Development of a Simple System for the Analysis of Water-Containing Biological Samples by TOF-SIMS

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In recent years, the needs of mass spectrometry for biological samples have rapidly increased. In this study, in order to analyze water-containing biological samples using time-of-flight secondary ion mass spectrometry (TOF-SIMS), a rapid-cooling method, sectioning system, and a sample introduction method that could avoid frost formation in plant biological samples were developed. It was confirmed that both frost formation and evaporation of volatile compounds were prevented by a rapid-cooling and new introduction method. Essential elements and H2O could be detected by this new rapid-cooling TOF-SIMS methodology. Therefore, SIMS analysis in the natural state became possible. [DOI: 10.1380/ejssnt.2016.131]

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I. INTRODUCTION

In recent years, the needs of mass spectrometry for biological samples have rapidly increased. The constituents of physiological tissues are commonly visualized by matrix assisted laser desorption ionization (MALDI) [1]. As a soft ionization technique, MALDI is capable of ionization large molecules without fragmentation; however, its spatial resolution is often limited by the size of the laser-spot [2]. Therefore, imaging of the anatomy of an individual cell has not been realized so far. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is another technique that can analyze tissue surface composition with a higher spatial resolution than MALDI when a focused ion beam (FIB) is used as the primary ion beam. In FIB-TOF-SIMS, atoms and molecules on the solid surface are sputtered by the scanning a focused primary ion beam over the analytical field-of-view. The sputtered secondary ions are then mass-separated by the time-of-flight mass spectrometer (TOF-MS), and mass-identification of these ions is carried out. Recently, TOF-SIMS analysis has been applied to biological samples. Imaging of biological samples with low damage and high sensitivity is typically done using an Ar cluster ion beam. However, it is difficult to focus the cluster ion beam on a small spot, and the spatial resolution is thus limited to a few μm [3, 4]. In this laboratory, high spatial resolution imaging, enough for single cell analysis, has been realized by developing an FIB-TOF-SIMS [5–7].

However, TOF-SIMS analysis of biological materials still faces some difficulties. Perhaps the most serious problem involves the evaporation of volatile compounds under the high vacuum conditions found in the TOF-SIMS apparatus, thus altering the samples from their natural living state. For scanning electron microscopy (SEM) or transmission electron microscopy (TEM) observation of such biological materials, freeze-drying or substitution methods are used successfully [8]. Unfortunately, in the case of SIMS, these conventional methods cannot be employed because they alter the original distribution of the components and change the composition through substitution [9]. To address this issue, a rapid-cooling method has been applied TOF-SIMS [10, 11]. For this method, a glove box and transportation system for the vacuum apparatus are generally required for preventing frost formation. In this study, a rapid-cooling step and sample introduction system for the TOF-SIMS apparatus were proposed; this system only used a sample holder and screw cap, and did not require a glove box and transportation system, making this a very simple and convenient method. However, rapid-cooling TOF-SIMS analysis of biological sample has some problems, including growth of ice crystals or non-adsorption of ambient air. Therefore, in this study, a contaminant-free TOF-SIMS analysis system was developed using a sample holder with a cap. A rapid-cooling TOF-SIMS analysis of a plant stem was carried out using the proposed sample introduction system. Rapid-cooling and sample preparation for the TOF-SIMS analysis of water-containing samples were evaluated with the aim of

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solving the above problems, e.g., formation of ice crystals and contaminant-free.

II. EXPERIMENTAL

A. Apparatus

The TOF-SIMS apparatus used in this study was originally developed by the authors [12]. It is equipped with a gallium FIB, an electron beam (EB) with a thermal field emitter for SEM observation, a TOF-MS with a two-stage reflectron and a sample stage with 5-axes of movement. The sample stage can be cooled to $-160^\circ C$ by circulation of liquid nitrogen.

