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Preparation of *Xenopus borealis* and *Xenopus tropicalis* Egg Extracts for Comparative Cell Biology and Evolutionary Studies

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Abstract

Cytoplasmic extracts prepared from eggs of the African clawed frog *Xenopus laevis* are extensively used to study various cellular events including the cell cycle, cytoskeleton dynamics, and cytoplasm organization, as well as the biology of membranous organelles and phase-separated non-membrane-bound structures. Recent development of extracts from eggs of other *Xenopus* allows interspecies comparisons that provide new insights into morphological and biological size variations and underlying mechanisms across evolution. Here, we describe methods to prepare cytoplasmic extracts from eggs of the allotetraploid Marsabit clawed frog, *Xenopus borealis*, and the diploid Western clawed frog, *Xenopus tropicalis*. We detail mixing and "hybrid" experiments that take advantage of the physiological but highly accessible nature of extracts to reveal the evolutionary relationships across species. These new developments create a robust and versatile toolbox to elucidate molecular, cell biological, and evolutionary questions in essential cellular processes.

Keywords

Xenopus ; *Xenopus borealis* ; *Xenopus tropicalis* ; Egg extracts; Mixed extracts; Hybrids

1 Introduction

Cell-free extracts from *Xenopus* eggs were first prepared by Lohka and Maller in 1985, who collected and crushed the eggs to fractionate their components and harvest undiluted cytoplasm [1, 2]. The authors took advantage of the well-established allotetraploid African clawed frog, *Xenopus laevis*, whose eggs are arrested in metaphase II of meiosis and stockpiled with maternal RNAs and proteins to support rapid early embryonic cell cycles and embryogenesis. Thus, once isolated, cytoplasmic extracts contain all the necessary biomolecules and machineries necessary to recapitulate the cell cycle in a test tube, including bipolar spindle assembly when a DNA source, such as sperm nuclei or DNA-

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coated beads, is added [1, 3]. Subsequent optimization of extract preparation by Andrew Murray and colleagues allowed induction of interphase and transit through multiple cell cycles in vitro, demonstrating the power and tractability of egg extracts to investigate central cell biological questions [2, 4, 5]. Notably, metaphase spindles, mitotic chromosomes, and interphase nuclei can all be formed in vitro, and a number of fundamental cellular processes, including DNA replication, chromosome condensation, and kinetochore formation, are fully supported in extracts [6, 7]. The extract system undergoes a simple cell cycle, mimicking the timing and functions of the early embryonic cleavages, lacking gap phases and oscillating rapidly between S and M phases. Moreover, the extract's accessible cell-free nature allows for unparalleled control over the cell cycle, as it can be arrested in either metaphase or interphase, and is amenable to biochemical and pharmacological manipulations, such as protein immunodepletions and small-molecule inhibitor treatments. Thus far, *X. laevis* egg extracts have been key to our understanding of the fundamental principles, including DNA replication [8], cyclin degradation [9], as well as microtubule dynamics and spindle assembly [3, 10–12].

While *X. laevis* egg extracts remain a system of choice to study a variety of cellular events in vitro, comparisons across multiple *Xenopus* species have provided a unique evolutionary window to examine conservation and divergence of these mechanisms. In vitro egg extract systems have now been developed and applied to a variety of species with different egg and genome sizes. Beginning in 2007, Brown and colleagues developed the egg extracts of the diploid Western clawed frog, *Xenopus tropicalis* [13]. Remarkably, spindles formed in egg extracts of this smaller frog were shorter in length compared to *X. laevis* due to cytoplasmic composition differences between species. Indeed, mixing *X. laevis* and *X. tropicalis* extracts produced spindles of intermediate size and morphological characteristics in a dose-dependent manner, regardless of the source of chromosomes, demonstrating that cytoplasmic factors are sufficient to scale subcellular structures and build the associated morphological architecture [13, 14]. Comparisons between these two species' egg extracts led to the discovery of numerous mechanisms of nuclear and spindle-size scaling [14–17]. More recently, we developed egg extracts from a third *Xenopus* species, the allotetraploid Marsabit clawed frog, *X. borealis* [18]. Intermediate in size, but more recently diverged from *X. laevis* than *X. tropicalis*, *X. borealis* thus occupies a unique place in evolutionary history that allows us to address the conservation of spindle morphology, assembly, and scaling mechanisms as well as chromosome biology across evolution [18–20].

These three *Xenopus* species egg extracts make it possible to question how various central cellular processes have evolved and adapted. In this protocol, we focus on the preparation of egg extracts from eggs of *X. borealis* and *X. tropicalis*, as *X. laevis* protocols have been extensively detailed elsewhere [21, 22]. In addition, we provide specific advice regarding frog care for *Xenopus* species. We also highlight two experimental schemes that have been key to our understanding of scaling and evolutionary divergence in cell cycle events: mixing cytoplasmic extracts and "hybrid" reactions.

