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Viewing the Future of IR through Molecular Histology: An Overview of Imaging Mass Spectrometry

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Imaging mass spectrometry (MS; IMS) is an emerging technology that originated at the close of the 20th century with the landmark work of Caprioli et al in 1997 (1). It combines the molecular sensitivity and specificity of MS with the spatial fidelity of classical histology (2,3). Similar to tissue microscopy, IMS is an ex vivo biomedical imaging modality that uses thin tissue sections mounted on glass or metal substrates before analysis. The most important differentiating aspect of IMS compared with traditional liquid chromatography (LC)/MS metabolomics or proteomics is that IMS is performed by collecting spectra from discrete locations across a surface without any previous chromatographic purification, thereby preserving spatial information normally lost by tissue homogenization and molecular extraction. IMS produces high-dimensional data with relative intensity values and x/y coordinates for every molecule detected. Molecular images are produced by generating heat maps of the observed ion intensity over the sample surface for a given molecular species. A single IMS experiment can produce tissue images for hundreds to thousands of molecular species and can be applied to nearly all types of molecules, including drugs, metabolites, lipids, glycans, peptides, nucleotides, and proteins. This overview will cover instrumentation and ionization techniques and the workflow, advantages, and limitations compared with existing histopathologic methods, and will briefly examine some technical aspects inherent in this powerful technology.

INSTRUMENTATION AND DATA ACQUISITION

All mass spectrometers consist of 3 main components: an ion source, a mass analyzer, and an ion detector. Ions produced are subjected to an electric and/or magnetic field in the mass analyzer and are deflected based on their unique mass-to-charge (m/z) ratio. Although any mass analyzer can be used for IMS, the instrumentation requires that the ion source is capable of interrogating the sample surface in a spatially defined manner. Of several methods used, the most common ion source for tissue imaging is matrix-assisted laser desorption ionization (MALDI).

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Appendix A can be found by accessing the online version of this article on www.jvir.org and clicking on the Supplemental Material tab.

As shown in Figure 1, MALDI IMS experiments are performed by cutting fresh frozen or fixed tissue into thin sections and flat-mounting them onto a slide. The sample is then coated with a MALDI matrix, a compound chosen to assist in desorption and ionization of endogenous molecules. Many compounds can function as the matrix, with some more suited for detection of particular analytes than others. Experience often guides selection. Application of matrix in a spatially uniform and reproducible way is critical. This is done by sublimation or with the use of a robotic sprayer. Although beyond the scope of this short overview, the MALDI matrix is usually a small organic compound that is chosen for its ability to (i) extract molecules of interest from the sample into the matrix layer during application, (ii) absorb energy from the laser strike, causing desorption into the gas phase, and (iii) facilitate efficient ionization of target molecules with minimal fragmentation.

During acquisition, spectra across a defined mass range are collected by laser irradiation at discrete x/y sample coordinates. The laser is most often stationary and the sample is translated (“rastered”) to generate spectra at all x/y coordinates across the specimen. Composite signal-intensity pixel maps corresponding to intensity of a particular mass peak are then plotted across the sampled area, creating images. Commercial IMS platforms can achieve spatial resolutions from 10 μm to 200 μm , and custom instruments have demonstrated imaging resolutions as fine as 1 μm . A single MALDI IMS experiment can produce a data set yielding thousands of images (one from each m/z peak in a spectrum). These can be compared with histologically stained sections of the tissue, providing multiplexed molecular information that complements classical histologic analysis.

DATA ANALYSIS AND EXPERIMENTAL DESIGN

An example MALDI IMS data set is shown in Figure 2, highlighting selected protein images generated from human clear-cell renal cell carcinoma (ccRCC) tissue in a discovery-based, top-down analysis. The data shown are just 4 of the > 100 proteins detected in this single MALDI time-of-flight IMS experiment. In view of the “big-data” characteristics of IMS, significant effort has been put toward data visualization and analysis. IMS data undergo a number of spectral preprocessing steps to minimize signal artifacts and variation resulting from instrumentation and sample preparation (eg, peak intensity normalization and m/z ratio calibration). To address the high degree of molecular complexity and data dimensionality, supervised and unsupervised machine learning approaches are commonly applied. Supervised approaches are generally “classification” tasks (eg, tumor class prediction) in which, for example, a pathologist will label data from a number of patients as representative of “healthy” or “diseased.” These data are then used to build a computational model that can predict healthy versus diseased status for other patients. Unsupervised machine learning approaches (eg, principal component analysis), alternatively, do not require any *a priori* knowledge of the sample and are simply tasked with discovering underlying trends in the data to help interpret highly complex IMS data.

Experimental design of IMS experiments generally falls into 2 categories: discovery-based and targeted experiments. In discovery-based experiments, the question(s) may be specific, but the molecules to be queried are unknown at the outset. This is typically the case, for example, in biomarker discovery or spatial proteomics analysis. In these workflows, IMS

data are first collected, and then supervised or unsupervised analysis methods are used to filter the most prominent peaks associated with diseased areas of tissues (ie, biomarkers) or proteomic signatures of various substructures within the tissue. When molecular signatures have been determined, ion images are generated. Such peaks are identified by querying molecular databases by using mass accuracy information (ie, putative identification) or through separate MS/MS fragmentation experiments (ie, structural identification). In targeted analysis, the specific molecule (eg, a drug), and therefore the mass, is known, and the m/z ratio can be predicted at the outset. Experiments are then tuned to maximize sensitivity and specificity for each target molecule, and questions are likely to be focused more on distribution, quantification, metabolism, and biologic effects. In some targeted experiments, MS/MS is performed during the imaging experiment to enable the spatial differentiation of structural isomers and maximize molecular specificity for the target molecules. When this is not the case, separate fragmentation experiments (eg, LC-MS/MS) are performed to validate identifications.