B. Rapid-cooling and sample introduction system

The specimen chamber of the apparatus was maintained at approximately $4 \times 10^{-6}$ Pa using a sputter ion pump. The biological samples were cooled to $-130^\circ C$ to prevent the evaporation of volatile compounds in vacuum. Moreover, it is important to prevent the contamination of ambient air during the sample introduction procedure under the cooled condition. Figure 1 shows the sample holder used to prevent both the evaporation of volatile compounds and the adhesion of contaminants from ambient air onto the sample surface [13].

The introduction procedure is as follows. First, the sample stage of the apparatus was cooled to $-160^\circ C$ by liquid nitrogen circulation. After placing a sample on the holder, the holder was cooled and capped in a liquid nitrogen bath. The capped sample holder was then introduced into the load-lock chamber. The load-lock chamber was vacuum pumped to a pressure of approximately $10^{-4}$ Pa. Next, the cap was removed under the vacuum condition using a rotational motion feedthrough from the atmosphere, and the sample holder was transferred to the sample stage in the specimen chamber. During this process, the interior pressure in the cap rose due to the evaporation of liquid nitrogen, which leaked through the threading. Since the interior pressure in the cap was always higher than the pressure of ambient air or vacuum in the load-lock chamber, any external gas containing contaminations could not penetrate into the interior, thus preventing frost adsorption on the sample surface. The sample introduction procedure is summarized in Fig. 1.

C. Sample preparation

In this study, plants stems were used as a biological sample to verify the proposed introduction system. For TOF-SIMS analysis of the interior structure of a biological sample, a sample preparation method that meets the necessary conditions, e.g., very flat sample surfaces, thin sectioning, and frozen condition, is also required; thus, evaluation of the sample preparation method is necessary. Generally, secondary ions are difficult to detect with high sensitivity on uneven surfaces due to the irregular extraction electric field. The conventional method of sectioning a sample by hand using a knife introduces a certain roughness to the sample surface. Therefore, a system that produces smoother sample surfaces by ensuring a consistent blade angle and speed has been developed. Figure 2 shows a schematic illustration of the sectioning stage. The system is equipped with a z-stage (TAR-34403, SIGMAKOKI Co., Ltd.) and a blade (MTBM-5H1, Kai Co., Ltd.). The manipulator stage was moved at $\sim 10$ mm/s, while the blade angle was $\sim 30^\circ$ with respect to the horizontal. A cross section of a plant stem was sectioned and analyzed while maintaining its natural structure using a combination of the above introduction system and sectioning stage. The plant was a weed (Metaplexis japonica) collected from within the...
university campus.

III. RESULTS AND DISCUSSIONS

A. Evaluation of the rapid-cooling method and sample introduction system

After the introduction of a frozen sample into the TOF-SIMS apparatus, the specimen chamber pressure was maintained below $4 \times 10^{-10}$ Pa while the temperature of the sample stage was reduced to $-160^\circ$C. The sublimation temperature of ice is approximately $-160^\circ$C at $1.18 \times 10^{-11}$ Pa [14]. It is very lower than pressure of the TOF-SIMS apparatus. Therefore, the evaporation of water was suppressed under this condition. The state of the sample was then compared with and without the cap of the sample holder in the load-lock chamber at $10^{-4}$ Pa. It has been confirmed that there was no frost formation on the sample surface using the proposed method as described in Section II.B. Based on these results, both frost formation and evaporation (sublimation) of water were successfully prevented.

B. Evaluation of sample preparation method

Figures 3(a) and (b) show SEM images of the sample surface cut without (conventional method) and with (current method) the sectioning stage, respectively. It was confirmed that the sample prepared by the conventional method had significant surface roughness. On the other hand, the current method using the sectioning stage produced a much smoother surface than the conventional method. From outside to inside, the plant stem consisted of epidermis, cortex, vascular system, and pith, as depicted in Fig. 4. In the SEM images (Fig. 3(b)), the hollows indicated by arrows are intercellular spaces for passage of air. If the ice in the other vessels, such as the pith in the center of the stem, contained artifacts due to water migration or frost formation, the intercellular space would also be filled with ice. The SEM image tells us there was no artifact in relation to the ice. Thus, the new sample preparation method combined with the sample introduction system prevents the introduction of artifacts in the plant section.

