Title:

MALDI mass spectrometry imaging of formalin-fixed paraffin-embedded tissues in clinical research

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Abstract

The molecular investigation of archived formalin-fixed, paraffin-embedded (FFPE) tissue samples provides the chance to obtain molecular patterns as indicatives for treatment and clinical end points. MALDI mass spectrometry imaging is capable of localizing molecules like proteins and peptides in tissue sections and became a favorite platform for the targeted and non-targeted approaches, especially in clinical investigations for biomarker research. In FFPE tissues the recovery of proteomic information is constrained by fixation-induced cross-links of proteins. The promising new insights obtained from FFPE in combination with the comprehensive patients' data caused much progress in the optimization of MS imaging protocols to investigate FFPE samples. This review presents the past and current research in MALDI MS imaging of FFPE tissues, demonstrating the improvement of analyses, their actual limitations, but also the promising future perspectives for histopathological and tissue-based research.

Key words

FFPE (formalin-fixed, paraffin-embedded), mass spectrometry imaging, MALDI, proteins, peptides

Introduction

Formalin fixation became a routine measure for the long-term storage of tissues in clinical settings after its first report in 1893 (Blum, 1893). It sustains the tissue integrity for years (Casadonte & Caprioli, 2011) and biomolecules like proteins, DNA, and RNA can be extracted (Ralton & Murray, 2011; Frankel, 2012). Numerous formalin-fixed, paraffin-embedded samples were archived over the years. These samples are well documented with information on patient treatments, treatment response, disease progression and other relevant clinical data. The retrospective analysis of biomolecules from FFPE samples enables the correlation of molecular patterns to clinical end points to personalize and optimize therapies.

The proteome is well suited to search for molecular markers and classifiers. Several proteins already assist cancer diagnosis using immunoassays (Hudler et al., 2014). In FFPE, proteins are conserved by dehydration and are cross-linked by methylene bridges, so the original proteomic information is maintained, but hard to reveal. Numerous original papers and reviews address the extraction of proteins from FFPE (Ergin et al., 2010; Klockenbusch et al., 2012; Magdeldin & Yamamoto, 2012; Nirmalan et al., 2008; Ralton & Murray, 2011) (Table 1). Many approaches apply an antigen retrieval to break the molecular bonds between proteins that can be subsequently extracted and analyzed by gel-based proteomics or liquid chromatography (Azimzadeh et al., 2010; Magdeldin & Yamamoto, 2012).

Mass spectrometry imaging (MSI) enables the localization of diverse molecular species (e.g. lipids, metabolites, drugs, peptides and proteins) in animal and human tissue (Casadonte & Caprioli, 2011; Römpp & Spengler, 2013). MALDI MS imaging is the acquisition of mass spectra directly from matrix-coated tissue, thereby sustaining the information of compound localizations. Spatial resolutions of 3 µm were reported (Römpp & Spengler, 2013) enabling a detailed correlation of MS data with histological features. Compound abundances can be visualized by heat maps on the histological images of the section. Spectra of specific sample regions can be selected after data acquisition, providing accurate annotation of tissues and substructures.

MSI can be used for targeted approaches such as the localization of a compound of interest (e.g. drugs, Prideaux and Stoeckli, 2012). In addition, MSI exhibits profiling character since the mass spectra provide information about numerous compounds detected simultaneously. This enables the analysis and localization of either single molecules as well as the description of complex molecular patterns derived from tissues.

Whole proteins as well as peptides can be detected by MALDI MSI. Several analyses of frozen samples demonstrated the suitability of MSI to obtain tissue-specific protein profiles (Seeley et al., 2011). In FFPE tissues, the cross-linking of proteins prevents their detection, so novel strategies had to be developed. The experience of immunohistochemistry and extraction-based proteomics of FFPE samples were of high benefit with the integration of an antigen-retrieval and a protein digestion step in the FFPE-MALDI-MSI workflow. The first protocols were reported in 2007 (Lemaire et al., 2007; Aoki et al., 2007) with many attempts in the optimization and variation of the protocol in the following years (Table 2). The studies demonstrated the feasibility to investigate archived FFPE samples by MALDI MS imaging and even a more than 100 years old specimen could be analyzed (Seeley et al., 2011).

Much attention is paid to the investigation of cancerous tissues. To date, diagnoses and tumor classifications are based on clinical information, morphology, immunohistochemistry, and molecular methods, which is complex, expensive, and time consuming (Casadonte & Caprioli, 2011). Tumors of the same histopathological features can have different clinical courses (Djidja et al., 2010). MALDI MS imaging of FFPE samples might enable molecular tumor classifications to assist diagnosis. The generation of tissue microarrays (TMA) from the FFPE biopsies allows high-throughput screening of tissues to define molecular patterns. This strategy was successfully applied for TMAs of breast and pancreatic cancer (Casadonte et al., 2014), pancreatic cancer (Djidja et al., 2010), lung tumors (Groseclose et al., 2008) and gastric cancer (Morita et al., 2010) (Table 2). MALDI MS imaging links the expert disciplines of pathology and mass spectrometry and provides a promising basis for the understanding of diseases on the molecular level and the detection of novel disease biomarkers for the optimization of treatments and therapies.

This review provides a summarizing view on MALDI MS imaging studies of FFPE samples performed to date. The main strategy is presented as a generalized workflow. The protocol is complemented by information about the molecular mechanisms as well as practical advice and

considerations for optimal results in MS performance and identifications. This review focuses on MS imaging approaches, but the comprehensive experience from LC-MS approaches is integrated to provide ideas for further optimization of the MSI protocols. In the last section, studies of FFPE tissues will be outlined to demonstrate the potential of MALDI MS imaging to distinguish between cancer types and subclasses.

