Mouse Intracytoplasmic Sperm Injection

Mutant Mouse Resource and Research Center

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1: ICSI pipette making

Materials:

- Borosilicate Glass OD. 1.0 mm, I.D.: 0.75mm, 10 cm length (Sutter Instrument Co. Cat. # B100-75-10.
- Model P-97 or P-1000 Flaming/Brown Micropipette Puller
- MF-900 Narishige Microforge
- Large Petri dishes labeled for mouse ICSI (Flatten a piece of modeling clay on the bottom of the dish, running along the diameter, to hold the pipettes).

Procedure:

Pulling Pipettes:

1. Turn the puller's Power on.
2. Press program <98> followed by <ENTR> on the keypad. This is the correct program for pulling ICSI pipettes on the P-1000 Flaming/Brown Micropipette Puller in the lab.
   Note: if your machine doesn't have a preset program for ICSI needle, please follow the user's manual to set one.
3. Insert Borosilicate Glass tube into the micropipette puller.
4. Press the <PULL> key on the keypad. The heating filament should turn on and the glass should separate in less than 10 seconds.
5. Loosen the clamping knobs and remove the pipettes from the puller bars.
6. Inspect the pulled needles for straightness, discard the pulled pipette(s) waving at the desired breaking point (please see below).

Breaking/Bending ICSI pipettes:

7. Turn on the power to the Narishige MF-900 microforge.
8. Looking through the eye piece at 10x, check to see if the filament is in view and whether there is a glass bead located on the peak of the filament. If the filament is not in view, then switch to 4x and place the filament in the middle of the view finder by using the three adjustment knobs on the right side.
9. Once the filament is in the proper location switch back to 10x and bring the filament into focus using the 3 adjustment knobs again.
10. Once in focus, move the filament to the bottom quarter of the viewing pane. This is where the filament and glass bead will remain for the rest of the procedure.

11. Insert the pulled capillary into the capillary holder. You will have to loosen the neck in order to insert the capillary. Be sure to insert the capillary slowly or you may break it.
12. With the capillary in place, place the capillary holder in its holder and tighten it. This will be the small vice on the side of the light source.
13. Adjust the vice, using the coarse control knobs located above and below the vice, so that the capillary is in view. It may also be necessary to move the vice to the proper location which is the left side of the light source. To do this, loosen the screw located on the metal ring around the light source and then physically move the vice to the proper location.
14. With the capillary in view, bring it into focus, and then move it so that the capillary is around 10 µm in width, using the coarse control knobs used before. At 10x each horizontal line represents 10 µm.
15. Now use the fine control knobs, located on the right-hand side of the microscope stage to bring the capillary to the desired width. For mouse ICSI pipettes between 6-7 µm is suggested.
16. For mouse ICSI tips you want to have a tip that is a little over half the distance between two lines at 10x. It is advisable to make several tips (no less than 5) for any one ICSI run since there will be variation in tips size and quality.
17. Now that you have your tip at the desired width, bring it down to the glass bead on the filament. Check to make sure that the capillary will sit directly on the tip of the glass bead and not in front of or behind it.
18. Using the coarse control knobs, raise the pipette up, out of your field of view. Press the pedal with your foot and observe how far the filament raises up. You want to lower your pipette ~2-4 marks (20-40 µm) above this point for cutting.
19. Check the silver knob on the right-hand side (Heater adjustment) and set it to 70-80, if not already set to that.
20. Using the foot pedal apply heat to the filament for a short time. The filament/glass bead should come up to meet the capillary. Release the foot pedal once the capillary has attached itself to the glass bead. This usually occurs when the capillary begins to bend just slightly. If the capillary does not attach to the glass bead, then repeat this step, however if the capillary has bent too much, or has melted such that the inner diameter is now too small, simply throw away the capillary and use a different one.
21. When the capillary is attached to the filament, raise the capillary with the coarse adjustment until it breaks free from the filament. The cut should be clean and smooth.
22. Now move the broken tip back to the middle of the view pane and then adjust the capillary so that it is about 20 µm thick (using the coarse adjustment).
23. Raise the pipette a little bit higher than mid-viewing field, and carefully apply heat (using foot pedal). This will cause the pipette to bend upward. Once the capillary reaches the second angle mark (located

on the right hand side of the viewing pane) discontinue heat. This will leave the pipette at a 20° -30° angle. (Each angle marks represents 10° at 10x.)

