Molecular Imaging Mass Spectrometry for Probing Neuronal Protein Dynamics in Neurodegenerative Disease Pathology

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Abstract

Recent advances in understanding basic pathological mechanisms of various neurological diseases relate directly to the development of new bioanalytical technologies that allow highly resolved, sensitive, specific and comprehensive molecular analysis, including molecular imaging in complex biological tissues and nervous tissue, in particular. Imaging mass spectrometry (IMS) is an emerging technique for molecular imaging, characterized by its high molecular specificity allowing comprehensive, spatial protein and peptide profiling *in situ*. IMS represents therefore a powerful approach for investigation spatio-temporal protein and peptide regulations in CNS-derived tissue and cells. Over the last two decades, IMS has gained immense impact in biomedical and preclinical research, however, its translation to clinical practice is still in its infancy. The goal of this review is to familiarize the reader with the methodological workflow, instrumental developments, methodological challenges and to give a concise overview of the major advances and applications of IMS based protein and peptide profiling with particular focus on neurodegenerative diseases.

1. Introduction

As the average age of the world's population is rising, the prevalence of age-related diseases is increasing accordingly, including neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Both diseases are characterized by progressive neuronal degeneration and accumulation of misfolded proteins into neurotoxic deposits. The major molecular mechanisms that account for neurodegeneration in these diseases are still not resolved, which in turn significantly hampers the development of curative treatment strategies. The limited understanding of the underlying pathomolecular mechanisms in neurodegenerative diseases relates directly to the lack of appropriate biochemical tools that feature the necessary sensitivity, specificity, spatial- and temporal resolution, respectively, in order to delineate molecular interactions mechanisms at cellular length scales.

Here, chemical imaging techniques are essential bioanalytical tools to gain in-depth understanding of molecular mechanism and interactions at the subcellular scale. Here different molecular imaging modalities involving, *e.g.*, immunohistochemistry, *in situ* hybridization, chemical probes and fluorescent microscopy as well as spectroscopic methods are employed to retrieve topographical and temporal information of molecular abundance distributions. Here, a major challenge lies on obtaining suitable temporal and spatial resolution while maintaining high molecular specificity and sensitivity.

Over the last 30 years, mass spectrometry (MS) has transformed biomedical research, especially for large scale, comprehensive protein profiling, termed proteomics (Aebersold & Mann 2003). The introduction of soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) (Karas & Hillenkamp 1988) and electrospray ionization (ESI) (Fenn *et al.* 1989) MS facilitated fast, sensitive, and chemically specific detection of intact large biomolecules including proteins and peptides.

Proteomic-based approaches for characterizing proteins and (neuro)peptides in tissue extracts, including brain tissue, are valuable tools for sensitive *in situ* peptide and protein identification and quantitation (Skold *et al.* 2002, Svensson *et al.* 2003, Svensson *et al.* 2007a). However, a major limitation in proteomics remains with respect to spatial resolution of analyte localisation, as spatial information within the respective tissue compartment is not obtained. Given the complexity of the human nervous system, the spatial information of protein and peptide distribution is of significant interest in order to resolve ongoing molecular mechanisms. Moreover, neuroactive peptide species are in numerous neuronal processes and their localization is therefore of essential relevance for identifying their role in various neuronal signaling pathways.

Imaging mass spectrometry (IMS) is a powerful approach for spatial profiling of molecular species in biological tissue and single cells. In contrast to common molecular biology imaging techniques, IMS does not require any *a priori* knowledge of the potential target species and is not dependent on antibody or primer availability and specificity. IMS features high molecular specificity and allows comprehensive, multiplexed detection, identification, and localization of hundreds of proteins, peptides and lipids in biological tissue samples (Caprioli *et al.* 1997, Cornett *et al.* 2007). Over the last 20 years, IMS has slowly evolved as a relevant, alternative approach in biomedical research for studying proteins, peptides, lipids, drugs and metabolites in disease pathology, pharmacotherapy, drug metabolisms as well as fundamental biological processes (McDonnell & Heeren 2007). The technique allows matching of histological features of a biological tissue sample to molecular localization patterns and can therefore also be referred to as molecular histology (Cornett *et al.* 2006, Schwamborn & Caprioli 2010).

MS imaging can be performed with different probes to desorb and ionize molecular species directly from a biological sample (Figure 1a). The most prominent IMS modalities include MALDI IMS, where a laser is used for ion desorption and ionization; time of flight

secondary ion mass spectrometry (ToF-SIMS) based IMS, in which an ion beam is employed to sputter molecular species into the gas phase (McDonnell & Heeren 2007); as well as desorption electrospray ionization (DESI) (Takats *et al.* 2004), where a focusing an electrospray onto the sample facilitates image data acquisition at atmospheric pressure, though at larger spatial resolution. The different imaging MS modalities have various strengths and limitations with respect to spatial resolution, selectivity and molecular information. Here, MALDI IMS is of particular relevance as it is amongst all the different IMS technologies, to date the only one that has been demonstrated for robust *in situ* neuroproteomics and neuropeptidomics (Hanrieder *et al.* 2013b, Hanrieder *et al.* 2015, Karlsson & Hanrieder 2017, Martin-Lorenzo *et al.* 2014).

The present review is therefore focused on MALDI IMS in neurodegenerative diseases. The aim is to provide a concise overview of the technology and its suitability for protein and peptide imaging in complex biological samples. Different methodological concepts and advances as well as challenges with respect to sample preparation, data acquisition, and validation are discussed. Finally, an overview of MALDI IMS application for probing spatial protein and peptide regulations in neurodegenerative disease pathology is provided with focus on Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and in particular Alzheimer's disease (AD).