C. TOF-SIMS analysis of a plant stem

After the introduction of a sectioned plant stem into the specimen chamber, TOF-SIMS analysis was carried out for a $100 \times 100 \mu m^2$ area in the frozen state, and then again at room temperature. Figure 5(a) shows the secondary ion maps of the stems in the frozen state. The imaging area was located around the center of the stem. As described in the literature [15], the $^{19}H_3O^+$ ion signal from ice is more prominent than the $^{18}H_2O^+$ signal. Among the obtained maps in Fig. 5(a), images for both $m/z = 18$ and 19 (corresponding to $^{18}H_2O^+$ and $^{19}H_3O^+$, respectively) show that water (ice) mainly existed within the vessels, whereas such images could not be obtained at room temperature (Fig. 5(b)). Furthermore, no ice-crystal was observed, at least at this magnification. Effective rapid-cooling systems typically employ propane as the cooling medium [16]. It is believed that the formation of a gas boundary layer between the sample surface and coolant decreases the cooling speed. The use of propane as a cooling medium prevents the formation of the gas boundary layer and the cooling speed is enhanced, resulting in rapid-cooling for the prevention of ice-crystal growth. In this experiment, liquid nitrogen was used as

![FIG. 2. Schematic of the instrument used for sectioning of plants in liquid nitrogen.](image_url)

![FIG. 3. Images of the cross-sections of the plant stem by SEM observation integrated in the TOF-SIMS. (a) The conventional method (without the section stage) and (b) current method (using the section stage).](image_url)

![FIG. 4. Schematic of the cross section of a typical plant stem.](image_url)
the cooling medium instead of propane. This was not the ideal condition, but there was no serious problem due to crystallization. Among the other constituents, one of the main inorganics, potassium, was found in both the plant vessels (ice) and vessel walls. This implies that the potassium in the ice originated as K\(^+\) ions in water while the plant was alive, whereas the potassium in the vessel walls is a structural component of the plant. In the image at room temperature, potassium in the ice disappeared upon evaporating the ice, while that in the vessel walls was still detected. Calcium was also clearly detected in the vessel walls. Calcium is one of the essential elements, and acts as a binder between the organic acids forming plant walls. Therefore, calcium was also detected at room temperature in the same manner. In addition to single atom species, water-attached ions were also identified in the frozen sample. In Fig. 5(a), the ions at \(m/z = 57\) are attributed to both K\(_2\)H\(_2\)O\(^+\) and CaOH\(^+\). Based on comparison of the images for K\(^+\) and Ca\(^+\), the image at \(m/z = 57\) should be correlated mainly with K\(_2\)H\(_2\)O\(^+\), because the spatial distribution is similar to that of K\(^+\). This interpretation is further supported by the significant decrease in \(m/z = 57\) ions when the sample loses water at room temperature. Only a weak signal of CaOH\(^+\) was recorded in the map. This result indicates that TOF-SIMS imaging of a water-containing sample has been successfully performed using a sample holder with a cap and introduction procedure that prevents frost.

IV. CONCLUSIONS

In this study, a rapid-cooling method, a sample introduction system that could prevent frost, and a sample sectioning stage were developed to analyze water-containing biological samples in their natural state under the high vacuum conditions used for TOF-SIMS. The sample surface produced by the developed method was kept free of contaminants, and more ions originating in H\(_2\)O were detected compared to that with the conventional introduction method (without cooling). From these results, it was confirmed that both the frost formation and sublimation of water were prevented. Therefore, SIMS analysis of biological samples in the natural state became possible.

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