

Chapter 2

Purification of Nuclei and Preparation of Nuclear Envelopes from Skeletal Muscle

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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 intransigent 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 because 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 ref. (*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, because 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 with 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 with kidney (*17*). Secondly, muscle fibers are extremely tough and homogenization procedures must be chosen with care 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 copurify with nuclei.

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 in which the muscle nuclei are floated on a 2.8M sucrose cushion instead of just being pelleted through 2M sucrose improved purity, because it separates dense contaminants such as any remaining myofibrils 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)

1. This procedure has been developed using the hind leg muscles of 6- to 10-week-old rats (e.g., Sprague-Dawley 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 six rats, although yields may vary a further two-fold in either direction (*see Note 1*).
3. Guillotine or equivalent local method for euthanizing animals.
4. Dissection scissors, forceps/ tweezers, scalpels, single-sided razor blades and bone scissors (kitchen scissors that will cut chicken bones are adequate).
5. Two beakers on ice, one containing phosphate-buffered saline (PBS) and one containing homogenization buffer.
6. Appropriate materials for covering surfaces during the procedure and for cleaning and waste disposal.
7. PBS: 4.3 mM sodium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, and 1.4 mM potassium phosphate, adjusted to pH 7.4 with HCl. Store at room temperature. Two hundred and fifty milliliters of PBS is sufficient for washing and collecting leg muscles from six rats.

2.2 Purification of Nuclei from Skeletal Muscle

2.2.1 Hardware

1. Standard meat mincer, as can be purchased in local grocery or hardware stores.
2. Potter-Elvehjem homogenizer with a motor-driven Teflon pestle providing 0.1- to 0.15-mm clearance and a drive motor capable of 1,000 rpm (e.g., Potter S

- homogenizer motor 853 3032, 60mL homogenizer cylinder 854 2600, and PFTE Plunger 854 3003 from Sartorius or equivalent).
3. Loose fitting (Wheaton type B pestle) glass Dounce homogenizer with a clearance of between ~0.1 and 0.15 mm.
 4. Swinging-bucket floor-model centrifuge and rotor capable of spinning 50-mL clear tubes to speeds of $27,000\times g$ for Percoll gradients (e.g., Beckman J-25 centrifuge with a JS 13.1 rotor and Nalgene 3110-9500 50-mL centrifuge tubes, *see Note 2*).
 5. Swinging-bucket ultracentrifuge and rotor capable of $82,000\times g$ (e.g., Beckman Coulter SW28 rotor with Beckman Coulter 344058 Ultra-Clear 25×89 -mm centrifuge tubes) for sucrose gradients.
 6. Standard light microscope, with phase contrast if possible; and glass slides and coverslips.
 7. Assorted 500–1,000 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 50×20 cm are required for 500 mL of muscle homogenate.
 10. Large-bore Luer-lock stainless steel needles (e.g., 14 gauge) of length greater than the 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 MgCl₂ (*see Note 4*).

1. Protease inhibitor cocktail, e.g., Sigma P8340 (*see Note 5*), should be freshly added to all solutions at a 1:250 dilution (*see Note 6*). If general protease cocktails are used, make certain that they do not contain ethylene-diamine tetraacetic acid (EDTA) because this interferes with steps of NE preparation (e.g., Mg²⁺ ions are required to stabilize chromatin and as a cofactor for DNase I).
2. Percoll (Sigma) should be diluted to a working stock of 81% (v/v) in 10 mM 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 dithiothreitol (DTT; from a 1 M solution in H₂O) immediately before use. For every gram of muscle, 2.5–5 mL of homogenization buffer is required (e.g., 100–200 g of minced muscle should be suspended in 500 mL of 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. For 100–200 g of starting material, 150 mL of Percoll gradient buffer is required.

