Sperm cryopreservation and in vitro fertilisation in Zebrafish facilities at King's College London

DIMITRA MANTZOROU, THOM BERRIMAN, WILL HAVELANGE, JACQUELINE GLOVER, SAM BERRY and BRUNO CORREIA DA SILVA

Zebrafish Facilities, Biological Services Unit, Hodgkin Building, King's College London, Guy's Campus, London SE1 1UL

Correspondence: dimitra.mantzorou@kcl.ac.uk

Winner of the young presenters prize at the 2019 West Middlesex Branch Technicians Symposium

Introduction

King's College London (KCL) Zebrafish Facilities have the capacity to house 50,000 zebrafish (*Danio rerio*). There are currently 491 distinct lines of Zebrafish on the racks however, not all lines are actively and constantly in use. This created the need to establish an in-house Sperm Cryopreservation and *In Vitro* Fertilisation programme. At the time of writing, KCL has successfully frozen 94 lines.

Sperm Cryopreservation is one of the most effective methods of large-scale and long-term storage of genetic material and offers multiple advantages, such as extension of the reproductive period of an individual male Zebrafish, the **Reduction** of the number of live Zebrafish in a facility, a significant drop in cost and space required for maintenance of live populations, prevention of loss of important and irreplaceable lines, and finally allows the re-introduction of any lost alleles back into the population.[1-4]

Sperm cryopreservation

Sperm cryopreservation is scheduled to take place every two weeks, on Wednesday mornings between 9.00 and 11.00. The first step to a successful Sperm Cryopreservation session is the selection of the male Zebrafish. Ideally, only male Zebrafish younger than 1.5 years old will be used for Sperm Cryopreservation, because older Zebrafish tend to produce sperm of impaired quantity and quality. Two weeks prior to a scheduled Sperm Cryopreservation session, the selected males are set up in breeding pairs with wildtype females to breed. The male Zebrafish that successfully fertilise eggs are considered to have good quality of sperm. These male Zebrafish are separated from the females, fed extra diet and are used in the next scheduled Sperm Cryopreservation session. The evening prior to Sperm Cryopreservation, gated breeding tanks are set up with 3 male Zebrafish, which have previously been selected, on one side and 2 female wildtype Zebrafish on the other side as seen in Figure 1 and food is withheld for 12 hours to avoid regurgitation.

Figure 1. Gated Zebrafish breeding tanks with 3 male Zebrafish on one side and 2 female Zebrafish on the other side.

Materials

The materials (Figure 2) needed for collection of the sperm are:

- a microscope

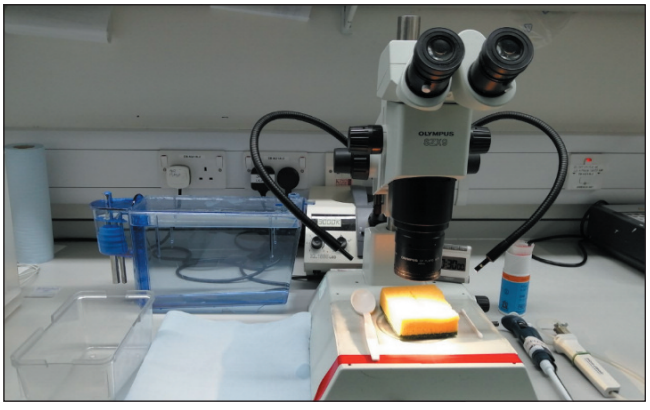
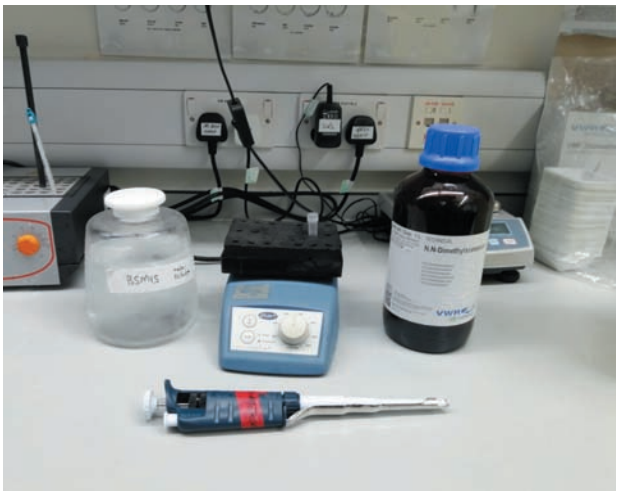


Figure 2. Set-up for sperm collection.

