Novel matrix strategies for improved ionization and spatial resolution using IR-MALDESI mass spectrometry imaging

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Abstract

In mass spectrometry imaging (MSI) applications of infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI), an exogenous ice layer is the gold standard for an energy-absorbing matrix. However, the formation of the ice matrix requires additional time and instrument hardware, so glycerol was investigated herein as an alternative to the ice matrix to potentially improve spatial resolution and ionization, while decreasing experiment time. Glycerol solutions of varying concentrations were sprayed over top of rat liver tissue sections for analysis by IR-MALDESI and compared to the typical ice matrix condition. Additionally, we tested if combining the ice matrix and glycerol matrix would further improve analyses. Matrix conditions were evaluated by comparing ion abundance of six lipid species, the laser ablation spot diameter, and number of METASPACE annotations. The ion abundances were also normalized to the volume of tissue ablated to correct for lower abundance values due to less ablated tissue. It was observed that utilizing a 50% glycerol matrix without ice provides improved spatial resolution with lipid abundances and annotations comparable to the ice matrix standard, while decreasing the time required to complete an IR-MALDESI tissue imaging experiment.

KEYWORDS
energy-absorbing matrix, glycerol, IR-MALDESI, mass spectrometry imaging

1 | INTRODUCTION

Infrared matrix-assisted laser desorption electrospray ionization is a hybrid ionization source combining matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), primarily used in mass spectrometry imaging applications. In IR-MALDESI, molecules are ablated by a mid-IR laser fired at the sample; the desorbed neutral molecules are then post-ionized by an orthogonal electrospray plume. Similar to MALDI, energy-absorbing matrices surround the analyte and facilitate molecular desorption from the surface during the ablation event. Therefore, selecting a matrix with high absorbance at the utilized laser wavelength is essential to maximizing laser efficiency and analyte desorption. The primary energy-absorbing matrix used for IR-MALDESI imaging applications is water in the form of a deposited ice layer matrix, in addition to the endogenous water that is abundant in biological tissues. Due to the high absorbance of water at 2.97 μm, the application of this exogenous matrix was shown to improve both ion abundance and spatial resolution and is currently the gold standard for MSI applications of IR-MALDESI.

While beneficial to tissue imaging, forming the ice matrix requires additional instrument hardware and time and is subject to the laboratory conditions (e.g., relative humidity). Glycerol was selected as a
promising new matrix for the following reasons: (1) It has previously produced positive results as a MALDI matrix for different analytes and for enhancing sensitivity with fast-atom bombardment (FAB); (2) it absorbs IR energy at the same wavelength of the source laser (2.97 μm) due to the O–H bond stretch; (3) it is stable at physiological conditions; and (4) it can be quickly and reproducibly applied to multiple tissues at once using a pneumatic matrix application sprayer. Using this sprayer, we have greater control over the matrix application process which helps to improve the coating homogeneity from the formation of the ice matrix. This also decreases experiment time since up to four matrices can be applied in approximately 30 min rather than one in the same amount of time with ice matrix formation. Lipids have been the focus of numerous works with IR-MALDESI due to their importance in human health and thus, we have selected this molecular class to evaluate the use of glycerol as an alternative to the ice matrix.

Specifically, we compared the effects of the glycerol matrix to the gold-standard ice matrix using metrics such as number of lipid annotations, ion abundance, and laser spot ablation dynamics during IR-MALDESI-MSI of rat liver tissue. Laser spot ablation dynamics are an important parameter to consider because the laser ablation influences the spatial resolution we can achieve and the ion abundance we can obtain. We report here a novel matrix strategy utilizing glycerol without the need for an ice matrix in order to achieve better experimental efficiency while maintaining the overall quality of lipid measurements relative to the ice matrix.

