1.0 Purpose:

To describe the procedure used for piezo-activated mouse intracellular sperm injection (ICSI) in mice.

Useful References:

Kimura, Y & Yanagimuach1 R (1995) Intracytoplasmic sperm injection in the mouse. Biol Reprod **52** (4), 709-720.

Stein and Schultz (2010) Intracytoplasmic sperm injection in the mouse. Methods in enzymology, **476**, 252-261 Chapter Fortheen – Elsevier

Yoshida and Perry (2007) Piezo-actuated mouse intracytoplasmic sperm injection. Nature Protocols, **2**(2), 296-304, Nature Protocols

2.0 Safety Requirements:

2.1 Staff working with any hazardous chemicals must have read and understood the relevant COSHH risk assessment.

2.2 Where PPE is specified this MUST be worn at ALL times.

2.3 Equipment provided must be used solely for the purpose and stored in an appropriate manner.

3.0 Equipment:

Piezo-actuated drill
Inverted microscope
CO_2 incubator
Dissecting microscope
Gilson pipette

4.0 Supplies:

Hyaluronidase
Holding needles
Injection needles
Gilson pipette tips
Ca^{2+} free M2 media
35mm petri dishes
Dissecting kit
PVP360

KSOM media
NIM media
Mineral oil
Frozen sperm samples
Oocyte donor females
PMSG and hCG hormones for superovulation

5.0 Procedure:

7.1 General Information

5.1.1 Mice should be handled wearing gloves at all times.

5.1.2 Where possible, mice should be contained in a Class II Type A/B3 Biohazard Safety Cabinets re-classified

5.2 Preparation and incubation of oocytes

5.2.1 To obtain large numbers of oocytes, superovulate female donor mice by intraperitoneal injection (26 G needle) of 5 IU PMSG followed 45–54h later by 5 IU hCG (inject 0.1 ml of each hormone per female). Typically (with standard mouse room light/dark cycles), administer hormone injections at 6–7pm. Females of 8–10 weeks give good yields, but the optimum age may vary with mouse strain.

5.2.2 12–15 h post-hCG injections, sacrifice the mice (5 per experiment is usually sufficient), collect their oviducts and place them in Ca^{2+} free M2 medium.

5.2.3 Working under the stereomicroscope at 15–20 X magnification on a temperature controlled heating stage at 37°C, hold the oviduct wall with one pair of forceps and tear the swollen ampulla open with another pair of forceps to release the oocyte-cumulus complex (COC). Tease the oocyte-cumulus complex from the oviduct. Discard the oviductal remnant and repeat the process for the remaining oviducts

5.2.4 Place the COCs in a droplet of Ca^{2+} free M2 medium/hyaluronidase (300µg/ml).

5.2.5 Leave the oocyte-cumulus masses in the same dish for 5 min to allow the hyaluronidase to digest the intercellular matrix. The cells will fall away from the complexes, leaving the oocytes on a carpet of cumulus cells. This step can be optionally accelerated by placing the dish on a stage heated at

37°C for up to 10 min.

5.2.6 Remove oocytes with a transfer pipette and place them in a drop (200µl) of Ca^{2+} free M2 medium (without hyaluronidase). Wash the oocytes by pipetting them 4–6 times. Repeat this washing procedure 3–4 times, each time in a fresh droplet (200µl) of Ca^{2+} free M2 medium.

5.2.7 Transfer the oocytes to a drop (200µl) of equilibrated culture medium (e.g. KSOM) under mineral oil in a 35mm dish, washing once to remove Ca^{2+} free M2 medium. Prepare the embryo culture and micromanipulation droplets (5–20µl) and overlay with mineral oil to prevent evaporation. Place the 35mm dish containing the oocytes in a humidified incubator 5% (v/v) CO_2/air mix at 37°C for at least 15 min until required. From oviduct to incubator, oocyte isolation should take less than 60 min.

Note: Oocytes may be incubated in humidified 5% (v/v) CO_2/air mix at 37°C for several hours (up to 20h post-hCG) prior to micromanipulation, but they are more typically injected before 18h post-hCG.