- a sponge with a slit
- a small tank
- anaesthetic solution
- analgesic solution
- a plastic spoon
- paper towels
- cotton swabs
- a post-anaesthesia recovery tank
- microcapillary tubes
- microcapillary tube pipette controller

The materials (Figure 3) needed for sperm freezing are:

- a 200µl Gilson pipette
- 2ml orange lid cryovials
- a Styrofoam box with ice
- 0.5ml microcentrifuge tubes
- freezing medium
- a box with Dry Ice – Ethanol Bath
- 15ml blue lid falcon tubes
- a Timer
- a cryovial freezing box
- a box with liquid Nitrogen
- a Liquid Nitrogen storage tank



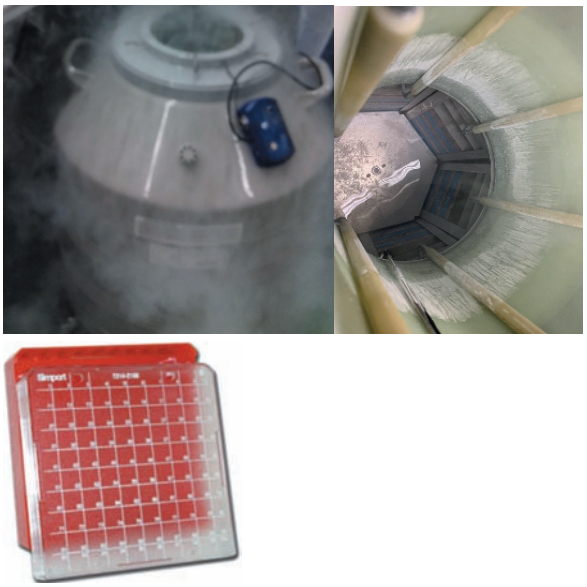


Figure 3. Materials used in sperm freezing.

Tip: *Only certified cryopreservation cryovials should be used to freeze the sperm at the correct rate.*

Sperm cryopreservation technique

The Cryopreservation technique is time-sensitive and requires two experienced technicians to run efficiently. The technicians carry out the following steps:

1. Technician 1 prepares the anaesthetic and the analgesic solution. Analgesia is added to the breeding tanks and to the recovery tank 30 minutes prior to the procedure. Stock solutions and dosage rates can be found in Table 1.
2. Technician 2 prepares a box with Dry Ice- Ethanol bath, the tubes with 145µl freezing medium solution (details on preparation can be found on Table 1), the box with liquid Nitrogen containing a cryovial freezing box, labels the cryovials and prepares the Record books.

Tip: *It is important that the freezing medium is prepared fresh before each Sperm Cryopreservation session.*

3. Technician 1 prepares the microscope set up as seen in Figure 2.
4. Once prepared, Technician 1 immerses 2-3 male Zebrafish to be anaesthetised into the anaesthetic solution. Once the fish lose balance and become immobile, the Technician checks that they are fully anaesthetised by gently touching with the spoon, monitoring for a reflex response.
5. Technician 1 removes one anaesthetised fish from the anaesthetic solution with the plastic spoon (head facing towards the handle of the spoon) and tilts the spoon slightly so that excess water is removed. The fish is dried carefully with paper

towel, especially around the urogenital pore and placed in the slit of the sponge.

6. Technician 1 gently spreads the anal fins with a cotton swab to expose the urogenital pore. A microcapillary tube, attached to the microcapillary tube pipette controller, is placed at the urogenital pore and gentle abdominal pressure is applied to obtain sperm (Figure 4).

