High-performance MALDI imaging analysis of on-tissue digested proteins in mammalian FFPE tissues using the Bruker rapifleX MALDI-TOF/TOF

In this application note, we describe a workflow for MALDI Mass Spectrometry Imaging (MSI) of proteins from a variety of FFPE tissues. The method comprises steps of paraffin removal and antigen retrieval followed by on-tissue digestion and deposition of MALDI matrix.

MALDI imaging of the resulting tryptic peptides is performed using the Bruker rapiflex MALDI-TOF/TOF instrument. SCiLS Lab statistical software was used to highlight regionally specific peptides which can be subsequently identified by direct interrogation with MALDI-TOF/TOF and database searching. An added dimension of molecular specificity is demonstrated by imaging the tissue using TOF/TOF mode and spatially mapping characteristic fragment ions of target peptides.

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Introduction

Formalin-fixation followed by paraffin embedding (FFPE) is the standard for preserving tissue samples. Many institutions such as hospitals and universities have assembled extensive tissue libraries with corresponding patient information. Therefore, FFPE tissue represents a particularly important type of sample to be dealt with in mass spectrometric imaging (MSI).

Formalin cross-links primary amine groups of protein backbones while paraffin embedding allows for easier sectioning and infinite storage of samples at room temperature. Both processes, however, are inherently incompatible with MALDI, but additional sample processing can be carried out to make FFPE samples amenable for MALDI imaging analysis:

- Paraffin must be removed as it would otherwise interfere with subsequent steps in the sample prep workflow as well as the ionization.
- Antigen retrieval (AR) must be performed to disrupt a portion of the cross-linked protein network to open cleavage sites for endoproteases such as trypsin.
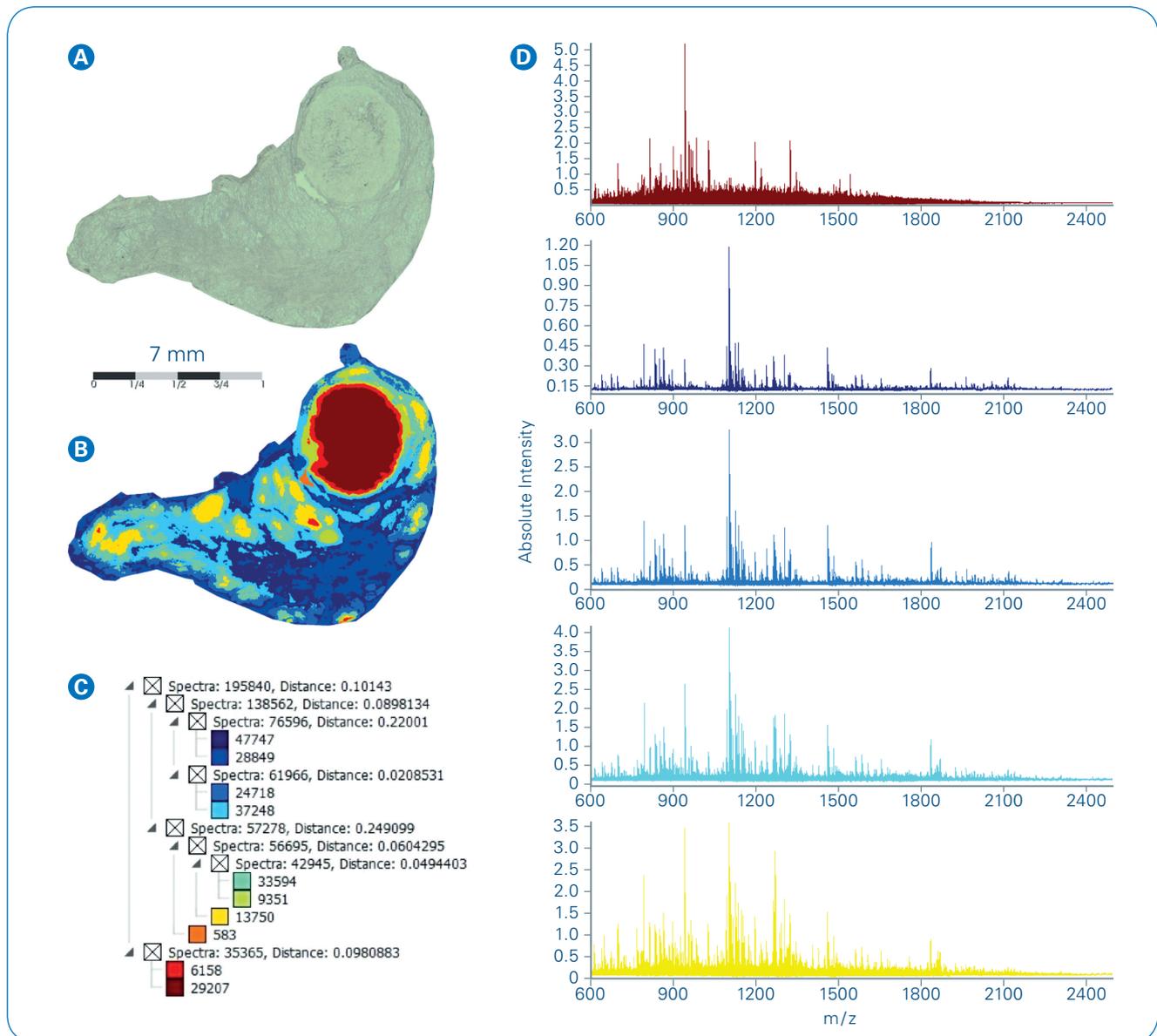

