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TITLE:

Sample Preparation for Metabolic Profiling Using MALDI Mass Spectrometry Imaging

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KEYWORDS:

MALDI, Mass Spectrometry, Metabolomics, Sample Preparation, Metabolism, brain, metabolite, imaging Techniques

SUMMARY:

The goal of this protocol is to provide detailed guidance on the sample preparation when planning for experiments using MALDI MSI to maximize metabolic and molecular detection in biological samples.

ABSTRACT:

Metabolomics, the study to identify and quantify small molecules and metabolites present in an experimental sample, has emerged as an important tool to investigate the biological activities during development and diseases. Metabolomics methods are widely employed in the study of cancer, nutrition/diet, diabetes, and other physiological and pathological conditions involving metabolic processes. An advantageous tool that aids in metabolomic profiling advocated in this

paper is matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI). Its ability to detect metabolites in situ without labeling, structural modifications, or other specialized reagents, such as those used in immunostaining, makes MALDI MSI a unique tool in advancing methodologies relevant in the field of metabolomics. An appropriate sample preparation process is critical to yield optimal results and will be the focus of this paper.

INTRODUCTION:

Metabolites, the intermediate or end product of metabolism, including nucleotides, amino or organic acids, lipids, are key components to biological function and processes. Metabolomics, the study of metabolites, allows for the exploration of their biochemical interactions and the understanding of their roles in the context of basic, translational, and clinical research. Metabolites are strongly associated with the phenotypes of organisms and provide information on biochemical activities that occur during cellular metabolism[1]. Therefore, in addition to genomics and proteomics, metabolomics has emerged as an important tool in understanding both physiological and pathological conditions. For instance, metabolomics is used in elucidating the mechanisms behind existing drugs as well as their tolerance. In drug development, xenobiotic metabolism is useful in assessing the activity or toxicity of metabolites across species, which later translates to supporting personalized medicine[2]. Despite the broad application of metabolomics, imaging of metabolites can be challenging due to metabolites' chemical reactivity, structural heterogeneity, and broad concentration range[3]. However, the concentrations of labile metabolites including high energy compounds, glucose, lactate, glycolytic, pentose shunt pathway, and TCA cycle intermediates, phospholipids, neurotransmitters, signaling compounds, can change within seconds and progress over minutes when tissue enzymes are active during tissue harvesting procedures, such as postmortem ischemia in brain harvesting[4-6]. To ensure accurate metabolomics data acquisition, appropriate and careful sample preparation is critical[7,8]. Current established platforms for measuring metabolites include NMR, enzyme assays, and mass spectrometry (including liquid and gas chromatography), the last of which is discussed further below.

MALDI-MSI is a state-of-the-art technique that allows for the analysis of complex samples through the detection of individual molecular species. MALDI MSI grants the benefit of being able to quickly and reproducibly measure various molecular compounds in biological samples. Mass spectrometry imaging further allows for the production of images that represent tissue biology based on its composite metabolites, and does so while preserving the spatial distribution of the metabolites in the sample[9]. MALDI's ability to detect analytes in a sample without the use of antibody labeling, structural modifications, or other specialized reagents, such as those used in immunostaining, coupled with its ability to monitor hundreds of molecules within a single experiment comprise just a few of the advantages MS imaging grants when it comes to metabolic profiling[10,11]. In addition to commonly used matrix such as 2,5-dihydroxybenzoic acid (DHB) and 9-aminoacridine (9-AA), recently discovered novel matrix N -(1-Naphthyl) Ethylenediamine Dihydrochloride (NEDC), which is well-suited for analysis of various low molecular weight metabolites, has further improved the application of MALDI MSI in metabolic profiling[12].

Despite the broad application of MALDI MSI, the high cost of the instrument and the complexity of the experimental procedure obstacle its wider implementation in individual research laboratories. Therefore, most of the MALDI MSI studies are supported through shared core facilities. The sample preparation, including slide preparation and matrix coating, is the most critical step in MALDI MSI. However, the slide preparation is normally carried out in individual researcher's laboratory, which creates potential variations in later MALDI MSI acquisition. Here we aim to provide a detailed protocol for the sample preparation of biological samples before proceeding to MALDI MSI measurements, and use a metabolomic profiling of developmental mouse brain as an example.

PROTOCOL:

The protocol follows the guidelines of City University of New York (CUNY) Advanced Science research Center (ASRC)'s institutional animal care and use committee (IACUC).

1. Harvest the tissue

1.1. Prepare the aluminum boat. Prepare a rectangle aluminum 10cm x 20 cm, fold in the middle to make 10 cm double layer square. Label the sample information on one side, and fold the other side to form a boat with bottom surface about 4 cm x 4 cm.

1.2. Precool the boat on the liquid nitrogen (LN_2) in a Styrofoam box.

NOTE: If the boat is too small, the sample might fall out of the boat and become over frozen by directly contacting LN_2, which may result in fragmentation during cryosectioning.

1.3. Euthanize the animal by cervical dislocation following institutional IACUC guidelines, immediately dissect out the tissue of interest.

1.3.1. Keep the interval between animal anesthesia and snap freezing as short as possible, to minimize the alteration of metabolites during tissue harvesting, especially the labial metabolites in the brain due to postmortem ischemia.

NOTE: Perfusion the animal with phosphate-buffered saline (PBS) will increase the duration of ischemia, further altering the concentrations of labile metabolites and exaggerating the artifactual results. Therefore, perfusion or washing the tissue with PBS is not recommended, unless the contamination of blood or body fluid is more concerned than metabolites deterioration or washout for individual project.

1.4. Immediately place the tissue into the aluminum boat floating on liquid nitrogen, close the lid of Styrofoam box, and freeze for 2-10 min depending on the size of the tissue: 2 min, 5 min, 7 min for postnatal day 1 (P1), P21, P60 mouse brain, respectively, and 10 min for P60 rat brain.

NOTE: Do not freeze the tissue for prolonged time (e.g., 5 min for P1 mouse brain) as the over-frozen might lead to tissue fragmentation during cryosectioning.

1.5. Remove the boat by forceps, fold the foil to wrap the tissue, transport on dry ice and store at -80 °C for later use. If followed by immediate sectioning, carry the samples on dry ice to the cryostat.

NOTE: To better preserve the metabolites, it is preferred to store the sample in intact tissue and section it right before proceeding to MALDI imaging. The tissue can be stored in -80 °C for up to 24 months.

2. **Cryosection the tissue**

NOTE: Wear gloves at all times when handling the Indium tinoxide (ITO) slides. Avoid direct breathing onto the slide or wear masks (optional) to prevent the contamination of human saliva onto the tissue section.

