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Current State and Future Challenges of Mass Spectrometry Imaging for Clinical Research

The ability of mass spectrometry imaging (MSI) to localize panels of biomolecules in tissues, without prior knowledge of their presence and in a label-free manner, has led to a rapid and substantial impact in clinical and pharmacological research, uncovering biomolecular changes associated with disease and providing low cost imaging of pharmaceuticals. This Feature article will give an introduction to the capabilities and role of MSI in the clinical analysis of patient tissues and discusses those improvements that are necessary for the progression of MSI toward routine clinical application.

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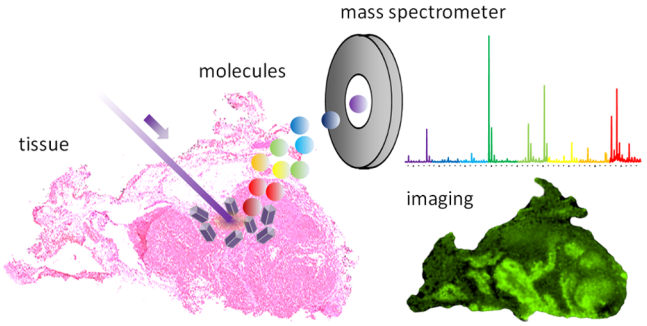

Mass spectrometry imaging (MSI) was first described almost 50 years ago using secondary ion mass spectrometry,[1] quickly followed by Hillenkamp and workers with the laser microprobe mass analyzer.[2] These techniques are highly adept at elemental and small molecule analysis and became established for semiconductor research. However, it was the pioneering work of Richard Caprioli in the late '90s, demonstrating how matrix-assisted laser desorption/ionization (MALDI) MSI could be applied to study the distributions of larger biomolecules in tissue sections, which led to the current clinical interest in MSI.[3−5]

MALDI is currently the dominant technique in clinical MSI, owing to its widespread commercial availability and its ability to analyze proteins (which can then be independently validated using clinically well-established methods). The lipid signatures generated by desorption electrospray ionization[6] (DESI) and rapid evaporative ionization mass spectrometry[7] (REIMS) have begun to demonstrate their potential for classifying patients and tissues. MALDI, DESI, and REIMS have been used in large-scale investigations with thorough statistical evaluation of the results. New MSI techniques continue to be developed and applied to clinical tissues; noteworthy examples include liquid junction surface sampling for metabolite analysis[8] and the recent mass cytometry work by Bodenmiller and workers for multiplex immunohistochemistry.[9]

MSI has been used in a multitude of clinically relevant studies, mostly in the fields of oncology, pathology, diagnostics, and surgery.[10] Furthermore, MSI can be a tool in the imaging of drugs and its metabolites. In this Feature article, we will focus on the current state of MSI in a clinical context. Established and emerging technologies will be discussed as well as recent imaging studies with clinical significance. Finally we will discuss the merits of MSI in a clinical setting and highlight those improvements we think are necessary for it to progress toward routine clinical application.

TECHNOLOGY

In a clinical setting, MSI is mostly concerned with the analysis of thin tissue sections from patient material where mass spectrometric data is acquired in a spatially resolved manner by aligning the ionization source to distinct spots on the sample's surface, Figure 1A−C. Images for each detected ion can be generated that represent the distribution of the analytes within the tissue and which can be compared with the tissue's underlying histological structure, Figure 1D. In practice, patient tissue samples, either fresh frozen or formalin fixed paraffin embedded are sectioned and mounted on glass slides. Depending on the experimental aim and method, different sample preparation steps are used, which may include tissue washing, digestion, or derivatization.

Tissues may be washed with a combination of organic or aqueous solvents to remove unwanted cofactors, such as salts, that negatively affect the ionization efficiency of the analytes, thereby increasing the sensitivity and quality of the imaging experiment.[11]

On-tissue digestion is a technique where proteases, most commonly trypsin, are used to digest proteins into small, more detectable, peptides while maintaining their original distribution. The enzyme is applied directly on the tissue and the resulting tryptic peptides are analyzed by MSI. On-tissue digestion is able to access larger proteins and assign identities by matching the detected peptides to those confidently identified in a separate extract-based protein identification experiment.[12] In a similar fashion the enzyme PNGase F has been used for in-tissue release

