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REVIEW

Future technology insight: mass spectrometry imaging as a tool in drug research and development

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In pharmaceutical research, understanding the biodistribution, accumulation and metabolism of drugs in tissue plays a key role during drug discovery and development. In particular, information regarding pharmacokinetics, pharmacodynamics and transport properties of compounds in tissues is crucial during early screening. Historically, the abundance and distribution of drugs have been assessed by well-established techniques such as quantitative whole-body autoradiography (WBA) or tissue homogenization with LC/MS analysis. However, WBA does not distinguish active drug from its metabolites and LC/MS, while highly sensitive, does not report spatial distribution. Mass spectrometry imaging (MSI) can discriminate drug and its metabolites and endogenous compounds, while simultaneously reporting their distribution. MSI data are influencing drug development and currently used in investigational studies in areas such as compound toxicity. In *in vivo* studies MSI results may soon be used to support new drug regulatory applications, although clinical trial MSI data will take longer to be validated for incorporation into submissions. We review the current and future applications of MSI, focussing on applications for drug discovery and development, with examples to highlight the impact of this promising technique in early drug screening. Recent sample preparation and analysis methods that enable effective MSI, including quantitative analysis of drugs from tissue sections will be summarized and key aspects of methodological protocols to increase the effectiveness of MSI analysis for previously undetectable targets addressed. These examples highlight how MSI has become a powerful tool in drug research and development and offers great potential in streamlining the drug discovery process.

Abbreviations

11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; ADME, absorption, distribution metabolism and excretion; DESI, desorption electrospray ionization; MALDI, matrix-assisted laser desorption ionization; MSI, mass spectrometry imaging; PD, pharmacodynamics; PK, pharmacokinetic; SIMS, secondary ion mass spectrometry; WBA, whole-body autoradiography

Tables of Links

TARGETS
Enzymes
Prostaglandin E synthase 1 (mPGES1)

LIGANDS
Imatinib
Oxaliplatin
Paclitaxel
Tiotropium

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

The goal of pharmaceutical research is to develop new chemical entities that are efficacious, but also safe. In recent years, significantly fewer new drugs have been licensed, with escalating costs of research and development (Scannell *et al.*, 2012). Notably, many compounds fail later in development, incurring substantial expense. Although technological advances have streamlined the discovery and development of new therapeutics, some important fundamental aspects of monitoring the fate of drugs *in vivo* remain inadequately addressed, contributing to high attrition rates.

Early knowledge of tissue distribution of drugs and their metabolites is extremely important in understanding pharmacological responses [pharmacokinetics (PK), pharmacodynamics (PD), drug transport, toxicity], and in predicting undesirable off-target effects (safety, drug–drug interactions) (Lanao and Fraile, 2005). While the physico-chemical properties of a compound (membrane permeability, protein binding, lipophilicity etc.) can be measured and modelled to predict biodistribution, tissue exposure has historically been inferred from surrogate measures such as concentrations of drug in plasma or tissue homogenates (Monro, 1990). While such methods enable high throughput screening during the discovery phase, reliance on circulating concentrations can prove erroneous when assessing tumour or blood–brain barrier penetration or highly localized delivery within multicellular tissues (Langer and Muller, 2004). Therefore techniques allowing histological assignment of drug distribution within tissue are required.

To date, drug distribution in tissues has been largely ascertained using whole-body autoradiography (WBA) or autoradioluminography. Such techniques detect radioisotopes integrated into drugs, typically ^{14}C or ^{3}H (Hahn, 1979); the radiolabel is quantified in dissected or whole animal tissue sections. However, as detection is based on the presence of a radioisotope, the techniques cannot distinguish parent drug from any metabolites retaining the label. Furthermore, during metabolism cleavage and ring scission may occur, so the radiolabel may no longer be associated with the phamacophore of the parent molecule and hence intensity of signal cannot be interpreted as a reflection of amounts of active drug. These factors restrict the value of WBA in predicting pharmacological and toxicological responses (Solon *et al.*, 2010; Castellino *et al.*, 2011). The associated cost in time and resources for synthesis also means that radio-isotopologues are not commonly generated during the discovery phase, but typically only once a drug progresses into development. Despite these limitations, the technique offers good specificity for quantifying 'drug-related material'. Its high sensitivity and large dynamic range make this approach the industry gold standard in compliance with the regulatory authorities (Solon, 2007). For most WBA studies, time-dependent appearance of drug-related material in tissue is quantified, with good spatial resolution (50–100 μm). Higher resolution (down to 0.05 μm) can be achieved using micro-autoradiography, even locating tracers between cells, but is technically challenging and not standardized (Stumpf, 2013).

When an antibody to the drug is available, immunostaining can be highly specific, but does not achieve high throughput, with typically only one analyte measured per histological section, albeit at high (near cellular) spatial resolution. Similarly, fluorescent tags allow highly specific visualization of one analyte with high spatial resolution (Giepmans *et al.*, 2006), although some dual-staining approaches exist. However, adding a fluorescence tag to the molecule may change distribution of the analyte into tissues. The use of clinical imaging techniques, such as PET and MRI to evaluate drug localization is very limited. (Artemov *et al.*, 1995; Aboagye and Bhujwalla, 1999; Glunde *et al.*, 2002; 2011; Giepmans *et al.*, 2006; Ackerstaff *et al.*, 2007; Glunde and Bhujwalla, 2007). MRI and PET target specific classes of molecules, but with low specificity and sensitivity. The spatial resolution of MRI varies from 1 cm^3 to 1 mm^3 (Zierhut *et al.*, 2010) and new PET instrumentation can achieve less than 1 mm^3 isotropic volume resolution (Lewellen, 2008). However, although these techniques are uniquely useful for *in vivo* imaging, it is again the time and resources required to generate probes or labels and lack of multiplexed analysis that limit the applicability of such techniques.

Mass spectrometry (MS) offers a label-free, multiplex alternative to evaluate the abundance of drugs in tissues, usually achieved by LC–MS/MS using sample homogenates. This commonly used technique can be quantitative, discriminates between parent and metabolites by mass and chromatographic retention time and is accepted by regulatory authorities. However, the main drawback of LC-MS/MS lies in the assumption of tissue homogeneity, which ignores the distinct compartments and functions within multi-cellular tissues (Mouton *et al.*, 2008). Mass spectrometry imaging (MSI) has come to prominence for PK/PD and toxicological studies by merging the benefits of the spatial resolution of autoradiography, with the specificity of tandem MS. In addition, concomitant assessment of spatial distribution of many analytes,

Table 1

Comparison of advantages and disadvantages of techniques assessing drug distribution

Methodology	Question answered	Advantages	Disadvantages
Autoradiography	Where and how much radioactivity?	Very high spatial resolution; reliable quantitation.	*Ex vivo*; requires radio-labelled drug; does not distinguish drug from metabolites.
Inmunohistochemistry	Where	Short processing time; easy interpretation; inexpensive	*Ex vivo*; requires antibodies, which vary in sensitivity and specificity; difficulties assigning; detection threshold; lack of standard scoring system
Fluorescence	Where	*In vivo* possible; reasonable cost	Not quantitative; poor resolution; autofluorescent interference
PET	Where, what and activity	*In vivo* possible; good resolution; can be coupled to CT X-ray, gamma camera	Expensive; short-lived isotopes; need cyclotron to produce isotopes
Coherent anti-Stokes Raman scattering microscopy (CARS)	Where and what	Label-free; sub-cellular spatial resolution	Not quantitative; poor selectivity; high background noise
Electrochemical atomic force microscopy (AFM)	Where and what	Label-free imaging; high resolution	Not quantitative; poor reproducibility; high background
MSI	Where and what	Multiplex; label-free imaging; good spatial resolution	Semi-quantitative; ion-suppression effects; complex analysis

CT, computed tomography.

across a wide mass range, means that both targeted (compound specific) and untargeted (toxicological investigations of biomolecules) studies can be routinely performed. Table 1 summarizes the relative merits of MSI versus other techniques.

Applications of MSI with a pharmaceutical focus are reviewed here, highlighting its benefits and associated technical challenges. The paper primarily addresses the most commonly employed MSI technology, matrix-assisted laser desorption ionization (MALDI), but reference is given to alternative methodologies coming online. Examples of applications for both exogenous and endogenous compound are presented to highlight its effectiveness to monitor both drug distribution and also the biological changes caused by drug target engagement or disease progression.

