

Video Article

A Cell-Free Assay Using *Xenopus laevis* Embryo Extracts to Study Mechanisms of Nuclear Size Regulation

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Abstract

A fundamental question in cell biology is how cell and organelle sizes are regulated. It has long been recognized that the size of the nucleus generally scales with the size of the cell, notably during embryogenesis when dramatic reductions in both cell and nuclear sizes occur. Mechanisms of nuclear size regulation are largely unknown and may be relevant to cancer where altered nuclear size is a key diagnostic and prognostic parameter. *In vivo* approaches to identifying nuclear size regulators are complicated by the essential and complex nature of nuclear function. The *in vitro* approach described here to study nuclear size control takes advantage of the normal reductions in nuclear size that occur during *Xenopus laevis* development. First, nuclei are assembled in *X. laevis* egg extract. Then, these nuclei are isolated and resuspended in cytoplasm from late stage embryos. After a 30 - 90 min incubation period, nuclear surface area decreases by 20 - 60%, providing a useful assay to identify cytoplasmic components present in late stage embryos that contribute to developmental nuclear size scaling. A major advantage of this approach is the relative facility with which the egg and embryo extracts can be biochemically manipulated, allowing for the identification of novel proteins and activities that regulate nuclear size. As with any *in vitro* approach, validation of results in an *in vivo* system is important, and microinjection of *X. laevis* embryos is particularly appropriate for these studies.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54173/>

Introduction

The sizes of cellular organelles typically scale with the size of the cell, and this has been perhaps best documented for the scaling of nuclear size with cell size¹⁻¹⁰. This is particularly true during embryogenesis and cell differentiation, when dramatic reductions in both cell and nuclear size are often observed^{11,12}. Furthermore, altered nuclear size is a key parameter in cancer diagnosis and prognosis¹³⁻¹⁷. Mechanisms that contribute to nuclear size regulation are largely unknown, in part due to the complexity and essential nature of nuclear structure and function. The method described here was developed as an *in vitro* assay for nuclear size scaling that is amenable to biochemical manipulation and elucidation of mechanisms of nuclear size regulation.

Xenopus laevis egg extract is a well-established system to recapitulate and study complex cellular processes in an *in vitro* context. These extracts have revealed new fundamental information about several cellular processes including the assembly and function of the mitotic spindle, endoplasmic reticulum, and nucleus¹⁸⁻²². One key advantage to the extract system is that *X. laevis* egg extracts represent a nearly undiluted cytoplasm whose composition can be easily altered, for instance through addition of recombinant proteins or immunodepletion. Furthermore, one is able to manipulate essential processes by employing treatments that might otherwise be lethal in an *in vivo* context. Modifications of the egg extract procedure allow for isolation of extracts from *X. laevis* embryos rather than eggs, and these embryo extracts are equally amenable to biochemical manipulation²³. During *X. laevis* development, the single-cell fertilized embryo (~1 mm diameter) undergoes a series of twelve rapid cell divisions (stages 1 - 8) to generate several thousand 50 μ m diameter and smaller cells, reaching a developmental stage termed the midblastula transition (MBT) or stage 8²⁴⁻²⁶. The MBT is characterized by the onset of zygotic transcription, cell migration, asynchronous cell divisions, acquisition of gap phases, and establishment of nuclear steady-state sizes rather than continual nuclear expansion as in the pre-MBT embryo. From stage 4 to gastrulation (stages 10.5 - 12), the volume of individual nuclei decreases by more than 10-fold¹¹.

Here, the goal is to identify mechanisms responsible for these reductions in nuclear size during developmental progression. The approach is to first assemble nuclei in *X. laevis* egg extract and to isolate those nuclei from the egg cytoplasm/extract. These nuclei are then resuspended in cytoplasm from late gastrula stage embryos. After an incubation period, the nuclei from egg extract become smaller in late stage embryo extract. We reasoned that this would be a useful assay for identifying cytoplasmic components present in late stage embryos that contribute to developmental nuclear size scaling. Using this assay, coupled with *in vivo* validation, we demonstrated that protein kinase C (PKC) contributes to developmental reductions in nuclear size in *X. laevis*²³.

Protocol

All *Xenopus* procedures and studies were conducted in compliance with the NRC Guide for the Care and Use of Laboratory Animals 8th edition. Protocols were approved by the University of Wyoming Institutional Animal Care and Use Committee (Assurance # A-3216-01).

