Chapter 6
High-Yield Isolation and Subcellular Proteomic Characterization of Nuclear and Subnuclear Structures from Trypanosomes

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Abstract The vast evolutionary distance between the Opisthokonta (animals and yeast) and the excavata (a major group of protists, including *Giardia* and *Trypanosoma*) presents a significant challenge to in silico functional genomics and ortholog identification. Subcellular proteomic identification of the constituents of highly enriched organelles can alleviate this problem by both providing localization evidence and yielding a manageably sized proteome for detailed in silico functional assignment. We describe a method for the high-yield isolation of nuclei from the kinetoplastid *Trypanosoma brucei*. We also describe the subsequent purification of subnuclear compartments, including the nuclear envelope and nucleolus. Finally, using several proteomic strategies, we survey the proteome of a subcellular structure or organelle, using the nuclear pore complex as an example.

1 Introduction

The excavate euglenozoid *Trypanosoma brucei*, a member of the class Kinetoplastida, is the etiologic agent of the African sleeping sickness (trypanosomiasis), a disease that is invariably fatal if untreated ***(1)***. African trypanosomiasis is endemic to the most rural and undeveloped regions within 36 sub-Saharan African countries, and the emergence of drug resistant strains represents a considerable public health and economic problem ***(2)***. Beyond world health concerns, the evolutionary distance of the trypanosomes from the major model systems amongst the higher eukaryotes is of great interest to comparative and evolutionary biology ***(3)***. Unfortunately, vast evolutionary distance impedes in silico functional assignment and, thus, only partial gene annotation can be achieved ***(4, 5)***. For example, approximately 40% of the open reading frames (ORFs) in the trypanosome genome are considered to be

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unique to the kinetoplastida, but there are indications that this number is a considerable overestimate due to limitations of BLAST algorithms ***(6)***.

T. brucei, in particular, has proven to be an excellent distal model system for evolutionary biology ***(7)***. First, trypanosomes are highly divergent from the Opisthokonts ***(8)***. Second, *T. brucei* is amenable to laboratory investigation, with major life stages available to in vitro culture, multiple expression systems available for genetic manipulation, and, significantly, a robust RNA interference (RNAi) system ***(9, 10)***. Third, the genome of *T. brucei* has been sequenced, and comparative data indicate a very high degree of similarity to the related parasites *Leishmania major* and the American trypanosome *T. cruzi*: hence, work in *T. brucei* is directly applicable to these additional pathogens ***(11)***. Here, several of these advantages are exploited in order to produce, in high yield, purified nuclei and then to survey the subnuclear structures to identify novel components ***(12)***.

The first step to producing subnuclear fractions is the isolation of nuclei away from the remainder of the cellular compartments. Once accomplished, these enriched nuclei may be further subfractionated to yield nucleoli, nuclear envelopes, or lipid-stripped nuclear envelopes (termed pore complex–lamina fraction (PCLF)). The subnuclear components are of high quality and suitable for further biochemistry and mass spectrometry. Nuclei from either the vector (procyclic) or the host (blood stream form) life stage may be isolated, providing access to life stage-dependent aspects. However, the procyclic stage is somewhat more convenient because these cells can be grown to higher density in in vitro culture.

2 Materials

2.1 The Isolation of Trypanosoma brucei Nuclei

1. PVP solution: 8% polyvinylpyrrolidone (PVP-40, Sigma-Aldrich, St. Louis, MO, USA), 11.5 m*M* KH_2PO_4, 8.5 m*M* K_2HPO_4, and 750 μ*M* $MgCl_2$. Adjust to pH 6.53 with concentrated H_3PO_4 (~15 μL for 1 L solution). Store at 4°C. (*See* **Note 1**).
2. Sucrose solutions (sucrose/PVP): store in sterile tubes at −20°C. (*See* **Note 2**).
   (a) 2.01 *M*: to 183.3 g sucrose, add PVP solution to a final weight of 338 g. Refractive Index (RI) = 1.4370.
   (b) 2.10 *M*: to 193 g sucrose, add PVP solution to a final weight of 340 g. RI = 1.4420.
   (c) 2.30 *M*: to 216 g sucrose, add PVP solution to a final weight of 340 g. RI = 1.4540.
3. Phosphate-buffered saline (PBS; PBS Tablets, Sigma-Aldrich). Chilled to 4°C.

