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Original Article

BioQuant: Data Processing Software for Simultaneous Imaging Analysis for Elements and Molecules Using Two Mass Spectrometers

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Simultaneous imaging analysis for both elements and molecules was conducted by introducing laser-induced sample particles into two separate mass spectrometers (*i.e.*, an inorganic spectrometer and an organic mass spectrometer) through the split-flow protocol. The timing of ion detections for elements and molecules can be different, reflecting the differences in mass scan rates, lengths for transport tubing, and flow rates of carrier gases, and thus, the timing of ion detections must be carefully aligned to discuss abundance correlations between elements and molecules. To achieve this, a new data processing software "BioQuant" was developed to correct the time difference of the signal intensities for components obtained by the two mass spectrometers. With the BioQuant software, signal intensity data obtained from several unit cells were merged into a newly defined unit cell, calculated by the common time intervals from both mass spectrometers. With the newly defined unit cells, correlation analysis between the elements and molecules can be conducted. Combination of the BioQuant software and laser ablation system connected to two separated mass spectrometers can become a benchmark technique for simultaneous imaging analysis for both the elements and molecules from single sample material.

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INTRODUCTION

The understanding of the function and behavior of metals in biological system on the level of single cells is the ultimate goal of metallomics.[1)] Laser ablation inductively coupled plasma (ICP) mass spectrometry (MS) is a sensitive and quantitative analytical tool for elemental mapping of biological samples for metallomics studies.[2–4)] The high kinetic temperature achieved by the ICP leads to effective ionization (90% or higher) of most elements, allowing quantitative analysis of major to trace elements.[5–7)] Despite the obvious success in rapid and sensitive elemental analysis, due to this high-kinetic temperature achieved by the atmospheric plasma ion source, both the molecular structure and status of elements (chemical form or oxidation states) would be lost. For elemental analysis, "hard ionization" using ICP ion sources is required to improve quantification, whereas for molecular analysis, "soft ionization" is required to reduce fragmentation of molecules.[8–10)] To achieve soft ionization, atmospheric pressure ion source using dielectric barrier discharge (DBD) was described.[11)] The ion source based on the DBD has several unique features, including (i) being an atmospheric pressure ion source, leading to the ion source to be acceptable of dry aerosols produced through laser ablation, (ii) high excitation temperature, and (iii) low kinetic temperature, obviating the risk of decomposition of organic compounds. Pseudomolecular ion ($[M+H]^+$) was mainly observed as the base peak (strongest ion signal within the obtained mass spectrum), although $[M+NH_4]^+$ as the base peak was also detected depending on the target analyte, indicating that the molecules were effectively ionized with minimum fragmentation. One of the great advantages achieved by the DBD ion source is that the ion source is powerful enough to ionize molecules in sample aerosols generated by the laser ablation technique, and thus, imaging analysis of molecules can be conducted.

In recent years, laser-induced sample particles could be detected by two separate mass spectrometers through the

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split-stream protocol. The laser ablation split stream (LASS) technique was first achieved by Yuan *et al.*,[12] simultaneously measuring isotopic ratios and trace element compositions with multiple collector and quadrupole ICP–MS, respectively. Ever since, to our knowledge, LASS has only been used for elemental analysis. By using the LASS concept, imaging analysis can be simultaneously achieved with two mass spectrometers. This suggests that simultaneous imaging analysis of elements and molecules can be made if laser-induced sample particles are introduced into two mass spectrometers equipped with DBD ion source and ICP ion sources, respectively, through the split-flow technique. Through this approach, information on possible functional linkage between metal ions and biomolecules can be derived from a single analysis.

In the split-stream technique, the timing of signal events observed for the two mass spectrometers is different. This reflects the differences in mass scan rate, monitoring mass ranges, lengths of transport tubing, flow rates of carrier gas, and/or data output frequency between the two mass spectrometers. This means that the size of unit cell defined by single data output is different between the mass spectrometers, and therefore, great care must be given in the correlation analysis for different components. Post-processing can be conducted by an imaging processing software, in which a plethora of in-house software for mass spectrometric image visualization has been developed.[13–15] Integration of independently measured organic and inorganic images has been reported, but highly sophisticated processes are required to identify and compare the shape of the sample.[16,17] However, in hybrid imaging, no complex mathematics is required to compare the measured data because the same sample particles are detected by the two mass spectrometers. The only data-processing procedure we should do is adjust the timing of the measured data. To take a full advantage of simultaneous imaging, a software that can manage hybrid imaging data is needed. In this paper, we introduce a new imaging software, BioQuant, to handle imaging data processing from different mass spectrometers. Here, the principles of the data processing method and basic performance achieved on hybrid imaging using organic and inorganic mass spectrometers through the split-stream technique are described.

