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Authors
Gibeaux, Romain
Heald, Rebecca

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Chapter 18

Generation of *Xenopus* Haploid, Triploid, and Hybrid Embryos

Romain Gibeaux and Rebecca Heald

Abstract

Frog species of the genus *Xenopus* are widely used for studies of cell and developmental biology, and recent genome sequencing has revealed interesting phylogenetic relationships. Here we describe methods to generate haploid, triploid, and hybrid species starting from eggs and sperm of *Xenopus laevis* and *Xenopus tropicalis* that enable investigation of how genome size and content affect physiology at the organismal, cellular, and subcellular levels.

Key words *Xenopus*, *Xenopus laevis*, *Xenopus tropicalis*, Fertilization, Embryology, Ploidy, Haploid, Triploid, Hybridization, Hybrid

1 Introduction

Although central to evolution, the biological consequences of changes in ploidy or genome hybridization are poorly understood. Amphibian genomes exist in a range of sizes and external fertilization in vitro has permitted manipulation of ploidy in various ways [1, 2]. *Xenopus* frog species are most widely studied and occupy a range of ploidies from diploid *Xenopus tropicalis* (2 N, 20 chromosomes) to dodecaploid *Xenopus longipes* (12 N, 108 chromosomes) [3]. Cross-fertilization between species is possible, as well as the generation of haploid and triploid embryos [4–10]. The most commonly used species, *Xenopus laevis*, is an allotetraploid with 36 chromosomes that arose through interspecific hybridization of diploid *X. tropicalis*-like progenitors that diverged from a common ancestor ~48 million years ago [11]. Interestingly, whereas the hybrid produced when *X. laevis* eggs are fertilized by *X. tropicalis* sperm ($l_e \times t_s$) is viable, the reverse hybrid ($t_e \times l_s$) dies prior to gastrulation [8, 9]. In this protocol, we describe simple and optimized methods to generate haploid, triploid, and hybrid embryos of the *Xenopus* species, *X. laevis* and *X. tropicalis* (Fig. 1). The protocols are organized according to maternal species. Sperm

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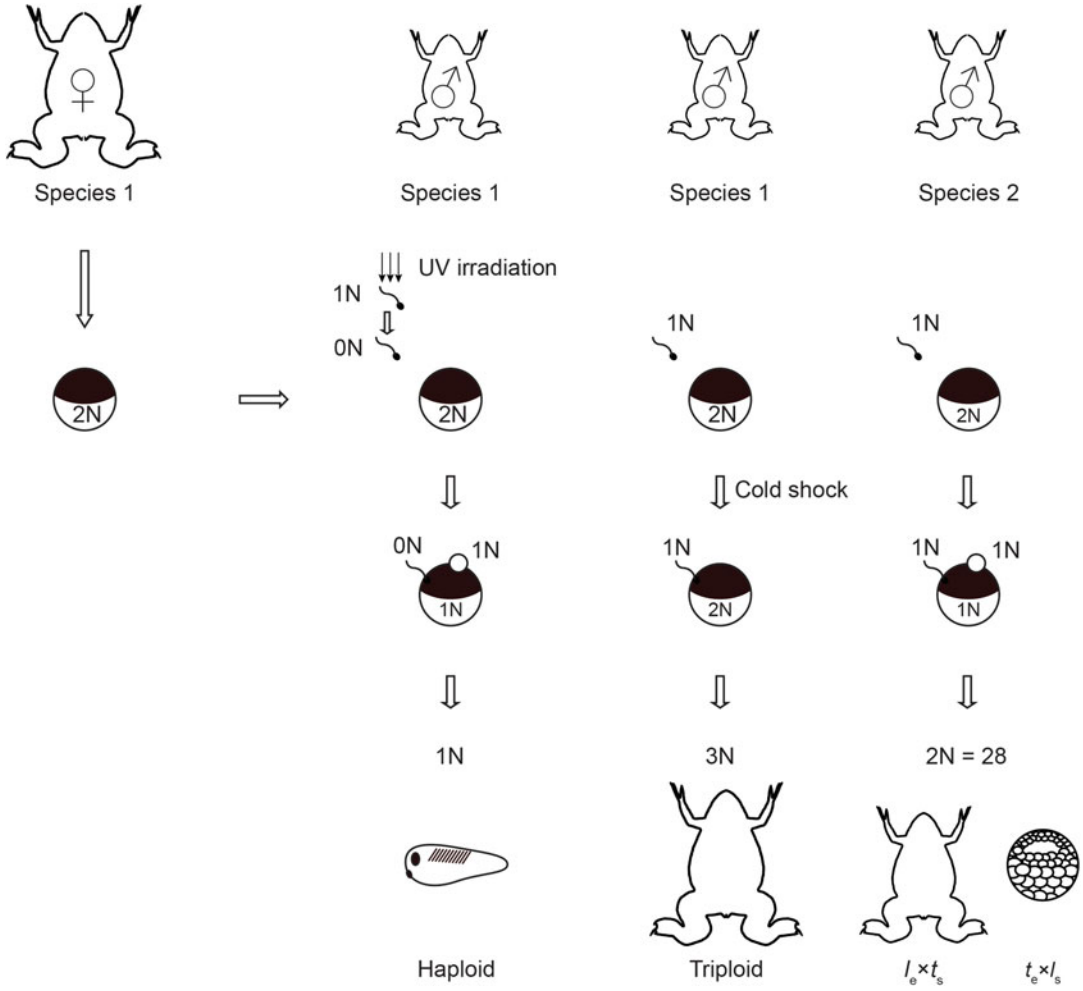