2. General Principle of Imaging Mass Spectrometry

For all imaging mass spectrometry techniques, the molecular intensity distribution in biological samples is obtained by sequential acquisition of mass spectra from predefined pixel array over a biological tissue section or a single cell. Single ion images are generated by plotting the intensity distribution of a distinct molecular species over the analyzed MS analysis array (Figure 1). The point-to-point distance defines the limit for lateral resolution of the IMS analysis. Spatial resolution is mainly limited by the probe (ion

beam, SIMS; or laser, MALDI); however, lateral diffusion during sample preparation has a major impact as well. In ToF-SIMS for instance, lateral resolution of down to 50-100 nm is achievable, which however compromises the mass resolution as well as the sensitivity (Benabdellah *et al.* 2009). Similarly, high spatial resolution MALDI IMS experiments of up to 1-10 um are limited by the sensitivity of the method to the respective compounds of interest (Benabdellah et al. 2009, Rompp *et al.* 2010, Kompauer *et al.* 2017).

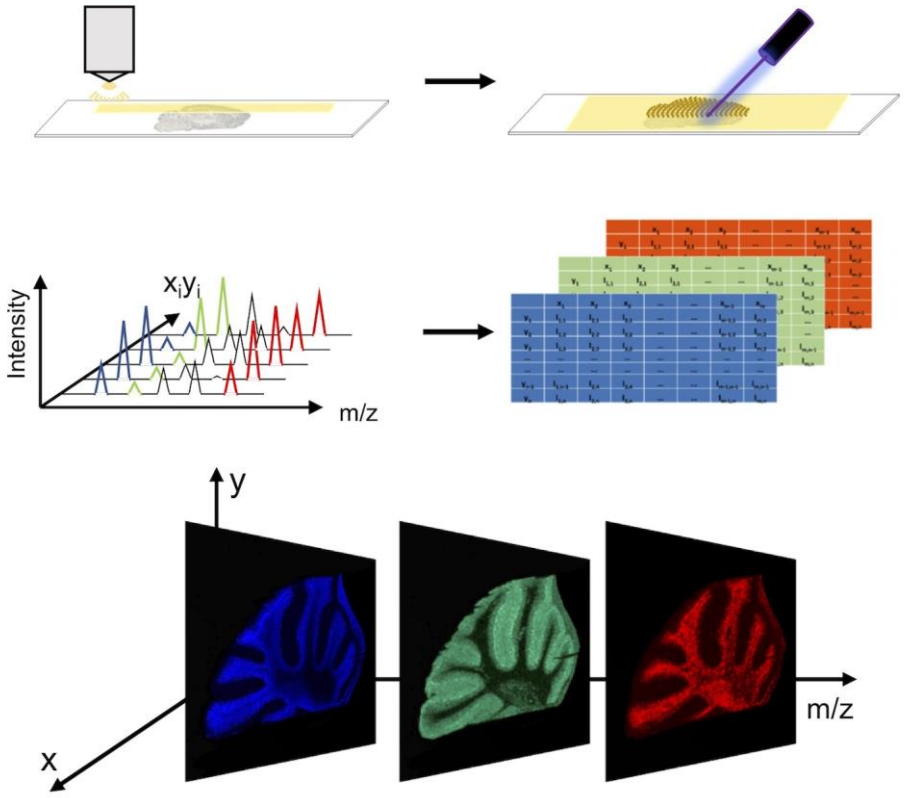

Figure 1: Principle of Imaging Mass Spectrometry. (a) I, Tissue sections are collected mounted on a target for imaging MS. II, For SIMS IMS, tissue sections are probed with an ion beam, generating low molecular weight secondary ions (m/z >1000Da). III, In contrast, MALDI IMS requires precoating with matrix (indicated in yellow) before systematic scanning with a laser probe. MALDI based ionization generates larger intact molecular species, including peptides and proteins. **(b)** One mass spectrum is acquired for every x_i,y_j coordinate of the scanned tissue section. **(c)** Single ion images are generated by mapping the intensity of an individual ion signal (m/z;rel.Int) over a the whole tissue slide.

Acquired images can involve enormous data amounts, as there is a complete spectrum for each pixel, hence these often comprise file sizes of multiple gigabytes. Therefore, spatial resolution and pixel number, respectively, need to be in a practical balance (Jones *et al.* 2012b). Although theoretically, one can carry out untargeted studies with IMS, in general, it is necessary to have target molecules in mind when choosing the matrix best suited to provide the required sensitivity. This is particularly relevant for protein and peptide analysis, where typically several tissue-washing steps are performed for analyte precipitation and lipid and salt removal (Seeley *et al.* 2008).

Analysis of imaging data can be performed using multiple multivariate statistical analysis (MVSA) tools, such as principal component analysis (PCA) and hierarchical cluster analysis (Deininger *et al.* 2008, Henderson *et al.* 2009). These represent excellent approaches for unbiased interrogation of molecular intensity distributions and potential localization to anatomical features of interest. This data handling approach allows segmentation of a biological sample in distinct regions of interest by detecting variances and correlations in the multivariate data that are encompassed in the MVSA-factors (PCA) and clusters. From the corresponding scores and loadings, the variables, i.e. mass peaks and their intensities, that are contributing the most to these variances can be deduced revealing histology associated chemical changes (Alexandrov 2012).

3. MALDI MS Imaging

MALDI MS has since its introduction (Karas & Hillenkamp 1988) significantly impacted the field of protein analysis in molecular biological and biomedical research. MALDI MS is a soft ionization technique permitting analysis of intact macromolecules including proteins and peptides. The technique is based on laser irradiation for desorption and ionization of molecular species assisted by a crystalline, UV radiation absorbing matrix (Figure 1). The technique is characterized by its superior mass range, sensitivity, high

resolution and mass accuracy as well its robustness, acquisition speed and insensitivity to sample impurities.

In 1997, a MALDI based approach was introduced that facilitated spatial profiling of large molecular species in mammalian tissue samples (Caprioli et al. 1997). The technique is particularly well suited for medium to large biomolecules including glycolipids, neuropeptides and proteins. Detection of small molecules such as drugs, metabolites and neurotransmitters is, however, hampered by interference of matrix cluster ions. This limitation can be overcome by matrix free laser desorption/ionization (LDI) approaches as well as other strategies including e.g. use of a stable isotope modified matrix or MS/MS methodologies just to name a few (Northen *et al.* 2007, Shariatgorji *et al.* 2012b, Nilsson *et al.* 2010).

