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Metabolic profiling directly from the Petri dish using nanoDESI imaging mass spectrometry

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Abstract

Understanding molecular interaction pathways in complex biological systems constitutes a treasure trove of knowledge that might facilitate the specific, chemical manipulation of the countless microbiological systems that occur throughout our world. However, there is a lack of methodologies that allow the direct investigation of chemical gradients and interactions in living biological systems, in real time. Here we report the use of nanospray desorption electrospray ionization (nanoDESI) imaging mass spectrometry for *in vivo* metabolic profiling of living bacterial colonies directly from the Petri dish with absolutely no sample preparation needed. Using this technique, we investigated single colonies of *Shewanella oneidensis* MR-1, *Bacillus subtilis* 3610, *Streptomyces coelicolor* A3(2) as well as a mixed biofilm of *S. oneidensis* MR-1 and *B. subtilis* 3610. Data from *B. subtilis* 3610 and *S. coelicolor* A3(2) provided a means of validation for the method while data from *S. oneidensis* MR-1 and the mixed biofilm showed a wide range of compounds that this bacterium uses for the dissimilatory reduction of extracellular metal oxides, including riboflavin, iron-bound heme and heme biosynthetic intermediates, and the siderophore putrebactin.

Introduction

The driving force behind most scientific inquiries is to better understand the natural world we live in. As a result of this pursuit new tools are constantly being developed to allow scientists to observe natural phenomena in the most direct and non-invasive way possible. This becomes increasingly important within the field of microbiology where the complex interactions between microbes and their environment, both living and non-living components, are governed by continuously changing chemical signals. These chemical signals occupy a wide chemical space, ranging from small metabolites to lipids to macrocyclic peptides, and allow for a balance to be reached between each microbial

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population and their surrounding environment through modulation of cellular processes.[1-4] It is the ability of these chemical signals, also referred to as secondary or specialized metabolites, to directly affect the physical and chemical development of microbial populations that makes them so valuable to study as these attributes can be exploited for their therapeutic and industrial potentials.[5-8] With the majority of pharmaceutical therapies originating from specialized metabolites[9] and solutions to many lingering industrial issues, such as biofouling, bioremediation of toxic environmental contaminants and production of biofuels, likely to be addressed through exploitation of natural microbial behavior, the development of research tools that allow these systems to be studied in more meaningful and accurate ways is necessary to progress the field.

Recently, we have shown that nanospray desorption electrospray ionization (nanoDESI) mass spectrometry[10] is capable of sampling a microbial colony directly from the Petri dish with absolutely no sample preparation required (**Figure 1A**).[11,12] By performing a nanoliter scale liquid extraction of the biological surface immediately followed by direct infusion into the mass spectrometer, nanoDESI ionization allows for rapid detection of local chemical signals at high sensitivity due to the small volume of the droplet maintaining high local concentration of the extracted metabolites. In addition, as mass spectrometry is capable of simultaneously detecting a wide range of discrete chemical signals without the need for chemical tags or labels, this allowed us to probe the native chemical environment of each microbial colony and identify novel metabolites that were previously undetected by other analytical means.

The nanoDESI source allowed us to not only profile a single bacterial colony over the course of time, but to also spatially profile two interacting bacteria by taking measurements across a grid of discreet locations on their surface.[11] While profiling a few locations within a microbial colony can already yield valuable information regarding chemical localization, imaging mass spectrometry or IMS, a technique able to profile thousands of discrete locations within the same colony, produces a spatially-resolved map of each compound thus enriching our understanding of chemical roles of these molecules. By rendering each chemical signal as a heat map which can be overlaid on top of an optical image of the microbial sample, comparisons between the chemical and physical characteristics of the colony can yield valuable information regarding complex chemical interactions present within the sample and how they affect phenotypic development, especially when two or more microbes co-exist.

