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Strategies and Considerations for Distributing and Recovering Mouse Lines

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Abstract

As more and more genetically modified mouse lines are being generated, it becomes increasingly common to share animal models among different research institutions. Live mice are routinely transferred between animal facilities. Due to various issues concerning animal welfare, intellectual property rights, colony health status and biohazard, significant paperwork and coordination are required before any animal travel can take place. Shipping fresh or frozen preimplantation embryos, gametes, or reproductive organs can bypass some of the issues associated with live animal transfer, but it requires the receiving facilities to be able to perform delicate and sometimes intricate procedures such as embryo transfer, *in vitro* fertilization (IVF), or ovary transplantation. Here, we summarize the general requirements for live animal transport and review some of the assisted reproductive technologies (ART) that can be applied to shipping and reviving mouse lines. Intended users of these methods should consult their institution's responsible official to find out whether each specific method is legal or appropriate in their own animal facilities.

1. Introduction

The laboratory mouse has remained a favored model organism in biomedical research for over a century. Especially since the development of transgenic mouse technology in the late 1970s and the knockout mouse technology in late 1980s, the number of useful mouse models has increased exponentially. Majority of these genetically modified mouse lines are being created by individual research laboratories and transgenic core facilities throughout the world, and large-scale production facilities now exist which aim at generating at least one null mutant mouse line for every gene. It is critically important that the research community can share these valuable genetic resources by acquiring mouse lines through transportation. Thus far, majority of the mouse line transfers occur as shipment of live animals, which is technically simple and reliable, but requires substantial paperwork and coordination. Mouse lines can also be transferred in the form of embryos, gametes, or dissected reproductive organs. These forms of transfers are generally more technically challenging because they involve the use of sometimes complicated micromanipulative and surgical procedures. But they are often easier to arrange for the actual transportation. With recent improvements in mouse sperm cryopreservation and *in vitro* fertilization (IVF) methods, archiving and delivering frozen sperm is bound to play an important role in future mouse line distribution (Ostermeier *et al.*, 2008). In fact, systematic archiving and distributing genetic modified mouse lines in the form of frozen sperm have already begun at large centers, such as the Jackson Laboratory (JAX) and Mutant Mouse Regional Resource Centers (MMRRC). Individual researchers are also encouraged to deposit their strains to the repositories to facilitate such efforts.

This chapter is intended to summarize the general considerations for animal transport, and to review some of the technologically proven methods that have been used to transfer and revive mouse lines. Different countries, states, or institutions may have specific

requirements and policies for animal transport, and the rules should therefore be conformed to when arranging for the actual transportation. We advise intended users of these methods to consult with their institutional animal care and use committee (ACUC) and animal facility manager about whether these methods are legal or appropriate for their specific situations.

2. Shipping Live Mice

Live animals can be transported domestically and internationally following proper procedures and regulations. Transferring live mice can avoid the technical challenging and sometimes problematic procedures, such as IVF and embryo transfer. Usually, a few breeding pairs of live mice can ensure the receiving institution to establish a sizeable colony within 3–6 months. A large group of ready-to-use mice can also be shipped to the receiving side for immediate study. Great majority of animal facilities should already be familiar with shipping and receiving live animals, and many of them have specially designated personnel to handle the paperwork and coordinate the shipment. The cost for the actual shipment is low (about 100–200 US dollars/crate) and many freight companies can provide the transportation. However, before the shipment can take place, substantial paperwork and careful coordination are required. Generally, the following issues need to be considered for each animal shipment.

2.1. Animal welfare issues

In the United States, transportation of most animal species is regulated by the United States Department of Agriculture (USDA) and individual state (http://www.aphis.usda.gov/import_export and State requirements therein). However, research mice and rats are generally exonerated but are governed by the Public Health Service (PHS) policy, the Humane Care and Use of Laboratory Animals (<http://grants.nih.gov/grants/olaw/references/phspol.htm>), if the research is funded by the PHS. Under the Health Research Extension Act of 1985, the PHS Policy mandates adherence to the guidelines in the *Guide for the Care and Use of Laboratory Animals* (the *Guide*) (NRC, 1996, http://www.nap.edu/openbook.php?record_id=5140&page=57). Specific policies regarding to rodent transportation on institutional level vary under this general guideline, for example, NIH has policy manual 3043-1 to regulate the introduction of rodents into NIH facilities.

Internationally, for countries within the World Organization for Animal Health, formerly known as the Office International des Epizooties (OIE; http://www.oie.int/eng/OIE/en_about.htm?e1d1), arrangements can be made to send and receive mice with health certificates. Unless they are known to carry zoonotic disease or diseases of risk to domestic or wild animals, rodents can be transported internationally with specific permits, bill of lading for transportation of animals, and commercial invoice (e.g., NIH1884-1). For a comprehensive review of live animal transport, see *Guidelines for the Humane Transportation of Research Animals* (2006) prepared by the Committee on Guidelines for the Humane Transportation of Laboratory Animals (http://books.nap.edu/openbook.php?record_id=11557&page=11).