1. Preparation of *X. laevis* Egg Extract (adapted from^{27,28})

- Prime female *X. laevis* frogs a minimum of three days and a maximum of two weeks before egg collection with a single 100 IU injection of pregnant mare serum gonadotropin (PMSG).
- One day before the experiment, inject primed frogs with 800 IU of human chorionic gonadotropin (HCG) and place at 16 °C in 1 L per frog of 1/3x Marc's modified ringers (MMR). See **Table 1** for all buffer compositions.
Note: Some frogs will not lay eggs or will lay poor quality eggs. Typically 2 - 3 frogs are injected to ensure at least one frog will lay sufficient numbers of good quality eggs. The average frog will lay enough eggs to produce at least 1 ml of egg extract.
- Prepare 1 L 1/3x MMR with cold distilled deionized water (ddH₂O), 500 ml buffer B, 1 L buffer C, 500 ml buffer D, and 100 ml buffer E.
- Transfer laid eggs to a glass crystallizing dish (150 x 75 mm). Using a plastic Pasteur pipet with the tip cut off, farm out white puffy activated eggs or lysed eggs. Only retain eggs with distinct and equal dark animal poles and white vegetal poles.
- Prepare 13 x 51 mm centrifuge tubes with 1 ml of Buffer E supplemented with 355 μM cytochalasin D added just prior to use in step 1.7.
- Dejelly and wash the eggs.
 - Wash eggs briefly with cold 1/3x MMR in an appropriately sized beaker, changing the buffer 3 - 4 times.
 - Remove the jelly coats from the eggs by incubation with Buffer B. This will take anywhere from 3 - 10 min. As the cloudy jelly coats are released from the eggs, change the buffer.
Note: Dejelling is complete when the eggs appear tightly packed in the corner of the dish when tilted and vegetal poles are oriented downwards. Eggs are much more fragile after dejelling, so do not incubate eggs with Buffer B for longer than necessary and subsequent washes need to be performed very carefully.
 - Wash eggs briefly with 3 - 4 changes of Buffer C.
 - Wash eggs briefly with 2 changes of Buffer D.
 - Wash eggs briefly with 2 changes of Buffer E.
- Fill centrifuge tubes with eggs using a wide bore glass pipette or plastic dropper. Aspirate off excess buffer. To pack the eggs and remove as much buffer as possible, centrifuge in swinging bucket rotor at 212 x g for 60 sec and then at 478 x g for 30 sec. Aspirate off excess buffer.
Note: It is important to remove as much excess buffer as possible at this step to ensure the egg extract is minimally diluted.
- In a swinging bucket rotor, ultracentrifuge under vacuum for 15 min at 12,000 x g and 4 °C to crush eggs open and separate into different layers, with the cytoplasm being the amber-colored layer in the middle. Remove centrifuge tubes and place immediately on ice.
- Collect the extract.
 - Using an 18 gauge needle and syringe, puncture the centrifuge tube just above the dark pigmented layer at the bottom of the tube and remove the middle amber-colored cytoplasmic layer. Do not collect any of the top lipid layer. Collect the cytoplasm in an appropriately sized tube.
Note: Generally, 1 frog produces at least 1 ml of extract.
 - Supplement cytoplasm with 1/1,000 the volume of 19.7 mM cytochalasin D, 1/1,000 the volume of LPC stock, and 1/50 the volume of 50x energy mix. Gently invert the tube to mix. Do not excessively pipet the extract.
 - Keep the extract on ice and use it within 3 hr of preparation for best results.

2. Preparation of Demembrated *X. laevis* Sperm (adapted from²⁹)

Note: The procedure volumes presented here are for up to 8 testes.

- Remove 0.05% benzocaine (10% benzocaine stock prepared in ethanol and diluted to 0.05% in frog tank water) from 4 °C and warm to room temperature. Anesthetize and sacrifice male *X. laevis* frogs in benzocaine solution at room temperature for 20 - 30 min. Ensure death by absence of heartbeat by examining and feeling the chest area, by lack of response to mechanical stimulation, and/or by decapitation.
- Make a U-shaped incision along the abdomen. Remove the tubulated mass of yellow fat bodies. At the top of the kidneys, locate the testes, which are oval-shaped pale pink masses about 1 cm in length²⁹. Excise the testes and roll them on blotting paper to remove blood and other tissue.
- Wash testes in Buffer T. Use a tight fitted pestle to mince testes with 1 ml of Buffer T in a 1.5 ml tube until homogenous (10 strokes or more). Centrifuge 5 - 10 sec in a mini centrifuge and collect the supernatant, removing large pieces of tissue. Repeat the centrifugation once more and collect the supernatant.
- Transfer the sperm-containing solution to a 15 ml plastic tube. Increase the volume to a total of 2 ml with Buffer T. Centrifuge at 1,411 x g for 10 min at 16 °C to pellet the sperm.
- Remove the supernatant and resuspend the pellet in 500 μl of Buffer T. Centrifuge at 1,411 x g for 10 min at 16 °C. Repeat this step once or twice as needed to clean the pellet of somatic and red blood cells.
- Resuspend the pellet in 100 μl Buffer T and 300 μl Buffer S. Incubate at room temperature for 5 min.
Note: Buffer S contains lyssolecithin, which is responsible for demembration.
- Add three volumes of Buffer R (prepared fresh before use). Invert tube exactly once. Centrifuge at 1,411 x g for 15 min at 16 °C.
- Resuspend the pellet in 400 μl Buffer R and then add an additional 2 ml of Buffer R. Centrifuge at 1,411 x g for 15 min at 16 °C.
- Resuspend the pellet in 50 μl Buffer T.
- Dilute 1 μl demembrated sperm nuclei with 9 μl Buffer T. Apply this dilution to a hemocytometer. Count the number of thin S-shaped sperm nuclei in one large 4 x 4 grid, and multiply that number by 100 to obtain the concentration of the stock in sperm nuclei per μl.

- Dilute the stock to 100,000 sperm nuclei per μl with Buffer T, resulting in a 200x stock. Prepare 3 - 5 μl aliquots. Flash freeze in liquid nitrogen and store at $-80\text{ }^{\circ}\text{C}$.

