REVIEW

Mass Spectrometry Imaging of Microbes

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Abstract : Microbes influence many aspects of human life from the environment to health, yet evaluating their biological processes at the chemical level can be problematic. Mass spectrometry imaging (MSI) enables direct evaluation of microbial chemical processes at the atomic to molecular levels without destruction of valuable two-dimensional information. MSI is a label-free method that allows multiplex spatiotemporal visualization of atomic- or molecular-level information of microbial and microbe-related samples. As a result, microbial MSI has become an important field for both mass spectrometrists and microbiologists. In this review, basic techniques for microbial MSI, such as ionization methods and analyzers, are explored. In addition, we discuss practical applications of microbial MSI and various data-processing techniques.

Keywords : Mass spectrometry Imaging, Microbes, Tissues, Infection

Introduction

Microbes reside in diverse environments, such as soil,[1] drinking water,[2] beneath the Antarctic ice sheet,[3] the Sahara,[4] and in the human body.[5] The commensal microbes present in various compartments of the human body are important for maintaining health and combatting invading pathogens.[6-8] To date, this research has focused on the complexity of microbial communities and their relationship to food[9] and energy[10] by exploring energy metabolism[11] and production of secondary metabolites.[12] Despite substantial advances in the understanding of specific microbial processes in recent years, many challenges remain.[13]

Spatiotemporally resolved mass spectral information is important for understanding the molecular interactions between microbes and their environment.[14] Therefore, tools that conserve this information can enable elucidation of the chemical landscape of a microbial community.

Traditionally, microscopic imaging techniques have been the gold standard in microbiology[15] as they provide molecular distribution data without discarding spatiotemporal information. For example, fluorescence microscopy is used to characterize host-pathogen interactions within tissue.[16,17] However, these targeted techniques require prior knowledge of specific molecules and the development of specific and sensitive reagents, such as probes or antibodies, limiting multiplexed imaging.[18] Discovery-based approaches, such as nuclear magnetic resonance[19] and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS),[20] have been used to identify and quantify molecules originating from microbes. Because those techniques do not require labeling and enable multiplex detection, they facilitate discovery of novel molecules originating from microbial communities.[21] However, the sample homogenization and extraction processes required destroy any spatial information.

Mass spectrometry imaging (MSI) combines the advantages of microscopic techniques and discovery-based approaches while enabling spatiotemporal analysis of complex microbial communities with multiplex detection.[22] The basic principle of MSI is image reconstruction using x,y-coordinate linked mass-spectral data as pixels.[23] The number of mass spectra within a data set depends on the size of a selected region of interest (ROI) and on the spatial resolution (x,y pitch).[24] After collecting mass spectra within the ROI, the data are merged into an averaged mass spectrum from which is generated an ion map of selected ions that are represented by the mass/charge ratio (*m/z*). The ion map displays the

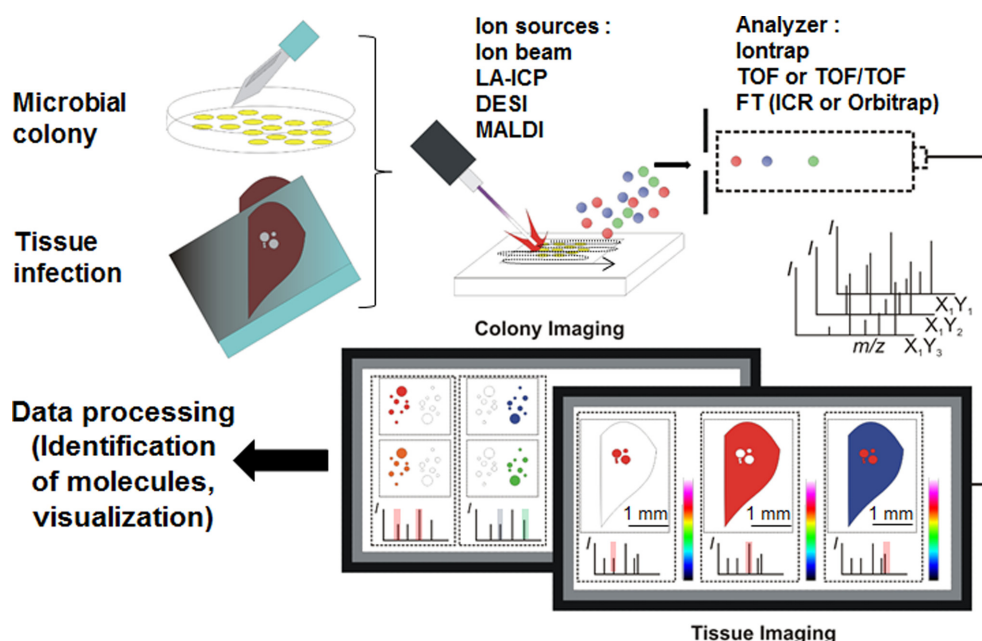Hyojik Yang, David R. Goodlett, Robert K. Ernst, and Alison J. Scott

