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Young Jin Lee
Iowa State University, yjlee@iastate.edu

David C. Perdian
Broward College

Zhihong Song
Ames Laboratory

Edward S. Yeung
Iowa State University

Basil J. Nikolau
Iowa State University, dimmas@iastate.edu

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Abstract

We discuss and illustrate recent advances that have been made to image the distribution of metabolites among cells and tissues of plants using different mass spectrometry technologies. These technologies include matrix-assisted laser desorption ionization, desorption electrospray ionization, and secondary ion mass spectrometry. These are relatively new technological applications of mass spectrometry and they are providing highly spatially resolved data concerning the cellular distribution of metabolites. We discuss the advantages and limitations of each of these mass spectrometric methods, and provide a description of the technical barriers that are currently limiting the technology to the level of single-cell resolution. However, we anticipate that advances in the next few years will increase the resolving power of the technology to provide unprecedented data on the distribution of metabolites at the subcellular level, which will increase our ability to decipher new knowledge concerning the spatial organization of metabolic processes in plants.

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HIGH-RESOLUTION MEASUREMENTS IN PLANT BIOLOGY

Use of mass spectrometry for imaging metabolites in plants

Young Jin Lee[1,2], David C. Perdian[3], Zhihong Song[2,4], Edward S. Yeung[1,2] and Basil J. Nikolau[2,4*]

[1]*Department of Chemistry, Iowa State University, Ames, IA 50011, USA,*

[2]*Ames Laboratory, US DOES, Ames, IA 50011, USA,*

[3]*Department of Natural Science, Broward College, Coconut Creek, FL 33066, USA, and*

[4]*Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA*

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*For correspondence (fax +1 515 294 0453; e-mail dimmas@iastate.edu).

SUMMARY

We discuss and illustrate recent advances that have been made to image the distribution of metabolites among cells and tissues of plants using different mass spectrometry technologies. These technologies include matrix-assisted laser desorption ionization, desorption electrospray ionization, and secondary ion mass spectrometry. These are relatively new technological applications of mass spectrometry and they are providing highly spatially resolved data concerning the cellular distribution of metabolites. We discuss the advantages and limitations of each of these mass spectrometric methods, and provide a description of the technical barriers that are currently limiting the technology to the level of single-cell resolution. However, we anticipate that advances in the next few years will increase the resolving power of the technology to provide unprecedented data on the distribution of metabolites at the subcellular level, which will increase our ability to decipher new knowledge concerning the spatial organization of metabolic processes in plants.

Keywords: mass-spectrometry, imaging, metabolites, metabolomics, high-spatial resolution.

INTRODUCTION

The multicellular nature of higher plants presents many challenges to deciphering how biological processes are distributed among different organs and tissues. At the macro level, resolvable by the naked eye, different plant organs can be readily distinguished by their different appearances regarding shape, color, and temporal patterns of development. Furthermore, additional heterogeneity can be resolved by microscopic examinations that reveal individual cellular and subcellular compartments.

Over the past 20 years many techniques have been developed that elucidate the distribution of macromolecules (DNA, mRNA and protein) among these different tissues and organs. Some of these techniques have built on older, traditional histological methods that visualize cellular structures (e.g. Glick, 1961). Many of these methods rely on the physical–chemical interactions between an external probe and an *in situ* macromolecule, the latter being visualized by the probe. Examples include the immuno-localization of proteins, the *in situ* generation of an enzyme-catalyzed product, and *in situ* hybridization to mRNA and DNA molecules. The products of these biomolecular interaction-reactions are then visualized through either radiological detection or via the localized generation of a molecule that provides a unique electromagnetic radiation signal [e.g. ultraviolet–visible (UV–VIS) absorbance, emission or fluorescence].

More recently, with the development of transgenic technologies it has become possible to infer the location of these macromolecules based upon the expression of the genes that encode proteins and mRNAs. In plants this technology became widespread with the use of the β-glucuronidase (Jefferson *et al.*, 1987; Gallagher, 1992), or luciferase encoding genes (Ow *et al.*, 1986), which are fused to transcription regulatory regions (i.e. promoter elements). Hence, the development of β-glucoronidase- or luciferase-mediated development of localized colored or light-emitting products was used to infer the cell and tissue specificity of the location of gene products (Koo *et al.*, 2007). Even more recently, jellyfish Green Fluorescent Protein (GFP) and its several variants that generate different fluorescence colors have also been used to visually infer the location of protein gene products at the cellular and subcellular levels (Chalfie *et al.*, 1994; Prasher, 1995; Tsien, 1998).

These high-resolution molecular visualization technologies provide data concerning the spatial distribution of macromolecules that underlie the molecular mechanisms differentiating tissues and organs at the cellular and subcellular levels. However, such highly spatially resolved data are much more difficult to gain at the level of small molecules that define the metabolic inter-conversions supporting the bioenergetics of living processes (e.g. the assimilation of inorganic carbon, nitrogen, and phosphorus into biologically relevant metabolites) or the assembly of complex cellular structures (e.g. cell wall, membranes, organelles, etc.). Metabolites are the products of biological processes catalyzed by enzymes. Therefore, although the location of an enzyme can be used to infer the location of the biosynthesis of the product(s) of these reactions, that is not necessarily the final location where that product of metabolism will accumulate. For example, tropane alkaloids are synthesized in roots but they are transported and accumulate in the leaves (De Luca and St Pierre, 2000). Another example is the biosynthesis of the indole alkaloid vindoline, which is divided among different compartments at both the cellular and subcellular levels (cytoplasm, endoplasmic reticulum, vacuole, and chloroplast of epidermal and mesophyll cells), and the final product accumulates in specialized storage or secretory cells (i.e. idioblast or laticifer cells) (De Luca and St Pierre, 2000; Facchini, 2001). Therefore, a technology that can visualize the location of metabolites at high-spatial resolution has multiple applications in enabling a mechanistic understanding of the molecular differentiation that gives rise to different biological structures defining different tissues and organs.

The collection of small molecules that are catalytically acted upon by enzymes has been collectively labeled the metabolome, and metabolomics and metabolite profiling is the technology that attempts to globally profile the metabolome. Analogous to genomics, transcriptomics and proteomics procedures are being developed to determine the metabolome of organisms (e.g. http://www.plantmetabolomics.org; (Fiehn, 2002; Hall *et al.*, 2002; Lei *et al.*, 2011; Nikolau and Wurtele, 2007). However, as with the proteinaceous and nucleic acid macromolecules, to fully realize the complexity of biological processes there is a need to determine the distribution of metabolites to high spatial resolution, to the level of single cells, and to even map their location to subcellular structures to extract biologically relevant information from metabolite distribution studies. Illustrative examples of the importance of asymmetric cellular distribution of metabolism is that of C-4 photosynthesis, in which different aspects of the process are distributed between two different photosynthetic cell types (Sage and Monson, 1999). Another, but less well-characterized, metabolic process that is asymmetrically distributed among different cellular and subcellular compartments is the metabolic trafficking and assembly of lipid molecules (Moore, 1993; Benning, 2008; Xu *et al.*, 2008). These molecules have carbon and energy storage functions, they define cellular boundaries, and act as a two-dimensional medium for the organization of complex processes such as photosynthesis and respiration.

