

Chapter 19

On Tissue Protein Identification Improvement by N-Terminal Peptide Derivatization

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Abstract

Identification of potential markers of a physiological stage (e.g., pathology) discovered using MALDI-MSI is an important step in the understanding of signaling pathways or for providing sets of diagnosis and prognosis markers for clinical applications. Classically, identification can be achieved by extraction from a piece of tissue and proteomics strategies. However, this induces loss of information especially for low-abundance proteins or proteins localized to a specific region of the tissue. In this respect, identification directly at the tissue level is an attractive alternative. Because the molecular charge states in MALDI are low, on tissue identification is possible using bottom-up MALDI-MSI strategies. Enzymatic digestion using an enzyme such as trypsin can be performed at the micro-scale level to generate peptide collections while avoiding these peptides to be delocalized. It is, therefore, possible to image proteins through the molecular images of their digested peptides. These peptides can also be used to retrieve information on protein sequences by performing MS/MS, although databank interrogation or de novo sequencing using MS/MS spectra does not always lead to a successful or confident identification because on tissue complexities render PMF data problematic. Identification can be improved by increasing MS/MS spectra quality and simplifying their interpretation. This can be achieved by derivatization of peptides. In fact, derivatization of peptides leads to increases in fragmentation yields and orients fragmentations toward a specific series of fragment ions. In this respect, N-terminal chemical derivatization has proven to be particularly efficient. N-terminal chemical derivatization of tryptic peptides has been developed to be performed at the tissue level after on tissue digestion. Specific focus is given to 4-sulfophenyl isothiocyanate (4-SPITC), 3-sulfobenzoic acid NHS ester (3-SBASE), and (*N*-succinimidylloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP) derivatizations. This provides a complete strategy for protein identification in a bottom-up MALDI-MSI approach and opens the way for novel biomarker identification.

Key words: Mass spectrometry imaging, de novo sequencing, derivatization, protein identification.

1. Introduction

MALDI-MSI has shown potential in the field of proteomics to study the distribution of proteins within tissue sections. Specifically, MALDI-MSI can be used to follow protein markers along physiological stages in order to provide information on signaling pathways. In some cases, MALDI-MSI is used to study the distribution of known proteins at different physiological stages. But often, MALDI-MSI leads to the discovery of molecular weight protein markers. In such cases, protein identification remains to be performed. Protein identification can be performed by classical proteomic strategies after extraction from the remaining tissue piece. However, the most straightforward strategy is to perform protein identification directly from the tissue section itself. As for classical proteomics, two approaches may be considered, namely “top-down” and “bottom-up” approaches. Top-down at the level of the tissue section is clearly the most direct way to protein identification since identification is performed directly by fragmentation into the gas phase of the intact molecule ions. Unfortunately, MALDI generates low charge stages, which make it not well-suited for most of the analyzers or ion activation methods. Therefore, MALDI-MSI “bottom-up” strategies were needed. Such strategies are less direct and require the optimization of enzymatic digestion at the tissue level (**Fig. 19.1**). On tissue digestions can be performed while maintaining localization of digested proteins using micro-spotting of the enzyme. A collection of digestion peptides are generated and protein localization can therefore be obtained indirectly by imaging digestion peptides. $[M+H]^+$ peptide ions are then available for structural elucidation via MS/MS using classical activation methods such as collision-induced dissociation (CID) or infrared multiphoton dissociation (IRMP) or metastable decay. On tissue MS/MS performed after on tissue digestion is accessible although it must be noted that fragmentation yields appear lower when working on tissue sections. This could be explained by a lower internal energy of the parent ion by a higher relaxation of energy during the desorption process itself. This is especially true for MALDI-TOF systems that often lead to weak fragmentation especially in the higher m/z range of the MS/MS spectrum and also leads to different series of fragments creating incomplete sets of series only giving access to small sequence tags. Peptide sequencing can be difficult to establish owing to the multitude of fragment ions generated during MS/MS experiments, like immonium ions (**1**), internal fragments, N-terminal ions (**2**, **3**), or C-terminal ions (**4**).

