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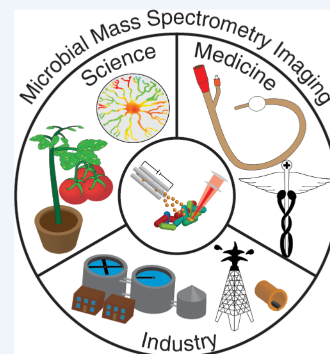
Mass Spectrometry Imaging of Complex Microbial Communities

Sage J. B. Dunham, Joseph F. Ellis, Bin Li, and Jonathan V. Sweedler*

Department of Chemistry and Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana−Champaign, Urbana, Illinois 61801, United States

CONSPECTUS: In the two decades since mass spectrometry imaging (MSI) was first applied to visualize the distribution of peptides across biological tissues and cells, the technique has become increasingly effective and reliable. MSI excels at providing complementary information to existing methods for molecular analysis—such as genomics, transcriptomics, and metabolomics—and stands apart from other chemical imaging modalities through its capability to generate information that is simultaneously multiplexed and chemically specific. Today a diverse family of MSI approaches are applied throughout the scientific community to study the distribution of proteins, peptides, and small-molecule metabolites across many biological models.



The inherent strengths of MSI make the technique valuable for studying microbial systems. Many microbes reside in surface-attached multicellular and multispecies communities, such as biofilms and motile colonies, where they work together to harness surrounding nutrients, fend off hostile organisms, and shield one another from adverse environmental conditions. These processes, as well as many others essential for microbial survival, are mediated through the production and utilization of a diverse assortment of chemicals. Although bacterial cells are generally only a few microns in diameter, the ecologies they influence can encompass entire ecosystems, and the chemical changes that they bring about can occur over time scales ranging from milliseconds to decades. Because of their incredible complexity, our understanding of and influence over microbial systems requires detailed scientific evaluations that yield both chemical and spatial information. MSI is well-positioned to fulfill these requirements. With small adaptations to existing methods, the technique can be applied to study a wide variety of chemical interactions, including those that occur inside single-species microbial communities, between cohabitating microbes, and between microbes and their hosts.

In recognition of this potential for scientific advancement, researchers have adapted MSI methodologies for the specific needs of the microbiology research community. As a result, workflows exist for imaging microbial systems with many of the common MSI ionization methods. Despite this progress, there is substantial room for improvements in instrumentation, sample preparation, and data interpretation. This Account provides a brief overview of the state of technology in microbial MSI, illuminates selected applications that demonstrate the potential of the technique, and highlights a series of development challenges that are needed to move the field forward. In the coming years, as microbial MSI becomes easier to use and more universally applicable, the technique will evolve into a fundamental tool widely applied throughout many divisions of science, medicine, and industry.

INTRODUCTION

Bacteria influence nearly every aspect of life on earth. The estimated one trillion bacterial species on our planet have successfully colonized most habitable environments, from the depths of the Mariana Trench to the limits of the stratosphere.[1−3] Far from behaving as isolated and independent organisms, bacteria usually reside in surface-bound multicellular communities where they work in concert to efficiently harness surrounding nutrients, protect one another from adverse environmental conditions, and launch coordinated expeditions in search of new territory. The influence of microbial communities on humankind is beyond dispute: bacteria, archaea, and fungi are both partners and adversaries to our health, and they interact with plants and animals to influence growth, vitality, disease, and many processes critical to life. In an affirmation of the significance of these multispecies ecosystems, our nation's newest national research initiative—the National Microbiome Initiative—seeks to develop a better understanding of complex microbial communities and their relationship to food, energy, and health.[4,5]

Dynamic molecular processes define many aspects of microbial life, including behavioral coordination, antibiotic resistance, and competition between groups. How do distinct single cell organisms coordinate their actions? Intra- and interspecies bacterial communication occurs via a density-dependent molecular exchange process termed quorum sensing, in which individual cells secrete and sequester small-molecule messengers and "sense" the surrounding population. As a result of altered translation of ancillary genes, quorum sensing leads to the coordinated onset of many complex behavioral patterns, including surface colonization, biofilm formation, virulence, and programed cell lysis.[6] For example, in the Gram-negative bacterium *Pseudomonas aeruginosa*, a well-studied model for biofilm formation, the interplay between two quorum-sensing

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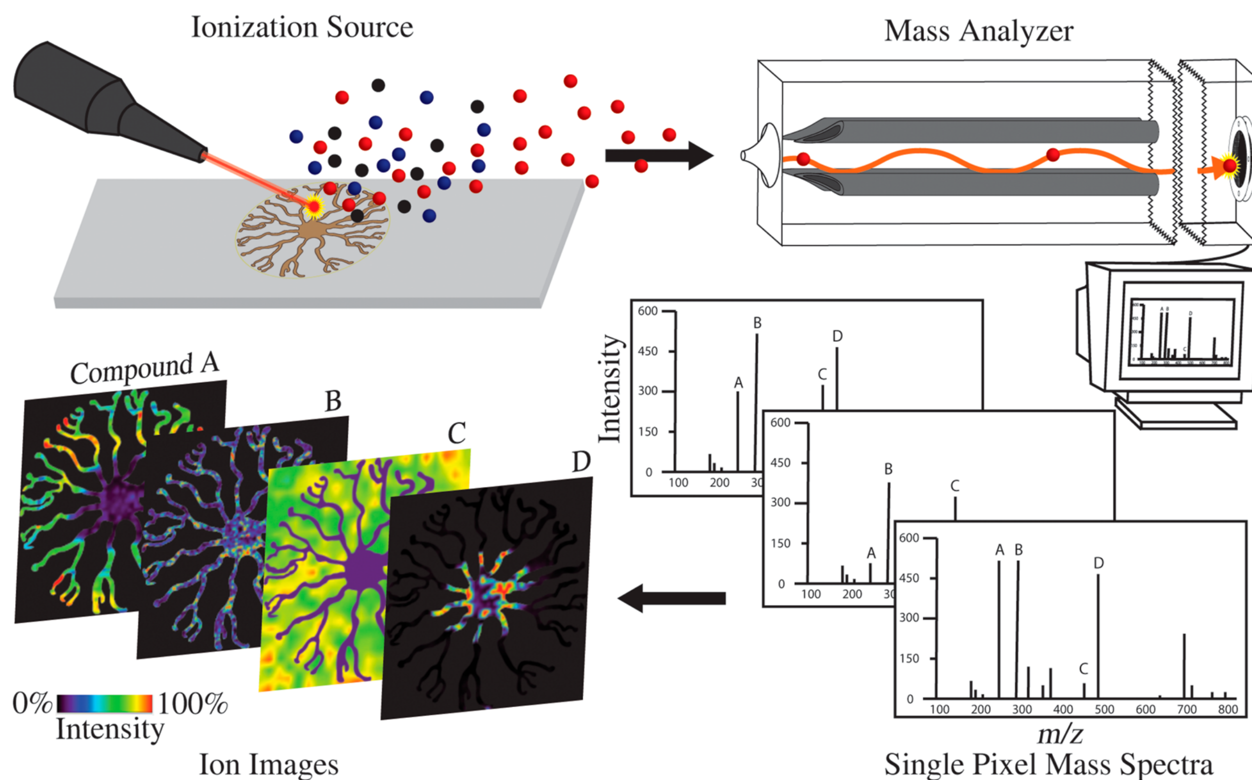

Figure 1. General overview of MSI. A focused microprobe desorbs molecules into the gas phase, where they are ionized and electrically focused into the mass analyzer. Subsequent mass-to-charge ratio (m/z) differentiation and detection produce a mass spectrum for each point across the sample. The abundance of each ion of interest is then plotted as a function of location to produce false-color ion images of specific molecular features.

molecules (acyl-homoserine lactones from the *las* and *rhl* systems) control the expression of up to 10% of the total genome.[7] Outside of cell−cell communication, lipids, proteins, polysaccharides, and extracellular DNA (collectively termed the "extracellular polymeric substance" or EPS) shape the local microenvironment and form a three-dimensional scaffolding, or biofilm, that supports colony survival. From these examples and others, it is apparent that molecular-level scientific evaluations are necessary both for understanding how microbial systems function and for influencing this function.