2.1. Before sectioning the tissue, gather the desired number of MALDI compatible ITO coated glass slides.

2.2. Test the conductivity of the slide using a voltmeter set to resistance. Label the side where a resistance measurement is read: this will be the side that the tissue sections adhere to. Always place the slide on a clean paper towel to avoid contamination.

2.3. Pre-cool the slides in a cryostat set to -20 °C.

2.4. If tissue samples were removed from the -80 °C, let the tissue equilibrate in the cryostat chamber for about 45-60 min depending on the size of the tissue. If samples are removing from dry ice, equilibrate it for about 30 min.

2.5. Thoroughly clean the cryostat with 70% ethanol. Pre-cool all the necessary tools including thin-tipped artist brush and forceps in the cryostat chamber.

2.6. Set the temperature of the cryostat chamber and specimen head according to the type of the tissue: -14 °C for liver, -20 °C for muscle and -25 °C for skin[9].

2.7. Mount the tissue to the chuck using cryo tissue embedding compound OCT, avoiding OCT from the region of interest.

2.8. Place a clean blade in the stage and lock. Adjust the position of the stage and the angle of the specimen to achieve the desired cutting angle.

NOTE: If different types/genotypes of tissue are to be cut, be sure to either reposition the sample so that a clean part of the blade is used , or change to a new blade before cutting the next sample to prevent cross-contamination.

2.9. Continue cutting until the region of interest (e.g., corpus callosum in the brain) is found. Be sure to keep the stage clean by brushing off extra pieces with an artist brush that has been equilibrated in the cryostat.

2.10. Once the desired region is revealed, cut smaller sections of 10-12 µm thickness. If the section tends to flake or fall apart easily, raise the temperature of the cryostat, staying in the -22 °C to -11 °C range. We have found that the optimal cutting temperature for brain tissue is -15 °C to – 18 °C.

2.11. Once a good section has been cut, adhere it to the ITO slide (operate in the cryostat chamber).

2.12. Transfer one section of the tissue onto ITO slides using the tip of artist brush.

2.13. Finger-warm the section by placing a finger under the slide to warm up the section to ensure secure mounting. The tissue section will first turn to transparent in 5-10 s and then turn opaque in about 30-60 s.

2.14. Carefully set the slide aside in the cryostat.

2.15. Repeat steps for other tissue samples, ensuring that each treatment of the tissue is placed evenly on the slide and is as aligned as possible.

2.16. Since the MALDI target can hold up to two slides, place the sections from multiple samples of the same cohort onto a single slide or onto two slides, to ensure they can be analyzed at the same time.

2.17. When finished, place ITO slides in a vacuum box and transfer to a desiccator with desiccant. Vacuum-dry the slide for 45-60 min.

NOTE: If a vacuum desiccator is not available in the lab, keep the slides under -20 °C all the time to avoid metabolites deterioration.

2.18. Slide storage and shipping: unless the sample is prepared by the MALDI imaging core facilities to proceed directly to MALDI imaging, store the slides at -80 °C or ship to core facilities or other MALDI research laboratories on dry ice.

2.19. To best preserve the samples, place the slides into the slide transporter, fill it with nitrogen (optional), seal with parafilm, place into a zip bag, which is then placed into another zip bag containing desiccant. Label the outer zip bag.

2.20. Proceed to storage at -80 °C (up to 6 months) or shipping with adequate dry ice.

3. **Matrix Preparation**

3.1. Prepare the matrix.

NOTE: All the reagents must be HPLC grade.

3.1.1. Prepare NEDC at a concentration of 10 mg/mL. Prepare 10 mL of matrix dissolved in a solvent of 70% methanol (100 mg of NEDC, 7 mL of methanol, 3 mL of H_2O).

3.1.2. Additionally prepare an extra 10 mL of 70% methanol:water solution to flush the sprayer system before filling in the matrix.

3.2. Once the slides are dehydrated in step 2.17, place "X" marks on the blank space of the glass slide outside of the tissue sections using a bold point silver marker, and then place a second "x" with a fine point black marker on top of the silver "X". The black "X" with a sharp contrast out of the silver background (the bold silver "X") will later serve as fiduciary marker for the later mass spectra acquisition in MALDI instrument.

3.3. Load the slide into the MALDI slide metal target. Use plastic cover and draw/outline where the samples are onto the plastic cover. Set aside.

3.4. Scan the image of the slide together with the MALDI target using flatbed scanner. The screws on the surface of the target will serve as the spacer to prevent the damage or contamination of the tissue section by the scanner. Preview and scan the selected slide area in 16-bit grayscale and 2400 dpi. Save the image for later use in MALDI MSI.

4. **Matrix deposition**

NOTE: There are multiple methods to apply an even layer of matrix in fine crystal size on to the MALDI slide, including sublimation, droplet inkjet printing, manual spray using artist airsprayer, automatic matrix sprayer[9]. We will use automatic matrix sprayer as an example in this protocol for its high reproducibility.

4.1. Start up: Turn on the matrix sprayer unit, being sure that the valve is positioned at **LOAD** and launch the sprayer software. Check that the exhaust fan is operating well. Do not start the solvent pump if the active venting is not functioning properly.

4.2. Confirm on the **Comms** tab that everything is communicating, and then start the solvent pump at .1 mL/min, with a backpressure of ~500 psi (or 3.4 MPa).

4.3. Start compressed air flow to the matrix sprayer by setting the nitrogen tank to 30 psi. Then, adjust the pressure regulator on the front of the sprayer to 10 psi, and set sprayer nozzle temperature as desired.

NOTE: If the air pressure in prayer is lower than 5 psi, the sprayer nozzle will not be able to heat up for protection.

4.4. With the valve still in the **LOAD** position, use a syringe to flush the loop with 7 mL of 70% methanol, and then fill the loop with 6 mL of matrix.

4.5. Place the tissue slides into the holders in the sprayer, taping down both ends to prevent movement, and to preserve a matrix-free edge to avoid the contamination of the metal clamp on MALDI target by the matrix.

NOTE: Testing matrix spray on a blank microscope slide first before proceeding to precious sample slides is highly recommended.

4.6. Check that the flow rate and temperature are stable to begin spraying.

4.7. Select desired method pre-tested using blank glass slide.

4.8. Press **Start**. This will set the nozzle temperature and adjust the pump flow rate to match the selected method. Switch the valve to from **Load** to **Spray** position then confirm by clicking **Continue**.

4.9. Allow the system to run, which will take 5-20 min per slide depending on the method. Switch the valve to from **Spray** to **Load** position then confirm by clicking **Continue** when finished.

4.10. Remove the slide(s) from the sprayer, examine the pattern of matrix coating under microscope to ensure an even layer of fine matrix crystal.

4.11. After matrix deposition, place the slide(s) into the metal MALDI holder for immediate use. If there is only one specimen slide, add another blank slide to fill the two spaces of the MALDI holder.