Figure 1. Workflow of a microbial MSI experiment using microbial colonies or infected tissues. Modified from: Yang, H. et al. J Am. Soc. Mass Spectrom. In press.

relative intensity of the selected peak as a false-color scale of each pixel in the ROI. Because MSI employs mass spectrometry, the number of ion-map images available within a data set is dependent on the mass tolerance window, which is dictated by the resolving power of the instrument and the peak shapes in the data.[13] Thus, higher resolving power instruments can generate more ion map images, theoretically because they yield smaller windows of mass tolerance error; in some experiments the total number of ion map images reaches into the hundreds.[25] These features render MSI suitable for biomarker discovery and characterizing the complex molecular interactions between microbes and their environment.

To demonstrate the ability of MSI to directly analyze microbes, two types of conventional samples are illustrated in Figure 1. Bacterial colonies on thin agar[26] and in microbially infected tissues[27] are typically used to demonstrate the capability of MSI. Analysis of microbial colony samples by MSI enables the discovery of novel antibiotics and antifungals.[28-30] Microbial virulence factors and immunological mediators have been characterized *in vivo* by analysis of infected tissues using MSI.[31,32] Animal models of infectious disease are the gold standard for testing, for instance, immune responses and the penetration efficiency of antibiotics or antifungals into microbial communities in tissue; the latter typically employs autoradiography.[33] Recently, MSI has been used as an alternative to autoradiography to evaluate the efficiency of drug penetration of infected tissues.[34-36] We discuss these topics further in the section.

The basic components of a mass spectrometer (MS) are an ionization source, analyzer, and detector. Individually, the ionization source is the most important determinant of the spatial resolution of, and the ion species detectable by, MSI,[37,38] whereas the analyzer is also an important consideration if the goal is to identify chemicals in complex biological samples.[39] This review focuses on several of the ionization techniques and analyzers used for microbial MSI.

Figure 1 illustrates the principle of microbial MSI. Currently, four ionization methods—ion beams for secondary ion mass spectrometry (SIMS), laser-ablation inductively coupled plasma (LA-ICP), desorption electrospray ionization (DESI), and matrix-assisted laser desorption/ionization (MALDI)—have been commercialized and are widely used for microbial MSI. Although other ionization methods are available, they are outside the scope of this review.[13] We discuss the advantages and disadvantages of the aforementioned four ionization techniques for microbial MSI below.