METHOD

Instrumentation: LASS–DBDI–MS/ICP–MS

A hybrid system of various analytical techniques was used to conduct simultaneous imaging. The instrumental setup is illustrated in Fig. 1, where laser ablation was used for *in situ* sampling from solid materials. A laser ablation system utilizing a Yb:KGW femtosecond laser equipped with Galvanometric optics (Jupiter Solid Nebulizer; ST Japan, Tokyo, Japan) was connected to two mass spectrometers in parallel. Details of the Galvanometric optics and system setup are described in our earlier papers.[18,19] The aerosols generated by the laser ablation were transported into two mass spectrometers by a mixture of helium gas (carrier gas) and argon gas (makeup gas). The carrier gas flow was then split into two streams with a Y-piece. A portion of the aerosols were transferred to an in-house dielectric barrier discharge ionization (DBDI) source connected to an organic mass spectrometer (QTRAP 5500; SCIEX, Framingham, MA, USA) for molecular analysis,[11] while the other portion of the aerosols were transferred to an ICP–MS (iCAP TQ; Thermo Fisher Scientific, Bremen, Germany) for elemental analysis.[19] A manual flow control pinch valve (Flow control pinch valve, PV-2; AS ONE, Osaka, Japan) was placed at the entrance of the tube of the ICP–MS, to allow a larger portion of aerosols to be transferred to the less-sensitive DBDI–MS. The optimal conditions were determined by comparing the detected signal intensities of both mass spectrometers. A detailed description and instrumentation on the DBDI source were reported by Khoo *et al.*[11]

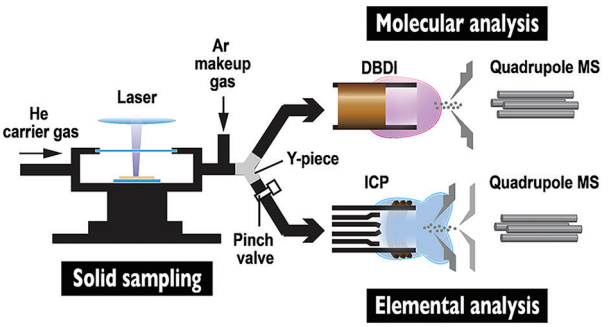

Fig. 1. Instrumental setup of the LASS–DBDI–MS/ICP–MS system. The aerosols were split into two streams with a Y-piece. A pinch valve was placed at the entrance of the ICP–MS to control the proportion of aerosols for both mass spectrometers. DBDI, dielectric barrier discharge ionization; ICP, inductively coupled plasma; LASS, laser ablation split stream; MS, mass spectrometry.

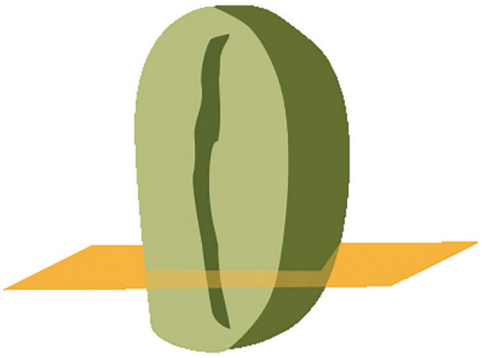

Fig. 2. Sample preparation of the coffee bean.

Sample preparation

The possibility of LASS–DBDI–MS/ICP–MS system for simultaneous imaging was evaluated using green coffee beans. The coffee bean was used for evaluation because their chemical composition is reported in various studies.[20,21] The sample for imaging was prepared by slicing the bean with a hand microtome and disposable blade, revealing a cross-section with a thickness of 50 μm. The bean was sliced in the direction as shown in Fig. 2. The sliced section is secured with a double-sided tape on a glass slide and then subjected to analysis.

Analytical procedure

Imaging analysis was conducted based on repeated line-profiling analysis across the sample. Laser conditions are fluence 3.8 J/cm^2, repetition rate 1000 Hz, raster speed 80 μm/s, vertical resolution 100 μm, and 67 lines with no gap between the lines.

