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RESEARCH ARTICLE

Improvement of Sensitivity and Reproducibility for Imaging of Endogenous Metabolites by Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry

Tomomi Morikawa-Ichinose,[1,2] Yoshinori Fujimura,[1,2] Fusa Murayama,[1,2] Yuzo Yamazaki,[3] Takushi Yamamoto,[3] Hiroyuki Wariishi,[1,2] Daisuke Miura[1,2,4]

[1]Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka, 812-8581, Japan
[2]Innovation Center for Medical Redox Navigation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan
[3]Analytical Applications Department, Global Application Development Center, Shimadzu Corporation, 1 Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto, 604-8511, Japan
[4]Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki 305-8566, Japan
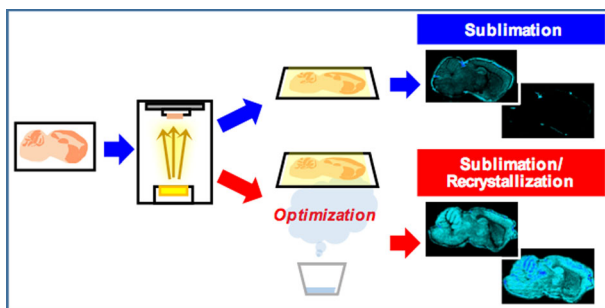
Abstract. Matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry imaging (MSI) is a powerful technique to visualize the distributions of biomolecules without any labeling. In MALDI-MSI experiments, the choice of matrix deposition method is important for acquiring favorable MSI data with high sensitivity and high reproducibility. Generally, manual or automated spray-coating and automated sublimation methods are used, but these methods have some drawbacks with respect to detection sensitivity, spatial resolution, and data reproducibility. Herein, we present an optimized matrix deposition method of sublimation coupled with recrystallization using 9-aminoacridine (9-AA) as a matrix capable of ionizing endogenous metabolites. The matrix recrystallization process after sublimation was optimized for the solvent concentration and reaction temperature for matrix–metabolite co-crystallization. This optimized method showed excellent reproducibility and spatial resolution compared to the automatic spray-coating method. Furthermore, the recrystallization step after sublimation remarkably improved the detectability of metabolites, including amino acids, nucleotide derivatives, and lipids, compared with the conventional sublimation method. To date, there have been no other reports of 9-AA-based sublimation combined with recrystallization. The present method provides an easy, sensitive, and reproducible matrix deposition method for MALDI-MSI of endogenous metabolites.

Keywords: MALDI-mass spectrometry imaging, Matrix sublimation system, Sample preparation method, Visualization of metabolite distribution

Correspondence to: Daisuke Miura; *e-mail:* daisuke.miura@aist.go.jp

Introduction

Elucidating the physiological functions of endogenous metabolites can provide important insights into disease processes and pathophysiology [1]. Spatiotemporal information for metabolites within tissues is indispensable for understanding metabolic pathways and biological systems. Matrix-

assisted laser desorption/ionization (MALDI)-mass spectrometry imaging (MSI) enables visualization of the distributions of a wide range of molecules present at a tissue surface without labeling. This technique is widely used for in situ imaging of analytes, including proteins, peptides, lipids, drugs, and metabolites [2, 3].

For MALDI-MSI, a matrix application step is used to co-crystallize the matrix with analytes over the sample surface [4]. In MALDI-MSI experiments, the choice of matrix and application method strongly influences the variety of ionized molecular species, the spatial resolution of the MSI, and experimental reproducibility [4, 5]. Potential limitations to spatial resolution can be introduced by the matrix crystal size [6]. Generally, matrix application is performed by spray-coating or sublimation method. These have some advantages and disadvantages in terms of detection sensitivity and reproducibility [7]. Manual spray-coating method using an airbrush is relatively fast and convenient, but controlling the velocity of the spray is very difficult and this results in inhomogeneous matrix deposition, variation in the thickness of the matrix layer, and reduced reproducibility. These issues are extremely dependent on operator skill. Automatic sprayer platforms have been developed, but some drawbacks against direct manual spraying have been reported. For example, insufficient matrix crystal core formation can result in insufficient crystal growth and poor reproducibility [8–10]. Unlike spraying, sublimation is a solvent-free matrix deposition method. The matrix powder in a sample preparation chamber under vacuum is heated to the boiling point of the matrix, and the sample positioned above the matrix is coated with a thin layer of very small matrix crystals. This strategy provides fast and uniform matrix deposition [11–13]. Although sublimation can produce an image with high spatial resolution, this method shows low sensitivity because of the absence of solvent in the matrix deposition process, which results in poor extraction efficiencies for some metabolites. In contrast, recrystallization and rewetting of the sample after sublimation procedures have been suggested in order to increase the extraction efficiency using the matrices such as 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), and sinapinic acid (SA) [8, 9, 11, 14, 15]. Although 9-aminoacridine (9-AA) is a suitable representative matrix for in situ metabolomic analysis, little is known about its recrystallization.

