In vitro fertilisation using freshly harvested sperm TYH + MBCD & GSH medium

This protocol is based on the work published in Takeo et al., (2011)

A. Preparation of sperm dispersal dishes

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

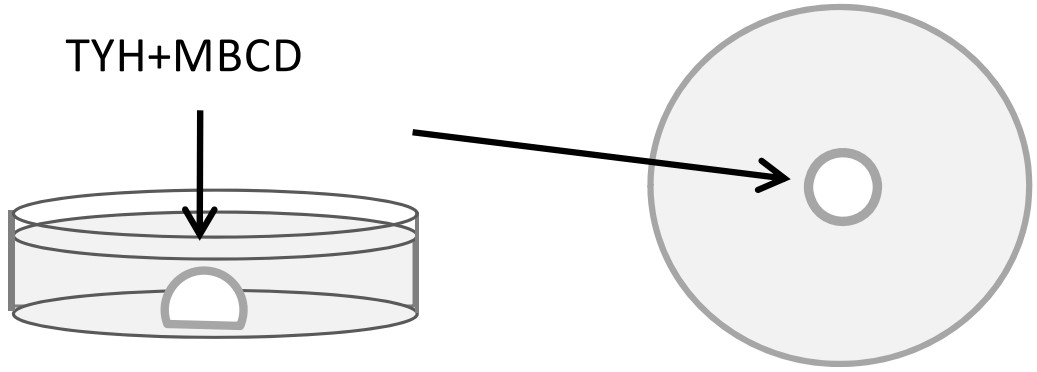

Fig. 1

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

B. Preparation of 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. Dissect the cauda epididymides and 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 watchmaker forceps gently tease out a small 'ball' of the sperm from the cauda epididymides and drag it into the sperm dispersal drop (Fig.2).

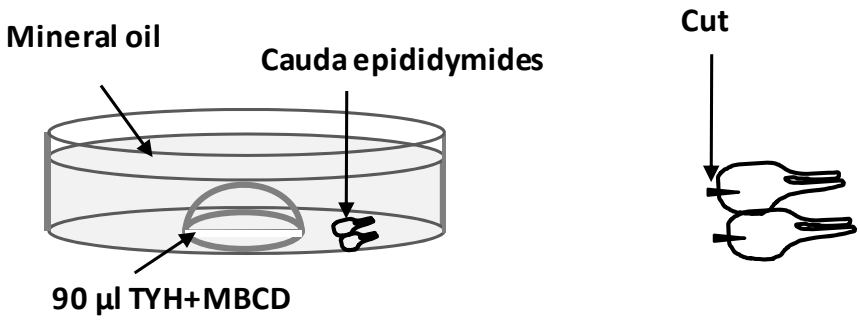

Fig. 2

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.

