Clinical Chemistry 65:4
510–513 (2019)

Q&A

Mass Spectrometry-Based Tissue Imaging: The Next Frontier in Clinical Diagnostics?

Moderators: Felix Leung[1] and Livia S. Eberlin[2*]
Experts: Kristina Schwamborn,[3] Ron M.A. Heeren,[4] Nicholas Winograd,[5] and R. Graham Cooks[6]

The diagnosis of tissue samples traditionally has been performed by anatomic pathologists using a combination of cellular staining and light microscopy. With these techniques, pathologists can characterize various tissue features including cell morphology, structure, and composition to subsequently confirm whether a disease process is present. Although the histopathologic "gold-standard" methods are invaluable for routine tissue diagnosis, the results can be subjective, owing to a combination of factors such as variability in staining quality, nature of the sample, and human interpretation, and inconclusive for diseases that present indistinguishable histologic features. There is a need for new technologies that can be used as complementary tools in pathology for objective tissue analysis and disease diagnosis.

Mass spectrometry (MS)[7] imaging has been heralded as an upcoming advance in tissue analysis. The ability of MS to rapidly identify a variety of biomolecules present in a sample is highly attractive to the clinical laboratory. Indeed, MS coupled to chromatographic separation techniques is currently used to detect and/or quantify small molecules such as pharmacological agents and hormones in blood and urine. The advent of MS techniques that allow direct tissue analysis, including MALDI-MS and secondary ion MS, has allowed laboratories to extend the use of MS beyond biofluids into tissue samples. Using MALDI MS, for example, thin tissue sections can be analyzed in a nontargeted manner for the abundance and spatial distribution of biomolecules. As such, MALDI-MS imaging is being increasingly applied in clinical research, especially in the context of cancer diagnostics based on tissue proteomic and lipidomic signatures.

The potential of MS in revolutionizing tissue analysis and diagnosis has driven several advances to accelerate its feasibility and utility in the clinical setting. The development of ambient ionization MS (AIMS), for example, has brought MS-based tissue imaging even closer to routine clinical use. Unlike MALDI and secondary ionization MS (SIMS), AIMS techniques, such as desorption electrospray ionization (DESI), allow for tissue analysis in an open environment at atmospheric pressure, providing real-time assessment of tissue molecular composition. In the envisioned situation, clinicians and laboratorians could perform rapid clinical assessment of tissue biopsies to improve and expedite diagnosis, as well as to potentially guide intraoperative tumor excision.

Here, we discuss the rapidly evolving field of MS-based tissue imaging with a few of the top experts in the field, as well as the challenges still faced in terms of implementing this technology in the clinical laboratory.

How is MS currently used with respect to tissue imaging and what are the limitations that are preventing its routine use in the clinical laboratory?

Kristina Schwamborn: Up until now, many MS imaging studies have addressed clinical questions mostly related to differentiating healthy from cancerous tissue. From my point of view, there are 3 limitations that still prevent clinicians and pathologists from integrating MS into their clinical routine, thus hampering its use in the clinical laboratory. Firstly, in many instances, the question addressed (e.g., healthy vs cancer) is fairly uncomplicated for trained pathologists even with a simple hematoxylin and eosin stain. Secondly, most studies have been based on com-

[1] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada; [2] Department of Chemistry, The University of Texas at Austin, Austin, TX; [3] Senior Physician, Institute of Pathology, Technische Universität München, Munich, Germany; [4] Director, The Maastricht MultiModal Molecular Imaging Institute, Maastricht University, Maastricht, the Netherlands; [5] Professor, Department of Chemistry, Pennsylvania State University, University Park, PA; [6] Professor, Department of Chemistry, Purdue University, West Lafayette, IN.

