

# Combined Whole-Mount *in Situ* Hybridization and Immunohistochemistry in Avian Embryos

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The whole-mount *in situ* hybridization process has revolutionized the study of gene expression in the embryo. This procedure allows extremely sensitive detection of RNA transcripts and excellent spatial resolution. Numerous experiments benefit from the detection of more than one marker molecule in the same experimental embryo. While antisense RNA probes are extremely useful and methods for two-color *in situ* hybridization are available, antibodies recognizing specific protein species can help to expand the range of markers detected. Here we present a protocol that permits the simultaneous localization of RNA transcripts and immunocytochemical localization of proteins in the chick embryo. © 2001 Academic Press

The avian embryo is a powerful system to study important problems in developmental biology, since it allows experimental embryology to be combined with molecular approaches. The availability of many specific antibodies and cDNA probes that can identify particular tissues and cell types has greatly enhanced the possibilities for analysis of the outcome of embryological manipulations in an objective way. Furthermore, methods for interfering with the level or location of gene expression, by the use of retroviral vectors or implantation of transfected homologous or heterologous cells, have been developed and are now used routinely to understand developmental mechanisms at the cellular and molecular levels. In this

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type of experiment, it is important to study the expression of different transcripts and/or proteins during normal development of the embryo and under experimental conditions and it has often proved advantageous to detect several of these probes simultaneously. In this article, we provide detailed protocols for whole-mount *in situ* hybridization using digoxigenin (DIG)-labeled antisense probes in combination with detection of proteins by immunocytochemistry.

## SPECIALIZED REAGENTS

### General Note on Solutions

To avoid degradation of RNA prior to hybridization, salt solutions used on Day 1 of the protocol should be autoclaved or prepared from autoclaved stock solutions in sterile water. All stocks for the hybridization solutions are prepared in ultrapure or diethyl pyrocarbonate (DEPC)-treated H<sub>2</sub>O. The SSC solution is DEPC-treated and autoclaved. There is no need for special treatment of the solutions used after the posthybridization washes.

Tris-buffered saline (TBS) is 50 mM Tris-HCl (pH 7.4), 200 mM NaCl. Tris-buffered saline-Tween 20 (TBST) is TBS containing 1% Tween 20.

Calcium and magnesium-free phosphate-buffered saline, pH 7.4 (CMF-PBS). Prepare a 20× stock solution containing 3 M NaCl, 160 mM Na<sub>2</sub>PO<sub>4</sub>, 340 mM NaH<sub>2</sub>PO<sub>4</sub>.

Fixative: 4% formaldehyde (w/v), 2 mM EGTA in

CMF-PBS. Dissolve the appropriate amount of paraformaldehyde in distilled H<sub>2</sub>O at 65–70°C by continuous stirring and adjusting the pH to 7.2–7.4 with 1 N NaOH (usually 2–4 drops per 100 ml). Add the appropriate amount of CMF-PBS and EGTA stock solutions and adjust the final volume. Cool down on ice before use. This solution can be used for 2–3 days if stored at 4°C in the dark.

### Preabsorbing Anti-DIG Antibody

The anti-digoxigenin-AP Fab fragments supplied by Boehringer Mannheim have given very reliable results at a working dilution of 1: 5000 to detect AP-labeled probes (e.g., 1  $\mu$ l of stock into 5 ml of blocking solution); however, the dilution should be tested for each batch and might even be reduced to 1:10,000. To preabsorb the antibody, weigh out 3 mg of chick embryo powder (see below) for every microliter of antibody from the stock solution, add 1 ml of Tris buffered saline, 1% Tween 20, pH 7.4 (TBST), vortex, and incubate for 30 min at 70°C. Vortex again and spin at 3000 rpm for 1 min in an Eppendorf centrifuge and discard the supernatant. Wash the pellet by vortexing and spinning with TBST until the supernatant is clear. This procedure removes the embryo-derived lipids that float at the top of the liquid. After the final wash, resuspend the pellet in 1 ml of blocking buffer, add the desired amount of antibody, and incubate for 2–3 h at room temperature with gentle shaking. Then spin down at 10,000 rpm for 3–5 min and discard the pellet. The supernatant contains the preabsorbed antibody and is now adjusted to the final volume with blocking buffer to give a final dilution of antibody of 1:5000. The antibody can be reused 15–20 times if stored properly at 4°C. To prevent bacterial contamination, add thimerosal to a final concentration of 0.01%.

