Paper

Three-Dimensional Analysis of Biological Samples using Dual FIB ToF-SIMS

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Secondary Ion Mass Spectrometry (SIMS) has been used as an analysis tool for biological samples using cluster ions of C_{60}^+, Bi_n^+, etc. However, because of bad focusing of cluster ions, accurate three-dimensional analysis is difficult. On the other hand, FIB (Focused Ion Beam) using Ga^+ ions can be focused to about 50 nm, and applied to samples of micro region such as biological samples. However, three-dimensional analysis is difficult because of surface damage by primary ions. Therefore, we have developed Shave-off section processing method. Shave-off scan is extremely unique section processing method. Raster scan of FIB is very slow in vertical direction compared with in horizontal direction. In this study, we evaluated surface damage in section processing and carried out three-dimensional analysis of biological sample using Dual FIB ToF-SIMS with Shave-off scan. We evaluated surface damage by primary ions and actually analyzed biological sample with Shave-off scan. We demonstrated the utility of this instrument by analyzing biological samples.

1. Introduction

Secondary Ion Mass Spectrometry (SIMS) has been widely used as an analysis tool for microelectronic devices and industrial materials. Three-dimensional analysis of biological samples has been also carried out by using cluster ions of C_{60}^+, Bi_n^+, etc [1]. However, accurate three-dimensional analysis is difficult because of problems such as cone formation and sputtering re-deposition on the section of the samples. Moreover, the spatial resolution has been limited because of bad focusing characteristics of cluster ions. On the other hand, FIB (Focused Ion Beam) using Ga^+ ions can be focused to about 50 nm, and applied to samples of micro region such as biological samples. However, three-dimensional analysis is difficult because of surface damage by primary ions. In section processing for three-dimensional analysis, surface was especially damaged by implanted primary ions. Because original structure was broken by the implanted primary ions, accurate three-dimensional analysis was complicated. Particularly, in the case of high-molecular material such as biological samples, detection of high-molecular ions is difficult because the bondings are broken by primary ions. As a result, analysis of the original structure is difficult.

We have developed Dual FIB ToF-SIMS for accurate three-dimensional analysis [2]. This instrument has two Ga ion guns and ToF-Mass spectrometer.

Shave-off scan is extremely unique section processing method [3]. Raster scan of FIB is very slow in vertical direction compared with in horizontal direction. Therefore, section of the sample is shaved off flatly and almost parallel to the raster and the axis in direction of FIB. This method has features as follows: 1) No sputtering re-deposition, 2) Implantation of Ga ion is suppressed. Therefore, this method can be applied for sample with irregular shape and/or different phases.

In this study, we compared with surface damage by primary ions in Shave-off section processing and conventional methods. And we actually analyzed biological sample with our instrument.

2. Experimental
2.1. Evaluation of surface damage in section processing

In conventional methods, the primary ions beam is irradiated in the vertical direction, therefore, primary ions are implanted inside of samples. As a
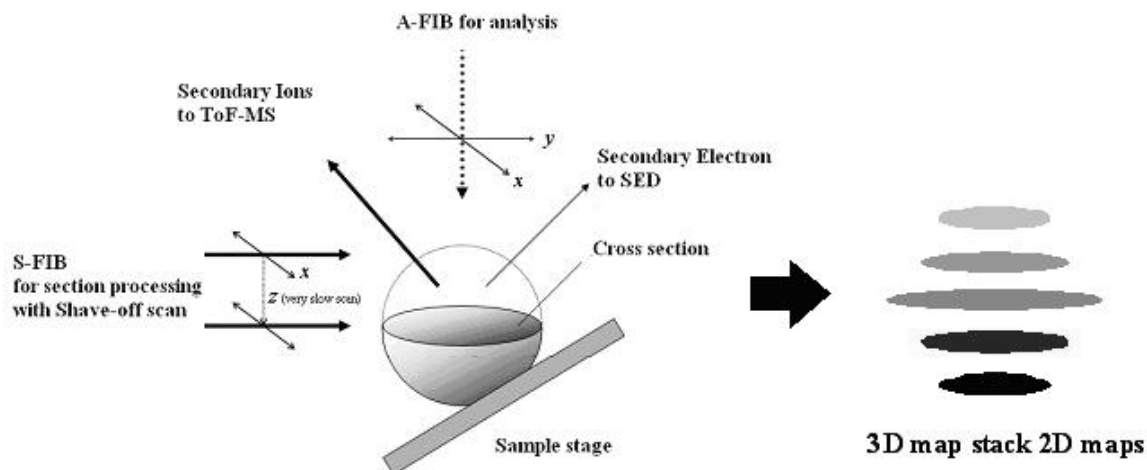
Fig.1. Schematic of Dual FIB ToF-SIMS instrument and 3D map.

