

Fresh Frozen Versus Formalin-Fixed Paraffin Embedded for Mass Spectrometry Imaging

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Abstract

Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry Imaging (MSI) is fast becoming an industry leading technique as a means of investigating the distributions of protein and peptide molecules directly from sections of tissue. Developing protocols for the analysis of FFPE tissue opens up numerous opportunities for novel biomarker discovery, due to the large number of tissue banks containing FFPE biopsy samples. This chapter reports the analysis of both fresh frozen and formalin-fixed paraffin-embedded (FFPE) tissues using such protocols.

Key words MALDI-MSI, Fresh frozen, Formalin-fixed paraffin embedded (FFPE), Peptides, Trypsin

1 Introduction

MALDI-MSI has been successfully used to elucidate the relative abundance and spatial distribution of a number of molecules; ranging from small molecules, peptides, and proteins. The first stage of the MSI workflow is sample collection (Fig. 1). Extra care and detail must be taken from this point through to imaging the sample, as it is crucial that the integrity in spatial localization of the molecules is maintained. It must be emphasized that delocalization and degradation of the analytes may be caused by poor handling and negligence in storage. To avoid such scenarios, it is generally advised that consolidated protocols are followed to ensure the samples are collected in the correct manner. Additionally, the orientation in which the organ is preserved is of great importance to the imaging experiment; disrupting the morphology can lead to ambiguity of results.

Snap-freezing using liquid nitrogen at the point of collection is the most common method employed [1]. Cracking and fragmentation of the tissue may occur as the tissue cools down at different rates. To counteract this, Schwartz et al. (2003) recommend wrapping the tissue loosely in aluminum foil to stabilize the cooling [2].

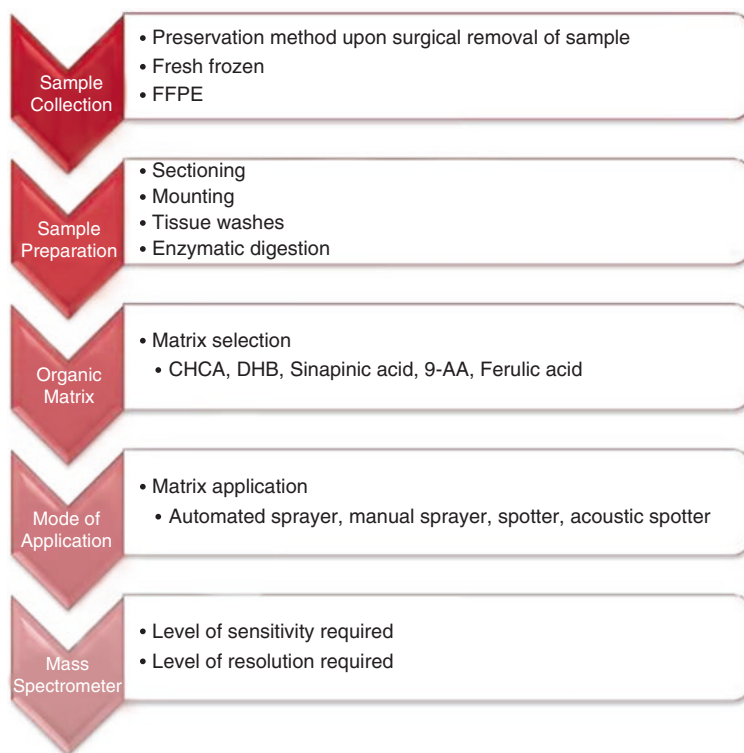


Fig. 1 Basic workflow of an imaging experiment with key focus areas; sample collection, sample preparation, choice of organic matrix, mode of application, and finally choice of mass spectrometer for analyses

On the other hand, Goodwin et al. (2008) mention that wrapping can distort the shape of the tissue, and therefore suggest a method of using ethanol or isopropanol at ≤ -70 °C as a solution [3]. We obtained best results when floating a weighing boat containing our tissue onto liquid nitrogen. Fresh frozen tissue may remain frozen at -80 °C without signs of degradation for at least a year.

The alternative to fresh frozen preparations is to formalin-fix paraffin-embed tissue. It was thought that this form of preservation could limit the type of analysis carried out to peptide analysis alone, as the cross-linking in FFPE may prevent the detection of analytes such as metabolites, lipids, and pharmaceutical compounds, however this is not the case [4–6]. Drexler et al. (2007) reported the detection of small molecules in their study which indicates that FFPE may not limit compound analysis [7]. More recently, the Walch group have successfully imaged metabolites from FFPE tissue blocks; their method involves the deparaffinization of FFPE tissue and matrix coating by 9-aminoacridine prior to MALDI-MSI [8].

