Visualization of small intact proteins in breast cancer FFPE tissue

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Abstract

Molecular visualization of metabolites, lipids, and proteins by Matrix Assisted Laser Desorption Ionization Mass Spectrometry Imaging (MALDI MSI) is becoming an in demand analytical approach to aid histopathological analysis of breast cancer. Particularly, proteins seem to play a role in cancer progression and specific proteins are currently used in the clinic for staging. Formalin-Fixed Paraffin Embedded(FFPE) tissues are ideal for correlating the molecular markers with clinical outcomes due to their long-term storage. So far, to obtain proteomic information by MSI from this kind of tissue, antigen retrieval and tryptic digestion steps are required. In this chapter, we present a protocol to spatially detect small proteins in tumor and necrotic regions of patient-derived breast cancer xenograft FFPE tissues without employing any on-tissue digestion. This protocol can be used for other kind of FFPE tissue following specific optimization of the sample preparation phases.

Key words
MALDI MSI, protein, breast cancer, FFPE tissue

Introduction

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging (MSI) is a well-known technique able to spatially resolve biological molecules such as metabolites, lipids and proteins. MALDI MSI is readily applied in clinical research where associations between molecular and clinical information is becoming important to improve patient outcomes in a variety of diseases such as cancer. For this purpose, there is ample availability of formalin-fixed and paraffin embedded (FFPE) tissue which is collected routinely in the clinic. Therefore, FFPE tissue is a potentially highly valuable source of patient material for new biomarker discovery using MALDI MSI. However, the steps involved in the FFPE process requires specific considerations for the sample preparation before MALDI MSI compared to fresh frozen tissue. The paraffin from the embedding of FFPE tissue typically needs to be removed by strong organic solvents before MSI analysis. Formalin, or formaldehyde, is the standard form of fixation agent used in the preservation of clinical samples, and functions by creating covalent cross-link bonds within and between proteins. For proteomic analysis, these cross-links need to be reversed through the process of antigen retrieval, where enzymes[1], reactives[2] or heat[3, 4] is applied. Intact proteins can be sensitive to degradation following the process of fixation and subsequent antigen retrieval. This is why fresh frozen tissue is more often used for intact protein analysis with MALDI MSI[5, 6], while FFPE tissue is used for detection of tryptic peptides[7, 8].

In the case of breast cancer research, previous MALDI MS profiling of fresh frozen tissue has demonstrated separation of the luminal, HER2+ and triple negative subtypes based on protein and lipid profiles[9]. The prospect of expanding this research on FFPE breast cancer tissue is of high interest due to the much higher availability of FFPE compared to fresh frozen material. Several protocols have been developed to use FFPE cancer tissue for MALDI MSI detection of tryptic peptides[7, 8], endogenous peptides[10], metabolites[11] and lipids[12]. Recently, it was also discovered that antigen retrieval is

beneficial for the detection of groups of lipids which are cross-linked to proteins[13]. Our group recently published and found that lipids, which are unlocked with antigen retrieval, show significant alterations on patient-derived breast cancer tissues treated with glutaminase inhibitors compared to control groups[14]. Using the same modified protocol, the intact proteins can also be detected on FFPE breast cancer tissue as we will present in this chapter.

We provide a detailed protocol to detect and map the spatial distribution of small intact proteins on FFPE breast cancer tissue sections. The protocol includes sectioning, washing and deparaffinization, antigen retrieval, matrix application and MALDI MSI (*see* **Figure 1**).

Materials

Tissue sectioning

- FFPE tissue block(s).
- Microtome
- Indium tin oxide (ITO) coated glass slides (dimensions 7.5x2.5 cm) or other conductive slides compatible with MALDI-TOF MSI.
- Incubation oven at 60 °C
- Poly-D-lysine (poly-D-lysine hydrobromide molecular weight 70,000-150,000 Da, 0.1 mg/mL)

Tissue washing

- Toluene, isopropanol, ethanol and water (*see* **Note 1**).
- Glass staining jars. Approximate inside dimensions of 70 x 30 x 30 mm (*see* **Note 2**).
- Metal tweezers for the transfer of the slides.

