Microsurgical Techniques in the Genetic Engineering of Mammalian Embryos at the Preimplantation Stage

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The genetic engineering of mammalian whole embryos has become one of the most popular and fruitful techniques in modern developmental biology, not only to investigate the normal development and pathogenesis of diseases but also to develop future gene-therapy applications for human genetic diseases.

Although the rapid advancement of molecular biological techniques has created a theoretical basis for genetically engineering mammalian embryos, it has become practical only after the establishment of the culture system for mammalian embryos, which normally develop (hidden from the human eye) in the oviduct and the uterus, the improvement of optic instruments, and finally, the development and establishment of microsurgical techniques.

In this article, we describe the microsurgical techniques that are utilized in the genetic manipulation of mouse embryos at the preimplantation stage as well as some related surgical techniques on the maternal organs.[1,2,3,4,5,6,7] The techniques described are the microinjection into the pronucleus and the enucleation of the pronucleus at the one-cell stage, the embryo transfer into the oviduct or the uterus of the pseudopregnant female mouse, and the transplantation of the ovary. A few other related techniques are also briefly mentioned.

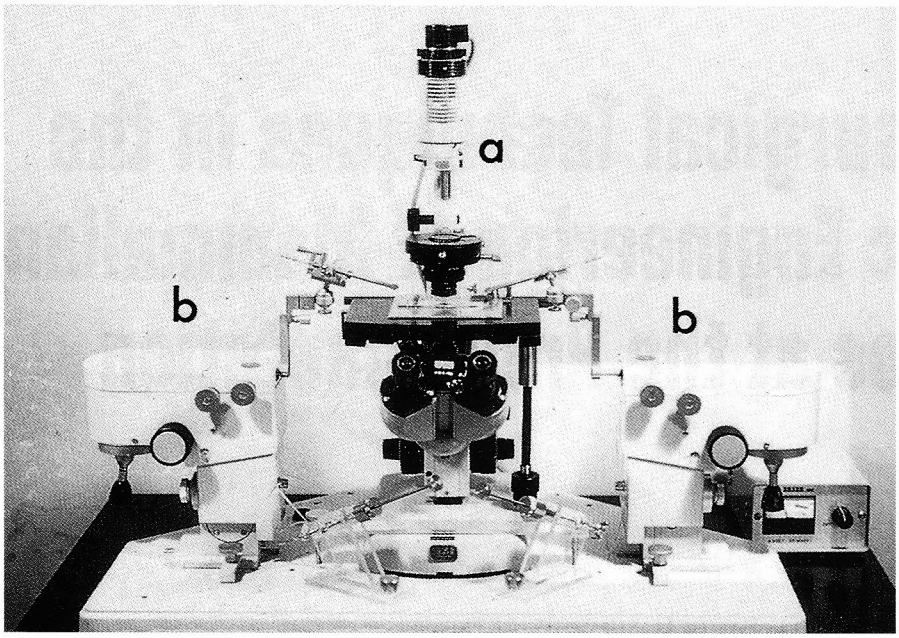


Figure 1. Setup for the micromanipulation of the preimplantation stage mouse embryos. a: Invert Scope D (Carl Zeiss), b: Micromanipulators (Leitz).

