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Protein identification by accurate mass matrix-assisted laser desorption/ionization imaging of tryptic peptides

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The spatial distribution of proteins in tissue sections can be used to identify potential markers for pathological processes. Tissue sections are often subjected to enzymatic digestion before matrix-assisted laser desorption/ionization (MALDI) imaging. This study is targeted at improving the on-tissue identification of tryptic peptides by accurate mass measurements and complementary off-line liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) analysis. Two adjacent mouse brain sections were analyzed in parallel. The first section was spotted with trypsin and analyzed by MALDI imaging. Direct on-tissue MS/MS experiments of this section resulted in the identification of 14 peptides (originating from 4 proteins). The second tissue section was homogenized, fractionated by ultracentrifugation and digested with trypsin prior to LC/ESI-MS/MS analysis. The number of identified peptides was increased to 153 (corresponding to 106 proteins) by matching imaged mass peaks to peptides which were identified in these LC/ESI-MS/MS experiments. All results (including MALDI imaging data) were based on accurate mass measurements (RMS <2 ppm) and allow a confident identification of tryptic peptides. Measurements based on lower accuracy would have led to ambiguous or misleading results. MS images of identified peptides were generated with a bin width (mass range used for image generation) of $\Delta m/z = 0.01$. The application of accurate mass measurements and additional LC/MS measurements increased both the quality and the number of peptide identifications. The advantages of this approach for the analysis of biological tissue sections are demonstrated and discussed in detail. Results indicate that accurate mass measurements are needed for confident identification and specific image generation of tryptic peptides in tissue sections. Copyright © 2011 John Wiley & Sons, Ltd.

Mass spectrometry imaging is a method of scanning a sample of interest and generating an image of the intensity distribution of a specific analyte signal. In matrix-assisted laser desorption/ionization (MALDI) mass spectrometry a focused laser beam is used to generate ions which are analyzed in the mass spectrometer. Mass spectral information is available for each individual position of the scanned area and selected ion images are generated for individual mass peaks. MALDI imaging has evolved from the first proof-of-principle[1] to a widely used analytical technique in recent years.[2] Numerous applications have been published, including the imaging of lipids,[3] peptides,[4,5] proteins,[6] and drug compounds.[7]

MALDI imaging has been used to correlate the spatial distribution of proteins to pathogenic processes.[8–10] A recent review of MALDI imaging of proteins for cancer research was published by McDonnell *et al.*[11] The authors report several interesting applications such as improved diagnostics and prognostics through the detection of marker proteins, but they also conclude that the identification of proteins is still challenging. The detection of intact proteins is limited by sensitivity and low fragmentation efficiency for vibrational activation in tandem mass spectrometry (MS/MS) mode.[11] Direct analysis under imaging conditions is also limited to proteins below 30 kDa in mass[12] and formalin-fixed paraffin-embedded (FFPE) tissue is not accessible. One possibility to overcome these problems is to digest proteins on tissue and to image the distribution of the resulting tryptic peptides.[9,13–15] Peptides, due to their lower mass, are easier to detect and to identify. However, these results are still limited to the most abundant proteins in most cases.[16] Typically, no more than 20 peptides are identified by direct MS/MS measurements. Another way to improve the identification of imaged compounds is the combination of MS imaging with complementary techniques such as liquid chromatography coupled to mass spectrometry (LC/MS). This approach has recently been applied to lipids,[17,18] peptides[18,19] and proteins.[10,20] Another approach to resolve the high complexity of tissue sections is the use of ion mobility as an additional separation step. This has been recently applied for the MS imaging of tryptic peptides.[9]

Accurate mass measurements greatly increase the reliability of compound identification. The highest mass accuracy reported so far for imaging of tryptic peptides was 30 ppm on a time-of-flight (TOF) system.[21] The unambiguous identification of proteins is especially important in applications where marker compounds for certain processes or conditions are investigated. Mass spectrometers with mass accuracies in the low-ppm range are routinely used in many bioanalytical applications. The highest mass accuracy and mass resolution are obtained by Fourier transform ion cyclotron (FTICR) mass spectrometers.[22,23] Another type of Fourier transform mass spectrometer is the Orbitrap system. An overview of the use

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of the Orbitrap system in general and applications in proteomics was published by Scigelova and Makarov.[24] The use of these mass analyzers in MALDI imaging is so far limited to very few examples.[4,25–27] These measurements show, however, that the full complexity of biological samples can only be resolved with high mass accuracy and high resolution mass analyzers. The use of these high mass resolution analyzers for MS imaging of proteins imaging has so far been hindered by limited mass range (Orbitrap) or limited sensitivity (FTICR). Tryptic digestion on tissue makes these systems applicable to protein identification in imaging experiments. The terms 'mass deviation', 'mass accuracy' and 'maximum mass deviation' are used according to the definition by Zubarev and Mann[28] in this article.

