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Methodologies to Study Implantation in Mice

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Summary

Pregnancy begins with fertilization of the ovulated oocyte by the sperm. After fertilization, the egg undergoes time-dependent mitotic division while trying to reach the blastocyst stage and the uterus for implantation. Uterine preparation for implantation is regulated by coordinated secretions and functions of ovarian sex steroids. The first sign of contact between the blastocyst and the uterus can be detected experimentally by an intravenous blue dye injection as early as the end of day 4 or the beginning of day 5 of pregnancy. This blastocyst–uterine attachment reaction leads to stromal decidual reaction only at sites of implantation. The process of implantation can be postponed and reinstated experimentally by manipulating ovarian estrogen secretion. Stromal decidualization can also be induced experimentally in the hormonally prepared uterus in response to stimuli other than the embryo. Fundamental biological questions surrounding these essential features of early pregnancy can be addressed through the application of various techniques and manipulation of this period of early pregnancy. This chapter describes the routine laboratory methodologies to study the events of early pregnancy, with special emphasis on the implantation process in mice.

Key Words: Mouse; blastocyst; implantation; ovariectomy; vasectomy; delayed implantation; embryo transfer.

1. Introduction

The mouse, as one of the most common laboratory animals, is widely used in basic biological research and could provide useful information that is relevant to human biology. This chapter focuses on some of the procedures for studying events of early pregnancy in mice. Following mating and fertilization, the embryo develops to the blastocyst stage. Attachment of the blastocyst into the uterine wall is an absolute requirement for further growth and collection of nutrients from the maternal vasculature. Hence, the implantation process is a critical event in the embryo's life and a central step to the establishment of placentation and pregnancy.

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The following description and methodologies are intended for investigators who wish to pursue research in various aspects of early pregnancy, with special reference to implantation-related processes. Studies on early pregnancy start with breeding to generate pregnant females. Ideally, experimental mice are maintained and bred in an institutional animal facility governed by the institutional animal care and use committee guidelines with the help of veterinarians who supervise the health and well being of animals. Natural breeding is routinely used for performing research on pregnancy.

1.1. The Reproductive Cycle in Mice

The reproductive cycle in mice is known as the estrous cycle. The estrous cycle is the time period from the onset of estrus until the onset of the next estrus. The length of the estrous cycle varies depending on the animal species. The average length of the estrous cycle is 4–5 d in mice, but it is highly variable. The estrus stage signifies a period when females show signs of mating behavior. Mice spontaneously ovulate during each estrous cycle. Females become cyclic when they reach puberty by 4 wk of age. The different phases of the estrous cycle in adult females are regulated by a functional hypothalamo–pituitary–ovarian axis. Sexual maturity is coincident with pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus with rising levels of circulating gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) from the pituitary. While rising levels of FSH trigger follicular growth and maturation, ovulation occurs under the influence of increasing levels of LH. These changes are reflected in ovarian steroid production during each cycle.

The stages of the estrous cycle are estrus, metaestrus, diestrus, and proestrus ***(1)***. These stages occur in each cycle and in a sequential manner. The day of estrus is usually designated as day 1 of the cycle. The stages of the estrous cycle are best determined by the cell types observed in the vaginal smear. Normally, vaginal smears should be examined in the morning (0800 to 0900 h). The estrous cycle is also divided into two ovarian phases: follicular phase and luteal phase. Follicular phase is the period of ovarian follicle development, and consists of proestrus and estrus. The luteal phase is the period of corpus luteum formation and function, and comprises metaestrus and diestrus. The uterus also undergoes hormonal changes during the estrous cycle. The uterus is distended during proestrus and estrus as a result of an increase in uterine vascular permeability and accumulation of fluid due to a higher level of circulating estrogen. The distention starts to decline in late estrus and it is no longer observed at diestrus. The wet and dry uterine weights are lowest at diestrus and heaviest at proestrus.

1.2. Breeding of Mice

Sexual maturity in females occurs earlier than in males. Whereas females are normally used for breeding at 45–50 d of age, males are not ready until 60 d of age. Sexual maturity may be delayed a week or so in both males and females depending on the strain of mice. Typically, males can breed successfully longer than females because spermatogenesis continues throughout life. Many male mice tend to become overweight with age, which may negatively affect their ability to breed successfully. It is often recommended to retire old males (9 to 10 mo of age) and set up cages with new males.

1.2.1. Effect of Light Cycle on the Reproductive Behavior of Mice

The light cycle controls the reproductive performance of females and males ***(2)***. Breeding conditions such as light cycle and timing must be carefully controlled and regulated. The artificial light–dark cycle of an animal facility is critical to the synchronous development of eggs. If female mice are purchased from a commercial supplier, they should be allowed approx 1 wk to adjust to the institutional animal room's light–dark cycle. The release of LH, a pituitary hormone that induces ovulation, is regulated by the light–dark cycle. The animal rooms are usually maintained at 12 h light:12 h dark or 14 h light:10 h dark cycles.

1.2.2. Copulatory Plug Formation

The presence of a vaginal plug in the morning following copulation with a male indicates successful mating. The ejaculate from the male's accessory sex glands forms a short-lived, whitish-looking or cream-yellow-colored plug in the vagina of a female. The presence of a vaginal plug only indicates successful mating, but does not always mean that a pregnancy will occur from this mating. It should also be noted that sometimes plugs fall out of the vagina; this may result in pregnancy that initially remains unnoticed, especially if checked late in the morning.

1.3. Early Pregnancy

After ovulation, eggs released by the ovaries enter the associated oviduct. Mouse ovaries are covered with the bursa (a thin membrane) and no egg can escape into the abdominal cavity. Fertilization of the egg occurs in the ampulla (ovarian end of the oviduct) after a successful mating. The egg completes its first maturation division by the time ovulation occurs. If the egg has not completed the first maturation division, it does so very quickly after ovulation. Female mice normally ovulate 8–10 ova in each cycle. Freshly ovulated eggs are surrounded by a mass of cumulus cells. The uterus in the mouse consists of

two horns (duplex). After mating, sperm travel through both uterine horns to reach the site of fertilization. They penetrate the cumulus cells to fertilize eggs. Usually, more than one sperm enters the perivitelline space. However, only one sperm penetrates and fertilizes the egg. After fertilization, the zygote divides mitotically to eventually reach the blastocyst stage. After mating, the mating stimulus triggers prolactin release from the pituitary, which leads to the formation of a functional corpus luteum in the ovary, blocking further ovulation and cyclicity to continue pregnancy.

1.4. Experimental Delay in Implantation

In many animals, implantation is delayed for an extended period, during which the blastocyst remains in a quiescent state called embryonic diapause ***(3,4)***. Delayed implantation in these animals seems to be a strategic plan for regulating the time of birth coincident with favorable environmental conditions. In some species, delayed implantation occurs under specific conditions. Mice show postpartum estrus immediately after parturition. If conception takes place immediately after parturition, embryos develop into blastocysts, but remain in a dormant state until the lactational stimuli from suckling pups are removed. In mice, implantation can be experimentally delayed by removing the ovarian source of steroids ***(5)***. The timing of normal blastocyst implantation is tightly controlled in mice. Normally, initiation of implantation occurs at night (2200–2300 h) of day 4 ***(6)***. Ovarian steroid hormones are necessary to prepare the endometrium for the process of implantation. In mice, both ovarian progesterone and estrogen are required for implantation. The ovary secretes a small amount of estrogen in addition to progesterone in the morning of day 4 of pregnancy in mice. This preimplantation estrogen secretion is an absolute requirement for blastocyst activation, preparation of the uterus, and initiation of the implantation process. Surgical removal of both ovaries in mice before the preimplantation ovarian estrogen secretion occurs on day 4 leads to delayed implantation.

