SUPPORTING INFORMATION:

Multilabel per-pixel quantitation in mass spectrometry imaging

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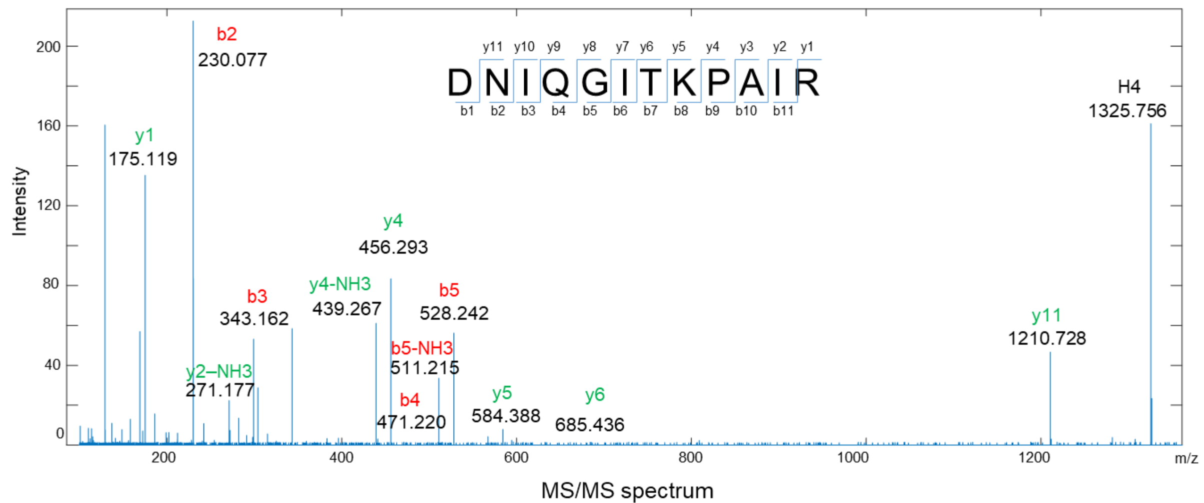
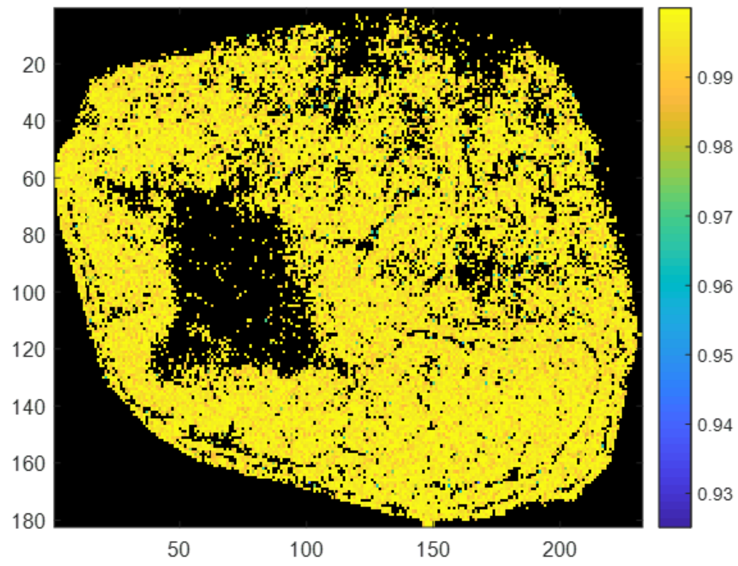
Supplementary Information Method 1: LC-MS sample preparation, instrumentation and data analysis.

Tissue material from six different regions was collected by scratching them off the tissue section on which multi-label MALDI-Q-MSI had already been performed. Peptides were extracted using 50% acetonitrile with 0.1% trifluroacetic acid. Samples were then centrifugated at 15,000 g and 4°C for 10 minutes and dried using Hetovac VR-1 (Heto Lab Equipment, Denmark) before desalting using Zip Tip (Millipore, The Netherlands). Peptide separation was performed on a Thermo Scientific (Dionex) Ultimate 3000 Rapid Separation UHPLC system equipped with a PepSep C18 analytical column (15 cm, ID 75 µm, 1.9 µm Reprosil, 120Å). Peptide samples were first desalted on an online installed C18 trapping column. After desalting peptides were separated on the analytical column with a 140 minute linear gradient from 5% to 35% acetonitrile with 0.1% formic acid at 300 nL/min flow rate. The UHPLC system was coupled to a Q-Exactive HF mass spectrometer (Thermo Scientific). Data-depedent-analysis (DDA) settings were as follows: full MS scan between *m/z* 250–1,250 at resolution of 120,000 followed by MS/MS scans of the top 15 most intense ions at a resolution of 15,000.