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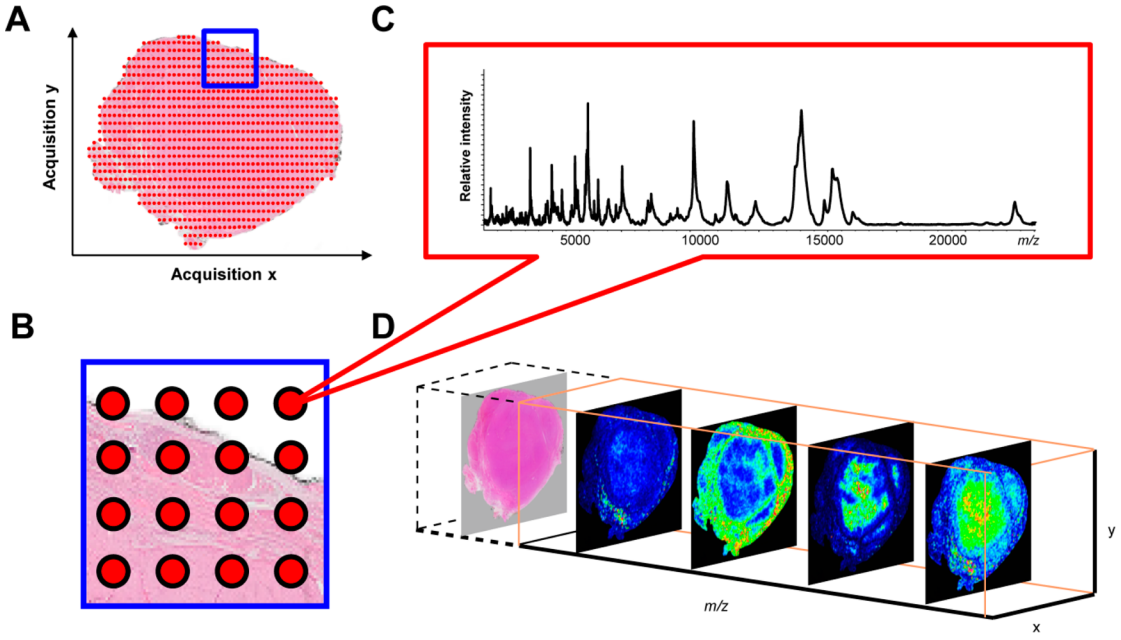

Figure 1. Principle of mass spectrometry imaging (MSI). MSI performs mass spectrometric experiments in a grid like manner on the surface of a tissue section (A); a magnification of a measurement region of part A is shown in part B. This results in a mass spectrum for each measurement spot (C), which allows the visualization of single mass signals (m/z) (D). As the sample stays intact, coregistration of the tissue's histology with the MSI images is possible (D).

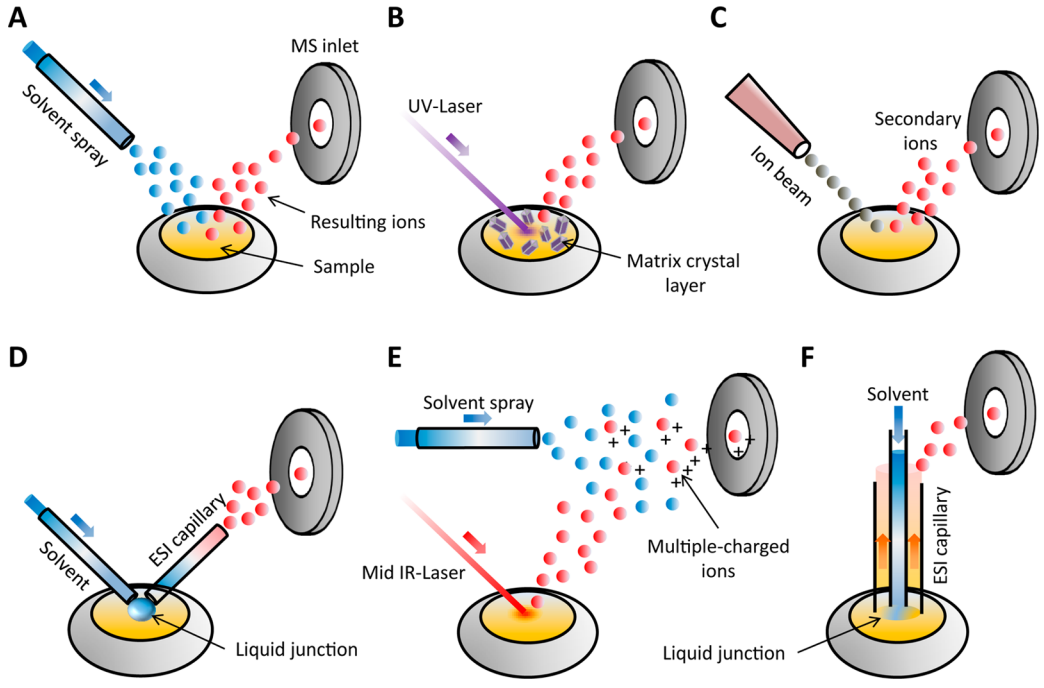

Figure 2. Working principle of different ionization sources in MSI. The upper row shows the established ionization sources: desorption electrospray ionization (DESI, A), matrix-assisted laser desorption/ionization (MALDI, B), and secondary ion mass spectrometry (SIMS, C). The lower row shows the emerging ionization technologies: nano-DESI (D), laser ablation electrospray ionization mass spectrometry (LAESI, E), and liquid extraction surface analysis (LESA, F). Abbreviations used: MS, mass spectrometer; UV, ultraviolet; ESI, electrospray ionization; IR, infrared.

of N-linked glycans, after which MALDI MSI could reveal their distribution.[13]

Chemical derivatization, a technique that changes the chemical properties of an analyte by adding or altering a functional group, has been adapted for MSI to measure low molecular weight analytes, for instance, metabolites and amino acids, which show poor ionization efficiency prior to derivatization.[14]

During mass spectrometric experiments, the analytes within the tissue have to be ionized. Depending on the experimental aims, several ionization methods can be employed which will be briefly introduced, Figure 2.

