Purification of nuclei and preparation of nuclear envelopes from skeletal muscle

Purification of Nuclei and Preparation of Nuclear Envelopes from Skeletal Muscle.

Gavin S. Wilkie and Eric C. Schirmer*

Wellcome Trust Centre for Cell Biology,
University of Edinburgh, Mayfield Road, Edinburgh. EH9 3JR, UK

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***corresponding author E.Schirmer@ed.ac.uk**
The Wellcome Trust Centre for Cell Biology,
University of Edinburgh,
Room 4.19, Michael Swann Building
Kings Buildings, Mayfield Road
Edinburgh EH9 3JR
UK
+44 131 650-7090 (office)
+44 131 650-7073 (lab)
+44 131 650-7360 (fax)

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Abstract

The nuclear envelope is a complex membrane-protein system that is notoriously difficult to purify because it has many connections to both nuclear and cytoplasmic components. This difficulty is compounded by the fact that the nature of these connections vary in different cell types and so methods must be significantly adapted according to the cell type from which nuclear envelopes are being purified. Here we present a detailed method for purification of nuclear envelopes from one of the most intransient tissues: skeletal muscle. We further note in the procedure how this method differs from that for other tissues. Identification of nuclear envelope-specific proteins is principally encumbered by endoplasmic reticulum contamination; therefore we also present a method to purify sarcoplasmic reticulum from muscle.

1. Introduction

The nuclear envelope (NE) is a double membrane system that includes a number of integral membrane proteins, nuclear pore complexes and the intermediate filament lamin polymer (1-3). Recently several inherited diseases, especially muscular dystrophies, have been associated with mutations in NE proteins (2,4), sparking renewed interest in the muscle NE (5). Purification of NEs from cell lines and tissues in general is encumbered with problems due to the high degree of connectivity between the nuclear membrane and other cellular structures on both sides. At the cytoplasmic face, the outer nuclear membrane is continuous with the endoplasmic reticulum and contains unique transmembrane proteins that connect it to the cytoskeleton (6-8). Inside the nucleus, the inner nuclear membrane proteins connect to lamins, and both lamins and integral membrane proteins bind directly to chromatin proteins and DNA (9,10). These connections vary in different cell types; so the tricks used to separate NEs from other cellular structures must be modified according to each tissue from whence the envelopes are being prepared.

In general, the first step in NE preparation is to isolate intact nuclei, taking advantage of their large mass relative to other organelles, either following homogenization of tissue in buffers lacking detergent or hypotonic lysis of cells (11). Subsequent dounce homogenization followed by pelleting through dense sucrose may reduce the amount of endoplasmic reticulum connected to the nuclei, but cannot fully eliminate it as the outer nuclear membrane, studded with ribosomes, is itself part of the endoplasmic reticulum. Treatment with enzymes to digest nucleic acids followed by salt washes and pelleting through sucrose cushions reduces the relative abundance of chromatin proteins (12-14).

We have previously elaborated procedures specific to purification of NEs from liver and from blood cells (15,16). These and other established procedures for the isolation of nuclei from soft tissues such as liver, kidney and brain cannot be successfully applied to skeletal muscle. Several methods for the purification of nuclei from skeletal muscle have been published previously [reviewed in (17)]. However they require Triton X-100 for the efficient release of nuclei from muscle fibers during homogenization: in the absence of detergent the yield drops by a factor of ten (18). These procedures cannot be used for isolation of NEs, as the detergent removes the nuclear membranes. The preparation of nuclei from skeletal muscle presents several problems unique to this tissue. Firstly, the number of nuclei in skeletal muscle is very low compared to that of other tissues, meaning that yields are comparatively low per gram of starting material. The DNA content of muscle is 17% that of liver and only 9% compared to kidney (17). Secondly, muscle fibers are extremely tough and homogenization procedures must be chosen with care in order to balance maximum disruption of the tissue with a minimum of damage to nuclei. Thirdly, the purification of nuclei from muscle homogenates on sucrose gradients often yields unsatisfactory results due to the presence of dense myofibrillar material derived from sarcomeres that tends to co-purify with nuclei.

In order to carry out a proteomic analysis of the NE of muscle nuclei, we have developed a procedure for preparing muscle NEs on a relatively large scale. Muscle tissue is initially broken down by mincing, and is then gently homogenized using a motorized dounce homogenizer (such as a Potter-Elvehjem). The resulting homogenate is filtered to remove the bulk of the fibrous material and poorly disrupted tissue pieces, and a crude nuclear pellet is obtained by low-speed centrifugation. Nuclei are initially purified from the dense myofibrillar material by isopycnic banding

in Percoll gradients (19). Ultracentrifugation through discontinuous sucrose gradients is then used to further purify the nuclei from other cytoplasmic components. However, we found that a three-step gradient where the muscle nuclei are floated on a 2.8 M sucrose cushion instead of being pelleted through 2 M sucrose improved purity, as it separates dense contaminants to the pellet (20). Finally, the nucleoplasmic contents are removed by enzymatic digestion of chromatin and salt washes, yielding a NE fraction highly enriched in lamins and integral NE proteins.

It is important to note that because of the large number of connections that NEs have to chromatin, the endoplasmic reticulum (sarcoplasmic reticulum in muscle), and cytoskeletal components, no method can truly “purify” NEs to homogeneity. However, subsequent purification steps may be employed to remove contaminants. Some further purification procedures are based on the biochemical properties of the nuclear lamina, which as an intermediate filament system remains insoluble in the presence of relatively high concentrations of salt and detergent (10). Other procedures rely on the solubility properties of membrane proteins (16,21,22), though with the possible loss of true NE proteins. Therefore, we also describe a method to purify sarcoplasmic reticulum membranes from the nuclear and mitochondrial - depleted muscle homogenate. This fraction can be used for a comparative analysis to subtract proteins that are not unique to the NE *in silico* (23).

2. Materials

2.1. Preparation of Tissue (Rodent Leg Muscle)

2.1.1. Animals

1. This procedure has been developed using the hind leg muscles of six to ten week-old rats (*e.g.* Sprague-Dawly or equivalent). However, it could easily be adapted to use some skeletal muscle types other than leg muscle, or different organisms such as mice.
2. Volumes in the protocol are given based on grams of muscle or millions of nuclei. To estimate how many animals to use: 20 – 30 g of muscle can be obtained from the hind limbs of one rat. We generally produce 10 – 20 million nuclei from 150 g of muscle obtained from 6 rats, although yields may vary a further 2-fold in either direction (*see* **Note 1**).

