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Imaging and spatially resolved quantification of drug distribution in tissues by mass spectrometry

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Mass spectrometry imaging (MSI) is a powerful label-free technique for visualizing drug and metabolite distributions in biological tissues. In this review, we discuss recent developments in MSI and spatial profiling technologies to visualize and quantify drug distributions in tissues. We also present recent examples of applications of these technologies for assessing drug distribution within tissues and individual cells. Finally, we focus on an emerging technique coupling laser capture microdissection (LCM) to quantitative mass spectrometry, which combines the respective advantages of imaging and conventional liquid chromatography mass spectrometry, and thus enables spatially resolved drug quantification.

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Introduction

To exert its desired activity, a drug must reach the biological target at its exact location (host cell or pathogen) at sufficient concentration. Drug and metabolite concentrations measured in plasma often provide poor representation of drug exposure at target tissues or cells, and are not always sufficient for accurate interpretation and understanding of efficacy within the body.

Mass spectrometry imaging (MSI) is a powerful label-free technique for the *in situ* analysis of drug and metabolite distributions within *ex vivo* tissues. MSI produces valuable spatial and molecule-specific information, complementary to that acquired by traditional analytical approaches, such as quantitative autoradiography (QWBA) and liquid chromatography mass spectrometry (LC/MS/MS) of tissue homogenates [1–4,5•]. Significant spatial information is lost during tissue homogenization before quantification of drugs by LC/MS/MS. Autoradiography offers high spatial detail, but lacks molecular specificity as only the label of administered radiolabeled drug is detected [6]. As MSI techniques detect drug and metabolite molecules directly, they offer the unique opportunity to spatially resolve metabolites from their parent drugs.

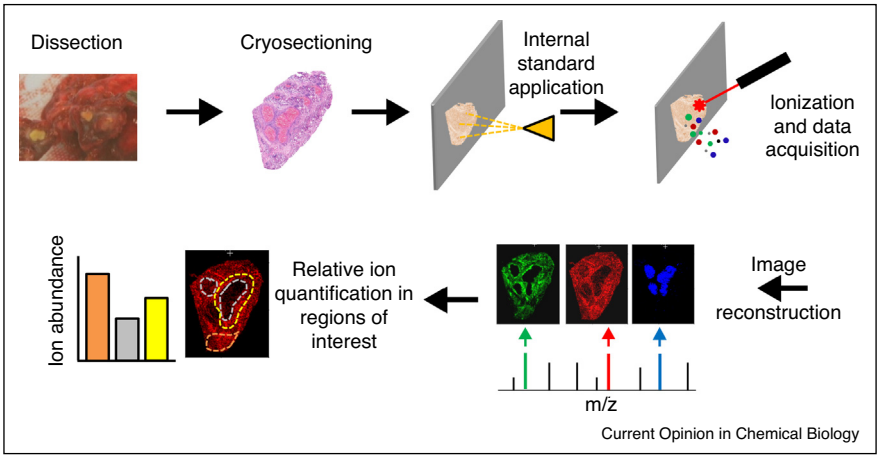
In a standard MSI experiment, an ionization beam (typically a laser, solvent stream, or primary ion source) is rastered across the tissue surface, and mass spectra are acquired at defined (x,y) coordinates. A two-dimensional (2D) ion distribution map is reconstructed using software, in which a mass spectrum is generated for each pixel. Correlating drug MS images with histologically stained adjacent tissue sections allows for accurate interpretation of drug delivery to specific tissues, cells and/or pathogens. A schematic representation of a typical MSI workflow applied to drug imaging in tissue is shown in Figure 1. MSI of drugs and other small molecules in tissues has been previously reviewed in depth [7–9]. The aim of this review is to discuss recent technological advances in the field, with particular focus on spatial resolution and quantification capabilities. A summary of the MS imaging and spatial profiling methods discussed in this paper including required sample preparation protocols is presented in Table 1.

