

Study of 3D Micro-Scale Analysis of Freeze-Non-Dried Biotissue

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It is considered difficult to analyze a biotissue (water, 70-80%) in its life-like state in three-dimension at micro-scale using conventional analysis methods such as SEM (Scanning Electron Microscopy), TEM (Transmission Electron Microscopy) or EPMA (Electron Probe Micro-analysis). In this article, a process of keeping the life-like state of the biotissue is introduced, and the possibility of analyzing the biotissue in its life-like state is also discussed. In the analysis procedure, a cooling stage was used to maintain a frozen state of the biotissue sample. Furthermore, an ice protection film method and water vapor supplement method were adopted to maintain a frozen state. A secondary electron image of ice induced by a gallium focused ion beam was successfully observed.

INTRODUCTION

Biotissue, with 70-80% of its component being water, makes it difficult to preserve their ultra structure for micro-scale analysis. Therefore, the analysis is usually performed after pretreatments (fixation, dehydration, embedding, etc.). However, these pretreatments cause some modification and denaturizing [1]. So the life-like state cannot be maintained in the analysis. And, it is also difficult to carry out three-dimensional (3D) micro-scale analysis. So, it is necessary to develop a new analyzing method for the 3D micro-scale analysis. In this study, we are developing a new method for element distribution analysis in 3D micro-scale of a biotissue in its life-like state, using Gallium Focused Ion Beam (Ga-FIB) and Secondary Ion Mass Spectrometry (SIMS).

SIMS with merits of ability to perform 3D analysis, high spatial resolution (nm order), high sensitivity (ppm to ppb order) and ability to perform quantitative analysis has been already realized by introducing Ga-FIB for application to 3D micro-scale analysis of solid and inorganic materials [2]. In order to apply such 3D analysis to biotissue samples it is necessary to develop a method of how to maintain the life-like state during the whole analysis procedure. In

other words, composition and structure should be kept the same in micro-scale as those when the biotissue was alive. Rapid freezing technique has been used to fix the life-like state of the biotissue [1]. In this article the method to keep the fixed biotissue unchanged throughout the steps of introduction to the analysis apparatus, evacuation, transfer to analysis position and actual microbeam analysis are discussed.

EXPERIMENTAL

Schematic diagram of the instrument used in this study is shown in Fig.1. The sample holder is

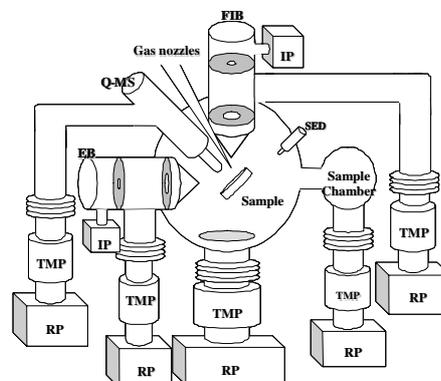


Fig.1 Apparatus diagram

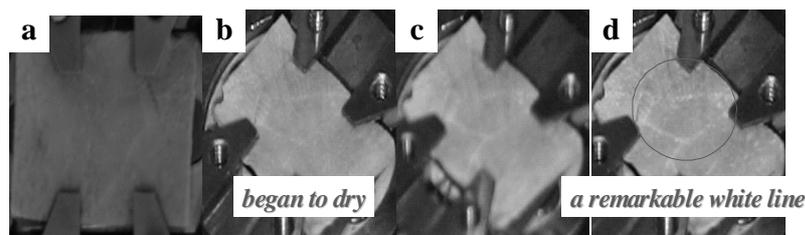


Fig.2 State change of a carrot slice without the protection film in a vacuum a Immediately after a freeze b After 1h c After 3h d After 4h.
 Carrot slice: with a thickness of about 100 μm ; rapid frozen by liquid N_2
 Sample stage: Preliminary cooled; temperature during b to f: About -110°C
 The pressure in main chamber: below 10^{-4} Pa