2.1.2. Hardware

1. Guillotine or equivalent local method for euthanizing animals.
2. Dissection scissors, forceps/ tweezers, scalpels, single-sided razor blades and bone scissors (kitchen scissors that will cut chicken bones are adequate).
3. Two beakers on ice - one containing Phosphate buffered saline (PBS) and one containing Homogenization buffer.
4. Appropriate materials for covering surfaces during procedure and for cleaning and waste disposal.

2.1.3. Solutions

1. PBS: 4.3 mM Sodium Phosphate, 137 mM Sodium Chloride, 2.7 mM Potassium Chloride, 1.4 mM Potassium Phosphate, adjusted to pH 7.4 with HCl. Store at room temperature. 250 ml of PBS is sufficient for washing and collecting leg muscles from 6 rats.

2.2. Purifiction of Nuclei from Skeletal Muscle

2.2.1. Hardware

1. Standard meat mincer as can be purchased in local grocery or hardware store.
2. Potter-Elvehjem homogenizer with a motor-driven Teflon pestle providing 0.1 to 0.15 mm clearance and drive motor capable of 1000 rpm (*e.g.* Potter S Homogenizer motor 853 3032, 60 ml homogenizer cylinder 854 2600, and PFTE Plunger 854 3003 from Sartorius or equivalent).
3. Loose fitting (Wheaton type B pestle) glass dounce homogenizer with clearance of between ~0.1 and 0.15 mm.
4. Swinging-bucket floor model centrifuge rotor capable of spinning 50 ml clear tubes to speeds of 27,000 x g for Percoll gradients (*e.g.* Beckman J-25 centrifuge with JS 13.1 rotor and Nalgene 3110-9500 50 ml centrifuge tubes, *see* **Note 2**).
5. Swinging-bucket ultracentrifuge rotor capable of 82,000 x g (*e.g.* Beckman Coulter SW28 rotor with Beckman Coulter 344058 Ultra-Clear 25 x 89 mm centrifuge tubes) for sucrose gradients.
6. Local standard light microscope, with phase contrast if possible. Glass slides and coverslips.
7. Assorted 500 – 1000 ml beakers, two funnels, twenty-five 50 ml centrifuge tubes, and several spatulas.
8. Two large ice buckets with ice for keeping all solutions and tubes cold.
9. Sterile cheesecloth for filtering homogenate (*see* **Note 3**). Two pieces of cheesecloth 50 x 20 cm are required for 500 ml of muscle homogenate.

10. Large bore luer lock stainless steel needles (*e.g.* 14 gauge) of greater length than centrifuge tubes and glass luer lock syringes to dispense dense sucrose solutions.

2.2.2. Solutions

Many of the solution names include the initials for the primary components: S for sucrose, H for HEPES, K for KCl, and M for $MgCl_2$ (*see* **Note 4**).

1. Protease inhibitor cocktail, *e.g.* Sigma P8340 (*see* **Note 5**), should be freshly added to all solutions at 1:250 dilution (*see* **Note 6**). If general protease cocktails are used, it is important to make certain that they do not contain EDTA as this harms many steps of NE preparation (*e.g.* Mg^{2+} ions are required to stabilize chromatin and as a co-factor for DNase I).
2. Percoll (*e.g.* Sigma 77237) should be diluted to a working stock of 81% (v/v) in 10mM HEPES, pH 7.4, 60 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 300 mM sucrose (*see* **Note 7**). Store at 4°C. Each Percoll gradient requires 13.3 ml of this buffered 81% Percoll solution and is sufficient for 100-200 g of starting material.
3. Homogenization buffer: 10 mM HEPES pH 7.4, 60 mM KCl, 0.5 mM Spermidine, 0.15 mM Spermine, 2 mM EDTA (*see* **Note 8**), 0.5 mM EGTA and 300 mM sucrose. Store at 4°C. Add protease inhibitors and 2 mM DTT (Dithiothreitol; from a 1 M solution in H_2O) immediately before use. 2.5 - 5 ml of Homogenization buffer is required for every gram of muscle (*e.g.* 100 – 200 g of minced muscle should be suspended in 500 ml of Homogenization buffer).
4. Percoll Gradient buffer: 10 mM HEPES pH 7.4, 60 mM KCl, 0.5 mM Spermidine, 0.15 mM Spermine, 0.1 mM EDTA, 0.1 mM EGTA and 300 mM

sucrose. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. 150 ml is required for 100 – 200 g of starting material.

5. HKM: 50 mM HEPES pH 7.4, 25 mM KCl, and 5 mM $MgCl_2$. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. 100 ml is required for 100 – 200 g of starting material.

6. 0.25 M SHKM: 250 mM sucrose, 50 mM HEPES pH 7.4, 25 mM KCl and 5 mM $MgCl_2$. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. 150 ml is required for 100 – 200 g of starting material.

7. 2.15 M SHKM: 2.15 M sucrose (from a 2.5 M sucrose stock, *see* **Note 9**), 50 mM HEPES pH 7.4, 25 mM KCl and 5 mM $MgCl_2$. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. 20 ml is required for 100 – 200 g of starting material.

8. 2.3 M SHKM: 2.3 M sucrose (from a 2.5 M sucrose stock, *see* **Note 9**), 50 mM HEPES pH 7.4, 25 mM KCl and 5 mM $MgCl_2$. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. 100 ml is required for 100 – 200 g of starting material.

9. 2.5 M sucrose: Dissolve 855.75 g of sucrose in a total volume of 1 liter of distilled water by stirring and heating. Store at 4°C. This stock can be used to make all working solutions that contain sucrose, except the 2.8 M SHKM.

10. 2.8 M SHKM: 2.8 M sucrose (*see* **Note 10**), 50 mM HEPES pH 7.4, 25 mM KCl and 5 mM $MgCl_2$. Store at room temperature. Add 2 mM DTT and protease inhibitors and chill on ice immediately before use. 20 ml is required for 100 – 200 g of starting material.