The *Guide* provides performance standards on the transportation of research animals. It states that all transportation of animals should be planned to minimize transit time and the risk of zoonoses, protect against environmental extremes (below 45 °F or above 85 °F), avoid overcrowding, provide food and water when indicated, and protect against physical trauma. Sick mice are not advised to travel, because of the stress involved during transportation. Animal distributors such as Harlan, Taconic, and Charles River Laboratories all provide shipment services. Other established ground and air shippers can be found at <http://laboratoryanimalsciencebuyersguide.com/results.php?>

category=Transportation&heading=202&category_id=2813. If traveling by air, the International Air Transport Association (IATA) Live Animals Regulations (LAR) provides guidelines for the packaging and documentations needed for the transport (<http://www.iata.org/ps/publications/live-animals.htm>). Ground transportation between airport and destinations should also be arranged to avoid any delay.

Use of laboratory animals for biomedical research is a privilege, not a right. Therefore, all major research institutions have specifically designated committee or office to evaluate and approve investigators' proposals for using animals. The mouse line to be sent should already be listed in the sender's animal study proposal before requesting the shipment. Most, if not all, receiving institutions also require the receiving investigators to add the mouse lines to be received in their animal protocols before they can access and use the animals for experimental studies. Therefore, it is advisable that the receiving investigators should start early to amend their animal protocols in order to avoid delays in receiving and using the new mouse lines.

2.2. Animal health status

One major challenge for operating an animal facility is to keep the animal colonies healthy and free of microorganism contamination. Keeping the mice disease-free is important not only for the welfare of the animals, but also for the validity of the research findings.

Experimental results obtained from sick or contaminated animals should not be trusted.

There are many mouse pathogens

(<http://www.radil.missouri.edu/info/dora/mousepag/mouse.htm>), and some are tolerated in certain research colonies (e.g., http://jaxmice.jax.org/health/agents_list.html). Most institutions have specific pathogen free (SPF) environment, while their pathogen lists may be different. During transportation, it is important to separate animals from different animal rooms to avoid cross-contamination. Improper care and monitoring during animal shipment can lead to contamination of mouse colonies and lost strains.

Most animal facilities have strict policies against the introduction of diseases or unwanted microorganisms into their animal rooms. Therefore, the receiving facilities usually require the sending animal facilities to provide direct or sentinel serological testing (<http://www.radil.missouri.edu/>) results for the past several months. Based on these health reports (e.g., NIH Form 1192), the receiving facility veterinarian can decide whether the animals should be imported directly, imported after certain period of quarantine, or they need to be cleaned up through rederivation before entering the facility. If quarantine is required, the receiving investigator must make sure there is enough quarantine space available on the day of receiving animals. Some strict animal facilities require that all animals, except from the proven vendors, to be rederived regardless of the health report.

2.3. Intellectual property rights

A good animal model may lead to scientific discoveries with significant commercial values, such as the development of new drugs or new diagnostic methods. Because of the unpredictability of scientific research, it is impossible to foresee which mouse lines are economically important and which ones are not. Therefore, most institutions require the receiving institutions to sign Material Transfer Agreements (MTA) on all mouse lines transferred to protect their intellectual properties. Often an Animal Transfer Agreement (ATA) is also required. The MTAs among most governmental agencies, universities, and nonprofit entities are quite generous, without too much limitation on the use of the animal models. However, almost all MTAs prohibit the receiving investigator from further distributing the mouse lines.

2.4. Biosafety concerns

Mice of different biosafety levels (<http://www.cdc.gov/OD/ohs/biosfty/bmbl4/bmbl4s3.htm>) should be sent and received with clear labels. Infectious disease carrying mice is subject to additional regulations imposed by the Centers for Disease Control and Prevention (CDC). CDC is part of the US Department of Health and Human Services (DHHS) and enforces regulations to prevent the introduction, transmission, or spread of communicable diseases in the United States. CDC regulates the importation of any animal or animal product capable of carrying a zoonotic disease (regulation 42CFR71.54) through permitting, registration, and quarantine. CDC may implement prohibitions on the importation of animals when new health risks to humans arise. The CDC Etiologic Agent Import Permit Program regulates the importation and subsequent transfer of animals or animal tissues within the US that contain etiologic agents. CDC defines etiologic agent as a viable microorganism or its toxin that may cause human disease, but does not specifically identify them. Contact information and permit applications for importation of etiologic agents are available at: <http://www.cdc.gov/od/eaipp/>.

Mice carrying diseases of risk to domestic or wild animals are also subject to regulations by the US Fish and Wildlife Service (<http://www.fws.gov/le/ImpExp/CommWildlifeImportExport.htm>), as they may be considered injurious species (http://www.fws.gov/contaminants/ANS/pdf_files/50CF_16_10-05.pdf). Mice carrying infectious agent are also subject to Department of Transportation (DOT) Hazardous Material (HAZMAT) regulation (<http://www.phmsa.dot.gov/hazmat/regs>), and if traveling by air, by the IATA Dangerous Goods Regulations (DGR) (<http://www.iata.org/ps/publications/elist.htm>).

