MALDI-imaging and Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy in an Invasive Aspergillosis Diagnostics

Tereza Juříková[1], Dominika Luptáková[3], Tomáš Pluháček[2], Miloš Petřík[2], Andrea Palyzová[1], Vladimír Havlíček[1] & Oldřich Benada[1]

[1]Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., 142 20, Prague, Czech Republic
[2]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Olomouc, CR
[3]Department of Pharmacology, Jessenius Faculty of Medicine, Comenius University Bratislava, BioMed Martin, Slovakia

Introduction

The invasive aspergillosis represents a life-threatening disease in immunocompromised patients. MALDI imaging mass spectrometry (MALDI-IMS) allows qualitative and quantitative detection and measurement of biologically important molecules and macromolecules as proteins, peptides, lipids or metabolites directly on the tissue sections. The resolution of MALDI-IMS is in the range of tens of microns and strongly depends on the laser beam energy and spot size of laser probe. Moreover, the MALDI-IMS data collection cause non-negligible sample damage (1). The average diameter of *Aspergillus* hyphae usually ranged between 3 and 6 µm that is far bellow the resolution limits of MALDI-IMS method. Although there are standard histological approaches for detection of fungal hyphae in the infected tissues as Grocott's methenamine silver staining (GMS) their resolution is not sufficient to precisely localize the fungal hyphae in the infected tissue. Therefore we started to develop a methodological approach to combine high resolution data from Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS) with images generated with MALDI-IMS or LA-ICP-MS imaging (2). This allowed us to monitor iron, silver and gold in specifically GMS-eosin stained *A. fumigatus* hyphae in the infected lung tissue.

Material and Methods

Tissue handling - The deeply LN_2 frozen native infected and control lung tissue samples were allowed to warm up to ~ -20 °C prior to the sectioning with a Leica cryomicrotome CM1950. 15 or 30 µm tissue slices were dedicated to SEM, MALDI-IMS or Laser Ablation Inductively Coupled Plasma Mass Spectrometry Imaging (LA-ICP-MS). The sections were thaw-mounted onto precooled ITO glass slides and vacuum-dried.

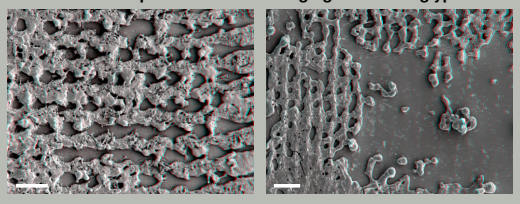
Mass spectrometry and data processing - **MALDI imaging:** Data were acquired on the 12T SolariX FTICR mass spectrometer (Bruker Daltonics, USA) equipped with a Smartbeam-II 1 kHz laser (Nd:YAG, 355 nm). The spot traces for *minimum* (~20 µm) or *small* (approx. 50 µm) laser diameter were collected five times in experiments with variable laser intensity from 15% to 50% with 5% step. **LA-ICP-MS:** An Analyte G2 LA system (Photon Machines, USA) equipped with an ArF excimer nanosecond laser (193 nm) was used for tissue ablation. Coupling of the LA and a 7700x ICP-MS (Agilent Technologies, Japan) was achieved with a Tygon^{R} (1.2 m × 4 mm) tubing. An octopole reaction cell in helium mode allowed us to overcome spectral interferences observed on ^{56}Fe.

Correlation of histological images and SEM images - For localization of infected areas in tissue sections, Grocott's staining and eosin counterstain was used. For precise localization of *Aspergillus* hyphae in SEM, optical image of stained tissue section was correlated with navigation montage image of SEM using Fiji TurboReg plugin.

Scanning electron microscopy - Dried 15 µm rat lung cryosections on ITO glass surface were examined in a Nova NanoSEM 450 (FEI, Czech Republic) high-resolution scanning electron microscope equipped with a CBS concentric backscatter detector for high-resolution imaging of nonconductive samples and an EDAX Octane Plus detector for EDS analysis and elemental mapping. The whole section was visualized using Navigation Montage option of the SEM software. The final image was then correlated with optical scan image of the same dried section. This allowed us to precisely select the areas for high-resolution imaging in the SEM and subsequent EDS analysis.

