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MALDI IMAGING OF FFPE TISSUES: APPLICATION TO MODEL ANIMALS OF PARKINSON DISEASE FOR BIOMARKER HUNTING

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Abstract:

A common technique for the long term storage of tissues in hospitals and clinical laboratories is preservation in formalin-fixed paraffin-embedded (FFPE) blocks. Such tissues stored for more than five years have not been useful for proteomic studies focused on biomarker discovery. Recently, MS-based proteomic analyses of FFPE showed positive results on blocks stored for less than 2 days. However, most samples are stored for more than one year, and thus our objective was to establish a novel strategy using as a model system 6-hydroxydopamine (6-OHDA) treated rat brain tissues stored in FFPE blocks for more than 9 years. We examined MALDI tissue profiling combining the use of automatic spotting of MALDI matrix with *in situ* tissue enzymatic digestion. On adjacent sections, the identification of compounds is carried by *in situ* tissue digestion followed by nanoLC/MS-MS analysis. The combination of these approaches provides MALDI direct analysis, MALDI/MS imaging as well as the localization of a large number of proteins. This method is validated since the analyses confirmed that ubiquitin, trans-elongation factor 1, hexokinase and the Neurofilament M are down-regulated as previously

ζ: Equal contribution

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shown in human or Parkinson animal models. In contrast, peroxiredoxin 6, F1 ATPase and α -enolase are up-regulated. In addition, we uncovered three novel putative biomarkers, the Trans elongation Factor 1 (eEF1) and the Collapsin response mediator 1 and 2 from protein libraries. Finally we validate the CRMP-2 protein using immunocytochemistry and MALDI imaging based on the different ions from tryptic digestion of the protein. The access to archived FFPE tissue using MALDI profiling and imaging opens a whole new area in clinical studies and biomarkers discovery from hospital biopsy libraries.

Keywords: MALDI direct analysis, MALDI Imaging, FFPE tissues, Parkinson, biomarkers

INTRODUCTION

Parkinson's disease (PD) is the second most common neurological disease after Alzheimer's disease. PD is characterized by a selective degeneration of dopaminergic neurons in the *substantia nigra pars compacta* and by cytoplasmic inclusions (Lewy Bodies) where specific proteins are stored like the α -synuclein¹. The clinical symptoms are severe motor dysfunctions, including rigidity, postural imbalance, slowness of movements, and uncontrollable tremor. Mutations in genes encoding α -synuclein, parkin and ubiquitin carboxy-terminal hydrolase L1 (UCH L1) have been identified in sporadic familial forms of PD². Several proteomic studies using 2D gel analyses in animal models and humans have identified biomarkers implicated in this pathology^{3, 4, 5-7} (Table 1). In Parkin knockdown models, pyruvate dehydrogenase; NADH ubiquinone oxyreductase 30 kDa, cytochrome c oxydase, peroxiredoxin 1, 2 and 6, lactoylglutathione lyase, vacuolar protein sorting 29, crystalline chain b and heterogeneous nuclear ribonucleoprotein 1 are all down regulated⁴. Similarly, in 6-OHDA animal models⁸, as well as in humans, α -enolase, β -actin, Lasp-1, neurofilament triplet L and M are also down-regulated³⁻⁶. In contrast, human peroxiredoxin 2, complexin I, fatty acid binding protein, L type calcium channel d subunit, mitochondrial complex III and ATP synthase D chain are up-regulated⁵. These results have been confirmed by Strey *et al.*⁷ in SOD1 gene studies, where α -enolase as well as HSP25, HSP27, phosphatidylinositol transfer protein, apolipoprotein E and ferritin heavy chain are up-regulated. These contradictory studies for peroxiredoxin 2 or profilin show the difficulty to get real biomarkers from these classical techniques. Molecular profiling of proteins and peptides can be performed directly on or near the surface of brain tissue sections with high specificity and sensitivity by utilizing matrix-assisted laser desorption ionization mass spectrometry (MALDI MS). This technique was first introduced by Caprioli *et al.*⁹ and adapted in other studies¹⁰. The first tissue profiling studies on 6-OHDA Parkinson model have been performed by Per Andr n group¹¹. From their studies, calmodulin, cytochrome c, cytochrome c oxydase appear to be down-regulated with the exception of ubiquitin¹¹.

In this study, we have been interested by looking to Parkinson 's biomarkers using 6-OHDA injected rat as model animals. Because brains available for this study has been stored after formalin fixation and paraffin embedding (FFPE) over 9 years more direct strategy was to perform *in situ* tissue enzymatic digestion prior to analysis. Indeed, formalin fixation induces proteins cross-linking inside the tissue by formation of methylene bridges. If such samples are extremely stables along the time and allow for high preservation of tissue integrity at the ultra cellular level, they raise analytical difficulties by the formation of a global protein network. Thus,

enzymatic digestion gives the opportunity for retrieving small proteins pieces that are possible to be analyzed by conventional technologies. Proteins identification and localization was obtained using two different and complementary strategies that are MALDI MS direct profiling of tissues and imaging with nanoLC-nanoESI MS. First strategy allows for quickly observing variation in proteins abundances looking to their digestion peptides by comparison of model animals brains with control brains whereas second strategy allow high throughput identification of proteins including proteins of interest. Such combined approach as allowed us to confirmed that Hexokinase and Neurofilament M proteins are down-regulated whereas by contrast, Collapsin Response Mediator Proteins 1 and 2 (CRMP1 and CRMP2), Peroxidoredoxine 6, F1 ATPase, Ubiquitin and α -Enolase are up-regulated. Moreover, we also uncovered that the Trans elongation Factor 1 (eEF1) is down-regulated..

EXPERIMENTAL

Material

α -cyano-4-hydroxycinnamic acid (HCCA), 2,5 dihydroxibenzoic acid (2,5-DHB), ammonium bicarbonate, trisma base, toluene, ethanol, Angiotensin II, Des-Arg-Bradykinin, Substance P, ACTH 18-39, ACTH 7-38 and bovine Insulin were obtained from Sigma-Aldrich and used as provided. Trypsin was from Promega. Trifluoroacetic acid (TFA) was purchased from Applied Biosystems. Acetonitrile p.a. and methanol p.a. from J.T. Baker.

Animal Surgery and Treatment.

Wistar male rats (180–200 g) were anesthetized with pentobarbital (50 mg/kg, i.p.) and infused over 8 min with 6-OHDA (8 mg in 4 ml of 0.05% ascorbic acid in saline) at coordinates A 5 23.8 mm, L 5 1.5 mm, H 5 28.5 mm¹².

Tissue fixation

Rats were sacrificed 4h after the last drug injection. Their brains were removed and fixed for 40 min at 48°C in 4%paraformaldehyde. The accuracy of the 6-OHDA lesion was checked by immunocytochemistry with an anti-tyrosine hydroxylase antibody according to a previously published procedure¹². These brain tissue blocks were then stored in a box at room temperature 9 years before used in this experimental plan procedure.

