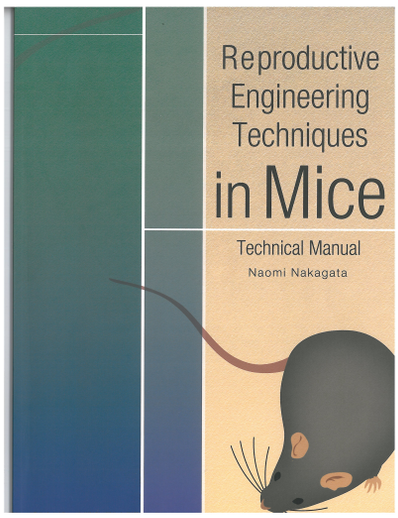
Reproductive Engineering Techniques **in Mice**



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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* Please see page 90 for details.

4-1 Preparation of Laser-microdissected Oocytes

Certain strains of cryopreserved spermatozoa, such as a number of inbred strains, may possess a low fertilizing ability. In order to overcome this impediment, we use oocytes which have been laser-microdissected for *in vitro* fertilization.

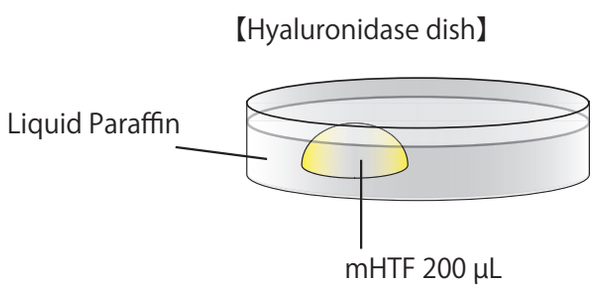
Materials and Equipment

1. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
2. mHTF
3. Liquid paraffin
4. Hyaluronidase in mHTF (Hyaluronidase, Cat. No. H-3506, Sigma)
5. The *Saturn 3* laser system (Research Instruments Ltd, Cornwall, UK)
6. Humidified incubator (37℃, 5% CO_2 in air)

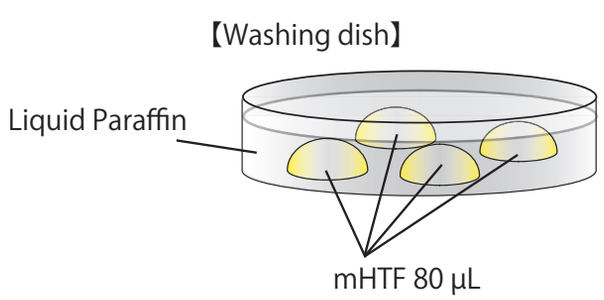
Procedures

Preparation of Sperm and Dishes

1. For IVF, sperm must be prepared in the methods described at the chapters of *In Vitro* Fertilization on page 8, *In Vitro* Fertilization using Epididymal Sperm Transported at Cold Temperature on page 18 and *In Vitro* Fertilization using Cryopreserved Spermatozoa on page 28.
2. Put a 200 µL drop of mHTF into a dish. Cover it with liquid paraffin and place them in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

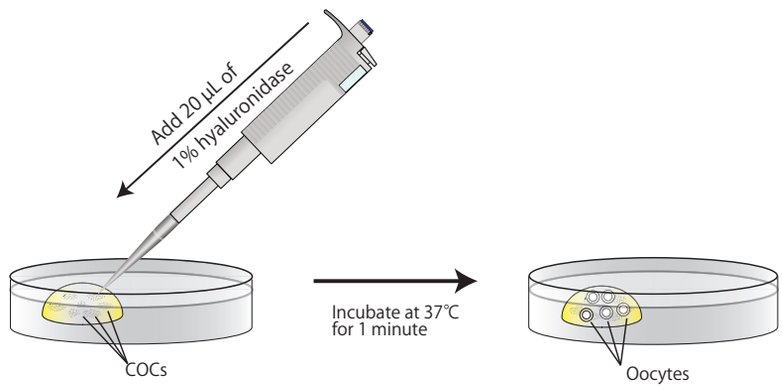


3. Put 4 drops (80 µL/drop) of mHTF into a dish. Cover them with liquid paraffin and place them in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

