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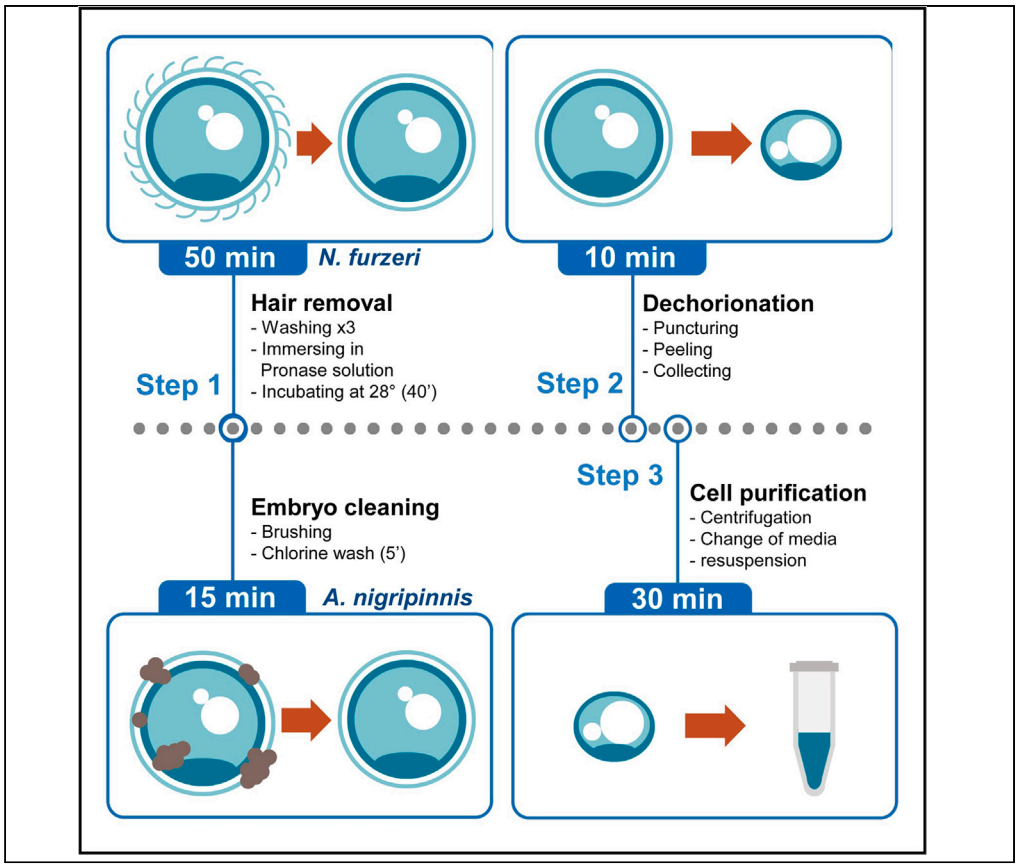
Protocol for extracting live blastoderm cells from embryos of annual killifish



The implementation of *in vitro* approaches using undifferentiated embryonic cells from annual killifish to complement existing *in vivo* developmental studies has been hindered by a lack of efficient isolation techniques. Here, we present a protocol to isolate annual killifish blastoderm cells, at the epiboly and early dispersion phase, from embryos. We describe steps for hair removal, embryo cleaning, dechorionation, and cell purification. This protocol may also be used to develop strategies to isolate cells from embryos presenting similar challenges.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps to extract live blastoderm cells from annual killifish embryo

Cleaning and decontamination of chorionic surface, cell isolation, and image acquisition

Extraction and purification of blastoderm cells for *in vitro* studies

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Protocol for extracting live blastoderm cells from embryos of annual killifish

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SUMMARY

The implementation of *in vitro* approaches using undifferentiated embryonic cells from annual killifish to complement existing *in vivo* developmental studies has been hindered by a lack of efficient isolation techniques. Here, we present a protocol to isolate annual killifish blastoderm cells, at the epiboly and early dispersion phase, from embryos. We describe steps for hair removal, embryo cleaning, dechorionation, and cell purification. This protocol may also be used to develop strategies to isolate cells from embryos presenting similar challenges.

BEFORE YOU BEGIN

Teleost fish are powerful and diverse animal models for investigating embryonic development, physiological function, and disease in an *in vivo* context.[1] The evolutionary adaptation of this group of animals to different ecological niches has resulted in contrasting developmental pathways to form the embryo. For example, in most teleost embryos studied to date, including the zebrafish (*Danio rerio*), the processes and movements that give rise to the embryo during gastrulation coexist with the movement of epiboly, by which both embryonic and extraembryonic cellular domains move from the animal pole to engulf the entire surface of the egg.[2–4] In contrast to this, annual killifish have evolved a developmental adaptation to the harsh environmental conditions of their habitats that include the temporal separation between the process of epiboly and gastrulation.[5–8] This shift results in embryonic cells first dispersing and then re-aggregating to form the embryonic axis by a mechanism not yet well understood[9,10] but which may involve self-organizing events such as those described in embryonic stem cell-based models.[11]

Research on embryonic development, mainly based on *in vivo* and *ex vivo* models (e.g., tissue explants), has recently been enriched by the emergence of *in vitro* approaches based on undifferentiated embryonic stem cells. These approaches allow to investigate cellular and developmental

mechanisms under controlled experimental conditions, as seen in well-studied mammalian species such as mouse (e.g.,[12]) and teleost models such as zebrafish (e.g.,[13–15]). In annual killifish, however, the isolation of embryonic cells has been a challenge because of the hardness of the chorion, the small perivitelline space, and the high internal pressure of the egg, which make it difficult to remove the chorion while leaving the embryo intact and alive. This work therefore aims to provide a robust method to isolate undifferentiated embryonic cells from annual killifish at the early stages of development as a first step for future implementation of *in vitro* studies. We focus on embryos of *Austrolebias nigripinnis (A. nigripinnis also known as by its common name blackfin pearlfish)* and *Nothobranchius furzeri* (*N. furzeri also known as turquoise killifish*), two annual killifish species recently used for research on embryonic development.[7,9,10] The developmental stage chosen for the isolation procedure is the dispersion phase, which involves epiboly and early-post epiboly stages. At this developmental phase that precedes gastrulation and formation of the embryonic axis, the deep cell layer of undifferentiated embryonic cells lies between the extraembryonic yolk and enveloping cell layer (EVL). The extraction protocol detailed here consists of 3 steps and it allows the isolation of both deep embryonic and EVL cells, which together form the so-called blastoderm. This protocol can also be generalized to isolate blastoderm cells from embryos of other teleosts with hard chorion. Similarly, we successfully applied this protocol without any specific modifications to extract viable cells from killifish embryos at earlier and later developmental stages than the ones presented in here (tested up to 24 h post epiboly). Possibly the same protocol could be adapted for stages after gastrulation. However, other challenges regarding separation and purification of cells derived from differentiated tissues (i.e., those derived from gastrulation) should be considered.

