Chapter 18

MALDI Direct Analysis and Imaging of Frozen Versus FFPE Tissues: What Strategy for Which Sample?

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Abstract

Significant advances have been made in the past decade in the field of mass spectrometry imaging with MALDI ion sources (MALDI-MSI). While MALDI-MSI has high potential in the field of biology and in the clinic, a challenge for MALDI-MSI has been to adapt itself to a greater range of sample types. In particular, much of the biological archived materials for pathology studies are tissue biopsies fixed with paraformaldehyde and embedded in paraffin (FFPE tissues) because of the high stability of such samples. Thus, there has been a need to develop strategies for analyzing FFPE samples as this would allow retrospective studies of past clinical cases on large cohorts of existing samples. Obviously, PAF fixation, by inducing protein cross-linking, causes problems for molecular analysis by MS. We developed on tissue digestion strategies for overcoming these difficulties and allowing molecular data to be retrieved from FFPE samples no matter how long they have been stored. These digestion strategies preserve localization from digested proteins making MALDI-MSI of proteins possible by monitoring the resulting peptides. We present methods and protocols for FFPE samples. These strategies have proven to be valuable for all tested FFPE samples and have opened archived tissues from hospital banks to MALDI-MSI.

Key words: Matrix-assisted laser desorption/ionization, time-of-fight, mass spectrometry, mass spectrometry imaging, formalin fixed and paraffin embedded, frozen tissue, on tissue digestion, proteins identification.

1. Introduction

After 10 years of development, MALDI-MSI has shown a great potential for applications to a variety of fields. In particular, applications are found in pharmacology for drug evaluations (localization of drugs and their metabolites) (1, 2) or in clinics for

S.S. Rubakhin, J.V. Sweedler (eds.), *Mass Spectrometry Imaging*, Methods in Molecular Biology 656,
DOI 10.1007/978-1-60761-746-4_18,

biomarkers discovery and study or molecular classification of pathologies (3–6). MALDI imaging was successfully carried out and demonstrated on frozen tissue sections after snap freezing procedures without fixation (7, 8). But the possibility of simultaneously detecting and localizing hundreds of different molecules in a sample without prior labeling become a tremendous challenge: how does one identify them? In fact, clinical applications require the identification and validation of target molecules (9).

Importantly, a vast number of samples contained in hospital tissue banks have proven to be incompatible with MS analysis and are thus had not been usable for MALDI-MSI. The majority of archived samples have been stored after fixation and paraffin embedding. The fixation step is necessary to increase sample stability and preserves tissue fine structures for observations after tissue sectioning. The most commonly used fixative is paraformaldehyde (PAF) or formalin. When PAF fixation is used prior to paraffin embedding, the resulting samples are called formalin fixed and paraffin embedded or FFPE. Because FFPE samples can be stored for decades, many samples are available from hospital tissue banks allowing creating larger cohorts even for rare pathologies or subpathologies (8, 10–12). In addition, the clinical outcomes of the donors and the pathologist diagnosis are known, making FFPE tissues a mine of information, especially if the imaged compounds can be identified. While PAF fixation is ideal for conservation and structure preservation, it causes difficulties for molecular analysis. In fact, PAF fixation induces molecular cross-linking, especially of peptides and proteins.

Chemical reaction processes involved in PAF fixation are extremely complex. Protocols and outcomes of PFA fixations have been extensively studied, but only few literature reports directly address the issues of the specific molecular chemical reactions underlying the fixation process (13–15). In a simplified way, we can state that PAF reacts principally with free amine groups by the formation of methylene bridges. The primary reaction of the aldehyde to the protein has fast kinetics (13–15). On the contrary, secondary reactions lead to the formation of the methylene bridge as a much slower process taking place over days (13–15). Thus, reactions still proceed even after paraffin embedding. Therefore, the proteins become further imprisoned over time as methylene bridges slowly form.

As proteins are all cross-linked together, MS analysis of such tissue samples after tissue sectioning and paraffin removal generates few peaks. As reactions still proceed inside the paraffin blocks, MS spectra generated from these samples are dependent on storage time. For recent FFPE blocks (~0–6 months), ion signals are retrieved (8). These signals are mainly observed in the peptide mass range and detailed study of the corresponding mass spectra shows that peaks have broadened profiles with +12 u.

mass adduct (16). Such phenomena were previously observed and described in other MS studies and are due to sub-reactions from PAF fixation. These sub-reactions also present slow kinetics and proceed after paraffin embedding. The intensity of +12 u. adducts peaks and their number increases with storage time as is observed in the MS spectra. More peptide signals are observed when using specific MALDI matrices such as the reactive matrix 2,4-dinitrophenylhydrazine (2,4-DNPH) (8). Surprisingly, for such a matrix, MS spectra do not contain the +12 u. adduct ions. This can be explained by assuming that 2,4-DNPH has a benefic effect by reacting with any free aldehyde groups (PAF) remaining in the tissues. For longer stored samples (>1 year), MS analyses do not allow the detection of exploitable signals even using reactive matrices such as 2,4-DNPH. Proteins cross-linking has also shown to be problematic for immunohistochemistry (IHC) experiments, by hampering antibody access to the epitope of the antigen. To overcome such problems, pathologists have extensively studied epitope unmasking procedures. Different antigen retrieval (AR) protocols compatible with IHC are now well known and described (17–23). One popular procedure is heating the sample at high pressure (17–23). But, if AR allows epitope unmasking, such procedures do not reverse protein cross-linking. AR can be used before MS analysis of tissues and can improve analysis, although this has not proved not sufficient as the cross-linked proteins render these samples difficult to use for direct analysis by MALDI and MALDI-MSI.

