Protocol development for on-tissue enzymatic digestion for direct protein analysis

Klára Ščupáková

Daily Supervisor: Dr. N. Ogrinc Potočnik (Postdoc at M4I); Co-supervisor: Prof. Dr. Ron M.A. Heeren (Director of M4I, SAB of O2I); HZ Mentor: Assoc. Prof. Kas Wannee

Image retrieved from Richard Caprioli and Reid Groseclose (Mass Spectrometry Research Center, Vanderbilt University, Nashville, TN).
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Final thesis report

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Mentor: Assoc. Prof. Kas Wannee

HZ University of Applied Sciences, Vlissingen
Omics2Image, BV, Amsterdam (O2I)
Maastricht MultiModal Imaging Institute, Maastricht (M4I)
Abstract

Over the last years, Mass Spectrometry Imaging (MSI) has proven to be a useful tool for direct in-situ analysis of thin tissue sections. This label free technique was quickly recognized and applied in different fields by demonstrating its advantages over other imaging techniques such as autoradiography, immunohistochemistry or positron emission tomography [1]. The MSI is capable of providing simultaneous distribution and identification of hundreds of different (unknown) compounds in a single imaging experiment [2-4].

In every successful MSI measurement, the sample preparation plays an essential part of the analysis. Therefore, it is necessary to optimize and adapt the procedures according to the tissue and analyte under investigation [5]. In this case, the protocol was developed for the analysis of rodent brain tissue and proteins/peptides within the tissue.

Different washing procedures, enzyme application techniques, incubation times, protease solutions, matrices and matrix application techniques were tested to define the optimal protocol. The chloroform/ethanol washing procedure was found to be the best in lowering the ion suppression effect. The optimal digestion was achieved with trypsin dissolved in ddH₂O, applied with SunCollect and incubated for 18 hrs. Good extraction/ionization of peptides during the MALDI-MSI experiments was achieved with α-CHCA matrix applied with SunCollect. All acquired spectra were baseline corrected, normalized by TIC, autoscaled and peak picked. Furthermore, Principal Component Analysis (PCA) and Discriminant Analysis (DA) were applied to uncover and visualize the variability within the data.

The developed protocol for on-tissue digestion demonstrated that it is feasible to localize and identify peptides/protein directly from brain tissue. More than 30 proteins could be identified from the wild type (WT) tissue. The optimized protocol was also used to study distribution of peptides/proteins within the Alzheimer mouse model (APP/PS1) brain tissue, showing subtle differences between the two models.
Acknowledgements

First of all, I would like to thank Dr. H.R. Poolman for the opportunity to perform my final thesis in his company. In addition, I am grateful to Prof. Dr. Ron M.A. Heeren for introducing me and incorporating me in his group and thus allowing me to gain invaluable experience. Moreover, I would like to share my gratitude for the interesting and challenging project that I have been assigned to work on.

Then, I would like to express my thankfulness to my daily supervisor, Dr. N. Ogrinc Potočnik. Her expertise, precious patience, assistance, positive energy and friendly attitude added significantly to my experience.

Lastly, I would like to share my special thanks to Dr. Tiffany Porta and the rest of my colleagues for their warm welcoming, sympathy and aid during my final thesis at Omics2Image and Maastricht MultiModal Molecular Imaging Institute.
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1. Introduction

Over the last years, Mass Spectrometry Imaging (MSI) has proven to be a useful tool for direct in-situ analysis of thin tissue sections. This powerful technique allows for a rapid label-free detection, localization and identification of a broad range of molecules directly from the tissue surface and so demonstrating its advantages over other imaging techniques such as autoradiography, immunohistochemistry or positron emission tomography [1–3,6,7]. MSI was first described by Caprioli et al. in 1997 [8] and from then on went through a rapid development in instrumentation, methodology, sample preparation and data processing [6–8].

The power of MSI was quickly recognized and became a powerful tool for studying the pathology of different diseases [4,7,9]. One of such examples is the M4I (Maastricht MultiModal Molecular Imaging) Institute, focusing its research on the development and application of Mass Spectrometry Imaging in biomedical sciences and nanomedicine. The projects carried out at M4I Institute, among others, involve MSI and multivariate data analysis for monitoring molecular changes in brain regions of transgenic mice. A transgenic technique is used in model mice to change the expression levels of specific proteins in the brain, resulting in a disease associated phenotype. Altered expression levels of proteins cause different biomolecular profiles, which are used to study differences between the wild type and transgenic mice. One of such examples is the study of Alzheimer’s disease (AD).

Alzheimer is a progressive neurological disorder characterized by loss of memory and cognitive impairment [10]. The prevalence of AD is 35 million worldwide and AD accounts for 2/3 of all cases of Dementia [11]. The slow progression, thus long duration of the disease and the high prevalence rate of AD imposes great financial and social impact on society [12]. The pathology of AD is complex. One of the most probable causes of the AD pathogenesis is suggested to be the accumulation, aggregation and decreased degradation of Aβ peptides. Research shows that the increased production of Aβ and its impaired clearance causes the aggregation of Aβ into oligomers and fibrillar amyloid plaques, which disrupt synaptic functions, signaling pathways, neuronal activities and make glial cells to release neurotoxic mediators [11]. The ability to localize and identify Aβ peptides within the brain tissue is critical for the study of AD pathology.

In order to study these large protein biomarkers, it is important to perform on-tissue enzyme digestion prior to analysis. The digestion induces peptide fragments and the proteins can be identified through the peptide mass fingerprint using tandem mass spectrometry (MS/MS). For these types of experiments trypsin is the most commonly used enzyme, although several other enzymes such as pepsin, elastase or chemotrypsin are available.

The aim of this project was to optimize and develop protocols for on-tissue enzymatic digestion for direct protein analysis of rodent brain tissue using MSI techniques. The study also investigated other parameters such as digestion efficiency, extraction and identification
of peptides from the tissue surface. Ultimately the developed protocol was tested in order to study the distribution of digested Amyloid-β (Aβ) peptides as potential biomarkers for amyloid plaque formation, which is a major hallmark of Alzheimer’s disease progression.

