

# Studying nuclear disassembly in vitro using *Xenopus* egg extract

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## Abstract

*Xenopus* egg extract provides an extremely powerful approach in the study of cell cycle regulated aspects of nuclear form and function. Each egg contains enough membrane and protein components to support multiple rounds of cell division. Remarkably, incubation of egg extract with DNA in the presence of an energy regeneration system is sufficient to induce formation of a nuclear envelope around DNA. In addition, these in vitro nuclei contain functional nuclear pore complexes, which form *de novo* and are capable of supporting nucleocytoplasmic transport. Mitotic entry can be induced by the addition of recombinant cyclin to an interphase extract. This initiates signaling that leads to disassembly of the nuclei. Thus, this cell-free system can be used to decipher events involved in mitotic remodeling of the nuclear envelope such as changes in nuclear pore permeability, dispersal of membrane, and disassembly of the lamina. Both general mechanisms and individual players required for orchestrating these events can be identified via biochemical manipulation of the egg extract. Here, we describe a procedure for the assembly and disassembly of in vitro nuclei, including the production of *Xenopus* egg extract and sperm chromatin DNA.

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## 1. Introduction

In eukaryotic cells, genomic DNA is separated from the cytoplasm by the nuclear envelope [1]. This envelope is both the boundary and passageway between the nuclear and cytoplasmic compartments. Selective trafficking occurs through nuclear pore complexes (NPCs), which facilitate movement of macromolecules between the two environments. The nuclear envelope consists of an outer membrane, which is continuous with the endoplasmic reticulum (ER), and an inner membrane. Directly beneath the inner membrane is the nuclear lamina, a meshwork of lamin proteins that contributes to the structural integrity of the nuclear envelope.

Higher eukaryotes have an open mitosis, in which nuclear structures are disassembled and then reassembled during the cell cycle. Nuclear envelope breakdown is initiated by activated Cdk1-cyclin B, also referred to as maturation

promoting factor. This factor is responsible for initiating the phosphorylation of nuclear pore proteins, lamin proteins, and inner nuclear membrane proteins. Phosphorylation is accompanied by changes in pore permeability, disassembly of the nuclear lamina, microtubule-mediated gathering and tearing of the nuclear envelope, and dispersal of nuclear membranes [1–7]. Although these basic events have been identified, we are still far from understanding all the factors necessary for nuclear disassembly and how these factors coordinate successful progression into mitosis.

In vitro studies of cell cycle processes and their regulation in eukaryotic cells have benefited extensively from *Xenopus laevis* egg extracts. Unfertilized *Xenopus* eggs are arrested at metaphase of the second meiotic division and contain large stores of soluble proteins and membranes [8]. When eggs are crushed under conditions that promote cyclin degradation, the resulting egg extract is “interphase” in nature. When DNA is introduced into such an extract, along with an energy regeneration system, functional nuclei form. Many dynamic processes are recapitulated in this

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system, including nuclear assembly, DNA replication, and nuclear transport. If the egg extract is not held in interphase by the presence of translation inhibitors, nuclei will also undergo disassembly as the extract cycles into mitosis. Alternatively, cyclin can be added to an extract arrested in interphase in order to initiate mitotic events. Indeed, this cell-free system has been utilized extensively by many labs to identify and characterize many cell cycle events. In our laboratory, cell cycle studies using this *in vitro* technique have focused on the mechanism and events surrounding nuclear envelope breakdown at mitosis.

There are several advantages to the use of this cell-free system. *Xenopus* eggs and sperm chromatin are relatively easy to obtain in large quantities, providing a constant and abundant source of protein, membranes, and DNA. Egg extracts and chromatin can also be stored for use in multiple experiments. These extracts are useful not only for functional studies but for biochemical dissection as well, providing multiple avenues by which to approach a particular question. The nuclear assembly and disassembly assay allows cell cycle-dependent events to be probed in a highly synchronous population. Particularly useful is the ability to introduce dominant-negative proteins, antibodies, or drugs in the assay to probe the role of particular proteins. The ability to immuno-deplete a protein and assess the effect on specific processes is an additional benefit of this system.

