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REVIEW

MALDI imaging mass spectrometry for direct tissue analysis: technological advancements and recent applications

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Abstract Matrix assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) is a method that allows the investigation of the molecular content of tissues within its morphological context. Since it is able to measure the distribution of hundreds of analytes at once, while being label free, this method has great potential which has been increasingly recognized in the field of tissue-based research. In the last few years, MALDI-IMS has been successfully used for the molecular assessment of tissue samples mainly in biomedical research and also in other scientific fields. The present article will give an update on the application of MALDI-IMS in clinical and preclinical research. It will also give an overview of the multitude of technical advancements of this method in recent years. This includes developments in instrumentation, sample preparation, computational data analysis and protein identification. It will also highlight a number of emerging fields for application of MALDI-IMS like drug imaging where MALDI-IMS is used for studying the spatial distribution of drugs in tissues.

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Introduction

Imaging mass spectrometry (IMS) is a method with enormous potential because it allows performing molecular analysis of tissue while retaining the information about the spatial distribution of different analytes, e.g., proteins, peptides, lipids or small molecules (drugs). During an IMS experiment, a mass spectrometric analysis is performed on a sample, but instead of taking a measurement from a single sample spot, a multitude of measurements are taken from a sample slice (usually a tissue slice) in a predefined raster. A mass spectrum for each individual measuring spot is acquired, a so-called pixel, resulting in a two-dimensional distribution map for each measured mass. The mass spectrometric approach allows analyzing the distribution of hundreds of analytes at once, without the need of labels or prior knowledge of the analytes.

The first IMS methods were developed over 40 years ago (Liebl 1967). One important aspect where these methods vary is the ionization method used to generate the ions that form the basis of the mass spectrometric measurement. Currently, there are three established methods of ionization used in IMS: secondary ion mass spectrometry (SIMS) (Liebl 1967), matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp 1988) and desorption electrospray ionization (DESI) (Takats et al. 2004; Wiseman et al. 2006). In addition to these three methods, multiple new ionization methods have been developed in recent years (Lewis et al. 2003; Nemes and Vertes 2010; Northen et al. 2007; Sampson et al. 2006), but currently none of these new methods have been used much,

owing also to the lack of commercially available instruments using these ionization methods. Each of these methods has its individual advantages and drawbacks, such as different lateral resolutions, mass ranges or the capability to analyze special samples.

In this review, we focus on MALDI-IMS in particular, instead of giving a broad overview, since MALDI is currently the most widespread ionization method for IMS on biological samples and multiple reviews already deal with the general topic of IMS (Amstalden van Hove et al. 2010b; Pol et al. 2010; Watrous et al. 2011).

As the name implies, samples are covered with a crystalline matrix (usually a light-absorbing organic acid with a low molecular weight) before mass spectrometry is performed on them. This matrix absorbs most of the energy of the irradiating laser used for ionization, leading to a so-called "soft ionization" of the analyte molecules. The exact mechanism of this soft ionization is not fully understood yet and still intensely studied, but it allows for the creation of larger ions such as peptides and proteins when compared with SIMS or DESI (Chang et al. 2007; Karas and Kruger 2003; Knochenmuss 2003, 2006, 2009). Importantly, the type of molecules observed in a mass spectrometric experiment not only depends on the ionization method employed, but also on the matrix type and the sample preparation. Special washing protocols of the sample can enhance or suppress the detection of specific molecules.

In direct tissue analysis by MALDI-IMS, matrix is applied over the entire tissue section either manually, by robotic spotting, nebulization or sublimation, and laser shots are subsequently performed across the tissue sample. In this context, the spatial resolution is limited by the size of the matrix crystals and the laser focus, and currently lies in the micrometer range (10–100 μm). The resulting ions are usually separated by a time-of-flight (TOF) mass analyzer according to their mass-to-charge values (*m/z*), which has shown to be most sensitive for the measurement of biomolecules with masses going up to 30 kDa (Seeley and Caprioli 2011). During the measurement, the sample is not damaged, facilitating the direct correlation of the mass spectrometric data with the morphological features of the tissue (Fig. 1).

The availability of the sample tissue after the measurement has made MALDI-IMS in the last few years a successful tool for analyzing the molecular content of tissue sections, especially in the field of cancer proteomics (Fig. 2). Here, protein profiles obtained by MALDI-IMS have been used for the molecular classification of tissue samples regarding disease state, risk stratification and

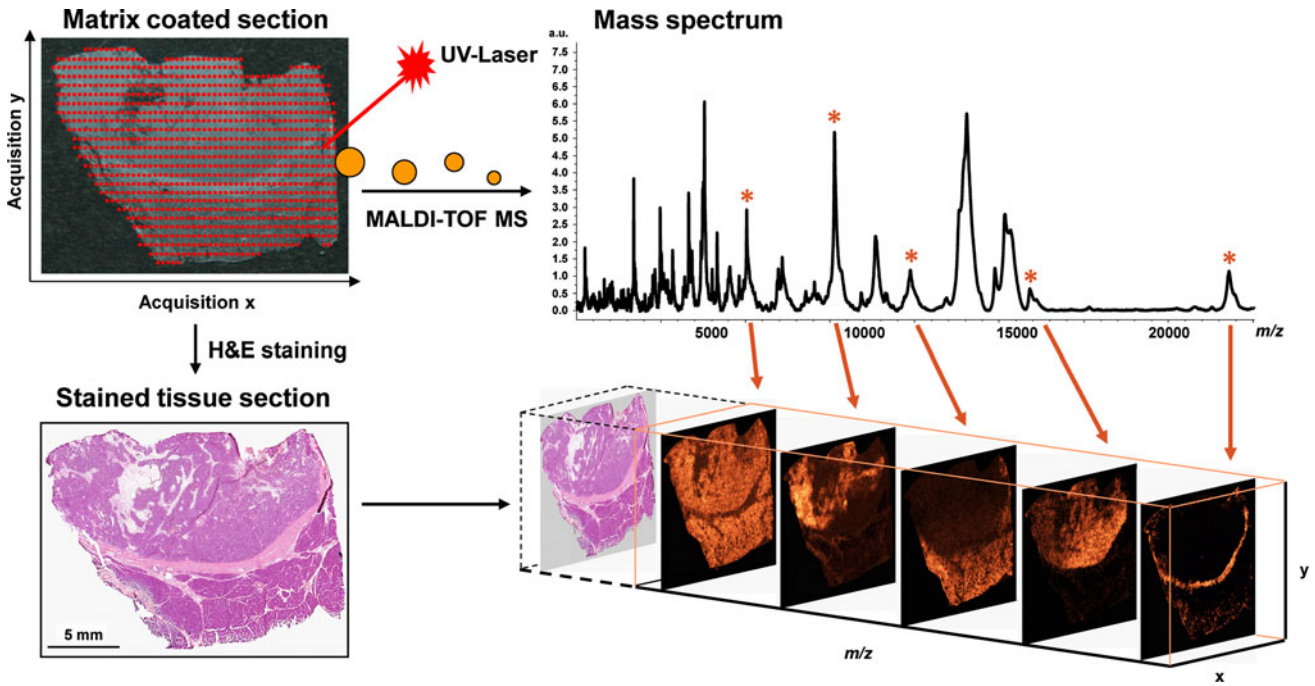

Fig. 1 Schematic depiction of the MALDI-IMS workflow. A tissue section is coated with matrix before multiple mass spectrometric measurements are taken in a predefined raster. After matrix elution, the section is stained conventionally by, for example, hematoxylin and eosin. Scanning and co-registering the sample to the spatially resolved mass spectrometric data allow visualizing the distribution of masses within their morphological context. *m/z* mass to charge, *H&E* hematoxylin and eosin, *MALDI* matrix-assisted laser desorption/ionization, *TOF* time of flight, *MS* mass spectrometry (Reprinted with kind permission from Springer Science+Business Media: Der Pathologe, In-situ-Proteomanalyse von Geweben mittels bildgebender Massenspektrometrie (MALDI Imaging), 30, 2009, pages 140–145, S. Rauser, H. Höfler and A. Walch, figure 1)