New strategies are then needed to analyze FFPE tissue sections in an efficient manner, especially an approach that is independent of storage time. We have developed an approach based on “tissue enzymatic digestion” allowing one to obtain peaks from pieces of proteins (**Fig. 18.1**). In this strategy, the protein is enzymatically digested and the resulting peptides are subsequently analyzed on the tissue. MALDI-MSI after on tissue enzymatic digestion is possible if the localization of the generated peptides is maintained, i.e., if enzyme can be precisely deposited and localized onto the tissue. This is achievable by dropping small droplets of enzyme in a control manner. For the highest reproducibility, this can be done via an automatic device. If digestion peptides locations are precisely maintained, then MALDI-MSI of proteins can be deduced from the images generated from the peptide ion signals. In addition, these peptides are also useful for protein identification. In fact, because MALDI produces ions with only a few charges, MALDI is not well suited for direct identification of proteins by the means of “top-down” approaches; thus, “bottom-up” strategies are more common. Here we digest the proteins and use MALDI-MSI bottom-up approaches by performing MS/MS experiments on the peptides from the tryptic digestion from the FFPE preserved proteins. Such strategies are

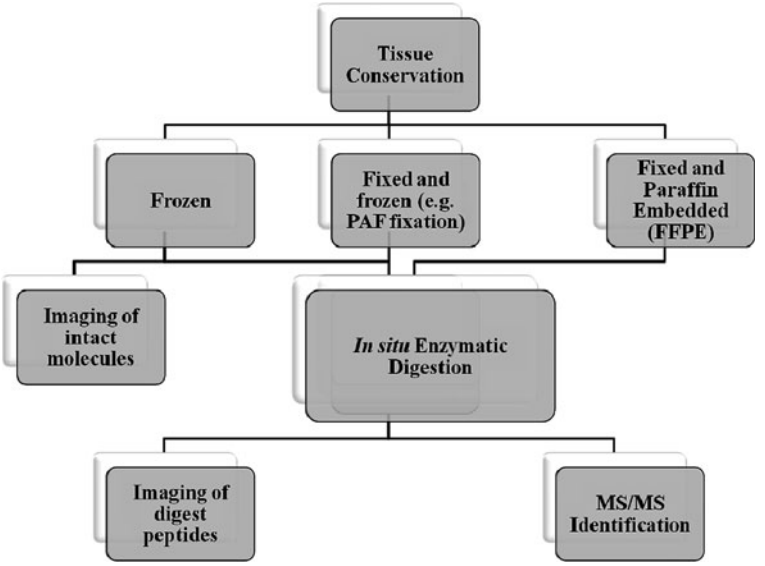

Fig. 18.1. Strategies for direct analysis and imaging mass spectrometry of FFPE, PAF, and frozen tissue section.

work with frozen samples when the proteins need to be identified (**Fig. 18.1**). In comparison with FFPE samples, frozen samples are simpler to analyze and image as they do not systematically require an on tissue digestion strategy. While frozen samples are easier for MALDI-MSI, they do not present the same stability. Degradation of the proteome can be observed for samples stored over long periods at –80°C.

In this chapter, we describe methods and protocols for direct analysis of frozen and FFPE tissue samples, including on tissue digestion for both frozen and FFPE samples for protein identification.

2. Materials

2.1. Preparation of Frozen Tissue Sections

2.1.1. Snap Frozen Tissues

1. Isopentane cooled at –45°C with dry ice. Vapors may cause drowsiness and dizziness, so work in a hood.

2.1.2. Tissue Cryosection and Thaw Mounted

1. Optimal cutting temperature polymer, OCT.
2. Indium tin oxide (ITO) coated glass slides or other holder compatible with mass spectrometry analysis.

3. A cryomicrotome, Leica CM150S (Leica Microsystems, Nanterre, France).

2.1.3. Pre-analysis Treatment – Tissue Fixation

1. Ethanol 75% (–20°C): 75 ml of absolute ethanol (≥99.8%) and water (HPLC grade) to 100 ml. Prepare fresh. Store at –20°C.
2. Ethanol 95% (–20°C): 95 ml of absolute ethanol (≥99.8%) and water (HPLC grade) to 100 ml. Prepare fresh. Store at –20°C.

2.1.4. Pre-analysis Treatment – Removal of Lipids

1. Chloroform (–20°C): 100 ml of chloroform (≥99.9%). Store at –20°C. Chloroform is harmful by inhalation, so work in the hood.

2.2. Preparation of FFPE Tissue Section

2.2.1. FFPE Tissue Section

1. Indium tin oxide (ITO) coated glass slides or other holder compatible with mass spectrometry.
2. Water: 100 ml of water (HPLC grade). Prepare fresh.
3. A microtome and a hotplate warm at 50°C.

