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CHAPTER 9

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I. Introduction

Detection of the temporal and spatial regulation of gene expression in embryos is essential for elucidating the developmental functions of genes and for elucidating the cell interactions that regulate tissue patterning and differentiation. Patterns of gene expression can be visualized by detecting the encoded protein product by immunocytochemistry or mRNA using *in situ* hybridization (Wilkinson 1992; Wilkinson and Nieto, 1993). The detection of protein has the advantage of being a more accurate guide to sites of gene action, because, due to translational regulation, RNA and protein expression do not always correlate. Furthermore, immunocytochemistry reveals the subcellular location of protein that can be an important clue to how it functions. However, the production of specific antibodies can be difficult and time-consuming. In contrast, specific probes for *in situ* hybridization to mRNA can easily be produced.

In situ hybridization to RNA involves a series of procedures:

1. synthesis of a labeled nucleic acid probe complementary to the target mRNA.
2. fixation and permeabilization of tissue (sectioned or whole embryo).
3. hybridization of probe to the tissue and washing to remove unhybridized probe.
4. detection of the probe.

Many types of probe and methods of labeling and visualization have been used for *in situ* hybridization to embryos. Hapten-labeled single-stranded RNA probes are most commonly used, as they enable high sensitivity, a single cell resolution of signal, the ability to visualize gene expression in whole embryos, and detection of multiple RNAs can be carried out (Lopez-Sanchez, 2004; Nieto *et al.*, 1996; Stern, 1998). Following hybridization and washing, the location of probe is detected with a hapten-binding protein conjugated to an enzyme. This latter enzyme catalyzes the conversion of a substrate to an insoluble, colored, or fluorescent product, and thus a signal is produced at the sites of the target mRNA.

A number of haptens and enzyme conjugates of hapten-binding proteins are available: haptens include digoxigenin (DIG), fluorescein, and dinitrophenol

(DNP) that can be detected with commercially available antibodies, and enzymes include alkaline phosphatase (AP) and horse radish peroxidase (HRP). For each of these enzymes, a variety of substrates can be used that yield different colored or fluorescent products. For detection of a single RNA, the reagents that have found widespread favor because of their high sensitivity and low backgrounds are DIG-labeled probes detected with an AP-conjugated anti-DIG antibody and the chromogenic substrate mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) plus 4-nitro blue tetrazolium chloride (NBT). For detection of two RNAs, a mixture of RNA probes labeled with DIG and fluorescein can be used followed by sequential detection with AP-conjugated antibodies to generate different colored products (Protocols 1–6). Alternatively, fluorescent products can be generated by using HRP-conjugated antibodies (Protocols 8–10).

For many purposes, the *in situ* hybridization of whole embryos is the method of choice as it is easier and provides a broader picture of the gene expression pattern than the hybridization of sections. If sections are required, it is less work to section embryos after whole-mount hybridization and signal detection than it is to prepare sections and then hybridize. However, the extent of penetration of reagents into tissues limits the size of embryos that can be used for whole-mount *in situ* hybridization. Although we have obtained low backgrounds with chick embryos up to stage HH25 (Hamburger and Hamilton, 1951), much stronger signals are obtained when the tissue is at the surface than when it is internal, and thus some sites of expression could be missed. This limitation can be alleviated to some extent by longer hybridization and washing steps. In addition, access of the reagents can be increased by bisecting embryos or dissecting out the tissue of interest prior to hybridization.

II. Solutions

TE buffer: 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0.

5 × transcription buffer: 200 mM Tris-HCl, pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl.

Nucleotide mix: 10 mM GTP, 10 mM ATP, 10 mM CTP, 6.5 mM UTP, 3.5 mM DIG-UTP or fluorescein-UTP, pH 7.5 (Roche).

Phosphate-buffered saline (PBS): prepared using Dulbecco "A" tablets (Oxoid) and treated with DEPC.

PBT: PBS, 0.1% Triton X-100 (Tx).

Proteinase K: 10 mg/ml stock in sterile H_2O.

Paraformaldehyde fixative: 4% paraformaldehyde in PBS. Heat at 65 °C with occasional agitation until dissolved, cool, and then filter. Use on the day of preparation. Note: take precautions with paraformaldehyde fumes that are toxic.

Hybridization solution: 50% formamide, 5 × SSC, 2% Roche blocking powder, 0.1% Tx, 50 mg/ml heparin, 1 mg/ml Torula yeast RNA, 1 mM EDTA, 0.1% CHAPS, DEPC-treated dH_2O.

20 × SSC stock solution: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.

KTBT: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 0.3% or 0.1% Tx.

NTMT: 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tx, 1 mM levamisole.

NBT stock solution: 75 mg/ml NBT (Roche) in 70% dimethylformamide.

BCIP stock solution: 50 mg/ml BCIP (Roche) in dimethylformamide.

