Hosted by the Frozen Embryo and Sperm Archive, MRC - Harwell

IVF recovery procedure incorporting methyl-β-cyclodextrin and reduced glutathione

This protocol is based on the work published by Takeo et al., (2011). The reagents can be prepared according to the protocols provided here or purchased separately or as part of a mouse in vitro fertilisation kit from Cosmo Bio Co., Ltd (www.cosmobio.co.jp)

A. Preparation of sperm dispersal dishes

NB: This medium is referred to as Fertiup®: PM, and can be purchased separately or as part of a mouse IVF kit from Cosmo Bio Co., Ltd (www.cosmobio.co.jp)

1. Pipette 90µl TYH + 0.75mM MBCD into the centre of a 35mm Petri Dish (Falcon 351008) (Fig.1).

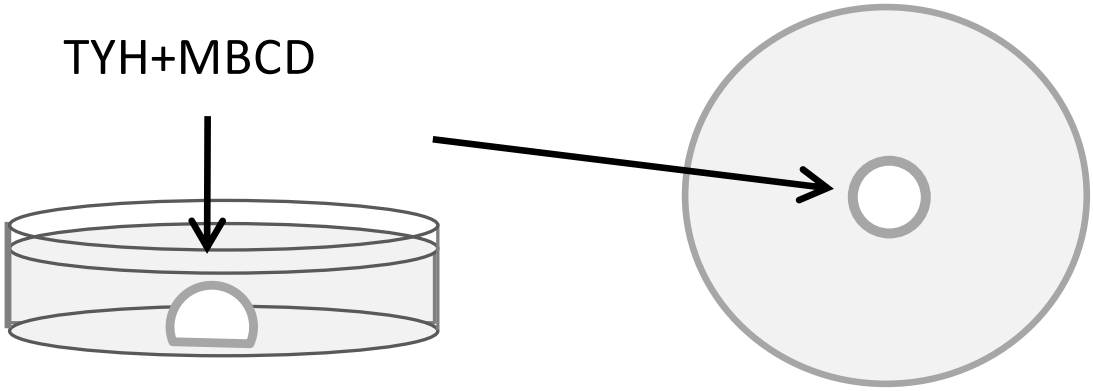

Fig. 1

2. Overlay with mineral oil and equilibrate for 10-20min at 37°C, in 5% CO_2 in air.

B. Thawing sperm samples frozen in gCPA

1. Remove the required straws from the storage tanks and place in a flask of liquid nitrogen.

2. Quickly transfer a straw into a 37°C water bath and leave for 10 minutes.

Note: To ensure warming of the frozen sperm, completely immerse the part of the straw containing the sperm in the water bath. Furthermore, frozen-thawed mouse sperm are sensitive to environmental changes. If the straw is not kept in the water bath long enough (10 minutes), the motility of the cryopreserved sperm will be reduced.

3. Carefully dry the straw with paper towel. Cut the heat-sealed end of straw and use a straw pusher to expel **only** the 10µl sperm suspension into the centre of the drop of 90µl TYH+MBCD pre-incubation medium (Fig. 2). Do not expel any of the HTF media contained within the straw.

Note: Do not disturb the dishes containing the frozen/thawed sperm until the sperm are moving rapidly within the medium. If the dishes are disturbed before the sperm start to move they will not recover full motility

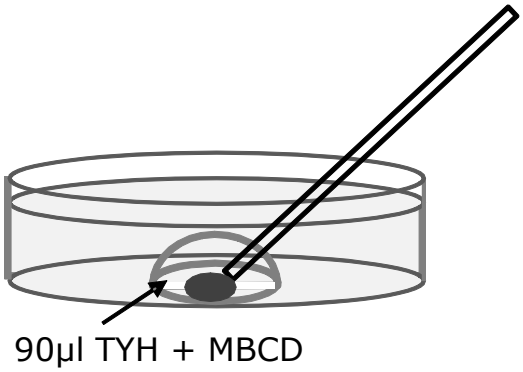

Fig. 2

4. Pre-incubate the frozen/thawed sperm in TYH+MBCD medium for 30min at 37ºC in the CO_2 incubator.

C. Preparation of freshly harvested sperm samples:

1. The selected male should be at least 8 weeks old, and not have been used for mating for at least 3 days before sperm collection.

