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Selected Protein Monitoring in Histological Sections by Targeted MALDI-FTICR in-source decay Imaging

David Calligaris[1,†], Rémi Longuespée[1], Delphine Debois[1], Daiki Asakawa[1,2], Andrei Turtoi[1,3], Vincent Castronovo[3], Agnès Noël[4], Virginie Bertrand[5], Marie-Claire De Pauw-Gillet[5], Edwin De Pauw[1]**

[1]Mass Spectrometry Laboratory, Systems Biology and Chemical Biology, GIGA-Research, University of Liege, 4000 Liege, Belgium.

[2]Graduate School in Nanobioscience, Mass Spectrometry Laboratory, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama, Japan.

[3]Metastasis Research Laboratory, GIGA Cancer, University of Liege, Bat, B23, CHU Sart Tilman Liège, 4000 Liège, Belgium.

[4]Laboratory of Tumor and Development Biology, GIGA-Cancer, University of Liege, Bat, B23, CHU Sart Tilman Liège, 4000 Liege, Belgium.

[5]Mammalian Cell Culture Laboratory, GIGA Systems Biology and Chemical Biology, GIGA-R, University of Liege, 4000 Liege Belgium

[†]Present address: Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

* Corresponding authors:
Dr. Calligaris David, Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, dcalligaris@partners.org.
Prof. De Pauw Edwin, University of Liege (ULg), GIGA-Research, Mass Spectrometry Laboratory, 4000 Liege, Belgium, e.depauw@ulg.ac.be, +324/3663415.

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Abbreviations: MALDI, FTICR, ISD, MSI, BSA, HSA, MBP, HNP-1

ABSTRACT

MALDI mass spectrometry imaging (MALDI MSI) is a rapidly growing method in biomedical research allowing molecular mapping of proteins on histological sections. The images can be analyzed in terms of spectral pattern to define regions of interest. However, the identification and the differential quantitative analysis of proteins require off line or *in situ* proteomic methods using enzymatic digestion. The rapid identification of biomarkers holds great promise for diagnostic research but the major obstacle is the absence of rapid and direct method to detect and identify with a sufficient dynamic range a set of specific biomarkers. In the current work, we present a proof of concept for a method allowing identifying simultaneously a set of selected biomarkers on histological slices with minimal sample treatment using in-source decay (ISD) MSI and MALDI-Fourier transform ion cyclotron resonance (FTICR). In the proposed method, known biomarkers are spotted next to the tissue of interest, the whole MALDI plate being coated with 1,5-DAN matrix. The latter enhances MALDI radical-induced ISD, providing large tags of the amino acid sequences. Comparative analysis of ISD fragments between the reference spots and the specimen in imaging mode allows for unambiguous identification of the selected biomarker while preserving full spatial resolution. Moreover, the high resolution/high mass accuracy provided by FTICR mass spectrometry allows the identification of proteins. Well-resolved peaks and precise measurements of masses and mass differences allow the construction of reliable sequence tags for proteins identification. The method will allow the use MALDI-FTICR MSI as method for rapid targeted biomarker detection in complement to classical histology.

INTRODUCTION

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) is a powerful method to reveal the two-dimensional spatial distribution of molecules in tissue sections. Essentially, a laser beam irradiates pixel-wise positions on the sample coated with a specific matrix[1]. A mass spectrum is associated to each pixel and hence an image of the drugs[2], metabolites[3], lipids[4-6], peptides[7,8] and proteins[9,10] distribution can readily be obtained. MALDI-MSI can therefore be considered as an emerging method to complement diverse histological staining or immunohistochemistry (IHC). In particularly the IHC is the building pillar of every clinical pathology laboratory, allowing for correlation of protein expression with histological features and is commonly used to detect clinically relevant biomarkers. Unfortunately, the methodology involves many sample preparation steps at which uncertainty can be introduced. In addition, each biomarker requires an antibody that can lead to unspecific reactions. MALDI MSI brings the advantage of preserving the histological information but the mass spectrum has a severe limitation in useful mass range when special detectors are not used.

Although MALDI-MSI methodology seems appropriate for protein biomarker studies as illustrated in previous works[11-13], the main obstacle remains however the limited applicability for *in situ* protein identification. The classical proteomics identification methods namely bottom-up and top-down approaches have been employed *in situ* to identify proteins by MALDI MSI. Bottom-up proteomics is most commonly used, involving enzymatic protein cleavage using enzymes such as trypsin. Digested peptides are then analyzed by MALDI PMF (peptide mass fingerprint) or MS/MS sequencing[14,15]. In the top-down approach, activation techniques (usually ETD and ECD) are used to fragment ions from intact proteins and hence provide sequence information[16]. One of the activation techniques particularly

suitable for MALDI instruments is the in-source decay (ISD)[17-19]. ISD occurs in the hot MALDI plume under a laser power 5-20% above analytes ionization threshold, leading to intermolecular transfer of hydrogen atoms between excited matrix molecules and carbonyl oxygen on the protein backbone[17,20,21]. The backbone is cleaved at N–$C_\alpha$bond, producing c/(z+1) fragment pairs. The (z+1) radicals undergo either gain of a hydrogen to form (z+2) or react with a matrix molecule[22]. C_α–C bonds cleavage can also be observed during MALDI-ISD fragmentation initiated by hydrogen abstraction, generating a- and x-seriesions[23,24]. This particular fragmentation yields a long series of peaks allowing for N- and C-terminal protein sequencing and reliable identification. MALDI-ISD is therefore often referred to as an alternative to Edman sequencing[19,25-27]. MALDI-ISD can be used within tissue sections[28,29] but the spectra become rapidly very difficult to analyze even when additional confirmation of the peptide sequence can be obtained using a pseudo MS/MS analysis of selected ISD fragments, named T^3-sequencing[19,30-33]. Using FTICR analyzers provides higher resolution and mass accuracy measurement compared to TOF analyzers and brings better sequence tags for the identification and the localization of proteins by MALDI-ISD MSI[34].

If the *in situ* identification of unknown proteins is very challenging, the detection of targeted proteins could be easier, knowing the sequence to be searched. In the current work, we present a proof of concept of a rapid targeted *in situ* method for the detection of selected biomarkers directly on histological slides, using MALDI-ISD MSI with a FTICR mass spectrometer. The method relies on the analogy of series of peaks between the sample and spots of the pure target proteins deposited in the sample vicinity. As the method is based on direct comparison of the MS spectra, it eliminates the need for complex computational software. To assess the validity of this approach various hypotheses had first to be verified. The first assumes that the ISD fragmentation patterns are the same when proteins are

deposited pure on a MALDI spot or localized in a tissue. The detection of peptide ions both in the pure protein spot and in the tissue sample by FTICR MS allowed to identify peaks belonging to the same fragmentation series presenting the same localization on images and led to an unambiguous identification of the original protein. The applicability of the method had then to be proven on protein mixtures. Finally, the method had to be applied on both frozen and paraffin embedded tissue sections. We show here that the proposed strategy allows detecting the presence of peptide/protein directly on histological tissue sections, opening new perspectives for the use of MALDI MSI for multiple biomarkers detection in pathology analysis.