As the difficulties presented in analyzing FFPE tissues (due to complex cross-linking) are now successfully being overcome,

further studies using the numerous samples in tissue banks would be of significant benefit [9]. Analysis would provide an insight into the histology of disease tissues with the potential to discover biomarkers by MALDI-MSI.

2 Materials

All solutions were prepared using ultrapure water (18 M Ω -cm at 25 °C) and analytical grade reagents with a purity of $\geq 99.5\%$. Reagents should be disposed of in compliance with health and safety regulations and in accordance with COSHH guidelines.

2.1 *Materials for Mass Spectrometry Sample Preparation and Acquisition*

1. Dewar of liquid nitrogen.
2. Carboxymethylcellulose (CMC).
3. Cork circles.
4. Cryostat chuck.
5. Surgipath X-tra adhesive precleaned micro slides.
6. Ethyl alcohol, 200 proof, ACS reagent $\geq 99.5\%$.
7. Chloroform $\geq 99.5\%$.
8. Trypsin Gold, Mass Spectrometry Grade.
9. Ammonium bicarbonate, BioUltra, $\geq 99.5\%$.
10. Octyl- α/β -glucoside 10 mM solution (OcGlu).
11. Methanol, CHROMASOLV®, for HPLC, $\geq 99.9\%$.
12. Formalin solution, neutral buffered, 10%.
13. Embedding cassettes.
14. Xylene substitute.
15. Embedding medium paraffin wax.
16. Hydrogen peroxide solution (H₂O₂).
17. Trizma base, $\geq 99.9\%$.
18. α -Cyano-4-hydroxycinnamic acid (CHCA), $\geq 98\%$ powder.
19. Aniline, ACS reagent $\geq 99.5\%$.
20. Acetonitrile, CHROMASOLV® Plus, for HPLC, $\geq 99.9\%$.
21. Phosphorus red, 99%.

2.2 *Reagents—Working Composition*

1. 2% Carboxymethylcellulose.
2. Ethanol solutions: Dilute 100% ethanol with ultrapure water to 90% and 70% (vol/vol).
3. Ammonium bicarbonate 50 mM: Dissolve 0.2 g ammonium bicarbonate in 50 mL ultrapure water. Check and adjust pH to 8.0. Solution can be stored for 1 month.

4. Trypsin solution 20 µg/mL: Add 1 mL ammonium bicarbonate (pH 8.0) directly to the trypsin 100 µg vial to give a 100 µg/mL stock solution. Agitate to ensure lyophilized trypsin is reconstituted. For the 20 µg/mL working solution, pipette 200 µL of the stock solution into a sterile 1.5 mL snap top Eppendorf and add 800 µL ammonium bicarbonate (pH 8.0).
5. Trypsin solution 20 µg/mL containing detergent 0.5%: Add 5 µL detergent solution (10 mM) to 995 µL trypsin solution 20 µg/mL.
6. Ethanol solutions for paraffin embedding: Dilute 100% ethanol with ultrapure water to 70%, 80%, and 95% (vol/vol).
7. MeOH: H₂O₂ 3% solution: Mix together 48.5 mL and 1.5 mL H₂O₂.
8. Tris-HCl buffer (1 L): Completely dissolve 1.21 g Trizma base in 1000 mL of ultrapure water. Adjust pH to 9.0 with 1 M NaOH or HCl as required (solution can be stored in a glass duran for up to 6 months).
9. MALDI matrix solution: Prepare 5 mg/mL CHCA in 50% (vol/vol) acetonitrile and 0.5% (vol/vol) trifluoroacetic acid in ultrapure water. Add equimolar amounts of aniline to the CHCA (i.e., 1 mL of 5 mg/mL CHCA solution contained 2.4 µL aniline). Sonicate in a sonic water bath for 5–10 minutes until matrix crystals have fully dissolved. Matrix containing aniline should be prepared fresh on the day of the experiment and not stored.
10. Phosphorus red calibrant: 10 mg/mL in 100% acetone. Vortex for 10–15 seconds. N.B. phosphorus red may not dissolve entirely.

3 Methods

3.1 Cryo-conservation of Tissue

1. Arrange the excised organ/biopsy into the correct orientation onto a regular weighing boat.
2. While wearing appropriate PPE, float the weighing boat containing the tissue on liquid nitrogen (*see Note 1*).
3. Take ~50 µL of 2% CMC and attach the frozen tissue to the cork circle, ensuring the tissue is level and in the correct orientation.
4. Place into the –20 °C freezer briefly to ensure that the CMC has frozen.
5. Mount the cork circle with tissue to a cryostat chuck. This can be done with ~50–100 µL 2% CMC solution.