2.2.2. FFPE Tissue Dewaxing

1. Xylene: 100 ml of xylene (≥99.9%). Xylene is harmful by inhalation, so work in the hood.
2. Ethanol 100%. Prepare fresh.
3. Ethanol 95%: 95 ml of absolute ethanol (99.8%) and water to 100 ml. Prepare fresh.
4. Ethanol 75%: 75 ml of absolute ethanol (≥99.8%) and water to 100 ml. Prepare fresh.
5. Ethanol 30%: 30 ml of absolute ethanol (≥99.8%) and water to 100 ml. Prepare fresh.
6. Water: 100 ml of water (HPLC grade). Prepare fresh.

2.3. On Tissue Digestion

2.3.1. Using a Microspotter

1. Trypsin, sequencing grade modified (Promega). Suspend in 20 mM NH_4HCO_3 buffer at 20 μg/ml (*see* **Note 1**).
2. Methanol 50%: 50 ml of absolute methanol and water to 100 ml. Prepare fresh. Methanol is toxic, so work in the hood.
3. Chemical Inkjet Printer CHIP-1000 (Shimadzu Biotech, Kyoto, Japan).

2.3.2. Using an Automatic Sprayer

1. Trypsin, sequencing grade modified (Promega). Resuspend in 20 mM NH_4HCO_3 buffer at 40 μg/ml (*see* **Note 1**).

2. Methanol 50%: 50 ml of absolute methanol and water to 100 ml. Prepare fresh. Methanol is toxic, so work in the hood.
3. ImagePrep (Bruker Daltonics, Bremen, Germany).

2.4. Matrix Deposition

2.4.1. For Protein Analysis Using a Microspotter

1. SA/ANI solution: 1 equivalent of aniline (ANI) was added to a solution containing 40 mg/ml of sinapinic acid (SA) in acetonitrile/aqueous TFA 0.1% (6:4, v/v) (*see* **Note 2**). Aniline and TFA are toxic, so work in the hood.
2. Chemical Inkjet Printer CHIP-1000 (Shimadzu Biotech, Kyoto, Japan).

2.4.2. For Protein Analysis Using an Automatic Sprayer

1. SA/ANI solution: 1 equivalent of aniline (ANI) was added to a solution containing 40 mg/ml of sinapinic acid (SA) in acetonitrile/aqueous TFA 0.1% (6:4, v/v) (*see* **Note 2**). Aniline and TFA are toxic, so work in the hood.
2. ImagePrep (Bruker Daltonics, Bremen, Germany).

2.4.3. For Peptide Analysis Using a Microspotter

1. HCCA/ANI solution: 1.5 equivalent of aniline (ANI) was added to a solution containing 10 mg/ml of α-Cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile/aqueous TFA 0.1% (6:4, v/v) (*see* **Note 3**). Aniline and TFA are toxic, so work in the hood.
2. Chemical Inkjet Printer CHIP-1000 (Shimadzu Biotech, Kyoto, Japan).

2.4.4. For Peptide Analysis Using an Automatic Sprayer

1. HCCA/ANI solution: 1.5 equivalent of aniline (ANI) was added to a solution containing 10 mg/ml of α-Cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile/aqueous TFA 0.1% (6:4, v/v) (*see* **Note 3**). Aniline and TFA are toxic, so work in the hood.
2. ImagePrep (Bruker Daltonics, Bremen, Germany).

2.5. Mass Spectrometry Analysis

2.5.1. MALDI-MSI Experiment

1. Peptide calibration standard II (Bruker Daltonics, Wissenbourg, France): angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39, somatostatin 28, bradykinin fragment 1–7, renin substrate tetradecapeptide porcine. Covered mass range: ~700–3,200 Da. Store at –20°C.

2. Protein Calibration Standard I (Bruker Daltonics, Wissenbourg, France): insulin, ubiquitin I, cytochrome C, myoglobin. Covered mass range: ~5,000–17,500 Da. Store at –20°C.
3. An Ultraflex II TOF–TOF equipped with a smartbeam laser and all the Flex software suite (FlexControl, FlexAnalysis, and FlexImaging) (Bruker Daltonics, Bremen, Germany).

2.5.2. MS/MS Analysis

1. An Ultraflex II TOF–TOF equipped with a smartbeam laser and all the Flex software suite (FlexControl, FlexAnalysis, and FlexImaging) (Bruker Daltonics, Bremen, Germany).
2. Biotools (Bruker Daltonics, Bremen, Germany).

3. Methods

In what follows, the methods are presented according to the MALDI-MSI workflow. We primarily describe the steps leading to tissue section preparation prior to MALDI-MSI which is an extremely important part of the method. In particular, snap frozen tissues require specific tissue treatment (washing procedures) prior to other steps. One of the procedures is fixation of tissues using ethanol (24). The second step removes small organic compounds (namely lipids) that hampers detection of peptides in the low m/z range by use of organic solvents. These steps are independent of each other, but are strongly recommended when performing in situ digestion and identification of proteins. We also describe how to prepare FFPE tissue sections for MALDI-MSI analysis. Because in situ digestion and/or matrix deposition must be performed with precision and reproducibility to prevent molecule delocalization, it is recommended that one use an automated deposition device. Two types of instruments are described (**Fig. 18.2**):

- An automatic microspotter delivering picoliter volumes of solution in defined coordinates allow the coverage of the entire surface of the tissue with an array of spot spaced by ~200 μm.
- An automatic sprayer can spray small droplets (~25 μm size in average) onto the top of the tissue section. In this case the tissue section is fully covered by the spray.

