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Multimodal Imaging Mass Spectrometry: Next Generation Molecular Mapping in Biology and Medicine

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ABSTRACT: Imaging mass spectrometry has become a mature molecular mapping technology that is used for molecular discovery in many medical and biological systems. While powerful by itself, imaging mass spectrometry can be complemented by the addition of other orthogonal, chemically informative imaging technologies to maximize the information gained from a single experiment and enable deeper understanding of biological processes. Within this review, we describe MALDI, SIMS, and DESI imaging mass spectrometric technologies and how these have been integrated with other analytical modalities such as microscopy, transcriptomics, spectroscopy, and electrochemistry in a field termed multimodal imaging. We explore the future of this field and discuss forthcoming developments that will bring new insights to help unravel the molecular complexities of biological systems, from single cells to functional tissue structures and organs.

I. Introduction

Imaging mass spectrometry (IMS) is a technology that enables the mapping of hundreds to thousands of molecules within biological systems.[1-6] The instruments within the field can measure a diverse array of sample types and chemical classes, ranging from low molecular weight metabolites[7-10] and signaling molecules[11-13] to lipids,[14-21] peptides,[22-24] and proteins.[25-29] A typical IMS experiment involves defining an area of the sample surface to be imaged followed by the desorption and ionization at multiple discrete locations. Each desorbed location, or pixel, is composed of an individual mass spectrum. A molecular image is generated for each signal recorded by plotting the ion intensity of that signal throughout the array of pixels generated from the sample. While there are many types of IMS technologies that have been reported in literature, we highlight here the three most common: matrix-assisted laser desorption/ionization (MALDI),[30,31] secondary ion mass spectrometry (SIMS),[32-34] and desorption electrospray ionization (DESI).[35-38] Additional information on other types of IMS technologies can be found in several recent reviews.[1,4,39]

Each IMS technology can be coupled to a variety of analytical approaches to enhance the information gained in a single experiment. Although IMS provides rich, chemically informative spectra and mapping capabilities, combining this unique mass specific technology with other analytical approaches can provide additional information for an analyzed sample. Combining two or more imaging modalities is termed multimodal imaging. The accrued advantages include enhanced discrimination between modality specific chemical and instrumental noise from biologically relevant chemical signals, improved sensitivity and specificity of chemical classes not easily analyzed by a single modality alone, and enhanced data mining capabilities. Combining IMS technologies with other analytical approaches provides a more effective means to probe the molecular complexity of biological systems. In this review, we summarize major advancements in the field of multimodal imaging mass spectrometry over the past few years and discuss the exciting future of this field.

II. Overview of Imaging Mass Spectrometry Technologies

a. Matrix-Assisted Laser Desorption/Ionization

MALDI is a common ionization method within the IMS field as it can visualize numerous molecular species over a broad mass range with great molecular diversity. For tissue analysis, sample preparation typically involves sectioning of tissues and thaw mounting these sections onto a glass slide or other target (e.g. conductive surface for high voltage ion sources) and subsequent application of an organic chemical matrix that aids in analyte desorption and ionization.[40] Spatial resolution is defined by the ablation area of the laser and the distance between pixels (pitch). A variety of matrices are used for specific molecular classes. Commonly used matrices include 2,5-dihydroxybenzoic acid[41] or cyano-4-hydroxycinnamic acid[42] for positive ion mode analysis of metabolites, lipids, and peptides. Additionally, 9-aminoacridine[43] is typically used for negative ion mode analysis of metabolites, lipids, and proteins, while 1,5-diaminonaphthalene[44] is employed for analysis of lipids in both ion polarities. Recently norharmane has been shown to be effective for the analysis of hydrophobic molecules and low

molecular weight metabolites.[45] Matrix deposition is often performed robotically to best balance reproducibility, analyte extraction, achieve small crystal size, and produce coating homogeneity, although sublimation, sieving, and airbrushing is also used in the field.[4,46]

As high spatial resolution MALDI IMS capabilities are developed that approach cellular and subcellular resolutions, challenges in sensitivity arise as the number of molecules sampled decreases with smaller pixel sizes. Ultimately, spatial resolution is dependent upon multiple factors such as matrix crystal size, laser focus, and stage motor step precision. Most modern MALDI IMS platforms utilize either a frequency tripled Nd:YAG (355 nm) or nitrogen gas laser (337.7 nm) and can achieve spatial resolutions of 5-20 µm using traditional front-side laser optics.[52-54] Improving laser focus beyond 5 µm can be achieved with more advanced setups using lower laser wavelengths (e.g. 213 nm) or changing laser geometries (e.g. transmission-mode), to minimize the effective spot size as described in the next several examples. For instance, Heiles et al. demonstrated a new source that integrates a 213 nm laser allowing for a ~3 µm footprint using front-side laser optics.[55] Alternatively, Zavalin and coworkers developed a transmission-mode geometry MALDI source, where the UV-laser is redirected to ablate the sample from the backside. By decoupling the laser and ion optics, higher numerical aperture objectives can be utilized without impeding the ion path, resulting in ~1 µm spot sizes.[56] However, a drawback of any high spatial resolution IMS experiment is a reduction in ion abundances; the Caprioli[57] and Dreisewerd[47] labs incorporated a secondary laser perpendicular to the primary ablation plume as a means to enhance ionization of transmission geometry setups. An example of these datasets is featured here (Figure 1A).