While the methods currently available for doing imaging mass spectrometry on microbial colonies provide valuable insight into the chemical environment of microbes, these methods perturb the sample to some extent during sample preparation.[13,14] For analysis by matrix assisted laser desorption ionization (MALDI) imaging mass spectrometry, the microbial sample must be excised from the Petri dish, placed on a MALDI target plate and covered with a large amount of matrix (a low molecular weight organic acid), which kills the microbial cells and can cause occlusion of metabolite signals due to the large amount of mass signals associated with the matrix in the lower mass range and cause matrix adducts to observed metabolite signals.[15] Analysis of microbial samples grown on agar medium by desorption electrospray ionization (DESI) mass spectrometry typically cannot be done directly due to the high pressure nebulizing gas ablating the colony during analysis and therefore requires indirect measurement, such as transferring secreted metabolites to a hydrophilic membrane for subsequent analysis.[16,17] Lastly, analysis by secondary ion mass spectrometry (SIMS) usually requires the sample to be excised, transferred to a conductive target plate, dried down prior to analysis and be extremely flat.[18] In addition, compounds above 1000 Da are difficult to ionize by SIMS without fragmentation induced by the sputtering process.[19] As nanoDESI is capable of overcoming many of these limitations, we

adapted our previously reported nanoDESI workflow for *in vivo* IMS analysis of living microbial colonies directly from the Petri dish.

Materials and Methods

Preparation of Bacterial Colonies

Colonies of *Streptomyces coelicolor* A3(2)[20] were prepared by inoculating 1[.μl of harvested spores onto ISP2 nutrient agar (7.5g agar, 5g malt extract, 2g yeast extract, and 2g dextrose in 500mL milli-Q water) and incubating at 28°C for 72 hours. Colonies of *Bacillus subtilis* 3610 and *Shewanella oneidensis* MR-1 were prepared by inoculating 1μl of cell stock into 4mL of LB broth and incubating at 28°C until OD_{600}=0.4. From this starter culture, 0.2μl of *B. subtilis* 3610 or 1μL of *S. oneidensis* MR-1 was inoculated onto ISP2 nutrient agar and allowed to grow at 28°C for 48 hours. Co-cultures of *B. subtilis* 3610 and *S. ondeidensis MR-1* were prepared by inoculating 1μl of S. oneidensis MR-1 onto ISP2 nutrient agar, allowing to dry, and then innoculating 0.2μ]L of B. subtilis 3610 at the same location and then letting grow for 48 hours at 28°C. Note that an additional benefit of nanoDESI IMS over MALDI and SIMS IMS is that the analysis is not limited by agar thickness and therefore can be performed on any agar depth desired in order to achieve desired chemo- and phenotypic growth of the microbial sample.

NanoDESI Instrument Setup

The nanoDESI source was constructed by modifying an existing Prosolia DESI source (www.Prosolia.com), which was equipped with a computer controlled x-y directional stage that could be programmed using the Omnispray 2D software (version 2.0.1). Both the primary and secondary fused silica capillaries were 150μm O.D. × 50μm I.D. with a contact angle of 60° between the secondary capillary and the sample surface. The droplet size using this configuration was approximately 250μm in diameter. For analysis of polar compounds, a solvent consisting of 65% acetonitrile and 35% water with 0.05% formic acid was used while a solvent consisting of 50% methanol, 35% acetonitrile and 15% toluene was used for analysis of non-polar compounds. Solvent was delivered at a rate of 800 nL/min with an applied voltage of 2 kV. The mass spectrometer was a Thermo LTQ-Orbitrap mass spectrometer equipped with CID capabilities and was operating with a tube lens voltage of 125 V, capillary voltage of 40 V and a capillary temperature of 200 °C. All analyses were performed in positive ion mode in the mass range of *m/z* 50-2000.

