TARGETED MASS spectrometry Imaging: Specific Targeting Mass Spectrometry imaging technologies from history to perspective

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Abstract

Since its introduction during the last decade, MALDI mass spectrometry imaging (MSI) is now a routine technique in biology. Nevertheless, a missing link exists in MALDI MSI. Lipids, peptides/proteins, metabolites and drugs can easily be mapped using MALDI-MSI, but this technique has not yet been used to map the transcriptome, which includes microRNA, siRNA and other components. This latter field of research is now one of the major fields in clinical research and needs to be explored using MALDI-MSI. To investigate the transcriptome, a novel imaging technique has been developed called Tag-Mass imaging mass spectrometry. The aim of this review is to discuss this technique from its history to its place in the future of mass spectrometric imaging.

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1. Introduction

Since its introduction in the late 1980s, MALDI has become a tool of choice for the analysis of biomolecules, especially for large compounds. With a growing need for molecular information about peptides and proteins, matrix-assisted laser desorption/ionization (termed MALDI) was quickly adopted for the direct analysis of tissues because of the convenience of this ion source for the analysis of crude samples. This highly sensitive method has allowed for the analysis of a wide range of molecules in their tissue-specific context and has also been used for the analysis of a single cell. Direct analysis offers the advantages of studying cells in their original context and avoiding lengthy purification and separation steps. To achieve these benefits, MALDI has taken advantage of knowledge of mass spectrometry techniques to improve direct tissue analysis, thus leading to the development of MALDI-Mass Spectrometric Imaging (MSI). As is the case with other desorption techniques, MALDI was developed with the help of physics. Whereas other fields that use mass spectrometry (e.g., chemistry) generally study solutions for which morphological aspects are not important, surface imaging is very common in physics. Indeed, a number of different imaging techniques have emerged, but thanks to the groups of B. Spengler (1994) in Germany and R.M. Caprioli (1997) in the United States, the MALDI-MSI methodology was developed in the late 1990s. These groups reported the reconstruction of ion density curves and the construction of molecular images of biomolecules in tissue samples. The development of MALDI-MSI was innovative for fundamental biology, leading to new clinical applications. Nevertheless, a missing link exists in MALDI-MSI. Lipids, peptides/proteins, metabolites, drugs can easily be mapped using MALDI-MSI; however, the transcriptome, which includes microRNA and other RNA-related molecules, has not yet been mapped in this way. Because the transcriptome is now a major area of clinical research, it would certainly benefit from the use of MALDI-MSI. To address this important topic, a novel imaging technique has been developed, namely targeted mass spectrometric imaging (Tag-Mass MSI). This
review discusses topics ranging from the history to the future of mass spectrometric imaging.

2. From lenses to Immunocytochemistry

The oldest lens made of polished glass has been dated back to 700 BC. This lens was discovered at Ninive, the last Assyrian establishment in Kurdistan. Ancient Roman writings have mentioned some enlarging tools such as balls full of water or an emerald sharpened like a concave lens. Since ancient times, human curiosity has encouraged us to observe the infinitely small. The first documented scientific usage of lenses appeared in the seventeenth century; before that time, all lenses were used to correct vision. The origin of the first microscope is difficult to ascertain because several types of microscopes were built during the same time period. However, Antonie van Leeuwenhoek and Robert Hooke have recorded observations of the highest quality. In 1625, Robert Hooke published drawings of plant cells, fungi and lice observations in the journal “Micrographia”. The magnification he used was approximately 160x. Using his microscope, Robert Hooke also successfully demonstrated the circulation of the nettle poison in his finger. At the same time, Francesco Stelluti published a book describing his observations using microscopes, and Marcello Malpighi was the first biologist to conduct research on the machinery and embryology of the liver using a microscope. Additionally, van Leeuwenhoek was the first to describe bacteria living in the mouth, which he named the “levende dierkens”, or living small animals. In 1650, the magnification of the microscope was increased due to the use of biconvex lenses, but chromatic aberrations caused by light dispersion (e.g., coma) limited the usable field of view. In the eighteenth century, John Dollond corrected this problem by adding a negative eyeball composed of two plane-convex lenses. In 1870, a microscope with an approximately 160-mm-long tube and optics developed by Hartnack was in standard use in Europe. Thereafter, Carl Zeiss and Ernst Abbe were the first to propose the wave theory of microscopic imaging. Combining his own efforts with the work of his predecessors, Abbe developed a mathematical treatment of the lens concept. Concurrent with the development of novel types of glasses, the resolving power of microscopes was pushed to the limit of what was physically possible. In 1890, microscopic images became more refined due to the development of coating techniques, and the field of view increased. Since that time, the basic microscope structure has not changed. To achieve higher resolution, it was necessary to develop the electron microscope, which was introduced in 1930 and has a magnification of 800,000x. Major advancements have been made in the microscope optics, mechanics and, most critically, the lighting. Since the invention of the microscope, an average magnification of 200x to 1500x has been used, which means that most of the objects studied are 200 to 400 times smaller than the image observed. Immediately prior to World War II, two technical advances in optics were introduced: the phase contrast (invented by Zernike) and the interference contrast. These advances allowed for the observation of almost transparent objects, such as living cells. The most recent microscope developments include the use of probes to detect an electric field using a tunnel effect, magnetic force or atomic force. The limits of magnification are now approximately 1 billion times, permitting the observation of the surface of objects on the atomic scale.
3. From Immunohistochemistry to MALDI Mass Spectrometry Imaging

In addition to the microscope, immunohistochemical techniques permitting the localization of antigens in tissues, cells, organisms, bacteria, viruses, etc. were introduced. Immunohistochemistry was developed in 1930. In 1940, Coons and collaborators developed an antibody labeled with the fluorescent tracer fluorescein (Coons et al., 1941). The development of novel fixation procedures (ethanol, methanol, acetone, picric acid and paraformaldehyde) (Gabe, 1964; Gabe and Saint Girons, 1967; Gabe, 1969, 1972; Petit and Sahli, 1975; Steinbach, 1977; Hayashi et al., 2004; Demeure et al., 2007) were then introduced along with a technique for embedding tissues in paraffin and procedures to retrieve antigens during immunocytochemistry studies (Curran and Gregory, 1980; Beckstead, 1994; Ino, 2004). Currently, multiplex immunocytochemistry using quantum dots (Brocke et al., 2003) and multiple antisera are being developed (Furuya et al., 2004). In summary, tissue imaging using a microscope allows us to localize several molecules of interest at a cellular or subcellular resolution. The current limit of this technology is the simultaneous detection of almost 50 different antigens. The major limitations of this technology are the tissue itself and the methods of tissue preservation. All developments made during the last seven decades regarding tissue preparation are now becoming a foundation for MALDI-MSI. Currently, we are interested in determining whether we can use this background knowledge to improve the technology and what contributions MALDI-MSI will make to this field of tissue imaging.

4. MALDI Mass Spectrometric Imaging (MSI)

MALDI has become a tool of choice for the analysis of biomolecules, especially large compounds. Because MALDI is easy to use with crude samples, it allows for the direct analysis of a wide range of molecules in the tissues. This property made MALDI-MSI an ideal technique to meet the growing need for molecular information about biomolecules. Various reports of the analysis of peptides from different invertebrate cells or organelles have been published since 1994 (Jimenez et al., 1994), and many of these concern the study of peptide processing from protein precursors. Taking advantage of the great sensitivity of MALDI mass spectrometry, the direct analysis of single cells was also successful and allowed for the study of specific cell types, including neurons (Jimenez et al., 1994; Li et al., 1994a; Li et al., 1994b; Li et al., 1994c; Jimenez et al., 1997; Jimenez et al., 1998; Li et al., 1998; Li et al., 1999; Rubakhin et al., 1999; Li et al., 2000a; Li et al., 2000b; Rubakhin et al., 2000; Li et al., 2001; Rubakhin et al., 2003; Jimenez et al., 2004; Jimenez et al., 2006; Rubakhin et al., 2006). To improve the direct analysis method, it was necessary to take advantage of the knowledge of other mass spectrometry techniques, such as laser desorption/ionization (LDI) (Verbueken et al., 1985) or secondary-ion mass spectrometry (SIMS) (Castaing, 1962), to achieve better MALDI-MSI results. As with other desorption techniques, MALDI was physics-based, wherein the imaging of sample surfaces is very common. MALDI-MSI was introduced in the middle of the 1990s (Spengler et al., 1994; Caprioli et al., 1997; Chaurand et al., 1999; Stoeckli et al., 1999). These groups reported
how the data acquired from biological samples can be processed to reconstruct ion density curves or molecular images of biomolecules in tissue samples.

4.1. MALDI imaging and the basis of protein molecular imaging

In certain ways, MALDI-MSI is a simple concept. In MALDI sources, ions are produced by laser irradiation of the sample, which, theoretically, is a homogeneous solid solution consisting of a large excess of matrix molecules and the analyte molecules. Our understanding of the processes that lead to ion formation in MALDI has increased since the introduction of this technique; however, we are still unable to fully describe the underlying mechanisms involved. Unfortunately, this incomplete understanding indicates that an important part of our knowledge is empirical; therefore, it is difficult to predict the properties of a good matrix. Currently, it is understood that short pulses of photon irradiation induce energy transfer to the matrix molecules (Karas and Kruger, 2003; Knochenmuss and Zenobi, 2003) to generate excited states. The relaxation of these high-energy states involves the dissipation of part of the energy to the analytes. Basic studies performed to examine the irradiated area of simple matrix crystals (a solution of a single molecule in a water/solvent mixture) demonstrated that the laser crater formed by the removal of material does not exceed the area of the incident beam (Fournier et al., 2002; Fournier et al., 2003). Thus, most of the laser energy is transferred deep inside the sample, with little surrounding spread. The irradiation of the sample at the punctate coordinate will then produce ions that define the impact area between the laser and the sample surface. Controlling the focus of the laser can change the resolution of this area. This control is an important consideration for MALDI-MSI because it allows for the correlation of the laser irradiation coordinates with the corresponding position in the sample. Thus, laser irradiation at regular steps across the entire sample allows for the generation of coordinated spectra that can be used for molecular image reconstitution. Each collected spectrum represents the average of several laser shots to obtain a statistical representation of the analyzed area. Thus, MALDI-MSI is basically a point-to-point analysis.

MALDI-MSI is composed of four main steps (Fig. 1).