Table 1. Isotopic ratio of obtained ions from each surface.

	Shave-off scan	Conventional methods
	$^{69}Ga^+ : {}^{71}Ga^+$	$^{69}Ga^+ : {}^{71}Ga^+$
Unprocessed surface	61.1 : 38.9	58.6 : 41.4
Processed surface	61.3 : 38.7	66.7 : 33.3
*Ratio (%)	0.5	**19.6**
Natural abundance	$^{69}Ga^+ : {}^{71}Ga^+ = 60.1 : 39.9$	

*Ratio: Ga^+ ions implanted in section processing to sum Ga^+ ions implanted ratio (%)

result, analysis of the original structure is difficult. Especially in the case of high-molecular material such as biological samples, detection of high-molecular ions is difficult because the bondings are broken by primary ions.

In this study, we compared surface damage by primary ions in section processing of Shave-off scan and conventional methods.
To evaluate surface damage by primary ions in section processing, we measured the amount of implanted $^{69}Ga^+$ ions in surface. Analytic FIB (A-FIB) included isotopes of $^{69}Ga^+$ and $^{71}Ga^+$ ions, and section processing FIB (S-FIB) included only $^{69}Ga^+$ ions.

We used Ni wire. At the first, surface was analyzed with A-FIB, then the surface was processed by S-FIB. After section processing, the surface was again analyzed**.** We carried out section processing by each method. Processing time, detected ion counts and beam current were constant in each method (8 h, 4×10^4 counts, 8 nA).

2.2. Three dimensional analysis of biological sample
2.2.1 Sample Preparation
We used lung of Xenopus laevis as model sample. Xenopus laevis was cut in the living state, the lung was extracted. The lung was rapidly washed

(ion-exchanged water) and cut off 5×5 mm. The lung was gently placed on Al plate (1×1 cm). Then the lung was frozen by plunging in liquid nitrogen and rapidly introduced to analysis chamber (4×10^{-5} Pa) through the preparation chamber. Beforehand, the analysis chamber was cooled at 150 K by liquid nitrogen.

2.2.2 ToF-SIMS analysis
ToF-SIMS analysis was performed using a Dual FIB ToF-SIMS instrument, the instrument has been described elsewhere [2].

Dual FIB ToF-SIMS has two Ga^+ ions guns and ToF- Mass spectrometer for the three-dimensional analysis. One Ga^+ ions gun (S-FIB, IOG25) carries out section processing by shave-off scan, and the other Ga^+ ions gun (A-FIB, Eiko Engineering) analyzes the section with ToF-Mass. The scheme of Dual FIB ToF-SIMS is depicted in Fig. 1.

2.2.3 Three-dimensional ion mapping
Position of the lung was found by Secondary Electron Image (SEI). In this study, we analyzed the region with size of 60×60 μm in the sample.

In the first place, the surface of the sample was etched by A-FIB and analyzed by ToF-Mass. Beam condition of A-FIB was 20 keV, 4pA (6×10^{11} ions/cm^2) and constant during analysis. Two dimensional (2D) maps were obtained by scanning A-FIB over the section of sample.

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For three-dimensional (3D) analysis, sample was shaved by S-FIB with Shave-off scan and new surface was processed. The processed surface was plat and parallel to S-FIB. After section processing, the surface was also analyzed by A-FIB and obtained 2D ions maps. Three-dimensional (3D) io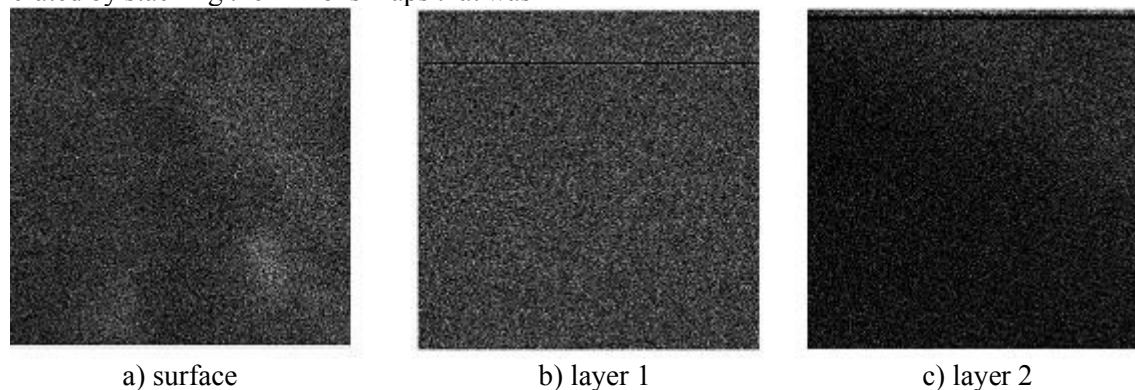ns map was generated by stacking the 2D ions maps that was obtained by repeating these section processing and analysis. The scheme is shown also in Fig. 1.

