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DIRECT ANALYSIS AND MALDI IMAGING ON FORMALIN FIXED PARAFFIN EMBEDDED TISSUE SECTIONS

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Abstract : Formalin fixation, generally followed by paraffin embedding, is the standard and well established processing method employed by pathologist. This treatment conserves and stabilizes biopsy samples for years. Analysis of FFPE tissues from biopsy libraries has been, so far, a challenge for proteomics biomarker studies. Herein, we present two methods for the direct analysis of formalin fixed paraffin embedded (FFPE) tissues by MALDI/MS. The first is based on the use of a reactive matrix, 2,4-dinitrophenylhydrazine, useful for FFPE tissues stored less than one year . The second approach is applicable for all FFPE tissues regardless of conservation time. The strategy is based on *in situ* enzymatic digestion of the tissue section after paraffin removal. *In situ* digestion can be performed on specific area of the tissue as well as on very small area (micro-digestion). Combining automated micro-digestion according a predefined tissue array with either *in situ* extraction prior to classical nanoLC/MS-MS analysis or automated micro-spotting if MALDI matrix according to the same array allows for

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both protein's identification in nanoLC-nanoESI and imaging by MALDI. Using adjacent tissue sections, it is, thus, possible to correlate protein identification and molecular imaging. These combined approaches, along with FFPE tissue analysis provides access to massive amounts of archived samples in the clinical pathology setting.

Abbreviations footnote: FFPE, formalin-fixed paraffin-embedded; nanoLC, nanoflow liquid chromatography; OCT, optimal cutting temperature compound; MALDI, Matrix Assisted Laser Desorption/Ionisation; MS, Mass Spectrometry; DNPH, 2,4-dinitrophenylhydrazine;HCCA, α-cyano-4-hydroxycinnamic acid; SA, Sinapinic Acid; ITO, Indium Teen Oxide

INTRODUCTION

Since its introduction in the mid 80's[1-3], MALDI mass spectrometry has become a powerful tool in biological research, especially in proteomics. Recently by Caprioli[4-6] and other groups[7-13] have developed MALDI techniques for direct tissue analysis and molecular imaging allowing the detection and localization of a large number of compounds directly from tissue sections in one acquisition. However, this novel MALDI application has only been successfully carried out on fresh frozen tissues, without fixation or tissue processing for conservation, except for ethanol fixation[14] or in direct analysis on invertebrates[15]. Nonetheless, a major source of tissue samples are FFPE tissues found in hospitals libraries. Even if FFPE tissues are extensively used for histological, immunohistochemical or ISH studies, such tissues have only been very few studied for obtaining molecular information either on DNA or proteins. MALDI was already used to identify SNP from DNA isolated from FFPE tissues[16]. However, for protein identification from FFPE tissues, approaches using either LC-MS/MS [17-19] or 2D gels electrophoresis were mainly reported in the literature[20-22]. These studies demonstrate that large numbers of proteins can be identified in profiling. They also shown that same proteins can be identified independently of the conservation used since identification is comparable for frozen and FFPE tissues[22]. Recent reviews summarized the advancements in proteomic of FFPE tissues [23, 24]. However, LC-MS/MS and 2D gel electrophoresis on protein extracts do not provided anatomical localization data of these peptides/proteins. Potentiality of MALDI-MS direct analysis and imaging on FFPE tissues was not investigated. Currently immunohistochemistry is the best technology for providing such information. The drawback of this approach requires a priori knowledge of the protein/peptide candidates and the production and use of specific antibodies. Simultaneous localization of several proteins/peptides in one experiment is tedious, generally providing

information on two to three markers at the same time. Thus, immunohistochemistry is more amendable for biomarker validation and less so for biomarker discovery.

MATERIAL and METHODS

Material

α-cyano-4-hydroxycinnamic acid (HCCA), sinapinic acid (SA), 2,4 dinitrophenylhydrazine (DNPH), ammonium bicarbonate, trisma base, xylene, ethanol, Angiotensin II, Des-Arg-Bradykinin, Substance P, ACTH 18-39, ACTH 7-38 and bovine Insulin (Sigma-Aldrich). Trypsin (Promega), Asp-N and Lys-C endopeptidases (Roche), Trifluoroacetic acid (Applied Biosystems.) Acetonitrile p.a. and methanol p.a. (J.T. Baker). Indium Teen Oxide (ITO) coated glass slides were from Bruker Daltonique (Wissembourg, France).

Tissue fixation

Adult male Wistar rats weighing 250-350g (animal welfare accreditation by the French ministry of the agriculture N° 04860) maintained under standard care were used. Five Animals were sacrificed by decapitation and immediately dissected to remove the brain. Tissues were frozen at -80°C for good conservation. Formalin fixation was obtained according to classical procedures. Briefly, after dehydratation in successive graduated ethanol bathes, tissue was fixed with 4% formalin in Tris-HCL buffer pH 7.4 during 24 hours. Tissues were, then, embedded in paraffin using xylene and strored in box at room temperature until use [25].

Tissue dewaxing and preparation

Tissue sections of 10µm were applied onto ITO (Indium Teen Oxided) coated conductive glass slides. Paraffin was removed by 2 baths of 5 minutes of xylene and lightly rehydrated with graded ethanol (100°, 96° and 70°) before drying at room temperature [25].

6 months stored FFPE tissues.

2,4 Dinitrophenylhydrazin (2,4-DNPH) or a mix of 2,4-DNPH and HCCA was used as matrix. 20-30µL of the mix was applied onto the tissue using a micropipette and dried at room temperature.

2 years stored FFPE tissues.

For direct analysis, several spots of 2µL of enzyme (trypsin 0.033µg/µL in 25mM Tris buffer pH 7.4) were performed at different spots on the tissue to obtain representative proteins/peptides profile. Enzymatic digestion was realized at room temperature after covering the tissue section to decrease liquid evaporation. Each 10 minutes, enzyme was again deposited on the same spots. After final digestion, tissue was rinsed with ethanol 80%. 30 µL of matrix was then applied on the tissue.

Functionalized Magnetic beads extraction of peptides from tissue

Extraction/purification of the sample was performed using Clinprot purification (C8/C3) system from Bruker Daltonics according to manufacturer's protocols adapted for tissue.

For a section of 2cm2, after enzymatic digestion, 15µL of binding solution was directly applied onto the tissue during 1 minute, then 15µL of magnetic bead was added on the slice. Extraction occurred during 10 minutes. During this step, beads and enzymatic result were mixed 3 times using a micropipette directly onto the tissue.

Then, digestion solution and beads were deposited in a polypropylene tube and washed 3 times using 500µL of H_2O / TFA 0,1%. Peptides were eluted with 30µL of ACN/H_2O 0,1% TFA. MALDI/MS analysis can be performed after mixing resulting solution with MALDI matrix (HCCA, 10mg, ACN/H_20 0,1% TFA (2/1 v/v)) or after solvent evaporation and dissolution in 10µL of water.

For nano LC-MS/MS identification, peptides were redissolved in H_2O/MEOH 0,1% formic acid (9/1 v/v) after elution and evaporation.

For MALDI imaging, spots of enzyme (trypsin at 0.1 µg/µL in ammonium bicarbonate 25 mM filtered in a 0.45 µm Pall GxF/GHP filter) were performed using a high accurate position automatic microspotter (MALDI Sun Collect Spotter, Gmbh germany) operated with a 75µm capillary column. Thus, the whole tissue section was micro-spotted with enzyme following a regular raster of spots of ~300 µm size. Flow rate was after optimization set to 300 nL/s, and 300 nL of trypsin was applied each second at different spots covering the surface of tissue. The program was repeated several times to increase time of digestion. 300 nL HCCA matrix was then spotted on the same spots as for the enzyme using the same program. No ethanol washing tissue step was used between trypsin and HCCA in that case.