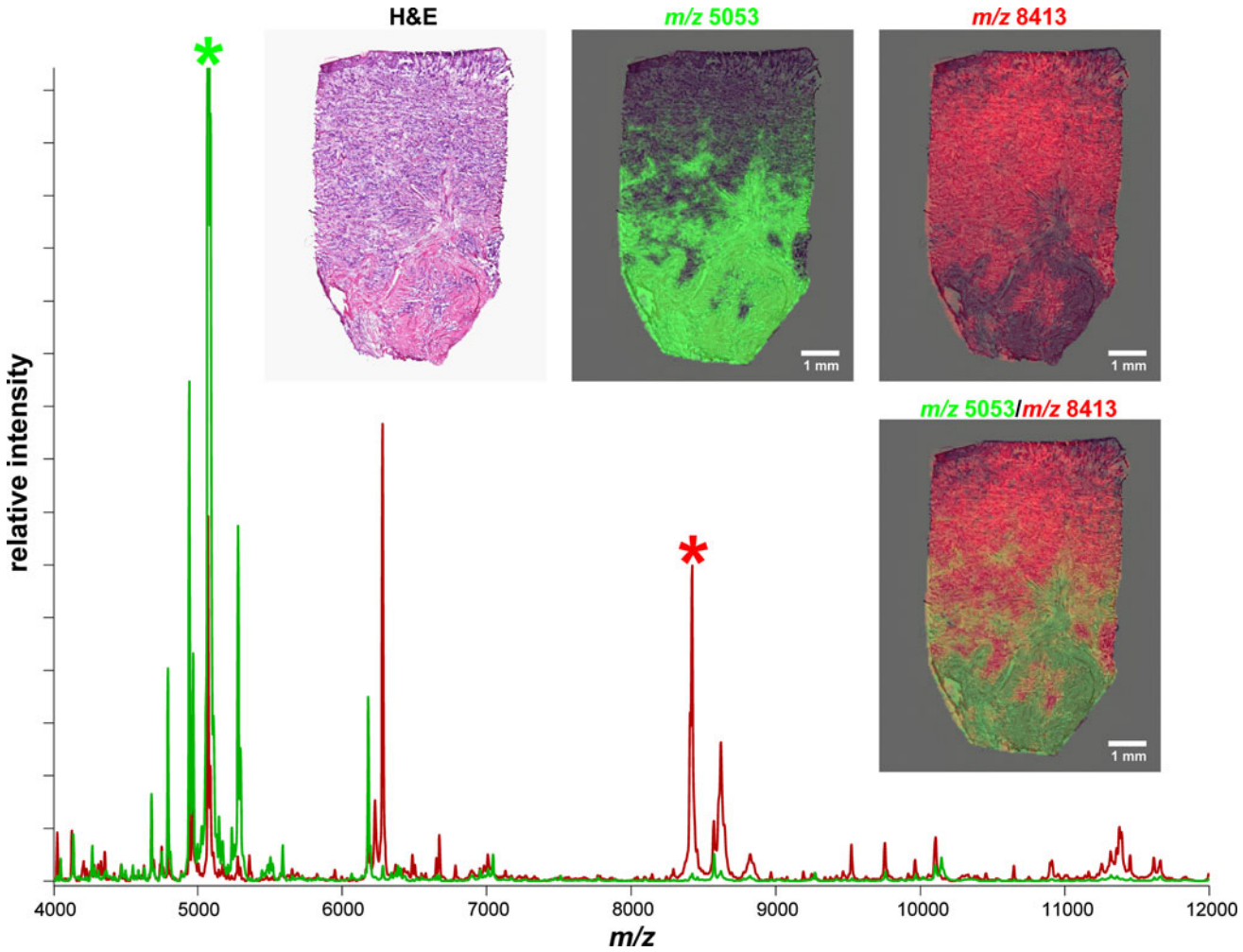

Fig. 2 MALDI-IMS of a breast cancer tissue section. The recorded mass spectra of the stroma region (*green*) and the tumor region (*red*) are compared within a breast cancer tissue section. Two specific protein signals for stroma and tumor at *m/z* 5,053 and *m/z* 8,413 (indicated by *asterisks* in the mass spectra) are visualized in *green* and *red*, respectively. *m/z* mass to charge, *H&E* hematoxylin and eosin

therapy response, as well as for the identification of disease biomarkers. Another medical field where MALDI-IMS is getting increasing attention is for studying the spatio-temporal behavior of drugs in animals and patients, as in pharmaceutical development, the assessment of the distribution of a drug candidate in targeted tissues is of central interest for the evaluation of its pharmacological properties (Signor et al. 2007). However, MALDI-IMS is not restricted to biomedical research only; it has proven to be useful in any other field where complex biological tissues have to be analyzed.

This increased interest in MALDI-IMS has led to notable technical advances that have been made over the last few years to improve instrumentation to increase the laser repetition rate, spatial resolution, sensitivity for higher mass ranges, mass resolving power and mass accuracy. As some limitations, such as mass range and lateral resolution, are crucially linked to sample preparation, developments in sample preparation have also been made especially regarding sample washing protocols, matrix choice and matrix application methods. Strong efforts have also been made to make formalin-fixed, paraffin-embedded (FFPE) tissue accessible for MALDI-IMS, as the ability to work with FFPE tissues will augment the number and quality of samples for clinical studies (Seeley and Caprioli 2011).

In addition, protocols for the identification of proteins are of special interest, as in a direct MALDI-IMS analysis only the molecular weights of the proteins are measured. This identification requires an additional step in MALDI-IMS studies. The possibilities to identify a protein of interest depend mainly on its chemical properties, but primarily on its mass and range from extract-based approaches to in situ identification.

In this review, we discuss the current state of the art of the MALDI-IMS technology and its scientific applications, especially within a clinical and preclinical context. Special emphasis will be given to the computational data analysis

part as it takes a key position in the analysis and interpretation of the results obtained by MALDI-IMS experiments. In general, we will place special focus on the advances that happened in the time since our last review that focused on this topic (Walch et al. 2008).

Current state of MALDI-IMS for the in situ analysis of tissue sections

Current technological state of the instrumentation

Instrumentation plays an important role in the achievable quality of results during MALDI-IMS. The technology used decides over the limitations on many important factors, such as lateral resolution, measuring time or mass range, and resolution. In the last couple of years, there have been advances in all technical aspects of MALDI-IMS, further improving the methods and its potential applications.

Lateral resolution

A current limitation of MALDI-IMS is the lateral resolution of the measurements, which currently go down to 50 μm in most measurements. The resolution is mainly limited by the matrix application step, due to the resulting matrix crystal size, which can range from about 100 nm to several micrometers, depending on the chosen method (Jaskolla et al. 2009). In addition, the laser focus has to be considered. While the size of the laser beam can be easily reduced, a reduction of the irradiated area directly results in a lower ion yield. The goal is to improve it to a level where the MALDI-IMS measurements are comparable to the information gained from classical histomorphological examination based on optical microscopy, without loss in signal quality. For this, it is crucial to reach at least a single cell resolution ($\sim$10 μm).

There have been two different approaches to improve the lateral resolution to a single cell level. The combination of MALDI-IMS with the "sample stretching" method proposed by Monroe et al. divides the sample tissue into cell-sized fragments, destroying the morphological information and is therefore rather interesting for single cell analyses (Monroe et al. 2006; Tucker et al. 2011; Zimmerman et al. 2008, 2010). The oversampling approach moves the laser over the sample in small increments and takes measurements with overlapping laser shots. It leaves the sample tissue intact, but has the problem that the signal quality is lower compared to MALDI-IMS without oversampling. (Jurchen et al. 2005; Li et al. 2007).

While these two methods allow effective improvement of the resolution, they both come with individual drawbacks as a trade-off. A promising new direction is the implementation of a new generation of lasers that allow for a smaller laser focus size, while achieving a higher ion yield compared to older lasers. Experiments performed by Lagarrigue et al. (2011) on rat testes with this new laser generation allowed to reach a lateral resolution of 20 μm while detecting ions in the 2-16 kDa range, so that it was possible to distinguish between different cell types within the testis by differentially expressed masses (Fig. 3). An even higher lateral resolution could be achieved by Chaurand et al. Using a home-built mass spectrometer, they were able to perform MALDI-IMS measurements of phospholipids at a cellular level, reaching resolutions between 5 and 25 μm (Chaurand et al. 2011).

