Myelin: Methods for Purification and Proteome Analysis

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Abstract

Molecular characterization of myelin is a prerequisite for understanding the normal structure of the axon/myelin-unit in the healthy nervous system and abnormalities in myelin-related disorders. However, reliable molecular profiles necessitate very pure myelin membranes, in particular when considering the power of highly sensitive “omics”-data acquisition methods. Here, we recapitulate the history and recent applications of myelin purification. We then provide our laboratory protocols for the biochemical isolation of a highly pure myelin-enriched fraction from mouse brains and for its proteomic analysis. We also supply methodological modifications when investigating posttranslational modifications, RNA, or myelin from peripheral nerves. Notably, technical advancements in solubilizing myelin are beneficial for gel-based and gel-free myelin proteome analyses. We conclude this article by exemplifying the exceptional power of label-free proteomics in the mass-spectrometric quantification of myelin proteins.

Key words Myelin, Oligodendrocyte, White matter, Density gradient ultracentrifugation, Mass spectrometry, Proteome/proteomics, Proteoform, Lipidome/lipidomics, Transcriptome/transcriptomics, Cyclic nucleotide phosphodiesterase (CNP), Proteolipid protein (PLP), Myelin basic protein (MBP), Cholesterol, Demyelination, Nerve conduction

1 Introduction

About 65 years ago, the emergence of novel lipid extraction methods enabled the first biochemical analyses of myelin. Notably, Marjorie Lees and Jordi Folch developed a protocol to extract lipids from nervous tissue using a chloroform-methanol mixture [1]. Compared to the previously used ethyl alcohol and ester, chloroform-methanol has allowed extracting a much wider range of lipid species, enhanced purity, and less impact on the chemical nature of the lipids. When the method was applied to rat brains, the lipid extracts also contained an abundant protein that like lipids was soluble in chloroform-methanol mixtures but insoluble in aqueous

solutions [2]. Termed proteolipid protein (PLP), this protein is the most abundant constituent of central nervous system myelin [3–6] and associated with lipids [7–11]. Indeed, expression of PLP is required to enrich membrane cholesterol to the high levels normally found in central myelin [12].

More systematic molecular characterization of the entire myelin sheath required a method to enrich myelin from nervous tissue to a level of purity that was not achievable by chloroform-methanol extraction. It took over 15 years until such a protocol had evolved from classical approaches to fractionate entire brains by density centrifugation. Initially, myelin was found as an often-unwelcome contaminant of the nuclear fraction [13, 14]. However, such approaches led to the insight that myelin membranes have the highest lipid content and thus the lowest hydrated density of any membrane in the nervous tissue. In an early attempt specifically directed to exploit this characteristic for enriching myelin from nervous tissue, Saul Korey and his colleagues homogenized subcortical white matter in low-density (0.25 M) sucrose; before ultracentrifugation the homogenate was layered over sucrose of high and intermediate density (1.75 M and 0.5 M) [15]. Most myelin accumulated at the interface between 0.25 M and 0.5 M sucrose. Yet, the fraction also contained considerable amounts of cell debris and axonal fragments that were reduced only to a certain extent during a second ultracentrifugation, in which myelin floated up in a single layer of 1.0 M sucrose.

A subsequent modification of this protocol resulted in improved purity by using 0.8 M instead of 1.0 M sucrose in the second (“floating-up”) ultracentrifugation and replacing buffered salt solutions by water in the washing steps [16]. The use of water, a hypotonic solution, represents an osmotic shock, which we now know contributes considerably to separating myelin from the axonal plasma membrane and other cellular debris. In a next stage of methodological evolution, brain tissue was homogenized in 0.32 M sucrose and layered over 0.66 M sucrose for ultracentrifugation; then two osmotic shocks were followed by a second ultracentrifugation using a similar “spin-down” gradient [17]. Importantly, the high purity of the resulting myelin fraction was carefully scrutinized. Indeed, the myelin fraction displayed a high lipid and proteolipid content, and when observed by electron microscopy contained a high proportion of intact-appearing, multilayered myelin sheaths. Equally important, the preparation appeared essentially free of nuclei, mitochondria, blood vessels, cell debris, endoplasmic reticulum, and axonal remnants.

Finally, William Norton and Shirley Poduslo combined the advantageous-appearing conditions of the earlier protocols. Rat brains homogenized in 0.32 M sucrose were layered on top of 0.85 M sucrose; most material sedimented during ultracentrifugation. Indeed, a discrete myelin fraction accumulated at the

interface, which was subsequently subjected to two osmotic shocks [18]. It is noteworthy that this protocol is commonly cited as “*the*” method to isolate myelin from nervous tissue despite the fact that it lacks a second ultracentrifugation step, the omission of which is disadvantageous for the purity [19] and enrichment [20] of myelin membranes as we now know. The most relevant procedural adaptations since 1973 thus include the reintroduction of the second ultracentrifugation step, as well as replacing the previously used Dounce homogenizer by a high-shear disperser (Ultra-Turrax) [21] and the supplementation of solutions with protease inhibitors.

Together, by taking advantage of its low density, myelin can be highly enriched from homogenized nervous tissue using sucrose density gradient ultracentrifugation. Other cellular compartments and debris are almost quantitatively removed. Indeed, the method has enabled a fair yield of highly purified myelin across a number of laboratories despite moderate procedural differences. Considering the long-standing interest in myelin’s composition of proteins [22–25] and lipids [26–28] it is not surprising that the myelin fraction has more recently been the subject of modern systematic approaches, thereby laying the foundations for myelin proteomics [4] and lipidomics [29]. Notably, a recent RNAseq-analysis of myelin purified from mouse brains at various ages has identified an unpredictably large transcript pool enriched in CNS myelin that is intimately interrelated to the myelin proteome but quite dissimilar to the transcriptomes of oligodendrocytes and brain regions [30]. The findings imply that the selective incorporation of transcripts into the myelin compartment enables both local adaptation and turnover of the myelin sheath. Yet, among the conceivable systematic approaches to myelin, proteome analysis has been most frequently used so far ([31] and references therein; [32–34]).

