AQUA-FAANG – Standard Operating Protocol: **Cell dissociation and slow freezing for ATAC-Seq using salmonid embryos**

Overview: This protocol describes a method used to obtain dissociated cells from salmonid embryos. There are developmental stage specific adjustments to this protocol, and it has been divided into two broad sections: 1. Blastulation and early gastrulation, 2. Mid Gastrula – Pharyngula (eyed stage onwards). Additional comments within sections describe adjustments for slightly different stages.

Consumables:

- 0.25% Trypsin EDTA
- 15ml Falcon tubes
- Rotator system
- Dissection microscope (optional)
- FBS (Heat inactivated)
- DMEM
- DMSO
- 40um filters
- 2ml cryotubes
- Mr Frosty cooling chamber
- Isopropyl alcohol

  Isopropyl alcohol is required to fill the Mr Frosty cooling chambers. Follow instructions provided with the chamber to fill and re-fill Isopropyl alcohol as required.

- Fixed angle centrifuge

1. **Blastulation and early gastrulation**

   Starting from DevMap SOP – Embryo extraction step 1.11

   1.1 Pipette up and down 10 times. Use a wide bore pipette tip and a p-1000 pipette.

   1.2 Vortex at a slow speed for 15 seconds.

   1.3 Observe solution, it should be homogeneous with no clumps. If clumps are observed, repeat step 1.1.

1.4 Transfer solution to a 2ml cryotube.

1.5 Add 10ul of Trypan blue to a 1.5ml Eppendorf tube

1.6 Pipette sample up and down 3 times with a bore tip p-1000 pipette set to 500ul.

This step, meant to homogenize sample before taking an aliquot for cell counting, only needs to be done with one replicate.

1.7 Take a 10ul aliquot for cell counting and add it to the tube with Trypan blue. Pipette up and down three times.

Trypan blue stain needs an incubation period of 5 minutes, leave tube on ice and continue to step 1.8

1.8 Add 100ul of DMSO. Invert tube 4 times.

1.9 Store in a pre-chilled Mr Frosty cooling chamber and transfer to a -80^{o}C freezer for up to 4hr

1.10 Assess cell numbers per ml and percentage of live cells using a haemocytometer.

1.11 Store cryopreserved samples at -80^{o}C

2. Mid Gastrula – Pharyngula (eyed stage onwards)

With the onset of epiboly, cells will form distinct layers and it will not be feasible to dissociate embryos by mechanical means. Instead, 0.25% Trypsin EDTA is used. FBS is used to stop trypsin activity.

Repeat each step for all replicates.

2.1 Starting from step 2.10 of the DevMap SOP - Embryo extraction protocol. Remove supernatant and add required volume of cold Trypsin EDTA. It may be required to use a 15ml Falcon tube for the trypsin digestion when higher volumes of Trypsin EDTA are required.

- *Mid gastrula: 1000ul for 30 gastrulas (33ul per gastrula)*
- *Early somitogenesis: 1000ul for 20 embryos (50ul per embryo)*
- *Mid somitogenesis: 2000ul for 10 embryos (200ul per embryo)*
- *Late somitogenesis: 2000ul for 10 embryos (200ul per embryo)*
- *Pharyngula (eyed stage onwards): 1000ul per embryo*

2.2 Rotate for required time, 50RPM at room temperature.

- *Mid gastrula: 5min*
- *Early somitogenesis: ~5min, monitor digestion and increase if necessary.*
- *Mid somitogenesis: ~10min.*
- *Late somitogenesis ~20min, observe after 15 minutes and decide if more time is required.*
- *Pharyngula (eyed stage onwards): ~30min – 1hr, constant monitoring of digestion required. Observe tube against bright background to better observe clumps.*

2.3 Use dissection microscope to estimate digestion progress. Go back to step 2.2 if clumps are observed.

2.4 Add 500ul of cold heat inactivated FBS per 1000ul of Trypsin EDTA.

If using a 15ml Falcon tube for digestion, split homogenate into 1.5ml Eppendorf tubes for fixed angle centrifugation.

2.5 Centrifuge for 6min, 300RCF, 4^{o}C

2.6 Remove supernatant and resuspend in 1000ul of PBS

2.7 Centrifuge for 6min, 300RCF, 4^{o}C.

2.8 Remove supernatant and resuspend in 900ul of cryopreservant solution without DMSO (45% DMEM, 55% FBS)

2.9 Pass homogenate through a 40um filter into a 2ml cryotube.

2.10 Add 10ul of Trypan blue to a 1.5ml Eppendorf tube

2.11 Pipette sample up and down 3 times with a bore tip p-1000 pipette set to 500ul.

This step, meant to homogenize sample before taking an aliquot for cell counting, only needs to be done with one replicate.

2.12 Take a 10ul aliquot for cell counting, and add it to the tube with Trypan blue. Pipette up and down three times.

2.13 *Trypan blue stain needs an incubation period of 5 minutes, leave tube on ice and continue to step 2.14.*

2.14 Add 100ul of DMSO, close cryotube and invert several times. (Perform this step in a timely manner for all replicates at the same time to minimize cytotoxicity of DMSO)

2.15 Place in Mr Frosty Cooling chamber and store at -80^{o}C for a minimum of 4hr.

2.16 Assess cell numbers per ml and percentage of live cells using a haemocytometer.

2.17 Store cryopreserved samples at -80^{o}C