論 文 の 内 容 の 要 旨

論文題目　Micro/Extended-nano Sampling Interface for Living Single Cell Analysis
(単一生細胞分析に向けたマイクロ・拡張ナノサンプリングインターフェース)

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1. Introduction

Cell analysis methods are important in general biology and medical fields, and many methodologies were developed. In particular, for understanding the real cellular process, the cell expression analysis (proteins and metabolites) are becoming important. In addition, it is recently recognized that cell analysis at single cell level is essential due to the cell heterogeneity in epigenetics, immunology, cancer, etc. As an ultimate goal, living cell analysis is required to study cellular processes of the specific single cell among same kinds of single cells. For this purpose, ultrasensitive analytical technologies are increasingly required. Currently, microfluidic technologies are recognized as one of the potential methods for analyzing proteins in/from single cells. However, the cell volume is much smaller than the volume of microchannels (nL to sub-nL). And the number of the analyte becomes 1-1000 molecules in nM-pM range, and countable molecule detection is required. That is why protein analysis in single cell is difficult.Previously, we developed novel ultrasensitive analytical devices using extended-nano (10-100 nm) space: high resolution chromatography and single molecule immunoassay devices. The volume of the extended-nano space is aL to fL, which is several orders smaller than the single cell volume (~pL). The extended-nano space is promising for single cell analysis. Based on these investigations, I conceive an idea that living single cell analysis becomes possible by analyzing small volume (fL) of samples from a living single cell using the extended-nano space. However, a great challenge exists to realize the micro/extended-nano sampling interface for single cell.

Based on the background, objective of this Ph.D study is to create extended-nano interface for living single cell analysis.

(1) Micro/Extended-nano Interface by Fusion of Lipid Bilayer (Chapter II)
(2) fL Sampling Method by Micro-nano Fluidic Engineering (Chapter III)
(3) Sampling Volume Control and Transport at fL (Chapter IV)

2. Micro/Extended-nano Interface by Fusion of Lipid Bilayer

2.1.Proposal of micro/extended-nano interface by fusion of lipid bilayer

Firstly, the principle of extended nano interface based on fusion of lipid bilayer is shown in Fig.1. Microchannel, single cell chamber, and extended-nano channel were connected each other. In the single cell chamber, a specific single cell can be selected and isolated by optical tweezers. A lipid bilayer is uniformly modified on the wall of the extended- nano channel by introducing vesicles (vesicle fusion methods). The cell membrane and modified lipid bilayer contact

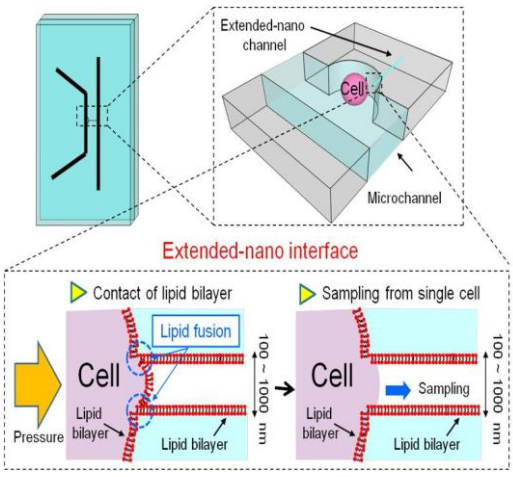

Fig.1. Principle of this study

by applied pressure. When the pressure is increased, the lipid bilayers on the cell and extended-nanochannel contact and form a new lipid bilayer after fusion by the molecular interaction. As a result, a hole with 100 nm size is formed, and the cytoplasm inside the single cell can be sampled by applying pressure without any leakage.

2.2. Design and fabrication of micro/extended-nano sampling interface

The microchip for cell fusion is designed and fabricated as shown in Fig. 2. The microchannels and single cell chamber were fabricated on a glass substrate by UV lithography and dry etching techniques. On another glass substrate, extended-nano channel was fabricated by electron beam lithography and dry etching. The two substrates were thermally bonded at 1080 ℃. Vesicle fusion method was utilized to modify lipid bilayer in extended-nano space. The vesicles were introduced into extended-nano channels. Vesicle interacted with surface of glass substrate, resulting in the rupture and uniform lipid bilayer modification on the surface.

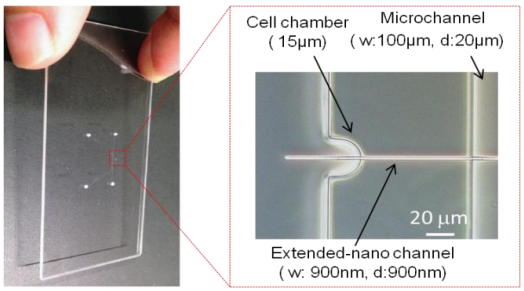

Fig.2. Photo of the fabricated microchip and expanded view of cell chamber and nanochannel.

