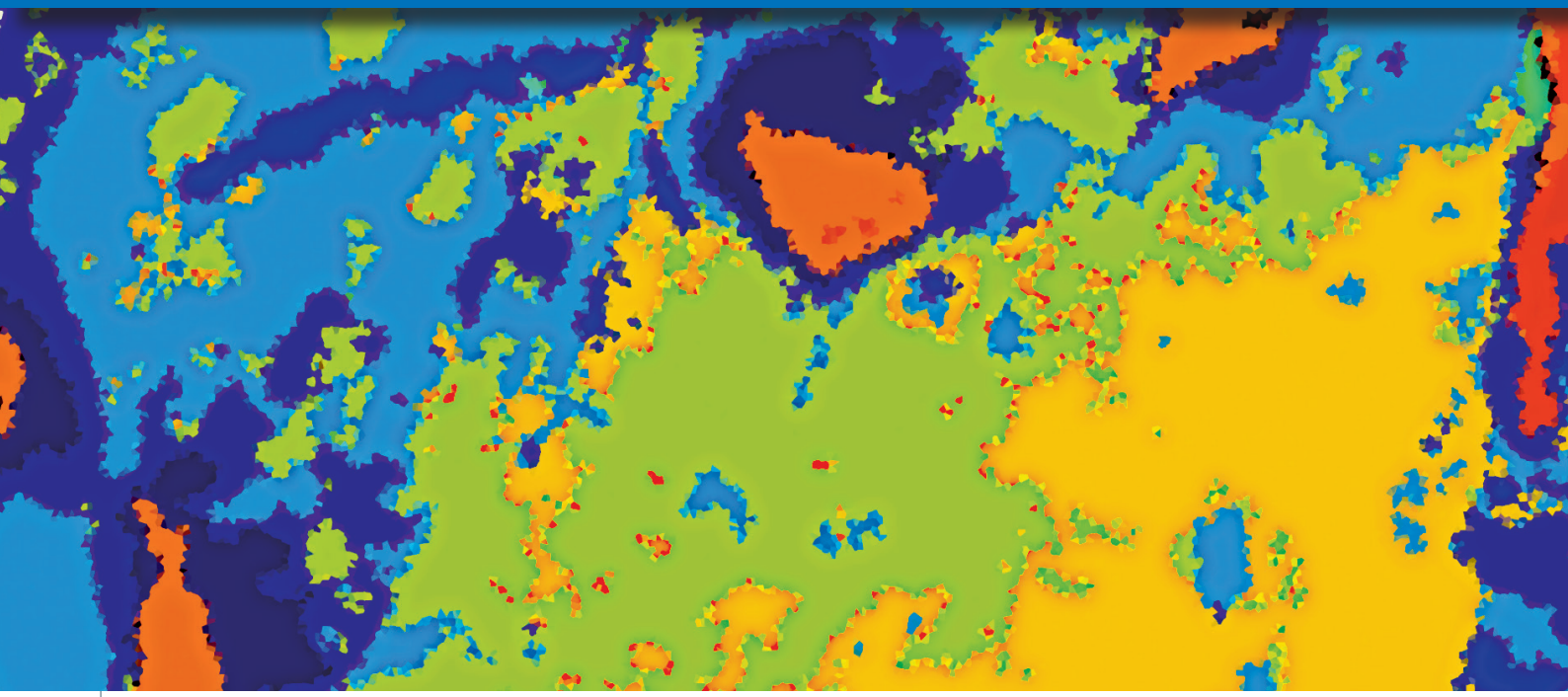

# High-speed MALDI-TOF/TOF imaging of mouse brain tissue performed on intact proteins and after on-tissue digestion

The unique flexibility of MALDI-TOF/TOF systems facilitates tissue imaging of intact proteins as well as of tryptic peptides resulting from on-tissue digestion. While intact protein imaging can provide information on the diversity of proteoforms, the on-tissue digestion approach benefits from significantly higher mass resolution and accuracy that can be achieved for tryptic peptides in reflector operation mode as well as from additional specificity provided by MS/MS analysis.