1- Tissue sectioning and preparation
2- Matrix deposition
3- Data acquisition
4- Data processing/image generation

The first step of MALDI-MSI involves tissue sectioning and preparation, which is performed using traditional procedures. The main concern is the preservation of tissue integrity while avoiding molecular composition changes, e.g., changes due to enzymatic activation. There is a subtle equilibrium that must be reached to preserve the molecular composition and molecular localization of the tissue from the time of dissection to the time of mounting on the experimental plate. As we will discuss later, the long-term storage of a tissue before analysis can be a problem. The second and third steps are based on MALDI-MS technology. The second step, which is crucial for analytical performance, consists of matrix deposition on the tissue section. It is known that the matrix is crucial for the quality of the
Fig. 1. A: The tissue preparation and analysis workflow for MALDI imaging. The resected organs or tissues were sliced with a microtome for FFPE tissues or with a cryostat for fresh frozen tissues. The sections were mounted on conductive slides, the matrix was applied dropwise onto the section, and the mass spectrometric analysis was performed over the area of the tissue. The positions and intensities of the ions are represented in a visual format called the “image”.

B: The corresponding PCA analysis of the tissue with normal and carcinomatous endometrium. The PC1/PC2, PC1/PC3, and PC2/PC3 analyses are shown, illustrating the best 2D-space separation of the ions.

As with any technology, we must understand the limitations of MALDI-MSI. By drawing a simple parallel with photography, it is very easy to identify where developmental efforts
should be directed. In photography, the expectation is to reflect an exact copy of a scene as observed by the human eye. The image must have enough definition to detect fine details and enough contrast to observe objects of different sizes, shapes, colors or brightness. Similarly, in MALDI-MSI, the pixel size and density in the image will define the acquisition of a highly resolved image. For MALDI-MSI, this limitation requires one to obtain the smallest irradiated area with the highest number of analysis spots while preserving analytical performance, i.e., a maximum of contiguous pixels should be used. For example, for a circular area, the distance between two spots should be minor relative to the diameter of the circle. Obtaining an exact molecular copy of whatever is being observed implies that we can analyze any class of molecule with the same capacities independently of its size, amount and localization within the system or origin. These are clearly challenges for MALDI-MSI because this technique must be able to deal with all samples independently of their preparation and conservation process and to analyze a complete range of biomolecules, such as peptides, proteins, lipids, sugars and oligonucleotides at very low levels. Comparing one of the initially published images that was obtained using MALDI-MSI to a recent image directly addresses some of these concerns. Now, a decade after its introduction, MALDI-MSI has greatly improved its capacities and has overcome some of its challenges because of the constant efforts of several research groups involved in this field.

4.2. MALDI-MSI, a continuously evolving technology

4.2.1. Sample preparation: Tissue Conservation and Imaging Strategies

4.2.1.1. Frozen tissues. For molecular imaging technologies, the preparation and conservation of samples are crucial. Proper conservation ensures that molecules do not move or undergo degradation. Several well-established conservation methods can result in compatibility issues for MALDI-MSI, which are mainly analytical difficulties (Lemaire et al., 2006b). The samples can be preserved using three methods after dissection, including direct freezing (in isopentane for better structure integrity), freezing after the use of a fixative (e.g., formalin, Bouin, Bouin Hollande) and conservation after fixation followed by embedding in paraffin (Lemaire et al., 2007a) (Fig. 2). The direct freezing approach results in the lowest number of analytical problems in the mass spectrometry analysis. However, frozen samples must be cut using a cryostat instrument and require storage in a freezer, preferably at -80 °C. During long-term preservation at -80 °C for more than 6 months, the lipids present in tissues move and mask the tissue sections. Specific washings, including washes with chloroform/methanol or acetone, are needed to remove these lipids (Lemaire et al., 2006b; Seeley et al., 2008).

4.2.1.2. Formalin-Fixed Paraffin-Embedded tissues. The most commonly used fixative for the long-term preservation of tissue sections is paraformaldehyde (PFA) or formalin (Fig. 2). When PFA fixation is used prior to embedding the tissues in paraffin, the resulting samples are called formalin-fixed paraffin-embedded (FFPE) tissues. The FFPE preparation not only increases sample stability over time but also results in better preservation of the fine structures of the tissue after tissue sectioning. The development of strategies for MALDI-MSI analyses also allows for retrospective studies of past clinical cases to be performed.
based on large collections of samples. Because FFPE samples can be stored for decades, many samples are available in hospital tissue banks, thus allowing for the creation of larger collections including rare pathologies or sub-pathologies (Lemaire et al., 2007a; Groseclose et al., 2008; Ronci et al., 2008; Stauber et al., 2008). However, although PFA fixation is ideal for conservation and structure preservation, it can also result in difficulties during the molecular analysis. For example, PFA fixation is known to induce molecular cross-linking, especially in peptides and proteins. The chemical reaction processes involved in PFA fixation are extremely complex. Although the protocols and outcomes of PFA fixation have been extensively studied, only a few literature reports directly address the molecular chemical reactions underlying the fixation process (Kieman, 2000; Plenat et al., 2001; Plenat et al., 2006). To simplify, we can state that PFA reacts principally with free amine groups, binding amino acids tightly via the formation of methylene bridges. The primary reaction of the aldehyde with the protein involves rapid kinetics (Kieman, 2000; Plenat et al., 2001; Plenat et al., 2006). In contrast, the second reaction leading to the formation of the methylene bridge is a much slower process that occurs over days (Kieman, 2000; Plenat et al., 2001; Plenat et al., 2006). Thus, reactions are still occurring even after the tissues are embedded in the paraffin such that the proteins become further imprisoned over time as a result of the slow formation of methylene bridges. An MS analysis of such tissue samples after tissue sectioning and paraffin removal has generated very few results because the proteins are all cross-linked together. Because chemical reactions can still occur inside the paraffin blocks, the MS spectra generated from these samples are dependent on the storage time. For recently

Fig. 2. Scheme of the tissue conservation procedure as a function of the type of tissue considered, i.e., fresh or fixed.
prepared FFPE blocks (~0–6 months), some ion signals can be retrieved (Lemaire et al., 2007a), which are mainly observed in the mass range of a peptide. A detailed study of the corresponding mass spectra indicates that the peaks exhibit broadened profiles with +12 amu adducts that increase with storage time as a result of the slow PFA secondary reaction (Redeker et al., 1998). However, more peptide signals could be observed when using very specific MALDI matrices, such as the reactive matrix 2,4-dinitrophenylhydrazine (2,4-DNPH) (Lemaire et al., 2007a). Surprisingly, with such a matrix, the MS spectra do not present any additional +12 amu adduct ions. This result could be explained by assuming that 2,4-DNPH reacts with the free aldehyde groups (PFA) remaining in the tissues. For samples stored for longer periods (> 1 y), the MS analyses do not allow for the retrieval of exploitable signals even with 2,4-DNPH. The cross-linking of proteins has also been shown to be problematic for immunohistochemistry (IHC) experiments because it hampers antibody access to the epitope of the antigen. To overcome such problems, pathologists have extensively studied epitope-unmasking procedures. Different antigen retrieval (AR) protocols that are compatible with IHC are now well known and have been described elsewhere (Shi et al., 1997; Taylor and Shi, 2000; Shi et al., 2001a,b; Shi et al., 2007a; Shi et al., 2007b; Xu et al., 2008). The most popular procedures include heating at high pressures (Shi et al., 1997; Taylor and Shi, 2000; Shi et al., 2001a,b; Shi et al., 2007a; Shi et al., 2007b; Xu et al., 2008) and the citric acid-antigen retrieval procedure (CAAR) (Gustafsson et al., 2010; Bonnel et al., 2011b). However, although AR protocols result in epitope-unmasking, such procedures do not reverse protein cross-linking. AR protocols can be used prior to the MS analysis of tissues to improve the quality of the results, but this method is generally not sufficient because of the cross-linking of the proteins that renders these samples very difficult to analyze directly using MALDI and MALDI-MSI. New strategies are thus needed to analyze FFPE tissue sections in a very efficient manner that is independent of the storage period. Recently, we have developed an approach based on the enzymatic digestion of tissues that allows for the retrieval of pieces of proteins. In this strategy, digested peptides are generated and subsequently analyzed on the tissue or in the images. MALDI-MSI analysis following on-tissue enzymatic digestion is possible if the localization of the generated peptides can be retained, i.e., if the enzyme can be precisely deposited and localized onto the tissue. This requirement can be met by applying small droplets of enzyme in a controlled manner, i.e., by means of a reproducible automatic device (Lemaire et al., 2007a). If the localization of the digested peptides is sufficiently precise, then a MALDI-MSI analysis of the intact proteins can be deduced from the images generated from the ion signals of the digested peptides. This generation of peptides is also extremely interesting for identification purposes.

### 4.2.1.3. High-Mass protein accessibility

Proteins exceeding 25 kDa in size are not routinely detected using MALDI-MSI. This issue represents a significant methodological limitation because the size of most classes of proteins exceeds 25 kDa. Two previous articles reported sample preparation methods that enable MSI to be used for proteins with higher masses (Grey et al., 2009; Leinweber et al., 2009). The first method used extensive water washing procedures to remove the abundant soluble proteins, which was followed by the automated application of a matrix solution containing a high percentage of an organic solvent. This sample preparation allowed for the detection of a 28 kDa integral crystalline
lens membrane protein (Grey et al., 2009). In the second approach, a matrix application protocol using Triton X-100 was shown to enable the detection of proteins ranging from m/z 25 000 to 50 000 (Leinweber et al., 2009). These observations suggest several reasons for the lack of sensitivity of high-mass proteins that can be exploited for the further development of sample preparation methods. The use of organic solvents or Triton X-100 suggests that high-mass proteins may not be detected because they are not efficiently solubilized in the matrix solution. Consequently, these larger proteins are not extracted from the tissue. The detection of high-mass proteins after the removal of abundant soluble proteins suggests competition with the abundant proteins for incorporation into the matrix crystals and/or ionization in the MALDI process (ion suppression effects) (Cohen and Chait, 1996). Ion suppression is most likely another effect that limits the routine detection of high-mass proteins. A new tissue sample preparation has been undertaken using hydrophobic solvents, e.g., hexafluoroisopropanol (1,1,1,3,3,3-hexafluoro-2-propanol, HFIP) (Redeby et al., 2004; Redeby et al., 2006) and 2,2,2-trifluoroethanol (TFE) (Redeby and Emmer, 2005). These solvents have previously been used to extract membrane proteins (Ferro et al., 2000; Chertov et al., 2004; Wang et al., 2007b) and high-mass proteins (Thompson et al., 2008) and could aid in detecting high-mass proteins directly from the tissue. Using this method, proteins that are approximately 70 kDa in size have been detected (Franck et al., 2010). Coupling this procedure with the use of a specific high-mass detector has allowed for the detection of proteins with a molecular mass exceeding 100 kDa. Increasing the mass range of the detection is a key for developing direct tissue proteomics and, especially, for any potential functional investigation. These data will make possible a novel approach in mass spectrometric imaging (van Remoortere et al., 2010).

