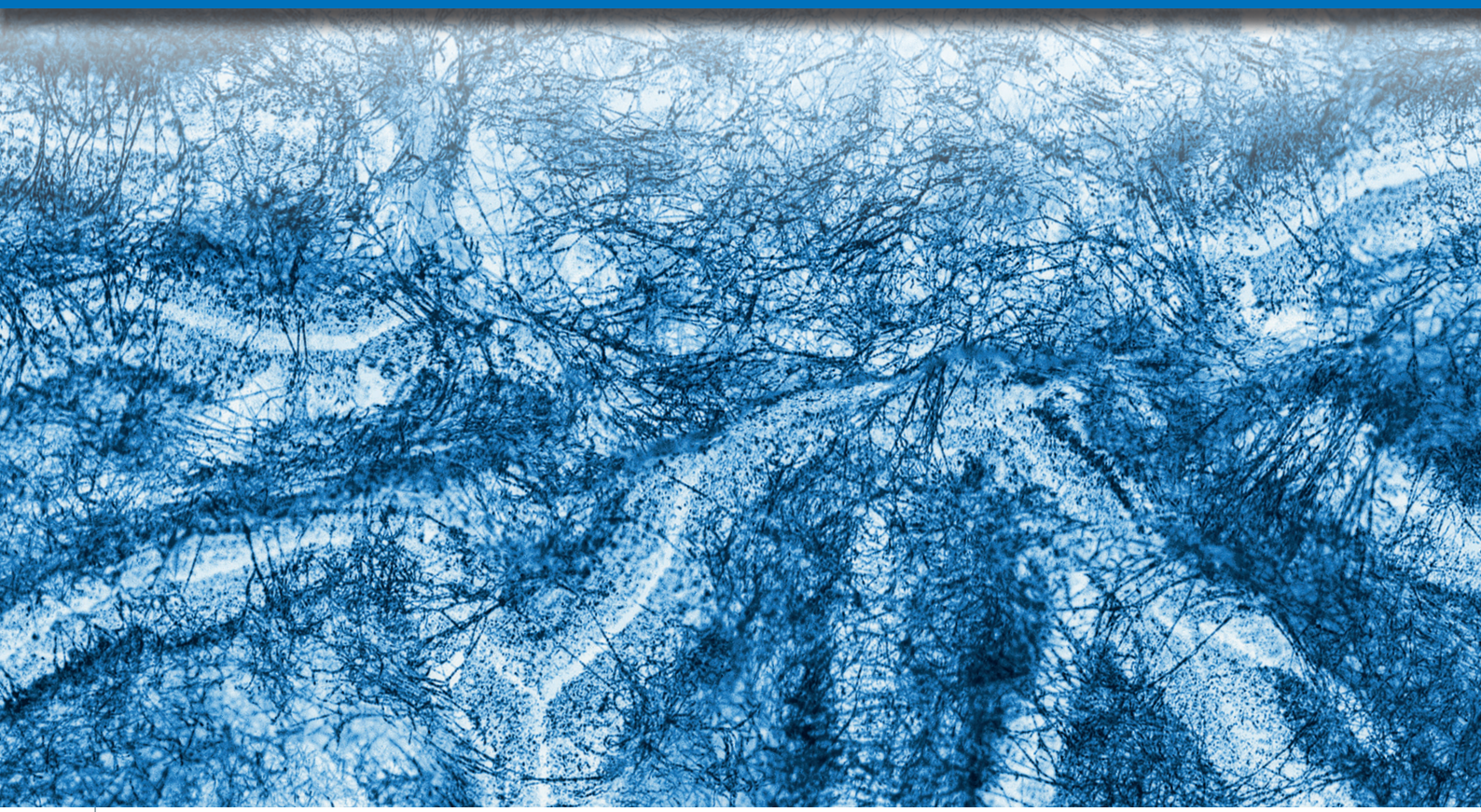

Large scale MALDI-TOF imaging of metabolites from filamentous fungi

MALDI-TOF imaging has proved a very useful research tool as it can rapidly produce high spatial resolution images with minimal sample preparation.

However, a major drawback of MALDI-TOF based imaging can be attributed to limitations in sample preparation such as matrix application and plating to avoid flaking into the instrument itself once it is exposed to high vacuum. Some filamentous fungi in particular have proven difficult to image with MALDI because either the fungal hypae projects deep into the agar, or non-uniform matrix application onto the colony results in some regions producing no spectra.[1] These issues as well as the dimensions of imaging on a 96 spot plate make fungal imaging challenging, thus we have optimized MALDI imaging of filamentous fungi on a 384 spot steel plate and seen a high level of consistency between imaging runs due to our improved sample preparation steps.