2. Sacrifice the male and dissect the cauda epididymides.

3. Clean off all adipose and vascular tissue. This is best achieved by placing the organs on a tissue and examining them under a dissecting microscope lit from above.

4. Place the cauda epipidymides into the oil next to the sperm dispersal drop and nick the apex of the cauda epididymides using miniature scissors. Using watchmakers forceps gently tease out a small 'ball' of the sperm from the cauda epididymides and drag it into the sperm dispersal drop (Fig. 3).

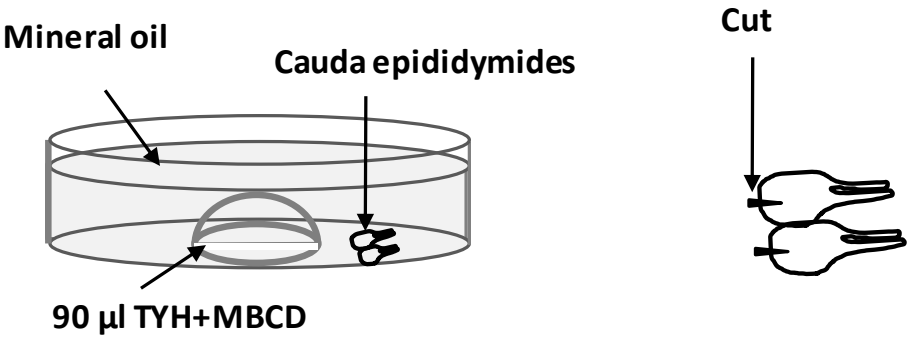

Fig. 3

5. Remove the tissue from the dish. Allow the sperm to disperse throughout the medium for approximately 60 minutes at 37ºC in the CO_2 incubator.

D. Preparation of fertilisation dish containg reduced glutathione (GSH – Sigma: G4251)

NB: This medium is referred to as CARD medium and can be purchased separately or as part of a mouse IVF kit from Cosmo Bio Co., Ltd (www.cosmobio.co.jp).

1. Take 1ml HTF medium and add it to a tube containing 30.7mg reduced glutathione (GSH). Close the lid, mix the medium and the powder in the tube (Fig. 4).

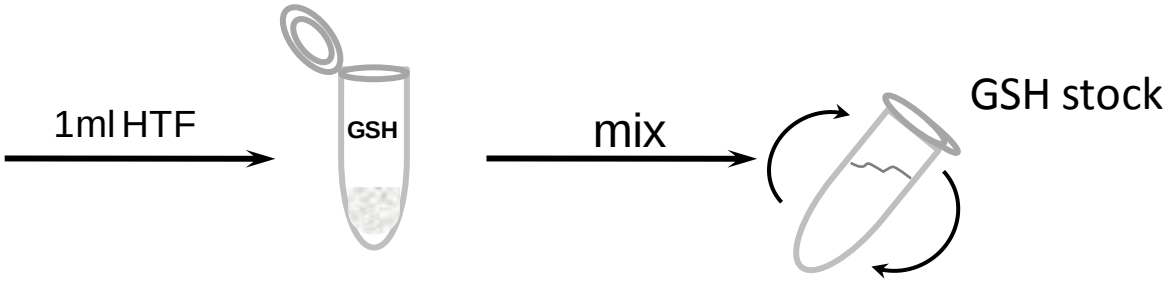

Fig. 4

2. **<u>For frozen/thawed sperm samples</u>,** take **50µl** of the GSH solution and add it to **5ml** HTF medium and mix them together gently (Final concentration 1mM GSH, Fig. 5).