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2 Materials

2.1 Xenopus *Frogs*

1. Marsabit clawed frog, *Xenopus borealis*, females.
2. Western clawed frog, *Xenopus tropicalis* females.

X. borealis and *X. tropicalis* frogs can be obtained from the National *Xenopus* Resource (NXR), Woods Hole, USA. In Europe, *X. tropicalis* frogs can be obtained from the European *Xenopus* Resource Centre (EXRC), Portsmouth, UK. Females are used for egg collection to make extracts, while males are used to prepare demembranated sperm chromatin [23].

2.2 Hormones

1. Pregnant mare serum gonadotropin (PMSG): 200 IU/mL PMSG in sterile MilliQ H_2O. Store at 4 °C.
2. Human chorionic gonadotropin hormone (HCG): 1000 IU/mL HCG in sterile MilliQ H_2O (e.g., HCG from Sigma lyophilized with buffer) or provided diluent (e.g., Chorulon HCG from MSD). Store at 4 °C for the time recommended by the provider.

2.3 Extract Preparation Buffers and Chemicals

1. 20× Marc's Modified Ringer solution (MMR): 2 M NaCl, 40 mM KCl, 40 mM $CaCl_2$, 20 mM $MgCl_2$, 100 mM HEPES (free acid), 2 mM EDTA, NaOH pH 7.8. Prepare 1 L in MilliQ H_2O and filter-sterilize.
2. 20× XB salts: 2 M KCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$. Prepare 1 L in MilliQ H_2O, and filter-sterilize. Store at 4 °C.
3. Dejellying solution for *X. borealis* eggs: 1× XB salts and 3% L-cysteine in MilliQ H_2O. Adjust to pH 7.8 with 10 N NaOH. Prepare 500 mL fresh just before use.
4. Dejellying solution for *X. tropicalis* eggs: 3% L-cysteine in MilliQ H_2O. Adjust to pH 7.8 with 10 N NaOH. Prepare 500 mL fresh just before use.
5. CSF-XB: 1× XB salts, 50 mM sucrose, 10 mM K-HEPES pH 7.8, 2 mM $MgCl_2$, 10 mM K-EGTA pH 7.8 in MilliQ H_2O. Adjust to pH 7.8 with 10 N KOH. Prepare 1 L fresh just before use.
6. LPC protease inhibitor cocktail: 10 mg/mL each of leupeptin, pepstatin, and chymostatin in DMSO. Aliquot and store at −20 °C.
7. Cytochalasin B or D: 10 mg/mL in DMSO. Aliquot and store at −20 °C.
8. 50× energy mix: 190 mM creatine phosphate, 25 mM adenosine triphosphate, 25 mM MgCl2, 2.5 mM K-EGTA pH 7.7 in MilliQ H_2O. Aliquot and store at −20 °C.

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9. CSF-XB+: CSF-XB supplemented with 10 μg/mL each of LPC. Prepare 50 mL fresh just before use by adding 50 μL 10 mg/mL LPC stock to 50 mL CSF-XB.

2.4 Extract Preparation Equipment

1. Square plastic containers for individual storage of *X. borealis* frogs (about 22 × 22 × 12 cm, 4–6 L capacity) with tight lids allowing air exchange.
2. Square glass containers for individual storage of *X. tropicalis* frogs (about 20 × 20 × 25 cm, 8–10 L capacity) with lids allowing air exchange.
3. Room or non-airtight incubator set to 16 °C for *X. borealis*.
4. Transfer pipettes, including wide bore and narrow stem transfer pipettes.
5. Thin-wall centrifuge tubes, 5 mL (SW-55 ultraclear thin-wall; Beckman).
6. Adaptor tubes, 13 mL (Sarstedt).
7. Clinical centrifuge set to 16 °C equipped with a swinging-bucket rotor.
8. High-speed floor centrifuge set to 16 °C equipped with a swinging-bucket rotor (e.g., Sorvall HB-6 or Beckman JS13.1) and corresponding rubber adaptors.

2.5 Extract Reactions

1. Demembranated sperm nuclei, prepared as detailed elsewhere [23].
2. 10× calcium solution, for interphase induction: 4 mM $CaCl_2$, 100 mM KCl, 1 mM $MgCl_2$.
3. Spindle fix: 1× MMR, 48% (w/w) glycerol, 11% (w/v) formaldehyde, 5 μg/mL Hoechst DNA dye.
4. Glass slides and square coverslips (18 × 18 or 22 × 22 mM) for fluorescence microscopy.

3 Methods

All *Xenopus* egg extracts described so far follow the same basic protocol flow. However, key differences exist in hormone doses, buffer recipes, and species-specific nuances that have been made for maximal frog health and experimental output [6, 13, 18, 21, 22, 24]. Please refer to Table 1 for a full comparison between *X. laevis*, *X. tropicalis*, and *X. borealis* egg extract protocols.

3.1 Frog Care Considerations

Xenopus frogs can be prone to stress during ovulation for egg extracts, particularly *X. tropicalis*. Here, we detail several considerations and signs to look out for during any frog handling and ovulation. Information about frog housing has been detailed elsewhere [25–27].

1. All primed frogs should be fasted after hormone injections to reduce the possibility of fecal matter contaminating the eggs.

