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Use of Sequential Chemical Extractions to Purify Nuclear Membrane Proteins for Proteomics Identification

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Abstract

The nuclear envelope (NE) is a double membrane system that is both a part of the endoplasmic reticulum and part of the nucleus. As its constituent proteins tend to be highly complexed with nuclear and cytoplasmic components, it is notoriously difficult to purify. Two methods can reduce this difficulty for the identification of nuclear membrane proteins: comparison to contaminating membranes and chemical extractions to enrich for certain groups of proteins. The purification of nuclear envelopes and contaminating microsomal membranes is described here along with procedures for chemical extraction using salt and detergent, chaotropes, or alkaline solutions. Each extraction method enriches for different combinations of nuclear envelope proteins. Finally, we describe the analysis of these fractions with MudPIT, a proteomics methodology that avoids gel extraction of bands to facilitate identification of minor proteins and membrane proteins that do not resolve well on gels. Together these three approaches can significantly increase the output of proteomics studies aimed at identifying the protein complement of subcellular membrane systems.

1. Introduction

At the core of the nuclear envelope (NE) is a double membrane system. One half of this system, the inner nuclear membrane (INM) faces the nucleus and contains many integral membrane proteins ***(1-3)***. Most of these appear to be type II integral proteins and several have been shown to bind chromatin ***(4, 5)*** and/ or the intermediate filament lamin polymer that lines the INM ***(6)***. The lamin polymer can also interact directly with the membrane via a farnesyl group at the C-terminus of B-type lamins ***(7)*** and a GNAERG group at the N-terminus of lamin C2 ***(8)***. The other half of this membrane system, the outer nuclear membrane (ONM), faces the cytoplasm and, studded with ribosomes, is clearly a subcompartment of the endoplasmic reticulum (ER) ***(9, 10)***. It contains some unique proteins that appear to connect the NE to cytoplasmic filament systems ***(11)***. These two lipid bilayers connect at the pore membrane (PoM) where they flow around the nuclear pore complexes (NPC), large macromolecular assemblies that regulate the directional trafficking of soluble molecules between the nucleus and cytoplasm ***(12, 13)***. Recent work suggests that the NPCs actively regulate trafficking of transmembrane proteins as well ***(14)***. Between the ONM and INM is the lumen of the NE, which may also contain unique proteins or functions: for example most of the mass of the integral NPC protein gp210 resides in the lumen ***(15)***. Together the ONM and associated transmembrane proteins, PoM and associated transmembrane proteins, lumen and associated proteins, INM and associated transmembrane proteins, and the connected lamin polymer constitute the NE.

Because of its inherent inner complexity and outer connectivity the NE cannot be purified to homogeneity, complicating efforts to identify the full complement of

NE proteins. We developed a subtractive proteomics approach specifically to identify membrane proteins unique to the NE. As there are no expected contaminating membrane structures within the nucleus, the major expected contaminant would be ER membranes: indeed single membrane vesicles likely to have been derived from vesiculated fractionated ER are observed in NE preparations by electron microscopy ***(16)***. Therefore, proteins appearing in both NEs and a separately analyzed ER fraction are discarded from the dataset ***(16)***. In contrast, contamination from soluble proteins could come from the ER, cytoplasmic filaments, or the nucleoplasm; thus it is not possible to choose a single contaminating fraction for subtraction. The ER-rich fraction was obtained by using standard protocols for purification of microsomal membranes that are made completely free of nuclear membrane because the large intact nuclei are readily pelleted before membranes are floated on sucrose cushions ***(17, 18)***. NEs are prepared by first isolating nuclei ***(19)***, then douncing to vesiculate other membranes that are removed by floating on sucrose cushions, and finally digesting chromatin to remove nucleoplasmic contents ***(20, 21)***.

To enrich for transmembrane proteins, NE and microsome fractions are extracted with either alkaline solutions ***(16)*** or chaotropes ***(22)***, which should solubilize cytoskeletal and chromatin components/ contaminants while leaving transmembrane proteins embedded in the insoluble membrane. To enrich for proteins associated with the intermediate filament lamin polymer NE fractions were extracted with salt and detergent, as the lamina (lamins and interacting transmembrane proteins) is defined biochemically by its general insolubility at NaCl and Triton X-100 concentrations of up to 1 M and 2% respectively. Detergents solubilize membrane proteins by mimicking the lipid-bilayer environment. At low concentrations they integrate into the lipid bilayer, but as the bilayer becomes saturated the membrane

disassembles to form mixed micelles. There is no general rule for which type of detergent is optimal to extract a particular membrane protein or type of membrane protein; thus one detergent may extract a subset of both contaminants and NE proteins while another may extract a different subset of both. Indeed, extraction characteristics differ even among lamin subtypes ***(23)***. The variability in what each chemical extracts is highlighted by the minimal overlap between the proteins identified using alkali extraction and those identified using salt/ detergent extraction, yet proteins in both datasets were strongly predicted to contain membrane spanning segments by computer prediction and concentrated at the NE when tested by transient transfection of tagged versions ***(16)***.

To minimize loss of membrane proteins that often do not resolve well on gels ***(24)***, proteins were identified using MultiDimensional Protein Identification Technology ***(25-27)*** in which the extracted NE fraction is directly digested without prior separation of proteins. The complex peptide mixture is then resolved on combined reverse phase and cation exchange microcapillary columns (LC/LC), eluting directly into ion trap tandem mass (MS/MS) spectrometers. MS/MS datasets are searched for peptide sequence information using SEQUEST against a database combining mammalian protein sequences ***(28)***. DTASelect is used to compile SEQUEST outputs into protein level information, and filter spectrum/ protein matches based on SEQUEST-defined parameters ***(29)***. Multiple runs are compared using CONTRAST ***(29)***. To identify transmembrane proteins within the dataset, additional computational algorithms are employed.

2. Materials*2.1. Preparation of Blood Lymphocytes*

2.1.1 Hardware

1. Buffy coats from local blood bank.

2. Tissue-culture laminar flow hood.

3. Standard supplies (centrifuge tubes, gloves, pipettes, scissors, tissue-culture flasks, etc.).

2.1.2 Solutions

1. Phosphate buffered saline (PBS): 4.3 mM sodium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, 1.4 mM potassium phosphate.

2. Ficoll-Hypaque 1077 density gradient medium.

3. RPMI medium: RPMI 1640 (e.g. Cambrex 09-774), 10% fetal bovine serum, 100 U potassium penicillin, 100 µg/ml streptomycin sulfate, 100 µg/ml gentamicin sulfate.

2.2. Preparation of Nuclear Envelopes

2.2.1. Hardware

1. Loose fitting (Wheaton type B pestle) 15 ml glass Dounce homogenizer with clearance of between ~0.1 and 0.15 mm (*see* **Note 1**).

2. Swinging-bucket rotor (e.g. Beckman Coulter SW28 rotor with Beckman Coulter 344058 Ultra-Clear 25 x 89 mm centrifuge tubes).

3. Local standard light microscope, glass slides, and coverslips.

4. Large bore luer lock stainless steel needles (14 gauge or larger) of greater length than centrifuge tubes and luer lock syringes.

2.2.2. Solutions

Sucrose solution names are defined by the initials for the primary components: S for sucrose, H for HEPES, K for KCl, and M for $MgCl_2$ (*see* **Note 2**).

1. Phosphate buffered saline (PBS): 4.3 mM sodium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, 1.4 mM potassium phosphate.

2. DNase resuspended at 10 U/µl in H_2O.

3. RNase resuspended in H_2O at 10 mg/ml and boiled for 20 min (*see* **Note 3**).

4. Protease inhibitors (*see* **Note 4**): all solutions require freshly added 1 mM AEBSF [4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride] from a 1 M solution in H_2O (*see* **Note 5**), 1 µg/ml aprotinin (from a 1 mg/ml stock in H_2O), 1 µM pepstatin A [from a 1 mM stock in DMSO (dimethyl sulfoxide)], 10 µM leupeptin hemisulfate (from a 10 mM stock in H_2O), and 10 µM 3,4-dichloro isocoumarin (e.g. Sigma D7910, from a 10 mM stock in DMSO). (*See* **Note 6**.)