Mass spectrometry imaging technologies

Matrix-assisted laser desorption ionization MS (MALDI-MS) remains the most widely applied ionization method for tissue imaging due to its sensitivity, large dynamic range of masses covered, and the relatively high availability of commercial MALDI mass spectrometers. Recent improvements in laser optics have enabled imaging of drug distribution in tissues at sub 10 μm lateral resolutions [10,11••], opening new avenues for subcellular drug localization. However, the increase in lateral resolution reduces sampling volume and ion yield, resulting in reduced analytical sensitivity.

The sensitivity in MALDI-MS imaging is often limited by ionization efficiency. *In situ* chemical derivatization of selected drugs has been shown to dramatically increase

Figure 1



Representative schematic of mass spectrometry imaging (MSI) workflow for drug imaging in biological tissue sections (adapted with permission from [4]. *m/z*: mass to charge ratio.

Table 1

Table summarizing common MS imaging and profiling techniques used for drug localization in tissues and cells. The speed of sample preparation, speed of MS image acquisition, and analytical sensitivity are rated from 1 to 5 (in order of increasing speed or sensitivity). Speed of MS image acquisition depends upon the pixel and overall tissue dimensions as well as MS scan/dwell time. Analytical sensitivity is heavily dependent upon ionization properties, which are highly drug specific. The sensitivity rating is therefore only meant as a guide and should be carefully evaluated for each drug analyzed

	MALDI-MSI	Laser ablation/ desorption-MSI	SIMS-MSI	DESI-MSI	Nano-DESI-MSI	LMJ-SS	LCM-ESI or LCM-LC/MS
Tissue preparation	Thin cryo-sections (5–50 μm)	Thin cryo-sections (10–50 μm)	Cultured cells or bacteria	Thin cryo-sections (5–50 μm), intact tissues	Thin cryo-sections (5–20 μm)	Thin cryo-sections (10–50 μm)	Thin cryo-sections (10–30 μm)
Requires matrix?	Yes	No	No	No	No	No	No
Ionization pressure	Vacuum or atmospheric	Vacuum or atmospheric	Vacuum	Atmospheric	Atmospheric	Atmospheric	Atmospheric
Speed of sample preparation	**	*****	*****	*****	*****	*****	**
Speed of MS acquisition	***	***	**	****	**	*****	**** (ESI) ** (LC/MS)
Sensitivity	***	**	*	**	**	****	*****
Lateral resolution	<10 μm	>100 μm	<250 nm	>200 μm	>10 μm	>500 μm	>20 μm
Imaging mode	Imaging	Imaging	Imaging	Imaging/profiling	Imaging	Profiling/imaging	Profiling
References	[1–4,5•,10, 11••,12,13, 26,27,29,43, 45,46,49]	[14,25]	[16••, 17••,18•]	[20•,21]	[22–24]	[28–32]	[33,34,25,36]

the ion yield [12,13], opening previously unsuitable classes of drugs to MSI. The addition of a laser-induced postionization step following initial MALDI ionization can enhance the analytical sensitivity for many small molecules by up to two orders of magnitude, even when sampling from pixels as small as 5 μm wide [11••]. A similar postionization method has been applied at lower lateral resolution in a quantitative matrix-free approach to image drug distributions in a mouse model [14]. In this study, the antibiotic drug acriflavine was imaged in *ex vivo* kidney tissue at a spatial resolution of 500 μm with a calculated limit of quantification (LOQ) of 500 nmol per mm^2 of tissue area.

Of all MSI ionization methods, secondary ion mass spectrometry (SIMS) offers the highest spatial resolving

capabilities (nanometer level), and, in contrast to MALDI, does not require the addition of an ionization-enhancing matrix. However, it suffers from lower analytical sensitivity than MALDI, and the resulting high induced in-source fragmentation of ionized molecules makes spectral interpretation difficult [15]. Whilst these factors have limited the utility of SIMS for drug imaging in tissues, improvements in sample preparation and instrument design (such as coupling to high resolution MS analyzers [16••]) has enabled highly detailed drug distribution within single cells and bacteria [17••,18•].