FFPE analysis by MALDI MS imaging: The common workflow

In the last years several MS imaging protocols whave been published that provide excellent guidelines for the recovery of peptides from FFPE tissues, including advice for critical steps and trouble shooting (Table 1). Reviewing the material and methods of the clinical studies (Table 2) a main workflow could be assigned, which is provided as figure 1 with an FFPE mouse brain as an example. The workflow includes deparaffination of sections, rehydration of the proteins, and antigen retrieval to break the protein cross-links (Fig. 1, box 2). Subsequently, proteins are digested to peptides (Fig. 1, box 3). Afterwards, the protocol follows the typical MALDI MS imaging procedure with matrix application, MSI data acquisition and data analysis, including protein identification approaches (Fig. 1, boxes 4 - 6). We would like to recapture the main steps of the workflow with background information on the challenges of FFPE samples and the respective considerations that should be made during analysis. Exemplary studies and results will be presented.

Deparaffination, rehydration, and antigen retrieval as FFPE-specific sample treatments

MALDI MS imaging can be performed on conventional FFPE sections of 3 – 8 µm layer thickness mounted on conductive, in most cases indium tin oxid (ITO) coated glass slides (Fig.1, box 1). A section thickness of 3 – 5 µm enables detailed histological investigations after the MALDI MSI workflow. Using 10 µm thick sections provides higher amounts of peptides and might be advantageous for spectra quality (Wisztorski et al., 2010).

Formalin-fixation as well as paraffin-embedding are both critical for mass spectrometry based analyses of proteins. Paraffin might cause ion suppression. Deparaffination is easy and straightforward with the incubation of the sample in xylene for several minutes (Casadonte & Caprioli, 2011). The formalin-fixation is more challenging, because dehydration, denaturation, crosslinking, precipitation, and agglutination of the proteins (Kiernan, 2000) prevent their detection by MALDI MS. The molecular principles of formalin-fixation are described elsewhere in detail (Kiernan, 2000; Nirmalan et al., 2008; Magdeldin & Yamamoto, 2012). In this review, we focus on the dehydration and the covalent modifications of proteins, namely Schiff base formation, methylol adduct formation, and methylene bridges (Fig. 1, box 2), which are of relevance for MALDI MS imaging.

The reconstitution of the proteins' hydrate environment is a requirement for successful downstream measures. Rehydration is performed by rinses in solutions of sequentially decreasing ethanol concentrations. The following antigen retrieval aims to break the methylene crosslinks of proteins by incubation of the slide in buffer at high temperatures. Recommended buffers vary in ion strength, pH (Guhl et al., 1998; Gustafsson et al., 2010) and might be improved by the addition of chelators (e.g. EDTA; Groseclose et al., 2008, Caprioli et al., 2008) or denaturating agents (Fröhlich et al., 2012). The addition of detergents, e.g. SDS, was applied in LC-MS based analyses (Yamashita, 2005), but especially SDS is prone to cause ion suppression effects and might therefore be critical in MALDI MS imaging approaches (Guo et al., 2007). Reviewing the recent studies, Tris (pH 9) and citrate (pH 6) buffers are the most frequently used buffers. Distinct protein patterns were observed in 1D gel electrophoresis using different buffers for protein extraction from FFPE mouse hearts (Azimzadeh et al., 2010), demonstrating the influence of the buffer composition on protein extractability. Similar scenarios are likely in MS imaging as observed by Gustafsson et al. (2010) during their investigation of FFPE ovarian cancer samples. In contrast to various successful applications of Tris buffer for antigen retrieval in other studies, Tris was less effective for this tissue. The use of citrate bufferimproved MS imaging results and allowed the detection and localization of numerous peptides (Gustafsson et al., 2010).

The antigen retrieval procedure can further be varied in its temperatures, incubation time, the application of high pressure or the addition of organic solvents like acetonitrile (Fowler et al., 2012; Kakimoto et al., 2012). Heat treatment seems to be essential for protein recovery with higher extraction efficiency after incubation at 100°C than at 70°C (Ergin et al., 2010). However, the high temperatures might bear negative aspects such as protein modifications (Guo et al., 2007). The molecular mechanisms behind the antigen retrieval procedure retrieval remain unclear, but are most likely based on the heat induced hydrolysis of the formalin-derived methylene cross-links (Gown, 2004) (Fig. 1, box 2). The choice of buffer and treatment conditions might be tissue specific and might also depend on the fixation time of the sample (Kiernan, 2000; Lemaire et al., 2007). A better understanding of the molecular mode of action of the antigen retrieval will ease the choice of the optimal protocol for the respective sample.

On-tissue digestion of proteins

Although the recovery of whole proteins from FFPE samples is feasible (Ergin et al., 2010; Fröhlich et al., 2012), the incomplete reversion of cross-links as well as other physical factors hinder their satisfactory detection by MALDI MS imaging. As a consequence, most researchers perform on-tissue protein digestion, maintaining tissue integrity and spatial information. Trypsin is the most frequently applied digestion enzyme and was used in all reviewed MALDI MS imaging studies listed here. It is well established in LC and gelbased proteomics and well characterized. However, other digestion enzymes, for example chymotrypsin, elastase, pepsin or PNGase, or

also enzyme combinations can be used (Cillero-Pastor & Heeren, 2014). Standardized and sufficient digestion is desirable, since it ensures reproducibility and eases peptide identification. The use of additives such as octyl-a/b-glucoside improved on-tissue digestion of FFPE samples (Djidja et al., 2009 a).

