Combined in situ procedures

Conlon, R. A. (1995) Combined immunocytochemistry and whole mount in situ hybridization to mouse embryos. In: Methods in Molecular Biology: Expression and Detection of Recombinant Genes (R. S. Tuan ed.) Humana Press, in press.Chapter 64. Methods for double detection of gene expression: Combined in situ hybridization and immunocytochemistry or histochemistry

Introduction

The distribution of two different molecules can be analyzed within the same embryo using the procedures described below. The protocol for combined whole mount in situ hybridization and immunocytochemistry allows for simultaneous detection of mRNA and protein. The protocol for combined whole mount in situ hybridization and b-galactosidase staining allows for simultaneous detection of mRNA and transgene-directed B-galactosidase expression. Simultaneous detection allows for the most direct comparison of expression patterns. These procedures are derived from protocols used in Drosophila (1) and mice (2, 3).

Materials

The required materials include those listed in Chapter 63 for whole mount in situ hybridization, plus the following reagents.

Combined protein and RNA detection

1. Primary antibody against the protein of interest.2. An appropriate secondary antibody conjugated to horse radish peroxidase.3. Blocking Reagent for nucleic acid hybridization (Boehringer Mannheim).4. DAB (30 mg/ml 3, 3Õ-diaminobenzidine tetrahydrochloride in 10 mM Tris pH 7.6 stored at -20 C in the dark in single use aliquots). Handle with extreme caution: DAB is carcinogenic.

Combined B-galactosidase and RNA detection

1. 100 mM EGTA pH 7.3 treated with 0.1% diethylpyrocarbonate and autoclaved.2. 1 M MgCl2 treated with 0.1% diethylpyrocarbonate and autoclaved.3. Fixative G, prepared fresh (0.2% glutaraldehyde, 2 mM MgCl2, 6 mM EGTA pH 7.3 in PBS).4. Wash G (PBT containing 2 mM MgCl2, treated with 0.1% v/v diethylpyrocarbonate and autoclaved).5. X-gal (25 mg/ml 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside in N,N-dimethylformamide stored at -20 C).6. Potassium ferricyanide (K3Fe(CN)6).7. Potassium ferrocyanide (K4Fe(CN)6¥3H2O).

Methods

Combined protein and RNA detection

Follow the protocol for whole mount in situ hybridization in Chapter 63 to step 14 on Day 2, then substitute the following for step 15 and all subsequent steps. Note that the anti-digoxigenin and secondary antibodies are

preabsorbed against the embryo powder whereas the primary antibody against the protein of interest is not.

Day 2

15. Wash with Wash 3 for 30 min at 50 C. (At this time, inactivate the embryo powder by heating a few milligrams of powder in 1 ml of TBST to 68 C for 30 min. Prepare a 1% w/v solution of Blocking Reagent (Boehringer Mannheim) in TBST. The reagent must be stirred and heated for some time to make a milky solution. Once in solution, cool to 4 C.)16. Rinse twice with Wash 4, then heat in Wash 4 at 68 C for 20 min.17. Incubate for at least 1 hour at room temperature in TBST containing 0.5 mg/ml freshly added levamisole and 1% Blocking Reagent. (At this time, preabsorb the anti-digoxigenin antibody by diluting to 1/5000 in cold TBST containing 0.5 mg/ml levamisole, 1% Blocking Reagent, and the heat-inactivated embryo powder. Rock the tube for 30 min at 4 C. Centrifuge the mixture at 10,000 g for 10 min at 4 C. Remove the supernatant containing the preabsorbed anti-digoxigenin antibody, and add the primary antibody against the protein of interest to the appropriate dilution.)18. Incubate the embryos with the antibodies overnight at 4 C.