5. HKM: 50 mM Hepes pH 7.4, 25 mM KCl, and 5 mM MgCl₂. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. For 100–200 g of starting material, 100 mL of HKM is required.
6. 0.25 M SHKM: 250 mM sucrose, 50 mM Hepes pH 7.4, 25 mM KCl, and 5 mM MgCl₂. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. For 100–200 g of starting material, 150 mL of 0.25 M SHKM is required.
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 MgCl₂. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. For 100–200 g of starting material, 20 mL of 2.15 M SHKM is required.
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 MgCl₂. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. For 100–200 g of starting material, 100 mL of 2.3 M SHKM is required.
9. 2.5 M sucrose: Dissolve 855.75 g of sucrose in a total volume of 1 L 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 MgCl₂. Store at room temperature. Add 2 mM DTT and protease inhibitors and chill on ice immediately before use. For 100–200 g of starting material, 20 mL of 2.8 M SHKM is required.

2.3 Preparation of Nuclear Envelopes

1. The same hardware is required as for preparation of nuclei, *see Section 2.2.1*.
2. DNase I (e.g., Sigma cat. D4527) at 10 U/μL in H₂O. Store at –20°C.
3. RNase A (e.g., Sigma cat. R4875) in H₂O at 10 mg/mL, boiled for 20 min. Store at –20°C.
4. 10% SHM buffer: 0.3 M sucrose, 10 mM Hepes pH 7.4, 2 mM MgCl₂, and 0.5 mM CaCl₂ (*see Note 4*). Store at 4°C. Add 2 mM DTT and protease inhibitors before use. For 20 million nuclei, 50 mL of 10% SHM buffer is required.
5. 30% SHKM buffer: 0.9 M sucrose, 10 mM Hepes pH 7.4, 25 mM KCl, and 2.5 mM MgCl₂. Store at 4°C. Add 2 mM DTT and protease inhibitors before use. Two milliliters of 30% SHKM buffer 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

1. The same hardware is required as for preparation of nuclei.
2. A Type 45 Ti fixed-angle ultracentrifuge rotor or equivalent that can provide 150,000×g, and matching tubes.

3. The same sucrose solutions used for preparation of nuclear envelopes can be used in preparing microsomes. In particular, 2.8M SHKM and 0.25M SHKM will be required.

2.5 *Determining the Purity/Quality of Fractions*

2.5.1 Fluorescent Staining of Chromatin

1. Hoechst 33342 or 4,6-diamidino-2-phenylindole (DAPI) (e.g., Molecular Probes) for fluorescent labeling of chromatin.
2. An epifluorescence microscope capable of at least $\times 400$ 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 Western Analysis of Fractions

1. Standard labware for sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blotting.
2. Antibodies to lamins, characterized integral NE proteins, and endoplasmic reticulum proteins. Among the best lamin antibodies are 119D5-F1 against lamin B1 (MAB3213; Chemicon Europe, Chandlers Ford, UK) and 131C3 against lamin A/C (MAB3538; Chemicon). For endoplasmic reticulum proteins, a number of companies sell antibodies to calnexin or calreticulin, but we do not have personal experience with these antibodies.
3. An assay system for estimating protein concentration in purified fractions (e.g., Bradford reagent and a spectrophotometer capable of measuring absorbance at 595 nm).

3 Methods

If NEs are being prepared from muscle, the entire procedure can take 8 or 9 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 killing and dissecting the animals should be done quickly, and it is very helpful to have assistance at this point.

1. Kill the rats according to local animal protocols.
2. Pull up the skin in the dorsal posterior area and cut through the 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 the skin 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 the hind legs by cutting through the hip joint with bone scissors.
5. Rinse legs in ice-cold PBS to wash off hair and blood.
6. Remove the muscle tissue from the bone using a single-sided razor blade. Grasp the 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 cleanup.

3.2 Purification of Muscle Nuclei

The first step in NE enrichment is the isolation of nuclei. Although 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 the nuclei. An extra Percoll gradient step is required to remove contaminating muscle fibers from the homogenate. Finally, the nuclei are spun through a three-step sucrose gradient in the ultracentrifuge (in contrast to the two-step sucrose gradients used in most other nuclear purification protocols). This floats microsomal membranes and pellets any dense contaminants, while allowing purified nuclei to collect on a 2.8M 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.