b. Secondary Ion Mass Spectrometry

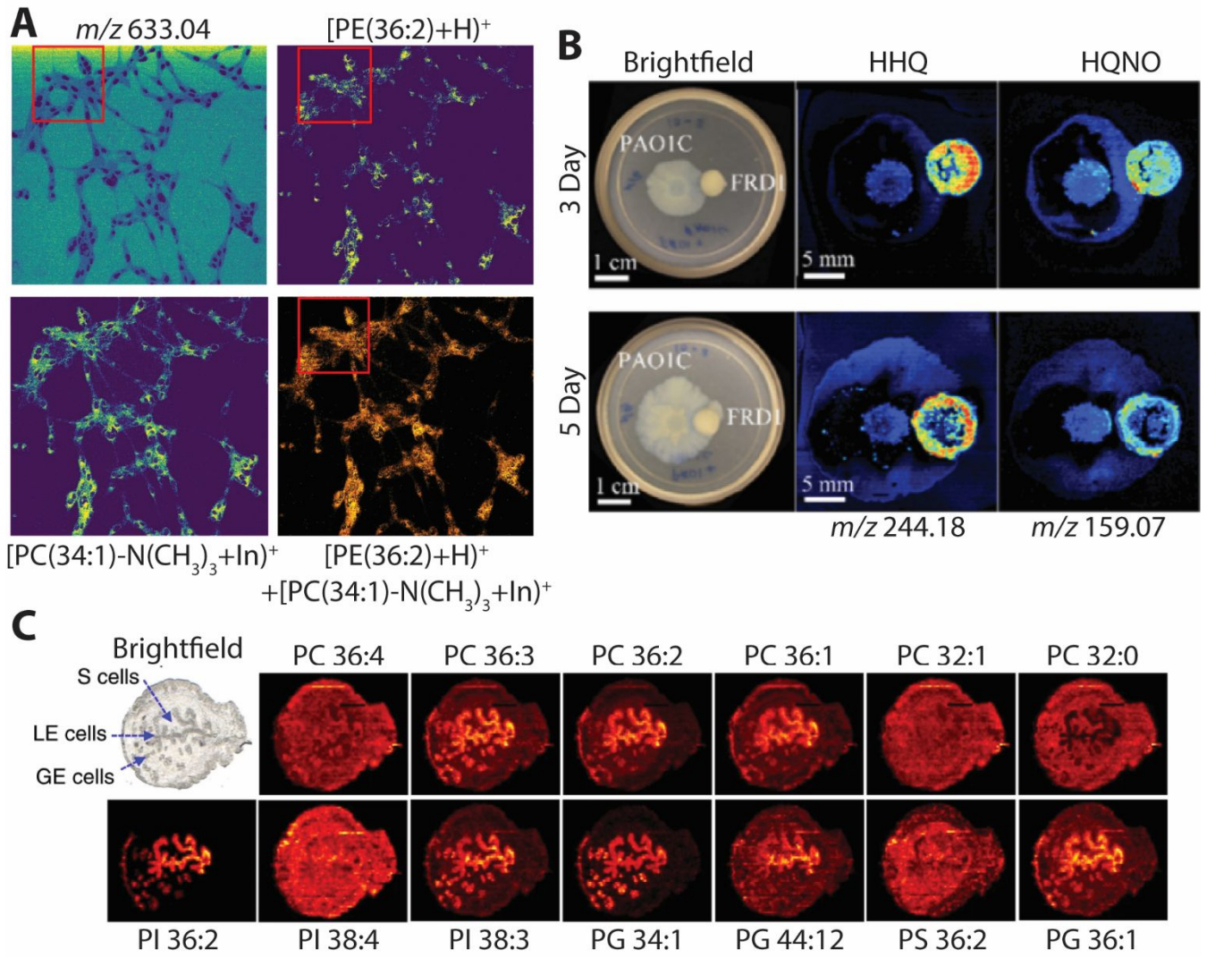

Figure 1. Selected developments within the IMS community. Image of a Vero B cell culture at 1 µm spatial resolution acquired with transition mode MALDI-2 IMS developed by the Dreiswerd lab (**A**). Panel A is adapted with permission from ref[47], Niehaus, M. *et al*. *Nat Methods* **16**, 925–931 (2019). Copyright 2019 Nature Publishing. SIMS image of a coculture of different *Pseudomonas aeruginosa* stains visualized with different signaling small molecules (**B**). Panel B is adapted with permission from ref[24,48], Cao, T. *et al*. *SPIE BiOS* **108630A** (2019). Copyright 2019 SPIE. Digital Library. Lipid images both positive and negative mode of the human eye using nanoDESI at 10 µm spatial resolution (**C**). Panel C is adapted with permission from ref[49-51], Yin, R. *et al*. *Nature Protocols* **14**, 3445-3470 (2019). Copyright 2019 Nature Publishing.

SIMS utilizes electrostatically focused primary ions (e.g. Bi^+, Cs^+, and O^-) or clusters (e.g. Au_3^+, C_{60}, and Ar) to impact the sample surface, causing a collisional cascade in the top few monolayers of the sample leading to the ejection of secondary ions.[2,34] Because of the high energy of the ion beam, analyte ions often undergo significant fragmentation and analysis is typically limited to molecular weight ions <2 kDa.[58] The energy of the ion beam plays a critical role in sampling and can be divided into two regimes: static and dynamic SIMS. Static SIMS is defined by low primary ion doses ($<10^{13}$ cm^{-2}) and beam currents (pA-nA) suitable for surface analysis of elements and molecules. Alternatively, dynamic SIMS has much higher primary ion doses ($>10^{13}$ cm^{-2}) and beam currents (mA), making it suitable for depth profiling and three dimensional imaging.[59] In general, sample preparation for SIMS analysis is minimal and consists of mounting tissues flatly onto conductive targets and drying prior to introduction into the source vacuum chamber.[60,61] Although not required, washing samples to remove salts from tissue prior to analysis can improve ion yield.[62,63]

The primary advantage of SIMS is its high spatial resolution capabilities, because ion beams can be tightly focused using electric fields (1 µm to 30 nm).[32] Similar to MALDI, SIMS spatial resolution is defined by the diameter of the ion beam at the surface and the pitch. SIMS has been used for cellular and subcellular analyses.[64-67] and for the determination of molecular profiles of various disorders including different cancer types[68] and cardiovascular disease.[69] It has also been used to monitor signaling between bacterial cocultures and distinct alkyl quinolone messengers between different stains of *Pseudomonas aeruginosa* (Figure 1B).[48] Additionally, SIMS was employed to visualize salt redistribution in brain tissue between healthy and stroke mice.[70] While effective for low molecular weight metabolite and lipid analyses, it has not been commonly applied to peptide and protein imaging studies.

c. Desorption Electrospray Ionization

Desorption electrospray ionization (DESI) is performed by spraying charged solvent droplets on the surface of the sample where the analytes are desorbed and ionized for subsequent detection by MS.[71] The imaging experiment is performed in a continuous raster sampling mode where the target is moved continuously under the DESI spray in a 'typwriter-like' motion. Spatial resolution is estimated by the target stage velocity, sampling rate of the mass spectrometer, number of spectra averaged for a single pixel, and distance between adjacent line scans. Although DESI has limited spatial resolution capabilities (~150 µm), it requires minimal to no sample preparation. Tissues sections are mounted onto glass slides and sampling is performed at ambient pressure. Recently, the Laskin lab developed a modified form of this sampling approach, termed nanoDESI, using a liquid microjunction to increase spatial resolution to ~10 µm (Figure 1C).[49] While nanoDESI is not discussed in a multimodal imaging context here, we eagerly anticipate the higher spatial resolution of nanoDESI being coupled to other modalities. Moreover, DESI and nanoDESI been used to map metabolite,[72-74] lipid,[75-78] and drug distributions in a variety of biological systems ranging from plants to diseased mammalian tissue.[79-81] Further, additives in DESI solvent can target specific molecules in the case of reactive DESI[82] or enhance extraction.[72]

DESI and nanoDESI IMS are minimally destructive techniques and have been used experimentally in surgical settings to enable intraoperative molecular assessment and aid in real-time intraoperative decisions.[36,83,84] Sans et al. molecularly characterized high-grade serous carcinoma, serous borderline ovarian tumors and normal ovarian tissue samples using DESI IMS.[85] They identified predictive markers of cancer aggressiveness and built classification models to enable diagnosis and prediction of high-grade serous carcinoma in comparison to normal tissue with a high certainty of ~96 %. Similar work from the Eberlin lab has led to the development of a handheld mass spectrometry device, the MasSpec Pen, which enables *in vivo* diagnostics during surgery.[86,87,88] This device has been used for classifying ovarian[89] and breast cancer[90] since it was originally developed. Because it can be operated at atmospheric pressure and requires minimal sample preparation, DESI holds promise for use in clinical and surgical settings.