MALDI Imaging causes the sample damage - mouse kidney tissue (1)

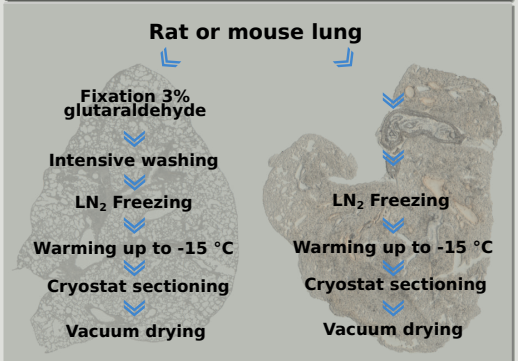
SEM of tissue samples after MALDI imaging - R-GB anaglyphs



Left: MALDI resolution 20 µm, laser beam intensity 30%,scalebar: 20 µm
Right: MALDI resolution 10 µm, laser beam intensity 30%, scalebar: 10 µm

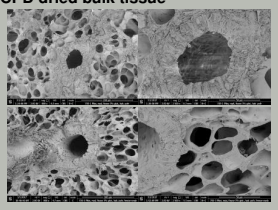
The sample preparation with respect to MALDI analysis and structural tissue preservation for high resolution imaging represents the main challenge.

Preparation of lung tissue for analyses



SEM control of tissue preservation

CPD dried bulk tissue

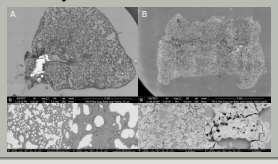


Razor cut surface of mice lung fixed in 3% glutaraldehyde. Compressed tissue artefacts are present.

LN_2 freeze-crashed surface of mice lung fixed in 3% glutaraldehyde. Well preserved structure of lung tissue.

Both samples were CPD-dried and coated with 3 nm of Pt.

Dried cryostat sections

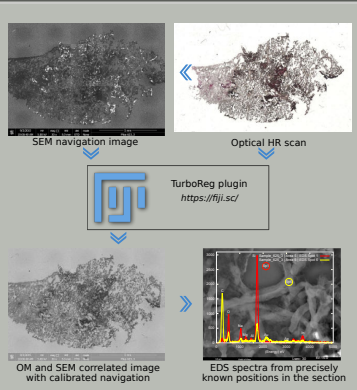


A. Mice lung fixed in 3% glutaraldehyde. Air-dried.

B. Unfixed mice lung. Dried in OsO_4 vapour.

A and B samples were mounted onto poly-l-lysine treated coverslips, dried and coated with 11 nm of carbon.

Correlation of SEM and Optical Images

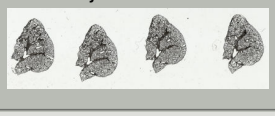


Cryostat serial sectioning of lung tissue

Unfixed tissue

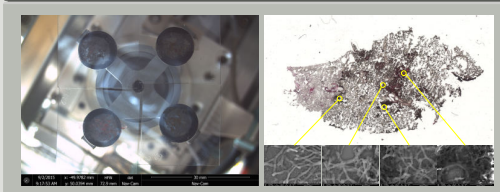
Signals in Mass Spectrometry Imaging are good, but structural preservation of the tissue is poor.

Glutaraldehyde fixed tissue



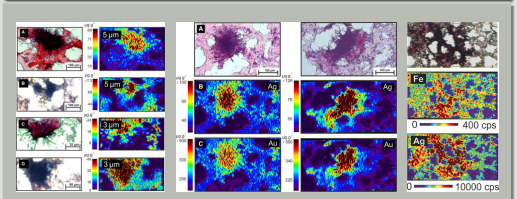
Structural details of the tissue are well preserved but fixation process still needs to be optimized to avoid noise in the Mass Spectrometry spectra

Air Dried Mouse Lung Cryostat Sections



Precise localization of *Aspergillus* hyphae in the section from infected lung tissue

LA-ICP-MS ^{107}Ag, ^{197}Au & ^{56}Fe distribution (2)



Left: ^{107}Ag distribution in the infected tissue sections with 5 or 3 µm laser focusing.
Middle: ^{107}Ag and ^{197}Au distribution in the infected tissue, 5 µm laser focusing.
Right: ^{107}Ag and ^{56}Fe distribution in the infected tissue, 10 µm laser focusing.

Conclusion

Ag-L and Au-M peaks in the EDS spectra collected from *Aspergillus* hyphae proved the specific accumulation of silver and gold into fungal bodies during the GMS-eosin staining procedure. LA-ICP-MS showed the colocalization of Fe with Ag and Au in *Aspergillus fumigatus* hyphae, however a high Fe background from hemoglobin decay products caused problems. Nevetheless, the low silver detection limit of LA-ICP-MS enabled us to decrease the actual laser spot size to 3–5 µm, which is near in size to the fungal hyphae diameter and to increase a resolution of the method.

Acknowledgement: The support from Czech Science Foundation under grant No. 16-20229S is gratefully acknowledged.

*References: 1. Krásný L, Benada O, Strnadová M, Lemr K. & Havlíček V (2015) Anal Bioanal Chem **407**(8):2141–2147*
*2. Pluháček T, Petřík M, Luptáková D, Benada O, Palyzová A, Lemr K. & Havlíček V (2016) Proteomics **16** (11–12):1785–1792*