ADVANTAGES AND LIMITATIONS

A significant advantage IMS brings is the “agnostic” nature of analysis. Because tissues are imaged by using a mass spectrometer, the technology is completely label-free. This dramatically widens the pool of potential analytes and enables investigations that would otherwise not be feasible with traditional tissue imaging modalities. No isotopic labels are required, no antibodies are used, and no radioactivity is involved. This eliminates the need for special synthesis of heavy labeled targets, and the logistics associated with unstable isotopes with short half-lives are not an issue. Intrinsic fluorescence or covalently attached fluorescent tags are likewise immaterial. Similarly, there is no dependence on antibodies for a specific antigen as needed in immuno-fluorescence or immunohistochemistry. This avoids the vagaries of antibody sourcing and the sensitivity variations that may occur as a result.

An important and very basic difference compared with most staining technologies is that all of the data are acquired in a single scan, which can be archived and computationally mined for further study at any future time as new questions arise. This unique multiplex advantage allows for thousands of metabolites/lipids and hundreds of proteins to be imaged in a single experiment. Additionally, IMS is unique in that it also allows for molecular modifications (eg, downstream metabolites, lipid modifications, and posttranslational protein modifications) to be visualized independently and simultaneously from unmodified precursors. For example, IMS is capable of detecting differences in spatial localization within tissue for a given protein and its various proteoforms. This is a substantial improvement over the traditional “one question/one slide/one stain” paradigm or even modern multiplexed immunofluorescence or immunohistochemistry experiments that use several antibodies on a single tissue. Finally, method development and sample preparation is relatively straightforward: frozen tissue requires no other preparation before matrix application for drug, metabolite, and lipid analysis.

A practical issue to bear in mind is that higher spatial resolution IMS comes at the price of increased acquisition time (more pixels are required for a given area) and decreased sensitivity (less material in a smaller pixel) in some cases. Fortunately, speed and sensitivity

are continuously being advanced by instrument vendors and research laboratories. For example, there are now high-performance commercial MALDI IMS platforms that can collect more than 50 pixels per second, allowing most IMS acquisitions on these instruments to be completed in less than 1 hour.

One aspect that newcomers to the field may have difficulty appreciating is ionization. Not all analytes will ionize efficiently, which affects sensitivity, and not all of those that do will ionize proportionately to the amount that is present relative to other species. Sensitivity may be low for targets that ionize poorly. Changing matrices may improve this, but the search for an optimal matrix may require more resources. In addition, although producing relative quantification imaging data is straightforward, the achievement of absolute quantitative results is not trivial. This requires significant method development, internal standards, and the generation of appropriate quantification curves. If absolute quantification is required, LC-MS is often used to confirm imaging results.

RELEVANCE TO INTERVENTIONAL RADIOLOGY

In the field of interventional radiology, IMS has the potential to improve understanding of biology, physiology, and pharmacology of interventions such as ablation, embolization, drug-coated balloon angioplasty, and drug-eluting stent placement. IMS could be used to visualize a drug delivered into tissue such as an antineoplastic or anti-inflammatory agent. Immunotherapies likewise will benefit, as IMS can depict drugs directly injected in tissue regardless of whether they are fluorescent. Biomarker discovery is another important area in which IMS will likely have an impact. The knowledge gained may well influence how therapies are delivered in the future and will likely lead to new therapies altogether.

FUTURE DIRECTIONS AND SUMMARY

Next-generation molecular imaging, an emerging area of research, is the synergistic fusion of IMS data with other imaging modalities by using machine learning. As recently demonstrated through the combination of IMS and microscopy, data-driven multimodal image fusion can predict molecular distributions in tissue by combining the molecular specificity and coverage of IMS with the spatial resolution of stained microscopy (4). This provides new potential for deeper data mining and predictive applications (eg, spatial sharpening and out-of-sample prediction) through the combination of complementary biomedical imaging technologies. Finally, the data set can be permanently archived and accessed for unlimited new queries without perturbing or consuming the original sample. Therefore, IMS is a powerful new tool that has untapped potential to make unique, substantial contributions covering multiple aspects of interventional radiology. The possibilities of IMS in the era of image-guided precision medicine are only just beginning to be appreciated.

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ABBREVIATIONS

ccRCC	clear-cell renal cell carcinoma
IMS	imaging mass spectrometry
LC	liquid chromatography
<i>m/z</i>	mass-to-charge

MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry

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Figure 1.

Overview of the MALDI IMS workflow. After sectioning and mounting, a matrix is applied, and a laser creates an ionization plume at each spot. Ions enter the mass analyzer, and spectra are acquired from the mixture at each location. Composite images are generated, and data are then analyzed.

Figure 2.

MALDI time-of-flight protein imaging data collected from human ccRCC tissue. **(a)** Hematoxylin and eosin–stained kidney tissue shows morphologic malformation resulting from ccRCC with clear visual differences between healthy and tumor regions of the tissue. **(b)** IMS data for Thymosin β 10 (m/z ratio, 4,940; yellow), Calcyclin (m/z ratio, 10,097; teal), Histone H2B (m/z ratio, 13,784; pink), and Hemoglobin subunit β (m/z ratio, 15,858; red). IMS data were collected on a Bruker rapifleX MALDI TissueTyper in positive ion mode. The image was collected at 30- μ m spatial resolution and has 115,047 total pixels. Samples were collected from patients undergoing nephrectomies and obtained through the

National Institutes of Health Cooperative Human Tissue Network and the Vanderbilt University Ingram Cancer Center-Human Tissue Acquisition and Pathology Resource.

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