ATAC-seq protocol part 1: Sampling, dissociation and cryopreservation of salmonid embryos

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This document describes the different steps followed for the sampling, dissociation and cryopreservation of salmonid embryos for subsequent ATAC-seq library preparation. I have divided the protocol into two sections: 'Embryo extraction' and 'Cell dissociation and cryopreservation'.

'Embryo extraction' describes the process of separating and cleaning embryos from other egg constituents, as well as pooling of embryos. The second section 'Cell dissociation and cryopreservation' describes steps to dissociate embryos into cell homogenates through trypsination and subsequent cryopreservation and storage at -80 ºC.

1. Embryo extraction

Overview: This section describes the method used to separate and clean salmonid embryos from other egg constituents, such as the chorion, oils and yolk which can interfere with trypsin digestions and downstream library preparation. Because this process is stage-dependent, the methodology varies as embryos mature, so in the interest of clarity, different sections are available for different developmental stages.

Consumables:

- Petri dishes
- Cold water from egg trays
- Watchmaker forceps
- 1.5ml Eppendorf tubes
- PBS
- DMEM
- FBS (heat inactivated)
- Vortex
- Fixed angle centrifuge
- Crushed ice
- Ice tray

1.1. Blastulation and early gastrulation.

During blastulation the cell mass is not very tightly bound to the yolk and can be easily separated mechanically. However, it is also very easy to lose or break cells. Because of this, the best approach is to dechorionated eggs under a dissection microscope, and use a bore tip pipette to transfer each blastula to an Eppendorf tube:

1.1.1. Place required number of eggs in a petri dish with cold water from egg trays on ice.

Required number of eggs is stage-dependant and will need optimization for non-salmonid species. See table 1 for numbers of eggs used for salmonids.

1.1.2. Place another petri dish with cold PBS in the stage of a dissection microscope.
1.1.3. Transfer one egg to the petri dish under the microscope.
1.1.4. Dechorionate using watchmaker forceps. Take care not to damage the blastula.
1.1.5. Use light mechanical pressure from the forceps to separate the blastula from the yolk.

For this step, a very careful and light scraping motion on the sides of the blastula seems to work best.

1.1.6. Using a P1000 pipette se to 200µl, with a bore tip pipette, transfer blastula to a 1.5ml Eppendorf tube on ice.
1.1.7. Repeat for required number of eggs for each replicate.

See table 1 for egg numbers used in salmonids for different stages.

1.1.8. Let blastulas decant to the bottom of the tube, then remove as much supernatant as possible and add 1000µl of fresh ice-cold PBS.
1.1.9. Proceed to section "**2. Cell dissociation and cryopreservation"**

1.2. Mid gastrulation onwards

At some point during gastrulation, normally on the onset of epiboly, cells will be more tightly bound to each other and to the yolk surface. This makes it more challenging to isolate embryos under the dissection microscope, but it also makes it easier to separate the gastrulas from the yolk without accidentally dissociating them. Because of this, eggs at this stage are dechorionated on a well of a six well plate, and vortexed to separate embryonic mass from yolk and oils.

1.2.1. Pre-chill swinging bucket centrifuge to 4°C.
1.2.2. Place required number of eggs for one biological replicate (see table 1) in a well of a six well plate, fill with de-chlorinated, aerated, cold water (the egg trays provide a good source). Place the six well plate on ice.
1.2.3. Using watchmaker forceps, transfer one egg into another well, along with 1ml of PBS.
1.2.4. Dechorionate egg and remove chorion, take care not to leave any embryonic contents inside chorion.
1.2.5. Repeat for all eggs in one biological replicate.
1.2.6. Transfer contents of well into a 1.5ml Eppendorf tube, on ice.
1.2.7. Vortex tube for ~10 seconds.
1.2.8. Centrifuge on fixed angle centrifuge, at 300RCF, for 6min at 4°C.
1.2.9. Remove supernatant and add 1000µl of fresh cold PBS.
1.2.10. Repeat steps 1.2.7.-1.2.9 once.
1.2.11. Proceed to section "**2. Cell dissociation and cryopreservation"**

Table 1: Number of eggs required for one replicate for each stage of development. We decided to use these numbers based on estimations of cell counts at each stage and required number of cells per assay. Cell numbers per stage will differ for non-salmonids, so these numbers will need to be adjusted accordingly.