Antigen retrieval

- Citric acid solution 10 mM at pH 5.95. For a 500 mL solution, 1.05 g of citric acid monohydrate is dissolved in 480 mL of MQ water. The pH is adjusted to 5.95 by adding ca. 13 mL of NaOH 1 M and then MQ water is further added to reach 500 mL in a volumetric flask (*see* **Note 3**).
- Pressure cooker (*see* **Note 4**).
- Trivet
- Plastic containers with a volume around 300 mL that can withstand temperatures up 120 °C

Matrix application

- Microscopic scanner
- Ultrasonic bath
- CHCA-ANI matrix solution: Dissolve 100 mg of α-cyano-4-hydroxy-cinnamic acid (CHCA) in 6.959 mL MeOH and sonicate for 3 min until CHCA is dissolved. Add 48 µL of aniline (ANI) and then sonicate for 5 min in order to form the ionic matrix[15] (*see* **Note 5**). Afterward, 2.983 mL of LC/MS grade water was added, and finally 10 µL of trifluoroacetic acid (TFA).
- PTFE syringe filters 0.45 µm for filtration of the matrix solution during the injection in the sprayer. This removes solid residues to avoid clogging of the sprayer.
- Pastic syringes (without silicone plunger), 5 mL and 20 mL.
- HTX sprayer M5 (or previous version)

MALDI MSI analysis

- Slide adapter
- Water-based correction marker

- Bruker RapifleX™ MALDI Tissuetyper™ (or MALDI TOF/TOF)
- ProteoMass™ Peptide and Protein MALDI-MS Calibration Kit (Supelco cat n. MSCAL1).

Methods

We describe in detail how to prepare FFPE tissue, in particular breast cancer tissues, in order to determine the localization of small proteins without employing any enzymatic digestion.

The preparation of FFPE tissue sections will be described since it is a crucial step for the sample preparation. Due to the weak adherence of FFPE tissue to ITO slides it is crucial to perform a pre-coating of the slides with poly-D-lysine and warm up the tissue to melt the paraffin and facilitate strong adherence to the ITO slides. The next step is the de-waxing and washing with progressive dilutions of ethanol. The last step is the antigen retrieval which allow the detection of intact proteins.

FFPE Tissue sectioning

With the exception of using different glass slides, FFPE blocks of breast cancer tissue are sectioned the same way as for classical histological staining and immunohistochemistry.

1. Prior to sectioning the ITO slides are cleaned by sonication first in hexane and then in methanol for 15 minutes each. Afterwards, the slides were coated with poly-D-lysine in order to improve the adherence of the tissue section on the slide (*see* **Note 6**).
2. FFPE tissue are sectioned at 4 µm thickness using a microtome at room temperature
3. The sections are transferred onto the conductive side of the ITO slide
4. The sections on the ITO slides are incubated at 60 °C for 1 hour to melt the paraffin and thereby improve the adherence of the tissue sections to the ITO slide.
5. The slides can be stored in a desiccator at room temperature or in the fridge at 4 °C until further use. The slides can be stored more than one year.

De-waxing and washing

Use the following procedure to dewax and partially rehydrate the tissue sections. Perform the washing steps in a fume hood (*see* **Note 7**).

1. Remove the paraffin by washing the tissue with toluene (HPLC grade) three times for 5 minutes
2. Wash with isopropanol (histological grade) for 5 minutes
3. Wash with EtOH 100% (HPLC grade) for 5 minutes
4. Wash with EtOH 90% for 5 minutes
5. Wash with EtOH 70% for 5 minutes
6. Let the tissue sections dry for 5 minutes flat on wipers in the fumehood with high air flow.

Antigen retrieval

Antigen retrieval is performed to reverse the cross-links created by the formalin fixation. This protocol uses a citric acid solution, high temperature and pressure for this purpose.