3. **<u>For freshly harvested sperm samples</u>,** take **10µl** of the GSH solution and add it to **4ml** HTF medium and mix them together gently (Final concentration 0.25mM GSH, Fig 5).

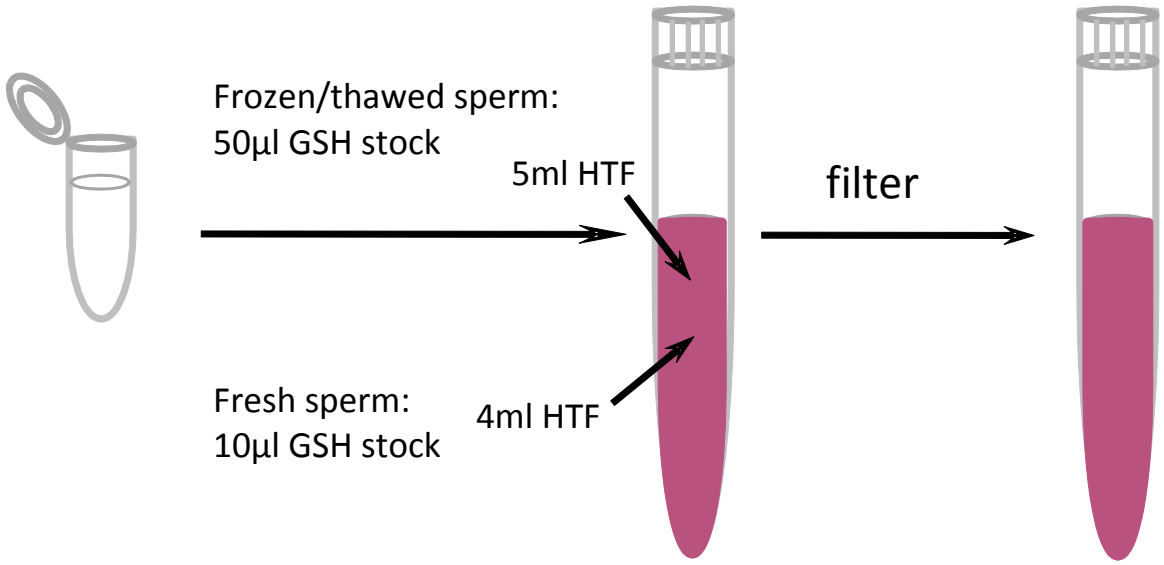

Fig. 5

4. Before use, filter the solution using 0.22µm syringe end filter.

5. Make a drop of the medium in a 35mm Petri Dish (Falcon 351008) (Fig. 6), and then place the dish in an incubator (37^{0}C, 5% CO_2 in air) for 10-20minutes. The volume of the drop should be **200µl** if using a freshly harvested sperm sample and **90µl** if using a frozen sperm sample.

