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Imaging mass spectrometry for natural products discovery: a review of ionization methods

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Abstract

Over the last decade, methods in imaging mass spectrometry (IMS) have progressively improved and diversified toward a variety of applications in natural products research. Because IMS allows for the spatial mapping of the production and distribution of biologically active molecules *in situ*, it facilitates phenotype and organelle driven discovery efforts. As practitioners of IMS for natural products discovery, we find one of the most important aspects of these experiments is the sample preparation and compatibility with different ionization sources that are available to a given researcher. As such, we have focused this mini review to cover types of ionization sources that have been used in natural products discovery applications and provided concrete examples of use for natural products discovery while discussing the advantages and limitations of each method. We aim for this article to serve as a resource to guide the broader natural product community interested in IMS toward the application/method that would best serve their natural product discovery needs given the sample and analyte(s) of interest. This mini review has been limited to applications using natural products and thus is not exhaustive of all possible ionization methods which have only been applied to image other types of samples such as mammalian tissues. Additionally, we briefly review how IMS has been coupled with other imaging platforms, such as microscopy, to enhance information outputs as well as offer our future perspectives on the incorporation of IMS in natural products discovery.

1. Introduction

The discovery of bioactive small molecules, or natural products (NPs), from chemically complex biological tissues can be approached using a variety of analytical methods, and often requires orthogonal techniques. Traditionally, NPs are extracted in bulk from their producing organism, concentrated, and separated based on polarity or affinity to certain solvents prior to further analyses and structure elucidation. While time-proven and effective, this approach is often laborious, sample and material intensive, and importantly, removes the molecules from their biological context. Over the last decade, various imaging mass spectrometry (IMS) methodologies have emerged as useful components of NP discovery

Conflicts of interest

There are no conflicts of interest to declare

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platforms, providing a flexible approach to interrogate biological samples directly for both known and unknown molecular features. The resulting datasets can be had in a fraction of the time of extraction-based methods and can provide the user with a molecular map of their sample allowing them to triage these features of interest by their proximity to- and relative intensities in- biologically relevant tissues or cultures.

At the forefront of a NP discovery effort, IMS can greatly refine the number of molecular features that a researcher is interested in pursuing. Using visible phenotypic information such as zones of inhibition or changes in morphology, researchers can pinpoint molecules localized at or proximal to the evidenced bioactivity. For example, IMS has been applied to various antagonistic microbial interactions where the interacting microbes are utilizing specialized metabolites to interact and compete for resources.[1, 2] Molecules produced in apparently antagonistic microbial interactions can be identified in the zones of inhibition between the producing and target organism. This approach has helped lead to the discovery of a number of novel compounds with antifungal and antibacterial activity[3] as well as those that induce shifts in target organism development and behavior.[4] Further, IMS can be used to characterize specific tissue localization of NPs *in situ* across the tree of life ranging from microorganisms[5] to plants[6–8] to animals.[9]

One of the main advantages of IMS is that it requires relatively minimal sample preparation compared to more traditional bioassay guided workflows and can provide flexibility to tailor the analysis to specific compound classes and sample types. The biggest challenges in any IMS experiment are in releasing the analytes of interest from the sample surface and ionizing them, thus the two major considerations when designing an IMS experiment revolves around 1) the physical qualities of the samples and 2) the types of analytes to be imaged (Figure 1). Some IMS methodologies require the sample to be completely dried and imbedded in thin layer of external matrix before being placed under vacuum. Some sample types, such as fresh leaves, cannot retain their structure under such conditions and may be better suited to other techniques which allow for imaging directly from the tissue under ambient conditions, although usually at the cost of decreased spatial resolution. For analyte considerations, if the analytes have a low molecular weight (100–1000 Da) or are relatively volatile or heat or light labile, they are likely best imaged with minimal sample handling and preparation, which can be facilitated by matrix-free and ambient ionization methods. Alternatively, for larger molecular weight molecules (>1000–2500 Da) that may be more difficult to extract from the sample surface with ambient methods, using a vacuum based method such as MALDI may help. Similarly, different ionization sources should be used for different purposes as each method has its own advantages and limitations in regard to sample preparation, spatial resolution, analyte fragmentation, and sample destruction. Here we attempt to provide the reader with a working understanding of each of the predominant IMS platforms used in modern NP discovery efforts, focusing specifically on the ionization methods that differentiate these methods and their applications (Figure 2).

Before reading this review, we'd like to provide the reader with some insight into the scope of this article and acknowledge its limitations. First, we'd like to emphasize to those new to mass spectrometry that although we focus here on the ionization of target molecules, this is just one of the many important considerations that should be taken into account when

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conducting any mass spectrometry experiment. Once the molecules are ionized they are introduced into any number of mass analyzers, each with their own advantages and limitations. Although incredibly important for the type of information gained about molecules of interest and eventual structure elucidation, the details necessary to understand these different mass analyzers and the interpretation of their spectra are beyond the scope of this article and can be found in an excellent book edited by Eckman *et al.*[18] A thorough review of sample preparation and IMS applications beyond natural products was recently reported by Buchberger *et al.* and can be used as a guide for those looking for further background reading.[19] Additionally, when reading any primary or review literature related to mass spectrometry, the reader will encounter an abundance of acronyms used to reduce the often complex descriptors of these technologies into digestible names. Similarly, this article is replete with common mass spectrometry acronyms which are listed above and in Table 1, which we hope can be used as a resource to new to the field researchers. Finally, because IMS data provides a completely orthogonal view to other imaging modalities, we also aim to highlight here how IMS is being integrated with other biological imaging methods to enhance informational outputs for researchers interested in correlating observable biological phenomena with their constituent chemical parts.

2. Overview of different ionization methods

2.1 Desorption electrospray ionization (DESI) and nanoDESI

DESI was first described in 2004 by Takats *et al.,* and was an early example of using ambient ionization to desorb analytes from a variety of surfaces.[20] DESI is closely related to electrospray ionization and results in the creation of multiply charged ions from a variety of biological surfaces. In order to desorb ions from an analyte on a biological surface, high voltage is applied to a desired solvent mixture with a nebulizing gas in parallel, creating a fine vapor of charged microdroplets. Once the resulting microdroplets come into contact with the analytes, ions are carried away towards the mass spectrometry inlet (Figure 3, Table 1) where they are detected and analyzed. In the first report of this methodology, γ-coniceine was shown to be present in a seed from *Conium maculatum,* although these metabolites weren't imaged. An extension of the DESI application to NP discovery using imaging was described by Lane *et al.* with the discovery of bromophycolides from damaged tissues in the red macroalga *Callophycus serratus.*[21] While DESI has proven powerful across a number of biological samples, some of the inherent drawbacks are that it is incompatible with specific microbial samples that are highly mucoid in nature and biosafety associated with aerosolizing microbial spores or the desired NP themselves. Additionally, like many extraction based approaches, the solvent selection will heavily bias the analytes that can be desorbed and ionized from any given sample. The solvent system will also impact the desired spatial resolution of the resulting image since very viscous solvents (such as high percentages of organics) will rapidly diffuse across a sample, which has somewhat limited the technique to solvents miscible with water, precluding some of the more non-polar organic molecules from detection in discovery platforms. An advantage of DESI is that there is relatively little destruction to the tissue/colony which allows for longitudinal studies and real time monitoring, but may also not permit the identification of NPs that are not readily imaged at the surface of the plant, microbe, or tissue.

