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Efficient Superovulation and Egg Collection from Mice

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Abstract

Superovulation is a method used to reduce the number of mice used per experiment by increasing the egg number. Conventionally, superovulation for obtaining mouse eggs involves the use of equine chorionic gonadotropin (eCG) for stimulation and human CG for induction. Female mice of the C57BL/6 inbred strain spontaneously ovulate approximately 10 eggs. The average number of eggs ovulated using the conventional superovulation method is approximately twice as high as that obtained by spontaneous ovulation. Here, we describe the conventional and non-conventional methods of intraperitoneal injection of superovulation reagents in mice and subsequent egg collection. The non-conventional superovulation method combining anti-inhibin serum (AIS) plus eCG for stimulation is more efficient than conventional superovulation. Appropriate intervals from each injection to sampling induce large numbers of high-quality eggs. Immediately after ovulation, eggs are surrounded by cumulus cells, forming an egg-cumulus complex. These cumulus cells are then removed from the egg-cumulus complex by treatment with hyaluronidase to obtain the exact number of eggs. This protocol is suitable for further manipulations such as intracytoplasmic sperm injection and cryopreservation of eggs, as well as for the analyses of responsivity to superovulation reagents in genetically modified mice obtained by genome editing.

Keywords: Mouse, Egg, Oocyte, Cumulus cells, Superovulation, Anti-inhibin serum, Chorionic Gonadotropin, Hyaluronidase

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Background

Superovulation, involving the stimulation of follicle development and induction of ovulation, is used to increase the number of eggs in animals, including mice. Conventionally, superovulation of mice involves the use of equine chorionic gonadotropin (eCG) that has a follicle-stimulating hormone (FSH)-like activity and human CG (hCG) for stimulation and induction, respectively. Anti-inhibin serum (AIS) neutralizes the function of inhibin, regulates FSH secretion, and increases the number of ovulated eggs (Wang *et al*., 2001). Thus, an efficient superovulation method involving the combined use of AIS with eCG for stimulation, increases the number and improves the quality of ovulated eggs, and reduces the number of mice used in animal experiments compared with the conventional superovulation method (Takeo and Nakagata, 2015). Furthermore, we and other researchers have compared the responsivity of multiple mouse strains to these superovulation reagents (Takeo and Nakagata, 2015; Shindo *et al*., 2021). Eggs obtained from superovulation reagent-treated female mice are used for subsequent applications such as intracytoplasmic sperm injection (ICSI) and cryopreservation of eggs. Considering that the demand for mouse egg manipulation will continue increasing to produce mouse models that reproduce human diseases and to perform *in vivo* functional studies using genome editing techniques in the future, as well as the application for *in vitro* fertilization, we describe an efficient protocol for the superovulation of mice and subsequent egg collection.

