Tissue Imaging by Mass Spectrometry: A Practical Guide for the Medicinal Chemist

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ABSTRACT: Understanding the tissue distribution of therapeutic molecules is often critical for assessing their efficacy and toxicity. Unfortunately, standard methods for monitoring localized drug distribution are resource-intensive and are typically performed late in the discovery process. As a result, early development efforts often progress without detailed information on the effect that changes in structure and/or formulation have on drug localization. Recent innovations in mass spectrometry (MS) provide new options for mapping the spatial distribution of drug in tissue and allow parallel detection of endogenous species. These advances are improving access to drug distribution data early in discovery and provide insight into local biochemical changes that are directly related to drug activity. The literature on these topics is voluminous, and the technology is advancing rapidly, offering a bewildering array of options for researchers who are new to the field. To guide medicinal chemists who wish to apply these methods in their research, this technology perspective provides our views on practical applications that are currently enabled by various MS imaging (MSI) approaches, along with recommendations for how best to implement these methods in pharmaceutical R&D.

KEYWORDS: Mass spectrometry imaging, drug discovery, MALDI

The pharmacological activity of a therapeutic molecule is governed by its level of target engagement, which is dependent on both its target affinity and concentration at the effect site. Measurements of plasma concentration provide a crude estimate of drug level in tissue, while more accurate information can be obtained from liquid chromatography–mass spectrometry (LC-MS) analysis on homogenized tissue to give an organ-level view of drug concentration. These approaches do not elucidate drug localization in specific organ substructures, and high-resolution analysis of drug distribution has historically relied on the use of radiolabeled compounds for performing whole body autoradiography (WBA) and microautoradiography (MARG). However, WBA and MARG are time-consuming and cannot distinguish drug from metabolites that contain the radioactive label. As a result, these methods constitute unwieldy tools for the early assessment of drug distribution and are typically performed late in a project life-cycle. In contrast, mass spectrometry imaging (MSI) can directly detect unlabeled molecules, eliminating bottlenecks associated with synthesis of radio-labeled compounds and enabling distribution mapping for drugs, metabolites, lipids, peptides, and proteins,¹ much earlier in drug development.

Although MSI represents a powerful set of tools for assessing molecular distribution in tissue, the required methods, equipment, and expertise can vary considerably depending upon study objectives. Literature reports provide detailed information on specific experiments and broad overviews of work in the field. However, there is a lack of practical

information about personnel and training requirements, capital and operating costs, and factors to consider when internalizing these technologies versus resourcing them externally. MSI is a complex field, and fit for purpose strategies are required for every organization and project. With these factors in mind, this technology perspective is designed to assist Medicinal Chemists in understanding how MSI can best be deployed to accelerate progress and optimize the probability of success for advancing candidate molecules in a pharmaceutical R&D environment. We will attempt to provide guidance on studies that are likely to yield actionable data in a reasonable timespan and will outline practical considerations for technology internalization, with a focus on commercially available MSI platforms.

TECHNOLOGY OVERVIEW

The direct MS analysis of tissue samples presents challenges not encountered in solution-phase studies. Traditionally, fractionation and LC separation are used to optimize detection sensitivity by minimizing the number of species sampled by the mass spectrometer at any one time. In contrast, direct analysis introduces thousands of ions into the mass spectrometer simultaneously, suppressing signals from compounds with poor ionization efficiencies and complicating mass assignments.

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Figure 1. (A) Overview of MSI technical capabilities. (B) MALDI image of a rat brain showing preferential distribution of a therapeutic molecule dosed at 1 mg/kg. (C) MIBI image of lung adenocarcinoma tissue showing the simultaneous detection of dsDNA (dark blue), CD8 (yellow), CD4 (pink), and FOXP3 (cyan) (image reproduced with permission from IONpath (www.ionpath.com)).