3. Add additional ice-cold homogenization buffer to minced muscle to achieve a final volume of between 2.5 and 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 a 55-mL Potter-Elvehjem homogenizer and homogenize at 900–1,000 rpm on ice, bringing the pestle to the bottom at least eight times (*see Note 11*). If the total volume exceeds 55 mL, homogenize in batches and collect the 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 a funnel. Pour 100–200 mL of crude homogenate through the cheesecloth and collect the 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 (Fig. 2.1; *see Note 12*). 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 (Fig. 2.1). This is rich with collagen, connective tissue, and large contractile filaments.
5. Transfer the filtrate to centrifuge tubes (e.g., Falcon conical centrifuge tubes, 50-mL capacity) and pellet nuclei at 1,000×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 because the pellets are very soft. Keep this supernatant if sarcoplasmic reticulum membranes (*see Section 3.4*) are to be prepared at the same time, because they are contained in this fraction (*see Note 13*).
7. Resuspend the crude nuclear pellets in ice-cold Percoll gradient buffer. Reduce the volume of the original homogenate fivefold (e.g., from 500 mL of muscle homogenate, resuspend the nuclear pellets in 100 mL of Percoll gradient buffer).
8. Pellet nuclei again at 1,000×g in a swinging bucket rotor for 10 min at 4°C. Remove the supernatant carefully, because the 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 greater than 40 mL. Centrifuge at 27,000×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 (Fig. 2.2a), which can be aspirated away with a pipette. Nuclei should band near the bottom of

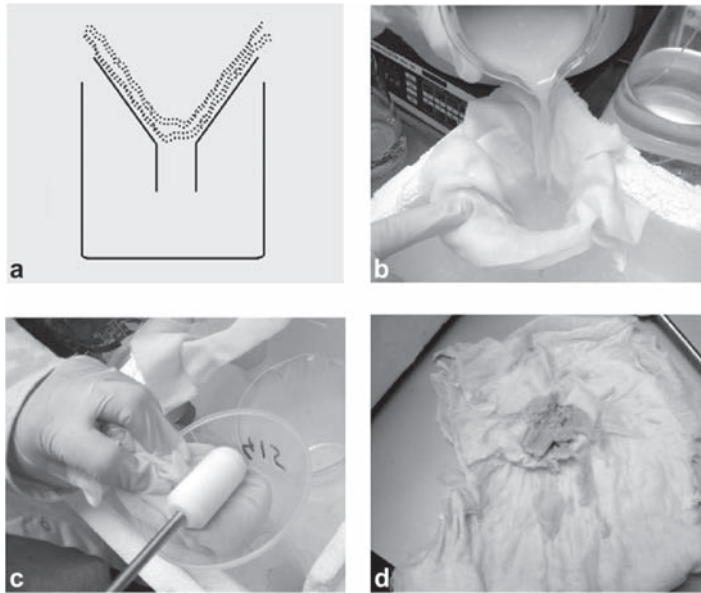


Fig. 2.1 Filtration of muscle homogenate. **a** In a cold room, arrange four layers of cheesecloth in a funnel suspended in a beaker. **b** 100–250 mL of homogenate is poured into the cheesecloth and allowed to drain into a beaker. **c** The cheesecloth is squeezed to recover remaining homogenate, using a pipette or similar tool to roll toward the bottom of the funnel. **d** A large amount of fibrous material should remain in the cheesecloth after all liquid has been recovered

