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Room-Temperature Storage of Zebrafish and Medaka Sperm Using Lactic Acid-Stabilized L-15 Medium

Yukiko Imai[1, *], Touma Asano[1], Toshiya Nishimura[2], Kazumasa Takemoto[3, $a], Akinori Kawamura[1] and Noriyoshi Sakai[3, 4, $b]

[1]Division of Life Science, Graduate School of Science and Engineering, Saitama University, Saitama, Japan
[2]Division of Marine Life Science, Hokkaido University, Hakodate, Japan
[3]Department of Genetics, School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), Mishima, Japan
[4]Department of Gene Function and Phenomics, National Institute of Genetics, Mishima, Japan
[$a]Present address: Department of Genome Editing, Institute of Biomedical Science, Kansai Medical University, Hirakata, Japan
[$b]Present address: Division of Applied Bioscience, Graduate School of Agriculture, Kyoto University, Kyoto, Japan
*For correspondence: yimai@mail.saitama-u.ac.jp

Abstract

Zebrafish offer numerous advantages as a vertebrate model because of their rapid development, high fecundity, transparent embryos, and ease of genetic manipulation. A wide variety of transgenic and mutant fish lines have been generated, and efficiently sharing these resources is crucial for advancing research. Zebrafish lines have typically been exchanged as early embryos, adult fish, or cryopreserved sperm, making transportation costly and logistically challenging. Here, we provide a protocol for preserving functional zebrafish sperm for more than 7 days at room temperature and subsequent in vitro fertilization using the preserved sperm. In this protocol, sperm collected either from the cloaca of an anesthetized male or from dissected testes is stored in L-15-based storage medium. Importantly, the storage medium, originally developed for zebrafish, is also applicable to medaka, another widely used vertebrate model. This sperm storage method allows researchers to ship sperm using low-cost methods and to investigate key factors for motility and fertilizing ability in those sperm.

Key features

- Provides conditions for room-temperature preservation of zebrafish and medaka sperm for at least one week, based on Takemoto et al. [1].
- Introduces an in vitro fertilization method to achieve high fertilization rates with sperm samples preserved at room temperature.
- Enables the easy exchange of fish lines across countries via low-cost postal shipping.

Keywords: Zebrafish, Medaka, Sperm, In vitro fertilization, Bioresource

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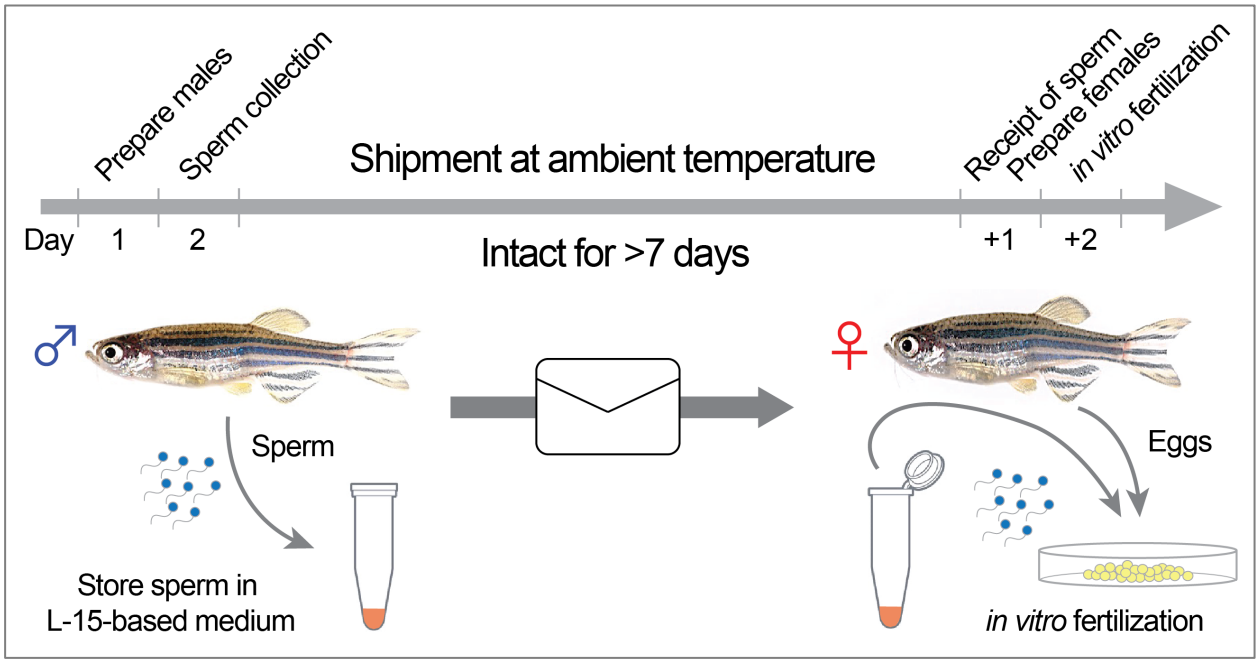
Graphical overview



Background

The zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are phylogenetically distant teleost fish. Both are powerful vertebrate models in biological research due to their genetic tractability, rapid development, and optical transparency during early embryogenesis. Both serve as an important platform for research in developmental biology, genetics, disease modeling, and toxicology [2–5]. As zebrafish and medaka research expands globally, the need for reliable and efficient methods to manage and share genetic lines has grown increasingly important.

Traditionally, zebrafish lines have been distributed by shipping fertilized embryos. However, this approach has several limitations. Embryos are highly sensitive to environmental factors such as temperature, oxygen levels, and mechanical stress, which can compromise their viability and development during transport [6]. Additionally, embryos can only be maintained in a closed container at certain developmental stages for a limited period of up to 3 days (e.g., ZIRC, https://zebrafish.org/wiki/protocols/shipping), making long-distance or international shipping logistically challenging and often costly. Alternatively, cryopreservation has been used for long-term storage of sperm in many fish species [7–9]. However, sperm cryopreservation requires skilled techniques and maintenance of frozen conditions during transport. Specialized containers and administrative procedures are also necessary for the shipment of liquid nitrogen. Moreover, frozen samples are at high risk of being ruined by poor handling or shipping.