Oocytes efficiently retain their fertilization capacity at least 22h post-hCG in vivo for hybrid strains, however for in bred strain oocytes, it may be much shorter.

5.3 Preparation of spermatozoa

5.3.1 Take a straw of frozen sperm e.g. C57BL/6J from the LN_2 container using a pair of long forceps, and placed in a small portable container filled with LN_2 and transported into the Lab.

5.3.2 Remove the selected sperm straw from the LN_2 and exposed to air for 10 seconds and placed in a water bath at 25°C for 5 min. After the sperm is completely thawed, cut off the two ends of the straw and eject the contents into a 0.5mL Eppendorf tube.

5.3.3 Spin the tube at 2000rpm for 5 min, then discard the supernatant and add 50µL of NIM medium to the pellet and vortexed for 10 seconds, repeat this procedure three times. The sperm is now ready to be used for ICSI.

5.3.4 Mix the sperm suspension with a PVP360 solution so that the final concentration of PVP360 is 5–15% (w/v). Mix using a pipette, such as a Gilson P200 or equivalent, equipped with a sterile wide bore tip.

Note: PVP360 acts as a high viscosity agent that retards movement of live sperm, making them easier to collect.

5.3.5 Using a transfer pipette, remove the MII oocytes from the CO_2 incubator and transfer them into a microinjection droplet (Ca^{2+} free M2 medium) on the microscope stage.

Note: Sperm in PVP360 suspensions at room temperature often lose their ability to support full development after 1 h. To overcome this problem make a fresh sperm-PVP360 suspension every 1 h. However, in the correct conditions spermatozoa can retain their ability to induce meiotic resumption for many hours when placed directly in a 20 µL droplet of $Ca2^{+}$ free M2 medium covered with mineral oil.

5.4 Piezo-actuated intracytoplasmic injection (ICSI)

5.4.1 If the sperm is intact (i.e., head, midpiece and tail are joined) decapitate the sperm to allow injection of the head alone. This minimises the volumes introduced into the oocyte. To do this bring a single sperm into sharp focus (200 X magnification) and draw its head into the microinjection pipette (inner tip diameter, 5.5–7.5µm; outer tip diameter, 6.5–8.5µm) so that the pipette lip touches the head-midpiece boundary. Apply several pulses with the piezo nit foot pedal.

5.4.2 For the initial attempts with the PMAS-CT150 (or equivalent) piezo unit, try intensity ¼ 3, frequency ¼ 6; as a general guide in piezo, use the lowest settings that work. The head and tail should separate.

Note: Alternative preparation protocols may be used e.g., freeze/thawing to decapitate sperm before the sperm before they are placed on the stage.

5.4.3 Draw one or more sperm heads into the microinjection pipette. With experience, multiple (5–10 or more) heads can be loaded within a single pipette at intervals 100µm; although only a single head is typically injected per oocyte. Collecting multiple samples removes the need to return to the sperm-PVP360 droplet each time between injections

5.4.4 Working at 40X magnification, move the microscope stage so that the pipette is in microinjection droplet containing Ca^{2+} free M2 medium and 10–15 oocytes. At this stage it is advisable to wash the pipette in a droplet to remove PVP360 solution from the needle's exterior.

5.4.5 Lower the holding pipette into the microinjection droplet and position the holding and microinjection pipettes so that their tips are in the centre of the field of view and on either side.

5.4.6 Use the microinjection needle to orientate the oocyte so that its MII plate is located straight up or down on the y-axis (i.e.12 o'clock or 6 o'clock).

Either orientation minimises the risk of damaging the MII plate during microinjection; even slight mechanical damage to the spindle (i.e. MII plate) will compromise normal development. Select an orientation that has the largest perivitelline space, i.e. the space between the plasma membrane and the zona pellucida.

5.4.7 At 200 X magnification, bring the oocyte plasma membrane into sharp focus. Use the fine z-axis control to move the pipettes up or down so that their ends are in focus.

5.4.8 Apply gentle suction within the holding pipette and push the oocyte against the holding pipette aperture with the microinjection pipette. Engage the oocyte on the holding pipette.

5.4.9 Advance the first sperm head so that it is 50–100µm from the pipette tip. Check the focus and touch the zona pellucida with the tip.

