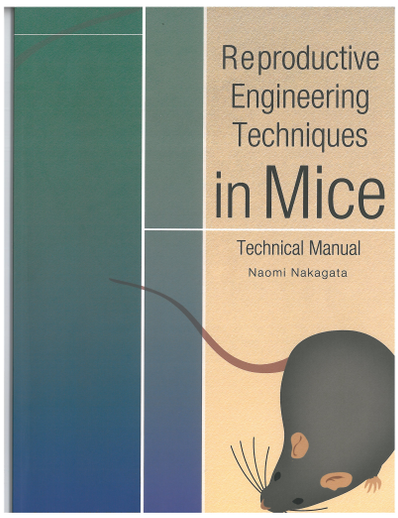
Reproductive Engineering Techniques **in Mice**



Technical Manual
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Preface

In recent years, the number of genetically engineered mice being produced has increased dramatically. Moreover, the rapid progress in the development of genome-editing (TALEN and CRISPR/Cas9) techniques for molecular biology research has been remarkable, so much so that a genetically engineered mouse strain can be produced easily in a few months. Production has been supported by reproductive engineering techniques such as *in vitro* fertilization, embryo and sperm cryopreservation, and embryo transfer techniques. Such techniques have become invaluable peripheral technologies, and their use is expanding rapidly.

This rapid expansion has led to the publication of many technical manuals relating to reproductive engineering techniques in mice hereto. However, mouse reproductive engineering techniques mainly involve delicate operations under a stereoscopic microscope, which means that a sufficiently detailed technical manual has not yet been published.

With that in mind, in this book we aim to create a mouse reproductive engineering technique manual that can be easily understood by anyone. We included a generous number of diagrams, photographs and videos in our manual, and explained each step of each reproductive engineering technique as clearly and thoroughly as we could. We sincerely hope that our manual will become the definitive guide for students, technicians, researchers and other people wishing to study mouse reproductive engineering techniques.

Naomi Nakagata

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6-1 Simple Vitrification of Mouse Embryos

Materials and Equipment

1. 1 M DMSO
2. DAP213
3. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
4. Filter unit (Millex-GV 0.22 µm Cat. No. SLGV013SL; MILLIPORE)
5. Gel loading tip (MBP Gel 200, Cat. No. 3621; Molecular BioProducts)
6. Transfer pipettes
7. Cryotubes (Cryogenic Vials Cat. No. MS-4501W; Sumitomo Bakelite, Japan is recommended. If you cannot get it, use Cat. No. 366656; NUNC.)
8. Micropipette
9. Vial canes
10. Nalgene Labtop Cooler (Cat. No. 5115-0012; NALGENE, USA)
11. Liquid nitrogen
12. Microscope
13. 0.25 M sucrose
14. KSOM/AA
15. Liquid paraffin

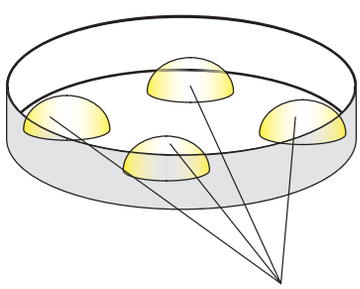
Procedures

Preparation of a Block Cooler and Cryotubes

1. A day before use, place a block cooler (Cat. No. 5115-0012; NALGENE, USA) in a freezer at -20℃.
2. About 10 minutes before commencing the vitrifying procedure, take the block cooler out of the freezer.
3. Stand some cryotubes in the block cooler. About 40 embryos / cryotube are easy to handle; in other words, when you want to vitrify 120 embryos, you need to stand 3 cryotubes in the block cooler.
4. Just before starting the procedure, check the temperature inside of the tubes is at 0℃.

Vitrification

1. Filter the 1 M DMSO and put 4 drops of it (~100 µL / drop) into a dish. One drop is to wash the embryos taken from the collection medium, while the others are to hold the washed embryos.