III. Combining Multiple Imaging Mass Spectrometry Technologies

Since each IMS technology has unique performance characteristics for different molecular classes, there is utility in coupling them together. Technologies such as tandem MS, microextractions, and ion mobility each add additional dimensions to the MS dataset, expanding upon the chemical information that can be obtain, either by enabling *de novo* identification or reducing spectral complexity.

a. Spatially Targeted Tandem MS

Tandem mass spectrometry enables *de novo* identification of molecules within complex samples.[91-93] Tandem MS is generally accomplished by performing isolation within one mass analyzer for subsequent fragmentation and transmittance into another mass analyzer for detection.[94] Serially performing these analyses allows for identification of discrete molecules within a biological sample.[95,96] In the context of imaging, pixels can be subdivided allowing for a precursor ion scan and subsequent MS/MS scans. This spatially targeted structural information comes at the cost of spatial resolution to accommodate multiple samplings. Additionally, it is often difficult to perform tandem MS analysis within an imaging experiment if the ion of interest is only present within a small number of disperse features or pixels.[17] Alternative means of targeting sample regions or locations for identifying key ions include multimodal image-guided surface sampling,[97,98] microprobe extraction for offline tandem MS,[99-101] and tandem MS on an orthogonal sample.[16,30] By using these various strategies, ions that are found in a small number of pixels or structural features can be targeted for analysis.

Tandem MS can be used within an imaging context to identify the differential localization of isomers or isobars within a tissue. Although tandem MS has been reported for several types of IMS technologies,[102-104] it is primarily used for SIMS imaging because most ion beams fragment analyte ions during the ionization process.[105-109] Any imaging mass spectrometer with MS/MS capabilities can generate fragment ion images[110,111] enabling direct visualization and differentiation of isomers and isobars within tissue without prior separation or

derivatization. Generally, these experiments are usually limited to surveying a small number of ions within a sample, since the tissue is partially consumed during analysis.

b. Multi-Imaging Mass Spectrometry Experiments

Each of the three types of IMS technologies discussed here provide different molecular coverage and spatial resolution. As a result, investigators have combined some of these to gain further functionality. SIMS and MALDI IMS have been combined to detect multiple chemical classes, such as low molecular weight metabolites and lipids within individual cells[97] and tissue.[113] Additionally, the combination of MALDI and SIMS has been used to analyze hair to enhance sensitivity and spatial resolution.[114,115] Both MALDI and SIMS sources are operated under vacuum using similar tissue preparation protocols. In some cases, SIMS analyses have been shown to be enhanced by the application of a MALDI matrix.[116,117] While less common, combining MALDI and DESI together enables analysis of different lipid species,[118] and co-mapping of lipids and proteins.[119] To our knowledge, there has not yet been a DESI and SIMS multimodal IMS experiment, likely because of the differences in sample preparation. Reaction additives can be employed for DESI analyses for quantitation and derivatization chemistries, bringing additional capabilities to any combination of technologies. Finally, DESI produces multiply charged ions that are often more amenable to tandem MS applications.

c. Microextraction

Microextraction protocols aim to remove key analytes from bulk material to reduce chemical complexity,[120] and target specific analytes. Both solid and liquid phase extraction techniques have been coupled to IMS for increased peak capacity and sensitivity. Solid phase microextraction (SPME) comprises a diverse set of solventless techniques that allow for *in vivo* analysis. An advantage of SPME devices is that most analytes are introduced into the MS system at once. Introducing ions concurrently increases sensitivity and signal-to-noise (S/N) compared to technologies that generate a transient signal.[121,122] Furthermore, SPME is used to separate an analyte of interest from bulk material, such as in trace analyte analysis.[123]

Liquid extractions, such as liquid microjunction (LMJ)[98,124]and liquid extraction surface analysis (LESA),[99,125] also enhance peak capacity and reduce ion suppression. Briefly, extraction solvents are dispensed onto a tissue surface and collected for subsequent liquid chromatography or capillary electrophoresis MS analysis.[52] Spatially targeted liquid extractions are advantageous because they can be coupled to different separation techniques, increasing both sensitivity and depth of coverage. For instance, Cahill et al. used an LMJ to image portions of a microfluidic device while it was functioning with future use aimed at biology-based microfluidic devices.[126] Typically, this technology utilizes relatively large areas for droplet placement. However, recently microLESA was combined with piezoelectric spotting of trypsin to achieve higher spatial resolution sampling than previously reported.[99,127] In addition, microLESA was integrated with autofluorescence microscopy to correlate protein signatures of murine kidney and *Staphylococcus aureus* abscesses without tissue staining.[99] As these techniques continue to improve in spatial resolution and automated platforms become available, combining IMS with spatially targeted microextractions will be adapted to supplement spatial information with deeper molecular coverage and added identification capabilities.

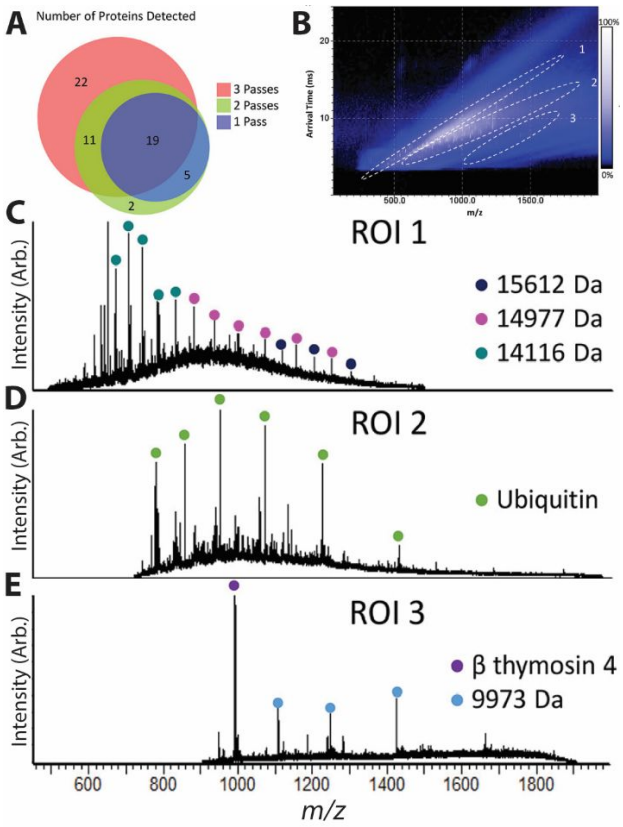

Figure 2. LESA and cIM analysis of a complex murine kidney protein extract. More proteins are detected after each additional cycle of cIM (A). IM heat map generated from a single cycle of cIM (B), where extracted mass spectra from different trend lines contain different protein signatures (C-E). This figure is adapted with permission from ref[112], Sisley, E.K. *et al*. *Analytical Chemistry* (2020). Copyright 2020 American Chemical Society.

d. Ion Mobility

Ion mobility separations add an orthogonal analytical dimension to IMS that reduce ion interference for improving peak capacity and specificity and aiding in identification of species. Additionally, drift time or collision cross sections (CCS) calculated with the use of standards can be used to help identify isomers and isobars. Ion mobility techniques that have been coupled to IMS include drift tube mass spectrometry (DTIMS),[128,129] traveling wave ion mobility spectrometry (TWIMS),[130,131] field asymmetric ion mobility spectrometry (FAIMS),[132-134] and—more recently—trapped ion mobility spectrometry (TIMS).[135-137]