In the following, we provide our laboratory protocols for myelin purification from the mouse central nervous system, as well as modifications for the application to peripheral nerves or for RNA analysis. We then focus on techniques for gel-based and gel-free myelin proteome analysis. We conclude this article by exemplifying the exceptional power of label-free proteomics in the relative quantification of myelin proteins. We refer the reader to original articles for the related topic of proteome analyses of isolated oligodendrocytes [35–38], to earlier review articles for proteome analysis in the context of white matter disorder [39, 40] and to general overviews about the mechanisms and functions of myelination [41–44], which we do not cover extensively.

2 Materials

2.1 Biochemical Purification of Central Nervous System Myelin

2.1.1 Preparations

(At Least One Day Ahead of the Experiment)

1. 500 mL ddH₂O.
2. 0.85 M sucrose: 29.09 g sucrose in 100 mL ddH₂O (sterile-filtered, cool to 4 °C).
3. 0.32 M sucrose: 10.95 g sucrose in 100 mL ddH₂O (sterile-filtered, cool to 4 °C).
4. Thermo Scientific WX+ Ultracentrifuge (or equivalent).
5. TH641 swing-out rotor (or equivalent).
6. 6 corresponding adaptors.

2.1.2 Preparations

(On the Day of the Experiment)

1. Thermo Scientific WX+ Ultracentrifuge (or equivalent).
2. Protease inhibitors to 50 mL 0.32 M sucrose (dissolve at 4 °C, then keep on ice).
3. Large ice bucket.

2.1.3 Tissue Preparation

1. 6 Beckman centrifuge tubes, Ultra-clear, 14 × 89 mm.
2. IKA T10 basic Ultra-Turrax (or equivalent).
3. 70% EtOH.
4. ddH₂O.

2.1.4 First Sucrose Density Gradient

1. 6 Beckman centrifuge tubes, Ultra-clear, 14 × 89 mm.

2.1.5 Washing

1. 6 Beckman centrifuge tubes, Ultra-clear, 14 × 89 mm.
2. Forceps.
3. Pasteur pipette.

2.1.6 First Osmotic Shock

1. Paper tissue.

2.1.7 Second Osmotic Shock

1. Paper tissue.
2. Protease inhibitors to 50 mL 0.32 M sucrose (dissolve at 4 °C, then keep on ice).

2.1.8 Second Sucrose Density Gradient

1. 6 Beckman centrifuge tubes on ice.
2. Paper tissue.
3. 10× TBS: 1370 mM NaCl, 200 mM Tris/HCl, pH 7.4, 4 °C.

2.1.9 Washing

1. 6 Beckman centrifuge tubes on ice.
2. Pasteur pipette.
3. 10× TBS: 1370 mM NaCl, 200 mM Tris/HCl, pH 7.4, 4 °C.
4. −80 °C freezer.

2.2 1D-Gel Separation of Myelin for Proteome Analysis

2.2.1 1D-SDS-PAGE

1. Precast Bis-Tris 4–12% gradient gels (8 × 8 cm, 1 mm gel thickness, NuPAGE, Thermo Fisher Scientific) OR Tris-glycine 8–16% gradient gels (7 × 8 cm, 1 mm gel thickness, TG PRiME, Serva).
2. Running buffer containing 2-(*N*-Morpholino)ethanesulfonic acid OR 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer system (NuPAGE gels) OR Tris-glycine buffer system (TG PRiME gels).
3. 5–10 µg per lane of purified myelin.

2.2.2 Gel Staining

1. Gel fixative: 40% (v/v) ethanol, 10% (v/v) acetic acid.
2. ddH₂O.
3. Dye stock solution: 2% (w/v) orthophosphoric acid, 10% (w/v) ammonium sulfate, 0.1% (w/v) Coomassie Brilliant Blue (CBB) G-250. Completely dissolve 20 g orthophosphoric acid and 100 g ammonium sulfate in 800 mL ddH₂O; add 1 g of CBB G-250 while stirring; fill up to 1 L with ddH₂O. Prepare stock solution ≥24 h before staining; do not filter. Dye working solution: 80% (v/v) dye stock solution, 20% (v/v) methanol (prepare dye working solution just before use; add methanol while stirring of the stock solution).

2.3 2D-IEF/SDS- PAGE Separation of Myelin for Proteome Analysis

2.3.1 Delipidation / Precipitation/Solubilization

1. 150 µL of purified myelin (typical protein concentration ~4 µg/µL).
2. 2 × 1.5 mL tube (preferably Eppendorf Safe-Lock).
3. vortex.
4. 600 µL methanol.
5. 300 µL chloroform.
6. 450 µL ddH₂O.
7. Centrifuge, e.g., Eppendorf 5415 with rotor F45-24-11 or equivalent.
8. Pipet.
9. Nitrogen stream.
10. Homogenization buffer: 30 mM Tris-HCl pH 9.0, 7 M urea, 2 M thiourea, 2% ASB-14.

2.3.2 Rehydration/ Isoelectric Focusing

1. IPG-strip (24 cm pH 3–12 NL, Serva).
2. IPGphor (GE Healthcare) or a Protean i12 (BioRad) IEF system.
3. 300 µg protein per IPG-strip.
4. Rehydration buffer I: 7 M urea, 2 M thiourea, 2% ASB-14, 1% ampholytes (Serva), 30 mM DTT.
5. Sonicator.

6. Shaker.
7. Rehydration buffer II: 7 M urea, 2 M thiourea, 2% ASB-14, 0.5% ampholytes (Serva), 1.2% DeStreak (GE Healthcare) or equivalent reagents based on organic disulfides.
8. Centrifuge (e.g., Eppendorf 5415 with rotor F45-24-11 or equivalent).
9. Mineral oil.
10. Water-wetted paper pads.

2.3.3 SDS-PAGE

10–15% acrylamide gradient gels (25.5 × 20 cm, gel thickness 0.65 mm).