To overcome these challenges, we have developed a room-temperature sperm storage method [1] that allows storage for at least one week and retains ~32% fertilization efficiency even after 28 days under optimal conditions, thereby facilitating the transport of zebrafish genetic resources. When combined with in vitro fertilization (IVF) techniques, room-temperature sperm preservation provides a practical solution for sharing zebrafish lines. Fertilization using eggs from lab-stock females minimizes the risk of pathogen transmission between facilities, and stored sperm can be readily used to cross with locally maintained lines, enabling rapid experimental crosses. Moreover, this method facilitates studies on sperm quality—such as fertilization capacity and motility—and allows researchers to experimentally dissociate mating behavior from fertilization success, a critical distinction for understanding reproductive mechanisms. Here, we present detailed protocols for sperm preservation and its use in IVF in zebrafish. Notably, this sperm preservation method has been successfully applied to medaka, and corresponding protocols for medaka are described.

Materials and reagents

Biological materials

1. Adult zebrafish males and females
This protocol has been successfully used with the India strain (provided by Dr. Y. Kishimoto, National Institute of Genetics, Japan), the AB strain (provided by Dr. U. Strähle, Karlsruher Institute of Technology, Germany), the RW strain (provided by RIKEN, NBRP zebrafish), and their derivative transgenic and mutant lines. It could be applied to other zebrafish strains. Sexually mature males with a reddish body color and females with a fuller abdomen are suitable for sperm and egg collection, respectively.
2. Adult medaka males and females
This protocol has also been applied to the medaka OK-Cab strain (MT830-NIBB, provided by NBRP medaka), and other medaka strains could be used as well. Sexually mature males and females (4–6-month-old) exhibiting secondary sex characteristics in the anal and dorsal fins are suitable for sperm and egg collection. *meioC* loss-of-function mutants, in which germ cells arrest at the early stage of spermatogonia [10], can be used as infertile males to mate with wild-type females for the collection of unfertilized eggs.

Reagents

1. L-15 medium, Leibovitz (Sigma, catalog number: L5520)
2. Penicillin-Streptomycin, 5,000 U/mL (Gibco, catalog number: 15070-063)
3. D(+)-Glucose (Wako, catalog number: 041-00595)
4. Fetal bovine serum (FBS), Mexican origin (Sigma, catalog number: 173012, lot number: 0001653205)
5. Bovine serum albumin (BSA), fraction V, cell culture glade (Sigma, catalog number: A9418)
6. HEPES, suitable for cell culture (Sigma, catalog number: H4034)
7. L-(+)-Lactic acid (Sigma, catalog number: L6402-1G)
8. 1 M NaOH (Wako, catalog number: 196-05375)
9. Tricaine (3-amino benzoic acid ethylester) (Sigma, catalog number: A-5040)
10. NaCl (Wako, catalog number: 191-01665)
11. KCl (Wako, catalog number: 160-03555)
12. $CaCl_2 \cdot 2H_2O$ (Wako, catalog number: 031-00435)
13. $MgSO_4 \cdot 7H_2O$ (Wako, catalog number: 131-00405)
14. 0.82% methylene blue solution (Japan Pet Design, JAN code: 4975677045615)
15. 70% ethanol (Amakasu Chemical Industry, catalog number: NKH)
16. Bleach solution (Oyalox, JAN code: 4987038730016)
17. $NaHCO_3$ (Wako, catalog number: 191-01305)

Solutions

1. 1 M HEPES-NaOH pH 7.9 (see Recipes)
2. 5% BSA (see Recipes)
3. 2 M glucose (see Recipes)
4. 1 M L-(+)-lactic acid (see Recipes)
5. 10 mM L-(+)-lactic acid (see Recipes)
6. Sperm preservation medium (see Recipes)
7. 500× tricaine (see Recipes)
8. 1× tricaine (see Recipes)
9. 50× E3 stock solution for zebrafish (see Recipes)
10. 1× E3 for zebrafish (see Recipes)
11. 20× solution A for medaka (see Recipes)
12. Solution B for medaka (see Recipes)
13. Balanced salt solution (BSS) for medaka (see Recipes)

Recipes

1. 1 M HEPES-NaOH pH 7.9

Reagent	Final concentration	Quantity or volume
HEPES	1 M	23.83 g
1 M NaOH	n/a	n/a
ddH_2O	n/a	Fill to 100 mL

Add 23.83 g of HEPES to ~80 mL of ddH_2O. Dissolve completely. Adjust pH to 7.9 with 1 M NaOH. Make the volume to 100 mL with ddH_2O. Filter sterilize using a 0.2 µm filter, aliquot, and store at -20 °C.

2. 5% BSA

Reagent	Final concentration	Quantity or volume
BSA	5%	2.5 g
ddH_2O	n/a	Fill to 50 mL

Add 2.5 g of BSA to ~40 mL of ddH_2O in a 50 mL conical tube. Allow the powder to settle without mixing to prevent clumping. Once the powder has swelled, gently mix by rotating the tube, then adjust the volume to 50 mL with ddH_2O. Filter sterilize using a 0.2 µm filter, aliquot, and store at -20 °C.

3. 2 M glucose

Reagent	Final concentration	Quantity or volume
D(+)-glucose	2 M	1.80 g
ddH_2O	n/a	Fill to 5 mL

Filter sterilize using a 0.2 µm filter and store at 4 °C. Warm up in a water bath if necessary.

4. 1 M L-(+)-lactic acid

Reagent	Final concentration	Quantity or volume
L-(+)-lactic acid	1 M	90 mg
ddH_2O	n/a	Up to 1 mL

Filter sterilize using a 0.2 µm filter. Store at -20 °C.

5. 10 mM L-(+)-lactic acid

Reagent	Final concentration	Quantity or volume
1 M L-(+)-lactic acid	10 mM	10 µL
Autoclaved ddH_2O	n/a	990 µL
Total	n/a	1 mL

Prepare in a sterile condition. Store at -20 °C.

