New Developments in MALDI Imaging for Pathology Proteomic Studies

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Abstract: With new emerging mass spectrometry technologies, it can now be demonstrated that *direct tissue analysis* is feasible using matrix-assisted laser desorption/ionization (MALDI) sources. A major advantage of direct MALDI analysis is to avoid time-consuming extraction, purification or separation steps, which have the potential for producing artifacts. Direct MALDI analysis of tissue sections enables the acquisition of cellular expression profiles while maintaining the *cellular and molecular integrity*. With automation and the ability to reconstruct complex spectral data using imaging software, it is now possible to produce *multiplex* imaging maps of selected biomolecules within tissue sections. Thus, direct MALDI spectral data obtained from tissue sections can be converted into imaging maps, a method now known as *MALDI-imaging*. MALDI-imaging combines the power of mass spectrometry, namely exquisite sensitivity and unequivocal structural information, within an intact and unaltered morphological context. Critical improvements to increase image resolution are presented in this manuscript e.g., solvent treatment, new solid ionic matrices, gold sputtering, nickel support or laser focalization. One of the most important developments is the ability to carry out either direct MALDI analysis or MALDI imaging on paraffin tissue sections, thus opening the path to an archival "gold-mine" of existing pathology samples to proteomic analysis. These developments provide new avenues for biomarker hunting and diagnostic follow-up in the clinical setting. Further developments in MALDI-imaging of specific targets provide an added dimension, as validated disease-marker-gene RNA transcripts can be analyzed along with their translation by targeting their specific protein products or metabolites. Disease/health states will thus be closely molecularly monitored at protein and nucleic acids levels, with a single technique. Taken together, MALDI imaging will become a key tool for pathology proteomic studies.

INTRODUCTION

In the era of proteomics and high throughput studies, need for new technologies presenting higher sensitivity, versatility and specificity are sought. Classic proteomics is now a widely used strategy for obtaining wide ranging protein information from tissues as demonstrated by growing literature in that field. In general, proteomic studies require homogenization of a sample as well as long, tedious and consuming extraction, purification and separation steps before characterization, resulting in the loss of the anatomical data. Over these past ten years, improvements of mass spectrometry instruments together with growing importance of this method for compound identification in the field of biology had lead to the development of direct analysis of tissue samples by the means of mass spectrometry. Mass spectrometry has become an analysis tool allowing identification of compounds directly from tissues without any extraction or separation, but adds one dimension to the analysis with some localization on tissue samples. Thus, in a single experiment, molecular information on hundreds of proteins can be retrieved. By incorporating automation of this method and data processing, molecular images can be obtained from tissue sections. A major advantage of this method, rely on the sensitivity of mass spectrometry instruments revealing hundred compound molecular images after one data set acquisition. MALDI ion sources are well suited for this application since it gives access to very different biomolecules families ranging from peptides, proteins to oligonucleotides, sugars or lipids with a spatial resolution at the cellular level. MALDI imaging was first introduced by Caprioli and collaborators [1-3] (Fig. **1**). Few studies have involved the still recent developments in MALDI imaging. Of note are the studies of Pr B. Spengler (Giessen, Germany) with the development of a new microprobe MALDI mass spectrometer combining the Laser Microprobe Mass Spectrometer concept with a more refocused laser beam to increase images spatial resolution (SMALDI or Scanning micro

probe matrix-assisted laser desorption ionization) [4]. This group has also developed MALDI imaging instruments with atmospheric pressure MALDI (AP-MALDI). Other important developments have been established by Pr. R.M.A. Heeren (FOHM institute, Netherlands) introducing the concept of imaging using an instrument in the microscope mode with a position detector allowing image reconstruction on the base of ion arrival position [5, 6]. Interesting applications on aging diseases have also been reported by M. Stoeckli and coll. (Novartis Pharma, Switzerland) [7]. MALDI imaging of lipids [8-11] is also under current development in the group of Pr. A. Woods. Neuroproteomics is well developed by the group of J. Sweedler [12-15] and our group has focused on developing tissue preparation methods as well as probe specific MALDI-imaging [16-18].

MALDI-imaging combines the power of mass spectrometry, namely exquisite sensitivity and unequivocal structural information, within an intact and unaltered morphological context. Furthermore, our unprecedented ability to carry out this powerful technique on paraffin tissue sections opens up an archival "gold-mine" of existing pathology samples to proteomic analysis.

