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Review

MATRIX ASSISTED LASER DESORPTION IONIZATION MASS SPECTROMETRY IMAGING FOR PEPTIDE AND PROTEIN ANALYSES: A CRITICAL REVIEW OF ON-TISSUE DIGESTION

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MATRIX ASSISTED LASER DESORPTION IONIZATION MASS SPECTROMETRY IMAGING FOR PEPTIDE AND PROTEIN ANALYSES: A CRITICAL REVIEW OF ON-TISSUE DIGESTION

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Running title: Enzymatic digestion in MALDI-MSI for biomarker discovery. **Review article**

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Abstract

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has established itself among the plethora of mass spectrometry applications. In the biomedical field, MALDI-MSI is being more frequently recognized as a new method for the discovery of biomarkers, targets of treatment, classification of diseased and healthy tissues or to predict the outcome of a pathology. The technology has been used to study the localization of proteolytic peptides directly on tissue sections. A direct correlation between the detected peptides and the distribution and identity of the original precursor protein is the ultimate goal of any MALDI-MSI experiment. Enzymatic digestion protocols are commonly used to reveal the protein signature of these complex tissues. Considerations that pertain to methods of sample preparation, on-tissue digestion, data analysis and visualization will be addressed. This review will also discuss selected applications of on-tissue digestion combined with the MALDI-MSI technology in biomedicine.

Key words: MALDI-MSI, enzymatic digestion, biomarker discovery, pathology, peptide, protein, imaging, technology

1. Introduction

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has become a powerful method to explore the diversity of the molecular complexity in biological samples [1]. The main advantage of this technique is the possibility to study different areas of a tissue section with a lateral resolution below 50 μm, with or without previous knowledge of the heterogeneity of the sample [2, 3]. The power of MALDI-MSI resides in the potential detection, identification and localization of lipids, proteins, drugs, metabolites and other compounds. Interdisciplinary usage of the technology in the last years covered diverse areas such as biology, chemistry or physics. In the biomedical field, the technology has been applied to a variety of pathologies such as ageing related disorders and many cancer types [4-6]. Mass spectrometry imaging (MSI), including techniques such as SIMS and MALDI-MSI, has been used as a method to detect new disease candidates and its use has increased exponentially in the last years. Figure 1 provides insight in the rapidly growing number of published papers using this methodology.

MALDI-MSI, as a spatially-resolved label-free bioanalytical technique for direct analysis of biological samples, was also recently introduced for analysis of 3D tissue specimens. New experimental and computational pipelines for molecular analysis of tissue specimens integrates 3D MALDI-MSI, magnetic resonance imaging (MRI), and histological staining-microscopy. This multimodal approach can be applied to address clinical questions such as proteomic analysis of heterogeneous diseased tissues [7, 8]. MALDI-MSI in combination with in-vivo MRI has been recently employed for monitoring inflammatory processes [9, 10]. The combination of both approaches provides a unique way to link the highly resolved anatomic information of MRI with the proteomic data provided by MALDI-MSI.

Proteins are the major molecular players in many of the pathways involved in defective cellular signaling cascades. Changes in their spatial distribution can be used to detect pathological processes in very small length scale areas [1]. An ideal MALDI-MSI experiment would combine the localization of a protein and its identification. In classical proteomics, two different approaches are widely used for this protein characterization: top down and bottom up.

- Top down. The identification of proteins is performed through mass measurement of the intact protein followed by tandem mass spectrometry (MS/MS) fragmentation [11]. Top down methodology relies on the fragmentation of the protein without any enzymatic treatment prior to sample analysis [12].
- Bottom up. Direct analysis of proteins in an imaging experiment is usually limited to proteins below 25 kDa, some groups have developed methods that partially overcome this problem [13-16]. On-tissue digestion of proteins and the visualization of the distribution of the resulting peptides is an effective solution to solve this problem. The multiple number of peptides generated from a single protein and the lower mass of peptides compared to proteins, lie at the basis of the efficiency of this approach [1]. The localization of the proteolytic peptides is preserved by specific methods of enzymatic application and the identification of the protein can be made by peptide mass fingerprint (PMF) and by MS/MS. In addition, posttranslational modifications of specific peptides can be studied directly from tissue [17].

At the moment no gold-standard on tissue digestion protocol exists, which merits the review of existing on-tissue digestion methodology.

2. MALDI-MSI for peptide analysis

In the following section, the workflow to perform a successful experiment for the detection of proteolytic peptides by MALDI-MSI is explained and reviewed.

The first important parameter that must be considered is a careful sample preparation. After this step, the tissue section will be introduced in the mass spectrometer and then, the peptides will be desorbed from discrete pixels at the surface in an ordered way. Each pixel is linked to the mass spectrum specific from a spatial coordinate. A plot of the intensities of a selected signal along the tissue will produce a map of the relative abundance of that compound over the imaged tissue [10]. For proteolytic peptide detection, several additional steps like washing the sample or the application of the enzyme that cleaves the proteins in targeted positions, must be considered. An example of the workflow followed for formalin fixed paraffin embedded (FFPE) samples is depicted in figure 2. Once the matrix is applied the sample is measured and subsequent statistical analyses must be performed in order to select candidates for suitable identification. A direct MS/MS fragmentation on the tissue or an extraction of peptides can be performed to identify the original protein. The co-registration of the imaging experiment with a classical staining image (like hematoxylin eosin staining) will be beneficial to discriminate areas with a specific peptide signature.

a. Sample preparation

Frozen tissues are known to best maintain their molecular properties. Tissue biopsies, organs or whole organisms should be frozen immediately after collection in order to preserve the morphology and integrity of the sample, minimize the degradation of the proteins through

endogenous enzymatic proteolysis, oxidation or changes in the metabolome [3]. Sample stabilization and standardization of sample handling can solve this problem [18]. Devices that utilize a combination of heat and pressure stop degradation immediately after sample excision. Once the sample has been stabilized the process of freezing the tissue can be performed in a safe way [18, 19].