2. Theoretical Background

2.1. Basic principles of on-tissue protein digestion MSI

Each MSI experiment is divided into three different parts, which play an important role in the collection and the interpretation of the analyzed data: sample preparation, MSI and data analysis. Each of the different parts has to be optimized according to the analytes in question. In our experiments the protocols must be adapted for the investigation of peptide biomarkers. The schematic representation of our MSI experiment is shown in Figure 1.

![Figure 1: Overview of MALDI-MSI experiment.](image-url)
Sample preparation is one of the most crucial steps in MSI analysis. It is of great importance to preserve the sample as much as possible to the in-vivo state. This is usually achieved by snap-freezing or embedding the biological tissue. The samples are sectioned into 5-20 μm thin slices by a cryo-microtome [6] and thaw mounted on the indium tin oxide (ITO)-coated or regular glass slides [13]. To prevent condensation and delocalization of molecules the sections are immediately dried in the vacuum desiccator. For peptide and protein analysis the sections need to be washed with organic solvents in order to remove unwanted salts and lipids which may suppress ionization [6,14,15]. The samples are then analyzed with the MSI instrument by desorbing and ionizing biomolecules from the sample surface. The ionization is achieved through exposure of the surface to a laser beam e.g. Matrix Assisted Laser Desorption/Ionization (MALDI), a primary beam e.g. Secondary Ion Mass Spectrometry (SIMS) or a charged droplet flux e.g. Desorption Electrospray Ionization (DESI) [6]. The SIMS method provides high spatial resolution whereas MALDI covers a broader mass range for biomolecular investigation. Contrary to MALDI and SIMS experiments which are performed under high vacuum, DESI can be used in ambient conditions. This brings several advantages, mainly in the area of sample preparation and stability [6,16]. However, the resolution and sensitivity of DESI are still limited. In order to cover the broad range of biomolecules studied the MALDI-MSI technique was the method of choice. It offers high sensitivity, tolerance for salts and other contaminants, wide mass range (> 30 000 Dalton (Da)) and little fragmentation [3,14].

In order to assure a proper on-tissue digestion, prior to MSI analysis, the sections must be coated with an enzyme. This can be achieved by either spotting or spraying. In the spotting technique small volumes (e.g. 100 pL) are deposited on an area (usually 100 μm) on the sample surface, lowering the risk of analyte diffusion and delocalization. However, the spatial resolution is limited to the spot size. With the spraying approach, the entire sample surface is covered in a homogenous layer of enzyme solution. Spraying is better when it comes to obtaining high spatial resolution, however, a risk of analyte delocalization is higher. The enzyme application is performed at room (RT) or at slightly elevated temperature (37°C) in a humid chamber followed by incubation period varying from couple of hours to overnight incubation time [2,5,17].

Prior to each MALDI experiment the sample has to be coated with the matrix solutions. The most commonly used matrices are organic acids which absorb the radiated laser at a specific wavelength. The choice of matrices depend on different factors, one of the most important being the analytes in question and their ionization affinity (positive or negative mode) [4,18]. The matrix is dissolved in volatile organic solvents, which help with the extraction of the molecules from the sample resulting in co-crystallization of the analyte into the matrix [18,19]. These crystals are then irradiated by the laser causing desorption and ionization of the compounds.

The ions are detected by a mass spectrometer according to their mass to charge (m/z) ratio. Numerous mass analyzers are available, e.g. Time-of-flight (TOF), Ion Trap (IT), Triple Quadrupole, etc. [6]. This study uses primarily MALDI-TOF-MS. The principle of TOF-MS is
based on the ion’s m/z ratio over a measured time. The ions are accelerated by an applied electric field. The time needed to reach from the accelerator to the detector for an ion is thus dependent on its m/z ratio [6,20]. The data acquired is in a form of a mass spectrum where the X-axis represents the m/z ratio and the Y-axis the intensity of the detected ion. The schematic representation of the MSI workflow is shown in Figure 2.

![Figure 2: Schematics of the different step necessary for the investigation of biomolecules in tissue samples using MALDI-MS.](image)

When using a MALDI-TOF for imaging, the matrix coated tissue sample is scanned in a pre-defined pattern. The pattern is a two dimensional area which is analyzed spot by spot and mass spectra are generated at each sampled location (Figure 3) [21,22].

![Figure 3: The Mass spectrometry imaging principle.[6]](image)
2.2. Data analysis

In a single MSI experiment large data sets (up to few gigabytes) are generated and thus intense data processing is required. Needless to say, the data processing and interpretation is complex and crucial for reliable and accurate results.

First, the obtained spectra are pre-processed to reduce the influence of instrumental and analytical variation and to extract the relevant biological information [1,5,23]. Smoothing and baseline correction is performed to eliminate the noise and background signal [24–26]. Second, the spectral normalization is carried out to minimize the spectrum-to-spectrum differences in peak intensity. The most common form is the Total Ion Count (TIC) normalization where all of the spectrum intensities are devided by the sum of all intensities (TIC) [26]. The following step in data pre-processing is autoscaling. Autoscaling is a process where each column of the data matrix is divided by its standard deviation and its mean is subtracted. That is done to avoid the domination of large-valued descriptors in the analysis. In other words, autoscaling removes unintentional weighting of variables that would otherwise happen [27,28]. Peak picking is an additional pre-processing step applied to assure the selection of m/z values corresponding to relevant peaks. In this way, the amount of data is significantly reduced by eliminating the m/z values related to noise and non-specific features [23,26].

Once the data is pre-processed, further statistical analysis is applied. Principal Component Analysis (PCA) is unsupervised statistical method describing the largest variances within the dataset. Its ability to reduce the dimensionality of a multivariate dataset (e.g. filter the data by detecting correlated noise such as matrix signal, salts, etc.) significantly helps the MSI data interpretation [24,25]. Contrary to PCA, Discriminant Analysis (DA) is supervised statistical method used to define classes within pre-defined groups – e.g. healthy vs diseased sample. In our case, the PCA-DA is combined to reveal a number of features that are correlated with a specific condition [5].