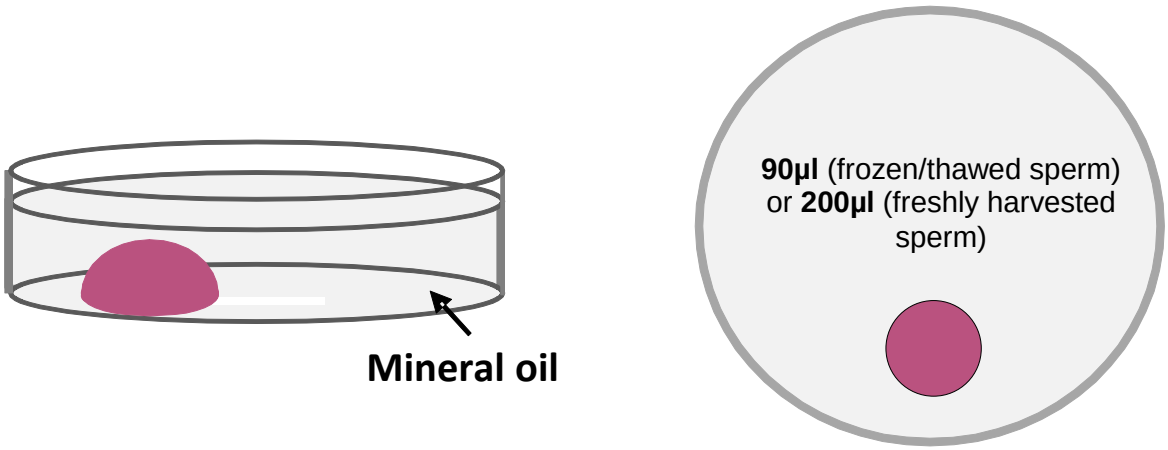

Fig. 6

E. Oocyte harvesting and *in vitro* fertilisation

1. Dissect the oviducts from three superovulated female mice (for superovulation methods, see the protocol for harvesting and cryopreservation of *in vivo* derived embryos and the table below). and transfer them into the mineral oil overlaying the pre-incubated fertilisation drop.

2. Under a dissecting microscope, hold each oviduct down with forceps and gently tear the swollen ampulla with a second pair of forceps to release the cumulus masses into the oil. Using the forceps, drag the clutches through the oil and into the fertilisation drop. Then remove the oviduct from the dish.

3. Add the sperm to the fertilisation drop:

   a. **Frozen/thawed sperm** must be equilibrated for **30 minutes** in the incubator before adding to the drop. Take a 10µl aliquot of the sperm suspension from the peripheral part of the pre-incubation drop. This region will contain the most motile sperm. Try to avoid aspirating any sperm debris (Fig. 7).

   b. **Freshly harvested sperm** should be allowed to disperse in the TYH + MBCD medium for approximately **60 minutes** in a 37ºC CO_2 incubator. Add 3-5µl freshly harvested sperm to the fertilisation drop, once again, avoiding aspirating any sperm debris (see Fig 7).

Note: This step should be performed gently under a light microscope and is best achieved using a wedge-shaped 10µl pipette tip (Axygen Inc; T-400). Following this procedure it is easy to collect good quality motile sperm without picking up dead sperm or cell debris.

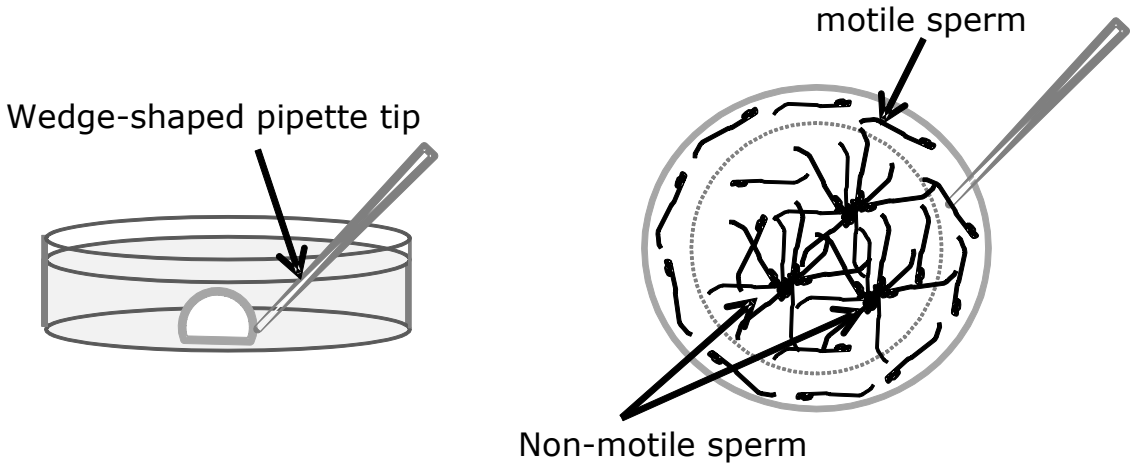

Fig. 7

4. Repeat steps 1-3 for each fertilisation dish in succession (i.e. complete all of the steps from collecting the oviducts to returning the fertilisation dishes to the incubator for one batch of females before starting the next batch). Aim to take no more than 5 minutes from collecting the oviducts to returning the fertilisation drop (including oocytes and sperm) to the incubator.