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In order to partially overcome the spatial resolution limitations of DESI, nanoDESI was developed by Julia Laskin.[22] In this case, the major innovation to the DESI platform was to create constant droplets from solvent exposed to high voltage using a fused capillary. This allows for a controlled desorption of analytes with the reported spatial resolutions ranging from 10–200 microns depending on the solvent mixture and biological source (Figure 3, Table 1) although the standard resolution reported in NP based experiments is 50–200 microns. NanoDESI was first applied in a discovery workflow to identify the molecules produced over time from *Bacillus subtilis* 3610 such as the sublancin, plipastatin, sporulation killing factor (SKF), and surfactin metabolite families.[23] Similar to DESI, nanoDESI allows for longitudinal analyses with imaging capabilities from both a macroscopic and microscopic perspective (mm to μm). The main advantage of DESI-based imaging is the minimal sample preparation. Profiling different solvents can also be beneficial in this system but requires significant user optimization in order to achieve the desired spatial resolution while also desorbing the desired ions from the analyte. Older DESI systems suffer from a lack of automation, though advances have been made to manufacture a more automated system, such as DESI 2D.

A recent example of the strength of DESI for a discovery workflow was described by Tata *et al.* in their examination of the fungal strain *Trichoderma harzianum* in co-culture with the cocoa tree pathogen *Moniliophthora roreri*.[24] As mentioned above, many samples are not compatible with the DESI ionization mechanism. Tata *et al.* sought to overcome the agar deformity caused by DESI to examine fungal co-cultures by using an imprinting method. In this case, DESI created divots in the agar surface which led to decreased ionization of NPs as it disrupts the geometry of the setup. Co-cultures were grown for three weeks and then imprinting was accomplished simply by applying tape for five seconds to the desired areas for analysis. Using DESI imaging, the authors were able to describe four phytopathogen-dependent NPs from *T. harzianum.* T39 butenolide, harzianolide, and sorbicillinol were all found to be produced by *T. harzianum* in the interaction zone of the co-cultures in response to the presence of *M. roreri.*[24] This example highlights the power of imaging for quickly identifying NPs for further studies.

2.2 Laser ablation electrospray ionization (LAESI)

Laser ablation (LA) is commonly used in mass spectrometry to release analytes from the surface of solid samples into the gas phase. In this method the target sample absorbs the energy of a mid-IR laser and excites free water which results in a small explosion, releasing a very small plume of particles into the air. Because most of these particles are neutral, post-ablation ionization is required in order to introduce charged molecules into the mass spectrometer. In LAESI, this ionization source is ESI, charged solvent microdroplets are sprayed tangentially to the laser plume, carrying charged analytes into the MS inlet (Figure 4B). This method was first introduced by Nemes and Vertes in 2007,[25] and has been applied to various samples including plants, microorganisms, and clinical samples.[26–28] One of the advantages of LAESI is that it is an ambient ionization method, meaning that the samples are kept at atmospheric pressure throughout the experiment. There are a number of obvious advantages to this, including direct analysis of samples without any need for prior desiccation or cryopreservation as is required of IMS methods performed under vacuum.

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One of the major limitations to LAESI is that it is not compatible with dried materials (such as older leaves) as the ablation depends on water molecules in the sample. This can be partially overcome by infiltrating tissue with water prior to imaging.[26] Like other non-chromatographic techniques, it can be difficult to differentiate isobaric ions, but this limitation has been addressed by coupling LAESI with MS/MS, allowing for the detection of distinct ions that are isobaric.[28] As a safety note, with live microorganisms, care should be taken when considering the aerosolization of spores or toxic natural products. Decontamination of the system and caution with the samples should be noted to minimize exposure.

Etalo *et al.* applied LAESI to image fresh plant tissue for molecular features, including luteolin deposition in orchid petals and pathogen-mediated degradation of the fungitoxic alkaloid α-tomatine, produced by tomatoes.[26] Because post-ablation ionization is carried out, LAESI was ideal for the plant tissue because there is no requirement for matrix application which can greatly reduce sample handling and preparation time. Finally, because the laser ablation removes material from the sample, LAESI can be used for 3D imaging, or "depth-profiling", of tissues or samples by repeatedly ablating from the same target location. This approach was used by Li *et al.* to show the diffusion patterns of streptomycin in agar media as well as the production of bacterial lipids at various depths of the bacterial colony.[27] In a recent example, Xu *et al.* have applied LAESI IMS to screen libraries of elicitors of cryptic gene clusters in a high throughput manner to discover new molecules.[3] In this case, the imaging occurs by visualizing the spectral output across 1000 conditions. So while there is not a spatial component to this use, it is an excellent application for discovery to visualize changes in hundreds to thousands of features across hundreds to thousands of conditions in a high throughput manner. This LAESI IMS platform resulted in the discovery of a novel lasso peptide, canucin A, using the elicitor kenpaullone.[3]

2.3 Matrix assisted laser desorption/ionization (MALDI)

Laser desorption ionization (LDI) is an ionization method similar to LA, in that it uses laser light in either the UV or IR spectrum to directly desorb compounds from the sample surface while simultaneously ionizing the samples. Matrix-assisted laser desorption/ionization (MALDI) is one of the most commonly used LDI techniques in NP discovery efforts, due to flexibility, ease of use, speed, and well-documented sample preparation techniques. This system requires the application and crystallization of a matrix of small organic acids to the biological sample either in a premixed droplet or directly to the sample surface. Typically, the matrix is composed of small organic acids such as α-cyano-4-hydroxycinnamic acid (CHCA – 189 Da) and/or 2,5-dihydroxybenzoic acid (DHB – 154 Da) which insulates the analytes from the laser while simultaneously inducing desorption and ionization. For compounds that are sensitive to acids or ionize better in negative mode, alternative matrices are available such as 9-aminoacridine (9-AA). For MALDI time-of-flight (TOF) imaging, it is imperative to evenly apply the matrix across the sample, with higher granularity matrix often affording higher spatial resolution imaging (Figure 4A). Matrix application can be done via sublimation, spraying with an artistic airbrush, an automated sprayer, or through a fine stainless steel sieve prior to sample desiccation on a MALDI plate or other conductive surface.[5, 29, 30] Because the laser energy is indirectly transferred to sample analytes via the

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UV-ionized matrix, MALDI is considered to be a soft ionization which results in little ion fragmentation with the resulting spectra mainly containing protonated molecules, or sodiated and potassiated adducts.