III. Single and Multiple Detection of RNA in Floating Sections or Whole-Mount Embryos

It is important that solutions used for processing the embryos prior to hybridization are ribonuclease-free to avoid the degradation of the cellular RNAs. We find it sufficient to autoclave the PBS used for making fixative and pretreatment solutions and to use disposable plastic tubes. In order to obtain low backgrounds, it is important that the washes are thorough, but do not damage the embryo. We use a variable speed rocking platform adjusted such that the embryos are gently agitated during prehybridization, hybridization, and washing steps; this is easier to achieve if the container is not completely full. For the high-temperature incubations, we place microtubes in a heater block turned on its side on a rocking platform. Alternatively, an incubator containing a rocking platform can be used. When changing solutions, allow the embryos to settle to the bottom of the container and leave some liquid above them otherwise surface tension can flatten them. A variety of different containers can be used, partly depending upon the equipment available. We use 7 ml flat-bottomed tubes for the fixation and pretreatment of embryos and 2 ml microtubes for hybridization, washing, and immunodetection.

The following protocol has been used extensively to detect RNA transcripts by *in situ* hybridization of embryos from different species such as chick (Fig. 1A–E), mouse, zebrafish, lizard, turtle, and amphioxus, as well as in adult tissues including human samples. This multiple detection of RNA can also be combined with immunohistochemical protocols (Fig. 1F and G) to detect protein expression.

A. Protocol 1: Preparation of Labeled RNA Probes

To detect one or two different RNAs, probes are synthesized labeled with one of two different haptens: DIG or fluorescein. The protocol used to synthesis each probe is to carry out *in vitro* transcription of the DNA template in the presence of ribonucleotides, to one of which DIG or fluorescein is conjugated (usually UTP).