Over recent years, interest in MALDI mass spectrometry imaging (MSI) has grown within the field of clinical research, driven by MALDI-TOF/TOF systems which offer a unique level of versatility with regard to tissue imaging applications. Inherently, MALDI-TOF mass spectrometers can analyze ions over a wide m/z range and, therefore, enable imaging of a wide spectrum of molecules, ranging from small drug compounds up to intact proteins of ca. 20-25 kDa. MALDI-MSI has been demonstrated to be highly capable of differentiating subtypes of tissues, for example tissues representing different stages of cancer, by analyzing their specific protein signatures [1].

*Keywords:*
*rapifleX; MALDI imaging; MALDI-MSI; proteins; mouse brain; digestion; TOF/TOF; MS/MS; peptide identification*

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MALDI-MSI of proteins can be performed with different approaches. Analysis of intact proteins can reveal the spatial distribution of individual proteoforms, e.g. of specific modification states that may be involved in certain biological pathways. Histones, as an example, are well known to appear in various, differentially regulated levels of site-specific methylation and acetylation, commonly referred to as the "histone code". Lahiri et al. nicely illustrated the ability of MALDI-TOF imaging as a method to analyze the spatial distribution of histones in rodent brain [2].

Bottom-up imaging of tryptic peptides obtained from on-tissue digested proteins complements intact protein imaging in that it can provide access to larger proteins that may be difficult to detect in intact form. In addition, analysis of digested peptides benefits from the significantly higher mass resolution and accuracy at which peptides can be analyzed in reflector MALDI-TOF operation mode. Finally, on-tissue MS/MS can provide additional specificity in peptide imaging. Here, we demonstrate these capabilities for MALDI imaging of proteins in fresh-frozen mouse brain tissue using both the top-down and bottom-up workflows.