MSI

MSI owes its origins to the field of physics, where it was used to study semiconductor surfaces (Benninghoven and Sichtermann, 1978). The introduction of MALDI-MSI in 1997 by Caprioli (*et al.*) triggered significant development and optimization of methodologies, instrumentation, and software for bioanalysis. The technologies were rapidly applied to pharmaceutical biodistribution analysis to align molecular maps of drug distribution with histology (Stoeckli *et al.*, 2007; Heeren and Chughtai, 2010; Nilsson *et al.*, 2010), and are now making important contributions in fields such as molecular histology, drug distribution and proteomics (Fletcher *et al.*, 2011). The ability to localize compounds allows MSI to tackle questions relating to complex distribution patterns such as blood–brain barrier penetration (Liu *et al.*, 2013).

MSI can detect endogenous and exogenous analytes across a wide mass range, from low molecular weight drugs and endogenous compounds through to lipids and large proteins, directly from tissue samples (Pierson *et al.*, 2004; Skold *et al.*, 2006; Groseclose *et al.*, 2008; Castellino *et al.*, 2011; Parson *et al.*, 2012; Weaver and Hummon, 2013). Importantly, MSI can, by mass measurement, differentiate between drugs and metabolites formed by Phase 1 or 2 metabolism, overcoming the limitations of non-specificity encountered with autoradiography. In addition, as labelled compounds are not required, animals may be dosed with multiple compounds of different mass (cassette dosing) (White and Manitpisitkul, 2001; Smith *et al.*, 2007). This offers the combined benefits of increasing throughput of compounds in discovery, enabling study of combination therapies and reducing the numbers of animals required in research and development.

To successfully, accurately and reproducibly analyse the abundance and distribution of a drug directly from tissue sections, and for the resultant data to be acceptable to regulatory authorities, a robust workflow is required. Here, the steps in sample preparation, processing and analysis required for successful pharmaceutical MSI are briefly described, with reference to pertinent in-depth reviews. Consideration is also given to commonly available mass analysers and ionization techniques.

Principles and instrumentation

MSI is a multi-step process involving sample preparation, analyte desorption and ionization, mass analysis, and image registration (Figure 1A). Briefly, tissue cryosections are placed,

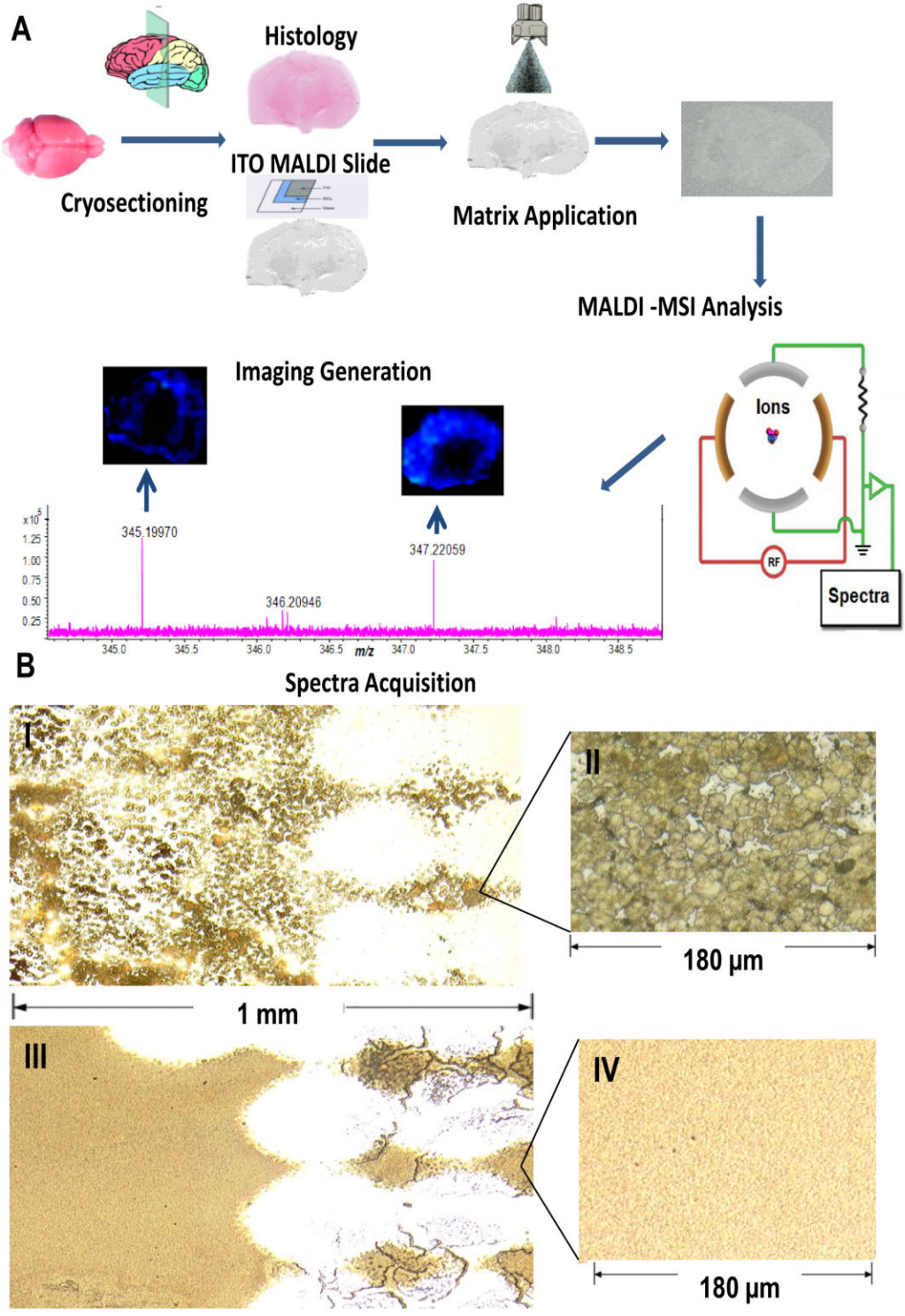

Figure 1

(A) MSI workflow. MSI involves sample preparation, analyte desorption and ionization, mass analysis and image registration. In brief, cryo-sections of tissue are coated with a suitable MALDI matrix and then introduced into an MSI instrument. A specified area of the tissue section is analysed and mass spectral information collected. The resulting ion distributions are presented as ion images. ITO, indium titanium oxide. (B) Matrix deposition by manual spraying and sublimation. Sublimation leads to deposition of smaller crystals (sub-µm) compared with a standard spray-coating, the latter achieving crystal sizes ~10–20 µm. Photograph (×25 magnifications) of a rat adrenal gland tissue section mounted on a conductive glass slide and coated with α-cyano-4-hydroxycinnamic acid (CHCA) matrix by (I) a pneumatic thin layer chromatography sprayer (2.61 mg, whole slide) (III) sublimation (2.74 mg, whole slide). Optical image of CHCA crystals (×40 magnification) (II) formed by spraying or (IV) sublimation.

commonly under vacuum, into an MS instrument and analyte molecules desorbed and ionized from the surface (Monroe *et al*., 2008). With ionization being performed from known positions, the collated mass spectra allow reconstruction of the distribution of molecules present, typically presented as 2D ion images showing relative abundance of selected molecular masses. The sample processing pipeline for MSI, while simple, offers multiple stages for optimization and modification (comprehensively reviewed, Goodwin, 2012).

Tissue preparation for MALDI-MSI analysis

Protocols for sample collection must ensure that histological integrity of tissue sections is retained so that spatial localization of target molecules is uncorrupted by analyte degradation/diffusion. Rapid proteolytic activity has been reported in tissue sections (Goodwin *et al*., 2008; 2012a). Put simply, the quality of MSIs are only as good as the starting tissue section. To achieve the highest quality sections, the following steps need to be optimized for specific target tissues.

Preparation of tissue sections

MSI is commonly conducted using snap-frozen cryosections cut from dissected organs rapidly frozen in liquid nitrogen, rather than conventionally embedded/fixed tissues. For analysis of small drug molecules, processes that delocalize the analytes must be avoided, specifically lengthy soaking in fixative or washing with ethanol, although these processes may be compatible with analysis of structural molecules, for example proteins. Even so, proteomic analysis from formalin-fixed paraffin-embedded tissues is fraught with difficulty, involving multistage approaches to reverse cross-linking and remove paraffin (Groseclose *et al*., 2008; Djidja *et al*., 2009; Casadonte and Caprioli, 2011).