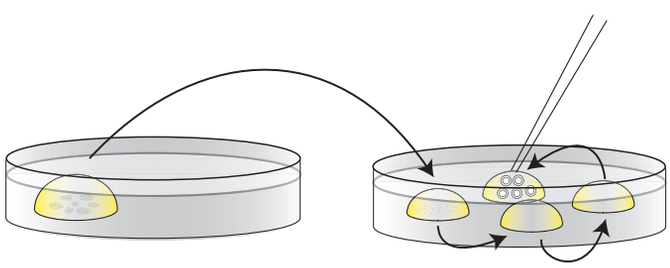


Preparation of Denuded Oocytes

1. Collect cumulus-oocyte complexes (COCs) from superovulated female mice and introduce them into a 200 µL drop of mHTF (Hyaluronidase dish).
(Please refer to the chapter of *In Vitro* Fertilization on page 6 and 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.

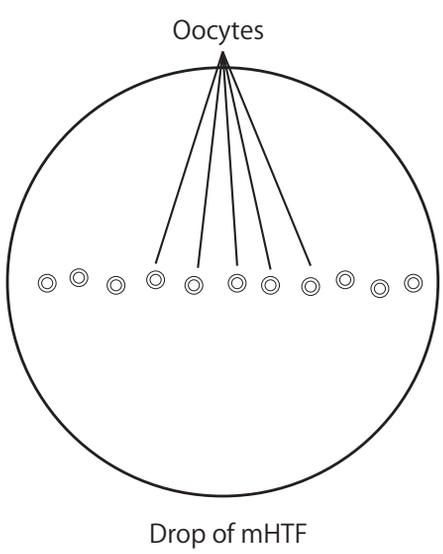


Comment

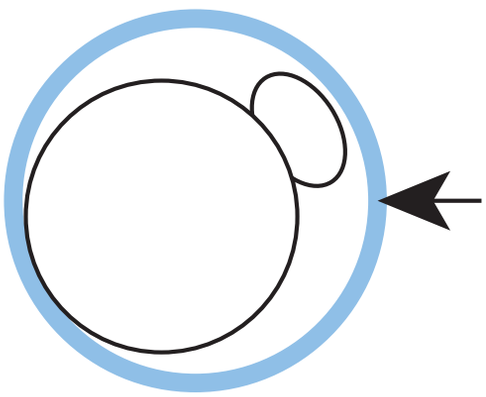
If some cumulus cells become attached to the zona pellucida of the oocytes, they can be removed by manipulating the glass capillary.

Dissection of the Zona Pellucida using a Laser

1. Put a 100 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.
2. Transfer fifty denuded oocytes into the drop of 100 µL mHTF.
3. Arrange the oocytes in a line along the bottom of the plastic dish.



4. Set the dish containing the oocytes on the *Saturn 3* laser system.
5. Target the zona pellucida at a spot adjacent to the first polar body and dissect it with a laser beam (see arrow).



[Dissection of Zona Pellucida using a Laser] No. 08-01

6. After dissecting the zona pellucida of all oocytes, transfer them into a drop of CARD MEDIUM® for fertilization. Place the dish in a CO_2 incubator.
(Please refer to the chapters of *In Vitro* Fertilization on page 6, *In Vitro* Fertilization using Epidydimal Sperm Transported at Cold Temperature on page 18 and *In Vitro* Fertilization using Cryopreserved Spermatozoa on page 26.)

Note

To avoid injuring the plasma membrane of the oocytes, aim the laser at the area with the widest distance between the plasma membrane and the zona pellucida.

Note

The diameter of the hole is 10-12.5 µm and the pulse length is 0.55-0.60 ms.

References

1. Kaneko T., Yanagi M., Nakashima T., and Nakagata N. 2006. The improvement in fertility of cryopreserved mouse spermatozoa showing low fertility using laser-microdissected oocytes. *Reprod. Med. Biol.* **5**(4): 249-254.
2. Anzai M., Nishiwaki M., Yanagi M., Nakashima T., Kaneko T., Taguchi Y., Tokoro M., Shin SW., Mitani T., Kato H., Matsumoto K., Nakagata N., and Iritani A. 2006. Application of laser-assisted zona drilling to *in vitro* fertilization of cryopreserved mouse oocytes with spermatozoa from a subfertile transgenic mouse. *J Reprod Dev.* **52**(5): 601-606.