3. Nuclear Assembly

- Supplement 100 μl of egg extract with 1.5 μl 35.5 mM cycloheximide (final concentration $\sim 0.5\text{ mM}$), 6 μl 20x calcium stock solution (final concentration $\sim 0.6\text{ mM}$), and 0.7 μl 200x sperm (final concentration $\sim 700\text{ sperm nuclei}/\mu\text{l}$). Scale the reaction volume up or down as necessary. Invert or gently tap to mix.
Note: Extract cycled from metaphase into interphase provides a more robust and reliable platform for nuclear assembly.
- Incubate in a water bath at $16 - 20\text{ }^{\circ}\text{C}$ for 90 min. Mix by gently tapping every 15 min to ensure nuclei remain suspended in the extract.
- Monitor progress of nuclear assembly at 45 min by preparing a squash as follows.
 - Pipet 2 μl of extract onto a glass slide and add 2 μl nucleus fix.
 - Overlay with a 22 mm^2 coverslip. Image on an epifluorescence microscope using water, oil, or air 20 - 100X objectives and a DAPI filter.
Note: Nuclei can be visualized by bright-field imaging, but are much easier to visualize by fluorescence.
- Use nuclei immediately or flash freeze aliquots in liquid nitrogen and store at $-80\text{ }^{\circ}\text{C}$.
Note: Adding 4% glycerol to the nuclei prior to freezing can help to maintain their integrity. Frozen nuclei are generally still viable and capable of nuclear import, however the freezing process can lead to disrupted or more structurally fragile nuclei. Nuclei up to a year after freezing can be used.

4. Preparation of *X. laevis* Embryo Extract

- Induce female *X. laevis* frogs to lay eggs as described in 1.1 and 1.2.
- Isolate testes from male *X. laevis* frogs as described in 2.2. Submerge testes in L15 media supplemented with 50 IU/ml of penicillin and streptomycin in a glass 35 x 10 mm Petri dish. Store at $4\text{ }^{\circ}\text{C}$ for up to two weeks.
- Collect and fertilize eggs.
 - Collect freshly laid eggs by holding frogs over a glass reusable Petri dish and promote egg laying by applying gentle pressure to the lower back near the cloaca. Squeezing the frog too hard can result in injury, leading to a bloated abdomen and requiring euthanasia²⁹.
 - Prop the dish at an approximate 45° angle, allow eggs to collect in the edge of the dish, remove all excess buffer, and add enough 1/3x MMR to barely cover the eggs. Fertilize eggs within 15 min of collection.
 - Use a tightly fitted pestle to macerate and homogenize 1/4 of a testis in a 1.5 ml tube with 500 μl of high salt Modified Barth's Saline (High Salt MBS). Add macerated testes to the eggs and allow for fertilization to occur at room temperature for 15 - 20 min, then flood eggs with 1/3x MMR.
Note: Effectiveness of testes decreases with storage time, therefore more testes tissue may be required for successful fertilization when using testes that have been stored for more than one week.
 - Confirm fertilization by checking for contraction of the dark pigmented animal pole and by rotation of the embryos with their animal poles facing up, usually about 20 - 30 min after addition of crushed testes. Expect the first cell cleavage to occur within 90 min.
 - Using a wide bore glass pipette diligently remove dead (white and puffy or uneven distribution of yolk and pigment), lysed, or unfertilized eggs (*i.e.*, not cleaved), as they will rapidly induce death in neighboring embryos.
 - Anywhere from 1 - 2.5 hr post-fertilization, dejelly embryos in 2 - 3% cysteine dissolved in 1/3 x MMR and adjusted to pH 7.9 with 10 N KOH. Perform two buffer changes that are each 3 - 4 min. Thoroughly wash the embryos with 6 - 10 brief changes of 1/3x MMR to remove all traces of cysteine.
Note: Dejelled embryos have a vitelline membrane and are not as fragile as dejellied eggs.
- Using a wide bore glass pipette, transfer healthy fertilized (*i.e.*, cleaved) embryos to a Petri dish containing fresh 1/3x MMR and allow them to develop to the desired stage at RT or, if slower development is preferred, $16\text{ }^{\circ}\text{C}$. Continue to remove dead or lysed embryos indicated by lack of first division or white puffy appearance.
- Verify the stage of the embryos by checking for nearly complete closure of the blastopore and by comparing to drawings of staged embryos available in reference materials²⁴.
Note: Embryos cultured at $16\text{ }^{\circ}\text{C}$ will reach stage 11.5 - 12 in approximately 12 hr.
- Incubate stage 11.5 - 12 embryos in fresh 1/3x MMR supplemented with 0.5 mM cycloheximide for 1 hr at RT, to arrest embryos in late interphase.
- Transfer with wide bore glass or plastic pipette a minimum of 15 (preferably 30 or more) interphase-arrested embryos to a microcentrifuge tube.
Note: 15 embryos are sufficient to produce approximately 20 μl of extract. Scale up as needed.
- Add 1 ml egg lysis buffer (ELB) supplemented with 1 μl of LPC stock. Gently invert to wash embryos, allow embryos to fall to the bottom of tube, and remove the buffer. Repeat this wash step two more times.
- Resuspend embryos in 500 μl of ELB plus 0.5 μl of LPC stock, 5 μl of 19.7 mM cycloheximide, and 5 μl of 35.5 mM cytochalasin D (to inhibit actin polymerization). Centrifuge at 200 x g for 1 min in a tabletop microcentrifuge.
- Remove excess buffer and use a pestle to thoroughly crush the embryos. Centrifuge in a swinging bucket rotor for 10 min at 10,000 x g and $16\text{ }^{\circ}\text{C}$.
- Puncture the lipid layer from the top with a 200 μl pipet tip. With a clean 200 μl pipet tip, remove the middle cytoplasmic amber-colored layer to an appropriate sized tube.
- Supplement embryo extract with 1/50 the volume of 50x energy mix (to provide an ATP regeneration system), 1/100 the volume of 35.5 mM cycloheximide, 1/500 the volume of 19.7 mM cytochalasin D, and 1/1,000 the volume of LPC stock.
- Prepare a squash as described in 3.3 to visualize endogenous embryonic nuclei in the extract.
Note: Embryo extract can be frozen with the endogenous nuclei at this point. Aliquot to reduce freeze/thaw cycles and store at $-80\text{ }^{\circ}\text{C}$.