Consecutive tissue sections for traditional histology are commonly collected allowing MSI and histology images to be aligned. If the morphological assessment is to be performed using the MSI section, histological stains used must be compatible with MSI, such as methylene blue or cresyl violet (Chaurand *et al*., 2004), that is stains not causing ion suppression. 'Ion suppression' is a common problem encountered in biological MS, whereby high abundance analytes (e.g. fixatives, embedding agents) ionize preferentially to low abundance species (drug of interest), depleting the signal and swamping mass analyser. Haematoxylin and eosin staining can also be performed after MSI analyses by washing off the MALDI matrix (described later) with a suitable solvent, as many histological features remain intact (Chughtai and Heeren, 2010).

Tissue sections are usually cut at 10–20 μm, comparable with the thickness of a mammalian cell, so that the majority of cells are cut open. This allows MALDI matrix to co-crystallize with cell contents. This depth is also suitable for histological assessment, although thicker than required for some techniques for example electron microscopy. In conventional histology, tissues are usually embedded using an optimal cutting temperature (OCT) polymer to allow easier and precise cryosectioning. However, the presence of OCT suppresses the MS ion signal (Todd *et al*., 2001). If samples cannot be mounted with just a small drop of water on the reverse side of the sample, then mounting on gelatine can be attempted. An alternative procedure using RCL2/CS100, a non-volatile and non-cross-linking fixative reagent introduced by Mange *et al*. (2009), can be used for embedding to permit precise cryosectioning.

For some instruments, the sample must be ionized from a conductive surface; a metal plate, a metal-coated microscope slide or adhesive double-sided conductive tape may be used (Schwartz *et al*., 2003; Crecelius *et al*., 2005). Cell debris and salts from the tissue surface (Todd *et al*., 2001) that can cause ion suppression or formation of unstable adducts can be removed by washing. Several solvents have been used, ethanol being preferred because of its fixative dehydration properties (Lemaire *et al*., 2007). This approach is suitable for proteins or structural components of tissue, but may lead to the removal of small molecules, particularly hydrophobic ones (e.g. steroids). Therefore, the washing step is target compound-dependent and usually avoided for small molecule analysis unless optimized specifically (Shariatgorji *et al*., 2012). Sections should be stored at –80°C. Prior to matrix application, samples should be dried under vacuum.

Matrix deposition

The role of the MALDI matrix is to absorb laser energy in the MALDI source, leading to the explosive desorption of analytes (often neutral species) held within matrix crystals, into the gas-phase without significant degradation (Karas, 1996). Uniform matrix application plays a critical role in the quality of reproducible, high-resolution MSI data and the process must be controlled to enable homogenous co-crystallization of the analytes from the tissue within the matrix on the section surface, without causing diffusion or disruption of morphology.

The matrix solution consists of: (i) an organic solvent (commonly methanol or acetonitrile), whose function is to rapidly extract target compounds from the tissue, allowing co-crystallization of the molecules of interest within the growing crystals of the matrix at the tissue surface (Amstalden van Hove *et al*., 2010); (ii) trifluoroacetic acid or another strong organic acid (when performing positive mode ionization) promoting the ionization of analytes by enriching the media with available protons; and (iii) an organic acid (matrix). Together these are chosen to achieve a suitable coverage capacity, homogeneity of crystallization and crystal sizes, suitable time of crystallization, manageable duration of analysis (in terms of vacuum stability), resistance to laser irradiation especially for high-frequency lasers, ionic yield in negative and positive mode and, where appropriate, enough fragmentation to use the post-source decay to collate structural information.

Those matrices most commonly used are in Table 2 (Heeren and Chughtai, 2010). In terms of ionization modes, the well-known proton donor matrices such as α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid promote ionization by forming proton cluster ions in positive ion mode. In negative mode, basic matrices such as 9-amino acridine (Hercules, 2002) and norharmane are good proton acceptors, favouring the formation of deprotonated analyte ions (Scott *et al*., 2014). The choice of matrix is influenced by the size of the analyte, as interference from the matrix by isobaric clusters/adducts in the same mass window can occur: most conventional matrices have molecular weights in the

Table 2

Matrices used to facilitate laser desorption ionization

	Abbreviation	Applications	References
Matrix for positive ion mode			
α-Cyano-4-hydroxycinnamic acid	CHCA	Therapeutic peptides and proteins; small molecules	Reyzer *et al.*, 2003 Cornett *et al.*, 2008
Sinapinic acid	SA	Large peptides and proteins (>10 kDa)	Meetani and Voorhees, 2005
2-(4-Hydroxyphenylazo)benzoic acid	HABA		Huwiler *et al.*, 2003
2,4,6-Trihydroxyacetophenone	THAP	Oligonucleotide mass < 3.5 kDa	Streletskii *et al.*, 2005
3-Hydroxypicolinic acid	HPA	Oligonucleotide mass > 3.5 kDa	Wu *et al.*, 1993
2,5-Dihydroxybenzoic acid	DHB	Therapeutic peptides and proteins; small molecules	Goodwin *et al.*, 2012a,b Gonnet *et al.*, 2003
Porphyrins	TPP	Small molecules	Ayorinde *et al.*, 1999
Fluorine C_{60}		Small molecules	Liu *et al.*, 2012
Matrix for negative ion mode			
9-Aminoacridine	9AA	Small molecules/lipids	Hercules, 2002
9H-Pyrido[3,4-b]indole	norharmane	Lipids and small molecules	Scott *et al.*, 2014
2-Mercaptobenzothiazole	MBT	Therapeutic peptides and small molecules	Zhou *et al.*, 2010
Titanium dioxide	TiO_2	Cyclodextrins	Chen and Chen, 2004
Matrix for dual mode			
1,8-bis(Dimethylamino)naphthalene	DMAN	Small molecules	Shroff and Svatos, 2009
Liquid ionics		Therapeutic peptides, small molecules and oligosaccharides	Crank and Armstrong, 2009
Solid ionics		Therapeutic proteins and big peptides	Crank and Armstrong, 2009
Nicotinic hydrazine		Oligonucleotides	Jiao *et al.*, 2014

same range as many drug-like molecules (100–300 Da). This issue can be overcome using high molecular weight (Ayorinde *et al.*, 1999), inorganic matrices (Chen and Chen, 2004) or novel deuterium-labelled matrices (Shariatgorji *et al.*, 2012).

Size of droplets and subsequent crystals formed by the different matrices influences the spatial resolution of imaging possible (Figure 1B). Matrix deposition can be performed as individual droplets (spotted) or as a homogeneous layer (coated). Coating can be performed, either spray-based (e.g. thin layer chromatography sprayer) or solvent-free (e.g. sublimation) depending on the spatial resolution that is required (Figure 1B). To limit diffusion of the analyte molecules during matrix deposition, alternative solvent-free matrices have been developed and successfully applied for small molecule MSI analysis (Goodwin *et al.*, 2010a,b). However, the main drawback of 'dry-coating' is lack of sensitivity because of poor extraction of analytes from the tissue (Goodwin *et al.*, 2011). The advantages and disadvantages of the most common deposition techniques are summarized in Table 3 (Kaletaş *et al.*, 2009).

MSI techniques

Many techniques are capable of performing MSI, with MALDI the most commonly used. MALDI is a development of laser desorption/ionization MS introduced in 1988 by Karas and Hillenkamp (1988). In traditional MALDI, the analyte is mixed with excess of chemical matrix at a molar ratio of 1:100–1000 (Dreisewerd, 2003), whereas for MALDI-MSI, the matrix is coated as an even layer as described earlier. MALDI is highly sensitive (attomole) and can ionize molecules across a wide mass range lending itself to applications from large biomolecules down to small molecular weight drugs, and as such, has been used for analysis of proteins, peptides, lipids and pharmaceutical compounds (Franck *et al.*, 2009). Best results are achieved with molecules with masses up to 25 kDa; imaging for proteins >25 kDa is challenging with few published examples (Kislinger *et al.*, 2005).

