Video Article

Optimal Preparation of Formalin Fixed Samples for Peptide Based Matrix Assisted Laser Desorption/Ionization Mass Spectrometry Imaging Workflows

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Abstract

The use of matrix-assisted laser desorption/ionization, mass spectrometry imaging (MALDI MSI) has rapidly expanded, since this technique analyzes a host of biomolecules from drugs and lipids to N-glycans. Although various sample preparation techniques exist, detecting peptides from formaldehyde preserved tissues remains one of the most difficult challenges for this type of mass spectrometric analysis. For this reason, we have created and optimized a robust methodology that preserves the spatial information contained within the sample, while eliciting the greatest number of ionizable peptides. We have also aimed to achieve this in a cost effective and simple way, thereby eliminating potential bias or preparation error, which can occur when using automated instrumentation. The end result is a reproducible and inexpensive protocol.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56778/>

Introduction

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) has been employed as an image based technique for two decades^{1,2}, analyzing a range of biomolecules including: lipids³, peptides^{2,4}, proteins^{2,5}, metabolites^{6,7}, N-glycans⁸, and synthetic molecules such as therapeutic drugs^{9,10}. The number of publications demonstrating the utility of this technique have grown significantly over the last decade^{6,11,12,13}. Certain molecules, such as lipids, are relatively easy to analyze via MALDI MSI, as they ionize readily due to their chemical nature and thus require little prior preparation³. However, for more difficult targets such as peptides, the steps required to effectively ionize these molecules are extensive and generally complicated¹⁴. There are currently very few publications that aim to address or demonstrate reproducibility in the methodologies that are employed to prepare tissue for this unique visual technique¹⁵. For this reason, we have compiled observations and implemented optimizations into a single, easy to implement, methodology that should require little to no modification, for the analysis of peptides from a formaldehyde cross-linked tissue source¹⁴.

In this manuscript, we have described a validated, low cost reproducible methodology for the detection and spatial mapping of peptides, generated from formalin-fixed frozen (FFF) and formalin-fixed paraffin-embedded (FFPE) tissue sections. This methodology does not require or rely on any specialized instrumentation³. Specifically, we address the many aspects of specialized sample preparation necessary to analyze peptides; steps such as antigen retrieval¹⁶ and matrix coating. Our protocol also utilizes inexpensive equipment and reagents, thereby making this methodology accessible to a wider community who would otherwise be unable to afford the alternate robotic apparatus¹⁷.

The reasoning behind developing a manual sample preparation method was two-fold: Firstly, the use of a sublimator creates a consistent and homogenous coating of matrix crystals that are ~1 µm in length¹⁸, something unachievable with more common spraying techniques. Secondly, the relatively small set up costs: the total cost of the custom apparatus was <\$1500 AUD. We note, in terms of cost effectiveness, the price per sample is far cheaper when there is no robotic machinery involved. The use of sublimation has been reported previously, however, to the best of our knowledge, step-by-step methodologies that describe this process and sample preparation have not been reported nor described in the literature.

This protocol is intended to assist researchers that have access to a MALDI mass spectrometer and who are intent on generating spatial information in relation to a bio-molecule of interest¹⁹. In essence, MALDI MSI is a form of histological screening that does not rely on antibodies or stains².

Protocol

CAUTION: All applicable safety precautions should be followed when performing this procedure, including the use of appropriate personal protective equipment (PPE) (e.g., lab coats, nitrile gloves, safety glasses, etc.)

1. Preparation of Reagents and Equipment

1. Preparation of solutions

1. To prepare 500 mL of Carnoy's fluid, mix 100% ethanol (EtOH), chloroform, and glacial acetic acid in a 6:3:1 v/v/v ratio in a clean glass Schott bottle.
2. To make liquid nitrocellulose (NC), dissolve NC membrane (commonly used in western blotting) in neat acetone, by gentle agitation, to a final concentration of 40 mg/mL.

2. Preparation of NC coated slides

1. Use a technique similar to the one used for the preparation of blood smears for histological examination²⁰ to prepare NC coated slides.
2. Pipette 40 μ L of liquid NC onto one edge of a conductive indium tin oxide (ITO) microscope slide. Using a regular glass microscope slide, drag the NC under surface tension across the slide to create an even thin film coating.
3. Allow the NC to dry at room temperature for 20 s and then store until needed at room temperature. Storage of samples under these conditions can be indefinite provided the tissues are maintained and free of dust.

NOTE: This method of preparation is better known as "thin film" and will produce a uniform coating that relies on the viscosity of the NC as well as its lack of surface tension to self-level on the slide. The only care that should be taken when preparing the slides is not moving too quickly, which will create streaks of NC rather than a complete coverage.

