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Bacterial biofilm sample preparation for spatial metabolomics

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Spatial metabolomics using mass spectrometry imaging (MSI) has become an important approach to study the surface of biological systems. MSI can probe bacterial metabolic processes through the direct analysis of bacterial colonies. In this review, we explore recent advancements made for bacterial metabolomics of primary and secondary metabolites using MSI, focusing on improvements in agar-based sample preparation and the use of membranes for improved sample preparation. The application of derivatization agents on bacterial samples enhances select metabolite signals and can aid analyte identification. Implementing dual imaging or multi-omics techniques also aids in identifying analytes and elucidating metabolic pathways active during the host–microbe interactions. Finally, we explore improvements towards robust three-dimensional protocols for whole colony MSI analysis. These advances enhance MSI analysis of bacterial samples and pose promising avenues for future studies.

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1. Introduction

Microorganisms, such as bacteria, produce key metabolites that are biosynthesized by the organism for survival. These metabolites are classified as primary, which are part of essential metabolic processes, and secondary, which are not required for survival and aren't universally present in microorganisms.^{1,2} Metabolomics can be harnessed to increase our knowledge of bacterial infections in animals and plants,^{3–5} to discover new natural products to combat bacterial infections,^{6,7} and to apply to the bioproduction of precious chemical materials.^{8,9} Primary metabolites are easily traceable and characterizable due to their involvement in key metabolic pathways. Secondary metabolites are less conspicuous as they are not necessary for survival, yet provide advantages for survival through many processes, such as defense mechanisms against other organisms or environmental stressor protection.¹⁰ Secondary metabolites can be targeted for biopharmaceutical purposes as they tend to have a similar structure to key metabolites or scaffolds used in primary metabolism and can be used as antibiotics, antitumor agents, immunosuppressants, and more.^{11,12} Further understanding of bacterial metabolomics can increase the function of these metabolites during infection or help elucidate new uses for possible natural products.

Both primary and secondary metabolites can be identified using genomic mapping and analytical methods, such as

nuclear magnetic resonance (NMR), mass spectrometry (MS), chromatographic separations, or their combination.^{13–17} Mass spectrometry imaging (MSI) has been an emerging tool with applications for many biological samples, including bacteria. MSI is a technique developed to study the spatial distribution of analytes of interest on a two-dimensional surface. MSI can be applied to a variety of molecular classes, including metabolites, peptides, lipids, proteins, and other areas (Fig. 1B).^{18,19} Its versatility for biological samples has made it a popular tool for the study of spatial localization on biological tissue slices,^{20–22} has been implemented in cancer-related studies,^{23–25} and has been applied to bacterial samples, including those involving metabolomics.^{26,27} The versatility of this technique has made it a useful tool for bacterial metabolomics for different studies, including pharmacokinetic effects of drugs on bacterial samples,²⁸ bacterial human infection profiles for primary and secondary metabolites,^{3,4,29} plant-microbe interactions,^{30,31} biofilm studies,^{29,32–34} among many others.

Multiple reviews have covered the instrumentation used for MSI and its application to material biofilms.^{35–39} Understanding the instrumentation is important as the ionization source can determine what molecules are detected and the spatial resolution of the imaging, while the mass analyzer dictates the detection limits, mass resolution, and other important figures of merit. As a few key points, the most common ionization sources used in MSI are matrix-assisted laser desorption ionization (MALDI) and secondary ion mass spectrometry (SIMS) (Fig. 1A).^{22,24,29,37,40,41} Because measurements take time, it is important to collect data from regions of interest (ROIs) and not the entire surface. Usually, the ROI is

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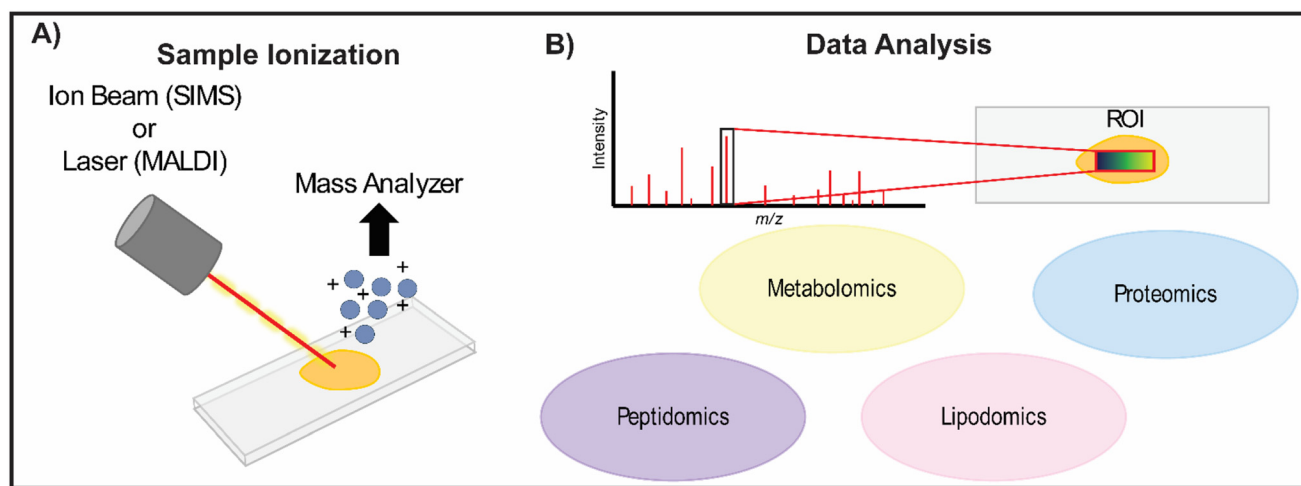


Fig. 1 MSI omics studies from collected samples. (A) Region(s) on the prepared sample are selected (the region of interest (ROI), and an ionization source – an ion beam (SIMS) or laser (MALDI) – is rastered across the ROI to create the MS image); (B) the collected data is then analyzed where the locations where spectra are obtained are overlaid onto the optical image. The collected m/z data is processed to visualize the spatial distributions of specific molecules.

selected based on information obtained from optical images. Besides the area to be imaged, the user needs to determine the spatial resolution needed, which dictates the step and raster dimensions. At each point, ions are sent into the mass spectrometer and eventually, the mass spectrum is produced from that point. The resultant mass spectra are then aligned to the appropriate locations, often involving the combination with an optical image, thus producing the MSI image.