5. Hypotonic lysis buffer: 10 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, and freshly added 2 mM DTT (dithiothreitol; from a 1 M solution in H_2O) and protease inhibitors.

6. 0.25 M SHKM: 50 mM HEPES pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, and freshly added 1 mM DTT and protease inhibitors.

7. 2.2 M SHKM: 2.2 M Sucrose, 50 mM HEPES pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, and freshly added 1 mM DTT and protease inhibitors (*see* **Note 7**).

8. 30% SHKM: 0.9 M Sucrose, 50 mM HEPES pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, and freshly added 2 mM DTT and protease inhibitors. This can also be prepared by mixing 67 ml 0.25 M SHKM with 33 ml 2.2 M SHKM.

9. 30% SHM buffer: 0.9 M sucrose, 10 mM HEPES pH7.4, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, and freshly added 2 mM DTT and protease inhibitors.

10. 10% SHM buffer: 0.3 M sucrose, 10 mM HEPES pH7.4, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, and freshly added 2 mM DTT and protease inhibitors.

2.3. Preparation of Microsomal Membranes

2.3.1. Hardware

1. The same hardware is required as for *Preparation of Nuclear Envelopes*.

2. A type 45 Ti fixed angle rotor or equivalent that can provide 150,000 x g and matching tubes (e.g. Beckman-Coulter 348176).

2.3.2. Solutions

The same sucrose solutions used for *Preparation of Nuclear Envelopes* can be used in preparing microsomes. In particular the 2.2 M SHKM, 0.25 M SHKM, and a mixture of the two to 1.86 M sucrose will be required.

2.4. Chemical Extractions

2.4.1. Hardware

1. TLA100.3 rotor for table-top ultracentrifuge or equivalent and corresponding tubes (e.g. Beckman-Coulter 343778 polycarbonate 11 x 34 mm tubes).

2.4.2. Solutions

1. Salt wash: 50 mM HEPES pH 8.0, 500 mM KCl, 5 mM $MgCl_2$ with freshly added 2 mM DTT and protease inhibitors.2. Alkaline extraction: 0.1 N NaOH, 1 mM DTT in H_2O.3. Salt/ Detergent extraction(i). Triton X-100 resuspended at 1% in a solution containing 25 mM HEPES pH 7.5, 400 mM NaCl.(ii). Octyl ß-D-glucopyranoside (also called n-Octyl glucoside) resuspended at 1% in a solution containing 25 mM HEPES pH 7.5, 400 mM NaCl.

(iii). Empigen BB resuspended at 0.3% in a solution containing 25 mM HEPES pH 7.5, 400 mM NaCl.

4. Chaotrope extraction: 0.1 M Na_2CO_3, 4 M urea.

2.5. Digestion of Proteins

2.5.1. Hardware

1. Eppendorf Thermo Mixer R and Thermo mixer R block for 1.5 ml tubes.

2. pH Indicator Strips, 7.5 to 14.

2.5.2. Solutions

1. 90% Formic Acid.

2. Cyanogen Bromide at 500 mg/ml in 90% Formic Acid.

3. Ammonium Hydroxide solution, NH_4OH, in water at 0.9 g/ml density.

4. Urea, solid.

5. HPLC grade water.

6. Tris(2-Carboxylethyl)-Phosphine Hydrochloride, TCEP (e.g. Pierce, 20490), as a 1 M stock in HPLC grade water, stored at -20^{o}C.

7. Iodoacetamide, IAM, made fresh weekly as a 500 mM stock in HLPC grade water, and stored at -20 ^{o}C.

8. Hydrochloric acid, HCl

9. Tris base, as a 1 M solution in HPLC grade water, pH adjusted to 8.5 with HCl, stored at 4^{o}C.

10. Endoproteinase LysC, sequencing grade, as a 1 µg/µl stock in in HPLC grade water, stored at -20^{o}C.

11. Calcium chloride as a 500 mM stock in HPLC grade water, stored at room temperature.

12. Trypsin, modified sequencing grade, as a 0.1 µg/µl stock in HPLC grade water, stored at -20^{o}C.

2.6. Microcapillary Column Preparation

2.6.1. Hardware

1. Laser Puller (e.g. Model P-2000 Sutter Instrument Co).

2. Polyimide coated fused silica, 50 μm i.d. x 365 μm o.d. (e.g. Polymicro Technologies, TSP 050375).

3. Polyimide coated fused silica, 100 μm i.d. x 365 μm o.d. (e.g. Polymicro Technologies, TSP 100375).

4. Polyimide coated fused silica, 250 μm i.d. x 365 μm o.d. (e.g. Polymicro Technologies, TSP250350).

5. Column Scribe (e.g. Chromatography Research Supplies, 205312).

6. M-520 Inline Micro Filter Assembly (e.g. UpChurch Scientific, M-520).

7. 0.5 μm PEEK Filter end fitting (e.g. UpChurch Scientific, M-120X).

8. Microtight 395 μm Sleeves (e.g. UpChurch Scientific, F-185X).

9. Pressurization Device (Brechbuehler, Inc., Houston, TX, or MTA for blueprints available by request from John Yates, Scripps Research Institute, La Jolla, CA).

10. Agilent 1100 series G1379A degasser, G1311A quaternary pump, G1329A autosampler, G1330B autosampler thermostat, and G1323B controller (Agilent Technologies, Palo Alto, CA).

2.6.2. Solutions

1. HPLC grade Methanol

2. HPLC grade Acetonitrile

3. 90% Formic Acid

4. HPLC grade water

5. Ammonium Acetate

6. C_{18} Aqua Reversed Phase, 5 μm (e.g. Phenomenex, bulk material, 04A-4299). (*See* **Note 8**.)

7. Partisphere Strong Cation Exchange, 5 µm (e.g. Whatman, WC4621-1507; *see* **Note 9**).

8. Buffer A: 5% Acetonitrile, 0.1% formic acid, in HPLC grade water.

2.7. Multidimensional Chromatography Coupled to Tandem Mass Spectrometry

2.7.1. Hardware

1. LCQ DECA-XP^{plus} tandem mass spectrometer (Thermo Electron, San Jose, CA).

2. Nano electrospray stage (e.g. Thermo Electron Nanospray II ion source or PicoView Source from New Objective).

3. MicroTee Assemblies (e.g. UpChurch Scientific, P-775).

4. Micro Ferrule for 360 µm OD tubing (e.g. UpChurch Scientific, F-152).

5. Gold wire 0.025 in diameter (e.g. Scientific Instrument Services, W352).

2.7.2. Solutions

1. Buffer A: 5% acetonitrile, 0.1% formic acid, in HPLC grade water.

2. Buffer B: 80% acetonitrile, 0.1% formic acid, in HPLC grade water.

3. Buffer C: 500 mM ammonium acetate, 5% acetonitrile, 0.1% formic acid, made with HPLC grade water and filtered.

2.8. Analysis of MS/MS Dataset

Linux Computer cluster (over 100 nodes) dedicated to SEQUEST analysis.

2.9. Determination of Transmembrane Proteins

Many computer algorithms that can be used to predict transmembrane proteins are freely available online and discussed elsewhere in this volume. Those we typically use are listed:

1. TMHMM “http://www.cbs.dtu.dk/services/TMHMM-2.0/”

2. TMPred “www.ch.embnet.org/software/TMPRED_form.html”

3. Methods

The first step in NE enrichment is the isolation of nuclei from other cellular proteins and membranes. Factors critical to this process are the size ratio of nucleus to cytoplasm, the concentration of cytoplasmic filament systems, and nuclear density, which all vary among different tissues. The protocol described here is one we have optimized for purifying NEs from human blood leukocytes (generally 60-80% lymphocytes) (*see* **Note 10**). We generally recover ~65 million NEs from one buffy coat (~100 million leukocytes; *see* **Note 11**).