2.4 Filter-Aided Sample Preparation (FASP) for Gel-Free Myelin Proteome Analysis

2.4.1 Procedure

Per sample:

1. 10 µg of purified myelin.
2. DTT: 1 M stock solution in water, store 200 µL aliquots at –20 °C.
3. Lysis buffer: 7 M urea, 2 M thiourea, 0.1 M Tris pH 8.5, store at –20 °C. Add 1% ASB-14 or 2% CHAPS and 10 mM DTT shortly before use.
4. Iodoacetamide: 450 mM stock solution in water, prepare shortly before use, dilute 1:10 in urea buffer w/o DTT.
5. Shaker at 37 °C.
6. Filter unit (e.g., Merck Millipore Microcon-30 kDa Centrifugal Filter or equivalent).
7. 100 µL 1% (v/v) formic acid.
8. Urea buffer: 8 M urea, 0.1 M Tris pH 8.5, store at –20 °C. Add 10 mM DTT just before use (do not add DTT when diluting iodoacetamide).
9. Centrifuge (11,000 × *g*, RT) or a vacuum manifold (Δ600 mbar, RT).
10. Sonicator.
11. 100 µL 45 mM iodoacetamide in urea buffer (prepare freshly just before use from 450 mM stock solution in water).
12. 3 × 200, 300 and 450 µL 50 mM ammonium bicarbonate, 10% (v/v) acetonitrile.
13. 40 µL of trypsin working solution (0.01 µg/µL), 50 mM ammonium bicarbonate/10% acetonitrile.
14. 40 µL 50 mM ammonium bicarbonate in water.
15. 40 µL 1% (v/v) trifluoroacetic acid (TFA) in water.
16. 1.5 mL low protein-binding tubes (e.g., Eppendorf LoBind or equivalent).

17. 2 μL Yeast Enolase 1 digestion standard OR Hi3 EColi standard (Waters) (100 fmol/ μL).
18. Glass autosampler vial for LC-MS analysis.

3 Methods

3.1 Biochemical Purification of Central Nervous System Myelin

3.1.1 Preparations (At Least One Day Ahead of the Experiment)

1. Cool 500 mL ddH₂O to 4 °C.
2. Prepare sucrose solutions, filter-sterilize them, and cool to 4 °C.
3. Cool rotor and adaptors to 4 °C.

3.1.2 Preparations (on the Day of the Experiment)

1. Cool centrifuge to 4 °C.
2. Add protease inhibitors to 50 mL 0.32 M sucrose (dissolve at 4 °C, then keep on ice).

3.1.3 Tissue Preparation

1. Cool 6 Beckman Centrifuge tubes on ice.
2. Freshly dissect brain or spinal cord of mice (*see* **Notes 1** and **2**).
3. Transfer half a brain or one spinal cord into 5 mL 0.32 M sucrose in one of the precooled Beckman centrifuge tubes.
4. Homogenize the tissue using IKA T10 basic Ultra-Turrax (Speed 3).
5. Clean the Ultra-Turrax thoroughly after each sample with 70% EtOH and ddH₂O (*see* **Note 3**).

3.1.4 First Sucrose Density Gradient

1. Place additional 6 Beckman centrifuge tubes on ice.
2. Transfer 6 mL of cold 0.85 M sucrose into each of the new tubes.
3. Carefully and slowly layer the homogenate (in 0.32 M sucrose) on top of the 0.85 M sucrose (Fig. 1) (*see* **Note 4**).
4. Fill one tube with cold 0.32 M sucrose up to 0.5 cm below the tube edge and tare the other tubes with 0.32 M sucrose exactly to the same weight (max. difference 0.01 g) (*see* **Note 5**).
5. Centrifuge at $75,000 \times g$ for 30 min at 4 °C (rotor TH641 at 24,400 rpm, acceleration 7, deceleration 7); always wait for vacuum.
6. Crudely purified myelin accumulates at the 0.32/0.85 M sucrose interface as fluffy cloud (Fig. 1). The crude myelin fraction appears whitish if mice were transcardially perfused with physiological salt solution or orange-to-reddish if not.

Fig. 1 To isolate CNS myelin, nervous tissue is first homogenized in 0.32 M sucrose (see **method 1**). The homogenate is layered over 0.85 M sucrose (image on the left) and subjected to sequential sucrose density gradient centrifugation, intermittently by osmotic shocks. During ultracentrifugation, the myelin-enriched light-weight membrane fraction accumulates at the interface

3.1.5 Washing

1. After each centrifugation step, place the adaptors on ice as vertically as possible.
2. Place 6 new Beckman centrifuge tubes on ice.
3. Carefully remove centrifuge tubes from the adaptor (forceps may help).
4. Collect the interface with a Pasteur pipette (be careful and try to collect as little of the 0.85 M sucrose as possible) and transfer it into the new tubes.
5. Add 1 mL of 4 °C cold ddH₂O and vortex.
6. Fill one tube with 4 °C cold ddH₂O up to 0.5 cm below the tube edge and tare the other tubes with ddH₂O.
7. Centrifuge at $75,000 \times g$ for 15 min at 4 °C (rotor: TH641 at 24,400 rpm; acceleration 9, deceleration 9); always wait for vacuum.

3.1.6 First Osmotic Shock

1. Discard the supernatant and remove remaining fluid with paper tissue.
2. Add 1 mL cold ddH₂O to each tube and resuspend the pellet (vortex).
3. Fill tubes with cold ddH₂O up to 0.5 cm below tube edge and incubate 10 min on ice.
4. In the meantime tare the tubes with ddH₂O.
5. Centrifuge at $12,000 \times g$ for 15 min at 4 °C (rotor TH641 at 9800 rpm; acceleration 9, deceleration 9); always wait for vacuum.

3.1.7 Second Osmotic Shock

1. Same procedure as for the first osmotic shock (*see* above).
2. During centrifugation, add protease inhibitors to 50 mL 0.32 M sucrose (dissolve at 4 °C, then keep on ice).