Materials and Reagents

1. Glass pipettes (Drummond Scientific Company, MICROCAPS®, catalog number: 1-000-0500)
2. 1.5 mL microcentrifuge tubes (WATSON, catalog number: 131-815C)
3. 50 mL tubes (Greiner, catalog number: 227261)
4. 10 mL plastic pipettes (FALCON, catalog number: 357551)
5. 1,000 µL pipette tips (WATSON, catalog number: 110-7-6C)
6. 200 µL pipette tips (WATSON, catalog number: 110-705C)
7. 10 µL pipette tips (WATSON, catalog number: 110-207C)
8. Kimwipes (NIPPON PAPER CRECIA Co., Ltd., catalog number: 62020)
9. Paper towels (ASKUL, catalog number: 1944368)
10. 35 mm dishes (IWAKI, catalog number: 1000-035)
11. 60 mm dishes (CORNING, catalog number: 351007)
12. Syringes with needles (Terumo Corporation, 1 mL syringe with 26-gauge 1/2-inch needle, catalog number: SS-01T2613S)
13. Disposable gloves (AXEL, catalog number: 61-7347-30)
14. C57BL/6N and C57BL/6J female mice (Japan SLC, Inc.): 4–6 and ≥10 weeks of age (excluding 7–9 weeks of age because they are unsuitable for superovulation)
15. eCG (ASKA Animal Health Co., Ltd., SEROTROPIN®, 1,000 IU × 10 vials, catalog number: No application), store at 4°C
16. AIS plus eCG (Kyudo Co., Ltd., CARD HyperOva®, 1 mL, catalog number: F021), store at -20°C
17. hCG (ASKA Animal Health Co., Ltd., Gonadotropin for animal, 3,000 IU × 5 vials, catalog number: No application), store at 4°C
18. Saline (Otsuka Pharmaceutical Co., Ltd., 20 mL × 50 vials, catalog number: Not applicable): Store at room temperature
19. CARD mHTF medium (Kyudo Co., Ltd., 2 mL, catalog number: GA017), store at 4°C
20. M2 medium (Merck, EmbryoMax® M2 Medium, catalog number: MR-015-D), store at -20°C
21. Hyaluronidase (Sigma-Aldrich, catalog number: H3506-100MG), store at -20°C
22. Liquid paraffin (Nacalai Tesque, Inc., Specially prepared reagent, catalog number: 26137-85): Store at room temperature and in the dark, away from sunlight
23. 70% ethanol (Yoshida Pharmaceutical Company, Ecosyoueta disinfectant solution, catalog number: 14987288980046)
24. Aluminum foil (AXEL, catalog number: 6-713-01)

Equipment

1. Protective equipment (*e.g.*, masks, goggles, and lab coats)
2. CO_2 incubator (ASTEC Co., Ltd., model: SCA-165DS)
3. Stereo microscope (Nikon, model: SMZ645)
4. Dry-heat sterilizer (Panasonic, model: MOV-212S-PJ)
5. Precision balance (AXEL, model: 1-1726-01)
6. Ampoule cutter (AXEL, model: 5-124-22)
7. Alcohol lamp (AXEL, model: 6-487-01)
8. Pipette controller (FALCON, model: 357469)
9. P-1000 pipette (GILSON, model: F120602)
10. P-200 pipette (GILSON, model: F123601)
11. Micropipette (Eppendorf, model: 4920000024)
12. Mouth pipette (Drummond, model: 2-040-000)
13. Filter (Millipore, model: SLGPR33RS)
14. Ampoule glass cutter (AXEL, model: 5-124-22)
15. Large straight scissors (Natsume Seisakusho Co., Ltd., model: B-3)
16. Small straight scissors (Natsume Seisakusho Co., Ltd., model: B-12)
17. Curved tip tweezers (AXEL, model: 6-531-19)
18. Tweezers (AXEL, model: 2-529-12)
19. Precision tweezers (DUMONT, model: NO.5-INOX)
20. Needle (AXEL, model: 2-013-01)
21. Dispenser for paraffin liquid (Nichiryo Co., Ltd., model: 00-DP-2B)
22. Mechanical tally counter (AXEL, model: 63-1584-34)
23. Plastic cages (Clea Japan, Inc., model: CL-0103-2 Mouse TPX)

Software

1. Microsoft Excel (Microsoft Corporation)

Procedure

A. Animals

Breed the C57BL/6 strain (2–4 mice per cage) under the following specific pathogen-free conditions: at 23 ± 1°C controlled temperature, 12 h light-dark cycles (light on at 8:00 and off at 20:00), and *ad libitum* access to food and water.

B. Preparation of eCG (7.5 IU/100 µL)

1. Open three vials containing eCG powder and two bottles of saline.
2. Add 2 mL of saline into each eCG vial using a P-1000 pipette with a 1,000 µL tip, mix by pipetting, and transfer the solution (a total volume of 6 mL) to a 50 mL tube.
3. Repeat Step B2 twice to collect all the eCG, *i.e.*, dissolve eCG powder (3,000 IU) in 18 mL of saline.
4. Add 22 mL of saline into the 50 mL tube using a pipette controller with a 10 mL plastic pipette to obtain an eCG working solution, *i.e.*, dissolve eCG powder (3,000 IU) in 40 mL of saline.
5. Prepare 1.2 mL aliquots (7.5 IU/100 µL per mouse) in 1.5 mL microcentrifuge tubes and store at -20°C until use, for up to six months.