It is not always possible to analyze freshly prepared samples. FFPE tissue blocks are a good source of samples since in general large bio-banks of these tissues are stored in hospitals for years. Although these type of samples are commonly used for DNA and protein studies, they require special protocols ensuring removal of embedding media like paraffin (incompatible with MS) and the digestion of cross-linked proteins to make them accessible for a correct identification [3]. For tissues like bone or whole body sections carboxymethylcellulose is often used. For soft tissues or cell cultures, gelatin facilitates the embedding and cutting process since its consistency is similar to these tissues [19-21].

i. Washing procedure to remove contaminants and other interferences

In order to succeed in the creation of a map of peptides specific to a tissue type, washing steps prior to the measurement are absolutely necessary. Lipids, salts and other environmental factors reduce the efficiency of the enzymatic digestion and the detection of the peptides. EtOH at different percentages is widely used [4, 20, 22, 23]. The time of incubation can vary between different tissues according to their composition. The highest total ion count (TIC) signal can be used as control to check the efficiency of the protocol. Isopropanol, glacial acetic acid, toluene or chloroform washings have been successfully used in frozen sections to remove salts and lipids from the tissue [24-26]. For FFPE tissues, removal of paraffin is crucial and samples must be washed

in toluene or xylene. The most used protocols are the classical procedures for antibody staining in immunohistochemistry experiments [27-29]. Antigen retrieval protocols that reduce the cross-linking among proteins induced by formaldehyde or glutaraldehyde include heating or enzymatic digestion and therefore facilitate the protein identification [5, 30]. For all these washing steps different methods to apply the solvent can be chosen. The most commons include pipetting or the use of a beaker. A wet tissue in contact with the samples can be used for very delicate tissue sections. This procedure uses solvent wetted fiber-free paper to enable local washing of tissue sections for mass spectrometry imaging and tissue profiling experiments [31]. The tissue-stamp maintains the organization of the majority of the molecules in fragile frozen sections while the use of a pipette or a beaker is more used for fixed or FFPE samples.

ii. The right enzyme

The application of the enzyme on the tissue or region of interest (ROI) will produce the proteolytic fragments of the proteins that will allow us to locate and identify the corresponding protein after a MALDI-MSI and MS/MS experiments. The optimization of the enzyme concentration, time of incubation or the buffers used in the protocol must be adapted according to the tissue to be analyzed.

- Trypsin: Although a number of alternative proteases have been successfully applied, for most studies the endopeptidase trypsin remains the most commonly used enzyme in the field of proteomics and MALDI-MSI. It efficiently and specifically cleaves C-terminally after the frequently occurring basic amino acids lysine and arginine and generates peptides compatible with MS. Different strategies have been suggested to improve the efficiency of trypsin digestion. Especially for FFPE samples, the addition of detergents

compatible with MS, like octylglucoside, improved the detection and identification of proteins such as histones [32]. These detergents break the cell membranes and expose the proteins to a better trypsin interaction [5, 6, 26]. Surfactants such as RapiGest (Waters) have been shown to improve the efficiency of the digestion without interfering with the MS analysis.

Other agents like Dithiothreitol (DTT) and iodoacetamide allow for the reduction of SH protein groups and facilitate the protein identification. The digestion process must be performed in a wet atmosphere to avoid the dehydratation of the enzyme/sample. The sample can be kept in a bath sealed (100% humidity) with parafilm or in a saturated atmosphere of MeOH/H_2O (1:1, v:v) [4, 33].

Another important parameter is the concentration of the trypsin which must be determined depending on the tissue to be analyzed [34]. In addition, Schober et al. pointed out the importance of the buffer in which the enzyme is dissolved which must be also carefully selected in order to achieve the best enzymatic efficiency [1]. Depending on the tissue to be analyzed, the incubation can be performed between 15 min to several hours as other authors have shown for rat brains or pancreatic tumor tissues [27, 34-37]. A longer incubation time will lead to smaller peptide fragments [37]. The dissolution of the trypsin in water or the incubation at lower temperatures are not the ideal conditions to perform an efficient digestion, however some authors have reported successful results without controlling these parameters [29]. One explanation is that the tremendous complexity of the tissue will determine the efficiency of the digestion reducing the number of proteotypical peptides to a greater extent than any of these other parameters [37].

Complementary studies that take advantage of other enzymes are extremely useful especially when peptide identification by tryptic digestion does not provide sufficient sequence information on the surface proteins. Other enzymes that have been used in MALDI-MSI for that purpose are briefly discussed in the following paragraph:

- Chymotrypsin: Chymotrypsin is a serine protease that hydrolyzes peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met, Leu) on the carboxyl end of the bond. Tucker et al. have showed that new peptides formed by chymotryptic protein digestion can be detected and identified using a combination of MALDI-MSI and offline liquid chromatography tandem mass spectrometric analysis [38]. Even though new peptides are detected with Chemotrypsin most species observed relate to high abundant proteins including actin, myelin or tubulin, also detected with trypsin.

- Elastase: Elastase cleaves C-terminal of alanine, valine, serine, glycine, leucine and isoleucine [20]. It is useful for the studies of tissues that are rich in elastin protein such as the skin.

- Pepsin: Pepsin cleaves at the C-term of phenylalanine, leucine, tyrosine and tryptophan. It has been reported that it is extremely useful for Hemoglobin, Lumican and Serum albumin detection in skin analyses [20].

- PGNase F. Amidase PNGase F is an enzyme used in the analyses of glycoproteins. The application of trypsin after PNGase F leads to the removal of the sugar moiety of the glycopeptides and therefore their ionization are improved leading to a better detection of glycoproteins [39].

iii. Choice of the matrix

The matrix selection mostly depends on several factors such as the sample type or molecular weight of the target to be analyzed [3]. Matrices are low molecular weight organic compounds that co-crystallize along with analyte molecules. It absorbs energy from the laser source and transfers the absorbed energy from the laser to analyte molecules helping in its ionization. The process of crystallization depends on the solvent, time of incubation and on the concentration of the matrix [3, 40]. These parameters will affect the crystal size. Ultimately, the choice of matrix crystal size is determined by a combination of the desorption and ionization technique used, the required spatial resolution and the required sensitivity [3]. If the droplets of the matrix solution are too large, then turbulence in the droplets leads to diffusion which limits the achievable lateral resolution. Just minimizing the droplet size increases the possible spatial resolution, but at the cost of the spectra quality because smaller droplets dry faster and are therefore less efficient in analyte extraction. Smaller crystals typically exhibit lower spectral signal-to-noise.