Multiple atmospheric pressure (AP) and matrix-free ionization methods have emerged to analyze drugs and metabolites within biological tissues [19]. Of these techniques, the most widely used ionization method for MSI is desorption electrospray ionization (DESI) [20•,21], in which drug ions are formed by directing a solvent stream to the surface of the tissue. DESI-MSI provides rapid image acquisition and has the potential for high spatial resolution when nano-DESI sources are utilized [22]. However, the spatial capabilities of this technique can be compromised by the complex surface morphology of biological tissues. Improvements in capillary design [23] and combined nano-DESI/shear force microscopy sources, as demonstrated for lipids, offer the potential for more routine drug imaging at higher spatial resolution (<50 μm) [24]. A comparison of MS images generated using multiple ionization methods at different lateral resolutions is shown in Figure 2.

Novel developments in matrix-free ambient ionization methods enable direct imaging of *ex vivo* tissues with minimal damage. Picosecond infrared laser ablation electrospray ionization (PIR-LAESI) has been applied to image the distribution of MRI contrast agent gadoteridol in mouse kidney tissues [25]. Although the 200 μm spatial resolution is suboptimal, a major advantage of this approach is the ability to image drug distribution in frozen tissue sections, reducing the potential for enzymatic degradation or tissue dehydration.

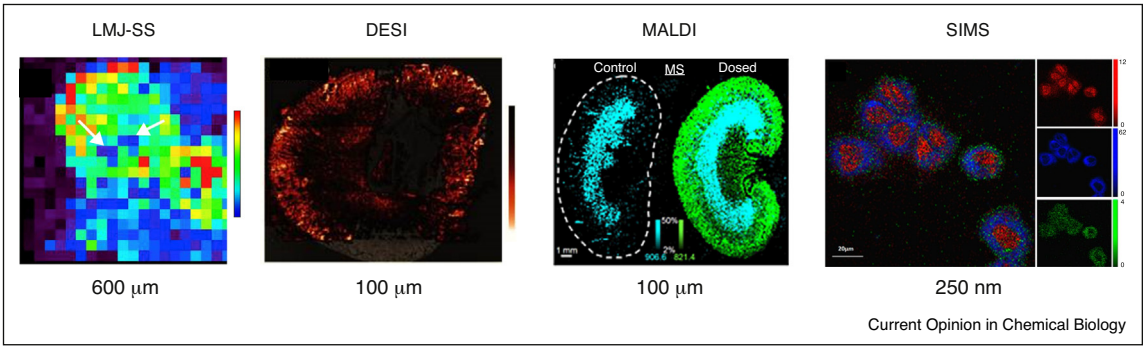
The speed of MSI acquisition is a key concern for pharmacokinetics (PK) studies in which large numbers of tissues from multiple PK timepoints and animal replicates are required. High repetition lasers and continuous raster acquisition scanning [26,27] have vastly decreased total MALDI-MS image acquisition time, creating the potential for routine high spatial resolution and high throughput imaging. For example, images of drug distributions in individual organs can be acquired at 100 μm lateral resolution in under 1 hour, a greater than 10-fold reduction in total image acquisition time. When images are acquired at this speed, the sample preparation process (tissue sectioning and matrix application) may become the rate-limiting step in generating the MALDI-MS image rather than the MS analysis.

Ever-increasing high mass accuracy and mass resolution capabilities of orbital trapping (Orbitrap) and Fourier-transform ion cyclotron resonance (FT-ICR) instruments have widened the range of applications for MS imaging of drugs and metabolites [10,4]. The high resolving power decreases the risk of unresolved spectral peaks from interfering matrix or endogenous species, and the high mass accuracy enables determination of elemental composition, aiding in identification of novel metabolites. In contrast to targeted tandem MS approaches, endogenous lipids, metabolites and peptides are simultaneously detected during full-scan high resolution imaging. This powerful approach can be applied to the discovery of biomarkers of inflammation, disease or therapeutic response, to be directly correlated to drug distribution at the tissue and cellular levels.