14. To remove nuclei from the embryo extract, dilute the extract with an equal volume of ELB containing 1/50 the volume of 50x energy mix, 1/100 the volume of 35.5 mM cyclohexamide, 1/500 the volume of 19.7 mM cytochalasin D, and 1/1,000 the volume of LPC stock. Centrifuge in a swinging bucket rotor at 17,000 x g for 15 min at 16 °C.
15. Collect the supernatant being careful to avoid any remaining lipid at the top. Do not disturb the pelleted nuclei at the bottom of the tube. Prepare a squash as described in 3.3 to ensure that most nuclei have been removed. Use the extract fresh or aliquot and store at -80 °C.
16. To heat inactivate embryo extract for control experiments, heat 30 - 100 µl aliquots of extract at 56 °C for 30 min using a thermal cycler. Allow heat inactivated extract to return to room temperature prior to use.

5. Nuclear Shrinking Assay and Immunofluorescence

1. Isolate nuclei assembled in egg extract by diluting 25 - 150 µl of pre-assembled nuclei in 1 ml ELB in a 1.5 ml tube. Centrifuge at 1,600 x g for 3 min at 4 °C in a tabletop microcentrifuge. Remove buffer, being careful not to disturb the fragile pellet of nuclei at the bottom of the tube.
2. Resuspend nuclei in a volume of embryo extract equal to the original volume of egg extract used in 5.1. Use ELB or heat inactivated extract for control reactions, as appropriate.
3. Gently tap the tube to break up the pellet and resuspend the nuclei. Incubate at room temperature for 90 min, gently tapping the tube to mix every 15 - 30 min. Note: Most of the nuclear shrinking occurs within the first 30 min.
4. Monitor progress of nuclear shrinking by preparing a squash.
 1. Pipet 2 µl of extract onto a glass slide and add 2 µl nucleus fix.
 2. Overlay with a 22 mm² coverslip. Image on an epifluorescence microscope.
5. Tap the tube to resuspend the nuclei just prior to adding 500 µl of fixative consisting of ELB, 15% glycerol, and 2.6% paraformaldehyde. Invert immediately, and place the tube on a rotator for 15 min at room temperature.
6. Prepare spin down tubes by outfitting 15 ml round bottom glass tubes with round bottom plastic inserts (design and schematics available upon request). Add 5 ml of nuclear cushion buffer. Drop a 12 mm circular coverslip into the tube, being sure it lays flat on top of the plastic insert.
7. Gently layer the solution containing fixed nuclei on top of the nuclear cushion using a wide-bore pipet tip. Centrifuge in a swinging bucket rotor for 15 min at 1,000 x g and 16 °C.
8. Use an aspirator to remove all of the buffer and pull the plastic insert to the top of the tube. Remove the coverslip with a pair of fine forceps, being careful to note the side of the coverslip onto which the nuclei were spun. Post-fix in cold methanol (stored at -20° C) for 5 min at RT.
9. Transfer the coverslip nucleus side up onto a sheet of plastic paraffin film lining a large plastic petri dish. Place wet disposable wipes along the side of the dish to prevent dehydration.
10. Rehydrate nuclei on the coverslip with 500 µl of PBS-NP40 (1x PBS plus 0.1% NP40) and aspirate after 5 - 10 sec.
11. Carefully layer 75 µl of PBS-3% Bovine Serum Albumin (BSA) onto the coverslip. Allow to block 1 hr at room temperature or overnight at 4 °C.
12. Remove blocking solution, and incubate with primary antibody (e.g., mAb414 against the nuclear pore complex, 1:1,000) diluted in PBS-3% BSA for 1 hr at room temperature or overnight at 4 °C. Wash with five immediate changes of PBS-NP40.
13. Incubate with secondary antibody diluted in PBS-3% BSA for 1 hr at RT. Wash with five immediate changes of PBS-NP40.
14. Incubate with 10 µg/ml Hoechst diluted in PBS-3% BSA for 5 min at RT. Wash five times with PBS-NP40. Remove all excess buffer.
15. Mount the coverslip onto a slide with 5 µl antifade mounting medium. Seal with clear nail polish. Image immediately using an epifluorescence microscope with 20 - 100x objectives or store at 4 °C for later imaging.
Note: Perform quantification as previously described²³.