The identification of peptides/proteins is commonly done through peptide mass fingerprint using tandem mass spectrometry (MS/MS). The tryptic peptides are most commonly observed in the mass range 800–3500 Da. Additionally, database searches and in-silico digestion (i.e. computer-based simulation of enzymatic digestion) are quick method to identify imaged peptides and asses the digestion efficiency. Important parameter in peptide identification is the threshold value (or mass tolerance window) used in database search, which is directly linked to the mass accuracy of the mass spectrometer [1,29].

Given the enormous computational challenge that MSI data imposes, software packages (such as MassLynx, DataCube explorer, Biomap, ClinProTools, FlexImaging, etc.) are available to perform all the different steps in pre-processing and data analysis [6].
2.3. Limitations of MALDI-MSI

The biggest advantages of MALDI-MSI are unfortunately directly linked to its limitations. The high sensitivity of MALDI-MSI demands the sample stability at room temperature as well as stability in high vacuum [6]. Therefore, any sample handling and preparations such as the sectioning procedure, washing steps as well as enzyme and matrix deposition must be performed attentively in order to prevent any sample damage and/or contamination and molecular delocalization [14,21,22]. The latter is also important for maintaining the spatial distribution of analytes in the MALDI experiment. The complexity of the biological samples is a major factor imposing a risk of biomolecules negatively effecting each other’s desorption and ionization efficiency. This phenomenon is called ion suppression [6,15] and it occurs when one analyte (e.g. lipids) is present in great excess and ionizes better than the analyte of interest (e.g. proteins/peptides). Ion suppression limits the number of detected molecules and thus decreases the quality of MALDI-MSI measurement. In order to overcome these limitations, great attention is paid to the protocols followed for sample pre-treatment.

3. Materials & Methods

3.1. Samples

The fresh frozen brain tissues of 24 months old male wild type (WT) Mus musculus and transgenic APP<sub>KM670/671NL/PS1</sub>1169 Mus musculus were obtained from the University of Antwerp, Bio-Imaging Lab, Antwerp, Belgium.

Sacrification of the animals:

The University of Antwerp, Bio-Imaging Lab, is in compliance with the European animal welfare regulations. The animals were injected with an anesthetic, causing an overdose. The abdomen was cut open and cardiac perfusion with ice-cold phosphate-buffered saline (PBS) was performed to replace the blood from the system. After the completed perfusion, the mice were decapitated and the brain was snap frozen in liquid nitrogen. The brain samples were stored at – 80 °C freezer.
## 3.2. Chemicals

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>Purity</th>
<th>Cas number</th>
</tr>
</thead>
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<td>Acetonitrile</td>
<td>Biosolve B.V.</td>
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<td>Ammonium bicarbonate</td>
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<td>Amyloid β peptide standard</td>
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<td>107761-42-2 131438-79-4</td>
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<td>Chloroform</td>
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<td>Entellan</td>
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<td>-------</td>
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<td>7723-14-0</td>
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<td>76-05-1</td>
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<tr>
<td>Trypsin</td>
<td>Sigma Aldrich</td>
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<td>T6567-5X20UG</td>
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<td>Water ULC/MS</td>
<td>Biosolve B.V.</td>
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<td>67-17-5</td>
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<tr>
<td>α-Cyano-4-hydroxycinnamic acid</td>
<td>Sigma Aldrich</td>
<td>99%</td>
<td>28166-41-8C</td>
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## 3.3. Consumables

<table>
<thead>
<tr>
<th>Name</th>
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<th>Type</th>
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<tbody>
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<td>Indium tin oxide -coated glass slides</td>
<td>Delta Technologies</td>
<td>CG-40IN-1115</td>
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<tr>
<td>Microscope Glass slides</td>
<td>Thermo Scientific</td>
<td>ISO 8037/1</td>
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## 3.4. Instrumentation

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<th>Type</th>
</tr>
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<tr>
<td>CHIP</td>
<td>Shimadzu</td>
<td>CHIP-1000</td>
</tr>
<tr>
<td>Cryostat</td>
<td>Leica</td>
<td>CM1860 UV</td>
</tr>
<tr>
<td>ImagePrep</td>
<td>Bruker Daltonics</td>
<td>----------</td>
</tr>
<tr>
<td>MALDI Spotter/Sprayer</td>
<td>SunChrom-SunCollect</td>
<td>----------</td>
</tr>
<tr>
<td>MALDI-TOF-MS</td>
<td>Waters</td>
<td>Synapt G2Si HDMS</td>
</tr>
<tr>
<td>Microscope</td>
<td>Leica</td>
<td>DM6000 B</td>
</tr>
<tr>
<td>MIRAX scan</td>
<td>Zeiss</td>
<td>----------</td>
</tr>
<tr>
<td>Scanner</td>
<td>CanoScan</td>
<td>9000F Mark II</td>
</tr>
</tbody>
</table>
3.5. Methods

3.5.1. Tissue collection

The MALDI-MSI is performed on thin intact tissue sections, thus optimal sectioning conditions are essential to avoid raptures and preserve the spatial distribution of molecules in question.

Coronal and sagittal sections of the brain tissue were obtained with a cutting instrument—Cryostat (Leica CM1860UV), Figure 4. The brain tissue (stored at -80 °C) was carefully mounted on the sample holder. The cryostat stage holding and blade temperature were set to -25°C and -21°C respectively. Twelve µm thick sections were obtained and thaw mounted onto glass slides/ITO-coated glass slides, which were kept at ambient temperatures during mounting. Immediately after sectioning the samples were stored at -80 °C for later use. The tissue sectioning process was performed according to standard laboratory practice which was also described by Kamila Chughtai et al. [6].

Figure 4: Sectioning instrument – Cryostat. Adapted from Leica. [36]
3.5.2. Tissue washing

For peptide and protein analysis the sections need to be washed with organic solvents in order to remove unwanted salts and lipids which may suppress ionization [6,14,15]. Brain tissue is rich in lipid species and therefore the ion suppression was expected.