3.1.8 Second Sucrose Density Gradient

1. Place 6 new Beckman centrifuge tubes on ice.
2. Discard the supernatant and remove remaining fluid with paper tissue.
3. Add 1 mL cold 0.32 M sucrose to each tube and resuspend the pellet (vortex).
4. Add additional 4 mL cold 0.32 M sucrose.
5. Transfer 6 mL of cold 0.85 M sucrose into each of the new tubes.
6. Carefully and slowly layer the crude myelin (in 0.32 M sucrose) on top of the 0.85 M sucrose.
7. Fill one tube with cold 0.32 M sucrose up to 0.5 cm below tube edge and tare the other tubes with 0.32 M sucrose to the same weight (max. difference 0.01 g).
8. Centrifuge at $75,000 \times g$ for 30 min at 4 °C (rotor TH641 at 24400 rpm, acceleration 7, deceleration 7); always wait for vacuum.
9. During centrifugation, dilute 10× TBS to 1× TBS and add protease inhibitors (dissolve at 4 °C, then keep on ice).
10. Purified myelin accumulates at the interface as a fluffy white cloud (Fig. 1).

3.1.9 Washing

1. Place 6 new Beckman Centrifuge tubes on ice.
2. Collect the interface with a Pasteur pipette and transfer it into the new tubes (on ice).
3. Fill one tube with cold ddH₂O up to 0.5 cm below tube edge and tare the other tubes with ddH₂O.
4. Centrifuge at $75,000 \times g$ for 15 min at 4 °C (rotor TH641 at 24400 rpm; acceleration 9, deceleration 9); always wait for vacuum.
5. Discard the supernatant and remove remaining fluid with paper tissue.
6. Dissolve pellet in 200 µL cold 1× TBS (supplemented with protease inhibitor).
7. Transfer myelin (in 1× TBS) to a new 1.5 mL Eppendorf tube and store at −80 °C (*see* Notes 6–9).

3.2 1D-Gel Separation of Myelin for Proteome Analysis

One-dimensional separation of myelin and immunoblot analysis (Fig. 2) can serve to test for the enrichment of myelin proteins and the diminishment of contaminants during the biochemical purification of myelin. When aiming to establish myelin protein

Fig. 2 Immunoblot illustrates that myelin proteins are enriched in purified myelin compared to brain lysate while markers for other cellular sources are diminished. The detected proteins localize to compact myelin (PLP/DM20, MBP), non-compact myelin (CNP, SIRT2), the oligodendroglial nuclear/cytoplasmic compartment (OLIG2), astrocytes (GFAP), microglia (AIF1/IBA1), the neuronal plasma membrane (GPM6A), axonal microtubules (TUBB3/TUJ1), and mitochondria (VDAC). The blot shows three biological replicates (male c57Bl6/N mice at postnatal day 75); equal amounts of brain lysate and myelin were loaded. *Figure modified from ref. [30]*

inventories, the use of various complementary proteomic approaches (e.g., with or without pre-fractionation of undigested proteins) will provide the most comprehensive, yet nonquantitative compilation of the protein constituents. One-dimensional (1D)-separation via SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) followed by in-gel tryptic digestion is possibly the simplest method to prepare myelin for the mass spectrometric identification of its proteins, including very hydrophobic transmembrane proteins (e.g., PLP, OSP/CLDN11) and proteins with extreme isoelectric points (e.g., MBP, MOBP). The comparatively low resolution provided by these gels is compensated by chromatographic separation of the proteolytic peptides prior to mass spectrometry (MS), an approach often referred to as “GeLC-MS.”

3.2.1 1D-SDS-PAGE

For separation of purified myelin, we have mainly used precast Bis-Tris 4–12% gradient gels (8 × 8 cm, 1 mm gel thickness, NuPAGE, Thermo Fisher Scientific) according to the manufacturer’s protocols. The running buffer containing 2-(N-Morpholino)

Fig. 3 Separation of purified CNS myelin in one-dimensional gels (*see method 2*). (a) Purified myelin was separated by SDS-PAGE using 4–12% Bis-Tris gels (precast, NuPAGE). Different buffer systems enhance separation in the low (MES buffer) or high (MOPS buffer) molecular weight range. Proteins were visualized by silver staining (S; load 0.5 μ g per lane) or colloidal Coomassie (C; 5 μ g loaded). PLP proteolipid protein, MBP myelin basic protein. Molecular weight standard is given in kDa. *Figure modified from ref. [31] with permission.* (b) More recently, we also use 8–16% Tris-glycine gels (precast, TG PRiME), yielding excellent separation of myelin

ethanesulfonic acid (MES) is recommended when aiming at good separation of myelin basic protein (MBP) and other small proteins, while the 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer system [45] enhances resolution in the molecular weight range above 25 kDa (Fig. 3a). More recently, we have also used Tris-glycine 8–16% gradient gels (7 $\times$ 8 cm, 1 mm gel thickness, TG PRiME, Serva), newly available precast gels related to the commonly used Laemmli system. These gels provide coverage of a broad molecular weight range and a remarkably high resolution for large proteins (Fig. 3b).

For subsequent mass spectrometric protein identification, typically 5–10 μ g per lane of purified myelin is separated by 1D-SDS-PAGE. If a higher protein load is desired, it may be required to perform delipidation (*see* Subheadings 2.3.1 and 3.3.1) before loading, thereby preventing lipid-related distortion of separation particularly in the low molecular weight range (i.e., <20 kDa).

To deplete soluble and membrane-associated proteins and thereby enrich membrane-spanning proteins, the myelin fraction may be subjected to consecutive high-salt and high-pH washing/centrifugation cycles prior to loading on the gel as described previously [20].

3.2.2 Gel Staining

For gel staining, we recommend colloidal Coomassie according to [46].

1. Fix gel with 40% (v/v) ethanol, 10% (v/v) acetic acid for at least 1 h.
2. Wash 3×10 min with ddH₂O.
3. Stain the gel in dye working solution at least overnight.
4. Transfer gel into a new box with ddH₂O, wash until all Coomassie particles are removed (*see* **Notes 10–13**).