Representative Results

Assembly of Nuclei in Egg Extract

The first steps of this protocol are to prepare *X. laevis* egg extract (Protocol 1) and demembrated sperm nuclei (Protocol 2). These reagents are then used to assemble nuclei *de novo* (Protocol 3). **Figure 1** shows some representative data. Addition of calcium drives the meiotically arrested egg extract into interphase, and the cycloheximide keeps the extract arrested in interphase. By 30 - 45 min after initiation of the reaction, the initially thin S-shaped sperm nuclei should begin to thicken into slug-shaped chromatin masses. Successful formation of an intact nuclear envelope can be assessed by import and retention of a fluorescently labeled import cargo, such as GST-GFP-NLS (data not shown), or by rim staining for the nuclear pore complex using mAb414 that recognizes FG-repeat containing nucleoporins (**Figure 1A**). After nuclear assembly occurs, nuclear shape should become more rounded and the nuclear volume should increase as the nuclear envelope expands and nuclear proteins are imported (**Figure 1B-C**). Failure to observe formation of an intact nuclear envelope or nuclear growth indicates a poor quality egg extract. In this case, it is best to start again with a new batch of eggs.

Removal of Endogenous Nuclei from Embryo Extract

The next step is to prepare a late stage embryo extract (Protocol 4). After initial preparation of the extract and prior to removal of nuclei, it is best to prepare a Hoechst-stained squash of a small aliquot of the extract. Visualization of many small embryonic nuclei is a good indication of success in preparing the extract (**Figure 2A**). As endogenous embryonic nuclei can interfere with the downstream analysis, it is also important to check an aliquot of the extract after removing nuclei by dilution and centrifugation. Compared to the first squash, very few nuclei should be left in the extract (**Figure 2B**). If there are still many nuclei present, a second round of centrifugation is required.

Nuclear Shrinking Assay

Figure 3 shows representative results for the nuclear shrinking assay (Protocol 5). Egg extract nuclei resuspended in late stage embryo extract become smaller over time (**Figure 3B**). A good negative control for the assay is to incubate nuclei with embryo extract that has been heat inactivated (**Figure 3A**). As nuclei fail to shrink in heat inactivated extract, this provides some confidence that observed nuclear shrinking with untreated embryo extract is not a consequence of osmotic effects. The extent of nuclear size reductions observed varies depending on the particular batch of egg extract nuclei as well as the quality of the embryo extract. In our experience, nuclear shrinking is always observed (e.g., in more than 40 different embryo extracts), however it is important to repeat these experiments multiple times with different nuclei and extracts to address the inherent variability of the assay. **Figure 4** shows a schematic of the entire protocol.

Nuclear Assembly - NPC

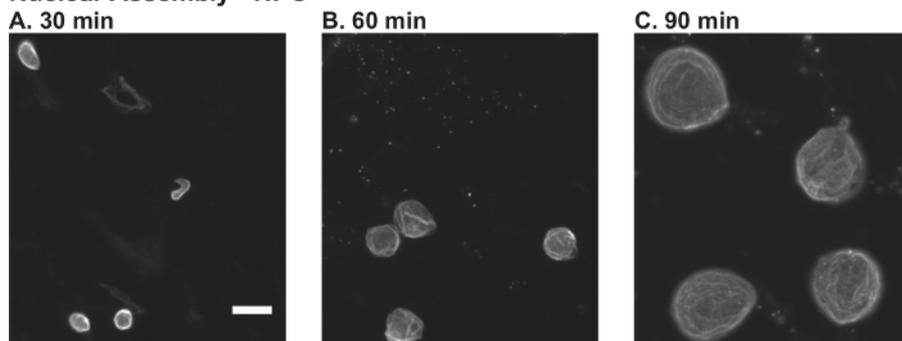


Figure 1. Nuclear Assembly Time Course in *X. laevis* Egg Extract. Nuclei were assembled *de novo* in *X. laevis* egg extract as described in Protocol 3. Aliquots from the same reaction were fixed and visualized by immunofluorescence using an antibody against the nuclear pore complex (NPC) (mAb414) at: **A**) 30 min, **B**) 60 min, and **C**) 90 min after the initiation of nuclear assembly. The immunofluorescence protocol is described in Protocol 5. The scale bar is 25 μ m. [Please click here to view a larger version of this figure.](#)