Figure 1: Spatial segmentation analysis of MALDI-MSI dataset from FFPE human thyroid tissue:
A *Optical image* **B** *Segmentation map* **C** *Segmentation tree* **D** *Mean spectra of main regions extracted from segmentation result*
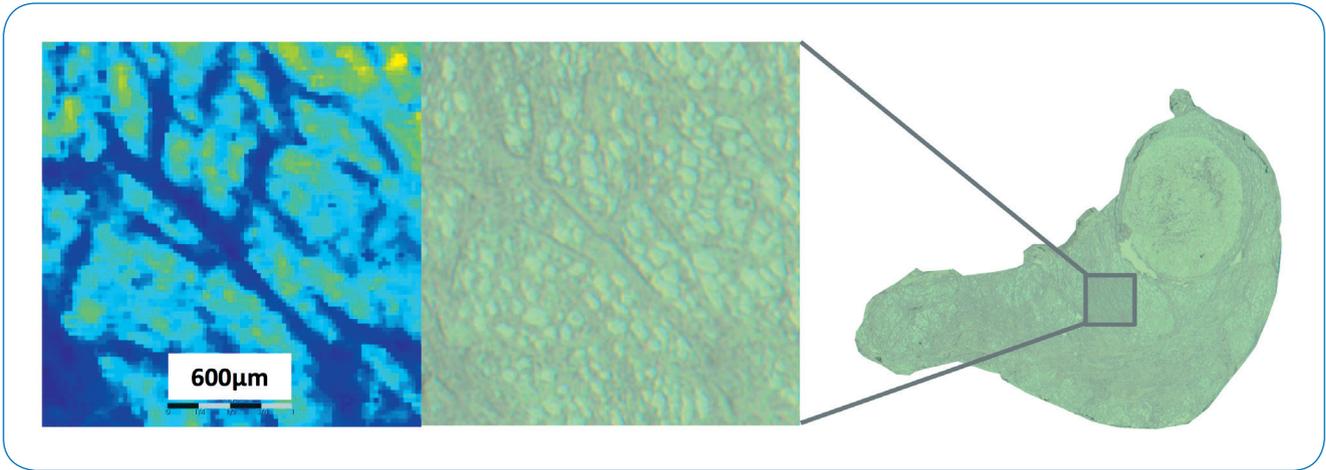
Figure 2: An expanded view of an ion image of m/z 644.4 from a region of human thyroid FFPE tissue to assess the MSI spatial resolution achieved using the sample analysis workflow described. Smallest structural features observed in the MSI dataset are approximately 40-50 µm, i.e. 2 pixels wide.

- Finally, enzymatic digestion is necessary to liberate peptides from the remaining cross-linked protein.