The quality of MALDI MS spectra of biomolecules is influenced not only by the choice of matrix molecules but also by a variety of other factors such as the solvent properties, matrix additives or temperature that will affect the analyte extraction [40]. The most used matrix for peptide detection is CHCA at 5-20 mg/mL and dissolved in different organic solvents like AcN, MeOH or EtOH with TFA (0.1-0.5%). The use of aniline (ANI) has been also reported as an efficient solid ionic matrix for the identification of digested actin or hemoglobin proteins among others [4, 5, 41, 42].

Despite the fact that CHCA is the most popular matrix for peptide ionization, Schober et. al have demonstrated that DHB is also a good choice to detect peptides in mouse brain (30 mg/mL 50:50 acetone/water/0.5 %TFA).

2,4 Dinitrophenylhydrazyn (2,4-DNPH) or a mixture of DNPH and CHCA have been reported as a matrix for peptide detection in FFPE tissues stored up to 2 years [37]. This combination is ideal for peptide extraction above 5000 kDa. Adducts corresponding to the formalin fixation (protein-$N{=}CH_2$) are suppressed with this matrix.

Complementary matrices target an increased protein sequence coverage. For instance peptide mapping with CHCA can yield more low-mass peptide ions (<2500 Da), while sinapinic acid (SA) may provide better coverage for the higher-mass peptides (>2500 Da) [35]. These experiments require different measurements to be conducted on adjacent tissue samples.

iv. Methods of application

- *Enzyme*

Different methods have been used for an efficient enzymatic digestion while maintaining the peptide distribution.

- Sprayer: Spray coating covers the tissue with a homogenous layer of a protease, allowing for on-tissue digestion. The method can either be carried out manually or by using an automated commercial system. Thin-layer chromatography (TLC) sprayer is a manual method to perform this type of deposition and it is both fast and cost effective however the reproducibility is very low. Automated commercial systems such as the ImagePrep™ (Bruker Daltonics Inc., MA, USA) or the TM-Sprayer™ (Leap Technologies, Inc.,

Carrboro, NC, USA), are more expensive methods but the variability is lower compared to manual methods [43]. The ImagePrep device utilizes vibrational nebulization of the solution with a piezo-electric spray head. The spray head ejects small droplets with an average diameter of 20 μm that are deposited onto the tissue [34]. A controlled atmosphere in the deposition chamber avoids the risk of analyte diffusion. In addition, the process of spraying and drying is monitored by an optical sensor that allows the evaluation of the tissue wetness, drying rate and matrix coverage. Another commercial device, the TM-sprayer uses a heated capillary with a pneumatic spray that moves in predefined patterns over the tissue sample. This makes the spray more reproducible than a manual pneumatic spray. In this case there is no control over parameters such as the drying rate and the wetness of the tissue during the spraying and for that reason the settings must be tuned in advance [44]. A reasonable compromise between the amount of material deposited and analyte migration must be optimized in every experiment.

Other devices like Suncollect (MALDI Sun Collect Spotter, Gmbh Germany) provide very reproducible results with a lateral resolution up to 200-300 μm [4, 5]. For trypsin deposition lower flow rates must be used compared to matrix application [27]. Over wetting must be avoided while maintaining humid environmental conditions. The adjustment of the flow of enzyme deposition must be adapted for each tissue type. Very low amounts of enzyme are consumed by this device. Programs can be repeated several times in order to increase the digestion quality [32, 37].

Bouschen et al. have developed a spraying method that allows the detection of tryptic peptides below 50 μm using a home build sprayer, combining high spatial resolution and high mass accuracy for the detection of proteins up to 40 kDa [45].

- Microspotting: Spotters apply small droplets of matrix/enzyme or other solutions in a predefined array [39]. However, spotting robots typically have a negative effect on the sample to preparation time, which increases with the tissue area and lateral resolution. Spotters provide image resolutions in the 150–500 μm range. If small droplets are deposited, the intensity of the signal drops dramatically because they do not extract the analyte as efficient as large ones [36, 46]. Therefore, spotting the enzyme limits the diffusion to the size of the droplet and can be used if no extremely high spatial resolution is required. Robotics available include the Portrait® (Labcyte Inc., Sunnyvale, CA, USA) and the TM iD™ (Leap Technologies). The CHIP-1000™ (Shimadzu Corp., Kyoto, Japan) is an example of a piezoelectric chemical inkjet printer, that spots about 5-20 nL of trypsin per spot in cycles of 30-40 rounds [28].
- Other methods of protein digestion involve tissue blotting through a PVDF membrane with trypsin immobilized on the surface. Peptides will be captured onto a second PVDF membrane maintaining the spatial distribution [47]. This procedure is referred to as the molecular scanner and the result obtained is the molecular distribution of the digested peptides by the trypsin as a "mirror". This method is based on a semi-dry electroblotting approach and has been suggested as a technology for the detection of proteins above 30 kDa and to reduce the complexity of the obtained spectra.
- Stretched sample method (SSM): The SSM was originally created as a method to simultaneously prepare many small tissue samples for profiling peptide content [38]. Samples are mounted on a substrate of glass spheres embedded in Parafilm M and stretched in each direction and then coated with matrix and inserted in a controlled chamber in order to condense water on the surface of the sample. This step increases the

analyte extraction and reduces at the same time its diffusion. Different enzymes can be attached to the glass beads to provide in situ digestion sites. This in situ enzymatic digestion is called modified bead stretched sample method (MBSSM) [38]. Actin beta, myelin basic protein or tubulin alpha chain were identified in spinal cord rat samples by this method.

- A novel implementation of *in situ* protein digestion supported by a graphene oxide-immobilized enzyme reactor (GO-IMER) in the MALDI-MSI workflow has been reported, which enables the simultaneous diagnostic identity and distribution attributes of the proteome on tissue [48].

- *Matrix*

The standard dried-droplet preparation is known to result in an efficient way of extracting analytes but this is not the ideal method for an imaging experiment because the spatial information is lost. Figure 3 shows the differences in peptide distribution in two sections digested with a sprayer (ImagePrep) or by the dried droplet method. Imageprep was used to spray trypsin in FFPE rat joints and after an overnight incubation, CHCA matrix was deposited by the same method. The peak with m/z 1790.9 was only spatially preserved when the ImagePrep was used.

