Protocol for preparing day 1 stage embryos for FACS (ver. 1; CLS)

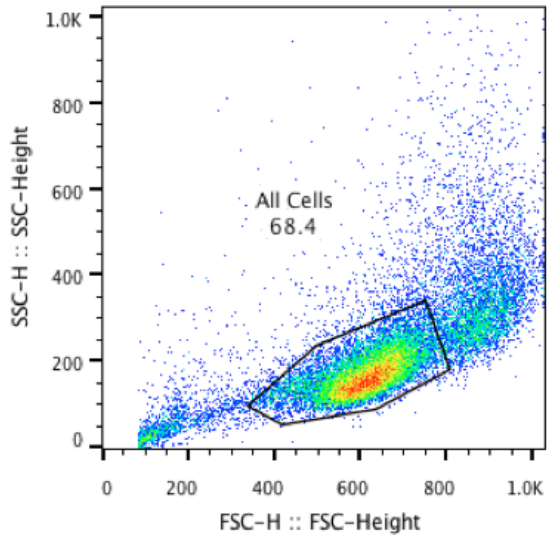
1. **Dechorionate embryos**
    a. Count embryos and transfer to a 15ml conical in E3.
    b. Adjust volume to 2ml for every 1000 embryos (2ml minimum; See Note 1)
    c. Add 17ul/ml of 30mg/ml Pronase (See note 2).
    d. Gently swirl or use transfer pipette to gently mix until chorions begin to come off.
    e. Wash 3x with E3, using a transfer pipette to gently mix, until chorions are removed.
2. **Deyolk embryos**
    a. Wash with 1ml of Ginzburg. Remove.
    b. Add 1ml of Ginzburg, and transfer embryos at 1000/ml to 1.5ml microfuge tube. **Do not pipette up and down in the 15ml conical**.
    c. Once in the 1.5ml tube, slowly pipette ~10x with a P1000 set to 900ul with minimal force (See note 3).
    d. Let deyolked embryos settle to bottom of tube. Remove the majority of the supernatant, taking care not to remove the deyolked embryos.
    e. Wash the embryos 3x with 1ml PBS. Let deyolked embryos settle to bottom of tube between washes.
    f. Centrifuge at 500g for 5min.
    g. Remove nearly all of the PBS. Freeze at -80 or immediately proceed to adding buffer A.
3. **Make nuclei and stain**
    a. If the deyolked embryos are frozen at -80, immediately transfer to ice and let sit for ~ 3 minutes.
    b. Resuspend embryos in 300ul buffer A by pipetting up and down with p1000 10X.
    c. Incubate the embryos in buffer A on ice for 5 minutes.
    d. Centrifuge in benchtop microfuge for 5 minutes at 3700 rpm.
    e. Remove buffer A supernatant.
    f. Resuspend nuclei in 300ul staining buffer by pipetting up and down with p200 10X.
4. **Visualize the nuclei under the microscope (Optional)**
    a. Pipette 10-20ul of cells onto a chamber slide.
5. **Stain and filter (300ul/1000 embryo final concentration)**
    a. Resuspend cells in 200ul/1000 embryos of cold staining solution (PBS containing 1%BSA, 50ug/ml PI, and 100ug/ml RNase A) by gently pipetting 5-10x with a P1000.
    b. Keep on ice until ready to perform FACS
    c. Immediately before performing FACS, pipette cells 5-10x with P1000 and filter through a 40um filter mesh into a FACS tube
    d. Wash filter with 200ul/1000 embryos

Note 1. You may find the rinsing steps easier if you do the pronase inclubation in a relatively large tube. For hundreds of embryos, a 15 ml conical tube may work best. For thousands of embryos, a 50 ml tube may be better.

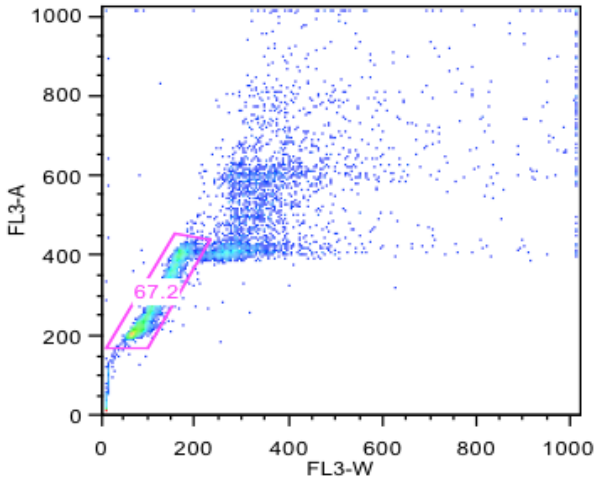
Note 2. The strength of the Pronase differs between batches. Therefore the concentration may need to be adjusted.

