CHAPTER 15

BIOPHOTONICS APPLIED TO PROTEOMICS

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Abstract: Since the completion of the human genome sequencing, our understanding of gene and protein function and their involvement in physiopathological states has increased dramatically, partly due to technological developments in photonics. Photonics is a very active area where new developments occur on a weekly basis, while established tools are adapted to fulfill the needs of other disciplines like genomics and proteomics. Biophotonics emerged at the interface of photonics and biology as a very straightforward and efficient approach to observe and manipulate living systems. In this chapter, we review the current applications of photonics and imaging to proteomics from 2D gels analysis to molecular imaging.

1. INTRODUCTION

The complete set of genes of an organism composes its genome, while the complete set of proteins of a cell or tissue constitutes its proteome. Whereas the genome of cells remains stable through their lifetime, the cellular proteome changes in response to the

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stimuli from its environment. Since the sequencing of the human genome, we have access to lists and databases of protein sequences encoded by genes. From these, we know the repertoire cells have to build their protein machinery. In most cases, we do not know what drives gene expression or how genes intervene in a physiopathological context. Proteomic analysis makes it possible to follow the quantitative variations of cellular protein expression through processes like tumorigenic transformation or in response to chemical and pharmacological agents. Thereby, proteomic analysis encompasses the whole set of complex interactions that occur within cells or tissues. The analytical instrumentation has to resolve and characterize protein isoforms that are due to genetic variations or post-translational modifications. Post-translational modifications like phosphorylation and proteolytic cleavages are essential to modulate the activity of proteins. One of the purposes of proteomic analysis is precisely to take the post-translational modifications into account. The characterization system relies mainly onto mass spectrometry and biophotonics technologies like MS Imaging, while the proteomic platform is based on two-dimensional electrophoresis techniques which are the only one to possess a sufficient resolution to distinguish post-translational variants.

2. TWO-DIMENSIONAL ELECTROPHORESIS

Two-dimensional electrophoresis was introduced about 30 years ago (O'Farell 1975), at this point, its separation power is still unequaled. Many polypeptidic species observed as distinct spots on the gel are the products of a single gene. This diversity is mainly due to post-translational modifications like cleavages or the addition of some chemical functions on specific amino-acids. As a rule, a chemical modification of an amino-acid is much more likely to affect the isoelectric point of a protein than its observed mass. In the case of phosphorylation and glycosylation, the native form of the protein and its modified form will appear as two separate spots on a two-dimensional gel. Other post-translational modifications like cystein acylation do not affect noticeably the physico-chemical parameters and are not detected by classical electrophoresis. The performance of isoelectric focusing in terms of separation (first dimension of two-dimensional electrophoresis) is constantly improving. Remarkable progresses have been achieved thanks to the introduction of immobilized pH gradients (Bjellqvist et al. 1982; Görg, et al. 2000) and the development of narrow-range high resolution pH gradients (Hoving et al. 2000, 2002). Allelic or post-translational variants that were previously invisible in classical electrophoresis are now easily resolved by isoelectric focusing on narrow-range gradients, even in very basic ranges. In a similar manner, progresses in protein extraction and solubilisation (Rabilloud 2000) have considerably increased the width of 2Dim electrophoresis analysis.

The main challenge in the area is to visualize as many proteins as possible while maintaining the density of polypeptidic spots in two-dimensional gels at a manageable level. We have to take into account that each gel maps the protein content of a single sample and that the goal of most experiment in proteomic analysis is to compare several samples, that is, several gels.

Polypeptidic spots located at the same position across a set of gel also share the same identity, as long as the spot intensity is not so high that it merges with its neighbours. The emergence of immobilized pH gradients allowed the development of overlapping narrow-range pH gradient that have a increased resolution (Hoving et al. 2000). This type of approach allows the visualisation of an increasing number of proteins and preserves the separation power, which brings an additional level of depth to proteomic analysis.

It is also of advantage to use a detection method that is both as sensitive and as linear as possible, in order to achieve a quantitative analysis possible and to limit the amount of sample consumed. In this respect, it is important to underscore that the quantitative precision of proteomic analysis is in the range of $\pm 10\%$, which permits the detection of small variations in protein amount with a good statistical confidence (usually twofold differences).

In spite of their sensitivity and linearity, methods based on radioactivity are not widely used to detect proteins. The most common methods for protein detection following two-dimensional electrophoresis rely either on colorants, heavy metals (mostly silver salts) or fluorescent probes. An extensive description and comparison of these different methods is out of the scope of the current article. Briefly, the detection sensitivity reaches 10 ng of protein for the methods based on organic colorants, 2–5 ng for those based on fluorescent probes, down to a fraction of a 1 ng for silver staining methods. However, the sensitivity of silver staining methods is variable from one protein to the other, and their dynamic range is quite limited as well. Methods based on organic colorants or fluorescent probes are far more homogeneous and linear: the dynamic range of fluorescence based methods spans more than three orders of magnitude. Moreover, whereas organic colorant or silver staining methods have seemingly reached their maximal sensitivity, fluorescence based methods still have some perspectives of improvement, especially if we consider the detection sensitivity (a few attomoles which corresponds to several femtograms).