Naturally, there are some limitations to this system as well. Egg quantity and egg extract quality can be variable. Also, although it provides a robust recapitulation of nuclear events during the cell cycle, the egg extract system is clearly a step away from the *in vivo* situation. Although it may initially be an advantage to study nuclear function without the full complexity of the cellular milieu, any findings should be examined in intact cells as well. It is also worth bearing in mind that egg extract most closely mimics cellular conditions within the early embryo, and thus some differences may arise when comparing to somatic cells. Overall, as egg extracts are amenable to manipulation in ways not possible in intact cells, the insight gained about basic mechanisms using these assays often provides valuable information not readily obtainable elsewhere.

## 2. Description of method

### 2.1. *Xenopus* interphase egg extracts

Cell-free extracts from *Xenopus* eggs are a useful tool in studying many events such as DNA replication, spindle assembly, nuclear assembly and disassembly, and nuclear import and export. There are various procedures for making egg extracts depending on the experimental question and strategy [9–13]. “Ultra-S” or high speed supernatants are more highly fractionated, CSF-arrested extracts hold conditions at meiosis rather than interphase, and “cycling” extracts permit several rounds of cell cycle driven by endogenous components. For our nuclear disassembly assay, we

use a “crude” interphase egg extract, adding in cycloheximide to prevent new cyclin synthesis. This extract has gone through only a low speed spin, and is sometimes referred to as an LSS, or “low speed supernatant” extract. The protocol below incorporates our experiences and findings into previously published procedures for generating a crude egg extract.

### 2.2. Materials

- 3–4 Female frogs (from Nasco, or other *Xenopus* distributor).
- 6 L Tubs and lids (with several breathing holes in lid).
- 250 ml Glass beakers.
- 14 ml Round-bottom Falcon tubes (Fisher, C/N 14-959-11B).
- 5 ml Round-bottom Falcon tubes (Fisher, C/N 14-959-11A).
- IEC Centra CL2 Clinical centrifuge (or equivalent).
- Beckman JS13.1 rotor.
- Beckman Avanti J-25 I centrifuge.
- 3 ml or 10 ml Syringes (BD Biosciences, C/N BD309585 or BD309604).
- 18 Gauge, 1 1/2 needle (Fisher C/N 305-196).
- Human chorionic gonadotrophin (HCG) (Sigma, C/N CG-10); 5 U/ $\mu$ l in sterile distilled water.
- 1 $\times$  MMR: 100 mM NaCl, 2 mM KCl, 1 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 0.1 mM EDTA, and 5 mM Hepes, pH 7.8.
- 0.25 $\times$  MMR.
- ELB (Egg lysis buffer): 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Hepes, pH 7.6, 250 mM sucrose, pH 7.4–7.5.
- 2% L-cysteine hydrochloride, pH 7.8–7.9 (prepare immediately before dejellying and pH with NaOH) (Fisher, C/N BP376-100).
- 1 M dithiothreitol (DTT) (Fisher, C/N BP172-5).
- Aprotinin and leupeptin (10 mg/ml) (Roche Applied Science, C/N 10 236 624 001 and 11 017 101 001).
- Cycloheximide in sterile distilled water (10 mg/ml) (Calbiochem, C/N 239764).
- Cytochalasin B in DMSO (5 mg/ml) (Calbiochem, C/N 250233).
- Glycerol.
- Liquid nitrogen.
- Standard plastic bulb transfer pipette (Fisher, C/N 13-711-7).
- 0.5 ml Microfuge tubes.

### 2.3. Frog care and maintenance

Frogs are stored in municipal water which has been run through a carbon filter, a water softener and a reverse osmosis filter. Synthetic ocean mix is added for sodium and other minerals, and sodium bicarbonate to adjust the pH to 6.9–7.1 and the conductivity to 1.0–1.5 micro siemens. We also test weekly for nitrite and ammonia levels, which represent the level of unprocessed waste. Frogs are stored at a temperature of between 17 and 20 °C.