In order to decrease the ion suppression effect of salts and lipids, the desiccated tissue sections were washed as follows:

- Submerge in 70% ethanol for 30 seconds
- Submerge in 70% ethanol for 30 seconds
- Submerge in chloroform for 30 seconds
- Submerge in 70% ethanol for 30 seconds

Finally, the sections were dried in a vacuum desiccator for at least 20 minutes before the on-tissue enzyme application.

3.5.3. Hematoxylin and Eosin staining for MALDI-MSI analyzed tissue

The MALDI-MS images were compared with microscopic images to facilitate alignment of the peptide distribution with the anatomy of the brain tissue. The H&E protocol used was established in the lab.

The staining protocol is the following:

1. Immerse the MALDI-MSI analyzed tissue section into 70% ethanol for matrix removal.
2. Follow series of ethanol washes: immerse the sample in 2x 100%, 2x 96%, 2x 70% ethanol for each time 3 min.
3. Immerse the section in distilled water for 3 min.
4. Stain the samples with filtered 0.1% Hematoxylin for 3 min. If Hematoxylin is stored, then it should be covered in foil, since it is light unstable.
5. Immerse in tap water for 3 min.
6. Immerse in 0.2% Eosin for 30 sec.
7. Wash in running tap water approx. 1 min until it stops streaking.
8. Equilibrate the sample for 1 minute in 100% ethanol.
9. Finally, dip in xylene for 30 sec.
10. Let the stained glass slides to dry. If necessary, carefully wipe off excess water with a kimwipe.
11. Mount the stained sections with glass cover slips using Entellan as mounting media.
12. Let it dry minimally 2 hrs before microscopy imaging.

After the staining and drying the glass slides were imaged with a Microscope using bright field light - Leica DM6000B and MIRAX scanner.
3.5.4. On-tissue enzymatic digestion

In order to assure a proper on-tissue digestion the sections must be coated with an enzyme. A number of devices for spotting e.g. CHIP as well as for spraying e.g. SunCollect are available. Several different techniques such as manual spotting, CHIP and SunCollect were tested. Furthermore, to achieve a proper digestion the enzyme concentration, technique of application, incubation time and conditions were evaluated. The optimized method is described in the following paragraph, whereas the description of other tested methods can be found in Appendix I.

![Figure 5: SunCollect Spray device. Adapted from Sunchrom. [37]](image)

The trypsin solution was prepared accordingly: 20 µg of trypsin was dissolved in 400 µL of ddH₂O to obtain final concentration of trypsin 0.05µg/µL. Furthermore, 5 µL of 10 mM Octyl-α/β glucoside solution was added to the trypsin solution. The enzyme was applied with SunCollect MALDI Sprayer (Figure 5) on the tissue sections as specified in Table 1.
Table 1: SunCollect settings for enzyme application.

<table>
<thead>
<tr>
<th>SunCollect settings for enzyme application</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vial X</td>
<td>0.3 mm</td>
</tr>
<tr>
<td>Vial Y</td>
<td>2.0 mm</td>
</tr>
<tr>
<td>Z</td>
<td>45.0 mm</td>
</tr>
<tr>
<td>Z-offset</td>
<td>0 mm</td>
</tr>
<tr>
<td>Number of layers</td>
<td>13</td>
</tr>
<tr>
<td>Flowrate layer 1</td>
<td>10 μL/min</td>
</tr>
<tr>
<td>Flowrate layer 2</td>
<td>15 μL/min</td>
</tr>
<tr>
<td>Flowrate layer 3</td>
<td>20 μL/min</td>
</tr>
<tr>
<td>Flowrate layer 4&gt;</td>
<td>25 μL/min</td>
</tr>
<tr>
<td>Speed X</td>
<td>Low (4)</td>
</tr>
<tr>
<td>Speed Y</td>
<td>Medium (1)</td>
</tr>
</tbody>
</table>

After enzyme deposition, the glass slides were incubated overnight (approx. 18 hours) at 37 °C in a high humidity chamber with 5% CO₂. The samples were finally placed in the vacuum desiccator in order to dry the tissue (approx. 20 minutes) prior to matrix application.

3.5.5. Matrix coating

The ionization in MALDI-MSI is enhanced by the MALDI matrix deposited on top of the tissue. As for the enzyme deposition, several devices are available and were tested for the matrix application such as ImagePrep or SunCollect. The employed method is described in the following paragraph, whereas other matrix application techniques are described in the Appendix I.

The SunCollect sprayer was used to apply the matrix on the tissue section. The dried sections were coated with α-Cyano-4-hydroxycinnamic acid (α-CHCA) solution. The solution was prepared with 10 mg/ml α-CHCA crystals dissolved in 50% acetonitrile (ACN) and 0.2% trifluoroacetic acid (TFA). The optimized sprayer settings are shown in table 2.
Table 2: SunCollect settings for matrix application.

<table>
<thead>
<tr>
<th>SunCollect settings for matrix application</th>
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</thead>
<tbody>
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<tr>
<td>Vial Y 2.0 mm</td>
</tr>
<tr>
<td>Z 45.0 mm</td>
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<tr>
<td>Z-offset 0 mm</td>
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<tr>
<td>Number of layers 10</td>
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<tr>
<td>Flowrate layer 1 10 µL/min</td>
</tr>
<tr>
<td>Flowrate layer 2 20 µL/min</td>
</tr>
<tr>
<td>Flowrate layer 3 25 µL/min</td>
</tr>
<tr>
<td>Flowrate layer 4&gt; 25 µL/min</td>
</tr>
<tr>
<td>Speed X Low (4)</td>
</tr>
<tr>
<td>Speed Y Medium (1)</td>
</tr>
</tbody>
</table>

3.5.6. MALDI-MSI

MALDI imaging software HDIImaging V4.1 (Waters) was used to create a pattern and select the area to be sampled. The horizontal and vertical step sizes between each sample spot were set e.g. 100 x 100 µm. All locations were stored inside a pattern-file and mass spectra were acquired from each predefined location. For acquisition a high-definition MS (HDMS) system (Waters, Etten-leur, NL) was used with MALDI option (Figure 6). The system was calibrated in positive ionization mode between m/z 100 to m/z 5000 by a red phosphorous standard. The data was acquired in sensitivity mode, positive polarity and mass range from 100 to 3500 Da. Quadrupole profile was set to 900. Other MS settings were set to default values.
3.5.7. Data Analysis