MALDI-MSI can detect multitudes of biomolecules generated as intact ions directly from localized pulsed laser spots; the laser beam is rastered across the matrix-covered tissue surface (Karas and Kruger, 2003). Matrix molecules absorb the laser energy, resulting in explosive desorption of matrix and analytes. The depth of ablation craters is estimated around 1 µm or more, depending on laser fluence (Knochenmuss, 2014). Although a traditionally slow process, the introduction of N_2 (337 nm) or neodymium-doped yttrium aluminium garnet (Nd:YAG) (355 nm) lasers with repetition rates of 200–5000 Hz and typical pulse lengths of 3 ns or less has shortened the data acquisition process (Dreisewerd, 2003; 2014). To attempt near cellular imaging, laser spot sizes have been reduced from >100 to <20 µm, with focusing to the

Table 3

Matrix deposition techniques

Technique	Advantages	Disadvantages
Acoustic multi-spotter	Uniform, fast, good reproducibility	Droplet application, limited spatial resolution
Electrospray deposition	Homogenous	Limited time for analyte matrix interaction
Pneumatic sprayer	Homogenous	Droplet size not constant
Image Prep®	Controlled conditions, automated, homogenous	Droplet size not constant, expensive
Dry-coating	Cheap, high purity matrix	Poor analyte–matrix extraction
Sublimation	Homogenous, reproducible, fast	Poor analyte–matrix extraction
Desktop inkjet printer	Uniform droplets (multichannel)	Slow, poor solvent compatibility, clogging

Table 4

Properties of mass analysers used in MSI

Analyser	MRP	Mass range	Detection	PAF (Hz)
Time of flight	10^3–10^4	0–300 KDa	Parallel	>10
FTICR-MS	10^4–10^6	20 Da–10 KDa	Parallel	>1
Linear ion traps	10^2–10^3	50 Da–5 KDa	Sequential	<10
Triple quadrupole	10^2–10^3	0–5 KDa	MRM/sequential	>100
Magnetic sector	10^2–10^3	0–5 KDa	Single ion/array	<1

FTICR, Fourier transform ion cyclotron resonance; MRM, multiple reaction monitoring; MRP, mass resolving power; PAF, pixel acquisition frequency; ToF, time of flight.

diameter of a single cell (~7 μm) reported (Holle *et al.*, 2006). However, although such improvements greatly enhance spatial resolution, they dramatically decrease sensitivity and are only suitable for highly abundant species (e.g. membrane lipids, Schober *et al.*, 2012; Anderson *et al.*, 2013).

Mass analysers

The ions generated by the approaches mentioned earlier are detected by their mass/charge ratios (*m/z*) and a range of mass analysers is available, each with benefits and limitations (Table 4; Jaroslav *et al.*, 2010). A single MSI experiment can yield multiple ion images within a narrow mass range of only a few hundred Da and therefore analysers with high mass accuracy and resolving power are desirable. This contrasts with tandem quadrupole systems commonly used for targeted analysis, but only resolving to unit mass [for implications of varying mass resolution, see Figure 2 (Heeren and Chughtai, 2010)].

The majority of MALDI imaging measurements are performed using time-of-flight (ToF) mass analysers because of their high detection efficiency and parallel detection, leading to high sensitivity. Ion-traps are gaining popularity, with three main trapped-ion mass analysers in existence; three-dimensional quadrupole ion traps (QIT) (Paul traps), Penning traps as used in Fourier ion cyclotron resonance (FTICR) mass spectrometers and Kingdon trap as used in the Orbitrap® mass spectrometers (Thermo Fisher, Palo Alto, CA, USA). All operate by storing ions in the trap and manipulating them using electrostatic, magnetic fields and radiofrequency in a series of carefully timed events (Douglas *et al.*, 2005). The traditional strategy for MALDI-MSI of pharmaceutical compounds is to perform the imaging experiment in MS/MS mode. All ions in the range of the targeted precursor are fragmented and the abundance of the target compound is determined from the measured intensity of one or more of its structurally significant fragment ions. High-performance instruments such as FTICR-MS offer new strategies not reliant on fragment ions, as their superior accurate mass resolving power allows identification by elemental composition (Grange *et al.*, 1996; Tolmachev *et al.*, 2006). Subsequent MS/MS measurements can be made at discrete pixel locations by complementary techniques to add insight into molecular structure and to determine if multiple isomers contribute to the image. This is only possible if each isomer has a unique MS/MS fragmentation pattern.

Recently, Bruker has introduced continuous accumulation of selected ions (CASI®) (Fuchser *et al.*, 2014), a technique showing very promising results with an increase in sensitivity up to 10 times in comparison with the broadband acquisition mode. This continuous mode of operation successfully separates the targeted ions (e.g. drug, metabolite, etc.) from the intense chemical background generated from the tissue, thereby lowering the limit of detection for the targeted species.

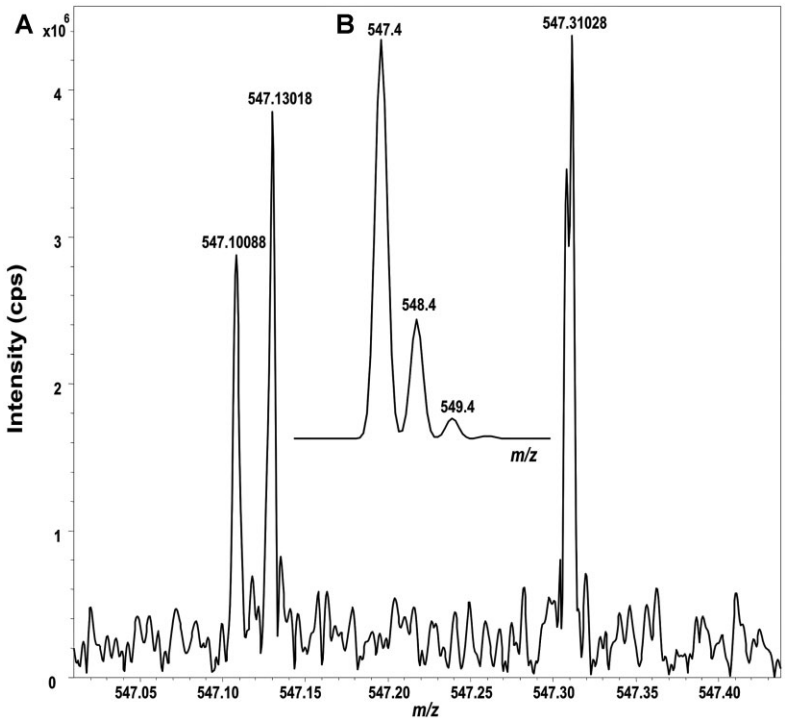

Figure 2

High-resolution versus low-resolution MS. High mass resolution is particularly important in tissue imaging because of the presence of many background ions. High resolving power allows distinction of low molecular weight analytes at low concentration from isobaric matrix interferents. Mass spectra of endogenous metabolites in a section of mouse brain, collected by (A) Fourier transform ion cyclotron resonance MS (FTICRMS) with high mass resolution, showing a mass window of 0.45 Da, with three distinct ionic species clustering at *m/z* 547 or (B, inset): a quadrupole MS with low mass resolution, showing a mass window of 6 Da; the same ions at *m/z* 547 cannot be distinguished. cps, count per second.

Data processing from spectra to pixels

Specialized software programs, including Biomap® (Novartis, Basel, Switzerland) and FlexImaging® (Bruker Daltonics, GmbH, Bremen, Germany), generate distribution images of ions of specific masses from MSI data. Data from each laser spot on the irradiated tissue, contribute mass spectral information about the species desorbed (Caprioli, 2007), and the relative intensity of a specific target molecule is plotted by pixel, producing a molecular map. The maps can then be aligned with histological information, commonly using an adjacent tissue section.

Quantitation by MSI

MSI has been generally considered a qualitative method (Chen *et al.*, 2008), although fundamentally, the signal generated by MSI is proportional to the relative abundance of the analyte. The challenges to quantitation arise in robust sample preparation and homogenous matrix application as both factors influence signal intensity. Application of a calibration curve onto control tissue, processed and analysed concomitantly, is the simplest way to obtain quantitative information (Nilsson *et al.*, 2010). However, as ion suppression of analyte signal can change across a complex tissue because of varying regional composition, analysis may be improved by inclusion of an internal standard (e.g. stable isotopomer) within the matrix (Atkinson *et al.*, 2007; Kallback *et al.*, 2012). Using this approach, tissue-specific correction factors can be invoked (Hamm *et al.*, 2012), allowing correction of analyte signal in every pixel sampled. Currently, data generated by MSI are cross-referenced to a gold standard quantitative technique. Concentrations measured by LC-MS/MS in adjacent tissue sections following homogenization have been shown to correlate well with data generated by MALDI-MSI (Reyzer *et al.*, 2003; Hankin and Murphy, 2010; Nilsson *et al.*, 2012; Cobice *et al.*, 2013).

