IVF protocol using unfrozen sperm collected from epididymides following shipment in Lifor at refrigerated temperatures (4-8°C).

This protocol is based on the work published in Takeo et al., (2012 & 2014). 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. Receiving the refrigerated package

1. On arrival the recipient should remove the aluminium lined box from the polystyrene container and place it in a refrigerator (4°C) if the IVF is not going to be performed immediately.

2. Shortly before setting up the IVF, open the aluminium lined box and retrieve the Eppendorf tube which contains the epididymides.

3. Allow epididymides to warm to room temperature by placing the Eppendorf tube on the bench for 30min before harvesting the sperm.

B. 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 (see Appendix B table 2) into the centre of a 60mm Petri Dish (Falcon 353004) (Fig.1)

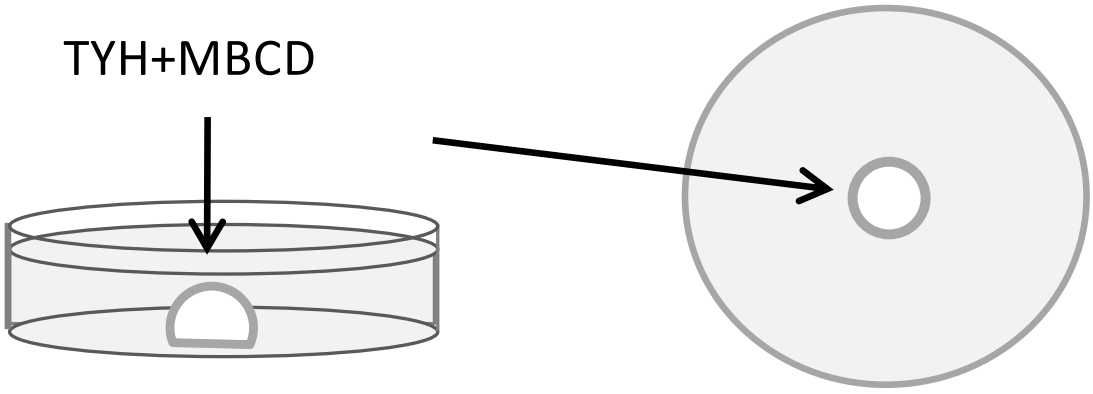

Fig. 1

2. Overlay with mineral oil or silicone fluid and equilibrate for 10-20min, or overnight, at 37°C, in 5% CO_2 incubator.

C. Preparation of fertilisation medium (CARD MEDIUM) containing 0.25mM 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. 2).

