



Small molecule MALDI MS imaging: Current technologies and future challenges



Paul J. Trim, Marten F. Snel *

Lysosomal Diseases Research Unit, South Australian Health and Medical Research Institute, North Terrace, Adelaide, South Australia, Australia

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ABSTRACT

Imaging of specific small molecules is particularly challenging using conventional optical microscopy techniques. This has led to the development of alternative imaging modalities, including mass spectrometry (MS)-based methods. This review aims to provide an overview of the technologies, methods and future directions of laser-based mass spectrometry imaging (MSI) of small molecules. In particular it will focus on matrix-assisted laser desorption/ionization (MALDI) as the ion source, although other laser mass spectrometry methods will also be discussed to provide context, both historical and current.

Small molecule MALDI MSI has been performed on a wide variety of instrument platforms: these are reviewed, as are the laser systems that are commonly used in this technique.

Instrumentation and methodology cross over in the areas of achieving optimal spatial resolution, a key parameter in obtaining meaningful data. Also discussed is sample preparation, which is pivotal in maintaining sample integrity, providing a true reflection of the distribution of analytes, spatial resolution and sensitivity.

Like all developing analytical techniques there are challenges to be overcome. Two of these are dealing with sample complexity and obtaining quantitative information from an imaging experiment. Both of these topics are addressed.

Finally, novel experiments including non-MALDI laser ionization techniques are highlighted and a future perspective on the role of MALDI MSI in the small molecule arena is provided.

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Abbreviations: MSI, Mass Spectrometry Imaging; IMS, Ion Mobility Separation; LDI, Laser Desorption/Ionization; MALDI, Matrix-Assisted Laser Desorption/Ionization; SIMS, Secondary Ion Mass Spectrometry; DESI, Desorption Electrospray Ionization; TOF, Time-Of-Flight; LAMMA, Laser Microprobe Mass Analyzer; Nd:YAG, Neodymium-Doped Yttrium Aluminum Garnet; Q-TOF, Quadrupole-TOF; QIT-TOF, Quadrupole Ion Trap TOF; FT-ICR, Fourier Transform Ion Cyclotron Resonance; APCI, Atmospheric Pressure Chemical Ionization; ICP, Inductively Coupled Plasma; ESI, Electrospray Ionization; FWHM, Full Width at Half Maximum; Nd:YVO₄, Neodymium-Doped Yttrium Ortho Vanadate; Nd:YLF, Neodymium-doped Yttrium Lithium Fluoride; LA, Laser Ablation; MALDESI, Matrix-Assisted Laser Desorption Electrospray Ionization.

* Corresponding author.

E-mail address: marten.snel@sahmri.com (M.F. Snel).

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1. Introduction

This review is focused on small molecule imaging using laser-based mass spectrometry (MS), in particular matrix-assisted laser desorption/ionization (MALDI). It will examine the role of instrumentation, methods, current challenges and a future perspective. To provide context, the rich history of laser-based mass spectrometry imaging (MSI) will also be discussed. Large molecule imaging or specific applications of MSI will not be covered as both have been extensively covered by previous reviews [1–4]. As will become apparent, small molecule imaging was the original form of laser-based MSI. It also has been an area that has seen much greater variety in terms of mass spectrometers, laser wavelengths and ionization methods used. This is largely due to the fact that mass spectrometers are at their most efficient when analyzing small molecules [5,6]. Small molecule MSI, the focus of this review, belongs to the wider field of MALDI MSI. MALDI MSI is an area of intense and growing research interest with more than 1500 peer reviewed publications on MALDI MSI at the time of writing (source PubMed, search term “MALDI imaging”). Where appropriate, the reader is referred to detailed reviews on specific aspects of this technique which are beyond the scope of this review.

2. What is MSI?

MSI is a form of surface analysis, where ions are generated from a surface and detected using a mass spectrometer. In laser-based

MSI, the surface of interest is irradiated with a laser. The laser energy desorbs atoms and molecules from the sample surface. Some of the desorbed species are ionized, either by the ablating laser or through a secondary process, e.g. post desorption laser ionization with a second laser. These ions are then mass analyzed to determine the chemical composition of the sample and to gain an appreciation of the amount of the molecular species present. The latter quantitative aspect of the experiment is challenging and will be discussed in greater detail later. Many different surfaces can be studied, e.g. polymers, semiconductors, plant or animal tissues [7–10].

What sets MSI apart from other laser desorption/ionization MS experiments is that spatial coordinates are assigned to the mass spectra generated, i.e. x- and y-coordinates. This spatial information can then be used to make an ion map of the surface under investigation, typically using a color scale to indicate ion intensity.

Two methods are employed to ensure data are recorded from a specific location. The most commonly used is a scanning technique, analogous to scanning electron microscopy. A small sample area is irradiated by the laser and coordinates and a mass spectrum are recorded for this locus. The sample is moved and the process is repeated many times to cover the desired area to be analyzed (microprobe analysis) [11].

In the other, ‘microscope’, approach a larger area is irradiated with the laser and a space-sensitive detector is utilized to provide the spatial information [12,13]. This approach is theoretically capable of much higher spatial resolution than scanning techniques,

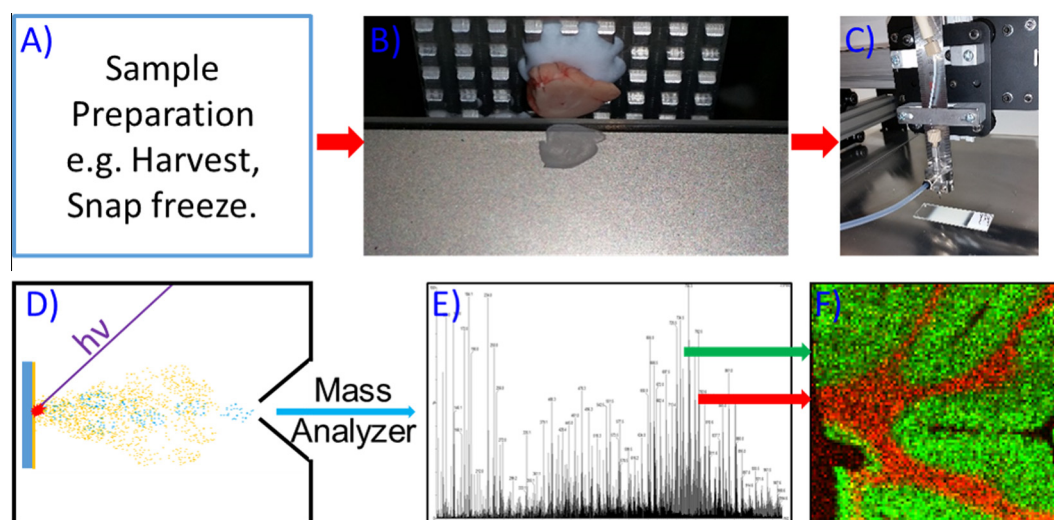


Fig. 1. Typical MALDI MSI workflow for tissue analysis. (A) Sample preparation including tissue harvesting and snap freezing. (B) Thin sections are cut using a cryotome. (C) MALDI matrix is applied. (D) Data acquisition using a mass spectrometer with a MALDI source. (E) Mass spectrum generated for each position. (F) Image generation and analysis.

however, it places some constraints on the MS hardware as the x- and y-positions of a molecule/ion need to be retained from the source to the detector.

The typical MALDI MSI workflow consists of harvesting and snap freezing tissue samples, which are then sectioned using a cryotome. In the next sample preparation step the light-absorbing MALDI matrix is applied to enhance ionization and laser ablation of molecules of interest. The prepared sample is mass analyzed and ion intensity maps are generated from the resultant data. This workflow is shown schematically in Fig. 1.

As well as laser-based methods, there are also other techniques that are well suited to MSI, the most prominent of which are secondary ion mass spectrometry (SIMS) and desorption electrospray ionization (DESI). These techniques are not covered in this review.

3. What is a Small Molecule?

The term “small molecules” means different things to different scientists and the meaning has also changed over time. It is therefore important to define what a small molecule is in this review. Here it is taken to be any molecule with a molecular weight under 2000 g/mol in its native form. This choice is based on the fact that this encompasses a large range of compounds e.g. small molecule drugs, lipids, plasticizers, metabolites, etc. It should be noted that the analysis of tryptic peptides generated through on-tissue digestion will not be included in this review. The interested reader is referred to the recent review by Cillero-Pastor and Heeren for more information on this extensively researched topic [14].