Complex biomedical questions cannot always be answered by a single analytical method. The reliability of combining data from different mass spectrometry methods can be significantly improved by using accurate mass measurements. We demonstrated this in a recent study that combined information from accurate mass MALDI-FTICRMS and LC/ESI-FTICRMS measurements in order to identify protein biomarkers.[29] In the following study we present the first application of Fourier transform mass spectrometry (FTMS) for imaging tryptic peptides. MALDI imaging experiments were complemented by LC/ESI-MS measurements to increase the number of identified peptides. It is important to note that all the MS measurements and the processing steps were based on accurate mass data (root mean square (RMS) <2 ppm). The advantages of this approach for the analysis of biological tissue sections are demonstrated and discussed for the example of mouse brain tissue.

EXPERIMENTAL

Materials

Water (HPLC grade), formic acid (FA) (puriss), trifluoroacetic acid (TFA) (for protein sequence analysis), 2,5-dihydroxybenzoic acid (DHB) (puriss) and 1,4-dithio-DL-threitol (DTT) (Ultra) were purchased from Fluka (Neu-Ulm, Germany). Acetonitrile (MeCN) (Uvasol®), ethanol (Uvasol®), acetone (Uvasol®), glacial acetic acid (Suprapur®), EDTA Titriplex (III) (reinst) and sodium carbonate (Na_2CO_3) (pro analysis) were purchased from Merck KGaA (Darmstadt, Germany). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Biotechnology Performance certified), ammonium bicarbonate (NH_4HCO_3) (ReagentPlus™), iodoacetamide (IAA) (Sigma Ultra), and Protease Inhibitor Cocktail (DMSO solution) were purchased from Sigma (Steinheim, Germany). Trypsin (sequencing grade modified trypsin, porcine) was purchased from Promega (Madison, WI, USA). Sucrose (molecular biology grade) was purchased from AppliChem (Darmstadt, Germany).

Tissue samples

The mouse brain tissue sections originated from a C57B6/N mouse. The mouse brain was dissected and immediately snap-frozen in liquid nitrogen. Coronal tissue sections were cut in sections of 20 µm thickness with a cryotome (HM500, Microm, Walldorf, Germany) at −20°C, thaw mounted on glass slides and stored at −80°C until analysis. Before use the tissue sections were placed in a desiccator for 30 min to allow the tissue sample to dry and equilibrate to room temperature.

MALDI imaging

Sample preparation

A series of ethanol/water washing steps as described by Aerni *et al.*[30] was applied to the brain tissue section to fix the sample and to remove salts, lipids and other contaminants. A final washing step in a solution of 90% ethanol, 9% glacial acetic acid and 1% deionized water for 30 s was applied.[31] The tissue sample was then dried under a gentle nitrogen stream. Subsequently, 0.2 µL trypsin solution (0.1 µg/µL in 30 mM NH_4HCO_3 buffer) per digestion spot was deposited manually on the tissue sample and allowed to dry (3 iterations per spot every 30 min). After 2.5 h digestion time at room temperature the sample was covered with DHB (30 mg/mL in 50:50 acetone/water/0.5% TFA) by a pneumatic sprayer, as described in Bouschen *et al.*[32]

Measurement

For MALDI MSI measurements a MALDI Orbitrap XL (Thermo Fisher Scientific GmbH, Bremen, Germany) was used. An area of 141 × 71 pixels with a pixel size of 100 µm × 100 µm was scanned. One scan per pixel in FTMS mode in the range of *m/z* 600–4000 with a mass resolution of R = 60000 at *m/z* 400 was acquired followed by two data-dependent MS/MS experiments of the two most intense peaks in the survey scan in ion trap (IT) mode on the same position. The isolation window for precursor ions was ±2.5 u. The fragmentation mode was collision-induced dissociation (CID) at a 'normalized' collision energy of 45%. Activation Q was 0.25 and the activation time was 30 ms. Wideband activation was used. Automatic gain control was activated. The software ImageQuest (Thermo Fisher Scientific GmbH) was used to generate MS images.

LC/ESI-MS/MS

An adjacent mouse brain section was prepared for LC/MS/MS by ultracentrifugation resulting in four subcellular fractions: cytosol, mitochondria/nuclei, endoplasmatic reticulum/golgi and plasma membranes. For more detailed information on the fractionation protocol, see Supporting Information 1. A tryptically digested homogenate of the mouse brain section without fractionation was analyzed as a reference sample.

Measurements

Measurements of the four ultracentrifugation samples and of the additional crude homogenate sample of the mouse brain section were accomplished on an Ultimate binary nano-high-performance liquid chromatography (nano-HPLC) pump/autosampler system for HPLC analysis (LCPackings/Dionex, Idstein, Germany). Volumes of 5 µL of the sample were pre-focused on a trap column (Dionex, C18 PepMap, i.d. 300 µm, length 5 mm) and separated on a fused-silica C18 PepMap100 capillary column (Dionex, 3 µm, 100 Å; i.d. 75 µm; length 150 mm). The flow rate was 0.2 µL/min. Solvent A was water containing 2% MeCN (v/v) and 0.1% formic acid (FA) (v/v). Solvent B was MeCN containing 20% water

(v/v) and 0.08% FA (v/v). Separation was performed as follows: first B was increased from 0% to 25% in 5 min, increased to 50% B in 25 min, further increased to 100% B in 4 min and maintained for 20 min to elute the strongly hydrophobic peptides and to clean the column. The gradient was then ramped down in 1 min to 0% solvent B for equilibration for 25 min. The nano-HPLC system was coupled to a nanoelectrospray interface of an LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific GmbH). Survey MS scans were measured with a mass accuracy of better than 2 ppm. The five most intense peaks in the survey scan were chosen for fragmentation in IT-MS mode with an exclusion time of 20 s. The isolation window for the precursor ion was ±2 u. CID was used for fragmentation in the IT at a normalized collision energy of 35%. The activation Q was 0.25 and the activation time was 30 ms. Each sample was measured three times.