3. PROTEOMICS ANALYSIS BY MASS SPECTROMETRY

Proteomics, analysis of a proteome (all proteins expressed by a genome at a given time in a given environment) is constituted of three parts, separation of proteins, enzymatic digestion and analysis of the generated peptide mixtures. The analysis of these peptides is always performed by mass spectrometry (MS). Indeed, two ionization modes, matrix-assisted laser desorption ionization (MALDI) and electrospray (ES) associated respectively to time-of-flight (TOF) and ion trap mass analyzers, are the methods of choice for proteomics analysis because they allowed analysis of peptides rapidly, with high sensibility and resolution. The domain of applications of the proteomics analysis is large and can include for example, the identification of proteins, the quantification of their variable expressions and the localization of their post-translational modifications.

Ionization by MALDI consists in the transfer of energy absorbed by a chemical matrix to sample embedded in this matrix thereby producing desorption of the

analyte molecules as ions into the gas phase. Ions formed into the gas phase are then pulsed toward the mass analyzer TOF. The combination of techniques such as two-dimensional polyacrylamide-gel electrophoresis, mass spectrometry with MALDI-TOF coupled with search in protein sequence database allows the identification of a protein (Peptide Mass Fingerprint, PMF) (Henzel et al. 1993). Indeed, the proteins separated by 2D gel are digested with a specific protease such as trypsin to generate peptide mixtures. The masses of the peptides originating from the protein spot are determined by MALDI-TOF. These peptide masses are then compared with those derived from a protein sequence database (such as SwissProt) in order to identify the protein. In some cases, this is not sufficient to identify a protein, therefore, determination of partial amino acids sequence of one or several peptides is necessary for the identification. To obtain these information, analysis by tandem mass spectrometry (MSMS) is mandatory. Generally, electrospray ionization coupled to MSMS analyzer is used. But, since few years, systems like TOFTOF analyzers coupled to MALDI source were also used to analyzed directly the same sample in MS and MSMS modes if the search with PMF does not give reasonable results.

Electrospray is a method which consist in the "desolvatation" and ionization of molecules in solvent under electric field. It takes place in a source at atmospheric pressure. In several proteomics applications, a miniaturize source with a 20–200 nl/min flow can be used, about it is termed nano-electrospray (Mann and Wilm 1995). The electrospray source is coupled to a mass analyzer which enables to obtain amino acids sequence tags of peptides. Determination of a partial amino acids sequence by mass spectrometry coupled with search into database can identified in general without any ambiguity the analyzed protein (Peptide Sequence Tag). In addition, manual interpretation enables *de novo* sequencing of a peptide which could not been matched in the database search. The analyzer used in these cases can be a triple quadrupole, an ionic trap, a hybrid triple quadrupole-TOF or a FT-ICR instrument (Fourier Transform – Ion Cyclotron Resonance Mass Spectrometry). The principle of tandem mass spectrometry is to isolate a peptidic ion, fragment it in general by collision with gas atoms (e.g. N_2 or Ar) and finally analyze the obtained fragment ions. In general, such analyses can be performed with a nano-electrospray source alone (Shevschenko et al. 1996) or coupled with HPLC separation. This technique is particularly used in case of complex peptide mixtures resulting from, for example, digested proteins separated by 1D gel electrophoresis, or originating from protein complexes, organ extracts or total cell extracts. This strategy is often used for the differential analysis of protein complexes (Pandey et al. 2000). In the case of complex peptide mixtures, a bi-dimensional chromatographic separation (cation exchange chromatography followed by C18 reverse phase chromatography) like the Multi-dimensional Protein Identification Technology or MUDPIT method (Link et al. 1999; Washburn et al. 2001) can be used to reduce the complexity of the initial mixture and therefore identify proteins which are present in low abundance and rarely observed in a regular analysis.

The domain of applications in proteomic analysis are abundant and diverse. This method can be used to understand important biological processes involved in cell

