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Publication Date

2022

DOI

10.1016/bs.mie.2021.11.014

Peer reviewed

HHS Public Access

Author manuscript

Methods Enzymol. Author manuscript; available in PMC 2023 January 01.

Published in final edited form as:

Methods Enzymol. 2022 ; 665: 281–304. doi:10.1016/bs.mie.2021.11.014.

Utilizing Imaging Mass Spectrometry to Analyze Microbial Biofilm Chemical Responses to Exogenous Compounds

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Abstract

Matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS) is an appealing label-free method for imaging biological samples which focuses on the spatial distribution of chemical signals. This approach has been used to study the chemical ecology of microbes and can be applied to study the chemical responses of microbes to treatment with exogenous compounds. Specific conjugated cholic acids such as taurocholic acid (TCA), have been shown in inhibit biofilm formation in the enteric pathogen *Vibrio cholerae* and MALDI-IMS can be used to directly observe the chemical responses of *V. cholerae* biofilm colonies to treatment with TCA. A major challenge of MALDI-IMS is optimizing the sample preparation and drying for a particular growth condition and microbial strain. Here we demonstrate how *V. cholerae* is cultured and prepared for MALDI-IMS analysis and highlight critical steps to ensure proper sample adherence to a MALDI target plate and maintain spatial distribution when applying this technique to any microbial strain. We additionally show how to use both manual interrogation and statistical analyses of MALDI-IMS data to establish the adequacy of the sample preparation protocol. This protocol can serve as a guideline for the development of sample preparation techniques and the acquisition of high quality MALDI-IMS data.

Keywords

Imaging mass spectrometry; matrix-assisted laser desorption/ionization; microbial biofilm

1. Introduction

1.1 Imaging Mass Spectrometry

Imaging mass spectrometry (IMS) is a rapidly growing field which includes a large variety of ionization options and sample preparation techniques to visualize the spatial distribution of chemical species present across a biological sample. Various modes of ionization such as matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI), and secondary ion mass spectrometry (SIMS) facilitate the detection of different

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analytes and support a variety of sample types in IMS experiments. These ionization methods can be used orthogonally within the same biological systems to study different chemical classes and spatial distributions (Spraker, Luu, and Sanchez 2019). MALDI is commonly used for IMS as it is fast, offers a high spatial resolution, and has been applied to many biological systems to detect a wide variety of compound classes, including small signaling molecules (Spraker et al. 2016, 2018; Eckelmann, Spiteller, and Kusari 2018; Shariatgorji et al. 2019), lipids (Angel et al. 2012; Puolitaival et al. 2008), peptides (Si et al. 2016; Kinnel et al. 2017), and larger proteins (Dilillo et al. 2017; Spraggins et al. 2016). MALDI involves the use of an organic matrix which is necessary for both the desorption of the analytes from the surface of the sample and the ionization process. The availability of various MALDI matrices which enhance the ionization of specific types of compounds, and the diversity of sample preparation methods contribute to the versatility of MALDI-IMS applications (Buchberger et al. 2018).

The spatial distribution of molecules across a biological sample provides vital information to generate hypotheses surrounding the biological functions of molecules and chemical cross talk between different cell types or microbial species. As a label-free technique, IMS can accurately detect chemical species across a sample, which can also be co-registered with other imaging modalities including autofluorescence microscopy (Patterson et al. 2018). MALDI-based mass spectrometers are becoming more user friendly, which has led to a rapid growth in unique application of IMS through the integration with other imaging tools (Spraker, Luu, and Sanchez 2019; Neumann et al. 2020).

1.2 Microbial Biofilms

Biofilms are made of extracellular polymeric substances (EPS) which are secreted biological matrices produced by microorganisms to protect the microbial community from adverse external environments (Conner et al. 2016). Biofilms play important roles in environmental pollution removal through metabolic processes, but also cause serious problems such as biofouling and antibiotic resistant infections (Flemming et al. 2016). Whether utilizing biofilms for beneficial processes or attempting to detach and disperse biofilms to treat infections, control over biofilm formation and growth is essential (Zhang et al. 2019). Various cellular processes are involved in the formation, maintenance, and dispersal of biofilm matrices to maintain the vitality of the microbial community and facilitate cell to cell communication between microorganisms. Cellular attachment, exopolymeric substances (EPS) secretion, and transfer and metabolism of molecular substrates in microbial biofilms have been studied using confocal laser scanning microscopy, atomic force microscopy, Raman spectroscopy, and infrared spectromicroscopy in addition to IMS (Zhang et al. 2019; Stenclova et al. 2019). Microprocesses are important for understanding biofilm formation and dispersal, and chemistry is an essential driver in many of these microprocesses. However, IMS is the only imaging technique which allows for the identification of specific chemical species present in a sample in a label-free, untargeted manner (Spraker, Luu, and Sanchez 2019; Grim, Luu, and Sanchez 2019; Cleary et al. 2017; Galey and Sanchez 2018).

We have previously utilized IMS to study the specialized metabolism response of the Gram-negative, biofilm forming *Pseudomonas aeruginosa*, to treatment with a biofilm

inhibitor, taurolithocholic acid (TLCA) (Condren et al. 2020). In this study, *P. aeruginosa* increased production of the siderophore pyochelin and motility factors, the rhamnolipids upon exposure to TLCA. These compounds were known to be associated with increased virulence, and when TLCA was used in a wax moth virulence assay, virulence was statically increased. Based on this evidence, we have detailed a protocol for applying this similar IMS based approach to another Gram-negative biofilm forming bacterium, *Vibrio cholerae*, which is responsive to taurocholic acid (TCA) as a biofilm inhibitor (Sanchez et al. 2016). This chemical microbiology approach allows one to rapidly analyze the chemistry of the colony on agar to identify statistically significant signals whose abundance is altered upon treatment with an exogenous small molecule inhibitor or therapeutic (Phelan, Fang, and Dorrestein 2015).

2. Sample Preparation

Determining the optimal sample preparation technique to use in a MALDI-IMS experiment is crucial to success, but highly dependent on the biological sample and agar medium used. Minor changes in sample preparation or drying conditions can lead to drastically different results in terms of measured chemistry and spatial distribution (R. J. A. Goodwin 2012). Furthermore, poor sample preparation can lead to flaking of the sample into the MALDI source, which may cause damage to the instrument and often requires specialized cleaning protocols to remove. Careful development of the MALDI matrix application and drying technique used is highly recommended any time a new type of biological sample is being prepared for IMS analysis. In the following section we will demonstrate our logic for developing a workflow for optimizing sample preparation of *V. cholerae* biofilm colonies grown on lysogeny broth (LB) agar plates, with and without the addition of an exogenous compound taurocholic acid (TCA), for MALDI-IMS (Figure 1). Because the sample preparation process is highly unique and customizable for different samples, we recommend further reading of previous work that has been published on preparing bacterial colonies grown on agar. (Yang et al. 2012; Hoffmann and Dorrestein 2015) The sample preparation process can be broken down into two main sections. Details to consider for growing biofilm colonies are provided (Figure 1A), followed by a step-by-step process for drying and preparing the samples for MALDI-IMS analysis (Figure 1B–E).Ther goal for these sections is to offer other researchers a roadmap for their own biological sample optimization needs.