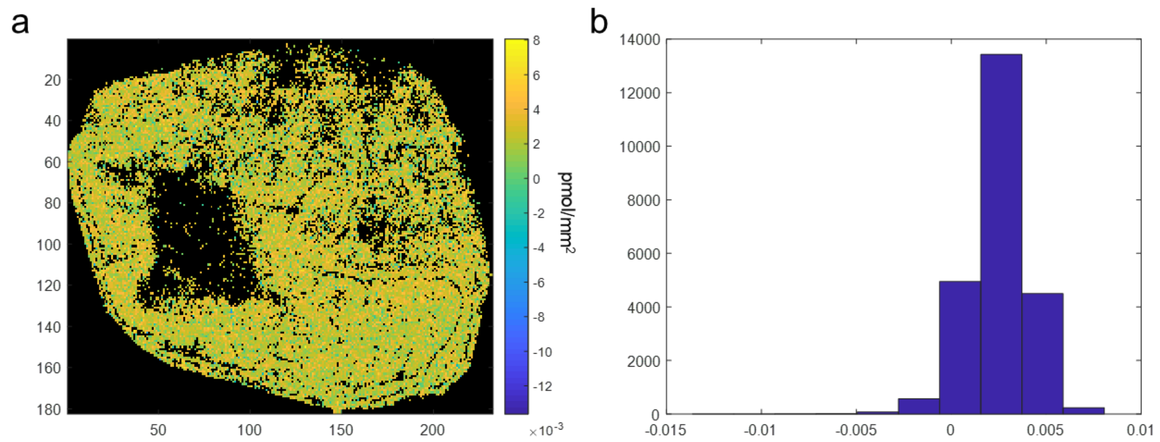
Peptides were identified with Proteome Discover 2.2. The search engine was used with the amino acids sequence of our target histone H4 peptide (H-DNIQGITKPAIR-OH), a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.02 Da.



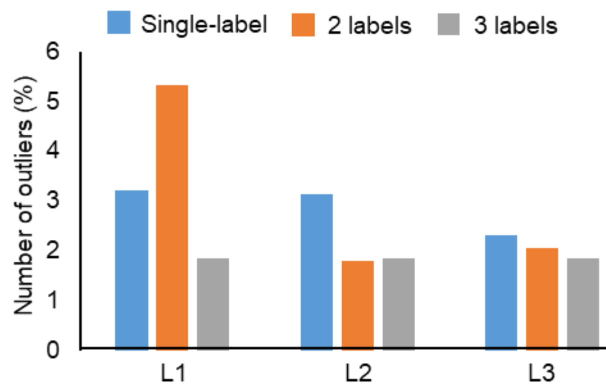
Supplementary Figure 1. MS/MS measurement of the histone H4 peptide with a theoretical precursor mass of *m/z* 1325.754. The MS/MS measurements were performed on tissue using a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Germany) equipped with a MALDI source (Spectroglyph LLC, WA, USA) within a mass range of *m/z* 100–1500, with a collision energy of 35 eV, a 0.5 Da isolation window, and an average of 25 scans. The y- and b-ion amino acid sequence ladder proves the detection of the peptide with the sequence “DNIQGITKPAIR” which is unique to histone H4.



