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Isolation of Amyloid Plaques and Neurofibrillary Tangles from Archived Alzheimer's Disease Tissue Using Laser-Capture Microdissection for Downstream Proteomics

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Abstract

Here, we describe a new method that allows localized proteomics of amyloid plaques and neurofibrillary tangles (NFTs), which are the two pathological hallmarks of Alzheimer's disease (AD). Amyloid plaques and NFTs are visualized using immunohistochemistry and microdissected from archived, formalin-fixed paraffin-embedded (FFPE) human tissue samples using laser-capture microdissection. The majority of human tissue specimens are FFPE; hence the use of this type of tissue is a particular advantage of this technique. Microdissected tissue samples are solubilized with formic acid and deparaffinized, reduced, alkylated, proteolytically digested, and desalted. The resulting protein content of plaques and NFTs is determined using label-free quantitative LC-MS. This results in the unbiased and simultaneous quantification of ~900 proteins in plaques and ~500 proteins in NFTs. This approach permits downstream pathway and network analysis, hence providing a comprehensive overview of pathological protein accumulation found in neuropathological features in AD.

Keywords

Amyloid plaques; Neurofibrillary tangles; Laser-capture microdissection; Mass spectrometry; Formalin-fixed paraffin embedded; Alzheimer's disease

1 Introduction

Amyloid plaques and neurofibrillary tangles (NFTs) are the two pathological hallmarks that define Alzheimer's disease (AD). While plaques and NFTs primarily consist of aggregated beta amyloid (A β) and tau respectively, targeted immunohistochemistry studies have shown that they also contain a diverse range of other proteins. Amyloid plaques contain many amyloid binding proteins (such as apolipoprotein E, clusterin, ubiquitin, etc.) and proteins found in astrocytes, microglia, and dystrophic neurites that surround and infiltrate plaques. NFTs contain phosphorylated tau binding proteins, such as heat shock proteins, cytoskeletal proteins, and kinases [1]. The presence and abundance of proteins besides A β in plaques and tau in NFTs may have an important role in the development or spread of these neuropathological features during disease. However, the methodology available to comprehensively characterize and quantify these proteins has been limited; restricted to

targeted immunohistochemistry studies or co-immunoprecipitation biochemical studies. Both of these techniques rely on targeted analysis and hence make discovery of novel plaque and NFT-associated proteins impossible.

Therefore, we have developed a localized proteomics method that combines laser-capture microdissection (LCM) and label-free quantitative mass spectrometry (LC-MS). This strategy allows the detection and quantification of plaque and NFT-associated proteins isolated from archived human brain tissue. The vast majority of human brain tissue specimens available for research are formalin-fixed, paraffin-embedded (FFPE) blocks of tissue collected at autopsy. Traditionally, this type of tissue has been neglected in proteomic research studies because it was thought to be only suitable for immunohistochemistry or histology. Researchers have avoided using FFPE tissue for proteomics in the past because of concerns that the protein crosslinks generated during FFPE processing will prevent the detection of proteins using mass spectrometry or lead to inferior results compared to those using frozen, unfixed tissue. However, we (and an increasing number of other researchers, primarily in the cancer field) have shown that this is not the case [2–6]. The method described below has been specifically optimized to use FFPE tissue because the abundance of human FFPE tissue specimens is a potential gold mine for future studies of human disease. A limited number of previous studies have performed proteomics on plaques and NFTs microdissected from human brain samples; however, these have all used frozen tissue [7–11]. The method we describe below is a significant improvement over the ones described previously, because it results in a greater number of proteins detected. We have also determined that an initial solubilization step with formic acid is essential for detection of multiple proteins present in plaques and NFTs that are of particular interest in AD (most notably A β and tau), while not affecting the detection of abundant soluble proteins [2].