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2. If *X. tropicalis* frogs are raised throughout their life in buffered 0.1× MMR, keeping them in this solution and at husbandry temperature throughout the experiment, including ovulation, does not impair egg and extract quality while reducing animal stress.

3. *X. tropicalis* frogs are sensitive to the container plastic quality. While some plastic tubs are used without any problem by some labs, we opted for glass containers here, for extra safety.

4. If frogs lay eggs or their water/buffer is cloudy from lysed eggs after the priming (first) hormone dose, check to see if the frog is still actively laying before deciding whether or not to proceed with a second boosting dose. Reduce the priming dose if this happens consistently.

5. Frogs can suffer from hyper-ovulation after hormone injections, leading to death within a few days post-ovulation. This is particularly true for *X. tropicalis*, and less of a concern with *X. laevis* and *X. borealis*. Occasionally, this is indicative of a "sensitive" batch of frogs, but if the problem is persistent, the boosting dose should be adjusted.

6. Frogs will continue to lay for 12–24 h after use and may suffer from post-experiment problems such as internal ovulation. Be sure to store them isolated and under observation overnight to a few days before returning them to their permanent housing systems. This prevents eggs and unhealthy or dead animals from clogging and contaminating the housing system water and flow.

7. It is recommended that female frogs are rested for specific intervals between ovulations to maintain the highest egg quality over time (*see* Table 1).

8. Depending on whether frog colonies are bred in-house or shipped from a supplier, care must be taken to acclimate frogs and adapt them slowly to their housing environment. For instance, *X. tropicalis* and *X. borealis* can be more sensitive than *X. laevis* to changes in conductivity and salt content in water. In addition, *X. tropicalis* frogs are particularly sensitive to *Batrachochytrium dendrobatidis* (*Bd*) chytrid fungal infections (Kitaoka and Heald, unpublished observations) and thus should be tested prior to introducing them into permanent housing systems.

9. Each frog species should have dedicated spaces in the lab (i.e., benches) and housing rooms. Care should be taken to ensure that *X. tropicalis* are separated from other species, but *X. laevis* and *X. borealis* can be housed together within the same recirculating system, though separate tanks should be used. Cross-contamination between species can be particularly dangerous as *X. laevis* and *X. borealis* are more tolerant of *Bd* chytrid fungus, while *X. tropicalis* are highly susceptible.

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3.2 *Preparation of* X. borealis *Egg Extracts*

1. Prime *X. borealis* females (usually 3–4) by injecting 0.3 mL (60 IU) of PMSG subcutaneously into the dorsal lymph sac using a 1 mL syringe and a 30-gauge needle, at least 48 h before boosting (*see* Note 1).

2. Boost *X. borealis* females on the evening preceding the experiment by injecting 0.3 mL (300 IU) of HCG to induce ovulation, 16–18 h before eggs are needed. Store each frog individually in 2 L of freshly prepared 0.5× MMR in their plastic container overnight at 16 °C (*see* Note 2).

3. The next morning, make sure that both the clinical and the high-speed centrifuges are set to 16 °C.

4. Just before collecting the eggs, prepare the dejellying solution and the CSF-XB. Set aside a 50 mL aliquot of CSF-XB (to later prepare the CSF-XB+), and, if possible, store all solutions at 16 °C until use. Allow frozen aliquots of Cytochalasin, LPC cocktail, and energy mix to thaw at room temperature.

5. Remove the females from their containers and analyze the egg quality to select batches of eggs to be used. Avoid batches that contain a significant amount of misshaped, lysed, or stringy eggs, and remove these from the chosen batches with a wide-bore transfer pipette (*see* Note 3). Retrieve the selected batches of eggs by gently pouring off the majority of 0.5× MMR from the tub and collecting eggs into a 500 mL glass beaker.

6. For the dejellying and washing steps **(steps 7–8)**, take extra care to avoid exposing the eggs to air and swirling them excessively. Remove misshaped and lysing eggs throughout these steps using a transfer pipette (*see* Note 4).

7. To dejelly the eggs, pour off as much 0.5× MMR as possible and replace with the *X. borealis* dejellying solution (about 1/3 of the prepared amount). Let sit for a few minutes, gently swirling a couple of times before exchanging. Repeat twice to use all the solution (500 mL). In about 6 min, the eggs should settle and pack with gravity without any space left in between. In addition, eggs will rotate so that the animal pole (pigmented hemisphere) will face upward once it has dejellied, indicating when the process is complete (*see* Note 5).

8. As soon as the eggs are dejellied, pour off the dejellying solution and start washing with the CSF-XB solution. Pour a generous amount of CSF-XB buffer into the beaker of eggs and allow the eggs to swirl and settle before proceeding to the next wash. Wash 3–4 times until all the solution (~1 L) is used. During

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1. Priming the female frogs improves egg yield and quality. While they may be usable for 2 weeks like *X. laevis*, we use *X. borealis* frogs within 1 week of priming.
2. Individual housing prevents mixing egg batches of different quality.
3. Egg quality is the primary and most important parameter as this will determine the extract quality. Careful analysis of eggs is thus highly recommended. Yet, egg strings can be used for *X. tropicalis*, if they do not contain any lysed eggs.
4. Exposure to air, especially once eggs are dejellied, will induce egg lysis and corrupt extract quality. Excessive swirling increases the risk of releasing the metaphase arrest and obtaining an interphasic or non-functional extract.
5. The dejellying step should not take more than 10 min. If eggs are not dejellied after 10 min, quality may not be good. Also, overexposure to the dejellying solution will lead to egg lysis.

