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A Rapid and Simple Procedure for the Isolation of Embryonic Cells from Fish Eggs

Vasily Golotin[1, 2, *], Anatoly Lyutikov[1], Tatiana Filatova[1], Vladimir Sharoyko[2] and Olga Apalikova[1]

[1]Saint Petersburg branch of "VNIRO", "GosNIORH" named after L.S. Berg, Saint-Petersburg, Russia
[2]First Saint-Petersburg State Medical University, Saint-Petersburg, Russia
*For correspondence: golotin@bk.ru

Abstract

Fertilized teleost fish eggs are a complex formation, in which dividing cells are located in a small point in the entire volume of eggs. Isolating embryonic cells can be considered a necessary step in the research of developmental peculiarities of fish cells at the earliest stages of embryogenesis before embryo formation. The main advantages of the offered protocol are rapid isolation, no enzymes, and overall low cost compared to other protocols. The protocol is suitable for the isolation of embryonic cells from medium-sized eggs at the stages of blastula or gastrula, for studies in a variety of applications (e.g., microscopy, flow cytometry, and other methods). Fertilized nelma eggs (Stenodus leucichthys nelma) are used in the protocol as a model.

Key features

- Fast and cheap isolation of cells from fish eggs at early stages (blastula or gastrula).
- Applicable for most of the methods for cell study (any staining, microscopy, flow cytometry, etc.).
- Can be applied to other teleost fish eggs with medium egg diameter of 3–4 mm.

Keywords: Aquaculture, *Stenodus leucichthys nelma*, Blastodisc, Cell isolation, Flow cytometry, Propidium iodide, Microscopy

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Graphical overview

Background

To date, many methods for isolating living cells from various tissues are used for different purposes. Mainly, living cells from tissues are disaggregated by trypsinization. This method is very common and involves the extraction of an embryo or tissue with further trypsinization (Durkin et al., 2013). Some other protocols are based on mechanical crushing of tissues or formed embryos to obtain cells (Fetherman et al., 2015).

When rapid testing of samples is required as soon as possible after egg fertilization, it is necessary to isolate cells directly from the blastodisc at the earliest stages of teleost fish embryogenesis before formation of embryo, larva, or fry. Cell isolation from fertilized eggs by the methods described above does not lead to the desired result, since the eggs contain huge amounts of various substances and a small number of cells relative to the weight of the egg. In addition, the cells in the blastodisc are not tightly connected with each other. That is why the use of trypsin (and other enzymes) is not ideal, with undesirable chemical effects on the cells that can lead to the destruction of cell membranes.

To date, we have only found one other protocol describing a similar procedure (Rieger, 2019). That protocol requires the presence of pronase from *Streptomyces griseus* for the dechorionization of embryos. The pronase only softens the chondrion, requiring additional washing to remove it from the embryo. Besides, the isolation procedure is complicated by particular features of enzymes, as most biological catalysts have narrow operating limits as well as a short shelf life. We aimed to ensure maximum simplicity and low cost of the isolation method with minimal requirements for laboratory equipment.

Materials and reagents

Biological materials

Fertilized fish eggs in the stages from blastodisc to the late gastrula.

Solutions

1. PBS soluble tablets (Sigma-Aldrich, catalog number: P4417), for cell isolation

2. 70% ethanol solution (Sigma-Aldrich, catalog number: 65348-85), for cell preservation
3. o-safranin ready-to-use solution 0.1% (Scientific Laboratory Supplies, catalog number: TMS-009-C), for protocol verification
4. Propidium iodide ready-to-use solution 1 mg/mL (Thermo Fisher Scientific, catalog number: P3566), for protocol verification
5. RNAse A ready-to-use solution 10 mg/mL (Thermo Fisher Scientific, catalog number: EN0531)
6. Phosphate-saline buffer solution (see Recipes)
7. 70% ethanol (see Recipes)

Recipes

1. **Phosphate-saline buffer solution**

Reagent	Final concentration	Quantity
PBS	150 mM total of all salts: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4	1 tablet
H_2O	n/a	Up to 100 mL
Total	n/a	100 mL

2. **70% ethanol**

Reagent	Final concentration	Quantity
Ethanol (95%)	70%	74 mL
H_2O	n/a	26 mL
Total	n/a	100 mL

Laboratory supplies

1. Microcentrifuge tube pestles (Sigma-Aldrich, catalog number: BAF199230001-100EA)
2. Conical bottom microcentrifuge tubes (Sigma-Aldrich, catalog number: HS4325-1000EA)
3. Centrifuge tubes (Sigma-Aldrich, catalog number: CLS430790)