Mass Spectrometry

MALDI-MS

MALDI-TOF mass spectra were performed on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA, USA) with delayed extraction (DE) and a 337nm pulsed nitrogen laser operating at 3 Hz and 2 ns pulse width. Either HCCA, SA, or 2,4-DNPH were used at concentrations of 10 mg/mL, 20 mg/mL and 4 mg/mL respectively, in ACN/0.1% TFA:H_2O (2:1, v/v). Matrices were applied onto the tissue using a micropipette (typically 20µL for a whole rat brain slice) and then dried at room temperature. External

calibration was performed using a mixed solution of peptides (Bradykinin 1.6 µM, Substance P 1.6 µM, ACTH 18-39 1.6 µM, ACTH 7-383 2 µM, bovine Insulin 4.8 µM and bovine Ubiquitin 4.8µM in H_2O). Slices were visualized in the mass spectrometer using a color CCD camera (SONY). Each recorded mass spectrum was resulting from the average of 200 laser shots on the area of interest. Acquisition parameters were set as follow:

-HCCA and 2,4 DNPH matrices (mass range 500-10000*):* acceleration voltage: 25kV, 1st grid voltage: 94%, guide-wire voltage: 0.05%, extraction delay time: 200ns.

MALDI-Imaging

For MALDI-IMS of 6 months stored fixed and paraffin embedded tissues, imaging was performed on an Ultraflex II TOF-TOF (Bruker Daltonics, Bremen, DE). After dewaxing, images were obtained in positive linear mode using a mixture of HCCA and 2,4 DNPH (1:1, v/v). 30µL of the mix was applied onto the tissue using a micropipette and dried at room temperature. Acquisition was realized using a 337 nm, pulsed nitrogen laser, with a repetition rate of 50 Hz. For images reconstruction the FlexImaging v. 1.0.6.0 software (Bruker Daltonics, Bremen, DE) was used. For positive mode, 12 000 points covering the whole slice with 100 laser shots per position were scanned. From each position the software measures an average mass spectrum with its coordinates on the slice.

For MALDI-IMS of 2 years stored fixed and paraffin embedded tissues, imaging was performed on the voyager DE STR, using MALDI Imaging Tools (M. Stoeckli, Novartis, Switzerland) for image acquisition and reconstruction by screening 8000 points on the tissue section (30 shots averaged by position). Images were overlaid with the picture of the tissue slice before experiments using PaintShop Pro X software.

MALDI-MS/MS

MALDI-MS/MS experiments of 2 years old FFPE tissue sections after in situ digestion of the whole tissue section was performed on an Ultraflex II TOF-TOF instrument (Bruker Daltonics, Bremen, DE) equipped with LIFT III cell and smart beam laser with a repetition rate up t 200 Hz. For MS/MS experiments parameters were set as follow: laser repetition rate was 100 Hz with 33% attenuation, ion source voltages were respectively 8 kV and 7.3 kV on MALDI sample plate and 1st electrode; LIFT cell was pulse from ground for electrode 1 and 2 to 19 kV and in the last step electrode 3 was decrease to 3.2 kV; reflector end voltage was set to 29.5 kV and mid-grid to 13.85 kV. For MS/MS experiments no collision gas was used. All protein identification in databanks on the TOF-TOF instrument were performed using the Biotool 3.0 interface (Bruker Daltonics, Bremmen, DE) connected to Mascot search engine and interrogating the Swissprot databank.

Nano RPLC MS/MS

Analyses were performed on an ion trap mass spectrometer (LCQ deca XP plus, Thermo electron). For each run, 0.5 µL of digest was injected with a Switchos Autosampler (Dionex Corporation) and separation performed on a reverse phase C18 silica bonded stationary phase (75 µm i.d., 150 mm long, 3 µm 100 Å pore size, Dionex Corporation). Samples were washed during 2 minutes at 10 µL/min with 100% mobile phase A (95% H2O, 5% ACN 0.1% formic acid). Peptides were eluted using a linear gradient of 1%/min mobile phase B (ACN 80%, H2O 20%, formic acid 0.08%) during 70 minutes at a flow rate of 0.200µL/min. The LCQ was operated in a data dependent MS/MS mode in which one MS full scan was followed by one MS/MS scan on the most abundant peptide molecular ion. Collision energy was set to 35%. The heated capillary temperature and electrospray voltage were 160°C and 1.5kV respectively.

Protein identification was performed with the MASCOT sequence query search program using the SwissProt database filtered for the taxonomy "rattus". A tolerance of 2Da for peptide and 0.8 Da for MS/MS was fixed. Only protein sequences with MOWSE score higher than 32 (indicating significant homology or identity) and identified in several samples representing 2 significant MS/MS were considered. Methionine oxydation was defined as variable modification.

RESULTS

Formalin fixation is known to induce methylene bridges formation between free amine groups, especially between amino groups of the lysine lateral chains or the N-terminal of proteins. The fixation process has also been described to continue during conservation of the sample. Thus, direct analysis and MALDI imaging were used in the present study in relation to conservation time of the tissue samples using rat brain FFPE tissue blocks as models.

In a first approach, FFPE tissues that were fixed for over 24 hours and stored for less than one year were examined and compared to fresh frozen tissue samples in terms of spectra quality (i.e. signal intensity and resolution, signal to noise ratio and mass range) by direct MALDI analysis. Sinapinic acid (SA) and α-cyano 4-hydroxycinnamic acid (HCCA) matrices were both tested and results compared with those of frozen tissues. For both matrices, decreased signal intensity and number of peptides/proteins detected were observed in FFPE tissues. Loss of signal was especially important in the higher mass range and in particular using HCCA as matrix. The best results in terms of signal intensity and mass range were obtained using SA. A comparison of direct MALDI analysis of FFPE tissues *vs* frozen tissues (Figure 1) with SA as the matrix shows that the same ions are observed on both mass spectra, although the signal intensity is slightly lower for FFPE tissues and the relative peak intensities differ. A most striking observation relies in the peak shape, where FFPE tissues present

multiple overlapping peaks resulting in loss of resolution and difficulties in mass measurement, whereas frozen samples present classical single peak shapes and optimal resolution. Multiple peaks showed a repetition of +12 Da to the $[M+H]^+$ ion generally observed (e.g. m/z 5493.08 and m/z 5505.47). The formation of these multiple peaks is directly connected to the process of formalin fixation and has previously been described[26, 27]. These adducts formation can be explained by a mechanism described by Metz et al [28] suggesting the formation of a Protein-N=CH2 compound (with ΔM=12). FFPE tissues can be examined using direct MALDI analysis but a decrease in signal intensity and resolution were observed. Protein cross-linking cannot be reversed resulting in observed difficulties. In addition, it is likely that non-reacted formalin molecules are also problematic for MALDI analysis.

Accordingly, we examined the possibility of neutralizing residual formalin molecules to improve the signal. In traditional analytical procedures, 2,4-DNPH was successfully used to detect the presence of ketones or aldehydes molecules in solution. Moreover, 2,4-DNPH was used as a MALDI matrix [29] by reacting with reactive function and especially free aldehydes. Here, we show that 2,4-DNPH can be used as matrix for direct tissue analysis (Figure 2). Used on FFPE tissues, this matrix provides excellent results. A large in signal intensity is observed for the m/z range corresponding to peptides greater than 5000 kDa and peak of adducts corresponding to protein-N=CH2 ions are suppressed (Figure 2). Adducts suppression allows for a much more precise m/z determination, and for peptide mass range MALDI direct analysis performances are similar to those of frozen tissues. Even though methylene bridges are very strong links, it is still likely that the 2,4-DNPH is able to neutralize aldehydes which have not reacted with NH_2 groups, resulting in increased signal of the unreacted species. While 2,4-DNPH performs well on tissues, for higher masses, direct analysis remains difficult. One of the major obstacles for the use of this matrix in MALDI imaging has been the

crystallization pattern. 2,4-DNPH crystallizes in long needles that do not properly cover the entire surface of the tissue. However, a mixture of 2,4 DNPH with HCCA in equal proportion preserves the benefit of the DNPH *i.e.* neutralization of formalin, while HCCA gives the desired even crystallization across the tissue surface. This matrix mix was then used to perform MALDI imaging on FFPE rat brain tissue sections stored for 6-12 months. As shown in Figure 3 for reconstructed images from data recorded in the cerebellum, specific localization of peptides is observed as given in examples for m/z 1075, 1594, 2726 and 5163 ions.