Despite these difficulties, strategies have been developed that allow direct analysis of solids with steadily decreasing analysis areas (spot-sizes), enabling MSI with moderate to high spatial resolution.^{2,3} Initial efforts utilized ion beams and lasers as surface probes, leading to the development of secondary ion MS (SIMS)⁴ and laser desorption MS,⁵ respectively. The energetic nature of these sources results in extensive fragmentation of organic species, limiting their early utility to elemental analysis.^{6,7} By the 1980s adjustments to primary ion flux^{8,9} and the development of cluster ion sources¹⁰ extended SIMS to the analysis of small organic molecules. Simultaneously, Karas and Hillenkamp discovered that addition of radiation-absorbing matrices to solid samples enabled the “soft” laser ionization of labile compounds, leading to the development of matrix-assisted laser desorption/ionization (MALDI) MS for the routine detection of large organic molecules.¹¹ The precise control of analysis area via primary ion and laser beams now enables tissue MSI to be routinely performed with spot-sizes of 0.5–3 μm for SIMS (optimum spot-size will depend on analyte abundance and primary ion source) and $\sim 20 \mu\text{m}$ for MALDI, on commercially available systems. Ion beams and lasers can be more tightly focused, but the resulting smaller analysis area reduces analyte levels below MS limits of detection. To compensate, signal amplification is employed in specialized MSI implementations that utilize lasers or ion beams as surface probes. These methods utilize antibodies to detect cellular proteins, and each antibody is

labeled with a unique metal tag. The elemental tags are detected by MS, enabling multiplexed analysis of >40 antibody–metal combinations.¹² Polymeric chelation molecules allow binding of ~ 100 metal atoms per antibody, improving the limit of detection (LOD) by 2 orders of magnitude.¹³ This allows imaging mass cytometry (IMC) to detect metal-tagged antibodies via laser ablation at spot-sizes of $\sim 1 \mu\text{m}$, while multiplexed ion-beam imaging (MIBI) can perform similar analyses at spot-sizes down to 0.2 μm .¹⁴

More recently, a family of ambient ionization methods have been developed as alternatives to ion beam and laser-based methods.³ Chief among these is desorption electrospray ionization (DESI),¹⁵ which utilizes a pneumatically assisted electrically charged solvent stream to desorb and ionize compounds directly from solid surfaces. DESI MS allows matrix-free analysis of small molecules, minimizing low-mass interferences observed in MALDI MS while enabling the detection of higher mass species than possible with SIMS. The solvent stream cannot be focused to low micrometer levels, so the analysis area is limited to $\sim 100 \mu\text{m}$ (Figure 1). In liquid extraction surface analysis (LESA)¹⁶ surface extraction is achieved by suspending a liquid droplet over the region of interest to create a liquid junction, and the droplet is coupled to nanospray ionization for extended MS analysis. The combination of extraction into a small ($\sim 1 \mu\text{L}$) volume and nanospray analysis provides excellent detection sensitivity, but the spot-size is limited to 1–2 mm. In Flowprobe analysis,¹⁷

Venturi assisted continuous flow and microextraction are coupled to a standard electrospray MS inlet. The liquid junction with the tissue surface varies in diameter with the extraction solvent, with typical spot-size in the 600 μm range. Since the LESA and Flowprobe methods sample larger surface areas than DESI, they generally provide greater analyte signal strength. LESA can also be combined with LC-MS, providing a useful microextraction tool when sample is limited. Additional details are shown in Figure 1.

The development of hybrid methods shows great promise in addressing technical challenges encountered with the techniques described above. An excellent example of this approach is illustrated by the development of infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) MS by Muddiman et al.¹⁸ In this method electrospray ionization is combined with IR MALDI, enabling the use of water/ice as the matrix and eliminating tissue distortion and low-mass interferences associated with standard MALDI experiments. A commercial source is not currently available. However, commercialization of this method will add important capabilities to the MSI toolbox.

Principal Applications of MSI. *Mapping the Tissue Distribution of Proteins.* Understanding protein tissue distribution has broad importance in drug discovery, and the high mass resolution afforded by the Fourier transform ion cyclotron resonance (FTICR) MALDI platform provides enhanced (but still limited) capabilities for direct protein identification.¹⁹ As an example, a recent study of animal models of chronic kidney disease (CKD) utilized MALDI FTICR MSI to detect increased albumin fragments in the tubules of diseased mice. Elevated levels were also observed in a urinary assay predictive of CKD,²⁰ illustrating the potential for MSI to increase confidence in low-level biofluid changes via correlation with markers observed directly in diseased tissue. Unfortunately, MALDI MSI is biased toward abundant species, and deep proteome coverage requires LC-MS analysis. Alberts and co-workers addressed this by using MALDI MSI to identify heterogeneous tissue regions, prior to excising spots of interest by laser capture microdissection (LMD) for micro-proteomic analysis.²¹ Although this workflow can be used to assess tissue heterogeneity, LMD is labor-intensive and requires large samples, which are frequently not available.

