APPLICATION NOTE

#61 | Matrix Density Adjustments for Imaging by AP/MALDI

Application & Background

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) is used to visualize the distribution of many different biological molecules in thin tissue slices, including metabolites. Atmospheric Pressure (AP)/MALDI allows for imaging analysis to be performed at semi-physiological conditions for softer ionization. This also allows for the use of matrices that will sublime when kept under vacuum for an extended time without the need for extremely thick matrix coatings. AP/MALDI sources are often coupled to high mass resolution spectrometers such as an orbital trapping mass spectrometer. An overview of this AP/MALDI set-up is shown in **Figure 1**.

Homogenous application of matrix is essential for any MALDI MSI experiment. Matrices must be uniformly deposited across the tissue surface as well as produce crystal sizes within the range of desired imaging resolution. The HTX M5 Sprayer allows for reproducible spray of matrix across slides while minimizing crystal size via a heated nozzle. Unlike vacuum MALDI, AP/MALDI does not cause matrix to sublime during analysis and thus will require different amounts of matrix to be applied to samples for optimal analyte signal without excess signal from matrix peaks. In this study, three key parameters were explored in regard to matrix and signal optimization: tissue visibility for teaching, matrix density, and slide surface choice.

The interaction of multiple parameters such as these often impacts the overall quality of an imaging run. When optimizing matrix application the choice of MALDI instrument, type of slide used, sample type, and expected analyte abundance/ionization efficiency must be accounted for. Parameters selected for vacuum MALDI will need to be adjusted when using an AP/MALDI source as explored in this study.

Experimental

Experimental Design

Frozen mouse kidney tissue was obtained from BioIVT and sectioned at 12 µm onto both glass (Fischer Scientific, Pittsburgh, PA) and ITO-coated (Delta Technologies, Loveland, CO) slides. The tissue sections were imaged without any washing.

Matrix Application

Alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix was prepared in 70% acetonitrile/0.1% trifluoroacetic acid and sonicated prior to spraying. Using the HTX M5 Sprayer, serial tissue sections were sprayed with CHCA matrix via the parameters shown below in **Table 1**. The number of passes was varied for each tissue to assess the effect of matrix density on metabolite signal and background matrix peaks. Matrix density was calculated via the following equation:

$$Matrix\ Density = \frac{\#\ of\ Passes \cdot Flow\ Rate \cdot Matrix\ Concentration}{Velocity \cdot Track\ Spacing}$$

Table 1. AP/MALDI matrix spray parameters used on the M5 Sprayer.

Solvent	70% ACN, 0.1% TFA
Concentration (mg/mL)	10
Flow rate (mL/min, FR)	0.120
Velocity (mm/min, V)	1200
Nozzle Temperature (°C)	75
Track Spacing (mm, TS)	3
Number of Passes (NP)	1-4
Nitrogen Pressure (psi)	10
Spray Pattern	CC
Drying Time (s)	0