Spatial resolution in MALDI IMS is essentially dependent on three factors: the laser beam focus, the matrix crystals, and lateral diffusion of analyte molecules occurring during sample preparation. Typically a practical spatial resolution of 10-30 µm can be achieved; however, different efforts to push these limits with respect to the probe have been reported (Jurchen *et al.* 2005, Spengler & Hubert 2002, Kompauer et al. 2017, Rompp et al. 2010). For high resolution MALDI (>5-10um), method sensitivity, data file size and acquisition time have to be considered. In addition, sample preparation is also an important factor affecting both the crystal size and lateral analyte diffusion eventually impacting spatial resolution.

3.1 Sample Preparation for MALDI imaging of proteins and peptides

For imaging MS of proteins, various target preparation parameters have an important impact on final data quality, particularly with respect to signal intensity, reproducibility, as well as lateral resolution. The sample preparation workflow in MALDI IMS comprises the whole chain of sample treatment starting with appropriate tissue retrieval and tissue

storage, tissue sectioning, sample wash, and final matrix application. Each of these steps is critical and might impact the final data significantly. In particular, matrix application is a cornerstone in MALDI IMS experimental workflow schemes and has to be tailored according to many factors, including tissue origin and molecular target.

3.1.1 Tissue Collection

As for all tissue imaging experiments, tissue retrieval from animal or human sources is critical to data quality. This is of particular relevance, since commonly used perfusion and fixation strategies cannot be applied in mass spectrometry due to interference of the polymeric fixation agents such as paraffin. Solutions to overcome these obstacles, including *in situ* trypsination following paraffin removal or alternatively use of the reactive matrix 2,4-dinitrophenylhydrazine have been presented (Lemaire *et al.* 2007). This approach, however, does not permit the analysis of intact neuropeptide due to enzymatic degradation. Fresh-frozen tissue samples are, instead, by far the most commonly used samples for IMS. In terms, of sample dissection such as for intact brain, quick performance is essential, since post mortem delays by as little as 3 min result in severe degradation of neuropeptides (Goodwin *et al.* 2008). One solution for overcoming this shortcoming, is heat stabilization, where quick sample heating to 95°C leads to efficient protease inactivation (Goodwin *et al.* 2010). This approach, however, impacts the sample morphology, which in turn hampers its suitability in IMS. This is primarily due to difficulties in maintaining tissue integrity during sectioning although an elegant workaround using collection on conductive carbon tape has been presented recently (Goodwin *et al.* 2012). For fresh frozen tissue, cryosections are collected and thaw mounted onto conductive glass slides. Here, the collected sections have to be dried immediately before storage at -80°C to prevent damage by water condensation during freezing (Hanrieder *et al.* 2012c).

3.1.2 Tissue washing

A further critical step in sample handling is the choice of appropriate washing protocols. Lipid species typically do not require any advanced washing steps, whereas, drugs, neuropeptides and proteins require optimized washing protocols for signal enhancement. These involve pH sensitive clean up as well as organic solvents for precipitation of peptides and proteins while washing off remaining lipids that potentially interfere with neuropeptide signals prior to matrix application (Shariatgorji *et al.* 2012a, Hanrieder et al. 2012c, Seeley et al. 2008). Several washing protocols have been evaluated for enhancing protein signal in MALDI imaging. Here, stepwise washing with gradient alcohol was found to give the most significant improvement in signal quality (Seeley et al. 2008, Martin-Lorenzo et al. 2014).

3.1.3 Matrix Application

In terms of matrix application, currently there are three approaches available, each with its own strengths and limitations. The most straightforward approach involves manual application of matrix solution employing an airbrush sprayer. While this is a quite cost effective solution, it is severely hampered by its lack in reproducibility as well its susceptibility to sample diffusion as well as limited extraction efficiency. A more controlled way to deposit matrix is to use a nebulizer-based sample application available in a commercial *ImagePrep* device (Bruker Daltonics, Bremen, Germany). Here, pneumatic aerosol formation and subsequent matrix application is monitored by a light scattering sensor below the sample glass to estimate matrix thickness. Although this is not as economical as the bare nebulizer, this technology has significantly advanced the aerosol-based matrix application in terms of reproducibility and signal quality. Further controlled means of nebulizer based matrix deposition are found in the *TM sprayer* (HTX Technologies Carrboro, NC, USA), the *SunCollect* (Sunchrome, Napa, CA, USA), or the

open access available iMatrixSpray (Stoeckli *et al.* 2014), where an airbrush head is repeatedly moved over the tissue section thereby applying layer by layer of matrix.

Still, one major disadvantage of this solution remains in its rather low extraction efficiency, as a consequence of reduced lateral diffusion. Another more controlled approach for sample application involves matrix droplet deposition by a chemical inkjet (*ChiP*, Shimadzu, Kyoto, Japan) or a pneumatic vertical spotter (*Portrait*, Labcite, Sunnyvale, CA). These approaches uses accurate deposition of distinct droplets in a geometrical patter thereby avoiding lateral diffusion of the analyte (Aerni *et al.* 2006). The spotting approach is particularly beneficial for low abundant species, including neuropeptides (Hanrieder *et al.* 2011), but is limited in terms of spatial resolution.

A plethora of different matrices has been reported for MALD IMS of various molecular species. Different matrices are well suited for different compounds and the choice depends largely on the targeted substance. Many advances in matrix developments have been aimed at overcoming various limitations of the standard compounds, including crystal size, reproducibility, interfering matrix cluster, mass range, signal quality. For protein analysis, the most commonly used matrices are 2,5-dihydroxy-benzoic acid (DHB), sinnapinic acid (SA), dihydroxy acetophenone (DHA) and 4-hydroxyl-alpha-cyano-cinnaminic acid (HCCA).