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this step, prepare the CSF-XB+ solution. Then, take out 1 mL of CSF-XB+ to fill each thin-wall centrifuge tubes and supplement with 10 μL of 10 mg/mL Cytochalasin (100 μg/mL final).

9. Returning to the eggs, pour off the CSF-XB solution and replace with the CSF-XB+ solution, taking care to ensure that all eggs remain submerged. Often, the beaker should remain tilted to keep all eggs submerged.

10. Cut a transfer pipette at the beginning of its larger diameter, and use it to carefully transfer the eggs to the thin-wall centrifuge tubes containing the Cytochalasin-supplemented CSF-XB+ without exposing the eggs to air or adding too much liquid to the tube.

11. Add about 200 μL of CSF-XB+ to the adaptor 13 mL tube, and without touching the eggs, use forceps to place the thin-wall centrifuge tubes containing the eggs inside it.

12. To pack the eggs, centrifuge the tubes in the clinical centrifuge at 500 g for 1 min at 16 °C, and then remove the excess buffer on top of the packed eggs using a narrow stem transfer pipette (*see* Note 6).

13. To crush the eggs, immediately transfer the tubes to the high-speed centrifuge and spin for 15 min at 17,000 × g at 16 °C (*see* Note 7).

14. After the spin, take the thin-wall centrifuge tubes out of the adaptor tubes. Using a 1 mL syringe and an 18-gauge needle, perforate the tube at the bottom of the cytoplasmic fraction (the second one out of three) and aspirate the isolated cytoplasm. Take care that only the cytoplasmic layer is aspirated, not the surrounding yolk or lipid layers as this will affect extract activity and quality. Remove the syringe from the needle and transfer the cytoplasm to a tube that was precooled at 16 °C.

15. Supplement the extract with the LPC cocktail (1:1000 dilution of stock), Cytochalasin (1:500 dilution), and energy mix (1:50 dilution). Mix by inverting the tube or flicking gently. Keep the extract at 16 °C (*see* Note 8). Extracts should be used freshly, but good quality extracts will last 4–8 h.

3.3 *Preparation of* X. tropicalis *Egg Extracts*

1. Prime *X. tropicalis* females (usually 5) by injecting 0.1 mL (10 IU) of HCG (HCG stock solution diluted 1:10 in MilliQ H_2O or diluent) subcutaneously into the dorsal lymph sac using a 1 mL syringe and a 30-gauge needle, about 20 h before boosting (*see* Note 1). Store the frogs in 30 L sodium bicarbonate-buffered 0.1× MMR in a glass tank overnight at husbandry temperature (*see* Note 9).

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6. The tube can be quickly tipped to 45° to remove as much buffer as possible. Do not hesitate to sacrifice a few eggs.
7. An extra minute can be added to make sure the eggs are centrifuged at full speed for 15 min.
8. This temperature was empirically determined as it was known to allow the conservation of both *X. laevis* and *X. tropicalis* egg extracts. Sensitivity of *X. borealis* extracts to cold was not determined. *X. borealis* extracts are stable at this temperature for at least 4 h.

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2. The next morning, boost *X. tropicalis* females by injecting 0.2 mL (200 IU) of HCG to induce ovulation, ~3–4 h before eggs are needed. Store each frog individually in 4 L of buffered 0.1× MMR in their glass container at husbandry temperature (*see* Notes 2 and 10).

3. Frogs should start laying about 3 h after boosting. Let the females lay for about 2 h, frequently watching for decreases in egg quality. When the needed amount of eggs is laid or at the first sign of quality decreases, start collecting the eggs (*see* Note 11).

4. During **step 3**, make sure that both the clinical and the high-speed centrifuges are set to 16 °C; prepare the dejellying solution and CSF-XB. Set aside a 50 mL aliquot of CSF-XB (to later prepare the CSF-XB+), and, if possible, store all solutions at 16 °C until use. Allow frozen aliquots of Cytochalasin, LPC cocktail, and energy mix to thaw at room temperature.

5. Remove the females from the containers and analyze egg quality to select batches of eggs to be used. Avoid batches that contain a significant amount of misshaped or lysed eggs (*see* Note 3). Retrieve the selected batches of eggs in a 400 mL glass beaker (*see* Note 12).

6. For the dejellying and washing steps **(steps 7–8)**, take extra care to avoid exposing the eggs to air and swirling them excessively (*see* Note 4). Remove misshaped and lysing eggs throughout using a transfer pipette.

7. To dejelly the eggs, pour off as much buffered 0.1× MMR as possible and replace with the *X. tropicalis* dejellying solution (about 1/3 of it). Let sit for a few minutes, gently swirling a couple of times before exchanging. Repeat twice to use all the solution (500 mL). In about 8 min, the eggs should settle and pack with gravity without any space left in between. In addition, eggs will rotate so that the animal pole (pigmented hemisphere) will face upward once it has dejellied, indicating when the process is complete (*see* Note 5).