1. Maintain fish in daylight cycles of 12 h between 17°C–20°C for A. nigripinnis and 26°C for *N. furzeri* in systems of aeration of recirculating water.
2. Set water conductivity to 400–500 μS and the pH to a range of 7–7.5.
3. Keep male and female fish in separate tanks with one fish per tank.
4. Feed the fish 3 times a day with newly hatched brine shrimps and freshwater live food (Eisenia foetida, Lumbricus variegatus).
5. Replace 50% of the tanks water with fresh water once a week.
6. For breeding, place the male in the female's tank along with a glass box filled with peat moss for A. nigripinnis and sand for *N. furzeri* for eggs deposition, which takes between 3–4 h.
7. The fertilized eggs were d by filtration of peat or sand in strainers with a pore size of 500 μm.
8. Recover fertilized eggs and transfer them to a Petri dish.
9. Maintain embryos in culture media E3 (S1) at a temperature of 25°C ± 2°C to match the best conditions for embryo development.

Institutional permissions

Adult annual killifish of the species *Austrolebias nigripinnis (A. nigripinnis)* and *Nothobranchius furzeri* (*N. furzeri*) was bred and maintained following the bioethical guidelines determined by the Ethics Committee of Universidad de Chile. All animal procedures were approved by the Bioethics Committee of the Faculty of Medicine, University of Chile (Cicua certificate number: 20385-Med-Uch).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
SYLGARD™ 184 Silicone Elastomer Kit (PDMS)	Dow Corning	Cat#101697
Pluronic F-127	Sigma-Aldrich	Cat#P2443

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NaCl	Winkler	Cat#SO-1455
KCl	Winkler	Cat#302407
$CaCl_2/2H_2O$	Winkler	Cat#CA-0520
$NaHCO_3$	Sigma-Aldrich	Cat#S5761-1KG
$MgCl_2/6H_2O$	Winkler	Cat#MA-0960
Tris base	Sigma-Aldrich	Cat#10708976001
EGTA	Sigma-Aldrich	Cat#E8145
Pronase	Sigma-Aldrich	Cat#10165921001
DMEM/F-12 powder (L-Glutamine – 15mM HEPES – without $NaHCO_3$ and without phenol red)	Sigma-Aldrich	Cat#D2906
Pen/strep	Gibco	Cat#15140122
10% Chlorine solution	Avalco	Cat#4201
Calcein AM	Thermo Fisher	Cat#C3099
Experimental models: Organisms/strains		
Austrolebias nigripinnis	[4,7]	NCBI:txid135282
Nothobranchius furzeri	[4,16]	NCBI:txid105023
Other		
Dumont Dumoxel Style 5/45 Tweezer, Biology Tips, Antimagnetic Stainless Steel, 109 mm	Spi Supplies	Cat#0T054A-XD
Leica S6 D Stereoscope	Leica Microsystems	https://www.leica-microsystems.com/products/light-microscopes/stereo-microscopes/p/leica-s6-d/
Thermo Scientific Heraeus Pico 17 microcentrifuge	Thermo Scientific	Cat#75002401
Nikon Eclipse Ti2 inverted microscope	Nikon instruments	https://www.microscope.healthcare.nikon.com/products/inverted-microscopes/eclipse-ti2-series
Weighing scale	Belltronic XU1002B	Cat#B-01-02-03-0401
Vacuum pump	Rocker 300	Cat#167300
Vacuum desiccator	Sp Bel-Art "space saver" polycarbonate vacuum desiccator	Cat#F42025-0000
Forced air drying oven	Biobase BOV-V65F	Cat#AO-BOV-V65F
Plasma cleaner	Tergeo - Pie Scientific	–
UV light source	Ossila UV ozone cleaner	Cat#L2002A3
Stereoscope	Leica S6D	Cat#S6D-PS
Cell culture centrifuge	Thermo Scientific Heraeus Pico 17	Cat#75002414
UV Ozone Cleaner	Ossila	Cat#L2002A3-UK
Tissue-culture treated culture dishes, 35 mm	Corning	Cat#CLS430165
Biopsy punch, sterile, 8 mm, 10/pk	Kruuse	Cat#273693
Disposable surgical blades size 10	ChannelMed	Cat#CJ6011
Style #2A Flat Tipped Tweezers, Antimagnetic Stainless Steel, High Precision, 120 mm	SPI-Swiss	Cat#0S2AP-XD
1.0 mL volume, flat bottom 9 × 38 mm soda glass tube with push in cap	Teklab	Cat#GL389
Pasteur pipettes	Nest	Cat#318212
Pipet-Lite™ XLS+ manual single-channel pipette, 20–200 µL	Rainin	Cat#17014391
Flat tipped No. 10 brush	Artel	Cat#20660140
15 mL Conical centrifuge tubes	Falcon	Cat#14-959-53A
Microtubes 3810×	Eppendorf	Cat#Z606340

MATERIALS AND EQUIPMENT

E3 media (S1)		
Reagent	Final concentration	Amount
NaCl	4.913 mM	580 mg
KCl	0.177 mM	26.67 mg
$CaCl_2/2H_2O$	0.650 mM	96.67 mg
$MgCl_2/6H_2O$	0.397 mM	163 mg
Milli-Q H_2O	N/A	2 L
Adjust pH to 7.3 using NaOH and HCl.		
Solution can be stored at 4° for up to 4 months if handled using aseptic techniques and conditions.		

Pluronic acid solution (S2)

Prepared in Milli-Q H_2O at a final concentration of 0.2% W/V.

- Add 0.2 g of Pluronic acid F127 to 100 m of Milli-Q H_2O.
- Store at room temperature up to 4 months.

Modified Yamamoto solution (S3)		
Reagent	Final concentration	Amount
NaCl	128 mM	7.5 g
KCl	2.683 mM	0.2 g
$CaCl_2/2H_2O$	2.717 mM	0.2 g
$MgCl_2/6H_2O$	0.098 mM	0.02 g
$NaHCO_3$	2.381 mM	0.2 g
Milli-Q H_2O	N/A	1 L
Adjust pH to 7.3 using NaOH and HCl.		
Solution can be stored at 4° for up to 4 months if handled using aseptic techniques and conditions.		

