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MALDI Imaging Combined with Laser Microdissection-Based Microproteomics for Protein Identification: Application to Intratumor Heterogeneity Studies

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Abstract

Matrix-assisted laser desorption ionization (MALDI) imaging is widely used for in situ proteomic mapping and finds multiple applications in pathology. However, low fragmentation yields in MALDI avoid an optimal identification of peptides from tissues. On the other hand, LMD-based microproteomic analyses allow for the identification of hundreds to thousands of proteins from small tissue regions. Herein, we present the combination of MALDI imaging and LMD-based microproteomic approaches for parallel identification. We illustrate the workflow with an application to intratumor heterogeneity studies.

Keywords Formalin-fixed and paraffin-embedded tissues, Histopathology, MALDI imaging, Intratumor heterogeneity, Protein identification, Laser microdissection, Microproteomics

1 Introduction

MALDI imaging is now recognized as a method of choice for applications in fundamental and clinical pathology [1]. It is now on the process to be used for routine diagnosis in pathological institutes. However, MALDI imaging suffers from inefficient fragmentation yields that limit its potential for peptide/protein identification. In parallel, other methods have been designed for the identification of thousands of proteins from small tissue regions. In particular, LMD-based microproteomics have been created for proteomic biomarker discovery from FFPE tissue regions containing less than 3000 cells [2]. Microproteomic dataset could be correlated to MALDI imaging ones for parallel protein identification [3, 4]. The combination of approaches is particularly adapted when the identification of compounds detected from small tissue regions by MALDI imaging is necessary. Laser microdissection also provides the unique advantage to allow one to collect tissue regions of any shape for further chemical processing. Identification of compounds with LMD-based microdissection method is then possible in any histological context. The combination of methods presented here is dedicated to formalin-fixed and paraffin-embedded (FFPE) tissues.

As MALDI imaging allows for molecular analyses in respect with microanatomical context, it is particularly adapted to study intratumor heterogeneity [5]. Segmentation algorithm allows for in situ classification of tissue subclusters on the basis of their molecular differences [5]. Hereafter, we illustrate the combination of MALDI imaging and LMD-based microproteomics for parallel identification from a single section, in the context of intratumor heterogeneity (ITH) studies [4].

This combination of methods also holds good promise for future biomarker discovery assays related to ITH studies.

2 Material

2.1 Material and Solutions

1. Indium tin oxide conductive glass slide (Bruker Daltonics, Bremen, Germany)
2. Citric acid (CA) 50 mM, CA 10 mM, pH 6.0
3. Tipp-Ex (Tipp-Ex, Frankfurt am Main, Germany)
4. Tissue scanner
5. α-Cyano-4-hydroxycinnamic acid (CHCA) solution 5 mg/mL
6. Spraying system. In this protocol, SunCollect sprayer (SunChrom, Friedrichsdorf, Germany)
7. MALDI mass spectrometer. In this protocol: UltrafleXtreme TOF/TOF (Bruker Daltonics, Bremen, Germany)
8. Peptide calibration standard I (Bruker Daltonics, Bremen, Germany)
9. Methanol
10. Polyethylene naphthalate (PEN) membrane glass slides for laser microdissection (Leica Microsystems, Wetzlar, Germany)
11. Laser microdissection device: Leica LMD 7000 (Leica Microsystems, Wetzlar, Germany)
12. Milli-Q H_2O
13. NaOH 1 M
14. RapiGest surfactant (Waters, Milford, MA) 0.1%
15. NH_4HCO_3 (ammonium bicarbonate) 500, 100, 50, and 25 mM
16. Dithiothreitol (DTT) 500, 131, and 171.6 mM
17. Iodoacetamide (IAM) 500 and 194.67 mM
18. Aluminum foils
19. Hydrochloric acid (HCl) 10 mM
20. Trypsin Gold, Mass Spectrometry Grade (Promega, Fitchburg, WI) 1 and 0.5 μg/μL
21. Acetonitrile (ACN) 100%
22. Trifluoroacetic acid (TFA) 10%