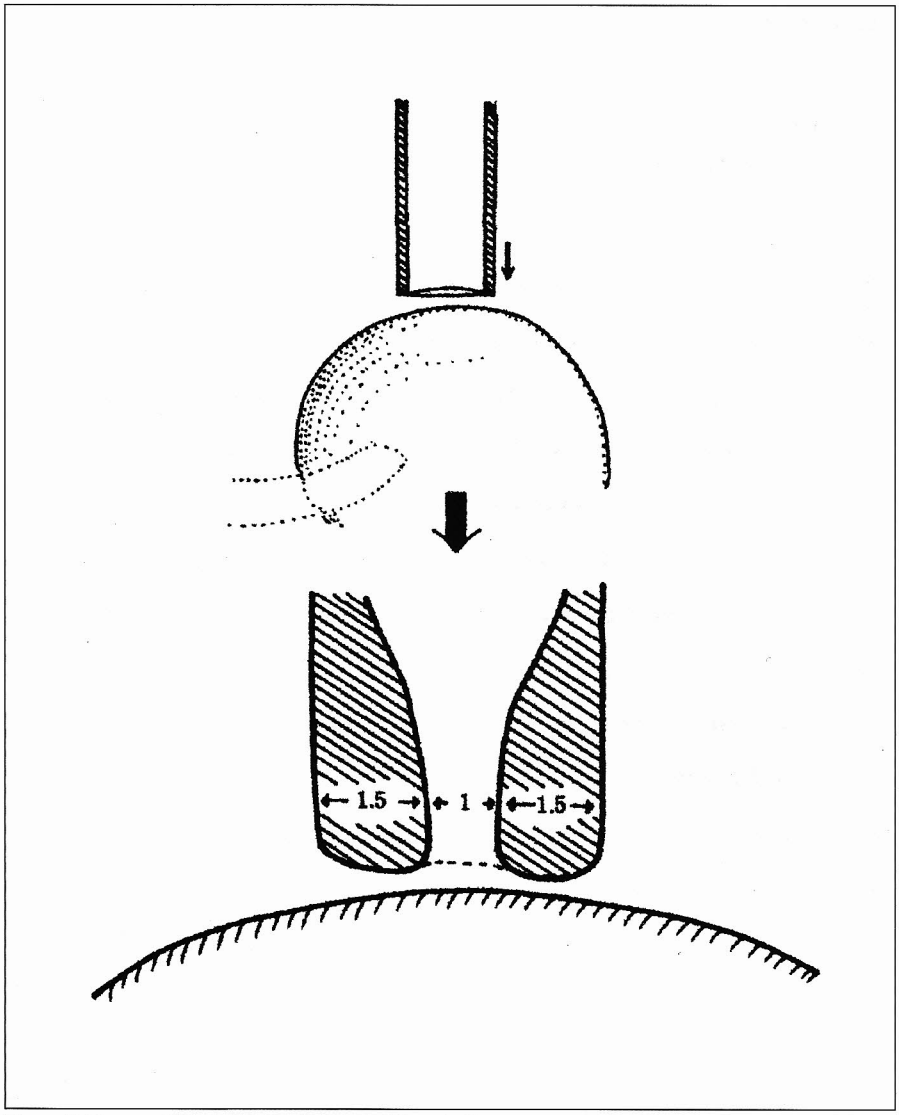


Figure 2. Flame-polishing of the end of the holding pipette.

MOUSE EMBRYO SYSTEM FOR GENETIC ENGINEERING

Although the mouse system needs finer or more intricate techniques and devices than other larger animal systems, it has definite advantages in biomedical research, especially in genetic investigations. There are a wide variety of established normal and abnormal (wild type and mutant) mouse strains and a wealth of background information on their genetics. The reproduction characteristics of mice, including a relatively short reproduction period (gestation period of 20 days, sexual maturation at about eight weeks of age) and larger litter sizes (around ten or more), are beneficial. In addition, their small size is an advantage, particularly since genetic analysis usually requires large specimen numbers. Although the whole system, including the recovery of good eggs and the culture of preimplantation-stage embryos, is important, we only describe procedures and setups directly related to the embryo manipulation.

MICROSURGERY ON ONE CELL STAGE MOUSE EMBRYOS

The microinjection into the pronucleus, the enucleation of the pronucleus, and the nuclear transplantation are all performed at the one-cell stage - at the pronucleus stage, when the male and female pronuclei, each derived from the sperm and the oocyte, have not yet fused to form the zygotic nucleus. Basically, embryos are placed in a drop of medium covered by paraffin oil on a glass slide, which is set on a high-quality light microscope of the inverted type. On one side of the microscope, embryos are held by a glass holding pipette and, on the other side, they are manipulated by a glass manipulation micropipette made for each operation.

a) Instruments and setup

Since mouse embryos from one cell to morula stages are only about 0.1 mm in diameter, the quality of the optics and of the micropipette is critical. Many different laboratories are using similar setups, but our system has a high efficiency and productivity.[1] A high-quality light microscope of the inverted type equipped with a Nomarski device (we have used two types: Invert Scope D, Carl Zeiss, Germany; IMT-2, Olympus, Tokyo, Japan) and a pair of fine micromanipula-

tors (Leitz, Germany) (Figure 1) are necessary to obtain stable results. Pipettes are made using a microelectrode puller (PN-3, Narishige Scientific Instrument Lab, Tokyo, Japan), a pipette grinder (MGC-II, Chatani Limited, Tokyo, Japan) and a microforge (DeFonbrune, France; MF-79, Narishige Scientific Limited, Tokyo, Japan). To make holding pipettes, hard-quality glass tubes (G1.2, Narishige Scientific Limited, Tokyo, Japan) are pulled by the puller and then cut with the microforge to make the edge about 90–95 μm in diameter. The edge is heat-blunted by the heated glass ball ("anvil") on the platinum filament so that smooth holding surface are made with an remnant orifice of about one fourth of the diameter to hold the egg by a negative pressure (Figure 2). Manipulation pipettes are pulled similarly but with a little stronger tapering since they are used at the very end of the needle and do not cut. For microinjection of DNA or other solutions, the tip of the needle is lightly ground at an angle of 30–45° between the needle and the grinder surface so that the diameter of the tip becomes 1–2 μm. Then the tip is washed through concentrated hydrogen sulfate, and then twice washed in distilled water with a positive pressure applied continuously to prevent the capillary action. Pipettes for enucleation are ground to make tips of about 5 μm in diameter and washed similarly. Holding and manipulation pipettes are finally bent using the microforge to about 15–30°, depending on the setup (Figure 3), so when they are set in the manipulator, the ends lie parallel to the bottom surface of the glass slide and the tip of the manipulation pipettes can be seen only in profile (Figure 4). Pipettes are connected with Hamilton syringes (0.5 ml, #81000, USA) by the polyethylene tube. The syringe to the pipette must be filled with paraffin oil without any air bubbles to ensure that the precise pressure is applied from the syringe to the tip of the pipette. The distal half of the holding pipette is filled with medium and the manipulation pipette is filled with paraffin oil to the distal end. Pipettes are placed in the manipulator set, and before the manipulation, their tips are properly positioned (and conveniently movable) in the center of the visual field of the medium. After the manipulation, embryos are transferred to the medium drops covered with paraffin oil and cultured *in vitro* until they are transferred to the oviduct or the uterus of pseudopregnant females.