The low practical mass range (20 kDa), limited spatial resolution (20 μm), and minimal ability to analyze formalin fixed paraffin embedded (FFPE) tissue are severe shortcomings for MALDI MSI of proteins. Difficulties associated with the analysis of FFPE tissue are of particular concern, since the vast majority of human disease samples are available in this format. Fortunately, rapid developments in IMC and MIBI are addressing these shortcomings.^{12–14} Although MIBI has advantages that include superior spatial resolution and the ability to perform repeat analyses on (precious) samples, it has been used less extensively than IMC due to its more recent introduction and the lack of a commercial platform. However, ongoing deployment of the commercial IMC platform continues to highlight applications for both technologies. Most recently, Bodenmiller used IMC to simultaneously image mRNAs, and their corresponding proteins, in breast cancer tissue. This study showed variations in mRNA/protein ratios at the cellular level,²² providing a preliminary roadmap for correlating these ratios with data on signaling networks to yield broad biological insights that can enhance/accelerate target validation efforts. The MIBI platform can easily be adapted to

studies of this nature, providing the potential to elucidate these complex relationships at the subcellular level. The ability of MIBI to perform repeat analysis on a single tissue section, and the expected introduction of a commercial instrument in 2019, suggest that its use will increase significantly in the near future.

Mapping the Tissue Distribution of Endogenous Small Molecules. There is increasing recognition that biological insight obtained from histomorphological methods can be enhanced by incorporating localized molecular analyses, and that MSI is an ideal method for bridging “omics” technologies with classical histology. The list of region-specific molecules identified by MALDI MSI continues to expand and includes markers for blood vessels,²³ skin layers,²⁴ and necrotic regions in tumors.²⁵ A recent study utilized MALDI MSI to identify molecular species uniquely distributed in the primary visual cortex, showing proof-of-concept for the use of MSI as an unbiased approach for molecular delineation of brain regions.²⁶ Initiatives of this type will define the spatial composition of various tissues, enhancing the ability to gain biological insight by detecting biochemical changes that coincide with drug localization. Recent studies have illustrated this capability by employing molecular probes to map esterase distribution,²⁷ histone methylation status,²⁸ and neuropeptide conversions,²⁹ providing localized data on target engagement that is not available by other methods.

Additional studies illustrate the potential for probing specific aspects of disease biology to facilitate early activities in drug discovery. In oncology, MALDI MSI screening of tumors for lactate heterogeneity identified models that provided accurate measurements of lactate modulation in studies of drug efficacy,³⁰ while analysis of mutant gliomas highlighted metabolic vulnerabilities that might be therapeutically exploited.³¹ Similarly, MSI analysis of atherosclerotic plaques revealed colocalization of pro-thrombotic phospholipids with relevant proteins and highlighted the lysophosphatidic acid pathway as a novel therapeutic target in atherosclerosis.³² In diabetes research, DESI MSI analysis of mouse pancreas showed that minor variations in fatty acid structure govern lipid localization to individual islets, providing potential biomarkers for monitoring the production of reactive oxygen species on these islets.³³ At the opposite end of the development spectrum, DESI MSI has been used in interoperative³⁴ and diagnostic³⁵ applications to distinguish tumor tissue/margins by accurately measuring small metabolites, carbohydrates, and lipids.

Mapping the Tissue Distribution of Drugs and Drug Metabolites. The high spatial resolution, mass resolution, and molecular weight range of MALDI FTICR make it the most useful MS approach for mapping drug distribution. This technique is readily applied in cases where drug levels are high, such as in preclinical toxicology, where localized analysis of drugs/metabolites has enabled mechanisms of lesion formation to be elucidated.³⁶ Likewise, it is an effective tool for determining formulation effects on the skin distribution of topically applied therapeutics^{24,37} and for assessing drug release from synthetic delivery systems such as polymeric microspheres³⁸ and liposomes³⁹ in tumor models. Sensitivity improvements are enabling MALDI MSI to be performed on a wider variety of model systems, with applications in oncology being especially prevalent. Recent work includes its use in mapping epertinib distribution in models of brain metastasis⁴⁰ and in determining irinotecan distribution in tumor spheroids⁴¹ and organoids.⁴² Subcellular imaging of metal

