Paper

Chemical imaging of biomolecules in skin using TOF-SIMS and multivariate analysis

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Chemical mapping of biomolecules in biological samples such as tissues and cells is crucial for biological, medical and biochemical fields. Although secondary ion images of biomolecules such as phosphocholine and cholesterol distributions on tissue samples are easily obtained with time-of-flight secondary ion mass spectrometry (TOF-SIMS), the observation of minor ingredients is often difficult. In this study TOF-SIMS imaging data of skin samples were analyzed with a multivariate analysis technique, multivariate curve resolution (MCR), in order to investigate appropriate analysis conditions. Mice middorsal skin was sliced with a microtome and placed on indium-tin oxide glass plates. The samples were measured with TOF-SIMS using Bi_3^+, and then the obtained data were analyzed with MCR under various analysis conditions for investigating appropriate conditions. As a result, it is indicated that MCR classifies TOF-SIMS raw data into some categories representing different features, which makes interpretation of TOF-SIMS data easier.

1. Introduction

Chemical imaging and depth profiling of biomolecules in biological samples such as tissues and cells are crucial for biological, medical and biochemical fields. For instance, chemical mapping of biological samples is useful for studying various biological phenomena such as hyperkeratosis caused by genetically modified glycosylation [1,2]. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is one of the most promising techniques due to its high spatial resolution and high sensitivity in terms of chemical microscopy. Applications of TOF-SIMS as a chemical microscope for biosamples, however, have been reported fewer than other methods such as TEM, SEM, IR and Raman spectroscopy and mass spectrometry so far, because interpretation of fragment ions from biomolecules is difficult in TOF-SIMS. In order to interpret TOF-SIMS data appropriately, several factors specific to secondary ion mass spectrometry (SIMS) such as secondary ion yield difference depending on a material and unclear fragmentation and secondary ion generation mechanisms should be considered.

Multivariate analysis (MVA) techniques have been applied to TOF-SIMS data analysis [3], and they accelerated TOF-SIMS application into life science fields. Principal component analysis (PCA) is the most popular MVA technique, it often provides useful information on outlines of data. However, PCA does not always provide straightforward interpretation of spectra because the procedure involves eigenanalysis to the data matrix, which rotates the spectra, sometimes showing unrealistic negative intensities [4]. In terms of extracting data related to a particular material, multivariate curve resolution (MCR) [4] and independent component analysis (ICA) [5] will be powerful. The objectives of this study are applying MCR to a complex sample such as tissues and investigating effective conditions of data treatments for MCR calculation and then evaluate possibility of MCR for TOF-SIMS studies.

The model samples of this study were mice skin specimens. The skin samples were measured with TOF-SIMS and then their TOF-SIMS data were analyzed using MCR.

2. Materials and Methods

Sample preparation

Mice mid-dorsal skin was removed and mounted in OCT compound (Sakura Finetek USA, CA) without fixation. The frozen specimens were cut with a cryostat CM1850 (Leica Microsystems GmbH, German) to obtain 6-μm-thick slices, and put on indium-tin oxide coated slide glass plates (SIGMA- Aldrich Co., St Louis, MO). Then the sliced specimens were completely air-dried.

TOF-SIMS measurement

Positive ion spectra of the samples were obtained with TOF-SIMS 5 (ION-TOF GmbH, Münster) with 25 keV Bi_3^+ primary ion sources. All measurements were acquired while maintaining the primary ion dose at less than 10^{12} ions/cm^2 to ensure static conditions. The secondary ion image raw data were obtained using a pixel density of 128 x 128 over 155 μm x 155 μm region of the samples.

MCR

Peaks of secondary ions were auto searched with the TOF-SIMS analysis software provided by ION-TOF GmbH, and then 276 peaks, ranging from m/z 15 to 756, were selected. Secondary ion images of the selected peaks were converted into binary image files (BIF) and they were transformed into matrix data using MIA_Toolbox (Eigenvector Research Inc., WA).

The matrix data were analyzed using the MCR program developed by S. Muto et al. [6]. The program is coded on Matlab (The MathWorks Inc., MA). The modified alternating least-squares (MALS) [7] was adopted to the program. The TOF-SIMS data were treated with Poisson-scaling (root mean scaling) [8-11] before the MCR calculations.

3. Results and Discussion

The mouse skin sample was analyzed in order to evaluate MCR features on dividing TOF-SIMS data of complex biological sample. MCR was performed on the same sample with a variety of the number of components from three to eleven which should be initially given for MCR calculation. In addition, principal component analysis (PCA) indicated three or four important components.

Fig. 1 shows typical secondary ion images of the sample. The skin sample exists in the area except for the upper area indicated by In^+ distribution. Distribution of phosphocholine, one of the main components of the skin, is obtained with m/z 86 and 184 secondary ions related to phosphocholine [12], which shows the distribution of the skin sample. Components whose total ion images are included in the skin sample area are related to the skin.