4.2.2. The MALDI matrix: The cornerstone of MALDI-MSI

4.2.2.1. What is the best matrix for MALDI-MSI? There is insufficient knowledge available regarding the MALDI mechanisms to properly use theoretical and predictive considerations to choosing appropriate matrices; thus, we depend solely on empirical experimental data to establish the best matrix. At least three types of matrices are known for the MALDI analysis of biomolecules: sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (2,5-DHB). Although all three of these matrices can be used for both peptide and protein analysis, SA is generally preferred for proteins in terms of signal intensity, resolution, signal-to-noise ratio and the number of detected compounds. CHCA is generally used for peptides, whereas 2,5-DHB offers a broader range for analysis and can be used for either peptides or proteins. For MALDI-MSI, the situation is more complex due to the interaction between the matrix and the tissue. Based on experimental data obtained following matrix deposition on tissue sections, CHCA appears to provide good signals for peptides up to m/z 5000, but only a few signals with weak intensities can be observed above this limit. SA yields better signals in the range of m/z 5000 to 30,000. However, although SA is better suited for high-mass proteins on tissues than it is for classical MALDI, the mass ranges are more limited on tissues. Thus, when comparing the classic solution analysis to the tissue analysis, proteins up to 100 kDa in size (e.g., antibodies) cannot be analyzed in tissues. This limitation in tissue sections is yet to be overcome. The most likely hypothesis to explain such a result is that it is difficult to readily extract high-mass proteins from tissues and incorporate them into the matrix.
crystals. The use of 2,5-DHB has been less than that of the other matrices, although it was initially employed for peptide/protein analysis because of the heterogeneous crystallization of this matrix. Indeed, spotted 2,5-DHB generally crystallizes in fine long needles at the rim of the spot, leaving small crystals in the inner region of the spots that contain high levels of salts. A MALDI signal is obtained by irradiating the rim of the sample. Moreover, 2,5-DHB is known to have “hot spots”, i.e., very strong signals in some parts of the crystals and no signals in other parts. This type of crystal behavior is very difficult to use for a MALDI direct tissue analysis for which signals must be reproducible for all studied spots of the sample. Nonetheless, satisfactory results may be obtained when 2,5-DHB is used in a spray or in a micro-spotting deposition procedure.

Although classic MALDI matrices are applicable for tissue analysis and have been shown to give good results, the search for new matrices that are better suited for MALDI-MSI remains a priority. Solid ionic matrices have been shown to provide good results in tissue analyses. Lemaire et al. (2006a) showed that novel solid ionic matrices were particularly well suited for the analysis of peptides up to 10 000 m/z. CHCA/aniline and CHCA/2-amino-4-methyl-5-nitropyridine were the best matrices for tissue analysis (compared with CHCA), and these matrices resulted in increased signal intensities, improved signal-to-noise ratios and a larger number of detected compounds. Similarly, a solid ionic matrix consisting of SA and aniline was reported to be the best matrix for protein detection (Franck et al., 2010). For lipids, liquid ionic matrices such as DHB/aniline can also be used (Meriaux et al., 2010). The principal advantage of these matrices is their homogeneous crystallization (Fig. 3), resulting in a very limited delocalization of peptides/proteins, although they are deposited without any specific system.

These studies also showed that ionic matrices are very stable under vacuum conditions and lead to very low material ablation rates under the source vacuum, thus allowing the sample to be used for an extended period inside the instrument to obtain several acquisitions with no decrease in performance.

Although classic MALDI matrices provide good performance, the analytical performance of MALDI-MSI must be maximized to increase the dynamic mass range and the number of analytes observed. Thus, the effort to find new matrices continues.

4.2.3. Which method is optimal for matrix deposition?

It is clear that adding a matrix on top of the tissue sample is a critical step in the sample preparation process, and several criteria must be fulfilled for successful MALDI-MSI. The first question is whether the solvent used for the matrix solubilization induces the delocalization of analytes during the crystallization process. In most cases, delocalization of the analytes does occur and must be limited. Deposition of the matrix solution onto a tissue section using a micropipette is thus not very satisfactory. Stoeckli et al. (2002) demonstrated that matrix crystals could migrate over 400 μm on the tissue section before solidification. In a typical preparation, the matrix crystals can range from 10 to 100 μm in size. Thus, the ideal matrix should have a minimal amount of solvent and produce the smallest size of crystals possible. This point is critical to obtain reliable image results and to achieve a better image resolution.

There are three different strategies to circumvent this problem: (1) spray the matrix solution, (2) deposit the matrix solution as discrete spots or (3) blot the analyte onto a
membrane via passive or active transfer. Blotting was one of the first strategies to be tested (Chaurand and Caprioli, 2002; Chaurand et al., 2002). With this method, the matrix can be easily deposited by adsorbing proteins on membranes. Conductive polyethylene membranes were successfully used for the direct analysis of a wide range of proteins up to 100 000 Da in size. The membrane transfer method is limited in terms of variability and transfer yields, especially when comparing different proteins. At present, analyses performed directly on the tissues are preferable to maximize the number of detected compounds and the sensitivity. The spraying technique and the application of discrete spots of the matrix are thus preferred. Spraying the matrix is now widely used because it is one of the easiest and cheapest methods for preparing samples for MALDI-MSI. Spraying is generally performed using a pneumatic sprayer, such as a TLC sprayer or an airbrush system. Such systems minimize the solvent quantities and the matrix crystal size, but they often lack reproducibility because the optimal spraying conditions are difficult to control. Because of the physicochemical proprieties of peptides/proteins, these species must be included in the matrix crystal for optimal ionization. This requirement leads to a paradox related to the correct spatial localization of molecules and their proper ionization. The proper incorporation of molecules into the matrix crystals

Fig. 3. The matrix deposition procedure as a function of the type of instrument used. A) Molecular images of a rat cerebellum section covered by CHCA/aniline using an automatic sprayer. B) Multiplex molecular image of 4 ions with different m/z values at the level of the rat cerebellum section covered by CHCA/aniline using an automatic sprayer. C) Multiplex molecular images of 2 ions of a rat brain section covered by SA/aniline using piezoelectric heads.
is better achieved with a reasonable amount of solvent; however, experimental data have confirmed that an important loss of signal is observed even with low amounts of solvent. Thus, if the spraying conditions are not well controlled, the sample will be either too “dry” (not enough solvent) and will not permit sufficient incorporation of the analytes into the matrix crystals or too “wet”, leading to delocalization and heterogeneous crystallization. The distance between the sprayer and the sample, the flow rate of the spray, the duration of a spray cycle and the number of cycles applied are difficult to control by hand. Therefore, even if the microscopic observation of the deposited matrix layer is satisfactory, the resulting mass spectra can be of poor quality in terms of peak intensity, signal-to-noise or number of detected compounds. A robotic sprayer system that offers very good reproducibility of the MALDI mass spectra (Fig. 3) was developed by Bruker Daltonics (Schuerenberg et al., 2007). This system monitors the growth of the matrix layer using an optical sensor that monitors the light scattered from the matrix crystals and combines a fine control of all of the parameters to optimize the spraying conditions. The average droplet size is very small (~25 μm) in this system. Recent developments have also improved the discrete deposition method, which is more complex because it requires the deposition of picoliter amounts of matrix according to a regular raster to cover the entire tissue section (Fig. 3). This strategy requires the use of a robotic system that can plot all of the matrix points. For example, to cover an entire 2 cm by 1 cm section of rat brain tissue, 5000 discrete matrix spots are required. Picoliter amounts of a matrix solution are generally insufficient to provide good extraction and incorporation of the analytes into the matrix crystals. Additionally, printing systems generally do not allow for the use of high concentrations of the matrix solution because this results in clogging of the system. It is therefore necessary to perform several deposition cycles to obtain good spectral quality, which results in an increased sample preparation time. Several commercial platforms are available to perform picoliter deposition of a solution. One of the simplest methods is to use a fused silicate capillary to generate a very small droplet that is gently deposited onto the samples, such as the systems used for nano-LC fraction collection. The solution volume and the spot size are then directly dependent on the internal diameter of the capillary. Piezoelectric systems, which are used for microarrays, can also be used for MALDI-MSI (Franck et al., 2009a). Finally, a system based on the acoustic ejection of solutions has the advantage of avoiding problems with clogs. Each of these strategies produces spot sizes ranging from 100 to 500 μm, which is sometimes higher than the typical resolution of 150-200 μm used for MALDI-MSI. The application of discrete spots can be quite time-consuming (several hours) and requires that the conditions are finely optimized for each solvent and matrix solution to obtain optimal MALDI spectra. Although it may appear that discrete deposition results in a lower resolution due to the spot size, this method does guarantee that the delocalization of a compound cannot exceed the size of the spots. Nonetheless, experimental results using discrete deposition do not exhibit any major differences in terms of image resolution when compared with the other matrix deposition methods. For a cheaper approach, an inkjet printer with ink cartridges filled with the matrix solution can be used for matrix application (Baluya et al., 2007). This approach yielded good results when monitoring lipids with 2,5-DHB as the matrix, but it was unsuccessful for the deposition of CHCA, which needed to be solubilized in a high percentage of acetonitrile. Alternatively, matrix application via sublimation is also suitable for MALDI imaging (Hankin et al., 2007). This method was
previously used to analyze polymers, and it has the advantage of being solvent-free. In this method, the matrix is applied by heating the solid matrix until sublimation occurs, and the matrix is then deposited by condensation onto the tissue sample. This strategy results in extremely fine and homogeneous layers of the matrix and avoids delocalization of the analytes, thus providing to highly resolved images. Although this method is well adapted for lipid detection, it is inappropriate for peptides and proteins because these latter molecules must be incorporated into the matrix crystals for the desorption/ionization process to be successful (i.e., peptides/proteins are too polar to be easily transferred into the gas phase).

4.2.4. Acquisition Time

The data acquisition time for the purpose of image reconstruction is a parameter sustained by the user with little choice. The acquisition time is principally dependent on the spatial resolution and the laser repetition rate, and it benefits from the developments of new lasers with higher performances.

