Brazilian Journal of Analytical Chemistry
2023, Volume 10, Issue 38, pp 128-137

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High Resolution in Mass and Space: AP-SMALDI coupled with Orbitrap Exploris Mass Spectrometer for MS Imaging

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This report was extracted from the Thermo Scientific Application Note 000659

Keywords: Orbitrap technology, tissue samples, high resolution accurate mass, high spatial resolution, SMALDI, lipids, metabolites, peptides, 3D-Surface Imaging

Objectives: High resolution in mass and space is achieved by Atmospheric-Pressure Scanning microprobe Matrix-Assisted Laser Desorption/Ionization (AP-SMALDI) coupled with Thermo Scientific™ Orbitrap™ mass spectrometers.

AP-SMALDI[5] AF ion source is a product of TransMIT GmbH, Giessen, Germany.

- The ion source has been adapted to the Thermo Scientific™ Orbitrap Exploris™ MS series products.
- AP-SMALDI[5] AF ion source has been adapted to both, S-Lens (Thermo Scientific™ Orbitrap Exploris™ 120 MS/Thermo Scientific™ Orbitrap Exploris™ 240 MS) and Ion Funnel (Thermo Scientific™ Orbitrap Exploris™ 480 MS) technology.
- The new instrumentation has been used to study compounds such as lipids, peptides, and metabolites in mouse brain, small parasites[1] and bacterial colonies using various Orbitrap[2] platforms.
- 3D-Surface topography imaging enables the study of non-flat biofilms with steep edges.