2.3 Preparation of Nuclear Envelopes

2.3.1. Hardware

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

2.3.2. Solutions

1. DNase I (*e.g.* Sigma DNase I D4527) resuspended at 10 U/ µl in H_2O. Store at -20°C.
2. RNase A (*e.g.* Sigma RNase A R4875) resuspended in H_2O at 10 mg/ ml and boiled for 20 min. Store at -20°C.
3. 10% SHM buffer: 0.3 M sucrose, 10 mM HEPES pH 7.4, 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (*see* **Note 4**). Store at 4°C. Add 2 mM DTT and protease inhibitors before use. 50 ml is required for 20 million nuclei.
4. 30% SHKM buffer: 0.9 M sucrose, 10 mM HEPES pH 7.4, 25 mM KCl and 2.5 mM $MgCl_2$. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. 2 ml is required for 20 million nuclei to underlay the 10% SHM and form a sucrose cushion during centrifugation.

2.4. Preparation of Sarcoplasmic Reticulum Membranes

2.4.1. Hardware

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

2. A Type 45 Ti fixed angle ultracentrifuge rotor or equivalent that can provide 150,000 x g and matching tubes.

2.4.2. Solutions

The same sucrose solutions used for *Preparation of Nuclear Envelope* can be used in preparing microsomes. In particular, the 2.8 M SHKM and 0.25 M SHKM will be required.

2.5. Determining the Purity/ Quality of Fractions

2.5.1. Reagents and hardware required for fluorescent staining of chromatin

1. Hoechst 33342 (*e.g.* Molecular Probes H-3570) or DAPI (*e.g.* Molecular Probes D1306) for fluorescent labeling of chromatin.
2. An epifluorescence microscope capable of at least 400 x magnification. Excitation and emission filters suitable for viewing DAPI or Hoechst 33342 fluorescence will be required (the excitation and emission spectra of these dyes are very similar).
3. Standard microscope slides and coverslips.

2.5.2. Reagents and hardware required for Western analysis of fractions

1. Standard labware for SDS-PAGE and Western blotting.
2. Antibodies to nuclear lamins, characterized integral NE proteins and endoplasmic reticulum proteins.
3. An assay system for estimating protein concentration in purified fractions (*e.g.* Bradford reagent, Sigma B6916 and a spectrophotometer capable of measuring absorbance at 595 nm).

3. Methods

If NEs are being prepared from muscle, the entire procedure can take eight or nine hours to complete. If several preparations of nuclei are required to generate sufficient material for NEs, it may be more practical to freeze the purified muscle nuclei and to prepare NEs from the accumulated nuclei at a later time.

3.1. Preparation of Tissue (Rodent leg muscle).

Most of the NE preparation procedure can be efficiently performed with one individual; however euthanizing and dissecting the animals should be done quickly, and it is very helpful to have assistance at this point.

1. Euthanize rats according to local animal protocols.
2. Pull up the skin in the dorsal posterior area and cut through skin and fur with scissors perpendicular to the length of the body. Make another incision along the backbone down the length of the body. Peel back to remove the fur and expose the hind legs.
3. Pull off extraneous fat and then cut along the muscle line delineating the hip with a razor blade. Likewise, in the anterior aspect cut underneath the pelvis so that the larger muscle mass is disconnected around the connection between the femur and hip bone.
4. Remove hind legs by cutting through hip joint with bone scissors.
5. Rinse legs in ice-cold PBS to wash off hair and blood.
6. Remove muscle tissue from bone using single sided razor blade. Grasp tissue with tweezers to avoid injury to hands. Cut up muscle tissue into small chunks

of up to 5 mm square and collect in a small volume (*e.g.* 50 ml) of ice-cold PBS. If possible, have several people working at this point so that one can begin processing the material while the other deals with disposal and clean-up.

3.2. Purification of Muscle Nuclei

The first step in NE enrichment is the isolation of nuclei. While release of nuclei can be achieved in cell suspensions by hypotonic lysis and with soft tissue by dounce homogenization, the fibrous nature of muscle and associated connective tissue requires that the tissue be broken down by mincing, followed by extensive douncing using a motorized Potter-Elvehjem homogenizer to release nuclei. An extra Percoll gradient step is required to remove contaminating muscle fibers from the homogenate. Finally, the nuclei are spun through a 3-step sucrose gradient in the ultracentrifuge (in contrast to the 2-step sucrose gradients used in most other nuclear purification protocols). This floats microsomal membranes and pellets any dense contaminants, whilst allowing purified nuclei to collect on a 2.8 M sucrose cushion.

1. Drain the PBS from the muscle tissue and weigh the material.
2. Use a meat mincer (*e.g.* a domestic hand mincer) to finely mince the muscle tissue. Pass the tissue through the mincer at least three times, using 50 ml of ice-cold Homogenization buffer to wash all the tissue through. Collect the minced muscle in a tray cooled on ice (*see* **Note 11**).
3. Add additional ice-cold Homogenization buffer to minced muscle to achieve a final volume of between 2.5 – 5 ml for every gram of muscle (*e.g.* 100 – 200 g of muscle should be suspended in 500 ml of Homogenization buffer) and mix thoroughly. Pour into 55 ml Potter-Elvehjem homogenizer and homogenize at 900 – 1000 rpm on ice, bringing the pestle to the bottom at least 8 times (*see* **Note 12**). If total volume exceeds 55 ml then homogenize in batches and collect homogenate on ice. Rinse the homogenizer with buffer when finished and add to

the homogenate. This combination of mincing and douncing releases a significant proportion of the nuclei.

4. In a 4°C room, fold cheesecloth into four layers and place in funnel. Pour 100 – 200 ml crude homogenate through cheesecloth and collect filtrate in beaker. As the flow slows, fold the cheesecloth over and roll a sterile pipette (or similar clean cylindrical tool) from top to bottom, to squeeze the remaining fluid out (Figure 1, *see* **Note 13**). Pour 25 ml of ice-cold homogenization buffer through the cheesecloth after the homogenate has drained to wash out any trapped nuclei. A large amount of solid material should remain in the cheesecloth (Figure 1). This is rich with collagen, connective tissue, and large contractile filaments.

5. Transfer filtrate to centrifuge tubes (*e.g.* Falcon™ Conical Centrifuge Tubes, 50 ml capacity) and pellet nuclei at 1,000 x g in a swinging bucket rotor (*e.g.* 2,000 rpm in a Beckman Coulter J6MI floor model centrifuge) for 10 min at 4°C.

6. Remove the supernatant carefully as the pellets are very soft. Keep this supernatant if sarcoplasmic reticulum membranes (*see* Section 3.4) are to be prepared at the same time, as they are contained in this fraction (*see* **Note 14**).