In this application note, we describe a protocol for preparing FFPE tissue sections for MALDI imaging analysis and apply it to various tissues (human thyroid; human breast cancer; mouse brain) using the rapifleX MALDI-TOF/TOF. Analyses of the MSI data using SciLS Lab reveals many of the digested peptides to have distributions specific to tissue pathology. Further, these peptides can be selected for fragmentation by TOF/TOF to identify the originating protein behind.
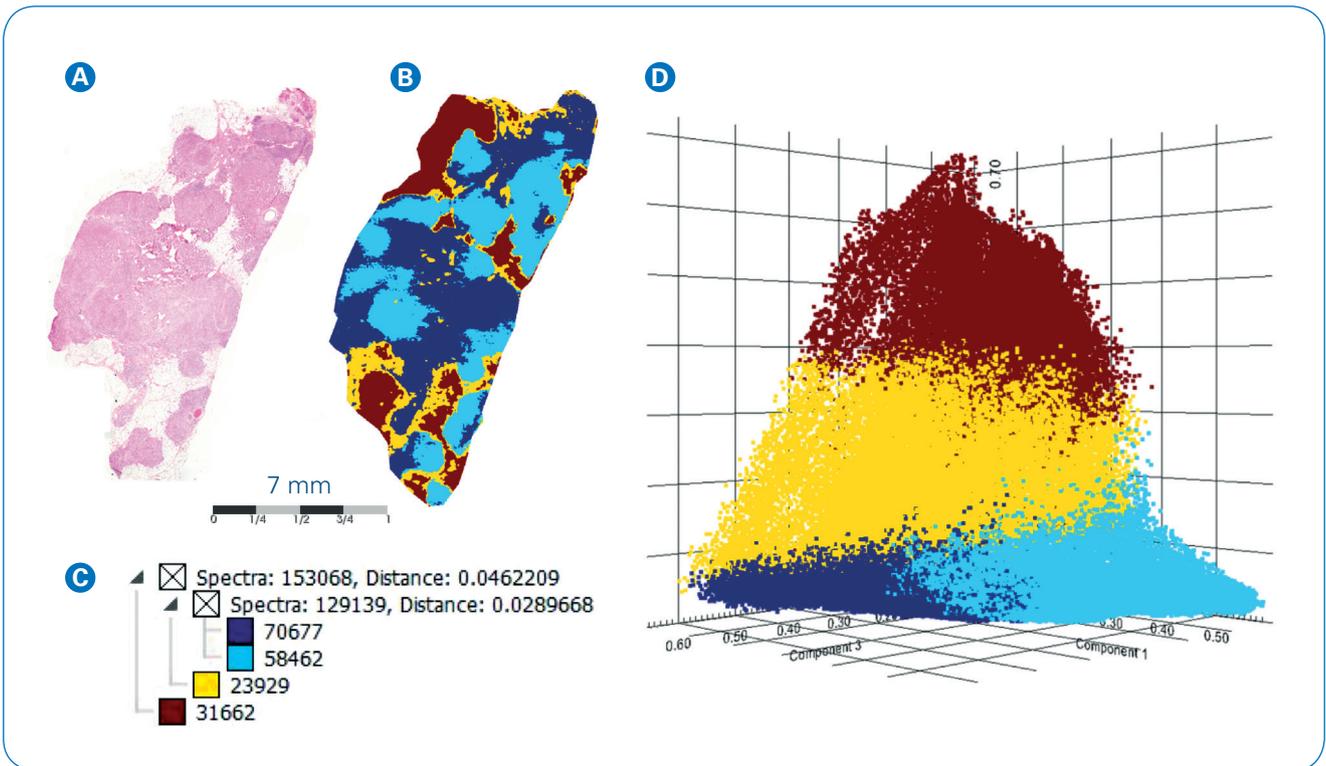

