NANOBLADE ARRAY FOR SPATIAL DISSECTION OF SINGLE CELLS AND TISSUES

D. Kagawa[1], M. Kusumoto[1], Y. Takemura[1], H. Takao[1], F. Shimokawa[1] and K. Terao[1,2]
[1]Kagawa University, Kagawa, JAPAN
[2]PRESTO, Saitama, JAPAN

ABSTRACT

We propose a novel method for sampling single cells and intracellular biomolecules without losing spatial information in tissues or in single cells, using Si nano-blade array device. The device has an array of thin blade structures ranging from 300 nm to tens of μm in thickness. The blade design is easily arrangeable in accordance with the target samples (single cells and tissues). Pushing the device physically against cultured cells or a sliced tissue allows us to dissect and keep them in the space walled by the blade structures. The biomolecules are extracted in each space without contaminating each other, and are collected with ligand molecules immobilized on the blades or a micropipette approaching from the backside of the device. The device allows the sampling of biomolecules extracted from tissue or single cells with spatial information, which is expected to realize the analysis of spatial distribution of biomolecules.

INTRODUCTION

Spatial distribution of biomolecules (ex. proteins, nucleic acids.) in a tissue and a cell governs a vast of biological processes, such as organ development and formation of cell polarity. Various methods have been employed to elucidate it, including fluorescence imaging technique and laser microdissection technique [1, 2]. In imaging technique, target molecules are labeled with fluorescent markers, which bring precise spatial information, though, it limits the quantification of molecules. Laser microdissection is a sampling technique to pick a small part of tissues by laser cutting, which realizes the use of various omics analysis methods. However, it depends on the skill of an operator and limits the throughput of the sampling. Thus, high-throughput sampling technique from tissues and cells is required for the further development of biological analysis. Therefore, we propose a method using Si nano-blade array device for sampling fractions with spatial information.

PRINCIPLE

Device

We targeted two types of bio-sample; subcellular regions in single cell and single cells in a tissue slice. Subcellular sampling should require high spatial resolution. Thus, the device for this aim is fabricated with the combination of the processes of EB (electron beam) lithography and Si deep etching (ICP-RIE). The device has an array of thin blade structures of around 300 nm thickness. The gap between the blades ranges from 2 μm to 10 μm, which defines the resolution of sampling. The blades are designed to have the depth of 15 μm, which is deeper than the thickness of the single cell size. Hereafter, the device is mentioned as "high-resolution type". On the other hand, sampling single cells from a tissue slice need rigid blades, and adequate space for collecting cells. Thus, the device for this application has an array of blade structures with tens of μm in thickness, whereas the depth of the blade is 300 μm for tissue cutting. This configuration allows the fabrication of through-holes surrounded by blades (hereafter, through-hole type). This enables us to approach to the cell samples from the backside of the device.

Sampling

The operation with the device includes two processes; dissection and analysis, for the quantitative measurements of intracellular or cellular spatial heterogeneity. First, target sample is dissected by cutting and segmented with the device. Next, fractions of the sample are analyzed with quantitative method for obtaining spatial data of heterogeneity. The dissection is done just by pushing the device physically against cultured cells or a sliced tissue, which allows us to separate and keep the fractions in the space walled by the blade structures. The biomolecules are expected to be extracted in each space without contaminating each other.

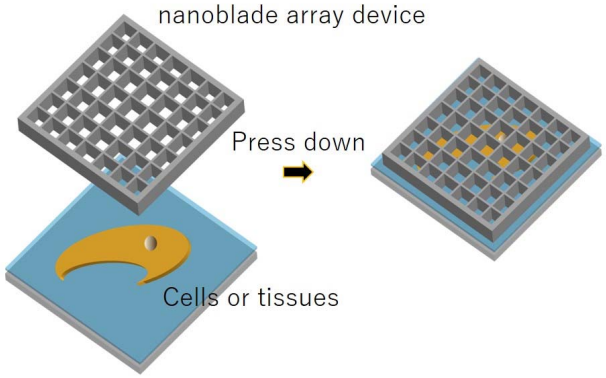

Figure 1: Principle of spatial dissection.