Measuring speed

An increase of resolution directly leads to an exponential increase in measurement time, since more measurements need to be taken for a sample of the same size. To keep measuring times to an applicable level, it is necessary to develop new ways to increase the throughput of MALDI-IMS systems. To achieve this, there has been development to increase the frequency of the used lasers, which allowed lowering the measurement time of tissue samples by up to 90% (Trim et al. 2010). In addition, an automated setup was developed to save time in between experiments, which included controlled sample storage, a sample loading robot and a MALDI-TOF/TOF mass spectrometer, all controlled by a single user interface. Such automated setups facilitate the use of MALDI-IMS for studies working on large amounts of samples (McDonnell et al. 2010c).

Mass range

Another field where important advancement could be made was the mass range of MALDI-IMS. MALDI-IMS works best in the mass range of small proteins and protein fragments. If the weight of the analyte lies below 500 Da, the data clarity suffers (Watrous et al. 2011), while masses exceeding 25 kDa are rarely detected during MALDI-IMS. Since this limitation excludes a great amount of interesting proteins, whose weight exceeds 25 kDa, efforts have been made to improve the mass range of imaging measurements.

Grey et al. showed that membrane proteins with a mass exceeding 25 kDa could be measured after repeatedly washing the sample to eliminate abundant soluble proteins and using a matrix with high amounts of organic solvent (Grey et al. 2009). While this approach works for membrane proteins that are anchored in the membrane, it is not

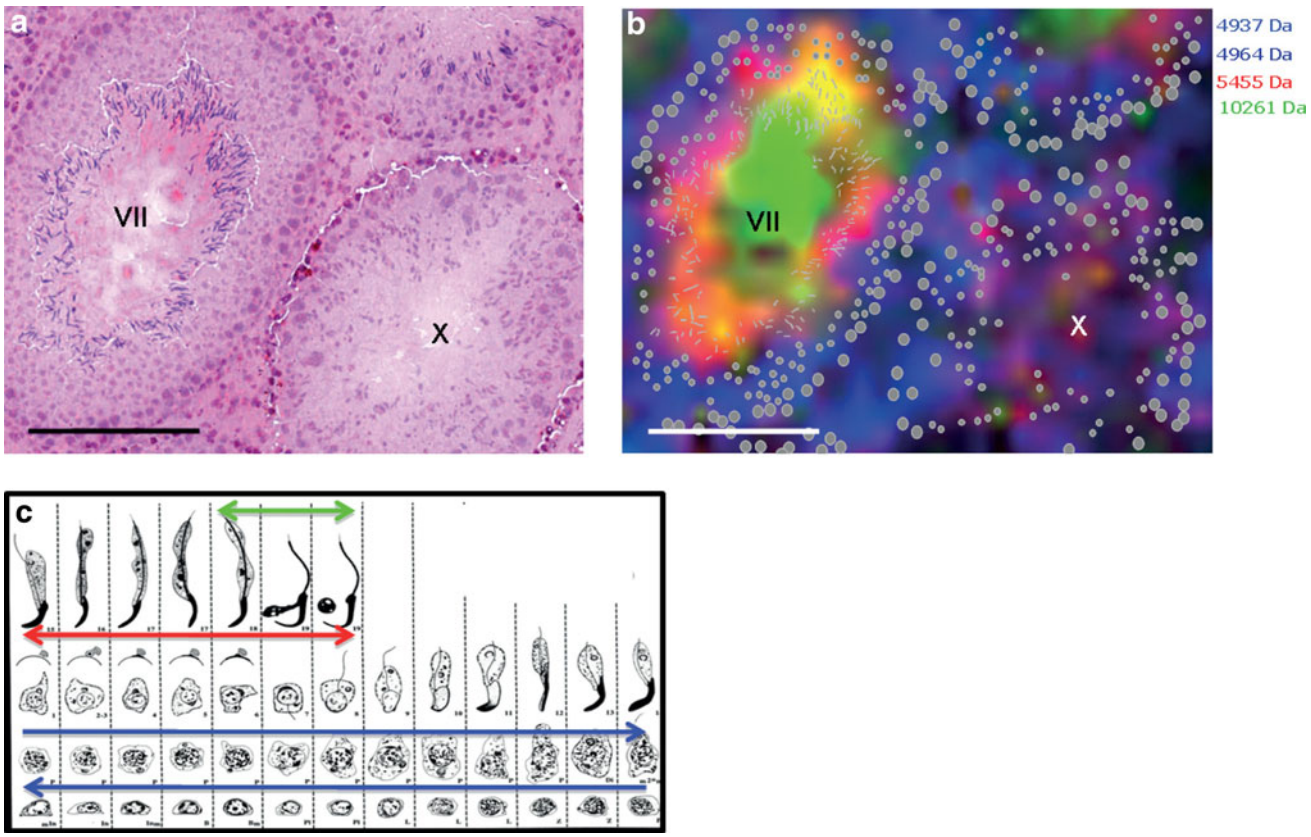

Fig. 3 Correlation between histology and molecular imaging at high resolution. Two seminiferous tubule sections are displayed that correspond to stage *VII* and stage *X* of the seminiferous epithelium classification. **a** Close-up of a transverse section of the testis of an adult rat stained with hematoxylin and eosin. *Scale bar* 200 µm. **b** Corresponding molecular image at a 20-µm lateral resolution. Four proteins in the stage *VII* section corresponding to *m/z* 4,937, 4,964, 5,455 and 10,261 could be assigned, respectively, to the basal compartment, (*m/z* 4,937 and 4,964) the adluminal compartment (*m/z* 5,455) and the lumen (*m/z* 10,261). The signal at *m/z* 10,261 can be matched with the presence of flagellae from step 18–19 spermatids. In the stage *X* section, which contains spermatids at early differentiation steps (step 10), no signal was observed for molecular species at *m/z* 5,455 and 10,261. In **c**, the *lower panel* shows summaries of the in situ localization of selected masses superimposed on the map of spermatogenesis. *Scale bar* 200 µm. Modified from original figure by Lagarrigue et al. (2011) (©2011 by American Society for Biochemistry and Molecular Biology)

suited to become a general approach, since most analytes would be simply eliminated from the sample during the on-tissue purification process.

An approach that could be generally used would be the improvement of the extraction of high mass proteins from the sample tissue by adding different organic solvents to the matrix (Franck et al. 2010; Leinweber et al. 2009). With this method, Franck et al. were able to detect proteins with a weight of up to 70 kDa directly from tissue (Franck et al. 2010).

Using a mass detector specifically designed for the detection of high mass ions, van Remoortere et al. (2010) were able to visualize masses of up to 70 kDa and detect even heavier ions directly from tissue. This approach has the advantage that it can be used with normal sample preparation without relying on additional solvents that may be difficult to handle (e.g., due to high chemical aggressiveness).

Mass resolution

The mass resolution is important to separate ions with a weight close to each other (e.g., different metabolites during small molecule imaging) and depends on the mass analyzer used for the MALDI-IMS measurement. While time-of-flight (TOF) mass analyzers have been the method of choice for MALDI-IMS, the development of new MALDI mass spectrometric imaging sources allows the use of high mass resolution and high mass accuracy mass analyzers, like Fourier transform ion cyclotron resonance (FTICR) and Fourier transform Orbitrap (FT-Orbitrap). (Amstalden van Hove et al. 2010b).