4-2 Partial Zona Dissection (PZD)

If you cannot utilize laser-microdissection instruments, you can dissect the zona pellucida of oocytes manually under a stereomicroscope.

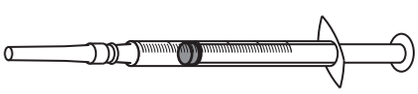
Materials and Equipment

1. Female mice superovulated with PMSG and hCG
   (Please refer to the chapter of *In Vitro* Fertilization on page 6.)
2. mHTF
3. Hyaluronidase in mHTF (Hyaluronidase, Cat. No. H-3506, Sigma)
4. 0.3 M sucrose (BSA-)
5. 0.3 M sucrose (BSA+)
6. Liquid paraffin
7. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
8. Tip (10-100 µL volume)
9. Micropipette

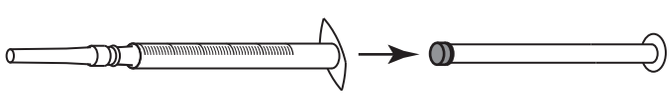
Procedures

Preparing the Needle for PZD

1. A 1mL disposable syringe with a 30 gauge needle should be altered for use in PZD as shown in the diagram below.

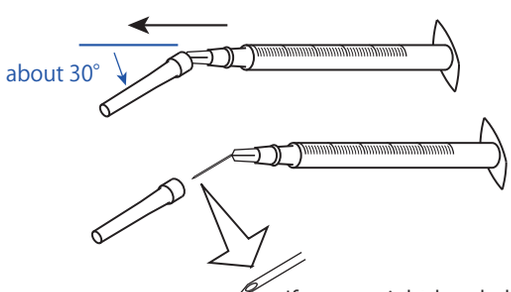


Take a 1 mL syringe with a 30 G needle.



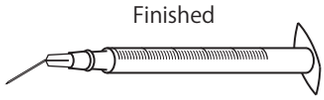
Remove and discard the plunger.

Remove the needle cover,
and use the cover to bend the needle about 30°.



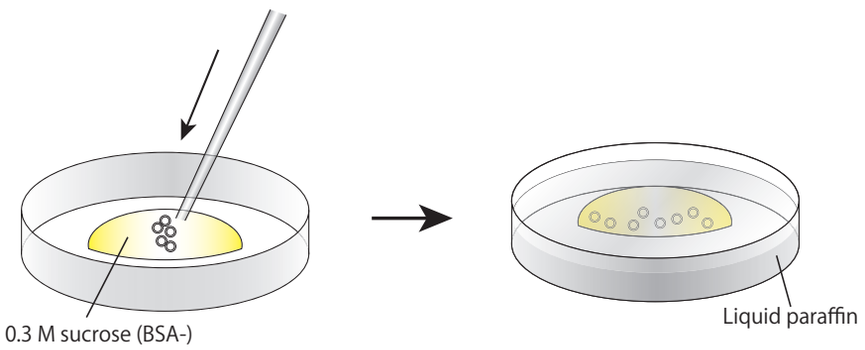
If you are right-handed,
the hole in the needle should face you
as shown in the diagram.

Finished

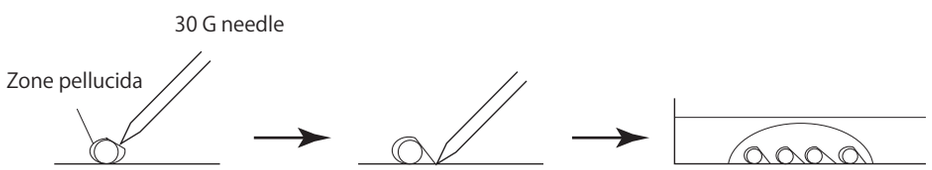


PZD

1. Collect oocytes from the oviducts of superovulated females 14-15 hours after injecting them with hCG. Denude the oocytes with hyaluronidase.
   (Please refer to the chapters of *In Vitro* Fertilization on page 9 and Preparation of Laser-microdissected Oocytes on page 37.)
2. Introduce the denuded oocytes into the upper part of a 100 µL drop of 0.3 M sucrose (BSA-) in a dish.
3. When the oocytes sink to the bottom of the dish, cover the drop with liquid paraffin.