23. Microtubes 0.6 mL
24. Kimwipes disposable (Kimberly-Clark, Dallas, TX)
25. Centrifuge
26. Thermoshaker
27. Antistatic gun Zerostat 3 (Sigma-Aldrich, St. Louis, MO)
28. Binocular microscope
29. Sonicator
30. Cleaning paper
31. Speed vacuum centrifuge
32. ZipTip (ZT) cartridge C18 2 μg, 5 μg (Millipore, Billerica, MA)
33. NH_4HCO_2 (ammonium hydroxide) 20 mM, pH 10
34. Formic acid 0.1% in water, 0.1% in acetonitrile
35. MassPREP digestion standard (MPDS) mixtures, mix 1 and 2 (Waters, Milford, MA)
36. Speed vacuum (Thermo Scientific, Waltham, MA)
37. Ultraperformance liquid chromatography (UPLC) nanoACQUITY 2D (Waters, Milford, MA)
38. Reverse phase (RP) XBridge BEH C18 5 μm column (300 μm × 50 mm) (Waters, Milford, MA)
39. Trap column Symmetry C_{18} 5 μm (180 μm × 20 mm) (Waters, Milford, MA)
40. Analytical column BEH C_{18} 1.7 μm (75 μm × 250 mm) (Waters, Milford, MA)
41. Orbitrap mass spectrometer Q Exactive or Q Exactive Plus (Thermo Fisher Scientific, Waltham, MA)

2.2 α-Cyano-4-hydroxycinnamic Acid Solution 5 mg/mL

1. Weight 5 mg of CHCA in a tube. Add 700 mL of acetonitrile and 300 mL of mQ water with 0.1% TFA.

2.3 Citric Acid Solutions

1. Citric acid stock solution (50 mM): weight 0.96 g of citric acid and dissolve in 99.94 mL Milli-Q water.
2. NaOH 1 M: weight 40 g of NaOH and dissolve in 1 L Milli-Q water.
3. Citric acid 10 mM, pH 6.0: transfer 20 mL of the stock solution (50 mM) to a 100 mL bottle and adjust to 50 mL with Milli-Q water. Adjust the pH to 6.0 by adding 1 M NaOH. Adjust the final volume to 100 mL with Milli-Q water.

2.4 NH_4HCO_3 (Ammonium Bicarbonate) Solutions

1. 500 mM. Weight 39.53 g of NH_4HCO_3 and dilute in 1 L Milli-Q water. Store at 4 °C in a glass bottle.
2. 100 mM. Dilute 5× the NH_4HCO_3 500 mM solution, NH_4HCO_3 500 mM/Milli-Q water 1:4. Store at 4 °C in a glass bottle.
3. 50 mM. Dilute 10× the NH_4HCO_3 500 mM solution, NH_4HCO_3 500 mM/Milli-Q water 1:9. Store at 4 °C in a glass bottle.
4. 25 mM. Dilute 20× the NH_4HCO_3 500 mM solution, NH_4HCO_3 500 mM/Milli-Q water 1:19. Store at 4 °C in a glass bottle.

2.5 DTT Solutions

1. 500 mM.

   Weight 0.7713 g of DTT. Solubilize with 10 mL of Milli-Q water. Aliquot in tubes with 100–500 µL and cover with aluminum foil. Freeze at −20 °C until use.
2. 131 mM in 50 mM NH_4HCO_3.

   Mix 10 µL of DTT 500 mM with 9.1 µL of Milli-Q water and 19.1 µL of 100 mM NH_4HCO_3 in a 0.6 mL microtube. Cover with an aluminum foil.
3. 171.6 mM in 50 mM NH_4HCO_3.
   Mix 10 µL of DTT 500 mM with 4.57 µL Milli-Q water and 14.57 µL of 100 mM NH_4HCO_3 in a 0.6 mL microtube. Cover with an aluminum foil.

2.6 IAM Solutions

1. 500 mM in water.

   Weight 1.85 g of IAM. Solubilize with 20 mL Milli-Q water. Aliquot tubes with 100–500 µL. Freeze at −20 °C until use.
2. 194.67 mM in 50 mM NH_4HCO_3.
   Mix 20 µL of IAM 500 mM with 5.68 µL Milli-Q water and 25.68 µL of 100 mM NH_4HCO_3 in a 0.6 mL microtube. Cover with an aluminum foil.

2.7 HCl Solutions

1. 1 M

   Commercial solutions of HCl are usually 12 M. Dilute 12 × the 12 M HCl solution with Milli-Q water to obtain a 1 M stock solution: HCl 12 M/Milli-Q water 1:11.
2. 10 mM
   Dilute 100× the 1 M HCl solution with Milli-Q water: HCl 1 M/Milli-Q water 1:99.

2.8 Trypsin Solutions

1. 1 µg/µL

   Reconstitute 100 µg of lyophilized trypsin in 100 µL of 10 mM HCl. Aliquot in tubes with 1, 2, or 3 µL depending on further use, and store at −20 °C until use.

2. 0.5 μg/μL
   Dilute 2× a trypsin stock solution (1 μg/μL) in 25 mM NH_4HCO_3. Depending on the number of samples to process, the amount of stock trypsin solution should be adapted.