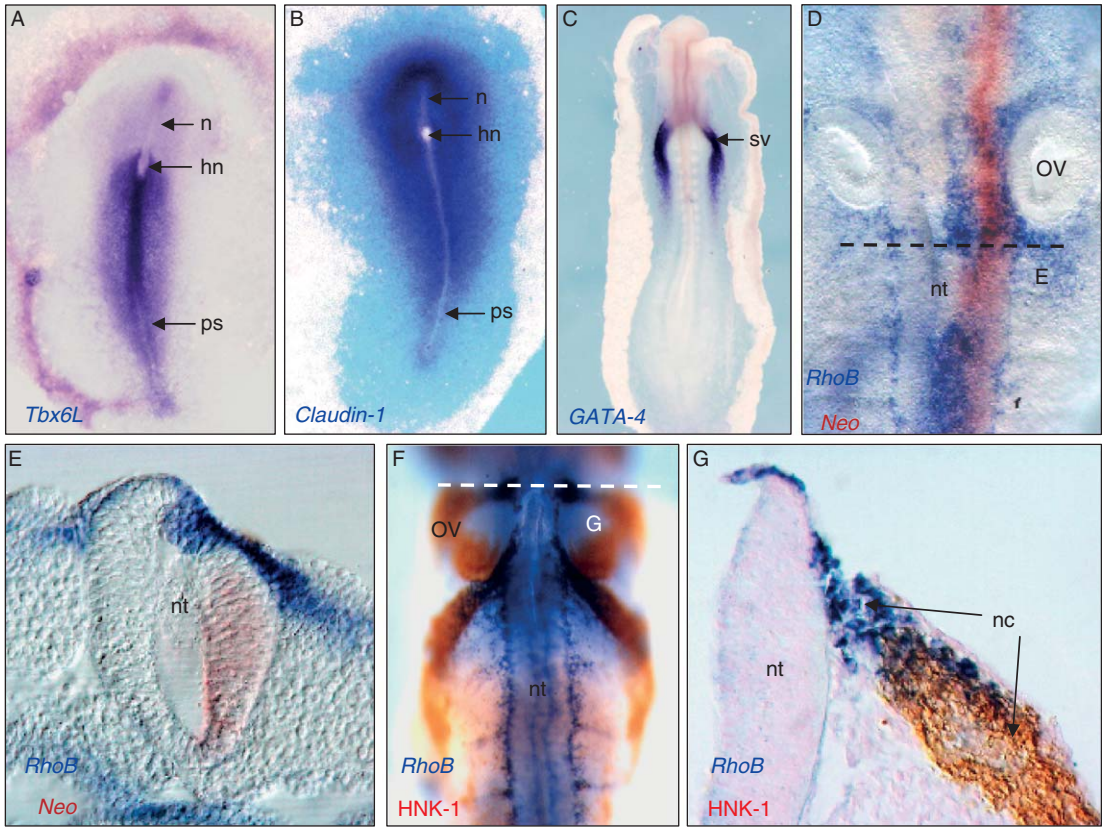

Fig. 1 Multiple detection of gene transcripts and proteins in chick embryos using nonfluorescent *in situ* hybridization and immunohistochemistry. (A) *Tbx6L* expression in the mesoderm of an HH6 chick embryo (B) *Claudin-1*-expression in the epiblast of an HH5 embryo. (C) *GATA-4* expression in the sinus venosus and the cardiogenic mesoderm of an HH10 embryo (D) Simultaneous detection of *RhoB* (blue) and *Neomycin* expression (red) shown in a dorsal view of the hindbrain at HH13. The *Neomycin* coding region was included in a plasmid vector electroporated in the right-hand side of the neural tube. (E) Vibratome section obtained from the embryo shown in (D) at the level of the dotted line. (F) Dorsal view of the posterior hindbrain region of an HH13 embryo hybridized with *RhoB* (blue) and immunostained for the HNK-1 carbohydrate (brown). (G) Section taken from the embryo shown in (F) at the level indicated by the dotted line (adapted from Del Barrio and Nieto, 2004) allows the visualization of different neural crest populations. Abbreviations: n, notochord; hn, hensen's node; nc, neural crest; nt, neural tube; ov, otic vesicle; ps, primitive streak; sv, sinus venosus.

The labeled RNA synthesized should be complementary or antisense to the target mRNA. When possible, long probes are recommended (around 0.5–2 kb) to increase specificity and signal strength. Before probe synthesis, the DNA template should be linearized at a restriction site located at the 5′ end of the cDNA (inside the cDNA or in the plasmid's multiple cloning site) using an enzyme that produces a blunt or 5′ overhanging end. After checking that linearization is complete,

the DNA is purified using either columns or phenol/chloroform extraction and ethanol precipitation. We generally redissolve the DNA at a concentration of approximately 1 μg/μl in TE buffer. Usually probes are synthesized using SP6, T7, or T3 RNA polymerases, the promoters for which are generally present in most commercial vectors. The transcription buffer is generally supplied with the enzymes.

1. Mix these reagents in the following order at room temperature:

   8.5 μl sterile distilled water
   4 μl 5 × transcription buffer
   2 μl 0.1 M dithiothreitol
   2 μl nucleotide mix
   1 μl linearized plasmid (1 μg/μl)
   0.5 μl RNasin (100 U/μl) (Promega)
   1 μl SP6, T7, or T3 RNA polymerase (10 U/μl)
2. Incubate at 37 °C for 2 h.
3. Add 2 μl of 3 M ammonium acetate and 50 μl ethanol, mix, and incubate at −20 °C for 2 h.
4. Spin for 10 min in a microfuge at 4 °C, wash the pellet with 70% ethanol, and air dry.
5. Redissolve the pellet in 20 μl ice-cold DEPC-treated dH_2O and 20 μl formamide.
6. Remove 2 μl aliquot and run on 1% agarose/TAE gel to check the amount of RNA synthesized.
7. Store the synthesized probe at −70 °C.

B. Protocol 2: Preparation of Embryos and Tissue Sections

A crucial step in this procedure is the way in which the embryos are obtained because the dissection has to be performed quickly in ice-cold PBS in order to minimize any degradation of RNA. With mouse (from E10.5) and chicken embryos that are at least 2 days of age, it is necessary to make a small hole in the cavities (like the dorsal hindbrain) to prevent probe or antibody trapping that can give rise to nonspecific signals. Paraformaldehyde fixation preserves the RNA transcripts, the embryonic tissues, and the general morphology of the embryos. Normally for chick embryos (up to stage HH17), a fixation time of 2 h is sufficient but frequently we use overnight fixation. During all these steps, the embryos or sections are kept on ice.

1. Dissect out the embryos in ice-cold DEPC-treated PBS.

2. Fix the embryos overnight at 4 °C in 4% paraformaldehyde prepared in DEPC-treated PBS. When working with vibratome sections, cut the sections at a thickness of 50–100 μm and refix the tissue sections in 4% paraformaldehyde.

3. Wash the embryos or sections with ice-cold PBT, twice for 5 min.

4. Wash the embryos with ice-cold 25%, 50%, 75% methanol in PBT for 5 min in each solution and then twice with 100% methanol. When dealing with bigger embryos extend the length of the washes to up to 15 min. The embryos or sections can be stored at –20 °C in 100% methanol, although it is better to store them after the prehybridization step.

C. Protocol 3: Prehybridization Treatments and Hybridization

The permeabilization steps that constitute the prehybridization process are important to improve the penetration of the probes and antibodies into the embryos and tissue sections. Proteinase K treatment partially digests cellular proteins and facilitates probe penetration into embryonic tissues. However, excessive digestion with proteinase K will alter embryonic morphology and it is not recommended for early embryos (up to stage 8 in the chick and E8 mouse embryos). A treatment with H_2O_2 is necessary to inactivate endogenous peroxidase activity. This step can be omitted if peroxidase will not be used later in the detection procedure. In the following protocol, the embryos are kept on ice, except for steps 4 and 6.