8. As soon as the eggs are dejellied, pour off the dejellying solution and start washing with the CSF-XB solution. Pour a generous amount of CSF-XB buffer into the beaker of eggs, and allow the eggs to swirl and settle before proceeding to the next wash. Wash 3–4 times until all the solution (~1 L) is used. Prepare the CSF-XB+ solution during this step. Then, take out 1 mL of CSF-XB+ to fill each thin-wall centrifuge tubes and supplement with 10 μL of 10 mg/mL Cytochalasin (100 μg/mL final).

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9. *X. tropicalis* frogs can be primed and boosted successfully in ddH_2O, which does not impair egg and extract quality.
10. The boosting dose for *X. tropicalis* can be increased up to 250 IU HCG, in our hands.
11. If high yield of extracts is an objective, one can accelerate egg release by simply holding the frog gently, allowing her to wiggle and kick her legs. Repeat 3–4 times at 15-min intervals. Be aware that this may cause animal stress, though it should not be detrimental if done gently.
12. *X. tropicalis* eggs stick to the container. Wear nitrile gloves and use your finger to gently sweep and detach the eggs. Collect them in one corner of the tub while carefully pouring excess water off. The eggs will settle and stick again, so keep them in suspension in water when collecting them into a clean beaker.

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9. Pour off the CSF-XB solution and replace with the CSF-XB+ solution, making sure to keep all eggs submerged by keeping the beaker angled if necessary.

10. Cut a transfer pipette at the beginning of its larger diameter, and use it to carefully transfer the eggs to the thin-wall centrifuge tubes containing the Cytochalasin-supplemented CSF-XB+ without exposing the eggs to air or adding too much liquid to the tube.

11. Add about 200 μL of CSF-XB+ to the adaptor 13 mL tube, and, without touching the eggs, use forceps to place the thin-wall centrifuge tubes containing the eggs inside it.

12. To pack the eggs, centrifuge the tubes in the clinical centrifuge at 500 g for 1 min at 16 °C, and then remove the excess of buffer on top of the packed eggs using a narrow stem transfer pipette (*see* Note 6).

13. To crush the eggs, immediately transfer the tubes to the high-speed centrifuge and spin for 15 min at 17,000 × g at 16 °C (*see* Note 7).

14. After the spin, take the thin-wall centrifuge tubes out of the adaptor tubes. Using a 1 mL syringe and an 18-gauge needle, perforate the tube at the bottom of the cytoplasmic fraction (the second one out of three) and aspirate the isolated cytoplasm. Take care that only the cytoplasmic layer is aspirated, not the surrounding yolk or lipid layers as this will affect the extract activity and quality. Separate the syringe from the needle, and transfer the cytoplasm to a tube that was precooled at 16 °C.

15. Supplement the extract with the LPC cocktail (1:1000 dilution of stock), Cytochalasin (1:500 dilution), and energy mix (1:50 dilution). Mix by inverting the tube or flicking gently. Keep the extract at 16 °C (*see* Note 13). Extracts should be used freshly, but good quality extracts will last 4–8 h.

3.4 *Mixing* Xenopus *Egg Extracts*

Xenopus egg extracts have been instrumental in understanding the mechanisms of spindle assembly and scaling. Importantly, the cell-free nature of extract cytoplasm uniquely allowed mixing experiments to demonstrate that cytoplasmic contents control size scaling and morphology [13, 14, 18]. Here, we provide the example of mixing *X. borealis* and *X. tropicalis* extracts and studying the consequence on spindle assembly and size (Fig. 1), but this experiment can apply to any two frog extracts or other cellular structures or organelles. All reactions are at room temperature.

1. Prepare *X. borealis* and *X. tropicalis* egg extracts as described above (*see* Subheadings 3.2 and 3.3). It is best to use both extracts as freshly as possible to ensure that both remain functional throughout the experiment.

2. Supplement extracts with 40 ng/μL of rhodamine-labelled tubulin to visualize microtubules.

[13] *X. tropicalis* extracts cannot be kept on ice. This will impair extract activity.

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3. Prepare mixing reactions in 1.5 mL microcentrifuge tubes. The total volume of extract reactions should be 20–50 μL.

4. First, pipette the appropriate amount of *X. borealis* extract using wide-bore P200 tips (Fig. 1a).

5. Add *X. tropicalis* extract in the appropriate proportions on top of *X. borealis* extract, pipetting with wide-bore P200 tips.

6. Mix by flicking the tube gently to ensure that both cytoplasmic extracts are fully merged.

7. Controls are internal to this experiment, as one sample should be 100% *X. borealis* extract and another should be 100% *X. tropicalis* extract (Fig. 1b).

8. Add 500–1000 *X. tropicalis* sperm nuclei per μL to each reaction (*see* Notes 14 and 15). Flick the tube gently to mix, as this will help the sperm disperse throughout the cytoplasm and prevent clumping of structures.

