

In Vitro Study of Nuclear Assembly and Nuclear Import Using *Xenopus* Egg Extracts

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Summary

Nuclear import is a critical process for the cell: molecules are selectively permitted into the nuclear interior where the sheltered genome resides. The process of nuclear import can be biochemically studied in vitro using nuclei reconstituted from *Xenopus* egg extract components and *Xenopus* sperm chromatin. This in vitro system allows for the visualization of nuclear import by monitoring the accumulation of fluorescent nuclear import substrates in the reconstituted nuclei. A powerful aspect of the system is that "biochemically mutant" nuclei can be readily generated, either by immunodepletion of proteins from or addition of proteins to the reaction. This ability allows ascertainment of the role of specific proteins in nuclear import.

Key Words: In vitro nuclear reconstitution; nuclear assembly; nuclear import; nuclear import substrate; nuclear pore; nuclear transport; nucleus; sperm chromatin; *Xenopus* egg extract.

1. Introduction

Study of the nucleus and its molecular functions has been greatly aided by a system capable of assembling nuclei in vitro (1–4). This system takes advantage of the fact that each *Xenopus* oocyte contains a very large nucleus with tens of millions of nuclear pores. As the interphase oocyte matures into the metaphase egg, it disassembles its large nucleus and pores into their component parts. Unfertilized *Xenopus* eggs thus serve as bountiful storehouses of disassembled nuclear components. In nature, these components would be used to assemble the nuclei needed for the thousands of cells formed in the early embryonic divisions.

Experimentally, when *Xenopus* eggs are lysed, the disassembled components can be combined with added deoxyribonucleic acid (DNA) or chromatin and will spontaneously form nuclear structures. *Xenopus* egg extract thus allows one to study the steps of nuclear assembly, including nuclear envelope and nuclear pore assembly. The assembled nuclei can further be used to study nuclear import in vitro. Transport is

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assayed by the accumulation of fluorescent nuclear import substrates in the reconstituted nuclei (5–8). The effect of individual cytosolic factors on nuclear import can be determined either by adding an excess amount of the protein to the extract or by removing the factor from the extract by immunodepletion (9,10).

In a different type of experiment, a specific structural component of the pore can be removed from the extract while in the disassembled state by immunodepletion (11–13). Assembly of nuclei lacking the component can then be performed in the immunodepleted egg extract. The resultant “biochemically mutant” nuclei can then be tested for function. The ability to assemble nuclei lacking a specific protein in vitro has been used to reveal proteins involved in nuclear import, nuclear envelope assembly, nuclear pore assembly, and DNA replication.

2. Materials

1. *Xenopus laevis* frogs, female and male.
2. Pregnant mare serum gonadotropin (12.5X stock; cat. no. 367222; Calbiochem, La Jolla, CA); 5000 U/2 mL sterile filtered water.
3. 1X Stock human chorionic gonadotropin (cat. no. CG-10; Sigma, St. Louis, MO); 10,000 U/10 mL sterile filtered water.
4. 27 × 0.5-in. Gage needles.
5. 18 × 1.5-in. Gage needles.
6. Plastic Pasteur pipet.
7. Egg lysis buffer (ELB): 250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 10 mM HEPES, pH 7.8.
8. Sucrose cushion: 1X ELB with 500 mM sucrose.
9. 10X Marc's Modified Ringer's solution (MMR) wash buffer: 1M NaCl, 20 mM KCl, 10 mM MgSO₄, 20 mM CaCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM HEPES, pH 7.8.
10. 2% Cysteine in water, pH 7.8.
11. 1000X Dithiothreitol (DTT): 1 M in water.
12. 1000X Cytochalasin B stock: 5 mg/mL in dimethylsulfoxide.
13. 200X Cycloheximide stock: 10 mg/mL in water.
14. 1000X Aprotinin/leupeptin stock: 10 mg/mL in water.
15. IEC clinical tabletop centrifuge.
16. Sorvall swinging bucket rotor (HB-4 or HB-6); adaptors for 18 × 100-mm tubes (cat. no. 00367; Sorvall, Asheville, NC).
17. Beckman TL100 ultracentrifuge with TLS55 rotor: 11 × 34-mm Ultra-Clear tubes (cat. no. 347356; Beckman, Fullerton, CA).
18. Tricaine methanesulfonate (MS-222; cat. no. E10521; Sigma).
19. 10X buffer X: 100 mM HEPES, pH 7.4, 0.8 M KCl, 150 mM NaCl, 50 mM MgCl₂, 10 mM ethylenediaminetetraacetic acid (EDTA).
20. Large-orifice 200-μL pipet tips (cat. no. 1011-8406; USA Scientific, Ocala, FL).
21. Energy mix stocks: 5 mg/mL creatine phosphokinase (cat. no. C3755; Sigma; in 10 mM HEPES, pH 7.5, 50% glycerol, 50 mM NaCl); 1 M phosphocreatine (cat. no. P7936; Sigma; in 10 mM KH₂PO₄, pH 7.0); 0.2 M adenosine triphosphate (cat. no. A2382; Sigma; in water, pH 7.0); store all frozen at –20°C.
22. Fix: 70 μL 16% paraformaldehyde plus 25 μL 1 M sucrose plus 1 μL 1 mg/mL Hoechst 33258 DNA dye (cat. no. 861405; Sigma).

