

Analysis of Nuclear Reconstitution, Nuclear Envelope Assembly, and Nuclear Pore Assembly Using *Xenopus In Vitro* Assays

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Abstract

The large and complex eukaryotic nucleus is the arbiter of DNA replication, RNA transcription, splicing, and ribosome assembly. With the advent of *in vitro* nuclear reconstitution extracts derived from *Xenopus* eggs in the 1980s, it became possible to assemble multiple nuclei *in vitro* around added DNA or chromatin

substrates. Such reconstituted nuclei contain a nuclear lamina, double nuclear membranes, nuclear pores, and are competent for DNA replication and nuclear import. *In vitro* nuclear reconstitution has allowed the assembly of “wild-type” and “biochemically mutant” nuclei in which the impact of individual components can be assessed. Here, we describe protocols for preparation of the nuclear reconstitution extract, nuclear reconstitution *in vitro*, assessment of nuclear membrane integrity, and a more specialized assay for nuclear pore assembly into preformed pore-free nuclear intermediates.

INTRODUCTION

In vitro assays allow one to alter or study reactions in more detail. The nuclear reconstitution extract derived from *Xenopus* eggs in essence serves as a miniature *in vitro* reactor for analyzing the biochemical reactions and properties encountered in the more organized and complex environment of intact cells (Forbes, Kirschner, & Newport, 1983; Lohka & Masui, 1983, 1984; Newport, 1987; Newport & Spann, 1987; Wilson & Newport, 1988).

Prior to the advent of nuclear reconstitution, an experimental attack on the structure and assembly of the eukaryotic nucleus was almost impossible. The tools available at the time included only nuclear isolation from cells, followed by crude fractionation via sequential enzymatic treatments such as DNase, RNase, high salt, and detergent. These approaches were reductive and ones for which the results obtained, in the form of a “nucleoskeleton” or purified proteins, were difficult to assess as being comparable or related to the original *in vivo* situation. Similarly, the knowledge of nuclear envelope components was greatly limited. Lamins had been discovered, but only one nucleoporin was known (gp210), no inner nuclear membrane proteins had been discovered, the connections between lamins and chromatin were in their infancy, and authentic nuclear transport had never been achieved. Nuclear localization signals had been identified, but the protein(s) that recognized the NLSs or even where such NLS recognition took place were unknown (Dingwall, Sharnick, & Laskey, 1982; Kalderon, Richardson, Markham, & Smith, 1984). It was assumed that there must be a receptor in the nuclear pore complex; even this was later proven not to be the case with the discovery of soluble nuclear import and export receptors (Adam, Lobl, Mitchell, & Gerace, 1989; Finlay, Newmeyer, Price, & Forbes, 1987; Gorlich, Prehn, Laskey, & Hartmann, 1994; Gorlich, Vogel, Mills, Hartmann, & Laskey, 1995; Newmeyer, Finlay, & Forbes, 1986; Newmeyer & Forbes, 1988). At some level, the nucleus of the time was almost a black box with *in vitro* replication, transcription, and splicing analysis in the test tube far outpacing what we knew of nuclear structure. With few methods for analysis, it seemed this situation would not soon change.

The advent of the ability to reconstitute nuclear structure, both *in vivo* and *in vitro*, by two separate groups in the 1980s thus represented a significant and serendipitous advance in the ability to analyze many nuclear structures and reactions

in a more natural manner. The ability of extracts to reconstitute nuclei stemmed largely from the fact that organisms with external fertilization and/or embryonic development, such as frogs and flies, construct their yolky eggs to be replete with all the components needed for many rounds of fast cell division and growth. Additionally, these stored cellular components are provided in a disassembled state and in millions of copies, enough to form several thousand cells in the early embryo, almost avoiding the need for early transcription. The last bit of evolutionary serendipity that made *Xenopus* egg extracts such a powerful tool was the discovery that mere addition of DNA—or chromatin for a faster reaction—was enough to set in motion reconstitution of hundreds of normal appearing nuclei, both within the early egg if the DNA was injected or within the test tube if DNA was added to egg extract. It became clear from these studies that much of the complex structure of the nucleus assembled spontaneously around DNA, one sequential step after another, and did so with complete disregard for eukaryotic DNA sequence (Forbes et al., 1983). Long linear dsDNA of heterogeneous sequence from any source would set nuclear reconstitution in motion (Blow & Laskey, 1986; Dasso & Newport, 1990; Dasso, Smythe, Milarski, Kornbluth, & Newport, 1992; Finlay & Forbes, 1990; Finlay et al., 1987; Forbes et al., 1983; Lohka & Masui, 1983, 1984; Newmeyer et al., 1986; Newmeyer & Wilson, 1991; Newport, 1987).

Newly laid *Xenopus laevis* eggs are naturally arrested in second meiotic metaphase by a Cytostatic Factor (CSF). This arrested meiotic stage is biochemically and physiologically very related to a mitotic state. Inducing fertilization, or mimicking fertilization by adding Ca^{++} , triggers *Xenopus* eggs to progress on to an interphase state (Andreuccetti, Denis-Donini, Burrini, & Campanella, 1984; Masui, 2001; Masui & Markert, 1971; Tunquist & Maller, 2003). Extract of such eggs is thus said to be in an *interphase* state. On the other hand, omission of Ca^{++} and lysis of the eggs in the presence of the Ca^{++} chelator EGTA arrests the cell cycle of the eggs in a mitotic state. Techniques for generating extracts that are biochemically considered either *interphase* or *mitotic* have been developed and used extensively (reviewed in Murray, 1991). DNA or chromatin can be added to crude interphase extracts to reconstitute nuclei *in vitro* or, alternately, added to a crude mitotic extract to induce spindle assembly *in vitro* (Dasso & Newport, 1990; Maresca & Heald, 2006). In addition, extracts prepared even more carefully can reproduce up to three cell-cycle rounds *in vitro* (Murray, 1991). Further fractionation of interphase extracts, separating the extract into soluble and membrane fractions, can also be performed. When added together, these fractions can be used to more precisely control and analyze the reconstitution of nuclear structure or specific nuclear functions *in vitro*. A full report of the efforts of all the excellent contributors to this field are far beyond the scope of this chapter, but early contributors and reviews include the following: Desai, Murray, Mitchison, & Walczak, 1999; Finlay et al., 1987; Forbes et al., 1983; Lohka & Maller, 1985; Lohka & Masui, 1983; Newmeyer & Forbes, 1988; Newmeyer & Wilson, 1991; Newport, 1987; Newport & Spann, 1987; Pfaller, Smythe, & Newport, 1991.

Xenopus egg cytosol, when properly prepared, is diluted so minimally that it contains a very high concentration of the soluble factors and proteins needed to assemble nuclei (≥ 40 mg/mL). This property of the extract confers the experimental advantage of being able to use a small volume for each reconstitution reaction (10 to 50 μ L). Another advantage is that a single batch of eggs laid by a *Xenopus* female frog can produce 2 to 8 mL of extract. Nuclear reconstitution using the *Xenopus* egg extract additionally enables one to independently study individual steps in the mechanism of assembly occurring during the process of nuclear formation (see, for example, Fichtman, Ramos, Rasala, Harel, & Forbes, 2010; Macaulay & Forbes, 1996). The nuclei assembled *in vitro* have been found to be comparable in fundamental architecture to nuclei *in vivo* in a number of aspects (Lau et al., 2009; Newmeyer & Wilson, 1991; Newport, 1987; Newport & Dunphy, 1992; Pfaller et al., 1991). Interestingly, endogenous nuclei can also be isolated from existing cells and added to the egg extract; mammalian nuclei can acquire additional *Xenopus* nuclear membrane and pore components, becoming hybrid in their nuclear envelope and protein composition (see, for example, Newmeyer & Forbes, 1988; Newmeyer et al., 1986). The mitotic disassembly of isolated nuclei derived from different cell types can also be studied by addition to mitotic egg extracts (Newport & Spann, 1987; Pfaller et al., 1991).

