Video Article

# Dithranol as a Matrix for Matrix Assisted Laser Desorption/Ionization Imaging on a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer

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## Abstract

Mass spectrometry imaging (MSI) determines the spatial localization and distribution patterns of compounds on the surface of a tissue section, mainly using MALDI (matrix assisted laser desorption/ionization)-based analytical techniques. New matrices for small-molecule MSI, which can improve the analysis of low-molecular weight (MW) compounds, are needed. These matrices should provide increased analyte signals while decreasing MALDI background signals. In addition, the use of ultrahigh-resolution instruments, such as Fourier transform ion cyclotron resonance (FTICR) mass spectrometers, has the ability to resolve analyte signals from matrix signals, and this can partially overcome many problems associated with the background originating from the MALDI matrix. The reduction in the intensities of the metastable matrix clusters by FTICR MS can also help to overcome some of the interferences associated with matrix peaks on other instruments. High-resolution instruments such as the FTICR mass spectrometers are advantageous as they can produce distribution patterns of many compounds simultaneously while still providing confidence in chemical identifications. Dithranol (DT; 1,8-dihydroxy-9,10-dihydroanthracen-9-one) has previously been reported as a MALDI matrix for tissue imaging. In this work, a protocol for the use of DT for MALDI imaging of endogenous lipids from the surfaces of mammalian tissue sections, by positive-ion MALDI-MS, on an ultrahigh-resolution hybrid quadrupole FTICR instrument has been provided.

## Video Link

The video component of this article can be found at https://www.jove.com/video/50733/

## Introduction

Mass spectrometry imaging (MSI) is an analytical technique for determining the spatial localization and distribution patterns of compounds on the surface of a tissue section[1,2]. Matrix assisted laser desorption/ionization (MALDI) MSI for the analysis of peptides and proteins has been used for over a decade and there have been great improvements in methods for sample preparation, detection sensitivity, spatial resolution, reproducibility and data processing[3,4]. By combining information from histologically stained sections and MSI experiments, pathologists are able to correlate the distributions of specific compounds with pathophysiologically interesting features[5].

The distribution patterns of small molecules, including exogenous drugs[6,7] and their metabolites[8-10] have also been interrogated by MALDI-MS tissue imaging[11]. Lipids are perhaps the most widely-studied class of compounds with MALDI imaging, both in the MS[12-17] and MS/MS[18] modes. The use of MALDI MSI for small molecule imaging has been limited by several factors: 1) MALDI matrices are themselves small molecules (typically m/z <500), which generate abundant ion signals. These abundant signals can suppress the ionization of small-molecule analytes and interfere with their detection[19,20]. Solvent-free matrix coating[21], matrix sublimation[22], and matrix precoated MALDI MS[23], among others, have been developed to improve MSI of small molecules.

New matrices that can improve the analysis of low-MW compounds are of great interest in small-molecule MSI. These matrices should provide increased analyte signals with decreased matrix signals. In the positive-ion mode, 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) are the two commonly used MALDI MS matrices for MSI[24]. The ideal matrix would form small crystals, so as to preserve the spatial localization of the analytes. DHB tends to form larger crystals, therefore applying the matrix using sublimation has been developed to partially overcome this problem, and has allowed the use of this matrix for sensitive imaging of phospholipids[22,25]. 9-Aminoacridine has been used for MSI of protic analytes in the positive-ion mode[26] and for nucleotides and phospholipids in the negative-ion mode[26-29]. 2-Mercaptobenzothiazole has been found to give efficient MALDI detection of lipids[30], and has been used for the imaging of mouse brain gangliosides[31]. The ultrahigh resolution of Fourier transform ion cyclotron resonance (FTICR) mass spectrometers can somewhat alleviate this problem by resolving analyte signals from matrix signals[32]. Another advantage of the use of FTICR-MS is that the intensities of the metastable matrix clusters are reduced[33], which also reduces these interferences[27].

 

The use of dithranol (DT; 1,8-dihydroxy-9,10-dihydroanthracen-9-one) as a MALDI matrix for tissue imaging has previously been reported[34]. In this current work, a detailed protocol is provided for the use of DT for the MSI of endogenous lipids on the surfaces of bovine lens tissue sections, in the positive-ion mode.