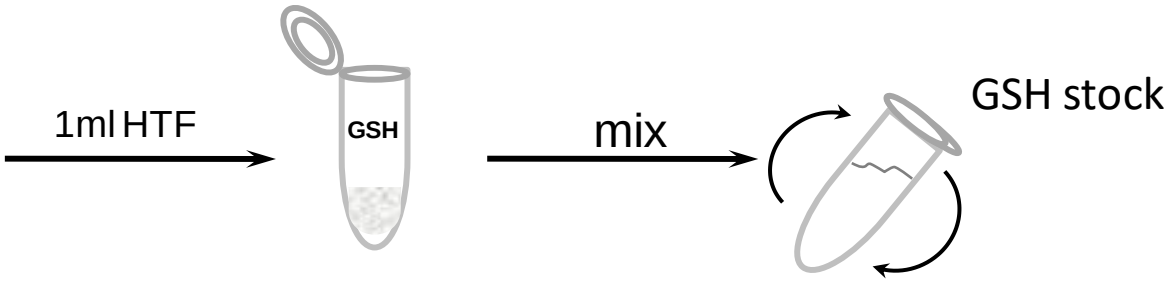

Fig. 2

2. Take 10µl of the GSH stock solution and add it to 4ml HTF medium and mix them together gently (Fig. 3).

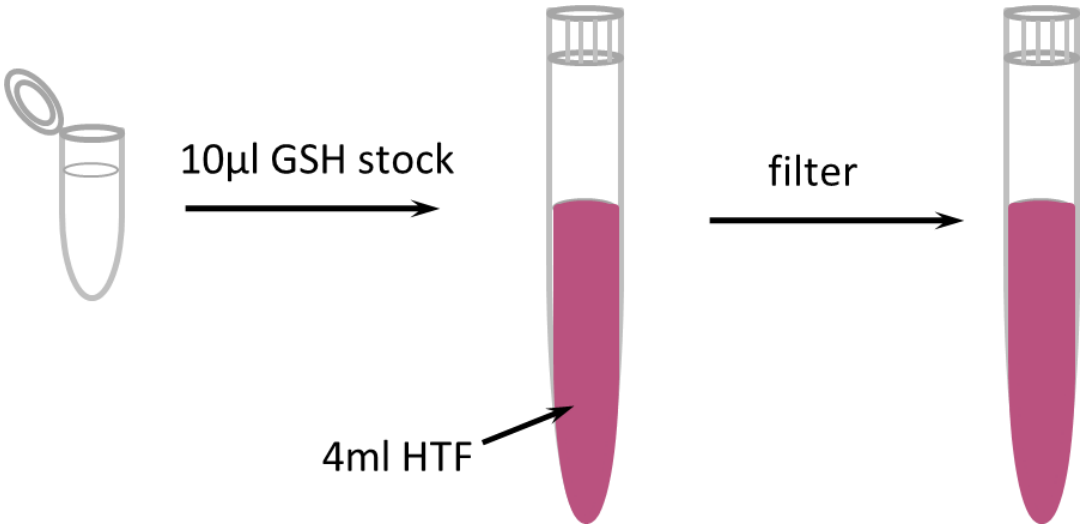

Fig. 3

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

4. Make a 200µl drop of the medium, slightly offset from the centre, in a 60mm Petri Dish (Falcon 353004) (Fig. 4).

5. Overlay with mineral oil or silicone fluid and equilibrate for 10-20min at 37°C, in 5% CO_2 incubator.

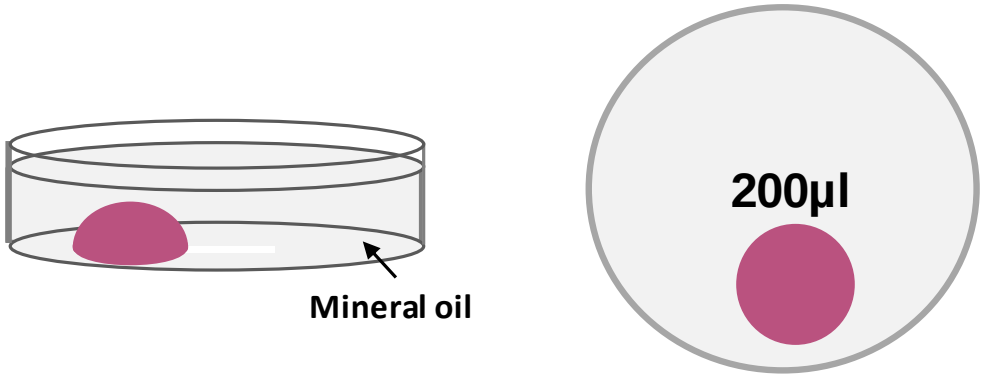

Fig. 4

D. Preparation of sperm samples

1. The cauda epididymides should be wiped free of any Lifor solution using paper tissue.

2. Wash each cauda epididymis through 4 x drops of MBCD. Steps 1 & 2 are very important, if you skip these two steps sperm motility will be compromised.

3. ***NOTE:*** To make a wash dish, place 4 x 80µl drops MBCD into a 60mm Petri Dish (Falcon 353004). There is no need to overlay with mineral oil (Fig. 5).

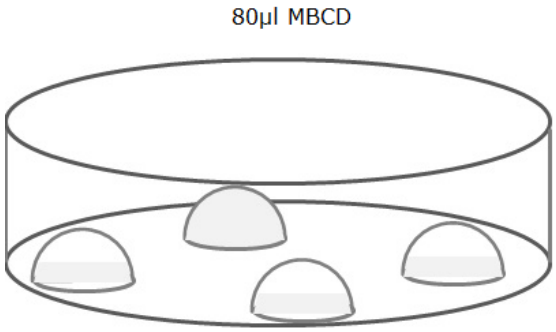

Fig. 5

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

5. 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. 6).

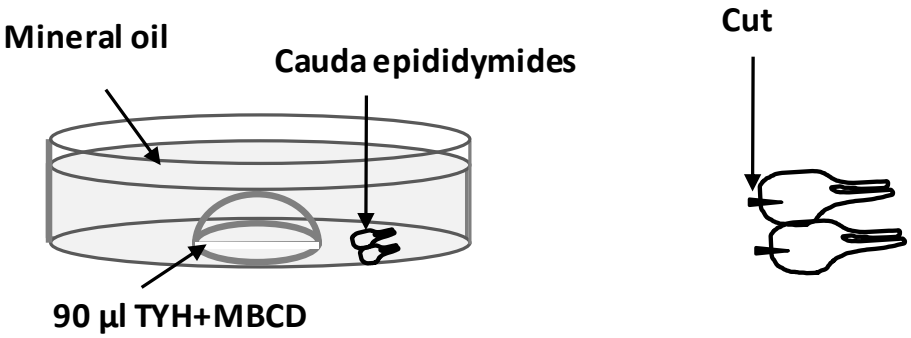

Fig. 6

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

E. <u>Oocyte harvesting</u>

1. Dissect the oviducts from three superovulated female mice (for superovulation method, see Appendix A) and transfer them into the mineral oil or silicone fluid 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. Repeat steps 1-2 for each fertilisation dish in succession. Aim to take no more than 5 minutes from collecting the oviducts to returning the fertilisation drop (including oocytes) to the incubator.

