HORMONE AND MEDIUM PREPARATION

PMSG (Intergonan)

Ressuspend the lyophilized powder (6000 IU) in 60mL of Dulbecco's PBS (Sigma).

Aliquot and store at -20ºC until use

Use: IP injection of 5IU/female, that is, 50uL of a stock solution (100IU/mL).

HCG (Chorulon)

Ressuspend the lyophilized powder (1500IU) in 15mL of Dulbecco's PBS (Sigma).

Aliquot and store at -20ºC until use

Use: IP injection of 5IU/female, that is, 50uL of a stock solution (100IU/mL).

Medium

M2 (100mL or 50mL, Sigma)

To a 100mL or 50mL flask, add 1000uL or 500uL of 100x Pen/strep (Sigma).

Filter with 0,22um filters

Aliquot into 15mL falcons and store at 4ºC

KSOM

To a 8mL flask add 80uL off 100x Pen/strep (Sigma).

Filter with 0,22um filters

Aliquot into 1,5mL eppendorf and store at -20ºC

Hyaluronidase

From bovine testes (Sigma)

Stock solution: ressuspend the lyophilized powder (30mg) in 3mL of M2 (Sigma) to prepare a stock solution at 10mg/mL

Aliquot 750uL into 1,5mL vials and store at -20ºC

Work solution: add 750uL of stock solution (at 10mg/mL) to a 50mL of M2 to prepare a work solution at 150ug/mL

Aliquot into 1,5mL vials and store at -20ºC until use.

Microinjection Buffer

8mM Tris-HCl pH 7,4

0,2 mM EDTA pH 8,0

Add 80uL of 1M Tris-HCl pH 7,5 and 4uL of 0,5M EDTA pH8,0 to "water for embryo transfer" (Sigma) to a final volume of 10 mL

It is also possible use these two combinations:

10mM Tris-HCl pH 7,4

0,15 mM EDTA pH 8,0

Or

10mM Tris-HCl pH 7,4

0,25 mM EDTA pH 7,4

Preparing DNA fragment to microinjection

Bacterial DNA removal:

-with the appropriate enzymes digest of DNA in 70uL, over night.

-run 0,5uL of the enzymatic reaction to check if digestion is ok. If it is ok,

-run 19,5 uL of enzymatic reaction to isolate the desired band.

-extract de DNA using the QUIAQUICK Gel Electrophoresis extraction kit, from Quiagen, and elute with 60uL of buffer EB.

-run 1uL of the isolated frangment.

-check the concentration in nanodrop. (the concentration must be higher than 30 ug/uL.

If not, **repeat**.

For microinjection: Dilute the isolated fragment to 3ug/uL in microinjection buffer (8mM Tris-HCl pH 7,4; 0,2 mM EDTA pH 8,0) and then filter using a 0,22um filter from milipore.

MICE CERVICAL DISLOCATION AND EXPOSURE OF REPRODUCTIVE TRACT

Procedure

1. Place the mouse on top of a cage, so that it grips the bars with its front paws. Break its neck humanely by applying firm pressure at the base of the skull (Fig. 4.3) while at the same time pulling backward on the tail. Alternatively, a spatula, pencil, or cage card holder can be used to apply pressure to the base of the skull. The CO2 inhalation method also may be used if necessary.
2. Lay the animal on its back on absorbent paper and soak it thoroughly in 70% ethanol from a squeeze bottle. This important step reduces the risk of contaminating the dissection with mouse hair.

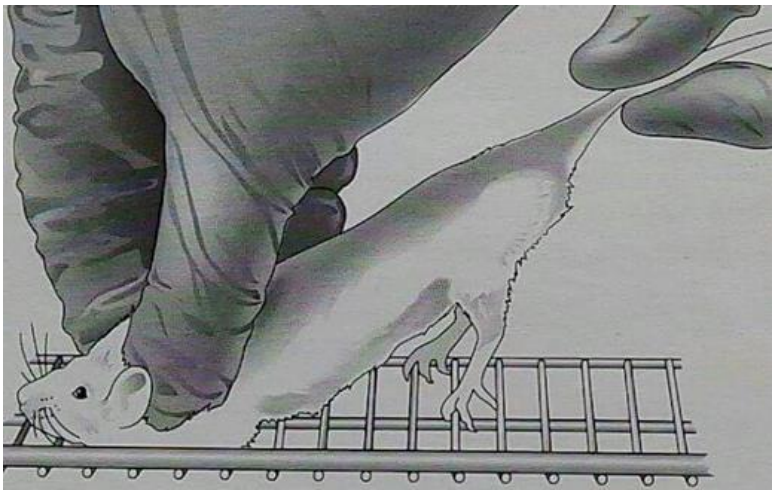


FIGURE 4.3. Method for quick and humane sacrifice of a mouse by cervical dislocation.

3. Pinch the skin and make a small lateral incision at the midline (the position is not critical) with regular surgical scissors (Fig. 4.4A). Holding the skin firmly above and below the incision, pull the skin toward the head and tail until the abdomen is completely exposed and the fur is well out of the way. Using the watchmaker's forceps and fine scissors, cut the body wall (peritoneum) as shown in Figure 4.4B. Push the coils of gut out of the way and locate the two horns of the uterus, the oviducts, and the ovaries (Fig.4.4C).

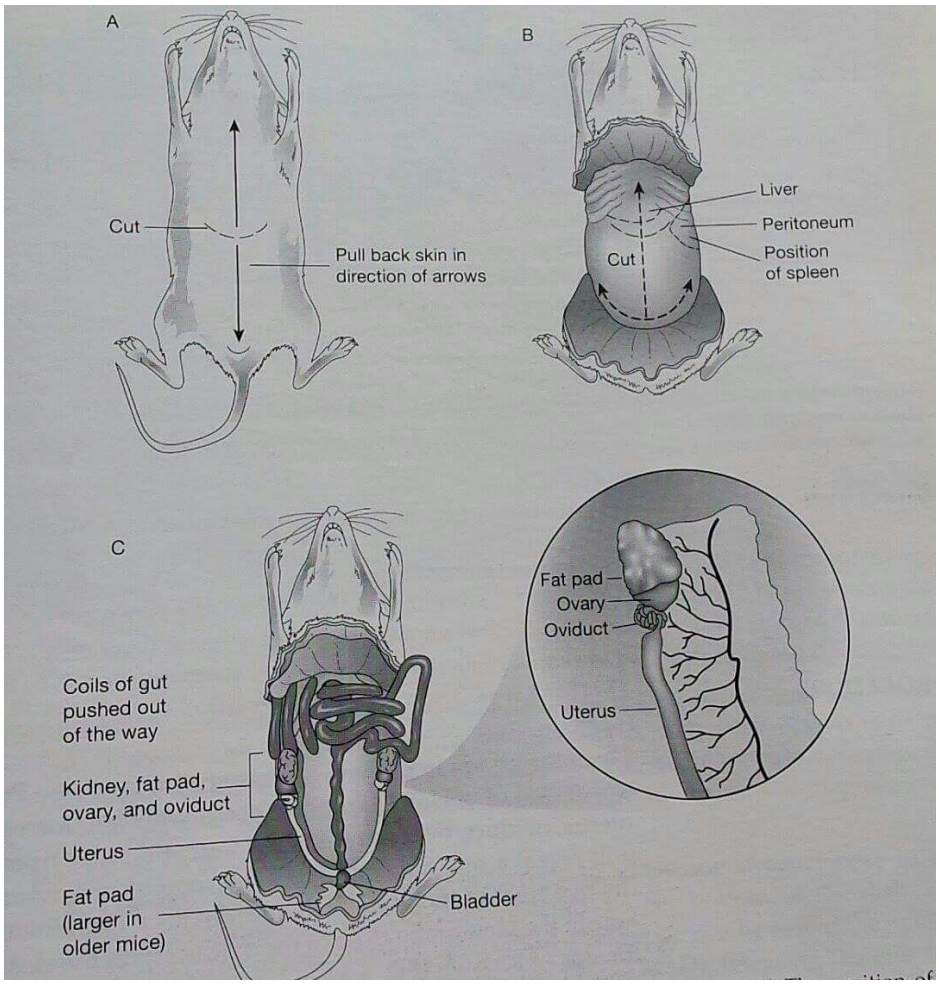


FIGURE 4.4. Dissection of reproductive organs of a female mouse. (A) The position of samall lateral incision in the skin is indicated by the dashed line. The skin is then pulled back in the direction of the solid arrows. (B) The body wall (peritoneum) is cut in the direction of the dashed arrows. (C) The alimentary tract displaced to reveal reproductive organs in the floor of the body cavity.