In this chapter, we describe our method of preparation of interphase *Xenopus* egg extract, sperm chromatin substrate, and applications using the extract. We describe a method for assembling nuclei *in vitro*. In more specialized protocols, we describe an assay for testing the integrity of the nuclear envelope and a second protocol for analyzing nuclear pore complex assembly into preformed, pore-free nuclear intermediates. The latter has the advantage of allowing one to study nuclear pore assembly separate from nuclear membrane assembly.

In Chapter 9, Eisenhardt et al. describe a slightly different *Xenopus* egg extract fractionation procedure and complementary protocols that allow the analysis of the function of transmembrane nucleoporins and the targeting of proteins to the inner nuclear membrane. In Chapter 2, Fichtman et al. use an anchored nuclei approach to visualize the NPCs by Field Emission Scanning Electron Microscopy.

8.1 MATERIALS

8.1.1 Equipment

1. 27 G \times 0.5-in. gauge needles (cat. no. 305109; BD Biosciences).
2. 1 mL syringes (1 mL BD Luer-Lok™ disposable syringe; cat. no. 309628; BD Biosciences).
3. Disposable 2-mL plastic transfer pipettes (cat. no. 357524; BD Biosciences).
4. Beckman TL100 ultracentrifuge with TLS55 rotor; 11 \times 34-mm Ultra-Clear tubes (cat. no. 347356; Beckman).

5. Tomy high-speed refrigerated centrifuge TX-160 with TMH-21 rotor.
6. 1.5-mL microcentrifuge tubes (1.5 mL MaxyClear Snaplock-lid Microcentrifuge Tube, Polypropylene; cat. no. MCT-150-A; Axygen).
7. 2-mL microcentrifuge tubes (2-mL MaxyClear Snaplock-lid Microcentrifuge Tube, Polypropylene; cat. no. MCT-200-C; Axygen).
8. Pushcap plastic 14 mL round bottom centrifuge tubes (cat. no. 14-959-11B; BD Biosciences).
9. 15-mL conical centrifuge tubes (cat. no. 14-959-70C; Corning Life Sciences).
10. 200 and 1000 μ L Pipetmen (classic pipetmen brands) and appropriate tips are used throughout.
11. Large orifice 200 μ L pipette tips (cat. no. 1011-8406; USA Scientific). (When not available, 2 mm of the extremity of normal 200- μ L pipette tips can be cut off with scissors.)
12. 18 \times 18 mm glass coverslips (Premium Cover Glass; cat. no. 12-548A; Fisher Scientific).
13. Zeiss Axioskop 2 microscope with a 63 \times objective, connected to a charge-coupled device camera; images captured on computer.

8.1.2 Reagents and buffers

1. *X. laevis* frogs: Three to five females for making fresh egg extracts (Section 8.2) and two males to make a stock of demembrated sperm chromatin (Section 8.3).

Note: Key housing conditions for our frog colony are: the frogs are maintained in an 18 °C environment, immersed in tap water supplemented with 100 mM NaCl, and 0.1 mM sodium thiosulfate (cat. no. 72049, Sigma). Large colonies of *X. laevis* (>20) require substantial amounts of water, which compels the use of tap water. However, frogs are very sensitive to heavy metals, detergents, chlorine, and organic solvents, so care should be taken to avoid these. To purify the tap water and deplete possible contaminants, sodium thiosulfate is used as a chelator for a broad range of heavy metals (mercury, cadmium, etc.) and as a potent dechlorinator. When double distilled water is used (as for fewer frogs), there is no need for adding sodium thiosulfate (Chum, Felt, Garner, & Green, 2013; Wu & Gerhart, 1991). (More information can be found at: <http://www.rspca.org.uk/ImageLocator/LocateAsset?asset=document&assetId=1232712646624&mode=prd>.)

2. Pregnant Mare Serum Gonadotropin (PMSG, 12.5 \times stock; cat. no. 367222; Calbiochem): 5000 U/2 mL in sterile filtered water.
3. 1 \times stock Human Chorionic Gonadotropin (HCG, cat. no. CG-10; Sigma): 10,000 U/10 mL in sterile filtered water.
4. Xenopus buffer (XB): 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 50 mM sucrose, pH 7.7, in distilled water.
5. Sucrose cushion: 1 \times XB with a final concentration of 500 mM sucrose.
6. Cysteine buffer: 2% cysteine in distilled water, pH 7.8.

7. Cytochalasin B stock: 10 mg/mL in dimethylsulfoxide (cat. no. 1955119; MP Biochemicals).
8. Cycloheximide stock: 10 mg/mL in distilled water (cell-culture grade, white powder, cat no. 0219452705; MP Biochemicals).
9. Protease inhibitors: Aprotinin and Leupeptin, combined in a 1000× stock solution of 10 mg/mL each in distilled water (cat no. A6106 and L2884, respectively; Sigma).
10. 10× Buffer-X for sperm: 100 mM HEPES, pH 7.4, 0.8 M KCl, 150 mM NaCl, 50 mM MgCl₂, 10 mM ethylenediaminetetraacetic acid in distilled water.
11. Bovine Serum Albumin (BSA) (cat. no. A7030; Sigma).
12. Triton X-100 (TX-100) (cat. no. T8787; Sigma).
13. DTT (DL-Dithiothreitol): 1 M stock solution in distilled water (keep stock aliquoted in 1 mL fractions and frozen at −20 °C) (cat. no. 43815; Sigma).
14. 20× Energy Mix stock in 1× XB buffer, containing:
 - 200 mM phosphocreatine (cat. no. P7936; Sigma; in 10 mM KH₂PO₄, pH 7.0),
 - 1.6 mg/mL creatine phosphokinase (cat. no. C3755; Sigma; in 10 mM HEPES, pH 7.5, 50% glycerol, 50 mM NaCl, and distilled water),
 - 20 mM ATP (Adenosine 5′-triphosphate disodium salt hydrate—cat. no. A2382; Sigma; in distilled water, pH 7.0),
 - 20 mM MgCl₂ (cat. no. M8266; Sigma; in distilled water), and
 - 100 μM EGTA (Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid—cat. no. E3889; Sigma; in distilled water, pH 7.5 with NaOH).
15. Anti-FG Nup antibody mAb414 (cat. no MMS-120P; Covance), directly labeled with an AnaTag™ HiLyte Fluor™ 555 Protein Labeling Kit (cat. no. 72045, Anaspec, CA) or purchased already labeled from Covance. Once labeled, the antibody is referred in the text as HF555-mAb 414.
16. Fixation buffer: 48% glycerol, 11% formaldehyde, and 10 mM HEPES, pH 7.5, 5 μg/mL Hoechst 33258 DNA dye (cat. no. 861405; Sigma) and freshly added 100 μg/mL DHCC (final concentration) (3,3-dihexyloxycarbocyanine iodide—cat. no. 318426; Sigma-Aldrich).

8.2 XENOPUS EGG EXTRACTS

Assembly of *in vitro* nuclei is performed using a high-speed cytosolic extract derived from *X. laevis* eggs. When demembrated sperm chromatin (from *X. laevis* males) and membranes (derived from the fractionation of *X. laevis* eggs; see below) are added to the cytosolic extracts (Newmeyer et al., 1986; Newport, 1987; Pfaller et al., 1991), reconstituted nuclei form and are fully functional in terms of nuclear import and DNA replication.

As an overview of the protocol below, induction of egg laying by *X. laevis* females is stimulated and precisely controlled by hormone injection (Section 8.2.1). The cytosol is then separated from the membranes by two steps of fractionation and centrifugation (Section 8.2.2). The resulting cytosolic layer is a highly concentrated fraction of soluble proteins (~40 mg/mL) and contains all components necessary for nuclear reconstitution in a cell-free environment. The *in vitro* assembly of nuclei (Section 8.4) initiates as soon as the cytosol is incubated with the membrane fraction, demembranated sperm chromatin (Section 8.3) and the energy regenerating system. Finally, individual steps in nuclear assembly can be dissected by separate protocols and techniques (Sections 8.5 and 8.6).