For FFPE rat brain tissues stored over 1 year, it is impossible to obtain good signal by direct analysis of tissue after paraffin removal (Figure 4). This phenomenon can be attributed to formalin reticulation which progresses with time, creating a particularly abundant protein network. For such tissues another analytical strategy must be developed. Considering that the methylene bond is a strong link, that is difficult to break without destroying the peptide backbone, we designed a biochemical approach using endopeptidase enzymatic digestion. Recently, this approach was successfully applied for protein identification from formalin fixed tissues using LC/MS/MS[17-19]. However, to our knowledge, direct digestion on FFPE tissues for direct MALDI-MS analysis has never been tested. Whole tissue digestion was carried out by covering the entire section with enzyme solution. Numerous signals (> 300) corresponding to digested peptides were observed, as presented in Figure 5 for a 15 min trypsin digestion. Interestingly, despite the fact that very low fragmentation yields are obtained (even on frozen sections), as previously described[30], enzymatically digested peptides present the classical behaviour of peptides toward fragmentation. Metastable decay experiments performed on observed peptide ions with a MALDI-TOF/TOF directly on the tissue after digestion, enabled us to identify several proteins, as illustrated for the fragmentation study of a peptide at m/z 1372.68 that was identified to be the haemoglobin α-

chain by data bank interrogation with an ion score of 18 fragments of MS/MS identified on 58 total fragments possible (mainly b and y fragments which is consistent with the experiments), confirming that the peptides correspond to digested peptides within the tissue. Other proteins such as β tubulin were also identified in the same way.

Several parameters, such as, the buffer used, the concentration of the enzyme, the temperature and time of digestion were tested. Tris or bicarbonate buffers gave similar results. In regards to temperature, digestion was preferably carried out at ambient temperature and no major difference was observed when compared with a digestion at 37°C, probably due to the strong concentration of enzyme used. Concerning the time of digestion, many signals were obtained with very short incubations (2 min) as well as longer ones (up to 3 hours) but depending upon the time of digestion, peptide profiles were very different, with much lower mass ions (or peptides) obtained for longer digestion time. These incubation times remain shorter than the traditional overnight digestions which could lead to signal saturation due to the abundance of detected peptides. In all cases, experiments were highly reproducible for identical incubation times and enzyme concentrations. Various enzymes were tested, mainly V8 protease, aspN and lys C endopeptidases. As expected, specific profiles were obtained depending upon the enzyme used (Figure 6). Other fixation procedures were studied (e.g. Bouin's protocol [25]), and successfully analyzed after enzymatic digestion (data not shown). It is thus possible to obtain and detect peptides/proteins directly from fixed tissues, by MALDI direct analysis after enzymatic digestion with various types of enzyme regardless of the fixation process used.

The same *in situ* digestion experiments can be carried out on micro-areas (up to 1 mm) by micro-depositing the enzyme solution on specific areas of the tissue using either a micro-pipette for larger spot or a very fine capillary. Digestions on very small areas give excellent results. Micro digestion represents several advantages including less complex

peptides/proteins mixtures since less of these compounds are present on smaller areas, better enzymatic hydrolysis yields and minimized delocalisation since at maximum delocalisation reach the dimension of the micro area. Comparison of digestion profiles obtained from two different regions of the same rat brain section after MALDI direct analysis shows that digestion peptides are very different thus validating the strategy.

Moreover, in order to obtain MALDI direct analysis of tissues compatible with histological procedures and in particular with histological staining methods that are classically used in hospitals for diagnosis of biopsy samples, two year old rat brain FFPE tissue sections were submitted to enzymatic digestion following coloration. Chaurand et al. have shown that many staining procedures were compatible with direct analysis, as well as imaging of frozen tissues[31]. The area of interest can be located then directly analyzed by mass spectrometry after matrix deposition. In our study, methylene blue, toluidine blue, and hematoxylin eosin safran (HES) staining were tested and compared to adjacent unstained tissue sections using direct MALDI analysis in the same regions after micro-spotting of the enzyme solutions. This procedure resulted in acceptable signal levels (Figure 7). The detection of peptides is slightly lower than for untreated tissue, in the mass range of 1000-1500 and 1800 2300 u, but is basically the same as observed in unstained sections. This phenomenon has also been noted during the study of frozen tissues by Chaurand *et al.*[5] and for FFPE tissues, a correlation between time and coloration of the tissue leads to a considerable reduction of spectral quality. Nevertheless, when traditional times of staining are used, very good signals, mainly equal to signals obtained from unstained sections, were observed for direct MALDI analysis after trypsic digestion.

In order to improve protein identification, extraction procedures after *in situ* digestion were tested. Various extraction procedures were examined (see Figure 8), including Tris buffer, H_2O, ethanol and direct deposition of functionalized magnetic beads (C8 or C3

functionalized silica beads). The mass spectra show that certain ions present very different abundance depending on the extraction conditions. These results provide evidence for the importance of the extraction step that orientates protein identification depending on the physico-chemical properties of the buffer or support used inducing a pre-fractionation of the very complex sample. The extraction of digestion peptides was followed by a purification step for desalting and buffer removal when needed (*i.e*, Tris buffer or ethanol extraction) before injection on a nanoLC-nanoESI system. Finally, different strategies can be used to obtain protein identification. The different strategies tested are schematically represented and summarized Figure 9. Basically, it is possible to work directly on tissue up to the step of digestion peptides extraction for performing the LC-MS and MS/MS analysis. Here, it is again possible to choose either to perform a global digestion of one part of the tissue section before extracting the resulting peptides either using EtOH, Tris buffer or functionalized magnetic beads. In another strategy, enzymatic digestion can be performed on micro-area (as previously presented) by rastering the tissue and using an automatic micro-spotter. In this case, digestion peptides can be extracted by covering the whole tissue with Tris buffer and pipetting it for analysis. It is also possible to scrap one part of the tissue section (e.g. the half), transfer the tissue pieces in a tube, performed the enzymatic digestion before analysis by nano-LC MS and MS/MS.

The methodology was validated by comparing the nano-HPLC profiles for the different strategies Comparable results were obtained for the same extraction protocols, demonstrating that *in situ* digestion and extraction yield identical results as *in vitro* ones. Figure 10 shows a chromatogram obtained from the total ion current of the mass spectrometer detector against the retention time obtained after on tissue digestion with trypsin and extraction using C8 functionalized silica beads of a whole part of the FFPE tissue. Measuring m/z of peptides for each chromatographic peak, performing automatic MS/MS experiments

on the most intense ones and using these data for databanks analysis, more than one hundred proteins were identified with this approach (Table 1). All proteins identified are proteins expected to be found in rat brain. From the databases, subcellular localization of these proteins was obtained and it was interesting to note that proteins from very different cellular compartments such as the cytoplasm, nuclear envelope or cytoskeleton are present demonstrating that "MALDI enzyme assisted direct analysis" digestion does not occur just at the surface of the cell but also produces an efficient hydrolysis within cells. Similarly, identified proteins are implicated in very different biological activities (e.g. enzyme, regulation, or signal transduction) demonstrating that this strategy allows for obtaining a large panel of protein functions. Moreover, detection of very high mass proteins directly from tissue e.g. Na^+/K^+ transporting ATPase (111kDa) or neural cell adhesion molecule (95kDa) was also possible. Validation of this strategy on *in situ* enzymatic digestion of FFPE tissues was carried out with a similar analysis by nanoLC-MS/MS of frozen tissues treated with trypsin, using the same digestion time and studying same area of the brain, resulted with very similar protein profiles.