NEW DEVELOPMENTS FOR ROUTINE HISTO-PROTEO-MICS IN PATHOLOGY

Histo-proteomics MALDI technology can be developed for drug biodistribution studies, biomarker discovery or for molecular mechanism discovery. At the present time, MALDI imaging can provide new avenues for clinical proteomic studies with the goal of characterizing cellular circuitry and to understand the impact of disease and therapy on cellular networks though analysis of tissue pathology samples. In fact more than just new clinical proteomic tool, MALDI-imaging has the potential to become a major method in the way tissue samples are investigated in pathology. MALDI-imaging histo-protomic is the application of proteomic technologies and bioinformatic tools to clinical material though tumor analysis. The translational nature of this technology provides unique challenges and yet unimagined opportunities that promise to transform the way disease is detected, treated, and managed. Rather

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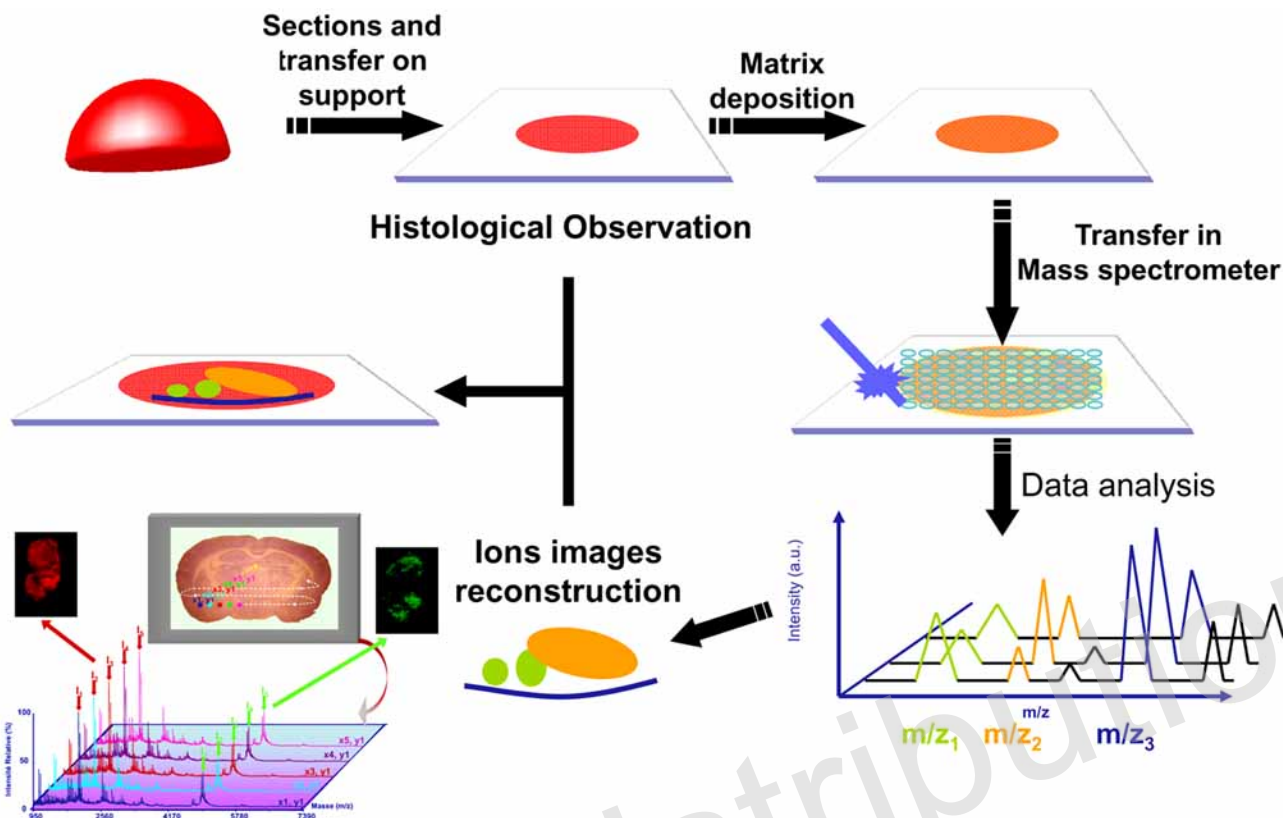
**Fig. (1).** Representative scheme of MALDI imaging technology.

Tissue sections from fresh organ or biopsy are laid out on the MALDI target sections first covered with a specific matrix depend to the nature of the bio-molecule under study. For peptides/proteins, very intense signals are obtained with α-cyano-4-hydroxycinnamic acid (α-CHCA) as a matrix. Sections are covered with α-CHCA (or another matrix) and are then introduced in the MALDI-TOF for analysis. Next, MALDI laser is used to scan each point of the surface area and the mass spectra representative of the peptides/proteins or lipids present in each point are analyzed. Automated analysis of the complete tissue is obtained by performing mass spectra every 10-50 μm, providing representative information of selected ions (each ion is a specific bio-molecule). Analysis is obtained within 2-6 hours and images are reconstructed using Flex-imaging software.

than focusing on genetic alterations that may lead to a particular disease, it is emerging that changes in multiple protein expression patterns are the most accurate way to identify diseases in their early stages and to determine the most effective course of treatment. Indeed, genome sequences do not provide information of post-translational modification events such as glycosylation, phosphory-lation, acylation or partial proteolysis. One of the most common objectives in proteomics is the study of protein expression patterns (e.g., protein profiling) associated with diseases. In contrast to existing diagnostic assays, which examine protein biomarkers one at a time, MALDI-based histo-proteomics provides powerful simultaneously detection of hundreds or even thousands of proteins in a single assay directly from the tissue. The effectiveness of such a proteomics approach in pathology hinges on two technological components: rapid, multiplex protein detection assays and data analysis systems to assimilate vast amounts of protein expression data from healthy and diseased individuals into clinically relevant data sets. In the present review, we envisage the application of state-of-the-art peptide/protein profiling directly on tissues to study of multi-factorial diseases and to develop new methods based on nanotechnologies for high-throughput proteome characterization. Based on accumulated knowledge, we conclude that whatever the disease's molecular origin (e.g., inherited/acquired abnormalities of intracellular control or of bi-directional communication networks exerting homeostatic control), the corresponding affected tissues are characterized by abnormal patterns of protein/peptide expression and/or secretion.