A major focus of the ion mobility field is developing higher resolving power platforms, so they can separate isomers and isobars. Park, Fernandez-Lima, and co-workers utilized a buffer gas and ion trapping by electric field gradient for the development of TIMS devices. They report resolving powers of ~200 but up to ~400.[138-140] One approach for increasing resolution is by increasing path length and/or number of passes

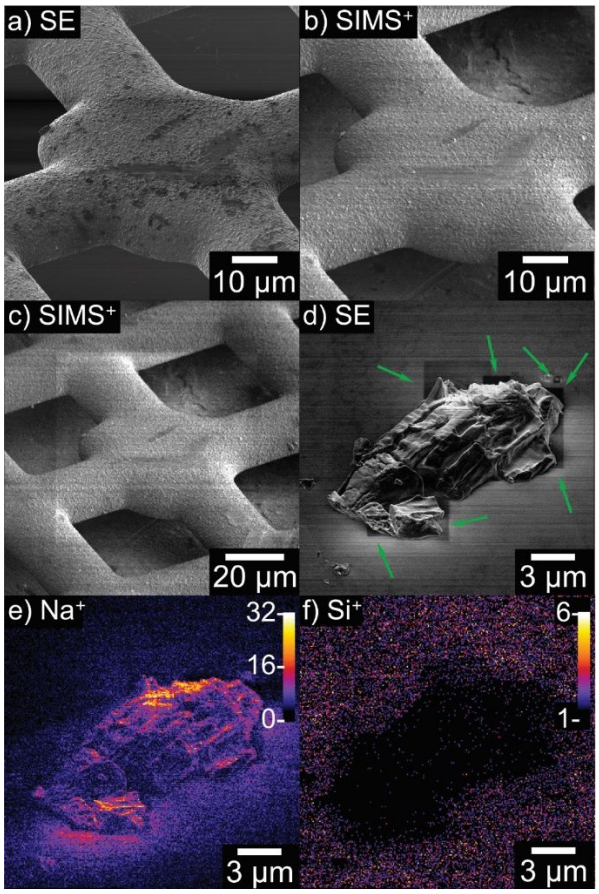

Figure 3. Images of a TEM grid (**A-C**) and NaCl salt (**D-F**) crystal generated from the SIMS helium microscope. The SIMS (**B,C,E,&F**) images are close to the resolution of the microscopy (**A&D**), demonstrating the power of this technique. This figure is adapted with permission from ref[149], Klingner, N. *et al*. Ultramicroscopy **198**, 10-17 (2019). Copyright 2019 Science Direct.

around the mobility device. Notable ion mobility advancements include the development of structures for lossless ion manipulation (SLIM) devices,[141,142] as well as the cyclic ion mobility (cIM).[143] SLIM devices utilize a separation path length of ~540 m[144] for achieving resolving powers over 1800. Using cIM, resolving power of 750 (with 100 passes) has been reported using a reverse sequence peptide pair. Although, sensitivity can be challenging in these devices as ions are lost radially over time. Recently cIM has been integrated with LESA for the improvement of S/N and the number of detected proteins from tissue samples (Figure 2).[112]

Beyond increasing peak capacity and specificity, trendlines within ion mobility heat maps can be used to identify different molecular species, classes, and subclasses.[145] For example, MALDI DTIMS IMS was used successfully to separately image a lipid species ([Phosphatidylcholine(34:2)+H]$^+$) from a closely isobaric peptide ion (RPPGFSP).[146] Škrášková et al. visualized multiply charged polysialylated gangliosides using DESI TWIMS.[118] Recently TIMS has been integrated with MALDI IMS to the separate and map isobaric lipid species directly from tissue.[147] Cooper and coworkers introduced a new cylindrical FAIMS device coupled to LESA IMS. This workflow improved the number of detected proteins from what was previously reported as much as 10 times in murine brain, testes, and kidney.[148] Clearly, ion mobility has great potential for IMS applications as it provides the ability to overcome the analytical challenges associated with direct sampling of complex biological tissues. Software is continually being developed to more efficiently process highly dimensional ion mobility IMS data.

IV. Integration with other technologies

Non-MS analytical technologies can be integrated with MS technologies to increase chemical coverage. Since each technology has distinctive advantages for different molecular classes, experiments that synergistically incorporate multiple technologies gain unique chemical information neither one can obtain alone. For example, many cell types and functional cell states are uniquely suited for analysis by specific methods, such as transcriptomics or immunostaining, and integrating these can add great utility to the molecular specificity of MS.

a. Microscopy

Microscopy is one of the oldest analytical approaches dating back to the early 17th century and is commonly applied to biological and clinical problems.[150-153] Overall, microscopy images are generated by capturing electromagnetic radiation or particle beams as they interact with the sample through reflection, refraction, or diffraction. A series of lenses and objectives focus the light/particles enabling imaging with high magnification.

Brightfield and histological stains, such as hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS), are used extensively in pathology to assess tissue integrity, health, and disease.[154-156] Stained tissues have been employed with IMS to connect molecular profiles to histological features of both healthy and diseased tissue, such as cancer[157-161] and functional disorders.[162,163] Recently, Basu et al. enabled IMS and histology to be rapidly correlated by incorporating matrix pre-coated slides, templates, and a 10 kHz laser.[164] Histological stains are ubiquitous within the scientific and medical field, so multimodal studies that combine IMS and stained microscopy improve interpretation and facilitate collaboration between the technologists and the biologists or physicians. Similarly, simple brightfield images can be used for correlation of IMS signals to specific tissue structures, particularly in cases that have features that are easily distinguished with brightfield microscopy.[165,166]

Fluorescence microscopy of both endogenous fluorophores[167,168] and tagged antibodies[169-171] or nucleic acids[172,173] have also been fundamental to our understanding of biological systems. For example, Vardi et al. used autofluorescence from chlorophyll and MALDI IMS to study lipid metabolism within algal plaques.[174] By targeting key proteins or genes, investigators can parse metabolic pathways and monitor how these change as a function of disease state or demographic. While exceptionally powerful and informative, these technologies are generally limited to studying peptides or proteins since there are few probes available for low molecular weight metabolites and lipids.[175,176] IMS has been readily coupled to fluorescence microscopy approaches to tie together cell-type specific immune[16,17,177,178] and transcript profiles[179-181] to metabolites detected by IMS.[182] Immunohistochemistry can be used to histologically classify and contextualize the chemical information obtained by IMS. Moreover, the combination of

both IMS and immunohistochemistry produce more rigorous classification schemes than either modality independently. Recently, several labs have combined immunohistochemistry and IMS to correlate protein or metabolite signals to specific tissue substructures.[183-186] This type of experiment is further extended by recent work where investigators coupled multiplexed immunohistochemistry with MALDI FT-ICR IMS to determine metabolic profiles of cancer cell, incorporating molecular classes.[187] The increase in the plexity of the immunofluorescence labels additionally enhances the specificity of chemical profiles created by MS analysis, as these immunofluorescence labels can be correlated to more specific tissue regions or cell types.