Another important aspect of the method described below is the use of LCM to isolate plaques and NFTs from human brain sections. Successful isolation of neuropathological features from human brain samples has been a technical roadblock that has prevented the comprehensive analysis of proteins specifically localized in plaques and NFTs. LCM bypasses this complication; using a precise laser to dissect microscopic regions of interest without affecting the protein content of the isolated tissue, therefore generating a tissue sample that contains the highest purity possible of plaques and NFTs. The time-consuming nature of LCM is one of the concerns with using this technique; therefore in our previous studies we optimized the minimal amount of tissue required for downstream LC-MS. We have shown that 1.5 mm² total tissue area microdissected from 8 μ m thick tissue sections is adequate for consistent LC-MS analysis [2]. It takes approximately 1.5 h to microdissect 1.5 mm² total tissue area of plaques and approximately 8 h to microdissect 1.5 mm² total tissue area of NFTs.

Analysis of the protein content of plaques and NFTs is performed using LC-MS. Previous studies have relied on targeted, semi-quantitative techniques such as immunohistochemistry, Western blot, or ELISA for protein detection. Unbiased, quantitative mass spectrometry has many benefits over these traditional techniques; (a) it does not rely on the availability, sensitivity, and specificity of antibodies, (b) it is untargeted and therefore allows the unbiased identification of novel proteins in AD pathogenesis, (c) it allows the concurrent

identification of hundreds of proteins at once, which then allows for pathway/network analysis of AD pathogenesis, giving a much richer picture of the molecular mechanisms that underlie AD.

We have optimized the mass spectrometry conditions described below to permit maximum protein identification using the minimal amount of microdissected tissue. The dynamic range of mass spectrometry is below the dynamic range of protein concentration found in most systems; therefore, the entire proteome cannot be detected by a single mass spectrometry experiment. Generally, fractionation or enrichment strategies are needed to increase the proteome coverage and allow comprehensive detection of the protein composition of a given system. Here, the use of LCM to isolate defined pathological features (plaques or NFTs) allows us to focus our analysis and thus greatly reduces the proteome complexity, enabling reasonable proteome coverage without the need of extensive fractionation. The microscopic amounts of tissue used for this technique means that a label-free quantitative strategy rather than multiplexed quantitation using isobaric tags is recommended. In our hands labeling with multiplexing reagents leads to less proteins being detected as the extra sample manipulation during labeling with the multiplexing reagents increases the sample loss. Furthermore, the microscopic amount of starting material makes the minimization of contamination and choice of tube for the collection of LCM dissected tissue critical (see Subheading 4). Tubes should be carefully tested in preliminary studies to ensure that they do exacerbate sample loss. We also recommend use of a sensitive mass spectrophotometer that offers high resolution and mass accuracy (<10 ppm).

2 Materials

2.1 Tissue Sectioning, Staining, and LCM

1. Microtome (Leica RM2255).
2. Fisher Tissue Flotation Bath.
3. FFPE archived tissue blocks.
4. PET membrane FrameSlides (Leica).
5. Xylenes (Histological Grade).
6. Ethanol (100%).
7. Staining containers (we use typical glass staining containers with a corresponding glass slide holder, but any staining containers suitable for histological staining will work).
8. Slide box (for drying and storing slides).
9. Laser-capture microdissection microscope (we use a Leica LMD6500).
10. Collection tubes (Axygen™ PCR tubes with 0.5 ml flat cap).
11. Phosphate-buffered saline (PBS).
12. ImmEdge Hydrophobic Barrier PAP Pen (Vector).

13. Normal goat serum (NGS; MP Biomedicals).
14. Hydrogen peroxide (H₂O₂; Fisher H323–500).
15. VECTASTAIN Elite ABC HRP Kit (Vector).
16. Metal enhanced DAB substrate Kit (Thermo Scientific).
17. 4G8 anti-A β primary antibody (BioLegend).
18. AT8 anti-phosphorylated tau primary antibody (Thermo Scientific).
19. Alex Fluor 488-conjugated goat anti-mouse IgG, Fc γ fragment specific (Jackson ImmunoResearch).
20. Biotinylated horse anti-mouse IgG antibody (Vector).