1.5. Artificial Decidualization

During normal pregnancy uterine stromal cells first proliferate and then differentiate to decidual cells in response to an implanting blastocyst ***(4)***. This process is known as decidualization. Decidualization starts following initiation of blastocyst implantation in mice. The decidua enlarges as the embryo grows. Decidual cells are characterized by the presence of polyploid nuclei, and glycogen and lipid in their cytoplasm. As the deciduum grows, it occupies the uterine lumen at the mesometrial side (dorsal to the embryo). The antimesometrial decidua is divided into two zones. A thin and dense cellular zone that immediately surrounds the blastocyst is known as the primary decidual

zone (PDZ). This is an avascular zone of the endometrium. The secondary decidual zone (SDZ) surrounds the PDZ. The SDZ is a broad, well-vascularized edematous zone.

The decidual response can also be induced experimentally without the presence of an embryo ***(4)***. However, a proper hormonally prepared uterus is needed for this purpose. The uterus of a pseudopregnant female is a preferred choice. The uterus of a progesterone-primed (at least 48 h), ovariectomized mouse can also be used for this purpose.

Methods pertinent to the investigation of early pregnancy in the mouse are described in this chapter.

2. Materials

2.1. Monitoring the Estrous Cycle

2.1.1. Collection of Vaginal Smears

1. Sexually mature female mice (45–50 d old).
2. Clean mouse cage with a wire-top cage cover.
3. Plastic dropper (Fisher Scientific, Hanover Park, IL, cat. no. 13-711-10).
4. Saline (0.9% Sodium chloride solution, Baxter Healthcare Corporation, Deerfield, IL, cat. no. 281324).
5. Glass slides (Fisher Scientific, cat. no. 12-518-104).

2.1.2. Identification of the Stage of Estrous Cycle

1. Glass slides (Fisher Scientific, cat. no. 12-518-104).
2. A compound microscope with a 10× and 40× objectives.

2.2. Breeding and Plug Checking

2.2.1. Natural Breeding

1. Sexually mature female mice (45–50 d old).
2. Sexually mature male mice (60 d old).
3. Mouse cages.

2.2.2. Checking Copulatory Plug

1. Female mice mated with fertile males.
2. A pair of curved forceps (Fine Science Tools, Inc., Foster City, CA, cat. no. 11152-10).

2.3. Early Pregnancy Determination

2.3.1. Noninvasive Method

Vaginal plug-positive mice.

2.3.2. Invasive Method

2.3.2.1. Embryo Collection From the Oviduct and Uterus

1. Vaginal plug-positive mice.
2. 70% ethanol (Aaper Alcohol & Chemical Co., Shelbyville, KY).
3. Paper towels.
4. Forceps (Fine Science Tools, Inc., cat. nos. 11151-10, 11153-10, and 11150-10).
5. Scissors (Fine Science Tools, Inc., cat. nos. 14558-11 and 15000-10).
6. Falcon Petri dish (Fisher Scientific, cat. no. 08-757-100B).
7. Whitten's culture medium (per/100 mL): 514 mg NaCl, 36 mg KCl, 16 mg KH_2PO_4, 29 mg $MgSO_4$, 190 mg $NaHCO_3$, 53 mg calcium lactate, 100 mg glucose, 2 mg penicillin, 2 mg streptomycin sulfate, 0.5 mg phenol red, 3.5 mg pyruvic acid (sodium salt), 0.37 mL lactic acid (sodium salt), and 300 mg bovine serum albumin.
8. BD brand disposable 3-mL syringe (Fisher Scientific, cat. no. 14-829-14B).
9. Hamilton 31-gauge steel needle (Fisher Scientific, cat. no. 14-815-619).
10. 27-gauge BD PrecisionGlide sterile disposable needle (Fisher Scientific, cat. no. 14-826-48).

2.3.2.2. Detection of Early Implantation Sites by Intravenous Dye Injection

1. Anesthesia (Avertin).
2. Paper towels.
3. BD PrecisionGlide 1-mL syringe with 27-gauge, one-half-inch needle (Becton Dickinson & Co., Franklin Lakes, NJ; cat. no. 309623)
4. Blue dye (Chicago Blue B or Evans blue, or pontamine blue from Sigma Chemical Co.).
5. Saline (0.9% sodium chloride, Baxter Healthcare Corporation, cat. no. 281324).
6. Warm water.

2.4. Experimental Delay in Implantation

2.4.1. Ovariectomy

1. Seminal plug-positive female mice (day 4 of pregnancy).
2. Anesthesia (Avertin).
3. Animal clipper (Fisher Scientific, cat. no. 01-305-10).
4. 70% Ethanol (Aaper Alcohol & Chemical Co.).
5. Povidone-Iodine solution (Aplicare, Inc., Branford, CT).
6. Forceps (Fine Science Tools, Inc., cat. nos. 11153-10 and 11150-10).
7. Scissors (Fine Science Tools, Inc., cat. no. 14558-11).
8. BD Autoclip wound clips (Fisher Scientific, cat. no. 01-804-5) and applier (Fisher Scientific, cat. no. 01-804).

2.4.2. Experimentally Delayed Implantation

1. Progesterone (Sigma Chemical Co., cat. no. P-1030)
2. Estradiol-17β (Sigma Chemical Co., cat. no. E-8875)

3. BD PrecisionGlide 1-mL syringe with 27-gauge, one-half-inch needle (Becton Dickinson & Co., cat. no. 309623).

2.5. Artificial Decidualization

2.5.1. Induction of Pseudopregnancy in Females Using Vasectomized Males

2.5.1.1. Preparation of Vasectomized Males

1. Sexually mature male (60 d old).
2. Anesthesia (Avertin).
3. Animal clipper (Fisher Scientific, cat. no. 01-305-10).
4. 70% Ethanol (Aaper Alcohol & Chemical Co.).
5. Povidone-Iodine solution (Aplicare, Inc.).
6. Forceps (Fine Science Tools, Inc., cat. nos. 11153-10 and 11150-10).
7. Scissors (Fine Science Tools, Inc., cat. no. 14558-11).
8. Ethicon nonabsorbable surgical suture (size 4.0; Ethicon, Inc., Somerville, NJ).
9. Slide Warmer (Lab-line Instruments, Inc., Melrose Park, IL; Model No. 26020).
10. BD Autoclip wound clips (Fisher Scientific, cat. no. 01-804-5) and applier (Fisher Scientific, cat. no. 01-804).
11. Saline (0.9% sodium chloride, Baxter Healthcare Corporation, cat. no. 281324).

2.5.1.2. Induction of Pseudopregnancy

1. Vasectomized male mice.
2. Sexually mature female mice (45–50 d old).
3. A pair of curved forceps for checking vaginal plugs (Fine Science Tools, Inc., cat. no. 11152-10).

2.5.1.3. Hormonal Priming of Ovariectomized Mice

1. Ovariectomized females rested for 10–15 d.
2. Sesame seed oil (Sigma Chemical Co., cat. no. S-3547).
3. BD PrecisionGlide 1-mL syringe with 27-gauge, one-half-inch needle (Becton Dickinson & Co., cat. no. 309623).
4. Progesterone (Sigma Chemical Co., cat. no. P-1030).
5. Estradiol-17β (Sigma Chemical Co., cat. no. E-8875).
6. Hotplate (Fisher Scientific, cat. no. 11-497-6A).