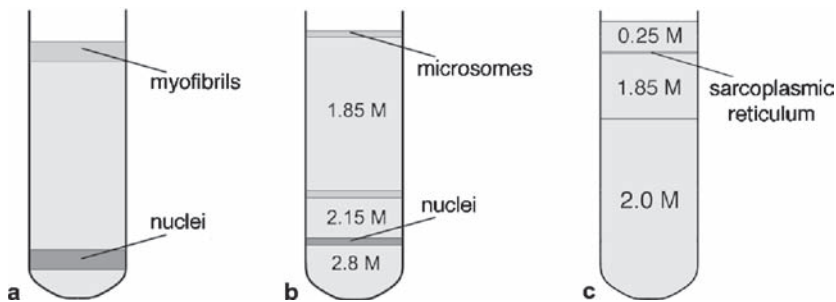
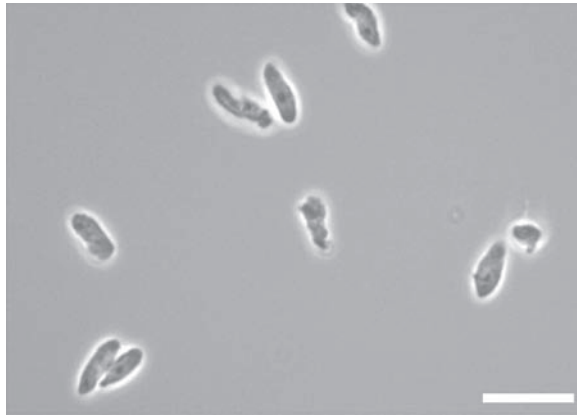


Fig. 2.2 Position of fractions in Percoll and sucrose gradients. **a** After centrifugation at $27,000 \times g$, the Percoll gradient separates nuclei and myofibrils. Nuclei form a layer at or near the bottom of the tube. **b** After centrifugation of the discontinuous sucrose gradient at $82,000 \times g$, purified nuclei will form a layer at the interface between $2.8 M$ and $2.15 M$ 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 interface, and any remaining dense myofibrils pellet to the bottom of the $2.8 M$ sucrose. **c** After centrifugation at $57,000 \times g$, microsomal membranes derived from sarcoplasmic reticulum collect at the interface of the $0.25 M$ and $1.85 M$ sucrose layers

the tube and can be collected with a clean pipette. Check under the microscope that the fraction collected is enriched in muscle nuclei, 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 tenfold with ice-cold 0.25 M SHKM and centrifuge in 50-mL conical tubes at $4,000\times g$ for 20 min at 4°C in a swinging bucket rotor. Remove the supernatant with care. The pink-colored pellet contains nuclei, microsomal membranes, and other cytoplasmic contaminants.
13. Resuspend the pelleted nuclei in 11 mL of ice-cold 0.25 M SHKM. Use a Dounce homogenizer with a loose-fitting pestle to break up any aggregated nuclei (*see Note 14*). Bring the 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 the nuclear suspension with 5 mL of ice-cold 2.15 M SHKM and then underlay this with 5 mL of ice-cold 2.8 M SHKM, using a Luer-lock syringe with a 14-gauge needle (*see Note 15*). Handle the tubes with care to avoid mixing the layers.
15. Balance the tubes and centrifuge in a SW28 swinging bucket ultracentrifuge rotor for 60 min at $82,000\times g$ (25,000 rpm) at 4°C (*see Note 16*).
16. The pink layer at the top of the tube (Fig. 2.2b) 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 (Fig. 2.2b). 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 17*).
17. Dilute the nuclei tenfold in ice-cold HKM buffer and centrifuge at $4,000\times g$ for 20 min at 4°C in a swinging bucket rotor.
18. The grey pellet (if visible) contains purified muscle nuclei. Aspirate the supernatant carefully, because the pellet may be soft. Resuspend the purified nuclei in 10 mL of ice-cold 0.25 M SHKM and use a hemocytometer and a phase contrast microscope to estimate the number of nuclei and to check their relative purity. The nuclei should be free of contaminants and should display the characteristic elongated shape of muscle nuclei (Fig. 2.3).
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 $4,000\times g$ 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 .

Fig. 2.3 Light micrograph showing purified muscle nuclei viewed with phase contrast at $\times 400$ magnification. Scale bar, $20\ \mu\text{m}$



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 with nuclei from soft tissues, at least in part because muscle nuclei do not swell appreciably in hypotonic 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 on grams of tissue.

