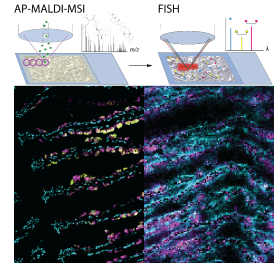


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🌐 **Spatial metabolomics of in situ, host-microbe interactions** (practical guide for combining MALDI-MSI and FISH microscopy on the same section)



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We use this protocol and it's working

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Abstract

Spatial metabolomics describes the location and chemistry of small molecules involved in metabolic phenotypes, defense molecules and chemical interactions in natural communities. Most current techniques are unable to spatially link the genotype and metabolic phenotype of microorganisms in situ at a scale relevant to microbial interactions. Here, we present a spatial metabolomics pipeline (metaFISH) that combines fluorescence in situ hybridization (FISH) microscopy and high-resolution atmospheric pressure mass spectrometry imaging (AP-MALDI-MSI) to image host-microbe symbioses and their metabolic interactions. metaFISH aligns and integrates metabolite and fluorescent images at the micrometer-scale for a spatial assignment of host and symbiont metabolites on the same tissue section. To illustrate the advantages of metaFISH, we mapped the spatial metabolome of a deep-sea mussel and its intracellular symbiotic bacteria at the scale of individual epithelial host cells. Our analytical pipeline revealed metabolic adaptations of the epithelial cells to the intracellular symbionts, a variation in metabolic phenotypes in one symbiont type, and novel symbiosis metabolites. metaFISH provides a culture-independent approach to link metabolic phenotypes to community members in situ – a powerful tool for microbiologists across fields.

Troubleshooting

1 Cryo-fixation and Embedding

a) Dissect tissue samples and submerge in precooled (4°C) 2% w/v carboxymethyl cellulose gel (CMC, $M_w \sim 700'000$, Sigma-Aldrich Chemie GmbH) and snap-freeze in liquid N_2

Note

- Make sure the sample is fully covered in CMC gel
- Avoid air bubbles in CMC gel
--> this will cause holes in the frozen block
- Store samples at -80°C in sealed bags/containers with a minimum volume of air
--> storage in unsealed containers causes freeze drying of the CMC over months, leaving a "bubble gum-like" embedding that cannot be sectioned

2 Cryo-sectioning

a) Trim CMC around sample (**be careful, use single edge 'safety razor blade'**) with the sample centered in the CMC block, leaving ca. 0.5 cm of CMC around the tissue

b) Section tissue at 10–15 μm (we used 10 μm) thickness with a cryostat (Leica CM3050 S, Leica Biosystems Nussloch GmbH) at a chamber temperature of -35 °C and object holder at -22 °C

Note

- Leave sample at least over night at ca. -20°C before sectioning
--> acclimatization of CMC allows for smoother sectioning
- Adjust cryostat chamber temperature to ca. 10°C colder than object holder temperature
--> this compensates for temperature fluctuations during opening and closing of the cryo-chamber

3 Thaw mounting of the cryo-sections



a) Thaw-mount cryo-sections **onto coated Polysine® slides (Thermo Scientific)**

Note

- **Using polysine coated slides is essential for cross-linking the tissue/bacterial cells during the PFA post-fixation to the glass slide and ensure proper adhesion during washing and hybridization steps.**
- Depending on the MALDI-MSI setup, conductive ITO-coated slides have to be used, which can also be coated with Polysine manually

b) Subsequently place slides with sections back into cryostat chamber

c) Store slides with tissue sections at -80°C in sealable slide containers with silica granules (e.g. LockMailer™ microscope slide jar, Sigma-Aldrich, Steinheim, Germany)

Note

- Silica granules prevent air moisture condensation on the tissue upon removal from the freezer or fridge
- Alternatively, slides can be stored in desiccators at room temperature

4 Marking and Microscopy



a) Warm up slide containers to room temperature

b) Mark glass slide very close to tissue with white paint (edding 751®, described previously¹) for orientation during image acquisition