All acquired mass spectra were combined into a single image-file of several gigabytes in size. The data were pre-processed and visualized using Masslynx (Waters), mMass [30] and BioMap (Novartis, Switzerland) software. The spectra were baseline corrected, normalized by TIC, autoscaled and peak picked. The peak picking was performed on peaks with threshold S/N (i.e., signal to noise ratio) ≥2. Furthermore, statistical analysis such as Principal Component Analysis (PCA) and Discriminant Analysis (DA) were applied to uncover and visualize the variability within the data. The software used here is the “in-house” developed ChemomeTricks toolbox [23]. The identification of imaged peptides was based on published literature, In-silico digestion and databases (Mascot, Uniprot). The peaks were assigned to identified peaks on the basis of max 0.5 Da tolerance. In-silico digest was performed using the following website: http://web.expasy.org/cgi-bin/peptide_cutter. The following parameters were used: Taxonomy *Mus Musculus*, enzyme Trypsin, number of missed cleavages 1, oxidation of methionine as variable modification, peptide mass rage 800-3500 Da and MALDI-TOF instrument [31].
4. Results & Discussion

4.5. Optimization of an MALDI imaging protocol to analyze proteins/peptides from fresh frozen brain tissue

In every successful MSI measurement, the sample preparation plays an essential part of the analysis. Therefore it is necessary to optimize and adapt the procedures according to the tissue and analyte under investigation. In this case, the protocol must be developed for the analysis of rodent brain tissue. The optimization was performed on control (WT) rodent brain sample. Once the optimization was complete, the same method is applied to the transgenic (APP/PS1) mouse model expressing amyloidosis.

4.5.1. Tissue preparation

The optimization process started by testing an available protocol for an on-tissue enzymatic digestion. The tissue sectioning process was carried out as described by Kamila Chughtai et al. [6]. The sections were then washed by immersion of the glass slide into 70% ethanol for 30 seconds (2x) and dried in vacuum desiccator. The 0.05 µg/µL trypsin solution was deposited on the tissue using the CHIP. After 18hrs incubation in humid chamber at 37 °C, the digestion was stopped by the matrix application. 10 mg/ml CHCA in 50% Acetonitrile, 50% water, 0.1% TFA matrix solution was applied onto the tissue using the ImagePrep. Further settings are described in Appendix I.

The MALDI-MSI analysis revealed strong ion suppression effect. When looking at the spectra displayed in Figure 7, high signal is observed in the mass range of 700-850 Da corresponding mainly to lipid species [31]. Since the brain composition consists 60% of lipids [32], the high ion suppression effect of the lipids is reasonable. Ion suppression limits the number of detected peptides/proteins and thus decreases the quality of MALDI-MSI measurement. This phenomenon is clearly observed in the spectrum (Figure 7) in the mass region corresponding to tryptic peptides, i.e. 800-3500 Da, where only a limited number of peaks are present.
Figure 7: MALDI-MSI spectrum of experiment 1 with the most dominant peaks corresponding to the phospholipids, namely the m/z 184 the phosphocholine (PC) head group and m/z 760 PC 34:1. Clear ion suppression effect observed.

Furthermore, the brain is composed of the white and grey matter. Besides their functional differences, their molecular profile differs as well. Thus three different regions of interest (ROIs) were selected, 1= white matter, 2= grey matter, 3= outside the tissue. It is clearly visible that different signal comes from each ROI. The spectra corresponding to each of the ROI and the corresponding MS Images of selected m/z = 1520 are presented in Figure 8.

Figure 8: Three different regions of interest were selected and compared. The variance in molecular composition between White and Grey matter is also projected in the spectra obtained.
The different signal pattern is clearly observed in different ROIs. In order to lower the strong ion suppression effect observed, the removal of the lipids plays an essential part of sample preparation. Different washing procedures were previously published with the ethanol wash being the most common organic solvent for salt and lipid removal. In order to enhance the lipid exclusion, additional solvents such as chloroform or ethanol/acetic acid/water (90/9/1) mixture were investigated. The washing procedure involving ethanol and chloroform steps was the most effective in removing salts and lipids, thus lowering the ion suppression effect, for fresh frozen brain tissue. The spectra of different washing procedures and their efficiency are shown in Figure 9.

![Figure 9: Comparison of the washing procedures, concluding that chloroform, is the most effective wash.](image)

The spectra in Figure 9 show a clear reduction of the ion suppression when using a chloroform wash. This can be clearly observed by the amount of peaks detected in the 800 - 3500 Da region after the tryptic digestion. In contrast, only a few peaks are detected in that region when using the ETOH/H2O or ETOH /acetic acid wash. Chloroform is a polar organic solvent, which is frequently used in lipid extraction techniques because it dissolves polar phospholipids [15] and therefore an effective solvent for on-tissue washing procedures.
4.5.2. On-tissue enzymatic digestion

After the washing procedure optimization the protocol needed to be optimized for the on-tissue digestion. Trypsin is the most commonly used enzyme due to the optimal size and charge of tryptic peptides, high proteolytic activity and cleavage specificity [33]. In order to assure a proper digestion the enzyme concentration, technique of application, incubation time and conditions were evaluated. Several devices for spotting e.g. CHIP as well as for spraying e.g. SunCollect are available. The investigation also covered the efficiency of each technique.

The optimization process started with enzyme application done by spotting device - CHIP. In the spotting technique small volumes (e.g. 100 pL) are deposited on an area (usually 100 µm) on the sample surface, lowering the risk of analyte diffusion and delocalization. However, the spatial resolution is limited to the spot size and the duration of the enzyme application is long (up to several hours). Successful on-tissue digestion using CHIP was previously reported, however it was performed on different tissue type. The molecular variation in tissue composition could be a possible explanation for the failed digestion in this experiment. The experiment was repeated, yet the digestion was not successful. As previously mentioned, the majority of tryptic peptides can be found in the mass range of 800-3500 Da, which was not the case in this experiment. As a proof of principle, manual deposition of trypsin on the tissue was performed. Since, the manual deposition resulted in efficient digestion, the conclusion was that the CHIP method does not allow for sufficient wet environment and thus the digestion is inefficient. Spectra from both experiments are displayed in Figure 10a.