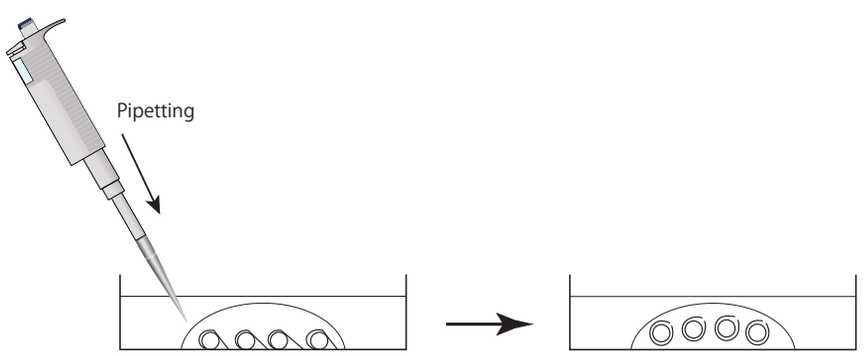


4. Under a stereomicroscope, partially dissect the zona pellucida (PZD) of the oocytes using a single downward motion with a 30 gauge needle.



[PZD] No. 09-01

5. Following PZD, neutralize the electrostatic attraction between the zona pellucida and dish surface by adding 20 µL of 0.3 M sucrose (BSA+) to the drop.
6. To remove PZD oocytes from the dish surface, spray sucrose solution on the oocytes using a micropipette.



7. Wash the PZD oocytes gently 3 times in CARD MEDIUM® to remove any leftover sucrose.

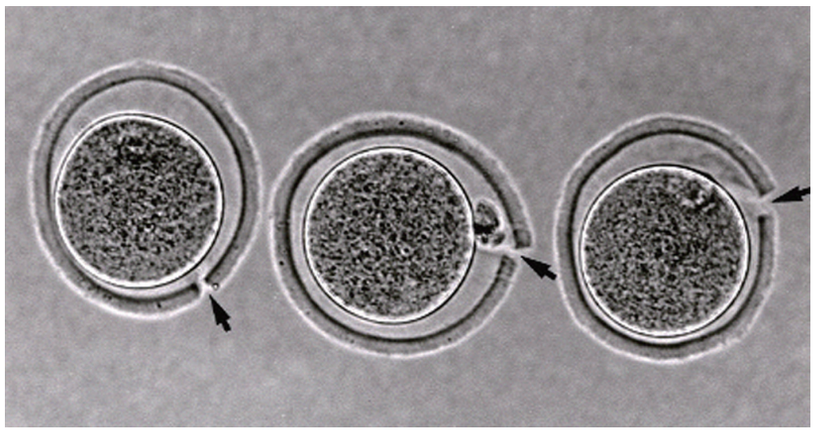
Comment

When the oocytes are placed into the 0.3 M sucrose (BSA-), the ooplasm shrinks due to osmotic forces and an electrostatic interaction occurs between the zona pellucida of the oocytes and the dish surface.
As a result, the perivitelline space widens and the oocytes become attached to the bottom of the dish.

Comment

The spray should be applied from the opposite side of the slit to prevent oocytes from escaping out of the zona pellucida.