9. Follow each reaction with fixed "squashes" as desired every 15 min, taking care to flick the tube at this time as well.

10. To "squash" samples, pipette 1 μL of spindle fix onto a glass slide, then pipette 1 μL of extract into the spindle fix. Carefully place a square coverslip on top – the extract should spread immediately across the coverslip. DNA and microtubules should be readily visible by fluorescence microscopy, allowing observation of the cell cycle progression as desired.

11. Conclude the experiment by spinning down the reactions onto coverslips if, for example, immunofluorescence analysis is desired, as detailed elsewhere [22] (*see* Note 15).

3.5 "Hybrid" Extract Reactions

Xenopus frogs provide a phenomenal, vertebrate model system to study speciation barriers and mechanisms of hybrid inviability that lead to embryonic lethality [20, 28]. While other model systems, particularly *Drosophila*, provide genetic insights to hybrid incompatibility [29, 30], the egg extract system opens the door for detailed molecular, cell biological, and evolutionary investigation of maternal vs. paternal contributions to hybrid embryos. Here, we add a twist to classical extract reactions by mix-and-matching sperm chromosomes and egg cytoplasmic extracts from different species. This mimics the early embryonic cell divisions in hybrids, which are powered exclusively by maternal cytoplasmic components and machinery but also need to handle "foreign" paternal sperm DNA (Fig. 2a–c). Thus, this system can reveal mismatches and molecular conflicts in hybrids. In this protocol, we investigate combining *X. tropicalis* egg extract with *X. borealis* sperm DNA, as described

14. Recent work has demonstrated that hybrids from *X. borealis* eggs and *X. tropicalis* sperm are viable and have no obvious cell cycle defects, while the reverse hybrid is inviable and has chromosomal defects [20, 28]. Here, we chose to add *X. tropicalis* sperm nuclei to assay spindle assembly without complicating our results with additional hybrid incompatibility concerns.

15. Centrifugation of extract reactions onto coverslips and their fixation are required for further processing as detailed elsewhere [6, 22] but will shrink the size of subcellular structures. This is not an issue if all experiments compared are spun down. Conversely, squashes do not change the size of structures, providing more accurate size measurements, but are not compatible with immunofluorescence.

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recently to investigate chromosomal defects in inviable hybrids [20]. However, other combinations of sperm chromosomes and egg extracts are bound to reveal new insights as well and have already been used to understand spindle assembly and scaling mechanisms across frog species [13, 14, 31].

1. Prepare *X. tropicalis* egg extract as described above (*see* Subheading 3.3).
2. Prepare reactions in 1.5 mL microcentrifuge tubes. The total volume of extract reactions should be 20–50 μL.
3. First, pipette the appropriate amount of *X. tropicalis* extract using wide-bore P200 tips.
4. Add 500–1000 *X. borealis* sperm nuclei per μL of reaction (*see* Note 16). Be sure to use *X. tropicalis* sperm nuclei in control reactions (Fig. 2a).
5. Mix by flicking the tube gently. This will help the sperm disperse throughout the cytoplasm and prevent clumping of structures.
6. Follow each reaction with fixed “squashes” every 15 min, taking care to flick the tube at this time as well (see **step 10** in Subheading 3.4).
7. Conclude the experiment by spinning down the reactions onto coverslips to analyze either spindles or individualized chromosomes, as detailed elsewhere [6].

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16. If induction into interphase is desired, add 1× calcium solution to release the extract from its metaphase arrest and flick the tube gently. Incubate the reaction for 5–6 min, and then add sperm nuclei.