b) Microinjection into the male pronucleus

A drop of the medium and a drop of the injection solution are prepared in the small well created on the glass slide and are covered with paraffin oil. Pronucleus-stage embryos are transferred to the drop of the medium. The number of embryos that are handled at one time depends on the skill of the manipulator, since there is a limit to the time embryos can stay outside the culture. The male pronucleus is larger than the female pronucleus and usually used for the microinjection of solutions. The glass slide with embryos and the injection solution are put on the manipulator set, and then the solution is aspirated into the injection pipette by the negative pressure applied by the syringe. Using the holding pipette, the embryo is held with its male pronucleus on the line between the ends of the manipulation and holding pipettes and at the periphery of the cytoplasm facing the manipulation pipette (Figure 4). Under a higher magnification view (x400 or higher), the position of the injection pipette is adjusted so that the tips of both pipettes, the zona pellucida and the male pronucleus align on the same focal plane. The pipette is then inserted. With a slight positive pressure in the syringe, we can see that the solution is injected into and expands the pronucleus. The injection pipette is quickly removed, and with the holding pipette under a lower magnification, the injected embryo is placed to one corner of the drop, an uninjected embryo is brought from the other corner and the injection procedure is repeated.

c) Enucleation from the pronucleus stage embryos

The basic setup and procedures are the same to that of microinjection. However, enucleation pipettes have a tip of about 5 μm and a nail has to be created at the very tip using the microforge to make the insertion of the thicker needle easy and thereby reducing the damage to the cell. The nail is created by lightly and briefly touching the very tip of the pipette's oval opening to the heated glass ball ("anvil") on the platinum wire, while a controlled amount of air flow is applied to prevent radiating heat to melt the tip of the pipette. Just before the enucleation in the drop of the medium, a small amount of the medium is aspirated into the pipette to create a meniscus of the medium, which serves as an indicator of the strength of the negative pressure applied during enucleation (Figure 4b). Pretreatment of embryos with cytochalasin B at 5 mg/ml at 37°C for 40 minutes to 1 hour is necessary to disorganize the cytoskeleton and minimize the damage to the cell due to the enucleation procedure. The ends of both pipettes, especially of the enucleation pipette, the male or female pronucleus, and the zona pellucida are accurately placed on the same plane of the focus (Figure 4a), and the enucleation pipette penetrates the zona and indents (not penetrates) the plasma membrane of the pronucleus (Figure 4b). By carefully applying negative pressure, the pronucleus, together with the surrounding plasma membrane and minimal accompanying cytoplasm, is aspirated into the pipette, and the pipette is slowly pulled out so that the membrane with the pronucleus (pronuclear karyoplast) is pinched off from the rest of the cell without breaking the continuity of the plasma membrane of both the vesicle and the cell (Figure 4c).