High-resolution type device has ligand molecules immobilized on its blade surface to capture specific biomolecules extracted from single cells. The captured biomolecules are measured by staining with fluorescent labelling etc. Ligand molecules are chemically immobilized on Si surface after nanofabrication of the device. Figure 2 shows the example of sandwich immunoassay. First, Si-tagged protein A and ligand molecules such as antibodies are immobilized on Si blade device successively. Then, a single cell is dissected using the device, when target biomolecules are captured on the ligand molecules on the blade surface. After releasing the device from the substrate, detection reagents such as antibodies are applied to bind to the target biomolecules. Finally, secondary reagents such as fluorescent-labelled antibodies are applied to detect target molecules. Target

biomolecule in the single cell are quantified by measuring fluorescence intensity in each section walled with the blade structures.

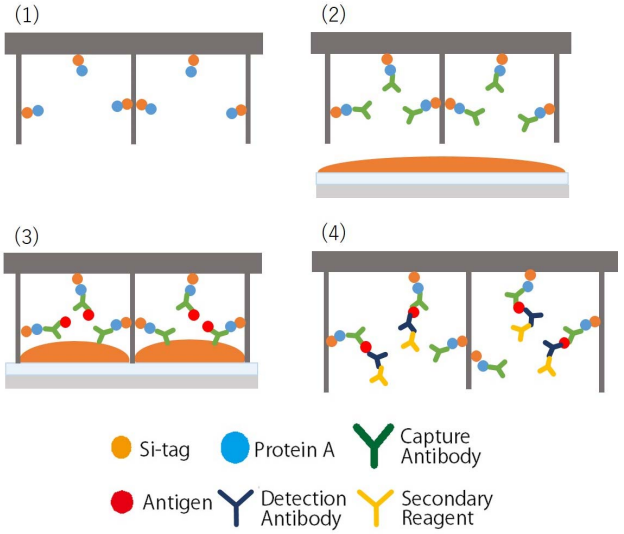

Figure 2: Detection method using sandwich immunoassay with high-resolution type (non-though-hole type). (1) and (2) Ligand molecules immobilized on the device. (3) Dissection process. (4) Detection process.

In case of a through-hole blade array for tissue dissection, a micropipette is accessible to the dissected samples from the backside of the device to pick up the fractions one by one (Figure 3). The micropipette connected with a micro pump allows dispensing/suction of solution into/from the section. We utilized single cell picking system (TOPick 1, Yodaka Corp.) for this purpose.

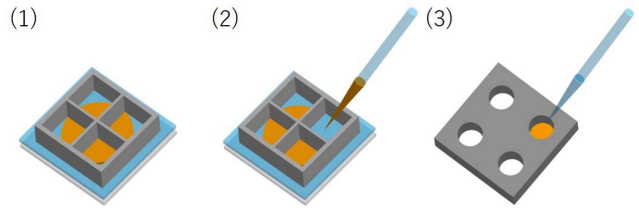

Figure 3: Sampling method of a fraction by though-hole type. (1) Dissection process. (2) Sampling using a micropipette. (3) Process of dispensing to a well plate.

FABRICATION

We fabricate high-resolution type and through-hole type with lithography process (Figure 4). High-resolution type has an array of nano-size-blade structures, which is characterized with two geometrical parameters; width of the blade and their gap. The blade device was fabricated by combination of EB-lithography and deep-RIE process of silicon substrate using EB resist as etching resist. (Figure 5a) Through-hole device has a300 µm depth of the blades. This requires deeper etching than high-resolution type. First, SiO_2 is formed on the Si wafer. Then, SiO_2 on Si substrate was patterned by maskless exposure system, where AZ-1500 was directly coated. After that, SiO_2 is patterned by BHF. Using deep-RIE process of silicon substrate, through holes walled with blades were formed. Finally, the device was cleaned with a piranha solution (H_2SO_4 : H_2O_2 = 3 : 1) for 10 min. (Figure 5 b)

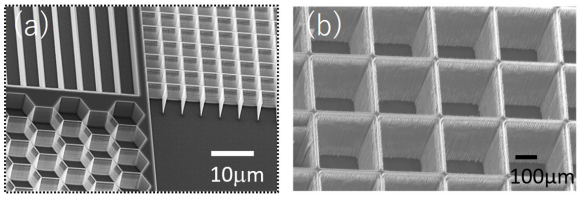

Figure 4: SEM images of the device. (a) High-resolution type. (b) Through-hole type.

