

## Direct fluorescence *in situ* hybridization to *Drosophila* embryos

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### Equipment and reagents

- ◆ Orbital shaker or roller
- ◆ Confocal microscope
- ◆ 100  $\mu\text{m}$  and 670  $\mu\text{m}$  nylon mesh sieves
- ◆ 4% formaldehyde in 1x PBS: made from a 40% stock solution of formaldehyde and 10x PBS
- ◆ 6% sodium hypochlorite solution
- ◆ Anti-DIG-AP: anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, make a working dilution in blocking solution
- ◆ Blocking solution: PBT containing 0.2 mg/ml BSA and 0.025% sodium azide
- ◆ Citifluor AF3 (Agar Scientific)
- ◆ dHybe: 50% formamide, 5x SSC, 100  $\mu\text{g}/\text{ml}$  yeast RNA, 0.1% Tween 20, 50  $\mu\text{g}/\text{ml}$  heparin, adjust to pH 6.5 with 1 M citric acid
- ◆ Heptane
- ◆ Methanol chilled on dry ice
- ◆ Pre-stain buffer: 100 mM Tris-HCl pH 8.2
- ◆ Vector™ Red staining solution (Vector Labs); mix stock solutions as described with kit

### A. Preparation of *Drosophila* embryos

- 1 Collect *Drosophila* embryos on yeasted agar plates in a population cage and age as required.
- 2 Wash off embryos with water and a soft paintbrush. Pour through a coarse (670  $\mu\text{m}$ ) nylon sieve to remove any dead flies. Collect the embryos in a fine (100  $\mu\text{m}$ ) nylon sieve.
- 3 Wash embryos from the sieve into a sterile 50 ml screw-capped, polypropylene centrifuge tube with 6% sodium hypochlorite solution. Dechorionate for about 2 min (the exact time will depend on the age and strength of the bleach solution).
- 4 Collect the dechorionated embryos in a fine sieve and rinse well with water to remove all traces of the bleach.
- 5 Transfer the embryos to a sterile 50 ml screw-capped, polypropylene centrifuge tube with 4% formaldehyde in PBS. Add an equal volume of heptane and mix on a roller for 30 min at room temperature.

- 6 Stop rolling and allow the embryos to settle at the interface. Remove most of both solutions with a pipette and replace with fresh fix and heptane. Incubate for a further 30 min on the roller. (Alternatively fix for 30 min with equal volumes of heptane and 10% formaldehyde in PBS.)
- 7 Remove the lower phase and replace with an equal volume of methanol at  $-20\text{ }^{\circ}\text{C}$ .<sup>b</sup> The embryos should pop out of their vitelline membranes within 1–2 min. They then fall to the bottom of the bottle.
- 8 Remove most of the methanol and replace with fresh cold methanol. Discard any embryos which have not sunk to the bottom of the tube.
- 9 Store the embryos in methanol at  $-20\text{ }^{\circ}\text{C}$ .

### **B. Pre-hybridization treatments of *Drosophila* embryos**

- 10 Rehydrate through a graded methanol/PBS series:
  - (a) 2 min in methanol:PBS, 7:3.
  - (b) 2 min in methanol:PBS, 5:5.
  - (c) 2 min in methanol:PBS, 3:7.
  - (d) 2 min in PBS.
- 11 Refix for a further 20 min in 4% formaldehyde in PBS.
- 12 Rinse with PBS. The embryos are now ready for hybridization without proteinase K treatment if hybridized at  $70\text{ }^{\circ}\text{C}$ .

### **C. Hybridization of *Drosophila* embryos**

- 13 Rinse five times in PBT.
- 14 Rinse in 1:1 PBT:dHybe.
- 15 Pre-hybridize in dHybe for at least 1 h at  $70\text{ }^{\circ}\text{C}$ .
- 16 Hybridize overnight at the appropriate temperature in 150–400  $\mu\text{l}$  of dHybe in 1.5 ml microcentrifuge tubes containing heat denatured probe. For double label *in situ* hybridizations mix digoxigenin and fluorescein probes. Use fluorescein antisense probes at a 1:50 dilution of a standard reaction that has been precipitated and redissolved in 100  $\mu\text{l}$  of DEPC water. (Note this is fourfold higher than recommended for detection of hybridized signals with antibodies.)

### **D. Post-hybridization washes of *Drosophila* embryos**

- 17 Rinse in dHybe at  $70\text{ }^{\circ}\text{C}$ .
- 18 Wash for 20 min in dHybe at  $70\text{ }^{\circ}\text{C}$ .

- 19 Wash in 1:1 PBT:dHybe for 20 min at 70 °C.
- 20 Wash in PBT four times for 20 min each at room temperature.
- 21 Mount a few embryos in 0.1 ml of Citifluor, spacing the coverslip and slide with tape, and sealing with nail polish. Examine under the microscope using epifluorescence with a fluorescein filter set.

### **E. Visualization of the digoxigenin labelled probe**

- 22 Block non-specific binding sites by washing in blocking solution for 20 min.
- 23 Incubate in a 1:2000 dilution of anti-DIG-AP for 3 h at room temperature on a gently rocking platform.
- 24 Rinse twice with PBT.
- 25 Wash five times with PBT over a period of 3 h, on a gently rocking platform.
- 26 Rinse twice with pre-stain buffer.
- 27 Transfer embryos to a 24-well microtitre plate, for easy observation of the staining reaction under a dissecting microscope.
- 28 Stain samples with 0.4 ml Vector™ Red staining solution for 10–30 min.
- 29 Stain until the colour is visible under the dissecting microscope, but not too intense. If the precipitate is too dense, it will quench the fluorescence from the fluorescein.
- 30 Stop the staining reaction by washing twice in PBT.
- 31 Mount in Citifluor.
- 32 View by epifluorescence microscopy. The Vector™ red precipitate fluoresces red with a rhodamine filter set giving a high signal-to-noise ratio. The fluorescein signal is seen with a fluorescein filter set but is very much weaker with a higher background. Higher resolution is obtained with the confocal microscope.

### **Notes**

- a Modified from Jowett, T. (1996). *Tissue in situ* hybridization: methods in animal development. Publ. Wiley and Sons, NY.
- b Better morphology is retained if the methanol is cooled to –70 °C on dry ice.