TISSUE PREPARATION

Our recent technological advancements in MALDI-imaging allow the identification of novel markers and *in situ* characterization from fresh sections/biopsy embedded in paraffin, including archived material.

As shown in the (Fig. **1**), tissue sections from fresh organ or biopsy are laid out on the MALDI target. Sections are first covered with a specific matrix depending on the nature of the bio-molecule under study [19]. For peptides, very intense signals are obtained with ionic matrices [20]. Sections are laid out on conductive glass (i.e., Nickel or ITO, (Fig. **2a**), treated with organic solvent to remove lipids [21] (Fig. **2b**), covered with a solid ionic matrix [20], overlayed with gold in order to remove charges effects [22] and are then introduced in the MALDI-TOF for analysis (Fig. **2c**). Next, the MALDI laser is used to scan each point of the designated surface area and the mass spectra representative of the biomolecules present in each point are analyzed. Automated analysis of the complete tissue is obtained by performing mass spectra every 50-200 μm, providing representative information of selected ions (each ion is a specific bio-molecule) [20, 23, 24]. Analysis is obtained within 2-6 hours and images are reconstructed using imaging software (Fig. **1**). We recently made a important advances with the development of specific new matrices, called ionic matrices, for high repetition laser (200 Hz). These ionic matrices advantageously prevent pep-tide delocalization after deposition, permit better coverage as well as low level ablation and the possibility to work in positive or nega-
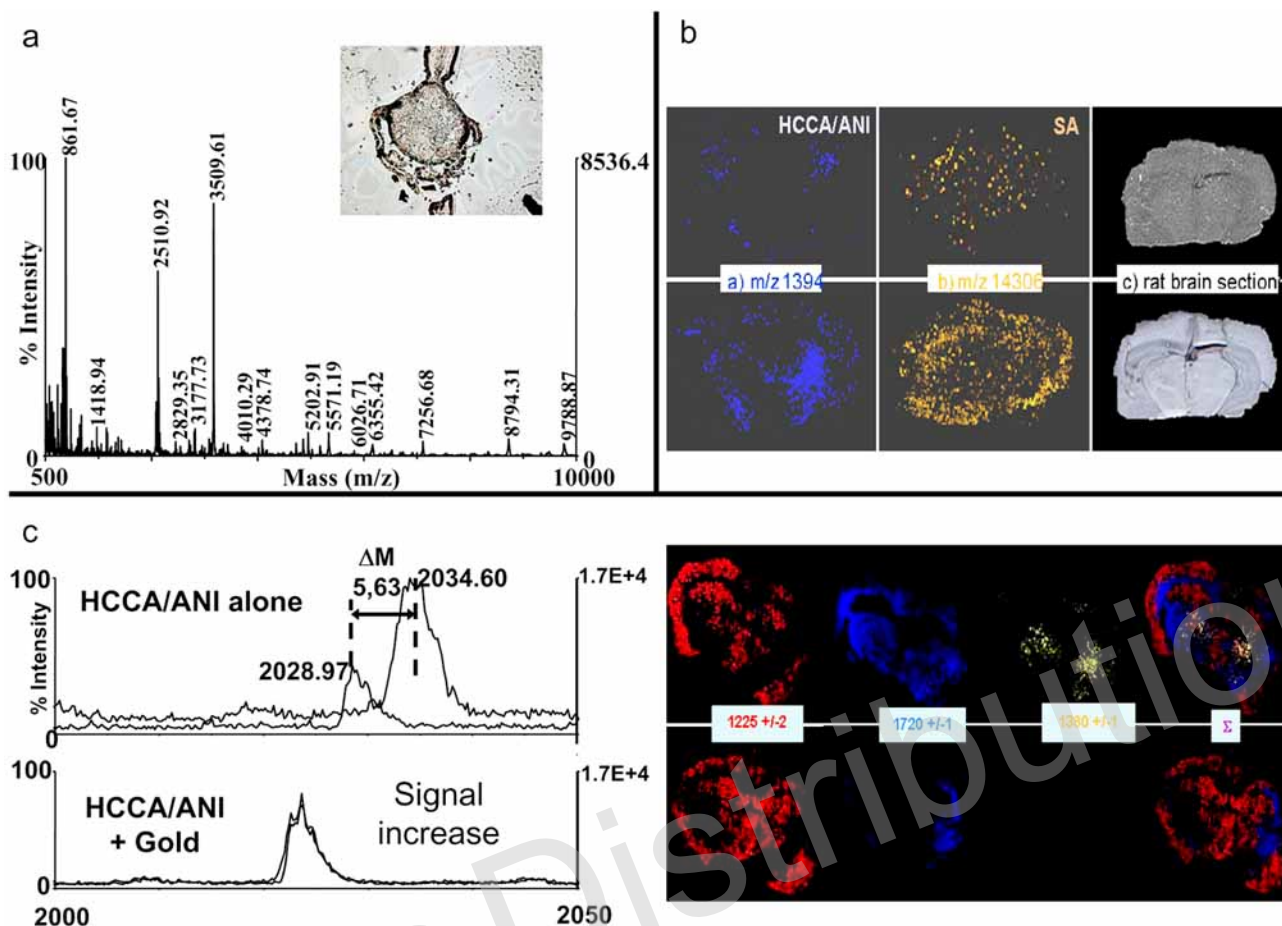
Fig. (2). Scheme of the MALDI imaging new developments.

