MALDI mass spectrometric imaging (MSI) is a powerful technique enabling analysis of the spatial distribution of a wide variety of molecules (such as dosed drugs, as well as endogenous lipids, metabolites, peptides, and proteins) in biological tissue sections. One potentially exciting application of this technology is towards the identification and subsequently determination of the spatial localization of peptide/protein biomarkers for a given disease state within tissue. In situ enzymatic digestion of proteins in tissue is becoming a more widespread technique for the identification of proteins using MSI. Successful implementation of MSI requires instrumentation capable of generating high-quality MS and MS/MS data that can be used to interrogate database search engines, enabling the identification of the peptide or protein. The AB SCIEX MALDI TOF/TOF™ systems are ideally suited for this purpose because of their superior sensitivity, mass resolution and mass accuracy in both MS and MS/MS acquisition modes.

This application note describes the use of in situ trypsin digestion on rat brain tissue sections, followed by matrix coating and subsequent MS and MS/MS analysis using the 4800 MALDI Plus TOF/TOF™ Analyzer. The same workflows can be performed on all AB SCIEX TOF/TOF™ systems. The tryptic peptides generated from in situ enzymatic digestion of the proteins present in brain tissue provided an extra dimension to the analysis because tryptic peptides are generally more easily analyzed and identified using mass spectrometric sequencing techniques and database searching than native, non-tryptic peptides.

Figure 1. Schematic Representation of the Workflow for Trypsin Digestion and Matrix Coating of Rat Brain Tissue.
Methods and Materials

Sample Preparation: Frozen wild-type Sprague Dawley rat brain tissue was sliced into coronal sections of 12 µm thickness using a Leica cryostat and thaw-mounted onto 44mm x 44mm stainless-steel OptiTOF™ plates. Mounted tissues were rinsed for 30 seconds in ice-cold 70% ethanol, then rinsed for 30 seconds in ice-cold 90% ethanol/9% glacial acetic acid. After tissue sections were dried in a vacuum chamber for 1 hour at room temperature, a trypsin solution (66 µg/mL in 10 mM ammonium bicarbonate/10% isopropanol) was deposited onto one half of the rat brain tissue slice using a Shimadzu Chemical Inkjet Printer (ChIP) 1000 system (Figure 1). The trypsin solution was applied in a grid pattern at a pitch (i.e., spot-to-spot spacing) of 250 µm x 250 µm, with a total amount of 10 nL deposited per spot over 20 iterations. After application of trypsin, the plate was placed in a humidification chamber at 37°C and incubated for 12 hours. MALDI matrix solution (6 mg/mL α-cyano-hydroxycinnamic acid [CHCA] in 50% acetonitrile/0.1% trifluoroacetic acid) was then spotted onto the entire tissue section also using the Shimadzu ChIP. The CHCA solution was applied at a 250 µm x 250 µm pitch, with a total amount of 10 nL deposited per spot over 20 iterations.

Mass Spectrometry: MS imaging was performed on the 4800 Plus MALDI TOF/TOF™ Analyzer at a laser pixel resolution of 100µm x 100µm using an MS Reflector Positive method scanning the mass range 1000 - 4000 amu, firing 50 laser shots per pixel. Imaging data was processed using TissueView™ software. Peptides of interest observed in the MS imaging scans were further analyzed in subsequent rounds of dedicated MS/MS imaging acquisitions, also at a laser pixel resolution of 100 µm x 100 µm using an MS/MS 1 V Positive method. MS/MS images were processed and peptide MS/MS spectra were exported into mass-to-charge/intensity lists using TissueView™ software. These mass-to-charge/intensity lists were submitted to database searching using the MASCOT search engine. Database searches were performed against the SwissProt database.
Advantages of In Situ Digestion for Tissue Imaging