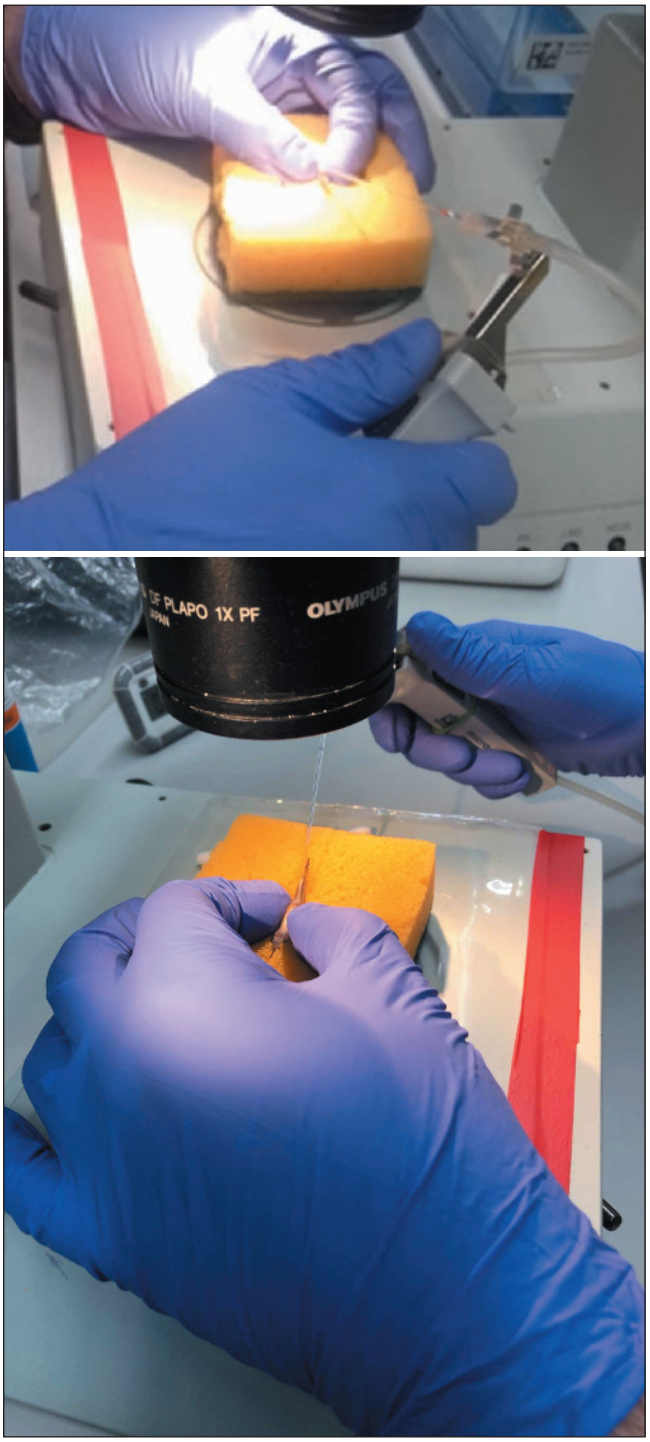


Figure 4. Collection of sperm from male Zebrafish.

7. Technician 1 transfers the fish into the recovery tank containing fresh system water with analgesia, monitoring the fish to ensure that it recovers fully. These steps are repeated for the next male Zebrafish.
8. Usually sperm is pooled together from 2-3 male Zebrafish with a target sperm volume of 3.3 µl and minimum accepted volume of 1.5 µl.[5]
9. Technician 2 hands Technician 1 a 0.5ml microcentrifuge tube containing 145µl of freezing medium, into which the sperm is expelled.
10. Technician 2 pipettes up and down once to mix and then aliquots 35µl of sample into four cryovials, then places each cryovial into a pre-chilled falcon tube in dry ice and sets a timer for 30 minutes.

Tip: *It should only take 30 seconds from the moment the sperm touches the freezing medium to the moment the cryovial is placed in the falcon tube in the dry ice.*

11. When the time elapses, Technician 2 quickly transfers the cryovials into the cryovial freezing box with liquid nitrogen.