Salt-Tris buffer (S4)		
Reagent	Final concentration	Amount
NaCl	0.128 M	7.5 g
KCl	2.683 mM	0.2 g
Tris base	50.025 mM	6.06 g
Milli-Q H_2O	N/A	1 L
Solution can be stored at 4° for up to 4 months if handled using aseptic techniques and conditions.		

Pronase solution (S5)		
Reagent	Final concentration	Amount
Pronase 10 mg/mL stock	2 mg/mL	100 µL
EGTA 0.1 M	1 µL/400 µL of Salt-tris buffer (S4)	1 µL
Salt-tris buffer (S4)	N/A	400 µL
To be freshly prepared before each procedure.		

DMEM/F-12 media (S6)

- Prepared in Milli-Q H2O following manufacturer guidelines.
- Media is complemented with 10mL of pen/strep solution (1/100 dilution).

- Adjust pH to 7.4 using NaOH.

Solution can be stored at 4° for up to 4 months if handled using aseptic techniques and conditions.

DMEM/F-12 media with EGTA (S7)
Prepared using DMEM/F-12 media (S6) with addition of EGTA 0.1 M.

- Add 19.01 g of EGTA powder to 500 mL of DMEM/F-12 media (S6).
- Adjust pH to 7.4 using NaOH or HCl.

Solution can be stored at 4° for up to 4 months if handled using aseptic techniques and conditions.

Chlorine solution (S8)
Prepared in Milli-Q H2O by dilution of the 10% chlorine solution to the final concentration of 100 ppm.

- Dilute 100 µL of 10% chlorine solution in 100 mL Milli-Q H2O.

Store at room temperature up to 4 months.

Calcein-AM loading solution (S9)
Prepare a stock solution for live cell staining at a concentration of 2 µM of Calcein-AM in DMEM/F-12 media (S6).

- Add 0.1 µL of Calcein-AM stock (2 mM) to 100 µL of DMEM/F-12 media (S6).
- Mix using the pipette for at least 5 times.

To be freshly prepared before each procedure.

STEP-BY-STEP METHOD DETAILS

The protocol we present here has been developed for isolation of blastoderm cells from annual killifish embryos and it is specifically optimized for *A. nigripinnis* and *N. furzeri*, that have a similar hard and resistant chorion.

Fabrication of bursting chambers

Timing: 6 h 20 min

The procedure presented here requires bursting of the eggs in what, hereafter, we will call "bursting chambers". These chambers are small (8 mm in diameter and approx. 5 mm in height), sterile, made of inert silicone (PDMS, polydimethylsiloxane) passivated with antifouling agent (pluronic acid). Bursting chambers are composed of two layers of PDMS (i.e., bottom layer and wells) which are fabricated using commonly available equipment (i.e., scale, vacuum chamber, oven, UV lamp, tweezers). For each device, 4 chambers are obtained (Figure 1).

Note: embryo is in a confined environment, thus facilitating its manipulation.

Note: after bursting, embryonic material is dispersed in a small volume and, thus, it can be easily collected.

Note: hydrophobic profile of PDMS and pluronic acid passivation provide an antifouling environment that prevents cell adhesion to the chamber.

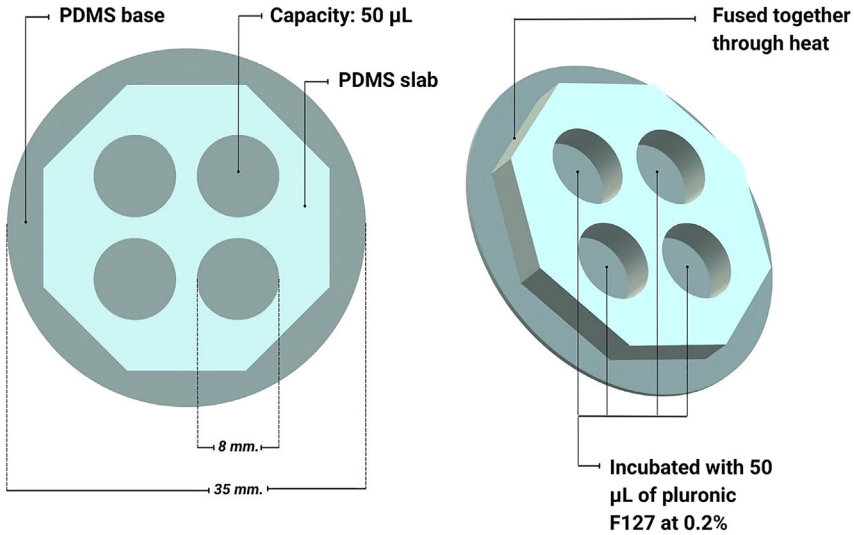

Figure 1. Specifics of the device for embryo bursting (bursting chambers)
Schematics show two different views of the device and report the details of its structure and dimensions. These chambers facilitate the collection of the material extracted from the egg during the dechorionation procedure.

1. Prepare petri dishes with a thin layer of PDMS that will serve as the bottom layer of the bursting chambers.
   a. Add 1.5 g of base and 0.1 g of crosslinker in a petri dish of 35 mm diameter.
   b. Carefully mix PDMS at a 15:1 ratio by stirring with a plastic disposable pipette (e.g., p1000 pipette tip or dropper pipette)

   ⚠ CRITICAL: mix carefully for at least 3 min. Make sure to stir all the mixture including that at the corners. The mixture will turn from transparent to milky white due to incorporation of air bubbles.

2. Prepare a thick slab of PDMS used to fabricate the wellś layer of the bursting chambers.
   a. Add 15 g of base and 1 g of crosslinker directly in a petri dish of 100 mm diameter.
   b. Carefully mix PDMS at a 15:1 ratio by stirring with a plastic disposable pipette (e.g., p1000 pipette tip or dropper pipette).

   ⚠ CRITICAL: mix carefully for at least 5 min. Make sure to stir all the mixture including that at the corners. The mixture will turn from transparent to milky white due to incorporation of air bubbles.

3. Place both PDMS containing dishes (prepared in step 1 and 2) in a vacuum chamber and apply a strong negative pressure (below 500 mmHg) for at least 5 min.

   Note: this step is needed to degas the mixture and eliminate bubbles formed during the previous mixing process.

   Note: alternatively, if vacuum chamber and negative pressure pump are not present, PDMS mixtures can be left for 30 min to allow gas bubbles to come to the surface. Thereafter, bubbles can be broken by strong mechanical action, e.g., firm knocking of the bottom of the petri against a table.

