

Chapter 18

Quantitative Neuroproteomics of the Synapse

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Abstract

An emerging way to study neuropsychiatric or neurodegenerative diseases is by performing proteomic analyses of brain tissues. Here, we describe methods used to isolate and identify the proteins associated with a sample of interest, such as the synapse, as well as to compare the levels of proteins in the sample under different conditions. These techniques, involving subcellular fractionation and modern quantitative proteomics using isotopic labels, can be used to understand the organization of neuronal compartments and the regulation of synaptic function under various conditions.

Key words: Neuroproteomics, subcellular fractionation, presynaptic terminal, mass spectrometry, quantitative proteomics, differential isotopic labelling.

1. Introduction

Neuroproteomics is the study of the proteome, or the collection of proteins encoded by the genes of an organism, in particular that of the central nervous system (CNS). With the development of new techniques and the improvement of those already available, it is now possible not only to identify proteins, but also to determine changes in their abundance under various conditions (1). This is particularly useful in understanding the physiological function of biological systems, as well as determining the functional implication of alterations in proteins in disturbed states, such as those induced by neurodegenerative disorders and/or drugs of abuse (2).

In the CNS, synapses are essential for the communication between neurons. Upon stimulation, a presynaptic neuron releases neurotransmitters that bind to receptors in the postsynaptic neuron, which in turn induces a series of events in response to the stimulus. One of the most fascinating properties of the CNS is synaptic plasticity or the ability to reconfigure and/or modulate synapses to accommodate for the wide variety of stimuli that they receive at any given time. The inability to respond adequately may lead to the development of neurodegenerative or addictive disorders.

Neuroproteomic studies have started to identify the proteins present in different compartments of the synapse, including synaptosomes (3) and presynaptic and postsynaptic terminals (4), mainly through subcellular fractionation protocols. This type of approach facilitates the analysis by reducing the complexity of the system. Moreover, it enriches synaptic compartments with less abundant proteins, which are commonly masked by those with the highest abundance. One way to take advantage of this methodology is to select a brain region, isolate the synaptic fraction of interest before and after a treatment (such as exposure to morphine) and identify the proteins in the fraction as well as their relative changes upon treatment (5).

The approach described in this chapter is divided into two major sections. **Section 3.1** describes the details for isolating a brain region and separating it into synaptic fractions using subcellular fractionation. It is expected that (at least) two samples that undergo separate treatments (such as exposure to morphine and a control) are prepared. **Section 3.2** details the steps required to isolate proteins, digest and differentially label them with appropriate isotopic labels and perform mass spectrometry to identify the peptides and hence the proteins from the original sample. The data analysis (also described) yields a list of proteins and specifies the relative change in protein levels upon treatment. Whilst we have used specific examples from our research such as working with synaptic proteins after animal exposure to morphine, these protocols are easily adaptable for use with other animal models, other brain regions or a range of treatments. In addition, while specific fractionation and mass spectrometric equipment have been used, other instrumental platforms can be used with appropriate modifications.

2. Materials

2.1. Sample Preparation

2.1.1. Tissue Acquisition and Storage

1. Dissection tools: forceps, razor blades.
2. Dry ice.
3. Ethanol, 70%.
4. Filter paper (Fisher Scientific, Fairlawn, NJ).

5. Isopropanol (Sigma Aldrich, St. Louis, MO).
6. Phosphate-buffered saline (PBS).

2.1.2. Fractionation

1. Sucrose (0.32 M): 5.4 g sucrose in 50 mL CaCl₂. Store at 4°C.
2. Sucrose (1 M): 17 g sucrose in 50 mL CaCl₂. Store at 4°C.
3. Sucrose (2 M): 34 g sucrose in 50 mL dH₂O. Store at 4°C.
4. Tris-HCl (1 M), pH 6.
5. Tris-HCl (20 mM)/Triton X-100 (1%), pH 8.
6. Protease inhibitors cocktail (Sigma Aldrich, St. Louis, MO).
7. Phosphatase inhibitors cocktail (Sigma Aldrich, St. Louis, MO).
8. Cold acetone (Sigma Aldrich, St. Louis, MO).
9. SDS, 0.1%.
10. SDS, 1%.
11. Ethanol, 70%.
12. Wheaton glass homogenizer, 7 mL.
13. Amicon Ultra-15 centrifugal filters (Millipore, Bedford, MA).

2.1.3. Protein Estimation

1. BCA protein assay kit (Pierce, Rockford, IL).
2. Bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO).
3. Microplate reader, 550 Model (Bio-Rad, Hercules, CA).

2.1.4. SDS-PAGE

1. Acrylamide/Bis solution, 40% (29:1 with 3.3% C) (Bio-Rad, Hercules, CA).
2. TEMED (Sigma Aldrich, St. Louis, MO).
3. Resolving Buffer (4X): 1.5 M Tris-HCl, pH 8.8; 0.4% (w/v) SDS.
4. Stacking buffer (4X): 0.5 M Tris-HCl, pH 6.8; 0.4% (w/v) SDS.
5. Ammonium persulfate, 10%.
6. Running buffer (1X): 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.
7. Transfer buffer (1X): 25 mM Tris, 192 mM glycine, 20% (v/v) methanol.
8. Pre-stained molecular weight markers (Dual Color Protein Standards, Bio-Rad, Hercules, CA).
9. Isopropanol (Sigma Aldrich, St. Louis, MO).