Fig. 1 Overview of ploidy manipulation in *Xenopus*. Irradiation of sperm is used to inactivate the paternal genome and generate haploid embryos. Haploid embryos develop as stunted tadpoles that never reach metamorphosis and exhibit the so-called haploid syndrome (*see* **Note 6**). Cold shock of fertilized eggs is used to block polar body extrusion and generate triploid embryos. Triploid embryos are viable and can develop to adult frogs (*see* **Note 8**). Fertilization of eggs with sperm of a different species produces hybrid embryos. Whereas the hybrid produced when *X. laevis* eggs are fertilized by *X. tropicalis* sperm ($l_e \times t_s$) is viable and develops to adult frog, the reverse hybrid ($t_e \times l_s$) dies prior to gastrulation

irradiation inactivates the paternal genome to generate haploids, while cold shock of eggs blocks polar body extrusion to generate triploid embryos. These are the simplest methods to perturb *Xenopus* embryo ploidy. Finally, we describe optimized methods to generate the viable hybrid obtained from the fertilization of *X. laevis* eggs with *X. tropicalis* sperm ($l_e \times t_s$) as well as the inviable hybrid obtained from the fertilization of *X. tropicalis* eggs with *X. laevis* sperm ($t_e \times l_s$), with reproducible fertilization efficiencies.

Thus, this protocol provides methods to prepare *Xenopus* embryos with varying genome sizes and content.

2 Materials

1. Pregnant mare serum gonadotropin (PMSG): 200 IU/mL PMSG in sterile Milli-Q H_2O. Store at 4 °C.
2. Human chorionic gonadotropin hormone (HCG): 1000 IU/mL HCG in sterile Milli-Q H_2O. Store at 4 °C.
3. 10× Modified Ringer's solution (MR): 1 M NaCl, 18 mM KCl, 20 mM $CaCl_2$, 10 mM $MgCl_2$, and 50 mM HEPES-NaOH pH 7.6. Prepare 500 mL in deionized H_2O and filter sterilize. Store at room temperature.
4. 20× Marc's modified Ringer's 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 deionized H_2O and filter sterilize. Store at room temperature.
5. 1.5% Agarose in 1/10× MMR: Add 1.5 g agarose to 100 mL 1/10× MMR and microwave until dissolved. Melt in microwave before using.
6. Dejellying solution: 3% L-Cysteine in Milli-Q H_2O. Adjust to pH 7.8 with 10 N NaOH. Prepare 100 mL fresh just before use.
7. 10% of fetal bovine serum in Leibovitz's L-15 Medium: Leibovitz's L-15 Medium is stored at 4 °C. FBS is stored at −20 °C as 500 μL aliquots. Prepare 1.1 mL of 10% FBS in Leibovitz's L-15 Medium fresh just before use.
8. Plastic containers for frogs (4 L for *X. laevis* and 6 L for *X. tropicalis*) with tight-fitting lids and holes punched for air exchange.
9. Room (16 °C) or large non-airtight incubator set to 16 °C for *X. laevis*.
10. 1 mL Syringes.
11. 30 gauge needles.
12. Forceps.
13. Dissection scissors.
14. Plastic pestle for 1.5 mL microfuge tube.
15. Petri dishes (glass, 6 cm diameter, with lids).
16. Plastic squeeze bottles.
17. Transfer pipets.
18. Dissection stereomicroscope.