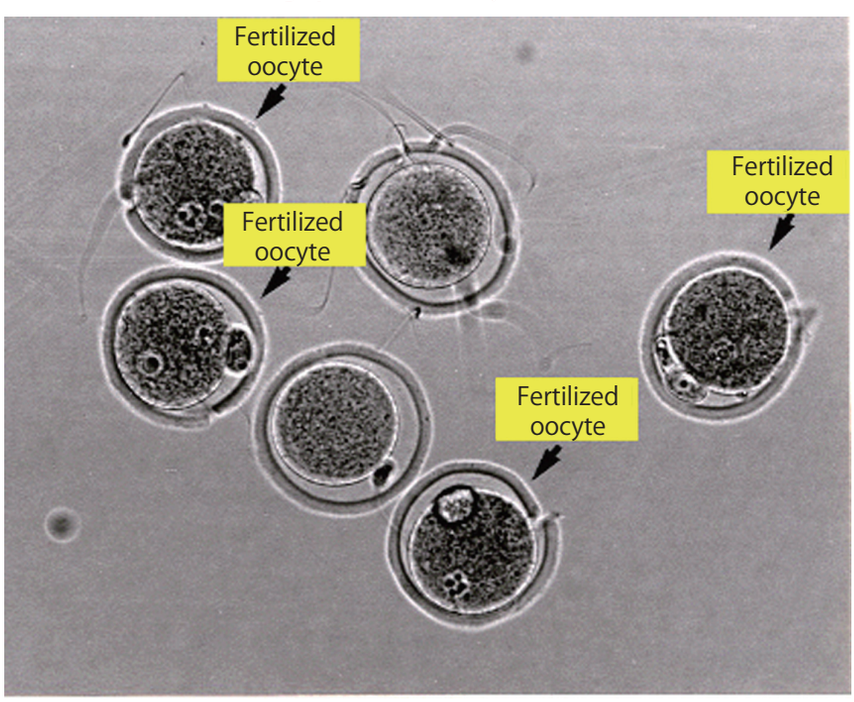
[Micrograph : PZD Oocytes]



In Vitro Fertilization and Embryo Transfer

1. Introduce the PZD oocytes into the CARD MEDIUM® containing the spermatozoa prepared previously (insemination).
   (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.)
2. Gently wash the fertilized oocytes twice in fresh mHTF 3 hours after insemination, then culture them for 3 days until they have developed to the early blastocyst stage.

[Micrograph : Fertilized Oocytes]



3. Transfer the early blastocysts into the uterine horns of a recipient on Day 3 of pseudopregnancy (Day 1 is the day on which vaginal plug is observed).
   Please refer to the chapter of Embryo Transfer into the Uterus on page 72.

References

1. Nakagata N., Okamoto M., Ueda O., and Suzuki H. 1997. The positive effect of partial zona-pellucida dissection on the *in vitro* fertilizing capacity of cryopreserved C57BL/6J transgenic mouse spermatozoa of low motility. *Biol. Reprod.* **57**: 1050-1055.

Comment

If 2-cell stage embryos are transferred to the oviducts of recipients on Day 1 of pseudopregnancy, the rate of development into live offspring will be very low.
This is because the blastomeres of the embryos escape from the zona pellucida via the peristaltic action of the oviduct when they travel through the oviduct to the uterus.

4-3 Collecting 2-Cell Stage Embryos

Materials and Equipment

1. Pair of watchmaker's #5 forceps
2. Fine scissors
3. KSOM/AA
4. Liquid paraffin
5. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
6. 1 mL syringe
7. Flushing needle (30 G blunt needle)
8. Transfer pipettes

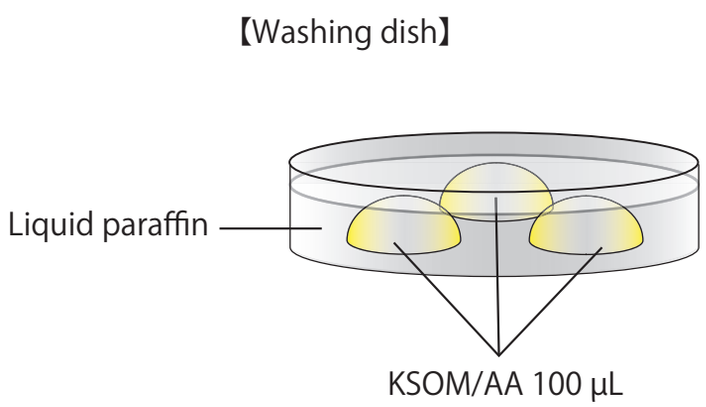
Procedures

Superovulation and Selection of Plugged Females

1. Inject females i. p. (8-12 weeks of age) with 7.5 IU of PMSG (14:00-18:00).
2. Inject females i. p. with 7.5 IU of hCG 48-52 hours after the PMSG injection. Following the hCG injection, allow females to mate with males.
3. Check for vaginal plugs the morning after mating.
(Please refer to the chapter of Embryo Transfer into the Oviduct on page 66.)