Current state of sample preparation

Another important aspect of MALDI-IMS is the sample preparation. On the one hand, one has to consider the

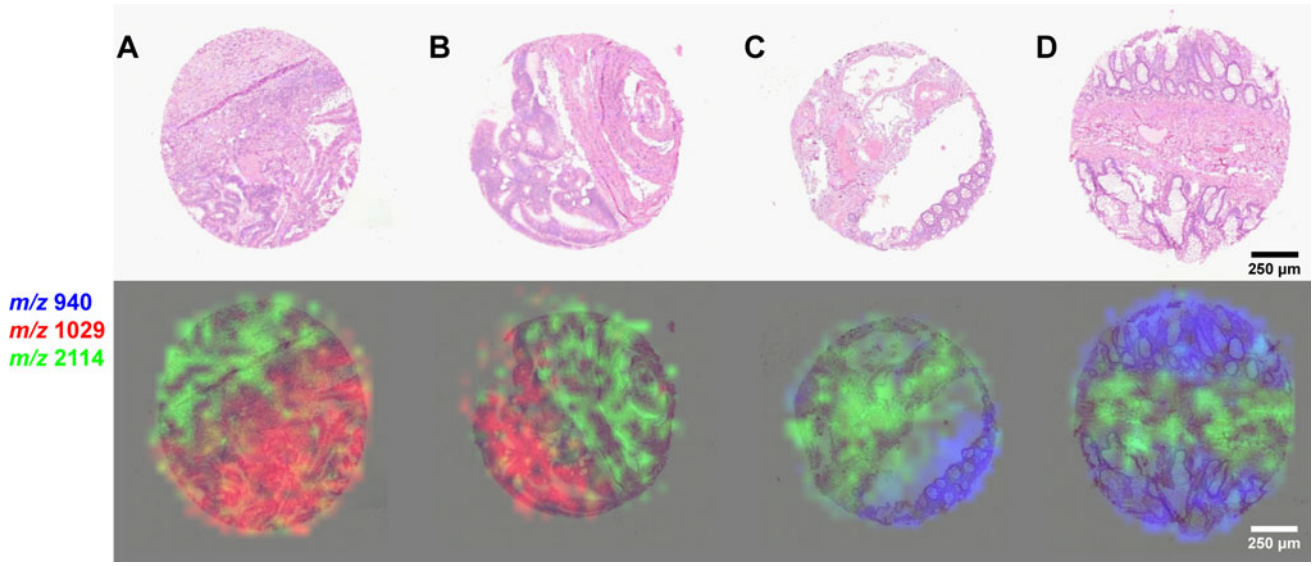

Fig. 4 Analysis of formalin-fixed, paraffin-embedded (FFPE) tissue by MALDI-IMS. An array of FFPE colon cancer (**a**, **b**) and healthy colon tissues (**c**, **d**) was measured by MALDI-IMS after on-tissue tryptic digestion. Visualized are the masses of three peptides specific for stroma and muscle tissue (*green*, *m/z* 2,114), normal epithelial cells (*blue*, *m/z* 940), and tumor cells (*red*, *m/z* 1029). *m/z* mass to charge

available sample material and how it is preserved. On the other hand, one needs to bear in mind what analytes should be measured and which matrix and form of matrix application are necessary to this end.

Measurement of formalin-fixed, paraffin-embedded tissue

Until recently, MALDI-IMS of tissues was limited to cryo-tissues, since the cross-linking between proteins in formalin-fixed, paraffin-embedded (FFPE) tissues made it impossible to ionize them properly. Recent experiments have shown that it is possible to circumvent this problem by performing an enzymatic digestion on the sample section to generate ionizable peptides (Fig. 4). To this end, the enzyme is applied using either spraying or spotting techniques to retain the localization of the analytes (Groseclose et al. 2008, 2010; Lemaire et al. 2007a; Ronci et al. 2008; Stauber et al. 2008). This has the great advantage of opening decade-old tissue archives up to the analysis by MALDI-IMS, but currently there is no way to analyze the distribution of whole proteins on FFPE tissues, since the enzymatic digestion still is a required step.

Measurement of alcohol-fixed tissues

A potential alternative to FFPE and cryo-tissue is the use of tissues preserved with an alcohol-based fixation. Such tissues are as easily handled as FFPE tissues, but the proteins within the samples are not cross-linked and thus measurable by MALDI-IMS, without the need for enzymatic digestion. The possibility of this could be shown for two different fixation methods: RCL2/CS100 (Belief et al. 2008; Mange et al. 2009) and PAXgene (Ergin et al. 2010). Since tissues preserved in this way are not only better suited for MALDI-IMS, but also for multiple other proteomic methods, it would be advantageous if they saw more widespread use in the future.

Matrix choice and matrix application

The choice of matrix and its deposition are crucial steps in the sample preparation for MALDI-IMS. The matrix is usually a small, light-absorbing organic acid that must not chemically react with the analytes. In addition, it should have a low sublimation rate since most MALDI-IMS measurements are performed in high vacuum or ultrahigh vacuum conditions. The choice of which matrix to use for an experiment depends on the analytes one wishes to measure, since each matrix is suited for the ionization of different molecular classes (e.g., lipids, peptides or small proteins). For an overview of which matrix is suited for which analyte, refer to Table 1 or the review by Amstalden van Hove et al. (2010b).

The matrix deposition needs to be homogenous and reproducible. Homogeneity of matrix deposition guarantees that local variations in desorption and ionization are kept to a minimum, while reproducibility is necessary for the comparability between different measurements (Amstalden van Hove et al. 2010b). In addition, it is important that the matrix deposition is neither too dry nor too wet. While a too dry matrix deposition would lead to an insufficient extraction of analyte molecules, a too wet

Table 1 An overview of analytes and their recommended matrices in MALDI-IMS

Analyte	Matrix
Peptides	2,5-Dihydroxybenzoic acid (DHB, gentisic acid)
	α-Cyano-4-hydroxycinnamic acid (HCCA)
	3-Hydroxypicolinic acid (3-HPA)
	2,4-Dinitrophenylhydrazine (2,4-DNPH)
Proteins	2,5-Dihydroxybenzoic acid (DHB, gentisic acid)
	α-Cyano-4-hydroxycinnamic acid (HCCA)
	3,5-Dimethoxy-4-hydroxycinnamic acid (SA, sinapinic acid)
Oligonucleotides	4,6-Trihydroxyacetophenone (THAP)
	3-Hydroxypicolinic acid (3-HPA)
Lipids	2,5-Dihydroxybenzoic acid (DHB, gentisic acid)
	2,6-Dihydroxyacetophenone (DHA)

deposition could lead to the diffusion of analyte molecule and thus loss of localization. Lastly, the size of the matrix crystals created by the deposition method decides over the maximal lateral resolution that can be attained. A more in-depth look at these aspects can be found elsewhere (Kaletas et al. 2009).

The matrix can be either applied manually or with an automated device. Manual methods are quick but have low reproducibility, so that they are best only used when establishing a new method. Applying matrix manually can be done in two different methods. One is by "dried-droplet", where the matrix is simply manually spotted onto the tissue with a pipette. This leads to a large spot with a high analyte extraction, but also strong diffusion within. Due to this, the "dried-droplet" method is not suited for imaging approaches, but useful for profiling experiments. The matrix can also be applied using a handheld thin layer chromatography sprayer or airbrush (Schwartz et al. 2003). While this generates a matrix that is usable for MALDI-IMS, its reproducibility is questionable.

Due to this, systematic multi-measurement studied should use automated devices to guarantee the comparability of different measurements. Automated methods of matrix application are based on the same two principles as the manual application: either the matrix is spotted or sprayed onto the sample. Chemical inkjet printers (Adams and Roy 1986; Nakanishi et al. 2005) or automated spotting devices (Aerni et al. 2006) apply small droplets of matrix solution in a precise, uniform and highly reproducible manner. In addition, it is possible to use these devices to precisely spot other solutions, such as enzyme solutions for tryptic digestion. On the other hand, this form of matrix application only allows for a lower resolution than spray coated samples, since the matrix is only on those distinct spots and not on the whole sample. Automated spray coaters work within a closed system and allow the control of the droplet size and the drying time between spraying bursts, so that the matrix crystallization can be optimized and easily reproduced.

In addition to these rather direct approaches, new methods of matrix deposition have been developed: matrix sublimation and dry coating. During matrix sublimation, the matrix is evaporated under low pressure and high temperature and condensed on the cold sample surface, leading to an enhanced purity of matrix and a uniform deposition with a small crystal size, but necessitating a long preparation time (Hankin et al. 2007; Yang and Caprioli 2011). For the dry-coating method, finely ground matrix is directly applied onto the sample, making this a fast and easily reproducible method (Goodwin et al. 2010a, b; Puolitaival et al. 2008; Trimpin et al. 2010). In addition, both methods also have the advantage of limiting the diffusion of analyte, due to both of them being solvent free.