Table 1. Cost Information for Various MSI Methods

	SIMS	MALDI FTICR	*DESI	*LESA	*Flowprobe	Imaging Mass Cytometry (IMC)	Multiplexed Ion Beam Imaging (MIBI)
Initial capital cost	\$1–2 million	>\$1 million	\$100,000	\$100,000	\$50,000	>\$1 million	>\$1 million
Service contract	\$20,000–55,000/yr	\$60,000–70,000/yr	~\$8,000/yr	~\$7,000/yr	\$5,000/yr	~\$80,000/yr	NA (commercial unit not yet available)
Expertise required	High	High	Moderate	Moderate	High	Moderate	High

*A compatible MS (cost = \$300–\$600k) must be coupled to the DESI, LESAs, and Flowprobe systems.

containing drugs can be achieved by exploiting the high spatial resolution of SIMS, as illustrated for the analysis of human cancer cells to detect TriplatinNC accumulation in the nucleus and cytoplasm.⁴³

A key advantage of MSI is its ability to map drug and metabolites simultaneously. The utility of this approach was illustrated by Castellino and co-workers, who used MALDI FTICR to map the brain distribution of fosdevirine and its metabolites in preclinical species.⁴⁴ This study revealed dissimilar localization of drug and metabolites in rabbit, pig, and monkey, and correlated these differences with neuro-behavioral changes. The tissue distribution data enabled investigators to formulate hypotheses that provided additional insight into preclinical results that were not predictive of the seizures observed in human subjects in a Phase IIb study. More recently, MALDI MSI has been used to map the distribution of sunitinib and its metabolites in syngeneic mouse tumor models.⁴⁵ This data revealed that drug was concentrated at the tumor periphery and major metabolites were colocalized with drug. These metabolites were undetectable in blood, providing the first direct evidence that an oral antiangiogenic drug is metabolized in the tumor compartment.

Quantitation enhances the utility of drug/metabolite imaging data, and method development in this area has focused on MALDI MSI. In one study LC-MS quantitation on adjacent tissue sections was used to determine total drug per section, allowing calculation of drug per pixel for corresponding MS images.⁴⁶ A second approach involved the use of tissue mimetic models, where preparation of homogenates with varying drug levels provided calibration curves that accounted for tissue-dependent matrix effects.⁴⁷ More elegant work applied a “pseudo internal standard” across organs to develop a normalization factor, the tissue extinction coefficient,⁴⁸ that enabled tissue and drug-specific ion suppression to be addressed. DESI quantitation faces challenges similar to those encountered in MALDI, while LESAs and Flowprobe MS are better suited to internal standard incorporation for robust quantitative analysis.

Practical Perspectives on the Use of MSI in Drug Discovery. Priorities in pharmaceutical R&D are centered on identifying promising targets and rapidly advancing drug candidates, and medicinal chemists in this environment seek tools that provide timely, critical-path data to address time-consuming bottlenecks. As with most new technologies, MSI may provide data that is interesting but not actionable, so the following discussion will focus on methods that are currently capable of accelerating key activities.

When initiating a discovery program, the early analysis of target prevalence can provide insight into the potential for achieving an adequate therapeutic window. Additionally, for targets in oncology, the assessment of target abundance across tumor types is used to determine the indication(s) that will be

pursued. With the increasing focus on treatment approaches that require a broad understanding of the disease micro-environment (e.g., immuno oncology), the emphasis on mapping multiple protein species continues to increase, testing the limits of traditional immunohistochemistry. Fortunately, multiplexed protein imaging via the IMC and MIBI platforms is poised to provide substantial workflow improvements for applications that rely on determining the distribution of multiple protein species. Data acquisition speeds, and the need for extensive image analysis, present throughput challenges that limit sample interrogation to several smaller regions of interest (typically 500 $\mu\text{m} \times 500 \mu\text{m}$) estimated to be representative of individual tissue samples. For screening large numbers of samples, multiplexed methods⁴⁹ that provide quantitative digital readouts can provide rapid assessments of protein abundance and efficient triage of samples that should undergo more detailed analysis by IMC or MIBI.