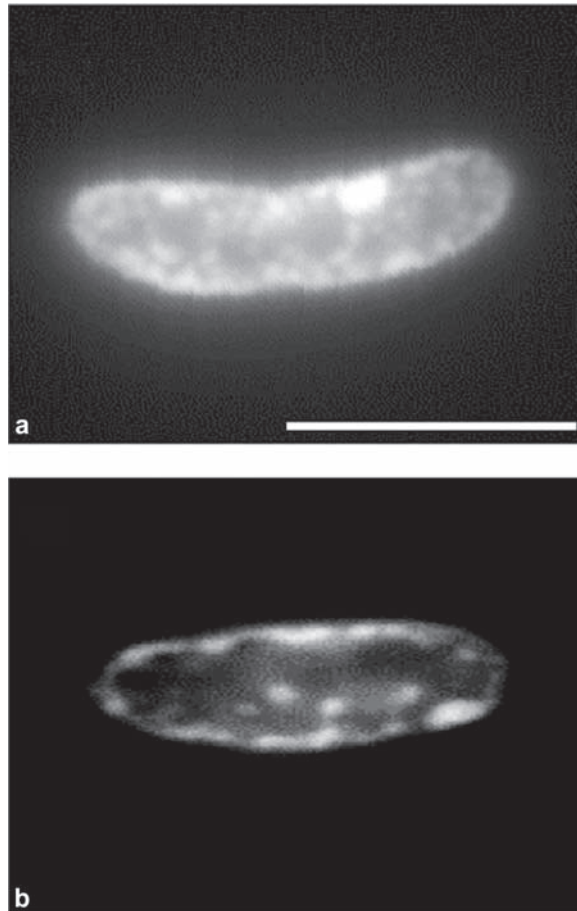
1. Resuspend purified muscle nuclei in ice-cold 10% SHM (*see Note 18*) at a concentration of 1–2 million nuclei/mL. Use a Dounce homogenizer with a loose-fitting pestle to break up any aggregated nuclei.
2. Withdraw a small sample of nuclei that will not be digested, for comparison with the digested material after salt washes.
3. Add DNase I to 10 U/mL and RNase A to 1.4 $\mu\text{g/mL}$ (*see Note 19*). Incubate on ice for 20 min to begin chromatin digestion (*see Note 20*).
4. Centrifuge at $4,000\times g$ for 20 min at 4°C in a swinging bucket rotor to pellet the nuclei. Discard the supernatant.
5. Resuspend the 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 U DNase I/mL and 5 μg RNase A/mL for 20 min on ice (*see Note 21*). In contrast to nuclei from other cell types, chromatin removal cannot be monitored by phase contrast microscopy (*see Note 22*).
6. Centrifuge at $4,000\times g$ for 20 min at 4°C in a swinging bucket rotor to pellet the nuclei. Discard the supernatant and resuspend nuclei in 10 mL of ice-cold 10% SHM supplemented with 300 mM NaCl to wash out digested chromatin (*see Note 23*).

7. Transfer NEs in 10% SHM/300 mM NaCl to a clear round-bottomed centrifuge tube (e.g., a 15-mL 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 24*) at $6,000\times g$ for 30 min at 4°C (e.g., 6,200 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, because it contains histones and chromatin (*see Note 25*). Aspirate the supernatant very carefully (do not decant by pouring) because the NE pellet is very soft.
9. Because, unlike in nuclei from most other tissues, no clear phase-dark to phase-lucent transition occurs in 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*). After washing in 300 mM NaCl, chromatin should be almost completely removed leaving only a small amount of staining closely associated with the NE (Fig. 2.4). The fluorescence intensity should also be greatly diminished in the digested sample compared with undigested nuclei. If a large amount of chromatin remains, pellet the nuclei and repeat the digestion (go back to Step 4).
10. Centrifuge at $4,000\times g$ 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 $4,000\times g$ 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 $6,000\times g$ 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 the muscle homogenate (step 6 in **Section 3.2**, Purification of Muscle Nuclei) and add EDTA to a final concentration of 0.5 mM to inhibit metalloproteases. Centrifuge at $10,000\times g$ 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 $100,000\times g$ for 45 min at 4°C (e.g., 36,000 rpm in a Beckman Type 45 Ti fixed angle ultracentrifuge rotor, *see Note 26*). This step pellets the microsomes derived from sarcoplasmic reticulum membranes, reducing the soluble protein