The application of MSI for bacterial spatial omics has been evolving since the first use of MSI in bacterial samples. Through improvements in sample preparation,^{36,42} applications for different ionization sources,^{29,35,43} and multimodal analysis using various optical and spectroscopic techniques,^{44–46} MSI metabolomics studies have been used to investigate both primary and secondary metabolites of a large number of bacteria or host–pathogen interactions.

In this review, we highlight advances made in MSI sample preparation and analysis, focusing on advances from the past three years. These advances have improved the quality of the collected data and expanded our knowledge of metabolites and their distribution in bacterial systems. We focus on several areas with rapid advances, including sample preparation improvements on agar surfaces to expand on the range of microbial samples amenable to MSI, derivatization agents applied for enhanced analyte identification, multi-omics combining MSI and *in situ* approaches, and three-dimensional MSI of bacterial samples.

2. Advances in agar-based sample preparation

Much of the original MSI of bacterial biofilms used bacterial cultures on hard surfaces, but much of the bacterial culturing

occurs on softer materials such as agar. With this as a driving force, the implementation of agar-based MSI analysis on bacterial samples has been advancing. Various aspects of the MSI sample preparation have been modified throughout the years, adding a repertoire of methods and techniques to prepare the agar sample for MSI analysis. Sample collection, matrix application, and sample drying are the most important aspects of this sample preparation (Fig. 2). Below, we will discuss current sample preparation methods and advances made to prepare agar samples for improved analyses.

2.1 Current preparation methods

As agar cultures are a popular form of bacterial growth, the development of methods and techniques to transfer these cultures for MSI analysis has improved our understanding of many processes involving growth and motility. Agar-based sample collection varies depending on the purpose of the study. Most samples are analyzed as a whole culture,^{35,42} with some exceptions, used for cross-sectioned images.⁴⁷ Other methods involve imprinting the live colony onto a conductive membrane^{48–50} or a MALDI-compatible filter^{51–53} to transfer the surface analytes onto a secondary surface for analysis. Analysis of colony impressions leads to lower analyte signal or lack of adhesion for some analytes, which makes whole culture analysis, as seen in agar culture MSI samples, a more effective approach. Bacterial cultures grown in agar are typically transferred to MALDI target plates or conductive indium titanium oxide (ITO) microscope slides (Fig. 2).³⁶ To allow for easier transfer and faster drying, some researchers use low agar volumes to create a thin layer that can be easily transferred to the MALDI plate or ITO slide. For cross-sectional MSI analysis, the colony is embedded in carboxymethylcellulose (CMC), gelatin, or a mixture and is subsequently frozen and sliced through cryosection for transfer and MSI analysis.⁴⁷



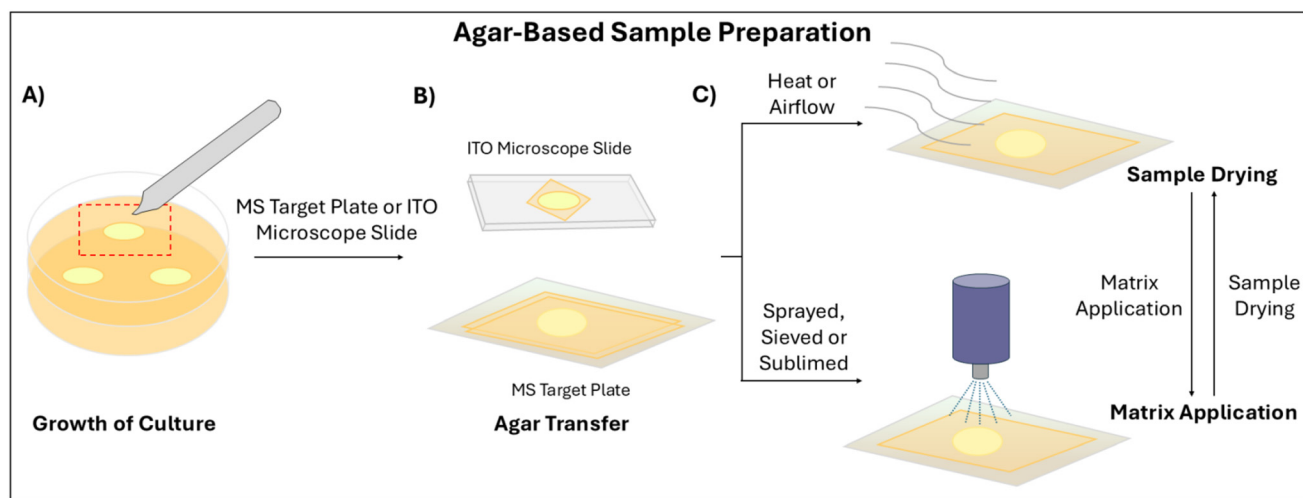


Fig. 2 Sample preparation workflow for agar-based MSI bacterial cultures. (A) Bacterial cultures are grown in agar conditions. Cuts in the agar can be made to isolate single colonies or colonies of interest; (B) colonies can then be transferred to a target plate or an ITO microscope slide for drying or matrix application; (C) agar sample is dried using forced airflow or heat followed by matrix application using a sprayer, a sublimator or a sieve. These steps have been used interchangeably.

Both sample collection methods yield intact bacterial colonies for MSI analysis.