Ionization Techniques

SIMS

Accelerated primary ion beams produce secondary analyte ions by surface sputtering, resulting in ejection of secondary ions.[40] Of the four ionization methods for microbial MSI, SIMS achieves the highest spatial resolution, on the order of nanometers. According to the total quantity of primary ions, there are two types of SIMS – dynamic and static. Dynamic SIMS removes a

substantial depth of molecules in the area bombarded by the ion beam,[41] due to the large quantity of primary ions produced. In contrast, static SIMS ejects surface ions from the uppermost atomic layer without significant damage to the underlying sample.[42] Dynamic SIMS is optimal for elemental analysis, such as monoatomic or diatomic ions, because of the high yield of source-induced fragmentation. Dynamic SIMS is also referred to as NanoSIMS because it can be configured for very high spatial resolution (< 50 nm), meaning that on the microbial scale of several to less than one-micron, sub-organismal features can be spatially resolved. By observing stable isotope-labeled small molecules at the single-cell level, the metabolic function of a molecule can be determined.[43,44] Dynamic SIMS sources are typically paired with a sector field analyzer, which combines electrostatic and magnetic components.[45] The most common analyzer for static SIMS is time-of-flight (TOF); therefore, static SIMS is commonly referred to as TOF-SIMS. Because static SIMS uses a relatively small number of primary ions (or atomic cluster-based primary ion guns) it results in less destructive ionization, reducing source-induced fragmentation.[46] Hence, TOF-SIMS is more suitable for analyzing fragile biomolecules that might fragment in the gas-phase prior to detection than dynamic SIMS. However, achieving a spatial resolution below 3 μm with TOF-SIMS is difficult due to the primary ion flux and focusing capacity.[47] Therefore, TOF-SIMS is mainly used for analyzing single colonies and biofilms.

LA-ICP

Maintaining metal ion homeostasis is critical for microbes and LA-ICP MSI has been used to directly observe the distributions of metal ions within microbial communities[48] and infected tissues.[49] This ionization technique involves ablation of a solid sample on a surface using a laser pulse (typically ultraviolet); the resulting ion plume is carried into a plasma source via a carrier gas, such as argon or an argon–helium mixture. All compounds are atomized and ionized in the ICP due to the high temperature ($\sim 10^4$ K) and ionized nature of plasma.[50] Therefore, LA-ICP MS is suitable for detection of all elements in the periodic table.[51] The operating spatial resolution can reach 1 to 100 μm.[52] Unlike SIMS or MALDI MSI, LA-ICP MSI does not require sample preparation, such as matrix deposition or a conductive surface. An example of the usefulness of LA-ICP-MSI for microbial pathogenesis lies in determination of the inflammation-associated shift in the distribution of metal elements in murine tissues infected with *Staphylococcus aureus*.[49] *S. aureus*-induced abscesses were found to be rich in calcium and devoid of manganese, iron, and zinc. Therefore, LA-ICP MSI can enhance our understanding of microbial pathogenesis at the atomic level.

DESI

DESI was developed in 2004 and is a surface-sampling ionization process that begins with a pneumatically assisted stream of charged solvent droplets projected onto a sample positioned a few millimeters from the sprayer tip.[53,54] The ejected droplets collide with the sample surface from which analytes are extracted and ionized. After desorption, the generated analyte ions are drawn into an MS inlet and through an electrospray ionization (ESI) source. However, the pneumatic pressure used in DESI hampers direct analysis of microbes on dehydrated agar because it can damage the agar surface and the microbial colony.[30] This can be overcome by imprinting the microbial colony or microbial community onto a hydrophilic membrane for surface sampling.[30] To circumvent imprinting and facilitate imaging of microbial colonies by DESI, NanoDESI MSI has been used.[55] NanoDESI MSI can be configured to visualize living microbial colonies on agar without the dehydration required for other ionization methods, including MALDI.[56]

