The coming of age of MASS SPECTROMETRY IMAGING

Mapping complex drug distribution/retention in tissues, aligned with efficacy and safety biomarker localisation is now possible using label-free mass spectrometry imaging technologies. While there remain challenges to be overcome, these molecular imaging techniques are starting to impact drug discovery and development. They have the potential to evolve beyond predominantly pre-clinical applications and to impact upon clinical monitoring and decision making.

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A significant challenge within pharmaceutical R&D is changing the attrition profile suffered in late development. This is central to building a sustainable industry that will deliver meaningful therapeutics that can address significant unmet medical needs. Critical to this, is improved PKPD modelling of efficacy and safety biomarker data, from pre-clinical models through to the patient. Improving the ability of pre-clinical and early clinical data to predict outcomes in the target population, will impact both compound investment decisions and the design and selection of candidates. The weakness of this paradigm, which is currently being applied widely across the industry, is that the biomarker relationships are almost exclusively being derived from plasma drug concentrations, the underlying assumption being that drug free-concentrations are equivalent in plasma and tissues. While in many cases this is a valid approach, there are clear instances where these assumptions do not hold. Furthermore, there are also limitations to the common bioanalytical techniques employed to overcome potential disconnects between plasma and tissue concentrations (see **Table 1**).

Improvements in our ability to determine drug, metabolite, and of both safety and efficacy biomarker levels within tissues, specific regions of tissues and even specific cells and organelles, both from *in vitro* systems and *in vivo* studies will have a major impact on the efficiency of the drug discovery process.

The potential for mass spectrometry imaging (MSI) to enable access to spatially-resolved drug and biomarker information has been increasingly discussed over the last decade, since the technique started to be used in a small number of Pharma[1-4]. Recent developments in the performance and efficiency of MS imaging platforms are starting to allow the wider uptake and use of MSI in drug

Table 1: Summary of the complications in relating plasma and tissue concentration for certain bioanalytical assays

ANALYSIS PARADIGM FOR UNDERSTANDING TISSUE DISTRIBUTION	LIMITATIONS AND COMPLICATIONS
Plasma as surrogate for tissue concentration	1. Poorly vascularised tissue 2. Complication of blood brain barrier (eg CNS PKPD) 3. Local delivery (eg targeting nanomedicines)
Label based assays	1. Selectivity (tracking label not molecule) 2. Time and cost associated in producing labelled compound 3. Tagged molecules having different physio-chemical properties
Tissue homogenisation	1. Loss of all spatial information 2. Blood contamination 3. Dilution of low abundant but localised compounds

discovery a reality[5]. Importantly, data can now be obtained at a throughput that means projects can be influenced in meaningful timescales. This level of multiplexed spatial information, enabling assessment of drug disposition and effect, related to precise cell types and tissue morphology is unprecedented.

The technology

The essential process of MSI is to sequentially sample small areas of a tissue of interest, and by determining the constituents of each sampling position, reconstruct images of the tissue based on the abundance of the tissue constituents of interest. As with all 'measuring technologies', the key factors that determine the analytical utility are the specificity, sensitivity and speed of analysis. However, in the case of imaging technologies there is an additional factor that not only impacts on the above, but also greatly affects the information that can be derived from the analysis – the spatial resolution, or pixel size of the image. For MSI there is a high interdependency between sensitivity and spatial resolution. Each experiment will involve balancing the required spatial resolution with the sensitivity of the mass spectrometer. Less analyte will be available for detection as the area sampled is reduced to increase the spatial resolution of the final images.