EXPERIMENTAL SECTION

Reagents

Purified matrix 1,5-diaminonaphthalene (1,5-DAN) and 1,1,1,3,3,3-Hexafluoro-2-propanol (Hexa Fluoro Iso Propanol, HFIP) were purchased from Sigma-Aldrich, Saint Louis, MO.

Peptide and proteins

Ubiquitin from bovine erythrocytes (Mr 8565 Da), α-crystallin from bovine eye lens (Mr 19778 Da), Bovine Serum Albumin (BSA, Mr 67161 Da), Human Serum Albumin (HSA, Mr 69321), MBP from mouse (MBP, Mr 14113 Da), β-casein from bovine milk (Mr 23568 Da), lysozyme from chicken egg (Mr 16228), myoglobin from equine heart (Mr 17072), cytochrome c from equine heart (Mr 11811), insulin from porcine pancreas (Mr 11664), β-lactoglobulin from bovine milk (Mr 19870) and HNP-1 (Mr3439.5 Da) were purchased from Sigma-Aldrich, Saint Louis, MO. All proteins solution were prepared at a concentration of 10 mg/ml in 0.1% Trifluoroacetic Acid (TFA).

Tissues

Harvesting of Tissues

A 6-months-old Balb C mouse was provided by the Central Animal Housing of Liège University. During intraperitoneal anesthesia with 60 mg/kg pentobarbital, mouse was dissected to extract the brain which was snap frozen by immersion in pre-cooled isopentane at -80°C for 1 min. Brain was kept at -80°C until required.

Eyes coming from pigs aged from 18 to 24 weeks were purchased from the slaughterhouse of Aubel (Detry S.A, Belgium). The eyes were dissected immediately after

harvesting to extract the lenses which were frozen on powdered dry ice for 5 min. Eye lenses were kept at -80°C until required.

Fresh liver metastasis tissue was collected from a 63-years-old female patient at the time of surgery. The patient was resected for rectal adenocarcinoma and associated liver metastasis at the same time, and had no treatment before the resection. The original primary tumor was poorly differentiated (grade 3) with nodal infiltrations. The primary tumor was K-Ras mutated. Sampled liver tissue was fixed overnight in paraformaldehyde solution (4%) and included in paraffin block.

All further tissue manipulations were done on dry ice to minimize the possibility of localized tissue warming.

Tissues Preparation

Mouse brain was placed at -25°C 1 h before use. Tissue sections were prepared on a Microm HM 500 O (Microm, Heidelberg, Germany) with the microtome chamber chilled at -25°C and the specimen holder at -23°C. 14µm thick coronal sections at Interaural 9.70 mm and Bregma 0.70 mm have been realized and thaw mounted onto ITO-coated microscopic slides (Bruker Daltonics, Bremen, Germany) adapted for MALDI mass spectrometry. Following thaw mounting of tissue sections, MALDI target slides were allowed to dry for 1 h in a desiccator.

Porcine eye lens were placed at -25°C 1 h before use. 18µm thick equatorial sections have been realized, thaw mounted onto ITO-coated microscopic slides and dried for 1 h in a dessiccator.

Liver tissue was sectioned at 10 µm thickness and placed on ITO conductive slides. Prior to MALDI-imaging analysis, the tissue was subjected to de-paraffination step in xylene bath

followed by methanol baths of decreasing concentration (100% - 50%). Next, the tissue was subjected to antigen retrieval step conducted in citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) bath at 95°C for 30 min. Finally, the slide was air-dried and coated with MALDI matrix.

Then, mouse, porcine eye lens and liver tissue sections were washed six times using the following protocol previously described[35]: 70% ethanol, 100% ethanol, Carnoy's fluid (60% ethanol, 30% chloroform, and 10% acetic acid), 100% ethanol, H2O, 100% ethanol. All rinse steps were carried out for 30 s except for the step with Carnoy's solution, which was for 2 min. Following washing, ITO slides were dried in a desiccator for 1 h before matrix deposition. Finally, 3 µL of MBP, α-crystallin or HNP-1 solutions (1 mg/ml in 0.1 % TFA) were spotted near mouse brain, porcine eye lens or normal/diseased liver sections and allowed to dry under vacuum for 10 min.

Protein spots preparation for MALDI-ISD MSI experiments

For the first MALDI-ISD MSI experiments on protein spots, 1pmol of β-casein, MBP, ubiquitin, BSA and α-crystallin were deposited on ITO-coated microscopic slides. Then, 2 pmol of each protein solution were mixed and deposited on the same ITO-coated microscopic slides. For the second experiments, 1 pmol of β-casein, MBP, ubiquitin, BSA and α-crystallin were deposited on a ITO-coated microscopic slides. Then, 2 pmol of MBP, BSA and α-crystallin, 1 pmol of ubiquitin and 0.2 pmol of β-casein were mixed and deposited on the same ITO-coated microscopic slides. Spots were then allowed to dry under vacuum for 10 min.

Protein mixture spots preparation for MALDI-ISD experiments

Spots of 1pmol of β-casein, MBP, ubiquitin, HSA, lysozyme, myoglobin, cytochrome c, insulin, β-lactoglobulin and α-crystallin were deposited on the same ITO-coated microscopic slides. Spots were then allowed to dry under vacuum for 10 min.

Matrix deposition for protein solutions and tissue MSI

1,5-DAN matrix was used for all experiments. For MALDI-ISD imaging, other MALDI matrices (2,5-dihydroxybenzoic acid, 2-Aminobenzamide and 2-aminobenzoic acid) were tested and does not allowed to obtain intense ISD fragments ions as observed with 1,5-DAN (data not shown). For all the experiments, 1,5-DAN matrix (6 mg/mL solution in ACN/0.2% TFA 70:30 vol/vol) deposition was performed with an ImagePrep automated sprayer device equipped with the new spray head (Bruker Daltonics) as previously described[29].

HFIP treatment and matrix deposition for MALDI-ISD analyzes of protein mixture spots and mouse brain tissue sections

For on tissue and protein spot solvent-based protein extraction, 10 µL of pure HFIP solution was manually dropped following a procedure adapted from Longuespée *et al.*[36]. For tissue sections, a first volume of 2 µL of HFIP was deposited in order to condition the protein tissular environment to the highly hydrophobic solvent HFIP. For protein extraction on tissue and protein spot, four droplets of 2 µL of HFIP were deposited, each followed by up and down manual pipetting. After the drying of the preparation, a droplet of 1,5-DAN (6 mg/mL solution in ACN/0.2% TFA 70:30 vol/vol) was deposited on the prepared area. The volume of this last droplet is adapted in function to the size of the area to analyze.

MALDI-FTICR mass spectrometry

Mass spectra were acquired using a SolariX FTMS (9.4 T) equipped with a ESI/MALDI Dual Ion Source including smartbeam™II laser (wavelength, 355 nm; focus setting, 'minimum'; repetition rate, 1000 Hz). MALDI MSI experiments were acquired with a pixel step size for the surface raster set to 150 µm in FlexImaging 3.0 software. Spectra were externally calibrated using a peptide mix calibration standard. For MALDI-ISD MSI and MALDI-ISD analyzes, spectra were acquired in positive ion mode from 120 and 4000 laser shots accumulated at each spot, respectively. The laser intensity was set to 20% with a frequency of 150 Hz. Q1 mass was fixed at *m/z* 750 and *m/z* 350 for protein spots and tissue sections analyzes, respectively. Ion cooling time and time of flight values were set to 0.02 s and 7 ms, respectively.