3.1. Preparation of Blood Lymphocytes

1. Buffy coats are obtained from a blood bank according to local permissions and protocols. If dealing with more than one buffy coat at a time it is important to keep them separate until Sect. 3.2.7, otherwise the mixed lymphocyte activation response can rapidly alter expression profiles and cells may aggregate, forming rosettes.

2. Open six 50 ml conical tubes (e.g. Falcon™ Conical Centrifuge Tubes, 50 ml capacity) in a laminar flow cabinet/ tissue culture hood (*see* **Note 12**).

3. Wipe scissors and blood bag with 70% EtOH. Cut one of the strands of tubing coming from the bag while holding upright. Tilt end of tube over first 50 ml conical tube and pour 12.5 ml into each tube using tilt angle to regulate gravity flow.

4. Dilute blood with 4 volumes PBS (50 ml total per tube that has a full 12.5 ml of blood). Calculate the total volume and divide by 15: this gives the number of tubes required for step 5.

5. Pour 15 ml of Ficoll-Hypaque 1077 media into fresh tubes.

6. Gently overlay with 15 ml of diluted blood (*see* **Note 13**).

7. Centrifuge at 400 x g for 30 min at RT.

8. Three layers will be obtained after centrifugation: the middle layer containing principally lymphocytes should appear cloudy white (*see* **Note 14**). Carefully dispense of the upper layer with a pipette. Collect all the intermediate phase (on average 4-6 ml) into a new tube, taking care to disrupt any clumps.

9. Dilute the material with 4 volumes of PBS and centrifuge at 250 x g for 10 min at RT (*see* **Note 15**).

10. Resuspend each pellet in 25 ml PBS, combine (two tubes into one), and centrifuge at 250 x g for 10 min at RT (*see* **Note 16**).

11. Resuspend each pellet in RPMI medium and transfer into 75 cm^2 tissue culture flasks (*see* **Note 17**).

12. One buffy coat will generally yield ~120 million leukocytes and can be seeded at between one and ten million cells per ml.

3.2. Preparation of Nuclear Envelopes

1. Count cell number and pellet (in 50 ml tubes) at 250 x g for 10 min at room temperature (*see* **Note 18**).

2. Carefully and completely decant supernatant and resuspend the pellet from each blood pack from step 1 in 0.5 ml ice cold PBS by gentle agitation (*see* **Note 19**).

Accumulate so each tube has ~25 million cells and add additional PBS to 30 ml (*see* **Note 20**).

3. Withdraw a 10 µl sample for microscopic analysis, then pellet the rest at 250 x g for 10 min at 4°C.

4. Pour off buffer carefully by inversion, carefully re-right the tube, and let PBS settle. Remove the remaining PBS with a pipette tip. Place pellets on ice and resuspend the first pellet in 7 ml ice cold hypotonic lysis buffer with freshly added DTT and protease inhibitors (*see* **Note 21**). Let incubate on ice for 10 min, following an aliquot under the microscope throughout (*see* **Note 22**). Good swelling is depicted in Figure 1: compare panel A (before swelling) with panel B (swelling). When such a state is reached, the Dounce step (6) should be performed.

5. Five minutes after resuspension of the first pellet, similarly resuspend the second pellet (*see* **Note 23**).

6. When the 10 min incubation is complete, cells should be moved to the 15 ml loose Dounce homogenizer and lysed by 10 vigorous strokes. Immediately add 1/10 volume of 2.2 M SHKM and 1/10 volume of 1 M KCl (*see* **Note 24**).

7. Remove to centrifuge tubes and underlay with a cushion of 1/10th volume 30% SHKM using a 14 gauge needle and syringe. Pellet nuclei at 2,000 x g in a swinging bucket rotor (e.g. 4,000 rpm in a Beckman Coulter J6-MC floor model centrifuge) for 20 min at 4°C.

8. Decant the supernatant and resuspend pellets in 11 ml 0.25 M SHKM. If they appear at all aggregated, give a few sharp strokes in the Dounce homogenizer with the loose pestle to uniformly resuspend them. Add 2.2 M SHKM to a final concentration of 1.9 M sucrose (39 ml). (*See* **Note 25**).

9. Dispense 25 ml into each SW28 ultracentrifuge tube and underlay with 5 ml of 2.3 M SHKM using a 14 gauge needle in a luer lock syringe (Figure 2).

10. Balance the tubes by exchange between the upper nuclei-enriched lysate phase and spin in the SW28 rotor for 2 h at 82,000 x g (25,000 rpm).

11. Move to a cold room and remove any floating white material with a spatula, then pour off the rest of the supernatant by rapid inversion. Keep the tubes upside down in the cold for 10 min to drain them. Then gently wipe out the inside walls of tubes with a folded kimwipe (or equivalent towel), being very careful not to touch the pellet (*see* **Note 26**).

12. Resuspend each pellet in a small volume (~2 ml) of 0.25 M SHKM with freshly added 2 mM DTT and protease inhibitors, being careful to avoid touching the walls.

13. Dounce to break the aggregates of nuclei and transfer into an ice-cooled 15 ml conical tube. Wash the homogenizer with 1 ml 0.25 M SHKM and add to the resuspended nuclei. Take a small aliquot for counting nuclei (step 14) and another to place on a slide for step 16.

14. Count nuclei using a hemocytometer and calculate the total number from the volume currently spinning in the centrifuge. The nuclei should be clean of contaminating cell fragments and appear as in Figure 3A.

15. Resuspend in 10% SHM with freshly added 2 mM DTT and protease inhibitors at 1.5 million nuclei/ml. Add 4 U/ml DNase and 1 µg/ml RNase and incubate at room temperature for 15 min. Transfer into ice cooled tubes and centrifuge at 6,000 x g (~5,000 rpm) in a floor model intermediate speed centrifuge with a swinging bucket rotor (e.g. Beckman-Coulter J6-MC) for 10 min at 4°C.

16. Resuspend the pellet in 10% SHM with freshly added 2 mM DTT and protease inhibitors at 3 million nuclei/ml. Dounce if necessary, transfer into a 15 ml conical

tube. Add 8 U/ml DNase and 2 µg/ml RNase and incubate at room temperature for 15 min. Observe digestion on the microscope in parallel. The phase grey of the nuclei should diminish (Figure 3, compare A and B).

17. When 90% of nuclei are no longer phase-grey, underlay the solution with 30% SHM with freshly added DTT and protease inhibitors. Spin for 30 min at 6,000 x g using a swinging bucket rotor (e.g. 5,000 rpm in a Beckman-Coulter floor model J6-MC centrifuge; *see* **Note 27**).

18. Carefully aspirate off the supernatant (do not decant by pouring) as the pellet will be very soft (*see* **Note 28**).

19. Resuspend the pellet in the same volume of 10% SHM. Withdraw a 10 µl sample and count NEs using a hemocytometer to calculate the yield of the prep: percentage yield is calculated as (final number of NEs/total number initial cells) x 100.

20. Although there is no time during the digestion to stain nuclei with DAPI or Hoechst dyes to visualize more directly the extent of chromatin digestion, if this is desired samples can be taken at this point and fixed for later staining. The loss of chromatin observed by phase contrast light microscopy (Figure 3A and B) can be more clearly observed by DNA staining (Figure 3C and D).

21. Aliquot the NEs to centrifuge tubes (chosen for desired storage method and concentration) and spin at 6,000 x g (5,000 rpm) for 10 min (no cushion) at 4°C (*see* **Note 29**).

22. Carefully aspirate the supernatants and immediately flash-freeze in liquid nitrogen and store at -80°C.

3.3. Microsomal Membrane Purification

Because lymphocytes have very little cytoplasm to produce microsomes, we instead used the HL-60 human cell line that can be induced into different blood cell lineages. Treatment with phorbol esters induces differentiation into megakaryocytes with associated attachment and spreading of the cells. We predicted that this would produce microsomes in sufficient quantity while being largely similar in protein composition to the lymphocyte ER.