3.3 2D-IEF/SDS-PAGE Separation of Myelin for Proteome Analysis

Two-dimensional (2D) separation of purified myelin provides highly reproducible protein maps [20, 47] and enables the resolution of different “proteoforms” (i.e., protein isoforms, splice variants, and posttranslationally modified species) of myelin proteins. Indeed, the combination of isoelectric focusing (IEF) as the first dimension with SDS-PAGE as the second dimension (2D-IEF/SDS-PAGE; Fig. 4a) continues to be valuable for myelin proteome analysis despite limitations with respect to the underrepresentation of hydrophobic, transmembrane, and basic proteins. The separation of very basic proteins, or more generally the resolving power in the first dimension, can be improved by using longer IEF strips with wider pH gradients (Fig. 4b).

Fig. 4 Myelin proteome maps based on two-dimensional gels (*see* **method 3**). (a) Purified myelin was separated two-dimensionally using isoelectric focusing (IEF) in a nonlinear pH-gradient (pH 3–10, 18 cm gel) as the first and SDS-PAGE (self-cast 8–16% gel, vertically run) as the second dimension. Proteins were stained using colloidal Coomassie and identified by MALDI-TOF-MS. Molecular weight standard is given in kDa. *Figure modified from ref. [20]*. (b) More recently, we have changed the separation conditions to a pH 3–12 gradient (24 cm gel) as the first and horizontally run 10–15% precast gels as the second dimension, thereby gaining improved separation of basic proteins. We note that proteoforms of myelin basic protein (MBP, encircled in red) are still hardly resolvable due to their extreme basicity. Yet, moving MBP out of the more populated part of the gel allows the detection of previously masked spots

3.3.1 Delipidation/ Precipitation/Solubilization

Prior to 2D-IEF/SDS-PAGE, purified myelin is first delipidated and precipitated with methanol/chloroform according to [48], as lipids and salts compromise the resolution during IEF. The solubility of hydrophobic and glycosylphosphatidylinositol (GPI)-anchored myelin proteins is greatly improved by the zwitterionic detergent amidosulfobetaine ASB-14 compared to the commonly used 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) [49], thereby enhancing their resolution by 2D-IEF/SDS-PAGE.

1. Thaw myelin sample on ice (the precipitation procedure itself is performed at room temperature (RT)).
2. Transfer 150 μL of purified myelin (typical protein concentration $\sim 4 \mu\text{g}/\mu\text{L}$) into a 1.5 mL tube (preferably Eppendorf Safe-Lock) (*see Note 14*).
3. Add 600 μL methanol; vortex.
4. Add 300 μL chloroform; vortex.
5. Add 450 μL ddH₂O.
6. Centrifuge at $9000 \times g$ for 1 min (proteins accumulate at the interface).
7. Discard upper phase by pipetting.
8. Add 450 μL methanol and vortex.
9. Centrifuge at $16,100 \times g$ for 5 min to pellet precipitated proteins.
10. Discard supernatant by pipetting.
11. Dry pellet (use nitrogen stream; do not dry entirely).
12. Add 50 μL homogenization buffer and solubilize carefully (ultrasonic bath or freezing/thawing cycles help to solubilize the pellet; no vortexing to prevent foaming); prevent from heating up above 37 °C to avoid protein carbamylation (*see Note 15*).
13. Centrifuge at $10,000 \times g$ for 2 min.
14. Transfer supernatant into a new 1.5 mL tube.
15. Determine protein concentration by 2D-Quant Kit (GE Healthcare) (*see Note 16*).

3.3.2 Rehydration/ Isoelectric Focusing

Here, we describe a novel protocol for focusing myelin proteins on 24 cm immobilized pH-gradient (IPG) strips of nonlinear pH 3–12. For our previous protocol employing 18 cm IPG-strips of nonlinear pH 3–10 the reader is referred to a previous methods article [50].

1. Take the volume equivalent of 300 μg protein per IPG-strip and mix with the same volume rehydration buffer I.
2. Sonicate for 2 min, shake gently (700 rpm) for 15 min at RT.

3. Fill up to 450 μL with rehydration buffer II (*see* **Note 17**).
4. Sonicate for 2 min, shake gently (700 rpm) for 30 min at RT.
5. Centrifuge $10,000 \times g$ for 1 min.
6. Transfer 450 μL of supernatant to focusing tray, apply IPG-strip facing the gel-side down. Let the strip re-swell for 15 min and cover slot with mineral oil.
7. After 12 h active rehydration at 50 V (70 μA per strip, 20 $^{\circ}\text{C}$), apply water-wetted paper pads to both electrodes (the cathode pad should be soaking to compensate water flow from cathode to anode in highly basic pH-gradients).

Focus for about 40 kVh (125 μA per strip, 20 $^{\circ}\text{C}$) by a ramped two-step protocol (0–8000 V /1 h, 8000 V/4 h) on an IPGphor (GE Healthcare) or a Protean i12 (BioRad) IEF system. The latter has the advantage that the program of choice is regulated for each strip individually.

3.3.3 SDS-PAGE

Here, we describe the use of precast gels polymerized on a non-fluorescent backing film, of which up to four can be run simultaneously in horizontal electrophoresis chambers (Serva HPE system). The major advantage of this high-performance electrophoresis system is the efficient cooling of the horizontal gels during the runs, which, together with other technical refinements, allows excellent reproducibility, spot resolution and detection sensitivity [51]. For our previous protocol employing self-cast 18×20 cm gels for the second dimension the reader is referred to a previous methods article [50].

We use 10–15% acrylamide gradient gels (25.5×20 cm, gel thickness 0.65 mm) according to the manufacturer's manual (Serva HPE system) and in the following only list our adaptations for the separation of myelin.

1. Increase the SDS content of the equilibration solution to 6%.
2. Use 6 mL cooling contact fluid (at least for the first generation HPE tower).
3. Run final step of electrophoresis 60 min longer (*see* **Notes 18 and 19**).

