Whole Mount Fluorescent Antibody Staining of *Drosophila* Embryos
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DAY ONE

1. Dechorionate, devitellinize, and fix embryos according to the in situ hybridization protocol. If embryos have been previously fixed and dehydrated and are in 70% ethanol, rehydrate by incubating in 50% ethanol for 5 minutes, then 30% ethanol for 5 minutes. Alternatively, if all fixed embryos are to be used for immunofluorescence, you can skip the dehydration step and continue directly to Step 2 below:

2. Wash embryos with 1XPBS + 0.1% Triton X-100 (PBS-Triton), 3 times 10 minutes at room temperature. Fill tube entirely with wash solution and place on nutator to wash. Remove final wash solution, leaving only enough solution to cover the embryos.

3. Block for 30 minutes, room temperature in P/T/S (1XPBS, 0.1% Triton X-100, 5% heat inactivated normal Goat serum) (to heat inactivate serum, incubate at 55^{o}C for 30 minutes, then sterile filter and freeze in aliquots at –20^{o}C.)

4. Replace blocking solution with primary antibody diluted in P/T/S according to the requirements of the individual antibody. Use only enough diluted antibody to cover the embryos. Incubate overnight in the refrigerator. Agitation is not necessary.

DAY TWO

1. Remove antibody and wash embryos at least 3 times 10 minutes with PBS-Triton. It is possible to wash over the course of many hours at room temperature, and this may lessen non-specific background staining.

2. Block as above (DAY ONE, Step 3)

3. Replace blocking solution with secondary antibody. Use the appropriate secondary antibody that will react specifically with your primary antibody.

PRIMARY	SECONDARY	
Rabbit	Goat anti-Rabbit	or Donkey anti-Rabbit (for triple labeling)
Mouse	Goat anti-Mouse	or Donkey anti-Mouse (for triple labeling)
Sheep	Donkey anti-Sheep	
Goat	Donkey anti-Goat	

For triple labeling or when using Sheep or Goat primary antibodies, you must block in Horse serum, not Goat serum!!!!!!

FLUOROPHORES

Molecular Probes Alexa Fluor 488=green
Molecular Probes Alexa Fluor 546 or Jackson CY3=red
Jackson CY5=blue

Dilute all secondary antibodies 1:100 in P/T/S.

4. Incubate in the dark, with tubes sitting vertically in a foil-covered rack on a nutator, 2 hours at room temperature.

5. Wash samples in the dark at least 3 times 10 minutes with PBS-Triton. It is possible to wash samples over the course of the day and this may substantially decrease background fluorescence.

6. Remove last wash and replace with 100 ul Vectashield (Vector Labs). Allow embryos to sink in Vectashield (1 hour room temperature or overnight at 4^{o}C).

7. Mount on glass slides with bridge coverslips, using additional Vectashield. Coverslip and view in the confocal. Store preparation at 4^{o}C; will be stable for weeks or months.