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Stretched Tissue Mounting for MALDI Mass Spectrometry Imaging

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Abstract

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) combines information-rich chemical detection with spatial localization of analytes. For a given instrumental platform and analyte class, the data acquired can represent a compromise between analyte extraction and spatial information. Here we introduce an improvement to the spatial resolution achievable with MALDI MSI conducted with standard mass spectrometric systems that also reduces analyte migration during matrix application. Tissue is placed directly on a stretchable membrane that when stretched, fragments the tissue into micron-sized pieces. Scanning electron microscopy analysis shows that this process produces fairly homogeneous distributions of small tissue fragments separated and surrounded by areas of hydrophobic membrane surface. MALDI matrix is then applied either by a robotic microspotter or an artist's airbrush. Rat spinal cord samples imaged with an instrumental resolution of 50–250 μm demonstrate lipid distributions with a 5-fold high spatial resolution (a 25-fold increase in pixel density) after stretching compared to tissues that were not stretched.

INTRODUCTION

Matrix assisted laser desorption/ionization (MALDI) mass spectrometry (MS) is a useful tool for the investigation of a variety of analyte classes including lipids,¹ peptides,² and proteins.³ In addition to obtaining mass spectra from compounds, the spatial localization of each analyte can be discerned by moving the sample under a focused laser beam.^{4–7} This technique, MALDI mass spectrometry imaging (MSI), generates information-rich, spatially-resolved maps in which each sampled position contains a full mass spectrum; multiple ion images can be extracted from these maps to localize specific analytes.⁴ MSI has been used for several decades in the analysis of semiconductors with ion sources such as secondary ion mass spectrometry (SIMS).⁸ More recently, MSI using SIMS,^{9–10} MALDI,^{1–2,11} and desorption electrospray ionization¹² MS have seen increasing use and application in biological and medical research.¹³

MSI of biological specimens is typically performed using thin tissue sections mounted to a microscope slide or metal target plate. For imaging small tissue features, the spatial resolution of the imaging technique becomes an important figure of merit. Spatial resolution can be limited by several factors including laser spot size, analyte concentration, and lateral analyte migration.^{11,14} Currently, the minimum laser spot size for an Nd:YAG laser used in commercially available MALDI MS instruments is on the order of 10 μm .¹⁵ There are some

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Supporting Information. Additional figures as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

methods currently available that increase spatial resolution for MSI, including oversampling^{14,16} and sample stretching.^{17–21} Custom instruments are able to reach spatial resolutions below 1 μm .²² Regardless, many instruments have laser spot sizes of 50 μm or larger, making spot size a limiting factor. Limited analyte amounts can also affect the effective spatial resolution. When using a 100 μm spot size, there is 100 times more material being analyzed than if the analysis were performed using a 10 μm spot size. This decrease in sample analysis area typically results in a concomitant reduction in signal intensity, which can lead to some analytes falling below the instrument's limit of detection.²³

Analyte migration is another key parameter to consider when determining effective spatial resolution.²⁴ The matrix application method is one of the most important factors that affects the degree of analyte migration and sensitivity that one achieves from a MALDI MSI experiment.²⁴ Matrix is commonly applied via printing,³ spraying² or sublimation.²⁵ When a MALDI matrix solution is applied, analyte molecules are extracted into the matrix layer while the matrix is solvated on the sample surface. However, solvated analytes also tend to redistribute laterally on the sample surface, thereby reducing the effective spatial resolution of the resulting image. A robotic chemical printer eliminates this migration by delivering an array of discrete droplets on the tissue. Moreover, because droplets can be reapplied to the same tissue location indefinitely, printing allows for extended extraction times without the redistribution of analytes beyond the radius wet by an individual droplet. However, spatial resolution becomes limited by the minimum droplet spacing achievable by the printer (typically >100 μm).^{3,19,24} Alternatively, an airbrush- or piezo-assisted spraying device can be used to coat the sample by producing small droplets (~20 μm), forming a thin layer of solution on the sample surface, resulting in small matrix crystal sizes.^{26–27} Because of the smaller droplet and matrix crystal sizes, spraying provides relatively high spatial resolution with low analyte redistribution.²⁴ In another approach, suitable for abundant, low molecular-weight species, a dry MALDI matrix can be sublimed onto the sample surface, effectively eliminating analyte redistribution.²⁵ These matrix application techniques represents a compromise between analyte extraction and lateral analyte migration. The appropriate method is selected according to the specific experimental goals and required spatial resolution, as well as sample type and target analyte class(es).