MALDI MS imaging aims at the localization of compounds; therefore, all diffusion has to be avoided during the application of the enzyme solution and digestion. The two most common ways to apply the digestion enzyme are spraying or spotting (Fig. 1, box 3). Both methods use repeating cycles of application, interrupted by incubation and drying. In the spotting technique, picoliter drops of trypsin solution are placed in a raster on the sample. The spatial resolution is limited by the distance and the diameter of the spots with raster sizes (center to center) of 200 µm (Stauber et al., 2008), 250 µm (Groseclose et al., 2008; Morita et al., 2010) or 300 µm (Lemaire et al., 2007) and spot diameters of about 200 µm (Wisztorski et al., 2010), for example. If higher spatial resolutions are demanded, trypsin spraying is advantageous. Spraying provides a uniform application with much smaller droplet sizes (e.g. 25 µm; Wisztorski et al., 2010). Protein digestion is carried out either during application between the spotting and spraying cycles (Groseclose et al., 2008; Casadonte & Caprioli, 2011) or after application of the enzyme by incubation of the slide in a humid atmosphere. Using the latter option, two or three hours at room temperature or at 37°C (as recommended for trypsin by providers) are sufficient (Casadonte & Caprioli, 2011).

Matrix application: Extraction of peptides from the tissue

The matrix aids the ionization of molecules, thereby enabling their detection by mass spectrometry. A common and well established matrix for peptides is □-cyano-4-hydroxycinnamic acid (CHCA) dissolved in a mixture of organic solvent and water with low percentages of trifluoroacetic acid (Cohen & Chait, 1996). This matrix was recommended in all protocols listed in table 2. The addition of aniline (ANI) was reported to be beneficial for spectra quality (Wisztorski et al., 2010). Also 2,5-dihydroxybenzoate (DHB) was used for peptide analysis (Groseclose et al., 2007). Interestingly, a mix of 2,4-dinitrophenylhydrazine (2,4-DNPH) and CHCA as matrix enabled the detection of undigested, intact proteins in freshly prepared FFPE samples (six months formalin-fixed, paraffin-embedded) without previous antigen retrieval. The reactive matrix seemed to break methylene cross-links and reduced other formalin-induced protein modifications like Schiff bases (Fig. 1, box 2). These effects of 2,4-DNPH were only observed in short-term stored FFPE samples (Lemaire et al., 2007; Wisztorski et al., 2010).

Like for trypsin application, the matrix might be spotted or sprayed on the sample with the same advantages of spraying over spotting regarding the spatial resolution. All overwetting of the sample has to be avoided to prevent peptide dislocations (Wisztorski et al., 2010). The matrix is applied in repeating cycles of spraying or spotting, incubation (extraction of peptides from the

tissue in the matrix solvent), and drying (co-crystallization of peptides with the matrix) (Fig. 1, box 4). In this manner the peptides are extracted sequentially in the accumulating matrix layer. Uniform matrix coating and crystallization on the sample surface should be investigated, because - in our experience - these macro- and microscopic inspections provide a first idea about preparation quality and reproducibility.

Data acquisition: MALDI MS imaging for spatially resolved detection of peptides

Matrix-assisted laser desorption ionization (MALDI) is the ionization of analytes from matrix-coated samples after excitation by a laser. The ions are detected by a mass analyzer, which is monitoring m/z values and their abundances as mass spectra. MALDI MS imaging is the sequential acquisition of mass spectra from a sample section in a tight raster (Fig. 1; box 5). A high spatial resolution is demanded to assign compounds to specific tissue regions. Many improvements were achieved in the laser focus and the raster size allowing MS imaging down to 3 µm resolution (Römpp & Spengler, 2013). On the other hand, this high spatial resolution means a smaller ionization area, and consequently less analytes and lower signal intensities. For tryptic digested FFPE samples, raster widths of 30 µm were published (Mahmoud et al., 2013); 50 - 150 µm are state-of-the-art and used in most MSI studies as a compromise between capabilities of devices, data amounts, and research purposes. To provide an idea for the spatial resolution, figure 2 presents a tissue microarray analyzed with 70 µm raster width.

Different mass spectrometers and ionization methods can be used for MS imaging, which was extensively reviewed by Pól et al. (2010) and Trim et al. (2012). Here we focus on matrix-assisted laser desorption ionization (MALDI MS), which is the most frequently applied technique for the analysis of FFPE tissues (Table 2). The ionization methods as well as the ion detection modes (e.g. ToF, FT-ICR, Orbitrap) define the range and the number of detected peptides. With respect to the identification of peptides, high mass resolution is demanded, as for example provided by FT-ICR or Orbitrap mass spectrometry with mass accuracies of 1 ppm or even better (Römpp & Spengler, 2013; Seeley et al., 2011).

The instrument settings should be optimized for favorable mass spectra. The optimization of spectra signals on the original sample area might impact the results, since matrix and analytes were removed by the laser shots. A defined standard tissue or parts of a consecutive section mounted next to the sample can serve for instrument adjustment to save the original sample until final measurements.

Analysis of MS Data: From Data to Results

The correlation of mass spectra to histological features (e.g. cancerous regions) is the main advantage of MALDI MS imaging. Hematoxylin and Eosin (H&E) staining can be performed prior

to MS imaging on the same (Fröhlich et al., 2012) or on a consecutive section if detailed investigation is needed to select MS measurement regions. Since prior H&E staining might cause interfering signals and ion suppressions in the range below m/z 500 (Fröhlich et al., 2012), the subsequent H&E staining of the MS imaged slides and co-registration to the acquired MS data might be preferable. Besides Hematoxylin and Eosin staining, other histological or immunohistochemical stainings are possible.