10. Loading buffer (6X): 60 mM Tris-HCl/SDS pH 6.8, 10% glycerol, 2% SDS, 20 mM DTT, 0.001% bromophenol blue.
11. Mini-Protean II electrophoresis cell gel system (Bio-Rad, Hercules, CA).

2.1.5. Western Blotting

1. Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE).
2. Primary antibody solution in Odyssey Blocking Buffer with 0.1% Tween-20 and 0.01% NaN₃.
3. Fluorescently labelled secondary antibody solution in Odyssey Blocking Buffer with 0.1% Tween-20 and 0.01% NaN₃.
4. Tris-buffered saline (1X) with Tween-20 (TBS-T): 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween-20.
5. Nitrocellulose membrane.
6. Chromatography paper (Fisher Scientific, Fairlawn, NJ).

2.2. Differential Isotopic Labelling and Mass Spectrometry

2.2.1. Protein Digestion and Stable Isotope Labelling

1. Solution of 1 M NaOH in water.
2. Phosphate buffer (pH 8.5).
3. Solution of 200 mM dithiothreitol (DTT) in 100 mM NH₄HCO₃ (pH 8–9).
4. Solution of 1 M iodoacetamide (IAM) in 100 mM NH₄HCO₃.
5. Digestion buffer: 50 mM NH₄HCO₃, 2 M urea (pH 8–10).
6. Sequencing-grade modified trypsin (Promega Co., Madison, WI).
7. Solution of 2 M succinic anhydride in dimethyl sulfoxide (DMSO) (SA-H) (Sigma Aldrich, St. Louis, MO).
8. Solution of 2 M succinic [²H₄] anhydride in DMSO (SA-D) (Sigma Aldrich, St. Louis, MO).
9. Solution of 2.5 M glycine.
10. Solution of 2 M hydroxylamine.

2.2.2. Sample Clean-Up

1. Protein desalting spin columns (Pierce, Rockford, IL).
2. Activating solution: 50% aqueous acetonitrile solution containing 0.1% formic acid (FA) and 0.01% trifluoroacetic acid (TFA) (*see Note 1*).
3. Equilibrating solution: 5% aqueous acetonitrile solution containing 0.1% formic acid and 0.01% trifluoroacetic acid.
4. Eluting solution: 70% aqueous acetonitrile solution containing 0.1% formic acid and 0.01% trifluoroacetic acid.

2.2.3. Liquid Chromatography/Mass Spectrometry

1. MicromassTM CapLC system (Micromass, Manchester, UK).
2. HCTUltra-PTM discovery systemTM (Bruker Daltonics, Billerica, MA).
3. Manual injector (Valco Instruments Co., Inc., Houston, TX).
4. OPTI-PAK[®] Trap Column 0.5 μ L C18 (Optimize Technologies, Inc., Oregon City, OR).
5. LC PackingsTM 300 μ m i.d. \times 15 cm, C18 PepMap100, 100 Å (LC Packings, San Francisco, CA).
6. Solvent A: 5% acetonitrile in water containing 0.1% formic acid and 0.01% trifluoroacetic acid.
7. Solvent B: 95% acetonitrile in water containing 0.1% formic acid and 0.01% trifluoroacetic acid.
8. Software: Bruker Daltonics Hystar, version 3.2 – integrates the capLC system and the mass spectrometer.
9. Software: Bruker Daltonics EsquireControl, version 6.1 – controls the mass spectrometer.

2.2.4. Data Analysis

1. Software: Bruker Daltonics DataAnalysis, version 3.4 – identifies, deconvolves and quantifies peptides.
2. Software: Bruker Daltonics Biotools, version 3.1 – catalogues and matches MS and MS/MS spectra of peptides with Mascot results.
3. Software: Mascot (Matrix Science, London, UK) – assigns peptide sequence based on the MS/MS spectra and database used.

3. Methods

3.1. Sample Preparation

3.1.1. Tissue Acquisition and Storage

This protocol is based on the use of rodent brain tissue. Once the animal is sacrificed by decapitation, the brain is rapidly removed and the regions of interest, such as striatum and hippocampus, are extracted (6).

1. Prior to tissue acquisition, place a Petri dish on ice.
2. Fill small tubes with isopropanol and place on dry ice. The microtubes used for tissue collection must fit inside these tubes.
3. Remove the brain and place it on a Petri dish lined with a filter paper soaked in ice-cold PBS (*see Notes 2 and 3*).
4. Locate the areas of interest and remove them carefully using clean forceps and razor blades (*see Note 4*).

5. Place each dissected area, separately, in a clean microtube and freeze immediately by placing the microtube on dry ice in the tube containing isopropanol. If using the dissected tissue immediately, keep the tubes on dry ice until ready to use; otherwise, store at -80°C .

3.1.2. Fractionation

Once the brain region of interest is collected, subcellular fractionation is performed to further simplify the sample for proteomic analysis. The following protocol allows the separation of various synaptic compartments, including synaptosomes, presynaptic and postsynaptic fractions (5, 7).

