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Smooth sectioning of biological samples by FIB-TOF-SIMS

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Spheroids, which are three-dimensionally cultured cells that resemble actual living organisms, have been attracting attention. FIB-TOF-SIMS (Focused Ion Beam Time-of-Flight Mass Spectrometry) is capable of simultaneous mass imaging of multiple elements without the need for labeling. FIB-TOF-SIMS is expected to process the spheroid and image the cross-sectional components. However, FIB processing of spheroids larger than 100 μm often results in uneven cross sections due to the so-called curtain effect. The unevenness of the cross-section affects the sputtering and hinders component imaging. In this experiment, we considered the processing of spheroids by FIB from multiple directions to suppress the curtain effect. The curtain effect was evaluated by comparing the processing from one direction and from multiple directions.

I. Introduction

Recently, "spheroids", which are three-dimensionally cultured cells similar to actual living organisms, have been attracting attention [1]. In addition, spheroids containing beads with internal channel-like structures for supplying oxygen and nutrients to the inside of the spheroid have been developed [2]. By creating spheroids with the same number of alginate hydrogel beads as cells, a network-like arrangement of hydrogel beads is created from the surface of the spheroid to the interior. Since the hydrogel beads are mostly water, oxygen, nutrients, and waste products can diffuse and move through the channel-like space formed by the hydrogel beads. In addition, alginate lyase can digest alginate hydrogel beads in a few minutes, creating an actual space. Spheroids have been applied to drug dosing experiments, and the distribution of drugs has mostly been observed by fluorescent labeling and confocal microscopy [3]. However, some drugs cannot be fluorescently labeled, and excessive labeling may alter the kinetics of the drug. Therefore, it is necessary to devise a new method to visualize drug distribution without using fluorescent labeling.

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The FIB-TOF-SIMS (focused ion beam time-of-flight mass spectrometry) system developed by our laboratory has the highest resolution of 40 nm, which enables simultaneous mass imaging of multiple elements without the need for labeling [4]. In FIB-TOF-SIMS, the spheroid can be processed by FIB and the cross section can be component imaged. However, FIB-TOF-SIMS requires analysis in a high vacuum, and biological samples such as spheroids are susceptible to moisture volatilization, which may alter their composition and structure [5]. FIB-TOF-SIMS is expected to provide a solution to this problem, since both processing and analysis can be performed in the instrument.

However, spheroids has diameters around 100 ~ 400 μm, in such cases so-called "curtain effect" occur. This is because that the FIB is not a parallel beam, but a focused beam. If the processing object is larger, the distance from the beam focal point to the edge of the object has a dispersion. So, the cross-section is not flat, but "wave interdigital" morphology. In this paper, we examined the solution for suppress the curtain effect by cross-sectioning from multiple angle of incidence of the FIB.

II. Experimental

A spheroid used here was made by mixing two-thousands of HuH-7 cells and that of hydrogel beads. The spheroid as fixed on a Si wafer using Ag-paste, and then transferred into the TOF-SIMS stage at room temperature. The FIB was installed at 45° with respect to the surface (substrate) normal. Therefore, in the first processing, the specimen stage was tilted to 45° so as to the FIB axis and substrate surface are parallel. As a result, the top part of the spheroid was sectioned. After that, the tilt was returned to 0°, and the processed cross section was observed by FIB-induced secondary electrons.

Next, the tilt was applied again at 45° and the rotation was set at 45° for machining from the second direction. After the second machining, the tilt was returned to 0° and the machined cross section was observed. This process was also carried out with rotation of 30° and -30° at tilt angle of 45°. The machining conditions are summarized in Table 1. The FIB used in this experiment was Ga^+, and the acceleration voltage was 30 kV.

Table 1: Processing conditions

	Beam current	Processing time	Processing width	Tilt	Rotation
First direction	1st 4.2 nA 2nd 8.4 nA	130 min 120 min	13 μm (thickness) 100 μm (width)	45°	0°
Second direction	1.86 nA	42 min	3 μm (thickness) 115 μm (width)	45°	45°
Third direction	1.86 nA	39 min	3 μm (thickness) 104 μm (width)	45°	30°
Fourth direction	1.86 nA	24 min	2 μm (thickness) 111 μm (width)	45°	-30°

III. Results and Discussion

In case of FIB processing of a large sample greater than several hundreds micrometers, so-called curtain effect becomes prominent [6]. Typical result is shown in Fig.1. The