Much of our existing knowledge about the chemical environment in microbial communities has arisen from genomics and transcriptomics, which enable genes and their expected products to be probed without prior knowledge of their identity. These studies are often followed by targeted analytical approaches, such as bioluminescence, fluorescence microscopy, or autoradiography, all of which provide temporal and spatial detail but require analyte preselection. Oftentimes, less targeted molecular characterization is required. Today's most chemically information-rich approaches include nuclear magnetic resonance, vibrational spectroscopy and, to a greater extent, offline chromatographic techniques such as liquid chromatography (LC) and gas chromatography coupled to mass spectrometry (GC−MS).

One attractive approach that complements traditional chemical measurement techniques by providing untargeted and highly multiplexed chemical imaging data is mass spectrometry imaging (MSI). In MSI, the sample is bombarded with a focused microprobe to induce desorption of chemical compounds into the gas phase, where they are ionized and discriminated on the basis of the mass-to-charge ratio (m/z) (Figure 1). The desorption and ionization process is sequentially repeated over the entire sample, and postprocessing of the position-correlated mass spectra provides a series of false-color ion maps that display the distribution and abundance of each ion.

Since the first applications of MSI to microbiology in the early 2000s,[8] the approach has been used in more than 100 studies ranging from quantitative subcellular imaging of symbiotic nitrogen fixation by bacteria inside shipworm gills[9] to profiling microbial metabolites across the human skin surface.[10] Because of its unique capability to interrogate complex samples with spatial and chemical specificity and its complementarity to genomic and transcriptomic measurements, MSI continues to offer unmatched molecular detail on ever more complex microbial ecologies.

This Account provides an overview of the instrumentation and sample preparation strategies for microbial MSI, highlights applications that demonstrate the enormous potential of the technique, and describes several existing measurement challenges and the potential routes by which these challenges can be overcome. Interested readers are referred to a comprehensive review of MSI[11] and its specific application to microbiology.[12]

CURRENT STATE OF TECHNOLOGY

Much as a surgeon's choice of scalpel is contingent upon the incision to be made, the microbiologist's choice of MSI sampling procedure and instrumentation should be determined by the constraints imposed by the specific sample under examination. A comprehensive survey of a chemically complex microbial community is not possible with a single MSI experiment, as any given measurement ensemble is capable of providing information on only a fraction of the molecules present. The details of the chosen sample preparation and MSI instrumentation determine *which* of these chemicals are observable.

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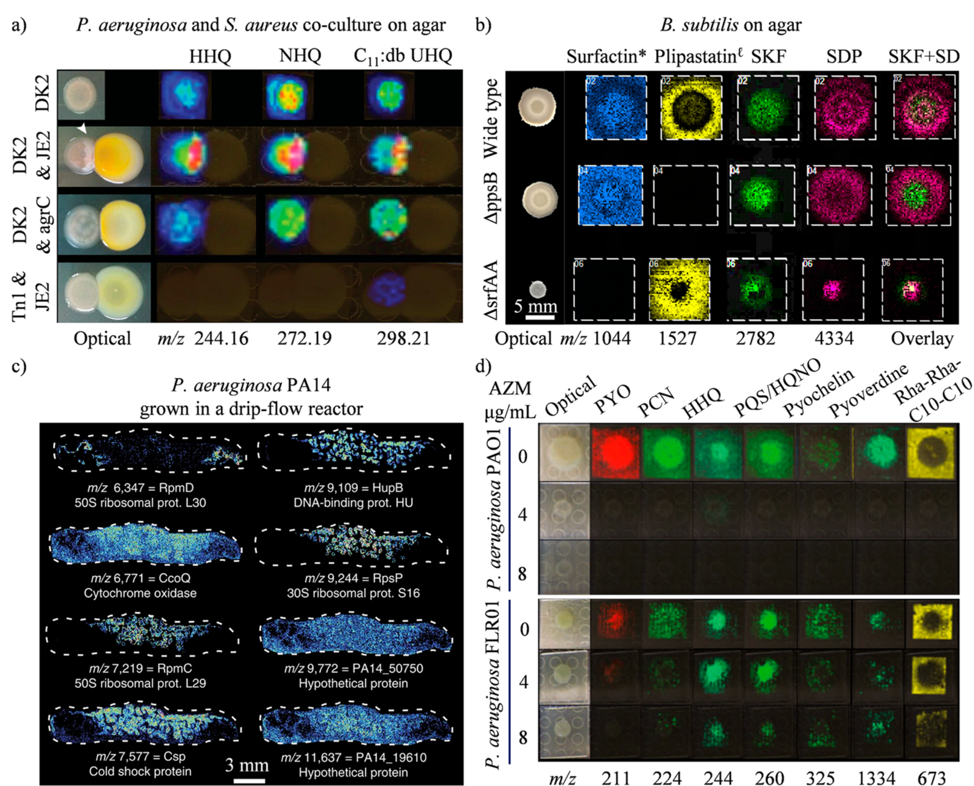

Figure 2. Examples of MALDI MSI in microbiology. (a) Alkyl quinolines produced by *P. aeruginosa* in the presence of *S. aureus*. Reprinted with permission from ref 15. Copyright 2016 Macmillan Publishers Ltd. (b) Surfactants and peptides produced by colony biofilms of *B. subtilis*. Adapted from ref 16. Copyright 2016 American Chemical Society. (c) Nutritionally dependent *P. aeruginosa* proteins from a heterogeneous biofilm grown in a drip-flow reactor. Adapted with permission from ref 17. Copyright 2016 Nature Publishing Group under a Creative Commons CC-BY license. (d) Chemical response of two strains of *P. aeruginosa* in the presence of the antibiotic azithromycin. Adapted with permission from ref 18. Copyright 2015 Springer. See the original references for more information on the specific bacterial strains used and the identities of all of the ions. *, Surfactin-C14; *I*, Plipastatin-C17-Val.