Fig. 2.4 Removal of chromatin from purified muscle nuclei. **a** Nucleus stained with Hoechst 33342 and imaged with an epifluorescence microscope at $\times 1,000$ magnification. Scale bar, $10\mu\text{m}$. **b** Muscle nucleus imaged exactly as in **(a)** but after digestion with DNase I and RNase A and washing in 300mM NaCl. The majority of chromatin has been removed, except that which is tightly associated with the nuclear envelope



content and lowering the volume required for subsequent fractionation in sucrose gradients.

3. Resuspend each crude microsomal pellet in a small volume (e.g., 5 mL) of ice-cold 0.25M SHKM by pipetting. Make the sucrose concentration up to 2M by adding 2.7 volumes of ice-cold 2.8M SHKM (e.g., add 13.5 mL of 2.8M 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 2M SHKM to SW28 ultracentrifuge tubes on ice. Overlay each tube with 7 mL of ice-cold 1.85M SHKM and 3 mL of 0.25M SHKM. Centrifuge at $57,000\times g$ 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 upward into the less dense sucrose, while 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 (Fig. 2.2c) and have a fluffy yellow-brown appearance, if visible. The microsomal band can be recovered by aspiration with a syringe, either by puncturing the tube with a needle or by inserting the needle through the upper phase.
6. Dilute the purified microsomes with four volumes of 0.25 M SHKM and pellet them at $152,000\times g$ 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 the microsomal pellets into preweighed tubes suitable for freezing (e.g., 1.5-mL Eppendorf tubes) and calculate the additional mass due 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 and washing with 300 mM NaCl (step 9 in **Section 3.3**). Place the samples in suitable small tubes (e.g., 1.5-mL Eppendorf tubes) and pellet at $5,000\times g$ for 10 min at 4°C in a microcentrifuge.
2. Resuspend the nuclei in a small volume (e.g., 50–100 μL) of 10% SHM containing DAPI or Hoechst 33342 (25 $\mu\text{g}/\text{mL}$). Mix well, pipette 5–10 μL of each sample onto a glass slide, and place a coverslip on top. Seal the edges with nail varnish.
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 (Fig. 2.4).

3.5.2 *Western Blot 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 (Fig. 2.5).

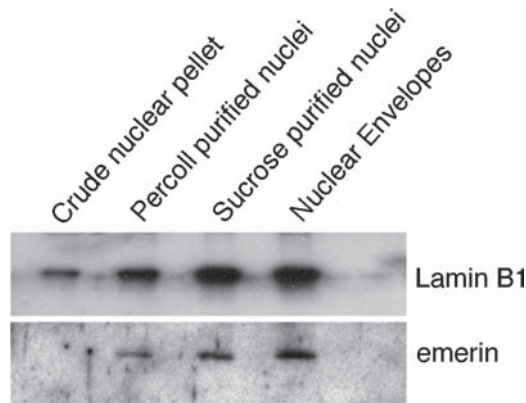


Fig. 2.5 Western blot of purified fractions. An equal amount of protein was loaded in each lane and subjected to SDS-PAGE and Western blot analysis. Lamin B1 (a component of the nuclear lamina) and emerlin (an integral inner nuclear membrane protein) are both enriched during the purification of muscle nuclear envelopes. Lanes show from left to right: crude nuclear pellet (1,000×g pellet of muscle homogenate); purified nuclei after the Percoll gradient; purified nuclei after the sucrose gradient; and purified nuclear envelopes after chromatin removal