In a recently developed methodology, tissue is placed on an array of beads that have been directly mounted onto a stretchable membrane.^{17–21,28–30} After stretching the membrane, the tissue adhering to the beads is fragmented into small, bead-sized samples surrounded by a hydrophobic substrate, which are then interrogated with MSI. Here, we demonstrate that the beads are not always needed—the tissue still fragments when placed directly on the hydrophobic membrane. These stretching approaches reduce cationic adducts and enhance MSI spatial resolution, especially in cases where raster spacing is limited to >50 μm by either the matrix application method or the laser spot size. We validate this technique by imaging lipids from the rat spinal cord, a well-studied and well-characterized neuronal model system.

EXPERIMENTAL SECTION

Chemicals

All chemicals were purchased from Sigma-Aldrich, St. Louis, MO and used without further modification.

Sample Preparation

Adult Sprague-Dawley male rats (University of Illinois at Urbana-Champaign) were sacrificed by decapitation and their spinal cords surgically removed. This procedure followed animal use protocols approved by the University of Illinois Institutional Animal

Care and Use Committee and in accordance with all state and federal regulations. The spinal cords were then flash frozen in liquid nitrogen and stored at -80°C until use. Each spinal cord was affixed to the sectioning stage using deionized water as the mounting media. Spinal cord sections of $14\text{ }\mu\text{m}$ thickness were prepared at -20°C using a Leica CM 3050 S cryostat (Leica Microsystems, Bannockburn, IL). Several adjacent sections were thaw-mounted onto indium-tin oxide (ITO)-coated glass microscope slides (Delta Technologies, Stillwater, MN) as control samples. Other sections were thaw-mounted directly onto Parafilm-M (Pechiny Plastic Packaging, Chicago, IL) substrates for stretched samples.

The Parafilm-M substrate samples were returned to the cryostat chamber and allowed to reach -20°C in order to refreeze the tissue sections. Each substrate was then placed face-down against a clean stainless steel block in the chamber, while pressure was applied using a second cold (-20°C) stainless steel block to provide better adhesion, which aids the stretching process. After removing the tissue from the chamber, a warm puff of air ($\sim 36^{\circ}\text{C}$) applied to the back of the substrate thaws the tissue and softens the Parafilm-M prior to stretching, as described previously.¹⁷ The substrates were then stretched at least 5-fold in each direction for an increase of at least 25-fold in area. Stretching was performed manually under visual control by monitoring the natural symmetry of the spinal cord in dorsal/ventral and lateral directions. Manual stretching is preferred over automated micromanipulator-based stretching because tactile feedback allows greater control and more effective determination of maximal Parafilm-M expansion.

Uniform stretching was determined by measuring the stretched tissue and comparing it to a serial section that was not stretched. After the MSI experiment, the images were independently adjusted in the x and y directions based on the stretching factor so that the final images had the same proportions as the native tissue. If greater control during manual stretching is required, the application of a grid design to the back of the Parafilm-M provides visual feedback regarding nonuniform expansion of the sample.

The stretched samples were then placed over ITO slides, which provides both a conductive surface that aids for MALDI analysis and the clear transparency needed for bright field microscopy imaging.^{19,21} The samples were stored at -80°C until use. Prior to use, all samples were dried under nitrogen gas. Samples for MALDI MSI were transferred to a chemical inkjet printer (ChIP-1000, Shimadzu, Tokyo, Japan) for matrix printing or were mounted onto a slide holder for matrix application via an artist's airbrush. Additional serial sections were kept for tissue fragment sizing experiments and as references for measuring the unstretched dimensions of tissue sections.