Equipment

1. Microcentrifuge (Thermo Fisher Scientific, catalog number: 75004081)
2. Microscope with 8–20× zoom lens for data validation (Thermo Fisher Scientific, catalog number: 4479672)
3. Vortex mixer (VELP Scientifica, catalog number: F202A0173)
4. Dissecting needles (Thermo Fisher Scientific, catalog number: 13-820-024)
5. Flow cytometer for data validation (Beckman Coulter, catalog number: CO9752)

Software and datasets

1. CytExpert software for flow cytometry (used for data validation)

Procedure

1. This protocol has been tested on fertilized nelma eggs of medium size (3–4 mm in diameter) among teleost fish (including zebrafish). Before the procedure, rinse the eggs with PBS: place the eggs in a centrifuge tube (15 mL) and pour 10–20 mL of cool PBS solution into it. Drain the liquid and repeat this step to prevent any

contamination that could affect further analysis. This step can also be done in any way available in the laboratory: using a tea strainer, beakers, gauze, etc.

2. Using a pestle, pop the eggs (or single egg) in the tube. It is better to add eggs and process them gradually. Do not homogenize nor strongly push the pestle to avoid damage to the cells: it is enough to break the shell. The sample volume from the processed eggs should not exceed 2/3 of the tube. A 1.5 mL centrifuge tube can be fitted with a different number of eggs, depending on their size. Thus, approximately 30 eggs of 3–4 mm diameter can be sequentially placed in the tube during the process.
3. Fill the tube with eggs with cold PBS and shake it on a vortex for 5 s or mechanically using a pestle or dissecting needle to release the contents of all eggs outside the visible shell.
4. Mechanically remove all shells from the tube using a dissecting needle. It is very important to ensure that there are no residual shells in the resulting translucent medium to avoid cell loss.
5. Centrifuge at 300–500× *g* for 5 min at 4 °C.
6. Discard the supernatant without affecting the precipitate. Add 1–1.5 mL of cold PBS and centrifuge again.
7. (Optional) Repeat step 6 1–2 times to wash the cells out of small debris and resuspend the pellet in 100 µL of cold PBS.

Following these steps, we obtain a pellet containing the cells. The volume of PBS can be changed as desired (50–500 µL). This suspension contains a certain amount of small suspended non-cellular particles. After the procedure, the total proportion of cells among other floating non-cellular particles in the suspension is at least 10%.

The cell suspension is ready to use for any analysis. It can also be fixed for long-term cell storage using standard protocols such as using chilled ethanol (Ciancio et al., 1988).

Data analysis

Flow cytometry and microscopy were used for protocol verification. The CytExpert software was used for all cytometry data.

Validation of protocol

Maturating nelma eggs (*Stenodus leucichthys nelma*) were used to validate the protocol (Figure 1A).

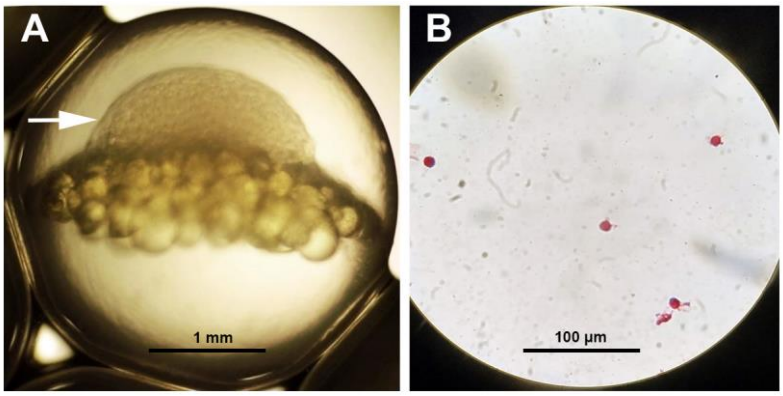

Figure 1. Maturating *Stenodus leucichthys nelma* egg. (A) White arrow shows the position of the blastodisc. (B) Isolated cells from the blastodisc stained with O-safranin.

Thirty fish eggs were processed by the protocol described above. Then, the cell-containing pellet was resuspended in 300 µL of PBS and fixated in 70% ethanol (Ciancio et al., 1988) to ensure long-term preservation prior to analysis. A small 10 µL aliquot of the obtained suspension was stained with safranin (Swain and De, 1990). The suspension

was air-dried at room temperature on a glass slide causing the cells to adhere to it. Then, the slide was immersed in a solution of safranin for 5 min and washed one time with PBS. The result was checked using a light microscope (Figure 1B).

