TECHNICAL NOTE

#38 Linking *m/z* Protein Images with Proteomics Data

Application

Moving forward from traditional histological and western blotting techniques, the visualization of biomolecules in tissues can now be supplemented by specific molecular identification and localization thanks to the highly sensitive discovery tool of MALDI Imaging Mass Spectrometry (MALDI IMS).

MALDI IMS consists of coating a fresh frozen or fixed tissue sample with a MALDI matrix that allows for desorption and ionization of the molecules present. A laser is then irradiated on the tissue at discrete points, thus generating an *m/z* spectrum across the x and y coordinates. Ion images can then be matched with an image of the same stained tissue, therefore granting valuable information of the spatial-anatomical distribution of the molecule of interest. In particular, MALDI IMS of intact proteins is of relevance, as many of the protein isoforms (proteoforms [1]) can be identified and spatially resolved.

Confirmation of protein identity remains to be one of the biggest challenges in proteomic research, with several strategies developed including bottom-up, top-down and indirect identification. The highest performance on mass resolution are provided by the Fourier transform ion cyclotron resonance (FTICR, [2]) and orbital trapping (Orbitrap, [3]) mass analyzers. However, due to their *m/z* range limitations, these instruments have not yet been utilized for MALDI IMS of intact proteins.

Intended Use Of This Technical Note

This technical note describes the utilization of MALDI FTICR MS for the generation of images of endogenous intact proteins of up to 12 KDa, with a mass accuracy of <5ppm. HTX, the manufacturers, and the users that have accepted to share their data do not guarantee the performance of the work-flow, and each lab is responsible for applying the corresponding health and safety regulations. For research purpose only.

Background

Immune cells have the capacity to attack pathogens via a wide range of strategies including reactive oxygen species (ROS) generation and chelation of metals that are essential for bacterial proliferation and antioxidant defense [4]. Calprotectin is a Zn and Mn-binding protein heterodimer, highly abundant in neutrophils, with antimicrobial and antifungal properties [5] based on the sensitation of the bacterium to superoxide oxidative damage

In the infectious foci, calprotectin is in close proximity to ROS-generating proteins, thereby being exposed to high levels of oxidative stress. Understanding the consequences of oxidative modifications on protein function of S100A8 is of high relevance to gain insights into host-pathogen interactions.

As a proof-of-concept case study, here the user was interested in imaging the various proteoforms of a particular subunit of calprotectin (S100A8), in kidney tissue from mice infected with Staphylococcus aureus.

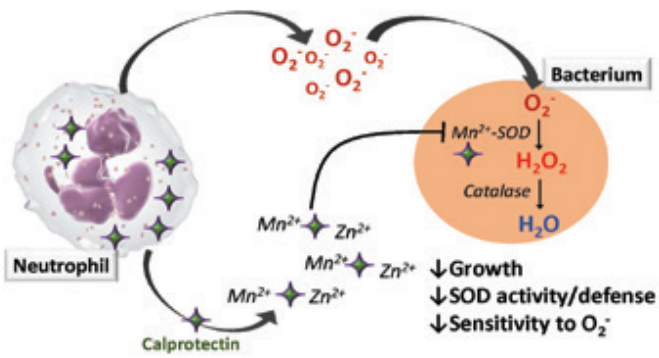

Figure 1. Adapted from [5], inhibition of bacterial processes through Mn^{2+} chelation by calprotectin. At the site of infection, neutrophils deliver a "double hit" to S. aureus by releasing calprotectin (crosses), which chelates Mn^{2+} and Zn^{2+}, thus sensitizing S. aureus to ROS generated by the neutrophil.

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Imaging Workflow

10 µm cryosections of mouse kidney were placed onto conductive ITO coated slides (Delta Technologies). Tissue was then washed with 70% EtOH for 30 sec, 100% EtOH for 30 sec, Carnoy fluid (6:3:1 EtOH: chloroform: acetic acid) for 2 min, 100% EtOH for 30 sec, H2O with 0.2% TFA for 30 sec, and 100% EtOH for 30 sec, and stored at -80 °C until IMS analysis.

Tissue sections were sprayed using an HTX TM-Sprayer (HTX Technologies), as described below:

Flow Rate	0.15 mL/min
Spray Nozzle Velocity	1300 mm/min
Spray Nozzle Temperature	30°C
Track Spacing	2 mm
Number of Passes	4, with offsets, rotations
Nitrogen Pressure	10 psi (0.7 bar)

Images were collected with a laser setting of ~50 µm and a pixel spacing of 75 µm in x and y axis, over a mass range of *m/z* 1,000 to 15,000. Calibration was performed externally using CsI clusters.