Spatial profiling technologies

When high spatial resolution is not required, discreet spatial profiling approaches are a rapid and sensitive alternative to MSI. Liquid microjunction surface sampling (LMJ-SS) technologies, such as discreet liquid

Figure 2



Example MS images from different imaging modalities displayed in order of spatial resolution capabilities. LMSS-MSI imaging of levofloxacin in rabbit pulmonary lesions using Flowprobe at 600 μmm lateral resolution [29], DESI-MSI of a novel tyrosine kinase inhibitor compound in rabbit kidney (100 μm resolution) [20•], MALDI-MSI of rifampicin in rabbit kidney (100 μm resolution) [26], and ToF-SIMS imaging of amiodarone (green channel) in single rat macrophages (250 nm resolution) [17••].

extraction surface analysis (LESA) [28] and continuous flow devices [29,30], offer significantly better extraction efficiencies and require simpler sample preparation than traditional MSI techniques [31]. They are typically used to spatially profile drugs at low resolution, rather than to generate full images of the tissue surface due to the relatively large sampling area (≥500 μm). In these techniques, extraction solvents are deposited on the tissue surface in the form of discreet droplets or continuous flow, which are subsequently aspirated and analyzed by ESI-MS.

While primarily utilized in spatial-profiling mode, LMJ-SS techniques can also be applied to MSI by sampling over the entire tissue surface rather than discreet locations [29,32]. Even accounting for the limited spatial resolving power, this approach has value for imaging drugs at concentrations that are too low to be detected by alternative, higher-spatial resolution approaches.

Coupling laser capture microdissection (LCM) with quantitative mass spectrometry to identify and isolate small tissue areas or cell populations is an emerging and innovative approach for drug localization [33,34,35••,36]. Whilst not a true imaging technique, it is suitable for routine absolute quantification and offers enhanced sensitivity/selectivity by adding chromatographic separation, two unique advantages over traditional MSI methods. The spatial resolving capabilities of LCM are dependent upon the ability to identify and dissect small regions and cell populations from tissues. Technology limiting factors include the focus of the cutting laser and the microscope objectives utilized. Identifying target cell populations can be challenging in unstained tissue sections viewed through bright-field microscopy, but may be aided by close alignment with a histologically stained serial reference section. Cahill *et al.* implemented an on-line LCM method for direct quantification of propranolol, a β-blocker used to treat high blood pressure disorders and migraine headaches, within small microdissected regions of brain, kidney and liver tissue (20 μm × 20 μm or 40 μm × 40 μm) [34]. They developed a hybrid optical microscopy/LA-liquid vortex capture probe to quantify the drug against an internal standard spiked into the solvent phase. Heterogeneous propranolol concentrations were quantified in the kidney (renal cortex 44 ± 12 nmol/g versus renal medulla 13.6 ± 1.9 nmol/g), whereas brain (39.9 ± 7.1 nmol/g) and liver (11.0 ± 2.3 nmol/g) concentrations were relatively uniform across the tissue.

The pathology of many infectious diseases is complex, leading to pathogen sequestration in niches that are partially disconnected from blood supply. This is true for infections as diverse as tuberculosis, HIV and fungal abscesses [37–40]. Such diseases present a significant pharmacokinetic/pharmacodynamic challenge to drug developers. To meet this challenge, our group has applied LCM of tissues coupled to off-line chromatographic separation (LCM-LC/MS/MS) in order to spatially quantify a range of drugs within infected tissues [35••,36]. A graphical schematic of this workflow is shown in Figure 3.

