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Emerging mass spectrometry techniques for the direct analysis of microbial colonies

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Abstract

One of the emerging areas in microbiology is detecting specialized metabolites produced by microbial colonies and communities with mass spectrometry. In this review/perspective, we illustrate the emerging mass spectrometry methodologies that enable the interrogation of specialized metabolites directly from microbial colonies. Mass spectrometry techniques such as imaging mass spectrometry and real-time mass spectrometry allow two and three dimensional visualization of the distribution of metabolites, often with minimal sample pretreatment. The speed in which molecules are captured using these methods requires the development of new molecular visualization tools such as molecular networking. Together, these tools are beginning to provide unprecedented insight into the chemical world that microbes experience.

Introduction

Historically, advances in microbiology closely follow the development of technologies. The invention of microscope in early 1660s by Anton van Leeuwenhoek (1632-1723) for example enabled the visualization of a hidden living world [1]. Similarly, modern techniques such as fluorescence microscopy revolutionized our understanding of membrane fluidity, calcium sensing, vesicle formation, distributions of proteins and many other important processes in microbiology [2-6]. Although mass spectrometry has been used to analyze microbiological samples for decades, it never gained a solid footing in the arsenal of routine tools used by microbiologists [7-19]. However, there is a resurgence in the adaptation of mass spectrometry towards the analysis of microbial samples due, in part, to the increased sensitivity, the increase in need to understand the mechanistic roles of microbial behavior at the chemical level, new instrumentation, ease of use of modern

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instruments and novel microbial and mass spectrometry compatible workflows that are being developed.

This year we are celebrating the 100-year anniversary of mass spectrometry. With the invention of cathode rays by Sir Joseph John Thomson (1856-1940), measuring the mass of atoms and molecules became possible [20]. Over the past few decades, as softer ionization methods have become available, entirely new ways were opened up to peek into the fascinating world of microbes. Liquid chromatography coupled with mass spectrometry (LC-MS) has been used to understanding the intricate complexities of microbial metabolism, protein-protein interactions and post-translational modifications [21-32].

However, LC-MS is not the only way to investigate microbial systems. Direct analysis of microbes can be accomplished without a separation step. The ability to directly analyze microbial colonies has led to strain identification workflows that are now approved for clinical use. Furthermore, recent mass spectrometry advances enabled the mapping of microbial molecules spatially, the observation of molecules produced by living microbial colonies and of microbial molecules at the single cell level.

Clinical use of microbial mass spectrometry

In the 1970s, the lab of Catherine Fenselau published their pioneering research using mass spectrometry to identify cultured bacteria from patients' specimens. It was realized that direct mass spectrometry of microbes gives rise to fingerprint type signatures that can be correlated to a database of known species and a microbe's fingerprints as obtained with a mass spectrometer. These methods have now advanced to the clinic [33,34]. This pioneering work set the stage of the development of clinical microbiology identification tools such as Biotyper and VITEK-MS. The Bruker in vitro diagnostics (IVD) matrix-assisted laser desorption/ionization (MALDI) Biotyper is a MALDI-time of flight (ToF)-mass spectrometry based platform for the identification of bacterial and yeast. Biotyper uses the unique molecular fingerprint such as the most abundant proteins, to identify the microbes [33,35,36]. The VITEK MS from bioMérieux is a similar platform as the Biotyper from Bruker [37]. The FDA clearance in 2013 for both of these microbial tests marks an exciting transition for clinical diagnostic mass spectrometry. It is likely that these and other mass spectrometry techniques will be widely introduced into clinic laboratories in a foreseeable future since they are faster, less labor intensive and cheaper than their counterparts to determine the phylogenetic branch that microorganisms belong to [38].

Direct analysis of microbial colonies in recent years has gone beyond microbial identification. Imaging mass spectrometry (IMS) and real-time mass spectrometry are two new and exciting areas (Table 1). These developments are also leading to the development of new data visualization tools, such as molecular networking. Due to the volume of data that is generated, it has given rise to the need for a molecular Genbank to compare and contrast the data.