6. Sperm preservation medium

Reagent	Final concentration	Quantity or volume
L-15 medium	n/a	727.5 µL
Autoclaved ddH_2O	n/a	100 µL
5% BSA (10×)	0.5%	100 µL
Penicillin-Streptomycin, 5,000 U/mL	50 U/mL	10 µL
1 M HEPES, pH 7.9	10 mM	10 µL
FBS	3%	30 µL
2 M glucose	25 mM	12.5 µL
10 mM L-(+)-lactic acid	0.1 mM	10 µL
Total	n/a	1 mL

Prepare on the day of sperm collection. Assemble all components in a sterile 1.5 mL tube on a clean bench. Ensure that the medium is prepared under sterile conditions to prevent contamination. The use of penicillin–streptomycin, which is commonly employed in cell culture media, is recommended as an antibiotic. Keep at room temperature (RT) until use.

7. 500× tricaine

Reagent	Final concentration	Quantity or volume
Tricaine	10%	5.0 g
ddH_2O	n/a	Up to 50 mL

Store as 1 mL aliquots at -20 °C.

8. 1× tricaine

Reagent	Final concentration	Quantity or volume
500× Tricaine	0.02%	200 µL
Fresh fish water	n/a	100 mL

Prepare in a 100 mL plastic beaker on the day of use. Mix by agitating the cup gently.

9. 50× E3 stock solution for zebrafish

Reagent	Final concentration	Quantity or volume
NaCl	250 mM	14.6 g
KCl	8.5 mM	0.63 g
$CaCl_2·2H_2O$	16.5 mM	2.43 g
$MgSO_4·7H_2O$	16.5 mM	4.07 g
1 M NaOH	n/a	to pH 7.2
dH_2O	n/a	Up to 1 L

Dissolve NaCl, KCl, $CaCl_2·2H_2O$, and $MgSO_4·7H_2O$ in ~800 mL of dH_2O. Adjust pH to 7.2 with 1 M NaOH. Autoclave and store at 4 °C.

10. 1× E3 for zebrafish

Reagent	Final concentration	Quantity or volume
NaCl	5 mM	20 mL of 50× E3 stock solution
KCl	0.17 mM	
$CaCl_2·2H_2O$	0.33 mM	
$MgSO_4·7H_2O$	0.33 mM	
0.82% Methylene blue solution	0.82 ppm	100 µL
dH_2O	n/a	Up to 1 L

Store at RT. Stable for a week.

11. 20× solution A for medaka

Reagent	Final concentration	Quantity or volume
NaCl	2.2 M	130 g
KCl	107 mM	8.0 g
$CaCl_2·2H_2O$	27.2 mM	4.0 g
$MgSO_4·7H_2O$	16.2 mM	4.0 g
dH_2O	n/a	Up to 1 L

Dissolve NaCl, KCl, $CaCl_2·2H_2O$, and $MgSO_4·7H_2O$ in 1 L of dH_2O. Autoclave and store at 4 °C.

12. Solution B for medaka

Reagent	Final concentration	Quantity or volume
$NaHCO_3$	600 mM (5%)	2.5 g
ddH_2O	n/a	Up to 50 mL

Add 2.5 g of $NaHCO_3$ to ~40 mL of ddH_2O in a 50 mL conical tube. Gently mix by rotating the tube, then adjust the volume to 50 mL with ddH_2O. Filter sterilize using a 0.2 µm filter.

13. Balanced salt solution (BSS) for medaka

Reagent	Final concentration	Quantity or volume
NaCl	111 mM	50 mL of 20× solution A
KCl	5.4 mM	
$CaCl_2·2H_2O$	1.4 mM	
$MgSO_4·7H_2O$	0.8 mM	
dH_2O	n/a	Up to 1 L
$NaHCO_3$	1.2 mM (0.01%)	2 mL of solution B

Dilute 20× solution A 20-fold with dH_2O, autoclave, and adjust pH to 8.3 by adding solution B. Store at 4 °C.

Laboratory supplies

1. 1.5 mL tube (Watson, catalog number: 131-7155C)
2. Filtration disc with pore size of 0.2 μm (Advantec, catalog number: DISMIC-25CS)
3. 50 mL syringe, slip tip (Terumo, catalog number: SS-50ESZ)
4. 0.6 mL microtube (Watson, catalog number: 130-806C)
5. 1 mL syringe, slip tip (Terumo, catalog number: SS-01T)
6. 5 mL syringe, slip tip (Terumo, catalog number: SS-05SZ)
7. 50 mL syringe, slip tip (Terumo, catalog number: SS-50ESZ)
8. 10 μL tip (Watson, catalog number: 110-207C)
9. 200 μL tip (Watson, catalog number: 110-705C)
10. 1,000 μL tip (Watson, catalog number: 110-706C)
11. 90-mm Petri dish (BMBio, catalog number: BME-SNS0002)
12. Cellulose sponge (Nihon Insole Industry, no catalog number)
13. 100 mL plastic beaker (As One Corp., catalog number: I-100)
14. Plastic container
15. Membrane forceps (Advantec, catalog number: FS-1)
16. Cell counter plate, Burker-Turk (Watson, catalog number: 177-212C)
17. Microscissors (Roboz, model: RS-5803)
18. Tweezers, no. 5 Inox 08 (Dumont, model: 0108-5-PO)
19. Homogenizer pestle for 1.5 mL tube (BIO-BIK, catalog number: 1005-39)
20. Kimwipe (Nippon Paper Crecia, catalog number: S-200)
21. Paper towel (Konya Paper, catalog number: 4952137011014)
22. 24-well plate (Falcon®, catalog number: 351147)
23. 60-mm Petri dish (Iwaki, catalog number: 1010-060)

Equipment

1. Stereomicroscope with epi-illumination (Olympus, model: SZ61-SZ2-ILST)
2. Stereomicroscope with trans-illumination (Evident, model: SZ61-SZX2-ILTS)
3. Inverted or upright microscope equipped with a 40× objective lens (Nikon, model: Eclipse TE2000-S, or equivalent)
4. Incubator (PHCbi, model: MIR-154-PJ)
5. Timer (any)

Procedure

A. Zebrafish: Sperm collection from anesthetized males

1. Preparation of male fish

One day before sperm collection, isolate adult males into a separate container outside of the housing system.