3.4 Filter-Aided Sample Preparation (FASP) for Gel-Free Myelin Proteome Analysis

The outstanding sensitivity of current mass spectrometers necessitates careful consideration of sample preparation. In particular, it is crucial that substances are efficiently removed (or at least diminished) that may interfere with chromatographic separation or electrospray ionization. Moreover, particle-free samples are required when direct injection setups without trapping columns are used to provide higher chromatographic separation [52].

In our previous studies [4, 34, 47] we have successfully subjected myelin to tryptic in-solution digest in the presence of the

acid-labile surfactant RapiGest (Waters) (for protocols *see* refs. [34, 50]). Indeed, this method has facilitated sufficiently robust solubilization and unfolding of proteins. Yet, in our current view the particular requirements of myelin samples are met even better by filter-aided sample preparation (FASP) [53]. FASP allows the depletion of soluble low molecular weight compounds (including detergents) from a complex protein mixture by washing it with urea buffer in an ultrafiltration device, which is followed by buffer exchange, direct on-filter digestion, and recovery of the proteolytic peptides by filtration (for overview *see* Fig. 5). The ultrafiltration devices are designed as spin columns and typically

Fig. 5 Schematic overview of filter-aided sample preparation (FASP). For details *see* method 4

operated by centrifugation. However, reasoning that vacuum-driven removal of liquids can enable the automation of FASP we have implemented the procedure into a liquid handling workstation, the customized hardware and software components of which will be described elsewhere. The procedure itself is adapted from a previously reported manual protocol [52]. We consider FASP automation as important toward even higher reproducibility of sample preparation that is essential for quantitative label-free proteome profiling.

3.4.1 Procedure

1. Solubilize and denature 10 μg of purified myelin in 150 μL Lysis buffer containing 1% (w/v) ASB-14 and 10 mM DTT, sonicate for 5 min, incubate for 30 min at 37 °C while shaking.
2. Dilute sample with 1500 μL Lysis buffer containing 2% (w/v) CHAPS to reduce ASB-14 concentration to below 0.1% (w/v).
3. In parallel, rinse the filter unit first with 100 μL of 1% (v/v) formic acid (FA) and then with 200 μL 8 M urea buffer/10 mM DTT. A centrifuge (11,000 $\times g$, RT) or a vacuum manifold ($\Delta 600$ mbar, RT) can be used in this and all following steps unless noted otherwise. In the following protocol, centrifugation/application of vacuum is referred to as “liquid removal.”
4. Transfer 220 μL of myelin to the filter unit, remove buffer on top of the filter by liquid removal. Repeat until all volume is applied to the filter unit. Always sonicate the solubilized sample for 5 min before transferring an aliquot onto the filter.
5. Wash 2 $\times$ with 200 μL urea buffer/10 mM DTT to deplete detergents by liquid removal.
6. Optional: Pause the procedure overnight. Cover the filter membrane with 100 μL urea buffer/10 mM DTT, cap the filter units and store in wet chamber at RT. Liquid removal is required before proceeding to the next step.
7. Alkylate cysteine residues by addition of 100 μL 45 mM iodoacetamide in urea buffer (prepare freshly just before use from 450 mM stock solution in water), incubate 20 min at RT in the dark, followed by liquid removal.
8. Wash 2 $\times$ with 200 μL urea buffer/10 mM DTT to quench excessive iodoacetamide, followed by liquid removal.
9. Wash 3 $\times$ with 200, 300, and 450 μL 50 mM ammonium bicarbonate/10% (v/v) acetonitrile for buffer exchange, followed by liquid removal.
10. Place filter unit onto centrifugation tube.
11. Wash 3 $\times$ with 200 μL 50 mM ammonium bicarbonate/10% (v/v) acetonitrile, followed by liquid removal. Centrifuge 30 min at 10,000 $\times g$ (RT).

12. Place filter unit onto new centrifugation tube.
13. Add 40 μL of trypsin working solution (0.01 $\mu\text{g}/\mu\text{L}$) in 50 mM ammonium bicarbonate/10% acetonitrile to the filter, shake once gently (manually) and digest overnight at 37 °C in a wet chamber.
14. Centrifuge the filter for 30 min at $11,000 \times g$ (RT) and collect the flow-through.
15. Add 40 μL 50 mM ammonium bicarbonate in water, centrifuge for 30 min at $11,000 \times g$ (RT) and collect the flow-through into the same tube.
16. Add 40 μL 1% (v/v) trifluoroacetic acid (TFA) in water, centrifuge for 30 min at $11,000 \times g$ (RT) and collect the flow-through into the same tube.
17. Combined flow-through is transferred to 1.5 mL low protein-binding tubes (e.g., Eppendorf LoBind or equivalent), document the volume.
18. Mix 2 μL Yeast Enolase 1 digestion standard or Hi3 EColi standard (Waters) (100 fmol/ μL) with 20 μL of sample in a glass autosampler vial for LC-MS analysis (*see* **Notes 20–24**).

3.5 Gel-Free Myelin Proteome Analysis and Mass Spectrometric Quantification of a Myelin Protein Using CNP as an Example

For myelin proteome analysis, gel-free (“shotgun”) approaches allow circumventing many of the limits of gel-based approaches, especially those of 2D-IEF/SDS-PAGE (*see* above). The replacement of gel-based separation of entire proteins by their tryptic cleavage and subsequent separation of the resulting peptides via high resolution liquid chromatography (LC) before interfacing with mass spectrometry (LC-MS) goes at the expense of information about protein size and charge. This is a disadvantage when analyzing “proteoforms,” i.e., protein isoforms, splice variants, or posttranslationally modified proteins. However, LC-MS is particularly well suited for quantitative and differential analyses of the myelin proteome with its many extraordinarily basic or hydrophobic proteins and its exceptional range of several orders of magnitude of protein abundance. Indeed, LC-MS enables the label-free quantification of proteins in complex samples such as myelin with the required dynamic range, yet it implements no bias against particular protein classes. Finally, only low amounts of starting material are required, about 100th compared to 2D-IEF/SDS-PAGE.