The acquired mass spectra pass through a pipeline of processing steps, for example baseline removal, denoising, smoothing, and normalization, which were described in detail by Nimesh et al. (2013), Alexandrov (2012), and Jones et al. (2012). The MS data set allows the selection of a single signal and the visualization of the intensity of this compound at each position of the sample (Fig. 2 and 3, m/z visualizations). This visualization of m/z values provides an insight into peptide localizations and abundances.

Hundreds of peaks are present in the mass spectra of peptide imaging. This large amount of detected signals together with the high dimensionality of data (localizations, m/z values, and peak intensities) makes it impossible to manually detect and annotate significant differences or specific biomarkers. Statistical tools are needed for the objective evaluation and classification of MS profiles (Jones et al., 2012). These are - amongst others - principal component analyses (PCA), hierarchical cluster analyses (HCA) (Deininger et al., 2010), receiver operation characteristics (ROC), probabilistic latent semantic analysis (pLSA), independent component analysis (ICA), co-localization analyses (Jones et al., 2011) and also machine learning approaches like support vector machine (SVM) (Lagarrigue et al., 2012) and random forrest (RF) (Hanselmann et al., 2009). Since each approach addresses a specific question, the application of several methods might be advantageous to cover a broad range of information. Jones et al. tested five multivariate analyses for the investigation and visualization of tumor heterogeneity (on frozen samples). The subsequent generation of agreement plots served to merge the segmentations that were obtained by each test. This cross validation of several statistical investigations certainly provides higher accuracy and reliability (Jones et al., 2011).

Some analyses do not need the connection of MS information to localizations, so limitations in the imaging software can be compensated by outsourcing calculation capacities. For example, in the MS imaging analysis of tissue microarrays, spectra of tissue regions can be exported, sorted in sample groups, and processed with common statistical tools (Fig. 2). Depending on the purpose of the study, different MS groups can be assigned. Figure 2 presents the analysis of colon tissues with the comparison of carcinoma and mucosa from 15 patients. Spectra processing and statistics are performed without exact MS position, but with group information (e.g. patient and region, Fig. 2). Comparisons of peptide peak intensities are performed by hypothesis tests, for example Kruskal–Wallis test, Fisher's exact test or Student's *t* test. Downstream data mining leads to the annotation of tissue-specific peptides, enabling classification and machine learning approaches, which can be tested on novel, independent TMAs or tissue sections. The

visualization of the revealed peptides on the original MS imaging data restores the positional information. Finally, disease specific features, e.g. carcinoma specific classifiers, can be correlated to patient data for the overall aim to detect novel biomarkers or descriptive signatures.

Distinctions of the tissues might not be provided by single peptides, but might be present in the combination of several peptides (tissue profiles). These distinctions can be obtained by multivariate approaches (HCA and PCA [Deininger et al. 2010]; pLSA), which can be applied to the original MSI data set, maintaining the spatial information. The evaluation of multivariate approaches can be complicated and non-intuitive (Alexandrov, 2012) and needs experience of the scientist to read out the relevant peptides of the discriminating profile. Additional complementary analyses like the export of spectra and statistics can assist the definition of indicative peptides for the revealed clusters. Figure 3 presents a hierarchical clustering of peptide patterns obtained from a human colon tissue section that underwent the typical FFPE sample workflow (Fig. 1) in our laboratory. The peptide signatures (clusters) separated the submucosa (orange), the mucosa (red), and the muscularis propria (green). The high amount of hemoglobin (m/z 1274.8, one exemplary peptide shown) provided additional subclusters of the blood vessels. Some m/z values (classifiers) were selected as examples for specific peptide localizations (Fig. 3 D). Multivariate approaches like the example presented in figure 3 are advantageous in MSI, since they involve the complete mass spectral information, reduce data dimensions, and obtain differences that are not easy to detect using univariate tests.

Current MS imaging workflows depend on relative abundances of peptides. Since most approaches aim at the detection of molecular markers, the absolute quantification of peptides would be desirable. The FFPE workflow includes many critical steps that will influence peptide intensities, e.g. tissue specificities, efficiency of antigen retrieval and trypsin digestion, followed by the conventional challenges of MSI, like heterogeneous peptide extraction during matrix application, device performance, and post-processing and normalization of data. Seeley et al. reported a high degree of reproducibility in MS imaging of peptides from FFPE-TMAs, demonstrating a solid basis for quantification attempts (Seeley et al., 2011). To our knowledge, no systematic study was performed to investigate the feasibility of absolute peptide quantification in FFPE tissues by MALDI MS imaging.

Identification of peptides: From unknown m/z values to proteins

After the detection of peptides and revealing potential hints to classifiers, the identification of these so far anonymous m/z values is desired. Peptides can be identified by direct on-tissue tandem mass spectrometry (MS/MS) or by the analyses of tissue extracts e.g. by direct spotting on MALDI targets or using liquid chromatography coupled to mass spectrometry (LC-MS/MS). The fragmentation patterns (MS/MS) of the peptides are subjected to database search. On-tissue MS/MS can be performed manually (Lazova et al., 2012) or in an automated way (Seeley et al.,

2011), which provides the identification of mainly high abundant proteins. Complementary LC-MS/MS revealed many more data base hits due to higher sensitivity and higher purity of MS/MS precursors. The identified peptides from LC-MS analyses can be assigned to the m/z values of MSI data as performed in several studies (Table 2, column 3).