19. Stratalinker UV-Crosslinker (Stratagene, San Diego, CA).
20. Rectangular ice bucket.
21. Rectangular plastic dish (~12 × 9 × 4 cm) with walls as thin as possible (e.g., pipette tip box lid).

3 Methods

3.1 Methods based on X. laevis Eggs

3.1.1 Preparation for X. laevis Egg Fertilization

1. Prime *X. laevis* females by injecting 0.5 mL (100 IU) of PMSG subcutaneously into the dorsal lymph sac using a 30 gauge needle and 1 mL syringe, at least 48 h before boosting (*see* **Note 1**).
2. As needed, prime one *X. laevis* male by injecting 0.5 mL (500 IU) of HCG or one *X. tropicalis* male by injecting 0.25 mL (250 IU) of HCG, about 24 h before dissection.
3. Carry out **steps 3–6** the day before fertilization. Boost *X. laevis* females by injecting 0.5 mL (500 IU) of HCG to induce ovulation (*see* **Note 2**). Store each frog individually in 2 L of freshly prepared 1× MMR in a 4 L plastic container overnight at 16 °C.
4. If applicable: Euthanize the *X. laevis* male and dissect to extract the testes. Clean testes free of fat or blood vessels, and store at 4 °C in a glass petri dish filled with 1× MR (*see* **Note 3**).
5. Prepare fresh 0.5–1 L of 1/10× MMR to be used for the preparation of petri dishes and fertilization.
6. Prepare fertilization petri dishes by coating the bottom with 5–10 mL melted 1.5% agarose in 1/10× MMR. Once solidified, cover the agarose with 1/10× MMR, and store the dishes at room temperature to prevent them from drying out.
7. Carry out **steps 7–9** on the day of fertilization. Analyze egg quality and select the females to be used. Avoid females that have laid lysed or stringy eggs (*see* **Note 4**).
8. Fill a plastic squeeze bottle with 1/10× MMR.
9. Discard the 1/10× MMR covering the agarose from the fertilization petri dishes.

3.1.2 Generation of Haploid X. laevis

1. Cut 1/2–2/3 of a testis using forceps and scissors and add it to 1.1 mL of Milli-Q H_2O in a 1.5 mL microcentrifuge tube.
2. For selected *X. laevis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization petri dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).

3. Gently swirl the dish to form a monolayer of eggs.
4. Prepare sperm solution by cutting the piece of testis into smaller, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle.
5. Spin down briefly using a benchtop centrifuge to pellet the tissues.
6. Collect 1 mL of supernatant, without pipetting any pieces of tissue, and transfer into a non-agarose-coated glass petri dish. Swirl to distribute the solution evenly.
7. Place the open dish into the UV-Crosslinker and irradiate the solution two times with 30,000 microjoules, swirling the solution between the two irradiations (*see* **Note 6**).
8. Retrieve the irradiated sperm and fertilize by depositing 0.5 mL of irradiated sperm solution on top of the eggs.
9. Use a clean plastic pestle to gently mix the eggs within the sperm solution and incubate for 10 min (*see* **Note 7**). Keep the dish slanted during this step to maximize the interaction of the sperm with the eggs.
10. Flood the fertilization dish with 1/10× MMR, making sure to submerge all eggs, and incubate for 10 min.
11. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.
12. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.1.3 Generation of Triploid X. laevis

1. Fill a rectangular ice bucket with slushy ice, and partially submerge a rectangular plastic dish and a 50 mL conical tube filled with 1/10× MMR (Fig. 2). Store at 4 °C for at least 2 h.
2. Prepare cold shock-ready fertilization petri dishes by coating the bottom with only ~1.5 mL of melted 1.5% agarose in 1/10× MMR, 1–2 h ahead.
3. Cut 1/3–1/2 of a testis using forceps and scissors and add it to 1 mL of Milli-Q H_2O in a 1.5 mL microcentrifuge tube.
4. For selected *X. laevis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
5. Gently swirl the dish to form a monolayer of eggs.