One powerful feature of TissueView™ software is the ability to perform comparative qualitative analysis between two or more sub-sections of a tissue slice. This is done by drawing Regions of Interest (ROIs) encompassing the desired regions of the tissue to be compared. Performing this type of analysis allowed the comparison between the undigested and trypsin-digested halves within a coronal rat brain section. Several peaks were observed either only in the undigested or only in the trypsin-digested half. Some examples of peaks found in the undigested half of the brain slice but not the digested half are shown in Figure 2. The ions with m/z values 2564.5 and 3065.7 are clearly visible in only the undigested half and also show strong localization to distinct regions of the brain. The fact that these peaks are not visible in the trypsin-digested half of the brain strongly suggests that these ions are native peptides that have undergone proteolysis by trypsin into smaller fragment peptides on the digested half.

Although identification of native peptides by MS/MS and database searching is feasible, it is generally more difficult than the identification of tryptic peptides – this is due to several factors. Native, non-tryptic peptides generally fragment and ionize less efficiently than tryptic peptides that carry a positive charge at the C-terminus. Other factors include the incomplete annotation of native peptides in many commonly-used proteomic databases, as well as the inherent difficulty of database search engines such as MASCOT in interpreting MS/MS data and assigning the correct amino acid sequence for undigested peptides. While tryptic digestion increases the complexity of biological material present in tissues through the conversion of proteins to a much larger number of peptides, the digestion of proteins/peptides in situ with proteases has the potential to lead to more confident identification of proteins present in tissue slices.

![Spatial Distribution of a Tryptic Peptide with m/z = 2141.2](image)

Figure 3. Spatial Distribution of a Tryptic Peptide with m/z = 2141.2. Shown is the extracted ion image (A), the extracted ion image overlayed with the tissue optical image (B), and the MS spectrum of the peptide (C). This peptide is clearly present in only the trypsin-digested half of the tissue.
Peptide Identification From MS/MS Imaging

Figure 3 highlights a peak that was observed only in the trypsin-digested half of the brain slice (m/z = 2141.2). This peak also displayed spatial localization to a distinct portion of the brain. To verify the spatial localization, as well as identify the peptide sequence, a dedicated MS/MS experiment was performed specifically for this precursor mass. As expected, the major fragment ion of the 2141.2 precursor (1797.2) displayed the same localization within the brain as the precursor, as shown in Figure 4.

Figure 4. Spatial Distribution of a Major MS/MS Ion Fragment (m/z = 1797.2) from the Precursor Tryptic Peptide with m/z = 2141.2 from Figure 3. Shown are the extracted ion image of the 1797.2 fragment (A) and the extracted ion image of the m/z 1797.2 fragment overlaid with the tissue optical image (B). Also shown for comparison is the extracted ion image of the m/z 2141.2 precursor overlaid with the tissue optical image from Figure 3 (C), demonstrating that the precursor and the fragment overlap within the tissue. Finally, the MS/MS spectrum for the m/z 2141.2 precursor is shown in (D). The mass-to-charge/intensity list from this spectrum was submitted for a MASCOT database search (Matrixscience, UK).

An averaged MS/MS spectrum for this precursor ion was generated by drawing an ROI around the region where the fragment ion intensity was highest. The exported mass-to-charge/intensity list from this MS/MS spectrum was submitted to MASCOT for database searching and as shown in Figure 5, this resulted in the assignment of the sequence TQDENPVHFFKNIVTPR to this peptide with high confidence. The origin of this peptide is Myelin Basic Protein (MBP), a protein that is abundant in nervous tissue. As shown in Figure 5, the MS/MS spectrum contains almost all matching y-ions and several b-ions from this sequence. This result successfully demonstrates this overall workflow for the identification of endogenous tissue proteins. This technique should therefore be applicable to the more widespread identification of tissue protein/peptide biomarkers, either exogenous or endogenous.
Conclusions

This work demonstrated the successful application of in situ trypsin digestion on tissue towards the identification of tissue-specific proteins/peptides. The sensitivity, resolution and mass accuracy of the 4800 Plus MALDI TOF/TOF™ Analyzer enables the generation of MS/MS data of high quality that is required for such an application.

References