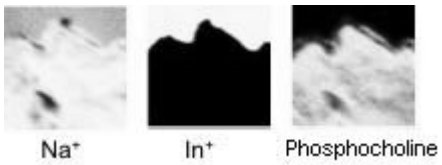

Fig. 1. Secondary ion images of *m/z* 23 (Na^+), 115 (In^+) and 86 and 184 (phosphocholine). Field of view is 155 μm.

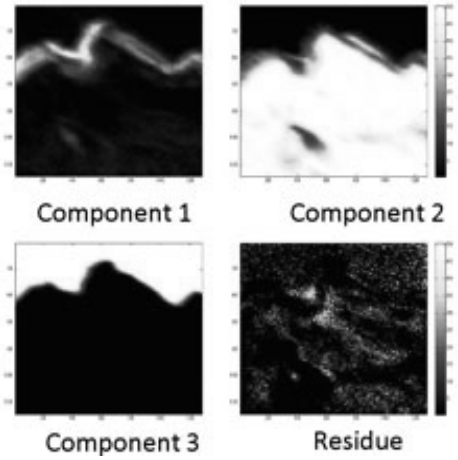

Fig. 2. Secondary ion image of each component extracted by MCR using Poisson-scaled data, when the number of components is assumed to be three. Field of view is 155 μm.

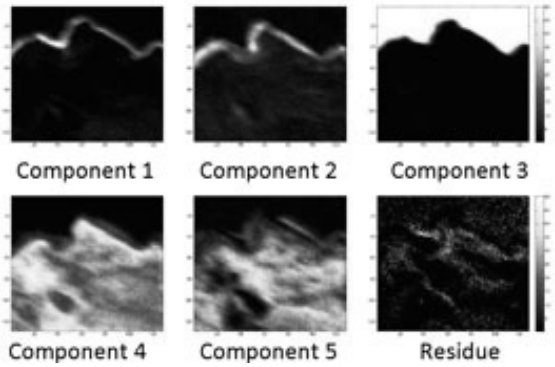

Fig. 3. Secondary ion image of each component extracted by MCR using Poisson-scaled data, when the number of components is assumed to be five. Field of view is 155 μm.

When the numbers of initially given components are varied from three to five, images representing the ITO substrate distribution are almost the same, and their spectra are very similar. As shown in the distribution of the component 2 of Fig. 2, TOF-SIMS data only related to the skin sample were extracted using MCR. The component 1 in Fig. 2 shows the interface between the ITO substrate and the skin, and the component 3 shows the distribution of the ITO substrate. The

extracted spectrum related to the component 2 in Fig. 2 is, however, still complicated.

In Fig. 3, two components were extracted from the skin sample area. The interface between the ITO substrate and the skin, as shown in the components 1 and 2 in Fig. 3, is emphasized because of the ITO electrode which has high conductivity. Since intensities of secondary ions on the interface are highly enhanced, they may not suggest appropriate information of materials there. Therefore components related to the interface were omitted.

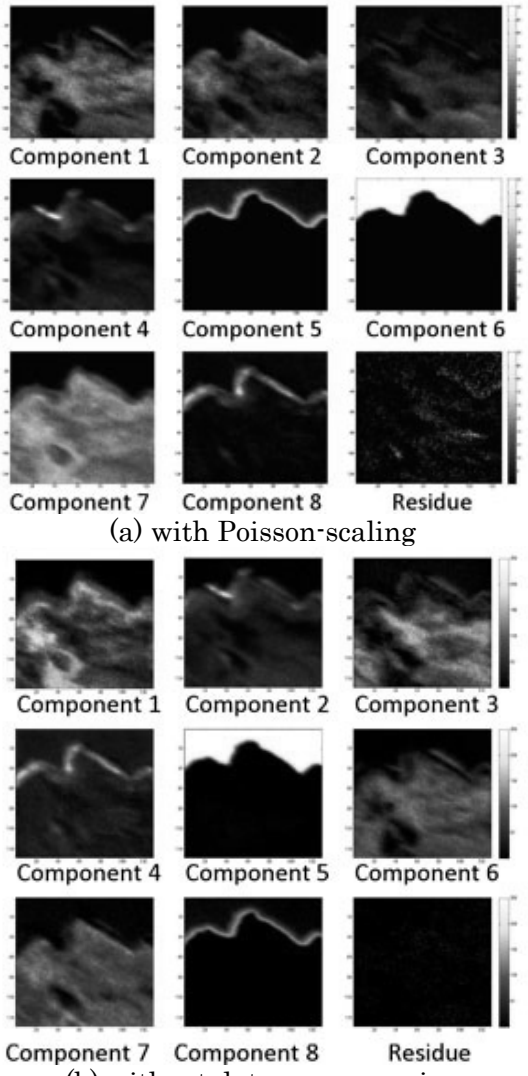

Fig. 4 Secondary ion image of each component extracted by MCR, when the number of components is assumed to be eight. Field of view is 120 μm.