2.2 LC-MS

1. Water, LC-MS grade.
2. Acetic acid, mass spectrometry grade.
3. Formic Acid LCMS grade (Thermo Scientific).
4. Trifluoroacetic Acid (TFA), sequencing grade (Thermo Scientific).
5. Ammonium bicarbonate.
6. Acetonitrile, LC-MS grade (Fisher).
7. Methanol, LC-MS grade (Fisher).
8. Ethanol, LC-MS grade (Acros).
9. Bench top centrifuge (Eppendorf).
10. Thermomixer R (Eppendorf).
11. Dithiothreitol (DTT).
12. Iodoacetamide (IA).
13. Sequencing grade trypsin (Promega).
14. Speed Vacc Concentrator.
15. Ultrasonic cleaner.
16. pH indicator strips pH 0–14.
17. POROS 50 R2 (Applied Biosystems). Weigh out 500 mg of POROS R2 50 μ m and place into a 15 ml falcon tube. Wash it with 10 ml of Optima LC/MS methanol. Vortex gently until all of the beads are in methanol. Let the beads settle to the bottom of the tube. Discard the methanol and wash with 10 ml of Optima LC/MS acetonitrile, followed by 95% acetonitrile in 0.1% TFA as above. Wash with 10 ml of Optima LC/MS water. Repeat the water wash step two more times. Resuspend the POROS beads in 10 ml of 10% ethanol (final concentration 50 mg/ml). Wrap the lid with parafilm and store at 4 °C. This POROS bead

slurry can be stored at 4 °C for about 2–3 months. Alternatively, C18 ZipTips (see below) or StageTips [12] can be used instead of the POROS beads.

18. C18 ZipTip (Millipore).
19. Autosampler vial (Fisher Scientific).
20. Autosampler vial caps (Fisher Scientific).

We used the following LC-MS setup for our studies; however, this can be replaced with other nano-LC columns and high-resolution mass spectrometers.

21. Easy spray PepMap column 75 µm × 50 cm (Thermo Scientific).
22. Acclaim PepMap 100 pre-column (Thermo Scientific).
23. Easy nLC 1000 HPLC system (Thermo Scientific).
24. QExactive Mass Spectrometer (Thermo Scientific).

3 Methods

3.1 FFPE Tissue Sectioning

1. Clean microtome and water bath with picopure water and RNase Away (Thermo Fisher) to minimize likelihood of contamination. *See Note 1*
2. Cut 8 µm thick sections from FFPE tissue blocks using a microtome. *See Note 2*
3. Place tissue sections onto the surface of a water bath.
4. Collect sections onto LCM-compatible PET slides.
5. Leave the slides to dry in a closed container (e.g., slide box) for at least 24 h to ensure that all residual water from the sectioning process has evaporated prior to staining (*see Note 3*).

3.2 Fluorescent Immunohistochemistry (Plaques)

1. Dewax sections in xylene (2 × 30 s), followed by a series of graded ethanol washes (100% ethanol (2 × 30 s), 95% ethanol (1 min), 70% ethanol (1 min)). *See Note 4*
2. Wash with PBS (3 × 5 min).
3. Outline sections with PAP Pen.
4. Wash with PBS (2 × 5 min). *See Note 5*

¹All the solutions should be prepared fresh and kept in clean, closed containers. Additionally, all surfaces, consumables, and containers should be clean and gloves should be worn throughout the process to prevent contamination from dust and other sources. This is because contaminating proteins (such as keratin found in dust) will interfere with and influence mass spectrometric analysis.

²The archival time of FFPE blocks does not appear to significantly alter downstream LC-MS. To date, we have successfully performed LC-MS on tissue microdissected from FFPE blocks archived at room temperature from 1 to 15 years. Even though we have not yet examined tissue blocks that have been archived for longer than 15 years it is likely that such tissue is still amenable for the analysis given the lack of difference between blocks stored for 1 and 15 years.

³The tissue has to be completely dry or it will detach from the slide during staining.

⁴Xylene and ethanol solutions can be reused three times (30 slides total). Solutions should be stored in sealed containers when not in use to prevent contamination with dust.