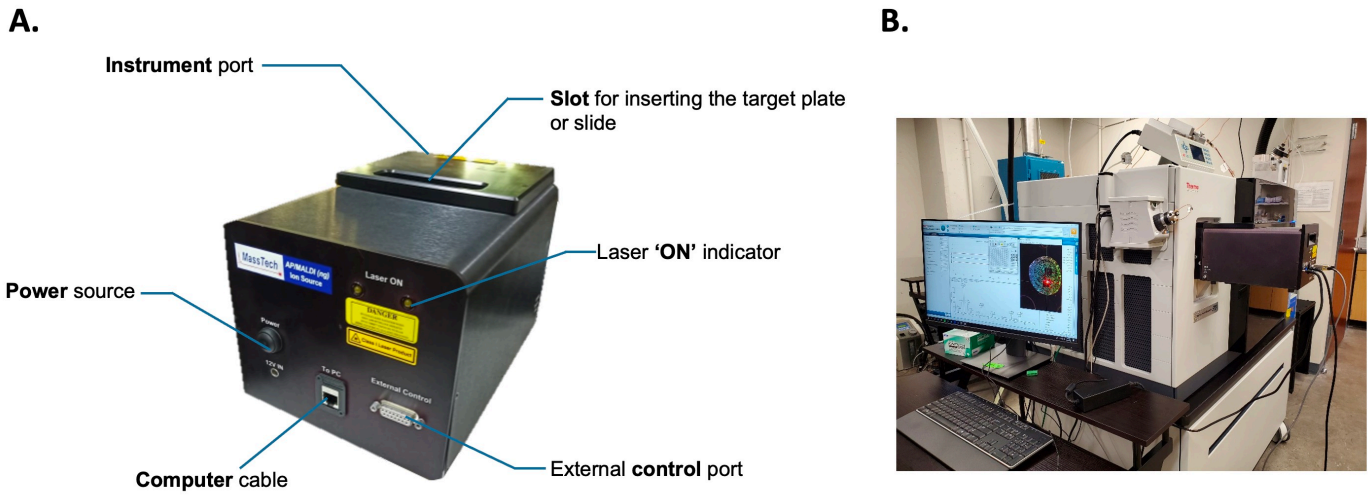

Figure 1. (A) Overview of MassTech AP/MALDI Source. **(B)** AP/MALDI source attached to Thermo Orbitrap MS.

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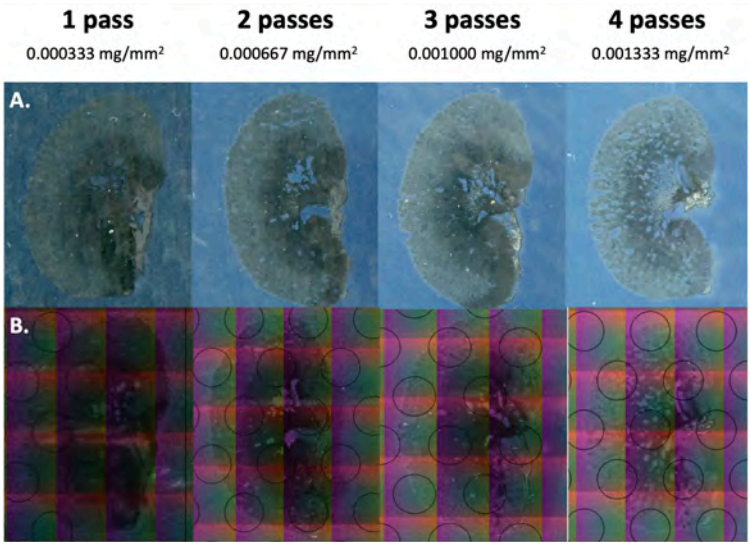


Figure 2. Visual images of tissues coated with varying amounts of matrix. Matrix density for each section is shown above. **(A)** Tissue sections scanned with a flatbed scanner. **(B)** Tissue sections visualized with AP/MALDI source camera. Black circles represent superimposed standard target plate, and each tissue image was stitched together from 20 individual images.

AP/MALDI MSI

All imaging experiments were performed on an AP/MALDI ion source (AP/MALDI(ng)UHR source, MassTech, Columbia, MD, USA) coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). AP/MALDI was performed in positive ion mode with a diode-pumped solid-state laser (λ = 355 nm) operating at a 8 kHz repetition rate with a laser spot size of approximately 15 µm. The AP/MALDI source operates in line scan with continuous acquisition. A pixel size of 100 µm was generated by moving the stage at a velocity of 0.11 mm/sec and collecting two spectra per pixel. The Orbitrap resolution was set to 120,000 at m/z 200 and 2.2 spectra were collected per second. The mass range for these experiments was set to *m/z 50-500.* Tissue samples were visualized using the AP/MALDI source camera for teaching, see **Figure 2B**.

Data Analysis

To evaluate the analyte signal from each experimental condition, .RAW files were opened in Thermo FreeStyle and an average spectrum generated from each whole tissue section. A spectrum of CHCA blank was used to determine prominent matrix peaks. All images files were imported together into SCiLS Lab MVS 2021b and normalized to root mean square. Peak selection was performed manually and images evaluated for differences due to matrix deposition density and/or the type of slide on which the sections were collected.

Results

Tissues were visualized using a flatbed scanner, **Figure 2A**, and the AP/MALDI source camera, **Figure 2B**. The source camera images are required for teaching of the tissue border in the AP/MALDI. Therefore, it is important for matrix coverage to not obscure the tissue image with a thick coating. **Figure 2B** depicts the stitched images from the source camera overlaid with a well plate template. As shown in **Figure 2B**, the tissue coloration got lighter and harder to see as more passes of matrix were applied, which led to difficulty in teaching and defining ROIs for data acquisition. Calculations of matrix density on each tissue are shown, with 0.000333 mg/mm^2 of CHCA matrix applied with each pass, **Figure 2**.