CAUTION: As liquid NC dries very quickly, it is essential to work quickly to avoid drying the solution before it has been properly spread. Care should also be taken when handling NC in its liquid state, as it is a highly energetic compound and should not contact a source of ignition to prevent incendiary explosion. NC also undergoes endothermic transitions when drying and can cause cold burns if allowed to come into prolonged contact with skin or gloved hands. To minimize all associated risks, only small amounts should be prepared at each time (<1 mL is recommended). Once dry it is stable at room temperature. However, NC can remain explosive when present in large quantities.

3. Creation of custom vapor chambers

1. Cut a piece of thick blotting paper with a pair of scissors big enough to fit the bottom half of a standard plastic petri dish (94 mm in diameter and 3 mm thick).
2. Cut the paper, leaving a rectangular strip in the center, the same size as a microscope slide, leaving enough excess so that the paper will maintain its position in the Petri dish (**Supplementary Figure 1**).

2. Preparation of Tissue Sections

1. FFPE Tissue²¹.

1. Select the desired tissue block and section it at 12 μ m thickness in a standard bench mounted microtome and float mount onto the NC pre-coated ITO slides.
2. Submerge the slides in fresh xylene to remove residual paraffin for 2 min, and repeat the operation a second time.
NOTE: If the paraffin block is especially pervasive or the tissue is relatively small compared to the paraffin block, the samples can be heated at 60 °C to melt the majority away prior to washing in xylene.
3. Wash the deparaffinized samples by submerging the slides in a graded solvent series: 70% v/v EtOH/water, 100% EtOH, Carnoy's fluid, 100% EtOH, ultra-pure water and 100% EtOH. The duration of each submersion should be 30 s, except for Carnoy's fluid, which must last 2 min.

CAUTION: Carnoy's fluid and xylene must be stored and used in a fume hood, as they are highly toxic if inhaled.

2. Formalin-Fixed Frozen (FFF) Tissue

NOTE: Samples should already be frozen, appropriately according to the sample type.

1. Equilibrate the tissue block at the operating temperature of the microtome for 20 min²².
NOTE: Equilibration temperature is dependent on the type of tissue.
2. Section the tissue using a microtome at 12 μ m and thaw mount the sections onto a pre-prepared NC coated ITO slide.
3. Store the slides in a vacuum desiccator on the bench in the dark.
NOTE: Samples can be stored for several weeks under these conditions.
4. Wash the samples in a graded solvent series as stated for FFPE tissue (2.1.3).
NOTE: There is no need for a xylene deparaffinisation step as the sample is not embedded.

3. Methylene Crosslink Hydrolysis

1. Load the sample slides (either FFF or FFPE) into a plastic slide box which is filled to the top with 20 mmol tris-HCl (pH 8.8). Seal the box and place it in a water bath, containing 500 mL of water, allowing the box to touch the bottom. Then heat it for 15 min at 120 °C in a pressure cooker which can achieve an operating pressure of 70 kPa.
2. Remove the slides, allow them to cool, and let them dry at ambient temperature for 15 min.

4. Tissue Digestion

1. Once hydrolyzed, coat the samples with 10 μL of trypsin solution (1 mg/mL) in ultra-pure water, by pipetting 10 μL of the solution onto the edge of the tissue section then, using the same pipette tip, drag the droplet across the whole surface of the tissue under surface tension.
NOTE: Avoid scratching the tissue surface with the pipette tip and over spreading the enzyme beyond the borders of the tissue edges.
2. Allow the samples to dry at ambient temperature.
3. Once dry, mount the samples inside the top of the previously constructed vapor chamber (tissue facing down) with autoclave tape on either edge of the slide (**Supplementary Figure 2**).
4. Pipette 600 μL of a solution containing a 1:1 v/v mix of 100% acetonitrile and 50 mM ammonium bicarbonate carefully onto the blotting paper finger in the bottom part of the petri dish until the finger appears to be evenly wet.
5. Place the top half of the vapor chamber on the bottom half, ensuring the paper finger and sample slide align perfectly, and seal the chamber around its equator with paraffin film.
6. Leave the sample overnight in a 37 °C incubator to allow complete digestion.