5. Block sections for 1 h at room temperature (RT) in 10% NGS diluted in PBS.
6. Incubate the slides overnight at 4 °C with 4G8 anti-A β primary antibody (1:1000 in 4% NGS diluted in PBS).
7. Wash the slides with PBS (3 $\times$ 5 min).
8. Incubate the sections with 488 fluorescent secondary antibody (1:500 in PBS) for 2 h at RT (*see* Note 6).
9. Wash with PBS (3 $\times$ 5 min).
10. Rinse with double distilled MilliQ water to remove residual salt from staining procedure.
11. Allow the slides to dry in a closed container (e.g., slide box) to avoid contamination with dust.

3.3 Peroxidase Immunohistochemistry (Neurofibrillary Tangles) (See Note 7)

1. Dewax sections in xylene (2 $\times$ 30 s), followed by a series of graded ethanol washes (100% ethanol (2 $\times$ 30 s), 95% ethanol (1 min), 70% ethanol (1 min))
2. Wash with PBS (3 $\times$ 5 min).
3. Outline sections with PAP Pen.
4. Incubate the slides with 0.3% H₂O₂ in PBS for 20 min.
5. Wash the slides PBS (3 $\times$ 5 min).
6. Block sections for 1 h at RT with 10% NGS diluted in PBS.
7. Incubate the slides overnight at 4 °C with AT8 primary antibody (1:500 in 4% NGS diluted in PBS).
8. Wash the slides with PBS (3 $\times$ 5 min).
9. Incubate sections with biotinylated anti-mouse IgG (1:1000 in PBS) for 2 h at RT.
10. 30 min prior to the end of incubation, make ABC solution by mixing 1 drop of solution A and 1 drop of solution B in 10 mL PBS. Slowly mix solution using a rotator until use.
11. Wash the slides with PBS (3 $\times$ 5 min).

⁵Extra precaution is needed when staining on LCM slides as tissue sections do not adhere as strongly as they do to traditional glass slides. Therefore, tissue is more likely to detach during vigorous washes. In addition, use of detergents such as Tween-20 or Triton-X can result in tissue sections detaching from slides. Therefore, we recommend that these detergents should not be used. Also, all the reagents should be freshly made to prevent contamination.

⁶All incubations and washes should be performed in an enclosed slide box to minimize photo-bleaching from this point onward.

⁷Either fluorescent and peroxidase immunohistochemistry can be used to stain plaques and NFTs. Fluorescent immunohistochemistry has the benefit of a faster staining protocol that uses fewer reagents; however, all LCM then has to be done in the dark to prevent photo-bleaching. The benefit of peroxidase immunohistochemistry is that LCM does not have to be done in the dark (which is beneficial when microdissecting smaller regions such as neurons, as this is more time consuming). Importantly, both fluorescent and peroxidase immunohistochemistry are compatible with downstream LC-MS and we have not observed any proteomic differences when interchanging these techniques.

12. Incubate the slides with ABC solution for 1 h at RT.
13. Wash the slides with PBS (3×5 min).
14. Incubate the slides with DAB solution (diluted 1:10 in a peroxidase solution provided in the kit) until appropriate color development. *See Note 8.*
15. Quickly rinse the slides with PBS.
16. Wash the slides with PBS (2×5 min) using a coplin jar.
17. Quickly rinse sections with ddH₂O.
18. Leave the slides to dry in a closed slide box to avoid contamination.

3.4 Laser-Capture Microdissection

1. Load the slides onto an LCM microscope with the tissue facing downward toward the collector (*see Note 9*).
2. Load two 0.5 ml collection tubes into the collector; the first tube containing nothing (this is the “waste tube”) and the second tube with 60 μ l of ddH₂O in the cap. Make sure to load tubes without touching the inside of the tube or the cap to prevent contamination.
3. Check the collection tube containing the water for contamination at 5 $\times$ magnification; contamination will be obvious as a fiber/hair or smaller particles floating on the water surface (*see Note 10*).
4. Locate your region of interest on the LCM slides at 5 $\times$ magnification and switch to 10 $\times$ magnification (plaques) or 20 $\times$ magnification (NFTs) for LCM (Fig. 1).
5. Position the waste collection tube under the slide and calibrate the laser using the “calibrate” function. It is important to calibrate the laser at the magnification that you will use to do LCM (in this case 10 $\times$ or 20 $\times$ magnification).
6. Optimize laser settings (*see Note 11*).
7. Position the second collection tube (contains water) under the slide, in preparation for LCM.