COLLECTING ZYGOTES AND REMOVING CUMULUS CELLS WITH HYALURONIDASE

Material and Equipment

Pregnant female mice sacrificed humanely

Embryo-handling pipette consisting of mouth or hand-held pipette assembly and pulled capillary

Forceps

Forceps, watchmaker's #5, two pairs

Microdrop culture dishes

Needles, 26-gauge

Organ culture dish (Falcon 3037)

Petri dishes, 35-mm, or embryological watch glasses

Scissors, fine

Stereomicroscope with transmitted and reflected or fiber optics *(optional)* illumination (preferably a ground-glass stage) with 20x and 40x magnification

Hyaluronidase

M2 medium

Procedure

1. Open the abdominal cavity as described above. Grasp the upper end of one of the uterine horns with fine forceps and gently pull the uterus, oviduct, ovary, and fat pad taut and away from the body cavity. This will reveal a fine membrane (the mesometrium), which connects the reproductive tract to be body wall and carries a prominent bood vessel. Poke a hole in the membrane close to the oviduct with the closed tips of a pair of fine forceps or scissors (Fig. 4.5A).

2. Pull the oviduct, ovary, and fat pad taut with fine forceps and cut between the oviduct and ovary with fine scissors as shown in Figure 4.5B. Do not be afraid to go close to the oviduct. Reposition the forceps and cut the uterus near the oviduct.
3. Transfer the oviduct and attached segment of uterus to a 35-mm petri dish or embryological watch glass containing M2 medium at room temperature. Oviducts from several mice can be collected in the same dish.

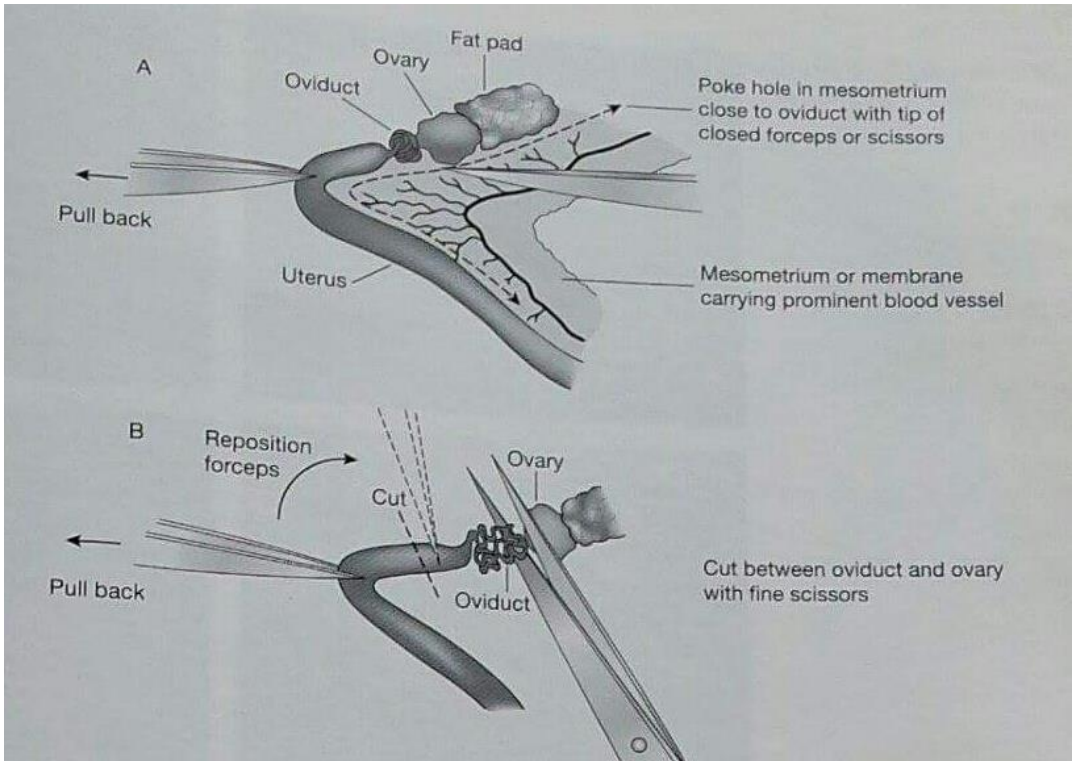


FIGURE 4.5. Dissection of the oviduct and end of the uterus are separated from the mesometrium with the closed tips of a pair of fine forceps. (B) A cut is made between the oviduct and the ovary. After repositioning the forceps, a second cut separates the oviduct from the uterus.

4. Newly ovulated oocytes, surrounded by cumulus cells, are found in the upper part of the ovidut (ampulla), which at this time (12 hours postovulation) is much enlarged. The fimbriated end of the oviduct (infundibulum) is also swollen during ovulation and can easily be located under 20x magnification in the stereomicroscope.
5. Transfer one oviduct at a time into another 35-mm petri dish containing hyaluronidase solution in M2 medium (~0.3 mg/ml) at room temperature (or 37ºC) and view through the stereomicroscope at 20x or 40x magnification.
6. Use one pair of watchmaker's forceps to grasp the oviduct next to the swollen infundihulum and hold it firmly on the bottom of the dish. Use another pair of watchmaker's forceps or a 26-gauge needle to tear the oviduct close to where the

zygotes are located, releasing the clutch of cumulus cells. If the zygotes do not flow out by themselves, use the forceps to push them out by gengly squeezing the oviduct. If they stick to the outside of the oviduct, allow the oviduct to sit for several minutes in the hyluronidase solution. Zygotes will be released as the digestion removes the sticky cumulus cells. If the zygotes stick to the forceps, simply lift the forceps out of the petri dish and they will be retained by the surface tension of the medium and will fall back to the bottom of the dish.

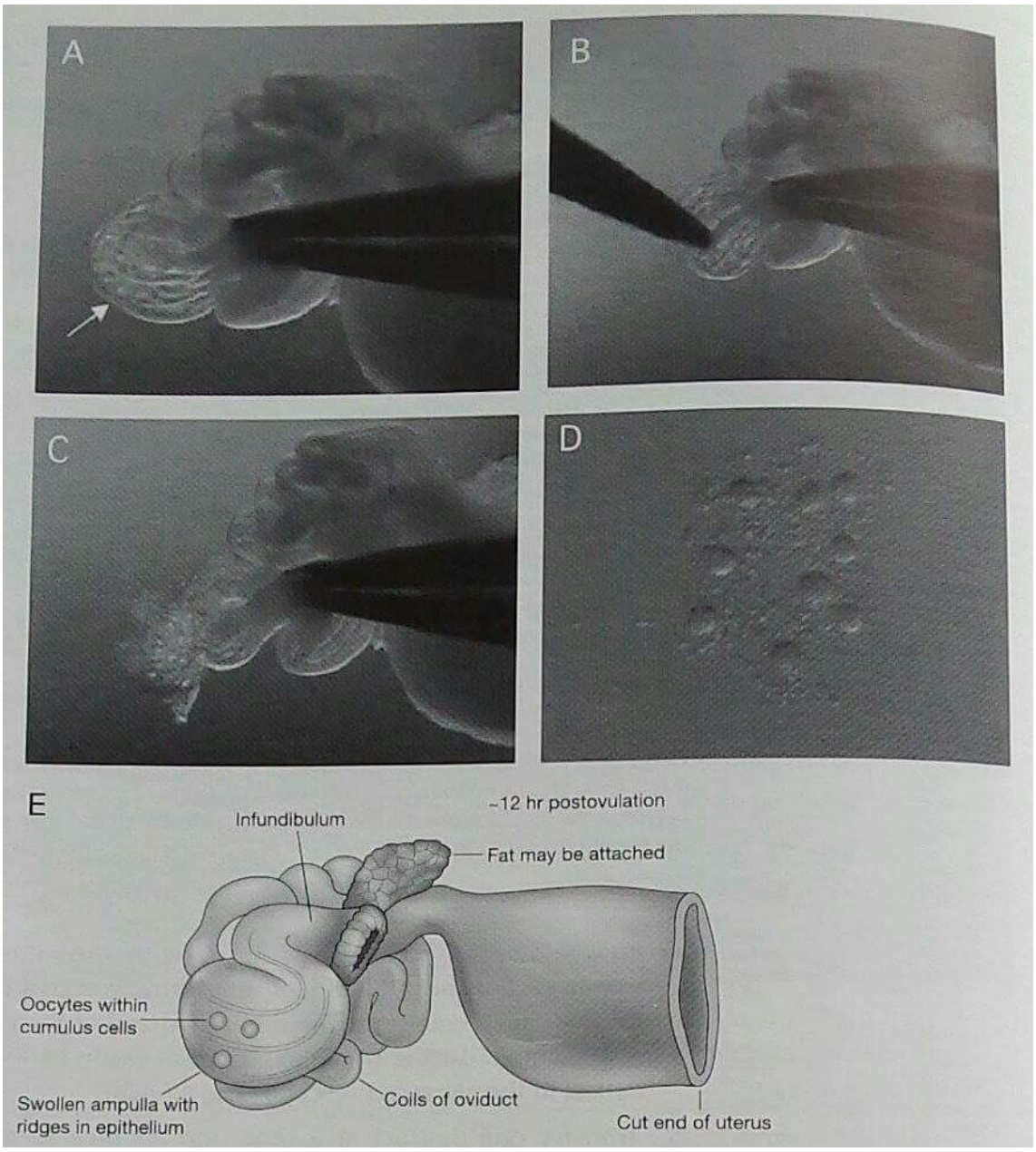


FIGURE 4.6. Isolation of zygotes from dissected oviduct. If the oviduct is removed soon after fertilization, the zygotes surrounded by cumulus (follicle) cells can be seen in the swollen upper part of the oviduct, the ampulla (A,E), and can be released by tearing the ampulla with fine forceps (B,C). (D) Cumulus-enclosed zygotes. (E) Diagram of procedure.