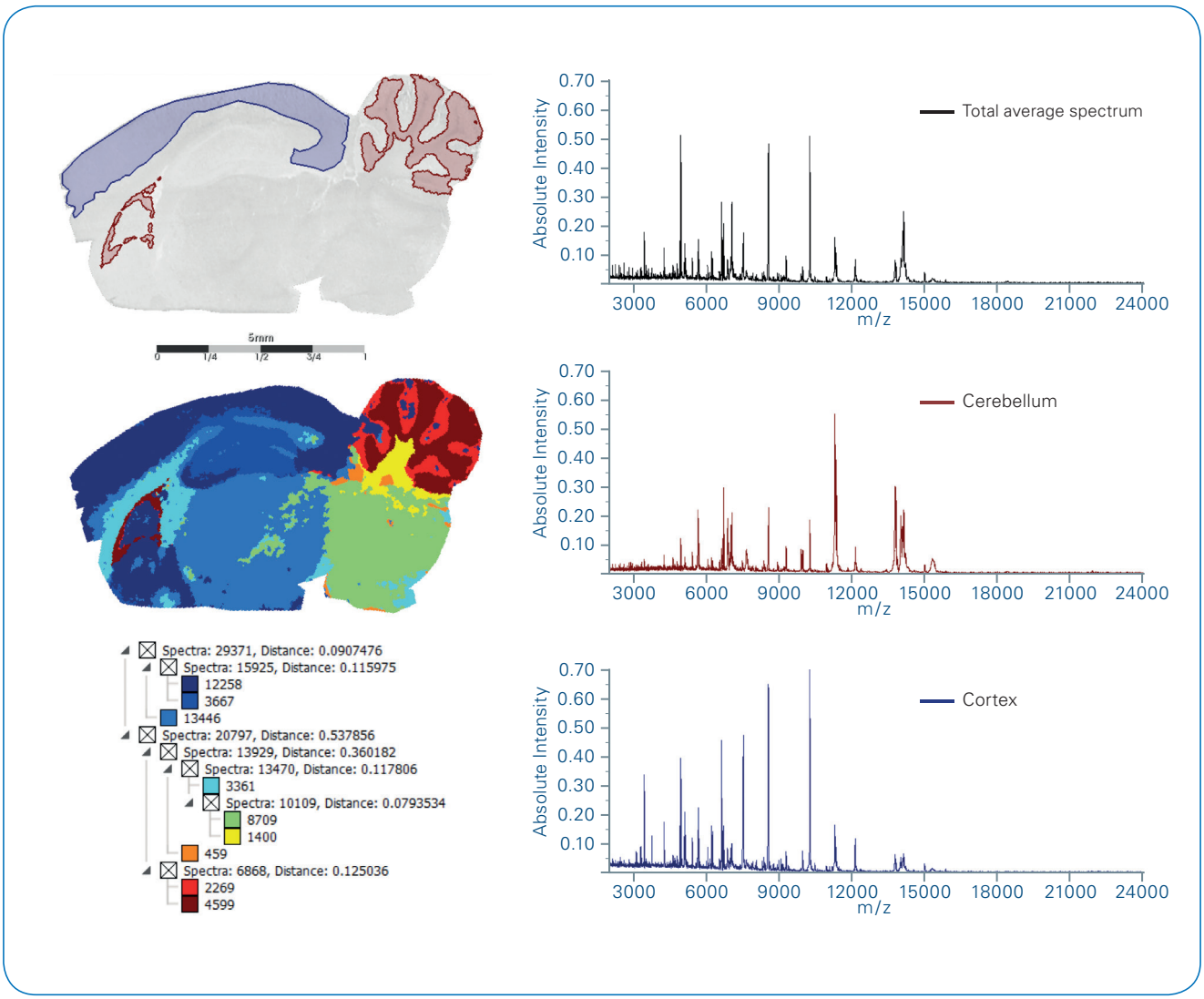



***Figure 1. Spatial segmentation of intact protein MALDI imaging data acquired from a sagittal mouse brain section.*** *Left-hand side: Optical image overlayed with 2 distinct regions as obtained from spatial segmentation (top); segmentation map (center); segmentation tree (bottom). Right-hand side: Average spectra representing total measurement region (top), cerebellum (center) and cortex (bottom) as yielded by spatial segmentation*