The main advantages of these devices are to limit delocalization of molecules. This depends on the solvents used, but they still must allow optimal extraction of the proteins and peptides, allowing enzyme activity and the incorporation of analytes into matrix crystals during matrix crystallization. These devices currently

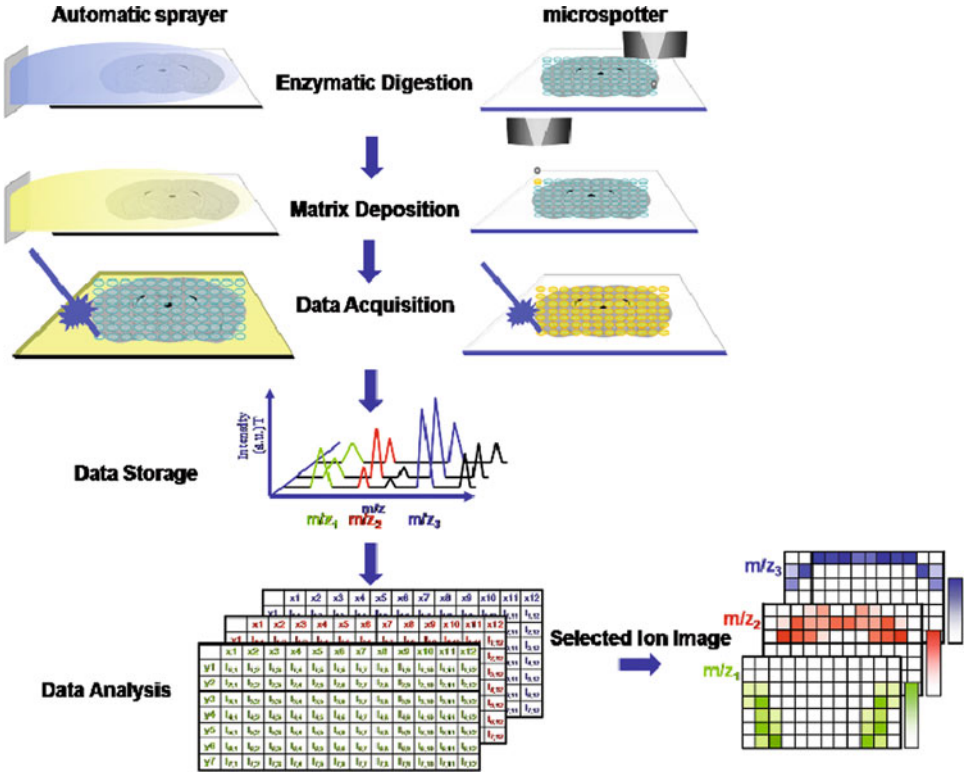

Fig. 18.2. Schematic representation of the strategies for in situ digestion using an automatic sprayer or microspotter devices.

represent the best compromise of minimizing lateral diffusion of analytes while maintaining enzymatic digestion and analyte incorporation efficiency (**Fig. 18.3**).

For matrix deposition using these automatic devices, it is recommended to use solid ionic matrices (25). In fact, solid ionic matrices have proven to be more efficient for MALDI-MSI. They have several advantages including higher salt tolerances, higher stabilities under vacuum conditions (lower sublimation rates because of higher sublimation temperatures), lower ablation volumes and thus produce higher spectral quality (increased number of detected ions, higher signal intensity). Moreover, these matrices are compatible with the automatic deposition devices. Classical matrices often lead to clogging problems of the systems when used at optimal analytical concentrations. If concentration of matrix solutions is decreased to reduce clogging, this dramatically decreases the resulting MS spectra quality (less detected signals with a lower average intensity). Overall, the classical matrices used with automated spotting devices do not currently offer a robust system for high-quality analyses of tissues. On the contrary, solid ionic matrices have demonstrated that they are well suited with automatic devices. In addition, solid ionic matrices present

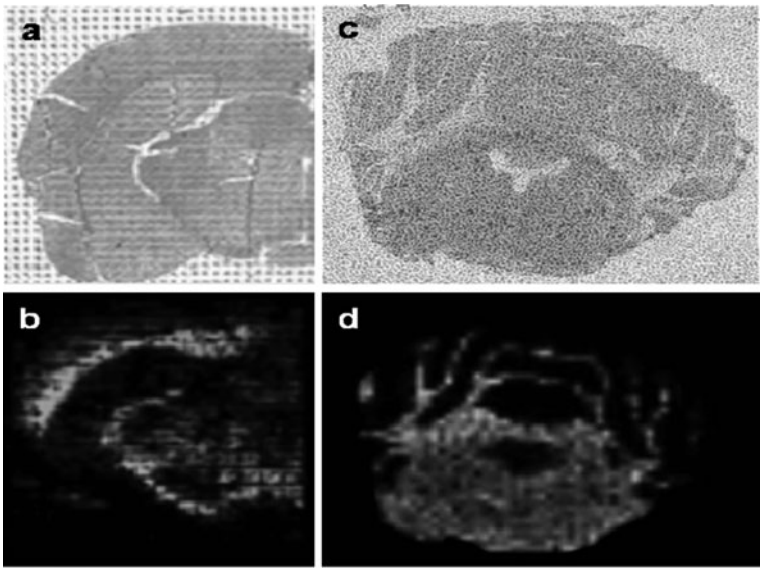

Fig. 18.3. Optical picture and MALDI image using microspotting (respectively **a** and **b**) or using an automatic sprayer (respectively **c** and **d**) for enzymatic digestion and matrix deposition.