As the CHIP method is time consuming, has limited spatial resolution and gives insufficient digestion, the investigation of enzyme deposition techniques moved to the SunCollect device. With the spraying approach of SunCollect, the entire sample surface is covered in a homogenous layer of the enzyme solution. This device allows for highly automated, fast, user friendly and reproducible method. Spraying is also better when it comes to obtaining high spatial resolution, however, the risk of analyte delocalization is higher.

The settings for on-tissue enzymatic digestion using the SunCollect were inspired by methods reported by Jonathan Stauber et al. [17], Bram Heijs et al.[29] and M. Reid Groseclose et al. [2]. From the previous experiments (CHIP, manual deposition), it was clear that the hydrated surface results in better on-tissue digestion. The settings of the SunCollect were adjusted to higher flow rates, namely to 25 µL/min. When applying the high flow rate there was an increase in the efficiency of the digestion. In order to limit the possible delocalization which can result from over-hydrating of the tissue surface as well as to lower the trypsin consumption, low flow rate, i.e. 5 µL/min, was tested. Unfortunately, the low flow rate resulted in poor digestion, confirming the importance of wet environment for effective tryptic digestion. The acquired spectra are shown in Figure 10b.
Figure 10: A – spectra shows the comparison between digestion efficiency when CHIP enzyme application method (dark blue) and manual enzyme deposition method (green) used. B – The data reveals the importance of wet environment for sufficient on-tissue digestion. The crucial parameter with SunCollect is the flow rate. High flow rate (orange) vs low flow rate (light blue) C - Summary of the three enzyme application techniques and their digestion efficiencies. Orange-SunCollect, blue-CHIP, green-manual.
A combined spectrum of different enzyme deposition methods and digestion efficiencies is present in Figure 10c. Evidently, manual enzyme deposition brought the most optimal digestion, however, as high throughput methods with better spatial resolution were required manual application was unacceptable. It was time consuming, not reproducible and not practical. On the other hand, SunCollect enzyme spraying technique reached almost as efficient digestion as the manual deposition method and in addition it offered automated, reproducible, fast and user friendly enzyme application.

Several trypsin solutions using various buffers and detergents were published for on-tissue enzymatic digestion. Here we tested trypsin dissolved in ddH$_2$O, in ddH$_2$O with addition of 10 mM Octyl-$\alpha/\beta$-glucoside and in 50 mM ammonium bicarbonate buffer with 10 mM Octyl-$\alpha/\beta$-glucosidase. The acquired spectra using different trypsin solutions are shown in Figure 11a. The spectra obtained show clear differences between the two solutions. The water solution spectrum shows a more efficient digestion and higher amount of peaks in the tryptic peptide mass area. Similar results were also obtained by Bram Heijs et al.[29]. Despite the fact that most of the trypsin suppliers recommend to dissolve the enzyme in buffer to create a perfect environment with optimal pH for the trypsin to work, our findings showed otherwise. This concludes that in order to achieve the optimal digestion on the rodent brain tissue, the trypsin should be dissolved in ddH$_2$O with the 10 mM Octyl-$\alpha/\beta$-glucoside addition.

![Figure 11](image-url)

**Figure 11:** In 11a, the two spectra compared display the two trypsin solutions (ddH$_2$O in blue, Ammonium buffer in green) tested for on-tissue enzymatic digestion. Obviously, trypsin dissolved in ddH$_2$O works better. In the part B of this figure, the influence of incubation time on the digestion efficiency is depicted. The green spectrum represents 18hrs of incubation time while orange spectrum shows the 5hrs. Our results confirm findings from a recent study performed by H. C. Diehl et al. [34], showing that overnight (i.e. 18hrs) incubation time is the most efficient for trypsin digestion.
Furthermore, incubation times and conditions play an important role as described in the literature. Therefore the next study evaluated two different incubation times - 5hrs and 18hrs, respectively. For this type of tissue, the most efficient incubation time was observed to be 18 hours in 37 °C in a high humidity chamber with 5% CO₂. Our findings are in agreement with a recent study performed by H. C. Diehl et al. [34], which showed that overnight (i.e. 18hrs) incubation time is the most efficient for trypsin digestion. The acquired spectra are shown in Figure 11b.

Here two incubation times were compared (5hrs vs. 18hrs) demonstrating that a longer incubation with trypsin increases the number detected of peptides. The complexity of the tissue could explain the fact that the enzyme needs a longer incubation time to ensure the good proteolytic activity.

4.5.3. Matrix application

Based on the literature study, the best matrix for protein/peptides in question are α-Cyano-4-hydroxycinnamic acid (CHCA), Sinapinic acid (SA) or 2,5-Dihydroxybenzoic acid (DHB) [2,5,6,29]. The proper crystallization of the matrix depends on the solvent, the matrix concentration and the matrix application technique. As for the enzyme deposition, several devices are available for matrix application such as ImagePrep or SunCollect. Both devices were tested and compared for better matrix deposition.

The spatial distribution of peptides/proteins in m/z range 800-3500 Da was analyzed, thus the matrix of choice in this study was α-CHCA. Different concentrations and solvent systems were tested. The best results were achieved with concentration of 10 mg/ml dissolved in 50% acetonitrile (ACN) and 0.2% trifluoroacetic acid (TFA). Our findings are consistent with a recent study performed by H. C. Diehl et al. [34], which showed that α-CHCA is the matrix of choice for peptide analysis.

Matrix deposition with ImagePrep, SunCollect as well as manual application was investigated. Efficient extraction of analyte, proper crystallization of matrix and homogenous deposition of matrix onto the tissue was achieved with the SunCollect. The ImagePrep method showed good results as well, however, it was more time consuming than SunCollect. Since the enzyme deposition is also performed with the SunCollect it is also the preferred method for matrix application.

