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Nucleus assembly and import in *Xenopus laevis* egg extract

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Abstract

Xenopus egg extract represents a powerful cell-free biochemical tool for studying organelle assembly and function. Large quantities of cytoplasm can be isolated, and biochemical manipulation of extract composition and cell cycle state is relatively straightforward. In this protocol, we describe the reconstitution of nuclear assembly by adding a chromatin source to interphasic *X. laevis* egg extract. Intact nuclei assemble within 30–45 minutes of initiating the reaction, followed by nuclear growth. We also describe methods for imaging and quantifying nuclear import kinetics. Recombinant proteins or small molecules of interest can be added to the extract prior to or after nuclear assembly, and immunodepletion allows for removal of specific proteins from the extract. This approach will continue to inform mechanisms of nuclear assembly, nuclear pore complex assembly and function, nucleocytoplasmic transport, DNA replication, nuclear envelope breakdown, and nuclear size and shape regulation.

MATERIALS

Reagents

Calcium stock solution (10 mM CaCl₂, 0.1 M KCl, 1 mM MgCl₂)

Store at –20 °C in 200 µL aliquots.

Cycloheximide stock solution (10 mg/mL in XB) <R>

Energy mix (190 mM creatine phosphate disodium, 25 mM ATP disodium salt, 25 mM MgCl₂)

Store at –20 °C in 100 µL aliquots.

FITC-Dextran stock solution (150 kD; 20 mg/mL in ddH₂O; Sigma-Aldrich 46946)

Store at –20 °C in 100 µL aliquots.

GFP-NLS stock solution

Recombinant GST-GFP-NLS is purified and dialyzed into 1x XB (Levy and Heald 2010).

Store at –80 °C in 10 µL aliquots. Our stock concentrations range from 2–28 mg/mL.

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Hoechst (10 mg/mL in ddH₂O).

Store at –20 °C in 40 µL aliquots.

Nuclear fix solution <R>

Oxygen scavenging mix (Aitken et al. 2008)

Sperm dilution buffer (SDB) (10 mM HEPES-KOH pH 7.8, 1 mM MgCl₂, 100 mM KCl, 150 mM sucrose)

Store at –20 °C in 1 mL aliquots.

Valap sealant (Cold Spring Harbor Protocols.rec082917)

XB buffer (10x) (1 M KCl, 1 mM CaCl₂, 10 mM MgCl₂, 500 mM sucrose, 100 mM HEPES-KOH pH 7.8)

Store at 4 °C and dilute to 1X with ddH₂O before use.

Equipment

Cotton tipped applicators

Epifluorescence microscope, equipped with DAPI and GFP/FITC filters and 20X or 40X objective Eppendorf tubes (0.65 mL and 1.5 mL)

Glass slides and 22 mm x 22 mm coverslips (clean slides and coverslips immediately before use with Kimwipes saturated with 95% ethanol and let dry)

Hot plate

Pap pen (Scientific Device Laboratory, 9804–2)

Software for image quantification (e.g. ImageJ, Metamorph, cellSens)

Water bath set between 16–20 °C

Wide bore pipet tips or normal pipet tips with the tips cut off (~1.5 mm diameter opening for 200 µL pipet tips, VWR 89079–456; ~2 mm diameter opening for 1 mL pipet tips, VWR 89049–162)

METHOD

Prepare *X. laevis* metaphase-arrested egg extract (Good and Heald, this volume) (Hannak and Heald 2006; Maresca and Heald 2006) and demembrated *Xenopus* sperm (Hazel and Gatlin, this volume) (Murray 1991).

Assemble nuclei

1. For every 100 μL of freshly prepared egg extract, add 2 μL energy mix, 1.5 μL cycloheximide stock solution, and 6 μL calcium stock solution in a 1.5 mL Eppendorf tube on ice. To mix, gently tap the tube or pipet using wide pore pipet tips. The reaction volume can be scaled between 25 – 500 μL . For smaller reactions, use of 0.65 mL Eppendorf tubes is preferred.

Note: Calcium induces progression of the metaphase-arrested extract into interphase, and cycloheximide keeps the extract arrested in interphase. Excessive pipetting or mixing can inactivate the extract. While we always add energy mix, some researchers do not. We suggest that the requirement for energy mix be determined empirically.