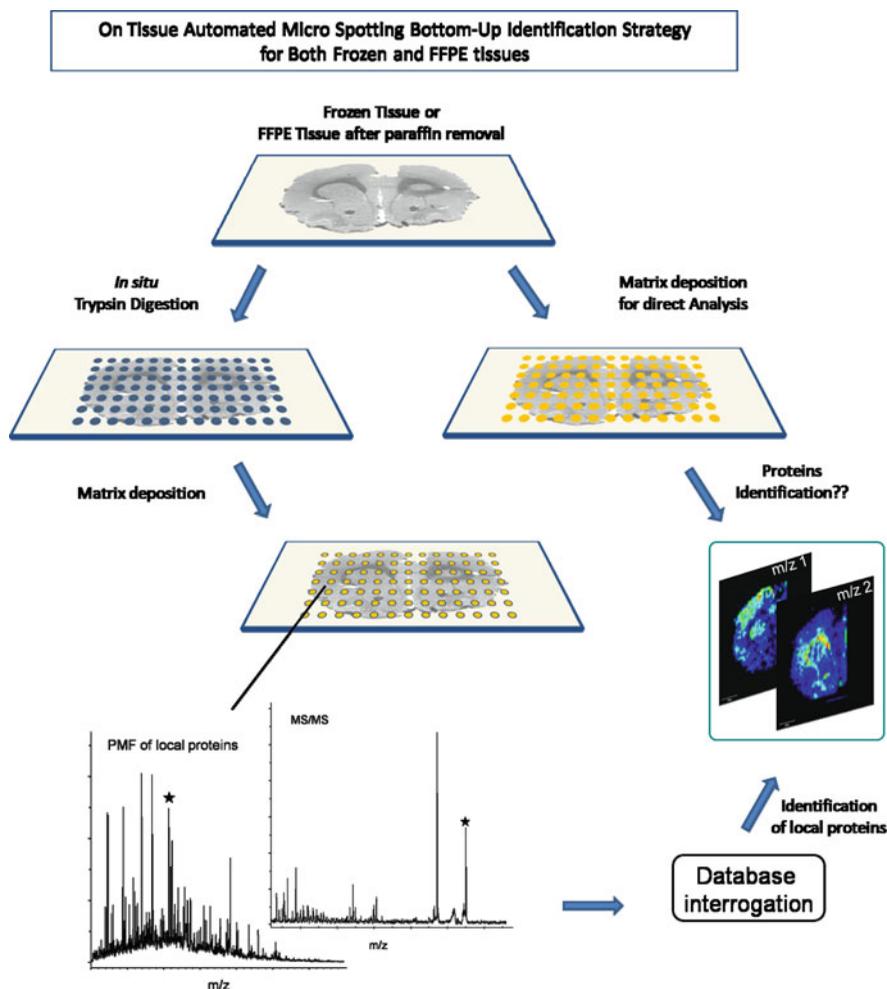


Fig. 19.1. Schematic workflow of MALDI-MSI “bottom-up” strategy used to perform protein identification.

Under classical identification strategies efforts were given to simplify data interpretation by increasing fragmentation ion yield and orienting fragmentations toward a specific series of fragment ions. In this respect, derivatization at the C- or N-terminal part of peptides by addition of positive or negative charges has proved to be an efficient strategy (5), especially for post-source decay (PSD) spectra. In general N-terminal modifications are easier to achieve because of the primary amine reactivity and have proved to be more efficient for fragmentations. Sulfonation at the N-terminal part of peptides seems especially advantageous when working on tryptic digestion peptides that are naturally positive at their C-termini. Keough and coworkers have shown a possible orientation of fragmentations toward the y_i^+ series of fragment ions by addition of chlorosulfonylactetyl chloride (6, 7)

or 2-sulfobenzoic acid cyclic anhydride (8) at the N-terminal side of peptides. Fragmentation orientation greatly eases MS/MS spectra interpretation allowing for de novo sequencing to be performed (9). This method was later on improved by using 3-sulfopropionic acid NHS ester as derivatization agents (10) to allow the reaction to be performed in aqueous phase. Alternatively, derivatization for liquid chromatography (11) and quantification with isotope coded (12) were proposed using derivative agents such as 4-sulfophenyl isothiocyanate (13–15). Such derivatizations are fast and easy, showing good reaction yields, although they often lead (according to the peptide sequence) to the loss of the derivative group as one of the fragmentation pathway lowering the benefits of derivatization. More recently, another water-compatible reagent, the 3-sulfobenzoic acid NHS ester, was introduced (16). This sulfonation agent has the advantage not to lead to the loss of the derivative group consecutively resulting in the observation of a complete and unique y ion series. Alternatively, addition of a positively charged group at the N-terminal part of peptides using (*N*-succinimidylloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP) was successfully used for fragmentation orientation (17). This derivatization has the advantage of being independent of the presence and/or position of basic amino acids (mainly arginine) in the peptide chain and leads to an orientation toward a_i^+ fragment ion series with the presence of a few b_i^+ , c_i^+ , and d_i^+ ions. TMPP is useful for peptide identification in bottom-up strategies.

Because MS/MS data interpretation from tissue sections is particularly complex, development of peptide derivatization is a good alternative to help protein identification. In fact, on tissue digestion leads to numerous digestion peptides that are not all belonging to the same protein and not separated. In this context, peptide mass fingerprint data interrogation is useless and only MS/MS data interrogation is used. This can render the identification difficult if MS/MS spectra do not contain enough information. Thereof, providing higher quality MS/MS spectra is important for easier and more confident identification of proteins from the tissue sections. Because derivatization of peptides increases fragmentation yields and above all channel fragmentation to specific pathways, protein identification is improved with MS/MS from on tissue experiments.