4. 1 *M* dithiothreitol (DTT). Store at −20°C in 200 µL aliquots.
5. 10% Triton X-100 (Sigma-Aldrich).
6. Protease inhibitor cocktail (PIC) (P8340, Sigma-Aldrich). Store at −20°C.
7. Solution P: 0.04% (w/v) pepstatin A and 1.8% phenylmethanesulfonyl fluoride (PMSF) (both from Sigma-Aldrich) in absolute (anhydrous) ethanol. Store at −20°C (*See* **Note 3**).
8. 0.3 *M* sucrose/PVP: dilute stock sucrose/PVP with PVP solution.
9. Lysis buffer (prepare fresh): 0.05% Triton X-100, 5 m*M* DTT, 1:100 solution P and 1:200 PIC in PVP solution. Twenty milliliters of lysis buffer is equivalent to 1 volume (*See* **Note 4**).
10. Underlay buffer (prepare fresh): 5 m*M* DTT, 1:100 solution P and 1:200 PIC in 0.3 *M* sucrose/PVP. Ten milliliters of underlay buffer is equivalent to 1 volume.
11. Resuspension buffer (prepare fresh): 5 m*M* DTT, 1:100 solution P and 1:200 PIC in 2.1 *M* sucrose/PVP. Eight milliliters of resuspension buffer is equivalent to 1 volume.

2.2 Subnuclear Fractionation

2.2.1 The Nuclear Envelope

1. 0.1 *M* bis-Tris-Cl, pH 6.50.
2. BT/Mg buffer: 0.01 *M* bis-Tris-Cl pH 6.50, 0.1 m*M* $MgCl_2$.
3. Shearing buffer (prepare fresh): 1 m*M* DTT, 1.0 mg/mL heparin, 20 µg/mL DNase I (Sigma-Aldrich, store stock at −20°C), 2 µg/mL RNase A (Sigma-Aldrich, store stock at −20°C), 1:100 solution P, and 1:200 PIC in BT/Mg buffer.
4. 2.10 *M* sucrose in 20% Accudenz (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) in BT/Mg buffer. Store at −20°C.
5. 2.50 *M* sucrose in BT/Mg buffer. The final refractive index should be 1.4533. All sucrose BT/Mg solutions should be stored at −20°C.
6. 2.25 *M* sucrose BT/Mg, by stock dilution.
7. 1.50 *M* sucrose BT/Mg, by stock dilution.

2.2.2 The Pore Complex–Lamina

1. Extraction buffer (prepare fresh): 1.5% Triton X-100, 1.5% sodium taurodeoxycholate, 1:100 solution P, and 1:200 PIC in BT/Mg buffer.
2. 2.50 *M* sucrose BT/Mg.
3. 1.75 *M* sucrose BT/Mg, by stock dilution.

2.2.3 The Nucleolus

1. Disruption buffer (prepare fresh): 10 m*M* bis-Tris-Cl pH 6.50, 0.6 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.34 *M* sucrose/BT, 0.05% Tween 20 (Pierce, Rockford, IL, USA), 1:100 solution P, and 1:200 PIC.
2. 2.50 *M* sucrose BT/Mg.
3. 2.25 *M* sucrose BT/Mg.
4. 1.75 *M* sucrose BT/Mg.

2.3 Biochemistry and Mass Spectrometry

2.3.1 Protein Precipitation

1. HPLC-grade methanol.

2.3.2 Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis (PAGE)–Mass Spectrometry (MS)

1. 1 *M* iodoacetamide. Store at −20°C in 100 μL aliquots.
2. NuPAGE Sample Reducing Agent (Invitrogen, Carlsbad, CA, USA).
3. NuPAGE lithium dodecyl sulfate (LDS) Sample Buffer (Invitrogen).
4. NuPAGE 3-(N-morpholino)propanesulfonic acid (MOPS) SDS Running Buffer (Invitrogen).
5. NuPAGE 10% and 4–12% bis-Tris gels (Invitrogen).
6. Novex 8% Tris-glycine gels (Invitrogen).
7. GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA).
8. 15- or 30-degree Feather MicroScalpel (Electron Microscopy Sciences, Hatfield, PA, USA).
9. Fine Point Diamond Tweezers (Electron Microscopy Sciences).
10. Destain solution: 25 mM ammonium bicarbonate in 50% acetonitrile.
11. Trypsin (bovine, modified, sequencing grade; Roche Applied Science, Indianapolis, IN, USA). Resuspend lyophilized trypsin to 1 μg/μL in 1 mM HCl. Store 1μg aliquots at −20°C.
12. Digestion buffer: 50 mM ammonium bicarbonate.
13. Poros R2 beads (Applied Biosystems, Foster City, CA, USA) (*See* **Note 5**).
14. Poros dilution buffer: 2% (v/v) trifluoroacetic acid, 5% (v/v) formic acid in water.
15. 0.1% trifluoroacetic acid.
16. Elution solution: 20% acetonitrile, 50% methanol, and 0.1% trifluoroacetic acid (aq).
17. ZipTips C18, size P10 (Millipore, Billerica, MA, USA). Prior to use, the ZipTips are washed and conditioned as follows:

(a) Wash twice with 10 µL 0.1% trifluoroacetic acid.
(b) Wash four times with 10 µL elution solution.
(c) Wash four times with 10 µL 0.1% trifluoroacetic acid.
(d) Retain 10 µL of 0.1% trifluoroacetic acid to wet the resin.