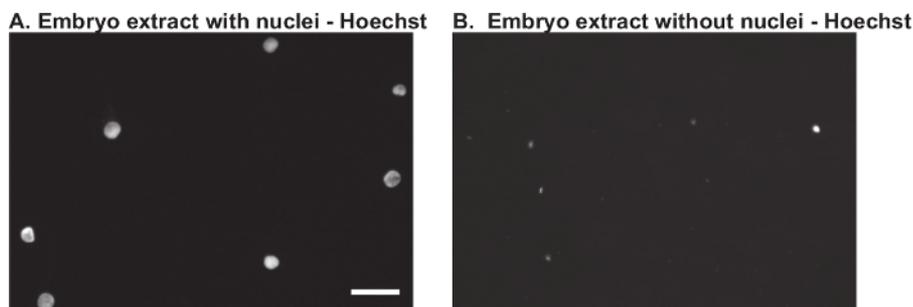
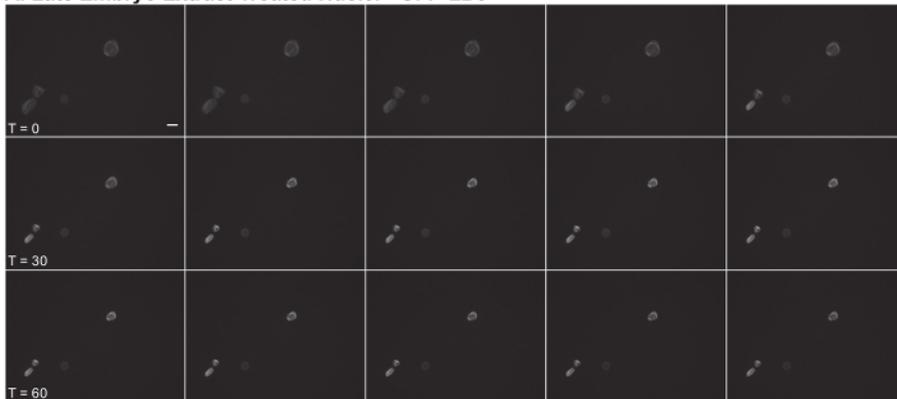
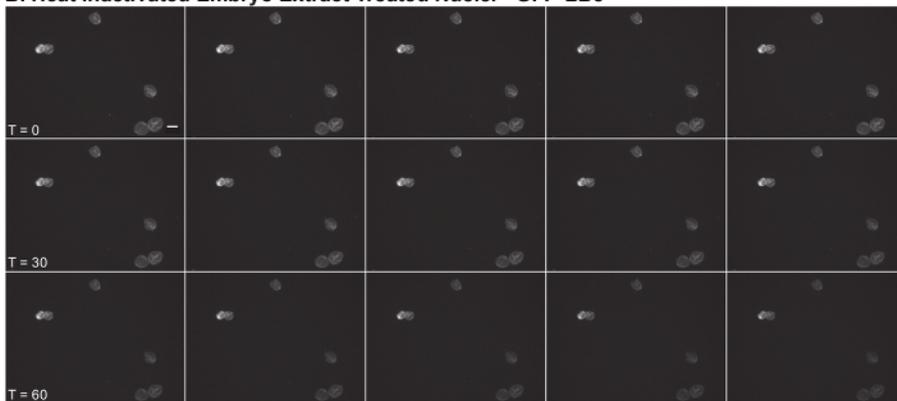


Figure 2. Removal of Nuclei from *X. laevis* Embryo Extract. *X. laevis* embryo extract was prepared from stage 11.5 - 12 embryos as described in Protocol 4. A small aliquot of extract was fixed and stained with Hoechst: **A**) prior to removal of nuclei, and **B**) after removal of nuclei by centrifugation. The scale bar is 25 μ m. [Please click here to view a larger version of this figure.](#)

A. Late Embryo Extract Treated Nuclei - GFP-LB3



B. Heat Inactivated Embryo Extract Treated Nuclei - GFP-LB3



C. Variability of Shrinking Assays with Nuclei of Different Starting Sizes

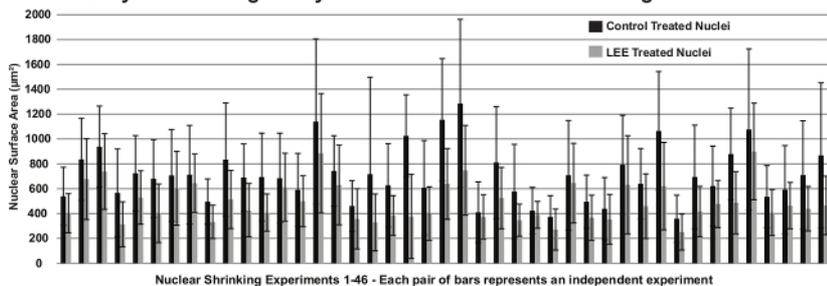


Figure 3. Representative Data from the Nuclear Shrinking Assay. Nuclei were assembled *de novo* in *X. laevis* egg extract supplemented with recombinant GFP-LB3 (to visualize the nuclear lamina). As described in Protocol 5, egg extract nuclei were isolated and resuspended in stage 11.5 - 12 embryo extract from which endogenous embryonic nuclei had been removed. Live time-lapse imaging was performed at 30 sec intervals for 90 min. Figure panels show images acquired at 6 min intervals for nuclei incubated in: **A)** late stage embryo extract (LEE), and **B)** heat inactivated (HI) embryo extract. Images from the time courses proceed from left-to-right. The scale bar is 25 µm. **C)** Nuclear shrinking data from 46 different egg and embryo extracts. Bars show the means for > 240 nuclei. Error bars are standard deviation. [Please click here to view a larger version of this figure.](#)