2 | METHODS

2.1 | Sample preparation

Wild-type, healthy rat liver tissue (NCSU Department of Biological Sciences) was equilibrated to −20°C and sectioned at 15 μm thickness using the Leica CM1950 cryostat (Buffalo Grove, IL, USA). The tissue sections were thaw mounted onto microscope slides (1 mm height, plain, Fisher Scientific, Pittsburgh, PA). Glycerol (Sigma Chemical Co.) solutions were applied to the tissue-mounted slides using a pneumatic sprayer (TM-Sprayer, HTX Technologies, Chapel Hill, NC, USA) with previously optimized standard spraying conditions. Full TM sprayer parameters are detailed in Table S1. A 10% glycerol solution and a 50% glycerol solution in 45:45 MeOH:H2O, respectively, were tested. LC/MS methanol and water were obtained from Fisher Chemical. Prepared slides that were not immediately analyzed were stored at −20°C. The use of animals and procedures was reviewed and approved by the NC State University Institutional Animal Care and Use Committee (IACUC).

2.2 | IR-MALDESI-MSI of rat liver tissue

All tissues were analyzed using the in-house built IR-MALDESI source coupled to an Exploris 240 Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) for MSI. To form the deposited layer ice matrix, the sample slide was placed on a Peltier-cooled stage within the source. The source enclosure was purged with nitrogen gas until the relative humidity of the enclosure was 10%. The sample stage was then cooled to −8°C and allowed to equilibrate at these conditions. The enclosure was then opened to allow ambient humidity to fill the enclosure and allow ice matrix to form. The enclosure was then closed and purged with nitrogen gas, and these conditions were maintained during analysis. Two rectangular regions of interest (ROI) (12 × 10 laser spots) were analyzed per tissue prior to ice matrix formation. Following ice matrix formation, two additional ROIs were analyzed for a total of four ROIs per tissue section.

A 2.97 μm laser (JGMA, Burlington, MA, USA) was used to ablate targeted tissue regions with one burst of 10 pulses (1 mJ/burst) at a rate of 10 kHz. Ablated neutral molecules were subsequently ionized within the orthogonal electrospray plume. The electrospray plume was generated by applying a voltage of 3.6 kV to the electrospray solvent containing 50:50 (v/v) ACN: H2O modified by 0.2% formic acid. The automatic gain function (AGC) was disabled, while the injection time was fixed to 15 ms to synchronize the timing of tissue ablation and ion collection while limiting the accumulation of ambient ions. Ions in positive ionization mode within the 200–1,000 m/z range were analyzed at 240,000 resolving power at m/z 200. Internal calibration at lock mass 202.0782 was used throughout analyses to achieve high mass measurement accuracy (2.5 ppm).

Immediately following imaging, tissues were stained using the Arcturus™ HistoGene™ Staining Solution (Thermo Fisher Scientific, Bremen, Germany). Laser ablation spot diameters were measured from stained tissue with a Leica LMD7000 microscope (Leica Microsystems, Buffalo Grove, IL, USA) and corresponding software.

2.3 | Data analysis

Raw spectra were viewed and analyzed directly in XCalibur. For imaging analysis, raw data files were converted from the .RAW file format to the mzML format using MSConvert, a tool from Proteowizard. The mzML file was converted to the imzML file format using imzMLConverter, which is the file type used to create images and extract ion abundance information in MSIReader v1.3i. These converted files were also uploaded to the METASPACE annotation platform to obtain putative identifications using the LipidMaps database.

3 | RESULTS AND DISCUSSION

The workflow for applying glycerol and investigating matrix conditions is summarized in Figure 1. Healthy, wild-type rat liver tissue was used in this study as it is a relatively homogenous model with minimal biological variability. Prepared glycerol solutions were evenly sprayed onto slides containing tissue sections using a pneumatic sprayer to create an energy-absorbing matrix around the tissue. Sections were
analyzed with IR-MALDESI coupled to an Exploris 240 Orbitrap mass spectrometer to achieve high resolving power and mass accuracy. For each condition tested, two rectangular ROIs were analyzed before and after ice matrix formation (four total ROIs per section) to minimize biological variability between technical replicates of the tested conditions.