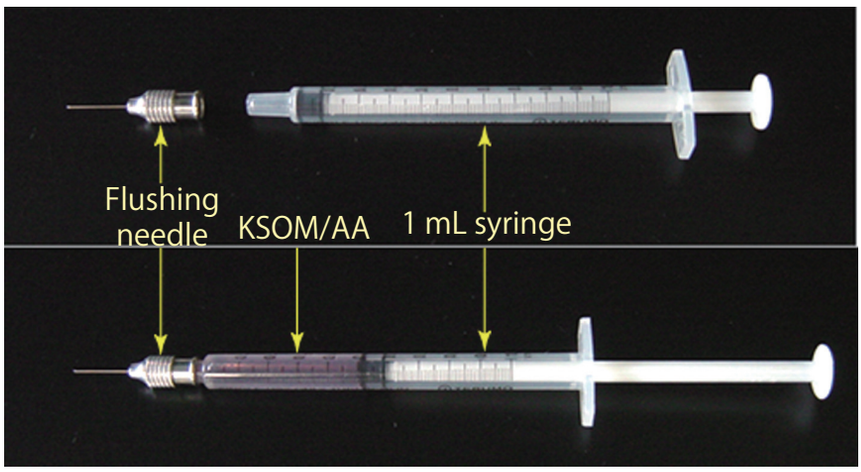
Dish Preparation

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.



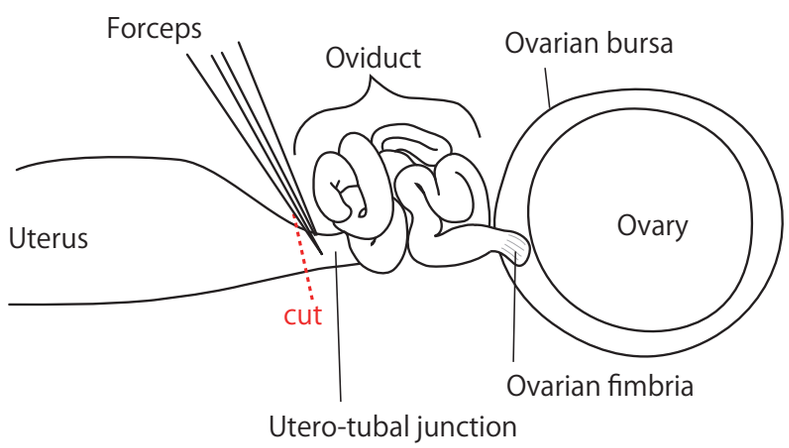
Preparation of Flushing Needle

1. Fill a syringe with KSOM/AA and connect it to a blunted flushing needle.
2. Test the syringe to ensure that it is free of air bubbles, and that the KSOM/AA is flowing smoothly.