4. Transfer the PDMS dishes in the oven and cure the PDMS mixture for 1 h at 80°C on a plane surface.

Note: this step is to form a solid layer inside the petri dish as the PDMS will crosslink.

Note: alternatively, if an oven is not present, PDMS mixtures can be cured overnight at room temperature.

5. Cut the thick layer of PDMS to form the wells for the bursting chambers.
   a. Remove the thick layer of PDMS (prepared in step 2) from the petri dish using a round-tip tweezer and place it on the lid of the same petri dish.
   b. Detach the PDMS from the dish by passing the tip of the tweezer along the border and then pulling it out.
   c. Cut its edges of the slab of PDMS using a bistoury.
   d. Punch 4 through holes of 8 mm diameter using an appropriate biopsy punch to form the wells of the bursting chamber.

⚠ CRITICAL: work in clean conditions and avoid getting dust and dirt on the PDSM to ensure good bonding in steps 6. Use clean tweezers to manipulate the PDMS. Bare hands would leave a layer of skin oil and gloves may leave powder on the PDMS surface.

Note: ideally an octagon with 4 equidistant through holes should result. Make sure the size of the octagon fits the 35 mm plate.

6. Mount the PDMS slab with the wells onto the thin PDMS layer in the dish (prepared in step 1).
   a. Gently press the slab against the surface of the dish using the tweezers to ensure adhesion and squeeze out air bubbles in between the two layers.
   b. Leave the device in the oven at 80°C for 3 h to improve bonding.

Note: as both slabs are made of PDSM at a ratio of 15:1, which is below the recommended 10:1 (saturating crosslinking ratio), the slabs are relatively soft and retain some adhesive properties. Thus, they should sufficiently adhere to each other. However, we recommend using a protocol for PDMS / PDMS bonding if a plasma cleaner is available (a detailed protocol can be found in[17]).

7. Fill the chambers with 50 μL each of Pluronic acid solution (S2) and incubate for 2 h at room temperature.

Note: pluronic acid will form an antifouling coating on the PDSM which prevents protein adsorption and inhibits cell adhesion.

Note: pluronic acid may leak underneath the chambers if the PDMS layers did not adhere properly (see step 6). This may occur if PDMS surfaces have been contaminated with dust or organic lipids from e.g., the operator's hands. In which case we suggest revising step 6 and ensuring clean working conditions.

8. Wash chambers 4 times using dH2O by adding 50 μL of Milli-Q H2O and removing it using a p200 pipette.
9. Let them dry and sterilize under UV light for 15 min before use.

Note: in our experience, the UV light of the biosafety cabinet would be sufficient. Do not use 70% ethanol as it may deteriorate the pluronic coating.

Note: we do not recommend reusing the device as after each use antifouling properties may deteriorate, and risk of contamination may increase.

Species specific pretreatments

Timing: *N. furzeri:* 50 min, *A. nigripinnis:* 15 min

Annual killifish eggs from different species have different chorion structures. Thus, different types of pretreatments are necessary to obtain a clean and sterile cell extract. The procedure below is only required for the specific species.

Hair removal for *N. furzeri*

Timing: 50 min

The surface of *N. furzeri*ś eggs is characterized by a coating of hairs to improve adherence to the substrate where the eggs are laid. Here, we present a procedure to enzymatically remove those hairs from the surface of the chorion. If hairs are not removed, they appear as contaminants in final cellś extract.

⚠ CRITICAL: all solutions and ambient temperature should be at 25°C ± 2°C.

⚠ CRITICAL: removal of the supernatants should be done carefully to avoid damaging or accidentally discarding the eggs. Best is to leave a small amount of solution on the eggs.

10. Use a plastic Pasteur pipette to gently place the eggs (one-by-one) inside a glass tube for a maximum of 5 eggs per tube.

⚠ CRITICAL: glass tubes are required as, after pronase treatment eggs tend to adhere to the walls of plastic Eppendorf tubes and can break when extracting them from the tubes.

11. Add at least 200 µL of modified Yamamoto solution (S3) and let the eggs incubate for 10 min, after which the solution must be removed. This washing step should be repeated 3 times.
12. Remove modified Yamamoto solution (S3) from the tube and add 200 µL of salt-tris buffer (S4) and incubate for 5 min.
13. Remove the salt-tris buffer (S4) from the tube and add 200 µL of pronase solution (S5) and incubate for 40 min at 25°C.

⚠ CRITICAL: if 40 min are not enough for hair digestion, incubate for another 30 minutes (maximum). Frequently, inspect the eggs using a stereoscope to confirm hair removal.

14. Remove pronase solution (S5) and wash the eggs 3 times using DMEM/F-12 media (S6) with EGTA.

Note: swelling of the perivitelline space can be expected.

Note: although we have not performed a systematic analysis of viability, the embryos after enzymatic hair removal look viable, and could potentially remain viable over time in E3/embryo medium if enzymatic treatment is washed out properly.

⚠ CRITICAL: when visualized under the stereoscope, eggs should look clean with a smooth surface (see Figures 2A and 2B). If their appearance is different, eggs should be discarded as longer pronase treatment can ultimately damage the embryo.

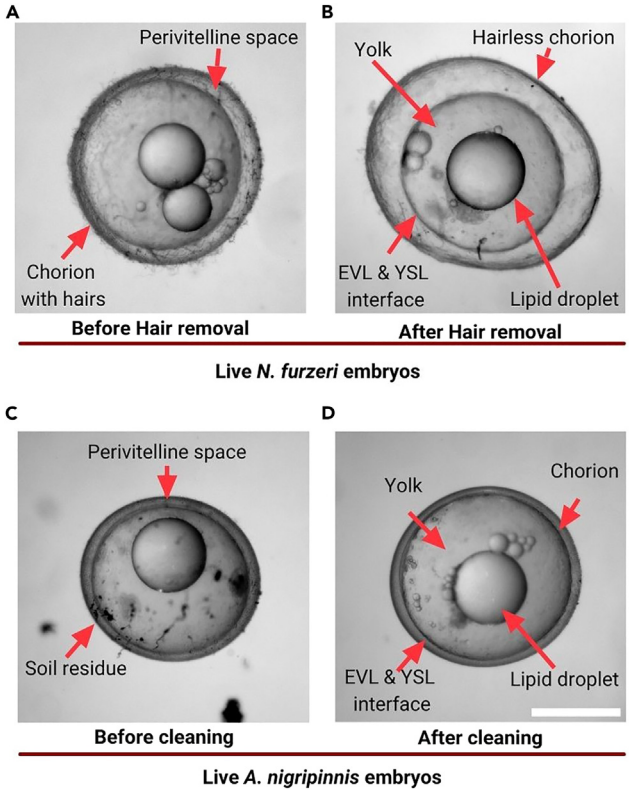

Figure 2. Result of the cleaning process of annual killifish eggs collected from the substrate

