Roadmap Practical 1

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Zebrafish (20 min)
2 groups of 4 trainees

1. Demonstration of sperm collection and then, retrieval of sperm by the trainees.
2. Demonstration of oocytes collection

   Put zebrafish oocytes in trout coelomic fluid (TCF)

   Observation of zebrafish oocytes under a binocular

Goldfish (20 min)
2 groups of 4 trainees

1. Demonstration of an injection of 0.5ml/kg Ovaprim (Syndel LTD, Canada), a synthetic salmonid GnRH with a dopaminergic inhibitor to stimulate sperm or oocytes production.
2. Demonstration of sperm and oocytes collection and then, retrieval of sperm and oocytes by the trainees.
3. Put goldfish oocytes in TCF (trout coelomic fluid)
   or GFR+STI (goldfish Ringer + soybean trypsin inhibitor)
   Leave some oocytes without any medium

Gametes quality (40 min)
1 group of 8 trainees

1. Fertilization of goldfish oocytes incubated in 3 different conditions
2. Motility
3. Counting of the number of spermatozoa

Gametes collection of goldfish and fertilization

Gametes collection of goldfish and fertilization

Two years-old goldfish are reared in water at 14°C under spring photoperiod.

Three days before gametes collection, fish are transferred into 20°C water.

Males and females are stimulated by one injection of 0.5ml/kg Ovaprim (Syndel LTD, Canada), a synthetic salmonid GnRH with a dopaminergic inhibitor.

Gametes are collected 16 hours after injection.

Equipment for gametes recovery

- SFMM : dilution buffer for sperm
- dechlorinated water (tap water left 24 hours in contact with air)
- Eppendorf 1.5 ml for sperm collection
- Petri dishes 5 cm of diameter for oocytes collection and 10 cm of diameter for fertilization
- Pastette transfer pipettes to recover the sperm

Composition of SFMM

SFMM preserves sperm, it remains still. This solution is at 290 mOsmol/kg and pH 8.

		Final conc. :	500 ml H_2O
NaCl	(MW=58.44 g/mol)	110 mM	3.2 g
KCl	(MW=74.55g/mol)	28.3 mM	1.05 g
$MgSO_4$, 2 H_2O	(MW=246.47 g/mol)	1.1 mM	0.15 g
$CaCl_2$, 2 H_2O	(MW=147.02 g/mol)	1.8 mM	0.13 g
Bicin	(MW=163.2 g/mol)	10 mM	0.82 g
Hepes sodium salt	(MW=260.3g/mol)	10 mM	1.3 g

Sperm collection

1) Catch a male with a net
   Goldfish is quiet outside the water and does not require an anesthesia
2) Wipe the papilla with a paper towel
3) Maintain the fish on the back.
   With one hand press gently flanks of the animal and at the same time, with the other hand, aspirate the sperm with a Pastette transfer pipette.
4) Put the collected sperm in an eppendorf 1.5ml
5) Dilute the sperm 1/5 in SFMM and keep on ice.

Oocytes collection

1) Catch a female with a net
   Goldfish is quiet outside the water and does not require an anesthesia
2) Wipe the papilla and the anal fin with a paper towel
3) Maintain the fish above a Petri dish (5 cm of diameter) and press gently flanks of the animal. Oocytes (1 mm of diameter) fall in the Petri dish slowly (hundreds of oocytes per female)
4) Keep at 12°C to 16°C

Oocyte dilution

Collect a spoon of eggs and mix them with about 1 mL

1) Goldfish Ringer

Composition from (Kagawa et al, Gen Comp Endocrinol 54, 139- 143 - 1984) with soybean trypsin inhibitor (0.5 mg/mL, Hsu et Goetz, Can J. Fish. Aquat. Sci. 50 932-935 - 1993)

NaCl	58.44 g/mol	125mM	7.3 g/L
$CaCl_2$ $2H_2O$	147.02 g/mol	2.4 mM	0.35 g/L
KCl	74.55 g/mol	2.4 mM	0.18 /L g
$MgSO_4$, $7H_2O$	246.48 g/mol	284 µM	0.07 /L g
$MgCl_2$ 6 H_2O	203.3 g/mol	890 µM	0.18 /L g
D glucose	180156 g/mol	5.55 mM	1 g/L
Hepes	238g/mol (pas H de Na)	4 mM	0.95 g/L