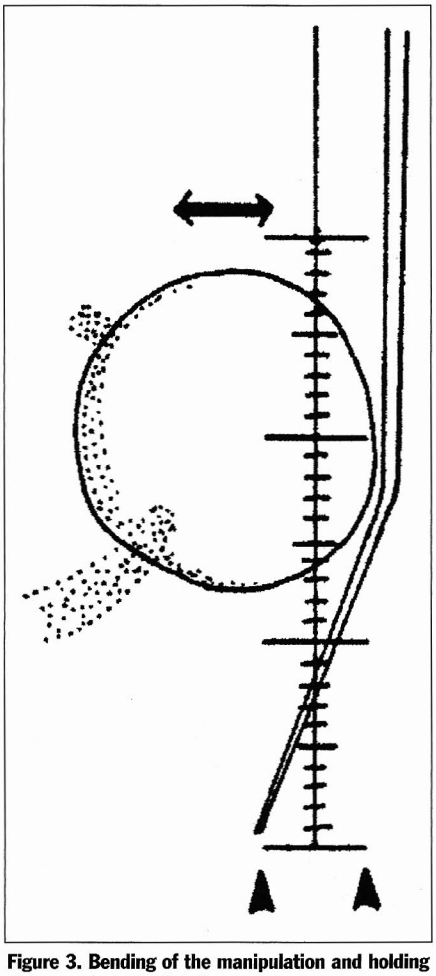
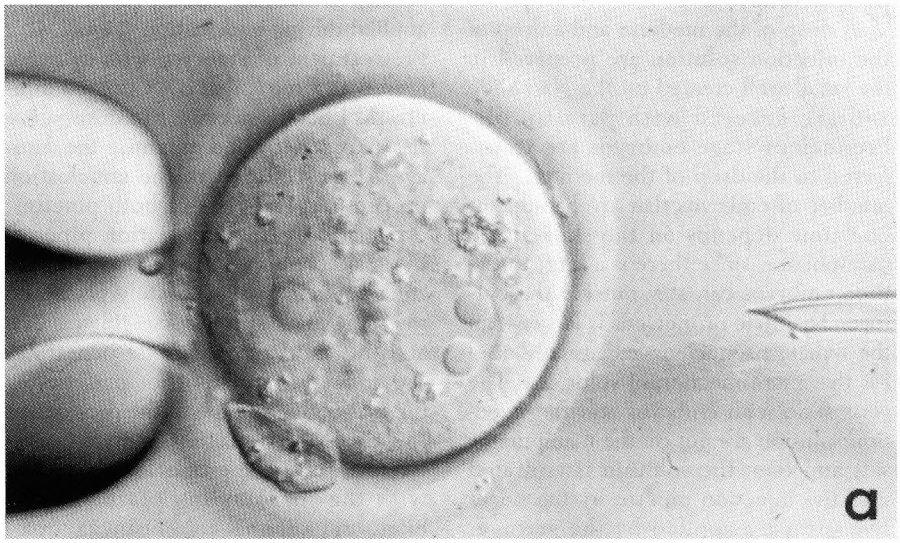
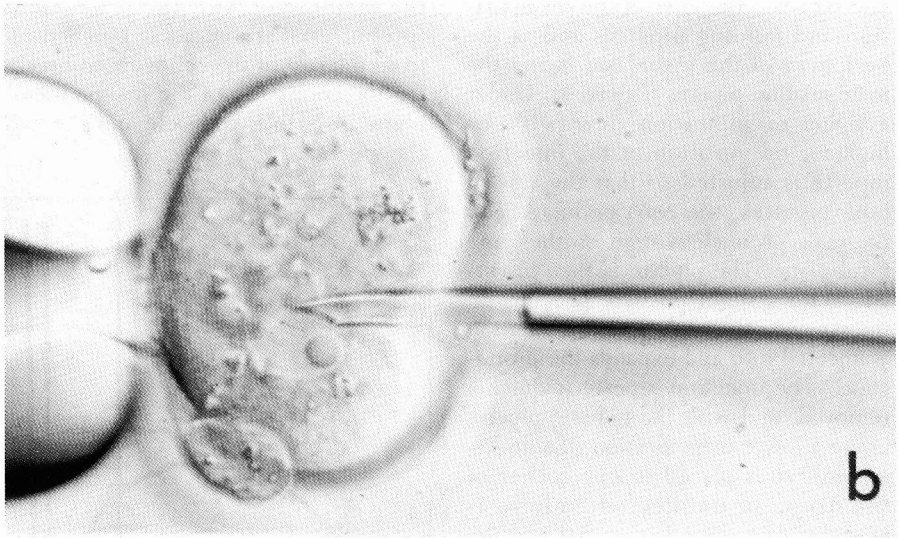


Figure 3. Bending of the manipulation and holding pipettes with the microforge.

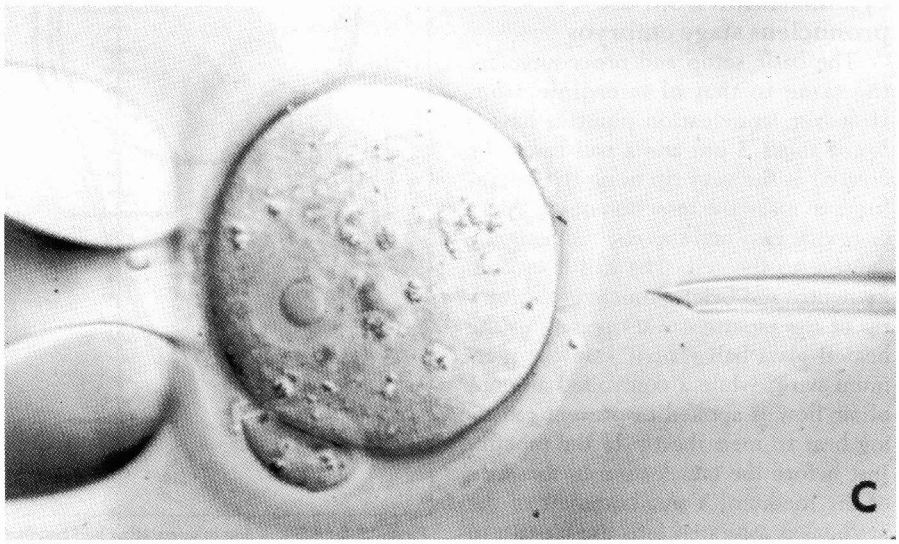
Figure 4. Enucleation of the male pronucleus from the one-cell stage mouse embryo.



a) Pipettes, the zona pellucida, the male pronucleus are aligned.



b) The manipulation pipette is inserted through the zona, indenting the plasma membrane to approach the male pronucleus.



c) The pronuclear karyoplast (see text) is removed from the embryo.

d) Other applications of the embryo manipulation

The nuclear transplantation is performed using exactly the same method described above for the enucleation, but with a few additional steps.[2] First, the host cell is enucleated. Next, the pronuclear karyoplast is removed from the donor cell and transferred through the previous site of the pipette penetration and into the perivitelline space, the space between the zona and the host cell so that the host egg cell and the donor pronuclear karyoplast attach each other. Then, using either electrofusion or co-injection of the pronuclear karyoplast with the Sendai virus, the membrane fuses and the nuclear transplantation is completed.