Beam Technologies. Currently the most popular technique in MSI, MALDI uses the application of a layer of matrix solution (the matrix being a small organic acid) to the tissue. The matrix

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solution extracts the analytes and incorporates them into the matrix crystals formed upon solvent evaporation. Molecular ions are then generated by a pulsed and focused laser beam aimed at the tissue surface, Figure 2B. The choice of matrix is important, as this influences the type of analytes that can be analyzed. Several different matrixes are available, allowing an array of molecular classes to be analyzed using the same technique, among which metabolites, lipids, peptides, and proteins are most often measured.

Desorption electrospray ionization (DESI) utilizes a combination of electrospray and desorption ionization. A beam of highly charged droplets, formed by an electrospray, is directed at the sample surface, where analytes are desorbed. The analyte-containing charged droplets evaporate during their transition to the mass spectrometer and thereby generate the sample-specific ions, Figure 2A. The technique has drawn much attention since it is applicable to solid, liquid, frozen, and gaseous samples. DESI MSI allows the analysis of a wide range of organic and biological compounds without requiring extensive sample preparation, at room temperature and under ambient conditions.[15]

Secondary ion mass spectrometry (SIMS) analyzes tissues by directing a focused ion beam (of so-called primary ions) at the sample. Secondary ions generated by the primary ions are then analyzed by the mass spectrometer, Figure 2C. SIMS is a destructive technique (the impact of the primary ions obliterates all molecules within a short radius of the impact site). In static SIMS, a small fraction of the surface, <1%, is irradiated by ions and so static SIMS-based MSI has been used in combination with other ionization techniques such as MALDI.[16] The advent of polyatomic primary ions has increased the sensitivity of SIMS for larger molecules. The high speed and ability to tightly focus ion beams has made SIMS synonymous with very high spatial resolution, allowing the investigation of single cells, subcellular organelles, and communities of cells.[17]

Emerging Technologies. New ionization techniques are continually developed by the mass spectrometry community, many of which are then applied to MSI. We will discuss a few of the more promising technologies, which are ambient based and allow the analysis of tissue sections at atmospheric pressure.[18]

Laser Ablation Electrospray Ionization mass spectrometry (LAESI) is a combination of mid-infrared laser ablation and electrospray ionization (ESI). Gas phase particles created by a mid-IR laser are ionized through the interaction with charged droplets from an ESI source, Figure 2E. LAESI is performed under atmospheric pressure and does not need the application of a matrix. Like DESI, it therefore overcomes many of the obstacles that traditional mass spectrometry techniques present, such as extensive sample preparation. LAESI has been used to measure a multitude of molecular classes, ranging from small molecules such as metabolites and lipids to larger biomolecules such as peptides and proteins.[19]

Liquid extraction surface analysis (LESA) and nano-DESI are both based on liquid microjunction surface-sampling (LMJ-SSP), a technique where a solvent flow through a system of aligned capillaries forms a liquid microjunction (or bridge) between the capillaries and the surface.[20] Localized analyte extraction from a tissue section can occur within this micro-junction; a flow of liquid thus enables the tissue to be sampled and the extracted analytes to be analyzed by ESI. The difference between LESA and nano-DESI concern the arrangement of capillaries and how the liquid is delivered. In LESA, a capillary or concentric capillary is used to extract analytes from a liquid junction at the sample surface,[21] after which the solvent is aspirated and ions are formed using ESI, Figure 2F. Nano-DESI is very similar to LESA except that the capillaries are not concentric and much smaller liquid bridges are reported,[8] maintained by using a very low flow rate of the liquid, Figure 2D. Both LESA and nano-DESI allow real-time atmospheric sampling and can be implemented in proteomics, lipidomics, and the analysis of drugs and metabolites.

The emergence of novel techniques allowing near-real-time analysis of patient material without extensive sample preparation and under ambient conditions should push MSI further in the fields of clinical research and diagnostics. For the interested reader Table 1 provides a cursory comparison of the different MSI ionization methods.