1. Fill the pressure cooker with deionized water up to a height of approximately 3 to 4 cm.
2. Insert the ITO slide into a plastic slide holder filled with citric acid 10 mM pH 5.95 and place these slide holders in the pressure cooker (*see* **Figure 2** and **Note 8**).
3. Switch on the heating plate under the pressure cooker with a power rating that allows to reach the full pressure in 20-25 minutes (*see* **Note 9**).
4. Wait until the vent on the pressure cooker lid pops up, indicating that full pressure is reached. Start the countdown for 2-45 min (*see* **Note 10**).
5. Turn off the heating plate and carefully move the pressure cooker on a trivet.
6. Wait until the pressure has reached ambient conditions/dropped before opening the lid. Be careful when opening the lid as both the lid is hot and hot steam may come out.

7. Take the slides out of the buffer solution and let them cool and dry for 10 minutes (*see* **Note 11**).
8. The slides can be stored in the desiccator or in the fridge until the further use (*see* **Note 12**).

Matrix application

The marix application used in this protocol includes a wet spray of the ionic matrix CHCA-ANI. The wet condition of the spray allows efficient extraction of proteins. The using of an ionic matrix has several advantages including higher signal intensity, signal-to-noise ratio and number of detected compounds. Moreover they show a high vacuum stability due to their high sublimation temperature[16]. Ionic matrices have shown to ensure more efficient detection of proteins and protein complexes as compared to solid crystalline matrices[17, 18].

1. Prior the matrix application, rehydrate the tissue sections with HPLC water by immersion into a glass coplin jar for 2 minutes (*see* **Note 13**).
2. Dry the sections for 10 minutes at room temperature under the fumehood with high air flow.
3. Add teaching points by marking the slides with water-based correction marker.
4. Scan the tissue section including the teaching points with a resolution of 5 µm/pixel by using a microscopic scanner.
5. Spot 1-2 µL of protein calibration standard on a free area of the slide and allow to dry. Mark the spot underneath the slide so you can locate it later.
6. Fill a plastic syringe with 6 mL of the prepared matrix (CHCA-ANI). Get rid of any air bubbles from the syringe as these may cause trouble with the pump and the matrix application (*see* **Note 14**) .
7. Attach a PTFE filter (0.45 micrometers) to the syringe and gently push the syringe plunger until you see liquid bulging out from the tip.

8. Make sure reservoir loop of the HTX-Sprayer is set to 'LOAD' and fill with ca. 5 mL of the prepared matrix solution
9. Set the flow rate of the isocratic pump to 0.06 mL/min, the temperature of the nozzle to 75 °C and the velocity to 1200 mm/min and the number of passes to 2.

   Turn on nitrogen-gas and adjust the pressure to 5 psi on the sprayer (*see* **Note 15**).
10. Turn the loop switch to 'SPRAY'.
11. Check that the nozzle is not clogged and the matrix is correctly deposited by running a blank slide. If the matrix deposition is homogenous, you can continue.
12. Place the sample slide and secure it with tape. Start the deposition method. The matrix deposition should be completed in less than 5 minutes per slide.
13. When finished, switch back to "LOAD", remove the slide, turn off the nitrogen gas. This automatically starts the cooling of the nozzle.
14. Clean the sprayer by first flushing the loop on "LOAD" position with 15 mL of 70 % MeOH, increasing the pump flow rate to to 0.5 mL/min, switching the valve to "SPRAY" and letting run for 6 minutes. Afterwards, switch the valve on "LOAD" and set the pump flow rate to 0.05 mL/min and, finally, fill the loop with 6 mL of 50% MeOH.
15. Check the surface morphology of the matrix applied on the slide with an optical microscope. The matrix should appear as a yellowish-transparent film with small crystallized zones (*see* **Figure 3**)

MALDI-MSI

For the detection of small intact proteins we recommend using positive ion mode, m/z range 2000-22200 and linear detector mode. Most experiments using the Bruker RapifleX™ MALDI Tissuetyper™ instuments use the reflector detector mode, as it provides improved mass resolution. However, large complex molecules, such as proteins, are very sensitive to breakage during the process of acceleration,

deceleration and reacceleration required for reflector mode. Linear detector mode only accelerates the analytes once and is therefore more gentle and increases the detection of intact proteins.