function and regulation (cell division, immune responses, etc.), to search markers characteristic of diseases for prognostic and diagnostic use, to identify target in drug discovery, etc (Burbaum and Tobal 2002; Petricoin et al. 2002; Seliger and Kellner 2002; Towbin et al. 2003). A comparative and quantitative analysis of proteins expressed, for example, in cells treated versus non-treated cells is often performed. Therefore, 2D-gel electrophoresis followed by proteins identification by mass spectrometry is still a widely used method (Towbin et al. 2003). Recently, an approach based on mass spectrometry and isotopic labeling of specific amino acids such as cysteine has been developed (Gygi et al. 1999). The chemical modification strategy ICAT (Isotope Coded Affinity Tag) (Griffin et al. 2001) uses a reagent which incorporate into a specific amino acid (cysteine) light or heavy isotopes of hydrogen (H/D), or in a more recent version light or heavy isotopes of carbon ($^{12}C/^{13}C$). These isotopic differences enable to compare protein abundance between two samples. The peptides containing such labeled cysteines are separated from the other peptides by affinity chromatography, which results in a reduction of the complexity of peptide mixtures. And finally these set of "marker" peptides are identified and quantified by capLC-ESMSMS or MALDI-TOF. This method reduces drastically the analyzed peptide complexity and enables therefore, identification of low abundance proteins. However, this reduction does not allow to obtain important information like the post-translational modifications. Also, quantitation relies often on one or two peptides per protein, which does not offer a good statistic. To overcome these problems two methods have recently emerged, SILAC and iTRAQ. SILAC (stable isotope labeling with amino acids in cell culture) is based on the introduction of an isotopically labeled amino acid (e.g. 13C6-Lysine) during cell culture resulting in full incorporation of this amino acid in each protein (Ong et al. 2002, 2003). However, the accurate quantitation with this approach is depending of the type of MS instruments used, for example, ion-trap type instruments are less accurate for quantitation purposes in comparison to quadrupole-time-of-flight or FT-ICR type instruments. Recently, a new quantitative method was developed, the isobaric peptide tagging system iTRAQ which label all primary amine, regardless of peptide class (Ross et al. 2004). The labeled isobaric peptides have the same mass and after fragmentation, these peptides generate diagnostic signature ions which enable easy quantitation in the low mass region. Because you do not lose important information with these two approaches, post-translational modifications are observed and accurate quantitation of their relative changes can be performed (Griffin et al. 2001; Ong et al. 2003).

Tandem mass spectrometry coupled to electrospray ion source allowed not only to identify proteins but also to characterize post-translational modifications of a protein (phosphorylation, acetylation, methylation, glycosylation, etc.). Indeed, the presence of such modifications induces an increase of the peptide molecular masses compared to the calculated masses based on the theoretical sequence, which often directly identifies the type of modification. In addition, tandem mass spectrometry allows in general precise localization of the modification at specific residue of the peptide. Analysis of modifications allows to understand biological mechanisms because several processes are controlled and/or induced by such modifications (Mann and Jensen 2003). Being

able to quantify post-translational modifications such as phosphorylation, is very important since it is reversible and therefore plays an important role in the regulation of the protein function.

Phosphorylation sites in eukaryotes are present mainly on serine and threonine, tyrosine phosphorylation represents only around 0.1% of all phosphorylated sites. It was estimated that approximately one third of proteins from eukaryotic cells are phosphorylated. To understand the regulation of protein activity by phosphorylation, deep analysis of the phosphorylation on protein are necessary (Loughrey Chen et al. 2002; Bonenfant et al. 2003). Such analysis is shown in Figure 1A. A protein extract from cancer cells was immuno-purified by a specific antibody against phosphotyrosine. The protein mixtures obtained were then enzymatically digested and the peptides generated were analyzed by nanoCalHPLC-MS/MS in automatic mode (reverse phase C18 HPLC column, 75 μm $\times$ 15 cm, coupled to a electrospray-quadrupole-TOF mass spectrometer). In this mode, a mass spectrum is first acquired, then the three most intense peptidic ions are selected and fragmented.

The cycle (1 MS scan+3 MSMS scans of selected precursor ions) is repeated during the entire chromatographic separation. For example, in Figure 1B, the peptidic ion at