In this review, we illustrate recent advances that are being made in applying mass spectrometry (MS) to identify the distribution of metabolites among different tissues and cells of plants. These are relatively new technological applications of MS (Chughtai and Heeren, 2010), and they are providing metabolic insights, which are asserting the ability to decipher new knowledge concerning the spatial organization of metabolic processes in plants (Sumner *et al.*, 2011).

MASS SPECTROMETRIC TECHNOLOGY FOR IMAGING MOLECULES

Figure 1 shows the overall scheme of a standard MS imaging (MSI) experiment. It comprises tissue preparation, matrix application [for matrix-assisted laser desorption ionization (MALDI) applications], MSI data acquisition, followed by data analysis and image construction. Acquisition of MS data is divided into two processes. The first process involves desorbing analyte molecules from the tissue followed by ionization to create positively or negatively charged ions. Desorption and ionization occurs in what is often referred to as the ionization source of the mass spectrometer. Depending on the probing beam that is used to interrogate tissue samples, a variety of ionization sources are available. Matrix-assisted laser desorption ionization (Caprioli *et al.*, 1997; Chaurand *et al.*, 1999; Stoeckli *et al.*, 2001), desorption electrospray ionization (DESI) (Takats *et al.*, 2004; Wiseman *et al.*, 2008; Manicke *et al.*, 2009; Mueller *et al.*, 2011), and secondary ion mass spectrometry (SIMS) (Colliver *et al.*, 1997; Vickerman, 2011) are the most common ionization sources, and they utilize a laser beam, a solvent stream, and an ion beam, respectively, as the probe. Laser ablation electrospray ionization (LAESI) (Nemes and Vertes, 2007; Nemes *et al.*, 2008, 2009) uses laser irradiation to desorb the analyte molecules and a solvent stream to ionize the molecules. Table 1 summarizes the major characteristics of these commonly used ionization sources.

Once analyte molecules have been desorbed from the tissue surface and ionized, they are introduced into a mass analyzer and sorted on the basis of their mass to charge ratios (m/z). Table 2 lists a few commercially available mass analyzers and some of their important characteristics for MALDI MSI experiments. Time-of-flight (TOF)-MS was initially the only analyzer that was available for MALDI MSI, but now a wider variety of mass analyzers are available for MSI from most manufacturers (Garrett *et al.*, 2007; Cornett *et al.*, 2008; Strupat *et al.*, 2009). Atmospheric pressure (AP) MALDI (Spengler and Hubert, 2002; Koestler *et al.*, 2008; Römpp *et al.*, 2010), DESI, and LAESI can be used with any mass spectrometer with an ESI source. Secondary ion mass

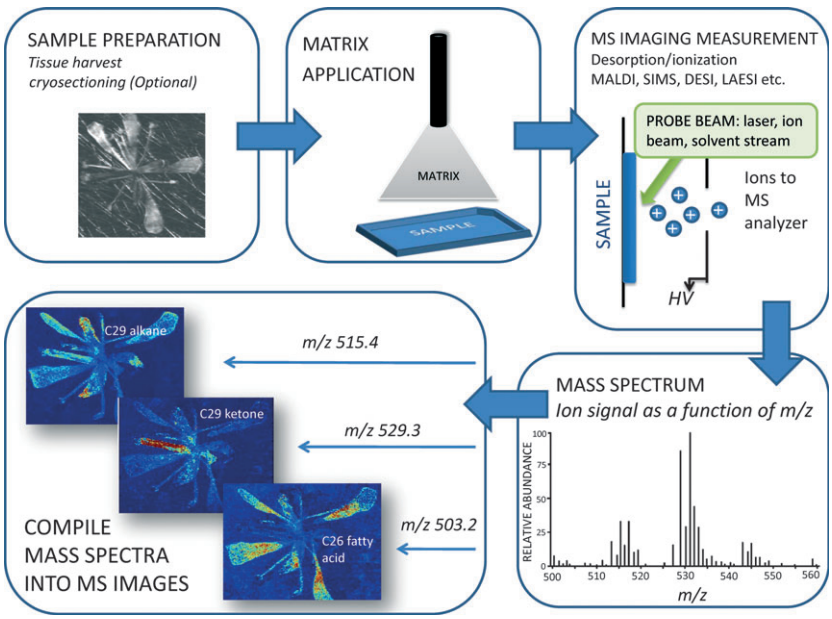

Figure 1. Experimental scheme for mass spectrometry imaging experiments.

Table 1 Common ionization methods for mass spectrometry imaging experiments

Ionization source	Probe beam	Ions	Pressure regime	Spatial resolution (μm)[a]
MALDI[b]	Laser beam	Singly charged	Vacuum or ambient	10–100
SIMS[c]	Ion beam	Singly charged	Vacuum	0.2–3
DESI[d]	Solvent stream	Singly or multiply charged	Ambient	200
LAESI[d]	IR laser beam[e]	Singly or multiply charged	Ambient	300–500

MALDI, matrix-assisted laser desorption ionization; SIMS, secondary ion mass spectrometry; DESI, desorption electrospray ionization; LAESI, laser ablation electrospray ionization; IR, infrared.
[a]Typical spatial resolution available with commercial ion source.
[b]MALDI sources are available from most vendors (see Table 2). Atmospheric pressure MALDI sources are available from MassTech (http://www.apmaldi.com) and can be used with most electrospray ionization mass spectrometers.
[c]Time-of-flight (TOF)-SIMS is available from Physical Electronics, Inc. (http://www.phi.com/) and Ion-TOF GmbH (http://www.iontof.com/).
[d]DESI sources are available from Prosolia, Inc. (http://www.prosolia.com/) and LAESI sources are available from Protea Biosciences, Inc. (https://proteabio.com/). They can be used with most commercial ESI mass spectrometers.
[e]It does not have to be used with an IR beam, but is best known and commercially available using an IR laser beam.

spectrometry is commonly used with a TOF analyzer, and recently its adaptation to a quadrupole (Q)-TOF has been demonstrated (Carado *et al.*, 2008). After a mass spectrum is acquired from an individual spot on the tissue, the sample plate is moved to a new position for the next spectral acquisition, and thousands or more spectra are obtained by rastering hundreds of *x* and *y* positions. The collected spectra are collated and chemical images of each ion of interest can be generated. More details about ionization, mass analysis, and image production are discussed in other review articles (Chughtai and Heeren, 2010), but basic information is described below along with some recent advances.

