

Double fluorescent *in situ* hybridization of zebrafish or *Drosophila* embryos^a

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Equipment and reagents

- ◆ Microscope equipped for epifluorescence with a rhodamine and DAPI filter set
- ◆ 0.5% Triton X-100 in PBS
- ◆ Pre-stain buffer: 100 mM Tris-HCl pH 8.2, 0.1% Tween 20
- ◆ Anti-DIG-AP: sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, 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
- ◆ AP inactivation solution: 0.1 M glycine-HCl pH 2.2, 0.1% Tween-20
- ◆ Blocking solution: 2 mg/ml BSA, 5% sheep serum, 1% DMSO, 0.1% Triton X-100 in PBS
- ◆ ELF™ pre-reaction buffer: 30 mM Tris-HCl pH 7.5, 150 mM NaCl
- ◆ ELF™ stop buffer: 25 mM EDTA, 0.05% Triton X-100 in PBS pH 7.2. Dissolve EDTA in PBS and check pH.
- ◆ ELF™-AP substrate kit (Molecular Probes): includes ELF™ reagent, reaction buffer, aqueous mounting medium
- ◆ Fast Red tablets (alkaline phosphatase substrate; Boehringer): each tablet contains 0.5 mg naphthol substrate, 2 mg Fast Red chromogen, and 0.4 mg levamisole. Store tablets at -20°C. Dissolve one tablet in 2 ml of 100 mM Tris-HCl pH 8.2. Use the solution within 30 min

Wear gloves and use plastic forceps to handle the tablets.

- ◆ 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
- ◆ Sigma *Fast*™ Fast Red (Sigma) dissolve a buffer tablet in water, add a stain tablet, and use immediately
- ◆ Vector™ Red staining solution (Vector Labs); mix stock solutions as described with kit

Method

- 1 After hybridisation and washing off the unbound probes as described in 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 wash with blocking solution.

- 2 Incubate for at least 60 min.
- 3 Incubate in a 1:2000 dilution (0.37 U/ml) of anti-FLU-AP in blocking solution overnight at 4 °C.
- 4 Wash embryos with PBT for 2 h (eight times for 15 min each).
- 5 Equilibrate with pre-stain buffer at room temperature by washing three times for 5 min each.
- 6 Stain embryos with Fast Red (Boehringer), Sigma *Fast*TM Fast Red, or VectorTM Red.
- 7 Stop the reaction by washing several times with PBT.
- 8 Inactivate the alkaline phosphatase activity by incubating in AP inactivation solution twice for 15 min each at room temperature.
- 9 Rinse four times for 5 min each with PBT.
- 10 Incubate in PFA fix for 20 min at room temperature.
- 11 Rinse five times for 5 min each with PBT.
- 12 Incubate embryos in blocking solution for 60 min.
- 13 Incubate in a 1:5000 dilution (0.15 U/ml) of anti-DIG-AP in blocking solution overnight at 4 °C.
- 14 Wash in 0.5% Triton X-100 in PBS for 2 h.^b
- 15 Wash three times for 5 min each at room temperature with the ELFTM pre-reaction buffer.
- 16 For staining incubate in a 1:20 dilution of the ELFTM substrate reagent at room temperature for 5 h, or a 1:100 dilution of the ELFTM substrate overnight at room temperature.^c
- 17 Monitor the staining reaction using a UV fluorescent microscope with a DAPI filter set at intervals after starting the reaction.
- 18 Stop the reaction by washing with ELFTM stop buffer.
- 19 Mount the tissue with the special aqueous mounting medium supplied with the kit from Molecular Probes.^d

Notes

- a Procedure modified from [Jowett, T. \(1996\). Tissue *in situ* hybridisation: methods in animal development. Publ. Wiley and Sons, NY. Jowett, T, and Yan, Y-L. \(1996\) *Trends Genet. Tech. Tips Online*. Jowett, T., Mancera, M., Amores, A., and Yan, Y-L.](#)

(1966) In *In situ hybridization: Laboratory Companion*. p. 91. (ed. M. Clark) Chapman and Hall. Jowett, T. and Yan, Y-L. (1966) In *A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis*. p.381. (ed. P. A. Krieg) Wiley-Liss, NY.

- b DMSO and Tween 20 in the wash solutions cause the final ELF crystals to be large.
- c This is far longer than recommended by Molecular Probes. With zebrafish embryos it is not necessary to add 1 mM levamisole.
- d This mountant preserves the ELF™ signal better than the usual glycerol-based aqueous mountants for fluorochromes.