2.3. Development of monitoring method for lipid fusion

In order to investigate the principle, monitoring method of lipid fusion process is essential. When the tight junction is realized (lipid fusion), the current is almost zero, and theresistance usually becomes GΩ level, while the value becomes MΩ level if there is leakage (no fusion). In order to investigate the resistance between the single cell and the extended-nanochannel, an equivalent circuit was designed as shown in Fig. 3. For electrical measurement, Ag/AgCl wire electrodes wires were inserted to the reservoirs. The resistances were calculated from current-voltage curves, and the results were compared for the extended-nano channels with and without lipid bilayer modification.

As a model sample, normal human aortic endothelial cells (HAECs) were used, the cytoplasm was stained with Calcein AM for observation. Cells were introduced into the microchannel at density of more than 1×10^{7} cells cm^{-2}. By applying ~5 kPa pressure, individual cells were introduced to the single cell chamber. In order to realize the fusion of lipid bilayer, higher pressure (70 kPa) was applied. The current-voltage curve is shown in Fig. 4. The resistance of the modified channel was 2.8 GΩ, while that of non-modified channel was 163 MΩ.

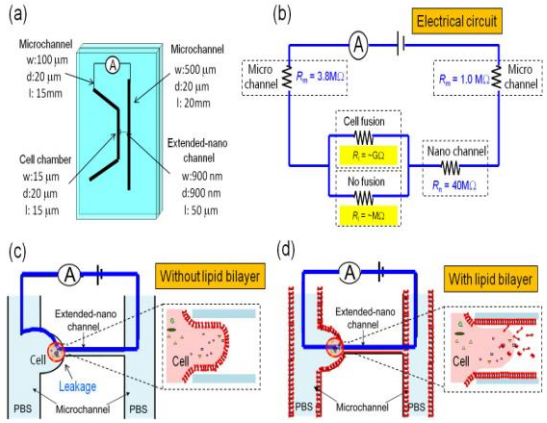

Fig.3. Principle of extended-nano patch clamp method: (a) chip design, (b) the equivalent circuit, (c) resistance for no cell fusion, (d) resistance for cell fusion.

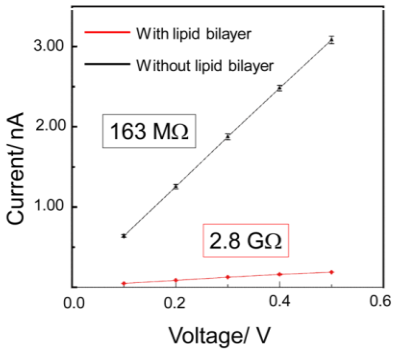

Fig.4. Voltage-current curve measured by the extended-nano patch clamp

Therefore, it can be concluded that tight junction between the cell and nanochannel was achieved. Based on these results, the working principle of the extended-nano interface was confirmed for the first time.

3. fL Sampling Method by Micro-nano Fluidic Engineering

3.1. Proposal of fL sampling method

For analytical methods, sampling plays important roles. In order to quantify the concentration of sample, which is number of analyte molecules divided by the volume, determination and regulation of the sample volume is essential. However, the volume is fL for single cell analysis, which is three order smaller than the conventional methods (pL). In addition, for living single cell analysis, we should sample from the cell without less damage. In order to solve these problems, I will propose the sampling method as follows. The fL is too small to be determined, and the nanochannel is used for the determination by measuring the length l of introduced sample and calculating the sampling volume = $l \times w \times d$ (w: channel width, d: channel depth). For regulating the sample volume, fluidic pressure is used because of the ability of controlling the pressure precisely. However, there is a possibility that w and d changes during the thermal bonding process (1080℃). Therefore, I investigate the deformation of the cross section of the nanochannel during bonding process.

3.2. Evaluation of cross section of extended-nano channel after bonding

In order to confirm the sampling volume, we verified the extend-nano channel cross section (= depth × width) before and after bonding. The extended-nano channel (w: 900 nm and d: 900 nm) was used. The substrate was cut before bonding, and the depth and width were measured by SEM. The other substrate was cut after bonding, the depth and width were measured by SEM. The SEM image of cross section is shown in Fig.5. Comparing with extended-nano channel before and after bonding, the depth and width was almost same (< 1% change). Therefore, the width and depth of the extended-nano channel before bonding can be used for the sampling volume estimation.

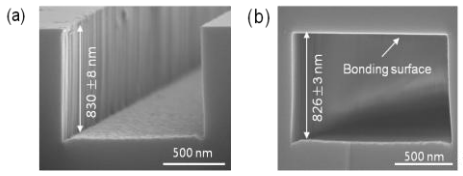

Fig. 5. SEM image of cross section of the extended-nano channel: (a) before bonding, (b) after bonding.

3.3. Confirmation of sampling from living single cell

The cytoplasm in the HAEC cell was visualized by a fluorescence microscope as shown in Fig. 6. When the extended-nano channel was modified with the lipid bilayers, the cytoplasm was introduced into the extended-nano channel (Fig. 6(a)). No leakage of the cytoplasm was observed in the single cell chamber. The cell kept the round shape, and the other part of the cell was not damaged. Cell was keeping good viability over long times (12h) after sampling. On the other hand, the introduction of the cytoplasm was not observed for the extended-nano channel without lipid bilayer modification (Fig. 6(b)). Even at the maximum pressure of 500 kPa, the cell was totally deformed, and the cytoplasm was not introduced. Therefore, the micro/extended-nano sampling was confirmed.