4.12. Clean up the system immediately after the usage following the manufacturer's instruction, to prevent the clogging of sprayer nozzle.

4.13. After the sample slide is coated with matrix, either proceed immediately to MALDI imaging instrument, or ship it with dry ice using the same double zip bag preparation described in step 2.19.

NOTE: Under emergency circumstance such as the MALDI instrument is not available for immediate usage, store the coated slide in vacuum condition or at -20 °C for up to 24 hrs, although the deterioration of some metabolites might happen during the storage, which has not been thoroughly studied.

REPRESENTATIVE RESULTS:

The representative experiment was performed according to the workflow shown in **Figure 1**. The developmental C57BL wildtype mouse brains of postnatal day 2, 21, 60 (adult) were harvested as described above following CUNY IACUC guidelines and were snap frozen for 2, 5 and 7 min, respectively, on an aluminum boat floating on liquid nitrogen. The frozen tissues were cryosectioned at 10 µm thickness sections at −15 °C set for both specimen head and the chamber. The tissue cryosections were then gently transferred onto the pre-cooled conductive side of ITO-coated glass slides for MALDI imaging. Mounted cryosections on ITO slides were desiccated in vacuum for 45 min at room temperature, followed by matrix deposition using automatic matrix sprayer. Matrix NEDC was used to detect metabolites, and a matrix solution of 10 mg/mL in methanol/water (70/30, v/v) was deposited at a flow rate of 0.1 mL/min and a nozzle temperature of 75 °C for 12 cycles with 5 s drying between each cycle. A spray velocity of 1300 mm/min, track spacing of 2 mm, N_2 gas pressure of 10 psi and flow rate of 3 L/min and nozzle height of 40 mm was used.

MALDI mass spectra were acquired in negative ion mode by MALDI time of flight (TOF) MSI instrument. 0.5-1 µL of red phosphorus (Pn clusters with n = 1 – 90) emulsion in methanol was deposited onto the ITO slides, next to the mounted tissues, and used to calibrate the instrument in the 100 – 1000 m/z mass range by applying quadratic calibration curve[13]. The laser spot diameters were focused to “Medium” modulated beam profile for 50 µm raster width. Spectra within the mass range from m/z 50 to 1000 were acquired at a 1000 Hz for 500 shots. Mass spectra data were recorded, and the imaging was further analyzed using advanced MALDI MSI data analysis software. Ion images were generated with root-mean square (RMS) normalization at a bin width of ±0.25 Da.

The results in **Figure 2** show output images from MALDI MSI data analysis software of m/z spectra selected from every 100 Dalton interval, clearly depicting the utility for identification of spectra from small molecule metabolites to high molecular weight lipids. Each row depicts respective ion heat maps containing both spatial and spectral information of a certain metabolite species across three tissues collected at postnatal day 2, 21, and 60. For each representative m/z, analysis of regional distribution and ion abundances can be used to compare relative amounts of corresponding species between different ages. A strength of the MALDI MSI methodology is the ability to discern the specificity of certain species identified by m/z to developmental milestones or specific anatomical structures. Some metabolites are observed to be enriched in P1 neonates (m/z 320.1), enriched in P60 adults (m/z 846.5), or uniformly distributed across ages (m/z 480.3); other molecular species are specifically enriched in gray matter (m/z 117.1; 524.3; 765.1), white matter (m/z 673.4; 906.6), or CSF/ventricles (m/z 239.0) (**Figure 2A**). The spatial distribution of representative metabolites including hypoxanthine (m/z 135.0), Glutamic acid (m/z 146.1), N-Acetyl-L-aspartic acid (m/z 174.0), Arachidonic acid (m/z 303.2), and several lipids such as lysophosphatidylethanolamine LPE (18:1) (m/z 478.3), LPE(20:4) (m/z 500.3), LPE (22:6) (m/z 524.3), phosphatidylethanolamine PE (44:10) (m/z 838.5), Phosphatidylinositol PI(38:4) (m/z 885.6) are also shown (**Figure 2B**).

FIGURE AND TABLE LEGENDS:

Figure 1. Workflow of MALDI- time of flight (TOF) mass spectrometry imaging. The snap frozen tissue is cryrosectioned in cryostat and mounted on ITO slide. →The slide with tissue sections is

coated with a fine layer of matrix using automatic matrix sprayer. → Mass spectra is collected by MALDI-TOF MSI instrument at a raster of 20-200um. → The data is analyzed, and the images are generated using advanced MALDI MSI data analysis software. Scale bar: 2 mm.

Figure 2. Representative output with selected m/z spectra from mass spectrometry acquired at 50 µm lateral resolution. (**A**) A heat map depicts the spatial distribution of specific metabolite species selected from every 100 Dalton m/z interval across the three developmental milestones identified at P1, P21 and P60. (**B**) The spatial distribution of representative metabolites at P1, P21 and P60, including: hypoxanthine, Glutamic acid, N-Acetyl-L-aspartic acid, Arachidonic acid, and different lipid species such as lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE), Phosphatidylinositol (PI). Scale bar, 500 µm.

DISCUSSION:

MALDI-Imaging (MALDI-MSI) is a label-free imaging technique that allows researchers to investigate the distribution of various biomolecules and their modifications in tissue, the molecular basis of pathology. Combined use of MALDI-MSI with traditional LC-MS approaches for tissue analysis provides the same molecular depth as traditional Omics workflows but which also retains the spatial relationship of those signals within the cellular network. The sample preparation is the most critical step in MALDI MSI and accounts for the variation in the final read outs of metabolomics studies carried out in different labs[4]. Here we provide a comprehensive yet practical protocol to standardize the sample preparation for metabolomic profiling using MALDI MSI, in the hope it will benefit a broad research community to implement MALDI MSI in their current and future research from basic biology to translational studies.

One must always bear in mind all precautions to minimize changes in the molecular profiles (both abundances and spatial distribution) during sample preparations and avoid contamination. Firstly, minimize the time between animal euthanasia and tissue harvesting, such as frozen in situ or heated by microwave fixation to inactivate enzymes in the brain to reduce the liability to postmortem ischemia[4-6]. Secondly, the snap freezing condition of the sample is critical. Inadequate freezing will cause the degradation and loss of the metabolites, while over freezing will lead to tissue fragmentation during cryosectioning. The freezing time should always be tested first according to previous reported studies, and the study of developmental mouse brain presented in this paper provides the reference points for rodent brain tissue. Thirdly, cutting of biological tissues and transferring their sections to the ITO slides will require adequate practice. It is important to note that while using the brush to pick up the section from the stage, the brush should be used delicately. Allow the bristles of brush to only come in contact with the edges of the tissue section to decrease the risk of contamination and section fragmentation. Flatten the section on the slide as much as possible, this will prevent the curling of the section during finger-warming. Fourthly, while mounting onto the ITO slide, make sure the entire section is well attached to the ITO slide as different regions of the tissue might require different time of finger-warming. For example, brain tumor tissue requires longer warming time than normal brain tissue. A poor mounting might lead to detachment and fragmentation of the tissue during MALDI-MS scanning. Bear in mind that the finger warming might enable enzyme action and metabolism causing artifactual changes of metabolites. Fifthly, a fine and uniform deposition of MALDI matrix plays an important role in achieving accurate spatial information and strong MALDI-MS signal. It is recommended to test the matrix spraying on a blank slide, and observe the crystal pattern under a microscope to verify proper coverage, before proceeding to the coating of precious specimen slide. And lastly, since an individual researcher executes the tissue harvesting and slide preparation at different speed, it would be ideal to have one person to handle the sample preparation for the specimen in the same cohort, to minimize the variation.