In dist Water (1 L final)

Adjust at pH 7.3 avec NaOH 5N Osmolality : 256 mOsm/kg

Add Soybean Trypsin inhibitor 0.5 mg/mL

2) Trout coelomic fluid
3) Keep them “Dry”

Fertilization

1) Put approximately 100 oocytes (the equivalent of a big drop) in a Petri dish 10 cm of diameter, with a spatula.
2) Put 15 µl of diluted sperm next to the oocytes.
3) Cover with dechlorinated water (10-15 ml) and shake gently the Petri dish to distribute eggs in the entire dish.
   Eggs stick on the dish after a few minutes.
4) Rinse with some dechlorinated water after 5 min.
5) Incubate eggs at 20°C
   2-cells stage after 1h and then, a division every 30 minutes
   Hatching after 4 days

Collection ZF gametes

Gametes collection of zebrafish

Composition of HBSS 300 (from Yang et al, 2007, 38, 128-136 Theriogenology)
HBSS300 (Hank's balanced salt solution) is a solution that keeps sperm of zebrafishimmobilized. This solution is at 300mOsmol/kg and pH7,5.

NaCl (58,44g/mol) 137mM
KCl (74,55g/mol) 5,4mM
$CaCl_2$, $2H_2O$ (147,02g/mol) 1,3mM
$MgSO_4$, $7H_2O$ (246,47g/mol) 1mM
Na_2HPO_4, $12H_2O$ (358,14g/mol) 0,25mM
KH_2PO_4 (136,1g/mol) 0,44mM
$NaHCO_3$ (84,01g/mol) 4,2mM
Glucose (180,16g/mol) 5,55mM

Sperm collection

1) Put 30µL of HBSS300 in 3 Eppendorf
2) put 250mL of the fish recycled water and add 250µL of phenoxy-2-ethanol
3) place 1 zebrafish in the anesthetic and wait for the zebrafish to turn on its side (should take 20-30 sec, if too fast, add some fish recycled water
4) With a little net, take the zebrafish and place it in a wet paper so that the fish mucus will not dry and will not be wiped out
5) with a little pipet, spray the male genital papilla with HBSS 300 to prevent spermatozoa from being activated by water
6) give a light pressure on the flanks of the animal (in the area of the middle of the body) and at the same time, aspirate with the pipet in the area of the genital papilla to recover sperm (white liquid, about 1µL recovered)
7) Place sperm into 30µL of HBSS300

Oocyte collection

1) For anesthetized female, wipe genital papilla, press lightly flanks of animal and recover eggs into a plastic spoon. **Attention : we are the afternoon and the females may give nothing or give white eggs.**

2) Put eggs in a Petri dish with a drop of trout coelomic fluid to avoid egg drying.

A beautiful spawn is a spawn with some coelomic fluid ("liquid spawn")
, yellow eggs, and intact eggs as checked under the binocular. Upon dilution in trout coelomic fluid, damaged eggs will spread chunks of white material.

Motility of goldfish or zebrafish sperm

Motility of goldfish or zebrafish sperm

Motility is a major factor of sperm quality.

Principle:

Sperm activation and estimation of the motility under a microscope

Equipment:

- Microscope
- Microscope slides
- Activation medium : water + 0.5 mg/ml BSA (Bovin Serum Albumin)
  BSA prevents spermatozoa from sticking on the slide
- SFMM
  SFMM preserves sperm, it remains still. This solution is at 290 mOsmol/kg and pH 8.

NaCl	(MW=58.44 g/mol)	110 mM
KCl	(MW=74.55g/mol)	28.3 mM
$MgSO_4$, 2 H_2O	(MW=246.47 g/mol)	1.1 mM
$CaCl_2$, 2 H_2O	(MW=147.02 g/mol)	1.8 mM
Bicin	(MW=163.2 g/mol)	10 mM
Hepes sodium salt	(MW=260.3g/mol)	10 mM

- HBSS300
  HBSS300 (Hank's balanced salt solution) is a solution that keeps sperm of zebrafish. This solution is at 300mOsmol/kg and pH7.5, the sperm is so immobilized.