Tissue dewaxing

Tissue sections of 10 μ m were obtained using a microtome (E.Leitz, Westlard, Germany) and applied onto ITO (Indium Teen Oxided) one side coated conductive glass slides. Paraffin was removed by 2 baths of 5 minutes of toluene and lightly rehydrated with graded ethanol (100°, 96°, 70° and 30°) bathes before drying at room temperature.

In situ Trypsin digestion

For MALDI direct analysis, 2 μ L of enzyme (trypsin 0.033 μ g/ μ L in 25mM Tris buffer pH 7.4) was added at different spots on the tissue to obtain representative proteins/peptides profiles. The digest is performed at room temperature; the tissue is covered with a cap to decrease liquid evaporation. Each 10 minutes, enzyme solution is added on the same spots. Digestion is stopped by rinsing the tissue with 80% cold ethanol (-20°C) to remove salts. The sections are allowed to dry at room temperature. 30 μ L of matrix solution (HCCA 20 mg in

ACN:H₂O TFA 0.1% (7:3, v/v) is then applied on the tissue using a micropipette in order to cover the whole tissue section and dried at room temperature.

For MALDI Imaging, Trypsin microspotting: spots of enzyme (trypsin at 0,05 µg/µL in water) were performed using a high accurate position automatic Chemical Inkjet Printer (CHIP-1000, Shimadzu Biotech, Kyoto, Japan). Thus, the whole tissue section was micro-spotted with enzyme following a regular raster of spots of ~200 µm size. The quantity of trypsin was after optimization set to 20 nL by 40 cycles of 500 pL on each spot position in order to cover the surface of tissue. 40 nL of ionic matrix was then spotted on the same position as for the enzyme using 40 cycles of 500 pL. The matrix preparation was described previously (ref). Briefly, just prior to use, 4,8 µL of Aniline (1 eq.) was added to the matrix solution (HCCA 10mg/mL in ACN/TFA 0,1%, 6:4, v/v) and sonicated during 2 minutes.

For nanoLC-ESI MS analysis, on a section of 2cm², *in situ* enzymatic digestion is performed by adding 15 µL of trypsin enzyme (0.033µg/µL in 25mM Tris buffer pH 7.4) for 1H hour at room temperature. After enzymatic digestion, purification of resulting digestion peptides was achieved by using reverse phase C₈ coated silica magnetic beads (ClinProts™, Bruker Daltonics, Germany) according to the protocol of the manufacturer modified for tissues. For this, 15µL of binding solution was directly applied onto the tissue during 1 minute, then 15µL of magnetic bead was added on the section. Extraction occurred during 10 minutes. During this step, beads and digested products were mixed 3 times using a micropipette directly onto the tissue. Digestion solution and beads were, then, deposited in a polypropylene tube and washed 3 times using 500 µL of H₂O/TFA 0.1%. Peptides were eluted from the beads with 30µL of ACN/H₂O (1:1, v/v) and solution was dried by vacuum centrifugation. For nano LC-MS/MS identification, peptides were re-dissolved in H₂O/MEOH 0.1% formic acid (9:1 v/v) after elution and evaporation.

Mass Spectrometry

MALDI MS direct analysis of tissue sections

Spectra were acquired on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA, USA) with delayed extraction (DE) and a 337nm pulsed nitrogen laser with a repetition of 3 Hz. External calibration was performed using a solution of standard peptides (bradykinin 1.6 µM, Substance P 1.6 µM, ACTH 18-39 1.6 µM, ACTH 7-38 3.2 µM, bovine Insulin 4.8 µM and bovine Ubiquitin 4.8 µM in H₂O). Slices were visualized in the mass

spectrometer using a color CCD camera (SONY). Each spectrum is the result of the average of 200 laser shots of the area of interest.

MALDI MS direct analysis of tissue sections after *in situ* automatic trypsin digestion

Trypsin fingerprint experiments of FFPE tissue sections after *in situ* digestion of the whole tissue section were performed on an Ultraflex II TOF-TOF instrument (Bruker Daltonics, Bremen, DE) equipped with LIFT III cell and Smartbeam laser with a repetition rate up to 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. Trypsin fingerprint protein identification in databanks were performed using the Biotoools 3.0 software (Bruker Daltonics, Bremmen, DE) connected to Mascot search engine and interrogating the NCBI, Swissprot and ESTs databanks with Oxydation as Variable modification, Monoisotopic as mass values, a peptide mass tolerance ± 0.1 Da with a peptide charge state of 1+, max missed cleavages of 2 and number of queries of 300.

MALDI MSI

For MALDI-MSI of 9 years stored fixed and paraffin embedded tissues, images were performed on an Ultraflex II TOF-TOF (Bruker Daltonics, Bremen, DE). After dewaxing, images were obtained in positive reflector mode after trypsin digestion and matrix deposition 20nL of the mix was applied onto the tissue using a microspotting as explained above and dried at room temperature. Acquisition was realized using Smartbeam laser, with a repetition rate of 100 Hz and a spatial resolution of 100x100 μm . For images reconstruction the FlexImaging v2.0 software (Bruker Daltonics, Bremen, DE) was used. For positive mode, 12 919 spots covering the whole slice with 500 laser shots per position were scanned. From each position the software measures an average mass spectrum with its coordinates on the slice.

Nano LC-nanoESI-IT MS and MS/MS

Analyses were performed on an ion trap mass spectrometer (LCQ deca XP plus, Thermo electron, Manchester, UK) equipped with a nano ESI ion source and on-line coupled to a nano HPLC system. 0.5 μL of digest were injected with a Switchos Autosampler (Dionex corporation) and separation performed on a C18 silica bonded stationary phase (75 μm i.d., 150mm long, 3 μm 100 Å pore size, Dionex). Samples were washed for 2 minutes at 10 $\mu\text{L}/\text{min}$ with 100% mobile phase A (95% H₂O, 5% ACN 0.1% formic acid). Peptides were then eluted using a linear gradient of 1%/min mobile phase B (ACN 80%, H₂O 20%, formic acid 0.08%) for 70 minutes at

a flow rate of 0.2 $\mu\text{L}/\text{min}$. 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 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 under MASCOT sequence query search program using SwissProt database filtered for the taxonomy "*Rattus norvegicus*". A tolerance of 2 Da for peptide and 0.8 Da for MS/MS was set. Only protein sequences with MOWSE score higher than 36 (indicating significant homology or identity) and identified in several samples representing at least 2 significant MS/MS were considered. Methionine oxidation and acetylation of N – terminal were defined as variable modification.