Ionization sources

The first mass spectrometer to be used for imaging applications was TOF-SIMS, initially developed for elemental species (Castaing and Slodzian, 1962; Liebl, 1967). Secondary ion mass spectrometry uses a primary ion beam to desorb and ionize analyte molecules, which are then referred to as secondary ions. The single biggest advantage of SIMS sources compared with other ionization methods is their spatial resolution. Spatial resolution values of less than 100 nm are regularly reported with SIMS methods (Ostrowski *et al.*, 2004; Piehowski *et al.*, 2008), which compares to a typical high resolution of 10–20 μm with MALDI. In SIMS analysis, the primary ions bombard the sample surface with very high energy (typically 5–40 keV) and in high-ion-current mode, which can sputter individual atoms from the sample surface for elemental analysis. At lower ion currents ($<10^{13}$ ions cm^{-2}) the surface damage is minimized, and the desorption/ionization of intact molecules on the surface becomes possible (Vickerman, 2011). This process,

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Table 2 Listing of commercially available mass analyzers for matrix-assisted laser desorption ionization mass spectrometry imaging

Mass analyzer	Manufacturer[a]	MS/MS	MS*n*	Resolution ($m/\Delta m$)[b]	Relative mass spectra acquisition speed[g]	Other notes[h]
TOF	Bruker Daltonics	No	No	2500/40 000[c]	Fast	High vacuum
TOF/TOF	Bruker Daltonics/AB Sciex	Yes	No	>26 000[d]	Fast	High vacuum
Ion trap – TOF	Shimadzu Scientific	Yes	Yes	10 000	Fast	Moderate pressure
Ion trap	Thermo Scientific	Yes	Yes	~1000	Medium	Moderate Pressure
Ion trap – Orbitrap	Thermo Scientific	Yes	Yes	100 000	Slow	Moderate Pressure
FTICR	Bruker Daltonics	Yes	Yes	>200 000[e]	Slow	Moderate pressure
IM-Q-TOF	Waters	Yes	No	13 000/40 000[f]	Fast	Ion mobility separation

MS/MS, tandem mass spectrometry; MS*n*, multiple MS/MS experiments; TOF, time-of-flight; FTICR, Fourier transform ion cyclotron resonance; IM-Q-TOF, ion mobility-quadrupole-time-of-flight.
[a]Manufacturer URLs: Bruker Daltonics, http://www.bdal.com/; AB Sciex, http://www.absciex.com/; Shimadzu Scientific, http://www.ssi.shimadzu.com/; Thermo Scientific, http://www.thermoscientific.com/; Waters, http://www.waters.com/.
[b]Current typical mass resolution to the best of our knowledge.
[c]Linear and reflectron mode, respectively.
[d]Second TOF in reflectron mode.
[e]This can vary depending on the magnetic field, data acquisition time, and (m/z).
[f]Sensitivity mode and high-resolution mode, respectively.
[g]Data acquisition time per pixel: fast, <0.1 sec; medium, 0.1–0.3 sec; slow, ≥1 sec.
[h]Source vacuum pressure: high vacuum, approximately 10^{-7} Torr; moderate pressure, 0.08–1 Torr.

called 'static SIMS', still creates a large number of molecular fragmented ions, and limits the size of the biological molecule that can be analyzed. Because of such high fragmentation and inherent low ionization efficiency, SIMS imaging has not been as widely used as other ionization sources for biological applications, in spite of its long history (Heeren *et al.*, 2009).

The technique that has received the most widespread use as the ionization source for MSI experiments is MALDI. The exact mechanism of MALDI is not well understood (Karas and Kruger, 2003), but it is generally accepted that the process involves photon absorption by the matrix, local surface heating, and explosion/desorption of the surface molecules. Ionization can occur during the desorption process or in the high-density gas plasma that is initially formed, called the laser plume (Jaskolla and Karas, 2011). Co-crystallization of the matrix with the analyte molecules is considered to be essential, particularly for macromolecules such as polypeptides and proteins. The choice of matrix depends on many factors including, but not limited to, the class of analyte molecules being investigated, the molecular weight of the analytes, and the sample composition. A comprehensive description of matrix choices and the factors that need to be considered in selecting the matrix has been reviewed (Hillenkamp and Peter-Katalini, 2007). In some instances, the analyte molecules or other native species have adequate absorption at the wavelength of the laser (Li *et al.*, 2006; Hoelscher *et al.*, 2009), and data acquisition can be performed without a matrix, simplifying the sample preparation process.

Both MALDI and SIMS create singly charged ions, usually protonated or deprotonated ions ($[M+H]^+$ or $[M-H]^-$), but sometimes other adduct ions can be formed (e.g. $[M+K]^+$ or $[M+Na]^+$). Because the majority of ions created by these techniques are singly charged, the mass range of the mass analyzer is an important consideration if the experiment calls for imaging of large biological molecules such as proteins. Recently, Trimpin and co-workers showed that MALDI can also produce multiply charged ions for peptides and proteins with specific matrices and instrument conditions (Trimpin *et al.*, 2009).

Desorption electrospray ionization desorbs and ionizes molecules from the surface of the sample by utilizing a plume of highly charged solvent molecules typically produced from a modified traditional ESI source (Dill *et al.*, 2009). This method is energetically very soft, and is able to desorb and ionize a large range of molecules in a plant tissue sample without the use of a matrix (Mueller *et al.*, 2011). In typical DESI experiments, the size of the ESI plume limits the spatial resolution of the technique. More recently, a nano-DESI source has been developed that could improve the spatial resolution of this technique (Roach *et al.*, 2010). Additional molecules can be added to the solvent which can enhance the desorption/ionization processes for certain classes of molecules (Badu-Tawiah and Cooks, 2010). The DESI ionization source is capable of producing multiply charged ions, $[M+nH]^{n+}$, which effectively lowers the (m/z) values of the macromolecular ions. Desorption electrospray ionization sources are especially useful for ionizing polar compounds, but more hydrophobic compounds can also be detected using appropriate solvents.

Another ionization method that is capable of producing multiply charged ions is LAESI. This technique separates the desorption and ionization process into two distinct steps (Nemes and Vertes, 2007). First, infrared (IR) laser photons focused on the sample surface are absorbed by native water

molecules and create an ablation event. The ablated molecules, mostly cytoplasmic metabolites inside the tissue, are post-ionized by electrospraying with appropriate solvents. As in DESI, LAESI has the advantage of producing multiply charged ions, and the experiment can be performed in the ambient atmospheric environment. Laser ablation electrospray ionization has a unique advantage in that it can easily achieve depth profiling; each ablation event removes 30–40 μm of material from the tissue surface. Multiple acquisition of two-dimensional MSI data provides a means of stacking three-dimensional views of the distribution of analyte molecules (Nemes *et al.*, 2009).

Secondary ion mass spectrometry and most MALDI ion sources operate in a vacuum chamber, which leads to the loss of volatile compounds and complicates issues associated with sample handling due to the removal of water from the sample. The DESI and LAESI techniques operate exclusively at atmospheric pressure (AP) and can minimize sample handling and associated issues. Recently, an AP MALDI technique has been developed that allows sampling in an ambient environment; however, this approach suffers from significant ion losses during the transfer of the ions from the source to the vacuum of the mass analyzer. Detailed mechanistic studies are being conducted on the formation of the laser plume in AP, and the transportation of the resulting ions into the mass analyzer to improve the ion transfer efficiency (Schmitz *et al.*, 2010, 2011). Various ambient ionization methods have been developed or adapted for MSI (e.g. femtosecond laser ablation followed by electrospray ionization, Judge *et al.*, 2011; low temperature plasma ionization, Liu *et al.*, 2010; IR laser ablation followed by metastable chemical ionization, Galhena *et al.*, 2010b). Continuing developments and applications are expected in MSI with ambient ionization conditions.

Mass analyzers

The second main component in MS data acquisition is the mass analyzer. Many different commercial mass analyzers are available for MALDI MSI (Table 2). There are several important characteristics that need to be considered in terms of selecting the appropriate analyzer for MSI: (i) mass resolution and tandem MS (MS/MS) capability, which is important for confident identification of analytes; (ii) spatial resolution, which is important for obtaining high-quality images; and (iii) MS scan speed, which enables acquisition of the image in a reasonable time frame. Tandem MS experiments fragment a selected 'precursor' ion and subsequently collect a mass spectrum of the fragment ions. Information garnered from MS/MS or multiple MS/MS experiments (i.e. MS*n*) can be used to provide structural information about the precursor ion. There is no single instrument that satisfactorily meets all these criteria, and therefore it is inevitable that an experimenter will have to sacrifice one or more of these characteristics. For example, high-resolution mass spectrometers [i.e. Fourier transform ion cyclotron resonance (FTICR) or Orbitrap] typically have slower scan speeds (see Table 2).