4. History of Laser-based MSI

Laser-based MSI is now often equated to MALDI MSI introduced in the mid 1990s [10,11]. The origins of this technique, however, go back at least another two decades. The key enabling technology for laser-based MSI was the laser microprobe mass analyzer (LAMMA), which was developed in the mid-1970s [15,16]. This technology employs a time-of-flight (TOF) mass analyzer coupled to a laser ion source equipped with an optical microscope. This allows for spatially selective mass analysis, i.e. selection of a specific region of a sample to be mass analyzed. The mass range of this technique is limited by laser ionization and ablation processes leading to fragmentation of larger molecules, and early work focused on elemental analysis. An example of a commercial instrument is the LAMMA 1000, which is a reflectron TOF MS equipped with a frequency quadrupled neodymium-doped yttrium aluminum

garnet (Nd:YAG) laser and an optical microscope [17]. This instrument was used for spatially-resolved MS analysis e.g. the radial distribution of sparteine and lupanine in the stems of *Cytisus scoparius* and *Lupinus polyhyllus*, respectively [18].

One of the first examples of what would now be considered laser-based MSI appeared in 1987 (N.B. SIMS-based MSI was developed in the 1960s). Wilk and Hercules successfully imaged the ion distribution of small molecules and elemental species, including several dyes and metal ions using a LAMMA mass spectrometer equipped with a frequency quadrupled Nd:YAG laser. The substrates imaged were electron microscope grids and integrated circuits, and results were presented in the form of an ion intensity map (see Fig. 2) [9]. What is striking about this work is the high spatial resolution, and spacing between pixels as low as 2.5 μm was demonstrated. Computational resources available at the time were one of the major limiting factors in the speed of analysis. At this stage, the experiments were limited by what molecules could be ablated and ionized by a laser. Much work was done to develop ever better laser systems. However, the big breakthrough was a novel form of sample preparation in which a small molecule, referred to as the matrix, was added in vast excess to samples of interest [19–22]. The matrix serves to absorb the energy from the laser, leading to rapid heating and expansion from the solid-phase into the gas-phase: the analyte molecules that were in contact with, or dissolved in the matrix are also entrained in this plume of material. Importantly, this process is so rapid that little energy transfer between the matrix and the analyte is observed allowing even very large molecules to be transferred into the gas phase intact [23]. For a full discussion of the desorption process in MALDI, the reader is referred to the review by Dreisewerd [24]. The most common MALDI matrices used also act as proton donors aiding in formation of positive ions. The ionization processes in MALDI are complex and no consensus on their exact nature has been reached. However, it now seems clear that some ions are pre-formed in the solid state, prior to laser irradiation, and that others are formed through gas-phase ion-molecule reactions in the desorption plume [25,26].

The first use of MALDI in an imaging context was reported by Spengler *et al.* at the 1994 conference of the American Society for Mass Spectrometry [11]. They imaged matrix crystals containing the neuropeptide Substance P at a lateral resolution of 1 μm . Three years later in a landmark proof of concept paper, Caprioli *et al.* described the use of MALDI MS in biological imaging [10]. Here, a MALDI matrix was applied to biological samples, e.g. human buccal mucosa cells, and endogenous proteins were measured and ion maps were produced. This group then went on to automate the

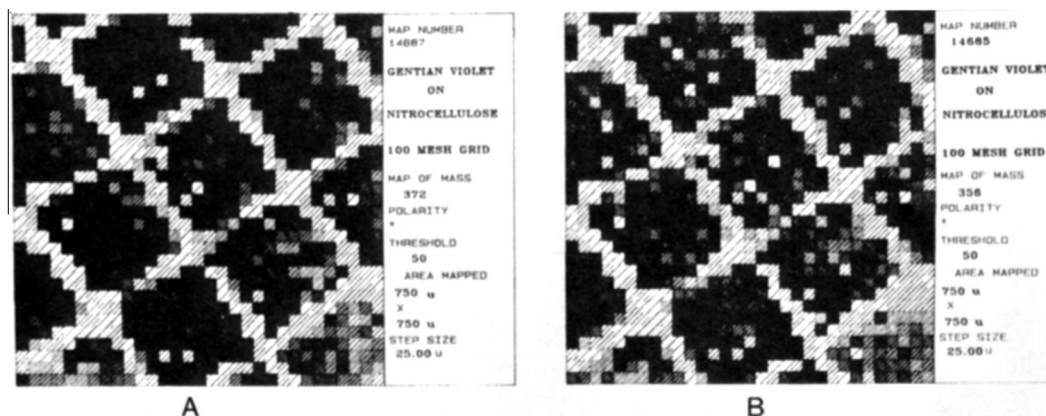


Fig. 2. Early example of LDI imaging from 1987. Depicted are: (A) Ion map of the quasi-molecular ion ($M+H$)⁺ of Gentian Violet at m/z 372; (B) Ion map of the fragment ion ($M+H-CH_2$)⁺ of Gentian Violet at m/z 358. The hole width of the mesh grid is 205 μm . The step size is 25 μm . (reprinted with permission from Anal. Chem. 59 (1987) 1819–1825 [9]. Copyright 2016 American Chemical Society).

Table 1
Typical performance characteristics of common, currently commercially available MS platforms and strengths and weaknesses of these instruments for small molecule imaging.

Geometry	TOF-TOF	Q-TOF	Linear ion trap	Orbitrap	FTMS
Mass accuracy (ppm) Mass resolving power (FWHM definition)	<5 (off-tissue) 40,000	<5 50,000	50–100 1000 (10,000 in UltraZoom mode)	<3 >100,000	<3 >100,000
Mass range (Da)	50–100,000	10–40,000	50–4000	50–4000	100–10,000
Laser shots per MS acquisition	Single	Multiple	Multiple	Multiple	Multiple
MS/MS capability	Post source decay, CID	CID	CID, MS ⁿ	CID, MS ⁿ	CID, SORI, etc.
Ion Mobility Separation	No	Yes (some models)	No	No	No
Decoupled source	No	Yes	Yes	Yes	Yes
Laser repetition rate (Hz)	Up to 10,000	2500	60	60	2000
Laser beam diameter on sample (µm)	10	50	60	60	10
Strengths	Acquisition speed; mass range; sensitivity	Mass accuracy; precursor ion selection; orthogonal separation with ion mobility	Sensitivity; CID and MS ⁿ for compound identification; precursor ion selection	Mass resolving power; mass accuracy; MS ⁿ for compound identification; precursor ion selection	Mass resolving power; mass accuracy; MS/MS modes; precursor ion selection
Weaknesses	Precursor ion selection window; fine control of MS/MS conditions; mass accuracy and resolution can be affected by sample surface morphology	Acquisition speed	Mass accuracy; mass resolving power; acquisition speed	Acquisition speed	Acquisition speed

MALDI MSI process [27]. They demonstrated the application of the technique to imaging of proteins and peptides in mouse brain and glioblastoma sections [28]. Much early MALDI MSI work focused on the analysis of intact proteins. However, the application areas have rapidly expanded to include a large number of small molecules, e.g. small molecule drugs, peptides, lipids and neurotransmitters [29–32]. To accomplish successful small molecule imaging, a variety of different mass analyzers are used, which will be discussed in the following section.

5. Instrumentation used in MSI

5.1. Mass Spectrometers

Most laser-based MSI work is performed on axial time-of-flight (TOF) instruments [10,27,28]. The pulsed ion source is ideally suited to the pulsed nature of TOF analysis. Large molecule MALDI MSI is almost exclusively performed using TOF and TOF/TOF analyzers primarily because this type of mass spectrometer can measure up to very high *m/z* values, which is critical as ions produced by MALDI are usually singly charged, although large molecules such as proteins do form some multiply-charged ions.

On the other hand, small molecule MSI is carried out on a vast array of different MS platforms, including axial TOF(/TOF) [10], quadrupole-TOF (Q-TOF) [33], quadrupole ion trap TOF (QIT-TOF) [34,35], ion traps [36,37], orbitraps [38], Fourier transform ion cyclotron resonance (FT-ICR) [39], triple quadrupole instruments [40] and ion mobility-enabled mass spectrometers [41,42]. This proliferation of analyzers has come about because the *m/z* range of interest is well suited to all of these geometries and each instrument type has desirable properties for the analysis of small molecules.