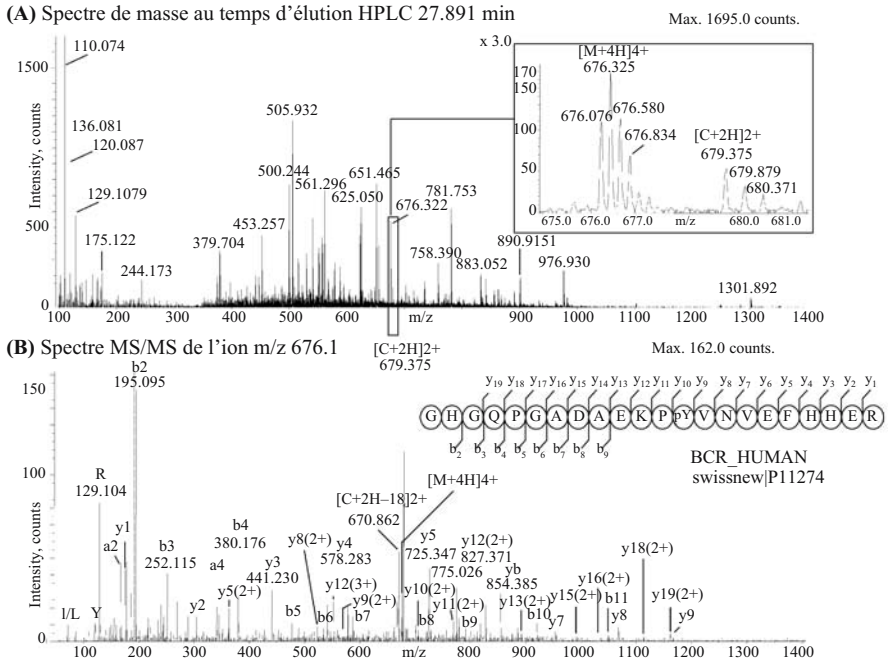

Figure 1. Example of identification and characterization of phosphorylation by mass spectrometry. (A) Mass spectrum of peptides generated after tryptic digestion of a protein extract of cancer cell lines. (B) MS/MS mass spectrum of the ion at *m*/*z* 676.076 corresponding to peptide 165–185 of BCR protein phosphorylated on tyrosine 177.

m/*z* 676.076 was selected and fragmented. The mass window for selecting precursor ion was relatively large in automatic mode, therefore in addition the peptidic ion at *m*/*z* 676.076, a peptidic ion doubly charged at *m*/*z* 679.375, was also selected. However, the ion fragments observed could almost all be attributed to peptidic ion at *m*/*z* 676.076 because these two parent ions had very different molecular masses and the fragmentation energy applied depend directly to the mass of the peptide. Finally, total sequencing of the peptide was obtained and the presence of y10 and y11 fragment ions localized the phosphorylation on tyrosine 177 of the BCR protein.

Mass spectrometry is the method of choice for identification of proteins, quantitation of proteins expression at a given time but also for characterization of post-translational modifications. Indeed, allowing direct analysis of peptides and proteins, the MALDI and ES ionization modes have been revolutionary in the field of mass spectrometry applied to biology.

4. MASS SPECTROMETRY IMAGING

A comprehensive analysis of a body fluid or a specific tissue, including target and/or biomarker identification often requires the analysis of only a group of cells or a small region in a tissue. In the drug discovery process, compound and metabolite biodistribution is a key information, which could benefit from the development of sophisticated molecular imaging techniques. *In vivo* methods, such as X-Ray, Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), Near-Infrared Fluorescent (NIRF) or bioluminescence imaging, needs reporters to give access to the localization of specific biomolecules in real-time. This requires the development of a tag for each analyte group, and therefore significantly reduces the number of detectable analytes for one experiment set, usually to just one. Besides, the results depend strongly on the specificity of the tag or the label.

In biomedical research, mass spectrometry (MS) is considered as the method of choice for the analysis of metabolites, peptides and proteins, due to its high specificity and unmatched sensitivity (as low as attomol level), especially since the development of soft ionization techniques. Introduced for biopolymer MS analysis in 1987, matrix-assisted laser desorption/ionization (MALDI) applies a weak organic acid (e.g. sinapinic acid, α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid) to the sample, and the resulting mixture interacts with UV laser pulses. The analyte being embedded in an excess of this so-called matrix, it is ionized by energy and proton transfer from the matrix to the analyte. The molecular weight of theses ions are typically determined using a time-of-flight (TOF) mass analyzer.

The emerging technology MALDI Mass Spectrometry Imaging (MSI) is an extension of MALDI-TOF analysis, since it allows the generation of spatially resolved distribution of analytes, simply by sequentially acquiring a mass spectrum at defined positions. It offers the flexibility of detection without reporter molecules, as well as sensitivity and resolution needed for biological tissues. This *ex-vivo* assay is based on the concept of applying MALDI TOF MS directly on tissue sections. A very important aspect in MALDI MSI on tissue sections is the high number of different analyte classes

which can be simultaneously analyzed, including proteins, lipids, carbohydrates and metal ions. This takes benefit of the high dynamic range of the method, even though some analytes are more efficiently ionized during MALDI process (Knochenmuss et al. 1996; Wang and Fitzgerald 2001).

4.1. Principles and Methods

Currently developed for many applications (Stoeckli et al. 1999; Stoeckli et al. 2001, 2002, 2003; Chaurand et al. 2004), MALDI MSI is achieved by rastering sequentially the surface of a defined area while acquiring a mass spectrum from every location (see Figure 2). A typical sample preparation for MSI involves the fixation of the sample, for example, tissue section, on a MALDI plate and the application of the matrix solution over the latter, either as a thin layer or as a spot pattern, to get co-crystallization of analytes with matrix while solvents evaporate. Once dried, the sample is introduced in the mass spectrometer, where, for each defined image position, short UV laser pulses are fired onto the surface to generate ions. Those are analyzed by the TOF instrument and a mass spectrum is acquired.