MALDI

MALDI was developed in 1985 and was first reported as an imaging modality in 1997 to visualize peptides and proteins in animal tissue samples.[57,58] Because of its ability to analyze a wide variety of molecules, approximately half of the microbial MSI publications involve MALDI MSI.[13] MALDI requires matrix deposition onto the sample surface and both application and matrix selection are major considerations for microbial MSI. The applied matrix absorbs laser energy, facilitating localized desorption/ionization of a microbial sample. The MALDI matrix and other sample preparation parameters are critical for optimal signal generation.[59] Generally, small aromatic carboxylic group-containing molecules—such as 2,5-dihydroxy benzoic acid (DHB), alpha-cyano-4-hydroxycinnamic acid (CHCA), and coumaric acid—act as a proton donor for positive-ion mode. By contrast, aromatic primary, secondary, and tertiary amine-containing molecules—such as 9-aminoacridine (9-AA), 1,5-diaminonaphthalene (DAN), and norharmane (NRM)—are used as the basic matrix in negative-ion mode.[60] The two microbial MSI workflows in Figure 1 both have unique considerations. To image microbial colonies cultured on agar, complete dehydration is essential.[61] Because of the matrix coating the sample surface, background signals in the small *m/z* region (> 500 Da) can hamper analysis of small-molecule metabolites.[62] This limitations can be overcome through the use of high-resolution mass analyzer[63] or development of alternative matrices to reduce background signals.[64] At present, microbial MSI of infected tissues typically uses a MALDI source due to its ability to detect diverse molecules and its high sensitivity. Although the pressures used for MALDI MSI range from atmospheric to high vacuum ($\sim 10^{-5}$ bar and below), high-vacuum conditions are typically employed to maximize sensitivity.[61] Unlike high vacuum ionization techniques (MALDI, SIMS)

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ambient ionization methods (ex: DESI, LA-ICP) do not require sample preparation, such as matrix deposition, complete desiccation, or a conductive surface. This feature makes them attractive options for diverse samples such as bacterial colonies on a growth medium where complete desiccation of thin agar can be burdensome. In terms of spatial resolution, high vacuum environments can generally provides higher resolution for each targeted ion species. For instance, SIMS and LA-ICP are both suitable for imaging of metal ions, but SIMS can readily generate below 1 μm imaging whereas LA-ICP routinely operates above the 10 μm range. DESI and MALDI are less destructive, making them appropriate choices for imaging complex biomolecules, with typical spatial resolutions range in the tens to hundreds of microns. DESI can range between 50 and 200 μm. On the other hand, MALDI is readily achieves 10 μm using a commercially available instrument without special modification. The critical selection of ionization method will be driven by the balance between molecular target, spatial resolution, and sample format.

Mass Analyzers for Microbial MSI

Several mass analyzers are used in MS; their design and use have been reviewed elsewhere.[65,66] Here, we highlight the analyzers commonly used for microbial MSI. TOF devices are the most common and are combined with a variety of ionization sources; for example, MALDI, TOF-SIMS, LA-ICP, and DESI.[67-69] TOF analyzers have advantages including a high scan rate (microseconds)[70] and high resolving power ($> 10^4$).[71] However, even the best-performing TOF analyzers do not provide sufficient resolving power and accuracy to identify unknown biomolecules in complex samples, such as a microbial colony or infected tissue, without additional steps. Therefore, full molecular identification typically involves the use of other mass analyzers that can provide high mass accuracy and tandem MS or MS^n for structural determination. Instruments capable of performing MS^n provide more in-depth structural information on specific molecules in a sample.[72] Ion-trap analyzers are particularly useful for tandem MS-based imaging of select precursor ions.[73,74] These analyzers allow structural determination by directly fragmenting the ion of interest in a microbial MSI experiment. In addition, sensitivity and selectivity can be significantly enhanced by tandem MS-based imaging. These analyzers are particularly useful for small metabolite imaging, and for quantification of medications because of the specificity of the targeted ions. Chumbley et al. reported quantification of the anti-tuberculosis drug, rifampicin in rat liver tissues by MALDI LIT MS.[75] Tandem MS imaging is now possible without ion-trap instruments using TOF analyzers, such as TOF/TOF (Bruker, Shimadzu) or quadrupole/TOF (Q/TOF, Waters). The limiting factor for multiplex