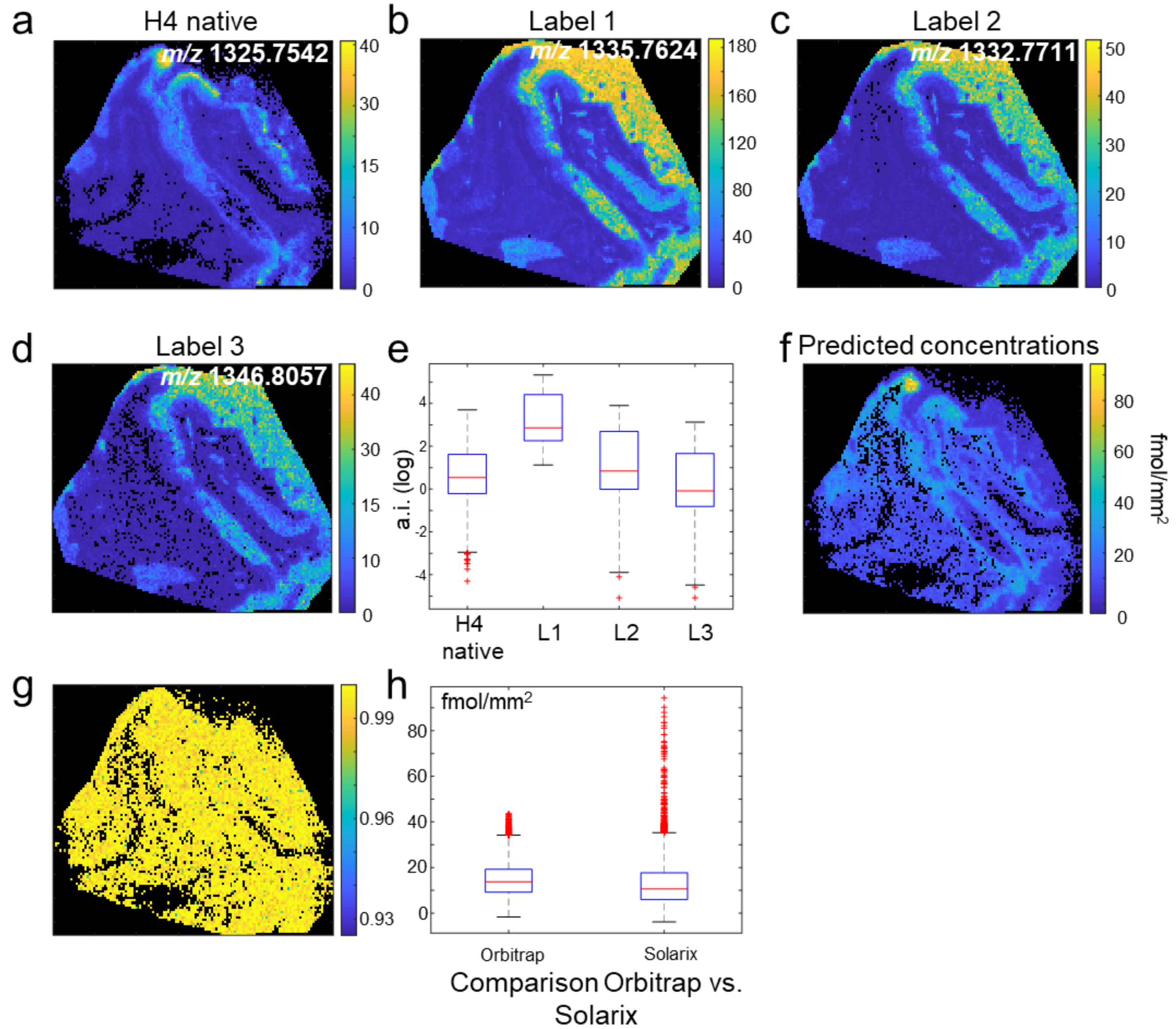
Supplementary Figure 2. R^2 values for every per-pixel regression of the dataset shown in Figure 3.