4. Molecular Identification

While IMS is a very powerful approach, there is a need for complementary validation strategies to fully exploit the potential of the technique. All generated IMS results have to be further validated to rule out false positive findings as a result of e.g. suppression effects. Universally, peak identification can be a sticking point when attempting to make convincing conclusions about these images considering the specificity of labeling strategies used in other methods. Most common validation approaches include *in situ*

MS/MS for top-down protein and peptide fragmentation (Debois *et al.* 2010, Groseclose *et al.* 2007); LC-MS/MS for proteomics and peptidomics in tissue extracts (Falth *et al.* 2007b); and common immunohistochemistry (Hanrieder *et al.* 2012a, Hanrieder et al. 2011, Ljungdahl *et al.* 2011).

This is of particular relevance for IMS of intact proteins since mass accuracy and resolution do not permit unequal identification of intact protein peaks and require extensive identification and validation strategies. Validation of peak identity and quantity can be achieved by targeted ESI- LC-MS/MS experiments of tissue extracts for intact lipids and neuropeptides as well as small intact proteins. Changes in protein concentration that are detected by IMS are commonly validated *in situ* using immuno-based methods. This is, however, largely dependent on antibody availability and specificity and difficult to compare in case of truncated protein isoforms. Protein peak identification protocols have been investigated thoroughly in previous studies (Groseclose et al. 2007, Andersson *et al.* 2008, Hanrieder et al. 2012a).

Most commonly, peptides and proteins are annotated by their accurate mass. Beyond accurate mass matching, protein and peptide sequence information are, however, needed for unequivocal peak annotation. The most convenient strategy would therefore involve direct *in situ* fragmentation and identification of peptides and proteins. This top-down approach is, however, very challenging for intact protein species, although significant contributions have been made to advance this strategy. Here, in order to aid in identification of neuropeptides *in situ*, Taban *et* al. have employed the high mass resolution of Fourier transform ion cyclotron resonance mass spectrometry (Taban *et al.* 2007) (Kiss *et al.* 2014).

Another commonly used, straightforward strategy for protein identification in MALDI imaging includes direct on-tissue digestion (Groseclose et al. 2007). Here MALDI IMS is followed by applying trypsin solution onto the same or a consecutive section followed by

imaging and fragmentation of the corresponding proteolytic peptides. Identified peptide species are then confirming protein identity by showing similar distribution pattern as the corresponding intact protein (Groseclose et al. 2007). This approach has due to its simplicity become a standard procedure in validation for protein distribution. However, it has limitations with respect to lateral diffusion as a consequence of additional liquid deposition. In addition, only one or two MS/MS spectra can be retrieved from a single spot, since this MALDI based MS/MS requires higher laser powers resulting in increased sample consumption.

A further convenient strategy to identify unknown protein peaks was based on tissue extraction and intact protein prefractionation using e.g. C8 reversed phase liquid chromatography (LC). This was followed by off-line fractionation and automatic MALDI MS interrogation of collected protein fractions (LC-MALDI). Based on the intact protein MALDI results, protein fractions that contained a protein peak of interest were then subjected to subsequent trypsination followed by identification using LC-ESI-MS/MS (Hanrieder et al. 2012a, Andersson et al. 2008).

As for peptide identification, LC-MS-based identification of tissue extracts, *i.e.*, peptidomics, is performed. Here, the most critical steps involve sample collection, peptide extraction, peptide fractionation and data analysis (Hanrieder *et al.* 2012b, Skold et al. 2002, Svensson et al. 2003). As mentioned above, fast tissue collection and snap freezing are essential to avoid peptide degradation (Svensson *et al.* 2007b, Goodwin et al. 2008). For peptide quantification experiments, it is essential to employ an internal standard in order to account for technical variation introduced through different extraction, purification and fractionation procedures. The peptide fractions are analyzed using standard LC-MS procedures commonly used for peptide identification in proteomics. Here, one-or two-dimensional HPLC based peptide separations are followed on- or offline by ESI (Svensson et al. 2003) or MALDI (Holtta *et al.* 2012) tandem mass spectrometry. The

acquired peptide fragmentation data are then compared to peptide databases containing in-silico generated or experimental peptide fragmentation data (Falth *et al.* 2006, Falth *et al.* 2007a, Falth et al. 2007b).

5. Data Analysis

A number of image data analysis methods have been used to extract meaningful information from spectral image data. MALDI image data from the analysis of brain and spinal cord samples have been evaluated with conventional region of interest (ROI) analysis (Eriksson *et al.* 2013, Weishaupt *et al.* 2015, Knittelfelder *et al.* 2014) and various multivariate image analysis techniques (Fonville *et al.* 2012, Maccarrone *et al.* 2017, Jones *et al.* 2012a, De Sio *et al.* 2015, Hanrieder *et al.* 2013a, Race *et al.* 2016, Karlsson *et al.* 2017). Conventional ROI approaches allow for data reduction and targeted analysis only of the selected regions within a single tissue sections. This approach does compromise on the full potential of the spatial component achieved through IMS, as the data from a single ROI are reduced to an average spectrum. It is, however, well suited for general comparisons of selected tissue regions, particularly when differences across such ROI are not present. The ROIs can be either annotated manually based on underlying morphological information or following multivariate image analysis approach to reveal such pathochemical features. Follow up univariate and multivariate approaches of the spectra from individual ROIs are then performed.

Multivariate data analysis (MVA) methods can independently be used for evaluation, exploration, and comparison of the IMS data. Here, such multivariate image analysis approaches have been shown to be powerful tools for unsupervised data mining for e.g. exploratory purposes, and for supervised classification as applied in, *e.g.*, biomarker discovery (Alexandrov 2012, Bonnel *et al.* 2011). Principal component analysis (PCA) (Fonville et al. 2012), hierarchical cluster analysis (HCA) (Maccarrone et al. 2017), and

non-negative matrix factorization (NMF) (Jones et al. 2012a) have been applied one at a time. However, many studies report combinations of MVA methods such as PCA and HCA (De Sio et al. 2015), PCA and maximum autocorrelation factor analysis (MAF) (Hanrieder et al. 2013a, Henderson et al. 2009), or PCA, NMF, MAF in combination with probabilistic latent semantic analysis (PLSA) (Race et al. 2016). Race et al. highlight how a combination of multivariate statistical tools in a complementary fashion aid interpretation of MALDI MS images of sagittal sections of rat brain (Race et al. 2016). Moreover, correlation analysis has been applied on MALDI image data (Fülöp *et al.* 2016) and in order to correlate information between MALDI and complementary imaging experiments, such as Raman imaging (Masyuko *et al.* 2013, Bocklitz *et al.* 2013).