3. Shipping Nonfrozen Embryos or Gametes

Freshly collected preimplantation stage embryos or sperms in epididymis can be shipped without cryopreservation. Some issues discussed for live animal transfer still apply to embryo and gamete shipment, such as ATA and MTA. However, the animal welfare issue associated with transportation is alleviated. It could translate into significant savings in both time and cost, particularly for international shipment. For contaminated mouse lines, the transportation of embryos or gametes offers an opportunity to clean them up through rederivation (Van Keuren and Saunders, 2004). Embryos from contaminated mouse lines can be washed at the sender and/or receiver's facility to be sanitized before transferring into clean surrogate mothers. Another advantage of shipping embryos is that the receiving institution does not have to have a "dirty" facility to house the infected mice. Many animal facilities actually require rederivation regardless of the health status to avoid bring in common pathogens such as mouse parvovirus virus (mpv), mouse hepatitis virus (mhv), helicobacter into their colonies.

The disadvantage of shipping embryos or gametes is that the receiving facilities must be able to revive the mouse line through embryo transfer for preimplantation embryos, or IVF (intracytoplasmic sperm injection (ICSI) if necessary) followed by embryo transfer for the sperm. Time coordination is critical for shipping fresh embryos or gametes.

3.1. Shipping fresh embryos

Preimplantation stage embryos ranging from two-cell stage to blastocyst can be shipped without freezing (Miyoshi *et al.*, 1992a), with blastocysts appear to be the most robust and commonly used. In fact, oviducts isolated from mated females, which contains two-cell embryos, can be shipped directly at low temperatures (Miyoshi *et al.*, 1992b). Shipping oviducts is particular useful if the sender's laboratory lacks the necessary skills to isolate

and handle the tiny early embryos. For mouse embryos, 0.75 *M* sucrose was found to improve the survival rate stored at 0 °C (Kasai *et al.*, 1983). Shipments can be made in regular test tubes with an appropriate thermal package, and a clear label so that it would not go through the X-rays. At the receiving institution, mouse line can be rederived through embryo transfer.

3.1.1. Brief protocols

- At sender's laboratory:
 1. Set up mating and check plug the next morning. Euthanize the plugged females on an appropriate day depending on the stage of embryos wanted as listed below:
 - Day 1 (the same day as checking plug): one-cell stage (fertilized eggs)
 - Day 2: two-cell stage embryos
 - Day 3: eight-cell and morula stage embryos
 - Day 4: blastocysts
 2. For one-cell stage embryos, dissect out oviducts and tear open the ampullae to release the cumulus mass into M2 medium (Millipore/Specialty Media, MR-015-D). Briefly treat the cumulus mass with hyaluronidase (Sigma, H4272) to isolate individual fertilized eggs.

For two-cell to morula stage embryos, flush oviducts with M2 medium to obtain embryos.

For blastocysts, dissect out uteri and flush the embryos out with M2 medium.

Two-cell through blastocyst stage embryos can also be obtained by culturing one-cell stage fertilized eggs in KSOM medium (Milli-pore/Specialty Media, MR-106-D) for 1–3 days in a 37 °C incubator with 5% CO₂.
 3. Wash the embryos in M2 medium with 0.75 *M* sucrose (Sigma, S7903), and then transfer them into a 1.5–2.0 ml cryovial containing M2. Cover the medium with mineral oil (Sigma, M8410).
 4. Early embryos inside oviducts can be shipped directly. In this case, the dissected oviducts from Day 2 or 3 are placed in a cryovial directly. Simply cover the tissue with mineral oil and the tube is ready to be shipped.
 5. Ship the tubes in a thermal package with refrigerated ice gel packs through express mail.
- At receiver's laboratory:
 1. Prepare pseudopregnant foster mothers 1–3 days before the anticipated embryo arrival.
 2. After receiving the tubes containing embryos, transfer all contents into a 35 mm tissue culture dish (NUNC 153066) using a plastic transfer pipette (Thomas Scientific, 7760M25). Wash the tube with M2 and transfer all wash media into the same 35 mm dish. Gently swirl the dish to concentrate the embryos into the center region of the dish. Under a

stereomicroscope, collect the embryos using a mouth pipette (Sigma, A5177, modified) and wash by passing through new M2 drops under mineral oil.

3. If whole oviducts were shipped, wash the oviducts in M2 to remove mineral oil. Then flush out embryos with M2 medium.
4. Implant the embryos into pseudopregnant foster mothers through embryo transfer. Blastocysts should be transferred into the uteri of 2.5 dpc (day post coitus) foster mothers, while two-cell, eight-cell, or morula stage embryos should be transferred into the oviducts of 0.5 dpc foster mothers. It is recommended to culture the embryos at 37 °C for 1–6 h before transferring into the foster mothers. Alternatively, two-cell through morula stage embryos can be cultured in KSOM till they become blastocysts, and then they are transferred into the uteri of 2.5 dpc foster mothers.