Because ionization efficiency and spatial resolution are dependent on matrix crystal formation, amongst other factors, this method is routinely carried out under vacuum and thus samples must be thoroughly dried before conducting the experiment. While this can be an issue for samples that are very sensitive to desiccation, MALDI has been routinely applied to numerous biological samples, including plants, microbes, and animal tissues and has proven to be one of the most utilized methodologies for imaging of NPs.[31–34] One of the biggest drawbacks of MALDI-IMS is that the small matrix ions dominate the spectrum making low molecular weight ions (<500 Da) difficult to detect. High mass resolving power when MALDI is coupled to FT-ICR or an Orbitrap as the mass analyzer can help overcome overlap in the ions.[35]

This approach was used to discover a novel bioactive lipopeptide produced by the plant pathogenic bacterium, *Ralstonia solanacearum*, which caused morphological shifts in fungi.[1, 2] The structure of ralsolamycin was fully elucidated by two different groups following its discovery by IMS.[36, 37] Further, MALDI-TOF IMS was used to detect the production of the fungal NP, bikaverin, as a local response to this lipopeptide.[2] Bikaverin was previously described as a weak antibiotic, but in this case, IMS revealed that *Fusarium fujikuroi* shuttles this NPs to the nearest hyphae exposed to *R. solanacearum*, which was the first instance a possible ecological role was described for bikaverin.[2] In both of these experiments, as is common with many MALDI instruments, a time-of-flight (TOF) mass spectrometer was used. TOF mass spectrometers provide a broad mass range for effective full-scan analyses, although they have limited mass resolving power compared to other hyphenated instruments such as MALDI FT-ICR, but are unmatched in speed when measuring samples with large surface areas. When large areas are required for a natural products discovery, such as a large fungal colony or whole leaf, it can be beneficial to image using a TOF mass analyzer which is non-scanning and ions are separated through space in the flight tube. Large areas coupled to a theoretically unlimited mass range allow for a deep interrogation of the sample at the sacrifice of high mass resolving power in a narrow mass range over small spatial areas which are common with FTICR and orbitrap based imaging experiments.

2.4 Surface-assisted laser desorption/ionization (SALDI)

Similar to MALDI, surface-assisted laser desorption/ionization (SALDI) is an LDI technique that utilizes UV lasers to simultaneously aerosolize and ionize analytes from biological tissues or microbial cultures. Instead of relying on an organic matrix to transfer laser energy to the analytes, SALDI uses a thin layer of graphite or nanomaterials such as nanostructured silicon (nSi) to directly adsorb molecules from a sample surface (Figure 4A). The sample is often subsequently removed from the surface and the imprinted molecular features on the nSi or graphite are then imaged.

While MALDI and SALDI share many of the same advantages regarding ease of sample preparation and straight forward acquisition methods, SALDI has a few advantages over MALDI for specific purposes. One of the major advantages of SALDI is that it has a very

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low background of matrix signals, which can make detecting molecules in the <600 Da range possible. Chen *et al.* used SALDI to discover the fungal NPs sterigamtocystin (325 Da), fellutamides (578–594 Da), and small dipeptides (245–261 Da) occurring in the antagonistic interaction zone between *Aspergillus* spp. and the wood pathogen *Phellinus noxius.*[16] Further, they demonstrated that many of these signals would have been difficult to detect using MALDI due to matrix contamination in the lower mass range. One limitation of SALDI can be incomplete transfer of the analytes of interest to the nSi or graphite from the biological sample, resulting in a failure to detect ions of interest, although one could envision adapting different transfer methods to capture microbial surface associated metabolites or other clever sample preparation techniques.

2.5 Liquid extraction surface analysis (LESA) and Liquid microjunction-surface sampling probes (LMJ-SSP)

When high spatial resolution is not a concern in a discovery effort, surface extraction methods can greatly expand one's ability to collect more detailed structural information (such as retention time and MS^n) due to the increased sample volumes. These liquid extraction-based methods can still be used to image an entire sample, albeit at a much lower spatial resolution that the laser based ionization sources, while also providing detailed structural information about the molecules across the sample. These methods differ from DESI in that the sample surface molecules aren't ionized *in situ* by charged solvent microdroplets. Rather, the sample analytes are extracted through the direct application of solvent to the sample surface, followed by rapid reuptake of the liquid solvent. The incorporation of the extraction step prior to analysis provides the ability to subject analytes to further separation through LC systems prior to MS analyses, which can be viewed as an advantage to this methodology when a very complex sample is the source for the discovery effort, such as a fungal co-culture interface. This can greatly improve the capacity for identification of features based on chromatographic information, and also facilitates the incorporation of any mass spectrometer that is compatible with liquid chromatography.

Liquid extraction surface analysis (LESA) was developed in 2010 and has been applied to a number of biological tissue types.[38] This method employs a robotic autosampler which moves to defined locations, pipettes small volumes of extraction solvent to and from the sample surface, then directly introduces the sample to the MS via direct infusion or to LC system (Figure 5). Another limitation of LESA is that the large extraction area tends to be destructive to the sample which can preclude the extension to longitudinal studies, and typically images are of spectra or extracted ion chromatograms over a large area of the tissue of microbial co-culture. An example of the type of imaging and discovery that can occur with this technique was the interaction of *Schizophyllum commune* with other fungi.[39] In co-cultures of *S. commune* with *Hypholoma fasciculare*, pigments were detected by LESA IMS to be indigo, indirubin, and isatin. In this case, LESA was critical to the discovery of these pigments from the co-culture because they were only observed at the interface and only at the surface, which may mean the ions would have been difficult to detect if the entire colony was subjected to extraction.

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Liquid microjunction surface sampling probes (LMJ-SSP) were developed after DESI and nanoDESI and use a coaxial tube geometry flowprobe system to continuously extract surface metabolites while traversing across the sample.[40] Like DESI/nanoDESI, LMJ-SSP allows for the direct analysis of biological tissues with virtually no need for prior sample preparation. Specialized metabolites from various microbial sources have been assessed using LMJ-SSP, and imaged in a similar fashion as that described by Xu *et al.* in the LAESI section.[3] In this case different fungal and bacterial strains were sampled and the resulting spectra were visualized to assess differences in NPs across all the cultures.[41] LMJ-SSP discovery examples are limited, but an advantage of this technology is that the probe can easily be unclogged by pressure whereas when a nanoDESI capillary clogs, a new capillary typically has to be installed.[15, 42, 43]

2.6 Direct analysis in real-time (DART)

Direct analysis in real-time (DART) is an ambient ionization method that allows for detection of analytes without any sample preparation.[44] DART uses gas, typically He or N_2 which is passed through a chamber where an electrical discharge creates electronic excited state atoms. These metastable atoms proceed by Penning ionization to interact with water molecules at atmospheric pressure to subsequently transfer protons to the NPs (Figure 6). DART has been adopted broadly where real-time, no-preparation, and ambient analyses of metabolites are needed such as in airport security, food quality assessment, and clinics. However, there are limited examples of DART to evaluate the localization of NPs *in situ* due largely to limited spatial resolution.[45] In IMS applications, Gromek *et al.* utilized DART to evaluate the spatial production of the bacterial NP indigoidine across a bacterial colony by manually sampling different locations across the bacterial sample and introducing the samples into the sample inlet, providing millimeter-scale resolution.[46] To achieve higher resolution imaging with DART, Fowble *et al.* coupled a DART source with UV laser ablation methods to evaluate the *in situ* distribution of the plant alkaloids atropine and scopolamine, as well as their biosynthetic precursors.[12] This approach provided 50 μm^2 resolution across an entire seed and showed that different biosynthetic precursors were spatially separate, suggesting spatial regulation of metabolite production.