In vitro fertilisation (IVF)

At King's College London, Zebrafish *In Vitro* Fertilisation (IVF) is used to assess if the Sperm Cryopreservation has been successful. It can also be used to recover a frozen line or to get a new generation when the parents do not spawn.

IVF is scheduled to take place in the main facility every two weeks alternating with Sperm Cryopreservation, on Wednesday mornings between 9.00 and 11.00. The first step to a successful IVF is the selection of the female Zebrafish. Three stocks of female wildtype Zebrafish are used in rotation for the IVF procedures. Two weeks prior to a scheduled IVF session, the selected females are set up in breeding pairs with wildtype males to breed. The female Zebrafish that successfully lay healthy fertilised eggs are selected. These female Zebrafish are separated from the males, fed extra diet and will be used in the next scheduled IVF. The evening prior to IVF, gated breeding tanks are set up with 3 female Zebrafish on one side and 2 male Zebrafish on the other side and food is withheld for 12 hours to avoid regurgitation.

The materials (Figure 5) needed for *In Vitro* Fertilisation are:

- a microscope
- a small tank
- anaesthetic solution
- analgesic solution
- a plastic spoon
- a post-anaesthesia recovery tank
- 90mm petri dishes
- 200µl AquaBoost™ OvaCoat solution per petri dish
- a fine brush
- paper towels
- a box with liquid Nitrogen

- a heat block set at 37˚C
- parafilm cut into squares
- E3 Medium solution
- fructose solution

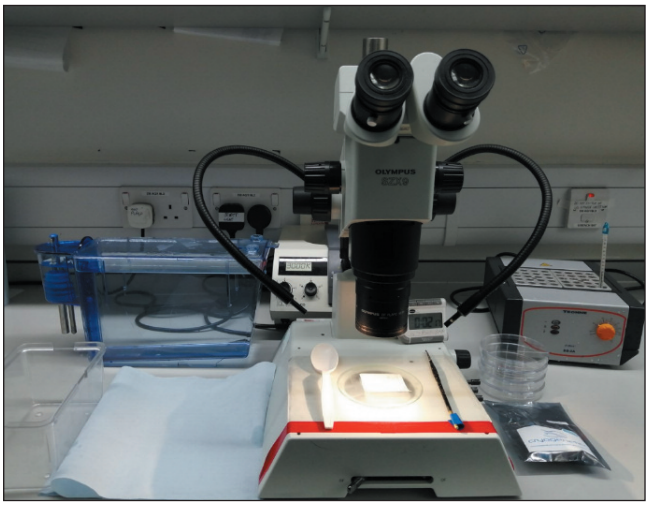


Figure 5. In vitro fertilisation set-up.

IVF technique

IVF is carried out by two experienced Technicians to run efficiently. The Technicians carry out the following steps:

1. Technician 1 prepares the anaesthetic and the analgesic solution. Analgesia is added to the breeding tanks and to the recovery tank 30 minutes prior to the procedure. Stock solutions and dosage rates can be found in Table 1.
2. Technician 2 prepares the E3 Medium solution, fructose solution, adds 200µl of OvaCoat solution to each petri dish and sets the heat-block at 37˚C.
3. Technician 1 prepares the microscope set up as seen in Figure 5.
4. Once prepared, Technician 1 immerses 2-3 Zebrafish to be anaesthetised into the anaesthetic solution (Figure 6). Once the fish lose balance and become immobile, the Technician checks that they are fully anaesthetised by gently touching with the spoon, monitoring for a reflex response.
5. Technician 1 removes one anaesthetised fish from the anaesthetic solution with the plastic spoon (head facing towards the handle of the spoon) and tilts the spoon slightly so that excess water is removed. The fish is dried carefully with some paper towel, especially around the urogenital pore and placed into a piece of parafilm (Figure 7).
6. Technician 1 then applies gentle abdominal pressure to obtain the eggs. Good egg clutches are transferred into the OvaCoat solution in the petri dish with a fine brush (Figure 7).