⁸Using this protocol we incubate slides with DAB solution for 90 s. However, optimization studies should be performed for each antibody to determine the optimal incubation time that provides the greatest discrimination between staining and background.

⁹The direction the tissue is facing is very important. LCM is still possible when the tissue is facing away from the collector; however, this appears to result greater static between the microdissected tissue and the underside of the slide, preventing some microdissected tissue from falling into the collector.

¹⁰We use plastic wrap to cover the LCM microscope when in use. This prevents airflow between the slide and the collector, ensuring that microdissected plaques/NFTs fall into the collector. In addition it also prevents contamination from dust.

¹¹Ideally, you should manipulate the laser power, aperture, and speed settings so that the laser line is as thin as possible, while still maintaining a consistent cut that results in plaques/NFTs falling cleanly into the collector. Together, the power and the aperture determine the thickness of the laser line, but power should be carefully monitored because too much power results in tissue damage. A lower speed results in a cleaner laser cut; however, the speed should be optimized to be as fast as possible to minimize LCM time. Specimen balance helps to ensure that plaques/NFTs fall cleanly into the collection tube; this function provides an extra boost of power at the end of the cut, which helps plaques/NFTs to detach cleanly from the tissue section. We have noticed that some tissue is more friable than others. The exact reason for this is unknown, but it could result from different formalin fixation times, length of time between staining and LCM or tissue archival time. Therefore, it is best to check that the laser settings are optimal for each tissue section.

8. Outline and microdissect plaques or NFTs (*see* Notes 12 and 13).
9. Repeat **step 8** until at least 2 mm² of plaques and 1.5 mm² total area of NFTs have been microdissected (approximately 750 plaques and 4000 NFTs) (*see* Notes 14 and 15).
10. Remove collection tube carefully from the holder, making sure not to touch the rim or the inside of the tube in the process. Firmly close the cap of the collection tube.
11. Spin down the collection tube at 14,000 × *g* for 2 min to transfer the water and the microdissected plaques/NFTs to the base of the tube.
12. Store the sample at −80 °C until sample preparation for LC-MS.

3.5 Formic Acid Solubilization

1. Thaw the sample tubes at room temperature. Add 50 µl of 100 mM ammonium bicarbonate 20% acetonitrile solution to the cap of the tube, pipet up and down 2–3 times and centrifuge the sample to collect solution at the bottom of the tube. Repeat this step two more times. If one sample is dissected into multiple tubes, then combine the samples into one tube as described in **steps 2–4** (*see* Note 16).
2. Add 50 µl of 100 mM ammonium bicarbonate, 20% acetonitrile solution to the cap of each tube. Pipette up and down 2–3 times making sure the cap is thoroughly washed.
3. Close the cap and spin down the tubes to collect the solution containing LCM dissected cells at the bottom of the tube. Transfer the solution from the two tubes to one.

¹²Microdissection of plaques identified using fluorescent immunohistochemistry must be performed in the dark to prevent photo-bleaching.

¹³In our experience, it is best to limit the combined area for one continuous cut to ~100,000 µm² for plaques and ~50,000 µm² for NFTs. This is because the LCM stage position memory gets progressively more altered as more fields of view are included, which could result in non-plaque or non-NFT tissue areas being collected.

¹⁴We performed optimization experiments to show that microdissection of at least 1.5 mm² total area from 8 µm thick sections is required for consistent downstream LC-MS [2]. A greater area results in marginally more comprehensive LC-MS results; therefore, 2 mm² area was microdissected for plaques. However, the additional time required to microdissect more NFTs was not justified by slightly better proteomic results; therefore, 1.5 mm² area was collected for these samples to minimize LCM time.