The covalent modification of proteins is of high impact for the identification of peptides from FFPE samples. Methylol adduct formation, Schiff base formation, and remaining methylene bonds (schematically indicated in figure 1) cause mass shifts of peptides (Magdeldin & Yamamoto, 2012). Therefore, the original peptide can be represented by several peaks: the unmodified $[M+H]^+$ and the adduct peaks of +12 Da (Schiff base) or +30 Da (methylol group) distance. The covalently bound groups might also prevent the access of trypsin to its cleavage sites, leading to insufficient enzymatic digestion. The possible modifications, as well as trypsin miscleavages, should be included in the database search, but this higher degree of freedom in database searches certainly provokes more false positive identifications. The number of false positive hits can be estimated by the search of MS data against a database generated from random sequences (Cillero-Pastor & Heeren, 2014). The review of Magdelin and Niigata (2012) provides excellent hints for protein extraction and database search of peptides recovered from FFPE samples.

Most of the so far identified proteins are highly abundant or ubiquitous proteins like actin, tubilin, albumin, collagen, hemoglobin, histones (Djidja et al., 2010) or vimentin (Lazova et al., 2012). There is a high demand to enhance sensitivity. Progress is apparent with the detection of e.g. cyclophilin A, antimicrobial peptides, and neuropeptides (Chansela et al., 2012) or HSPB 27, ribonucleoproteins, filamin, and SH3L1 (Casadonte et al., 2014). Sensitivity might be increased by the simultaneous application of several digestion enzymes, enabling higher sequence coverage and the localization of post-translational modifications (Cillero-Pastor & Heeren, 2014). The analysis of posttranslational modifications (PTMs), for example phosphorylations or glycosylations, might be of high importance for pathological considerations. PTMs influence protein folding, interactions of proteins with other cell compounds, protein localizations, and the activity of enzymes (Vidal, 2011). Formalin preserves these modifications at least partially (Magdeldin & Yamamoto, 2012), as for example demonstrated by Ostasiewicz et al. in LC-MS analyses (Ostasiewicz et al., 2010). Mapping of differential post-translational modifications of a protein within distinct tissues might be possible using MALDI MS imaging. This would provide additional benefits for pathology regarding the role of PTMs in many diseases (Vidal, 2011).

The application of MALDI MS imaging in clinical research

The number of available MALDI MS imaging studies of FFPE samples is limited, which is certainly due to the recent establishment of suitable protocols. Interest in the investigation of clinical samples is large and archived FFPE tissues promise important new knowledge for the

understanding of various diseases. It can be expected that the amount of literature will increase in the next years. Next, we would like to present some results and findings that were obtained in the last six years of MALDI MS imaging research on FFPE samples.

The analysis of tissue microarrays provides the possibility for high throughput screenings. More than a hundred needle core biopsies can be arranged in one paraffin block, integrating diverse sampling groups in one measurement and providing large case numbers for statistics (Djidja et al., 2010). The overview in table 2 demonstrates that all TMA investigations addressed the distinction and classification of cancerous tissues.

Djidja et al. classified FFPE pancreatic tumors by peptide profiles using principal component analysis – discriminant analysis (PCA-DA) (Djidja et al., 2010). The authors were able to discriminate tumors of different stages and obtained additional tumor classifications that were not assigned by pathological classifications. These molecular classifications demonstrated the capability of MALDI MS imaging for the detection of molecular signatures that might be of relevance in clinical diagnostics. Several characteristic peptides were assigned to each tumor class. The subsequent investigation of pancreatic tumor sections revealed the localization of these classifier peptides within tumor regions, but also demonstrated molecular heterogeneity within the tumor regions. This tumor heterogeneity is one of the most challenging items in cancer research and MALDI MS imaging is a well suited method for *de novo* discovery of molecular intra-tumoral heterogeneity (Schöne et al., 2013).

The analysis of the colon cancer TMA presented in figure 2 can be generalized to a common strategy that is a) acquisition of MS data, b) annotation of MS groups, c) statistical evaluation of data, d) extraction of classification features by data mining, and e) test of classifier candidates on novel data sets. This pipeline was applied by several groups (Table 2). Casadonte et al. extended the analyses to track metastases (Casadonte et al., 2014). First, they defined the distinct peptide patterns of breast and pancreatic cancerous tissues (TMAs training set). With the validated classifiers they could assign liver metastases to their origin of either breast or pancreatic cancer. This study raises promising future perspectives for the application of MALDI MS imaging to retrack metastases to their original cancerous tissue using peptide signatures as a kind of barcode.

Tissue sections provide larger sampling areas than the TMA needle cores. Consequently, in most cases, more distinct tissue types and substructures are represented and can be analyzed for molecular differences. Lazova et al. investigated dermal tumors and the tumor microenvironments to differentiate between benign Spitz nevi (SN) and Spitzoid malignant melanomas (SMM). Using generic algorithm (GA) classifiers on a training set, they could distinguish SN from SMM tumors by their peptide profiles with 29 of 30 (97%) for SN and 26 of 29 (90%) for SMM correct classifications. The tumor environment was of higher heterogeneity, but still 28 of 31 (90%) and 16 of 24 SN (64%) SMM and SN were classified correctly, respectively (Lazova et al., 2012). This study demonstrated the value of MS imaging for molecular classifications to assist diagnoses.

MS imaging studies mostly focused on the investigation of cancerous tissue biopsies, covering pancreatic cancer, breast cancer, ovarian cancer, colon cancer, and skin melanomas for the localization of peptides in specific cancerous regions. Studies on rat brain served to define Parkinson disease related biomarkers (Table 2). Many of these studies addressed the optimization of FFPE protocols, however, improvements in the recovery of peptides and also in downstream data analysis are still needed. For example, no spatial segmentation or a similar on-tissue cluster analysis was published for FFPE, although these analyses are clearly capable of distinguishing tissue-specific spectral compositions, as shown in figure 3. There is much progress in MS imaging of FFPE samples. The improvement of data quality, together with the application of appropriate statistics and data mining will certainly provide impressive and exciting novel discoveries within the next years.