Figure 3: MALDI-MSI analysis of FFPE human breast cancer tissue:
A *Co-registered H&E image of tissue section stained post-MALDI* **B** *Spatial segmentation map* **C** *Corresponding segmentation tree* **D** *PLSA Scores plot from the four main segmentation regions*
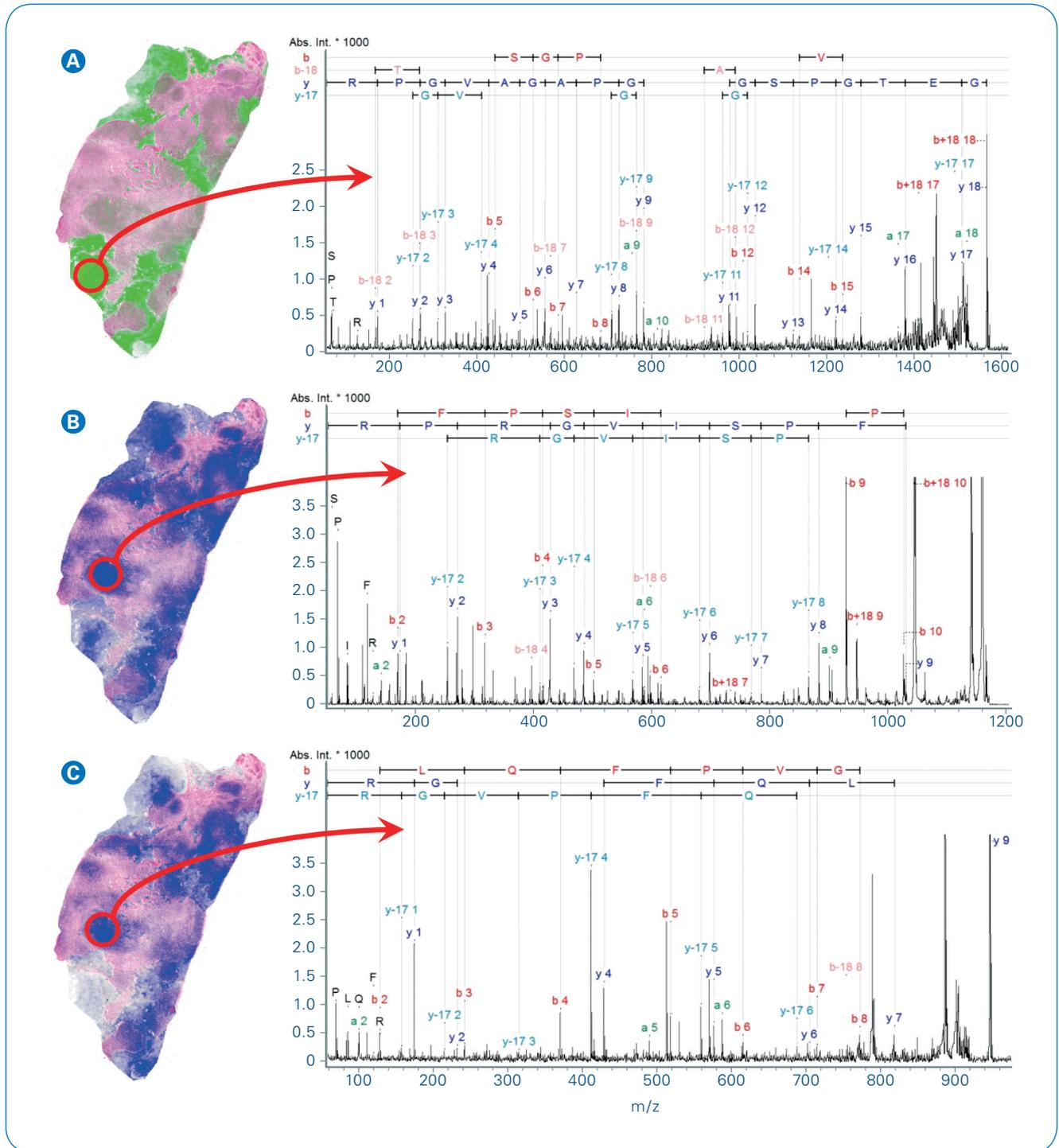
Figure 4: Selected MALDI ion images from FFPE human breast cancer tissue overlayed with co-registered H&E. Peptide identification was achieved by MALDI-MS/MS directly from tissue.

A m/z 1562.8; GETGPSGPVGPAGAVGPR from Collagen alpha 2 **B** m/z 1198.7; AVFPSIVGRPR from Actin, cytoplasmic 1 **C** m/z 944.5; AGLQFPVGR from Histone H2AV

Experimental

Samples

FFPE tissue sections were mounted on conductive slides precoated with polylysine. Thickness of tissue sections was as follows: human thyroid 6 μm; human breast cancer 3 μm; mouse brain 5 μm. Human thyroid sections were provided by Prof. Fulvio Magni, University of Milano-Bicocca, Italy. Human breast cancer sections were obtained from Dr. Zeljko Debeljak, KBC Osijek, Croatia.

Sample preparation

Paraffin removal was carried out applying the following protocol:
Slides were vertically oriented and heated at 85 °C for 15 min; slides were rinsed in a succession of baths: xylene (5 min, 2x; first bath was performed immediately after removing slide from oven); isopropanol (5 min, 1x); ethanol gradient (100%, 96%, 70%, 50%, 5 min each); water (5 min, 1x).