Note

- Using toothpicks or needles to create markings often appear very large under the microscope and cannot be matched as precisely
--> attaching a short hair (e.g. eye brow) or animal hair to the tip of a toothpick creates a perfect small brush that also takes up the paint and creates µm-scale markings
- **It is essential that the white marker is insoluble by the organic solvents used to spray the ionization matrix to avoid diffusion/ leakage into the tissue**
(e.g. edding 751® works with acetone, methanol, ethanol and acetonitrile)

c) Acquire overview images of the marked tissue in bright-field and autofluorescence of the tissue (if possible)

Note

- Printouts of the whole tissue section including the markings are very useful for orientation/ navigation to the often small areas (e.g. specific cells) that will be measured with high-resolution MALDI-MSI
- For MALDI-MSI setups where the ROI is determined directly on the tissue, the marker is visible below the ionization matrix and helps to navigate on the tissue to determine the ROI
- The marker has a specific molecular signature and autofluorescence, which can help with the virtual alignment of microscopy and MS-images

5 Matrix application

- a) Dissolve 30 mg ml⁻¹ of 2,5-dihydroxybenzoic acid (DHB; 98% purity, Sigma-Aldrich, Steinheim, Germany) in acetone/water (1:1 v/v) containing 0.1% trifluoroacetic acid (TFA)
- b) Apply 100 µl of the matrix solution with an ultrafine pneumatic sprayer system with N₂ gas (SMALDIprep, TransMIT GmbH, Giessen, Germany)² to the tissue section
- c) To locate the field of view and facilitate laser focusing, we drew lines around the matrix-covered tissue section using a red marker

Note

- There are many different matrix chemicals, solvents and matrix application systems, ranging from sieves to pneumatic sprayers and sublimation devices.
--> Important for high-resolution MALDI-MSI is to find a good trade-off between crystal size (**crystals must be smaller than the laser spot**) and the extraction/ metabolite signal
- Questions, which will help choosing a suitable matrix/spaying setup (<https://www.ncbi.nlm.nih.gov/pubmed/29682685>):
--> Ionization mode: negative or positive? Target molecules: polar or non-polar? Target structures: Do I really need high-spatial resolution? If yes, which spot/crystal size is needed?

6 (AP)-MALDI-MSI

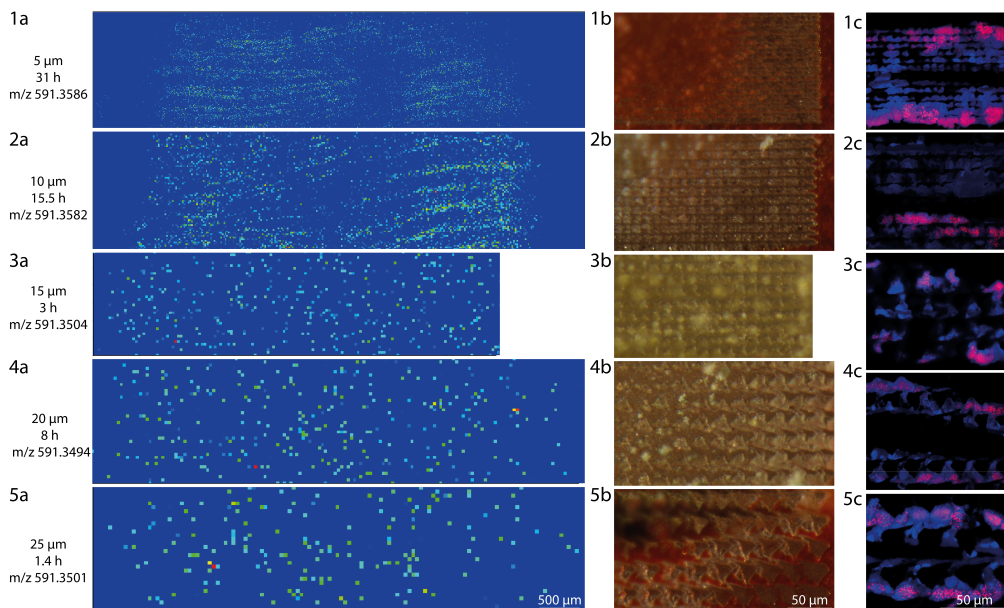
- a) Atmospheric pressure (AP)-MALDI-MSI measurements were carried out at an experimental ion source setup³, coupled to a Fourier transform orbital trapping mass