1. One hour before starting the procedure, place the SW28 rotor and tube holders in the ultracentrifuge (Beckmann L7-65), set to 4°C , 28,000 RPM ($141,000\times g$), 3 h, and turn ON power and vacuum. This allows the centrifuge and tube holders to reach the desired temperature before the samples are ready to be centrifuged.
2. Place centrifuge tubes (Beckman Polyallomer) on ice until ready to use.
3. Weigh total brain samples (at least 200 mg tissue) (*see Note 5*).
4. Using a glass homogenizer, homogenize the tissue ~ 25 times in 3 mL 0.32 M sucrose solution (*see Note 6*), 30 μL protease inhibitor cocktail (100X) and 30 μL phosphatase inhibitor cocktail (100X). Store a 200- μL aliquot of this homogenate and transfer the rest to a 50-mL tube (*see Note 7*).
5. Add 12 mL 2 M sucrose solution and 5 mL 0.1 mM CaCl_2 to the homogenate. This will form a solution with a concentration of 1.25 M sucrose. Mix well (do not vortex) and transfer to an ultracentrifuge tube.
6. Using a 10-mL plastic pipette, overlay slowly and carefully with 1 M sucrose solution until the tube is almost full. Repeat for every sample to be fractionated. This will create a sucrose gradient that allows the isolation of synaptosomes.
7. Place the centrifuge tubes in the tube holders and balance with a 1 M sucrose solution before placing them in the centrifuge.
8. Centrifuge in the SW28 rotor at 28,000 RPM for 3 h at 4°C (*see Note 8*).
9. After centrifugation, discard myelin (viscous layer floating on top) by suctioning with a glass pipette (**Fig. 18.1**). Harvest the synaptosome band ($\sim 3\text{--}4$ mL) located at the interface between the 1.25 and 1 M sucrose layers. Record the

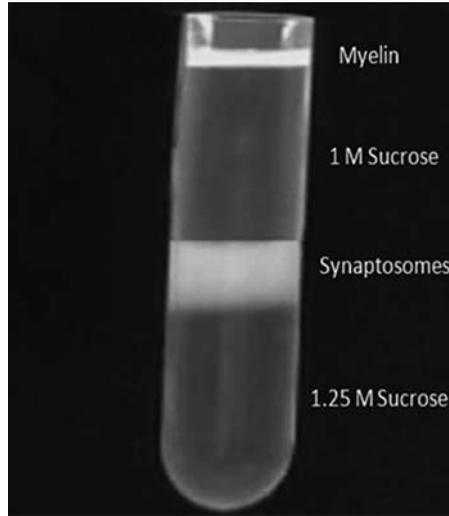


Fig. 18.1. Illustration of layers formed after sucrose gradient centrifugation to isolate synaptosomes.

- amount harvested, take a 500- μ L aliquot of the synaptosomes fraction and store at -80°C . Transfer the rest to 50-mL polycarbonate tubes (samples can be stored overnight at -20°C after this step) (*see Note 9*).
10. Add 0.1 mM CaCl_2 at 10X volume of synaptosomes, 1 M Tris-HCl buffer, pH 6 at 1/50th final volume, 10% Triton X-100 at 1/10th final volume and 100X protease and phosphatase inhibitors. Mix by inversion and incubate on shaker (horizontally in ice box or at 4°C at a moderate speed for 20 min; if using the VWR orbital shaker OS-500, the speed should be 2.5).
 11. Weigh and balance tubes with CaCl_2 . Centrifuge at 18,500 RPM ($40,000\times g$) on the SS-34 rotor at 4°C for 20 min to pellet synaptic junctions. (This protocol assumes the use of the Sorvall RC 5C Plus centrifuge.)
 12. Pour out the supernatant and resuspend the pellet in 2 mL 20 mM Tris-HCl/1% Triton X-100 buffer, pH 8, and 20 μ L each of the protease and phosphatase inhibitor cocktails (100X). Homogenize on ice. In this step it is suggested to suck the pellet into the pipette with 1 mL of buffer, transfer it to the homogenizer and then add 1 mL of buffer to the tube to wash any residue that might have been left. Then, transfer to the homogenizer and add the protease and phosphatase inhibitors. Homogenize and transfer back to the polycarbonate tube. Take a 200- μ L aliquot and store at -80°C . This aliquot contains the synaptic junctions.

13. Add 18 mL of 20 mM Tris-HCl/1% Triton X-100 buffer, pH 8, 180 μ L each protease and phosphatase inhibitor cocktails (100X) to the tube containing the synaptic junctions.
14. Mix by inversion and incubate on shaker (horizontally in ice box or at 4°C, vigorously) for 20 min. Weigh and balance with 20 mM Tris-HCl/1% Triton X-100 buffer, pH 8.
15. Centrifuge at 18,500 RPM (40,000 $\times g$) on the SS-34 rotor at 4°C for 20 min to pellet the postsynaptic density (PSD) fraction. Pour supernatant containing the presynaptic fraction into a separate 50-mL Falcon tube and store PSD pellet at -80°C.
16. While the samples are in the centrifuge, pre-rinse Amicon Ultra-15 centrifugal filters with 10 mL dH₂O. Centrifuge at 3,500 RPM for ~15 min. Discard the flow-through.
17. Add 10 mL of the supernatant containing the presynaptic fraction to the pre-rinsed centrifugal filter and centrifuge at 3,500 RPM, 4°C, for ~20–30 min.
18. Pipette up and down to homogenize the concentrated supernatant. Repeat previous step with the remaining 10-mL supernatant. This step allows concentration of the supernatant containing the presynaptic membrane fraction from 20 to 1 mL.
19. Pour concentrated supernatant into glass centrifuge tube. Add ice-cold acetone to 10X volume to precipitate proteins. Cover with paraffin and store overnight at -20°C (*see Note 10*).
20. To pellet the presynaptic membrane fraction, centrifuge the concentrated supernatant stored in acetone at 11,500 RPM (15,000 $\times g$) at 4°C for 30 min in the SS-34 rotor. If the tubes do not fit properly in the rotor, use rubber tube holders to avoid breaking the glass. Discard supernatant. Let presynaptic pellet air dry and store at -80°C.
21. If using the samples for immunoblotting, dissolve the PSD pellet in 1% SDS and the presynaptic pellet in 0.1% SDS (200 μ L each).
22. Add 2 μ L protease and phosphatase inhibitor cocktails (100X). Vortex and spin down in the microcentrifuge.
23. Sonicate the samples at 3 Watts by moving the tubes ten times up and down in the sonicator probe.
24. Store the samples at -80°C until ready to use (*see Note 11*).