Once the bacterial culture has been transferred to the slide or target plate, the protocol diverges between sample drying or matrix application. This is due to the practicality of method development, and the MSI analysis may yield similar results with either approach. Several types of matrix application on agar samples are performed: sieving the matrix on the agar prior to drying,^{36,42} spraying solubilized matrix on the dried agar sample,^{54–56} and sublimating the matrix onto the dried agar sample (Fig. 2).^{46,57} Sieving is commonly used for agar-based MSI samples, while spraying post-drying has been on the rise as more uniform spraying instruments and techniques have been made available to researchers in the past decade.^{54,56} Sublimation has been less common as it requires a vacuum chamber and unusually involves positioning the sample upside down, which could lead to detachment and loss in the chamber. The method of choice depends on resources and equipment availability, which may be why sieving is the most common matrix application method, as it requires a sieve and the matrix. The sublimation method requires a sublimating chamber, a vacuum pump, and the matrix, and the spraying method requires an airbrush or an automated sprayer, solvent, and matrix. For agar-based samples being analyzed in a MALDI-MS, any of the three methods mentioned above can be used as part of the sample preparation, as they all are effective for MSI analysis.

Finally, sample drying is often achieved through two different methods: drying through heat³⁶ and forced airflow (Fig. 2).^{54,55} The former is the most common drying method used for MSI analysis of agar cultures and is commonly applied with the sieving method for matrix application.^{36,42} It consists of incubating the sample post-transfer onto the MALDI target plate or the ITO slide. This is typically done at

37 °C³⁶ or at higher temperatures.⁵⁴ This process involved incubating the sample from 2 to 6 h, depending on its thickness. Unfortunately, this allows for further growth of the sample on the agar during the drying incubation time. Drying the agar samples using forced airflow is typically done at room temperature inside a chamber similar to a desiccator.⁵⁶ Similar to the heat drying method, this method could take 1 to 8 h, depending on the pressure applied to the chamber. Sometimes this leads to uneven drying, which could lead to culture growth on one side of the agar with long drying times at low air pressure or agar cracking or flaking with short drying times at high air pressure.⁵⁸ Although growth could still be observed post-drying using both methods, their protocol feasibility makes them convenient drying methods for agar-based MSI samples.

An underexplored step in the preparation of bacterial MSI samples is the application of internal standards or calibration standards for quantification. Although standards can be spotted outside of the sample location on the microscope slide or target plate, the different sample properties of the slide *versus* the colonies or agar suggest such standards will not allow the correlation of peak intensities observed from standards to those from analytes in the bacterial colonies. This is due to ionization differences between analytes in agar and the media as well as ion suppression observed from agar surfaces and complex environments such as colonies or microbiome.^{42,59} Internal standards and calibration standards have been implemented with varying degrees of success for tissue MSI analysis and effectively for LC-MS extract analyses.^{57,60–62} We were unable to find examples of calibration or internal standards for MSI from microbial agar samples. Alternatively, isotopically labeled molecules such as ¹³C-glucose can be added to the media to monitor the production of metabolites from the starting material. Approaches to



reduce analyte interference when sampling from agar are needed to enable effective use of standards for quantitation.

The sample preparation methods mentioned above describe multiple sample preparation processes that can be used for agar-based MSI samples. Improvements and advances in these methods will improve the data quality collected during acquisition. An improvement we have yet to see in this area is bacterial MSI analysis on still hydrated agar cultures (not yet dried). An advantage of this is the possible identification of volatile or degradable metabolites that can be lost during the drying process. An optimally prepared MSI agar culture allows for the analysis of metabolites and other molecules on the surface without loss of signal from environmental factors. Another common issue found during sample preparation with some bacterial cultures involves significant topological features, sometimes referred to as rugged or rough surfaces. *Mycobacterium tuberculosis*,⁶³ *Bacillus subtilis*,⁶⁴ and *Streptomyces ambofaciens*⁶⁵ are some examples of bacteria with heterogeneous colonies. Due to the surface irregularity on the colony, matrix application and sample drying affect the outcome of the MSI analysis, leading to a loss of signal during data acquisition.⁶⁵ In the next section, we discuss two advances in bacterial sample preparation that address these two areas.

2.2 Advances using commercial membranes for faster or improved sample preparation for bacterial MSI metabolomics

As previously mentioned, sample drying could lead to loss of signal of volatile or degraded metabolites. An alternative explored by Müller *et al.* involves blotting the surface of grown colonies onto a membrane to collect surface analytes and perform MSI on the collected membrane, which shortens sample preparation.⁵⁹ They then compare the acquired MSI data with already established protocols.⁵⁹ This process allows for the removal of the sample transfer and subsequent drying processes from the sample preparation protocol, just leaving matrix application on the sample. The membrane used during the process is called desorption/ionization using through-hole alumina membrane (DIUTHAME), it is commercially available and is also a substitute for matrix, removing the matrix application from the sample preparation protocol. It allows for both positive and negative mode analysis. Reduction in noise caused by the matrix and the agar sample was observed on the collected data using the DIUTHAME membrane. Overall, this sample preparation substitute shows promising results for the data acquisition of MSI of bacterial colonies.

The authors compare the DIUTHAME membrane MSI analysis, which is one of the surface-assisted laser desorption/ionization (SALDI)-MSI⁶⁶ approaches. SALDI-MSI often uses a porous nanosubstrate surface of silicon, carbon, or metal-based nanomaterials on which the sample is deposited.^{66–69} The application of DIUTHAME for colony transfer eliminates the nanosubstrate preparation, as it is commercially available. Secondary metabolites are identified in both SALDI-MSI and MALDI-MSI. Background was reduced in both positive and negative modes. Reduction of mass shift was also observed

when comparing both techniques. One of the most notable mass shift reductions was observed with fengycins for *Bacillus velezensis*, where no shift was observed using SALDI-MSI, while a -12.7 to -25.4 shift was observed using MALDI-MSI.⁵⁹ Although this method is an effective sample preparation protocol, it does have limitations. During the study, a loss of signal on a portion of the imprinted colonies was observed. This may be due to regions where the impression of the colony onto the membrane was not performed optimally. This is a possible issue that arises with flat colonies or bacteria with thicker surfaces, which prevents the membrane from covering the whole colony. Another possibility is selective transfer of certain analytes onto the DIUTHAME membrane, while others are less effectively transferred due to different membrane interactions. This may lead to a loss of signal from those analytes that are present. Finally, lower analyte intensity is observed in SALDI-MSI over MALDI-MSI, which could lead to issues with LODs for less abundant analytes. Despite these issues, it is promising for bacterial MSI and metabolomic studies. Perhaps further optimization will alleviate several of the limitations leading to increased use of this technique.