spectrometer (Q Exactive™ HF, Thermo Fisher Scientific GmbH, Bremen, Germany)

b) The setup contained a 337 nm nitrogen laser, operated at 60 Hz for 500 ms, resulting in 30 shots per pixel

Note

- Different setups use different laser energies and repetition rates and often the tissue can show different levels of destruction
- > Shooting grids onto the same tissue with different laser energies and using FISH afterwards, helps to determine the suitable trade-off between destructivity of the MALDI-laser, the FISH signals after MALDI-MSI and the necessary spatial resolution



Different measurements at different laser energies (a) (done with commercially available AP-SMALDI-10 source, connected to an orbitrap QExactive Plus), and spatial resolutions (see holes in matrix in column b) and FISH signals afterwards, see column (c) (DNA stained with DAPI (blue) and bacteria stained with a general bacterial monoFISH probe with a Cy3 fluorophore (red)).

--> Large spotsizes + high laser energies show that almost whole bacteriocytes are ablated, whereas small laser spotsized, in combination with high-spatial resolutions result in lower destructivity and better FISH signals.

c) AP-MALDI-MSI was performed in positive mode for a mass detection range of 400–1200 Da and a mass resolving power of 240,000 (at 200 *m/z*)

d) A specifically developed AP-MALDI-MSI autofocus that was based on a laser triangulation system allowed us to avoid oversampling when imaging at a step size of 3 μm ^{4, 5}

Note

- The combination of small laser spot size, atmospheric pressure source, low repetition rate/ tissue ablation resulted in low destructivity and allowed us to sufficiently preserve tissue for FISH on the same tissue section

e) Record measured sample surface with a stereomicroscope (e.g. SMZ25, Nikon, Düsseldorf, Germany) after AP-MALDI-MSI

Note

- An overview image of the tissue section and the ROI, measured with MALDI-MSI is useful to find the same ROI when imaging the section with the CLSM and when matching fluorescence and MSI images
- microscopy images allow to record and measure the distance between ablation spots and to determine the real resolution (also a control for oversampling)

7 Post fixation



a) Use tissue tip or cotton swab, wetted with 70% ethanol to carefully wipe away matrix and red marker around the tissue

Note

- Removing excess matrix and red pen before submerging the slide into fixative
--> prevents diffusion of red color into the tissue

b) Submerge glass slide with matrix-covered tissue section in a 2% PFA/PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4) solution for one hour at room temperature

Note

- DHB matrix is water soluble and therefore suited for a simultaneous matrix removal/fixation step.
- When using matrices, which require stronger organic solvents to dissolve, the tissue can be fixed first in 2% PFA/PBS and the matrix can be removed by washing the slide in 96% ethanol afterwards

c) Wash off fixative by submerging slide 2×20 min in PBS at room temperature

d) Carefully dip slide into 96% Ethanol and air dry afterwards

Note

- avoid rapid movement and of the glass slide in the solutions, which could cause damage to the tissue sections despite fixation

8 Fluorescence in situ hybridization (FISH)

the protocol was modified after a previous study⁶

a) Encircle dried section with a liquid blocker (PAP-Pen, Science Services) on the glass slide to prevent leakage of the hybridization mixture during incubation

b) For the hybridization mixture use 5 ng μl^{-1} of probe in hybridization buffer (35% formamide (v/v), NaCl 0.9 M, 0.02 M Tris-HCl (pH 7.5), 10% dextran sulfate (w/v), 0.02% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) Blocking Reagent (Roche, Basel, Switzerland))