1. Take small samples of material from throughout the purification procedure, for example, 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 6,000×g for 10 min at 4°C in a microcentrifuge. Discard the supernatant containing soluble proteins.
3. Nuclear lamins and transmembrane proteins tend to be insoluble. Resuspend the pellets in a small volume (e.g., 50 µL) of PBS + 0.1% (v/v) 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 6,000×g for 2 min. Transfer the supernatant to a new tube.
5. Estimate the protein concentration of each sample (e.g., by Bradford assay), and normalize the samples to contain equal amounts of total protein.
6. Resolve proteins by electrophoresis 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 (Fig. 2.5).

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 produce 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). We have found that a swinging bucket rotor (e.g., Beckman JS13.1) gives superior separation of myofibrils and nuclei.
3. Muslin can be used in place of cheesecloth if not chemically treated: make certain to ask the supplier.
4. The 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, reducing 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. Phenylmethylsulfonyl fluoride (PMSF) or 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (both from Sigma-Aldrich), a protease inhibitor with similar activity to PMSF but water soluble and less toxic, may be used to inhibit proteases in the initial homogenization step to reduce costs, because the volume is likely to be high.
7. Do not add spermine or spermidine to the 81% Percoll solution, because this tends to encourage the formation of a precipitate. The spermine and spermidine contained in the homogenization and gradient buffers 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 because chromatin is stabilized with spermine/spermidine.
9. The 2.3 M SHKM can be prepared by adding 230 mL of a 2.5 M sucrose stock to 12.5 mL of 1 M Hepes, 6.25 mL of 1 M KCl, and 1 mL of 1 M MgCl_2 , and freshly added 2 mM DTT and protease inhibitors. The 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 HKM, if required.
10. The 2.8 M SHKM can be prepared by dissolving 240 g of sucrose in a total volume of 250 mL 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 deteriorate rapidly due to precipitation of sucrose, which 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. This requires a reasonable amount of physical strength and one must take care to keep the homogenizer parallel 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.
 12. It is important to let most of the liquid drain through the cheesecloth before beginning squeezing, because 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.
 13. If the intent is to prepare sarcoplasmic reticulum membranes at a later time, the postnuclear 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.
 14. Use of a Dounce homogenizer at this step is important to reduce traces of sarcoplasmic reticulum membrane still adhering to the nuclei.
 15. 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 procedure can be performed in 30 sec. It is important to use a Luer-lock syringe because the viscosity of the solution can produce a high pressure on the connection.
 16. 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.
 17. The nuclei collected from the interface of the 2.15 *M*/2.8 *M* sucrose layer should be examined under a microscope to check their 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 to efficiently purify nuclei from different muscle types or from organisms other than rats.
 18. 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, in contrast to muscle or blood nuclei for those familiar with these procedures.

19. Micrococcal (S7) nuclease (e.g., Worthington cat. 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 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$, respectively.
20. Chromatin digestion continues during centrifugation steps. Therefore, the centrifugation step should proceed even in the absence of any evident chromatin removal.
21. Two subsequent digestions appear to be necessary to break chromatin into small enough pieces to be readily removed through the still-intact NEs.
22. In contrast to liver or lymphocyte nuclei, the grey appearance of muscle nuclei under phase contrast microscopy does not change, because chromatin appears to remain in myonuclei after nuclease digestion (*see Note 23*).
23. The 300 mM NaCl wash is essential to wash chromatin out of nuclei. However, do not add stock 5 M NaCl directly to nuclei in 10% SHM because 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 Hoechst 33342 or DAPI after the 300 mM NaCl wash step (Fig. 2.4).
24. It is important to use a swinging bucket rotor when spinning the NEs through the sucrose cushion at this point to float any chromatin that is released away from the NEs.
25. The supernatant may appear cloudy, but this is mostly chromatin that should give a dark, worm-like appearance under the microscope.
26. The type 45 Ti rotor tubes must be filled near to the top or they can collapse.

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