Data processing

Database search

LC/ESI-MS/MS and MALDI-MS/MS data were searched against the SwissProt[33] *mus musculus* database (06/2/2009) using Proteome Discoverer 1.1 (Thermo Fisher Scientific GmbH) based on the SEQUEST search algorithm. The mass tolerance for precursor ions was set to 2 ppm, and the mass tolerance for product ions was set to 0.8 u. Two missed cleavages were allowed in order to account for incomplete digestion. Methionine oxidation was allowed as variable modification. Peptides with a "peptide probability" (SEQUEST parameter) of 20 and higher were considered as significant identifications. The false discovery rate, as determined by a reversed database search, was 3.4%.

In silico digestion

An *in silico* digestion (enzyme: trypsin) of the SwissProt *mus musculus* database was performed using the MS FIT tool of Protein Prospector.[34] No missed cleavages were allowed.

Comparison of MSI and LC/ESI-MS/MS data

Selected ion images were created for identification of the on-tissue tryptic peptides from mouse brain. The *m/z* values of the MALDI imaging spectrum averaged over the whole area were compared with the list of *m/z* values of peptides identified by database search of the LC/ESI-MS/MS measurements. The software used for comparison called "Data Comparison" was developed by us in Delphi (Borland Developer Studio 2006™ for Windows™). The software compares two peak lists. Matches within a given *m/z* tolerance are stored in a *.csv- file. A maximum mass deviation of 3 ppm between the *m/z* values of the MALDI imaging peaks and the masses of the ions from the peptides identified by LC/ESI-MS/MS was allowed in this study.

RESULTS AND DISCUSSION

This study was targeted at the identification of proteins in MALDI imaging by a combination of on-tissue tryptic digestion and off-line LC/MS/MS analysis of the corresponding proteome. In a first step MS/MS spectra were acquired directly on-tissue during the MS imaging experiment. In a parallel approach an adjacent mouse brain section was homogenized, digested and analyzed by LC/ESI-MS/MS in order to identify tryptic peptides that were present in the investigated tissue. The list of these identified peptides was then used to assign peptide sequences to mass peaks detected in the MS imaging experiment. The workflow of this parallel approach is illustrated in Fig. 1.

MALDI imaging and on-tissue MS/MS

MS images of 141 × 71 pixels at a pixel size of 100 µm were acquired from the mouse brain section. More than 3000 distinct peaks in the mass range *m/z* 600–4000 were detected in this measurement. Figure 2(a) shows an optical image of the measured mouse brain section before sample preparation. The shape of the deposited trypsin spots is indicated by the black lines. The trypsin was spotted in a coarse pattern (approximately 1000 µm in diameter) in this initial experiment. The spatial resolution of the tryptic peptides was limited to the enzyme spot size. The results and implications of this study with respect to accurate mass measurements, however, are equally relevant for experiments with smaller trypsin spots. The digest might be less effective to some degree at smaller trypsin spot sizes, but the MALDI imaging experiments were performed with a pixel size of 100 µm. This is a commonly used pixel size in the MALDI imaging of peptides/proteins, so the MS measurements would not be different. Mass accuracy in orbital trapping mass spectrometers is also less dependent on intensity than in TOF instruments.[35] We have demonstrated in previous studies that low-ppm mass accuracy (<2 ppm RMS) can be obtained even at a pixel size of 5 or 10 µm for lipids,[26] drug compounds[36] and (endogenous) peptides.[26,37]

Figures 2(b)–2(f) show selected ion images (bin size $\Delta m/z$ = 0.01 units) of peptides which were identified by on-tissue MS/MS measurements. Actin and tubulin are homogeneously distributed as expected by their biological function. These images demonstrate that tryptic digestion was successful over the whole sample. The myelin peptides on the other hand show a more distinct spatial distribution. On-tissue MS/MS measurements led to the identification of 14 peptides, corresponding to four proteins. A table of these peptides is provided in the Supporting Information 2. All these peptides belong to high-abundance proteins such as myelin, tubulin and actin. These results demonstrate that direct on-tissue identification of resulting peptides was rather inefficient. The limited number of identified peptides is in agreement with the results of previous studies: direct on-tissue MS/MS measurements are obviously able to identify highly abundant proteins only.[13–15,38] Many more potential peptide peaks are detected, but they cannot be identified due to the limited sensitivity of the MS/MS experiments. We therefore performed additional LC/ESI-MS/MS measurements in our study.