2.1 Equipment and Supplies

1. Optima grade methanol
2. Milli-Q water
3. Microbiology culturing supplies
4. Lysogeny Broth (LB) growth medium (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar for solid media only)
5. Thin LB agar plates (10 mL for 100 mm diameter, 3 mL for 60 mm diameter)
6. Taurocholic acid (≥98%, Sigma-Aldrich)

7. Sterilization filters (0.22 μm)
8. Microbiological incubator for plates
9. Shaker incubator for liquid cultures
10. Lab oven
11. Unpolished stainless steel MALDI target plates
12. Razor blades
13. Metal spatula
14. Kimwipes
15. Mortar and pestle
16. α-Cyano-4-hydroxycinnamic acid (CHCA, >98% purity Sigma) was recrystallized by dissolving CHCA in warm ethanol, filtering, adding two volumes of deionized water, and letting it stand under refrigeration overnight. CHCA was filtered as a yellow solid.
17. 2,5-Dihydroxybenzoic acid (DHB, >98% purity Sigma) was recrystallized by dissolving DHB in hot deionized water and filtering through a fritted glass filter. Collect thin buff-colored crystals by filtration.
18. Three-inch diameter, 53 μm stainless steel sieve, pan and lid (Hogentogler Inc)
19. Camera for taking photos of samples
20. Air flow / tubing (for removing excess matrix)
21. Mass calibrant (any calibrant may be used)
22. Vacuum desiccator
23. MALDI mass spectrometer (Bruker timsTOF fleX mass spectrometer)

Optional: Lights to brighten sample for photos, HTX TM Sprayer

2.2 Growing biofilm colonies

Maintaining the biofilm morphology of *V. cholerae* grown on agar plates requires consistent growth conditions. While the specific conditions are arbitrary, it is vital to consistently use the same growth conditions because factors such as salt content, pH, humidity, and temperature can greatly affect biofilm morphology. (Conner et al. 2016) For example, *V. cholerae* biofilms have different morphologies when grown at 30°C or 37°C, (Conner et al. 2016) therefore it is important to treat such samples as different biological conditions for comparison, as opposed to biological replicates. Below is a protocol for growing *V. cholerae* biofilms specifically for MALDI-IMS analysis; most of the growth conditions described may be modified, except for the use of ‘thin’ agar plates. The *V. cholerae* strain (O1 El Tor A1552, rugose variant, Rif^r) in these studies overproduces the biofilm matrix (Yildiz and Schoolnik 1999).

1. Streak *V. cholerae* from frozen stock onto an LB agar plate, and store in an incubator at 30°C.

2. After 20–30 hours, inoculate 5 mL of liquid LB media with a single colony of *V. cholerae*. Grow this culture overnight (12–14 hours) in a shaker incubator at 30°C.

3. Dilute liquid *V. cholerae* cultures to an OD_{600} of 0.1 in fresh LB media prior to inoculating agar plates for imaging. This will maintain consistency in growth between samples prepared on different days and from different liquid cultures.

4. Carefully spot 5 μL of the *V. cholerae* culture onto a thin LB agar plate to avoid bubbles depositing on the surface which ultimately pop and spread the microbe across the plate rather than allowing a single colony to form. The plate can be either 100 mm or 60 mm in diameter with 10 mL or 3 mL of media, respectively. It is important to pour these plates with consistent volumes using a serological pipet. The thickness of the agar greatly impacts the drying time of the sample, and thin agar samples dry more consistently flat. Additionally, larger solid media volumes dry to an overall thicker height and are not compatible with the height requirements dictated for use with commercial mass spectrometers (Yang et al. 2012). After spotting the plate with liquid culture, let the plate stand for 20–30 minutes before handling or inverting. This allows the spot to dry and for the bacteria to adhere to the plate. Plates handled or inverted right away lead to smaller colonies on the periphery of the plate which ultimately may lead to colony-to-colony crosstalk that can interfere with the interpretation of the resulting data following IMS as the method captures diffusible chemical signals.

   Note: When testing the effects of an exogenous compound such as TCA, agar plates containing a specific concentration of TCA must be inoculated alongside negative control plates containing the same volume of the solvent vehicle used. Exogenous treatments with small molecules can be added via sterile filtration to an aliquot of autoclaved liquid agar media in a conical centrifuge tube. First, dissolve TCA in Optima grade methanol to a concentration of 0.5 M and sterile filter the solution into a sterile vial or a microcentrifuge tube for storage. After autoclaving the LB medium with agar, the medium is cooled to 55°C before adding the appropriate amount of TCA to achieve the desired final concentration (1 mM was used in these experiments). This is vital to ensure that TCA does not degrade due to the heat generated during sterilization. Then, add the same volume of sterile filtered methanol to the negative control LB media. Be sure to mix the medium in the conical centrifuge tube gently to ensure even distribution of the compound when the plates are poured. Allow plates to dry for at least 24 hours prior to inoculation. Long term storage of agar plates at room temperature or in refrigeration is not recommended because differences in humidity can influence motility and biofilm formation.

5. Store the plates in an incubator at 30°C until analysis. Typically, samples are analyzed after 72–96 hours of growth to ensure adequate production of the biofilm matrix and availability of chemistry for ionization. As with any

experiment, MALDI-IMS analysis should be performed at different time points to optimize the experiment for the specific research goals.

2.3 Preparing biofilm colonies for MALDI-IMS analysis

There are four main requirements that IMS sample preparation must meet. First, the sample must be completely dry before entering the MALDI source which is under vacuum. Second, the sample must be completely flat, or at least as flat as possible. Height differences in the prepared sample result in decreased mass accuracy across the sample, artifacts in ion intensity, and 'dead spots' or empty spectra in locations where the sample height is not uniform. Third, the sample must be fully adhered to the MALDI target plate. Sample 'flaking' off of the MALDI target plate can be caused by bubbles under the agar sample, over-drying the sample, or inadequate MALDI matrix deposition and can result in the sample flaking or breaking off into the MALDI source. Finally, the MALDI matrix must be evenly distributed across the sample and co-crystallize with metabolites present in the sample. MALDI-IMS sample preparation is a highly curated process which is unique to different biological samples and growth media. Because of this, there is no shortage of original sample preparation techniques described throughout the literature. (Yang et al. 2012; Hoffmann and Dorrestein 2015; R. J. Goodwin et al. 2010; Gemperline, Rawson, and Li 2014; Vergeiner et al. 2014) The following step-by-step protocol was developed specifically for working with *V. cholerae* grown on thin LB agar plates (Figure 1). With careful observations and testing, this protocol can be adapted to any microbial organism grown on thin agar media.

1. Carefully excise the colony and approximately 2–3 cm of the surrounding agar from the plate using a razor blade (Figure 1B). Excising clean edges of the agar is important here to maintain consistent adherence of the edges of the agar to the MALDI target plate and prevent sample flaking under vacuum. The blade may be sterilized with 70% ethanol between uses or disposed of in the appropriate biological hazardous waste sharps container.

2. Slowly lift the excised colony and place on the MALDI target plate using a clean spatula. This step requires careful and slow placement of the colony to ensure two major factors are met once the sample is placed. First is that the sample maintains essentially the same spatial distribution and is not deformed, crushed, or cut excessively. Second is that no air bubbles are trapped between the agar and the MALDI target plate (Figure 1B). Air bubbles trapped under the agar prevent the sample from fully adhering to the plate and can often lead to flaking once the sample has dried (Figure 1E). Place a piece of agar with no bacterial colony in the same manner as a negative control for background signals present in the medium. Take a photo of the target plate with agar and colonies (Figure 1B).

3. Apply CHCA:DBH matrix to the MALDI target plate using a 53 μm stainless steel sieve (Figure 1C). Prepare approximately 0.3 g of dry CHCA:DBH matrix and crush the matrix using a mortar and pestle for each MALDI target plate to be analyzed. Ensure even matrix coating across the sample by checking the sample frequently during sieving.

4. Dry the samples onto the target plate in an oven at 37°C (Figure 1D). This process may take 2–6 hours depending on the sample, ambient moisture, and how quickly the matrix is absorbed. Check the samples regularly, at approximately one hour intervals, to ensure even matrix absorbance by the sample. Matrix can be reapplied using the sieve method throughout the drying process as needed. This is a particular concern for highly mucoid bacterial colonies which readily absorb the matrix (Spraker et al. 2016). The sample is typically flat and fully adhered to the plate when it is fully dry. It is important here to not leave the sample in the oven for extended amounts of time, such as overnight, as over drying the sample frequently leads to flaking.

5. Remove excess matrix from the sample and the back of the MALDI target plate using a gentle stream of air. Inspect the sample carefully for bubbles and flaking edges.

6. Spot a calibrant onto the MALDI target plate in a position that is not covered by agar or sample. Any calibrant may be used, but these are typically selected based on the mass range of interest.

7. If you are concerned about potential flaking of the sample, it can be left under vacuum for an hour or longer in a simple vacuum dessicator to ensure that the sample can be exposed to low pressure without flaking prior to introducing the sample into the MALDI source.

8. Take a final photograph of the prepared sample prior to MALDI-IMS analysis (Figure 1E). This photo is necessary for serving as a guide to select the regions of interest on the MALDI target plate to be analyzed. Teach points are used in FlexImaging and other commercial software used for IMS data acquisition to identify the regions of interest on the target plate (Figure 2). It is important that the photo is centered, straight, and not blurry as shown in Figure 2. This will ensure the best match between the sample photo and MALDI-IMS analysis and allow for accurate spatial recognition of signals produced by the biofilm colonies and co-registration of the optical image with the resulting ion images.