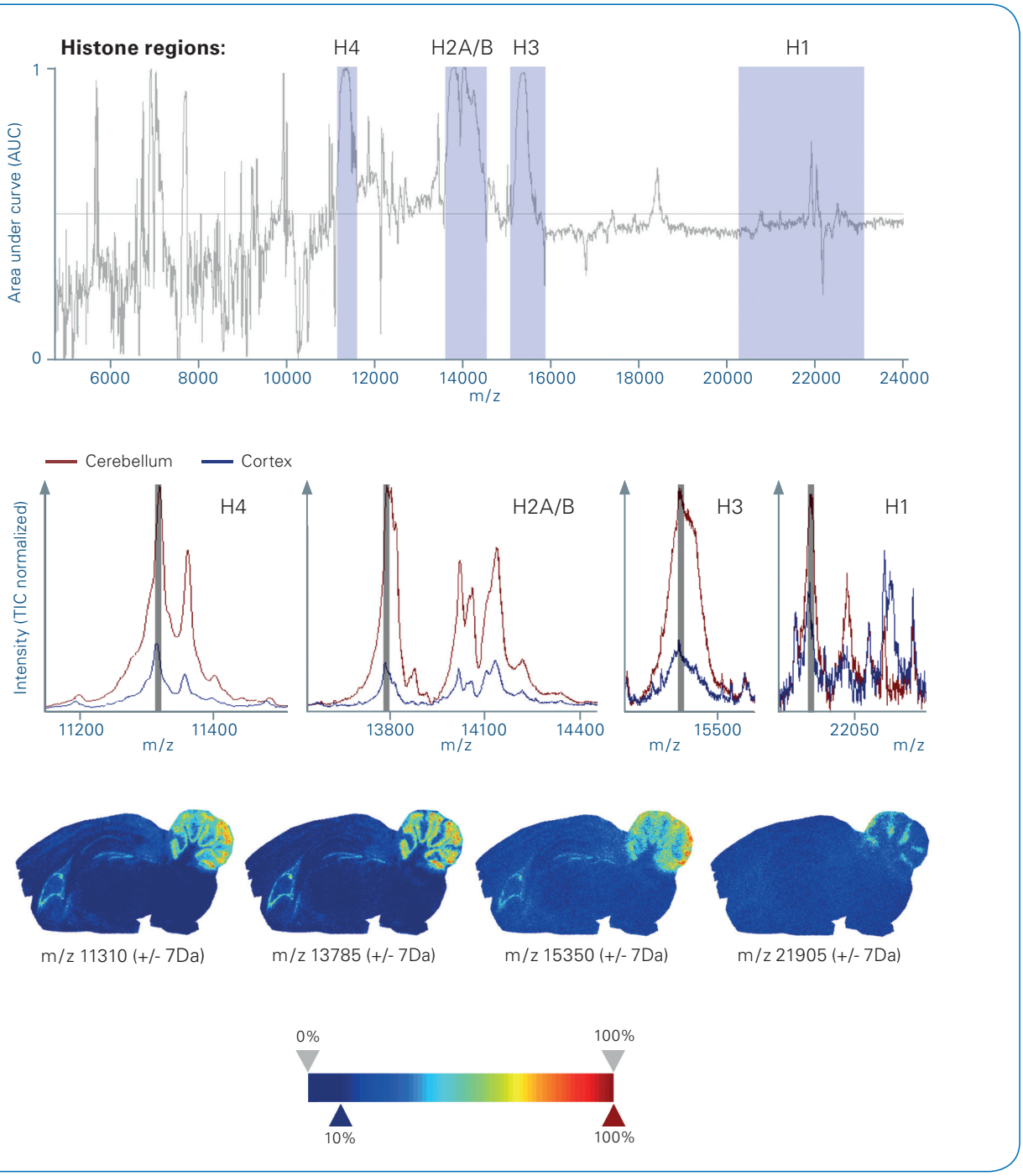



***Figure 2. ROC analysis of intact protein MALDI imaging data , comparing cerebellum with cortex as obtained from segmentation analysis.***
*Top: ROC plot. Grey-colored m/z ranges indicate histone regions; Center: Overlayed average spectra representing cerebellum (brown) and cortex (blue) zoomed into histone-specific m/z regions; Bottom: Ion images of example m/z features representing various histone m/z region*