¹⁵Dissection of 2 mm² of plaques takes approximately 2 h of continuous LCM time, which can be done in one session. However, LCM of 1.5 mm² NFTs takes approximately 8 h. This length of time is problematic because the water in the collection cap evaporates after approximately 6 h. We have found that using dry collection tubes results in significantly reduced protein identification using LC-MS, possibly due to increased adhesion between the microdissected tissue and the collection cap. Therefore, we recommend that NFT samples are collected into three separate tubes, each containing ~500,000 µm² total area, which are then combined prior to peptide extraction. The number of tubes that will be combined prior to peptide extraction should be consistent across all samples used in an experiment.

¹⁶As discussed in Drummond et al. (2017) [13], the type of tubes used for LCM collection is critical as microdissected tissue can adhere to the collection cap, and the extent of this adhesion depends on the type of tube used. This is primarily a concern when analyzing microdissected single cells (e.g., NFTs) in comparison to larger tissue areas (e.g., amyloid plaques) as smaller microdissected areas are more likely to adhere to the collection cap. The LCM requires flat-cap 0.5 ml tubes, and only specific types of tubes physically fit into the LCM collector. While optimizing this method, we compared the effect of adhesion and consequent peptide identification using three different types of tubes (supplied by Biozym, Axygen, and Thermo Fisher). In our experience the tissue collected in Biozym and Axygen tubes yielded a larger number of proteins identified in comparison to the Thermo Fisher tubes. Therefore, we recommend that investigators conduct preliminary experiments to evaluate the suitability of collection tubes used prior to large-scale experiments. Due to the limited availability of Biozym tubes, we recommend Axygen™ PCR tubes with 0.5 ml flat cap, which are a comparative alternative in our hands. We also recommend washing tubes prior to use with methanol followed by acetonitrile and three final washes with water (all LC-MS grade) to minimize keratin and polymer contamination.

4. Add another 50 μ l of 100 mM ammonium bicarbonate, 20% acetonitrile solution into the sample tubes 1b and 1c. Vortex the tubes and transfer the solution from tubes 1b and 1c to 1a.
5. Incubate the sample at 95 °C for 1 h, followed by incubation at 65 °C for 2 h in a preheated heat block to deparaffinize and reverse crosslink the proteins (*see Note 17*).
6. At the end of incubation cool the sample to room temperature. Spin the sample at $14,500 \times g$ for 3 min to collect the condensed solution in the cap into the tube.
7. Put the sample in a SpeedVacc concentrator to dry.
8. Prepare a fresh solution of 70% Formic acid (LC-MS grade) by dissolving it into water (LC-MS grade) (*see Note 18*).
9. Add 90 μ l of formic acid to the dried sample, vortex well and incubate overnight at room temperature.
10. At the end of incubation sonicate the samples for 5 min using a water bath sonicator with vortexing three times in between (*see Note 19*).
11. Completely dry the samples using a SpeedVac concentrator (*see Note 20*).

3.6 Reduction-Alkylation and Digestion

1. Resuspend the sample into 150 μ l of 100 mM Ammonium bicarbonate solution, pH 8.0.
2. Prepare a fresh solution of 0.2 M DTT by dissolving 3 mg of DTT in 100 μ l of ammonium bicarbonate. Add 2 μ l of 0.2 M DTT solution to the sample and vortex gently (*see Note 21*).
3. Incubate the samples in a thermomixer at 57 °C for 1 h.
4. During the last 5 min of reduction, prepare a fresh solution of 0.5 M iodoacetamide by dissolving 9 mg of iodoacetamide in 100 μ l of 100 mM ammonium bicarbonate and cover the tube with a foil to avoid light exposure (*see Note 22*).

¹⁷Paraffin and intact crosslinks in the tissue will be detrimental for the downstream LC-MS analysis. The simple heating steps ensure that any residual paraffin is removed and it also reverses the formaldehyde crosslinks sufficiently to facilitate analysis by LC-MS.