different physicochemical properties than classical matrices. And in particular their surface tension is lower. Thus, it is possible to avoid clogging problems using solid ionic matrices while working at the optimum analytical concentration. By using solid ionic matrices with automated devices, we have a robust system that can be used in an automate fashion without any control giving higher printing (more defined spots) and analytical quality.

In the last part of methods, mass spectrometry analyses of tissue sections (FFPE or Frozen) after in situ digestion and matrix deposition are described. Typically on tissue digestion produces complex MS spectra depending on the local composition of the tissue (**Fig. 18.4**). MS/MS experiments can be performed by selecting a parent ion to achieve structural information. Identification is generally obtained by database interrogation using search algorithm such as MASCOT. Because of spectral complexity, the approach used is generally to first identify one the fragment peptides of a protein and then, based on the protein sequence, search for the presence of the other digestion fragments of the putative protein. If the additional fragments are present, they are submitted to MS/MS experiments to confirm that their fragmentation pattern fits with the expected peptide sequence. In parallel, localization of the different digestion peptides expected to be part of the same protein is generated in order to confirm identification; all digestion fragments of a single protein should present the same spatial distribution in the tissue (**Fig. 18.5**).

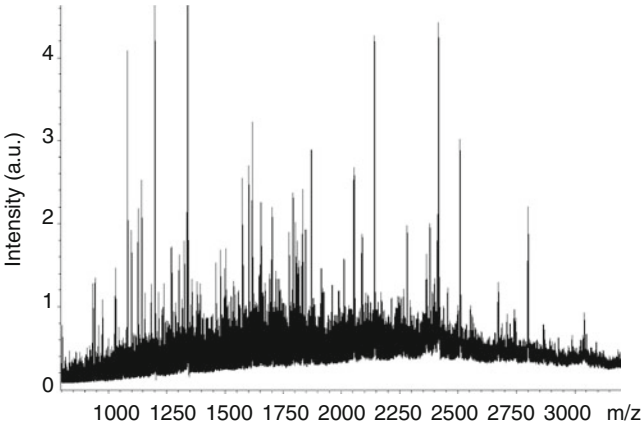

Fig. 18.4. Example of a typical mass spectrum recorded on a rat brain tissue section after in situ enzymatic digestion of a FFPE or frozen tissue.

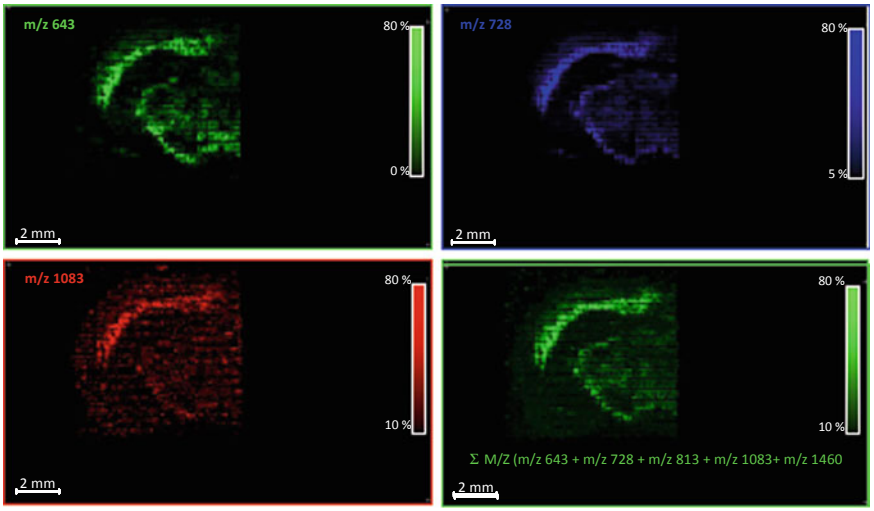

Fig. 18.5. Example of MALDI images of digest fragments of the same protein (here CRMP2) recorded from FFPE tissue section stored for 10 year after in situ enzymatic digestion of a 6-OHDA injected rat brain. Reprinted from (10).

3.1. Preparation of Frozen Tissue Section

3.1.1. Tissue Snap Frozen

1. The organ is dissected and rinsed with a saline solution suitable for the tissue. This removes blood and other tissue fragments from the surface. Alternative: prior to killing, the animal can be perfused with the saline solution to remove blood inside the organ.
2. Morphology of the organ needs to be carefully maintained. Thus, the tissue should not be placed in a tube or wrapped

in an aluminum foil to avoid deformation of the organ (otherwise, it adapts to the outlines of the container).