Another current issue with bacterial MSI involves the analysis of 'rugged' colonies. Three-dimensional morphology prevents complete analysis of the colony due to an uneven distribution of the matrix on the colony surface or an absence of a flat surface for the MSI analysis. Slimani *et al.* delve into a membrane-based method to improve MSI analysis on colonies with a rugged morphology for bacterial metabolomic studies.⁶⁵ The sample preparation during this study is modified for heterogeneous colonies from *S. ambofaciens* using a commercial cellophane membrane that is placed on the agar surface prior to inoculation. This membrane allows for metabolite diffusion into the agar that can be identified through MSI. It substitutes the rugged heterogeneous surface for a flat surface after removal, making it easier for matrix deposition and MSI analysis. An advantage this technique has over the general agar MSI approach is the reduction of agar deformation produced during the drying step often caused by bacterial growth during drying.⁶⁵ Another advantage to the general approach involved the identification of secreted metabolites that could be obscured by the colony during MSI, creating dark regions in the MS image where these analytes are expected (Fig. 3). This increases the ability to identify metabolites not easily observed on whole colony MSI, which would be an advantageous technique to do with surface colony MSI as a way to identify more metabolites not typically found in the immediate colony surface.

The researchers were able to identify a siderophore for *S. ambofaciens*, desferrioxamine E, which is typically obscured by the colony during the general colony MSI approach (Fig. 3A) and spiramycin, a natural product produced by *S. ambofaciens*, also obscured during MSI analysis (Fig. 3B).⁶⁵ About 61% of the detected metabolites were found using both the general and the membrane approach, and 33% were found only on the membrane-based approach.⁶⁵ Unfortunately, this technique doesn't follow the colony morphology as well as the