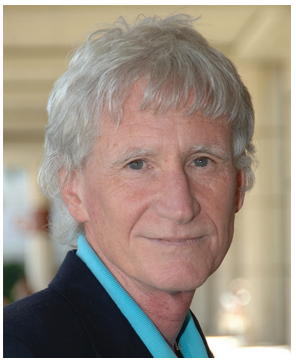
* Address correspondence to this author at: Department of Chemistry, University of Texas at Austin, 105 E 24th St., Austin, TX 78712. E-mail liviase@utexas.edu.
Received November 26, 2018; accepted January 11, 2019.

[7] Nonstandard abbreviations: MS, mass spectrometry; AIMS, ambient ionization MS; SIMS, secondary ionization MS; DESI, desorption electrospray ionization; FFPE, formalin-fixed, paraffin-embedded.

paratively small cohorts, thus limiting the statistical power. Thirdly, the majority of studies have been performed in a single laboratory, thus questioning reproducibility of the results in external laboratories.

Photo courtesy of Harry Heuts.

Ron Heeren: MS imaging is the main segue into molecular pathology for rapid tissue-based diagnostics. Whether it is disease staging, tumor margin assessment, therapy prediction, or cellular phenotyping, it is evident that MS imaging is making a stronger and stronger impact on the clinical decision-making process. The breadth of molecular information it can uncover in a single experiment is still unsurpassed. In my mind, the current limitation is still throughput. Although we are capable of generating hundreds of pixels per minute at this stage, we are still far away from the speeds of 1 slide per minute currently used in routine digital optical pathology. As speed, resolution, and sensitivity are intimately linked, these are all MS imaging parameters that merit combined improvement.



Nicholas Winograd: MS imaging determines spatial localization of molecular species directly from the sample—something not possible with tissue homogenates and extractions. The big limitation of any MS-based assay is the need for validated biomarkers.

Graham Cooks: One needs to be careful not to underestimate the power of existing methods. Immunohistochemistry gives very valuable molecular information, typically at the nucleic acid level, and is itself a rapidly developing diagnostic method. The information provided by MS is acquired rapidly and has great molecular detail, but it is often not quantitative. It is not clear to me that there are significant limitations which prevent more widespread use.

What are the major preanalytical concerns for MS tissue imaging? For example, what are the analytical issues surrounding analysis of formalin-fixed, paraffin-embedded (FFPE) tissues and what strategies are being investigated to adapt MS imaging to FFPE tissues?

Kristina Schwamborn: Many MS imaging studies have demonstrated that FFPE samples can generate reliable results similar to fresh, frozen tissue samples especially with the advance of on-tissue (in situ tissue) tryptic digestion. However, there are still two major points of concern regarding analysis of FFPE samples. Firstly, due to additional sample preparation steps (e.g., antigen retrieval and on-tissue digestion), a considerable amount of variation can be introduced, rendering interlaboratory data difficult to compare. Secondly, the additional sample preparation steps can significantly prolong the turnaround time, thus limiting the clinical utility if time-to-result is critical.

Ron Heeren: FFPE analysis with MS imaging is considered indispensable for its clinical implementation. The availability of historical collections of preserved and "fixed" tissue is almost a molecular museum of humanity. It has the potential of being an enormous information resource for clinical diagnostics, in particular for retrospective studies. One of the major challenges is the fact that different organizations prepare and store their FFPE material very differently. As recently pointed out in a round robin study, it is difficult to mitigate this preanalytical heterogeneity, which is convolved with the tissue heterogeneity that MS imaging researchers are looking to understand and put to use. The additional challenge is that small molecules are imaged with more difficulties in FFPE due to the process of paraffin removal and washing, which can concomitantly remove small compounds of interest. Recent development of direct metabolite and direct neuropeptide imaging seem to offer potential new avenues, but these have not been picked up by the community at large. More surface-sensitive methods such as SIMS suffer from extreme sensitivity to surface contamination, as they only examine the top 50 nm of a surface. This is where most of the embedding material ends up after sectioning. Sputter cleaning protocols offer amelioration of the problem but increase the analysis time. AIMS technologies could be helpful in this regard but still have to prove themselves for this purpose. FFPE material is demonstrated to be useful for abundant protein and glycan analysis using a variety of enzymes. Multiplexed enzyme strategies are routes that various researchers are investigating. Ultimately, FFPE material is

the substrate of choice for immunohistochemistry. MS imaging, particularly imaging mass cytometry, offers an approach for targeted, multiplexed protein imaging. This is an area in which I expect extensive development, as it already has been demonstrated to be strongly complementary to the discovery-oriented, untargeted incarnations of MS imaging.