3. Snap freezing procedures are applied for tissue preservation to maintain tissue morphology and to prevent ice crystal formation and cell explosion. In fact, different cooling rates for some parts of the organ or direct dipping of the organ into liquid nitrogen lead to the formation of cracks and fragmentation of the tissue. Therefore, the use of isopentane cooled at –45°C with dry ice is recommended. Freezing time is dependent on the size of the organ. It is preferable not to use an embedding media. For very small organs or surgical pieces, cutting without embedding material increases deformations and damages of the tissue sections. In such a case, a solution containing nonpolymeric compounds such as 10% gelatin solutions help to obtain high-quality tissue section. Tissue is embedded in 10% gelatin directly after dissection and frozen as previously described.
4. After snap freezing, tissue is removed from isopentane and stored at –80°C. We heartily recommend not surpassing a storage period of 6 months. After 6 months of storage, variations in the molecular profiles are observed if no sample stabilization procedure is performed. Preferentially, tissues should be analyzed a few days or weeks after snap freezing.

3.1.2. Tissue Cryosection and Thaw Mounted

1. The use of cryopreservative solutions containing organic polymers such as optimal cutting temperature (OCT) polymer should be restricted to the attachment of the tissue to the sample holder and not used for complete embedding of the tissue. Moreover, all parts of the cryostat in contact with the tissue must be cleaned to prevent any contamination between two different samples or with the polymer containing solution. In the case of contact between the tissue and cryopreservative solutions containing polymers, MS spectra will be dominated by polymer signals such as PEG.
2. Tissue is placed in the cryostat during sufficient time before sectioning for slow warming of the sample to the cryostat temperature. If the tissue is too cold, poor quality sections are obtained.
3. 10 μm thickness tissue sections are cut using cryomicrotome at –20°C. Different tissue types may need other temperature settings. 10 μm thickness is optimal (*see* **Note 4**).
4. Collect the tissue sections onto ITO glass slides pre-cooled at –20°C. Transfer is performed by applying the cooled ITO slide onto the section. The cuts thus stick on the cold slide (*see* **Note 5**). Adhesion of the frozen sections to the glass slides is obtained by heating, by putting fingers under the

slide, or by placing the slide at room temperature. This transfer procedure, contrary to classical thaw mounting, prevents formation of ice crystals at the surface of the cryostat microtome cutting plate.

5. Mounted sections are stored in a sealed container at –80°C until their use.

3.1.3. Pre-analysis Treatment – Tissue Fixation

1. A closed container store at –80°C is warmed at room temperature in a vacuum desiccator to prevent water condensation at the surface of the frozen slide.
2. After complete drying, the ITO slide is washed. Washing steps are optional and dependent on the molecules to be analyzed. Careful washing is crucial for conserving spatial localization of molecules.
3. For analysis of small molecules like lipids or drugs, no washing steps are used. For macromolecules such as peptides or proteins, washing procedures are generally used. Washing is performed by immersing the glass slide softly in ice-cold 75% ethanol during 30 s. No agitation or shake is needed. This step washes out salts, cells fragments, or residual fluids.
4. Take the slide out and remove the excess of liquid around the section. A stream of nitrogen over the surface could help to remove excess of ethanol.
5. The ITO glass slide is then placed in a vacuum desiccator to complete dry of the tissue. The time of drying is dependent on the size of the section.
6. *Optional:* A second bath of fresh ice-cold 75% ethanol during 30 s followed by a complete drying under vacuum desiccator can be achieved.
7. After complete drying, the sample is dipped into cold 95% ethanol during 30 s. No agitation or shake is needed. This step prevents degradation of proteome by dehydration and fixation of the tissue.
8. The slide is completely dried as in Steps 4 and 5.

3.1.4. Pre-analysis Treatment – Lipid Removal

1. After complete drying, immerse the glass slide softly in ice-cold chloroform during 30 s (*see* **Note 6**). No agitation or shake is needed. This step removes lipids (especially phospholipids) present in high concentration in the tissue (components of cell membranes) and may cause signal suppression in MS spectra (26).
2. Take the slide out and place it in the vacuum desiccator for complete drying of the tissue.

3.2. Preparation of FFPE Tissue Section

3.2.1. FFPE Tissue Section

1. 10 μm thickness FFPE tissue sections are cut using a microtome at room temperature. Paraffin block can be cooled down –20°C prior sectioning to facilitate tissue sectioning.
2. Sections are transferred onto a conductive ITO glass slide on top of a water droplet.
3. Glass slide is warmed up on a hotplate to leave the cuts unfolds.
4. Excess of water is removed and glass slide is stored in an incubator at 30°C during 20 min for good adherence. Subsequently obtained glass slides with FFPE tissue sections can be stored over months at room temperature.

3.2.2. FFPE Tissue Dewaxing

1. After complete drying, the glass slide is gently dipped into a bath of xylene during 5 min. This procedure is repeated two times. No agitation or shaking is needed.
2. The slide is then washed in stepwise immersion, 5 min duration each, into 100% ethanol twice, 95% ethanol, 75% ethanol, and 30% ethanol for rehydration of tissue sections.
3. The ITO glass slide is placed in the vacuum desiccator for complete drying of the sections.