1. Seed 1 x 10^7 HL-60 cells per 10 cm tissue culture dish.

2. Add PMA (Phorbol 12-myristate acetate; Calbiochem 524400) from a 5 mg/ml stock in acetone to a final concentration of 1 µg/ml.

3. When the cells have adhered and spread (2-3 days), collect by scraping with a teflon cell scraper, transfer into a centrifuge tube, and pellet at 250 x g for 10 min.

4. Wash once in PBS and collect again by centrifugation.

5. Follow steps 4-7 in *Preparation of Nuclear Envelopes (section 3.2).*

6. Recover the supernatant from *Preparation of Nuclear Envelopes* step 7 and add EDTA to 0.5 mM to inhibit metalloproteinases. Centrifuge at 10,000 x g for 15 min to remove mitochondria (*see* **Note 30**).

7. Mix the post-mitochondrial supernatant with 5 volumes (e.g. 1 ml + 5 ml) of 2.2 M SHKM (with 0.5 mM EDTA, 2 mM DTT, and protease inhibitors) to achieve a final sucrose concentration of roughly 2 M.

8. Float the microsomes by pouring 20 ml of the diluted membranes in each SW28 rotor tube and overlay with 6 ml of 1.86 M SHKM with 0.5 mM EDTA, 2 mM DTT, and protease inhibitors. Finally, overlay this with 2.3 ml of 0.25 M SHKM with 0.5 mM EDTA, 2 mM DTT, and protease inhibitors. Centrifuge at 57,000 x g (21,000 rpm) in type 45 Ti rotor for 4 h (*see* **Note 31**).

9. The microsomes will be in the interface beween the 1.86 M and 0.25 M sucrose layers and should appear largely white and translucent. There will be material in and above the 0.25 M layer, which should be discarded; thus it is best to remove material by tube puncture from the side though a syringe inserted between the layers from the top.

10. Dilute the membranes with 4 volumes of 0.25 M SHKM (with 0.5 mM EDTA, freshly added DTT and protease inhibitors) and pellet at 152,000 x g (44,000 rpm in a type 45 Ti, 48,000 rpm in a type 50 Ti, or 60,000 rpm in a TLA100.3 rotor) for 75 min (*see* **Note 32**). There should be a translucent pellet with the appearance of a fat droplet in the corner of the tube. Decant supernatant and store at -80°C.

3.4. Chemical Extractions

It is wise to check an aliquot of the NE preparation by Coomassie blue stained SDS-PAGE as "Purified" NEs often contain a prominant histone band indicating a large degree of chromatin contamination. If this is the case, they may be salt washed prior to further chemical extraction. Formulas for the chemicals used to extract are depicted in Figure 4.

3.4.1. Salt wash

1. To salt wash, resuspend the NE pellet in the salt wash solution at ~5 million NEs/ml and let stand on ice 15 min with occasional mixing.

2. Pellet by centrifugation at 20,000 x g (~14,000 rpm) in a cooled microcentrifuge for 30 min and decant supernatant.

3.4.2. Alkali (NaOH) extraction

1. Resuspend NEs (no more than 10 million/ml or an equivalent amount of microsomes, *see* **Note 33**) in 0.1 M NaOH on ice.

2. Transfer immediately to TLA100.3 ultracentrifuge tubes and pellet insoluble material at 104,000 x g (50,000 rpm) for 35 min at 4°C (*see* **Note 34**).

3. Wash the pellet quickly with double distilled H_2O (*see* **Note 35**).

4. Either freeze at -80°C or directly process for mass spectrometry.

3.4.3. Extraction with salt and detergent

1. Resuspend NE pellet in any salt/ detergent buffer at no more than 10 million NEs/ ml and incubate on ice for 15 min with occasional mixing.

2. Pellet by centrifugation at 20,000 x g (~14,000 rpm) in a cooled microcentrifuge for 30 min and decant supernatant.

3. Wash the pellet with the same buffer minus detergent, re-pellet, and either freeze at -80°C or directly process to digest for mass spectrometry.

3.4.4. Chaotrope extraction

1. Resuspend the pellet in chaotrope buffer and incubate on ice for 15 min.

2. Pellet the insoluble material in a TLA100.3 table top ultracentrifuge rotor or equivalent at 104,000 x g (50,000 rpm) for 35 min at 4°C.

3. Wash the pellet with double distilled H_2O and either freeze at -80°C or directly process to digest for mass spectrometry.

3.5. Digestion of Proteins

As described in ***(26)***, the membrane pellets were first partially solubilized in formic acid and cyanogen bromide to chemically cleave large portions of proteins, before digesting these larger peptides with endoproteinase Lys-C and trypsin.

1. On day one, resuspend the dried membrane pellets in 100 µl of CNBr at 500 mg/ml in 90% formic acid, mix by pipetting and leave under a fume hood overnight in the dark (for example, in a cardboard freezer box).

2. On day two, transfer 100 µl of partially solubilized membranes to a 15 ml conical tube on ice (*see* **Note 36**), and add NH_4OH drop by drop to neutralize formic acid until there is no more bubbling and the pH is ~8.5 (check after every 100 µl added with 1 µl using pH indicator strips). Once the appropriate pH is reached, the sample can be transferred back to a 1.5 ml tube. The final volume should be around 500 µl, i.e. a 3- to 5-fold dilution.

3. Add solid urea to 8M (taking into account the fact that adding urea will cause the volume to increase to about 800 µl) and then add TCEP to a final concentration of 5 mM. Allow the reduction to proceed at room temperature for 30 min, then add IAM to 20 mM, and let the carboxiamidomethylation of free cysteines proceed for 30 min at room temperature in the dark.

4. Re-check the pH at this step using pH indicator strips and adjust with 1 M Tris-HCl pH 8.5 if necessary before adding endoproteinase Lys-C at 1 µg/µl for an estimated enzyme to protein ratio of 1:100 (w/w). Let the digestion proceed at 37°C, for at least 6 h.

5. Dilute the digestion mix to 2 M urea by adding 0.1 M Tris-HCl, pH 8.5. (*See* **Note 37**.) Add $CaCl_2$ to 2 mM, then trypsin at 0.1 µg/µl for an estimated enzyme to protein ratio of 1:100 (w/w). Let the reaction proceed overnight at 37°C.

6. On day three, quench the reaction by adding formic acid to 5%. Either store the peptide mixtures at -20°C or load directly onto microcapillary columns.

3.6. *Microcapillary Column Preparation*

The large final sample volume (3 x 1150 µl) would take a very long time to load onto traditional 100 µm columns, so we use the "split" column approach (Figure 6), in which the sample is loaded onto larger diameter open-ended columns packed with

reverse phase and strong cation exchange resins, before being connected to a resolving 100 µm column packed with reverse phase (see ***(30)***) for a detailed description of the following steps).

3.6.1. Single-Phase Fused-Silica 100 µm Microcapillary Column

1. Place 40 cm of 100 µm i.d. x 365 µm o.d. fused silica into P-2000 laser puller and pull tip to about a 5 µm opening.

2. Make a slurry of 5 µm C_{18} Reverse Phase (Aqua Phenomenex), at 15 mg/ml in 500 µl methanol.

3. Pack fused silica column with 8-9 cm of 5 µm C_{18} RP using high pressure loading device.

4. Wash with methanol for at least 10 min.

5. Equilibrate in Buffer A for at least 30 min.

3.6.2. Double-Phase Fused-Silica 250 µm Microcapillary Column

1. Connect two 250 µm i.d. x 365 µm o.d. fused silica capillaries (about 30 cm) with a 2 µm filtered union.

2. Make slurries of 5 µm C_{18} Reverse Phase (Aqua Phenomenex) and of 5 µm strong cation exchange material (Partisphere SCX, Whatman), both at 15 mg/ml in 500 µl methanol.