C. Preparation of fertilisation dish containing 1mM reduced glutathione (GSH – Sigma: G4251)

1. Take 1ml of high calcium HTF medium (Table 2) 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. 3).

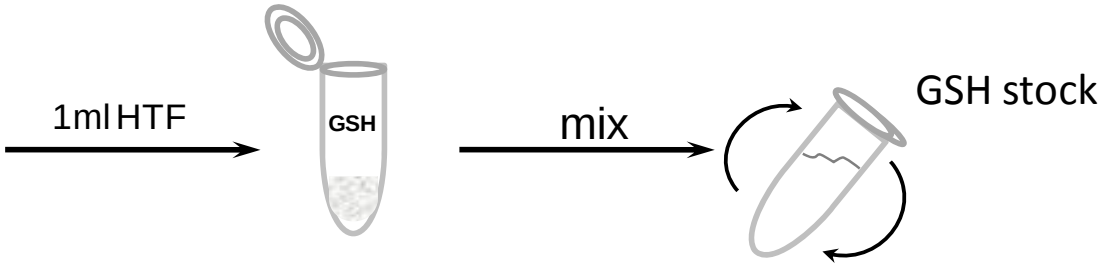

Fig.3

2. Take 50µl of the GSH solution and add it to 5ml HTF medium and mix them together gently (Fig. 4).

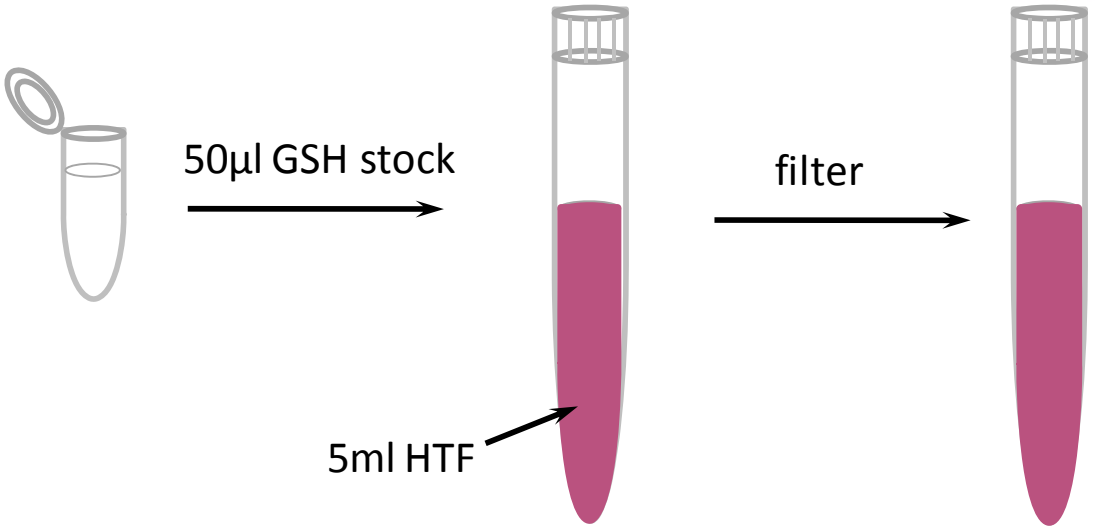

Fig. 4

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

4. Place a 200µl drop of the GSH solution into a 35mm Petri Dish (Falcon 351008), and then place the dish in an incubator (37ºC, 5%CO_2 in air) for 10-20minutes (Fig 5).

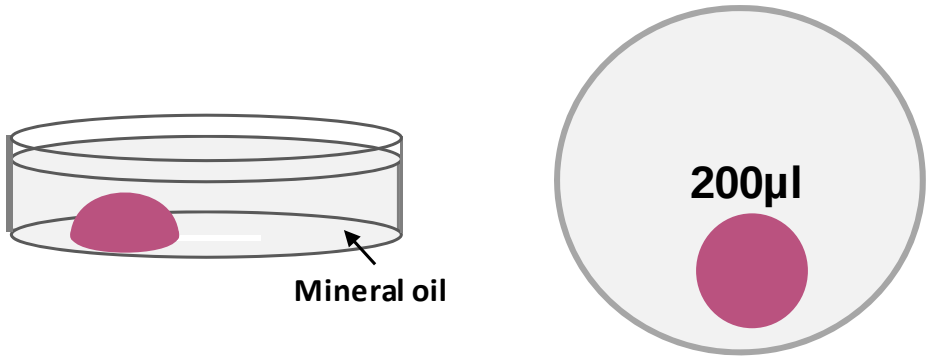

Fig.5

D. Harvesting oocytes

1. Dissect the oviducts from three superovulated female mice and transfer them into the mineral oil overlaying the pre-incubated fertilisation drop.

2. Using a microscope to aid dissection, 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 cumulus masses through the oil and into the fertilisation drop. Then remove the oviduct from the dish.

3. After transferring the cumulus masses into the fertilisation drop, add 3-5µl of pre-incubated sperm.

Note: The sperm should have been equilibrated for 60 minutes in the incubator before it is added to the fertilisation drop. Take the 3-5µl 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. 6).

This step should be performed under a dissecting microscope using a wedge-shaped 10µl pipette tip (Axygen Inc; T-400) to gently aspirate the sperm. By following this procedure it is easy to collect good quality motile sperm without picking up dead sperm or cell debris.