Deposition methods such as sprayer or microspotting are widely used for matrix deposition, however other methods like sublimation increase the spatial resolution [41].

Matrix sublimation is a process by which solid matrix is sublimed upon application of heat in a flat-bottom condenser at reduced pressure [43]. Sublimation gives a homogenous crystal layer over the tissue with an increased S/N ratio. This method is more efficient than manual deposition in

terms of reproducibility and is one of the most cost-effective techniques. In order to adapt this approach to peptide and protein detection, a recrystallization step is needed when using sublimation [45]. In fact Bouschen et al. have combined matrix sublimation with matrix recrystallization to create a new matrix deposition method termed vapor deposition/recrystallization [45]. The matrix must be first applied to the surface of the tissue by sublimation. For recrystallization, the samples are introduced in a wet chamber over a 24–72 h period. In a paper recently published by Yang and Caprioli, a spatial resolution of 10 μm was achieved using this method for imaging proteins up to 30 kDa in size [49].

b. Instrumentation

The majority of the published papers analyze peptides with mass ranges from 100-4000 Da, therefore time of flight analyzers (ToF) based on ToF-ToF, Q-ToF, ion mobility ToF and ion-trap-ToF or Fourier transform analyzers (Orbitrap and FTICRMS) are the most used instruments [50]. High quality MALDI-MSI can be performed in the majority of the mass spectrometers, sample preparation and data analysis are the limiting factors. In principle TOF analyzers have a mass resolving power R=30000 and mass accuracies better than 5 ppm, however the structure of the sample and differences in height result in a lower mass resolution and mass accuracy for the linear and reflectron ToF system [40].

Instruments like FT-ICR or LTQ orbitraps are strongly recommended to solve these questions [40]. The mass accuracy obtained by FTICR or LTQ-orbitrap instruments typically outperforms other instrumentation [51-53]. Schober et. al have combined the power of identifying peptides with mass tolerances of 3 ppm and the information about their spatial localization [24]. Only by using instrumentation with high resolving power, a peptide from SNAP-91 protein with a theoretical

m/z 727.3174 could be distinguished from the isotopic isomer of the myelin peptide HGFLPR *m/z* 727.4080.

Another interesting analytical tool for the investigation of structural and conformational properties of biomolecules is ion mobility separation (IMS) coupled with mass spectrometry [27]. It allows the separation of ions according to their collision cross sections and/or charge. IMS adds a gas-phase separation dimension (drift time) comparable to the retention time (RT) in LC and gas chromatography (GC) and it is especially interesting for MALDI-MSI where no previous peptide/protein separation is performed [26]. The combination of IMS with MALDI-MSI helps the separation of molecules with a very similar *m/z* values. The use of MALDI-IMS-MSI has already been reported for the investigation of the protein distribution within FFPE breast and pancreatic tumor tissue sections after in situ digestion [5, 32]. The combination of IMS and MALDI-MSI improved the identification of proteins directly from the tissue [27, 32]. For example, separation between collagen I alpha 2 chain and unknown compounds, was only possible because of the use of IMS. Stauber et al. have showed that thanks to IMS technology, peptides from tubulin and ubiquitin were actually separated subsequently allowing observation of a different localization of both proteins and also improved identification scores [26].

c. Data processing, visualization and interpretation

Data processing and interpretation is complex and it is very important for accurate and reliable results. As in classical proteomics workflows, the spectra require preprocessing for baseline correction, noise removal, realignment of the *m/z* channels and peak selection to reduce the experimental variance. In addition the matrix signal produces a high number of *m/z* values that are not typically useful for data analyses. Exclusion lists that contain matrix peaks or autolytic

peptide trypsin values can increase the quality of the measured spectra avoiding the acquisition of those peaks. Systematic variation across the imaged tissue and therefore background subtraction and normalization are essential to obtain reliable images [54]. Thus, normalization processes are often needed in order to compare different samples/region of interest (ROI). Usually a matrix peak or the TIC are used for the intensity normalization, however this must be taken into account for data interpretation [6]. Nevertheless modern mass spectrometers include automated algorithms specifically for these purposes.

Some software packages provided by manufacturers of the instruments perform all the different steps. For instance FlexImaging™ and ClinProTools (Bruker Daltonics GmbH, Bremen, Germany,www.bdal.com) software is used for acquisition and evaluation of MALDI-TOF and TOF/TOF imaging data [3]. This software allows the visualization of the distribution of any ion detected during an imaging experiment. Several ROIs are defined according to the morphology of the tissue and representative spectra of each ROI can be exported to ClinProTools for further analysis. The spectra of different groups or ROIs can be compared and subsequent statistical analyses will reveal the molecules that classify different groups [28].

MassLynx (Waters) also allows baseline subtraction, automatic peak detection and other data processing steps, but complementary software is necessary to visualize the spectrum distribution. Raw data can be converted to imZML or the Biomap format. Biomap (Novartis, Basel, Switzerland, www.maldi-msi.org) is an image processing application originally created for the evaluation of MRI data in biomedical research [3]. It allows for displaying the mass spectrum from selected single points or ROI visualizing its distribution along the tissue.

Other software like Datacube Explorer and its version for 3D data, Volume explorer, offer both an image-based as well as a spectrum based view of the data, with an easy way to dynamically scroll through the masses in a dataset [3].

MSI produces large datasets that contain valuable information that is difficult to analyze in order to extract the relevant peptide information. One of the most used approaches to extract the interesting features from a dataset is Principal component analyses (PCA), a statistical method commonly used to reduce the dimensionality of a multivariate data set. Only the principal components that describe a defined amount of variance, e.g. 80% are retained and used for further analyses. In this manner the uncorrelated noise is discarded. In addition PCA can find differences in a non-supervised way [55]. Djidja et al. used this method to generate a classification model based on sample clusters defined by PCA [56]. In this work, peptide profiles obtained from MALDI–MSI obtained from adenocarcinoma cores were separated into two data sets: a training data set (to generate a model of classification) and a second set used as a test dataset for the validation of the classification model. Supervised methods like discriminant analyses (DA), are often used to define a classification from a observational predefined groups. Our group has used this approach to reveal the peptide signature of diseased and healthy cartilage [6].