8.2.1 Obtaining eggs from frogs: Priming and induction of egg laying

To *prime* frogs for ovulation, the hormone PMSG is used. This hormone stimulates the growth of oocytes and allows them to reach an adequate size. Only eggs with a certain size will be released during the eventual laying process. Such oocytes are in “Stage VI” of the “Dumont classification” and show a clearly differentiated brown animal hemisphere and a white vegetal hemisphere, separated by an unpigmented equatorial band (Dumont, 1972). The injection of HCG stimulates the production of progesterone, which in turn activates a complex signaling pathway leading the oocytes to enter meiosis (Ferrell, 1999). The cell-cycle progresses until it is stopped in metaphase of the second meiotic division by a CSF; this state is referred to as “CSF arrest” (Masui, 2001; Tunquist & Maller, 2003). This arrest prevents the oocytes from progressing further in the cell cycle as they await laying. Only CSF-arrested eggs will be laid (when laid, they are then called “eggs”). Approximately, 24 h after HCG injection the frogs will lay their eggs naturally.

In the wild, laid eggs resume the cell cycle when fertilized by sperm. *In vitro*, we induce interphase entry in a population of laid eggs by calcium stimulation, which mimics the burst of calcium that occurs upon fertilization. Calcium is stored in a cortical network of smooth endoplasmic reticulum (ER) vesicles. Simple crushing of the eggs at the beginning of the protocol releases calcium from these specialized storage vesicles and initiates interphase (Andreuccetti et al., 1984; Murray, 1991). A synchronized interphase extract can then be prepared.

PROTOCOL

1. Each female frog is primed with 0.5 mL PMSG (mix 1 mL from a 12.5 × stock with 11.5 mL sterile distilled water) 2–3 days before inducing the laying process itself, but up to 2 weeks ahead is acceptable (Wu & Gerhart, 1991).
Approximately, 2 weeks post-PMSG injection, any unlaid eggs acquire a proapoptotic phenotype (von Ahsen & Newmeyer, 2000).
2. Frogs are injected in the thigh region (back side), between the skin and the muscle (subcutaneous injection), using a 27 × 0.5 in. gauge needle, then placed in a container of 100 mM NaCl in distilled water at 18–22 °C.

3. Approximately, 24 h before egg laying is desired, frogs are injected subcutaneously with 0.5 mL of HCG stock solution (i.e., 500 Units of HCG are injected per frog).

Note: When collected, the eggs should be as fresh as possible for extract preparation. Usually, the HCG injection is done in the morning so that the eggs can be collected 24 h later, i.e., the next morning. Then, the remainder of the second day can be used for preparing the extract.

4. At the time of injection, place each HCG-injected frog in a separate 10 L container filled with 2 L of distilled water containing 100 mM NaCl at 18 °C (laying solution). Approximately, 24 h later the eggs are collected by pouring off the majority of laying solution and proceeding to Step 5.

Note: The 18 °C temperature is ideal for housing the frogs. Inducing the frogs to lay at 18 °C in a laying solution containing 100 mM NaCl is also crucial to preserve good quality in the eggs. After laying, the frogs should rest for 2 to 3 days in a large container of 100 mM NaCl in distilled water. This step is important to allow the frogs to finish laying any remaining eggs (which are discarded) before placing them back in their long-term housing (Wu & Gerhart, 1991).

5. The eggs of each frog (a batch) are kept separate. Each batch is placed in a 250-mL beaker. The eggs must remain immersed in a small volume of laying solution at all times to lower the risk of lysis or any kind of damage during handling and transportation (4 cm of laying solution above the top of the egg layer is sufficient).

Note: Eggs within the same batch are likely to be very similar. However, different batches of eggs may differ in quality. It is therefore not good to mix eggs from different frogs as this may increase variability in extract quality and reduce success.

8.2.2 Preparation and fractionation of *Xenopus* egg extracts

To separate the extract into soluble and membranes fractions, the following protocol is used. The goal is, by a series of centrifugations, to separate and purify: (1) a lighter fraction, which is the clear cytosol that contains soluble proteins and (2) a heavier and cloudy fraction that contains the intracellular membranes (ER and Golgi membranes). Note that the plasma membrane and yolk and pigment granules are removed in an initial low-speed centrifugation step, while mitochondria and glycogen polymers are removed in the second higher-speed centrifugation step. This type of interphase extract is sometimes referred to in publications as “US” or “Ultra S” (for “ultracentrifuged S-phase extract”).

PROTOCOL

Steps 4–17 should be carried out on ice unless stated otherwise.

1. Solutions are made fresh the same morning as the extract:
 - a. 2% cysteine, pH 7.7–7.8
 - b. The wash and lysis buffer used is $1 \times$ XB buffer.

2. The first step consists of removing the gelatinous layer around the eggs by treating with a 2% cysteine solution.
 - a. The excess water is removed from the collected eggs in the 250-mL beaker, making sure that the eggs remain submerged in water (1 cm). At this point, the jelly-coated eggs do not physically touch each other and occupy a large space in the beaker.
 - b. Pour ~100 mL of 2% cysteine, pH 7.7–7.8, into the beaker.
 - c. Very gently swirl the eggs in the beaker with 2–3 swirls.
 - d. Once the eggs have settled, gently remove the excess solution and repeat Steps 2a–c twice more until the jelly is completely removed. The eggs become very fragile without their gelatinous layer, thus pouring, swirling, and removing the solutions must be performed with care. Once dejellied, the eggs are physically in contact with one another and occupy a smaller space in the beaker.

Note: This step must be done in relatively short time, i.e., DO NOT let the eggs remain in contact with the 2% cysteine solution >5–6 min total as this will damage the eggs, which will become white, swell, and lyse. Remove every white or nonconventionally shaped egg from the beaker (some are laid this way) using a 2-mL plastic transfer pipette. Dying or damaged eggs release proteolytic enzymes that may trigger a cascade of reactions leading to the death of the entire egg population. Thus, it is necessary to remove the dying eggs during the 5–6 min period.

3. Proceed immediately to the following step once the eggs have settled: wash 3 to 4 times with $1 \times \text{XB}$. For this:
 - a. Pour off *as much as possible* of the 2% cysteine buffer. Immediately, add 50–100 mL of $1 \times \text{XB}$ to the beaker and swirl very gently.
 - b. Once the eggs have settled, remove the excess solution and repeat this wash twice more.

Note: The cysteine solution has a very specific smell of sulfur. After the last wash, this smell should not be perceptible. If cysteine remains, it may cause problems in future experiments that use the egg extract.

4. Prepare 2-mL plastic microcentrifuge tubes with snaplock lids that have been prechilled on ice for a few minutes and then filled with 0.5 mL of $1 \times \text{XB}$ buffer. The $1 \times \text{XB}$ will act as a “cushion,” ensuring that the fragile eggs do not fall abruptly to the bottom of the tube.
5. Using a 2-mL disposable plastic transfer pipette (with the tip cut off 2 cm with scissors to accommodate the 1 mm eggs), carefully transfer the eggs from the beaker into the 2-mL tubes. (The tube should be filled to no more than 90% of its capacity with eggs plus solution to avoid crushing the eggs with the lid.)
6. Once the eggs have settled in the tube, this time remove as much as possible of the $1 \times \text{XB}$ solution above the eggs. Leaving too much solution will dilute the extract (Fig. 8.1A).
7. Add 100 μL of 10 mM cycloheximide to the top of the each tube. The next centrifugation step will mix the cycloheximide into the future extract.

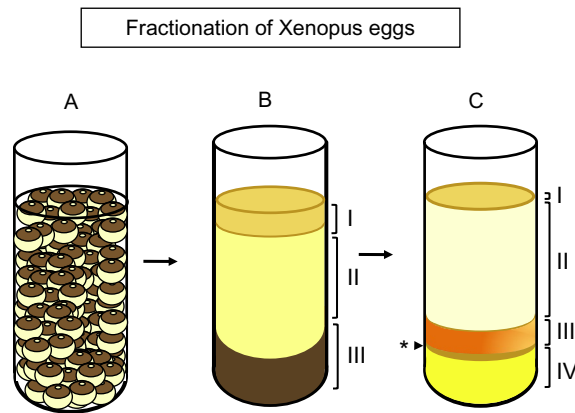


FIGURE 8.1 Fractionation of *Xenopus* Eggs

(A) *Xenopus* eggs prior to the first centrifugation, which will crush and fractionate the eggs. (B) The egg extract fractionated after the first step of centrifugation. Three distinct layers are formed: the yellow lipid layer at the top (I), the intermediate golden crude extract layer (cytosol and membranes) (II), and the black pigment and yolk granule pellet (III). (C) The crude extract after high-speed centrifugation fractionates into four major layers. From the top to the bottom of the tube (I) a thin yellow layer containing remaining lipids, (II) a clear layer (soluble fraction), (III) a cloudy yellow membrane layer, and (IV) a gelatinous pellet with dark and light layers comprised mitochondria, glycogen, and residual pigment and yolk granules. Asterisk indicates the light brown layer containing mitochondria.