2.5.1.4. Induction of Decidualization by Artificial Means

1. Day 4 pseudopregnant female or ovariectomized progesterone-treated female mice.
2. Anesthesia (Avertin).
3. Animal clipper (Fisher Scientific, cat. no. 01-305-10).
4. 70% Ethanol (Aaper Alcohol & Chemical Co.).
5. Povidone-Iodine solution (Aplicare, Inc.).
6. Forceps (Fine Science Tools, Inc., cat. nos. 11153-10 and 11150-10).
7. Scissors (Fine Science Tools, Inc., cat. no. 14558-11).

8. BD PrecisionGlide 1-mL syringe with 27-gauge, one-half-inch needle (Becton Dickinson & Co., cat. no. 309623).
9. Sesame seed oil (Sigma Chemical Co., cat. no. S-3547).
10. BD Autoclip wound clips (Fisher Scientific, cat. no. 01-804-5) and applier (Fisher Scientific, cat. no. 01-804).

2.6. Intrauterine Blastocyst Transfer

1. Day 4 pseudopregnant or progesterone-treated ovariectomized mice.
2. Anesthesia (Avertin).
3. Paper towels.
4. Animal clipper (Fisher Scientific, cat. no. 01-305-10).
5. 70% Ethanol (Aaper Alcohol & Chemical Co.).
6. Povidone-Iodine solution (Aplicare, Inc.).
7. Forceps (Fine Science Tools, Inc., cat. nos. 11153-10 and 11150-10).
8. Scissors (Fine Science Tools, Inc., cat. nos. 14558-11).
9. 23-gauge BD PrecisionGlide needle (Fisher Scientific, cat. no. 14-826A).
10. BD Autoclip wound clips (Fisher Scientific, cat. no. 01-804-5) and applier (Fisher Scientific, cat. no. 01-804).
11. Slide Warmer (Lab-line Instruments, Inc., Model No. 26020).
12. Saline (0.9% sodium chloride, Baxter Healthcare Corporation, cat. no. 281324).
13. Serrefine clip (Fine Science Tools, Inc., cat. no. 18050-35).
14. 1-mL Hamilton pipet controller syringe (Hamilton Company, Reno, NA; cat. no. 84001).
15. 6-in thin capillary (1 mm diameter) glass pipet (World Precision Instruments, Inc., Sarasota, FL., cat. no. TW 100-6).
16. Popper 16-gauge steel needle (Fisher Scientific, cat. no. 14-825-16J).

2.7. Commonly Used Anesthetics

1. Injectable anesthetic, Avertin: (components: Avertin [2,2,2-tribromoethanol, Sigma Aldrich Chemie GmbH, Steinheim, Germany; cat. no. T4,840-2] and tert-amyl alcohol, Fisher Scientific, cat. no. A730-1]).
2. Short-lasting inhalant anesthetic, Isoflurane (Minrad, Inc., Buffalo, NY).

2.8. Common Injection Techniques

1. Mice.
2. 70% Ethanol (Aaper Alcohol & Chemical Co.).
3. Clean cage with a cage top.
4. BD PrecisionGlide 1-mL syringe with 27-gauge, one-half-inch needle (Becton Dickinson & Co., cat. no. 309623).

2.9. Euthanasia

2.9.1. Cervical Dislocation

1. Mice.
2. Clean cage with a cage top.

2.9.2 Inhalants

2.9.2.1. Carbon Dioxide

1. Carbon dioxide cylinder (local gas supplier).
2. A cage specifically designed for killing mice.

2.9.2.2. Isoflurane

1. Isoflurane (Minrad, Inc.).
2. Cotton wool (Absorbent Cotton Co., Inc., Valley Park, MO) or gauze (Kendall Healthcare Products Co., Mansfield, MA).
3. Bell jar or a scew cap glass container (Fisher Scientific).

3. Methods

3.1. Monitoring the Mouse Estrous Cycle

3.1.1. Collection of Vaginal Smears

1. Grasp the tail of a mouse with the thumb and forefinger of one hand.
2. Place the mouse on the top of the cage cover (wire top). As the mouse attempts to move forward, quickly grasp the loose skin at the back of the neck using the thumb and forefinger of the other hand. The head of the mouse will be immobilized, if the skin is held properly.
3. Lift the mouse in your hand and secure the tail between the small finger and the palm of the same hand.
4. Keep the face of the mouse up and locate the vagina.
5. Fill a plastic dropper with a small amount of saline (0.05 to 0.1 mL 0.9% NaCl) and insert the tip superficially, but not deeply, into the vagina.
6. Gently squeeze the bulb of the dropper to release saline inside the vagina. Slowly release the pressure on the bulb to aspirate the vaginal lavage inside the dropper.

3.1.2. Identification of the Stage of the Estrous Cycle

1. Place a drop of aspirated vaginal fluid on a glass slide.
2. Unstained material is observed under a light microscope with 10× and 40× objective lenses to identify the stage of the estrous cycle.
3. The following criteria are used to identify the specific stage of the cycle:
   a. The estrus stage vaginal smear contains anucleated cornified cells (irregular shaped cells).
   b. The metaestrus stage vaginal smear is composed of a mixture of cornified epithelial cells and leukocytes.
   c. At diestrus, the smear contains predominantly leukocytes.
   d. At proestrus, the smear shows a predominance of nucleated epithelial cells.

3.2. Breeding and Plug Checking

3.2.1. Natural Breeding

1. In general, 2–3 mature females irrespective of their estrous cycle are placed into a male's cage for breeding in the afternoon (1600–1800 h).

2. If not bred, the same females should be used for breeding 3–4 d in a row because the pairing with males helps to synchronize the cycle in females. Males copulate with females at proestrus at around the midpoint of the dark cycle.
3. After a successful mating, the male should be given a rest of 2–3 d.

3.2.2. Checking Copulatory Plugs

1. A method for holding the mouse has already been described in the section on collection of vaginal smear (*see* **Subheading 3.1.1.**).
2. Hold the mouse in one hand with its face up.
3. Locate the vagina and use a small pair of curved forceps to spread the lips of the vulva to identify the plug.
4. When gently touched with a pair of forceps, the plug feels solid and blocks the vagina. It is a common practice to check the plug early in the morning, before 0900 h. The presence of a plug in the vagina is usually considered day 1 of pregnancy (*see* **Note 1**).

3.3. Detection of Early Pregnancy

3.3.1. Noninvasive Method

1. The presence of a plug in the vagina is defined as day 1 of pregnancy (*see* **Note 2**).
2. Implantation sites that look like a string of pearls in both uterine horns can be detected by palpating the abdomen from day 8 onward (*see* **Note 3**).

3.3.2. Invasive Method

Dating of early pregnancy starts from the time of fertile mating to the time of implantation occurring late on day 4 or early on day 5 ***(5)***. Timing can be determined by assessing the developmental stage of the preimplantation embryo. Developmental stages of preimplantation embryo and their location in the reproductive tract are described in **Table 1**. Mice are normally sacrificed to collect preimplantation embryos.

3.3.2.1. Preparation of Mice for Excision of Oviducts and Uterine Horns

1. Place a euthanized mouse on its back (supine position) on a paper towel.
2. Swab the belly with 70% ethanol.
3. Holding the lower half of the abdominal skin with a pair of forceps, make a small lateral incision on the skin just below the forceps with a pair of scissors.
4. Holding the skin above the incision with your thumb and forefinger of one hand and below the cut with other hand, pull the skin towards head and tail to expose abdominal muscle.
5. Lift the abdominal wall muscle up with a pair of forceps and cut the abdominal muscle from the midline laterally on both sides to expose internal organs.
6. Push aside the intestine to visualize uteri, oviducts, and ovaries.