3.1.3. Protein Estimation

This protocol uses the Pierce[®] BCA Protein Assay for the protein estimation, which uses bicinchoninic acid for colorimetric detection and quantitation of proteins. It allows the detection of protein amounts ranging from 0.02 to 20 μg .

To determine protein amounts of the samples, it is necessary to use a common protein as a reference. This protocol uses bovine serum albumin (BSA) as the reference standard (*see* **Table 18.1** for a guide in preparing the standards). The BSA stock used for the preparation of these curves has a concentration of 1 mg/mL.

1. Use the following formula to determine the amount of working reagent (WR) needed for the estimation:

$$\begin{aligned} \text{Volume of WR} &= (\# \text{ standards} + \# \text{ unknowns}) \\ &\quad \times (\# \text{ replicates}) \times (200 \mu\text{L}) \end{aligned}$$

2. Mix 50 parts of BCA Reagent A with 1 part of BCA Reagent B (*see* **Note 12**).

Table 18.1
Preparation of BSA Standard Curve

| Tube | Volume of dH ₂ O | Volume of BSA (1 mg/ml) | Total protein amt in 20 μl |
|---|-----------------------------|-----------------------------------|---------------------------------------|
| <i>Standard curve (working range: 0.5–10 μg)</i> | | | |
| 1 | 80 μL | 0 | 0 |
| 2 | 78 μL | 2 μL | 0.5 μg |
| 3 | 76 μL | 4 μL | 1 μg |
| 4 | 72 μL | 8 μL | 2 μg |
| 5 | 64 μL | 16 μL | 4 μg |
| 6 | 56 μL | 24 μL | 6 μg |
| 7 | 48 μL | 32 μL | 8 μg |
| 8 | 40 μL | 40 μL | 10 μg |
| <i>Standard curve (working range: 0.005–0.5 μg)</i> | | | |
| 1 | 195 μL | 5 μL Stock BSA | 0.5 μg |
| 2 | 100 μL | 100 μL Tube 1 dilution | 0.250 μg |
| 3 | 100 μL | 100 μL Tube 2 dilution | 0.125 μg |
| 4 | 120 μL | 80 μL Tube 3 dilution | 0.05 μg |
| 5 | 100 μL | 100 μL Tube 4 dilution | 0.025 μg |
| 6 | 100 μL | 100 μL Tube 5 dilution | 0.0125 μg |
| 7 | 120 μL | 80 μL Tube 6 dilution | 0.005 μg |
| 8 | 200 μL | 0 | 0 |

3. This protocol uses a microplate, which allows using a smaller volume of the sample for the protein estimation (*see Note 13*).
4. Add 20 μL of each standard to the microplate wells.
5. Add 200 μL of the WR solution to each well and mix on a plate shaker for 30 s.
6. Cover the plate with paraffin and incubate at 37°C for 30 min (*see Notes 14 and 15*).
7. Cool plate to room temperature.
8. Measure the absorbance at approximately 562 nm on a plate reader.
9. Prepare a standard curve by plotting the average BSA standards absorbance versus its protein amount in micrograms and use this curve to estimate the concentration of your samples.

3.1.4. SDS-PAGE

These instructions assume the use of a Mini-Protean II electrophoresis cell gel system. It is critical that the glass plates for the gels are scrubbed clean with a detergent after use and rinsed extensively with distilled water.

1. For electrophoresis, prepare two 1.5-mm thick, 7.5% gels by mixing 3.75 mL of 4X resolving buffer (pH 8.8) with 2.8 mL of 40% acrylamide/bis solution, 8.45 mL water, 50 μL 10% ammonium persulfate solution and 7.5 μL TEMED. Pour the gel, leaving space for a stacking gel, and overlay with water-saturated isopropanol. The gel should polymerize in about 45 min.
2. After the gel is polymerized, pour off the isopropanol and rinse the top of the gel twice with water. Blot with paper to remove excess water before adding stacking gel.
3. Prepare the stacking gel by mixing 1.56 mL of 4X stacking buffer (pH 6.8) with 0.56 mL acrylamide/bis solution, 4.13 mL water, 33 μL ammonium persulfate solution and 7.5 μL TEMED. Use about 3 mL of this to pour the stack for each gel and insert the appropriate comb. The stacking gel should polymerize within 30 min.
4. Prepare 1X running buffer by adding 6 g Tris-HCl and 28.8 g glycine to 2L H₂O. Add 20 mL 10% SDS solution and adjust pH to 8–8.9.
5. Once the stacking gel has set, carefully remove the comb and use a syringe to wash the wells with water and then running buffer.
6. Prepare samples in 1% SDS + 6X loading buffer (DTT added). Spin down samples and boil at 100°C for 5 min. Keep samples at 4°C until ready to use.