NanoDESI Imaging MS Experiments

Imaging experiments were conducted by mounting the Petri dish containing the sample onto the nanoDESI sample stage using double sided tape. The motion of the sample stage was automated using Omnispray 2D software while the mass spectrometer automated data collection was performed using Thermo Xcalibur software. Similar to DESI imaging, nanoDESI imaging was performed using continuous line scans with a 1 mm step size between line scans. Typical experiments were conducted using a fixed accumulation time in the ion trap of 200 ms and acquiring 5 microscans for each spectrum while moving the sample at a speed of 200 μm/sec. At this scan rate we estimate, based on previous work[21], that only the top few monolayers of cells are being sampled. However, the sampling depth will be affected by the hydrophobicity of the colony surface and the type of solvent used. In all cases, the sampling depth never appeared to penetrate the full depth of the bacterial colony as signals from the agar media were never observed when sampling the colony surface. The resulting images were acquired with a spatial resolution of 1 mm × 1 mm. To preserve the spatial integrity of the chemical signals, each line scan was performed starting at the agar end of the sample and moving toward the microbial colony with a 30 second delay between each line scan to prevent carry over between lines. Lastly, while previous

work has shown that discrete sampling of microbial colonies by nanoDESI using these solvents does not affect the overall growth and development of the microbes[11], performing nanoDESI IMS on the entire microbial colony will halt or alter growth of the microbe. Therefore the microbes are alive during sampling, but if still viable will likely exhibit altered phenotypes for future measurements after the initial IMS measurements have been taken. As a result, IMS analysis of each colony was only performed a single time.

Imaging mass spectrometry using nanoDESI is complicated by the necessity of z-axis control of the probe as the vertical tolerance between the tips of the fused silica capillaries at the liquid junction and the sample surface (between 5-500 μm depending on the size of the droplet) is usually less than the overall height of the bacterial colony. This means that the height of the probe must be adjusted to follow the contour of the sample in order to prevent clogging, due to collision with the sample, or loss of signal, due to the droplet losing contact with the sample surface. Therefore, with a relatively flat sample, such as a tissue section, the spatial resolution of nanoDESI IMS can be as low as 10 μm using pulled capillaries[22] while the complex surface morphology of intact microbes required a larger droplet to be used resulting in poorer spatial resolution but allowing for more tolerance along the z-axis. Even with a larger droplet, the vertical position of the droplet had to be manually adjusted during each line scan to follow the contour of the bacterial colony (**Figure 1B**). For microbial colonies that exhibited high variations in their contour, such as *S. coelicolor* which displayed a cobblestone-like morphology, the sample was slightly dried by leaving it exposed to ambient air in a laminar flow hood for 10-15 minutes prior to analysis in order to dehydrate and therefore flatten the sample. Note that colonies of *Bacillus subtilis*, *Shewanella oneidensis* as well as their mixed biofilm, did not have to be dehydrated prior to analysis due to their native flat surface contour. To determine if this drying time affected the detectable mass signals from colonies of *S. coelicolor,* both hydrated and dehydrated colonies of *S. coelicolor* were sampled by nanoDESI line scans with the resulting data showing no significant differences in mass signals between the two preparation techniques (**Figure S1**).

IMS Data Processing

Once collected, the raw imaging data was loaded into MATLAB software (The Mathworks Inc., Natick, MA, USA) and was processed by using total ion current (TIC) normalization, removal of abnormally intense pixels (a.k.a. hot spots) and image denoising (**Figure S2**). This was done by first converting the spectra to mzXML format using Prosolia's Firefly conversion software before importing into MATLAB. The rest of the processing was done using custom made MATLAB scripts. Each spectrum was loaded using the “mzxmlread” MATLAB routine (Bioinformatics Toolbox) and binned to 10,000 bins. After normalization to total ion count (TIC), each spectrum was baseline corrected using “msbackadj” MATLAB routine (Bioinformatics toolbox) with regression method “pchip”, smoothing method “lowess”, and window size 200 m/z-values. Abnormally intense pixels (a.k.a. hot spots) were removed by computing the 99-95% quantile and setting higher values to the quantile value (a.k.a. quantile filtering). Furthermore, each mz-image was denoised using the image convolution filter with Gaussian function of a span of 5 pixels.