Antigen retrieval was performed in a NXGEN decloaking chamber (Biocare Medical) at 110 °C for 20 min using only deionized water as bath. Processed slides were gently cooled down by stepwise partial exchange of the hot bath solvent and were dried under vacuum.

Deposition of trypsin (porcine sequencing grade; Promega) was performed using a HTX TM-sprayer connected to a syringe pump. The deposition method used the following parameters: trypsin solution 25 ng/μl in 20 mM ammonium bicarbonate buffer; nozzle height 40 mm; nitrogen pressure 10 psi; nozzle temperature 30 °C; flow rate 0.03 ml/min; z-arm velocity 750 mm/min; number of passes 8; moving pattern CC; track spacing 2 mm; drying time 0 s.

Sections coated with trypsin were incubated for 2 hours at 50 °C under humidity-saturated atmosphere. After incubation, slides were dried under vacuum for 30 minutes.

Deposition of HCCA matrix was performed on a HTX TM-sprayer connected to an isocratic LC pump applying the following method parameters: HCCA solution 10 mg/ml in ACN:H_2O 70:30, 1% TFA; nozzle height 40 mm; nitrogen pressure 10 psi; nozzle temperature 75 °C; flow rate 0.12 ml/min; z-arm velocity 1200 mm/min; number of passes 4; moving pattern HH; track spacing 3 mm; drying time 0 s.

After MALDI data acquisition, sections were prepared for histological staining by removing the remaining MALDI matrix by washing in 70% ethanol for 2 min and subsequent dip-washing in deionized water.
Haematoxylin and Eosin (H&E) staining was performed according to common protocols.

Data acquisition

Prior to deposition of MALDI matrix, slide-mounted tissue sections were scanned on a Reflecta MF-5000 medium format scanner at a resolution of 3200 dpi.
Additional high-resolution optical images of H&E stained tissue sections were acquired on a MIRAX DESK scanner (3DHISTECH) after finishing MALDI data acquisition.

All MALDI-MS imaging data were acquired on a Bruker rapifleX MALDI-TOF/TOF system in positive reflector mode. The following MS image acquisition parameters were applied:

- Human thyroid:
  20 μm pixel size; 300 laser shots per pixel; m/z 600 – 2500 detection range
- Human breast cancer:
  25 μm pixel size; 400 laser shots per pixel; m/z 700 – 3200 detection range
- Mouse brain:
  20 μm pixel size; 400 laser shots per pixel; m/z 700 – 2400 detection range

MALDI-MS/MS spectra of selected peptides were acquired directly from tissue in positive MS/MS mode without the use of collision gas. The number of laser shots accumulated per spectrum varied depending on signal to noise ratio of each peptide. MS/MS imaging of selected precursor peptides was performed using 40 μm pixel size and 1000 laser shots per pixel.

Data analysis

Analyses of imaging data, including co-registration of H&E stained optical images, was performed in SCiLS Lab software. Calculations were performed on datasets normalized to Total Ion Count (TIC).

Results and Discussion

The FFPE sample processing workflow described above was applied to a variety of tissue samples and data analyzed for spatial resolution, correlation with tissue architecture and ability to identify proteins. Figure 1 shows results obtained from MALDI-MSI of human thyroid FFPE tissue. The dataset was spatially segmented in SCiLS Lab using an unsupervised multivariate statistical analysis method. The segmentation map (Figure 1B) attributes similar digest spectra to a common color and the color mapping reveals tissue heterogeneity as function of protein fingerprint, i.e. as reflected in the individual peptide profiles resulting from on-tissue digestion. The segmentation tree shown in Figure 1C, provides an overview over the hierarchical structure of pixel cohorts within the MSI dataset, i.e. describes the level of similarity/dissimilarity between various pixel cohorts. Figure 1D presents in a side-by-side comparison the mean spectra representing five main branches (i.e. proposed tissue subtypes) from the segmentation result.