The often complex nature of the samples analyzed and the general lack of up-front/pre-ionization separation techniques mean that instrument parameters such as mass accuracy and mass resolving power become very important in dealing with sample complexity and analysis of near isobaric species. It is easier to achieve both of these instrument performance characteristics when the source of the mass spectrometer is decoupled from the analyzer. This is not the case for axial TOF instruments, where the source forms part of the flight tube. Measurement accuracy in these systems can be impaired by the thickness of the sample to be analyzed and by the lack of conductance of samples although appropriate sample preparation can minimize these effects, e.g. the use of conductive indium tin oxide-coated microscope slides [43]. In most other MALDI mass spectrometer designs the link between ionization source and mass analyzer is indirect making it easier to achieve high mass accuracy and mass resolving power [44].

Typical performance characteristics of different mass spectrometers available commercially have been summarized in Table 1. Each of the instrument characteristics has a bearing on the suitability of a mass spectrometer for a particular experiment or how they are used in a specific context. For instance, if ions of similar mass are present in a sample, instruments with high resolving power such as FTMS or orbitrap instruments may be able to separate these ions in a conventional MS experiment. Lower resolving instruments such as ion traps or Q-TOFs may need to be operated in MS/MS or MSⁿ mode to achieve separation of near isobaric species and ion mobility-enabled systems may be able to use this additional dimension of separation to reach the same goal.

The mass spectrometers used most commonly in small molecule MALDI MSI will be discussed in the following paragraphs.

5.1.1. Axial TOF and TOF/TOF

Axial MALDI-TOF mass spectrometers were the original systems used for MALDI MSI and remain the dominant form of instrumentation [10]. The TOF mass analyzer has a simple design:

an evacuated tube with a pulsed ion source and a detector; there is usually also an ion mirror (reflectron) to improve instrument mass resolving power. Determination of m/z is by measurement of the time it takes for an ion to traverse the distance from source to detector. TOF detectors are extremely sensitive and are therefore well suited to MALDI MSI where small numbers of ions are produced for each pixel. Limitations of this technology for small molecule imaging are in the limited MS/MS capability relative to other platforms, e.g. collision-induced dissociation on a Q-TOF mass spectrometer, and the lower mass resolving power and mass accuracy compared to orbitrap and FT-ICR instruments.

The popularity of this type of instrument has led to a number of commercial companies developing and producing TOF-based MALDI MSI systems, including hardware and software. At the time of writing, the latest commercial TOF instrument has a laser focus of 5 μm and a laser repetition rate of 10 kHz [45,46]. Despite these impressive specifications, investigator-led instrument development is still on-going: for instance, Zavalin *et al.* recently demonstrated a new transmission geometry MALDI source with 1- μm laser spot diameter [47]. The capability of this system was demonstrated by imaging an axon fiber at a step-size of 2.5 μm .

5.1.2. Q-TOF Mass Spectrometers

The first non-axial TOF mass analyzer to be pushed into service for MALDI MSI was the Q-TOF [29]. These instruments are a flexible imaging platform with good MS/MS capabilities. The source is decoupled from the mass analyzer ensuring optimal mass accuracy when tissue samples are being analyzed. The quadrupole makes it possible to perform MS/MS imaging experiments and the mass resolving power and mass accuracy of modern orthogonal acceleration TOF systems are in the region of 40,000 (FWHM definition) and below 5 ppm, respectively. The imaging capabilities of this type of instrument have been shown by a number of groups [29,33,48,49].

Reyzer *et al.* used the MS/MS capability of a Q-TOF instrument to distinguish a small molecule drug compound, SCG 226374, from MALDI matrix ions [29]. This work elegantly demonstrates the advantage of MS/MS in analyzing small molecules in a complex matrix. The precursor ion cannot be detected reliably by MS only owing to interference from near isobaric species. However, when the ion of interest is selected using the quadrupole and then fragmented using CID, fragment ions are generated that are specific to the drug compound and free from interference.

Bunch *et al.* showed MALDI MSI using a Q-TOF instrument equipped with a nitrogen laser in the analysis of a small molecule drug in skin tissue blots on cellulose membranes [33]. Pesticide imaging of soya plants was also demonstrated [50]. Again, greater specificity can be achieved through the use of MS/MS. An example of which was the measurement of clozapine in rat brain tissue: the data obtained using MALDI MSI were validated by comparison with autoradiography of the same compound [48].

A significant improvement in the acquisition speed of MALDI-Q-TOF MSI experiments was demonstrated by Trim *et al.* using a 20 kHz Nd:YVO₄ (yttrium ortho vanadate) solid-state laser directed onto the tissue section using a fiber optic where the beam profile of the laser was modified to improve MALDI performance [51]. The MALDI source region of a commercial MALDI-Q-TOF instrument was recently modified by improving the ion optics and source pressure control, making it possible to achieve a laser spot size of ca 10 μm , enabling high spatial resolution imaging [49]. Another technological advance in MALDI-Q-TOF MSI experiments is the introduction of ion mobility separation devices, which is described in detail in Section 5.2 [41,42,52,53].

5.1.3. Ion Traps and Hybrid Linear Ion Trap/Orbitraps

The ground work to enable laser MSI using ion trap instruments was done by the Yost group [36,37]. They developed quadrupole

and linear ion trap instruments equipped with laser desorption sources, the latter based on a commercial Finnigan LTQ [36,37]. These researchers went on to perform MALDI MSI experiments using this technology [44,54]. Although ion traps have fairly low mass resolving power and mass accuracy, one of the stand-out features of this technique is its sensitivity, for example Garrett *et al.* showed that tissue sections could still be imaged after 30 previous experiments had been performed on them [44]. In the same manuscript the utility of serial fragmentation steps (MSⁿ) was illustrated through detailed characterization of a phosphatidylserine species.

An extension of the use of ion traps is the use of hybrid linear ion trap/orbitrap instruments in laser-based MSI (hereafter shortened to orbitrap). The high mass accuracy and mass resolving power make orbitraps attractive instruments in the small molecule space. Imaging became possible on this platform when an intermediate pressure laser ion source was developed for it [38]. The imaging capability of this system was demonstrated by Landgraf *et al.* by imaging lipids in spinal cord sections [55]. The high mass resolving power and exact mass capability of the orbitrap in conjunction with the MS/MS (and MSⁿ) capability of the linear ion trap combined to give excellent discriminating power between near isobaric compounds, which is very important in the small molecule space. A further finding was a reduction in matrix cluster-related ions when the instrument was used in orbitrap mode. The authors tentatively ascribed this observation to additional cooling and longer dwell time of the ions within the instrument leading to a loss of cluster ions. An early application of orbitrap-based imaging was the imaging of neuropeptides in lobster brain and insect brains [30,56].

A method for greatly increasing analysis speed at high spatial resolution and acquiring data in different MS and ionization modes from the same tissue sample was demonstrated by the Lee group [57–59]. For example, by acquiring data in a mixed mode, high spatial resolution (step size of 10 μm) for linear ion trap acquisition and lower spatial resolution (step size of 20 μm) for the slower orbitrap measurements was possible, resulting in an estimated maximum time saving of 75–80% [57].

Another noteworthy advance in orbitrap imaging was the introduction of a custom-built MALDI source for very high spatial resolution imaging [60]. The source design was previously implemented on an FT-ICR mass spectrometer [61] and was also employed on an orbitrap to image single cells with 7 μm pixel size [60]. Korte *et al.* also demonstrated a custom-built MALDI source with a <3 μm laser spot size [62].

Orbitraps have been coupled to alternative laser ablation sources. For example, Herdering *et al.* used a commercial laser ablation source and introduced the ablated material into the mass spectrometer via a modified APCI (atmospheric pressure chemical ionization) source [63]. They went on to show that it was possible to split the flow of material from the source and simultaneously deliver sample to an ICP (inductively coupled plasma) MS system, thus obtaining elemental and molecular information from the same tissue [64]. A further example of a new laser-based ionization source for MSI on the orbitrap is the MALDESI source. In this case a thin layer of ice is deposited on the sample to be imaged. The water acts like a MALDI matrix. Ablation is by means of an infrared (IR) laser rapidly heating the water and causing an expanding plume of material [65]. Ionization is proposed to be an electrospray ionization (ESI)-like process. An advantage of this approach is that it is carried out at atmospheric pressure using a modified ESI source. Imaging of lipids using this system was demonstrated on dried spots of human serum. The water was doped with silver nitrate to provide Ag⁺ ions for adduction to lipids [65].