The most widely used instrument for MSI is MALDI-TOF. The operating principle behind a TOF mass analyzer is relatively straightforward. Ions are pulse-injected into a flight tube, which is field-free. The velocity of the ions inside the tube (v) depends on the (m/z) values of the ions [$v = (2\ zV/m)^{1/2}$, where V is the accelerating voltage], and thus the corresponding arrival time of the ions at the mass detector is given by $t = L/v$, where L is the length of the flight tube. Mass spectra can be obtained by measuring the arrival time of each ion at the detector; ions with low (m/z) values arrive earlier, and ions with high (m/z) values arrive later. The MALDI technique interfaces very well with TOF MS because both are pulse-based (i.e. the laser pulse can be used for both ion production and initiation of time measurement). Another advantage of TOF for MSI is the scan speed. TOF MS is able to record a mass spectrum at unparalleled speeds (as fast as 100 Hz), reducing data acquisition time in MSI. Commercial TOF mass analyzers provide mass resolution up to 40 000 ($m\ \Delta m^{-1}$) in reflective mode, while maintaining fast scan speeds. Tandem MS experiments cannot be performed with a single TOF mass analyzer. To perform MS/MS experiments, a TOF/TOF or a Q-TOF hybrid mass spectrometer is required. In both cases an additional component, either another flight tube (in the case of TOF/TOF) or a quadrupole mass analyzer (in the case of Q-TOF), allows the system to select precursor ions for the subsequent fragmentation and fragment analysis in TOF-MS. Mass resolution for precursor selection is limited in TOF/TOF, but it offers the advantage of high-energy fragmentation, which is usually not available with other MS/MS instruments.

Ion-storage based mass analyzers have also been coupled with a MALDI source. These include an ion trap (IT) (Garrett and Yost, 2006), FTICR (Taban *et al.*, 2007), or Orbitrap (Landgraf *et al.*, 2009). In these types of analyzers, ions are stored inside the cell and their m/z values are determined by extracting individual ions (in IT) or through image current measurement (in FTICR and Orbitrap). Alternatively, the stored ions can be subjected to MS/MS analysis. Unlike other typical tandem mass analyzers in which selection of the precursor ion, fragmentation, and measurement of the (m/z) values of the fragments occur at different physical locations (MS/MS in space), MS/MS in ITs occur within the same space by sequential processes of precursor ion selection, fragmentation, and fragment spectrum acquisition (MS/MS in time). Excellent sensitivity and the ability to conduct multiple fragmentation MS cycles (i.e. MS*n*) are unique advantages of IT mass analyzers. Another very practical advantage of the IT technique, which has particular application in MSI, is the fact that there is almost no surface charging effect because low voltage is needed for ion

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extraction, so non-conducting surfaces (e.g. a glass slide) or thick tissue samples can be used in a MALDI IT.

The operation principles of Fourier transform (FT) type mass analyzers (i.e. FTICR and Orbitrap) are similar. Both analyzers trap ions in a cyclotron or orbiting motion inside a cell, either with magnetic or electric fields. Cyclotron or orbiting frequency is a function of (m/z), and Fourier transformation of ion current measured as a function of time provides the mass spectrum. Orbitrap and FTICR mass spectrometers provide ultrahigh mass resolving power, 100 000 or greater. However, this is at the expense of scan speed, which typically is 1 sec or longer. With a higher magnetic field, FTICR can perform mass analysis at a resolving power exceeding 1 000 000, but such analyzers store and measure ions with an even longer time. The slow scan speed makes imaging with FTICR challenging for large tissue samples, but it may be well suited for specialized applications. For example, the high mass resolving power of FTICR is able to distinguish ions with an m/z difference of only 0.001, even with extremely complex spectra (Kaiser *et al.*, 2011).

Most MSI data are obtained in 'microprobe' mode, sampling one small area of the tissue at a time, then moving the MALDI plate to the next spot. An alternative approach is 'microscope' mode (Luxembourg *et al.*, 2006; Harada *et al.*, 2009), where a large area is sampled simultaneously and the spatial information of the ions is preserved during the ion flight, and recorded simultaneously with a position-sensitive detector. This approach has several advantages. First, the spatial resolution is not limited by the probe beam size, but rather by the detector pixel size, ion optical magnification, and the perfection of the ion optics. These parameters can be easily controlled to provide spatial resolution down to a few microns. Second, data acquisition is much faster, because data can be simultaneously acquired from hundreds or thousands of pixels. Currently, several technical barriers limit the wide application of this data acquisition mode. First, only TOF or magnetic sector type mass separation allows the preservation of the original spatial information of the ions; hence, high-resolution MS or MS/MS is not possible. Second, it has a limited field of view (i.e. 80×80 pixels), which limits sampling to small areas only. In addition, the current detectors for microscope mode have a limited response time and dynamic range. After the removal of these limitations, this technique will find its niche in imaging applications where fast data acquisition and ultrahigh spatial resolution are needed.

Software for data analysis

There are currently two major issues regarding the software for analyzing MSI data. One issue is that each commercial instrument typically uses a unique software platform and there is no consensus on how to integrate image data obtained with different instruments. This can be especially problematic when a research group has multiple MSI instruments or when image data have to be exchanged with other research groups. A common format for MSI data has been developed for this purpose, imzML (http://www.maldi-msi.org) (Römpp *et al.*, 2011), but as yet it is not commonly used by researchers.

Another extremely useful goal of several software ventures is to extract common or previously unknown image features through automatic analysis of large data sets (Amstalden van Hove *et al.*, 2010; McDonnell *et al.*, 2010). Currently, most analysis of imaging data is performed manually, which is not only a time-consuming process but may also result in missing important image features. The automated analysis of large imaging data sets requires a huge computational resource, and their full utilization needs not only software developments but probably also developments in hardware.

TECHNICAL BARRIERS, PITFALLS, AND RECENT ADVANCES

Several barriers currently limit the full potential of MSI technology. There also are a few pitfalls that should be carefully considered for newcomers to MSI. Here we discuss some of these issues and recent efforts that have been made to overcome them. This discussion is primarily focused on MALDI MSI, but some of these issues are also relevant to other ionization techniques.