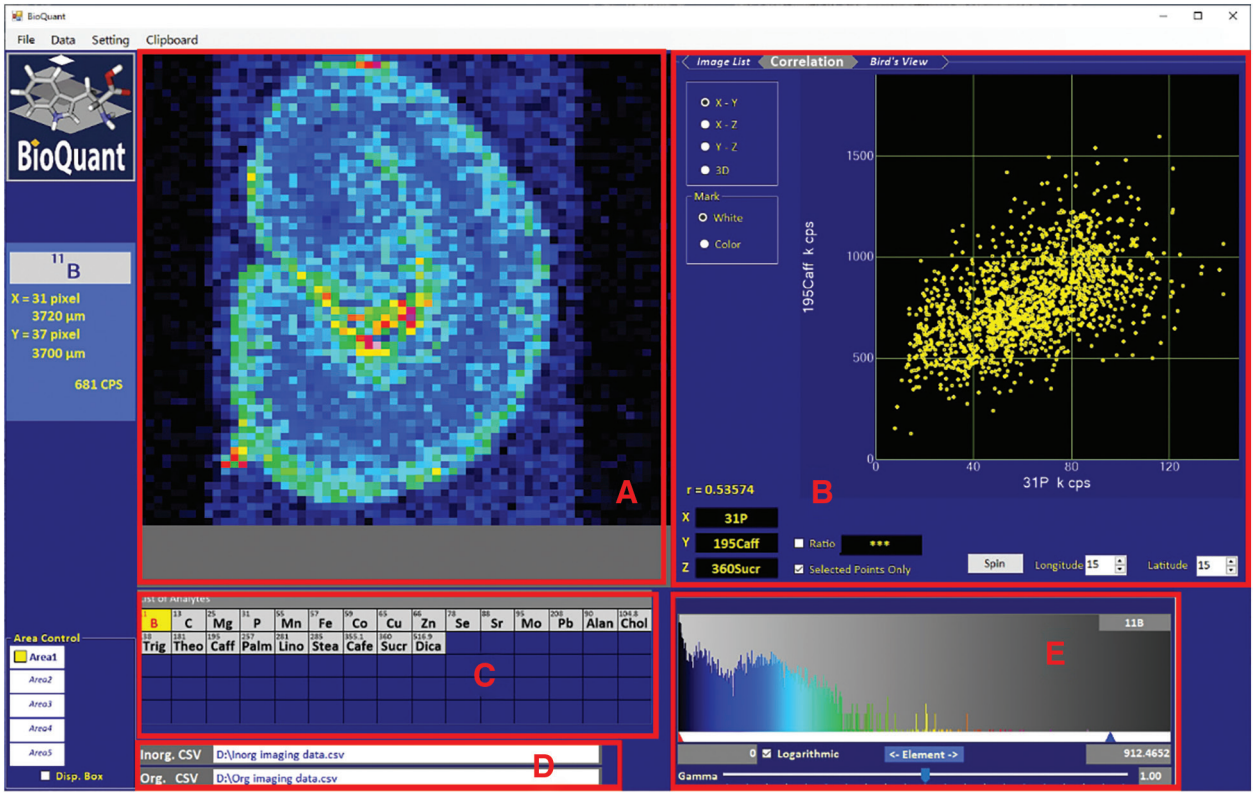

Fig. 3. The main window of BioQuant is composed of several panels. The constructed image is displayed in Panel A, and Panel B shows results of data processing, such as correlation analysis. Elements or molecules can be selected by using Panel C. The loaded data files from the mass spectrometer are shown in Panel D. Contrast of displayed image can be adjusted by using Panel E.

The data acquisition of the mass spectrometers was conducted with the following procedures. Molecular imaging was conducted with the multiple reaction monitoring mode, with nitrogen as the collision gas. Prior to imaging, the optimization of the collision energy and identification of the highest abundant fragment ions produced during collision-induced dissociation was performed. Eleven analytes were monitored for molecular analysis. For elemental imaging, an analysis was conducted using the kinetic energy discrimination mode with helium gas as the collision gas to remove polyatomic interferences. A total of 13 analytes were monitored for elemental analysis. Dwell time of each analyte in both mass spectrometers was set to 50 ms, and the resulting data output frequency was 0.6675 s in ICP–MS and 0.6052 s in DBDI–MS.

Overview of software "BioQuant"

BioQuant was developed with Microsoft Visual Studio 2022 operating on Windows OS. This software is constructed to integrate the line-profile data measured by simultaneous hybrid imaging analysis. BioQuant does not support spot analysis because no position adjustment is required in spot analysis. The main window of BioQuant is shown in Fig. 3. The basic structure of this software is identical to that of iQuant2[3] for easy operation. The most important feature of the BioQuant is the ability to accept two time-based signal intensity profiles produced by two mass spectrometers (Fig. 3D). The names of analytes measured by organic-MS are manually assigned at comma separated values (CSV) file-loading process based on observed *m/z* values. As the first step, the loaded two files will be individually processed to images, and then they will be reconstructed to merge into a unified data set.