Stage	Blastulation	Early gastrula	Mid – late gastrula	Early somito-genesis	Mid somito-genesis	Late somito-genesis	Pharyngula
Number of embryos	40	30	30	20	10	10	5

2. Cell dissociation and cryopreservation

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: 2.1. Blastulation and early gastrulation, 2.2. 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

2.1. Blastulation and early gastrulation

Starting from step 1.1.9. of this protocol.

2.1.1. Pipette up and down 10 times. Use a wide bore pipette tip and a P1000 pipette.
2.1.2. Vortex at a slow speed for 15 seconds.
2.1.3. Observe solution, it should be homogeneous with no clumps. If clumps are observed, repeat step 2.1.1.
2.1.4. Transfer solution to a 2ml cryotube.
2.1.5. Add 10µl of Trypan blue to a 1.5ml Eppendorf tube.
2.1.6. Pipette sample up and down 3 times with a bore tip P1000 pipette set to 500µl,

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

2.1.7. Take a 10µl aliquot for cell counting and add it to the tube with Trypan blue. Pipette up and down three times.
2.1.8. Assess cell numbers per ml and percentage of live cells using a haemocytometer.
2.1.9. Centrifuge samples at 300RCF for 6min at 4^{o}C
2.1.10. Carefully remove supernatant.
2.1.11. Add 150µl of cold FBS, 122µl of cold DMEM, and 30ul of DMSO to samples.
2.1.12. Pipette up and down 3 times to resuspend cell pellet.
2.1.13. Store in a pre-chilled Mr Frosty cooling chamber and transfer to a -80^{o}C freezer for a minimum of 4hr
2.1.14. Store cryopreserved samples at -80^{o}C.

2.2. 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.

2.2.1. Starting from step 1.2.11 of this protocol. Remove supernatant and add required volume of cold Trypisn 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: 1000*µl for 30 gastrulas (33ul per gastrula)
- *Early somitogenesis: 1000*µl for 20 embryos (50ul per embryo)
- *Mid somitogenesis: 2000µl for 10 embryos (200ul per embryo)*
- *Late somitogenesis: 2000µl for 10 embryos (200ul per embryo)*
- *Pharyngula (eyed stage onwards): 1000*µl per embryo.

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

Required RPM may vary depending on the diameter of the rotator used. Aim for inverting the tubes 1-2 times per second. If RPM is too high, liquid will stay at the bottom of the tube due to centrifugal force and mixing will be inefficient.

- *Mid gastrula: 5min*
- *Early somitogenesis: ~5min, monitor digestion and increase as 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.*

2.2.3. Use dissection microscope to estimate digestion progress. Go back to step 2.2.2. if clumps are observed.
2.2.4. Add 500µl of cold FBS per 1000µl of Trypsin EDTA
If using a 15ml Falcon tube for digestion, split homogenate into 1.5ml Eppendorf tubes for fixed angle centrifugation.
2.2.5. Centrifuge for 6min, 300RCF, 4°C.
2.2.6. Remove supernatant and resuspend in 1000µl of cold PBS.
2.2.7. Centrifuge for 6min, 300RCF, 4°C.
2.2.8. Remove supernatant and resuspend in 300µl of cryopreservant solution without DMSO (45% DMEM, 55% FBS - 122µl of DMEM and 150µl of FBS)
2.2.9. Pass homogenate through a 40µm filter into a 2ml cryotube.
2.2.10. Add 10ul of Trypan blue to a 1.5ml Eppendorf tube.
2.2.11. Pipette sample up and down 3 times with a bore tip P1000 pipette set to 500µl.

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

2.2.12. Take a 10µl aliquot for cell counting, and add it to the tube with Trypan blue. Pipette up and down three times.
2.2.13. Assess cell numbers per ml and percentage of live cells using a haemocytometer.
2.2.14. 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.

2.2.15. Place in Mr Frosty cooling chamber and store at -80°C for a minimum of 4hr.
2.2.16. Store cryopreserved samples at -80°C.