5.1.4. FT-ICR Mass Spectrometers

Like orbitraps, FT-ICR mass spectrometers have performance characteristics that make them highly suited to the analysis of

small molecules against a complex chemical background, as encountered in small molecule imaging. This type of instrument is capable of ultra-high mass resolving power: a resolving power of over 24,000,000 using the FWHM (full width at half maximum) definition was shown on a reserpine isotope [66]. A number of fragmentation modes are available on FT-ICR systems, for example sustained off-resonance irradiation collision-induced dissociation (SORI-CID), infrared multiphoton dissociation (IRMPD), and surface-induced dissociation (SID) [67]. The first proof-of-concept of MALDI-FT-ICR MSI was shown by the Heeren group in collaboration with the instrument manufacturer Bruker Daltonik [39]. In this example the FT-ICR MS was compared to a TOF MS for the analysis of neuropeptides using different hemispheres of the same rat brain tissue section. With the superior mass resolving power of the FT-ICR it was easily possible to baseline separate three compounds with m/z 1293.6771, 1293.7527 and 1293.9119 and show that each of these chemical species had distinct spatial distributions in tissue; this was not possible in the TOF experiment. They also demonstrated that SORI-CID, IRMPD and CID can be used in direct tissue analysis.

Three groups introduced their own custom-built laser imaging sources for FT-ICR instruments [61,68,69]: Sampson *et al.* introduced a MALDESI source [69]; and Koestler and co-workers designed a high spatial resolution MALDI source [61]. Both sources have since been applied to LTQ orbitraps and were described above. The MALDESI source has been up-dated to operate with an IR laser, where ice/water can act as the laser energy-absorbing matrix [70]. The imaging source developed by Smith *et al.* incorporates a method for continually recalibrating the mass spectrometer by sampling a physically separate reference sample, storing the reference ions and combining these with ions generated from the analyte sample [68].

Mass calibration in FT-ICR MSI experiments was also tackled by the Muddiman group through the use of ambient background ions as internal references, thus yielding <1 ppm deviation from the theoretical m/z values for two near isobaric lipid species [71].

5.1.5. QIT-TOF Instruments

In QIT-TOF instruments the first mass analyzer is a quadrupole ion trap and the second is an in-axis TOF analyzer [34,35]. The MALDI source is maintained at high vacuum ($ca\ 1 \times 10^{-5}$ mbar).

The Setou group has demonstrated imaging on this instrument in a number of biological systems, including mouse brain tissue, retina, kidneys and Japanese rice fish as well as TLC plates [72–76]. Analytes included lipids, metabolites and peptides. MSⁿ analysis was performed on tissue using lipid characterization as an example. Harada *et al.* introduced an atmospheric pressure MALDI source equipped with a 1 kHz Nd:YAG laser to a QIT-TOF [77]. Using this source spatial resolution of 10 μ m was shown, and direct imaging of ginger rhizome using an LDI approach permitted MSI of a number of terpenes, 6-gingerol, disaccharides, and several other unidentified small molecules.

5.1.6. Triple Quadrupoles

A commercial offering that had great potential, but is no longer available was the FlashQuant™ system, which consisted of a laser desorption ion source coupled to a triple quadrupole [40]. The strength of this set-up was in targeted analysis. Single/multiple reaction monitoring (SRM/MRM) mode experiments performed on triple quadrupoles are the standard method for quantifying small molecules against a complex biological background. This is especially relevant for drug quantitation in the pharmaceutical industry. The method works as follows: the first quadrupole is set to the m/z of the drug/metabolite; the selected ions are fragmented in the collision cell; and the next quadrupole transmission is set to the m/z value of a fragment ion from the precursor ion.

This method is highly specific (especially when multiple transitions are measured) and very sensitive because of its high duty-cycle (percentage of time during which the instrument measures a specific compound). The drawback of this approach is that no information is gathered on other chemical species making it unsuitable for untargeted discovery experiments. The potential of this type of geometry in MSI was demonstrated by Hopfgartner *et al.* in small molecule drug imaging in rat whole-body sections [78]: it was possible using a 1 kHz Nd:YAG laser to image a drug dosed at 0.5 mg/kg in SRM mode in less than 15 min. Another application of this instrument was the forensic analysis of single hairs for the presence of specific small molecules, *e.g.* cocaine [79,80].

5.2. Ion mobility separation

Ion mobility separation (IMS) is a post-ionization separation technique analogous to electrophoresis. Ions are propelled by an electric field through a stationary phase, in IMS the stationary phase is a gas rather than a gel. Separation is on the basis of the collisional cross-section. Ions of a given charge state with a large cross-section will have a longer drift time than smaller ions. IMS mass spectrometry of small molecules was reviewed by Laphorn *et al.*, and a protocol for IMS MSI of lipids was published by Woods and Jackson [81,82].

The first application of IMS to MALDI MSI was shown by McLean *et al.* and Jackson *et al.* [41,42]. Both groups used an instrument equipped with a drift tube mobility cell located between the source and analyzer of an orthogonal TOF instrument and were able to demonstrate class separation between lipids and peptides. McLean and co-workers illustrated the usefulness of IMS by the separation of near isobaric peptides and lipids based on differences in their gas-phase collisional cross-section and hence their mobility. Jackson and colleagues went on to show the utility of negative ion mode IMS for MALDI MSI of more acidic lipid species [52].

Using a traveling wave IMS device located between the quadrupole and TOF analyzer of a Q-TOF instrument, Trim *et al.* demonstrated the separation of vinblastine (a small molecule drug) from an interfering lipid ion in imaging a whole body section of a rat [53].

It should be noted that while IMS has much promise as an orthogonal separation technique for MALDI MSI, its usefulness at present is limited by the resolving power of this technique.

5.3. Lasers used in MALDI MSI

Although lasers form an integral part of the mass spectrometer, they are worthy of a separate discussion as they play a key part in MALDI MSI. Laser irradiation is used to desorb molecules from the sample surface and also either directly or indirectly provide the energy required for ionization. They determine the type of analyte that can be measured and are one of the limiting factors in spatial resolution and speed of data acquisition [83]. Over the years, many laser systems covering a large part of the electromagnetic spectrum have been utilized. There are some common themes: typically pulsed lasers are used with pulse widths in the order of ns, and peak power in the μ J–mJ range.

The first MALDI experiments used a frequency quadrupled Nd:YAG laser (wavelength 266 nm) with a solid matrix and a nitrogen laser (wavelength 337 nm) with a glycerol/cobalt particle matrix [19,22]. Subsequently Beavis *et al.* showed that the frequency tripled Nd:YAG (wavelength 355 nm) was suitable for use with solid matrices as a MALDI laser [84]. Many MALDI matrices have been developed that work with IR or ultraviolet (UV) lasers [85–87]. The latter have established themselves as the most