2.7 Infrared matrix assisted laser desorption electrospray ionization (IR-MALDESI)

Infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) is a hybrid MS method, harnessing the advantageous aspects of both MALDI and ESI experimental platforms.[47] Similar to MALDI, IR-MALDESI relies on a matrix and laser to desorb molecules from the sample surface. This method is unique in that the matrix for IR-MALDESI is ice cyrtsals (as opposed to water in the LAESI applications). Ice matrix is utilized in IR-MALDESI when researchers utilized relatively low humidity (~10%) and a Peltier cooled sample plate to deposit a thin ice layer over sectioned tissues in addition to the water found throughout the biological tissue section. Care should be taken to avoid depositing ice under ambient conditions as this was noted to create water droplets rather than ice crystals. The use of ice eases sample preparation and reduces matrix contamination in MS datasets. In addition to the ice matrix on top of the sample, the ice throughout a thin section leads to the ionization of an entire volume, thereby creating voxels instead of pixels, which all of the other methods described above create. Additionally, using ice as the matrix,

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this methodology is capable of ionizing molecules from high salinity environments, which is typically incompatible with all other described methods. Once the molecules are desorbed throughout the designated cubed area from the biological sample, standard ESI post-ionization is carried out prior to detection in the mass analyzer (Figure 4B). While IR-MALDESI has been predominantly used for analysis of tissue samples, it has gained attention recently for its applications in NP research. The Muddiman group, has detected small- and large-molecular features in cyanobacteria[48] and even in the high salinity environment of the pickle![49, 50]

In a discovery workflow, fermented cucumbers were imaged using IR-MALDESI to identify three triterpenoid lipids at the surface of the fermented cucumber. Incredibly, 50 micron sections of the pickle which was stored in a 1M brine solution yielded the identification of β-sitosterol, stigmasterol, and lupeol in various volume units of the cucumber section.[49] This analysis was accomplished with no sample preparation aside from freezing the fermented cucumber section for the IR-MALDESI analysis. In further work with the pickle, Fideler *et al.* identified bioactive peptides in pickle sections and correlated these results with the presence of lactic acid bacteria by comparing acidified versus fermented cucumbers.[50] Lactic acid bacteria in fermented foods have long been thought to produce bioactive peptides, but this is the first direct instance in which these peptides were found on a fermented food, although more work is needed to directly demonstrate that these peptides were produced by the lactic acid bacteria.[50] IR-MALDESI has yet to be commercialized but is a highly promising imaging modality for samples from high salinity environments, like sponges, and can be interfaced with a number of mass analyzers, much like the DESI source, allowing for high mass accuracy measurements or tandem mass spectrometry experiments to gain more information about NPs in a single experiment.

2.8 Secondary ion mass spectrometry (SIMS)

Secondary-ion mass spectrometry (SIMS) imaging was the first IMS method developed to evaluate and image sample surface analytes.[51] SIMS relies on a focused primary ion beam directed at the sample surface. When the primary ions collide with molecules in the sample, the primary ions transfer their charge to sample molecules generating secondary ions, which are ejected from the sample surface and introduced into the mass analyzer. Because of the high energy state of the primary ion beam, the detected analytes often represent fragments of the original intact molecule, meaning that SIMS experiments are often best employed when studying low molecular weight molecules or known molecules with characteristic secondary ion fragmentation patterns (Figure 7). Because SIMS is a high-vacuum technique, sample preparation usually involves fixation of the cells either chemically or cryogenically in order to maintain tissue integrity, which may alter the molecular composition of the sample. Additionally, because the primary ion beam is relatively non-destructive and doesn't penetrate and eject ions from deep within the sample, SIMS techniques are limited exclusively to the outer-most analytes of the sample. Despite these limitations, SIMS has been used in the identification of bioactive molecules from plant tissues[52] and microbial biofilms.[17, 53] It is worth noting that the main limitation of this method is interpreting the secondary ions. In a clever study of biofilms, Li *et al.* paired MALDI IMS with SIMS in two paired sample sets. Although the SIMS experiment yielded a richer spectrum with more

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distinct ions, the researchers noted that their mass defect suggested that they were inorganic in composition.[53] Therefore, SIMS may not provide the necessary information for NP discovery platforms, but does produce rich spectra, which may assist in other types of biological investigations.

3. Open-Source data platforms for IMS analyses

Although these methods are not the focus of this review as most commercial IMS platforms have proprietary software to facilitate data analysis, it is worth mentioning the open-source software platforms that can facilitate IMS analyses as those new to the field may need to process their datasets external to the lab where the data were acquired, and thus may not have access to the proprietary software. Because each company's software generates its own raw data file types which don't lend themselves to open source software platforms, a growing need for inter-lab collaborations has driven the development of many open-source tools for IMS analyses. Toward this goal, an open and standard format for IMS datasets has been implemented as the imzML format.[56] Recently a number of open-source IMS analysis tools that utilize the imzML format (as well as other formats) have been developed, although they have been underutilized in natural products discovery efforts and have instead been primarily used for the imaging of endogenous metabolites and proteins in mammalian tissues.[57–61] One exception is the use of CycloBranch for the *de novo* peptide sequencing and dereplication of microbial siderophores from IMS datasets.[62] As the natural products community relies more on IMS for compound discovery, these tools will likely see increased use. We encourage readers of this article to explore these open access tools so that a more robust open-source community may develop around IMS workflows. A list of these tools is summarized in Table 2.

4. Multimodal imaging

Visualizing the molecular topography of an intact sample is one of the most significant advantages to IMS, thus incorporating it with other imaging techniques such as macro- and microscopic imaging can provide a wealth of information (Figure 8). In order for the localization data to be meaningful, the data from the mass spectrometer needs to be aligned, or "coregistered", with the orthogonal imaging data. This often requires computational or clever experimental design to locate specific points on orthogonal images, which are oftentimes on a different spatial scale, than the IMS dataset, and the computational capacity to align hundreds to thousands of individual MS data-points to these images.