Tissue preparation

Sample preparation is a very delicate procedure and needs careful attention at each step. Primarily, it should be designed not to degrade the molecules of interest or alter their original physical distribution. Recent reviews provide thorough details of common sample preparation procedures (Chughtai and Heeren, 2010; Kaspar *et al.*, 2011). Although there are common issues with imaging technologies for peptides and proteins, adaptations are necessary for imaging of plant metabolites. Sample handling for MSI of metabolites is more difficult than for peptides or proteins because metabolites can be easily turned over metabolically, diffuse from the sample, or be removed during sample preparation. For example, lipids can be easily removed with the use of organic solvents; this is of particular significance because organic solvents are often used in preparing biological samples for localizing peptides and proteins. The use of any 'washing' or 'rinsing' steps in the sample preparation procedures risks the removal of small metabolite molecules from the sample, and is not usually recommended. Once harvested, plant tissue samples should be either freeze-dried (for cryo-sectioning) or immediately dried directly on the imaging plate in a vacuum desiccator to minimize metabolite degradation. The effect of histological tissue fixation is not well studied for plant MSI applications and the use of fresh tissue is advised. In a preliminary experiment

that applied MSI to the imaging of metabolites in cottonseed tissues, we (in collaboration with Professor Kent Chapman's lab at the University of North Texas, Denton, TX, USA) have found that fixing the tissue with paraformaldehyde minimized tissue tearing and preserved the original distribution of major lipids during cryo-sectioning. Polymer based materials (e.g. OCT polymer) that are commonly used in optical imaging cannot be used for most MSI applications because they diffuse into the soft tissues and generate significant contaminant ions and suppress other ions in mass spectrometric data acquisition. Ice and gelatin have been suggested as an alternative supporting material (Chen *et al.*, 2009; Chughtai and Heeren, 2010). In our experience, both ice and gelatin work quite well in cryo-sectioning of plant tissues, although slicing plant tissues with a thickness of less than 15 μm is somewhat challenging. Thicker tissue slices (>20 μm) can be used for MSI, except in the case of TOF-MS where good electrical conductivity is essential. However, extra caution is necessary as it takes longer to dry such thicker tissue sections and they tend to warp during the drying process.

A method that circumvents the difficulty associated with tissue preparation is indirect MSI, which imprints tissue molecules on to polytetrafluoroethylene (PTFE) or Teflon surfaces, from where they are analyzed by MS (Mueller *et al.*, 2011; Thunig *et al.*, 2011). This approach offers several advantages in that by using tissue cross-sections access to internal metabolites become available, and lengthy and cumbersome sample preparation procedures can be avoided. However, the loss of some high spatial resolution will be unavoidable during this procedure because disruption of cell integrity is inevitable as metabolites are probably 'smeared' during the imprinting process.

Spatial resolution and sensitivity

Spatial resolution and chemical sensitivity are among the most important parameters that define the quality of MSI data. However, these are two parameters whose optimization is mutually incompatible. Specifically, as the sampling size is reduced to achieve increased resolution, the number of molecules available on the sampling surface diminishes, reducing the sensitivity and eventually limiting the spatial resolution.

Spatial resolution is mostly determined by the size of the probing beam. In DESI applications, the probing solvent beam cannot be focused to a diameter of less than about 200 μm. Nano-DESI was recently developed, which appears to have a spatial resolution of approximately 12 μm (Roach *et al.*, 2010). During IR ablation in LAESI applications, tissue surfaces of approximately 300 μm diameter are sampled at a time (Nemes *et al.*, 2008). Recent development of etched tip optical fibers allows sampling of a small size of approximately 20 μm in LAESI (Shrestha and Vertes, 2009), but its application in MSI has not been reported. Ion beams can be focused to a very narrow beam size, 0.1 μm or smaller. Thus, SIMS imaging has been applied to image subcellular structures (Roddy *et al.*, 2002; Piehowski *et al.*, 2009). However, limited sensitivity due to low ionization yield and in-source fragmentation has often prohibited chemical imaging, with the exception of abundant membrane lipids. Many new advances are currently being made, such as 'matrix enhanced' SIMS (ME-SIMS) (Adriaensen *et al.*, 2005; Fitzgerald *et al.*, 2010) and polyatomic ion beams (e.g. C60, Baker *et al.*, 2006; or Ar_n (n = 60 – 3000), Rabbani *et al.*, 2011); however, a significant breakthrough in sensitivity is needed for wide biological applications.

Currently we consider MALDI MSI to be the most promising technology for high-resolution MSI. Laser beam can be reduced to a size of approximately 1 μm, albeit sensitivity could be a serious bottleneck. In application to plants, the smallest spatial resolution that has been achieved is about 10 μm (Hoelscher *et al.*, 2009; Jun *et al.* 2010b). This is primarily due to the combination of limited sensitivity and the difficulty of producing a smaller beam diameter with commercial instruments. However, there are opportunities to overcome these limitations and achieve resolution down to about 1 μm; for example, 5-μm resolution has been achieved with mouse tissues (Römpp *et al.*, 2010).

Figure 2 demonstrates how spatial resolution and ion signal can influence the quality of MSI by comparing three images of the distribution of the C29 alkane on the surfaces of an Arabidopsis flower obtained with different laser beam sizes and data acquisition methods. The images compare the resolution obtained using laser beam sizes of 100, 50, and 12 μm, and also explore the use of an oversampling method to increase the effective resolution obtained with the larger laser beam. The oversampling method allows higher effective spatial resolution (of 50 μm; Figure 2a) with the 100-μm laser beam. In this method the beam spot is moved in small raster steps (smaller than the beam diameter), and ideally if all molecules present at each sampled spot are removed only the 'new area' that is sampled in the next raster step will contribute to the image, thus enhancing the resolution of the resulting image. However, in reality there

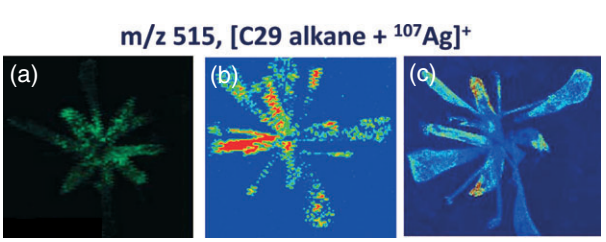

Figure 2. Distribution of C29 alkane on organ surfaces of an Arabidopsis flower.
(a) Spatial resolution of 50 μm with 100-μm laser beam using the oversampling method.
(b) Spatial resolution of 50 μm with 50-μm laser beam.
(c) Spatial resolution of 12 μm with 12-μm laser beam.

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always are some tailing laser beam profiles that will deplete some of the molecules in the next spot, particularly with the multiple laser shots needed to remove all molecules at each spot, degrading the image quality. This concept is illustrated in Figure 2a,b, both obtained with 50-μm spatial resolution but one was obtained with an actual beam of 50 μm (Figure 2b) while the other was obtained with the oversampling method using a 100-μm beam (Figure 2a); the image obtained with the actual 50-μm beam shows a much better quality. For example, the stamens are very thin and barely seen in Figure 2a, while they are clearly seen in Figure 2b. Image quality is further improved by using the smaller laser beam size (12 μm) (compare Figure 2c,b), and one can readily observe the fine structure of the anther and single pollen grains at the tip of the carpel in Figure 2c. Major surface lipids in Figure 2c have ion signals of 10^4 counts or higher on a single pixel (12×12 μm) mass spectrum, indicating that sufficient sensitivity is obtained even with this small sampling size. If the sampling size becomes too small and there are not enough ion signals on each pixel, the image quality will start to degrade.

Chemical identification

Another obvious limitation in MSI, compared with other metabolite profiling technologies [e.g. gas chromatography (GC)-MS or liquid chromatography (LC)-MS], is the absence of chromatographic separation, and accordingly lack of confidence in the chemical identifications. A common approach to circumvent this issue is in-parallel GC- or LC-MS analysis of homogenized tissue or extracted metabolites. However, one should keep in mind the MSI samples only very thin surface layers (<1 μm in MALDI) and there might be significant difference in the sample composition or concentration between the surface and bulk analysis. Moreover, isobaric ions or structural isomers can further confound chemical identification, and although they can be separated via chromatography that is not possible in typical MSI. In general, chemical identification in MSI is inherently more difficult than in metabolite profiling, and there is probably no best practice to overcome all these limitations that will fit every application. Several approaches will need to be used in combination to increase the confidence in the identification of unknown metabolite assignments.