2.9 RapiGest SF Surfactant 0.1%

1. Reconstitute one vial (1 mg) in 1 mL NH_4HCO_3 50 mM. Aliquot in tubes with 100 μL and store at −20 °C before use.

2.10 TFA 10%

1. Mix 10 mL of TFA with 90 mL of Milli-Q water. Store at 4 °C in a glass bottle.

2.11 ZipTip (ZT) Solutions

1. ZT Solution 1 (ACN + 0.1% TFA): mix 999 μL ACN with 1 μL TFA in a tube.
2. ZT Solution 2 (ACN/H_2O 1:1 + 0.1% TFA): mix 499.5 μL ACN with 499.5 μL Milli-Q H_2O and 1 μL TFA in a tube.
3. ZT Solution 3 (H_2O + 0.1% TFA): mix 999 μL H_2O with 1 μL TFA in a tube.
4. Larger volumes can also be prepared and stored in glass bottles at 4 °C.

2.12 20 mM NH_4HCO_2 (Ammonium Formate) Solution pH 10

1. 200 mM ammonium formate pH 10 stock solution: NH_4OH (ammonium hydroxide) commercial solutions are usually 25%. For 1 L of solution, use a 1 L graduated cylinder and place 850–900 mL of Milli-Q H_2O. Add 15.4 mL of NH_4OH 25%. Mix well. Add 1.62 mL HCO_2H (formic acid). Mix well. Adjust the pH to 10 with NH_4OH 25% or formic acid. Adjust the volume to 1 L with Milli-Q H_2O. Store in a glass bottle at 4 °C.
2. 20 mM ammonium formate pH 10: for 1 L of solution, mix 100 mL of 200 mM ammonium formate pH 10 stock solution with 900 mL of Milli-Q H_2O. Check the pH. Store in glass bottles at 4 °C.

2.13 0.1% Formic Acid in Water

Mix 1 mL of formic acid with 999 mL of Milli-Q H_2O.

2.14 0.1% Formic Acid in ACN

Mix 1 mL of formic acid with 999 mL of ACN.

3 Methods

3.1 MALDI Imaging

3.1.1 Tissue Sectioning

1. After surgery, immerse the tissue overnight in 10% formalin, dehydrate and embed in paraffin using well-known procedures widely used among institutes of pathology [6]. Cut 5 μm-thick sections from the FFPE block and deposit on one ITO glass slide on the conductive side.
2. Perform three serial 5 μm sections and deposit on LMD membrane slides.

3.1.2 Antigen Retrieval

1. Place the ITO glass slide with the section to analyze by MALDI imaging in a histological holder.
2. Fill a recipient that can contain glass histological holder with CA 10 mM pH 6.
3. Preheat at 98–99 °C (near boiling buffer) and immerse the histological holder with the glass slides for 60 min.
4. Remove the histological holder and place it in a recipient full of mQ water. Replace the water three times.
5. Remove the sections from the histological holder and let the sections dry.

3.1.3 Tissue Scanning

1. Apply teach marks using Tipp-Ex on corners around the tissue.
2. Perform high-resolution scan (1200–2400 dpi).

3.1.4 Trypsin Spraying and Incubation

1. Wash the system with the mixture ACN/mQ water 7:3.
2. Spray 200 µL of 25 mM NH_4HCO_3.
3. Use an aliquot of 10 µL of trypsin 1 µg/µL and dilute in 200 µL of 25 mM NH_4HCO_3.
4. Spray the volume of trypsin solution over the tissue section on a surface equivalent to the half of an ITO section at a flow rate of 10 µL/min.
5. Place the section in a humidity chamber preheated at 37°C and place the humidity chamber in an incubator at 37 °C for 4 h (see **Note 1**).

3.1.5 Matrix Spraying

1. Fill the spaying system with CHCA 5 mg/mL. Spray the surface of the tissue at a flow rate of 20 µL/min for 15 passages. Remove the section and deposit 0.5 µL of peptide calibration standard on an area covered with matrix.
2. Wash the system with the mixture ACN/mQ water 7:3.

3.1.6 Data Acquisition

1. Hold the glass slide to the tissue target and insert it in the MALDI instrument.
2. In FlexControl, select the method recommended by the constructor for peptide mass range in the positive mode (see **Note 2**).
3. Calibrate the method with the peptide calibration standard and save it.
4. In FlexImaging, create a new imaging run, select the calibrated method, and select the scan of the tissue section and perform teaching. Adjust the raster step to the tissue of interest as well as the number of laser shots per position.
5. Define an area covering the tissue section and perform the run.

3.1.7 Matrix Removal and Histological Staining

1. Remove CHCA from the tissue section analyzed by MALDI imaging by immersion in two successive baths of methanol. Let the section dry for 2 min.
2. Perform hematoxylin and eosin (HE) staining from the section as described before [2] and perform a high-resolution scan of the section.