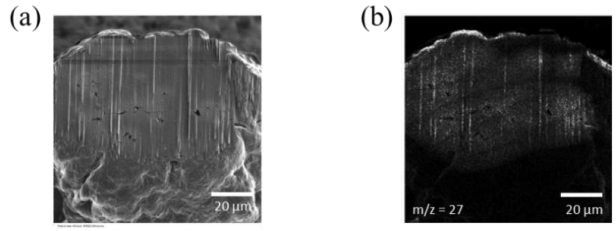

Figure 1: Curtain effect generation.

sample was a spheroid greater than 100 μm, and the cross-sectioning width was identical. As shown in Fig1(a), wave interdigital feature was seen in FIB-induced secondary electron image. Understandably, TOF-SIMS image ($C_2H_3^+$, in this case) also exhibit the same effect. It is clear that the smoothing of cross section is essential for highly-reliable imaging.

We considered whether it would be possible to perform a thin finishing process on the cross section cut out from multiple directions after an FIB processing from a direction. The angle of incidence of the FIB in our laboratory is 45° to the sample stage. If the angle of incidence is set to 0° and the specimen stage rotated, it is possible to process the same plane from any direction. Therefore, we devised a eucentric rotation system where the specimen stage is tilted at 45° and rotated at the same angle. The eucentric rotation system is shown in Fig. 2. In this study, we used the eucentric rotation system to verify FIB processing from not only one direction, but from various directions, and to evaluate the occurrence of the curtain effect. Since the quick freezing method for water-containing biological sample is still under investigation, we first evaluated the processing at room temperature.

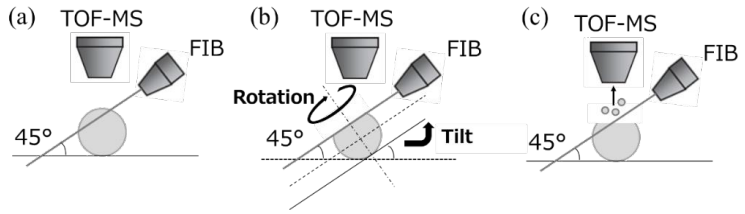

Figure 2: Eucentric rotation system in this study

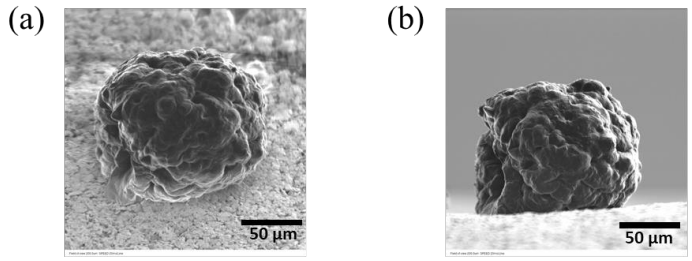

Figure 3: Secondary ion images before processing. (a) tilt at 0° and (b) tilt at 45°.

The eucentric rotation and processing method was applied to a spheroid with a diameter around 150 μm. The secondary ion images of the spheroid before processing at tilt angle of 0° and 45°, respectively in Fig.3(a) and (b). The shape of the spheroid was not a complete sphere but uneven shape. This causes curtain effect. As the first processing,

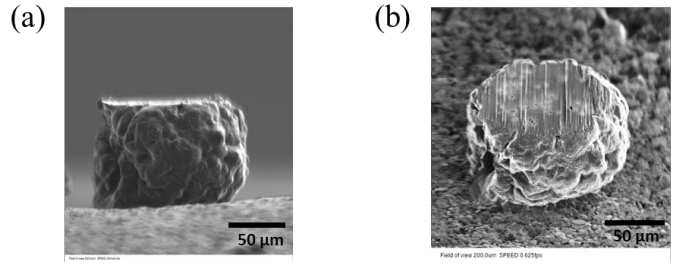

Figure 4: Secondary ion images after processing. (a) a view of the finished machining with the tilt at 45°. (b) a cross-sectional view with the tilt set to 0°.

cross-sectioning of the spheroid with a width of 100 μm. As shown in Fig.4, a cross section was obtained, but a strong curtain effect occur. This was mainly due to the unevenness of the initial shape of the spheroid.