23. 18 × 18 mm glass cover slips.
24. Zeiss Axioskop 2 microscope: 63× objective, connected to charge-coupled device (CCD) camera, images captured on computer.
25. Human serum albumin (HSA; cat. no. 126658; Calbiochem).
26. Tetramethylrhodamine isothiocyanate Isomer R (TRITC; cat. no. T3163; Sigma).
27. Synthetic nuclear localization sequence (NLS) peptide (CTPPKKKRKV).
28. MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; cat. no. 22310; Pierce, Rockford, IL).
29. Transport substrate buffer: 20 mM HEPES, pH 7.5, 50 mM KCl.
30. Desalting column: a Poly-Prep chromatography column (0.8 × 4 cm; cat. no. 731-1550; Bio-Rad, Hercules, CA) filled with 10 mL P-6 DG desalting gel (cat. no. 150-0738; Bio-Rad) hydrated with phosphate-buffered saline (PBS).
31. Bio-Rad protein assay: dye reagent concentrate (cat. no. 500-0006; Bio-Rad).
32. Protein A-Sepharose beads (cat. no. 17-528001; Amersham, Piscataway, NJ).

3. Methods

3.1. *Xenopus* Egg Extracts

In vitro-assembled nuclei can be reconstituted using cytoplasmic extracts of the eggs of female *X. laevis*, combined with chromatin from the demembrated sperm of male *X. laevis*. The reconstituted nuclei are capable of nuclear import as well as a complete round of DNA replication.

Female frogs are induced to lay unfertilized eggs (**Subheading 3.1.1.**). These eggs are then fractionated to obtain cytosol and membrane fractions (**Subheading 3.1.2.**). The cytosol fraction is a concentrated solution of egg proteins (~40 mg/mL). The high concentration of protein in *Xenopus* egg cytosol greatly facilitates biochemical studies, such as assaying the effect on nuclear assembly and nuclear import (**Subheadings 3.3. and 3.4.**) and immunodepletions to remove specific proteins of interest (**Subheading 3.5.**). The membrane fraction, when combined with the cytosol fraction, contributes membranes for forming nuclear membranes, endoplasmic reticulum networks, and annulate lamellae (cytoplasmic membrane stacks with nuclear pore complexes; **14–16**).

3.1.1. Priming Frogs and Inducing Egg Laying

To prime frogs for ovulation and to induce egg laying, female frogs are injected with hormones. After the frogs are used for egg laying, they are allowed to rest for approx 3 to 6 mo before being used again (*see* **Note 1**).

1. Prime each female frog with 0.5 mL pregnant mare serum gonadotropin (dilute 1 mL stock with 11.5 mL water to make a working dilution) 2 d to 2 wk before you wish to induce egg laying (*see* **Note 2**). Inject the frogs in the thigh region, underneath the skin, using a 27 × 0.5 in. gauge needle. Place them in a container with distilled water at room temperature.
2. Schedule the chorionic gonadotropin injection to induce egg laying approx 24 h before you wish to collect eggs. Preferably do this injection in the morning so that the eggs can be collected the following morning, and the remainder of the second day will be used for fractionating the egg extracts. Inject 0.5 mL human chorionic gonadotropin per frog in the thigh region underneath the skin using a 27 × 0.5 in. gauge needle. Place each frog in a separate container filled with distilled water containing 30 mM NaCl at room temperature (*see* **Note 3**).

3. After frogs have laid their eggs, transfer the frogs into a large storage bin of water. Keep the frogs in the storage bin overnight to allow them to finish laying eggs before returning them to their long-term housing. Collect laid eggs in 500-mL glass beakers. Do not mix eggs from different frogs.

3.1.2. Fractionation of *Xenopus* Egg Extracts

Two major fractions are isolated from *Xenopus* eggs: (1) the clear cytosol, which contains soluble proteins of the disassembled nuclei and soluble cytoplasmic proteins; and (2) the cloudy membrane fraction, which consists of endoplasmic reticulum, Golgi, and nuclear membranes (**12**).