A few MS approaches have been developed to improve the confidence in identification of compounds in MSI. Accurate mass filtering, i.e. MS image production with very narrow mass tolerance (e.g. ±0.01 Da), can be performed for the data set acquired with high-resolution mass spectrometers to separate isobaric ions. For example, C29 alkane and C28 aldehyde have a mass differences of only 0.036 Da, but they were successfully imaged separately in MSI with an Orbitrap (Jun*, et al.* 2010b). Tandem MS imaging provides an additional dimension of separation and can selectively produce a chemical image of interest, even allowing for separation of structural isomers (Perdian and Lee, 2010).

Recently, a completely different dimension of separation, based on gas phase ion mobility, has become available in MSI using ion-mobility separation (McLean *et al.*, 2007). For example, this approach can separate peptide ions from lipids in MSI, utilizing their slight difference in gas phase sizes (McLean *et al.*, 2007). Despite the potential of 'gas-phase ion chromatography' for MSI, the current bottleneck to applying this new technique includes the low resolution in ion mobility and the limitation in drift time predictions. Recent development of differential-mobility separation, also known as field asymmetric ion mobility spectrometry (FAIMS), in DESI-MS might provide high specificity in MSI (Galhena *et al.*, 2010a); however, it is limited to 'targeted analysis', as in MS/MS imaging, and data concerning the non-targeted ions are lost.

Data acquisition time

As technological advances are being made to increase the spatial resolution by sampling and gathering data with smaller and smaller beam sizes, data acquisition time has vastly increased and has become a serious bottleneck in high-spatial-resolution MSI. It is especially significant when both high-spatial- and high-mass-resolution MSI is attempted at the same time (i.e. HR^2MSI) (Römpp *et al.*, 2011). For example, if it takes 30 min to obtain an image with 50-μm spatial resolution, it will take 12.5 h to gather the same image with 10 μm spatial resolution; a daunting but still doable task. However, if one incorporates the additional time required to acquire high-resolution MS data (i.e. use of FTICR or Orbitrap), estimated at more than three times longer than for low-resolution MS, scanning of the same area will take 40 h or more. This is a near impossible task considering the long-term instability of laser beams and other instrument conditions. There is room for improvement in the near future, for example the pre-movement of the MALDI plate while data acquisition is being completed. The newly commercialized Q-Exactive Orbitrap allows MS scan speeds as fast as 12 Hz with mass resolving power of up to 140 000; hence, its MALDI version (not yet commercially available) may be 10 times faster than the current MALDI Orbitrap at similar or higher mass resolution.

Although mass resolution is not as high, TOF-MS is often a popular choice for MSI because of its high speed. Further improvements of mass resolution in TOF-MS could be another solution for HR^2MSI. Vestal has developed high-speed TOF-MS for MSI using a laser repetition rate of 3 kHz and continuous raster sampling; with such improvements an image of a rat brain section could be obtained in 10 min with an effective sampling rate of 30 pixels sec^{-1} (Spraggins and Caprioli, 2011).

As an alternative approach to reducing data acquisition time in HR^{2}MSI, we have recently developed 'multiplex MSI' using a linear IT-Orbitrap mass spectrometer (Figure 3) (Perdian and Lee, 2010). In this approach, each raster area is divided into a few spiral steps and MS data are acquired simultaneously with both spectrometers. While the Orbitrap scan (with slower scan speed, but at high mass resolution) is acquiring data from the center spiral position (1 in Figure 3a), multiple IT MS scans (with a faster scan speed, but at low mass resolution) are acquiring data from the other spiral steps (2–9 in Figure 3a). Because the two mass spectrometers are scanning in parallel (Figure 3b), there is a saving of 50% or more in data acquisition time. Essentially, high-spatial-resolution information can be extracted from low-mass-resolution IT data, and high-mass-resolution information can be obtained at low spatial resolution using Orbitrap (FT-MS), and significantly reducing total data acquisition time. In addition, we can replace some IT-MS scans with MS*n* scans to obtain the distribution of each structural isomer as illustrated in Figure 3d. In this example, heterogeneous distribution of structural isomers of the glycosylated flavonoids kaempferol-hexose and quercetin-rhamnose were distinguished using MS/MS scans, which were concurrently obtained with the IT and Orbitrap scans.

Quantification

Mass spectrometry is a quantitative analytical technique. Ion abundances in a mass spectrum are, in general, proportional to the analyte concentrations. Ionization efficiencies, however, can be greatly affected by many factors that often preclude quantification without appropriate standards that are ionized in the same conditions. This is an issue that is even more troublesome for MSI because (i) desorption efficiencies are also influenced by a variety of factors and (ii) tissue surface characteristics is another factor that influences desorption and ionization efficiencies. Hence, chemical images obtained by MSI should be regarded as semi-quantitative, and it is essential to normalize ion signals against matrix peaks or an endogenous internal compound(s) to minimize spot-to-spot variation.

Quantitative MALDI assays can be performed for biomolecules in complex biological extracts spotted on the MALDI plate (Bucknall *et al.*, 2002), which suggests the feasibility of quantitative MSI. To date, however, a good solution for correcting for different signal responses depending on tissue types has not been developed. Efforts have recently been made toward quantitative MSI, and most of these have focused on imaging drugs to replace or complement whole-body autoradiography (Wang *et al.*, 2005). One successful method is the co-analysis by LC-MS/MS of tissue extracts from adjacent tissue slices, and comparing these quantitative data with ion signals from MSI of the adjoining tissue section. Using this approach, Koeniger and co-workers successfully achieved linear correlation between ion counts in MSI and LC-MS/MS quantification for wide concentration range of a drug compound in rat liver sections (Koeniger *et al.*, 2011). However, this approach can be applied only for

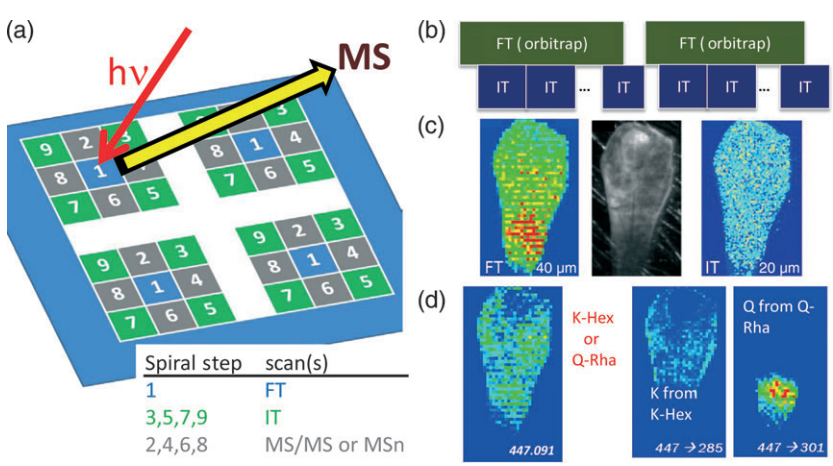

Figure 3. Multiplex mass spectromatry (MS) imaging to save data acquisition time while providing rich chemical information (Perdian and Lee, 2010).
(a) Illustration of multiplex MS imaging data acquisition method. Each raster step of matrix-assisted laser desorption ionization plate movement is composed of multiple spiral steps, nine steps in the given example; one Fourier transform (FT) scan at the center position and eight ion-trap (IT) scans [four IT MS scans and four multiple MS/MS (MS*n*) scans].
(b) A simplified workflow of the data acquisition method. The first set of ions from spiral step 1 is injected into the Orbitrap and the subsequent sets of ions are injected into the IT for MS or MS*n* scans.
(c) With a 10-μm spiral step size and 40-μm raster size, and the data acquisition scheme in (a), a FT image is obtained at 40-μm resolution and an IT image is obtained at 20-μm resolution.
(d) Differentiation of structural isomers of K-Hex (kaempferol-hexose) and Q-Rha (quercetin-rhamnose) using tandem MS imaging, which could not have been distinguished in accurate mass chemical image (right).