In this study, we attempted to optimize the 9-AA deposition method of sublimation coupled with recrystallization reaction with solvent treatment, for visualizing the distributions of endogenous metabolites. The matrix vapor deposition system iMLayer (Shimadzu), which can automatically monitor the temperature and thickness of a matrix deposited during sublimation, was used. Our procedure for matrix sublimation and recrystallization on a tissue sample (mouse brain) consisted of two steps. The first step was formation of the matrix layer on mouse brain sections by sublimation. The next step was co-crystallization of the matrix and analyte on the tissue, with exposure to solvent vapor to promote recrystallization of the matrix using an original and convenient sample preparation chamber. We evaluated our proposed procedure for its ability to provide MSI data with high sensitivity, good spatial resolution, and excellent reproducibility.

Experimental

Materials

9-AA was obtained from Merck Schuchardt (Hohenbrunn, Germany). Indium tin oxide (ITO)-coated glass slides were purchased from Matsunami Glass (Osaka, Japan). Methanol and metabolite standards were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animal Protocol

All the procedures and animal care protocols were approved by the Animal Care and Use Committee of Kyushu University and carried out in accordance with the Guidelines for Animal Experiments, Kyushu University. Five-week-old male C57BL/6N mice were purchased from Kyudo Co. (Tosu, Japan). The mice were housed in a temperature- and humidity-controlled room and fed a commercial diet and water ad libitum. Optimum cutting temperature polymer was used to fix each tissue block. Extirpated tissue samples were immediately frozen and stored at − 80 °C until required for analysis.

Matrix Deposition by Automatic Sprayer

9-AA solution was deposited on the brain sections using SunCollect (SunChrom, Friedrichsdorf, Germany) spraying device. Sixty layers of 5 mg/mL 9-AA in methanol/water (80:20, v/v) solution were sprayed as previously described (16, 17) with slight modifications. Flow rate was set to 20 μL/min, and 600 μL of solution was sprayed.

Matrix Sublimation and Recrystallization Procedures

Brain tissues were sectioned sagittally (10 μm thickness) with a cryostat and thaw-mounted onto ITO-coated glass slides. For matrix deposition, 9-AA (600 mg) was sublimated at 220 °C under vacuum (5×10^{-2} Pa). An iMLayer (Shimadzu, Kyoto, Japan), a matrix deposition device that can control the temperature and calculate the thickness of a deposited matrix during sublimation, was used to monitor the thickness of the matrix layer and ensure it was 0.5 μm. After sublimation, methanol was vaporized at some temperatures to promote extraction of metabolites from the tissue and recrystallization. A convenient recrystallization chamber was constructed using a 300-mL beaker, heating block, and glove box (Figure 1). We regulated both the humidity and temperature in the glove box (28 °C, 30% humidity). The 9-AA-sublimated slide was attached to a piece of nonwoven fabric and placed on the beaker. The beaker was then placed on a preheated heating block for 90 s at various temperatures. A small piece of filter paper (2 cm × 6 cm) was placed in the bottom of the beaker, and 300 μL of methanol was
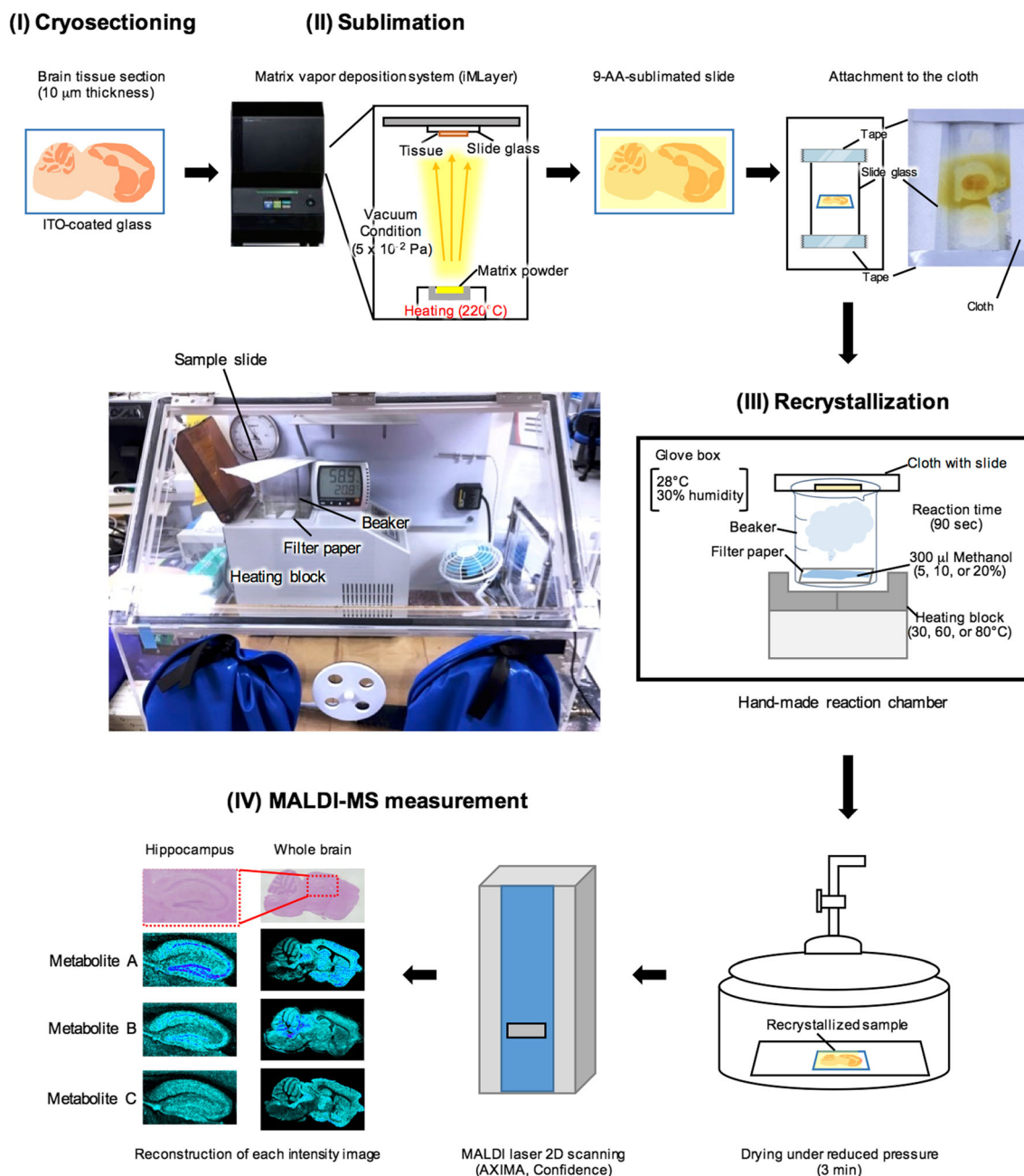
Figure 1. Workflow of sublimation coupled with recrystallization method in MSI experiment. **(I)** Brain tissues were sectioned sagittally (10 μm thickness) with a cryostat. **(II)** 9-AA was sublimated at 220 °C and the thickness of matrix was monitored at 0.5 μm using iMLayer. After sublimation, a 9-AA-sublimated slide was attached to the cloth and placed on the beaker in the reaction chamber. **(III)** Methanol pipetted onto the filter paper in the bottom of the beaker was vaporized on a preheated heating block. Both humidity and atmospheric temperature were checked by the thermo-hygrometer in the chamber during the recrystallization reaction. Sample was dried in the desiccator after the recrystallization. **(IV)** MSI data was acquired in negative ionization mode, and the signals between *m/z* 100 and 1000 were collected