Immunofluorescence imaging has been correlated with MALDI IMS[178] and fluorescence in situ hybridization (FISH) with SIMS.[179] We could not find an example of either being used as part of DESI multimodal studies. This is interesting because there are no experimental considerations preventing IHC or FISH from being combined with all the IMS modalities discussed here. However, technical challenges in co-registering modalities with dramatic differences in spatial resolution may be driving this perceived disparity. Patterson et al. have developed combination experimental and computational pipelines using autofluorescence microscopy to overcome this challenge for multimodal MALDI IMS studies.[167,168] It is anticipated that fluorescence microscopy will become an important correlative technology in IMS studies.

Particle-based microscopy, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM), offer very high spatial resolution because it is not diffraction limited. A series of ion optics are used to focus ions towards a sample. The ions then interact with the sample and the emerging electrons and various energy conversion products (e.g. secondary electrons, X-rays, light) are captured as an image. Because nanoSIMS and electron microscopies have similar sample and operational requirements, they have often been used on the same sample.[189] TOF-SIMS has been integrated into a helium ion microscope for 8 nm imaging with the potential for extremely high resolution molecular imaging of biological samples (Figure 3).[149] For example, nanoSIMS and TEM have been combined to map dopamine distributions in dense core vesicles, providing key insight into vesicle loading and nanocompartmentalization.[190] Another study involves integration of fluorescence microscopy, SIMS, high-energy resolution x-ray photoelectron spectroscopy, and SEM on the same plant root to study bacterial growth and infection on this root.[191] The authors combined these four modalities because of the simple and compatible sample preparation involved for each. Beyond registration for enhanced localization, particle-based microscopy is also readily used to assess MALDI matrix application for ensuring matrix coverage and crystal size.[192,193]

b. Spectroscopy

Spectroscopic imaging includes a suite of optical approaches, such as infrared (IR)[194] and Raman, that provide unique spectra of complex chemical mixtures, creating reproducible chemical profiles of different physiological regions and disease states.[195] Spectroscopy is used in a wide array of biological studies,[196-201] because the approaches are generally non-destructive, label free, and capable of high spatial resolutions (diffraction limited, 250 nm). While each spectroscopic approach activates different, well characterized molecular modes, it is often difficult to correlate spectroscopic signatures of complex mixtures to discrete chemicals. Rather, they provide general information on bond types and functional groups for the entire chemical mixture.[202-205] As such, spectroscopy has been coupled to a variety of MS technologies, including IMS, to provide more detailed molecular descriptions of samples.[206-208] Because spectroscopic analysis is label free and nondestructive, both modalities can be performed on the same tissue section.[209]

Raman and IMS have been correlated to study bacteria,[210-212] plants,[213] single cells,[214] and mammalian organs.[215,216] Fourier transform infrared microscopy (FT-IR) has similarly been coupled to IMS for many biological studies.[209,217,218] Recently, Rabe et al. developed a method for FT-IR guided MALDI IMS (Figure 4). By coupling these two technologies together, the authors reduced data load and acquisition time by >90%.[188] Magnetic resonance imaging has also been used to compliment molecular specificity of IMS with dynamic, whole organ imaging.[219-224] While MALDI IMS has primarily been coupled to spectroscopic measurements, we foresee DESI increasingly becoming incorporated with Raman and magnetic resonance imaging. DESI does not have many tissue preparation requirements and can theoretically be directly integrated within either approach. Moreover, Raman and magnetic resonance spectra are not saturated when water is

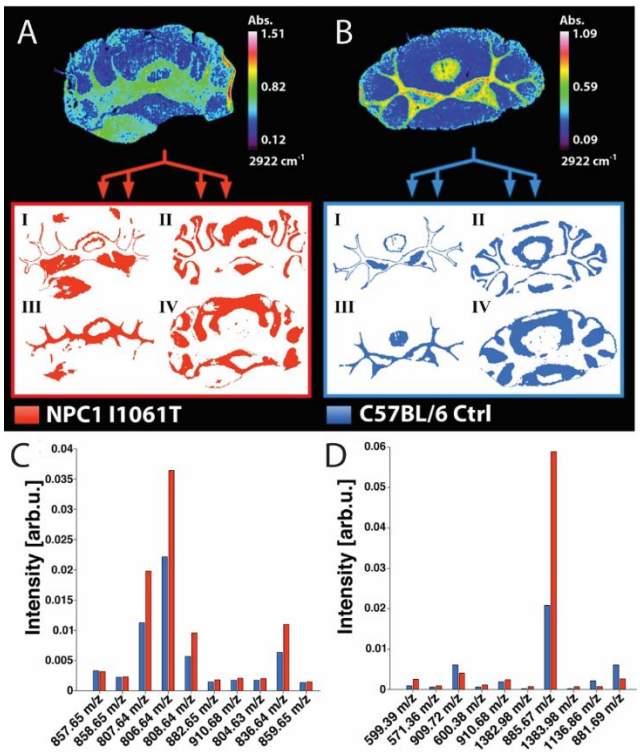

Figure 4. MALDI IMS lipid profiles obtained after FT-IR generated segmentation. Murine brain is differentially segmented based on absorbance of 2922 cm^{-1} based on disease (**A**) and control mice (**B**). Using this segmentation, lipid profiles can be generated for different masks for chemical differentiation (**C-D**). This figure is adapted with permission from ref[188], Rabe, J. A. *et al. Scientific Reports* **8**, 313 (2018). Copyright 2018 Nature Publishing.

present, unlike IR which readily is absorbed by water, making them ideal candidates for multimodal DESI experiments.

c. Transcriptomics

Transcriptomics technologies measure gene expression through the extraction and amplification of nucleic acids, most generally ribonucleic acids.[225-227] Because of the exponential amplification that can occur, this group of technologies are highly sensitive and has been the driving force behind cell typing and classification. There is great interest in coupling IMS with transcriptomics analysis as it would enable simultaneous correlation of gene expression to gene products and biproducts. A majority of the literature combining mass spectrometry and transcriptomics measurements has involved liquid chromatography for bulk proteomics and/or metabolomics.[228,229] Towards an imaging context, investigators probed an insect, *Carausius morosus*, for neuropeptide content with MALDI MS and correlated this to bulk sequencing to uncover peptides with no known homology.[230] Although this was not an imaging application, the sample preparation was performed in such a way that this workflow could be adapted to an imaging workflow. As an additional step towards combining IMS with transcriptomics, the Knepper et al. microdissected kidney tubules and performed RNA-seq and proteomics.[231] This methodology incorporates spatial information of the microdissected structures, which is an important step towards coupling IMS and transcriptomics directly. Moreover, there has been some exciting work where transcriptomics and proteomics IMS information has been correlated on different bulk samples.[232] Work combining two approaches on the same sample provided insight into the link between fatty acids and immunity within breast cancer[233] and role of liver X receptors in male reproduction.[234] While only a few examples exist, the combination of transcriptomics and IMS will continue to increase in the coming years and has the potential to uncover new connections that span the central dogma of molecular biology.

d. Electrochemistry

Electrochemistry has been vital in the study of electroactive signaling molecules, such as dopamine, epinephrine, serotonin, and histamine within biological systems.[235-237] This is partly because it is capable of absolute quantitation of femto- to zeptomole amounts of analyte at μs temporal resolutions . It is highly selective and naturally applicable to the analysis of many low molecular weight metabolites, such as neurotransmitters.[238] nanoSIMS/nanoSIMS and electrochemistry are compatible and have been used together often to study nonbiological[239] and biological samples alike.[238,240,241] Ewing et al. have quantitated L-DOPA concentrations in vesicles and other organelles using nanoSIMS and validated these concentrations with electrochemistry.[242] This is particularly important because it enables quantitation of low molecular weight metabolites at high spatial resolutions. Further, Larsson and coworkers quantitated octopamine release during different stimulations using nanoSIMS and an embedded electrode.[243] Investigators in the IMS field have also adapted many technologies and developments from electrochemical studies,[244] ranging from nanopipettes to electrochemical principals of ion generation. Adopting materials, analyses, or processes from other fields is an effective, time saving process that expedites scientific advancements.