The specificity of MSI derives from the resolving power of mass spectrometers to detect thousands of individual compounds in a single analysis. Mass spectrometers of sufficient power can identify compounds with a very high degree of certainty on solely the basis of the molecular mass. In addition, a target molecule can be induced to break apart and the resulting fragment molecular masses can be used to confirm identification of the original molecules. However, before becoming concerned with mass analyser sensitivity or specificity, analytes need to be transferred from the sample surface and into the gas phase (only molecules in the gas phase are detectable). There are a wide range of 'ionisation techniques' that can be employed that have different efficiencies for the range of chemotypes of interest, which may include elements, small molecules, lipids, peptide and even intact proteins. The ionisation method, to a large degree, also determines the spatial resolution. Currently there are three ionisation methods that lead the field, with the most widely utilised being matrix-assisted laser desorption ionisation (MALDI). This can enable detection of a wide range of analytes, from small molecules up to proteins, at a maximum spatial resolution of circa 5µm. An older method of sample ionisation is secondary ion mass spectrometry (SIMS) where a primary ion beam delivers the highest spatial resolution (within the nm range) but with a limited mass range. The newest method for sample ionisation is desorption electrospray ionisation (DESI). While this offers neither the highest spatial resolution (currently ~50µm) nor largest mass range (small molecules and lipids) it is proving effective in the breadth of endogenous analytes that can be detected.

Once the gigabytes of spectral data have been collected, analysis of the image will need to be performed. In the simplest form this is achieved by plotting the relative abundance of a selected mass at each sample position, generating a 2D map of

the distribution of the target molecule. Simple on-tissue calibration spotting can elevate the information generated from relative abundance to more quantitive data. The use of stable-labelled standard allows quantitation of endogenous compounds or more accurate quantitation by removing localised ion suppression effects. Basic image analysis is readily performed using instrument vendor software. More advanced image analysis can include co-registration of MSI data to traditional imaging modalities such as immunohistochemistry. However, additional value from MSI data can be obtained when multivariate statistical interrogation is performed, to better understand complex

CORE TECHNOLOGY	VARIATION	KEY ATTRIBUTES	POTENTIAL LIMITATIONS
SIMS (Secondary ion mass spectrometry)	TOF-SIMS	High spatial resolution medium spectral resolution SIMS platform Range of ion beam sources suitable for softer, larger molecule ionisation Applied to range of pharmaceutical applications	Sensitivity
	Nano-SIMS	Highest spatial resolution MSI platform Subcellular resolution Lower spectral resolution Most effective using labelled target High capital investment required More complex sample preparation required	Mass range
	3D Nano-SIMS	Newly developed system combining high mass resolving power with high spatial resolution Increased acquisition speeds Simple sample preparation	Under development
MALDI (matrix assisted laser desorption ionisation)	TOF/TOF	Higher speed and spatial resolution Small molecule to protein analysis Most widely available MALDI systems Requires matrix application but allows optimisation for drug or biomarker target	Mass resolution
	FTICR	Highest mass resolving power Slower data acquisition but ideal for identification of unknown lower molecular weight targets Typically most expensive MALDI system	Speed
	AP-MALDI	High spatial resolution Atmospheric pressure interface	Speed
	Ion mobility	Added resolving power but gas phase separation Separates by molecule cross section area rather than just on mass and change	Efficacy small molecules
DESI (Desorption electrospray ionisation)		Newest MSI platform Electrospray interface makes compatible with many mass analysers Increasingly applied to pharmaceutical research Effective at drug and endogenous target detection Speed and spatial resolution improving but not currently matching best performing MALDI No sample preparation following tissue sectioning	Speed and source robustness

Table 2: Summary of the core technologies for MSI analysis detailing major attributes and limitations