The acquisition of the mass spectra for peptide and protein imaging is done in our lab on commercial MALDI TOF instruments (Voyager sSTR, 4700 Proteomics Analyzer: Applied Biosystems, Framingham, MA) equipped with a Nd:YAG laser. The acquisition of the data is controlled by an in-house designed software (MALDI MS Imaging Tool, MMSIT). The area to be scanned is specified as a pattern of points, equidistant from each other. The distance is chosen referring to the diameter of the

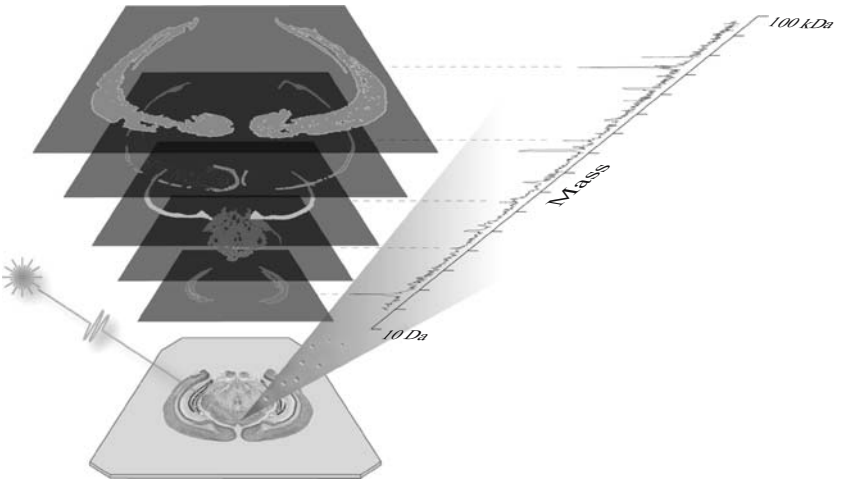

Figure 2. MALDI mass spectrometric imaging. A laser is rastered over a tissue sample while acquiring a complete mass spectrum from each position, allowing to generate molecular images for multiple analytes.

laser spot, here 50 μm – the smallest realistic raster step size – and depending on the lateral resolution required. To limit the data file size, sections or membranes have been imaged with a resolution of 100 μm. Data are recorded without compression by the MMSIT in the Analyze Image 7.5 data format (Mayo Foundation, Rochester, MN) and raw files are transferred to an image processing application named "BioMap" (Stoeckli et al. 2002). This software is based on IDL (Research Systems, Boulder, CO) and provides comprehensive tools for MS image analysis. From each data set, multiple images can be extracted, representing the spatial distribution of the analytes of interest. The same data can be used to compare mass spectra from multiple regions in the same sections. It also allows to select single points or regions of interest (ROIs) on the generated image and to display the corresponding mass spectrum.

4.2. Compound Imaging

Since drug imaging focuses on the low mass range (up to 1000 Da), the resulting MSI distribution can be skewed by an overlapping with other signals from the biological medium. Furthermore, matrix-related signal interference in this mass range is a common shortcoming in MS analysis. To improve MSI analysis accuracy and confidence, a "filtering" of mass signals is then required: for this purpose, specified molecular ions, for example, the drug molecular ion or one of its metabolites, can be fragmented. Fragmentation pattern being highly specific to a defined analyte, the analysis confidence is thus greatly improved. Usually, sections are coated with α-cyano-4-hydroxycinnamic acid (CHCA), known to enhance fragmentation. MSI analysis is so carried out in the so-called MS/MS mode, that is, by the acquisition of mass spectra resulting from the fragmentation of a given parent ion. The distribution is subsequently calculated in Biomap by selecting the mass of one or multiple identified fragment ion of the drug.

The example given in Figure 3 (**see colour insert**) refers to an *in-vitro* experiment studying the uptake of an antiflammatory drug into articular cartilage. For MS/MS drug imaging, two types of joint bovine cartilage have been treated by incubation in an anti-inflammatory drug solution. This procedure mimics the *in-vivo* situation in which the drug is directly injected into the joint synovial fluid. Figure 3 represents MS/MS images from bovine joint cartilage pieces incubated with compound solution. In the control section, that is, coming from an unresponsive cartilage (left panel), no signal related to the compound could be found in the image. Contrary to the control, drug-related signals were observed from cartilage sample (see Figure 3 right panel), indicating that the compound penetrates into the tissue. Signals were obtained from many localized regions, at the edge of the sample but also large areas inside the section. The presence of some isolated signals can be explained by the diffusion that can be induced by matrix application. Matrix coating is a crucial step in MSI: in this sole step, the solution must be able to extract analytes from tissue in order to get them cocrystallized with matrix but without inducing any lateral diffusion. The latter is responsible of the spatial signal artifacts, as illustrated in the right panel of 4.