7. Add the running buffer to the upper and lower chambers of the gel unit and load 36 μL of each sample in a well. Include one well for pre-stained molecular weight markers.
8. Complete the assembly of the gel unit and connect to a power supply. The gel can be run at 100 V for about 1.45 h or until dye front reaches the bottom of the gel.
9. A few minutes before the SDS-PAGE ends, soak filter papers, fiber pads and the nitrocellulose membrane needed for the transfer in cold 1X transfer buffer.
10. When the SDS-PAGE ends, remove the gel from the apparatus and discard the portion corresponding to the stacking gel.
11. Prepare the transfer assembly as follows: On the dark side of the cassette place a fiber pad, two sheets of 3 MM filter paper, the resolving gel, the nitrocellulose membrane, two additional sheets of 3 MM filter paper and a fiber pad. After placing the nitrocellulose membrane on top of the gel, make sure that there are no bubbles between the gel and the membrane. If there are bubbles, remove them by rolling a glass tube on top of the assembly (*see Note 16*).
13. Close and lock the cassette carefully to avoid moving the gel and/or the membrane out of place.
14. Place the cassette on its module and put in inside the transfer tank together with the cooling block.
15. Fill the tank with transfer buffer. Since the transfer generates a lot of heat, we suggest using cold transfer buffer (4°C) to help dissipate the heat.
16. Close the lid firmly and connect to a power supply.
17. Transfer at 30 V overnight at room temperature or at 100 V for 1.75–2 h on ice.
18. At the end of the run, remove the cassette from the transfer apparatus and take out the assembly so that the gel is on top and the membrane is behind the gel; cut the membrane to the size and shape of the gel. At this point, the molecular weight marker should be visible on the membrane. Since the colours of the molecular weight markers can fade over time, make a cut in one corner of the membrane to remember the orientation of the samples.

3.1.5. Western Blotting

To validate the fractionation protocol, Western blotting analysis can be performed using the various aliquots obtained during the procedure. Antibodies for presynaptically and postsynaptically enriched proteins, such as Syntaxin 1 and PSD95 respectively, can be used to confirm the separation of the subcellular fractions (**Fig. 18.2**). Since the quality of the sample is critical for accuracy

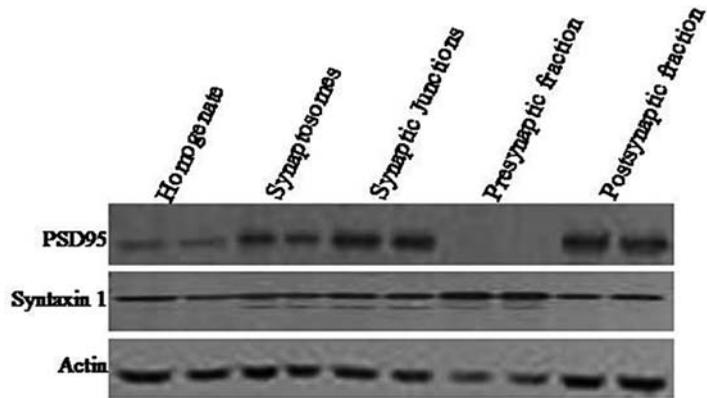


Fig. 18.2. Biochemical validation of the fractionation protocol. To demonstrate the enrichment of proteins in each fraction, equal amounts of protein from each fraction were separated by SDS-PAGE and probed with antibodies for presynaptically (Syntaxin 1) and postsynaptically (PSD95) enriched proteins.

of the data, it is recommended to perform this validation prior to proteomic analysis. The following protocol is based on the use of the Odyssey Infrared Imaging System; therefore, we suggest blocking the membrane in Odyssey Blocking Buffer.

1. Block the membrane in Odyssey Blocking Buffer for 1 h at room temperature on a rocking platform.
2. Add the primary antibody to the membrane and incubate either for 1 h at room temperature or overnight at 4°C with gentle shaking. Add enough antibody solution to cover the whole membrane. Dilution of the antibody solution and incubation time may vary for different antibodies; therefore, it is necessary to optimize these conditions for each particular case (*see Note 17*).
3. After incubation with the primary antibody, remove the antibody and wash the membrane three times, 5–10 min each, with TBS + 0.1% Tween-20.
4. Incubate the membrane with the secondary antibody solution for 1 h at room temperature on a rocking platform. Increasing the incubation time can lead to an increase in background. From this point on, the membrane should be protected from light (*see Note 18*).
5. Remove the secondary antibody solution and wash the membrane three times, 10–15 min each, with TBS + 0.1% Tween-20.
6. Rinse the membrane with TBS to remove residual Tween-20.
7. Scan the membrane using the Li-Cor imaging system.