V. Future of the Field

Mass spectrometry technologies have rapidly advanced with improved sampling, ion transmission, and detector sensitivity enabling highly sensitive and specific analysis of biological tissues. Under favorable conditions, MS can detect concentrations as low as 10 zeptomoles or approximately 6000 molecules.[6] This sensitivity is sufficient for probing most molecules within biological systems and yet there is still much we do not understand. Nevertheless, improvements in the molecular, spatial, temporal, and biological specificity are required to answer remaining questions. IMS is one available tool that provides untargeted, highly multiplexed molecular analysis but only through the combination with other technologies can we achieve specificity in all these areas mentioned above. Multimodal imaging experiments can significantly improve performance characteristics including structural identification, throughput, cell type specificity, and dynamic range of MS-based chemical profiles. The present article has summarized the current literature surrounding multimodal IMS and the development of the field to further explore remaining biological questions.

A major task facing multimodal approaches involves efforts to fully integrate the several datasets to enable deeper data mining. This is particularly difficult within an imaging regime because the various technologies can have dramatically different spatial resolutions, data structures, and chemical information. Different computation methods for addressing differences within spatial resolution include various methods of up sampling the data, performing more refined data fusion, as well as other experimental approaches.[167,168,245-248] While these are capable of connecting modalities that are similar in resolution, significant experimental and technological capabilities are required to avoid the introduction of artifacts.[209] While many imaging modalities are within an order of magnitude of one another, this will influence the capacity to combine lower spatial resolution IMS technologies like DESI with higher resolution molecular imaging approaches, like SEM.

Moreover, technologies provide both overlapping and orthogonal information that is often difficult to correlate. For instance, transcriptomics measures gene expression of different biochemical pathways that result with peptide and metabolomic products. Ideally, a highly expressed gene would indicate the presence of a specific metabolite and IMS would detect this same metabolite in high abundance within the same regions, but this is not always the case. There many transport, degradation, and modification pathways that impede this correlation and create complex datasets that are difficult to interpret. Additional compounding technical factors, such as matrix effects, differences in ionization efficiencies, and limited dynamic range add to this complexity. While transcriptomics is used as an example here, a similar scenario applies to other multimodal approaches, such as protein abundances between IMS and immunofluorescence experiments or lipid analysis by Raman spectroscopy and IMS. While challenging, future multimodal experiments will slowly begin to unravel the complex relationships between the data produced by orthogonal technologies. In connection with this, new machine learning

algorithms and approaches will be essential for untangling the abundance of chemical information obtained with multimodal IMS. This will inevitably lead to a more complete picture of biological systems and pathways.

Finally, improving sample preparation and workflows will undoubtedly improve the quality and reproducibility of collected data, particularly as these technologies enter the rigorous domain of medicine and clinical trials. Many technologies cannot be combined "out of the box" and so there are often tradeoffs made to enable multimodal analysis. Although, the gained information from the suboptimal combination of the approaches is often greater than the data of either technique alone. Developing methods that enable multiple imaging modalities to be performed optimally and with minimal spatial compromise will dramatically improve the ability to integrate and discover connections between multimodal datasets. Optimizing the ways and methods behind combining the different approaches is a clear path forward in the field.

In summary, multimodal IMS is a remarkably diverse endeavor that incorporates the best attributes from a variety of scientific disciplines. In the future, multimodal IMS technologies will progressively become more common as the scientific community begins to study more complex biological and medicinal questions. Such studies have the potential to bring together genomic, proteomic, and metabolomic imaging technologies to provide unprecedented insights into biology and medicine.

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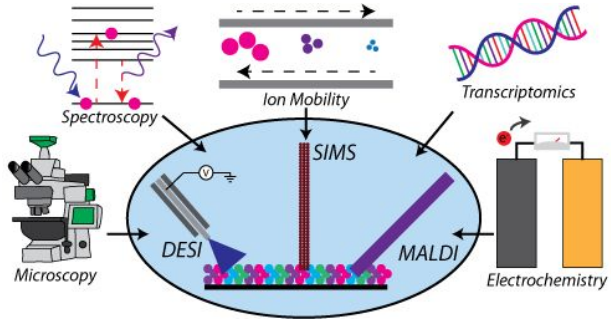
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DESI
MALDI
Microscopy
Electrochemistry