pipetted onto the paper to create vapor for the recrystallization process. We investigated the effects of both the methanol concentration and temperature of the heating block on the recrystallization reaction. Serial sections of brain tissue were subjected to this process with either a 5%, 10%, or 20% (*v/v*) methanol solution at 60 °C or a 5% methanol solution at 30 °C, 60 °C, or 80 °C. The resulting samples were dried under

vacuum for 3 min before performing the MSI experiment. The drying time was determined according to the previously reported methods [16, 17]. Prior to optimization of the detailed recrystallization condition, we examined which is a better solvent, methanol or acetone, because they have been used for solvent-based matrix application methods of 9-AA [2, 18]. Methanol treatment provided clearer distribution images

of the endogenous metabolites compared to acetone treatment (Figure S1); thus, we used methanol as the solvent in this study.

MSI Analysis

In this study, we used three MS instruments. A single reflectron-type MALDI-time-of-flight (TOF)-MS (AXIMA Confidence, Shimadzu) and TOF/TOF-type (AXIMA Performance, Shimadzu) instruments were used for MSI, and a quadrupole ion trap (QIT)-type TOF-MS instrument (AXIMA QIT, Shimadzu) was used for identification of metabolites by MS/MS analysis as previously described [2, 19]. MS/MS spectra obtained from tissue surface were compared to that of each metabolite standards or databases. These instruments were equipped with a 337-nm nitrogen laser. In the MSI experiments, the data were acquired in negative ionization mode with 50 μm iteration (10 laser shots per data point), and the signals between m/z 100 and 1000 were collected. To compare the three different matrix application methods, MSI was performed under the same laser power conditions. Acquired MSI data were normalized with the total ion count using SIMedit software (Shimadzu), and obtained data were processed with the freely available BioMap software (http://www.maldi-msi.org). This software was used for creation of two-dimensional ion-density maps, adjustment of the color scale, and quantification of the ion intensities [20, 21]. MS images of each selected metabolite peak are generated using a 0.05-Da mass window. The signal intensities for the imaging data in the figures are represented as normalized intensities.