3.2. Differential Isotopic Labelling and Mass Spectrometry

3.2.1. Protein Digestion and Stable Isotope Labelling

1. Dissolve 30 μg of extracted proteins in 50 mM NaHCO_3 solution to a final volume of 100 μL .
2. Add 5 μL of 200 mM DTT solution to the protein extracts to reduce the disulphide bonds and incubate the solution in a water bath at 40°C for 1 h.
3. To alkylate the reduced proteins, add 5 μL of the 1 M IAM solution and incubate in the dark (cover the vial using aluminium foil) for 40 min.
4. Quench the unreacted IAM using 3 μL of DTT solution and incubate for 1 h at room temperature.
5. Add 500 μL (5 X by volume) of ice-cold acetone to precipitate the alkylated extracts and store at 4°C overnight.
6. Centrifuge the precipitate at 15,000 RPM for 40 min at 4°C and remove the supernatant.
7. Dissolve the pellet in 20 μL of digestion buffer.
8. Add 0.3 μg of trypsin to the dissolved proteins and incubate for 3 h at 40°C.
9. Following digestion, adjust the pH of the solution to 8–9 using 5–10 μL of 1 M NaOH solution.
10. React the contents of the tubes using the isotopic labels (SA-H or SA-D, *see Note 19*). For duplicate measurements, ensure that the duplicates are labelled in forward and reverse fashion (Sample A with SA-H/Sample B with SA-D, and then Sample A with SA-D/Sample B with SA-H).
11. Add 5 μL of SA-H/SA-D solution based on the label and incubate at room temperature for 10 min with intermittent vortex and centrifuge.
12. Using 0.1 μL of the solution and pH paper, check the pH. Adjust the pH to ~ 9 using 1 μL of the 1 M NaOH solution (*see Note 20*).
13. Repeat Steps 11 and 12 three times for each sample.
14. Add 5 μL of the 2.5 M glycine solution to the mixture and incubate for 20 min at room temperature or at 4°C overnight.
15. Add 1 μL of the 1 M NaOH solution followed by 2 μL of 2 M hydroxylamine solution and incubate at room temperature for 15 min.
16. Repeat Step 15 again for each sample.
17. Combine the light- and heavy-labelled samples that are to be compared.
18. Vortex the samples and centrifuge using a microcentrifuge.

19. Store the samples at -20°C or proceed to sample clean-up. **Figure 18.3** illustrates the general steps used to prepare the sample, including the protein digestion and isotope labelling, prior to sample clean-up and analysis.

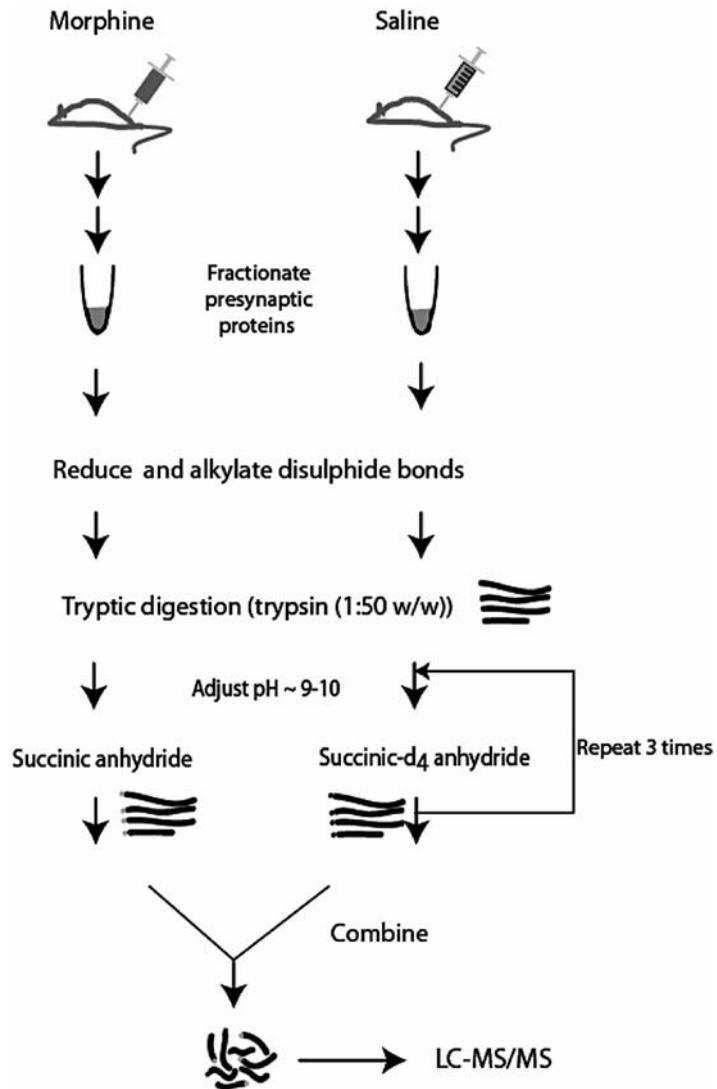


Fig. 18.3. Flow chart illustrating sample processing steps for protein digestion and stable isotopic labelling prior to LC-MS analysis.

3.2.2. Sample Clean-Up

1. Place the spin columns in the centrifuge with 2-mL centrifuge tubes and add 200 μL of activating solution. Centrifuge for 3 min at 1,500 RPM.
2. Repeat Step 1.
3. Add 200 μL of the equilibrating solution to the spin column and centrifuge for 3 min at 1,500 RPM.