Ionization and Sample Preparation in Microbial MSI

Often the first choice made when designing a microbial MSI experiment is the ionization method, as this decision influences the obtainable chemical coverage, the achievable spatial resolution, and the requirements for sample preparation. Of the wide array of ionization approaches reported in the literature, only three are commercially available and commonly used in microbiology. These three approaches utilize focused probes of light, primary ions, or electrospray solvents to facilitate the process of desorption and ionization and are termed matrix-assisted laser desorption/ionization (MALDI), secondary ion mass spectrometry (SIMS), and desorption electrospray ionization (DESI), respectively.

Matrix-Assisted Laser Desorption/Ionization. Representing approximately half of all microbial MSI publications, MALDI offers the most comprehensive coverage of molecular species. Lipids, peptides, and proteins are all accessible with the proper matrix selection, and the technique commonly achieves a spatial resolution of better than 100 μm for microbial samples. The ultimate achievable spatial resolution in an MSI experiment—generally defined as the measure of how closely two objects can be and still be resolved—is a function of not only instrument parameters (e.g., microprobe size, raster width, sensitivity) but also properties inherent to the sample itself, such as feature size and chemical abundance. Although uncommon, resolutions of better than 5 μm have been demonstrated in MALDI applications outside of microbiology by using specially adapted ion optics and matrix application procedures.[13,14]

Important applications of MALDI MSI in microbiology include the visualization of chemical interactions between different species of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Figure 2a),[15] characterization of surfactants and peptides produced by different strains of *Bacillus subtilis* (Figure 2b),[16] imaging of nutritionally dependent *P. aeruginosa* proteins produced in a heterogeneous drip-flow reactor (Figure 2c),[17] and characterization of the chemical response of *P. aeruginosa* to the addition of the antibiotic azithromycin (Figure 2d).[18] These studies and many others illustrate the potential of MALDI MSI in both fundamental biological discovery and medical research.

Because of their high water content and the propensity for analytes to migrate during handling, microbial samples cultivated on agar are challenging to analyze with MSI. These challenges are often exacerbated by the MALDI matrix application process. One simple method developed specifically for agar-bound microbes is to apply dry matrix to the hydrated colony with a sieve followed by oven drying at 37 °C.[19] Recently, spray-based matrix application to both hydrated[20] and dry[21] agar samples was shown to provide reproducible ion images. Another challenge with agar is that thick, nonconductive samples can become electrically charged under laser irradiation, potentially leading to signal decay over the duration of an image.[19] This impediment can be overcome by using agar with an ultimate dry thickness of less than 50 μm or potentially by sputter-coating the sample with a few nanometers of a conductive material prior to imaging. These examples illustrate that method optimization is often required when using the unique samples present in microbiology.

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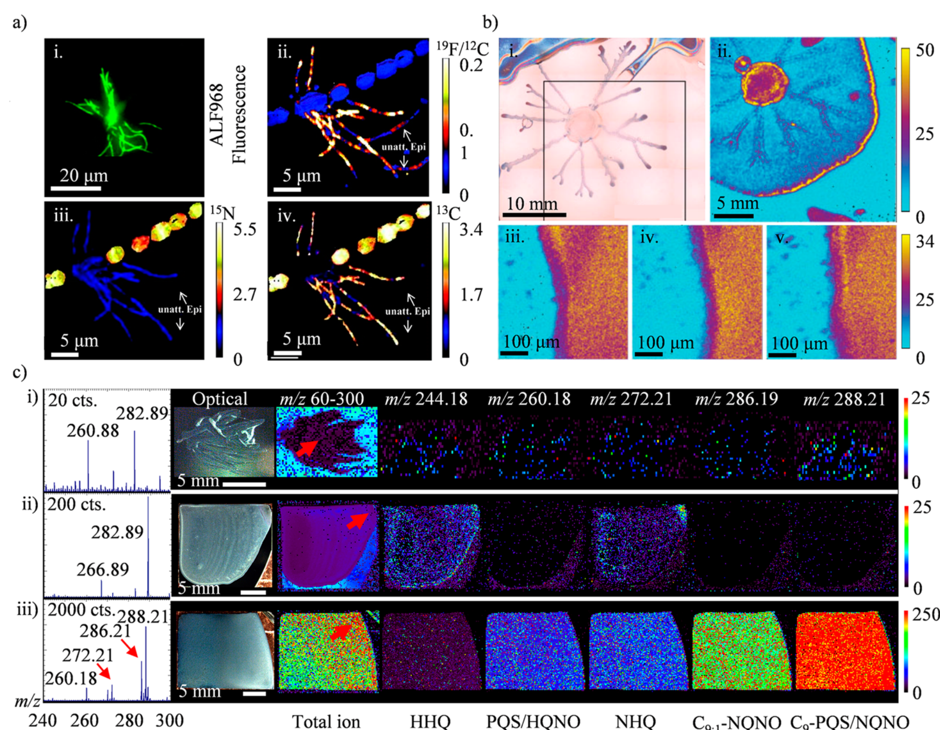

Figure 3. Examples of SIMS imaging in microbiology. (a) (i) FISH and (ii−iv) NanoSIMS imaging of the filamentous cyanobacterium *Anabaena* sp. and *Rhizobium* sp. In (ii), *Rhizobium* sp. is labeled with fluorine using ALF968 dye, while in (iii) and (iv) *Anabaena sp.* metabolically incorporates ^{15}N-dinitrogen and ^{13}C-bicarbonate. Adapted with permission from ref 24. Copyright 2008 American Society for Microbiology. (b) Bi_3^+-TOF-SIMS imaging of a *B. subtilis* swarming community imprinted onto a silicon wafer. (i) Microscopy image of the community prior to imprinting. (ii) Low- and (iii−v) high-resolution TOF-SIMS images of the sum of all surfactant ions. Adapted with permission from ref 26. Copyright 2008 John Wiley and Sons. (c) Quinolones and quinolines produced by static (i) *P. aeruginosa* microcolonies, (ii) planktonic culture, and (iii) 7 h biofilms. Mass spectra are averages of four pixels from the regions indicated by the red arrows. Reproduced with permission from ref 28. Copyright 2015 Royal Society of Chemistry.

Although issues are certain to arise during the adaptation process, many of the traditional methods for applying MALDI matrices to animal tissues are applicable to dehydrated microbial communities. Researchers are also encouraged to look to "matrix-free" methods for laser desorption/ionization (LDI), which primarily utilize UV-absorbing nanoparticles, metal overlays, or nanostructured surfaces.[22] Whether adapting existing approaches or developing entirely new methods, one should take care to perform the appropriate control and replicate experiments. As the title of Richard Goodwin's excellent 2012 review suggests, "Small mistakes [in MSI sample preparation] can lead to big consequences."[23]

Secondary Ion Mass Spectrometry. Ion beams can be focused to exceptionally small spot sizes, allowing SIMS to achieve a lateral resolution of better than 50 nm for monatomic and diatomic secondary ions such as C^-, S^-, and CN^-. This approach (dubbed NanoSIMS) is especially powerful when coupled to metabolic function experiments through stable isotope labeling or cytogenetic identification via fluorescence in situ hybridization (FISH). A recent example of NanoSIMS with FISH metabolic labeling is shown in Figure 3a.[24]