Keywords: MALDI, Fungi, Imaging, Microbiology, Autoflex Speed, 384 spot ground steel MALDI target plate, TM Sprayer

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Introduction

Molecular imaging provides contextual information about biological samples by producing a map of the spatial distribution of molecules found in a sample. Imaging is routinely performed on tissue sections and intact bacterial and fungal colonies by applying matrix directly over wet microbial colonies or cryo-sectioned tissue. Imaging of intact microbial colonies can provide information about metabolic exchange between species, which is important as we consider entire microbiomes rather than isolated species.[2] In general, there are many factors to consider when designing a MALDI-TOF imaging experiment when microbial colonies are being utilized.

In previous reports for agar-based microbial imaging, bacteria or fungi are grown on thin agar plates (~10-11 mL of agar in a 90 mm Petri dish) and whole agar pieces are excised and directly transferred onto non-polished steel MALDI target plates, coated with matrix, and desiccated. *Important note: use of larger volumes of agar or smaller Petri dish will produce a film that is too thick and can result in damage to the MALDI-TOF.* Yang et al. describe many techniques to minimize sample flaking, including using non-polished MALDI plates, optimizing culture media (such as adjusting overall salt and agar content), and matrix application of a 1:1 mixture of CHCA and DBA using a 53 micron stainless steel sieve with a 3" diameter.[3] However these applications were limited to 96 spot steel target plates and are not compatible with the larger 384 spot target plates due to the inconsistencies with attempting to coat a large target plate in a larger diameter sieve.

We report here the most efficient of these suggested techniques for reproducible imaging of filamentous fungi and media with both high lipid and salt content on a 384-well ground steel plate. Imaging on fungus sometimes proves difficult as hyphae tend to pull agar pieces away from the MALDI plate as it dries, and therefore steps need to be taken to encourage adherence to the plate. Typically, sieving matrix over a piece of agar helps the piece to adhere to the plate but can only reliably be done on a 96 spot steel plate due to the diameter size constraints of the stainless-steel sieves and the amount of matrix that would be required for a large sieve to ensure an even coating of the microbial colonies. Larger plate area is especially useful for filamentous fungi as they tend to spread across agar rapidly and large areas are needed to properly visualize excreted versus colony-associated metabolites. Additionally, space to include a variety of conditions allows the user to directly compare metabolite intensities for each condition in one imaging run. Particularly if a user is interested in detecting metabolites that are involved in interactions between bacteria and fungi and/or those that are secreted outward compared to colony associated it is important to retain the spatial separation of colonies. To adapt this imaging process to a 384 spot ground steel plate, optimization of application of the agar pieces is reported here.

Experimental

First, the TM sprayer (HTX Technologies) was used to evenly coat a 384 spot ground steel target plate

Table 1. TM Sprayer matrix density = (# of passes x flow rate x matrix concentration) / (velocity x track spacing)

TM Sprayer Parameters	
Parameter	**Setting**
Temperature	30°C
Number of Passes	9
Flow Rate	0.2 mL/min
Velocity	1100 mm/min
Track Spacing	3 mm
Pattern	CC (crisscross)
Gas Flow Pressure	10 psi
Gas Flow Rate	3 L/min
Drying Time	0 seconds
Nozzle Height	40 mm
Matrix	1:1 DHB:CHCA in 90% ACN, 0.1% TFA
Matrix Concentration	5 mg/mL
Matrix Density	0.002727 mg/mm^2

with 1:1 CHCA:DHB MALDI matrix (Table 1). This matrix was chosen as it is reported to enhance adhesion to the steel plate.[3] Figure 1 displays the plate throughout this process. The density of matrix applied can be easily calculated given the equation provided by HTX (equation 1). Ground steel provides more surface area for adherence than the polished steel plates and this technique therefore works best with ground steel plates. A coating density of at least 2.7 µg/mm^2 has been found to sufficiently adhere agar pieces to the steel plate

After spraying the target plate, agar sections with bacterial and fungal colonies and co-cultures were excised from Petri plates and placed onto the pre-sprayed steel plate. Contact between the agar and pre-coated matrix secures sections in place and transfer of matrix onto the underside of the agar occurs immediately when sections are touched to the plate. Therefore, agar sections were carefully placed to