*3.1.8 Image Segmentation and Determination of Heterogeneous Compounds (See **Note 3**)*

1. Load the images in the last version of the software SCiLS Lab (SCiLS, Bremen, Germany) and perform baseline removal using the convolution algorithm.
2. Normalize the spectra using total ion count (TIC) algorithm.
3. Perform peak peaking from the area of interest.
4. Perform segmentation. Analyze all spectra using bisecting k-means algorithm. Select the peaks picked as a mass range, 0.2 Da as interval width value and TIC as a normalization method. The process will result in a segmented image as shown in Fig. 1 where the molecular subclusters are highlighted in different colors.
5. In SCiLS Lab, create regions from the heterogeneous clusters and increase the transparency. Load the HE scan after MALDI and superimpose with the MALDI segmented dataset. This will result in the correlation of histological morphologies with molecular clusters as shown in Fig. 2a.
6. Create from the MALDI imaging dataset a small region in the heterogeneous clusters. Perform a principal component analysis (PCA) to compare the molecular content between the two regions. Use the peak picked as *m/z* interval and the following parameters: TIC normalization, interval width value 0.2 Da, five PC for PCA, and analysis from individual spectra.
7. Select *m/z* of interest at the edges on component 1. These *m/z* values are the most discriminant between the regions the spectra used for PCA (Fig. 2c).
8. Verify the intensity trends of the peaks of interest between the selected clusters by MALDI imaging and from the average spectra of the clusters (Fig. 2d and 2e).

At this step, enough information is collected from MALDI imaging to proceed to LMD-based microproteomic procedure.

3.2 MALDI Imaging-Guided LMD-Based Microproteomics (See Note 4)

3.2.1 Laser Microdissection

1. From the HE-stained section after MALDI imaging, perform cell counting using digital pathology software such as QuPath (https://qupath.github.io/) or Cytomine (http://www.cytomine.be/) [7]. Cytomine allows sharing online annotations and comments of the tissues between different institutions.
2. Localize the regions of interest (heterogeneous regions) by comparing the MALDI segmented images superimposed with

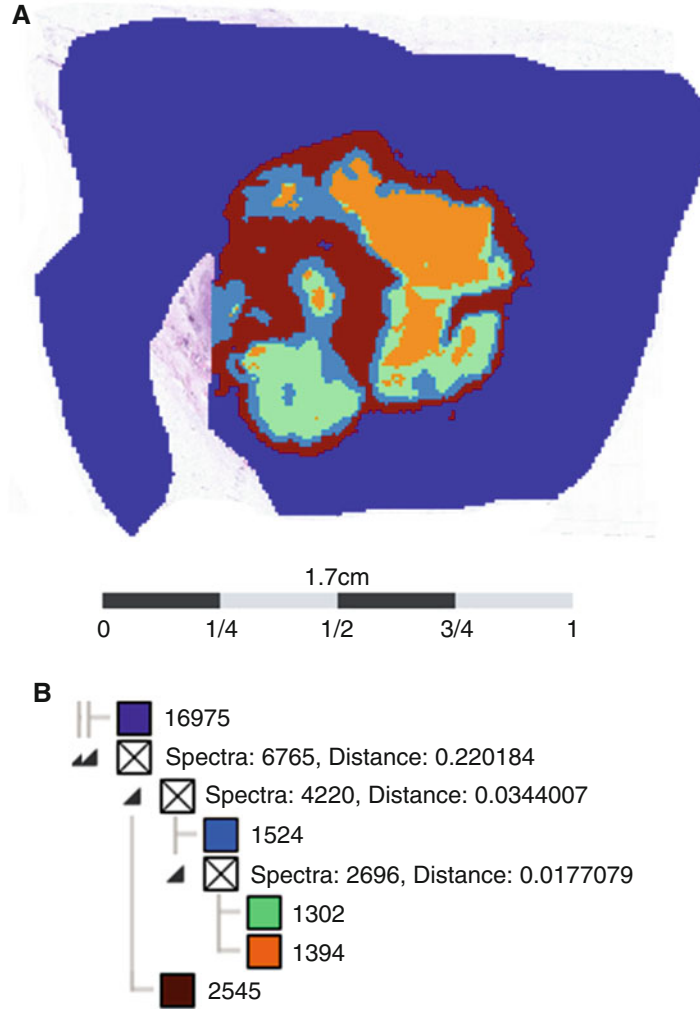

Fig. 1 MALDI imaging segmented dataset. (**a**) Molecular cluster from the breast cancer tissue section. (**b**) Hierarchy of the clusters. The *dark blue* cluster corresponds to the areas around the tumor. The *brown* cluster corresponds to the tumor. A subcluster in *light blue* is composed of two subclusters in *orange* and *green*, selected figure 3, inset **a**. Adapted with permission from [4]

the HE scan in SCiLS Lab, and the morphology of the tissue in bright field observation through the laser microdissector (see **Note 5**). Select areas from the clusters of interest containing the same number of cells on the basis of the counts from QuPath. A number of 3500 cells for cancer samples are recommended.