Top row illustrates the comparison of the surface of the chorion of *N. furzeri eggs* before and after the hair removal.
(A) the surface of *N. furzeri eggs* is covered with hairs (arrows).
(B) a hairless egg after pronase treatment is shown and the different components of the egg are indicated. Bottom row illustrates the aspect of A. nigripinnis *eggs* before and after the cleaning from soil residues and sterilization with chlorine solution.
(C) the chorion of the egg laid in soil is covered with visible impurities (arrows).
(D) a clean egg without the presence of sediments is shown and the different components of the egg are indicated. Images were taken using a Leica S6 D stereo microscope at a 4× magnification. Scale bar = 500 μm.

Debris cleaning for *A. nigripinnis*

Timing: 15 min

A. nigripinnis eggs are laid in mud, thus their chorion is typically covered in soil and microorganisms. The following steps are needed to ensure proper removal of soil residue and elimination of contaminants, such as bacteria.

⚠ CRITICAL: all solutions and ambient temperature should be at 25°C ± 2°C.

⚠ CRITICAL: removal of the supernatants should be done carefully to avoid damaging or accidentally discarding the eggs. Best is to leave a small amount of solution on the eggs.

15. Remove soil from the embryo surface mechanically.
    a. Use a plastic Pasteur pipette to gently place one egg on a dry clean wipe.
    b. Use a paintbrush dipped in DMEM/F-12 media (S6) to carefully move and rotate the egg. Brushing and rotation are aimed at removing as much soil residue as possible.

c. Once clean, use a plastic Pasteur pipette to collect the egg and place it in a 15 mL falcon tube with 1 mL DMEM/F-12 media (S6) and repeat for all the eggs.

⚠ CRITICAL: if the egg deflates or breaks during this process the chorion was damaged before and it should be discarded as it would not survive the next steps.

Note: to ensure proper cleaning this process should be performed for at least 2 min and at least 1 mL media should be used to brush off the soil. While the eggs can resist long dehydration times, we don't recommend exceeding 10 minutes of dry condition in order to avoid triggering undesired signaling cascades (e.g., Annual killifish may enter diapause).

16. Place around 10–15 eggs in a 15 mL falcon tube, remove the DMEM/F-12 media (S6) and add 5 mL of chlorine solution (S8) for 5 min to disinfect the eggs.

⚠ CRITICAL: higher concentrations or longer incubation times may damage the embryo and reduce cellular viability.

17. Remove the chlorine solution (S8) and wash the eggs 3 times using DMEM/F-12 media with EGTA (S7).

⚠ CRITICAL: eggs should look relatively smooth under a stereoscope (see Figures 2C and 2D). If their appearance is different, eggs should be discarded.

Removal of the chorion

Timing: 1 h and 10 min per embryo

Opening and removal of the chorion is arguably the most critical step of this protocol because of the chorion's rheological properties, small perivitelline space, and the high hydrostatic pressure it holds. Example videos (Methods video S1 and S2) and images of the critical steps (Figure 3) are provided.

Note: Mishandling of the steps below may cause the embryo to burst uncontrollably, which in turn may lead to loss of cells due to dispersion and cell death due to high shear forces.

⚠ CRITICAL: all solutions and ambient temperature should be set at 25°C ± 2°C.

18. Place one egg in the bursting chamber and add 50 μL of DMEM/F-12 media with EGTA (S7) and place the bursting chamber under a stereoscope chamber for high precision manipulation of the egg (Figure 3, Plating).

Note: bursting chamber must be filled to the brim, but not overfilled to avoid formation of a convex meniscus that may affect proper visualization of the egg under the stereoscope. On the other hand, a concave meniscus can displace the egg to the bottom corners of the bursting chamber.

19. CRITICAL STEP: Chorion puncturing (Figure 3, Puncturing and Methods video S1). Two high precision tweezers with a very fine tip need to be used simultaneously in this step.
    a. Gently but firmly hold the egg using the tweezer in the non-dominant hand.
    b. Use the tip of the other tweezer to gently puncture the chorion and release the internal pressure.

⚠ CRITICAL: a gentle touch with the tip of the tweezer is sufficient to puncture the chorion. If too much pressure is applied, the embryo will burst out causing cell dispersal and cell death.