Presently, lasers with repetition rates of up to 1000 Hz are available for commercial MALDI-TOF systems. However, the scan rate can also be limited by the mass analyzer duty cycle time and the number of duty cycles to be performed for good spectral quality. Only one cycle per laser shot is required for TOF analyzers, and the limitation in speed is linked to the capacity of the TDC (time-to-digital converter) system. Therefore, if a very broad mass range must be recorded, the repetition of laser shots may become faster than the time-to-digital conversion. In such cases, it is necessary to decrease either the acquisition mass range or the laser repetition rate. Using an ion trap (IT), the limitations of this method include the number of duty cycles necessary to record a mass spectrum.

MALDI-MSI typically works in a microprobe manner; therefore, the acquisition time is dependent on the size of the sample. The collection time of MALDI-MSI data can range from 15 min to greater than 24 hours depending on the size of the sample, the number of points to be acquired (i.e., the spatial resolution), the laser repetition rate and the time required to move the sample between each analysis point. For example, a $15 \times 10$ mm rat brain section with a raster of $100 \mu m$ in the $x$ and $y$ directions will result in $150 \times 100$ spots (i.e., 15,000 spots to be analyzed). Using 300 laser shots for each spot to ensure good spectral quality and a laser repetition rate of 10 Hz, the acquisition time would be >20 hours. Using a laser repetition rate of 1000 Hz, the same spectra could be obtained in less than 1 hour. If better images are required, which can be achieved by decreasing the footstep between two points or by increasing the number of laser shots to be averaged, then the acquisition time will again increase. If MALDI-MSI is to be an important application in the pharmaceutical industry or in health-related research, the throughput must be optimized. The latest generation of MALDI-TOF/TOF spectrometers is now equipped with lasers with a repetition rate of 1000 Hz, and the next-generation MALDI laser could be 5000 Hz to 10,000 Hz.

4.2.5. Image Resolution

As mentioned earlier, the image resolution is dependent on the size of the image pixels and the number of pixels in the area studied. The number of pixels is limited by the precision of the $(x,y)$ table that controls the sample plate, which result in very high precision that ensures good reproducibility within a sample of 1-2 $\mu m$. The image resolution is also limited by
the data processing capacities. Indeed, each pixel that is added to a sequence corresponds to the data of a full mass spectrum that contains thousands of values for intensity vs. m/z. The amount of data collected will thus depend on the mass analyzer used for the MALDI-MSI sequence. Extremely large amounts of data are generated for an imaging sequence regardless of the type of instrument used. For example, an image of an entire rat brain section with a raster of $100 \times 100 \mu m$ (approximately 1.5-2 cm by 1-1.5 cm) can result in more than 6 Go of data. Such a large data set requires a significant amount of computational power, which is not always provided by a standard mass spectrometer workstation.

Another challenge that must be overcome to improve MALDI-MSI resolution is to decrease the size of the pixel, i.e., to decrease the area irradiated by the laser. For a typical MALDI analysis, the irradiated area is not of great concern and does not need to be minimized. Thus, most existing systems, both home-made and commercial instruments, are generally equipped with a laser that can focus on an irradiated area with a diameter of approximately 100-150 \mu m, which produces images with a resolution (R) of 10000 pixels/cm$^2$. This resolution is a good starting point in the field of mass spectrometry, but it is insufficient for biologists because this resolution corresponds at least to an irradiation area of $5 \times 5$ cells. To image a single cell using MALDI-MSI, a resolution of 20-25 \mu m would be required. Because the number of pixels (N) is proportional to $R^2$, doubling the resolution to collect the maximum amount of information leads to 4 times the number of pixels and data to be processed. The only direct way to decrease the size of the irradiated area is to use a better-focused laser beam, which may be a challenge but may be achievable for laser physicists. A very high-resolution system was developed by Spengler and Hubert (2002) for MALDI-MSI, namely the SMALDI (scanning microprobe MALDI). Using a very specific set-up of lenses that were incorporated both outside and inside the instrument along the laser beam pathway, a minimal resolution of $\sim 0.5 \mu m$ per pixel is theoretically reachable. Experimentally, the resolution of the SMALDI system was very close to this theoretical value under classic conditions of laser flux (a measure of the energy delivered per unit area) and using a homogeneous laser beam. The resolution obtained (5 \mu m for lipids) is close to the size of an average cell (Römpf et al., 2010). The next step would be to develop systems capable of sub-cellular imaging and the relatively fine observation of organelles. Although such fine resolution is possible, obtaining data for peptides and proteins in tissue sections at such a resolution is another matter. To date, the best images have been obtained with an average resolution of 20 \mu m for highly abundant proteins (Lagarrigue et al., 2011). The first consideration is that decreasing the pixel size will also decrease the copy number of the ionized molecule being analyzed. Considering a fixed limit of detection, the amount of proteins available on such a surface is likely too low to be detected. Basic studies have shown a drastic decrease in the ion yield when the size of the irradiated area is decreased (Dreisewerd et al., 1995). A 35-50 \mu m laser raster is reasonable, but the ion yields are well below their limits. MALDI is well known for its poor ability to produce ions because only a small fraction of the molecules ejected from the solid will reach the detector of the mass analyzer. Although our knowledge of MALDI processes has increased considerably over the past decade, we have not yet been able to increase the ion yield. Thus, the resolution of MALDI-MSI will only progress with increases in the fundamental understanding in this field. Presently, dedicated systems for MALDI-MSI provide better focusing systems for the laser beam, which are capable of reaching a pixel size as low as 10 \mu m and should at
least provide pixels 30-50 μm in size while maintaining good analytical capacities (Holle et al., 2006). Recently, Chaurand et al. (2007) designed a new system that allows for the collection of protein images at a resolution of 10 μm. Their experimental set-up combines a very carefully drawn system consisting of a focusing lens and an iris aperture to finely control the laser beam size. The source geometry also uses co-axial illumination of the sample, which reduces the radial distributions of the ejected molecules and ions formed, thus increasing the uptake of the ion into the mass analyzer. Although, such systems are currently in a developmental phase and are not commercially available, other systems have been developed to increase the image resolution using alternate methods. One such alternative was proposed by Sweedler and collaborators (see: Jurchen et al., 2005) and involves the overlap of pixels during the acquisition of data. In this method, the sample is irradiated sufficiently to remove all matrix material from a spot, and the sample is then moved by only a fraction of the irradiated area diameter (e.g., half the diameter in a simple case). Thus, for consecutive spots, only half of the new material and ions can originate from an area that was not previously irradiated. This very simple method increases the spatial resolution up to approximately 25 μm and can be used on all existing instruments. However, this methodology results in a significant increase in acquisition time. Other methods designed to be applicable for all instruments are currently under study. Other authors have proposed the use of mask systems (Fournier et al., 2007; Wisztorski et al., 2007a,b) that involve covering the tissue with a mask, thus presenting a network of apertures of defined size and eliminating the need for a very complex system of laser focusing. The size of these apertures would limit the laser beam and control the size of the irradiated area. These systems reduce the pixel size up to 30 μm but maintain a sufficiently high ion yield for analysis of peptides and proteins. Mask systems are of great interest because they lead to an increase in the ion yield according to the specific shape of the apertures. Finally, Heeren et al. (see: Luxembourg et al., 2004) proposed a very different MALDI-MSI system that operates in ion microprobe mode: the so-called “stigmatic MALDI”. This specific instrument geometry is closely related to the old systems of the laser microprobe mass analyzer (LMMA) and is based on a correlation of the arrival positions of the ions with their original positions on the sample. In such a system, the laser is defocused to irradiate an area of approximately 200 × 200 μm with a very specific arrangement of extraction lenses and a position-sensitive detector that independently treats data from each channel of its surface. Thus, the arrival positions of the ions allow for the calculation of their initial positions on the sample. A very fine lateral resolution of 4 μm can be achieved for an area of 200 × 200 μm² in a timeframe of less than 1 ms because only one-shot acquisition is needed for this surface. Reconstructing an entire image with high resolution would only require the contiguous acquisitions of small areas of the sample followed by the summation of these acquisitions. This technique also has great potential, as shown by the recently published reports (Altelaar et al., 2007) on the distribution of peptides in the rat pituitary tissues. This unique system has not yet been established for high-throughput applications.

4.2.6. MALDI-MSI and bioinformatics

As with all imaging technologies, data processing is vital for MALDI-MSI. Various types of software exist for image reconstruction, but there is no unique software for imaging. Even in the early years of MALDI-MSI, automation of the acquisition of data was proposed for
most instruments. However, this automation was not suitable, and considerable time was lost in setting up the acquisition. Moreover, no tools were available for post-acquisition data processing. The simplest software would have to perform several tasks: acquisition of data using the specific instrumental settings according to a defined raster, creation of an average spectrum from all of the collected spectra, extraction of the intensity at a specific m/z value upon user inquiry and a report of the m/z intensities using a color scale as a function of the corresponding (x,y) coordinates. The first usable software able to perform these tasks was “Biomap” developed Martin Rausch with MSI additions by Markus Stoeckli. It allows full automation of the data acquisition for different MALDI-TOF instruments. “Biomap” is dedicated software that reconstructs images from whatever MALDI data are obtained. It offers many different functionalities and is available free of charge at http://www.maldi-msi.org/.

Optimal data processing can be the result of very different solutions that have the final goal of producing images that are closest to reality. Several questions must be answered when processing MALID-MSI data if the aim is to represent reality: Is reality better described by measuring peak intensity or peak areas? Must the mass spectra data be normalized? Must a minimum threshold of intensity be defined? Must a maximum intensity be defined? How well can mass calibration be performed? Can image resolution be increased by extrapolation of a signal between two data points? What algorithms should be used? Can better tools be designed for biological applications using classification parameters? Overall, two different processing issues must be distinguished, including the need for the data processing to describe reality and the use of statistics for sample classification when performing a differential display analysis. Optimization of this work is balanced between increasing the extraction of information and reducing false information.

The influence of data processing in the classically used data process are discussed in a paper by Norris et al. (see: Klerk et al., 2007). Statistical analyses and clustering are already used to analyze proteomics data. Clustering methods were used in MALDI-MSI experiments and direct analyses to compare human glioma and other brain tumors with normal brain samples (Schwartz et al., 2004). This study demonstrated that tumor tissue could be easily discriminated from non-tumor tissue based on its very unique protein profiles, which were extracted using a statistical analysis. Moreover, a combination of molecular profiling and clustering also differentiated the different grades of tumors. An assessment of protein patterns in specific diseases was reviewed by Chaurand et al. (2004). Multivariable analyses and clustering can also be used to find regions of interest (ROI). A ROI represents the area on the sample in which some of the analyzed molecules are differentially expressed compared with other samples. An image can thus be reconstructed from the entire signal to identify the spatial correlation of the mass spectra with the tissue localization (McCombie et al., 2005). Recently, researchers demonstrated the applicability of principal component analysis (PCA) algorithms for MALDI-MSI (Van de Plas et al., 2007; Deininger et al., 2008; Walch et al., 2008).