Table 1
Dating of Early Pregnancy Depending on the Developmental Stage of Preimplantation Embryos*

Day of pregnancy	Developmental stage of embryos	Reproductive tract
1	One-cell zygote	Oviduct
2	Two-cell	Oviduct
3 (0100–0400 h)	Four-cell	Oviduct
3 (0500–1400 h)	Eight-cell	Oviduct
3 (1500–2300 h)	Morula	Oviduct
4	Blastocyst	Uterus

*The time of embryonic development may be slow or fast, depending on the strain of mice and light-dark cycle of an animal facility.

3.3.2.2. Excision and Flushing of Oviducts

1. Using a pair of curved forceps, grasp the uterine horn just below the utero-tubal junction and cut the horn just below the pair of forceps (**Fig. 1**).
2. Then lift the uterine horn and clean the underlying fat pad and mesentery that are attached to the oviduct using a pair of iris scissors.
3. Separate the oviduct from the ovary using the same pair of scissors and place the oviduct in a Petri dish with a drop of Whitten's media for flushing to recover embryos.
4. Repeat this procedure for the contralateral oviduct.
5. Place a fresh Petri dish on the stage of a stereo dissecting microscope.
6. Attach a 31-gauge needle with a blunt end that has been bent in the middle to form a 120° angle to a 1-mL or a 3-mL plastic syringe filled with Whitten's medium.
7. Using a pair of forceps, place an oviduct on the dish under the microscope and manipulate the oviduct to locate the fimbriated end of the oviduct known as the infundibulum.
8. Hold the infundibulum loosely between the forceps and insert the needle inside the oviduct (**Fig. 2**).
9. Holding both the needle and the oviduct together, gently squeeze the syringe to pass the Whitten's medium through the oviduct. This procedure should inflate the oviduct and flush the embryos from the oviduct into the dish.
10. Examine the flushing under the microscope to identify developmental stage of embryos.

3.3.2.3. Excision and Flushing of Uteri

1. Using a pair of forceps, grasp one uterine horn just above the cervical bifurcation and cut below the point of holding with a pair of scissors (**Fig. 3**).
2. Pull up the uterine horn and trim the horn free of fat and mesentery with a pair of scissors (**Fig. 3**).

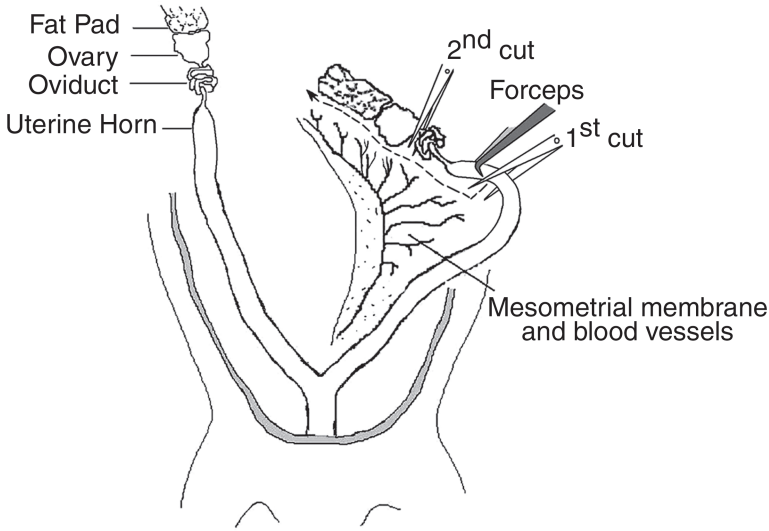

Fig. 1. Schematic representation to show excision of the mouse oviduct. The method of excision of mouse oviduct is described under **Subheading 3.3.2.2.**

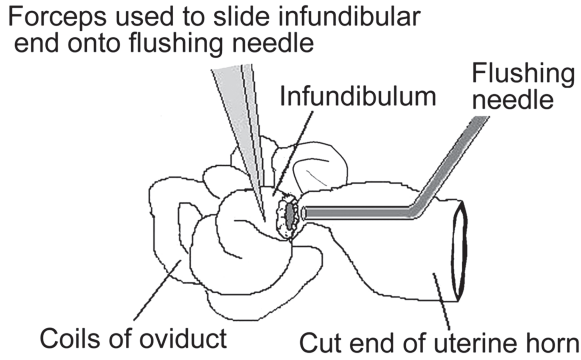

Fig. 2. Schematic representation to show flushing of the mouse oviduct. The method of flushing oviducts is described under **Subheading 3.3.2.2.** The oviduct is flushed to recover preimplantation embryos.

3. Cut the other end of the horn just below the utero–tubal junction and keep the uterine horn in a clean moistened tissue paper to absorb blood.
4. Hold one of the uterine horns at the utero–tubal junction and insert the tip of a 27-gauge needle, with a 3-mL plastic syringe filled with Whitten's medium attached, inside the uterine lumen (**Fig. 4**).

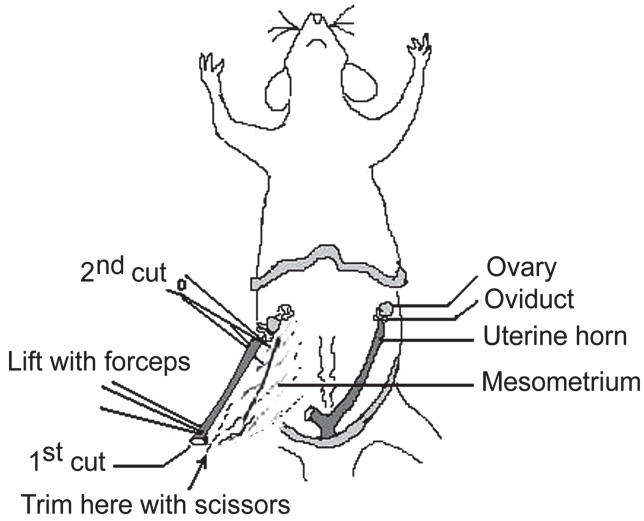

Fig. 3. Schematic representation to show excision of uterine horns. The method of excision of mouse uterus is described under **Subheading 3.3.2.3.**

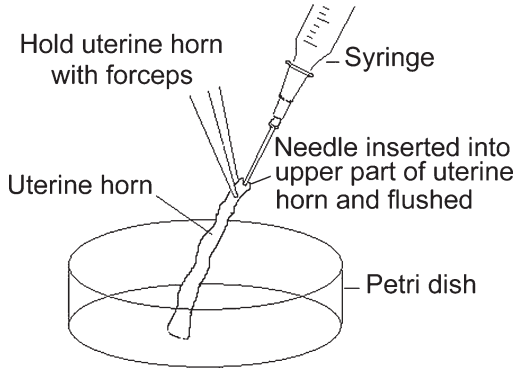

Fig. 4. Schematic representation to show flushing of the mouse uterine horn to recover blastocysts. The method of flushing uterine horns is described under **Subheading 3.3.2.3.**

5. Holding the needle and the uterine horn together, push the plunger of the syringe to flush the uterine luminal contents into a Petri dish. It is important to flush gently (**Fig. 4**).
6. Repeat this procedure to excise and flush the other uterine horn.
7. Check uterine flushings under a stereomicroscope for the presence of appropriate stages of developing embryos.