Analytical Reproducibility of Imaging Analysis

We validated the analytical reproducibility of the metabolite MSI. After full scan imaging of tissue sections, the MSI data were normalized as described above. After normalization, the averaged spectral data in regions of interest (ROIs) of 4×4 pixels were established in part of the cerebral cortex using BioMap software. Each average intensity from the averaged mass spectrum of each sample was determined within the selected ROIs. We compared the intensities for the three matrix deposition methods (spray-coating, sublimation, and sublimation with recrystallization) ($n = 3$).

Hematoxylin and Eosin Staining

The tissues sections were fixed in 4% formaldehyde and stained with hematoxylin and eosin (H&E) according to standard protocol.

Results and Discussion

Development of Sample Preparation Method of 9--AA Sublimation and Recrystallization

Previously, we developed an in situ metabolomic MSI technique for mouse brain tissue using 9-AA as a matrix and the spray-coating method for matrix deposition [2]. This matrix was used for detecting endogenous metabolites, including nucleotide derivatives and central metabolic pathway metabolites, redox-related metabolites, lipids, and amino acids in negative ionization mode. There were only a few matrix-derived peaks in the mass range of m/z 200–500, and 9-AA was the most suitable matrix for the simultaneous detection of high and low molecular weight compounds that contains phosphate, sulfate, and carboxyl group [2]. Manual spray-coating method is relatively fast and simple, but data quality is predominantly dependent on operator skill. On the other hand, the automatic sprayer system can provide reproducible results because of its automatic operation system. Thus, it is expected to become a part of routine matrix deposition system. Sublimation method is performed automatically and can produce
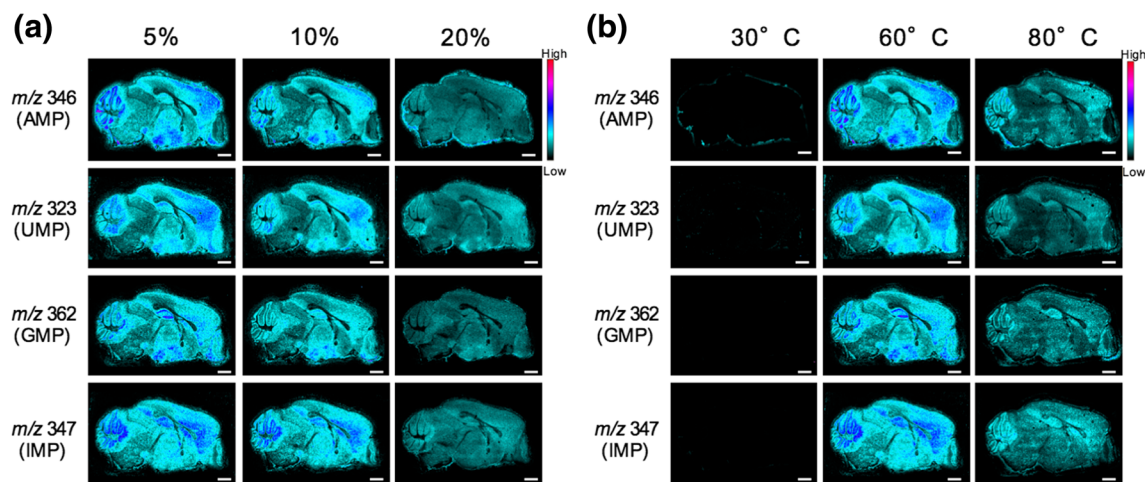

Figure 2. Optimization of sublimation coupled with recrystallization method using 9-AA. Sagittally sectioned mouse brain was used for investigating recrystallization conditions after 9-AA sublimation. Matrix application was performed by spray-coating, sublimation, or sublimation coupled with recrystallization method. Ion images were acquired in negative ionization mode with 50 μm iteration (10 shots/data point). Scale bar = 1.0 mm. (a) The suitable solvent concentration was investigated. After 9-AA sublimation, the recrystallization reaction was performed at 60 °C for 90 s using 5 %, 10 %, or 20 % (v/v) methanol. (b) The suitable temperature was investigated. After 9-AA sublimation, the recrystallization was performed at 30 °C, 60 °C, or 80 °C for 90 s using 5% methanol

a homogeneous matrix layer across the tissue section [7, 13, 22]. This method yields highly reproducible results but has low-detection sensitivity because of the absence of a solvent in the matrix deposition process, which results in insufficient extraction of metabolites from the surface of the sample. A method using sublimation of 9-AA has been reported, but the issue of the detection sensitivity was not resolved [23]. To overcome this, we investigated 9-AA sublimation for metabolite imaging using sagittal sections of mouse brain with an added recrystallization step and optimized the recrystallization conditions focusing on the solvent concentration and temperature. After sublimation, a 9-AA-coated glass slide was exposed to 5–20% methanol vapor at 30–80 °C for 90 s in a reaction chamber constructed in our laboratory (Figure 1). Ion images of four representative 9-AA-sensitive metabolites were observed at all methanol concentrations at 60 °C (Figure 2a). However, although 5% and 10% methanol provided clear images with good contrast, 20% methanol did not. Using 5% methanol, we then investigated the effect of altering the temperature on visualization of the metabolites (Figure 2b). At 30 °C, signals were not observed for the four metabolites. At 60 °C or above, the distributions of these metabolites were visualized, with the best contrast obtained at 60 °C. In consideration of the boiling point of methanol at atmospheric pressure (64.7 °C), our results suggest that lower temperatures (e.g., 30 °C) and higher temperatures (e.g., 80 °C) are not suitable for clear detection of the representative 9-AA-sensitive metabolites (nucleotides). In addition, a higher methanol concentration (e.g., 20%) may inhibit co-crystallization between the matrix and metabolites, because such a solvent condition increases the risk of an inadvertent diffusion of analytes [22]. These observations indicate that strict control of both the temperature and solvent concentration in the reaction chamber (Figure 1) is indispensable for optimization of the recrystallization process. In subsequent experiments, the sublimation and recrystallization method was performed with 5% methanol at 60 °C for 90 s after 9-AA sublimation.