a- Mass spectra of leech ganglia on Nickel Glasses for tissue application on conductive and transparent supports (inset represent a leech ganglia section on nickel Glass).

b- Solvents washing for removing lipids acoding to [21].

c- Gold sputtering of the slices for removing charge effects. Comparison was performed with HCCA/ANI matrix in present or absence of gold (Left panel). All spectra are obtained in a laser energy close to the minimal value for ions production and are obtained in a laser energy close to the minimal value for ions production. 15 shots are realized with an increasing of 15% of the laser fluence (no acquisition) and immediately after a spectrum is recorded in the same conditions of laser energy. Right panel represents MALDI-IMS using MALDI TOF/TOF in reflector mode at 50 Hz repetition rate with ionic matrix HCCA/ANI. The image have been reconstructed with FlexImaging software and represent the superposition of the repartition of m/z = 1225 (blue), m/z = 1720 (red) and m/z = 1380 (yellow).

tive modes [20]. One such matrix, namely ANI (aniline/HCCA) is highly stable under vacuum and has high resistance to repetitive laser shots (Fig. **3**).

The use of archived material in paraffin blocks from hospital pathology departments thus represents a major source of catalogued existing information. However, the major hurdle to analyse such samples is the cross-linking due to formalin fixation and embedded in paraffin (FFPE tissue). Two approaches have been established depending on the age of the tissue blocks. In the case of tissues stored less than 6 months, an active matrix, namely 2,4-dinitrophenylhydrazine (DNPH) is used. DNPH neutralizes formalin excess and allows analysis of embedded tissues such as those from paraffin sections (Fig. **4a**) [25]. In the case of tissue blocks stored more than 6 months, micro-digestion with tryspin must be performed. On adjacent sections, one is used for protein characterization and the other for a raster image using a microspotter for spotting the trypsin (200 μm) then the matrices at the same level. The section used for the protein characterization is treated with trypsin after paraffin removal and rehydratation. The digest is then injected

in nanoLC-nanoESI/IT MS trap for peptide characterization. The ions of the protein shown to change between normal or pathology are then examined on the adjacent slices in order to obtain an image of its localization. All ions deriving from the same protein after digestion can then be localized and the same cellular localization is obtained validating the methodology (Fig. **4b**) [16, 25].