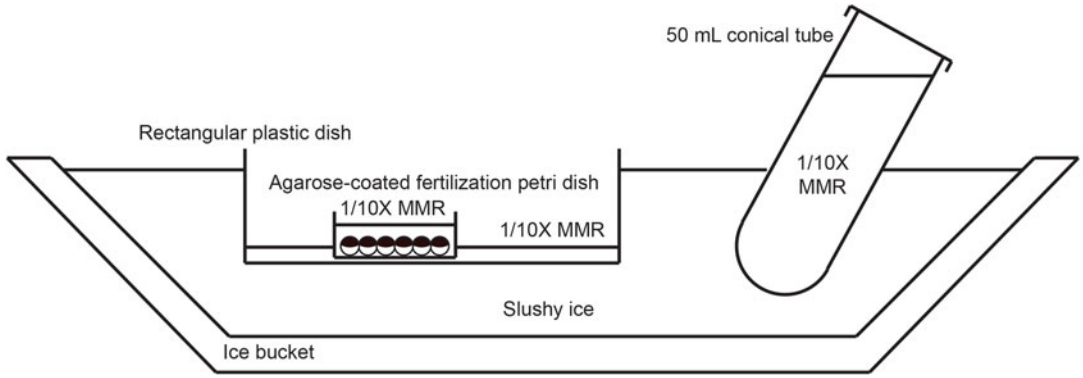

Fig. 2 Cold-shock treatment apparatus. The rectangular ice bucket is filled with slushy ice. A rectangular plastic dish and a 50 mL conical tube filled with 1/10× MMR are partially submerged and stored at 4 °C for at least 2 h. To proceed with the cold shock, the fertilization petri dish (coated with 1.5 mL of 1.5% agarose in 1/10× MMR) containing the fertilized eggs and ice-cold 1/10× MMR is placed, without its lid, in the cooled plastic dish, and the rest of the ice-cold 1/10× MMR is used to fill the cooled plastic dish

6. Prepare sperm solution by cutting the piece of testis into smaller, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle.
7. Fertilize the eggs by transferring the 1 mL of sperm solution on top of the eggs.
8. Use a clean plastic pestle to gently mix the eggs within the sperm solution. Make sure that the eggs make a monolayer and incubate for 10 min (*see* **Note 7**).
9. Flood the fertilization dish with 1/10× MMR, making sure to submerge all eggs, and incubate for 3 min.
10. Exactly 13 min after sperm addition, rapidly remove the 1/10× MMR using a plastic transfer pipette, pour ice-cold 1/10 MMR from the 50 mL conical tube, place the fertilization petri dish in the cooled plastic dish, and add the rest of the ice-cold 1/10× MMR to the cooled plastic dish (Fig. 2). Incubate for 15 min. Leave the lid of the fertilization petri dish at room temperature (*see* **Note 8**).
11. Remove the petri dish from the plastic dish, and exchange the cold 1/10× MMR for room-temperature 1/10× MMR. Close the fertilization petri dish with its lid left at room temperature, and let the temperature equilibrate on the bench for 25–30 min.
12. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.

13. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.1.4 Generation of $|_e \times t_s$ Hybrids

1. Euthanize the *X. tropicalis* male and dissect to extract the testes. Clean testes free of fat and blood vessels, and store at room temperature in 1 mL of L15 supplemented with 10% FBS until they are used for fertilization (*see* **Note 9**).
2. For selected *X. laevis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
3. Gently swirl the dish to form a monolayer of eggs.
4. Transfer one testis in 1 mL of Milli-Q H_2O and prepare the sperm solution by cutting the testis into small, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle.
5. Fertilize by depositing 1 mL of sperm solution on top of the eggs (*see* **Note 10**).
6. Use a clean plastic pestle to gently mix the eggs within the sperm solution, and incubate for 10 min (*see* **Note 7**). Keep the dish slanted during this step to maximize the interaction of the sperm with the eggs.
7. Flood the fertilization dish with 1/10× MMR, making sure to submerge all eggs, and incubate for 10 min.
8. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.
9. Remove the dejellying solution and wash 3–5 times with 1/10X MMR.

3.2 Methods based on X. tropicalis Eggs

3.2.1 Preparation for X. tropicalis Egg Fertilization

1. Carry out **steps 1–5** the day before fertilization. As applicable: Prime one *X. laevis* male by injecting 0.5 mL (500 IU) of HCG or one *X. tropicalis* male by injecting 0.25 mL (250 IU) of HCG, about 24 h before dissection.
2. Prime *X. tropicalis* females by injecting 0.25 mL (25 IU) of HCG subcutaneously into the dorsal lymph sac using a 30 gauge needle and 1 mL syringe, 20 h before boosting (*see* **Note 11**).
3. If applicable: Euthanize the *X. laevis* male and dissect to extract the testes. Clean testes free of fat and blood vessels, and store at 4 °C in a glass petri dish filled with 1× MR (*see* **Note 12**).