RESULTS AND DISCUSSION

Brain tissue blocks of five 6-OHDA treated *versus* 5 untreated rats were systematically studied and compared for biomarkers hunting. The FFPE brains had been stored for 9 years before use in the present report. Formalin fixation provokes the formation of protein-nucleic acid and protein-protein cross-linking in the intracellular environment arising from the reactivity of formaldehyde with the side-chains of lysyl, arginyl, tyrosyl, aspartyl, histidyl, and seryl residues¹³. The methylene bonds are difficult to break without destroying peptide backbone. To solve this problem, we used endopeptidases directly on tissue sections to break down the protein network and retrieve digestion peptides that can also be used for protein identification.

In a first strategy, digestion of the whole tissue section followed by extraction of the resulting solution from the tissue, purification, separation and analysis on a nanoLC-nanoESI-IT system is used. By plotting intensity vs. retention time vs. M/z differences between our two physiological states can easily be observed. Comparison of nanoLC profiles from Parkinson animal models compared to controls reflects the presence of numerous proteins among which most are common and a few of them are specific to each condition has exemplified by the plots presented Figure 1. For each peak observed on the TIC chromatogram an MS is performed followed by MS/MS on the most abundant ions. More than 100 hundreds proteins can be identified for each type of samples using this method. Among all the identified proteins some of the proteins are known to be present a low concentration. Interestingly, such FFPE tissue seem to bring an easier access to lowly abundant proteins than samples stored frozen without treatment, probably because the protein cross-linking network render some proteins more accessible that they are in the normal tissue.

In a second step we were interested by comparing the results of the classical proteomic “Bottom-up” approach with the direct analysis by MALDI. MALDI direct analysis of the tissue after digestion allows for identifying several proteins by peptide mass fingerprint and confirmation of the modulation of the previously identified proteins. As given in example in Figure 2 for Neurofilament Triplet M protein is clearly observed in the control samples whereas this protein is almost not observed in the 6-OHDA treated samples. Table 2 gives examples of proteins that were identified in the MALDI direct analysis approach. Considering the sub cellular localization of these compounds we can confirm that MALDI direct analysis after *in situ* digestion allows the detection of proteins from different compartment such as cytosol, nuclear envelope and cytoskeleton and not just from the cell surface. Various functional proteins were detected including enzymes or signal transduction molecules or regulators. The technique allows the detection of very high mass compounds directly from the tissue like vimentin, neuromodulin, neurogenesisin.

Considering, the data obtained by MALDI and ESI; in the case of the animal treated with 6-OHDA, the results obtained (Table 3) are in agreement with those obtained from 6-OHDA rat brain frozen tissue section by Pierson et al.¹¹ (Table 1). 6-OHDA is known to inhibit the mitochondrial transport chain and this, along with the resulting production of reactive oxygen species contributing to neuronal death¹⁴. This agent appears to induce neuronal death by activating transcription as well as DNA repair enzymes¹⁵. When activated, the signal transcription pathways initiated by PERK and IRE1a induce a characteristic set of genes encoding ER chaperones and nuclear transcription factors that lead to reduction of ER stress or to death¹⁶.

Based on these data, comparison of the proteins present or absent in both conditions revealed the presence of specific proteins which have been identified in the Parkinson model, such as Hexokinase (2-7-1-1), Neurofilament M protein, Peroxydoxin 6, F1 ATPase and α -Enolase (Tables 1 and 3).

Neurofilament M protein has been described as down-regulated^{17 5} and its down regulation is correlated with decreased mRNA during the disease and is dependant upon disease severity¹⁷. Our results using direct analysis of FFPE tissues support these findings.

Due to 6-OHDA treatment provoking oxidative stress, several molecules have been found to be up-regulated under these conditions, such as Peroxydoxin 6, known as an anti-oxidative protein. Similar results were found Strey et al.⁷ using classical proteomic studies. In contrast, peroxydoxin 2 is described as being down-regulated in the parkin knockout model but is upregulated in human Parkinson’s disease studies. These data reaffirm the need to consider multiplex biomarkers for

pathology. α -enolase was also increased in our study as previously observed elsewhere in the case of Parkinson's or Alzheimer's disease^{3,7}). This molecule is known to be the target of specific oxidation or nitrozylation³. We also observed upregulation of F1 ATPase as previously demonstrated by Seo et al.¹⁸

The ubiquitin complex was observed to be upregulated in our study on FFPE tissue as shown by Pierson et al.¹¹. It is known that high levels of ubiquitin and ubiquitinated proteins are present in Lewy bodies indicating that protein degradation is impaired in PD². Proteins conjugated with a chain of ubiquitin moieties are targeted to the ubiquitin-proteasome system complex, where they undergo proteolytic degradation. Genetic studies of PD have identified mutations in the genes coding for proteins involved in the ubiquitin-proteasome degradation pathway^{19,20}.

Interestingly, results obtained from 6-OHDA treated animals after trypsin digestion and nano-LC nanoESI-IT MS and MS/MS experiments revealed a novel biomarker, the collapsin response mediator protein 2. Figure 3 presents the results of identification for CRMP2 proteins. Several digestion fragments of this protein are found in nanoESI (Figure 3a) and databanks interrogation using the MS/MS experiments confirm this identification with attribution of the MS/MS data (as exemplified for ion at m/z 508.59) that is clearly consistent with ion fragment series expected for such instruments (Figure 3b). MALDI direct analysis experiments give good agreement with the previous identification even if digestion fragments observed in MALDI are different from those observed in nanoESI excision of one peptide as highlighted in the CRMP2 sequence (Figure 3c). MS/MS performed directly on the tissue section in MALDI confirmed that the observed fragments would be consistent with digestion peptides of CRMP2 protein. Globally, all the data combined together give identification to this up-regulated protein as the CRMP2 splice variant B of the CRMP2 protein (*Rattus norvegicus*, P47492).

Two splice variants have been recently found²¹. Indeed, the CRMP2A is the long N-terminal isoform (75 kDa) and induce oriented microtubule patterns in cultured fibroblasts, a pattern also observed in axons. Conversely, CRMP2B, the shortest variant (64 kDa) induces disorientation of microtubule patterns in cultured fibroblasts and reduces axon length when over-expressed in retinal explants²¹. In adult brain, it is known that expression of CRMPs is dramatically down regulated²². In our study, an increase in of CRMP2B in 6-OHDA treated animals has been observed which is in agreement with previous molecular data.²³ To go deeper inside the understanding of the role CRMP2 we have consecutively performed MALDI Imaging of CRMP2 in the 6-OHDA treated samples to obtain the localization of the protein. Figure 3 presents the MALDI molecular images obtained from such samples and reconstructed using different ions corresponding to different digestion fragments of the protein. All these ions globally

present an equivalent localization in the rat brain section, even if some of them present more contrasted images. Last image is a composite image taking into account the signal of all ions corresponding to identify digestion fragments of CRMP2 for a global view. The most striking feature is the important localization of the protein in very specific regions of the brain with a highly contrasted signal. This is especially true for the *corpus callosum* where ions are always found to be very intense. Figure 4 presents the map of the corresponding section and compared the MALDI images from the 6-OHDA treated animals to the expression of the CRMP2 mRNA in normal adult mouse. As found in the literature for the rat, this variant of CRMP2 is normally not present in the *corpus callosum*. It is located predominantly in dendrites of specific neuronal populations, such as cortical pyramidal neurons, hippocampal CA1 pyramidal cells, or Purkinje cerebellar cells²⁴. Thus localization in *Corpus callosum* is in line with an involvement in neurodegenerative diseases. In fact, *Corpus callosum* is known to be a brain area implicated in dementia in a lot of neurodegenerative diseases²⁵.