Note: Cycloheximide will prevent any further protein synthesis (i.e., cyclin synthesis) and keep the future extract in an interphase state.

8. Spin the 2 mL microcentrifuge tubes containing the eggs in a TMH-21 horizontal bucket rotor for 15 min, 17,360 rcf (15,000 rpm) at 4 °C using a table top refrigerated centrifuge (Tomy TX-160). Egg lysis during centrifugation will release stored calcium, which sends the extract into interphase.

THE EGGS WILL FRACTIONATE INTO THREE DISTINCT LAYERS (SEE FIG. 8.1B)

- I. A top opaque bright yellow lipid layer.
 - II. A middle cloudy golden layer consisting of *crude* cytosol (i.e., the soluble part plus membranes).
 - III. A bottom dark layer formed largely of black pigment granules and yolk granules.
9. Using a 200- μ L pipette tip (only the tip) as a tool, scoop out and discard as much as possible of the top lipid layer.

10. Next, recover the crude cytosol layer gently using a 1000- μ L pipette tip and pipette and transfer it into a 2-mL ultracentrifuge tube (Beckman Ultra-Clear tubes—11 \times 34 mm).

Note: Even if some remaining lipids and pigment granules are collected with the crude cytosol, these will be separated away during the next centrifugation step. An average of 1 mL of crude extract is recovered per \sim 2 mL of lysed eggs.
11. Add Cytochalasin B to the crude extract to a final concentration of 50 μ g/mL. Mix by pipetting the extract gently up and down a few times, using a 1000 μ L tip and pipette.

Note: Cytochalasin B treatment decreases the viscosity of the crude cytosol by depolymerizing the cytosolic actin network that would otherwise form in this fraction.
12. To separate the cytosol from the membranes:
 - a. Combine the crude cytosols (from the same original batch of eggs) into new Ultra-Clear tubes (11 \times 34 mm). Fill each Ultra-Clear tube with 2 mL of crude extract (if possible). Repeat this step for the cytosol from each batch of eggs.

Note: The tube may collapse during centrifugation if the final volume is less than 1.5 mL/tube.
 - b. Spin down the crude cytosolic extract using a TLS55 Beckman rotor with a TL-100 Beckman ultracentrifuge at 260,000 rcf (55,000 rpm) for 90 min at 4 $^{\circ}$ C.

THE CYTOSOL WILL FRACTIONATE INTO FOUR DISTINCT LAYERS (FIG. 8.1C)

- I. A thin topmost layer of residual lipids.
 - II. An upper clear yellow cytosolic layer.
 - III. An underlying cloudy yellow layer comprised membranes.
 - IV. A gelatinous bottom pellet with a hard consistency, composed of alternating golden and dark layers. These contain mitochondria (a dark layer just underneath the cloudy membrane layer), glycogen (a clear golden layer), and any remaining pigment granules (a bottom dark layer).
13. Recovery of the clear cytosol and membranes:
 - a. First, remove and discard the top thin lipid layer very gently by aspiration with a glass Pasteur pipette and vacuum.
 - b. The clear cytosol layer should then be recovered with a 200 μ L tip and pipette. A gentle swirl while pipetting aids in preventing aspiration of the cloudy membrane fraction. Avoid collecting too much of the cloudy membrane layer while recovering the cytosol. The clear cytosol should be transferred into a new Ultra-Clear tube (11 \times 34 mm) and stored on ice until Step 14.

- c. The cloudy membrane layer (approximately 250–500 μL) should next be collected using a large orifice 200- μL tip and pipette, followed by placement of the cytosol into a 14-mL round bottom push-cap tube and storage on ice until Step 16.

Note: When collecting the membrane fraction, avoid the dark-brown viscous layer just underneath as it contains mitochondria. After the *in vitro* nuclear reconstitution assay, the nuclear envelopes are often detected by staining with DHCC dye. If too many mitochondria are present, the DHCC will stain the mitochondrial membranes and the surface occupied by the coverslip will be covered by a myriad of tiny dots (i.e., the mitochondria). In addition, leaving excess mitochondria in the extract is undesirable as the mitochondria could trigger entry into an apoptotic state (von Ahsen & Newmeyer, 2000).

14. To the cytosolic fraction (Step 13c, stored on ice), add Aprotinin and Leupeptin to a final concentration of 10 $\mu\text{g}/\text{mL}$ each. To mix, pipet the extract up and down using a 1,000 μL tip and pipette.
15. In order to remove residual lipids and membranes from the clear cytosol collected and treated in Steps 13 and 14, the cytosol should be spun a second time, now at 260,000 rcf (55,000 rpm) for 30 min at 4 $^{\circ}\text{C}$ using a TLS55 rotor (in 11 \times 34 mm Ultra-Clear tubes) and a TL100 Beckman ultracentrifuge. The ultracentrifuged cytosol should then be aliquoted into 100 μL aliquots, quickly frozen in liquid nitrogen and stored at -80°C
16. The membrane layer from Step 13 should next be salt-washed using 1 \times XB buffer supplemented with 0.5 M KCl (high-salt XB buffer) to remove much of the proteins bound to the membranes. For washing:
 - a. Transfer the membranes (\sim 250–500 μL) into a 14-mL push-cap tube.
 - b. Fill the tube with 8 mL of high-salt XB buffer.
 - c. Pipet the mixture of membranes and high-salt XB buffer gently up and down using a large orifice 1000- μL tip and pipette.
17. Purify the salt-washed membranes through a sucrose cushion. To do this:
 - a. Place 2 mL of the mixture of membranes and high-salt XB buffer in a new Ultra-Clear tube (11 \times 34 mm).
 - b. Using a 200- μL pipette and tip, go to the bottom of the tube and slowly add 400 μL of a sucrose cushion (500 mM sucrose in 1 \times XB).

Note: The salt-washed membranes will be on top of the sucrose cushion at this point.

- c. Spin the mixture at 70,000 rcf (30,000 rpm) for 30 min at 4 $^{\circ}\text{C}$ using a TLS55 rotor and a TL100 Beckman ultracentrifuge.
- d. After this step of centrifugation, the membranes will be in a loose pellet at the bottom of the tube. Remove most of the liquid over the membranes, leaving a minimal amount of liquid at the bottom of the tube with the pellet of membranes.

Note: Leaving too much liquid may dilute the membranes. Leaving a small amount of the liquid, however, is required to enable one to more readily pipet up the membrane pellet (e.g., leave \sim 20 μL of the top layer over the pellet).

- e. Flick the tube to mix the liquid and membranes; then use a large orifice 200 μ L tip and pipette to recover the membranes. The resuspended membranes should then be aliquoted into 5 μ L fractions), quickly frozen in liquid nitrogen and stored at -80°C . We define this as approximately a 20 \times membrane stock and use it accordingly.

8.3 DEMEMBRATED SPERM CHROMATIN

The source of DNA used for *in vitro* nuclear reconstitution experiments is sperm chromatin prepared from male *Xenopus* frog testes. These demembrated sperm contain a packet of chromatin composed of all the sperm chromosomes. This chromatin can form a functional nucleus when added to egg cytosol supplemented with membranes and is a convenient way to store and add chromatin. Thus, sperm chromatin is commonly used as a source of chromatin for *in vitro* nuclear reconstitution; however, other sources of DNA or chromatin can also be used (Newport, 1987).