For each protein fragmented by MALDI-ISD, monoisotopic masses of an acquired spectrum selected from FlexImaging 3.0 were labelled using DataAnalysis 4.0 with the SNAP peak-picking algorithm and exported in .bsc format. Spectra in .bsc format were then permanently assigned as ISD-type in BioTools 3.2 software. A default approach was used to assign peaks with a mass tolerance comprised between 3 and 6 ppm. For all proteins, the sequence was transferred from Sequence Editor 3.2 and sequence tags were automatically determined and annotated on the spectrum. Results were interpreted in combination with a local intranet Mascot™ (Matrix Science, London) server. For top-down sequencing searching, the "virtual precursor" approach has been used. A high mass c ion is automatically selected as virtual precursor for the MS/MS ion search. 1 Da is added to the mass of the c ion for database searching. All MALDI-ISD spectra were searched against the NCBI-nr database. No enzyme cleavage was specified and protein N-terminus acetylation, or more of the following modification were set as variable: lysine acetylation and serine/threonine

phosphorylation. Charge state of +1 were considered for parent ions. Mass tolerance was set to 0.1 % for parent ion masses and ±0.1 Da for fragment ion masses. Identification of proteins were validated for a Mascot score of 30 or above.

RESULTS AND DISCUSSION

In situ identification of proteins on tissue sections requires either their localized extraction or their digestion with enzymes, conducing to a loss of lateral resolution. MS/MS is required to sequence the peptides but is difficult to perform due to the large number of peaks making very difficult the precursor selection. Unsupervised image analysis is still time consuming and brings only the definition of regions of interest. When looking for the presence of target proteins for biomarkers detection in tissue slices, a faster and simpler protocol would be helpful to improve the throughput. *In situ* protein identification by MALDI-ISD does not require treating tissue sections[29]. However, in the current workflow, the identification of proteins from mixtures by MALDI-ISD still requires MS/MS sequencing of an ISD fragment by T^3-sequencing[30]. This last step is generally easy to perform from ISD fragments of pure proteins but is difficult in complex samples. In the proposed strategy, we assign fragments by comparison with series of peaks observed in the spectra of spots of pure target proteins deposited besides the tissue section. In addition, we use localization information of fragments to reconstitute series belonging to the same protein. The presence of a fragment in an area of the sample as well as in the spot of a target protein simplifies the analysis of the complex spectra in targeted analysis mode. We hope, with that strategy, to increase the dynamic range and speed-up the image analysis.

To develop this method, the following questions should first find answers:

1 Can MALDI-ISD MSI detect *in situ* a target protein finding a sequence tag similar to that obtained for the spot of pure proteins?

2 Can MALDI-FTICR ISD be used to directly identify several proteins in a tissue section?

1 Can MALDI-ISD MSI detect *in situ* a target protein finding a sequence tag similar to that obtained for the spot of pure proteins?

Here we show on a model system that FTICR MS allows identifying proteins by MALDI-ISD MSI by the comparison with peaks obtained from a pure protein spot. Figure 1 shows a MALDI-ISD MSI experiment performed on pure MBP and α-crystallin solutions spots. As evidenced in Figure 1A, it is possible to assign specifically ISD fragment ion peaks of each protein deposited from synthetic solutions. Identification by ISD can be obtained, by comparison between ISD ion images of a given protein. As shown on upper panels of Figure 1A, c_{10} and c_{14} ions images of MBP (red spots) and $^{\alpha A}c_{10}$ and $^{\alpha B}c_{16}$ ions images of α-crystallin (blue spots) are identical and confirm the identification. MS/MS from an intense ISD fragment is therefore not required anymore for a reliable identification. In the latter, the protein can be identified by Mascot search from a mass spectrum selected from a pixel with maximum intensity (identifications with a score of 1021 for MBP and of 312 for α-crystallin in Figure 1B, respectively).

The same experiment was performed on histological tissue sections to find correlations between the ISD ion images. The “co-localization of fragments and MALDI-ISD profile matching” is interesting to qualitatively detect in a tissue section a set of peak belonging to the same protein as the one spotted near the tissue. For example, a MALDI-ISD MSI experiment was performed on a horizontal mouse brain section and on a pure MBP solution spot filled near the tissue section (Figure 2A). A signal, both within the white matter of the central nervous system where is localized this protein and the MBP spot, is observed in the c_{15} ion image (upper right panel of Figure 2A) and signal is correlated with hematoxylin and eosin (H/E) staining (upper left panel of Figure 2A). As shown in Figure S2, all the selected ISD ions of the MBP (c_{10}, $c_{15}$and c_{19}) triggers the same signal both within the white matter of

the central nervous system where is localized this protein and the MBP spot. Mascot searches results for a mass spectrum selected from a pixel with maximum intensity confirmed the identification of the MBP with a score of 1002 (Figure 2A and S1A). Alternatively, a list of peaks taken from the tissue and similar to peaks in the reference spot can confirm the identification using database search. We performed the same experiment on a porcine eye lens section and a spot of αA/B-crystallin solution (Figure S3). A signal is simultaneously observed on the ion images of the c_n ISD ions of the αA- (data not shown) and αB-crystallin (c_{11} ion for example, Figure S3) isotypes, both in the tissue section and the αA/B-crystallin spot. On the contrary, no signal is observed for the αA/B-crystallin spot on the ion images of the c_n ISD ions of the γ-crystallin (c_{14} ion as example, Figure S3). As previously presented by Han *et al.*, same localization of the crystallin isotypes is observed[37]. Ion images of c_{11} ion of αB-crystallin and c_{14} ion of γ-crystallin is correlated with H/E staining image (Figure S3A). αB-crystallin is localized in the periphery of the eye lens whereas γ-crystallin is in the central part. Mascot searches results for mass spectra selected from pixels with maximum intensity confirmed the identification of the αA/B-crystallin and γ-crystallin isotypes with scores of 123 and 91, respectively (Figure S3). We demonstrated here that it is possible to identify isotypes of a given protein and observe their expression within a tissue section without any doubt.

To consider if this approach could complement histopathology analysis based on native molecular signatures in tumors and the surrounding tissues, we applied it to identify unambiguously a known tumor necrosis biomarker, HNP-1. HNP-1 is a peptide that belongs to the α-defensin family. It is synthesized in neutrophil precursor cells and is released by mature circulating neutrophils at inflammatory sites[38-40]. Several studies have shown that HNP-1 is overexpressed by eosinophils[41] and neutrophils[42,43] in cancer cells like renal cell

carcinoma[44] but also oral squamous cell carcinoma or colorectal cancer[43] and is associated with tumor growth and proliferation. Another study led by MALDI MSI has indicated that HNP-1 is mostly expressed in the necrotic tissue area of cancer tissue so that it can be assimilated as a tumor necrosis biomarker[45]. Figure 2B shows a MALDI-ISD MSI experiment performed on a liver tissue section of a patient with colorectal cancer liver metastasis and two spots of HNP-1 (positive control) and MBP (negative control) solutions. In the same way, a signal is observed for both the necrotic core of the liver tissue section and the spot of HNP-1 for c_7, c_{10} and c_{13} ISD ions (upper right panel of Figure 2B and Figure S4A). These results are entirely consistent with a H/E staining previously done on this tissue section (upper left panel of Figure 2B) and a Mascot searches result for a mass spectrum selected from a pixel with maximum intensity in the necrotic core that confirmed the identification of HNP-1 with scores of 1581 (lower panel of Figure 2B and Figure S1B). On the contrary, as expected, signals for the c_8, c_{14} and c_{17} ISD ions of the MBP (negative control) are not observed in the tissue section (Figure S4B).