TOC Graphic

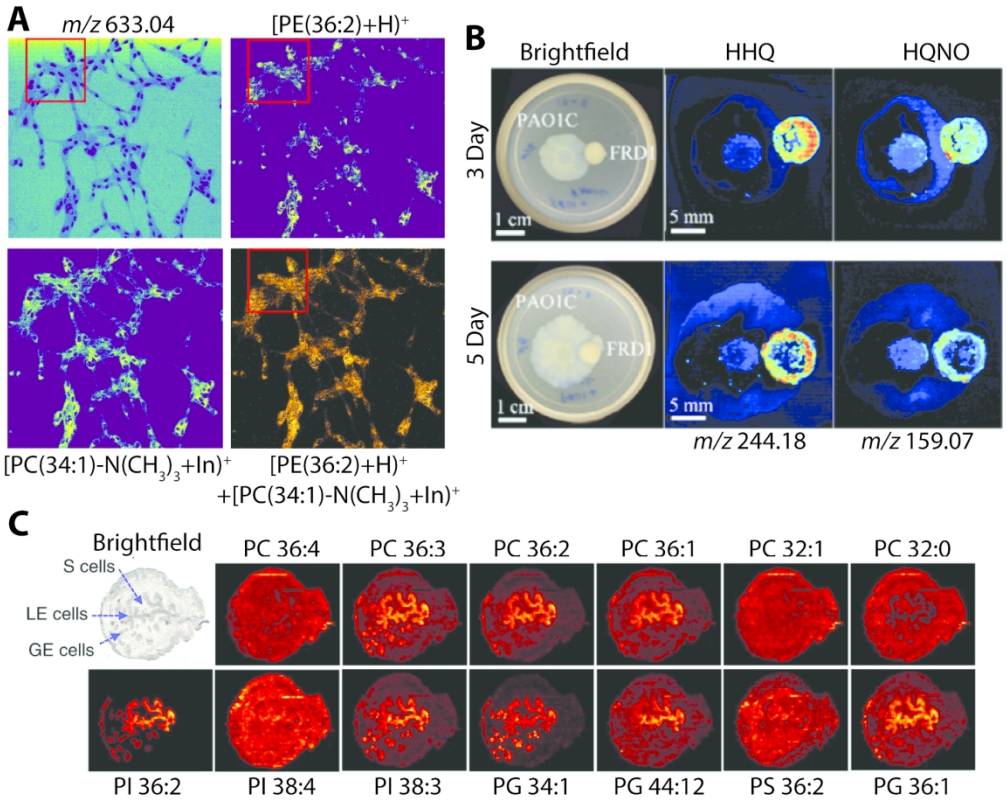


Figure 1. Selected developments within the IMS community. Image of a Vero B cell culture at 1 µm spatial resolution acquired with transition mode MALDI-2 IMS developed by the Dreiswerd lab (A). Panel A is adapted with permission from ref47, Niehaus, M. et al. Nat Methods 16, 925–931 (2019). Copyright 2019 Nature Publishing. SIMS image of a coculture of different Pseudomonas aeruginosa stains visualized with different signaling small molecules (B). Panel B is adapted with permission from ref24,48, Cao, T. et al. SPIE BiOS 108630A (2019). Copyright 2019 SPIE. Digital Library. Lipid images both positive and negative mode of the human eye using nanoDESI at 10 µm spatial resolution (C). Panel C is adapted with permission from ref49-51, Yin, R. et al. Nature Protocols 14, 3445-3470 (2019). Copyright 2019 Nature Publishing.

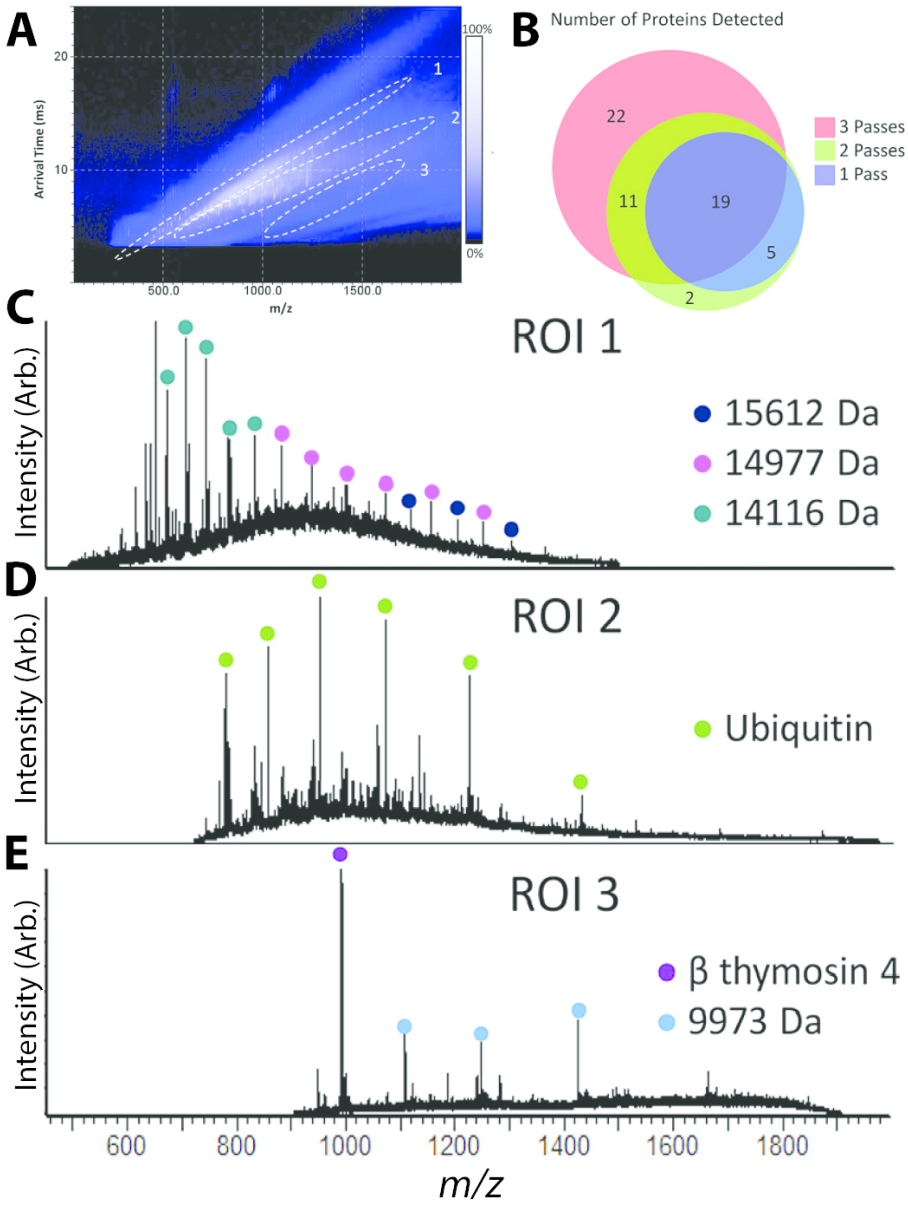


Figure 2. LESA and cIM analysis of a complex murine kidney protein extract. More proteins are detected after each additional cycle of cIM (A). IM heat map generated from a single cycle of cIM (B), where extracted mass spectra from different trend lines contain different pro-tein signatures (C-E). This figure is adapted with permission from ref109, Sisley, E.K. et al. Analytical Chemistry (2020). Copyright 2020 American Chemical Society.