18. 2,5-dihydroxybenzoic acid (DHB; Lancaster, Pelham, NH, USA). Prepare a saturated solution in elution solution at room temperature. The saturated solution is diluted to 40% (v/v) saturated DHB just prior to use. Saturated DHB may be stored at 4°C. However, DHB may partially precipitate in storage to form an insoluble pellet. Prior to use, allow the solution to warm to room temperature and add more solid DHB to saturation before diluting.

2.3.3 High-Performance Liquid Chromatography (HPLC)–Mass Spectrometry

1. Digestion buffer: 50 m*M* ammonium bicarbonate.
2. Trypsin: *see* **Section 2.3.2**.
3. Quench solution: 10% trifluoroacetic acid.
4. Mobile phase A: 5% acetonitrile, 0.1% trifluoroacetic acid.
5. Mobile phase B: 95% acetonitrile, 0.1% trifluoroacetic acid.

2.3.4 Hydroxyapatite Chromatography

1. Macro-Prep ceramic hydroxyapatite (HA) type I, 40 µm (Bio-Rad Laboratories, Hercules, CA, USA).
2. 10% SDS.
3. Wash buffer: 200 m*M* Na_2HPO_4. Do not adjust pH.
4. HA sample buffer: 10 m*M* Tris, 10 m*M* DTT, and 2% SDS. Store at 4°C.
5. HA loading buffer: 10 m*M* NaH_2PO_4 pH 6.8, and 0.1 m*M* $CaCl_2$.
6. Mobile phase A: 1 m*M* DTT, and 0.1 m*M* $CaCl_2$. Store at 4°C.
7. Mobile phase B: 1 *M* NaH_2PO_4 pH 6.8, and 1 m*M* DTT. Store at 4°C.
8. Poly-Prep chromatography columns (Bio-Rad).

2.3.5 Chemical Extraction

1. Solution P: *see* **Section 2.1.7**.
2. Salt and detergent extraction buffer: 400 m*M* NaCl and 1% (w/v) β-octylglucoside in 25 m*M* Hepes, pH 7.5.
3. Base extraction buffer: 100 m*M* NaOH.
4. Heparin extraction buffer: 10 mg/mL heparin in BT/Mg buffer.
5. 1 *M* sucrose BT/Mg.

3 Methods

Either the blood stream form (BSF) or procyclic life stage may be used with the following protocol, with similar yields. Procyclic cells are generally easier to culture because they do not require infection of animals to achieve the requisite number of cells for the isolation. At least 4×10^{10} cells are needed, which allows for two separate gradients with 2×10^{10} cells in each. One must be careful not to exceed 2×10^{10} cells in each gradient to maximize efficiency. Unless otherwise noted, cells and lysates must be kept on ice and pelleted in a refrigerated centrifuge at 4°C. The entire protocol is represented as a flow diagram in Fig. 6.1. If desired, the enrichment of the nucleus and subnuclear components may be monitored by Western blotting and thin section electron microscopy (EM) ***(12)***.