The analysis of IMS data usually requires preprocessing of the raw spectral data in order to reduce experimental variance within the data set and thereby prepare them for subsequent statistical analysis. Except for additional spatial coordinates, spectral image data do not differ much from single measurement data and thus, similar preprocessing procedures can be applied. The most important spectra processing steps include baseline correction, intensity normalization, smoothing, recalibration and alignment of spectra (Norris *et al.* 2007). The latter is of particular importance when subjecting the data from several IMS experiments to MVA methods for data analysis.

For processing, visualization and evaluation of IMS data, a number of commercial software and freeware packages have been developed. Commercially available IMS software packages include flexImaging (Bruker Daltonics, Bremen, Germany), HD Imaging (Waters Corp., Manchester, UK), ImageQuest (Thermo Scientific, San Jose, USA), MALDIVision (Premier Biosoft, Palo Alto, USA), MIA_Toolbox (Eigenvector Research, Inc., Wenatchee, USA), Quantinetix (Imabiotech, Loos, France), SCiLS Lab (Bruker Daltonics, Bremen, Germany), and TissueView (AB Sciex, Concord, Canada). Many of the available freeware/open access IMS software packages are toolboxes for

Matlab or R packages including Cardinal (Bemis *et al.* 2015), DataCube Explorer (Klinkert *et al.* 2014), MALDIquant (Gibb & Strimmer 2012), *massPix* (Bond *et al.* 2017), Mirion (Paschke *et al.* 2013), msIQuant (Källback *et al.* 2016), MSiReader (Robichaud *et al.* 2013), Omnispect (Parry *et al.* 2013), OpenMSI (Rübel *et al.* 2013), rMSI (Ràfols *et al.* 2017), SpectralAnalysis (Race et al. 2016) and SpectViewer (Marie-France *et al.* 2014). All of these enable qualitative IMS analysis and most include data preprocessing algorithms, but only few have been developed for quantitative IMS analysis (MALDIquant, msIQuant, Quantinetix). A limited number of packages exhibit capacities for multivariate analysis of image data, in various extents (Cardinal, *massPix*, MIA_Toolbox, SCiLS Lab, SpectralAnalysis).

6. MALDI IMS Applications to Investigate Neurodegenerative Disease Pathology

6.1 Parkinson's disease

Imaging MS has great potential in exploring biomolecular mechanisms underlying neurodegenerative disorders. Previously, MALDI IMS was successfully employed to delineate neuropeptide mediated motor control signaling circuits in PD and L-DOPA pharmacotherapy induced dyskinesia (Hanrieder et al. 2012c, Hanrieder et al. 2011, Ljungdahl et al. 2011) (Figure 2a). *In situ* neuropeptide analysis is however challenging as commonly used antibody-based techniques are significantly limited by antibody specificity and availability. This is particularly relevant for opioid peptides that differ in only a few C-terminal amino acids, which compromises the reliability of immunohistochemistry results significantly. Further limitations of IHC include the inherent limited throughput, as well as well-known quantification issues. Imaging MS based neuropeptide detection has significant advantages over immuno-based techniques particularly with respect to molecular specificity. A major consideration for imaging MS of biological tissue samples is the inherent biological variation within the sample groups. An elegant way to avoid the

effects intra-sample group biological variation is to use hemispheric disease models such as a unilateral nigrostriatal lesion with 6-hydroxy-dopamine (6-OHDA) as a model for Parkinson's disease (Ungerstedt 1968). These animals develop PD pathology only on one side and the other side can serve as an internal control accounting for variation as a result of suppression effects that are a common challenge in mass spectrometry. Using this model, MALDI IMS has been employed to analyze spatial regulations of dynorphin neuropeptides in L-DOPA induced dyskinesia in experimental PD (Hanrieder et al. 2011, Ljungdahl et al. 2011) (Figure 2a). Elevated levels of prodynorphin the neuropeptide precursor protein have been previously associated with L-DOPA induced dyskinesia (LID). Due to lack of specific antibodies, the distinct prodynorphin processing products could not be characterized using conventional techniques. MALDI imaging was used to demonstrate that dynorphin B and alpha neoendorphin were significantly elevated in the dorsal lateral striatum in the high dyskinetic group but not for low diskinetic animals. In addition, both dynorphin species correlated positively with LID severity.

In another MALDI IMS study to probe *in situ* protein regulations in the 6-OHDA model of experimental PD, ubiquitin, trans-elongation factor 1, hexokinase, and neurofilament M were found down regulated in PD (Stauber *et al.* 2008). The technique has further been successfully applied for protein imaging in another neurotoxin induced model of PD. Here, IMS was employed to study striatal protein localization in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injected rats and MALDI imaging revealed a striatal decrease of the neuronal calmodulin binding protein Pep19, which suggests that altered calcium homeostasis might be associated with neuronal cell death in PD (Skold *et al.* 2006).