When the initially given number of components increases in MCR analysis up to ten, more components are extracted from the skin area with the increase of the number of components. The images obtained by MCR with and without Poisson-scaling are clearly different when the number of components is assumed to be more than seven, though the images and spectra of pure components obtained with and without Poisson-scaling are almost the same assuming less than eight components. For instance, the images of components 2 and 7 with Poisson-scaling (Fig. 4 (a)) were not obtained using the data without preprocessing (Fig. 4 (b)). Even if similar images are obtained, their spectra are different. Table 1 shows typical peaks related to phosphocholine, m/z 86 and 184, which are mainly detected from tissue samples and the peak related to In, m/z 115, which is one of main materials in the ITO glass substrate. Although the image of component 1 in Fig. 4 (a) and that of component 6 in Fig.4 (b) are very similar, their spectra are different. This tendency becomes higher with the larger assumed number of components.

Tale 1. Intensities of typical peaks on TOF-SIMS spectra of Fig. 4 data.

	(a)	(b)	(a)	(b)
m/z	Component 1	Component 6	Component 4	Component 2
15.03	0.0341	0.0011	0.0000	0.0101
22.99	0.1426	0.0000	0.0001	0.2125
86.11	0.0980	0.2254	0.0154	0.1517
114.91	0.0000	0.0000	0.0000	0.0000
184.09	0.1221	0.4743	0.0628	0.1047

Fig. 5 shows comparison between images obtained with and without Poisson-scaling when the number of components is assumed to be nine. In the results without data preprocessing, the ITO glass substrate area is divided into three components, components 6, 7 and 9. This division does not provide essential information on the sample. Without data preprocessing, high intensity peaks may contribute to results more than real importance. It is indicated that data preprocessing such as Poisson-scaling is necessary when targets of analysis are minor ingredients.

When the number of components is assumed to be more than ten, stable results are not obtained. In other words, the solution of MCR did not converge with more than ten components. Therefore, the number of components of this sample should be less than eleven. The results obtained with Poisson-scaling are better than those without data pre-processing because they classified the skin sample into more categories than without data pre-processing, which helps interpretation of complex samples.

Since biological samples contain numerous materials, it is difficult to extract every pure component. MCR is, however, useful for obtaining less complex data containing important

information regarding target materials because it can classify a tissue sample into several categories representing different materials which can be evaluated with distribution data. For example, since the distribution of the component 3 in Fig. 5 (a) shown high intensity on the edge of the skin, this component may suggest particular materials which concentrates on the most upper surface of skin. The component 6 may be related to general ingredients in skin because its distribution spreads over the skin sample area. Thus important information in terms of chemistry or biology can be indicated by analyzing total ion images and spectra of the components obtained with MCR.

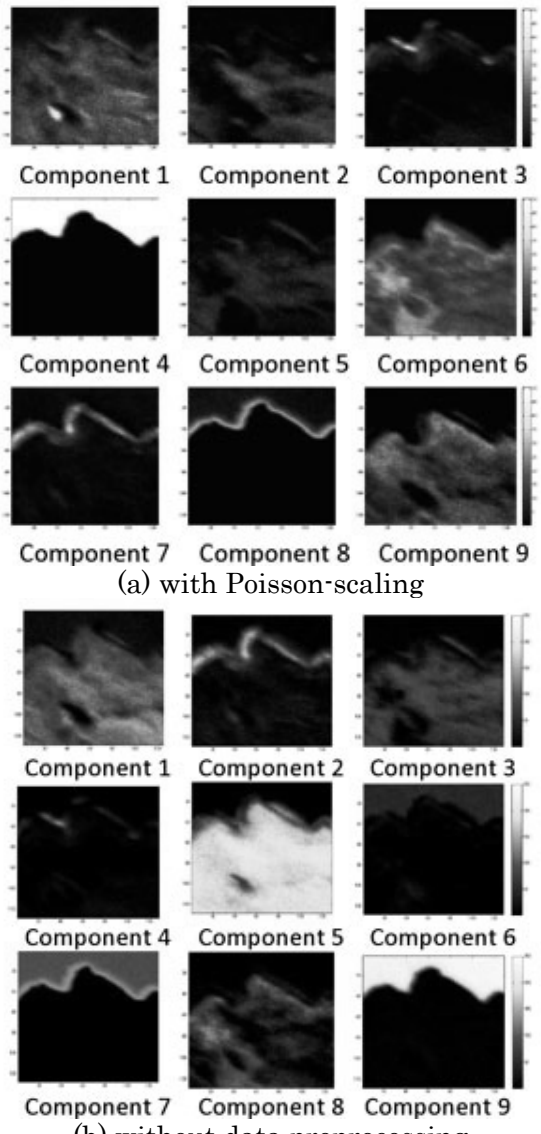

Fig. 5. Secondary ion image of each component extracted by MCR, when the number of components is assumed to be nine. Field of view is 155 μm.

In summary, the TOF-SIMS data of the complex skin sample were divided into at most six categories. By analyzing a spectrum and a secondary ion image of each component, the skin sample can be evaluated using less complex information than that of the original TOF-SIMS data. Further study on application of MCR to TOF-SIMS data is necessary for characterizing important ingredients in tissues generating weak secondary ions.

4 Conclusions

It is indicated that MCR is effective to simplify TOF-SIMS data of complex samples containing a variety of materials such as biological tissues. Poisson-scaling is effective to reduce influence of strong peaks which often suppress important minor peaks.

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