It is important to emphasize that there is not currently a requirement to know tissue concentrations of drugs or metabolites for regulatory submission. WBA is used during regulatory submissions, to agencies such as the FDA, to assess fetal exposure and to compare data of animal biodistribution to mass balance studies of absorption, distribution metabolism and excretion (ADME) of radio-labelled drug in humans. Therefore, MSI data collected during drug discovery and development is and will be used in an explanatory and exploratory way. Clinical MSI-based diagnostic approaches will require technique, instrumentation and database approval from regulatory agencies. While we cannot provide references for MSI-based methods currently achieving this status, aligned MS profiling and tissue-based assays are progressing into this area (Schafer *et al.*, 2009; Marko *et al.*, 2012).

Application of MSI in drug development

Application of MSI in biomedical science has expanded significantly since first described by Caprioli *et al*. in 1997 with many diverse publications ranging from disease pathology, single-cell analysis and drug distribution (Chaurand *et al*., 2001; Cornett *et al*., 2008; Schober *et al*., 2012). Here, examples are chosen to highlight the significant impact MSI has made to small molecule drug discovery and development.

Drug distribution

PK analysis to assess ADME of drug-like molecules in either animals or human is mandatory in new drug applications submitted for final approval by the regulatory authorities (Rohner *et al*., 2005; Rubakhin *et al*., 2005; Greer *et al*., 2011). The first publication of MSI to assess tissue distribution of small drug-like molecules was by Troendle (*et al*., 1999) in which the anti-neoplastic drug paclitaxel was imaged in human ovarian tumour. The spatial distribution of the parent drug and its endogenous metabolites was assessed; these species could not have been distinguished by WBA. Since then, several applications of imaging of drugs and their metabolites by MSI have been reported (Reyzer *et al*., 2003; Goodwin and Pitt, 2010; Prideaux and Stoeckli, 2012).

The report by Nilsson *et al*. (2012) clearly demonstrates the added value of MSI in PK analysis. *In vivo* transport of tiotropium (an anticholinergic bronchodilator drug) into rat lung was studied using MALDI-ToF MSI. Analysis at high spatial resolution allowed accurate localization of drug to specific histological tissue compartments, achieved by systematic point by point MS and MS/MS sampling at 200 μm intervals (Figure 3A). A recent extension of this work (A. Nilsson, unpublished), presented here shows tiotropium distribution monitored at near cellular resolution (20 μm rastering) at 6 and 120 min following inhalation (Figure 3). At the earliest time point, tiotropium was localized to the major airways and especially airway walls, whereas already at 120 min post inhalation, the drug is distributed more widely into the parenchyma. Co-registration of images from histologically stained sections with MSI data allows superimposition of drug distribution with endogenous markers of specific compartments such as bronchioles, blood vessels, airways and epithelial layers (Fehniger *et al*., 2014).

Localization of drugs in tumours by MSI has been particularly important in oncology research, demonstrating targeted delivery. MALDI-FTICR-MSI enabled the anti-tumour drug, imatinib, and its des-methyl metabolite to be localized in a mouse glioma (Cornett *et al*., 2008). Another anti-cancer agent, oxaliplatin, was imaged in rat liver, spleen and muscle (Bouslimani *et al*., 2010), and in another study, a pro-drug banoxatrone (AQ4N) and its active form were imaged by MALDI-MSI in lung tumour xenografts (Atkinson *et al*., 2007).

Lastly the technique has also allowed efficient assessment of brain penetration of drugs, for example clozapine distributes in rat brain and other tissues, including the lungs (Hsieh *et al*., 2006; Wiseman *et al*., 2008; Yanes *et al*., 2009), kidneys and testes (Goodwin *et al*., 2010a,b) in close agreement with WBA.

Investigatory toxicology

In contrast to targeted analysis of specific drugs, MALDI MSI also has great potential to reveal endogenous molecular changes associated with toxicological or pharmacological events (Lalowski *et al*., 2013) in a 'hypothesis–free' manner. The use for MSI for investigatory toxicology is highlighted by Nilsson *et al*. (2012) who studied biomarkers of renal damage following treatment with inhibitors of prostaglandin E synthase 1. The composition of renal crystalline deposits was identified when other bioanalysis methods failed. This untargeted MSI analysis aligned to histology also revealed molecular changes that co-localized to pathologically defined, damaged regions of the kidney (Figure 4). Indeed off-target effects of drugs may be predicted using whole-body MSI offering the possibility to localize both parent and metabolites in every organ within a whole-body section (Stoeckli *et al*., 2007). This technique permits comparison and cross-validation of quantitative WBA. Sample preparation of whole-body sections is challenging and has been optimized by Goodwin *et al*. (2010a) using conductive carbon tape for mounting the large specimen. Figure 5 shows an example of use of MSI during whole adult rat tissue sectioning of animals embedded in carboxymethyl cellulose.

Broadening the scope of MSI by on-tissue derivatization

Ionization of the drug/metabolites by desorption and ionization process is an absolute requirement for effective detection of compounds in tissue sections by MALDI-MSI. Careful matrix selection and solvent composition can help achieve the desired sensitivity; however, some drugs are recalcitrant to these processes, lacking chargeable moieties, for example many steroids do not readily ionize and are difficult to detect by MS.

Chemical derivatization is an extensively used strategy for improving the detection of poorly ionizable molecules like steroids for both atmospheric pressure chemical ionization and electrospray analysis in LC/MS analysis (Quirke and Van Berkel, 2001; Higashi *et al*., 2005). However, the exciting possibility of MSI in combination with on-tissue chemical derivatization (OTCD) is still in its infancy with few cases reported (Chacon *et al*., 2011; Manier *et al*., 2011). Chacon *et al*. (2011) demonstrated the use of OTCD with a low MW scavenger of levuglandins (LGs), 3-methoxysalicylamine (3-MoSA). LGs are highly reactive towards primary amines, forming adducts with proteins and DNA; a process linked to oxidative injury, inflammation and the progression of Alzheimer's disease (Boutaud *et al*., 2006). The study of the distribution of 3-MoSA on intact tissue by MALDI-MSI is impeded by matrix interference and low sensitivity. Derivatization of 3-MoSA with 1,1'-thiocarbonyldiimidazole (TCDI) resulted in a readily ionizable oxothiazolidine derivative. TCDI treatment of tissue from mice dosed with 3-MoSA allowed successful PK profiling of this drug in multiple organs.

A recent study by Cobice *et al*. (2013) applied OTCD in conjunction with MSI, to image ketosteroids, mediators of diverse cellular responses in stress, reproduction and metabolism and common pharmaceutical targets. The approach, using Girard T as a derivatization reagent (Wheeler and Rosado-Lojo, 1962), offered exciting advances in mapping