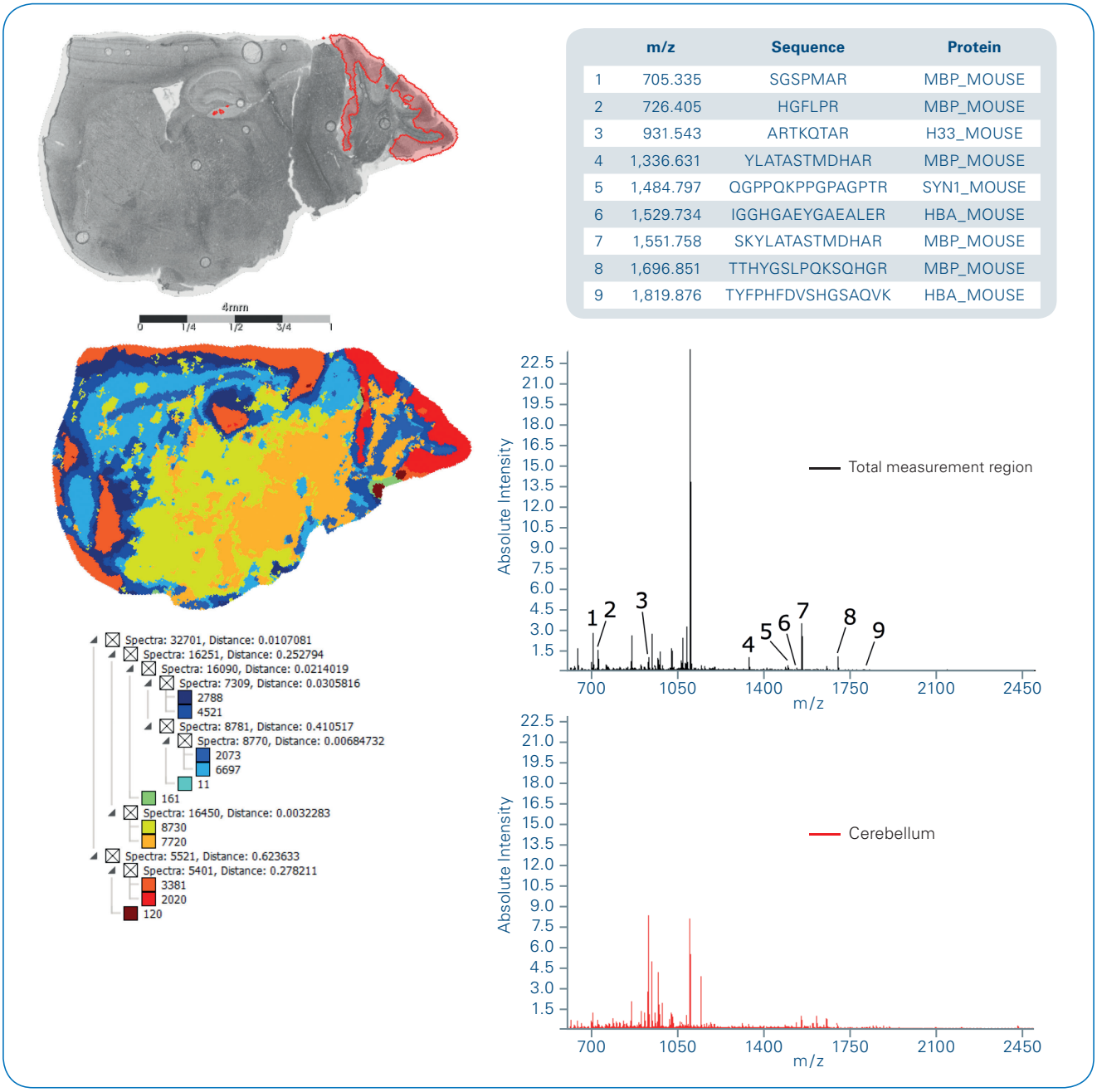
## Experimental

### Samples

Sagittal sections of fresh-frozen mouse brain tissue (10 µm thickness) were generated using a Leica CM 1900 UV cryotome. Sections were thaw-mounted on Bruker conductive slides and further dehydrated under vacuum for 30 minutes. Slides for intact protein analysis were precoated with polylysine prior to mounting tissue sections.

### Sample preparation
### Intact protein analysis

All slide-mounted tissue sections were washed applying the Carnoy protocol. This included the following steps: 70% ethanol (30 s); 100% ethanol (30 s); Carnoy buffer (ethanol : chloroform : acetic acid 6:3:1; 2 min); 100% ethanol (30 s); 0.2% TFA in water (30 s); 100% ethanol (30 s). Washed sections were dried under vacuum for 30 minutes.
Deposition of MALDI matrix sinapinic acid (SA) was performed utilizing




| | m/z | Sequence | Protein |
|---|---|---|---|
| 1 | 705.335 | SGSPMAR | MBP_MOUSE |
| 2 | 726.405 | HGFLPR | MBP_MOUSE |
| 3 | 931.543 | ARTKQTAR | H33_MOUSE |
| 4 | 1,336.631 | YLATASTMDHAR | MBP_MOUSE |
| 5 | 1,484.797 | QGPPQKPPGPAGPTR | SYN1_MOUSE |
| 6 | 1,529.734 | IGGHGAEYGAEALER | HBA_MOUSE |
| 7 | 1,551.758 | SKYLATASTMDHAR | MBP_MOUSE |
| 8 | 1,696.851 | TTHYGSLPQKSQHGR | MBP_MOUSE |
| 9 | 1,819.876 | TYFPHFDVSHGSAQVK | HBA_MOUSE |

***Figure 3. Spatial segmentation of tryptic on-tissue digest MALDI imaging data obtained from a sagittal mouse brain section.*** *Left-hand side: Optical image overlayed with cerebellum region extracted from segmentation result (top); segmentation map (center); segmentation tree (bottom). Right-hand side: Average spectra representing total measurement region (top) and cerebellum as yielded from segmentation (bottom). Inset lists peptides that were identified by MS/MS from tissue directly.*