PROTOCOL

Steps 1 to 6 are carried out at room temperature (unless stated otherwise).

Steps 7 to 12 are carried out on ice or at 4°C (unless stated otherwise).

1. The Buffer-X solutions used below contain high concentrations of sucrose, which takes considerable time to dissolve. It is recommended that these solutions be prepared the day before the sperm prep.
 - a. Buffer-X + 0.2 M sucrose (prepare 100 mL)
 - b. Buffer-X + 0.2 M sucrose + 0.4% TX-100 + 1 mM DTT + 1 μ g/mL Aprotinin/Leupeptin (prepare 2 mL)
 - c. Buffer-X + 0.2 M sucrose + 3% BSA + 1 mM DTT + 1 μ g/mL Aprotinin/Leupeptin (prepare 10 mL)
 - d. Buffer-X + 0.5 M sucrose + 3% BSA + 1 mM DTT + 1 μ g/mL Aprotinin/Leupeptin (prepare 5 mL)
 - e. Buffer-X + 2 M sucrose (prepare 5 mL)
 - f. Buffer-X + 2.3 M sucrose (prepare 10 mL)
 - g. Buffer-X + 2.5 M sucrose (prepare 5 mL)
2. Two male frogs are sufficient to yield a large amount of demembrated sperm chromatin, enough for ~ 500 reconstitution reactions. For a frog euthanasia procedure and recovery of testes, please refer to Chan and Forbes (2006).
3. Cleaning the testes:
 - a. Place the testes obtained in a Petri dish containing Buffer-X + 0.2 M sucrose.
 - b. Remove any remaining blood, blood vessels, and fat from the testes with forceps.
 - c. Move the testes into a new Petri dish containing Buffer-X + 0.2 M sucrose.
 - d. Mince the testes with a small scalpel into very small pieces (1–2 mm). Transfer the minced testes and all the buffer from the Petri dish used in Step c to a 15-mL conical tube.

- e. Rinse this Petri dish (Step c) with 2 mL of fresh Buffer-X + 0.2 M sucrose. **KEEP** and transfer the wash to the same 15-mL conical tube.

Note: The washes contain some freed sperm released when testes were minced.

4. To release additional sperm from the testes:
 - a. Mash the minced testes that have settled to the bottom of the conical tube in Step 3, using a thin glass stirring rod or a thin spatula.
 - b. Vortex the conical tube vigorously for 1 min to maximize the release of sperm.
 - c. Pellet the larger pieces of tissue at 100 rcf (1,000 rpm) for 10 s, at RT, in a low-speed, swinging-bucket clinical tabletop centrifuge.
 - d. Recover and transfer the supernatant into one new 15-mL conical tube (i.e. **KEEP** and set aside for further use)—**Store on ice**.
 - e. **KEEP the pellet**, and add 2–4 mL of Buffer-X + 0.2 M sucrose to this pellet.
 - f. Start over at Step 4a and repeat Step 4 until the supernatant becomes clear (see note below).

Note: The supernatants are cloudy when they contain freed sperm. Keep the pellet after every centrifugation. The pellet still contains plenty of trapped sperm. Step 4 must be repeated until the supernatant becomes clear. The turbidity of supernatants indicates the presence of sperm. Depending on the testes quality, Step 4 may be repeated 3–10 times (there is no rule for this; the turbidity is the only reliable indicator).

5. Combine all the supernatants collected in Step 4d together (they contain the freed sperm in suspension).

Note: Depending on how many times Step 4 has been repeated, more than one 15-mL conical tube may be required for collecting the supernatants.
6. To remove remaining large pieces of tissues collected with the sperm in suspension:
 - a. Spin down the combined supernatants in Step 5 at 185 rcf (1350 rpm) for 1 min using a clinical tabletop centrifuge
 - b. The supernatant is recovered and transferred to a new 14-mL round bottom tube. (**KEEP**)
 - c. This pellet is of no use: **DISCARD**
7. The freed sperm in suspension (recovered in Step 6) will be, now pelleted to remove damaged sperm, contaminant cells and undesired particles. For this:
 - a. Spin down the supernatant at 2,600 rcf (4,000 rpm) for 10 min at 4 °C (using a Sorvall HB6 swinging-bucket rotor).
 - b. **KEEP the pellet**, which now contains the freed sperm.
 - c. **DISCARD the supernatant** that contains damaged sperm and other useless particles.
 - d. Resuspend the sperm pellet in 0.8 mL of Buffer-X + 2 M sucrose.

Note: Red blood cells are major contaminants of sperm. Step 7 does not remove the red blood cells from the sperm (for this, see next step).

8. The red blood cells are separated from the sperm by spinning the sperm solution through a sucrose gradient. For this:

- a. Take 4 Ultra-Clear tubes (11 × 34 mm) and add to each one:
 - i. 0.25 mL of Buffer-X + 2.5 M sucrose.
 - ii. Slowly overlay with 1.7 mL of Buffer-X + 2.3 M sucrose.
 - iii. Finally overlay this with 0.2 mL of the resuspended sperm solution.
 - b. Spin, using a TL100 Beckman ultracentrifuge with a TLS-55 rotor at 93,000 rcf (33,000 rpm) for 25 min at 4 °C.
 - c. DISCARD the top half of the gradient by aspiration (it contains the red blood cells).
 - d. KEEP and transfer the lower half of the gradient to a new 14-mL round bottom tube (**this lower half contains the sperm**).
 - e. To recover any remaining sperm possibly left in the previous set of four Ultra-Clear tubes: add to each tube, 1 mL of Buffer-X + 0.2 M sucrose and rinse each tube walls with this. Keep and transfer the washes into the same 14-mL round bottom tube used in Step 8d.
 - f. Repeat two more times Step e and combine the washes into the same 14-mL round bottom tube used in Step 8d.
 - g. Now dilute the sperm solution (obtained in 8f) with Buffer-X + 0.2 M sucrose to reach a volume of 12 mL of solution.
9. Next pellet the sperm in suspension (from Step 8g):
- a. Spin the tube at 4,000 rcf (5,000 rpm) for 10 min at 4 °C using a Sorvall HB6 swinging-bucket rotor.
 - b. KEEP the pellet on ice (i.e., sperm) and DISCARD the supernatant.
10. The demembration process consists in removing the membranes that surround the sperm chromatin. To do this:
- a. Resuspend the pellet obtained in Step 9b with 1 mL of Buffer-X + 0.2 M sucrose + 0.4% TX-100, 1 × Aprotinin/Leupeptin + 1 mM DTT.
 - b. Incubate for 30 min on ice.
11. The TX-100 and solubilized membranes are removed by a series of wash/spins:
- a. In two 1.5-mL microcentrifuge tubes, add 0.5 mL of Buffer-X + 0.5 M sucrose + 3% BSA + 1 × Aprotinin/Leupeptin + 1 mM DTT (this will serve as a cushion).
 - b. Overlay each sucrose cushion with 0.5 mL of the demembrated sperm mixture obtained in Step 10.
 - c. Spin the tubes in a clinical tabletop centrifuge at 360 rcf (1,900 RPM) for 10 min.
 - d. KEEP the pellets (they contains the sperm) and DISCARD the supernatant.
 - e. Resuspend each sperm chromatin pellet with 100 µL of Buffer-X + 0.2 M sucrose + 3% BSA + 1 × Aprotinin/Leupeptin + 1 mM DTT.
 - f. Transfer the sperm chromatin into two new 1.5 mL microcentrifuge tubes and add to each 900 µL of Buffer-X + 0.2 M sucrose + 3% BSA + 1 × Aprotinin/Leupeptin + 1 mM DTT.
 - g. Spin down the tubes in a clinical tabletop centrifuge at 360 rcf (1,900 rpm) for 10 min.

- h. Repeat Steps d to g twice more.
 - i. Resuspend the TX-100-free pellet of sperm chromatin in 0.5 mL Buffer-X + 0.2 M sucrose + 3% BSA + 1 × Aprotinin/Leupeptin + 1 mM DTT and store on ice.
12. The sperm chromatin packets (SpC; each being the nuclear contents of one sperm) can be counted using a hemocytometer. After counting, the sperm chromatin should be diluted to a final concentration of 50,000 Units of sperm chromatin/ μL (SpC/ μL). For dilution, use the same buffer as in Step 11i. This is then aliquoted into 10 μL fractions, quickly frozen in liquid nitrogen and stored at -80°C . This mix, once stored at -80°C , is stable for a number of years.