¹⁸Amyloid plaques and NFTs are primarily composed of aggregates of beta amyloid and tau respectively. The use of 70% formic acid is essential to solubilize aggregated proteins (particularly fibrillized A β and tau).

¹⁹Only use water bath sonication. Use of probe type sonicators will result in protein loss, which cannot be afforded when using such a small amount of starting material.

²⁰The pH of the sample has to be 7.5 to allow successful reduction and alkylation by DTT and iodoacetamide, respectively, and digestion by trypsin. Always verify that the pH is 7.5 before proceeding with the alkylation and digestion step.

²¹DTT denatures protein by reducing the disulfide bonds of proteins and thus facilitates complete digestion and improved protein coverage by LC-MS. The pH of the reaction should be 7.5–8.0. Alternative reducing agents, for example tris(2-carboxyethyl) phosphine (TCEP), can be used as well. This reagent works at lower pH, however, we found no difference when using TCEP versus DTT.

²²Alkylation of free cysteines prevents re-forming of cysteine disulfide bonds during the digestion steps. Disulfide bond formation would prevent detection of these peptides in the sample using standard search algorithms. Iodoacetamide is light sensitive. We recommend that iodoacetamide is prepared directly prior to use and that the reaction is performed in the dark. The pH of the alkylation reaction should be between 7.5 and 8.0 to ensure complete derivatization of cysteines while minimizing alkylation of other side chains. The concentration of iodoacetamide should be about 2.5 times the amount of DTT as each DTT molecule has two reaction sites for

5. Add 2 μ l of 0.5 M iodoacetamide solution to the sample tube and vortex gently. Keep the samples at room temperature in the dark for 45 min.
6. Dilute the stock of sequencing grade-modified trypsin to a final concentration of 50 ng/ μ l using 100 mM ammonium bicarbonate. Add 4 μ l of diluted trypsin to the sample. Pipette up and down to mix the enzyme into the solution and incubate the sample overnight at room temperature with gentle agitation.
7. Spin the sample at $14,500 \times g$ for 1 min.
8. Quench the trypsin by adding trifluoroacetic acid (TFA) at a 0.2% final concentration.
9. Add 15 μ l of R2 POROS beads slurry to the sample. Incubate the sample at 4 °C for 2 h (*see* Note 23).
10. Place the C18 ZipTip onto an adaptor in a 1.5 mL Eppendorf tube.
11. Add 30 μ l of LC-MS grade methanol and spin for 30 s using a bench top centrifuge at 1000 rpm, or until the solution passes through.
12. Repeat the **step 11** once using LC-MS grade acetonitrile and three times using 0.1% TFA.
13. Load 50 μ l of the peptide sample to the C18 ZipTip and spin the sample at ~ 100 – $170 \times g$. Do not load more than 50 μ l solution on a ZipTip at a time. G force should not be increased above $190 \times g$.
14. Repeat **step 13** until entire sample is loaded onto the C18 ZipTip.
15. Add 50 μ l of 0.1% TFA to the empty sample tube. Vortex the sample to get residual POROS beads in the solution. Load the wash solution on the corresponding ZipTip and spin the sample for 30 s.
16. Repeat the **step 15** two more times.
17. Pipette 200 μ l of 0.5% acetic acid solution into a fresh Eppendorf tube. To further thoroughly rinse the POROS beads in the ZipTip, aspirate 0.5% acetic acid through the ZipTip and pipette 8–10 times making sure the POROS beads move up and down (*see* Note 24).
18. Transfer the ZipTip on the top of a new 0.5 mL Eppendorf tube to elute the peptides. Make sure that the tube is labeled with a sample identifier.
19. Add 40 μ l of 40% acetonitrile, 0.5% acetic acid solution to the ZipTip, and spin at 1000 rpm for 1 min to elute the peptides.

iodoacetamide. An excess of iodoacetamide or higher pH can cause alkylating lysine side chains, N-termini, methionine, histidine, aspartate, and glutamate. This can reduce the sensitivity in the LC-MS analysis as the individual peptide signal is split over the different alkylated forms.

