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Mass Spectrometry Imaging for Spatial Toxicology Research

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ABSTRACT

The spatial information of xenobiotics distribution, metabolism, and toxicity mechanisms in situ has drawn increasing attention in both pharmaceutical and environmental toxicology research to aid drug development and environmental risk assessments. Mass spectrometry imaging (MSI) provides a label-free, multiplexed, and high-throughput tool to characterize xenobiotics, their metabolites, and endogenous molecules in situ with spatial resolution, providing knowledge on spatially resolved absorption, distribution, metabolism, excretion, and toxicity on the molecular level. In this perspective, we briefly summarize applications of MSI in toxicology on xenobiotic distribution and metabolism, quantification, toxicity mechanisms, and biomarker discovery. We identified several challenges regarding how we can fully harness the power of MSI in both fundamental toxicology research and regulatory practices. First, how can we increase the coverage, sensitivity, and specificity in detecting xenobiotics and their metabolites in complex biological matrices? Second, how can we link the spatial molecular information of xenobiotics to toxicity consequences to understand toxicity mechanisms, predict exposure outcomes, and aid biomarker discovery? Finally, how can we standardize the MSI experiment and data analysis workflow to provide robust conclusions for regulation and drug development? With these questions in mind, we provide our perspectives on the future directions of MSI as a promising tool in spatial toxicology research.

1 | Spatial Toxicology: Elucidating Xenobiotic-Biological Interactions in Spatial Context

The absorption, distribution, metabolism, excretion, and toxicity (ADMET) of xenobiotics represent key research topics in pharmacology and toxicology. The concept of ADMET is mostly used in pharmaceutical toxicology to evaluate drugs, whereas it is also applicable to toxicology of environmental pollutants and toxins [1]. Measurements of xenobiotics and their metabolites, as well as endogenous biomolecules, have enabled in-depth elucidation of ADMET mechanisms at the molecular level and facilitate drug development and risk assessment. In recent years, the spatial contexts of toxicity responses in tissues and cells have attracted increasing attentions. Biological organisms are highly heterogeneous across scales. Molecules, organelles, and cells of different functions form spatially organized compartments to carry out specialized biological functions and interact with each other in spatial context, such as ligand-receptor interactions and cell-to-cell signaling. Xenobiotics entering the body are not uniformly distributed throughout the body but have preferential localizations in specific tissues and cells. The spatial heterogeneity of biological organisms also results in different xenobiotic metabolism and toxicity at different locations. We define the term "spatial toxicology," derived from the term "spatial biology," as the subfield of toxicology focusing on elucidating the ADMET mechanisms in biological organisms in spatial context. Compared to measuring bulk, homogenized samples, spatial toxicology investigates xenobiotic-biological interactions in situ from samples where the tissue and cellular architectures are preserved. Spatial toxicology approaches can

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be used to reveal highly localized effects in specific regions of the tissues, offering unique and significant insights into how xenobiotics interact with the highly heterogeneous biological organisms.

2 | Mass Spectrometry Imaging in Spatial Toxicology

Many tools are available to study the ADMET mechanisms in situ with spatial resolution, such as whole-body autoradiography [2], positron emission tomography (PET) [3], spectroscopy [4], and recently, spatial transcriptomics [5]. Mass spectrometry imaging (MSI) provides label-free, highly multiplexed, and high-throughput measurements to characterize xenobiotics, their metabolites, and endogenous molecules in situ on various types of samples [6–11]. Matrix-assisted laser desorption/ionization MS (MALDI-MS) imaging uses highly focused laser pulses to desorb and ionize chemical matrix and sample materials to achieve chemical imaging. It has been the most commonly applied MSI method for a wide range of molecules. Desorption electrospray ionization (DESI), an emerging MSI technique in recent years, achieves spatial resolution by directing a flow of electrospray onto the sample surface. Secondary ion mass spectrometry (SIMS) is a less commonly used MSI technique but provides unique capabilities in 3D depth profiling by using a focused primary ion beam onto the sample surface and generating secondary ions of samples for measurements. With a proper ion beam source, SIMS can achieve submicron spatial resolution, whereas the nanoSIMS further pushes the limit to as small as 50 nm of lateral resolution [12]. Finally, although not frequently mentioned in the field of MSI, laser ablation inductively coupled plasma MS (LA-ICP-MS) is a specialized tool in elemental imaging and plays key roles in studying heavy metal exposure and biological metal homeostasis [13].