As shown in of Figure S3, this highlights that this approach could be used for targeted analysis of known biomarkers. For that purpose, it is essential that several potential biomarkers, with an increased dynamic range can be identified by MALDI-ISD.

2 Can MALDI-FTICR ISD be used to directly identify several proteins in a tissue section?

Figure 3 shows a MALDI-ISD MSI experiment performed on five spots of ubiquitin, α-crystallin, BSA, MBP and β-casein pure solutions and an additional spot corresponding to an equimolar mixture of these proteins (called “the 5-proteins mixture” further in the text). According to the signals observed on the ion images of Figure 3 (c_{11} and $(z+2)_{15}$ for ubiquitin,

c_{14} and c_{16} for α-crystallin, c_{12} and c_{24} for BSA, c_{10} and c_{12} for MBP, and y_{17} and y_{19} for β-casein), it is possible to monitor five proteins by MALDI-FTICR ISD MSI. Moreover, the spots of pure protein solutions provide a direct identification, by MALDI-ISD and without MS/MS sequencing, of the proteins present in the mixture. For each protein, sequence tags have been identified from mass spectra selected from pixels of high intensity (Figure S5) and Mascot searches confirmed their identification except for the α-crystallin that present a Mascot score under the identity threshold (Figure S6). As shown in Figure S5B, most of the ISD peaks of the α-crystallin are slightly above the baseline and can explain this low Mascot score. This low yield of ISD fragments can be explained by either fragmentation or ionization yield that can be affected by the presence of specific amino acids residues and the accessibility for the hydrogen radical[17, 46-49]. A deeper understanding of the ISD process is mandatory to fully exploit the method.

To determine if the relative protein concentration can favor the ISD fragmentation of the α-crystallin, we performed the same experiment with different molar ratios of ubiquitin and β-casein 10-times and 2-times lower in the "5-proteins mixture", respectively (Figure 4). According to the signals observed on the ion images of Figure 4 (c_{20} and $(z+2)_{19}$ for ubiquitin,c_{14} and c_{16} for α-crystallin, c_{10} and c_{11} for BSA, c_8 and c_{11} for MBP, and y_{17} and y_{19} for β-casein), it is possible to identify five proteins by MALDI-FTICR ISD MSI. MALDI-ISD allowed to determine sequence tags of α-crystallin, BSA and MBP from mass spectra selected from pixels with maximum intensity for each protein in the "5-proteins mixture" (Figure S7) for their identification by Mascot searches (Figure S8). However, no sequence tags long enough to allow the identification of ubiquitin and β-casein by Mascot searches were found.

A major issue of the top-down ISD for *in situ* identification is the high dynamic range required to detect low abundance proteins. It is now well known that a simple ISD analysis of a tissue, without any prior tissue preparation can only give access to the identification of the major proteins, then reducing the usefulness of the method[29, 50]. For example, the ISD on tissue analysis of brain sections mostly allows the tagging of MBP. Here we describe a method that can guide the user to increase the number of proteins identified. However, the relative amount of some proteins still remains problematic since only identification of the most prevalent proteins can be addressed using this method. This could be detrimental for histopathological applications since the amount of some markers of interest, even if overexpressed in the pathological context, could have an expression under the most prevalent proteins. We then speculate that the use of chemical sample preparation, specifically applied to tissue section could allow the identification of protein by MALDI-ISD by their extraction and their unmasking at the surface of the tissue section. Then, the disadvantage of the low amount of the proteins of interest can be overcome by the exposition of these proteins at the surface of the tissue, and the 1,5-DAN matrix. Longuespée *et al.* previously described a method involving the use of the highly hydrophobic solvent HFIP that was used for the detection of the C-terminal fragment of Reg-Alpha, which was not detected without this specific procedure[36]. They previously revealed that this procedure allowed extending the mass range for *in situ* protein analysis until 70 kDa[51]. We then decided to adapt this method to this present issue in order to allow the unmasking of the proteins present in tissues. We then deposited this solvent on a mouse brain tissue section in separated step in order to condition the tissue to the highly hydrophobic property of the solvent and to address the problem of the spreading of the solvent. We speculate that the first drop allows the exposition of the most hydrophobic parts of the proteins. The next

drops are then necessary to extract these proteins at the surface of the tissue for their incorporation in 1,5-DAN matrix crystals. The Figure 5A illustrates the comparison between ISD analyzes of serial mouse brain sections with and without prior tissue preparation. This shows that the number of peaks is greatly improved using this method (supplementary peaks are annotated with red arrows on the lower mass spectrum of Figure 5A). Supplementary tags have been determined from these supplementary peaks but only one allowed the identification of a protein by MASCOT searches (data not shown). As shown in Figure 5B, the hemoglobin has been identified from an ISD fragment tag by MASCOT searches. This protein, which is known to be secondly detected in rodent brain sections is here detected, exclusively in the context of a HFIP extraction procedure. The same approach has been applied on an equimolar mixture of ten proteins (Figure S9). As shown in Figure 5A, supplementary ISD ions are detected using the HFIP extraction procedure (supplementary peaks are annotated with red arrows on the lower mass spectrum of Figure S9). Moreover, MALDI-ISD data acquired with a FTICR MS are sufficiently accurate (mass accuracy measurement below 2 ppm) for the identification of the ten proteins based on MASCOT searches from ISD fragment tags (Figure S10, S11, S12 and S13). These experiments show that some improvements are possible to allow the multiplexed *in situ* protein identification using chemical procedures for on-tissue protein extraction. These chemical procedures could then be combined to this presented issue in order to guide the identification of many proteins.

CONCLUSION

MALDI MSI is now a proven new diagnostic tool complementary to histochemistry when analyzing molecular signatures to define regions of interest. It can lead to differentiate different compartments such as the tumor and the surrounding tissues using spectral patterns. MALDI-ISD has proven to be an efficient method for the N- and C-terminal sequencing of proteins present in tissue sections and for biomarkers identification. Coupling MSI for localization and MALDI-FTICR ISD for identification by comparison with spots of pure target proteins could allow a fast-targeted detection and identification of known proteins by comparison with side deposited spots of target proteins. These can have been identified by any other approach if they are available as a pure protein. Combined with software dedicated to protein identification from ISD data, the proposed method can be the basis of a new diagnostic tool complementary to histopathology analysis based on native molecular signatures in tumors and the surrounding tissues. Further improvements will tend to increase the yield of ISD fragmentation of protein present in tissue sections. For this, specific treatments such as protein extraction procedure were used here to allow a better exposition of the proteins to the matrix crystals and allow the identification of additional proteins. A special attention will be devoted to keep intact of the lateral distribution that could be affected during the use of solvents.

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Figure 1.MALDI-FTICR ISD MSI of MBP and α-crystallin pure protein solutions.