Comparison of the Detection Sensitivity and Analytical Reproducibility Among the Three Matrix Deposition Methods

Next, we investigated the three different matrix application methods for their detection sensitivity, spatial resolution, and reproducibility using mouse brain sections. Generally, the spray-coating method is performed manually or automatically. Manual spray-coating is dependent on user's skill, whereas an automatic sprayer system is user-independent mode. In this study, we compared an automatic spray-coating method with sublimation methods. Previously, the distributions of central pathway metabolites, nucleotide derivatives, and lipids were visualized using an automatic matrix sprayer in mouse brain or kidney tissues [24, 25]. Based on this methodology, we tried to visualize the distribution of nucleotide derivatives and lipids. MSI experiments were performed under the same laser power with a raster pitch of 50 μm. The averaged mass spectra for the three methods showed different patterns (Figure 3). In the mass
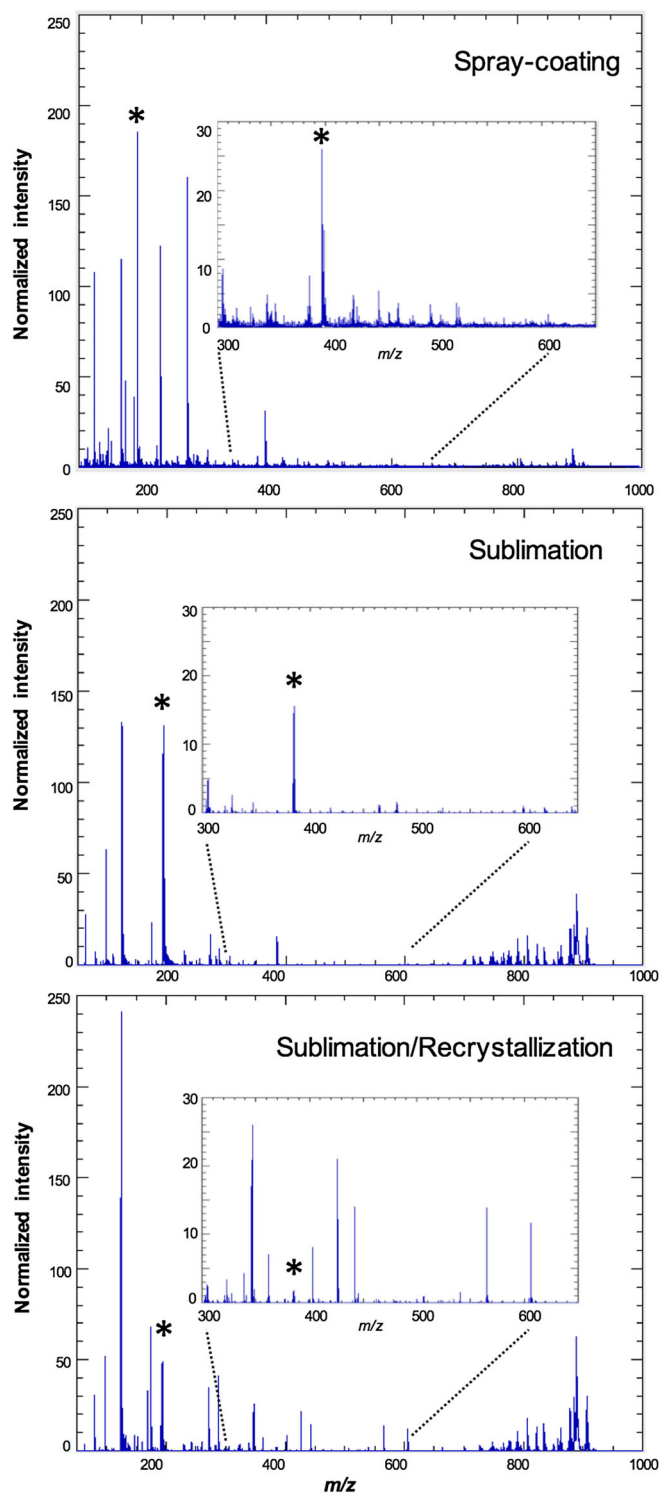