3. Pack first with 3 to 4 cm of Partisphere SCX, followed by 2 to 3 cm of Aqua RP.

4. Wash with methanol for at least 10 min.

5. Equilibrate in buffer A for at least 30 min.

3.6.3. Off-Line Loading and Desalting

1. Spin samples down at 14,000 rpm for 30 min and transfer to a new tube.

2. Load sample to 250 µm column.

3. Wash with Buffer A (1.5 ml).

4. Connect 250 µm i.d. column to equilibrated 100 µm double-phase column.

3.7. Multidimensional Chromatography Coupled to Tandem Mass Spectrometry

3.7.1. Chromatography

1. Install the loaded and washed split-3-phase microcapillary column on the nanoelectrospray stage (Figure 6D), connecting it with the quaternary HPLC pump using a microtee.

2. Cut the overflow 50 µm fused silica capillary to the appropriate length (about 40 cm) to have a flow rate at the tip of the column of about 200-300 nl/min (i.e. back pressure of ~40 bars), while the HPLC flow rate is kept constant at 0.1 ml/min throughout the chromatography.

3. Engage a 12-step chromatography run (24 h) on samples with the gradient parameters described in Table 1. In such sequences of chromatographic events, peptides are sequentially eluted from the SCX resin to the RP resin by increasing salt steps (increase in Buffer C concentration), followed by organic gradients (increase in Buffer B concentration). For the last chromatography step, wash in high salt with 100% Buffer C, followed by the acetonitrile gradient (repeated twice).

4. Apply a 2.4 kV voltage to the eluting peptides via a gold wire connecting the microtee and the mass spectrometer.

3.7.2. Tandem Mass Spectrometry

Chromatographic gradients and MS/MS data acquisition are controlled by the Xcalibur™ data system. For runs performed on LCQ-Deca tandem mass spectrometers, the acquisition scheme is a cycle of one full MS scan (from 400 to 1600 m/z), followed by three MS/MS events at 35% collision energy on the top three most intense ions. Dynamic exclusion is enabled for 5 min, allowing ions of lesser

intensities to be analyzed. This cycle is repeated continuously throughout the chromatography. One RAW file is generated for each chromatographic step and needs to be converted into a dat file using the XCalibur file converter function.

3.8. Analysis of MS/MS Dataset

1. Convert each dat file into a ms2 file ***(31)*** using extract-ms followed by 2to3 ***(32)*** to remove spectra of poor quality and assign a charge state to the precursor ion when possible.

2. Use SEQUEST ***(28)*** to search the MS/MS datasets against a database of sequences downloaded from NCBI (National Center for Biotechnology Information). We combined 27,960 human, 26,180 mouse, 21,205 rat, 21,909 chimp, 5,373 orangutan, 903 gorilla and 2,777 macaque protein sequences on 2005-02-17, and complemented with 172 sequences from usual contaminants (human keratins, IgGs…). (*See* **Note 38**.)

3. Bring together the peptide information contained in the SEQUEST output files and organize these into protein level information using DTASelect ***(29)***. DTASelect is also used to select and sort peptide/spectrum matches passing the criteria defined in Table 2. In particular, the validity of peptide/spectrum matches is assessed using the SEQUEST-defined parameters, cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltCn). Spectra/peptide matches are only retained if they have a DeltCn of at least 0.08 and minimum XCorr of 1.8 for singly-, 2.5 for doubly-, and 3.5 for triply-charged spectra. In addition, the peptides have to be at least 7 amino acids long and the preceding residue in the protein sequence has to be a methionine, an arginine or a lysine (to account for the combined cyanogen bromide/trypsin digestion protocol).

4. Compare protein lists from multiple runs using CONTRAST ***(29)***. Although some argue that at least two peptides should be recovered for higher confidence, we allow single peptide identifications in the final dataset because some well-characterized NETs were also detected by single peptides in previous studies, likely indicating their lower abundance ***(16)***.

5. Additional utilities of DTASelect/CONTRAST are used to create subset databases in Fasta format containing only the proteins in the final list and to generate tab-delimited text files that can be consolidated into relational databases using MSAccess.

3.9. Determination of Transmembrane Proteins

Use Fasta format protein sequence files to search for predicted transmembrane segments using various algorithms. These are detailed elsewhere in this volume (*see* **Note 39**).

4. Notes

1. In section 3.2.2 the number of leukocytes aliquoted for each Dounce step should be correspondingly altered if a smaller or larger Dounce homogenizer is used.

2. $MgCl_2$ concentration in the original procedure was 5 mM throughout; however if NEs are being prepared for viewing by electron microscopy, dropping the concentration through most of the procedure to 0.1 mM will yield better structure. If this is done, the $MgCl_2$ must be increased back to 5 mM, or to 2 mM with 0.5 mM $CaCl_2$ as we use, during DNase and RNase treatment for the enzymes to function properly.

3. This may seem counter-intuitive as the goal of the procedure is to degrade both RNA and DNA, but these enzymes are commonly prepared from bovine pancreas and therefore may have contaminating proteases unless recombinant proteins are used.

4. The optimal protease inhibitors will vary according to the tissue being investigated; so it is important to search the literature to determine what proteases are present at high concentrations in the tissue of choice. The choice for blood covers a wide general range (inhibiting serine, trypsin, cysteine, and aspartic proteases), but also includes coumarin which inhibits granzyme B which is particularly abundant in blood cells.

5. We had traditionally used PMSF (phenylmethylsulfonyl fluoride) because it is much less expensive and large volumes are needed for the procedure. However, we have found that some batches tend to precipitate and form crystals when added to the solutions and when this happens we have observed nuclei/ NEs aggregating on these crystals under the microscope. Thus, if using PMSF crystal formation should be tested for each batch prior to its use in the procedure. When used, it can be added from a 100 mM stock in EtOH.

6. If general protease cocktails are used, it is important to make certain that they do not contain EDTA as this has been reported to negatively affect NE preparation.

7. The solution can be prepared by adding 220 ml of a 2.5 M (85%) sucrose stock to 12.5 ml 1 M HEPES, 6.25 ml 1 M KCl, 1.25 ml 1 M $MgCl_2$, and 10 ml H_2O.

8. Phenomenex now recommends the use of “Synergi Hydro-RP as an improved alternative to Aqua 125Å”.

9. Bulk material is not available. The resin is extracted from the HPLC column (cut in half with hacksaw), washed with methanol, dried, and stored as a powder.

10. Chapters detailing modifications of the NE protocol for rodent liver and muscle are being prepared for other volumes in the *Methods in Molecular Biology* series.

11. As with most protocols there is an optimal middle ground with too little or too much starting material resulting in lower yields. Because of the timing of centrifuge steps and layering gradients, one person can only easily manage two buffy coats at a time; however, four can easily be processed in a day in two sets. It is useful to increase the total number of blood packs used for a NE prep as it is difficult to see the nuclear pellets after pelleting through the sucrose cushions, but the use of too much starting material can saturate the sucrose gradients. The maximum capacity of the SW28 rotor used in this procedure would be approximately eight buffy coats.

12. This is an excess to ensure that a sufficient number of tubes are open to accommodate the volume in the bag (which is variable); only four are usually required.

13. It is helpful when layering to tilt the tube at a 45° angle and direct the pipette tip at a right angle to the side of the tube so that the force of the flow is distributed over a wide area of the wall of the tube.

14. Sometimes the cells will be clumped and sometimes also have a red tinge. Neither invalidates the prep. However, if cells are in large clumps extra care should be made to disrupt them when resuspending, or rosette formation may occur in the next pelleting step and reduce yields. The red tinge is due to erythrocyte contamination: if it is critical to remove all erythrocytes, then another ficoll gradient may be engaged after washing the cells with PBS and pelleting.

15. The supernatant will appear cloudy: this is because it is dense with platelets and does not reflect a loss of leukocytes/ lymphoctyes.