Note: Healthy zebrafish aged 5–12 months post-fertilization (mpf) are ideal for the best outcomes. Males with a reddish body color are suitable for sperm collection. Prepare as many males as possible, as not all males release sperm.

Critical: Do not feed the fish from the afternoon of the previous day until the time of sperm collection.

2. Preparation of the sperm preservation medium
a. Prepare the sperm preservation medium under a clean bench (see Recipe 6). Aliquot 40 µL of the sperm preservation medium into the required number of sterile 1.5 mL tubes. For example, if you plan to collect from two males, prepare two tubes.
b. Keep the sperm preservation medium at RT (~25 °C) until use.
c. Arrange the bench with all required equipment, as shown in Figure 1.

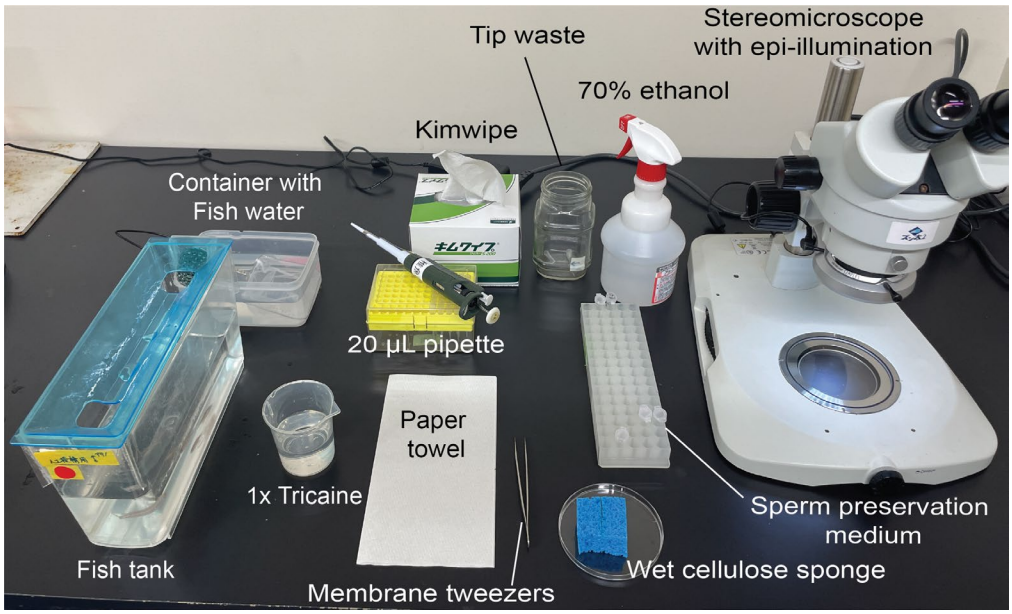


Figure 1. Preparation for sperm collection from zebrafish

3. Sperm collection
a. Transfer a male fish in 1× tricaine (see Recipe 8). Anesthetize until the fish stops swimming and the gill movements slow down.
b. Place the fish on a paper towel using a membrane forceps and remove excess water from the fish body (Video 1). This step is important to avoid contamination.

Video 1. Preparation of male zebrafish for sperm collection. Removal of excess water from an anesthetized male zebrafish, followed by disinfection of the cloaca with a Kimwipe moistened with 70% ethanol.

c. Place the fish with the ventral side up on a cellulose sponge pre-wetted with fish water. Use a slit-cut cellulose sponge (Video 1).
d. Wipe the cloaca with a Kimwipe moistened with 70% ethanol (Figure 2) by gently moving the pelvic fins aside (Video 1).

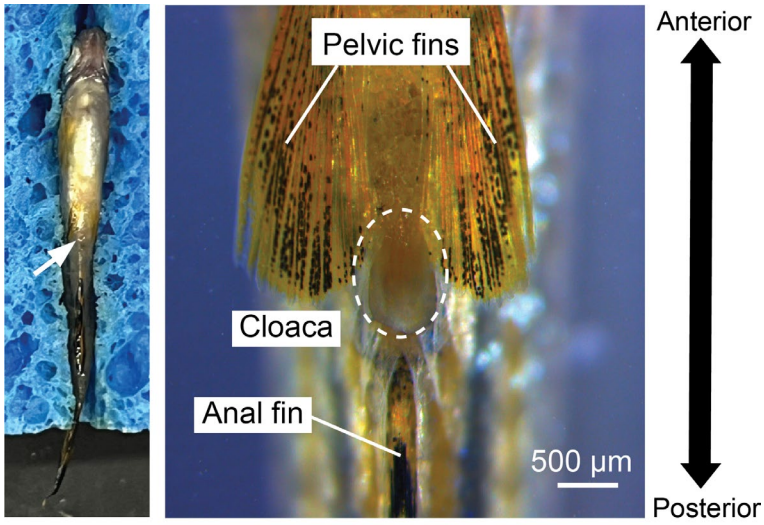


Figure 2. Sperm collection from a zebrafish male. The position of the cloaca is shown on an image of the whole zebrafish male body (left, white arrow) and on a magnified image (right). The tip of a 20 µL tip must be placed on the cloaca (circled with a broken line).

e. Set a pipette to 20 µL and place the tip of a 20 µL pipette in the cloaca. Aspirate sperm by gentle abdominal massage (Video 2). Sperm can be pipetted as a few microliters of white liquid. It is important to use a narrow-bore 200 µL pipette tip for efficient sperm collection. If the tip is too wide, it may not effectively aspirate sperm from the cloaca.
Note: Not all males will release sperm. If no sperm is obtained after 2 or 3 attempts, switch to another fish. Do not squeeze or apply excessive pressure to force sperm out.

Video 2. Sperm collection from a zebrafish male. Aspirating sperm from the cloaca of male zebrafish using a 20 µL pipette.

f. Gently suspend the collected sperm in 40 µL of sperm preservation medium (sperm suspension), confirming that the solution turns cloudy.
g. Repeat steps A3a–f until sufficient sperm is obtained. Sperm from several males of the same genotype may be pooled in the same aliquot. See step C1 for sperm counting and the expected amount of sperm.
Note: The numbers of required males depend on the sperm volume and concentration obtained from each male.