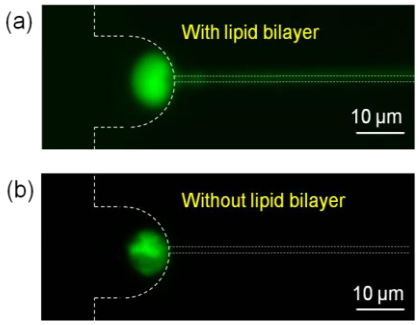

Fig. 6. Fluorescent images of cell fusion (a) with lipid bilayer (b) without lipid bilayer.

4. Sampling Volume Control and Transport at fL

4.1 Proposal of fL Sampling Control and Transport Method by Laplace Valve

In order to realize fL sampling control and transport, the two issues need to be solved. The first is the design of the fL sampling volume control after introduce to extended-nano channel. The second, the fL sampling volume transport to detection chamber with control fluidic speed. Nano Laplace valve, it is essential to the controlling, and the tranport for fL volume in extended nano-channels. The use of different channel sizes was designed in the integration of the micro extended-nano fluidic device as shown in (Fig.7). The fL sampling volume was controlled and transported by Laplace-valve. Based on the chapter 3, the sampling volume was evaluated in extended-nano channel. Therefore, fL sample volume could be measured depending on L (Length) after controlling by nano Laplace valve. The fL samples will transport to transport-chamber for fluidic speed control before into detection chamber.

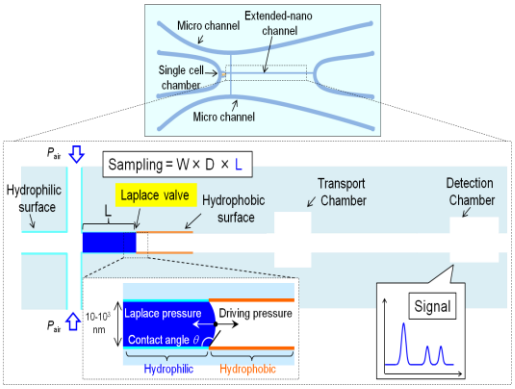

Fig. 7. Proposal of fL Sampling Control by Laplace valve

4.2 Demonstration fL sampling volume control and transport

Based on the proposal of fL sampling control and transport was described in section 4.1. There was a successful fabrication of nano Laplace valve using nano-fabrication techniques, and a fluorine coating pattering on the extended-nano surface. Femtoliter scale liquid handling had been demonstrated in the following way (Fig. 8).11.7 fL sampling volume control and transport was demonstrated. This method offers a general platform that is able to manipulate ultra small volume liquid in extended-nano channel.

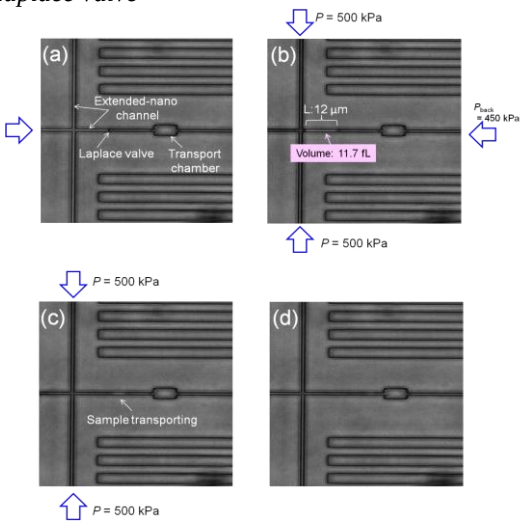

Fig. 8. Demonstration of fL sampling volume control and transport: (a) after sample introduction and sample stopped by Laplace valve and (b) 11.7 fL sampling volume was controlled and (d) fL sampling was transporting to transport chamber and (d) 11.7fL sampling volume was transported to chamber.

5. Conclusion

The micro/extended-nano interface was realized for the first time. Micro/extended-nano interface was realized by fusion of lipid bilayer. Chemical principle was utilized to make a hole on the cell surface and connect the hole with the extended-nano channel. Extended-nano patch clamp method was developed for monitoring fusion of lipid bilayers, and GΩ sealing was demonstrated. The extended-nano channel cross section was investigated before and after bonding, and almost no deformation was observed. In sampling experiments, femtoliter sampling from living single cell was demonstrated. A total integration micro/extended-nano fluidics device was designed and fabricated. The fL sampling control and transport of a 11.7 fL sample liquid was demonstrated. The demonstrated methodology would be applied to the analysis of living single cell analysis. This new sampling method allows ultrasensitive analysis of living single cells and contributes to cell biology and tissue engineering.