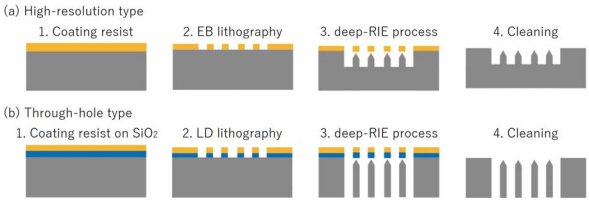

Figure 5: Process flow of device. (a) Fabrication of high-resolution type. (b) Fabrication of through-hole type.

DISSECTION

Cell culture substrate

We developed a cell culture substrate for the dissection process. A typical plastic culture dish is not suitable for our experiments, because its inflexible feature prevents reliable contact and its thick bottom limits the use of high-numerical aperture objective lens. Thus, we fabricated substrate for dissection by using PDMS, collagen gel and glass substrate. The substrate improves cell adhesion and flexibility required for the contact between the blades and the substrate. First, glass substrate is cleaned with a piranha solution (H_2SO_4 : H_2O_2 = 3 : 1) for 10 min. Rectangular frame made of PDMS was bonded to the glass substrate to form a well structure, where collagen gel was coated (base layer). We cultured Hela cells on it as test sample. Then, the cytoplasm of the cells is fluorescently stained by cell tracker green CMFDA.

Experimental setup

We set the experimental equipment having a stage moving in z-axis on an inverted optical microscope, where the nanoblade array device was mounted. The z-stage moves to press the device vertically against the Hela cells cultured on the substrate described above, when the cells were monitored through the microscope. To improve contact between nanoblade array device and cell culture substrate, nanoblade array device is mounted on z-axis stage through a silicone rubber sheet.

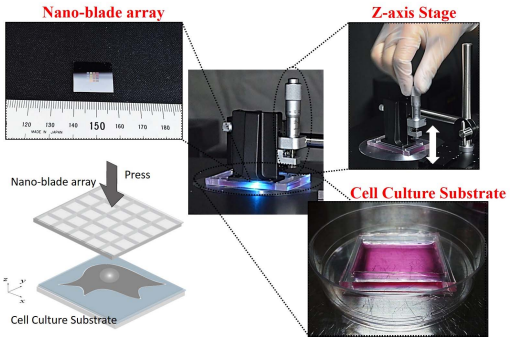

Figure 6: Experiment setup for dissection.

Result of single cell dissection

The fluorescence image shows successful dissection of a single cell into the several regions using grid nanoblade array device (Figure 7). As shown the figure, cells were dissected into small pieces in accordance with the size of the gap of nanoblade (2.5 µm, 5.0 µm, 7.5 µm, 10.0 µm), indicating that the cells were separated at their initial positions. This method proposed here will allow the dissection of single cells into subcellular fractions, keeping the spatial information. It should be noted that the device is reusable after dissection process by washing with piranha solution (H_2SO_4 : H_2O_2 = 3 : 1) for 5 min.