   Note: When defining sample regions for analysis select a region that is well within the edges of the agar surrounding each colony (Figure 3). This helps prevent flaking in the event that the agar edges are not fully adhered, and helps keep the ion intensity at the edges of the sample region more consistent.

3. MALDI-IMS Data Acquisition

There are relatively few parameters to optimize in MALDI-MS compared to other ionization modes such as ESI. For IMS applications, settings such as laser power, shots, and frequency can be optimized similarly to standard MALDI-MS experiments. Laser power is arbitrary and cannot be compared between instruments but gives a general idea of how much power is being used. Keeping the laser power low is ideal for maintaining the longevity of the laser. If higher than 50% laser power is regularly required for ionization of certain samples, other parameters such as the laser pitch and size should be adjusted. Sample preparation

challenges such as sample height and inadequate matrix deposition can also contribute to necessitating higher laser power. We used a laser power of 30% at 1000 Hz frequency with 200 shots per raster point.

A region of interest (ROI) is selected as the sample area to be analyzed by laser ablation across a user indicated raster width (Figure 3). In these experiments, a 500 μm raster width was employed. The laser size should be adjusted to approximately match the raster width used to prevent over or under-sampling. The laser pitch and the laser size are important in IMS applications to prevent over or under sampling. Ideally, the laser size will match the raster width to optimize both sensitivity and spatial resolution.

4. Data Analysis

The distribution of a given ion can be visualized as a heat map of ion intensity across the sample area as an ion image (Figure 4). Ion images can be generated in most commercial IMS software such as SCiLS Lab, but can also be generated using open-source software such as MSi reader and Cardinal. (Bokhart et al. 2018; Robichaud et al. 2013; Bemis et al. 2015; Luu et al. 2020) Manual selection of ion images that have biologically relevant spatial distributions is commonly used to identify signals for further investigation and statistical analysis. Generally, these ion images serve to determine the success of an experiment in terms of adequate sample preparation, and can help identify sample preparation artifacts such as ‘hot’ raster points or pixels.

One main goal of IMS is to identify signals that have interesting spatial distributions, meaning they originate from the biological system being studied and are not present in the negative controls. Signals may also localize to one portion of the biological sample. When viewing ion images from IMS data, it is important to ensure that the data is normalized prior to inferring spatial distributions of ions. Uneven deposition of MALDI matrix across the sample area can result in an artifact of higher ion intensity where a thicker layer of matrix resides. This can be seen in Figure 4, where the top right side of the agar control region has a higher intensity for both *m/z* 335.1030 and *m/z* 568.1355 prior to root mean square (RMS) normalization to the maximum peak intensity in the total ion chromatogram (TIC). These ions also appear to have higher intensity around the outside of the bacterial colonies prior to normalization, implying that they may originate from the agar medium. Both *m/z* 335.1030 and *m/z* 568.1355 are ions associated with the CHCA matrix, and should not have such pronounced spatial distributions across the sample area regardless of the spatial distribution of the biological sample. These artifacts commonly arise from applying MALDI matrix by sieving and may be misinterpreted as biologically relevant due to apparent spatial distribution, however, applying RMS normalization resolves this by generating more evenly distributed ion images for ions originating from the matrix (Figure 4.1). Ion images of matrix ions can be observed initially to assess the presence of hot spots and intensity artifacts in the data, as well as to ensure that the normalization function used is appropriate.

Following normalization, ion images can be manually selected on the basis of biologically relevant spatial distribution (Figure 5). It is important to have both a negative agar control and some region of agar surrounding the bacterial colonies to assist in differentiating

agar medium related signals from those that are biologically produced. Figure 5 shows a comparison of a signal derived from the agar (*m/z* 688.0137) which is present in the negative agar control, a signal that is secreted by *V. cholerae* (*m/z* 461.2712), which is not present in the negative agar control. We also identified signals that are localized within the bacterial colony (Figure 5). One ion, *m/z* 399.1449, is localized across the entire bacterial colony, while *m/z* 634.3791 resides only along the outer edge of the colony where more rugosity can be seen in the optical image of the biofilm (Figure 5). Localization of *m/z* signals not only shows differences between the bacterial colony and agar medium, but also shows localization specifically with the edge of the biofilm colony, indicating metabolic differences associated with the spatial organization of growing biofilms. We identified one ion (*m/z* 982.3090) that was present when *V. cholerae* was grown in the presence of TCA, but was not present in the control samples containing only the methanol vehicle (Figure 5). Identifying consistent spatial distribution of ions across biological replicates can assist in determining the reliability of signals and assessing the success of sample preparation.

While the spatial distribution of ions is one of the greatest assets of using IMS analyses, the high dimensionality of these data results in large file sizes and requires special software to facilitate data analysis. Proprietary software is commonly used to visualize and analyze IMS data, however open-source platforms are being actively developed to provide rigorous data analysis options using generic data formats such as '.imzML'. (Schramm et al. 2012) These tools have been reviewed elsewhere, and can be used to apply complementary analyses to large datasets. (Buchberger et al. 2018; Luu et al. 2020; Spraker, Luu, and Sanchez 2019) Here we demonstrate unsupervised segmentation processing and expected results using SCiLS Lab, but similar statistical processes can be applied in open-source platforms such as Cardinal and MSiReader. (Bemis et al. 2015; Bokhart et al. 2018) While supervised statistical methods are commonly used when working with discrete classes of data for categorization of new data into biologically relevant categories, unsupervised methods can be used as an untargeted approach to identify chemical signals contributing to variation in the dataset. (Buchberger et al. 2018) An unsupervised segmentation analysis groups similar spectra together based on intensity and spatial distribution to identify co-localized *m/z* values that contribute to the spectral uniqueness of specific spatial regions. Biofilm colonies in particular have spatial variation due to metabolic variations across the center and edges of the biofilm, and these differences can be visualized using unsupervised segmentation.

The segments in Figure 6 and Figure 7 were generated with SCiLS lab using a list of *m/z* peaks above an intensity threshold of 500, bisecting k-means correlation, and weak denoising. This analysis initially segments the image into two regions which roughly align with the bacterial colony and agar control (Figure 6A). Each segment or region generated can be further segmented based on spectral similarity for further biological interpretation (figure 6B,C). These data show that the biofilm colony (red region, Figure 6A) has distinct spectral differences from the agar control, which groups as a segment with the agar surrounding the edges of the colony (green region, Figure 6A). Initially, the center of the bacterial colonies more consistently grouped with the agar region (green region, Figure 6A). However, after further segmentation, the center of the bacterial colonies group as a distinct region from both the agar control and edges of the biofilm colony (blue regions, Figure 6B,C). A similar trend was also observed in the *V. cholerae* samples that were treated with 1

mM TCA (Figure 7). These segments can be further investigated in the software to identify the specific *m/z* signals that contribute to the grouping of each spatial region.

Further statistical analysis would be required to determine whether a signal upregulated in a specific region, or between biological conditions. Selected ions can then be subjected to univariate statistical analysis to answer specific biological questions, while multivariate analysis can be applied to spatial groupings such as segmentation, partial least squares (PLS), and principal component analysis (PCA) to assess the reliability of the spatial classification. Initial observation of statistical groupings using segmentation can assist in the sample preparation optimization process and method development in general, followed by more advanced analyses for subsequent datasets. Poor sample preparation and intensity artifacts due to uneven sample height or matrix deposition can lead to artifacts in segmentation that are unrelated to biologically relevant spatial regions (Zink et al. 2019). An initial critical assessment of the ion images and segmentation of MALDI-IMS datasets was essential to sample preparation optimization and method development.

Subsequent compound identification for testing biological hypotheses based on the IMS data is necessary, however this is a common challenge in all mass spectrometry metabolomics, and MADLI-IMS is no exception. Databases such as METLIN (Smith et al. 2005; Guijas et al. 2018), and the NP atlas (van Santen et al. 2019) can be used to search for *m/z* values and dereplicate known unknowns and prioritize novel compounds. Although there are not MALDI-specific mass spectral databases available and most databases focus heavily on LC-MS datasets, many databases exist for specific compound classes and biological sources that can assist in dereplication. The Global Natural Products Social molecular networking (GNPS) platform has also been used with MALDI-IMS coupled to MS/MS fragmentation to dereplicate and identify relevant chemistry (Wang et al. 2016; Ibáñez et al. 2019).