Combination of MALDI imaging and LC/ESI-MS/MS measurements

In the next step a mouse brain tissue section, adjacent to the one used for MALDI imaging, was analyzed by LC/ESI-MS/MS in order to identify the proteins present in this tissue. This section was homogenized and tryptically digested in solution prior to analysis (see Supporting Information 1 for

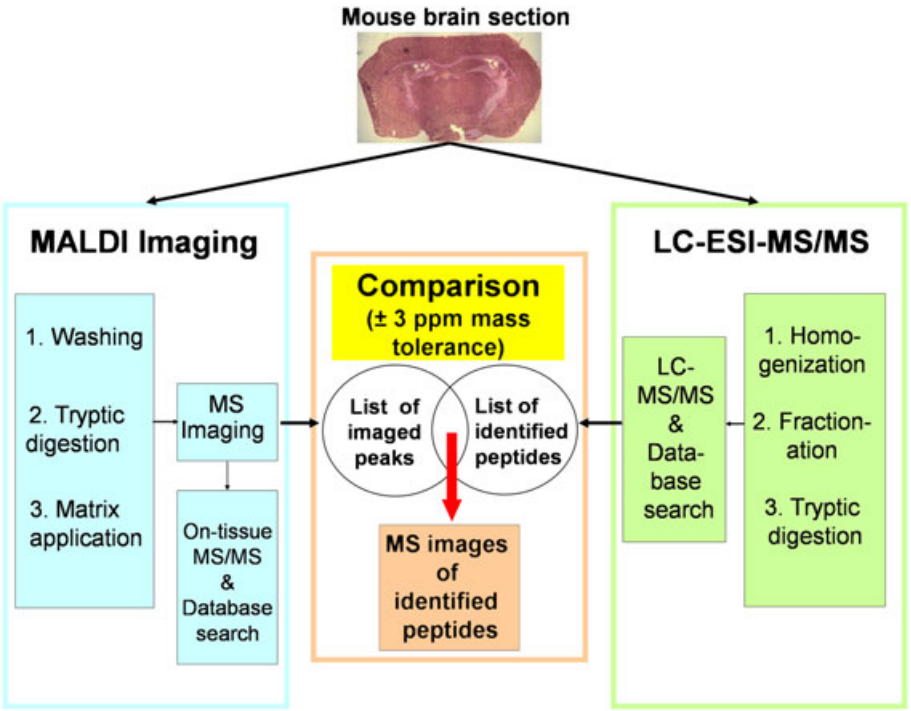

Figure 1. Workflow of the protein identification in MS imaging experiments. MALDI imaging and LC/ESI-MS were performed in parallel and results were combined in order to increase the number of identified peptides in the imaging experiment.

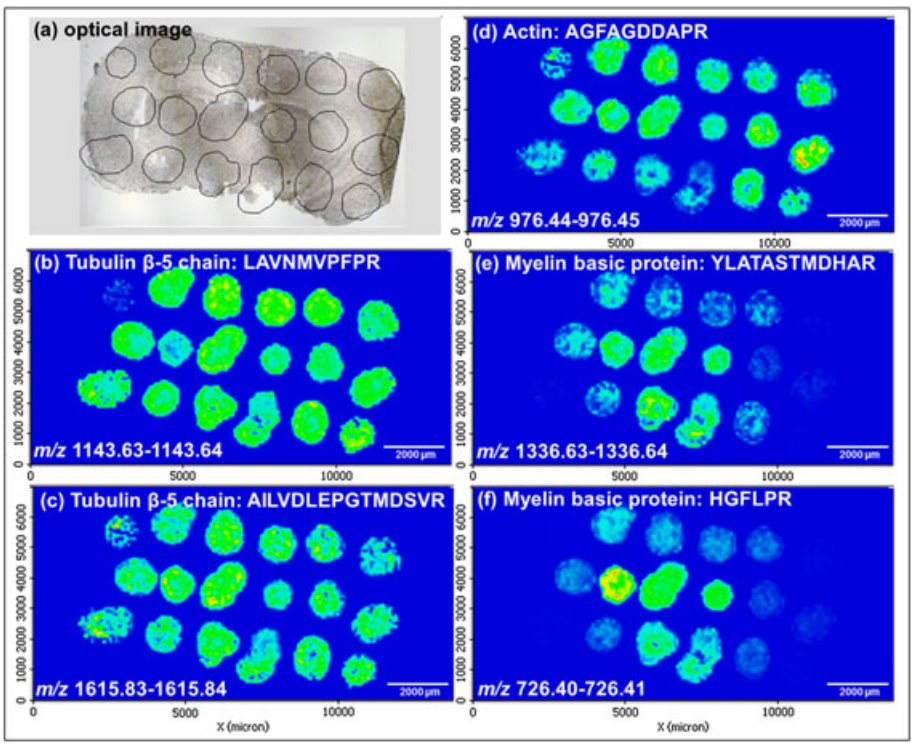

Figure 2. Coronal mouse brain section. (a) Optical image before sample preparation, trypsin spots indicated in black. (b)–(f) selected ion images of MALDI imaging experiment (pixel size 100 μm, image size 141 × 71 pixels).