24. Repeat until you have the desired number of tips.

2. Realiquoting mouse sperm for ICSI

Materials:

- Sperm Cryo media (out of -20 freezer)
- Frozen straw of Sperm
- 35mm Petri dish (Falcon 1008)
- 5 sterile straws and weights
- Syringe to load the realiquoted sperm into straws
- Scissors
- Straw Heat sealer
- Pipette

Procedure:

1. First, you will need to make your 5 straws to put the realiquoted sperm in. Also, make a place for them in the proper Dewar. You will need a weight in the proper end of each straw.
2. You will also need a barcode on each straw; use the same barcode number that was used when the sperm was frozen. Make sure you put MS, on straw for "Mouse Sperm," with a fine tip marker write ICSI on the straw label; this way when someone sees it, they know it is for ICSI. Also write the male number on each label (see step 3 for choose males).
3. You will need to put a tube of Sperm Cryo Media in the bead bath to thaw.
4. Search for the appropriate straw in the Rodent Resource Centers cryopreserved inventory database; pick the straw/male with the best motility. To find the motility go back to the binders where the case material is filed.
5. Once you have retrieved the straw put it in the bead bath for two minutes.
6. While the straw is thawing get your other supplies ready at one of the warming plates in the lab. Scissors, syringe to load sperm, 35 mm Petri dish (put on warmer, so it is warm when you expel sperm), straws, and vial of sperm cryo media.
7. Take the straw out of the bead bath, cut the end opposite of the weight first. Hold the straw over the 35mm dish and cut above the fluid and below the weight and barcode, it will expel into the dish.
8. Once expelled in the dish add 1.2 ml of sperm cryo media. This will give a final volume of 1.5ml, since there is .3ml of sperm in the straw prior to expelling it.

9. Mix solution in dish by swirling the dish around or mixing with a 1000ul pipette.
10. Load your 5 straws with the diluted sperm; try to get an even amount in all 5 straws.
11. Once the straws are loaded with sperm you will freeze them. You will freeze them just like you do in the mouse Sperm Cryopreservation Protocol. Refer to the protocol if you don't remember how to freeze the straws.
12. Once straws are frozen put them in the cane you made earlier and put them in the proper Dewar based on what Dewar you added the cane to in the database.
13. If you will be doing ICSI in the upcoming week, you may want to take two straws and put them in a separate cane to store in the Temp Dewar in the lab.

3: Preparing Frozen mouse sperm sample for ICSI

Purpose: to thaw a straw of cryopreserved mouse sperm and prepare sample for use in ICSI.

Materials:

- 37°C water bath
- Kimwipes
- 15ml round-bottom tube (or a tube that will fit into your centrifuge)
- 15ml standard centrifuge tube (with orange cap)
- Scissors
- Syringe with straw aspiration device attached to tip
- FHM.
- Sonicator (Fisher Scientific Sonic Dismembrator)
- Centrifuge (Thermoforma High Performance Centrifuge)

Procedure:

1. Remove straw from liquid nitrogen and immerse in 37°C water bath until sample is completely thawed (about 15 seconds).
2. Remove straw from water bath, and wipe with kimwipe.
3. Cut the sealed tip off the end of the straw that is opposite of the end with the weight and label.
4. Holding the straw vertically, with the open end in a 15 ml round-bottom tube, cut the other end of the straw (below the weight, and just above the top of the sample). This will cause most of the sperm sample to fall out of the straw, into the tube.
5. Expel the rest of the sample from the straw with the syringe/aspiration device.
6. Slowly add 3.0 ml FHM, by pipetting 1.0 ml into the tube every 10-15 seconds.