### Preparation of Chick Embryo Powder

To prepare embryo powder, we generally use a mixture of different stages, however, if young embryos are used the yield is very low. There does not seem to be a disadvantage in using powder from older embryos, even if younger ones are used in the assay. Homogenize embryos in a minimal volume of ice-cold CMF-PBS using a homogenizer or a syringe. Add 4 vol of ice-cold acetone, mix, and incubate on ice for 30 min. Centrifuge at 10,000g for 10 min, discard the supernatant, wash the pellet once with ice-cold

acetone, and spin again. Spread the pellet out on Whatman filter paper and grind to a fine powder using a pestle. Air dry and store at 4°C.

### Silicone Rubber Coated Dishes

Sylgard 184 (Dow Corning) is a clear silicone rubber that polymerizes by mixing two components (rubber solution:accelerator/catalyst, 9:1). After mixing them, pour into Petri dishes to a depth of 2–5 mm. Stand the dishes at room temperature for 2 h to allow air bubbles to dissipate and then incubate at 55°C until polymerized (2 h to overnight). The dishes can be stored indefinitely.

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## METHOD

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### Preparation of Embryos

Embryos are collected in CMF-PBS, cleaned from remaining yolk, and carefully removed from the vitelline membrane. Stretching out the embryos in a flat state before fixation may be achieved in two different ways, depending on their age. Young embryos (up to about 24 h old) are transferred to a plastic Petri dish in a small drop of CMF-PBS. All the liquid, except for a thin film, is carefully removed using a Pasteur pipet and fixative is gently and immediately added dropwise directly onto the embryo. After 10–15 min, transfer the embryos into glass scintillation vials. Older embryos (2–5 days) are best pinned out in CMF-PBS on silicon rubber-coated (Sylgard 184, Dow Corning) dishes, using insect pins (0.1 mm). The saline is then removed and replaced with fixative. To avoid trapping of color detection reagents in embryonic cavities during the *in situ* procedure, these cavities may be perforated using fine insect pins at this stage. Particular problems have been observed in heart, eye, gut, and brain (the latter can be opened in the midline using a fine surgical blade if necessary). After 10–15 minutes, cut out the embryos using a surgical blade, leaving the extraembryonic area behind, and then transfer to glass scintillation vials.

The initial fixation is crucial for the quality of the final *in situ* hybridization signal. We have obtained best results by fixing for 4–5 h at room temperature or overnight at 4°C. The fixative should not be more than 2–3 days old and should be stored in the dark at 4°C after preparation. After fixation replace the fixative with 100% methanol and store the embryos at –20°C. We find, however, that best results are

obtained when the embryos are processed through Day 1 of the *in situ* hybridization procedure (see below) within a few days of fixation. Once in hybridization solution the embryos can be stored at  $-20^{\circ}\text{C}$  for extended periods.

#### Day 1: Rehydration, Prehybridization, and Hybridization

On the first day of the *in situ* process, start by rehydrating the embryos through 75, 50, and 25% methanol in CMF-PBS, 0.1% Tween (PBT), allowing them to settle between steps, and then wash twice for 10 min in PBT. Embryos more than 2–3 days old should be bleached in 6%  $\text{H}_2\text{O}_2$  in PBT for 1 h with gentle rocking and then washed three times for 10 min each in PBT. Bleaching removes eye pigmentation and also seems to reduce general background staining. For the last wash, measure the volume of PBT and add proteinase K to a final concentration of 10  $\mu\text{g}/\text{ml}$  (i.e., a 1000-fold dilution of a 10 mg/ml stock solution). Young embryos (up to Stage 5–6) and embryos that have been grown in New culture (1) are incubated in proteinase K solution for 15 min; whole older embryos are incubated for 30 min at room temperature. Occasionally rotate the vials gently during the incubation to ensure that the entire inner surface and the lid of the vial become exposed to proteinase K, which will help to destroy RNase on the glass surface. Carefully remove the proteinase K solution, rinse the embryos twice in PBT, and postfix for 20–30 min in Fixative with 0.1% glutaraldehyde at room temperature. It is essential to include glutaraldehyde at this step, because embryos will otherwise disintegrate during the hybridization procedure.