4. Prepare fresh 0.5–1 L of 1/10× MMR to be used for the preparation of the dishes and fertilization.
5. Prepare fertilization dishes by coating their bottom with 5–10 mL melted 1.5% agarose in 1/10× MMR. Once solidified, cover the agarose with 1/10× MMR, and store the dishes at room temperature.
6. Carry out **steps 6–9** on the day of fertilization. Boost *X. tropicalis* females by injecting 0.25 mL (250 IU) of HCG to induce ovulation. Store each frog individually in 2–3 L of deionized H_2O in a 6 L plastic container at room temperature.
7. Analyze egg quality and select the females to be used. Avoid females that have laid lysing or stringy eggs (*see* **Note 4**). Always use the frogs shortly after first eggs are laid (*see* **Note 13**).
8. Prepare plastic squeeze bottles filled with Milli-Q H_2O and 1/10× MMR.
9. Discard the 1/10× MMR covering the agarose from the fertilization petri dishes.

3.2.2 Generation of Haploid X. tropicalis

1. Euthanize the *X. tropicalis* male and dissect to extract the testes. Clean testes free of fat and blood vessels, and place in 1.1 mL of L15 supplemented with 10% FBS (*see* **Note 9**).
2. For selected *X. tropicalis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization petri dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
3. Gently swirl the dish to form a monolayer of eggs.
4. Prepare sperm solution by cutting the testes into small, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle within the L15 + 10% FBS.
5. Spin down briefly using a benchtop centrifuge to pellet the tissues.
6. Collect 1 mL of supernatant, without pipetting any pieces of tissue, and transfer into a non-agarose-coated glass petri dish. Swirl to distribute the solution evenly.
7. Place the open dish in the UV-Crosslinker, and irradiate the solution once with 50,000 microjoules (*see* **Note 6**).
8. Retrieve the irradiated sperm and fertilize by depositing 0.5 mL of irradiated sperm solution on top of the eggs.
9. Use a clean plastic pestle to gently mix the eggs within the sperm solution, and incubate for 4–5 min (*see* **Note 7**). Keep

the dish slanted during this step to maximize the interaction of the sperm with the eggs.

10. Flood the fertilization dish with Milli-Q H_2O, making sure to submerge all eggs, and incubate for 10 min.
11. Exchange the Mili-Q H_2O for 1/10× MMR, and incubate for 10 min.
12. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.
13. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.2.3 Generation of Triploid *X. tropicalis*

1. Fill a rectangular ice bucket with slushy ice, partially submerge a rectangular plastic dish and a 50 mL conical tube filled with 1/10× MMR (Fig. 2). Store at 4 °C for at least 2 h.
2. Prepare cold shock-ready fertilization petri dishes by coating the bottom with only ~1.5 mL of melted 1.5% agarose in 1/10× MMR, 1–2 h ahead.
3. Euthanize the *X. tropicalis* male and dissect to extract the testes. Clean testes free of fat and blood vessels, and place both in 1 mL of L15 supplemented with 10% FBS (*see* **Note 9**).
4. For selected *X. tropicalis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
5. Gently swirl the dish to form a monolayer of eggs.
6. Prepare sperm solution by cutting the testes into small, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle within the L15 + 10% FBS.
7. Fertilize the eggs by transferring 0.5 mL of sperm solution on top of the eggs.
8. Use a clean plastic pestle to gently mix the eggs within the sperm solution. Make sure that the eggs form a monolayer and incubate for 4 min (*see* **Note 7**).
9. Flood the fertilization dish with Milli-Q H_2O, making sure to submerge all eggs, and incubate for 5 min.
10. Exactly 9 min after sperm addition, rapidly remove the Milli-Q H_2O using a plastic transfer pipette and pour ice-cold 1/10 MMR from the 50 mL conical tube, place the petri dish in the cooled plastic dish, and add the rest of the ice-cold 1/10 MMR

to the cooled plastic dish (Fig. 2). Incubate for 10 min. Leave the lid of the fertilization petri dish at room temperature (*see* **Note 8**).