These developments in the histo-proteomics of MALDI-imaging for archived materials could lead to the creation of a national disease marker database, and allow the elaboration of early diagnostics for various pathologies as well as a follow up in disease progression. MALDI-imaging has the potential applications for drug development. In this context MALDI-imaging can be used to analyze drug metabolic pathways directly in tissues (e.g., through in situ multiplex analysis of metabolites), providing important information in order to understand secondary effects and unexpected feedback loops. In order to provide specific examples of the versatility and power of MALDI-imaging, we briefly present preliminary data on a well established animal model of Parkinson's disease, where mice are treated with 6 hydroxydopamine (6-OHDA). In
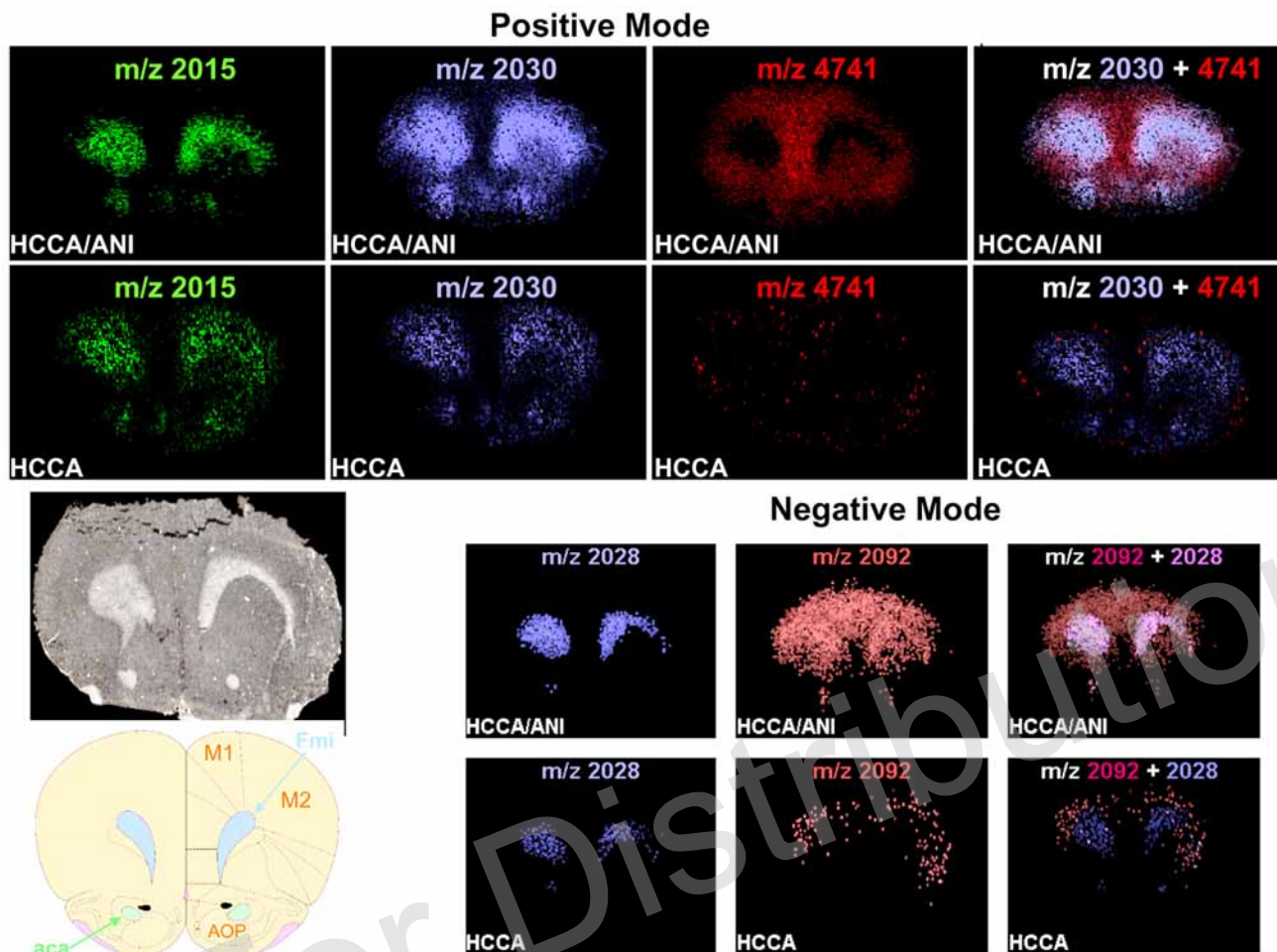
Fig. (3). MALDI-IMS using MALDI TOF-TOF in reflector mode at 50-Hz repetition rate with ionic matrixes CHCA/ANI and CHCA in positive (**a**) and negative modes (**b**). MALDI imaging can be compared with rat brain anatomy (**c**). For CHCA/ANI and CHCA, acquisitions in both polarities were performed on the same rat brain cut. Images have been reconstructed with the same parameters for ionic matrix and CHCA using FlexImaging software and represent the repartition of a m/z in the tissue slice. Images with two colors correspond to the superposition of two m/z images (aforceps minor of corpus callosum, banterior commissure, and c,dmortor cortex) With permission from analytical chemistry [20].

neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, post-mortem tissues are used to identify disease markers. A well-studied animal model of Parkinson's disease is the rat model subjected to 6-OHDA treatment. Direct MALDI analysis on FFPE brain tissue sections (stored for 9 years) from treated and control rats were carried out with an enzymatic digestion followed by a tandem mass spectrometry (MS-MS) analyses and then subjecting the entire fragmentation spectra to the Mascot™ interface software (Matrix Science Ltd). A total of eight markers were considered noteworthy, either down- or up-regulated, five of which have previously been described in Parkinson's disease literature (see Table **1**). This demonstrates the feasibility of this technique and its great potential in future histo-proteomics to discover new biomarkers from archived tissues from hospital library.

SPECIFIC MALDI IMAGING

In order to add a dimension of specificity to MALDI-imaging, we have developed designed probes directed against specific targets. This strategy has been developed for various biomolecules, from mRNA and peptides/proteins [26]. In order to specifically amplify signals, we have developed the concept of "Tag-Mass". Tag-Mass is a novel method that uses labeled probes for specific