We note that the particular procedure of myelin proteome analysis will greatly depend on the locally available mass spectrometer and associated data acquisition and quantification method. For our investigations, we have decided for separation using nanoscale ultraperformance liquid chromatography (nanoUPLC) with quadrupole time of flight (QTOF)-mass spectrometers that allow a data-independent acquisition workflow with alternating low-energy and

elevated-energy scans (e.g., QTOF-Premier or Synapt G2S, Waters). We refer the reader to previous articles for an overview about data-independent acquisition methods [54] or for a detailed protocol [52]. To determine the absolute abundance of myelin proteins, we utilized the data post-processing software ISO-Quant [55], which quantifies the validated mass spectra of the three most abundant peptides per protein according to the TOP3 method [56]. To compare the abundance of a myelin protein between two states (e.g., between mutant and control mice), its relative abundance was considered changed if significant according to two-tailed t-test and exceeding a threshold of 25%. This approach has enabled robust and reliable differential analysis of the entire myelin proteome in various mouse models of myelin-related pathology [34].

To exemplify the power of gel-free mass spectrometry for the differential quantification of myelin proteins, we have assessed the abundance of cyclic nucleotide phosphodiesterase (CNP) [57, 58], the third-most abundant CNS myelin protein [4]. Heterozygous *Cnp*^{+/-} mice display a catatonia-depression syndrome upon aging [59]; yet their myelin appears morphologically normal. *Cnp*^{+/-} mice provide a defined model in which the halved gene dosage should theoretically lead to a halved abundance of CNP in myelin. Indeed, when using the workflow outlined above, the abundance of CNP in myelin purified from *Cnp*^{+/-} mice compared to wild-type littermates is 50.7% ($\pm 0.4\%$). The remarkable exactness of the quantification and the excellent sample-to-sample reproducibility (see Fig. 6) allows high confidence in unexpected findings yielded by differential myelin proteome analyses, notwithstanding that relevant-appearing results require validation by independent methods.

In conclusion, the method to purify myelin from nervous tissue has changed only moderately since the 1960s and 1970s, reflecting its efficiency and the excellent degree of myelin membrane enrichment that can be gained reliably and reproducibly. More recently, it was possible to combine myelin purification with modern “omics”-techniques to systematically determine molecular composition at the level of mRNA [30], lipids [29], and proteins. With respect to lipids, carbohydrates, and posttranslational modifications, their capabilities are not yet exploited to their full extent. However, myelin proteome analysis has proven a valuable resource for myelin research, as it has facilitated selecting proteins for functional investigations in mutant mice, revising the abundance of myelin proteins [4, 47], calculating molar ratios [60–62], analyzing models of myelin-related disorders [20, 39, 47, 63–65], discovering disease-causing genes [66], and identifying a novel molecular scaffold in the myelin sheath [34]. We believe that systematic approaches to myelin involving “omics”-techniques hold great promise for improving our understanding

Fig. 6 Quantification of myelin proteins by label-free mass spectrometry using CNP as an example. (a, b) One-dimensional SDS-PAGE separation and silver staining (a) or immunoblotting (b) of myelin purified from mouse brains at P75 indicate that the abundance of CNP in myelin is reduced in heterozygous *Cnp*^{+/-} mice compared to wild-type littermates. Sirtuin 2 (SIRT2) served as a loading control. CNP cyclic nucleotide

of the development, structure and function of the axon/myelin-unit in the context of the healthy and the pathological nervous system.

4 Notes

1. Optional: When dissecting the tissue earlier, store at -80°C or keep frozen on dry ice until just before homogenization.
2. Optional: Animals may be transcardially perfused with physiological salt solution (HBSS, PBS), thereby clearing capillaries and reducing contamination of the myelin fraction with proteins derived from blood.
3. Optional: Store 400 μL of the homogenate for later use (-80°C), e.g., for control by immunoblot analysis as exemplified in Fig. 2.
4. Do not add the homogenate drop by drop; best phase separation is achieved when the homogenate flows down slowly along the tube wall. The tube is placed vertically on ice; the pipette is tilted by 45° .
5. The tubes have to be weighed with adaptor and lid. Dry the centrifuge tubes before placing them into the adaptor. Dry the adaptors before placing them in the rotor.
6. From half a mouse brain, about 600 μg of purified myelin can be expected. For measuring protein concentrations, we recommend a Lowry assay (e.g., Biorad DC Protein Assay) or the precipitation-based assay kits 2-D Quant (GE Healthcare) or SingleQuant (Serva). Myelin should be well homogenized, e.g., using a Teflon pestle that fits exactly with 1.5 mL Eppendorf tubes (e.g., Thomas Scientific).

Fig. 6 (continued) phosphodiesterase. Arrowheads point at protein isoforms. **(c)** Differential myelin proteome analysis quantifies the abundance of CNP in myelin purified from heterozygous *Cnp*^{+/-} mice as 50.7% ($\pm 0.4\%$) compared to wild-type littermates. The bar graph shows individual data points for three independent experiments with three biological replicates per genotype and four technical replicates per biological replicate. For details, see **method 5**. PPM parts per million. **(d)** Venn diagram comparing the number of proteins identified in myelin by MS^E (as used for quantification in Fig. 6c) with those identified by high-definition MS^E (HDMS^E). The higher proteome depth of HDMS^E owing to the ion mobility option, which may be considered as a gas phase electrophoresis-like device that equips modern mass spectrometers with an additional level of peptide separation. As this compresses the dynamic range of quantification, highly abundant proteins such as PLP, MBP, and CNP are more reliably quantified by MS^E (i.e., without ion mobility separation) at the cost of the number of identified proteins. **(e)** Venn diagram comparing the proteins identified in three biological replicates of myelin by HDMS^E (left image) or MS^E (right image). Note that both approaches allow excellent sample-to-sample reproducibility

7. Myelin samples to be compared in one experiment should be purified in parallel, i.e., in one rotor run.
8. Quality control may include electron microscopy [20] and immunoblot (Fig. 2).
9. Myelin purified according to this protocol can be used for protein and lipid analyses, RNA extraction, enzymatic assays, electron microscopy, and other applications.

