Chimera mouse production by blastocyst injection

Preparation of blastocyst donors

1. Set up matings between C57BL6$^{cBrd/cBrd}$ females in oestrus with fertile C57BL6$^{cBrd/cBrd}$ males 4 days before the planned blastocyst injection date.
2. The following day, check the females for copulation plugs. Store plugged females in waiting for the injection date.

Preparation of pseudo-pregnant recipients for uterine transfer

1. Set up matings between CBA;C57BL6/J F1 hybrid females with vasectomised males 3 days before the planned injection date.
2. The following day, check the females for copulation plugs. Store plugged females in waiting for the injection date.

Preparation of embryonic stem (ES) cells for blastocyst injection

1. Thaw ES cells by placing a freezing vial in a heated block at 37 ^{o}C until fully defrosted (~ 2 – 3 min).
2. Gently pipette the cell suspension into a 15 ml Falcon tube and add 7 ml of M15 a few drops at a time while mixing gently.
3. Spin at 1300 for 5 minutes, aspirate off supernatant, resuspend the cell pellet gently in 3 ml of M15, and add cell suspension to feeder plate (3ml/well of a 6-well plate).
4. **On the day of the injection**, change media 0.5 to 2 hours before adding trypsin to the cells.
5. Aspirate off media from well with desired clone for microinjection.
6. Wash the well with 1 ml of PBS, aspirate off PBS (repeat twice), and add trypsin to the well (200 µl/well of a 24-well plate, 500 µl/well for a 6-well plate). Incubate at 37 ^{o}C for 15 min.
7. In the meantime label a sterile 1.5 ml Eppendorf tube with: Gene name, clone ID, microinjection number, and date.
8. Look under microscope to ensure that cells have separated and lifted up from the bottom of the plate.
9. Add M15 to the well to inactivate trypsin (1.5 ml/well 24 well, 3ml 6 well). Pipette up and down several times to resuspend cells.
10. Transfer cell suspension into a Falcon tube and centrifuge for 2 min at 2000 rpm.
11. Aspirate off media, put 0.5-1 ml of microinjection media into the Falcon tube and resuspend cells.
12. Transfer cell suspension from Falcon tube into sterilised 1.5ml Eppendorf tube and put on ice while transferring to microinjection station.

Flushing of Blastocysts

1. Sacrifice by cervical dislocation the C57Bl6/J$^{cBrd/cBrd}$ females that were plugged 3.5 days before the day of the injection.
2. Open the abdominal cavity and remove the uterus, grasp it with fine forceps just above the cervix (located behind the bladder) and cut across the cervix with fine scissors. Pull the uterus upward to stretch the mesometrium and use fine scissors to trim this membrane away close to

the wall of the uterine horns. Then cut between the oviduct and the ovary, keeping the utero-tubal junction intact.

3. Place the uterus in a small volume of M2 medium in a 35-mm plastic tissue culture dish. The next step depends on which way the uterus will be flushed:
    a. **Option 1 (from Cervix toward Oviduct).** Because the utero-tubal junction acts as a valve, cut it lengthwise to allow flushing. Insert the needle into the cut cervix and slide it into the base of each horn. Flush each horn with ~0.5 ml of M2 medium using a 26-gauge hypodermic needle and syringe.
    b. **Option 2 (toward Cervix).** Cut each uterine horn near the cervix. Insert a 26-gauge needle into the upper part of the uterus near the utero-tubal junction and flush each horn toward the cervix.
4. Use a pipette to pick up the embryos and wash them through several drops of fresh M2 medium to rinse off the debris. Then, transfer the embryos to a microdrop culture dish at 37 °C, 5% CO_2 until needed.