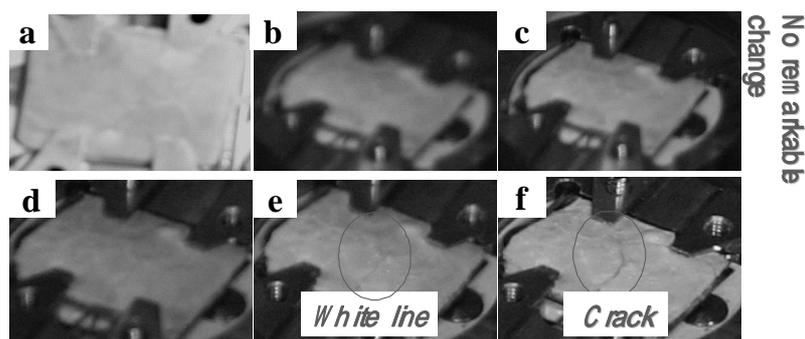


Fig.3 State change of a carrot slice with the protection film in a vacuum a Immediately after ice film formation b After 3h c After 4h d After 5.5h e After 6h f After 8h.
 Carrot slice: with a thickness of about 100 μm ; rapid frozen by liquid N_2
 Sample stage: Preliminary cooled; temperature from b to f: About -110°C

first introduced into Sample Chamber. After pre-evacuation of Sample Chamber the sample holder is transferred into Main Chamber. The sample stage (not shown) has capability to cool the sample holder down to -120 degree C. Main Chamber is equipped with a field emission electron gun, a gallium focused ion gun, a secondary electron detector, a gas nozzle, etc. Each gun is differentially pumped to keep the pressure in the beam source part sufficiently low.

Slices of carrot with thickness of about 100 μm were used as samples of biotissue in this study. Liquid N_2 was used as coolant. Details of procedure are described in Results and Discussion.

RESULTS AND DISCUSSION

A slice of carrot held on the sample holder was first frozen in liquid N_2 , then, introduced into Main Chamber through Sample Chamber. It took about 4 minutes for the sample to be transferred to the sample stage. When the sample was transferred to the sample stage at -110 degree C under 10^{-4} Pa, no change was

observed macroscopically. Figure 2b shows the photograph at 1 hour after taking out the sample from liquid N_2 . At that time the sample began to dry. After 3 hours the sample seemed to have lost more water (Fig. 2c), and thin white lines appeared on the sample surface. After 4 hours clear white lines appeared on the sample. From these observations the dryness of the sample during evacuation of the main chamber cannot be ignored. In addition there was still possibility to begin to dry microscopically during sample introduction and transfer.

In order to avoid dryness problem during sample introduction and transfer, protection film method was examined. A biotissue sample was frozen by liquid N_2 , then, one drop of 3-time-distilled water was poured on it and the sample was again immersed in liquid N_2 . Then, a thin ice film was formed on the surface of the biotissue sample. Figure 3a shows the photograph of the sample taken just after being covered by an ice protection film. When the sample was transferred to the sample stage at -110 degree C

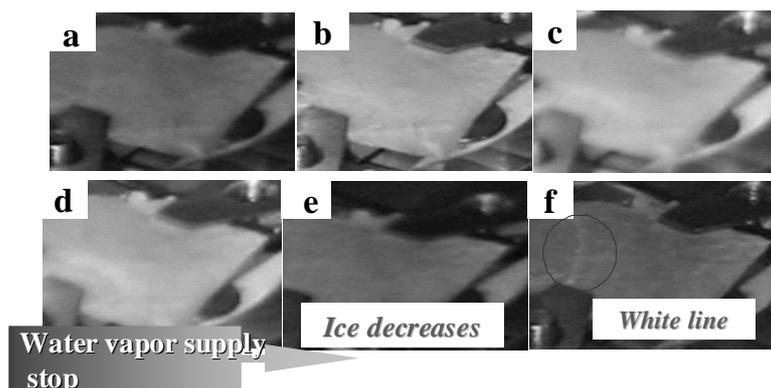


Fig.4 State change of a carrot slice which has a protection film while filling up water vapor a Immediately after ice film formation b After 4h c After 5.5h d After 6h e After 8h f After 9h.
 Carrot slice: With a thickness of about 100 μm ; rapid frozen by liquid N_2 ; with ice protection film
 Sample stage: Preliminary cooled; temperature from b to f: About -110°C
 The pressure in main chamber: $1.2 \times 10^{-4} \sim 8.1 \times 10^{-4} \text{ Pa}$
 Water vapor filling up time: 6h