Conclusion

The investigation of archived FFPE tissues by MALDI MS imaging provides the unique chance to correlate molecular patterns to patient data. Special focus was paid to cancer research in the last years. MALDI MS imaging offers the capacity for novel molecular tumor classifications with different peptides as classifiers that might bear biomarker potential. In this review we described the challenges of formalin fixation. The improvement of FFPE samples' MS imaging protocols and MS instrumentation provided much progress. Expanding the range of detected peptides will certainly enable the description of further classifiers. The biggest challenge of FFPE tissue analyses is the reversal of protein cross-links, so rehydration and antigen retrieval exhibit a high potential for the optimization of results. After optimal protein recovery, the protein digestion will be much more efficient or might even be skipped to image undigested proteins (Fröhlich et al., 2012; Wisztorski et al., 2010). Enhancing the amount of recovered peptides will increase the number of features to be analyzed, demanding appropriate bioinformatic tools for their analysis. Statistics and data mining analyses are used for the comparison of spectra groups obtained from FFPE samples. More complex multivariate analyses such as localization-based hierarchical clustering (Fig. 3), PCA, and pLSA, which are currently available and frequently applied on frozen tissues (Jones et al., 2011), are certainly of the same benefit for FFPE, although they remain to be applied.

MS imaging is a highly dynamic field and these developments will also be beneficial for FFPE tissue analyses. Archived FFPE samples provide the possibility of retrospective analyses to correlate molecular patterns to patient history. This dataset has the potential for the detection of markers for disease and treatment responses, raising hope for optimized, personalized therapy.

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Tables

Table 1: Publications of detailed protocols and manuals for MALDI MS imaging of FFPE sample sections and selected reviews for further information about the molecular background of formaldehyde fixation. Protocols: The main characteristic steps of antigen retrieval, trypsin / matrix application and the MS instrumentation are presented as suggested in the different protocols. FFPE Reviews: The list of selected reviews about formalin fixation is complemented by a very short summary of contents and specificities.

	Antigen retrieval	Trypsin / Matrix application	MS device	Reference
Protocols for MALDI MS imaging of FFPE samples	10 mM Tris-HCl, pH 9	spotting	MALDI-ToF/ToF	(Casadonte & Caprioli, 2011)
	10 mM Citrate, pH 6	spraying	MALDI-ToF/ToF	(Gustafsson et al., 2013)
	none	spotting or spraying	MALDI-ToF/ToF	(Wisztorski et al., 2010,)
Reviews about form-aldehyde fixation	Principles of formalin fixation, list of antigen retrieval strategies for MSI, LC-MS and gel based proteomics, analyses of protein-protein and protein-DNA interactions			(Klockenbusch et al., 2012)
	Overview on the fixation principles of diverse fixatives incl. formaldehyde / formalin, basic information for FFPE fixation mechanisms			(Kiernan, 2000)
	Molecular background of protein fixation and resulting protein modifications, providing important hints for peptide identification			(Magdeldin & Yamamoto, 2012)
	Overview on the formalin-fixation and modifications of proteins, focus on liquid extraction procedures, quantification, and data evaluation; chapter on MALDI MSI of FFPE			(Nirmalan et al., 2008)
	Overview on protein extraction procedures for different analytical approaches. Focus on protein identification rates and problems of identification, chapter on MALDI MSI			(Ralton & Murray, 2011)

Table 2: Overview of MALDI MS imaging studies of FFPE tissues. Column 1 presents the analyzed specimen and tissue arrangement (tissue microarrays in the first four studies and tissue sections in all other approaches). Column 2 provides a short summary of the content of the study, column 3 summarizes the proteins and peptide identification approach and the number of identifications. *) Abbreviation provided at the end of the table.

Tissue type	Content of the study	Identified proteins and peptides	Reference
Breast and pancreatic cancer (TMA*), liver metastases (sections)	Description and distinction of breast and pancreatic tumor peptide profiles, classification of liver metastasis to their origin (breast or pancreas) using SVM*	LC-MS/MS* 6 peptides	Casadonte et al., 2014
Pancreatic adenocarcinoma (TMA* + sections)	Molecular classification of pancreatic tumors by peptide patterns using PCA-DA*, distinction of tumor states that were pathologically of the same class	on-tissue MALDI-MS/MS 63 peptides of 21 proteins	Djidja et al., 2010
Lung tumor biopsy (TMA*)	Classification / distinction of adenocarcinoma and squamous cell carcinoma by SVM* algorithms	on-tissue MALDI-MS/MS ~ 50 proteins	Groseclose et al., 2008
Human gastric carcinoma (TMA*)	Description of 53 and 14 discriminating, cancer-specific peptides, molecular distinction of histological differences of cancerous tissue; group discrimination by t-test, one-way ANOVA, Fisher's PLSD*, additional immunohistochemistry	on-tissue MALDI-MS/MS 2 peptides of 2 proteins	Morita et al., 2010
Pancreatic adenocarcinoma (sections)	Distribution of Grp78* (tumor biomarker) by MALDI MSI and immunofluorescence	on-tissue MALDI-MS/MS: 1 (Grp78) + 26 peptides of 16 proteins	Djidja et al., 2009 b
Human breast adenocarcinoma (sections)	Optimization of the protocol for FFPE treatment samples and detection of peptides	on-tissue MS/MS 23 peptides of 13 proteins	Djidja et al., 2009 a
Rat kidney, frozen and FFPE (sections)	Investigation of the kidney microstructure by MALDI-MSI of lipids in fresh tissue and undigested proteins in FFPE tissue	9 peptides	Fröhlich et al., 2012
Human ovarian cancer (sections)	Workflow for MSI of FFPE with citrate buffer for antigen retrieval, comprehensive peptide identification	on-tissue MALDI-MS/MS 2 proteins; HPLC-MS/MS*: 106 (67 in MSI)	Gustafsson et al., 2010
Human colon carcinoma (sections)	Optimization of antigen retrieval (SSP: swelling and steaming pretreatment): High temperature, high pressure, and addition of acetonitrile for AG retrieval	on-tissue MALDI-MS/MS 1 peptide / protein	Kakimoto et al., 2012
Human skin melanomas (sections)	Differentiation of Spitz nevi (SN) from Spitzoid malignant melanomas (SMM) and SN / SMM microenvironments using a generic algorithm classifier	on-tissue MALDI-MS/MS: 2 proteins	Lazova et al., 2012
Rat brain, frozen and FFPE (sections)	Comparison of MSI quality between frozen, short-term and long-term formalin fixation; 2,4-DNPH*-matrix for fresh fixed tissues without antigen retrieval, MS imaging of whole proteins in FFPE	on-tissue MALDI-MS/MS nanoLC-MS/MS*: > 100; 21 proteins detected in MSI	Lemaire et al., 2007
human placenta (sections)	Imaging of the EGFR* (cancer drug target), epiregulin, and amphiregulin in FFPE human placental tissue, comparison to immunohistochemistry	8 peptides of 3 proteins	Mahmoud et al., 2013
Ovarian cancer (TMA* + sections)	Generation of a peptide reference table by nanoLC-MS/MS* for the identification of peptides in MALDI MSI	nanoLC-MS/MS*: 840 proteins in total	Meding et al., 2013