Blastocyst injection

1. Pre-cool the injection chamber on the microscope stage to 10°C.
2. Set up depression slide with a drop of injection medium covered with mineral oil. Using a mouth pipette, introduce a few hundred ES cells onto the depression slide filling, at most, half the drop and allow them to settle on the bottom.
3. Use a transfer pipette to transfer the expanded blastocysts in groups of ~10 onto the depression slide.
4. Transfer injection slide to the microscope stage. Align injection needle and holding capillary so that they are on the injection slide.
5. Using high-power magnification, select individual cells carefully on the basis of size (small, compared with the feeder cells) and shape (uniformly round, compared with more ragged or "rough" feeder cells). Draw up the required number of cells into the injection needle and position them near the tip in a minimal amount of medium.
6. Immobilize a single blastocyst by applying suction to the holding pipette and move it toward the centre of the microscope field.
7. Position the embryo with the inner cell mass (ICM) at either the 6 or 12 o'clock position. If the ICM is difficult to visualize, it is most probably located on the side nearest or farthest away from the optics. In this case, the blastocyst should be turned around with the help of the injection needle until the ICM can be seen clearly.
8. Align the tip of the injection needle in the same focal plane as the midpoint, or equator, of the blastocyst. Touch the end of the injection needle gently to the surface of the embryo. This will make a small indentation in the embryo, which will give an indication of the position of the tip of the needle relative to the surface of the blastocyst. Do not damage the zona pellucida.
9. With a single, swift, continuous movement, introduce the loaded injection needle into the blastocoel cavity, at a junction between two trophoblast cells. This will minimize the damage to the embryo and will make successful penetration much easier. Do not touch the ICM with the injection needle. If the first attempt to penetrate the trophoblast layer is unsuccessful, and the blastocyst is not collapsed, insert the needle at the same position with a swifter movement. The blastocyst should be discarded if it collapses without successful injection.

10. Slowly expel the cells inside the blastocyst cavity. Do not insert any oil bubbles or lysed (dark) cells into the blastocyst.
11. Withdraw the injection needle slowly but not fully so that just the tip is in. This will allow the blastocyst to collapse without expelling the ES-cells. The ES-cells will come into close contact with the surface of the ICM.
12. Place injected blastocysts back into incubator (culture dish) and leave to re expand before Embryo Transfer.

Uterine transfer

1. Prepare for surgery the female mice derived from the CBAxC57BL6F1 cross to vasectomised males.
    a. Weigh the recipient mouse and anesthetise with Ketamine/Xylazine by intra-peritoneal injection.
    b. Once anaesthetised give mouse 0.1 ml of Vetergesic analgesic by subcutaneous injection.
    c. Place mouse on heat pad under stereomicroscope.
    d. Load a transfer pipette with embryos. Take up a small amount of M2 medium into the transfer pipette, then a small air bubble, then M2 medium, and then a second air bubble. Repeat until good control is reached with reduction of capillary action.
2. Draw up the blastocysts in a minimal volume of medium (filling ~ 5-7 mm of the pipette).
3. Rest the pipette next to the microscope, making sure that the tip of the pipette does not touch anything. Be careful not to disturb the pipette. Place mouth pipette on a clean tissue.
4. To expose the uterus, wipe the back of the recipient mouse with tissues soaked in 70% ethanol and then make a single small longitudinal incision (<1 cm) in the skin with fine dissection scissors in the midline at the level of the last rib. Wipe the incision with 70% ethanol-soaked tissue to remove any loose hairs.
5. Slide the skin to the left or right until the incision is over the ovary (orange-pink) or fat pad (white), both of which are visible through the body wall. Then pick up the body wall with watchmaker's forceps and make a small incision (avoiding larger blood vessels) just over the ovary with micro-scissors. Stretch the incision with blunt forceps to widen hole.
6. To transfer the embryos, use blunt fine forceps to pick up the ovarian fat pad and pull out the attached left ovary, oviduct, and upper part of the uterus. Clip a Serrefine clamp onto the fat pad and lay it down over the middle of the back, so that the ovary, oviduct, and uterus remain outside the body wall.
7. Hold the top of the uterus gently with blunt fine forceps and use a 27 gauge needle with the bevel facing up to make a hole in the uterus a few millimeters down from the utero-tubal junction. Avoid small blood vessels in the uterine wall. If bleeding occurs, use tissue to remove the blood. To test whether the needle has entered the lumen, pull it out slightly. If it slides easily, the needle has penetrated the lumen. Do not move the needle too much or the wall of the uterus may be lacerated. Keep the needle parallel to the horn.
8. Watching the hole made by the needle, pull out the needle and insert ~5 mm of the prepared transfer pipette containing the blastocysts into the hole. Blow gently on the transfer pipette until the air bubble closest to the blastocysts are at the tip of the pipette and all of the blastocysts have been expelled. Watch the movement of air bubbles in the pipette and remove the pipette when the first bubble reaches the opening in the uterus.

9. Unclip the Serrefine clamp. Use blunt fine forceps to pick up the fat pad and place the uterus, oviduct, and ovary back inside the body cavity.
10. Close the skin with wound clips.
11. At the end of the procedure, place the mouse in a clean cage in the recovery cabinet which is kept at 30°C. Keep the mouse here until it has recovered from anaesthetic.