The protocol provided above details standard procedures, which can be tailored towards the needs of particular experiments. For example, a cryo-sectioning gel OCT, which is normally used in cryo-sectioning of the sample, can be further utilized as a mounting glue to the tissue chuck (as in the study described above). Prior studies have shown that the polymer component in OCT causes strong ion suppression[14]. However, embedding the sample may be unavoidable in the cases where the tissue is too fragile to be cut without additional support from a polymer gel. In order to combat signal suppression in these cases, the tissues may need to be washed with serial washing in 70% ethanol and 95% ethanol to remove residual OCT for the detection of proteins or lipids, while the washing is not recommended for the detection of small molecular metabolites[9].

MALDI MSI has grown increasingly relevant in both the research laboratory and clinical practice setting. For instance, MALDI MSI has recently proved useful in studies of proteomics in order to characterize the phenotypic functional status of an organism[15], and in acting as an agent for microbial identification and diagnosis of subsequent disease[16]. While MALDI MSI supports a wide range of applications, there are some limitations associated with relying solely on this technique, especially when it comes to differentiating between similar species or metabolites, and the identification of specific targets. Another challenge is the quantification of the metabolites concentration according to MALDI MSI signals. It is often assumed that ion abundances in MALDI MSI spectra and the spatial distribution (or relative abundances) of corresponding molecular species across dissected tissues are well correlated. However, one should always bear in mind that the relationship between ion intensity and the amount of corresponding molecular species is complicated by numerous factors including, but not limited to, effects of ion suppression, changes in tissue structure, ion-molecule reactions[17]. Techniques that make use of internal standards can be implemented for absolute quantification (µmol/g tissue) in MALDI-MSI[18]. These two challenges are typically addressed with the combined workflow of MALDI MSI with liquid chromatography tandem MS(LC-MS/MS) techniques, whereby MALDI-MS allows for the mapping of the region of interest, which is later subjected to microextraction and LC-MS/MS to provide more information for identifying the metabolite[19].

MS-based imaging methods have been developed in recent years as an alternative modality to previous techniques for imaging small molecule metabolites. With the advancements and growing popularity of MALDI MSI, it is expected that MALDI imaging will become a new standard tool for visualizing small molecules. Imaging of lipid and endogenous small molecules (e.g. neurotransmitters and metabolites) in the biological context, as well as imaging of xenobiotics for new pharmaceutical agents development are of particular interest. These three areas are expected to have rapid advancements with application of MALDI MSI in the near future[20].

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DISCLOSURES:

The authors declare no competing financial interests.