6. When needed, thaw the eCG solution completely at room temperature and mix well before use.

C. Preparation of hCG (7.5 IU/100 µL)

1. Open a vial containing hCG powder and two bottles of saline.
2. Add 2 mL of saline into the hCG vial using the P-1000 pipette with a new 1,000 µL tip, mix by pipetting, and transfer the solution to a new 50 mL tube.
3. Repeat Step C2 twice to collect all the hCG, *i.e.*, dissolve hCG powder (3,000 IU) in 6 mL of saline.
4. Add 34 mL of saline into the 50 mL tube using the pipette controller with a new 10 mL plastic pipette to obtain the hCG working solution, *i.e.*, dissolve hCG powder (3,000 IU) in 40 mL of saline.
5. Prepare 1.2 mL aliquots (7.5 IU/100 µL per mouse) in 1.5 mL microcentrifuge tubes and store at -20°C until use, for up to six months.
6. When needed, thaw the hCG solution completely at room temperature and mix well before use.

D. Preparation of M2 medium containing hyaluronidase

1. Measure 0.5 mg of hyaluronidase and add to a new 50 mL tube.
2. Thaw M2 medium and add 50 mL to the tube containing hyaluronidase (1% solution).
3. Mix thoroughly by gentle inversion.
4. Prepare 1 mL aliquots in 1.5 mL microcentrifuge tubes and store at -20°C until use.
5. When needed, thaw the solution completely at room temperature and mix well before use.
   Note: Hyaluronidase is sterile but non-filtered because this reagent is adjusted on a clean bench.

E. Preparation of glass pipettes for egg handling (see Video 1)

1. Cut the glass pipettes in the middle using an ampoule glass cutter (Figure 1A).
2. Heat the cut edge of glass pipettes with the flame of an alcohol lamp to smooth.
3. Hold both sides of the glass pipette with one hand and a pair of curved tip tweezers.
4. Heat the middle of the pipette until it is softened slightly (Figure 1B), remove from the flame, and immediately pull both ends horizontally.
5. Cut the excess part of the pipette, heat, and smooth the tip slightly. The smooth tip is less likely to damage the eggs and dishes.
6. Check the tip under a stereomicroscope (Figure 1C) and choose glass pipettes with an inner diameter of approximately 150–200 µm.
7. Wrap the glass pipettes with aluminum foil (Figure 1D), dry-heat sterilize (180°C, 30 min), and store in a new 15 mL tube at room temperature.
   Note: Pipettes with proper inner diameter enable to handle eggs easily. Multiple glass pipettes should be prepared for one experiment to replace the pipette with a new one when operation is not easy (e.g., cumulus cells adhere to the tip).

Video 1. Making glass pipettes.