Environmental Scanning Electron Microscopy (ESEM) Analysis

Three stretched samples on ITO-coated microscope slides were mounted individually in a sputter coater (DeskJet II TSC, Denton Vacuum LLC, Moorestown, NJ) and coated with approximately 7 nm-thick AuPd. Slides were affixed to an ESEM sample mount using carbon tape; a small amount of silver paint was applied on the edges of the slides to make electrical contact between the sample surface and the mount. Samples were imaged on a Philips XL30 ESEM-FEG (FEI Co., Hillsboro, OR) operating in standard high-vacuum mode with a beam energy of 10 kV and a working distance of 11.6 mm using the Robinson Series 6 scintillator-type backscattered electron detector for improved relief contrast of the tissue surface. The collected images were analyzed using the "Threshold" and "Analyze Particle" functions within ImageJ, version 1.43u (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/ij/>), in order to isolate the tissue fragments and determine their average size, as summarized in Table 1.

ChIP-1000 Matrix Application

Rat spinal cord samples, both stretched and unstretched, were individually loaded into the ChIP-1000 printer while nitrogen gas was flowing into the printing chamber and onto a modified stainless steel sample holder plate. An optical image of each spinal cord section was obtained using the on-board optical scanner. The ChIP-1000 was then used to deposit a MALDI matrix of 10 nL of 2,5-dihydroxybenzoic acid (DHB, 25 mg/mL) at 250 μ m center-to-center spacing between each spot by repeatedly depositing multiple bursts, each being five $\sim$ 100 pL drops across the entire tissue sample. The ChIP-1000 was found to be capable of reproducible printing at a minimum of 250 μ m spacing for the unstretched tissues and was used for all subsequent samples.

Airbrush Matrix Application

Bright field microscopy images were taken of each sample with an AxioVert 200 fluorescence light microscope (Carl Zeiss, Inc., Jena, Germany) for alignment within the MALDI instrument. Samples were then spray-coated with 3–5 mL of a 25 mg/mL solution of DHB in 50:50 acetone:water using an airbrush held at a distance of 15 cm, propelled by compressed nitrogen. Multiple coats were applied until the sample exhibited thin, homogeneous DHB crystals across the tissue section, as monitored using a low power light microscope.

MALDI Mass Spectrometry

The mass spectra were collected in positive ion mode on an UltrafleXtreme MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency tripled Nd:YAG solid state laser using the FlexImaging software package (Bruker Daltonics). At each raster position, 250 spectra were acquired at 500 Hz, summed, and saved for analysis. MSI was performed using varying raster spacing (50, 100 or 200 μ m) for sprayed samples, as noted in Table 2. All ChIP-1000 printed samples were imaged at 250 μ m spacing to align with the printed matrix pattern. The laser spot size was adjusted to match the step size being used. The largest setting possible was used in all cases using a 50 μ m spot size for the 50 μ m raster, and a 95 μ m spot size for the 100 μ m raster. The laser was defocused to its largest spot size ($\sim$ 150 μ m) for imaging the 200 μ m and 250 μ m step size samples. As is common during MALDI MSI data analysis, images were normalized using the normalization function built into FlexImaging to account for differences in matrix application and/or ion yield. Analyte identification was based on mass matches to lipids previously identified within the rat spinal cord.^{1,12,31} Four rat spinal cords were serially sectioned, with multiple sections used for analyses; at least six MSI experiments from each combination of matrix application and mounting technique were performed.

RESULTS AND DISCUSSION

Approaches aimed at improving spatial resolution and analyte extraction/incorporation into MALDI matrix crystals are important as MSI strives to achieve higher spatial resolutions while maintaining its coverage of the metabolome, peptidome, or proteome. Whereas many improvements have been focused on the optics and instrumentation, we have concentrated our efforts on sample preparation.

Here we describe an approach whereby spinal cord tissue was directly thaw-mounted onto a membrane and stretched, fragmenting the sample into pieces separated by relatively broad hydrophobic areas of membrane surface (see Figure 1). Spinal cord is well suited as a model system as it is chemically well characterized, contains larger neuron soma, and has regions of heavily myelinated processes that are more difficult to fragment than other central nervous system tissue. Manual stretching of the membrane may produce some regional

differences, disproportionate stretching and/or rotational repositioning of individual tissue pieces; however, with practice, the user can reduce these effects. After one is comfortable with stretching tissues on membranes, the technique becomes quick and robust.