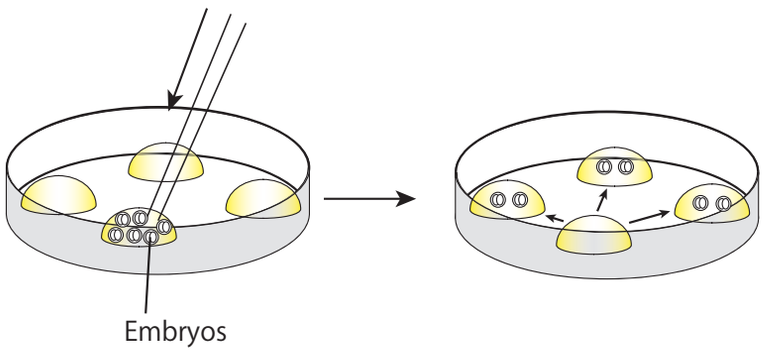


1 M DMSO 100 µL

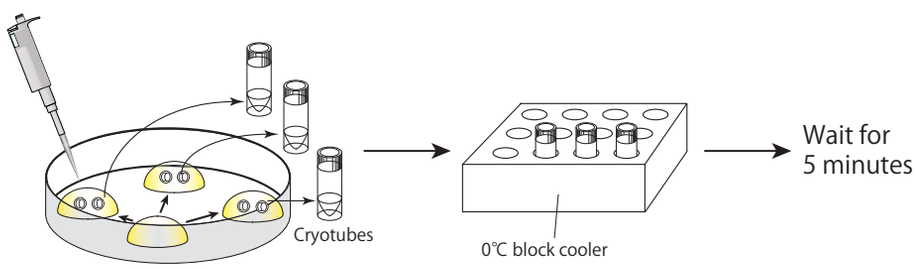
Comment

Crushed ice can be used instead of a block cooler.

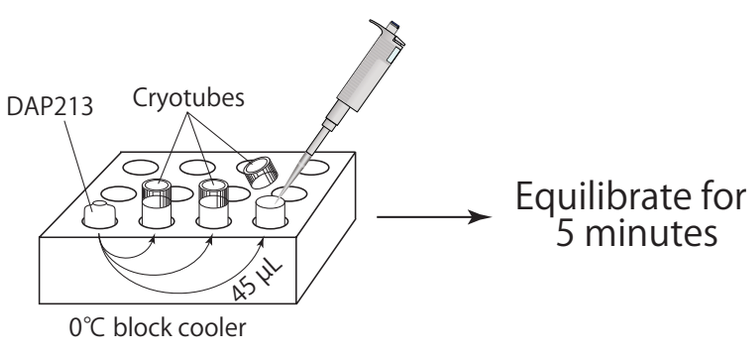
2. Place a group of embryos into one of the 4 drops to rinse them of the collection medium. Divide the rinsed embryos equally between the other drops. These aliquots will eventually be transferred to a storage vial. For example, if one were to collect 120 embryos and vitrify them in 40-embryo aliquots, the embryos would first be placed together in the rinse drop and then divided equally among the three drops.



3. Using a 20 µL pipette and a gel-loading tip, transfer the embryos contained within 5 µL of 1 M DMSO solution into a cryotube. Once transferred, put the cryotube into the block cooler at 0℃ and wait for 5 minutes.



4. Add 45 µL of cryoprotective solution (DAP213) at 0℃ into the cryotube and equilibrate for 5 minutes in the 0℃ block cooler.



Note

It is possible to keep the cryotubes in the block cooler at 0℃ for longer than 5 minutes (<20 minutes).