3.3.2.4. Determination of Early Implantation Sites by Intravenous Dye Injection

Implantation sites in mice can be detected as early as late at night on day 4 (2200–2300 h) and onward, considering the presence of a copulatory plug as day 1 of pregnancy. This is achieved by intravenous injection of a macromolecular blue dye solution, normally via a tail vein ***(7)***.

1. Fill a 1-mL syringe attached to a 27-gauge needle with 1% blue dye solution (Chicago Blue B, Evans blue, or pontamine blue) avoiding any air bubbles inside the syringe.
2. After a mouse is anesthetized, dilate the tail veins by the application of a paper towel soaked in warm water.
3. Locate one of the two lateral veins in the tail (veins are located on both sides of the central artery) and place the mouse on that side.
4. Hold the tail gently between the thumb and forefinger and keep the tail parallel to the body of the mouse (**Fig. 5**).
5. Align the needle (bevel side up) with the plane of the vein. Insert the needle into the vein and slowly inject the desired amount of dye (0.1 mL/mouse, 0.25 mL/rat). As a result of increased capillary permeability in the endometrial bed at the sites of implantation, the dye bound with the serum proteins accumulates in the interstitial space at the sites of blastocysts, showing distinct blue bands (**Fig. 6**). Chicago Blue B dye has been used for many years to identify implantation sites (*see* **Note 4**).
6. Animals are sacrificed 3–5 min after dye injection to identify blue bands in the uterus. Identification of uterine implantation sites from day 6 onward does not require blue-dye injection. Visual observation of prominent intermittent swellings in the uterus indicates that blastocyst implantation is in progress.

3.4. Experimental Delay in Implantation

3.4.1. Ovariectomy

1. Under anesthesia, shave the lumbar dorsum bilaterally and place day 4 pregnant animals in a prone position (face down).
2. Clean the exposed skin of the back with a 10% povidone-iodine scrub followed by 70% alcohol for aseptic surgery.
3. Make a midline skin incision near the abdominal cavity (on the back).
4. Turn the animal to one side (left or right). Pull the skin incision laterally away from the spine and make an incision on the abdominal muscle to locate the para-ovarian fatty tissue (light whitish-yellow-colored fat) (**Fig. 3**).
5. Lift out the para-ovarian fatty tissue and excise the ovary by making a sharp cut between the oviduct and the ovary.
6. Rejoin the fat tissue with the oviduct in the abdominal cavity.
7. Repeat the same procedure on the other side. If the incisions are small, there is no need to close the incisions in the abdominal muscle.
8. Close the midline skin incision using wound clips.

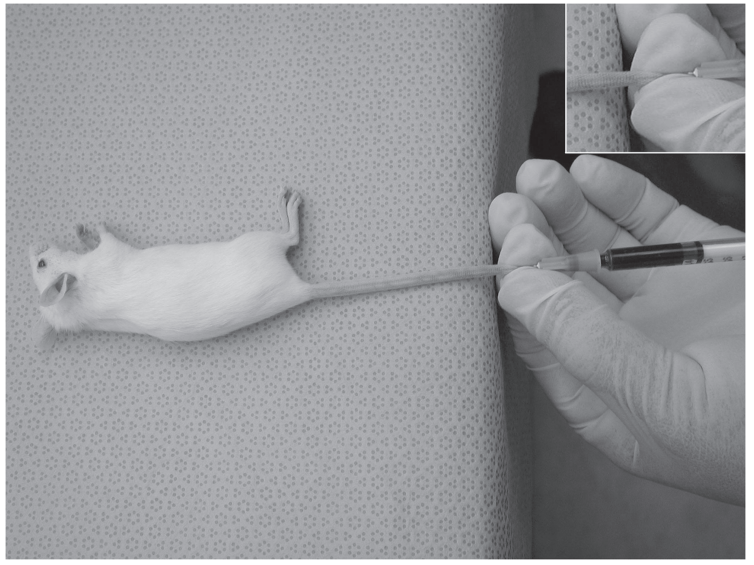

Fig. 5 (*see* companion CD for color version). Detection of implantation sites by intravenous injection of macromolecular dye into tail vein. The method of intravenous Chicago Blue B dye injection into tail vein is described under **Subheading 3.3.2.4.**

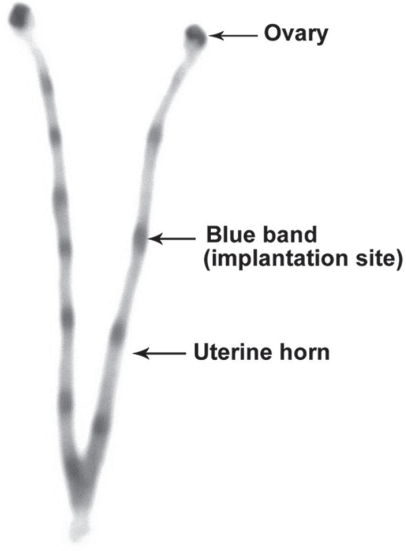

Fig. 6 (*see* companion CD for color version). Implantation sites in uterine horns on day 5 of pregnancy as detected by intravenous Chicago blue B dye injection. The method of intravenous Chicago Blue B dye injection into tail vein is described under **Subheading 3.3.2.4.**

3.4.2. Experimentally Delayed Implantation

During experimentally delayed implantation, blastocysts enter a state of dormancy or diapause and the uterus enters a neutral state ***(3,4)***. This condition can be maintained for several days and sometimes even weeks by continuing daily progesterone (1 or 2 mg/mouse) injection. However, the number of dormant blastocysts is gradually reduced if this delayed condition is maintained for a long time. Uterine luminal closure and blastocyst apposition occur during delayed implantation, but the attachment and invasion of trophoblast cells and decidual transformation of uterine stromal cells do not occur. Activation of the dormant blastocyst and implantation can be achieved by giving an injection (subcutaneous) of estradiol-17β (3–25 ng/0.1 mL of sesame seed oil). The implantation sites in the uterus can be detected by tail-vein injection of blue dye (*see* **Subheading 3.3.2.4.**) 18–24 h after estradiol-17β injection ***(8)*** (*see* **Note 5**).

3.5. Artificial Decidualization

3.5.1. Induction of Pseudopregnancy in Females Using Vasectomized Males

Regular cyclic females are mated with vasectomized infertile males to induce pseudopregnancy.

3.5.1.1. Vasectomy

Vasectomy is performed on fertile male mice at 6–8 wk of age. Surgical resection of the vas deferens eliminates sperm from the ejaculate.

1. Place an anesthetized male on the table in supine position (face up).
2. Shave the lower half of the abdomen (anterior to the genital) to remove hair, swab, and make a midline ventral incision (one-half of an inch) in the skin anterior to the genitalia.
3. Using a pair of curved forceps and scissors, pull the abdominal muscle up and cut the muscle in the midline (a white line in the muscle). Wipe the incision site with a lint-free tissue dampened with saline to remove excess cut hair.
4. Push the left scrotum with fingers so that the testis moves up into the abdominal cavity. The testis will be out together with the vas deferens.
5. Grasp the left vas deferens gently with forceps and lift a section clear of the incision. Tuck the curved forceps underneath the vas deferens and allow them to spread. Maintaining this position, use the suture to make two firm knots in the vas deferens, about 4–5 mm apart, tying both knots firmly.
6. Cut out a section of vas deferens between the knots.
7. Place all tissues back inside the abdominal cavity and push the testis back into the scrotum. Repeat the procedure on the right side of the body.
8. When both sides have been done, sew up the incision in the body wall with separate sutures.