Section processing was carried out 20 keV Ga^+ ions, beam current of 7 nA. The beam condition was constant during section processing in each layer.
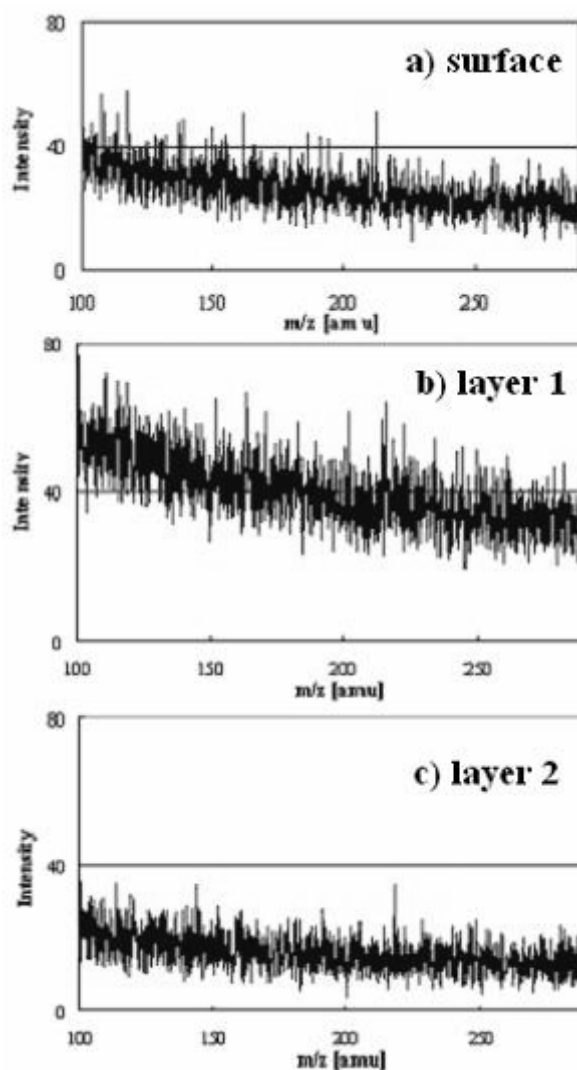.

a) surface   b) layer 1   c) layer 2

Fig. 2. 2D maps of entire ions in each layer.

3. Results and discussion

3.1. Evaluate surface damage in section processing

Table 1 shows isotopic ratios of obtained Ga^+ ions from processed and unprocessed surfaces. From Table 1, it can be seen that isotopic ratios of Ga^+ ions were unchanged between processed and unprocessed surface in the Shave-off scan. In the conventional method, however, ratios of $^{69}Ga^+$ ions were extremely raised. Moreover, we calculated amount of implanted $^{69}Ga^+$ ions in section processing. Because A-FIB has isotopic of $^{69}Ga^+$ and $^{71}Ga^+$ ions, isotopic ratio of implanted Ga^+ ions should close to the natural abundance ($^{69}Ga^+$: $^{71}Ga^+$ = 60 : 40). Before section processing, actually the isotopic ratio of detected Ga^+ ions neared to the natural abundance ($^{69}Ga^+$: $^{71}Ga^+$ = 61 : 39, $^{69}Ga^+$: $^{71}Ga^+$ = 58 : 42). After section processing, if ratio of implanted Ga^+ ions changed, it can be considered that Ga^+ ions implanted in section processing. Therefore, rate of implanted Ga^+ ions was compared in unprocessed and processed surface. Formula is followed;

$$(X \times B - A \times Y) / (1+B)$$

Here, X = $^{69}Ga^+$ in processed surface, Y = $^{71}Ga^+$ in processed, A = $^{69}Ga^+$ in unprocessed, B = $^{71}Ga^+$ in unprocessed surface.