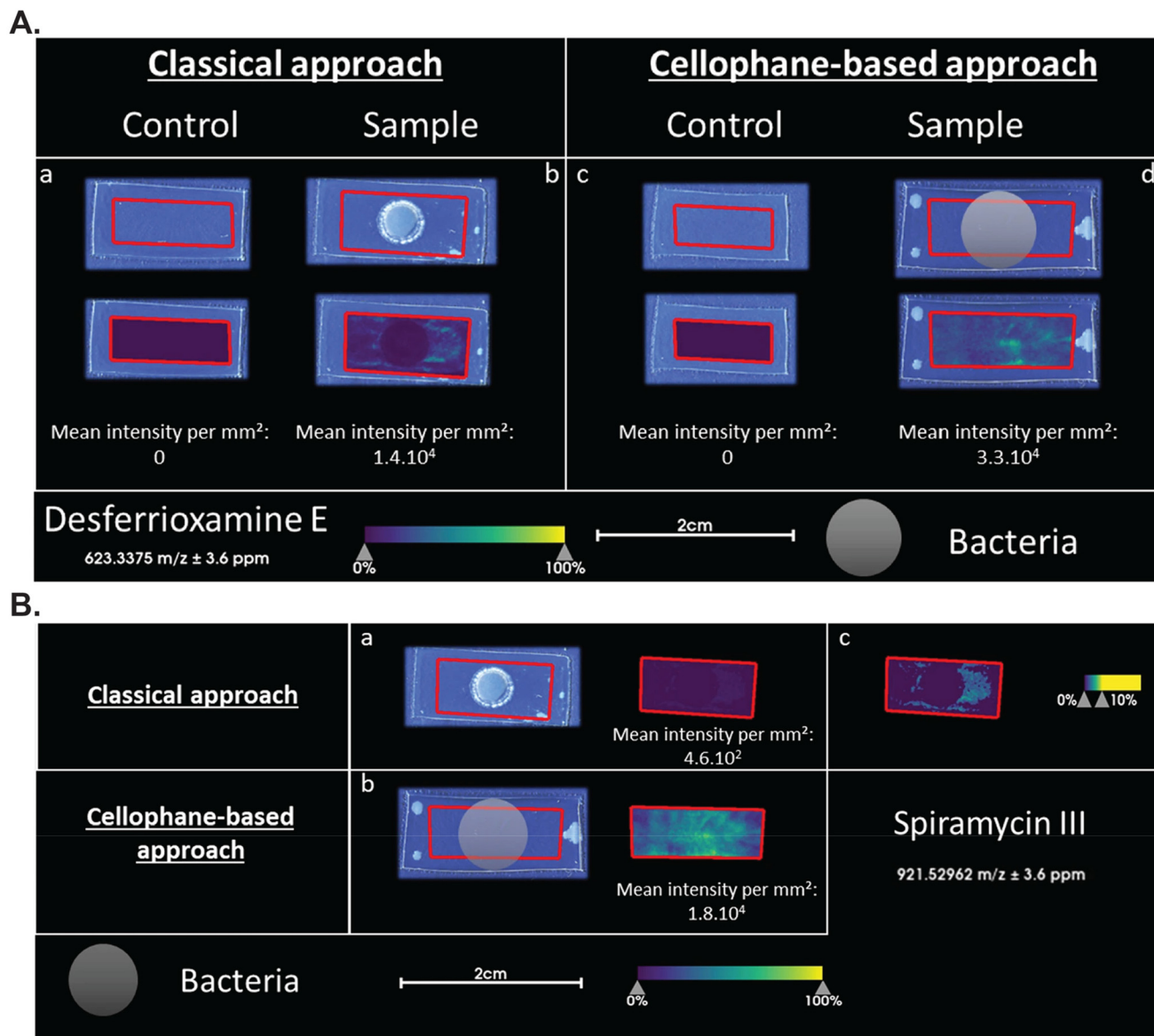


Fig. 3 Cellophane membrane MSI data. A, Spatial distribution of desferrioxamine E using the classical MALDI-MSI approach (a and b) and cellophane-based MSI approach (c and d); B, spatial distribution of spiramycin III using the classical MSI approach (a) and the cellophane-based approach (b), MS image with reduced color gradient at 10% (c). Analytical and Bioanalytical Chemistry, Farès Slimani, Laurence Hotel, Aurélie Deveau, Bertrand Aigle, Patrick Chaïmbault & Vincent Carré, 416, Springer Nature, 202 reproduced with permission from SNCSC.

DIUTHAME method, as the metabolites are distributed throughout the agar over the specific region where the colony grew (Fig. 3), which renders this method less useful for colony spatial metabolomics using MSI. This method is advantageous for studies where an untargeted or targeted metabolomics profile is needed for a certain bacterium without needing the spatial location of these metabolites, which can be performed with the general MSI approach to cover as many metabolites produced by the bacteria as possible. Both membrane-based methods provide ample advantages to the sample preparation and data collection of bacterial colonies for metabolomics studies, although they do come with some limitations.

2.3 Live bacterial colony analysis using a liquid micro-junction surface sampling probe

As sample preparation for bacterial colonies on agar tends to take a long time, MSI instrumentation that allows for immediate analysis post-incubation is optimal. As metabolite changes can occur during the entire period prior to MSI, minimizing this time has advantages for unstable and volatile analytes. Yu *et al.* report a protocol development using a liquid micro-junction surface sampling probe (LMJ-SSP) for MSI analysis.⁷⁰ This method allows for the analysis of bacterial colonies directly from their surface. The LMJ-SSP dispenses liquid, in this case



methanol, onto the colony surface, which is then aspirated and analyzed through electrospray ionization (ESI)-MS. Through a stepwise solvent collection in a designated area, an image can be created from the observed ions, creating an MS image. This method has been used to identify natural products in *Pseudoalteromonas*, which are marine bacteria.

Using a multivariate principal component analysis (PCA) scoring system, the authors reduced the data from traditional MSI into a color-coded image that designates the mass range with molecules of interest within their screened colonies.⁷⁰ The PCA scores reduced the number of m/z values of interest from 13 000 to 500, which is more manageable when screening unknown bacterial colonies for metabolites and natural products. Unfortunately, the spatial resolution using the LMJ-SSP method is ~ 1 mm, while MALDI can reach down to 5 μm . Overall, Yu *et al.* demonstrated an effective protocol for rapidly screening large datasets from bacterial colonies by applying a LMJ-SSP methodology, which was successfully used to identify natural product candidates.⁷⁰

3. Derivatization and dual imaging methods for improvements on metabolite identification

Some molecular classes are difficult to measure with MSI, and derivatization is a common practice in bacterial mass spectrometry to selectively enhance the detection of specific classes.^{71–73} Derivatization allows for enhanced signals on analytes with low or no ionization, and increasing the number of analytes identified during data acquisition. The molecular complexity of biological molecules suggests that derivatization reagents for specific classes/functional groups will be an effective approach. The principle of using derivatization agents for MSI has been previously explored, it mainly targets on-tissue mammalian measurements over bacterial applications.^{19,74–76} The current bacterial sample derivatization methods often target large molecules over smaller metabolites.^{74,77} Although not yet tested with derivatized samples, another improvement for MSI bacterial metabolomics includes dual imaging techniques for metabolite identification on tissue samples to distinguish between microbe and host analyte signals. In this section, we will discuss derivatization and dual imaging approaches that have improved MSI analysis of bacterial metabolites.

3.1 MSI signal enhancement through agar-based microbial derivatization of carbonyl-containing analytes

MSI signal enhancement can be achieved using multiple methods, including chemical derivatization of samples, leveraging the functional groups of the molecules of interest. A lack of agar-based derivatization examples for bacterial MSI analysis has been surprising compared to on-tissue derivatization agents demonstrated for MSI analysis.^{74–76,78} 4-(2-((4-Bromophenethyl)dimethylammonio)ethoxy)benzenaminium

dibromide (4-APEBA) has previously been applied to on-tissue MSI analysis to derivatize carbonyl-containing compounds of interest.⁷⁹ This derivatizing agent can also be applied to agar-based microbial MSI analysis, as demonstrated by Veličković *et al.*⁸⁰ A *Bacillus subtilis* and *Fusarium* sp. co-culture was used as proof-of-concept for testing the practicality of 4-APEBA as a derivatization agent. Over 300 carbonyl-containing compounds were identified on the co-culture MSI data, including aldehydes, ketones, carboxylic acids, lactones, esters, and others.⁸⁰ Many of these functional groups tend to have poor ionization due to their inclination to negatively ionize, which makes signal identification difficult, as most MALDI-MSI sample acquisitions are developed and performed in positive mode.⁸⁰ Applying 4-APEBA as a derivatization agent for bacterial samples increases the identification of carbonyl-containing analytes in MSI, as the derivatized product allows for positive ionization of molecules of interest. This technique increases the MSI scope of bacterial agar-based samples by enhancing the signal of primary and secondary metabolites that contain carbonyl functional groups.