In order to verify the results and additionally show the real presence of isolated cells, an alcohol suspension of the obtained cells (300 µL) was stained with propidium iodide (Riccardi and Nicoletti, 2006). The alcohol was washed off the cells twice with PBS by centrifugation at 300× *g* for 5 min; cells were then resuspended in 100 µL of PBS (+0.1 µg/mL RNase A and 0.01 µg/mL propidium iodide). After 30 min of incubation in the dark at 4 °C, stained cells were analyzed using a flow cytometer (channel PE) (Figure 2). The flow cytometer analyzed 500,000 events. Using a software interface, side scatter, and forward scatter data, only single cells were selected for analysis. The histogram along the PE channel clearly showed peaks corresponding to the cell cycle of isolated cells (Figure 2A). The vertical line on the dot plot (Figure 2B) showed the position corresponding to the G1 peak of the cell cycle in the histogram (Figure 2A). On the dot plot diagram (Figure 2B) for the same channel, there is size cell dispersion (y-axis) that corresponds to the stage of embryogenesis (small cell blastula) where cells acquire different sizes (Figure 2B). According to our data, approximately 100,000 cells were isolated (Figure 2B) from 30 eggs (Figure 1A), which was quite commensurate with other known methods.

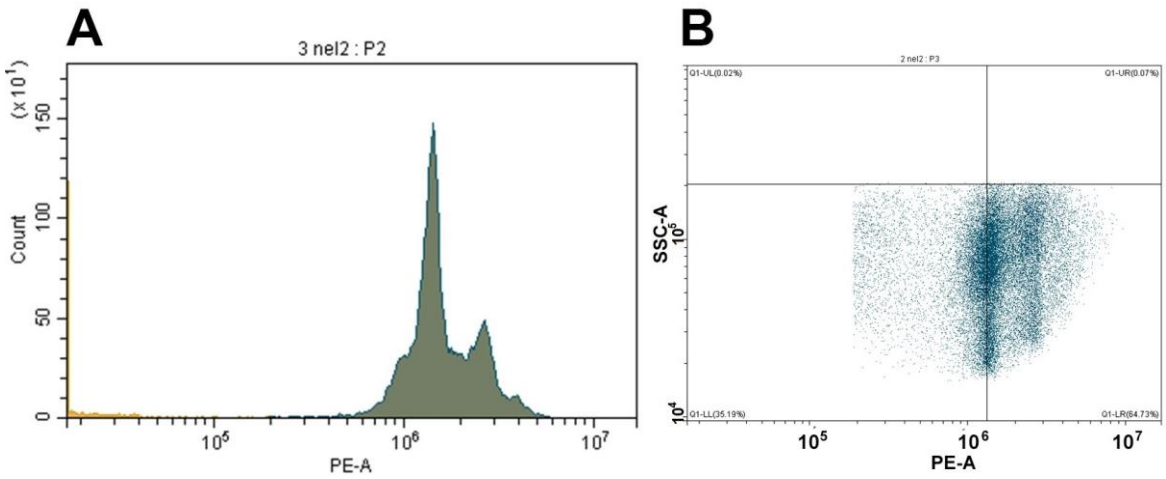

Figure 2. Results of flow cytofluorometry (PE channel). Cell cycle histogram (A) and dot plot (B) showing the heterogeneity of the cell analyzed from small cell blastula.

Thus, the protocol was tested in two independent ways: visual (microscopic) and photometric. The procedure efficiency for cell isolation from fish egg blastodiscs was proved. The protocol can be applied to most teleost fish species with middle-sized eggs at early maturating stages (blastula, gastrula); this protocol may be suitable for other representatives of teleost fish with different diameters of eggs [large (salmon) or small (zebrafish)].

General notes and troubleshooting

General notes

Before you start: to isolate cells from the blastodisc, it is enough to have developing eggs at the stage of large cell blastula. Based on specific goals, it is important to mind the number of cells needed for analysis. If it is necessary to analyze each egg individually, it is important to know the total number of required cells in the developing egg. For examining a specific number of eggs (if general statistics on the target analysis is needed), the stage of development of the eggs is not so important.

Troubleshooting

No cells after procedure:
1. Take more eggs for processing and repeat the procedure.
2. Ensure that you have taken the developing eggs. An embryo should be visible under a light microscope.
3. Remember that in the first step of the isolation protocol, after rinsing the eggs, it is sufficient to gently pop the eggs. Do not completely homogenize.

Acknowledgments

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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