microbial MSI by tandem MS is that single compounds are manually determined, although parallelized data-dependent imaging methods are becoming more widely available for multiplexed tandem MS MSI.[76] High-resolution mass spectrometry (HRMS) microbial MSI enables observation and identification of molecules using accurate mass assignments in multiplex without tandem MS, although fragmentation analysis is still required for complete molecular identification.[77,78] For example, Fourier-transform instruments such as ion cyclotron resonance (ICR)[79] or orbitrap MS[80] achieve high resolving power and accuracy for determining the chemical formulae of ions in mass spectra without MS/MS. However, the acquisition time of HRMS-based MSI (1 pixel/sec) is considerably slower than that of TOF (40 pixels/sec).[81] Given that each analyzer type have advantages and disadvantages, the mass analyzer used for microbial MSI should be selected based on the aim of the experiment.

Applications

Single-cell microbial MSI

Microbes range from nanometers to millimeters in diameter. For instance, a mycoplasma cell is approximately 200 nm across,[82] but *Thiomargarita namibiensis* can be 0.75 mm in diameter.[83] Most microorganisms are around 0.5 to 5 μm in diameter, smaller than eukaryotic cells (~ 10 μm).[84] Given that static and dynamic SIMS can provide sufficient spatial resolution at the nanometer scale, they are suitable for studying individual microbial cells.[69,85] *Tetrahymena thermophila*, a eukaryotic microorganism was imaged by static SIMS during membrane fusion at the single-cell level.[86] The results revealed that the phospholipid composition at the mating junction varies considerably. For instance, an ion with an *m/z* of 184.1, originating from the phosphatidylcholine headgroup, was highly abundant in the cell body but not at the conjugation junction. In contrast, an ion with an *m/z* of 124.1, derived from 2-aminoethylphosphonolipid was one of the predominant ions at the conjugation junction but not in the cell body.[86] Another example of single-cell microbial MSI used dynamic SIMS MSI on the nitrogen-fixing bacterium, *Teredinibacter turnerae* (*T. turnerae*) during polymicrobial culture with *Enterococcus faecalis* (*E. faecalis,* non-N$_2$-fixing bacteria) following labeling with ^{15}N.[87] Briefly, *T. turnerae* and *E. faecalis* were cultured separately for 120 hours in a heavy isotope (^{15}N) atmosphere to label the components of the nitrogen cycle.[87,88]

The results showed that *T. turnerae* selectively generated a $^{12}C^{15}N^-$ signal. However, this ion was absent in the *E. faecalis* control because the control is non-N$_2$-fixing bacteria.[87] Recent developments of the other ionization techniques discussed here (MALDI, LA-ICP, and DESI) have allowed spatial resolutions of around 0.6, 1, and 10 μm, respectively.[52,89,90] Therefore, imaging of single

Table 1. Ionization techniques and mass analyzers for microbial MSI samples.

Ion Source	Analyzer	Limit of Spatial Resolution (nm)	Detected Ions	Types of Sample	Degree of fragmentation
Ion beam	Dynamic SIMS: electrostatic and magnetic sector[43]	35[43]	atom : majority of elements, small molecule : CN, C$_2$, O$_2$[43,87]	Single cells[87]	Very severe
	Static SIMS: TOF[47]	100[47]	Atoms : majority of elements, small molecules to high molecular mass lipids (e.g : gangliosides, cardiolipin)[47,86]	Single cells[86], monoculture	Severe
MALDI	Ion trap,[72] TOF,[58] TOF/TOF,[97] Q/TOF,[98] FT (ICR[99] or Orbitrap[89])	600[89]	small molecule to macromolecules (e.g Fatty acid, glycan, peptides, proteins)[100]	Monoculture,[91] coculture,[101] infected tissue[31,49,102]	Minor
DESI	Ion trap,[53] Q/TOF,[103] FT (ICR[104] or Orbitrap[105])	10,000[90]	small molecule to macromolecules (e.g Fatty acid, glycan, peptides, proteins)[100]	Monoculture,[94] coculture[103]	Minor
LA-ICP	TOF,[49] Quadrupole[106]	1,000[52]	atom : theoretically all elements[52]	Monoculture,[107] infected tissue[49]	Very severe

microbial cells remains a challenge with those three modalities but not with SIMS.