The FFPE sample preparation workflow described here involves a number of steps e.g., incubation with the enzyme and matrix deposition, that bear a certain risk of artificial delocalization of peptides. To assess the spatial resolution that can be achieved using the workflow, Figure 2 shows a zoomed view on the human thyroid tissue section along with the corresponding ion image of m/z feature 644.4. The architectural features visible in this ion image are highly consistent with the structures visible on the corresponding optical image. Further examination reveals that the minimum dimension of structural feature that can be resolved in the image is 40-50 µm, i.e. 2 pixels wide. In a second example, the workflow

was used to image a section of FFPE human breast cancer tissue and results are presented in Figure 3. As before, spatial segmentation analysis of the breast cancer tissue produced the map given in Figure 3B. Together with the corresponding hierarchical cluster tree (Figure 3C), and the overlayed H&E image (Figure 3A), the segmentation map indicates a clear differentiation between section areas that represent low density, i.e. fatty and connective tissue (yellow and brown colors) and the higher density tumor tissue (light and dark blue colors), respectively. To cross-validate the segmentation results, the dataset was subjected to Probabilistic Latent Semantic Analysis (PLSA), another method of unsupervised multivariate data analysis that deconvolutes pixel spectra into contributing molecular signatures. On the resulting PLSA scores plot, shown in Figure 3D, the image pixels differentiate into virtually non-overlapping clusters that correspond to the four main branches of the segmentation tree (i.e. low density tissue colored yellow/brown vs. higher density tumor tissue colored light/dark blue). The striking consistency observed when comparing the results obtained from two independent statistical analysis methods illustrates the high level of confidence featured by the MALDI-MSI data.

Statistical analysis of MSI data can yield a number of putative tissue marker ions. The next step is to identify the putative peptide and the protein from which it derived. First choice for such task is tandem-mass spectrometry of the peptides directly from tissue. Figure 4 shows MALDI-TOF/TOF fragment ion spectra of three peptides that were acquired directly from tissue regions of highest local appearance. Precursor m/z 1562.8 (Figure 4A) was selected for MS/MS analysis as an example of peptides showing high relative

intensity in the low density tissue regions, whereas the other two precursors (m/z 1198.7, m/z 944.5, Figure 4B-C) were detected at elevated relative peak intensities in the higher density tumor tissue. MASCOT ion searches identified all three peptides at highly significant ion score values.