The last challenge was to attempt MALDI imaging of FFPE tissues of greater than 2 years using the enzymatic digestion strategy. Digesting the whole tissue section was possible but would have produced a possible delocalization of certain peptides liberated in the digestion process. Different solutions were tested to limit this phenomenon including a vaporization of matrix, ionic matrices [30] or the use of an automatic spotter which allows the deposition of micro-droplets of matrix point by point[32]. Studies clearly demonstrate that micro-spotting method is the best suited for MALDI imaging. Rastering the tissue section with automatic micro-spotting of trypsin, enzyme results in digest peptides profiles of the whole sample with a limited delocalization to within the size of the matrix spot (i.e. 300 µm). For these studies classically used Tris buffer was replaced by bicarbonate buffer to avoid

desalting steps. Digestion was efficient only after several spottings of the enzyme (2 or 3 depending on the deposited volume) on the same spots thus requiring a high accuracy positioning micro-spotter. After digestion, matrix solution was then spotted on the same spots as enzyme and the tissue section was submitted to MALDI analysis, recording the images again following the same path of deposition. From the data recorded, images of numerous proteins could be indirectly obtained by looking to their corresponding digestion peptides images. Images of proteins identified by the LC-MS/MS experiments can be obtained such as histone H3.3 with m/z 1033 ion, NCAM 1 with m/z 1521 ion, myelin protein with two different digestion fragments at m/z 1803 and 1340 and malate dehydrogenase with m/z 1103 ions as presented Figure 11. Each of these proteins, as observed from the MALDI images, has very different distributions in the rat brain tissue section. Images reconstructed on different ions corresponding to different digestion fragments give similar images, showing the validity of the presented strategy. Moreover, using similar automatic micro digestion, followed by micro deposition of the matrix, it was possible to verify that the same protein localization was obtained for FFPE tissues as with frozen sections, as shown for m/z 1103 ion repartition corresponding to a digestion peptide of malate dehydrogenase protein.

DISCUSSION

FFPE is so far the most convenient method for pathologist to conserve samples in hospital tissue banks. However, FFPE induces proteins cross-linking and provokes difficulties for MALDI direct analysis. Moreover, cross-linking continues in time, thus different analytical strategies must be developed. For conservation time below 1 year, direct analysis remains possible, but notable loss in resolution and signal are observed especially for higher mass proteins. Using the 2,4-DNPH reactive matrix, signal of peptides (less than 5 kDa) can be retrieved with a normal resolution. When mixing 2,4-DNPH reactive matrix to HCCA,

very good matrix crystallization patterns are obtained and MALDI imaging of the peptides/proteins can be directly performed on such tissues, with comparable results to frozen conserved samples.

For longer stored tissue (> 1 year) very few signals are obtained from the FFPE tissues and another strategy was developed based on tissue enzymatic digestion. This approach gives abundant signal for MALDI direct analysis of 2 years old FFPE rat brain tissues and was shown to be compatible with classical histology colorations. Protein identification was also possible by extraction after the digestion and analysis by LC-MS/MS. "MALDI-MS enzyme assisted direct analysis" allows the detection of many proteins with different subcellular location, biological activities, or molecular mass including high molecular weight compounds. By combining enzymatic cleavage on tissue with high accuracy automatic spotting of enzyme and matrix, and ESI identification of peptides/proteins, we performed for the first time MALDI imaging on 2 years old archived FFPE rat brain tissues, and successfully obtained the expected localization of several identified proteins. The localization of various proteins on frozen tissue is identical when compared to FFPE tissue as in the case of malate dehydrogenase.

These results provide access to archived tissues for proteomic studies using MALDI-MS direct analysis and imaging experiments for localization of a large number of compounds in a single experiment.

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FIGURE LEGENDS

FIGURE 1 : 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 (each experiment were conducted 5 times).

FIGURE 2 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 (each experiment were conducted 5 times).

FIGURE 3 MALDI molecular images reconstructed from the data recorded on a <1year old FFPE tissues performed on MALDI-TOF/TOF with mix 2,4-DNPH and HCCA as matrix (each experiment were conducted 5 times).

FIGURE 4 MALDI mass spectrum in the linear positive mode of the direct analysis of a >1year old FFPE tissue (here 2 years) using sinapinic acid as matrix (each experiment were conducted 5 times).

FIGURE 5: 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) and MALDI metastable decay spectrum resulting from the fragmentation of the m/z 1572.68 parent ion using MALDI-TOF/TOF instrument (each experiment were conducted 5 times).

FIGURE 6: Compared MALDI mass spectra in the linear positive mode of the direct analysis of a 2 years old FFPE rat brain tissue sections after *in situ* trypsin or V8-protease digestion using HCCA as matrix (each experiment were conducted 5 times).

FIGURE 7: Compared MALDI mass spectra in the linear positive mode of the direct analysis of a 2 years old FFPE rat brain tissue sections after *in situ* trypsin digestion or HES staining followed by *in situ* trypsin digestion using HCCA as matrix (each experiment were conducted 5 times).

FIGURE 8: Compared MALDI mass spectra in the linear positive mode of the direct analysis of a 2 years old FFPE rat brain tissue sections after *in situ* trypsin digestion followed by extraction of digestion peptides by H2O, C8-functionalized silica magnetic beads or C3-functionalized silica magnetic beads using HCCA as matrix (each experiment were conducted 5 times).

FIGURE 9: schematic representation of the different strategies explored for obtaining identification and/or images of proteins directly from FFPE tissues independently of storage length.

FIGURE 10: nanoLC chromatogram of C8-functionalized silica magnetic beads peptides extracts on a 2 years old FFPE rat brain tissue sections after *in situ* trypsin digestion recorded during nanoLC-MS/MS analysis (each experiment were conducted 5 times).

FIGURE 11: 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 using HCCA as matrix and compared to rat brain picture and morphology (each experiment were conducted 5 times).

TABLE 1: Table of proteins matching by interrogation of Swissprot databank based on the whole proteins digestion data obtained by direct MALDI analysis of *in situ* digestion of the whole tissue section of a 2 years old FFPE rat brain tissue. Results includes protein score and percentage of sequence coverage.

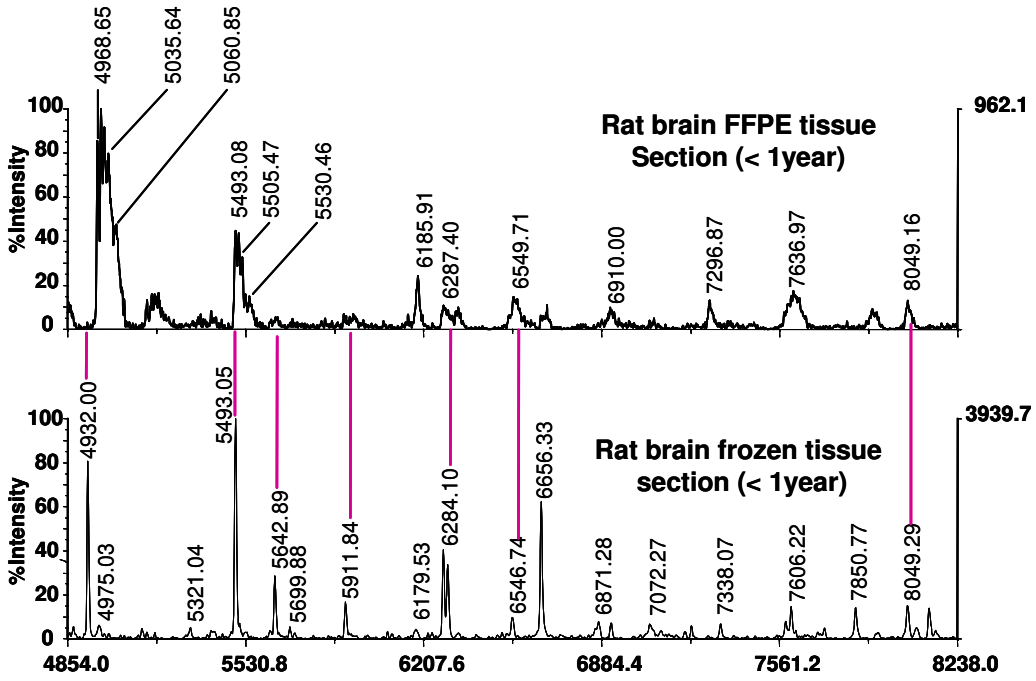
Rat brain FFPE tissue
Section (< 1year)
Rat brain frozen tissue
section (< 1year)
%Intensity
962.1
3939.7
4968.65
5035.64
5060.85
5493.08
5505.47
5530.46
6185.91
6287.40
6549.71
6910.00
7296.87
7636.97
8049.16
4932.00
4975.03
5321.04
5493.05
5642.89
5699.88
5911.84
6179.53
6284.10
6546.74
6656.33
6871.28
7072.27
7338.07
7606.22
7850.77
8049.29
4854.0
5530.8
6207.6
6884.4
7561.2
8238.0