1. Prepare these solutions fresh at room temperature before collecting eggs (*see Note 4*):
 - a. 2% Cysteine, pH 7.8: 100 mL per frog.
 - b. 1/4 X MMR wash buffer (prepared from 10X stock): 300 mL per frog.
 - c. ELB containing 1 mM DTT and 50 µg/mL cycloheximide: prepare 100 mL per frog.
2. Pour out the excess water from the collected eggs (*see Note 5*). At this point, the eggs are treated with 2% cysteine, pH 7.8, to remove the gelatinous coat around the eggs. For this, add 100 mL 2% cysteine, pH 7.8, into each beaker of eggs. Gently mix with a plastic Pasteur pipet until the eggs settle to bottom of beaker and the gelatinous coats of the eggs are removed. The eggs will occupy a smaller volume when dejellied. Let the eggs sit in the cysteine solution for a maximum of 5 min. If the gelatinous coats are not removed after 5 min, replace the solution with fresh cysteine solution. Pour out the cysteine solution after the eggs are dejellied. The eggs are very fragile after the gelatinous coat is removed, so use care in pouring solutions out of and into the beaker.
3. Wash the dejellied eggs three times with 1/4 X MMR wash buffer by swirling gently and pouring out the wash buffer.
4. Next, incubate the eggs in ELB with DTT and cycloheximide for 5 min. Cycloheximide inhibits protein synthesis, which includes inhibiting new cyclin synthesis. The eggs and the resulting extract are thus arrested in an interphase state.
5. Pour the eggs into a 14-mL plastic round-bottom tube. Remove as much of the buffer as possible with a plastic Pasteur pipet. This prevents the dilution of the extract. Spin in an IEC clinical tabletop centrifuge at 185 rcf (1350 rpm) for 30 s to pack the eggs (**Fig. 1A**). Keep the lysed eggs on ice at all times after this spin. Again, remove as much buffer from on top of the lysed eggs as possible. Add aprotinin/leupeptin and cytochalasin B to a final concentration of 1X to the top of the tubes of packed eggs to prevent protein degradation. No mixing is necessary.
6. Next, spin the packed eggs in a swinging bucket rotor (HB-4 or HB-6) at 13200 rcf (9000 rpm) for 20 min at 4°C. The eggs will separate into distinct layers: a top bright yellow layer that contains lipids, a middle golden layer containing cytoplasm and membranes, and a bottom dark layer that consists of pigment and yolk granules (**Fig. 1B**).
7. Using an 18 × 1.5 in. needle, puncture the tube just above the bottom dark layer (**Fig. 1B**). Quickly place a preassembled 10-mL syringe with an 18 × 1.5 in. needle into the hole and collect the middle golden layer and a bit of the top lipid layer for a total of approx 9.6 mL. This is termed *crude egg extract* and can be used directly for nuclear assembly as described in **refs. 18 and 19**. Transfer the crude extract to Ultra-Clear tubes (11 × 34 mm) for the TLS55 rotor (Beckman TL100 ultracentrifuge). Each tube holds a maximum of 2.4 mL. Spin the extract in a TLS55 rotor at 260,000 rcf (55,000 rpm) for 1.5 h at 2°C.

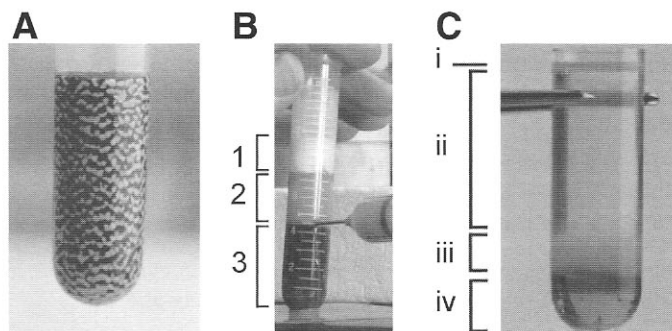


Fig. 1. (A) *Xenopus* eggs are packed. Notice that the vegetal (light) and animal (dark) poles of each egg are aligned in the same direction. (B) The eggs are separated into distinct fractions after centrifugation: the yellow lipid layer at the top (1), followed by the golden crude extract (2), which contains cytosol and membranes, and the black pigments and yolk granules at the bottom (3). A syringe is used to collect the crude extract by puncturing through the tube right above the bottom dark layer. (C) High-speed centrifugation of the golden crude extract results in the separation of the lipids (i, topmost thin yellow layer) from the cytosol (ii, clear layer) and membranes (iii, cloudy yellow layer). The gelatinous pellet with dark and clear layers at the bottom consists of mitochondria and glycogen (iv).

