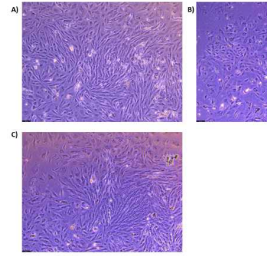


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Primary cell cultures from Atlantic salmon alevin head tissue

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We are still developing and optimizing this protocol

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Abstract

Introduction

Salmonids are the third most farmed fin fish species. And while the industry keeps growing, a variety of different pathogens continue to threaten its sustainability. Gene manipulation is quickly becoming a reliable way of creating genetically-resistant salmon stocks, especially when dealing with viral diseases such as SAV or ISAV. Methods of vector delivery into salmonid embryos, such as CRISPR/Cas9, are now well established, but the means to test the resulting embryo's disease resistance currently relies on live pathogen challenges in whole animals, which are logistically complex, expensive and challenging when it comes welfare. This can be somewhat overcome by producing primary cell culture from edited animals and exposing these cultures to pathogens. However, the techniques for doing this are either not well characterized or not well represented in the literature.

Atlantic salmon lags behind other salmonid species when it comes to cell cultures established from embryos or post-hatch alevins (juvenile salmon). In this study, we have developed a protocol to culture primary cells from Atlantic salmon alevins, which allows for the testing of *in-vivo* gene edited embryos in an *in-vitro* environment, without the need to expose whole fish to disease, along with all the associated animal welfare issues.

Methodology:

Alevins were sacrificed and dechorionated. The fish were each split into sections and decontaminated using an antibiotic mix. The tissues were chemically dissociated using trypsin and seeded into cell culture plates using various novel media mixes. The cells were kept in an incubator at 20°C. One media change was performed every 2-4 days.

Results:

Our alevin cell cultures have similar or better survival rates when compared to other salmonid species embryonal or pre-first feed fish cell cultures. The cells, which are able to form a monolayer in just over two weeks after seeding, remain viable and proliferative for up to four weeks (Figure 1).

Image Attribution

Figure 1) Primary cell cultures from alevin tissue, section A-head. The black bar at the bottom represents 100µm. **A)** Monolayer of cells three weeks post-seeding. Cells were dissociated with trypsin and grown in media M1. **B)** Cells three weeks post-seeding. Cells were dissociated with trypsin and grown in media M2. **C)** Monolayer of cells four weeks post-seeding. Cells were dissociated mechanically using a cell sieve and grown in media M1.



Materials

IVF:

- Atlantic salmon eggs
- Atlantic salmon milt (frozen or fresh)
- Glutathione solution (1mM, pH 8) - 0.307g of L-Glutathione powder reduced (cat no. G4251-5G, Sigma-Aldrich) per liter of distilled water
- Dry ice
- 15cm dishes

CELL MEDIA:

- Leibovitz's L-15 Medium GlutaMAXTM Supplement (Thermo Fisher Scientific)
- 20% FBS (Gibco)
- 1% Antibiotic-Antimycotic (100X) (Thermo Fisher Scientific)
- 1:1000 2-Mercaptoethanol 50uM (Sigma-Aldrich)
- 1% D-(+)-Glucose solution (Sigma Aldrich)
- 2% MEM Non-Essential Amino Acids Solution (100X) (Gibco),
- 10mM Glutathione Reduced (200mM stock, Fisher Chemical)

CELL CULTURING:

- Scalpel blades
- Falcon tubes
- Cell filters (40 µm and 70 µm)
- 24-well sterile cell culture plates
- Iodine solution
- PBS solution
- 0.5% sodium hypochlorite solution
- Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific)
- Leibovitz's L-15 Medium GlutaMAXTM Supplement (L15/GlutaMax, Thermo Fisher Scientific)
- Antibiotic-Antimycotic (100X) (a/a, Thermo Fisher Scientific)
- Trypsin-EDTA (0.25%), phenol red (Gibco)

Atlantic salmon egg IVF and incubation

15w

- 1 Atlantic salmon eggs were sourced from two different females (~4000 ova per female) and stored in the fridge at 4°C.

Milt straws were retrieved from liquid nitrogen storage and kept on dry ice until used. *In-vitro* fertilisation (IVF) was done in 15cm dishes (two dishes of eggs per IVF per female, approx. 800 eggs per female per IVF) in ovarian fluid by adding half a straw of milt (thawed for 20 seconds in a 37°C water bath) to each dish and manually swirling the eggs to ensure the milt gets distributed to all eggs equally. The eggs were incubated at 4°C for 2 minutes then washed three times with Glutathione solution (1mM, pH 8).

