Fixing Embryos for In Situ Hybridization
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1. A few days in advance, set up a large fly cage with a large number of healthy flies raised under optimal, low-density conditions. To do this, make up live yeast paste by mixing baker's yeast with water and making a smooth paste of even consistency that is neither too dry nor too wet. Spread a small amount of yeast paste in the center of a grape juice agar plate (100mm diameter).
2. Feed the cage once a day for 2 days, discarding old plates in the fly waste container in the fly room.
3. The evening before the first embryo collection, change the plate at about 6PM. The next morning, change the cage to a new plate and process the embryos on the old plate as described below. First, use a spatula or kimwipe to remove the large blob of yeast paste in the center of the plate and any adult flies that are lodged in this.
4. Flood the plate with 1XPBS dispensed from a wash bottle, and using a gloved finger, gently dislodge the embryos from the plate into the wash solution. Set up an embryo basket (consisting of a sawed-off 50 ml screw cap tube with a piece of nylon attached by the hollowed-out lid of the tube) in a small Tupperware container. Using a funnel, transfer all the embryos from the plate into the basket, using a stream of 1XPBS to ensure that as many embryos as possible are transferred.
5. Rinse the embryos briefly with tap distilled water.
6. Make up 50 ml of 50% bleach (25 ml of Chlorox bleach and 25 ml of tap distilled water).
7. Transfer the basket into a 100 ml beaker and gently pour the 50% bleach over the embryos. Place beaker with embryos on a shaker and gently shake for 4 minutes to remove the chorion (egg shell).
8. After dechorionation, remove basket from beaker and thoroughly remove all traces of bleach by rinsing basket under a stream of tap distilled water. If a paper towel turns pink when it contacts the basket, there are still traces of bleach and you should continue rinsing the embryos.
9. Meanwhile, make up Fixative solution and transfer to 20 ml glass scintillation vial. You will need 15 ml of Fixative for each Genotype.
10. Disassemble basket and gently dry the bottom. Use a paint brush to transfer all dechorionated embryos to the fix. Embryos will settle at the interface between the heptane (top layer) and the aqueous fixative (bottom layer).
11. Tape scintillation vial to rotary platform shaker and shake at maximum speed for 20 minutes. Make sure you tape vial well so it does not fly off the shaker during this fixation period!
12. Using a transfer pipette, remove as much fixative (bottom layer) as possible, without removing any embryos.
13. Add 7.5 ml methanol to scintillation vial and shake vial very hard continuously for 30 seconds to remove the vitelline membranes.
14. Allow the vial to stand on the bench and the heptane : methanol phases to separate. All embryos that settle to the bottom of the vial are devitellinized. All embryos that stick to the side or remain at the heptane interface are either old, not devitellinized, or damaged and should be discarded in step 15 and avoided in later transfer steps.
15. Remove all the heptane and most of the methanol, being sure not to disturb the embryos at the bottom of the vial. Remove and discard as many damaged embryos at the heptane interface as possible.

16. Rinse embryos twice with 5 ml Methanol/EGTA
17. After the second rinse, transfer embryos to smaller glass vial. Do not transfer embryos that are stuck to the side of the vial, only those that have settled to the bottom!
18. Rehydrate embryos by equilibrating with a series of the following mixtures of Methanol/EGTA and 4% Paraformaldehyde/1XPBS. Allow embryos to settle in these solutions for 5 minutes each solution.

Methanol/EGTA		4% Paraformaldehyde/1XPBS
7 ml	:	3 ml
5 ml	:	5 ml
3 ml	:	7 ml

19. Refix embryos in 4% Paraformaldehyde/1XPBS for 20 minutes. Place embryos on nutator.
20. Wash embryos 2 X 5 minutes in 1X PBS on nutator.
21. Dehydrate embryos with an ascending ethanol series:

    30% ethanol in distilled water 5 minutes
    50% ethanol in distilled water 5 minutes
    70% ethanol in distilled water 5 minutes

Allow embryos to stand on bench and settle.
22. At this point, embryos can be stored at –20oC for months until they are required for in situ hybridization experiments.

SOLUTIONS

50% Bleach
25 ml Chlorox bleach
25 ml tap distilled water

Fixative
7.5 ml 4% paraformaldehyde in 1XPBS **RNAse FREE PREPARATION**
15 ul 1 M MgSO4
15 ul 1 M EGTA
7.5 ml n-heptane

mix these amounts in a 15 ml tube and transfer to a 20 ml glass scintillation vial.

Methanol/EGTA
45 ml methanol
5 ml 1 M EGTA