We describe here on tissue N-terminal chemical derivatization of peptides performed after on tissue enzymatic digestion. Since N-terminal derivatization has proven to be more efficient than the corresponding C-terminal reactions in solution, focus has been given to N-terminal chemical derivatizations. More specifically, among the tested derivatization approaches,

three derivatization protocols appear more compatible and efficient when performed at the tissue level, namely 4-sulfo-phenyl isothiocyanate (4-SPITC), 3-sulfo-benzoic acid NHS ester (3-SBASE), and (*N*-Succinimidyl-oxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP) (18). These derivatives promote efficient charge-site-initiated cleavage of backbone amide bonds enabling selective detection of only a single series of fragment ions that contain either the original C-terminus of the peptide (y_i^+ ions) for 4-SPITC and 3-SBASE or the N-terminus of the peptide (TMPP). All three derivatization reagents have been shown to ease identification by databank interrogation (increase in the identification scores) and with de novo sequencing. Often peptide sequences can also be manually obtained if desired. We also notice that TMPP derivatization is a bit easier to perform at a micro-scale level using a robot than 4-SPITC and 3-SBASE mainly because the room temperature reactions for TMPP enable molecular images to be performed after the derivatization step. However, molecular images after on tissue derivatization are rarely required, which makes all three derivatization approaches available for identification. We describe here procedures for on tissue digestion using trypsin as well as N-terminal chemical derivatization using 4-SPITC, 3-SBASE, and TMPP. For 4-SPITC and 3-SBASE global on tissue derivatization protocols are given whereas micro-spotted derivatization protocol is provided for TMPP.

2. Materials

2.1. Preparation of Frozen Tissue Section

1. Optimal cutting temperature polymer, OCT.
2. Indium tin oxide (ITO)-coated glass slides or other holders compatible with mass spectrometry.
3. A cryomicrotome, Leica CM150S (Leica Microsystems, Nanterre, France).
4. Ethanol 75% (-20°C): 75 ml of absolute ethanol ($\geq 99.8\%$) and HPLC-grade water to 100 ml. Prepare fresh and store at -20°C .
5. Ethanol 95% (-20°C): 95 ml of absolute ethanol ($\geq 99.8\%$) and HPLC-grade water to 100 ml. Prepare fresh and store at -20°C .
6. Chloroform (-20°C): 100 ml of chloroform ($\geq 99.9\%$). Store at -20°C . Chloroform is harmful by inhalation, so work in the hood.

2.2. In Situ Enzymatic Digestion

2.2.1. Using a Micropipette

1. Trypsin, sequencing grade modified (Promega, Charbonnieres, France). Suspend in 20 mM NH_4HCO_3 buffer at 40 $\mu\text{g}/\text{ml}$ (*see Note 1*).
2. Methanol 50%: 50 ml of absolute methanol completed with water up to 100 ml. Prepare fresh. Methanol is toxic, so work in the hood.

2.2.2. Using a Microspotter

1. Trypsin, sequencing grade modified (Promega, Charbonnieres, France). Suspend in 20 mM NH_4HCO_3 buffer at 40 $\mu\text{g}/\text{ml}$ (*see Note 1*).
2. Methanol 50%: 50 ml of absolute methanol completed with water up 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. In Situ N-Terminal Derivatization

2.3.1. Synthesis of 3-Sulfobenzoic Acid Succinimidyl Ester (3-SBASE)

1. DMSO: 10 ml of dimethylsulfoxide.
2. 3-SBA: 1 g of 3-sulfobenzoic acid, purity: 98%. 3-SBA is irritating to the respiratory system, so work in the hood.
3. DCC: 1.38 g of *N,N'*-dicyclohexylcarbodiimide, purity 99%. DCC is toxic, so work in the hood.
4. NHS: 770 mg of *N*-hydroxysuccinimide, purity 98%.
5. Cold acetone: 60 ml of acetone ($\geq 99.9\%$), store at 4°C. Vapors may cause drowsiness and dizziness, so work in the hood.

2.3.2. On Tissue N-Terminal Derivatization with 4-SPITC

1. 20 mg/ml 4-SPITC in pure water.
2. 50% methanol: 50 ml of absolute methanol completed with water up to 100 ml. Prepare fresh. Methanol is toxic, so work in the hood.

2.3.3. On Tissue N-Terminal Derivatization with 3-SBASE

1. 20 mg/ml 3-SBASE in pure water.
2. 50% methanol: 50 ml of absolute methanol completed with water up to 100 ml. Prepare fresh. Methanol is toxic, so work in the hood.

2.3.4. On Tissue N-Terminal Derivatization with TMPP

1. TMPP: 1 mg/ml (*N*-succinimidylloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide, puriss. p.a., for protein sequence analysis (by MALDI-MS), $\geq 98.5\%$ in acetonitrile/ H_2O (3:7, v/v).
2. Solution of acetonitrile/ H_2O /TEA (triethylamine) (30:69:1, v/v/v). Prepare fresh (*see Note 2*).
3. Chemical Inkjet Printer CHIP-1000 (Shimadzu Biotech, Kyoto, Japan).

2.4. Matrix Deposition for Peptide Analysis

2.4.1. Using Micropipette

HCCA/ANI solution: 1.5 equivalents of aniline (ANI) were added to a solution containing 20 mg/ml of α -cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile/aqueous 0.1% TFA (6:4, v/v) (*see* **Note 3**). Aniline and TFA are toxic, so work in the hood.

2.4.2. Using a Microspotter

1. HCCA/ANI solution: 1.5 equivalents of aniline (ANI) were added to a solution containing 20 mg/ml of α -cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile/aqueous 0.1% TFA (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).

