition ratio, 10%. In a series of experiments at the optimal condition, bilayer formation occurred immediately or within 10

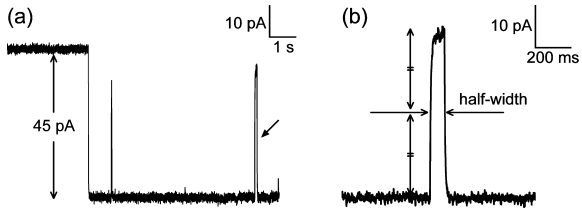

Figure 3. (a) Typical signal of αHL nanopore formation at the device. Applied voltage was −80 mV at cis-side negative. The increase of conductance with the formed nanopore is presented as negative. (b) Expanded trace of the spike indicated with the arrow in panel a, representing the translocation event of the analyte at 0.1 mg/mL poly(U).

min following injection of the last buffer. The experimental findings correlated well with the hypothesis that mixed alcohol diffuses into the buffer and accelerates the process of bilayer formation. Note that the number and size of the apertures on the parylene film related to the optimal ratio. In this paper we chose nine apertures with 55 μm in diameter because the increase of BLM area that is proportional to the number of apertures increases the probability of membrane protein incorporation while the diameter stabilizes the BLM when compared to the larger ones.[10]

Mixing the solvents was successful with respect to reproducibility and rapid formation of BLM. In former works BLM formation was controlled by the application of hydrostatic pressure between the cis- and trans-chambers,[6,26] yet the pressure-driven system may have difficulty when introduced to miniaturized, arrayed devices. In contrast we note the advantage of the solvent-driven approach that is able to adapt its condition flexibly to changes in device design.

Multichannel Recording of α-Hemolysin Nanopore Currents. Figure 3 shows a single signal of the αHL nanopore with an analyte, monitored by the array device. As described above the electrical current increases in a steplike pattern together with the number of αHL nanopore incorporation. Here we observed the step height of 45 pA at −80 mV of cis-side negative, i.e., 560 pS, corresponding to the conductance of a single nanopore.[27,28] This result implies that there is no influence of the mixed 1-hexanol on the nanopore signals although the hexanol may affect some kinds of ion channels. The translocation of analytes appears as rectangular spikes that reduce nanopore conductance (see Figure 3b).

Multichannel, simultaneous recordings of the signals are shown in Figure 4. In this example we obtained four signals within a short period of time (ca. a few minutes). At the level of individual wells, the reproducibility of αHL incorporation is promising. We, however, still face difficulty in simultaneous observation of the signals from multiple wells because the device cannot strictly control the timing of either BLM formation or αHL incorporation. Therefore, certain intervals of several minutes often arise on incorporation-timing among the wells. Nonetheless, we were able to obtain up to four concurrent signals out of the eight wells and achieved even larger numbers of successful nanopore formation in total. Additionally we found that there is no obvious cross-talk between the wells.

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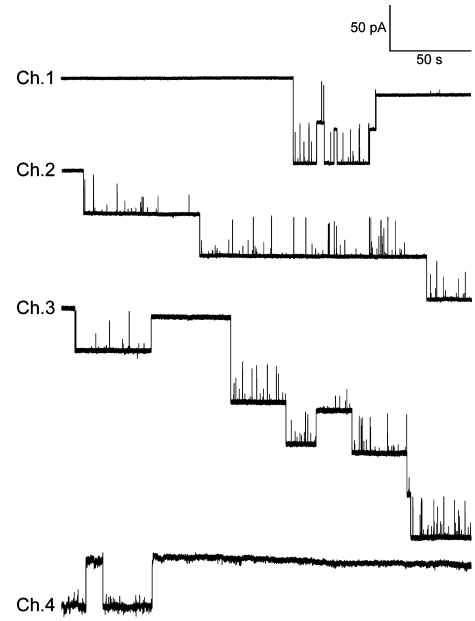

Figure 4. Four simultaneously obtained αHL signals with multichannel monitoring. At half of the eight wells we succeeded in synchronous nanopore formation. The applied voltage was −80 mV, and the analyte was 0.1 mg/mL poly(U).

From the viewpoint of application, we prefer to keep the state incorporating a single nanopore at each well to avoid overlapping spikes coming from the translocation of analytes as well as to suppress the noise level of the baseline that increases with decreasing membrane resistance. Undesired αHL incorporation can be lowered by optimizing αHL concentration or by using the trans-side channel for injecting and flushing of αHL solution. The latter, however, changes the orientation of the anisotropic αHL nanopore at the BLM that will affect the manner of translocation of analytes.

Regarding BLM stability we observed stable membranes for more than a few hours. The experimental results also indicated that αHL molecules are preferentially distributed into the BLM from the cis-side buffer by using the optimal mixture of solvents and form nanopores swiftly. This rapid formation of nanopores helps to realize multichannel measurements of single-molecule translocations.

Potential of the α-Hemolysin Nanopore Array Device. An RNA analogue of poly(U) and a short ssDNA were used as two representative analytes. The poly(U) has a broad range of molecular weights whose average is larger than 100 kDa (300 nt). On the other hand, the synthesized ssDNA contains a short, defined sequence of 19 nt. We compared the translocation of these two analytes to measure the potential of the multichannel system.