Utilization of MSI for toxicology research started in the early 2000s, concurrent with the development and commercialization of MSI instrumentations, and has steadily increased since then, as shown by a keyword search query to the PubMed (Figure 1). A large fraction of MSI's application in toxicology is for drug development. Drug distribution is an indispensable part in the pharmaceutical R&D pipeline, as drugs needs to be distributed to their intended target site in the right form for desired effects. A handful of reviews have summarized in detail the application of MSI in pharmaceutical research and drug development [14–28]. On the other hand, the application of MSI in environmental toxicology is less common, and MSI has not been incorporated in the pipeline of toxicity risk assessment for environmental contaminants. Two recent reviews summarized examples of MSI application in environmental sciences, which included MSI analysis for environmental contaminants [29, 30]. LA-ICP-MS has been used to visualize the localization of nano/microsized particulates and heavy metals in rodent tissues and wheat grains [31–34]. MSI has also been applied to study the spatial distribution and toxicity of pesticides in plants, honeybees, and zebrafish among other organisms [35–38], and recently, to investigate the spatial distribution of per- and polyfluoroalkyl substances (PFAS), a category of emerging contaminants, in zebrafish and rodent models [39–43]. The application of MSI in the area of environmental toxicology is still rising.

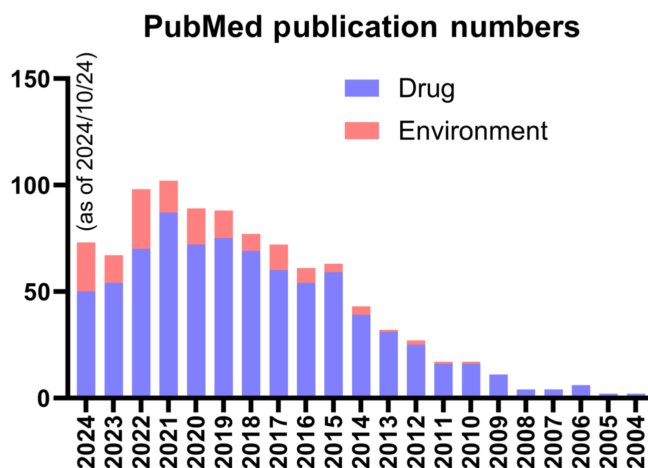

FIGURE 1 | PubMed search results using searching query of ("mass spectrometry imaging"[Title/Abstract] OR "imaging mass spectrometry"[Title/Abstract]) AND ("drug"[Title/Abstract] OR "pharmaceutical"[Title/Abstract]) for drug and ("mass spectrometry imaging"[Title/Abstract] OR "imaging mass spectrometry"[Title/Abstract]) AND ("environmental"[Title/Abstract] OR "pollutant"[Title/Abstract] OR "contaminant"[Title/Abstract]) for environment.

To date, MSI has been an emerging and powerful tool in toxicology to study xenobiotic distribution and metabolism, quantification, toxicity mechanisms, and biomarker discovery, whereas the application is mainly limited in laboratory studies without being fully incorporated into the pipelines for drug discovery and risk assessment. In this perspective, we identify three challenges regarding how we can fully harness the power of MSI in fundamental toxicology research, drug discovery, and regulatory practices. The challenges are as follows: (1) How can we increase the coverage, sensitivity, and specificity in detection of xenobiotics and their metabolites in complex biological matrices? (2) How can we link the spatial distribution to toxicity consequences so that we can understand toxicity mechanisms, predict exposure outcomes, and aid biomarker discovery in spatial context? (3) How can we standardize the MSI experiment and data analysis workflow to provide robust conclusions for regulation and drug development? With these questions in mind, we provide our perspectives on the future directions of using MSI as a promising tool in toxicology research.