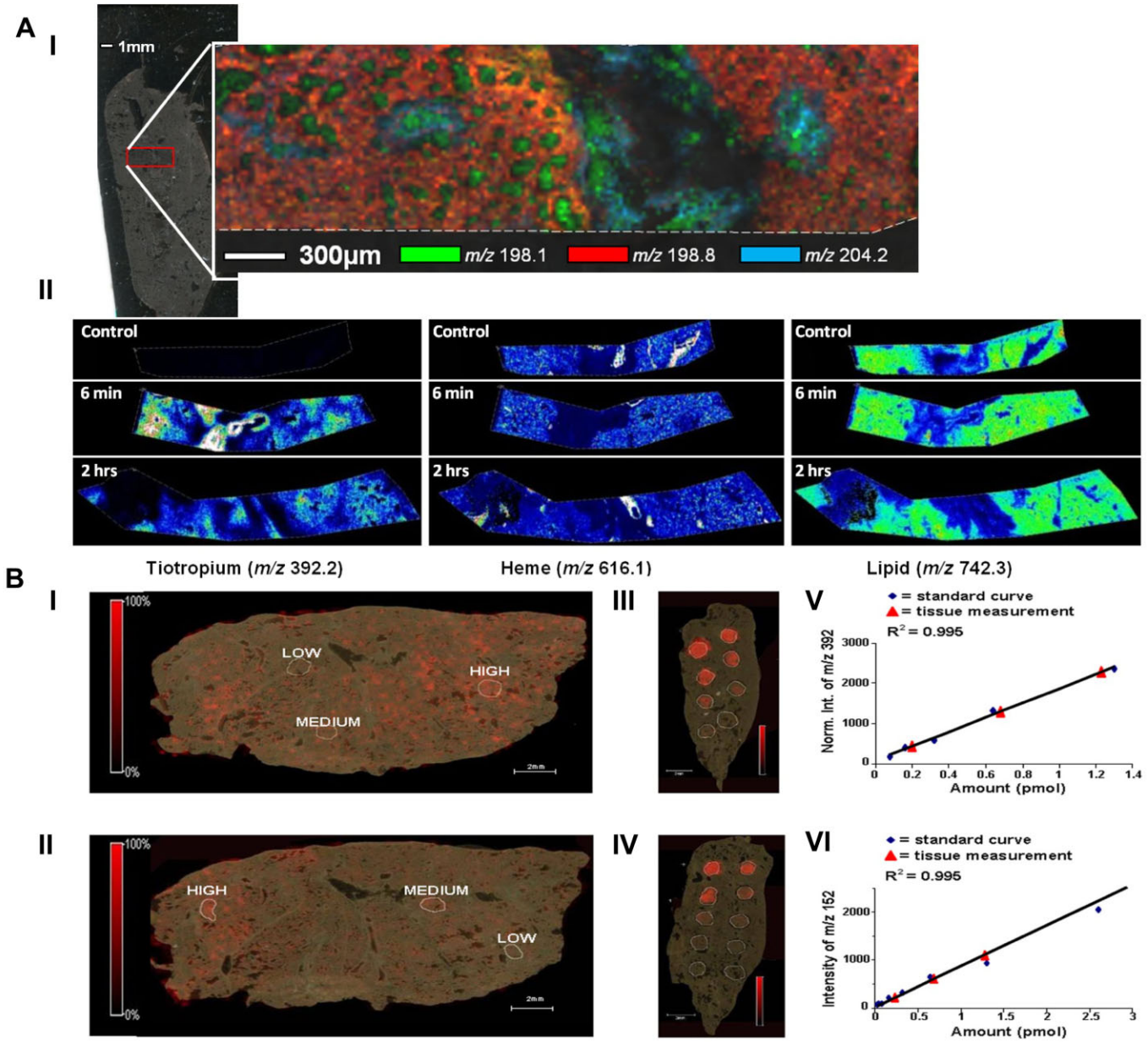

Figure 3

(A) Tiotropium distribution in lung analysed at 20 µm spatial resolution using MALDI-ToF/ToF. Optical insert shows area of lung analysed. (I) Multiple endogenous masses simultaneously displayed to produce a molecular histological images that helps define structure of tissue and airways, (II) masses associated with drug, haem (marker for blood) and endogenous lipid (marker for tissue) from tissues sections at 6 and 120 min post inhalation of drug. (B) Quantitation and analysis of distribution of tiotropium in lung tissue from rats dosed with the drug (I, II) by comparison with drug-standard samples spotted on control tissue (III, IV). Amounts measured by quantitative experiments performed in MS mode (I, III, V) matched very well with analyses in MS/MS mode monitoring the product ion of *m/z* 152 (II, IV, VI) even though sensitivity was greater using MS/MS. The abundance of tiotropium quantified by MSI also matched very well with amounts measured by conventional LC-MS/MS analysis of tissue extracts. Adapted from Nilsson *et al.* (2010).

the distribution of ketosteroids in rodent tissues, including brain and adrenal gland. Signal intensity was considerably increased (10^4-fold) after derivatization, allowing MSI to be applied during PK/PD analysis of an inhibitor of the enzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1). This enzyme generates active glucocorticoid hormones in brain and metabolic tissues and is an attractive target to protect against glucocorticoid-induced neuronal damage and metabolic disease, respectively, with ageing (Sooy *et al.*, 2010). Using MSI time-dependent alterations, the ratio of active to inactive glucocorticoids were demonstrated in brain and liver following dosing with a novel 11β-HSD1 inhibitor (Figure 6). Relative quantitation was achieved using ratio of substrate and product of the enzyme and the data correlated well with

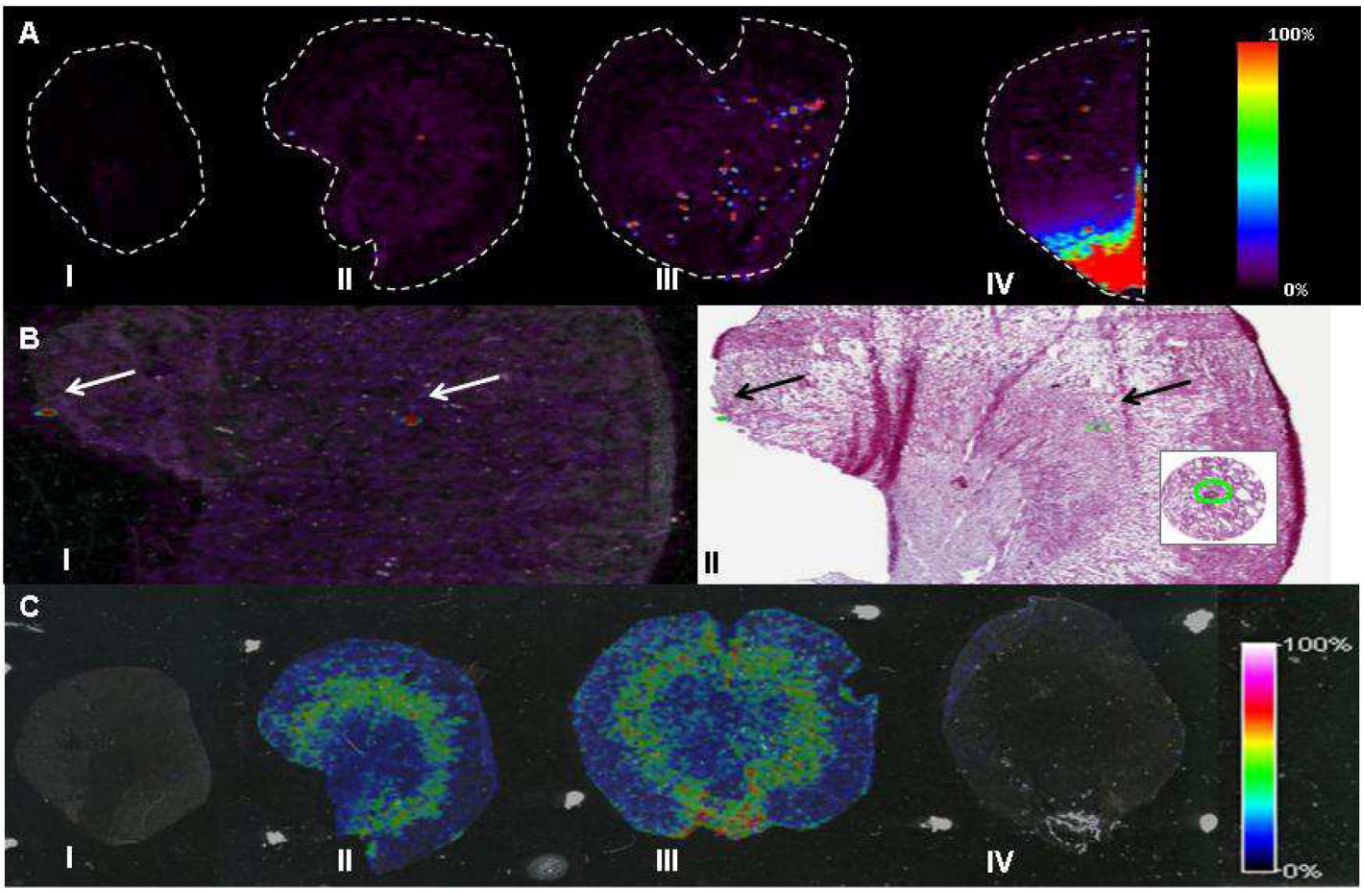

Figure 4

Detection of crystalline deposits in kidney by MALDI-TOF MSI. (A) Distribution of the drug metabolite, bisulphonamide at *m/z* 235.11. (i) vehicle control; (ii) tissue from animal receiving drug with low crystal load; (iii) tissue from animal receiving drug with high crystal load; (iv) tissue from animals receiving further drug with high crystal load. (B) (i) Localization of bisulphonamide matched the locations of crystals annotated by pathologists; (ii) sections stained with haematoxylin and eosin post MALDI analysis. (C) Un-targeted analysis by MSI revealed molecular changes associated with areas in the kidney described as damaged by the pathologists (i, iv) optical images; (ii, iii) endogenous metabolite *m/z* 437.31. Data were acquired at 100 μm spatial resolution. Adapted from Nilsson *et al.* (2012).

measurements by LC-MS/MS in whole-brain homogenates (Figure 6). Further OTCD approaches are required to broaden the spectrum of analytes currently readily detected by MALDI-MSI.