4.2.7. MALDI-MSI: a general technology for all types of biomolecules?

Cells contain a large variety of molecules that constitute families ranging from peptides/proteins, oligonucleotides (DNA, RNA), saccharides, lipids, salts and small organic compounds, such as neurotransmitters, ATP, ADP and NO. Each of these families are themselves composed of molecules that can exhibit a large range of physico-chemical properties
in terms of polarity, hydrophobicity, solubility, molecular mass, and acid/base properties. A perfect molecular imaging technique should be able to equally determine the distribution of all of these compounds. To date, none of the tools available is capable of such a task. However, a full understanding of living systems requires a better knowledge of the interactions between these different families of biomolecules. It is thus worthwhile to use mass spectrometry to analyze these biomolecules.

4.2.7.1. Imaging of peptides and proteins. Due to its capacity to generate ions from compounds of various polarities and its ability to analyze molecules with very high molecular masses, MALDI-MSI is naturally well designed for monitoring peptides and proteins. Moreover, the analysis of peptides and proteins is currently the main field of application for such an ion source. The specificity of MALDI compared with other MSI techniques, such as SIMS-MSI, which affords very high resolution (1 μm lateral resolution routinely) but which is only well adapted for studying small organic compounds, such as lipids, and thus does not allow for peptide or protein analysis. Thus, it was natural that initial efforts were dedicated to improve MALDI-MSI in the field of proteomics. However, even in proteomics, MALDI-MSI has limitations, one of which is the mass range, which was observed in the direct MALDI-TOF/TOF analysis of proteins from tissue sections. These proteins had masses up to approximately 30 000-35 000 Da, which is outside the classic MALDI-TOF/TOF range. The reasons for this limitation are not clear. Reasonable hypotheses include difficulties in incorporating high-mass proteins in the matrix crystals because of their solubility in the solvent used for the matrix solubilization and a lower laser/matrix energy transfer to these proteins in tissue samples that does not allow the desorption process to occur.

4.2.7.2. Imaging of lipids. Small endogenous compounds can be difficult to analyze using MALDI for either practical or fundamental reasons. The matrix produces numerous and highly abundant signals in the Mw < 1000 Da spectral region that can interfere with the signals arising from the compounds of interest in this molecular range. However, some of these compounds are non-polar and exhibit low ionization rates. Saccharides are generally very difficult to analyze using MALDI and have not yet been analyzed using MALDI-MSI.

Besides proteins, lipids comprise the family of endogenous molecules that are the most studied using MALDI-MSI. Many efforts have been made by the groups of Woods (see: Jackson et al., 2005a,b; Jackson et al., 2005c; Wang et al., 2005; Wang et al., 2007a) and Yost (see: Garrett 2007; Hsieh et al., 2007) to improve the direct analysis of lipids using MALDI. The identification of lipids from tissue sections is very complicated because there are only very small variations in the masses of different lipids, even those with very different compositions. For example, phosphatidylserine (PS) 40:6 (Mw = 835.54 Da) and sulfatide (ST) 20:0 (Mw = 835.59 Da) are from very different families of lipids, but they exhibit a mass difference of only 0.05 Da. Moreover, several lipids present the same Mw and the same atomic composition but vary structurally because of differences in the position of an unsaturated bond. Moreover, the molecular weights of lipids generally range from 100 to 1000 Da, which, as previously explained, is where matrix interference occurs. The imaging protocols used for classic MALDI, e.g., using 2,5-DHB as the matrix or using more specific
matrices, such as DHAP (2,6-dihydroxyacetophenone) or ATT (6-aza-2-thiothymine), has also been shown to be successful for the direct analysis of lipids. However, in the case of DHAP, the matrix was shown to be unstable under vacuum. Therefore, this matrix is not well suited for MALDI-MSI experiments (Wang et al., 2007a; Franck et al., 2009a). However, it was shown that the addition of heptafluorobutyric acid (HFBA) to the matrix solution increases the stability of DHAP under vacuum, thus making MALDI-MSI experiments with this matrix possible (Colsch and Woods, 2010). Recently, several groups have shown that the use of ionic matrices could improve the detection of some classes of lipids including gangliosides (Chan et al., 2009) and phospholipids (Meriaux et al., 2010). Regardless of the matrix used, the analyses were conducted in both positive and negative modes depending on the class of lipids being analyzed.

Great care must be taken for the lipids during matrix deposition because such small organic compounds are easily spread out on the tissue section. For this reason, the matrix is generally deposited using a pneumatic spray system or an airbrush to obtain a repartition of homogenous crystals and to avoid large quantities of solvents. Other strategies that were found to be efficient because they provided a homogeneous matrix coverage on tissues include solvent-free procedures (Puolitaival et al., 2008; Bouschen et al., 2010; Trimpin, 2010) and matrix application via sublimation (Hankin et al., 2007; Bouschen et al., 2010). With respect to microspotting preparations, the use of liquid ionic matrices was found to greatly decrease the time needed for sample preparation, which is generally a significant issue with this procedure (Meriaux et al., 2010).

Finally, lipid imaging generally requires an MS² fragmentation analysis to confirm the identification and real assignment. As mentioned previously, lipids are very small in mass and are well adapted for a variety of mass analyzers. Thus, different instruments have been used for lipid analysis including Q-TOF, TOF-TOF, Ion Trap (IT), IM-o-TOF, orbitrap and FT-ICR. All analyzers that can function in MS² mode are suitable for lipids. The ion trap instrument does not result in high precision of the mass data, but it allows for MSⁿ fragmentation sequences and easy identification via structural elucidation. Conversely, FT-ICR provides highly resolved peaks with a high precision, but the instruments themselves remain very expensive and difficult to use. Recently, imaging and the direct identification of lipids were performed using an LTQ-Orbitrap instrument, leading to the detection of lipids with a very high mass-resolving power. Moreover, MSⁿ experiments were performed with a sub-ppm mass accuracy, allowing for a more accurate assignment of the lipids (Römpp et al., 2010).

4.2.7.3. Imaging of drugs. Imaging techniques for small exogenous compounds, such as drugs, have recently been developed (Garrett et al., 2007; Schwartz et al., 2003; Reyzer et al., 2010; Bonnel et al., 2011a). Drugs are compounds that generally yield good MALDI data, although the same analytical problems observed for lipids can also be encountered with drugs. For drugs, the major difficulty is interference due to the matrix ions because the matrix peaks sometimes completely overlap with the drug peaks. Therefore, MS² is required to determine which part of the signal should be attributed to the drug. However, the ease of MALDI-MSI and its capacity to detect, identify and image both drugs and metabolites largely outweighs these analytical difficulties.
4.2.7.4. Imaging of oligonucleotides. Oligonucleotides are also very difficult to analyze using MALDI because they contain phosphate groups that complex with salts and induce a high instability during ionization. This situation leads to the observation of large and very weak peaks, and the signal intensity decreases significantly as the oligonucleotide mass increases. Under such conditions, the imaging of mRNA using MALDI is compromised, although some progress was made in this field by studying oligonucleotides under infrared (IR)-MALDI conditions.

4.3. One-point Images: Identification the Major point

The direct identification of biomolecules is the key to increasing the potential of MALDI-MSI. The most straightforward strategy consists of identifying molecules directly from tissue sections without the need for any procedures to extract and separate the molecules. In the case of small compounds including lipids and drugs, in situ identification can easily be achieved with the use of devices that provide high resolving power and MSn capabilities. However, strategies for the direct identification of proteins have yet to be developed. The ideal procedure would involve the fragmentation of an intact protein on a time scale compatible with the mass spectrometer. For example, using an FT-ICR instrument equipped with an electro spray ionization (ESI) source, an electron capture dissociation (ECD) process (McLafferty et al., 1998; Zubarev et al., 2000) could fragment the intact protein, resulting in a highly charged state that is not compatible with MALDI sources in which even ions generated from proteins have a low charge.

Considering the instrumental specificities of the MALDI-TOF instrument, in-source decay (ISD) (Reiber et al., 1998a; Reiber et al., 1998b) is the only approach that allows for “Top-down” experiments to be performed. The second strategy requires the development of an in situ enzymatic digestion using micro-spotter, thus allowing for a “Bottom-up” experiment (Fig. 4).

4.3.1. Top-down strategy

Fragmentation along the protein backbone in the MALDI source was first observed by the team of Brown and Lennon (see: Reiber et al., 1998a; Reiber et al., 1998b). The time scale between the ionization of the proteins and the extraction of the proteins from the source is large enough for the proteins to fragment and form z and c fragment ions, according to Roepstorff’s nomenclature (Roepstorff and Fohlman, 1984). The N or C-terminal moiety of the protein is then easily achieved; therefore, databank interrogation can be used to identify the corresponding protein. The main drawback of in source decay (ISD) is the lack of selectivity of the precursor ion. For this reason, the protein of interest must be purified to avoid detection of ISD fragment ions from several proteins in the same mass spectrum. However, because ISD fragment ions are detected as intact ions, a pseudo MS3 analysis of these fragments, called T3 sequencing (Raska et al., 2002), can be performed, thus allowing the N-terminal or the C-terminal moiety of the protein to be sequenced. Moreover, this strategy can be adapted for MALDI-MSI experiments in which a mixture of proteins is detected on each pixel. The choice of the matrix is a very important parameter for the success of an ISD experiment. In fact, ISD involves the transfer of a radical proton from the matrix
to the proteins, and 2,5-DHB was found to be efficient in performing this function (Reiber et al., 1998a). Recently, Demeure et al. (2007) showed that 1,5-diaminonaphthalene (1,5-DAN) was more efficient for ISD experiments because it provided a better fragmentation yield. Very recently, Debois et al. (2010) introduced the in situ identification of proteins directly from porcine eye lens and rat brain tissue sections using the ISD strategy. The beta-crystalline B2 protein was identified directly from a porcine eye lens by investigating the ISD fragment ions following data bank interrogation and T3 sequencing. Although software has recently been developed for on-tissue ISD identification (Demeure et al., 2011), this strategy suffers from an inability to study FFPE tissues containing cross-linked proteins. In contrast, the Bottom-Up strategy allows for the detection and the identification of proteins regardless of the mode of preservation used.