Nicholas Winograd: Intact proteins are not able to be imaged with FFPE tissues. However, analyte retrieval and subsequent on-tissue digestion will allow tryptic peptide imaging.

Graham Cooks: A significant concern is to know where imaging is taking place. One method is to cross index with preoperative MRI. Formalin fixing changes the chemical nature of the sample, while paraffin embedding adds large amounts of matrix that contribute to the mass spectra. Neither is a first choice for the mass spectrometrist. There might be workarounds but the current direction of MS imaging to explore fresh tissue in depth seems particularly appropriate.

MS tissue imaging often revolves around protein and peptide analysis. How has the technology evolved beyond this and what are the challenges with analyzing nonprotein biomolecules?

Kristina Schwamborn: Nonambient as well as ambient ionization technologies have demonstrated the utility of analyzing small molecules such as lipids and metabolites to characterize tissue samples. Notably, studies using lipid profiles have achieved excellent classification results. However, apart from the same challenges that protein-based MS analyses are facing (e.g., reproducibility between different laboratories), validation of nonprotein biomarkers is less "straightforward." For example, validation of lipid biomarkers is more complex than protein biomarkers and validation of metabolite biomarkers is difficult due to rapid changes depending on ischemia time.

Ron Heeren: Looking at the literature, lipid and, more recently, metabolite imaging have all played an equally important role throughout the development of MS imaging. The challenges with nonprotein biomolecules are similar to those experienced with protein analysis. Any sample treatment potentially removes or delocalizes compounds, but, because small molecules are more susceptible to solvent effects, this effect might have a bigger impact. The use of fresh frozen sections has a clear advantage there, especially for intraoperative imaging diagnostics. New protocols that enable the analysis of peptides and metabolites directly from FFPE material without on tissue digestion are slowly making their way into the field.

Nicholas Winograd: The high abundance, low mass, and easy ionization of lipids have made lipid imaging just as popular as protein analysis in the last decade. The biggest disadvantage is that the most abundant species detected, such as dipalmitoyl phosphatidylcholine—PC(16:0/16:0), are not often the most biologically interesting species. Drug localization is also a major application.

Graham Cooks: The evolution has been towards analysis of small molecules, lipids and metabolites, which provide more direct contemporaneous information on the disease state of the individual.

What are the benefits and limitations of MALDI MS-based tissue imaging and how do alternative methods such as secondary ion MS and AIMS compare to MALDI MS?

Kristina Schwamborn: MALDI MS offers the capability of analyzing multiple proteins/peptides/lipids/metabolites in parallel without the need for targeting specific molecules. Thus, in a single experiment (using a single tissue section), a panel of potential biomarkers can be discovered without relying on antibodies. As a result, more tissue can be conserved for potential additional testing such as genomic analyses in the clinical workup. However, compared to traditional (immuno)histochemical analyses that characterize tissues beyond the level of a single cell, most MS imaging approaches are unable to provide that level of resolution. SIMS, however, can attain that level of resolution.

Ron Heeren: The main drawback of MALDI remains the fact that a matrix needs to be applied. The application process can result in delocalization, limit spatial resolution, and, for some matrices, results in a nonstable surface prone to evaporation of the matrix during the MS imaging experiment. SIMS and IMS technologies do not suffer from these phenomena. SIMS has the main advantage of extreme spatial resolution and new beam technologies can now also desorb and ionize larger molecules. The AIMS technologies offer molecular imaging capabilities for nonvacuum compatible samples and, as such, open up completely new imaging application domains.