3 | Comprehensive Spatial Mapping of Xenobiotics and Their Metabolism in Situ

One great advantage of MSI in toxicology is its capability to colocalize the xenobiotics and their metabolites in situ with high spatial resolution. Compared to quantitative whole-body autoradiography (qWBA), a standard tool to study drug ADMET in pharmacology, MSI can unambiguously identify xenobiotics and their metabolites with high spatial resolution and is label-free, eliminating the use of radioactively labeled compounds [44, 45]. As an example, using MALDI-MS imaging, Sun et al. [46] showed differential distribution of the drug

pirfenidone and its metabolites in mouse lung and kidney, providing valuable information on drug metabolism in situ in relation to histological features. However, it seems that MSI cannot fully replace qWBA yet due to challenges in quantification in complex tissue matrices (being discussed later) and in effective ionization and identification of xenobiotics and their metabolites.

Many xenobiotic molecules, particularly environmental pollutants, are hard to ionize by common MALDI- and ESI-based MS imaging techniques, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDE), and other persistent organic pollutants (POPs). Due to their nonpolar or low-polar properties and high volatility, these pollutants are commonly analyzed with gas chromatography (GC)-MS with electron ionization (EI) and chemical ionization (CI) techniques [47, 48], whereas unfortunately, EI and CI are not compatible with imaging. Several reports showed that graphene or graphene oxide films can be used as a MALDI matrix for the detection of PAH [49], octachlorodibenzo-*p*-dioxin [50], and nitro-PAH [51]. A recent work by Huang et al. [52] used tetraphenyl phosphonium chloride (Ph_4PCl) as an additive to enhance the electrospray ionization of polyhalogenated compounds. The additive was applied to an air flow-assisted ionization source to map the spatial distribution of chlorinated paraffins and hexabromocyclododecane in exposed zebrafish. Multiphoton laser desorption/ionization has also shown ionization of PCBs and PAHs [53, 54]. Overall, both laser-based and electrospray-based ionization have successfully shown MS imaging for nonpolar and low-polar xenobiotics by developing novel matrices, manipulating laser configurations, and exploring new additives.

In addition, xenobiotics and their metabolism products may have distinct physiochemical properties, making it challenging to have comprehensive coverage for both precursor and products in one MSI run. For example, POPs like PCBs can be metabolized in vivo to hydroxylated forms like OH-PCBs. These metabolites are more polar and commonly analyzed via liquid chromatography (LC)-MS with ESI. Therefore, it is possible to use ESI-based ionization such as DESI to image the POP hydroxylated metabolites in tissues. Recently, Zheng et al. [55] showed that atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) can efficiently ionize POPs such as PAHs and PCBs as well as their hydroxylated metabolites. Thus, although not commercially available, a UV laser ablation (LA)-APCI-MS setup [56] holds great potential to spatially map POPs and their metabolites simultaneously. Notably, the previously mentioned work using Ph_4PCl as an ESI additive achieved simultaneous detection of polyhalogenated xenobiotics and endogenous metabolites of distinct properties, making it a highly promising method for mapping xenobiotics, their metabolites, and endogenous molecules on one sample [52]. It should be noted that the high volatility of many POPs will require careful design of sample preparation for imaging; for example, the common vacuum drying step in MALDI-MSI sample preparation may cause the loss of volatile molecules. Compared to (halogenated) hydrocarbons, drug molecules are usually easier to ionize using MALDI and ESI. MSI of drugs and their metabolites have been reported using MALD-MS imaging [44, 46, 57–59], although the differences in ionization efficiency should be considered to derive quantitative models of drug ADMET.