## Protocol

# 1. Tissue Sectioning

1. Flash-freeze the issue specimens, once harvested, using liquid nitrogen, ship them on dry ice (if shipping is required), and store them at -80 °C until tissue sectioning. (If commercial samples are used, ensure that the samples are prepared in this manner.)
2. Cut organs to a manageable size to fit the MALDI target. Trim off any unwanted parts of the organ. For this study described here, bovine calf lenses were decapsulated using a previously-described procedure[35] before tissue sectioning.
3. Remove whole organs from the -80 °C freezer and fix them onto a cryogenic tissue cutting stage. To fix a bovine calf lens, place one or two drops of water on the tissue-cutting stage of a cryostat. Quickly place the lens in the water before it solidifies. Alternatively, optimal cutting temperature (OCT) compounds can also be used to fix a tissue onto the cutting stage. If OCT compounds are used, a minimal amount of OCT should be applied and care should be taken to ensure that the cut tissue sections will not be contaminated with OCT compounds which can interfere with ionization and detection of the analytes[5,36,37].
4. Allow the temperature inside the cryostat to equilibrate to -18 °C. Colder or warmer temperatures can be used for softer or harder tissues, respectively. Then cut the tissue equatorially into 20 µm thick slices. Use 10-15 µm thick slices for most tissues; however, because of the fragile nature of the bovine lens tissue, 20 µm thick slices were used. For bovine lens tissue, discard the first tissue sections and only use slices which are close to or at the equatorial plane.
5. If an ocular lens tissue is imaged, use 1.5 µl of formic acid (98% in purity, LC-MS grade) to prewet the surface of an indium tin oxide (ITO)-coated glass slide.
6. Carefully transfer the tissue sections to the surface of the ITO-coated glass microscopic slide inside the cryostat. The tissue section will quickly thaw and will become tightly affixed to the slide surface. Usually, multiple tissue sections can be mounted onto a same ITO-coated slide in this way.
7. Lyophilize the slide for 15 min before MALDI matrix application.
8. For matrix testing, dissolve the individual matrices in appropriate solvents. Manually spot 1 µl of each matrix solution onto the tissue section. Additionally, spot a small-molecule calibration standard onto the tissue for verification of MALDI sensitivity.
9. Add three teaching marks to the ITO coated glass slide by writing on the nonconductive surface of the ITO coated glass slide with a correction-fluid pen. Take an optical image of the tissue slide using a flatbed scanner and save it in an appropriate format such as tiff or jpg.

# 2. Matrix Coating

### 2.1. Automated Matrix Coating

1. Apply matrix solutions, which contain acetonitrile or mixed acetonitrile/water as solvents, automatically to the surfaces of the tissue sections using a Bruker ImagePrep or a similar electronic matrix sprayer.
2. Cover the edges of the front surface of the ITO-coated glass slide with tape so that the matrix does not coat the edges of the slide. This ensures that the teaching marks on the opposite surface can be used for tissue slide alignment. Do not cover the edges of the slide with matrix as they are used as contact points to maintain the electrical conductivity of the ITO-coated slide.
3. Coat the glass slide by using twenty cycles of matrix coating (2-sec spray, 30-sec incubation, and 60-sec drying time for each cycle).

### 2.2. Manual Matrix Coating

1. If organic solvents (*e.g.* chloroform and ethyl acetate) that are incompatible with the manufacturing materials of the electronic matrix sprayer are required, use a pneumatically-assisted airbrush sprayer to apply the matrix. Add the prepared matrix solution to the solvent reservoir of the airbrush gun and apply a gentle flow of pressurized nitrogen gas to prime the spray.
2. Cover the edges of the front surface of the ITO-coated glass slides with tape so that the matrix does not coat the edges of the slide. This ensures that the teaching marks on the opposite surface can be used for tissue slide alignment. Do not cover the edges of the slide with matrix as they are used as contact points to maintain the electrical conductivity of the ITO-coated slide.
3. After stable and fine sprays have been observed, manually spray the matrix so that it completely coats the tissue section. Apply the minimum amount of matrix solution required to barely wet the surface during each cycle to prevent possible analyte delocalization. In general, use approximately 10 cycles of matrix spray to coat a tissue section; the number of cycles is dependent on the tissue type and matrix composition.

# 3. MALDI MS

1. Prepare a mass calibration solution by diluting the "ES Tuning Mix" standard solution by a factor of 1:200 in 60:40 isopropanol:water (containing 0.1% formic acid in the final mixture).
2. Introduce 2 µl/min of the diluted "ES Tuning Mix" solution into the dual-mode electrospray ionization (ESI)/MALDI ion source on the FTICR mass spectrometer, from the ESI side.
3. Operate the FTICR instrument in the positive-ion ESI mode, with broadband detection and a data acquisition size of 1,024 kb/sec. Typical ESI parameters are capillary electrospray voltage, 3,900 V; spray shield voltage, 3,600 V; nebulizer gas ($N_2$) flow, 2 L/min; dry gas ($N_2$) flow and temperature, 4 L/min and 200 °C; skimmer 1 voltage, 15 V; time of flight (TOF), 0.009 sec; collision gas (Ar) flow, 0.4 L/s; source ion accumulation time, 0.1 sec; and collision cell ion accumulation time, 0.2 sec. Tune the FTICR operation parameters in order to maximize the analytical sensitivity over the mass range from m/z 200 to 1,400, while maintaining good time-domain free-induction decay (FID) signals.