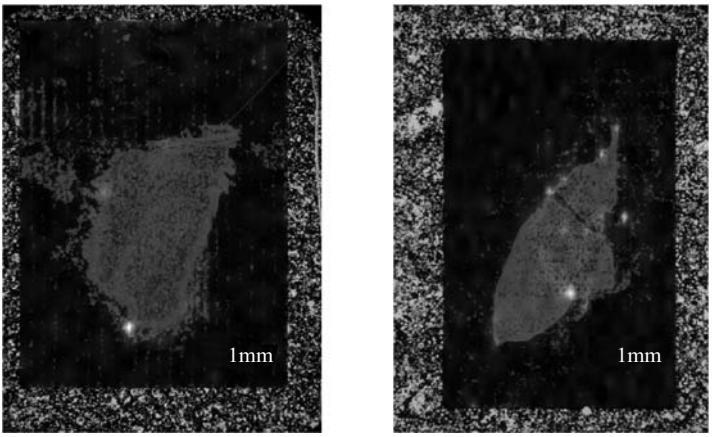

Figure 3. MS/MS molecular images of sections from bovine cartilage immersed in compound solution (left: control; right: drug). The images represent the penetration of the drug into the tissue (signal in red).

4.3. Peptide Imaging

In the mass range from 1 to 10 kD), MSI benefits of the best sensitivity of MALDI TOF instrumentation. Peptide imaging is therefore the optimal application of MSI. To get the maximum information from the tissue sections, sample preparation is a critical step and sections should be carefully processed. First, dissected tissue samples are snap frozen on a metal plate cooled with dry ice and stored at $-80°C$ prior to further processing. Sections are then cut on a cryostat to a thickness of 10 μm and are directly deposited on metal plates compatible with the MALDI MS instrument. The tissue is first fixed by immersing it in 80% ethanol solution for 30 s and dried under vacuum. The matrix coating is achieved by directly depositing 10 $\mu l/cm^2$ of a freshly prepared solution. A slow crystallization is achieved by keeping the plate at 4°C for one hour. This process results in lower noise mass spectra, as already pointed out by Stoeckli and co-workers (Stoeckli et al. 2002). An internal calibrant, typically a peptide in the same mass range as the analyte of interest, can optionally be added to the matrix solution. Hence, the matrix coating can be assessed and a baseline correction can be performed over the scanned area using BioMap.

Coronal mouse brain sections have been processed as abovementioned and mapped by MSI analysis. The most intense MS signals have been selected, the distribution of the corresponding analytes being presented in Figure 4. Depending on the selected mass, different patterns corresponding each to one peptide distribution can be observed. These peptides are either present on the whole area (see Figure 4, A), or specific to an anatomic part of the brain, for example, Figure 4C, D and E. Some distributions are confined to specific brain regions, as pointed out by molecular images B and E or C and F in Figure 4. One key issue for MALDI MSI is the matrix coating. Figure 4c illustrates this problem: the analyte signal, mainly detected in the parietal

meninges, is unevenly distributed on the two brain hemispheres. The use of an internal standard for signal normalization is one of the means to overcome this problem. Besides, MS intensities do not necessarily reflect concentration of analytes: the fact that some intensity distortions could occur due to the tissue nature which influences ionization efficiency and could cause ion suppression effect has to be taken into account.

Referring to their specific molecular images, analytes of similar molecular weights can be resolved thanks to mismatched spatial distributions, as illustrated by the peaks A and E of the global mass spectrum in Figure 4. The match of these peptide images to the anatomical images clearly demonstrates that spatial information is preserved during the sample preparation and imaging process and illustrates the relevance of the information provided by MSI: peptide identification and localization on tissue slices is the first step to potentially point out novel disease biomarkers and drug targets.

Once a biomarker has been identified, it can be used to evaluate the action of a drug on a specific pathology. One of the pathological features of Alzheimer's disease (AD) is the presence of amyloid deposits in senile plaques and in blood vessel walls. These deposits are mainly composed of amyloid beta (Aβ) peptide fragments of 4–5 kDa molecular mass, all derived from amyloid precursor protein (APP). The spatial analysis of Aß fragments, which are assumed to be linked to Alzheimer's disease pathogenesis, takes full advantage of MALDI MSI. For analysis, brain sections from APP23 transgenic mice – featured by an enhanced enzymatic degradation of APP were prepared as described above. These mice develop massive Aβ deposits in cortical and hippocampal structures upon aging.

A single scan of a brain section allows simultaneous localization of the different peptides, which were identified based on their masstocharge (m/z) ratio. As illustrated in Figure 5 (**see colour insert**), the Aβ(1–40) is by far the most abundant amyloid peptide. Two very intense regions are located in the parietal and the occipital cortical lobe and the third one close to the low part of Sylvian fissure, that is, in the hippocampus region. Referring to Figure 5, the normalized distributions of Aβ-(1–40) and Aβ-(1–42) show that they are the most abundant amyloid peptides. In agreement with these results, it has been reported that vascular amyloids were essentially composed of Aβ(1–40) and Aβ(1–42), the major peptide in aqueous cerebral cortical extracts from AD brains being the Aβ(1–40) proteolytic fragment of APP (Mori et al. 1992), while the insoluble amyloid Aβ(1–42) peptide was primarily in senile plaque cores (Miller et al. 1993; Roher et al. 1993). Thus, MSI gives access to the levels of known targets but also allows the mapping of the different targets with accuracy, which is not possible when whole-brain extracts are analyzed.

