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DEVELOPMENT OF A METHOD FOR SIMS ANALYSIS OF HYDRATED SINGLE CELL

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Bioimaging which can visualize the dynamics of components *in vivo* is one of the notable techniques in the field of life-science. In order to prevent the evaporation of volatile components and change in shape due to vacuum introduction of cells, we developed a freezing mechanism and it is possible to analyze hydrated single cells by TOF-SIMS. We examined a method for preparing samples close to *in vivo* conditions.

I. Introduction

Recent years, the needs of imaging for biological samples have been increased rapidly, particularly in life-science. The visualization of components in biological tissue is mainly performed by fluorescence microscope and matrix assisted laser desorption ionization (MALDI) so far. However, since they use a light as a probe (exciting source), their spatial resolution is limited by the wavelength. On the other hand, time-of-flight mass spectrometry (TOF-SIMS) we used is an analytical method using an ion beam for analytical probe on solid surfaces. When a focused ion beam (FIB) is used for primary ion beam in TOF-SIMS, several tens nanometer lateral resolution can be realized. Atoms and molecules on the solid surface are sputtered by the irradiation of FIB. In principle, TOF-SIMS analysis requires introduction of a sample into a high vacuum chamber. As a result, water and other volatile components in the sample evaporate rapidly, and the shape should be changed. As a countermeasure of these problems, we have developed a methodlogy to freeze the sample rapidly and introduce it into the vacuum chamber without contamination from ambient. Hereby the imaging of hydrated samples with TOF-SIMS has been realized. *In vivo*, a cell exchanges ions necessary for vital activities inside and outside the biological membrane of the cell. The collected or cultured biological sample exist in ionic liquid (buffer solution) of the same composition as *in vivo*. In this study, we used red blood cells (RBC) of *Xenopus laevis* which is easy to judge the cell shape. Then we examined the influence of buffer solution on freeze TOF - SIMS analysis and sample condition with less artifact.

II. Apparatus

In this research, we used a TOF-SIMS apparatus developed by Sakamoto *et al.*[1)] In order to obtain high spatial resolution, it is equipped with a focused ion beam (FIB) consisting of gallium liquid metal ion source, and Ga^+ ions are accelerated with 30 keV energy. It is also equipped with an electron beam (EB) for SEM observation for nondestructive observation and a sample stage of 5 axes motion (X, Y, Z, Tilt, and Rotation), enabling accurate cross

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section processing of the sample using the FIB. The inside of the specimen chamber is kept at high vacuum (10^{-6} Pa). Therefore, moisture and volatile components contained in the sample evaporate at room temperature, but countermeasures were taken by cryo-fixation and introduction of cooling mechanism. In a liquid nitrogen bath, the sample was frozen and then the sample folder was shield with a screw cap. There is a small gap in the cap, and the vaporized nitrogen leaks from inside the cap to the ambient. This prevent air pollution on the sample during sample transportation to the SIMS apparatus. After introducing the sample holder with the cap into the load lock chamber, the chamber pressure was reduced by a vacuum pump. When the pressure of the load lock chamber was reached at 10^{-4} Pa, the cap was removed. The sample stage was cooled at about -160 ° C by the circulation of liquid nitrogen, and the frozen state of the sample can be maintained.

III. Experimental

Blood harvested from *Xenopus laevis* washed with KRP (Krebs-Ringer Phosphate) buffer solution was used as a sample. The harvested erythrocytes were stored in the buffer to maintain cell activity. In order to know the influence the buffer solution on the TOF-SIMS analysis, we conducted the experiment under the following protocol.