B. Zebrafish: Sperm collection from dissected testes

Note: If sufficient sperm cannot be obtained using the methodology from section A, sperm can be collected from dissected testes using the following protocol, adapted from [11]. Before dissection, prepare male fish and the sperm preservation medium as described in steps A1–2, but aliquot 100 µL (instead of 40 µL) of the medium into each 1.5 mL tube.

1. Dissection of testes
a. Euthanize a male in 1× tricaine.
b. Place the fish on a paper towel using membrane forceps and gently blot excess water from the body.
c. Spray 70% ethanol onto the body surface and position the fish ventral side up on a cellulose sponge pre-wetted with water.
d. Using microscissors, make a V-shaped incision in the ventral skin starting just above the cloaca and extending cranially toward the pectoral fins (Figure 3A). Lift and fold the incised skin cranially to expose the abdominal cavity.
e. Cut the intestine just above the cloaca and move it cranially to expose the testes (Figure 3B).
f. Grasp and remove the swim bladder with tweezers.
g. Sterilize tweezers by spraying with 70% ethanol. Using the tweezers, pinch both testes near the base of the cloaca (Figure 3B, arrow). While lifting them cranially, carefully detach the testes from the surrounding fat and connective tissue.

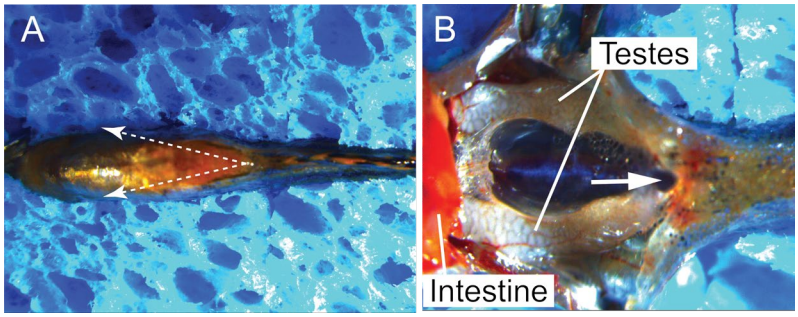
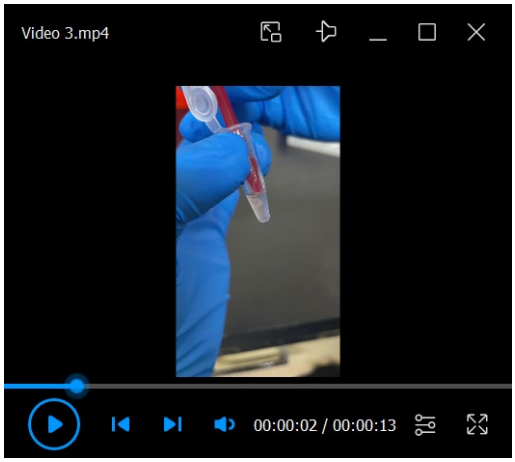


Figure 3. Dissection of zebrafish testes. (A) A euthanized zebrafish male placed on a cellulose sponge. The dashed lines indicate the cutting sites. (B) A pair of testes exposed after the removal of the intestine. The arrow indicates the point to grasp with tweezers when removing the testes.

2. Extraction of sperm from testes
a. Place the testes into a 1.5 mL tube containing 100 µL of sperm preservation solution. Gently squeeze the testes using a homogenizer pestle to release sperm (Video 3).
Caution: Avoid over-homogenization, as excessive somatic cell contamination may deplete components of the preservation medium and mechanically shear the flagella, thereby reducing sperm viability.
b. Transfer the cloudy sperm-containing supernatant to a new sterile tube using a pipette, avoiding tissue debris.



Video 3. Sperm extraction from dissected testes. Squeezing gently a pair of testes in 100 µL of sperm preservation medium.

C. Zebrafish: Sperm storage

1. Sperm counting

a. Mix the sperm suspension by gently tapping the tube. Pipette 1 µL of the suspension and mix with 9 µL of fresh sperm preservation medium by pipetting and tapping. Apply 6 µL of the 1:10 diluted sperm suspension to a cell counter plate. Count the number of sperm cells using an inverted or upright microscope equipped with a 40× objective lens. Sperm yield differs among individuals, ranging from approximately 6×10^5 to 5×10^6 per male when using the method described in section A (Table 1).

Table 1. Sperm yield per male. Sperm amounts obtained from single adult males are shown in ascending order. Sperm collection and counting were performed as described in section A and step C1, respectively.

Male ID	Total sperm amount per male	Mean	SD
1	6.2×10^5		
2	6.6×10^5		
3	7.2×10^5		
4	1.2×10^6		
5	1.6×10^6	2.2×10^6	1.6×10^6
6	1.6×10^6		
7	2.7×10^6		
8	3.2×10^6		
9	4.0×10^6		
10	5.4×10^6		

b. Based on the count, adjust the concentration of the sperm suspension to approximately 7.5×10^4 sperm/µL. Higher concentrations are acceptable but should not exceed 1.5×10^5 sperm/µL.

2. Sperm storage

a. Prepare 20 µL aliquots of sperm suspension in sterile 0.6 mL microtubes and keep the tubes at room temperature. Additionally, prepare 20 µL of fresh sperm preservation medium in a separate 0.6 mL tube as a control for contamination.

Note: When contamination occurs, the medium color turns yellow over time. Do not use contaminated sperm suspension for in vitro fertilization. Sperm activity after preservation depends on temperature, storage duration, and sperm concentration. In our experience, sperm solutions retained approximately 32% fertilization efficiency even after 28 days of storage at the optimal condition [1]. See General note 3 for the shipment and receipt of sperm samples.

D. Zebrafish: in vitro fertilization (IVF)

1. Preparation of female fish

a. One day before IVF, isolate adult females in a separate container outside of the housing system.

Note: Healthy zebrafish aged 5–12 mpf are ideal for the best outcomes. Females with a visibly fuller abdomen are suitable for egg collection. Prepare as many females as possible, as not all females produce eggs of good quality.

Critical: Do not feed the fish from the afternoon of the previous day until the time of egg collection.