#### 2.4. Protocol

1. In preparation for egg laying, frogs are primed initially with 100  $\mu$ l of 1.6 U/ $\mu$ l HCG subcutaneously in dorsal lymph sacs (a shallow injection near top of thigh). After injection, frogs are placed into tanks containing 100 mM NaCl to reduce the potential for infection at the site of injection. At least seven days are needed to allow HCG priming to take effect and frogs should be used within one month.
2. A day prior to making egg extracts, inject frogs subcutaneously in dorsal lymph sacs with 100  $\mu$ l of 5 U/ $\mu$ l HCG. Place each injected frog in its own tank of frog water supplemented with 100 mM NaCl at 17–22 °C.
3. After 16–22 h, move frogs into new tanks and collect eggs from each frog in separate beakers, pouring off the excess frog water. We generally keep eggs from each frog separate throughout the entire process or at least until the crushing step, since the egg quality can vary between frogs. Eggs should be fairly uniform in appearance with well defined hemispheres. Bloated, fluffy, or strings of eggs are not ideal and should be discarded with a plastic transfer pipette.
4. After removing excess water and bad eggs, add freshly made 2% cysteine for no longer than 5 min, swirling gently to facilitate the dejelling process. Generally, about 100 ml of cysteine are used for every 25 ml of eggs. When eggs are completely dejellied, they pack tightly against each other and often the buffer gets cloudy and contaminated with sediment.
5. Pour cysteine off and wash eggs three times with 0.25  $\times$  MMR and once with 1  $\times$  MMR, swirling between each wash and allowing eggs to settle before pouring off the buffer. These washes should be completed as quickly as possible, so that eggs do not sit long in the cysteine or MMR buffers.
6. Rinse eggs once with ELB, pour off buffer and add fresh ELB. At this point, any bad eggs should be removed with a plastic pipette. Bad eggs include those that are activated (mostly white) or fluffy and/or floating. Rinse once more with ELB.
7. Exchange eggs into ELB supplemented with 1 mM DTT and 50  $\mu$ g/ml cycloheximide. Decant as much ELB as possible before swirling the eggs gently into the remaining buffer and transferring them to a 14 ml round-bottom tube (divide evenly between several tubes if necessary). At this point, good batches of eggs can be combined, if desired. After the eggs settle, remove excess buffer from the top of the tube with plastic transfer pipette.
8. Spin eggs in a clinical centrifuge for 10–15 s at 800 rpm ( $\sim$ 110g) and remove excess buffer that collects on top with a plastic transfer pipette to avoid dilution of the egg extract. Pipette tips or a syringe with needle can also be used to aspirate excess buffer, but care must be taken to avoid breaking or losing the eggs.
9. Add aprotinin, leupeptin, and cytochalasin B (each to a final concentration of 5  $\mu$ g/ml, estimated from the volume of packed eggs) to the top of the eggs in the tube and centrifuge in a Beckman JS13.1 rotor at 10,000 rpm (15,680g) for 15 min at 4 °C [note: the rotor itself is held at room temperature until the spin starts]. This spin serves to crush the eggs and several layers will form in the tube. The top will be a yellow lipid cap, followed by the crude cytoplasmic layer that is generally tan to brown. Underneath that will be a thin layer of pigment granules and dark yolk beneath that (Fig. 1).
10. To retrieve the crude egg extract, an 18 gauge needle is attached to a 3 or 10 ml syringe, depending on the volume to be collected, and is inserted directly into the side of the round-bottom tube above the pigment granules (see red arrowhead, Fig. 1), being careful not to puncture both sides. If the needle gets plugged with plastic in this process, remove it and promptly place a new needle with syringe into the same hole—only a few drops of extract will be lost. With the bevel side of the needle facing up, the egg extract can be carefully taken up into the syringe stopping when the lipid layer begins to be taken up. The syringe is also used to measure the amount of crude extract obtained.
11. Transfer the crude extract into a chilled 14 ml round-bottom tube. As before, add aprotinin, leupeptin, and cytochalasin B (each to a 5  $\mu$ g/ml final concentration) to the top of the sample and centrifuge as above in Beckman JS13.1 rotor at 10,000 rpm (15,680g) for 15 min at 4 °C.
12. Aspirate off any lipid remaining on the top and repeat the syringe extraction or simply remove crude extract with pipette tip and transfer into a clean, chilled 5 or 14 ml round-bottom tube. Add glycerol to 5% final concentration and mix by rotating at 4 °C until well combined (5–10 min).
13. Make single-use aliquots of less than 100  $\mu$ l in 0.5 ml tubes on liquid nitrogen. Store in liquid nitrogen. Aliquots can also be stored at  $-80$  °C, with a shorter shelf-life. Yields will vary but typically a good yield is 2–4 ml of egg extract per frog.