Figure 3. Comparison of averaged mass spectra acquired from MSI analysis using different matrix deposition methods. Whole averaged mass spectra are acquired from the mouse brain section coated with 9-AA by either spray-coating, sublimation, or sublimation coupled with recrystallization method (m/z 100–1000). Asterisk mark indicates major matrix-derived peaks (matrix: 9-AA). Enlarged view of the averaged mass spectra is shown in the range from m/z 300 to 650

range *m/z* 100–1000, we detected 20 metabolite-derived peaks with the automatic spray-coating method, 19 peaks with the sublimation method, and 40 peaks with the sublimation and recrystallization method. In the sublimation and recrystallization method, the number of peaks with high signal intensities was larger than for the two other methods. Representative ion images of cerebral metabolites are shown in Figure 4. Several nucleotides and derivatives, including adenosine monophosphate (AMP) (*m/z* 346.06 [M–H]$^-$), adenosine diphosphate (ADP) (*m/z* 426.02 [M–H]$^-$), uridine monophosphate (UMP) (*m/z* 323.03 [M–H]$^-$), uridine diphosphate (UDP) (*m/z* 402.99 [M–H]$^-$), UDP-*N*-acetylglucosamine (UDP-*N*-AG) (*m/z* 606.07 [M–H]$^-$), and guanosine monophosphate (GMP) (*m/z* 362.05 [M–H]$^-$), had low signal intensities, and clear distribution patterns were not observed in the spray-coating method. Although *N*-acetylaspartate (NAA) (*m/z* 174.04, [M–H]$^-$) was detected, spatial resolution was low. It has been reported that the detection sensitivity of nucleotide derivatives was relatively lower than that of NAA [24–26]. Therefore, further optimization of the present methodology may be required for improving the detection sensitivity. In the sublimation method, the nucleotides and derivatives were not detected, even if MSI experiments were performed under the higher laser power conditions. Unlike the spray-coating method, the sublimation method did detect lipids such as phosphatidylinositol (PI) (18:0–20:4) (*m/z* 885.55 [M–H]$^-$) and sulfatide (d18:1–C24h:1) (*m/z* 904.76 [M–H]$^-$). These results indicate that the automatic spray-coating method is not suitable for detection of highly polar metabolites, such as nucleotide derivatives. In comparison with these two methods, the sublimation and recrystallization method showed the best detection and provided clear images with high contrast for all 12 metabolites. These findings suggest that the optimized recrystallization process is remarkably effective for overcoming the drawbacks of the conventional sublimation method to achieve excellent detection and clear visualization of endogenous metabolites. To examine the detection sensitivity and analytical reproducibility of the MSI data for the three methods, we used normalized intensities calculated from averaged mass spectra within selected ROIs of the cerebral cortex (Figure 5a). In this experiment, we compared triplicate data from serial tissue sections for the three methods (Figure 5b). In the spray-coating data, the signal intensities of the nucleotide derivatives and NAA were clearly lower than those from the sublimation and recrystallization methods. With the sublimation method, the intensity of lipids such as sulfatide was higher than those obtained with the spray-coating method. With the sublimation and recrystallization method, both nucleotide derivatives and lipids showed higher intensities than with the sublimation method. Additionally, the sublimation and recrystallization method provided the high reproducibility for detection of each metabolite with sustained higher intensities than the other two methods. The similar tendency was also observed in the other cerebral regions, such as hypothalamus, thalamus, mesencephalon, medulla oblongata, and olfactory lobe (Figures S2–6). The spray-coating method showed the lowest sensitivity and reproducibility among the three methods in the cerebral cortex region. In the other cerebral regions, the spray-coating method showed high reproducibility, but the similar tendency of low-detection sensitivity was also observed in those regions (Figures S2–6). It has been reported that automated spray-coating system have some drawbacks with respect to detection sensitivity and spatial resolution [9]. On the other hand, highly sensitive visualization of endogenous metabolites has been reported using the manual spray-coating method [2, 8, 19]. This method is relatively fast and convenient, but it is considered that data quality is dependent on operator skill. In this study, we attempted to perform MSI experiments using
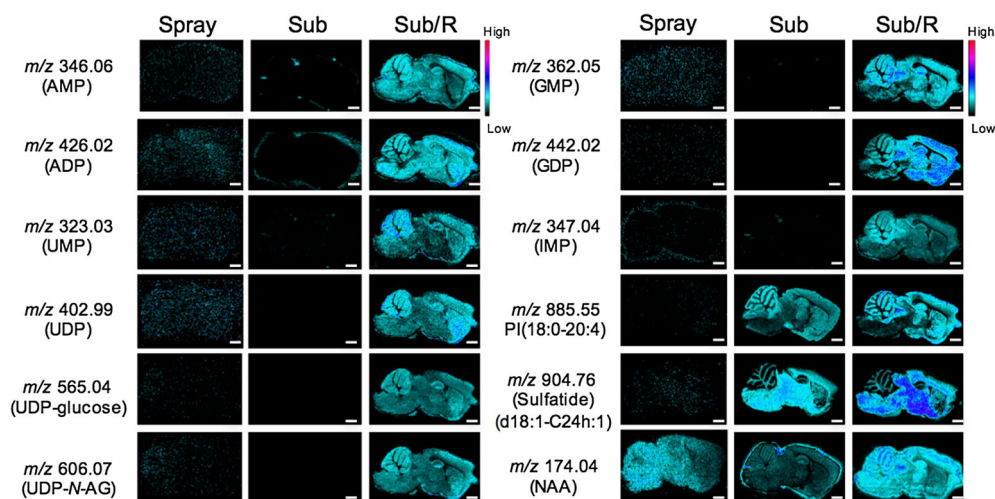