Prior to further examination of the system with these analytes, we ensured the consistency of signals obtained by multichannel measurements. We examined if the conductance values of the single nanopore and the resultant translocations of the analytes are equivalent between the wells. By comparing the statistic distribution of the half-width of the blocking peaks, we confirmed that these wells are able to be treated as equal.

First we compared the translocation events of the two analytes. As shown in Figure 5 the statistic distribution of the translocation clearly revealed the difference of the analytes. The polydispersity of poly(U) lengths is reflected by a broad distribution, whereas

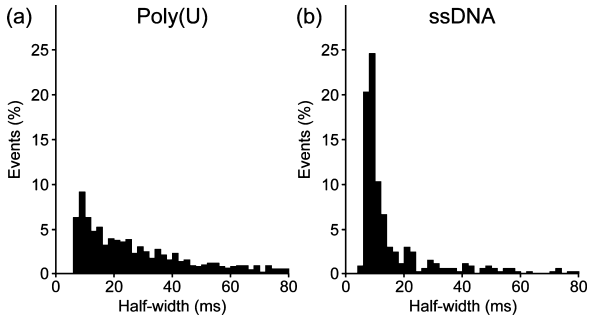

Figure 5. Histograms of the half-widths of blocking peaks by translocation of the analyte at (a) 0.5 mg/mL poly(U) and (b) 10 μM ssDNA. The applied voltage was −120 mV.

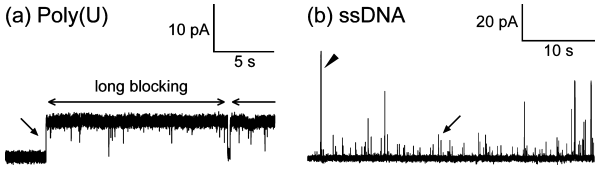

Figure 6. (a) Example of the long blocking events of the nanopore (0.1 mg/mL poly(U), −80 mV). Note that the nanopore is partially blocked (at the arrow) where the current decrease is smaller than the translocation event. (b) Bouncing spikes with short peak amplitudes shown with the arrow. A translocation peak is also indicated with the arrowhead (10 μM ssDNA, −120 mV).

the monodispersed ssDNA showed such a narrow band. There were also long blocking events seen with poly(U), representing that the nanopore is probably clogged by a tangled molecule(s) (see Figure 6a). Note that poly(U) contains molecules longer than 100 kDa, and we confirmed that the blockades are released when reversing the applied voltage. Meanwhile we observed few long blocking events with the ssDNA, but spikes with shorter peak amplitudes (Figure 6b). The latter was caused by collision or transient partial entry of the analytes to the nanopore but not translocation.[24,29,30]

We also examined the concentration dependence of the poly(U) analyte. Translocation events with 0.05, 0.1, and 0.5 mg/mL were monitored with the array device. As shown in Figure 7, the frequency of translocation increased with an increase in concentration and evaluated as 0.3, 0.5, and 1.4 Hz at 0.05, 0.1, and 0.5 mg/mL, respectively. The estimated values were obtained from at least three individual experiments. One reason that the frequency was not proportional to the concentration is that analyte concentration was saturated against the number of nanopores; the rate-determining step shifted from the diffusion of the analytes to the translocation at the nanopores.[31,32] The other reason is the decrease of the apparent concentration due to an increase of tangled polymers. On the other hand, the statistical distribution

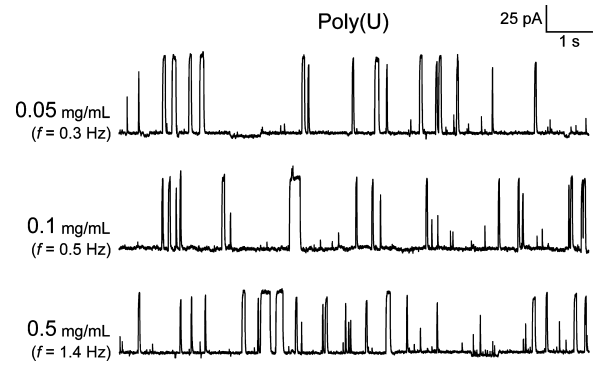

Figure 7. Probability of translocation events increases with an increase in concentration of poly(U). The frequency of the translocation events is noted in each condition. The different concentrations of poly(U) were examined at each well in the device. The applied voltage was −120 mV.

of the translocation events in Figure 5a was not significantly influenced by the concentration since each spike corresponded to the translocation of a single analyte (data not shown).

CONCLUSIONS

In this study, we presented a microarray system for membrane nanopore studies. The adjusted composition of the organic solvent of lipids enabled rapid BLM formation and αHL incorporation, which in turn realized the first implementation of multichannel and simultaneous monitoring of the nanopore ionic current. The results demonstrated that the developed system succeeded in scaling up the number of the measuring chambers. We believe that our method will overcome the data-throughput problem that is one of the major hurdles for the practical realization of a nanopore-based DNA sequencer. Furthermore, the same approach can be applied to electrophysiological research of membrane ion-channel proteins and accordingly contribute to the increase of throughput on drug screening. We, however, have to settle the problems on the data processing, which restricted the sampling rate and the recording duration, caused by the increased data size with increasing the number of simultaneous recordings.

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SUPPORTING INFORMATION AVAILABLE

Description of the evaluation method of BLM thickness. This material is available free of charge via the Internet at http://pubs.acs.org.

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