#### 2.5. Notes on procedure

1. Our IACUC-approved protocol requires that frogs be returned to a filtered tank system no more than 24 h from the time they were put into the transfer tank. Frogs then recover for at least 30 days before reuse. For best results, wait at least 60+ days for recuperation. The usual fertile lifespan for the *Xenopus* is 2–3 years, if they are allowed 3–4 months of off time.
2. Success of egg extracts in further application is essentially defined by the quality of the eggs after all washes. Although this has not been exhaustively examined, we generally abandon egg batches that have spontaneously activated during the course of washing (visualized by

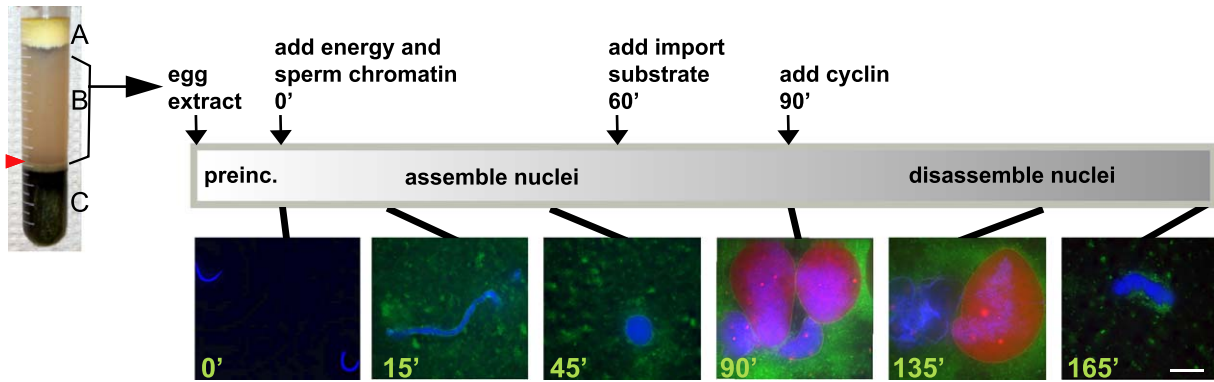


Fig. 1. Timeline for the nuclear assembly and disassembly assay. *Xenopus* eggs are fractionated into lipids (A), crude egg extract (B), and pigment granules and yolk proteins (C). To collect the crude extract, a syringe is inserted into the bottom of the crude layer (indicated by the red arrowhead). For the assembly assay, egg extract is first pre-incubated with protein or drug of interest. In order to initiate assembly, an energy regeneration system and sperm chromatin DNA are added to the extract (0'). Early in assembly the DNA (blue) decondenses and membrane vesicles (green) bind to the DNA (15'). As assembly progresses, the DNA becomes more rounded and vesicles fuse to form a nuclear envelope (45'). Import substrate (red) is added at 60 min. In the next 30 min, the import substrate accumulates within the nuclei, which continue to add more membrane leading to significant growth in size (90'). After 90 min of assembly, cyclin is added to shift the extract into mitosis. Breakdown begins sometime around 135' and is complete at 165' as is indicated by condensation of the DNA, dispersal of the membrane, and release of the import substrate. These images were acquired with a Deltavision microscope and deconvolved for 10 cycles. The scale bar indicates 15  $\mu$ M.

hyper-retraction of the pigment) or have more than 5% bad eggs.

- Since a calcium-dependent signal must be activated during the crushing step that ultimately leads to cyclin degradation, we do not chill the eggs, the buffer, or the rotor prior to the crushing step. The centrifuge is chilled so that by the end of the spin, the egg lysate is cold and we keep it cold from this point on. An alternative method is to pre-activate the eggs prior to crushing, either by incubation with calcium ionophore or electrical stimulation [13].
- It is important to remove as much excess buffer as possible from the eggs after the packing step but before crushing. Alternatively, one can also spin eggs through the oil, Versilube F-50, to help pack the eggs and separate them from the buffer [14]. Since this is not easily obtained, we use Nyosil M25 (TAI Lubricants Inc., Hockessin, DE), which is a commercially available equivalent. We have found, however, that this is not necessary if time and care is taken to remove as much buffer as possible with a transfer pipette.
- When obtaining the crude extract layer after the egg crushing step, it is preferable to sacrifice complete recovery of this fraction to avoid taking the pigmented layer directly below.
- To aliquot extracts on liquid nitrogen, we set up an open-bottom rack on top of a shallow Styrofoam container filled with enough liquid nitrogen to submerge the bottoms of the tubes. We then aliquot the egg extract into the partially submerged tubes and transfer directly into storage in liquid nitrogen.
- Crude egg extracts have a limited time in which they are functional for disassembly assays. In our hands, we have found that good crude egg extracts can last for approximately 3 months when stored in liquid nitrogen. Some