Following tissue stretching, ESEM analysis was performed to characterize the resulting tissue fragments. As shown in Figure 2, individual ESEM images of both gray and white matter were analyzed by manually thresholding each one in order to differentiate tissue fragments from “stretch marks” formed on the membrane surface during sample preparation. The tissue fragments were fit to an elliptical approximation using the long axis as the “fragment size” and from this parameter a fragment-size distribution was determined. The thresholding data, which were collected from imaging three separate regions of gray matter and three regions of white matter from three spinal cord sections using ESEM, are summarized in Table 1. Tissue fragments were found to be relatively homogenous with an average size of 14.1 μm and 12.9 μm for gray matter and white matter, respectively. Furthermore, only 2.0% and 0.01% of the gray and white matter fragments, respectively, were found to be larger than 50 μm .

After stretching but prior to MALDI MSI analysis, MALDI matrix was applied to the tissue surface, allowing for analyte extraction and co-crystallization with the matrix. Two matrix application methods were employed in this study: chemical inkjet printing and spraying via an artist’s airbrush. Chemical inkjet printers have become a popular matrix application technique for MALDI MSI studies.^{3,19,31–32} An advantage is the ability to apply precise volumes of matrix and/or reagent solutions to the tissue surface. Additionally, by repeated applying matrix to specific spots, the extraction time can be extended *ad infinitum*, allowing for enhanced analyte extraction into the matrix layer without decreasing spatial resolution. When using commercial chemical inkjet printers, the spatial resolution is defined by the obtainable spot-to-spot spacing, which is typically $\sim 200\ \mu\text{m}$.^{3,19,33} Our results demonstrate improvements in spatial resolution in imaging experiments where samples were prepared using the commercially available ChIP-1000 printer. The ion images in Figure 3, panels A and D, depict the localization of phosphatidylcholine (PC) 38:4 m/z 810.6, demonstrating the resolution enhancement of this method. MALDI matrix spot-to-spot spacing of 250 μm for both the unstretched (Figure 3A) and stretched samples (Figure 3D) was achieved reproducibly. Additional selected ion images, including images for several PC and phosphatidylethanolamine (PE) lipids are shown in Figures S1 and S2, for the unstretched and stretched data sets, respectively. The spatial resolution achieved by combining the stretching method with chemical printing was 44 μm compared to the 250 μm resolution used during image acquisition.

Spraying MALDI matrix using an artist’s airbrush, piezoelectric device or nebulizer is also an effective approach for MALDI MSI sample preparation.^{2,34–35} Spraying matrix deposits small droplets on the sample surface and can be repeated iteratively in order to apply enough matrix for analysis. An advantage to working with tissue that is surrounded by regions of hydrophobicity is the reduction of unwanted lateral analyte redistribution and the elimination of cation adducts.^{17,30} An unstretched spinal cord section was analyzed using a 50 μm raster pattern to provide an example of a typical MALDI MSI experiment for comparison with the stretched method. These results for PC 38:4 are shown in Figure 3B and in a zoomed-in image in Figure 3C. Additional lipid images are shown in supporting Figure S3A and as zoomed-in images in Figure S3B. Two stretched samples were imaged using 200 μm and 100 μm raster patterns and images for PC 38:4 are shown in Figure 3E and 3F, respectively. Additional ion images are shown in Figure S4 and S5 for rasters of 200 μm and 100 μm , respectively; these produce effective spatial resolutions of 30 μm and 16 μm using the 200 μm and 100 μm spacing.

The results from each of the imaging experiments are summarized in Table 2, in which parameters including tissue dimension, stretched area enhancement, effective pixel size, and pixel density are compared. When stretched printed samples were compared to unstretched printed samples, the effective spatial resolution improved from 250 μm to 44 μm , representing a 5.7-fold improvement in spatial resolution and an increase of over 32-fold in pixel density, allowing for the visualization of smaller features within a tissue section while retaining control over matrix application. Spatial resolution improvements were also observed for samples that were sprayed with matrix. The sprayed stretched samples were imaged at 100 μm and 200 μm , achieving spatial resolutions of 15 μm and 30 μm , respectively, while unstretched samples were imaged at 50 μm . This represents improvements in spatial resolution of 3.1-fold and 1.6-fold, respectively, while using a laser spot size of 100 μm . An additional set of samples analyzed in the same fashion are shown in Figure S6. When comparing images, the spatial ion distributions are similar, although the signal intensities vary between samples. Representative individual mass spectra from sprayed samples, both stretched and unstretched, are shown in Figure S7. These spectra demonstrate that the mass resolutions and signals are comparable using both methods. Stretched tissue mounting is a sampling approach that can be applied to any MSI platform and matrix application technique for tissue imaging.