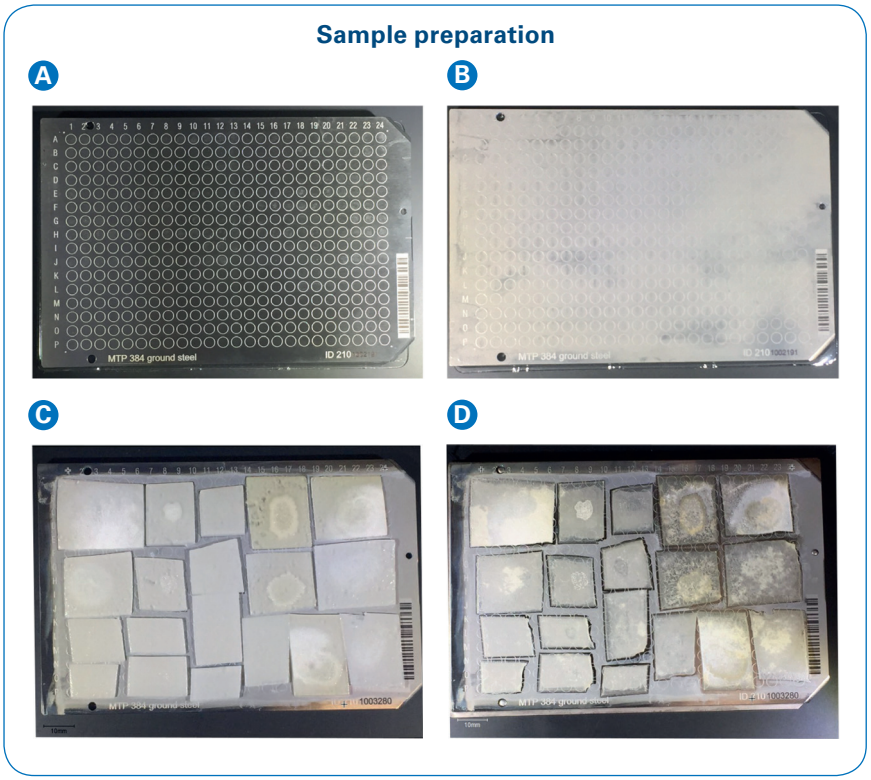

Figure 1: MALDI imaging of multiple conditions on a 384-well plate. Samples to be imaged are prepared as described in experimental section. A A Bruker 384 spot ground steel plate was used B CHCA:DHB 1:1 MALDI matrix (density of 2.7 µg/mm^2) was sprayed over the plate C fungal and bacterial colonies were excised from a petri dish and intact agar slices placed onto the sprayed plate D after desiccation the agar pieces were sprayed again with MALDI matrix and ready for analysis.