2.5. Mass Spectrometry Analysis

2.5.1. MALDI-MSI Experiment

1. Peptide calibration standard II (Bruker Daltonics, Wissembourg, 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. 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. 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 situ enzymatic digestion creates a library of peptides from the proteins present at that spot. These peptides can be fragmented using MS/MS; using this peptide fragmentation data and the appropriate database, the peptide can be identified. Using this information, the corresponding protein from which it came can be identified at the tissue level. This strategy can be performed at the scale of an entire tissue section, manually using micropipette deposition providing a fast and sensitive detection of peptides generated from protein digestion. However, this strategy does not provide information on the spatial distribution of the proteins in the tissue. Derivatization can also be performed at the micro-scale level and can be used to prevent migration/delocalization of compounds within the tissue, via an automatic device system such as automatic micro-spotting. Here, a protocol is described

for the automatic piezoelectric micro-spotting using the CHIP-1000 system. The micro-spotting device allows the deposition of the different reagents including trypsin and matrix solutions at the surface of the whole tissue sections or at a predefined location. Using this strategy of defined deposition, protein and peptides are not delocalized, thus providing the opportunity for imaging peptides after the derivatization step.

Figure 19.1 presents the global workflow for on tissue protein identification in a bottom-up strategy including chemical derivatization at a micro-scale level. The peptides that are characterized can be submitted to databank interrogation after MS/MS experiments to retrieve the protein identification. However, databank identification is not always successful nor does it provide highly confident assignments. To improve protein identification, N-terminal derivatization is used to facilitate peptide fragmentations toward a specific series of fragment ions. It is thereof possible to increase the information content of the resulting MS/MS spectra. 4-SPITC and 3-SBASE derivatizations use sulfonic acid reagents and provide complete y_i^+ series of ions for a peptide, ideally without missing cleavages. We notice that for 4-SPITC, loss of the 4-SPITC group is often observed as one fragmentation pathway in the MS/MS spectra, and this can slightly reduce the resulting MS/MS spectral quality. In addition, spots (i.e., missing cleavages) in the y_i^+ series will be observed if a basic amino acid is present in the peptide sequence. For these reasons, care must be taken with on tissue enzymatic digestion. Because of the higher optimum reaction temperatures (35–60°C), 4-SPTIC and 3-SBASE are a bit more difficult to use than TMPP for micro-spotting.

Figure 19.2 shows an example of 3-SBASE derivatization obtained from a rat brain tissue section. After interrogation of the database with a search algorithm like MASCOT, the score achieved is at least twofold better than the score obtained for the unmodified peptides as shown in **Table 19.1**. **Figure 19.3** presents TMPP derivatizations used in this micro-spotting strategy. Using MS/MS, the derivatized peptides provide an abundant series of a^+ ions. As shown in **Fig. 19.3**, this reagent is easily compatible with MALDI imaging.

3.1. Preparation of Frozen Tissue Section

1. 10- μm thick tissue sections are obtained from snap-frozen tissue using a cryomicrotome at -20°C . The tissue is placed in the cryomicrotome ~ 15 min prior to sectioning. If the tissue temperature is too low, tissue sections can pleat and crack. If the temperature is too high, tissue sections will roll up.
2. The tissue sections are then applied onto ITO-coated conductive glass slides and placed in a desiccator under vacuum for a minimum of 30 min to dry out the tissue sections.

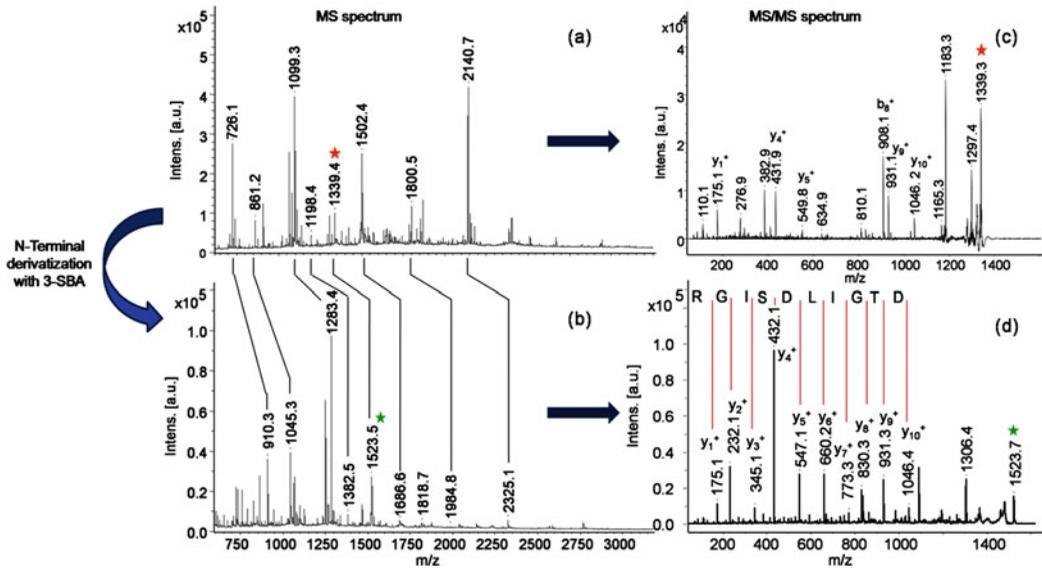


Fig. 19.2. (a–b) MALDI-MS spectra recorded on a rat brain tissue section after on tissue trypsin digestion (a) and on a tissue with trypsin digestion followed by derivatization with 3-SBA (b). (c–d) MALDI-MS/MS spectra of one of the peptides generated from digest (m/z 1,339.3) (c) and the same peptide after derivatization with 3-SBA (m/z 1,523.7) (d).