5. Alternative sample preparation techniques

5.1 MALDI Matrix Application using Sprayer

The main challenge with sieving MALDI matrix is a lack of control over the matrix deposition resulting in ‘hot’ raster points or pixels. Spraying is a popular matrix deposition technique used to circumvent uneven application and has been shown to result in a more uniform deposition of matrix as well as more reproducible and consistent signals (Anderton et al. 2016). Because sieved samples sometimes suffer from uneven matrix deposition due to the multiple rounds of sieving necessary to co-crystallize the MALDI and biofilm matrices, we opted to try a spray method with these samples as well. Dissolved MALDI matrix is typically sprayed onto a pre-dried sample, as opposed to sieving which is applied to a wet sample. To apply a spray method to the sample, an agar sample was placed on the MALDI target plate as described in section 2.3 and then dried in a lab oven at 37°C for one hour. Figure 8 depicts *V. cholerae* colonies and agar control samples when wet (Figure 8A), after drying for one hour (Figure 8B), and after applying 1:1 CHCA:DHB matrix using a HTX TM sprayer (Figure 8C). These samples dried noticeably faster than when the matrix was applied prior to drying, and had shrunk in size (Figure 8A–C). The ion images in Figure 8D depict *m/z* 688.1037, which is the same agar-associated ion depicted in the properly prepared samples in Figure 5. Raster points where the optical image is displayed as opposed

to an ion intensity represents spectra where no data were acquired, also referred to as a 'dead spot'. With most of the negative agar control containing dead spots, these samples were not adequate for analysis, even though some signals can be detected on the colonies in Figure 8D.

Overall, spraying resulted in a consistent layer of crystallized matrix across the biofilm colony, but given the overall shrunken size and lack of ionization, these samples were not compatible with this matrix application method. Generally, sieving is accepted as a preferable method for applying MALDI matrix to bacterial colonies grown on agar. However, spraying has been used with bacterial colonies and shown to improve image resolution in some circumstances, (Hoffmann and Dorrestein 2015) so this method should be considered and tested as an alternative to sieving.

5.2 Changing Growth Medium Ingredient Concentrations

The concentration of any growth medium ingredient can be modified to optimize experimental conditions for IMS analysis, keeping in mind that changing the medium will also likely affect the resulting biology. We tested whether decreasing the concentration of NaCl in the LB agar media would improve the drying of the agar directly onto the MALDI target plate (Figure 9). We found that the LB agar containing a low amount of NaCl (0.25%) showed less overall shrinkage (Figure 9B), but the sample area was still resistant to crystallization with the matrix (Figure 9C). Overall, the spraying method was not compatible with the LB agar used in our experiments, however, altering media ingredients could be used to optimize sample preparation in a number of ways. Besides optimizing the actual drying process of the sample, changing the concentration of growth medium can be used to minimize background signals from complex media ingredients and reduce ion suppression from high salt content.

6. Conclusion

We have presented a rationale for adjusting both microbial growth conditions and mass spectrometry sample preparation to compare metabolite changes across the model Gram-negative *V. cholerae* in response to treatment with an exogenous small molecule, TCA. We emphasize the importance of careful sample preparation to ensure reproducible results and the critical use of statistical analysis to demonstrate biologically relevant spatial distribution of chemical signals. This chemical microbiology approach offers a unique method for identifying spatially relevant metabolite changes for further study. This will serve as a foundation for setting up and testing ideal conditions for identifying biologically relevant chemical signals in microbial biofilms and other complex biological systems treated with exogenous compounds.

Acknowledgments

This chapter was supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under Award Number R01GM125943 (LMS), the National Cancer Institute of the NIH under Award Number R01CA240423 (LMS), the National Institute of Allergy and Infectious Diseases of the NIH under Award Number R01AI102584 (FHY), and by the National Science Foundation grant2128044 (LMS).

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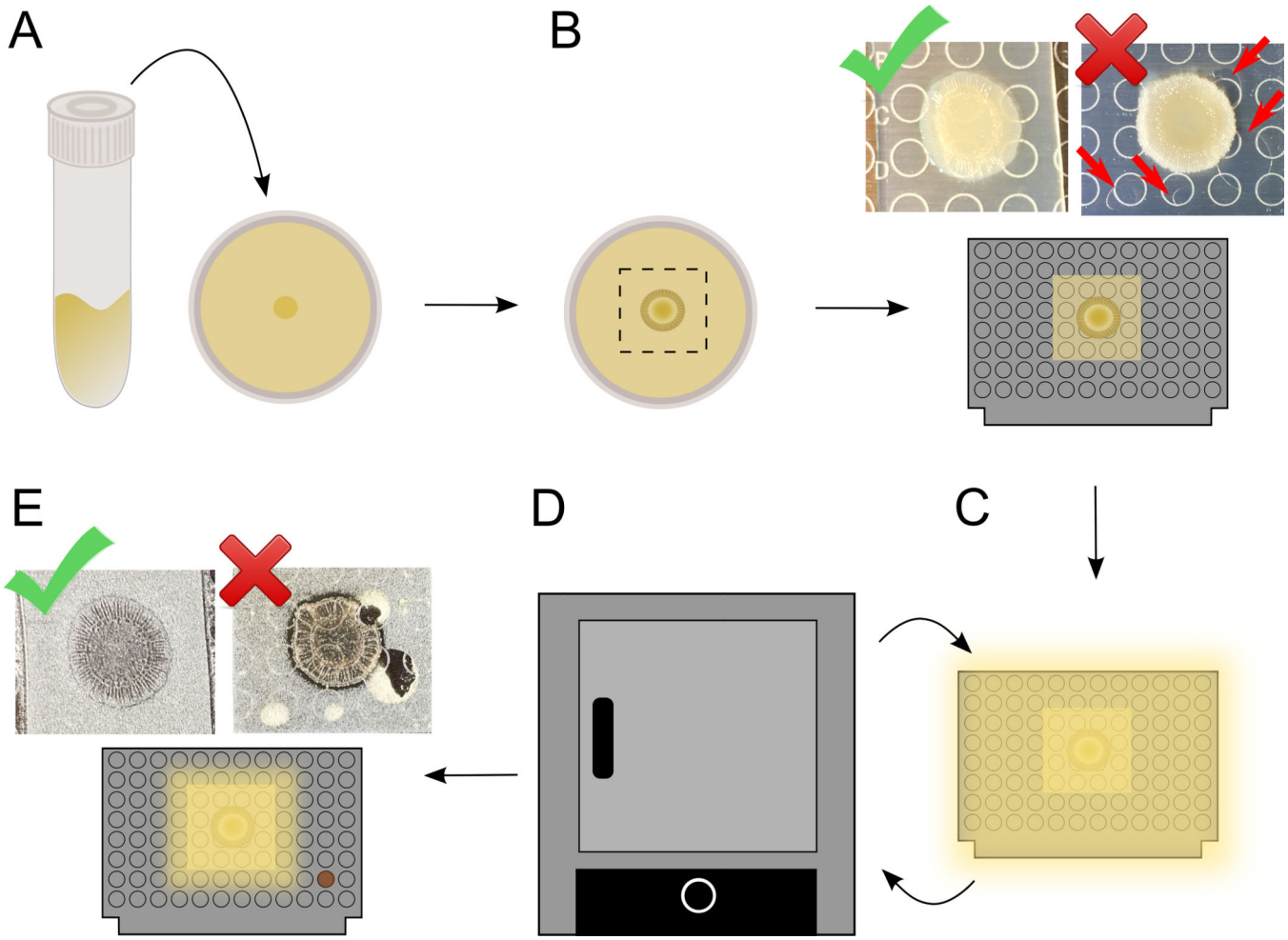

Figure 1:

Overview of protocol for MALDI-IMS sample preparation (A) Inoculate a thin LB agar plate with 5 μL of *V. cholerae* overnight culture as described in section 2.2. (B) After three or four days of growth, carefully excise bacterial colony biofilm and surrounding agar from the culture plate and place on a MALDI target plate. Photographs show examples of a well placed agar bacterial colony (left) and a poorly placed agar bacterial colony (right), with red arrows indicating bubbles under the agar surface. (C) Add 1:1 CHCA:DHB MALDI matrix to the sample using a 53 μm stainless steel sieve and (D) dry the sample in a lab oven set to 37 °C. Regularly check the sample throughout the drying process (2–6 hours) repeating the sieving and drying steps as necessary. (E) When the sample is fully dried remove excess MALDI matrix and spot a mass calibrant in a clean section of the plate. Take a photograph of the final sample prior to analysis. Photos indicate a fully dried sample that is flat and well-adhered to the MALDI target plate (left) and a sample that is not well-adhered due to air bubbles that dried under the agar resulting in flaking (right).