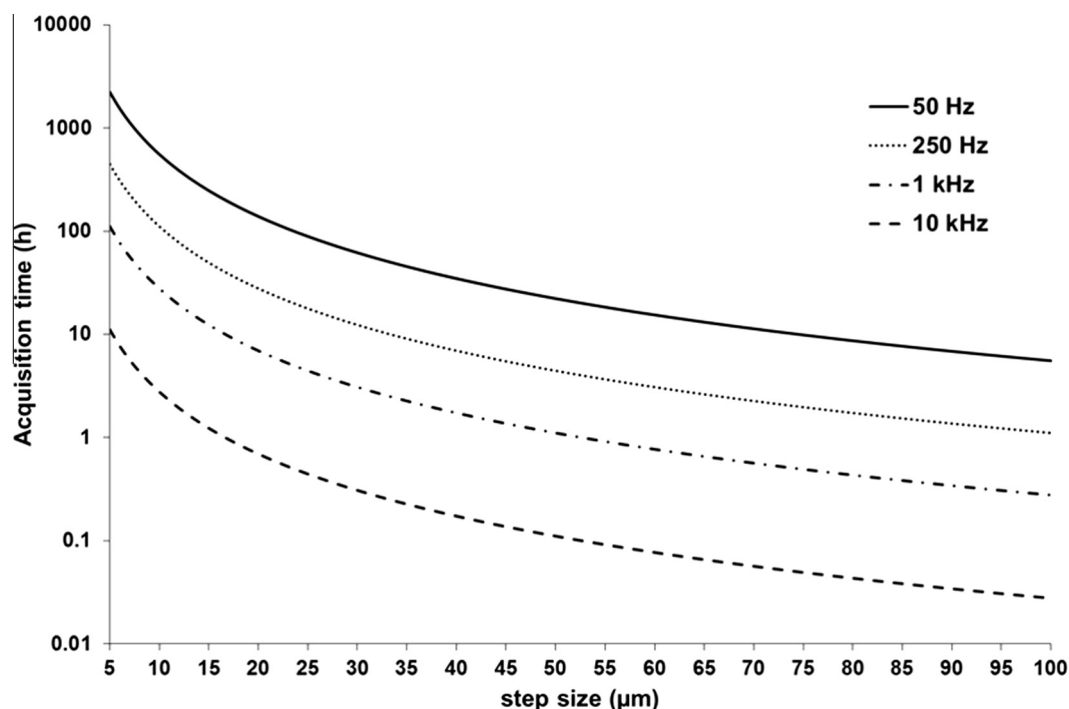


Fig. 3. Modeling of theoretical image acquisition time versus laser step size at four laser firing rates. An imaged area of 1 cm^2 and 100 laser shots per position were used. The acquisition time shown in this figure does not take into account the time needed for the stage to move between positions, processing time or limitations imposed by instrument scan speed.

commonly used. This is partially a reflection of the types of lasers typically fitted on commercial MALDI mass spectrometers.

Both the frequency tripled Nd:YAG and nitrogen lasers have remained the dominant laser systems in commercial mass spectrometers until very recently. Early MALDI-TOF mass spectrometers mostly made use of nitrogen lasers, as these were the most cost-effective option. This type of laser has, however, become much less common, probably largely due to the limited maximum repetition rate of these systems, which is *ca* 50 Hz. At the same time the cost of solid-state lasers, such as the Nd:YAG has decreased, and repetition rates of over 1 kHz are achievable. It is worth noting that in transitioning from nitrogen to YAG lasers, the beam profile of the solid-state lasers, *i.e.* the spatial energy distribution of the laser beam, was found to be “too Gaussian” (the nitrogen laser beam profile has several maxima). This led to the development of optics that emulate the nitrogen laser beam profile on a Nd:YAG laser [88] or the use a fiber-optic and mechanical movement to disrupt the beam [51].

The need for higher data acquisition speed is growing with demand for high resolution imaging. To illustrate the problem with higher spatial resolution, theoretical acquisition times are shown for a 1 cm^2 sample as a function of imaging resolution for a number of different laser repetition rates in Fig. 3. The 1 cm^2 sample area was chosen as it is approximately equivalent to a coronal mouse brain section. In this model 100 laser shots per pixel were used. It should be noted that this is purely a measurement of time when the laser is in operation and in real-life instruments there could be additional time-consuming steps such as moving sample stages and data handling. From this desktop study, it can clearly be seen that even at moderate laser step sizes of $50 \mu\text{m}$, acquisition at 250 Hz takes 4.4 h and at this repetition rate acquisition at $20 \mu\text{m}$ would take more than a day. The aim of many groups now is to achieve cellular level resolution and for this step sizes between 1 and $10 \mu\text{m}$ are required. As can be seen, this is only practical at very high repetition rates. In this illustration $5 \mu\text{m}$

imaging could be accomplished in 11.1 h using a 10 kHz laser (compared to 18.5 and 92.5 days on 250 Hz and 50 Hz systems, respectively).

Experimental work on implementing higher repetition rate lasers has been on-going for some years now [51,78] and 1–2.5 kHz lasers are now offered by most MALDI instrument vendors.

The optimal number of laser shots per pixel is dependent on many factors including choice of MALDI matrix, nature of the analyte, analyte concentration, sample thickness and matrix coating depth but typically are in the 50–200 shots per pixel.

The repetition rates are now so high that new bottlenecks in imaging analysis speed are emerging, for example the time it takes to physically move the sample between laser positions and the speed at which the mass analyzer can obtain a spectrum. To deal with the former issue, Trim *et al.* used continuous raster imaging with a variable repetition rate Nd:YVO₄ laser set to 10 kHz to image a sagittal rat brain section at $150 \mu\text{m}$ resolution in 37 min [51]. Prentice and colleagues recently showed the use of a continually moving sample stage, *i.e.* not stopping laser firing or sample stage movement between pixels [83]: they utilized a 5 kHz neodymium-doped yttrium lithium fluoride (Nd:YLF) laser (349 nm) on a TOF/TOF instrument and were able to acquire up to 13 pixels per second (*N.B.* each pixel is made up of multiple laser shots). At the 2015 meeting of the American Society for Mass Spectrometry in St. Louis, MO, the instrument manufacturer Bruker Daltonik launched an axial MALDI TOF system equipped with a 10 kHz laser system [45,46]. This instrument addresses many of the issues encountered in high speed analysis, *e.g.* the movement of sample relative to the laser is accomplished by moving both the target plate and the laser beam, thus speeding up the movement process. The laser beam is focused to $5 \mu\text{m}$ and the instrument and processing software were optimized. The high speed imaging capability of this system was illustrated by imaging a coronal rat brain section (area 1.2 cm^2) with a lateral resolution of $10 \mu\text{m}$, 200 laser shots per pixel in *ca* 10.25 h. The limiting factor

in axial TOF measurement is now the speed of the mass analyzer. At 10 kHz, the time between laser shots is 100 μ s, which is approaching the minimum time required for a typical TOF measurement. The scan time constraints are not as rigid on other geometries, such as trapping instruments or orthogonal TOF systems, as ions from multiple laser shots can be accumulated prior to mass measurement, however there are still minimum scan times.

A potentially highly significant development in the use of lasers in MALDI MSI was recently published by Soltwisch *et al.* [89]. They demonstrated that by using a second laser to photo-activate the plume generated from the initial MALDI step it was possible to increase the ion yield of several classes of membrane lipids by up to two orders of magnitude. It also made possible the visualization of lipid-soluble vitamins.

6. Spatial resolution

As mentioned in the previous section, the lateral resolution of MSI is a very important consideration. Of course, the required resolution depends on the question that is being asked. For instance, when determining the distribution of a small molecule drug in whole-body sections a spacing between measurements of millimeters may be adequate. For many biological questions, however, the aim is to measure individual cells or even sub-cellular features, which require distances between observations in the low micrometers. There are several barriers to achieving very high lateral resolution: sample preparation, laser focus, laser wavelength, time and data handling/storage. A detailed review of high resolution imaging was recently published [90].

Much work has been done to push laser-based MSI to ever higher spatial resolution. The ideal scanning laser imaging system uses a highly focused laser beam that is rapidly moved relative to the sample. A number of sources have been designed to accomplish this, many of which have been discussed in Section 5 [45–47,49,57,60–62,68,69,77,89].

One early example is the scanning microprobe MALDI MS source built by Spengler and Hubert, in which the lasers can be focused to very fine spots [91]. Using double-focusing laser optics and a piezo scanning table they demonstrated an effective focus diameter of 0.45 μ m using a Nd:YLF laser and <0.7 μ m using a nitrogen laser. Images were successfully acquired at a step size of 0.5 μ m for two small molecule dyes. The same group also developed an atmospheric pressure MALDI ion source with a similarly focused laser beam [61]. This source was interfaced to a linear ion trap FT-ICR hybrid mass spectrometry system.

Optimization for spatial resolution of laser systems on commercially available systems has recently advanced with the introduction of a new axial MALDI-TOF system with a highly focused (5 μ m) laser beam and optimized for analysis speeds up to 10 kHz [45,46].

Another approach to increase spatial resolution in MALDI MSI to better than the spot size of the laser is oversampling [92,93]. Although not often explicitly stated in the literature, it is reasonable to assume that this technique is frequently used. In this method, the laser is stepped over the sample surface in increments that are smaller than the beam diameter. This method relies on completely ablating all matrix material at each step so that on the next step signal is only obtained from the part of the sample that has a matrix coating [93]. It was possible to image hexosylceramide in spleen tissue in a mouse model of Gaucher disease at a step size of 15 μ m with a laser spot of ca 150 μ m [92].