Thus, in PD, the CRMP (intracellular protein mediating Semaphorin3A) and the mitochondrial stress protein HSP60 mRNA encoding these proteins are up-regulated. Antibodies directed against collapsin-1 (Sema3A, that functions in signaling growth cone collapse, chemorepulsion and neuronal apoptosis during early development of the central nervous system) provided marked and prolonged protection of several neuronal cell types from dopamine-induced apoptosis²³. It has been hypothesized that these proteins are positive mediators of DA-induced neuronal apoptosis in PD. In PD, nigral neuronal death could be due to excessive oxidative stress generated by auto- and enzymatic oxidation of the endogenous neurotransmitter dopamine (DA), the formation of neuromelanin and the presence of high concentrations of iron. DA toxicity is mediated through its oxidative metabolites, whereas thiol-containing antioxidants provided marked protection against DA toxicity, ascorbic acid accelerated DA-induced death. In Alzheimer's disease, the CRMP-2 is known to be implicated in neurite degeneration, acting on the assembly and polymerization of microtubules²⁶. Accumulation of Sema3A overlaps the appearance of phosphorylated MAP1B and tau in many neurons, suggesting that Sema3A signaling at some level may be coupled to these previously identified cytoskeletal markers of neurodegeneration²⁷. The hippocampus of patients with AD express phosphorylated MAP1B, collapsin-response mediator protein 2 (CRMP-2), Plexins A1 and A2, and a processed form of Sema3A²⁷.

Thus, we speculate that CRMP factors are good biomarkers for neurodegenerative diseases like PD or AD.

While our methodology is validated by confirming previously discovered molecules implicated in PD, we have also uncovered previously undescribed changes, such as the downregulation of Trans-elongation factor 1 (eEF1) in 6-OHDA samples. Studies on regulatory factors of heat shock factor (HSF-1) modulation and their involvement in the age-associated attenuation have demonstrated an attenuated response to stress which is characteristic of senescence. Heat shock (HS), a significant form of stress, is delayed and reduced in aging organisms. The proteins I-HSF [HSF inhibitor], eEF1 and a large non-coding RNA (HSR) participate in regulation and activation of HSF-1 in early stages of heat shock gene transcription. It is proposed that structural changes in any one or combination of these factors in response to heat shock may contribute to the age-associated attenuation in response to stress ²⁸. These results are in agreement with the fact that 6-OHDA treatment provokes a down regulation of the eEF1. Moreover, 6-OHDA provokes an oxidative stress stimulating HSP expression as demonstrated by Strey et al. ⁷ and as it is down regulated in these conditions. Finally, eEF1 is present in the dendrites of neurons that exhibit synaptic plasticity and its translation is locally regulated ²⁹. In the hippocampus, the dendritic mRNAs is highly expressed in cell bodies and is also concentrated in the zone of termination of commissural/associational afferents in the inner molecular layer, suggesting that mRNA localization is in some way related to the distribution of different types of synapses ³⁰. In the case of the 6-OHDA model, this treatment provokes a decrease of synaptic plasticity and thus a down regulation of eEF1 levels.

In conclusion, using nine year old FFPE tissues, we confirmed that molecules acting in oxidative stress are regulated as already demonstrated in various proteomic studies on human or fresh frozen tissues in 6-OHDA animal model. New biomarkers were found such as eEF1 and CRMP. This confirms the high interest to use this FFPE tissues stored in hospital tissue libraries for biomarkers hunting or validation in the case of clinical studies.

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FIGURE LEGENDS

Figure 1: Comparison of 3D Intensity vs. M/Z vs. retention time plots reconstructed from the data collected on the nanoLC-nanoESI-IT MS experiments after *in situ* trypsin digestion and extraction of peptides for **a)** control animals and **b)** 6-OHDA treated model animals. First 3D view corresponds to retention time 0-60 min vs M/z 700-800 whereas second view corresponds to retention time 30-33 min vs. M/z 200-1800.

Figure 2: Superposition of six mass spectra obtained by MALDI direct analysis in the same region of FFPE tissue sections after *in situ* trypsin digestion for three 6-OHDA treated animals versus three control animals. Arrows indicate ion at m/z 1770.3 identified as a digestion fragment of Neurofilament Triplet M protein which is a potential biomarker.

Figure 3: MALDI molecular images reconstructed from the data collected after acquisition on a 6-OHDA treated rat brain section originally conserved after formalin fixation and paraffin embedding over 9 years after *in situ* automatic trypsin digestion. a) Image of ion at m/z 643, b) m/z 728, c) 1083 and d) composite image of all ions corresponding to digestion fragments of CRMP2 protein and detected in the MALDI experiment.

Figure 4: a) composite MALDI molecular image reconstructed using all detected digestion fragments of CRMP2 protein on a 6-OHDA treated FFPE tissue rat brain section after paraffin removal and *in situ* trypsin digestion. b) Optical image of the tissue section after automatic spotting of trypsin and solid ionic matrix HCCA/ANI, c) Map of the corresponding tissue section with different region indicated (Bregma Index) and d) Expression of Dpysl2 mRNA (coding for CRMP2 protein) in mouse brain (data from Allen Brain Atlas <http://www.brain-map.org>)

Table 1: Comparison of Parkinson Biomarkers identified using different proteomics approaches on different models

Model	Proteomic Approach	Protein	Modification	References
Parkin -/-	2D Gel	Pyruvate Deshydrogenase NADH Ubiquinone Oxyreductase 24 kDa NADH Ubiquinone Oxyreductase 30 kDa Cytochrome c Oxydase Peroxiredoxin 1 Peroxiredoxin 2 Peroxiredoxin 6 Lactoylglutathione Lyase Profilin Vacuolor Protein Sorting 29 α -Crystallin Chain b Heterogeneous Nuclear Ribonucleoprotein 1 Lasp-1	Down-regulated Up-regulated Down-regulated Down-regulated Down-regulated Down-regulated Down-regulated Down-regulated Down-regulated Down-regulated Down-regulated Down-regulated	4
		α -enolase β Actin	Up-regulated Down-regulated	3
6-OHDA	MDA*	Calmodulin Cytochrome C Cytochrome C oxydase Ubiquitin	Down-regulated Down-regulated Down-regulated Up-regulated	11
Human	Blood	Serum Creatine Kinase	Up-regulated	31
	2D Gel	Neurofilament Triplet L Neurofilament Triplet M Peroxiredoxin 2 Mitochondrial Complex III ATP Synthase D chain Profilin L Type Calcium Chanel d Subunit Fattu Acid Binding Protein PEP 19	Down-regulated Down-regulated Up-regulated Up-regulated Up-regulated Up-regulated Up-regulated Up-regulated Up-regulated	5
MTP	MDA	α -Enolase	Down-regulated	32
G93 A (gene SOD1)	2D Gel	HSP 25 HSP 27 Phosphatidylinositol Transger Protein a Apolipoprotein E Ferritin Heavy Chain	Up-regulated Up-regulated Up-regulated Up-regulated Up-regulated	7