RESULTS

Image construction with conventional method

The raw data from both mass spectrometers were converted and the output was two respective CSV files. The two files were then inputted into BioQuant and processed with the conventional method, which is to just simply use laser conditions and each mass spectrometer condition individually, for time–position translation. Data from ICP–MS and DBDI–MS were independently processed to images, and the examples of the obtained image (^{31}P [*m/z* 31] by ICP–MS and caffeine [*m/z* 195 → *m/z* 138] by DBDI–MS) are shown in Fig. 4. These analytes are the major component of coffee bean and useful to identify the location of bean. The shape of coffee bean was easily identified and the distributions of phosphorus and caffeine can be visually compared. Figure 4A shows that phosphorus is concentrated around the region near the surface of the green coffee bean. A similar tendency can be observed from the image of caffeine (Fig. 4B), but more detailed discussion cannot be done from such approximated visual observations.

In order to analyze the distribution of materials in objective ways, numerical data processing is required. Unfortunately, it is not easy to numerically analyze hybrid imaging data, because the spatial resolution of data is different for each mass spectrometer. For example, in the present hybrid measurements, although dwell time for both mass spectrometers was set to 50 ms, the data output frequency of

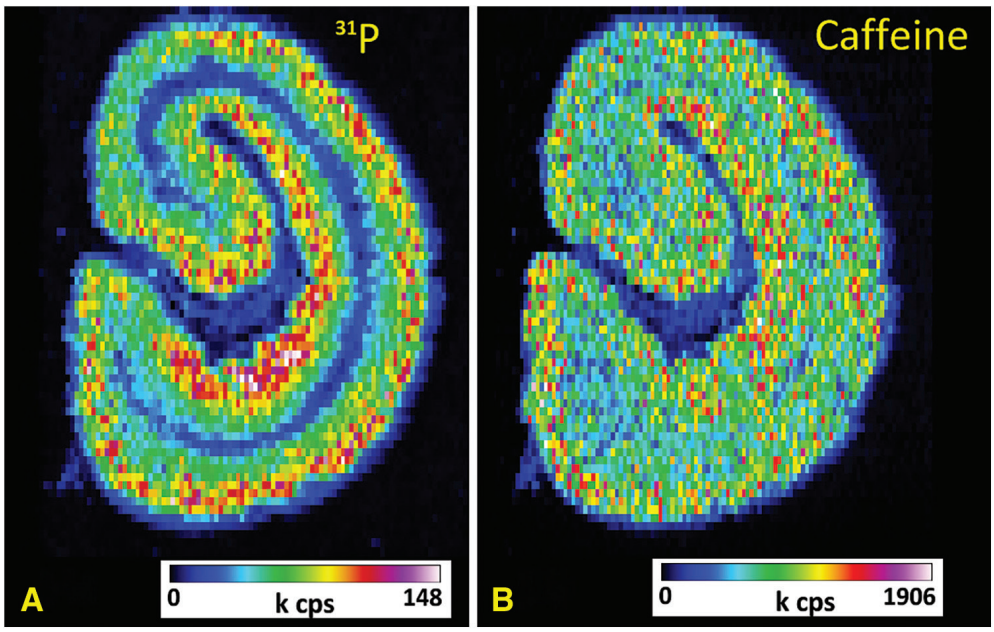

Fig. 4. Images of a coffee bean acquired by the hybrid imaging technique. Image of (A) ^{31}P was measured by ICP–MS and (B) caffeine was measured by DBDI–MS. Data measured by both MS were independently processed by BioQuant. DBDI, dielectric barrier discharge ionization; ICP, inductively coupled plasma; MS, mass spectrometry.

DBDI–MS (0.6052 s with 11 analytes) is shorter than that of ICP–MS (0.6675 s with 13 analytes). As a result, the horizontal size of unit cell of image is approximately 53 μm in the ICP–MS image, while being around 48 μm in the DBDI–MS image. Hence, in order to perform numerical analysis of the hybrid imaging data, rearrangement of acquired data is necessary. In the following sections, we described data processing methods of our new imaging software.