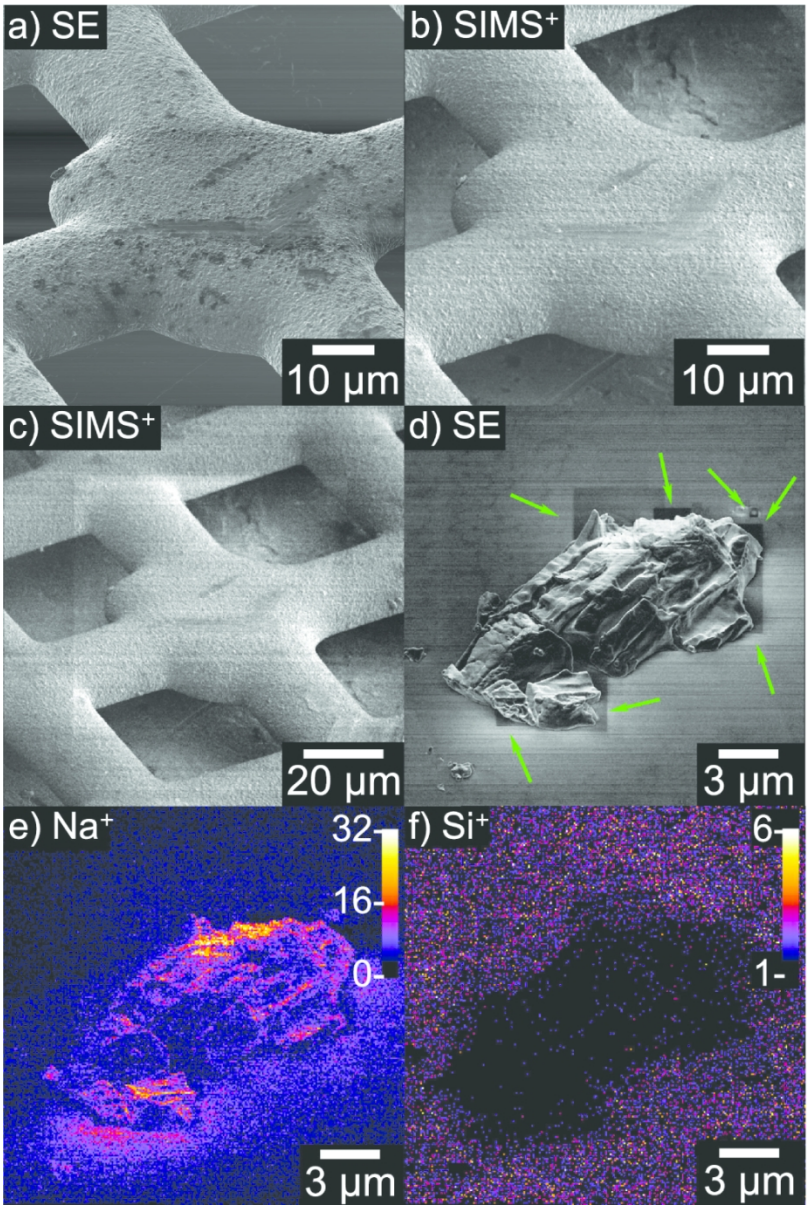


Figure 3. Images of a TEM grid (A-C) and NaCl salt (D-F) crystal generated from the SIMS helium microscope. The SIMS (B,C,E,&F) images are close to the resolution of the microscopy (A&D), demonstrating the power of this technique. This figure is adapted with per-mission from ref145, Klingner, N. et al. Ultramicroscopy 198, 10-17 (2019). Copyright 2019 Science Direct.

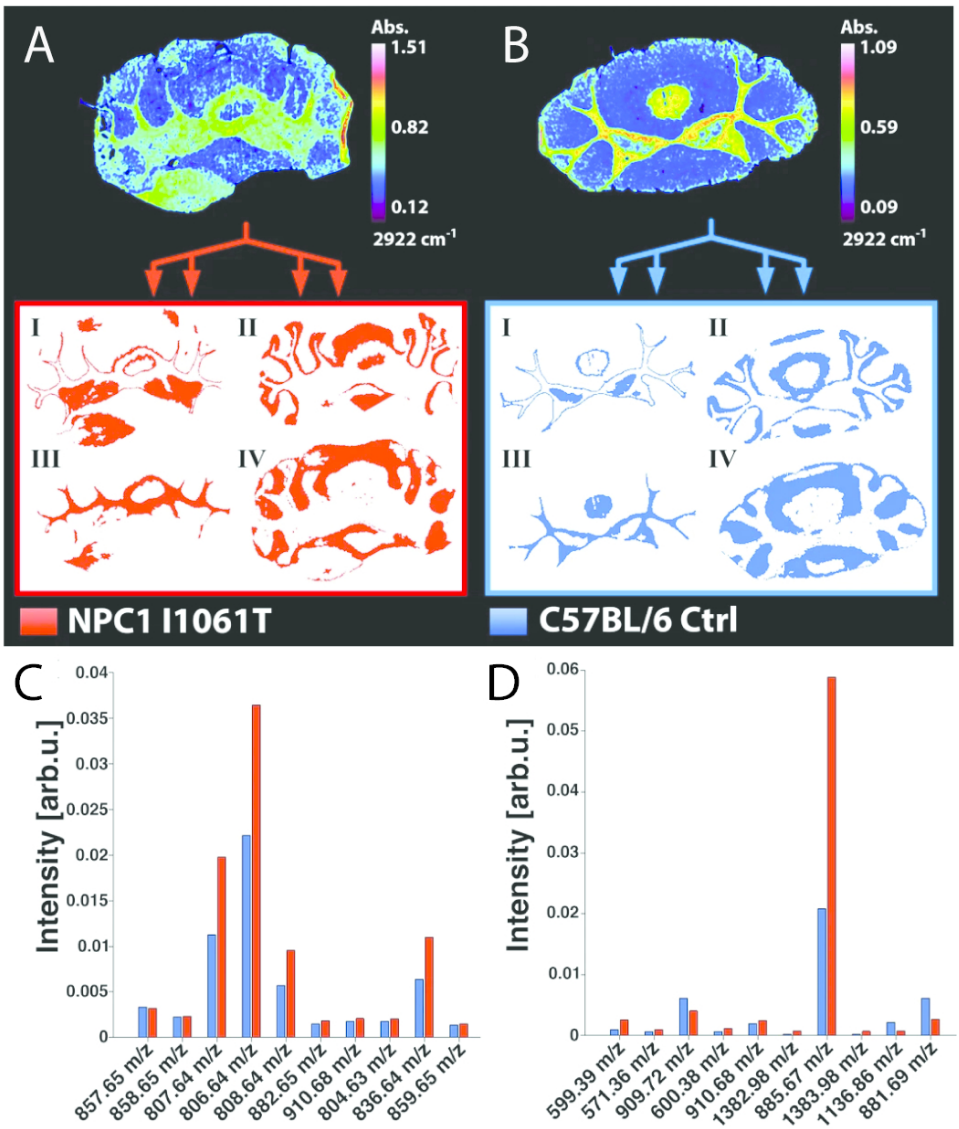
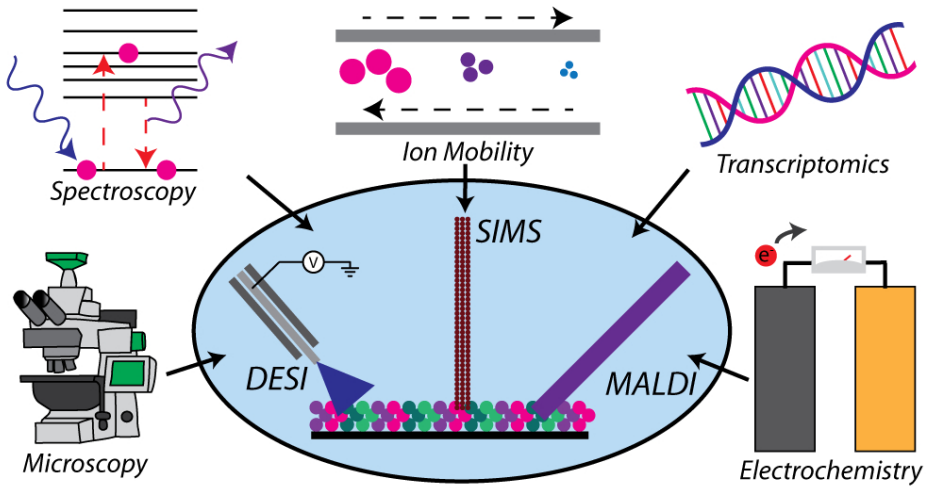


Figure 4. MALDI IMS lipid profiles obtained after FT-IR generated seg-mentation. Murine brain is differentially segmented based on absorbance of 2922 cm-1 based on disease (A) and control mice (B). Using this segmenta-tion, lipid profiles can be generated for different masks for chemical dif-ferentiation (C-D). This figure is adapted with permission from ref182, Rabe, J. A. et al. Scientific Reports 8, 313 (2018). Copyright 2018 Nature Publishing.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