Microbial imaging mass spectrometry

In microbiology, phenotypic changes are often observed using optical or fluorescence microscopy. However, the chemical signals responsible for these changes are poorly characterized. The study of microbial metabolites production is hindered by the lack of tools that can connect the chemotypes with observed phenotypes. Imaging mass spectrometry (IMS) is able to provide two-dimensional (2D) and three-dimensional (3D) visualization of surface metabolites and lipids directly from microbial colonies.

While imaging mass spectrometry was demonstrated with secondary-ion mass spectrometry (SIMS) in the 1960s [39], the use of IMS in microbiology has only been carried out in the last decade [40]. We will not be comprehensive in this article but we will address some of the key advances and highlight their utility. In a typical imaging mass spectrometry experiment, the desorption and ionization of the molecules from the sample occurs when a desorption probe hits the surface of a sample at defined positions controlled by x-y stage, dislodging ions that are then “weighed” in a mass spectrometer (Figure 1).

It results in a mass spectrum for each position - a graph where you have a mass-to-charge ratio on the x-axis and intensity on the y-axis. Eventually the collection of mass spectra at each position will be shown as a single image, with each specific mass displayed as a false-color gradient, showing the specific molecular distribution and relative abundance of the metabolites on sample. The ions for IMS can be generated with a variety of ion sources (Table 1). Here we highlight SIMS-IMS and MALDI-IMS. They have been used to image microbial systems, although imprinting techniques have enabled imaging mass spectrometry with other ion sources [41-44].

In SIMS, an analyte signal is produced from the sample surface by shooting a high-energy primary ion beam (e.g. gold, cesium clusters or bulky balls) and then producing secondary ions that will be guided into mass spectrometry detector through sputtering [45]. Although two main SIMS methods exist, we generalize both of them due to space limitation of this article. For a more thorough description of the methods see reference 37 and the references therein. SIMS is attractive for imaging because there is no need for extra matrix to increase ionization and can get an imaging spatial resolution of less than one micrometer. However, the requirement of ultra-high vacuum limits its throughput. Furthermore, the high-energy primary ion beam used to facilitate the ionization process limits the size of the molecules that can be detected [46]. Even in the gentlest SIMS method, most molecules that are detected are fragmented into fragment ions, charged atoms or atomic clusters. Due to the fragmented nature of the resulting ions, it is challenging to understand the origin of the signal detected, therefore creative use of isotopes and methods such as fluorescence in situ hybridization (FISH) can be adapted to gain insight into the spatial organization of microbial communities and their chemistries [47-51]. We anticipate that a solution to the high-energy beam will emerge in the next 20 years in the SIMS community to provide mostly intact molecular ions that would make SIMS results easier to interpret. Also, SIMS can be used for depth-profiling, in effect creating a 3D image [46]. Some molecules however can be observed intact with SIMS [52-54]. It has been possible to study intact membrane lipids and proteins by ToF-SIMS at the single cell level [55,56] and the analysis of unlabeled

molecular components in lipid monolayers [57,58]. It is for these reasons that SIMS IMS is being explored as a tool in microbiology as it is the highest spatial resolution IMS that currently exists [59-64].