Combination of PCA and complementary statistical approaches like the t-test is absolutely necessary since PCA-DA only gives a number of features that are correlated with a specific condition. In this sense, Djijdja et al. have shown a number of peptides given by DA that were actually increased in tumor samples validating the results found by PCA-DA. Wilcoxon/Kruskal Wallis or Mann–Whitney U-test have also been used since they are implemented in many software packages and are useful to compare different ROI from non- Gaussian Populations [28, 54, 57].

- Quantitation

MALDI-MSI has been used to generate qualitative and semi quantitative data, however true quantitation is still requires further development. The high degree of heterogeneity in tissues and cells leads to dramatic changes in the local environment across the sample. This complicates the quantitation of peptide abundance in a specific region [58]. Ion suppression effects, image distortion due to crystallization or inhomogeneous matrix deposition should be corrected through the application of internal standards. However, the use of internal standards to validate differences in analyte abundance is a method only suitable for a specific analyte/sample combination such as studies of drug or synthetic peptide distribution [19, 59, 60]. The simultaneous detection and quantitation of all peptides from a tissue would require isotopically labeled standards for all peptides as well as a significant increase in the peak capacity of the mass spectrum to resolve every component [54]. MALDI-MSI allows to image hundred of molecules in a single analysis which makes challenging the use of internal standards. In addition, the quantitation of all peptides would require calibration curves throughout the tissue. There is an additional challenge: the standard must be homogenously distributed on the surface of the tissue section [61]. For all these reasons, MALDI-MSI of peptides and proteins cannot be considered quantitative. MALDI-MSI experiments are limited to the comparison of the ion intensities between different regions or samples, after a number of data preprocessing and normalization steps [54]. Regions of interest are often defined based on relative intensities, setting a threshold to define their limits. Nevertheless MALDI-MSI methodology can reveal valuable information. In fact, the technology has been employed for the discovery of disease markers in tissue [4, 6]. Parallel techniques must be used to identify and to validate those markers that are linked to a specific pathology [54].

d. Identification and Validation

- Identification

The correlation of the peptide distribution with its identity is the last goal of any MALDI-MSI experiment [39]. Identification of the imaged peptides can be done in the same slide after performing an imaging experiment or in an adjacent section. This allows optimal correlation between the observed *m/z* and its identity. Direct MS/MS on the tissue is the ideal method, however on-tissue protein identification can be laborious and since there is no peptide/protein separation, the identification is often difficult to achieve. When this is possible, a profiling MS/MS or a directed MS/MS experiment can be performed in the area of interest. A profiling MS/MS experiment will retrieve information of the peptides that can be detected in a specific region. This method is useful when high-resolution images are not needed. It has the advantage of rapid acquisition times and smaller data files. The easiest and quickest way of performing these type of experiments is to digest discrete spots by manual application with a pipette [62]. However on-tissue MS/MS does not yield more than 20 peptides by direct measurements as a result of signal depletion [24, 34]. Directed MS/MS in a specific region will fragment masses that are relevant in that area and that have been previously selected by statistical analyses. This can be performed in imaging mode as well, and result in peptide fragment ions with the same distribution as the precursor peptide ion. The limitations of this approach are that not all the precursor peaks can be selected and fragmented because of signal depletion in a single pixel. Moreover the major peaks belong to the most abundant proteins (actin, tubulin, ubiquitin or histones,) and many peaks that correspond to less abundant proteins, are not detected because of the ion suppression and due to the complexity of the samples. In addition, if the sample is too complex the searching database scores will not be good enough to confirm the ID of a protein when directed MS/MS in a specific

region is performed. For all these reasons, many groups have already moved toward a complementary strategy that involves the extraction of digested proteins from the tissue by classical proteomic methodologies [63]. LC-MS identification of tryptic peptides generated form parallel tissue sections analyzed by MALDI-MS allows the creation of peptide databases tissue-specific that help in the identification of the peptides that are detected in an imaging experiment. Accurate mass and time (AMT) tag strategies also enables identification of thousands of peptides in a single analysis by comparing accurate molecular mass and LC elution time information from the analysis to a reference database [64]. Tissue homogenates can be additionally submitted to further separation by SDS PAGE and subsequent enzymatic protein digestion on gel bands.

Identification of proteins has been accomplished in normal or diseased regions of an eye suffering from pseudoexfoliation syndrome, a disease that produces glaucoma [17]. In this work, the parallel identification was improved by peptide extraction in liquid phase from the tissue followed by ZipTip cleaning in combination with direct tissue digestion. The combination of both methods permits the identification of a higher number of proteins plus the validation of some of the peptides found by the orthogonal tissue preparation. However, due to the different sample processing it is common to find different *m/z* values or different peptides in both methods. In addition, ion suppression, efficient extraction and sample complexity are factors that negatively influence the ionization of different peptides. For this reason, the combination of MALDI-MSI and LC-ESI requires more than one peptide to assume correct identifications.

Tissue excision by laser capture microdissection (LCM) and peptide extraction from specific areas offer an ideal combination of localization and protein identification [65]. Figure 4 shows an example of four identified peptides in serous ovarian cancer sample by LC-MS/MS after LCM.

Gustafssson et al. have performed in situ MS/MS identification combined with nLC/MS/MS in FFPE cancer samples. Laser microdissection technique is extremely useful to study the protein signature of very specific tissue areas after performing a MALDI-MSI [66].

Other methods include on tissue digestion in combination with the hydrogel microreactor method to digest and identify proteins by traditional peptide analyses such as liquid chromatography tandem MS (LC-MS/MS) (figure 5). By this technique hydrogels containing trypsin are incubated with the tissue identifying proteins above 55 kDa. This method provide a similar number of identifications found by LC-MALDI-FTICR or TOF/TOF [63]. Future modifications of the technique will try to keep the tissue morphology; the loss of the tissue into the hydrogel is a critical step that must be carefully controlled.

The method called liquid extraction surface analysis (LESA) has been demonstrated for the extraction of tryptic peptides in small spots and subsequent analyses using on line LC-ESI-MS under high spectral resolution conditions [2]. From a single digestion spot of 300 μm, corresponding to 500-700 cells, 2000 proteins can be identified by this approach. This technology is faster than the LCM strategy leading to the identification of a larger number of proteins with respect to the number of cells studied [2]. Another possibility is to dispense a small amount of solvent on a specific position and concentrate several extraction cycles in order to collect all solutions in the same vial [67]. The technique is compatible with further peptide separation by gel, LC or CE. However, improvements in the diameter of the spot must be undertaken because each spot represents an average of 600-700 μm, extracting at once 10000-12000 cells, for that reason the spatial resolution is a limitation.