8.4 *IN VITRO* RECONSTITUTION OF NUCLEI

Reconstitution of nuclei using high-speed *Xenopus* egg extract, membranes, and sperm chromatin is described in this paragraph. A standard reaction contains ~ 20 μL of high-speed cytosol, 1 μL of purified membranes, and $\sim 3,000$ SpC/ μL of final reaction. Some adjustments may be required to determine the best ratio of membranes to cytosol, varying from 0.5 to 2 μL per 20 μL of cytosol, using the considerations in [Chan and Forbes \(2006\)](#) as a guide. To visualize the proceedings of the major points described in the following protocol, an elegant and didactic video can be found online ([Cross & Powers, 2008](#)). Addition of an ATP-regenerating system provides the energy necessary to fuel the reconstitution reaction.

PROTOCOL

The protocol described below is sufficient for approximately five reactions of *in vitro* nuclear reconstitution.

From Step 1 to 5, the reactions must be carried out on ice.

1. Defrost slowly on ice
 - a. 1 tube of cytosol (100 μL aliquot).
 - b. 1 tube of membranes (5 μL aliquot).
 - c. 1 tube of sperm chromatin (50,000 SpC/ μL).
 - d. 1 tube of $20\times$ ATP-regeneration mix.
2. Add the cytosol directly to a tube containing the membranes and resuspend the membranes gently using a large orifice 200- μL tip and pipette.
3. Separate the mixture of membranes plus cytosol into five 1.5-mL microcentrifuge tubes. To each tube, add 1 μL of $20\times$ ATP-regeneration mix.

Note: At this point in the protocol, any test protein or molecule can be added to study their effect on nuclear membrane or nuclear pore assembly (see [Sections 8.5 and 8.6](#)).
4. LASTLY, to each tube add 1.5 μL from a stock solution of 50,000 SpC/ μL to give a final concentration of sperm chromatin at $\sim 3,000$ SpC/ μL of reaction.

5. Flick the tube gently five times to mix the sperm chromatin with the membrane/cytosol mixture. Avoid making bubbles while mixing.
6. Let the reaction incubate for 60 min at room temperature on the bench.
7. To determine if a new batch of membranes and cytosol assemble nuclei properly, one can assess the formation of nuclear pore complexes by immunofluorescence. For this, at $t=45$ min, we add 1 μL of a directly labeled HF555-mAb 414 antibody to an aliquot of the reaction (the antibody dilution is usually 1/50 in the reaction) and mix by gently flicking the tube once or twice.

Note: The monoclonal antibody mAb414 recognizes at least four *Xenopus* FG-nucleoporins (FG-Nups): Nup358, Nup214, Nup153, and Nup62 (Lau et al., 2009). When nuclear pores have been assembled in the nuclear envelope, the antibody will reveal a well-defined punctuate stain on the nuclear rim (mAb414 antibody can be directly labeled using a labeling kit for proteins—See Section 8.1 or bought pre-labeled, Covance, CA). A red-labeled antibody is important to use here if the nuclear membranes will be visualized in green using DHCC dye (as below).
8. Just before the end of the incubation, place a 1 μL drop of fixation buffer containing 100 $\mu\text{g}/\text{mL}$ of DHCC on a glass slide (one slide per reaction).
9. At $t=60$ min, using a large orifice pipette tip, take a 2 μL aliquot of a reaction and place directly on the drop of fixation buffer. (This is done for each reaction.)
10. Overlay the reaction aliquot/fixation buffer mixture with a 18×18 mm square coverslip and seal the edges of the coverslip with clear nail polish. Let dry!

Note: The fixation buffer/assembly reaction between the glass slide and the coverslip will automatically blend together and spread completely over the surface occupied by the coverslip by capillary action. Maximum care should be taken to gently lay on the coverslip, starting at one edge. Do not press hard on the coverslip and also try to prevent the coverslip from sliding, as this will result in damaged (sheared) nuclei. It is important to keep the tubes and slides in the dark until they can be visualized in Step 11 to avoid bleaching. The samples cannot be stored at 4 $^{\circ}\text{C}$ overnight. The most reliable and clearest results are obtained when visualization is made just after the end of the experiment.
11. Visualization of the assembled nuclei can be performed using a regular fluorescence microscope, ideally using a $63 \times$ objective.

FINAL NOTE FOR SECTION 8.4

“Good” *in vitro* reconstituted nuclei have the following characteristics: a round shape, decondensed DNA (showing dark and bright areas upon Hoechst DNA staining), a smooth continuous nuclear envelope when stained by DHCC, and a homogeneous dot-like nuclear rim stain with HF555-mAb414 antibody (staining the FG-Nups). The size of the reconstituted nuclei is usually between 10 and 20 μm (see Control in Figs. 8.2 and 8.3). Smaller or larger nuclei can indicate aberrant assembly.

8.5 ASSAYING ASSEMBLY AND INTEGRITY OF THE NUCLEAR ENVELOPE

The following assay is done to assay nuclear membrane assembly and integrity in the presence or absence of a component to be tested for an effect on membrane assembly. Normally, at the end of mitosis *in vivo*, membranes are recruited to the chromatin primarily from ER sheets. These then fuse side to side to surround the set of chromosomes with an intact double nuclear membrane (Anderson & Hetzer, 2007; Antonin, Ellenberg, & Dultz, 2008; Kutay & Hetzer, 2008). *In vitro*, in *Xenopus* egg extract, nuclei assemble spontaneously: a double nuclear membrane with nuclear pores forms around added chromatin (Forbes et al., 1983; Hetzer et al., 2001; Lohka & Masui, 1984; Newport, 1987). In this *in vitro* system, purified membrane vesicles, formed during fractionation of the extract, are the source of the membrane/ER-derived vesicles for the future nuclear membranes. When one observes nuclei very early in assembly *in vitro* or, alternately, if the vesicle-vesicle fusion machinery is blocked artificially, the nuclear membrane shows a discontinuous pattern when stained by fluorescent DHCC dye. Blocking vesicle-vesicle fusion can be achieved, for example, by the addition of GTP-gamma-S, a nonhydrolyzable analogue of GTP. GTP-gamma-S allows vesicles to be recruited to the DNA/chromatin, but prevents their fusion to form a continuous nuclear envelope (Boman, Delannoy, & Wilson, 1992; Hetzer, Bilbao-Cortes, Walther, Gruss, & Mattaj, 2000; Macaulay & Forbes, 1996). The gaps between the nonfused vesicles render the unfused nuclear membranes permeable to large macromolecules that cannot normally diffuse through fully fused nuclear membranes containing nuclear pore complexes. In contrast, the addition of BAPTA (a calcium chelator) to the reaction results in a fully sealed double nuclear membrane without nuclear pores. BAPTA thus creates intermediates that show a continuous and impermeable nuclear envelope, but no mature nuclear pores (Macaulay & Forbes, 1996). These intermediates are later used in Section 8.6.

To determine the integrity of the nuclear envelope, its ability to exclude 70 kDa rhodamine-labeled dextran is assayed. The 70 kDa dextran is of a size that is too large to diffuse through normal nuclear pores and will not pass through completely formed intact nuclear membranes; 70 kDa dextran will thus appear excluded from nuclei that have achieved complete nuclear membrane enclosure. However, this 70 kDa dextran can diffuse into nuclei if there are gaps in the nuclear membranes or, alternately, in cases where nuclear membrane assembly never proceeded beyond the vesicle-binding stage, such as in GTP-gamma-S reactions (Lau et al., 2009).

PROTOCOL

1. Initiate nuclear assembly reactions, as described in Section 8.4 through Step 3.
2. Add a protein or molecule of interest to an assembly reaction, while adding a similar amount of the same buffer or solvent to a control reaction. Mix the reaction by gently flicking the tube five times. Let incubate for 10–15 min at RT.