In the next, the processed cross sections from the first direction to the fourth direction are shown in Fig. 5. Here, the cross-sectioning from the fourth direction was observed

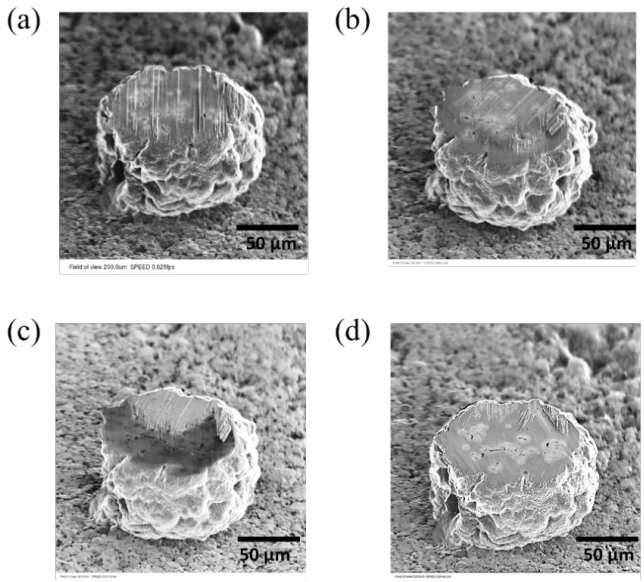

Figure 5: Processed cross section from the first to the fourth direction. (a) shows the machined cross section in the first direction (rotation 0°), (b) in the second direction (rotation 45°), (c) in the third direction (rotation 30°), and (d) in the fourth direction (rotation -30°).

after the rotation was returned to 0° in order to compare it with the cross section of the first direction.

In addition, we compared the processed cross sections of the first direction and fourth direction. First, the secondary ion image of the enlarged cross section is shown in Figure 6. The comparison of the secondary ion images clearly shows that the curtain effect is suppressed.

Next, using ImageJ software, each cross-section was inverse Fourier transformed and the high frequency portions were removed, as shown in Figure 7. This clearly shows the curtain effect. The lines drawn in Fig. 7 were then profiled by luminance. It is shown in Figure 8. Calculating the RMS (root mean square) of the data in Figure 8, we found that RMS = 9.5 for the first directional section and RMS = 4.3 for the fourth directional section. These values indicate that the variation of luminance has been greatly reduced. This means that the unevenness caused by the curtain effect has been suppressed. This is thought to be due to the smoothing of the convex part of the unevenness by grinding from multiple directions.

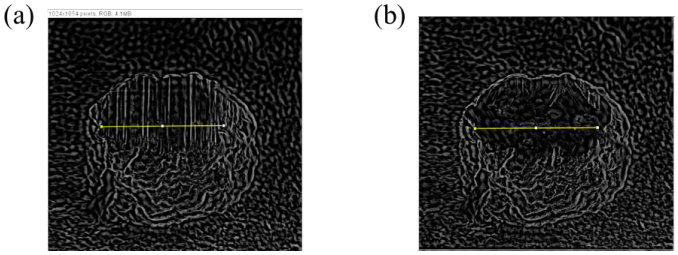

Figure 7: Machined cross-section of the first direction and the fourth direction (high-pass filter). (a) high-pass filtered image for the first directional processed cross section, and (b) high-pass filtered image for the fourth directional processed cross section.

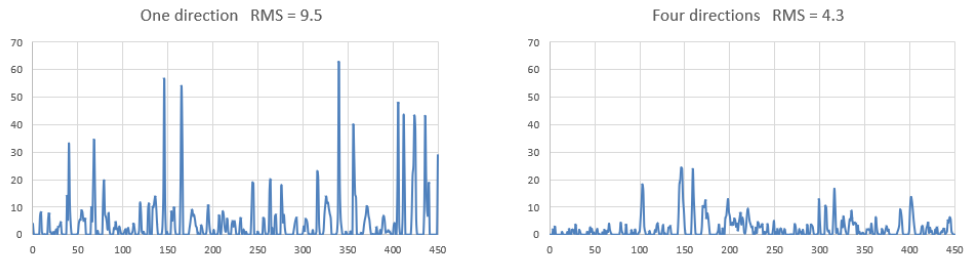

Figure 8: Unevenness profiles for the first direction and the fourth direction. The luminance profile of the horizontal line part of Figure 7.

IV. Conclusions

From the results of this study, we succeeded in machining a smooth cross-section of biological specimens larger than 100 μm by scraping the convex part of the unevenness

from multiple directions using the eucentric rotation system. In addition, we were able to quantitatively demonstrate the suppression of unevenness due to the curtain effect by comparing the results with numerical values using RMS. In near future, it will be necessary to perform processing under frozen conditions, which is necessary for biological specimens, and to evaluate the processed cross-section.

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