Adjustment of image position

Since transport time of aerosol streams to each mass spectrometer is different, adjustment of horizontal position is inevitable in the hybrid imaging technique. An example of "image shift" found in hybrid imaging is shown in Fig. 5, which displayed in the "Image Position Adjusting Window" of BioQuant.

In the present system setup, elapsed time for aerosol transportation to the DBDI–MS is shorter than that for the ICP–MS, and therefore, the signal intensities for elements and molecules released from single ablation point will be recorded in different elapsed times. In Fig. 5, ^{13}C (green) and sucrose (red) are displayed because these analytes were detected in almost all sample area. Without any corrections for the time delay, the resulting image for sucrose positioned toward the left-hand side than the image for ^{13}C (Fig. 5). To achieve reliable correlation analysis for both the elements and molecules, these inconsistencies should be corrected.

Guide signals, which are signals observed at the same timing in both mass spectrometers, are required to adjust the position of images. In the present hybrid imaging, signals of ^{13}C and sucrose were used because increase in signal intensities at the beginning of laser ablation was found in these species. In BioQuant, adjustment of image position can be done by mouse operation on the image, or the parameter "Time Shift" can be directly inputted. "Time Shift" will be adjusted until the image and line graph are aligned.

Adjustment of spatial resolution of image

For the numerical analysis of hybrid imaging, spatial "size" of data in all images should be the same. The difference of data resolution in ICP–MS and DBDI–MS in the present hybrid imaging is schematically shown in bar graphs (Fig. 6). The width of a bar represents the data output frequency of each mass spectrometer, while the measured intensity indicates the bar height. We can easily point out that the position of data unit of ^{13}C and sucrose does not match each other. Moreover, in the present measurement, both the mass spectrometers measured each analyte for 50 ms by changing the target mass, and consequently, data output frequency of a single scan will be longer than 600 ms (acquisition of 11 or 13 analytes in total). Hence, if we precisely describe the measurements as a bar graph, the width of the bar shown in Fig. 6 should be very narrow, and there will be significant "vacant" time in each measurement cycle. These complex situations make it difficult to analyze in a numerical way.

For a typical imaging using line analysis, the aerosols from a point are partly mixed with those from the neighboring points, leading to the actual spatial resolution to be not as "high" as calculated from data output frequency of mass spectrometer and laser scan speed. As a result, moving average values are sometimes adopted to construct the images, instead of "raw" signal intensities. By considering this kind of situation, for the hybrid imaging, it seems better to choose "long time span" for a unit cell of the image; lower limit of time span was tentatively set in BioQuant as double of data output frequency of mass spectrometer.

In the present analysis, we chose 1.5 s, and the averaged signal intensity of this time interval is calculated to use for integrated data (Fig. 6B). As a consequence of that, spatial resolution of horizontal direction becomes 120 μm for the hybrid imaging, while the resolution of vertical direction is unchanged.