PRESENT APPLICATIONS

MSI transfers the discovery capabilities of mass spectrometry to tissue samples by enabling the simultaneous and unlabeled imaging of multiple molecules in tissue sections, Figure 1. As MSI does not require any labeling it is especially suited for discovery purposes where the targets are *a priori* unknown. However, one of the most important features of MSI is that the tissue's integrity is not harmed. This allows a microscopic image of the tissue, i.e., its histological image, to be registered with the MSI data. The direct comparison of the MSI data and the underlying structure of the tissue facilitate the investigator to

(1) Compare the molecular distributions with the tissue's microscopic structure (histology), Figure 1D.

(2) Extraction of mass spectral molecular profiles from known, defined histological entities, e.g., tumor cells, Figure 3A,C.

(3) Annotation of tissues based on the molecular profiles generated by MSI, Figure 3B,D,E.

Biomarker Discovery/Patient Classification. Until now the above-mentioned capabilities have been exploited for the molecular analysis of clinical tissues, principally for the determination of specific biomarkers or biomarker profiles. Biomarkers are measurable indicators of a disease or disease phenotype and are essential building blocks for personalized medicine. Clinical tissue samples, particularly cancer, often exhibit a high cellular heterogeneity by containing invasive tumor cells, so-called tumor cells *in situ* (low grade nonmalignant cells), adipose tissue, connective tissue, and inflammatory cells. Cancer tissues may also be characterized by necrotic (dead) tissue and hypoxic (low oxygen content) tissue. Different tissue samples, whether from different patients or sections of the same tumor, contain an unknown variation in the contributions of these different cell/tissue types. As these different cell types are characterized by different molecular signatures, the cellular/tissue heterogeneity comprises a significant, and largely uncharacterized, source of molecular variability for any tissue-extract-based analysis.

The combination of MSI and histology enables the extraction of molecular profiles from specific regions of tissue or histopathological entities, Figure 3A. This process, a virtual microdissection, circumvents the high cellular heterogeneity and thereby increases the specificity of the data. It is this high cellular specificity that forms the foundation of MSI's ability to identify biomarkers (and thus clinical MSI is best performed in close collaboration with expert pathologists or other specialists in the disease anatomy/biology).

The virtual microdissection can be performed retrospectively on a complete MSI data set (which describes the spatio-molecular characteristics of the full tissue section) or prior to MSI data acquisition so that MS profiles are only obtained from

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Table 1. Overview of the Main Techniques Used for Clinical MSI

	Ionization	Pretreatment	Molecular Classes	Lateral Resolution	Availability
MALDI	Laser ablation and desorption/Ionization within the ablated plume	Application of matrix solution	Dependent on the matrix; metabolites, lipids, neurotransmitters, peptides or proteins	Commercial instruments <20 μm	Widespread commercial availability and support
SIMS	Sputtering of sample with a focused primary ion beam	Not necessary, matrix/metal coatings used to increase yield of molecular ions	Static SIMS (<1% of surface analyzed by primary ion beam) − elements, fatty acids and lipids. Dynamic SIMS − elements	Static SIMS > 1 μm Dynamic SIMS < 1 μm	Widespread commercial availability and support
DESI	Molecules collected from surface by impinging droplets then ionized by an electrospray like mechanism	None	Lipids, peptides and proteins from standards. Mostly lipids and small metabolites from tissue	Generally >100 μm	Commercialized (Prosolia)
LAESI	Gas-phase particles generated by laser ablation ionized through capture by an electropsray	None	Metabolites, peptides and proteins	<200 μm for imaging applications <50 μm in cell-by-cell LAESI	Commercialized (Protea Biosciences)
Nano-DESI	Surface molecules sampled by liquid bridge then ionization by nanoESI	None	Dependent on solvent composition: metabolites, peptides and proteins	<10 μm reported. Dependent on size of liquid bridge	No
LESA	Surface molecules sampled by liquid bridge then ionization by micro/nanoESI	None	Dependent on solvent composition: metabolites, peptides and proteins	>200 μm	Commercialized (LESA − Advion; Flowprobe − Prosolia)
MassTag/ TAMSIM	Antibodies functionalized with mass spectrometric reporter groups	Addition of functionalized antibodies (+ MALDI matrix for MassTag)	Proteins	Commercial instruments <20 μm	Experiments can be performed on commercial MALDI instruments
Mass cytometry	Antibodies functionalized with lanthanide containing polymers	Addition of functionalized antibodies	Proteins	<1 μm (using an LA-ICP instrument or dynamic SIMS)	Experiments can be performed on commercial LA-ICP and SIMS instruments

specific regions of interest (MS-profiling), Figure 4D. The only difference concerns the timing of the histopathological analysis and the ability to retrospectively analyze the data (with a virtual microdissection performed after the acquisition of a complete MSI data set it is possible to select different histological regions for subsequent statistical analysis). Either MSI or MS-histology-driven profiling can provide cell-type specific molecular profiles representative for each patient. These can then be compared with the clinical data of the patients (prognosis, therapy response, metastasis) using specific statistical tests (e.g., Kaplan−Meier survival analysis, Wilcoxon rank-sum test for categorical tests), Figure 3A,C. For example a MSI study on malignant melanoma found 12 protein signals associated with patient survival and another three associated with recurrence.[22]