7. Allow the zygotes to incubate in the hyaluronidase solution for several minutes until the cumulus cells fall off. If necessary, pipette them up and down a few times, but do not leave them in the hyaluronidase solution for more than a few minutes after the cumulus cells are shed, because this may be harmful. Although ~0.3mg/ml solution of hyaluronidase is usually recommended, more concentrated hyaluronidase solutions of 0.5-1 mg/ml that require a shorter incubation time of less than a minute may also be used.
8. Use pipettes to pick up the zygotes and transfer them to a petri dish containing several drops of fresh M2 medium to rinse off the hyaluronidase solution, cumulus cells, and debris. Then transfer the zygotes to a microdrop culture dish, rinse through several drops of equilibrated medium, and keep at 37ºC, 5% CO2 until needed. An organ culture center well dish with equilibrated embryo culture medium may be used as an alternative for short-term culture, i.e., before microinjection.

COLLECTING TWO-CELL-TO COMPACTED MORULA-STAGE EMBRYOS

Material and Equipment

Pregnant female mice (20-60 hours d.p.c.) sacrificed humanely

Embryo-handling pipette consisting of mouth or hand-help pipette assembly and pilled capillary

Flushing needle

Forceps, fine

Forceps, watchmaker's #5, two pairs

Microdrop culture dish

Organ culture dish (Falcon 3037) (*optional)*

Petri dishes (35-mm) or embryological watch glasses

Scissors, fine

Stereomicroscope with transmitted and reflected or fiber optics *(optional)* illumination (preferably a grounp-glass stage) with 20x and 40x magnification

Syringe, 1-cc

Ethanol, 70%

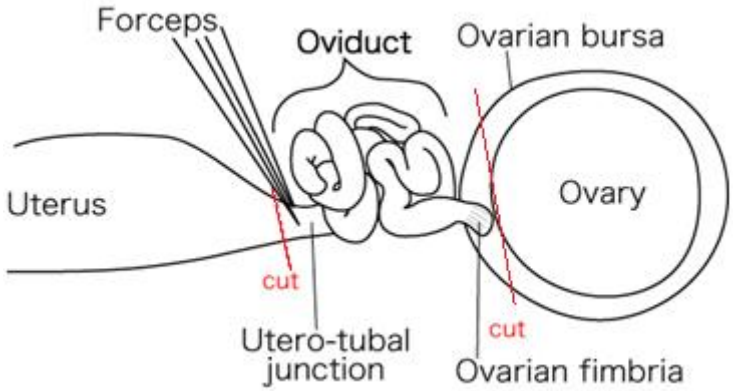
M2 medium at room temperature

Procedure

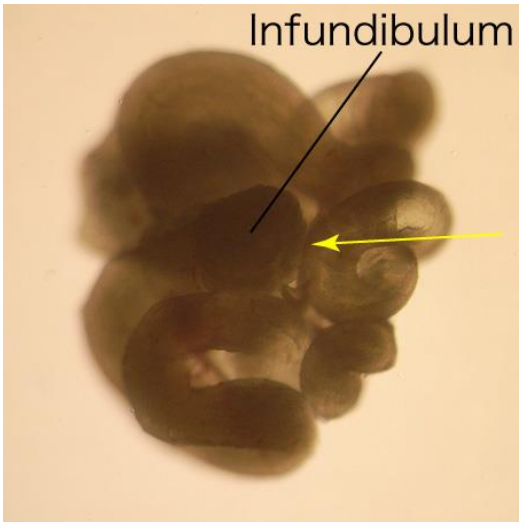
1. To reduce the risk of tearing the oviduct, cut the end of a 30- or 32-gauge hypodermic needle and grind it to a blunt tip (Fig. 4.7 A). It is also possible just to grind the sharp tip of the needle without cutting it to create a smaller beveled tip that may prove useful for flushing the oviducts from very young females. Sterilize the needle by flushing it with 70% ethanol immediately before use.
2. Open the abdominal cavity as described above. Grasp the upper end of one of the uterine horns with fine forceps and gently pull the uterus, oviduct, ovary, and fat pad taut and away from the body cavity. This will reveal a fine membrane (the mesometrium), which connects the reproductive tract to the body wall and carries a

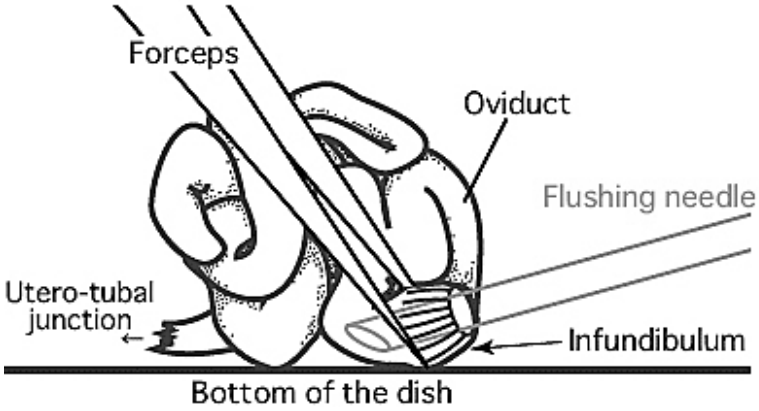
prominent blood vessel. Poke a hole in the membrane close to the oviduct with the closed tips of a pair of fine forceps or scissors.

3. Pull the oviduct, ovary,, and fat pad taut with fine forceps and cut between the oviduct and ovary with fine scissors. Do not be afraid to go close to the oviduct. Reposition the forceps and cut the uterus near the oviduct, leaving at least 1cm of the upper part of the uterus attached if the collection is taking place on 2.5 dpc.



4. Transfer de oviduct and attached segment of uterus to a 35-mm petri dish or embryological watch glass containing M2 medium at room temperature. Oviducts from several mice can be collected in the same dish. Place dish under stereomicroscope.
5. Test the syringe to be sure that it is free of air bubbles and that the M2 medium is flowing smoothly before inserting the needle.
6. The opening of the oviduct (infundibulum) at this time is no longer swollen and must be located within the coils of the oviduct. Use fine forceps to slide the end of the oviduct onto the bottom of the dish to hold it in place. Flush the oviduct with ~ 0.1 mil of M2 medium.





7. Use pipettes to pick up the embryos and wash them though several drops of fresh M2 medium to rinse off the debris.
8. Transfer the embryos to a microdrop culture dish, rinse through several drops of equilibrated medium, and keep at 37ºC, 5% CO2 until needed. An organ culture dish with equilibrated embryo culture medium may be used as an alternative for short-term incubation.

COLLECTING BLASTOCYSTS

Material and Equipament

Pregnant female mice (3.5-4.5 dpc) sacrificed humanely (see Protocol 4.8)

Embryo-handling pipette consisting of mouth or hand-help pipette assembly and pulled capillary (see Protocol 4.6 and 4.7)

Forceps, fine

Hypodermic needle, 26-gauge

Microdrop culture dish (Falcom 3037) *(optional)*

Scissors, fine

Stereomicroscope with transmitted and reflected or fiber optics (optional) illumination (preferably a ground-glass satage) with 20x and 40x magnification

Syringe, 1-or 2-ml

Tissue culture dishes, 35-mm sterile plastic or embryological watch glasses

M2 medium

Procedure

1. Open the abdominal cavity to remove the uterus, grasp it with fine forceps just above the cervix (located behind the bladder) and cut across the cervix with fine scissors. Pull the uterus upward to stretch the mesometrium and use fine scissors to trim this membrane away close to the wall of the uterine horns. Then cut between the oviduct and the ovary, keeping the utero-tubal junction intact.
2. Place the uterus in a small volume of M2 medium in a 35-mm plastic tissue culture dish.
3. The next step depends on which way the uterus will be flushed:

   Option 1 (from Cervix toward Oviduct)

   - Because the utero-tubal junction acts as a valve, it should be cut lengthwise to allow flushing.