1. Rehydrate the sections or embryos through 75%, 50%, 25% methanol in PBT and then wash twice with PBT.

2. If required, incubate the sections or embryos in 1% hydrogen peroxide in PBT for 20 min. For embryos from stage HH12 or for whole organs, incubation can be extended up to 1 h.

3. Wash the sections or embryos with PBT three times for 5 min.

4. Treat the sections or embryos with 10 μg/ml proteinase K in PBT for 15 min at room temperature. The appropriate treatment depends on the age of the embryos. Proteinase K should not be used for embryos up to stage HH8 (particularly if they have been cultured *in vitro*), while 5–7 min treatment is appropriate for embryos up to stage HH12. For vibratome sections, we recommend a 3 min digestion. Under no circumstances should the digestion be extended for longer than 20 min.

5. Wash the sections or embryos for 5 min each with freshly prepared 2 mg/ml glycine in PBT and twice with PBT. This step is optional.

6. Refix the sections or embryos with fresh 4% paraformaldehyde in PBT for 20 min.

7. Wash the sections or embryos twice for 5 min with PBT.

8. Add 1 ml of the hybridization solution and transfer the embryos to a 2 ml screw-capped tube. Incubate at 60 °C for 5 min to equilibrate.

9. Replace the hybridization solution with fresh solution and incubate the material overnight at 60 °C with gentle agitation. The sections or embryos can be stored indefinitely in this solution at −20 °C.

10. Denature the anti-DIG and/or antifluorescein probes by heating them at 65–70 °C for 3 min.

11. Remove the hybridization mix and add fresh solution containing around 2 µl of each RNA probe per milliliter of hybridization mix (equivalent to 1–5 µg of the purified probe). The volume used should be sufficient to cover the embryos or sections.

12. Incubate overnight at 60 °C with gentle agitation. For higher stringency, hybridization can be carried out at higher temperatures, provided the probe is longer than 400 bp.

D. Protocol 4: Posthybridization Washes and Signal Detection

Following hybridization, embryos and sections should be washed at moderate stringency to remove the unhybridized probe. The bound probe is then detected with an AP-conjugated antihapten antibody. The washes after antibody binding serve to remove the unbound antibodies. Overnight washing is not always necessary but it helps to reduce background in older embryos or organs. The embryos are then incubated with a chromogenic substrate for AP. This produces a colored precipitate at the site where the probe RNA and antibody have bound to the target RNA. The time required for the color reaction to develop normally varies from 30 min to 48 h and depends on several factors such as the quantity of target RNA, probe quality, and reagent penetration. With some probes, the reaction may even take longer than 48 h to visualize the reaction product in whole-mount embryos or tissue sections, but care must be taken so that the background levels of staining do not prevail or reach unacceptable levels. After visualization of the staining of the material, embryos or sections can be stored for a long time in PFA 4% in PBS or glycerol 50% + sodium azide 0.02% in PBS at 4 °C.

1. Wash twice for 5 min with 2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation.

2. Wash three times for 30 min each with 2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation. For older embryos, increase the length and number of washes.

3. Wash three times for 30 min each with 0.2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation.

4. Wash three times for 5 min each with KTBT (0.1% Tx) at room temperature.

5. Block the sections or embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx), for 2–3 h at 4 °C.

6. Incubate with 1/1000 dilution of AP-conjugated anti-DIG antibody (Roche) in blocking solution. The antibody can be preabsorbed for 1 h with embryo powder (prepared as in Protocol 5), but this step is optional. To preabsorb the antibody with embryo powder, incubate 3 mg of embryo powder in KTBT

(0.1% Tx) for 30 min at 70 °C. Allow the mixture to reach room temperature and preabsorb the desired amount of anti-DIG antibody for 1 h at 4 °C. Spin in a microfuge at 12,000 rpm for 1 min at 4 °C and dilute the supernatant in the appropriate amount of blocking solution.

7. Incubate the sections or embryos with antibody overnight at 4 °C.
8. Wash the sections or embryos in KTBT (0.3% Tx) eight times for 1 h each wash at room temperature.
9. Leave washing overnight in KTBT (0.3% Tx) at room temperature.
10. Wash the sections or embryos three times with NTMT, 15 min each wash at room temperature.
11. Incubate with AP substrate (3 µl/ml NBT + 2.3 µl/ml BCIP in NTMT) at room temperature until a blue precipitate is readily apparent.
12. Wash in KTBT (0.3% Tx) twice, 5–10 min each wash at room temperature. If you need to develop the reaction longer, the sections or embryos can be left overnight in KTBT and the staining resumed at step 10 on the following day.