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Figure 1

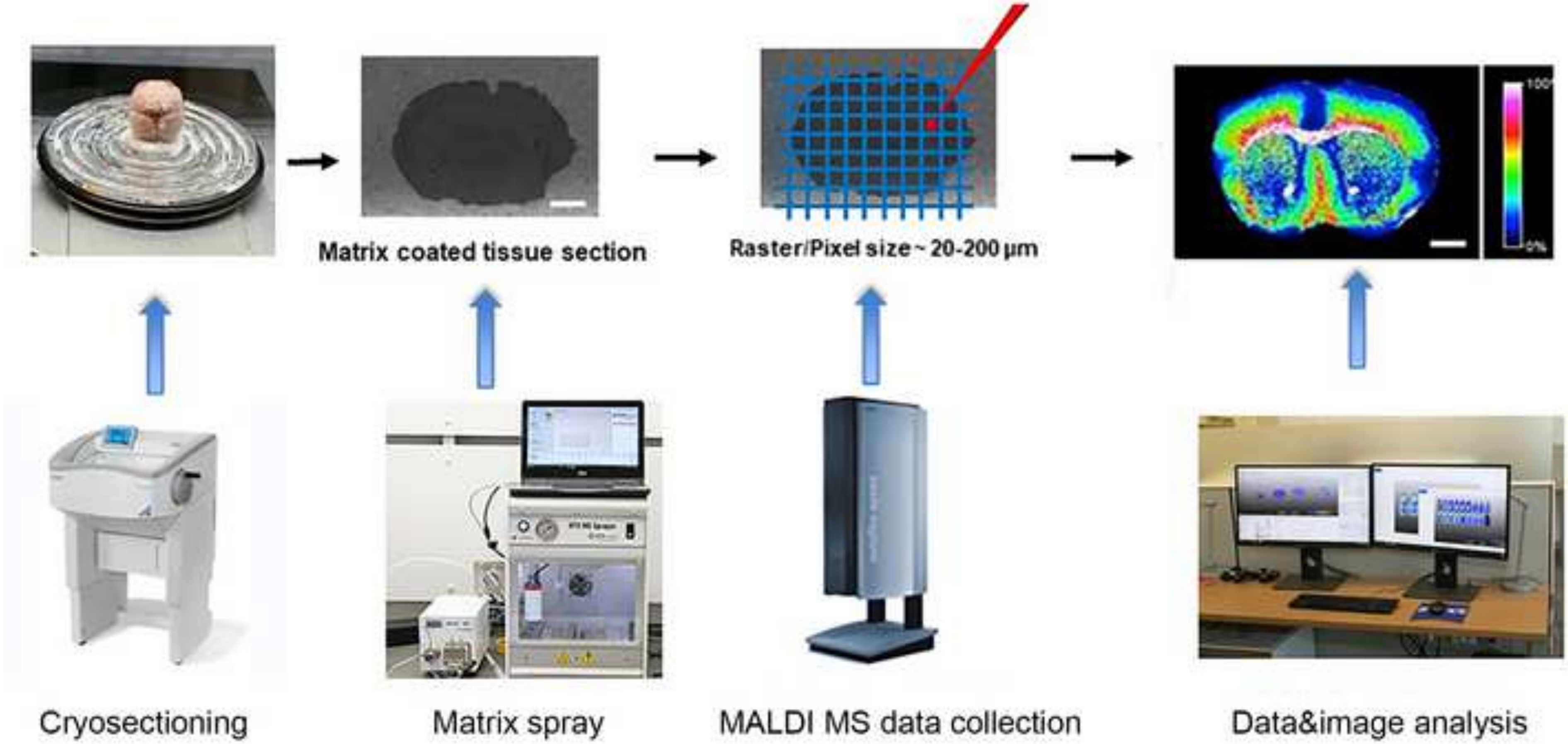
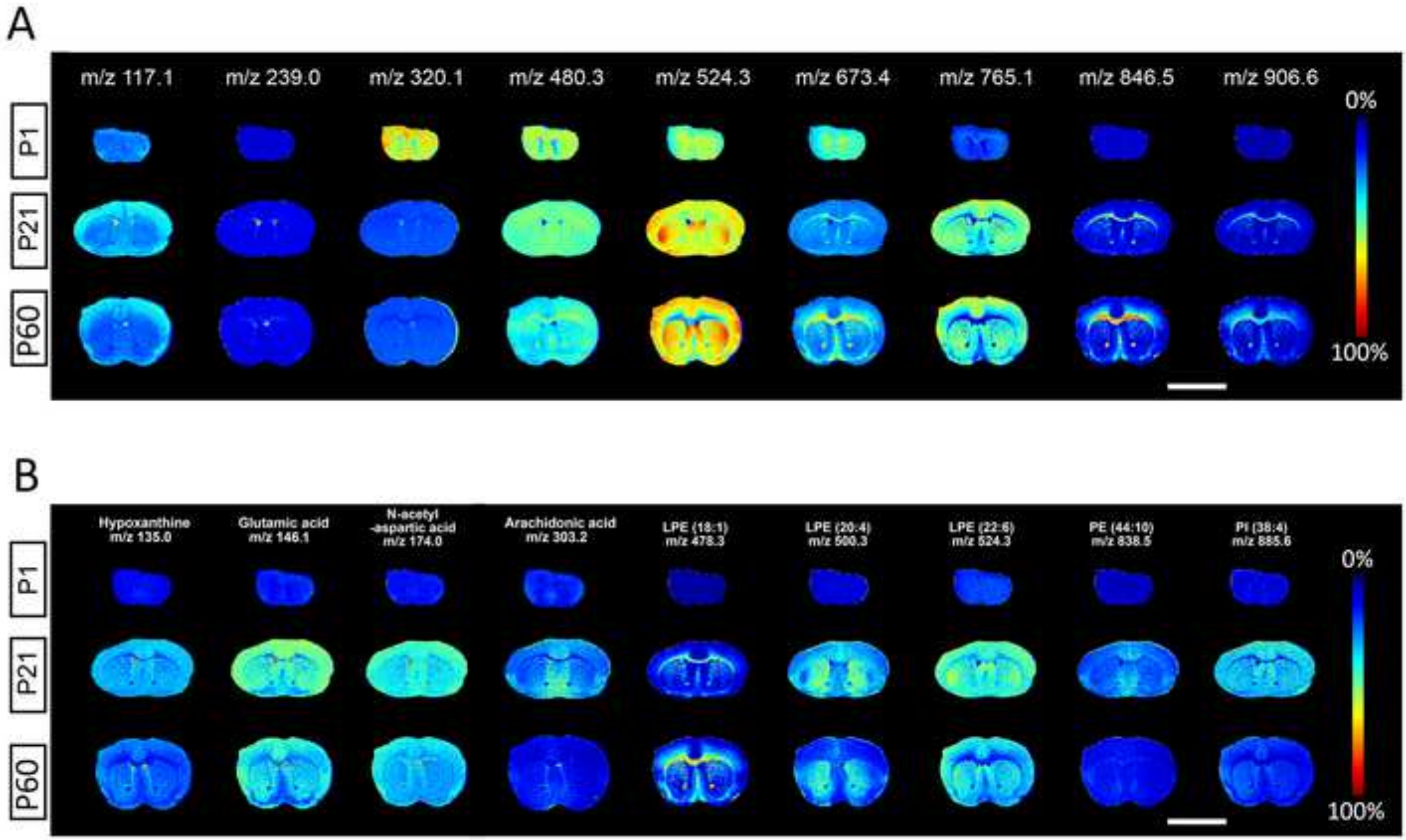

Matrix coated tissue section
Raster/Pixel size ~ 20-200 µm
100%
0%
Cryosectioning
Matrix spray
MALDI MS data collection
Data&image analysis

Figure 2


A
m/z 117.1
m/z 239.0
m/z 320.1
m/z 480.3
m/z 524.3
m/z 673.4
m/z 765.1
m/z 846.5
m/z 906.6
P1
P21
P60
0%
100%
B
Hypoxanthine m/z 135.0
Glutamic acid m/z 146.1
N-acetyl -aspartic acid m/z 174.0
Arachidonic acid m/z 303.2
LPE (18:1) m/z 478.3
LPE (20:4) m/z 500.3
LPE (22:6) m/z 524.3
PE (44:10) m/z 838.5
PI (38:4) m/z 885.6
P1
P21
P60
0%
100%

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Andwin Scientific Tissue-Tek CRYO-OCT Compound	Fisher Scientific	14-373-65	
Artist brush MSC #5 1/8 X 9/16 TRIM RED SABLE	Fisher Scientific	50-111-2302	
Autoflex speed MALDI-TOF MS system	Bruker Daltonics Inc		MALDI-TOF MS instrument
BD Syringe with Luer-Lok Tips	Fisher Scientific	14-823-16E	
BD Vacutainer General Use Syringe Needles	Fisher Scientific	23-021-020	
Bruker Daltonics GLASS SLIDES MALDI IMAGNG	Fisher Scientific	NC0380464	
Drierite, with indicator, 8 mesh, ACROS Organics		AC219095000	
Epson Perfection V600 Photo Scanner	Amazon	Perfection V600	
Fisherbrand 5-Place Slide Mailer	Fisher Scientific	HS15986	
Fisherbrand Digital Auto-Range Multimeter	Fisher Scientific	01-241-1	
FlexImaging v3.0	Bruker Daltonics Inc		Bruker MS imaging analysis software
HPLC Grade Methanol	Fisher Scientific	MMX04751	
HPLC Grade Water	Fisher Scientific	W5-1	
HTX M5 Sprayer	HTX Technologies, LLC		Automatic heated matrix sprayer
Kimberly-Clark Professional Kimtech Science Kimwipes Delicate Task Wipers	Fisher Scientific	06-666A	
MSC Ziploc Freezer Bag	Fisher Scientific	50-111-3769	
N -(1-Naphthyl) Ethylenediamine Dihydrochloride (NEDC)	Millipore Sigma Aldrich	222488	
SCiLS Lab (2015b)	SCiLS Lab		Advanced MALDI MSI data analysis software
Thermo Scientific CryoStar NX50 Cryostat	Fisher Thermo Scientific	95-713-0	
Thermo Scientific Nalgene Transparent Polycarbonate Classic Design Desiccator	Fisher Scientific	08-642-7	

Point-by-point response to reviewer comments

We thank all reviewers and editors for their thoughtful comments. Here we provide our point-by-point response.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

→ We have thoroughly proofread the manuscript and define all the abbreviations at the first use.