Although SIMS has been around for much longer, MALDI imaging mass spectrometry, a method championed by the Caprioli lab, is the most widely used IMS technique at this time [65-74]. MALDI-IMS is applied in characterizing specialized metabolites from microbial monocultures or tracking the metabolite transfer within polymicrobial colonies and biofilms [25,75,76]. In MALDI, the sample is first covered with a layer of matrix. The matrix is a UV absorbing organic acid such as 2,5-dihydroxybenzoic acid or α -cyano-4-hydroxycinnamic acid. The matrix facilitates the desorption and ionization of compounds from the sample surface. MALDI mass spectrometry has reported to desorb molecules with m/z up to 110kDa and higher [77]. In microbial imaging, the thickness of agar media is limited to 0.5-1.5 mm before applying matrix [76]. This is a physical limitation due to the insertion of the MALDI target plate into the instrument and there is not much space between the vacuum chamber and the MALDI plate during insertion. Emerging MALDI-IMS into studying specialized metabolites production is having a significant impact how microbes in culture are studied. For example, MALDI-IMS is a good tool to visualize the production of specialized metabolites as a result of interspecies interactions. For example, when *Streptomyces coelicolor* with other actinomycetes resulted in the production of many specialized metabolites that were not produced when it was grown alone. Many of those specialized metabolites are interaction specific [74,78]. Another example is the direct visualization of metabolic biotransformation of metabolites between *P.aeruginosa* and *A. fumigatus*. *A.fumigatus* converted *P.aeruginosa* phenazine metabolites such as phenazine-1-carboxylic acid (PCA) into other phenazine compounds such as 1-hydroxyphenazine (1-HP) with enhanced toxicity in side-by-side interaction [25]. Not only in interspecies interactions can we find new molecules even from well studied system IMS gives new molecular information. For example, MALDI-IMS was used to identify the *Bacillus subtilis* cannibalism factors [76]. MALDI-IMS is not limited to 2D. MALDI-IMS can define the chemistry that is found underneath microbial colonies. 3D microbial MALDI-IMS captures metabolic exchange factors but also capture chemical processes such as nutrient depletion and the alteration of the chemical environment on which the microbes grow [78,79]. With the most common MALDI and SIMS IMS instrument set-ups, however, the samples are under vacuum limiting real-time analysis.

Real-Time Mass Spectrometry

Another tool introduced in 2012 to directly analyze microbial colonies at the molecular level is real-time or live colony mass spectrometry [80]. Real-time mass spectrometry has a long history. As one of the most established real-time mass spectrometry techniques, membrane-introduction mass spectrometry (MIMS) was invented in the 1980s [82-84]. MIMS is used to detect liquid or gaseous samples by introducing ions into a mass spectrometer through semi-permeable membrane between the samples and the mass spectrometer. Aside from separating analytes from high and low-pressure environments of a mass spectrometer, the membrane also provides as pre-concentration of to provide increased sensitivity. Another approach to real-time analysis is proton-transfer-reaction mass spectrometry (PTR-MS),

which is based on the use of chemical ionization (CI) [85-87] for the analysis of volatile compounds. Aside from monitoring bio-reactions, these methods have not yet been employed in studying the chemical make up of microbes. Only recently have ambient ionization methods been used to capture molecules from microbes in real-time. These ambient methods are nanospray desorption electrospray ionization (nanoDESI) [81,88-91], ambient electrospray ionization flow-probe [92-94] and rapid evaporative ionization mass spectrometry (REIMS) [95,96]. They all show great potential in direct analysis of living colonies, due to the minimal sample preparation required and to the broader range of sample types and agar surfaces that can be analyzed when compared with vacuum based methods.

NanoDESI is an ambient pressure ionization method that relies on liquid extraction and ionization. NanoDESI is designed for real-time analysis of molecules on substrates through the creation of a solvent bridge between the sample surface and two capillaries [88]. More importantly, nanoDESI is able to extract metabolites from the surface sample for mass spectrometric analysis without damaging the entire sample [80]. This allows the same colony to be sampled more than once using nanoDESI, gaining a time-lapse view of metabolites production and the roles of those metabolites in the colony. NanoDESI can be used to rapidly profile the metabolites from samples [97], assist in the characterization of specialized metabolites from microbial colonies [90] and to capture metabolites production involved in interspecies interactions [98]. However, nanoDESI has some drawbacks. Since nanoDESI is dependent upon a solvent bridge, complete recovery of the solvent utilized can be hindered due to the differences in hydrophobicity for some of the microbial colonies, leading to low overall signal intensity. NanoDESI imaging is possible. This has been demonstrated with tissues [99,100] and metabolic profiling of living bacterial colonies directly from Petri dish. Although possible, when aiming to perform imaging, the transfer capillary may clog as it moves along the surface due to surface relief of a microbial colony [91]. A possible solution of this limitation would be to develop an automated height sensor.