4-APEBA was used to confirm metabolite interactions between the two species used in the study.⁸⁰ The agar sample was divided between *B. subtilis*, *Fusarium* sp., and the intersection between these two species. Some metabolites, such as hexosamine and hydroxypentonic acid, were found only in the intersection region of both species, while other analytes, including glutamate and citrate, were found in all regions of the sample.⁸⁰ This derivatization technique also helped identify aliphatic fatty acids and amino acids, which increases its use for primary and secondary metabolomic studies. A recurring issue with metabolomic studies is the differentiation of isomeric analytes. This issue can be resolved using tandem MS (MS/MS) to identify isomers through their fragmentation pattern³⁹ or trapped ion mobility (TIMS) to separate the isomers.⁸¹ 4-APEBA can also be used to resolve isomers.⁸⁰ During the derivatization process, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is applied to the sample prior to 4-APEBA to activate the agent against carboxylic acid, as 4-APEBA only reacts with aldehydes and ketones in the absence of EDC. This distinction can be used as an advantage to select aldehyde or ketone isomers over carboxylic acids. In the study, malate, a carboxylic acid, is compared to dehydrothreonate, a ketone, in the presence of 4-APEBA and the presence or absence of EDC.⁸⁰ No signal at an m/z of 479.1164 is observed in the MSI data for the co-culture sample, indicating no aldehyde or ketone is present. With the addition of EDC, 479.1164 m/z signal enhancement indicates the presence of a carboxylic acid, in this case, malate. Leveraging this property is useful for future metabolomic studies.

3.2 Harnessing *in situ* hybridization for multi-omics imaging analysis

MSI is often used to study host–microbe interactions to understand the effects this interaction has on the surface of a plant or tissue. Unfortunately, discerning analytes between the host and the microbe, on occasion, tends to be difficult. This can



be circumvented through the use of databases to identify analytes during the sample analysis, although some analytes might be difficult to identify as they are only present during the host-microbe interaction. Correlation between gene transcription and protein translation with collected MSI metabolomics data could allow for an increased understanding of metabolic processes and the identification of the origin of the analyte of interest. Fluorescence *in situ* hybridization (FISH) is a technique that uses fluorescent probes to label DNA or RNA sequences, typically based on sequencing data or used in untargeted studies.^{82–84} Used with MSI, FISH can be applied to host-microbe or microbe-microbe interactions to identify *in situ* mechanisms and pathways involved in these multispecies systems. Bourceau *et al.* harnessed the property of 16S ribosomal RNA (rRNA) as a marker for bacterial species identification^{85–87} using FISH and applied it to the MSI of *Bathymodiolus* mussels, which live in symbiosis with

gammaproteobacteria.^{57,88,89} This combination of spatial metabolomics and FISH labeling protocol is termed metaFISH by the authors.⁹⁰ A detailed protocol for this technique was published by the authors (Fig. 4), describing the lengthy sample preparation where samples are sectioned, analyzed using MSI, washed and fixed for hybridization, stained with fluorescent dyes, and analyzed using fluorescence microscopy.⁵⁷ The MSI collected data is then integrated with the FISH data to correlate observed metabolites to the localization of the bacterial symbionts on the tissue.⁵⁷ Cryoembedding of agar-based samples along with the application of the metaFISH protocol could enhance omics data acquisition on bacterial colonies by enabling the consecutive use of MSI and FISH without separate sample preparation, thereby streamlining analysis and potentially accelerating our understanding of the processing occurring in solid growth conditions.

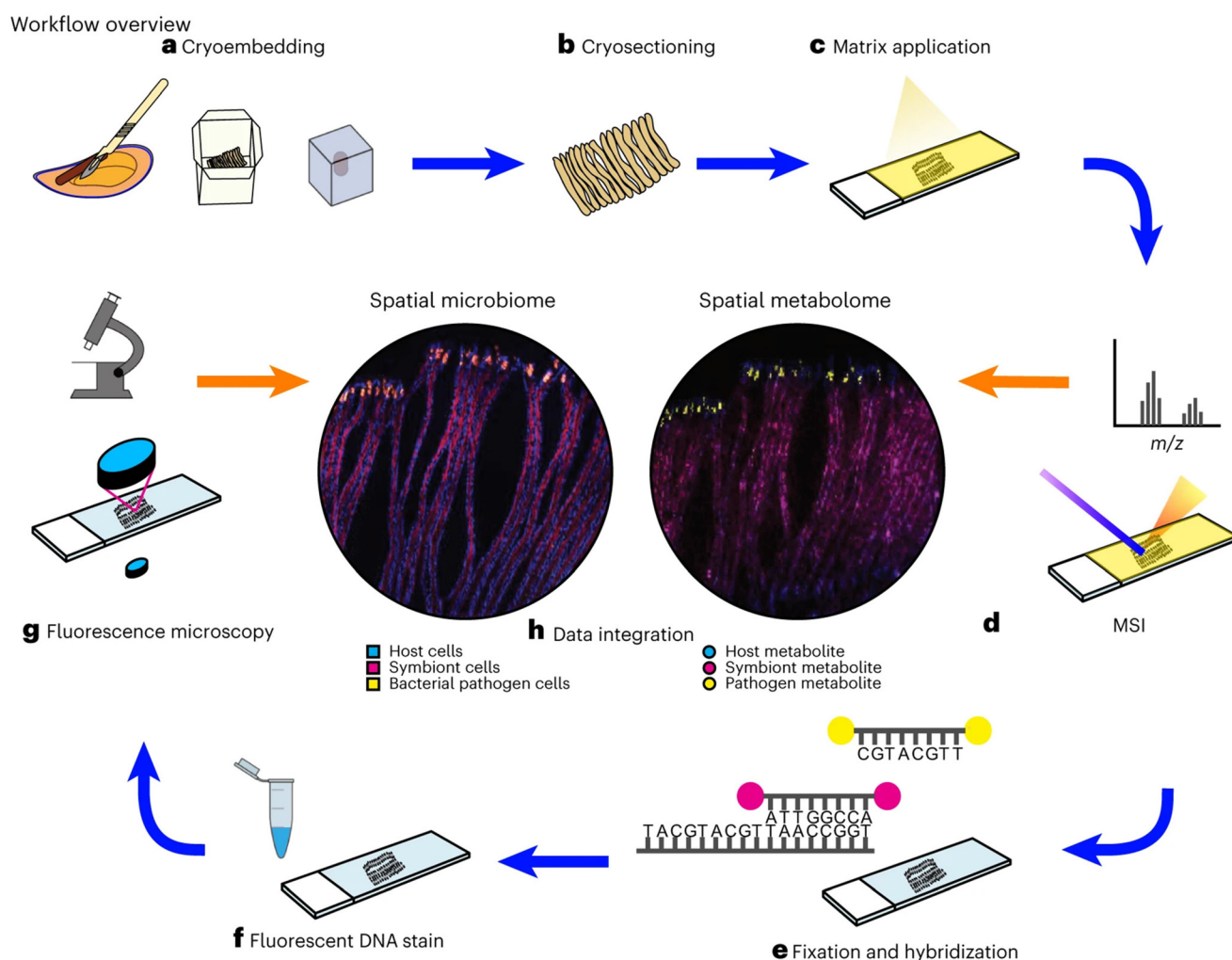
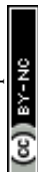