Results and Discussion

Analysis of the *S. coelicolor* test system

Using Streptomyces coelicolor A3(2) as a test system, due to its prolific secretion of well characterized secondary metabolites[11,20], a single colony was removed from the incubator, dried in open air for 10-15 minutes in order to flatten the colony, and placed directly under the nanoDESI source for analysis. Due to the surface of colonies of S. coelicolor being very hydrophobic, a more non-polar solvent (50% methanol : 35% acetonitrile : 15% toluene)

was used in order to allow greater penetration into the colony. However, mass signals for known molecules were not observed due to the solvent not being compatible with the molecules solubility (data not shown). Therefore a more polar solvent was used (65% acetonitrile : 35% water with 0.05% formic acid), which resulted in very little surface penetration as seen by the very high contact angle exhibited by the droplet. Despite the low surface penetration, signal for expected molecules was very good due to the fact that they are secreted into the extracellular space. Compared to similar analysis by DESI and MALDI IMS experiments[8,17], the range of molecules observed by nanoDESI was greater than DESI and on par with MALDI based experiments. In addition, a wide range of lipids were observed by nanoDESI using the non-polar solvent, which were not observed by MALDI or DESI analysis. This also alludes to nanoDESI have comparable detection limits to that of MALDI, which are in the low nanomolar range for microbial analysis. However, these detection limits will vary greatly depending on the nature of the analyte and the complexity and composition of the extracellular matrix of each microbial colony.

IMS data from *S. coelicolor* A3(2) using the polar solvent showed expected results (**Figure 1C and S3** for additional IMS signals) with the red pigment prodiginine localized to the colony while calcium dependent antibiotic (CDA) and the purple pigment actinorhodin showing signal both in the colony and the surrounding agar (See **Figures S8-S10** for tandem mass spectra).[8,17,23] Signal for agar polysaccharides (See **Figure S11** for tandem mass spectrum) also exhibited expected results with high signal intensity in the surrounding agar and no signal from the colony.[11] Surprisingly, a range of lipids including phosphocholine (See **Figure S12** for tandem mass spectrum) showed high abundance around the periphery of the colony and seemed to be localized to non-pigmented areas of the colony. The observation of actinorhodin was unexpected as this molecule would likely be observed only in negative ion mode mass spectrometry due to its acidic nature; however, nanoDESI analysis directly from the Petri dish showed high prevalence of sodium and potassium adducts observed allowing detection of molecules in positive ion mode which would normally be observed in negative ion mode.

As accurate analysis of IMS data depends upon consistent measurements to be taken at each sampling location about the sample surface, the ion current for selected mass signals was plotted for three separate line scans within the sample (**Figure 1C**). The resulting plots showed that the ion current for the selected mass signals was consistent between the three line scans and was consistent with the plotted IMS images. Plotting of the parent mass spectrum at three sampling locations (extracted from the line scan data) again showed that the changes in ion current for each mass signal were due to changes in the intensity of the discrete mass and not due to an overall change in spectrum intensities (**Figure 1C**). Together this shows that nanoDESI IMS is capable of generating reproducible and accurate data from a biological sample.

Analysis of *S. oneidensis* MR-1

We next wanted to determine if nanoDESI IMS would be a useful discovery tool by analyzing a microbe previously unexplored by IMS. The shewanellae are an impressive family of marine and soil bacteria that are able to produce omega-3 fatty acids, flourish at low temperatures, and have the ability to efficiently reduce a wide range of metals, dehalogenate halogenated organic compounds, and break down cyclic nitramines.[24,25] This incredible respiratory diversity has made many *Shewanella* strains the center of both bioremediation and fuel cell research.[26-29] Specifically, the ability of many *Shewanella* species to utilize a wide range of final electron acceptors, including Fe, Mn, U, As, V, Cr, dimethyl sulfoxide (DMSO), carbon tetrachloride, and trimethylamine-N-oxide (TMAO), is of great interest in both industrial and academic research.[24,25] However, despite the great

promise of studying such an organism, little research has been conducted into the metabolites produced by *Shewanella* strains with only a handful of compounds reported.