Nicholas Winograd: Obviously the improved spatial resolution of SIMS is a great advantage over MALDI. Depending on the ion source, submicron imaging is achievable along with depth profiling with a resolution of tens of nanometers. Considering the practical limitations of MALDI with the laser footprint and matrix cluster size that usually limit it to 5- or 10-micron spatial resolution, there is no practical way to perform depth profiling with MALDI. However, most SIMS ion sources induce a de-

gree of prompt fragmentation, effectively limiting the mass range to below *m/z* 1000. The advent of the gas cluster ion beams has markedly improved this, extending the mass range to above *m/z* 2000 and a spatial resolution in the range of 1 micron.

Graham Cooks: The fact that this is a useful question is a testament to the shared advantages of the various MS imaging methods. The list of those common advantages is long. Beyond that, MALDI has excellent spatial resolution, a large body of experienced practitioners, and solid data handling methodology and archival database. Ambient methods have advantages of speed and convenience in working in open air.

The ability to perform real-time MS imaging analysis for clinical use would require considerable information technology support due to the data generated from the MS and the need to document the relevant information into the laboratory and/or health information system. What challenges do you foresee in these regards to implementing MS-based imaging into clinical use?

Kristina Schwamborn: In my point of view, there are still 3 major challenges that real-time MS imaging faces. Firstly, on-tissue resolution might not be sufficient for all types of tumors since some do not grow as bulk tumors but rather as single dispersed cells. Secondly, providing only a tumor-free margin is not always sufficient for adequate tumor resection. Occasionally, resection of specific tumors will require a certain distance (e.g., 0.5 cm) between the tumor-free margin and the actual surgical resection margin. Thirdly, regarding biocomputational analysis, most classification models are trained for a certain number of conditions (e.g., healthy vs tumor type A vs tumor type B). However, the presented tumor may not always belong to any of the pretrained categories (e.g., lymphoma or sarcoma instead of adenocarcinoma) and, thus, may not be classified properly potentially leading to inappropriate treatment.

Ron Heeren: One of the challenges is the "big data" challenge. As the MS imaging technologies are improving resolution and throughput, the data sizes are increasing exponentially. Real-time MS-imaging analysis also requires the real-time consultation of large databases or high-speed computing. Although approaches exist that can build classification models to assist this process, these are still in their infancy. The challenge of implementing new data science strategies for real-time clinical diagnostics is only just beginning to be addressed. Innovations in the field of machine learning and artificial intelligence that will positively affect the clinical implementation of MS imaging are imminent. They will pave the way for full integration of MS imaging as a multiplexed clinical assay in digital pathology.

Nicholas Winograd: Again, validated biomarkers are needed for MS-based assays. Near real-time analysis has been done with a turnaround time of 30 minutes or less. "Real time" MS imaging would depend largely on the mode of ionization—there has been some progress with direct-ionization probes (i.e., DESI, direct analysis in real time, liquid extraction surface analysis). Such techniques have less fine spatial resolution than MALDI or SIMS, but still fine enough for practical problems such as tumor margins.

Graham Cooks: The challenges here are large but they are being encountered at a time of unprecedented advances in data processing online, in data storage, and manipulation through sophisticated multivariate methods. The attention being given to "big data" is encouragement that the challenges will be met.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: R.M.A. Heeren, Maastricht University.
Consultant or Advisory Role: None declared.
Stock Ownership: R.M.A. Heeren, The Dutch Screening Group.
Honoraria: None declared.
Research Funding: R.M.A. Heeren, The LINK program of the Dutch province of Limburg funding to institution.
Expert Testimony: None declared.
Patents: L.S. Eberlin, US 15/692,167, US 62/591,179, US 62/640,385.

Previously published online at DOI: 10.1373/clinchem.2018.289694