5. Matrix Coating via Sublimation

1. Once digested, weigh the sample slide on a 5-digit microanalytical balance.
2. Mount the slide onto the cooling finger of the sublimation apparatus and secure it with copper tape, in the same way as described for the vapor chamber (**Supplementary Figure 3**).
NOTE: To ensure that the slide is contacted evenly by the cooling finger, a layer of copper tape is placed on the bottom of the cooling finger to level the surface. This ensures that the slide is evenly cooled which leads to even deposition of matrix.
3. Place 300 mg of α -cyano-4-hydroxycinnamic acid (CHCA) matrix into a glass petri dish at the bottom of the chamber and spread evenly to create a thin layer of matrix crystals.
4. Assemble the sublimator and secure the two halves with the horseshoe clamp. Suspend the assembled unit 15-20 cm above a sand bath, preheated at 220 °C, by placing it in a metal ring connected to a retort stand.
5. Connect the chamber to the vacuum source, engage the vacuum, and allow it to stabilize down to ~25 mTorr for 5 min.
6. Pack the cooling finger to the top with ice and add 50 mL of water. Allow the apparatus to settle for a further 5 min before proceeding.
7. Lower the chamber onto the surface of the sand, ensuring the sand completely contacts the bottom of the chamber, and leave it for 45 min to create an ideal coating of 0.22 mg/cm².
CAUTION: Care should be taken when handling glassware at high vacuum, as any damage to the apparatus, while evacuated, can result in catastrophic failure of the glass chamber.
8. After 45 min, remove the chamber from the sand bath by raising the metal ring and vent the chamber.
NOTE: Once the chamber has been vented, the slide must be removed very quickly as water in the air will begin to condense on the freezing finger and sample slide.
9. Remove the slide and weigh it to ensure the desired coating has been achieved.
NOTE: If insufficient coating has been achieved, simply repeat the sublimation process for the slide until the desired coating thickness has been achieved.

6. Recrystallization

1. Once sublimated, mount the sample slide inside the top of the previously constructed vapor chamber, as described earlier (tissue facing down).
2. Pipette 600 μL of a solution containing a 1:1 (v/v) mix of 100% acetonitrile and 0.1% v/v trifluoroacetic acid in water carefully onto the blotting paper in the bottom part of the petri dish to ensure an even coating.
3. Assemble the chamber, ensuring the paper tab and microscope slide align perfectly, and leave in a 37 °C incubator for 1 h.
Caution: DO NOT seal the chamber.
NOTE: Once recrystallized, the normally yellow hue of the matrix should change to white, look less shiny, and appear very even. This indicates that recrystallization has been performed correctly.
4. The sample is now ready for analysis.

7. Instrumentation

1. Scan the slide in a flatbed scanner to create a digital image.
2. Load the sample into the mass spectrometer and then analyze using the appropriate software platform.
3. Analyze the samples in positive ion reflectron mode with a mass range of 750-3,500 Da, with a spot resolution that is applicable to the desired result. In most cases, 20-50 μm raster width is sufficient, however as little as 10 μm can be used¹⁵.
CAUTION: MALDI UV lasers are a source of ionizing radiation and should not be viewed directly. Ensure that the laser is contained within instrument shielding prior to operation.

Representative Results

If followed correctly, this protocol produces images that clearly represent the gross morphology of the tissue without any scratches or other deformations (**Figure 1**). The ideal validation for a correctly performed sample preparation, is the ability to distinguish between different physical structures by changing the molecule being viewed (**Figure 2**).

A good guide for determining if samples have been incorrectly prepared is to check for the presence of delocalization or matrix aggregation; peptide signatures that extend beyond the borders of the physical tissue are perfect indicators that molecules have moved during sample preparation. This is explained in detail in O'Rourke and Padula (2017)¹⁵.

The produced peptide spectrum should contain a high abundance of discrete molecules that are well distributed across the chosen mass range^{1,23} (this is largely sample dependent, however, it is not unusual to see in excess of 50 discrete peptide signatures from FFPE tissue) (Figure 3)¹⁸.

Figure 1: A correctly prepared sample of FFF human brain tissue. This processed tissue specimen has been imaged at 50 μm and shows good macro structure and a clear difference between white matter (WM) and grey matter (GM). Successfully prepared samples will show clear differentiation of different tissue locations. Here, there is a clear region of up-regulation of the peptide (represented by mass-to-charge ratio, M/Z) 1085 in the WM region of panel A. Differentiation is further demonstrated by the absence of that defined shape in panel B followed by its return in panel C. By assaying the distribution of three different molecules on the same tissue section and showing that some are uniformly distributed, while others are limited to discrete physical locations, we can see that sample preparation has been performed correctly. [Please click here to view a larger version of this figure.](#)

Figure 2: An incorrectly prepared section of FFF human brain tissue. This specimen has been imaged at 50 μm but shows a large-scale level of delocalization. The patterns of the molecules present across panels 1, 2 and 3 clearly demonstrate that, unlike **Figure 1**, there is no clear definition between biological regions or differences in the abundance of the molecules displayed. Since the images are the same, irrespective of the molecules selected for display, we can conclude that it has been delocalized due to being incorrectly prepared. Since this is brain tissue, this result does not make logical, biological sense. [Please click here to view a larger version of this figure.](#)