Typically, the ICR operation parameters are sidekick voltage, 8 V; sidekick offset voltage, 8 V; excitation amplification of 10; excitation pulse time, 0.01-0.015 sec; front trap plate voltage, 1.5 V; back trap plate voltage, 1.6 V and analyzer entrance voltage, -4 V. After a set of FTICR operation parameters has been determined, acquire the ESI mass spectra and calibrate the instrument using the reference masses of the standard compounds in the "ES Tuning Mix" solution.

4. To tune the instrument for MALDI operation, dissolve several 1 µl aliquots of a mixed terfenadine and reserpine standard solution in the matrix solution at a concentration of 1 µM each, and spot these solutions directly onto one of the tissue sections (*i.e.* a test tissue section) which has been mounted on an ITO-coated slide. Place the ITO-coated slide into a tissue slide adaptor (*i.e.* a special MALDI target) and load the adaptor from the MALDI side into the dual ESI/MALDI ion source. Optimize the appropriate MALDI operating parameters for the laser power and the number of laser shots for MALDI signal accumulation for each mass scan, *etc.* Typical MALDI operation parameters are: laser shots, 50; and a MALDI plate voltage of 300 V.
5. After tuning, calibrating, and optimizing the instrument for MALDI-MSI experiments, align the physical location of a tissue section to be imaged with its recorded optical image within the imaging software. Use the three "correction-fluid" marks, which had been previously put on the opposite side of the ITO-coated slide surface (step 1.9), for this alignment using a three-point triangulation method.
6. Perform a simultaneous ESI and MALDI operation so that each mass spectrum contains the reference mass peaks of the "ES Tuning Mix" solution for post-acquisition internal mass calibration. This will result in the most accurate mass measurement during MALDI MS. To do this, first attenuate the ESI signal by decreasing the capillary voltage until the MALDI signals dominate the spectra while the ESI calibrant signals are still high enough for internal mass calibration.
7. Next, set up an automated rastering method for laser irradiation. Define the tissue regions to be imaged and set the appropriate laser raster step size. Note that smaller raster step sizes provide higher resolution tissue images, but require a significantly longer mass spectral acquisition time and more data storage space. The number of the image pixels is dependent on the laser raster step size to set up and the tissue size. For a typical bovine lens which has a 1 $cm^2$ tissue size, a tissue image is typically composed of *ca.* 5,000 pixels if a laser raster step size of 200 µm is used on an FTICR instrument. Use a "random spot" analysis, as this prevents location-based bias due to gradual signal attenuation during the experiment.

## 4. Data Analysis

1. Calibrate the MALDI mass spectra using internal calibration for the initial comparison and to select peaks for MS/MS. De-isotope and select the monoisotopic peaks as previously described, using a customized VBA script[38].
2. Export the resulting monoisotopic peak lists and input the measured m/z values into the METLIN[39] and/or the HMDB[40] metabolome databases for mass matching with the library entries. Consider the $(M+H)^+$, $(M+Na)^+$, and $(M+K)^+$ ions during the database searches, with an allowable mass error of ±1 ppm.
3. Generate MALDI images for all of the lipid entities detected across the entire tissue section using image-analysis software, with a mass filter width of 1 ppm at the peak apex.
4. Once images have been generated for all m/z values that match database entries, generate images for all other peaks as well to look for unique distribution patterns that can be investigated later.