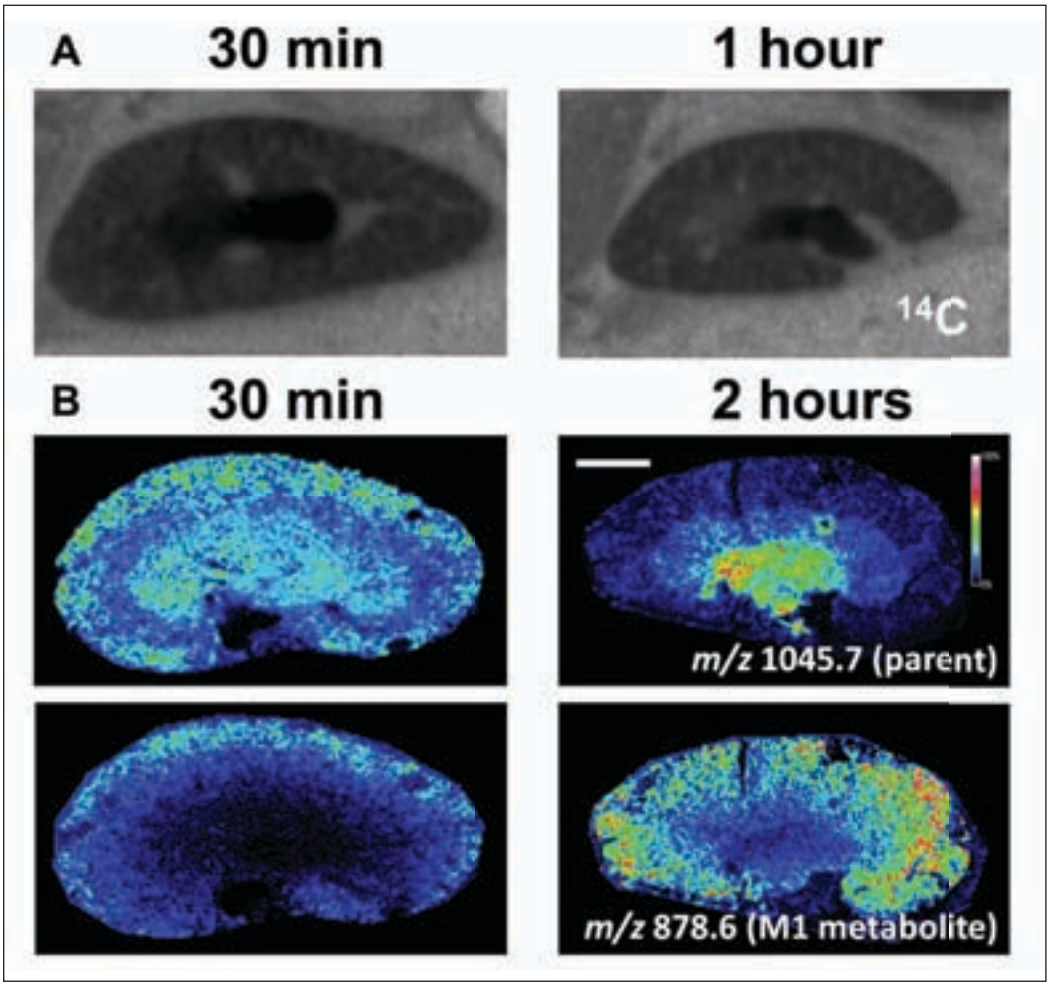

Figure 1
Matrix-assisted laser desorption ionisation mass spectrometry and autoradiogram images of distribution of AZD2820 in mouse kidney. Mass spectrometry imaging data collected at 50µm spatial resolution. Presented on heat map, scale inserted. Scale bar 2mm. Reprinted with permission from J. Biomol. Screen. 2016 Feb;21(2):187-93). Copyright 2016 Society for Laboratory Automation and Screening[6]

molecular interplay occurring at the cellular level. Such approaches generate new challenges, including increased data handling and processing, which can produce new bottlenecks in MSI analysis.

The opportunity

The recent advancements in key technologies (**Table 2**) mean that the contributions MS imaging makes to drug discovery/development projects is rapidly evolving. Ten years ago low spatial resolution (~100µm) images of administered compounds in samples were acquired over several hours before being manually aligned with optical images to describe the basic distribution of a compound. Some advocated that the primary use of MSI was as a label-free alternative to whole-body autoradiography, in which the broad distribution and retention across the body of drug and metabolites is assessed. Although the spatial resolution (~300µm), and speed of analysis (~24h) were consistent with this ambition, two key weaknesses – inherent to MSI – actually make this an inappropriate use of the technology. A WBA study is of most value when detecting tissues in which there is prolonged retention/accumulation of drug-related compounds, either due to covalent binding to, or high non-covalent affinity for, tissue constituents. The inappropriateness of MSI as an alternative, comes firstly from the fact that MSI is unable to detect covalently bound materials, and secondly that a well-designed WBA study is likely to be far more sensitive than MSI.