protocols suggest using crude extracts fresh. This alternative is fine but it is worth noting that the kinetics of disassembly will likely be faster than in an extract that has been frozen.

### 3. Demembrated sperm chromatin

*Xenopus* males are an excellent source of chromatin for use in nuclear disassembly assays. Male frogs are smaller in size and generally darker in pigment than their female counterparts. A benefit of using *Xenopus* sperm as a source of DNA is that, as external fertilizers, their testes contain large stores of easily harvested sperm. In addition, they are obtained from the same suppliers as females and can be housed similarly to females (though in separate tanks from females). Since each frog will yield about  $1\text{--}2 \times 10^7$  sperm heads and a single nuclear assembly reaction requires only 100–1000 sperm heads per microliter of egg extract, this protocol should provide an ample supply. The protocol below has been adapted from a previous protocol [15].

#### 3.1. Materials

- 2–4 Male *X. laevis* frogs (from Nasco, or other *Xenopus* distributor).
- 60 mm Glass petri dish.
- Dissection scissors (Fine Science Tools C/N 14068-12).
- 2 Pairs sharp forceps (Fine Science Tools C/N 11231-30 or 11021-12).
- Hemacytometer (Hausser Scientific C/N 1492).
- Liquid nitrogen.
- 15 ml Conical polypropylene tubes (Fisher 14-959-49B).
- 14 ml Round-bottom Falcon tubes (Fisher 14-959-11B).
- IEC Centra CL2 clinical centrifuge (or equivalent).
- Beckman TL-100 ultracentrifuge.

- Beckman ultraclear thin-wall centrifuge tubes (TLS-55, 2.5 ml) (Beckman, C/N 347356).
- Beckman JS 13.1 swinging bucket rotor (or equivalent).
- 1.5 ml Microfuge tubes.
- Eppendorf centrifuge 5415C (or equivalent).
- Ultra-pure sucrose (Fisher, C/N BP220-1).
- Triton X-100 (10%) (Fisher, C/N BP151-100).
- Aprotinin and leupeptin (10 mg/ml) (Roche Applied Science, C/N 10 236 624 001 and 11 017 101 001).
- Dithiothreitol (DTT) (1 M) (Fisher, C/N BP172-5).
- 10% Ethyl *p*-aminobenzoate (Benzocaine) solubilized in EtOH (Sigma, C/N E-1501).
- Bovine serum albumin (BSA) (Fisher, C/N BP1605-100).
- 0.5 ml Microfuge tubes.
- 10× Extraction Buffer: 100 mM Hepes, pH 7.4, 800 mM KCl, 150 mM NaCl, 50 mM MgCl<sub>2</sub>, 10 mM EDTA.

### 3.2. Reagent preparation

It is easiest to prepare reagents and solutions the day before and store at 4 °C. It will take a while for the sucrose to completely dissolve into the buffers, and rotation at room temperature for several hours is not uncommon, particularly for the 2.5 M sucrose buffer. Prepare 50 ml of 1× Buffer for use in preparation of other buffers. Also prepare 20 ml of 2.5 M sucrose buffer by adding 17.12 g of ultrapure sucrose to 2 ml 10× Buffer plus ddH<sub>2</sub>O to a final volume of 20 ml. Prepare the remaining buffers as outlined (Table 1). Buffers should be made without aprotinin/leupeptin, BSA, and DTT, adding these reagents just before using.

### 3.3. Protocol

1. Anesthetize male frogs by immersion in 0.05% benzocaine for 15 min, until frog is limp.
2. Lay the frog on its back. Using dissection scissors, cut through the skin first from the center of the abdomen in a semi-circle up both sides. Repeat incision into the peritoneum.
3. Lift flap up, push liver aside, and clip heart.
4. By removing the yellowish fatty material, the testes should emerge on either side of the midline. Testes are small, oval, and pink/tan in coloration, and are

attached by a small amount of tissue to the surrounding fatty material.