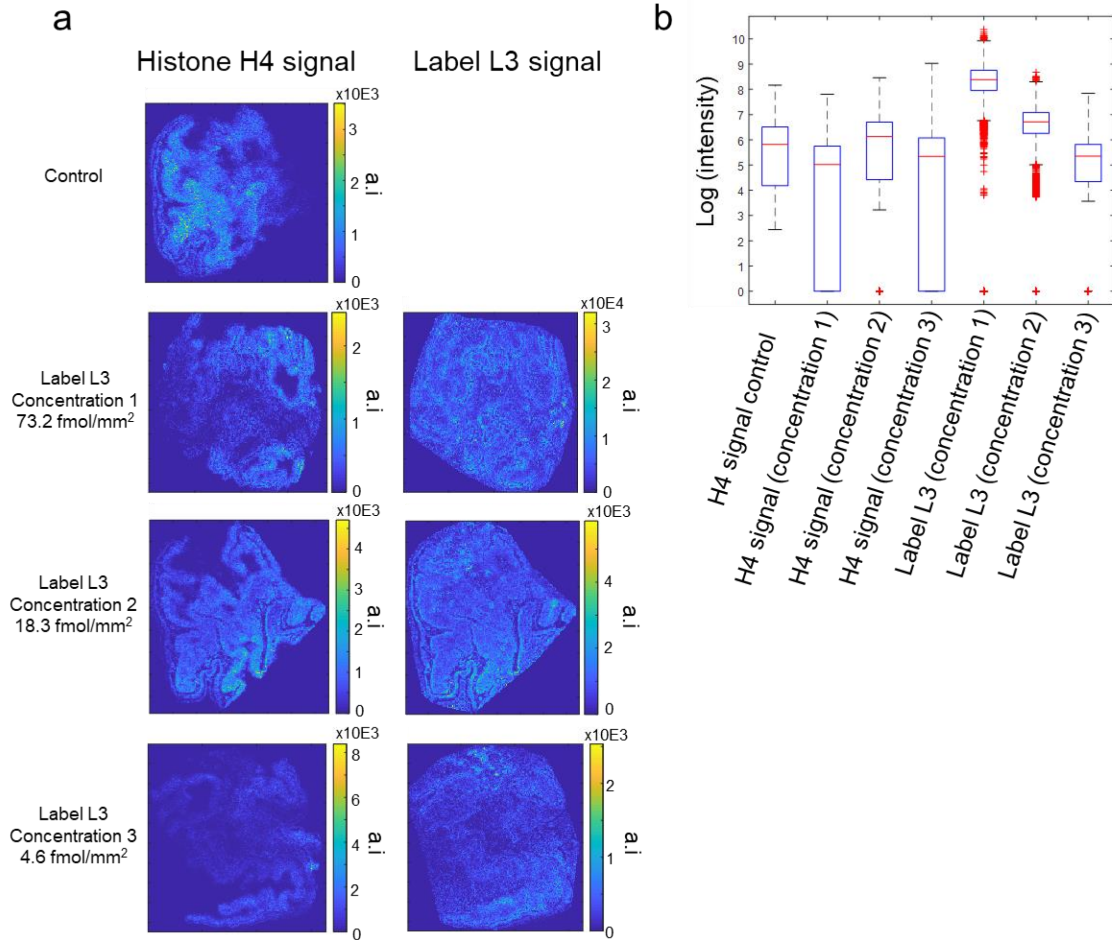
Supplementary Figure 3. Theoretical minimum detectable concentrations ($c_{min,i}$) for every pixel i (a). The c_{min} values were calculated from the linear regression functions ($y_i = c_i * m_i + t_i$) where the intensity y_i was set to 0 and the function solved for c_i to obtain $c_{min,i}$. The values mostly range between 0 and 5 fmol/mm^2 (b).



Supplementary Figure 4. Outliers were removed from the cross-valudation results of the three approaches. A data point is considered an outlier if it is is more than three scaled median absolute deviations (MAD) away from the median.



Supplementary Figure 5. The multi-label quantitation approach using an FT-ICR mass analyzer. The sample was prepared as described in the manuscript and analyzed using a Solarix (Bruker, Germany) with a mass resolution of 512k FWHM at *m/z* 400. The molecular ions [M+H]+ of histone H4, labels L1, L2, and L3 are shown in (a), (b), (c), and (d). The three labels sprayed in previously optimized concentrations enabled to cover the whole intensity range of the target histone H4 peptide (e). The absolute concentrations of peptide histone H4 were calculated and mapped for every individual pixel (f). R^2 values were close to 1, showing that linear regression was already a suited model for the number of available data points (here three labels) (g). The comparison of the predicted concentrations by Orbitrap and Solarix mass spectrometers shows that both mass analyzers delivered similar results (h). The outliers in the Solarix dataset seem to be caused by the hotspot at the top of the sample. Importantly, general differences in predicted concentrations might be also attributed to the digestion since both samples were prepared separately from each other.



Supplementary Figure 6. Investigation of the effect of different sprayed amounts of label L3 on the intensity of the histone H4 peptide. Label L3 was sprayed in three decreasing concentrations, 73.2 (1), 18.3 (2), 04.6 (3) fmol/mm^2, on three consecutive pig colon tissue sections; one section was left unsprayed as control. Images of the spatial distribution of the target histone H4 peptide and label L3 are shown in (a). The signal intensities of the target histone H4 peptide for the different sprayed amounts of L3 are displayed in (b). The results show no effect of L3 abundance on the ionization efficiency of the target compound.