Similar to nanoDESI, an ambient electrospray ionization flow-probe technique is able to analyze living microbes from a Petri dish. The flow-probe technique shares the same advantages as nanoDESI including no sample preparation or purification. If desired, it can even provide a spatiotemporal profile of molecular signals directly from the surface of a colony. This flow-probe technique is based on the coaxial tube geometry liquid microjunction surface sampling probe (LMJ-SSP). The major difference of LMJ-SSP from nanoDESI is using pneumatic nebulizer instead of two nanospray capillaries. The flow-probe reduces the difficulty of removing clogs by allowing the nebulizer valve to be temporarily blocked so the nebulizer gas could flush the transfer tube [92].

Finally, another creative real-time technique is called rapid evaporative ionization mass spectrometry (REIMS). This method was reported as we were writing this article. REIMS is designed specifically for real-time, *in vivo* analysis of biological tissues in the surgical environment [96]. REIMS could detect mass spectral fingerprints of microbes from intact bacterial cells upon subjecting the bacterial biomass to a radiofrequency (RF) electrical current [95]. Since the REIMS profile of intact bacteria can be obtained in 2-3 seconds without any sample preparation, and combined with multi-variable analysis, REIMS also has the potential to be used as a bacterial identification strategy similar to the Biotyper and

VITEX-MS platforms. Further development would be using REIMS to detect bacteria directly from human biofluids or tissue matrices, although it likely requires the specific understanding of the chemistry that belongs to specific microbes and a way to visualize it. Standard multivariable analysis probably would not work in such biofluid unless the majority of the biofluid composition is one or two microbes.

Real-time mass spectrometry techniques, as described above, can sample a living microbial community. More importantly, since real-time mass spectrometry enables rapid profiling under ambient conditions, it can be used in high-throughput screens of biological and clinical samples, be used for species classification and for the analysis of specialized metabolites (e.g. virulence factors, quorum sensors, antibiotics, etc). However enabling high throughput and information rich data acquisition also means that new methods for visualizing the data must be developed, something that is in its infancy right now.

Molecular Networking

One of the largest challenges with microbial analysis of live colonies using ambient ionization methods is that the data acquisition is easy and fast. This means lots of data is generated and new informatics methods to visualize this data needs to be developed. Akin to the need for a revolution in sequencing informatics 20 years ago a similar revolution is needed in the analysis of mass spectrometry data. It is expected that the next 10 years we will see a significant advancements in the data analysis tools of mass spectrometry information. One such method is molecular networking, a powerful MS based data analysis tool that enables the detection and visualization of related compounds via MS/MS fragmentation spectra relationships within a dataset, as well as analysis individual MS/MS signals by creating a chemical diversity map of detected molecules without the need to know the structure [80]. Currently, the chemistry of most virulence factors, and other specialized metabolites from microbes are studied by monitoring individual molecular species, which requires a significant amount of time to understand and interpret the information. Unlike traditional metabolomics, specialized metabolites are not common to every microbe and therefore one standard workflow may not work. The MS/MS based molecular networking is a powerful strategy to guide the molecular characterization based on the MS/MS fragmentation patterns of molecules that are detected as ions [80,101,102] (Figure 2). Molecular networking is able to visualize related compounds based on the similarities of MS/MS spectra and enables a more global analysis of the microbial metabolites, perhaps an apt comparison is metagenomic analysis. The repertoire of specialized metabolites is more complex than originally thought and changes significantly when a bacterium is in contact with other organisms [25,78]. It is expected that MS/MS based molecular networking will also provide a view of the composition of many uncharacterized microbial factors [103-105].

