

Technical Report

Improving the Signal Intensity of Cryosections Using a Conductive Adhesive Film in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging

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The matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) technique was used to obtain the molecular images of cryosections without labeling. Although MALDI-MSI has been widely used to detect small molecules from biological tissues, issues remain due to the technical process of cryosectioning and limited mass spectrometry parameters. The use of a conductive adhesive film is a unique method to obtain high-quality sections from cutting tissue, such as bone, muscle, adipose tissue, and whole body of mice or fish, and we have reported the utilization of the film for MALDI-MSI in previous. However, some signal of the small molecules using the conductive adhesive films was still lower than on the indium tin oxide (ITO) glass slide. Here, the sample preparation and analytical conditions for MALDI-MSI using an advanced conductive adhesive film were optimized to obtain strong signals from whole mice heads. The effects of tissue thickness and laser ionization power on signal intensity were verified using MALDI-MSI. The phospholipid signal intensity was measured for samples with three tissue thicknesses (5, 10, and 20 μm); compared to the signals from the samples on the ITO glass slides, the signals with conductive adhesive films exhibited significantly higher intensities when a laser with a higher range of power was used to ionize the small molecules. Thus, the technique using the advanced conductive adhesive film showed an improvement in MALDI-MSI analysis.



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INTRODUCTION

The matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) technique is utilized to demonstrate the molecular localization on cryosections obtained from unlabeled biological specimens and plants.¹⁻³⁾

For cryosectioning, the preparation of a 2–6- μm tissue thickness sample on a conductive material, in this case, an indium tin oxide (ITO) glass slide, is recommended for MALDI-MSI analysis.⁴⁾ However, high-quality tissue cryosections are difficult to cut from bones, adipose tissues, plants, and whole-body mice with maintenance of their shape and form while

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placing them on glass slides.^{5,6)} Adhesive conductive films have been developed to resolve these problems.⁷⁻⁹⁾ Although we have reported the evaluation of our conductive adhesive film (Cryofilm type MS1), the signal of the small molecules on the tissue section with the films by MALDI-MSI was not observed or was lower than the original intensity obtained from the cryosection on the ITO glass slide.¹⁰⁾ Therefore, the conditions of MALDI-MSI need to be optimized to observe localization by improving the signal intensities, especially for small molecules in the tissue section. Here, we optimized the tissue thickness of cryosectioning, laser power for ionization, and MS parameters to utilize the advanced conductive adhesive film (Cryofilm type MS2) by comparing the original signal intensity detected on the ITO glass slide for the small molecule analysis by MALDI-MSI.

EXPERIMENTAL

Chemical and reagents

Methanol was purchased from Kanto Chemical (Tokyo, Japan). α -Cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma-Aldrich (Tokyo, Japan) and used as a matrix. All other chemical standards were obtained from common commercial sources.

Animal

Mice (ICR strain, 9 days old, male) were purchased from CLEA Japan (Tokyo, Japan). The animal protocols were approved by the Institutional Animal Care and Use Committee of Tsurumi University.

Preparing cryosections

The cryosectioning protocol followed a previous publication.¹⁰⁾ All the devices for preparing cryosections were included in a kit purchased from SECTION-LAB Co. Ltd. (Kanagawa, Japan). Frozen samples were sliced with a disposable tungsten carbide blade, and Cryofilm type 3C (16UF) was used as a nonconductive adhesive film for histological observation. For the MALDI-MSI analysis, a conductive adhesive film (Cryofilm type MS2) was purchased from SECTION-LAB Co. Ltd. and used. The copper foil was purchased from SEKISUI Co. Ltd. (Tokyo, Japan).

Whole heads were collected from mice that were sacrificed under anesthesia using isoflurane and immediately frozen in hexane-dry ice. Then, the samples were embedded in SCEM (SECTION-LAB Co. Ltd.), frozen immediately in hexane-dry ice, and cut until the objective surface was observed by setting on the cryostat (CM3050; Leica, Tokyo, Japan).