A. Average mass spectrum of a MALDI-FTICR ISD MSI analysis of MBP and α-crystallin spots. ISD fragments of each protein are annotated (in red for MBP and blue for α-crystallin) on the average mass spectrum. Right inset show isotopic patterns of $^{\alpha B}c_{14}$ and c_{16} ions and associated imaging signal of αB-crystallin and MBP, respectively. Left inset shows an overlap of the ion images corresponding to the c_{10} ions of MBP (in red) and αA-crystallin (in blue).

B. ISD mass spectra selected from protein spots with monoisotopically assigned fragment ions of MBP (upper spectrum) and α-crystallin (lower spectrum). Insets indicate Mascot searches results for both ISD mass spectra.

Figure 2.MALDI-FTICR ISD MSI on tissue sections and pure protein/peptide solutions.

A. Left upper panel shows an ion image of c_{15} ISD ion of MBP in a mouse brain tissue section and a MBP spot. Right upper panel shows hematoxylin/eosin staining of a serial tissue section. Dotted lines delineate the anterior commissure, anterior part (*aca*), the external capsule (*ec*) and the cingulum (*cg*). *CPu* corresponds to the caudate putamen (striatum); *ec*, the external capsule; *MS*, the medial septal nucleus; *Pir*, the piriform cortex; *E*, the ependyma and sub ependymal layer. Lower panel corresponds to an ISD mass spectra selected from a pixel with maximum intensity (white arrow) with monoisotopically assigned fragment ions of MBP. Inset indicates Mascot searches result for this ISD mass spectrum.

B. Left upper panel shows an ion image of c_{10} ISD ion of HNP-1 on a liver tissue section with colorectal cancer metastasis and two spots of MBP and HNP-1. Right upper panel shows hematoxylin/eosin staining of a serial tissue section. Dotted lines delineate necrosis "*N*" area. Lower panel correspond to an ISD mass spectra selected from the necrosis area of the liver tissue section (white arrow) with monoisotopically assigned fragment ions of MBP. Inset

indicates Mascot searches result for this ISD mass spectrum.

Figure 3.MALDI-FTICR ISD MSI of an equimolar 5-protein mixture and pure protein solutions.

Ion images correspond to the signal acquired, on an equimolar 5-protein mixture and pure protein solutions, for the ISD ions of ubiquitin (c_{11} and $(z+2)_{15}$), α-crystallin (c_{14} and c_{16}), BSA (c_{12} and c_{24}), MBP (c_{10} and c_{12}) and β-casein (y_{17} and y_{19}). Numbers indicate the position of each pure protein solutions (1: ubiquitin, 2: α-crystallin, 3: BSA, 4: MBP and 5: β-casein).

Figure 4.MALDI-FTICR ISD MSI of a non-equimolar 5-protein mixture and pure protein solutions.

Ion images correspond to the signal acquired, on an equimolar 5-protein mixture and pure protein solutions, for the ISD ions of ubiquitin (c_{20} and $(z+2)_{19}$), α-crystallin (c_{14} and c_{16}), BSA (c_{10} and c_{11}), MBP (c_{8} and c_{11}) and β-casein (y_{17} and y_{19}). Numbers indicate the position of each pure protein solutions (1: ubiquitin, 2: α-crystallin, 3: BSA, 4: MBP and 5: β-casein).

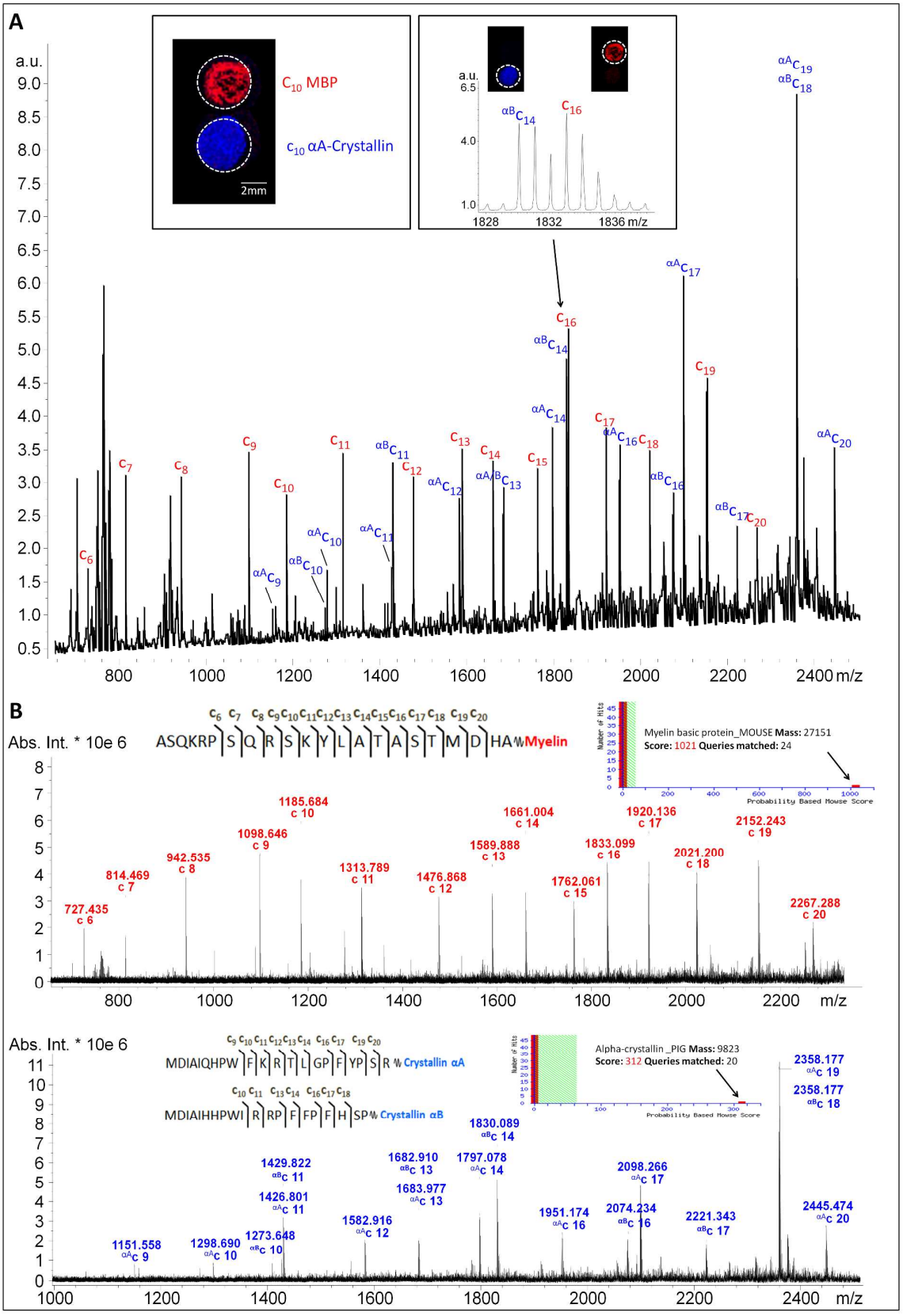
At the difference of analysis of Figure 3, quantities of ubiquitin and β-casein are respectively 10-times and 2-times lower in 5-protein mixture for this experiment.