8. After centrifugation, separate cytosol and membrane fractions become apparent, topped with a thin lipid layer (see **Note 6**; **Fig. 1C**). Loosen the topmost yellow lipid layer with a thin micropipet tip and then aspirate this layer off using a large-orifice pipet tip. Next, remove the layer of clear cytosol with a 200- μ L pipet tip, avoiding the cloudy membrane layer below, by gently swirling while pipeting up. Collect all of the clear cytosol into a tube. Next, pipet the cloudy pale yellow membrane layer with a large-orifice pipet tip into a 15-mL plastic conical tube, avoiding the dark brown layer beneath it, which contains mitochondria. To salt wash the membranes, fill the tube to a total volume of 8 mL with a final concentration of 1X ELB and 0.5 M KCl. Keep the tube of salt-washed membranes on ice while the cytosol goes through another spin in the next step.
9. Spin the cytosol in Ultra-Clear tubes (11 \times 34 mm) at 260,000 rcf (55,000 rpm) for 30 min at 2°C in a TLS55 rotor to remove the majority of residual contaminating membranes (note that some will still remain; see Discussion in Harel, A. et al., 2003, **ref. 9**). Avoid pipeting up the residual membrane pellet at the bottom. Aliquot the cleared cytosol into 100- μ L aliquots and quick-freeze it in liquid nitrogen. Store at -80°C.
10. Next, the salt-washed membranes are isolated through a sucrose cushion. After placing 400 μ L sucrose cushion in each of four Ultra-Clear tubes (11 \times 34 mm), slowly layer 2 mL of the salt-washed membranes on top of the cushion. Spin at 77,000 rcf (30,000 rpm) for 30 min at 2°C in a TL100 ultracentrifuge. Remove most of the supernatant by suctioning, leaving the minimum amount of liquid (~10–20 μ L) at the bottom of the tube to allow membranes to be pipetted up. Any further dilution of the membranes at this stage will affect the nuclear reconstitution reaction because the cytosol is sensitive to dilution. Flick the tube to loosen the membranes. With the tube tilted, pipet up the membranes with a large-orifice pipet tip. Leave behind the dark-color pellet at the bottom of the tube. Aliquot the concentrated membranes (to be used as an ~20X stock) into 10- μ L aliquots and quick-freeze it in liquid nitrogen. Store at -80°C.

3.2. Demembrated Sperm Chromatin

Sperm chromatin from male frogs is used as a source of DNA for nuclear reconstitution reactions (3). Each sperm, when demembrated, releases a "packet" of chromatin containing all the chromosomes. These chromosomes remain associated and are capable of forming a functional nucleus that can import and replicate its DNA when incubated with egg cytosol and membranes.

All procedures should be carried out at room temperature unless stated otherwise.

1. Prepare these solutions a day before preparing the sperm chromatin because the high-concentration sucrose solutions take time to dissolve. Store at 4°C.

100 mL Buffer X plus 0.2 M sucrose

2 mL buffer X-0.2 M sucrose plus 0.4% TX-100 (add aprotinin/leupeptin and DTT fresh)

10 mL buffer X-0.2 M sucrose plus 3% BSA (add aprotinin/leupeptin and DTT fresh)

5 mL buffer X-0.5 M sucrose plus 3% BSA (add aprotinin/leupeptin and DTT fresh)

5 mL buffer X-2.0 M sucrose

10 mL buffer X-2.3 M sucrose

5 mL buffer X-2.5 M sucrose

2. Place four or five male frogs in an ice bath. The frogs are anesthetized in 1 g/L tricaine methanesulfonate dissolved in water, buffered with NaHCO₃ to a final pH of 7.0 to 7.5. Then, cervical dislocation is performed by a sharp blow to the neck, and the brain stem is ruptured by double pithing. Place the frog on its back and cut through the abdomen to expose the organs. Isolate the testes, which are white, oval-shaped structures about 0.5 to 1 cm long. Blot the blood off the testes by rolling on paper towels. Place the testes in a small Petri dish containing buffer X-0.2 M sucrose. Remove as much blood and fat as possible before mincing the testes in fresh buffer X-0.2 M sucrose. Mince the testes into small, 1- to 2-mm pieces by pulling with two pairs of sharp forceps. Transfer the minced testes and buffer into a 15-mL plastic conical tube. Rinse the Petri dish with 2 to 3 mL buffer X-0.2 M sucrose into the same conical tube.
3. The release of sperm from the testes is maximized by mashing the testes with a spatula in the conical tube. In addition, vortex vigorously for 1 min. Pellet the larger pieces of tissue at 100 rcf (1000 rpm) for 10 s in an IEC clinical tabletop centrifuge. Remove the supernatant containing released sperm to a new 15-mL conical tube. Add 2 to 3 mL of buffer X-0.2 M sucrose to the pellet, vortex for 1 min, and recentrifuge as above. Combine the supernatants into the 15-mL conical tube and repeat the extraction of the pellets two or three times until the supernatant is no longer cloudy. Centrifuge the combined supernatants in the 15-mL conical tube at 185 rcf (1350 rpm) for 50 s to pellet any remaining pieces of tissue. Transfer the supernatant, containing released sperm, to a 14-mL plastic, round-bottom tube. Pellet the sperm in a Sorvall swinging bucket rotor at 2600 rcf (4000 rpm) for 10 min at 4°C. Resuspend the sperm pellet very well in 0.8 mL of buffer X-2 M sucrose.
4. To separate the sperm from red blood cells, prepare sucrose step gradients in four Ultra-Clear tubes (11 × 34 mm) for the TLS55 rotor. To each tube add 0.25 mL buffer X-2.5 M sucrose. Slowly and gradually overlay with 1.7 mL buffer X-2.3 M sucrose. Be sure not to disturb the interface between the buffer X-2.5 M sucrose and buffer X-2.3 M sucrose layers. Next, overlay the sucrose gradient in each tube with 0.2 mL of the resuspended sperm. Stir the interface well between the sperm and the buffer X-2.3 M sucrose layer