Tip: *Big egg clutches improve fertilisation rates.*

7. The eggs can stay hydrated in the OvaCoat solution without activation for 30 minutes. This gives enough time to perform IVF with frozen sperm.
8. Technician 2 removes the excess OvaCoat solution from the eggs with some paper towel, carefully.

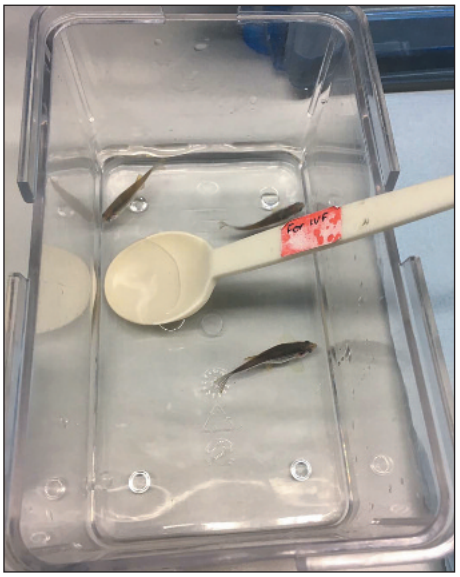


Figure 6. Female Zebrafish immersed in a tank with anaesthetic solution.

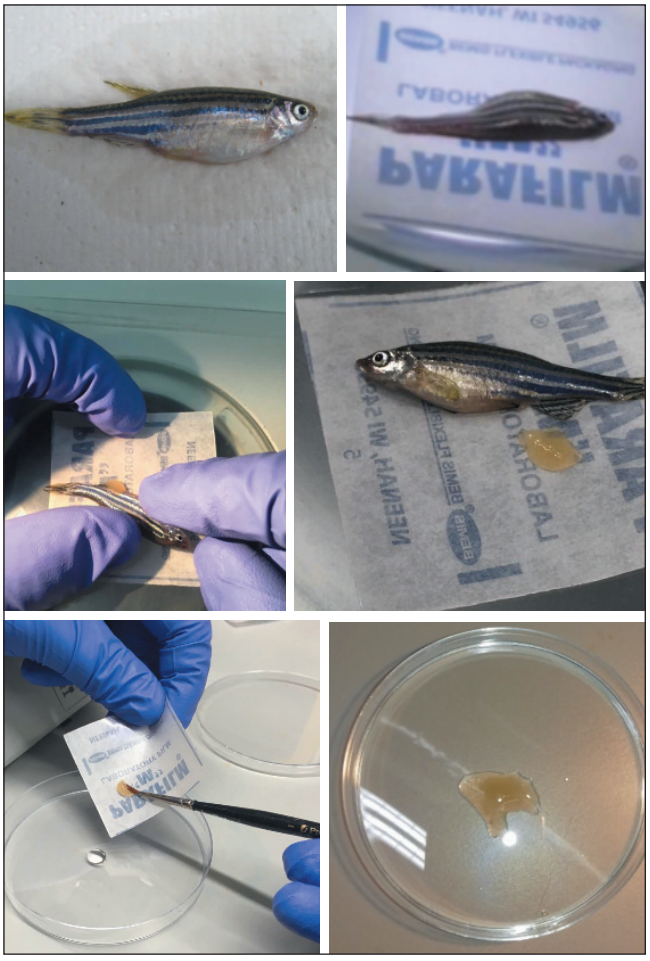


Figure 7. Collection of eggs from an anaesthetised female fish.

Anaesthetic Solution	The stock solution of 0.4% MS222 (Tricaine methanesulfonate) is made up with: 1000ml RO water 4g MS222 powder 8g sodium bicarbonate $NaHCO_3$
	For anaesthesia: To make a 0.02% anaesthetic solution: Dilute to a 1:20 solution e.g. add 10ml of 0.4% MS222 to 190ml System Water
Analgesic Solution	530mg Lidocaine hydrochloride powder into 1L of system water. This analgesia stock solution is ready to use at a dose rate of 7ml per litre.
Freezing Medium	10% DMA (N,N-dimethylacetamide;*TOXIC)* final in BSMIS Add 200µl of DMA to 1.8ml BSMIS and vortex for 10 minutes
BSMIS	4.383g NaCl 5.218g KCl 0.218g $CaCl_2$-$2H_2O$ 0.246g $MgCl_2$-$6H_2O$20ml 1M Tris pH 8.0
Fructose solution	0.5% Fructose in 1XE3 medium 0.5g Fructose in 100ml 1X E3 Medium
E3 Medium	16.5ml 60X E3 made up to 1L distilled water 60x E3 Medium: 17.4g NaCl 0.8g KCl 2.9g $CaCl_2$-$2H_2O$ 4.89g $MgCl_2$-$6H_2O$ In 1L distilled water pH 7.2
OvaCoat Solution	AquaBoost™ OvaCoat from Cryogenetics AS