3.2. Shipping fresh sperm in epididymis

Intact epididymis was tested as a short-term storage and transportation method for mouse spermatozoa (Mochida *et al.*, 2005; Sankai *et al.*, 2001). Certain strains have reduced survival rate due to cold temperature. For C57BL/6, it was determined that 7 °C is the optimal low temperature for nonfrozen sperm storage (Mochida *et al.*, 2005). Strains therefore should be tested at low temperature conditions for survival rate before arranging for such transport. When high concentrations of B6C3F1 sperm ($4 \times 10^5 \text{ ml}^{-1}$) were stored at different temperatures, it was found that sperms retained motility at 22 °C better than at 4 or 37 °C, and can fertilized eggs after storage up to 3 days albeit at a very low rate (Sato *et al.*, 2001). We have stored epididymides from 129S6 and C57BL/6 hybrid stains at 4 °C overnight, and were still able to achieve acceptable IVF rate. Sending mouse embryos will entail less work for the receiving end, while nonfrozen transport of epididymis seems to be the simplest for the sender.

3.2.1. Brief protocols

- At sender's laboratory:
 1. Euthanize two 3- to 6-month-old male mice, and quickly dissect out epididymides and vasa deferentia. Place epididymides and vasa deferentia from each mouse into a separate 1.5–2.0 ml sterile tube. Add mineral oil into the tube to prevent the tissues from drying out.
 2. Place the tubes in a thermal package with refrigerated ice gel packs and send through express mail.
- At receiver's laboratory:
 1. Three days before the scheduled IVF, choose 5–10 young donor females (3–6 weeks). At 8 p.m., inject 5 IU pregnant mare's serum gonadotropin (PMSG; Sigma G4527) intraperitoneally per mouse.
 2. After receiving the package containing the epididymal tissue, store the tubes at 4 °C until they are used for IVF the next morning.
 3. One day before the scheduled IVF, 48 h after PMSG injection, inject 5 IU human chorionic gonadotropin (hCG; Sigma C8554) per mouse intraperitoneally into the same female mice.
 4. On the day of IVF, at 8 a.m., ~12 h after hCG injection, take the epididymides and vasa deferentia out of the tubes and place them in a 35

mm tissue culture dish containing 1 ml of IVF medium (MVF medium, Cook's Medical, K-RVFE-50).

5. Tear open the epididymis with Dumont tweezers and squeeze the sperms out of vas deferens.
6. Incubate at 37 °C (Cook's Medical, K-MINC-1000-US) equilibrated with 5% O₂, 5% CO₂, 90% N₂ (Robert Oxygen premixed gas, R0133502001) for ~60 min to allow sperm swim out of the tissue and to be capacitated.
7. Euthanize the superovulated females, and quickly dissect out ampullae. Try to keep the eggs at 37 °C at all time, and keep the time between egg collection and fertilization as short as possible.
8. Tear open the ampulla with a 29½ G needle and release the egg clutches.
9. Use a wide mouth pipette, transfer 10 µl capacitated sperm suspension into 0.5 ml IVF medium covered under mineral oil. Transfer egg clutches from about three females into the drop.
10. Incubate the dish containing sperm and eggs for 4–6 h at 37 °C with 5% O₂, 5% CO₂, and 90% N₂.
11. Wash the eggs in three drops of M2 medium, and then culture them in KSOM medium overnight in a 37 °C incubator with 5% CO₂.
12. On the same day as IVF, set up recipient females with vasectomized males.
13. On the day after IVF, check plug and transfer the embryos that have been fertilized (two-cell) into the plugged pseudopregnant females through oviduct transfer.

While cold temperature can induce sperm capacitation (Fuller and Whittingham, 1997), it can cause the disassembly of meiotic spindle of the oocytes (Pickering and Johnson, 1987). The oocytes are not normally used as a form of animal transfer, also because they only remain competent for IFV for a short period of time after ovulation (~13 h after hCG injection for most mouse strains) due to zona hardening. However, with the use of chemically- (Gordon and Talansky, 1986), laser- (Tadir *et al.*, 1991) or piezo-assisted (Kawase *et al.*, 2002) partial zona dissection (PZD) method or with ICSI (Markert, 1983; Yoshida and Perry, 2007), one can overcome the zona hardening and increase the efficiency of IVF. Fresh oocytes beyond the 13-h window can presumably be shipped and used to recover mouse lines for successful IVF, providing they do not undergo apoptosis. In fact, oocytes rescued from postmortem mouse (Schroeder *et al.*, 1991) and equine ovaries that had been store at room temperature (Ribeiro *et al.*, 2008) can still give rise to normal embryos. Not a suggested way to ship animals, but nonetheless useful in desperate situations to rescue important mouse lines.