Highly focused ion beams induce molecular fragmentation that limits the observable mass range and causes damage accumulation at or just below the sample surface. To partially alleviate these effects, polyatomic ions (e.g., Bi_3^+ or C_{60}^+) or large gas clusters (e.g., Ar_n^+ or $(H_2O)_n^+$) are often employed as projectile sources for biomolecular SIMS imaging. These ion sources have extended the usable mass range of SIMS to above m/z 2000 and enabled 3D imaging; however, limitations in primary ion flux and focusing capacity currently restrict the achievable lateral resolution to about 3 μm for lipids.[25]

In principle, SIMS imaging requires little to no sample preparation, but the specimen must be dry and vacuum-stable, and, depending on the ion optics, it may also need to be conductive and microscopically flat. These limitations have historically prevented direct SIMS imaging of microbial communities on agar—an important growth substrate required for many microbiology experiments. Debois et al.[26] circumvented issues with charging and surface architecture by imprint-transferring surfactants produced by swarming *B. subtilis* onto a silicon wafer (Figure 3b). Many researchers avoid agar by cultivating samples directly on conductive silicon wafers, which can be dehydrated and analyzed directly.[27,28] Figure 3c shows a series of SIMS images of alkyl quinolone signaling molecules collected from *P. aeruginosa* communities grown on or transferred to silicon wafers.[28] The advent of new SIMS instruments with orthogonal mass analyzers should reduce the impact of topographical variations and conductivity and therefore allow direct SIMS imaging of agar-bound communities.[29]

As with MALDI, there is considerable interest in sample treatments that enhance SIMS ion yields. Because of an overall increase in ion availability, these protocols are also expected to produce improvements in molecular coverage and spatial resolution. Using C_{60}^+-SIMS, our lab demonstrated that a thin layer of gold selectively enhances the yields of quinolone cell-to-cell signaling molecules and rhamnolipid biosurfactants in *P. aeruginosa* bacterial biofilms while simultaneously suppressing background ions from the cell-culture medium,[30] and previously

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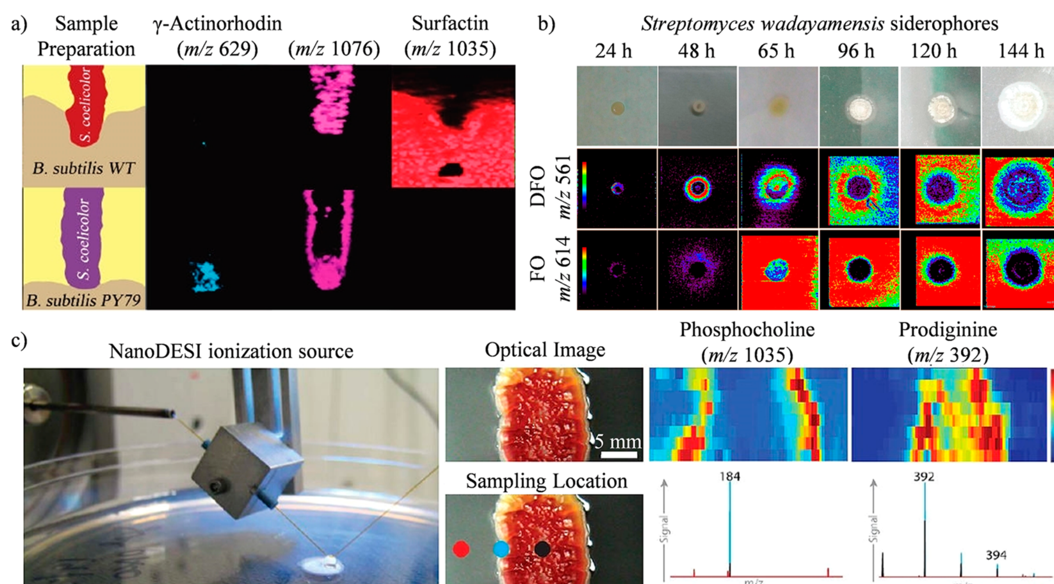

Figure 4. Examples of DESI MSI in microbiology. (a) Imprint imaging of interacting communities of *B. subtilis* and *S. coelicolor*. Adapted from ref 32. Copyright 2010 American Chemical Society. (b) Ion images of iron-scavenging siderophores at different times during the growth of *Streptomyces wadayamensis*. Samples were grown on thin agar and vacuum-desiccated prior to imaging. Adapted from ref 34. Copyright 2015 American Chemical Society. (c) NanoDESI liquid microjunction probe design and ion images of living *S. coelicolor* colonies on agar. Adapted from ref 35. Copyright 2013 American Chemical Society.

we employed a similar tactic for enhanced ionization with monatomic (Au^+) primary ion beams.[29]

Desorption Electrospray Ionization. Although DESI is an ambient ionization method and should therefore be applicable for direct imaging of hydrated (and live) microbial samples, the technique works best on hard, uniform surfaces.[31] This experimental constraint makes it difficult to image many sample types without prior dehydration. As with SIMS and MALDI, imprinting onto an amenable substrate prior to DESI imaging has been shown to be effective. An example of imprint DESI MSI used to visualize interacting communities of *B. subtilis* and *Streptomyces coelicolar* is presented in Figure 4a.[32] A number of blotting surfaces, such as polytetrafluoroethylene (PTFE), porous Teflon, TLC plates, C-18 beads, and cellulose membranes, have been reported as viable substrates for indirect DESI MSI.[33] The simplest approach to DESI imaging, and the one that is reported to offer the best results, is to image following cultivation on thin agar and vacuum desiccation (Figure 4b).[34]

Direct MSI of living colonies has been accomplished through the use of "nanospray" DESI, an ionization approach that implements a small liquid microjunction as an extraction probe. NanoDESI has been applied to image living communities of *Shewanella oneidensis*, *B. subtilis*, and *S. coelicolor* as well as mixed biofilms (Figure 4c).[35] While topographical irregularities are a complicating factor for all ionization modalities, they can be especially challenging for NanoDESI, as they disrupt the liquid microjunction. This complication can be overcome by way of a feedback mechanism that adjusts the sample-to-microjunction distance on the basis of the local topography.[35]

Mass Analyzers for Microbial MSI

The choice of mass analyzer is as important as the modality of ionization, as this selection will determine the range of detectable ions and the confidence of the chemical assignments. Important considerations include mass accuracy (deviation between the theoretical mass and the measured mass), mass resolving power or resolution (smallest difference between two peaks such that the valley between is discernible by a specified fraction of the peak height), mass range (minimum and maximum detectable m/z values), data acquisition speed, and tandem-MS (MS/MS) capabilities, with the details on specific figures of merit being similar to those for other MSI applications.[36]

As is the case for all MS methods, identification of isomers, which share elemental compositions (and therefore m/z values), and isobars, which differ by small fractions of an m/z value, can be exceptionally challenging. Isobars can be distinguished using instruments with high mass accuracy and high mass resolving power, such as Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap mass spectrometers, which are capable of differentiating mass deviations smaller than 1 ppm. Although high-resolving-power analyzers are well-suited for differentiating isobars and for determining elemental composition through isotopic fine structure, they are relatively slow when it comes to acquiring the hundreds of thousands of mass spectra that can be present in a single MS image. For example, to achieve a mass resolving power of 50 000 (full width at half-maximum) at m/z 1000, a typical Orbitrap or FT-ICR instrument requires an acquisition time of about 1 s/pixel, while a time-of-flight (TOF) analyzer can achieve a similar resolving power in less than 0.1 s/pixel. Additionally, the large data files arising from images obtained at high mass resolving power can quickly become cumbersome, resulting in images of several terabytes. For these reasons, most commercial imaging instruments are equipped with TOF analyzers, which have the added benefit of compatibility with pulsed microprobes, such as lasers and ion beams.