identification in MALDI-imaging. As shown in (Fig. **5a**) the concept is schematized with an oligonucleotide sequence (or probe) that hybridizes mRNA in a tissue section as is done in standard *in situ* hybridization techniques. An oligonucleotide probe sequence is attached to a photo-cleavable group linked to a "Tag" marker which is an amino acid sequence of defined mass. This "Tag" marker can be modified to generate "Tags" of different known masses. Following hybridization of the Tag-Mass probe to the tissue section, MALDI analysis is performed as previously described, however, the pulse laser will cleave the photo-cleavable group to yield a Tag of a defined mass. The signals obtained for specific bio-molecules in Tag-Mass will be much higher and will yield unique signatures. Our results with a first generation of Tag-Mass probes have established a proof-of-principal, as we can detect hybridized mRNAs on tissue sections. We then developed a second generation Tag-Mass, with a modified uracil base that bears the photo-cleavable linker, allowing us to perform multiplex *in situ* hybridization using MALDI technology (Fig. **5b**). Our results also establish the concept that a photo-cleavable linker can be used in other application with antibodies, lectin or aptamers for use in Tagged-specific MALDI-imaging. The development of "Universal Tags" for direct *in situ* tissue analysis by MALDI-TOF mass spectrometry is a significant
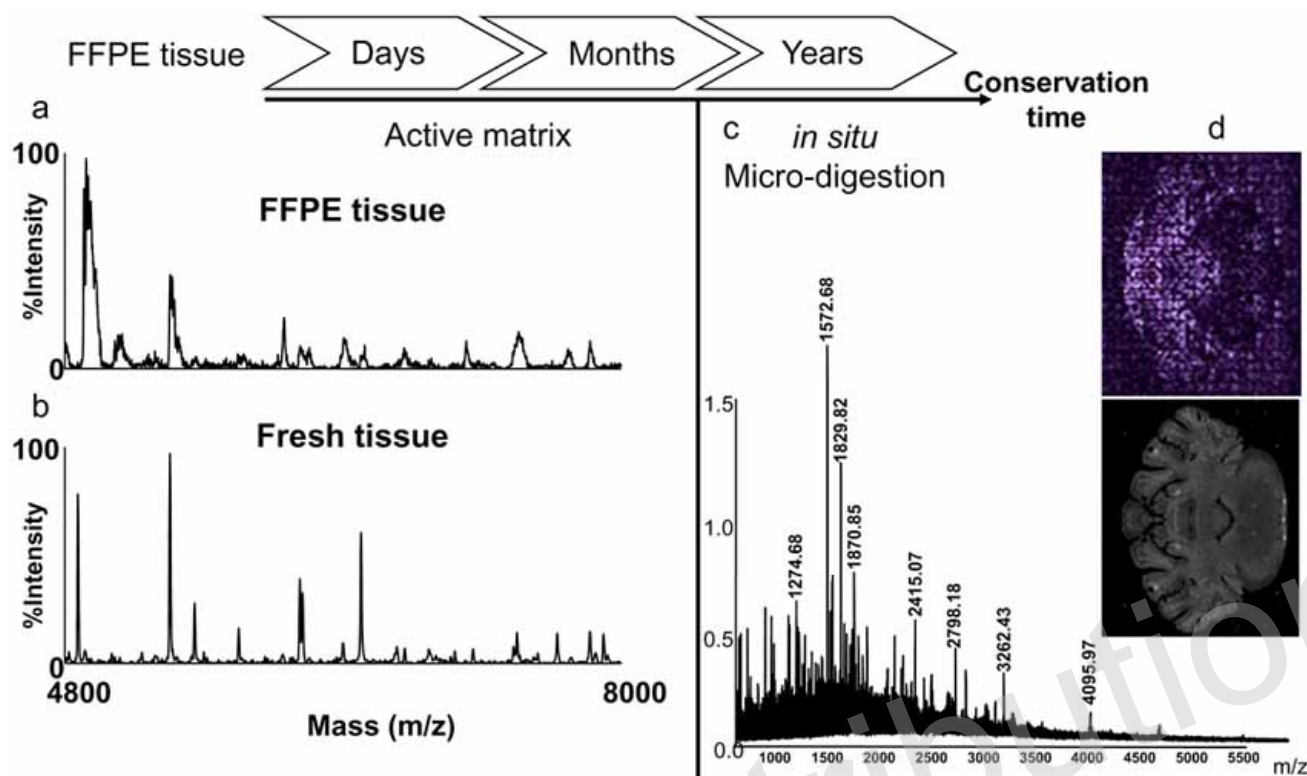
Fig. (4). Strategies used for FFPE tissue (a) stored less than 6 months, (b) stored more than 6 months.

a: Compared MALDI mass spectra in the linear positive mode of the direct analysis of a <1year old FFPE and fresh frozen rat brain tissues recorded in the same region With sinapinic acid as matrix.

b: MALDI mass spectrum in the linear positive mode of the direct analysis of a <1year old FFPE tissue using 2,4-DNPH as matrix. Zooming compared this spectrum to the one recorded in the same conditions and in the same region of the rat brain of a fresh frozen tissue.

c: MALDI mass spectrum in the linear positive mode of the direct analysis of a 2 years old FFPE tissue section after *in situ* trypsin digestion of the whole tissue section (15 min).

d: MALDI molecular images reconstructed from the data recorded on the 2 years old FFPE rat brain tissue section after micro-spotted *in situ* trypsin digestion followed by extraction and performed on MALDI-TOF-TOF/TOF using HCCA as matrix and compared to rat brain picture and morphology.

Table 1.