3.2 Sectioning and In Situ Digestion of Fresh Frozen Tissue Sections

1. Fresh frozen rat brain tissue sections should be cut to 10 µm thickness using a Leica CM3050 cryostat chilled to –20 °C.
2. Thaw-mount sections onto Surgipath X-tra adhesive glass slides and store in glass slide holders at –80 °C (*see Note 2*).

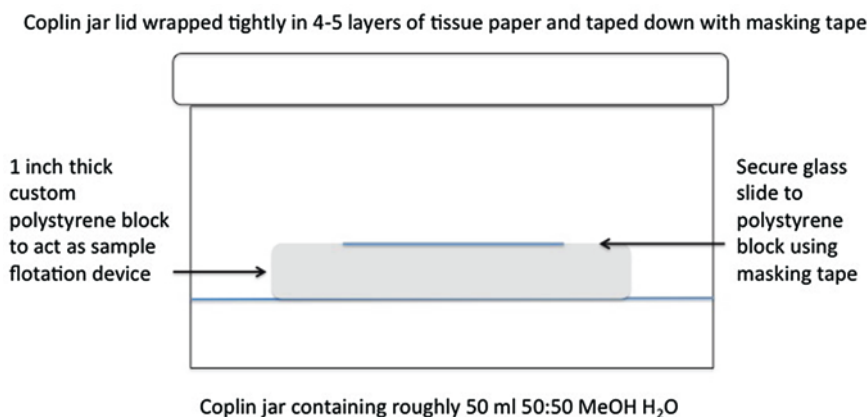


Fig. 2 Humidity chamber setup using a glass coplin jar containing 50:50 MeOH:water and a polystyrene floatation device to hold the sample. Once the lid is placed on the jar, carefully wrap the seal with parafilm to allow the humidity to remain within the chamber

3. Before digestion with trypsin, pretreat tissue sections with wash steps of 70% and 90% ethanol (1 minute each) and chloroform (10 seconds) (*see Note 3*).
4. Spray 20 µg/mL trypsin solution containing 0.5% 10 mM OcGlu onto the tissue sections using the SunCollect (SunChrom GmbH, Friedrichsdorf, Germany) automated sprayer (seven layers at a flow rate of 1.5 µL/minute and a nitrogen pressure of 3 bar).
5. Incubate tissue sections in a parafilm-covered coplin jar containing the humidity chamber solution; 50% MeOH (Fig. 2), for 3 hours at 37 °C.

3.3 Tissue Fixation and Embedding

1. Cover the excised tissue with 10% formalin, ensuring the tissue is fully submerged in the fixative. Fix for 24 hours at room temperature.
2. Place the fixed tissues into embedding cassettes, taking care to place the tissue in the correct orientation.
3. Dehydrate the tissue in a series of EtOH concentrations. Place the cassette containing tissue in 70% EtOH for 1 hour, 80% EtOH for 1 hour, 95% EtOH for 1 hour, and finally 100% EtOH for 1 hour.
4. Place the tissue into 100% xylene substitute for 1 hour. Repeat this two more times (therefore totalling 3 hours in xylene substitute).
5. Add liquid paraffin wax to the cassettes and wait for 2 hours (58–60 °C) (*see Note 4*). Top up with more paraffin wax if necessary.

3.4 Sectioning and In Situ Digestion of FFPE Tissue Sections

1. Prior to sectioning place the blocks in the –20 °C freezer for 1 hour (*see Note 5*). Trim the paraffin blocks on a Leica microtome; sections were cut to 3 µm thickness.

2. Gently place paraffin ribbons (*see Note 5*) on a float bath heated to 30 °C. After separating individual sections, mount onto Surgipath X-tra adhesive micro slides.
3. Air dry sections for 30 minutes and then place into cardboard slide holders. Place holders in a 40 °C oven overnight and store the following day in plastic slide containers.
4. Prior to trypsin digestion, deparaffinization and antigen retrieval of FFPE tissue is crucial. Place the glass slide in 100% xylene substitute for 5 minutes. Repeat using fresh xylene substitute for another 5 minutes.
5. Hydrate the tissue sections in a series of EtOH solutions. Place the glass slide in 100% EtOH for 3 minutes, 95% for 3 minutes, and 70% for 3 minutes.
6. Place the slide into a block to prevent protein degradation. Immerse in ~50 mL MeOH: ultrapure water (3%) for 15 minutes at room temperature.
7. For antigen retrieval, place glass slide in a 50 mL falcon tube and fill with Tris-HCl buffer. Place in a preheated water bath (97 °C) for 30 minutes (*see Note 6*).
8. Rinse in ultrapure water.
9. 20 µg/mL trypsin solution containing 0.5% 10 mM OcGlu was sprayed onto the tissue sections using the SunCollect automated sprayer (seven layers at a flow rate of 1.5 µL/minutes and a nitrogen pressure of 3 bar).
10. Tissue sections were incubated in a parafilm-covered coplin jar containing the humidity chamber solution; 50% MeOH (Fig. 2), for 3 hours at 37 °C.