### 3.1 Laser spot size analysis

Immediately following IR-MALDESI analysis, tissues were stained, and the laser ablation spot sizes were measured under a microscope. We measured 10 spots from each ROI for a total of 20 spots per matrix condition ablated at a fixed applied laser energy (1 mJ/burst). The average spot size measurements for each condition are summarized in Figure 2. We observed similar spot diameters (~145–157 μm) between conditions except for the tissue where the 50% glycerol solution was applied without the ice matrix (121 μm), leading us to speculate that this matrix affects the spot diameter required to reach the laser fluence threshold needed for tissue desorption. Laser fluence and its connection to spot size have been previously studied in a UV-MALDI application using organic matrices where it was empirically determined that smaller spot sizes require higher laser fluence. Spatial resolution is limited by either the smallest laser spot size or the smallest spot-to-spot distance physically achievable by the translation stage. Achieving a smaller spot size is ideal because it translates to a greater achievable spatial resolution if not limited by physical movement. The 20% decrease in laser ablation spot diameter from 151 to 121 μm allows for a decrease in the spot-to-spot distance between laser ablation events. For example, in an ROI covering the same area,
that reduction in spot-to-spot distance could result in an approximately 59% increase in the number of scans from 2,500 to 3,969. This may increase spatial accuracy and precision of generated images, which is of high importance in some applications (e.g., single cell analysis). Improved images of chemical localization in tissues can enhance resolution of important morphological features. The use of 50% glycerol as an energy absorbing matrix may provide this advantage; however, it was next necessary to investigate whether this resulted in a loss in sensitivity (e.g., number of annotations).

3.2 Ion abundance using glycerol matrices

To compare ion abundance of the glycerol matrices to the gold-standard ice matrix, we selected six different highly investigated lipid species across multiple main classes within the same lipid category due to their high abundance and relatively homogeneous spatial distribution across rat liver tissue. These lipids included cholesterol ([M + H\(^+\)]/C\(_{10}H_2O\], m/z 369.3516), three phosphatidylcholines (PC(34:2) [M + H\(^+\)], m/z 785.5698, PC(36:2) [M + H\(^+\)], m/z 786.6010, PC(38:4) [M + H\(^+\)], m/z 810.6003), and two phosphatidylethanolamines (PE(36:2) [M + H\(^+\)], m/z 744.5538, PE(38:4) [M + H\(^+\)], m/z 768.5535).

Heatmaps of the abundances measured for these species are shown in Figure 3 across each matrix condition. We included data from control tissues (i.e., no sprayed glycerol) from both the start and end of the analysis day to account for and visualize analytical variability that may have occurred throughout the experiment. As expected, adding any exogenous matrix, glycerol or ice, in all cases improved abundance from the control (Figure 3A). While the ice matrix shows considerable abundance improvement over the control tissue without a matrix, the 50% glycerol without ice condition has the highest abundance among all matrices without ice applied. The 50% glycerol without ice matrix specifically improved abundances of cholesterol and the PEs (Figure 3B), with abundances of the latter matching those from the ice matrix samples. This suggests that the 50% glycerol is an improvement over no exogenous matrix and can work as an effective energy-absorbing matrix that is comparable to the ice matrix, which allows for the detection of some lipids under these conditions.

**FIGURE 3** Abundance heatmaps for each ROI in each matrix condition. (A) Key to sample layout and summed abundance data across all six lipid species. (B) Heatmaps for each m/z that was studied shown individually. Cividisblack colormap was used since it has a linear scale and is color vision deficiency (CVD) friendly.
3.3 | Lipid annotations using METASPACE

To broadly compare the quality of lipid data collected and investigate the previous finding further, we utilized the METASPACE annotation platform to pull out lipid annotations from the LipidMaps database at a 10% false discovery rate (FDR). METASPACE calculates a metabolite-signal match (MSM) score for each annotation that is reported, and this score signifies the likelihood that the signal corresponds to the ion. This strategy provides more confidence in the annotations collected and may be used as a metric in determining the overall quality of data collected. The METASPACE annotations are summarized in Figure 4. These results were organized in sampling.