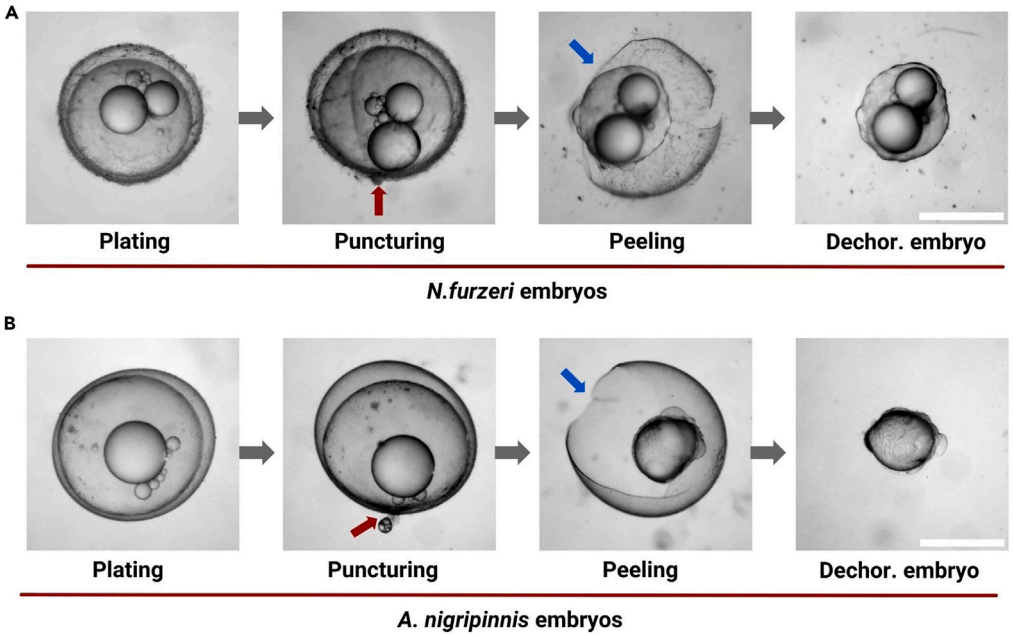

Figure 3. Critical steps in the dechorionation process of annual killifish eggs

(A and B) Images in A and B depict the aspect at different steps of one *N. furzeri* and one *A. nigripinnis* egg, respectively. For both: eggs placed inside a bursting chamber (plating) are punctured using a pair of fine-pointed tweezers (puncturing). The eggs are grabbed and gently punctured using the tip to partly release the high pressure (Methods video S1). After partial deflation of the egg and enlargement of the perivitelline space, the chorion is carefully peeled off using the tweezers from the opposite side of the puncture until detaching all the chorion from the egg (peeling – Methods video S2). The chorion is broken into pieces and discarded. The resulting dechorionated embryo is shown in the panel Dechor. Embryo. Images and suppl. Movies were taken using a Leica S6 D stereo microscope at a 4× magnification. Scale bar = 500 μm.

⚠ CRITICAL: the embryo tends to adhere to the tweezers. Care must be taken not to accidentally remove the egg from the chamber.

Note: a small amount of the eggś content will be released (red arrows in Figure 3). Eventually, flow of fluid due to high pressure will move the EVL toward the puncture and obstruct the hole.

20. CRITICAL STEP: opening and peeling of the chorion (Figure 3, peeling and Methods video S2).
    a. Gently grab the chorion with both tweezers on the opposite side of the puncture and pull the chorion apart to create a small opening (approximately $^1/_4$ of the diameter of the embryo).

Note: while grabbing the chorion, part of the embryo's contents will protrude through the opening made when puncturing and a small amount of the yolk content may be released. Be sure to not let the chorion membrane cut into the embryo, releasing the tweezers if the bending of the chorion produces extensive deformation of the embryo.

    b. Using the tweezers, gently grab opposite sides of the small opening made in step 20a and pull the whole chorion apart to release the embryo (blue arrows in Figure 3).
    c. In the process, the chorion will break into small pieces which can be removed from the chamber to leave only the dechorionated embryo in the chamber.

Note: be sure to not let the chorion membrane cut into the embryo, releasing the tweezers if the bending of the chorion produces extensive deformation of the embryo.

21. CRITICAL STEP: collection of the dechorionated embryo). Remove the content of the bursting chamber using a p200 micropipette and place it in a sterile Eppendorf 1.5 mL tube.

⚠ CRITICAL: approximately 5 mm of the tip of the p200 micropipette must be cut to allow the passage of large objects and to reduce the sheer force during pipetting.

Note: in our experience the dechorionated embryo does not adhere to the pipette tip.

22. Wash twice the well with additional 50 µL DMEM/F-12 media with EGTA (S7) to collect all the embryonic material in the chamber. Place all the media collected in the same Eppendorf tube.
23. Repeat the protocol for all the embryos and pool the extracts together to a maximum of five dechorionated embryos per Eppendorf tube.

⚠ CRITICAL: in our experience, 5 embryos per tube represents the optimal number that allows the formation of a visible pellet after centrifugation. Collecting more embryos per tube results in accumulation of an excessive amount of material from the yolk that is consequently spun down with the cells.

Blastoderm cells isolation

Timing: 30 min

This step allows the purification of cells of the blastoderm (deep embryonic and EVL cells) from the lipids of the yolk.

⚠ CRITICAL: all solutions and ambient temperature should be at 25 ± 2°C.

24. Gently mix the DMEM/F-12 media with EGTA (S7) containing the dechorionated embryos using a p200 pipette set to 50 µL to separate the lipid droplets in the yolk from the rest of the embryo.

⚠ CRITICAL: it is mandatory to use DMEM/F-12 media with EGTA (S7) in this step to dissociate Ca^{2+}-dependent cell-cell adhesions and separate aggregates into single cells.

⚠ CRITICAL: a sufficient shear stress should be applied to separate the lipid droplets from the yolk. However, excessive flow may cause cell damage. In our experience, slow pipetting for approximately 10 times using an intact p200 pipette tip should yield the desired outcome, which is achieved when droplets of lipids floating on top media can be seen.

25. Use a cell culture centrifuge to pellet the blastoderm cells. Place the Eppendorf tubes in the centrifuge and spin at 200 × *g* for 5 min.

Note: in case of problems with cell viability, centrifugation can be done using lower centrifugal forces for longer time, e.g., 100 × *g* for 15 min.

26. Discard the supernatant using a p200 pipette by making sure not to disturb the pellet at the bottom of the tube.

⚠ CRITICAL: removal of the supernatants should be done carefully to avoid removal of the isolated cells.

Note: a small pellet should be visible. For this reason, it is important to centrifuge together the extract of at least 3 embryos.

Note: ideally, the supernatant should be removed from the air-liquid interface to remove the lipids and other light components of the yolk.

27. Add 300 μL of DMEM/F-12 media (S6) and gently resuspend the solution using a p200 pipette. Repeat centrifugation and resuspension DMEM/F-12 media (S6) to remove undesired embryonic residues and reintroduce physiological levels of Ca^{2+}.

⚠ CRITICAL: as more impurities and materials are removed during the washes, the pellet may become less apparent. Hence, even more care should be taken when removing the supernatant.

28. After the last centrifugation, resuspend the pellet in 100 μL of DMEM/F-12 media (S6) using a P200 pipette. The cells can be used for the experiment.

Note: while cells are viable for at least 6 h, experiments should be conducted as soon as this step is finished as, in general, it can be expected that embryonic cells may genetically and phenotypically drift when outside their physiological environment.

Assessment of cell viability and cell isolation yield

Timing: 30 min

Count of viable cells can be measured by Calcein-AM assay. This assay serves the double scope of simultaneously assessing the ability of cells to de-esterify and retain the dye. As result, only viable cells with functional metabolic activity (de-esterase activity) and intact cell membrane are visible in green fluorescence and can be counted using standard cell counting methods (Neubauer chamber).

⚠ CRITICAL: all solutions and ambient temperature should be at 25°C ± 2°C.

⚠ CRITICAL: Higher concentrations of Calcein-AM dye have been tested up to 10 μM in DMEM/F-12 media (S6) showing staining without affecting cell viability, but fluorescence is observable already at 2 μM

29. Centrifuge the cell suspension obtained in step 28 of previous session at 200 × *g* for 5 min and discard the supernatant using a p200 pipette.

⚠ CRITICAL: removal of the supernatants should be done carefully to avoid removal of the isolated cells.