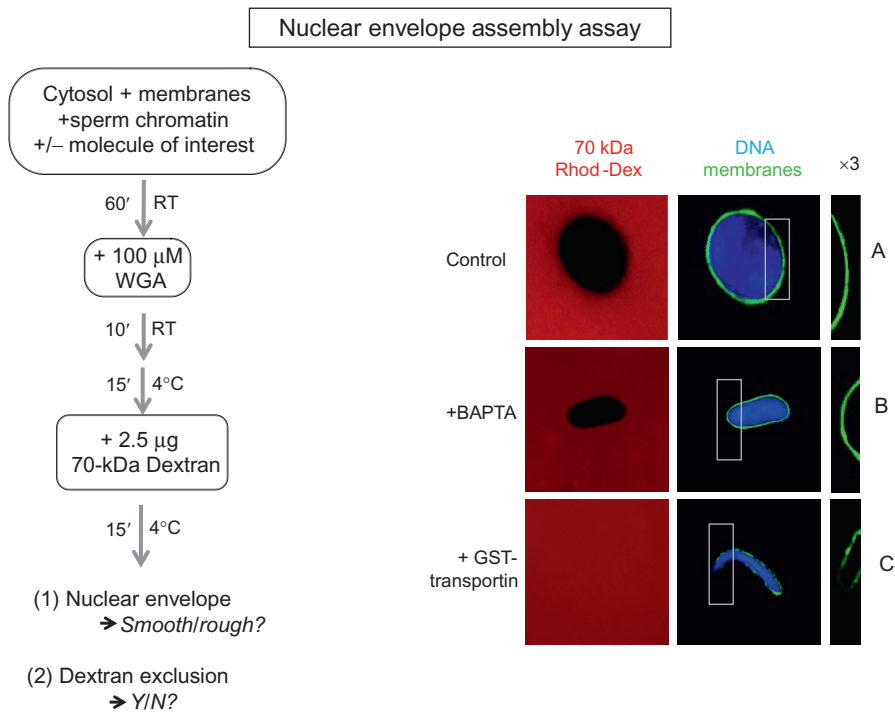


FIGURE 8.2 Nuclear Membrane Assembly and Integrity

High-speed interphase *Xenopus* egg extract is mixed with sperm chromatin and membranes and allowed to incubate at room temperature for 60 min. Here, nuclei formed in the presence and absence of a potential inhibitor of membrane fusion are shown. The recombinant protein added to test its role on nuclear membrane assembly and integrity is GST-transportin (as shown in Lau et al., 2009). The integrity of the resulting nuclear envelope was examined with two assays: exclusion of 70 kDa rhodamine dextran (red) and staining with the membrane dye DHCC (green). The latter reveals the presence or absence of a continuous green nuclear envelope. Chromatin is stained with Hoechst DNA stain (blue). For each condition shown, the nuclear envelope is also shown magnified threefold for better viewing (3×; right column). (A) The control consists of addition of a protein that does not affect membrane assembly and integrity (here 20 μM GST is used). A smooth nuclear envelope staining is observed and the representative nucleus shown is impermeable to 70 kDa dextran. (B) A similar intact phenotype is observed when 8 mM BAPTA is added to the reaction. (C) Addition of 20 μM GST-transportin prevents vesicle-vesicle fusion, which results in a permeable nuclear envelope. The phenotype is characterized by a rough nuclear envelope staining around the DNA as well as by a permeable nuclear envelope, as determined by the absence of exclusion of the 70 kDa dextran.

Note: The volume of the compound of interest to be tested should not exceed 1/5 of the final volume of the reaction. Empirically, 1–2 μL volume of the added compound is preferred.

3. To each tube, add 1 μL of $20\times$ ATP-regenerating system.
4. To each tube, add a final concentration of sperm chromatin of $\sim 3,000$ SpC/ μL of reaction. Mix gently by flicking the tubes 3 to 5 times.
5. Allow assembly for 60 min at room temperature.
6. At $t=60$ min, add 100 μM of WGA (final concentration) to each tube.

Note: Even though fully formed nuclear pores are expected to exclude 70 kDa rhodamine dextran, lectin WGA can be added after nuclear reconstitution to ensure a tight seal of the nuclear pores, as WGA inhibits transport through the nuclear pore (Finlay et al., 1987, 1989). In cases where the reconstituted nuclei will also be assessed for nuclear import or other functions, WGA addition should be omitted.

7. Mix by gently flicking the tube several times.
8. Let incubate for 10 min at RT.
9. Transfer the tubes to ice for 15 min.
10. Add to each tube the equivalent of 2.5 μg of 70 kDa rhodamine-labeled dextran. (Do not exceed 1–2 μL in volume.) Mix by gently flicking the tube 5 times and let incubate on ice for 15 min.

Note: Exclusion of rhodamine-labeled 70 kDa dextran (70 kDa Rhod-Dex) appears as if the interior of an intact nucleus resembles a *black disc* surrounded by a homogeneous red background. Adding too much 70 kDa rhodamine-labeled dextran should be avoided as this results in excessive background noise above and below the excluding nuclei obscuring microscopic observation. The ideal 70 kDa rhodamine-labeled dextran concentration for use can be identified using control or BAPTA nuclear reconstitution reactions.

11. After incubation on ice, the reaction should be diluted 1:1 in XB buffer containing 7.4% formaldehyde to fix the nuclei.
12. On a glass slide, place 1 μL of the fixation buffer above containing 100 $\mu\text{g}/\text{mL}$ of DHCC and 5 $\mu\text{g}/\text{mL}$ Hoechst. Place 3 μL of the assembly reaction onto this drop of fixation buffer, using a large orifice 200 μL tip. Then, for visualization, follow Steps 10 and 11 of Section 8.4. (Other methods for determining nuclear membrane integrity can be found, for example, in Fichtman et al, 2010 and in the Anderson paper.)

Typical results obtained are depicted in Fig. 8.2.

8.6 A NUCLEAR PORE COMPLEX ASSEMBLY ASSAY USING PORE-FREE NUCLEAR INTERMEDIATES

The effect of a protein or molecule of interest on nuclear pore assembly can be assayed using nuclear intermediate structures that contain complete nuclear membranes, but lack mature nuclear pores (pore-free nuclear intermediates). For this, an *in vitro* nuclear assembly reaction is first performed in the presence of the calcium