METHODS

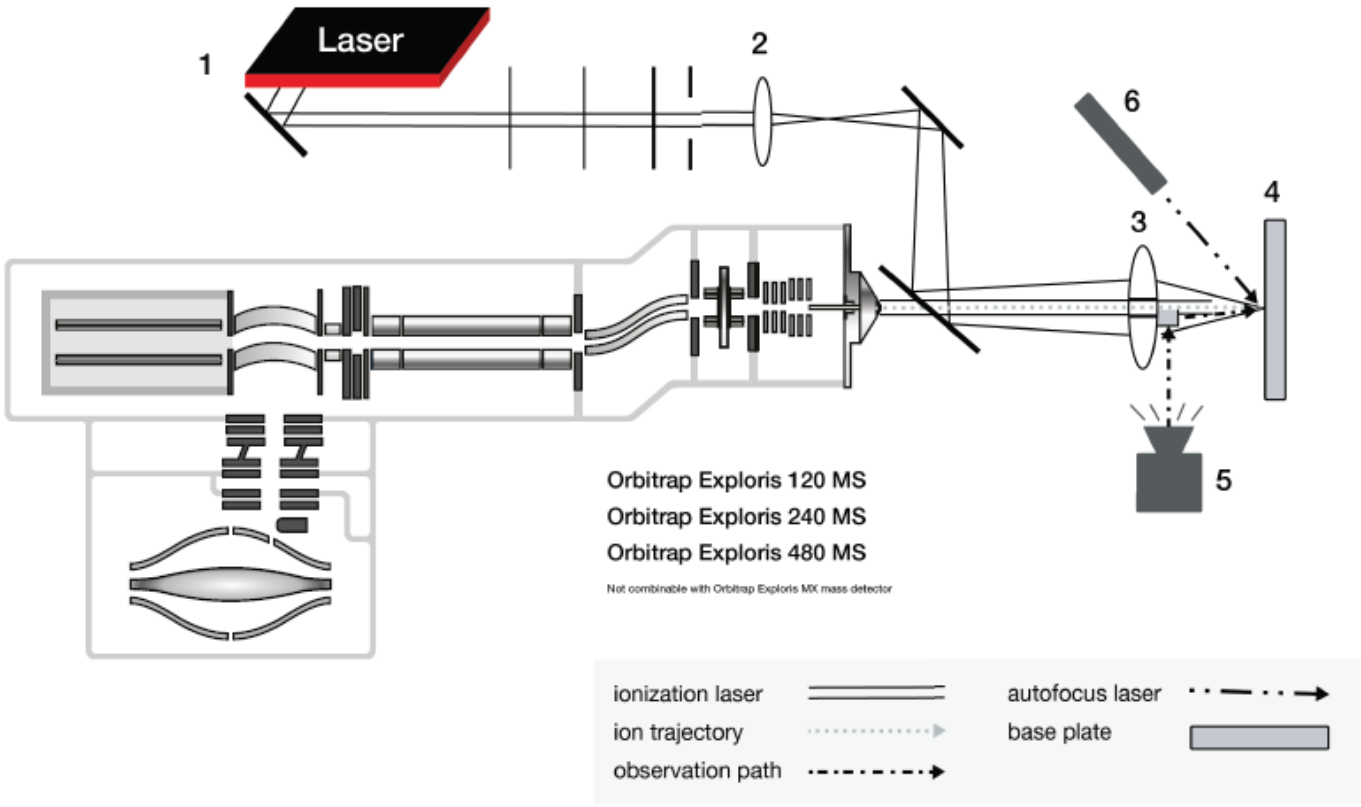

Figure 1. Schematic diagram of TransMIT's AP-SMALDI⁵ AF ion source mounted to Orbitrap Exploris 120 MS, Orbitrap Exploris 240 MS with S-Lens interface or Orbitrap Exploris 480 MS with electrodynamic ion funnel interface — here represented by Orbitrap Exploris 240 MS.

The AP-SMALDI⁵ AF ion source is schematically shown in detail: Starting at (1), the laser beam passes through a focusing lens (2) and two mirrors directing the light through an objective lens (3) close to the sample. The objective lens (3) focuses the pulsed laser light orthogonally to the tissue sample (base plate, 4). An off-axis camera (5) allows for sample inspection while the autofocusing laser (6) ensures the analyzing laser is always in focus.

Technical details of the AP-SMALDI⁵ AF ion source setup coupled to Orbitrap mass spectrometry for MS Imaging application are found in the paper by M. Kompauer, S. Heiles, B. Spengler, Nature Methods 14 (2017) 1156-1158 [3] – DOI: https://doi.org/10.1038/nmeth.4433.

Here matrix-covered samples (see schematics of SMALDIPrep device in Figure 2) are imaged using Atmospheric-Pressure Scanning microprobe MALDI mass spectrometry (AP-SMALDI-MS). Laser spot size is 5 µm in diameter. Various step sizes are used for scanning, resulting in respective pixel resolutions. Different imaging modes, such as 2D Pixel Mode, 2D Line Mode, Full Pixel Mode or 3D-Surface Imaging Mode are applicable. 3D-Surface Imaging Mode [3] is employed to analyze sample topography of non-flat objects.

Note: The source mounts to Orbitrap Exploris 120 MS, Orbitrap Exploris 240 MS with S-Lens interface, as well as to Orbitrap Exploris 480 MS with electrodynamic ion funnel interface.
Note: AP SMALDI⁵ AF ion source is a laser class 1 product.

Also refer to the Technical Note TN000660 for application work with Thermo Scientific Orbitrap Exploris MS and AP-SMALDI⁵ AF ion source.

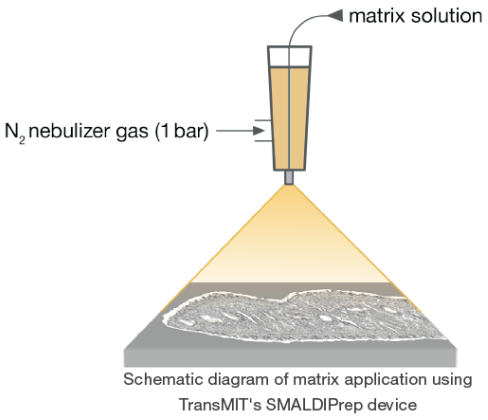

Figure 2. Schematic diagram of matrix application using the SMALDIPrep device — an automated ultrafine nebulizer for matrix deposition in MS Imaging applications. The SMALDIPrep device delivers crystal sizes of below 5 µm; it uses predefined and editable spraying methods.

For the applications in the context of this Technical Note, gelatin-embedded, treated *Fasciola* worms [4] and healthy mouse brain were cryosectioned (20 µm thickness) and spray-coated with matrix using the SMALDIPrep device. Matrix-specific and application-dependent protocols are provided to optimize results for spot size and tissue type in the application of interest. Here, matrices 2,5-dihydroxybenzoic acid (2,5-DHB), 1,5-diaminonaphthalin (DAN) and alpha-cyano-4-hydroxycinnamic acid (CHCA) were applied to the different tissue types and analytical questions; for details refer to the individual figure captions in the Results section further down.