*MDA: MALDI Direct Analysis MS

Table 2: Examples of proteins detected by MALDI direct analysis MS from the FFPE rat brain tissues after *in situ* enzymatic digestion, with the corresponding score, sequence coverage and matched peptides in the protein sequence

Protein	Match score	Mass (Da)	Databank	Sequence coverage (%)	Peptides (red) matched in the proteins sequence
Chordin-Like Protein 1 Precursor (Neuralin-1): Neurogenesis-1	39	50526	Swissprot	48	MEGIKYIASL VFFFVFLAS KTEPVKHSET YCMFQDKK YR VGEKWHPYLE PYGLVYCVNC ICSENGNVLC SRVRC PTLHC LSPVHIPHLC CPRCPDSLPP MNSKVT SKSC EYNGTTYQHG ELFIAEGLFQ NR QPNQCSQC SCSEGNVYCG LKTCPKLTCA FPVSV PDS CC RVCRGDGELS WEHSDADIFR QPANREARHS YLRSPYDPPP SRQAGGLPRF AGSRSHR GAV IDSQQASGTI VQIVINNKHK HGQVCVSN GK TYSHGESWHP NLRAFGIVEC VLCTCNVTKQ ECKK I HCPNR YPCYYPQKLD GKCKVCPEE PPSQNFDSKG SFCGEETMPV YEAVL VEDGE TARKVALETE KPPQVEVHVW TIRKGI LQHF HIEKISKEMF GGLHHFK LVT RTTMNQWKIF AEGEAQLSQM CSSR VCRTEL EDLVQVLYLE RPEKD HCR
Neuromodulin (Axonal Membrane Protein GAP-43)	24	23589	Swissprot	47	MLCCMRRTKQ VEKND EDQKI EQDGVK PEDK AHKAATK IQA SFRGHITR KK LKDEK GDAP AAEAEAKEKD DAPVADGVEK KEGDGSATTD AAPATSPKAE EP SKAGDAPS EEKKGEGDAA PSEEKAGSAE TESAAK ATTD NSPSSKAEDG PAKEEPKQAD VPAAVT DAAA TTPAAEDAAK AAQPPT TAE SSQAE EEKEA VDEAKPKESA RQDEG KEDPE ADQ EHA
Dystrophin-Associated Glycoprotein (43DAG)	50	41643	NCBI	47	MQPASDSRWR VTGLQ SEPT QTLAHS GFLS EGWRGRGKAW PSQV SPTPV PERKR GLRRA KMAAAAAAAAA ATE QQGSNGP VKKS MREKAV ERRNVNKEHN SNF KAGY IP I DEDRLHKTGL RGRKGNL AI C VIVLL F ILAV INLLITLVIW AVIR IGPNGC DSLEF HESGL LRFKQ VSDMG I I HPLYK STV GRRRNENLVI TGNNQ P IVFQ QGT TKLS VEK N KT SITSDIG MQFFD PRTQ N ILFSTDY ETH EFHLP SGVKS LNVQ K ASTER I T SNATSDLN IKVD GRAIVR GNEGV FIM GK TIEFHMRGDV ELKAEN S IIL NGTVMV S PTR LPSSSGD QS GSGDWVRYKL CMCADG T LFK VQV T SHNMGC QVSDN P CGNT H
Vimentin	54	53569	NCBI	42	STRSVSS SSY RRM FGSG TS SRP SSNRSYV TTSTR Y SLG SALRP S TSRS LYSS S PGGAY VTRSSAV R LR SSM P GV RLLQ DSVDF SLADA I N TEF K N TRT NEKVELQ E LN DRFANYIDKV RFLEQ Q NKIL LAELEQ L KGQ GKS R LGD LYE EEM REL R QV DQL TNDKARV EVERD NLAED I M R LREK LQ E EML Q R EEA S TLQ SFRQ D VD NAS LARLDLE RKVESLQ E EI AFLK K LHDEE IQELQ A QIQE QHVQIDVDVS KPDL T AALRD VRQ Q Y ESVAA KNL QEA E EWY KSK FADLSEA

					ANRNNDALRQ AKQESNEYRR QVQSLTCEVD ALKGTNESLE RQMREMEENF ALEAANYQDT IGRLQDEIQN MKEEMARHLR EYQDLLNVKM ALDIEIATYR KLEGEESRI SLPLPNFSSL NLRETNLESL PLVDTHSKRT LLIKTIVETRD GQVINETSQH HDDLE
UI-R-BJ2-bqp-d-02-0-UI.s1 UI-R-BJ2 Rattus Norvegicus cDNA Clone	58	17842	EST	83	RSPSRQSW_M RMPCHRSKA TILCAWCSPA ATRTWTLCEL CGPQEISRHR WTLQWPSMTC LWSWTF_ILS TRKPPCGSWT CAPLCCPKLR NFCRASM RAM SRLGVPPSS_ SCSGSYPSSL TFWQPRPLWV WTSAERRGCI SVDSASSSSR ASVASSRAGR V_AA
UI-R-DZ1-cnf-g-02-0-UI.s1 NCI_CGAP_DZ1 Rattus norvegicus cDNA clone	54	16280	EST	78	NGTMSPAITS CPSHVKRAQW PVENPQRWSM PEPLGRRKIA MRSAPWCGTS VLRALSSAMC PPSGVSPVGT GKSLES LAQT PTPSTGYRS GA_DPHGGAA PAWPTERSFH NVPRMLSPAA SHADRASHST WCLLVAFCHI RNPLKKEKKK KK
AGENCOURT_2 8540227 NIH_MGC_249 Rattus norvegicus cDNA clone	52	26005	EST	57	SAEMVDSLQT SPKSQWCAEP R_PEVVFCLS HLEGCIDVWV NSCPLXWTGL PD_SHMG_SG LHG PSSCWPA GCPSSCSPS_ FARWSVS_TL PPARSCQNGW LCHVASAGAS PRRTHPAPCP HRGSI PVRLG R_MYTEHLSG PSEGADCGF_ TQTRRRQPSV PSGSCPSWHR SQ_RCQSDPR PRYSGPQWAQ HVRG_VSSLS SGTSAFRWG_ MVPQSQASW_ SAAVRPAVEL CSCRGVADGC RTCGWCNPPG PRS