To determine if nanoDESI IMS could provide information into the respiratory activity of a *Shewanella* strain, we grew a single colony of *Shewanella oneidensis* MR-1, a strain capable of reducing heavy metal oxides under aerobic and anaerobic conditions, on ISP-2 nutrient agar under aerobic conditions for 36 hours at 28 °C. Initial nanoDESI measurements of the colony using a polar solvent (65% acetonitrile : 35% water with 0.05% formic acid) produced no signals unique to the colony; however, using a non-polar solvent (50% methanol : 35% acetonitrile : 15% toluene) produced a massive number of signals unique to the colony (**Figure S4**). This is probably due to the large amount of extracellular polymeric substances produced by *S. oneidensis* MR-1, which is characteristic of this strain.

The resulting mass spectrometry images of a single colony of *S. oneidensis* MR-1 showed many signals localized to the colony, but surprisingly very few of them appeared to be secreted into the surrounding medium (**Figure 2A and S5**). Of the signals observed, compounds relating to respiration and nutrient allocation could be annotated. Putrebactin, a siderophore common among most *Shewanella* strains[30], could be seen localized evenly throughout the microbial colony while riboflavin, a compound involved in electron shuttling and necessary for reduction of heavy metal oxides[31], was also observed and exhibited localization mainly on the colony interior (See **Figures S13-S14** for tandem mass spectra).

Of particular interest were MS/MS data that matched to a family of porphyrin compounds that appeared to be secreted into the surrounding media (**Figure 2A and S15-S19** for confirmation by parent and tandem mass spectra). These compounds included iron bound heme (*m/z* 616) as well as heme biosynthetic intermediates coproporphyrin (*m/z* 655) and protoporphyrin IX (*m/z* 563).[32] While these molecules have never been reported from MR-1 in their free form, *Shewanella* has intact heme biosynthesis pathways (www.biocyc.org) and is known for having the formidable ability of dissimilatory reduction of extracellular iron oxides using multi-heme cytochromes.[24,33,34] In fact, some *Shewanella* genomes can encode upwards of 42 different cytochromes with some localized to the outer cellular membrane (*e.g.* the decaheme protein MtrC).[34-36]

Analysis of S. oneidensis – B. subtilis mixed biofilm

As previously stated, microbes rarely exist in nature alone and routinely have their metabolic output altered through interaction of neighboring microbes. As such, we prepared a mixed biofilm of *S. oneidensis* MR-1 and *Bacillus subtilis* 3610, a fairly ubiquitous bacterium whose secreted secondary metabolites have been shown to alter the metabolic output of neighboring organisms (**Figure 2A**).[5-7] The resulting imaging mass spectrometry data showed that signals for many of the ions observed in the MR-1 colony alone, including riboflavin, were observed within the mixed biofilm and were localized to only areas where the MR-1 colony was exposed and therefore did not appear to be absorbed by the 3610 colony. Putrebactin however was not observed in the mixed biofilm along with a large number of unknown molecules with masses below *m/z* 500 (**Figure S6**). Signal for iron-bound heme and coproporphyrin could be seen throughout the mixed biofilm as well as the surrounding media while signal for protoporphyrin IX was only seen on the outer edges of the colony of the mixed biofilm. Note that iron-bound heme and coproporphyrin were detected in the single colony of *B. subtilis* 3610, but at much lower intensities than MR-1. Changes in metabolic output within the *B. subtilis* colony could also be observed with signal for plipastatin, an anti-fungal cyclic lipopeptide, not being observed within the mixed biofilm while signal for surfactin, an anti-microbial cyclic lipopeptide, and subtilosin, a macrocyclic peptide, were observed in both the mixed biofilm and control *B. subtilis*

colonies (See **Figure S7** for all observed IMS signals and **Figures S20-S22** for tandem mass spectra).