In conclusion, these advanced mass spectrometry techniques are capable of direct analysis of microbial communities with little or no sample preparation, even from live samples. Methods such as imaging mass spectrometry and real-time mass spectrometry are simplifying the otherwise time-consuming and labor-intensive sample preparation processes. They provide an avenue to widely apply mass spectrometry techniques towards clinical and

biotechnological studies and take inventory of the diversity of specialized metabolites produced by microbes. Combined with novel informatics approaches, the application of these mass spectrometry methods to microbiology are an exciting development that will impact how microbes are characterized in the future at the molecular level.

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Highlights

- Mass spectrometry techniques are powerful tools in studying specialized metabolites production from microbial colonies.
- Direct analysis of microbial colonies can be used for clinical strain identification.
- Microbial imaging mass spectrometry is able to visualize surface and secreted metabolites from microbial colonies.
- Real-time mass spectrometry techniques enable the direct mass spectrometric interrogation of living microbial colonies.
- Tandem mass spectrometry based molecular networking and emerging algorithms enable global molecular analysis of microbial metabolites.

Figure 1. MALDI imaging mass spectrometry workflow

Figure 2.

Generation and visualization of molecular network. A) Workflow of generation of molecular networking. The cosine score of MS spectra alignment between 0 to 1 as well as the thickness of edges in network represent the similarity of certain spectra. Score in 1 or thick edge means high spectral similarity. Certain cut-off of cosine score (normally 0.5) usually applied. B) Cytoscape visualization of molecular networking. The number labeled on each node represent the parent mass. Different colors represent different samples that data generated from. C) Compound identification via molecular networking. Different colors represent different mass-to-charge ratio (m/z). [102]

Table 1
Overview of novel mass spectrometry techniques used in microbiology

Techniques		Representative Results
Microbial Imaging Mass Spectrometry	<div> <p>Static SIMS</p> <p>Pulsed primary ion beam</p> <p>TOF or qTOF MS</p> <p>Near-surface sample analysis</p> <p>HV</p> </div> <div> <p>Dynamic SIMS</p> <p>Continuous primary ion beam</p> <p>Deep profiling available</p> <p>HV</p> </div>	Study intact membrane lipids and proteins at single cell level [55,56].
	<p>MALDI IMS</p> <p>Pulsed UV/Laser</p> <p>TOF or ion-trap based MS</p> <p>Matrix-assisted sample</p> <p>HV</p>	Capture the metabolic transformation in polymicrobial community [75,76].
	<p>NanoDESI / NanoDESI IMS</p> <p>solvent bridge on the surface of colony</p> <p>Solvent</p> <p>Solvent with analyte</p> <p>HV</p> <p>Primary capillary</p> <p>Secondary capillary</p> <p>ion-trap based MS</p> <p>Analysis directly on a Petri dish</p>	Monitoring of intact molecular species under ambient at single colony directly from Petri dish [91].
Microbial Real-Time Mass Spectrometry		High-throughput identification and characterization molecules secreted from bacteria colonies [80,90,97,98].

Techniques	Representative Results
<p data-bbox="235 722 386 877">Microbial Real-Time Mass Spectrometry (cont.)</p> <p data-bbox="396 321 894 352">Ambient Electrospray Ionization Flow-Probe</p>	<p data-bbox="1256 541 1528 604">Real-time metabolomics on living organism [92].</p>
<p data-bbox="396 793 472 821">REIMS</p>	<p data-bbox="1247 993 1539 1115">Rapid, sample preparation free species-level identification of intact bacteria [95].</p>

SIMS, secondary ion imaging mass spectrometry; MALDI, matrix-assisted laser desorption ionization; NanoDESI, nanospray desorption electrospray ionization; REIMS, rapid evaporative ionization mass spectrometry.