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 β-galactosidase staining allows for simultaneous detection of mRNA and transgene-directed β-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 for whole mount *in situ* hybridization, plus the following reagents.

Combined protein and RNA detection

1. High Salt Wash (500 mM NaCl, 10 mM Pipes pH 6.8, 1 mM EDTA, 0.1% Tween 20, autoclaved).
2. TBST (137 mM NaCl, 25 mM Tris-HCl pH 7.6, 3 mM KCl, 0.1% Tween 20, autoclaved).
3. Primary antibody against the protein of interest.
4. An appropriate secondary antibody conjugated to horse radish peroxidase.
5. Anti-digoxigenin antibody, alkaline phosphatase-conjugated (Boehringer Mannheim). Store at 4°C.
6. Blocking Reagent for nucleic acid hybridization (Boehringer Mannheim).
7. DAB (30 mg/ml 3, 3'-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.
8. BCIP (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt in 100% N,N-dimethylformamide, stored at -20°C in the dark).

9. NBT (75 mg/ml nitroblue tetrazolium salt in 70% N,N-dimethylformamide, stored at -20°C in the dark).
10. NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20).
11. PBTE (PBS containing 0.1% Tween 20 and 1 mM EDTA, autoclaved).
12. 50% glycerol/50% PBTE.
13. 80% glycerol/20% PBTE.
14. Sodium azide.

Combined β-galactosidase and RNA detection

1. 100 mM EGTA pH 7.3 treated with 0.1% diethylpyrocarbonate and autoclaved.
2. 1 M $MgCl_2$ treated with 0.1% diethylpyrocarbonate and autoclaved.
3. Fixative G, prepared fresh (0.2% glutaraldehyde, 2 mM $MgCl_2$, 6 mM EGTA pH 7.3 in PBS).
4. Wash G (PBT containing 2 mM $MgCl_2$, treated with 0.1% v/v diethylpyrocarbonate and autoclaved).
1. X-gal (25 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside in N,N-dimethylformamide stored at -20°C).
6. Potassium ferricyanide ($K_3Fe(CN)_6$).
7. Potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$).

Methods

Combined protein and RNA detection

Follow the protocol of your choice for whole mount *in situ* hybridization until the point at which the antibody to digoxigenin is to be added.

Day 1

1. 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.
2. Rinse the embryos twice with High Salt Wash, then heat in High Salt Wash at 68°C for 20 minutes.

3. Incubate for at least 1 hour at room temperature in TBST containing 1% Blocking Reagent.
4. Incubate the embryos with the antibodies overnight at 4°C. The anti-digoxigenin antibody should be diluted to 1/5000, and the primary antibody against the protein of interest should be diluted to its working concentration.

Day 2

5. Rinse briefly three times with TBST, then wash 5 or 6 times, one hour each, at room temperature in the same buffer.
6. Incubate for at least 1 hour at room temperature in TBST containing 1% Blocking Reagent.
7. Incubate with the secondary antibody overnight at 4°C.

Day 3

8. Rinse three times with TBST, then wash 5 or 6 times, one hour each, at room temperature in the same buffer.
9. Incubate for 20 minutes in the dark with TBST containing 0.3 mg/ml DAB.
10. In the dark, develop the peroxidase reaction by adding hydrogen peroxide to 0.03%. The reaction typically generates signal for the first 10 or 15 minutes, and then background staining begins to become evident. Stop the reaction by rinsing with TBST.
11. Wash twice for 20 minutes each at room temperature with NTMT.
8. Incubate with the alkaline phosphatase color reagents (4.5 µl/ml NBT and 3.5 µl/ml BCIP 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.
13. 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 β-galactosidase and RNA detection

The procedure for β-galactosidase staining decreases the sensitivity of the *in situ* hybridization procedure somewhat, so this combined procedure works best for prevalent target mRNAs.

Embryo preparation, β-galactosidase staining and storage

1. 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 minutes on ice.
3. Rinse three times with Wash G. Wash with Wash G for 60 minutes 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 ß-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).
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 by rehydrating the embryos through a methanol series. **Modify the *in situ* hybridization procedure by eliminating the glutaraldehyde in the fixation after protease digestion.**

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 $NiCl_2$ or $CoCl_2$. 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 β-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

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3. Dollé, P., Lohnes, D., Mark, M., Ruberte, E., Leroy, P., Morriss-Kay, G. and R. A. Chambon, 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.