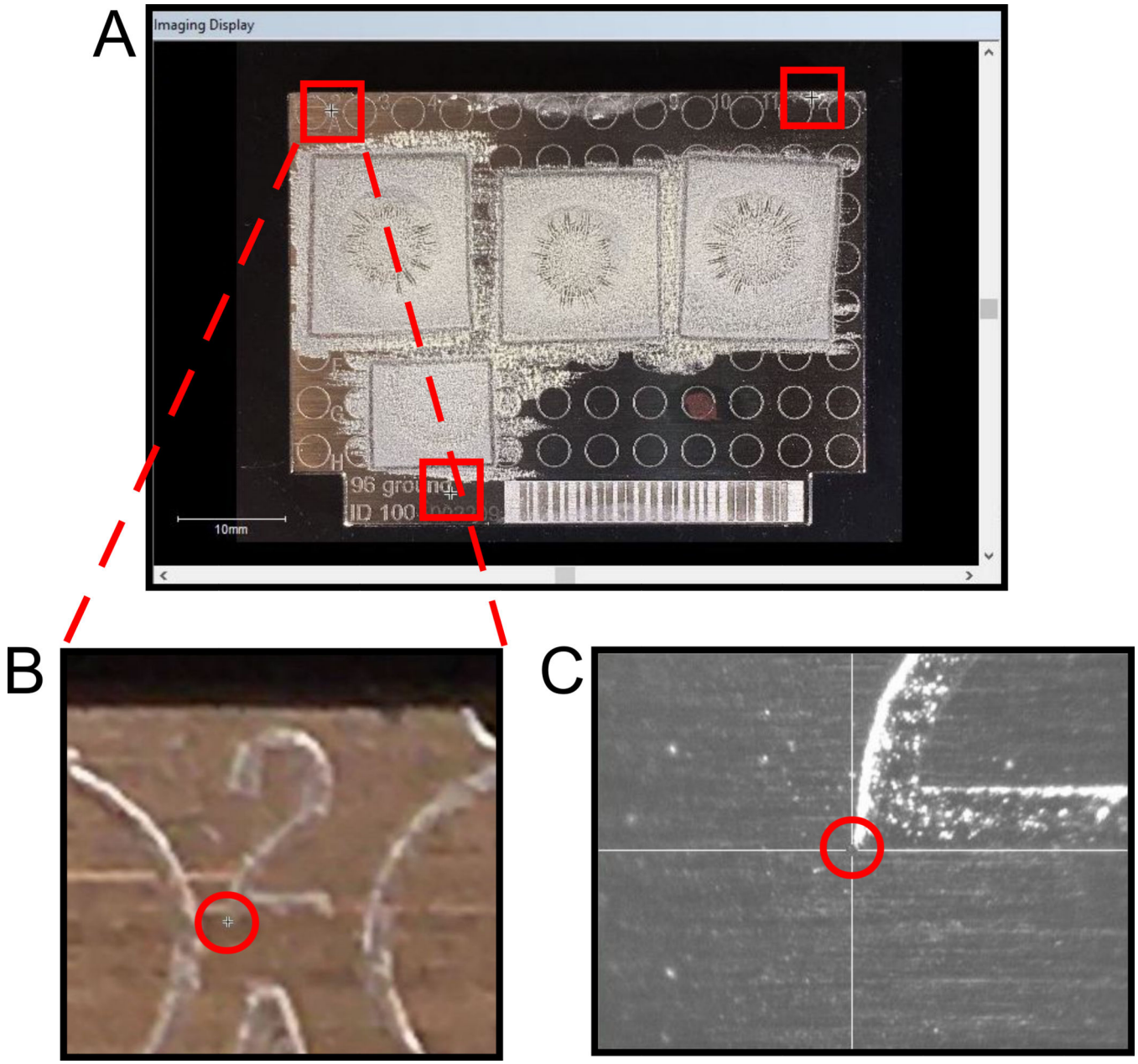

Figure 2:

Using teach points to align optical image and MALDI laser (A) Screenshot showing the optical image of a prepared sample uploaded in FlexImaging. The optical image needs to be clear and straight in order to place teach points. Red squares highlight the teach points placed in three positions on the image. These positions are selected near separate edges of the target plate to assist in alignment across the entire image. (B) Inset of the top left teach point from (A). Red circle indicates the position of the teach point at the bottom corner of the column label '2'. It is important to use sharp clear corners for alignment and to clear excess matrix from these areas. (C) Image showing the view of the target plate from the

MALDI source camera. The center crosshair on this view needs to match up with the teach point in (B).

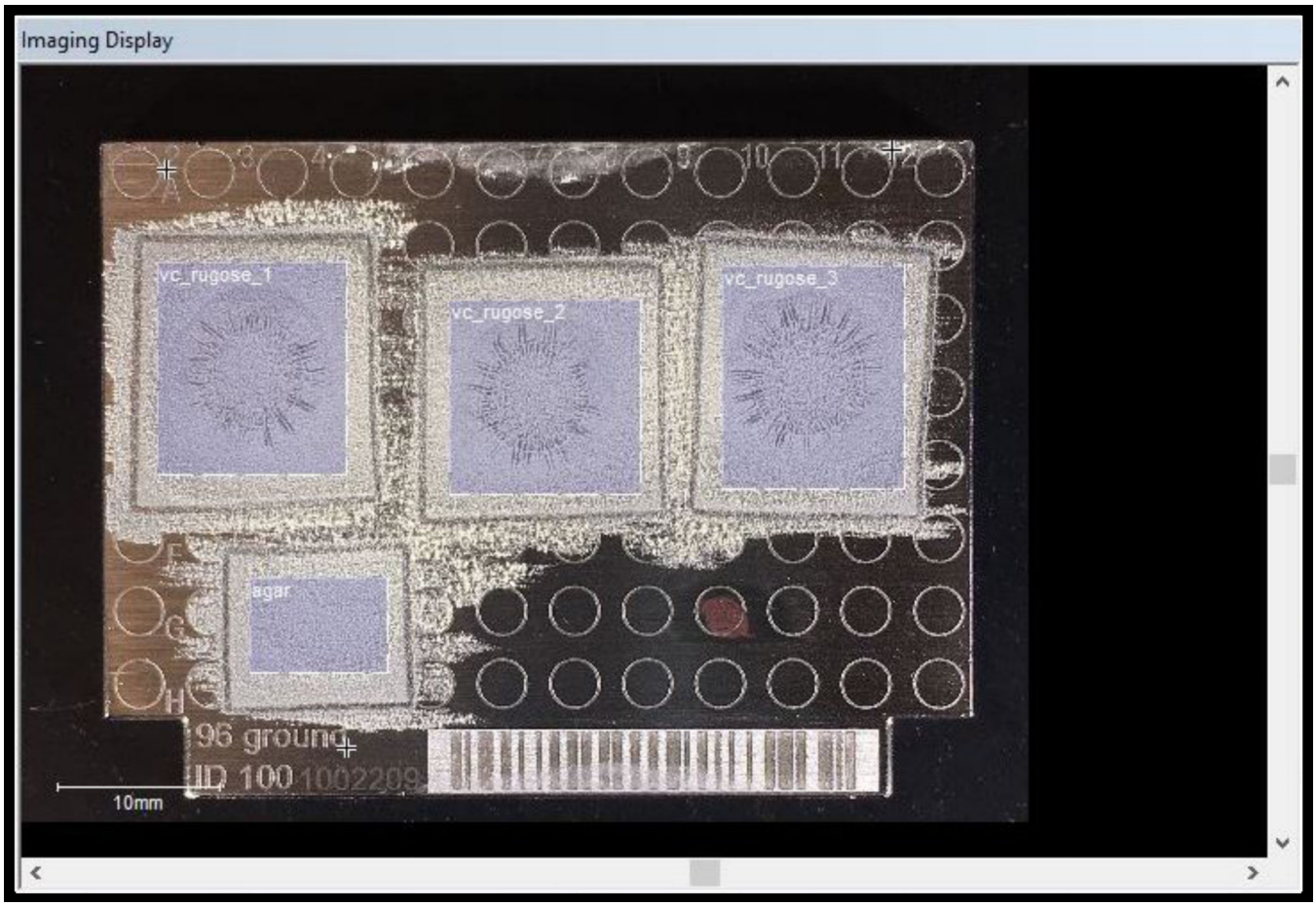

Figure 3:

Optical image of the MALDI target plate uploaded into FlexImaging, showing the three teach points and four regions of interest for IMS analysis. Within each purple rectangle, the MALDI laser will raster across the entire area at 500 μm steps. Regions of interest can be selected in any part of the sample, however it is better to leave some space between the edge of the region analyzed and the edge of the agar sample as shown here. Consistent ablation of the edge of the agar sample by the laser can lead to sample flaking, and data collected close to the sample edge can suffer from other edge effects such as decreased ion intensity.