The same strategies have been used to aid therapy decision by identifying biomarkers to predict drug response. Reyzer et al. were the first to demonstrate that the molecular signatures detected by MSI could be indicative of treatment response.[23] Using a mouse mammary tumor virus/HER2 transgenic mouse and treatment with the erbB receptor inhibitors OSI-774 and Herceptin, it was found that tumor reduction was predicted by a >80% reduction in thymosin β4 and ubiquitin levels and that these effects were time- and dose-dependent. In a separate study on esophageal adenocarcinoma, Walch and workers identified low levels of the mitochondrial protein COX7A2 as being indicative of poor survival[24] but, importantly, as also responding favorably to neoadjuvant chemotherapy,[25] Figure 4A. Follow-up work using electron microscopy and esophageal cell lines, treated with COX7A2 specific siRNA, demonstrated that lower levels of COX7A2 led to defective mitochondria and an increased sensitivity to cisplatin based chemotherapy.[25]

An important advantage of MSI is the fact that it can analyze small amounts of tissue, a routine diagnostic protocol for many diseases. This potential has been shown in a study using endoscopic biopsies of gastric cancer, in which the molecular protein signatures were able to distinguish healthy from diseased tissue and also to separate early stage from more advanced stage cancer, which is an important determinant for further therapy.[26]

Tissue micro arrays (TMAs), which contain needle core biopsies from entire patient cohorts, have been analyzed by MALDI MSI. The analysis of clinical tissue samples always involves histopathological analysis of the tissues when selecting which tissue blocks and which patients to include in a study. To create a TMA, the pathologist has already selected from each patient's tissue blocks tissue regions that are most representative/comparable. Accordingly, a TMA already incorporates an additional step of histological specification (which is otherwise performed during virtual microdissection of the MSI data set). As MSI acquires a molecular profile from each pixel, the histological specification can be increased to match the spatial resolution of the MSI analysis for yet further cellular specification (but requires a highly detailed histopathological annotation). An average mass spectrum from each patient's biopsy, or resulting from a combined MSI-histology analysis of each biopsy for improved cellular specification, can be submitted to statistical testing. Caprioli and workers have demonstrated how MALDI MSI, performed using TMAs and verified with additional independent TMAs, can aid the sometimes diagnostically challenging differentiation of Spitz nevus (a benign melanocytic lesion) from Spitz melanoma.[27] Dekker et al. recently demonstrated that the biomarkers detected by MSI could be robust to differences in patient series, MSI methodology, MSI tissue preparation method, and measurement location.[28] Such

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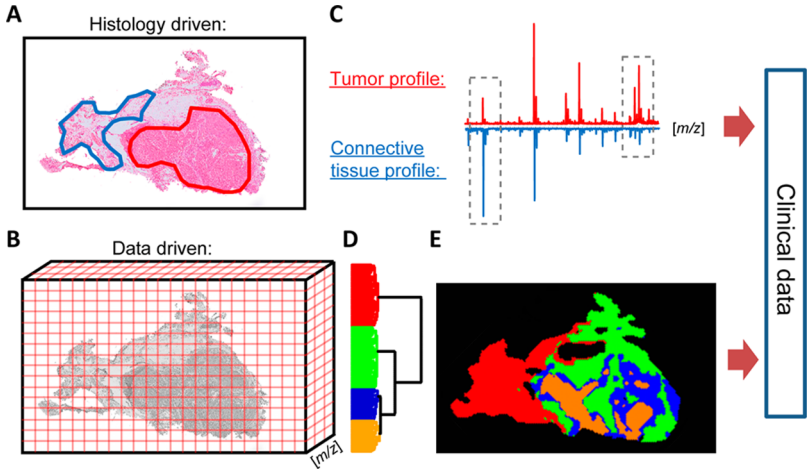

Figure 3. Data analysis approaches in MSI. In the histology-driven approach, histological information (A) is used to obtain cell-type specific molecular profiles (C). In the data-driven approach, the multivariate mass spectral data (m/z) (B) is used to annotate the tissue by clustering the single pixels using unsupervised statistical methods (D). The result is a segmented image where pixels with similar spectral profiles are visualized in the same color (E). The information obtained by either approach can be tested for correlation with the clinical data of the samples.