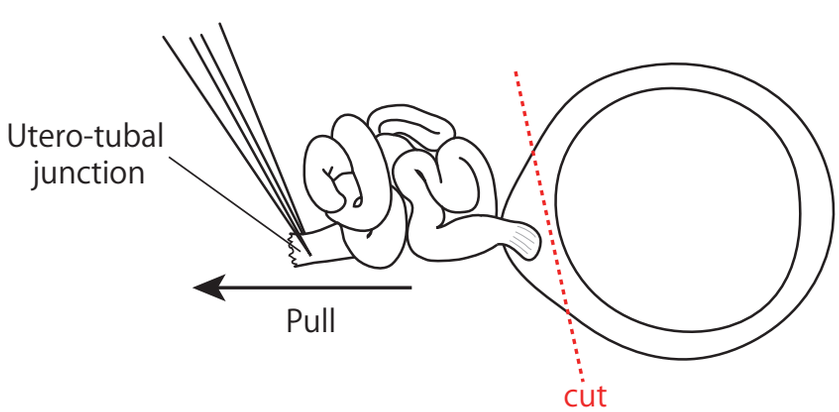


Collecting Embryos

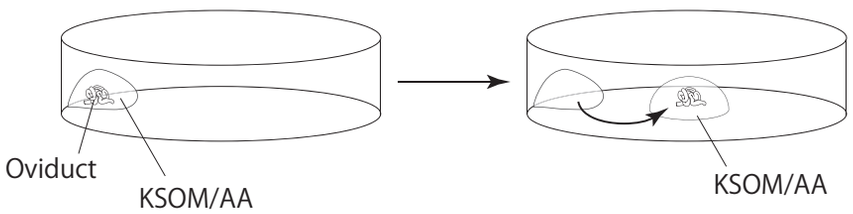
1. One day after a vaginal plug has been observed, remove the uteruses, oviducts and ovaries from the females, and place them on sterile filter paper.
(Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Pinch the utero-tubal junction and make a cut on the uterus side of it.



3. Pull the utero-tubal junction to separate the infundibulum from the ovary, then cut the ovarian bursa.

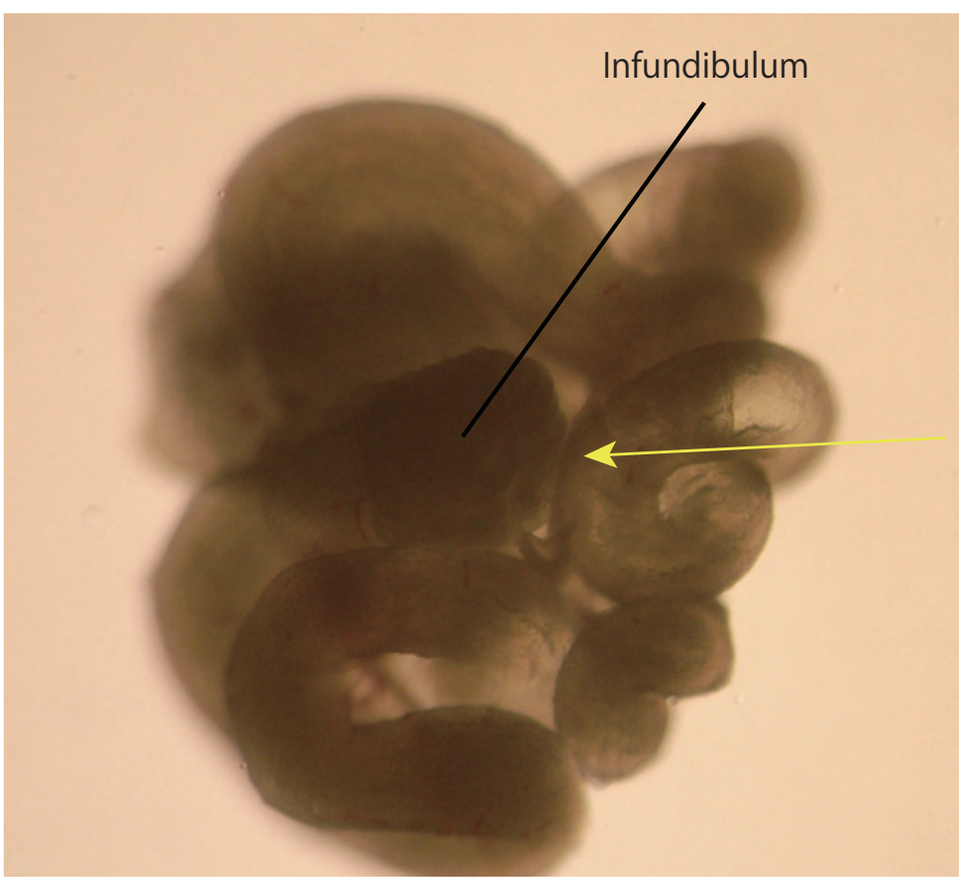


4. After washing the oviduct in a drop of KSOM/AA, put it into another drop of KSOM/AA.



5. Look closely at the oviduct to find the infundibulum, which is a tube of a larger diameter than the oviduct.
6. Set the oviduct so that the flushing needle can be easily inserted.

[Micrograph : An Oviduct before Flushing]



7. Hold the infundibulum against the bottom of the dish using a pair of forceps, and insert the needle into it.

Note

Because the infundibulum is usually hidden within the coils of the oviduct, you need to rotate the oviduct tenderly with forceps within the drop and look closely to find it.