3. Perform microdissection: we used the Leica LMD 7000 (Leica Microsystems, Wetzlar, Germany) with the following laser settings: laser wavelength, 349 nm; pulse energy, 52J; numerical aperture, 15; specimen balance, 14; laser head current, 100%; pulse frequency, 174 Hz; and focus offset, 50. The settings

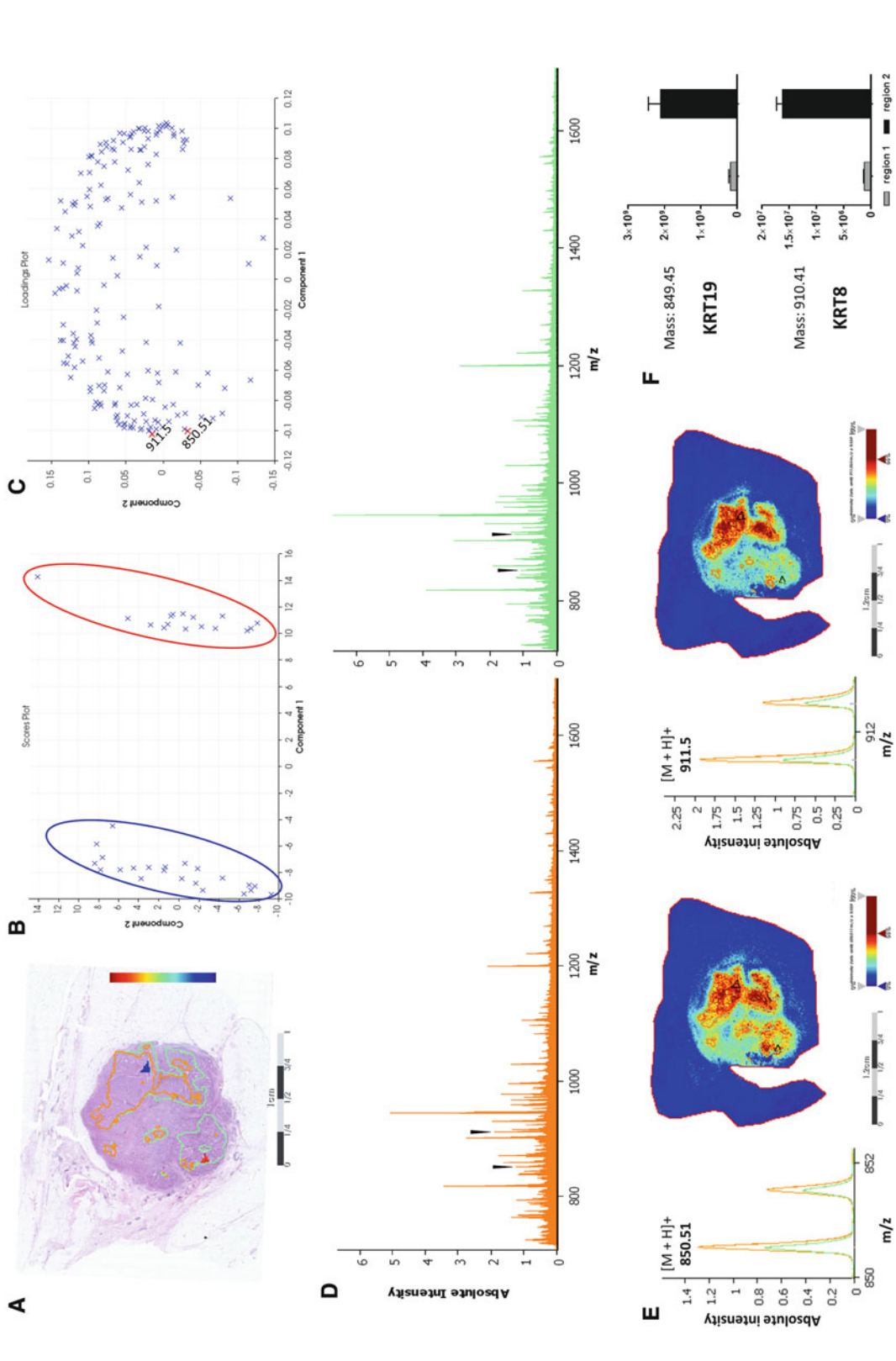

Fig. 2 Identification of heterogeneous peptide is a breast tumor. (**a**) Superimposition of the HE section after MALDI with the segmented dataset. The *green* and *orange* clusters are outlined. (**b**) Scores plot of the PCA analysis of the subset of spectra in the heterogeneous clusters. The selected area corresponds to the one selected for parallel

should be adapted to the tissue type. Three collection replicates should be performed, from the serial sections collected on LMD membrane slides (see **Note 6**).