As little metabolomic analysis of *S. oneidensis* MR-1 has been done beyond reporting of electron shuttling small molecules, we decided to employ our newly developed molecular networking data analysis tool[11] to help identify some of the unknown signals from the IMS data and to determine if *S. oneidensis* shared common metabolites with other bacteria (**Figure 2B**). The purpose behind determining whether MR-1 shares common mass signals with these bacteria is that these model microbial systems have been highly characterized by mass spectrometry and therefore determining the identity of an unknown ion present in MR-1 becomes much easier if it is shared with one of these model bacteria since it allows us to open up our database search space and increases the chance of finding a reference spectrum. Molecular networking is a method of organizing the massive amounts of MS/MS data generated in a tandem mass spectrometry study by determining the similarity in fragmentation patterns between every possible pair of MS/MS spectra within the experimental data sets and visually displaying these similarities as a molecular network within the program Cytoscape (www.cytoscape.org). Within these networks, each circular node essentially represents a single compound and the similarity between the fragmentation pattern of this compound and other compounds present within the data is shown as a connecting line with another node. The strength of similarity is visualized by the thickness of the connecting edges between nodes, with highly similar MS/MS spectra resulting in thicker connecting lines.

To determine if *S. oneidensis* MR-1 produced common compounds with other ubiquitous bacteria, data dependent tandem mass spectrometry data from individually grown colonies of *S. oneidensis* MR-1, *B. subtilis* 3610, *S. coelicolor* A3(2) and *Pseudomonas aeruginosa* PAO1 were all networked together and nodes were colored based on which bacteria the compounds originated from. Compounds unique to MR-1 (orange nodes), compounds not present in MR-1 (white nodes) and compounds present in MR-1 and other bacteria (green nodes) could all be visualized simultaneously with clusters of green/orange nodes being targeted for further investigation. Upon inspection of the raw MS/MS spectra that composed these nodes, many families of lipids were identified to be present within MR-1, including phosphocholines (PC), phosphatidylethanolamines (PE), ceramides, phosphatidylglycerols (PG), a wide range of diacylglycerols (DAG) and fatty acids (FA) (See **Figures S23-S31** for confirmation by molecular networking and tandem mass spectrometry). Upon inspection of compounds unique to MR1 (clusters of orange nodes), possible heme analogs could also be observed clustered around heme and its biosynthetic intermediates while another cluster hinted at a possible lipid family of molecules around *m/z* 1300-1400, whose parent masses are consistent with known cardiolipin compounds reported in bacteria (See **Figure S32-S33** for parent mass spectra). Lastly, as an internal control of sorts, mass signals for the agar polysaccharide present in the growth media from all four samples could be seen clustered together within a single region of the network. In the nanoDESI IMS data, many of these signals common between *B. subtilis* 3610 and *S. oneidensis* MR1, such as PE and some fatty acids, could be observed within the images of both control colonies as well as the mixed biofilm (**Figure 2A**). Ultimately, the use of nanoDESI IMS on a living colony of *S. oneidensis* MR-1 allowed for the detection of a wide range of lipids, peptides, and small molecules all within a single data set from a single bacterial colony, giving new insight into metabolic capacity and chemical characteristics of this increasingly important microbe.