7. Resuspend crude nuclear pellets in ice-cold Percoll gradient buffer. Reduce the volume of the original homogenate by 5-fold (*e.g.* from 500 ml of muscle homogenate, resuspend nuclear pellets in 100 ml of Percoll gradient buffer).

8. Pellet nuclei again at 1,000 x g in a swinging bucket rotor for 10 min at 4°C. Remove supernatant carefully, as pellets are soft. This step concentrates nuclei away from much of the mitochondria and vesiculated cell membranes that pellet

at higher speeds, and washes away additional collagen. However, the nuclei are still heavily contaminated with these structures and with myofibrils.

9. Resuspend crude nuclear pellets in 26.7 ml of ice-cold Percoll gradient buffer. Add Percoll to a final concentration of 27% v/v (*e.g.* add 13.3 ml of 81% Percoll in gradient buffer).

10. Transfer to a clear round-bottomed centrifuge tube with a capacity of more than 40 ml. Centrifuge at 27,000 x g for 30 min at 4°C, preferably in a swinging bucket rotor (*see* **Note 2**). The Percoll gradient is self-forming under these conditions.

11. Myofibrils will form a layer at the top of the tube (Figure 2A), which can be aspirated away with a pipette. Nuclei should band near the bottom of the tube and can be collected with a clean pipette. Check that the fraction collected is enriched in muscle nuclei under microscope, and ensure that any nuclei pelleted at the bottom of the tube are collected. This step is necessary because many of the myofibrils broken off from the muscle tissue during homogenization have a similar mass or size to nuclei, but a different density (19). The isopycnic gradient floats the less dense myofibrillar material.

12. Dilute nuclei 10-fold with ice-cold 0.25 M SHKM and centrifuge in 50 ml conical tubes at 4000 x g for 20 min at 4°C in a swinging bucket rotor. Remove supernatant with care. Pink-colored pellet contains nuclei, microsomal membranes and other cytoplasmic contaminants.

13. Resuspend pelleted nuclei in 11 ml of ice-cold 0.25 M SHKM. Use a dounce homogenizer with loose-fitting pestle to break up any aggregated nuclei (*see*

Note 15). Bring volume to 50 ml by adding 39 ml of ice-cold 2.3 M SHKM, thus adjusting the sucrose concentration to 1.85 M.

14. Pipette 25 ml of nuclei in 1.85 M SHKM into each of two SW28 ultracentrifuge tubes on ice. Underlay each tube with 5 ml of ice-cold 2.15 M SHKM then a further 5 ml of ice-cold 2.8 M SHKM using a luer-lock syringe with a 14-gauge needle (*see* **Note 16**). Handle tubes with care to avoid mixing layers.

15. Balance tubes from top and centrifuge in SW28 swinging bucket ultracentrifuge rotor for 60 min at 82,000 x g (25,000 rpm) at 4°C (*see* **Note 17**).

16. The pink layer at top of tube (Figure 2B) contains microsomal membranes derived from sarcoplasmic reticulum, and should be aspirated away with a pipette. Muscle nuclei should be visible as a grey band at the interface of the 2.8 M and 2.15 M sucrose layers (Figure 2B). Nuclei can be collected using a luer-lock syringe with a 14-gauge needle by inserting the needle through the upper sucrose layers and aspirating the nuclei from the 2.15 M / 2.8 M interface. It is important to use a microscope to determine the content of different fractions at this stage (*see* **Note 18**).

17. Dilute nuclei 10-fold in ice-cold HKM buffer and centrifuge at 4000 x g for 20 min at 4°C in a swinging bucket rotor.

18. Grey pellet (if visible) contains purified muscle nuclei. Aspirate the supernatant carefully, as the pellet may be soft. Resuspend purified nuclei in 10 ml of ice-cold 0.25 M SHKM and use a haemocytometer and a phase-contrast microscope to estimate the number of nuclei isolated and to check relative purity. Nuclei should be free of contaminants, and should display the characteristic elongated shape of muscle nuclei (Figure 3).

19. If required, purified nuclei can be stored at this stage by freezing in SHKM buffer containing at least 1 M sucrose as a cryoprotectant. Pellet the nuclei at 4000 x g for 20 min at 4°C in a swinging bucket rotor, and resuspend in 0.5 ml of ice-cold 0.25 M SHKM. Add 0.5 ml of 2.15 M SHKM, mix well and snap freeze in liquid nitrogen. Store at -80°C.

3.3. Preparation of Nuclear Envelopes

The critical step in the preparation of NEs is the removal of nucleoplasmic contents from the purified nuclei. This is achieved by enzymatic digestion of DNA and RNA, to break chromatin up into pieces small enough to be washed out of nuclei. This is more difficult to achieve for muscle nuclei compared to nuclei from soft tissues, at least in part because the muscle nuclei do not swell appreciably in hyptonic buffer. Salt washes are an absolute requirement to wash digested chromatin from muscle nuclei, and care should be taken to ensure efficient chromatin removal using a fluorescent stain such as Hoechst 33342 or DAPI (*see* Section 3.5.1).

From this point in the procedure, amounts are based on millions of nuclei, rather than grams of tissue.

1. Resuspend purified muscle nuclei in ice-cold 10% SHM (*see* **Note 19**) at a concentration of 1-2 million nuclei / ml. Use dounce homogenizer with loose fitting pestle to break up any aggregated nuclei.
2. Withdraw a small sample to the side that will not be digested, for comparison with the digested material after salt washes.
3. Add DNase I to 10 Units / ml and RNase A to 1.4 μg / ml (*see* **Note 20**). Incubate on ice for 20 min to begin chromatin digestion (*see* **Note 21**).
4. Centrifuge at 4000 x g for 20 min at 4°C in a swinging bucket rotor to pellet nuclei. Discard supernatant.
5. Resuspend nuclei in ice-cold 10% SHM at a concentration of 2-4 million nuclei / ml. Do a second digestion of chromatin, but now with 50 Units / ml of DNase I

and 5 μg / ml RNase A, for 20 min on ice (*see* **Note 22**). In contrast to nuclei from other cell types, chromatin removal cannot be monitored by phase-contrast microscopy (*see* **Note 23**).

6. Centrifuge at 4000 x g for 20 min at 4°C in a swinging bucket rotor to pellet nuclei. Discard supernatant and resuspend nuclei in 10 ml of ice-cold 10% SHM supplemented with 300 mM NaCl to wash out digested chromatin (*see* **Note 24**).