1. Place the sample slide into a target plate and insert into the Rapiflex.
2. Perform the detector check for linear detector mode on the matrix region and update with the recommended voltage value.
3. Save the method in linear positive mode.
4. Perform the target profile height detection for optimal laser focus.
5. Open flexImaging and set it up for a new imaging run.
6. In flexControl, calibrate the correct laser navigation and position between flexImaging and flexControl using the correction marker marks as teaching points.
7. Choose the laser geometry “M5” in flexControl and set the pixel size to 50 µm, or your preferred resolution (*see* **Note 16**).
8. Test the method on the tissue regions by changing the number of laser shots and the laser energy until sufficient signal appears in the *m/z* range between 2000 and 22200 (*see* **Figure 4 a** and **b**) with an intensity lower than 15000 a.i.. In general the number of shots per pixel/position is 500 and the laser energy is 60% at an optimized delay time (*see* **Note 17**).
9. Calibrate the instrument with protein standards spotted beside the tissue section.
10. In flexImaging, select the tissue regions you want to measure along with a small off-tissue, matrix-only, region as a control.
11. Start the MSI acquisition.
12. For histological analysis, stain the tissue section with haematoxylin-eosin saffron (HES) or H&E. You can use any standard staining protocol that works on your tissue. Stain within two weeks after MALDI MSI acquisition

13. Assess the MSI run in flexImaging and analyse the results with SCiLS Lab. An example of the results is shown in **Figure 4**.

Notes

1. Although the purity of the solvent is not an essential factor for protein analysis since the analysis will be performed in high mass range that generally is a contamination free region, HPLC grade solvents are employed in this protocol.
2. The dimensions of the glass jar and therefore the volume of the solvents used for all the washes is a crucial step of the sample preparation because large volumes can lead to artefacts of the analyte distributions. Moreover, the volume of toluene used is also sufficient to remove all the paraffin traces that might lead to ion suppression in MALDI-TOF. The glass jars are rinsed in Ethanol 70%, washed with water and then let to dry in the fumehood.
3. Different antigen retrieval buffers, at different pH, could also be used and may lead to recovering of different proteins.
4. It is possible to use any kind of pressure cooker, however it is important that the pressure and the temperature do not drastically change during the antigen retrieval. Therefore check carefully all the valves before use. Additionally, other systems which enable temperatures of 97 °C to be reached and maintained can be also employed depending on the tissue type.
5. Ionic matrices are organic salts formed by an acid-base reaction and they can be solid or liquid based on the molar ratio[16]. Adding equimolar amounts of ANI in respect to CHCA results in a liquid ionic matrix.
6. Dilute 5 mg of poly-D-lysine in 50 mL HPLC grade H_2O. Place the ITO slides on a disposable wiper. Apply on the conductive side of each ITO slide 2-3 droplets of the poly-D-lysine solution using a glass pipette. Distribute evenly using a glass spreader. Let dry at room temperature overnight.

7. The slides need to be handled with outmost care to avoid any tissue detachment during the immersion. Do not repetitively dip the slides and no agitation or shaking is needed. Having a sufficient volume of the washing solvent is also an important factor, for this reason, we recommend to use only one slide at a time per glass staining jar.
8. Be sure that the ITO slides are fully covered by the buffer
9. We advice to test different power settings depending on the pressure cooker type and set-up.
10. The time of the antigen retrieval is dependent on the tissue type. Optimal time for antigen retrieval has to be determined for each tissue type individually to balance potential loss of tissue from using a too long and reduced efficiency of reversing the fixation using a too short heating step. Therefore, we suggest to test the antigen retrieval of your tissue at different time points starting at 2 minutes up to 45 minutes, which are the typical time spans used for an antigen retrieval in immunohitochemistry[4]. In our case, using xenograft breast cancer tissue, we did not observe any loss of tissue after 30 minutes of antigen retrieval treatment. In contrast, we observed loss of tissue for clinical human breast cancer FFPE sections after 30 minutes and had to reduce the antigen retrieval down to 2 minutes.
11. The required time for cooling the slides is tissue dependent and the user needs to optimize the cooling method in order to avoid the partial/complete detachment of the tissue sections.
12. The slide can be stored at maximum overnight
13. This step is crucial because without rehydratation, protein and lipids are not detectable by MALDI-MSI.
14. For applying matrix on a reduced amount of the slides, volume of 6 mL might not be required. Therefore, whilst the loop has to be full, the sprayer sparys the last solution entered into the loop first. Thus, if the method only requires 2 mL of matrix, 3 mL of the solvent solution (used to dissolve the matrix) can be injected prior to the 3 mL of the matrix solution which is enough for