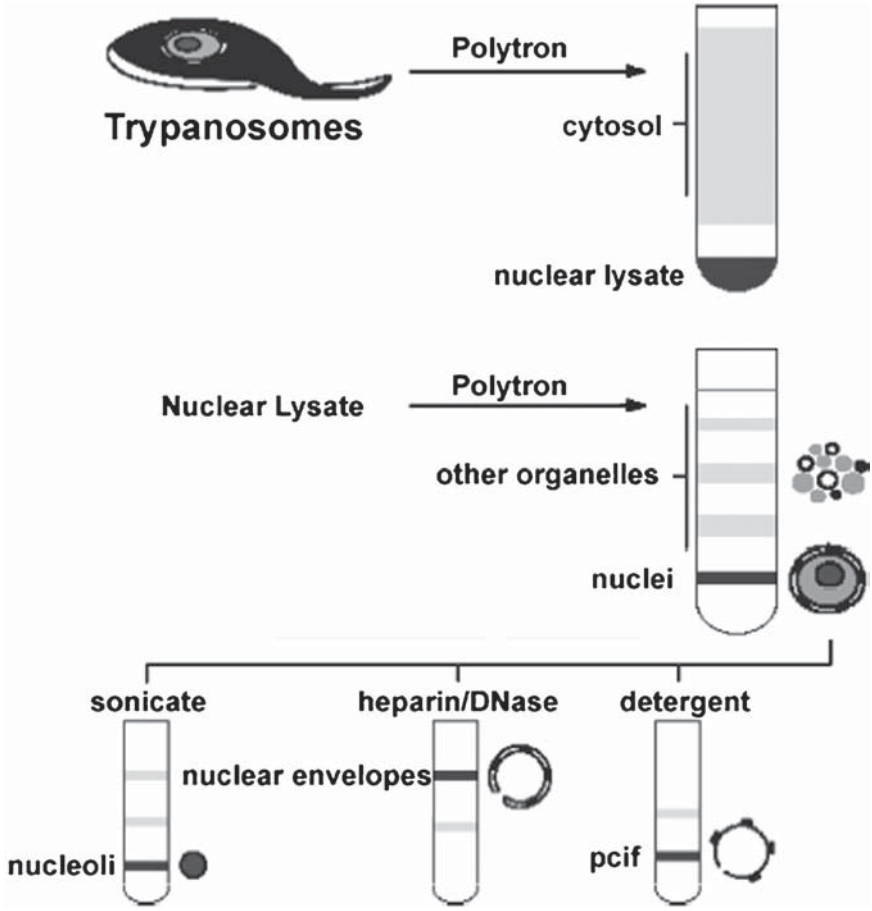

Fig. 6.1 Generalized flow diagram of nuclear isolation protocol. Regions containing isolated nuclear and subnuclear components are shaded *dark gray*

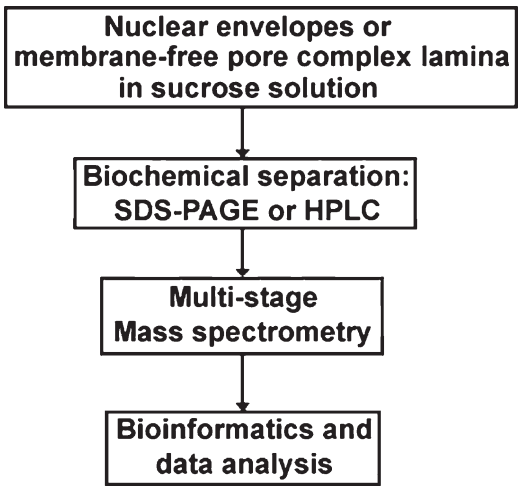

Fig. 6.2 Flow chart for post-isolation biochemistry, proteomics, and analysis

Subcellular proteomics is a robust tool to identify proteins that co-enrich with a particular organelle ***(13)***. In organisms such as *T. brucei*, whose evolutionary distance challenges functional genomics, localization information provides compelling additional functional evidence ***(14)***. Although subcellular proteomics significantly surmounts the many difficulties observed with whole cell proteomics, analysis of subcellular structures remains hindered by complexity (several hundred distinct proteins) and their dynamic range (several orders of magnitude). To overcome these challenges, we have employed a multipronged approach using separation and chemical extraction techniques and multistage mass spectrometry to identify more than 300 proteins that co-enrich with the nuclear envelope (Fig. 6.2). There is an array of bioinformatic algorithms that can be employed to predict which proteins colocalize to the nuclear envelope in vivo; however, a description of their use is beyond the scope of this chapter.

3.1 Nuclear Isolation

1. Gently pellet the cells at 1,700×*g* for 10 min. Discard the supernatant and resuspend the pellet with roughly 25 mL of prechilled PBS. After a second centrifugation, resuspend the pellet in 25 mL of prechilled PBS and transfer the cells to a Sorvall HB-4 tube. Pellet by centrifugation once again (1,800×g, 15 min, Sorvall HB-4 rotor) and discard the supernatant. (*See* **Note 6**).