Figure 1

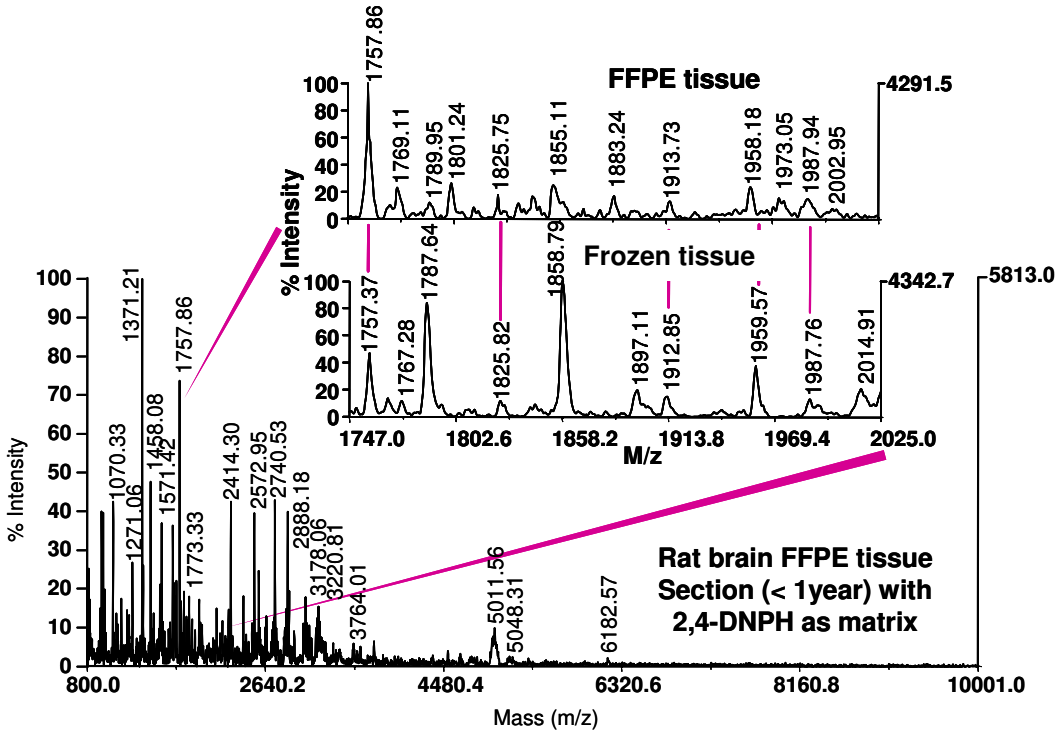
FFPE tissue
Frozen tissue
% Intensity
M/z
Mass (m/z)
Rat brain FFPE tissue
Section (< 1year) with
2,4-DNPH as matrix

Figure 2

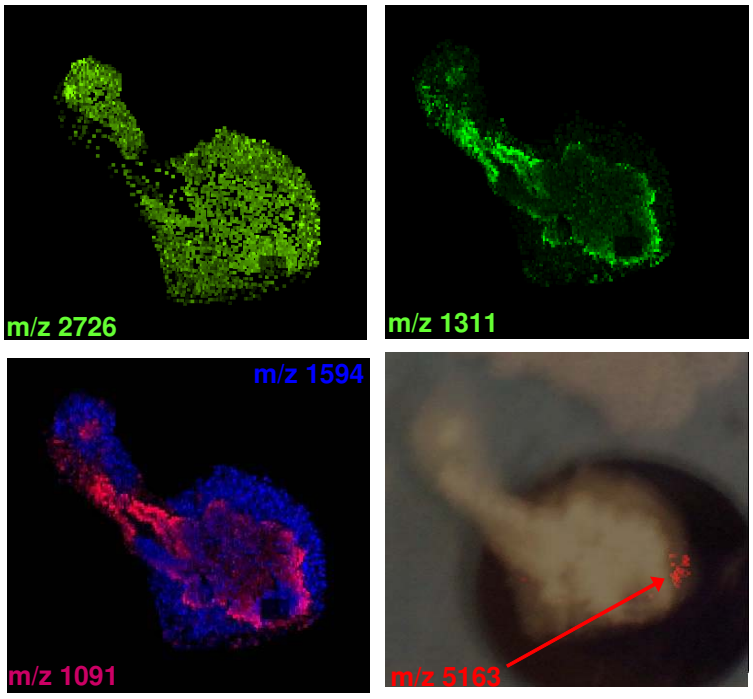

Figure 3

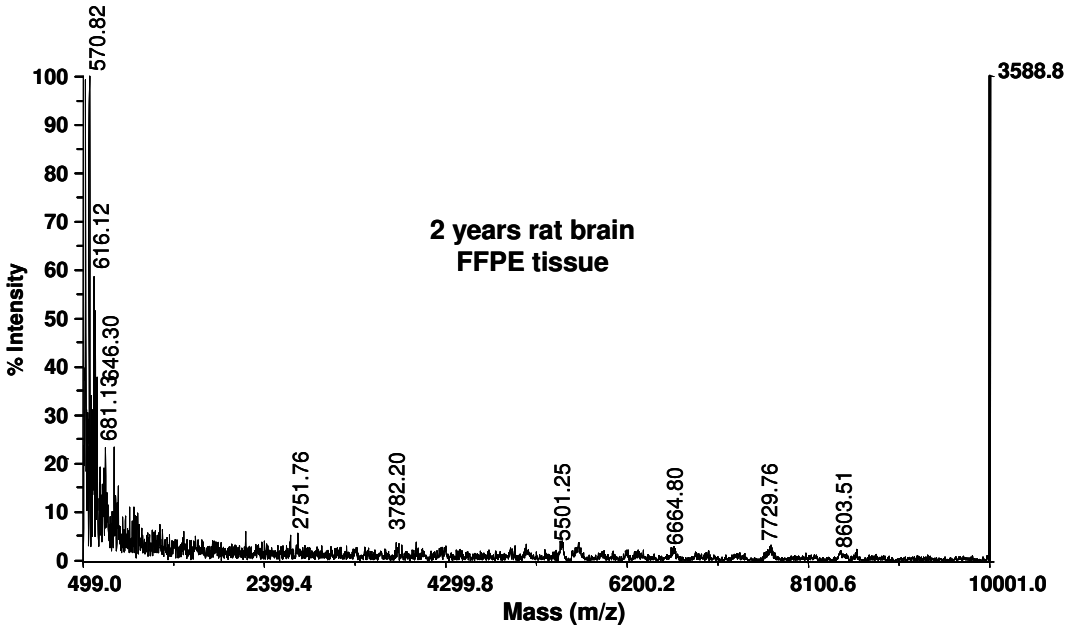
2 years rat brain
FFPE tissue
% Intensity
Mass (m/z)
570.82
616.12
646.30
681.13
2751.76
3782.20
5501.25
6664.80
7729.76
8603.51
3588.8
100
90
80
70
60
50
40
30
20
10
0
499.0
2399.4
4299.8
6200.2
8100.6
10001.0