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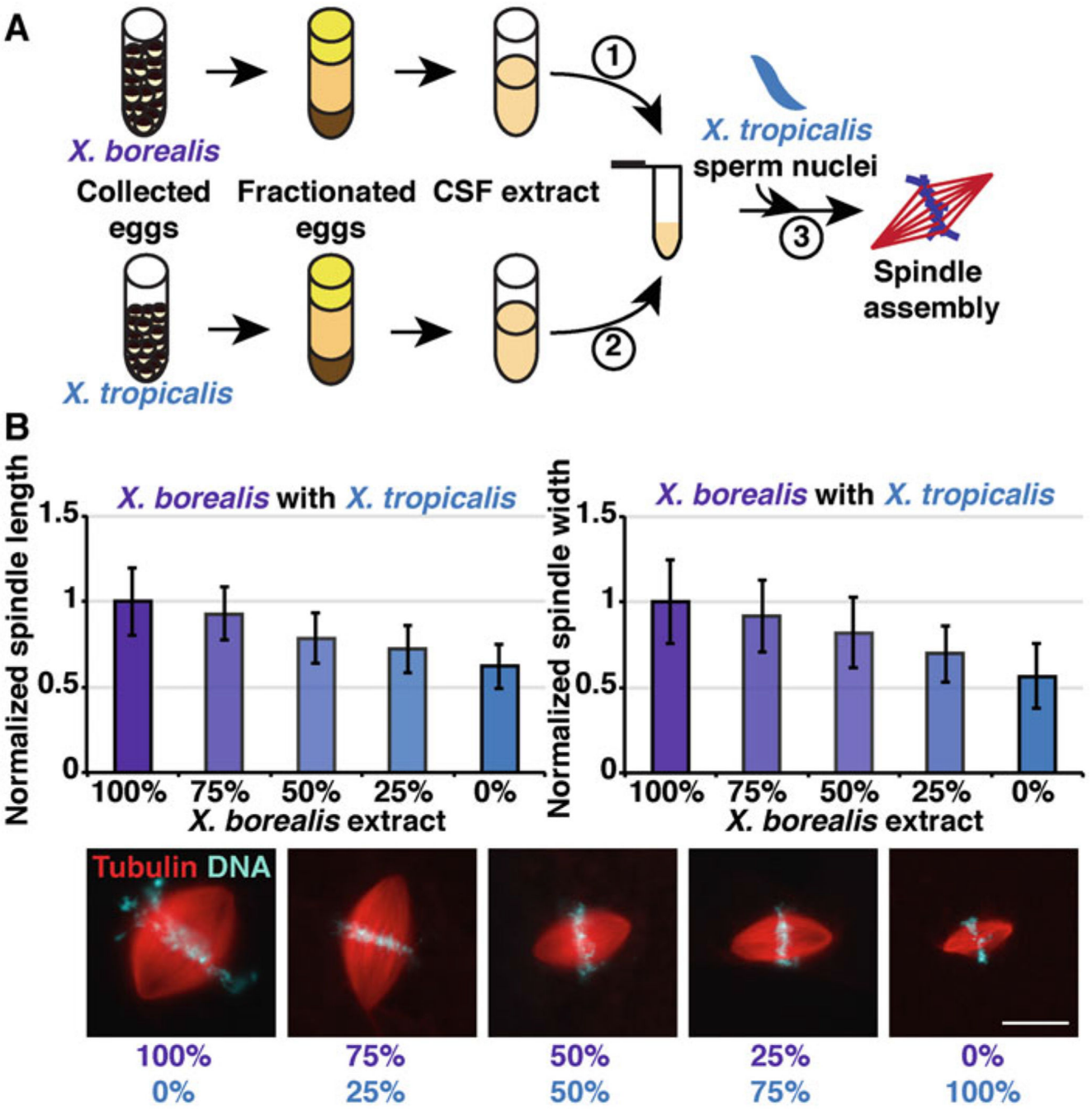


Fig. 1.

Mixing extracts uniquely demonstrates size control via cytoplasmic origin. (**a**) Experimental schematic of a mixing experiment. (**b**) Mixing *X. borealis* and *X. tropicalis* extract shrinks spindle size as more *X. tropicalis* extract is added. Scale is 20 μm. (Reproduced from Kitaoka et al. with permission from John Wiley and Sons [18])

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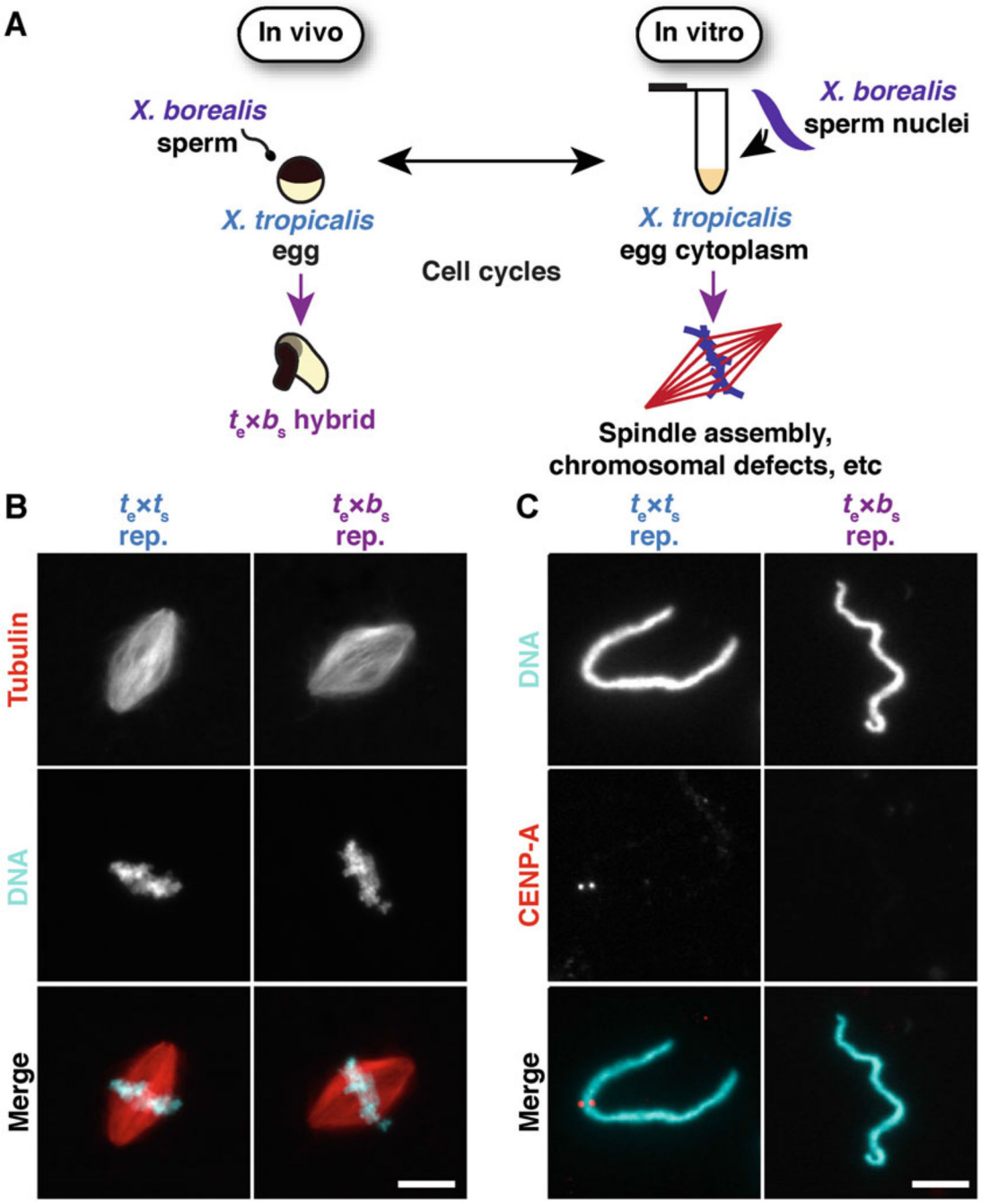