2. To lyse the cells, add 1 volume per 2×10^{10} cells of lysis buffer to the pellet and immediately disrupt the cells with a Polytron homogenizer (PTA-10, GlenMills, Clifton, NJ, USA) with 1 min bursts. Because the appropriate speed setting may vary between homogenizer models, start with setting #4 and increase in increments of 0.5 until cell lysis is achieved. The lysis should be conducted in a cold room to keep the homogenizer probe and cellular material cooled. Five minutes total homogenization time at the final setting is usually sufficient for an acceptable 70–90% cell lysis, with progress being monitored by phase contrast microscopy. (*See* **Note 4**).
3. Once acceptable cell lysis has been achieved, underlay the equivalent of 2×10^{10} cells with 1 volume of underlay buffer and centrifuge for 20 min at 10,500×g in a Sorvall HB-4 rotor. Decant the supernatant (which contains the crude cytosol) and store at −80°C.
4. The pellet should then be immediately resuspended by homogenization. Add 1 volume of resuspension buffer and homogenize with the Polytron (setting 4.5–5) in 1 min bursts. Monitor the progress with phase contrast light microscopy; all cells should now be lysed (a significant proportion of the total cell lysis can actually occur at this stage), and the nuclei will be visible in the field as many small gray spheres and ovoids. Usually, 4 min is sufficient to achieve full dispersion.
5. Prepare the gradient. Into a Beckman SW-28 centrifuge tube, add the following: 8 mL of 2.30 *M* sucrose/PVP, 8 mL of 2.10 *M* sucrose/PVP, and 8 mL of 2.01 *M* sucrose/PVP (*see* **Note 7**).
6. Carefully add the crude nuclear material on top of the gradient (the portion of the gradient that contains the crude material is designated as "S"). Afterwards, fill to within 5 mm of the brim with PVP solution to prevent collapse. In a Beckman ultracentrifuge and SW-28 rotor, spin the gradient at 141,000×g for 3 h.
7. Subcellular material may be found at the interfaces. Each interface (PVP/S, S/2.01, 2.01/2.1, and 2.1/2.3) should be collected and stored at −80°C for possible future study. Most of the nuclei settle at the 2.10/2.30 interface. The quality of the nuclei can be checked by phase contrast light microscopy. (*See* **Note 8**).
8. The concentration of the nuclei is measured by optical density: 1 OD_{260} is equivalent to about 10^8 nuclei. (*See* **Note 9**).

3.2 Subnuclear Fractionation

3.2.1 Nuclear Envelope

1. To a measured volume of 300 ODs of purified nuclei, add the equivalent of 0.2 volumes of PVP solution and vortex for 1–2 min until the solution is homogenous.

2. In a Beckman Ty50.2Ti rotor, pellet the nuclei by centrifugation at 193,000×g for 1 h. Decant the supernatant.
3. Resuspend the pellet in 3 mL (per 100 OD of nuclei) of shearing buffer and shear the nuclear envelopes by vigorous vortexing for 1 full minute after the last traces of the pellet disappear.
4. After shearing, let the tube stand for 5 min at room temperature.
5. Add 10 mL of 2.10 *M* sucrose in 20% Accudenz in BT/Mg buffer and mix well by vortexing.
6. Transfer the mixture to an SW-28 centrifuge tube and overlay with 12 mL of 2.25 *M* sucrose in BT/Mg and 10 mL of 1.50 *M* sucrose in BT/Mg. Top with BT/Mg to within 5 mm of the brim.
7. Spin the gradient at 141,000×g for 4 h.
8. Collect all interfaces. The nuclear envelopes float up to the 1.50 *M*/2.25 *M* interface. Their quality can be checked by microscopy; they appear as faint "C" structures by phase contrast light microscopy.

3.2.2 Nuclear Pore Complex–Lamina

1. To 1 volume of nuclear envelopes add the equivalent of 2 volumes of extraction buffer, and vortex for 5 min at room temperature. Allow the mixture to then incubate at room temperature for 25 min.
2. Prepare the gradient. In a Beckman SW-55 centrifuge tube, add 1 mL of 2.50 *M* sucrose in BT/Mg and then 1 mL of 1.75 *M* sucrose in BT/Mg.
3. Carefully overlay the extracted nuclear envelope mixture on the top of the gradient. Spin the gradient at 240,000×g for 30 min in a SW55Ti rotor.
4. Collect each interface fraction. The pore complex–lamina settles at the 1.75 *M*/2.50 *M* interface.

3.2.3 Isolation of the Nucleolus

1. To one volume of 50 OD_{260} of purified nuclei, add 0.2 volumes of PVP solution and vortex for 1–2 min until the solution is homogenous.
2. The nuclei are then pelleted in a type 80 rotor at 170,000×g for 1 h. Decant the supernatant.
3. 1 mL of disruption buffer is added to the pellet and the nuclei are disrupted by sonication with a microprobe in 6 sec bursts in the cold room. Progress between bursts is monitored by phase contrast microscopy (1,000×). Generally, six bursts is sufficient to achieve >99% disruption, releasing the small dark gray nucleoli.
4. The disrupted nuclei are then thoroughly mixed 1:1 (v/v) with 1.75 *M* sucrose in BT/Mg.
5. The mixture is then layered on top of the following gradient in a Beckman SW-55 centrifuge tube: 1 mL 2.50 *M* sucrose in BT/Mg buffer, 1.5 mL of 2.25 *M* sucrose in BT/Mg buffer, and 1.5 mL of 1.75 *M* sucrose in BT/Mg buffer.

6. The gradient is then centrifuged at 240,000×*g* for 2 h in a SW55Ti rotor. Collect all interface fractions. Nucleoli collect at the 2.00 *M*/2.50 *M* interface.

3.3 Biochemistry and Mass Spectrometry

Sections 3.3.2 and **3.3.3** are general methodologies and may be used in conjunction with either hydroxyapatite chromatography or chemical extraction.