2. IVF with RT-stored sperm

a. Perform IVF in the morning, during the natural spawning period of zebrafish (typically within 2 h after the lights turn on). Set up a bench as shown in Figure 4. Prepare a 1,000-µL pipette fitted with a tip and pre-load it with 800 µL of E3 medium. Set a timer for 90 s.

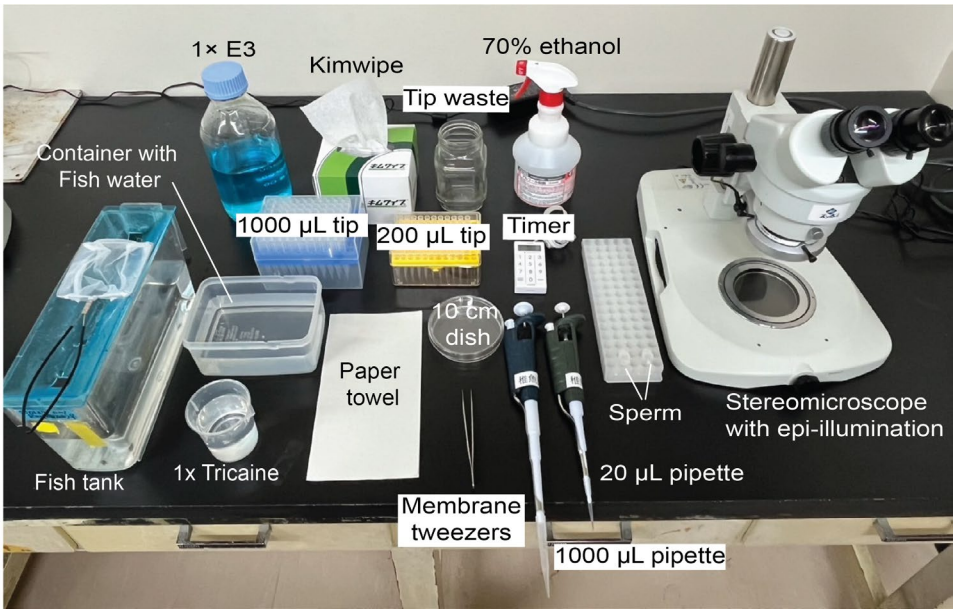
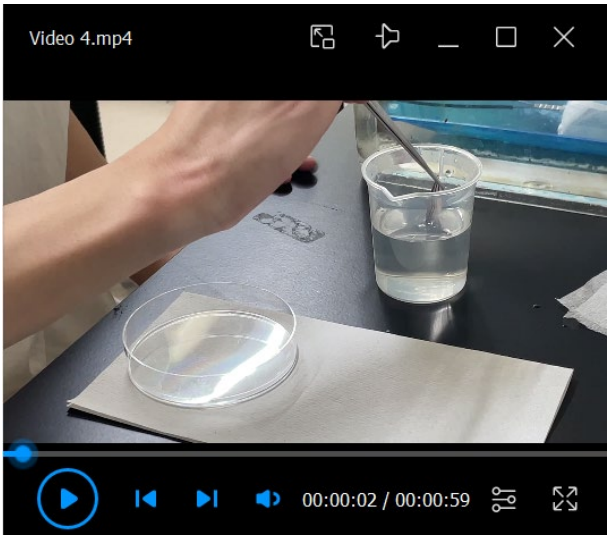


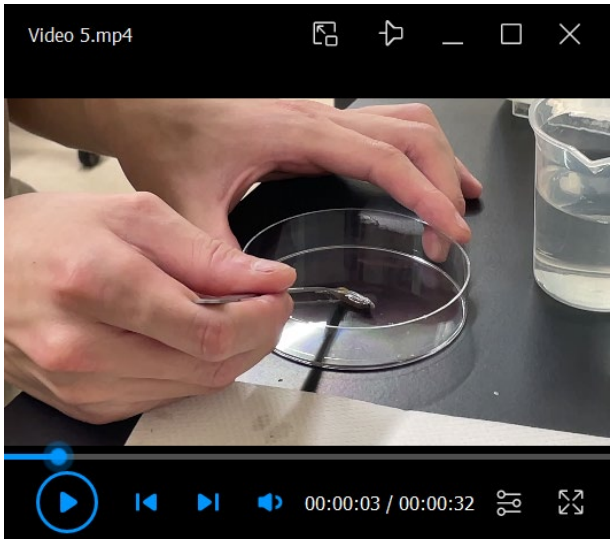
Figure 4. Preparation for in vitro fertilization (IVF) of zebrafish

b. Anesthetize a female fish in 1× tricaine.
c. Remove the fish from the anesthetic solution and gently blot it dry with a Kimwipe, paying particular attention to thoroughly drying the cloaca area (Video 4). Do not apply excessive pressure, as eggs may be unintentionally released during wiping.
Caution: Completely remove surface water. Residual water can prematurely activate eggs and interfere with fertilization. Insufficient drying can reduce fertilization rates to nearly zero.



Video 4. Egg collection from a female zebrafish. Gently remove excess water and squeeze eggs from an anesthetized female zebrafish.

d. Place the female fish on a 90-mm Petri dish and gently press the abdomen with your finger to release the eggs, stroking from the pectoral fins toward the tail (Video 5). If the female is ready to spawn, eggs will be released easily with minimal pressure. If no eggs are released with gentle pressing, do not force it, as excessive pressure may injure the fish. Avoid touching the eggs directly to prevent damage.
Note: Egg quality can be evaluated at this point. Good, viable eggs are uniform in size, yellowish, and translucent (Figure 5A), whereas nonviable eggs are whitish with a ruptured look, and are often released with excessive fluid (Figure 5B, C).



Video 5. In vitro fertilization (IVF) with zebrafish eggs. IVF of zebrafish eggs using sperm suspension.