Fig. 2.

Using "hybrid" reactions to understand maternal vs. paternal contributions to hybrid inviability. (**a**) Experimental schematic of "hybrid" reactions, demonstrating how the extract can mimic the early cell cycles in hybrid embryos. (**b**) *X. tropicalis* (left) and *X. borealis* (right) sperm DNA can both induce spindle assembly in *X. tropicalis* egg extract. (**c**) "Hybrid" reactions can reveal conflicts between species, such as loss of the centromeric histone CENP-A from replicated *X. borealis* chromosomes in *X. tropicalis* extract (right). Scale is 10 μm. Nomenclature denotes maternal egg or egg extract as

subscript e and paternal sperm chromosomes as subscript s. (Reproduced from Kitaoka et al. with permission from Cell Press [20])

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Table 1

Comparison between 3 *Xenopus* species' egg extract protocols

	X. laevis	***X. tropicalis***	***X. borealis***
Priming	100 IU PMSG, at least 48 h and up to 2 weeks before boost; or 15 IU hCG, 72 to 30 h before boost	10 IU hCG, 20 h before boost	60 IU PMSG, at least 48 h and up to 1 week before boost
Boosting	500 IU hCG, or up to 750 IU if primed with hCG, the evening before the experiment, 16–18 h	200–250 IU hCG, the morning of the experiment, ~3–4 h before	300 IU hCG, the evening before the experiment, 16–18 h
Egg laying conditions	1× MMR, 16 °C Overnight	ddH2O or buffered 0.1× MMR, 23 °C Frogs will start laying ~3 h after boosting. Can be helped/sped along by gently holding females to promote kicking 3–4 times at 15-min intervals	0.5× MMR, 16 °C Overnight
Egg collection	Pour off excess 1× MMR, until all eggs can be transferred to a clean beaker	Eggs will stick to the tub (glass or plastic). Use a gloved hand to gently sweep and detach the eggs. Collect them in one corner of the tub while carefully pouring excess water off. Collect them into a clean beaker	Pour off excess 0.5× MMR, until all eggs can be transferred to a clean beaker
CSF-XB	100 mM KCl 0.1 mM $CaCl_2$ *2 mM $MgCl_2$* 50 mM sucrose *5 mM K-EGTA* 10 mM K-HEPES pH 7.8	100 mM KCl 0.1 mM $CaCl_2$ *3 mM $MgCl_2$* 50 mM sucrose *10 mM K-EGTA* 10 mM K-HEPES pH 7.8	100 mM KCl 0.1 mM $CaCl_2$ *3 mM $MgCl_2$* 50 mM sucrose *10 mM K-EGTA* 10 mM K-HEPES pH 7.8
Dejelly	2% L-cysteine, 1× XB salts, in ddH_2O, pH 7.7–7.8 with NaOH	3% L-cysteine, in ddH_2O, pH 7.7–7.8 with NaOH	3% L-cysteine, 1× XB salts, in ddH_2O, pH 7.7–7.8 with NaOH
Approx. extract yield per frog	1 mL	200 μL	600 μL
Egg extract storage	4 °C, on ice	16 °C	16 °C
Extract reaction timing	CSF spindles in 60 min Cycled nuclei in 45 min	CSF spindles in 30 min Cycled nuclei in 30 min	CSF spindles in 30–45 min Cycled nuclei in 45 min
Resting interval between ovulations	6 months	3 months	4 months

Differences between *X. laevis*, *X. tropicalis*, and *X. borealis* egg extract protocols are often subtle but important for frog health and egg/extract quality