30. Resuspend the cell pellet in 10 μL DMEM/F-12 media containing Calcein AM (S9). Incubate for 20 min at 25°C.

Note: longer incubation times may result in accumulation of dye inside cell organelles. In case staining is not clearly visible, it is best to increase the concentration to a maximum of 5 μM rather than increase the incubation time.

31. After 20 min, pipette 10 μL of cell solution into a Neubauer chamber with a glass cover on top.

Note: the solution will enter by capillarity between the chamber and the cover glass, thus distributing the cells within grid of the counting chamber (Figure 4A, left).

Note: if the cover glass was properly placed the entire counting surface will be filled.

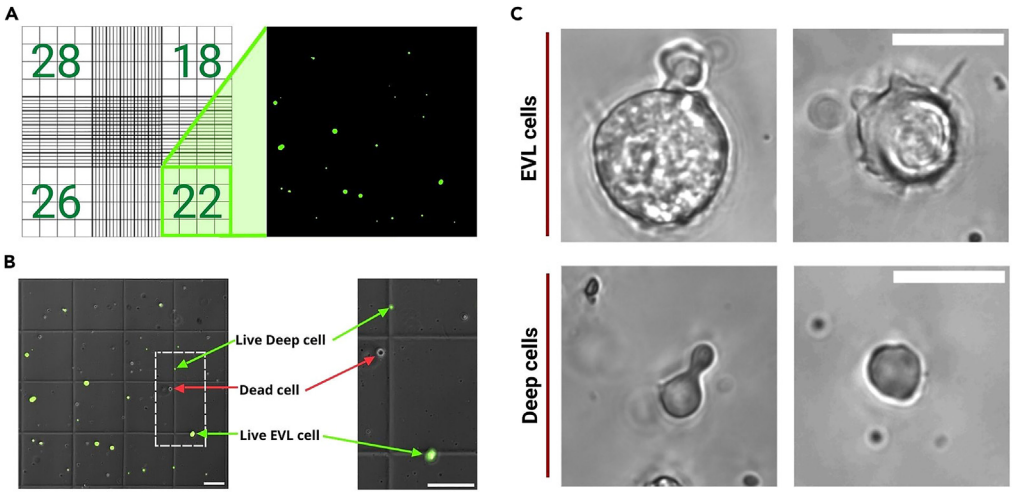

Figure 4. Yield of *A. nigripinnis* blastoderm cells (deep embryonic and EVL cells) extracted at early post epiboly stages

(A) Quantification of cell viability after extraction using the Neubauer chamber. Live cells appear green as they retain Calcein-AM fluorophore. Large cells are EVL cells and small are deep cells.

(B) Exemplary images of a quadrant of the Neubauer chamber (left) and enlarged view (right) illustrate how to recognize EVL and deep cells and how to distinguish between live cells (those that retain Calcein-AM – green arrows) and dead cells (without fluorescence – red arrow). Images were acquired using a Nikon Ti2 eclipse inverted microscope with a 10× air objective with 0.30 NA. Scale bar: 100 μm.

(C) Comparison of cell size allows distinguishing EVL (diameter above 10 μm) from deep cells (about 5 μm in diameter). Live cells also typically display cellular activities such as presence of cellular extrusion. Images were acquired using a Nikon Ti2 eclipse inverted microscope with a 40× air objective with 0.95 NA. Scale bar: 20 μm.

32. Using a fluorescence microscope, count the number of cells with green fluorescence in each quadrant (typically 4 quadrants are used for counting) and calculate the average cell number per quadrant (Figure 4A).

Note: by counting the number of non-fluorescence cells the ratio between viable and non-viable cells can be obtained (Figure 4B). This may be needed to understand whether the operator needs to be more careful when handling of embryo and/or cells (e.g. cells may be damaged by excessive mechanical manipulation or when exposed to the wrong temperatures).

33. The total number of cells per embryo is calculated by the following formulas:
    a. Average number of counted cells / Volume of each quadrant (0.1 μL) = Number cells / μL.
    b. Number of cell / μL * total volume (10 μL) = total number of cells.
    c. Total number of cells / number of embryos (5) = number of cells per embryo.

Note: The number in brackets are those to use if following this procedure where we used all the material isolated to accurately count the cells. For routine check of cell viability and yield we suggest using a fraction of the cell suspension obtained in step 28 of previous session. Obviously, the calculations should take this into account.

EXPECTED OUTCOMES

The most important outcome of the protocol is to obtain viable cells. From this protocol, we selectively isolate 2 types of cells: cells from the enveloping layer (EVL cells) and deep embryonic cells (deep cells). These cell types can be easily distinguished due to their size difference with EVL cell having a diameter above 10 μm and deep cells averaging 5 μm in diameter (typical appearance

Table 1. Summary of the cell extraction from *N. furzeri* and *A. nigripinnis* embryos

	Annual killifish embryo	
	A. nigripinnis	*N. furzeri*
Blastoderm cell per embryo (range depending on the developmental stage)	500–1000	500–1000
Live cells obtained from extraction	405 ± 46	350 ± 14
Yield percentage (range considering the different developmental stages)	81[a] ± 9.2 % - 40.5[b] ± 4.6 %	70[a] ± 2.8 % - 35[b] ± 1.4 %

Outcome of the protocol is reported as the number of viable cells extracted per embryo (counted as described above – Figures 3A and 3B) and as an estimate of the yield per embryo (expressed as a percentage).

[a]Considering 500 cells per embryo.

[b]Considering 1000 cells per embryo.

of viable cells is shown by phase contract microscopy in Figure 4C). Cells are characterized by well contrasted margins (denoting high cellular turgor) and by protrusive activities in the form of highly dynamic filopodia and blebs. Typically, the yield we obtain in our preparation is between 81 and 40.5% for *A. nigripinnis* and 70 to 35% for *N. furzeri* (Table 1). This is calculated as follow: Considering that embryonic cells are in a relatively fast replication phase during epiboly to early dispersion, the number of blastoderm cells ranges between 500 to 1000 cells per embryo. To evaluate the yield of our protocol we counted viable cells (see assessment of cell viability and cell isolation yield). Using our protocol, we obtain on average 350 cells from *N. furzeri* and 405 cells from *A. nigripinnis* from each embryo.