Figure 4

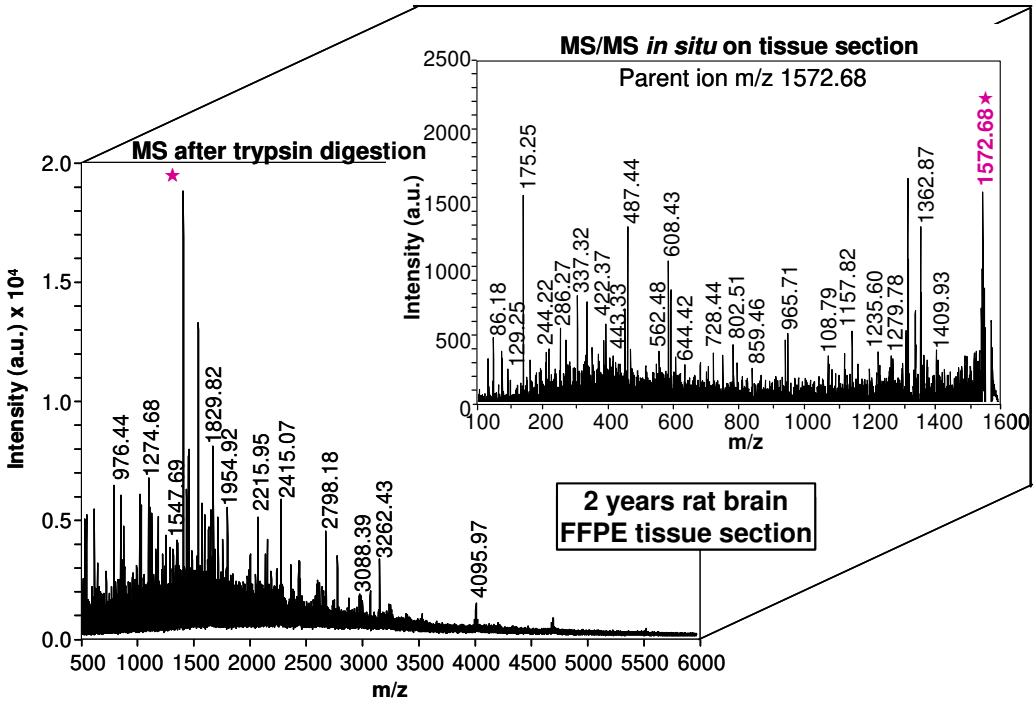
MS after trypsin digestion
Intensity (a.u.) x 10⁴
m/z
976.44
1274.68
1547.69
1829.82
1954.92
2215.95
2415.07
2798.18
3088.39
3262.43
4095.97
MS/MS in situ on tissue section
Parent ion m/z 1572.68
Intensity (a.u.)
m/z
86.18
129.25
175.25
244.22
286.27
337.32
422.37
443.33
487.44
562.48
608.43
644.42
728.44
802.51
859.46
965.71
1108.79
1157.82
1235.60
1279.78
1362.87
1409.93
1572.68
2 years rat brain
FFPE tissue section

Figure 5

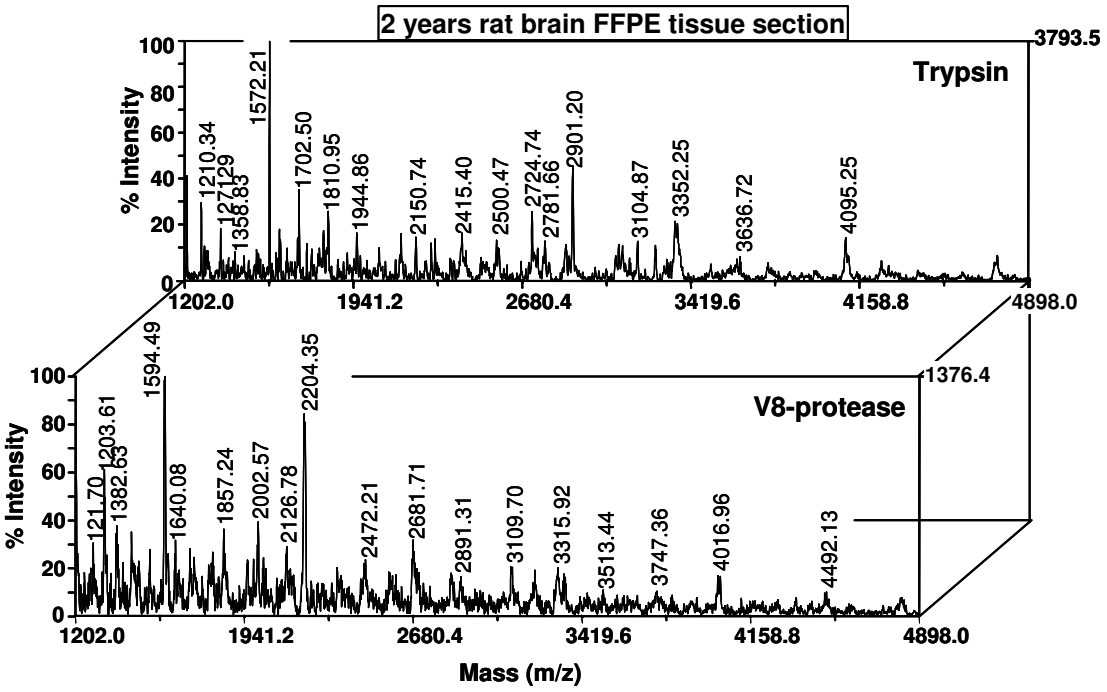
2 years rat brain FFPE tissue section
Trypsin
% Intensity
100
80
60
40
20
0
1572.21
1210.34
1271.29
1358.83
1702.50
1810.95
1944.86
2150.74
2415.40
2500.47
2724.74
2781.66
2901.20
3104.87
3352.25
3636.72
4095.25
3793.5
1202.0
1941.2
2680.4
3419.6
4158.8
4898.0
V8-protease
1594.49
2204.35
1376.4
1203.61
1382.63
121.70
1640.08
1857.24
2002.57
2126.78
2472.21
2681.71
2891.31
3109.70
3315.92
3513.44
3747.36
4016.96
4492.13
Mass (m/z)

Figure 6

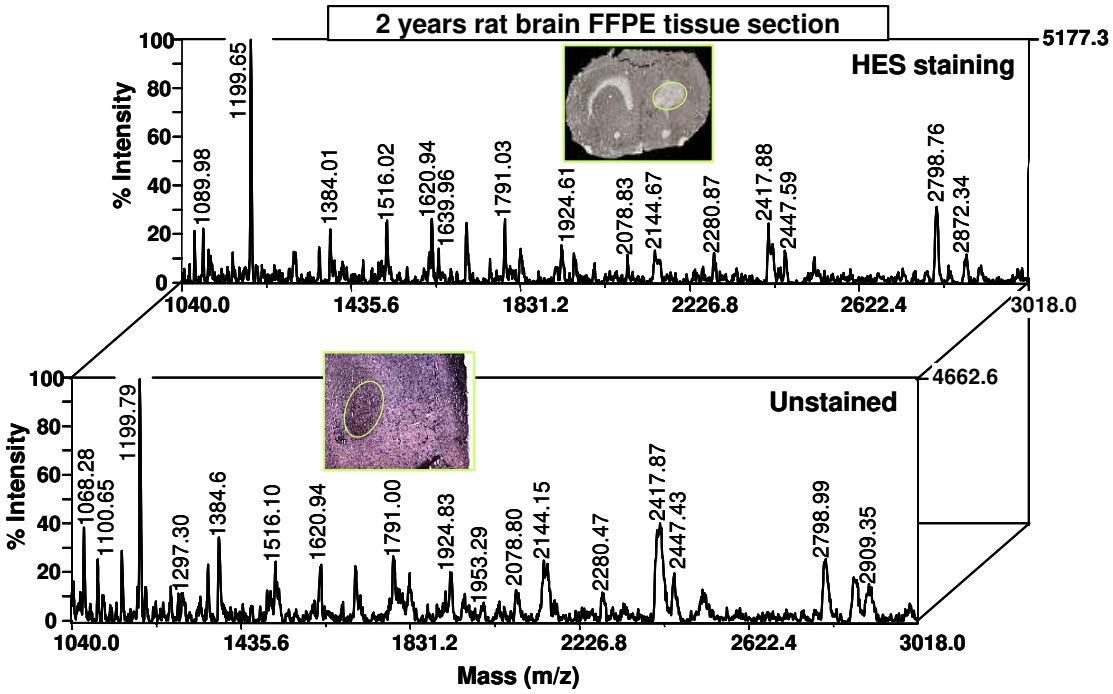
2 years rat brain FFPE tissue section
HES staining
Unstained
% Intensity
Mass (m/z)
5177.3
4662.6
1040.0
1435.6
1831.2
2226.8
2622.4
3018.0
1089.98
1199.65
1384.01
1516.02
1620.94
1639.96
1791.03
1924.61
2078.83
2144.67
2280.87
2417.88
2447.59
2798.76
2872.34
1068.28
1100.65
1199.79
1297.30
1384.6
1516.10
1620.94
1791.00
1924.83
1953.29
2078.80
2144.15
2280.47
2417.87
2447.43
2798.99
2909.35