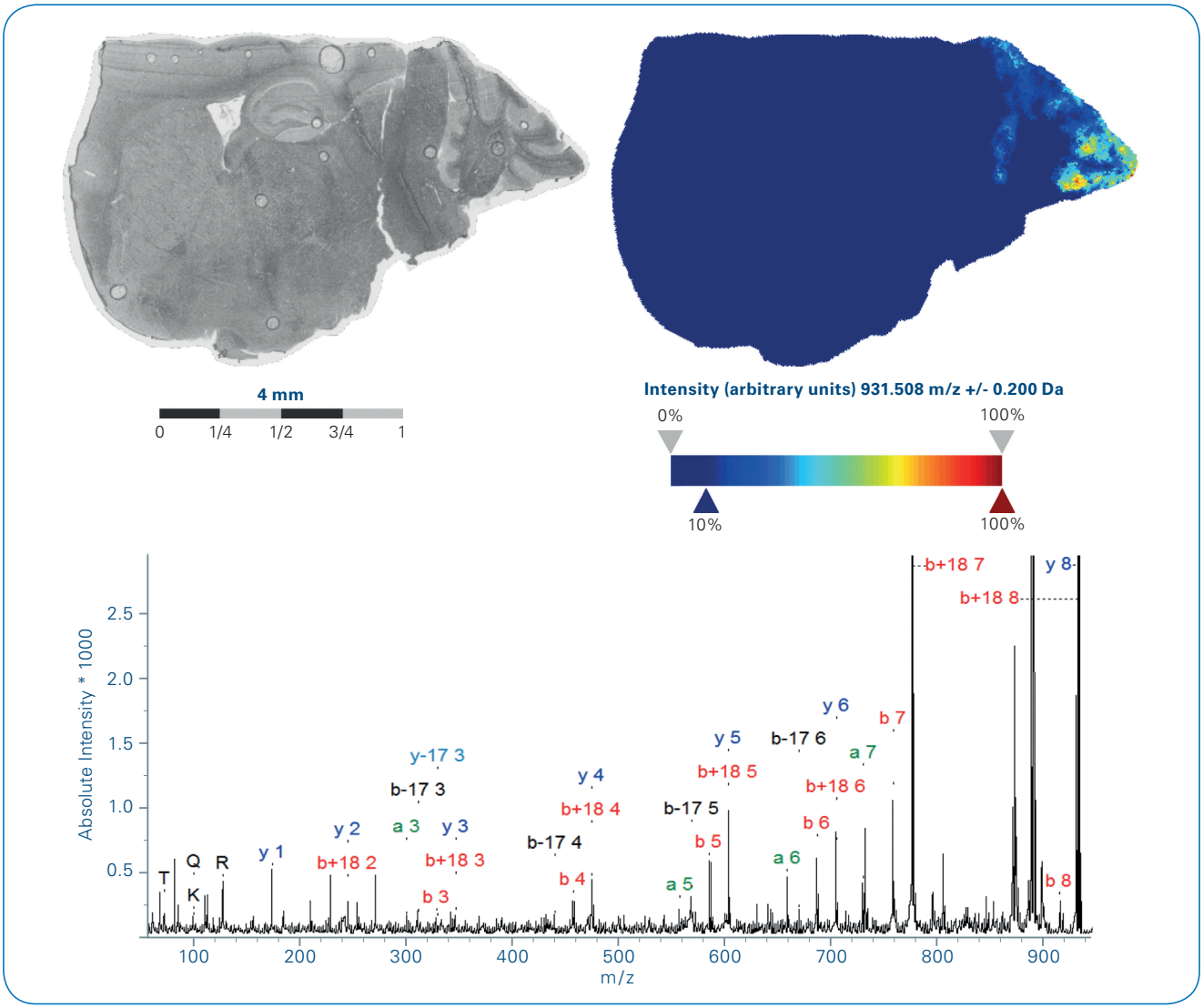



***Figure 4.*** *MALDI-MS ion image of tryptic peptide m/z 931.5 +/- 0.2Da (top). MALDI-MS/MS acquired from tissue directly from the region of highest local abundance allowed to identify peptide sequence ARTKQTAR from Histone 3.3 (bottom).*

a HTX TM-sprayer (HTX Technologies, LLC) connected to an isocratic LC pump applying the following method parameters: SA solution 10 mg/ml in ACN:$H_2O$ 50:50, 0.1% TFA; nozzle height 40 mm; nitrogen pressure 10 psi; nozzle temperature 75°C; flow rate 0.15 ml/min; z-arm velocity 1000 mm/min; number of passes 6; moving pattern HH; track spacing 2 mm; drying time 0 s.

### On-tissue tryptic digestion

Deposition of trypsin (porcine sequencing grade; Promega) was performed on a HTX TM-sprayer connected to a syringe pump for enzyme supply. 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.

### Data acquisition

### Optical images

Slide-mounted, washed tissue sections were scanned on a Reflecta MF-5000 medium format slide scanner at a resolution of 3200 dpi prior to applying matrix.