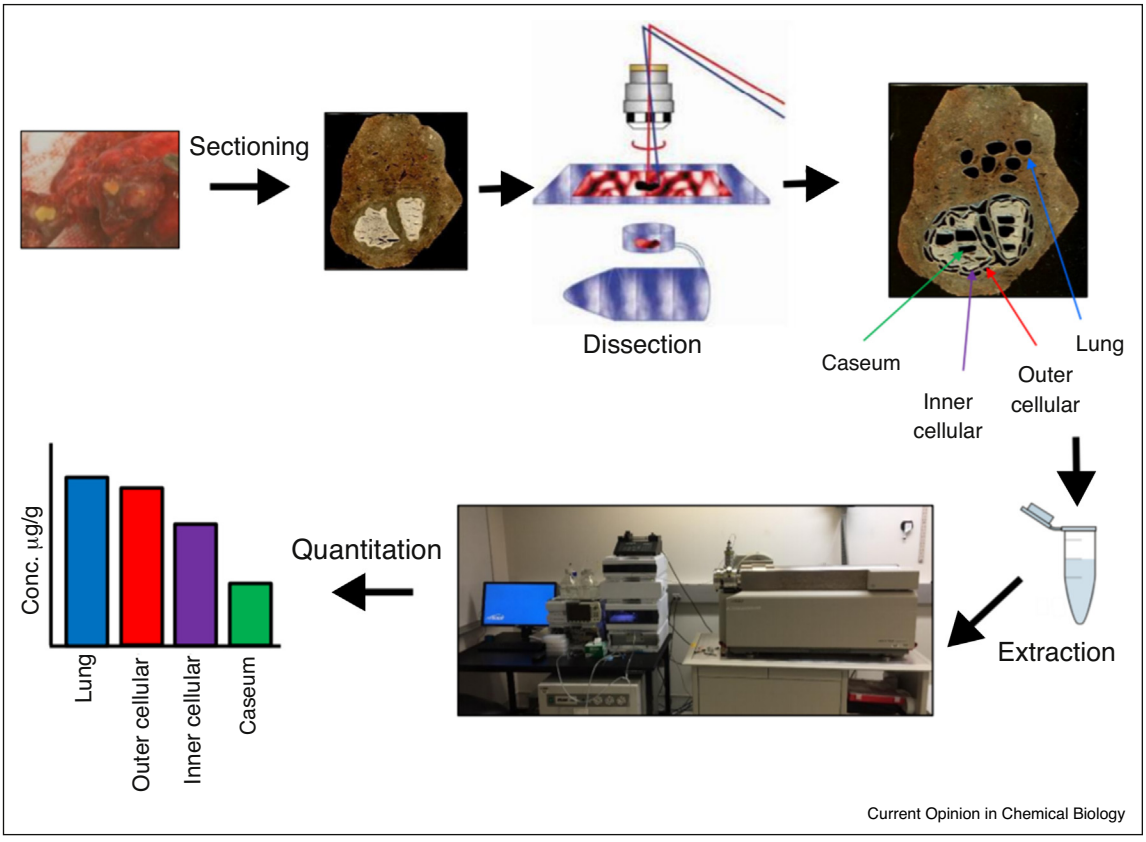
Quantification

Qualitative MSI provides valuable information regarding the penetration of drugs and metabolites into specific tissues and cells. However, absolute drug quantification is required to determine whether drugs are reaching target populations (pathogens or cells) at adequate concentrations to achieve a therapeutic response. Absolute quantification of drugs in biological tissues by MALDI-MSI is challenging for numerous reasons, which have been described and discussed in detail in an excellent review article by Rzagalinski and Volmer [41••]. Primary among these is drug ion suppression by competing abundant endogenous species, such as lipids, salts and metabolites. This suppression is highly heterogenous across different tissue areas and cell populations. Normalizing drug or metabolite ion signal against an internal standard (preferably a stable-labeled drug or structurally similar analog) compensates for ion suppression, matrix effects, and variances in tissue morphology [1,4,5•,35••].. However, this does not correct for differences in drug extraction efficiencies from heterogeneous cell populations across the tissue. Depositing the internal standard under the tissue section has been proposed as a method for improved normalization of extraction efficiency [42]. In a separate study, adding the standard to the tissue surface resulted in data which correlated more closely to drug quantified by LCM/MS/MS [43].