Sample staining

One of the great advantages of MALDI-IMS is that the tissue sample is not destroyed during the measurement. Deutskens et al. took advantage of this and developed a protocol that allows staining of the tissue sample after it has been subjected to MALDI-IMS. For this, the matrix is dissolved after the measurement by washing the sample with an alcohol solution before staining it with hematoxylin and eosin (Deutskens et al. 2011; Schwamborn et al. 2007). This staining protocol allows for the perfect correlation of mass spectrometric imaging data with morphological features and is a great advancement compared to the methods used before, where either a follow-up tissue slice was stained and co-registered, or the sample was stained before the MALDI measurement (Chaurand et al. 2004). The co-registration of sequential sections has the drawback that while its morphology resembles the one of the measured sample, it is not identical and morphological information is lost. On the other hand, this method is still valuable to co-register alternative or multiple different stainings to a single MALDI measurement (e.g., immunohistochemical staining). The pre-measurement staining allows gaining information about the sample, which might be useful for the measurement, such as the localization of special structures of interest, but there is a risk that the staining may negatively impact on the MALDI process.

Data processing, statistical analysis and visualization

The computational and statistical analysis of MALDI-IMS data is a key step in the study process. While computational data processing methods have to consider the special nature of MALDI-IMS data, subsequent statistical tools, which

can be divided into supervised and unsupervised approaches, lead the investigator to conclusions about the data set.

These data sets may consist of several thousand of spatially resolved mass spectra, each of which contains hundreds or thousands of mass signals (McDonnell et al. 2010b). With fast advances in instrumentation with regard to spatial resolution at higher laser repetition rates, improvements in mass resolution and additional separating dimensions, the amount of data has increased similarly. Furthermore, to improve statistical reliability in preclinical research studies, a minimum number of 50–60 samples have been proposed (Seeley and Caprioli 2011) and the number of patients involved has been raised now to around 100 (Balluff et al. 2010; Cazares et al. 2009; Djidja et al. 2010; Hardesty et al. 2011). The analysis of these large data sets needs efficient and sophisticated computational methods, ranging from basic spectra processing to advanced data mining algorithms, which should both take the special nature of MALDI-IMS data into account.

Watrous et al. showed that a simple graphical test can be used to check for the quality of an imaging data set before further processing, which usually includes baseline subtraction, normalization, peak picking and realignment with the aim to reduce experimental variance within or between single data sets (Norris et al. 2007; Watrous et al. 2011). The pre-processing step is crucial as results have shown to be sensitive to data preparation parameters such as recalibration and peak picking criteria (Agar et al. 2010). Another critical step which has been widely discussed is the normalization of spectra (Norris et al. 2007; Watrous et al. 2011). Deininger et al. proposed to normalize on the median or the noise level rather than on the commonly used total ion count, which was found to be significantly more susceptible to artifact generation (Deininger et al. 2011). Efforts have also been made to reduce the large file sizes by a factor of over 300 through extraction of the relevant features for MALDI-IMS data sets (McDonnell et al. 2010b).

For clinical studies, the subsequent analytical goals can be twofold: (1) the identification of molecular patterns for diagnosis, prognosis or response prediction by classification of patients into different classes, (2) the identification of biomarkers characteristic to the assessed classes. The first step in this analysis pipeline is usually to extract the mass spectrometric data from histologically interesting regions with designated states (e.g., healthy, tumor or responder). After extraction of the corresponding spectra, the mass signals (features) of these spectra are tested for correlation with given biological or clinical end points. At this point, it is important to mention that several of the testing techniques have been imported from the gene expression analysis community, which has already developed many sophisticated statistical methods to select discriminant features from high-dimensional data sets (Efron 2004). Such techniques that have already been employed in the field of MALDI-IMS are the significance analysis of microarrays (Bauer et al. 2010; Hardesty et al. 2011; Tusher et al. 2001), the permutation t test (Kim et al. 2010), the weighted flexible compound covariate method (Schwartz et al. 2005) and the receiver operating characteristics analysis (Balluff et al. 2010). If classical Mann–Whitney U test/Kruskal–Wallis test or t test/ANOVA is applied, the resulting significance values should be corrected by multiple testing methods such as Benjamini–Hochberg to control for the false discovery rate of features (Clarke et al. 2008; Kim et al. 2010).

Discriminating protein profiles have been used in several studies for supervised classification of unknown tissues using several classification models, including support vector machines for lymphomas, random forests for gastric cancer, discriminant analysis for ovary cancer and neuronal networks for breast cancer patients (Balluff et al. 2010; Rauser et al. 2010b; Schwamborn et al. 2010; Lemaire et al. 2007b). In the context of classification, wrapper and embedded feature selection methods deserve special attention as they try to find the optimal feature subset for a given classification model (Saeys et al. 2007). Genetic algorithms, which belong to the family of wrapper methods, have already been successfully employed in MALDI-IMS studies (Cazares et al. 2009; Hardesty et al. 2011; Schwamborn et al. 2010). An example for an embedded feature selection method is the random forest algorithm, which has been applied for a pixel-based classification of different tissue types within breast cancer xenograft sections of mice (Hanselmann et al. 2009).

If one wants to investigate the molecular composition of a tissue sample without prior knowledge, multivariate unsupervised methods are employed to decompose its different elements. This usually includes dimension-reducing methods such as principal components analysis, probabilistic latent semantic analysis or image denoising algorithms, followed by clustering algorithms where spectra are grouped together by their similarity (Alexandrov et al. 2010; Deininger et al. 2008; Hanselmann et al. 2008). This results in an image where pixels, representing the spectra from the same cluster, will be given the same color. Deininger et al. (2008) and Alexandrov et al. (2010) have shown that these procedures can be used to investigate histologically invisible molecular heterogeneities within gastric and neuroendocrine tumor tissues, respectively. Efforts have also been made to improve the quality of these resulting images through filtering (McDonnell et al. 2008; Hanselmann et al. 2009) and denoising algorithms (Alexandrov et al. 2010).

Unfortunately, most of the presented methods have not been implemented yet in the commercially available

software packages of mass spectrometry vendors and must therefore be computed externally. However, a step-by-step incorporation has been taking place, as for example FlexImaging in its latest version 3.0 (Bruker Daltonik, Bremen, Germany) is now offering different normalization procedures. An overview over available software tools and recent developments can be found on the website http://www.maldi-msi.org.

Protein identification

While MALDI-IMS allows measuring hundreds of masses at once, it has the limitation that these masses are just numbers with no name associated to them. While this is an important question, up to now no standardized and universally applicable protocols of identification exist. Due to this, there have been multiple approaches that were developed to take care of this important challenge.

Bottom-up and top-down protein identification

Bottom-up and top-down are two approaches of protein identification used in conventional mass spectrometry. During the bottom-up method, the proteins to be identified are digested and the resulting peptides are then fragmented for identification by mass spectrometry. Such an approach was also used for protein identification in conjunction with MALDI-IMS. It consisted of performing a tryptic digestion on the tissue sample and used the generated peptides for the identification process (Djidja et al. 2009; Groseclose et al. 2008). The challenge of this approach is that the complexity of the peptide mixture on the tissue impairs the quality and clarity of the generated mass spectra, which makes identification difficult.

For the top-down method of protein identification, the protein itself is fragmented and the resulting ions are used to identify it. This approach of protein identification was used by Rauser et al. It consisted of taking tissue that was used in the MALDI-IMS experiments and isolating proteins whose mass corresponded to the masses of interest found during the MALDI-IMS measurement. These isolated proteins were then identified by conventional top-down mass spectrometry (Rauser et al. 2010b). While this approach has the advantage that the isolated proteins are identified with a high confidence, the isolation process is tedious, complicated and it is sometimes impossible to isolate the proteins of interest for identification.

On-tissue identification

The preferable method of protein identification is to directly identify the measured ions without any further preparation steps in between, which may falsify the identification results. Therefore, new methods of protein identification directly from tissue have been developed that allow the direct identification of masses measured during MALDI-IMS, without the need for far-reaching additional experiments.

Applying tandem mass spectrometry (MS/MS), by which ions of interest are selected and fragmented for identification, directly on the tissue, Minerva et al. (2008) where able to identify small proteins with a mass below 3.5 kDa. While this is a direct approach, it is not suited for universal application due to the limitation to low mass proteins and peptides.