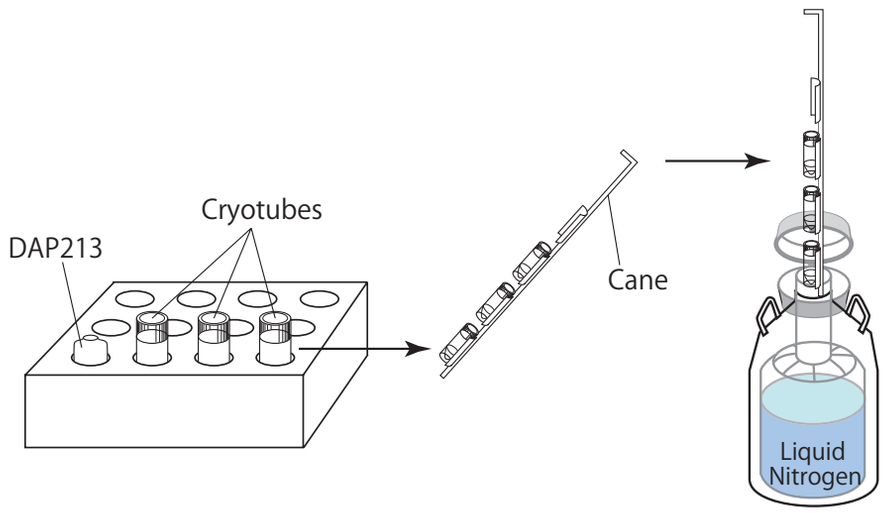
Note

If the embryos are pushed together in the center of the drop, it is easy to suck them all up in 5 µL of the 1 M DMSO solution.

Note

Do not fasten the caps too tightly after adding the DAP213, or they will be too difficult to remove quickly when samples are recovered from the freezer.

5. Quickly set the cryotubes on a cane and plunge the samples directly into liquid nitrogen.

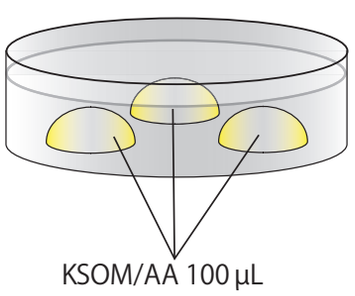


[Vitrifying Embryos] No. 13-01

Preparation for Thawing

1. Put 3 drops (100 µL/drop) of KSOM/AA into a dish and cover them with liquid paraffin. Place the dish in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

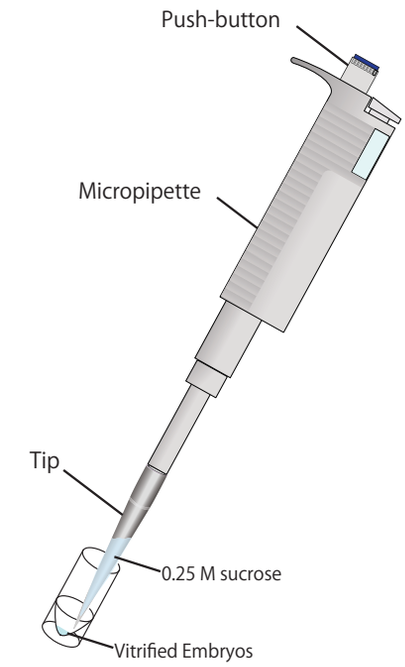
【Washing dish】



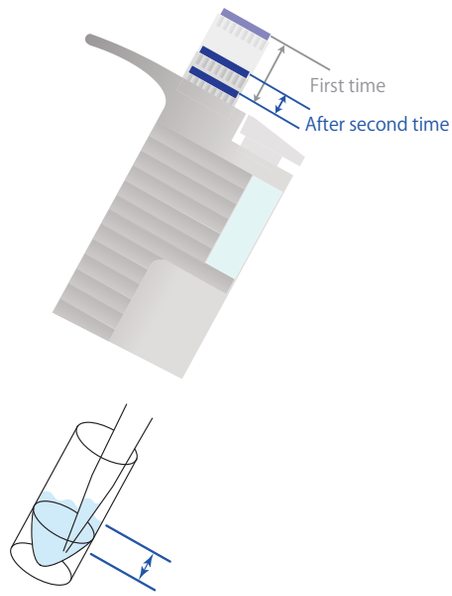
2. Warm 0.25 M sucrose in an incubator (37℃, 5% CO_2 in air) before use.