Identification of isomers is more challenging and often cannot be accomplished with MS alone. MS/MS fragmentation offers some clues, as it allows for comparison of the fragmentation spectrum of the unknown analyte to those from an analytical standard or an online repository, e.g., METLIN (metlin.scripps.edu), KNApSAck (kanaya.naist.jp/KNApSAcK), Lipidomics Gateway (lipidmaps.org), and the RIKEN MS^n spectral database (spectra.psc.riken.jp). Many available MSI instruments, includ-

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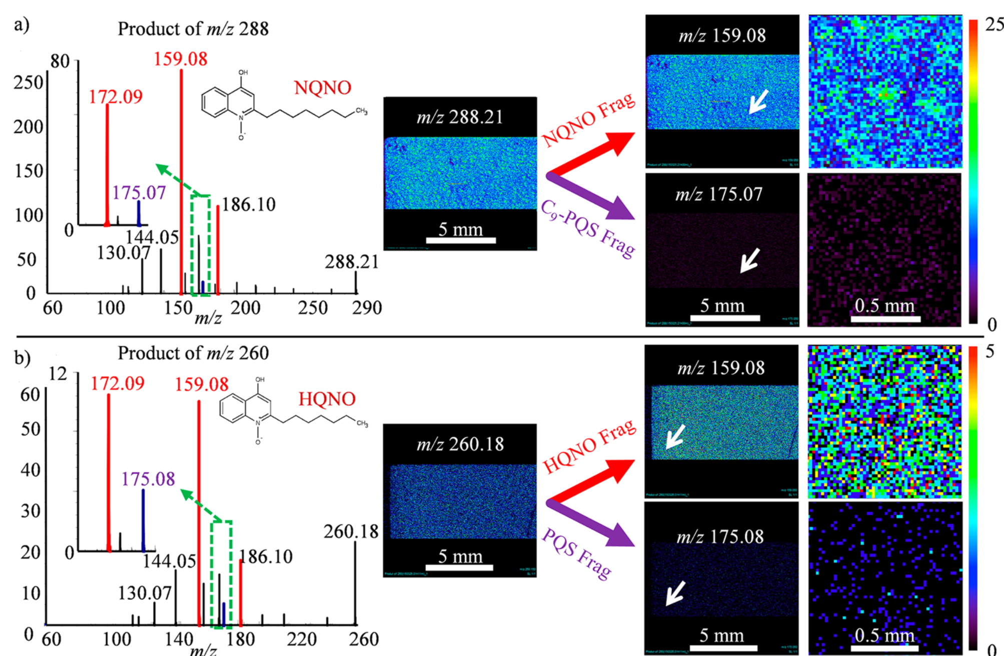

Figure 5. C_{60}-SIMS product ion imaging to differentiate the PQS/HQNO and C_9-PQS/NQNO isomeric pairs on two adjacent regions of a *P. aeruginosa* biofilm. (a) Product of *m*/*z* 288 for C_9-PQS and NQNO. (b) Product of *m*/*z* 260 for PQS and HQNO. Fragments arising from PQS and C_9-PQS are shown in purple, while those arising from *N*-oxides are shown in red. The white arrows indicate the approximate locations of the subsequent higher-magnification images. Reproduced with permission from ref 28. Copyright 2015 Royal Society of Chemistry.

ing hybrid TOF configurations, such as a quadrupole TOF (QTOF) or a TOF/TOF, are capable of MS/MS. SIMS instrument manufacturers have been slow to adapt to the imperatives of the biological imaging community, and most instruments are made with either magnetic sector or single-stage TOF analyzers, which are relatively low resolution, strongly influenced by topography, and not capable of MS/MS. Promising developments include the release of a novel ion bunching-TOF-SIMS by Ionoptika Ltd., a TOF/TOF-SIMS from Physical Electronics, and an Orbitrap/TOF-SIMS from ionTOF and Thermo Scientific, all of which are equipped with MS/MS capabilities.

Isobars and isomers are particularly challenging for imaging experiments, as a single *m*/*z* value can arise from different compounds. One approach to resolve this issue is to use MS/MS in conjunction with MSI. In an example relevant to microbiology, our lab applied SIMS MS/MS imaging to map the distribution of two isomeric analyte pairs, *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone) and 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) and the nine-carbon variants C_9-PQS and NQNO, across static *P. aeruginosa* biofilms (Figure 5). MS/MS imaging showed the *N*-oxide species to be localized in high-abundance zones throughout the sample (visible as high-intensity spots in the MS/MS images for the *m*/*z* 159.08 peak of NQNO (Figure 5a) and HQNO (Figure 5b)), whereas C_9-PQS and PQS were at lower abundance and distributed more evenly.[28]

While MS/MS imaging has obvious utility for isomeric imaging, the technique is limited in terms of throughput, as—with some notable exceptions[37] —only a single precursor ion can be examined with each image collection. One possibility for retaining the multiplexed capabilities of MSI while improving chemical specificity is to incorporate an ion mobility (IM) drift cell into a traditional MSI instrument. In IM-MS, gas-phase ions are separated on the basis of their collisional cross sections prior to MS detection, allowing some isomers and isobars to be differentiated by shape. Commercial IM-MSI instruments have been available for several years, and multiple applications have been demonstrated for tissue imaging.[38] In an example with relevance to microbiology, Li and co-workers[39] complemented MSI with IM-MS to identify and image molecules related to growth, metabolism, and antibiotic inhibition in bacterial colonies. IM-MSI is a leading technology with potential to alleviate the chemical ambiguity in imaging data.

Another attractive approach for increasing chemical coverage and specificity is to use a combination of several complementary imaging approaches to analyze the same or similar samples. Such combinatorial approaches are commonly termed “correlated” or “multimodal” imaging. For example, when studying multispecies communities with NanoSIMS it is often advantageous to incorporate FISH and/or electron microscopy. This allows accurate pairing of chemical information with cell identity and colony morphology.[24] Our lab has also found it advantageous to image microbial samples with a combination of MS and confocal Raman microscopy, a nondestructive vibrational imaging technique that provides information on the composition of functional groups and is applicable to living communites.[27,28] We anticipate multimodal imaging approaches to become increasingly necessary as biological inquiries grow in complexity.

■ PREVAILING CHALLENGES FOR MICROBIAL MSI

Of the tools available to the modern scientist, MSI is uniquely capable of untargeted interrogation of chemically complex systems with a high degree of spatial and chemical specificity. Since its introduction, the utility of MSI has been affirmed by numerous applications and their resulting discoveries. Despite this progress, there remains significant room for improvement in terms of both the technical aspects of the methods and the systems to which these methods can be applied. The following

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developmental challenges will help overcome many existing barriers in microbial MSI.