E. Protocol 5: Preparation of Embryo Powder

1. Homogenize embryos (12.5–14.5 days mouse embryos or 4–5 days chick embryos) in a minimum volume of ice-cold PBS.
2. Add four volumes of ice-cold acetone to the homogenate, mix and incubate on ice for 30 min.
3. Centrifuge at 10,000 × *g* for 10 min and remove supernatant. Wash the pellet with ice-cold acetone and repeat the centrifugation.
4. Spread the pellet out and grind it into a fine powder on a sheet of filter paper. Air-dry the powder and store it at 4 °C.

F. Protocol 6: Detection of the Second RNA

To detect a second RNA, one of the two probes is labeled with fluorescein and detected with AP-conjugated antifluorescein and a substrate that yields a distinct color from that generated for the DIG-labeled probe detected above (Fig. 1D and E). It is essential to inactivate the AP activity of the anti-DIG antibody prior to the detection of the fluorescein-labeled probe. This inactivation is carried out by acid treatment. Note that the INT/BCIP precipitate generated in the second color reaction is not very stable and can disappear if extensively washed.

1. Incubate the sections or embryos in 0.1 M glycine in PBS, pH 2.2, two times for 10 min each at room temperature.

2. Wash the sections or embryos in KTBT (0.1% Tx) three times for 5 min each at room temperature.
3. Block the sections or embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx), for 2–3 h at 4 °C.
4. Incubate overnight at 4 °C with antifluorescein antibody (Roche) at 1/3000 dilution.
5. Incubate the sections or embryos with antibody overnight at 4 °C.
6. Wash the sections or embryos in KTBT (0.3% Tx) eight times for 1 h each wash at room temperature.
7. Leave washing overnight in KTBT (0.3% Tx) at room temperature.
8. Wash the sections or embryos three times with NTMT, 15 min each wash at room temperature.
9. Incubate with INT/BCIP 75 µl /10 ml NTMT until a red-brown precipitate appears.
10. Wash in KTBT (0.3% Tx) twice, 5–10 min each wash at room temperature.
11. Wash in PBS and store embryos or sections in 4% paraformaldehyde or 50% glycerol at 4 °C.

G. Protocol 7: Immunodetection of Protein

To detect protein expression after *in situ* hybridization follow protocol 6 up to step 10 and then continue as indicated below (Fig. 1F and G).

1. Block again the embryos by incubating with 10% goat serum in KTBT for 2–3 h.
2. Incubate embryos overnight at 4 °C with an antibody against the protein you wish to detect at an appropriate dilution in 10% goat serum in KTBT. By inclusion of 0.01% azide at this step, incubation can go up to three days.
3. Wash the embryos three times for 5 min with KTBT.
4. Wash the embryos 12 times for 20 min with KTBT.
5. Incubate with a biotinilated, anti-IgG secondary antibody specific for the species of the primary antibody overnight at 4 °C in blocking solution.
6. Wash the embryos three times for 5 min with KTBT.
7. Wash the embryos 10 times for 20 min with KTBT.
8. Incubate with ABC kit Vectastain (Vector Laboratories) for 3 h and wash overnight.
9. Wash in 0.5 mg/ml diaminobenzidine in KTBT for 30 min.
10. Develop in the same solution containing 0.03% H_2O_2 at room temperature. This reaction is usually extremely fast and is completed in less than 2 min.

IV. Photography and Sectioning

Low-power photographs of the embryos can be taken on a dissecting microscope with overhead illumination (Fig. 1A–C). We usually place the embryo on a 3% agarose/PBS gel (in a 35 mm petri dish) that gives us a nice light blue background. Alternatively, embryos in 50% glycerol can be mounted under a coverslip in order to take photographs at higher magnification (Fig. 1D). Place two drops of petroleum jelly or silicon grease about 1 cm apart on a microscope slide, pipette the embryo in the middle, and orientate as desired. Lower a coverslip on top, gently pushing it down as required. Depending on the site of expression, it can be very useful to partially dissect embryo tissues to improve the observation and help focusing the structures of interest.

Embryos can then be embedded in gelatin/albumin or paraffin wax to be sectioned on a vibratome (Fig. 1E) or microtome (Fig. 1G), respectively. The dehydration steps used to embed in wax can produce a loss of signal, especially of INT/BCIP precipitates. Vibratome sectioning is the preferred method to analyze embryos subjected to double detection of mRNAs including INT/BCIP precipitates and paraffin sectioning when signals are resistant to treatments for embedding.

V. Whole-Mount Fluorescent *In Situ* Hybridization

The use of fluorescent *in situ* hybridization has two major advantages. One is that very good cellular resolution can be obtained, enabling the detection of coexpression or complementary expression of genes in adjacent territories. Second, it is possible to simultaneously detect up to three different messenger RNAs in whole-mount embryos (Fig. 2A) or sections. In addition, this multiple detection can be combined with fluorescent immunodetection of proteins (Fig. 2B and C). The use of a confocal microscope capable of analyzing many different excitation wavelengths is indispensable to obtain sufficient cellular resolution, although it is also possible to obtain very good images using a conventional fluorescence microscope.