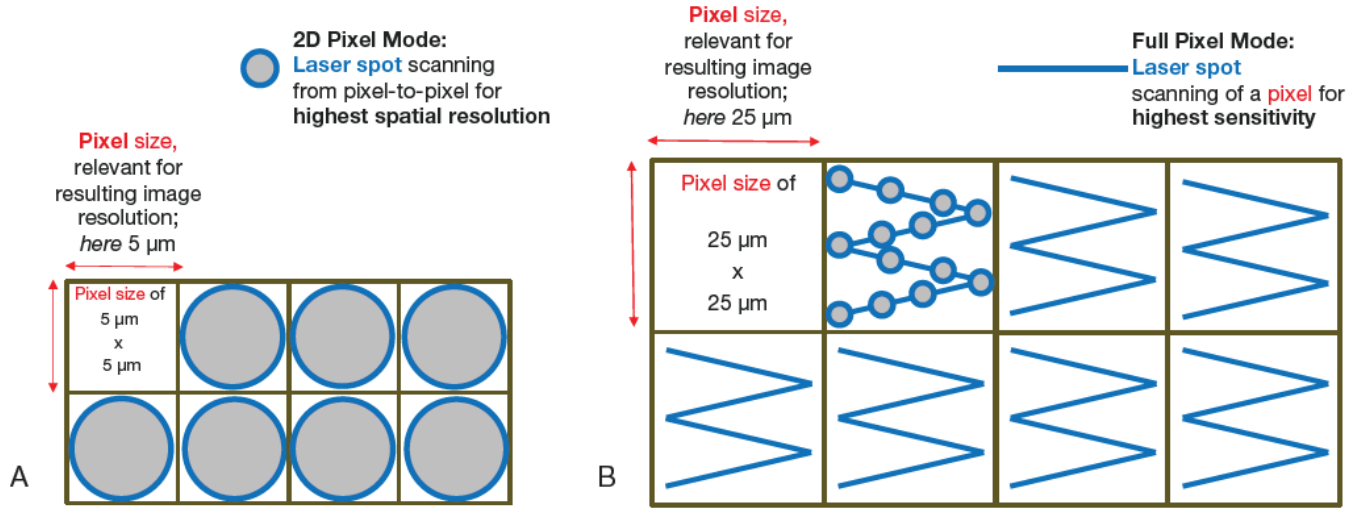

Figure 3. Various acquistion modes of the AP-SMALDI⁵ AF ion source are accessible[5]; a selection of MS Imaging acquisition modes is shown here.

A) 2D Pixel Mode with pixel size of 5 µm x 5 µm, applying a laser spot size of 5 µm – resulting in an image resolution of 5 x 5 µm². Here, each pixel is accessed by applying laser pulses to the center of the individual pixel and acquiring the corresponding MS scan from this location/pixel. Note: Orbitrap Exploris method setup allows to combine different scan types acquired from a given pixel, and e.g. MS and MS² images can be overlaid; such approach can be used for identification (MS) and confirmation (targeted MS²) by a single acquisition; data not shown here.

B) Full Pixel Mode with a pixel size of ≥ 25 µm x 25 µm applying a laser spot size of 5 µm – resulting in an image resolution of ≥ 25 x 25 µm². Here, each pixel is scanned by multiple laser pulses with a spot size of 5 µm. Meandering pulses ablate material across the pixel — resulting in a highly sensitive MS scan with an image resolution of ≥ 25 µm. Note that the schematically shown laser spot in Figure 3B is not to scale in order to facilitate illustration of the ablation raster.

RESULTS

High Spatial Resolution Imaging – 2D Pixel Mode – Lipids – 5 µm spatial resolution