4. Archiving and Distributing Mouse Lines Through Cryopreservation

Mouse lines should be cryopreserved as soon as possible to prevent accidental loss, genetic drift, and to reduce cost associate with the maintaining of live animals (Landel, 2005; Shaw and Nakagata, 2002). They can be frozen as sperm (Ostermeier *et al.*, 2008; Polge *et al.*, 1949; Tada *et al.*, 1990), oocytes (Nakagata, 1989; Tsunoda *et al.*, 1976; Whittingham, 1971, 1977; Whittingham *et al.*, 1972), embryos (Miyoshi *et al.*, 1992b; Whittingham, 1971; Whittingham *et al.*, 1972), ovary (Agca, 2000; Candy *et al.*, 2000; Cox *et al.*, 1996; Harp *et al.*, 1994; Migishima *et al.*, 2003; Sztein *et al.*, 1998), and testis (Ogonuki *et al.*, 2006; Ohta *et al.*, 2008). Many large animal resource centers, such as the JAX

(<http://cryo.jax.org/index.html>; <http://jaxservices.jax.org/cryopreservation/index.html>), MMRRC (<http://www.mmrrc.org/>), and European Mouse Mutant Cell Repository (EuMMCR; <http://www.eummc.org/>), are systematically archiving mutant mouse lines through cryopreservation. Many public and commercial repositories also offer custom cryopreservation services for individual investigators.

The cryopreserved embryos, gametes, or gonads can be conveniently shipped worldwide for distribution and reconstitution of mouse lines. Although sperm was successfully shipped at -80°C (Okamoto *et al.*, 2001), it is recommended that cryopreserved material be shipped in liquid nitrogen dry shippers, such as Cryo Express (CX) series from Taylor-Wharton (<http://www.taylorwharton.com/default.aspx?pageid=72>) and Vapor Shipper containers from MVE (http://www.chartbiomed.com/p_vapor_shippers.cfm; <http://www.cryodepot.com/shippers.htm>). Dry shippers are designed to maintain the liquid nitrogen temperature at approximately -190°C , yet to prevent the spill of liquid nitrogen if tipped over. It is important to completely saturate the absorbent material in the dry shipper with liquid nitrogen before put in the samples.

4.1. Freezing and shipping oocytes and preimplantation embryos

Preimplantation stage embryos from thousands of mouse lines have been cryopreserved, which can be shipped around the world to reconstitute live mice through embryo transfer. A major drawback for embryo cryopreservation is that a relatively large breeding colony is required in order to collect enough embryos (usually 200–500) to safely store and distribute the line. The cryopreservation methods can generally be categorized into two groups, the slow equilibration method and the rapid nonequilibration method, with corresponding thawing procedures. The slow method relies on the use of a controlled rate freezer to cool the embryos at a slow rate of $\sim 1^{\circ}\text{C}/\text{min}$. It is very reliable and the cryoprotectant used in this method is relatively nontoxic. On the other hand, the rapid nonequilibration method, commonly called vitrification, uses high concentration ($>5\text{ M}$) of cryoprotectant and an extremely fast ($>1000^{\circ}\text{C}/\text{min}$) cooling speed. It solidifies the solution inside the embryos into a glass state without forming ice crystals. The vitrification method is fast and does not require sophisticated instruments, and thus easy to adopt for laboratories not commonly doing cryopreservations.

4.1.1. Brief vitrification protocol

- At sender's laboratory:
 1. Wash freshly isolated embryos in PBS containing 1 *M* DMSO (Sigma D2650).
 2. Transfer embryos in 5 μl of 1 *M* DMSO solution to a freezing tube (NUNC 366656) and incubate at 0°C (Nalgene Labtop Cooler 5115-0012) for 5 min.
 3. Add 45 μl cold cryoprotective solution DAP213 (PBS containing 2 *M* DMSO, 1 *M* acetamide, 3 *M* propylene glycerol) into the freezing tube and further incubate for 5 min on ice.
 4. Quickly fix the tube to a cane and plunge samples directly into liquid nitrogen in a Dewar container. Ship the tube containing the embryos in liquid nitrogen dry shipper through express mail.
- At receiver's laboratory:
 1. Take the tube out of the dry shipper and leave it at room temperature for 30 s.

2. Add 0.9 ml PBS containing 0.25 M sucrose prewarmed at 37 °C into the freezing tube and thaw the samples quickly by pipetting. Transfer the contents into a 35 mm culture dish. For morulae before the blastocyst stage, pipet gently so that the blastomeres will not be dislodged.
3. Wash the tube with the same solution and combine. Recover the embryos and transfer to M2 or KSOM medium. Wash after 10 min of incubation or 60 min for blastocysts.
4. Transfer to recipient females according to protocols in Section 3.1.1.

Oocytes can be vitrified similarly by first free them from cumulus cells by hyaluronidase treatment, and incubate in Human Tubal Fluid (HTF) medium containing 20% FCS for 10–20 min to prevent zona hardening (George *et al.*, 1992). After thawing, follow protocols described in Section 3.2.1 for IVF.