For hematoxylin-eosin (H&E) staining, a nonconductive adhesive film (Cryofilm type 3C) was placed on the objective surface, and the tissue sections (4- μ m thickness) were collected with the film and fixed with paraformaldehyde for 30 sec. After H&E staining, the section was flipped over and placed on a glass slide for microscopic observation.

To perform MALDI-MSI analysis, a conductive adhesive film (Cryofilm type MS2) was pasted on the cut surface, and tissue sections (5- μ m, 10- μ m, or 20- μ m thickness) were collected with the film. To maintain the flatness of the section, the section was fixed on the glass slide with photopolymerization resin (SCMM-IM1; SECTION-LAB Co. Ltd.). Firstly, the section was placed on a glass slide on which a drop of resin facing upward the surface. The sample was then cured by irradiating

with ultraviolet (UV) light from the side of the glass slide to attach the conductive adhesive film to the glass slide.

MALDI-MSI analysis

CHCA was deposited (0.7- μ m thickness, iMLayer; Shimadzu, Kyoto, Japan) on the surface of the tissue section, and the samples were immediately analyzed with MALDI-MSI (iMScope TRIO, Shimadzu). The data were collected through the microscopic system and digitally processed using imaging software (Imaging MS Solution; Shimadzu).

RESULTS AND DISCUSSION

Quality of cryosection

We initially observed the quality of the tissue sections using the films. Figure 1 shows optical images of the surface of the mouse head before sectioning (Fig. 1A, left panel), the section stained with H&E (Fig. 1B, middle panel), and the section prepared with the conductive adhesive film (Fig. 1C, right panel). The H&E-stained section showed that tissues such as the brain, tongue, secretory gland, teeth, and hair were perfectly preserved. The section clearly shows each cell in these tissues. The section with conductive adhesive film shows the same high quality as the H&E-stained section.

For MALDI-MSI analysis, the most important step is to produce tissue sections while completely maintaining the tissue components and accurate morphology. Furthermore, the section must touch the conductive material surface directly to increase the signal intensity. To satisfy these requirements, some methods using conductive tape have been established previously.^{11,12)} Although various types of conductive tapes are commercially available for industrial purposes, the adhesive strength is limited by the low temperature of cryosectioning. Moreover, it is difficult to prepare the frozen sections while retaining their morphology using previous adhesive tapes, especially given the need to maintain temperatures lower than -30°C to prepare frozen sections. The present conductive adhesive film maintains the adhesive strength even at a lower temperature (-40°C), and complete frozen sections could be prepared from a sample containing all tissues (organs). Indeed, high-quality tissue sections are essential when attempting to observe the localization of small areas. For instance, the conductive adhesive film was utilized in the preparation of frozen section of plant samples as well as animal samples.¹²⁾ Therefore, the conductive adhesive film was utilized for molecular imaging by MALDI-MSI analysis.

Effect of conductive materials on signal intensity

We next examined the conductivity of the adhesive film by comparing the representative signal intensities derived from the deposited matrix reagent (CHCA) without tissue sectioning to determine the effect on the signal intensity from the nonconductive adhesive film, conductive adhesive film, copper foil, and an ITO glass slide (Fig. 2A). The MALDI parameters are shown in Table 1 (Exp. 1). The signal intensities at mass-to-charge ratio (m/z) values of 190.1, 335.1, and 379.1 derived from CHCA on the copper foil, conductive adhesive film and an ITO glass slide were higher than those on the nonconductive adhesive film (Fig. 2B). However, the conductive adhesive film maintained enough conductivity to exhibit higher signal intensities by MALDI-MSI analysis.