The "injection chimera" is created using the same setup on blastocysts.[2] Chimeras are animals composed of multiple genetically different cells. During the course of the "knock out mice" production, creation of chimeras between the embryonic stem (ES) cells, in which a specific gene is removed by the homologous recombination, and the host blastocyst is one of the critical steps. The blastocyst stage embryo, which already has a differentiated inner cell mass, the future embryo proper, trophoblasts, the future part of the placenta, and the blastocele, is held by the holding pipette so that the inner cell mass is located at the side of the holding pipette. The donor cells are aspirated into the injection pipette, introduced into the blastocele and placed onto the inner cell mass.

EMBRYO TRANSFER

Manipulated embryos are transferred for further development to either the oviduct or the uterus of the pseudopregnant female mouse, depending on the developmental stage of the embryo at the time of transfer. Embryos at one- and two-cell stages are transferred to the oviduct on pseudopregnant day 1, whereas blastocysts are transferred to the uterus on day 3. Pseudopregnant females are prepared by mating females that are at the natural or hormonally induced estrous stage of the estrous cycle with vasectomized males and confirming the vaginal plug on the next morning, 0 a.m. of which day is designated as pseudopregnant day 0.

a) Oviduct transfer

To obtain good and reproducible results, good and uniform pipettes,

avoidance of bleeding, and a short operation time are necessary. With experience, the whole procedure described below can be done within 10 minutes and more than 80% of transplanted embryos develop into newborn pups, in the case of unmanipulated embryos. We prepare special pipettes for the oviduct transfer. First, capillary tubes with an external diameter of 150–200 μm are pulled manually by heating hard glass tubes of 4 mm in diameter, cut at about 12 cm in length. They are sterilized and fixed with beeswax in a holder, which is made by cutting the thin-calibered portion of a Pasteur pipette, leaving about 3.5 cm of the capillary protruding from the holder. The end of the capillary is flame-polished, and at about 2 cm from the end, the capillary is bent at about 135° by heating with a small flame (Figure 5). Embryos are transferred from the culture dish, which has the paraffin oil cover to prevent evaporation, to the new dish that doesn't have the paraffin oil cover. This new dish is equilibrated in the CO_2 incubator and is kept in the incubator until the transfer. About ten embryos are transferred to one side of the oviduct, and one dish is prepared per pseudopregnant female host. The instruments are sterilized by wiping with cotton balls soaked in ethanol and flamed. Mice are anesthetized with the intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories) at 30 mg/kg body weight. The hairs on the back are trimmed and the back is wiped with ethanol-soaked cotton balls. A skin incision, about 1 cm, is made with the dissecting scissors on the midline of the back at the level of the last rib. The skin is moved until the incision is over the white fat pad, which surrounds the ovary on the left side. A small incision is made on the body wall over the left ovary. Next, turn the head of the mouse to the left, and using the blunt forceps,

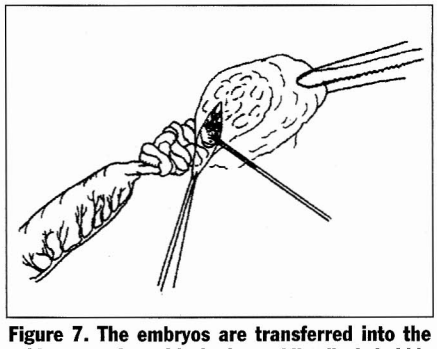


Figure 7. The embryos are transferred into the oviduct together with the lower (distal) air bubble as the indicator.