CONCLUSIONS

MSI is valuable for discovery studies because prior knowledge of analyte identification is not required. Here a robust sample preparation method allows for enhanced spatial resolution compatible with a range of matrix application techniques. The images shown here demonstrate enhanced spatial resolution using existing matrix application protocols. Future applications will expand this methodology to include the analysis of peptides and proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A schematic of the technique used to stretch and image tissue sections. (Top left) thin tissue sections are serially cut; (bottom left) the sections are thaw mounted, frozen and pressed onto the membrane surface to provide better adhesion, which aids the stretching process; (right) stretching produces a final sample with at least a 25-fold increase in tissue section area resulting from at least a 5-fold increase in the x and y directions.

Figure 2. ESEM images of stretched spinal cord sections. (Bottom) Stretched image showing the tissue fragments. (Top and Middle) Images processed to allow automated fragment identification, and illustrate that most tissue features are $<50\ \mu\text{m}$ in their longest direction. Arrows point toward representative tissue pieces.

Figure 3.

The improvement in spatial resolution using this approach is shown in images of PC 38:4 (m/z 810.6) distributions in the rat spinal cord. Tissue size is given in parenthesis after each panel's description. Top row: native tissue samples (without stretching): (A) ChIP-1000 printed matrix application analyzed at 250 μm resolution (5.0×3.5 mm), (B) sprayed matrix application analyzed at 50 μm resolution (4.3×3.1 mm), and (C) a zoomed in image from (B) showing the right dorsal region (1.9×2.5 mm). Bottom row: stretched tissue samples imaged with (D) ChIP-1000 matrix printing at 250 μm spacing (29.5×18.8 mm), (E) sprayed matrix application analyzed at 200 μm spacing (31.9×18.1 mm), and (F) at 100 μm raster spacing (25.5×15.3 mm; shown is the right dorsal portion measuring 12.1×8.4 mm). The images are shown in thermal color scale where white and red represent high intensity and blue and black represent low intensity. Additional ion images can be found in the Supporting Information.

Table 1

A summary of tissue fragment size based on ESEM imaging of stretched samples demonstrates that the fragment size is sufficiently small to image samples at 50 μm spatial resolution.

Image	Fragments counted	Fragments longer than 50 microns (count/percentage)	Avg. length (μm)
Gray 1	283	4/1.4%	13.2
Gray 2	204	9/4.4%	16
Gray 3	205	1/0.5%	13.2
Total Gray	692	14/2%	14.1
White 1	133	1/0.8%	12.3
White 2	217	3/1.4%	14.3
White 3	250	0/0%	12.1
Total White	600	4/0.01%	12.9
Total Fragments	1292	18/1.4%	13.5

Comparative analysis of different MALDI MSI sample preparation approaches showing the improvement in imaging spatial resolution that was obtained.

Table 2

Sample Type (Imaging raster step)	Prestretched X - (mm)	Prestretched Y - (mm)	Stretched X - (mm)	Stretched Y - (mm)	Area Enhancement	Effective Pixel Size (µm)	Pixel Density (pixel/mm ²)
Printed (250 µm)	5	3.5	NA	NA	NA	250	16
Stretched Printed (250 µm)	5	3.5	29.5	18.8	31.6	44	506
Sprayed (50 µm)	4.3	3.1	NA	NA	NA	50	400
Stretched Sprayed (200 µm)	4.3	3.1	31.9	18.1	43.3	30	1,083
Stretched Sprayed (100 µm)	3.9	2.5	25.5	15.3	40.0	16	4,001
Stretched Sprayed* (50 µm)	3.9	2.5	25.5	15.3	40.0	7.9	16,004

* Results shown are from the image shown in the Supporting Information, Figure S6.