## 5. Confirmation of the Identities of the Imaged Lipids

1. Confirm the identities of the high abundance lipids, which have characteristic fragment ions that can be detected using the FTICR instrument (*e.g.* 184.073 for phospholipids), by MALDI-MS/MS. Perform MALDI-MS/MS using collision-induced dissociation (CID) directly on the tissue.
2. For those lipid species that cannot be directly confirmed by MALDI-MS/MS, use a UPLC system coupled to a q-TOF mass spectrometer[34].
    1. Manually dissect aliquots containing ~10 mg of tissue from the area where the species of interest was localized. Place these tissue aliquots into 2 ml centrifuge tubes.
    2. Homogenize each tissue aliquot in 250 µl of water, using a mixer mill with two 5 mm stainless steel metal balls.
    3. Add 1 ml of chloroform-methanol solution (1:3, v/v), and vortex the tubes. Next, centrifuge the tubes using a microcentrifuge at 12,000 x g for 10 min.
    4. Collect the supernatants and dry them in a rotary speed-vacuum concentrator.
    5. Dissolve the residues in 100 µl of 30:70 isopropanol:water. Inject a 10-µl aliquot onto the UPLC column for separation using gradient elution.
    6. Use the chromatographic conditions for on-line lipid LC-MS/MS, which have been published previously[34].
    7. Generate extracted ion current (XIC) chromatograms using the theoretical m/z values, with a window of ±50 ppm around the theoretical masses.
    8. If authentic compounds for those lipids are available, match the retention times of the authentic compounds with those of the corresponding XIC peaks from the tissue samples. If the compounds are the same, the retention times and the MS/MS spectra should match.
3. If an authentic compound is unavailable, use the fragmentation pattern of the detected lipid to match a standard MS/MS spectrum from a metabolome database such as METLIN or HMDB. Use *de novo* mass spectral interpretation to determine a possible structure for the lipid.

## Representative Results

Tissue samples that are sectioned and thaw mounted onto the ITO coated glass slides should be intact, without visible tearing. For many tissues, direct tissue thaw mounting onto an ITO coated glass slide is acceptable. For some specific tissues such as bovine lens, extensive tearing of the tissue is often seen when direct thaw mounting is used (**Figure 1a**). Precoating of the ITO glass slide with ethanol or formic acid (**Figure 1b**) helps to maintain the integrity of the tissue sections during tissue mounting.

Both the choice of matrix and the selection of solvent are important factors influencing the quality of the MALDI spectra. When an appropriate MALDI MS spectrum is acquired from the tissue section, the mass spectrum is usually dense with lipid signals within the mass detection range

(**Figure 2a**). A matrix and a solvent should be chosen so that they have polarities similar to the analytes of interest, because the MALDI process requires a solid-phase solution of the analyte in the matrix crystals. Generally the best analyte signal intensities come from the use of MALDI matrices with solubilities similar to that of the desired analytes[41,42]. **Figure 2a** shows an example of a spectrum produced with an efficient matrix solvent (70% ACN with 0.01% TFA), and **Figure 2b** shows a poor choice of matrix and solvent (70% MeOH with 0.01% TFA) for dithranol.

One of the benefits of a dual-mode electrospray ionization (ESI)/MALDI ion source is that it allows the addition of ESI calibrant signals while simultaneously acquiring MALDI spectra without interfering with the ablation process. These ESI calibrant signals allow for internal mass calibration to provide high mass accuracy with mass error of <0.5 ppm[38]. As the ESI signal of the standard "ES Tuning Mix" solution can be an order of magnitude stronger than the MALDI signals of the analytes from the tissue, the ESI-derived calibrant signals must be attenuated. Calibrant signals should be visible and of sufficient intensity for calibration of the spectra, but should not dominate the spectra.

Once the set of mass spectra from a MALDI-MSI experiment has been acquired, the image for each of the detected ions can be generated, with each pixel representing a laser irradiation spot from the surface of a tissue section. Combining all of the individual pixels with different ion intensities across the tissue section from a MALDI MSI experiment reflects the ionization of target analytes within the tissue[1]. This can, in turn, provide information about the relative concentrations of the analytes in different portions of the tissue section (**Figure 3b**). Care must be taken in the processing of the data since many factors can affect what is seen and how the data is interpreted. In most experiments, the data is normalized to the total ion current (TIC) within each spectrum. Without this normalization, areas with better analyte-matrix co-crystallization (*i.e.* so-called "hot spots") could cause stronger signals for the analytes and this would skew the data by providing information that may not correlate well with the actual relative concentrations of the analytes (**Figures 3c-3d**).

Tissue preparation can also dramatically change the image that is generated. If the sample is "too wet" (*i.e.* too much solvent was applied), then the analytes will delocalize on the tissue and much of the spatial information will be lost (**Figure 3f**). The method of data acquisition is also important in the final image obtained. As MALDI experiments on untreated tissue sections are inherently "dirty", the sensitivity of the instrument may decrease over time. For short experiments this decrease may not be apparent; however, it can be a problem for longer experiments or particularly dirty samples. If data is acquired linearly across the sample this can lead to a locational bias as specific regions of the tissue section will be analyzed after the sensitivity of the instrument has decreased. Therefore, using random spots for all data acquisitions is recommended. Although this method takes more time, it helps to remove or minimize this bias in the data.