4.1 Histological

Although IMS has traditionally been used for the evaluation of surface-associated metabolites (with the exception of IR-MALDESI which are volume based measurements), various approaches have been used to evaluate metabolite production at different depths within a sample. For assessment of tissue samples, one of the most common approaches is to section the tissue manually using microtomy. While historically effective for mammalian tissues and use with other imaging methods such as microscopy and histology, this requires a substantial amount of sample handling, such as embedding into gelatin or resin, and also requires a thorough technical understanding of the sample preparation methodology to avoid

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artifacts caused by tissue damage. Le Pogam *et al.* found that hand sectioning of lichen tissue at 40–100 μm helped preserve morphological features, improving spatial mapping of metabolites to these features.[63] Garg *et al.* also successfully employed embedding a small portion of a lichen in gelatin followed by sectioning to reveal the striation of NPs by IMS across the sun-exposed versus middle and bottom layer of the lichen.[64]

4.2 Electron microscopy/ Atomic force microscopy

Microscopy provides a high spatial resolution (nm-μm) compared to IMS but is often dependent on tissue specific dyes or other target specific labeling methods. Inversely, IMS provides relatively low spatial resolution (10+ μm) but the capacity to detect specific molecules in an untargeted fashion. Thus, microscopy and IMS couple nicely to increase understanding of various metabolite distributions at the microscale. When mapping the biosynthetic topography of the seed of the tropical plant *Datura leichhardtii,* IMS was paired with electron microscopy to improve correlations between different seed tissues and alkaloid biosynthetic precursor distributions at a pixel resolution of approximately 50 μm^2.[12] Even greater resolution was reported in the combination of SIMS imaging with atomic force microscopy (AMF), with the AMF dataset providing sub-micron resolution and the IMS dataset providing 2 μm spatial resolution of phenazine production by *Pseudomonas aeruginosa*.[65]

4.3 Fluorescence microscopy

Fluorophores are molecules that can be excited by light of a certain wavelength, then re-emit light of a different wavelength. Microscopic imaging using fluorophores provides researchers with the opportunity to specifically localize certain proteins or molecules in a tissue sample by imaging the spatial re-emission of light. By co-localizing specific fluorescent signals with IMS data, researchers are able to glean chemical information about specific fluorescently labeled sites in their tissue samples. This approach has been incredibly useful to link metabolites to their producing organisms in an ecologically relevant context and to elucidate the biosynthetic capacity of unculturable microorganisms. MALDI-IMS and fluorescence in-situ hybridization (FISH) was recently reviewed by Kaltenpoth *et al.* and the group specifically highlighted symbiotic microbe production of antibiotics on the cocoons of bee-wolf larvae.[66] Similarly, Lackner *et al.* used FISH to label lab-unculturable bacterial symbionts of a marine sponge to demonstrate the correlation between these cryptic symbionts and the production of the NPs cyclotheonamide A and onnamide A (Figure 8).[67] Aside from using fluorophores to image specific biosynthetic organisms in complex symbioses, fluorescence microscopy and IMS strategies have been combined to monitor bacterial gene regulation in response to NPs. For example, a fluorescent protein gene was integrated downstream of a promoter of a major biofilm factor gene, thus when biofilms were produced, the bacterial cells would be labeled with an endogenous fluorophore. When combined with MALDI-IMS, Bleich *et al.* showed that the fluorescent signal colocalized with the distribution of the bioactive thiazolyl peptides, thiocillins, indicating that these bacterial NPs contribute to interspecies signaling and regulation of biofilm development.[68]

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5. Future outlook

Mass spectrometry is already an essential part of nearly all NP discovery efforts, and over the last decade IMS has increasingly played a role in understanding when and where NPs are produced in a biologically or ecologically relevant context. As highlighted in this article, IMS methods are applicable across a diversity of biological systems and tissue types, and can be used to differentiate producing organisms from complex poly-organismal systems. While DESI and MALDI are still the most commonly applied methodologies in NP research, new IMS modalities have recently emerged providing novel orthogonal approaches to investigating different analytes of interest from complex samples.

Like most imaging methods, increased spatial resolving power is always essential to gaining deeper insights within the context of a biological sample. Although advances in sub-cellular[69] and single-cell imaging[70] have been achieved with IMS, they have not been applied directly to NP research as they are not yet widely commercially available. The unfortunate trade-off of increasing spatial resolution in IMS is the reduction of total ions created from a sample surface, which effectively reduces sensitivity, so only the most abundant ions can be detected at high spatial resolution. A promising alternative approach to improving spatial mapping of IMS data without increasing sampling frequency is the use of data-smoothing algorithms, which use correlations to visible microscopic features to predict the actual distributions of detected molecules.[71] Although these methods haven’t yet been applied to NP research, but they may provide new opportunities to identify new chemoanatomical features.

Aside from improving spatial resolution, improved confidence in compound identification is critical to any NP discovery effort. Many IMS methodologies were recently employed on tandem high mass resolving power mass spectrometers, and although MS^n aides in compound identification and dereplication it is often insufficient to elucidate the structures of unknown molecules and some constitutional isomers and must be used in combination with other analytical methods such as 2D-NMR or X-ray crystallography to complete the structure elucidation. Recently, ion mobility mass spectrometry has been incorporated into an IMS experiment to resolve isobaric species and low-abundance molecules.[72] Additionally, some researchers are utilizing multiple mass analyzers within a single IMS experiment to enhance the informational content collected from each pixel. For example, model tripartite peatland cultures (fungus, moss, and cyanobacteria) were explored using multi-modal IMS which combined MALDI and LESA-MS^2 experimentation, providing high spatial resolution and highly accurate structural information to help resolve isobaric species.[73] Combined approaches such as this will undoubtedly aid in overcoming limitations of individual IMS modalities and provide a broader assessment of molecular features via orthogonal data acquisitions.

Although a majority of the examples given here focused on the use of IMS for NP discovery, these methodologies have historically been applied in a targeted fashion to clinically relevant tissues, one could envision applying IMS to study NP distributions and metabolism in mammalian tissues, enhancing drug development pipelines.[74] As such, we believe that advancements in IMS in the next five years will be driven primarily by creative biological

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questions and different sample preparations. Tissue applications have become relatively standard but not all biological applications utilize an intact tissue or microbial colony. The expansion of IMS to other fields will rely on adapting the existing techniques to be compatible across biological sample types and questions. An exciting example of this was our adaptation of the microbial agar based sampling preparation to 3D cultures of cells in co-culture with healthy explants in agarose to explore chemical communication in metastasis.[75] As IMS becomes more widely adopted and some of the recent innovations in ionization techniques highlighted herein become more widely available, it is likely to impact NP research from discovery to pharmaceutical development.

Acknowledgements

This publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R01GM125943 and by the National Science Foundation grant 1817955. We would also like to acknowledge Roger Linington and Shannon Cornett for helpful discussions.