under 10^{-4} Pa , the ice film was still covered the surface of the sample. Figures 3b and 3c show the photograph at 3 and 4 hours after ice film formation, respectively. After 4 hours the ice film still covered the surface of the sample. At 5.5 hours after ice film formation the sample seemed to begin to dry (Fig. 3d). After 6 hours thin white lines appeared on the sample surface. (Fig.3e). After 8 hours the white lines changed to cracks (Fig. 3f). From these observations validity and limitation of the ice protection film were obvious. The protection film prevents against sample dehydration during sample introduction and transfer by sublimating the ice film itself. However, the film cannot keep the sample non-dried for longer than, e.g., 4 hours. Equilibrium vapor pressure of water on ice at -110°C is sufficiently lower than 10^{-4} Pa . Therefore, the temperature of the sample surface might be higher than that of the sample holder because of low thermal conductivity of the sample or between the sample and the sample holder. Anyway, in order to avoid dehydration of the sample at the period of the analysis, the partial pressure of water on the sample surface must be raised to the equilibrium vapor pressure at the actual temperature of the sample surface.

In order to raise water vapor pressure just above the sample surface, the water vapor was slowly jetted to sample directly from about 1mm distance using a gas nozzle. A carrot section with a thickness of about 100 micrometers with a protection film was held on

the sample stage which carried out preliminary cooling. Just after the sample was set on the sample stage, the water vapor jet started. The pressure in Main Chamber was raised from $1.2 \times 10^{-4} \text{ Pa}$ to $8.1 \times 10^{-4} \text{ Pa}$. Figures 4b, 4c and 4d show the photograph taken after 4, 5.5 and 6 hours, respectively. Under the water vapor jet the ice film was stably covered the sample surface. After taking the photograph of Fig. 4d the water vapor jet was stopped. Figure 4e shows the photograph taken after 8 hours (2 hours after stopping the jet). The ice film on the sample surface seemed to be reduced in thickness but still existed. After 9 hours (3 hours after stopping the jet) thin white lines were observed on the sample surface. Therefore, by water vapor supplement, it is possible to keep a frozen biotissue sample un-dried under high vacuum.

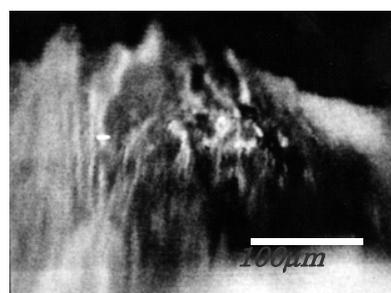


Fig.5 SEM image of the ice on the surface of a metal plate in a vacuum induced by Ga-FIB. Accelerate Voltage: 15.00kV, Sample current: 20pA, Sample stage temperature: -90°C Main chamber pressure: $3.0 \times 10^{-4} \sim 4.6 \times 10^{-5} \text{ Pa}$

Microbeam analysis of non-conductive material sometimes suffers from problem of charge-up. Therefore, it is important to examine whether a frozen biotissue sample can be observed with a microbeam of charged particles. A metal plate was cooled by liquid N₂ as low as possible, and then an ice film was produced on the surface by blowing distilled water. This sample was introduced in equipment and a secondary electron image induced by Ga-FIB was observed. Ga-FIB was operated under the condition of 15 kV acceleration and 20 pA current. During this procedure, the sample stage was kept at about -90 degree C. The pressure of Main Chamber was 3×10^{-4} Pa without water vapor jet. Figure 5 shows the secondary electron image of ice on aluminum plate. Under this condition no charge-up problem was encountered. This proved that it was possible to observe biotissue in the life-like state by

our equipment

CONCLUSIONS

We developed a method of how to keep the life-like state of the biotissue by our equipment and proved that it was possible to observe biotissue in the life-like state by our equipment. We can thereby conclude that it is possible to carry out the study of 3D micro-scale analysis of freeze-non-dried biotissue in its life-like state by using our equipment and our analysis method.

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