- Database searching

PMF and the peptide sequence information obtained from MS/MS analysis, provide the basis for protein identification. Tandem MS/MS provides amino acid sequence information on peptide fragments from a specific peptide. The protein will be identified with high confidence only if two or three peptides are sequenced from that protein [28].

One of the most used search engines is Mascot (www.matrixscience.com) which uses mass spectrometry data to identify proteins from primary sequence databases. PMF or MS/MS data search can be performed to identify an unknown protein from a digested sample. For MS/MS analysis the experimental mass values are then compared with calculated peptide mass or fragment ion mass values, obtained by applying cleavage rules to the entries in a comprehensive primary sequence database. By using an appropriate scoring algorithm, the closest match or matches can be identified. If the sequence database does not contain the unknown protein, then the aim is to pull out those entries which exhibit the closest homology, often equivalent proteins from related species. The sequence databases that can be searched on the Mascot server are MSDB, NCBInr, SwissProt and dbEST.

Peak smoothing, baseline correction and local mass correction are important steps to improve the database searching as they determine the quality of the data submitted to a database search. Other important search parameters are the parent and fragment ions mass tolerance that depends on the instrument used [5, 20, 68]. Different missed cleavages or protein modifications (acetylation, oxidation, phosphorylation or carbamidomethylation), can be included in the search according to the sample treatment [36].

Other freely available algorithms such as X!Tandem and OMSSA can also be used for protein identification [28]. X!Tandem allows the cross validation with Peptide Prophet, similar to Protein Blast used with Mascot [27]. Proteome Discoverer 1.1 (Thermo Fisher Scientific GmbH) is based on the SEQUEST search algorithm. Peptides with a certain "peptide probability" (SEQUEST parameter) are usually considered as significant identifications. The false discovery rate is an important way to reduce the false positive assumptions. It is recommended to repeat the search, using identical search parameters, against a database in which the sequences have been reversed or randomised. The number of matches that are found is an excellent estimate of the number of false positives that are present in the results from the real or "target" database.

- Validation

Once the identity of a protein is confirmed, immunohistochemistry studies can complement this information. If we assume that the localization of a peptide corresponds to the same distribution as the intact protein, the validation with an antibody that recognizes different peptides/sequences can be employed to confirm the MALDI-MSI results [6]. In addition antibodies designed to bind only a region of a protein can validate the distribution of a specific peptide rather than the entire protein [56].

Western blots have been used to validate new biomarkers for preneoplastic pancreatic lesions and early pancreatic ductal adenocarcinoma (PDAC) found by MSI. Intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasm (IPMN) were distinguished from normal pancreatic tissue and PDAC by 26 significant *m/z*-species. In this work, albumin and thymosin-beta 4 were identified by liquid chromatography and tandem mass spectrometry (LC-MS/MS), and further

validated by immunohistochemistry, western blot and also other molecular biology techniques like quantitative RT-PCR and ELISA [57].

Therefore immunohistochemistry and other classical techniques used in the field of molecular biology offer alternative methods to confirm the identity/abundance/distribution and also add information about subcellular localization of proteins [56].

3. Challenges and perspectives

The main challenge of any MALDI-MSI experiment is the identification of the features extracted by statistical analyses. The considerable number of MS/MS spectra returning no significant matches in the database search is probably due to the high molecular complexity of the tissue surface after enzymatic digestion leading to isobaric overlap of peptides or other biomolecules [3].

The efficiency of the digestion affects the intensity of the tryptic peptides. Local differences in the ionization efficiency (depending on the *m/z* or sequence) influence the peptide signal observed. An initial step to check for instance the efficiency of the enzymatic digestion involves the determination of endogenous peptides present throughout the tissue prior to digestion. This not only provides a higher level of confidence in assessing those peaks in the digest spectra as being generated from proteolysis, but also provides information about the presence of endogenous peptides in different regions of a tissue [39].

In addition, MS/MS spectra generated from MALDI ionization processes are often more complex to analyze because of the different types of fragment series generated during the fragmentation [32]. Another option to overcome the difficulties in the interpretation of the spectra already mentioned is the derivatization of peptides. Derivatization for the orientation of fragmentation patterns towards a specific series of fragment ions facilitates the peptide identification [33, 42].

In addition, exclusion lists that contain matrix adducts and autolytic trypsin peaks are employed to reduce the amount of data to be analyzed [5].

Protocols and methods that remove high abundant proteins from the sample must be developed in order to detect low abundant proteins that could also have with an important role in the prognosis/diagnosis of a disease. The use of enzymes such as Lysine C, V8 protease or AspN employed in classical proteomics could be applied in combination with trypsin. These enzymes will yield additional fragments and therefore increase the completeness of the digestion [69]. For instance, Lys-N has been shown to be complementary in terms of protein sequence coverage and mapping of post-translational modifications [70, 71].

Other authors have followed strategies orthogonally to MALDI-MSI in an attempt to compare the protein signature in different regions of biological samples. For instance Petyuk et al. have cut a sample brain in several cubes of approximately 1.5 mm^3. Proteins were extracted, digested and analyzed by LC-MS/MS and the images were reconstructed according the IDs from each sample cube [72]. More recently, Franck et al. have shown that a tissue section can be mounted on parafilm and then cut manually. All pieces are then subjected to an enzymatic digestion and the molecular images are reconstructed based on the quantitation of the identified proteins by a label free approach [73]. In both strategies, major as well as minor proteins were detected and localized although the lateral resolution is limited. In addition, these approaches do not allow for the detection of unknown areas based on their peptide composition. MALDI-MSI has a clear advantage in the classification of the heterogeneity of a sample when it is unknown in advance.

In spite of all these challenges, currently MALDI-MSI provides information that can be very useful for the patient classification. It has revealed new potential prognostic/diagnostic markers

of several disease pathologies. “Bottom-up” MALDI-MSI strategies allow the study of protein distribution (based on their digested peptides) and their identification. New methods that improve the correlation of the imaged peptides and protein identification must be adapted to increase the sensitivity and reliability in the field of biomarker discovery.