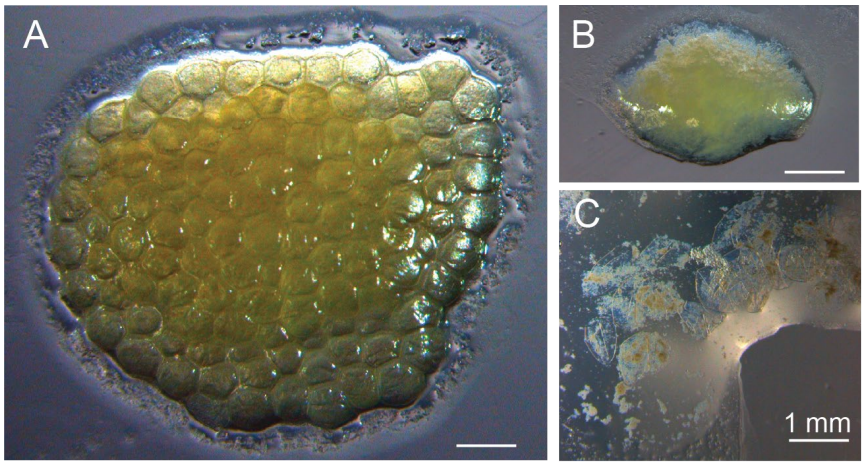


Figure 5. Zebrafish eggs with different qualities. (A) Good and viable eggs with a yellowish, translucent appearance. (B) Poor-quality eggs with a whitish and ruptured appearance. (C) Completely ruptured eggs released with watery fluid. Scale bar = 1 mm.

e. Immediately apply 20 µL of sperm suspension directly onto the eggs.
f. Immediately add 800 µL of 1× E3 for zebrafish to activate the sperm and initiate fertilization. Incubate for 90 s. Gently agitate to disperse the eggs. If the eggs adhere to the plastic dish, use a glass dish instead.
g. Fill the 90-mm Petri dish to approximately half its volume with 1× E3 for zebrafish. Gently agitate to disperse the eggs and ensure they have all settled at the bottom. Under a stereomicroscope with transmitted light, activated eggs can be recognized by chorion expansion, resulting in its detachment from the oocyte (elevation of the fertilization envelope).
h. Incubate the eggs at 28 °C for 4–5 h. In the afternoon, observe the eggs under a stereomicroscope with transmitted light and separate fertilized eggs from unfertilized ones. If required, count the total and fertilized eggs to calculate the fertilization rate.

E. Medaka: Sperm collection from dissected testes

1. Dissection of testes
a. Euthanize a male by immersion in ice-cold water.
b. Place the fish in a 90-mm Petri dish using membrane forceps and remove excess water from the body surface.
c. Using microscissors, make a midline incision from the cloaca to the heart. Sever the vertebral column to ensure euthanasia.
d. While holding the ventral skin with one tweezers, remove the internal organs, including the intestine, liver, and fat body,

using another pair of tweezers.
e. Using tweezers, grasp the anterior mesorchium that connects the testis to the dorsal body wall. Gently peel the testis posteriorly and then pinch it near its base close to the cloaca using microscissors.
f. Place the dissected testis in a 60-mm Petri dish containing BSS. Carefully trim away any adhering connective tissues and fat body using microscissors.
g. For sterilization, place the testis in a 1.5 mL tube containing 0.5% bleach (0.03% sodium hypochlorite) in BSS for 30 s, then rinse twice with fresh BSS to remove residual bleach.

2. Extraction of sperm from testes
a. Place the testis into the lid of a 1.5 mL tube containing 66 µL of sperm preservation medium. Gently tear the testis with tweezers to release sperm.
Caution: Avoid excessive crushing of the testis, as somatic cell contamination may deplete components of the preservation medium and reduce sperm viability.

F. Medaka: sperm storage

1. Sperm counting
a. Pipette 1 µL of sperm suspension and mix with 9 µL of fresh sperm preservation medium by gently pipetting and tapping. Apply 6 µL of the 1:10 diluted sperm suspension to a cell counter plate and count the number of sperm cells.
b. Based on the count, dilute the sperm suspension to approximately 0.45×10^3 sperm/µL. The working concentration can range from 0.20 to 1.0×10^3 sperm/µL.

2. Sperm storage
a. Prepare 100 µL aliquots of the sperm suspension in sterile 1.5 mL tubes. Store the tubes at room temperature (23 °C) or at 4 °C.
Note: When contamination occurs, the medium color turns yellow. Do not use contaminated sperm suspension for IVF. In medaka, sperm storage at 4 °C for one week is more favorable for maintaining fertility compared with storage at room temperature. The concentration of lactic acid in the preservation medium can be increased up to 0.2 mM to suppress somatic cell proliferation.

G. Medaka: in vitro fertilization

1. Preparation of unfertilized eggs
a. In the evening before IVF, place 5–6 wild-type adult females and 2–3 infertile males in the same tank containing 1.5 L of BSS.
Note: Healthy medaka aged 4–6 months post-hatching (mph) are ideal for optimal outcomes. Females with a swollen abdomen are suitable for egg collection. meioC *mutant medaka, in which germ cells arrest at the early stage of spermatogonia, can be used as infertile males [10]. When mutant fish are not available, infertile males can be generated by knockdown of the* dnd1 *and/or* nanos3 *genes during embryogenesis [12]. Even in the absence of infertile males, unfertilized eggs can be obtained by gently pressing the female abdomen with a finger, as described in section D.*
b. In the morning, allow the fish to mate and collect the unfertilized eggs attached to the abdomens of females using tweezers.
c. Place the eggs into a 90-mm Petri dish containing BSS and gently untangle the attachment filaments with tweezers to isolate individual eggs.
Critical: Handle unfertilized eggs very carefully to avoid any mechanical stimulation, as this may trigger egg activation and elevation of the fertilization envelope. After medaka spawn their eggs, it is preferable to collect unfertilized eggs and perform fertilization experiments as soon as possible; however, we have confirmed that a high fertilization rate is maintained within 30 min.

2. IVF with stored sperm
a. Transfer the unfertilized eggs into a 24-well plate, placing 20–30 eggs in each well.
b. Remove the BSS as much as possible and collect the eggs on one side of the well.
c. Apply 100 µL of sperm suspension directly onto the eggs and incubate at room temperature for 15 min.
d. Remove sperm suspension and fill the well to approximately half its volume with BSS. Gently agitate to disperse the eggs and ensure that they have all settled at the bottom. Under a stereomicroscope with transmitted light, activated eggs can be identified by the elevation of the fertilization envelope.

e. Incubate the eggs at 28 °C for 4–5 h. In the afternoon, observe the eggs under a stereomicroscope with transmitted light, and separate fertilized from unfertilized ones. If needed, count the total and fertilized eggs to calculate the fertilization rate.