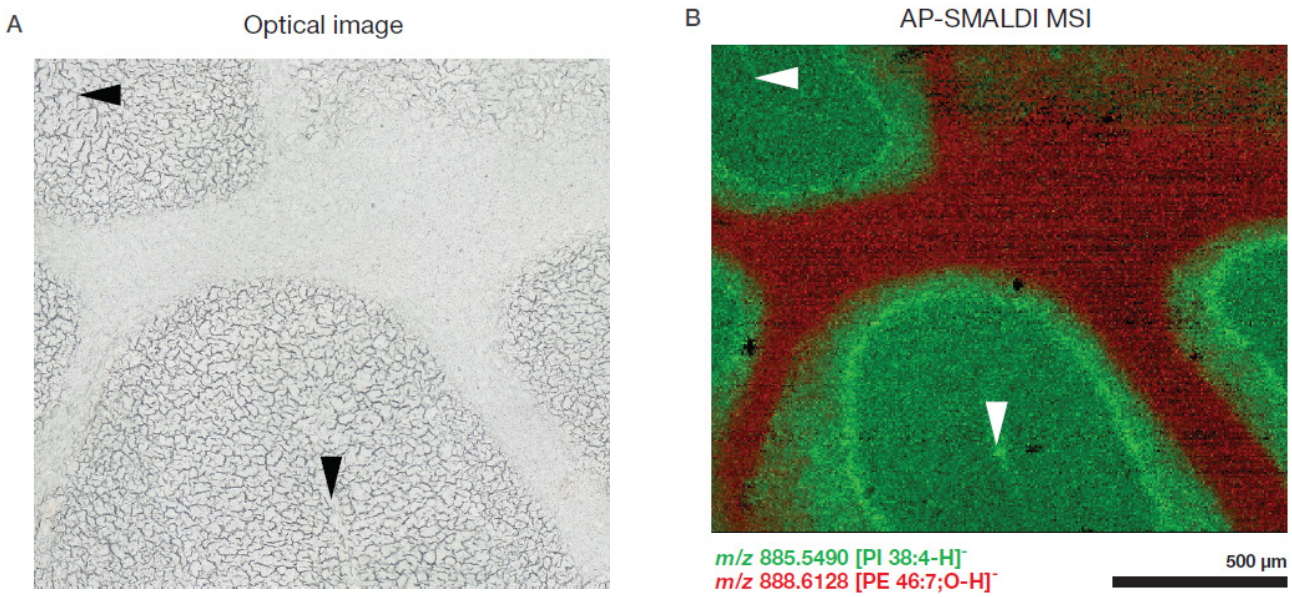

Figure 5. 2D Pixel Mode for high spatial resolution. Analysis of healthy mouse brain tissue section showing the distribution of two phospholipids from different lipid classes.

A: Optical Image

B: MS Image with two *m/z* values extracted:
lipid PI 38:4, $[M-H]^-$ at *m/z* 885.5490
lipid PE 46:7;O, $[M-H]^-$ at *m/z* 888.6128

For B: MS Image obtained using AP-SMALDI[5] AF ion source and Orbitrap Exploris 240 mass spectrometer; matrix applied: 1,5-diaminonaphthalin (DAN).

Source settings for Imaging: acquisition in 2D Pixel Mode, applying a laser spot diameter of 5 µm, and a pixel size of 5 µm x 5 µm.

Mass Spec settings for raw file acquisition: full scan, negative ion mode, *m/z* 600 – 1000, mass resolution set to 240,000 @ *m/z* 200, resulting in a measurement speed of 1.6 pixels/s.

For A and B: Find the arrows in the figure above; arrows (from top to bottom): enhanced levels of phosphatidyl inositol PI 38:4 were observed in these areas / compartments of the tissue: Prepyramidal fissure (ppf), Interposed Nucleus (IP), Presimple fissure (psf)

High Sensitivity Imaging – Full Pixel Mode – Lipids – 25 µm spatial resolution

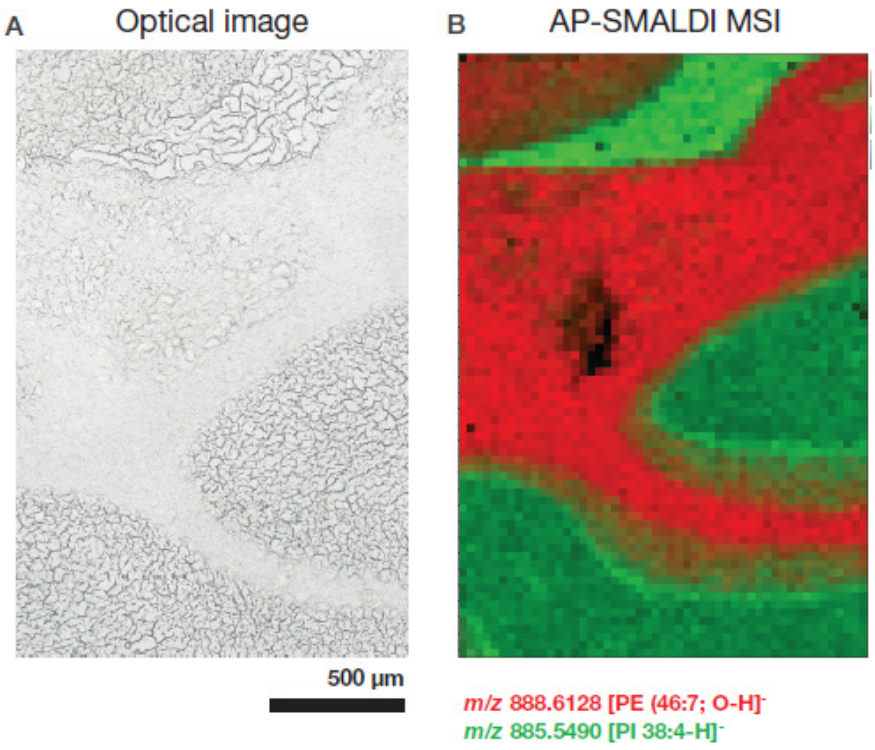

Figure 6. Full Pixel Mode for high imaging sensitivity. Analysis of a small area of healthy mouse brain tissue section. Imaging of different phospholipid classes.