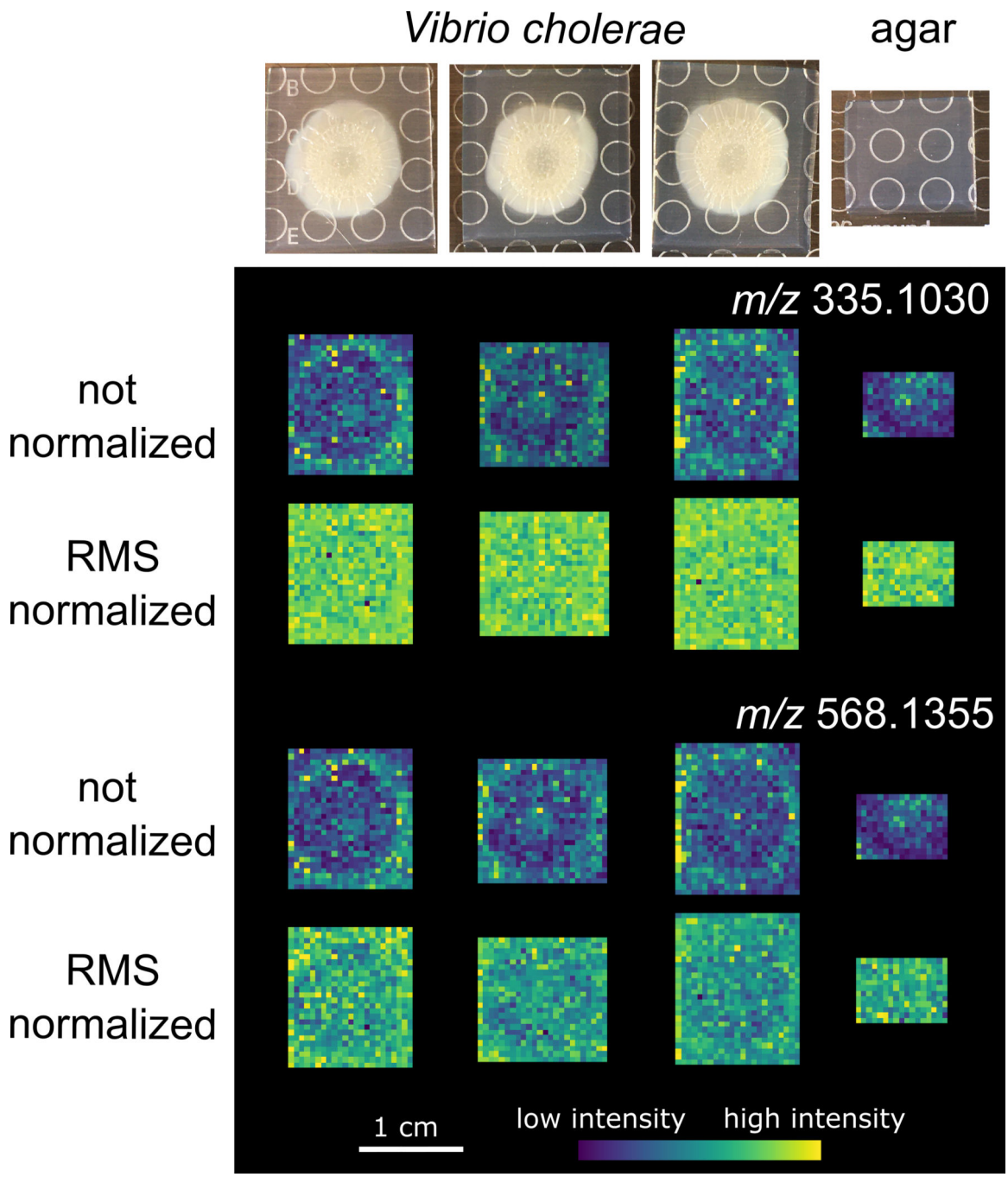

Figure 4:

Optical images of *V. cholerae* colonies and agar control and ion images with mass filters applied for the MALDI matrix ions *m/z* 335.1030 and *m/z* 568.1355 before and after RMS normalization is applied. Ion images that have not been normalized show artificial intensity gradients around the bacterial colonies and on the agar control due to differences in matrix deposition across the sample.

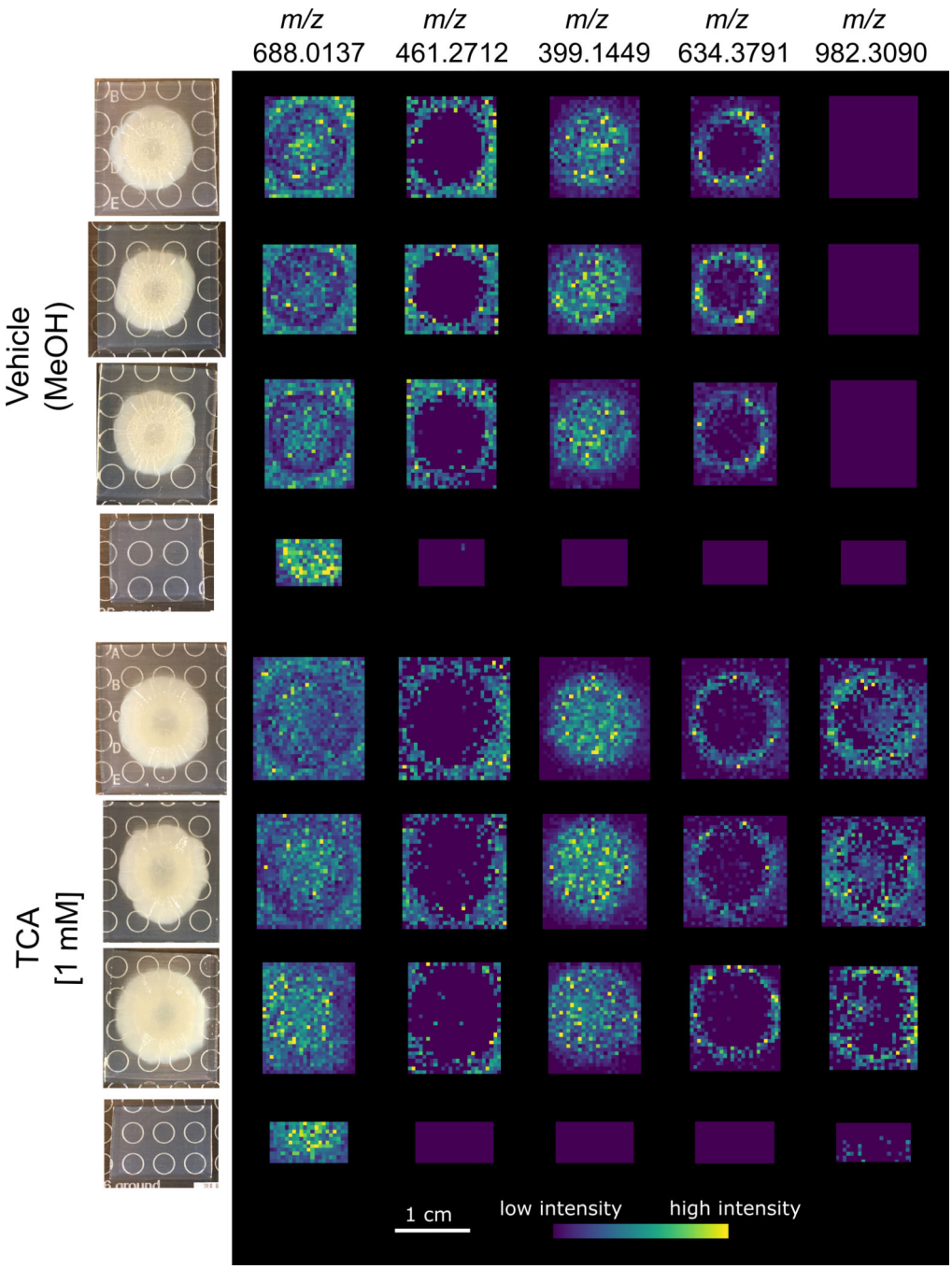

Figure 5:

Optical and ion images for *V. cholerae* grown on LB agar with an methanol vehicle control (top) and 1 mM TCA (bottom). Each ion was selected to show a different spatial distribution. Agar-associated signals such as *m/z* 688.0137, and signals secreted by the bacterial colony such as *m/z* 461.2712 can be differentiated by comparing the negative agar controls. All other signals we selected for having biologically derived spatial distributions and little to no signal in the negative agar controls.