Optional: When posttranslational modifications are to be analyzed, the supplementation of solutions with respective inhibitors may be beneficial. For example, add phosphatase inhibitors (e.g., PhosStop cocktail tablets, Roche) or deacetylase inhibitors (10 mM Nicotinamide (Sigma-Aldrich), 0.5 μ M Trichostatin A (Sigma-Aldrich)) to 0.32 M sucrose and $1 \times$ TBS.

If aiming to isolate RNA from purified myelin [30], work under RNase-free conditions is advantageous. Clean the bench, all materials, skull, and brain with RNase AWAY (Sigma-Aldrich); use RNase-free sucrose (e.g., Sigma-Aldrich) and DEPC-treated ddH₂O; after the last washing step dissolve myelin in QIAzol (Qiagen) instead of TBS; wear a mask and gloves at all times.

Subfractions of low, medium, and high density can be isolated from purified myelin [67–71]. Note that it is speculative if these subfractions correspond to distinct subcompartments of myelin.

Aiming to further purify the myelin fraction, purified myelin was subjected to two additional osmotic shocks and a third ultracentrifugation step using a “floating-up” protocol [67]. For the floating-up gradient, myelin was resuspended in 0.83 M sucrose and overlaid with 0.3 M sucrose; during ultracentrifugation myelin accumulated at the interface. Note that in our experience this procedure does not yield better purity but results in considerable material loss and should thus be omitted.

The purification of peripheral myelin requires different sucrose concentrations (0.27 M and 0.83 M sucrose) and only one gradient ultracentrifugation step [21, 47, 67]. A yield of up to 200 μ g of purified myelin can be expected when pooling 30 sciatic nerves from 15 adult mice. Note that the achievable factor of enrichment is lower compared to central myelin.

Myelin has been purified from other species ([72, 73] and references therein), including rats [74, 75], humans [29, 32, 76], clawed frogs [5], and zebrafish [77–79]. Note that sucrose concentrations may require adaptation if the density of myelin in a species differs from that in mice.

10. Only glass containers or plastic containers specified for gel staining should be used because low-quality plastic may emit substances that impede mass spectrometric identification.
11. Stained gels can be imaged using a near-infrared fluorescence scanner [80, 81] or a conventional flatbed scanner.
12. For mass spectrometric protein identification, manually cut out individual bands immediately after gel staining. Alternatively, excise entire gel lanes with a custom-made punching tool, resulting in 24 uniformly sized gel bands that can be subjected to automated in-gel digestion in microplate format [82]. Store gel bands or slices in adequate vials or microplates at -20°C until further processed.
13. Wearing gloves and a hair net during these procedures minimizes the contamination of the samples with human keratins.
14. 150 μL is the maximal input volume for 1.5 mL tubes. If upscaling is desired, we recommend 5 mL Eppendorf Safe-Lock tubes and the corresponding fixed-angle rotor as the procedure appears to require the specific tube geometry for proper function.
15. Optional: These buffer conditions also allow labeling with fluorescent dyes for quantitative comparison of proteomes by 2D difference gel electrophoresis (2D-DIGE). *See* refs. [83, 84] for protocols, as well as refs. [20, 47] for application to central and peripheral myelin, respectively.
16. For myelin delipidation/precipitation, expect protein loss of up to 50%.
17. This is a typical buffer composition for anodal cup-loading, which is commonly favored for focusing in highly basic gradients. Importantly, conventional cup-loading in our hands was incompatible with myelin, likely due to aggregation. However, the addition of DeStreak diminishes horizontal streaking also after rehydration loading, at least for separation of myelin proteins in broad pH-gradients of up to pH 12.
18. After gel staining (*see* Subheadings 2.2.2 and 3.2.2), cylindric gel spots are excised manually and subjected to automated in-gel digestion in microplate format, followed by analysis on a MALDI-TOF mass spectrometer [85].
19. As the improved coverage of membrane proteins comes at the cost of resolution, we currently see no major advantages in replacing IEF as the first dimension by size-dependent separation in presence of the cationic detergent 16-BAC (16-benzyl-dimethyl-*n*-hexadecylammonium chloride). For a detailed protocol of subjecting CNS myelin to 2D-16-BAC/SDS-PAGE we refer the reader to a previous methods article [50].

20. Pre-solubilization of myelin with ASB-14 under reducing conditions is crucial to increase accessibility of proteins for subsequent alkylation and enzymatic proteolysis. ASB-14 instead of CHAPS was chosen based on prior work [49] and our subsequent positive experience from 2D-IEF/SDS-PAGE (*see* Subheading 3.3). Although used in the original FASP protocol [53], we omit the anionic detergent SDS because potential remains of SDS (but not of the zwitterionic detergents ASB-14 and CHAPS) necessitate sample cleanup prior to injection to protect chromatography columns. Dilution with CHAPS buffer prevents clogging of the filter membrane.
21. Centrifugation is preferred when quantitative liquid removal is desired, as it cannot be entirely excluded that liquid remains within the membrane support in the bottom part of the filter unit after vacuum application.
22. Tryptic digestion in the presence of 10% acetonitrile facilitates the solubility of proteins after removal of urea and of nascent hydrophobic peptides.
23. Stepwise recovery of the tryptic peptides under different pH conditions is intended to increase peptide yield and leads to a sample that is ready to inject without further concentration or cleanup.
24. Centrifugation/vacuum application times may vary considerably depending on the protein input. A typical timing for FASP digestion of myelin is around 24 h with manual centrifugation being somewhat faster than automated vacuum-driven operation. However, considering that the latter is largely a walk-away process, its robustness and reproducibility clearly compensate the higher time need.

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