A: Optical Image

B: MS Image with two *m/z* values extracted:
lipid PI 38:4, $[M-H]^-$ at *m/z* 885.5490
lipid PE 46:7;O, $[M-H]^-$ at *m/z* 888.6128

For B: MS Image obtained using AP-SMALDI⁵ AF ion source and Orbitrap Exploris 240 mass spectrometer; matrix applied: 2,5-DHB. Compounds were detected by accumulating ions from several ablation spots within the pixel area and collective detection. This is especially useful for low-abundant substances.[5]

Source settings for Imaging: acquisition in Full Pixel Mode, applying a laser spot diameter of 5 µm, and a pixel size of 25 µm x 25 µm.

Mass Spec settings for raw file acquisition: full scan, positive ion mode, *m/z* 600 – 1000, mass resolution set to 240,000 @ *m/z* 200. Measurement speed of 1.6 pixels/s.

High Spatial Resolution Imaging – 2D Pixel Mode – Drug uptake in liver fluke – 10 µm spatial resolution

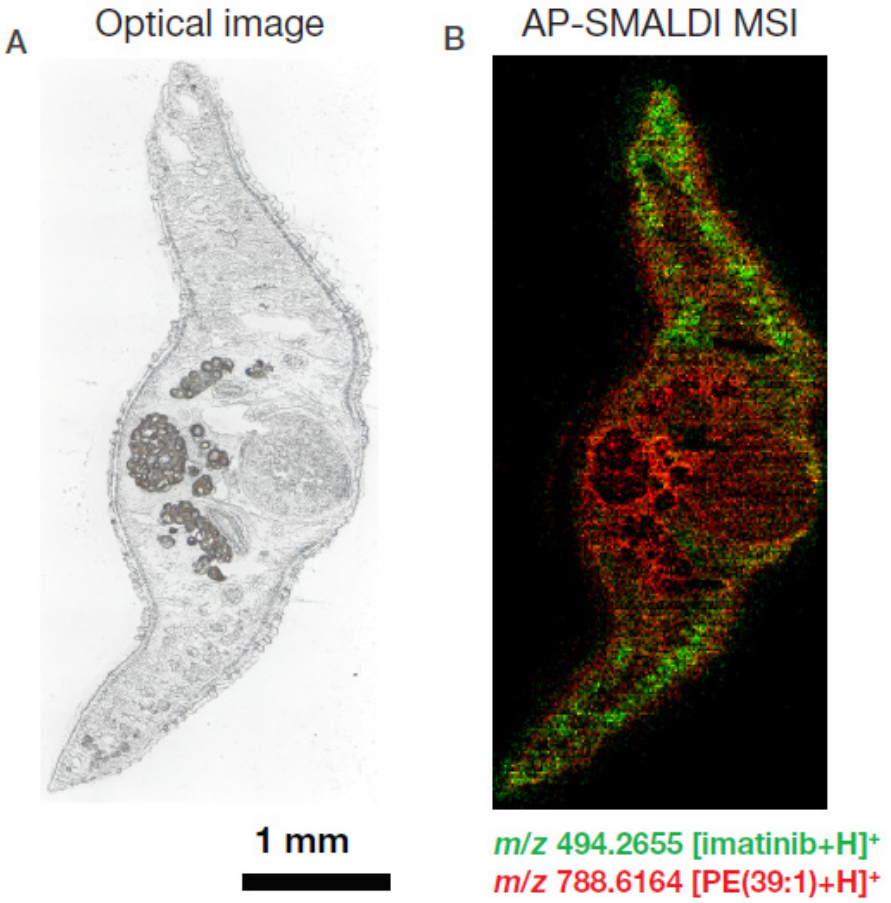

Figure 7. 2D Pixel Mode. Analysis of a cross section of a drug-dosed liver fluke *Fasciola hepatica* (worm). Imaging of anthelminthic drug imatinib in *Fasciola hepatica*. The data interpretation suggests uptake routes both orally and through the tegument (=„skin“) of the worm.