9. Close up the skin with two to three auto clips. The mouse should be wrapped in a tissue to keep it warm (loss of body heat is common in abdominal surgery) or, alternatively, placed on a heating pad, and allowed to recover. Animals that are placed under anesthetic should always be supervised and monitored until fully awake.
10. Following the operation, the mice are allowed to recover for 2 wk before being test-bred to confirm their sterility. One or two female mice are placed with the vasectomized male and are checked for plugs the following morning. Plug-positive females are sacrificed on day 2, and their oviducts are flushed with saline. The eggs should be at unfertilized, one-cell stage. The presence of two-cell embryos would indicate incomplete vasectomy.

3.5.1.2. Induction of Pseudopregnancy

The uterine environment of most laboratory animals becomes receptive to implantation only after mating. Vaginal–cervical stimulation during mating results in ovarian hormonal changes that alter the estrous cycle in preparation for a possible pregnancy. Pseudopregnancy can be achieved in one of two ways: (1) by mating a female to a sterile (vasectomized) male or (2) by mechanical stimulation of the vagina and cervix with a rod or vibrating tool. Currently, natural mating of a mature female with a vasectomized male (1:1) is a preferred choice of producing pseudopregnant females. Detection of a copulatory plug in the vagina of a female mated with a vasectomized male is defined as day 1 of pseudopregnancy. Procedures for breeding and checking plugs are as described previously (*see also* **Note 6**).

3.5.1.3. Hormonal Priming of Ovariectomized Mice

The method of ovariectomy is described earlier (*see* **Subheading 3.4.1.**). In general, cyclic young females are ovariectomized and rested for a couple of weeks to eliminate ovarian steroids from the circulation. After recovery, ovarian steroid hormones are administered to prepare the uterus for a chemical or physical stimulus. Either progesterone alone or a regimen of progesterone and estrogen-17β supplementation are used to sensitize the uterus for the decidual cell reaction ***(9,10)***.

1. Treatment schedule of progesterone alone: ovariectomized mice receive progesterone (1 mg/d/mouse) injection (subcutaneous) for 3 d (days 1–3). The induction of deciduoma is initiated on day 4 by infusing oil inside the uterine lumen. Daily progesterone injection is continued after the stimulus to maintain the decidualization response ***(9)***.
2. Treatment schedule of estradiol-17β and progesterone: ovariectomized mice receive injections of estradiol-17β (100 ng/d/mouse) for 3 d (days 1–3), no treatment on days 4 and 5, progesterone (1 mg/d/mouse) plus estradiol-17β (10 ng/d/

mouse) on days 6–8. The induction of deciduoma is initiated on day 8 by infusing oil inside the uterine lumen ***(10)***. Daily progesterone (1 mg/d/mouse) injection is continued after the stimulus to maintain the decidualization response.

3.5.1.4. Induction of Decidualization by Artificial Means

Artificial decidualization is usually induced by injecting sesame seed (or corn) oil or by placing a silk thread inside the uterus. Injection of sesame seed oil inside the uterus is a less invasive procedure and a preferred method to induce decidualization.

1. Fill a plastic 1-mL syringe attached to a 27-gauge needle with sesame seed oil.
2. Prepare the female and expose one uterine horn as previously described (*see* **Subheading 3.4.1.**).
3. Hold the uterine horn with a pair of forceps very close to the tip (slightly below the utero–tubal junction) and slowly inject about 20 μL of oil inside the uterine lumen. Because the luminal fluid volume is very low, the uterine horn will temporarily swell during the course of oil injection (*see* **Note 7**).

3.6. Intrauterine Blastocyst Transfer

3.6.1. Induction of Pseudopregnancy in Females

The method of inducing pseudopregnancy in females is described in an earlier section (*see* **Subheading 3.5.1.2.**). The pseudopregnant mouse contributes a womb for the transfer embryos. Commercially available outbred albino CD-1 females are a great choice for recipient mice.

3.6.2. Hormonal Priming of Ovariectomized Mouse for Embryo Transfer

These females are often used to study the role of the steroid hormones, progesterone and estrogen, for preparation of the uterus for implantation. The method of performing ovariectomy and the preparation of progesterone-treated ovariectomized mice are described above (*see* **Subheadings 3.4.1.** and **3.5.1.3.**).

1. Ovariectomized females start to receive daily subcutaneous injection of progesterone (1 or 2 mg/mouse/0.1 mL of sesame seed oil) for two consecutive days before they are ready to receive a blastocyst. Progesterone injections make the uterus achieve the prereceptive state.
2. This progesterone-primed prereceptive uterus achieves receptivity in response to a single subcutaneous injection of estradiol-17β (25 ng/mouse/0.1 mL sesame seed oil) ***(10)***. It has been established that a minimum of 48 h of uterine exposure to progesterone is necessary before an injection of estradiol-17β is provided in order to attain uterine receptivity in mice.
3. The blastocyst transfer is usually performed on the third day at the time of the third progesterone injection and an estradiol-17β injection.

4. Implantation of the blastocyst can be checked by blue-dye methods 24 h after the blastocyst transfer and estrogen injection.

3.6.3. Preparation of a Blastocyst Transfer Pipet

1. Take a 6-inch thin capillary (1 mm diameter) glass pipet and rotate it in a fine flame approx 1 inch from one end. As soon as the glass becomes soft, withdraw the glass from the heat and quickly pull both ends apart.
2. Check the diameter of the pulled pipet under a microscope and break the end at a place where the diameter will be greater (approx 200 μm) than the size of a blastocyst. One can use an oilstone to mark the breaking point of the glass for an even tip.
3. Fire-polish the tip of the pipet by quickly touching the flame (an uneven tip may damage the blastocyst and the uterus).
4. Bend the tube (at a 120–130° angle) over a flame about one-half of an inch from the unpulled end.
5. Place the unpulled end of the pipet inside a 16-gauge steel needle and seal it (make it air-tight) with "super" glue. The leakage of solution can be tested by passing water through the needle.
6. This pipet-and-needle assembly is then fitted to special 1-mL Hamilton pipet-controller syringe. This special syringe has a plunger assembly with a thumb-wheel cap inside of a glass barrel. Pull approx 0.2 mL of sterile water into the pipet barrel by pulling the pipet-controller plunger assembly. Fill the inside plunger with water by turning the plunger thumb-wheel counterclockwise (all the way to the end). Avoid drawing any bubbles inside the syringe. Connect the luer tip of the pipet-controller syringe barrel and the embryo transfer pipet. Turn the plunger thumb-wheel clockwise to push the water into the transfer pipet. Fix the water level in the middle of the transfer pipet. The transfer pipet is now ready for loading blastocysts.

3.6.4. The Blastocyst Transfer Technique

1. Prepare a pseudopregnant or hormonally treated ovariectomized mouse as described above (*see* **Subheadings 3.5.1.2.** and **3.5.1.3.**).
2. Anesthetize the mouse with avertin administered intraperitoneally.
3. Place the anesthetized mouse on a clean piece of tissue paper on a clean table in a prone position (head down).
4. Shave its lower back and both sides of the abdomen. Swab the shaven area with a 10% povidone-iodine scrub and 70% ethanol.
5. Turn the mouse toward one side. Using a pair of forceps, hold the skin of the abdomen and make a small cut (approx 1 cm long) with a pair of scissors.
6. Next, hold and slightly pull the abdominal muscle and make a small incision in the abdominal muscle, avoiding the blood vessels of the muscle. If the cut has been made in the right place, the ovarian fat pad is easily visible. If not, slightly lift the edge of the body wall and try to locate the fat pad.
7. Once the fat pad is located, gently lift the fat pad out the body. Ovary, oviducts, and a part of the uterus will come out with the fat pad (**Fig. 7**).