7. Transfer NEs in 10% SHM / 300mM NaCl to a clear round-bottomed centrifuge tube (*e.g.* 15ml glass Corex tube) and underlay with 0.15 volumes of 30 % SHKM using a luer-lock syringe with a 14-gauge needle. Handle tubes with care to avoid mixing layers. Centrifuge in a swinging bucket rotor (*see* **Note 25**) at 6000 x g for 30 min at 4°C (*e.g.* 6200 rpm in a Beckman JS 13.1 rotor or 5,000 rpm in a Beckman-Coulter floor model J6MI centrifuge). This step floats digested chromatin away from the NEs, which pellet through the 30% sucrose cushion.

8. The supernatant may be cloudy as it contains histones and chromatin (*see* **Note 26**). Aspirate the supernatant very carefully (do not decant by pouring) as the NE pellet is very soft.

9. As, unlike nuclei from most other tissues, no clear phase dark to phase lucent transition occurs for muscle nuclei during chromatin digestion, it is important to set aside a small sample to test for chromatin digestion by staining with a fluorescent chromatin marker such as Hoechst 33342 or DAPI (*see* Section 3.5.1). Chromatin should be almost completely removed from nuclei, leaving only a small amount of staining closely associated with the NE (Figure 4). Fluorescence intensity should also be greatly diminished in the digested sample,

compared to undigested nuclei. If a large amount of chromatin remains, pellet nuclei and repeat digestion (go back to Step 4).

10. Centrifuge at 4000 x g for 20 min at 4°C in a swinging bucket rotor to pellet nuclei. Discard supernatant and resuspend nuclei in 10 ml of ice-cold 10% SHM supplemented with 400 mM KCl. This step washes away any remaining digested chromatin.

11. Centrifuge at 4000 x g for 20 min at 4°C in a swinging bucket rotor to pellet nuclei. Resuspend purified NEs in a small volume of ice-cold 10% SHM and aliquot to tubes suitable for storage (*e.g.* 1.5 ml eppendorf tubes). Centrifuge at 6000 x g for 30 min at 4°C.

12. Carefully aspirate the supernatant and freeze the NE pellets immediately in liquid nitrogen. Store at -80°C.

3.4. Preparation of Microsomes derived from Sarcoplasmic Reticulum Membranes

1. Take the supernatant after pelleting of nuclei from muscle homogenate (step 6 in *3.2 Purification of Muscle Nuclei*) and add EDTA to a final concentration of 0.5 mM to inhibit metalloproteases. Centrifuge at 10,000 x g at 4°C for 20 min (*e.g.* 8,000 rpm in a Beckman JA14 centrifuge rotor) to pellet mitochondria.

2. Transfer the post-mitochondrial supernatant to ultracentrifuge tubes and centrifuge at 100,000 x g for 45 min at 4°C (*e.g.* 36,000 rpm in a Beckman Type 45 Ti fixed angle ultracentrifuge rotor, *see* **Note 27**). This step to pellets the microsomes derived from sarcoplasmic reticulum membranes, reducing the soluble protein content and lowering the volume required for subsequent fractionation in sucrose gradients.

3. Resuspend each crude microsomal pellet in a small volume of ice-cold 0.25 M SHKM (*e.g.* 5 ml) by pipetting. Make the sucrose concentration up to 2 M by adding 2.7 volumes of ice-cold 2.8 M SHKM (*e.g.* add 13.5 ml of 2.8 M SHKM to 5 ml of resuspended microsomes and mix thoroughly). Alternatively, freeze the crude microsomal pellet at -80°C at this stage.

4. Aliquot 28 ml of microsomal extract in 2 M SHKM to SW28 ultracentrifuge tubes on ice. Overlay each tube with 7 ml of ice-cold 1.85 M SHKM and 3 ml of 0.25 M SHKM. Centrifuge at 57,000 x g for 4 h at 4°C (*e.g.* 21,000 rpm in a SW28 swinging bucket ultracentrifuge rotor). This step forces the microsomes to float upwards into the less dense sucrose, whilst pelleting other contaminants derived from muscle cell cytoplasm.

5. The microsomes will be found at the interphase between the 1.85 M sucrose layer and the uppermost 0.25 M sucrose layer (Figure 2C), and will have a fluffy

yellow-brown appearance, if visible. The microsomal band can be recovered by aspiration with a syringe, either by tube puncture with a needle or by inserting the needle through the upper phase.

6. Dilute the purified microsomes with 4 volumes of 0.25M SHKM and pellet them at 152,000 x g in an ultracentrifuge (*e.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.
7. Discard the supernatant and scrape the microsomal pellet out of the ultracentrifuge tube using a clean spatula. The microsomal pellets should have an orange / brown, toffee-like appearance. Aliquot into pre-weighed tubes suitable for freezing (*e.g.* 1.5 ml eppendorf tubes) and calculate the additional mass of to the microsomes by weighing.
8. Freeze the microsomes in liquid nitrogen, and store at -80°C.

3.5. Determining the Purity/ Quality of Fractions

3.5.1. Fluorescent staining of chromatin

Staining of chromatin with fluorescent dyes allows the efficiency of chromatin removal during NE preparation to be monitored with high sensitivity using an epifluorescence microscope.

1. Take a small sample of nuclei before chromatin digestion (Step 2 in Section 3.3), and a second sample after chromatin digestion washing with 300 mM NaCl (step 9 in section 3.3). Place samples in suitable small tubes (e.g. 1.5 ml eppendorf tubes) and pellet at 5000 x g for 10 min at 4°C in a microfuge.

2. Resuspend nuclei in a small volume (e.g. 50 – 100 µl) of 10 % SHM containing 5 µg / ml Hoechst 33342. Mix well, pipette 5 – 10 µl of each sample onto a glass slide and place a coverslip on top. Seal edges with nail varnish. Fixation is not required for staining with Hoechst 33342. However, if using DAPI samples should first be fixed with 4% formaldehyde for 5 min, then pelleted as above and rinsed in PBS before staining.

3. View samples on an epifluorescence microscope, using a filter set appropriate for DAPI excitation and emission (this is also suitable for Hoechst 33342). The fluorescence intensity of the sample should be much fainter after chromatin digestion, with only a few areas of chromatin evident around the nuclear rim and little fluorescence remaining in the nucleoplasm (Figure 4).

3.5.2. Western analysis of fractions

Western blotting can be used to track the partitioning of known NE or microsomal membrane proteins during fractionation of muscle nuclei and in subsequent extraction of NEs. Nuclear lamins and NE transmembrane proteins should become significantly enriched during the procedure (Figure 5).