A: Optical Image

B: MS Image with two *m/z* values extracted:
drug compound imatinib, $[M+H]^+$ at *m/z* 494.2655
endogenous metabolite lipid PE(39:1), $[M+H]^+$ at *m/z* 788.6164.

For B: AP-SMALDI⁵ AF ion source and Orbitrap Exploris 240 mass spectrometer; matrix applied: 2,5-DHB.

Source settings for Imaging: acquisition in 2D Pixel Mode, applying a laser spot diameter of 5 µm, and a pixel size of 10 µm x 10 µm.

Mass Spec settings for raw file acquisition: full scan, positive ion mode, *m/z* 250 – 1000, mass resolution set to 240,000 @ *m/z* 200. Measurement speed of 1.6 pixels/s.

High Spatial Resolution Imaging – 2D Pixel Mode – on-tissue tryptic digest – 25 µm spatial resolution

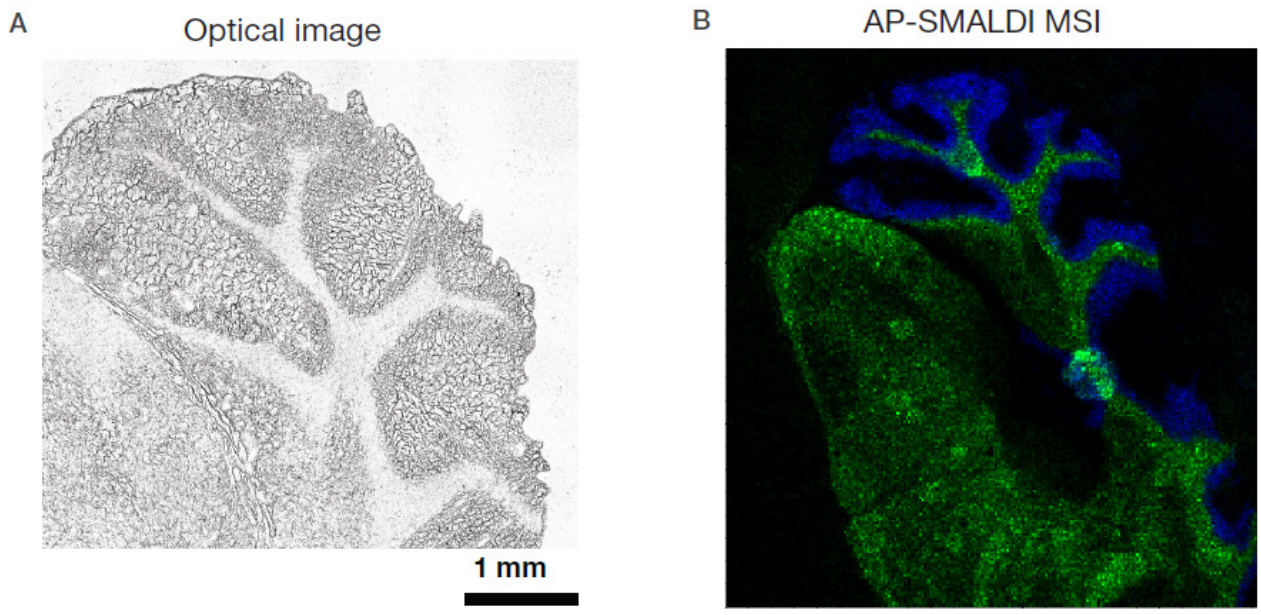

Figure 8. 2D Pixel Mode. Analysis of peptides after on-tissue tryptic digestion of mouse brain – executed by a 2 h incubation at 37 °C with trypsin prior to matrix application. Peptide in green corresponds to myelin basic protein; peptide in blue corresponds to a protein abundant in granular layer.

A: Optical Image

B: MS Image with two *m/z* values extracted:
Asn Ile Val Thr Pro Arg, $[M+H]^+$ at *m/z* 726.4041
Arg Leu Gln Asn, $[M+H]^+$ at *m/z* 530.3042

For B: MS Image obtained using AP-SMALDI[5] AF ion source and hybrid Orbitrap mass spectrometer (here: Q Exactive HF); matrix applied: CHCA. Source settings for Imaging: acquisition in 2D Pixel Mode, applying a laser spot diameter of 5 µm, and a pixel size of 25 µm x 25 µm.

Mass Spec settings for raw file acquisition: full scan, positive ion mode, *m/z* 250 – 1000, mass resolution set to 240,000 @ *m/z* 200. Measurement speed of 1.6 pixels/s.