NaCl	(58,44g/mol)	137mM
KCl	(74,55g/mol)	5,4mM
$CaCl_2$, $2H_2O$	(147,02g/mol)	1,3mM
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Na_2HPO_4, $12H_2O$	(358,14g/mol)	0,25mM
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Glucose	(180,16g/mol)	5,55mM

Sperm dilution

1) Sperm of goldfish (1/5 in SFMM) is at 2.10^9 spz/ml

   Dilute it 10 times: 10 µl of sperm + 90 µl of SFMM

2) Sperm of zebrafish (1/10) in HBSS300 is at 1.10^9 spz/ml

   Dilute it 5 times: 20 µl of sperm + 80 µl of HBSS300

Motility

1) Put 20 µl of activation medium (water + 0.5 mg/ml BSA) on a microscope slide
2) Add 2 µl of diluted sperm and mixed gently with the tip of the pipette
3) Observe directly under a microscope (objective X20) : the cell density should be around 100 / frame
4) Estimate the percentage of motility
   For accurate result, this must be done in duplicate
   It's also possible to estimate duration, speed and type of movement

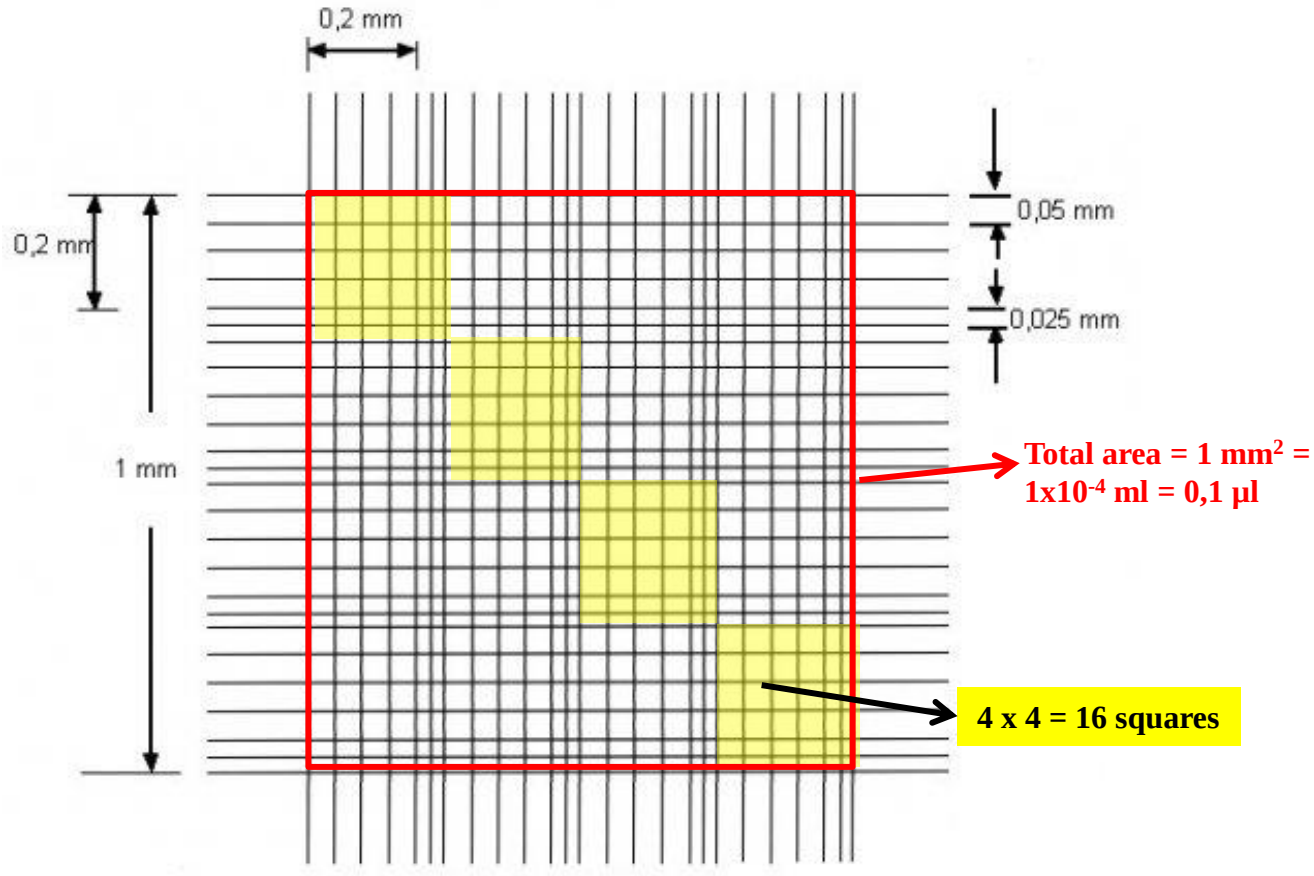
Thoma counting chamber

Thoma counting chamber

GF sperm : 1/5 in SFMM
ZF sperm : 1/10 in HBSS300

1) Dilute the sperm in an appropriate medium :

1/250 in SFFM for goldfish sperm
1/125 in HBSS300 for zebrafish sperm



2) To obtain a reliable counting, it is necessary to have from 20 to 50 cells by yellow square.

According to the density of spz, count the number of cells in a few yellow squares

If you count 4 squares : n x 4 x 10^4 x dilution factor = spz / ml
8 squares : n x 2 x 10^4 x dilution factor = spz / ml
…

Analysis of Ploidy from Goldfish samples by Flow cytometry using CyStain UV Ploidy

Analysis of Ploidy from Goldfish samples by Flow cytometry using CyStain UV Ploidy

alevin ***sperm*** ***embryos***

Principle:
The aim of the practical session is to determine for each sample the DNA contents of their cells : haploid (n chromosomes), diploide (2n) or triploide (obtained on embryos when eggs are of poor quality).

The samples are dissociated in the CyStain solution containing a detergent (to lyse cells) and a fluorescent dye (Dapi) to stain the nuclear DNA. Samples preparation is filtered and analysed by flow cytometry.

Staining kit: CyStain UV ploidy (PARTEC)
Ref : 05-5001 (500ml for 250 tests)
=>ready to use staining solution
=>stored between +2°C and +8°C