3.2.2 Preprocessing

This step consists in collecting the tissue pieces from the cap to the bottom of the tubes to improve monitoring during processing.

- During laser microdissection, the tissue pieces are collected in tube caps. Store the tubes at 4 °C before use, in a clean environment.
- Use the antistatic gun to get rid of static electricity during sample preprocessing.
- Centrifuge the tube at 15,000 × *g* during 5 min at room temperature (RT) and dispose the tubes in a clean holder.
- Check for the presence of the tissue piece(s) at the bottom of the tubes using a binocular microscope.

3.2.3 Antigen Retrieval

This step consists in getting rid of the methylene bridges linking proteins induced by formalin fixation.

- Gently add 20 μL of 10 mM pH 6.0 citrate buffer on the tissue piece at the bottom of the tube without touching it with the tip.
- Centrifuge at 15,000 × *g* for 5 min at RT. If it is still visible, ensure yourself that the tissue is properly immersed in citric acid.
- Sonicate the immersed tissue pieces for 10 min in a holder that avoids overheating of the tubes. This step allows removing air bubbles that may stay at the surface of the tissue.
- Incubate in a thermoshaker at 99 °C for 1 h at 800 RPM. Quickly spin the tubes every 10 min in order to collect the condensed water from the cap to the bottom of the tubes.
- Collect the tubes in a clean holder and let these cool at RT.
- Add 2.2 μL of RapiGest 0.1% in the samples to obtain a final 0.01% concentration and spin the samples at 21,000 × *g* for 2 min. Quickly spin.
- Shake at 800 RPM for 10 min at RT. Quickly spin.
- Add 2 μL of 500 mM NH_4HCO_3 to all the samples. Agitate for 10 min at 800 RPM and centrifuge the tubes (21,000 × *g*, 1 min). Check that the pH of the samples is 7.

Fig. 2 (continued) laser microdissection. (**c**) Loadings plot of the PCA analysis. *m/z* 911.5 and *m/z* 850.5 are highlighted. (**d**) Average mass spectrum from the *orange* and *green* clusters. (**e**) MALDI images of the *m/z* 911.5 and *m/z* 850.5. (**f**) Identification of the *m/z* 911.5 and *m/z* 850.1 after correlation with the LMD-based microproteomic dataset. Reprinted with permission from [4]

*3.2.4 Reduction-Alkylation-Reduction (See **Note 7**)*

This step consists in getting rid of disulfide bridges still existing within proteins after antigen retrieval.

1. Reduction (reduction of the disulfide bridges and liberation of the thiol groups): the samples have now a volume of 24.2 µL. Add 2 µL of 131 mM DTT in 50 mM NH_4HCO_3 to obtain a final concentration of 10 mM DTT. Incubate the samples for 40 min at 56 °C while shaking at 800 RPM. Quickly spin.
2. Alkylation (blocking thiol groups with IAM): the samples have now a volume of 26.2 µL. Let the samples cool at RT and add 3 µL of 194.67 mM IAM in 50 mM NH_4HCO_3 to obtain a final concentration of 20 mM IAM. Incubate at RT for 30 min while shaking at 800 RPM. Quickly spin.
3. Reduction (eliminate IAM in excess): the samples have a volume of 29.2 µL. Add 2 µL of 171.6 mM DTT in 50 mM NH_4HCO_3 to add a concentration of 11 mM DTT. Incubate for 10 min at RT (24 °C) while shaking at 800 rpm. Quickly spin.

3.2.5 Digestion

This step consists in digesting the proteins from the tissue piece.