with a Pasteur pipet tip. Spin in the TL100 ultracentrifuge at 93,000 rcf (33,000 rpm) for 25 min at 2°C.

5. Aspirate off the top half of the gradient containing contaminating red blood cells, which will fractionate at the interface between the 2 *M* and 2.3 *M* sucrose layers. Carefully transfer the entire lower half of the gradient containing the sperm to a 14-mL round-bottom tube. Be careful not to take any red blood cells left from the top half of the gradient. Rinse the bottom half of the tube with 1 mL buffer X–0.2 *M* sucrose three times and combine the washes with the sperm in the 14-mL round-bottom tube.
6. Dilute the sperm solution to a total volume of 12 mL with buffer X–0.2 *M* sucrose. Pellet the sperm by centrifugation in a Sorvall swinging bucket rotor at 4000 rcf (5000 rpm) for 10 min. The membranes surrounding the sperm and its nucleus are then removed by resuspending the sperm pellet in 1 mL buffer X, 0.2 *M* sucrose, 0.4% Triton X-100, 1X aprotinin/leupeptin, 1 mM DTT for 30 min on ice, with occasional agitation of the solution.
7. After demembranation of the sperm, residual Triton X-100 is removed by a series of spins. Prepare two 1.5-mL tubes containing 0.5 mL Buffer X with 0.5*M* sucrose, 3% BSA, 1X aprotinin/leupeptin, and 1 mM DTT. Overlay each sucrose cushion with half the demembranated sperm solution. Centrifuge in an IEC clinical tabletop centrifuge at 360 rcf (1900 rpm) for 10 min at room temperature.
8. Remove the supernatant and resuspend the sperm pellet in 0.1 mL buffer X, 0.2 *M* sucrose, 3% BSA, 1X aprotinin/leupeptin, and 1 mM DTT. Avoid the sides of the tubes, which may have residual Triton X-100. Transfer the sperm chromatin into a new 1.5-mL tube, dilute to 1 mL with the same buffer mix, and centrifuge in an IEC clinical tabletop centrifuge at 360 rcf (1900 rpm) for 10 min at room temperature. Repeat this wash two times.
9. Resuspend the sperm chromatin pellet in 0.5 mL buffer X with 0.2 *M* sucrose, 3% BSA, 1X aprotinin/leupeptin, and 1 mM DTT. Count the sperm using a hemocytometer. Dilute to 150,000 U sperm chromatin/microliter and 50,000 U sperm chromatin/microliter and freeze 5- μ L to 10- μ L aliquots in liquid nitrogen. Store at –80°C.

3.3. Reconstitution of Nuclei

In vitro nuclear reconstitution reactions using *Xenopus* egg extract and sperm chromatin are generally set up as described next (20). However, with different batches of extracts, the ratio of cytosol to membranes used to form functional nuclei may vary because of differences in preparing the extract and variation between the eggs of different frogs. Each batch of extract should be tested for the optimum ratio of cytosol to membranes to form nuclei. The shape and size of the nuclei, along with the functional test of nuclear import, are indications of how robust the nuclear reconstitution reaction is. The standard is to start with a ratio of 1 μ L of membranes to 20 μ L of cytosol and then to vary the amount of membranes from 0.5 to 2 μ L. The amount of sperm chromatin added can also be varied.

In this in vitro system, exogenous proteins can be added at the start of the reaction up to 10% of the final volume. Further dilution of the reaction, however, may decrease the extract's ability to form nuclei.