3.5 Matrix Deposition

1. Spray five layers of 5 mg/mL CHCA containing aniline onto the samples previously digested with trypsin, using the SunCollect autosprayer at a flow rate of 1.5 µL/minute and a nitrogen pressure of 2.5 bar (*see Note 7*).
2. Ideally, samples should be analyzed on the same day as spraying the matrix. If overnight storage is required due to unforeseen circumstances, keep sample in a sealed petri dish at +4 °C.

3.6 Instrumentation and Data Acquisition

1. Spot 0.5 µL phosphorus red calibrant onto a clean MALDI target plate. Calibrate the SYNAPT™ G2 HDMS mass spectrometer (Waters Corporation, Manchester, UK) using the phosphorus red spot across the 600–2800 Da mass range.
2. Scan the sample sprayed with matrix on a traditional flat-bed scanner and save the image as a .jpeg.
3. Follow the dialog box instructions for a “*new image pattern*,” upload the .jpeg within the high-definition imaging (HDI) software to allow coregistration and image setup (*see Note 8*).

4. Load the sample into the instrument, and acquire MALDI-IMS-MS images in positive ion mode from 600 to 2800 at a mass resolution of 10,000 FWHM.
5. Operate the instrument with a 1 kHz Nd:YAG laser and set the laser energy to 220 arbitrary units on the instrument.
6. Perform image acquisition at a spatial resolution of 100 μm \times 100 μm for the imaging of tissues.

3.7 Data Analysis

1. Process mass spectrometry imaging data using the HDI software (Waters Corporation, Manchester, UK).
2. It is also possible to use open-source software such as Biomap (Novartis) (*see* **Note 9**).
3. HDI software has a number of features including normalization, color gradients, and the option to create overlays of different ions. N.B. Please see the user manual for an extensive guide.

4 Notes

1. Floating the tissue on liquid nitrogen rather than submerging minimizes the chances of freeze fracturing. This allows the tissue to freeze evenly throughout without damaging. At this stage, the tissue can be covered in paraffin and stored in an air tight container if required at a later date.
2. Thaw mounting: once a section has been cut using the cryostat the tissue section is attached to the glass slide electrostatically; holding the slide close over the section allows it to attach to the glass slide. Rubbing the underside with a gloved finger allows the section to adhere to the slide.
3. Place the 70% and 90% EtOH in a 50 mL falcon tube if one slide is being analyzed at a time. If more than one slide is to be washed at once use a multislide rack in a glass coplin jar. Ensure that the chloroform is always placed in a glass vessel.
4. Melt the paraffin wax pellets at 58–60 °C in a glass beaker.
5. Placing FFPE blocks in the –20 °C freezer for 1 hour before sectioning enables easier sectioning and the production of ribbons. This is a consecutive number of sections (usually 5–10) all attached together. Once the ribbon is placed in the float bath the individual sections can be detached with forceps easily.
6. Carrying out antigen retrieval in a water bath as opposed to a microwave eliminates the increased production of bubbles, which can result in the tissue section detaching from the glass slide.

7. Ensure the Sun Collect has been flushed through with 100% ACN at a flow rate of 2 μ L/minute for 15 minutes before matrix is flushed through. This allows any matrix crystals blocking capillaries to be dissolved.
8. The HDI software includes automated image processing. This processing feature can be selected during image setup.
9. Images acquired using the SYNAPTTM can be converted using ImageConverter to be viewed on open-source software such as Biomap (Novartis).

Acknowledgments

The author gratefully acknowledges Dr Simona Francese for her support and guidance, and GlaxoSmithKline for the BBSRC Industrial Case Award funding.

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Imaging Mass Spectrometry

Methods and Protocols

Cole, L. (Ed.)

2017, X, 204 p. 84 illus., 68 illus. in color., Hardcover

ISBN: 978-1-4939-7050-6

A product of Humana Press