3. The tissue is fixed. The slide is immersed softly in an ice-cold 75% ethanol bath for 30 s and dried in the vacuum desiccator. After complete drying, the sample is dipped into cold 95% ethanol for 30 s and then dried completely with the vacuum desiccator. This important step removes salts contained in the tissue and prevents sample degradation by dehydration.
4. The lipids are removed. The sample is washed by immersion of the tissue section in a chloroform bath for 30 s to remove abundant phospholipids; these lipids lead to highly abundant signals in the 500–1,500 mass range hampering the observation of peptide signals, generating ionization suppression effects and poor matrix crystallization efficiency (*see Note 4*).

3.2. In Situ Enzymatic Digestion

3.2.1. Using a Micropipette

1. Use a tissue section after washing step (step 4 of 3.1). Whole tissue section is covered by the solution of trypsin using a micropipette. $10 \mu\text{l}/\text{cm}^2$ is used (according to the tissue section size).
2. Tissue sections are then incubated for 2 h at 37°C in an atmosphere saturated with $\text{MeOH}/\text{H}_2\text{O}$ (1:1, v/v).
3. The slide is placed in the vacuum desiccator to completely dry out the tissue prior to matrix deposition.

3.2.2. Using a Microspotter

1. Microspotter like the chemical Inkjet Printer, CHIP-1000, can be used. CHIP-1000 is a piezoelectric solvent delivery

Table 19.1

Comparison of identification scores as found from MASCOT interrogation using Swiss-Prot database and *R. norvegicus* taxonomy for four peptides before and after automated derivatization with 3-SBASE

	<i>m/z</i> observed	<i>m/z</i> expected	Ion score	Rank	Delta	Expect	Sequence
Peptide	643.35	642.32	NI	NI	NI	NI	RPSQR myelin basic protein
Peptide + derivati- zation (3- SBASE)	827.9		46	1	-0.0314	0.0064	
Peptide	726.23	725.22	28	2	-0.1765	0.044	HGFLPR myelin basic protein
Peptide + derivati- zation (3- SBASE)	910.1		30	1	-0.3046	0.029	
Peptide	1,339.24	1,339.71	42	1	-0.4706	0.0015	HRDTGILDS IGR myelin basic protein
Peptide + derivati- zation (3- SBASE)	1,523.7		93	1	-0.277	9.6×10^{-19}	
Peptide	1,701.92	1,701.91	57		0.0162	6.2×10^{-5}	AVFVDLEPTV IDEVR tubu- lin alpha-1A chain
Peptide + derivati- zation (3- SBASE)			142		-0.0058	1.1×10^{-12}	

system, able to deliver picoliter volumes of reagents at defined locations of a surface. The instrument is equipped with an onboard scanning device for imaging the area of interest and thus precisely define and select the print location. An array of micro-spots covering a specific area or the entire tissue section can be defined. Spots of 150 μm in diameter are generated. Micro-spots are spaced by 250 μm .

2. The slide is then loaded in the microspotter and scanned to precisely define the array to be printed with trypsin enzyme. Selection of the whole tissue section, one location, or several locations is possible.