Fig. 4 metaFISH protocol for MSI and FISH correlation. a, the sample is embedded for sectioning; b, the frozen tissue is cryosectioned; c, matrix is applied to sample; d, MSI data acquisition of the sample; e, matrix is removed and the tissue is fixed and hybridized for FISH analysis; f, fluorescent dye is applied to the slide; g, FISH data is collected using fluorescence microscopy; h, FISH and MSI data are integrated to produce the metaFISH image. Nature Protocols, Patric Bourceau *et al.*, 18, Springer Nature, 2024, reproduced with permission from SNCSC.



4. Three-dimensional MSI analysis on bacterial colonies

MSI is a two-dimensional molecular imaging technique using an optical image of a sample to assign analyte intensity information per pixel to identify the spatial localization of the sample surface. 2D MS images taken of different levels of the same samples can be stacked together to create a three-dimensional image. 3D MSI has previously been used for metabolomic studies on brain slices⁹¹ and *Pseudomonas aeruginosa* biofilm samples.⁹² Typically, 3D MSI data is collected with a 3D OrbiSIMS instrument, combining the functionality of a SIMS with an orbitrap mass analyzer.^{91,92} Recently, efforts to apply 3D MSI to whole bacterial colonies using the more accessible MALDI-MSI have been made.⁹³ The sample preparation and MSI analysis for this technique will be discussed below.

4.1 Whole colony three-dimensional MSI analysis for biofilm studies

As mentioned previously, the bacterial MSI sample preparation involves drying the agar prior to sample acquisition.³⁶ This changes the topography of the culture, making a planar surface optimal for 2D MSI analysis.²⁹ This is a disadvantage for MSI of biofilm-producing bacteria, as biofilm is composed of a complex polymeric matrix with chemical heterogeneity between the matrix layers. This heterogeneity could lead to

missing information found too deep within the colony to detect during 2D MSI analysis of biofilm-producing bacteria. The development of a 3D MSI technique allows for the layered analysis of whole colonies and improves our ability to measure these biofilms and their chemical environment during growth (Fig. 5A). Moisture-assisted cryosection (MACS) was developed by Shen *et al.* to prevent compound dislocation during sectioning as molecules residing in the biofilm matrix may diffuse into the embedding solution, leading to signal displacement or loss. Other aspects of the sample collections were optimized, including section thickness used for MSI studies, as dislocation of signals was observed throughout thicker sections, and sectioning direction, since perpendicular slicing of the collected biofilm sample would crumble during sectioning while frozen. The optimized MACS MSI method was applied to *P. aeruginosa* to recreate a 3D rendition of MSI analysis (Fig. 5A).⁹³

The layering of the images from the collected biofilm sections revealed heterogeneity in the analyzed matrix with molecule distribution throughout the biofilm layer (Fig. 5Ba).⁹³ The authors label the volume of the colony to better identify the accumulation of analytes in regions of interest (Fig. 5Bb and c). The application of MS/MS on the 3D MSI rendition to identify isomers and their localization throughout the matrix layers resulted in the identification of quorum-sensing isomers and their distribution throughout the matrix.⁹³ This methodology was also applied to study antibiotic diffusion in biofilm. 24 h