Figure 7

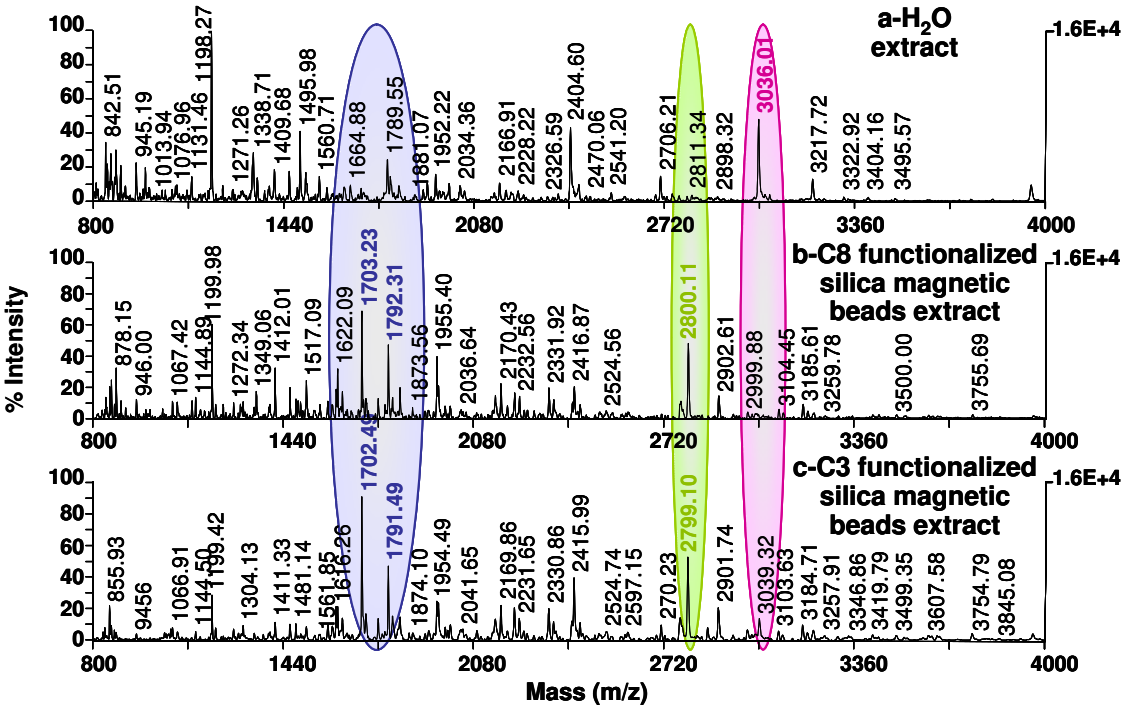
a-H2O extract
b-C8 functionalized silica magnetic beads extract
c-C3 functionalized silica magnetic beads extract
% Intensity
Mass (m/z)
1.6E+4
842.51
945.19
1013.94
1076.96
1131.46
1198.27
1271.26
1338.71
1409.68
1495.98
1560.71
1664.88
1789.55
1881.07
1952.22
2034.36
2166.91
2228.22
2326.59
2404.60
2470.06
2541.20
2706.21
2811.34
2898.32
3036.01
3217.72
3322.92
3404.16
3495.57
878.15
946.00
1067.42
1144.89
1199.98
1272.34
1349.06
1412.01
1517.09
1622.09
1703.23
1792.31
1873.56
1955.40
2036.64
2170.43
2232.56
2331.92
2416.87
2524.56
2800.11
2902.61
2999.88
3104.45
3185.61
3259.78
3500.00
3755.69
855.93
9456
1066.91
1144.50
1199.42
1304.13
1411.33
1481.14
1561.85
1616.26
1702.49
1791.49
1874.10
1954.49
2041.65
2169.86
2231.65
2330.86
2415.99
2524.74
2597.15
270.23
2799.10
2901.74
3039.32
3103.63
3184.71
3257.91
3346.86
3419.79
3499.35
3607.58
3754.79
3845.08
100
80
60
40
20
0
800
1440
2080
2720
3360
4000

Figure 8

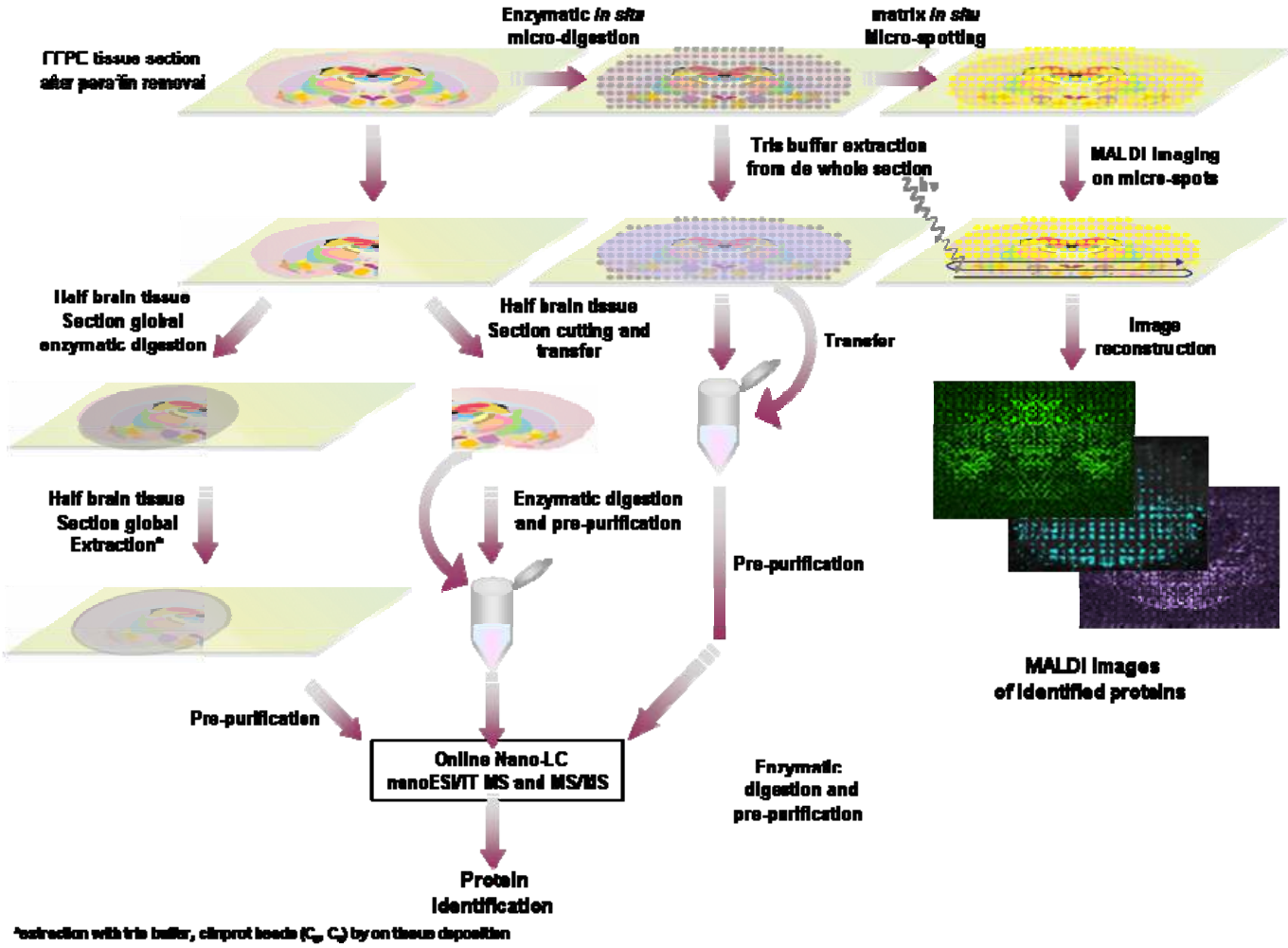
FFPC tissue section
after paraffin removal
Enzymatic in situ
micro-digestion
matrix in situ
Micro-spotting
Tris buffer extraction
from de whole section
MALDI Imaging
on micro-spots
Half brain tissue
Section global
enzymatic digestion
Half brain tissue
Section cutting and
transfer
Transfer
Image
reconstruction
Half brain tissue
Section global
Extraction*
Enzymatic digestion
and pre-purification
Pre-purification
MALDI Images
of identified proteins
Pre-purification
Online Nano-LC
nanoESI/IT MS and MS/MS
Enzymatic
digestion and
pre-purification
Protein
Identification
*extraction with tris buffer, cinprot beads (C8, C4) by on tissue deposition

