

## Zebrafish or *Drosophila* two colour whole mount *in situ* hybridization staining with DAB and BCIP/NBT<sup>a</sup>

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### Reagents

- ◆ 3% hydrogen peroxide: made fresh from a 30% stock solution stored at 4 °C
- ◆ Anti-DIG-HRP: sheep anti-digoxigenin Fab fragments conjugated with horse-radish peroxidase, make a working dilution in blocking solution
- ◆ Anti-FLU-AP: sheep anti-fluorescein Fab fragments conjugated with alkaline phosphatase, make a working dilution in blocking solution
- ◆ Blocking solution for antibodies: 1x PBS, 0.1% Tween-20, 2 mg/ml BSA (BDH), 5% sheep serum (Gibco BRL), 1% dimethylsulphoxide DMSO (Merck-BDH)
- ◆ DAB staining solution: 0.5 mg/ml diaminobenzidine in PBT.

**DAB is a potent carcinogen. All contaminated disposable materials should be incinerated and glassware soaked in 6% sodium hypochlorite solution**

- ◆ NBT/BCIP staining solution. **NBT** (4-nitroblue tetrazolium chloride, Boehringer): dissolve at 75 mg/ml in 70% dimethylformamide. **BCIP** 5-bromo-4-chloro-3-indolyl-phosphate also known as X-phosphate 4-toluidine salt: dissolve at 50 mg/ml in dimethylformamide. Store both solutions in aliquots at -20°C. To make the staining solution add 4.5 µl of 75 mg/ml NBT in 70% dimethylformamide and 3.5 µl of 50 mg/ml BCIP in dimethylformamide to 1 ml of NTMT buffer
- ◆ NTMT buffer: 100mM NaCl, 100mM Tris-HCl pH 9.5, 50mM MgCl<sub>2</sub>, 0.1% Tween-20 (if necessary, add levamisole to 5mM). Make from concentrated stock solutions on the day of use (the pH will decrease on storage, due to absorption of carbon dioxide)
- ◆ PBS (phosphate-buffered saline): 130 mM NaCl; 7 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O; 3 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O. For 10x PBS mix 75.97 g NaCl, 12.46 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 4.80 g NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O. Dissolve in less than 1 l of distilled water; adjust to pH 7.0 and final volume of 1 l, sterilise by autoclaving
- ◆ PBT: PBS, 0.1% Tween 20
- ◆ PFA fix: paraformaldehyde is dissolved in PBS at 65°C. If it does not readily dissolve add a drop or two of 1 M NaOH solution to pH 7.5. It should be cooled to 4°C and used within 2 days

### Method

- 1 Carry out probe synthesis, tissue fixation and pre-treatment, hybridization, and washing as described in [Synthesis of DIG or fluorescein labelled RNA probes](#), [Fixation and pre-treatment of embryos for whole mount hybridization](#), and

[Whole mount hybridization, washing, and detection of probe \(method 1\)](#). After steps 1 - 5 in [Whole mount hybridization, washing, and detection of probe \(method 1\)](#) replace the washing solution with blocking solution, and incubate at room temperature for at least 1 h on an orbital shaker.

- 2 Incubate for 2 h in pre-absorbed<sup>b</sup> anti-DIG–HRP in blocking solution at a dilution of 1:200 (0.75 U/ml).
- 3 Wash for 2 h in PBT (eight times for 15 min each).
- 4 Incubate for 2 min in DAB staining solution.
- 5 Add 1/1000 volume of 3% hydrogen peroxide to each incubation. Monitor the staining reaction and stop by rinsing thoroughly with PBT.
- 6 Replace the PBT with blocking solution and incubate at room temperature for up to 1 h on an orbital shaker.
- 7 Incubate for 2 h in anti-FLU–AP at a dilution of 1:2000 (0.375 U/ml) in blocking solution.
- 8 Wash for 2 h with PBT (eight times for 15 min each).
- 9 Wash three times for 5 min each in **freshly prepared** NTMT buffer.
- 10 Stain with NBT/BCIP staining solution.
- 11 Stop the reaction by washing with PBT.<sup>c</sup>
- 12 Fix the stain in PFA fix in PBS overnight.<sup>d</sup>

## Notes

- a This protocol is modified from [Jowett, T. and Lettice, L. \(1994\) \*Trends Genet.\* 10: 73-74](#) and [Jowett, T. \(1996\). \*Tissue in situ hybridization: methods in animal development\*. Publ. Wiley and Sons, NY.](#)
- b Pre-absorption is not necessary for *Drosophila* embryos or for zebrafish embryos which are less than 30 h old.
- c The staining reaction can take from 10 min to several hours. If the staining reaction is to take several hours, it is convenient to perform all the antibody washes the day before staining and leave the embryos in PBT overnight. This allows a full working day to monitor the development of the stain.
- d If the stain is not fixed, prolonged exposure to light can cause a dark background to develop.