As shown in our previous paper[34], when compared to CHCA and DHB, DT enabled the detection of additional lipid species, while the lipids detected with CHCA and DBH could still be detected.

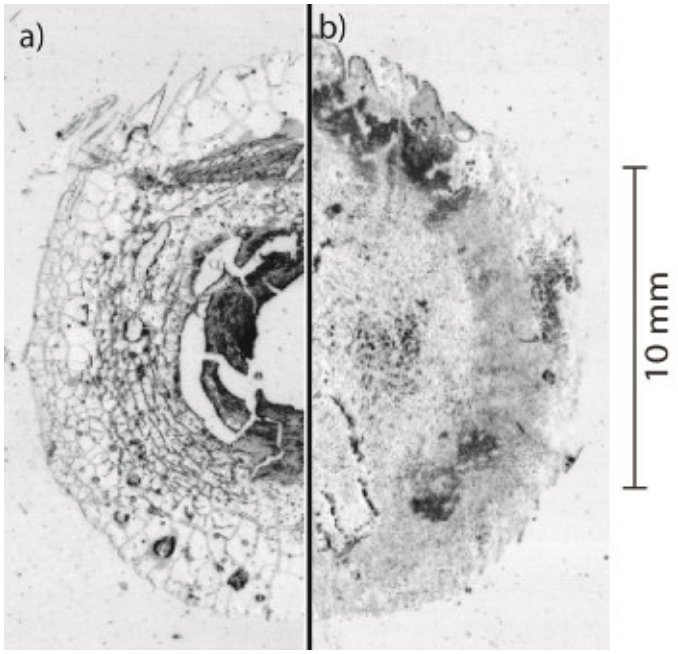


**Figure 1. Tissue mounting and cutting.** Comparative optical images of two bovine calf lens tissue sections (20-µm thick), without formic acid prewetting (**a**), and with formic acid prewetting (**b**), mounted on the ITO-coated glass slides.

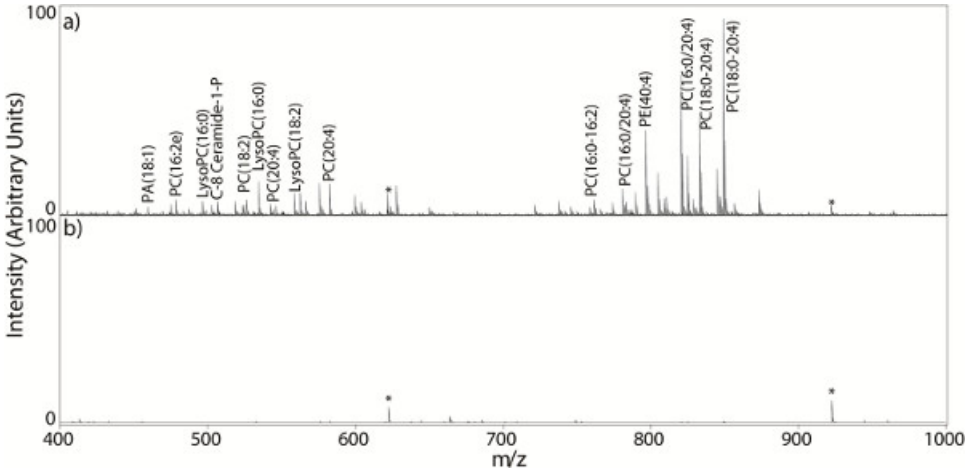


**Figure 2. Mass Spectra.** MALDI MS spectra acquired directly from a tissue section: **a**) an ideal densely populated mass spectrum with lipid signals (70% ACN with 0.01% TFA); **b**) a mass spectrum generated from a tissue section coated with a poor choice of solvent (70% MeOH with 0.01% TFA). Click here to view larger figure.