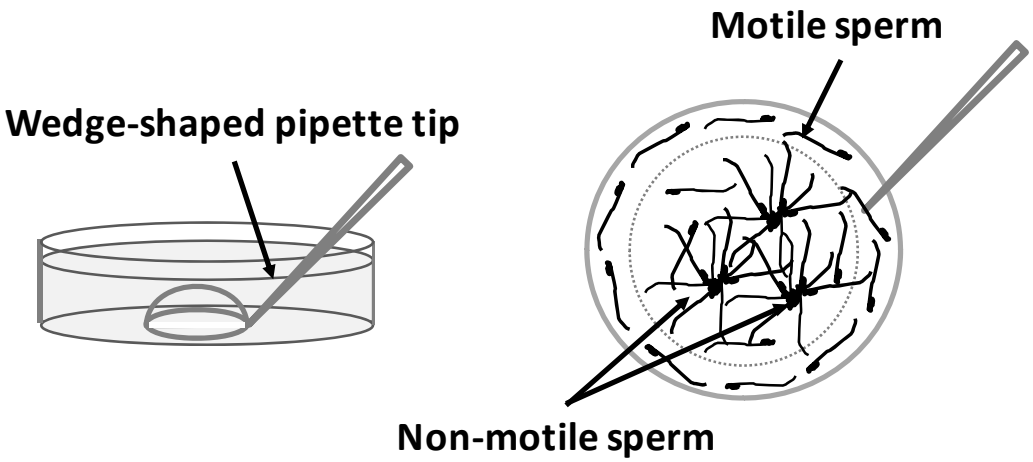

Fig. 6

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 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 the cumulus cells are being removed from the oocytes. 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 fertilised 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. 7). Place the dishes in an incubator (37°C) for at least 3hrs or overnight.

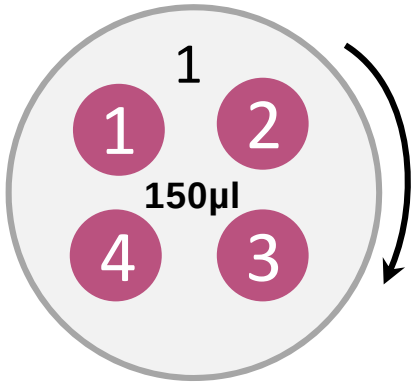

Fig. 7

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

6. 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.

G. Composition of Sperm Preincubation Medium (TYH +MBCD)

Table 1

Reagent Name	mg/100ml	Vendor	Cat. Number
NaCl	697.6	Sigma	S-5886
KCl	35.6	Sigma	P-5405
$MgSO_4 \cdot 7H_2O$	29.3	Sigma	M-7774
KH_2PO_4	16.2	Sigma	P-5655
$NaHCO_3$	210.6	Sigma	S-5761
Na-Pyruvate	5.5	Sigma	P-4562
Glucose	100.0	Sigma	G-6152
$CaCl_2 \cdot 2H_2O$	25.1	Sigma	C-5670
Methyl-β-cyclodextrin	98.3	Sigma	C-4555
Penicillin G	7.5	Sigma	P-4687
Streptomycin	5.0	Sigma	S-1277
Polyvinylalcohol	100.0	Sigma	P-8136

H. Composition of high calcium HTF Medium

Table 2

Reagent Name	mg/100ml	Vendor	Cat. Number
NaCl	593.8	Sigma	S-5886
KCl	35.0	Sigma	P-5405
$MgSO_4 \cdot 7H_2O$	4.9	Sigma	M-7774
KH_2PO_4	5.4	Sigma	P-5655
$CaCl_2 \cdot 2H_2O$	**75.5**	Sigma	C-5670
$NaHCO_3$	210.0	Sigma	S-5761
Glucose	50.0	Sigma	G-6152
Na-lactate (ml)*	0.34	Sigma	L-7900
Na-Pyruvate	3.7	Sigma	P-4562
Penicillin G	7.5	Sigma	P-4687
Streptomycin	5.0	Sigma	S-1277
BSA (Albumin Bovine Serum, Fraction V, Fatty Acid-Free)	400.0	Merck/Calbiochem	126575
0.5% Phenol Red (ml)*	0.04	Sigma	P-0290

*Indicates volume of reagent

I. References

1. Takeo T, Hoshii T, Kondo Y, Toyodome H, Arima H, Yamamura K, Irie T, Nakagata N. Methyl-beta-cyclodextrin improves fertilizing ability of C57BL/6 mouse sperm after freezing and thawing by facilitating cholesterol efflux from the cells. Biol Reprod. 2008 Mar; 78(3):546-51.

2. Takeo T, Nakagata N. Combination medium of cryoprotective agents containing L-glutamine and methyl-{beta}-cyclodextrin in a preincubation medium yields a high fertilization rate for cryopreserved C57BL/6J mouse sperm. Lab Anim. 2010 Apr; 44(2):132-7.

3. Takeo T, Nakagata N. Reduced glutathione enhances fertility of frozen/thawed C57BL/6 mouse sperm after exposure to methyl-Beta-cyclodextrin. Biol Reprod. 2011 Nov; 85(5):1066-72.