Recovering Vitrified Embryos

1. Remove the required sample from the liquid nitrogen and open the cryotube cap. Discard any liquid nitrogen in the tube and allow it to stand at room temperature for 30 seconds.
2. Add 0.9 mL of 0.25 M sucrose (preheated to 37℃) to the cryotube and warm the sample quickly via pipetting. When pipetting, take care not to generate large amounts of bubbles and to not physically damage embryos by pipetting too quickly. Once warmed, transfer the contents of the cryotube into a culture dish.



The tip must not touch the bottom of the cryotube. If it does so, the 0.25 M sucrose in the tip will freeze and you will not be able to inject the 0.25 M sucrose into the cryotube.



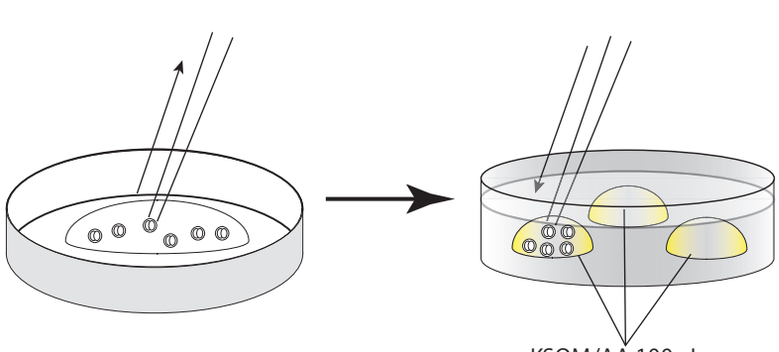
On the first press, the full volume of 0.9 mL of 0.25 M sucrose will be expelled.
On subsequent presses, around 10 amounts of 0.25 M sucrose which you have sucked up will be expelled. Pipetting smaller volumes in this way prevents bubbles from generating in the 0.25 M sucrose.

3. Place 0.4-0.5 mL of 0.25 M sucrose into the cryotube, and transfer the contents into the plastic dish. This further dilutes the cryoprotectant and ensures that all of the embryos have been transferred.

[Recovering Vitrified Embryos] No. 13-02

[Pipetting to Recover Vitrified Embryos] No. 13-03

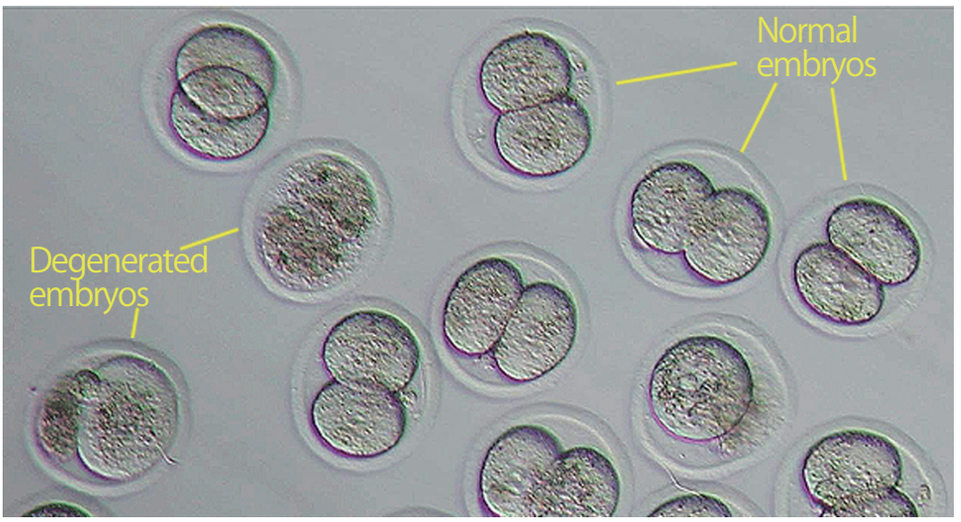
4. Aspirate the embryos from the liquid and carefully transfer them into a drop of KSOM/AA (washing dish), then keep them in an incubator (37℃, 5% CO_2 in air).