Precautions:
- use glowes and lab coat to manipulate this kit (contains DAPI, a nucleic acid intercallant)
- use specific trash to dispose Dapi-stained materials (tips, tubes...)

Preparation of samples:

1st step = sample dilaceration

- **Alevin**

- Place 2 alevins into a petri dish containing 1ml of CyStain UV Ploidy
- Chop the alevin with scalpel.
- Mix by pipetting up and down (10 times)with a P1000
- Incubate about 1min at RT in the dark

- **Sperm**

- Put 2ul of pure sperm into an eppendorf containing 1ml of CyStain UV Ploidy.
- Incubate about 1min at RT in the dark

- **Embryos**

- Put 5 embryos into the eppendorf containing 500ul of CyStain UV Ploidy
- Mix by pipetting up and down.

- Incubate about 1min at RT in the dark

2nd step = sample Filtration

- Filter each sample preparation through a 17um filter adapted to the 1ml seringle

- store filtered samples on ice

Viability assay of Goldfish spermatozoa by Flow Cytometry using Propidium Iodide

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Principle :
The Propidium iodide (PI) is a fluorescent DNA stain (intercalating dye), used to evaluate cell viability. PI is membrane impermeant and it is excluded from the cells with an intact plasma membrane (PM). PI is therefore only labelling the dead cells in a population.

DNA staining by propidium iodide 12 µM
PI : fluorochrome excitable by a blue laser 488nm
λmax excitation = 535nm
λmax emission = 617nm

dilution 1/20 from the 2.4 mM stock solution(prepared in PBS)
stored between +2°C and +8°C

Precautions when manipulating intercalating agent:
PI is suspected to be mutagenic and toxic for reproduction
- use gloves and lab coat
- use specific trash to dispose of PI-stained materials (tips, tubes...)

2 types of sperm sample to analyse in flow cytometry

=> fresh sperm
=> sperm stored for 3 days at 4°C in SFMM (seminal fluid mineral-like medium)

1st step : Preparation of sperm samples
- Dilute fresh and stored sperm: 2 µl of sperm + 1ml of SFMM.

- Filter all sperm preparation through a 17µm filter adapted to the 1ml syringe
- store it on ice

2nd step : Sperm DNA staining
- Add 2µl of PI 12 µM to each sperm sample (fresh and stored)

- store it on ice and in the dark for 2-3 min prior to flow cytometry analysis