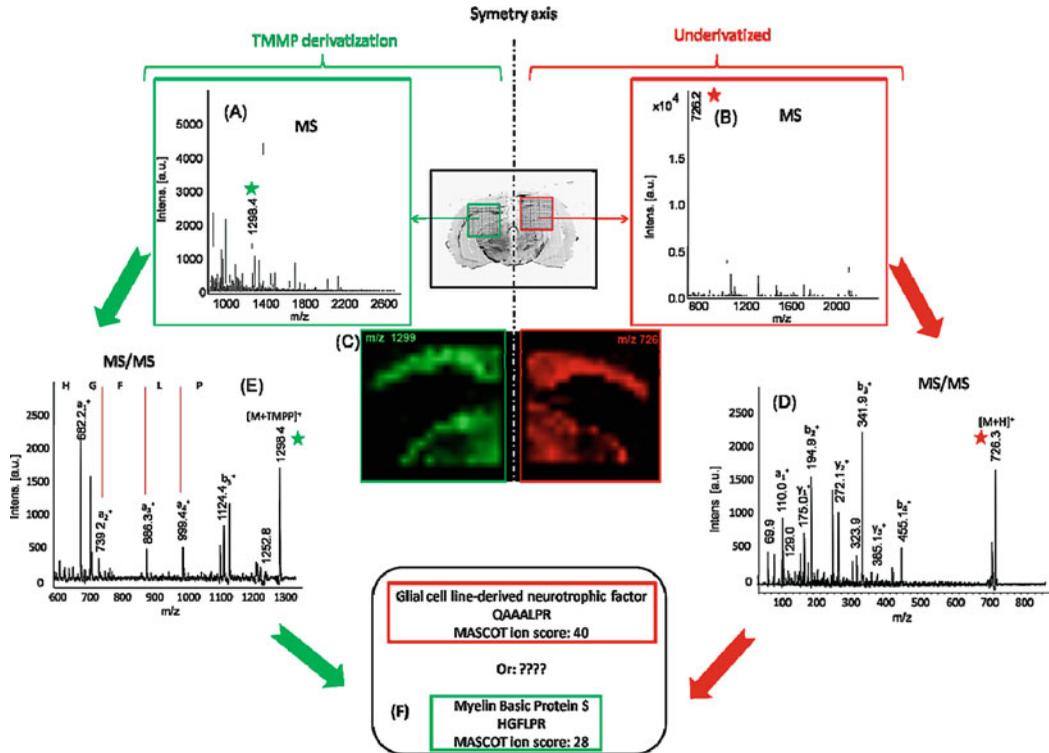


Fig. 19.3. (a–b) MALDI-MS spectra recorded after (a) enzymatic digestion followed by TMPP derivatization and (b) enzymatic digestion. (c) Images of the digestion peptide at m/z 726 and its corresponding TMPP derivative at m/z 1,299 showing an identical distribution for both ions. (d–e) MALDI-MS/MS spectra recorded from a rat brain tissue section in the same area after (d) on tissue trypsin digestion (e) and on tissue trypsin digestion followed by TMPP derivatization. (f) Identification results from the databank using *Rattus norvegicus* taxonomy.

3. A total of 20 nl of trypsin solution is applied on each spot. This is obtained by dropping off five droplets of approximately 100 μ l of solution at each spot per cycle. 40 iterations are thus necessary to obtain the final volume.
4. Tissue sections are then incubated at 37°C for 2 h in a MeOH/H₂O (1:1, v/v) saturated atmosphere.
5. After incubation, the slide is placed in the vacuum desiccator for complete drying of the tissue section prior to matrix deposition.

3.3. In Situ N-Terminal Derivatization

3.3.1. On Tissue N-Terminal Derivatization with 4-SPITC

1. Whole tissue trypsin digestions are performed by applying 10 μ l/cm² (according to the tissue section size) of trypsin solution using a micropipette at the surface of the section.
2. Tissue sections are then incubated for 2 h at 37°C in MeOH/H₂O (1:1, v/v) saturated atmosphere.
3. The slide is placed in the vacuum desiccator to let the tissue dry completely prior to 4-SPITC deposition.

4. Manual derivatization is performed by applying the solution of 4-SPITC in pure water. In fact, no buffer is required since NH_4HCO_3 is still present on the tissue after trypsin deposition (*see* step 1). Volume of solution is adjusted according to the size of the tissue section or the size of the area to be studied within a tissue section ($\sim 10 \mu\text{l}/\text{cm}^2$).
5. Tissue sections are then incubated for 1 h at 55°C in $\text{MeOH}/\text{H}_2\text{O}$ (1:1, v/v) saturated atmosphere.
6. *Optional step:* The tissue can be washed with cold 95% EtOH for 20 s to remove salts from buffer solutions to avoid signal loss due to poor ionization of derivate peptides. This procedure must be performed carefully to avoid peptide losses. Thus, washing steps in cold 95% EtOH must not exceed 20 s.
7. Place the slide in the vacuum desiccator for complete drying prior to matrix deposition.

3.3.2. Synthesis of 3-Sulfobenzoic Acid Succinimidyl Ester (3-SBASE)

1. 1.0 g of 3-SBA is completely dissolved in 10 ml of DMSO.
2. 1.5 molar excess of NHS is then added to the solution up to complete dissolution.
3. 1.5 molar excess of DCC is then added to the solution up to complete dissolution.
4. The mixture is stirred overnight at room temperature.
5. The precipitated dicyclohexylurea (DCU), a side product of the reaction, is filtered and discarded.
6. After filtration, 60 ml of cold acetone (4°C) is slowly added to precipitate 3-SBASE.
7. The product is then washed four times with cold acetone and dried under vacuum.

3.3.3. On Tissue N-Terminal Derivatization with 3-SBASE

1. Whole tissue trypsin digestions are performed by applying $10 \mu\text{l}/\text{cm}^2$ (according to the tissue section size) of trypsin solution using a micropipette at the surface of the section.
2. Tissue sections are then incubated for 2 h at 37°C in $\text{MeOH}/\text{H}_2\text{O}$ (1:1, v/v) saturated atmosphere.
3. The slide is placed in the vacuum desiccator to let the tissue dry completely prior to 3-SBASE deposition.
4. Manual derivatization is performed by applying the solution of 3-SBASE in pure water. In fact, no buffer is required since NH_4HCO_3 is still present on the tissue after trypsin deposition (*see* step 1). Volume of solution is adjusted according to the size of the tissue section or the size of the area to be studied within a tissue section ($\sim 10 \mu\text{l}/\text{cm}^2$).
5. Tissue sections are then incubated for 1 h at 37°C in $\text{MeOH}/\text{H}_2\text{O}$ (1:1, v/v) saturated atmosphere.