Conclusions and Future Directions

The enormously complex surface chemistry of living, breathing biological systems necessitates that accurate chemical analysis be performed in the most direct approach

available in order to preserve the native chemistry inherent within the system. Results from complicated sample preparation protocols and indirect chemical measurements can be too far removed from the original system to be correctly interpreted and are usually too narrow in chemical scope to fully appreciate the synergistic nature of interacting compounds from different molecular classes. It was with this motivation that we set out to develop, and herein report, a novel *in vivo* imaging technique that allows for the direct analysis of living biological specimens using the power of mass spectrometry. Utilizing a recently developed surface sampling technique known as nanoDESI mass spectrometry, we have shown it is possible to perform metabolic profiling directly from the Petri dish in order to better understand the molecular interactions within a single microbial colony and between neighboring colonies, which is key to developing a fundamental understanding of how these amazing little organisms can perform specialized tasks that we currently have no means of solving.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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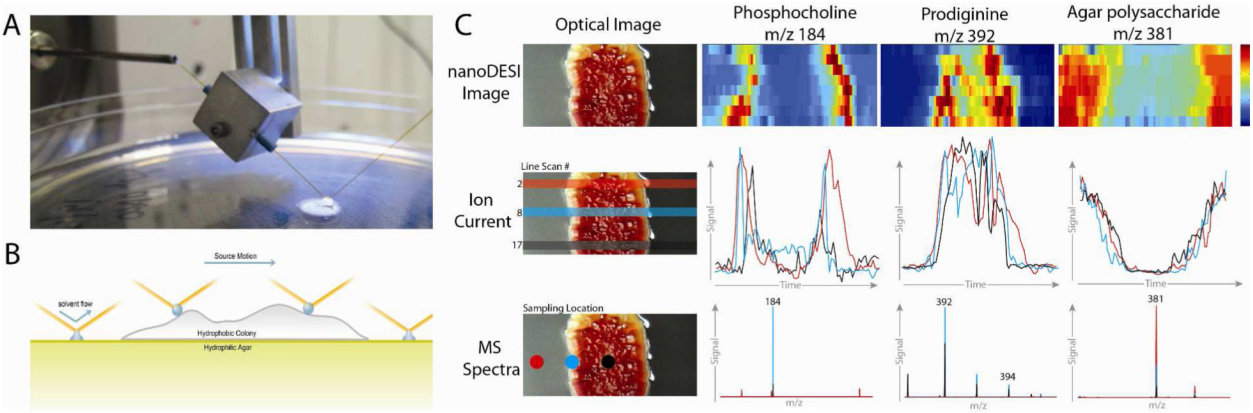

Figure 1.

General overview of nanoDESI imaging mass spectrometry (IMS). A) An optical image of a nanoDESI ionization source whereby the solvent of choice is delivered via the primary capillary to the liquid junction forming a droplet between the primary and a second capillary. This droplet, when placed on a chemical surface, will desorb analyte which is then carried into the mass spectrometry by the self-aspirating secondary capillary. B) Cartoon showing necessity of z-axis movement when scanning across a microbial colony. If the z-position is kept constant during scanning, the droplet will likely either lose contact with the surface or the capillary will clog due to collision with the bacterial colony. C) NanoDESI IMS data from *S. coelicolor* A3(2). The upper row shows an optical image of the bacterial colony that was imaged and the corresponding nanoDESI IMS images for phosphocholine, the red pigment prodiginine and a polysaccharide from the agar media. The middle row shows the location of the three line scans that were used to plot the ion current for the three selected masses. Lastly, the lower row shows an overlay of the MS spectrum at the three sampling locations shown. White scale bar indicates 5 mm.