Figure 4. MSI of metabolites in mouse brain tissue sections using three matrix deposition methods. C57BL/6N mouse brain was sectioned sagittally (10 μm thickness) with a cryostat and then thaw-mounted onto an ITO-coated glass slide. The matrix as applied by spray-coating, sublimation, or sublimation and recrystallization. Ion images were acquired in negative ionization mode with 50 μm iteration (10 shots per data point). Scale bar = 1.0 mm. Abbreviations: Spray, spray-coating; Sub, sublimation; and Sub/R, sublimation and recrystallization
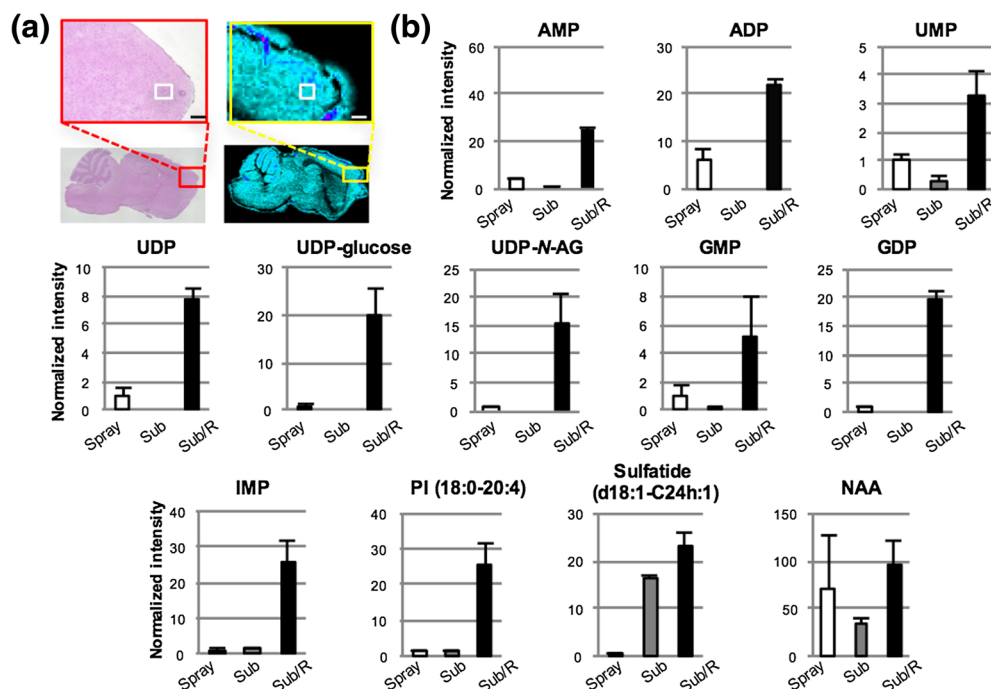
Figure 5. Analytical reproducibility of MSI of metabolites in the mouse cerebral cortex region. (**a**) The left panel shows H&E staining of the mouse brain section. The upper image is an enlargement of the cerebral cortex region. Scale bar = 200 μm. The right panel is a MSI image of a mouse brain section. Scale bar = 200 μm. (**b**) A 9-AA-coated brain tissue section was used to compare the three matrix deposition methods for ion intensity and validation of analytical reproducibility. After full scan imaging of the tissue section, the MSI data were normalized as described in the "Experimental" section. After normalization, the averaged spectral data in a 4 × 4 pixel ROI (white square indicated in panel **a**) in the cerebral cortex were acquired, and then the normalized intensity of each metabolite was collected. Data are shown as means ± standard deviations for triplicate measurements using serial tissue sections (n = 3). Abbreviations: Spray, spray-coating; Sub, sublimation; and Sub/R, sublimation and recrystallization