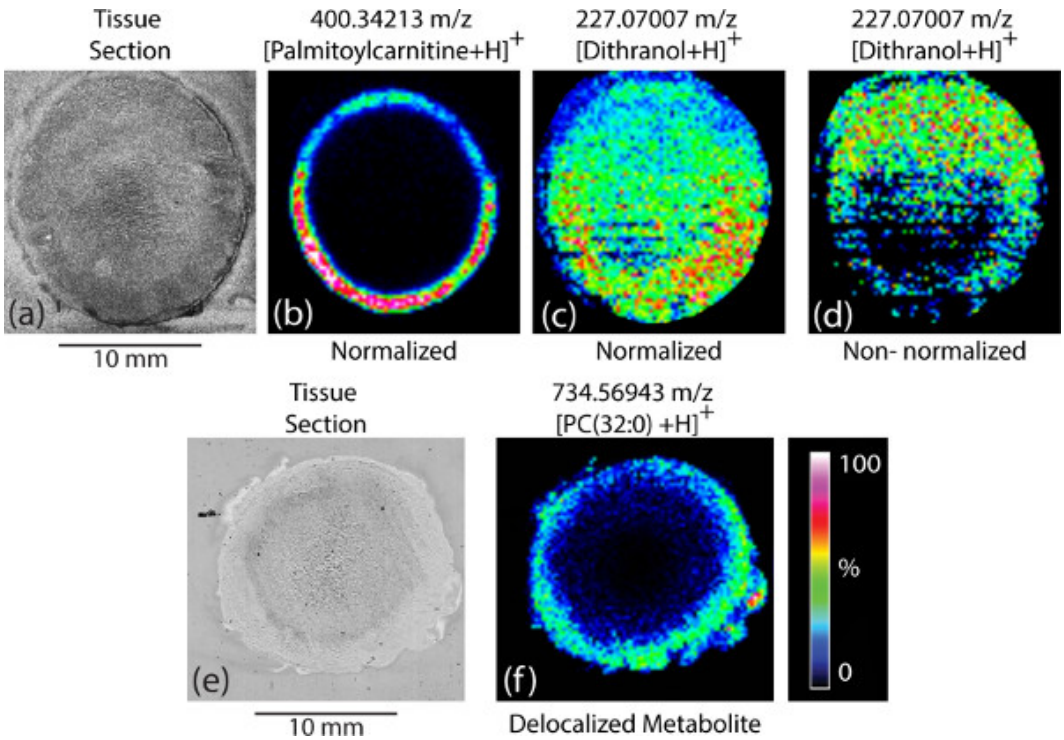


**Figure 3. MALDI MSI images.** Representative MALDI MSI images: **a**) a bovine lens tissue section; **b**) an MSI image of the same tissue section; **c**) an MSI image showing a background ion; **d**) a nonnormalized ion map of the same ion **e**) a bovine lens tissue section; and **f**) an ion map showing a partially delocalized analyte due to overwetting.

## Discussion

The most important considerations for successful MALDI MSI are: 1) tissue preparation; 2) matrix choice; 3) matrix application; and 4) data interpretation and analysis. When the sample and the matrix are appropriately prepared, the MS data acquisition is automated. The data analysis from this type of experiment is quite labor-intensive.

Appropriate tissue preparation is crucial for successful MALDI MSI experiments. The source of the tissue and the handling can have a large impact on the final analysis. The samples must be immediately flash frozen in liquid nitrogen and stored at -80 °C, and they should not be stored for an extended period of time, as some metabolites can be unstable even at -80 °C.

For many mammalian tissues, 10-15 µm thick tissue slices have been recommended for MALDI MSI. In these experiments, bovine calf lenses were cut equatorially into 20 µm thick tissue slices following lens decapsulation using a previously-described procedure[35]. The thicker bovine calf lens tissue sections were used because it was found difficult to maintain the integrity of a lens tissue section both during tissue cutting and during

tissue mounting. The lens is a spherically symmetrical tissue along its equatorial plane, so only slices which were close to, or at, the equatorial plane were collected.

Due to the difficulty in maintaining the tissue integrity during tissue cutting and mounting, prewetting using a solvent such as ethanol (for proteins and peptides[35]) or formic acid (for lipids) can be used. It should be also noted that, for protein and peptide imaging, the samples are often washed with an organic solvent to remove small molecules including lipids and OCT used for mounting; however, a washing step should only be done with solvents that do not dissolve the analytes of interest, and should be avoided for small molecule analysis.

The choice of matrix is also crucial for all MALDI experiments; however, on-tissue matrix performance may not be the same as matrix performance with a purified standard. For example, at minimum laser power, DT generated abundant DT-related background signals from the tissue-free matrix spots and these signals were assigned as the DT oligomers and their corresponding sodium and potassium adducts; however, on tissue, many of these signals were not observed, indicating that testing of different matrices on the specific sample of choice may be important in selecting the proper matrix for a given MALDI MSI analysis. DT is seldom used for lipid analysis due to the reported high background generated by the matrix; however, when compared with DHB and CHCA for the on-tissue profiling of lipids by MALDI-FTICR MS, DT produced favorable results. Thus this matrix could be a potentially useful matrix for MALDI tissue imaging using this instrument[34].