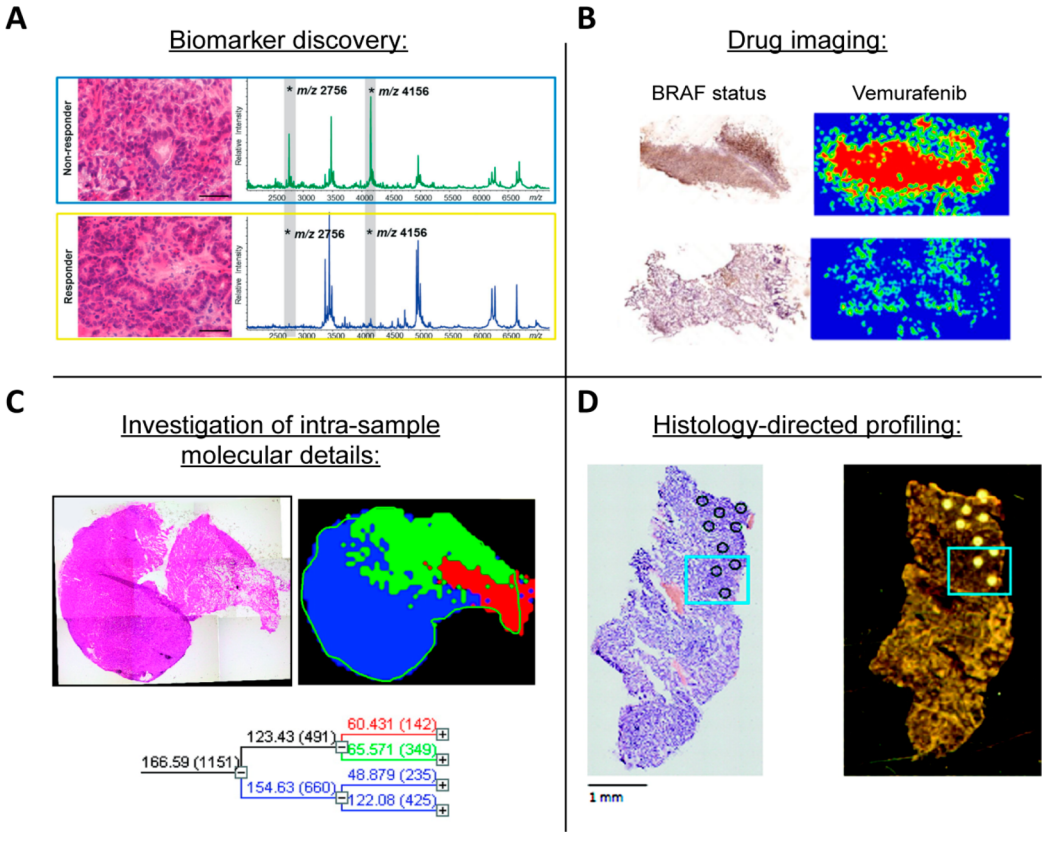

Figure 4. Examples of MSI in clinical research. MSI offers high specificity for biomarker discovery by providing cell-type specific molecular profiles. Comparing profiles from responders and nonresponders has led to the identification of biomarker candidates (A; reprinted with permission from ref 42. Copyright 2013 John Wiley & Sons, Ltd.). In drug imaging, the localization of the drug can be correlated with histological details or other data such as the molecular status of the tissue. The example shows colocalization of BRAF positive melanoma and the drug vemurafenib (B; adapted with permission from ref 35. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim). MSI facilitates investigating intrasample molecular details beyond histology. Clustering of MSI data can pinpoint molecular different regions in homogeneously looking tumor areas (C; adapted from ref 43. Copyright 2008 American Chemical Society). Acquisition of MSI spectra can be sped up by a histology-guided measurement (MS profiling). This process holds great potential for implementation into clinical practice (D; adapted from ref 44. Copyright 2010 American Chemical Society).

robustness is necessary for MSI to become a reliable diagnostic tool rather than a discovery tool that is then validated using more established techniques. The great commercial success of the MALDI based Biotyper technology, used for rapid bacterial identification, also demonstrates that with suitable procedures MALDI can generate robust classifiers.

Molecular Histology. Biomarker discovery experiments are typically defined by the tissue's histology. Such an approach

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functions well for tumors with relatively well-differentiated histologies but are less practical for histologically heterogeneous tumors and does not take into account the intratumor heterogeneity present in many tumors. Multiregion sequencing has demonstrated that the branched evolution of tumors is reflected in the tumor's heterogeneity and specific clones within the primary tumor play a critical role in the development of drug resistance and metastasis.[29] MSI offers an excellent way to examine biomolecular intratumor heterogeneity by annotating the tissues based on the measured molecular profiles, Figure 3B,D,E. A recent example of MSI combined with advanced statistical clustering methods was able to not only reveal tumor subpopulations based on their different spectral profiles but also to point out those with clinical importance,[30] Figure 4C.

The above examples are almost exclusively based on MALDI MSI of proteins, primarily because of the wide availability of MALDI instruments and the ability to use immunohistochemistry to confirm protein distributions. The use of different tissue preparations now enables MSI analysis of lipids, metabolites, neurotransmitters, and peptides in tissues and has begun to show potential for (pre)clinical research. For example MSI of these molecular classes has been used to investigate spatiotemporal changes in rodent models of stroke, epilepsy, migraine, and Parkinson's disease, revealing region-specific changes in molecular content. A recent report by Abdelmoula et al. demonstrated how MSI data from rodent models may be automatically registered to curated tissue atlases, thereby automatically placing the MSI results within their correct neurological context.[31] Such developments are key to the wider application of MSI for brain disorders.