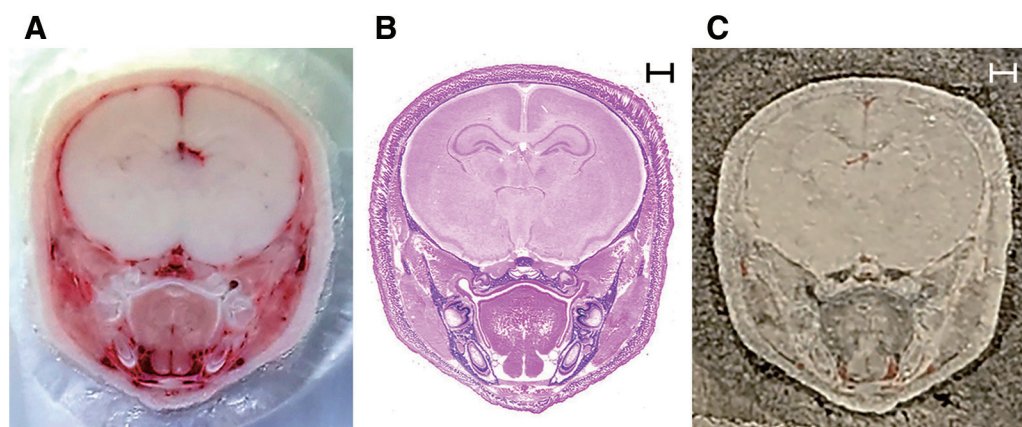


Fig. 1. Optical images. (A) Cut surface of the head of a 9-day-old mouse. (B) H&E-stained section (4 μm thickness). The section was obtained with a nonconductive adhesive film (Cryofilm type 3C). Scale bar=1 mm. (C) Section (10 μm thickness) made with a conductive adhesive film (Cryofilm type MS2). Scale bar=1 mm. H&E, hematoxylin–eosin.

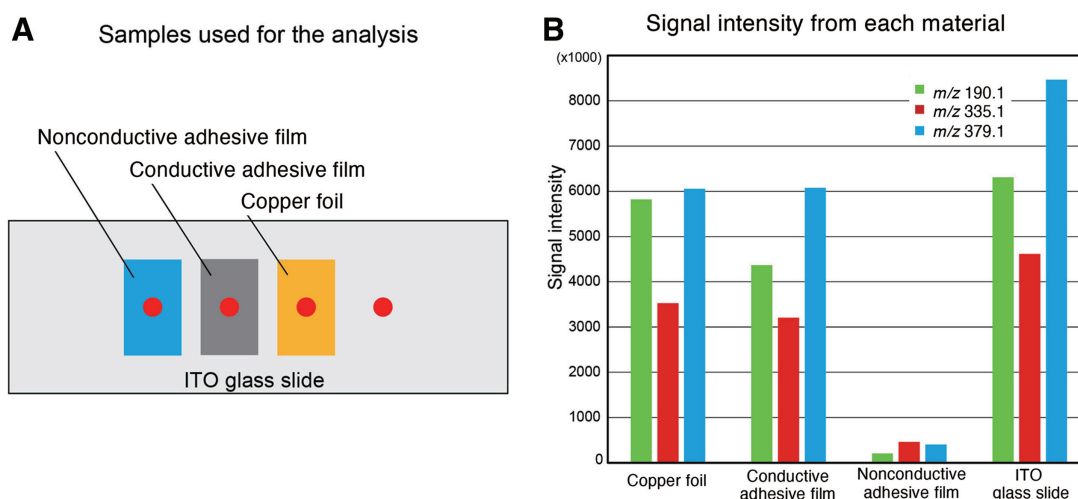


Fig. 2. Effect of conductive materials on signal intensity. (A) Placement of each sample. The red areas on the nonconductive adhesive film (Cryofilm type 3C), the conductive adhesive film (Cryofilm type MS2), copper foil and the ITO glass slide show the analyzed areas. (B) Signal intensities at *m/z* values of 190.1, 335.1, and 379.1 from CHCA by MALDI-MSI. CHCA, α-cyano-4-hydroxycinnamic acid; ITO, indium tin oxide; MALDI-MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging.

Table 1. The parameters of the experiments by MALDI-MSI.