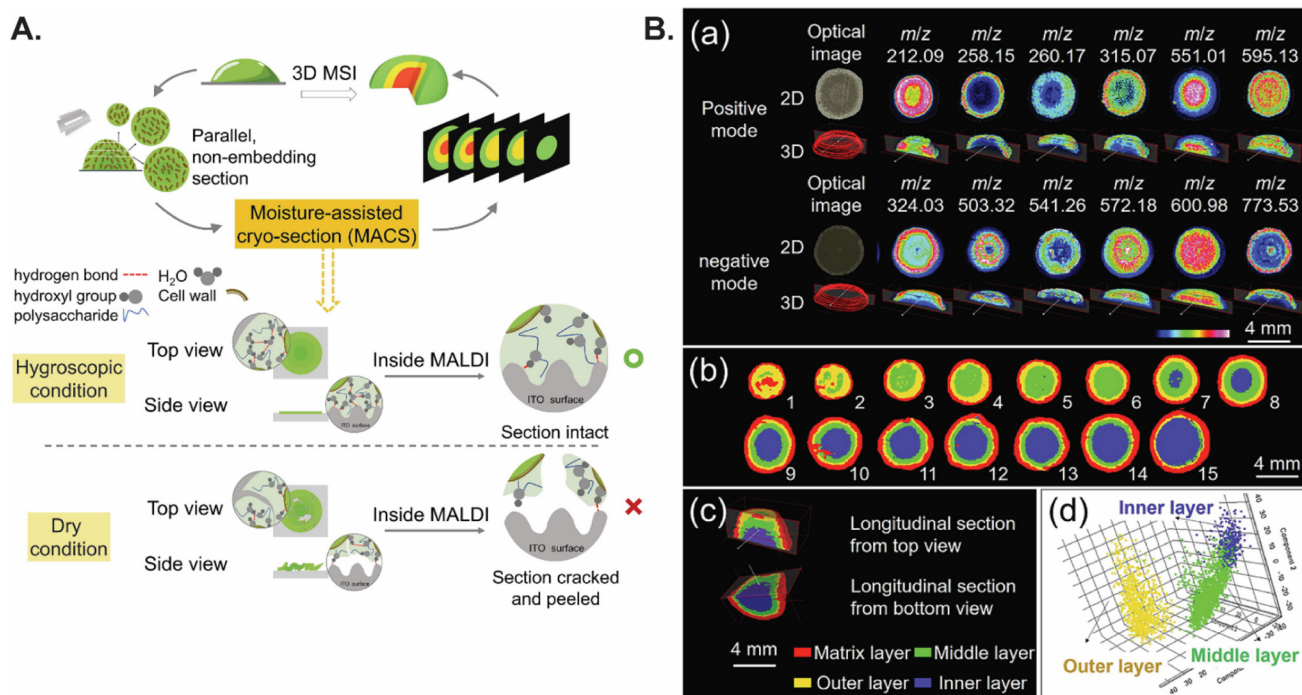


Fig. 5 3D MSI of *P. aeruginosa* biofilm. A, MACS MSI workflow; B, 2D and 3D MS images for *P. aeruginosa* metabolites (a); 2D view of each collected biofilm section (b); 3D biofilm colony volume (c); principal component analysis of different sections of the biofilm (d). Reprinted from *J. Adv. Res.*, in press, Shen, Y.; Wang, Y.; Wang, J.; Xie, P.; Xie, C.; Chen, Y.; Banaei, N.; Ren, K.; Cai, Z., High-Resolution 3D Spatial Distribution of Complex Microbial Colonies Revealed by Mass Spectrometry Imaging., Copyright 2024, with permission from Elsevier.



old *P. aeruginosa* colonies were transferred to a plate containing tobramycin, and 3D MSI was acquired at various time-points for 48 h. The distribution of the antibiotic was observed on the top layer of the biofilm matrix after 48 h, while the inner matrix shows a lower tobramycin signal intensity, indicating low diffusion into that zone. The MACS method was also applied to *Staphylococcus aureus* and in a co-culture with *P. aeruginosa*, although no 3D MSI was performed on the cultures. 3D MSI is a powerful tool to study biofilm and other matrix-related bacterial environments. The MACS method has proved to be a useful technique to collect intact sections of bacterial samples that can then be layered to create a 3D rendition of a bacterial structure. This could be applied to multiple host-microbe systems and be practical for metabolomic studies of biofilm-producing bacteria or microbial interactions on a variety of surfaces.

5. Challenges and future perspectives

Metabolomic studies of bacterial systems have led to new discoveries in natural product research,⁹⁴ understanding of host-microbe interactions^{95–97} and adaptation processes,⁹⁸ among other functionalities. Advancements in sample preparation, data acquisition, and data processing have improved data analysis and analyte identification. The application of MSI techniques to microbial metabolomics has been a great effort at improving our understanding of pathways and interactions on surfaces.^{29,36,42}

Here, we reviewed recent advances in several areas of sample preparation and molecular identification that enhance the application of MSI to microbial spatial metabolomics. Although the techniques are advantageous in the bacterial MSI metabolomics field, there are further advances to improve sample preparation and data analysis. First, sample preparation is dependent on the growth of the bacterial culture on an agar surface, which is typically grown on Petri dishes.^{35,36,42} The transfer process to the MS target plate perturbs the culture prior to drying, which could lead to displacement or loss of signal post-drying. Protocols to transfer a colony to an MS target plate involve perturbations and impression methods.⁵⁹ Matrix application is also needed for MALDI-MSI experiments, which adds an extra step during the sample preparation process. Substituting matrices for derivatization agents that are easily ionized enhances the signal and may shorten sample preparation. 2-Fluoro-1-methyl pyridinium (FMP)⁹⁹ derivatives have been applied to tissues to derivatize primary and secondary amines (FMP-10).¹⁰⁰ A push to identify and use other derivatization agents outside of 4-APEBA that can target other functional groups outside of carbonyls is needed, as it would increase the scope of the metabolites observed during bacterial MSI studies. Finally, the functionality of 3D MSI on bacterial cultures seems very promising as it could help elucidate many questions related to growth, colonization, and behavior. 3D MSI has been previously used to study *P. aeruginosa* biofilm

using a cryo-orbiSIMS for 3D analysis of the biofilm surface.⁹² The MACS protocol has a more in-depth study as it layers MS images collected from the colony slice to render a 3D image. More complex morphological cultures as those observed in swarming cultures^{101–103} might need additional optimization of the sample preparation, as the grown cultures are larger by area on the agar surface and might be more difficult to section. Attempts at using MACS for larger colonies should be made to expand its use to more complex systems. We expect these areas and others require additional developments for agar-based sample preparation for MSI. The pace of the development of MSI protocols for microbial MSI is fast, and we expect new developments in the coming years. After all, advances in bacterial MSI metabolomics in the recent past have improved the capabilities of spatial metabolomics.^{57,59,65,80,93}

Bacterial sample preparation for MSI studies has evolved over the years.^{29,36,42} Microbe metabolomics studies have been conducted using varying spectroscopic techniques through chemical extraction methods that collect the analytes of interest from bacterial culture.^{13–17} This sometimes leads to cell disruption or the collection of undesirable compounds without proper purification. These improvements for MSI analysis of agar-based bacterial cultures or host-bacterial interaction can be applied to study surfaces without perturbing the cell and adding background analytes, allowing for a streamlined analysis. The application of MSI to bacterial metabolomics can be a powerful tool for the field and should be promoted.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

No data was generated during the writing process of this minireview.

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