Mass Spectrometry-based Acronyms

ESI	Electrospray ionization
DESI	Desorption electrospray ionization
LAESI	Laser ablation electrospray ionization
MALDI	Matrix assisted laser desorption/ionization
SALDI	Surface-assisted laser desorption/ionization
LESA	Liquid extraction surface analysis
LMJ-SSP	Liquid microjunction-surface sampling probes
LA-ICP	Laser ablation inductively coupled plasma
DART	Direct analysis in real time
IR-MALDESI	Infrared matrix assisted laser desorption electrospray ionization
SIMS	Secondary ion mass spectrometry
DIMS	Direct injection mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
TOF	Time-of-flight
FT-ICR	Fourier-transform ion cyclotron resonance
IMS	Imaging mass spectrometry (as known as MSI in other reviews)

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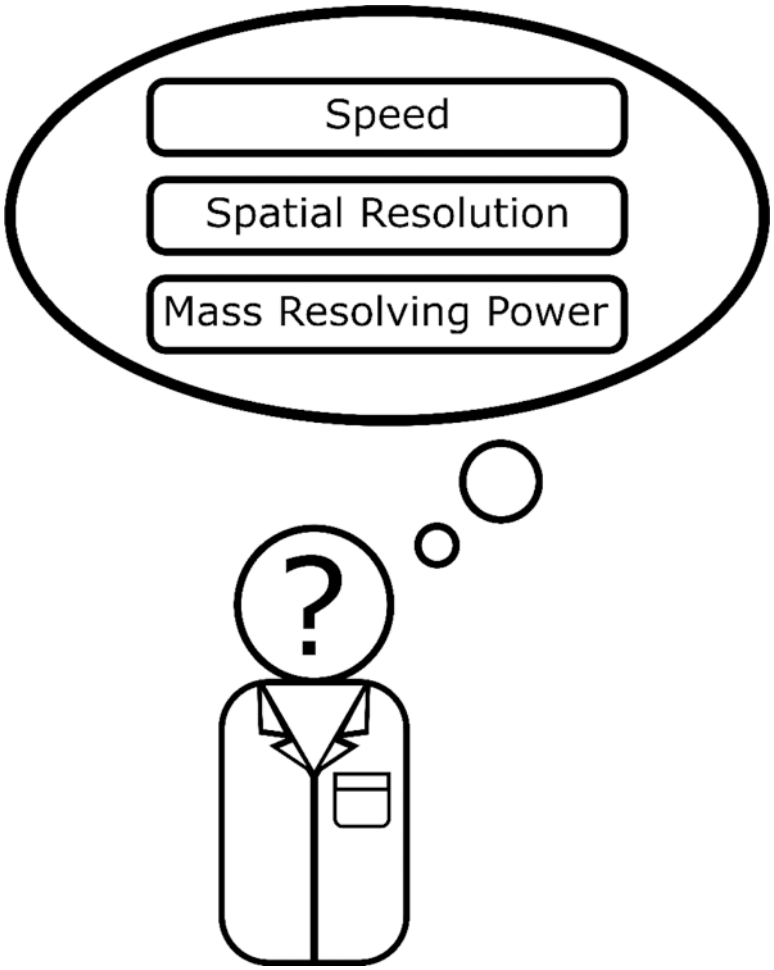

Figure 1.
When performing imaging mass spectrometry experiments, one must decide on the proper instrument to use depending on various factors including but not limited to speed, spatial resolution, and mass resolving power.

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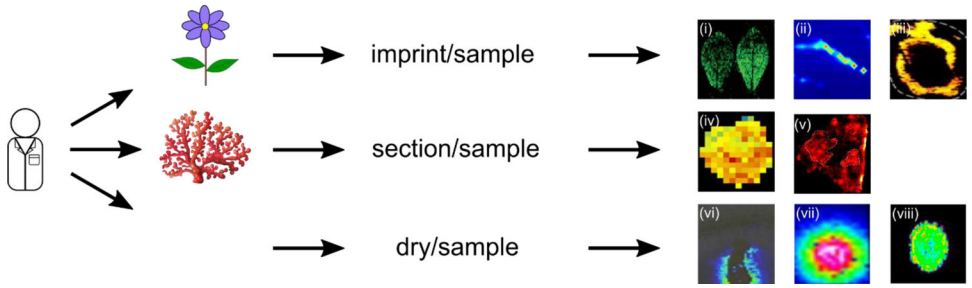

Figure 2.

Imaging mass spectrometry can be used to identify spatial distribution of metabolites from a variety of samples, such as plants, animals, and microbes. (i) DESI-IMS of *Lotus japonicus* cyd1, MG-20, and cyd2–2 leaves showing *m/z* 286. Figure adapted with permission under Creative Commons Attribution 3.0 Unported (CC BY 3.0).[10] (ii) LAESI-IMS on pansy leaf showing elphinidin-3-p-coumaroylr-hamnosylglucoside-5-glucoside (*m/z* 919.3). Reprinted (adapted) with permission from Vaikkinen *et al.* Copyright 2013 American Chemical Society.[11] (iii) DART-IMS on *Datura leichhardtii* seed showing *m/z* 163.0753. Reprinted (adapted) with permission from Fowble *et al.* Copyright 2017 American Chemical Society.[12] (iv) LESA-IMS on rat liver tissue sections of moxifloxacin (*m/z* 402.1823) at 2 hours. Figure adapted with permission under Creative Commons International 4.0 (CC BY 4.0).[13] (v) IR-MALDESI-IMS on cancerous hen ovarian tissue sections showing glutathione (*m/z* 306.0766). The analyst online by Society of Public Analysts (Great Britain) Nazari *et al.* Reproduced with permission of Royal Society of Chemistry.[14] (vi) MALDI-TOF-IMS on *Glutamicibacter arilaetensis* and *Penicillium #12* co-culture for *m/z* 655.2. Figure adapted with permission under Creative Commons International 4.0 (CC BY 4.0).[15] (vii) SALDI-IMS on *Aspergillus* 3Y of fellutamide C sodium adduct (*m/z* 580). Reprinted (adapted) with permission from Chen *et al.* Copyright 2018 American Chemical Society.[16] (viii) SIMS imaging on *Pseudomonas aeruginosa* PAO1C biofilm at 72 hours (*m/z* 288.20). Reprinted (adapted) with permission from Dunham *et al.* Copyright 2018 American Chemical Society.[17]

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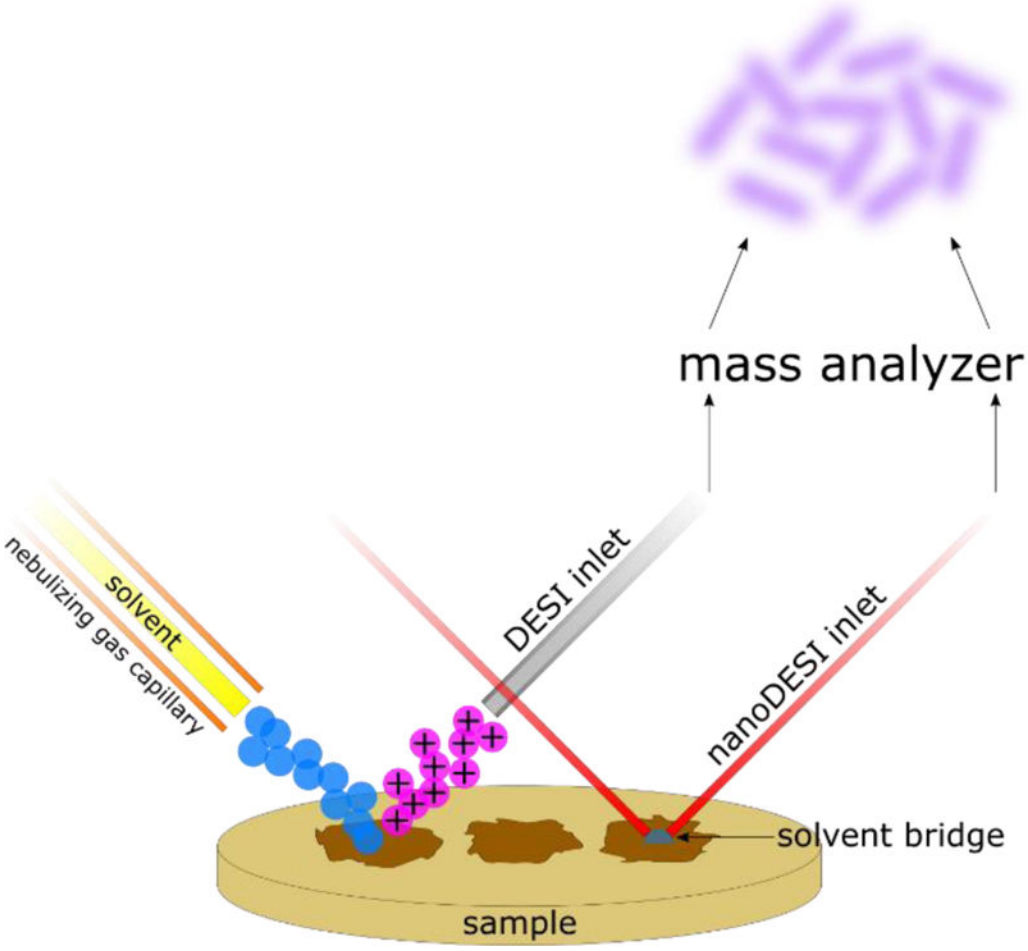