Figure 9

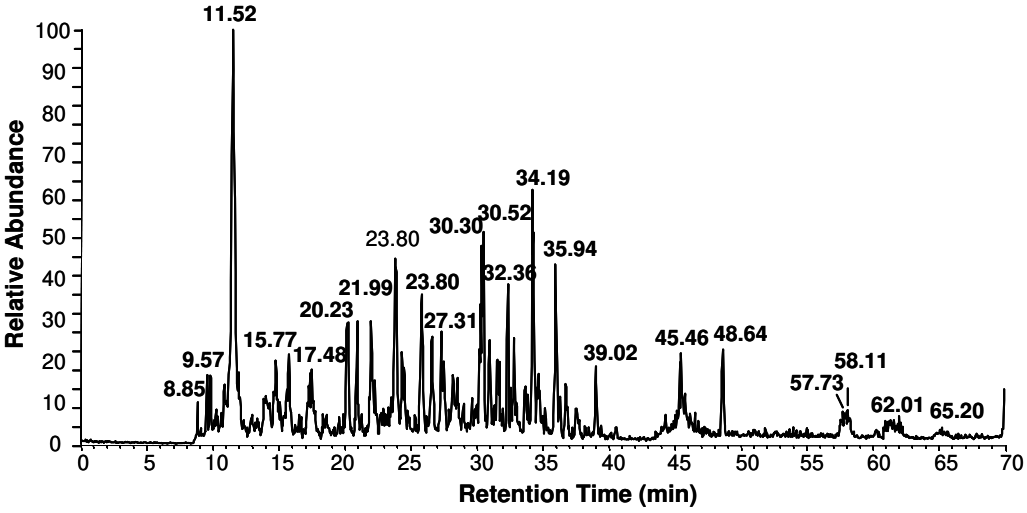
11.52
34.19
30.30
30.52
23.80
35.94
32.36
23.80
21.99
20.23
27.31
15.77
17.48
9.57
8.85
39.02
45.46
48.64
58.11
57.73
62.01
65.20
Relative Abundance
Retention Time (min)

Figure 10

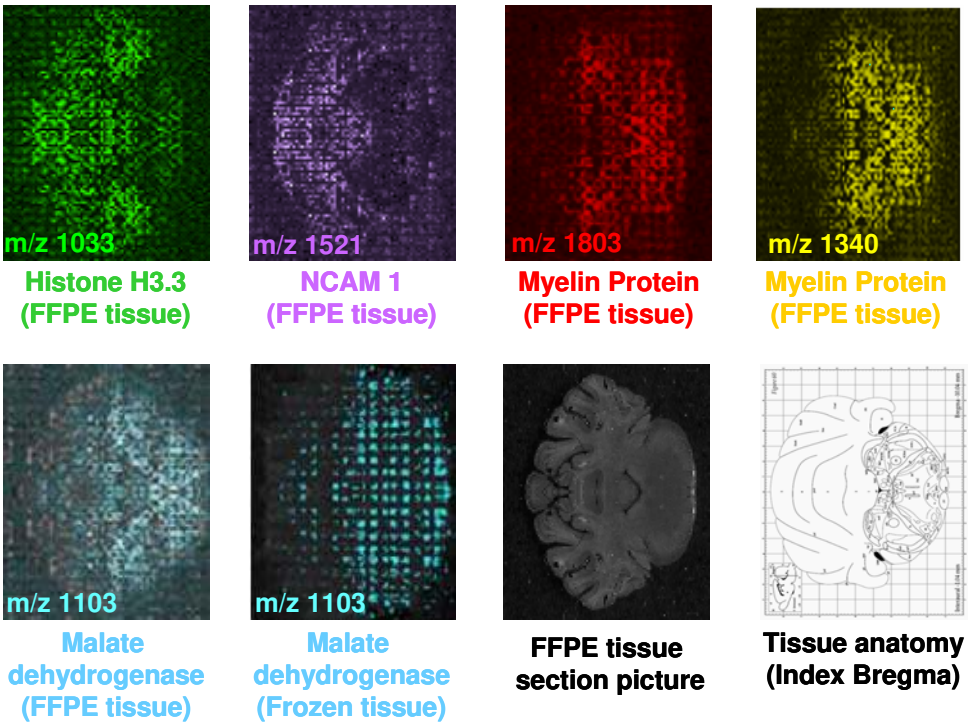
m/z 1033
Histone H3.3
(FFPE tissue)
m/z 1521
NCAM 1
(FFPE tissue)
m/z 1803
Myelin Protein
(FFPE tissue)
m/z 1340
Myelin Protein
(FFPE tissue)
m/z 1103
Malate
dehydrogenase
(FFPE tissue)
m/z 1103
Malate
dehydrogenase
(Frozen tissue)
FFPE tissue
section picture
Tissue anatomy
(Index Bregma)

Figure 11

Measured M/z Direct MALDI analysis	Calculated m/z identified in ESI	% variation	Protein	Accession Number	Mass (Da)	Match Score	Molecular function	Subcellular location
1016.2	1015.5	0.06	Enolase	P04764	46967	173	Enzyme	Cytoplasm
1033.0	1032.6	0.04	Histone H3.3	P84245	15187	71	-	Nuclear
1144.1	1144.6	0.04	Tubulin β Chain	P69897	49639	433	Structural protein	Microtubule
1188.9	1188.6	0.02	Actin	P60711	41710	312	Cell mobility	Cytoplasm
1303.8	1303.6	0.01	Na+/K+ Transporting aptase	P06687	111620	282	Enzyme	Cytoplasm
1340.0	1339.7	0.02	Malate dehydrogenase	O88989	36461	152	Enzyme	Cytoplasm
1370.0	1369.7	0.02	GLyceraldehyde 3P dehydrogenase	P04797	35682	134	Enzyme	Cytoplasm
1394.2	1393.7	0.03	Neurofilament triplet L	P19527	61167	145	Maintenance of neuronal caliber	Cytoskeleton, nuclear envelope
1490.1	1490.7	0.04	Protein kinase C inhibitor protien	P63102	27754	111	Intracellular signaling, signal transduction, cell cycle	Cytoplasm
1516.1	1516.1	0.00	HSC 71 kDa	P63018	70827	295	Molecular chaperone	Cytoplasm
1520.0	1519.1	0.02	Neural cell adhesion molecular	P13596	94599	67	-	-
1548.6	1548.7	0.00	Elongation factor 1-α	P62630	50082	62	-	Nuclear
1616.0	1615.8	0.01	Syntaxin 1β	P61265	33224	63	Regulating protein	Cytoplasm and membrane associated
1621.1	1621.8	0.04	Triosephosphate isomerase	P48500	26773	48	Enzyme	Cytoplasm
1683.6	1683.9	0.01	Phospho glycerate mutase 1	P25113	28497	55	Enzyme	Cytosol
1748.0	1747.8	0.01	Fructose biphosphate aldolase	P05065	39196	113	Enzyme	Cytoplasm
1759.8	1759.8	0.00	Histone H2B	Q00715	13851	109	-	Nuclear
1770.6	1770.8	0.01	Neurofilament triplet M	P12839	95603	51	Maintenance of neuronal caliber	Cytoskeleton, nuclear envelope
1804.7	1804.9	0.01	Myelin basic protein S	P02688	14071	172	-	-
1830.3	1829.9	0.02	DRP2	P47942	62239	231	Axon elaboration	Membrane associated
1884.5	1893.9	0.03	Internexin	P23565	56082	180	Maitenance of neuronal caliber	Cytoskeleton, nuclear envelope

Table 1:

SYNOPSIS

FFPE tissues represent a very important source of information on pathologies. However, such kinds of samples are very difficult to cope with for proteomic studies. MALDI imaging is an emerging technology that adds a dimension of localization to classical proteomic. In the present article are presented the different strategies that can be used to performed direct tissue analysis and MALDI imaging of such FFPE tissues depending on their conservation time.