The fluorescent whole-mount *in situ* hybridization protocol is very similar to the general protocol described above but it contains a few modifications that mainly affect the type of antibodies and reagents used to visualize the hybridized RNA probe. In the following protocol, we shall describe the triple localization of RNAs and their immunodetection in whole-mount embryos. The order in which the different probes are detected may be important when strong and weak signals are expected. Normally the stronger signal is detected with the fluorescein-conjugated probe and revealed with FITC-tyramide, while the weakest signal should be visualized with the DIG-conjugated probe and Cy3-tyramide. The protocol presented here is modified from Denkers *et al.* (2004) with an additional step to detect proteins by immunochemistry. It is important to keep embryos protected from light throughout the entire *in situ* hybridization process, especially during and after developing.

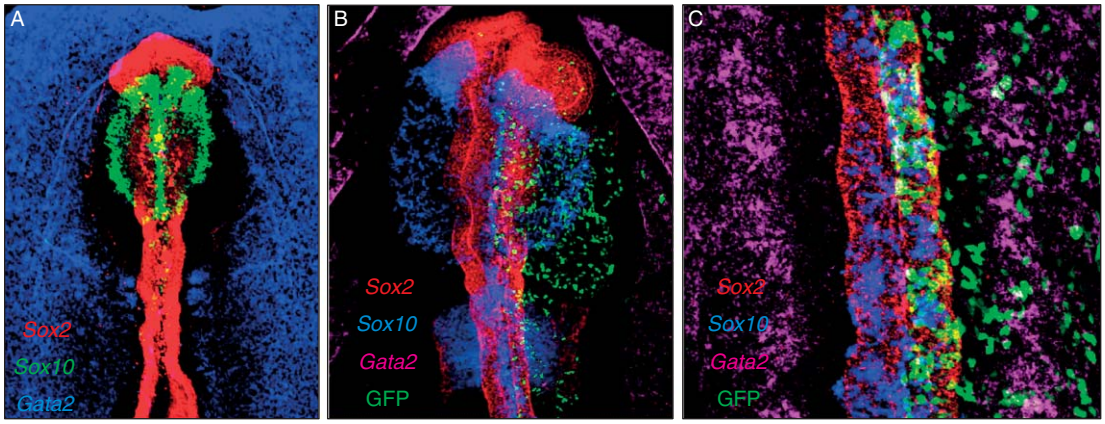

Fig. 2 Multiple detection of gene transcripts and proteins in chick embryos using fluorescent *in situ* hybridization and immunohistochemistry. (A) Three-color *in situ* hybridization in a HH9 chick embryo for the epidermal marker *Gata2* (blue, artificial color), the neural marker *Sox2* (red), and the neural crest marker *Sox10* (green). (B) Three color *in situ* hybridization in the head of an HH9 embryo for *Gata2* (magenta, artificial color), *Sox2* (red), and *Sox10* (blue). This embryo was electroporated with a plasmid encoding the green fluorescent protein (GFP). Cells expressing the GFP protein were detected by immunohistochemistry (green). (C) A higher power image of a similar embryo showing the trunk region of an HH9 embryo. Images were acquired using a Leica DM IRE2 confocal inverted microscope.

To detect three different RNAs, three probes are synthesized with different epitopes: DIG-UTP, fluorescein-UTP, and DNP-UTP. The protocol for probe synthesis is as described in Protocol 1, using a nucleotide mix of 10 mM GTP, 10 mM ATP, 10 mM CTP, 6.5 mM UTP, 3.5 mM DIG-UTP or fluorescein-UTP (Roche), or DNP-UTP (Perkin Elmer). The preparation of embryos, prehybridization, and hybridization are all carried out as described in Protocols 2 and 3, with the use of three probes in the hybridization solution.

A. Protocol 8: Posthybridization Washes and Signal Detection

1. Wash twice for 5 min with 2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation.
2. Wash three times for 30 min each with 2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation. For older embryos, increase the length and number of washes.
3. Wash three times for 30 min each with 0.2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation.
4. Wash three times for 5 min each with KTBT (0.1% Tx) at room temperature.
5. Block the sections or embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx), for 2–3 h at 4 °C.