Exp.	1	2	3
Pitch (μm)	40, 40	100, 100	100, 100
Ionization	Positive	Positive	Positive
<i>m/z</i> range	100–600	600–900	600–900
Count/pixel	1	1	1
Sample voltage (kV)	3.5	3.5	3.5
Detector voltage (kV)	1.7	2.1	2.1
Number of irradiation (shots)	100	100	50
Repetition frequency (Hz)	1000	1000	1000
Irradiation diameter (μm)	25	25	25
Lazar power* (%)	45	54	48, 51, 54, 57, 60

*Percentage for maximum output power of the laser system. MALDI-MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging.

In general, as mentioned above, it is essential to prepare the tissue section directly on the conductive material to increase the signal intensity for MALDI-MSI analysis. The relationship between the value of material conductivity and

the signal intensity may be needed to clarify the strength of its conductivity. In this study, we therefore compared the signal intensity obtained in MALDI-MSI using copper foil, ITO glass slides, conductive adhesive film, and non-conductive adhesive film. However, it is difficult to measure the exact conductivity value of the conductive adhesive film due to the technical issue, the signal intensity was clearly higher to that of the ITO glass slide under the same analysis by MALDI-MS.

In general, the metals and glues used for such tapes sometimes affected the signal intensity of ionized molecules by MALDI-MSI. For instance, a double-sided tape with gold deposition for conductivity has also been reported.¹³⁾ Although the deposition of gold on the cryosection could improve the signal intensity, it should also be noted that optimizing the thickness of the gold layer is technically difficult, as the surface of gold deposition may have some clusters with matrix on the cryosection, reducing the reliability of their distribution.^{14,15)} Previously, the nonflat surface of the section was found to accept the signal intensity and visualized image obtained by MALDI-MSI.¹⁶⁾ Therefore, it is important to