Normalization to an internal standard is the most widely used approach for both relative and absolute quantification of drugs in tissues by MSI. An alternative approach for quantitative whole-body MALDI MSI, where drug concentrations in tissues are measured at the organ level and high spatial detail is not required, has been validated based on a calculated tissue-specific ionization efficiency factor [44]. In this approach, a control (untreated whole-body section is homogeneously sprayed or spiked with the drug solution, and then imaged. From the resulting MS image, a numerical coefficient can be calculated from regions of interest encompassing individual organs, which future MS images of the same drug can be normalized against. An expanded version of this approach involving an additional normalization step applied to a dilution series of drug standards deposited adjacent to the tissue, termed tissue extinction coefficient (TEC), was used to accurately quantify propranolol and olanzapine in whole body rodent sections [45]. Propranolol concentrations ranged between 5.5 and 17.7 μg/g in the analyzed organs (brain, kidney, and lung) with an acceptable inter-sample variability of 16%. The concentrations closely matched those previously determined by QWBA.

A 'mimetic tissue model' for normalization and quantification has been introduced by Groseclose and Castellino

Figure 3



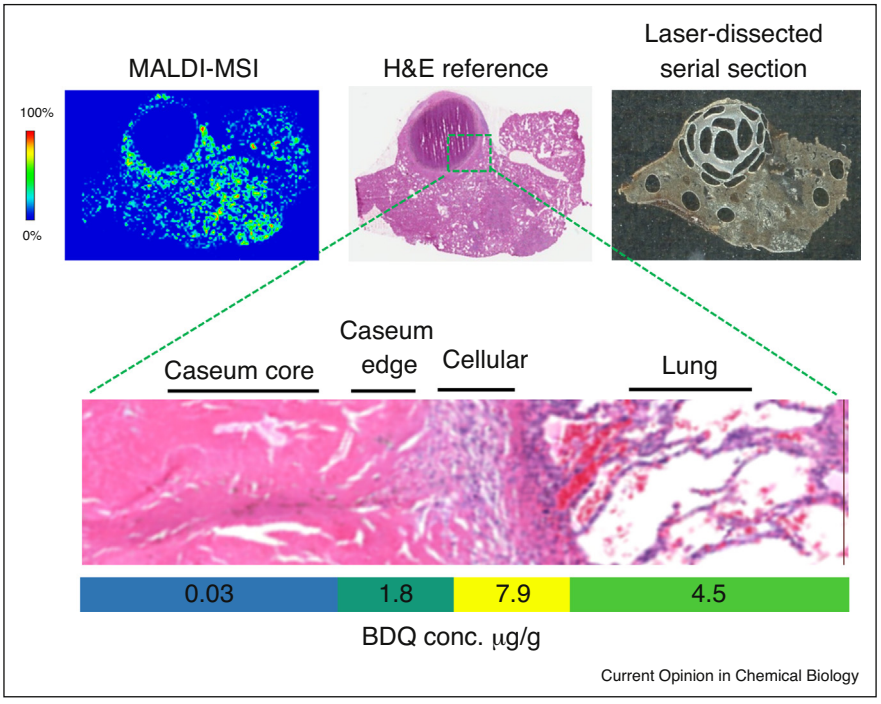
Representative schematic of LCM-LC/MS/MS workflow for spatially resolved quantification of drugs in biological tissue sections (adapted with permission from [25]).

