

Embryo Dissociation for Fluorescence-Activated Cell Sorting

This procedure describes the dissociation of zebrafish embryos into individual cells and is based on the Lawson Lab's protocol (<http://lawsonlab.umassmed.edu/reagents.html>).

Reagents

Ringer's solution (116 mM NaCl, 2.6 mM KCl, 5 mM HEPES, pH 7.0, 50 mL, sterile)

(Isotonic solution to minimize membrane rupture)

5.8 mL 1M NaCl

145 μ L 1M KCl

500 μ L 0.5M HEPES, pH 7.0

43.5 mL sterile water

Protease solution (0.25% trypsin, 1 mM EDTA, pH 8.0, PBS, 50 mL, sterile)

5 mL 2.5% trypsin (no phenol red)

100 μ L 0.5 M EDTA, pH 8.0

5 mL 10X PBS

40 mL sterile water

Stop solution (6X, 30% calf serum, 6 mM CaCl₂, PBS, 10 mL, sterile)

3 mL calf serum

30 μ L of 2 M CaCl₂

1 mL 10X PBS

5.9 mL sterile water

Filter through syringe filter, aliquot 200 μ L/tube, and keep frozen

Suspension media (1% calf serum, 0.8 mM CaCl₂, 50 U/mL penicillin, 0.05 mg/mL streptomycin, DMEM, 50 mL, sterile)

20 μ L of 2M CaCl₂

500 μ L 100X penicillin/streptomycin

500 μ L calf serum

48.6 mL DMEM (no phenol red, Gibco 21063)

Hank's Balanced Salt Solution (HBSS)

Collagenase P powder

15 x 60 cm dishes, agar coated

Pronase in E3 medium

40- μ m Falcon cell strainers

24-well plate

FACS tubes

1.5 mL microcentrifuge tube adapter for FACS tubes

Siliconized 1.5 mL microcentrifuge tubes

TRIzol

Procedure

- 1) Dechorionate all embryos with pronase in E3 medium. Gastrulation-stage embryos should be dechorinated in agar-coated 15 x 60 cm dishes.
- 2) Wash embryos with E3 medium
- 3) Dilute collagenase P to 100 mg/mL in HBSS. (Note: gastrulation-stage embryos do not require collagenase P for dissociation. Embryos require collagenase P for complete dissociation after mid-somitogenesis. Requirement for collagenase P during early somitogenesis has not yet been determined.)
- 4) Fill a 24-well plate with 1.2 mL of protease solution per well (one well per group of 30-40 embryos) and warm the plate in a 28 °C incubator for approximately 10 min.
- 5) With a flamed pipette, transfer embryos to a 1.5-mL microcentrifuge tube. Remove as much E3 as possible. Add 100 µL of calcium-free Ringer's solution. Gently pipette up and down with P200 to de-yolk (approximately 15 times). Let sit in Ringer's solution for approximately 5 min.
- 6) Transfer embryos to pre-warmed protease solution in the 24-well plate.
- 7) Add 27 µL of collagenase P in HBSS (see note in step 2)
- 8) Incubate at 28 °C for 15 min, homogenizing every 5 min with a P1000.
- 9) Add 200 µL of 6X stop solution to each well. Mix with a pipette. Should have a total of 1.5 mL of solution per well.
- 10) Transfer to a labeled 1.5 mL microcentrifuge tube and spin down for 5 min at 350 x g in the cold.
- 11) Remove supernatant with suction, leaving approximately 100 µL of solution.
- 12) Add 1 mL of chilled suspension solution, vortex, and spin down for 5 min at 350 x g in the cold.
- 13) Remove supernatant with suction, leaving approximately 100 µL of solution. Add 700 µL of chilled suspension solution, vortex, and pass 200 µL aliquots through a 40-µm cell strainer into a FACS tube. Keep cells on ice.
- 14) To get cell counts mix 20 µL of cells with 20 µL trypan blue and count on the hemocytometer.

Sort cells into 1 mL of TRIzol in siliconized 1.5 mL-tubes. Keep cells on ice during sorting. Freeze trizol samples at -80 °C or proceed with RNA isolation protocol.

If re-analysis is desired, sort into suspension media.

Sorting tips

- 1) Zebrafish cells settle readily to the bottom of the FACS tube. Use the sample mixer in the FACS machine and periodically stop the sort, remove the tube and vortex, and resume the sort.
- 2) Sort through a 100-µm nozzle. This is especially important for re-analysis, as the 70-µm nozzle shears the dissociated cells.

- 3) Reduce signal threshold to 2000. This will allow the sorter to pick up more embryo debris and keep it out of the sorting gate.
- 4) Suggested gating conditions:
 - a) FSCA vs FSCH can separate yolk particles and cell debris from live cells. This gate will also remove doublets.
 - b) FSCA vs SSCA can serve as a crude viability gate and further cut down on damaged cells and junk. Alternatively use a viability gate such as propidium iodide.
 - c) Fluorescence gates (as required for experiment).