Analysis of optimal matrix density for signal intensity must balance the analyte peak signal intensity compared to the matrix peak signal intensity. While increasing the amount of matrix can amplify analyte signal, this also can lead to more intense matrix peaks in the spectra. Matrix peaks are observed in most all MALDI experiments and the amount of matrix should be limited to the amount needed for efficient ionization of the analytes. Too much matrix signal may also lead to space charging effects in trapping mass spectrometers.

The effects of increased matrix density on signal intensity of both analytes and matrix are shown in **Figures 3-4**. Prominent matrix peaks are indicated with red arrows, and, as expected, were observed to increase with the number of matrix passes. The overall signal intensity of analyte and matrix was higher on ITO slides, **Figure 3**, compared to glass slides, **Figure 4**. The maximum analyte signal intensity for each slide type is indicated on the

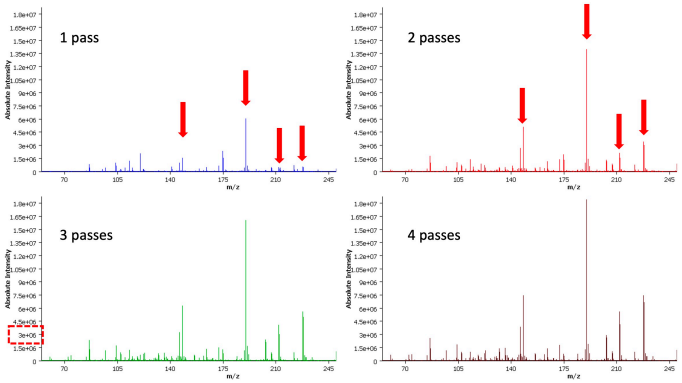


Figure 3. Spectra from samples placed on ITO-coated slides receiving 1-4 passes of matrix.

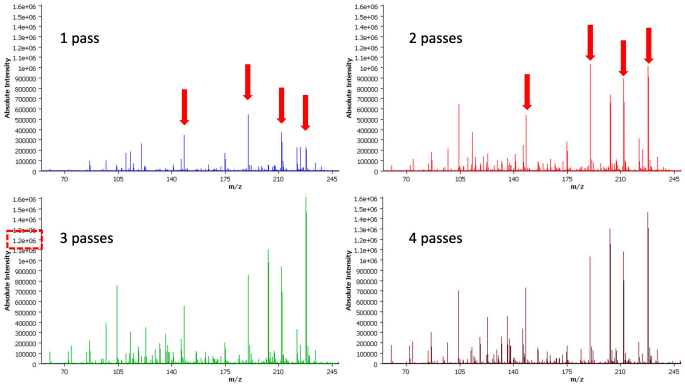


Figure 4. Spectra from samples placed on glass slides receiving 1-4 passes of matrix.

Results (cont.)

spectra with a red box, with absolute intensity of about $3x10^6$ on ITO and $1.2x10^6$ on glass. However, slide choice did affect the amplification of matrix peak signals. While higher overall signal was obtained from the ITO slides, the primary increase in signal was observed from matrix peaks rather than analyte peaks. This differed from samples prepared on glass slides, which did not show the same magnitude of increased matrix signal.

Metabolite ion images of kidney sections are shown in **Figure 5**. When comparing *m/z* images on kidneys sprayed with 1-4 passes of matrix on 2 different slide types, several trends emerged. For almost all metabolites and tissue sections, 1 pass of CHCA matrix was not sufficient to produce an ion image. This is likely due to low extraction of the analyte by the matrix as well as limited matrix crystals to co-crystallize with the analytes. **Figures 3-4** also depict this trend with most significant difference in signal intensities observed moving from 1 to 2 passes of matrix. Certain low abundance metabolites, such as spermidine (*m/z* 146.1663) and phosphocholine (*m/z* 184.0708), did not produce spatially resolved ion images until at least 3 passes were applied, corresponding to a matrix density of 0.001 mg/mm^2. This highlights the balance of depositing ample matrix for analyte signal while not causing excessive background from matrix peaks. In addition, the choice of slide surface did impact the signal intensity for certain analytes. The majority of metabolites were detected with higher abundance after normalization on the glass slide compared to ITO slide. Researchers should take into account analyte abundance when optimizing matrix density and slide type for a given set of experiments.