In this way, we achieved a fast, automated, user friendly and highly reproducible method for both enzyme and matrix deposition.
4.5.4. Implementation of developed protocol

Once the protocol for on-tissue enzymatic digestion and MALDI-MSI was successfully optimized, we implemented the protocol to study the endogenous proteins/peptides in the mouse brain tissue. With the optimized protocol, numerous peptides were identified from wild type (WT) mouse brain tissue. The acquired spectrum was pre-processed including baseline correction, normalization, autoscaling and peak picking. The processed spectrum is shown in Figure 12.

![Optimized protocol applied to WT brain tissue. Numerous peaks in the m/z range of 800-2000 Da demonstrate successful on-tissue enzymatic digestion.](image)

The high amount of peaks in the spectrum shows a successful implementation of the developed protocol on WT mouse brain tissue section. The next step involved peptide identification. Previously reported literature and Mascot search engine or other databases were used for tentative protein assignment [29,34].

Table 3 summarizes all of the identified peptides from digested WT brain tissue. The peaks were assigned with a max. mass tolerance window of 0,14 Da.
Table 3: Summary of identified peptides from wild type mouse brain tissue using on-tissue digestion and MALDI-MSI.

<table>
<thead>
<tr>
<th>MALDI-TOF-MS m/z</th>
<th>Error Da</th>
<th>UniProt ID</th>
<th>MALDI-TOF-MS m/z</th>
<th>Error Da</th>
<th>UniProt ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>951,4465</td>
<td>-0.018</td>
<td>ALDOA_MOUSE</td>
<td>1529,7145</td>
<td>-0.017</td>
<td>HBA_MOUSE</td>
</tr>
<tr>
<td>966,4429</td>
<td>-0.048</td>
<td>TAU_MOUSE</td>
<td>1615,7505</td>
<td>-0.041</td>
<td>STX1B_MOUSE</td>
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<tr>
<td>976,4554</td>
<td>0.006</td>
<td>ACTB_MOUSE</td>
<td>1615,7505</td>
<td>-0.086</td>
<td>TBB5_MOUSE</td>
</tr>
<tr>
<td>1003,4693</td>
<td>0.021</td>
<td>COX7C_MOUSE</td>
<td>1615,7505</td>
<td>-0.086</td>
<td>TBB2B_MOUSE</td>
</tr>
<tr>
<td>1003,4693</td>
<td>-0.015</td>
<td>MAP1A_MOUSE</td>
<td>1615,7505</td>
<td>-0.085</td>
<td>TBB2A_MOUSE</td>
</tr>
<tr>
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<td>CNTN1_MOUSE</td>
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<td>ADDB_MOUSE</td>
</tr>
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<td>1718,8715</td>
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<td>EDF1_MOUSE</td>
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<td>1346,6230</td>
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<td>ADDB_MOUSE</td>
<td>1750,7999</td>
<td>-0.048</td>
<td>AT2B2_MOUSE</td>
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<tr>
<td>1346,6230</td>
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<td>MAP6_MOUSE</td>
<td>1756,8156</td>
<td>-0.104</td>
<td>HBB1_MOUSE</td>
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<td>-0.043</td>
<td>ACTB_MOUSE</td>
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<tr>
<td>1377,6418</td>
<td>-0.075</td>
<td>ALDOA_MOUSE</td>
<td>1868,8578</td>
<td>-0.057</td>
<td>ACON_MOUSE</td>
</tr>
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<td>1527,7144</td>
<td>-0.044</td>
<td>CX7A2_MOUSE</td>
<td>1953,9479</td>
<td>-0.112</td>
<td>ACTB_MOUSE</td>
</tr>
</tbody>
</table>

To demonstrate the power of MALDI-MSI, spatial distribution of peptides within the brain tissue were visualized using BioMap software. The intensity maps of certain m/z and their distribution within the tissue are depicted in Figure 13.
Figure 13: Images of some ions and peptides obtained from MALDI-MSI and their distribution within the sagittal section of the WT brain tissue.

Moreover, the tissue sections were stained with H&E staining solutions and imaged under microscope for better visualization of the brain’s morphology. Allen Brain Atlas (ABA) (http://www.brain-map.org) was used as neuroanatomical reference. The ABA reference and the H&E stained section are displayed in Figure 14.

Figure 14: Sagittal brain neuroanatomy adapted from ABA[35] and a H&E stained tissue section.
The MS image of m/z 1198 when compared with the H&E stained section, clearly showed that this ion is mainly localized to the Olfactory Bulb, Anterior Olfactory nucleus, Caudate Putamen and Ventral Striatum. On the other hand, the other selected ions showed rather homogeneous intensities throughout the whole tissue section. However, due to the delocalization of analytes, the comparison and subsequent data interpretation with the respect to the morphology of the tissue was difficult and thus imprecise. Therefore, repetition of the experiment with lower flow rate preventing the delocalization is required. The optimal flow rate which imposes no delocalization effects and still results in efficient digestion remains to be determined.

4.6. Imaging of endogenous amyloid-β peptides and amyloid-β plaques in an APP/PS1 mouse model

The developed protocol could be applied to study various brain disorders. One of such examples is the study of endogenous Amyloid-β peptides and amyloid-β plaques in an APP/PS1 mouse model. Here, the aim was to digest and thus extract the Aβ peptides in order to visualize one of the hallmarks of AD progression.

The previously optimized protocol was applied to the APP/PS1 brain section. The spectra comparison between the digested versus non-digested tissue is shown in Figure 15.
Figure 15: The optimized on-tissue enzymatic digestion and MALDI-MSI workflow was applied to the APP/PS1 tissue section. The differences between digested (green) and not digested (blue) brain tissue are evident, confirming the success of the protocol development.