2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 234 ('medium"…)-238 (…v3.0)

→ We have revised the text.

3. As you will need to show this protocol for filming the video, please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's animal research ethics committee.

→ We have added the statement "The protocol follows the guidelines of City University of New York (CUNY) Advanced Science research Center (ASRC)'s institutional animal care and use committee (IACUC)."before the numbered protocol steps.

4. 1.3: Please specify the euthanasia method, but do not highlight it.

→ We have specified "cervical dislocation" as the method of animal euthanasia

5. 1.4: What do you mean by perfusion with PBS is not necessary? Do you mean the tissue need not be washed with PBS? Please clarify. Are there indicators that readers/viewers can rely on to ensure that the tissue is not "over-frozen"?

→ As reviewer#1 also pointed out that perfusion with PBS may cause metabolite washout, we have changed the "perfusion with PBS is not necessary" to "perfusion the animal with phosphate-buffered saline (PBS) or washing the tissue with PBS is not recommended, unless the contamination of blood or body fluid is more concerned than metabolites deterioration or washout for individual project."

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Kimwipes, Drierite, TM sprayer, HTX M5 Sprayer, HTX Technologies, LLC, Bruker ImagePrep, Bruker Autoflex Speed, FlexImaging, SciLS, etc

→ We have modified the text according to the policy.

7. 2.6: What do you mean by “set the temperature…the tissue8”?

→ “8” is a typo, should be superscripted for reference #8.

8. 2.10: Please give an example of “region of interest” in a tissue sample.

→ “Corpus callosum” has been added as an example of “region of interest”.

9. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

→ Done

10. As we are a methods journal, please add the significance of this technique with respect to existing methods to the discussion.

→ We have added the significance to the discussion part: “MALDI-Imaging (MALDI-MSI) is a label-free imaging technique that allows researchers to investigate the distribution of various biomolecules and their modifications in tissue, the molecular basis of pathology. Combined use of MALDI-MSI with traditional LC-MS approaches for tissue analysis provides the same molecular depth as traditional Omics workflows but which also retains the spatial relationship of those signals within the cellular network. The sample preparation is the most critical step in MALDI MSI and accounts for the variation in the final read outs of metabolomics studies carried out in different labs[4]. Here we provide a comprehensive yet practical protocol to standardize the sample preparation for metabolomic profiling using MALDI MSI, in the hope it will benefit a broad research community to implement MALDI MSI in their current and future research from basic biology to translational studies.”

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.

→ Fixed

12. Figure 1: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

→ Scale bar added to figure 1.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors provide a step-by-step protocol to process tissue samples for metabolic imaging.

Major Concerns:

While instructive, the authors fail to address the absolutely critical aspect of tissue harvest, postmortem ischemia. The procedures described in this article are inadequate for this purpose and will lead to artifacts that invalidate the analysis. The authors must discuss in detail the fact that concentrations of labile metabolites can change within seconds and progress over minutes when tissue enzymes are active. Labile metabolites include high energy compounds, glucose, lactate, glycolytic, pentose shunt pathway, and TCA cycle intermediates, phospholipids, neurotransmitters, signaling compounds, and other metabolites.

This means that the tissue must be frozen in situ or heated by microwave fixation to inactivate enzymes. Subsequent processing will depend on the enzyme inactivation method. Several papers have discussed aspects of this problem and these and other relevant reviews need to be cited. Most references below refer to brain, but ischemia applies to all tissues that must be harvested by freeze clamping to prevent ischemic changes. There are more reviews dealing with this issue for heart and liver and other tissues and other classes of compounds.

Dienel GA. Metabolomic and Imaging Mass Spectrometric Assays of Labile Brain Metabolites: Critical Importance of Brain Harvest Procedures. Neurochem Res. 2020 Sep 19. doi: 10.1007/s11064-020-03124-w.

Dienel GA. Metabolomic Assays of Postmortem Brain Extracts: Pitfalls in Extrapolation of Concentrations of Glucose and Amino Acids to Metabolic Dysregulation In Vivo in Neurological Diseases. Neurochem Res. 2019 Oct;44(10):2239-2260. doi: 10.1007/s11064-018-2611-y.

Wasek B et al. The use of microwave irradiation for quantitative analysis of neurotransmitters in the mouse brain. J Neurosci Methods. 2018 Sep 1;307:188-193. doi: 10.1016/j.jneumeth.2018.05.016.

Andres, DA et al. Improved workflow for mass spectrometry-based metabolomics analysis of the heart J Biol Chem. 2020 Feb 28;295(9):2676-2686. doi: 10.1074/jbc.RA119.011081.

→ We are grateful to reviewer#1's constructive comments, and have added the suggested reference to the manuscript.

Minor Concerns:
The manuscript would be improved by addressing the following points:

1. line 66 - include lability to postmortem ischemia as indicated above.
→ We have added the information of postmortem ischemia to the introduction.

2, line 105-11. These instructions need to incorporate the issue of postmortem ischemia and address effects of anesthesia. Note that perfusion with PBS may cause metabolite washout and it increases the duration of ischemia, further altering the concentrations of labile metabolites and exaggerating the artifactual results.
→ We have added the information to step 1.3 according to reviewer#1's valuable suggestion.
3. define all abbreviations at first use: PBS, line 110; ITO line 119; OCT, line 134; NEDC, line 170, and so on.
→ Fixed.
4. line 133 - what does the 8 refer to?
→ "8" is a typo, should be superscripted for reference #8.

5. line 159-160. Placing slices in a vacuum box at room temperature will allow active enzymes to metabolize tissue components. Since the tissue has now been freeze-thawed, cell membranes will be broken, and subcellular and cellular compartments will be disrupted, allowing more artifactual metabolism of metabolites, lipids, proteins, nucleic acids, and other compounds. Here the slides must be kept at -20oC or below to prevent this problem.

→ We thank reviewer pointing out the potential issue with placing slide in vacuum box. If it is intact cell tissue, we would also recommend keeping frozen at -20 °C. However, after cryosectioning into 10-12 um slices, most of the cells (>20 um diameter) in the sections will not be able to retain intact and will have the cell contents exposed. Therefore, we recommend to dehydrate the slide immediately to preserve the metabolite before shipping or storing at -80 °C. We have added in step 2.18 :"NOTE: if vacuum desiccator is not available in the lab, keep the slides under -20 °C all the time to avoid metabolites deterioration."