stabilising the spray and then the deposition. This procedure helps to minimise the waste of chemical.

15. The nozzle temperature will not increase unless nitrogen gas is flowing
16. To avoid oversampling on the border of each pixel, the pixel size is set somewhat smaller for a final pixel size of 50 µm in flexControl, so that it matches the final 50 µm in flexImaging. Generally, the laser geometry affects mainly the speed acquisition but we observed that using “M5” as laser geometry, the quality of the protein mass spectra is improved. However, we recommend to check both “single” and “M5” laser scan for obtaining high quality mass spectra that are specific for your sample.
17. Delay time crucially affects the sensitivity of high mass range compounds such as proteins, therefore we suggest to test different delay times in order to obtain the best sensitivity and accuracy[19]. Since the delay time also impacts the mass accuracy, the calibration must be reperformed each time the delay time is optimized/modified.

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Figures

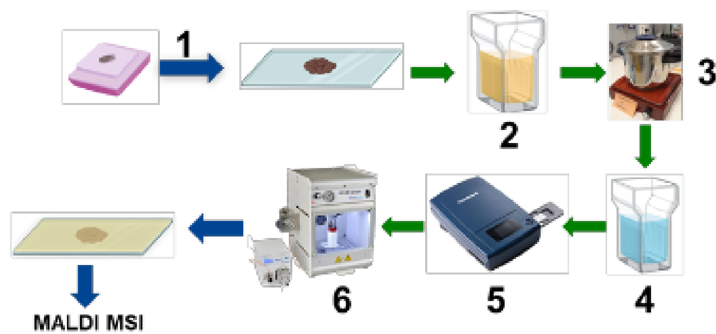

Figure 1. Workflow for sample preparation of FFPE tissues for detection of intact proteins by MALDI-MSI. FFPE tissue blocks are first (1) sectioned, sections are then (2) washed and deparaffinized, followed by (3) antigen retrieval, (4) rinsing and drying, (5) microscopically scanned, (6) covered with matrix and (7) analyzed with MALDI MSI. Adapted from Denti et al. [14]

Figure 2. Set-up of the ITO slides inside of the pressure cooker.

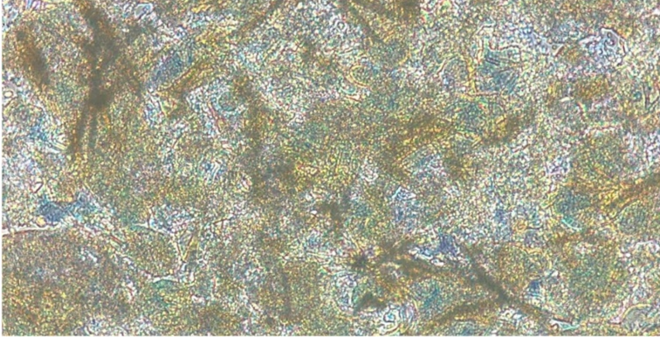

Figure 3. Surface morphology of CHCA-ANI matrix applied on ITO slide observed by optical microscope (40X). The matrix appear as a transparent-yellowish film with darker crystallized areas.