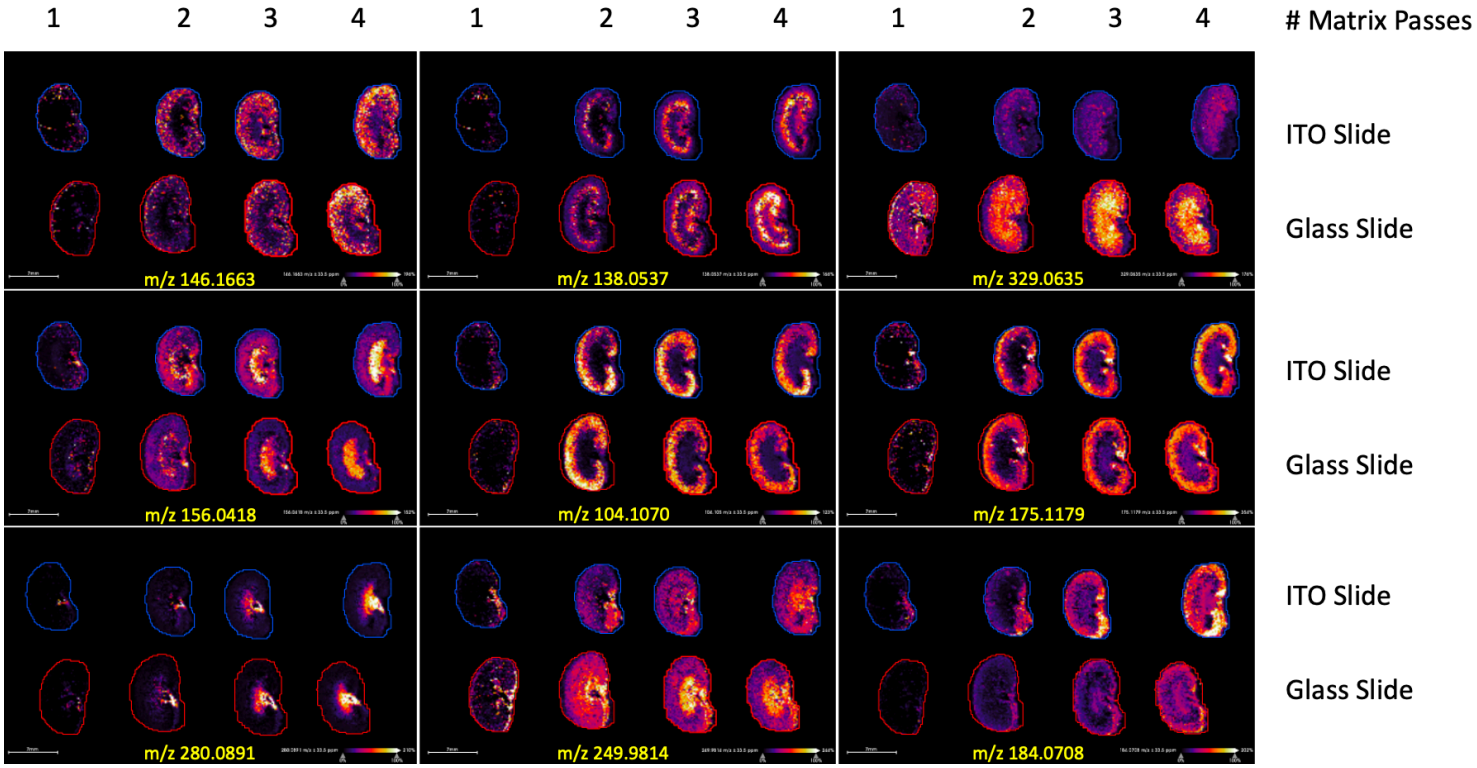


Figure 5. Effect of matrix density on imaging of metabolites on kidney sections.