Challenge #1: Robust Sampling Protocols and Ionization Methods That Enable Interrogation of Samples in Their Endogenous Chemical and Physical States

Most imaging experiments are currently conducted while the sample is under vacuum, which requires prior desiccation. Nonetheless, a number of promising ionization approaches operate under ambient conditions and should be adaptable to examine living microbial communities. Beyond NanoDESI, several other liquid extraction techniques are in use (as recently reviewed by Laskin and Lanekoff[40]). Ambient SIMS and MALDI have also been demonstrated. TOF-SIMS has been used to examine hydrated *P. aeruginosa* biofilms in a unique vacuum-compatible microfluidic reactor[41] as well as HeLa cells in their frozen-hydrated state.[42] Another promising method is to use infrared lasers with MALDI, which rely on water as the matrix and therefore allow for hydrated imaging.[43] To be applicable for a broader segment of scientists, these ambient ionization approaches need to become more robust and either be incorporated into commercial instruments or made available as affordable off-the-shelf attachments.

Challenge #2: Relative and Absolute Quantitation

In perhaps what represents the biggest challenge for the MSI community as a whole, it has been repeatedly shown that small sample differences—in terms of morphology, local salt concentration, or hardness—dramatically affect ionization efficiency and, as a result, the observed molecular distribution.[19,44] Difficulties can also arise when comparing the concentration of one analyte to that of another, as small differences in chemical structure can lead to large variations in ionization efficiency. As an example, the same molecule localized to different nanoenvironments, whether associated with a protein complex inside the cell or within a specific subdomain of the EPS, can exhibit large differences in extraction and ionization yields, and these differences cannot be easily recapitulated with externally applied standards. For these reasons, it is often necessary to combine MSI with other molecular imaging modalities or to follow MSI with localized extraction and quantitative analysis via an established approach such as LC−MS.[18] While it is conceivable to perform secondary verification procedures with every imaging experiment, this process is prohibitively time-consuming and expensive. Thus, there is a substantial need for imaging methods that are inherently quantitative and not reliant on secondary verification. Outside of NanoSIMS, we are aware of no published report on quantitative microbial MSI, but many existing approaches, including those used for tissue imaging[45] and drug penetrance studies,[46] should be adaptable.

Challenge #3: Specialized Sampling Protocols for Low-Abundance or Difficult-to-Access Chemicals or Chemical Classes

In its current state, MSI samples only a tiny fraction of the available chemical information in a microbial community. Although many important compounds can be easily studied using existing approaches, some analytes—particularly those that are low in concentration, labile, or difficult to ionize—require specialized procedures. We expect that these efforts will focus on in situ derivatization to target specific analyte classes and enhance ionization,[47,48] the use of nanomaterials for SIMS and LDI,[22] and the development of novel organic matrices.

Challenge #4: Improving Instrumentation and Sampling Protocols To Enable Routine Access to Chemical Environments across Many Dimensions of Space and Time

The chemical dynamics of microbial ecosystems occurs over many orders of magnitude in both space and time. On one end of the spectrum, microbes interact with their environment on a massive scale, influencing the chemistries of oceans, soils, and our built environments. On the other hand, comprehending microbial communities as a whole often requires understanding the microscopic contributions of individual bacteria on millisecond time scales. Studying these diverse systems will require a collection of innovative analytical approaches. We need to draw on the emerging field of 3D chemical cartography[10] to map the chemical contributions of bacteria across massive environments. We should develop our analytical platforms to improve sensitivity and spatial resolution (which often go hand-in-hand) for routine submicron chemical imaging.

Challenge #5: Developing and Nurturing Collaborations among Scientists from Diverse Fields

Directly addressing the grand challenges within the microbiome requires collaboration among scientists with vastly different expertise. This is even more important when the system under study is composed not only of microbes but also of microbes interacting with some component of their environment, whether it be natural (e.g., soil, plants, animals, decomposing vegetation, minerals) or a human construct (e.g., water pipes, oil pipelines, implanted medical devices, food processing machinery). These collaborations need to be established both among scientists with different analytical skills—such as spectrometrists, spectroscopists, and geneticists—and at a broader intellectual level among physicians, industrial scientists, and academics.

■ CONCLUDING REMARKS

We expect multiple research areas to gain from microbial MSI in the coming years. There is great potential for fundamental biological discovery and for understanding cellular heterogeneity, cell-to-cell signaling, the general chemical dynamics of single and multispecies microbial communities, and the interactions of the microbiome with its host. As an example, with over two million deaths per year caused by bacterial infections and close to 70% of pathogenic bacteria currently resistant to standard antibiotics, there is a critical need to elucidate the various mechanisms behind antibiotic resistance in order to develop new treatment strategies.[49]

Similarly, we need to further our understanding of the complex chemical interactions that occur in plant root/microbial communities; the changing environmental challenges impacting agriculture and bioenergy require enhanced approaches to understand these complex ecologies. In addition to bacteria, our understanding of the chemical processes underlying other microbial communities, including yeast, fungi, and archaea, could greatly benefit from the discovery power of MSI. As microbial MSI methods continue to become more rigorous, simpler to implement, and more accepted by a broader audience, the technique will become an essential chemical imaging tool ubiquitously employed by microbiologists in diverse sectors of science and industry.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1 217-244-7359. E-mail: jsweedle@illinois.edu.

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ORCID

Jonathan V. Sweedler: 0000-0003-3107-9922

Notes

The authors declare no competing financial interest.

Biographies

Sage J. B. Dunham earned his B.S. in chemistry from Westminster College of Salt Lake City in 2012, and he is currently a chemistry Ph.D. candidate at the University of Illinois at Urbana−Champaign (UIUC). His research is focused on developing mass spectrometry imaging instrumentation and sampling strategies for molecular neuroscience and microbial chemistry.

Joseph F. Ellis joined UIUC in 2015 to pursue a Ph.D. in analytical chemistry. He received his B.S. in chemistry from Texas Tech University in Lubbock, Texas, where he researched computational quantum chemistry under Professor Bill Poirier. His Ph.D. research focuses on developing methods for mass spectrometry imaging of quorum-sensing compounds in bacterial biofilms.

Bin Li received his B.S. and M.S. in traditional herbal medicine from China Pharmaceutical University and his Ph.D. in analytical biosciences from the University of Copenhagen. In 2013, he was a visiting scholar in Bernhard Spengler's lab at Giessen University. He is currently a postdoctoral fellow in Jonathan Sweedler's group at UIUC. His research focuses on the development and application of mass spectrometry imaging techniques in plant science and microbiology.

Jonathan V. Sweedler is the James R. Eiszner Family Endowed Chair in Chemistry and Director of the School of Chemical Sciences at UIUC and the Editor-in-Chief of *Analytical Chemistry*. His research group creates new measurement approaches to characterize the molecular content of individual cells, tissues, and environments and applies these approaches to understanding cell−cell signaling throughout the domains of life.

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■ REFERENCES

(1) Kato, C.; Li, L.; Nogi, Y.; Nakamura, Y.; Tamaoka, J.; Horikoshi, K. Extremely barophilic bacteria isolated from the Mariana Trench, Challenger Deep, at a depth of 11,000 meters. *Appl. Environ. Microbiol.* **1998**, *64*, 1510−1513.