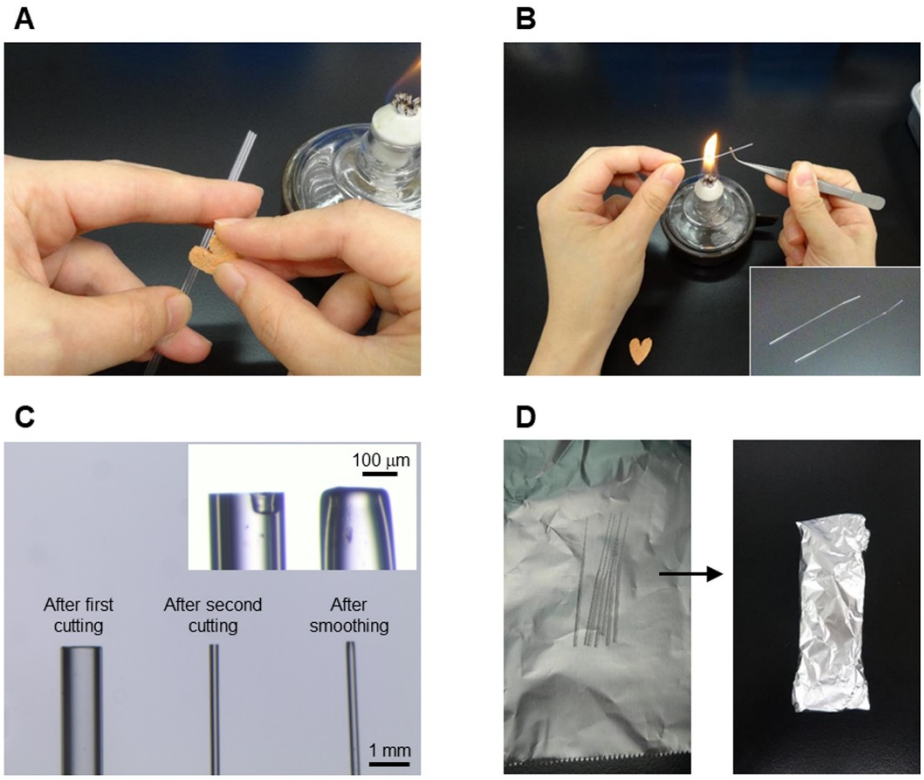

Figure 1. Making glass pipettes.
(A) Cut glass pipettes. (B) Heat and soften a glass pipette. Insert, pulled glass pipettes. (C) Tips of glass pipettes. Insert, an enlarged view of the pipette tips. (D) Wrap glass pipettes.

F. Superovulation

1. Thaw eCG or CARD HyperOva® solutions at room temperature and mix well.

2. Aspirate the required amount of eCG or HyperOva® (100 µL per mouse) with a syringe; for example, aspirate 1 mL of eCG or HyperOva® for 10 female mice (Figure 2A).
3. Hold a mouse and inject 100 µL of eCG or HyperOva® into the peritoneal cavity (Figure 2B and 2C).
4. Return mice to their cages.
5. At 48 h after injecting eCG or HyperOva®, inject 100 µL of hCG in the same manner (Figure 2A, 2B, and 2C).
6. Return mice to their cages.
   Note: Either wrong dosage or inadequate timing of hormone injection reduces the number of normal eggs ovulated.