16. If the supernatant still appears cloudy, this step can be repeated.

17. The pellet resuspends with less clumping when using a small volume (i.e. 5-7 ml) before transferring cells into 75 cm^2 tissue culture flasks.

18. If processing bloods from multiple donors, it is important to keep each blood separate until after the hypotonic lysis.

19. Care must be taken to remove as much supernatant as possible in order to reduce highly concentrated proteins from serum that could block swelling in step 4.

20. The cells are distributed thus, so each tube can be successively resuspended in hypotonic lysis buffer in step 4 to maintain an identical swell time for each douncing.

21. As many protease inhibitors are short-lived, it is important to add them fresh to buffers shortly before use throughout the procedure.

22. We have noted that each blood takes a different amount of time for the cells to swell. Thus 10 min is a guideline, but not an absolute.

23. It is important to stagger the pellets because protracted incubation of the cells in hypotonic buffer will also lyse nuclei.

24. This serves to stabilize the nuclei as otherwise they also will swell and lyse during the subsequent steps, particularly the long incubation in the sucrose gradients.

25. The 50 ml volume assumes that cells from 1-2 buffy coats are being processed. If more are being processed, the pellets should be resuspended so that (based on initial cell counts and assuming full recovery thus far) no less than 50 million and no more than 200 million nuclei are loaded per SW28 tube (25 ml).

26. Contaminating ER and other membranes that are rich in proteases have floated in the sucrose away from the nuclear pellet and these now line the walls of the tube. Therefore it is important to avoid contact between the protease rich walls and the pellet. It is also important to keep the tubes inverted in the cold room after pouring

off the supernatant until the sides can be wiped in order to prevent the remaining film collecting by gravity in the bottom of the tube.

27. It is very important to use a swinging bucket rotor when spinning through the sucrose cushion at this point in order to float any chromatin that is released away from the NEs. In a fixed-angle rotor the cushion will be distributed thinly and NEs will have more chromatin contamination.

28. The supernatant may appear slightly cloudy, but this is mostly chromatin that has been ejected and should give a dark, worm-like appearance under the microscope quite distinct from NEs.

29. Faster speeds with shorter times may be used in microcentrifuge tubes provided that the NEs are not intended for ultrastructural analysis.

30. The pellet here will typically be much larger than the nuclear pellet.

31. Although the membranes are floated better using a swinging bucket rotor, a fixed angle rotor can also be used (e.g. type 45 Ti). In this case, volumes should be increased to 35 ml of the diluted membranes, 9.5 ml of the 1.86 M SHKM, and 3 ml of the 0.25 M SHKM. This final volume fills the tubes sufficiently to minimize the chance of tube collapse, while minimizing the chance of leakage into the rotor. Centrifuge at 57,000 x g (~27,000 rpm) for 5 h.

32. The type 45 Ti rotor tubes must be filled to close to the top or they can collapse.

33. Microsome protein levels comprise a less predictable fraction of the starting material as compared to NEs. Therefore amounts are determined by either Coomassie staining on SDS-PAGE or by an alternative protein quantitation assay.

34. It is critical to start the centrifugation step immediately since, loss of membrane proteins was observed even after just 10 min of additional incubation on ice.

35. The pellet should be very hard, so there is no need to recentrifuge after adding the H_2O.

36. Tansfering from an 1.5 ml eppendorf tube to a larger volume tube is to avoid losing sample during the bubbling that occurs because of the neutralization process.

37. At this step, because of the large volume (~850 µl), the sample will have to be split into three aliqots of ~1150 µl in 1.5 ml eppendorf tubes.

38. This makes for a pretty redundant list of proteins, but not all protein sequences have been predicted in the annotated human genome and sometimes missing proteins will appear in another related organism.

39. We have used TMHMM since the second version has become available, but used TMPred earlier because the first version of TMHMM failed correctly to predict several of the well-characterized NETs. It is wise to compare the results from different algorithms and to search for multimers of beta barrels as well as transmembrane helices.

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Legends to Figures and Tables

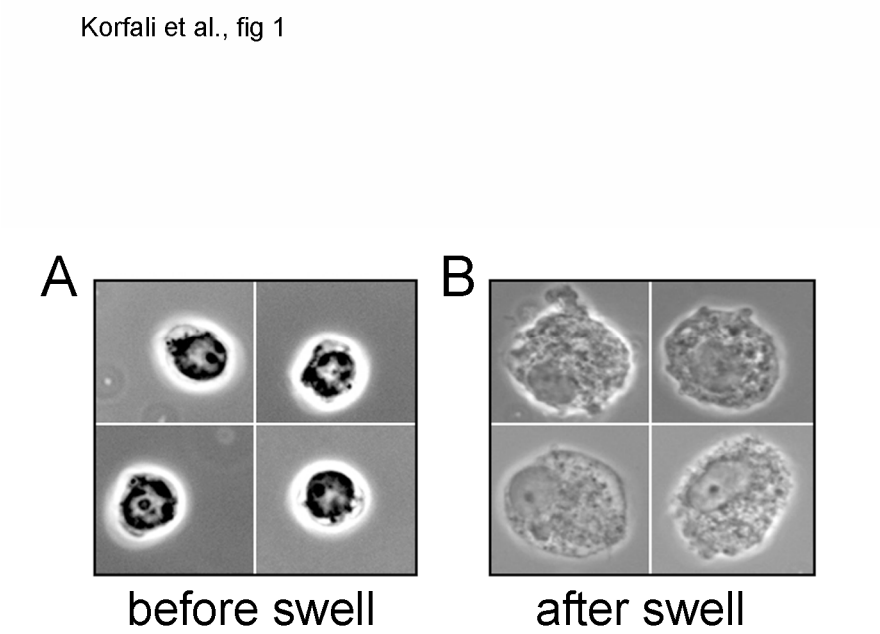

Fig. 1. Swelling of lymphoctyes for hypotonic lysis. A. Cells prior to treatment. Nuclei make the greater part of cell mass. B. The same cells after swelling in hypotonic lysis buffer. Note that some cells have burst even without Dounce homogenization. It is critical to Dounce the cells quickly at this stage and make the solution isotonic or the nuclei will also swell and burst.

Korfali et al., fig 2

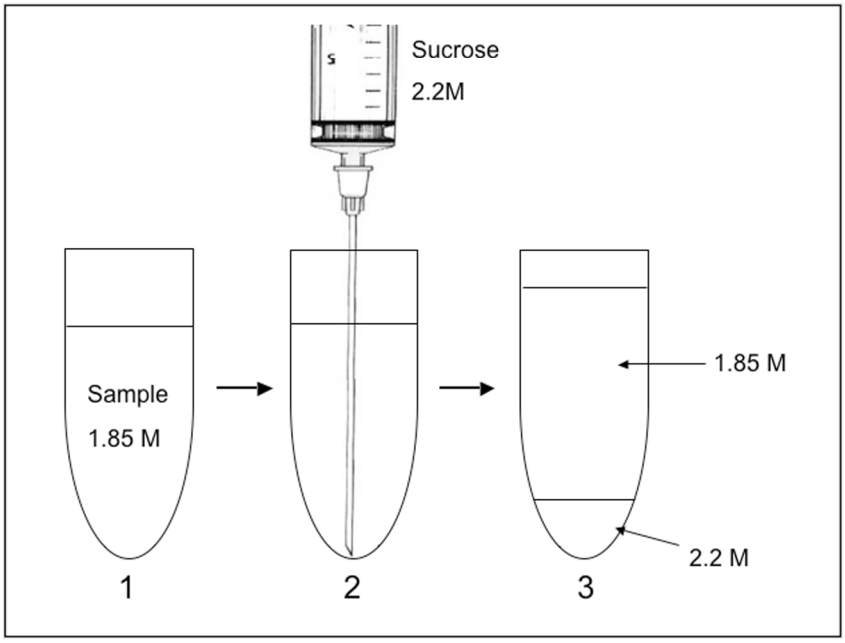

Fig. 2. Schematic detailing the addition of sucrose cushions. After distributing the nuclei-enriched lysate into SW28 ultracentrifuge tubes, a long large bore needle is inserted to the base of the tube to underlay with high sucrose buffer. Because of the high viscosity of the 2.2 M sucrose solution, it is important to use a needle of at least 14 gauge: it takes several minutes to underlay each tube if an 18 gauge needle is used compared to 30 seconds with a 14 gauge. It is important to use a luer lock syringe because the viscosity of the solution can produce high pressure on the connection.