4.3.2. Bottom-up Strategy

The second approach that allows for the direct in situ identification of proteins is based on the classical Bottom-Up strategy. Basically, a solution of the enzyme is deposited on a region of interest or on an entire tissue section using a micro-spotter. Peptides are then generated from the digestion of proteins, allowing for the localization and the identification of the corresponding proteins after the MALDI-MSI and MS² experiments. This strategy,
introduced by Lemaire et al. (2007a), has been used to detect and identify proteins directly from FFPE tissue sections. Caprioli et al. (see: Groseclose et al., 2007) improved the procedure using a micro-spotter, thus leading to a better and more reproducible application of trypsin on a fresh rat brain tissue section. Several teams have used this procedure for clinical applications, including cancer research (Groseclose et al., 2008; Ronci et al., 2008; Djidja et al., 2009) and animal models of Parkinson disease (Stauber et al., 2008) from FFPE tissue sections. To date, the Bottom-Up strategy has been clearly demonstrated to be able to retrieve information from FFPE tissues. However, many efforts have been undertaken to improve the analysis of FFPE tissues that are difficult to process using in situ enzymatic digestion because of the residual hydrophobicity of the FFPE tissue after paraffin removal. Several strategies involving antigen retrieval were then developed and resulted in an improvement of the in situ enzymatic digestion of FFPE tissues (Gustafsson et al., 2010) and therefore, the detection and the identification of proteins.

However, due to the presence of many fragment ions from different precursors after MS² experiments, the corresponding protein is sometimes not identifiable using databank interrogation. Moreover, a mixture of digested proteins is detected at each position; therefore, no clear peptide mass fingerprint (PMF) can be observed from any specific protein. This result implies that the identification of proteins is based on the fragmentation of one or two peptides without any PMF. This result may explain why the corresponding protein is sometimes not clearly identified. To overcome this drawback, an in situ N-terminal derivatization strategy was recently introduced after in situ enzymatic digestion to orient the fragmentation toward a unique series (Franck et al., 2009c). Using this approach, the MS² spectra are easier to interpret, and the protein assignment is greatly improved. It was shown that N-succinimidyloxycarbonylmethyl-tris (2,4,6-trimethoxyphenyl) phosphonium bromide (TMPP) is a better derivatization candidate because it allows for a rapid N-terminal derivatization of tryptic peptides at room temperature. This reagent leads to the detection of a strong ai+ series of fragment ions after MS² experiments.

4.3.3. Liquid-microjunction micro-extraction strategy

A novel strategy consists of combining MALDI-MSI and LC-MS/MS in a single workflow to improve protein identification. Basically, proteins are digested in situ and then extracted prior to nano-LC separation, which is followed by an MS² analysis for databank interrogation (Stauber et al., 2008). This approach has demonstrated clear improvement in the number of identified proteins, but it was found to result in the loss of information about the localization of certain identified proteins because it is performed at the level of one or half of a tissue section. It was recently shown that intact proteins could be extracted from a tissue section prior to fractionation by ultracentrifugation. Moreover, in combination with high-resolution and very high-accuracy mass spectrometry, this approach has allowed for the detection of approximately one hundred proteins (Schober et al., 2011). A combination of protein extraction from tissues and MALDI-MSI data using various proteomics-based approaches, including Electron-transfer dissociation (ETD) fragmentation on intact proteins, was used for clinical applications in oncology and has led to the identification of several markers (Rauser et al., 2010; Elsner et al., 2012; Nipp et al., 2012). It is clear that these strategies are helpful for protein identification, but information about the localization of the proteins within the sample is lost. Moreover, the proteins of interest may be
diluted in the complex extracts, which contain large amounts of abundant proteins. This problem is especially true if the region of interest is small in comparison to the size of the sample. Thus, there is a crucial need to develop a method that combines MALDI-MSI and LC-MS/MS to retain spatial information and avoid the dilution of low-abundance proteins. Recently, surface sampling using liquid microjunctions was introduced, demonstrating a possible liquid-microjunction micro-extraction technique that can be coupled to any ionization source (Van Berkel et al., 2008). The success of these methods as alternatives to typical ambient surface sampling/ionization sources is due to their greater extraction efficiency and larger surface sampling areas. For example, it has been shown that the metabolites of a drug could be detected using liquid-microjunction extraction, whereas these same metabolites were not detected using desorption electrospray ionization (DESI) (Kertesz and Van Berkel, 2010a). In a method called liquid extraction surface analysis (LESA) (Kertesz and Van Berkel, 2010b), the solvent can be brought into contact with the sample surface as a single droplet whose size and formation can be controlled by the instrument. Because the sample extraction is independent of the ionization process, the liquid extraction surface sampling techniques permit further treatment of samples using coupled devices prior to their introduction into the mass analyzer. Currently, LESA has been demonstrated to be useful for the quantification of drugs on dried blood spots (DBS), the profiling of the total drug distribution in whole body tissue sections, and the extraction and quantification of samples on MALDI spots (Kertesz and Van Berkel, 2010b). This technique has also recently been used to directly and rapidly identifies intact hemoglobin variants from DBS samples that are used for the neonatal screening of sickle cell and other hemoglobin-related diseases (Edwards et al., 2011). LESA has also been used to correlate the results obtained from MS imaging experiments (Marshall et al., 2011). Our group recently developed a new strategy to identify proteins from tissue sections that involves correlation of the spatial localization of the proteins based on liquid micro-junctions formed after on-tissue extraction and off-tissue LC-MS and MS² analyses. Specifically, we have evaluated the on-tissue enzymatic digestion of proteins with discrete localization, and this was followed by tissue micro-extraction and subsequent analysis using on-line nano-LC-ESI MS under high-spectral-resolution conditions. The results show that we can identify 1781 proteins from an original digestion spot of approximately 300 μm, which corresponds to 500-700 cells. All of the investigations were conducted using a MALDI-LTQ Orbitrap that provides a very high mass accuracy (<2 ppm), which is an important parameter for the unambiguous identification of proteins using the shotgun proteomics approach. This strategy will be integrated into the MALDI-MSI workflow to perform supervised profiling identification using defined cell groups with specific molecular profiles. This new strategy is more robust and faster than the laser capture microdissection (LCM) strategy and leads to the identification of a larger number of proteins with respect to the number of cells studied (Fournier et al., unpublished data, Fig. 5).

5. Photocleavable link and mass spectrometry

Biological processes involve many different signaling pathways from various classes of molecules. Multiplex techniques are necessary to provide tools for the diagnosis and
Fig. 5. The liquid junction micro-extraction procedure for identifying peptides and proteins from tissue sections.

A: Protein identification from two different location within the rat brain section, i.e., thalamic and cerebellar nuclei.

B: On-tissue micro-extraction strategy for the identification of biomolecules using liquid micro-junction surface sampling. The micro-extraction consists of 5 steps: (1) solvent sampling, (2) solvent dispensing on the tissue section via the liquid micro-junction, (3) analyte extraction via liquid micro-junction to the tissue, (4) sampling of the extracted molecules, and (5) dispensing of the extract into a tube. Steps 1-5 can be reiterated several times to reach the optimized extraction yields.

C: Comparison of the MALDI-TOF MS spectra recorded in the linear positive mode from 2 consecutive sagittal tissue sections from rat brains at two distinct locations in two regions (1, 2) of the tissue after micro-extraction under the optimized conditions for the tryptic digestion peptides: a) micro-extraction of the undigested areas and b) micro-extraction after on-tissue digestion on 300-µm spots. The black circles indicate the micro-extraction areas that are approximately 771 µm in diameter, and the red circles define the position of the 300-µm trypsin digestion spots.

prognosis of diseases. Tissue microarrays (TMAs) are being used more frequently to analyze a large number of diseased tissues, but new, fast and reproducible multiplex techniques are still indispensable (Walch et al., 2008). However, such technologies do not allow for the correlation of mRNA expression with the corresponding protein regulation, i.e., the correlation of the transcriptome with the proteome, which is of special interest for increasing our understanding of biological mechanisms. This correlation is an essential aspect of an analysis when studying pathologies to achieve earlier diagnosis by taking into account
the non-coding RNAs that are emerging as important mediators of epigenetic regulation. Significant effort has been devoted to screening for miRNA signatures and identifying functional and clinical links between non-coding RNA expression patterns and various normal and disturbed physiological states (Diao et al., 2010; Enkelmann et al., 2011). However, some specific classes of biomolecules, such as oligonucleotides, are still barely accessible for direct analysis using MALDI. Ideally, oligonucleotides should be directly detected from tissues, although their large size, low abundance in cells and the associated salt adducts and gas phase instability render their analysis difficult (Nordhoff et al., 1994). To circumvent these problems, Olejnik et al. (1999) developed a new approach that combines the affinity purification and MALDI-MS analysis of biomolecules based on the use of photocleavable linkers. This method relies on the design of photocleavable linkers (PC-linkers) that can be incorporated into the target molecule either chemically (Olejnik et al., 1996, 1998a; Lemaire et al., 2005) or enzymatically (Olejnik et al., 1998b) using transcription (Hahner et al., 1999; Olejnik et al., 1999) or PCR (Stauber et al., 2009; Stauber et al., 2010). PC-linkers can be efficiently cleaved photochemically to release the target analyte with the aid of the near-UV desorption/ionization laser pulses used for MALDI (Fig. 6).