Validation of protocol

This protocol has been used and validated in the following research article:
• Takemoto et al. [1]. In Vitro Storage of Functional Sperm at Room Temperature in Zebrafish and Medaka. *Zebrafish* (Figure 2D for zebrafish and Figure 4 for medaka).

In the original paper, we examined various sperm storage conditions, including the medium components, temperature, and storage duration. The optimal result was obtained with the medium components described in this protocol. When zebrafish sperm samples were stored at 23 °C, the fertilization ratio was 92.2% ± 3.3% (mean ± SD) after 7 days, 70.0% ± 23.2% after 18 days, and 32.8% ± 5.0% after 28 days of storage. In medaka, sperm storage at 4 °C showed the best fertilization ratio of 97.5% ± 2.5% after 8 days, compared to 45.5% ± 29.2% after 7 days at 23 °C.

We successfully obtained fertilized zebrafish eggs by IVF after 5–10 days of international shipments (Japan to Israel, Japan to the United States, and Israel to Japan) of sperm samples using the method described here. In these cases, fertilization rates ranged from approximately 12% to 82% (mean ± SD = 44% ± 23%), yielding sufficient numbers of fertilized eggs for subsequent maintenance of the mutant lines.

General notes and troubleshooting

General notes

1. Preparation of solutions
In this protocol, dH_2O stands for single-distilled water, whereas ddH_2O stands for double-distilled water. ddH_2O is used for stock solutions for the sperm preservation medium, and dH_2O is used for solutions related to embryo care.

2. Prevention of suffocation
When blotting off excess water from a fish body, avoid touching the gill area. A significant amount of water remains within the gills, and if this is absorbed with a paper towel, there is a high risk of suffocation or impaired recovery. It is sufficient to remove moisture from the area posterior to the pectoral fin. If necessary, revival may be possible using artificial respiration and cardiac massage, as described in Troubleshooting 1.

3. Shipment and receipt of sperm samples
Upon shipment, seal the 0.6 mL tubes containing the sperm solution with Parafilm to ensure secure closure. The tubes can be enclosed in a small box, a conical tube, or wrapped in bubble wrap for protection before being placed in an envelope. After receiving the sperm samples, it is recommended to keep them at 23–28 °C until IVF. If necessary, centrifuge the sperm solution at low speed (no more than 240× *g*) to correct the liquid at the bottom of the tube. We do not recommend testing sperm quality in order to avoid the loss of sperm samples, as using a larger amount of sperm generally results in better IVF outcomes. If necessary, sperm viability can be estimated by observing sperm motility immediately after adding E3 (for zebrafish) or BSS (for medaka). Under an inverted microscope, intact sperm are observed swimming actively.

Troubleshooting

1. Fish fails to recover from anesthesia
If the fish does not regain consciousness after anesthesia, perform artificial respiration. Unconscious fish are typically found upside down; in such cases, place a plastic pipette near the lower jaw or just behind the gills and gently pipette water repeatedly. Alternatively, grasp the caudal fin with membrane tweezers and slowly move the fish back and forth in the water. This promotes water flow through the gills and increases the chance of recovery. Be aware that if too much water has been removed from the gill area, the operculum (gill cover) may fail to open properly. As noted earlier, avoid touching the gills when wiping excess water from the body.

2. Contamination of sperm suspension
If feces are present at the cloaca before wiping off water and collecting sperm, remove them with a Kimwipe. After removing the feces and blotting excess water from the body, wipe the cloacal area with a Kimwipe moistened with 70% ethanol to ensure clean sperm collection. Avoid excessive wiping as it may damage the fish. It is recommended to withhold feeding from the afternoon of the previous day. It is recommended to prepare sperm preservation medium and to dilute collected sperm in a clean bench to prevent contamination of the sperm suspension.

3. Sperm concentration is too low
As mentioned above, sperm can be collected from several males and pooled into a single 20 µL aliquot. If the sperm suspension needs to be concentrated, centrifuge at 240× *g* for 10 min. If sperm quantity or concentration is known to be low, using half the volume of 1× E3 (400 µL) for zebrafish at step D2f may improve fertilization efficiency.

4. Female does not spawn
Females typically spawn every 2–4 days. Identify females likely to spawn by selecting those with a swollen lower abdomen when viewed from above the evening before IVF. Separate these females from males the day before the fertilization. On the day of fertilization, place the female together with a male and observe spawning behavior to confirm that she is ready to spawn. If the female is separated immediately after the first egg is spawned, she is likely to continue releasing eggs.

5. Poor quality of eggs
Perform IVF within 2 h after the lights turn on, as egg quality declines thereafter. When females are kept separated from males for extended periods, their eggs may become overripe, resulting in poor quality. Natural mating or gentle squeezing can help expel overripe eggs, following the same steps described in steps D2b–d. After squeezing, allow at least one week before reusing these females for IVF experiments.

6. Low fertility rates after IVF
Low fertility rates can result from poor-quality sperm or inefficient IVF procedures. Poor sperm quality is often due to contamination (see Troubleshooting 2), low sperm concentration (see Troubleshooting 3), or flagellar breakage caused by rough handling. Avoid excessive pipetting of the sperm suspension and use a cut pipette tip to minimize mechanical damage. Make sure to thoroughly remove excess water from the fish to prevent premature activation of sperm or eggs. During IVF, immediately add 1× E3 medium for zebrafish after adding the sperm suspension to activate fertilization.

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Competing interests

The authors declare no competing or financial interests.

Ethical considerations

The use of zebrafish and medaka for experimental purposes was approved by the animal care and use committee of Saitama University (approval number R7-A-1-24), the committee on laboratory animal care and use at the National Institute of Genetics (approval identification numbers 28–13, 29–13, and 30–6), and the committee at Nagoya University (approval number 11).

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