4.2. Freezing and shipping sperm

With recent improvements in methodology, cryopreservation of spermatozoa is quickly becoming the favored method for archiving and distributing mouse lines. Unlike the cryopreservation of preimplantation embryos, which requires many donor mice, sperm cryopreservation requires only two breeding aged males. Therefore, much less “front-end” work is required for safely store and distribute mouse lines. Recent work by Ostermeier *et al.* (2008) at the JAX showed that sperm from all tested strains can be cryopreserved and revived although their efficiencies are still strain-dependent. One disadvantage of sperm cryopreservation is that only one haploid gamete is preserved, which must be combined with another haploid gamete (egg) in order to fully reconstitute the mouse line. This can be problematic if the full genome of a mouse line with extensively backcrossing or complicated breeding history is desired. IVF is needed at the receiving end to recover the strain. For strains with low recovery rate, assisted reproductive technologies (ART) such as PZD and ICSI can be used to increase efficiency. With ICSI, even freeze-dried sperm can be used to achieve fertilization, which may prove to be the most cost effective way to archive large number of mouse lines (Wakayama and Yanagimachi, 1998).

4.2.1. Brief protocols

- At sender’s laboratory:
 1. Prepare cryoprotective medium (CPM) with distilled water (Invitrogen #15230-162) containing 18% raffinose (Sigma R7630), 3% skim milk (BD Diagnostics #232100), and 478 μ M monothioglycerol (MTG; Sigma M6145). Warm the water to 40 °C to facilitate the dissolving of chemicals. Spin at $13,000 \times g$ for 15 min to sediment debris, and filter sterilize supernatant with a 0.22 μ m filter cup (Milli-pore, SCGPU02RE). CPM can be aliquoted and stored at –80 °C for up to 6 months.
 2. Euthanize two males, preferably 3–6 months old, that have been single housed for more than 48 h.
 3. Dissect out vasa deferentia and epididymides. Tear open epididymis and squeeze out semen from vas deferens in 1 ml CPM in a 3.5 cm petri dish.
 4. Incubated the dish at 37 °C for 10 min to allow sperm to swim out.
 5. Load the sperm suspension into labeled straws (IMV, AAA201, 005565) as 4×0.5 cm segments spaced by air with straw attached to a 1 ml Monoject syringe.

6. Seal the straw with a heat sealer (American International Electric, AIE-305HD) and place them in labeled straw cassettes (Zanders Medical Supplies, 16980/0601).
 7. Place the cassettes on top of a Styrofoam raft floating on liquid nitrogen (6–9 cm deep) in a ThermoSafe (Thermo Fisher, 11-676-13) for 10 min.
 8. Plunge the cassettes in liquid nitrogen. Ship the straw in liquid nitrogen dry shipper through express mail.
- At receiver's laboratory:

The straw containing frozen sperm should be thawed quickly at 37 °C about 1 h prior to the scheduled IVF procedure. Perform IVF and embryo transfer according to the procedures described in Section 3.2.1 to revive the mouse lines.

4.3. Reviving mouse lines through transplantation of fresh or cryopreserved gonads

Orthotopic transplant of ovary had been done to produce live offspring (Robertson, 1940). Ovaries have been successfully frozen either by slow freezing (Candy *et al.*, 2000; Cox *et al.*, 1996; Gunasena *et al.*, 1997; Parrott, 1960; Szein *et al.*, 1998) or vitrification (Kagabu and Umezu, 2000; Migishima *et al.*, 2003; Takahashi *et al.*, 2001), and transplanted after thawing into immunologically compatible recipient to generate live pups. Even postmortem ovaries were successfully cryopreserved and transplanted to rescue the mouse line (Takahashi *et al.*, 2001).

Spermatogonia were successfully transferred to produce mature spermatozoa (Brinster and Zimmermann, 1994) and transmitted through germ line (Brinster and Avarbock, 1994). Cryopreserved germline cells were later demonstrated to do the same (Avarbock *et al.*, 1996). If immature germ-cells have to be used for transfer, this spermatogonial transplant technique can be deployed. Recently, spermatozoa and spermatids retrieved from frozen testis and epididymis were used successfully in ICSI to produce normal offspring (Ogonuki *et al.*, 2006). The organs or even the whole body were simply frozen without any cryoprotectant. The authors found that potassium-rich Ca^{2+} - and Mg^{2+} -free nucleus isolation medium (NIM) medium always gave better results for ICSI. Similarly, spermatozoa retrieved from frozen testicular sections were also successfully used in ICSI to produce live born mice (Ohta *et al.*, 2008), confirming frozen testis as a valid source for spermatozoa to be used in ICSI, and providing another way to archive large number of mouse lines.