- Insert the needle into the cut cervix and slide it into the base of each horn to flush. Flush each horn with ~0.2 ml of M2 medium using a 26-gauge hypodermic needle and a 1-or 2-ml syringe.

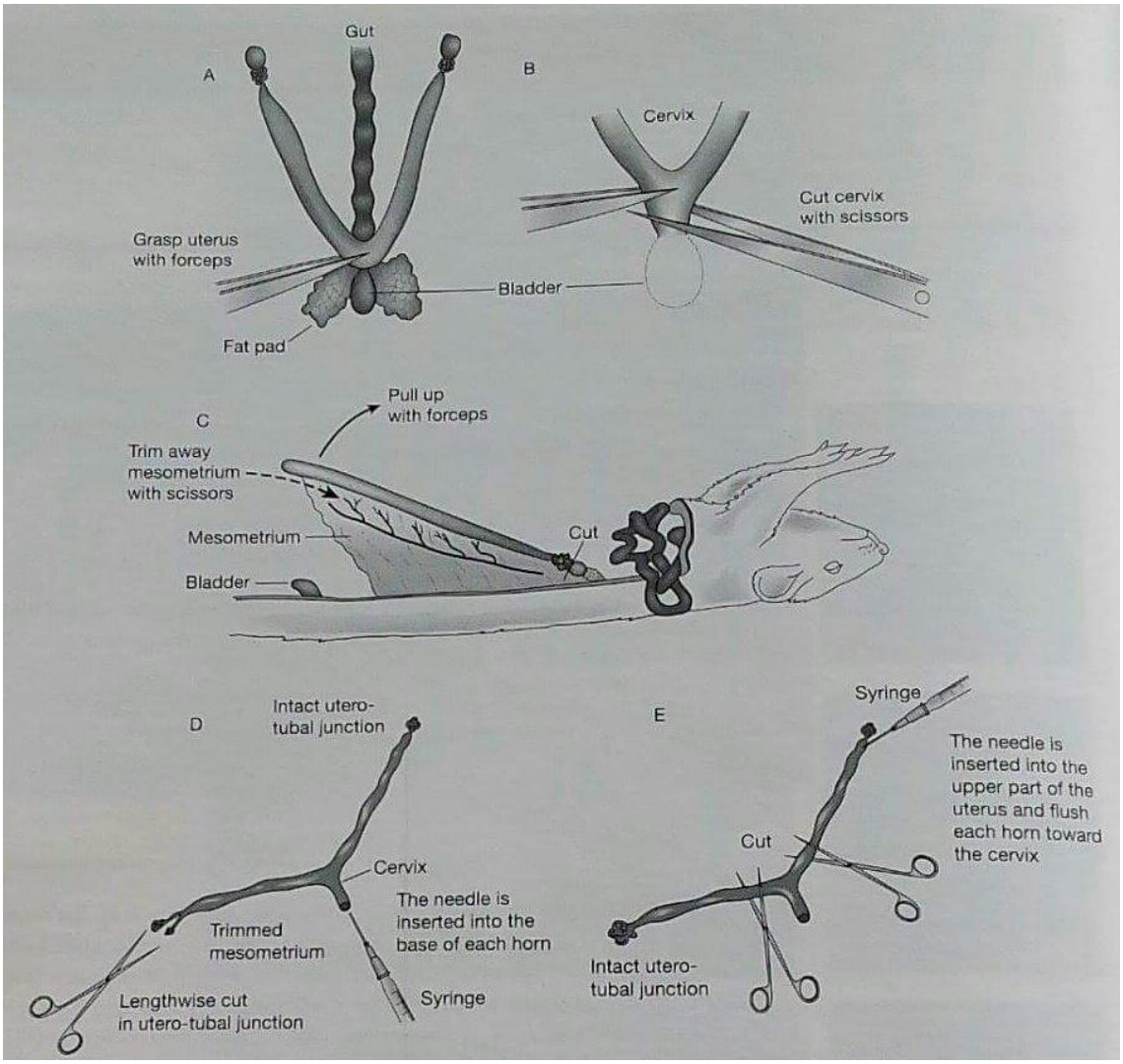


FIGURE 4.9. Dissection for flushing embryos from the uterus (A, B) The uterus is removed by cutting across the cervix. (C) The membrane (mesomtrium) holding the uterus to the body wall is trimmed away, and the uterus is cut, keeping the junction with the oviduct intact. (D) The uterus is placed in a small volume of M2 medium, utero-tubal junction is a cut lengthwise, and each horn is flushed from the cervix. (E) The uterus is cut near the cervix, the needle is inserted into the upper part of the uterus, and each horn is flushed toward the cervix.

Option 2

- Cut each horn near the cervix.
- Insert a 26-gauge needle into the upper part of the uterus near utero-tubal junction and flush each horn toward the cervix. It is also possible to flush both oviduct and uterus through the infundibulum.

4. Use a pipette to pick up the embryos and wash them through several drops of fresh M2 medium to rinse off the debris. Then transfer the embryos to a microdrop culture dish at 37ºC, 5% CO2 until needed.

Microinjection of mouse zygotes

Material and Equipament

Microinjection setup

Fertilized mouse embryos 0,5

KSOM

M2

Procedure

1. Use an embryo pipette to transfer a group of fertilized oocytes into the injection chamber. The number of zygotes to be moved into the microinjection drop should be determined by skills of the injector and quality of the setup. Do not attempt to work with more zygotes than can be injected within 20-30 min

2. examine the zygotes under high power, making sure that two pronuclei are visible and that the morphology is good. Discard all zygotes that appear abnormal.

3. To ensure that the injection pipette is not closed at the tip or clogged, place the tip of the microinjection pipette close to a zygote in the same horizontal plane as the midplane of the zygote. Apply pressure using the regulator of the injector

    a. If the pipette is open, a stream of DNA will move the zygote away from the tip of the injection pipette

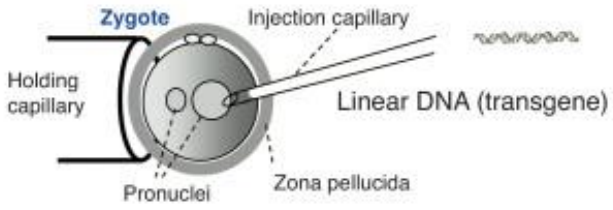
    b. If the pipette tip is closed or clogged, flush DNA with high power through the injection pipette by using the “clear “ function on FemtoJet. Repeat the test. If the tip is still not open, tip it carefully on the holding pipette and so break up to a larger tip diameter. If the diameter becomes too large, or tip is still not open, discard the pipette and use a new one.

4. To prepare a zygote for microinjection, place the tip of the holding pipette next to the zygote and suck it onto the end of the pipette by applying a negative pressure to the pressure control unit.

5. Focus the microscope to locate the pronuclei.

    a. If it is necessary to reorient the zygote to place the pronucleus in a better position, release the zygote from the holding pipette, use the microinjection pipette and/or the holding pipette to rotate slightly, and then suck the zygote back onto the holding pipette.

6. When satisfied with the position of the zygote, give the syringe controlling the holding pipette an extra twist to be sure the zygote is held firmly. Either of the two pronuclei may be injected.
7. Refocus on the pronucleus to be injected.
8. Move the injection pipette to a 3o'clock position without changing its vertical level. Push the injection pipette through the zona pellucida, into the cytoplas, and toward the pronucleus. Make sure that both the tip of the pipette and the outline of the pronucleus remain in focus.
    a. Avoid touching the nucleoli as they are very sticky and will adhere to the pipette.



9. If the pronucleus swells visibly, it ha been successfully injected! Quickly pull the pipette out of the zygote.

Microinjection of Blastocyst

Material and Equipament

Blastocysts

30 ml of injection media

Filtered light white mineral oil (Sigma M-3516 embryo-tested or equivalent)

DPBS

ES cell suspension (1 × 106/ml)

Holding pipette

Injection pipette

Dissecting microscope (10×/23 eye pieces, 1.0 x objective, 0.6 to 6.6× zoom, with base illumination)

Microinjection setup

Procedure

Transferring embryos to microinjection dish

Blastocysts are more tolerant to pH and temperature changes than that of other earlier stage embryos such as zygotes. Cooling the temperature will delay the onset of blastocyst hatching. Keeping blastocysts in the cold injection chamber too long might be detrimental to their vitality.

1. Take the dish containing the blastocysts with mineral oil and injection media out of the incubator.
2. With a clean and polished transfer pipette, mouth pipette light mineral oil to the hub of the transfer pipette.
3. Load the blastocysts inside the transfer pipette from the dish containing the blastocysts.