Once a program has been initiated, the selection of appropriate in vivo models is critical to enabling rapid candidate progression. The target distribution observed in these models frequently varies versus reference information, and proposed models must be extensively validated prior to implementation. As described in the previous section, IMC and MIBI represent effective tools for making these assessments. Spatial profiling of the metabolome by MALDI MSI may also be helpful in assessing the suitability of various model systems for specific applications.³⁰ Once chemistry efforts are underway, the ability of MSI to map unlabeled compounds provides an efficient route to mapping drug and metabolite distribution in organs of interest. These studies can be of critical importance in cases where the therapeutic window depends on optimized drug distribution (e.g., oncology). It is important to recognize that the limit of detection for a MALDI MSI experiment is generally 20–50-fold higher than that for a comparable optimized LC-MS/MS analysis on homogenized tissue.⁴⁶ As a result, MS imaging can be problematic for indications where drug penetration into target tissue is restricted. For example, assessing drug distribution in brain is a relevant topic in neuroscience, and the spatial resolution of MALDI MSI makes it well-suited for mapping structural features of therapeutic interest. Although some brain-penetrant compounds can be imaged by MALDI MSI,^{44,50} the low blood–brain barrier permeability of many molecules requires alternate approaches, as shown in cases where compounds undetectable by MALDI gave robust MS signals utilizing LESAs⁵¹ and DESI.²³ This trade-off between spatial resolution and sensitivity requires a careful assessment of research objectives prior to initiating resource-intensive MSI studies.

The cost of bringing technologies in-house is also an important consideration for most organizations. For example, SIMS has outstanding spatial resolution but narrow utility for tissue imaging. Capital and operating costs are high, and a user

with extensive training is required to keep the system running optimally (Table 1), suggesting that internalizing this capability will be of interest to only a small number of organizations. In many cases, studies of interest can be outsourced to one of the many excellent contract or academic laboratories that exist. MALDI FTICR MS is the most broadly useful method for tissue imaging, but it also has high capital and operating costs, and extensive user expertise is required. DESI MSI provides spatial resolution inferior to that of MALDI MSI but is relatively inexpensive and can be easily added to (and removed from) existing MS systems present in most pharmaceutical laboratories. Operating costs are low, and skilled MS analysts can be trained on DESI methods in a reasonable amount of time. LESA and Flowprobe methods provide cost profiles similar to that of DESI (Table 1). They have spatial resolution inferior to that of MALDI or DESI MSI, but the larger analysis areas correspond to higher analyte signal levels (Figure 1). For organizations with intermittent tissue imaging requirements, on-site MS instrumentation can be combined as needed with DESI, LESA, and Flowprobe to provide relatively low-cost routes to obtaining baseline MSI data on small molecules. These methods will provide an effective triage mechanism for assessing projects that should be sent to contract laboratories for higher resolution imaging by MALDI FTICR MS.

SUMMARY

Tissue imaging by mass spectrometry comprises an expanding family of technologies that are capable of imaging a wide-variety of molecular species. However, MSI for each class of molecules requires specialized methods and expertise, and instrument requirements often vary considerably. MALDI FTICR is the most versatile approach to MSI and can be used to map the spatial distribution of drugs, drug metabolites, endogenous small molecules, and proteins. The most promising applications to date have revolved around mapping the distribution of drugs and their metabolites. Efforts to develop comprehensive maps of endogenous molecules in various animal models are ongoing and are poised to provide important resources for understanding disease biology. For tissue imaging of small molecules, DESI, LESA, and Flowprobe MS offer lower-cost alternatives, although these methods cannot achieve the spatial resolution possible with MALDI MSI. For the more specialized application of multiplexed protein mapping in FFPE tissue, the MIBI and IMC platforms are poised to revolutionize both workflows and information content from these large sample repositories. With all of these methods it is important to recognize that access to samples, appropriate sample preparation, and data analysis represent the largest bottlenecks to timely acquisition of high-quality and relevant data. An organizational commitment to allocating personnel for front-end sample preparation and back-end data analysis is needed prior to investing in MSI technology. This requires a careful evaluation by leaders in the appropriate therapeutic, analytical, and medicinal chemistry teams to determine if adequate support is available to ensure an acceptable return on investments in MSI capabilities.

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Notes

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ABBREVIATIONS

MS, mass spectrometry; MSI, mass spectrometry imaging; LC-MS, liquid chromatography–mass spectrometry; WBA, whole body autoradiography; MARG, micro autoradiography; SIMS, secondary ion mass spectrometry; MALDI, matrix-assisted laser desorption ionization; LOD, limit of detection; IMC, imaging mass cytometry; MIBI, multiplexed ion-beam imaging; DESI, desorption electro-spray ionization; LESA, liquid extraction surface analysis; FTICR, Fourier transform ion cyclotron resonance; LMD, laser capture microdissection; FFPE, formalin fixed paraffin embedded

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