Successful implementation of the developed on-tissue enzymatic digestion protocol was accomplished and thus the distribution of peptides/proteins within the APP/PS1 brain tissue was studied. To further unravel the complex pathology of AD, the digested WT tissue and the digested APP/PS1 tissue were compared. Discriminant Analysis was performed on the two pre-processed data sets to uncover any variances. Remarkably, it seems like there are distinct differences between the APP/PS1 and WT tissue (Figure 16). In the higher mass, the following peaks were only present in the APP/PS1 spectrum: 2101.1, 2237.1, 1173.1, 2299.1, 2365.2, 2670.3, 2671.2 and 2901.6 m/z. Whereas, in the mass range 1000-1900 Da, the 1009.5, 1105.5, 1169.5, 1252.6, 1416.7, 1431.7, 1481.7, 1528.7, 1594.7, 1594.7, 1674.8, 1734.8, and 1900.9 m/z were observed only in the WT spectrum and the 1030.5, 1067.5, 1260.6, 1303.6, 1318.6, 1385.7, 1516.7, 1563.8, 1615.8, 1701.8, 1790.8 and 1830.8 m/z were unique to the APP/PS1 spectrum. The differences in the peaks observed specially in the peptide mass range might be due to the digestion of the Aβ plaques which are solely present in the APP/PS1 model or due to other pathological changes in the diseased phenotype.
Figure 16: The digested WT brain tissue was compared to the digested APP/PS1 brain tissue using PCA-DA methods. The figure above depicts the first discriminant function which describes the two data sets. The positive loadings correspond with the WT, whereas the negative loadings represent the APP/PS1. Numerous differences can be found between the two tissue types; several peaks dominate one spectrum while others are mainly present in the second spectrum.

However, careful data interpretation and peak identification must be performed before any conclusion can be made. Nevertheless, the peptide peaks obtained from the digested APP/PS1 tissue were compared with an in-silico digestion of Amyloid-β protein. Several peaks could be assigned with a max. mass tolerance window of 0.36 Da, possibly showing the presence of Amyloid-β plaques in the APP/PS1 tissue. These candidate peaks are listed in Table 4.
Table 4: Candidate peaks from the APP/PS1 tissue that could be assigned to Amyloid-β tryptic peptide peaks.

<table>
<thead>
<tr>
<th>MALDI-TOF-MS</th>
<th>Error</th>
<th>Peptide cutter</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z</td>
<td></td>
<td>m/z</td>
</tr>
<tr>
<td>1144,6</td>
<td>0,36</td>
<td>1144,24</td>
</tr>
<tr>
<td>1236,6</td>
<td>0,17</td>
<td>1236,43</td>
</tr>
<tr>
<td>1325,6</td>
<td>0,12</td>
<td>1325,48</td>
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<tr>
<td>1386,7</td>
<td>0,18</td>
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<td>1472,7</td>
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</tr>
<tr>
<td>1704,8</td>
<td>-0,19</td>
<td>1704,99</td>
</tr>
</tbody>
</table>

To confirm the identity of these peaks MS/MS should be performed and further experiments must be carried out.

5. Conclusion

The aim of this project was to optimize and develop protocols for on-tissue enzymatic digestion for direct protein analysis of rodent brain tissue using MALDI-MSI. First, the optimized washing procedure using a chloroform wash lowered the ion suppression effect and prepared the tissue section for further on-tissue enzymatic digestion. Second, the evaluated enzyme deposition technique with SunCollect together with the optimized enzyme solution in ddH₂O with the 10 mM Octyl-α/β-glucoside addition and 18hrs incubation time resulted in efficient on-tissue digestion. Third, the optimal matrix application conditions with SunCollect were determined, showing that the most important parameter is the flow rate. These improved settings facilitated good extraction/ionization of peptides during the MALDI-MSI experiments. Finally, the here successfully developed protocol for on-tissue digestion has demonstrated that it is feasible to localize and identify peptides/protein directly from the brain tissue. At the end, more than 30 proteins could be identified from the WT mouse tissue. Successful implementation of the developed on-tissue enzymatic digestion protocol was accomplished and thus the distribution of peptides/proteins within the APP/PS1 brain tissue was studied. Subtle differences between the APP/PS1 and WT tissue were observed. However, the experiments must be repeated and thorough data analysis must be performed before any conclusions can be made.
6. Future Experiments

Future plans can be divided into three categories: sample preparation, identification of APP/PS1 specific peptides/proteins and validation of obtained data. Firstly, the enzyme application can be still improved. It has been observed that when high flow rate is used to apply the enzyme the risk of analyte delocalization effect is high and delocalization occurs. On the other hand, when the flow rate was set lower, the digestion was not efficient. The optimal flow rate, less delocalization effect and still efficient digestion, remains to be determined.

Secondly, the experiments comparing WT and APP/PS1 brain tissue must be repeated in order to confidently draw any conclusions. The differences among the two tissue types must be investigated and APP/PS1 specific peptides/proteins must be identified. Lastly, the distribution of endogenous amyloid-β peptides as well as amyloid-β plaques in the APP/PS1 brain tissue must be investigated to provide insights into the complex AD pathology. Once, the successful digestion, extraction and visualization of the Aβ from the tissue is achieved, the findings must be validated, which can be performed for example by using immunohistochemical staining. In addition, the ratio between the free endogenous Amyloid-β peptides and the Amyloid-β peptides aggregated in the plaques must be studied to further understand the mechanisms of AD disease.
7. References


Appendix I

Experimental setting of experiment 1:

Tissue type: Wild type mouse brain tissue

Tissue sectioning: section thickness - 12 µm, cryostat stage holding and blade temperature were set to -25°C and -21°C. Tissue section was thaw mounted on glass slide.

Washing: The glass slide was immersed 2 x 30 sec in 70% Ethanol.

Trypsin: 20 µg of trypsin dissolved in 400µL of ddH₂O – final trypsin concentration 0.05 µg/µL. Trypsin application performed with CHIP-1000. CHIP settings: 150µm spacing scheme, in total 40 cycles with 5nL/drop of enzyme deposited at each location, humid chamber at 37 °C.

Matrix: 10 mg/mL CHCA in 50% acetonitrile, 50% ddH₂O and 0.1% TFA. Matrix deposited with ImagePrep in 3 cycles.

MALDI-MSI settings:

Sensitivity mode
Positive polarity
Acquisition range: 100 - 3500 m/z
Quadrupole profile – 900 m/z
Laser energy -250 nm
Laser step size - 150µm