Experimental Summary

Tissue type	Mouse kidney
Preservation	Mouse kidney
Tissue cut	10 µm thickness
Matrix deposition	DHA (15 mg/mL) in 8:1:1 acetone:H_2O: acetic acid
MALDI Laser	Smartbeam II 2KHz Nd:YAG
Acquisition mode	Linear positive ion

Instrumentation and Supplies

Cryostat	Cryostar™ Thermo NX70
MALDI plate	ITO coated slides
Matrix	Fisher Scientific #114810250
Matrix Sprayer	HTX TM-Sprayer™
MALDI MS	BRUKER Solarix 15T FTICR MS
Imaging software	BRUKER Daltonics flexImaging 4.1

Results and MALDI MS Images

Fig. 2 shows MALDI FTICR IMS of intact proteins obtained from a mouse kidney infected with S. aureus. Data were collected with a resolving power of ~75,000 at *m/z* 5,000, resulting in 2552 peaks detected between *m/z* 2,000 and 12,000. Ions up to *m/z* ~12,000 were detected with high sensitivity. The average mass spectrum of the entire data set for a wild type (Wt), and a calprotectin-knock-out (KO) mouse infected with S. aureus are shown in Fig. 2A. The protein subunit S100A8 from the heterodimer calprotectin was detected at *m/z* 10,1643.03 ([M+H]+1, -2.1 ppm) only in the Wt, and was identified by top-down fragmentation.

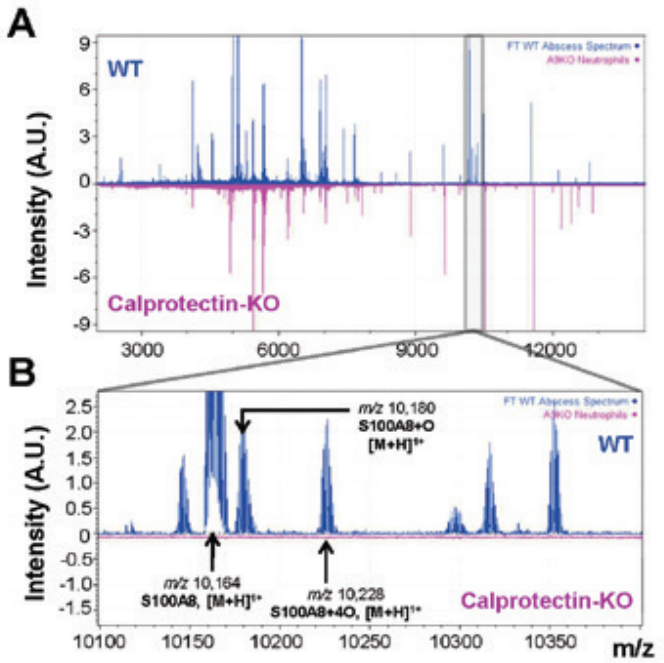

Figure 2. MALDI FTICR IMS of intact proteins from mouse kidney tissue of wild-type (blue), or calprotectin-KO (pink), infected with S. aureus. Images were taken from a lesion where neutrophils accumulate, in positive ion mode.

A closer view within the *m/z* range 10,160 – 10,320 allowed to identify several potential proteoforms of S100A8. Fig. 2B shows the S100A8 peak, as well as two of its oxidation products S100A8+O (*m/z* 10,180.07, [M+H]1+), and S100A8+4O (*m/z* 10,228.00, [M+H]1+).

A traditional H&E stain of the tissue is presented in Fig. 3A for spatial correlation. The ion images for a series of biologically-relevant proteins (ubiquitin, thymosin, histones H4 and H2A1) and S100A8 are shown in Fig. 3B. Note the higher localization of S100A8 in the infectious loci.

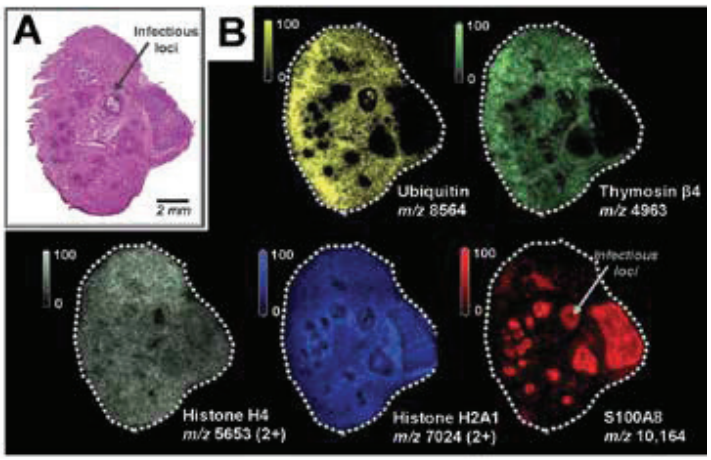

Figure 3. Selected MALDI FTICR MS ion images of intact proteins from mouse kidney infected with S. aureus. *m/z* 8,564 (ubiquitin, yellow), *m/z* 4,963 (thymosin 4, green), *m/z* 5,653 (histone H4, grey), *m/z* 7,024 (histone H2A1, blue), *m/z* 10,164 (S100A8, red). ~75 µm spatial resolution. Acquisition time ~4 hrs. Scale bar 2 mm.