Breast cancer (MSI) and tissue surrogates (LC-MS/MS) (sections)	Optimization of the antigen retrieval protocol on tissue surrogates, application on breast cancer tissue sections, PCA* for the distinction of tissue types	nUPLC-MS/MS*: 137 (frozen sample); 70, 22, and 59 in FFPE	Caprioli et al., 2008
Rat brain (sections)	MSI of long-term stored (> 9 years) FFPE rat brains for the detection of Parkinson disease biomarkers, additional immunohistochemistry, detailed workflow for identification of peptides	nanoLC-MS/MS*: > 100 proteins	Stauber et al., 2008
Central nervous system of shrimps (sections)	Localization of neuropeptides and proteins in the central nervous system, feasibility study for application in neurogenerative diseases	nanoLC-MS/MS*: 28 peptides of 5 proteins, 29 peptides of 7 neuropeptide classes	Chansela et al., 2012

Abbreviations

2,4-DNPH	2,4-dinitrophenylhydrazine
EGFR	Epidermal growth factor receptor
(Fisher's) PLSD	(Fisher's) protected least significant difference
Grp78	Glucose related protein
HPLC-MS/MS	High performance liquid chromatography – tandem mass spectrometry
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
nUPLC-MS/MS	nano ultra performance liquid chromatography – tandem mass spectrometry
PCA(-DA)	Principal component analysis (– discriminant analysis)
SVM	Support vector machine
TMA	Tissue microarray

Figure legends

Figure 1: Schematic view of the workflow of MALDI MS imaging of peptides recovered from FFPE tissues as performed in our laboratory. Images of FFPE mouse brain samples serve to emphasize the influence of each treatment at the macroscopic level. Schemes display the glass slide and the sample as well as the molecular level. The protocol is variable, e.g. the Hematoxylin and Eosin stain (H&E) might be performed in advance of MS imaging (Fröhlich et al., 2012; Lemaire et al., 2007). ITO: Indium tin oxide coated glass slide, CH_2: methylene bridge, M: methylol adduct, Sc: Schiff base; x, y: distance of the laser raster spots; MS^2: MS/MS of peptides for identification (ID).

Figure 2: Schematic workflow of a MALDI MS imaging analysis of a tissue microarray (TMA). The TMA contains needle core biopsies of 15 colon cancer patients with tissues from cancerous (red) and healthy mucosa areas (green). The biopsies of patient 6 are presented enlarged with the laser shot positions (raster width 70 µm) in grey on the H&E stain. Distinct tissue regions can be annotated in the Imaging software (mucosa vs. carcinoma) and spectra exported as MS groups for each of the 30 samples. MS processing and statistics can be performed in outsourced programs, e.g. ClinProTools (Casadonte et al., 2014), Mascot Destiller™ and MarkerView™ (Djidja et al., 2010) or MATLAB™ as used in our laboratory. Hypothesis testing provides peptides for data mining (e.g. classifications and machine learning) that are tested and validated on a novel data set. Extracted peptide candidates can be visualized on the MSI dataset; one characteristic signal of each group (carcinoma and mucosa) is shown as an example. The visualization of the statistical results re-connects the external (non-localized) MS calculations to detailed localization information.

Figure 3: Analysis of a formalin-fixed, paraffin-embedded colon tissue section by MALDI MS imaging after deparaffination, antigen retrieval, trypsin digestion, and matrix application. MS data were acquired using a MALDI-ToF with a raster width of 150 µm, revealing 9230 mass spectra on the sample area. A) Hematoxylin & Eosin stain of the tissue after MSI. B) The hierarchical cluster analysis (HCA) of peptides visualized the different peptide profiles of the tissue (Cluster map) with clear distinctions of mucosa, muscle tissue, and submucosa. Blood vessels provided their own clusters (blue / green). Pink and yellow clusters were localized at tissue bounderies. C) The cluster tree provides the distances between the MS clusters in relative units with the number of mass spectra of the respective cluster in brackets. D) Localization of selected peptides (m/z represents the $[M+H]^+$ ion) on the tissue, visualized by intensity heat maps. Data kindly provided by Dr. Stephan Meding, University of Adelaide, Australia.