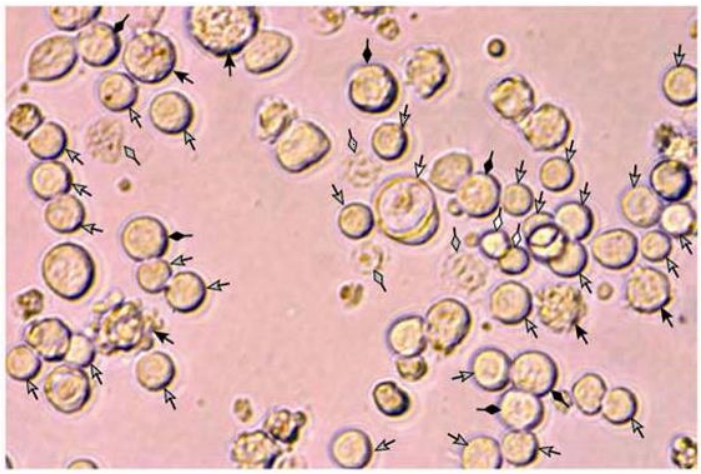
4. Take the transfer pipette and gently expel the embryos onto the chamber of the injection dish. Only transfer the number of blastocysts to the injection dish that can be injected within a half hour.

Injection of ES cells

When first learning ES cell injections, 200× magnification should be used. Focus on the outer ring of trophoblast cells and then the tip of the injection pipette before attempting to inject inside the blastocoel cavity. This ensures that the injection pipette is in the correct plane of the z-axis, being approximately in the center of the blastocoel cavity. After becoming proficient in ES cell injections, a tapping technique, which looks at indentations on the blastocyst, can be employed to make sure the injection pipette is in the right plane. 100× will work well with this procedure and has the advantage of a broad field of view to collect the ES cells, and changing the objective is not required. The general range for the number of ES cells that can be injected inside a blastocyst is considered to be between 10 and 20. The preference is to inject 15 ES cells, which balances the chance of germline transmission versus normal homeostasis in embryonic development. It is advisable not to inject more than 20 ES cells in that it is thought to be detrimental to the developing mouse embryo.

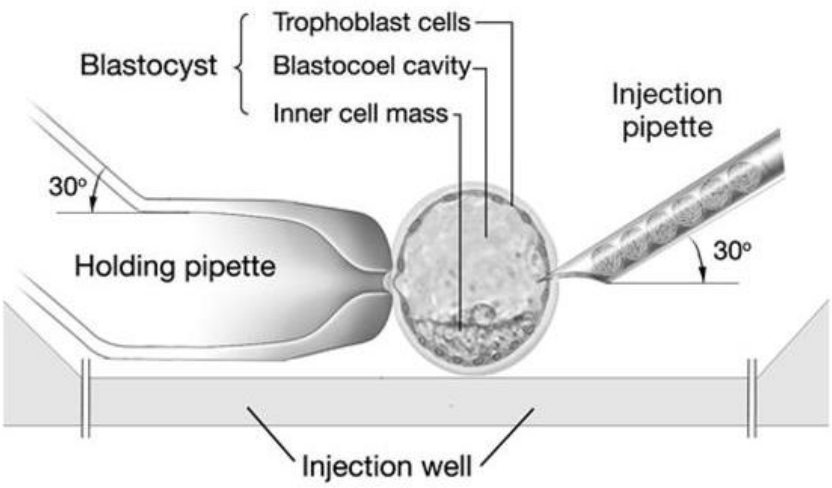
1. Load 15 ES cells inside the injection pipette with as little injection media as possible.

Note: The cells do not have to be touching each other when inside the injection pipette. If the cells are touching, there is a possibility that the cells may stick to each other, and many of the cells may come out of the blastocyst when withdrawing the injection pipette.



2. Orient the blastocyst so that the inner cell mass (ICM) is in the 6 o'clock position.

3. With the holding, uptake injection media inside until the blastocyst is held securely to the holding pipette. Lower the holding pipette until the blastocyst is just touching the bottom of the injection dish.

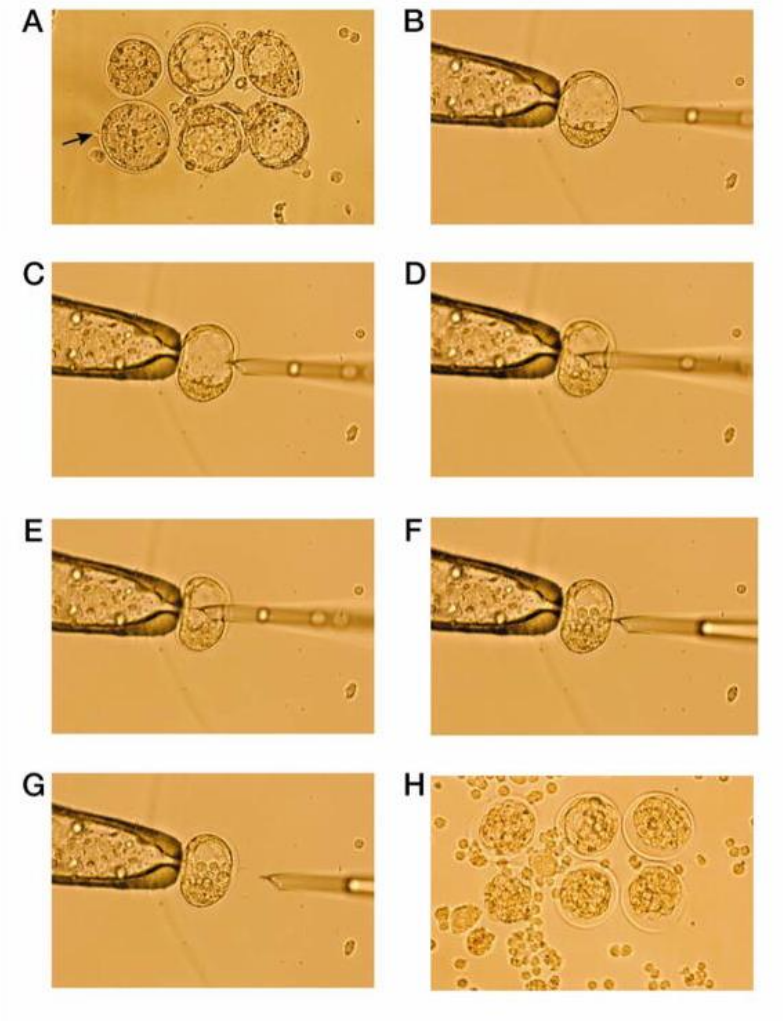


4. Focus on the zona and outer trophoblast layers of the blastocyst, and then move the injection pipette so it is also in focus. This puts the injection pipette in the correct plane of the z-axis.

Note: It is better to inject at the junction between the trophoblast cells, reducing damage to the embryo. Sometimes this junction may be hard to see.

5. With a quick, short popping and jabbing motion, insert the injection pipette inside the blastocyst cavity, being careful not to touch the ICM. Pull back the injection pipette slightly after inserting into the blastocoel cavity.
6. Gently expel the ES cells inside the blastocoel cavity onto the ICM, being careful not to inject oil into the blastocoel cavity.
7. Withdraw the injection pipette slowly out of the blastocoel cavity, preventing the expulsion of the ES cells outside of the blastocyst. Allow the injection site to close before fully withdrawing the injection pipette. Letting some media escape will enhance the settling of the injected ES cells onto the ICM.

Note: When injecting, consider the pressure inside the blastocoel cavity. If too much media is injected inside the blastocyst or too much holding force is applied by the holding pipette, the injected ES cells may be forced out of the blastocoel cavity.



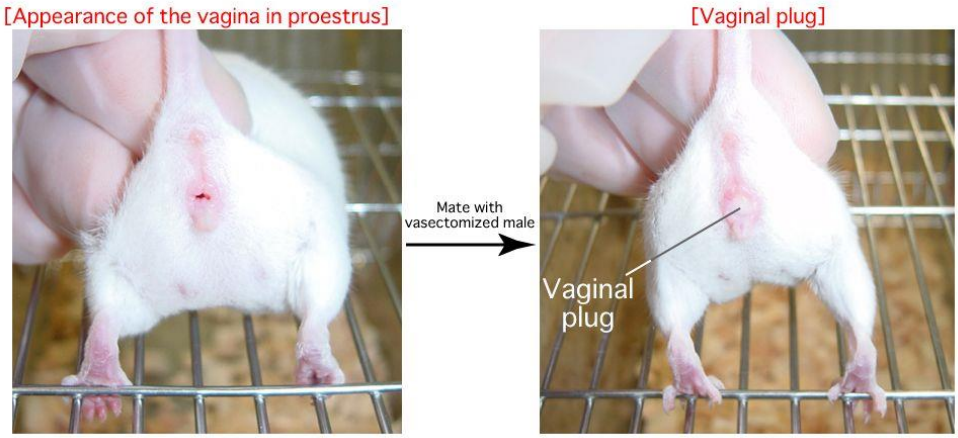
8. After injection of the blastocyst, move it to an area away from the uninjected ones on the injection dish.
9. When all blastocysts on the injection dish have been injected, with a transfer pipette place the injected blastocysts on a microdrop dish with only media and oil and put back into the incubator.
10. Allow the blastocysts to reexpand in culture. Reexpansion of the blastocyst is a possible sign of its vitality.