c) Use a negative control for unspecific binding, labeled with Cy3, for hybridization on a subsequent tissue section during the same FISH experiment

d) Hybridize tissue sections and controls with 20-30 μl of hybridization mixture for 2 h in a saturated formamide-water atmosphere at 46 °C (amounts of hybridization mixture should be sufficient to generously cover tissue section)

e) Wash the tissue sections on the slides for 15 min in prewarmed buffer (48°C) (0.1 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, 0.005 M EDTA)

f) Dip slides in PBS buffer and 96% ethanol speed up the drying process. Subsequently, the DNA of host and symbiotic bacteria was stained with 4',6-diamidino-2-phenylindole

(DAPI) for 3×10 min at room temperature. For microscopy, sections were mounted with VECTASHIELD®

Note

- Before choosing the fluorescence dyes for the probes, testing the autofluorescence of the tissue
--> if possible, avoid choosing probes that lie within the maximum of the autofluorescence of the tissue, this will result in better signal-to-noise values for the probe signal
- We used specific 16S rRNA probes to target the symbiotic bacteria containing two fluorophores, at the 5'- and 3'-end of the oligonucleotide for increased sensitivity (biomers.net GmbH, Ulm, Germany)⁷.
--> stronger signals based on the two fluorophores
- We chose probes that are directly connected to fluorophores unlike catalyzed reporter deposition (CARD) FISH probes
--> additional steps of the CARD FISH protocol such as permeabilizations, activation, inactivation and a second hybridization can cause stress to the tissue and increase the danger of dislocating/ damaging the post-fixed tissue
- To further increase the fluorescence signal or label multiple bacterial phylotypes probes with four fluorescence labels could be used in the same protocol⁸

9 Widefield microscopy

a) If possible, acquire overview tile-scans of the sample in bright field and fluorescent channel after MALDI-MSI, using an (automated tile scanning) epifluorescence microscope

Note

- This step is not essential but substantially helps to determine the region, which was measured with MALDI-MSI to guide the CLSM acquisition
- If fluorescence dyes are sensitive to bleaching, only acquire a quick overview image at a low magnification objective lens and short exposure times in the bright field channel
--> Comparing the overview bright field microscopy image acquired before MALDI-MSI and FISH with the overview acquired afterwards shows potential changes in the tissue integrity e.g. moved/ washed away tissue pieces

10 Confocal Laser Scanning Microscopy (CLSM)

- a) Image ROI, imaged with AP-MALDI-MSI with a confocal laser scanning microscope (CLSM) (Zeiss LSM 780)
- b) When selecting the ROI for CLSM, include tissue areas around region measured with MALDI-MSI (ca. one tile as a margin around ROI)
- c) Determine top and bottom plane of the tissue section on at least two diagonal corners of the ROI to compensate for potential tilting and inconsistencies of the slide and tissue section thickness, respectively
- d) Record of multiple z-layers throughout the tissue section

Note

- Using the CLSM provides an increased resolution, stronger excitation and the possibility of virtual z-stacking, to determine the fluorescence signals throughout the tissue section
- For imaging bacteria in tissue sections (tile scanning and z-stacking), we found the magnification of a 20x lens at the CLSM as best trade off, between imaging speed and resolution
- Particularly for smaller MALDI-MSI ROIs, higher magnifications could be used
--> For dyes that quickly bleach, remember that higher magnifications require more tiles in x y **and** z, increasing acquisition times substantially

comment

- 11 Although we have not tested different FISH approaches, the possibility of labeling specific genes and transcripts could be particularly useful when working on host-microbe or microbe-microbe interactions.

Horizontal gene transfer (HGT) often plays an essential role in such interactions and could change the metabolic capabilities of individual organisms.

Consequently, to better interpret metabolite distributions, investigating the genomes through additional omics approaches such as DNA sequencing can show, if the production of specific metabolites is restricted to one partner or even bacterial subpopulations in the symbiosis⁹. Using this information to design specific probes for individual genes would in combination with MALDI-MSI allow to locate gene specific metabolic phenotypes.