²³This step is performed to desalt the samples. Salts in the sample interfere with the ionization process and mass spectrometric detection. This leads to reduced sensitivity, which results in compromised peptide detection. C18 reverse phase columns are most commonly used for peptide desalting. Instead of POROS beads any C18 reverse material (i.e., stage tips or spin columns) can be used as well.

²⁴If you are using alternative C18 clean-up methods such as stage tips or spin columns, ensure efficient desalting by adding extra wash steps with 0.5% acetic acid.

20. Add 40 μ l of 80% acetonitrile, 0.5% acetic acid solution to the ZipTip and spin for 30 s at 1000 rpm to further elute the peptides.
21. Put the samples in a SpeedVac concentrator to remove organic solvent from the peptide mixture.
22. Reconstitute the dry samples in 15 μ l of 0.5% acetic acid solution. Vortex the sample thoroughly.
23. Analyze 5 μ l of the NFTs and 3 μ l of AD plaque sample using LC-MS.

3.7 Mass Spectrometric Analysis

The samples were analyzed on LC-MS as described in [2] with some modifications described below.

1. One third of each sample was loaded onto the EASY spray 50 cm C18 analytical HPLC column with <2 μ m bead size equilibrated in solvent A (2% acetonitrile, 0.5% acetic acid) using an EASY-nLC 1000 HPLC. The peptides were gradient eluted directly into the QExactive mass spectrometer (Thermo Scientific) using a 1 h gradient from 2% to 31% solvent B (90% acetonitrile, 0.5% acetic acid), followed by 10 min from 31% to 40% solvent B and 10 min from 40% to 100% solvent B (*see* Note 25).
2. The QExactive mass spectrometer was operated in data-dependent mode. The mass spectrometer acquired high-resolution full MS spectra with a resolution of 70,000, an AGC target of 1e6 with a maximum ion time of 120 ms, and scan range of 400–1500 m/z . Following each full MS, 20 data-dependent high-resolution HCD MS/MS spectra were acquired using the following instrument parameters: AGC target of 5e4 with a maximum ion time of 120 ms, one microscan, 2 m/z isolation window, fixed first mass of 150 m/z , Normalized Collision Energy (NCE) of 27 and dynamic exclusion of 30 s (*see* Note 26).
3. The resulting MS/MS spectra were searched against the Uniprot Human reference proteome database using Sequest within Proteome Discoverer. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine as a variable modification. The mass tolerance was set to 10 ppm for both MS1 and MS/MS searches. FDR filtration was done at 1% FDR using a standard target-decoy database approach. Proteins identified with less than two unique peptides were excluded from analysis (*see* Note 27).

²⁵Using a Q Exactive or Lumos mass spectrometer with 50 cm Easy spray columns and a 1 h gradient is sufficient for comprehensive detection of plaque and NFT proteins. Longer acquisition time did not significantly affect the number of proteins identified. However, the acquisition time and HPLC gradient should be optimized for each sample type.

²⁶The mass spectrometer acquisition parameters should be optimized based on the MS system being used. Generally, these samples are much less complex and contain less total protein than the typical proteomics sample. While for a typical proteomics experiment everything is optimized with speed in mind, care should be taken here to ensure sufficient ion signal for the MS/MS spectrum.

²⁷Even though the formaldehyde crosslinks are reversible, the workflow should be monitored for efficient reversal by checking for formylation on amino acid side chains, missed cleavages due to formylation on lysine residues that prevent cleavage by trypsin, as well as oxidation of methionine. We found the level of formylation in all tissues that we examined to be negligible, but we always include oxidized methionine as a reversible modification during data searching.

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Fig. 1. Schematic of the method used to perform localized proteomics of plaques and neurofibrillary tangles. A β plaques and NFTs are microdissected using a LMD6500 microscope (Leica), which uses a precise laser to microdissect regions of interest. Microdissected plaques or NFTs fall into the collection cap by gravity. Representative images before and after LCM show the precision of LCM to isolate plaques (arrows; fluorescent green staining) and NFTs (arrows; brown staining). The plaques and NFTs proteomes are then identified and quantified using LC-MS