A very elegant methodology for improving spatial resolution was demonstrated by Monroe *et al.* [94]. Thin sections of animal tissue were deposited on a surface consisting of an array of small glass beads embedded in Parafilm. The film was stretched, increas-

ing the spatial separation of parts of the tissue: no special conditions were required of the MALDI source as the spatial separation was increased using physical means prior to analysis. The spatial dimension of the experiment was given by the size of the beads, which was ca 40 μ m. A drawback of this technique was the lack of reproducibility with which the beads were positioned post-stretching, making automation more challenging.

A promising method to improving lateral resolution and acquisition speed is the use of space-sensitive detectors, so called microscope imaging [95]. This method irradiates a large area with the laser. Soltwisch *et al.* used a 150 $\times$ 300 μ m laser spot and relied on maintaining the x-, y-spatial distribution of the generated ions from source to a space-sensitive detector [95]. In this way pixel sizes of less than 1 μ m were shown, less than the wavelength of the MALDI source's IR laser, which was 2.94 μ m. This detector has also been successfully retrofitted on a commercial MALDI-TOF system [96]. This alternative approach has the potential to drastically reduce acquisition times for high resolution imaging.

7. Sample preparation

Even with the best hardware, sample preparation may be the bottleneck in achieving optimal lateral resolution and sensitivity. Sample preparation has a major bearing on the spatial resolution and sensitivity of MSI experiments. One of the most important considerations is whether or not the sample preparation results in delocalization of the analyte of interest, *i.e.* whether its measured spatial position reflects the original location or not. Mobility of a molecule depends on the molecule, the nature of the sample, sample handling and preparation.

Generally, but not always, small molecules are more mobile than large molecules. For example thin tissue sections are often washed using water and alcohols for protein MSI without any obvious delocalization of the protein. In this same washing process lipids and other small compounds are washed out of the sample, completely altering their spatial position [97]. Signor and co-workers observed that the spatial distribution of erlotinib in liver and spleen using MALDI MS/MS imaging on a Q-TOF instrument was different to that seen for autoradiography [98]. The authors attributed the differences to sample preparation issues, leading to delocalization and redistribution of analyte molecules.

The nature of the sample refers to what medium the compounds of interest are in. For instance if the sample under investigation is a meteorite, small molecules will be less mobile than if the sample is a cryo-cut tissue section. In these examples, the water content of the two samples is very different. In a dry mineral, matrix molecules will be fairly immobile, whereas in a thin tissue section molecules will move freely in the water-containing parts of the cell, *e.g.* the cytoplasm. It is possible and even intended that cells are ruptured during cutting, which will result in some delocalization of cytoplasmic components.

Improper sample handling may also result in distortion of the spatial distribution of compounds. A good example of which is condensation forming on samples when they are removed from a freezer. In some instances this may make a sample unusable. This can be overcome by carefully bringing a sample up to room temperature in a very dry atmosphere, *e.g.* a vacuum desiccator.

For tissue analysis, thought also needs to be given to how tissue is sectioned. The most successful approach appears to be very thin (5–15 μ m) sections cut using a cryotome. It is very important to avoid embedding tissue in optimal cutting temperature compound (OCT), as OCT is a mixture of synthetic polymers that are very easily detected by mass spectrometry and cause much interference in mass analysis, especially for small molecules. If embedding is necessary, MS-compatible systems have been developed, *e.g.*

carboxymethylcellulose, gelatin and poly(N-[2-hydroxypropyl]methacrylamide) [56,99–101].

For axial TOF analysis, the use of a conductive sample holder is advisable. If post-imaging optical microscopy is required, conductive microscope slides are a practical solution, e.g. indium tin oxide (ITO)-coated slides [43].

A consideration when working with biological samples such as tissue is whether any *post-mortem* changes occur, e.g. through enzyme activity. Heating of tissue is an effective way to stop enzyme activity and can therefore prevent degradation of biomolecules, aiding in the analysis of small endogenous molecules, e.g. neuropeptides [102].

Matrix addition adds a further layer of complexity to sample preparation for MALDI MSI. For the MALDI process to work, analytes need to interact with the photoexcited matrix. Fortunately, complete embedding of the analyte in matrix crystals does not seem to be a prerequisite to the MALDI process as has previously been suggested, at least not for all analyte classes. In this way it is possible for MALDI to occur from surfaces that are coated with MALDI matrix. Many different methods for matrix coating have been proposed, which can broadly be grouped into two categories based on the form the matrix takes: solvent-free and in-solution.

In-solution matrix delivery allows some extraction of analyte from the sample through leaching into the solution phase prior to matrix crystallization. The earliest MALDI MSI experiment relied on either electrospraying the matrix solution, or applying the matrix solution in large volumes to soak into the sample [11].

Both of these approaches have been further refined and have strengths and weaknesses. Deposition of matrix droplets onto sample has the advantage that the sample is wetted well and hence the analyte can be effectively extracted from the sample into the matrix droplet. This technique benefits greatly from automation, as it is difficult to manually place sub-microliter volumes on a surface, let alone produce a grid of these spots for building up an image. However, manual spotting can still be a useful tool in tissue profiling [103]. An early example of an automated spotting system is the Labcyte acoustic reagent spotter [104]: using this system 180–200 μm spots were reproducibly placed on tissue sections. An attractive feature of spotting techniques is the possibility of performing additional on-sample chemistry, for instance enzymatic digestion [105,106]. The big disadvantage of this mode of sample preparation is the limitation on spatial resolution of the experiment, which is determined by the size and spacing of the matrix/reagent droplets, typically hundreds of micrometers.

Spraying methods also wet the sample surface. However, the droplets are much smaller than those produced using droplet techniques, and samples prepared using this method can be used for high spatial resolution imaging. Spray can be produced in different ways but aerosol sprays are the most common, and these methods are similar to those developed for MALDI sample preparation for synthetic polymers [44,107,108].

The least expensive sprayers are either commercially available airbrushes or solvent sprayers for thin layer chromatography development. The matrix solution is usually sprayed in several coats until the desired thickness of matrix has been deposited on the sample. It is possible to achieve excellent results using this method. However, like all manual techniques, it is highly operator- and experience-dependent. This has implications for reproducibility and inter-sample comparisons. Recent years have seen a proliferation of automated systems, both investigator-built and commercial [109–113]. Different approaches exist for producing the small matrix droplets required, with the same end result: a controllable, optimizable and operator-independent means of coating samples with MALDI matrix droplets.

As well as solution-based methods two solvent-free techniques have been developed, namely matrix sublimation and matrix

dry-coating [114,115]. Both of these approaches are appealing as they are experimentally simple and relatively cheap.

The sublimation method uses a sublimation apparatus that can be sourced commercially. The matrix and sample are placed in the vacuum chamber of the apparatus. The chamber is evacuated and the sample is cooled while the matrix is heated. This leads to sublimation of the heated matrix, which then condenses on the cooled sample surface. The technique yields very high resolution images, illustrated by the imaging of phospholipids at cellular resolution in salamander retina [116].

The dry-coating technique is conceptually one of the simplest sample preparation protocols. Puolitaival and co-workers used 2,5-dihydroxybenzoic acid crystals, which were crushed in a mortar and pestle and dusted onto the sample through a 20 μm sieve filter [115]. Any excess matrix was removed prior to analysis. Using this technique high quality phospholipid images were obtained from brain sections. Trimpin *et al.* have subsequently automated this process using a ball mill: pre-crushed matrix and ball bearings are loaded onto a mesh which is held over the sample in a custom-built box. The whole assembly is shaken and matrix is automatically dusted onto the sample [117].

For readers interested in sample preparation, the recent reviews by Goodwin (2012) and Thomas and Chaurand (2014) are recommended [118,119].