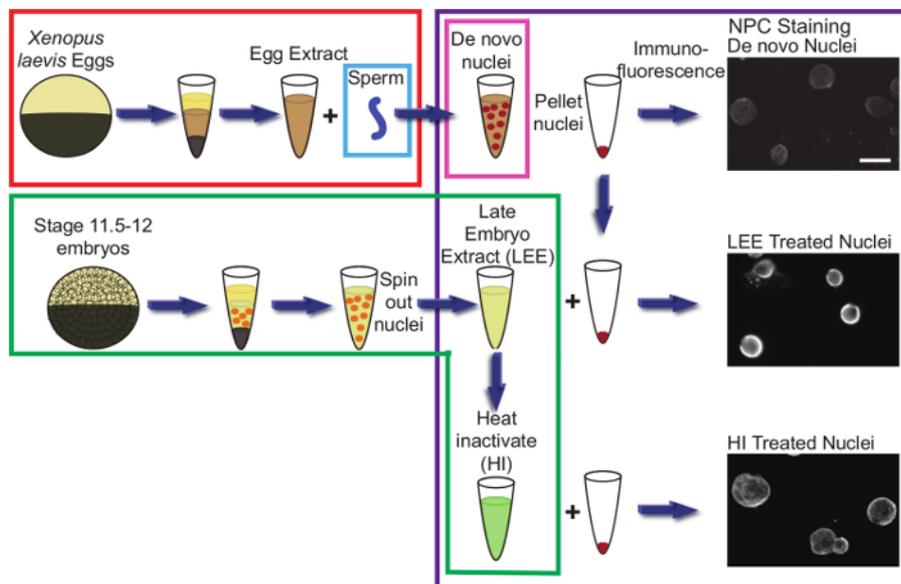


Figure 4. Schematic of the Nuclear Shrinking Assay. Each colored box represents one protocol. Red indicates preparation of *X. laevis* egg extract (Protocol 1). Blue indicates preparation of demembranated *X. laevis* sperm (Protocol 2). Pink indicates *de novo* nuclear assembly in egg extract (Protocol 3). Green indicates preparation of *X. laevis* embryo extract (Protocol 3). Purple indicates the nuclear shrinking assay protocol and immunofluorescence (Protocol 5). Portions of this figure have been reused from²³. [Please click here to view a larger version of this figure.](#)

Buffer name	Buffer composition
Marc's Modified Ringers (MMR), 1/3x	33 mM NaCl, 0.7 mM KCl, 0.3 mM MgSO ₄ , 0.7 mM CaCl ₂ , 1.7 mM HEPES, pH 7.4
Extract buffer (XB), 10x	1 M KCl, 1 mM CaCl ₂ , 10 mM MgCl ₂ , 500 mM sucrose, 100 mM HEPES, pH 7.8 (pH adjusted with 10 N KOH, stored at 4 °C)
Buffer B	4% Cysteine dissolved in 0.8x XB (prepared with cold ddH ₂ O, pH adjusted to 7.8 with 10 N NaOH)
Buffer C	Mix 100 ml of 10x XB with 900 ml of cold ddH ₂ O
Buffer D	Buffer C plus 5 mM EGTA and 800 μM MgCl ₂
LPC stock, 1,000x	10 mg/ml each of leupeptin, pepstatin, and chymostatin dissolved in DMSO (stored in aliquots at -20 °C)
Buffer E	100 ml Buffer D plus 100 μl LPC stock
Energy mix, 50x	190 mM creatine phosphate disodium, 25 mM ATP disodium salt, 25 mM magnesium chloride
Buffer T	15 mM PIPES, 15 mM NaCl, 5 mM EDTA, 7 mM MgCl ₂ , 80 mM KCl, 0.2 M sucrose, pH 7.4 (filter sterilize and stored at 4 °C)
Buffer S	20 mM maltose and 0.05% lysolecithin prepared in Buffer T
Buffer R	Buffer T plus 3% bovine serum albumin
Calcium stock solution, 20x	10 mM CaCl ₂ , 0.1 M KCl, 1 mM MgCl ₂ (stored at -20 °C)
Nucleus fix	125 μl 2 M sucrose, 12.5 μl 1 M HEPES pH 7.9, 250 μl 37% formaldehyde, 112 μl ddH ₂ O, 0.5 μl 10 mg/ml Hoechst (stored at room temperature for up to two weeks)
Modified Barth's Saline (MBS), 10x	880 mM NaCl, 10 mM KCl, 50 mM HEPES, 25 mM NaHCO ₃ , pH 7.8
High Salt MBS	1x MBS plus 0.7 mM CaCl ₂ and 20 mM NaCl
Egg lysis buffer, ELB	250 mM sucrose, 50 mM KCl, 2.5 mM MgCl ₂ , 10 mM HEPES, pH 7.8
Nuclear cushion buffer	1x XB, 0.2 M sucrose, 25% glycerol (filter sterilized and stored at 4 °C)

Table 1. Buffers. Compositions of all buffers mentioned in this protocol are described in this table.

Discussion

Here is presented a novel method to study mechanisms of nuclear size regulation during *X. laevis* development. Developmental progression is associated with dramatic changes in cell physiology, metabolism, division rates, and migration, as well as alterations in the sizes of cells and intracellular structures. These varied processes are complex and essential, so it is difficult to study just one of these aspects of development in an *in vivo* setting. The *X. laevis* embryo extract and nuclear shrinking assay described here allow for the study of size regulatory mechanisms in an *in vitro* context, circumventing the complex signals and physical constraints of the *in vivo* system. Furthermore, the open nature of the extract allows for its facile biochemical manipulation.