Note 3. Monitor the embryos between rounds of pipetting. The embryos with yolk attached should be visible by eye. Repeat pipetting until all embryos are deyolked. A total of 10x pipetting should be sufficient.

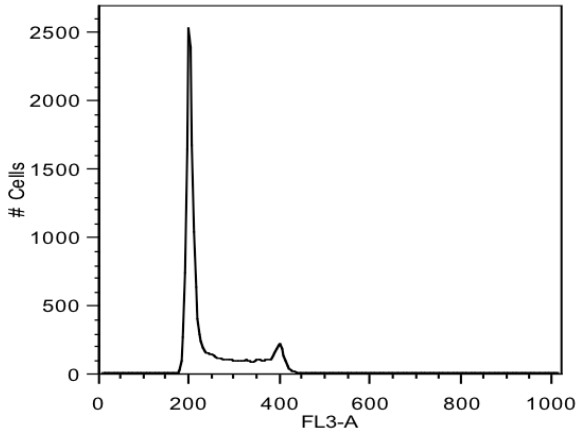
6. **Fixation**
    a. Determine volume of 100% EtOH needed to fix cells at a final concentration of 70% EtOH (7ml EtOH / 3ml cells in PBS)
    b. Slowly layer cold ethanol (-20C) on top of the ice-cold cell suspension
    c. Swirl tube to mix, vortex briefly on low setting, gently invert tube to mix.
    d. Place at -20C overnight.
7. **Wash cells**
    a. Centrifuge cells at 1400g for 5 minutes.
    b. Gently resuspend cells in 1000embryos/ml PBS/BSA by pipetting 5-10x with a P1000 (if cells are difficult to resuspend then allow to incubate first). **Do not force resuspension**.
    c. Centrifuge cells at 1400g for 10 minutes. Ensure the majority of cells have pelleted. If not centrifuge longer.
    d. If cells were EDU pulsed, go to step 8, if not then go to step 9.
8. **Click-it reaction**
    a. Gently resuspend cells in 175ul of PBS/BSA for every 1000 embryos by pipetting 5-10x with a P1000.
    b. Make a master mix containing the following per sample:
        i. 4ul of room-temp, light-protected, 100mM CuSO4
        ii. 0.2ul of Alexa Fluor 647 (A647).
        iii. 20.8ul of 0.5M ascorbic acid
    c. Add 25ul of master mix to each sample, mix by briefly vortexing on low speed and allow reaction to proceed at room temperature for 1 hour. (Additional reactions may be successful at increasing signal if necessary).
    d. Add 1ml/1000embryos of PBS/BSA and pipette 10x with a P1000 to wash cells, then centrifuge at 1400g for 10 minutes.
9. **Stain and filter (300ul/1000 embryo final concentration)**
    a. Resuspend cells in 200ul/1000 embryos of cold staining solution (PBS containing 1%BSA, 50ug/ml PI, and 100ug/ml RNase A) by gently pipetting 5-10x with a P1000.
    b. Keep on ice until ready to perform FACS
    c. Immediately before performing FACS, pipette cells 5-10x with P1000 and filter through a 40um filter mesh into a FACS tube
    d. Wash filter with 200ul/1000 embryos
10. **FACS analysis**
    a. Equilibrate instrument with staining solution prior to and between each sample analysis
    b. Run samples at a low speed with optimal total counts of 200-300 cells/sec.
    c. For PI alone, FSC x SSC should identify a population of cells distinguishable from debris:



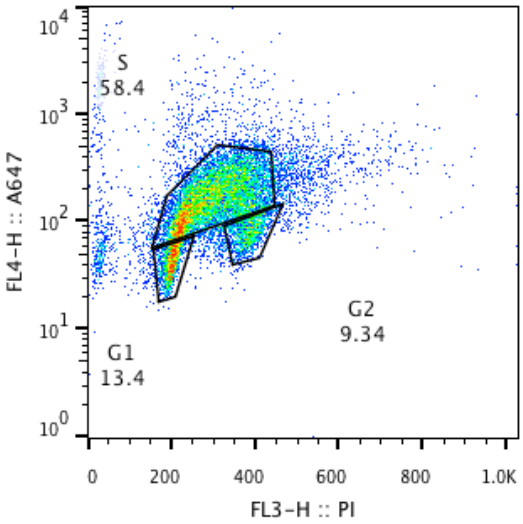
d. This can be cleaned up using FL3-A x FL3-W:



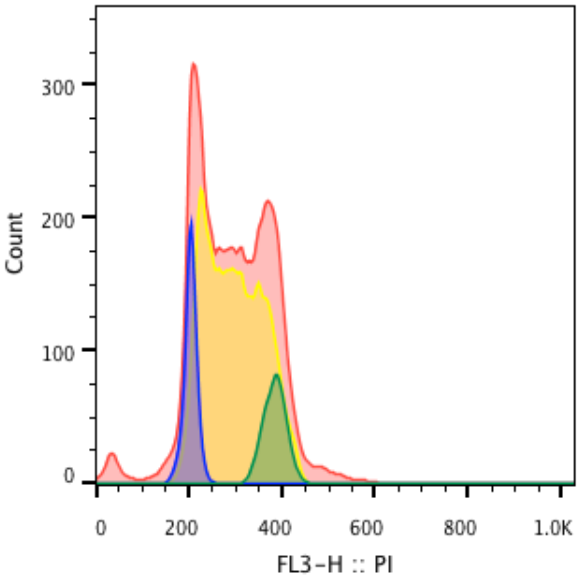
e. An ideal PI histogram (24hr embryos) of FL3A might look something like this:



f. If an EDU pulse was performed, after FSC x SSC, EDU positive cells can be identified by FL4-H:A647 x FL3-H: PI



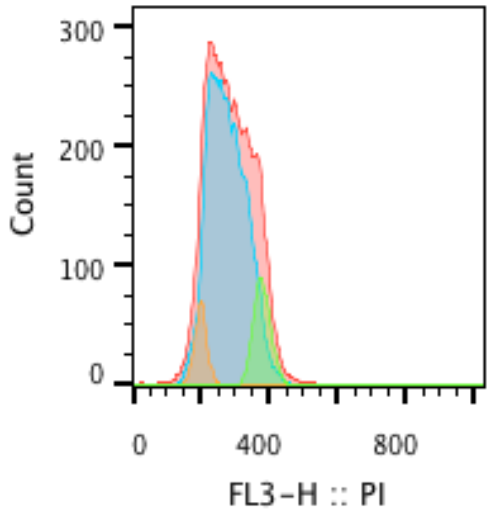
g. In the early embryos, this might yield a cell cycle profile something like this:



	Sample Name	Subset Name	Count
	500uM 15min.001	G2	1306
	500uM 15min.001	G1	1553
	500uM 15min.001	S	8504
	500uM 15min.001	FSC SSC	12964

h. Or this

	Sample Name	Subset Name	Count
	500uM.022	G2-M	1418
	500uM.022	G1	768
	500uM.022	S	9822
	500uM.022	All Cells	13200



Courtney/Tyler Protocol for EDU FACS on Early Embryos (optimized with 5hpf embryos) 4-8-2020

1. **EMBRYOS:** Dome-Shield-Bud. Start with 125-150 embryos per sample

2. **Dechorionate**. Add 25 ul of pronase (20mg/ml stock in -20) in 1ml E3. Place on rocker for 2 min. Using a cut transfer pipet, gently pipet up and down until chorions begin to come off. Once a few chorions are coming off, remove pronase solution and start washing in E3. Repeat 4X or more until chorions are removed.

3. **Deyolk**: Remove E3 from embryos and add 1ml of 1/2X Ginzburg Fish Ringer Soluion (stored in 4C). Shake at 11 rpm (room temp) for 5 min. Spin cells at 500g for 5 min. Remove supernatant.

4. **Isolate Nuclei**: Resuspend cell pellet in 300ul Buffer A (with 0.1% triton). 5 min on ice. Spin nuclei at 1300g in microfuge.

5. **ADD EDU:** Add EDU at 100mM concentration (5ul/ml) for 5-15 min at room temp.

6. **WASH** in 1ml PBS.

7. **CLICK-it RXN**.: Add 200ul of Click-it reaction mix (made fresh)

8. **WASH** in 1ml PBS

9. Resuspend in 300-400ul of **Staining buffer:** PBS+ 0.1%Triton + SYBR Green (1:10000) and RNASE (100 ug/ml)

100mM EDU labeled embryos at 5hpf. From top to bottom. 5 min pulse, 10 min pulse, 15 min pulse and 15 min with no A647