1. Quick-thaw a tube of cytosol (100 μ L), a tube of membranes (10 μ L), and a tube of sperm chromatin (~50,000 U sperm chromatin/microliter) at room temperature. Also, thaw components for the energy regeneration system at room temperature. Immediately place all tubes on ice after thawing.

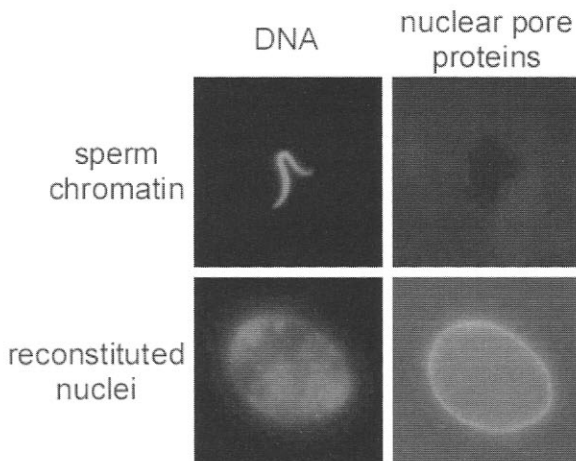


Fig. 2. Immunofluorescence of sperm chromatin and reconstituted nuclei, stained with Hoechst DNA dye and a tetramethylrhodamine -directly labeled monoclonal antibody against nuclear pore proteins (TAMRA-mAb414). mAb414 recognizes four nuclear pore proteins: Nup358, Nup214, Nup153, and Nup62. After incubation with cytosol, membranes, and energy, the sperm chromatin decondenses and acquires a nuclear envelope containing nuclear pores.

2. Prepare a fresh energy mix from the stock solutions using a ratio of 1 μL creatine kinase to 0.5 μL phosphocreatine to 0.5 μL adenosine triphosphate. Keep the energy mix on ice.
3. For each reaction, mix 20 μL cytosol with 1 μL energy mix and 2 μL ELB (or 2 μL exogenous protein) in a 0.5-mL tube. Then add 1 μL membranes. Immediately after adding the membranes, pipet the reaction four or five times to mix the membranes well (*see Note 7*). Avoid introducing air bubbles into the reaction. Sharp tapping of the tube on a hard surface helps remove air bubbles. Keep the reactions on ice until all the components have been added.
4. Last, add 1 μL sperm chromatin ($\sim 50,000$ U sperm chromatin/microliter) using a large-orifice pipet tip. After adding the sperm chromatin, any pipeting of the reaction is to be done with large-orifice pipet tips to prevent the shearing of the chromatin. Pipet up and down slowly, with the pipet set to about half the volume of the reaction. Let the reaction incubate at room temperature for 40 min to 1 h for complete nuclear assembly.
5. If reactions are to be assayed for nuclear import, *see Subheading 3.4*. If nuclei are only to be assessed for assembly and the growth of the nuclei, prepare fix and keep on ice. Aliquots of 2 μL can be removed from the reaction at intervals and mixed with 0.5 μL fix on a slide to assess the progress of assembly. Care should be taken when putting on the 18×18 mm glass cover slip so as not to damage the reconstituted nuclei (*see Note 8*). If samples are to be viewed later, fix the sample at a ratio of 2 μL fix to 8 μL sample (ideal volume for mixing with pipet) on ice. The fixed samples (2.5 μL /cover slip) should be viewed as soon as possible, within 1 to 2 h on ice. The samples cannot be stored at 4°C overnight. View the nuclei on a fluorescence microscope with a $63\times$ objective (*see Note 9; Fig. 2*).

3.4. Assaying Nuclear Import

Reconstituted nuclei are capable of robust nuclear import. This is assayed by the accumulation of nuclear import substrates in the reconstituted nuclei. Nuclear import substrates commonly used are either fluorescently labeled or green fluorescent protein (GFP) fusion proteins containing NLSs (7,8).

3.4.1. Preparing Nuclear Import Substrate

Nuclear import substrates are expressed recombinantly (GFP-nucleoplasmin) or, alternatively, synthesized by crosslinking NLS peptides to fluorescently labeled carrier protein (TRITC-NLS-HSA) (5,6). Both substrates are actively imported into reconstituted nuclei.

GFP fusion import substrates are expressed in bacteria, then purified via the protein's tag, such as a 6-His tag. Alternatively, NLS peptides can be covalently coupled onto carrier proteins that are conjugated to a fluorophore. The NLS peptide-carrier protein conjugate can be labeled with different fluorophores. The method to generate the second type of nuclear import substrate, specifically, NLS-HSA-TRITC, is described next.