The following protocol is modified from a procedure originally developed by D. Henrique, D. Ish-Horowitz, and P. Ingham. In this version, hybridization is performed under high-stringency conditions (low pH, low salt, and high temperature), which greatly simplifies the posthybridization washes by using identical solutions and conditions.

Remove the fixative, and rinse the embryos twice with PBT and once with hybridization solution. Add fresh hybridization solution and prehybridize in a water bath at  $70^{\circ}\text{C}$  for 3 h. Remove the solution and replace it with DIG-labeled antisense RNA probe diluted in hybridization buffer (for a final concentration of about 0.5–1  $\mu\text{g}/\text{ml}$ ) and then hybridize overnight. For all probes greater than 400 nucleotides in

length, hybridize at  $70^{\circ}\text{C}$ . For shorter probes, especially if they are A/T-rich, it may be necessary to optimize the hybridization temperature to achieve a good signal-to-background ratio. As a guide, probes of 250–300 nucleotides may require a hybridization temperature of about  $65^{\circ}\text{C}$ .

#### Day 2: Posthybridization Washes and Antibody Incubation

At this stage the embryos, especially young or cultured embryos, are fragile and must be handled with care. Use a Pasteur pipet (fire-polished if necessary), to change solutions and avoid sucking the embryos into the pipet. Make sure that all the liquid is removed at every step to allow efficient washing. To achieve this, tilt the vial and rotate slowly while removing the solution, until the embryos become lightly attached to the wall of the tube. Immediately add new liquid to the opposite side of the vial to ensure that the embryos do not dry out.

Remove the probe and store at  $-20^{\circ}\text{C}$  for reuse (we find that probe can be reused at least 20 times over a period of a year). Rinse the embryos three times with prewarmed hybridization solution. Then wash three times for 30–45 min in hybridization solution at the hybridization temperature and once for 20 min with prewarmed TBST: hybridization solution (1:1) at the hybridization temperature. Remove the vials from the water bath, rinse three times with TBST, and wash three times for 30 min each with TBST, while rocking horizontally. The vials should be completely filled with liquid to reduce violent agitation which may severely damage or destroy the embryos.

After the TBST washes, embryos are incubated first in a solution with high protein content (blocking solution) to reduce nonspecific antibody binding, and then with anti-DIG antibody solution overnight. For the blocking reaction, remove the TBST, add sufficient blocking solution to cover the embryos, and then incubate for 2–3 h at room temperature, rocking in a upright position. During this time, preabsorb alkaline phosphatase-coupled anti-DIG antibody (anti-digoxigenin-AP Fab fragments, Boehringer Mannheim) with embryo powder to remove nonspecific binding activity (see method above). After 3 h, remove the blocking buffer from the embryos, replace with anti-DIG antibody (1:5000 in blocking solution), and incubate overnight at  $4^{\circ}\text{C}$ .

### Day 3: Postantibody Washes and Detection

#### *Postantibody Washes*

The following morning, remove the antibody solution and store for reuse (at 4°C). We find that diluted antibody can be used at least 15–20 times without loss of sensitivity. Rinse the embryos three times with TBST and then wash three times in TBST for a total of 30–60 min at room temperature, rocking horizontally. Several methods for the visualization of alkaline phosphatase activity are described below. In our hands, NBT–BCIP is generally the substrate of choice since it produces very reliable results, little background, and strong signals, even for transcripts expressed at low levels.