This issue also applies to the text on lines 218-230 and 274. Snap freezing does not prevent postmortem ischemia that takes place between death and tissue freezing. The duration is critical and the longer it is the greater the magnitude of artifactual changes. This is a critical point that must be strongly emphasized. Line 283 - Finger warming could be expected to enable enzyme action and metabolism. Caution here must be noted.

→ We have added the notes about the importance of timely operation and caution of postmortem metabolites changes during finger warming and elaborated in the discussion.

6. line 229 subscript for N2

→ Fixed

7. line 233 - explain how MS spectra are calibrated with red phosphorus.

→ 0.5-1 uL of red phosphorus (Pn clusters with n = 1 – 90) emulsion in methanol was deposited onto the ITO slides, next to the mounted tissues, and used to calibrate the instrument in the 100 – 1000 m/z mass range by applying quadratic calibration curve (Sladkova et al., 2019).

8. line 300. washing tissue with ethanol (state the concentration or % in what other solvent) may be expected to wash out some soluble metabolites and cause losses that are not taken into account.

→ We have added "washed with serial washing in 70% ethanol and 95% ethanol to remove residual OCT"

9. line 244-246 and 260-266. Explain clearly what is relative abundance - relative to what? How are these relative values calibrated? How are they quantified? Quantification in relative terms is very ambiguous, it does not provide any information about the absolute concentrations (umol/g tissue) of any metabolite or among metabolites, and does not allow evaluation of validity of the measured concentrations. These are critical issues that can completely undermine the usefulness of MS imaging (see cited references above). A careful, detailed discussion of quantification of these data is required.

→ We have added detailed discussion about data quantification in the discussion part: "It is often assumed that ion abundances in MALDI MSI spectra and the spatial distribution (or relative abundances) of corresponding molecular species across dissected tissues are well correlated. However, one should always bear in mind that the relationship between ion intensity and the amount of corresponding molecular species is complicated by numerous factors including, but not limited to, effects of ion suppression, changes in tissue structure, ion-molecule reactions (Hankin et al., 2010). Techniques such as using internal standards can be implemented for absolute quantification (µmol/g tissue) in MALDI-

MSI (Prentice et al., 2017). These two challenges are typically addressed with the combined workflow of MALDI MSI with liquid chromatography tandem MS(LC-MS/MS) techniques, whereby MALDI-MS allows for the mapping of the region of interest, which is later subjected to microextraction and LC-MS/MS to provide more information for identifying the metabolite (Quanico et al., 2017)."

10. Fig. 2 has a color scale from 0-100%. This is useless and provides no information about the absolute or even relative concentrations of different compounds. What is 0 and what is 100%? How are the various metabolites related to each other among the different images? Identify the metabolites with the m/z values provided.

→ The heat map represents the relative abundance of individual metabolites across the samples, or relative abundance of different metabolites in the same anatomical regions. We have added additional representative metabolites in figure 2B, including hypoxanthine (m/z 135.0), Glutamic acid (m/z 146.1), N-Acetyl-L-aspartic acid (m/z 174.0), Arachidonic acid (m/z 303.2), and several lipids such as lysophosphatidylethanolamine LPE (18:1) (m/z 478.3), LPE(20:4) (m/z 500.3), LPE (22:6) (m/z 524.3), phosphatidylethanolamine PE (44:10) (m/z 838.5), Phosphatidylinositol PI(38:4) (m/z 885.6).

Reviewer #2:

Manuscript Summary:

This manuscript describes basic technology for the beginners of MALDI-based imaging mass spectrometry (MALDI-IMS). In analyzing tissue samples using MALDI-IMS, the most critical step must be sample preparation.

Authors are well trained for this technology and each chapters are properly described.

→We are very grateful to the reviewer's highly positive comments of our protocol and work.

Major Concerns:

Altough this draft is accurate and helpful for the beginners of MALDI-IMS, advancements of the field is so rapid and the latter part of this manuscript is only available for those whose laboratory is equipped with the same MALDI MS machine as well as TM-Sprayer, and even an analytical software must be identical. Furthermore, any specialized knowledge to avoid post mortem deterioration of the samples were not described.

→ We thank reviewer#2 for the valuable comments. The issue to avoid post mortem deterioration of the samples has been addressed together with reviewer#1's comments.

We do aware that MALDI MS machine and TM sprayer are not common for general biomedical research labs. Based on our interactions with the users and with other MALDI imaging facilities, we would speculate that for 100% MALDI imaging, ~80% of the slide preparation are carried out in individual research lab, ~50% of matrix spray are using TM-Sprayer, and ~30% of MALDI imagined are performed in Bruker MALDI machine (with several models available, and maybe only ~10% or less are using the same model Autoflex as our core). Therefore, our protocol covers 4 parts: Part 1&2 targets general MALDI imaging users, and part 3&4 is for more specialized audience: staff in the MALDI imaging core or laboratories, and users would like to do self-operation in MALDI imaging core.

Minor Concerns:
108: freeze for 2-10 min : is hard to follow. Are there any indices to settle an adequate time.

→ We added the example of "2 min, 5 min, 7 min for postnatal day 1 (P1), P21, P60 mouse brain, respectively, and 10min for P60 rat brain."
110: for prolonged time: How long? Are there any help to know an exact time?

→ We added an example of 5 min for P1 mouse brain would be prolonged, which will cause the fracture of the frozen tissue and fragmentation during cryosection according to our experience. The optimized time for each specimen depends on the type and size of the tissue, which would be best tested and determined by individual users. However, here we provided some examples of rodent brain and hope it can be served as a reference for other type of tissues.

117: Is it true?? 3-6 months must be so diverse and it depends on the analytes.

→ Indeed, the sooner the specimen be processed, the better the MS signal. However, in many circumstances, the MALDI instrument will not be available immediately, and the sample has to be stored at -80 °C for a few months. According to our experience, some tissue deteriorates faster, e.g. fly brain vs. mouse brain; so does some analytes, e.g. labile metabolites vs. lipids.

130: ethanol: what percentage of this? → 70% ethanol.
137: 90-degree cutting angle: exactly 90 degree?

→ We changed it to "desired cutting angle" to avoid confusion. The cutting angle will depend on the placement angle of the specimen.

183: Fleximaging is a commercial based name of the software, which should be avoided in the text. In the loatter part of this manuscript, this problem is often found.
such as Brauker Autoflex Speed (232)
Flex Imaging (237)
SCiLS(238)
→ We have modified the text according to the policy.

Reviewer #3:
Review of Veerasammy et al "Sample preparation for Metabolic Imaging using MALDI Imaging Mass Spectrometry.

