Original protocol used in Covassin et al. (2006) Dev. Biol. 299:551-562

Cell disruption for flow sorting
modified from Zebrafish Book protocol

You will need:
Fetal Calf Serum
1M CaCl2

Calcium free Ringer' solution
116 mM NaCl
2.9 mM KCl
5 mM HEPES, pH 7.2.

Suspension medium
Colorless (no phenol red) Leibovitz medium L-15, contains 0.3 mM glutamine (from GIBCO)
0.8 mM CaCl2
Pen 50 U/mL / Strep 0.05mg/mL (our stock is 10X)
1 % FCS

Culture medium
L15
0.3 mM glutamine
0.8 mM CaCl2
Pen/Strep

Embryo Extract
- Chill 200 3 day embryos after removing from chorions.
- Rinse in 0.5% chilled bleach for 2 min and then in zero calcium Ringer for 2 min.
- Transfer to a Dounce homogenizer with a minimum of liquid and homogenize well.
- Resuspend in 1 ml Culture Medium
- Store at -20°C

Protease solution
0.25% trypsin (trypsin we have is 10X, 2.5%)
1mM EDTA
in PBS, pH=8

Collection medium
Culture medium
10% FCS
10% Embryo Extract

Trizol (Invitrogen)

Do not forget the GFP- control (GFP- fish)!!

Protocol
Grow embryos in egg water.

Dechorionate with pronase.

When embryos reach desired developmental stage, transfer them in 1.5mL tube and rinse them for 15min in calcium free Ringer.

During this time, get rid of yolk:
Cut very end of 200uL tip and make 150uL aliquots in 1.5mL tubes; for each do 3-5 gentle up and downs (make sure all the yolks are gone). Pool embryos, rinse well with Ringer.

Transfer into small culture dish with 5-10mL of Protease solution. Incubate at 28C. Every 10min homogenize well doing up and downs with 200uL tip. Monitor dissociation with microscope; it takes 30-50min.

Add 1-2mM Cacl2 and 5-10%FCS to stop reaction
Centrifuge 3min at 3000rpm.
Discard supernatant and rinse cells in 10mL suspension solution. Centrifuge again.

Discard sup and resuspend cells in suspension medium $10*10^6$ cells/mL. At 24hpf, we approximate 25,000 cells/embryo.
For the control, if less than $10*10^6$ cells, resuspend in 1mL.

Bring cells to flow sorting facility (5^{th} floor Medical School Building, out of the elevator right and right), keeping them warm.
Bring along, some suspension buffer, in case she needs to dilute cells, and collection buffer (5mL/sample).

The experiment is done sterile <u>at room temperature</u>. She collects all GFP+ and in some cases she has to collect GFP- as well (at least as much as GFP+).

When you get the samples back, centrifuge, discard sup, resuspend cells in 250uL of Trizol and transfer in 1.5mL tube. Store at –80C.

Proceed to total RNA isolation.