Real-Time Tissue Classification. DESI MSI, the direct analysis of a tissue section by imparting an electrospray onto the tissue surface, can generate rich lipid profiles that have been used to classify tumors and tumor borders. In particular, it has demonstrated a marked adeptness to classify brain tumor patients, estimate tumor cell density, and reveal underlying intratumor heterogeneity.[6] The rapid analysis enabled by DESI, which does not require the tissue preprocessing of MALDI, has opened new vistas in the clinical application of MSI. Working under a cutting-edge surgical team it has been used to rapidly classify tissues following their excision; when these tissues are excised while recording the stereotactic coordinates of the sample it enables the *quasi in situ* classification of tumor and tumor margins.[32]

Takáts and workers have pioneered the *real-time, in situ* surgical use of mass spectrometry. Following unsuccessful attempts to utilize DESI for *real-time, in situ* classification of tissues they asked if the ions generated by, e.g., an electrosurgical scalpel, may be used instead of the ionization techniques more commonly used in mass spectrometry. This method, originally termed rapid evaporative ionization mass spectrometry (REIMS) but now commonly referred to as the *iKnife*, has demonstrated exquisite sensitivity and specificity for classifying tumors (once a suitable database of reference spectra has been used to create the classifier).[7]

Drug Imaging. The combination of MSI and histology is now extensively used for pharmacological research, which simultaneously images the distributions of the drug compounds and their metabolites.[33] With suitable calibration curves MSI has been shown to provide quantitative measurements of drug compound concentrations. Evidence of drug or drug metabolite accumulation is used to filter away lead compounds earlier, and more cheaply, in the preclinical phase (as accumulation is associated with toxicity). As with the above biomarker examples, pharmacological MSI is increasingly integrated with histology in order to localize the compounds within the often highly heterogeneous tumor tissue microenvironments. Recently, MSI was combined with digital image analysis of the tissue sections to relate pharmacokinetics to histological features, in this case the degree of microvascularization.[34] In another example it was demonstrated how MSI was used to trace a targeted medicine for malignant melanoma to metastatic lymph nodes tumors and provided evidence of concomitant BRAF overexpression and localization of the drug targeting BRAF, Figure 4B.[35] The ability to determine drug uptake at the target sites, in the context of the tissue's histology, provides important opportunities for understanding the mode of action of drug activity within the disease microenvironment and may well prove essential to understand the success/failure of a drug for treating different cancers/patients.

EMERGING TRENDS

Imaging Mass Cytometry. The emergence of imaging mass cytometry, highly multiplexed immunohistochemistry based on lanthanide-labeled antibodies and elemental mass spectrometry, enables panels of proteins to be simultaneously imaged in clinical biopsies. A proof-of-concept experiment on a small breast cancer TMA, assessing the distributions of 32 proteins including HER2-NEU, estrogen receptor, and progesterone receptor, could delineate the clinically recognized subtypes.[9] Furthermore, additional apparent subpopulations, e.g., HER2+ subpopulations that differed in cytokeratin 8/18, E-cadherin, -catenin, and c-MYC expression were identified. The Human Protein Atlas, a database of protein expression in human tissues, contains 99 candidate protein biomarkers for breast cancer (and similar numbers for other tumors) that have been assessed by multiple antibodies. Accordingly, the scope for identifying molecular signatures that stratify patients according to subtype, metastatic risk, recurrence, and treatment response is promising.

Imaging mass cytometry offers new capabilities for multiplexed, quantitative immunohistochemistry by exploiting the quantitative ability of laser-ablation-inductively coupled plasma mass spectrometry (LA-ICPMS). The difficulties associated with the availability and cost of antibodies (and antibody panels) may limit its use as a discovery technique but it is expected that the improved quantitation and multiplex capabilities, and its ready integration into established diagnostic practices (where antibody based assays are routine), will lead to the rapid identification of novel, multiplex biomarker profiles. The clinical impact of these profiles will determine if imaging mass cytometry establishes itself as a routine clinical tool.

International Validation. Published results strongly indicate that MSI has great potential to develop into a valuable clinical technique that can complement established histological and histochemical methods. While true, it requires biomarkers or classifiers to be rigorously reproduced in multiple biomedical centers. The wide success of the MALDI MS Biotyper platform, an instrument that identifies microorganisms using the same type of direct mass spectral analysis as MSI and which now enjoys European and FDA approval, demonstrates the potential of direct mass spectrometry for classifying complex cellular structures. A crucial aspect during the development of the Biotyper was a set of standardized sample preparation and data acquisition protocols and a database of microorganism-specific reference spectra generated using a specific mass spectrometer with defined performance criteria.[36] The success of the *iKnife* for

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in situ classification of tissues during surgery is similarly based on a reference database of spectra.[7]