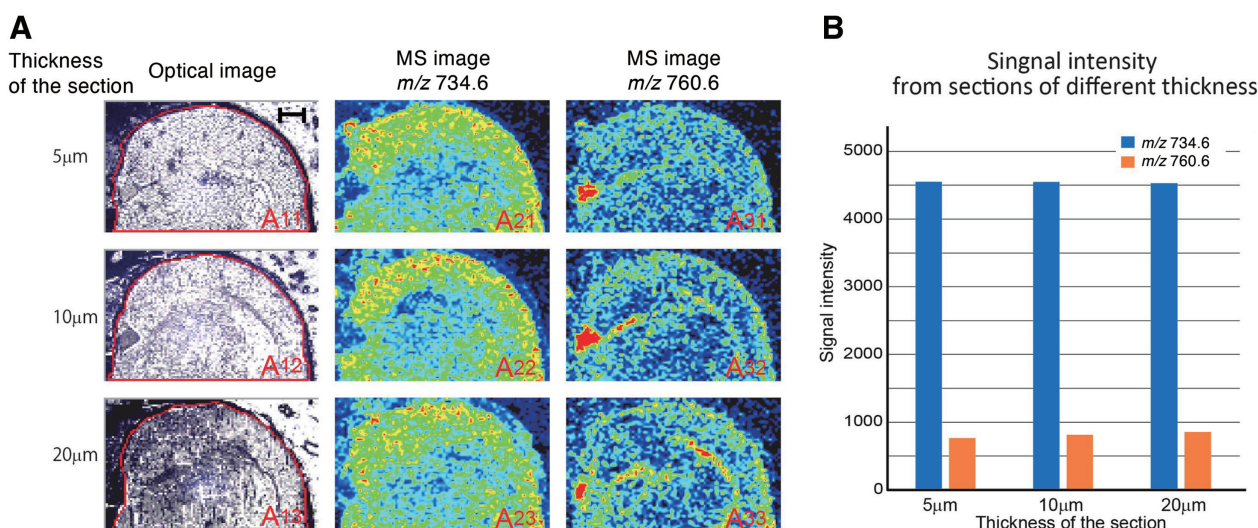


Fig. 3. Effect of tissue thickness on signal intensity by MALDI-MSI. (A) Optical images (A11, A12, A13) of the section used for MALDI-MSI, the visualized images of m/z values of 734.6 (A21, A22, A23) and 760.6 (A31, A32, A33) detected on a brain section. The sections were made with conductive adhesive film (Cryofilm type MS2), and the section thickness was 5 μm (A11, A21, A31), 10 μm (A12, A22, A32), and 20 μm (A13, A23, A33). Scale bar=1 mm. (B) Histograms of the average signal intensities at m/z values of 734.6 and 760.6 from each area surrounded by the red line (A11, A12, A13). MALDI-MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging; MS: mass spectrometry.

show the utility of the conductive adhesive film, and we will measure the tissue surface after cryosectioning in a future study. In addition, we could not observe the decrease in signal intensity affected by the ion suppression derived from the film materials. We surmise that the conductive adhesive film may be one of the best films for MALDI-MSI analysis.

Effect of tissue thickness on signal intensity

We next evaluated how the tissue thickness of cryosections affected the signal intensity by MALDI-MSI. The optical images and representative molecular images at m/z values of 734.6 and 760.6 were derived from phospholipids detected on the brain section by the MALDI-MSI analysis; these images are shown as left, middle, and right panels, respectively (Fig. 3A). The MALDI-MSI parameters are listed in Table 1 (Exp. 2). Notably, the images showing the relative intensity of the molecules for the 5- μm , 10- μm , and 20- μm thick sections made with the conductive adhesive films were similar. We then calculated the average signal intensity of the molecules in the area surrounded by the red line in each visualized image (Fig. 3B), and the histograms indicated that the signal intensities of the small molecules were not affected by the thickness of the cryosection with the use of the conductive adhesive film MALDI-MSI analysis.

In general, the signal intensities obtained by MALDI-MSI analysis for the samples using ITO glass slides were affected by the tissue thickness due to the loss of conductivity with a thick tissue section.⁴⁾ The strength of the conductivity may be affected by the tightness of adhesion with the tissue surface, as reported previously.¹²⁾ The conductive adhesive film has strong adhesion at lower temperatures and can be applied to sections of various thicknesses for MALDI-MSI analysis.

Effect of laser power on signal intensity

Finally, we examined the effect of laser power on the visualized images and signal intensities by MALDI-MSI with sections on the conductive adhesive film and ITO glass slide. Figure 4 shows the optical images of the mouse head obtained by H&E staining with a 4- μm thickness with

nonconductive adhesive films (Fig. 4A) and the serial sections with a 10- μm thickness for their representative molecular images with conductive adhesive films (Fig. 4B). We also prepared serial sections by a general technique of using ITO glass slides to compare the effect of laser power on the signal intensities (Fig. 4C). The MALDI parameters are listed in Table 1 (Exp. 3).

Optical images of the serial sections from mouse heads prepared on the ITO glass slides were of lower quality and exhibited different shapes due to distorted and missing parts of the head because of technical issues. In contrast, the sections prepared on the conductive adhesive film maintained the form of the head, and the images of molecules visualized by MALDI-MSI were overlaid with the image obtained by H&E staining (Fig. 4D).

The average intensities of the m/z values of 734.6 and 760.6 detected by MALDI-MSI using five different laser powers (48, 51, 54, 57, and 60) were calculated in the tissue area of the red line with and without conductive adhesive film on the ITO glass slides and plotted on the graphs in Fig. 4E. As a result, the highest intensity and the clearest image of the molecule was obtained at laser power 54.