Markers	m/z	Mascot Score (%)	Known in Parkinson's Disease Literature
Downregulated	Ubc-Ubiquitin	78	?
	Trans elongation Factor 1 (eEF1)	66	?
	Hexokinase (2-7-1-1)	76	Pastosis *et al*. Pharm. Res. 1995 31, 361-369
	Neurofilament M protein	57	Basso *et al*. Proteomics 2004 12, 3943-3952
Upregulated	Peroxidoxin 6	65	Basso *et al*. Proteomics 2004 12, 3943-3952
	Collapsin response Mediateur Protein	70	?
	F1 ATPase	81	Seo *et al*. Human Gen Ther. 2004 15, 887-895
	Enolase alpha	62	Poon *et al*. Neurobiol Des. 2005 18, 492-498

achievement toward specific molecular diagnosis. Thus we antici-pate targeting specific disease-marker-gene RNA transcripts, fol-lowing their expression within tissues and then confirming their translation by targeting their specific protein products or metabo-lites. Disease/health states will thus be closely molecularly moni-tored.

IMPROVING IMAGE QUALITY

The desorption/ionization process is mediated in MALDI analysis by the irradiation of the sample by the laser beam, for a fixed sample, the analyzed region is limited to the area irradiated by the laser beam. It is then possible to perform analyses on various positions of the sample and to obtain from each position a spectrum representing ionic species present in that position. Thus, by shifting the laser beam of a regular pitch defined by the user, the whole sample may be scanned, and a database comprising all spectra and their coordinates may be generated, which allows us to construct an expression map of any compound of known m/z ratio in the ana-lyzed sample. UV lasers used in MALDI imaging, and especially commercialized N_2 lasers emitting at 337 nm, have a laser beam
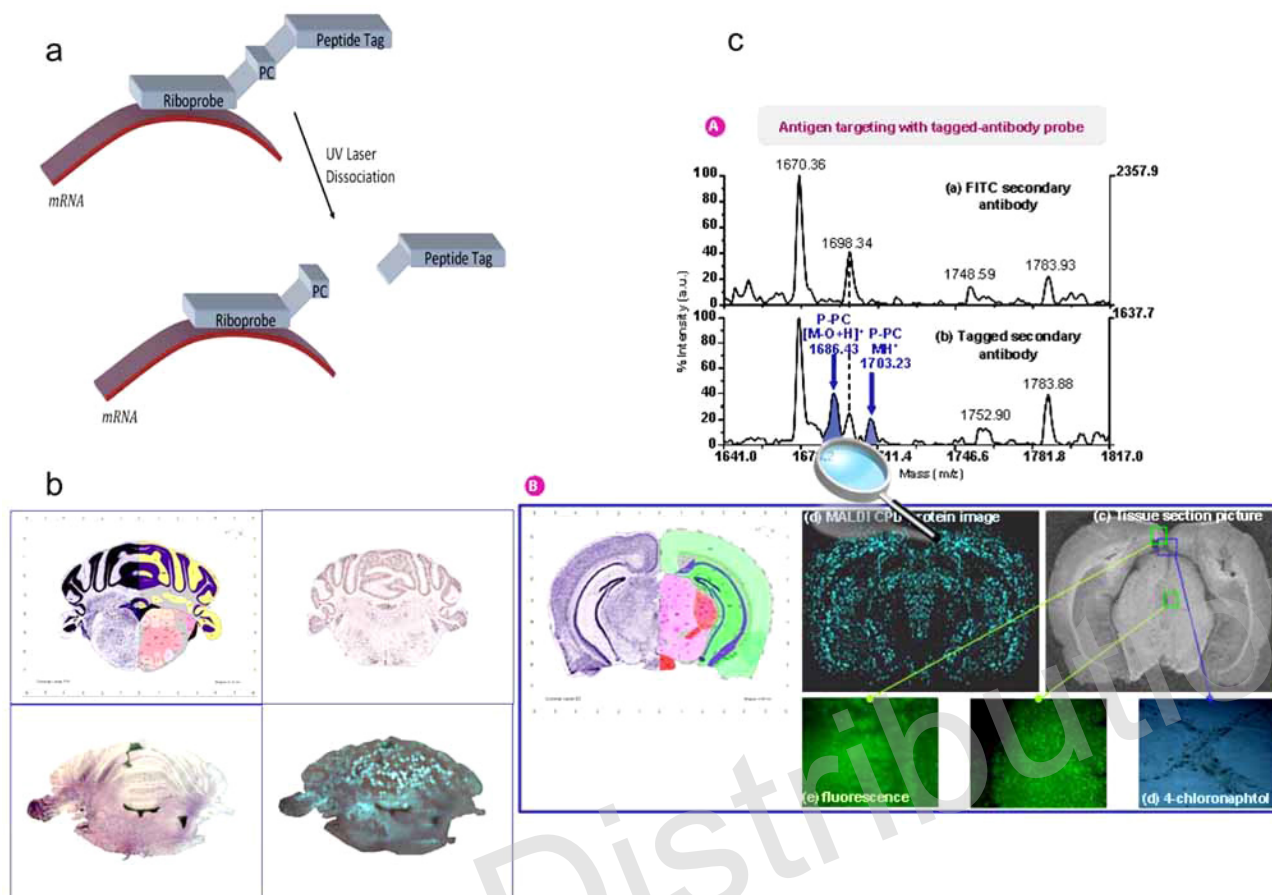
Fig. (5). Scheme of the tag-mass probe concept (**a**). MALDI molecular image reconstructed from data recorded with a proenkephalin tagged probe compared to mouse barin morphology and digoxigenin in situ hybridization data of mouse proenkephalin gene repartition (**b**) Molecular image of the carboxypeptidase D, a membrane protein of 180 kDa detected with a tagged antibody and compared to the same experiments with fluorescein and chloronaphtal revelation (**c**) [26].