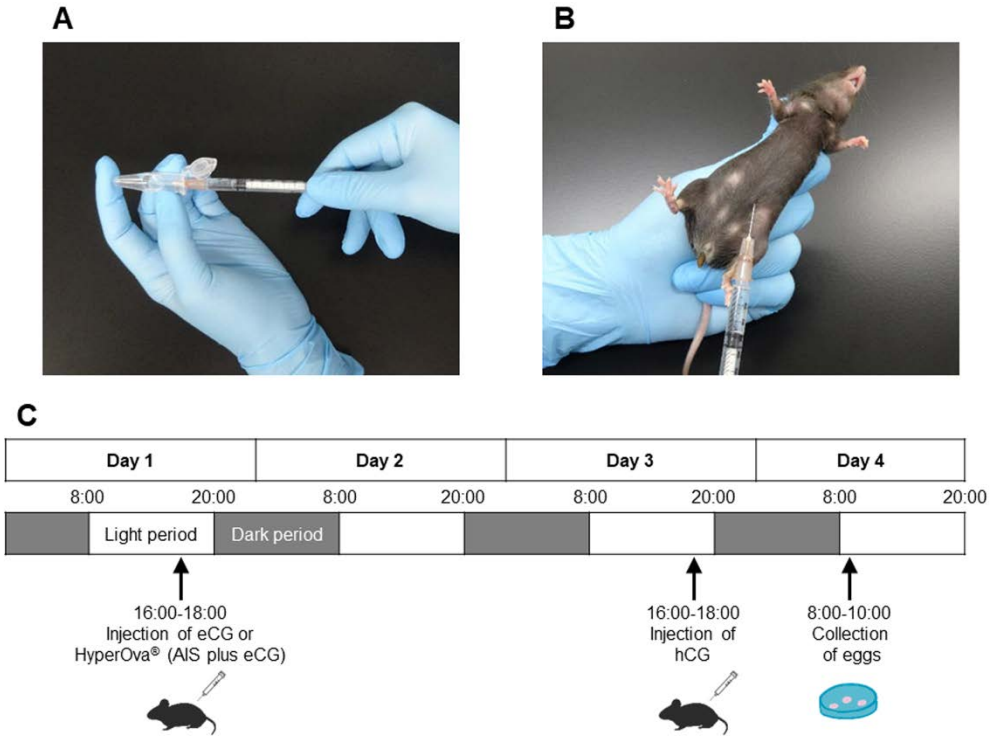

Figure 2. Superovulation procedure and egg collection.
(A) Filling a syringe. (B) Intraperitoneal injection of female mice. (C) Time schedule of superovulation reagent injections and egg collection. AIS, anti-inhibin serum; eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin.

G. Preparation of dishes

1. Add paraffin liquid to an empty 35 mm dish (Figure 3A and 3B).
2. Place four and six CARD mHTF medium drops (100 µL per drop) in two 60 mm dishes and cover these drops with paraffin liquid (Figure 3A, 3C, 3D, and 3E).
3. Incubate these dishes in a CO_2 incubator at 37°C with 5% CO_2 for at least 30 min before collecting the egg-cumulus complex (Figure 3F).
4. Thaw the M2 medium containing hyaluronidase and place four drops (200 µL per drop) in a new 60 mm dish (Figure 3G and 3H).
   Note: Prepare this just before washing the eggs. There is no need to cover M2 medium drops with paraffin liquid and to place them in the CO_2 incubator. As M2 medium is a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-based medium, it can be used for a long time at room temperature outside the CO_2 incubator (Carter et al., 1993).