(2) Wainwright, M.; Wickramasinghe, N. C.; Narlikar, J. V.; Rajaratnam, P. Microorganisms cultured from stratospheric air samples obtained at 41 km. *FEMS Microbiol. Lett.* **2003**, *218*, 161−165.

(3) Locey, K. J.; Lennon, J. T. Scaling laws predict global microbial diversity. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 5970−5975.

(4) Biteen, J. S.; Blainey, P. C.; Cardon, Z. G.; Chun, M.; Church, G. M.; Dorrestein, P. C.; Fraser, S. E.; Gilbert, J. A.; Jansson, J. K.; Knight, R.; Miller, J. F.; Ozcan, A.; Prather, K. A.; Quake, S. R.; Ruby, E. G.; Silver, P. A.; Taha, S.; van den Engh, G.; Weiss, P. S.; Wong, G. C. L.; Wright, A. T.; Young, T. D. Tools for the microbiome: nano and beyond. *ACS Nano* **2016**, *10*, 6−37.

(5) FACT SHEET: Announcing the National Microbiome Initiative. https://www.whitehouse.gov/the-press-office/2016/05/12/fact-sheet-announcing-national-microbiome-initiative (accessed Aug 15, 2016).

(6) Miller, M. B.; Bassler, B. L. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **2001**, *55*, 165−199.

(7) Wagner, V. E.; Bushnell, D.; Passador, L.; Brooks, A. I.; Iglewski, B. H. Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* **2003**, *185*, 2080−2095.

(8) Cliff, J. B.; Gaspar, D. J.; Bottomley, P. J.; Myrold, D. D. Exploration of inorganic C and N assimilation by soil microbes with time-of-flight secondary ion mass spectrometry. *Appl. Environ. Microbiol.* **2002**, *68*, 4067−4073.

(9) Lechene, C. P.; Luyten, Y.; McMahon, G.; Distel, D. L. Quantitative imaging of nitrogen fixation by individual bacteria within animal cells. *Science* **2007**, *317*, 1563−1566.

(10) Bouslimani, A.; Porto, C.; Rath, C. M.; Wang, M.; Guo, Y.; Gonzalez, A.; Berg-Lyon, D.; Ackermann, G.; Moeller Christensen, G. J.; Nakatsuji, T.; Zhang, L.; Borkowski, A. W.; Meehan, M. J.; Dorrestein, K.; Gallo, R. L.; Bandeira, N.; Knight, R.; Alexandrov, T.; Dorrestein, P. C. Molecular cartography of the human skin surface in 3D. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E2120−E2129.

(11) Rubakhin, S. S.; Sweedler, J. V. *Mass Spectrometry Imaging*, 1st ed.; Humana Press: New York, 2010.

(12) Fang, J.; Dorrestein, P. C. Emerging mass spectrometry techniques for the direct analysis of microbial colonies. *Curr. Opin. Microbiol.* **2014**, *19*, 120−129.

(13) Zavalin, A.; Yang, J.; Hayden, K.; Vestal, M.; Caprioli, R. M. Tissue protein imaging at 1 μm laser spot diameter for high spatial resolution and high imaging speed using transmission geometry MALDI TOF MS. *Anal. Bioanal. Chem.* **2015**, *407*, 2337−2342.

(14) Kompauer, M.; Heiles, S.; Spengler, B. Atmospheric pressure MALDI mass spectrometry imaging of tissues and cells at 1.4-μm lateral resolution. *Nat. Methods* **2016**, DOI: 10.1038/nmeth.4071.

(15) Frydenlund Michelsen, C.; Hossein Khademi, S. M.; Krogh Johansen, H.; Ingmer, H.; Dorrestein, P. C.; Jelsbak, L. Evolution of metabolic divergence in Pseudomonas aeruginosa during long-term infection facilitates a proto-cooperative interspecies interaction. *ISME J.* **2016**, *10*, 1323−1336.

(16) Si, T.; Li, B.; Zhang, K.; Xu, Y.; Zhao, H.; Sweedler, J. V. Characterization of Bacillus subtilis colony biofilms via mass spectrometry and fluorescence imaging. *J. Proteome Res.* **2016**, *15*, 1955−1962.

(17) Wakeman, C. A.; Moore, J. L.; Noto, M. J.; Zhang, Y.; Singleton, M. D.; Prentice, B. M.; Gilston, B. A.; Doster, R. S.; Gaddy, J. A.; Chazin, W. J.; Caprioli, R. M.; Skaar, E. P. The innate immune protein calprotectin promotes Pseudomonas aeruginosa and Staphylococcus aureus interaction. *Nat. Commun.* **2016**, *7*, 11951.

(18) Phelan, V. V.; Fang, J.; Dorrestein, P. C. Mass spectrometry analysis of Pseudomonas aeruginosa treated with Azithromycin. *J. Am. Soc. Mass Spectrom.* **2015**, *26*, 873−877.

(19) Yang, J. Y.; Phelan, V. V.; Simkovsky, R.; Watrous, J. D.; Trial, R. M.; Fleming, T. C.; Wenter, R.; Moore, B. S.; Golden, S. S.; Pogliano, K.; Dorrestein, P. C. Primer on agar-based microbial imaging mass spectrometry. *J. Bacteriol.* **2012**, *194*, 6023−6028.

(20) Li, B.; Comi, T. J.; Si, T.; Dunham, S. J. B.; Sweedler, J. V. A one-step matrix application method for MALDI mass spectrometry imaging of bacterial colony biofilms. *J. Mass Spectrom.* **2016**, *51*, 1030−1035.

(21) Hoffmann, T.; Dorrestein, P. C. Homogeneous matrix deposition on dried agar for MALDI imaging mass spectrometry of microbial cultures. *J. Am. Soc. Mass Spectrom.* **2015**, *26*, 1959−1962.

(22) Peterson, D. S. Matrix-free methods for laser desorption/ionization mass spectrometry. *Mass Spectrom. Rev.* **2007**, *26*, 19−34.

(23) Goodwin, R. J. A. Sample preparation for mass spectrometry imaging: Small mistakes can lead to big consequences. *J. Proteomics* **2012**, *75*, 4893−4911.

(24) Behrens, S.; Lösekann, T.; Pett-Ridge, J.; Weber, P. K.; Ng, W.-O.; Stevenson, B. S.; Hutcheon, I. D.; Relman, D. A.; Spormann, A. M. Linking microbial phylogeny to metabolic activity at the single-cell level

DOI: 10.1021/acs.accounts.6b00503
Acc. Chem. Res. 2017, 50, 96−104

by using enhanced element labeling-catalyzed reporter deposition fluorescence in situ hybridization (EL-FISH) and NanoSIMS. *Appl. Environ. Microbiol.* **2008**, *74*, 3143−3150.

(25) Fletcher, J. S. Latest applications of 3D ToF-SIMS bio-imaging. *Biointerphases* **2015**, *10*, 018902.

(26) Debois, D.; Hamze, K.; Guérineau, V.; Le Caër, J.-P.; Holland, I. B.; Lopes, P.; Ouazzani, J.; Séror, S. J.; Brunelle, A.; Laprévote, O. In situ localisation and quantification of surfactins in a Bacillus subtilis swarming community by imaging mass spectrometry. *Proteomics* **2008**, *8*, 3682−3691.