Without a pre-ionization separation such as chromatography, MS imaging of xenobiotics also faces the challenges of ion suppression from abundant biological molecules, and such effect is sample-dependent. As an example, Li et al. [43] showed detection of similar perfluorooctane sulfonate (PFOS) levels in the kidney, spleen, heart, and brain of dosed animals using LC-MS/MS, but the MALDI-MS imaging results only showed PFOS distribution in kidney, spleen, and heart, not in brain, indicating a tissue-dependent ion suppression on PFOS detection in MALDI-MS imaging. Possible solutions include increasing ionization efficiencies with secondary ionization, developing new MALDI matrices, and on-tissue derivatization. In general, ionization of xenobiotics represents a challenge for comprehensively mapping and colocalizing the spatial distribution of xenobiotic and their metabolites, and future efforts are needed in this direction to enable more effective, efficient, and biological matrix-tolerating ionization and sample preparation methods.

Finally, the isomerism of xenobiotics and their metabolites also poses challenges in the chromatography-free MSI techniques, as mass analyzers cannot simply differentiate isomers with the same chemical formula. Isomers of xenobiotics and their metabolites widely exist. As an example, branched chain PFAS may take up to 60% in abiotic environmental samples and show differential accumulation patterns as well as health effects in biological organisms [60]. Ion mobility spectrometry (IMS), a gas-phase separation technique, represents a powerful tool for isomer separation post-ionization [61, 62]. Recently, Zheng et al. [55] demonstrated ion mobility separation of parent and metabolized PAH, PCB, and PBDE isomers using drift tube IMS, and our group demonstrated the separation of PFOS branched vs linear isomers using MALDI-MS coupled with trapped ion mobility spectrometry (TIMS) [63]. With the development of IMS, we expect combination of MSI and IMS to be applied to resolve the isomerism of xenobiotics and their metabolites in situ.

4 | Uncovering Toxicity Mechanisms and Biomarkers in Highly Heterogeneous Tissues

MS imaging is an emerging tool to understand spatially resolved toxicity mechanisms [37, 41, 64–68]. LC-MS-based proteomics, lipidomics, and metabolomics are widely used to understand the changes of endogenous biomolecules upon xenobiotic exposure; however, the spatial information was lost during sample preparation. MS imaging helps to identify features that changed their levels at different regions. By atmospheric pressure-MALDI-MS imaging, Zeng et al. [68] resolved changes of endogenous lipids in different regions of kidney (cortex, medulla, and juxtamedullary cortex) after acute cadmium exposure. Another work by Liu et al. [37] on MS imaging of zebrafish showed differential patterns of lipids in zebrafish eyes after fipronil exposure. Spatially resolved isotope tracing with MS imaging was also used to discover mechanisms of action of a central nervous system drug [69]. These reports showcase that MS imaging can pinpoint the molecular changes with spatial resolution in highly heterogeneous tissues. It should be noted that compared to LC-MS, MS imaging is less quantitative due to the biological matrix

interference, crystal heterogeneity (MALDI only), and analyte-dependent variations in ionization. Therefore, researchers need to be careful when quantitatively comparing the signal intensities in two tissues sections. Conclusions should be confirmed with enough biological replicates. Several ways to minimize sample-to-sample variation include matching the sectioning plane of control and exposed tissues, mounting the control and exposed sections on the same slide, performing MS imaging in one instrumental run, and randomizing the order of imaging runs.