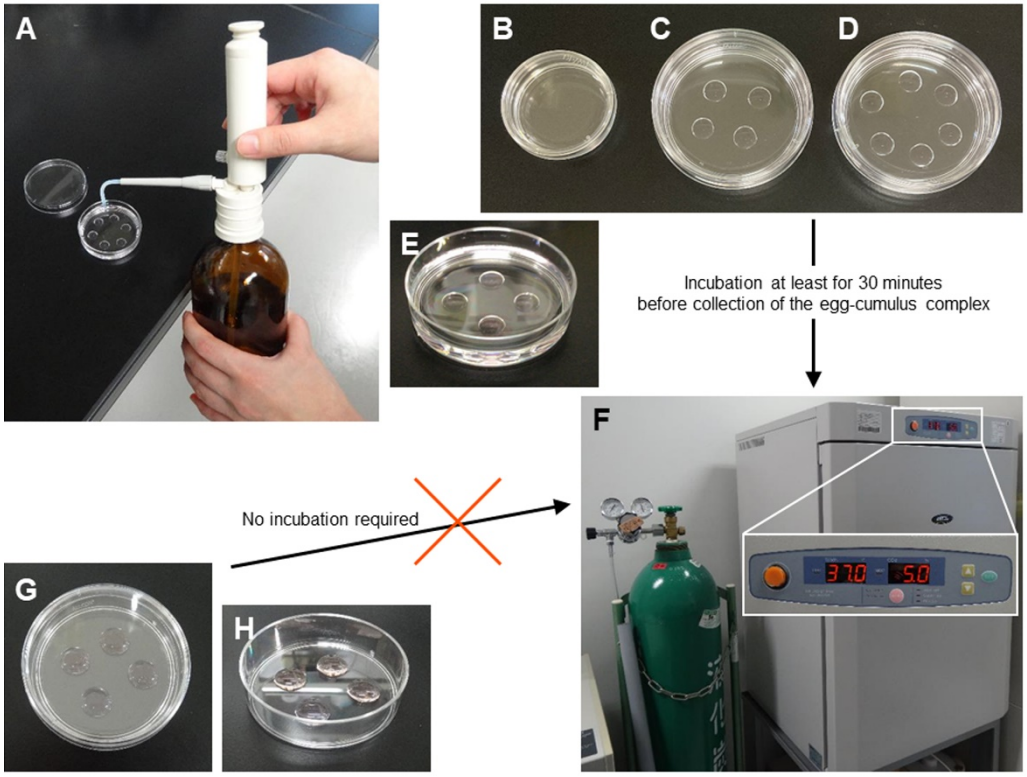

Figure 3. Dishes utilized in this experiment.
(A) Dispensing paraffin liquid. (B) Keeping dish for isolated oviducts. (C, E) Dish for collecting the egg-cumulus complex. (D) Dish for washing eggs. (F) CO_2 incubator. (G, H) Dish for removing cumulus cells from the egg.

H. Collection of the egg-cumulus complex

1. Place scissors and tweezers on the bench (Figure 4A) and wear gloves (and other protective equipment as necessary).
2. At 14–16 h after injecting hCG, sacrifice female mice (Figure 2C).
3. Disinfect the abdomen with 70% ethanol and cut the abdominal skin of mice using large scissors.
4. Pull two sides of the cut skin and access the mouse peritoneum.
5. Cut the peritoneum using small scissors.
6. Locate the V-shaped uterus, oviducts, and ovaries by shifting the position of the gut and internal organs.
7. Hold the utero-tubal junction with a pair of precision tweezers, cut the utero-tubal and isthmic-ampullary junctions, and separate the oviducts from the uterus (Figure 5A and 5B).
8. Clean the oviducts by removing adipose tissue, blood, and tissue fluid, and transfer oviducts to the keeping dish moved from the CO_2 incubator.
   Note: Reduce the exposure time of the collecting dish to room temperature using the keeping dish.
9. Move the collecting dish from the CO_2 incubator to the bench and transfer oviducts to liquid paraffin on the collecting dish using the pair of precision tweezers and place the oviducts on the bottom of this dish.
10. Take out the egg-cumulus complex from the ampulla using a needle under the stereomicroscope (Video 2 and Figure 4B) and add the egg-cumulus complex to one drop of CARD mHTF medium on the collecting dish (Figure 6A and 6B). Superovulation treatment induces an average of 20–50 eggs per female mouse (Shindo *et al*., 2021), and one drop includes <100 eggs.
11. Move the collecting dish to the CO_2 incubator.
12. Repeat Steps H2–H11 for all female mice.
    Note: Prepare the CARD mHTF medium drops corresponding to the number of superovulated mice.

