Localization and Identification of Peptides from Tissue using high-speed MALDI-TOF/TOF mass spectrometry

Introduction
MALDI Imaging is an analytical method for the detection of potential biomarkers directly from tissue sections that has gained popularity over the past decade. The development protocols for spatially resolved digests are of particular interest. Most importantly, on-tissue digestion allows the analysis of peptides from formalin-fixed paraffin-embedded (FFPE) tissue, the most common type of sample in clinical pathology. In addition, it allows detection of larger proteins using peptides as proxies and facilitates biomarker ID by means of MS/MS.

We established our previously described ImageID workflow (combining LC-MALDI and MALDI Imaging) on a novel MALDI-TOF/TOF instrument with a 10 kHz laser. Acquisition time is reduced by a factor of 5-10, allowing an entire experiment to be completed in a single workday. At this rate, ImageID becomes a powerful tool to evaluate and optimize on-tissue digestion protocols.

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MALDI imaging  TM-Sprayer
LC-MALDI  flexlImaging software
FFPE tissue  flexlImaging software
Methods

Tissue sections (fresh-frozen or FFPE) were mounted on ITO-slides. Two serial sections were mounted on the same slide to conduct imaging and LC-MALDI experiments in parallel on comparable samples. Trypsin for spatially resolved digestion was applied using a TM-Sprayer (HTX Technologies, USA) or ImagePrep (Bruker Daltonik, Germany). After digestion, one section was coated with matrix using the same device and imaged on a rapifleX™ (Bruker Daltonik).

Peptides were extracted from the neighboring section with 20µl of 0.1% TFA and separated by nano-LC (using an 60 min gradient split into 384 fractions). The LC-MALDI analysis was conducted on a rapifleX™ in TOF/TOF configuration. Imaging and LC-MALDI data were combined based on intact peptide masses after statistical recalibration of all spectra using the ImageID software, resulting in annotated and localized peptides (Fig. 1). In addition, selected peptide IDs were confirmed by on-tissue MS/MS (Fig. 3).

Fig. 1: ImageID workflow. After matching LC-MALDI and MS Imaging data, results are available as flexImaging filter lists, grouping all peptides detected for each individual protein.

Typical experiment time is 8 hours, not including antigen-retrieval for FFPE tissue. (4h for sample preparation, 1h for MALDI Imaging (Rat brain coronal section at 50 µm pixel size ~35,000 MS spectra) and 3h for LC-MALDI (384 fractions, ~5000 MS/MS spectra).
Results

Fig. 2: **ImageID results.** LC-MALDI analysis of peptides extracted from a coronal rat brain section achieved a 97.9% percent sequence coverage of Myelin basic protein, an abundant brain protein, with 42 peptides identified (A). 30 of these peptides were also matched to the corresponding MALDI image. Distribution of six peptides (B-G) is shown, which were all confirmed by on-tissue MS/MS.

For added confidence, we calculated the Pearson correlation matrix for these six images, indicating a high co-localization of all peptides (H).

Fig. 3: On-tissue MS/MS. Peptides identified by LC-MALDI (A, B) were confirmed by on-tissue measurements. A consecutive section was digested under identical conditions and peptide distributions visualized by a quick (15 min) imaging experiment at 100 µm pixel size (C, D). Although not clearly separated in the overall spectrum (E), two peptides (Δm/z = 3.077) could be clearly discriminated by on-tissue MS/MS at high intensity pixels (F) using the rapifleX (G,H).

Tab. 1: Mass accuracy on-tissue. Identifying peptides by ImageID allowed us to assess mass accuracy in MS mode.

With external calibration, the rapifleX achieves a solid mass accuracy of ~25 ppm. Statistical recalibration (according to Mann et al., 1993) corrects larger errors improving mass accuracy to ~7ppm on average. All calculations were performed on the average spectra of an entire imaging run.

<table>
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<th>Calculated Peptide m/z</th>
<th>External Calibration</th>
<th>Δ m/z</th>
<th>Δ ppm</th>
<th>Statistical Recalibration</th>
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<th>Δ ppm</th>
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Fig. 4: Fresh-frozen vs. FFPE tissue. ImageID is a powerful tool to evaluate on-tissue digests. Here, we compare fresh-frozen and FFPE rat brain tissue. Except for antigen retrieval (20 min at 110 °C, pH 6), digest and matrix application (using a TM-Sprayer) were identical. The lower number of peptides generated on FFPE tissue (376 or 37% compared to fresh-frozen) indicates digestion is less efficient (A, B). In addition, antigen retrieval introduces further delocalization of peptides as seen by the slight loss of lateral resolution in the FFPE experiment (D, F) when compared to fresh-frozen (C, E). Matching LC-MALDI data to the imaging data using ImageID, we could assign only 23% (235, fresh-frozen) and 57% (214, FFPE) of LC-MALDI peptides to peaks in the imaging data, indicating that extraction and incorporation of peptides during matrix application requires improvement.

Fig. 5: ImagePrep vs. TM-Sprayer. Comparison of on-tissue digestion and matrix application using serial sections of human breast cancer tissue (FFPE). Best practice protocols were used on both devices by experienced users, all MS data was acquired under identical conditions on a rapifleX. Digestion and matrix application steps appear to be less efficient using the TM-Sprayer, indicated by the lower number of peptides identified by LC-MALDI (370 peptides or 49% compared to ImagePrep) (A,B) and the lower percentage of peptides matched to the imaging run (136 or 37% vs. 399 (53%). At higher peptide masses, the ImagePrep shows more and higher intensity signals (C, D). In contrast, the TM-Sprayer provides a substantial improvement in lateral resolution. Peptide distributions (E, F) can be matched to corresponding tissue features more precisely (G, H).
Summary

We have transferred the ImageID workflow to the next generation MALDI TOF/TOF platform. Good mass accuracy in MS mode, combined with high quality imaging and LC-MALDI data allows confident matching of peptide IDs and distributions. In addition, the high MS/MS performance of the rapifleX™ facilitates direct analysis of peptides from tissue, for further validation of ImageID matches.

We have used ImageID to evaluate the performance of on-tissue digestion protocols, decoupling the digestion and the matrix deposition step of the protocol using the number of identified and assigned peptides respectively as useful readouts.

Acknowledgment

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Conclusions

- The ImageID workflow was adapted to rapifleX™
- The entire workflow including tissue imaging and peptide identification was completed in one workday
- On-tissue MS/MS was used to validate ImageID results
- The ImageID workflow is an efficient tool for on-tissue digest evaluation