Particularly, the signals from the cerebrum and parotid gland on the visualized images, which are indicated by the arrows in Fig. 4D (1) (left panel) and 4D (3) (right panel), increased with the laser power from 48 to 54 with the conductive adhesive film but decreased with the laser power from 57 and greater; additionally, clearer images were observed for the samples with the films on the ITO glass slides by MALDI-MSI than those without films on the ITO glass slides. In addition, the average intensity of the m/z value of 760.6 was optimal at laser power 54, and the localized signal could be observed from the ophthalmic glands using the conductive adhesive film. Although we reported the utilization of the previous conductive adhesive film (Cryofilm type MS1) for MALDI-MSI, the signal intensity of the small molecules (including m/z value of 734.6 and 760.6) was lower than ITO glass slides due to the less strength of adhesion at -40°C .⁹⁾ The adhesive strength and the lower temperature

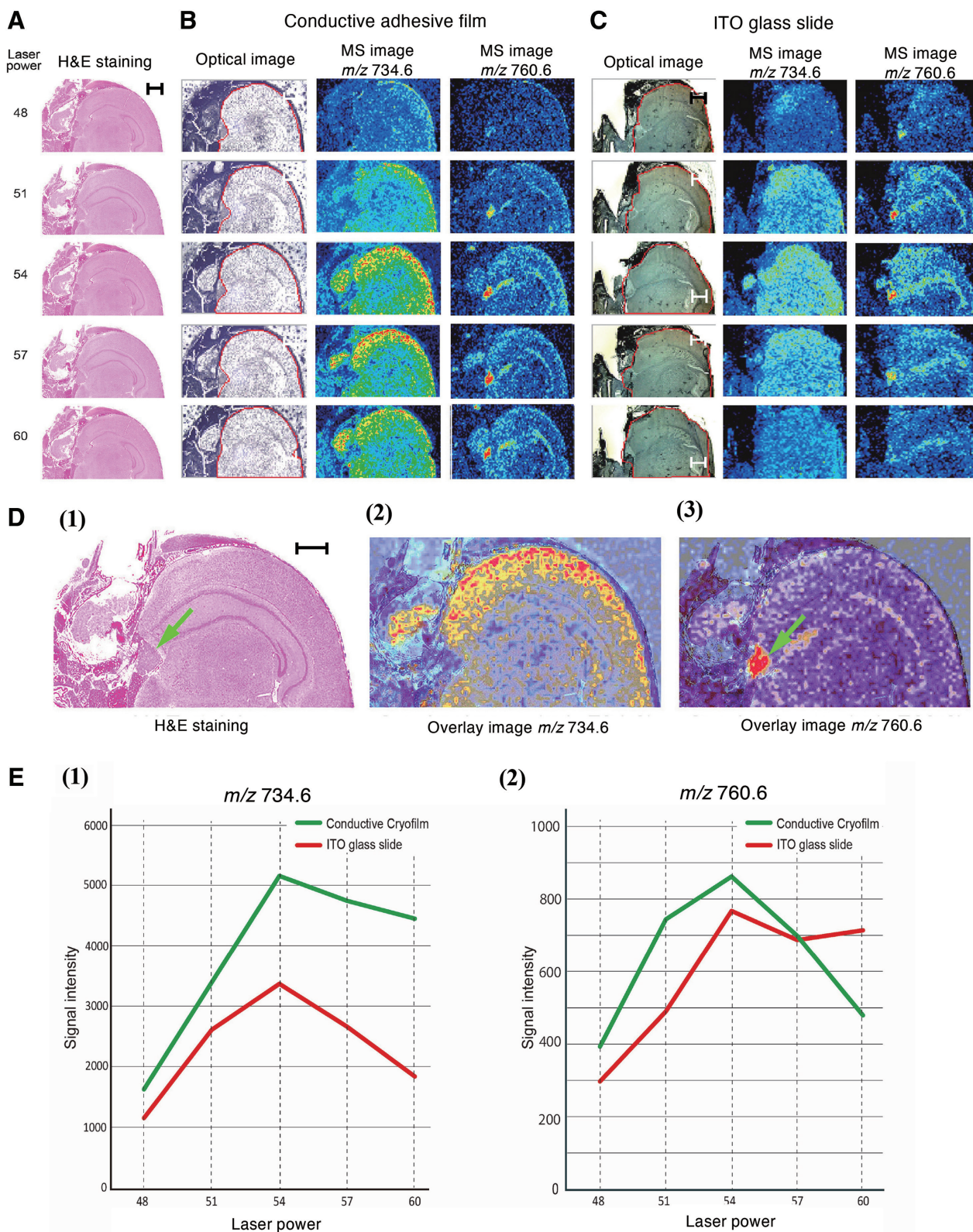


Fig. 4. Effect of laser power for the signal intensities by MALDI-MSI. (A) Sections of the mouse head were made with nonconductive adhesive films (Cryofilm type 3C) and stained with H&E. Scale bar=1 mm. (B) Sections (10 μ m thickness) were serially made with a conductive adhesive film (Cryofilm type MS2). The left panel of (B) shows optical images of sections taken with MALDI, and the middle and the right panels of (B) are the visualized images of the m/z values of 734.6 and 760.6, respectively. (C) Sections (10 μ m thickness) serially made on an ITO glass slide by a conventional technique. The left panel of (C) shows optical images of sections taken with MALDI, and the middle and the right panels of (C) show visualized images of m/z values of 734.6 and 760.6, respectively. (D) Expansion of the images. H&E-stained (D[1], left panel) and overlaid with each visualized image of the m/z values of 734.6 (D[2], middle panel) and 760.6 (D[3], right panel). Arrows in D(1) and D(3) indicate the same tissue area. (E) Plots of the average intensities surrounded by red lines in each visualized image of m/z values of 734.6 (E[1], left panel) and 760.6 (E[2], right panel) detected by MALDI-MSI at five different laser powers of 48, 51, 54, 57, and 60 with a conductive adhesive film and ITO glass slides. H&E, hematoxylin–eosin; ITO, indium tin oxide; MALDI-MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging; MS: mass spectrometry.