2. Add demembrated *Xenopus* sperm nuclei at 1000/ μL (e.g. 1 μL of a 100,000 sperm nuclei/ μL stock for a 100 μL reaction). If necessary, sperm can first be diluted in SDB or extract.
3. Incubate the reaction in a water bath at 16–20 $^{\circ}\text{C}$. Invert or tap gently every 15 minutes to ensure nuclei remain suspended in the extract. Initial nuclear assembly usually occurs within 30–45 min.

Note: For the subsequent steps, it is best to use wide bore pipet tips to avoid damaging the nuclei.

Image nuclei

4. Pipet 2 μL of the nuclear assembly reaction onto a glass slide, directly pipet 2 μL of nuclear fix solution into the extract droplet, and overlay with a 22 mm x 22 mm coverslip.
5. Image nuclei using the DAPI filter on an epifluorescence microscope. A high proportion of round chromatin masses is indicative of successful nuclear assembly, while S-shaped or elongated slug-shaped chromatin indicates that longer incubation is required or that the extract quality is poor (Fig. 1A).
6. To test if nuclei are intact, pipet 2 μL of the nuclear assembly reaction onto a glass slide, add 0.5 μL of FITC-Dextran (150 kD) stock solution, and overlay with a 22 mm x 22 mm coverslip. Seal the coverslip using a Pap pen or Valap to minimize extract flow. Image nuclei using the GFP/FITC filter. Intact nuclei should exclude the FITC signal due to the diffusion size exclusion limit of the nuclear pore complex (Fig. 1B and 1F).

Note: FITC-dextran can photobleach rapidly depending on the light source. In our experience, use of a light-emitting diode (LED) light source and reduction of the light intensity result in negligible photobleaching.

7. To test if nuclei are functional, pipet 2 μL of the nuclear assembly reaction onto a glass slide, add 0.5 μL of GFP-NLS stock solution, and overlay with a 22 mm x 22 mm coverslip. Image nuclei using the GFP/FITC filter. Functional nuclei should show intranuclear GFP-NLS signal (Fig. 1C and 1F).

Note: Nuclei generally begin to exhibit intranuclear GFP-NLS signal around 20–30 minutes after initiating the reaction. Nuclei continue to grow over time, with large round nuclei observed by 75–90 minutes (Fig. 1A and 1C). Nuclei can also be fixed, spun onto coverslips, and processed for immunofluorescence (Edens and Levy 2016).

Image nuclear import

8. Add GFP-NLS to the nuclear assembly reaction; we generally use a final concentration of 0.04–0.14 $\mu\text{g}/\mu\text{L}$. Gently tap or invert the tube to mix.

Note: The concentration of GFP-NLS added to the reaction can vary, but the added volume should not exceed 10% of the reaction volume. Other fluorescently labeled import cargos of interest can also be used.
9. Immediately pipet 5 μL of the reaction onto a clean glass slide and overlay with a 22 mm x 22 mm coverslip. Seal the coverslip using a Pap pen or Valap.

Note: Melt Valap using a hot plate and apply with cotton tipped applicators.
10. Image using the GFP/FITC filter at 30 sec intervals using the same exposure time for ~60 min. To minimize photodamage, oxygen scavenging mix can be added to the reaction immediately before imaging (Movie 1, Fig. 1C). To correct for photobleaching during quantification, acquire a similar time-lapse using extract without sperm added.

Quantify nuclear import

11. Using appropriate software (e.g. Metamorph), set the image distance calibration based on the objective used. Threshold nuclei, and quantify average nuclear GFP-NLS intensity (i.e. total integrated intensity divided by area) at each time point.
12. For the time-lapse without nuclei, select a region in the middle and quantify average GFP-NLS intensity over time to obtain the rate of photobleaching, which is the slope (m) (Fig. 1E).
13. To correct for photobleaching, add back the appropriate nuclear GFP-NLS signal intensity as calculated by: $I(t)_{\text{corr}} = I(t)_{\text{meas}} + m \cdot t$, where $I(t)_{\text{corr}}$ is the corrected intensity at time t and $I(t)_{\text{meas}}$ is the original measured intensity at time t . Plot I_{corr} as a function of time (Fig. 1D).