8. Sample complexity – Seeing the tree for the forest

With the marked improvements in base instrument sensitivity in the last decade the limiting factor in detecting analytes is increasingly the presence of chemical interference at the same or similar m/z as the analyte of interest, i.e. sample complexity. This is a particular problem for direct analysis techniques like MSI, where the availability of orthogonal dimensions of separation, such as HPLC or GC is limited. In the case of small molecule MALDI MSI, this problem is exacerbated by the addition of the matrix, which is present in high abundance and will give rise to several very intense signals in the low mass range. MALDI also yields ions from a large number of different molecular species when compared to straight laser desorption experiments for example. In MS experiments, dimensions of separation can be separated into two groups, either internal to the mass spectrometer (post-ionization) or external to the mass spectrometer (pre-ionization).

The pre-ionization options are limited in small molecule laser MSI where the aim is to minimize disruption to spatial distribution, although some sample preparation protocols have been reported that enhance the detection of particular analytes. In a recent example Shariatgorji *et al.* showed that pyrylium salts could be used as a reactive matrix for the detection of dopamine, which is not usually detectable by MALDI MSI [120].

A number of instrumental, post-ionization, strategies exist to deal with high sample complexity: high mass resolving power, high mass accuracy, MS/MS, MS^n and ion mobility separation.

A real-life example of sample complexity is shown in Fig. 4. Twelve MS signals were found in a 270 mDa window, containing the analyte, and one of these was separated by only 8.3 mDa from the analyte [121]. Ultrahigh mass resolving power (0.01 Da window) was used to produce an ion map of the M3 metabolite of atorvastatin in rat liver tissue free from interference from ions of similar m/z values (Fig. 4B left). For illustration, an image was also produced using a larger (1 Da) mass window (Fig. 4B right) which shows a very different intensity distribution due to a mixed ion population.

A recent example of the use of MS/MS was the analysis of neuropeptides from crustaceans using an orbitrap mass spectrometer [122]. In this elegant set of experiments complexity was reduced in a number of different ways by employing different scan conditions and scan types for four or nine pixels in a pattern. The

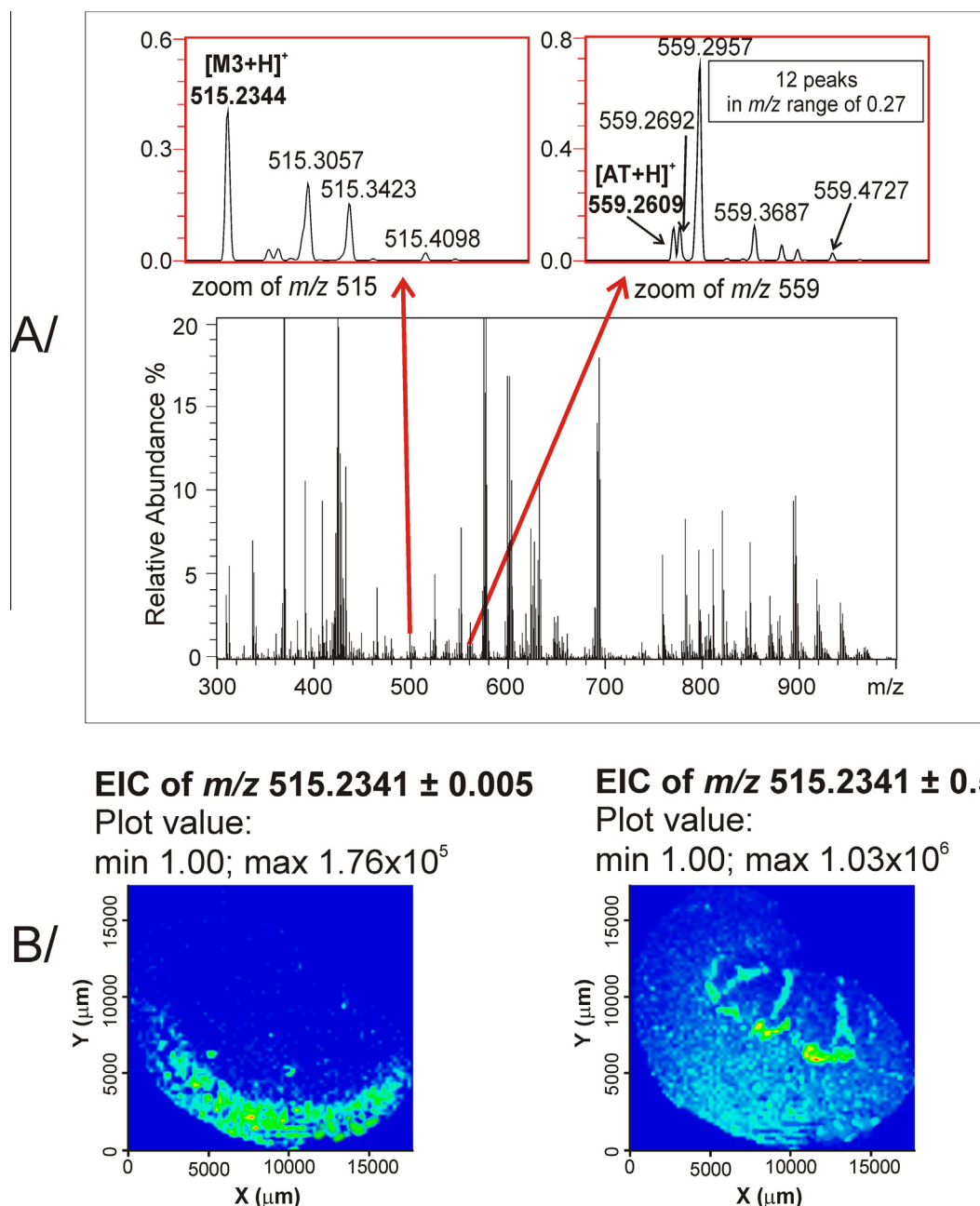


Fig. 4. Illustration of complexity in small molecule MSI of tissue containing the small molecule drug atorvastatin (AT) and one of its metabolites (M3). (A) Averaged full scan MS of rat liver tissue slice (Resolution = 100,000 (FWHM)) and zoom of m/z regions around m/z 515 and 559; N.B. complex chemical background. (B) Ion image of m/z 515 [M3+H]⁺ in rat kidney section with two different mass tolerances of $\Delta m/z$ 0.005 and $\Delta m/z$ 0.5. The ‘true’ ion map of M3 can only be seen in the $\Delta m/z$ 0.005 image (modified and reprinted with permission from reference [121]).

acquisition modes included targeted MS/MS, DDA (data-dependent acquisition) MS/MS, full m/z range MS and limited m/z range MS. The additional specificity of MS/MS has also been extensively drawn on in small molecule drug analysis in tissue [3,83].

9. Is MSI quantitative?

The aim of MSI experiments is to quantitatively determine the spatial distribution of particular molecules. However, almost all laser-based MSI techniques have limitations to the accuracy of quantitation. This is predominantly due to ionization efficiency, ion suppression, and matrix effects caused by sample heterogeneity. In MALDI MSI, this is further complicated due to matrix/analyte co-crystallization, extraction efficiency, crystal size, and uneven

matrix coating. Ion suppression is not limited to imaging-based MS techniques, but has also been observed using the ‘gold standard’ quantitative method of LC–MS [123,124]. An extensive review of quantitative MSI has recently been published by Ellis *et al.*, 2014 [125].

Some of the common techniques employed in overcoming the inherent issues with quantitation in MSI will now briefly be discussed.

Internal standards have been employed extensively to correct for ion suppression effects in MALDI MSI. Doping of the matrix with a labelled (normally deuterated) analog of the molecule of interest is currently thought to be the best method to account for ion suppression in MALDI MSI [126]. Using this technique the physicochemical properties of the analyte of interest are mimicked

by the labelled internal standard and spatial matrix effects can be accounted for because the matrix is applied uniformly across the tissue section. Källback *et al.* showed that normalization of imipramine and tiotropium in lung tissue to imipramine-D₃ spiked into the matrix could effectively compensate for areas of missing tissue [126]. The obvious limitation of this method of MALDI MS image normalization is the extraction efficiency of the analyte of interest from the tissue compared to a standard sprayed over the tissue.

As with other analytical techniques standard curves are required for quantitation in MSI. These are commonly prepared using one of two methods: tissue homogenates spiked with known standards [127,128] or spotting a standard dilution series onto/under a control tissue section prior to analysis [129]. Using these techniques it is common to see standard curves for laser imaging mass spectrometry with $R^2 > 0.9$.