3.3.1 Protein Precipitation

1. Prior to biochemistry and mass spectrometry, the proteins need to be recovered from the sucrose solutions. To one volume of nuclear or subnuclear material, add 5 volumes of HPLC-grade methanol and incubate for 4 h at 4°C. (*See* **Note 10**).
2. Spin at 3,300×*g* in a Beckman GH-3.8 for 15 min at 4°C.
3. Remove and discard the supernatant. Resuspend the pellet with 500 µL of 90% methanol and transfer to a microcentrifuge tube, if necessary, then incubate for 1 h at 4°C.
4. Spin the suspension one final time in a microcentrifuge (16,000×*g*, 15 min, 4°C). Discard the supernatant.

3.3.2 SDS-PAGE-MS

1. Resuspend the pellet in 20 µL of LDS sample buffer, 8 µL of sample reducing agent, and 52 µL of water. After mixing, heat to 70°C for 10 min and allow to cool to room temperature.
2. To alkylate the reduced cysteines, add iodoacetamide to a final concentration of 100 m*M* and allow the reaction to proceed, in the dark, for 30 min.
3. Prepare gel, MOPS running buffer, and gel assembly following the manufacturer's instructions. By using several different gradients, one can increase resolution in specific mass ranges. For example, a Novex 8% Tris-glycine gel offers high mass resolution whereas NuPAGE 10% bis-Tris gels offer superior low mass resolution.
4. Load 20 µL of alkylated sample onto each gel and run at a constant 125 V for 5 min followed by a constant 200 V for 45 min.
5. Fix the gel in 50% methanol and 7% acetic acid for 15 min and wash extensively. Stain with GelCode Blue stain and document by photography or digital flatbed scanning.
6. On a white shallow plate or glass pane, use a MicroScalpel to excise 2 mm-wide bands running down the entire gel lane; roughly 35 bands can be excised from a 10 cm gel. Using the fine point tweezers or MicroScapel, dice the excised gel bands into 1 mm cubes. Transfer the cubes to a microcentrifuge tube. (*See* **Note 11**).

7. Completely destain the gel pieces to remove all traces of stain and detergent. To the gel pieces, add 500 µL of destain solution and agitate (medium setting) at 4°C with a vertical vortexer (Tomy Mixer; Tomy Seiko Co., Tokyo, Japan). Replace the solution every 30 min for up to 4 h.
8. Add 100 µL acetonitrile. The gel pieces will dehydrate and turn white. Aspirate the acetonitrile after 10 min and leave the tubes open for several minutes to allow the last traces to evaporate.
9. Resuspend a trypsin aliquot in digestion buffer to a final concentration of 50 ng/µL. Add ≥100 ng trypsin to dehydrated gel pieces. Allow the pieces to swell and become translucent (~10 min) and then add 40 µL of digestion buffer. Incubate at 37°C for 4 h.
10. To 1 volume of Poros R2 bead slurry, add 9 volumes of Poros dilution buffer. Add 40 µL of these diluted Poros beads to the gel pieces. In a vertical vortexer, agitate (medium setting) at 4°C for 4 h. The beads will extract the peptides from the gel pieces and digestion buffer.
11. Transfer the 80 µL peptide/bead mixture into a washed and conditioned ZipTip from the top and, using a syringe, discard the supernatant (*See* **Note 12**). Add 20 µL of 0.1% trifluoroacetic acid to the gel pieces. Transfer the wash solution to the ZipTip from the top and expel wash the solution using a syringe.
12. Wash the Poros Beads twice more using 20 µL of 0.1% trifluoroacetic acid.
13. Slowly elute peptides onto a matrix-assisted laser desorption ionization (MALDI) plate with 2.5 µL 40% DHB in elution solution. When the spot is completely dry (less than 30 min), analyze by mass spectrometry ***(15, 16)***.

3.3.3 HPLC-MS

1. Resuspend the protein sample pellet in 50 µL of 50 m*M* ammonium bicarbonate. Sonication may be required to fully dissolve the pellet.
2. Add 250 ng of trypsin. Incubate at 37°C for 12 h.
3. Add another 250 ng of trypsin. Incubate at 37°C for 12 h.
4. Quench the reaction by the addition of 0.1% trifluoroacetic acid (final concentration).
5. Load an appropriate volume of the peptide solution onto a C-18 reversed phase column and elute under the following conditions: 25% B (95% acetonitrile, 0.1% trifluoroacetic acid) for 5 min, 25–100% B in 40 min. (*See* **Note 13**)
6. Analyze the eluate by online electrospray ionization–MS/MS (Finnigan LCQ series, ThermoElectron Corp., San Jose, CA, USA).