5.4.10 Introduce a small negative pressure within the microinjection pipette and apply piezo pulses (start with intensity 3, frequency 6) while gently pushing the microinjection pipette toward the holding pipette. The tip should rapidly pass through the zona, emerging into the perivitelline space. Stop pushing as soon as this happens.

Note: Failure to control the tip risks subjecting the plasma membrane to high intensity piezo, resulting in rapid cell lysis.

5.4.11 The zona core should be just visible (it is difficult to see without experience) entering the pipette as the tip penetrates into the perivitelline space. Although the oocyte cytoplasm can tolerate the presence of a zona plug, the plug can interfere with injection. Eject the zona plug into the perivitelline space prior to injection by placing the pipette tip against the plasma membrane (without piezo pulses) and gently increasing the pressure inside the pipette. When the core emerges from the pipette, it produces an impression (‘dent’) in the plasma membrane.

5.4.12 Dislodge the protruding core by wiping the tip against the oocyte (gentle movement mainly along the y-axis). Sustain positive pressure until a sperm head is 10–50 mm from the tip.

5.4.13 Ensure that the plasma membrane is in sharp focus. Making fine-adjusts to the tip to ensure that it is in focus.

Note: Avoid the first polar body, which can be displaced; it is not usually attached to the oocyte.

5.4.14 Maintain a slight positive pressure within the pipette.

5.4.15 Steadily advance the tip towards the opposite side of the oocyte, where it is anchored to the holding pipette. Stop when the tip has advanced 95% of the oocyte diameter. This produces a deep invagination in the oocyte plasma membrane, which is now stretched around the microinjection needle. Advancing the needle tip takes 2–5 sec.

5.4.16 Change the piezo pre-set channel to a gentler setting e.g. intensity 1, frequency 1. While maintaining zero set pressure (or a very small negative pressure) within the microinjection pipette, apply a single piezo pulse.

5.4.17 Ensure that the oocyte plasma membrane visibly relaxes along the shaft of the needle. This is a good indication that the membrane has been punctured.

5.4.18 Deposit the sperm head in the cytoplasm by applying a small positive pressure in the microinjection pipette. Introduce the smallest possible volume of extraneous medium while injecting the head. **Do not suck cytoplasm into the pipette.**

5.4.19 As soon as the sperm has been deposited, withdraw the needle smoothly from the oocyte. The membrane should return to its original position and seal. If the plasma membrane clings to the pipette, pause briefly during withdrawal. The interval from zona penetration to needle withdrawal should take 25–35 sec.

5.4.20 Release the injected oocyte by gently applying positive pressure within the holding pipette. Any minor distortion caused by holding the oocyte rapidly disappears. Allow oocytes at least 5 min (some workers prefer 10–30 min) to recover before being returned to the CO_2 incubator.

5.4.21 Fatal mechanical trauma to the oocyte during micromanipulation usually results in the onset of lysis within 30 sec. Remove dead eggs from the survivors during transfer to the CO_2 incubator. Wash the oocytes 4 times in equilibrated culture medium to remove Ca^{2+} free M2 medium (HEPES is toxic at 37^{o}C) before incubation. Remove dead all oocytes during this step transfer. For optimal development, limit the time the oocytes are out of the incubator to 40 mins.

5.4.22 Examine the outcomes of injections at 200X magnification, 5–8 h later. Oocyte activation produces a cytoplasm that is darker and more heterogeneous, and the plasma membrane is no longer smooth but irregular. The MII ‘bump’ will have disappeared and there will be a

second polar body. The first polar body has usually degenerated by now, although a remnant often remains. Male and female pronuclei will be visible, sometimes containing multiple pronucleoli whose numbers decrease with time. Visible light damages these structures, so for further culture, examine zygotes rapidly, wash them 4–6 times in CO_2-equilibrated culture medium (e.g. KSOM) and return them to the CO_2 incubator.

5.4.23 Next day, wash 2-cell embryos once in CO_2-equilibrated culture medium (removing any dead or ghost oocytes) and return them to the CO_2 incubator to monitor in vitro development further or transfer them to a surrogate mother.