Efficient co-crystallization of the matrix and the analyte is a prerequisite for high-sensitivity MALDI-MS analysis. Thus the solid-phase solubility of an analyte in the matrix is important in the MALDI process[41,42]. MALDI matrices with solubilities similar to those of the desired analytes have been reported to produce the strongest analyte signal intensities. Because DT is a weak organic acid, as well as a very hydrophobic organic compound, based on classical Brønsted-Lowry acid-base neutralization theory[43] and the theory of solubility, it is expected to favor the ionization of positively-charged and less-polar compounds. In fact, we found that polar lipids dominated the detected compounds in the positive-ion mode when DT was used as the matrix.

The solvents used for preparing a matrix solution also play an important role in direct tissue analysis by MALDI-MS. The pH has been implicated as an important factor in the effectiveness of a matrix, and TFA is a common additive used with CHCA and DHB. However, with DT, the addition of an acid or base modifier had little effect on the resulting data. Because of the hydrophobicity of DT and its limited solubility in polar solvents, a lipophilic organic solvent is recommended. When analyzing lipids, a mixture of chloroform-methanol (2:1, v:v) with 1% formic acid, which is a typical organic solvent for lipid extraction, was used. We postulated that this provides better co-crystallization of lipids with DT. The lipophilic nature of the solvent may also prevent the solubilization of other compounds such as proteins and salts, as demonstrated in previous liquid extraction surface analysis mass spectrometry (LESA-MS) experiments[44]. This would lead to better crystallization of the matrix and lipids with fewer contaminants. The solvent system that is selected for analysis should maximize the solubility of the matrix and the desired analytes (lipids) while minimizing the solubility of unwanted contaminants (salts and proteins/peptides).

The coating of the matrix on the surface of the tissue should be as uniform as possible. To maximize the spatial resolution of the image, the crystal size should be as small as possible[45]. Using an electronic matrix sprayer, the matrix can be coated uniformly and reproducibly with a small crystal size. This is the preferred method in our laboratory. It is much preferred to manual application as it reduces spot size, homogeneity, and reproducibility. However, many of the solvents that are useful for MSI of small molecules are not compatible with the materials used in manufacturing the automated matrix sprayer. Although not yet implemented in our laboratory, sublimation based matrix application has been recommended for lipid analysis[22]. This method provides improved (*i.e.* reduced) spot size, homogeneity, and reproducibility and this should probably be the method of choice when the matrix selected is amiable to this method.

For coating of matrices containing solvents incompatible with the automated matrix sprayer (including chloroform), an alternative method of matrix coating is to use an air brush gun. For these matrix solutions, we used a pneumatically assisted airbrush sprayer. Although use of the air brush gun is not an ideal method, it may be the only method that can be used for solvents and matrices which are incompatible with the other methods, and- with training and experience- it can generate very uniform matrix coating. When applying the matrix solution manually, only the minimum amount of liquid must be applied during each cycle to barely wet the surface of the tissue. Too much liquid could potentially delocalize analytes due to the organic solvents. There are reproducibility issues with manual application and experience in coating the slide with this method is essential for success. Due to the manual nature of the air brush gun matrix application, care must be taken to ensure that a uniform coating is made; an inhomogeneous coating can lead to skewed data, which is representative of the matrix coating and not the analyte localization.

When conducting MALDI MSI experiments, initial identification of the detected analytes is usually based on metabolome database searching of the measured accurate masses which are usually only available when a high resolution instrument is used[38]. The dual ESI/MALDI ion source on the Apex-Qe 12-Tesla hybrid quadrupole-FTICR mass spectrometer allows for the addition of ESI-generated signals for use as the internal mass calibrant peaks to each MALDI mass spectrum, without affecting the MALDI desorption/ionization process. The use of internal mass calibration is crucial for high mass measurement accuracy in MALDI-FTICR. External calibration cannot fully take into account the space charge effects within the ICR cell[46-48]. The tuning and calibration of the FTICR is crucial for success. On this type of instrument a parameter called the "Time of Flight" (TOF), which is the time it takes for an ion to travel from the collision cell to the analyzer (the ICR cell) is one of the most important user-defined settings that influence the detection sensitivity of an FTICR instrument. Within a given mass range (*i.e.* m/z 200-1,400), a lower TOF favors detection of the lower m/z ions and a higher TOF favors detection of higher m/z ions. Thus, for high-sensitivity detection of both low and high m/z ions within the detection mass range, a TOF value of 9 msec is desirable.