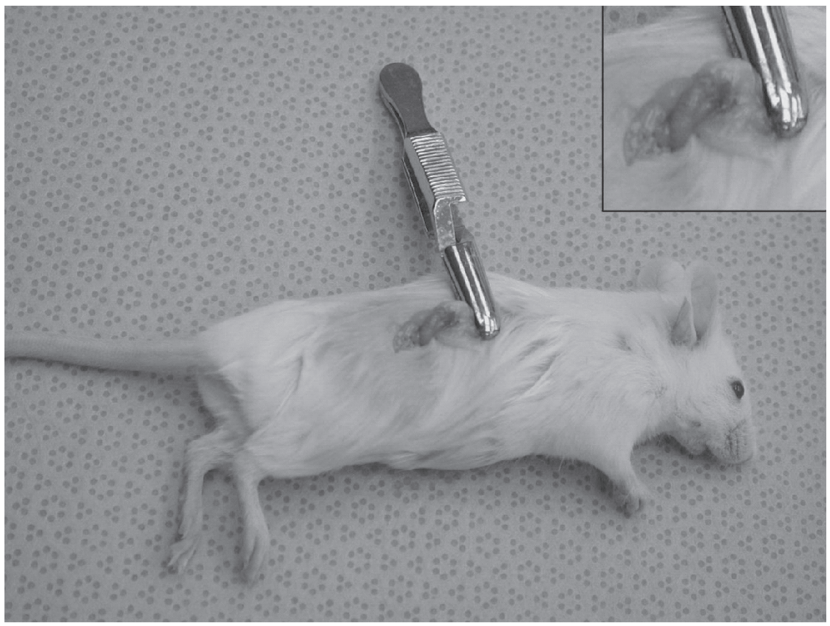

Fig. 7 (*see* companion CD for color version). Schematic representation to show exteriorization of utero-tubal junction in the anesthetized mouse. The method of exteriorization of utero–tubal junction is described under **Subheading 3.6.4.**

8. Attach a serrefine clip to the fat pad (do not to clip the ovary). In the absence of the ovary in the hormone-treated ovariectomized mice, clip the fat and mesentery near the oviduct. Try not to touch the uterine horns during this procedure.
9. Locate the tip and gently hold the uterine horn with a pair of forceps approx 1 cm below the utero–tubal junction (**Fig. 7**).
10. Five to seven blastocysts must be transferred to each horn. Take up a minute amount of embryo culture medium (Whitten's media) in the very tip of the transfer pipet by moving the plunger cap counterclockwise. Next, make a small bubble by taking up a little air. Then take up some more medium—roughly the same volume as you hope to transfer the blastocysts in. Take up another bubble, the same size as before. Then take up blastocysts in the smallest possible volume of medium, lining them up side by side in the transfer pipet (*see* **Note 8**).
11. Once the pipet is loaded and the uterine horn positioned, gently grasp the top of the uterine horn inside a pair of forceps.
12. While still holding the horn with one hand, use the other hand to gently insert a 26-gauge hypodermic needle through the uterine wall (close to the oviduct) and into the lumen (**Fig. 8**). Choose an area of the horn that is relatively devoid of blood vessels because blood will clot in the tip of the pipet and block it. Remove

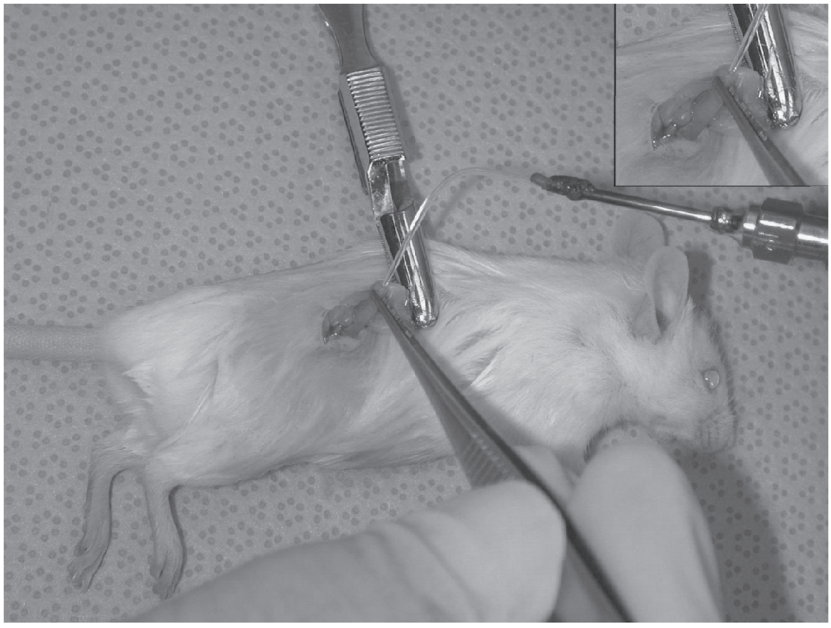

Fig. 8 (*see* companion CD for color version). Insertion of a transfer pipette into the uterine horn for embryo transfer. The method of blastocyst transfer inside uterine lumen is described under **Subheading 3.6.4.**

the needle and carefully (so as not to lose the site of the hole), without averting your eyes, pick up the loaded transfer pipet. Gently insert the transfer pipet tip about 3 mm into the uterine lumen (**Fig. 8**). Gently release the blastocysts into the uterus by turning the plunger cap clockwise. Be careful not to allow any air into the uterus.

13. Once the transfer is complete, quickly rinse the transfer pipet in some Whitten's medium and check to see if there were any blastocysts stuck in the transfer pipet. If there were, transfer these blastocysts again.
14. With the transfer complete, the serrefine clip can now be removed and the uterine horn gently eased back into the body. Do not touch the uterus, but ease it back by lifting the edges of the incision in the body wall and allowing the horn to fall back in, without actually handling it.
15. This procedure is then repeated on the other uterine horn.
16. The incision in the body wall is not sutured. The skin is closed with Michelle clips—two per incision is usually sufficient. Once surgery is complete, the mouse is placed in a clean cage and warmed to facilitate recovery (*see* **Note 9**).

3.7. Chemicals Commonly Used to Anesthetize the Mouse

There are three components of anesthesia: analgesia (pain relief), amnesia (loss of memory), and immobilization (no movement). Because general anesthetics affect the central nervous system and anesthesia is required by law to prevent pain and distress in research animals, it must not be taken lightly. It is not necessary to withhold food and water from rodents prior to anesthesia.

3.7.1. Avertin (A Commonly Used Injectable Anesthetic)

1. Preparation of an Avertin (components: Avertin [2,2,2-tribromoethanol] and tert-amyl alcohol) stock solution (1.6 g/mL):
   a. Add 15.5 mL T-amyl alcohol to 25 g of Avertin in a dark bottle.
   b. Stir on magnetic stirrer until the avertin is dissolved (approx 12 h).
   c. Store in the dark at room temperature (*see* **Note 10**).
2. Preparation of an Avertin working solution (20 mg/mL):
   a. Mix 0.5 mL Avertin stock solution and 39.5 mL normal saline in a beaker.
   b. Seal container with parafilm, wrap in foil to exclude light, and stir on magnetic stirrer for about 12 h or until dissolved.
   c. Avertin working solutions must be kept refrigerated in a dark bottle until used and should be replaced at least every month.
   d. Sterilize through 0.2-μm filter and store at 4°C. Working solutions should be replaced at least every month.
3. Dosages (0.4–0.6 mg/g body weight). 0.45–0.75 mL/mouse administered intraperitoneally (*see* **Note 11**).