4.4. Protein Imaging

As mentioned above, MSI for tissue section analysis is based on MALDI-TOF technology. This latter allows very sensitive detection of a wide variety of compounds, with masses up to 10 kDa. For biomolecules of larger molecular weights, MSI is no longer very sensitive due to detection limitations, but also due to the MALDI process

itself as well as the sample complexity, that is, tissue section. However, a major part of interesting proteins has their molecular weight well above 20 kDa, which means that this target mass range is currently out of reach for MSI tissue section analysis. To overcome this limitation, the molecular scanner, introduced by Bienvenut and coworkers (Bienvenut et al. 1999) for 2D gel electrophoresis post-processing, has

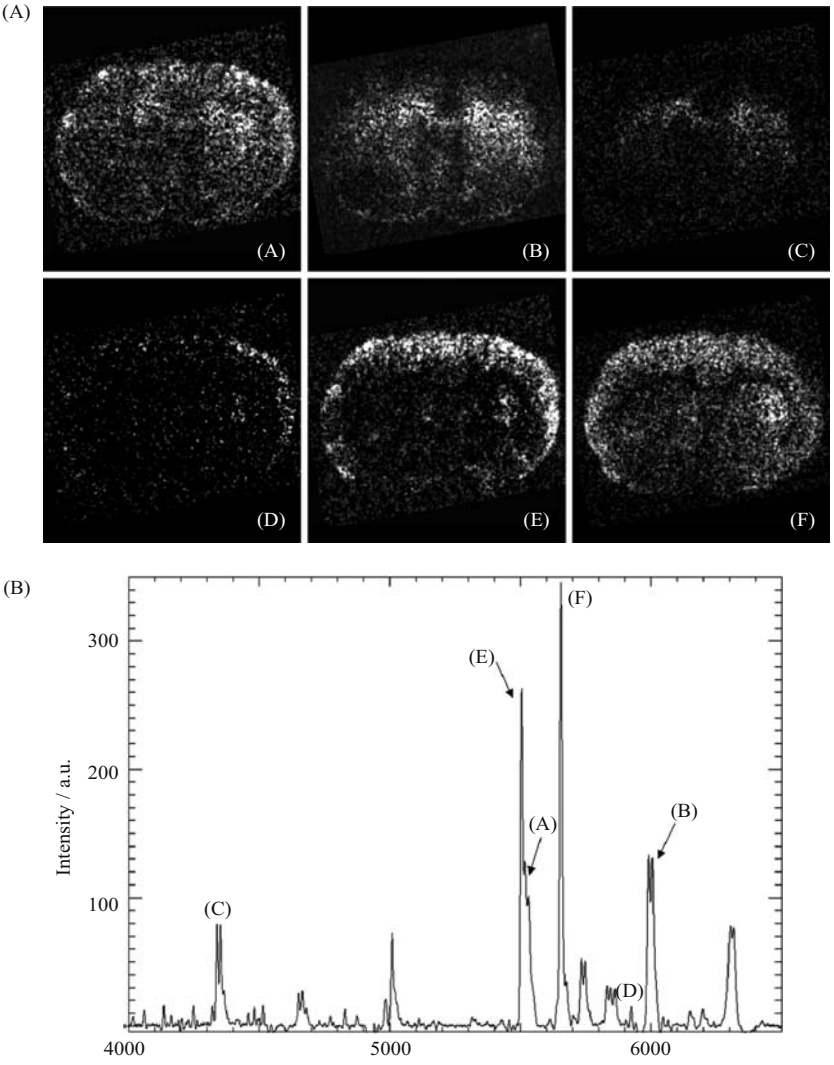

Figure 4. MSI of mouse brain coronal section. Molecular images for (A) *m*/*z* 5506; (B) *m*/*z* 6006; (C) *m*/*z* 4358; (D) *m*/*z* 5923; (E) *m*/*z* 5498; (F) *m*/*z* 5656; Average mass spectrum calculated over the mouse brain section area.

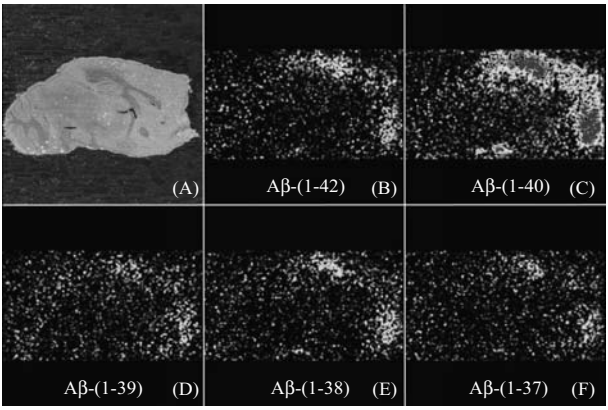

Figure 5. MSI on sagittal AD brain section. (A) Optical image of the sagittal AD brain section; (B) Aβ-(1–42) molecular image (*m*/*z* 4515.1); (C) Aβ-(1–40) molecular image (*m*/*z* 4330.9); (D) Aβ-(1–39) molecular image (*m*/*z* 4231.7); (E) Aβ-(1–38) molecular image (*m*/*z* 4132.6); (F) Aβ-(1–37) molecular image (*m*/*z* 4075.5).