5. Cut the testes free of adherent tissue with either forceps or scissors. Place testes in a 60 mm glass petri dish, containing a small amount (~3 ml) of cold extraction buffer (no supplements).
6. Shred testes into very small pieces of a few mm<sup>2</sup> or less with 2 pairs of sharp forceps, and transfer them with buffer into a 15 ml conical tube.
7. Vortex the minced testes vigorously and pellet the larger pieces by gentle centrifugation in a clinical centrifuge at ~1000 rpm (~200g) for 10–20 s.
8. Remove the supernatant containing the sperm to a new 15 ml conical tube and add 3 ml of extraction buffer supplemented with 200 mM sucrose to the testes pellet. Vortex and spin as before.
9. Collect supernatants together and repeat the process with the testes pellet 2–3 times or until the supernatant is no longer that cloudy.
10. Centrifuge the combined supernatants at 2600 rpm (~1200g) in a clinical centrifuge for 50 s to pellet any remaining pieces of tissue. Transfer the supernatants to a 14 ml round-bottom tube and pellet sperm at 4100 rpm (~2600g) for 10 min in Beckman JS13.1 rotor.
11. Prepare sucrose gradients for sperm separation as follows: Add 0.2 ml 2.5 M sucrose extraction buffer into each of four 2.5 ml tubes (Beckman TLS55 ultraclear thin-wall). Overlay with 1.7 ml of 2.3 M sucrose extraction buffer.
12. Resuspend sperm pellet in 0.8 ml of 2 M sucrose extraction buffer and overlay gently on top of the sucrose gradients (0.2 ml per tube). Stir the interface between the sperm and top sucrose layer by gently swirling with a pipette tip. Centrifuge sucrose gradients at 33,000 rpm (~73,000g) in a swinging bucket TLS55 rotor for the TL100 table top ultracentrifuge for 25 min at 4 °C.
13. Aspirate the top half of the gradient, which contains the majority of contaminating red blood cells. The majority of sperm heads are on top of the 2.5 M sucrose cushion; however, it is best to keep the entire lower half of the gradient and remove this into a 14 ml round-bottom tube.
14. Dilute the sperm to 12 ml with 0.2 M sucrose extraction buffer and pellet the sperm by centrifugation in a swinging bucket rotor at 5100 rpm (~4100g) in a Beckman JS13.1 swinging bucket rotor for 10 min at 4 °C.
15. Decant supernatant and resuspend the pellet in 1 ml of 0.2 M sucrose extraction buffer containing 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM DTT, and 0.4% Triton X-100. Incubate on ice for 30 min, being careful to avoid a longer incubation. This step will demembranate the sperm.
16. Prepare sucrose cushions in two 1.5 ml microfuge tubes as follows: Add 0.5 ml of 0.5 M sucrose extraction buffer with 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM DTT, and 3% BSA. Overlay each sucrose

Table 1  
Buffer preparation for demembranated sperm chromatin protocol

Buffer	2.5 M Sucrose buffer (ml)	1× Buffer (ml)
2.3 M Sucrose	9.2	0.8
2.0 M Sucrose	2	0.5
0.5 M Sucrose <sup>a</sup>	0.5	2
0.2 M Sucrose	4	46
0.2 M Sucrose <sup>b</sup>	Use 1.80 ml of 0.2 M sucrose buffer	—
0.2 M sucrose <sup>c</sup>	Use 3.00 ml of 0.2 M sucrose buffer	—

<sup>a</sup> Add 1.25 µl aprotinin and leupeptin, 2.5 µl DTT, and 75 mg BSA fresh.

<sup>b</sup> Add 1 µl aprotinin and leupeptin, 2 µl DTT, and 75 µl 10% Triton X-100 fresh.

<sup>c</sup> Add 1.5 µl aprotinin and leupeptin, 3 µl DTT, and 90 mg BSA fresh.

cushion with half of the sperm prep and centrifuge in a microcentrifuge (such as Eppendorf Centrifuge 5415C) at ~3300 rpm (870g) for 10 min at room temperature to pellet the sperm.