Table 1. Solutions used in sperm cryopreservation and in vitro fertilisation.

9. Technician 2 removes one cryovial from the Liquid Nitrogen, opens the cap, tips out any liquid Nitrogen within the vial and places the cryovial into position in the heat block.
10. Just before the sperm is fully defrosted, Technician 2 removes the cryovial from the heat-block and adds 400µl of fructose solution. The activated sperm is immediately added to the eggs and a timer is set for 2 minutes.

Tip: *The defrosted sperm should be added to the eggs as fast as possible.*

11. After 2 minutes, Technician 2 floods the dish with E3 Medium solution and the eggs are transferred to the incubator at 28.5˚C. Later in the afternoon, the Technicians check the petri dishes, removing any debris and splitting the dishes in half to reduce the embryo density in each petri dish.
12. The following morning, Technicians 1 and 2 check the eggs under the microscope and record the fertilisation rate. For sperm to be successfully preserved, we look for a minimum of 10% fertilisation rate (minimum of 100 fertilised embryos out of 1,000 eggs). At KCL in 2019, the average fertilisation rate from IVF procedures was 25.9%.

Summary

In keeping with the principles of the 3Rs, the Sperm Cryopreservation Programme at KCL has significantly reduced the number of live Zebrafish in the facilities, with a subsequent decrease in cost and space needed for the maintenance of live populations. Moreover, Sperm Cryopreservation acts as a gene bank and a back-up that prevents the loss of important irreplaceable lines and allows the re-introduction of any lost alleles back into the population. To date KCL have successfully frozen 94 lines. Cryopreservation success is evaluated through In Vitro Fertilisation, which can also be used to recover a frozen line or to get new generations when parents no longer spawn. Success depends on experience, attention to detail and time management.

References

1. **Carmichael, C., Westerfield, M.** and **Varga, Z.M.** (2009). Cryopreservation and in vitro fertilization at the Zebrafish International Resource Center. *Methods in Molecular Biology (Clifton, N.J.)*, 546, 45-65. http://doi.org/10.1007/978-1-60327-977-2_4.
2. **Dooley, C.M., Scahill, C., Fényes, F., Kettleborough, R.N., Stemple, D.L.** and **Busch-Nentwich, E.M.** (2013). Multi-allelic phenotyping – a systematic approach for the simultaneous analysis of multiple induced mutations. *Methods*, 62, pp. 197-206. doi: 10.1016/j.ymeth.2013.04.013.
3. **Matthews, J.L., Murphy, J.M., Carmichael, C., Yang, H., Tiersch, T., Westerfield, M.** and **Varga, Z.M.** (2018). Changes to Extender, Cryoprotective Medium, and In Vitro Fertilization Improve Zebrafish Sperm Cryopreservation. *Zebrafish*. 15(3), 279-290. doi: 10.1089/zeb.2017.1521. Epub 2018 Jan 25.
4. **Yang, H.** and **Tiersch, T.R.** (2009). Current status of sperm cryopreservation in biomedical research fish models: Zebrafish, medaka, and Xiphophorus. *Comp. Biochem. Phys. C.* 149, 224-232. doi: 10.1016/j.cbpc.2008.07.005
5. **Draper, B.W.** and **Moens, C.B.A.** (2009). High-Throughput Method for Zebrafish Sperm Cryopreservation and In Vitro Fertilization. *J. Vis. Exp.* (29), e1395, doi:10.3791/1395.