#### *Detection with NBT-BCIP (Blue)*

After the postantibody washes, wash twice for 10 min each in NTMT developing solution (100 mM NaCl, 100 mM Tris–HCl, pH 9.5, 50 mM MgCl<sub>2</sub>, 0.1% Tween 20) and then incubate the embryos in the dark in NTMT containing 4.5  $\mu$ l NBT and 3.5  $\mu$ l BCIP per 1.5 ml of NTMT. Rock the vials gently with the vial upright. Depending on the target sequence, the color reaction may take from 5 min to several days to develop. If there is only faint staining after 3–4 h at room temperature, vials should be incubated at 4°C overnight to slow the reaction. The next day, incubation may be continued at room temperature. If there is still no visible signal, or the signal remains faint, after the entire next day, the vials can be left at room temperature for the next night. When a dark blue color has developed, the reaction is stopped by washing twice for 10 min each in CMF–PBS. The reduced pH of this solution intensifies the signal and turns it even darker blue. The embryos are now postfixed and stored in 4% formaldehyde in CMF–PBS.

#### *Detection with Red or Fluorescent Substrates*

Fast Red TR produces a red precipitate that also fluoresces when viewed with rhodamine optics. Apparently, fluorescent detection provides greater sensitivity and therefore allows weaker signals to be visualized. There are several companies that supply Fast Red, each with slightly different properties. A kit called Vector Red can be obtained from Vector Labs, Fast Red tablets from Boehringer Mannheim, and tablets together with buffer from Sigma. In general, Fast Red from all these sources produces a considerable amount of yellow-orange background, especially in the yolky extraembryonic region of the embryo. In our hands, the Vector kit gives the best

results (provided the kit is reasonably new), while background is worst with the Boehringer Mannheim substrate. We recommend the use of Fast Red only for the detection of *very* highly expressed transcripts.

After the postantibody TBST washes (see section above), wash the embryos twice in 100 mM Tris–HCl, pH 8.2, and add the substrate as described by the manufacturer. The developing time can be from several minutes to days depending on the source of the substrate. The Vector reagent is generally fastest to produce a signal. The reaction is stopped by washing the embryos several times in CMF–PBS.

#### *Detection with ELF (Fluorescent)*

Molecular Probes has introduced a fluorescent substrate called ELF for detection of alkaline phosphatase activity, available as two different kits. The first, allowing detection of biotinylated molecules using streptavidin–alkaline phosphatase, produces too high a background in chick embryos, which contain high levels of endogenous biotin and avidin. The second kit, however, produces useful results with chick embryos and contains all the solutions necessary, including buffers and Hoechst 33342 dye as a counterstain.

Since ELF is observed to produce large crystals in the presence of Tween 20 (2), either wash the embryos in CMF–PBS, 0.5% Triton X-100, three times for 10 min each after the postantibody washes, or replace TBST in the postantibody washes by CMF–PBS, 0.5% Triton X-100 directly. Then follow the manufacturer's instructions. Just before developing the color reaction, place one or two embryos into a cavity slide in the prereaction solution and replace the liquid with substrate working solution. Do not cover with a coverslip, because it prevents mixing of the substrate components. Incubate at room temperature and check occasionally (but not too frequently, because this leads to photobleaching of the fluorochrome) under the fluorescence microscope with ultraviolet illumination (350–380 nm excitation, about 460 nm emission; DAPI/Hoechst filters are suitable) until the desired signal has developed. According to the manufacturer a signal should develop within 10 min to 1 h; however, weak signals may take 4–6 h. If no signal has developed after 2 h, the reaction can be accelerated by incubation at 37°C in a humid chamber. Stop the reaction in CMF–PBS, 25 mM EDTA, 0.05% Triton X-100, and postfix the embryos in 2% formaldehyde in CMF–PBS for 20 min (longer

fixation increases background and autofluorescence). Finally, mount the embryo samples using the materials supplied in the kit.

#### *Detection with other Chromogens*

The color reaction substrate 5-bromo-6-chloro-3-indolylphosphate (Molecular Probes, or Magenta-Phos from Biosynth AG) is an analog of BCIP that produces a magenta color when used as an alkaline phosphate substrate. The resulting color is weaker than that of NBT-BCIP and is therefore not very useful for double-color analysis if the expression patterns of the target sequences overlap. In addition, it is less sensitive than NBT-BCIP and is therefore recommended only for detection of highly expressed transcripts.