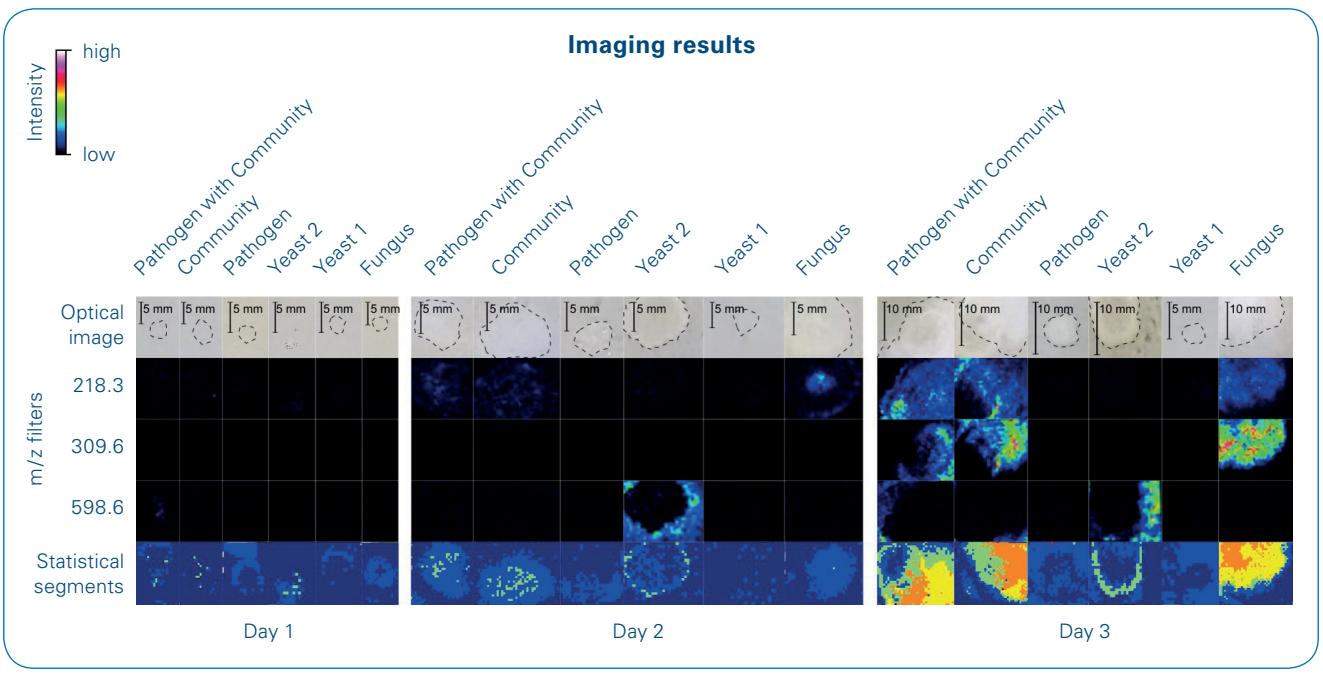

Figure 2: MALDI imaging and SCiLS statistical segmentation results. Optical image shows the wet agar piece before drying with dashed outlines of colonies. Colonies are not easily visualized in Day 1 but still produce small molecular signatures. m/z filters are applied to show spatial distribution of metabolites within the sample. Intensity of m/z filters correlates to metabolite abundance. Statistical segments were determined by SCiLS software based on co-localization of m/z peaks, and coloration is not related to intensity but simply denotes different regions.

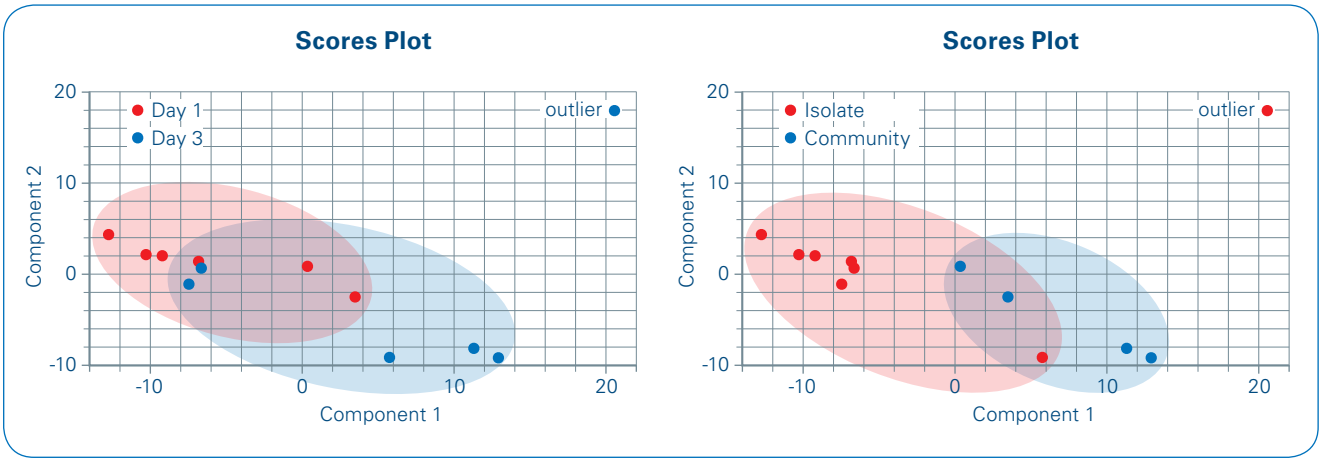

Figure 3: Principle Component Analysis (PCA) of IMS data. Statistical segmentation can be used to evaluate differences among seemingly similar segments, such as those of Day 1 and Day 3. PCA allows visualization of variance in data to determine whether time or microbial partners have the greatest effect on metabolite signatures. These plots were generated using the light blue segments detected in days 1 and 3 from each condition as those segments correlate spatially with the location of colonies, and each point can be considered to represent a metabolomic profile. The plot on the right shows somewhat greater divergence of conditions which indicates that metabolomic profiles tend to be similar based on the microbes present more so than whether they grew for 1 or 3 days. The outlier corresponds to peaks detected in Yeast 2 on Day 3 which was found to produce many unique metabolites.

avoid any movement from the initial placement. The hyphae of filamentous fungi extend from the plate creating a fluffy surface, and to create a more uniformly flat surface and prevent the spores from entering the MALDI-TOF MS, a sterile swab was dabbed in a raster-like manner over the colony area to flatten and remove the hyphal networks. Dabbing the swab rather than swiping helps to retain the original sample morphology.