Compared to MS imaging, LC-MS-based omics approach provides more quantitative analysis and more comprehensive coverage of molecules. Thus, combination of both methods represents a strategy for comprehensive investigations on toxicity mechanisms. Using zebrafish as a model, Ma et al. [64] identified affected metabolites and molecular pathways after indoxacarb exposure with the LC-MS metabolomics data, followed by MS imaging to confirm changes of the identified metabolites in situ in zebrafish livers. A similar approach was used for mouse models after cadmium exposure [67]. In both studies, MS imaging was used to confirm the results from LC-MS metabolomics by showing decreased signal intensities in tissues. An interesting thinking of reversing their roles is to use MSI to identify spatially resolved features as potential biomarkers for xenobiotic exposure and then use (ideally spatial) LC-MS to quantitatively confirm the identity of potential biomarkers with standards, retention time, and fragmentation patterns. This approach focuses on using MSI as the main tool for biomarker discovery, thus reducing the possibility of missing low-abundant but highly localized metabolites using LC-MS approaches. This approach requires researchers to ensure a good coverage of molecular profiles in MSI analysis. For example, multiple matrices and different polarities can be used to increase coverage for MALDI-MSI [68]. In addition, spatial sampling approaches, such as laser capture microdissection (LCM) [70] or liquid microextraction [65], together with small-volume LC-MS analysis, may be considered to catch up with the spatial resolution of MSI when two approaches are integrated.

Another exciting development is the application of MS imaging in protein biomarker discovery for xenobiotic exposure. Meistermann et al. [65] demonstrated the application of MALDI-MSI in the spatial profiling of proteins in kidneys and discovered a protein, transthyretin, as a biomarker for gentamicin nephrotoxicity. The protein signals found in MSI were also confirmed by liquid microextraction on tissue surface followed by LC-MS. An intriguing recent study used MALDI-MSI to monitor drug target engagement by measuring histone poly acetylation, identified by mass shifts, under histone deacetylase drug treatment [66]. Both studies focused on protein biomarkers. Protein MSI is challenging as tandem MS-based sequencing is necessary for protein identification, posing challenges in ionization efficiency, instrument capabilities, and data processing [71]. Thus, to use it as a biomarker discovery tool, integration with LC-MS-based proteomics should be considered for protein identification.

The potential of MSI in understanding spatially resolved toxicity mechanisms has yet to be fully harnessed. In addition to what has been demonstrated, we provide our perspective on its potential future developments. First, MSI can be multiplexed and/or coupled with other imaging modalities to provide multidimensional pictures of xenobiotic toxicity in biological organisms. These developments of multimodal imaging include multiplexing different MSI techniques [72] and/or combining MSI with other imaging modalities such as histopathology and immunohistochemical staining [73], in situ fluorescence hybridization [74], and infrared spectroscopic imaging [75]. Such multimodal combinations will help to link the spatial molecular features detected by MSI to phenotypes (e.g., pathological changes and cell types) and biological endpoints (e.g., gene expression). Secondly, MSI can be applied in causative mechanistic studies, such as profiling xenobiotics and their metabolism after blocking xenobiotic receptors and/or genetically manipulating model organisms. Finally, MSI can be used to elucidate subcellular toxicity mechanisms by further pushing the resolution and sensitivity to resolve the spatial distribution of xenobiotics and endogenous molecules in subcellular compartments, which will be a significant milestone for spatial toxicology studies.

5 | Standardizing MSI Practice for Regulatory and R&D Purposes

Although MSI has proven a powerful tool for spatial toxicology research, it has not yet been systematically incorporated into the pipelines of regulatory and R&D processes. Standardization of MSI practices is mentioned by several reviews as a necessity for MSI to be validated to achieve the metrology and standard needed for regulatory submissions [14, 18, 23]. Many variables exist in MSI workflow from sample preparation to instrumentation. For example, in MALDI-MSI analysis, sample preparation includes tissue freezing, cryo-sectioning, mounting, drying, and matrix application, and instrumental analysis can be done on mass spectrometers with different configurations with tunable laser intensity/profile and ion optics. Whereas these variations make MSI highly tunable and adaptable for various research questions, they also pose challenges in standardizing MSI practices for regulatory and R&D purposes. Guidelines of using LC-MS/MS for measuring drugs and environmental contaminants in different sample types have been developed and validated by authorities such as US Food and Drug Administration and Environmental Protection Agency. MSI methods will need to be similarly standardized in all aspects including sample type, sample preparation, instrumental parameters, and data analysis. And their reproducibility, accuracy, precision, specificity, and sensitivity need to be validated across instruments and sites, in order to make MSI results acceptable for regulatory submissions. Studies on multiplatform and multisite comparisons are valuable in the efforts for standardization. Boskamp et al. [76] tested the site-to-site reproducibility of MALDI-MSI by using a single human teratoma sample and a tissue microarray of tumor samples and comparing MALDI-MSI results obtained in two independent labs with varying protocols. They found that a cross-normalization strategy, which captured and matched the statistical distribution of spectral intensities, can significantly reduce intersample and interlab batch effects and also cross-protocol variations. This cross-normalization represents a promising data preprocessing step for MSI standardization to minimize site-to-site variations. Future multiplatform and multisite research will keep shedding light on the key factors in optimizing MSI standardization.