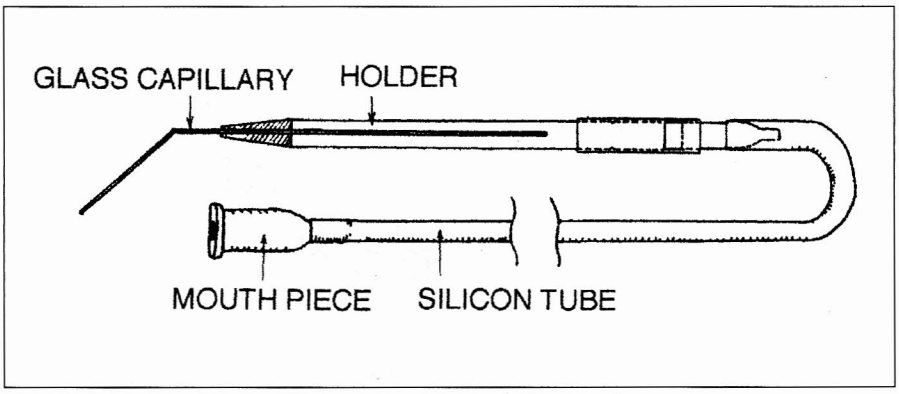


Figure 5. The pipette for the oviduct transfer connected with the mouth piece

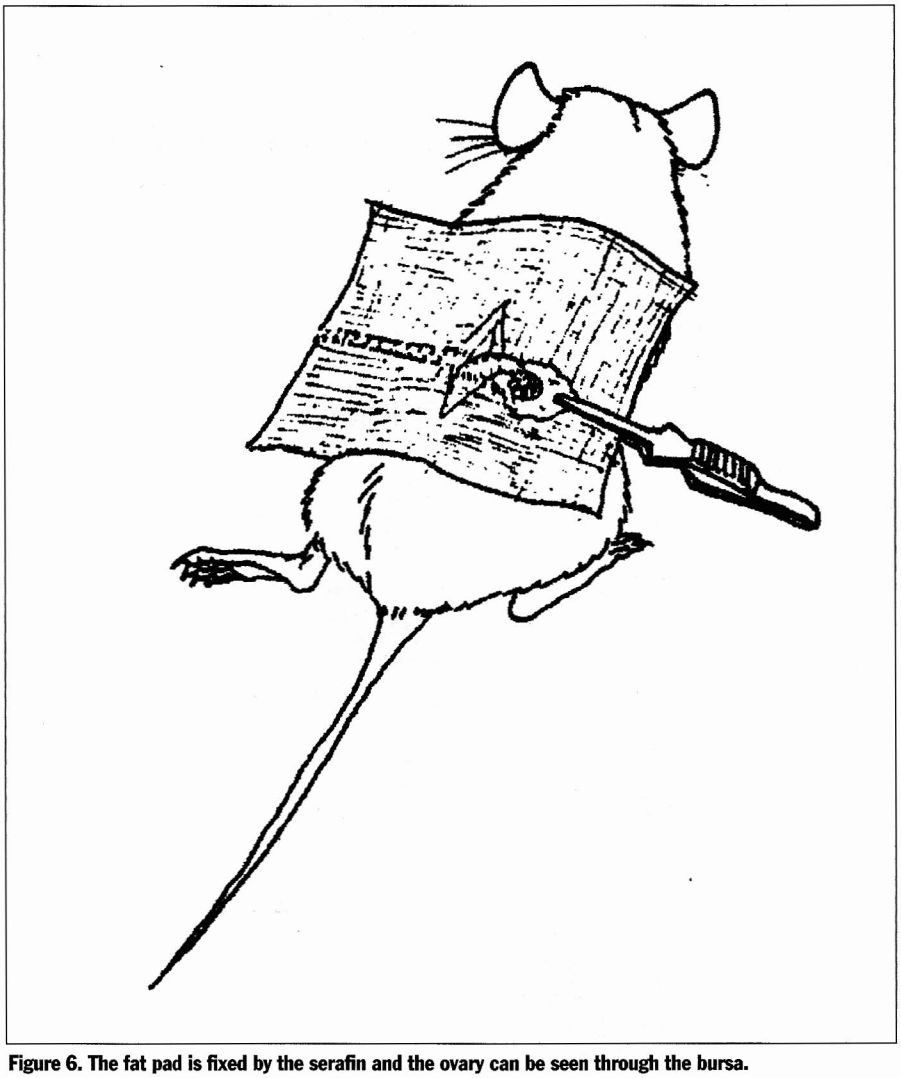


Figure 6. The fat pad is fixed by the serafin and the ovary can be seen through the bursa.

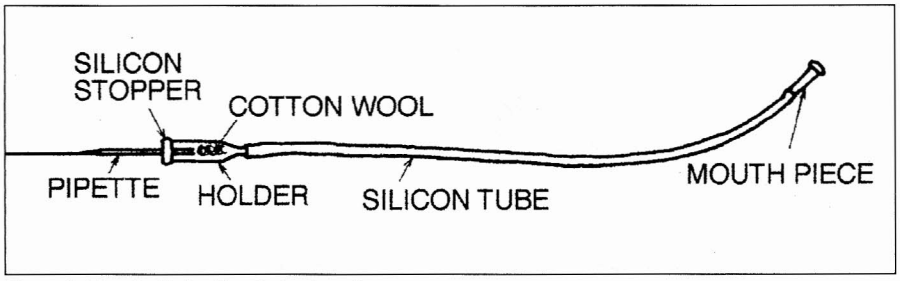


Figure 8. The pipette for the uterine transfer.

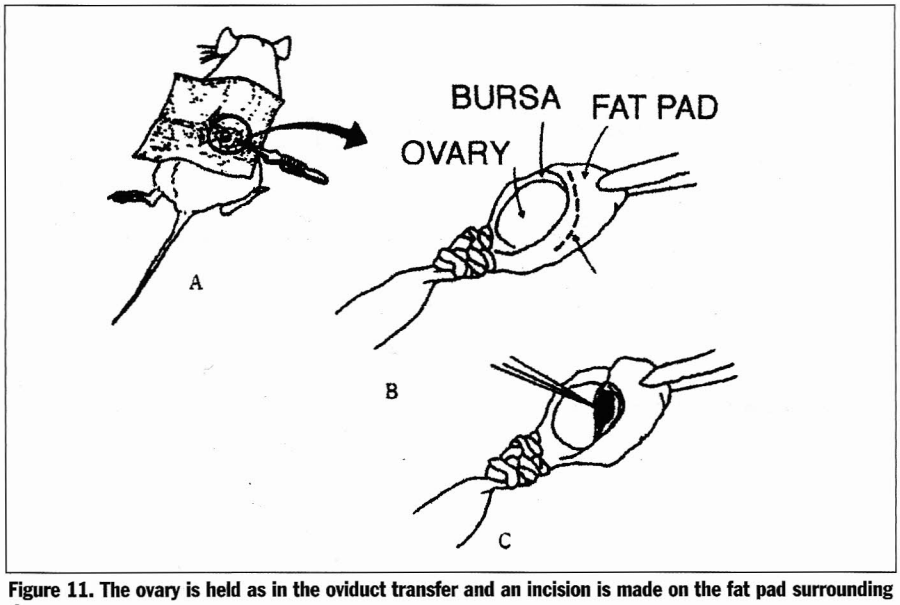


Figure 11. The ovary is held as in the oviduct transfer and an incision is made on the fat pad surrounding the ovary.