1. Take small samples of material from throughout the purification procedure. For example, take samples of raw homogenate, crude nuclear pellet, nuclei after Percoll gradient, nuclei after sucrose gradient and purified NEs. Place samples into suitable small tubes (e.g. 1.5 ml eppendorf tubes).
2. Pellet samples at 6000 x g for 10 min at 4°C in a microfuge. Discard supernatant containing soluble proteins.
3. Nuclear lamins and transmembrane proteins tend to be insoluble. Resuspend pellets in a small volume (e.g. 50 µl) of PBS + 0.1 % Triton X-100 by pipetting. Then add 1.3 volumes of 8 M Urea, bringing the final Urea concentration to 6 M (e.g. add 66 µl of 8 M Urea to 50 µl of sample in PBS + 0.1 % Triton X-100). Mix thoroughly by pipetting.
4. Pellet insoluble material by centrifugation at 6000 x g for 2 mins. Transfer supernatant to new tube.
5. Estimate the protein concentration of each sample (*e.g.* by Bradford assay), and normalize samples to contain equal amounts of total protein.
6. Resolve proteins on a 10 % SDS-PAGE gel, loading an equal amount of protein in each lane.

7. Carry out a Western Blot (24) of the gel with antibodies against nuclear lamins and integral NE proteins. These should show significant enrichment during the purification procedure (Figure 5).

4. Notes

1. As with most protocols there is an optimal middle-ground with too little or too much starting material resulting in lower yields. In our hands, six rats produces optimal yields without saturating two sucrose gradients in a Beckman-Coulter SW28 rotor.

2. The original protocol for Percoll gradient centrifugation (19) uses a fixed angle rotor (*e.g.* Beckman JA20 rotor at 15,000 rpm). We have found that a swinging bucket rotor (*e.g.* Beckman JS13.1 rotor at 13,000 rpm) gives superior separation of myofibrils and nuclei.

3. Muslin can be used in place of cheesecloth if not chemically treated: make certain to ask supplier.

4. $MgCl_2$ concentration in the original NE purification procedure was 5 mM throughout (11,25). 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. During DNase I digestion, it is important to increase the $MgCl_2$ concentration back to 2 mM, supplemented with 0.5 mM $CaCl_2$ for the enzyme to function efficiently.

5. Protease inhibitors should be tailored to individual tissues according to their most abundant proteases.

6. PMSF or AEBSF may be used to inhibit proteases in the initial homogenization step to reduce costs, as the volume is likely to be high.

7. Do not add spermine or spermidine to the 81% Percoll solution, as it tends to encourage the formation of a precipitate. The spermine and spermidine contained

in the Homogenization and Gradient buffer are sufficient to stabilize the chromatin.

8. EDTA and EGTA in the Homogenization buffer and Percoll Gradient buffer are essential to remove the endogenous Ca^{2+} released during homogenization of muscle tissue, which can cause the contraction of sarcomeres. Contraction leads to a broader distribution of myofibrils in the Percoll gradient, thus contaminating the band of nuclei (19). During this step, the absence of Mg^{2+} ions is not detrimental as chromatin is stabilized with spermine / spermidine.

9. The 2.3 M SHKM can be prepared by adding 230 ml of an 2.5 M sucrose stock to 12.5 ml 1 M HEPES, 6.25 ml 1 M KCl, and 1 ml 1 M $MgCl_2$, and freshly added 2 mM DTT and protease inhibitors. 2.3 M SHKM can be stored at 4°C. Lesser concentrations of sucrose can be obtained by mixing the 2.3 M SHKM with the HKM, if required.

10. The 2.8 M SHKM can be prepared by dissolving 240 g of sucrose in a total volume of 250ml of distilled water containing 5 ml of 1 M HEPES (pH 7.4), 6.25 ml of 1 M KCl and 1.25 ml of 1 M $MgCl_2$. Vigorous stirring and heating are required to dissolve the sucrose. This stock solution tends to go off rapidly due to precipitation of sucrose. This can be minimized by storage at room temperature or even at 37°C. However, it is best not to use the 2.8 M SHKM for preparing 2.3 M or 2.15 M SHKM stocks by dilution with HKM. A working 2.8 M SHKM solution can be prepared by mixing DTT and protease inhibitors and chilling on ice immediately before use.

11. To avoid loss of material during mincing, the void volume of the mincer can be minimized with addition of parafilm before blade.

12. This requires a reasonable amount of physical strength and one must take care to keep the homogenizer straight with the direction of the pestle or the homogenizer can break. Only start the motor when the pestle is at least partly inserted into the homogenizer tube. Never stop the pestle rotation while it is inserted inside the homogenizer with liquid, or this also can become stuck or break due to the vacuum produced during homogenization.

13. It is important to let most of the liquid drain through the cheesecloth before beginning squeezing, as the homogenate can easily spray out or spill into the filtrate when pressure is applied. Alternatively, if gloves are sterile and powder-free, it is possible to lift the cheesecloth and twist it to produce squeezing.

14. If sarcoplasmic reticulum membranes are intended to be prepared at a later time, the post-nuclear supernatant (*see* section 3.4.1) can be frozen at -80°C after a high speed spin to pellet mitochondria. It is important to remove mitochondria because they might otherwise fragment, contaminating the sarcoplasmic reticulum fraction with mitochondrial membranes.

15. Use of dounce homogenizer at this step is important to reduce traces of sarcoplasmic reticulum membrane still adhering to nuclei.

16. Due to the high viscosity of the 2.8 M sucrose solution, it takes several minutes to underlay each tube if an 18 gauge needle is used. In contrast, with the wide bore size of the 14 gauge needle, this same procedure can be performed in 30 seconds. It is important to use a luer lock syringe because the viscosity of the solution can produce high pressure on the connection.

17. Nuclei from different tissues have distinct densities; thus the concentration of sucrose in buffers may need to be altered or centrifugation steps lengthened if

nuclei are to be isolated from muscles other than leg muscles, or species other than rats.

18. The nuclei collected from the interface of the 2.15 M / 2.8 M sucrose layer should be examined under a microscope to check purity. Any material that pellets through the 2.8 M sucrose cushion should be resuspended in a small volume of 0.25 M SHKM and examined under the microscope, to ensure that it does not contain significant numbers of nuclei. Similarly, material from the 1.85 M / 2.15 M sucrose interface should be checked for absence of nuclei. Sucrose concentrations may need to be modified in order to efficiently purify nuclei from different muscle types or from organisms other than rats.