Stauber et al. used ion mobility separation mass spectrometry in conjunction with MALDI-IMS. This method adds a so-called drift time as separating dimension, which is comparable to the separation by chromatographic methods. The combination of mass with drift time as selection criteria enables the separation of isobaric peptide contributions to a fragmentation spectrum. Using this method, it was possible to identify peptides on tryptic digested tissue with a high sensitivity (Stauber et al. 2010b).

Debois et al. on the other hand used in-source decay (ISD) for on-tissue identification. ISD takes advantage of a fragmentation process that occurs right after the impact of the laser shot. While this generates complex mass spectra, it has the distinct advantage of having no mass limit, thus not necessitating a tryptic digestion performed on the sample tissue before the measurement and allowing the implementation of top-down sequencing approaches (Debois et al. 2010).

MALDI-IMS for the detection of specific target molecules by labeling

The method of choice to visualize the distribution of proteins in a tissue is immunohistochemistry (IHC), but it is limited by the availability of specific antibodies and its ability to only visualize one or two analytes on a single tissue sample. An alternative method may be the detection of specific analytes by labeling and detection with IMS. First described by Thiery et al. TArgeted multiplex Mass Spectrometry IMaging (TAMSIM) allows the direct visualization of a target structure on a tissue sample (Thiery et al. 2007). To this end, a molecular tag with a known mass is attached to a probe (e.g., an oligonucleotide or an antibody) by a photo-cleavable linker. During the MALDI measurement, the linker is exposed to the UV laser used for ionization and thus cleaved, releasing the tag, which can then be measured (Lemaire et al. 2007c; Stauber et al. 2010a; Thiery et al. 2008). The released tag can even be detected without the use of a matrix, which leads to an easier sample preparation and a better signal-to-noise ratio.

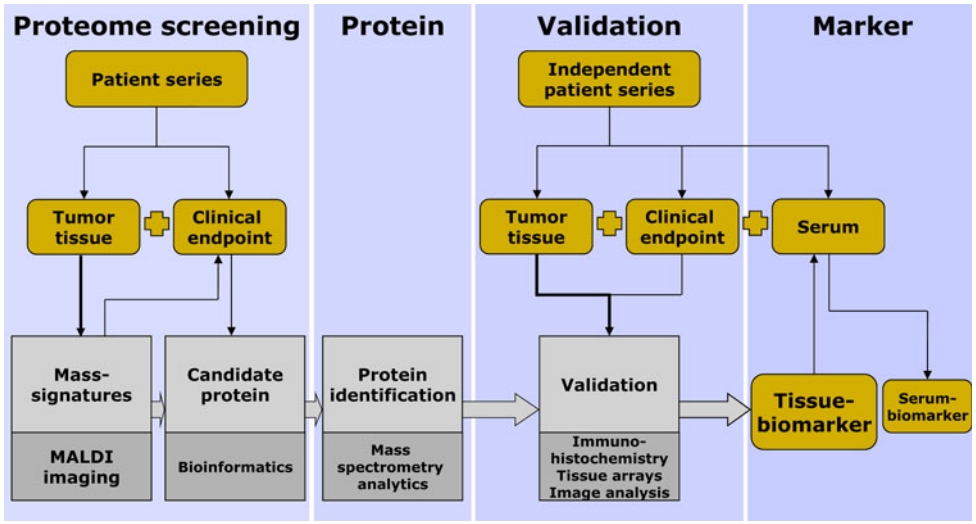

Fig. 5 MALDI-IMS based biomarker identification workflow. As an unlabeled, multiplexing, and morphology-driven approach, MALDI-IMS is the ideal starting point to discover candidate protein signals for a given clinical end point. After protein analytic identification of a candidate signal, it should be confirmed in an independent validation patient cohort by a different technique such as immunohistochemistry before being considered as a reliable tissue biomarker candidate. Subsequent studies on serum may proof its potential as a non-invasive biomarker

This, in addition to the fact that there is no signal quenching between different tags, as is the case for fluorescence tags, allows simultaneous detection of multiple molecules at femtomole levels (Lemaire et al. 2007c; Thiery et al. 2008). Furthermore, TAMSIM can be easily combined with other methods of analysis, such as immunohistochemistry, due to the minor alterations of the tissue that occur during the preparation and works on tissues independent of the fixation method used (Cazares et al. 2011). TAMSIM has great potential since it allows to measure analytes that are usually not accessible by MALDI-IMS or IHC, such as sugars (Cazares et al. 2011). In addition, it can be used to analyze the distribution of multiple analytes of different types on a single sample, allowing for deeper analysis of tissues and further enhancing the multiplexing capabilities of MALDI-IMS.

Clinical and preclinical application of MALDI-IMS to tissue analysis

In the field of cancer research, MALDI-IMS has been applied in numerous clinical and preclinical studies to a variety of tumor types, among others brain, breast, lung, ovarian, prostate and gastrointestinal cancers (McDonnell et al. 2010a). In this context, MALDI-IMS protein profiles are investigated for correlation with clinical end points, such as disease stage, survival or therapy response. Thus, different types of studies can be distinguished: diagnostic studies, prognostic and drug response studies, and tumor biology studies, which, for example, focus on elucidating the nature of tumor margins or intra-tumor heterogeneity. The role of MALDI-IMS for a successful and reliable biomarker discovery in tissues is depicted in Fig. 5.

In addition, special interest is increasing in applying MALDI-IMS for the imaging of small molecules such as drugs in tissues, since it offers several advantages to traditional drug imaging techniques.

Molecular tissue classification by MALDI-IMS

The application of MALDI-IMS for diagnostic purposes to distinguish between healthy and tumor tissue, different tumor stages or tumor subtypes has become the focus of several studies with the aim of finding better markers to aid in diagnosis.

Schwamborn et al. (2010) employed MALDI-IMS for diagnosis and classification of Hodgkin lymphoma with the aim of offering an alternative to immunohistochemical and molecular analysis. Comparing lymph nodes of classical Hodgkin lymphoma with lymph nodes of inflammatory changes resulted in a classification model that could distinguish the lymph nodes with an overall accuracy of 86.65%. In a study by Kim et al. (2010) histology-directed MALDI profiling of 63 gastric cancer and 43 healthy endoscopic biopsies found a protein profile, which classified samples in the validation set with high predictive values as either cancerous or healthy. Furthermore, the protein profile could distinguish pathologic AJCC (American Joint Committee on Cancer) stage Ia from more advanced stage patients (Ib or higher). This shows that useful molecular profiles can be obtained by MALDI-IMS

from even the smallest amounts of unprocessed fresh frozen tissue samples, such as biopsies for assisting in the diagnosis of cancers. In breast and gastric cancer determination of HER2, receptor status is part of standard diagnostics in a clinical setting and crucially important for decision making if patients are eligible for trastuzumab treatment. Two studies showed that a protein profile exists, which is able to accurately predict HER2 status in both breast and gastric cancer patients, respectively (Balluff et al. 2010; Rauser et al. 2010b). This protein pattern consists of seven signals and reaches similar correct classification rates for HER2 status in breast (accuracy: 89%) and gastric cancer patients (accuracy: 90%), suggesting that molecular changes of HER2 overexpression seem to be independent of the tumor entity (Balluff et al. 2010). One of the protein signals could be identified as CRIP1, which was strongly associated with HER2 overexpression (*m/z* 8,404) (Rauser et al. 2010b). In a large-scale study on prostate cancer comparing 34 prostate cancer and 41 benign fresh frozen prostate tissues, investigators found an expression profile which was able to discriminate 81% of the samples correctly (Cazares et al. 2009). One of the overexpressed peptides in prostate cancer (*m/z* 4,355) was identified as MEKK2. This identification together with the identification of CRIP1 mentioned above shows that molecular detail being measured by MALDI-IMS is not restricted to high-abundant proteins. Preparation protocols have been established (see "Current state of sample preparation") to increase the number of measurable molecules (Franck et al. 2010). In a study on ovarian cancer, these preparations were performed sequentially on the same samples to investigate first peptides, then proteins, followed by high mass proteins, to find molecular signatures for diagnosis (El Ayed et al. 2010). Using such a strategy, two new markers could be identified, that is, fragment C-terminal of the PSME1 (Reg-Alpha) and mucin-9.