Therefore the role of MSI is not as a multiplex, label-free, replacement for WBA but as a set of powerful investigatory tools that complement HPLC-based tissue analysis. Current and emerging technologies have radically expanded the utility of MSI. For example, advances in SIMS technologies have enabled sub-cellular imaging capable of describing compound distribution in organelles, meaning that understanding and optimising compound efficacy and safety based on organelle access/retention/accumulation is feasible. At a cellular resolution (5-50µm), sample analysis by rapid

MSI techniques are increasing the speed that mass spectrometers can delineate molecular events in tissues in minutes. This is allowing statistically powered studies to be performed, moving MSI beyond drug distribution assessment, and establishing a novel means of quantifying safety and efficacy biomarkers in tissues, with concomitant pathology and drug/metabolite localisation. Furthermore, the ability of the mass spectrometer to simultaneously collect untargeted full spectrum data, allows datasets to be revisited, once new molecular targets are identified. Such analysis can be used for the following levels of analysis. (1) Morphological definition of the tissue section, based on a training dataset, equivalent to an H&E image; (2) Refined definition of morphology based on endogenous molecules, eg regions of hypoxic/necrotic/tumour/stroma based on immunostaining training set; (3) Localisation of drug and metabolites, overlaid with morphological information; (4) Localisation of biomarkers of efficacy, aligned to drug/metabolite localisation; (5) Metabolomic analysis of treated/untreated tissues used to gain insights on mechanisms of toxicity/efficacy.