Note: All blastocysts should be injected within a half hour of putting them on the injection dish, and the ES cell clones should be injected within 2 hours after putting them on the injection dish. If both pipettes are flushed out with oil, they have the potential to be reused for another injection day.

EMBRYO TRANSFER INTO THE OVIDUCT

Material and Equipment

1.Female mice on Day 1 of pseudopregnancy (the day on which a vaginal plug is observed)

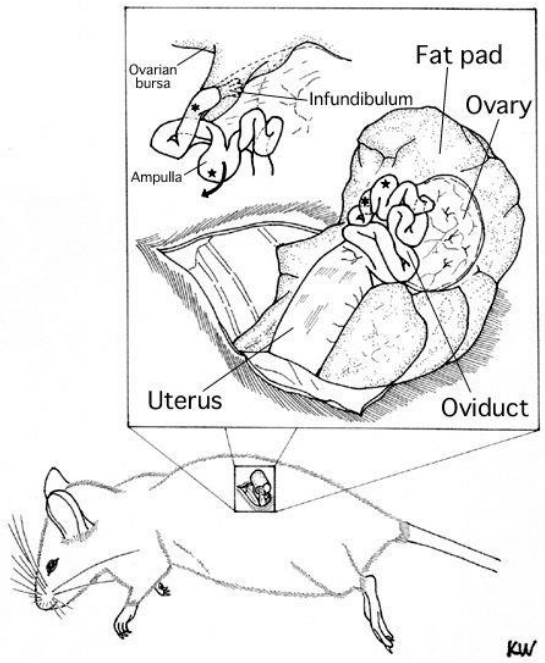


2. Micro-spring scissors (5mm blade)
3. Pair of watchmaker's #5 forceps
4. Serrefine clamp
5. Wound clip (Autoclip 9mm; Clay Adams 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams 427630)
6. Plastic dish (35mm X 10mm Cat.No.430588; CORNING)
7. Glass capillaries for embryo transfer and handling

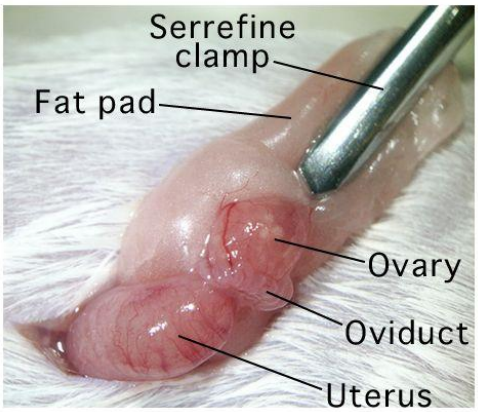
Procedure

Preparation of Mice

1. Anesthetize a female mouse.
2. Pull out the ovary, oviduct, and part of the uterine horn as per the conventional procedure.

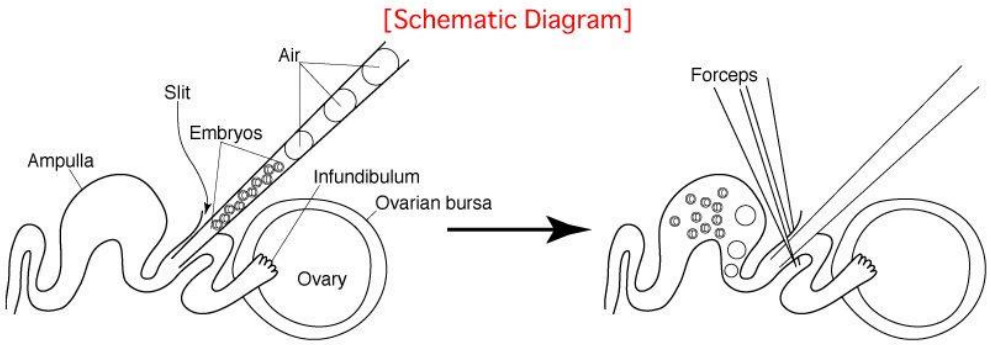


3. Clip a serrefine clamp onto the fat pad which is attached to the ovarian bursa.
Positioning of Oviduct

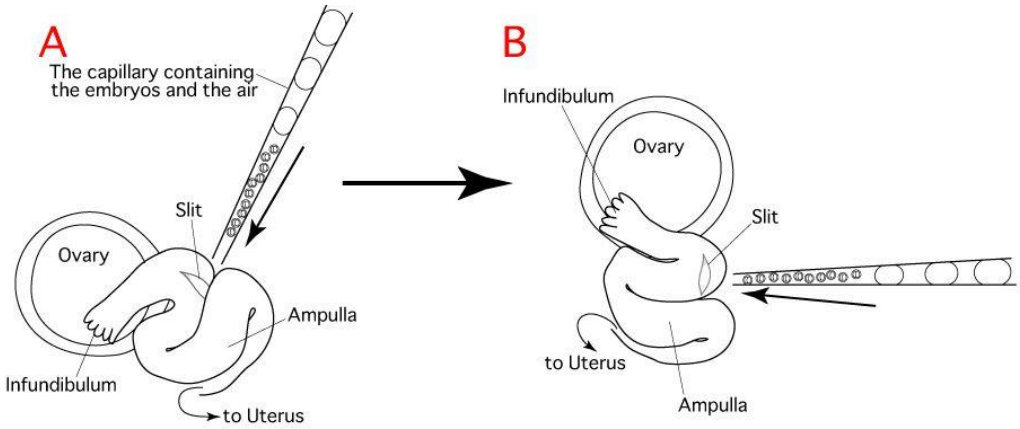


Positioning of Oviduct

As indicated in the diagram below, embryo transfer into the oviduct is carried out by cutting the oviduct, inserting a capillary thereinto and expelling embryos towards the ampulla.



Unfortunately, the oviducts of mice are small and the ducts are folded in a complicated manner, as we can see in the schematic diagram of an exteriorized oviduct below (A).
This makes it very difficult to insert the capillary into the oviduct towards the ampulla, because the insertion is made from above.
To make this procedure easier, position the oviduct by changing the position of the serrefine clamp and the mouse before starting the operation (B).

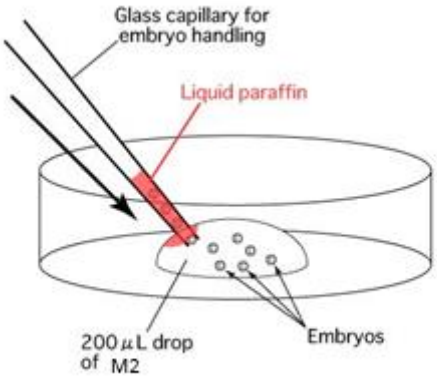


1. Observe the oviduct under a stereomicroscope and confirm the position of the infundibulum and ampulla using the tip of a set of forceps, or by changing the position of the serrefine clamp.
2. Position the oviduct by changing the position of the serrefine clamp and the mouse.

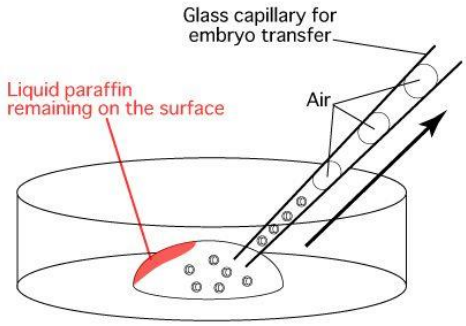
Note: Because the folds in the oviduct vary between each mouse, look closely and adjust the position of the oviduct to make easier to work on.

Preparation of Embryos and Glass Capillary

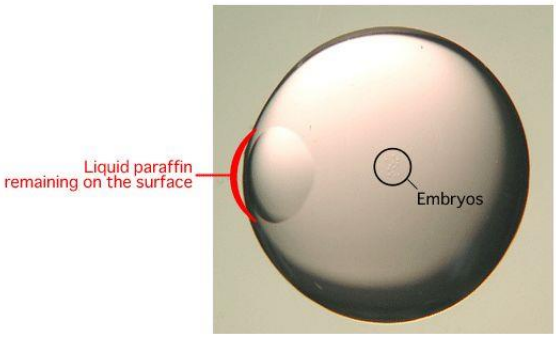
1. Make a 200μL drop of M2 in a dish (without liquid paraffin), and introduce 20 embryos into the drop.