Even though many successful studies have been reported using fresh frozen tissues (Seeley and Caprioli 2011), efforts in sample preparation protocols have been made to make the analysis of FFPE tissues by MALDI-IMS mass spectrometry feasible (Djidja et al. 2009; Gustafsson et al. 2010; Lemaire et al. 2007a; Ronci et al. 2008), even on tissue microarrays (TMAs) which are commonly used in cancer studies for high-throughput analysis (Lam et al. 2004). The first to show proof-of-principle results on that were Groseclose et al. (2008) by using TMAs for the classification of lung squamous cells and adenocarcinoma. In a similar proof-of-principle study, Morita et al. (2010) proved that the molecular information obtained by MALDI-IMS on FFPE-TMAs was able to discriminate gastric cancer samples of different differentiation grades. One advantage in analyzing FFPE tissues is that the digestion process generates hundreds of tryptic peptides in a mass range (*m/z* 500–3,000) amenable for sequence analysis by MALDI-IMS directly from their location in the tissue. For instance, Groseclose et al. (2008) could identify about 50 proteins. An additional peptide separation mechanism to the mass spectrometric measurement, like the ion mobility separation, has led to the identification of over 100 proteins in a study by Djidja et al. They intended to classify pancreatic cancer samples on TMA using an unsupervised multivariate method on the basis of the measured peptide profiles (Djidja et al. 2010).

MALDI-IMS for prognosis and therapy response prediction

Studies on prognosis by MALDI-IMS have been so far scarce. This may be due to the fact that follow-up data are rarely available for fresh frozen tissues. However, two early studies showed the usefulness of MALDI-IMS profiling to obtain protein signatures from non-small-cell lung cancer and gliomas for correlation with patient survival (Schwartz et al. 2005; Yanagisawa et al. 2003). In a recent study, Hardesty et al. (2011) investigated 69 stage III melanoma lymph node metastases by MALDI-IMS mass spectrometry with the aim to molecularly sub-classify these patients with respect to survival and recurrence. They found 12 survival and 3 recurrence-associated protein signals that were combined to generate a molecular signature to group patients into poor and favorable groups for recurrence and survival.

The capability of MALDI-IMS to obtain molecular signatures directly from the tumor, which is expected to react with the administered drug, makes it a particularly exciting application to determine the susceptibility and response to therapeutic agents in tumor and adjacent tissue (Rauser et al. 2010a). An early study on transgenic mice with mammary tumors showed that differences in the levels of several proteins between mice treated with erlotinib only and mice treated with both erlotinib and trastuzumab could be measured by MALDI-IMS (Reyzer et al. 2004). In a very recent study, MALDI-IMS was used to find markers that may indicate a pathologic response of breast cancer patients to neoadjuvant taxane and radiation therapy (Bauer et al. 2010). As taxane response rates vary between 8 and 31%, the identification of markers for therapy response would play an important role for taxane administration (Bauer et al. 2010). The proteomic analysis in this study revealed that the overexpression of α-defensins is an indicator for pathologic response. Another study by Cole et al. (2011) investigated the effect of treatment with CA-4-P, a vascular disrupting agent in late stage clinical trials, on fibrosarcoma mice models by MALDI-IMS. The results showed a clear increase in the signal

levels of certain peptides arising from hemoglobin with time after treatment with CA-4-P.

MALDI-IMS for investigation of tumor biology

MALDI-IMS is useful to analyze intra-sample molecular details either with (supervised) or without (unsupervised) the incorporation of histomorphological information. Deininger et al. (2008) showed that the application of unsupervised statistical methods allows to investigate histologically invisible, but present molecular heterogeneities within gastric cancers, which may reveal tumor subclones. A supervised analysis on myxofibrosarcomas revealed that single tissue section of intermediate-grade tumors could resemble regions with protein profiles similar to either high- or low-grade tumors, thus reflecting intra-tumor heterogeneity (Willems et al. 2010). This opens the way for specific and cancer-related in situ biomarker analysis and identification. In a histology-driven way, other groups have employed MALDI-IMS to study the proteome of renal cell carcinoma tumor margins (Oppenheimer et al. 2010), hepatocellular carcinoma and corresponding non-tumor junction sections (Han et al. 2011), and ovarian cancer interface zones (Kang et al. 2010).

Drug imaging (small molecule imaging)

In pharmaceutical discovery and development, the assessment of the distribution of a drug candidate in targeted tissues can be of central interest for the evaluation of its pharmaceutical properties (Signor et al. 2007). Initial studies by MALDI-IMS have shown the possibility of detecting not only the distribution of the drug itself, but also the simultaneous distribution of its individual metabolites, even in whole-body tissue sections (Khatib-Shahidi et al. 2006; Schwamborn and Caprioli 2010; Stoeckli et al. 2007). Thus, the technology and instrumentation is gaining enormous recognition in academic research, as well as pharmaceutical laboratories, as an important tool for the imaging of compound distribution directly from biological tissue (Prideaux et al. 2010). In comparison to other invasive drug imaging modalities, such as SIMS or autoradiography, MALDI-IMS offers several benefits. It is a label-free approach, which allows the simultaneous measurement of drug(s) and its metabolites, usually the unambiguous identification of name and structure of molecules, low costs in money and time, and a high sensitivity (Stoeckli et al. 2007; Solon et al. 2010; Nilsson et al. 2010; Signor et al. 2007; MacAleese et al. 2009) (Fig. 6).

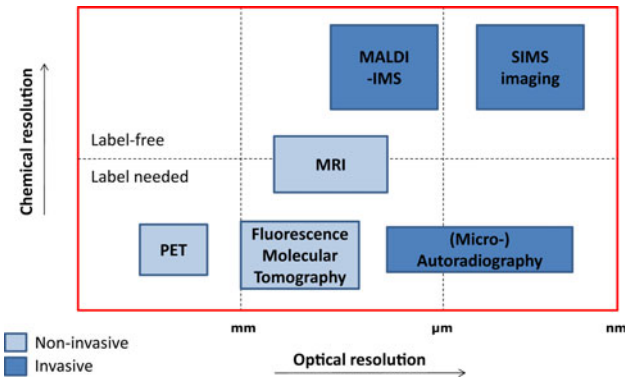

Fig. 6 Comparison of different techniques for drug imaging (small molecule imaging). While the main advantage of positron emission tomography (*PET*), magnetic resonance imaging (*MRI*) or fluorescence molecular tomography is the applicability to living objects, MALDI-IMS offers a higher spatial and chemical resolution, i.e., the possibility to distinguish the parent compound from its metabolite by its mass. *SIMS* secondary ion mass spectrometry

First applications provided good correlation to traditional autoradiography results (Hsieh et al. 2006; Signor et al. 2007; Trim et al. 2008), but disclosed also the current limitations of MALDI-IMS for small molecule imaging because of signal suppression, ionization deficiency and ion separability (Stoeckli et al. 2007; Sugiura and Setou 2010). To improve the detection of drugs on human cancer tissues, Marko-Varga et al. (2011) studied the effect of different solvent pre-treatments of tissue surfaces on signal intensities of MALDI-IMS readout. They concluded that specific experimental conditions are needed to be optimized, not only for any given drug but also tissue type. Other groups increased the ionization of the target molecule by adapting the matrix composition (Atkinson et al. 2007) or the application procedure (Goodwin et al. 2010b). Chemical derivatization may be employed to increase ion yields (Manier et al. 2011). This approach may be also useful if the target molecules are within the low-molecular weight region (less than *m/z* 1,000), since there is a strong chemical background in this mass range, created by ions from compounds endogenous to the tissue sample, such as matrix clusters or lipids (Cornett et al. 2008). The separation from this chemical background has been so far mainly achieved by using MS/MS measurements. Hereby, the ion of interest is selected and fragmented. The quantification and reconstruction of the MALDI-IMS image is then done on a specific fragment of the drug. Other techniques have been proposed. Cornett et al. (2008) employed high-resolution MALDI-FTICR for tissue imaging by separating four ions with different distributions within a mass range of only 0.02 Dalton. In contrast, Trim et al. (2008) separated the anti-cancer drug, vinblastine, from an endogenous isobaric lipid by ion mobility separation-MALDI-IMS in whole-body tissue sections. In another study by Prideaux et al. (2011) multiple reaction monitoring, which allows the specific measurement of selected ion transitions of low-molecular weight compounds at extremely high speed, has

been utilized to image the distribution of moxifloxacin in tuberculosis-infected rabbit lungs and granulomatous lesions. In addition, this work addressed the problem of matrix heterogeneities in tissues by adding an internal standard to the matrix solution. This problem especially arises where the tissue to be analyzed contains many different structures, like whole-body sections, as matrix crystallization will differ according to the physical properties of the different tissue structure (Prideaux et al. 2011; Stoeckli et al. 2007).