Figure captions

Figure 1. Evolution of the number of publications associated to “mass spectrometry imaging”, “MALDI imaging” or “SIMS imaging” using the citation search engine ISI Web of Science.

Figure 2. Schematic workflow of MALDI-MSI “bottom-up” strategy used to perform protein identification in formalin fixed paraffin embedded (FFPE). Reproduced from [42] with permission.

Figure 3. The importance of the devices used for enzymatic and matrix application. Trypsin and matrix deposition with the dry-droplet method (left). A high diffusion of the peak *m/z* 1790.9 is observed. Trypsin and matrix deposition with the Imageprep improves and preserves the localization of peptides in FPPE rat joints (center). Hematoxylin-eosin staining (right).

Figure 4. MALDI-MSI of four identified peptides in an ovarian cancer sample. (A) Hematoxylin-eosin staining of the analyzed section. (B−E) Intensity maps of four proteolytic peptides corresponding to four different proteins. (B) and (C) are predominately expressed in tissue adjacent to the cancer cell regions. (D) and (E) are predominately expressed in cancer cells. All four peptides could be identified by assignment of their mass using LC−MS/MS. Reproduced from [65] with permission.

Figure 5: Hydrogel-mediated proteomic digestion and extraction workflow beginning with (a) hydrogel synthesis on a laser printed piece of chromatography paper, (b) on-tissue placement of gel for proteolysis and incubation, (c) solvent extraction in aqueous and organic solvents, and analysis of reconstituted extracts with (d) MALDI MS and/or (e) LC-MS/MS. Reproduced from [63] with permission.

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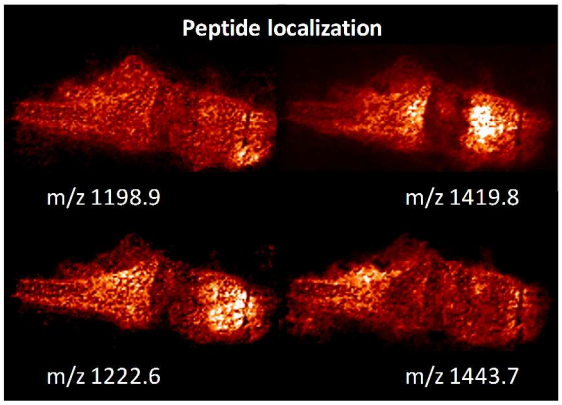
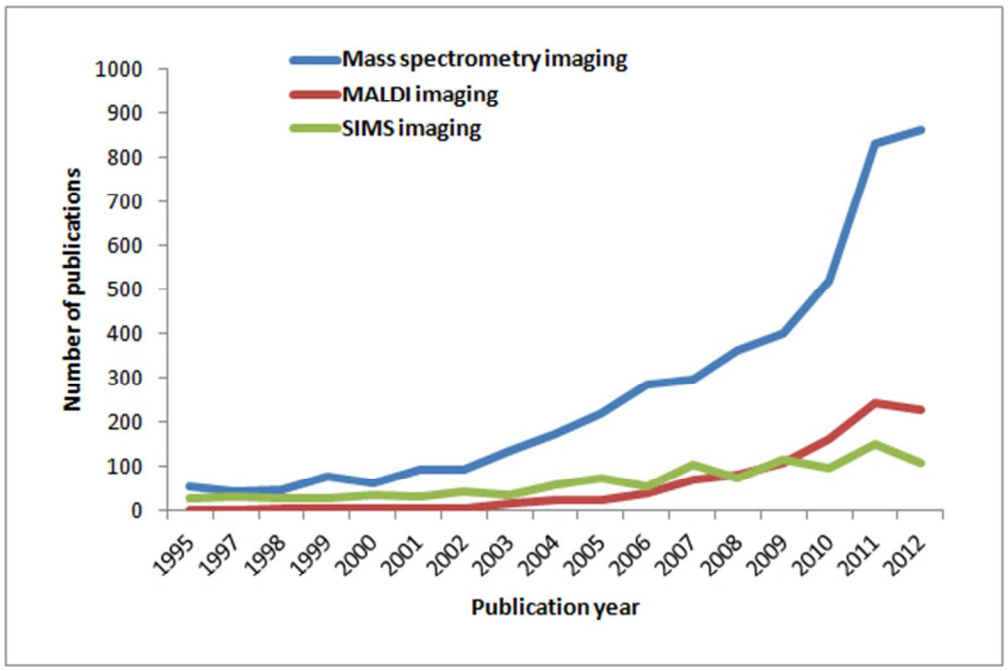
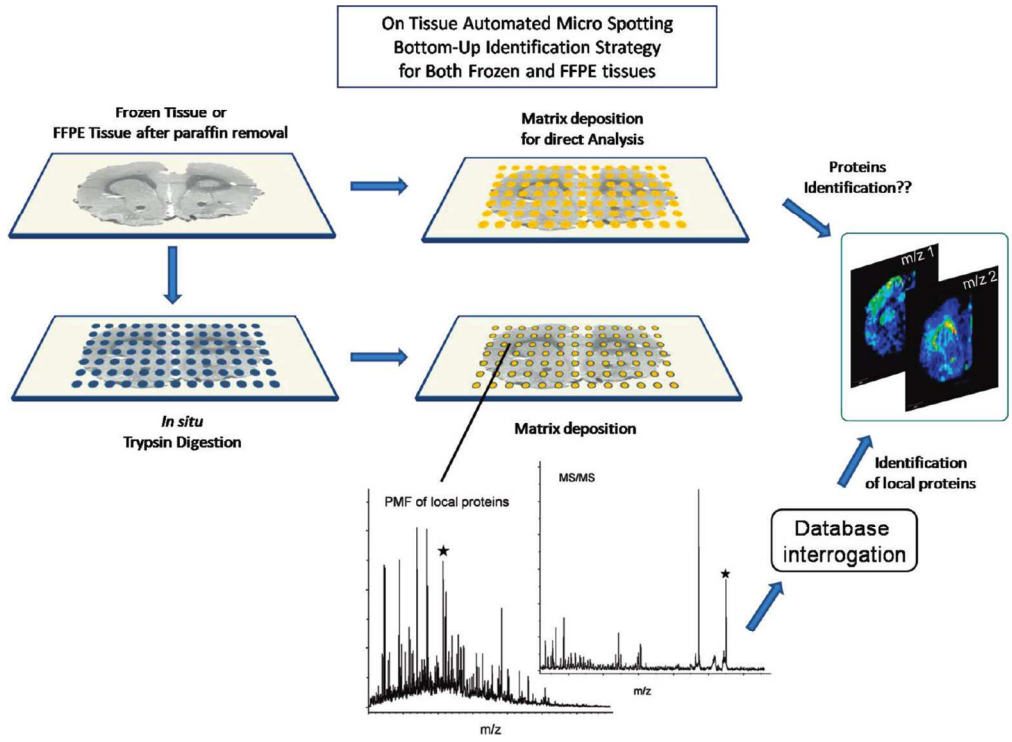
TOC