Figure 3: The global mass spectrum of the image displayed in Figure 1. The spectrum contains an abundance of peptide signatures across the lower end of the mass range, with several high mass peptide peaks present as well. [Please click here to view a larger version of this figure.](#)

Supplementary Figure 1: Schematic for cutting blotting paper used in vapor chamber. Thick blotting paper is cut into a circular shape with a diameter of 94 mm. A rectangular tab is also cut out to correspond with the size of a standard glass microscope slide. [Please click here to view a larger version of this figure.](#)

Supplementary Figure 2: Assembly schematic of vapor chamber. This schematic shows how the vapor chamber should be assembled with specific note of how the sample slides, blotting paper and adhesive tape all correspond with each other. [Please click here to view a larger version of this figure.](#)

Supplementary Figure 3: Schematic of sublimation chamber assembly: This figure shows how the sample slide, sublimator, and heating apparatus are arranged to allow reproducible sublimation. [Please click here to view a larger version of this figure.](#)

Discussion

This protocol was designed to maximize the generation of ionizable molecular species while eliminating delocalization of analytes. The key factors involve using the same overriding principle when applying matrix, digesting the sample, or recrystallizing after sublimation²⁴; namely, that an even deposition of vapor, matrix or otherwise, needs to be created and maintained. Pipetting solvent washes for recrystallization and digestion, evenly underneath the sample, prevents any individual area receiving more solvent vapor than anywhere else. This is important for preventing the formation of visible condensation and overall inhibits delocalization of the surface molecules as well as ensuring that the sample is evenly digested and recrystallized. It is imperative to achieve an even deposition of matrix, as too little will show a relatively low intensity and diversity of species, and too much will suppress the ions from the sample, also lowering intensity and diversity of species²⁵. To achieve an even deposition of matrix, ensure that an even, thin layer of matrix crystals of a homogenous size, are placed directly below the footprint of the suspended microscope slide. If the layer is uneven or too thin, then sublimation of the matrix will proceed too slowly or will be uneven²⁶. The potential issues with manual application of solvent and enzyme comes down to the individual experience of the user. Some level of "practice" is needed in order to ensure a repeatable result, and there may be some initial accidental incorrect preparation. However, if care is taken to ensure that the final image is in accordance with the properties of the "good" sample we have provided, then any improperly prepared samples will not lead to false biological conclusions.

In order to effectively digest a tissue sample preserved in formaldehyde (FFPE or FFF), it is essential to remove as many of the methylene crosslinks as possible prior to trypsin deposition¹⁶. This is easily achieved by heating the sample in a pressure cooker at >100 °C, for a short duration, without actually causing bubbles that can mechanically dissociate the sample. Tissue can easily be lost when treated in such a way,

so in order to combat this, NC slides are employed. While not critical, it does ensure the physical integrity of the sample when subjected to heat, pressure, and organic solvent. Digestion of the sample is then achieved by applying the enzyme in a state that prevents its activation and then allowing it to dry, *i.e.* suspended in ultra-pure water. The vapor chamber then follows the same principle as recrystallization, providing enough of a humid environment to allow localized movement of the enzyme to encounter and cleave the protein backbone, but not enough "wetness" to allow the resulting peptides to drift significantly from their initial location²⁶. Pipetting directly on the slide will not delocalize any surface analytes, due to the effects of residual crosslinking and the general insolubility of large proteins in water.

Although we have focused on the application of this methodology to peptides, it can be just as easily applied to workflows for the analysis of metabolites, lipids, and intact proteins. Some steps would need to be removed or augmented; intact protein imaging does not require any proteolytic cleavage steps, but the fundamental workflow of sample sectioning and mounting followed by matrix sublimation, recrystallization, and analysis can be applied to almost any sample type. Our intention for developing this protocol and ensuring its reliability and robustness through extensive empirical investigations, was to create a method that requires little or no modification, that uses inexpensive equipment, and is amenable to a wide community with varying degrees of instrumentation and biochemical skill. We hope that this opens new avenues of investigation to laboratories that would have otherwise dismissed this technique as too complicated or expensive. We are also confident that we have provided the first definitive sample preparation method for peptide MSI. At the time of writing, most methodologies have been largely instrumentation-based, with a particular emphasis on the ease of use of robotic spraying based methodologies^{27,28,29}. There has also been little in the way of reliable methylene hydrolysis methodologies with conjecture as to the correct procedure and apparatus to be used.

In conclusion, we consider that the application of the above methodology to FFPE and FFF tissues, will result in greater confidence in the efficacy of MSI for the analysis of peptides.

Disclosures

The authors have no conflict of interest or commercial interest to disclose

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