been evaluated and further developed for tissue sections. The molecular scanner is based on the principle of protein identification by peptide mass fingerprint, that is, the analysis of the digestion of one protein. The protocol is very similar to a semi-dry electroblotting transfer, except that the starting material is a tissue section and that a so-called trypsin membrane is implemented in the sandwich between the section and the capture membrane. This membrane, in which trypsin has been covalently bound, allows the digestion of proteins while they are migrating towards the capture membrane. Once tryptic peptides are generated, they will also migrate under the action of the electric field and will be entrapped on the capture membrane through hydrophobic interactions. Thus, this latter is afterwards analyzed by MSI, resulting in molecular images of tryptic peptides, with lower mass, and so allows identification of proteins through peptide mass fingerprint analysis.

The molecular scanner approach benefits from MS/MS imaging similar to drug imaging. It helps to reduce analysis complexity while keeping a high level of confidence in the identification of the proteins. Indeed, the transblotting imposes the separation between proteins and the other components of the tissue. However, some proteins are more easily extracted from the tissue section than others, even with the use of detergent. Therefore, only a part of the whole protein set within a tissue preparation can be effectively analyzed. Nevertheless, as illustrated for a mouse brain slice in Figure 6, distributions of a large number of peptides can be measured while preserving the spatial information. Even if the explored mass range stays below 4 kDa, it should be kept in mind that these peptides are digest products of proteins. In Figure 6, the distributions corresponding to m/z 2324 and 3099 are similar, and may come from the same protein. To perform protein identification by database searching with a sufficiently high degree of confidence, at least four peptides issued from one single

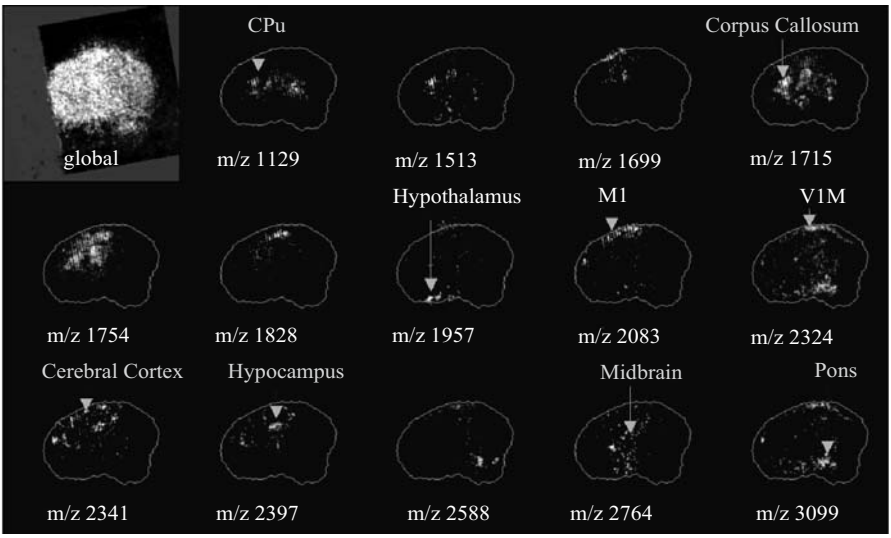

Figure 6. Mass spectrometry imaging of a capture membrane processed with molecular scanner on a mouse brain (CPu = caudate putamen (striatum); M1 = primary motor cortex; V1M = primary visual cortex, monocular region).

protein digestion are needed. If this requirement is not fulfilled, subsequent peptide analysis by MS/MS provides significant amino acid sequence information, allowing unambiguous protein identification.

5. CONCLUSIONS

MALDI MS and MALDI MSI takes full advantage of the high sensitivity of Biophotonics and mass spectrometry together with its ability to detect simultaneously a wide range of compounds, regardless from their nature and mass. Key issues in MALDI MSI that need to be overcome are analyte dependant ionization efficiency, suppression effects and cross-linking, and all of them are more or less related to sample processing and matrix coating. This step is indeed crucial since tissue sections or membranes should be wet enough to extract a part of the biological material and make it available to be co-crystallized with matrix. However, if the support becomes too wet when matrix solution is applied, biomolecules can be delocalized or even stripped off. As highlighted by the studies presented here, MALDI MSI can be used to track biomarkers involved in disease states, such as peptides or proteins, but also to map drug/tissue interactions, even though only a fraction of the whole set of biomolecules will actually be analyzed.

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