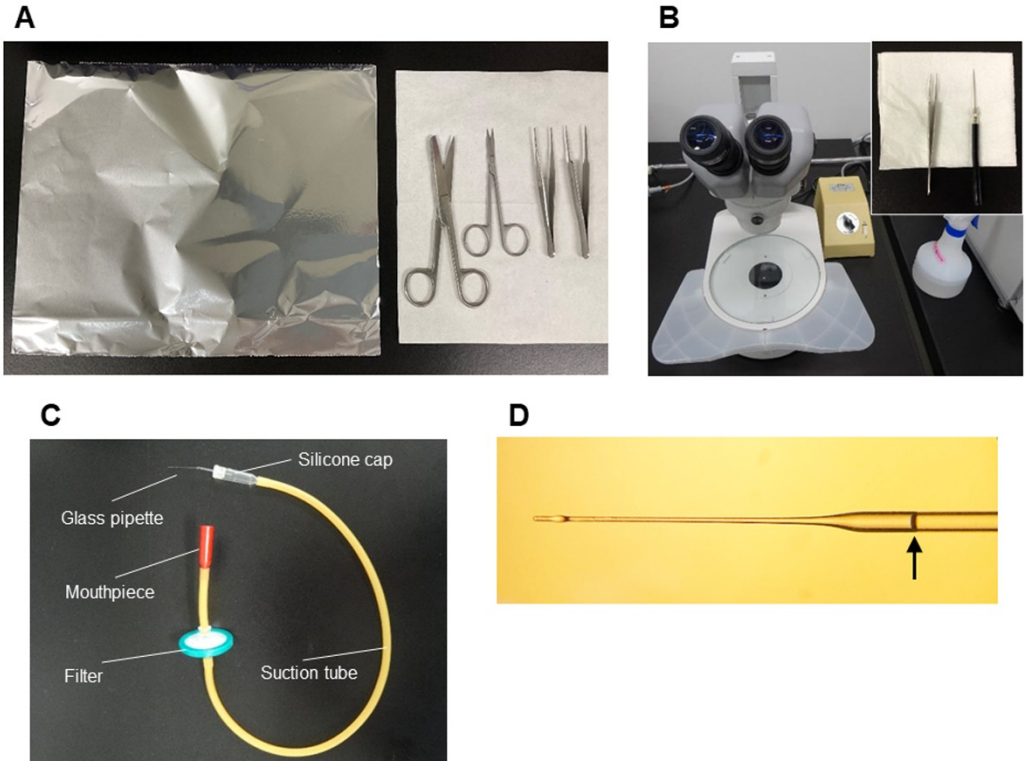

Figure 4. Tools utilized in this experiment.
(A) Anatomical set for abdominal exploration. (B) Stereo microscope. Insert, handling set for the egg-cumulus complex. (C) Mouth pipetting set for egg transfer. (D) Glass pipette with aspirated medium. Arrow, liquid surface of the medium.

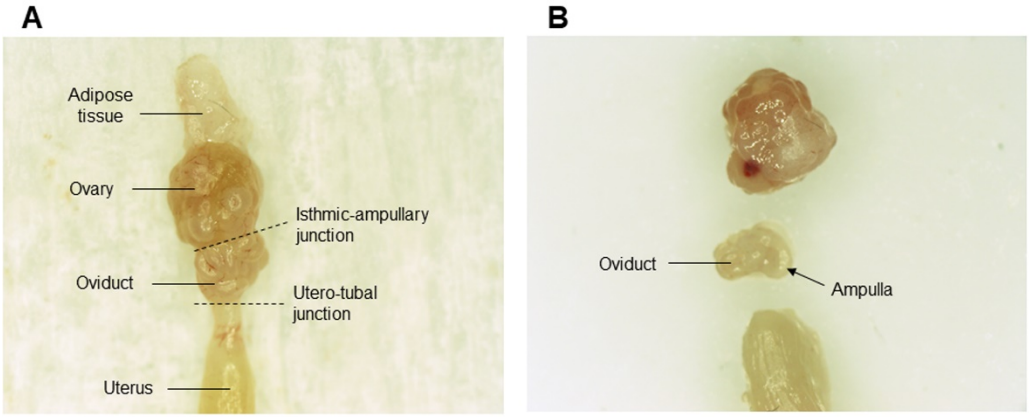

Figure 5. Pictures of ovary, oviduct, and uterus.
(A) Serial view of ovary, oviduct, and uterus. Each junction is represented by dashed lines. (B) Separated view of the oviduct. Arrow indicates the ampulla.

Video 2. Removing the egg-cumulus complex from the ampulla.

I. Removing cumulus cells and counting eggs

1. Move the collecting and washing dishes from the CO_2 incubator to the bench.
2. Aspirate the M2 medium containing hyaluronidase using a mouth pipetting set (Figure 4C), and transfer the egg-cumulus complex from the CARD mHTF medium drop on the collecting dish to the drop of M2 medium with hyaluronidase on the removing dish.
   Note: Aspirate the M2 medium containing hyaluronidase until the wide part of the glass pipette for easy handling of the eggs (Figure 4D).
3. Remove cumulus cells from the egg-cumulus complex by mouth pipetting and transfer the eggs to the drops of CARD mHTF medium on the washing dish (Figure 6C).
4. Wash the eggs by moving them between three drops of CARD mHTF medium on the washing dish.
5. Repeat steps I2–I4 for all egg-cumulus complexes.
6. Count the number of normal eggs per drop using a mechanical tally counter and record the results in Microsoft Excel. Normal and abnormal eggs are easily distinguished by morphology (Figure 6D and 6E).
   Note: If the number of normal eggs is reduced, check the expiration date for solutions used, the timing of superovulation, and whether the age of mice is suitable for superovulation.