Figure 5. MALDI-FTICR ISD analyzes on mouse brain tissue sections treated or not with HFIP.

A. MALDI-FTICR ISD analyzes of serial mouse brain tissue sections treated (lower mass spectrum) or not with HFIP (upper mass spectrum). c and (z+2) on the upper mass spectrum correspond to ISD fragments of MBP. Red arrows on the lower mass spectrum indicate peaks corresponding to supplementary ISD ions detected after HFIP extraction.

B. Upper panel shows ISD mass spectrum with monoisotopically assigned fragment ions of hemoglobin. Lower panel corresponds to Mascot search results for hemoglobin identification. Sequence coverage of protein termini is indicated in red.

Fig. 1


A
a.u.
C10 MBP
c10 αA-Crystallin
2mm
1828
1832
1836 m/z
B
Abs. Int. * 10e 6
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Myelin basic protein_MOUSE Mass: 27151
Score: 1021 Queries matched: 24
Probability Based Mouse Score
m/z
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MDIAIQHPWFKRTLGPFYPSRW Crystallin αA
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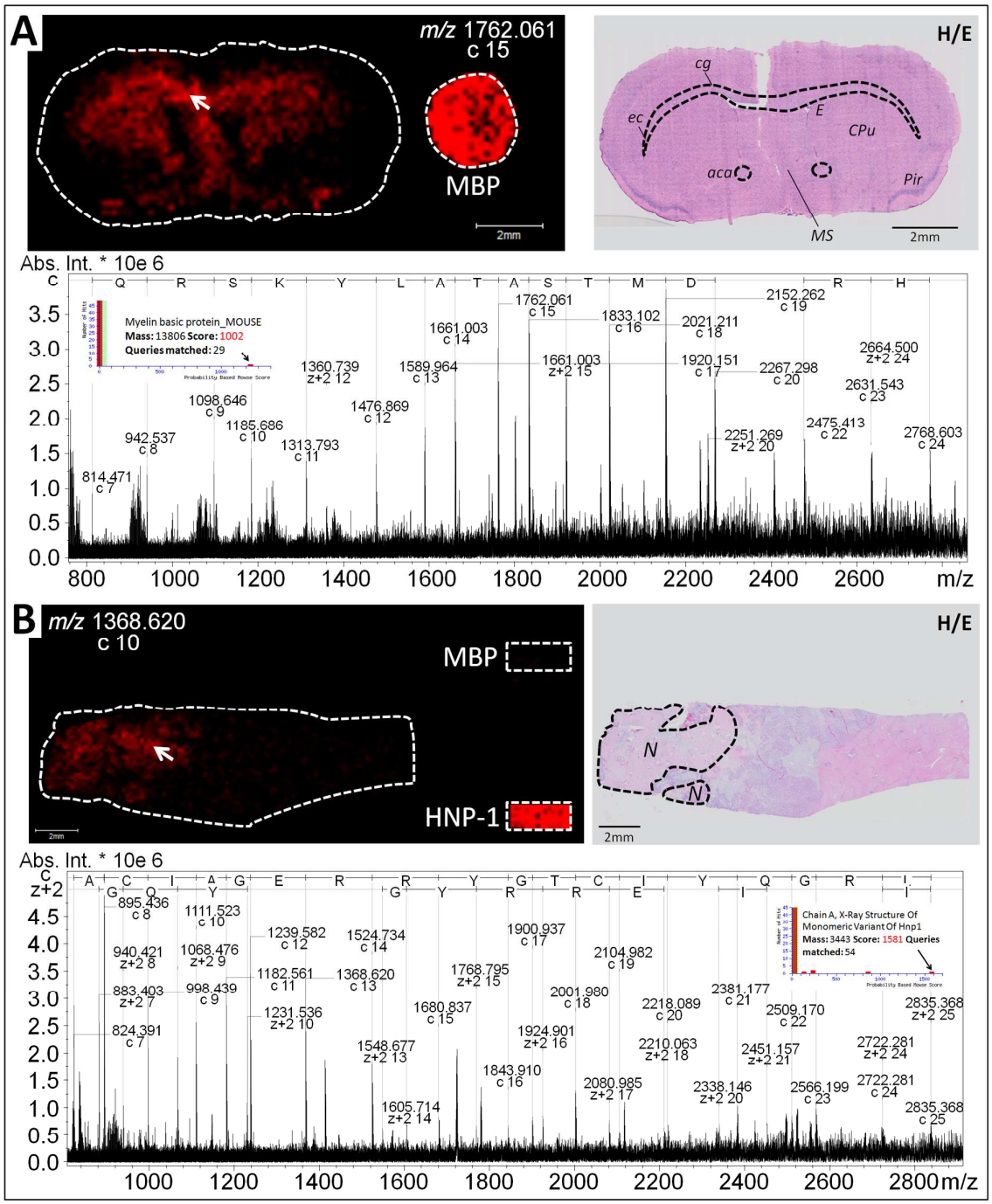
Score: 312 Queries matched: 20
Probability Based Mouse Score
m/z

Fig. 2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c 15
MBP
2mm
H/E
cg
ec
E
CPu
aca
Pir
MS
2mm
Abs. Int. * 10e 6
Myelin basic protein_MOUSE
Mass: 13806 Score: 1002
Queries matched: 29
m/z
B
m/z 1368.620
c 10
MBP
HNP-1
H/E
N
N
2mm
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Chain A, X-Ray Structure Of Monomeric Variant Of Hnp1
Mass: 3443 Score: 1581 Queries matched: 54
m/z