1. To conjugate the fluorescent TRITC group to HSA, incubate 0.5 mL HSA (10 mg/mL in 0.1 M Na₂CO₃) with 30 μ L of 1 mg/mL TRITC at room temperature for 1 h in the dark. All subsequent reactions should be done in the dark, by covering the reaction with foil, to prevent the quenching of the fluorophore TRITC. Next, add 0.25 mL of 0.1 M sodium phosphate and 250 μ L MBS (10 mg/mL in dimethyl formamide) and incubate for 1 h at room temperature with rocking. This will quench the unreacted TRITC in the reaction.
2. Unreacted TRITC and MBS are removed by running the sample through a desalting column. Set up a desalting column with 10 mL P-6 DG desalting gel and equilibrate it with 0.1M sodium phosphate. Load the reaction onto the top of the column and use 0.1M sodium phosphate to run the column. Collect 1 mL for the first fraction, followed by 0.25-mL fractions. Pool the fractions of HSA-TRITC that have the most protein as determined by the Bio-Rad protein assay.
3. Dilute 2.5 mg of the synthesized NLS peptide (CTPPKKKRKV) with 475 μ L of 0.1 M sodium phosphate, pH 6.0. Add the NLS peptide solution drop by drop into the pooled fractions of HSA-TRITC in **step 2**. The solution may become turbid as more peptide is added, and precipitate can be seen after adding in the NLS solution. Incubate at room temperature with rocking for 1 h.
4. Uncoupled NLS peptides are then removed from TRITC-NLS-HSA by running the reaction through two desalting columns. Spin the reaction in an IEC clinical tabletop centrifuge at 2800 rpm for 1 min. Split the supernatant in two. Equilibrate two desalting columns (each with 10 mL P-6 DG desalting gel) with transport substrate buffer. Run each half of the supernatant over a separate column. Use 0.1M sodium phosphate to run the columns. Collect 1 mL for the first fraction, followed by 0.25-mL fractions. Pool fractions that have the most protein as determined by the Bio-Rad protein assay. Concentrate the transport substrate to a concentration of 1 mg/mL. Aliquot into 5- to 10- μ L aliquots and quick-freeze with liquid nitrogen. Store at -80°C .

3.4.2. Assaying Nuclear Import in Reconstituted Nuclei

1. Reconstitute nuclei as in **Subheading 3.3.**, up to **step 4**. After 40 min to 1 h, functional nuclei are formed.

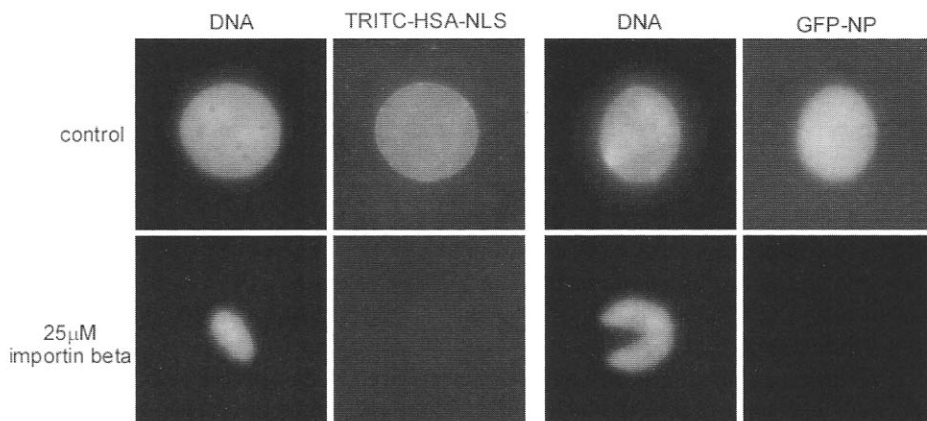


Fig. 3. Nuclear import of fluorescently labeled HSA conjugated to NLS peptide (TRITC-HSA-NLS) as well as GFP-nucleoplasmin (GFP-NP) are seen in control nuclei. The addition of 25 mM importin beta at the beginning of an assembly reaction, however, blocks the assembly of nuclear pores and thus prevents nuclear import (9).

2. Add 1.5 to 2 μ g import substrate to 10 μ L of the reconstituted nuclei. Classical nuclear import substrates include GFP-nucleoplasmin or TRITC-NLS-HSA. Mix the transport reaction gently two or three times with large-orifice pipet tips. Cover the tubes with foil to prevent the fluorescence of the import substrate from being quenched. Incubate the reaction at room temperature. Substantial import should be seen by 40 min.
3. Prepare fix and keep on ice. After the reaction is completed, fix the sample at a ratio of 2 μ L fix to 8 μ L sample. Keep the fixed nuclei on ice. View the nuclei with a fluorescence microscope as soon as possible (Fig. 3).