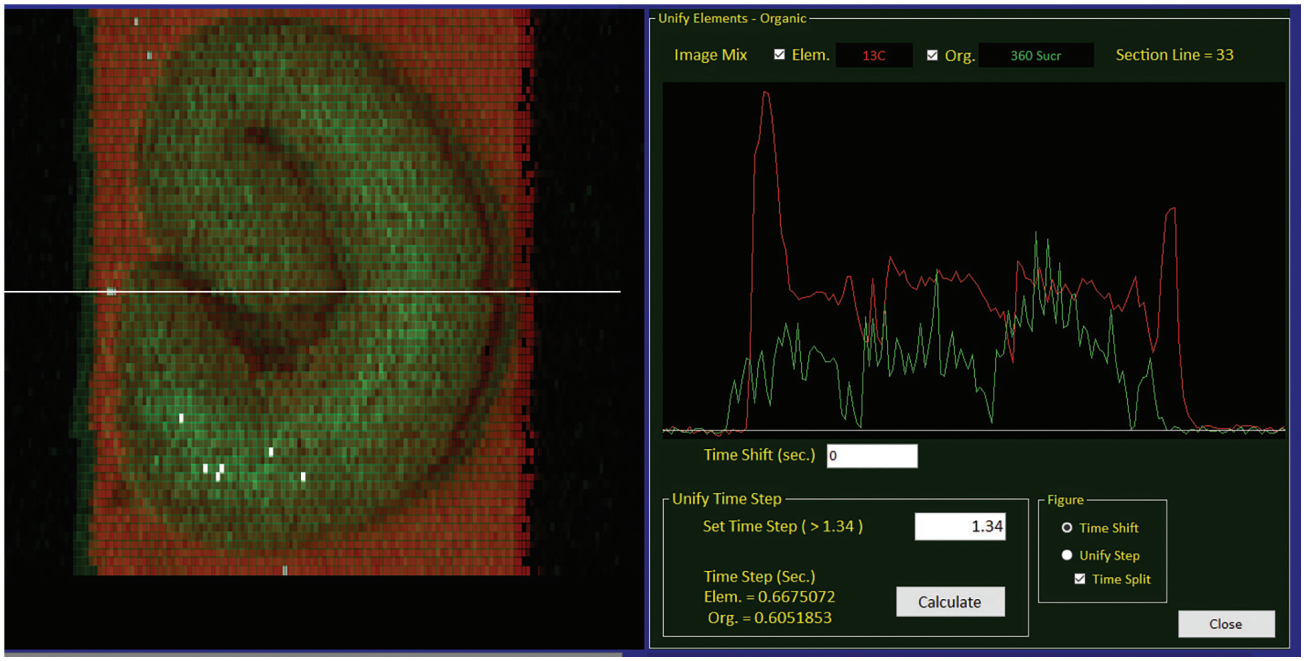

Fig. 5. Image Position Adjusting Window of BioQuant. The left panel displays the temporary overlapped image of sucrose by DBDI–MS (green) and ^{13}C by ICP–MS (red). The right panel shows the signal intensities of each analyte (*i.e.*, sucrose in green and ^{13}C in red) from the white line on the overlapping image. The horizontal position of the entire organic image for organic compounds can be adjusted with mouse operation or direct input of "Time Shift." DBDI, dielectric barrier discharge ionization; ICP, inductively coupled plasma; MS, mass spectrometry.

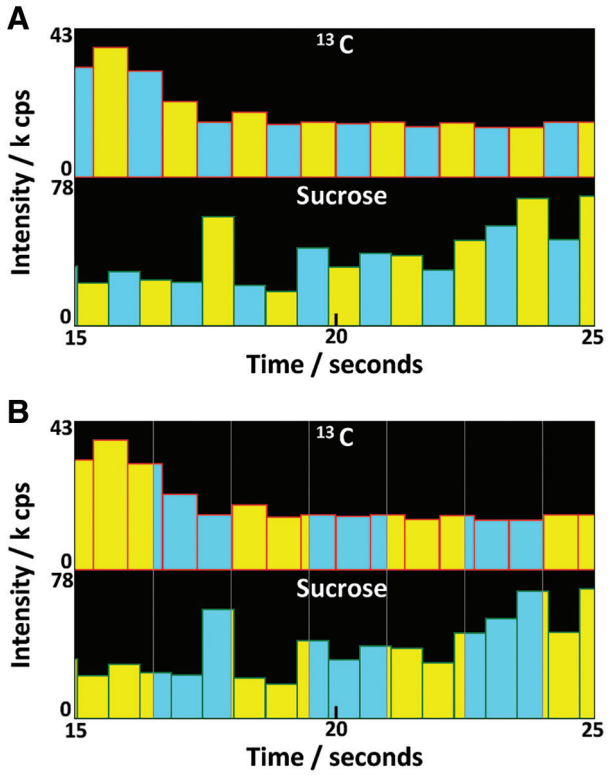

Fig. 6. Bar graphs of signal intensities measured by hybrid imaging. Data output frequency was 0.6675 s in ICP–MS and 0.6052 s in organic-MS. (A) In the general imaging, data output frequency is converted to with the unit cell of image, and each intensity value corresponds to a single unit cell. In order to unify the size of unit cell in hybrid imaging, a common time interval is defined for integrated imaging. (B) The common time span of 1.5 s is applied to each bar graph; the signal intensities of yellow and light blue regions shown in this figure are averaged and used as intensity of a new unit cell. ICP, inductively coupled plasma; MS, mass spectrometry.

DISCUSSION

Integrated hybrid imaging analysis

Since BioQuant is still under construction, functions of Panel B (Fig. 3) are limited. Some analysis must be done by using some other imaging software at this time, with output as the reconstructed data. However, some analyzing functions of BioQuant are already operational and the examples of preliminary data analysis are described below. Further detailed analyses of green coffee bean are discussed elsewhere.[22)]

The reconstructed images of ^{31}P (m/z 31), caffeine (m/z 195 → m/z 138), and sucrose (m/z 360 → m/z 163) are shown in Fig. 7. The unit cell size of the image is unified in all images, and signal intensities of ions in each cell can be easily compared. As mentioned previously, phosphorus and caffeine show similar distribution behaviors within the coffee bean. In the case of sucrose, this analyte concentrates at the interior of the bean.