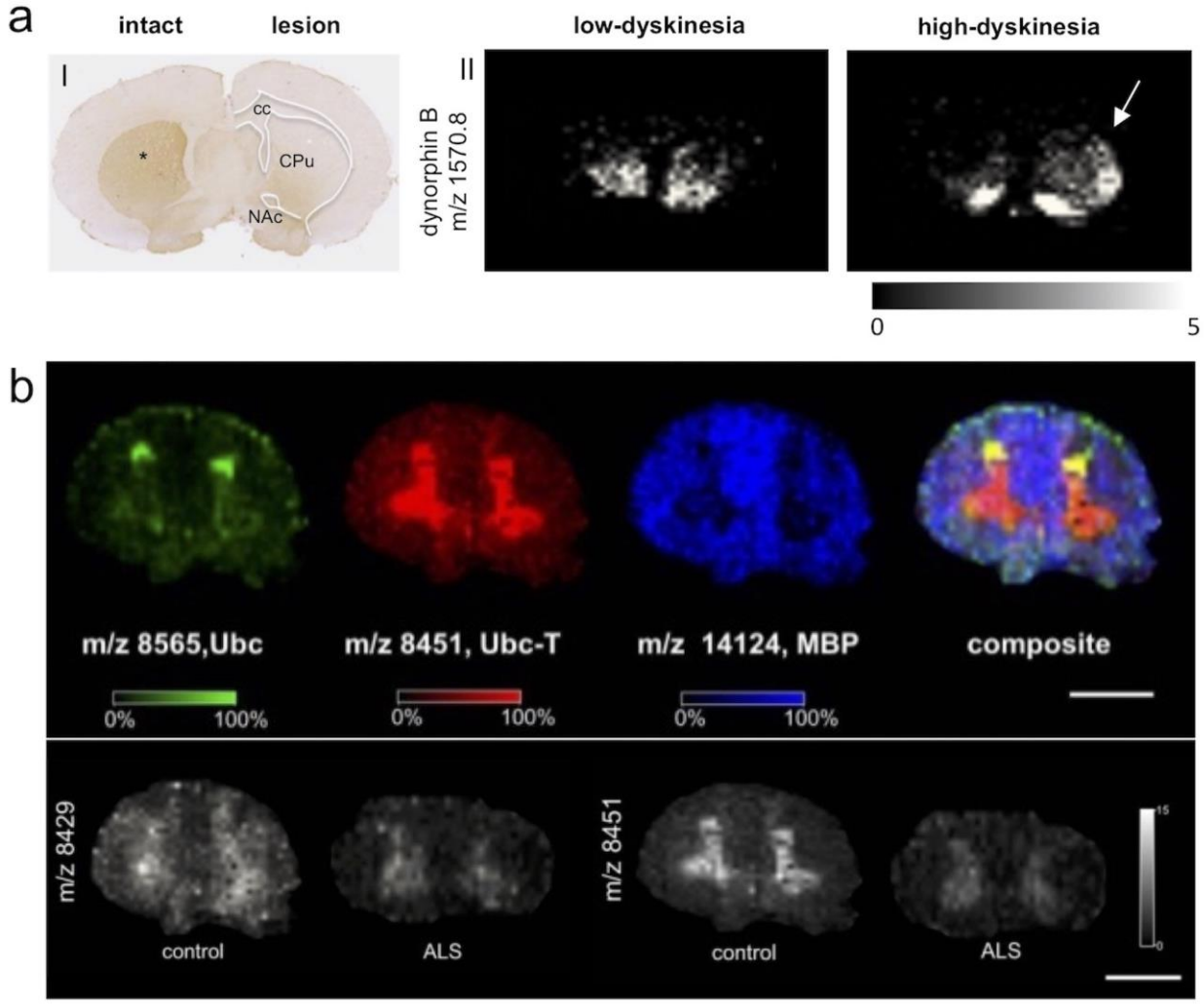

Figure 2: Delineating peptide and protein dynamics in neurodegenerative diseases using Imaging Mass Spectrometry. **(a)** IMS of neuropeptides in L-DOPA induced dyskinesia in experimental Parkinson's disease (PD). **aI**. Unilateral 6-OHDA injection leads to dopamine depletion (as illustrated by tyrosine hydroxylase,TH-immunostaining *). L-DOPA therapy results in two distinct groups with low and high-dyskinesia. **II** Dynorphin peptides, incl. dynorphin B, displayed a significant ($p>0.01$) relative increased in the dorso-lateral striatum of high- (HD) compared to low -dyskinetic (LD) animals and lesion controls (untreated PD animals) and correlated positively with dyskinesia severity. **(b)**. Imaging MS of spinal cord proteins reveals characteristic protein distribution patterns that are well in line with anatomical regions. This includes e.g. the grey matter (truncated ubiquitin 1-74 (Ubc-T)), the dorsal horn of the grey matter (ubiquitin, Ubc) and white matter (myelin basic protein, MBP). Multivariate statistics reveal significant decrease for two protein peaks, including Ubc-T (m/z 8451) in the grey matter of ALS patients compared to controls.

6.2 Amyotrophic lateral sclerosis

MALDI Imaging has been successfully employed to image intact protein species analysis of post mortem spinal cord sections of patients that suffered from ALS (Figure 2b). (Hanrieder et al. 2012a) Using this approach, characteristic protein distribution patterns were observed that were well in line with major histological regions of interest (ROI). MAF-based multivariate analysis was performed on spectral data extracted from these anatomical ROI's. Further statistics on the spectral data using partial least square-discriminant analysis (PLS-DA) suggested an ALS associated decrease of an unknown peak in the grey matter. Using extensive bottom up proteomics based on two-dimensional liquid chromatography, this peak could be identified as truncated ubiquitin (Ubc 1-74, Ubc-T), where two C-terminal glycine residues were removed (Figure 2b) suggesting an altered protein turnover in ALS. These findings are of particular relevance, since *in situ* differentiation of C-terminal sequence truncations cannot be achieved with antibody-based techniques due to limited specificity. Therefore, these data highlight the potential of imaging MS for studies on intact peptide and protein regulations *in situ*. In another study on ALS, MALDI IMS was employed to identify spatial protein changes in brain tissue. In a transgenic mouse model of ALS based on transfection with human mutant SOD1–G93A, MALDI IMS revealed selective accumulation and aggregation of hSODG93A protein in the facial and trigeminal nuclei. Moreover, statistical analysis revealed an up regulation of the 40S ribosomal protein S19 (RPS19) in the parenchyma and reactive glial cells in the facial nuclei of hSOD1G93A mice as compared to transgenic hSOD1WT and non-transgenic animals (Acquadro *et al.* 2014).