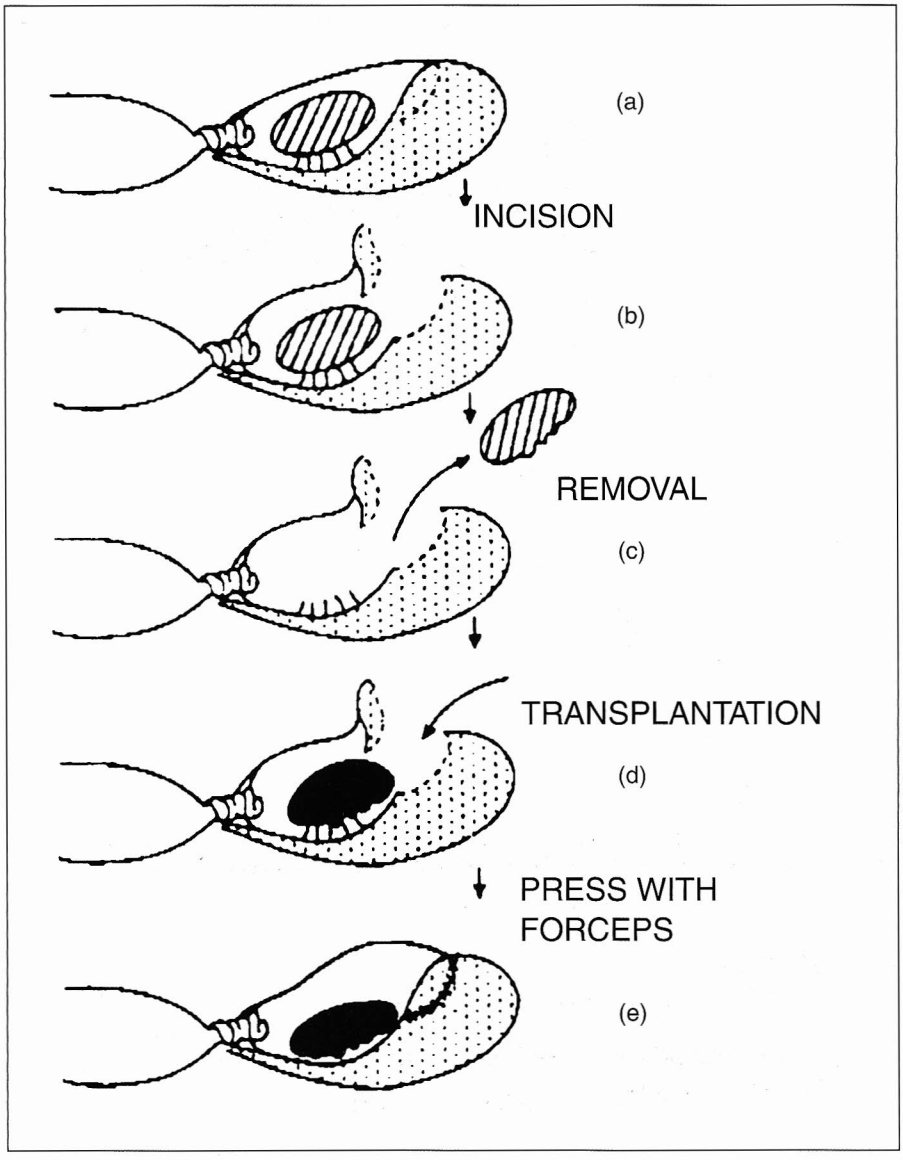


Figure 12. The donor ovary is inserted into the bursa so that the dissected surfaces of the donor and the host face to each other.

pull out the fat pad together with the ovary, oviduct, and uterus. The fat pad is put on a sheet of sterilized gauze and clipped by the arterial clamp (serafine) so that the ovary can be seen through the bursa (Figure 6). The mouse is put under the stereomicroscope (OME, Olympus, Tokyo, Japan) with its head at the top. The embryos are loaded into the pipette. First, the medium is aspirated by the capillary action to about 10 mm, then two tiny air bubbles are made - the lower of which serves as the indicator of a successful injection of embryos - and the embryos are drawn up with a minimum amount of the medium. The bursa is held with watchmaker's forceps, and using the microscissors, a small incision is made on its surface with care not to cut the vasculature. In case of bleeding, the blood has to be absorbed with small cotton balls. By slightly pulling the cut edge of the bursa to separate the oviduct from the ovary, the infundibulum is identified; the pipette is then inserted to 2–3 mm depth and blown slowly until the first (lower or distal) air bubble has entered the oviduct (Figure 7). The mouse is removed from the microscope and the serafin and the gauze are removed. Both sides of the incised back muscles are slowly lifted

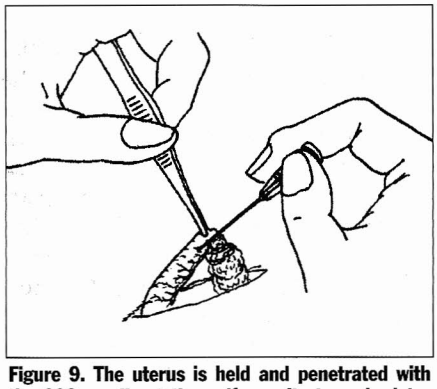


Figure 9. The uterus is held and penetrated with the 26G needle at the uniform site to make later identification easier to insert the pipette.