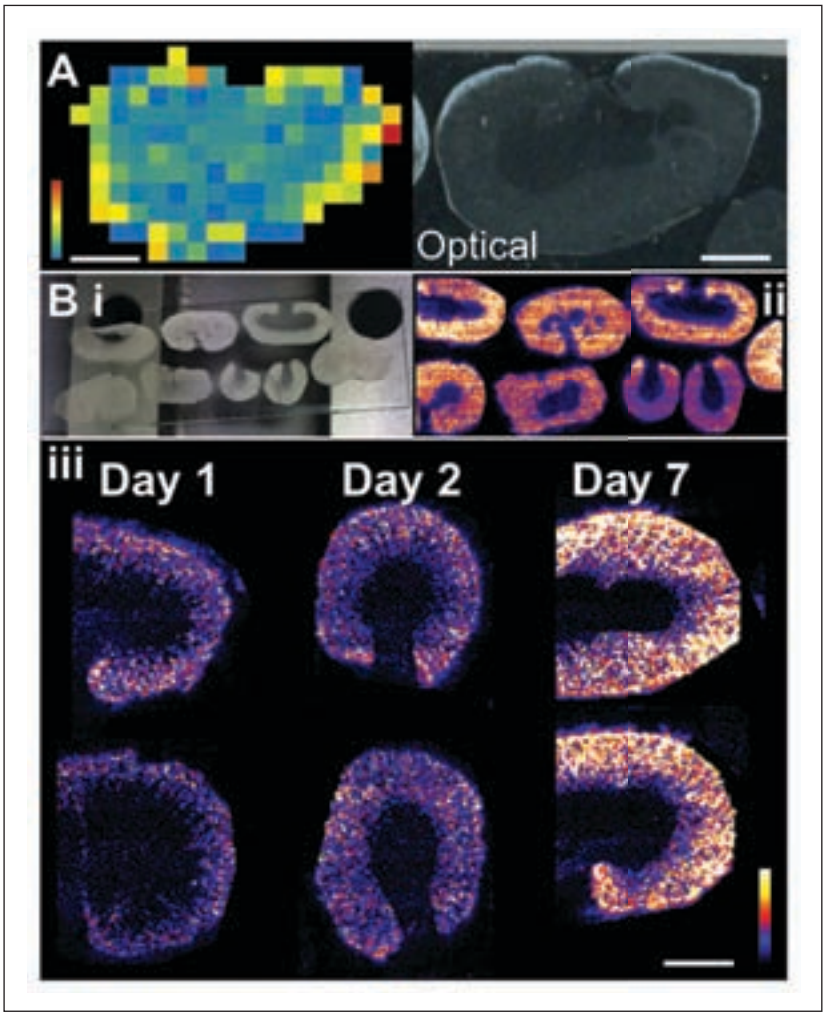

Figure 2: MSI images for polymyxin B1 displaying relative abundance distribution in rat kidney tissue sections. (A) LESA MSI at 1,000µm spatial resolution (B) MALDI MSI example of endogenous compound marker (m/z 853.70. (B iii) Relative abundance distribution of polymyxin B1 [M + H)+ at m/z 1203.8 (scale bar = 5mm). Reprinted with permission from Res. Toxicol. 2015 Sep 21;28(9):1823-30. Copyright 2015 American Chemical Society[7]

What drug discovery issues can MSI resolve?

As stated earlier, improved PKPD modelling of translatable efficacy and safety biomarker data is a cornerstone of the industry's bid to improve its attrition metrics. Much of this data, especially the drug concentrations that underpin the models, are based on measurements made in plasma. In many cases, either because free plasma concentrations are a reasonable surrogate of the 'effect compartment', or because some empirical correction factors are applied, the modelling is improving translation to the clinic. However, there are obvious examples where plasma concentrations are not able to be used to model outcome – and this is where MSI-based tissue measurements, with a focus on the target cells can improve understanding, design and decision making in drug projects. Key areas of focus can be seen as falling into three broad categories:

1. **Non-equilibrium conditions**
   a. Low permeability
   b. Limited blood flow
   c. Tissue targeting delivery systems (eg nanoparticles)
   d. Sites of Local delivery

2. **When free intracellular concentrations cannot be assumed**
   a. Active efflux/uptake
   b. Rapid Tissue Metabolism

3. **Biomarker determination**
   a. Biomarkers response specific to morphology/cell types
   b. MSI inherently the most sensitive technique for analysis
   c. Biomarkers unstable in standard tissue homogenisation

Large capital investments without direct experience of a technology carry a high element of risk, particularly in a rapidly-changing field or when there are a wide range of technology options. So how do researchers new to the field best gain first-hand experience of the potential of MSI to impact