4. Repeat Step 3.
5. Discard the solution in the 2-mL centrifuge tube and replace a fresh tube.
6. Add labelled mixture to the spin column and centrifuge for 3 min at 1500 RPM.
7. Reload the eluate to the spin column and centrifuge for 3 min at 1500 RPM.
8. Save the eluate and replace a fresh tube. Label the eppendorf tubes each time a fresh tube is placed to avoid confusion.
9. Add 200 μL of equilibrating solution to wash the salts and centrifuge at 1500 RPM for 3 min.
10. Repeat wash Step 9.
11. Replace with a fresh eluate-collecting tube.
12. Add 30 μL of eluting solution and centrifuge at 1500 RPM for 3 min.
13. Repeat Step 12 twice.
14. Remove the organic solvents in the eluate in a speed vacuum system for 20 min.
15. Reconstitute the residue in 15 μL of equilibrating solution; this solution is directly used for further mass spectrometric analysis.

3.2.3. *Liquid Chromatography/Mass Spectrometry (see Note 21)*

1. Setup for chromatography using a solvent gradient of solvent A and solvent B; the 70 min gradient run for LC separation includes three steps: 5–80% solvent B in 15–55 min (linear); 80% solvent B for 55–60 min (isocratic); 80–5% solvent B in 60–65 min (linear).
2. Inject samples using a manual injector (Valco Instruments), load onto a trap column (PepMapTM, C18, 5 μm , 100 \AA , LC Packings) using solvent A and wash for 5 min.
3. Elute the trapped peptides in reverse direction onto a reverse-phased capillary column (LC PackingsTM 300 μm i.d. \times 15 cm, C18 PepMap100, 100 \AA) using a solvent gradient at 2 $\mu\text{L}/\text{min}$ flow rate.
4. Use an electrospray system for the chromatographic eluate, with nitrogen as the nebulizing gas, at 15 psi and dry the solvents using heated nitrogen gas (dry temperature 190°C) at 8 L/min.
5. Set the mass spectrometer parameters to a target mass of m/z 600, the ion charge control value 200,000 and scan mode set as “standard enhanced” with a speed of 8100 $m/z/s$.

6. Perform MS data acquisition and the subsequent tandem MS (CID) of selected peaks in a data-dependent manner using Esquire Control (Bruker). For each MS scan, select three peptides to be fragmented for 300–500 ms, based on their charge (preferably +2) and intensity.
7. Set the dynamic exclusion of previously fragmented precursor ions to two spectra for a period of 60 s. Perform MS and MS/MS scans in the range of m/z 300–1500 and 50–2000, respectively. An example set of labelled mass spectra (and associated MS/MS spectra) are shown in Fig. 18.4.

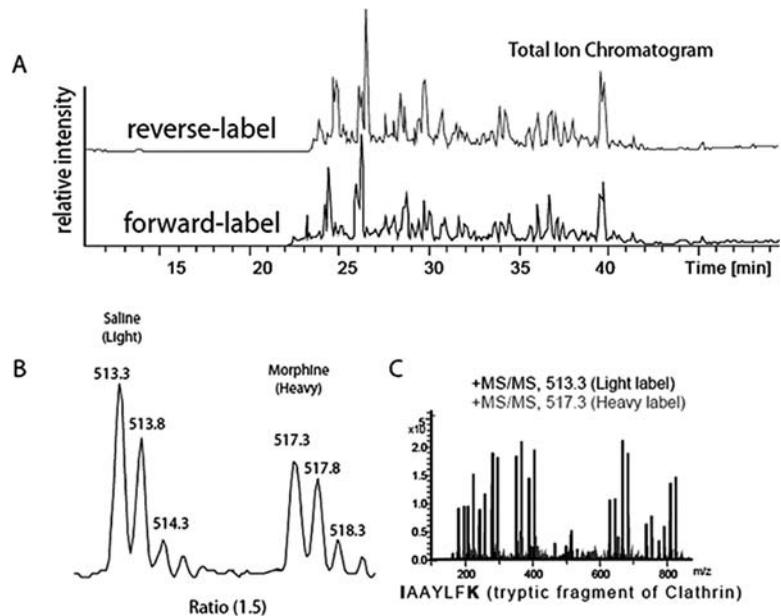


Fig. 18.4. Quantitative proteomic analysis. **A**. Representative total ion**** chromatogram from two samples labelled in forward and reverse fashion. **B**. Combined mass spectra in the range m/z 513–519 showing the difference in intensities of the tryptic fragment “IAAYLFK.” **C**. Tandem mass spectra of light (m/z 513.3) and heavy (m/z 517.3) labelled tryptic fragment “IAAYLFK.” The fragment shown here contains two sites for the labels: N-terminal and ϵ -amine of lysine residue.

3.2.4. Data Analysis

1. Process the data using the Data analysis software (Bruker). Search the mass spectral data obtained between 20 and 45 min of the LC run for compounds using an automated search option (parameters – intensity threshold of 10^5 ; retention time window – 0.7 min; fragments qualified by “amino acids”).
2. Deconvolve the short-listed compounds with their respective MS/MS scans using the automated feature.
3. Export the deconvoluted spectra to the Biotools software (Bruker) for database searching using an in-house Mascot database search engine. Typical values include a mass tolerance of 0.1% for the MS and 0.5 Da for the MS/MS.