5. Conclusion

Sharing genetically modified mouse lines is becoming increasingly important for biomedical research. So far, shipping live animals is the most widely used method for sharing mouse models. Breeding pairs can be transported among different animal facilities after necessary paperwork and coordination. As more and more animal facilities are becoming capable of performing embryo transfers and IVF procedures, shipping fresh or frozen embryos/gametes is becoming an important alternative method, particularly for international transfers where shipment of live animals can be cumbersome. Coordinated large-scale efforts are currently underway to cryopreserve and bank essentially all genetically modified mouse lines. With the recent improvements in methods, sperm cryopreservation is becoming the favored method for archiving mouse lines. Therefore, it is anticipated that shipping cryopreserved sperm will become more popular for distributing mouse lines in the future. At the present time, the efficiency and reliability of mouse sperm cryopreservation and IVF are generally acceptable, but they still depend upon the genetic background of the mice, and demand the involved personnel to strictly follow the specified experimental conditions and timelines.

Consequently, experienced staff with proven equipment setup is required in order to reliably recover the mouse line. Hopefully, further improvement in mouse sperm cryopreservation and IVF methods will reduce the technical challenge and make this method more robust and dependable.

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References

- Agca Y. Cryopreservation of oocyte and ovarian tissue. *ILAR J.* 2000; 41:207–220. [PubMed: 11123181]
- Avarbock MR, Brinster CJ, Brinster RL. Reconstitution of spermatogenesis from frozen spermatogonial stem cells. *Nat Med.* 1996; 2:693–696. [PubMed: 8640563]
- Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. *Proc Natl Acad Sci USA.* 1994; 91:11303–11307. [PubMed: 7972054]
- Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA.* 1994; 91:11298–11302. [PubMed: 7972053]
- Candy CJ, Wood MJ, Whittingham DG. Restoration of a normal reproductive lifespan after grafting of cryopreserved mouse ovaries. *Hum Reprod.* 2000; 15:1300–1304. [PubMed: 10831559]
- Cox SL, Shaw J, Jenkin G. Transplantation of cryopreserved fetal ovarian tissue to adult recipients in mice. *J Reprod Fertil.* 1996; 107:315–322. [PubMed: 8882299]
- Fuller SJ, Whittingham DG. Capacitation-like changes occur in mouse spermatozoa cooled to low temperatures. *Mol Reprod Dev.* 1997; 46:318–324. [PubMed: 9041134]
- George MA, Johnson MH, Vincent C. Use of fetal bovine serum to protect against zona hardening during preparation of mouse oocytes for cryopreservation. *Hum Reprod.* 1992; 7:408–412. [PubMed: 1587950]
- Gordon JW, Talansky BE. Assisted fertilization by zona drilling: A mouse model for correction of oligospermia. *J Exp Zool.* 1986; 239:347–354. [PubMed: 3760806]
- Gunaseena KT, Villines PM, Critser ES, Critser JK. Live births after autologous transplant of cryopreserved mouse ovaries. *Hum Reprod.* 1997; 12:101–106. [PubMed: 9043912]
- Harp R, Leibach J, Black J, Keldahl C, Karow A. Cryopreservation of murine ovarian tissue. *Cryobiology.* 1994; 31:336–343. [PubMed: 7924392]
- Kagabu S, Umezumi M. Transplantation of cryopreserved mouse, Chinese hamster, rabbit, Japanese monkey and rat ovaries into rat recipients. *Exp Anim.* 2000; 49:17–21. [PubMed: 10803357]
- Kasai M, Niwa K, Iritani A. Protective effect of sucrose on the survival of mouse and rat embryos stored at 0 degree C. *J Reprod Fertil.* 1983; 68:377–380. [PubMed: 6864652]
- Kawase Y, Iwata T, Ueda O, Kamada N, Tachibe T, Aoki Y, Jishage K, Suzuki H. Effect of partial incision of the zona pellucida by piezo-micromanipulator for in vitro fertilization using frozen-thawed mouse spermatozoa on the developmental rate of embryos transferred at the 2-cell stage. *Biol Reprod.* 2002; 66:381–385. [PubMed: 11804952]
- Landel CP. Archiving mouse strains by cryopreservation. *Lab Anim (NY).* 2005; 34:50–57. [PubMed: 15806091]
- Markert CL. Fertilization of mammalian eggs by sperm injection. *J Exp Zool.* 1983; 228:195–201. [PubMed: 6663257]
- Migishima F, Suzuki-Migishima R, Song SY, Kuramochi T, Azuma S, Nishijima M, Yokoyama M. Successful cryopreservation of mouse ovaries by vitrification. *Biol Reprod.* 2003; 68:881–887. [PubMed: 12604638]
- Miyoshi I, Ishikawa K, Kasai M, Kasai N. A practical transport system for mouse embryos cryopreserved by simple vitrification. *Lab Anim Sci.* 1992a; 42:323–325. [PubMed: 1320171]
- Miyoshi I, Ishikawa K, Kasai M, Kasai N. Useful short-range transport of mouse embryos by means of a nonfreezing technique. *Lab Anim Sci.* 1992b; 42:198–201. [PubMed: 1318455]