6.3 Alzheimer's disease

Given the distinct of MALDI IMS for comprehensive, *in situ* peptide imaging, the technology is a particular powerful tool to probe beta-amyloid (Aβ) peptide dynamics in

Alzheimer's disease. Here, Aβ truncations in individual plaques have been characterized in APP23 transgenic mice (Fig. 3a,b) (Seeley & Caprioli 2008, Stoeckli *et al.* 2002). The authors reported a significantly higher content of Aβ 1-40 than Aβ 1-42. Similarly, our group reported a comprehensive study on profiling brain-wide Aβ profiles in transgenic animals carrying the Swedish and Arctic mutation of APP (tgArcSwe) (Figure 3) (Carlred *et al.* 2016). In this study, we employed a multivariate image analysis approach to outline pathological features that constitute Aβ plaque pathology and reveal the associated Aβ profiles in individual deposits with different areas of the brain. Moreover, other plaque associated proteins were identified within the same experiment, including microglial derived macrophage inhibitory factor (MIF).

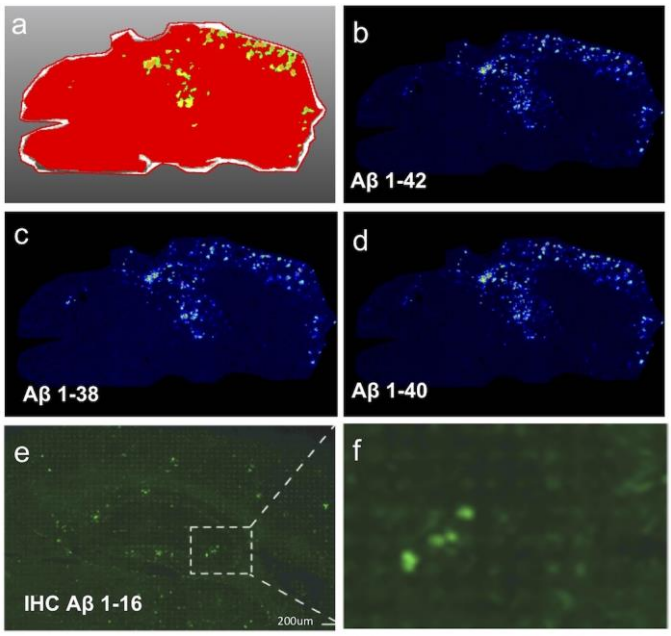

Figure 3. MALDI Imaging of Aβ peptides in transgenic AD mice. IMS experiments were performed for characterizing the plaque pathology in 18month old transgenic Alzheimer mice (tgAPPArcSwe). **a)** Image analysis using hierarchical cluster analysis (HCA, bisecting k-means) delineates histological features resembling plaque pathology (yellow,green). **b-d)** Inspection of the corresponding variables in the clusters that cause this difference, reveals major Aβ species. **d)** The IMS staining experiments were complemented with immunohistochemistry (IHC) towards Aβ on the same section to verify the Aβ identity of these plaques in general (e: scale 1mm; f: 200um).

Using the same animal model, we expanded the MALDI IMS toolbox towards multimodal, three-step, imaging MS of both lipids and proteins on the same imaging array on the same tissue section using a novel, histology compatible setup (Kaya *et al.* 2017a, Kaya *et al.* 2017b). The resulting trimodal MALDI IMS dataset revealed lipid and Aβ peptide correlates such as several sphingolipids, phosphoinositols and lysophosphatidic acids that have all been previously mechanistically implicated in AD pathology (Figure 4).