3.3.4 Hydroxyapatite Chromatography

1. In a 50-mL centrifuge tube, wash 7.5 mL of hydroxyapatite (HA) with 20 mL of wash buffer. Allow the hydroxyapatite to settle and aspirate the wash solution and suspended fine particles. Continue to wash 3 times with 20 mL loading

buffer, aspirating the loading buffer and suspended fine particles after each wash. To the final volume of hydroxyapatite, add 4 volumes of loading buffer supplemented with 0.1% SDS.
2. After methanol precipitation (*see* **Section 3.3.1**), resuspend the pellet in HA sample buffer and heat at 60°C for 10 min. Store unused sample solution at −20°C.
3. Dilute 1 volume of sample with 19 volumes of HA loading buffer.
4. Add conditioned HA beads to diluted sample. Roughly 2 mL of bead slurry is required for less than 0.5 mL of sample. Incubate mixture for 30 min, keeping the hydroxyapatite suspended by mechanical rotation or rocking.
5. Pour the mixture into a Poly-Prep chromatography column and collect the flow through. Wash the beads with 4 mL of 0.1% SDS in loading buffer. Collect the wash to monitor protein binding.
6. Elute proteins from the hydroxyapatite. All elution buffers are prepared from appropriate volumes of mobile phase A and mobile phase B, and SDS is added just before use at a final concentration of 0.1%. Add in successive order to the column: 300, 325, 350, 375, 400, and 500 m*M* NaH_2PO_4. Four milliliters of elution buffer is sufficient. (*See* **Note 14**).
7. Adjust the final volume of eluate to 10 mL and precipitate with sodium deoxycholic acid/trichloroacetic acid. (*See* **Note 15**).
8. The proteins may be analyzed by SDS-PAGE-MS or HPLC-MS, as described. An example of hydroxyapatite chromatography coupled to SDS-PAGE is shown in Fig. 6.3.

3.3.5 Chemical Extraction

Perform all extractions in Beckman TLA-55 centrifuge tubes.

1. Salt and detergent extraction:

   To one volume of nuclear envelopes (in sucrose solution) add 9 volumes of salt and detergent extraction buffer with solution P (1:100) and mix completely by vortexing.

2. Base extraction:

   To one volume of nuclear envelopes (in sucrose solution) add 9 volumes of base extraction buffer with solution P (1:100) and mix completely by vortexing.

3. Heparin extraction:

   To one volume of nuclear envelopes (in sucrose solution) add 9 volumes of heparin extraction buffer with solution P (1:100) and mix completely by vortexing.

4. Incubate the extractions on ice for 1 h.

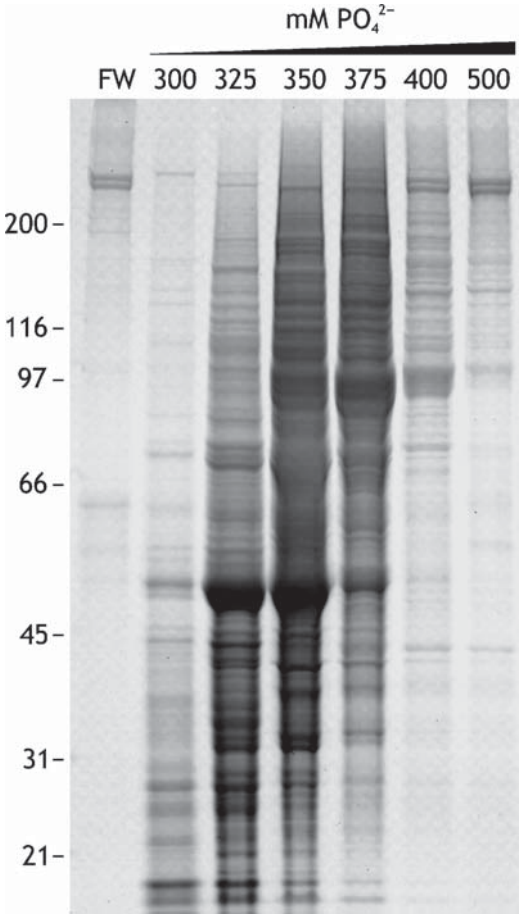

Fig. 6.3 Separation by SDS-PAGE of proteins in the nuclear envelope fraction. Each lane represents a protein fraction eluted from the hydroxyapatite column; the concentration of the elution buffer is indicated above each lane. Molecular weight markers are indicated to the left of the gel. *FW*, combined flow through and wash

5. Underlay the mixtures with 1 *M* sucrose in BT/Mg buffer with solution P (1:100).
6. Pellet by centrifugation at 103,000×*g* for 35 min.
7. Extracted proteins are retained in the supernatant. Carefully transfer the supernatant to a fresh tube and precipitate the proteins (*See* **Note 15**).
8. Wash the non-extracted pellet in 1 mL acetone and transfer the suspension to a microcentrifuge tube, if necessary, and incubate overnight at −20°C. Recover the pellet by centrifugation (16,000×g, 4°C, 1 h).
9. Both the extracted proteins and the pellet can be analyzed by either SDS-PAGE-MS or HPLC-MS, as above.