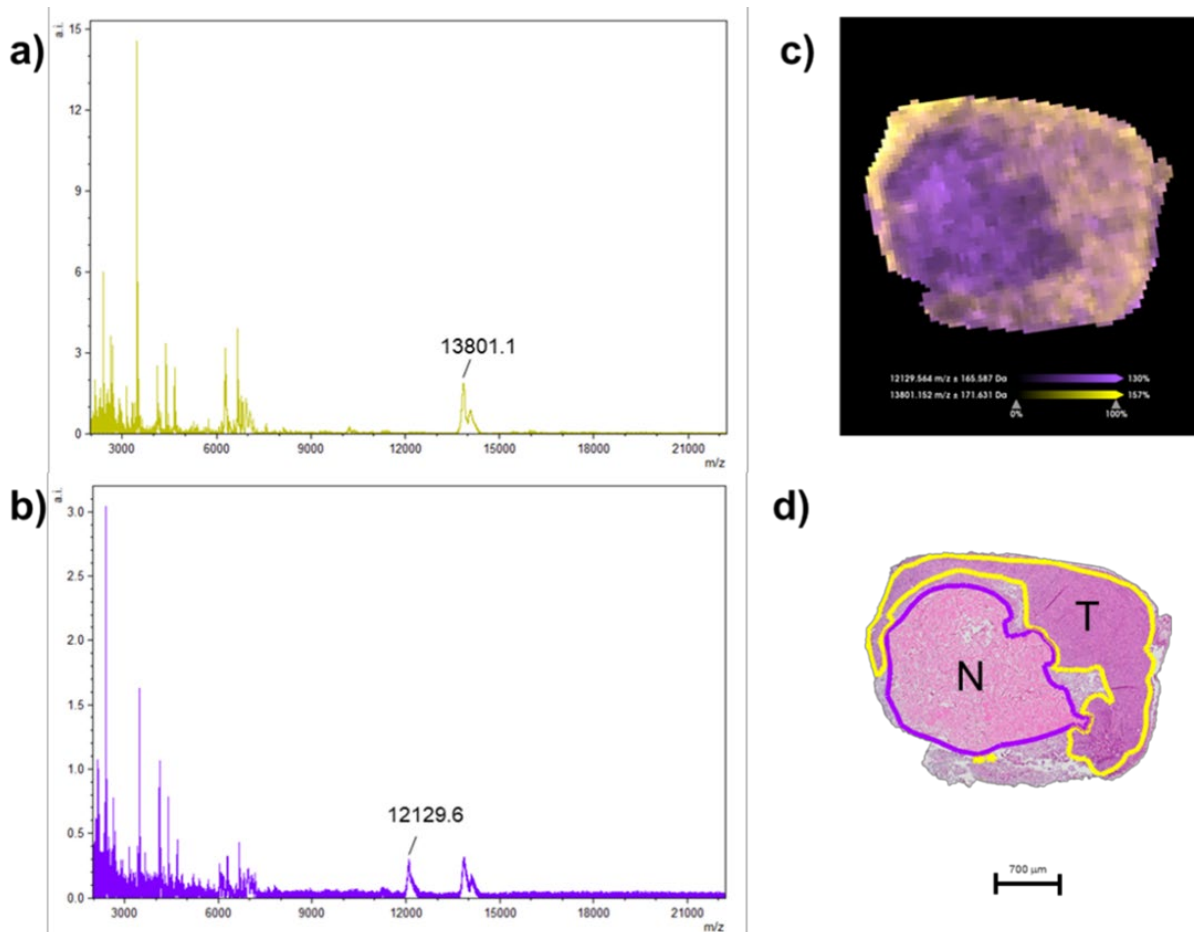

Figure 4. MALDI MSI of small intact proteins in breast cancer patient derived from xenograft tissue section. Overall average mass spectrum of a) tumor and b) necrotic regions. c) Dual ion map of the *m/z* 13801 (yellow) and 12129 (violet) showing co-localization with tumor (T) and necrotic region (N), respectively, with d) HE stained tissue section.