Achieving quantitative MSI (qMSI) also represents a challenge in standardization. Quantification of xenobiotics and endogenous metabolites is important for studies in xenobiotic ADMET and biomarker discovery. However, qMSI is intrinsically hard due to the spatial heterogeneity of biological matrices on tissue, which affect the analyte ionization efficiency ("matrix effects") and extraction efficiency in situ. Internal standards can be used for relative quantification [77]. For absolute quantification in qMSI, several strategies have been developed to build calibration curves, including in-solution, on-tissue (under-tissue as a variant), and in-tissue [78, 79]. In-solution calibration curves are collected from standards directly spotted onto the target plate/slide, whereas on-tissue strategy spots the standards onto (or under) an untreated, blank tissue section. Compared to in-solution, the on-tissue strategy helps to minimize matrix effects. The in-tissue strategy addresses both matrix effect and analyte extraction efficiency by creating tissue mimetics that are spiked and mixed with different concentrations of standards. However, it is the most time- and sample-consuming strategy. Balancing the pros and cons, on-tissue calibration curves are currently the most common qMSI strategy. Recently, an intriguing machine learning-based virtual calibration qMSI strategy was reported to map the drug distribution in whole-animal sections, which is highly heterogeneous and hard to perform on-tissue strategy [80]. The authors implemented machine learning-based regression models to predict calibration factors for correcting matrix effects and extractability based on endogenous metabolite signals. They successfully demonstrated pharmacokinetic evaluation of drugs in whole-animal sections. This method is yet to be tested in more scenarios, whereas it holds high potential for quantification of xenobiotics and their metabolites in highly heterogeneous samples. Finally, the selection of mass spectrometer also affects the results of quantification. MSI experiments using DESI showed that a triple quadrupole MS provided overall best performance compared to other quadrupole time-of-flight instruments [81]. In general, qMSI is an important part in developing MSI standardization and should be systematically validated for regulatory and R&D purposes.

6 | Outlook

Spatial toxicology represents a subfield of toxicology that investigates xenobiotic ADMET mechanisms in spatial context in cells and tissues. It provides significant insights into xenobiotic-biological interactions in highly heterogeneous biological organisms. Compared to measurement on the ensemble averages of bulk samples, spatial toxicology approaches provide the opportunity to reveal highly localized effects in specific regions in tissues or even cells, aiding accurate assessments of drug safety/efficacy and pollutant risks. MSI, a label-free, highly multiplexed, and high-throughput analysis to measure xenobiotics, their metabolites, and endogenous biological molecules, is a powerful tool to study spatial toxicology. With the development of MSI methodology, instrumentation, and data analysis, together with the integration with other analytical modalities, MSI holds high potential to play a major role for future spatial toxicology research, and standardization of MSI practices will further help this methodology to be validated and integrated for regulatory and R&D practices.

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Conflicts of Interest

The author declares no conflicts of interest.

Data Availability Statement

The dataset used to plot Figure 1 will be available upon request to the corresponding author. No other datasets were generated or analyzed in the current manuscript.

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