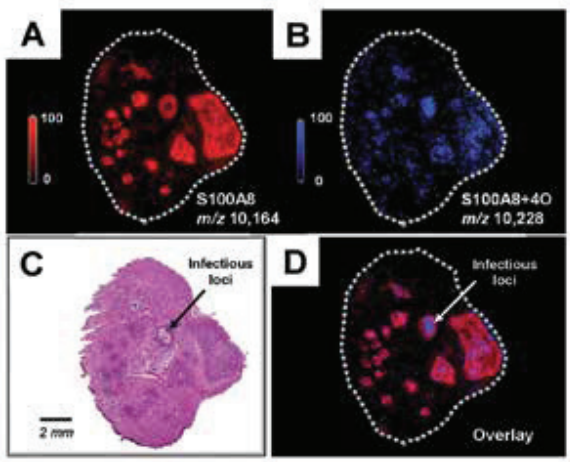

Figure 4. Selected MALDI FTICR MS ion images of (A) intact S100A8 (red, m/z 10,164) and (B) its oxidation product S100A8+4O (blue, m/z 10,228) from mouse kidney infected with S. aureus. (C) Shows the H&E stain, same as Fig 3A. (D) Two color overlay; m/z 10,164 (red), m/z 10,228 (blue). ~75 µm spatial resolution. Acquisition time 2 sec / pixel, total ~4 hrs. Scale bar 2 mm.

Representative ion images for S100A8 and one post-translational oxidation product are shown in Fig. 4A and B, respectively. By comparing the H&E stained micrograph with the MALDI FTICR IMS overlay data (Fig. 4D), the oxidative form of S100A8 shows to accumulate near the center of the infectious foci, suggesting highly oxidative processes occur at the host-pathogen interface.

Finally, to confirm that the observed ion at *m/z* 10,228.00 is an oxidation proteoform of S100A8, as well as to identify the specific AA residues oxidized, top-down and bottom-up strategies were employed for identification (Fig. 5). The tryptic peptide shown in Fig 5 was bottom-up fragmented and presented oxidation of the methionine 37 and cysteine 42 residues.

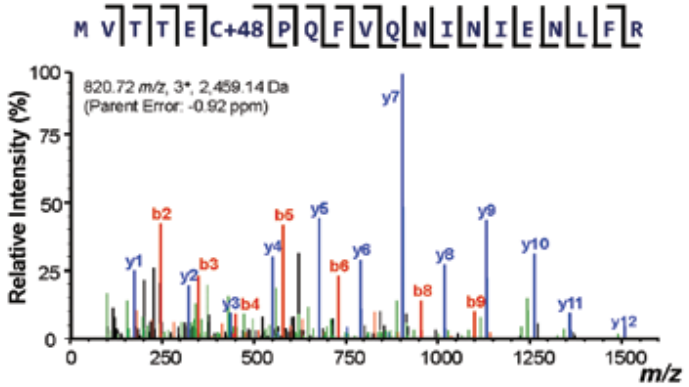

Figure 5. . LC-MS/MS results for S100A8 – M37O/C42O3. High-mass resolution bottom-up fragmentation data for the tryptic peptide shown above.

MALDI FTICR IMS experiments such as the ones presented in this study are highly valuable for immunological biology given that post-translational modification of proteins (i.e. oxidation) play a key role in the immune response.

Conclusions

Protein identification coupled to MALDI IMS has remained a significant challenge, due to the limited mass accuracy and low resolving power. MALDI FTICR IMS significantly advances he field of molecular imaging by combining MALDI IMS and LC-MS/MS at their optimum performance.

Notes

References

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Acknowledgements

This Technical Note, including figures and illustrations, was produced by Maria J. Torres, East Carolina Diabetes and Obesity Institute, East Carolina University, based on a consultation with Jessica Moore, co-author of the study published in [7]. Tissue images and MS data were provided courtesy of Jessica Moore, and Jeff Spraggins, Mass Spectrometry Research Center, Department of Chemistry, Vanderbilt University School of Medicine, Nashville, TN, USA.