MSI comes in many forms, each utilizing a vast range of different mass analyzer technologies. This ranges from laser based ionization techniques such as LDI, MALDI, and LA-ICP MSI, to charged particle/droplet beam based analyses such as SIMS and DESI, to liquid extraction based methods (LESA, nano-DESI, flowprobe). As a technology being developed principally by academic analytical chemists, the focus has remained on technical development rather than standardization. Nevertheless, the analysis of patient tissue samples from multiple institutions and a recent comparison of MALDI MSI of breast cancer tumor samples performed completely independently in two centers (different tissue collections, different tissue preparation methods, different MSI methods, and different practitioners) demonstrated that the biomarkers detected by MSI can be robust to small methodological differences (provided they are detected).[28] The recent report by Golf et al. of a cross platform normalization procedure, enabling data sets to be compared even if they were recorded on different instruments and utilize different mass analyzers (provided both systems detect the same species) is a welcome development.[37]

The most important aspect in clinical MSI research is to acquire high-quality MSI data sets. Improvements in instrument quality and robustness, automated sample preparation devices, and intuitive data analysis routines have all contributed to the increasing use and impact of MSI in biomedical research. The recent movement toward standardized reporting,[38] public web resources (http://www.maldi-msi.org), open data formats,[39] open data repositories,[40] and even open source instrumentation[41] are important developments for the further consolidation of the field toward standardized methods. Nevertheless the transdisciplinary nature of clinical MSI, spanning mass spectrometry (MSI and liquid chromatography−tandem mass spectrometry (LC−MS/MS) based identification), histopathology, and biostatistics means some basic training is still required. The MSI training programs organized by COST Action BM1104 and Vanderbilt's Advanced Imaging Mass Spectrometry (AIMS) courses are beginning to provide the targeted training necessary for the broad base of trained personnel for its widespread clinical application.

■ CONCLUDING REMARKS

For the successful biomedical application of MSI, it is essential that the experimenters keep the analytical capability of the technique in mind. For label-free MSI (i.e., excluding mass cytometry), the limited sample volume available in each pixel and the absence of an explicit biomolecular purification/separation step means MSI analyzes the more abundant components. Nevertheless, the success and independent validation of MSI-based diagnostics supports the notion that these molecular profiles can be robust surrogate markers of underlying biological differences. This has been further supported by transcriptomic and LC−MS/MS analysis of tissue regions highlighted by MSI. In this paradigm, MSI is used as a molecular histological tool to highlight tissue regions with distinct molecular signatures, which can then be further investigated using additional bioanalytical methods.

These characteristics of the MSI experiment mean it is better suited to classification/biomarker type studies than, e.g., in-depth protein analysis for elucidating signaling pathways. Accordingly most clinical applications have been and continue to be pathology focused, in which the combination of MSI and histology allows the signatures of highly specific cell types to be compared. More recently, the power of MSI has been used to develop novel clinical applications, such as imaging endogenous metabolites and neurotransmitters and the detection of intratumor heterogeneity.

The different implementations of MSI demonstrate a high potential clinical impact (and it is essential that MSI focuses on novel capabilities rather than seeking to replace established assays). Rapid and even *in situ* classification of tissues in the operating theater; biomarkers or biomarker profiles for new pathological assays (differential diagnosis, prognosis, and response to therapy); and the ability to image metabolites and neurotransmitters, molecular classes that have previously been largely intractable. Each of these applications has the potential to be transformative; for basic biomedical research, routine pathology diagnostics or *in-surgery* identification of tissues. MSI developments have been driven largely by academic analytical chemists and so method development has taken center stage; for it to become an established clinical technique in its own right the field must develop standardized methods and perform large-scale multicenter validation studies to demonstrate its clinical utility and significance.

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Benjamin Balluff is an assistant professor for imaging bioinformatics at the Maastricht MultiModal Molecular Imaging Institute, Maastricht University, The Netherlands. He did his Ph.D. at the Institute of Pathology of the Helmholtz Zentrum München, Germany, on the application of mass spectrometry imaging (MSI) in cancer research. Later, at the Center of Proteomics and Metabolomics, Leiden University Medical Center, he focused on the computational analysis of MSI datasets for the study of intratumor heterogeneity.

Professor Judith V. M. G. Bovée is a pathologist at the pathology department of the LUMC, her primary specialization being bone and soft-tissue tumors. Her research focuses on elucidating the mechanisms of tumor emergence in bone and soft-tissue as well as finding new treatment and diagnostic options as most malignant sarcomas can only be treated by surgical resection. This research is performed within a number of different collaborations, both on a national and international level.

Professor Hans Morreau is staff member and attending pathologist at the department of pathology of the LUMC since 1997. His clinical tasks include the molecular diagnostic aspects of endocrine and familial diseases and responsibility for the molecular diagnostic unit within the department of pathology. His research is focused on the familial basis of endocrine disease and the refinement of diagnosis using molecular insights.

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