Currently, absolute quantitation of an analyte on a pixel by pixel resolution is a complex but important area of research for many groups. One of the main challenges is the ability to validate quantitative information with the spatial resolution achievable using laser-based MSI techniques. The use of suitable calibration standards is essential using spiked control samples.

Only with further development and in the course of additional validation of data on a pixel-by-pixel resolution will laser-based MSI be truly quantitative. Until then quantification on a regional level with appropriate internal standards and calibration standards is the state-of-the-art in quantitative MSI.

10. Noteworthy and novel laser-based MSI experiments

Although this review is predominantly concerned with MALDI MSI, a brief overview of other laser-based MSI techniques is provided to provide some appreciation of the wider field of laser-based MSI of which MALDI MSI is only one part.

Several groups have designed means of exploiting the inherent benefits of the spatial resolution of laser-based MSI with the sensitivity and charging characteristics of ESI.

Laser ablation electrospray ionization (LAESI) was first reported by Nemes and Vertes [130]. However, they did not present any MSI data. The discrete ablation of material (*ca* 350 μm) using an IR laser and subsequent ionization of the ablated material by ESI lends itself perfectly to MSI, as demonstrated later by the same group imaging a variegated leaf pattern [131]. Using scanning electron microscopy, they imaged the ablation crater caused by the IR laser and, using flash shadowgraphy, showed the interaction of the laser-ablated sample plume and the electrospray solvent plume.

The use of MALDESI has been reported by Sampson *et al.* (2006) [132]. IR-MALDESI has recently been used by the same group to image cholesterol at a spatial resolution of 10 μm in cervical tissue by oversampling [89].

An alternative to using the plume of both the ESI source and laser ablation has been demonstrated by several groups using solvent to capture the plume generated by laser ablation for MSI applications. The captured material is then transferred to an ESI source to generate the mass spectra [133,134]. Ovchinnikova *et al.* used a liquid vortex capture probe to collect the laser ablation plume and transfer the sample directly into the ESI probe of a triple quadrupole mass spectrometer [134]. Using a scanning approach, images were produced from a stamped ink pattern at 2.5 $\mu\text{m} \times 2.5 \mu\text{m}$ pixel size and the distribution of PC 34:1 ion in brain tissue with a pixel size of 12 $\mu\text{m} \times 20 \mu\text{m}$ [134]. Solvent capture methods have also allowed the separation of complex samples by on-line HPLC post-laser ablation [135]. However, this technique results in impractical sampling times for imaging.

Although protein and large molecule MSI is not within the scope of this review it is worthwhile describing MALDI methods that can produce multiply charged ions. MALDI predominantly

produces singly charged ions, making peptide and protein imaging difficult except on axial TOF or TOF/TOF instruments. Several methods have been shown to produce multiply charged ions similar to ESI. König *et al.* showed that the yield of multiply charged ions could be greatly enhanced through the use of a liquid glycerol matrix and atmospheric pressure (AP)-MALDI using an IR laser [136]. The sensitivity of this technique was markedly improved to enable the analysis of peptides in the mid-femtomole range and the use of more common UV lasers was demonstrated by Cramer and co-workers [137]. Trimpin and colleagues discovered that it is also possible to generate a high yield of multiply charged ions from solid matrices and termed this laserspray ionization or field-free transmission geometry (FF-TG) AP-MALDI [138,139]. Using laserspray ionization the same group has shown charge states of up to 13+ for lysozyme, potentially opening up the possibility of intact protein imaging without the need for enzymatic digestion. Inutan *et al.* have shown the analysis of multiply charged peptides generated in a vacuum MALDI source of a commercially available Q-TOF instrument equipped with traveling wave IMS: the peptides were ion mobility-separated according to the different charge states. However, a fundamental difference was the use of a reflectron geometry compared to the previously reported transmission geometry [140].

11. Conclusions and future Perspective

Nils Bohr famously said that: “prediction is very difficult, especially if it’s about the future”. With this caveat, we will nevertheless venture an opinion on the future development of small molecule MALDI MSI. With the technological and methodological advances seen to date, there is no reason why this imaging modality will not develop into a standard tool for visualizing the distribution of some classes of molecules over the next 5 to 10 years. Most of the technical requirements have either already been met or are rapidly being developed.

Three areas that are likely to come on-line soon as routine techniques are lipid and endogenous small molecule imaging in a histological/molecular biology context, and small molecule drug imaging in the development of new pharmaceutical agents.

11.1. Lipid imaging

One reason for the general adoption of a new technique over existing technology is an unmet need. This condition exists in the field of lipid imaging. Lipids are an important group of biomolecules that show great promise as biomarkers of disease, *e.g.* in identifying tumor margins. Traditional techniques, for instance optical microscopy perform well in imaging large biomolecules that can either be tagged with fluorescent proteins or stained with specific antibodies. This approach is much less successful with small molecules. MALDI MSI of lipids has been widely demonstrated and works well for a number of lipid classes. For general application of this technique several boxes need to be ticked. Experimentally the requirements are: sufficient sensitivity, specificity, reproducibility, speed of analysis and spatial resolution. This review shows that all of these points have been or are being addressed for MALDI MSI of small molecules, the most recent focus having been on speed of analysis, which is now in the minutes to hours per sample, and lateral resolution which has reached the cellular level, a major benchmark for the biomedical community.

When engaging broader user groups who are not necessarily MS experts other aspects also become important to the general acceptance of a technique. These include cost of analysis and ease-of-use. With the latest high-speed instrumentation, the costs per sample should drop significantly and will hopefully soon be

comparable to the cost of other techniques such as confocal microscopy. Ease-of-use is a difficult issue to deal with, but the success of bacterial typing using MALDI-TOF MS and neonatal screening using triple quadrupole systems shows that mass spectrometry is a technique that can be used highly successfully in routine analysis. One of the bottlenecks is likely to be sample preparation, although at least for lipid analysis this seems to be a problem that will be solved in the near future. The 'dry' sample preparation protocols such as dry-coating and sublimation in particular promise a simple method for reproducibly preparing large numbers of samples. Another difficulty is dealing with large multi-dimensional datasets. This area has not specifically been covered in this review. However, a number of commercial and non-commercial initiatives are working on these issues and they are likely to become easier to manage with continuing improvements in computer hardware.

11.2. Endogenous small molecules

Like lipids, endogenous small molecules, e.g. metabolites and neurotransmitters, are difficult to image using traditional techniques and there is great utility in being able to measure these compounds. We therefore predict that MALDI MSI has a large role to play in future in routine analysis of these compounds. The adoption of this method is, however, likely to lag behind MALDI MSI lipid analysis.

As well as those faced by MSI of lipids, MSI of metabolites/neurotransmitters has some specific challenges that may slow down its general implementation. These include rapid *post-mortem* modification of the sample through residual enzyme activity, high mobility of the analytes owing to small size and/or hydrophilicity, and lower ionization efficiency. Some very elegant work is being done to address these challenges, including sample heating to stop enzyme activity and on-tissue derivatization to enhance ionization. It will, however, take time to fully automate these methods and make them ready for widespread use.

11.3. Xenobiotics

Although small molecule drug imaging requires most of the advancements mentioned above there is likely to be rapid growth in MALDI MSI throughout the drug development pipeline, eventually replacing whole body autoradiography (WBA) for drug distribution studies. There are several major benefits of using MSI compared to WBA. For example MALDI MSI can distinguish between the drug compound and its metabolites and provide separate spatial distributions of each and unlike WBA no expensive radiolabeled drug analogs are required. Furthermore, acquisition time is significantly different between WBA which is commonly 4–7 days, compared to MALDI MSI with 1–24 h for a whole body rat section [141]. However, one of the benefits of WBA is the sensitivity for a radiolabel remains consistent irrespective of the compound it is conjugated to. Whereas the sensitivity for MALDI MSI is very compound-specific depending on its ionization and detection efficiencies.

11.4. Conclusions

Since its initial development in the mid-1990s, MALDI MSI of small molecules has grown into a flourishing research area. In this article the wide range of instrumentation currently used to perform MALDI MSI on small molecules was reviewed. It also showed strategies for dealing with the challenges of obtaining optimal spatial resolution, selectivity and quantitation. A wider perspective on laser-based MSI was provided looking at novel experiments in this area. Finally, a future perspective was offered in which an attempt was made to predict the maturing of MALDI MSI into more general use as a new imaging modality.

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