### MALDI-MS imaging

All MALDI-MS imaging data were acquired on a Bruker rapifleX TOF/TOF system.
Intact protein spectra from m/z 2,000 up to m/z 24,000 were acquired in positive linear TOF mode. A pixel size of 30 µm without oversampling was realized by operating the smartbeam 3D laser in "Single" mode (i.e. 5 µm beam focus) with a beam scan of 30 µm in both x and y dimension. Individual pixel spectra were accumulated from 600 laser shots. MS imaging data from m/z 600 up to m/z 2,400 was acquired from digested tissue sections in positive reflector TOF mode. Pixel size (30 µm) and laser operation mode applied (Single) were identical to the settings used in intact protein analysis. Each spectrum consisted of 400 laser shots.
Fragment spectra of selected peptides were acquired on-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 ratios achieved for individual peptides.
MS/MS imaging of selected precursor peptides was performed at a pixel size of 30 µm (same settings as above). Fragment spectra were accumulated from 400 laser shots per pixel.

### Data analysis

All imaging data analysis was performed using SCiLS Lab software. Total Ion Count (TIC) was applied as normalization method for all datasets.

## Results and Discussion

Figures 1 and 2 display results obtained from intact protein imaging of mouse brain. In a first data analysis step, the dataset was subjected to spatial segmentation using an unsupervised multivariate statistical analysis method [3]. The resulting segmentation map, shown in Figure 1, reflects the molecular similarity within various anatomical regions in mouse brain, for example cerebellum, cortex and others. Also shown in Figure 1 are region-specific protein profiles (TIC normalized) from various mouse brain regions. Two distinct clusters that resulted from spatial segmentation analysis, representing mouse brain cortex and cerebellum were further investigated by supervised univariate statistics, i.e. Receiver Operating Characteristics (ROC), to identify which proteins most strongly differentiate between cortex or cerebellum. The results are shown in Figure 2. A plot of area under curve (AUC) versus m/z (Figure 2, top) reveals features at m/z positions that match various histones, indicating significant differences in the expression level of these proteins between cortex and cerebellum. The differences in the spatial distribution of histones