3.3. On Tissue Digestion

3.3.1. Using a Microspotter

1. Microspotter like the chemical Inkjet Printer, CHIP-1000 (Shimadzu Biotech, Kyoto, Japan) can be used. CHIP-1000 is a piezoelectric solvent delivery system able to deliver picoliter volumes of reagents to define locations of various surfaces. It is equipped with an on board scanning device, which allows imaging the area of interest and easily selecting the right print location. An array of microspots that cover a specific area or the entire tissue section can be defined. Spots of 200 μm in diameter were spaced by 250 μm.
2. An ITO slide after washing step for frozen tissue or dewaxing for FFPE tissue is used.
3. On each defined spot, 40 nl of trypsin solution is applied. Five droplets of 100 pl are deposited at each spot per cycle, then 80 iterations are necessary to obtain the total volume.
4. The ITO glass slide is then incubated at 37°C for 2 h in a humidity chamber containing 50% methanol in water.
5. The ITO glass slide is placed in the vacuum desiccator to completely dry the tissue prior to matrix deposition.

3.3.2. Using an Automatic Sprayer

1. Automatic and control sprayer like the ImagePrep (Bruker Daltonics, Bremen, Germany) can be used for on tissue digestion. The ImagePrep system is equipped with an optical sensor that monitors light scattering from the solution deposited at the surface of the slide to control all relevant preparation parameters in real-time, namely deposition periods and intervals, wetness, drying rate.
2. A method with different step of spraying, incubation, and drying phase is used. For trypsin deposition, the spray time needs to be defined depending on the surface of the tissue section. Spray time is generally less than 2 s.
3. A particular attention is required to set correctly the drying time to achieve a complete drying of the section before a new spray cycle. If the time is too short, the section will be too wet and a delocalization of molecules may be observed. Normally, 40 cycles of spray are sufficient for efficient digestion of a rat brain section.
4. For FFPE tissue, a larger amount of trypsin is required to achieve a better digestion. In this case the number of cycle is increased.
5. The ITO glass slide is then incubated at 37°C for 2 h in a humidity chamber containing 50% methanol in water.
6. The ITO glass slide is placed in the vacuum desiccator for complete dry of the tissue prior to matrix deposition.

3.4. Matrix Deposition

3.4.1. For Protein Analysis Using a Microspotter

1. An ITO slide after washing step for frozen tissue is used.
2. On each defined spot, 20 nl of SA/ANI solution is applied. Five droplets of 100 pl are deposited at each spot per cycle, then 40 iterations are necessary to obtain the total volume.
3. Check matrix coverage using an optical microscope (*see* **Note 7**).
4. A rapid MS analysis on one spot is recommended to verify that a sufficient amount of matrix is deposited. Increase of iterations number may improve MSI when signal intensity appears to low.

3.4.2. For Protein Analysis Using an Automatic Sprayer

1. An ITO slide after washing step for frozen tissue is used.
2. A method with different steps of spraying, incubation, and drying phase is required. The ImagePrep method for SA/ANI deposition is based on the normal SA method included in the ImagePrep. Optimization is necessary for

each different type of tissue. Briefly, the spray time is around 2 s (depending on the surface of the tissue section). An incubation time of 30 s (except for initialization phase: 10 s) allows an efficient extraction of proteins. A particular attention is required to set correctly the drying time to achieve complete crystallization on the tissue section. If the time is too short, the section will be too wet and a delocalization of molecules will be observed. The minimum drying time is around 45 s.

3. Check matrix coverage using an optical microscope (*see* **Note 7**).
4. A rapid MS analysis at one position can be performed to check out that a sufficient amount of matrix has been deposited. If not, some cycles of the last phase of deposition can be done again and may improve MSI when signal intensity is too low.

3.4.3. For Peptide Analysis Using a Microspotter

1. An ITO slide after washing step for frozen tissues or digestion for FFPE or frozen tissues is used.
2. On each defined spot, 20 nl of HCCA/ANI solution is applied. Five droplets of 100 pl are deposited at each spot per cycle, then 40 iterations are necessary to obtain the total volume. For slides after digestion, the matrix is deposited with the same array than the one used for trypsin deposition. In this case matrix is deposited exactly at the same position than the trypsin.
3. Check matrix coverage using an optical microscope (*see* **Note 7**).
4. A rapid MS analysis on one spot is recommended to verify that a sufficient amount of matrix is deposited. Increasing the number of iterations may improve MSI when signal intensity appears to low.

3.4.4. For Peptides Analysis Using an Automatic Sprayer

1. An ITO slide after washing step for frozen tissue or digestion for FFPE or frozen tissues is used.
2. A method with different step of spraying, incubation, and drying phase is needed. The ImagePrep method for HCCA/ANI deposition is based on the normal HCCA method included in the ImagePrep. Optimization is required for each type of tissue. Briefly, the spray time is around 2 s (depending on the surface of tissue section). An incubation time of 20 s (except for initialization phase: 10 s) allow an effective extraction of proteins. A particular attention is drawn to correctly set the drying time for complete crystallization on the tissue section. If the time is too short,

the section will be too wet and a delocalization of molecules will be observed. The minimum drying time is around 120 s.