Figure 3.

DESI experiments make use of a stream of charged solvent droplets generated from a solvent capillary flowing adjacent to a capillary containing nebulizing gas. The analyte is taken up by the charged solvent upon contact with the sample mounted on a non-conductive surface. Subsequent collisions with other charged droplets results in formation of multiply charged ions. These ions are taken up by the mass spectrometry inlet. NanoDESI experiments work similarly to DESI but use a fused capillary for both solvent and nebulizing gas. A second capillary that leads directly to the mass spectrometer inlet is placed near the sample, and a solvent is formed between the two capillaries.

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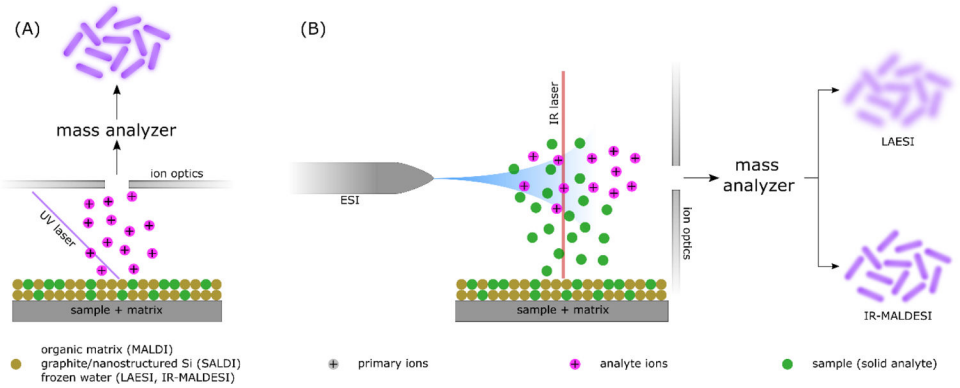

Figure 4.

(A) In MALDI and SALDI experiments, matrix is applied to a sample and allowed to co-crystallize on a conductive surface. A UV laser shot at the sample/matrix facilitates soft ionization by transferring energy from the laser to the analyte to form ions that are guided to the mass spectrometer for analysis. (B) LAESI and IR-MALDESI experiments have samples that contain frozen water. An IR laser shot at the sample releases/desorbs the analyte, which is then ionized by ESI before being taken up by the mass spectrometer for analysis.

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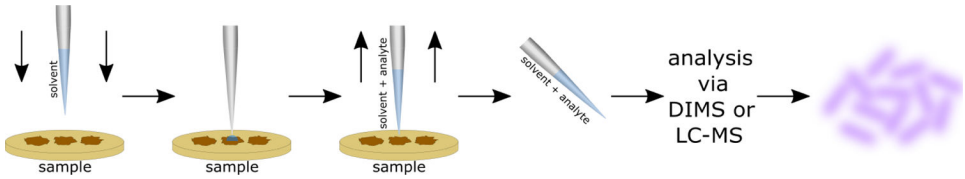

Figure 5.

In LESA and LMJ-SSP experiments, liquid solvent is applied to a sample surface and quickly taken up to perform an extraction of surface analytes. This solvent/analyte mixture can be separated or analyzed via direct injection mass spectrometry (DIMS) or injection into an LC-MS system.

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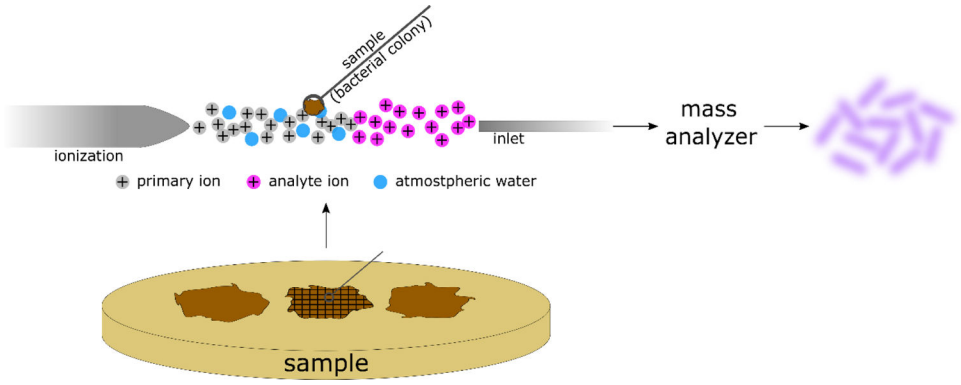

Figure 6.

DART experiments apply an ion plasma stream directly to a sample to facilitate ionization without the need for sample preparation, such as direct sampling of microbes from a solid agar culture. The resulting analyte ions produced during ionization are taken up by the mass spectrometer inlet for analysis.