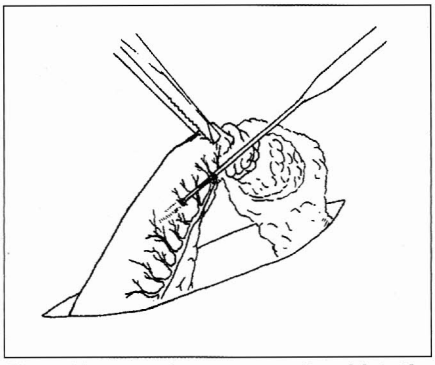


Figure 10. The embryos are transferred into the uterine lumen together with the air bubble.

up with the forceps so that the uterus, oviduct and ovary passively return inside the body wall. By positioning the mouse in the opposite orientation, embryos are transferred similarly to the right oviduct. The skin is closed with wound clips (the suture of the incisions on the body wall is optional). Until the mouse has recovered from the anesthesia (starts to move), she is kept in a warm place, either in the cage under the filament light or on the hot plate at 37°C.

b) Uterine transfer

When embryos are cultured until the morula or blastocyst stage, according to the experimental plan, they are transferred to the uterine horns of the pseudopregnant female of pseudopregnant day 3 (1 day earlier than that of the embryos). The basic setup and procedures are similar to those in the oviduct transfer. However, we use simpler pipettes and do not need dissection microscopes. The whole procedure discussed below also can be finished within 10 minutes, and when the unmanipulated blastocysts of gestational day 4 are transferred to the uterus of pseudopregnant day 3, more than 80% of them develop to term.

The pipettes are basically the same as those used in usual embryo handling, such as collection or transfer from medium to medium. They are pulled from capillary tubes (plain hematocrit tube 75 mm, Drummond) by using a small flame of the gas burner. They are scratched with an ampul-cutter and broken to give them an external diameter of 150–200 mm (Figure 8). For the uterine transfer, the end of the pipettes are flame-polished to ensure a smooth penetration through the uterine wall. Until the incision is made on the body wall, the same procedures are employed as those in the oviduct transfer. Embryos are drawn up with a small amount of medium (up to 5 mm) into the transfer pipette, and below that one tiny air bubble - the indicator of a successful transfer. The fat pad, together with the ovary and the uterine horn, is pulled out. The area near the junction of the oviduct and the uterine horn on the left side is gently held with a blunt forceps, and the uterine wall is penetrated by a 26G needle to the lumen (Figure 9). To make it easier to later identify the hole, it is important to uniform the sites held by the forceps and penetrated by the needle. In addition, slight bleeding helps identifying the hole. The pipette is inserted through the hole and embryos are slowly blown out together with the air bubble, which is small enough not to cause any trouble for embryo development (Figure 10). Embryos are also transferred to the right uterine horn, and hereafter, the mouse is handled similarly to the oviduct transfer.

Transfer of the ovary

This technique is utilized to obtain offspring and to maintain the genetic line of some mice, such as those transgenic mice that have potent oocytes but cannot normally produce offspring due to various types of reproductive disorders.[8] It is also very useful to analyze the causal relationships between the genetic predisposition of the embryos per se and the maternal environment in the pathogenesis of diseases.[4,9] The donor and the host have to be histocompatible, and they also have to have genetic markers to confirm later whether the pups are from the donor ovary or the remnant host ovary. The same procedures are employed as those in the oviduct transfer up until the point where the fat pad surrounding the left ovary is held together by the serafin. After the skin incision, the donor ovary, in this case, is removed and the body wall of the host is cut. To obtain the donor ovary, when the donor is younger than two weeks of age, the uterus, oviduct and ovary are dissected *en bloc* into the medium, and the ovary is separated with #5 watchmaker's forceps under a dissection microscope. On the fat pad covering the bursa, an incision of about one-fourth of the total margin is made with the microscissors (Figure 11). While gently pulling up the cut edge of the bursa with the #5 forceps in the left hand, dissect the host ovary with the scissors in the right hand (Figures 11,12). The donor ovary is inserted into the empty bursa so that the dissected (vascular) surface of the donor ovary faces that of the donor (Figure 12). The incision on the fat pad is closed by pressing together the fat on both sides with the watchmaker's forceps. When the bursa can be completely closed, it is filled with blood and swollen (Figure 12). As in the oviduct and uterine transfer, the ovary is put back inside the body wall; the right ovary is operated, and hereafter, the mouse is handled as in the embryo transfer. Two weeks after the operation, the mice mated with males and started to produce offspring. STI

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