(27) Lanni, E. J.; Masyuko, R. N.; Driscoll, C. M.; Dunham, S. J. B.; Shrout, J. D.; Bohn, P. W.; Sweedler, J. V. Correlated imaging with C60-SIMS and confocal raman microscopy: visualization of cell-scale molecular distributions in bacterial biofilms. *Anal. Chem.* **2014**, *86*, 10885−10891.

(28) Baig, N. F.; Dunham, S. J. B.; Morales-Soto, N.; Shrout, J. D.; Sweedler, J. V.; Bohn, P. W. Multimodal chemical imaging of molecular messengers in emerging Pseudomonas aeruginosa bacterial communities. *Analyst* **2015**, *140*, 6544−6552.

(29) Lanni, E. J.; Dunham, S. J. B.; Nemes, P.; Rubakhin, S. S.; Sweedler, J. V. Biomolecular imaging with a C60-SIMS/MALDI dual ion source hybrid mass spectrometer: instrumentation, matrix enhancement, and single cell analysis. *J. Am. Soc. Mass Spectrom.* **2014**, *25*, 1897−1907.

(30) Dunham, S. J. B.; Comi, T. J.; Ko, K.; Li, B.; Baig, N. F.; Morales-Soto, N.; Shrout, J. D.; Bohn, P. W.; Sweedler, J. V. Metal-assisted polyatomic SIMS and laser desorption/ionization for enhanced small molecule imaging of bacterial biofilms. *Biointerphases* **2016**, *11*, 02A325.

(31) Venter, A.; Sojka, P. E.; Cooks, R. G. Droplet dynamics and ionization mechanisms in desorption electrospray ionization mass spectrometry. *Anal. Chem.* **2006**, *78*, 8549−8555.

(32) Watrous, J.; Hendricks, N.; Meehan, M.; Dorrestein, P. C. Capturing bacterial metabolic exchange using thin film desorption electrospray ionization-imaging mass spectrometry. *Anal. Chem.* **2010**, *82*, 1598−1600.

(33) Cabral, E. C.; Mirabelli, M. F.; Perez, C. J.; Ifa, D. R. Blotting assisted by heating and solvent extraction for DESI-MS imaging. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 956−965.

(34) Angolini, C. F. F.; Vendramini, P. H.; Araújo, F. D. S.; Araújo, W. L.; Augusti, R.; Eberlin, M. N.; de Oliveira, L. G. Direct protocol for ambient mass spectrometry imaging on agar culture. *Anal. Chem.* **2015**, *87*, 6925−6930.

(35) Watrous, J.; Roach, P.; Heath, B.; Alexandrov, T.; Laskin, J.; Dorrestein, P. C. Metabolic profiling directly from the Petri dish using nanospray desorption electrospray ionization imaging mass spectrometry. *Anal. Chem.* **2013**, *85*, 10385−10391.

(36) *Mass Spectrometry: Principles and Applications*, 3rd ed.; de Hoffmann, E., Stroobant, V., Eds.; John Wiley and Sons: Chichester, U.K., 2007.

(37) Feenstra, A. D.; Hansen, R. L.; Lee, Y. J. Multi-matrix, dual polarity, tandem mass spectrometry imaging strategy applied to a germinated maize seed: toward mass spectrometry imaging of an untargeted metabolome. *Analyst* **2015**, *140*, 7293−7304.

(38) Trim, P. J.; Henson, C. M.; Avery, J. L.; McEwen, A.; Snel, M. F.; Claude, E.; Marshall, P. S.; West, A.; Princivalle, A. P.; Clench, M. R. Matrix-assisted laser desorption/ionization-ion mobility separation-mass spectrometry imaging of Vinblastine in whole body tissue sections. *Anal. Chem.* **2008**, *80*, 8628−8634.

(39) Li, H.; Balan, P.; Vertes, A. Molecular imaging of growth, metabolism, and antibiotic inhibition in bacterial colonies by laser ablation electrospray ionization mass spectrometry. *Angew. Chem.* **2016**, *128*, 15259−15263.

(40) Laskin, J.; Lanekoff, I. Ambient mass spectrometry imaging using direct liquid extraction techniques. *Anal. Chem.* **2016**, *88*, 52−73.

(41) Hua, X.; Yu, X.-Y.; Wang, Z.; Yang, L.; Liu, B.; Zhu, Z.; Tucker, A. E.; Chrisler, W. B.; Hill, E. A.; Thevuthasan, T.; Lin, Y.; Liu, S.; Marshall, M. J. In situ molecular imaging of a hydrated biofilm in a microfluidic reactor by ToF-SIMS. *Analyst* **2014**, *139*, 1609−1613.

(42) Piwowar, A. M.; Keskin, S.; Delgado, M. O.; Shen, K.; Hue, J. J.; Lanekoff, I.; Ewing, A. G.; Winograd, N. C60-ToF SIMS imaging of frozen hydrated HeLa cells. *Surf. Interface Anal.* **2013**, *45*, 302−304.

(43) Berkenkamp, S.; Karas, M.; Hillenkamp, F. Ice as a matrix for IR-matrix-assisted laser desorption/ionization: mass spectra from a protein single crystal. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 7003−7007.

(44) Lanekoff, I.; Stevens, S. L.; Stenzel-Poore, M. P.; Laskin, J. Matrix effects in biological mass spectrometry imaging: identification and compensation. *Analyst* **2014**, *139*, 3528−3532.

(45) Groseclose, M. R.; Castellino, S. A mimetic tissue model for the quantification of drug distributions by MALDI imaging mass spectrometry. *Anal. Chem.* **2013**, *85*, 10099−10106.

(46) Prideaux, B.; Dartois, V.; Staab, D.; Weiner, D. M.; Goh, A.; Via, L. E.; Barry Iii, C. E.; Stoeckli, M. High-sensitivity MALDI-MRM-MS imaging of Moxifloxacin distribution in tuberculosis-infected rabbit lungs and Granulomatous lesions. *Anal. Chem.* **2011**, *83*, 2112−2118.

(47) Shariatgorji, M.; Strittmatter, N.; Nilsson, A.; Källback, P.; Alvarsson, A.; Zhang, X.; Vallianatou, T.; Svenningsson, P.; Goodwin, R. J. A.; Andren, P. E. Simultaneous imaging of multiple neurotransmitters and neuroactive substances in the brain by desorption electrospray ionization mass spectrometry. *NeuroImage* **2016**, *136*, 129−138.

(48) Wu, Q.; Comi, T. J.; Li, B.; Rubakhin, S. S.; Sweedler, J. V. On-tissue derivatization via electrospray deposition for matrix-assisted laser desorption/ionization mass spectrometry imaging of endogenous fatty acids in rat brain tissues. *Anal. Chem.* **2016**, *88*, 5988−5995.

(49) Berdy, J. Thoughts and facts about antibiotics: where we are now and where we are heading. *J. Antibiot.* **2012**, *65*, 441−441.

DOI: 10.1021/acs.accounts.6b00503
Acc. Chem. Res. 2017, 50, 96−104