The eggs were left to incubate at 6-8°C in a freshwater recirculating system until they hatched (~3 months). The hatched alevins were further grown for ~2-3 weeks.

Cell media preparation

1h

- 2 Before the onset of experiments, cell media was prepared. Below are the two formulations that sustained the growth and proliferation of cells the best:

2.1 Media M1:

- Leibovitz's L-15 Medium, GlutaMAX™ Supplement (L-15/GlutaMax) (Thermo Fisher Scientific)
- 20% Fetal Bovine Serum (FBS) (Gibco) inactivated for 30 minutes at 56°C
- 1% Antibiotic-Antimycotic (a/a) (100X) (Thermo Fisher Scientific)
- 1:1000 2-Mercaptoethanol 50uM (Sigma-Aldrich)

2.2 Media M2:

- Leibovitz's L-15 Medium (L-15) (Thermo Fisher Scientific)
- 20% non-heat inactivated FBS (NHI-FBS)(Gibco)
- 1% D-(+)-Glucose solution (Sigma Aldrich)
- 2% MEM Non-Essential Amino Acids Solution (100X) (Gibco)
- 1% Antibiotic-Antimycotic (100X) (Thermo Fisher Scientific)
- 10mM Glutathione Reduced (200mM stock, Fisher Chemical)
- 1:1000 2-Mercaptoethanol 50uM (Sigma-Aldrich)

Alevin and tissue sterilisation

2h



- 3 18-21 days old alevins were collected from the system and brought to the tissue culture lab. Fish were killed by decapitation with a sharp blade and then placed into freshly prepared media made out of 1:1 Dulbecco's Modified Eagle Medium (DMEM)(Thermo Fisher Scientific) and L-15/GlutaMax, supplemented with 1% a/a.

Fish were washed in media once, then moved into a new falcon tube containing 1:100 Iodine in PBS with 1% a/a for 2 minutes. The iodine solution was removed, and fish were washed three times with DMEM supplemented with 1% a/a. The media was removed and 50mL of 0.5% sodium hypochlorite solution prepared in DMEM supplemented with 1% a/a was added into the tube. The falcon tube was shaken on a rocking platform at 120 rpm for 20 minutes, the liquid was drained and the fish were washed three times in PBS supplemented with 1% a/a.

After the alevins were sterilised, they were dechorionated and cut into four parts (see comment → for below protocol use section A-head). From here, the tissues underwent different dissociation protocols as seen in the next section and were then seeded in media.

Tissue dissociation and cell seeding

1h

- 4 Below are the two dissociation methods that allowed for the best cell survival and proliferation:

4.1 Chemical dissociation with trypsin:

The head section was cut into small explants and put into a falcon tube with 5mL of Trypsin-EDTA (0.25%), phenol red (Gibco) and placed onto a shaker platform at 120 rpm for 10 minutes. The tubes were then shaken vigorously by hand and DMEM supplemented with 1% a/a was added on top of the Trypsin-EDTA at a ratio of 1:1. The cell and tissue mix was then strained through a 40 µm filter into a new tube which was then centrifuged at 900 rpm for 4 minutes. The supernatant was discarded and the cell pellet was resuspended in 1.7 mL of DMEM supplemented with 1% a/a. 200 µl of the cell suspension was seeded into wells of a 24-well plate and topped up with 1mL of one of the two different media.

4.2 Mechanical dissociation with cell sieve:

Alevin head section was chopped into small pieces with a scalpel and passed through a 70 µm cell filter using DMEM supplemented with 1% a/a to wash down the tissue. 200 µl of the resulting mechanically-dissociated cell suspension was seeded into wells of a 24-well plate and topped up with 1mL of one of the two different media.



Cell culture care

4w

- 5 All empty wells from the plates were filled with 500µl of PBS solution to prevent excessive media evaporation inside the incubator. All cell plates were sealed with parafilm and incubated at 20°C in the dark at ambient levels of CO₂.

A third of the media was changed at 24h and 48h post-seeding. Following that, a third of a media was changed every 3 to 4 days. If required, the cells were washed with PBS to help remove dead cells and debris.

Protocol references

First version of the protocol was developed internally by Dr. Remi Gratacap during his time working at the University of Edinburgh. Following that, the protocol saw additional changes from Dr. Maeve Ballantyne and Dr. Jin Yehwa during their time at the University of Edinburgh. This secondary version of the protocol was carried forward and became the basis of the current version following several changes and optimisation steps.