17. Remove the supernatant and carefully resuspend the sperm pellet in 0.1 ml of 0.2 M sucrose extraction buffer with 3% BSA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 1 mM DTT. Avoid washing residual Triton X-100 from the sides of the tube. Transfer the resuspended sperm to a clean microfuge tube.
18. Further resuspend the sperm chromatin pellet up to approximately 0.25 ml of 0.2 M sucrose extraction buffer with 3% BSA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 1 mM DTT.
19. To count the sperm, make a 1:500 dilution of an aliquot of the sperm and load on a hemacytometer. Count sperm at 10× magnification. Sperm will appear black, thin, and curvy or curly. After counting, dilute the sperm appropriately to 50,000–100,000 sperm/µl.
20. Mix diluted sperm gently but thoroughly. Make 5 µl aliquots, snap freeze in liquid nitrogen, and store at –80°C. Typical yield is between 1 and 2 × 10<sup>7</sup> sperm/frog.

#### 3.4. Notes on procedure

1. Dissection of frogs is accompanied by a large amount of blood and other fluids. We generally lay frogs on paper towels or bench paper, which we can use afterwards to wrap the frog before putting into a rubber glove (hold frog with gloved hand and invert glove) for disposal in the animal facility.
2. Taking time to mince testes well can significantly increase the yield of sperm chromatin obtained.
3. Sperm are much more susceptible to shearing after the demembration step and care should be taken to avoid over-pipetting or excessive handling of the sperm after that point.
4. Sperm chromatin has a tendency to settle during the aliquoting process, so should be mixed by inversion throughout aliquoting.

### 4. Nuclear assembly/disassembly assay

Our protocol for the assembly and disassembly of nuclei (Fig. 1) has evolved from several earlier protocols [15,16]. The nuclei are first assembled from interphase egg extracts containing cycloheximide in order to prevent the synthesis of cyclin, required for mitotic entry. We then initiate mitosis by adding exogenous cyclin, which associates with and activates the mitotic kinase Cdk1. The form of recombinant cyclin B that we use (Δ90) results in a stable shift into mitosis since it lacks the destruction box required for degradation by the proteasome.

#### 4.1. Materials

- *Xenopus* crude egg extract.

- Sperm chromatin DNA (100–1000 sc/µl of egg extract).
- 5 mg/ml Creatine Kinase (Calbiochem, C/N 2384); Resuspend in 50% glycerol, 10 mM Hepes, pH 7.8, 100 mM NaCl.
- 0.2 M ATP (Calbiochem, C/N 1191).
- 1 M Phosphocreatine (Calbiochem, C/N 2380); Resuspend in 10 mM filtered potassium phosphate buffer, pH 7.0.
- ELB (egg lysis buffer): 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Hepes, pH 7.6, 250 mM sucrose.
- 0.5 ml Microfuge tubes.
- Pipette tips with ~3 mm removed from the end with a razor blade.
- NLS-RITC import substrate [15].
- 16% Paraformaldehyde (Polysciences, C/N 18814).
- Δ90 Cyclin [17].
- Fix: 12% paraformaldehyde, 0.2 M sucrose, 10 mM Hepes, pH 7.8, 1 µg/ml Bisbenzimidazole H 33258 Hoechst (Calbiochem, C/N 382061), and 10 µg/ml 3,3'-dihexyloxycarbocyanine (DHCC; Fisher, C/N AC407601000).
- Microscope (such as Zeiss Axiophot).