more details). More than 1100 peptides, corresponding to 500 proteins, were identified in this sample. The data from the MALDI imaging (tissue section) and the LC/MS/MS experiment (homogenate sample) were combined as shown in Fig. 1. Mass peaks detected in the MALDI imaging experiment were compared with the ions from the peptides identified in the homogenized sample. For this purpose a peak list of the spectrum averaged over the complete MALDI image was generated. If a peak of the imaging experiment matched a mass entry in the list of identified peptides within a mass tolerance of ±3 ppm, the corresponding image was assigned to that peptide (see also discussion of Fig. 6 for more details). This approach led to the identification of numerous additional peptides in the MS imaging experiment and thus to additional identified images. Figure 3 shows a mass spectrum averaged over one digestion spot as obtained by MALDI imaging. Selected peaks were labeled with the sequence of the assigned peptide and accession number of the corresponding protein. The mass deviation of the peptides detected on tissue was generally below 2 ppm.

An overview of all the identified peptides is shown in Fig. 4. Peptide signal intensities averaged over the complete MALDI image were plotted against their *m/z* values. The identified peptides had $[M+H]^+$ ions in the mass range of *m/z* 900–2100. The peak intensities varied by more than two orders of magnitude. Peptide images identified by direct on-tissue MS/MS were indicated by red markers. These peptides had relatively high signal intensities and low *m/z* values, whereas peptides of higher masses or lower signal intensities could only be identified by additional LC/MS analysis. This demonstrates the advantage of a combined MS imaging and LC/MS approach, providing identification of low-abundance peptides that cannot be identified directly by on-tissue MS/MS measurements. This difference is mainly due to the lower limit of detection of the LC/MS/MS method and the higher fragmentation efficiency in CID experiments of multiply charged ions in electrospray ionization. The combination of MALDI imaging and LC/ESI-MS/MS led to the identification of more than 147 peptides corresponding to 101 proteins. More than three-quarters of these proteins have a molecular weight of more than 30 kDa and would therefore not be accessible to a top-down approach measuring intact proteins directly. The ionization efficiencies for peptides can be different in MALDI and ESI and thus a confident identification requires more than one peptide per protein. Twenty-four proteins were detected with two or more peptides and can be considered as reasonable matches. In 77 cases only one peptide was detected per protein and the assignment to a certain protein is thus only tentative. These numbers are in line with those from comparable studies using on-tissue identification of peptides and proteins, which usually identify no more than 50 peptides and less than 20 proteins including several proteins which are only identified by one peptide.[9,15] This demonstrates the difficulty in identifying proteins in MALDI imaging experiments (compared with bulk sample analysis by LC/MS) and the need to improve methodologies in this field.

All the peptides in our study were detected with a mass deviation of less than 3 ppm in the MALDI imaging experiment. A comparison of the number of peptides and proteins identified by direct on-tissue MS/MS and the combined approach is shown in Fig. 5. The high number of identified peptides in the combined approach was only possible because of the dedicated sample preparation protocol (including ultracentrifugation) that was used for LC/MS/MS analysis. Analysis without additional fractionation steps (crude homogenate), for comparison, resulted in the identification of 60 peptides only. Details of both preparation methods are described in Supporting Information 1. A table of all identified proteins of the MALDI imaging measurement and the corresponding tryptic peptides is provided in Supporting Information 3. These results show that complementary LC/MS/MS measurements are an effective way to identify peptides in

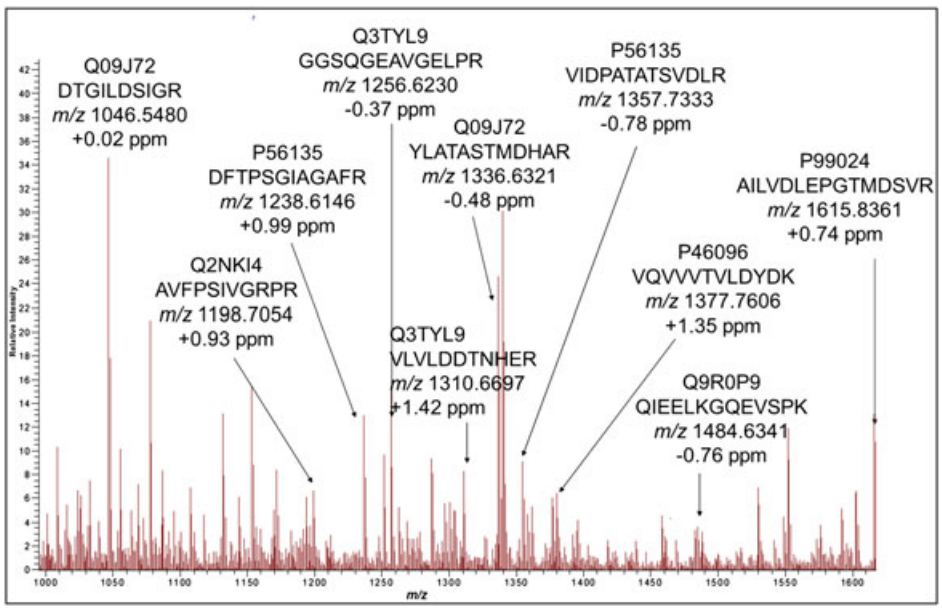

Figure 3. Mass spectrum (full scan) averaged over one trypsin spot on the mouse brain tissue section as obtained by MALDI imaging. Selected identified peptides are labeled with amino acid sequence, accession number of corresponding protein and mass deviation.