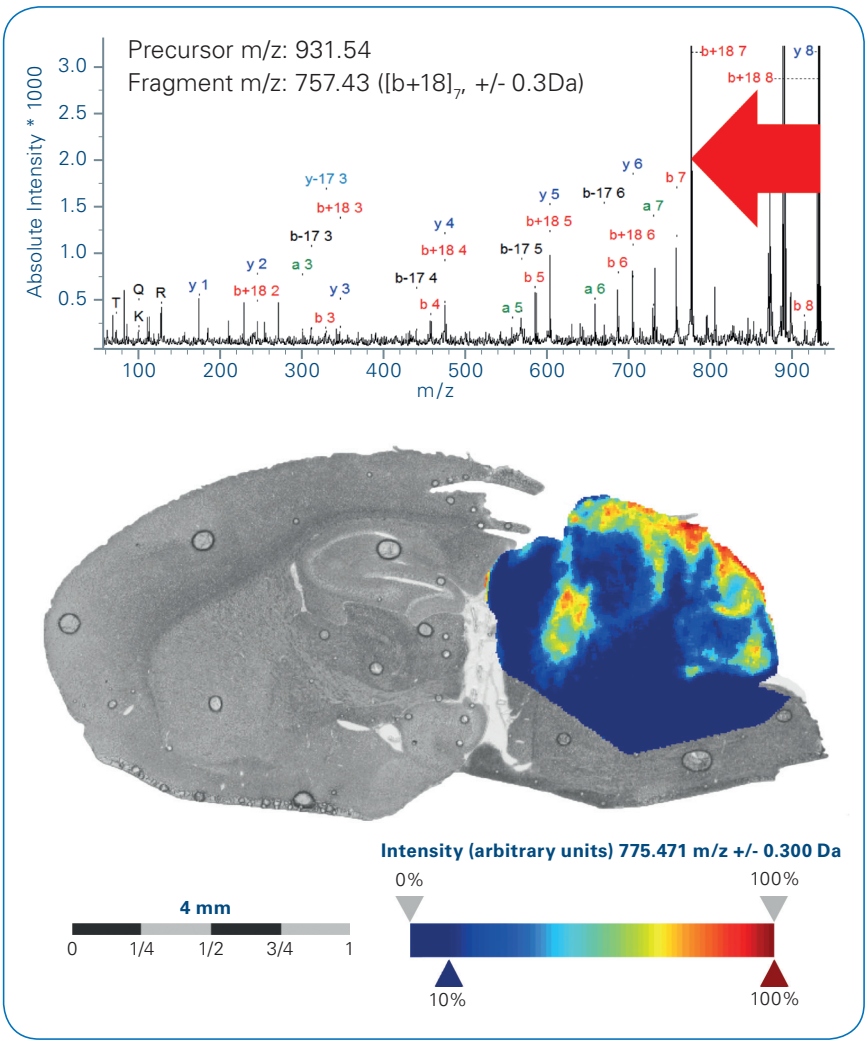



***Figure 5. MALDI-MS/MS imaging of precursor m/z 931.5 representing tryptic peptide ARTKQTAR from Histone 3.3.*** *The red-colored arrow inserted in the annotated MS/MS spectrum (top) indicates the most abundant fragment ion [b+18]7 utilized for generation of the MS/MS ion image (bottom).*

detected by ROC analysis are further highlighted when overlaying the corresponding TIC normalized mean spectra of the two regions representing either cerebellum or cortex (Figure 2, center). The cerebellum mean spectrum indicates significantly increased abundances of the respective histones when compared to the cortex mean spectrum. It should be noted that the partially resolved fine structure of the MS signals reflects the histones´ tissue-specific modification profile, which represents a unique result featured by the top-down MALDI-TOF imaging approach. From each of the four histone regions, one exemplar m/z channel has been selected and the respective ion images have been generated (Figure 2, bottom). These images again confirm the ROC result and indicate the distinct co-localization of these protein species in the cerebellum region.

The top-down protein imaging results presented here are in full agreement with data reported earlier by Lahiri et al. [2], who performed the analysis on a Bruker Ultraflex III instrument. However, using a rapifleX MALDI-TOF/TOF system, the analysis could be performed at nearly double the pixel resolution (30 µm on rapifleX versus 50 µm on ultraflex) and, at the same time, at dramatically increased acquisition speed, i.e. approximately 14 times faster.

Figures 3-5 summarize the results obtained from peptide imaging of a fresh-frozen mouse brain section after tryptic digestion. Spatial segmentation of the peptide dataset shows spectral similarity reflecting the anatomical structures present in mouse brain (Figure 3). In particular, the cluster colored red in the segmentation map clearly represents the cerebellum region (which has been covered only in part when preparing this tissue section). For a number of arbitrarily selected m/z features detected in the MS imaging data, TOF/TOF analysis was performed directly from tissue. The peptide sequences identified by MASCOT ion search are annotated in Figure 3. One of the identified peptides, m/z 931.54, ARTKQTAR, originates from histone 3.3 (*Mus musculus*). The MS ion image of this peptide (+/- 0.2 Da) is given in Figure 4, together with its annotated MS/MS spectrum acquired directly from tissue from a region of the peptide´s highest abundance. The localization of this histone 3.3 peptide within the cerebellum region is highly consistent with the spatial distribution observed for the H3 histones in the intact protein imaging experiment described before. To add further proof to these results, an MS/MS image was acquired in which precursor m/z 931.54 was isolated and the spatial distribution of its most abundant fragment ([b+18]7, m/z 775.4) is shown in Figure 5. The resulting MS/MS image again shows a distinct localization of this particular histone 3.3 peptide to the cerebellum region, which is well in accordance to the intact protein as well as peptide MS imaging data shown above.

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## Conclusions

The rapifleX MALDI-TOF/TOF instrument represents a uniquely versatile platform enabling MALDI tissue imaging of proteins at various stages:

- Top-down intact protein imaging
- Bottom-up MS based peptide imaging after trypsin digestion with subsequent identification of peptides by direct on-tissue MS/MS
- Bottom-up MS/MS based fragment imaging of selected tryptic peptides.

These workflows can provide information that complement each other. While the top-down approach is unique in its ability to monitor individual proteoforms, bottom-up MS and MS/MS imaging of on-tissue digested peptides adds additional dimensions of specificity and allows for the resolution of overlapping isobaric or nearly isobaric peptides by means of MS/MS.

The capabilities of the rapifleX for all of these MALDI imaging approaches have been shown here by analyzing the spatial distribution of histones and further proteins in mouse brain tissue. In combination with the HTX TM-sprayer, and sophisticated tools for data analysis, i.e. SCiLS Lab software, the rapifleX represents the ultimate system for high-speed and high-definition MALDI tissue imaging.

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