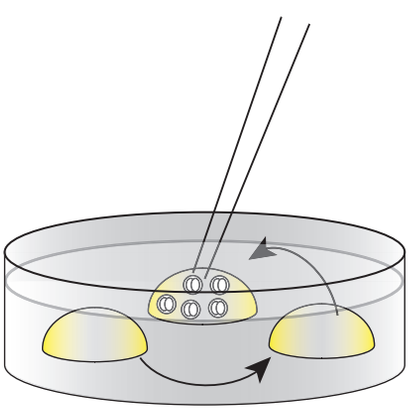
Note

It is very important to warm the sample quickly to avoid damaging the embryos due to the toxicity of the cryoprotective solution (DAP213).

1. **[Micrograph : Embryos Recovered after Vitrification]**



5. After 10 minutes, wash the embryos with 2 cycles of fresh KSOM/AA (washing dish).



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1. Nakagata N. 1989. High survival rate of unfertilized mouse oocytes after vitrification. *J. Reprod. Fert.* **87**: 479-483.
2. Nakagata N. 1993. Production of normal young following transfer of mouse embryos obtained by *in vitro* fertilization between cryopreserved gametes. *J. Reprod. Fert.* **99**: 77-80.
3. Nakagata N. 1995. Studies on cryopreservation of embryos and gametes in mice. *Exp. Anim.* **44**: 1-8.
4. Nakao K., Nakagata N., and Katsuki M. 1997. Simple and effcient procedure for cryopreservation of mouse embryos by simple vitrification. *Exp. Anim.* **46**: 231-234.

6-2 Simple Vitrification of Mouse Oocytes

Materials and Equipment

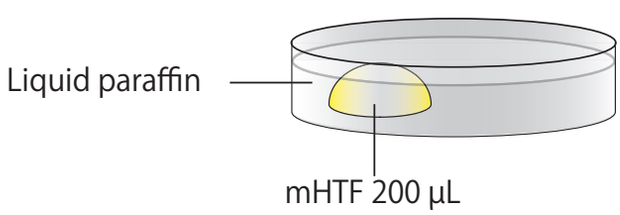
1. Female mice superovulated with PMSG and hCG
2. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
3. Liquid paraffin
4. Micropipette
5. Pipette tips
6. mHTF
7. 1% Hyaluronidase in mHTF
8. Fetal bovine serum (FBS Cat. No. 26140-087; Gibco)
9. Filter unit (Millex-GV 0.22 µm Cat. No. SLGV013SL; MILLIPORE)
10. Glass capillaries for embryo handling
11. Humidified incubator (37℃ 5% CO_2 in air)
12. Materials and equipment used for vitrification and warming of embryos (For washing warmed oocytes, mHTF drops are used.)
    (Please refer to the chapter of Simple Vitrification of Mouse Embryos on page 54.)

Procedures

Preparation of Dishes

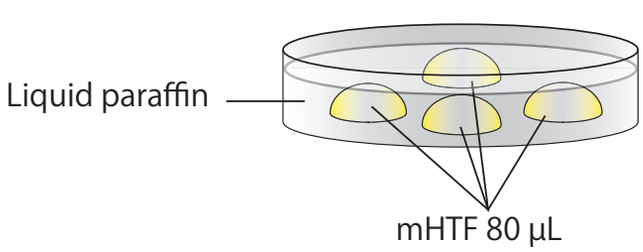
1. Put a 200 µL drop of mHTF into a dish. Cover it with liquid paraffin and place it in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