Microbial monoculture MSI

All types of MSI techniques are suitable for the visualization of a microbial colony. For instance, Tong Si *et al.* demonstrated that MALDI MSI enables visualization of the peptide distribution in a monoculture of *Bacillus subtilis* (*B. subtilis*).[91] Plipastatins, which have antibiotic activity, were highly localized in the extracellular region (outside the colony) on the agar plate. However, sporulation-delaying protein (SDP) and sporulation killing factor (SKF), the cannibalism toxins of *B. subtilis*, were highly concentrated in the inner region of the bacterial colony. In addition, the expression of SDP and SKF was markedly affected by the composition of the growth medium. For example, the authors did not detect SDP and SKF when they used ISP2 medium.[92] In contrast, they detected the cannibalism toxins in the colony using MSgg medium. While ISP-2 is a nutrient rich medium, MSgg is minimal nutrient containing medium.[93] Moreover, MSI showed that genetically modified strains did not produce surfactins or plipasptatins on agar.[91] Another application of MSI was discovery of secondary metabolites in colonies of *Streptomyces mashuensis*, ATCC 2934 using a DESI source.[94] Using DESI MSI, Pereira et al. detected and visualized lysolipin I and lienomycin and tested their antibiotic and antifungal activities by conventional disc diffusion assays. The identification of new antibiotics from bacteria is important because of the emergence of multidrug-resistant microbes.[95] New antibiotics and antifungals can be discovered by microbial MSI of two interacting organisms without the need for conventional disc-diffusion assays. Finally, monospecies biofilms have also been characterized using MSI. Eric *et al*. analyzed

Pseudomonas aeruginosa biofilms by SIMS and confocal Raman microscopy—the chemical spatial information yielded by the two techniques showed a high level of agreement. This is also covered in the section.

MSI of microbial cocultures

In nature, microbes are typically found in polymicrobial communities, in which multiple species interact, compete for and potentially share metal ions, metabolites, peptides, and macromolecules.[13] Polymicrobial communities can be reproduced *in vitro* and can be visualized using microbial MSI. For instance, using MALDI MSI, Holzlechner *et al.* visualized the distribution of the metabolites produced by two fungi on a potato-dextrose agar-coated indium-tin oxide slide.[101] *Trichoderma atroviride* (*T. atroviride*) inhibited the growth of *Rhizoctonia solani* (*R. solani*) by direct mycoparasitism. *T. atroviride and R. solani* were cocultured and mycoparasite–host interaction-related molecules, such as peptaibols (which have antifungal activity), were identified. The McLean group also visualized the predator-prey system of *Myxococcus xanthus DK1622* (*M. xanthus*) and *E. coli* using DESI MSI.[103] The results showed the utility of microporous membrane scaffolds for spatiochemical analysis of interacting microbes using DESI MSI. By DESI MSI, they visualized myxovirescin A in the area of interaction between *M. xanthus* and *E. coli*. Myxovirescin A is involved in predation of other bacteria and has antibiotic activity in the yellow phase of *M. xanthus*.[103] *M. xanthus* can have two phases to produce colonies that contain yellow or tan cells. Yellow phase of *M. xanthus* tends to lyse in liquid during stationary phase.[108] By employing a coculture system, investigation of the molecular networking in metabolomics of interspecies is possible using microbial MSI. For example, Watrous et al.

evaluated tandem MS and MSI for analyzing the molecular networks of microbial colonies using cocultures of *Streptomyces coelicolor* and *Bacillus subtilis*.[109] Characterization of samples by tandem MS and DESI MSI enhanced our understanding of the spatiotemporal dynamics of metabolite production in microbial communities. Moreover, MSI enables characterization and visualization of natural products in microbial communities, such as the soil microbiome.[65,110]