3.7.2. Commonly Used Short-Lasting Inhalant Anesthetic (Isoflurane)

1. Approximately 1 mL isoflurane is placed on a cotton ball in a bell jar or screw-top glass jar.
2. The mouse is then inserted inside the jar and removed when it is fully unconscious (unconsciousness can be judged by pinching the toe).
3. Duration of these anesthetics is 30 s to 2 min.
4. A nose cone containing a small amount of anesthetic is placed in front of the nose to maintain the depth of anesthesia.

3.8. Injection Techniques in the Mouse

Four types of injections are commonly used in mice: subcutaneous (SC), intraperitoneal (IP), intravenous (IV), and intramuscular (IM). Animals must be adequately restrained or anesthetized to receive injection.

3.8.1. Subcutaneous Injection (Anesthesia Not Required)

1. Grasp the base of the tail with the thumb and forefinger of one hand.
2. Place the mouse on the top of the cage cover (wire top). Clear the back of the neck with an alcohol swab.

3. Hold the syringe attached to a 27-gauge needle parallel to the head.
4. As the mouse attempts to move forward, quickly insert the tip of the needle in the scruff (loose skin on the back of the neck) at a very shallow angle and lift the skin with the needle to avoid underlying muscle. Now inject the solution and remove the needle slowly to avoid leaking. The larger the volume of injected solution, the greater the likelihood of leakage. It is also advisable to pinch the injection side with your thumb and forefinger to prevent leaking.

3.8.2. Intraperitoneal Injection (Anesthesia Not Required)

1. The procedures for grabbing and holding the mouse are described earlier.
2. Clean the injection site of one side of the abdomen with an alcohol swab. The caudal left abdominal quadrant is the preferred place for IP injection in order to avoid the cecum on the right.
3. Tilt the animal toward its head in order to allow the abdominal contents to fall away from the injection site.
4. Quickly insert a 27-gauge needle attached to a 1-mL sterile syringe containing the drug down through the abdominal wall to the peritoneal cavity and inject the animal. It is not uncommon to inject volumes up to 1 mL by this route.

3.8.3. Intravenous Injection (Anesthesia Required)

This method is similar to the method of injection of blue dye through tail vein (**Fig. 1**).

3.8.4. Intramuscular Injection (Anesthesia Not Required)

1. Grasp the mouse as described in the methodology for subcutaneous injection.
2. The quadricep muscle and the posterior thigh are acceptable sites for intramuscular injections. Clean the injection sites with an alcohol swab.
3. Insert the needle through the skin into the muscle and inject the desired amount.

3.9. Euthanasia

Cervical dislocation is the most common method of killing mice. However, mice can be killed using inhalants.

3.9.1. Cervical Dislocation

1. Hold the mouse at the base of the tail with the thumb and forefinger of one hand.
2. Keep the mouse on the cage top. As the mouse tries to move forward, quickly place the thumb and the forefinger of other hand behind the skull and hold firmly on the cage top.
3. Next, pull the tail in the direction away from the body. This will dislocate the neck. This should be performed very quickly.

3.9.2. Inhalants

3.9.2.1. Carbon Dioxide

Carbon dioxide inhalation is the most efficient and acceptable method of euthanasia.

1. A mouse cage with a solid lid is connected to a carbon dioxide gas cylinder.
2. Place the mice in the cage and cover the cage with a lid.
3. Open the carbon dioxide cylinder and fill the cage with gas.
4. The animals will die within 1–2 min.

3.9.2.2. Isoflurane

Isoflurane overdose can also be used to kill mice.

1. A cotton wool or gauze soaked with isoflurane is placed inside a bell jar or a screw-cap glass container.
2. Place the mouse inside the jar. The mice will die within 1 min (*see* **Note 12**).

4. Notes

1. The process of detecting copulatory plugs should be performed gently because stimulation of the vagina may induce pseudopregnancy.
2. Pregnant mice usually do not breed when placed with male mice. There is no other visual or noninvasive method for definitive identification of early pregnancy.
3. Abdominal distention is apparent in most mice by day 8 or later depending on the litter size and degree of swelling of the implantation sites.
4. This is a relatively simple procedure but requires practice. If the needle is inside the vein, injection will be smooth. If the syringe plunger does not move smoothly and resistance is felt while injecting or swelling around the injection site occurs, withdraw the needle and try again slightly above the first injection site (proximal to the body). It is always advisable to start injecting from the tip of the tail. After several attempts, it is advisable to change the needle because the tip becomes blunt.
5. The purpose of describing delayed implantation is that this model provides a powerful tool to examine steroid hormone regulation of uterine and embryonic changes with respect to embryo–uterine interactions during implantation.
6. The pseudopregnant female will display the hormonal profile of a normal pregnant female for several days after mating. The hormonal milieu of pseudopregnancy begins to differ from pregnancy after 7 to 8 d as a result of the absence of a developing embryo inside the uterus.
7. Injection of too much oil inside the lumen may migrate to the other uterine horn and cause decidualization. Prominent swelling of the uterus will indicate the extent of stromal cell decidualization in response to the artificial stimulus. Swelling of the uterus due to decidualization will be visible 48 h after the oil injection. The intraluminal oil injection to a pseudopregnant mouse uterus on day

4 afternoons (1300–1400 h) will yield the best results. If ovariectomized mice are used, animals should be exposed to progesterone (SC injection) for at least 48 h (daily injection of 2 mg progesterone per 0.1 mL sesame seed oil per day for 2 d) before the injection of oil inside the uterine lumen. These animals must also be maintained with daily progesterone injection after the induction of decidualization.

8. Loading blastocysts into the transfer pipet will take some practice. If it is likely to take more than a few minutes to load the transfer pipet, then do not expose the uterine horn until the pipet has been loaded. This prevents drying out and further trauma to the uterine horn. Alternatively, the uterine horn, ovary, and so on may be moistened repeatedly with a sterile cotton bud and saline.
9. Animal welfare guidelines recommend that all vertebrates undergoing procedures that might cause more than momentary pain or distress be treated with analgesics, unless it can be scientifically justified that the treatment will interfere with the experimental procedure. Analgesics should be given immediately after the surgery. A simple skin incision may only require 24 h of analgesic treatment. Rodents subjected to abdominal surgery or similar procedures normally require analgesic for the first 12 h. It is not appropriate to wait until signs of pain or distress are demonstrated before administering analgesics. In rodents, the signs of pain following surgery are manifested as decreases in food and water consumption. Consult your veterinarian and animal care committee for specific postoperative care in your institute, because these procedures are based on institutional rules and regulations.
10. The Avertin stock solution is light-sensitive and hydroscopic. Keep Avertin in a dark bottle at room temperature. The Avertin stock solution is quite stable at room temperature
11. It will take about 3–5 min for the mouse to become fully anesthetized (lack of toe-pinch reflex). An additional 0.1–0.2 mL can be administered if required. The mouse will remain anesthetized for approx 15–20 min and recover within 30–60 min. Keep the mouse warm during recovery. The effective dosage is dependent upon the weight of the mouse.
12. Isoflurane should be used in a fume hood to minimize the risk of exposure to the gas by the operator.

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