【Oocyte dish】

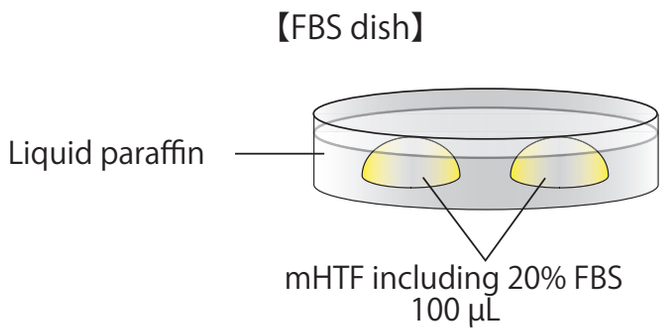


2. Put 4 drops (80 µL/drop) of mHTF into a dish. Cover them with liquid paraffin and place the dish in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

【Washing dish】

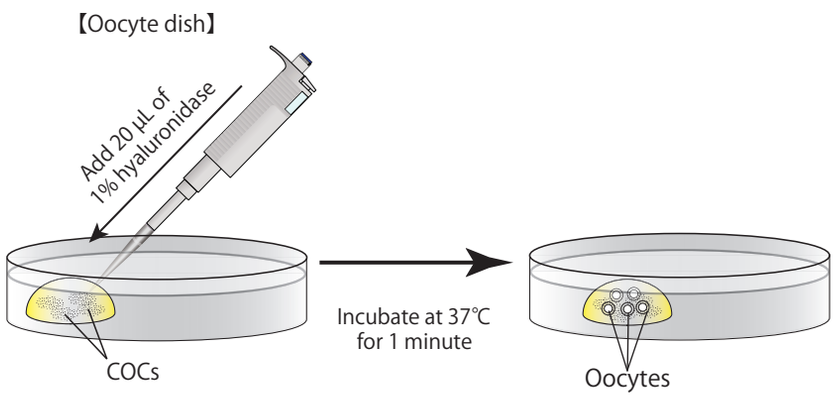


3. Prepare some mHTF including 20% FBS, and sterilize it using a filter. Put 2 drops (100 µL/ drop) of the medium into a dish. Cover them with liquid paraffin and place the dish in the incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

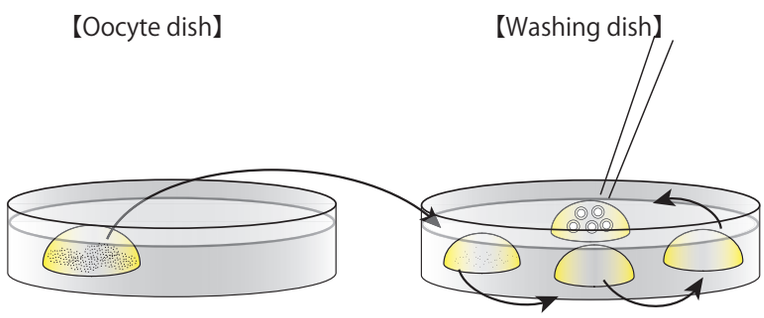


Preparation of Denuded Oocytes

1. Collect cumulus-oocyte complexes (COCs) from superovulated female mice and introduce them into a 200 µL drop of mHTF (Oocyte dish). (Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Add 20 µL of 1% hyaluronidase to the drop of mHTF containing the COCs, and keep the dish in an incubator (37℃, 5% CO_2 in air) for 1 minute.



3. Promptly collect and transfer the oocytes into a 80 µL drop of mHTF (Washing dish), and in turn wash them in the drops in the washing dish.



Note

Be sure to carry out all operations, from sacrificing the female and removing her oviducts to introducing the COCs into a drop of mHTF (Oocyte dish), in the shortest time possible (within 30 seconds).