19. The 10% SHM is hypotonic, which helps to wash nucleoplasmic contents out of nuclei. However, muscle nuclei do not appear to swell appreciably in this buffer. This is in contrast to muscle or blood nuclei for those familiar with those procedures.

20. Micrococcal (S7) nuclease (*e.g.* Worthington LS004797) can be used in place of DNase I / RNase A for chromatin digestion. Micrococcal nuclease digests both DNA and RNA, and requires a buffer containing Ca^{2+} and slightly higher pH for optimum activity. We found that a buffer containing 0.3 M sucrose, 10 mM HEPES (pH 8.2), 1.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 2 mM DTT and protease inhibitors worked well for chromatin removal using two subsequent digestions with micrococcal nuclease at 5 μg / ml and 20 μg/ ml, respectively.

21. Chromatin digestion continues during centrifugation steps. Therefore, the centrifugation step should proceed even in the absence of any evident chromatin removal.

22. Two subsequent digestions appear to be necessary to break chromatin into small enough pieces to be readily removed through the still-intact NEs.

23. In contrast to liver or lymphocyte nuclei, grey appearance of muscle nuclei under phase contrast microscopy does not change, as chromatin appears to remain in myonuclei after digestion.

24. The 300mM NaCl wash is essential to wash chromatin out of nuclei. However, do not add stock 5 M NaCl directly to nuclei in 10% SHM, as the concentrated salt can locally affect the stability of the nuclear lamina, thus damaging NEs. Chromatin removal can be monitored using a fluorescent stain such as Hoesht 33342 or DAPI after the 300mM NaCl wash step (Figure 4).

25. It is important to use a swinging bucket rotor when spinning the NEs through the sucrose cushion at this point in order to float any chromatin that is released away from the NEs.

26. The supernatant may appear cloudy, but this is mostly chromatin that should give a dark, worm-like appearance under the microscope.

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

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References

1. Hetzer, M. W., Walther, T. C., and Mattaj, I. W. (2005) Pushing the envelope: structure, function, and dynamics of the nuclear periphery. *Annu Rev Cell Dev Biol* **21**, 347-380
2. Mattout, A., Dechat, T., Adam, S. A., Goldman, R. D., and Gruenbaum, Y. (2006) Nuclear lamins, diseases and aging. *Curr Opin Cell Biol* **18**(3), 335-341
3. Schirmer, E. C., and Gerace, L. (2005) The nuclear membrane proteome: extending the envelope. *Trends Biochem Sci* **30**(10), 551-558
4. Roux, K. J., and Burke, B. (2006) Nuclear envelope defects in muscular dystrophy. *Biochim Biophys Acta*
5. Wilkie, G. S., and Schirmer, E. C. (2006) Guilt by association: the nuclear envelope proteome and disease. *Mol Cell Proteomics* **5**(10), 1865-1875
6. Starr, D. A., and Han, M. (2002) Role of ANC-1 in tethering nuclei to the actin cytoskeleton. *Science* **298**(5592), 406-409
7. Crisp, M., Liu, Q., Roux, K., Rattner, J. B., Shanahan, C., Burke, B., Stahl, P. D., and Hodzic, D. (2006) Coupling of the nucleus and cytoplasm: role of the LINC complex. *J Cell Biol* **172**(1), 41-53
8. Houben, F., Ramaekers, F. C., Snoeckx, L. H., and Broers, J. L. (2006) Role of nuclear lamina-cytoskeleton interactions in the maintenance of cellular strength. *Biochim Biophys Acta* **(**in press**)**, doi:10.1016/j.bbamcr.2006.1009.1018
9. Mattout-Drubezki, A., and Gruenbaum, Y. (2003) Dynamic interactions of nuclear lamina proteins with chromatin and transcriptional machinery. *Cell Mol Life Sci* **60**(10), 2053-2063

10. Stuurman, N., Heins, S., and Aebi, U. (1998) Nuclear lamins: their structure, assembly, and interactions. *J Struct Biol* **122**(1-2), 42-66

11. Blobel, G., and Potter, V. R. (1966) Nuclei from rat liver: isolation method that combines purity with high yield. *Science* **154**(757), 1662-1665

12. Aaronson, R. P., and Blobel, G. (1975) Isolation of nuclear pore complexes in association with a lamina. *Proc Natl Acad Sci U S A* **72**(3), 1007-1011

13. Dwyer, N., and Blobel, G. (1976) A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. *J Cell Biol* **70**(3), 581-591

14. Gerace, L., Ottaviano, Y., and Kondor-Koch, C. (1982) Identification of a major polypeptide of the nuclear pore complex. *J Cell Biol* **95**(3), 826-837

15. Florens, L., Korfali, N., and Schirmer, E. C. (2006) Subcellular Fractionation and Proteomics of Nuclear Envelopes. In: Methods in Molecular Biology pp (in press). Humana, Totowa, NJ

16. Korfali, N., Fairley, E. A., Swanson, S. K., Florens, L., and Schirmer, E. C. (2006) Use of Sequential Chemical Extractions to Purify Nuclear Membrane Proteins for Proteomics Identification. In: Methods in Molecular Biology pp (in press).

17. Kuehl, L. (1977) Isolation of skeletal muscle nuclei. *Methods Cell Biol* **15**, 79-88

18. Kuehl, L. (1975) Isolation of skeletal muscle nuclei. *Exp Cell Res* **91**(2), 441-448

19. Hahn, C. G., and Covault, J. (1990) Isolation of transcriptionally active nuclei from striated muscle using Percoll density gradients. *Anal Biochem* **190**(2), 193-197

20. Liew, C. C., Jackowski, G., Ma, T., and Sole, M. J. (1983) Nonenzymatic separation of myocardial cell nuclei from whole heart tissue. *Am J Physiol* **244**(1), C3-10

21. Dreger, M., Bengtsson, L., Schoneberg, T., Otto, H., and Hucho, F. (2001) Nuclear envelope proteomics: novel integral membrane proteins of the inner nuclear membrane. *Proc Natl Acad Sci U S A* **98**(21), 11943-11948.