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the quantification of exogeneous compounds, and thus cannot be applied for the MSI of plant metabolites. Pre-spotting or spraying of internal standards on the tissue samples has been tested for quantification (Reyzer *et al.*, 2003; Hsieh *et al.*, 2006; Landgraf *et al.*, 2011). Finding signal differences of 2–10-fold between top deposition versus bottom deposition indicates that this method has the potential for further development and verification is needed (Chen *et al.*, 2007; Koeniger *et al.*, 2011).

The matrix

Application of the matrix is an additional step that needs to be considered in MALDI-based MSI. The choice of matrix and its application method need to be carefully considered as it may introduce artifacts in imaging data. Matrix crystal sizes should be smaller than the probing laser beam size to ensure homogeneity in ion signals. The crystal size of the matrix material is dependent on the type of matrix, the application method, and the tissue surface, and needs to be carefully optimized for each matrix material. Spraying of the matrix material introduces issues associated with the solvent; for example, solvent-based matrix application has the risk of re-localizing metabolites. In addition, the applied matrix material could crystallize as the solvent evaporates. Solvent-free methods for applying the matrix (sublimation, Hankin *et al.*, 2007; dried particle spraying, Puolitaival *et al.*, 2008), or spraying of small droplets (e.g. an oscillating capillary nebulizer, Chen *et al.*, 2008) have been developed to minimize these problems.

The optimum matrix is greatly dependent on the class of molecule that is being analyzed. Well-known examples include α-cyano-4-hydroxycinnamic acid for peptides, sinnapinic acid for proteins, and 2,5-dihydroxybenzoic acid (DHB) for most lipids or sugars. There have been many studies regarding the fundamental mechanism of the MALDI process (Zenobi and Knochenmuss, 1998; Karas and Kruger, 2003; Jaskolla and Karas, 2011) and attempts at the rational design of a matrix based on a statistical approach (Meier *et al.*, 2007) or Brönstead acidity–basicity (Shroff *et al.*, 2009); however, such a systematic approach has not as yet demonstrated the ability to cover the chemical diversity that is present in the plant metabolome. New matrices (Edwards and Kennedy, 2005; Astigarraga *et al.*, 2008; Taira *et al.*, 2008; Shrivas *et al.*, 2010) or new applications of old matrices (Franck *et al.*, 2009; Sugiura and Setou, 2009; Manier *et al.*, 2011; Shanta *et al.*, 2011) are still being continuously developed.

As an example, we illustrate the issues that one needs to consider in choosing a matrix, and the method for applying this matrix, by discussing the use of DHB as a matrix in MALDI imaging. 2,5-Dihydroxybenzoic acid is one of the best matrices for oligosaccharides and lipid molecules; however it suffers from the fact that it forms non-homogeneous, large crystals when air-sprayed (>100 μm). This can be overcome if DHB is applied by sublimation, in which case crystals of approximately 1 μm size can be routinely obtained (Hankin *et al.*, 2007). To further improve the reproducibility and minimize the tissue surface dependence, pre-coating of DHB on the MALDI plate before tissue mounting can also be considered (Manier *et al.*, 2011).

Another issue that needs to be considered is that most organic matrices produce a significant level of contaminating peaks in the low mass range (<700 Da), produced by the ionization of the matrix and related clusters, and these interfere or suppress the signals from low-mass metabolites. Several nanoparticle-based matrices (e.g. organic matrices coated on nanoparticles or colloid-based matrices, or ionic liquids; Chiang *et al.*, 2011) have been successfully used to overcome this limitation. A nanostructured surface as a matrix (as is used in nanostructure-initiator mass spectrometry (NIMS); Northen *et al.*, 2007) was also successful in overcoming this problem, but such substrates are not commercially available yet, and these methods require very thin tissue samples (≤5 μm). These nanostructured substrates have the additional advantage of no matrix crystal size effect.

While the use of a matrix in MSI increases the complexity of the technology because it introduces a number of issues that need to be considered, it offers several advantages with regard to altering the surface and gas-phase chemistry in laser irradiation, and specifically enhancing the desorption and ionization of certain compounds while suppressing others. Thus, the optimum matrix can be utilized to overcome some of selectivity and sensitivity problems associated with MSI. Namely, the selection of the appropriate matrix could dramatically improve the sensitivity of a particular class of ions of specific interest. This is particularly important for high-spatial-resolution MSI, at the level of single cells or subcellular localization, where very limited number of molecules are available in a very small sampling volume. For example, assuming that analytes occur in plant tissues in a concentration ranging between 10^{-3} and 10^{-6} M, and MSI is querying a 1 μm diameter area of the tissue to a depth of 0.2 μm, we will be sampling a volume that contains only 10^2–10^5 molecules for each analyte. Therefore it is important to optimize the chemical sensitivity of the mass spectrometer. The yoctomole level of sensitivity achieved by NIMS (Northen *et al.*, 2007) suggests that the choice of desorption and ionization matrix can be further optimized to detect such extremely small numbers of molecules.

Another advantage with the use of a matrix that has been often ignored is based on the fact that the matrix is typically present in large amounts compared with the analytes. When a laser beam is radiated to a spot to form the laser plume, matrix is the most dominant species both on the surface and in the gas phase. We expect that this 'matrix effect' will minimize the desorption/ionization difference between the different types of tissue surfaces. It has been well known in

SIMS that the surface characteristics can greatly influence ionization efficiencies (Jones *et al.*, 2007). However, such a dramatic result has not been reported in MALDI MSI. In fact, good correlation between ion-signals from MSI and LC-MS/MS has recently been reported in the relative abundance of phosphatidylcholine in rat brain sections, and this is regardless of tissue type (Hankin and Murphy, 2010). Further in-depth studies are needed to validate the promise of MALDI MSI as a method of choice for quantitative MSI.