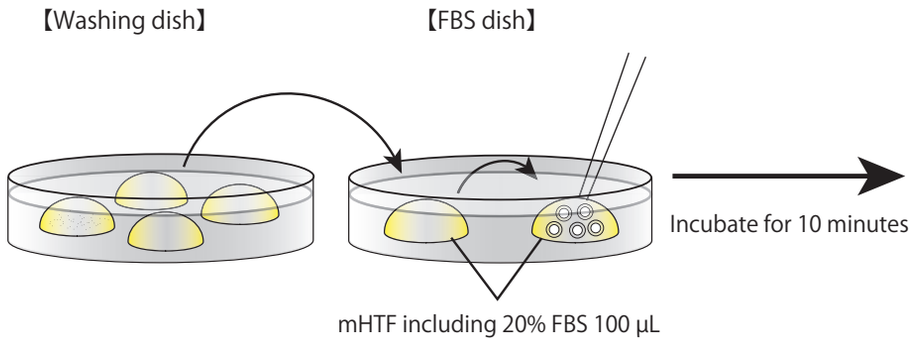
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

Comment

If some cumulus cells become attached to the zona pellucida of the oocytes, they can be removed by manipulating the glass capillary.

Culturing Oocytes in a Drop Containing FBS

1. Transfer the oocytes into the first drop in the FBS dish to rinse. Then, transfer them into the second drop to incubate (37℃, 5% CO_2 in air) for 10 minutes.



Simple Vitrification of Mouse Oocytes

1. The oocytes can be vitrified using the simple vitrification method for embryos, after removing cumulus cells and culturing them in a drop containing FBS.
   Moreover, the warming method is the same as for embryos. (Please refer to the chapter of Simple Vitrification of Mouse Embryos on page 54.)

In vitro Fertilization using Vitrified-Warmed Oocytes

1. The vitrified-warmed oocytes can be used for *in vitro* fertilization using fresh, cold temperature transported and frozen-thawed spermatozoa.
   Please refer to the chapters of *In Vitro* Fertilization on page 6, *In Vitro* Fertilization using Epididymal Sperm Transported at Cold Temperature on page 18 and *In Vitro* Fertilization using Cryopreserved Spermatozoa on page 26.

References

1. Nakagata N., Takeo T., Fukumoto K., Kondo T., Haruguchi Y., Takeshita Y., Nakamuta Y., Matsunaga H., Tsuchiyama S., Ishizuka Y., Araki K. 2013. Applications of cryopreserved unfertilized mouse oocytes for *in vitro* fertilization. *Cryobiology.* **67**(2):188-92.

Comment

FBS can prevent zona hardening in the oocyte during vitrifying and warming.

Note

There are three different methods of preparing CARD MEDIUM®, depending on whether *in vitro* fertilization will be carried out using fresh, frozen-thawed or cold-temperature transported spermatozoa. Please refer to the CARD MEDIUM® instruction manual.

6-3 Vitrification and Transplantation of Mouse Ovaries

Materials and Equipment

1. 35-mm sterile plastic tissue culture dishes
2. mWM
3. Donor: Female mouse (1 day to 30 weeks old)
4. Recipient: Four-week-old female mouse (a strain that is histocompatible with the transplanted ovary)
5. Anesthetic
6. Micro-spring scissors (5 mm blade)
7. Pair of watchmaker's #5 forceps
8. Wound clip (Autoclip 9 mm; Clay Adams Cat. No. 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams Cat. No. 427630)
9. Hot plate (37℃)

Procedures

Collection of Ovaries

1. Sacrifice a donor female and remove her ovaries.
   (Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Place the ovaries in a dish containing an adequate amount of mWM.