Topographic analysis – 3D-Surface Imaging Mode – Bacillus subtilis – 5 µm spatial resolution

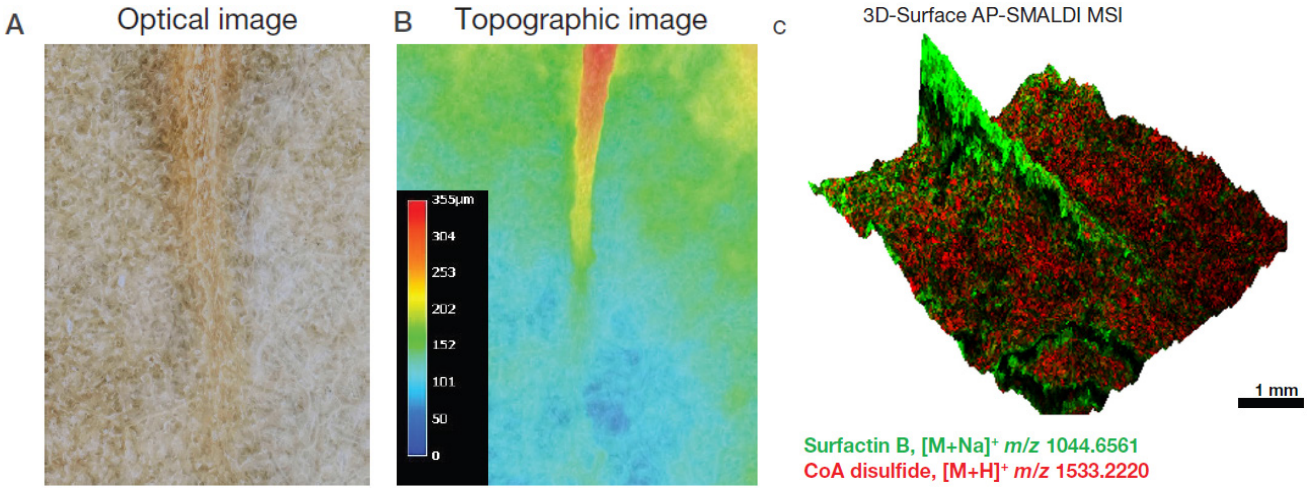

Figure 9. 3D-Surface Imaging of bacterial colonies which typically show up as 3-dimensional structures. Bacterial colonies (*Bacillus subtilis* (wildtype)) were grown on millipore filters.

A: Optical Image

B: Topographic image

C: 3D-Surface AP-SMALDI MS Image with two *m/z* values extracted:
Surfactin B, $[M+Na]^+$ at *m/z* 1044.6561
CoA disulfide, $[M+H]^+$ at *m/z* 1533.2220

For C: 3D-Surface MS Image obtained using AP-SMALDI^5 AF ion source and and hybrid Orbitrap mass spectrometer (here: Q Exactive HF MS); matrix applied: 2,5-DHB.

Source settings for Imaging: acquisition in 3D-Surface Imaging Mode, applying a laser spot diameter of 5 µm, and a pixel size of 5 µm x 5 µm.

Mass Spec settings for raw file acquisition: full scan, positive ion mode, *m/z* 400 – 1600, mass resolution set to 240,000 @ *m/z* 200.

Measurement speed of 1.0 pixels/s. Note that speed is slightly lower in 3D-Surface Imaging Mode.

The lipopeptide surfactin B (green) was found to be primarily located in the mature biofilm, which showed height variations up to 300 µm. Motile bacteria are dispersed into the environment after disruption of the biofilm matrix. Surfactin is essential for further biofilm formation. In contrast, CoA disulfide (red) is important for various metabolic processes and was primarily found in the region of biofilm maturation.