Troubleshooting some steps of the protocol may be necessary. Problems with nuclear assembly and growth can result from poor quality eggs, incomplete demembration of sperm nuclei, use of sperm nuclei at too high of a concentration, or incubating nuclear assembly reactions at too low of a temperature. Removal of endogenous embryonic nuclei from late embryo extract can be difficult due to the small size of these nuclei. This step in the protocol may require more than one round of centrifugation or the use of alternate rotors to completely remove all nuclei. Failure of nuclei to become smaller in the shrinking assay may be due to poor quality embryos, starting with nuclei that are too small, or too low of an incubation temperature. Poor quality eggs and embryos are indicated by a large proportion of white puffy activated eggs, unequal distribution of yolk and pigment granules, and eggs laid in strings. Spinning down fixed nuclei onto coverslips occasionally causes fragmentation of the nuclei, which can be avoided by decreasing the centrifugation speed or increasing the glycerol and sucrose concentrations in the nucleus cushion buffer. Troubleshooting antibody selection and dilution for immunofluorescence of nuclei is also often necessary. Critical steps within these protocols include: using high quality eggs and embryos, removing activated and lysed eggs and embryos, ensuring sperm nuclei are fully demembrated and appropriately diluted, monitoring nuclear assembly and growth, correctly staging embryos, completely removing endogenous embryonic nuclei from late stage embryo extracts, centrifuging fixed nuclei onto glass coverslips, and immunofluorescent staining of nuclei for visualization and quantification.

Nuclear size differences have long been noted between different species, cell types, disease states, and stages of embryonic development, indicating that nuclear size is tightly regulated¹⁻¹⁰. Cancer of the prostate, breast, and ovary, to name a few, are all diagnosed and staged based on graded increases in nuclear size^{14,30-32}. It is unclear to what extent changes in nuclear size are a cause or consequence of disease, and whether normal scaling of nuclear size contributes to proper development. Answering these functional questions necessitates an understanding of the mechanisms that control nuclear size. The assay presented here provides one approach to identifying novel pathways and proteins that regulate nuclear size, with potential relevance to cancer and development.

The *Xenopus* egg and embryo extract systems provide particularly powerful approaches to investigate mechanisms of organelle size regulation. While *Xenopus* extracts have been used for decades to study various aspects of cell cycle regulation and organelle assembly and dynamics, these extracts have more recently provided insights into size regulation of the nucleus^{3,23,33}, mitotic spindle³⁴⁻³⁶, and chromosomes³⁷⁻³⁹. One of the major advantages of working with *X. laevis* embryo extracts is that they can be isolated from different stages of development, representing cytoplasm from differently sized cells containing nuclei of differing sizes. The method for investigating nuclear re-sizing presented here is physiologically relevant and offers a simplified system with which to identify mechanisms of nuclear size regulation. One complication of studying size regulation *in vivo* is that it is difficult to distinguish effects of spatial constraints and volume-limited components on size versus developmentally regulated changes in the expression or activities of size-controlling factors⁴⁰. The approach described here focuses on developmentally controlled nuclear size regulators, using a robust nuclear re-sizing assay that is amenable to biochemical dissection.

Using the methods presented here, we identified protein kinase C (PKC) as a novel regulator of nuclear size. Furthermore, we showed that PKC activity and nuclear localization increase during *X. laevis* development^{23,41}. A key limitation to the nuclear shrinking assay is that it is performed entirely *in vitro*. While *Xenopus* extract systems provide a simplified platform to discover new factors that regulate size, it is important that these results are validated *in vivo*. There are a variety of different approaches to corroborate physiological significance. *X. laevis* embryos can be microinjected with mRNA or morpholinos to alter the levels of nuclear scaling factors identified in extract, and then the effects on nuclear size can be quantified. Treatment of embryos with cell-permeable small molecule inhibitors or activators is also possible. For instance, we observed nuclear shrinking in live embryos treated with a phorbol ester, and this shrinking occurred within interphase without a requirement for nuclear envelope breakdown and passage through mitosis²³. Another approach is to alter the levels of nuclear scaling factors in tissue culture cells to assess conservation of nuclear size control mechanisms outside of *Xenopus*.

What are the functional implications of altered nuclear size? One possibility is that nuclear size influences chromosome and chromatin organization, thereby altering gene expression. This idea can be directly tested as mechanisms of nuclear size regulation are further elucidated. Interestingly, PKC signaling and nuclear size are both routinely deregulated in cancer cells, often representing more aggressive metastatic disease^{13,14,42,43}. Whether there is a direct relationship between deregulated PKC signaling and altered nuclear size in cancer remains to be shown. There is still much to be learned about nuclear size regulation, and the assay presented here, or variants of it, may still yield new information about mechanisms of nuclear size control. Furthermore, this assay could easily be adapted for the study of scaling mechanisms of other organelles and intracellular structures such as the mitotic spindle, endoplasmic reticulum, chromosomes, and Golgi, to name a few.

Disclosures

The authors have nothing to disclose.

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