manual spray-coating method (Figures S7 and S8). With the manual spray-coating method, some nucleotides showed higher intensities than with the automated method (Figures 4, 6, S7, and S8). In comparison of this manual method with the sublimation and recrystallization method, however, higher intensities of both nucleotide derivatives and lipids were
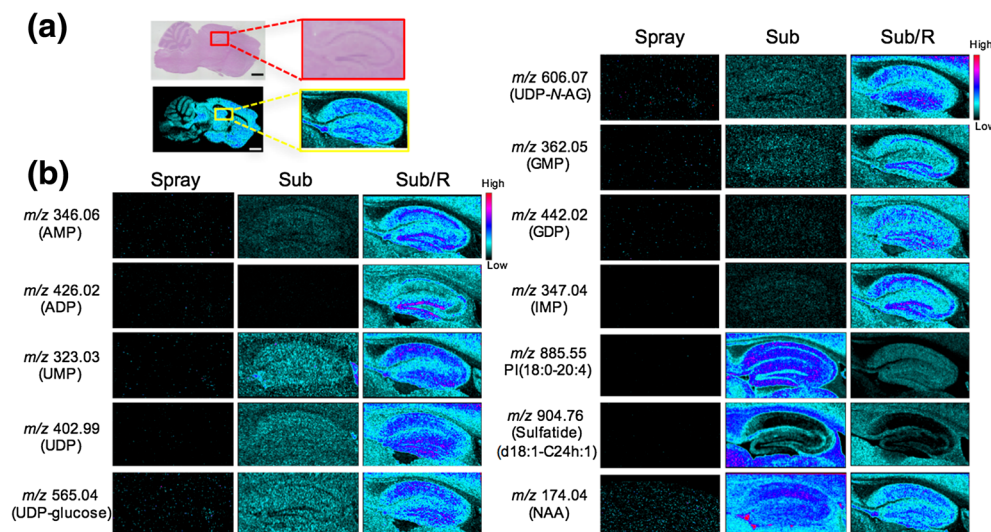

Figure 6. High spatial resolution images of endogenous metabolites in the hippocampus. (**a**) The upper left panel shows H&E staining of the mouse brain section. Scale bar = 1 mm. The lower left panel is a MSI image of the mouse brain section. The upper and lower right images are enlargement of the hippocampal region in H&E staining and MSI images. Scale bar = 1 mm. (**b**) Comparison of MSI data in the mouse hippocampus region obtained with spray-coating, sublimation, or sublimation and recrystallization for matrix deposition. Ion images were acquired in negative ionization mode with 20 μm iteration (10 shots per data point). Abbreviations: Spray, spray-coating; Sub, sublimation; Sub/R, sublimation and recrystallization

observed in the sublimation and recrystallization method (Figures S7, and S8). Taken together, these results suggest that the sublimation and recrystallization method is an effective system for acquiring metabolite MSI data with high sensitivity and reproducibility.

The Effect of the Optimized Recrystallization on Spatial Resolution

Spatial resolution is a major challenge in MALDI-MSI [27]. This is limited by the choice of matrix and the deposition method, which determines the matrix crystal size and homogeneity [28]. Homogeneous matrix deposition keeps local variations in desorption and ionization processes to a minimum [29]. Herein, we evaluated the effects of the three matrix application methods on the spatial resolution. MSI analysis was performed with a raster pitch of 20 μm in the hippocampus region of the sagittal brain sections (Figure 6a). Although peaks of nucleotide derivatives (AMP, ADP, UMP, UDP, GMP, GDP, and IMP) were detected, distribution images of those compounds were not acquired with the spray-coating method (Figure 6b). By contrast, clear images with good contrast were observed for the lipids with the sublimation method, and the signal intensities were higher than those obtained with the spray-coating method. The sublimation and recrystallization method provided clearer distribution images of the metabolites, except for the lipids, and much higher intensities than the other methods (Figure 6b). The optimized method improved both the detection sensitivity and signal-to-noise ratio compared with the two methods. Additional recrystallization steps after sublimation reportedly improve detection sensitivity [14]. Recent studies have provided interesting sample preparation protocols for animal and plant tissues [8, 9], but these approaches are not effective for all metabolites and matrices [14]. Although 9-AA is a suitable representative matrix for in situ metabolomic analysis, little is known about its recrystallization. The preparation of a homogenous matrix layer and highly efficient analyte extraction from the tissue surface are required to obtain reliable MSI data with high sensitivity and good spatial resolution. The sublimation with recrystallization approach developed herein produces a homogenous matrix layer automatically and achieves favorable analyte extraction to yield reproducible MSI data with high sensitivity and spatial resolution.

Conclusions

Spatial information for endogenous metabolites within tissues is indispensable for elucidating the mechanisms of physiological regulation and the functions of biomolecules. MALDI-MSI can simultaneously visualize multiple molecules, and the choice of matrix and its deposition method is key to good detection sensitivity and data quality. However, to date, an optimum matrix deposition method, especially the one using sublimation with 9-AA, has not been developed. We established a 9-AA sublimation method with coupling to a recrystallization step, which was optimized for the solvent

concentration and reaction temperature. This methodology is very simple but provides excellent detection sensitivity for endogenous metabolites and yields highly reproducible data. The present sample preparation method and its optimization strategy could be applied to tissue sections from different types of organisms and other matrices with suitable modification. This method could be used to obtain precise information about the distributions of endogenous molecules, which could be applied to biological discovery and biomarker development.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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