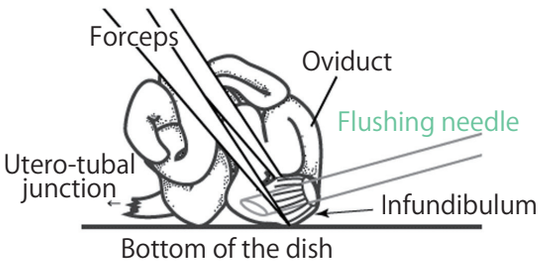
Comment

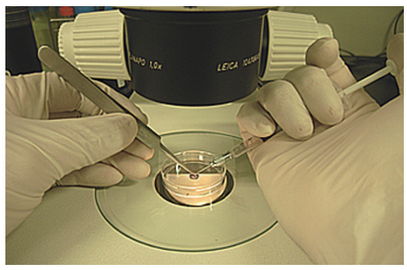
If you are right-handed, position the oviduct as shown in the diagram on the left. It is easy to insert the needle into the infundibulum from this position (as indicated by the yellow arrow).

Note

The infundibulum is extremely fragile, so use the forceps gently.

8. Push the plunger and slowly flush the oviduct with KSOM/AA.

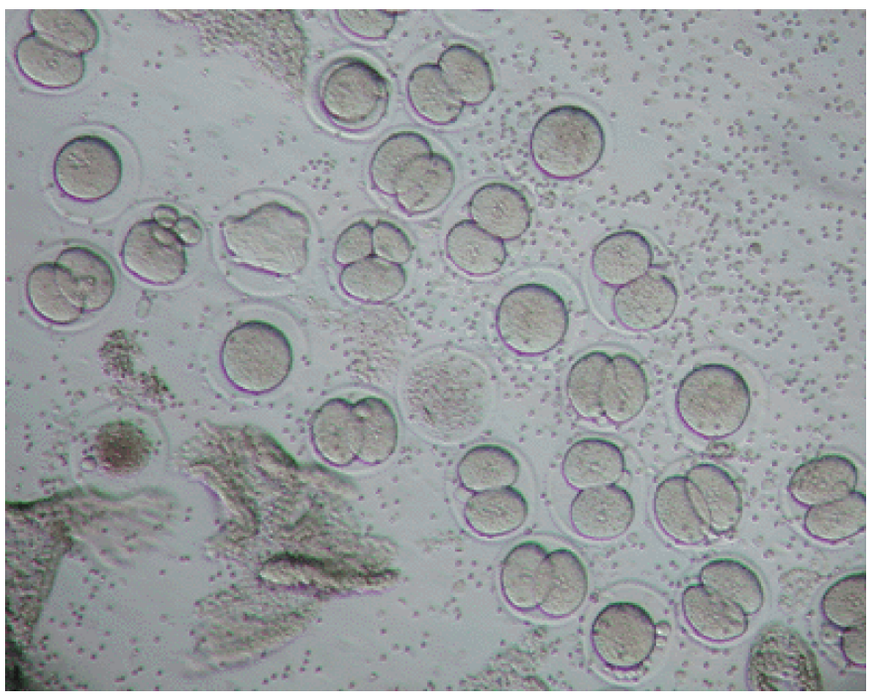




[Flushing] No. 10-01

9. Aspirate the embryos using pipettes and wash them in several drops of fresh KSOM/AA (washing dish).

[Micrograph : After Flushing of Oviduct]



Note

When flushing is successful, you should see the oviduct puffing up as you inject medium.
If you cannot find the infundibulum, do not blindly pinch the oviduct. Doing so may crush the infundibulum, which will prevent you from inserting the needle.

Note

Be sure to carry out all operations, from sacrificing the female through to flushing her oviducts, in the shortest time possible (within 5 minutes).
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly flush its oviducts before moving on to the next mouse.

References

1. Nagy A., Gertsenstein M., Vintersten K., and Behringer R. 2003. Manipulating the Mouse Embryo, A Laboratory Manual (Third edition). *Cold Spring Harbor Laboratory Press.* ISBN 0-87969-591-9.