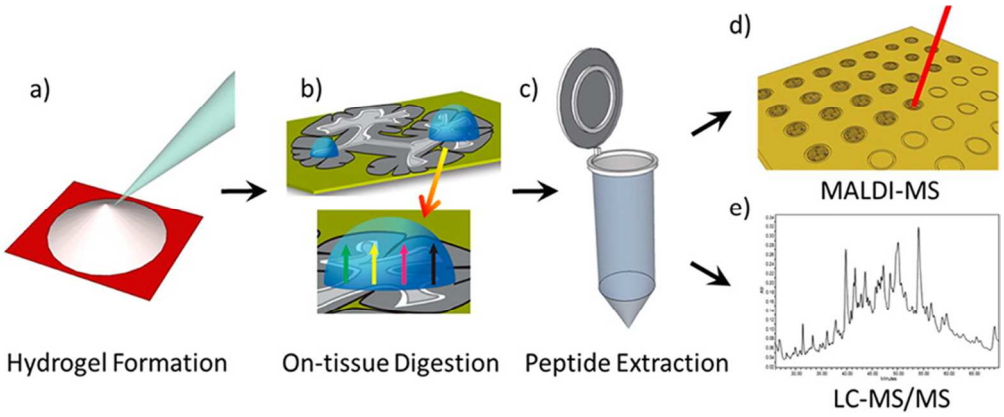




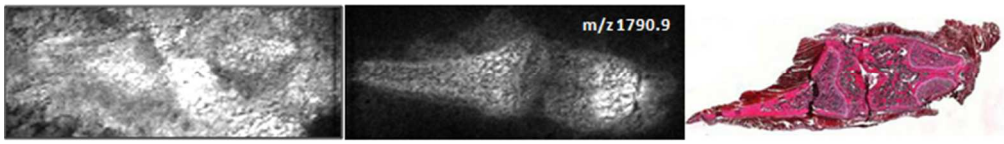
Evolution of the number of publications associated to "mass spectrometry imaging", "MALDI imaging" or "SIMS imaging" using the citation search engine ISI Web of Science.
49x33mm (300 x 300 DPI)



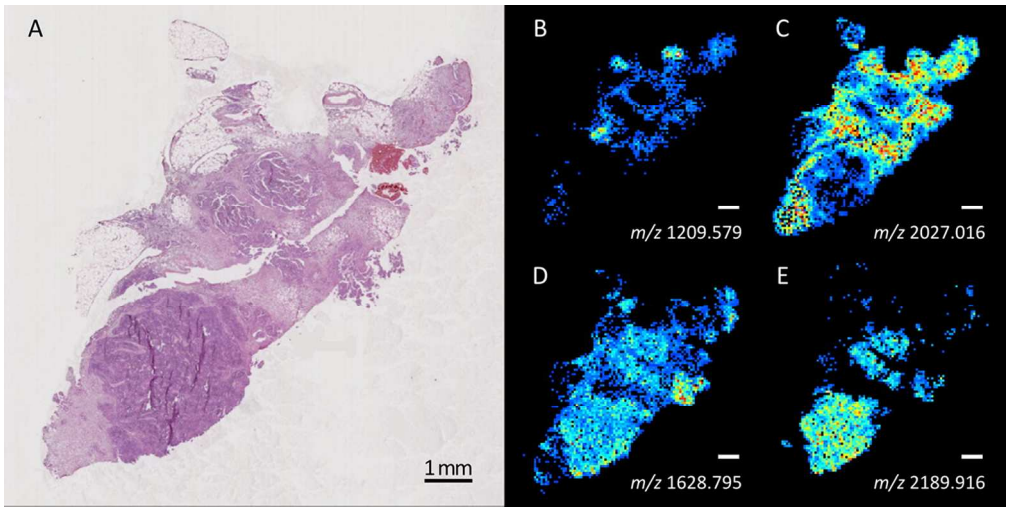
Schematic workflow of MALDI-MSI "bottom-up" strategy used to perform protein identification in formalin fixed paraffin embedded (FFPE). Reproduced from [63] with permission.
127x93mm (300 x 300 DPI)



Hydrogel-mediated proteomic digestion and extraction workflow beginning with (a) hydrogel synthesis on a laser printed piece of chromatography paper, (b) on-tissue placement of gel for proteolysis and incubation, (c) solvent extraction in aqueous and organic solvents, and analysis of reconstituted extracts with (d) MALDI MS and/or (e) LC-MS/MS. Reproduced from 47 with permission.
84x34mm (300 x 300 DPI)



The importance of the devices used for enzymatic and matrix application. Trypsin and matrix deposition with the dry-droplet method (left). A high diffusion of the peak m/z 1790.9 is observed. Trypsin and matrix deposition with the Imageprep improves and preserves the localization of peptides in FPPE rat joints (center). Hematoxylin-eosin staining (right).



MALDI imaging of four identified peptides in an ovarian cancer sample. (A) Hematoxylin-eosin staining of the analyzed section. (B–E) Intensity maps of four proteolytic peptides corresponding to four different proteins. (B) and (C) are predominately expressed in tissue adjacent to the cancer cell regions. (D) and (E) are predominately expressed in cancer cells. All four peptides could be identified by assignment of their mass using LC–MS/MS. Reproduced from 60 with permission.
119x59mm (300 x 300 DPI)