![Figure 4: Combined number of METASPACE annotations and unique annotations for each matrix condition at 10% FDR. The data are presented top to bottom in the order in which data were collected. The number of annotations from each ROI was added and duplicates removed. The most annotations and identifications at this confidence level come from the 50% glycerol without ice condition.](image)

**FIGURE 4** Combined number of METASPACE annotations and unique annotations for each matrix condition at 10% FDR. The data are presented top to bottom in the order in which data were collected. The number of annotations from each ROI was added and duplicates removed. The most annotations and identifications at this confidence level come from the 50% glycerol without ice condition.

![Figure 5: (A) Scatter plot of summed abundances versus ablation volume emphasizing the volume and abundance difference for the 50% glycerol without ice matrix condition. (B) Summary of ion abundance for each matrix condition normalized to volume of ablated tissue. This was calculated based on the average laser ablation spot diameter, tissue thickness, and the average abundance for each matrix condition. The ice matrix and 50% glycerol conditions had the highest abundances per cubic micrometer of ablated tissue.](image)

**FIGURE 5** (A) Scatter plot of summed abundances versus ablation volume emphasizing the volume and abundance difference for the 50% glycerol without ice matrix condition. (B) Summary of ion abundance for each matrix condition normalized to volume of ablated tissue. This was calculated based on the average laser ablation spot diameter, tissue thickness, and the average abundance for each matrix condition. The ice matrix and 50% glycerol conditions had the highest abundances per cubic micrometer of ablated tissue.
order, and no bias was observed due to the order in which they were analyzed. The 50% glycerol without ice matrix gave the most LipidMaps annotation matches closely followed by the ice matrix with and without 50% glycerol. For unique identifications, the 50% glycerol condition had 36 unique annotation without the ice matrix and 33 unique combinations combined with the ice matrix, one and three fewer than the ice matrix alone. Given both total and unique annotations, we can conclude that the 50% glycerol matrix allows for the identification of a wide variety of lipids and also facilitates the detection of lipids not identified in other matrices.

3.4 Evaluating ion abundance with tissue ablation volume

We have identified the 50% glycerol without ice matrix as the condition providing the highest spatial resolution with increased ion abundance from control tissues. The ion abundances obtained from the ice matrix without glycerol appear greater across each of the six lipids, but considering the smaller spot size and that all the tissue sections were ablated all the way through, we estimated that less volume of sample was ablated for analysis when applying 50% glycerol without ice matrix. We summed the total ion abundance of the six ions of interest for each matrix condition and plotted it against the volume of tissue section that was ablated (Figure 5A). We then normalized the sum abundance of each matrix condition to the volume of tissue ablated (sum abundance divided by volume of tissue ablated) in Figure 5B. While Figure 5A shows the uncorrected sum abundances of the 50% glycerol without ice matrix appeared lower than those of the ice matrix, Figure 5B shows this condition is equivalent to those of the ice matrices when all sum abundances were normalized to their corresponding ablated volume (likely due to lower volume ablated). Thus, when considering and normalizing to the volume of tissue ablated, the abundance of the 50% glycerol without ice matrix condition showed similar performance to the ice matrix. This provides more evidence that the 50% glycerol condition without ice matrix is an improvement over no applied matrix and is comparable to the ice matrix, yielding equivalently qualitative results and ion abundances.

4 CONCLUSIONS

In this work, we presented a novel matrix strategy utilizing glycerol as the energy-absorbing matrix for IR-MALDESI-MSI. We evaluated the new matrix by comparing it to the deposited ice layer matrix that is fundamental to IR-MALDESI measurements in terms of improving spatial resolution and ion abundance. We found that the 50% glycerol matrix without the additional ice layer increases ionization over the tissues without an applied matrix and resulted in improved spatial resolution with comparable lipid abundances and annotations when compared to the ice matrix. This matrix design does not require the formation of the ice matrix that increases the amount of control during matrix application and time efficiency.

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DATA AVAILABILITY STATEMENT

Fundamental study: Once accepted, we will place our data on METASPACE site.

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