22. Cronshaw, J., Krutchinsky, A., Zhang, W., Chait, B., and Matunis, M. (2002) Proteomic analysis of the mammalian nuclear pore complex. *J Cell Biol* **158**(5), 915-927

23. Schirmer, E. C., Florens, L., Guan, T., Yates, J. R. r., and Gerace, L. (2003) Nuclear membrane proteins with potential disease links found by subtractive proteomics. *Science* **301**(5638), 1380-1382

24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, New York

25. Aaronson, R. P., and Blobel, G. (1974) On the attachment of the nuclear pore complex. *J Cell Biol* **62**(3), 746-754

Figure Legends

Figure 1

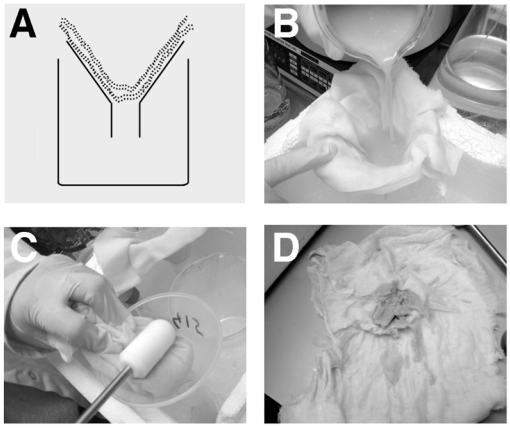

Figure 1. Filtration of muscle homogentate

(A) In a cold room, arrange 4 layers of cheesecloth in a funnel suspended in a beaker.

(B) 100 – 250 ml of homogenate is poured into cheesecloth and allowed to drain into beaker.

(C) Cheesecloth is squeezed to recover remaining homogenate, using a pipette or similar to roll towards bottom of funnel.

(D) A large amount of fibrous material should remain in cheesecloth after all liquid has been recovered.

Figure 2

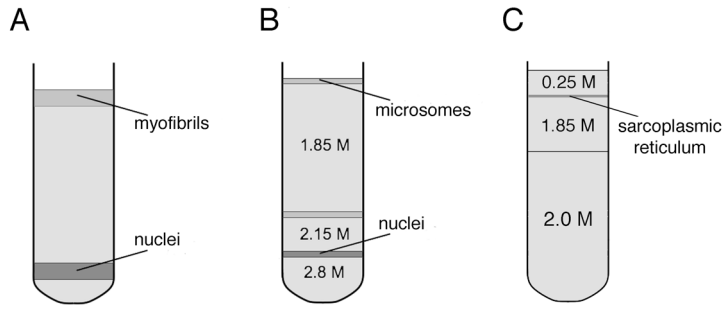

Figure 2. Position of fractions in Percoll and sucrose gradients.

(A) After centrifugation at 27,000 x g, the Percoll gradient separates nuclei and myofibrils. Nuclei form a layer at or near the bottom of the tube.

(B) After centrifugation of the discontinuous sucrose gradient at 82,000 x g, purified nuclei will form a layer at the interface of the 2.8 M / 2.15 M sucrose. Other cellular components are separated by the gradient – microsomes float on top of the 1.85 M sucrose, cytoplasmic contaminants form a layer at the 1.85 / 2.15 M sucrose and any remaining dense myofibrils pellet to the bottom of the 2.8 M sucrose.

(C) After centrifugation at 57,000 x g, microsomal membranes derived from sarcoplasmic reticulum collect at the interface of the 0.25 M / 1.85 M sucrose layers.

Figure 3

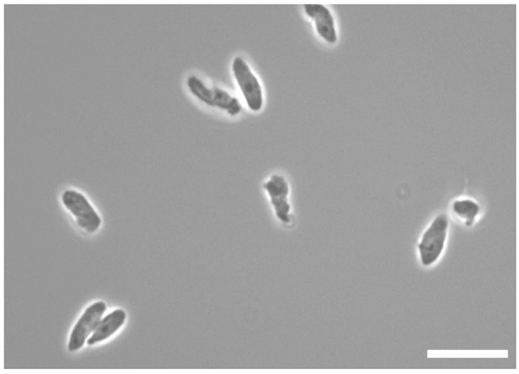

Figure 3. Light micrograph showing purified muscle nuclei viewed with phase-contrast at 400 x magnification. Scale Bar = 20 μm.

Figure 4

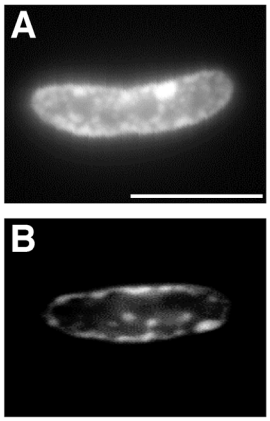

Figure 4. Removal of chromatin from purified muscle nuclei.

(A) Muscle nucleus stained with Hoesht 33342 and imaged with epifluorescence microscope at 1000 x magnification. Scale Bar = 10 μm.

(B) Muscle nucleus imaged exactly as in (A) but after digestion with DNase I / RNase A and washing in 300 mM NaCl. The majority of chromatin has been removed, except that which is tightly associated with the nuclear envelope.

Figure 5

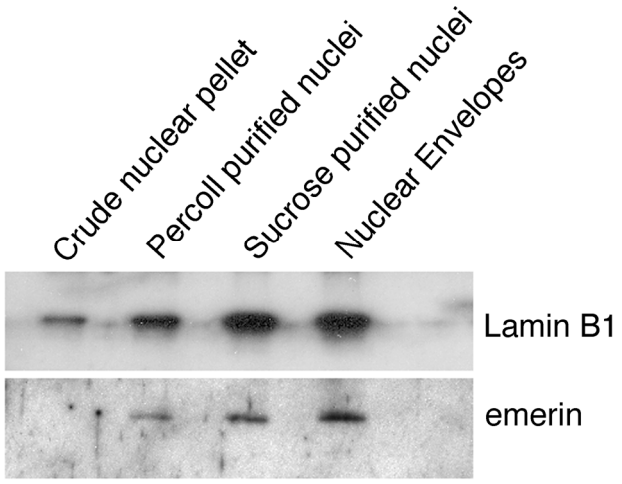

Figure 5. Western blot of purified fractions.

An equal amount of total protein was loaded in each lane and subjected to SDS-PAGE and Western analysis. Lamin B1 (a component of the nuclear lamina) and emerin (an integral inner nuclear membrane protein) are both enriched during the purification of muscle nuclear envelopes.

1. Crude nuclear pellet (1000 x g pellet of muscle homogenate).
2. Purified nuclei after Percoll gradient.
3. Purified nuclei after sucrose gradient.
4. Purified nuclear envelopes after chromatin removal.