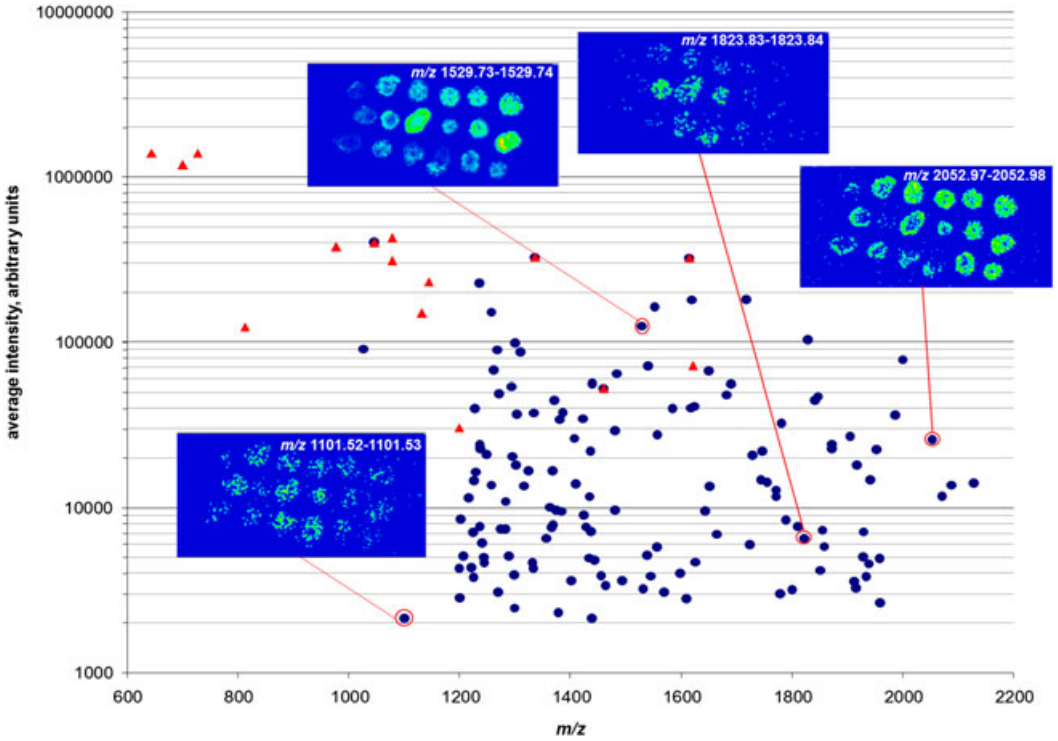

Figure 4. Averaged signal intensities of all identified tryptic peptides assigned to MALDI images. Ion images (bin width $\Delta m/z = 0.01$ units) of selected peptides are shown to demonstrate corresponding image qualities.

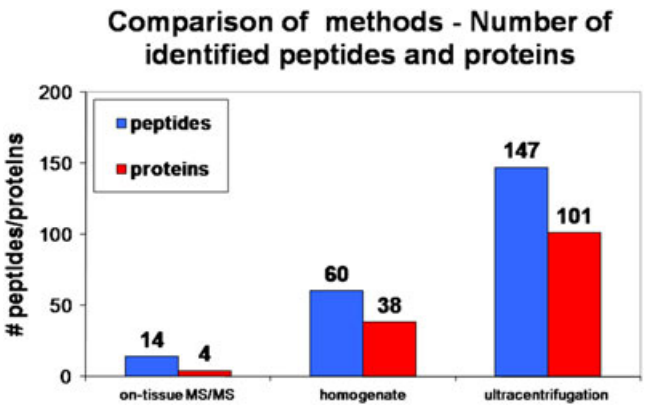

Figure 5. Number of peptides and proteins identified in MALDI imaging by on-tissue MS/MS and by combination with off-line LC/ESI-MS/MS measurements. Different sample preparation protocols were used for the two LC/MS/MS experiments.

MALDI imaging experiments and that an extended preparation protocol significantly increases the number of identified peptides. Mass spectrometry imaging, on the other hand, does not include chromatographic separation; consequently, MS measurements should be as accurate and specific as possible in order to obtain reliable results.