Table 3: Differential protein found after tryptic digestion by comparing 6-OHDA FFPE tissues to control FFPE tissues of rat brains using either MALDI-MS direct analysis or nanoLC-nanoESI-IT MS on analogue zones

Markers	Protein	m/z MDA*	m/z ESI	Mascot score (%)	Detected on heathy zone (number)	Detected on dammaged zone (number)	Known in Parkinson's Disease literature
Down-regulated	Trans elongation Factor 1 (eEF1)	1025.5	1025.8	66	3/5	0/5	
	Hexokinase (2-7-1-1)	1399.9	1400.5	76	3/5	0/5	33
	Neurofilament M Protein	1770.6	1770.8	57	4/5	0/5	5
Up-regulated	Peroxidoxin 6	1499.9	1498.8	65	0/5	4/5	5
	F1 ATPase		2007.3	81	0/5	4/5	18
	α -Enolase	3062.9	3063.6	62	0/5	3/5	34
	Ubc-Ubiquitin	647.4		78	0/5	4/5	11
	CRMP2	1083	1083.6.	70	0/5	4/5	

*MDA: MALDI Direct Analysis MS

Table 4: Data for the identification CRMP2 protein (MW_{avg}=62270.62 u., Score 245) in 6-OHDA injected rat brain FFPE tissues after trypsin digestion and nano-LC nanoESI-IT MS and MS/MS experiments **a)** peptide mass fingerprint identification data **b)** peptide sequence tag (MS/MS) identification data for the M/z 508.59 ion assigned as fragment SAAEVIAQAR of CRMP2 and **c)** CRMP2 protein (*Rattus norvegicus*, P47492) sequence. Red indicates parts of the sequence found in the nanoLC-nanoESI-IT MS experiments, in blue those obtained during MALDI direct analysis and in purple the common ones.

a)

Observed M/z ([M+H] ⁺ , [M+2H] ²⁺ or [M+3H] ³⁺)	Experimental Mw monoisotopic	Calculated Mw monoisotopic	Peptide Position	Peptide
508.590	1015.166	1014.546	259-268	SAAEVIAQAR
543.064	1084.114	1083.629	441-451	GSPLVVISQGK
562.215	1683.623	1681.863	452-467	IVLEDGTLHVTEGSGR
571.065	1140.116	1139.601	472-480	KPFPDFVYK
571.066	1710.178	1710.913	468-480	YIPRKFPDFVYK
648.020	1294.025	1293.686	64-75	MVIPGGIDVHTR
675.321	674.314	674.360	566-572	ANITSLG
834.047	2499.119	2498.160	271-293	GTVVYGEPIASLGTGDSHYWSK
863.482	1724.950	1724.804	375-390	MDENQFVAVTSTNAAK
896.971	1791.928	1791.827	346-361	DNFTLIPEGTNGTEER
956.396	1910.777	1910.015	174-189	FQLTDSQIYEVLSVIR
959.045	1916.076	1914.957	401-418	ISVGSADLVIWDPDSVK

b)

Peptide Sequence SAAEVIAQAR M/z observed 508.590 ([M+H] ⁺) Ion Score 63		
	Matched ions M/z	Ion assignment
1	229.129	[y ₂ -17] ⁺
2	230.113	b ₃ ⁺
3	246.156	y ₂ ⁺
4	341.146	[b ₃ -18] ⁺
5	359.156	b ₄ ⁺
6	374.215	y ₃ ⁺
7	440.214	[b ₅ -18] ⁺
8	445.252	y ₄ ⁺
9	458.224	b ₅ ⁺
10	553.298	[b ₆ -18] ⁺
11	558.336	y ₅ ⁺
12	571.309	b ₆ ⁺
13	642.346	b ₇ ⁺
14	657.404	y ₆ ⁺
15	752.394	[b ₈ -18] ⁺
16	769.420	[y ₇ -17] ⁺
17	770.404	b ₈ ⁺
18	840.457	[y ₈ -17] ⁺
19	841.441	b ₉ ⁺
20	857.484	y ₈ ⁺

c)

1	MSYQGKKNIP	RITSDRLLIK	GGKIVNDDQS	FYADIYMEDG
41	LIKQIGENLI	VPGGVK ^T IEA	HSRMVIPGGI	DVHTRFQMPD
81	QGMTSADDF	QGTKAALAGG	TTMIIDHVVP	EPGTSLLAAF
121	DQWREWADSK	SCCDYSLHVD	ITEWHKGIQE	EMEALVKDHG
161	VNSFLVYMAF	KDRFQLTDSQ	IYEVLSVIRD	IGAIQVHAE
201	NGDIIAEEQQ	RILDLGITGP	EGHVLSRPEE	VEAEAVNRSI
241	TIANQTNCPL	YVTKVMSKSA	AEVIAQARKK	GTVVYGEPI ^T
281	ASLGTGDSHY	WSKNWAKAAA	FVTSPPLSPD	PTTPDFLNSL
321	LSCGDLQVTG	SAHCTFN ^T TAQ	KAVGKDNFTL	IPEGTNGTEE
361	RMSVIWDKAV	VTGKMDENQF	VAVTSTNAAK	VFNL ^Y PRKGR
401	ISVGSADLV	IWDPDSVKTI	SAKTHNSALE	YNIFEGMECR
441	GSPLVVISQG	KIVLEDGTLH	VTEGSGRYIP	RKFPDFVYK
481	RIKARSRLAE	LRGVPRGLYD	GPVCEVSVTP	KTVTPASSAK
521	TSPAKQQAPP	VRNLHQSGFS	LSGAQIDDNI	PRRTTQRIVA
561	PPGGRANITS	LG		