Another challenge in drug imaging is the quantification of MALDI-IMS data. Some studies addressed this issue by spotting a dilution series of the compound of interest directly on the tissue sections or carrier to obtain a linear standard curve (Hsieh et al. 2006; Nilsson et al. 2010), but most combined liquid chromatography coupled with MS/MS for the quantification and confirmation of molecular species in tissue sections (Goodwin et al. 2010b; Manier et al. 2011; Prideaux et al. 2011; Reyzer et al. 2003; Signor et al. 2007). However, quantification of MALDI-IMS data is still an ongoing technical challenge, due to the lack of appropriate calibration and internal standards, but novel approaches have been proposed (Koeniger et al. 2011).

Despite these current challenges, MALDI-IMS has so far been successfully applied in several studies for analyzing pharmaceutical compounds in cancer (Atkinson et al. 2007; Bouslimani et al. 2010; Marko-Varga et al. 2011; Signor et al. 2007; Trim et al. 2008), lung diseases (Manier et al. 2011; Nilsson et al. 2010; Prideaux et al. 2011), and brain diseases (Hsieh et al. 2006; Khatib-Shahidi et al. 2006; Koeniger et al. 2011). Bouslimani et al. (2010) observed by MALDI-IMS that oxaliplatin and its metabolites showed an almost exclusive presence at the margins of rat kidneys, whereas the previously applied heat treatment was expected to increase the uptake of the drug into the kidney. Human tumor xenograft in mouse was investigated for spatial distribution of banoxatrone in hypoxic regions (Atkinson et al. 2007). It was found that reduced banoxatrone was confined to hypoxic regions of the tumor. To the best of our knowledge, all drug imaging studies have been performed on animal tissues so far. However, one study investigated the properties of erlotinib and gefitinib in different human lung cancer tissues by depositing the drugs retrospectively onto the tissue sections (Marko-Varga et al. 2011). The most important observation was that the signal intensities of the drugs were higher within regions of stroma than in tumor areas. In a non-oncologic context, distribution of the antibacterial agent moxifloxacin in tuberculosis-infected rabbit lungs was studied by MALDI-IMS (Prideaux et al. 2011). Moxifloxacin was observed to accumulate in the granulomas with highest levels between the time points 1.5 and 3.25 h, but at the same time showing inhomogeneous distributions within granulomas.

These examples demonstrate that MALDI-IMS is an attractive technology for the simultaneous exploration of the spatial distribution of drugs and its metabolites in tissue sections for pharmacological studies. In addition, it allows correlating reactions of endogenous metabolites or proteins in response to drug administration (Reyzer et al. 2004; Sugiura and Setou 2010).

Imaging of lipids

Lipidomics is a new field of scientific studies that has only recently emerged (Wolf and Quinn 2008). It deals with the distribution of lipids, their biological role (e.g., as signaling molecules, neurotransmitters or precursors for second messengers) and influence on cellular function (e.g., apoptosis, proliferation and inflammation). MALDI-MSI experiments on lipids have proven the feasibility for studying how lipid functions correlate with the tissue histology (Pol et al. 2010, 2011). A detailed overview of the current state of MALDI-IMS in this field can be found elsewhere (Fernandez et al. 2011; Goto-Inoue et al. 2011). While there are already studies dealing with the imaging of lipids to improve the visualization and identification of phospholipids (Shanta et al. 2011; Shrivas et al. 2010) and other fatty acids (Hayasaka et al. 2010), there is only a very small number of clinic related studies (Shimma et al. 2007).

In unpublished but reported work from Puolitaival and Caprioli, higher abundance of linoleic acid-containing phospholipids was found in clear cell renal cell carcinoma in comparison to normal regions, where sphingomyelins were more abundant (Schwamborn and Caprioli 2010). That lipid profiles can also be used to separate cancers with different grades has been recently shown by Willems et al. (2010) by performing MALDI-IMS on myxoid liposarcomas. While analysis of high-grade tumors revealed higher levels of phosphocholines, low-grade tumors showed predominant expression of PPARγ-induced fatty acids, such as triacylglycerols. Until now, MALDI-IMS has barely started to scratch the surface of lipidomics, but the above-mentioned examples show that it has already proven its usefulness for elucidating the function of lipids in cancer biology (Fernandez et al. 2011).

Conclusion and perspective

MALDI-IMS has emerged in the last few years as a useful tool for analyzing the molecular content of tissue sections. The application of MALDI-IMS has been focused so far on biomedical questions. But MALDI-IMS has also

demonstrated versatility for analyzing morphological complex tissues of other research areas such as plant tissues (Kaspar et al. 2011), complex cell cultures (Zimmerman et al. 2011), animal surfaces (Schoenian et al. 2011) or human biological systems, like the human ocular lens (Ronci et al. 2011). Therefore, MALDI-IMS may gain also more importance in the analysis of complex biological tissues like in the field of systems biology.

The examples reported in this review show that this technology can be successfully used for investigating the molecular make-up of a tissue, revealing disease-specific profiles or biomarkers, and for studying the spatio-temporal behavior of small molecules such as drugs in animals and patients. One can envision the future application of MALDI-IMS to several challenges in predictive and diagnostic pathology. Molecular profiles obtained by MALDI-IMS may be used for molecular classification to complement histological diagnostics, to predict prognosis, response prediction or metastasis potential. The application of MALDI-IMS in clinical routine is feasible, as MALDI analyses are quick, cheap and even possible on the smallest amounts of tissue-like biopsies. Together with genomics and perhaps additional molecular information describing the state of lipids and metabolites, MALDI-IMS has the potential to provide individual medicine, where each patient's disease is unique at the molecular level and will be treated accordingly (Rauser et al. 2010a).

In addition, protocols are now compatible with FFPE tissue samples, the standard embedding technique in clinical routines. The ability to work with FFPE tissues, and therefore even with tissue microarrays, greatly increases the number and quality of samples and throughput for study (Seeley and Caprioli 2011). Another interesting technical development is the antibody-directed measurement of multiple molecular parameters in a single slide at the same time by MALDI-IMS (Cazares et al. 2011). This multiplexed assay could prove beneficial for diagnostics in clinical pathology.

Recognizing the enormous potential of MALDI-IMS, notable technical and methodological advances have been made over the last few years. This includes, on the one hand, developments in instrumentation like laser repetition rates, spatial resolution, ion separation methods, mass resolving power, and mass accuracy, as well as, on the other hand, developments in sample preparation as outlined before.

One important issue which still has to be solved is the identification of proteins of interest resulting from MALDI-IMS studies. While large-scale identifications have been possible when in situ enzymatic digestion is involved, identification of larger proteins (above 4.5 kDa) remains difficult. Single successful identifications of larger proteins have been reported so far, but routine protocols have not been established yet.

Other developments include the integration of MALDI-IMS data with current clinical imaging techniques, such as magnetic resonance imaging, positron emission tomography and X-ray computed tomography. The ability to relate proteomic information with in vivo studies, together with efforts toward three-dimensional image construction by MALDI-IMS (Sinha et al. 2008), promises to gain a deeper insight into the molecular mechanisms of health and disease, as already shown in a recent study on xenograft breast cancer by Amstalden van Hove et al. (2010a).

Despite ongoing developments of this technology, it has reached a level of standardization, robustness and accessibility where it is now not confined to proteomics specialists anymore and may be routinely accessible to users who are interested in tissue-based research.

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