With an estimated 10,000 proteins expressed in each cell, on-tissue digestion produces a highly complex mixture of analytes. We should expect cases in which multiple nominally-isobaric peptides will cause undesired interferences in MS ion images. To eliminate such interferences, the complete imaging experiment can be performed in MS/MS mode to confirm the results obtained from MS imaging. Figure 5 shows TOF/TOF imaging results obtained from FFPE mouse brain tissue. Figure 5B shows the MS ion image of m/z feature 1198.7, which was identified using MALDI-MS/MS directly from tissue as peptide AVFPSIVGRPR from Actin, cytoplasmic 1 (Figure 5C). A subsequent MS/MS imaging experiment was made to confirm the distribution of this peptide. The peptide´s most abundant fragment ion $[b+18]_{10}$ at m/z 1042.4 (Figure 5E) yields an ion image (Figure 5D) that is consistent with the MS image obtained for m/z 1198.7. The consistency observed between MS and MS/MS imaging results indicates the dominance of the identified actin peptide in its contribution to the MS ion image and excludes any significant interference from other isobaric peptides.
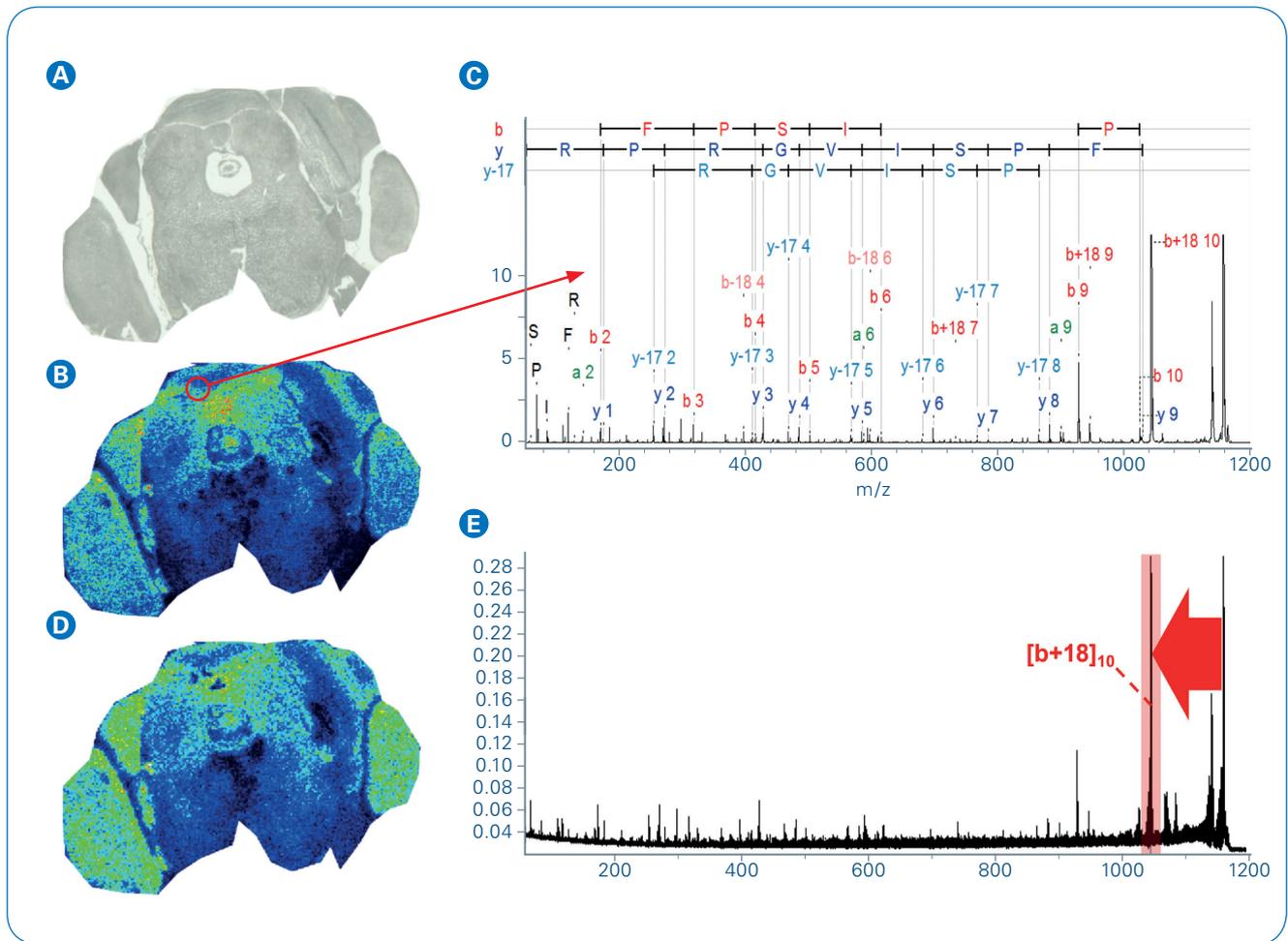
Acknowledgements

Figure 5: MALDI-MSI analysis of FFPE mouse brain tissue:
A Optical image **B** MALDI-MS ion image of m/z 1198.7 **C** Annotated MALDI-TOF/TOF spectrum of precursor m/z 1198.7 that was acquired directly from tissue and identified as peptide AVFPSIVGRPR from Actin, cytoplasmic 1 **D** MALDI-MS/MS ion image of m/z 1042.6, the most abundant fragment ion ([b+18]10) from precursor m/z 1198.7 **E** The mean spectrum from dataset D, red arrow indicates the MS/MS transition used to generate the MS/MS ion image

Conclusions

- A dedicated workflow has been described for preparing samples for MALDI imaging and sophisticated data analysis of FFPE tissue samples, the most commonly found type of tissue sample. Using the protocol for deparaffinization, antigen retrieval and on-tissue digestion, FFPE tissue can be made compatible with both MALDI MS and MS/MS imaging facilitated by the new Bruker rapifleX MALDI-TOF/TOF instrument. Spatial feature resolution of 40-50 μm, limited by delocalization effects inherent in the upfront sample preparation workflow, can be achieved. RapifleX data acquisition is much faster than on any previously available MALDI-MS instrument and integration with SCiLS Lab software allows one to extract highly confident and detail-rich information from the dataset.

- The outstanding MS/MS capabilities of the rapifleX instrument enable straightforward identification of peptides from tissue directly. The instrument's ability to perform fast MS/MS imaging adds another dimension of specificity and allows one to investigate potential interferences that may be induced by the presence of multiple isobaric peptides.

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