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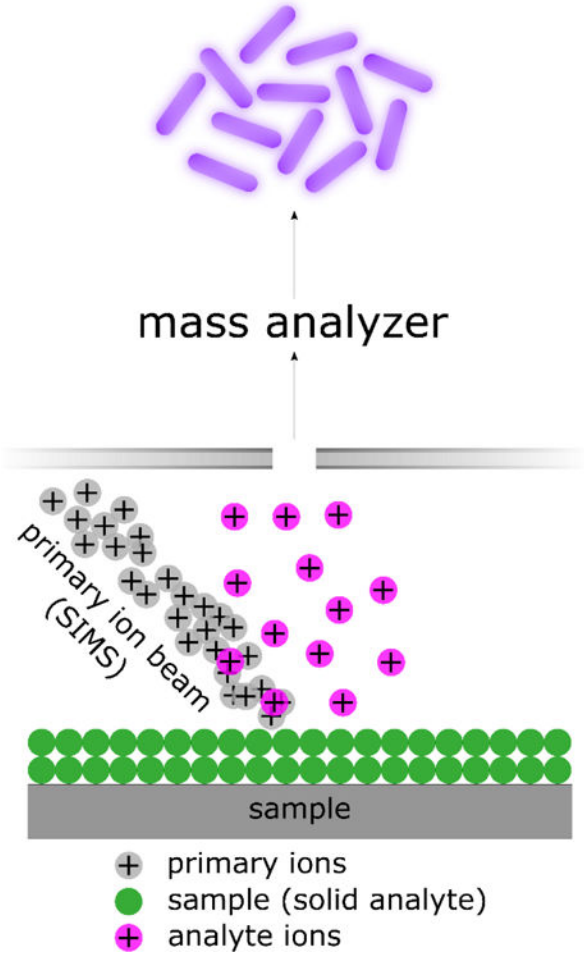

Figure 7.

SIMS experiments use a UV laser shot at the sample to facilitate soft ionization by transferring energy from the laser to the analyte to form ions. Collision between the primary ions and sample result in formation of analyte ions. Ions generated by this technique are taken up by the mass spectrometer inlet.

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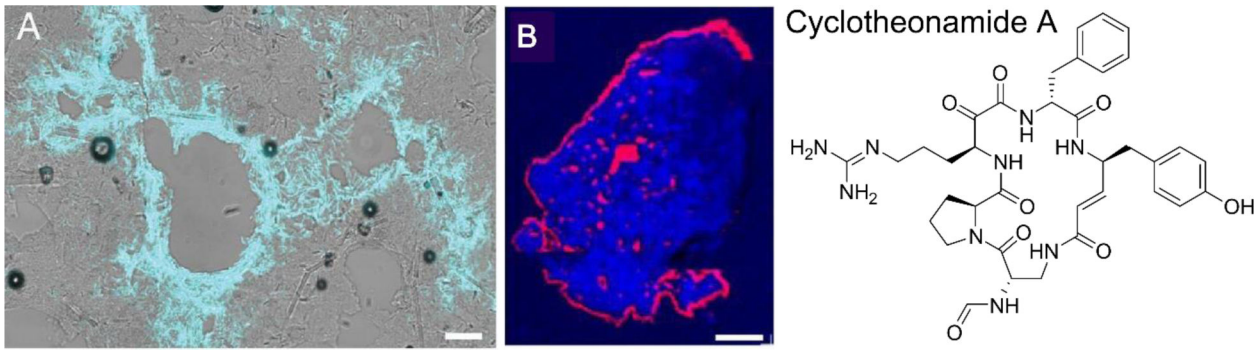

Figure 8.

Example of CARD-FISH coupled with MALDI-LTQ-Oribtrap IMS to identify the microbial source of natural product producing organisms which have not been cultured in the laboratory but are readily viable in sponge tissues in the environment. A) CARD-FISH of *Ca*. Entotheonella B) MALDI LTQ-Oribtrap IMS ion image of cyclotheonamide A demonstarting that while *Ca*. Entotheonella is localized to the outer edges of the pores of the sponge, the compound accumulates in the pores. Reprinted (adapted) with permission from Lackner *et al.* Copyright (2017) National Academy of Sciences.

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Table 1.

Ionization source characteristics.

Information about the resolution, type of information standardly collected, and sample considerations for each of the ionization sources reviewed herein. Pixels are two-dimensional datapoints, Voxels are three-dimensional datapoints. Abbreviations: DESI – Desorption Electrospray Ionization; LAESI – Laser Ablation Electrospray Ionization; MALDI – Matrix Assisted Laser Desorption/Ionization; SALDI – Surface Assisted Laser Desorption/Ionization; SIMS – Secondary Ion Mass Spectrometry; LESA – Liquid Extraction Surface Analysis; LA-DART - Laser Ablation Direct Analysis in Real-Time; IR-MALDESI - Infrared Matrix Assisted Laser Desorption Electrospray Ionization. *It is worth noting that this lateral resolution has not been achieved using a natural products based sample.[54] **This lateral resolution was achieved using a radio frequency plasma oxygen primary ion source and has identified elements in biological samples but has yet to be applied to a natural products based sample.[55]

Ionization source	Spatial resolution	Molecular information notes	Sample type/limitations considerations
DESI/nanoDESI (ambient)	150 microns/50microns routinely 10–15 microns*	Pixel	Solid, frozen liquid
LAESI (ambient)	150 microns	Voxel	Fresh sample
MALDI (vacuum)	5 microns	Pixel	Dried sample in matrix/matrix signal interference
SALDI (vacuum)	10 microns	Pixel	Sample transfer is necessary
SIMS (high vacuum)	5 microns – 0.037 microns**	Secondary ions, Pixel	Dried sample/May ionize both inorganic and organic molecules which may complicate discovery efforts Can requires extensive sample preparation due to high vacuum environment
LESA (ambient)	1 mm	Pixel	Fresh sample/Low spatial resolution
LA-DART (ambient)	50 microns	Pixel	Fresh sample/Low spatial resolution
IR-MALDESI (ambient)	50–100 microns	Voxel	Frozen sample/Compatible with high salt content

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Table 2.

A table highlighting open source software compatible with imaging mass spectrometry datasets, note vendor proprietary software is not included as this is instrument dependent, whereas many of these open source platforms are compatible with many different types of datasets.

Name	Function	Website	Documentation	Availability	Ref.
Cardinal	R package for imaging mass spectrometry preprocessing, statistical analysis, and visualization	https://bioconductor.org/packages/release/bioc/html/Cardinal.html	https://bioconductor.org/packages/release/bioc/vignettes/Cardinal/inst/doc/Cardinal-2-guide.html	Offline	57
MSiReader v1.0	MATLAB/standalone tool for imaging mass spectrometry quantification, quality assessment, and visualization	https://msireader.wordpress.ncsu.edu/	http://bitly.com/MSiReaderUsersManual	Offline	58
microMS	Python based tool for single-cell microscopy-based imaging mass spectrometry data analysis	http://neuroproteomics.scs.illinois.edu/microMS.htm	http://neuroproteomics.scs.illinois.edu/Site/microms/microMS_UserGuide.pdf	Offline	59
msIQuant	C++ based tool for imaging mass spectrometry preprocessing, quantitation, and visualization	https://ms-imaging.org/wp/paquan/	Included in installation files	Offline	60
OmniSpect	MATLAB based tool/web server for imaging mass spectrometry visualization and statistical analysis	http://cs.appstate.edu/omnispect/ (web server) https://github.com/rmparry7/omnispect/ (standalone)	https://github.com/rmparry7/omnispect/blob/master/README.txt (standalone)	Online/ Offline	61
CycloBranch	Cross platform tool for dereplication and *de novo* sequencing of nonribosomal peptides and polyketides	https://github.com/rmparry7/omnispect/blob/master/README.txt	https://ms.biomed.cas.cz/cyclobranch/docs/html/userguide.html	Offline	62