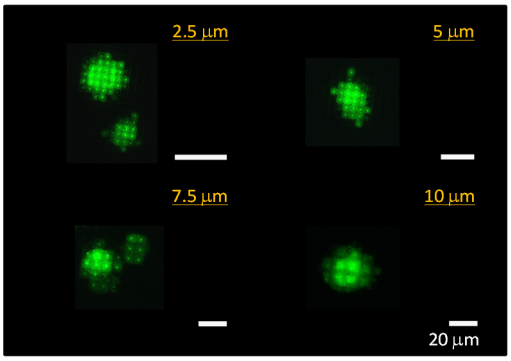

Figure 7: Fluorescence images after cell dissection. Upper right numbers show the size of the blade gap

LIGAND IMMOBILIZATION

Materials

To demonstrate the detection of biomolecules, we immobilized antibodies as ligands to capture specific proteins [3]. We evaluated the several pairs of antibodies commercially available for sandwich immunoassay. We targeted three proteins. BCL2-associated athanogene 1 (BAG1), Endoglin (ENG) and minichromosome maintenance complex component 4 (MCM4). The oncogene BCL2 is a membrane protein that blocks a step in a pathway leading to apoptosis or programmed cell death. The protein encoded by this gene binds to BCL2 and is referred to as BCL2-associated athanogene [4]. ENG is a homodimeric transmembrane protein, which is a major glycoprotein of the vascular endothelium [5]. MCM4 is one of the highly conserved mini-chromosome maintenance proteins (MCM) that are essential for the initiation of eukaryotic genome replication [6].

We selected the antibody pairs of BAG1, ENG and MCM4. BAG1 (Human), ENG (Human) and MCM4 (Human) matched antibody pairs are Abnova H00000573-AP51,H00002022-AP11 and H00004173-AP22, respectively.

Protein detection

Surface of nanoblade device is immersed in 15 µL 0.5 mg/ml Si-tagged protein A solution dissolved with 25 mM Tris-HCL buffer (pH 8.0), 0.5M NaCl, 0.5% (v/v) Tween 20. After incubating for 10 min at room temperature, nanoblade device is washed by 0.2% PBST (1xPBS with 0.2% Tween-20). 2.5 µg/mL antibody/ PBS is dispensed onto the nanoblade device. After incubating overnight, the nanoblade device is washed by 0.2 % PBST. 5% BSA/PBST is dispensed onto it, blocking the surface from nonspecific adsorption. Aftter incubating for 2 hours at room temperature, the nanoblade device is washed by 0.2% PBST. Solution extracted from Hela cells containing various proteins is dispensed onto the device. After incubating at 4ºC overnight, the nanoblade device is washed. Then, 1 µg/mL detection antibody solution was dispensed onto the surface and incubated for 2 h at room temperature. Followed by washing, Proteins are detected by applying Goat anti-Rabbit IgG H&L(FITC) (Abcam ab6717) for BAG1 and Goat Anti-Mouse IgG H&L (FITC) (Abcam ab6785) for ENG and MCM4 on the nanoblade device. After 1 h incubation at room temperature, we carried out protein measurement by fluorescence imaging.

Detection results

We immobilized antibody molecules on the blade surface as ligands to capture specific proteins in the sample. To check the process, we detected proteins (BAG1, ENG, and MCM4) in the lysate of Hela cells by sandwich immunoassay. The results show the successful immobilization of the ligand molecules (Figure 8). Although BAG1 shows high background, ENG and MCM4 proteins are successfully detected. Figure 9 shows the average intensity of the device surface obtained from the sandwich immunoassay. The fluorescence intensity of ENG (condition 2) increases two times higher than that of the negative control (condition 1). Also, the intensity of MCM4 increases higher than the negative control. Although BAG1 shows high background, ENG and MCM4 proteins are successfully detected.

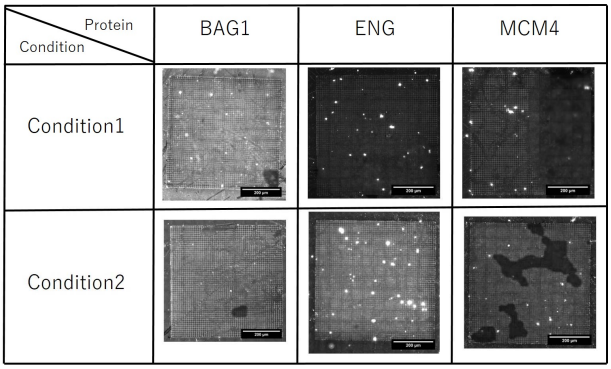

Figure 8: Sandwich immunoassay using nanoblade device Condition 1 shows negative control (w/o cell lysate). Condition 2 shows the results of cell lysate application.