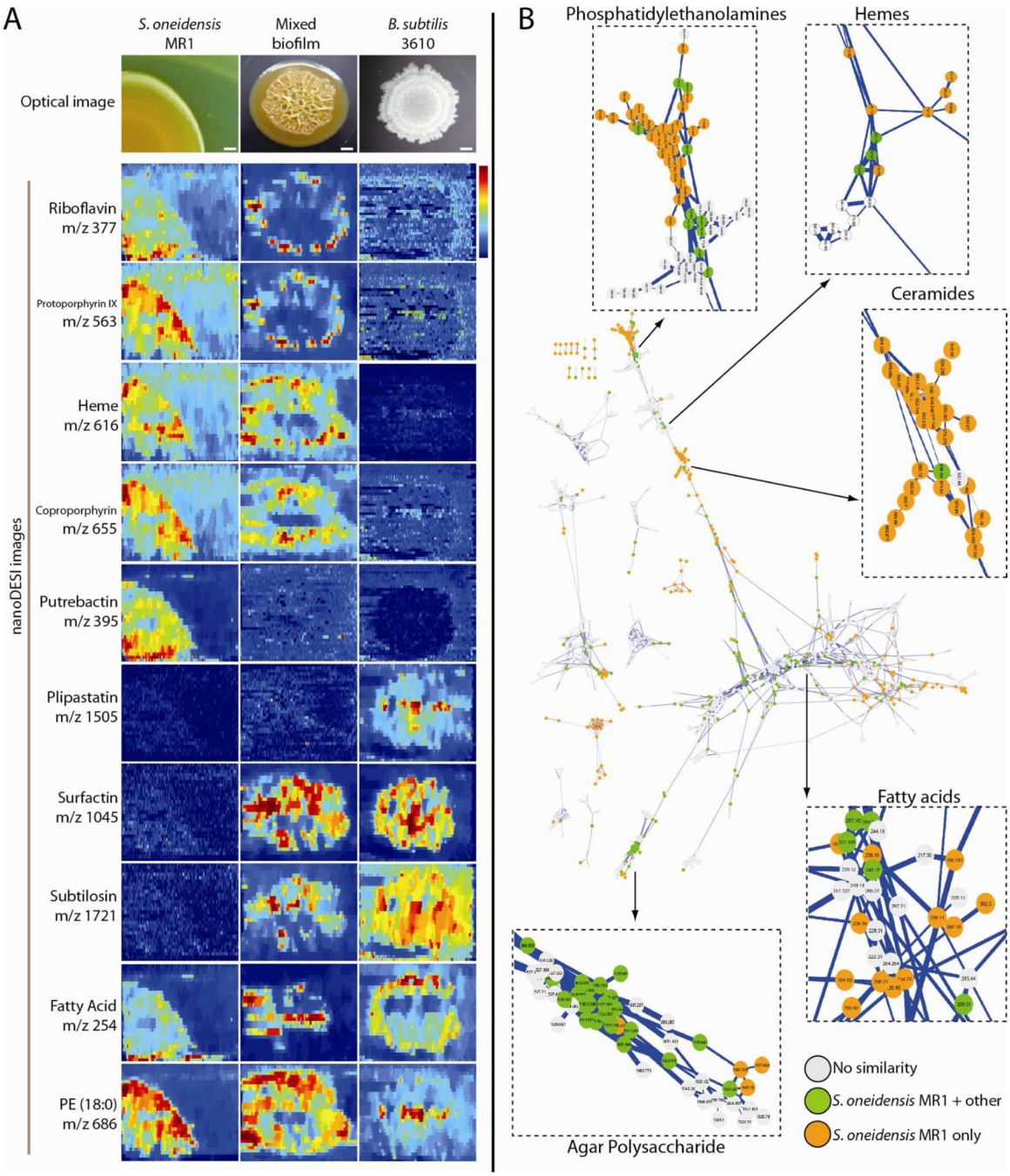

Figure 2.

Results of nanoDESI IMS and molecular networking on *S. oneidensis* MR-1 and *B. subtilis* 3610. A) Result of nanoDESI IMS analysis of a single colony of *S. oneidensis* MR-1 (left column), *B. subtilis* 3610 (right column) and a mixed biofilm of the two (middle column). White scale bars indicate 1 mm. B) Molecular networking results where data dependent tandem mass spectrometry data from *S. oneidensis* MR-1 was compared against MS/MS data sets from *B. subtilis* 3610, *S. coelicolor* A3(2), and *P. aeruginosa* PAO1. White nodes represent compounds not produced by MR-1, orange nodes represent compounds produced only by MR-1 and green nodes represent compounds produced by MR-1 and at least one

other bacterium. The entire network is shown in the middle with zoomed portions shown on the perimeter of the network.