(a) 1 μl of RBC suspension was applied to the substrate with a syringe and frozen.
(b) 1 μl of RBC suspension was applied to the substrate with a syringe. While observing with an optical microscope, dried until the fringe pattern was seen around RBC. Then it was frozen.
(c) The RBC suspension was placed in a container and RBC was precipitated. KRP buffer was removed from the container. The precipitated RBC was scooped with a toothpick and stretched to the substrate. Then it was frozen.(Fig.1)

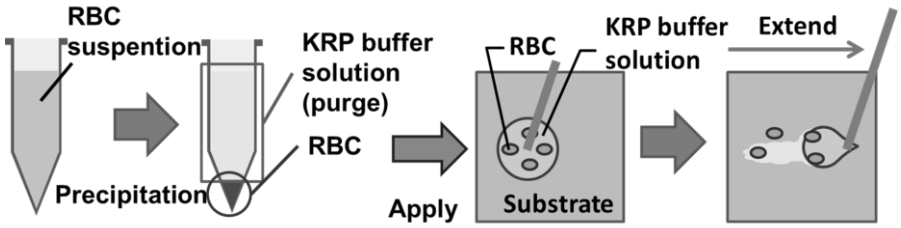

Fig.1 Protocol of the sample extend.

In addition, since we used a sample containing liquid, it was considered that it needs to know the influence on the sample due to the properties of the substrate. In (c) in the protocol, which has the lowest moisture among the above-mentioned conditions, the experiment was conducted with the Si substrate subjected to the hydrophilic treatment and the ordinary Si substrate.

IV. Result and discussion

The first thing is the result of (a). It is probably the most *in vivo* condition because it was frozen while it was in the buffer solution. But, the surface is covered with buffer solution and we could find RBC. In addition, because charge-up effect was serious, it was not suitable for TOF-SIMS analysis. Next is the result of (b). Figure 2 shows the images of

the dried RBC suspension under frozen conditions. Compared with (a), the position of erythrocytes can be cleared. However, when SIMS imaging was performed, ice (H_2O) and NaCl were separated as shown in the Figure 2. This phenomenon occurs when crystals of solute caused by drying extrude water. In addition, an electric potential gradient occurs due to the concentration of the buffer solution, and there was concern about the outflow of the cytoplasm.

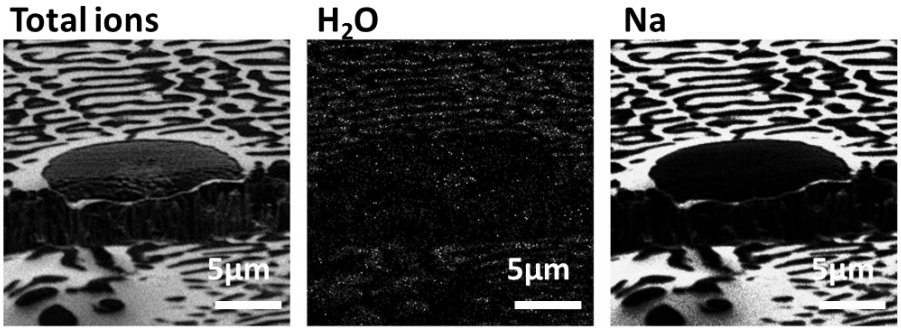

Fig.2 Images of dryed RBC suspension

As for the result of (c), Fig.3 shows the images of RBC extended precipitation under frozen conditions. Since there were few surrounding buffers, solute crystals also did not grow large. Distribution of Fe ions derived from hemoglobin. It can be seen that water-related ions were also retained by freezing fixation in case of (c) method.