section area generally ranging between 75x75 µm² and 200x200 µm² with a classical focalization system. For tissue imaging, the minimum distance between two points will have to be superior to the laser beam diameter, resulting in an image definition of at most the laser beam diameter (thus at best 75x75 µm), which corresponds to the irradiation of several cells in the tissue sample. Ideally, the image definition of a tissue sample should be or the order of a cell diameter (10-20 µm for small cells). In fact, the decrease by a factor 10 (10 µm steps) will increase imaging resolution.

Two types of solutions have been proposed to increase image resolution. The first, done by Caprioli team, is based on the different methods for matrix deposition to obtain more homogeneous microcrystalline layers and reduce peptide dispersion in the tissues (peptide dilution and delocalisation). An acoustic reagent multispotter has been developed to provide improved reproducibility for depositing matrix onto a sample surface [27]. For tissue sections, matrix spots of 180-200 microm in diameter were obtained and a procedure is described for generating coordinate files readable by a mass spectrometer to permit automated profile acquisition. Mass spectral quality and reproducibility was found to be better than that obtained with manual pipet spotting [27].

The second solution, developed by our group, implies a smaller diameter of the laser beam in order to get a diameter of the beam close to single cell dimensions. For this position, conductive masks have been developed to decrease the area irradiated by the laser beam for MALDI analysis in order to get a more precise localiza-

tion of the biomarkers in the tissue. Moreover, improving the beam focusing will get a homogeneous [28] energy profile laser beam (flat top laser) (Fig. **6**).

CONCLUSION

The future impact of MALDI-imaging can be liked to the formidable advancements made several years ago in the field of nuclear magnetic resonance (NMR) or in the development of the polymerase chain reaction (PCR). NMR has led to the development of magnetic resonance imaging (MRI) in the clinical setting for the diagnosis of various pathologies, while PCR revolutionized molecular biology and clinical diagnosis. Likewise, we foresee that MALDI-imaging will have an enormous impact in the clinical setting for the diagnosis and research leading to treatments in pathology proteomics.
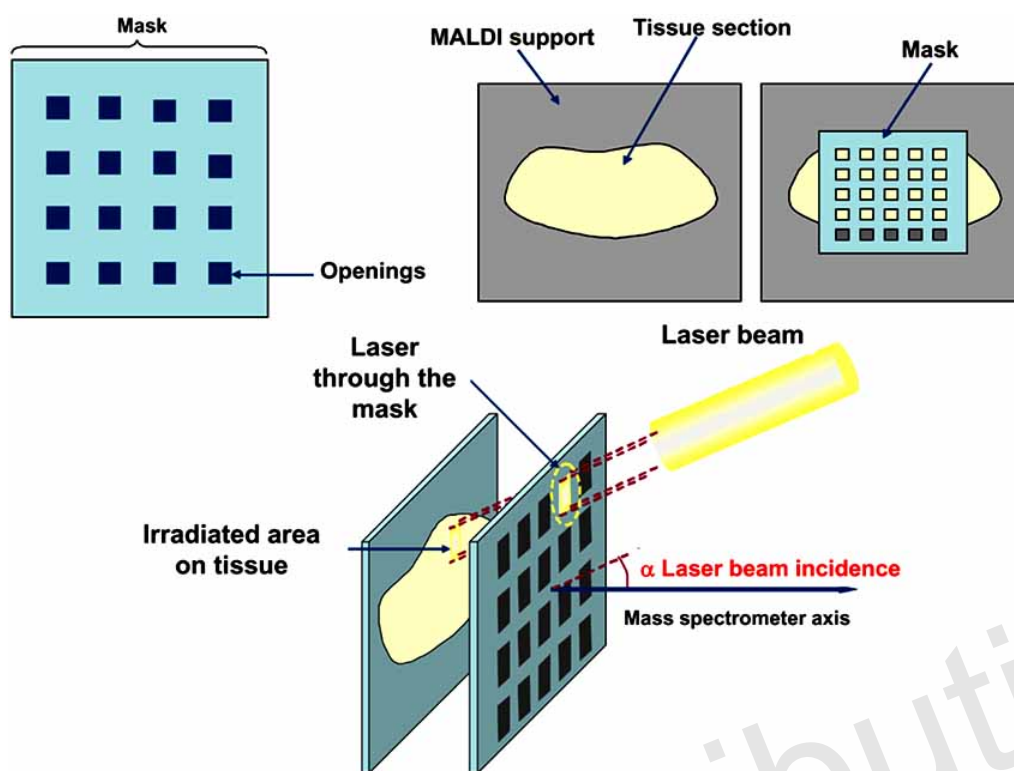
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Fig. (6). Scheme of laser beam reduction through masks. Reduction is now 30 x 30 μm [28]

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