MSI of infected tissue

Currently, MALDI MSI mainly uses for directly analyzing tissue and visualizing the distribution of target molecules.[58] Other MSI techniques are also readily applied to tissue samples.[100] Analyzing tissue using MSI enables characterization of the fundamental molecular basis for host-pathogen interactions.[111] Microbial pathogenesis is the ability of microbes, or their components, to cause infection in a host. Moreover, evaluation of microbial pathogenesis at the molecular level is critical for understanding infectious diseases.[111] Pathogens often produce unique molecular signatures during host invasion and infection. These are generally referred to as virulence factors and are key components of pathogenesis. Virulence factors are often related to colonization, nutrient and cofactor acquisition from the host, and immunosuppression. Lipid A, the acylated moiety of lipopolysaccharide, is a virulence factor of Gram-negative bacteria.[112] Using MALDI MSI, our group demonstrated the distribution of *Francisella novicida* lipid A in infected spleen tissue.[31] The virulence factor was highly localized in splenic red pulp. In contrast, arachidonic acid-containing phospholipids, originating from splenic white pulp host cells, were depleted during *F. novicida* infection. The MSI results were also used for mechanistic characterization of the role of the cyclooxygenase-2–dependent lipid inflammatory pathway in lethality in *F. novicida* due to the overproduction of proinflammatory effectors such as prostaglandin E2. The glycolipids, phosphatidyl-myo-inositol mannosides (PIMs), were also visualized by MALDI MSI in *Mycobacterium tuberculosis* (*M. tuberculosis*) lesions in lung granuloma tissues.[102] PIMs are important virulence factors during the infection cycle of *M. tuberculosis* and are highly localized in *M. tuberculosis* lesions. Moreover, MALDI MSI enabled qualitative evaluation of the penetration efficiency of anti-TB drugs to lesions by facilitating observation of their colocalization with PIMs.

Evaluation of the penetration efficiency of antimicrobials, such as anti-TB drugs and antifungals to infected tissue using MSI is an area of active pharmacologic research. For instance, Prideaux et al. demonstrated that rifampicin and pyrazinamide efficiently penetrate sites of TB infection in lung lesions.[34] Moreover, the concentration of drug in the lesion area was evaluated by offline laser capture microdissection (LCM) and HPLC-MS/MS. The antifungal compounds, echinocandin[35]

and isavuconazole,[36] were also visualized in fungal-infected tissues. Therefore, MSI enables evaluation of the distribution and pharmacokinetics of drugs in infected tissue.

Visualization and characterization of factors produced by host cells during infection is another important application of microbial MSI.[111] Using MALDI MSI, the Skaar group demonstrated that calprotectin was highly colocalized with the immune response to *Pseudomonas aeruginosa* (*P. aeruginosa*) and *S. aureus*.[48,113] Calprotectin accounts for 60% of the soluble cytosolic protein in neutrophils and exerts a bacteriostatic effect by sequestering manganese and zinc ions. A recent study of *S. aureus*-infected tissue by MSI demonstrated a paucity of calprotectin signals in the bacterial nidus in tissue abscesses.[49] Also, an intense signal of δ-hemolysin from *S. aureus* was detected in the bacterial nidus. These data suggest considerable molecular heterogeneity at the host–pathogen interface and the need for further MALDI MSI-based studies of tissue infection. Table 1. summarizes the ionization techniques and mass analyzers used for microbial MSI, with the ion species that can be detected, and the spatial resolutions achieved.

Data Processing

Identification of chemical species by database searching

After MSI, several processes are performed to generate chemical information and visualize the data. Targeted approaches, such as visualization of antibiotics and antifungals, do not require database searching as the chemical information is known. Visualization of metal ions by dynamic SIMS or LA-ICP MSI also does not require database searching because the ion *m/z* values are determined by the mass analyzer. However, to obtain molecular information from the mass spectra using untargeted approaches, peak annotation by searching against a database is necessary. Because microbial MSI typically aims to detect microbial metabolites, we briefly describe some of the search engines for microbial metabolites. One such search engine is the Human Metabolome Database (HMDB),[114] which also encompasses yeast[115] and *E. coli*[116] metabolomes. Additional search engines, such as PAMDB[117] and MassBank,[118] also allow searching of MS or tandem MS-based information. To identify the chemical species represented by a given ion via database searching, HPLC-MS/MS can provide supporting information for annotation.[119] This offline combination of MSI and HPLC-MS/MS is also useful for quantification of microbial molecules.