6. Incubate the embryos with HRP-coupled antifluorescein antibody (1/500, Perkin Elmer) in blocking solution overnight at 4 °C.
7. Wash the embryos in KTBT (0.3% Tx) three times, 5 min each at room temperature.
8. Wash the embryos in KTBT (0.3% Tx) 10 times, 20 min each at room temperature.
9. Wash the embryos for 1 min with the amplification buffer supplied by the manufacturer in the TSA kit.
10. Incubate the embryos with amplification buffer including freshly added Cy3-labeled tyramide (1:100, Perkin Elmer) for up to 1 h at room temperature.
11. Wash the embryos in KTBT (0.3% Tx) three times for 5 min each at room temperature.
12. Incubate the embryos for 45 min with 1% H_2O_2 in KTBT (0.3% Tx) to inactivate the HRP activity.
13. Wash the embryos in KTBT (0.3% Tx) three times for 5 min each at room temperature.

B. Protocol 9: Detection of the Second RNA

1. Block the embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx) for 2–3 h at 4 °C.
2. Incubate overnight at 4 °C with HRP-coupled anti-DIG antibody (1/1000, Roche).
3. Wash in KTBT (0.3% Tx) three times, 5 min each at room temperature.
4. Wash the embryos in KTBT (0.3% Tx) 10 times, 20 min each at room temperature.
5. Wash the embryos for 1 min with the amplification buffer supplied by the manufacturer in the TSA kit.
6. Incubate the embryos with amplification buffer including freshly added FITC- labeled tyramide (1:100, Perkin Elmer) for up to 1 h at room temperature.
7. Wash in KTBT (0.3% Tx) twice for 5–10 min each at room temperature.

C. Protocol 10: Detection of the Third RNA

1. Block the embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx) for 2–3 h at 4 °C.
2. Incubate overnight at 4 °C with HRP-coupled anti-DNP antibody (1/500, Perkin Elmer).
3. Wash in KTBT (0.3% Tx) three times, 5 min each at room temperature.
4. Wash the embryos in KTBT (0.3% Tx) 10 times, 20 min each at room temperature.

5. Wash the embryos for 1 min with the amplification buffer supplied by the manufacturer in the TSA kit.
6. Incubate the embryos with amplification buffer including freshly added Cy5-labeled tyramide (1:100, Perkin Elmer) for up to 1 h at room temperature.
7. Wash in KTBT (0.3% Tx) twice for 5–10 min each at room temperature.

D. Protocol 11: Immunodetection of Protein

1. Block the embryos by incubating with 10% goat serum in KTBT for 2–3 h.
2. Incubate embryos overnight at 4 °C with an antibody against the protein you wish to detect. To avoid possible cross-reaction of secondary antibody with the HRP-coupled mouse antibodies used previously, do not allow antibodies to be produced in mouse.
3. Wash the embryos three times for 5 min with KTBT.
4. Wash the embryos 12 times for 20 min with KTBT.
5. Incubate the embryos for 3 h with an Alexa 405-conjugated anti-IgG specific for the species of the primary antibody (1/500, Molecular Probe) in blocking solution.
6. Wash the embryos three times for 5 min with KTBT.
7. Wash the embryos 10 times for 20 min with KTBT.
8. Wash the embryos with PBS and store in the dark at 4 °C in 4% paraformaldehyde or in 50% glycerol.

VI. Photography and Sectioning

For image acquisition, embryos are maintained in 50% glycerol. Low-power photographs of the embryos can be taken on a dissecting microscope with epifluorescence illumination. In addition, embryos can be mounted as previously described to take photographs at higher magnification with a fluorescence microscope. For confocal image acquisition, a microscope with laser emission at 405 nm (UV), 488 nm (FITC), 541 or 561 nm (Cy3), and 633 nm (Cy5) is necessary to get four color images (Fig. 2B and C). Early embryos in glycerol (50%) should be placed without a coverslip in a 35 mm petri dish, the plastic base of which has been substituted by a glass microscope cover. After photographing, the embryos can then be embedded in gelatin/albumin and sectioned using a vibratome. Although both gelatin and albumin autofluoresce, this does not interfere with confocal image acquisition.

VII. *In Situ* Hybridization to Tissue Sections

The method for *in situ* hybridization to tissue sections involves steps identical (probe preparation, embryo fixation) or with simple adaptations (pretreatments, hybridization, washing, and the immunocytochemical detection of probe) to those

used in whole-mount hybridization. As described for whole mounts, precautions should be taken to avoid ribonucleases degrading cellular RNA prior to hybridization. In addition to using autoclaved PBS, we avoid using any slide holders that have been exposed to ribonucleases.

A. Protocol 12: Preparation of Tissue Sections and Subbed Slides

In the method described below, tissue sections are prepared by embedding fixed embryos in paraffin wax, cutting sections, and drying them onto slides that have been subbed with TESPA. An alternative is to cut cryostat sections. To maximize signal, it may be advantageous to cut thick sections.