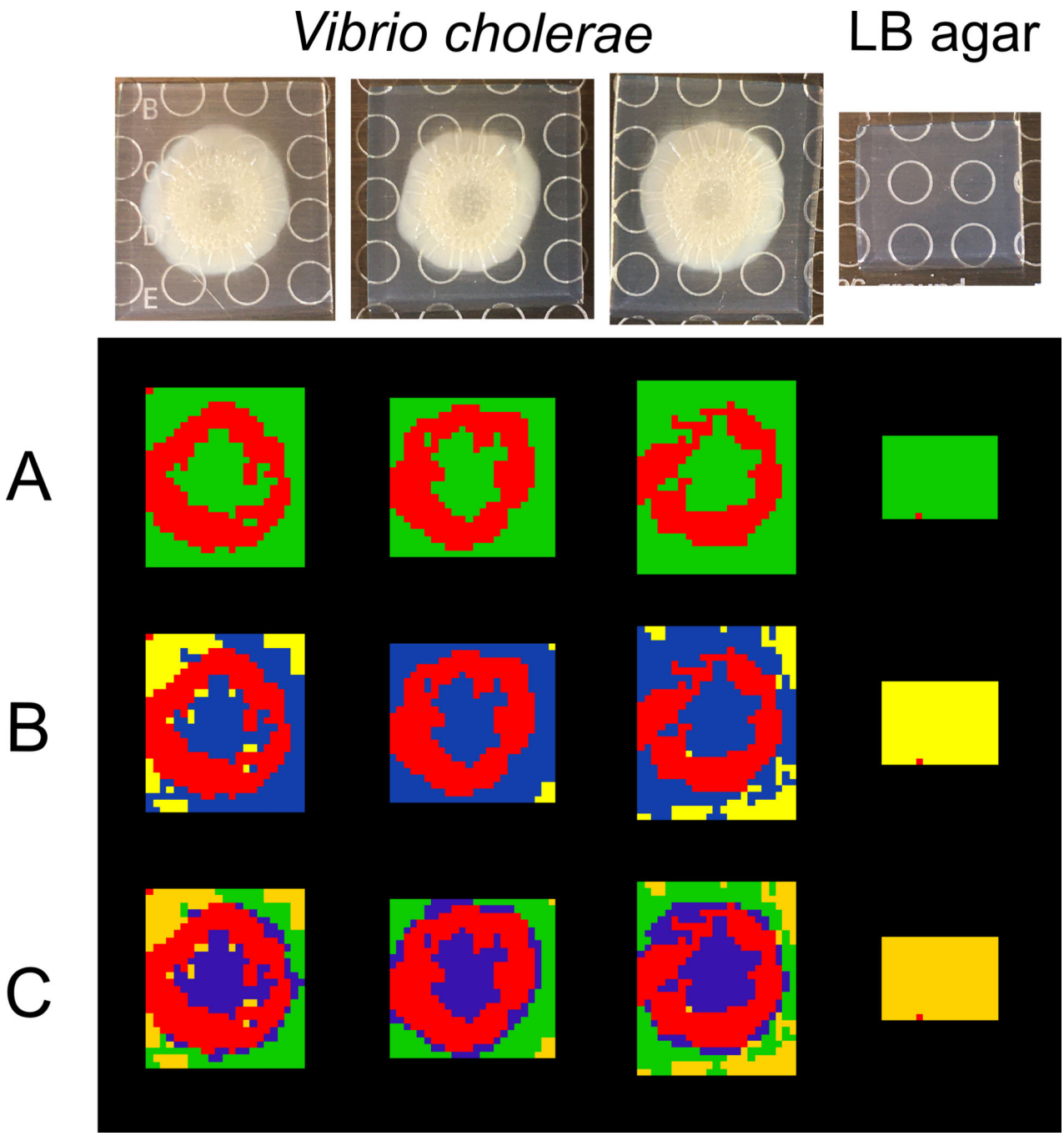

Figure 6:

Unsupervised segmentation of *V. cholerae* treated with methanol vehicle. (A) Initial segmentations splits the image into two regions. The red region correlates with the edge of the bacterial colony, while the green region groups the center of the bacterial colony with the LB agar. (B) By further segmenting the green region from (A), a blue region is generated which differentiates the center and edges of the bacterial colony from the agar control (yellow region). (C) By further segmenting the blue region from (B), the center of

the bacterial colonies is differentiated from the area directly surrounding the bacterial colony (green region) and the negative agar control (orange region).

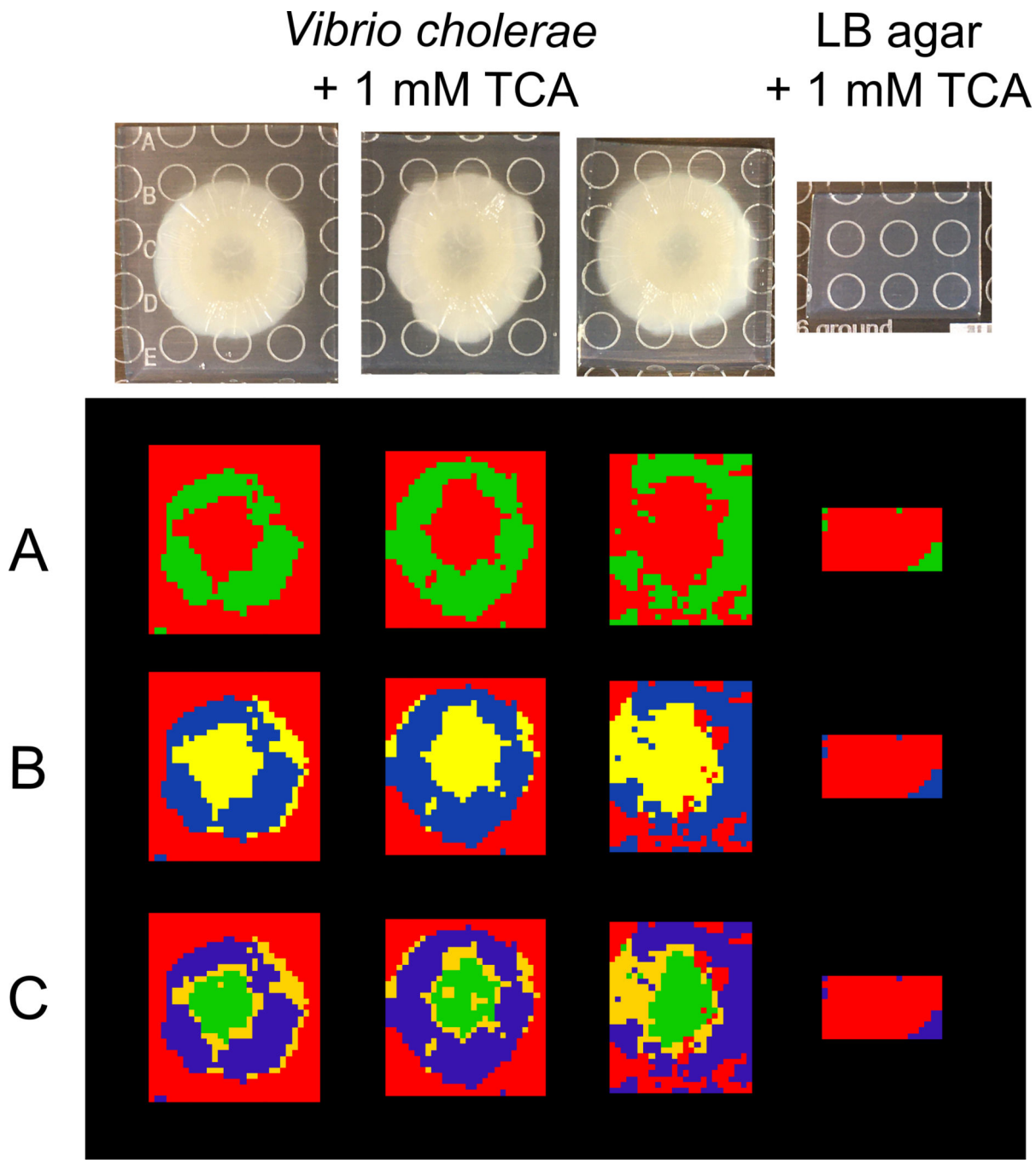

Figure 7:

Unsupervised segmentation of *V. cholerae* treated with 1 mM TCA. (A) Initial segmentation splits the image into two regions. The green region correlates with the edge of the bacterial colony, while the red region groups the center of the bacterial colony with the LB agar. (B) By further segmenting the red region from (A), a yellow region is generated which differentiates the center and edges of the bacterial colony from the agar control (red region). (C) By further segmenting the yellow region from (B), the center of the bacterial colonies (green region) is differentiated from the area directly surrounding the bacterial colony

(orange region) and the negative agar control (red region). The third replicate colony in this experiment demonstrated poor spatial segmentation compared to the other two replicates, however the segmentation in this replicate still tracks well with the three general regions of inner colony, outer colony, and agar.

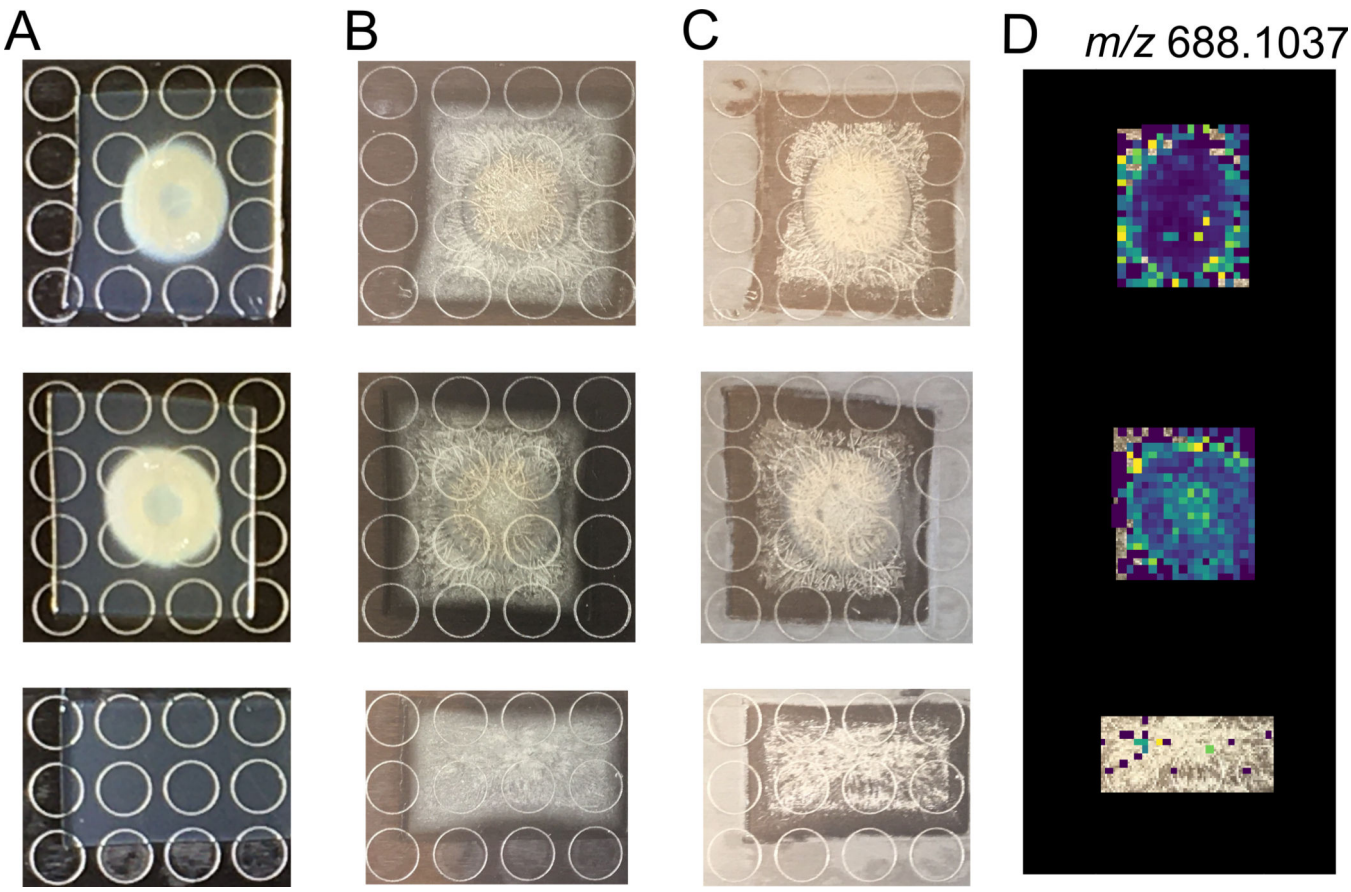

Figure 8:

Example of applying CHCA:DHB matrix to *V. cholerae* colonies by using a TM sprayer. (A) V. cholerae colonies and LB agar control on MALDI target plate prior to drying. (B) *V. cholerae* colonies and LB agar control fully dried for one hour at 37°C. (C) Dried sample from (B) after application of CHCA:DHB matrix using a TM sprayer. (D) Ion images of *m/z* 688.1037, which was identified as an agar-associated ion in Figure 5. Raster points where the optical image shows are 'dead' spots or empty spectra where no data was acquired.

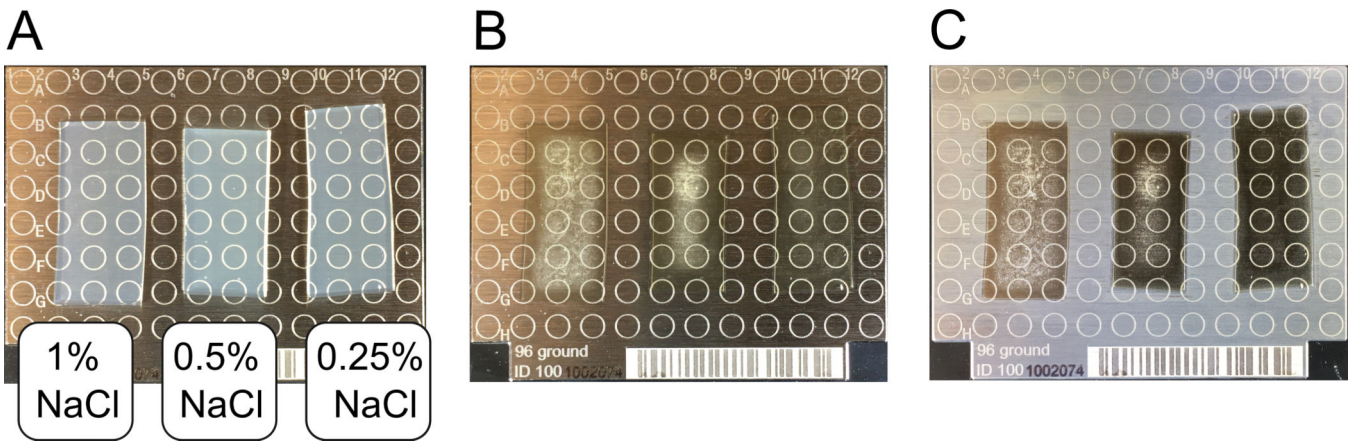

Figure 9:

Process of drying LB agar media for spray application of CHCA:DHB matrix. (A) Wet LB agar media containing different concentrations of NaCl on a MALDI target plate. (B) LB agar media after drying at 37°C for one hour. (C) Dried LB agar media with CHCA:DHB matrix applied using a TM sprayer. The area where the agar sits is darker, showing that insufficient matrix crystallization occurs within the sample area.