[46]. Control tissue homogenates are spiked with increasing concentrations of the drug, frozen inside an encasing block, cryosectioned, and coated with matrix (following the same protocol as *ex vivo* dosed tissues). The tissue homogenates are used to generate a standard curve from which actual drug concentrations in tissue are calculated. While the approach has shown excellent correlation with concentrations analyzed by traditional LC/MS/MS approaches, it is time consuming and requires substantial amounts of control tissue homogenate (particularly problematic for tissues with highly heterogeneous morphology).

LMJ-SS methods are highly efficient at extracting drug from cells due to the relatively large volume of solvent applied to the tissue surface. However, these techniques still suffer from tissue-specific ionization suppression due to the lack of on-line chromatographic separation [32]. Applying a deuterated standard homogeneously to the tissue surface, or spiking directly into the extraction solvent, has been shown to improve the reproducibility of spatial quantification of drugs by LESA [47•].

LCM coupled to mass spectrometry is a compelling choice for absolute quantification of drugs within tissue structures. It is a full-extraction method, in both on-line and off-line modes, maximizing analytical sensitivity and minimizing the variation of extraction observed due to heterogeneity in tissue density and cellular composition. Additionally, if coupled with LC separation, matrix-related effects are eliminated. A comparison of MALDI-MSI and LCM-LC/MS/MS techniques applied to assess bedaquiline (BDQ) distribution in mouse tuberculosis (TB) lesions is shown in Figure 4. MALDI-MSI provides a clear visualization of the relative partitioning of BDQ within the lung and various lesion compartments. However, drug signal is not detected within the caseum core. The ability of a TB drug to penetrate the non-vascularized caseum is of critical importance for sterilizing the large quantity of bacteria residing within. Owing to the high potency of BDQ (minimum inhibitory concentration of 0.06 µg/g [48]) and the limited sensitivity of MALDI-MSI (limit of detection 1.2 µg/g), efficacious but undetected drug concentrations could still be reaching the caseum [49]. The highly sensitive and fully

Figure 4



Comparison of MALDI-MSI and LCM-LC/MS/MS approaches for visualizing and quantifying bedaquiline concentrations in mouse pulmonary lesions, following a single 25 mg/kg oral dose. The drug signals determined by the two methods correlate well. However, the additional sensitivity of LCM-LC/MS/MS enabled accurate quantification of BDQ within the caseum core, which was not detected by MALDI-MSI.

quantitative LCM-LC/MS/MS approach (LOQ 5 ng/g) delivered absolute BDQ concentrations in all lesion compartments, which can now be related to the concentrations required to kill replicating and non-replicating *Mycobacterium tuberculosis* bacilli [50,51].

Conclusion

MSI is a rapidly evolving and powerful label-free technique that is gaining increasing traction in pharmaceutical research. This technology provides accurate evaluation of drug and metabolite distribution *in vivo*, critical for understanding and interpreting treatment responses. Recent developments in MSI, such as increases in spatial resolving power and sensitivity, are opening new avenues for imaging drugs at exquisite detail within tissues and cells. However, MSI remains a technique best applied as a complementary approach to existing technologies.

Absolute drug quantification is required to determine whether drugs reach tissues and cells at efficacious concentrations. While significant advances have been made in quantitative MSI, questions remain regarding its routine application to quantify drug localization in tissues. Further optimization of sample preparation techniques and ionization technologies will enhance quantitative capabilities. LC/MS and autoradiography are likely to remain the gold standard for the foreseeable future.

Combining high spatial resolution MSI with lower-resolution, fully quantitative methods (such as LCM-LC/MS/MS) in adjacent tissue sections, produces both spatially detailed and quantitative data. This new approach is a game changer in the field of tissue-centric pharmacokinetics/pharmacodynamics, and an ideal tool to avoid costly drug development attritions due to inadequate drug penetration to the site of action. In addition to infectious diseases, the methodology is broadly applicable to a wide spectrum of disorders including diseases of the central nervous and cardiovascular system, and cancer.

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