Fig. 3

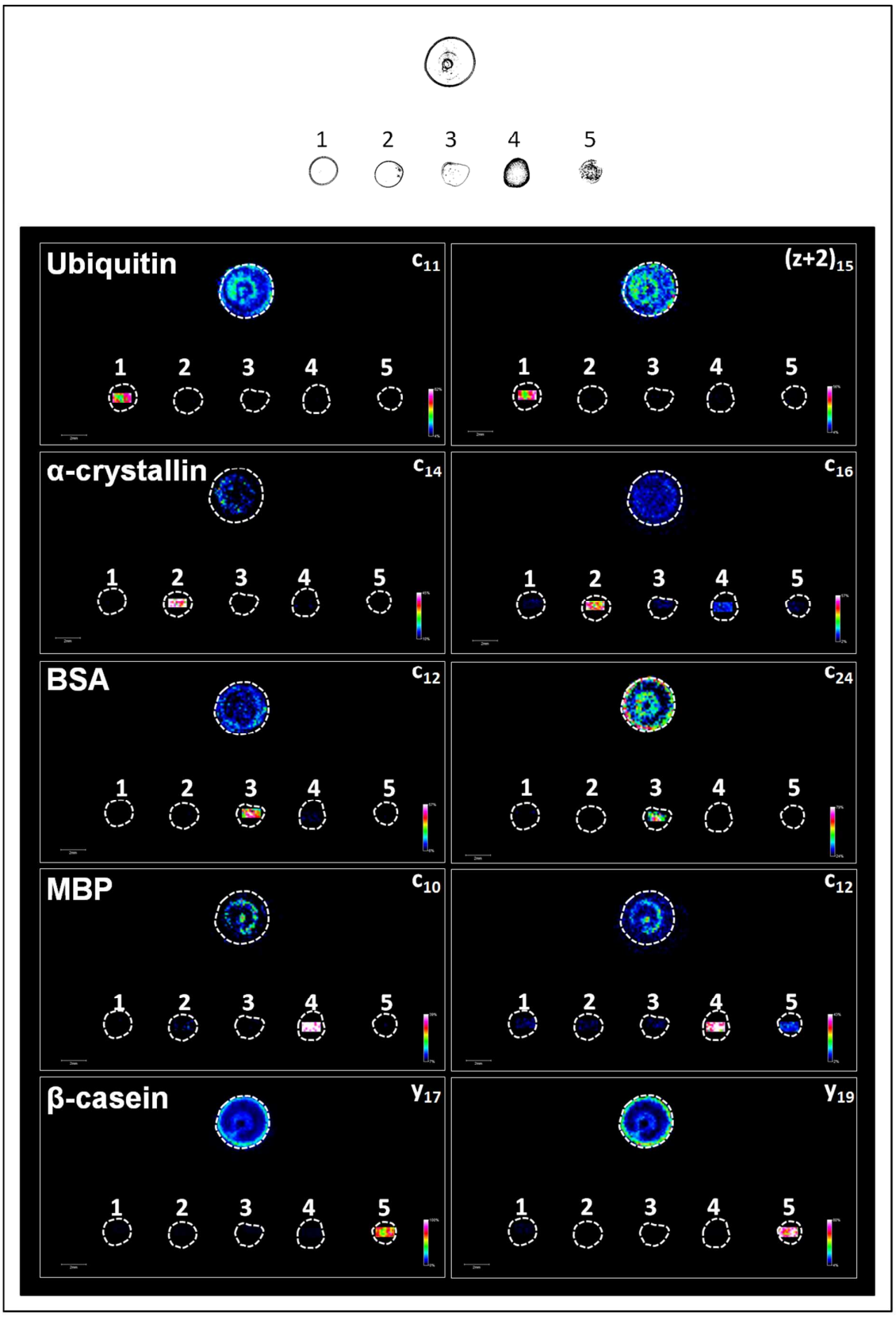
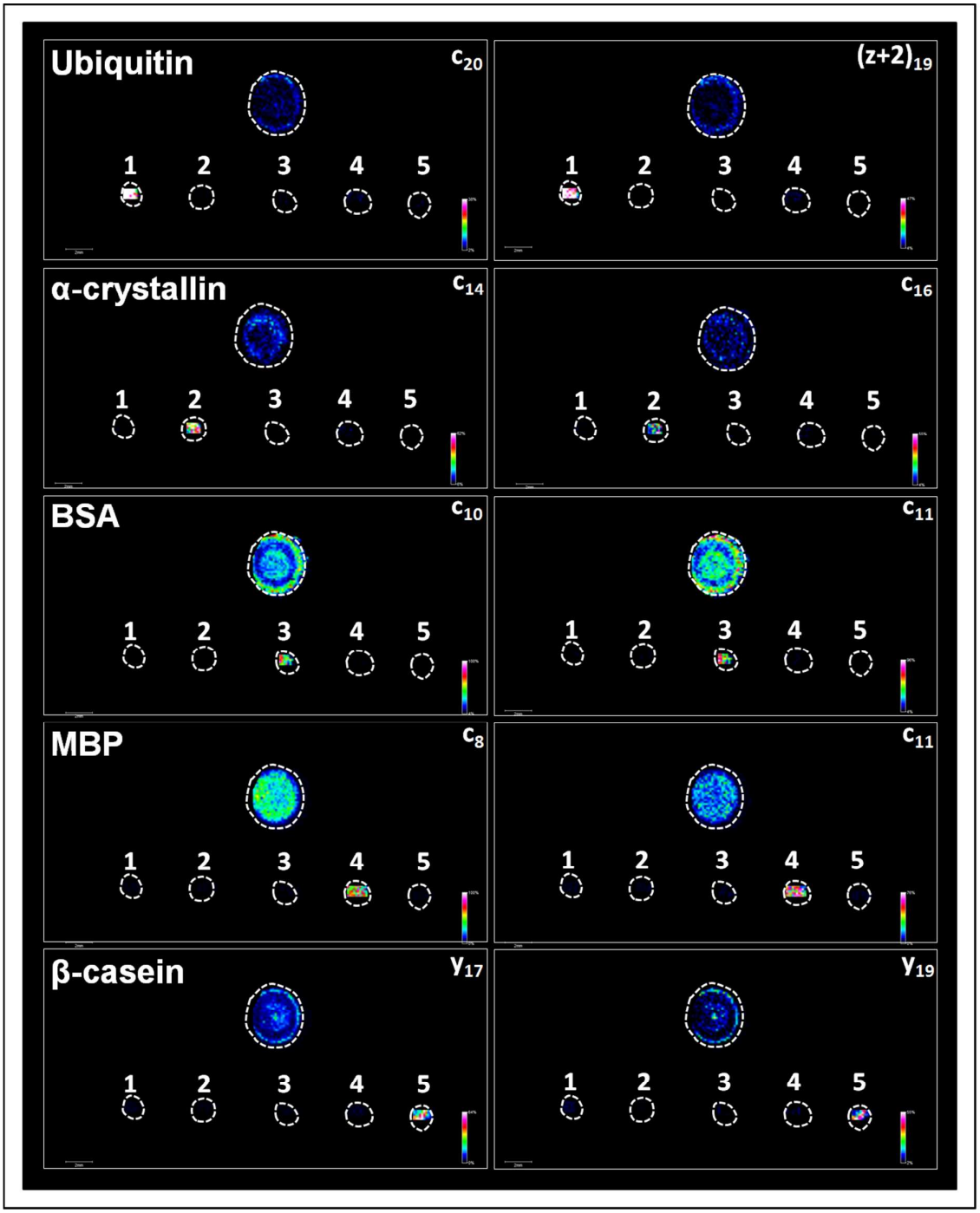

1 2 3 4 5
Ubiquitin
c_{11}
$(z+2)_{15}$
α-crystallin
c_{14}
c_{16}
BSA
c_{12}
c_{24}
MBP
c_{10}
c_{12}
β-casein
y_{17}
y_{19}