Figures

Figure 1

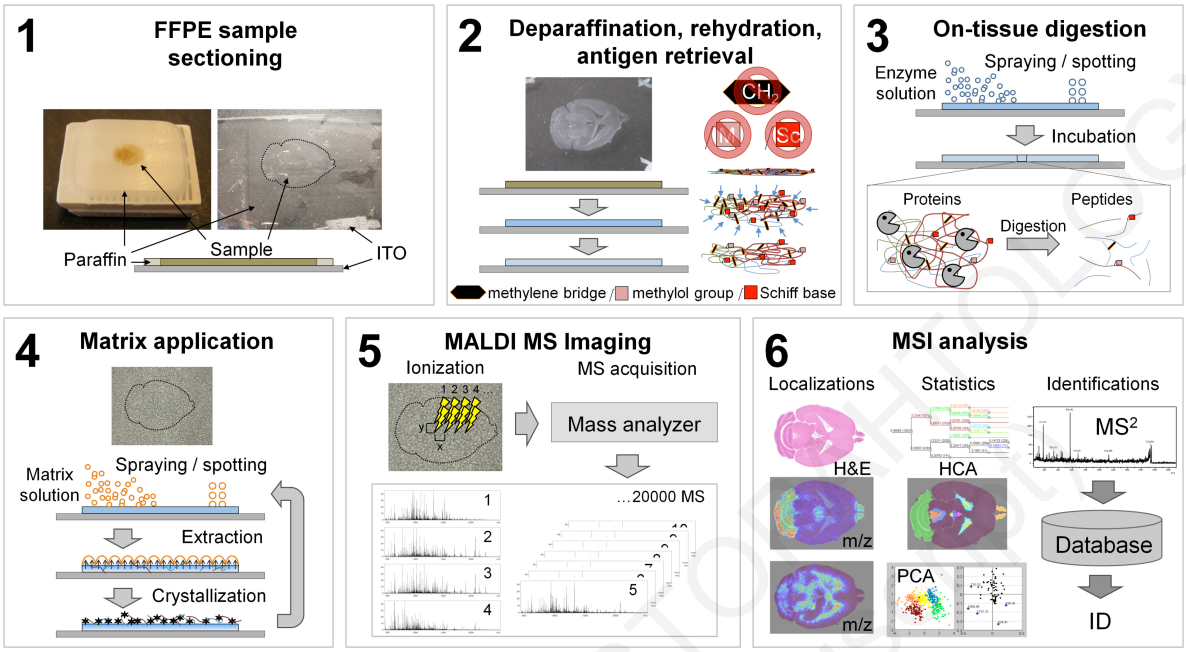

Figure 2

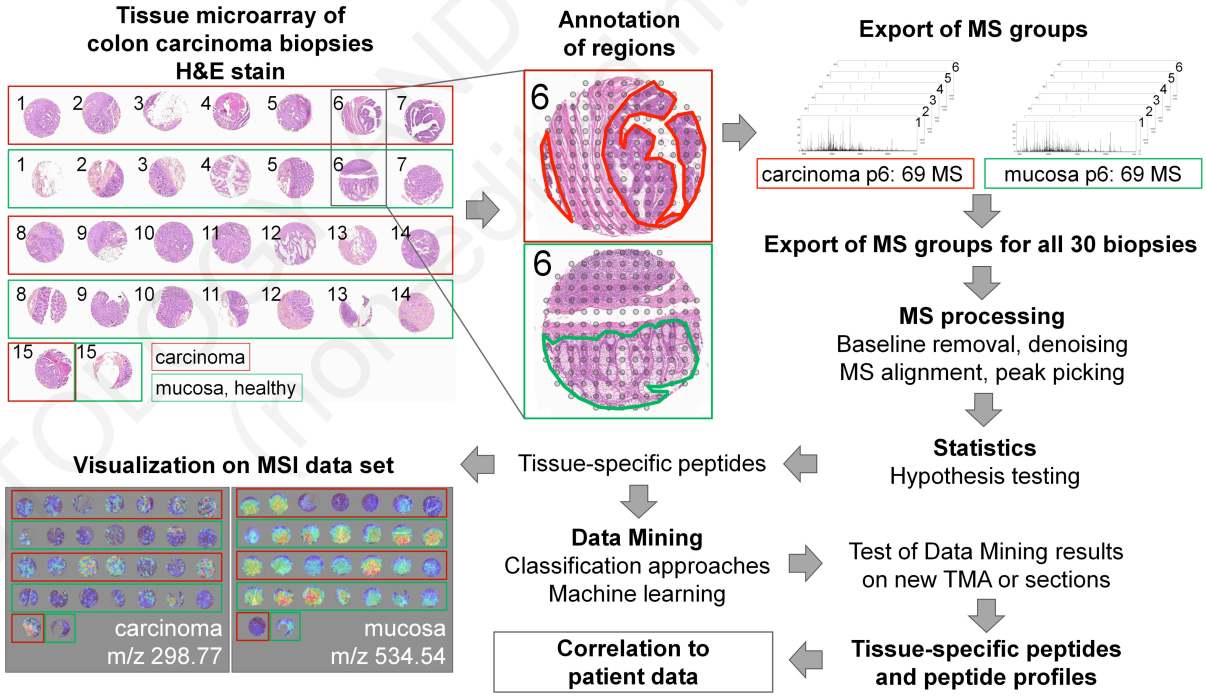

Figure 3