Korfali et al., fig 3

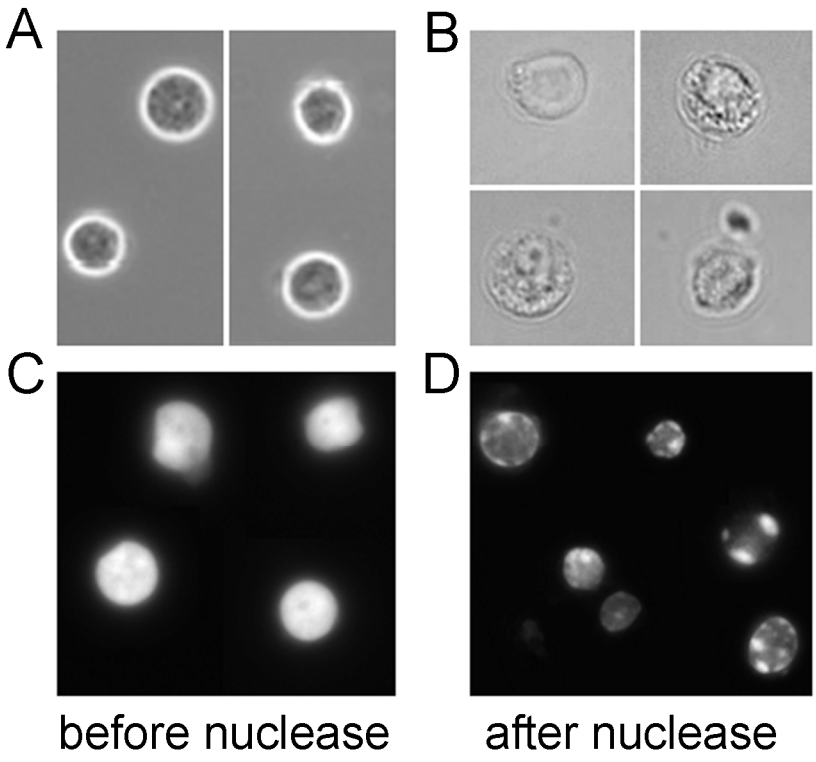

Fig. 3. Removal of nucleoplasmic contents to make NEs. A. Isolated nuclei before nuclease treatment. B. Nuclei after chromatin digestion. Note the change from phase dense to phase lucent. When the cells have reached this stage, the digestion is complete and pelleting yields the final NE fraction. C and D. DNA staining. Isolated nuclei (C) and NEs (D) are also depicted by fluorescence microscopy after staining with Hoechst 33342. Most of the DNA staining has disappeared from the chromatin-digested NEs.

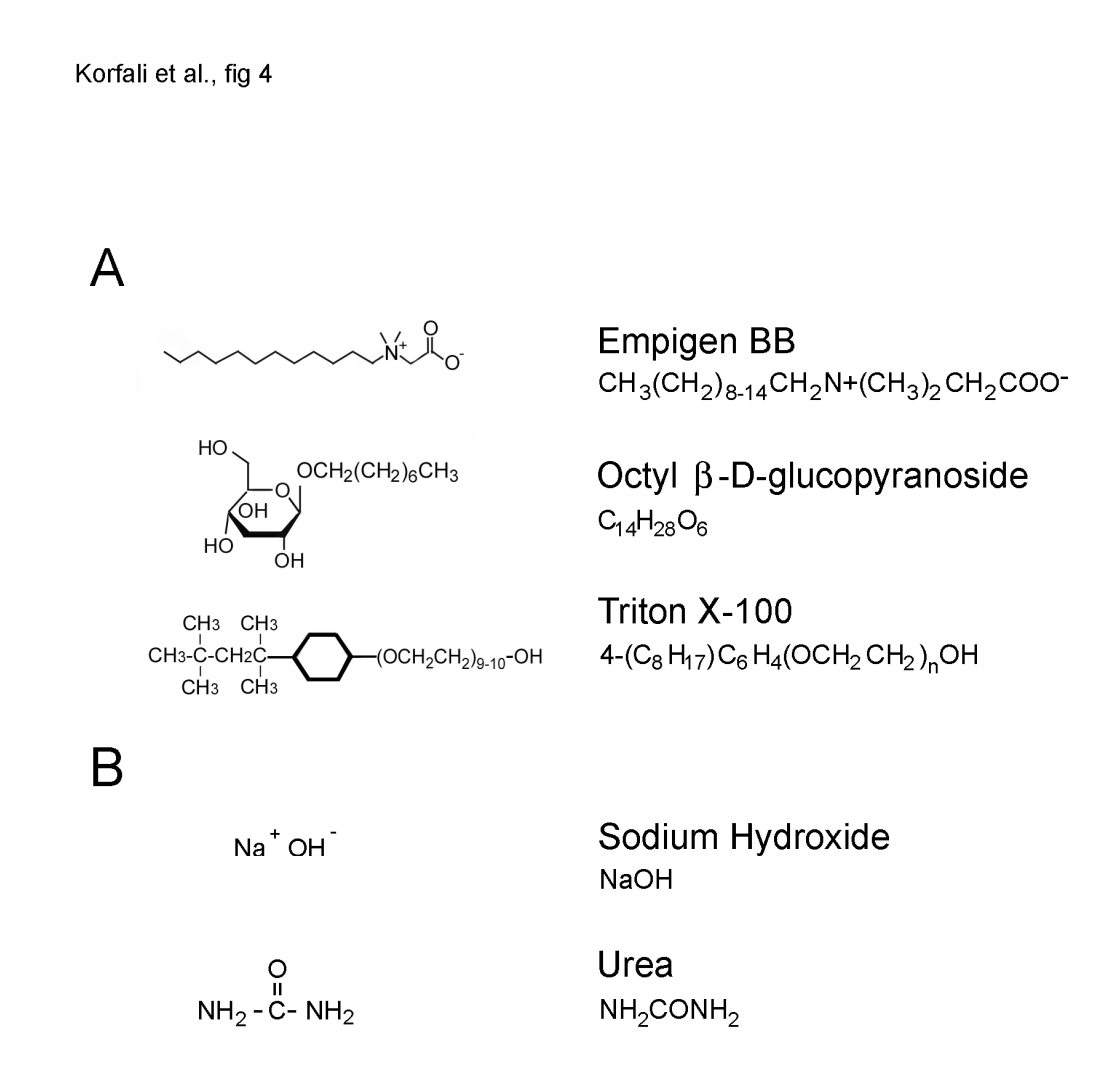

Fig. 4. Structures of chemicals used to extract NEs. A. The three detergents used have very different structures. Empigen BB is a zwitterionic detergent that was used in a recent proteomics study, where it was reported to preferentially solubilize nuclear pore complex core proteins ***(33)***. As a Zwitterionic detergent it has both properties of non-ionic and ionic detergents. Both octyl ß-D-glucopyranoside (also called octyl glucoside) and Triton X-100 are non-ionic detergents. Octyl glucoside has a glycosidic head group, while Triton X-100 has a polyoxyethylene head group. The non-ionic detergents are generally thought to be better suited to break lipid-protein interactions than protein-protein interactions. They also have the advantage of being

unaffected by the concentration of salt, which enables further removal of chromatin contaminants from the NEs. B. Alkaline and chaotrope stuctures for NaOH and urea respectively.