LIMITATIONS

The quality of the eggs is a critical factor in the success of the protocol, and it is highly dependent on the health and nutrition of the parent fish. This may vary from crossbreed to crossbreed. Thus, great care of the maintenance of the aquarium should be taken. Egg quality is not easy to perceive by simple inspection, although some signs during the procedure may indicate that eggs may not be viable or in bad condition. If the egg looks brown, rugged, or detached from the chorion under the stereoscope (see Figure 5), it is likely that the egg is in poor quality, and it is recommended to discard it. Another problem may arise during steps involving mechanical manipulation (e.g., cleaning of the surface) or changes in pH (pronase treatment) when the eggs may lose turgor. In this case, it is likely that the chorion is weak, and the embryo is not in suitable conditions for cell extraction. Whereas, if the egg withstands the cleaning process without deflating and has a translucent appearance, it can be used for extraction. Currently, this protocol allows for the purification of both types of blastoderm cells (deep and EVL cells), which we qualitatively distinguish based on their size. Typically, deep embryonic cells are less than half of the size of EVL cells. Other steps shall be developed in order to separate the two cell populations (e.g., IgG-coated magnetic beads, microfluidic sorting).

TROUBLESHOOTING

Problem 1

Fabrication of bursting chambers: step 7 – When incubating the bursting chambers with pluronic acid, solution may leak underneath the top layer. This signifies improper attachment of the top layer with the bottom one.

Potential solution

If pluronic acid gets in between the two slabs of PDMS, it will not be possible to use that device and needs to be discarded. Modifications to the protocol for fabrication include:

1. Make sure to eliminate all bubbles that may remain on the interface by pressing down on the slab using a pair of tweezers with curved tips.

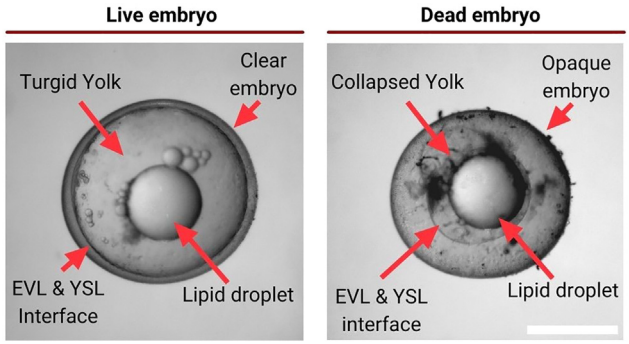

Figure 5. Comparison of structures of the living and of the dead annual killifish egg

Left panel – a live egg is shown. Arrow pointing at characteristic structures that should be clearly visible in healthy embryos. Right panel – a dead egg is shown. Arrow pointing at markers for corrupted and degraded structures. Main differences to be noticed are the clarity and turgor of the yolk and organization of the different structures of the egg. Images were taken using a Leica S6 D stereo microscope at a 4× magnification. Scale bar = 500 μm.

2. Be sure to work with PDMS in a dust free environment and avoid touching the PDMS with bare hands. Dust and lipids from the skin may prevent bonding. Cleaning of the surfaces can be done using a Magic Transparent Tape (3M).
3. Use a PDMS-PDMS bonding protocol using a plasma cleaner.

Problem 2

Hair removal for *N. furzeri*: step 14 – After pronase treatment some hairs may still be on the surface of the chorion. This leads to a final extract that may contain an unacceptable amount of debris that can interfere with the experiment.

Potential solution

A pair of fine tip tweezers can be used to gently scrape the surface over the chorion in order to remove the hairs that might be leftover. Great care should be taken not to mechanically damage the egg.

> *Note:* use a clean 35 mm petri dish to do this mechanical hair removal, then wash the egg three times with DMEM/F-12 media with EGTA before introducing it into the bursting chamber, to washing off the removed hairs.

Problem 3

Debris cleaning for *A. nigripinnis*: step 15 – During debris cleaning for *A. nigripinnis*, mechanical pressure of the paintbrush against the egg might partially or completely damage the chorion, which results in non-viable cells.

Potential solution

Damaged embryos should be discarded and a paintbrush with softer hairs should be used for this procedure. In our case, we use a paintbrush size 12 with flat tip and synthetic hog bristles. This paintbrush is on the stiff side but when operated with care and moved tangentially to the surface of the egg can deliver the right outcome.

> *Note:* make sure not to move the bristles in a way that may puncture the chorion unintentionally.

Problem 4

Removal of the chorion: step 18 – Quality and viability of the egg is a prime criterion to extract healthy blastoderm cells. Thus, visual inspection of the egg should be performed carefully to

distinguish good eggs (clear and turgid) from the bed ones (opaque, deflated and collapsed yolk, no visible perivitelline space and irregular internal structures).

Potential solution

Mechanical and chemical damage to the eggs can come from the species-specific pretreatment. Care should be taken in all steps where mechanical manipulation is required including transport of eggs from the animal facility to the laboratory. Reducing times and concentration of chemical treatments (pronase and chlorine) can also provide a solution to the problem.

Problem 5

Removal of the chorion: Critical step 19 – During Removal of the chorion, the wrong puncturing technique may cause the embryo to burst, causing a loss of structure integrity and material to be expelled from the egg under high pressure conditions, which may damage the cells.

Potential solution

There is no solution to this problem. Proper technique should be learned. However, in our experience, it is still possible to continue with the procedure if the damage is limited and the large majority of the egg content is still present. In this case, proceed with ¨Removal of the chorion: Critical step 20¨ paying attention not to further damage the embryo with the tip of the tweezers.

> *Note:* any type of mechanical damage should be avoided by following the recommended procedure.

Problem 6

Blastoderm cells isolation: Step 26 – When extracting the supernatant, it can be difficult to identify the pellet and it can be easily removed if not careful, losing the extracted cells in the process.

Potential solution

Several possible solutions in addition to the one indicates in the protocol (i.e., do not remove all the supernatant from the Eppendorf tube) should be considered:

1. Increase the number of embryos pulled together in order to increase the amount of material and make the pellet more visible.
2. Increase the time of centrifugation up to 15 min (important do not increase the speed as higher forces can damage the cells).
3. In case the tube has been accidentally shaken after centrifugation, gently resuspend the cells and proceed with a new centrifugation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Cristina Bertocchi (cbertocchi@bio.puc.cl) and Andrea Ravasio (and.ravasio@gmail.com).

Materials availability

No newly generated materials are associated with this protocol.

Data and code availability

This protocol does not include datasets and analysis.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102344.

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AUTHOR CONTRIBUTIONS

S.V., N.S., C.G.L., and M.M. performed the laboratory work. A.R., C.B., M.C., and M.L.C. provided reagents and analytical tools. S.V., C.B., and A.R. designed the study. S.V., M.L.C., C.B., and A.R. wrote the manuscript. All authors discussed the protocol and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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