The correlations of signal intensity between these species are shown in Fig. 8. In these figures, data points of the coffee bean were selected by specifying a polygon (displayed as red polygon in Fig. 7C) on the image panel of BioQuant and plotted on the graphs. A weak positive correlation is found between phosphorus and caffeine (correlation coefficient r=0.536, Fig. 8A), while negative correlations may be suggested for phosphorus–sucrose (r=−0.353, Fig. 8B). No obvious correlation can be found in caffeine–sucrose (r=−0.171, Fig. 8C).

Color mixing images of ^{31}P–caffeine and ^{31}P–sucrose are shown in Fig. 9. This color mixing image is different from the overlapping image used in "Image Position Adjusting Window" shown in Fig. 5. In the case of overlapping image, the red image and the green image are overlapped by using transparency function of Microsoft Visual Studio, and red or green colors are preserved on the created image. On the other hand, in the case of color mixing, the observed intensities

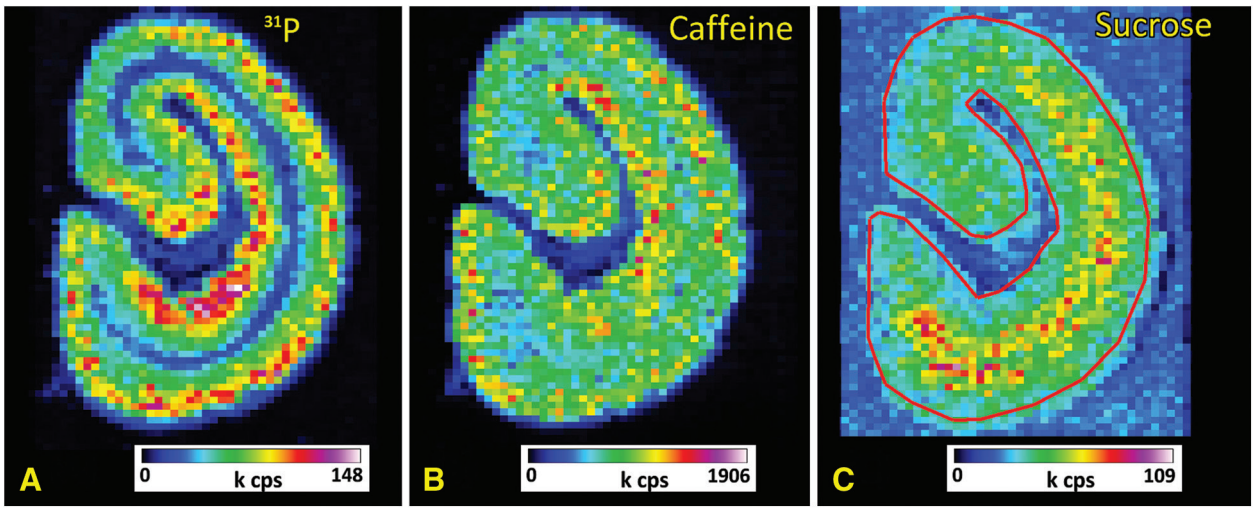

Fig. 7. Images of (A) ^{31}P, (B) caffeine, and (C) sucrose, which are all created in the same unit cell size (120 μm horizontal spatial resolution). The red-colored polygon shown in (C) is used for data point selection of correlation analysis shown in Fig. 8.