Korfali et al., fig 5

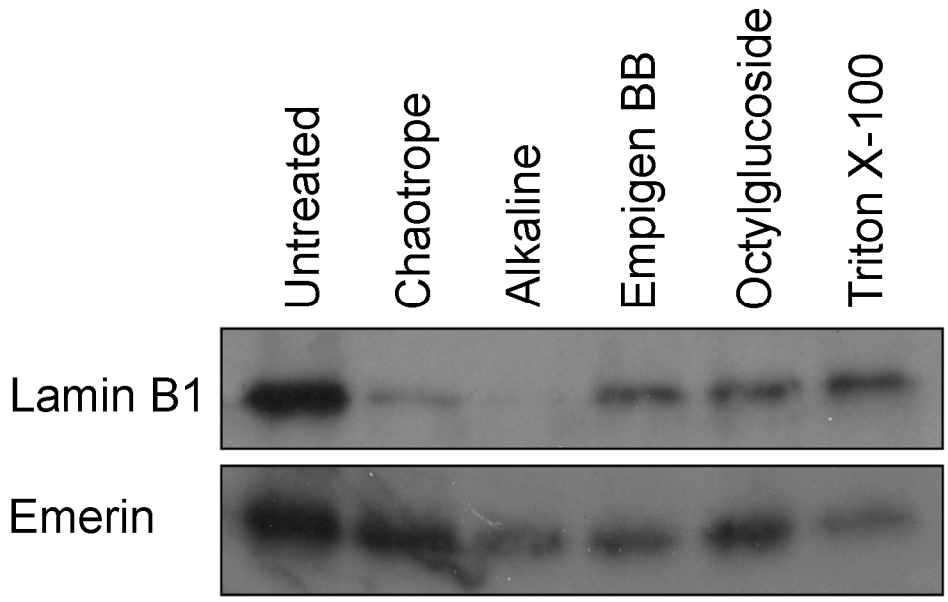

Fig. 5. Comparison of leukocyte/ lymphocyte NEs extracted with Na_2CO_3/ urea (chaotrope), NaOH (alkaline), Empigen BB, ß-octyl glucoside, or Triton X-100. Extracted proteins were resolved by SDS-PAGE (loading 0.5 million starting NEs per lane on BioRad 15-well mini-gels), transferred to PVDF membranes, and reacted with antibodies to either lamin B1 or the integral membrane proteins of the inner nuclear membrane emerin. The chaotrope and alkaline treatments removed most lamins, while detergents had little effect. In contrast, chaotrope and alkaline treatments had little effect on emerin, yet each detergent extracted this integral protein to a different degree with the most protein remaining after extraction with octylglucoside. More emerin was removed by Triton X-100 extraction than by any other treatment. This parallels results from the first proteomic analysis of NETs in which emerin was not identified in a Triton-extracted fraction, but was in the chaotrope-extracted fraction

(22). In that same study, another integral inner nuclear membrane protein (LBR) was lost from the chaotrope-extracted fraction and retained in the Triton-extracted fraction.

Korfali et al., fig 6

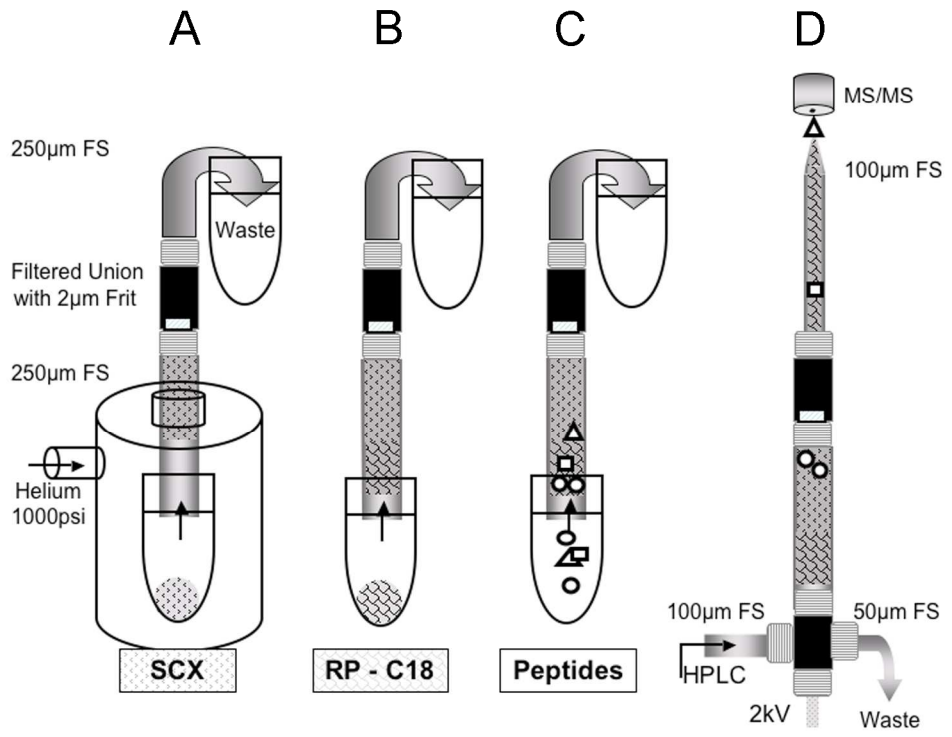

Fig. 6. Split-3-phase microcapillary column: Packing, Loading and Setup in LC/LC/MSfMS Mode. A. Two 250 µm fused silica tubing are connected via a filtered union. One end of the set up (on the frit side) is inserted into a high pressure deviced and packed using Helium pressure with SCX material in a slurry. B. The column is loaded with a slurry of Aqua C-18 RP, then washed with Methanol and Buffer A. C. The complex peptide mixture is pressure-loaded onto the 250 µm column. D. A pulled 100 µm single phase column is connected to the loaded and washed 2-phase column, and installed in-line with a quaternary HPLC pump and a tandem mass spectrometer.

Table 1: Gradient profiles for a 12-step MudPIT chromatography

Step #	Time (min)	Buffer A (%)	Buffer B (%)	Buffer C (%)
1	0	100	0	0
1	16	60	40	0
1	17	0	100	0
1	20	0	100	0
2 through 10	0	100	0	0
2 through 10	3	100	0	0
2 through 10	3.1	98	0	X*
2 through 10	5	98	0	X*
2 through 10	5.1	100	0	0
2 through 10	10	100	0	0
2 through 10	10.1	100	0	0
2 through 10	25	85	15	0
2 through 10	117	55	45	0
11 and 12	0	100	0	0
11 and 12	2	100	0	0
11 and 12	2.1	0	0	100
11 and 12	22	0	0	100
11 and 12	22.1	100	0	0
11 and 12	27	100	0	0
11 and 12	37	80	20	0
11 and 12	85	30	70	0
11 and 12	90	0	10	0
11 and 12	90.1	0	10	0
11 and 12	95	0	10	0
11 and 12	95.1	100	0	0
11 and 12	97	100	0	0

* X is equal to 5, 10, 15, 20, 30, 40, 50, 60 and 80 % C is steps 2 through 10, respectively.

Table 2: Filtering criteria applied to spectrum/peptide matches and proteins

Parameter	Value
Minimum +1 XCorr	1.8
Minimum +2 XCorr	2.5
Minimum +3 XCorr	3.5
Minimum DeltCN	0.08
Minimum charge state	1
Maximum charge state	3
Maximum Sp rank	10
Tryptic status requirement	Any
Multiple, ambiguous IDs allowed	FALSE
Preceding residue must be one of	KRM
Minimum sequence length	7
Maximum sequence length	100
Purge duplicate peptides by protein	XCorr
Include only loci with unique peptide	FALSE
Remove subset proteins	FALSE
Exclude protein names matching	Contaminant
Exclude protein descriptions matching	KERATIN
Minimum redundancy for low coverage loci	10
Minimum peptides per locus	1

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