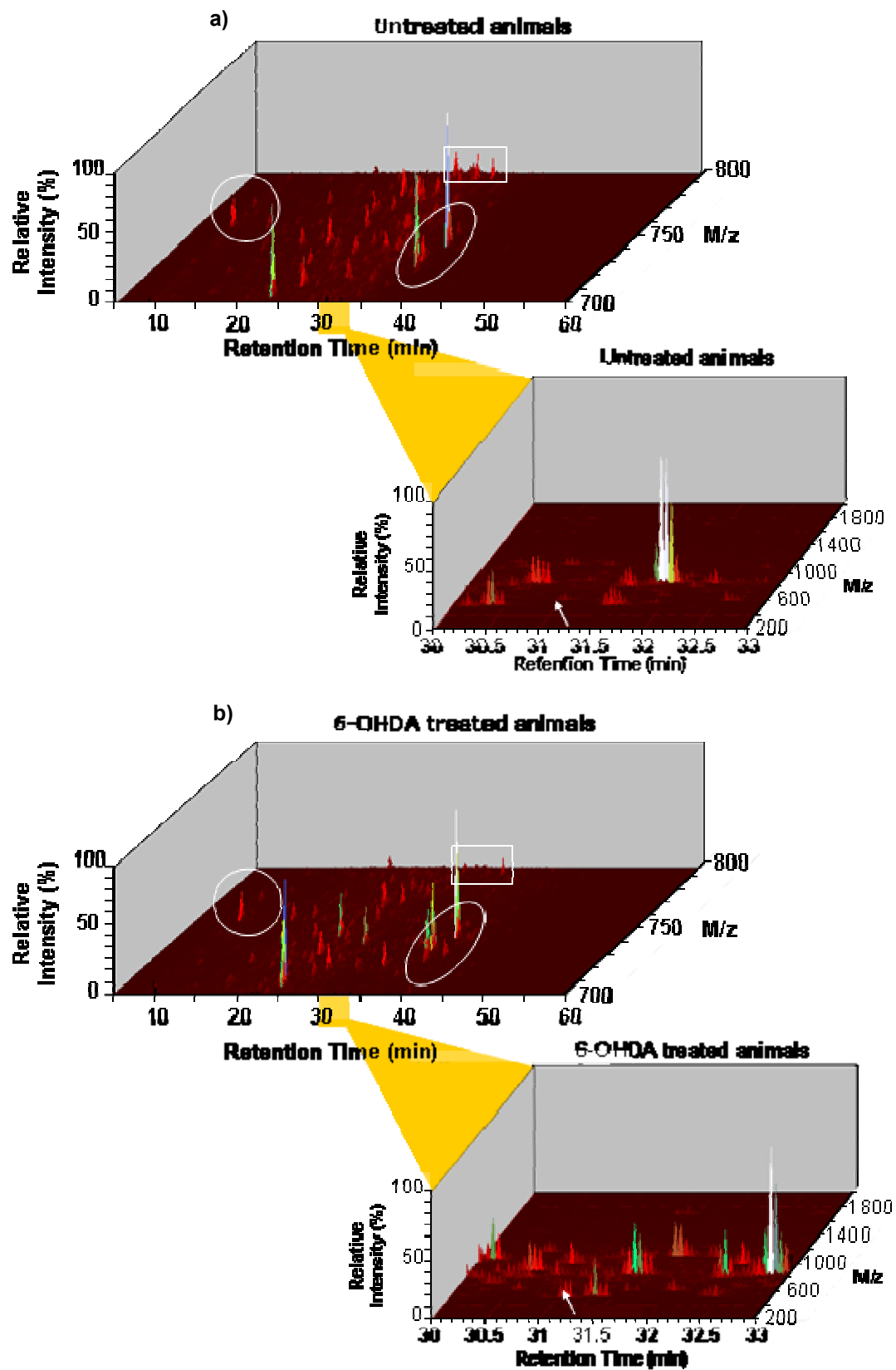


Figure 1

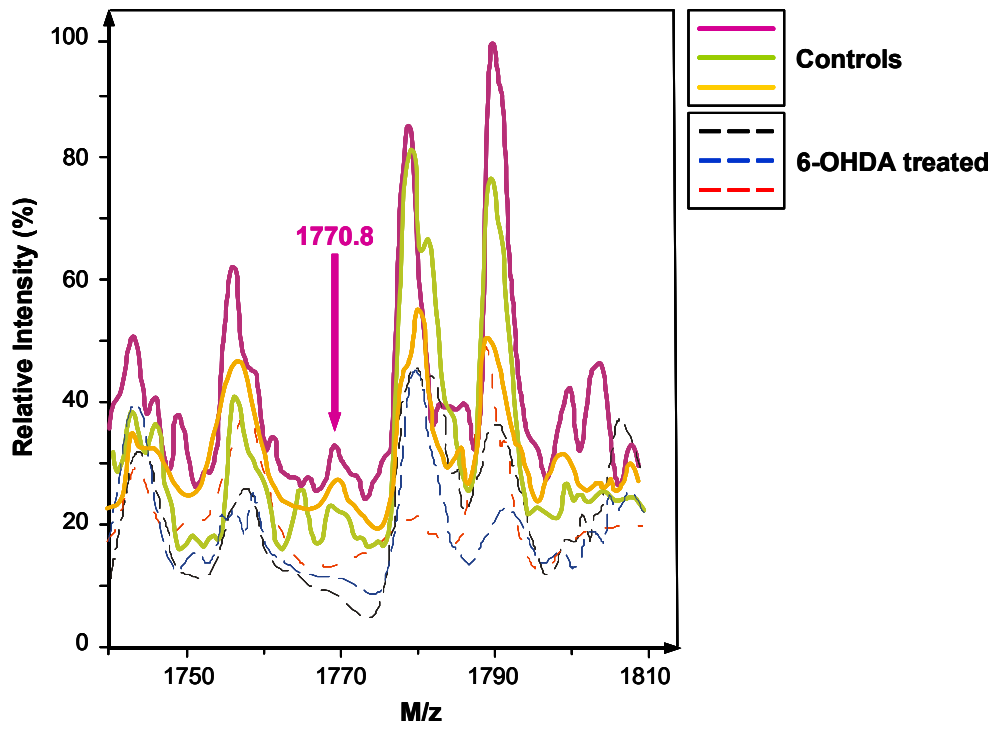


Figure 2

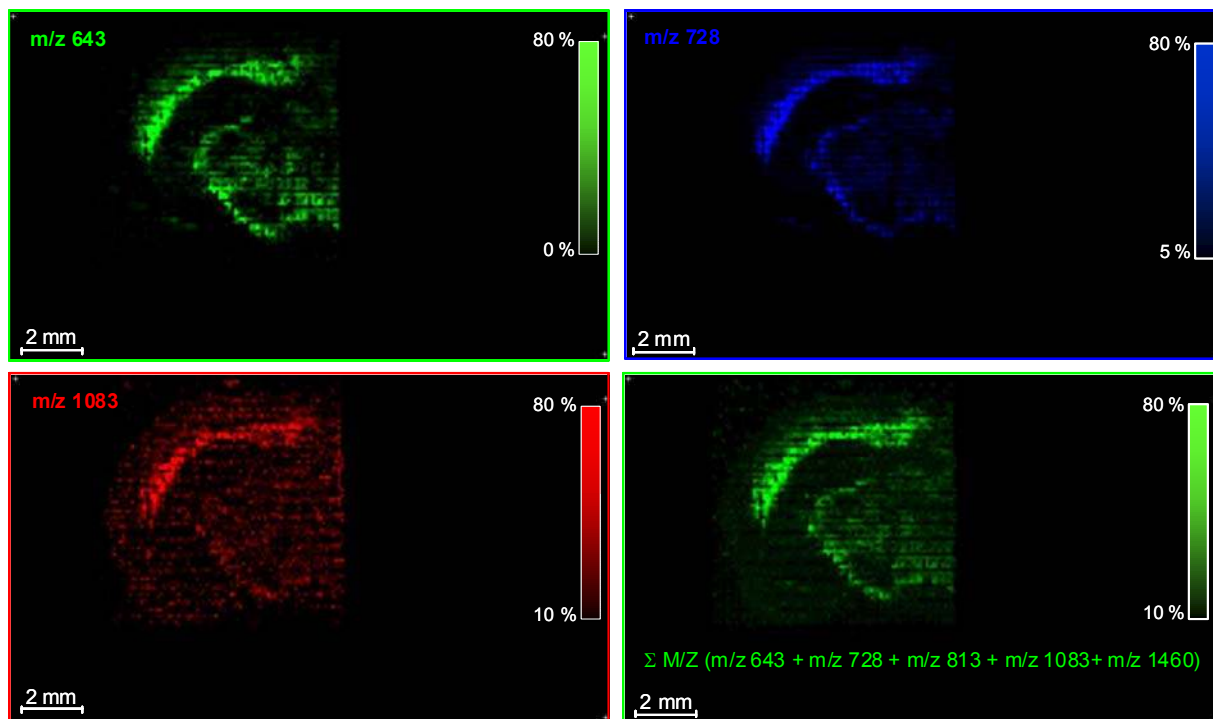