3.5. Assaying Nuclear Import in Nuclei Depleted of Specific Proteins

The function of particular proteins in nuclear assembly, nuclear pore assembly, or nuclear import can be elucidated by the removal of specific proteins from the cytosol by immunodepletion, followed by addition of membranes and sperm chromatin. The immunodepleted nuclei can then be assayed for various functions simultaneously, such as nuclear import, or for the ability to assemble a correct nucleus (11–13).

1. Incubate 300 to 600 μ g affinity-purified antibody to a protein of interest (or an equal amount of preimmune antibody) with 60 μ L protein A-Sepharose at 4°C overnight. Block the antibody beads with 20 mg/mL BSA in PBS buffer for 30 min at room temperature and wash two or three times with ELB buffer.
2. Immunodeplete or mock deplete 200 μ L of cytosol by incubating them with 30 μ L of the antibody-protein A-Sepharose two consecutive times, each time for 1 h at 4°C.
3. The immunodepleted and mock-depleted cytosol can be quickly frozen with liquid nitrogen in 20- μ L aliquots and stored at –80°C (see Note 10).
4. The immunodepleted or mock-depleted cytosols can then be used to form nuclei as in Subheading 3.3. Nuclear import can be assayed as described in Subheading 3.4.

4. Notes

1. The quality of extract varies with the time of the year. In the summer months, the frogs do not lay eggs as well. It is advisable, if possible, to make egg extracts during cooler months and freeze them for later use.
2. It is ideal for frogs to be primed with pregnant mare serum gonadotropin at least 2 to 3 d before injecting with chorionic gonadotropin to induce egg laying. If frogs are primed more than 2 wk before egg laying, apoptotic extracts will result (17).
3. The temperature of the distilled water plus 30 mM NaCl that is used for housing the frogs after the chorionic gonadotropin injection should not be warmer than room temperature. If the distilled water is warmer than room temperature, place the salt in the water and then cool the water by placing securely tied plastic bags filled with ice into the water. After the water has reached room temperature, remove the plastic bags with ice.
4. On the morning that the frogs have laid their eggs, check the number of frogs that have laid and the approximate volume of eggs each has laid. With these numbers in hand, the amount of each solution (2% cysteine, pH 7.8, 1/4 X MMR wash buffer, ELB containing DTT and cyclohexamide) needed for processing the eggs can more easily and accurately be prepared.
5. Avoid processing batches of eggs for which a majority of eggs that are entirely white (a sign of the eggs having been activated) or have lysed (causing the solution around them to be cloudy) at any stage before the eggs are lysed by centrifugation. In our experience, eggs that have activated before packing have generated extracts that did not assemble nuclei well or were apoptotic.
6. After spinning in the ultracentrifuge, the outside of the 11 × 34 mm Ultra-Clear tubes may fog up. To clearly see the boundary between layers after centrifugation, use a squirt bottle of ethanol to run ethanol on the outside of the 11 × 34 mm Ultra-Clear tubes (not close to the top of the tube) to remove condensation that is clouding the outside of the tube. Use a Kimwipe to remove excess ethanol from the side of the tube. Hold the tube up to a source of light to see the distinct layers in the tube.
7. In reconstituting nuclei, the mixing of the reaction is crucial for achieving homogeneity. After the membranes are added, the reaction needs to be mixed immediately, otherwise the membranes will clump in the reaction. We pipet the reaction around five times until there are no visible clumps of membranes, which are yellow color compared to the clear cytosol.
8. The preparation of the slide affects the appearance of the nuclei. Nuclei are more fragile than cells, thus care needs to be taken in the manner in which the samples are prepared on the slide. After the fixed sample (2.5 μ L final volume) is placed on a glass slide, place one edge of the glass cover slip next to the droplet of sample at a 45° angle. Slowly lower the cover slip so the solution is wicked up to encompass the area underneath the cover slip. If a bubble is developing, raise the cover slip slowly, keeping one edge of the cover slip on the slide at all times, and then lower the cover slip again. To avoid drying of the samples under the cover slip, only prepare one slide at a time for viewing (with a maximum of two cover slips per slide).
9. When nuclei are placed between the slide and cover slip, there may be regions on the slide where the nuclei have dried (when an air bubble is present over the nuclei) or, alternately, have been broken by the cover slip. These defects may also occur after long periods of fixing. Such nuclei are not to be included in your data set, especially in assaying for import, as the presence of import substrate in these nuclei is not reflective of the actual amount of import. When stained with a DNA dye, nuclei that have dried look flat or two dimensional; nuclei that have broken appear wrinkled or collapsed.
10. Immunodepleted extracts are to be freeze-thawed once only.

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