After plating, sections were dried in a 30°C oven until the sample was completely desiccated. Desiccation time varies according to sample type; e.g. filamentous fungi tend to dry more quickly than bacteria and defined media tends to dry more quickly than complex media. The pre-coating of matrix acts as a 'glue' to adhere the agar pieces firmly in place and prevents deformity of the fungal sections while drying and under high vacuum in the autoflex speed MALDI-TOF. Excess matrix can be carefully swabbed from the edges of the plate to allow visualization of letters and numbers for accurately registering the image in flexImaging and to minimize the amount of matrix the source encounters. After samples were completely dried, another coating of at least 2.7 µg/mm^2 density of matrix was applied using the same spraying method and parameters listed for pre-coating. Application of the matrix after desiccation decreases the likelihood of uneven matrix application due to regions of different polarities, and the spray method of application helps to further flatten any hyphae that extrude from the desiccated fungal sections.

Results

The area of a 384-well plate allows four times the area of a 96-well plate for sample plating, increasing the number of biological conditions to be analyzed. Imaging of multiple conditions and time points can be directly compared in one imaging run (Figure 2). Visualization of different time points shows how some signals (m/z 309.6) may not appear in earlier growth stages while others (m/z 598.6) are detected close to the colony at day 3 but diffuse through the agar by day 7. The number of different conditions also allows the user to perform studies on microbial consortia (community, or pathogen and community) alongside time points. This allows the user to visualize time points with maximum production of metabolites while determining the source of metabolite production. For example, as in Figure 2 including individual controls for each microbe that constitute the complete community quickly points out that 2 is responsible for production of m/z 598.6. Also, the inclusion of day 3 and day 7 demonstrates that production of m/z 598.6 is delayed when other microbes are present, and m/z 218.3 is present as early as day 1 during growth and steadily increases. Using a quarter of the space to image in this run would limit the user to analyzing only one day, choosing which isolated species to include, or losing some spatial information by taking smaller agar pieces. Statistical segments were determined by SCiLS software based on co-localization of m/z peaks.

The morphology of fungal colonies differs from bacterial colonies in that different stages of fungal life cycles

are represented in an imaging run and the simple adjustments to sample preparation described here allow space to image sufficient area for complete analyses. This is evidenced by the increased segmentation seen in fungal colonies on day 7 (Figure 2), indicating regions of metabolite signatures that did not exist on days 1 and 3, and may not be detected if smaller pieces of colonies were used. The pre-coating of matrix is an essential experimental step when imaging fungus as desiccated pieces inevitably chip (flake) from the plate when no coating is applied or less than the 2.7 µg/mm^2 density is attempted and this can be catastrophic in a high voltage TOF source.

The imaging run showed here contained 18 different conditions for a total of 13,121 spectra as opposed to three to five conditions with an upper limit of about 4,000 spectra that are typically analyzed on a 96-well plate at a spatial resolution of 500 microns. The increase in the amount of data collected is timely as statistical tools such as the SCiLS software suite have made management of such large data sets trivial in comparison to manual data curation and analysis. Larger data sets also increase confidence in the statistical analyses; as seen in the data segments, day 7 shows the best segmentation of fungal spots and fungal signals likely contribute most to the overall metabolite signatures of communities. With sufficient samples however, statistical tests can be used to point out trends that may not be apparent by visual inspection (Figure 3). Based on the PCA analysis of metabolomic signatures, the user might choose to exclude day 1 and perhaps add different media types, a time point between days 3 and 7, or pairwise combinations of individual community members.

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Conclusion

- Large-scale MALDI imaging of filamentous fungi in this manner has consistently proved successful. The increase in number of conditions analyzed or area of sample in one imaging run using the 384-well plate has made imaging of fungi simple and efficient. Images obtained by this method produce molecular images with high resolution and accurate representation of fungal morphology. One could envision adding additional time points if only 2-3 conditions are deemed important or adding additional strains for studies involving more complex microbial communities. Alternative media types or plating techniques could also be evaluated in a large-scale imaging run such as this. The advances in statistical tools available for MALDI imaging nicely complements the increase in sample area.

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