Figure 3

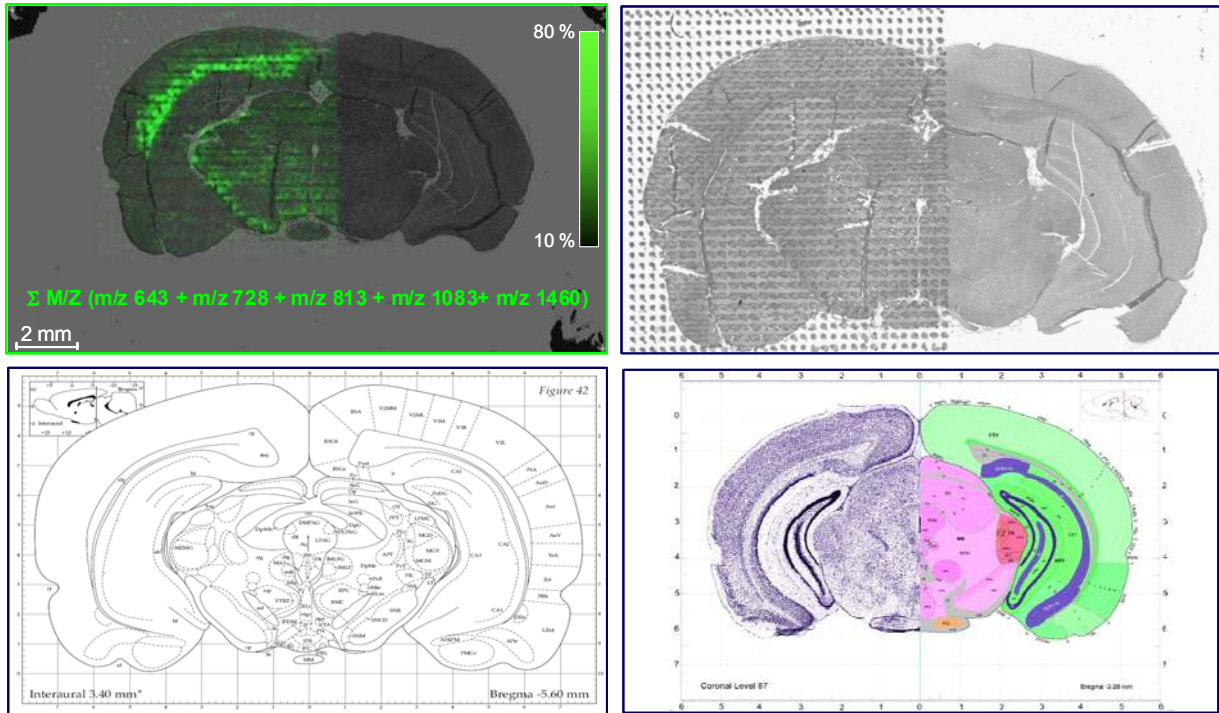
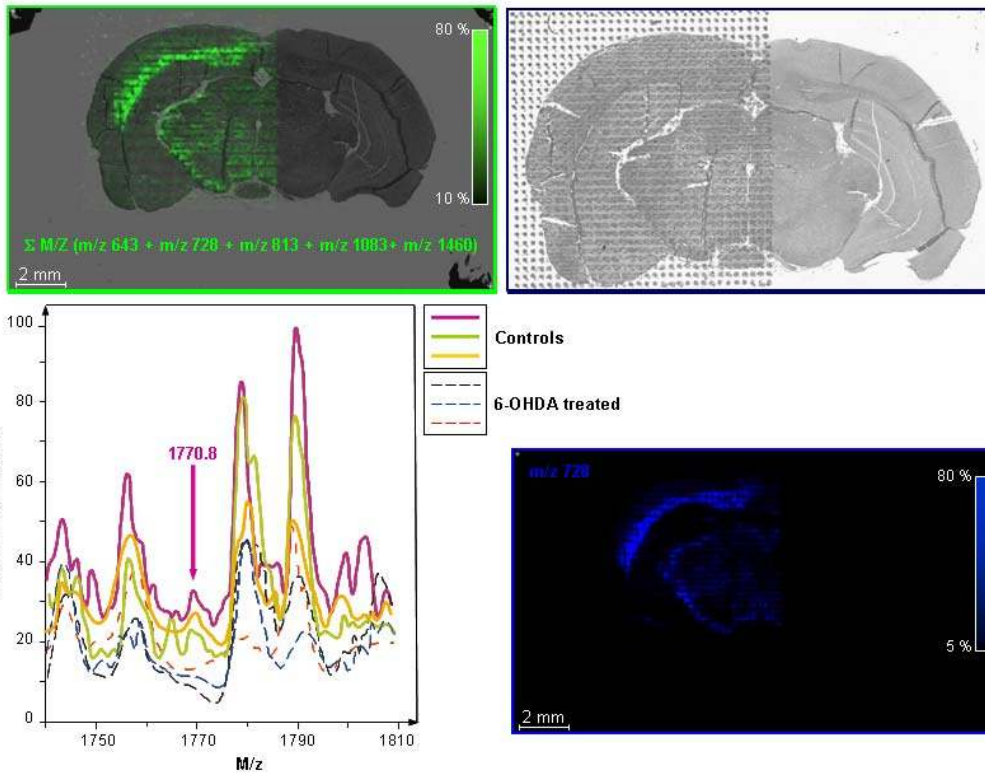


Figure 4



Synopsis