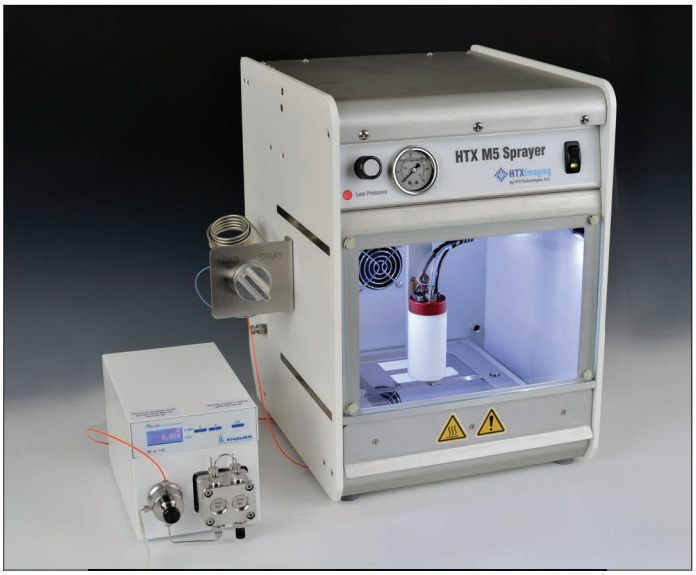
Conclusions

We demonstrate the use of the HTX M5 Sprayer for optimized matrix application in AP/MALDI experiments. While vacuum MALDI is more commonly used in imaging experiments, AP/MALDI offers the benefit of imaging at near-physiological condition, which leads to less fractionation of analytes and matrix sublimation. Because of the pressure difference, AP/MALDI imaging requires an adjustment of matrix parameters from vacuum MALDI imaging. While the optimal amount of matrix will differ based on analyte and matrix choice, a trend across the board was observed of about half as much matrix required for AP/MALDI as used in vacuum MALDI.

The HTX M5 Sprayer produces a uniform and highly reproducible matrix coating for MALDI imaging experiments. The amount of matrix applied to samples is easily calculated and adjusted via parameters such as matrix concentration, flow rate, number of passes, track spacing, and nozzle velocity. These parameters can be fine-tuned for optimal data quality including spatial resolution, analyte signal intensity, and decreased matrix background.

HTX M5 Sprayer™ Tissue MALDI Sample Preparation System

The images and data presented here were provided by Dr. Erin Seeley of the University of Texas at Austin, Austin, Texas, USA.



HTX M5 Sprayer™ System is an Automated MALDI Matrix Deposition System Offering **High Reproducibility** and **Superior Data Quality** for Imaging Mass Spectrometry

The HTX M5 Sprayer™ is an easy-to-use, versatile spraying system that provides automated processes for sample preparation in imaging mass spectrometry.

The proprietary spray technology of the HTX M5 Sprayer™ guarantees a very fine, uniform and consistent matrix coating crucial for high-resolution imaging and relative quantification of analytes.

The unique ability to control liquid and propulsion gas temperature creates a fine solution mist that can be deposited in a precise and adjustable pattern over all or part of any MALDI plate.

Spray characteristics (wet or dry) are easily adjustable via the intuitive operator interface. Users can create and save methods for reproducible operation.

Key Characteristics

- Proprietary technology providing very small matrix droplets (<5 microns)
- High flow rate and fast sample prep (2 to 18 minutes per slide)
- Highly consistent matrix deposition across entire sample area (+/- 3% by weight)
- Unique use of temperature and nitrogen flow to control evaporation rate and matrix crystal formation
- More than 30 validated protocols covering trypsin and most matrices (e.g.: SA, CHCA, DHB, DAN, 9-AA, DHA, CMBT, THAP)
- Validated protocols for Trypsin digestion of FFPE
- Continuous matrix coverage as needed for high-resolution imaging
- Rugged operation and easy clean-up

Addressing the Matrix Deposition Challenge

The main challenge when preparing samples for MALDI Mass Spectrometry Imaging is to balance the positive effects of the matrix solution penetrating the tissue and co-crystallizing with the analyte, and the negative effects of analytes delocalization.

The all-new M5 chassis, high velocity stage and heated sample holder drawer contribute to a greater user experience and expanded process capabilities including:

- Faster and drier deposition capability
- On-tray trypsin digestion capability
- On-tray sample re-hydration

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