Future perspectives

MALDI-MSI is an emerging tool to enhance the understanding of the distribution of drug, metabolite and endogenous biomarkers directly in tissue sections. Its ability to co-localize drug/metabolite distribution with histological information has been transformational in drug development and can be achieved at meaningful levels of spatial resolution. The power to differentiate parent drug from metabolites concomitantly without the need for labels offers significant improvements over alternative bioassays and may help answer questions about access in target and off-target tissues early in the drug development process, highlighting potential toxicological problems. Despite the extensive use of MSI in pharmaceutical companies, the reported use of MSI for pharmaceutical research is limited as publication requires full disclosure of compound structures. Many papers report only method development using non-proprietary compounds (Prideaux and Stoeckli, 2012). While it may be many years before related publications appear, data from MSI experiments are significantly influencing industrial project decisions and it is only a matter of time before MSI experiments support regulatory submission.

However, before MALDI-MSI is accepted as a routine quantitative tool, issues such as regional ion-suppression, analyte or post-mortem tissue degradation and reproducibility of sample preparation still need to be addressed. Salt and lipid removal prior to the analysis of peptides and proteins simplifies the chemical environment and efforts in this direction continue (Lemaire *et al.*, 2007; Mange *et al.*, 2009). Unfortunately, these protocols are not suitable for many low MW compounds and other approaches such as heat stabili-

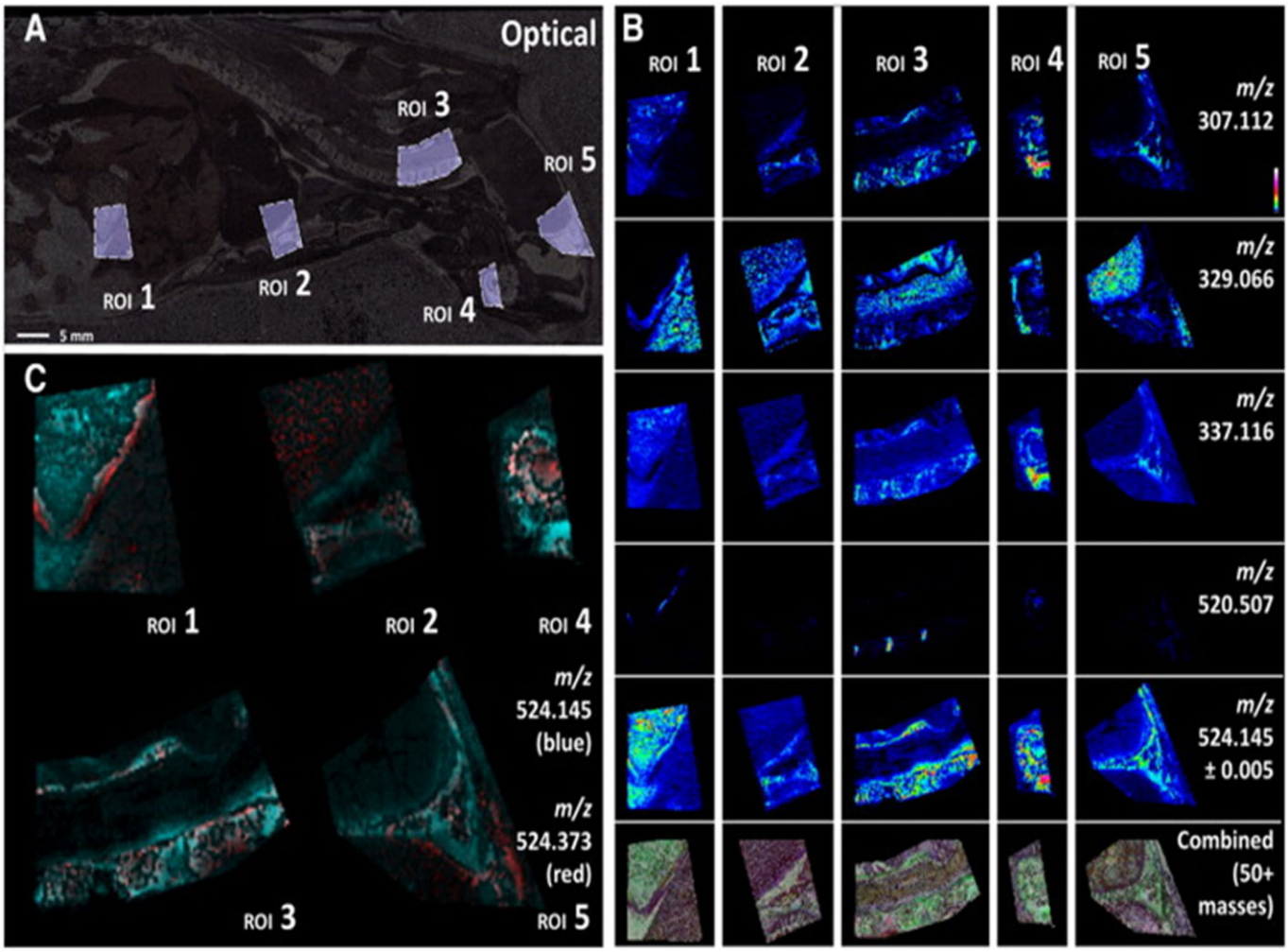

Figure 5

Whole-body MALDI-MSI using conductive adhesive carbon tape to maintain electric conductivity required for some mass spectrometers. High spatial (100 μm resolution) and spectral distribution mapping of a selection of regions of interest (ROI), 1 (abdominal area), 2–3 (thoracic area), 4–5 (head) from carbon tape mounted rat tissue section on conductive plastic, analysed by FTICR-MSI. (A) Optical image with ROI marked. (B) MSI distributions of multiple ROI for a range of endogenous compounds (C) multiple masses displayed simultaneously. Reprinted from Goodwin *et al.* (2012b), with permission.

zation are currently being explored (Goodwin *et al.*, 2012b). The problem of ion suppression is exacerbated with poorly ionizable molecules, where MSI is seriously hindered by poor limits of detection. For robust data, tissue preparation must be consistent and matrix coverage uniform. Conventional manual deposition techniques such as pneumatic TLC sprayer or artistic airbrush may lead to incorrect heat maps generation because of uneven deposition or hot spots. However, uniform coating can be achieved using the highly precise automatic matrix deposition systems now available.

In addition, industry and researchers will further develop and refine hardware and software to make MSI experiments more robust and easier to implement in high-throughput. Iterative technical improvements in ionization and mass analysers will increase the sensitivity and speed of MSI experiments. However, managing sample size and desired spatial resolution can be slow, requiring substantial computing resources. Dataset sizes can approach or exceed several gigabytes, with processing times reaching several hours. Nevertheless, if the mass range of the compounds and region of interest are known, the dataset may be vastly simplified. Because of these restrictions, it is often impractical to perform many MALDI MSI experiments and it can be advantageous initially to rapidly profile in lower mass resolution or low-density raster patterns to specify a region for confirmatory high-density imaging.