The benefit of high mass resolving power (R = 60000) and high mass accuracy measurements (RMS <2 ppm) in MS imaging experiments is illustrated in Fig. 6. Figure 6(a) shows the average mass spectrum of the imaging experiment in the range of *m/z* 1256.624 ± 100 ppm. Figure 6(b) shows the *m/z* values of the $[M+H]^+$ ions of peptides that were identified by LC/MS/MS measurements (in the selected mass window). The two peaks detected by MALDI imaging (Fig. 6(a)) match with masses of peptides that were identified in the mouse brain tissue by LC/MS/MS (Fig. 6(b)). Therefore, the corresponding images were assigned to these two peptides. The two matched peaks correspond to tryptic peptides of the proteins dynamin-1 (P39053) and Hsp90aa1 (A0PJ91). An unequivocal match of imaging and LC/MS/MS data is only possible with a mass accuracy in the low-ppm range for both measurement modes, while less accurate measurements would lead to ambiguous assignments. For example, at 30 or 100 ppm mass accuracy, more than one possibility of linking the two measurements would exist and this would obstruct a valid interpretation of the imaging results. False positive identifications are still possible, but the risk is greatly reduced by using high mass accuracy MALDI imaging and high mass accuracy LC/MS/MS data of adjacent tissue sections. This identification problem is even more severe if no additional information such as LC/MS/MS data is available for peptide identification. A database search that is solely based on the *m/z* values of imaged peaks would result in a large number of possible assignments. Figure 6(c) shows the *m/z* values of the $[M+H]^+$ ions of all theoretical tryptic peptides of mouse proteins in the mass range *m/z* 1256.50 to 1256.75, as obtained by *in silico* digestion of the *mus musculus* database (SwissProt) using the web-based program MSFit of the ProteinProspector platform.[34] More than 160 tryptic peptides (no modification, no missed cleavages) were found to lie within ±100 ppm of the peptide peak at *m/z* 1235.624. This number would increase significantly if a more realistic scenario was chosen and missed cleavages and/or variable modifications such as oxidation or phosphorylation were taken into account.

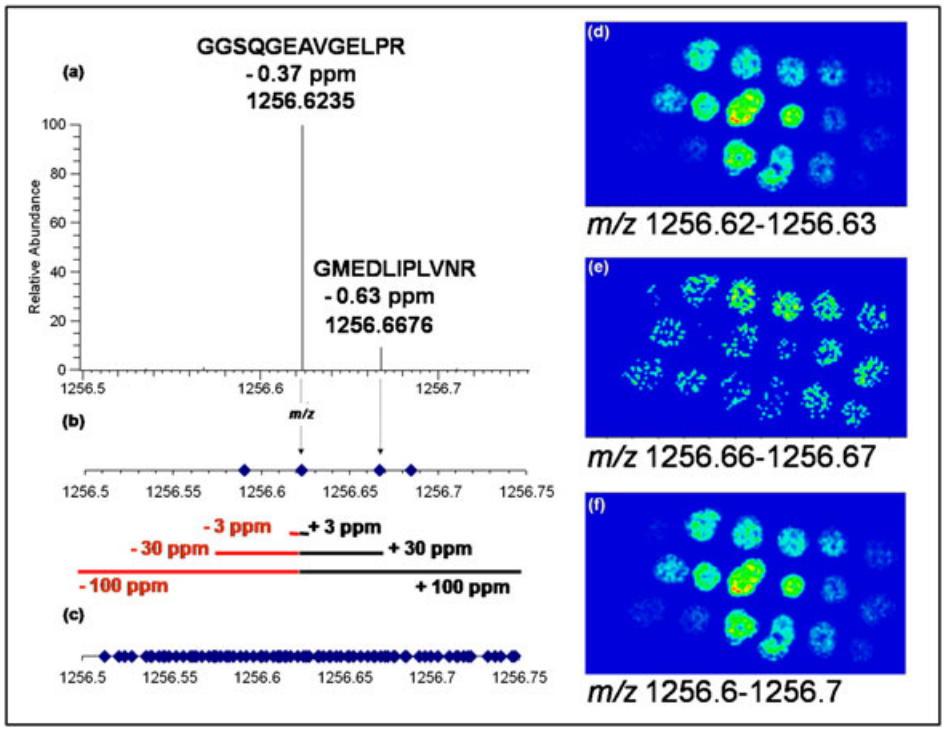

Figure 6. Demonstration of necessity of high mass accuracy in imaging experiments. (a) Section of the averaged mass spectrum of high mass accuracy MS imaging of mouse brain tissue, containing two identified tryptic peptides. (b) *m/z* values of all tryptic peptides identified in homogenate LC/ESI-MS/MS measurements in the displayed mass range (*m/z* 1256.50 to 1256.75). (c) *m/z* values of all theoretical tryptic peptides in the same mass range (*in silico* digest of *mus musculus* data base). (d)–(e) MALDI ion images of the two identified peptides with an imaging bin width of $\Delta m/z = 0.01$. (f) MALDI ion image with a bin width of $\Delta m/z = 0.1$ including both identified peptides.

These considerations show that peptide identification without accurate mass and/or without complementary approaches such as bulk identification methods can easily produce misleading results. Especially in the case of tryptically digested biological tissue sections of high complexity, reliable identification of proteins significantly benefits from high mass accuracy of measurements. It is important to note again that both the imaging and the LC/MS/MS measurement have to be performed at high mass resolution and high mass accuracy.