endurance of the advanced version of the film (Cryofilm type MS2) are improved, and their signal intensity could be the higher level of the ITO glass slide.

Both signal intensities of the m/z value of 734.6 and 760.6 from the tissue sections with conductive adhesive films were 2–3 times higher than those on ITO glass slides at the optimal laser power of 54. The value of laser power is the percentage of the maximum output power of the laser system, and the optimal condition depends on the laser system at each MALDI-MSI facility. A higher laser energy generally enables us to increase the signal intensity in MALDI-MSI. However, over the certain energy, the UV laser causes decreased intensity of the target molecules on the tissue section due to thermal decomposition. We, therefore, recommend optimization of the laser power and its related parameters to enhance the ion intensity using the conductive adhesive film for MALDI-MSI.

In this study, the signal intensity of small molecules was stable and higher detected than that of the ITO glass slide without consideration of its tissue thickness. Moreover, the optimal laser power condition can be obtained with the higher signal intensity using the advanced version of the conductive adhesive film. We surmised that the present technical report must be referred for the researchers to use the film.

CONCLUSION

We examined the performance of the conductive adhesive film by MALDI-MSI analysis. In the mouse head analysis, almost perfect cryosections could routinely be made with the conductive adhesive film. The signal intensities of the representative molecules with m/z values of 734.6 and 760.6 observed from the tissue sections with the films were 2–3 times higher than those obtained by cryosection using the general protocol on the ITO glass slide at the optimal laser power by MALDI-MSI. In addition, tissue thickness was not a limitation when the conductive adhesive film was used, whereas an 8- μm thickness was needed in the general protocol that utilized ITO glass slides. Therefore, the conductive adhesive film is very useful for MALDI-MSI analysis.

Author Contributions

Conceptualization, D.S., J.A., M.Y., and T.K.; methodology, D.S., R.S., Ko.K., Ku.K., and T.K.; software, D.S., Ko.K., R.S., and T.K.; writing—original draft preparation, D.S. and T.K.; writing—review and editing, D.S., M.Y., A.U., and T.K.; supervision, J.A., M.Y., and T.K.; and funding acquisition, D.S., J.A., M.Y., and T.K. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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