Veerasammy et al demonstrates a method for MALDI IMS sample prep for analysis of small metabolites on a Bruker Autoflex. In particular, the authors detail a protocol that they have adapted for sample prep of C57BL mouse brains. While their protocol is suitable for someone who has prepped MALDI IMS samples before, more details are needed to be a stand-alone protocol for a new MALDI IMS user who then could adapt it to their lab.
→ We are grateful to reviewer's valuable comments below to greatly improve our protocol to be served as a detailed guidance for MALDI imaging beginners.

Major Comments:
* What metabolites can be seen with this method? Smaller lipids? What species of lipids do the m/z values seen by this method correlate to?

→ Ceramides and phosphor-lipids can be detected in this set of experiment. However, the representative result we used in this manuscript does not include a thorough lipidomic study. In another study ongoing in our facility, MALDI imaging can detect DAG, TAG, PE, PC and PS.

* Throughout the protocol, the authors switch between brain and tissue. For consistency and application to other labs, change all "brain" to "tissue." →Fixed.
* Is "finger warming" required for the protocol? In other MALDI IMS protocols, this is not included in sample prep → The "finger warming" method is highly recommended by MALDI MSI specialist from Bruker company according to their communication with MALDI MSI customers across United States. We have successfully performed metabolomic imaging in multiple projects using "finger warming" method.
* Reference styles are inconsistent →Fixed.
* Table of Materials has inconsistent style →Fixed.

Minor Comments:
* Line 82: text mainly used "imaging mass spectrometry "but "MS imaging" is used here.

→IMS changed to MSI through out of the manuscript according to the suggestion of reviewer#4.
* Line 92: I would argue sample prep typically doesn't happen in the individual's lab. Clarification here with either slide prep or whole prep is necessary especially when this protocol details matrix preparation which is typically automated. → Agreed with the reviewer, have changed the sample prep to slide prep.
* Line 102: what does cap refer to? Box lid? Cap of jar? →Styrofoam box lid
* Line 110: Clarification on "prolonged time"→added an example of "5 min for P1 mouse brain"
* Line 127: change brain to tissue →Fixed.
* Line 134: Examples of cryostat temperatures per type of tissue would be useful. If cryostat dependent, indicate in the text →Fixed
* Line 146: Indicate temperature for other tissues, similar to line 134 →Added the example of -14 °C for liver, -20 °C for muscle and -25 °C for skin
* Line 149: artist brush? Terms are inconsistent in text →Fixed.
* Line 150: is finger warming→Fixed.
* Line 154: brain to tissue→Fixed.
* Line 157: is "coherent" the right word? Cohort? →Changed to "cohort".
* Line 160: does thickness influence length of drying in dessicator? →Not really. The drying time has some flexibility, and the range 45-60 min works on our hands.
* Line 166: include "label outer bag" here and remove from Line 167. →Fixed.
* Line 171: (v:v) refers to equal parts. Later in text, (v/v) is used. →Fixed.
* Line 171: 3ml 30% H20 is confusing. Perhaps write it as "(100 mg NEDC in 7ml Methanol; 3ml H2O (70%: 30%))"→Fixed.
* Line 174: dehydrate for how long? →Fixed.
* Line 177: include for Bruker MALDI instruments→Fixed.

* Line 199: how much 70% Methanol is used for flush the loop? →7 mL
* Line 204: include range in line→Removed “within the range” to avoid confusion
* Line 209: what prompts are on the machine? → Removed “prompts” to avoid confusion
* Line 216: how are slides stored? →Storage condition added.
* Line 221: commas around "respectively"→Fixed.
* Line 243: what species of lipids are seen? → Ceramides and phosphor-lipids can be detected in this set of experiment. However, the representative result we used in this manuscript does not include a thorough lipidomic study. In another study ongoing in our facility, MALDI imaging can detect DAG, TAG, PE, PC, PI and PS.
* Lines 249-252: which P2, P21, P60 relate to the terms "neonates" and "adults"? →changed to P1 neonates and P60 adults

Reviewer #4:

Manuscript Summary:

The manuscript is a very useful and interesting protocol for MALDI-MSI procedures for metabolome analysis directly on tissue section. This protocol fits perfectly in a time in which MSI is becoming a routine tool for several biological fields and therefore established protocols are strongly required.

→We are really grateful to the reviewer’s appreciation of the significance in publishing an established protocols for MALDI MSI.

Major Concerns:

Is there any publication in which the preserving of metabolites in matrix-coated tissue slides in dry-ice is proven after the shipment? Moreover how long time should be the shipment in order to avoid degradation of metabolites?

→ Thank the reviewer for a thorough thought of the preserving matrix-coated slides. Indeed, the most common practice is that the user sends the intact frozen tissue or cryosection slides in dry ice to the MALDI lab, and the MALDI lab will perform the matrix spray immediately followed by MALDI MSI. Although we could not find the specific publications on the metabolomic study using the matrix coated tissue slide shipped in dry ice. In practice, our facility has sent the matrix coated slides to Bruker company laboratory in Boston to be tested on some of their new models of MALDI instrument. The shorter of the transportation the better, and overnight shipment is necessary. Or sometimes the MALDI instrument malfunctioned broke down right after we sprayed the precious sample slide, and we had to store the matrix coated slide in -20 °C until the instrument fixed the next day. According to our experience, matrix-coated slide stored in dry ice or -20 °C for 24 hrs can still yield good signals for some metabolites.

Minor Concerns:

The mass spectrometry imaging society decided that MALDI-MSI is the most appropriated acronym for mass specotrometry imaging and not imaging mass spectrometry (IMS).

→ Thank the reviewer pointed out the updated acronym for MALDI MSI. We have replaced all the IMS to MSI in the manuscript.

In the spray application, I would add a sentence regarding performing a test spray on an empty ITO slide before to spray the real sample in order to check the matrix homogeneity and avoid waste of precious sample.

→ Thank the reviewer for the valuable suggestion, as we also follow this practice indicated in step 4.7. We have added notes to the step 4.5 to make it clearer: “NOTE: testing matrix spray on a blank microscope slide first before proceeding to precious sample slides is highly recommended”. But we recommend to use regular microscope slide instead of using more costly ITO slide for the testing.

on the step 3.4 difficult to understand why the authors are using two "x" (one white and one black) over each other for the teaching points, can the authors explain better this step?

→ When the glass slide is scanned in the flatbed scanner, the background will be black and the black marker will be hard to see. While using a silver marker to add a white background for black “X” will create a sharp contrast for the fiduciary marker.

Mass spectrum of only matrix and of the tissue should be provided in order to give the idea to the user of good mass spec acquisition.

→ We appreciate the reviewer’s suggestion of providing examples of good mass spectrum. However, as the mass spectrum of each specific anatomical spot, or with different matrix coating, varies greatly, it is difficult to set a standard of good mass spectrum. The best way to get the idea of good mass spectrum is during the practice of specific research projects, e.g. comparing different sample preparation conditions or choosing different matrix.