1. Dissect the embryos and fix them in 4% paraformaldehyde in PBS, overnight at 4 °C.
2. Wash the embryos with PBS, twice for 10 min.
3. Dehydrate by taking embryos through methanol series in PBT (25% methanol, 50% methanol, then 75% methanol) then twice in 100% methanol, for 10 min each. Later stage embryos should be washed for longer to ensure complete dehydration.
4. Equilibrate embryos with toluene, three times for 20 min, then with molten paraffin wax at 60 °C, three times for 20 min, occasionally agitating the vial. Take precautions to avoid breathing toluene fumes.
5. Transfer the embryos to glass embryo dishes (preheated to 60 °C), orientate them with a warmed needle under a dissection microscope and allow the wax to set. Paraffin wax blocks can be stored indefinitely at 4 °C until required for use.
6. On a microtome, cut 6 μm sections as ribbons that are then floated on a bath of distilled water at 50 °C until the creases disappear and collected on TESPA-subbed slides.
7. Dry the sections onto the slides at 37 °C overnight. They can be stored desiccated at 4 °C.

TESPA-subbed slides are prepared as follows:

1. Dip the slides in 10% HCl/70% ethanol, followed by distilled water and 95% ethanol, for 1 min each, and then air-dry.
2. Dip the slides in 2% TESPA (3-aminopropyltriethoxysilane) in acetone for 10 sec.
3. Wash twice with acetone, and then with distilled water.
4. Dry at 37 °C.

B. Protocol 13: Prehybridization Treatments

Prior to hybridization, the sections are dewaxed, permeabilized by proteinase K treatment followed by refixation, and dehydrated. The probe is then spread over the sections under a coverslip. This protocol does not include a prehybridization

blocking step. The same general considerations apply as for whole-mount hybridizations, with the additional factor that overdigestion with proteinase can lead to the sections falling off the slides. Except where otherwise stated, we place the slides in holders suitable for 250 ml slide dishes and use 200–250 ml of the solutions.

1. Dewax the slides in Histoclear, twice for 10 min, and then place them in 100% methanol for 2 min to remove most of the Histoclear.
2. Transfer the slides through 100% methanol (twice), 75%, 50%, and 25% methanol/PBT for 1–2 min in each solution, then wash twice in PBS for 5 min.
3. Immerse the slides in fresh 4% paraformaldehyde in PBS for 20 min.
4. Wash the slides with PBS, three times for 5 min.
5. Drain the slides and place horizontally on the bench. Overlay the sections with 10 μg/ml proteinase K (freshly diluted in PBS from a 10 mg/ml stock in dH_2O) and leave for 5 min.
6. Shake off excess liquid and wash the slides with PBS for 5 min.
7. Repeat the fixation of step 3; the same solution can be used.
8. Wash the slides twice with PBS for 5 min. Dehydrate by passing through 25%, 50%, 75% methanol/PBT, then twice in 100% methanol, for 1–2 min in each solution. Allow to air-dry.
9. Apply the hybridization mix to the slide adjacent to the sections ($\sim$5 μl/cm^2 of coverslip is sufficient) and gently lower a clean coverslip so that the mix is spread over the sections. Hybridization mix and probe are made exactly as described for whole mounts.
10. Place the slides horizontally in a box containing tissue paper soaked in 50% formamide, 5 × SSC, seal the box, and incubate overnight at 55–65 °C.

C. Protocol 14: Posthybridization Washing and Immunocytochemical Detection

The slides are washed and immunocytochemistry carried out under identical conditions as described for whole-mount hybridization. It may be possible to reduce the times given for these steps without affecting background.

1. Place the slides in a slide rack and immerse in prewarmed 2 × SSC, 0.1% CHAPS at 55–65 °C until the coverslips fall off. Gentle encouragement with forceps may be necessary.
2. Wash with 2 × SSC, 0.1% CHAPS, twice for 30 min at 55–65 °C.
3. Wash with 0.2 × SSC, 0.1% CHAPS, twice for 30 min at 55–65 °C.
4. Wash with KTBT, twice for 10 min at room temp.
5. Quickly drain each slide and place horizontally in a sandwich box containing moist tissue paper. Take care that the sections do not become dry, and quickly overlay them with 20% sheep serum in KTBT. Seal the box and incubate for 2–3 h.

6. If desired, the antibody can be preabsorbed as described in Protocol 4.
7. Remove the 20% serum from the embryos, replace with the diluted antibody and incubate in a moist box at 4 °C overnight.
8. Wash with KTBT for 5 min, three times, and then for 30 min, three times.
9. Wash with NTM, three times for 5 min.
10. Incubate in the dark with NTM containing 4.5 μl NBT, 3.5 μl BCIP per milliliter.
11. Occasionally monitor, and when sufficient signal has developed, stop the color reaction by washing with PBT.
12. Fix the signal by immersing the slides in 4% paraformaldehyde in PBS for 2 h, dehydrate quickly through a graded methanol series followed by Histo-clear, then mount under a coverslip using Permount mounting agent.

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