1. The samples have a volume of 31.2 µL. Add 4.26 µL of 0.5 µg/µL trypsin to obtain a final concentration of about 60 µg/mL. Quickly spin.
2. Incubate at 37 °C overnight while shaking at 800 rpm. Quickly spin.
3. The samples have a volume of 35.46 µL. Add 2.26 µL of 0.5 µg/µL trypsin to increase the concentration by about 30 µg/mL. Quickly spin.
4. The samples have now a volume of 37.72 µL. Add 150.88 µL of ACN to obtain a solution of 80% ACN. Quickly spin.
5. Incubate at 37 °C for 3 h while shaking at 800 RPM. Quickly spin.
6. The samples have a volume of 188.6 µL. Stop digestion by adding 10 µL of TFA 10% to obtain a final concentration of about 0.5% TFA in the tube. Agitate for 10 min at 800 RPM and centrifuge the tubes (21,000 × *g*, 1 min). Check the pH of the solution (pH < 3).
7. Incubate for 45 min at 37 °C while shaking at 800 rpm in order to cleave RapiGest.
8. Centrifuge the samples at 21,000 × *g* for 10 min, at 4 °C.
9. Collect the supernatant and transfer in new identified 0.6 mL tubes.
10. Evaporate the samples using the speed vacuum. Store the samples at 4 °C if necessary for 1 week maximum. If the storage time exceeds 1 week, store the samples at −20 °C.

3.2.6 Purification/ Desalting/Concentration of the Samples

This step consists in purifying, desalting, and concentrating the proteolytic digests obtained from the tissues by solid phase extraction (SPE).

1. Prepare the three solutions described in Sect. 2.11.
2. If the samples were dried, resuspend in 20 µL Milli-Q H_2O with 0.1% TFA (ZT Solution 3). If the SPE is performed just after the digestion, transfer the necessary volume of sample in another 0.6 mL tube and adjust to 0.1% TFA, with maximum 5% of organic solvent, in an ideal volume of 20 µL. Depending on the expected amount of peptides resulting from the digestion process, use ZT C_{18} 5 µg or ZT C_{18} 2 µg (µZT).
3. Set the micropipette to 10 µL. Wash the ZT with Solution 1: take and withdraw in the waste. Repeat three times.
4. Activate the ZT by taking Solution 2. Withdraw in the waste. Repeat three times.
5. Equilibrate the ZT: take Solution 3 and withdraw in the waste. Repeat three times.
6. Load the sample. Adjust the sample to the used ZT. If the expected concentration of peptides in the sample is too high (>5 µg when ZT −5 µg are used, >2 µg when ZT −2 µg are used), dilute with a 0.1% TFA solution. Pipet ten times to load the peptides to the ZT. If the sample is diluted or if the volume of sample is higher than 20 µL, pipet 25 times.
7. Wash the samples: take Solution 3 and withdraw in the waste. Repeat five times.
8. Elute the sample: take Solution 2 and collect in a new microtube. The peptides are present in the solution in the new microtube. Repeat once. The proteolytic digest is then concentrated in 40 µL of ACN/H_2O 1:1 and 0.1% TFA.
9. Evaporate the sample completely with the speed vacuum.
10. Proceed to the subsequent LC-MS/MS analysis or store the sample at 4 °C for 1 week maximum, at −20 °C if the analysis cannot be performed within 1 week.

*3.2.7 Two-Dimensional NanoLC-Orbitrap Mass Spectrometry Analysis (See **Note 8**)*

1. Resuspend the samples in 10–11 µL of appropriate buffer for 2D LC-MS and MPDS mix in order to get a final concentration of 50 fmol alcohol dehydrogenase (ADH) per volume of injection (9 µL). Use different MPDS mix solutions (1 or 2) in samples of different types. For example, if two conditions are compared (A and B), use MPDS mix 1 for samples from condition A and MPDS mix 2 for samples from condition B. This will allow to further control that the ratio of proteins from MPDS mixes 1 and 2 is the one expected.

2. 9 μL of the samples is injected in the 2D LC-MS system of choice (UPLC nanoACQUITY 2D (Waters, Milford, MA) controlled by MassLynx in our case) coupled to a Q Exactive Plus, controlled by XCalibur.
3. The samples are then injected in a two-dimensional RP/RP system, with a first dimension in a high pH (pH 10) and a second dimension in a low pH (pH 3). The peptides are loaded on the high-pH column [XBridge BEH C18 5 μm (300 μm × 50 mm)] at 2 μL/min (20 mM ammonium formate solution adjusted to pH 10), and three elution steps (15 min each) with the following percentages of ACN are realized: 13.3% (fraction 1), 19% (fraction 2), and 65% (fraction 3).
4. The eluate from the "high-pH" column is then diluted ten times with acidified water before being loaded on the trap column [Symmetry C18 5 μm (180 μm × 20 mm)] and separated on the "low-pH" analytical column [BEH C18 1.7 μm (75 μm × 250 mm)]. The gradient on the low-pH column is 140 min long with the following settings: flow rate of 250 nL/min, solvent A (0.1% formic acid in water), and solvent B (0.1% formic acid in acetonitrile) with a linear gradient as follows: 0 min, 99% A; 5 min, 93% A; and 140 min, 65% A. Cleaning and re-equilibration steps then take place during the following 40 min (total run of 180 min).
5. The LC eluent is then directly electrosprayed from the analytical column at 2.1 kV through the liquid junction of the nanospray source. The chromatography system was coupled to a Thermo Scientific Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA), programmed for data-dependent acquisition mode, with the following settings:
   - Top ten (data-dependent acquisition)
   - Parameters for MS: mass range, *m/z* 400 to 1750; resolution, 70,000; AGC target, 1×10^6; maximum injection time, 200 ms
   - Parameters for MS/MS: isolation window, *m/z* 2.0; stepped normalized collision energy (NCE), 21.2, 25, 28.8; resolution, 17,500; AGC target, 1×10^5; maximum injection time, 200 ms; underfill ratio, 1.0%; dynamic exclusion, 10 s