6. Targeted MALDI Mass Spectrometry Imaging (MSI)

Our team has proposed a new concept using PC-linkers for the multiplex, specific detection and localization of biomolecules. We have especially focused on mRNA and proteins for transcriptome/proteome correlations using MALDI-MSI (Lemaire et al., 2005; Fournier et al., 2006; Stauber et al., 2006). This concept, called Tag-Mass, relies on affinity detection using a specifically designed probe that is later detected using mass spectrometry (Lemaire et al., 2007c). Tag-Mass offers more selectivity and higher sensitivity during MALDI-MSI to specifically track known markers of physiological stages in collections of samples (Stauber et al., 2006; Lemaire et al., 2007c; Franck et al., 2009b). The Tag-Mass method is an affinity-based strategy wherein a probe is directed against a specific target. The probe bears a reporter group (Tag) that can be later imaged using MALDI-MSI to report the localization of the probe (Stauber et al., 2006; Lemaire et al., 2007c; Franck et al., 2009b). The reporter is designed to be a molecule of known molecular mass that is easily detectable under MALDI conditions but that does not correspond to an endogenous compound. To indirectly image a probe, the reporter must be linked to the probe and released in the final step immediately prior to or during the MALDI sequence. During Tag-Mass, the reporter group, which involves a photocleavable moiety that binds the reporter to the probe, is released via photodissociation as a result of the MALDI laser irradiation (Fig. 7A). Thus, the reporter is detached from the probe during the MALDI-MSI acquisition. Many different reporters can be used for this purpose, but peptides were often used. The photocleavable linker is selected to present a specific absorption band in the UV range at a wavelength (340 nm) that is very close to that of MALDI lasers (i.e., 337-355 nm). Thus, after hybridization of the modified probe to its target, a classical MALDI-MSI sequence is performed. At a specific point during the acquisition, the presence of a probe will be indicated by the presence of the reporter that was released due to the MALDI laser irradiation, which is evident by the observation of a peak at the m/z value expected for the reporter (Fig. 7B). Reconstruction
Fig. 6. An outline of the photo-cleavage matrix-assisted laser desorption/ionization (PC-MALDI) method. Step 1: A reaction mixture containing DNA modified with a photocleavable affinity tag (T) is applied to the affinity support (S) and selectively bound; Step 2: Washing with a MALDI-compatible buffer removes contaminants and salts; Step 3: A UV light source, such as a UV-MALDI laser, cleaves the analyte, which is then analyzed in the mass spectrometer (Olejnik et al., 1999).

of the reporter molecular image then yields the image of the probe, i.e., the image of the targeted molecule (Stauber et al., 2006; Lemaire et al., 2007c; Franck et al., 2009b). Such a concept is compatible with all types of probes including mRNA probes, antibody probes, lectins and aptamers, which can be used to selectively obtain images of mRNA, antigens, oligosaccharides (including glycosylated proteins) and drugs, respectively, as targets. In the Tag-Mass workflow, MALDI-MSI is combined with hybridization techniques including in situ hybridization (ISH) and IHC (Stauber et al., 2006; Lemaire et al., 2007c; Franck et al., 2009b) (Fig. 8). The procedures for imaging different targets (primer, antibody, dUTP) are presented in the legend of Fig. 8.
Fig. 7. A: Schematic representation of the reporter released via photodissociation as a result of irradiation from the MALDI laser using a photocleavable-reporter system coupled to the probe. B: Scheme of the photocleavable linker/tag system for indirect detection following photodissociation via the MALDI UV laser. Specific tagged probes (see inset for the structure of the deoxyuracil-tagged nucleotide that was used to amplify the tagged riboprobe for in situ hybridization (ISH) to access the transcriptome) were hybridized to the tissue before the linker that was coupled to the reporter (PC-peptide) was released by photocleavage upon irradiation from the MALDI laser. The PC-peptide was then detected using mass spectrometry. Different reporters or different targets can be used simultaneously to perform multiplex analyses.
Fig. 8. Workflow of the multiplex-specific MALDI MSI (Tag-Mass) method using either a tagged primer at the 5′ end, tagged riboprobes containing tagged-dUTP, a tagged-antibody to study the peptidome and proteome, a tagged lectin to study the glycome, or a tagged aptamer for drug or metabolite targeting. The figure represents a molecular image of mannose and glucose in ovarian cancer using tagged-lectin (concavalin A type IV). The mass spectrum in the inset indicates the detection of the PC-peptide couple following the on-tissue laser shots. The procedure for the synthesis of these different targets is as follows: Photocleavable tagged oligonucleotide. The peptide was synthesized using a Symphony system (Protein Technologies, Inc.) and purified on a Delta-Pak C18, 15 μm, 100 Å column (Waters). The oligonucleotide was synthesized from the 3′ end to the 5′ end using an Expedite instrument (Applied BioSystem). The amine functional group with the photocleavable linker was added to the 5′ region before cleavage and deprotection. These steps were performed using a 28% NH₄OH solution over 24 h in the dark. The amino oligonucleotide was then purified on a Delta-Pak C18, 15 μm, 300 Å column (Waters). The amino moiety of the oligonucleotide was coupled to a heterobifunctional reagent comprising a maleimide moiety. The maleimido oligonucleotide was solubilized in water and added to 1.2 equivalents of peptide in solution. The mixture was stirred for 16 h. The oligo-peptide conjugate was then purified on a Delta-Pak C18, 15 μm, 300 Å column (Waters) and characterized using MALDI-MS.

Photocleavable tagged antibody. The peptides were custom-made by Eurogentec S.A. using solid-phase peptide synthesis (SPPS) on a 0.25 mmol scale using Fmoc (9-fluorenylmethyloxycarbonyl amino-terminal protection) standard synthesis protocols (4 equiv. of Fmoc-AA) with double-coupling reactions (two reactions for 40 min each) using TBTU/NMM, which is used as an activator on a Symphony synthesizer (Rainin Instrument Co., Woburn, MA). The photocleavable linker (4 equiv.) was introduced manually using DIPCDI/DIPEA (2 h) as an activator. The product was purified using RP-HPLC on a Waters (Milford, MA) Delta-Pak C18 [15 μm, 100 Å, 25 mm × 100 mm] column using a Waters liquid chromatography system consisting of a Model 600 solvent delivery pump, a Rheodine injector and an automated gradient controller (Solvent A,
Fig. 8 (Continued). H₂O/0.125% TFA; Solvent B, CH₃CN/0.1% TFA; Gradients, 5-15% to 30-60% B over 20 min). The products were detected using a Model M2487 variable wavelength UV detector connected to the Waters Millennium software control unit. Quality control was performed using an analytical RP-HPLC system with a Waters Delta-Pak C18 [5 μm, 100 Å, 150 mm x 3.9 mm] column (Solvent A, H₂O/0.125% TFA; Solvent B, CH₃CN/0.1% TFA; Gradient, 100% A to 60% B over 20 min) using a Waters Alliance 2690 Separation Module equipped with a Waters 996 Photodiode Array Detector and a MALDI-TOF MS. The antibody was functionalized with the photolinker-derivatized peptide A as follows: a solution of 0.5 mg of MBS in 300 μL of DMF was added to a solution of 4 mg of goat anti-rabbit IgG in 2 mL of PBS and mixed for 30 min. The solution was then desalted on a PD 10 column using 50 mM phosphate buffer at pH 6. To this desalted activated IgG, a solution of 1 mg of the photocleavable-derivatized peptide in 300 μL of DMF and 1 mL of PBS was added and stirred for 3 h at room temperature. The reaction mixture was then dialyzed overnight against PBS (membrane cutoff 12-14 000).

Synthesis of dUTP-peptide conjugates with a photocleavable linker. To prepare this triphosphate, an Fmoc-protected CPG resin was required. The succinylate was prepared from GT115A (100 mg). The sample was relatively pure but contained a small amount (by TLC) of a higher mobility nontritylated compound. Because it was not possible to purify the succinate, the reaction was modified slightly. A 1.5 equiv. aliquot was used because the exact purity of the product was undetermined. Based on a comparison of the intensity of the components on a TLC plate (visualized by UV irradiation at 254 nm) and the intensity of the DMT cation upon treatment with HCl fumes, it was concluded that the reaction did not proceed to completion (the completion was more than 50% as determined by TLC). Since the nonsuccinyalted product would not react, the this mixture. The resin was prepared, but the loading was very low (5.4 μmol g⁻¹, 180 mg). The resin was detritylated using 2% TCA/DCM and washed with DCM until the DMT cation was completely removed. The resin was then washed (under argon) and soaked in 1:3 pyr/DMF (0.4 mL) for 5 min before a 0.1 M solution of Eckstein’s reagent in dioxane was added (0.1 mL). The reaction was allowed to stand for 15 min. The resin was then washed (dioxane, MeCN) and dried (under argon). The resin was again soaked in a solution of 0.5 M bis(tributylammonium)pyrophosphate in anhydrous DMF and tri-α-butylamine for 20 min, and the resin was then washed (DMF, MeCN) and dried (under argon). The product was oxidized (iodine/water/pyridine/THF for 30 min), washed (MeCN) and dried (under argon). The product was then washed with DCI, after which a solution of DCI/photolabile amino linker CEP (1:1, 0.5 mL) was added, and the reaction was allowed to stand for 20 min. The solution was removed, and the resin was washed (MeCN) and dried (under argon). A mixture of cap A/cap B (1:1, 0.5 mL) was added, and the resin was washed for 5 min before removing the capping reagents and washing and drying the resin as described above. The product was oxidized (I₂/THF/pyr/H₂O, 5 min), and the resin was washed and dried as described above. The product was then cleaved from the resin with cNH₂OH at room temperature for 30 min and purified using anion exchange HPLC on a Dionex NucleoPac100 HPLC column with the following solvent system: Buffer A, 0.1 M NH₄Cl with 10% acetonitrile; Buffer B, 1 M NH₄Cl with 10% acetonitrile; flow rate 2.5 mL/min using 6Triphos.mth. This purification yielded 3 fractions (A, 7 min; B, 7.9 min; and C, 10.3 min). All 3 fractions were lyophilized overnight before being desalted by reverse-phase (RP) HPLC using the following solvents: Buffer A, water; Buffer B, acetonitrile; flow rate 4 mL/min. The 3 fractions were again lyophilized overnight before being suspended in 200 μL of water. An MS analysis indicated that CMM661A pk 1 was definitely not the desired triphosphate, but either CMM661 pk 2 or 3 might be the desired product (both exhibited very similar MS profiles). CMM662A was formed from
6.1. Tag-Mass MSI vs. TAM-SIM

Two types of photolinkers and reporters developed by two different teams.