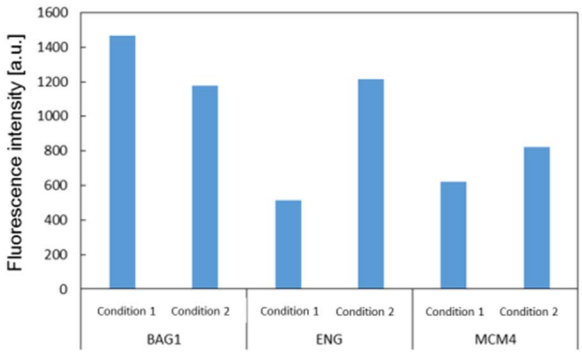

Figure 9: Fluorescence intensity in sandwich immunoassay using nanoblade device.

THROUGH-HOLE TYPE DEVICE

We also evaluated the through-hole type device by using a micro pipette, and demonstrated injection and suction of aqueous solution inside the space walled by blades. We set up the experimental equipment having a stage moving in x, y, z-axis, where the through-hole nanoblade array device was mounted to press the device vertically against the flexible substrate (Figure 10). Dispensing and suction of aqueous solution inside the space walled by blades was monitored through the optical microscope. Figure 11 shows the result, which indicates successful injection and suction of solution in each section.

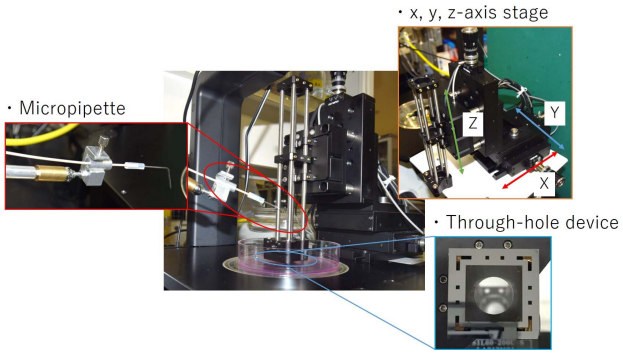

Figure 10: Experimental setup for dissection using through- hole nanoblade device.

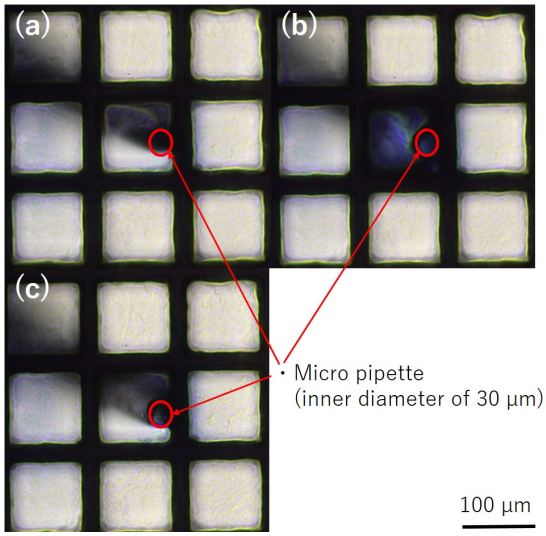

Figure 11: Time series of injection and suction of water in nanoblade array device. (a) Before injection of water. (b) After injection. (c) After suction of water.

CONCLUSIONS

We propose a novel method for sampling single cells and intracellular biomolecules without losing spatial information in tissues or in single cells, using Si nano-blade array device. We fabricated two types of nanoblade device (high-resonance type and through-hole type). We successfully dissected single cells into subcellular fractions, immobilized antibody molecules on the blade surface as ligands to capture specific proteins in the sample, and demonstrated injection and suction of aqueous solution inside the space walled by blades using through-hole type device.

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CONTACT

*K. Terao, e-mail; terao@eng.kagawa-u.ac.jp