#### 4.2. Procedure

1. For each reaction, 28 µl of freshly thawed crude egg extract can be pre-incubated if desired with antibody, recombinant protein fragment, or drug (this volume should be less than 10% the volume of the reaction) for 15–30 min at room temperature in 0.5 ml microfuge tube.
2. Make up an energy regeneration mix: 0.5 M phosphocreatine, 50 mM ATP, and 1.25 mg/ml creatine phosphokinase, to maintain ATP in the reaction. The components of the energy mix should be stored in small aliquots to avoid multiple freeze thaw cycles.
3. Add 1 µl of energy mix and 2 µl of sperm chromatin to initiate in vitro nuclear assembly. The sperm chromatin is pre-diluted in ELB to obtain the desired concentration. The pre-diluted sperm chromatin should be thoroughly mixed to avoid clumping, but after the sperm chromatin is added to the egg extract all mixing must be done with a cut pipette tip in a gentle manner.
4. After 60 min of assembly, add in 1 µl of NLS-RITC import substrate and store the tubes in the dark. At this time, a closed nuclear envelope containing nuclear pore complexes has formed around the sperm chromatin. Import of the nuclear localization signal containing substrate will test for efficient nuclear assembly and also serves as an indicator of nuclear envelope breakdown.
5. After 90 min of assembly, a 9 µl interphase sample is fixed in 3 µl of 16% paraformaldehyde. These interphase samples are taken with a cut pipette tip and are gently mixed. Then recombinant cyclin is added to the reaction to induce disassembly. We titrate the cyclin such that the amount added triggers nuclear envelope breakdown after ~75–90 min. After cyclin addition, the reaction is carefully mixed once with a cut pipette tip.

6. 75–90 min after cyclin addition, 9  $\mu$ l samples are fixed in 3  $\mu$ l of 16% paraformaldehyde. As the nuclei undergo disassembly, they become very fragile so mixing is avoided. Mitotic time points are taken with a cut pipette tip from the middle of the sample and mixed only once in paraformaldehyde.
7. From each fixed timepoint, 4  $\mu$ l is added to 1  $\mu$ l of fix on a microscope slide. A coverslip is placed very carefully over the sample (lower slowly at an angle using forceps with very fine tips). These samples can be imaged with fluorescence microscopy using a set-up such as a Zeiss Axiophot optimally with a Plan-Apochromat 63 $\times$ /1.40 Oil objective (Zeiss, C/N 440762) or Deltavision system. Disassembly of the nuclei is visualized by condensation of the DNA (blue, Hoechst), loss of the import substrate (red), and dispersal of the membrane (green, DHCC) (Fig. 1).
8. To quantitate disassembly, the number of nuclei in two 4  $\mu$ l aliquots of each fixed sample is determined and averaged. The percent of intact nuclei at a given mitotic timepoint is the average number of nuclei at that time divided by the average number of nuclei present at the interphase timepoint, multiplied by 100. When nuclei are quantitated, the lower amount of sperm chromatin (100 sperm chromatin/ $\mu$ l of egg extract) is added to the reaction.

#### 4.3. Notes on Procedure

1. An egg extract of high quality with efficient import is required. Egg extract and sperm chromatin freeze-thawed more than once will not work in the assay. The kinetics with which disassembly occurs can vary between egg extracts. To see efficient protection from disassembly, we prefer slower kinetics, with the process of disassembly taking 75–90 min. The kinetics of disassembly can be controlled to some degree by adjusting the amount of cyclin added.
2. The import substrate can be added at later time points. It takes 20–30 min depending on the egg extract to achieve significant accumulation within the nuclei.
3. The potential inhibitor of disassembly may be added to the reaction at a later time point if there is a concern that assembly may be inhibited. In addition, we have found that imidazole disrupts assembly and glycine interferes with fixation in paraformaldehyde, so recombinant protein and antibodies that are to be added to the reaction are dialyzed into PBS or ELB.
4. Rather than adding exogenous cyclin, disassembly of the assembly reaction can also be induced by adding mitotic egg extract, which is made in the presence of a calcium chelator and a general phosphatase inhibitor [14].
5. To process samples for immunofluorescence, nuclei can be fixed in 2 mM Ethylene glycol bis[succinimidylsuccinate] (Pierce, C/N 21565) or 4% paraformaldehyde and spun through a 30% sucrose cushion onto a coverslip. Alternatively, they can be fixed onto a coverslip through a quick-freeze in liquid nitrogen followed by a methanol

fixation [18]. These samples can also be fixed for electron microscopy [19]. To maximize the number of nuclei present, undiluted sperm chromatin (1000 sperm chromatin/ $\mu$ l of egg extract) is used for these techniques.

#### 5. Concluding remarks

The mechanisms required for nuclear envelope breakdown are not well understood. This assay is a powerful biochemical tool to dissect the steps required for this process. Mechanisms of interest can easily be interfered with by the addition of drugs, depletion of proteins, or the addition of recombinant proteins. This assay has enabled us to identify a requirement for the nuclear pore and COPI complex in the remodeling of the membrane during mitotic nuclear disassembly [18,20].

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