High-quality mass spectral data are not only necessary for confident identification, but also for reliable representation of spatial features. The two peptide peaks shown in Fig. 6(a) differ in mass by only 0.04 u, but they show significantly different spatial distributions. Selected ion images generated with a bin width of $\Delta m/z = 0.01$ (e.g. *m/z* 1256.62–1256.63) for both peaks are shown in Figs. 6(d) and 6(e). If an imaging bin width of $\Delta m/z = 0.1$ or higher were used, the spatial distribution of the less-abundant peptide would have been obscured, as demonstrated in Fig. 6(f) (bin width of $\Delta m/z = 0.1$). We have recently shown similar behavior for the MALDI imaging analysis of phospholipids[26] and neuropeptides.[37] Unresolved isobaric peaks also interfere with quantitative evaluation because multiple compounds might contribute to changes in the intensity of a certain mass signal. Consequently, the signal of a potential marker compound might be masked by neighboring peaks. Markers which are of pathological relevance, e.g. because they are involved in signaling processes, are often present at low concentrations and are thus easily masked by highly abundant house-keeping proteins.

The effect of various mass tolerance settings for the comparison of MALDI imaging and LC/ESI-MS/MS analysis is shown in Fig. 7. The number of matching peptides increases rapidly with increasing mass tolerance. Therefore, parameters have to be chosen carefully in order to find an optimum between the number of identified peptides and the rate of false positive identifications. A scatter plot of mass deviation values in the MALDI imaging experiment is shown in Supporting Information 4 for the example of the peptide TTHYGSLPQK at *m/z* 1131.579. This distribution is based on more than 1200 individual measurements of the peptide peak. The mass accuracy was 1.4 ppm root mean square (RMS). The vast majority of measurements show a deviation of 3 ppm or less. In order to account for some variability, we decided to consider only peptide matches with less than 3 ppm mass deviation as identified peptides in our study. These matches are correct with a higher probability than for values typically used for identification in MALDI imaging experiments (30–200 ppm). These results also indicate that mass peaks that deviate by more than 10 ppm from the theoretical peptide mass peak actually originate from a different peptide. Consequently, the vast majority of peptide matches with a mass deviation of more than 10 ppm are erroneously assigned and would lead to false positive identifications.

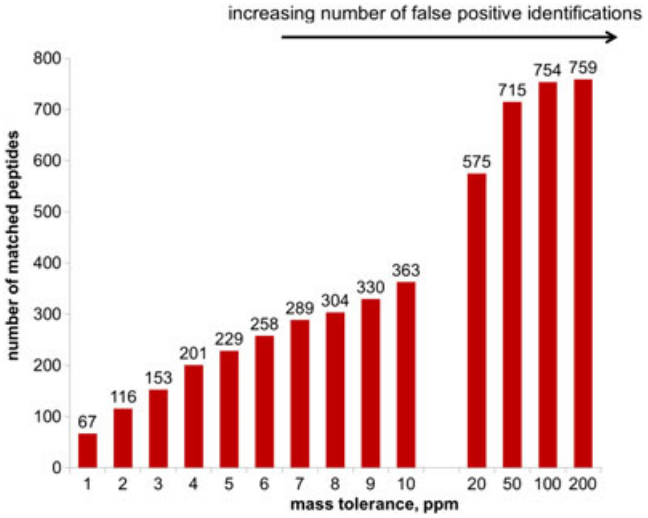

Figure 7. Number of the matched peptides versus mass tolerance used for matching.

In the light of these results, the identification results of previous studies have to be considered with care if no on-tissue MS/MS confirmation was performed. Identification can also be confirmed by comparing the spatial distribution of tryptic peptides originating from the same protein. However, higher mass accuracy measurements lead to more reliable results in any case. More frequent mass calibration steps, internal standards and/or post-run recalibration can be used to achieve this goal. This is especially important for linear TOF instruments as they are sensitive to height differences of the sample which cannot be entirely eliminated in biological tissue sections.

CONCLUSIONS

On-tissue tryptic digestion of proteins is an established method that can overcome some of the limitations (e.g. due to limited mass range) of top-down measurements. Identification by direct on-tissue MS/MS measurements, however, is usually limited to the most abundant peptides. The combination of LC/MS/MS and imaging experiments is used to make less-abundant peptides accessible. This approach is still challenging and needs to be improved with respect to sensitivity and reliability. In this work we demonstrated that additional preparation steps for the homogenate sample significantly improve the number of identified peptides. We also showed that high-quality mass spectral data are necessary in order to combine these two methods in an unambiguous way. False positive hits cannot be ruled out completely, but the probability is significantly reduced by the use of accurate mass measurements. Reliable determination of spatial distributions of tryptic peptides in tissue is only possible with high-resolution mass spectrometry.

In recent years accurate mass measurements have become routine in many bioanalytical applications. They have significantly increased the reliability of classical proteomics experiments. A similar development for MS imaging is highly desirable in order to improve the selectivity and reliability of this rapidly growing analytical technique. This is especially important for highly complex samples such as biological tissue sections because no separation steps (e.g. chromatography) are available prior to direct on-tissue analysis. Therefore, MS imaging experiments should always be designed to deliver the highest mass accuracy possible (for a given instrumental setup). We are currently working on improving the spatial resolution of our digestion method by reducing the spot size and on the analysis of clinically relevant samples. The considerations and strategies concerning LC/MS/MS and accurate mass measurements discussed above are highly relevant for these experiments and will help to improve results both qualitatively and quantitatively.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

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