5. When all oocytes have been added to the fertilisation drops, remove one dish from the incubator and assess the sperm motility and concentration again. Also observe whether cumulus cells are being removed. If the motility and concentration of sperm is poor and few cumulus cells are being removed, it may be necessary to add more of the sperm suspension to each fertilisation drop.

6. Incubate the dishes at 37°C, in 5% CO_2 in air for approximately 3-4hrs to allow fertilisation to occur.

E. Washing and culturing the fertilized oocytes

1. Prepare the wash drops by placing 4 X 150µl drops of HTF (without GSH) in a 35mm culture dish (Falcon 351008) and cover with mineral oil (Fig. 8). Place the dishes in an incubator (37°C) for at least 3hrs or overnight.

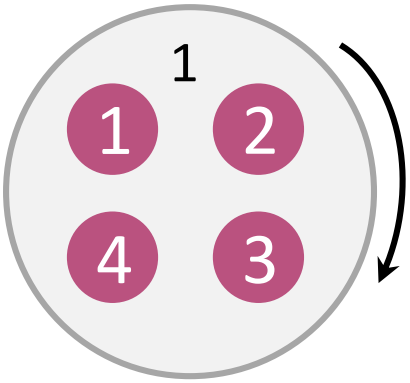

Fig. 8

2. Take the oocytes through three washes (drops 1, 2 & 3) to remove the cell debris, degenerating oocytes and dead sperm.

3. Move the presumptive zygotes into drop 4 and culture them overnight.

4. Next morning, separate the 2-cell embryos from the unfertilised or degenerating oocytes. Place all the 2-cell embryos in drop 4 and the 1-cell or degenerated oocytes/embryos in drop 3.

5. Prepare a drop of hyaluronidase solution (300µg/ml made up in M2) in a Falcon 351008 petri dish. **NB: The hyaluronidase washing step is only required when it is necessary to remove the adherent cumulus cells from the zona pellucida.**

6. Collect the 2-cell embryos into a drop of M2 in a Falcon 351008 petri dish.

7. Transfer these embryos into the drop of hyaluronidase.

8. Incubate at 37°C for a few minutes with gentle agitation at intervals using a glass pipette until any adherent cells or sperm have fallen off.

9. Wash the embryos through two drops of M2.

10. Either transfer the 2-cell embryos to the oviducts of 0.5 day pseudopregnant foster mothers, or:

11. Prepare the 2-cell embryos for cryopreservation according to a standard protocol for *in vivo* derived embryos, or:

7. Culture the embryos in KSOM, plus amino acids

Timetable of events for IVF

Day -3 (e.g. Saturday)	**Day -1** (e.g. Monday)	**Day 0** (e.g. Tuesday)	**Day 1** (e.g. Wednesday)
	Label dishes for oocyte harvest,fertilisation/wash/ culture and sperm dispersal (if using a freshly harvested sample). Prepare the sperm pre-incubation dishes and wash dishes but not the fertilisation dishes.	07:45 Thaw cryopreserved sperm sample, or collect and disperse freshly harvested sperm. Make up a fresh batch of high calcium HTF + GSH and prepare the fertilisation dishes.	Morning: score the IVF success (2-cell vs others).
Superovulate between nine and thirty 3-4 week old females by injecting 0.1ml (5iu) PMS at 16.00-17.00.	Induce ovulation in the females by injecting 0.1ml (2.5 to 5iu) hCG at 16.00-17.00	08:00-09:00 Harvest oocytes and place into diluted sperm preparation.	Prepare the 2-cell embryos for cryopreservation, embryo transfer or culture.
		Between 12:30 and 14.30, wash the presumptive zygotes and place into culture drops.	

This timetable assumes that the mice are exposed to 12 hours of darkness between 19:00 and 07:00.