2. Aspirate air and medium in alternate intervals of 2-3mm into a glass capillary in preparation for embryo transfer. Draw ten embryos into the glass capillary.



Comment: When the glass capillary is first inserted into the drop, some liquid paraffin will remain on the outer surface of the drop as shown below. The embryos should be drawn into the glass capillary from the opposite side of the drop to avoid sucking up any liquid paraffin. Evidence suggests that liquid paraffin which passes into the oviduct may have adverse effects on the development of the embryos into offspring.



Embryo Transfer

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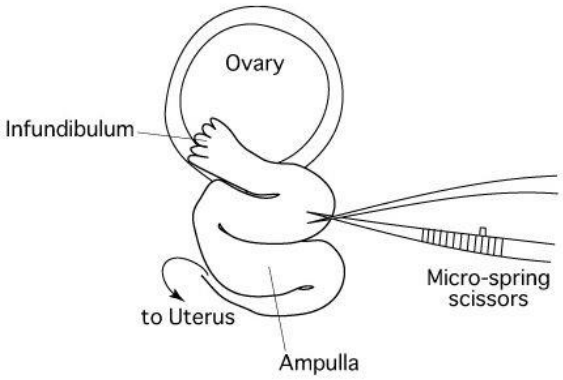
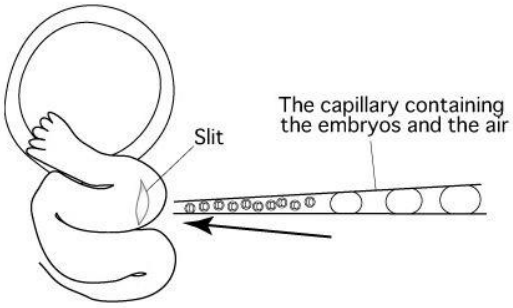
1. Using a pair of watchmaker's #5 forceps and micro-spring scissors, dissect the wall of the oviduct between the infundibulum and ampulla.



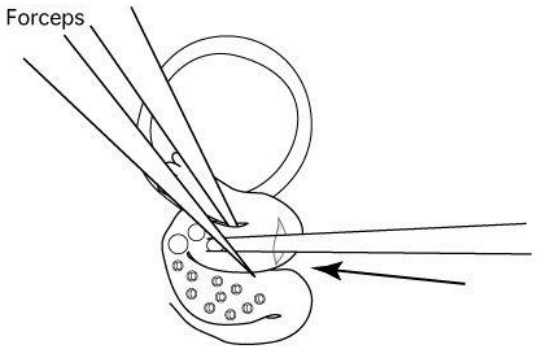
2. Insert the tip of the capillary containing the embryos into the slit, then push the capillary further into the slit towards the ampulla.



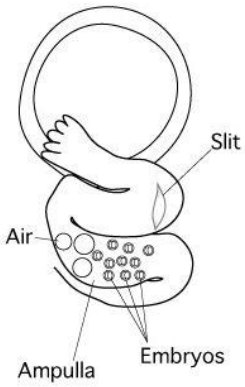
3. Use the forceps to hold the portion of the oviduct into which the capillary was inserted.
4. Expel the embryos and 2-3 of the air bubbles into the ampulla.

Comment: If performed successfully, you should be able to see air bubbles through the wall of the ampulla.

Note: If you cannot expel the embryos and the air bubbles into the oviduct, pull the capillary back out just a little from the slit and expel them again.

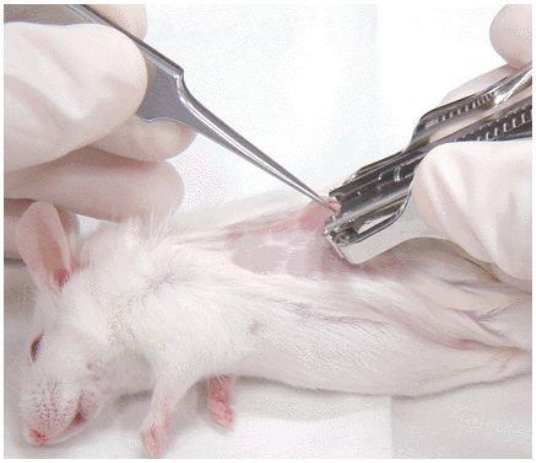


5. Withdraw the capillary gently from the slit.



Note: Transfer the embryos after adjusting the position and direction of the oviduct. If the oviduct is aligned parallel to the capillary, then it will be easier to insert the capillary into the oviduct.

6. Push the ovary, oviduct and uterine horn back into the abdomen and close the wound using wound clips.



7. Repeat the process to transfer the remaining 10 embryos into the other oviduct as described above.
8. Keep the mice warm on a 37°C warming plate until the mouse recovers from the effects of the anesthesia.

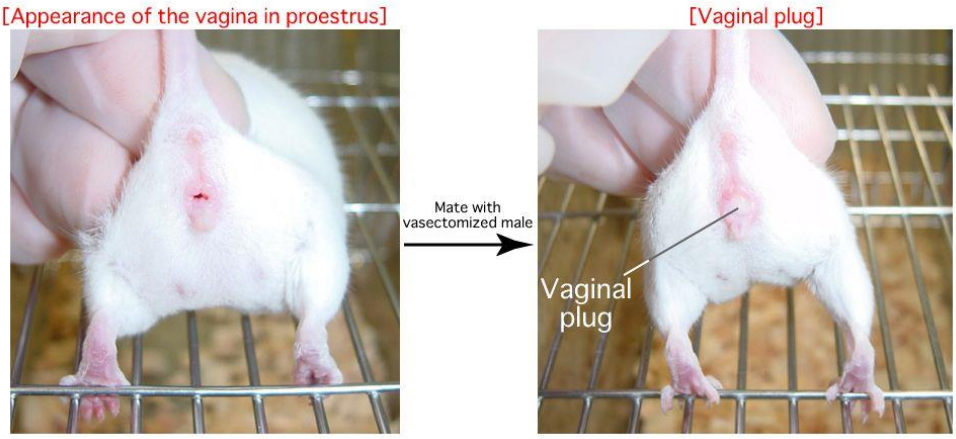
References

Nakagata N. 1992. Embryo transfer through the wall of the fallopian tube in mice. Exp. Anim. 41: 387-388.
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.

EMBRYO TRANSFER INTO THE UTERUS

Material and Equipment

1.Female mice on Day 2,5 of pseudopregnancy (the day on which a vaginal plug is observed)

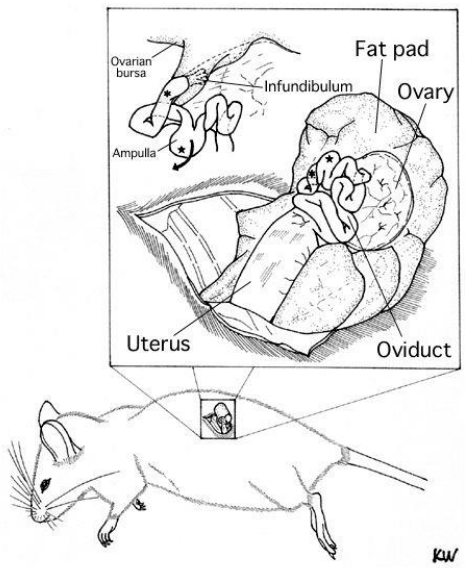


2. Micro-spring scissors (5mm blade)
3. Pair of watchmaker's #5 forceps
4. Serrefine clamp
5. Wound clip (Autoclip 9mm; Clay Adams 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams 427630)
6. Plastic dish (35mm X 10mm Cat.No.430588; CORNING)
7. Glass capillaries for embryo transfer and handling

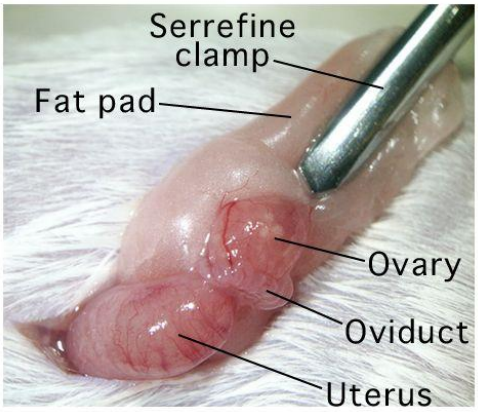
Procedure

Preparation of Mice

1. Anesthetize a female mouse.
2. Pull out the ovary, oviduct, and part of the uterine horn as per the conventional procedure.