These calculate results were shown in Table 1. In the calculated results, we found that amount of implanted Ga^+ ions (19.6%) in the conventional methods was more than implanted Ga^+ ions (0.5%) in the Shave-off scan. It shows that far less amount of Ga^+ ions was implanted to inside of the samples in the Shave-off section processing compared to in the

Fig. 3. Spectra of ions (m/z 100-300).

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conventional methods. These results clearly indicated that Shave-off section processing can suppress surface damage by primary ions. From these result, it appear that Dual FIB ToF-SIMS can be used biological samples by shave-off scan.

3.2. Three dimensional analysis of biological sample

Figure 2 shows obtained the 2D maps of total ions from each layer. The analysis size was 60×60μm.

Each layer was shaved from surface in depth direction by 5μm. After section processing finished, the processed surfaces (layer 1, layer 2) were analyzed. In Fig.2, the processed surfaces were entirely dark compared with the unprocessed surface, because lesser ion was detected from the processed surfaces than the unprocessed surface. Moreover, detected ion from layer 2 was lesser than from layer 1. The longer the Shave-off section processing is carried out, the fewer the ion counts emitted from the processed surface change.

We guess that decrease of ion counts in processed surfaces have been occurred by charge-up during shave-off section processing.

In section processing, +charges were accumulated on surface by ion beam of high current irradiating for a long time. It is wildly known that decrease of ion counts were occurred on charged surface [4]. From these result, decrease of ion counts were caused by charge-up in surface.

The peaks (140, 170, 220, etc.) from amino acid were detected in each layers. It is wildly known that these peaks were detected biological samples [2]. However, the separation of these peaks from noise was difficult because entire ion intensity was very low. And, because ion counts were very few, distribution of these ions could not be obtained.

Spectra of these ions from each layer were shown in Fig. 3. Intensity of these peaks was few in the layer 2, compared with surface and layer 1. And total ion counts were also few in processed surface, compared with total ion counts in unprocessed surface. Because of charge-up on surface, it was considered that decrease of ion counts in processed surface occurred. We guess that charge-up have occurred by primary ions in processing.

Obtained 3D map of total ions was shown in Fig. 4. The sample was shaved 10 μm in depth direction.

4. Conclusion

In this study, we evaluated primary ions damage in section processing and analyzed the lung of Xenopus laevis as biological model samples with Shave-off scan.

To compare with each processing method, implanted Ga^+ ions was fewer in Shave-off scan (0.5%) than in conventional methods (19%). These results clearly indicated that Shave-off section processing can suppress surface damage by primary ions.

Moreover, we actually analyzed the rapidly cooled lung as biological model sample. The peaks (140, 170, 220, etc.) from amino acid were detected from each layer. However, the separation of these peaks from noise was difficult because entire ion intensity was very low.

And, the less ions were detected from the processed surface compared with the unprocessed surface. Decrease of ion counts was occurred by charge-up during section processing.

We demonstrated the analysis of biological sample without surface damage in section processing. In case of insulant materials such as biological samples, however, the less ions were detected from the processed surface by Shave-off section processing owing to charge-up. And, the less ion count makes it difficult to distinguish signal from noise.

We concluded that the more accurate analysis of biological samples will be achieved by neutralizing charge-up of the processed surface.

Fig. 4. 3D map of the lung (60×60×10 μm).

5. Reference

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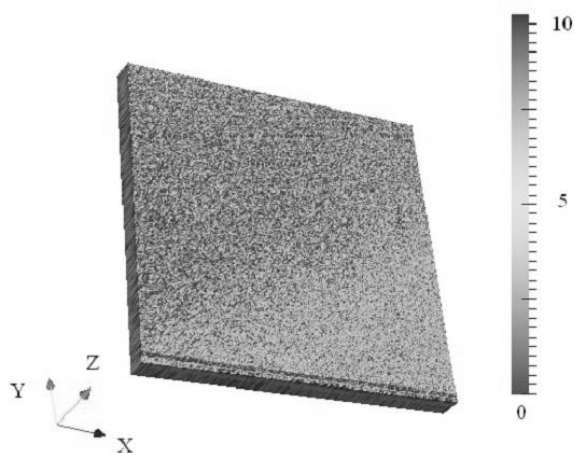
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