4. **The oocytes should be exposed to the GSH in the fertilisation drop for at least 30mins before adding the sperm suspension**.

F. ***In vitro*** **fertilisation**

1. The sperm should be allowed to disperse in the TYH + MBCD medium for 60 minutes in a 37ºC CO_2 incubator before adding to the fertilisation drop. Take a 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. 7).

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

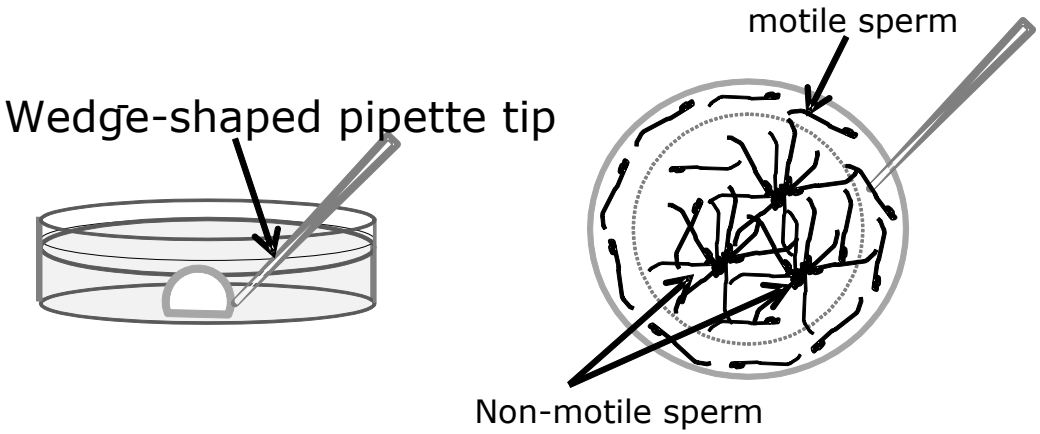

Fig. 7

2. When the sperm suspension has been added to all of the fertilisation drops return the dishes to the incubator. One dish can be removed from the incubator following 10mins incubation to assess the sperm motility and concentration again. It is also possible to 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. Return the dish(es) to the incubator.

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

G. Washing and culturing the fertilised oocytes

1. Prepare the wash drops by placing 4 x 150µl drops of HTF (without GSH) in a 60mm culture dish (Falcon 353004). Overlay with mineral oil or silicone fluid (Fig. 8). Equilibrate the dishes in an incubator (37˚C) for 10-20min or overnight.

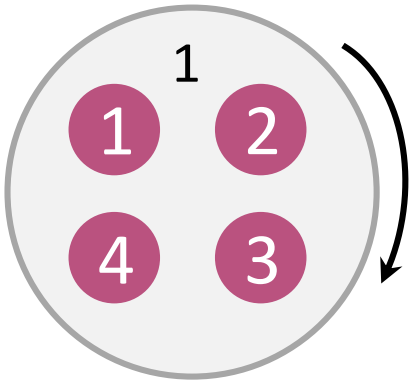

Fig. 8

2. Collect all oocytes from the fertilisation drop and take the oocytes through three washes (drops 1, 2 & 3) to remove the cell debris, degenerating oocytes and dead sperm. Degenerating oocytes can be left in drop 1.

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

H. Scoring the IVF

1. 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.

2. Wash the embryos through two drops of M2.

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

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

5. Culture the embryos in KSOM, plus amino acids.

Appendix A - 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)
	Prepare sperm pre- incubation and wash dishes for IVF.	07:00 Remove eipididymides from refrigerator and allow to warm to room temperature for 30mins.	Morning: score the IVF success (2-cell vs others).
Superovulate females by injecting 0.1ml (5iu) PMS at 16.00-17.00.	Induce ovulation in the females by injecting 0.1ml (5iu) hCG at 16.00-17.00.	Prepare GSH and fertilisation dishes for IVF.	Prepare the 2-cell embryos for cryopreservation, embryo transfer or culture.
		Harvest sperm sample. Pre-incubate for 60mins.	
		Harvest oocytes into fertilisation media.	
		Add sperm suspension to fertilisation drops containing oocytes.	
		12:30 Wash the presumptive zygotes and culture overnight.	

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