Day 3

19. Rinse three times with TBST containing freshly added 0.5 mg/ml levamisole, then wash 5 or 6 times, one hour each, at room temperature in the same buffer. (Once again, inactivate some embryo powder and prepare a 1% solution of Blocking Reagent in TBST) 20. Incubate for at least 1 hour at room temperature in TBST containing 0.5 mg/ml freshly added levamisole and 1% Blocking Reagent. (At this time, preabsorb the secondary antibody by diluting to the appropriate concentration in cold TBST containing 0.5 mg/ml levamisole, 1% Blocking Reagent and the heat-inactivated embryo powder. Rock the tube for 30 min at 4 C. Centrifuge the mixture at 10,000 g for 10 min at 4 C. The preabsorbed secondary antibody is in the supernatant.)21. Incubate with the secondary antibody overnight at 4 C. 22. Rinse three times with TBST containing freshly added 0.5 mg/ml levamisole, then wash 5 or 6 times, one hour each, at room temperature in the same buffer. 23. Incubate for 20 min in the dark with TBST containing freshly added 0.5 mg/ml levamisole and 0.3 mg/ml DAB.24. Develop the peroxidase reaction by adding hydrogen peroxide to 0.03%. The reaction typically generates signal for the first 10 or 15 min, and then background staining begins to become evident. Stop the reaction by rinsing with TBST containing 0.5 mg/ml levamisole.25. Wash twice for 20 min each at room temperature with NTMT containing freshly added 0.5 mg/ml levamisole.26. Incubate with the alkaline phosphatase color reagents (4.5 ml/ml NBT, 3.5 ml/ml BCIP and 0.5 mg/ml levamisole in NTMT). For most messages the color reaction needs to continue overnight at room temperature. Do not agitate the embryos during the overnight color reaction. Protect from light. 27. Stop the color reaction with 3 rinses with PBTE. Clear the embryos by passing the embryos into 1:1 glycerol/PBTE for one hour, then into 4:1 glycerol/PBTE with 0.02% sodium azide. The peroxidase reaction product fades with exposure to light. Store at 4 C in the dark.

Combined B-galactosidase and RNA detection

The procedure for B-galactosidase staining decreases the sensitivity of the in situ hybridization procedure somewhat, so this combined procedure works best for prevalent target mRNAs.Embryo preparation, B-galactosidase staining and storage1. Dissect gestational day 6 to 10 embryos free from extraembryonic tissues in cold PBS. A small puncture hole must be made in the

anterior neural tube of day 9 and 10 embryos.2. Fix in 10 ml of fresh cold Fixative G for 10 min on ice.3. Rinse three times with Wash G. Wash with Wash G for 60 min at 4 C.4. Transfer to a 2 ml plastic screw-cap tube. Incubate in freshly made staining solution (1 mg/ml X-gal, 2 mg/ml potassium ferrocyanide, 1.6 mg/ml potassium ferricyanide in Wash G) at 37 C until desired staining intensity is achieved. The incubation period can vary from minutes to hours depending on the level of expression of b-galactosidase. Use the minimum incubation period possible.5. Rinse twice with PBT. Fix for 2 hours at 4 C in fresh Fixative (4% paraformaldehyde in PBS, Chapter 63).6. Rinse three times with cold PBT. Change directly into 100% methanol, invert the tube several times to mix. Store at -20 C, or proceed to step 7.7. Treat with a 5:1 mixture of 100% methanol and 30% hydrogen peroxide for 2 to 3 hours at room temperature. Rinse three times in methanol. Store at -20 C.8. The detection of RNA by in situ hybridization may be resumed at step 5 of Day 1 of Hybridization in Chapter 63.

Notes

1. The peroxidase reaction products may be intensified by addition of metal salts to the reaction. If this is desired, make a 0.3% w/v stock solution of NiCl2 or CoCl2. Add to the DAB staining solution for a final concentration of 0.03%, filter, and use immediately.2. The accumulated background from two combined procedures may obscure signal somewhat. Better visualization may be possible with a stronger clearing agent, for example 1:2 benzyl alcohol/benzyl benzoate (BABB). In glass or polypropylene tubes, dehydrate the embryos quickly through an alcohol series to 100% ethanol. Transfer to 1:1 100% ethanol/BABB until the embryos sink, then into BABB. BABB dissolves polystyrene so the embryos must be observed in glass dishes. BABB also slowly dissolves the colored reaction products of alkaline phosphatase and b-galactosidase, so the embryos cannot be kept in this clearing agent for very long. Reverse the solvent series to return the embryos to an aqueous storage solution.3. The combined procedures give their best results when the probed expression patterns are largely non-overlapping, since it is difficult to distinguish double-labeled cells.

References

1. Cubas, P., de Celis, J.-F., Campuzano, S. and J. Modolell, (1991). Proneural clusters of achaete-scute expression and the generation of sensory organs in the Dcrosophila imaginal wing disc. Genes Dev. 5, 996-1008.2. Davis, C. A., Holmyard, D. P., Millen, K. J. and Joyner, A. L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an En-specific antiserum. Development 111, 287-298.3. Conlon, R. A. and Rossant, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. Development 116, 357-368.Ronald A. Conlon, Department of Genetics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4955TEL 216-368-1826; FAX 216-368-3432; EMAIL rac14@po.cwru.edu