3.3 Data Processing for Protein Identification

The data processing we propose is adapted for the identification of compound from a single section (see **Note 9**).

3.3.1 Label-Free Quantification (LFQ)

1. Use the last version of MaxQuant software. Load the samples from the two heterogeneous regions to compare. Three technical replicates per region of interest should be processed. For identification, use Andromeda search engine with the last release of Uniprot human database for interrogation. Use the following

parameters, N-ter acetylation and oxidation of methionines, as variable modifications and carbamidomethylation of the cysteines (see **Note 10**) as fixed modification. Set the maximum number of miscleavages at two and the minimal length for identification at seven amino acids and at least two peptides required for identification, including one unique peptide. Check "LFQ" for data normalization. Set the maximum ratio count for LFQ at 2. Set the main search tolerance at 4.5 ppm. Set peptide spectrum match (PSM) and false discovery rate (FDR) at 0.01.

2. For detailed explanations on the different parameters for MaxQuant and an informed choice of these for data processing setup, please refer to [8].

3.3.2 Correlation of Peptide Masses with Identifications

1. After MaxQuant analysis, a "combined" folder is created and located in the folder of the first raw file loaded in the software.
2. In the subfolder "txt," open the file "peptides" in Excel.
3. In the column "Mass," search for the mass of interest without the decimals (e.g., 910 for 910.5) (see **Note 11**).
4. Report the corresponding values from the column "Intensity."
5. Calculate the mean intensities and the standard deviation between the triplicates.
6. Compare the intensity trends between the heterogeneous regions with the one obtained by MALDI imaging, as shown in Fig. 2f (see **Note 12**).

4 Notes

1. Incubation chambers can have different forms such as a tip box or a lunch box. The chambers should be hermetically sealed. We recommend using tissue papers moistened with a fixed volume of water to create the humidity.
2. The optimal method parameters greatly depend on the instrument used. FT-ICR instruments require a specific training to select the appropriate ionization settings.
3. The segmentation process is described to study intratumor heterogeneity from a single tissue section. In the future, studies of intratumor heterogeneity would require finding common clusters between tissues and correlate those with prognostic values of patients, as described in [5].
4. As mentioned in **Note 3**, LMD-based microproteomic method can be used in the context of identification from single sections as presented here, but also to compare regions originating from large patient cohorts. In this case, a biomarker discovery assay should be performed, as described in [2, 9].

5. Usually, it is easy to recognize the limits of regions of interest in tissues such as breast tumors, based on the morphology. In the future, more histologically homogenous tissue would require more sophisticated tools to correlate the localization from MALDI imaging data to bright field observation through the laser microdissector.
6. The LMD-based microproteomic method may lead to some technical variability, as for any proteomic method and especially when less abundant peptides are quantified. For the identification of heterogeneous peptides from single sections, technical triplicates should be used.
7. In the context of a biomarker discovery assay, this step is recommended. In the present context, it can be skipped in order to mimic the chemical preparation for MALDI imaging. Indeed, no reduction/alkylation/reduction step is performed for MALDI imaging.
8. For a biomarker discovery assay, it is recommended to run pre-fractionated samples or to use 2D LC-MS/MS to increase the number of protein identifications. For identification from single sections, 1D LC-MS/MS is sufficient for correlation.
9. Data processing for a biomarker discovery assay is described elsewhere [2, 9].
10. This modification should not be included when no reduction/alkylation/reduction is performed. When this step is performed, the user has to make sure that the mass of the identified peptide for the correlation with MALDI imaging dataset does not bear this modification.
11. A slight mass shift is expected between values from MALDI imaging values and LC-MS/MS.
12. Many identification candidates are expected for a given peptide, but in most cases, only one will show the same intensity trends between tissues.

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Conflict of Interest

The authors have declared no conflict of interest.

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