Fig. 4


Ubiquitin
c_{20}
$(z+2)_{19}$
α-crystallin
c_{14}
c_{16}
BSA
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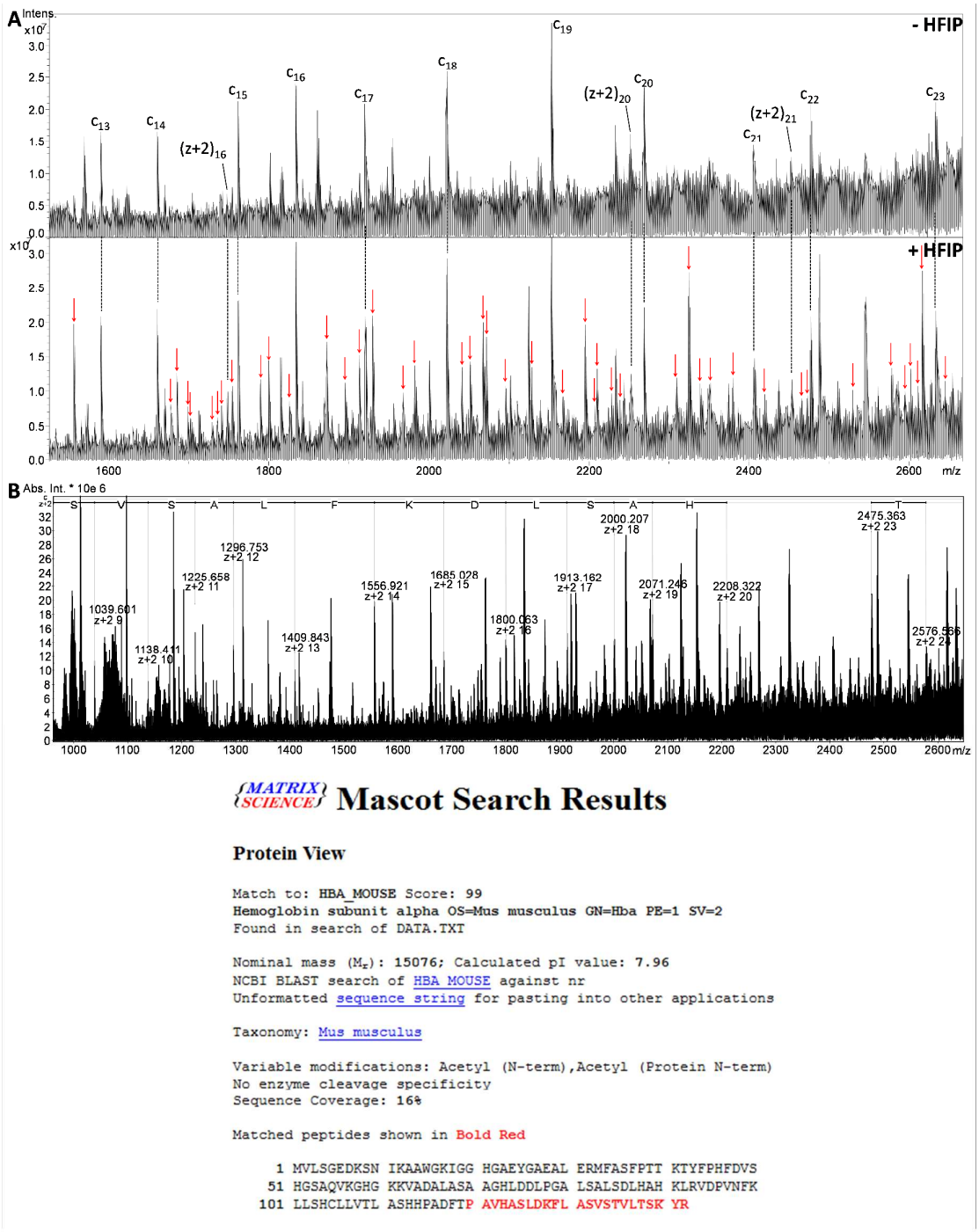
c_{11}
MBP
c_{8}
c_{11}
β-casein
y_{17}
y_{19}
1 2 3 4 5

Fig. 5



MATRIX SCIENCE **Mascot Search Results**

Protein View

Match to: HBA_MOUSE Score: 99
Hemoglobin subunit alpha OS=Mus musculus GN=Hba PE=1 SV=2
Found in search of DATA.TXT

Nominal mass (M_r): 15076; Calculated pI value: 7.96
NCBI BLAST search of HBA_MOUSE against nr
Unformatted sequence string for pasting into other applications

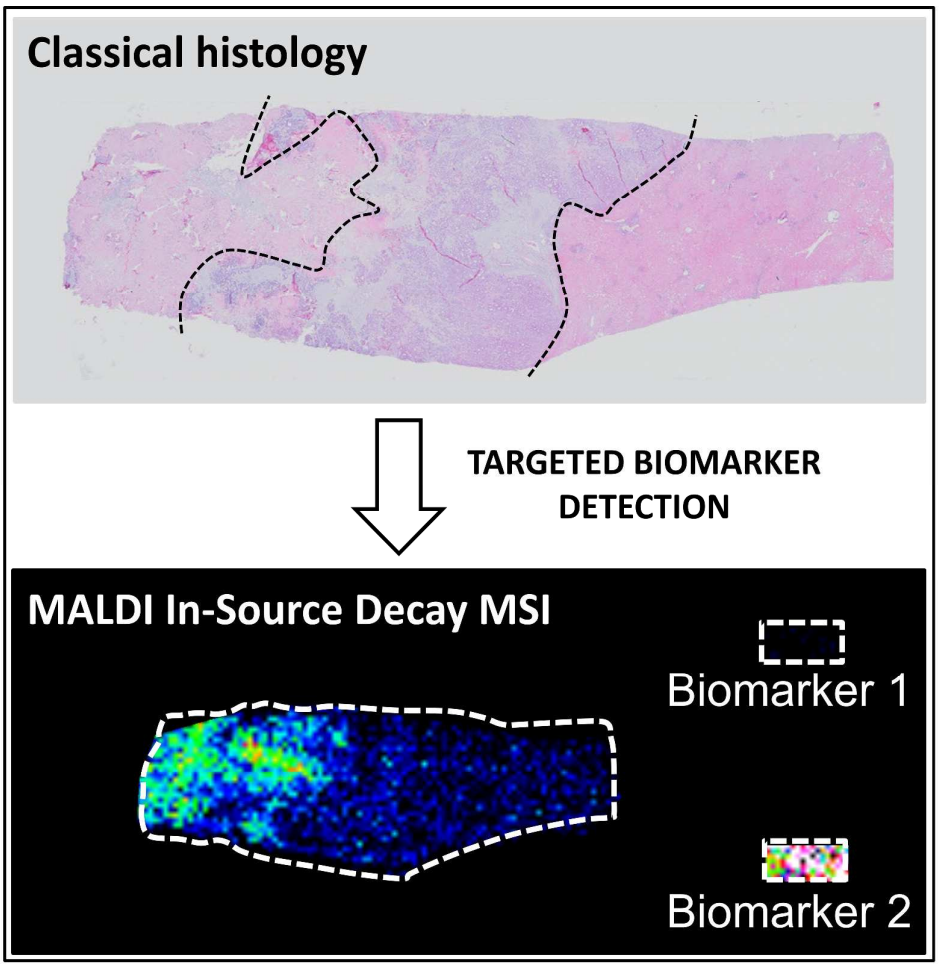
Taxonomy: Mus musculus

Variable modifications: Acetyl (N-term),Acetyl (Protein N-term)
No enzyme cleavage specificity
Sequence Coverage: 16%

Matched peptides shown in Bold Red

```
     1 MVLSGEDKSN IKAAWGKIGG HGAEYGAEAL ERMFASFPTT KTYFPHFDVS
    51 HGSAQVKGHG KKVADALASA AGHLDDLPGA LSALSDLHAH KLRVDPVNFK
   101 LLSHCLLVTL ASHHPADFTP AVHASLDKFL ASVSTVLTSK YR
```

For TOC only


Classical histology
TARGETED BIOMARKER DETECTION
MALDI In-Source Decay MSI
Biomarker 1
Biomarker 2