For a practical experiment, a trade-off must be made between reasonable data acquisition size, mass resolution, and the time spent on acquiring MS imaging data. For an FTICR MS experiment, the size of the acquired data file and the mass resolution are dependent on the data acquisition size of the free induction decay (FID) signal. A higher data acquisition size will result in a higher mass resolution and a larger data file size. However, a higher data acquisition size also causes a slower MS scan rate. As a trade-off, it is recommended that a data acquisition size of 1,024 kb/sec be used on the FTICR. A lower data acquisition size and a corresponding lower mass resolution do not allow the separation of some isobaric ion species.

The laser raster step size must also be chosen so that it is small enough to provide a good pixel resolution for MS images of the analytes of interest; however, a very small raster step size can make the data files unmanageable considering an MS imaging data file is composed of

thousands of MALDI mass spectra. Given the relatively large size of bovine lenses, *ca.* 1.2 -1.5 cm in diameter, we used a raster step size of 250 µm. Using a 1,024 kb/sec data acquisition size and a 250 µm raster step size, our sample dataset was approximately 60 Gb. During the data acquisition, a random spot analysis should be used, as this prevents location-based bias due to gradual signal attenuation; however, random spot requires significantly longer time to acquire the data.

Because the MALDI MSI datasets acquired on our FTICR MS instrument include accurate mass data, the extracted m/z can be searched against metabolome databases such as METLIN and/or HMDB. Using a ±1 ppm window the initial assignments of many ion signals to metabolites are of high confidence. However, because many species have the same chemical formula, confirmation of the ID must often be done using alternate means. Thus, MS/MS experiments, and comparison of the MS/MS spectra with previously published data should be performed for a confident identification. MALDI-MS/MS spectra can sometimes be acquired directly on the FTICR instrument, but for many lipid molecules, their abundances and/or ionization efficiencies are insufficient for obtaining useful MS/MS data, and enrichment and purification prior to LC-MS/MS are required. In these analyses, most of the observed lipids were polar phospholipids, and were assigned as PCs, PEs, SMs, PSs, PGs, PAs, and ceramide phosphates (CerPs); other lipid molecules identified include sterol lipids, acyl carnitines, and glycerides. Based on the properties of the solvent and matrix, this is to be expected. The MS/MS spectra of PCs contain a prominent peak at m/z 184.073, which has been attributed to the polar PC head group, phosphocholine, as well as additional structurally important information, which can give unambiguous identification of the molecules. Additionally, using this method, many sterol lipids are detected; however, most cannot be unambiguously assigned to unique identities, even with MS/MS data. Strong potassium adducts generally predominate the spectra, but protonated and sodiated adducts can also be detected.

Because the MALDI MS process is only able to provide *relative* abundance information based on the local ionization efficiency within each pixel, care must be taken when interpreting MALDI MSI data without stable isotope labeled internal standards[49]. Furthermore, for confidence in the localization results, confirmation of any distribution patterns detected must be done using alternative methods. Performing LC-MS/MS on dissected tissue samples for confirmation is recommended.

Based on the measured accurate masses in the low mass range chemical formulas can be generated for a given m/z; within a 1 ppm mass error and allowing an unlimited number of C, H, O, and N, and a maximum of 2 S and 2 P often only a single elemental composition is possible. This m/z can also be searched against the HMDB and the METLIN databases, which may yield potential candidate compounds. Unfortunately, on the FTICR MS the low-mass cut-off is *ca.* 130 Da, which may make it difficult to directly perform MS/MS. Thus, confirmation using another system is often required.

Q-TOF LC-MS/MS experiments are commonly conducted on tissue samples which have been manually dissected from specific areas of the tissues of interest. Using the described lipid extraction method, XICs can be generated for the target compounds and MS/MS can be acquired for confirmation. Either comparison to an authentic standard or *de novo* structural elucidation is required before there can be a confident chemical assignment.

Dithranol has been used to explore lipid distribution patterns in bovine calf lens and also tested on rat liver, heart, and kidney tissues[34]. MALDI MSI can be used for the diagnosis of human disease states and it is already being used for pathologic analysis[50]. With the development of more robust and rapid technologies for MALDI MSI, the spatial localization of specific compounds may be useful information for a pathologist. Once an analyte can be routinely imaged using a MALDI MSI-based method, it can be used for diagnostic purposes. In fact, tissue imaging could be performed in a hospital setting, with an instrument located next to the operating room, where, as has been already demonstrated[51], it could be used to accurately determine the margins of tumors.

## Disclosures

We have nothing to disclose.

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