Include in the search parameters a fixed modification for cysteine (carbamidomethyl) and variable modifications for methionine (Met-oxidized), lysine and N-terminal amines (succinic anhydride, succinic [$^2\text{H}_4$] anhydride).

4. Consider peptides identified with a Mascot score of ≥ 50 for each protein for further analysis. Manually inspect MS/MS scans for identified peptides to match the fragment ions.
5. Create the list of positively identified proteins, their corresponding tryptic peptide masses and their retention time (*see Note 22*).
6. As each of the identified peptides elute across a span of several MS scans, manually combine appropriate scans and calculate the associated ratio of peak intensities between the heavy and light versions. During data acquisition, in several cases, only one of the labelled peptides may have been selected for fragmentation. In such cases, peptide peak pairs can be manually identified and quantified; heavy and light isotope-labelled peak pairs are separated by 4 Da for singly charged ions, 2 Da for doubly charged ions and 1.3 Da for triply charged ions (*see Note 23*).
7. Obtain the average ratios of all the tryptic peptides corresponding to each specific protein.
8. Create the final list of proteins with the average peak intensity ratios representing the two treatments.

4. Notes

1. TFA is known to improve resolution of chromatographic separations through ion-pairing; however, when used at $>0.1\%$, TFA suppresses analyte ionization in a MS run. Formic acid is known to increase analyte ionization. Commercial solvents (Fischer Scientific, Pittsburg, PA) used in this study for sample clean-up and MS analysis have 0.1% FA and 0.01% TFA that is optimum for both ion-pairing and ionization.
2. Change the filter paper each time a different brain sample is dissected.
3. Once an animal is sacrificed, brain dissection should be performed on ice as quickly as possible to avoid protein degradation.
4. Clean dissection tools with 70% ethanol during dissection to avoid contamination of the samples.
5. Everything must be kept on ice at all times, unless otherwise specified.

6. We recommend using fresh solutions and buffers during the fractionation experiment. They may be prepared the day before the experiment starts and stored at 4°C.
7. If larger quantities of tissue are used for the fractionation experiment, amounts of buffers and solutions should be increased in proportion to the amount of tissue.
8. We recommend increasing the centrifugation time during the generation of the sucrose gradient to 4 h or more when using higher amounts of tissue. This allows for a better separation of the layers.
9. When treating the synaptosomes with 1 mM Tris-HCl buffer, pH 6, in order to pellet the synaptic junctions it may be necessary to split the volume of the synaptosomes into more than one centrifuge tube due to the limitation of the total volume that can be added to the tubes. This experiment assumes the use of the Sorvall RC 5C Plus centrifuge and the SS-34 rotor, which holds tubes with a maximum capacity of ~30 mL.
10. Make sure to store the sample with acetone in an appropriate freezer. After covering the tube, make a small hole in the paraffin covering the tube to avoid accumulation of vapours from the acetone.
11. Avoid freezing and thawing the samples too many times, as this can compromise the integrity of the proteins in the samples.
12. It is important to prepare enough WR in order to add 200 µL to each reaction. The equation in this section allows you to determine the amount of solution needed for all the standards and unknowns. To account for pipetting errors, add one extra reaction to the calculations.
13. The sample-to-WR ratio is approximately 1:8 (v/v), with a range of sample volume between 10 and 25 µL. Depending on the abundance of the proteins in the unknown samples, it might be necessary to make dilutions of the samples prior to adding the specific volume to the microplate.
14. Increasing the incubation time and/or temperature can lower the detection level.
15. Colour development continues even after cooling to room temperature, although at a slower rate; therefore, reading of the plate should be performed as quickly as possible.
16. The transfer protocol assumes the use of the Mini Trans-Blot cell transfer system.
17. When using the Odyssey Infrared Imaging System, primary antibodies can be diluted in Odyssey Blocking Buffer with 0.1% Tween-20 and 0.01% NaN₃. Optimum dilution

depends on the antibody; therefore, we suggest determining it individually for each antibody of interest.

18. Two different fluorescently labelled secondary antibodies can be used with the Odyssey System, the Cy5.5 and the IRDye800TM. These secondary antibodies should be diluted in Odyssey Blocking Buffer with 0.1% Tween-20 and 0.01% NaN₃, at a range of 1:2,000–1:10,000, although lower concentrations can be used to detect very small amounts of protein. The diluted secondary antibody can be stored at 4°C and reused.
19. Assuming there are two treatment groups (e.g. morphine or saline treatment), the samples are paired so that one treatment group receives the heavy or light isotopic label (SA-D or SA-H) (8–10). Divide the samples so that each aliquot contains 10–30 µg of protein. Greater or lesser amounts of protein can be used with the appropriate scaling of reagents (11).
20. When adjusting the pH of the sample solutions using 1 M NaOH, use the same amount of solution from samples to be compared, i.e. maintain a constant volume between samples.
21. The peptide separation and mass spectrometric analysis described here were performed using a capLCTM system coupled to an HCTUltra-PTM Discovery system ion-trap mass spectrometer equipped with an electrospray ionization source and the Hystar program was used to define the HPLC and mass spectrometry methods that were used to develop the solvent gradient and MS tune method and integrate the instruments used (12). Other MS and LC systems can be used with appropriate modifications to these protocols (13–15).
22. Protein identification should be based on two or more tryptic peptides.
23. Discard the ratio of peak pairs with overlapping peaks peptides originating from other “unknown peaks.”

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