4 Notes

1. To minimize the risk of contamination when preparing reagents, it is imperative to use the highest reagent quality available. With the exception of polymeric, high density, acidic or basic solutions, aqueous buffers should be filtered with a 0.22-μm-pore syringe or bottle filter. These precautions are especially important when this protocol is coupled to mass spectrometry in order to avoid exogenous protein and dust contamination. All aqueous solutions are made with high-quality filtered water with a measured resistivity of 18.2 MΩ-cm.
2. In a large dish of hot water atop a stirring hot plate, the appropriate amount of sucrose is dissolved into PVP solution in a 500 mL beaker by constant stirring. Once the sucrose has completely dissolved, remove from heat, cover, and allow to cool to room temperature. While continuously stirring the solution, adjust the RI to within 0.0003 by slowly adding PVP solution.
3. For best results, slowly dissolve each peptide inhibitor sequentially into room temperature ethanol.
4. Protease inhibitors become unstable at room temperature at working concentrations. On the bench, solution P must be kept on ice whereas the PIC may be left thawed. We recommend the addition of the protease inhibitors to the working solutions at the last possible moment.
5. A working Poros R2 bead slurry is made as follows:
   (a) 500 mg of Poros R2 beads are washed sequentially with 10 mL of 1) methanol, 2) 80% acetonitrile, and then 3) 20% ethanol.
   (b) Resuspend the washed beads in 20% ethanol at a final concentration of 50 mg/mL.
6. The rotors and centrifuge tube described herein may be replaced by others of similar capacities and rotational velocity. Conversion formulas are widely available.
7. To reduce the viscosity of the sucrose solutions, allow the solutions to completely warm to room temperature. Wide-bore pipets and pipette tips (made by cutting off ~3 mm from the point of the tip) also facilitate handling sucrose solutions. Before use, all sucrose solutions should be supplemented with solution P (1:100) and PIC (1:200).
8. Unloading the gradient will be easier if the interfaces are marked with a permanent marker before centrifugation. Collect the topmost fill layer and halfway through the first sucrose layer. Then, starting from the top, collect from halfway through the upper sucrose layer, through the interface, and continue to collect until halfway through the lower sucrose layer. The material at the interface may be gently dislodged with a pipette tip, if necessary.
9. Add 10 μL of nuclei to 1 mL of 1% SDS. Measure the absorbance at 260 nm against a blank of 1% SDS. Multiply the value by 100 to obtain the OD.
10. It is recommended to precipitate a 1 mL aliquot of the subnuclear fraction and determine the protein concentration using a suitable assay such as Bradford or bicinchoninic acid.

11. To avoid contamination from dust, clean all tools and surfaces with Windex cleaner. As an alternative to manual slicing, one can use a Mickle gel slicer (Brinkman Instruments, Westbury, NY, USA) which can mechanically slice the gel lane at 1 mm intervals.
12. After conditioning, the ZipTips are always loaded from the top. By doing so, the Poros beads settle behind the ZipTip resin, which acts as a frit. The supernatant can be easily expelled from the tip by air using a 1mL syringe fitted with a cut P200 pipette tip such that the ZipTip can form a seal around the pipette tip adaptor. When washing or eluting from the beads, it is imperative that solutions are loaded from the top.
13. For our work, we used an Ultimate HPLC system (LC Packings-Dionex, Sunnyvale, CA, USA) with a C18 column (0.18×250 mm, 1.8 µL/min).
14. The elution can be hastened by use of air pressure, although this is not necessary. After a fraction has been collected, allow the fine particles to settle and pour the eluate to a fresh tube to avoid contamination from proteins still bound to the fine particulates. It is essential to add SDS just before use, otherwise it may begin to precipitate.
15. When the proteins are present at relatively dilute concentrations, we recommend precipitation by sodium deoxycholic acid/trichloroacetic acid. Dilute the protein suspension with water to 1 mL (small scale) or 10 mL (large scale). Add 100 µL or 1 mL, respectively, of 0.3% sodium deoxycholic acid, mix well, and add an equivalent volume of 72% trichloroacetic acid. Incubate at 4°C for 1 h and then spin at maximum rotor speed (1 h, 4°C). Wash the pellet in 1 mL of acetone and transfer to a microcentrifuge tube, if necessary, and incubate overnight at −20°C. Recover the pellet by centrifugation in a microcentrifuge (16,000×*g*, 4°C, 1 h).

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