Vitrification

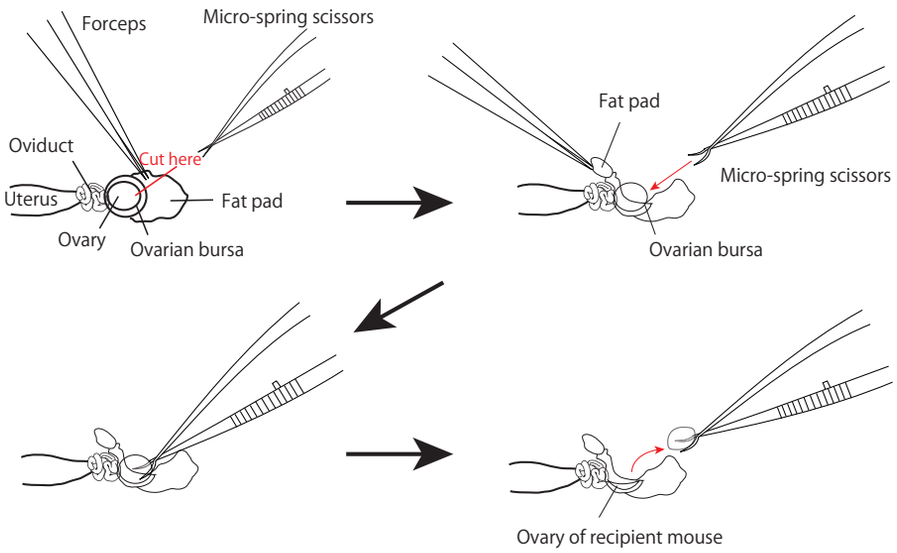
1. Ovaries can be cryopreserved following the same method used for embryos.
   (Please refer to the chapter of Simple Vitrification of Mouse Embryos on page 54.)

Transplantation

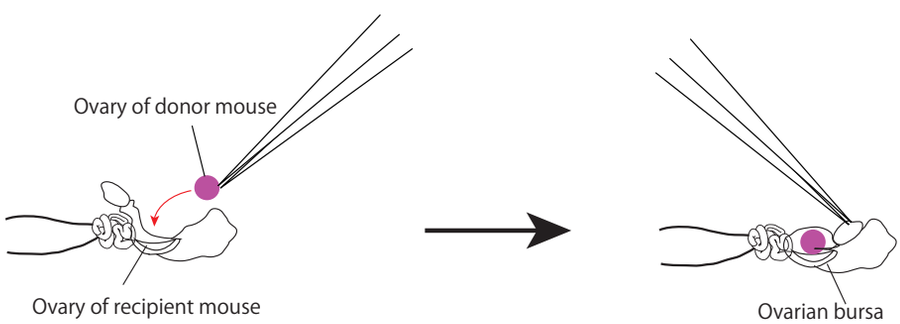
1. Anesthetize a recipient mouse.
2. Pull out the ovary, oviduct and part of the uterine horn as per the conventional procedure.
   (Please refer to the chapter of Embryo Transfer into the Oviduct on page 67.)
3. Using the micro-spring scissors, cut open approximately 1/4 of the recipient's ovarian bursa and some of the surrounding fat pad. Lift up the fat to reveal the ovary.
4. Cut off 1/2 - 2/3 of the ovary using the micro-spring scissors (curved blade).

Comment

By using micro-spring scissors with a curved blade, it becomes easier to place the donor ovary on the residual recipient ovary.



5. Insert the donor ovary into the residual ovary of the recipient, and cover it with the ovarian bursa.



[Transplantation of Mouse Ovary] No. 15-01

6. Push the ovary, oviduct, and part of the uterine horn back into the abdomen, and close the wound using wound clips.
7. Repeat the process for the ovary on the other side of the recipient mouse as described above.
8. Keep the mouse warm on a 37℃ warming plate until the mouse recovers from the effects of the anesthesia.

References

1. Migishima F., Suzuki-Migishima R., Song S.Y., Kuramochi T., Azuma S., Nishijima M., and Yokoyama M. 2003. Successful cryopreservation of mouse ovaries by vitrification. *Biol. Reprod.* **68**: 881-887.
2. Tsuchiyama S., and Nakagata N. 2009. Cryopreservation of ovaries from elderly female mice. *Exp. Anim.* **58**(3) Suppl: 248.