11. Remove the fertilization dish from the plastic dish, and exchange the cold 1/10× MMR for room-temperature 1/10× MMR. Close the fertilization petri dish with its lid left at room temperature, and let the temperature equilibrate on the bench for 20 min.
12. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.
13. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.2.4 Generation of $t_e \times l_s$ Hybrids

1. Cut 1/2 of a *X. laevis* testis using forceps and scissors, and place it in 0.5 mL of L15 + 10% FBS in a 1.5 mL microcentrifuge tube.
2. For selected *X. tropicalis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
3. Gently swirl the dish to form a monolayer of eggs.
4. Prepare sperm solution by cutting the piece of testis into smaller, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle within the L15 + 10% FBS.
5. Fertilize by depositing 0.5 mL of sperm solution on top of the eggs (*see* **Note 10**).
6. Use a clean plastic pestle to gently mix the eggs within the sperm solution and incubate for 5 min (*see* **Note 7**). Keep the dish slanted during this step to maximize the interaction of the sperm with the eggs.
7. Flood the fertilization dish with Milli-Q H_2O, making sure to submerge all eggs, and incubate for 10 min.
8. Exchange the Milli-Q H_2O for 1/10× MMR, and incubate for 10 min.
9. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.

10. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.3 General Postfertilization Embryo Care

1. Let the embryos develop at 23 °C to stages 2–3 (*see* **Note 14**). Observe the embryos under a dissection stereomicroscope and sort them to remove unfertilized eggs and dying embryos using plastic transfer pipettes. As soon as the sorting is complete, transfer healthy embryos to fresh 1/10× MMR in a fresh fertilization dish in order to keep the embryos in a dish that has not been exposed to dejellying solution.
2. Let the embryos develop at 23 °C until the desired developmental stage (*see* **Note 15**). Observe the embryos 2–3 times a day and remove dying ones as soon as possible. Make sure to exchange for fresh 1/10× MMR every 10–14 h.

4 Notes

1. Priming the *X. laevis* female frogs increases egg yield and quality. Primed frogs should be used within two weeks.
2. *X. laevis* female boosting is usually performed the evening preceding the experiment, 16–18 h before eggs are needed.
3. Properly stored *X. laevis* testes can be used up to two weeks after dissection, but fertilization efficiency may decrease over time.
4. Egg quality is the primary and most important parameter as this will determine the efficiency of the fertilization. Careful analysis of egg quality is thus highly recommended.
5. Skin secretions from the frog will cause eggs to lyse. It is thus important to remove any liquid that has dripped off of the frog during squeezing from the fertilization dish.
6. The sperm irradiation method to produce *Xenopus* haploids is derived from experiments originally performed with *X. laevis* [4]. However, haploids can also be produced by irradiation of the eggs rather than the sperm [5]. Haploid embryos develop as stunted tadpoles that never reach metamorphosis. They exhibit the so-called haploid syndrome, which can be recognized easily by a shortened body axis, microcephaly, poorly formed gut, and edema [12, 13]. Note that the irradiation of sperm is the key step in the generation of haploids and, although we found the presented irradiation doses to be the most efficient, one can tweak this parameter if necessary.
7. Due to the robustness of wild-type fertilizations, control fertilizations with nonirradiated sperm (for haploid experiments), non-cold-shock-treated zygotes (for triploid experiments), or sperm from the same species (for hybrid experiments) can be

conducted with the same incubation procedures and timings as the ones optimized for the corresponding experiments with good fertilization efficiencies.

8. The cold-shock method to produce triploid embryos is derived from experiments originally performed with *X. laevis* [6]. Alternatively, triploids can be produced by exposing the zygote to hydrostatic pressure [7]. Triploid embryos are viable and can develop normally beyond metamorphosis [6]. Note that the key parameters for the success of the cold-shock treatment are the starting time (number of minutes postfertilization) and the duration of the shock, which can be adjusted if necessary.
9. *X. tropicalis* testes are always prepared immediately before use to ensure the highest fertilization efficiency possible.
10. Cross-fertilizations have different efficiencies. Fertilization efficiency of the viable $l_e \times t_s$ hybrid is high (close to 100%), while the $t_e \times l_s$ fertilization is much less efficient. With the optimized protocol presented here, we could occasionally reach 60–70% efficiency but, on average, one might expect only 10% efficiency.
11. Priming the *X. tropicalis* female frogs increases egg yield, as well as reproducibility in timing of laying.
12. Although properly stored *X. laevis* testes can be used up to two weeks, recently extracted testes (1–3 days) are preferred for cross-fertilization.
13. With the recommended hormone dosage and injection timing, ovulation usually occurs 3–3.5 h after boosting.
14. Embryos are staged according to Nieuwkoop and Faber [14].
15. Confirmation of changes in ploidy can be assessed in various ways, including counting the number of nucleoli, karyotyping, or genotyping if one uses parental frogs of different strains. In addition, by analyzing the size of the nucleus relative to the size of the cell using immunofluorescence, it is possible to discriminate the different ploidy of embryos starting from late stage 9 to stage 10 [15].

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