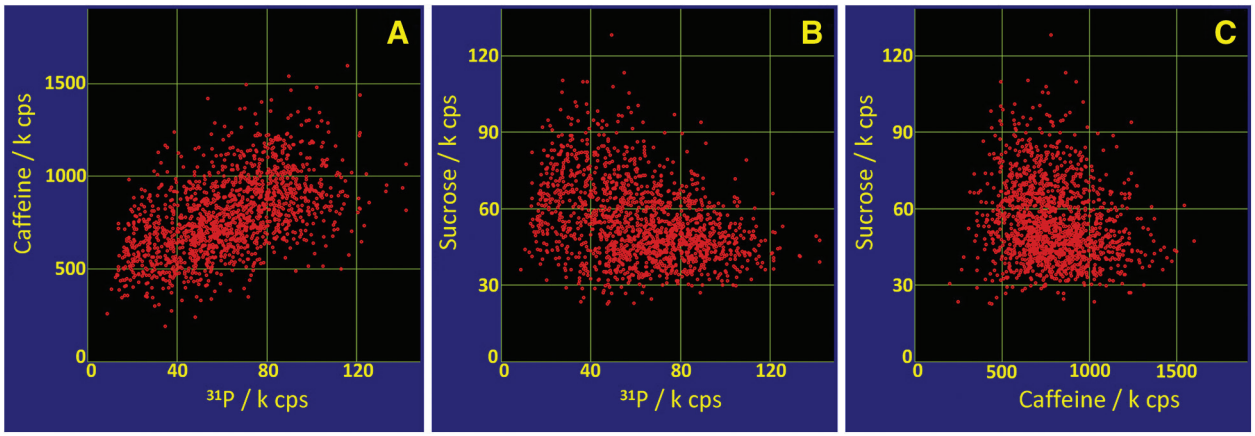

Fig. 8. Correlation graph of (A) ^{31}P–caffeine, (B) ^{31}P–sucrose, and (C) caffeine–sucrose. A weak positive correlation can be found in ^{31}P–caffeine (correlation coefficient r=0.536), while negative correlation may be suggested in ^{31}P–sucrose (r=−0.353). No obvious correlation is found in caffeine–sucrose (r=−0.171).

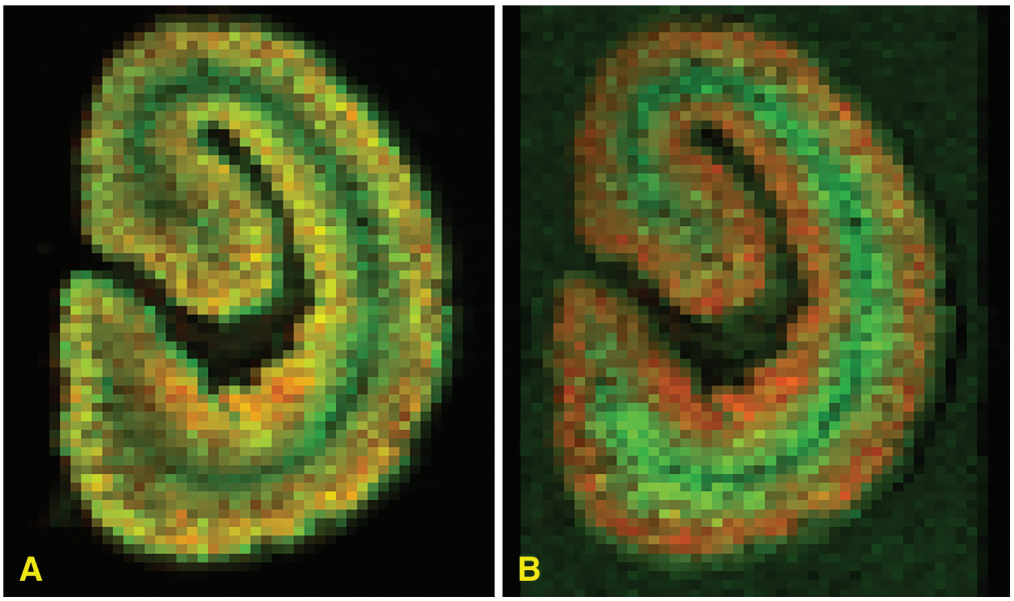

Fig. 9. Color mix images of (A) ^{31}P (red)–caffeine (green) and (B) ^{31}P (red)–sucrose (green). Yellowish colored cells indicate that both components exist at that cell, while reddish- or greenish-only colored cell shows that concentration of either component is prominent.

are converted to the brightness of red, green, and blue components, and a new color is created. For example, if the red component and the green component are equally high, then the color will be yellow. The color mixing image of ^{31}P (red) and caffeine (green) suggests weak correlation between these components (Fig. 9A). Yellowish cells can be found around the area near the surface of the coffee bean. Negative correlation is found in the mixed color image of ^{31}P (red) and sucrose (green). The surface area of the bean is red colored, while the central part is filled with green color (Fig. 9B).

CONCLUSION

Simultaneous hybrid imaging technique is a powerful tool to compare the distribution behavior of components that cannot be measured by a single mass spectrometer. Since two different mass spectrometers operate independently, rearrangement of measured data is required to analyze all data together. To achieve this, a new data processing software BioQuant is developed, and signal intensity data from several unit cells are merged to a newly defined unit cell with the common time intervals for both mass spectrometers. After these corrections, spatial resolution of the image becomes a little lower, but integrated numerical processing, such as correlation analysis, is possible.

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