6.1.1. Tag-Mass MSI

Our team developed the Tag-Mass concept in 2004 based on the 4-[4-[1-(Fmoc-amino)ethyl]-2-methoxy-5-nitrophenoxy]butanoic acid (photocleavable linker) coupled to a known peptide such as bradykinin (Figs. 7 and 8). Tag-Mass can also be used for semi-quantification under multiplex conditions if a reporter presenting the same physicochemical properties, i.e., the same analytical behavior, is used. For example, isotopically labeled reporters, such as differentially deuterated peptides, can be used. This concept can be extended by seeking alternate methods of releasing the reporter moiety, e.g. chemically induced release or prompt fragmentation pathways (i.e., before the end of the delay time period). The Tag-Mass concept was successful at detecting high-mass membrane proteins such as carboxypeptidase D (Fig. 8), ovarian biomarkers in ovarian cancer in multiplex (Franck et al., 2009b; El Ayed et al., 2010) and intracellular enzymes (cathepsins-cystatins) in leech immune cells (Stauber et al., 2010). Such a photocleavable linker was used to develop tagged primers that can be used in PCR (Lemaire et al., 2007c). A second generation of tagged oligonucleotides has been generated and has allowed for the synthesis of modified uracil nucleotides (tagged-UTP) that can be easily used in PCR or for riboprobe synthesis. This tagged-UTP requires a specific synthesis to keep both the 3′ and 5′ termini of the nucleotide bases free. Thus, the modified nucleotide can be used during the probe amplification process. Previous experiments using modified primers, which were directly modified by the addition of a photocleavable-reporter system, were used to detect proenkephalin expression in rat brains (Fig. 8) (Lemaire et al., 2007c; Stauber et al., 2009; Stauber et al., 2010). This approach revealed several disadvantages, including a lack of sensitivity (only one reporter per probe), a high cost (a specific synthesis is required for each mRNA to be localized) and the inability to amplify the probe via in vitro translation (because of the blockage of one terminus of the primer by the tagging agent). The development of modified uracil nucleotides is thus a significant advance. Modified nucleotides are available for the construction of all probes; the sensitivity of the probe is increased by the incorporation of several reporters in the probe sequence (amplification of the signal), and probes can be obtained via in vitro translation. Moreover, this method can be applied to both transcriptome and proteome molecular imaging in the same tissue section using classical MALDI-MSI. A comparison between classical MALDI-MSI and tagged-multiplex

Fig. 8 (Continued). CMM661A pk 2, and CMM663A was formed from CMM661A pk 3. Both samples were then used in the subsequent reaction. A bicarbonate buffer (10 μL) and the maleimide NHS ester (50 μL) were added to each sample, and the reactions were agitated overnight. The samples were diluted with milliQ water (500 μL) and filtered. The samples were purified by RP-HPLC (buffer A, 0.1 M TEAA; buffer B, MeCN; flow rate 4 mL/min) with MeCN50.0. The peptide was coupled in these fractions.
Fig. 9. Workflow of multiplex TAM-SIM.

A: Four different primary antibodies were incubated with the sample to form complexes with the antigen in the tissue. Secondary antibodies, each carrying a specific mass tag, were specifically attached to each primary antibody. The slide containing the tissue section was mounted on a target plate and introduced into the source of the mass spectrometer. A pulsed UV laser cleaved the tags from their antibodies and released them into the gas phase. The m/z values of the antibodies were determined using a time-of-flight analyzer. The acquisition of the peak intensities over thousands of spots and mass spectra were used to reconstitute images at specific molecular weight values, each of which corresponds to the localization of a specific antigen. This procedure is identical to that used for Tag-Mass except for the fact that Tag-Mass uses matrix to amplify the signal to achieve better sensitivity, and Tag-Mass is a MALDI-MS procedure, whereas TAM-SIM does not use a matrix but instead uses laser desorption/ionization mass spectrometry. B: Conjugation of a mass tag to an antibody, photocleavage of the mass tag-conjugated antibody, and laser desorption. The tagging reagent contains an NHS-ester as the reactive group for covalent attachment to the primary amine groups of an antibody. In the mass spectrometer, the trityl groups absorb UV light, resulting in the cleavage of the C–S bond, creation of a stable carbocation and release of the tag.

MALDI-MSI (Fig. 9) reveals the complementarity of these techniques. This new development now makes it possible to track mRNA or nonsense mRNA, such as miRNA, which are normally difficult to analyze using MALDI.

6.1.2. TAM-SIM

The second reporter designed to be observable under LDI conditions, TAM-SIM, was described in 2007 (Fig. 9) and is based on an N-hydroxysuccinimide (NHS) linker coupled to 2,5-dioxopyrrolidin-1-yl-3-[(3-(6-(tertbutylamino)-6-oxohex-1-ynyl)-4-methoxyphenyl)
Fig. 10. Image comparison between Tag-Mass and TAM-SIM.
The molecular images presented here that were obtained using Tag-Mass incorporated both the relevant tagged antibody and tagged dUTP. One image corresponds to the molecular image obtained using a tagged antibody. A molecular image of a 160-kDa membrane protein corresponding to the carboxypeptidase D localized in the rat brain is presented in A. B and C correspond to the transcriptome of the proenkephalin in the rat brain using either PC-tagged dUTP or deoxygenin dUTP in a mouse brain obtained from the brain atlas. A similar localization is observed in the two pictures. D corresponds to a TAM-SIM image of cells that were immunoreacted with a polyclonal anti-synaptophysin antibody in a healthy human pancreas using a monoclonal rabbit anti-synaptophysin conjugated with the tag, which was detected at 498 m/z. The false-colored green areas in the section indicate the presence of the tag, thus indicating synaptophysin-positive cells. E & F correspond to images of cells that were immunoreacted with a monoclonal rabbit anti-human somatostatin antibody (E) or a multiplex of 3 antibodies (anti-calcitonin, anti-somatostatin and anti-synaptophysin) (F) in the Islets of Langerhans using TAM-SIM technology.
Fig. 11. Comparison between MALDI-MSI, Tag-Mass imaging and LA-ICP-MSI.

A. A molecular image obtained using MALDI MSI of a specific biomarker detected in ovarian cancer, the C-terminal fragment of the immunoprotease activator PA28 alpha. The red color (a) corresponds to the ion at m/z 9744 in the tumor biopsy. The inset (b) corresponds to the detection of this biomarker at m/z 9744 using Tag-Mass MSI with an antibody directed against this biomarker in ovarian cancer (green color). (c) A comparison between Tag-Mass MSI and HES staining in a zoomed region. (d) HES staining of the ovarian cancer biopsy section.

B. Immunohistochemical staining of 3 μm breast cancer tissue sections, which are positive for Her2 (scale bar, 2 mm), using a holmium-labeled antibody and analyzed using LA-ICPMS. Laser spot size, 200 μm; scan speed, 150 μm s⁻¹; repetition rate, 10 Hz; laser energy, 35%.

present on the surface under study. For example, gold-labeled secondary antibodies are useful for imaging antigens via LA-ICP-MS at a spatial resolution below 10 μm. The ionization techniques used for this method result in significant fragmentation yields, and the reporter element appears as a fragmentation product.

6.2. Tag-Mass MSI vs. LA-ICP-MS

Gold clusters can be detected using LA-ICP-MS. Compared with single atoms, metal clusters increase the sensitivity based on the number of metal atoms in the cluster. Thus, gold cluster-labeled antibodies similar to those used in electronic microscopy immunogold technology (Molinari et al., 1984; He et al., 2001) can also be used in LA-ICP-MS (Becker et al., 2009; Becker et al., 2010) (Fig. 11). This method is suitable for any protein presenting an epitope that is recognized by an available primary antibody. The applications of this
technique include transgenic mouse models of Alzheimer’s disease (Hutchinson et al., 2005) and cancer (Seuma et al., 2008). Multiplex analyses have also been performed (Seuma et al., 2008). In fact, the distribution of several breast cancer-associated proteins (Her 2, CK 7, and MUC 1) has been studied using multiple line rastering of tissue sections and the detection of relevant lanthanide-tagged antibodies bound to the tissue (Giesen et al., 2011). Compared with optical microscopy, the LA technique offers extremely high sensitivity and sufficiently good resolution to make fine scale feature mapping at the cellular level possible (Seuma et al., 2008). The size of the laser spot is 200 µm in LA-ICP-MS (Becker et al., 2009) and 20 µm (corresponding to the size of a cell) in MALDI-MS and LDI-MS (Thiery et al., 2008) (Fig. 11).

7. Discussion

Fig. 11 shows that the three technologies discussed above are complementary. LA-ICP-MS provides an overview of the localization of a specific target, whereas MALDI-MSI and LDI-MSI provide localization information at the cellular level. Tag-Mass or TAM-SIM images can be clearly correlated with histological staining. The difference between these techniques is in the level of the signal intensity and the multiplex ability. Significantly, the Tag-Mass signal is higher than that of TAM-SIM due to the greater sensitivity of the matrix-assisted ionization. In fact, Tag-Mass can be used to track low-abundance proteins or to investigate some proteins involved in biological processes or mechanisms. Moreover, Tag-Mass technology presents the ability to perform highly multiplexed studies due to its ability to incorporate a variety of tag-like peptides or inorganic compounds (e.g., an 8 poly (amidoamine) dendrimer with 1024 amino groups (Yang et al., 2012)). Conversely, TAM-SIM multiplexing is limited to the rare monoatomic elements that are available. MALDI matrices and coumarins can also be used with the Tag-Mass method. Cellular localization can now be achieved using Tag-Mass as a result of the novel development by Spengler and co-workers of the SMALDI approach (Römpp et al., 2010). Laser spots smaller than 5 µm can be achieved, and the use of this technology with Tag-Mass MSI will provide access to minor proteins with a very high spatial resolution and a high accuracy at the single cell level (Fig. 11). Another advantage of the Tag-Mass technology is the possibility to change the linker type according to the nature of the target under investigation. In fact, tagged deoxy-nucleotides can be used in PCR or qPCR for transcriptomics, antibodies can be used to detect antigens, lectins can be used for glycomics, aptamers can be used to detect drugs, ligands can be used to detect receptors and enzyme substrates can be used for enzyme tracking, as recently demonstrated by Caprioli and colleagues (Yang et al.). Clear results have already been obtained for transcriptomics, glycomics and the detection of antibodies using the Tag-Mass technology (Lemaire 2005; Fournier et al., 2006; Stauber et al., 2006; Lemaire et al., 2007b; Lemaire et al., 2007c; Franck et al., 2009b; Stauber et al., 2009; El Ayed et al., 2010; Stauber et al., 2010). An on-tissue microarray can be performed to simultaneously obtain molecular information. Targeted-MSI revolutionized the pharmacological domain with the introduction of whole-body MSI, which makes on-tissue target quantification possible. Targeted MSI can also be used to complement MALDI-MSI, especially for clinical research. Fig. 11 shows a good example of this complementarity.
Using a potential ovarian cancer biomarker, the C-terminal fragment of the activator of the immunoproteasome PA28 alpha, it is possible to detect the protein and the mRNA encoding this protein in the same tissue section. This result reveals that the level of transcription is related to the abundance of the protein fragment. These data reflect the fact that cross-validation between the transcriptome and the proteome can be performed on the same tissue. Thus, this novel technology associated with classical IHC offers the ability to obtain a quick answer and a better molecular diagnosis for clinical surgery. Research using click chemistry to develop a novel generation of Tag-MASS reporters and linkers associated with novel signal amplification technology is now in progress. Moreover, this technology can also be applied to clinical diagnosis using enzyme-linked immunosorbent assays (ELISA) or PCR for biomarker quantification (Lemaire et al., 2007c; Stauber et al., 2009; Stauber et al., 2010). Several companies have recently developed their own “Tag-Mass” technology, such as the MassCode PCR available from Agilent or the isobaric TNT mass tags available from Trillion. This newly developed technology will soon be available in laboratories and will likely have significant impacts on the diagnosis and prognosis of diseases and on patient compliance.

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