6. *Optional step:* The tissue can be washed with cold 95% EtOH for 20 s to remove salts from buffer solutions to avoid signal loss due to poor ionization of derivatized peptides. This procedure must be performed carefully to avoid peptide losses. Thus, washing steps in cold 95% EtOH must not exceed 20 s.
7. Place the slide in the vacuum desiccator for complete drying prior to matrix deposition.

3.3.4. On Tissue N-Terminal Derivatization with TMPP

1. TMPP is deposited by micro-spotting using the piezoelectric device (CHIP-1000). The derivatization must follow the same array as the trypsin one. For trypsin digestion follow the procedure. The automated micro-spotting is performed either following the global scheme of the array or by dividing the total array into smaller areas of 10×10 spots which will each be printed one after the other to increase the yield of the derivatization reaction.
2. 10 nl of a solution of TMPP is applied at each spot. Five droplets of approximately 100 pl are deposited at each spot per cycle. 20 iterations are thus necessary to obtain the final volume on each spot.
3. 20 nl of a solution of ACN/H₂O/TEA is then spotted allowing reaction of derivatization and preventing the use of buffers like NH₄HCO₃ which could induce poor ionization of derivatized peptides (*see Note 2*). Five droplets of approximately 100 pl are deposited at each spot per cycle. 40 iterations are thus necessary to obtain the final volume on each spot.
4. The slide is then placed in the vacuum desiccator for complete drying of the tissue section prior to matrix deposition.

3.4. Matrix Deposition for Peptide Analyses

3.4.1. Using a Micropipette

1. Whole tissue matrix deposition is performed by applying a solution of HCCA/ANI using a micropipette (~10–20 μ l according to the surface to cover). Careful attention must be drawn on this step to avoid touching the tissue with the micropipette tip and not induce tissue damages.
2. Check matrix coverage using an optical microscope (*see Note 5*).
3. Perform a rapid MS analysis of one spot to check that a sufficient amount of matrix is deposited.

3.4.2. Using a Microspotter

1. 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 20 iterations are necessary to obtain the total volume.
2. Check matrix coverage using an optical microscope (*see Note 5*).

3. A rapid MS analysis at one spot is recommended to check through signal intensity that a sufficient amount of matrix has been deposited. Increasing the number of iterations may improve MSI when signal intensity seems too low but too much matrix may decrease S/N ratio by increasing the background noise and decreasing ionization.

3.5. Mass Spectrometry Analysis

3.5.1. MALDI-MSI Experiments

1. 0.5 μl of calibration solution is applied near the tissue section and mixed with 0.5 μl of HCCA/ANI matrix solution.
2. The mass spectrometer is calibrated with the calibration spot.
3. Using FlexImaging and after defining teach points, an area of interest is selected on the tissue section.
4. The distance between each measurement point is set. Distance between measurement points is dependent on the method used for matrix deposition. For piezoelectric deposition, spots are generally spaced by 250 μm center to center. If required, the same array used for matrix deposition can be used for acquisition. Due to the size of the spot it is also possible to accumulate spectra at different positions within one spot.
5. In FlexControl, the method for peptide analysis involves using positive reflectron mode and a total of 500 spectra acquired at each position at a laser frequency of 100 Hz.
6. Images are saved and reconstructed using FlexImaging 2.1.

3.5.2. MS/MS Analysis

1. Ultraflex II TOF–TOF is equipped with LIFT III cell. For each MS/MS spectrum, 5,000 total shots are averaged including 1,000 shots for the parent ion and 4,000 for fragments.
2. Peptides are identified by searching MS/MS spectra against an appropriate database using Biotoools software for MAS-COT (Matrix Science) interrogation. For MALDI data, peptide mass tolerance is set at 0.5 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 total sequence of this protein is used for *in silico* digestion and other digest fragments of this protein are searched in the total spectrum. MS/MS fragmentation is performed to confirm the identification of the proteins based on these fragments.
4. Using FlexImaging, digestion peptide distribution within the tissue is generated to check that all fragments of the same protein really provide 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. For TMPP derivatization, instead of using a solution containing ACN/H₂O/TEA (30/69/1), a solution of ACN/NH₄HCO₃ (pH=10) (3:7, v/v) can be used, but poor ionization of derivatized peptides can be observed due to the presence of salts.
3. Prior to deposition, 2 μ l 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 long and fine white crystals as shown by Lemaire et al. (*see* Table 2a in ref. (19)).
4. Other organic solvents could be used to perform this step. More information can be obtained in ref. (20, 21). You must be careful not to increase the number of washing steps to avoid delocalization of the peptides.
5. To check matrix coverage, you need to ensure that the crystallization is dense, uniform, and composed of small crystals.