MS method application – 3D-Surface Imaging Mode – Full scan – targeted SIM scan

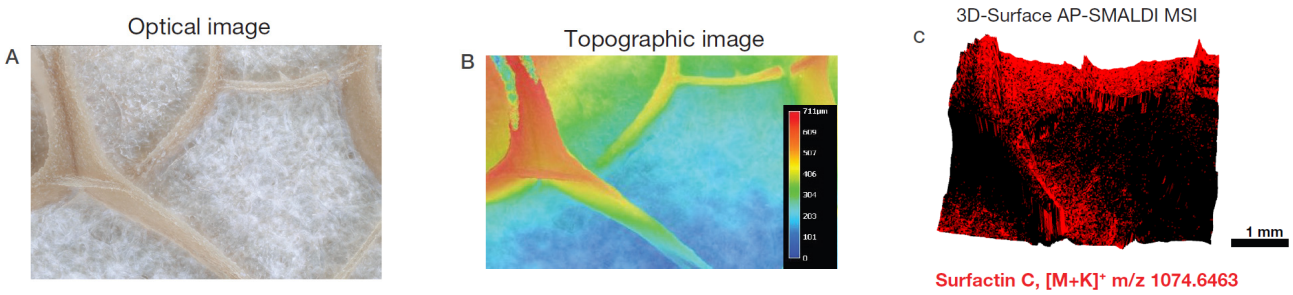

Figure 10. Analysis and 3D-Surface Imaging of bacterial colonies with applying a MS Method. For this application, two scan types were combined in a data acquisition method. A full scan and a targeted SIM scan (tSIM) were set up in Method Editor Application of the Orbitrap Exploris Instrument Control Software. In the AP-SMALDI⁵ AF ion source Instrument Control Software the user can select whether the two scans are acquired from the identical pixel or from adjacent pixels. The spectral information in the raw data file is merged with the spatial information from the autofocus feature and stored in imzML format [6]. As described in Figure 9, bacterial colonies (***Bacillus subtilis*** (wildtype)) were grown on millipore filters.

A: Optical Image

B: Topographic image

C: MS Image with *m/z* value extracted:
Surfactin C, $[M+K]^+$ at *m/z* 1074.6463

For C: 3D-Surface MS Image obtained using AP-SMALDI⁵ AF ion source and Orbitrap Exploris 240 mass spectrometer; matrix applied: CHCA.

Source settings for Imaging: acquisition in 3D-Surface Imaging Mode, applying a laser spot diameter of 5 µm, and a pixel size of 20 µm x 20 µm. Mass Spec settings for raw file acquisition: full scan, positive ion mode, *m/z* 400 – 1200, mass resolution set to 120,000 @ *m/z* 200.

tSIM scan @ *m/z* 1074.64 mass resolution set to 60,000 @ *m/z* 200.

Measurement speed of 1.0 pixels/s.

Sharp edges of up to 600 µm height difference are resolved in 3D-Surface Imaging Mode.

SUMMARY

The AP-SMALDI-Orbitrap setup allows for high spatial resolution along with high mass resolution:

a. Matrix application with crystal size below 5 µm,
b. MALDI ion production with laser spot sizes of 5 µm,
c. Subsequent Orbitrap detection (w/ or w/o an MS method applied), and
d. Data post-processing of raw data (position and MS) files for the generation of MS images.

Various tools are used to ensure high resolution in mass and space:

1. TransMIT's SMALDIPrep device delivers matrix crystal sizes below 5 µm and is, therefore, perfectly suited to go along with the 5 µm laser spot size of the AP-SMALDI⁵ AF ion source.

2. TransMIT's AP-SMALDI⁵ AF ion source coupled with Thermo Scientific Orbitrap Exploris mass spectrometers delivers information from pixels down to 5 µm in size and better than 1.5 ppm mass accuracy (internal or user-defined lock mass applied).
3. TransMIT's AP-SMALDI⁵ AF ion source and its SMALDIControl software allow to choose among different imaging acquisition modes: in the context of this Tech Note, the high-sensitivity (Full Pixel Mode) and the high-resolution imaging mode (2D Pixel Mode) as well as the 3D-Surface Imaging Mode are employed and demonstrate their respective performances.
4. Along with the Thermo Scientific Orbitrap Exploris Series Instrument Control Software, the user can combine different scan types in a method and apply it to the MS Imaging acquisition.
5. TransMIT's Mirion MS Image Generation Software with its full imzML integration is applied to generate images in post-processing manner.

CONCLUSION

Herein, application data are presented as follows:

- Drug uptake in parasites were studied for *Fasciola hepatica* liver flukes, indicating the routes of compound uptake – 2D Pixel Mode
- On-tissue tryptic digestion was investigated to image corresponding peptides at a pixel resolution of 25 µm – 2D Pixel Mode
- Study of lipid distribution in mouse brain sections – 2D Pixel Mode for high lateral resolution and Full Pixel Mode for high sensitivity data
- Bacterial colonies grown on biofilms and selected endogenous metabolites were studied using 3D-Surface Imaging Mode.

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Ethics approval

Animal experiments were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No 123; revised Appendix A) and the German Animal Welfare act. The experiments were approved by the Regional Council (Regierungspraesidium) Giessen (V54-19c20 15 h 02 GI 18/10 Nr. A16/2018).

Acknowledgements

Financial support by the LOEWE Centre for Novel Drug Targets against Poverty-Related and Neglected Tropical Infectious Diseases (DRUID) is gratefully acknowledged.

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