MASS SPECTROMETRY IMAGING IN PLANT BIOLOGY APPLICATIONS

Only recently has MSI started to be applied to the solution of biologically relevant questions. This is primarily because it relies on sophisticated MS instruments that are currently within the purview of analytical chemistry groups. However, with the increasing success of the new imaging technologies, collaborations among analytical chemists and biologists are developing, and these are providing for increasingly sophisticated experiments to address specific biological questions. Initial efforts in MSI evaluated the ability of the technology to locate and measure the relative abundance of metabolite molecules at higher and higher spatial resolution. These studies that focused on establishing the technology concentrated on metabolites that are readily accessible for analysis and/or are highly abundant. For example, the imaging of surface lipid molecules that compose the cuticle layer of terrestrial plants (Sluszny *et al.*, 2005; Cha *et al.*, 2008, 2009; Vrkoslav *et al.*, 2010) and are therefore readily accessible for analysis, or imaging the distribution of highly abundant metabolites, for example glucosinolates that are abundant in the Brassicaceae (Shroff *et al.*, 2008). These types of studies have established that MSI technology has the resolving power to measure the location of metabolites to near single-cell resolution (i.e. approximately 10 μm). Using this technology, researchers have explored the asymmetric distribution of a number of different metabolites, including phospholipids in rice seeds (Zaima *et al.*, 2010), γ-aminobutyric acid in eggplant (Goto-Inoue *et al.*, 2010), carbohydrates such as cellulose, fructans (Jung *et al.*, 2010; Li *et al.*, 2010), and complex secondary metabolites such as diterpene glycosides (Jackson *et al.*, 2009).

The ability to image the location of molecules to individual cells is providing unprecedented data concerning the location of metabolites. With this demonstrated capability it is becoming important that the new technology is appropriately validated. Namely, does MSI truly represent the location of metabolites at their *in situ* location, and is the image response intensity linearly related to the abundance of the metabolite being imaged? To address these questions, researchers are using MSI technology to determine the location of metabolites whose distribution can also be measured via an independent technology having equal resolving power. These validation experiments are being conducted with metabolites that are inherently visible either in the UV–VIS region of the electromagnetic spectrum or are fluorescent and can be visualized by microscopy. Moreover, the location of many of these metabolites is known, and they accumulate in discrete, readily recognizable cellular structures. Examples, include flavonoids and anthocyanins which are colored (Cha *et al.*, 2008; Nemes *et al.*, 2008; Hoelscher *et al.*, 2009; Shrestha and Vertes, 2009; Korte *et al.*, 2011; Shrestha *et al.*, 2011), and hypericin, a polyketide that accumulates in discrete glands of certain *Hypericum* species (Hoelscher *et al.*, 2009).

Another validation strategy is to integrate genetically induced changes in the metabolome of the tissue and visualize this known difference, and further enhance the spatial resolution of the biochemical alteration of the genetic lesion via MSI. Examples of such studies include the changes in the surface lipids associated with the *eceriferum* mutants of Arabidopsis (Cha *et al.*, 2008, 2009; Jun *et al.*, 2010a), or changes in flavonoid profiles of Arabidopsis petals in *transparent testa* mutants (Cha *et al.*, 2008; Korte *et al.*, 2011), and in chimeric and variegated leaf tissues of the zebra plant (*Aphelendar squarrosa*) (Hoelscher *et al.*, 2009; Nemes *et al.*, 2009). The results of these types of experiments indicate that MSI technology accurately locates the distribution of metabolites, and that resolution is currently sufficient to visualize their locations to a single cell. However, whether the responsiveness of the imaging technology is directly proportional to the abundance of the metabolite is still challenging to determine; namely it is still unclear if the imaging technology is quantitative.

COMPARISON OF MS IMAGING WITH OTHER OPTICAL IMAGING TECHNOLOGIES

Optical imaging has long been a staple for biologists studying the morphology and structure of cells and their sub-compartments. Bright-field, dark-field and differential interference microscopies display physical features and are therefore non-specific at the molecular level. Fluorescence microscopy reveals some chemical information when the species are inherently fluorescent (Tan *et al.*, 1995) or if chemically specific labeling can be implemented (Hogan and Yeung, 1992). Still, the exact chemical components giving rise to the signals are often not definitively identified, since fluorescence labeling is based on chemical functionality and not the molecules as a whole. The exceptions are labeling via fluorescent antibodies or via genetically engineered co-expression of proteins (Griffin *et al.*, 1998). In contrast, MSI offers chemical specificity and typically does not require special sample modifications.

For the practitioner, MSI is preferred over optical imaging for several reasons. Mass spectrometry imaging offers obvious advantages wherever the exact chemical structure of the molecule is unknown. With high-resolution MS or

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MS*n*, the molecular structure can even be determined *a priori*. Optical imaging of metabolites (e.g. by fluorescence labeling) is often precluded because it involves manipulations of chemical samples, which are either not desirable or not feasible. In the former case, the local distribution and/or the concentration ratios of the molecules can be drastically affected by the sample manipulation. This includes the spatial diffusion of the molecules and, more importantly, the unavoidable biochemical response of the living cell to the introduction of the label. In the latter case, molecules without reactive functional groups, such as lipids, tend to be excluded due to the inability to chemically modify and tag these molecules. When a complex mixture of analytes is under investigation, MSI is suitable because it can simultaneously display a large number of molecular species, while fluorescence multiplexing is limited because of substantial spectral overlap. Finally, MSI offers the potential for a very high depth resolution to be achieved. For example, surface-desorbed molecules could in principle be resolved to a level of only a few molecular layers by MSI. In comparison, standard optical microscopy can resolve depths of around 1 μm (the Rayleigh range of visible light) and evanescent-field microscopes can resolve tens of nanometers.

On the other hand several issues disadvantage MSI relative to optical imaging. To date, for example, MSI cannot compete with optical imaging wherever temporal information is required. Optical imaging by standard charge-coupled device (CCD) cameras can easily achieve 30-ms time resolution (video rate) and can be pushed to the 1-ms range with sub-frame imaging. The limitation in MSI is from rastering of the sample to acquire data one pixel at a time even though the mass analysis proceeds much faster. Furthermore, if the sample needs to be placed in a vacuum, such as in MALDI, only a snapshot in time can be obtained. Mass spectrometry imaging in microscopic mode in the ambient environment brings the possibility of *in vivo* imaging with high temporal resolution. Yet the destructive nature of mass spectrometric data acquisition will significantly limit this application. Another limitation in gaining temporal information with MSI is the need for very high chemical sensitivity. Detectable quantities in MS are still a far cry from the single-molecule level of fluorescence (Lu *et al.*, 1998) or chemiluminescence (Kang *et al.*, 2010). The main barriers are the efficiencies of ionization after vaporization and the collection efficiencies from the laser-generated plume. We have already discussed the issue of high lateral resolution; optical microscopes can readily deliver resolutions of 200 nm, which is the diffraction limit of visible light, and in special cases even 50-nm resolution has been achieved. The in-plane resolution of MSI thus far is in the several micron range, partly because of sensitivity and partly because of the available optics for coupling into the vacuum chamber (the exception is TOF-SIMS imaging where, as discussed above, other issues such as in-source fragmentation and low ionization yield are limiting its wide application). Finally, there is the issue of sample preservation; MS is inherently a destructive technique while optical imaging can be non-intrusive. A corollary is that multiple measurements cannot be made on the same sample to enhance the signal-to-noise ratio in the MS image.

SUMMARY

Mass spectrometry imaging is a rapidly growing technology, and its development has been mostly driven by the need in the biomedical sciences for biomarker discovery and drug localization. As the technology is becoming increasingly accessible, it has opportunities to make an impact in plant biology, particularly for the localization of metabolites at high spatial resolution. Currently, there are still many challenges to overcome in order for this technology to become widely adapted. We foresee many exciting developments and applications that will be made in the next few years and it will become one of routine biochemical tools by the end of the decade.

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