3. Check matrix coverage using an optical microscope (*see* **Note 7**).
4. A rapid MS analysis at one position can be performed to check out that a sufficient amount of matrix has been deposited. If not, some cycles of the last phase of deposition can be done again and may improve MSI when signal intensity is too low.

3.5. Mass Spectrometry Analysis

3.5.1. Mass Spectrometry Analysis for Protein MALDI-MSI (For Frozen Tissue Analysis Exclusively)

1. 0.5 μl of protein calibration solution is deposited near to the tissue section and mix with 0.5 μl of SA/ANI solution.
2. The mass spectrometer is calibrated with the calibration spot.
3. Using FlexImaging an area of interest is selected on the tissue after definition of the teaching points.
4. The distance between each measurement point is set. Distance between measurement points is dependent on the method used for matrix deposition:

   With Chip-1000 deposition, the spots are generally spaced by 250 μm center to center. It is possible to define the same raster than the one defined during matrix deposition. Due to the size of the spot it is possible to accumulate spectra at different position in the same spot. This increase statistics and reduce spot-to-spot variability.

   With ImagePrep deposition, distance between two measurements can be chosen by the user. Generally the resolution is around 100 μm.
5. In FlexControl, the adequate methods for proteins analysis is set in positive linear mode and a total of 500 spectra are acquired at each position at a laser frequency of 100 Hz.
6. The images are saved and reconstructed using FlexImaging 2.1.

3.5.2. Mass Spectrometry Analysis for Peptide MALDI-MSI (For Nondigest Frozen Tissue and Digest Frozen or FFPE Tissue)

1. 0.5 μl of peptide calibration solution is deposited near to the tissue section and mix with 0.5 μl of ANI solution.
2. The mass spectrometer is calibrated with the calibration spot.
3. Using FlexImaging an area of interest is selected on the tissue after definition of the teaching points.

4. The distance between each measurement point is set. Distance between measurement points depends on the method used for matrix deposition.
   4.1. With Chip-1000, deposition spots are generally spaced by 250 μm center to center. It is possible to define the same raster than for matrix deposition. Due to the size of the spots spectra can be accumulated at different positions in the same spot.
   4.2. With ImagePrep deposition, distance between two measurements is chosen by users. Generally the resolution is around 100 μm.
5. In FlexControl, the adequate methods for peptides analysis are set in positive reflector mode and a total of 500 spectra are acquired at each position at a laser frequency of 100 Hz. Although, negative reflectron mode can also be used for specific class of peptides.
6. The images are saved and reconstructed using FlexImaging 2.1.

3.5.3. MS/MS Analysis

1. Ultraflex II TOF–TOF is equipped with a LIFT III cell. For each MS/MS spectrum, 5,000 total shots are averaged including 1,000 for parent ions and 4,000 for fragments.
2. Peptides are identified by searching MS/MS spectra against an appropriate database using Biotools software for MASCOT (Matrix Science) interrogation. For MALDI data, peptide mass tolerance is set at 2 Da and MS/MS tolerance at 1 Da. Oxidation of methionine is selected as variable modification.
3. When a peptide is identified as a digest fragment of a protein, the protein sequence is used for in silico digestion. Expected digestion fragments of this protein are then search in the total MS spectrum. For the observed digestion fragments of the protein, MS/MS is performed to confirm that the peptide match to the expected sequence.
4. Using FlexImaging, molecular images of predicted digest fragments for one protein find in the data are realized to check out that they do present the same localization.

4. Notes

1. In some cases, the trypsin can be suspended in various solutions. For example, trypsin in water can be used for frozen sections for which the pH at the tissue surface is close to

the optimal pH value required for enzyme efficiency. Mix of water:methanol (1:1 v/v) can also be used with trypsin to achieve better extraction and permit a better access of cleavage sites to the enzyme. Use of water or water/methanol is recommended when using CHIP-1000 device for easier stabilization of droplets ejection.

2. Prior to deposition, 2 μl of matrix solution could be deposited with a micropipette on a classical MALDI sample plate to check the crystallization. Crystallization is expected close to that of SA, with homogenous smalls and fines white crystals.
3. Prior to deposition, 2 μl of matrix solution could be deposited with a micropipette on a classical MALDI sample plate to check the crystallization. Crystallization is expected to be uniform, with longs and fines white crystals as shown in table 2a of (25).
4. Smaller sections have not enough molecules for extraction and thicker sections may cause problems of conductivity (due to the insulating nature of tissues) and charge effects by charge accumulation at the sample surface during MALDI analysis. Charge effects will decrease spectral quality in axial TOF configuration instruments resulting in a progressive peak shifting toward the high m/z ratio.
5. Care must be taken of air bubbles formation at the surface of the tissue section that may leads to artifacts during MS analysis.
6. Other organic solvents could be used to perform this step. More information could be obtained in (26, 27). You must be careful not to increase the number of washing steps to avoid molecules delocalization.
7. To check matrix coverage, you need to ensure that the crystallization is dense, uniform and composed of small crystals.

Acknowledgments

This study was supported by grants from the Centre National de la Recherche Scientifique (DPI), Ministère de L'Enseignement Supérieur et de la Recherche, the Agence Nationale de la Recherche PCV (To IF).

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