7. Immerse the metal wand of the sonicator in the middle of the FHM/sperm solution, and sonicate the sample for approximately 5 seconds, on the "20" setting. This will cause the sperm heads and tails to separate.
8. Transfer the sonicated solution from the round-bottom tube to a 15ml centrifuge tube (with orange cap). ***This is done because the round-bottom tubes will become stuck in the centrifuge, but the sonicator wand cannot reach the sample if it is originally placed in the orange-capped centrifuge tube.***
9. Place the 15ml centrifuge tube, containing the sonicated sample, into the Thermoforma centrifuge. Make sure to properly counterbalance the sample tube by placing another 15ml centrifuge tube (containing an equal volume of water) opposite the sample tube.
10. Centrifuge the sample for 10 minutes at 500 G at 23 degrees Celsius.
11. After spinning, slowly pipette approximately 3.1ml supernatant from the top of the sample. You will most likely NOT see a sperm pellet.
12. Mix the remaining sample (about 200 µl) by slowly pipetting up and down.

4: Preparation of Petri Dish for Mouse Intracytoplasmic Sperm Injection

Materials:

- 10% PVP
- FHM
- 100x15mm petri dish (Falcon 1029)
- 35x15mm petri dish
- P20 Eppendorf pipettor
- Sterile mineral oil
- Pipette-Aid
- 10 mL sterile pipette

Procedure:

1. Place all materials in laminar flow hood with air circulating. Wear nitrile gloves.
2. Place 10 µL drops of FHM and 10 µL drops of 10% PVP in lid of dish (see figure 6 below). Discard bottom portion of dish.
3. Using a pipette-aid and 10 mL pipette, cover the drops with sterile, filtered mineral oil.

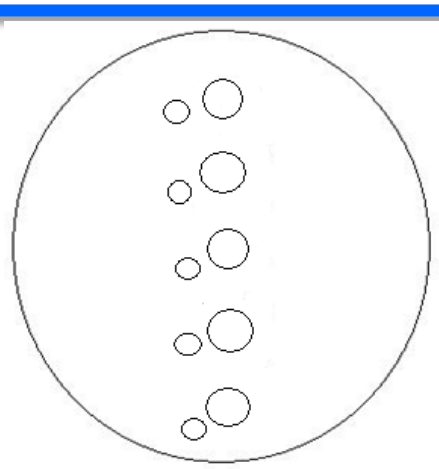

Figure 6. ICSI Injection Dish: FHM in center drop and 10% PVP to the left. Each series of two drops should be close to each other, without touching.

4: Mouse Intracytoplasmic Sperm Injection (ICSI)

Materials:

- PMSG (PROSPEC, 1000 IU)
- HCG (Calbiochem; Catalog #230734; 1mg)
- CO_2 euthanasia chamber
- Dissection tools (small scissors, forceps, etc.)
- FHM
- Small petri dishes
- Hyaluronidase (diluted to 1mg/mL; Sigma)
- Dissecting microscope with heated stage
- Eppendorf Vacu-Tip (Holding pipette)
- Injection pipette (2-3µm inner diameter)
- Injection dish
- P20 pipettor
- Recovery dish (FHM in a 35 mL dish)
- KSOM culture dishes (pre-equilibrated in 5% $C0_2$ incubator)
- Oocyte donors
- Sperm aliquot (prepared for ICSI)

Procedure:

1. Inject immature female donor mice with PMSG (25 IU/mL) at 0.3 IU/g at 12-2 pm.

2. Inject each mouse IP with HCG (25 IU/mL) at 0.3 IU/g 50.30-54.30 hours post PMSG injection (4:30-6:30 pm). **DO NOT MATE THE FEMALES**!
3. Oocytes are collected approximately 16-17 hours post HCG injection.
4. Euthanize female donors in the CO_2 chamber, followed by cervical dislocation or diaphragm dissection after respiration has ceased.
5. Carefully dissect out both oviducts from each female and place in a small petri dish containing warm FHM (placed on 37°C heating block).
6. Tear the oviducts in drops of hyaluronidase, under the heated-stage dissecting microscope (set at 37°C), using fine forceps and an insulin syringe needle. Carefully "tease" out the oocyte clutches and remove the rest of the oviduct from the drop.
7. Pick up the oocytes with a hand or mouth pipette and move them to a clean, pre-warmed drop of FHM drop.
8. Get a pre-equilibrated KSOM dish from the incubator and transfer the oocytes into it. Wash through the four outer drops before placing in the center drop.
9. Place the KSOM culture dish with the oocytes back into the incubator.

Setting up the micromanipulator

1. Swing out the manipulator arm and insert the holding pipette into the air tram holder by loosening the grip at the end of the holder. Insert the holding pipette gently until you feel it hit the back of the holder. Tighten the holder so that the tip is firmly secure.
2. Load your ICSI needle of Approx. 1/3 of the capillary with Fluorinert™ FC-770 by backfilling the needle with a Hamilton gastight syringe.
3. Loosen the grip at the end of the holder and insert your -loaded ICSI pipette in the same manner as you did your holding pipette.
4. Once the tip is secured check to see if it is level. Then swing the manipulation arm back into place.
5. Slowly and carefully slide your manipulation dish onto the stage of the scope. (Make sure that tips will clear the top of the dish).
6. Find and focus on one of your manipulation drops and bring in your holding pipette into this drop.
7. Straighten the holder as needed and then expel the media inside of the tip until the bubble meets the end. Then draw up fresh media and home the tip.
8. Now focus on the 10% PVP drop to the left of your manipulation drop and lower the injection tip into this drop.
9. Adjust for straightness and then bring the FC-770 to the edge of the tip and expel some of the FC-770 into the drop. Then suck up fresh PVP and move to your tip to your manipulation drop.

10. Move the tip to a greater height in the manipulation drop and then focus on the tip. Follow the pipette up from the tip and focus as needed. Depending on which way you focus this will tell you how to adjust the tip for straightness.
11. Once the tip is straight and level the manipulator is set up and ready for ICSI.

Oocyte injection

1. Using the P20 pipette, place 1-3 µl of sperm into the FHM drop (the same drop that the oocytes will go in) and pipette up and down to mix.
2. Make sure you have enough sperm heads in the drop, then add ~5 oocytes to the drop.
3. Move the injection pipette into the drop. Swing in the 20X objective of the inverted microscope and refocus.
4. Pick up a sperm by sucking up the sperm head into the tip of your injection pipette (it should not go all the way in, if it does, your pipette is too big).
5. Lower the holding pipette and injection pipette into view, on the same visual plane as the oocyte.
6. Position the oocyte so that the metaphase plate (usually close to the polar body) is at the 12 or 6 o'clock position and pick up by applying gentle suction with the holding pipette.
7. Slightly raise the holding pipette (and the oocyte) from the bottom of the dish to prepare for injection.
8. Move the tip of the injection pipette (containing the sperm head) to the zona of the oocyte.
9. Apply a small amount of negative pressure to the zona (with the injection pipette) and carefully cut through the zona by using the "high" setting of the piezo (set at ~4 intensity and 4 speed). **Note:** If it is difficult to cut through the zona of several oocytes, you should change your pipette.
10. When the injection tip is through the zona, place the tip against the oolemma.
11. Using the foot pedal, switch the piezo to the "low" setting (intensity 1-2, speed 1-2).
12. Gently push the injection pipette (with the sperm at the edge) into the center of the oocyte until almost touching the opposite zona (closest to holding pipette).
13. Carefully apply a single low pulse to the oocyte. The cytoplasm should visually "relax", indicating that you have punctured the oolemma.
14. Repeat until you have injected all of the oocytes (~5) in your manipulation drop and then transfer them in pre-equilibrated KSOM drops in a 35 mm culture dish and placed the dish back in the incubator.