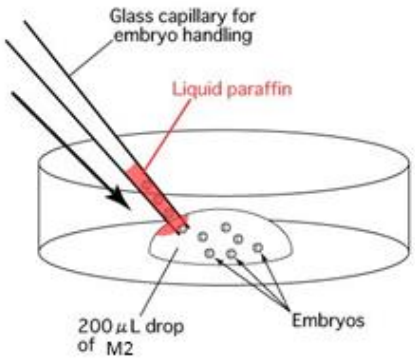


3. Clip a serrefine clamp onto the fat pad which is attached to the ovarian bursa.
Positioning of Oviduct

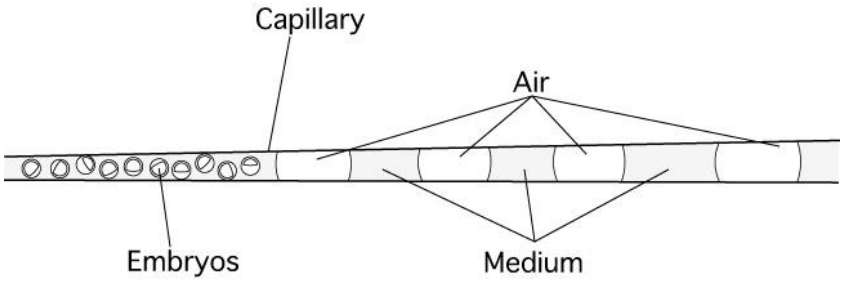


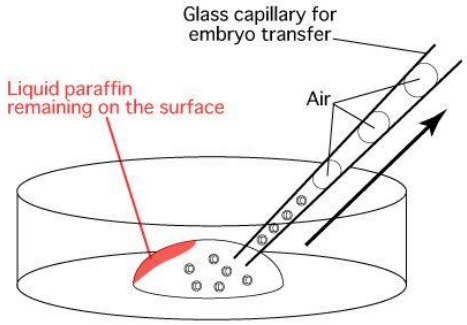
Preparation of Embryos and Glass Capillary

1. Make a 200µL drop of M2 in a dish (without liquid paraffin), and introduce 20 embryos into the drop.

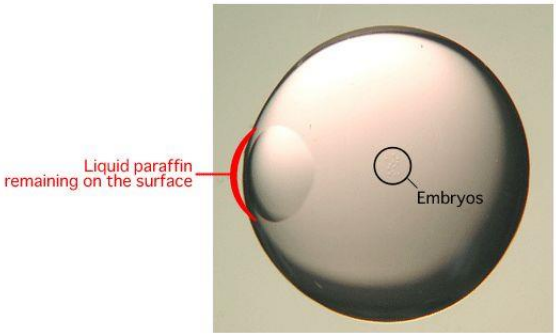


2. Aspirate air and medium in alternate intervals of 2-3mm into a glass capillary in preparation for embryo transfer. Draw ten embryos into the glass capillary.





Comment: When the glass capillary is first inserted into the drop, some liquid paraffin will remain on the outer surface of the drop as shown below. The embryos should be drawn into the glass capillary from the opposite side of the drop to avoid sucking up any liquid paraffin. Evidence suggests that liquid paraffin which passes into the oviduct may have adverse effects on the development of the embryos into offspring.

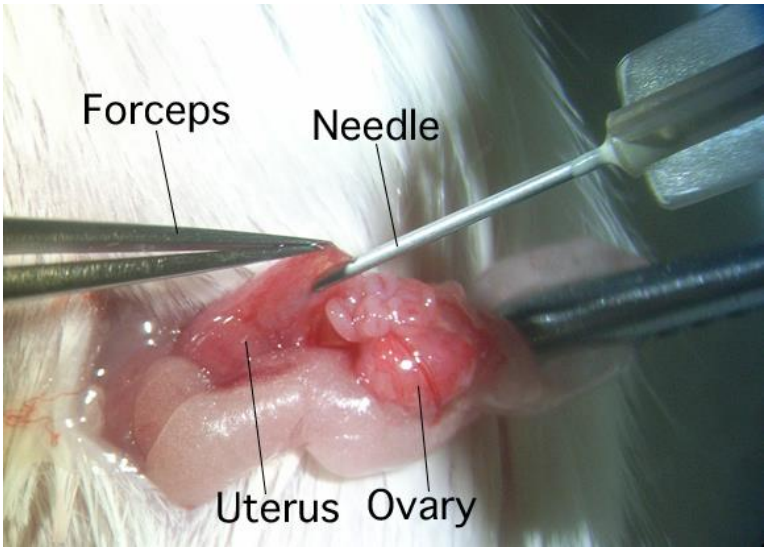


Embryo Transfer

1. use blunt fine forceps to pick up the ovarian fat pad and pull out the attached left ovary, oviduct and upper part of the uterus. Clip a Serrefine clamp onto the fat pad and lay it down over middle of the back, so that the ovary, oviduct and uterus remain outside the body wall.

2. Gently hold the top of the uterus with blunt fine forceps and use a 26- or 30-gauge ½-inch needle with the bevel facing up to make a hole in uterus a few millimeters down from the utero-tubal junction.

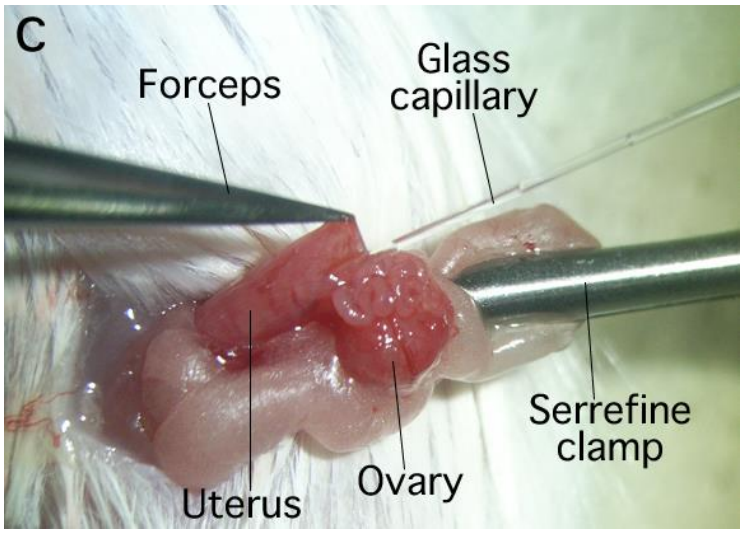
Note: avoid the small blood vessels in the uterine wall. If bleeding occurs, use a tissue to removal the blood.



3. Make sure that the needle has entered the uterine lumen and has not become lodged in the wall of the uterus.
    a. To test whether the needle has entered the lumen, pull it out slightly. If it slides easily, the needle has penetrated the lumen.

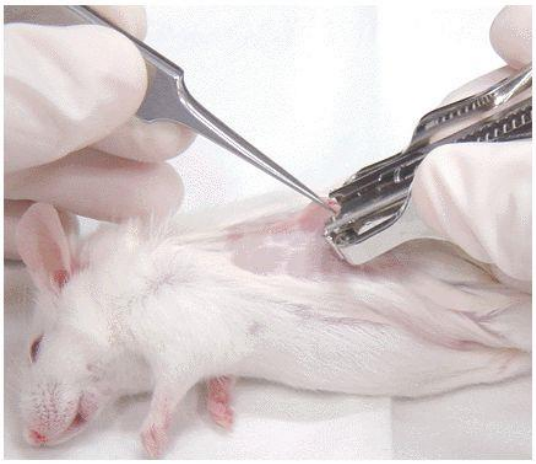
Attention: do not move the needle too much or the wall of the uterus may be lacerated. Watch the angle and depth of the needle's penetration and keep it parallel to the horn.

4. Keeping a eye on the hole made by needle, pull out the needle and insert -5mm of the prepared transfer pipette containing the blastocysts into the hole. Blow gently on the transfer pipette until the air bubbles closest to the blastocysts are at the tip of the pipette and all the blastocyst have been expelled. Watch the movement of the air bubbles in the pipette and remove the pipette when first bubble reaches the opening in the uterus.



Note: transferring too much bubbles into the uterus should be avoid because they might interfere with implantation.

5. Unclip the Serrefine clamp and use blunt fine forceps to pick up the fat pad and place the uterus, oviduct and ovary back inside the inside the body cavity. Sew up the body wall with one or two stitches and close the skin with wound clips.



6. At the end of the procedure, place a mousse in a clean cage and keep it warm on a warming plate until the mouse recovers from an injected anesthetic.

7. Repeat the process to transfer the remaining 10 embryos into the other oviduct as described above.

8. Keep the mice warm on a 37°C warming plate until the mouse recovers from the effects of the anesthesia.