References

1. Ambihapathy, K., Yalcin, T., Leung, H. W., Harrison, A. G. (1997) Pathways to immonium ions in the fragmentation of protonated peptides. *J Mass Spectrom*, **32**, 209–215.
2. Kaufmann, R., Kirsch, D., Spengler, B. (1994) Sequencing of Peptides in a time-of-flight mass spectrometer – evaluation of postsource decay following matrix-assisted laser-desorption ionization (MALDI). *Int J Mass Spectrom Ion Processes*, **131**, 355–385.
3. Yalcin, T., Csizmadia, I. G., Peterson, M. R., Harrison, A. G. (1996) The structure and fragmentation of *B-n* ($n \geq 3$) ions in peptide spectra. *J Am Soc Mass Spectrom*, **7**, 233–242.
4. Biemann, K. (1990) Sequencing of peptides by tandem mass spectrometry and high-energy collision-induced dissociation. *Methods Enzymol*, **193**, 455–479.
5. Roth, K. D., Huang, Z. H., Sadagopan, N., Watson, J. T. (1998) Charge derivatization of peptides for analysis by mass spectrometry. *Mass Spectrom Rev*, **17**, 255–274.
6. Keough, T., Youngquist, R. S., Lacey, M. P. (1999) A method for high-sensitivity peptide sequencing using postsource decay matrix-assisted laser desorption ionization mass spectrometry. *Proc Natl Acad Sci U S A*, **96**, 7131–7136.
7. Keough, T., Lacey, M. P., Youngquist, R. S. (2000) Derivatization procedures to facilitate de novo sequencing of lysine-terminated tryptic peptides using postsource decay matrix-assisted laser desorption/ionization

- mass spectrometry. *Rapid Commun Mass Spectrom*, **14**, 2348–2356.
8. Samyn, B., Debyser, G., Sergeant, K., Devreese, B., Van Beeumen, J. (2004) A case study of de novo sequence analysis of N-sulfonated peptides by MALDI TOF/TOF mass spectrometry. *J Am Soc Mass Spectrom*, **15**, 1838–1852.
 9. Keough, T., Youngquist, R. S., Lacey, M. P. (2003) Sulfonic acid derivatives for peptide sequencing by MALDI MS. *Anal Chem*, **75**, 156A–165A.
 10. Keough, T., Lacey, M. P., Strife, R. J. (2001) Atmospheric pressure matrix-assisted laser desorption/ionization ion trap mass spectrometry of sulfonic acid derivatized tryptic peptides. *Rapid Commun Mass Spectrom*, **15**, 2227–2239.
 11. Lee, Y. H., Kim, M. S., Choie, W. S., Min, H. K., Lee, S. W. (2004) Highly informative proteome analysis by combining improved N-terminal sulfonation for de novo peptide sequencing and online capillary reverse-phase liquid chromatography/tandem mass spectrometry. *Proteomics*, **4**, 1684–1694.
 12. Lee, Y. H., Han, H., Chang, S. B., Lee, S. W. (2004) Isotope-coded N-terminal sulfonation of peptides allows quantitative proteomic analysis with increased de novo peptide sequencing capability. *Rapid Commun Mass Spectrom*, **18**, 3019–3027.
 13. Gevaert, K., Demol, H., Martens, L., Hoorelbeke, B., Puype, M., Goethals, M., Van Damme, J., De Boeck, S., Vandekerckhove, J. (2001) Protein identification based on matrix assisted laser desorption/ionization-post source decay-mass spectrometry. *Electrophoresis*, **22**, 1645–1651.
 14. Marekov, L. N., Steinert, P. M. (2003) Charge derivatization by 4-sulfophenyl isothiocyanate enhances peptide sequencing by post-source decay matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Mass Spectrom*, **38**, 373–377.
 15. Wang, D., Kalb, S. R., Cotter, R. J. (2004) Improved procedures for N-terminal sulfonation of peptides for matrix-assisted laser desorption/ionization post-source decay peptide sequencing. *Rapid Commun Mass Spectrom*, **18**, 96–102.
 16. Alley, W. R., Jr., Mechref, Y., Klouckova, I., Novotny, M. V. (2007) Improved collision-induced dissociation analysis of peptides by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry through 3-sulfobenzoic acid succinimidyl ester labeling. *J Proteome Res*, **6**, 124–132.
 17. Huang, Z. H., Wu, J., Roth, K. D., Yang, Y., Gage, D. A., Watson, J. T. (1997) A picomole-scale method for charge derivatization of peptides for sequence analysis by mass spectrometry. *Anal Chem*, **69**, 137–144.
 18. Franck, J., El Ayed, M., Wisztorski, M., Salzet, M., Fournier, I. (2009) On tissue N-terminal peptide derivatizations for enhancing proteins identification in bottom-up Imaging strategies. *Anal Chem*, **81**, 8305–8317.
 19. Lemaire, R., Tabet, J. C., Ducoroy, P., Hendra, J. B., Salzet, M., Fournier, I. (2006) Solid ionic matrixes for direct tissue analysis and MALDI imaging. *Anal Chem*, **78**, 809–819.
 20. Lemaire, R., Wisztorski, M., Desmons, A., Tabet, J. C., Day, R., Salzet, M., Fournier, I. (2006) MALDI-MS direct tissue analysis of proteins: improving signal sensitivity using organic treatments. *Anal Chem*, **78**, 7145–7153.
 21. Seeley, E. H., Oppenheimer, S. R., Mi, D., Chaurand, P., Caprioli, R. M. (2008) Enhancement of protein sensitivity for MALDI imaging mass spectrometry after chemical treatment of tissue sections. *J Am Soc Mass Spectrom*, **19**, 1069–1077.