Limitations of spatial resolution also need to be addressed if cellular imaging is to be achieved. The alternative ionization methods, secondary ion MS (SIMS) and desorption electrospray ionization (DESI) may hold the key and are proving advantageous under specific circumstances depending on the target analyte and its abundance. Nano-structure initiator MS (NIMS) (Yanes *et al.*, 2009) and laser ablation electrospray ionization (LAESI) (Nemes and Vertes, 2010), among others, may also broaden the scope for application of the MSI in drug development. More detailed technical reviews have been

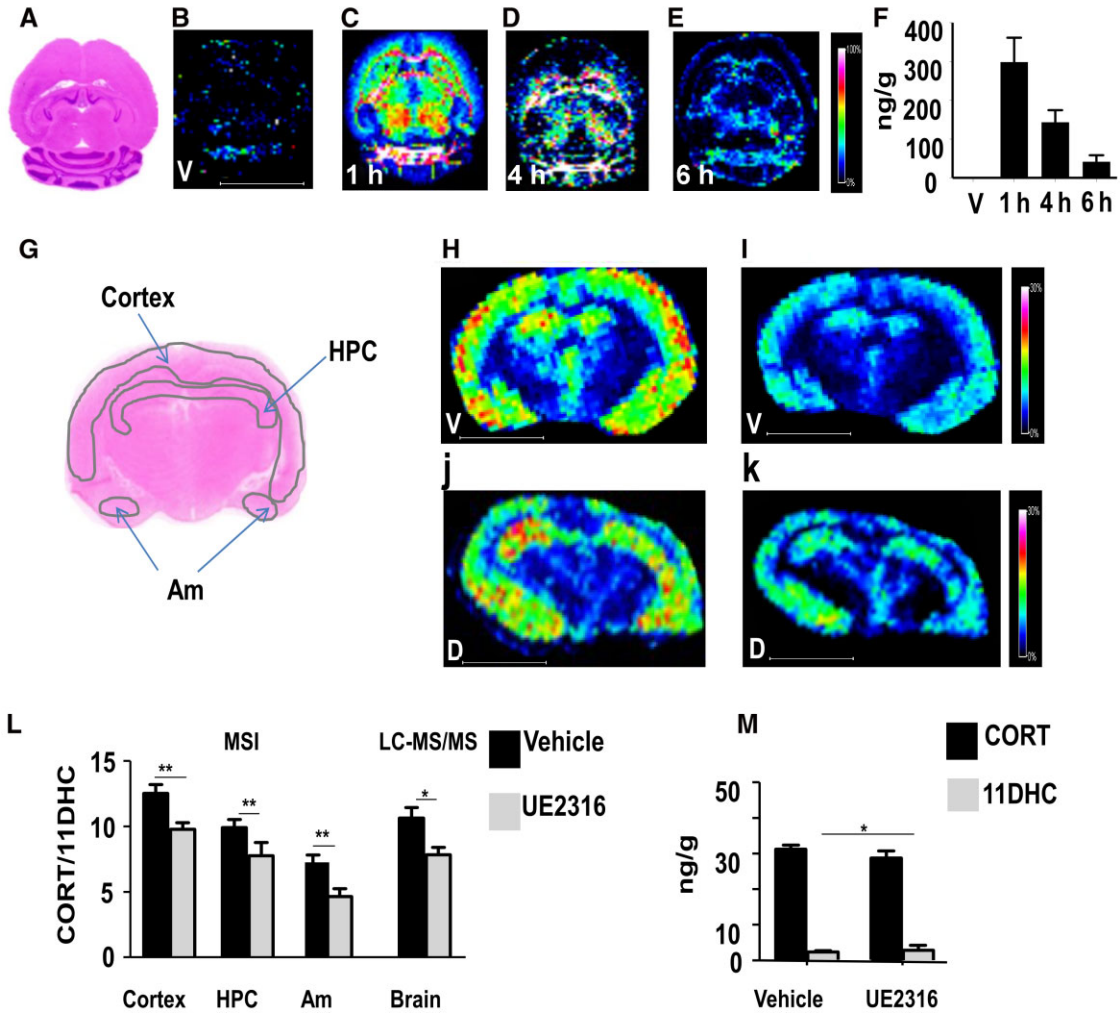

Figure 6

Effect of pharmacological inhibition of the enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), with a low MW inhibitor (UE2316) in C57BL6 mice. Girard T (GirT) derivatives [corticosterone (CORT, active steroid and enzyme product) and 11-dehydrocorticosterone (11DHC, inert metabolite and enzyme substrate)] were distributed across the cortex, hippocampus (HPC) and amygdala (Am) (A) Histological image of horizontal cryosection of murine brain stained with haematoxylin and eosin (H&E). (B–E) MSI heat map distribution of m/z 390.084 ± 0.025 Da representing UE2316 in brain over a 6 h time course in mice receiving vehicle (V) or UE2316. (F) Amounts of UE2316 in whole brain measured by liquid chromatography tandem MS (LC-MS/MS) demonstrating good agreement with MSI. (G) Histological image of coronal cryosection of murine brain stained with H&E showing the outline of the MSI regions of interest (ROIs) (cortex, HPC, Am). (H, J) MSI heat map of GirT-CORT at m/z 460.317 ± 0.005 Da brain from mice receiving Vehicle (H) or UE2316 (D; 1 h post dose) (J). (I, K) GirT-11DHC at m/z 458.301 ± 0.025 Da in brain from mice receiving vehicle (I) or UE2316 (K). Signal intensity is depicted by colour on the scale shown. Scale bar (2 mm). (L) A significant decline ($P < 0.01$, overall between groups) in CORT/11DHC ratios was observed across the ROIs by MSI in the brain after administration of UE2316, showing good agreement with data generated by LC-MS/MS in whole brain. Data are mean ± SEM; $n = 12$. *$P < 0.05$, **$P < 0.01$; two-way ANOVA for MSI and Student's t-test for LC-MS/MS. Reprinted with permission from Cobice *et al.*, 2013.

provided by Amstalden van Hove *et al*. (2010) and Vickerman (2011).

SIMS can achieve higher spatial resolution than MALDI without matrix application, hence reducing opportunities for diffusion. SIMS employs a primary ion beam (e.g. metal ions) to produce secondary ions from the sample surface (Jones *et al*., 2007), focussed as sharply as 50 nm, depending on the primary ion beam current, molecular weight, and its charge state (Altelaar *et al*., 2005). With higher spatial resolution and precision than laser-based imaging, SIMS offers exciting opportunities in molecular pharmacology (Nygren *et al*., 2007), for example, imaging of abundant elements and small organic molecules in cellular organelles (Altelaar *et al*., 2007). Ion suppression plays a minor role for absolute quantitation in comparison with MALDI. However, the main disadvantages are the energetic desorption process resulting in significant fragmentation, the mass range is limited to ions lower than 1000 Da and access to this technology is still very limited.

DESI, an atmospheric pressure ionization method developed by Cooks in 2004 (Takats *et al*., 2004), is derived from a combination of two MS ionization methods: electrospray and desorption ionization. Although improvements are still

required, it holds promise and is already being applied to study endogenous molecules and drug metabolites (Wiseman *et al.*, 2006). DESI uses energetic, charged electrosprayed solvent droplets to release the molecules from the surface (Takats *et al.*, 2004), offering the unique possibility of *in situ* and real-time analysis (Dill *et al.*, 2009). As a matrix-free technique, DESI does not suffer from matrix-analyte co-crystallization issues and serves as an alternative platform when compounds do not ionize in MALDI. DESI can routinely achieve at least 400 µm spatial resolution (Ifa *et al.*, 2007). However, a recent spray design theoretically predicts lateral resolution of 40 µm (Ifa *et al.*, 2007) and a new development in nano-DESI is said to resolve down to 10 µm (Laskin *et al.*, 2012). The VAMAS (Versailles Project on Advanced Materials and Standards) study of 20 laboratories from 10 countries showed that reproducibility of measurements could be achieved within 20%. However variability in spray and sample stage design played a significant factor in causing larger inter-laboratory differences (Gurdak *et al.*, 2014) and further work is required to improve the robustness of the technique. Reproducibility and robustness of any analytical technique is significant when undertaking multi-site analysis or if the study is performed over an extended period. However, emerging technologies can readily be utilized in standard investigatory studies where two samples are compared side by side. Findings can then be further validated with more stringent techniques.

To conclude, pharmaceutical companies are increasingly aware of the disconnection between outcomes in clinical programmes based on preclinical data. MSI can aid the refinement of pre-clinical studies and an increasing use of *in situ* MSI for analysis of clinical samples is predicted.

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Conflict of interest

The authors do not have any conflict of interest. The company employing R. G., P. E. and A. N. does not sell any of the devices or drugs mentioned in this paper.

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