TROUBLESHOOTING

Problem (Step 5): Poor nuclear assembly.

Solution: This is generally indicative of poor quality egg extract and the best course of action is to repeat the experiment with a new extract. Alternatively, the sperm stock solution may be too concentrated or sperm demembration may have been incomplete, requiring dilution or new preparation of the sperm stock, respectively.

DISCUSSION

This method describes the *in vitro* assembly of nuclei in *Xenopus* egg extract and quantification of nuclear import kinetics. While GFP-NLS informs bulk nuclear import kinetics, specific import cargos of interest can be fluorescently tagged and their import similarly measured using this approach. It is worth noting that this protocol can also be performed with *X. tropicalis* egg extract, as well as extracts prepared from staged *Xenopus* embryos that allow one to study the structure and function of endogenous embryonic nuclei (Good and Heald, this volume) (Levy and Heald 2010; Edens and Levy 2014). *Xenopus* extracts have provided mechanistic insights into nuclear assembly (Anderson and Hetzer 2007), nuclear pore complex assembly and function (D'Angelo et al. 2006), nucleocytoplasmic transport (Gorlich et al. 1994), DNA replication (Blow and Laskey 1986), nuclear envelope breakdown (Muhlhauser and Kutay 2007), and nuclear size and shape regulation (Jevtic et al. 2015; Vukovic et al. 2016). Mechanistic aspects of these various processes can be easily assessed due to the open biochemical nature of the extract, which can be supplemented with recombinant proteins, small molecules, or neutralizing antibodies and/or immunodepleted of specific proteins (Hannak and Heald 2006; Maresca and Heald 2006). For these experiments, proteins and antibodies should be dialyzed into XB buffer to be compatible with the extract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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RECIPES

Cycloheximide stock solution

Prepare 10 mL of 1x XB and warm slightly in microwave. Add 100 mg cycloheximide and vortex to dissolve. Syringe filter to sterilize. Store at -20°C in 100 μL aliquots.

Nuclear fix solution

Combine 125 μL 2 M sucrose, 12.5 μL 1 M HEPES-KOH pH 7.8, 250 μL 37% formaldehyde, 112 μL ddH₂O and 0.5 μL Hoechst stock solution (10 mg/mL). Nuclear fix can be stored at room temperature for several months.

Figure 1. Nuclear assembly and import quantification.

(A) Nuclei were assembled in *X. laevis* egg extract, fixed at different time points with nuclear fix solution, and visualized with Hoechst. (B) Nuclei were incubated with FITC-Dextran (150 kD), and intact nuclei excluding FITC signal are indicated with arrows. (C) GFP-NLS (0.04 $\mu\text{g}/\mu\text{l}$) was added to nuclei 30 minutes after the initiation of nuclear assembly. A slide was prepared and GFP-NLS images were acquired every 30 seconds with the same exposure time. Also see Movie 1. (D) For the nucleus indicated with an arrow in (C), nuclear GFP-NLS fluorescence intensity per unit area was measured at each time point using Metamorph (orange points), starting 30 minutes after the initiation of nuclear assembly. Using the trend line shown in (E), nuclear GFP-NLS fluorescence intensity per unit area was corrected for photobleaching (blue points), as described in the protocol. (E) Imaging and quantification were performed as in (C) and (D) except that the extract contained no nuclei. These data reflect the extent of photobleaching. The data (blue points)

were fit by linear regression (orange line). **(F)** Sperm nuclei in *X. laevis* egg extract were visualized 10 minutes after initiating the reaction. While sperm nuclei initially exclude FITC-dextran (arrows), they fail to import GFP-NLS. After complete nuclear assembly, only intact nuclei will exclude FITC-dextran and show intranuclear GFP-NLS signal, while damaged nuclei will fail to both exclude FITC-dextran and accumulate imported GFP-NLS. Scale bars, 20 μ m. All *Xenopus* procedures and studies were conducted in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. Protocols were approved by the University of Wyoming Institutional Animal Care and Use Committee (Assurance # A-3216-01).