- Mochida K, Ohkawa M, Inoue K, Valdez DM Jr, Kasai M, Ogura A. Birth of mice after in vitro fertilization using C57BL/6 sperm transported within epididymides at refrigerated temperatures. *Theriogenology*. 2005; 64:135–143. [PubMed: 15935849]
- Nakagata N. High survival rate of unfertilized mouse oocytes after vitrification. *J Reprod Fertil*. 1989; 87:479–483. [PubMed: 2600904]
- Ogonuki N, Mochida K, Miki H, Inoue K, Fray M, Iwaki T, Moriwaki K, Obata Y, Morozumi K, Yanagimachi R, Ogura A. Spermatozoa and spermatids retrieved from frozen reproductive organs or frozen whole bodies of male mice can produce normal offspring. *Proc Natl Acad Sci USA*. 2006; 103:13098–13103. [PubMed: 16920794]
- Ohta H, Sakaide Y, Wakayama T. The birth of mice from testicular spermatozoa retrieved from frozen testicular sections. *Biol Reprod*. 2008; 78:807–811. [PubMed: 18199885]
- Okamoto M, Nakagata N, Toyoda Y. Cryopreservation and transport of mouse spermatozoa at –79 degrees C. *Exp Anim*. 2001; 50:83–86. [PubMed: 11326428]
- Ostermeier GC, Wiles MV, Farley JS, Taft RA. Conserving, distributing and managing genetically modified mouse lines by sperm cryopreservation. *PLoS ONE*. 2008; 3:e2792. [PubMed: 18665210]
- Parrott DMV. The fertility of mice with orthotopic ovarian grafts derived from frozen tissue. *J Reprod Fertil*. 1960; 1:230–241.
- Pickering SJ, Johnson MH. The influence of cooling on the organization of the meiotic spindle of the mouse oocyte. *Hum Reprod*. 1987; 2:207–216. [PubMed: 3597741]
- Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*. 1949; 164:666. [PubMed: 18143360]
- Ribeiro BI, Love LB, Choi YH, Hinrichs K. Transport of equine ovaries for assisted reproduction. *Anim Reprod Sci*. 2008; 108:171–179. [PubMed: 17888596]
- Robertson G. Ovarian transplantation in the house mouse. *Proc Soc Exp Biol Med*. 1940; 44:302–304.
- Sankai T, Tsuchiya H, Ogonuki N. Short-term nonfrozen storage of mouse epididymal spermatozoa. *Theriogenology*. 2001; 55:1759–1768. [PubMed: 11393225]
- Sato M, Ishikawa A, Nagashima A, Watanabe T, Tada N, Kimura M. Prolonged survival of mouse epididymal spermatozoa stored at room temperature. *Genesis*. 2001; 31:147–155. [PubMed: 11783005]
- Schroeder AC, Johnston D, Eppig JJ. Reversal of postmortem degeneration of mouse oocytes during meiotic maturation in vitro. *J Exp Zool*. 1991; 258:240–245. [PubMed: 2022948]
- Shaw JM, Nakagata N. Cryopreservation of transgenic mouse lines. *Methods Mol Biol*. 2002; 180:207–228. [PubMed: 11873651]
- Sztejn J, Sweet H, Farley J, Mobraaten L. Cryopreservation and orthotopic transplantation of mouse ovaries: New approach in gamete banking. *Biol Reprod*. 1998; 58:1071–1074. [PubMed: 9546742]
- Tada N, Sato M, Yamanoi J, Mizorogi T, Kasai K, Ogawa S. Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol. *J Reprod Fertil*. 1990; 89:511–516. [PubMed: 2401978]
- Tadir Y, Wright WH, Vafa O, Liaw LH, Asch R, Berns MW. Micromanipulation of gametes using laser microbeams. *Hum Reprod*. 1991; 6:1011–1016. [PubMed: 1761652]
- Takahashi E, Miyoshi I, Nagasu T. Rescue of a transgenic mouse line by transplantation of a frozen-thawed ovary obtained postmortem. *Contemp Top Lab Anim Sci*. 2001; 40:28–31. [PubMed: 11451392]
- Tsunoda Y, Parkening TA, Chang MC. In vitro fertilization of mouse and hamster eggs after freezing and thawing. *Experientia*. 1976; 32:223–224. [PubMed: 944640]
- Van Keuren ML, Saunders TL. Rederivation of transgenic and gene-targeted mice by embryo transfer. *Transgenic Res*. 2004; 13:363–371. [PubMed: 15517995]
- Wakayama T, Yanagimachi R. Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nat Biotechnol*. 1998; 16:639–641. [PubMed: 9661196]
- Whittingham DG. Survival of mouse embryos after freezing and thawing. *Nature*. 1971; 233:125–126. [PubMed: 12058753]
- Whittingham DG. Fertilization in vitro and development to term of unfertilized mouse oocytes previously stored at –196 degrees C. *J Reprod Fertil*. 1977; 49:89–94. [PubMed: 833794]

- Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos frozen to -196 degrees and -269 degrees C. *Science*. 1972; 178:411–414. [PubMed: 5077328]
- Yoshida N, Perry AC. Piezo-actuated mouse intracytoplasmic sperm injection (ICSI). *Nat Protoc*. 2007; 2:296–304. [PubMed: 17406589]