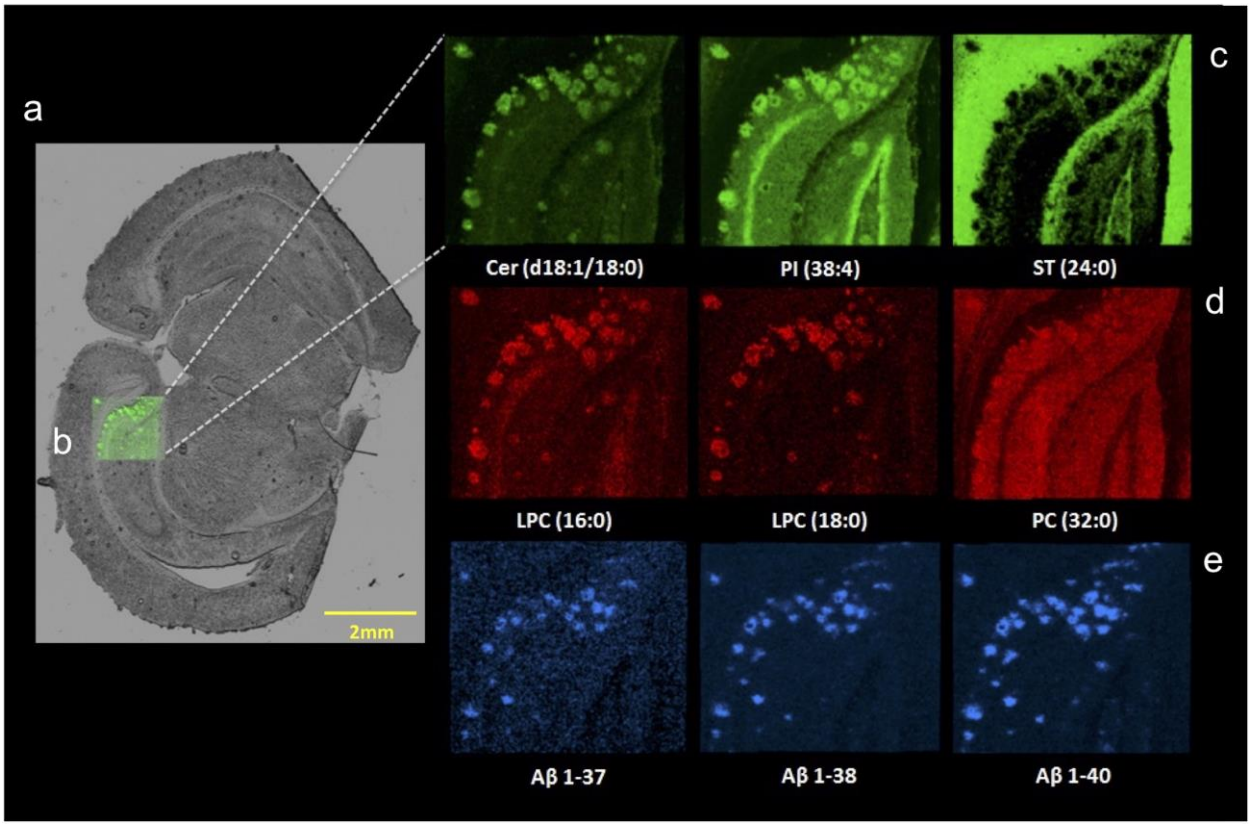

Figure 4. Trimodal MALDI Imaging. Hippocampal amyloid plaque-associated lipids in dual polarity and peptides **a)** on a coronal mice brain tissue section of transgenic AD mice (tgArcSwe) were revealed by multimodal MALDI-IMS. **b)** The hippocampal region analyzed with 10µm spatial resolution. **c)** Ion images of lipids: ceramides (Cer d18:1/18:0, m/z 564.6), phosphatidylinositols (PI 38:4, m/z 885.6), sulfatides (ST 24:0, m/z 890.6) in negative (green) and **d)** lysophosphatidylcholines (LPC 16:0, m/z 496.3, LPC 18:0, m/z 524.3) and posphatidylcholines (PC 32:0, m/z 734.6) in positive (red) polarities with **e)** subsequent amyloid-β (Aβ 1-37, m/z 4002.7, Aβ 1-38, m/z 4060.3, Aβ 1-40, m/z 4257.6) peptide (blue) ion images in the same imaging region.

In another study on triple-transgenic animal model of AD (3xtg), MALDI IMS revealed an AD pathology-associated decrease of neurogranin (NGR). This is of particular interest, as the synaptic protein NGR, has been demonstrated to be a potential biomarker reflection AD pathology associated neurodegeneration in CSF (Kvartsberg *et al.* 2015). Finally, working on 5xFAD mice, Schwartz et al. reported intriguing results on enhancing low abundant Aβ signals such as Aβ1-22 and Aβ1-26 using optimized parameters for random projections PCA (RP-PCA) based multivariate image analysis (Schwartz *et al.* 2015).

The potential for probing Aβ pathology in human AD tissue was recently demonstrated by two groups, where amyloid peptide profiles of individual plaques were delineated (Kelley *et al.* 2016a, Kelley *et al.* 2016b, Kakuda *et al.* 2017). Here, Kakuda et al. demonstrated the particular potential of MALDI IMS to provide novel insight in *in situ* amyloid pathology. In detail, their study demonstrated that Aβ1–42 and Aβ1–43 were selectively deposited in senile plaques while full-length Aβ peptides such as Aβ1–36, 1–37, 1–38, 1–39, 1–40, and Aβ1–41 were deposited in leptomeningeal blood vessels. Moreover, they managed to visualize distinct depositions of N-terminal truncated Aβ40 and Aβ42, including pyroglutamate modified at Glu-3 (N3pE) (Figure 5). Moreover, they demonstrated that one single amino acid alteration at the C-terminus between Aβ1–42 and Aβ1–41 results in profound changes in their distribution pattern, which can potentially be attributed to the difference in the self-aggregation ability amongst Aβ1–40, Aβ1–41, and Aβ1–42.

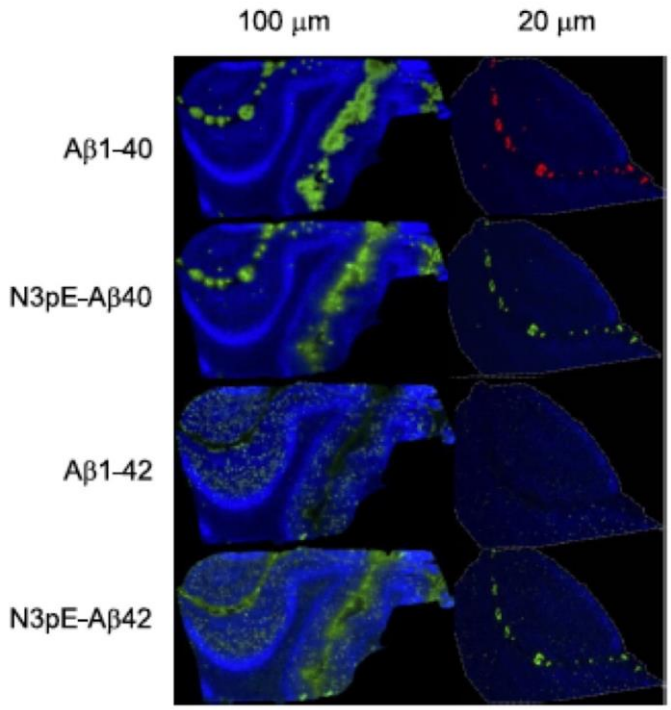

Figure 5. MALDI imaging of amyloid pathology in human AD tissue. Aβ1–40/42 and N3pE-Aβ40/42 were detected at a 100 μm and 20 μm resolution IMS, respectively. Aβ1–40 and N3pE-Aβ40 preferentially deposited in leptomeningeal blood vessels and arterioles, while Aβ1–42 and N3pE-Aβ42 deposited in the cerebral parenchyma.

7. Perspectives and Conclusions

The developments and applications of MS imaging presented here highlight the immense potential of the technique for clinical research. Major challenges associated with sample preparation, sample throughput and protein identification are more and more being overcome by recent developments. Exciting instrumental developments with respect to spatial and mass resolution will raise even more interest from the biomedical community. Moreover, standardized protocols and data formats significantly facilitate IMS-based applications in clinical settings. IMS-based findings are likely to further our understanding of mechanisms involved in neurodegeneration, as well as inform the development of

pathology-associated biofluid- and imaging-based biomarkers for neurodegenerative diseases.

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