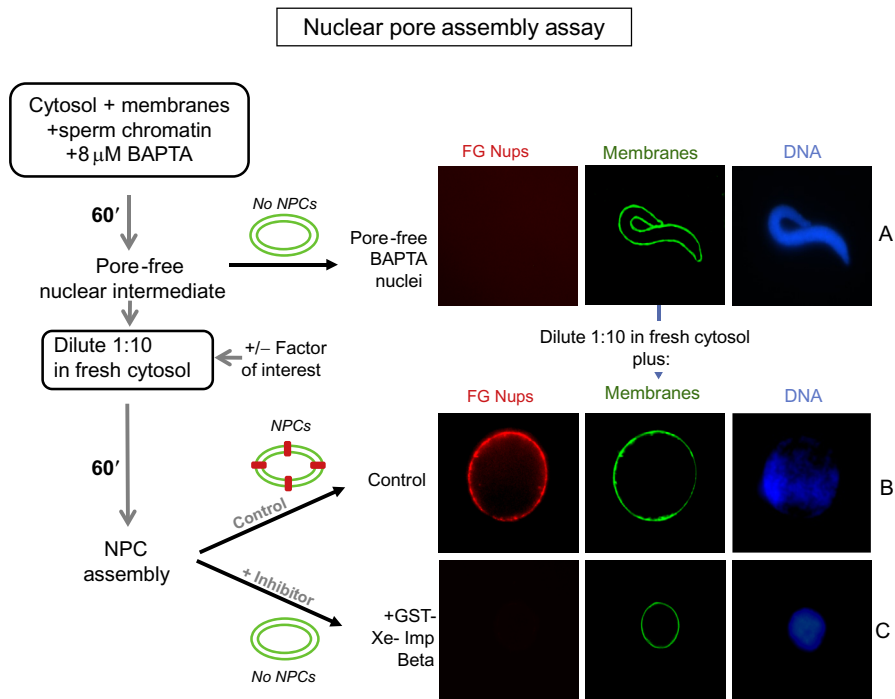


FIGURE 8.3 Nuclear Pore Assembly Assay

A schematic representation of the assay is depicted at the left. (A) To assemble pore-free nuclear intermediates, sperm chromatin, interphase egg extract, and membranes are incubated in the presence of 8 mM BAPTA for 60 min (Macaulay & Forbes, 1996). The BAPTA intermediate is quite small due to the lack of nuclear pores and resulting lack of nuclear import of vital nuclear proteins. The presence of nuclear pores is detected by staining for FG-nucleoporins (mAb414-TRITC; red), while membranes are detected with DHCC (green), and DNA with Hoechst dye (blue). (B and C) Nuclear pore assembly into these intermediates was tested under different conditions by diluting an aliquot of the pore-free intermediates 1:10 into fresh BAPTA-free cytosol and incubating for a further 60 min. (B) In the control condition, where no inhibitory protein or molecule was added (or when a control protein such as MBP is added), nuclear pore assembly occurred, as seen by the red FG-Nup rim. (C) Addition of 20 μM GST-*Xenopus* importin beta inhibits nuclear pore assembly, as seen by the lack of FG-Nups at the rim (Harel, Chan, et al., 2003).

chelator BAPTA (Harel, Chan, et al., 2003; Lau et al., 2009; Macaulay & Forbes, 1996). If these BAPTA intermediates are diluted 1:10 into fresh BAPTA-free cytosol, nuclear pore assembly ensues in the pore-free nuclear intermediates shortly afterward. The newly formed nuclear pore complexes can be detected via staining of their FG-Nups using a directly labeled fluorescent antibody, such as mAb414. If, however, a protein or molecule that blocks NPC assembly is added, the pore-free nuclear intermediates will remain free of nuclear pores and fail to stain with mAb414. The protocol for this assay is described below (see Fig. 8.3).

Note before starting the protocol: For this type of experiment, pore-free BAPTA nuclear intermediates are first assembled in 20 μL of cytosol for 60 min as described below. Then 5 μL of the BAPTA reaction containing pore-free intermediates are diluted into 45 μL of fresh BAPTA-free cytosol with energy mix, plus or minus a putative inhibitor. Therefore, from one 20 μL -BAPTA reaction, 4 nuclear pore assembly reactions can be performed.

PROTOCOL

1. Initiate the desired number of *in vitro* nuclear assembly reactions: relating to the note above and by following instructions described in [Section 8.4](#) through Step 3.
2. At that point, add BAPTA to each tube to a final concentration of 8 μM from the BAPTA stock solution described in [Section 8.1](#). Mix by flicking the tube gently 5 times.
3. To each tube, add 1 μL of 20 \times ATP-regenerating system.
4. To each tube, add the desired amount of demembranated sperm chromatin needed to achieve 3,000 SpC Units/ μL of reaction. Mix by flicking gently.
5. Incubate for 60 min at room temperature.

Note: At $t = 40$ min, in order to verify that successful formation of pore-free nuclear intermediates has occurred, check a 5 μL aliquot for the lack of FG-Nups using the HF555-mAb414 antibody and DHCC (as described in Steps 7–11 [Section 8.4](#)). The DNA in BAPTA nuclear intermediates should appear slightly decondensed and look “worm-shaped” or like a very small oblong/ellipsoidal structure. These BAPTA pore-free nuclear intermediates should also show a smooth and continuous DHCC staining of the nuclear membrane, with no mAb414 staining of FG-Nups.

6. At $t = 60$ min, dilute the BAPTA reactions 1:10 into fresh cytosol (BAPTA-free cytosol) as follows:
 - a. A few minutes before $t = 60$ min for the BAPTA reaction above, prepare a new 1.5-mL microcentrifuge tube containing: 45 μL freshly defrosted cytosol (important) and 2.5 μL of 20 \times Energy mix for each experimental condition or control planned. Add the desired amount of protein or molecule of interest for testing for an effect on nuclear pore assembly to the appropriate tubes. Mix each tube by gently vortexing for 1 s or flicking a few times.

Note: The compound of interest should be added to the fresh cytosol before proceeding to Step 6b, as it is known that NPC assembly commences as soon as the pore-free nuclear intermediate reaction is diluted into fresh control cytosol.
 - b. Take 5 μL from the 60' BAPTA reaction of Step 5 and add it to each of the prepared tubes from Step 6a. Mix the reactions by flicking the tubes twice, very gently (the BAPTA nuclear intermediates are extremely fragile).
7. Let the reactions proceed for an additional 60 min at room temperature.
8. To visualize the effects of the tested proteins or molecules on the assembly of nuclear pores follow the steps as described in [Section 8.4](#), Steps 7–11.

Representative results using this technique are shown in [Fig. 8.3](#).

ADDITIONAL NOTES FOR SECTION 8.6

1. If the protein/molecule added to the reaction does not block NPC assembly, the pore-free BAPTA intermediates, once diluted 1:10 into fresh cytosol, should convert into *regularly shaped* large nuclei, as are described in the last note of [Section 8.4](#).
2. The *concentration* of nuclei present in the final reaction, after dilution of the BAPTA reaction 1:10 into fresh cytosol, will be 1/10th the normal 3,000 SpC/ μ L. However, do not compensate for this by adding excess SpC/ μ L in the initial reaction because this will overload the reaction's capacity for correct nuclear assembly. One must compensate by observing more microscopic fields per assay.

CONCLUSION

In the 1980s, upon observing nuclear reconstitution *in vivo* and *in vitro* in the *Xenopus* egg or its extract, scientists realized that the cell was capable of performing a true molecular *tour de force*. Now it is the *modus operandi* for examining nuclear assembly in a cell-free environment. Greater mastery of this technique has opened up a wide field for developing new approaches derived from the *in vitro* reconstitution assay itself. Two assays we describe in this chapter, based on the first protocol, allow one to study independently nuclear membrane and nuclear pore assembly events. To determine the role of a factor in such assembly events, a protein or a molecule of interest can be added for testing on the *in vitro* reaction. One is not limited, however, to the sole addition of factors. Depletion techniques are another available option ([Finlay & Forbes, 1990](#)). Immunodepletion is one of the most used and has been used, to cite just a few examples, to characterize the absolute requirement of the Nup 107–160 complex, the pore-targeting protein ELYS, the GLFG nucleoporin Nup 98, and the nuclear pore scaffold protein Nup188 for nuclear pore assembly ([Franz et al., 2007](#); [Harel, Orjalo, et al., 2003](#); [Powers, Macaulay, Masiarz, & Forbes, 1995](#); [Theerthagiri, Eisenhardt, Schwarz, & Antonin, 2010](#); [Walther et al., 2003](#)). Nuclear reconstitution following specific protein immunodepletion has similarly been used to analyze the requirement for different transmembrane nucleoporins (see [Chapter 9](#)). *In vitro* nuclear reconstitution assays have also been widely used to study nuclear transport ([Chan & Forbes, 2006](#); [Lachish-Zalait et al., 2009](#); [Savulescu et al., 2011](#)), nuclear membrane and NPC assembly ([Anderson & Hetzer, 2007](#); [Fichtman et al., 2010](#)), DNA replication ([Gillespie, Gambus, & Blow, 2012](#); [Gillespie, Li, & Blow, 2001](#); [Walter & Newport, 1997](#)), apoptosis ([von Ahse & Newmeyer, 2000](#)), nuclear breakdown and reassembly mechanisms at mitosis ([Galy et al., 2008](#); [Newport & Spann, 1987](#)), pol III transcription from nuclei composed of recombinant tRNA genomes ([Ullman & Forbes, 1995](#)), and many other areas. High-resolution scanning electron microscopy has also had a strong impact on the study of *in vitro* nuclear assembly, as described in [Chapter 2](#). All together, these discoveries have shown that the *Xenopus* egg extract system used for *in vitro* nuclear reconstitution is a powerful tool for studying the nucleus and its mechanisms of regulation.

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