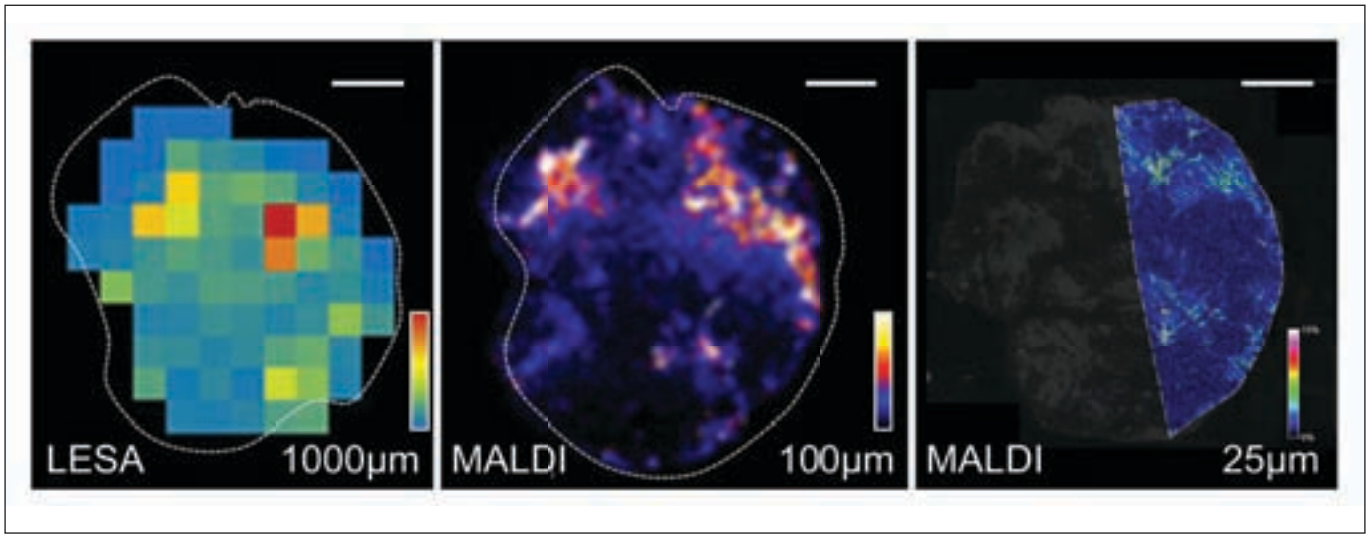

Figure 3: MS reveals relative abundance of [M+H]+ metabolite. (A) LESA analysis of a metabolite [M+H] + of AZD1152 (m/z 415.2) at a spatial resolution of 1,000µm. (B) MALDI analysis of [M+H] + for AZD2811 at m/z 508 at 100µm resolution. (C) MALDI analysis of [M+H] + in adjacent tumour sections analysed at spatial resolution of 25µm Scale bars, 2mm. Reprinted with permission from Sci. Transl. Med. 2016 Feb 10;8(325):325ra17. Copyright 2016 American Association for the Advancement of Science[8]

projects? It is not as complicated as it once was. Studies can be conducted in collaboration with contract research organisations or an ever-increasing number of expert academic groups with in-depth experience in application of MSI to investigatory drug research. Such relations can be an effective way to introduce MSI data into projects, or to supplement in-house capabilities. A mutual understanding of the key questions to be addressed in the study and of technical matters as sample collection/preparation are critical to success. Knowledgeable and technically competent scientists, who can assess the suitability of MSI to address the project issues at hand, have a deep understanding of both the hardware and data analysis challenges, and who can find innovative solutions to problems, are at the core of scientific endeavour. MSI scientists are being trained by the expanding number of academic centres, with MSI expertise, and this has/will provide talent for the future. Training in-house bioanalytical chemists with expertise in LCMS can also prove effective.

Mass spectrometers are an expensive capital investment (typically $200,000-600,000) and imaging interfaces are an additional cost ($100,000-200,000). Repurposing existing equipment, whose specifications are compatible for use in MSI, will reduce capital investments. Additional investment in data transfer/storage systems may also be required to accommodate the large volumes of data generated. The recent advances of commercially viable products have the potential to put the technology in the mainstream of drug discovery/development.

What challenges remain?

As could be anticipated, challenges still remain for MSI, particularly concerning robust quantification and improved sensitivity at low spatial resolution. For example, sub-cellular imaging, while attractive, is currently far from being a routinely applied technology. It is, however, being further developed and implemented by a number of specialist groups that work with Pharma to undertake bespoke studies.

The role of MSI data in regulatory submissions is often discussed. While in principle there should be no issues, questions could arise concerning standardisation of validation protocols for both analytical methods and quantification. Consequently, both due to the lack of a requirement for MSI data for drug registration, and due to the nature of the information/understanding that can be obtained to shape drug hunting projects, MSI is probably best applied to supporting internal decision making in early discovery. With recently developed, multi-vendor technologies, cross-platform data analysis is still a challenge. Especially in larger MSI research groups, wherein a single set of study samples might be analysed by several MSI systems to take advantage of systems' optimum analytical properties.

A final challenge, which really is a huge opportunity, is the integration of MSI to other molecular imaging modalities (MRI, CT, PET, IHC) that offer

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in vivo and *ex vivo* imaging that complements and is complemented by MSI. This is particularly true when wanting to design/develop new tracer, contrast or labelled compounds.

Summary

Advances in mass spectrometry imaging have the potential to revolutionise our understanding of important preclinical efficacy and safety issues. While not a high throughput technique, it is extremely data rich and can readily operate within the overall experimental timelines of drug discovery and development studies. However, at the current technology level, MSI still needs to be targeted at areas of most benefit within a project portfolio, where fully understanding drug and/biomarker distribution within tissues, adds real value. DDW

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