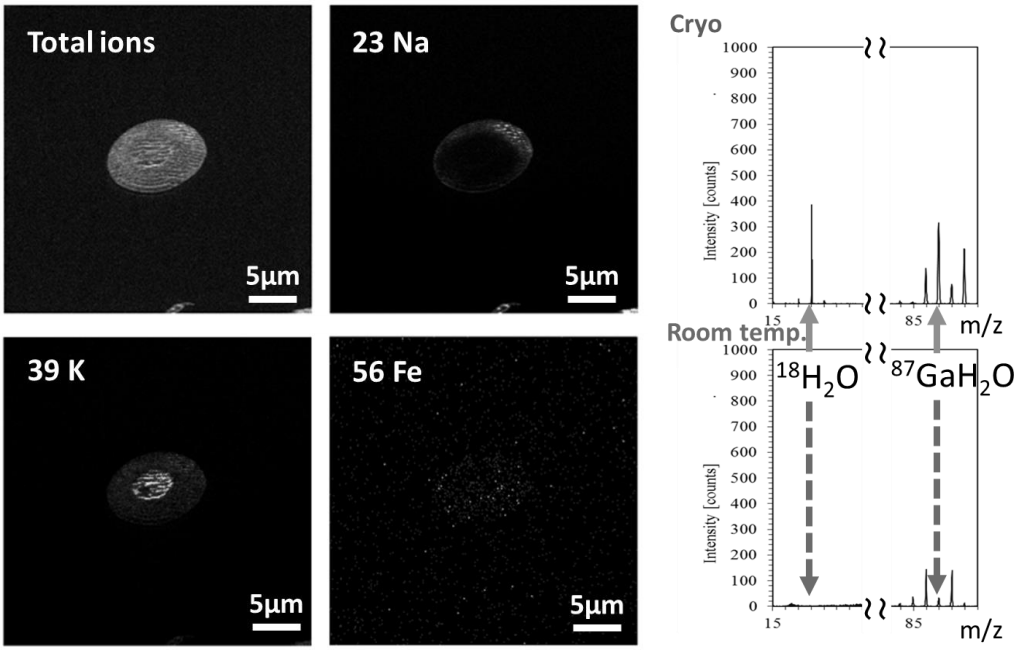

Fig.3 Image of RBC extended precipitation and water related spectrum.

Finally, it needs consider the influence of the properties of the substrate. Figure 4 shows an SEM image of RBC precipitate stretched on a hydrophobic substrate and a hydrophilic substrate. Hydrophobicity is less adhesion of buffer solution, but the number of RBCs is also small as well. On the other hand, the hydrophilic substrate has a large number of RBCs, but buffer solution adheres a lot and some solute crystals were also seen. This means that hydrophilic substrate is suitable for this kind of cell analysis.

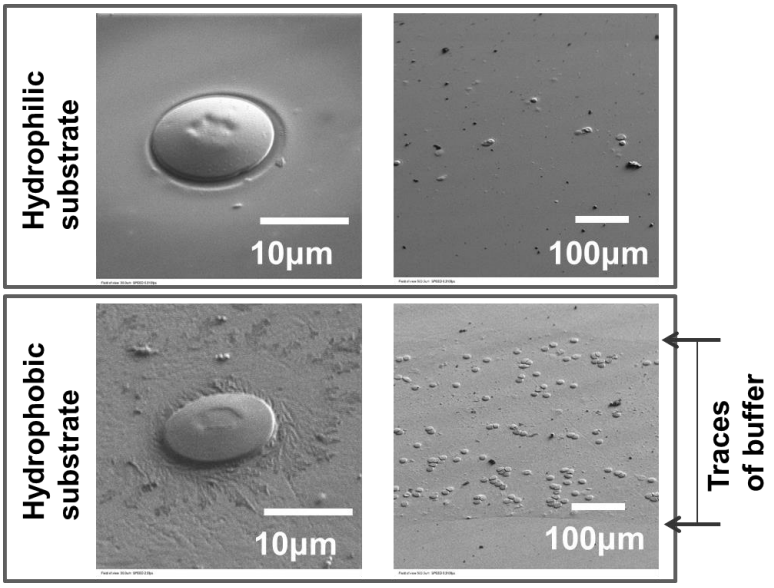

Fig.4 Influence on substrate by properties of substrate

V. Conclusion

The influence of the buffer solution was mainly to confirm the sample surface, crystal growth by crystal growth, and drying. The membrane potential changes due to the Inspissation, resulting in the influx of ions and the outflow of H_2O. It is known that the presence of water is important for ion channel operation. Also, with the buffer solution, the freezing rate also decreases as the heat capacity increases. Therefore, it is desirable that less buffer solution be used. The properties of the substrate are considered to be suitable for a hydrophobic substrate with little buffer solution adhesion, as the concentration of the buffer solution and pressure due to crystals occur.

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