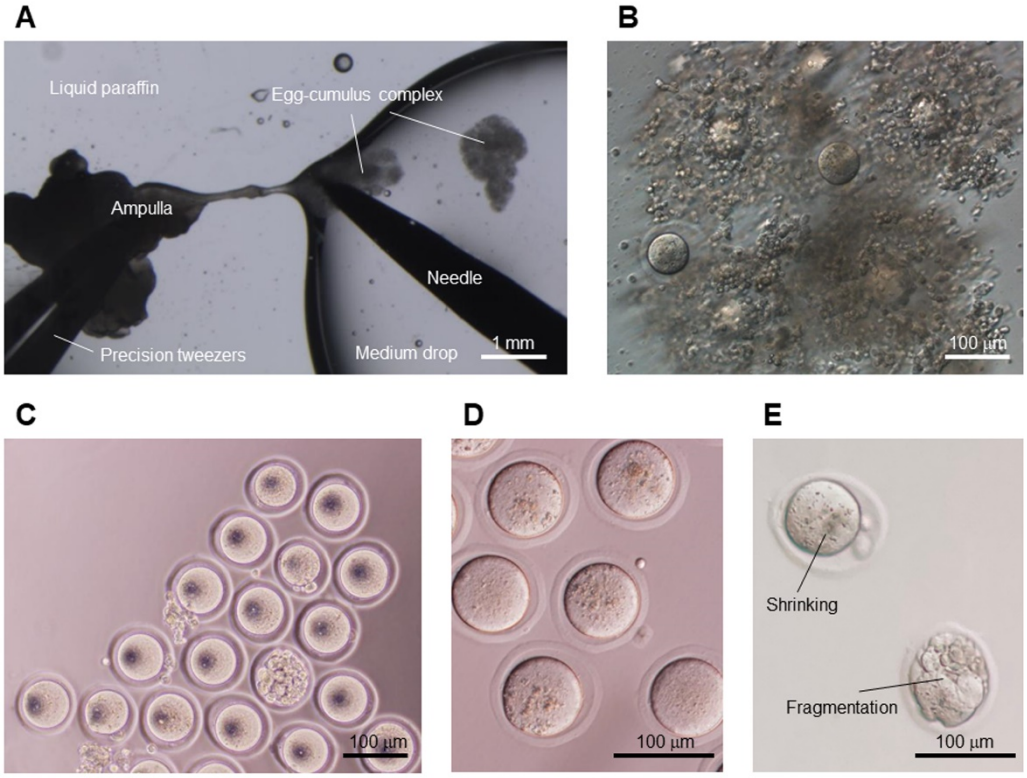

Figure 6. Eggs manipulation.
(A) Removing the egg-cumulus complex from the ampulla. This drop contains 47 eggs. (B) The egg-cumulus complex before hyaluronidase treatment. (C) Eggs after hyaluronidase treatment. (D) Normal eggs. (E) Abnormal eggs.

Data analysis

Perform Student's *t*-test or Mann-Whitney's *U*-test using Microsoft Excel (Shindo *et al*., 2021).

Notes

1. Inject twice the amount (200 µL per mouse) of superovulation reagents into mice with a body weight of 35 g or more.
2. Apply one drop for each mouse if you need to count the number of eggs per mouse.
3. Reduce the exposure time of eggs to room temperature to avoid a reduction in developmental efficiency when performing ICSI.
4. Take pictures with a microscope camera if necessary.

Acknowledgments

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Competing interests

The authors declare no competing interests.

Ethics

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the National Research Institute for Child Health and Development (experimental numbers, A2005-007 and A2020-005).

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