Visualization

Visualization of single or multiple *m/z* values based on the intensity from the mass spectrum of each pixel is possible using the specific vendors' software; however, some functions for visualizing the experimental data are

Table 2. Websites for database searching and software for visualization of MSI data.

Databases for Peak Identification	Visualization Software	
	Open Source/In-House	Commercial
Yeast metabolome[115] (http://www.ymdb.ca/)	MSI Reader[133]	HDI Imaging[125]
E. coli metabolome[116] (http://ecmdb.ca/)	Cardinal[125]	ImageQuest[126]
P. aeruginosa metabolome[117] (http://pseudomonas.umaryland.edu/)	msIQuant[134]	SciLS Lab[127]
MassBank[118] (https://massbank.eu/MassBank/Search)	OpenMSI[135]	4000ImagView[128]

not fully supported. For instance, image segmentation[120] to simplify MSI data into more meaningful and easier to interpret data is not supported. Image segmentation of tissue or colonies of two bacterial taxa in two-dimensional space based on the similarity of pixel mass spectra requires statistical analysis and specific software. In MSI-based segmentation, unsupervised analysis methods, such as principal component analysis (PCA), hierarchical clustering, and K-means, are typically used.[121,122] To process MSI data for segmentation, a common data format, imzML, is required.[123] This is a vendor-neutral data format, which facilitates the sharing of MSI data and their visualization into various software packages without restriction to a proprietary vendor.[124] MSI data obtained using the above-mentioned ionization techniques can be converted into imzML. Several free software packages are available for visualizing ion maps of MSI data but few for segmentation. For instance, Cardinal, R-based open-source software, allows statistical analysis and segmentation, which enables PCA or spatial shrunken centroids.[125] However, open-source software often requires coding skills, and is generally not user-friendly. SCiLS Lab (Bruker Daltonics) is commercial software that enables comprehensive statistical analysis with various visualization techniques, including a segmentation method for MSI data. The graphical user interface of the software facilitates its use by those unfamiliar with MSI or who lack coding experience.

Another example of a visualization technique is image co-registration; *i.e.*, selection of one image as the reference to which all other images are aligned.[24] This technique is used in medical imaging, such as magnetic resonance imaging (MRI) and computed tomography. There are many open-source software packages for image co-registration of MSI data based on python[126] and R.[127] MATLAB-based image co-registration, which is commercial software, is widely used.[128] Multimodal imaging of cells and tissues is enabled by combining MSI with other visualization techniques[129], such as MRI[130] and infra-red,[131] fluorescence,[126] and Raman microscopy.[132] Moreover, two MSI techniques, such as LA-ICP and MALDI, can be combined. For

example, integration of MALDI MSI and LA-ICP-MS data enabled spatial localization of calprotectin in relation to nutrient metals in *S. aureus*-infected mouse tissues.[49] Image co-registration has also been used to show colocalization of proteins with calcium ions. Table 2 lists the websites and software used for database searching and visualization of MSI data, respectively.

Conclusions and Outlook

Microbial MSI enables the discovery of bioactive compounds, investigation of host-pathogen interactions, and evaluation of the penetration efficiency of antibiotics and antifungals. Microbial MSI can be tailored to the application in question because the various techniques available provide atomic- or molecular-level information from the single cell to the tissue scale. As microbial MSI gains greater acceptance by analytical chemists and microbiologists, it will likely become the gold standard imaging tool in various fields of science and sectors of industry.

Acknowledgments

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