We have, however, found that the resulting color can be shifted further to the red when Magenta-Phos is used in combination with Tetrazolium Red (Sigma), which also slightly improves the sensitivity. To achieve this result, substitute Magenta-Phos for BCIP and substitute Tetrazolium Red for NBT in the method described above.

Using 6-chloro-3-indolylphosphate (Molecular Probes, or Salmon Phos from Biosynth AG) results in a pink color product. A green precipitate can be obtained by using BCIP only (without NBT). While this results in a considerable loss of sensitivity, it provides a better contrast in combination with other substrates such as Fast Red or Magenta-Phos when performing double *in situ* hybridization.

#### *Combination of in Situ Hybridization with Immunocytochemistry*

In many cases, it is useful to combine the detection of a transcript by *in situ* hybridization with the detection of a particular antigen using a specific antibody. We have successfully used two different approaches depending on the nature of the antigen and the expression level of the transcript. Both procedures are described in detail; however, it is important to note that the protocol may have to be modified slightly to take into account the sensitivity of the antibody to particular fixation methods and also to accommodate the specific types and concentrations of detergent and blocking agents.

#### *Immunostaining after in Situ Hybridization*

This procedure has been used successfully to detect nuclear or cytoskeleton-associated antigens following *in situ* hybridization. However, it is not suitable

for cell surface antigens (and probably not for soluble intracellular antigens either, although we have not tested this specifically), which may be destroyed during proteinase K treatment and may be removed by the high detergent concentrations in the hybridization and washing solutions.

To use this method, embryos may be processed through the *in situ* hybridization procedure using more or less the normal method. One alteration worth considering, however, is minimizing the time of proteinase K treatment to improve the detection of the antigen. We find that if the transcript to be detected in the *in situ* hybridization step is expressed at high levels, proteinase K treatment can be reduced to 3 min or even omitted altogether (for preprimitive streak stages) without significant loss of hybridization signal. After completing the color reaction, fix the embryos in 4% formaldehyde in CMF-PBS, rocking horizontally, and block for 1 h at room temperature in the appropriate blocking solution. For most nuclear antigens we use 1% goat serum in CMF-PBS containing 0.5–1% Triton X-100. Incubate the embryos in primary antibody diluted in blocking buffer for at least 2 days at 4°C. Then wash five times (for 30–45 min each) in CMF-PBS, 0.1% Triton X-100, shaking gently, and incubate in horseradish peroxidase (HRP)-labeled secondary antibody diluted in blocking buffer for another 2 days. Wash as described above, followed by two 10-min washes in 100 mM Tris-HCl, pH 7.4. Making sure to protect the embryos from excess exposure to light, incubate the embryos for 10 min in 100 mM Tris-HCl, pH 7.4, containing 0.5 mg/ml 3,3'-diaminobenzidine (DAB). Then add H<sub>2</sub>O<sub>2</sub> to a final concentration of 0.03% to start the color reaction. Development can take from 5 min to 1 h, after which the general background increases. If no color reaction is observed after 20–30 min, add more H<sub>2</sub>O<sub>2</sub> to double the final concentration. Stop the reaction by several 10-min washes in distilled water and store the embryos in 4% formaldehyde in CMF-PBS.

#### *Immunostaining before the in Situ Hybridization Reaction*

To detect cell surface antigens it is necessary to perform the whole mount immunostaining procedure prior to *in situ* hybridization. In our hands, the *in situ* hybridization part of the protocol works very reliably for detection of transcripts expressed at relatively high levels, but is much less reliable for transcripts with low levels of expression. During the immunohistochemistry procedure we prefer to use

autoclaved glass and plasticware and to wear gloves at all times to protect against RNase contamination. Fix the embryos as usual in freshly prepared 4% formaldehyde, 2 mM EGTA in CMF-PBS. We have also used 100% methanol or ethanol for fixation for antigens sensitive to formaldehyde. Follow the normal whole-mount immunostaining protocol established for the appropriate antibody, but using 1 M LiCl, 0.05% Tween 20 in CMF-PBS as the blocking and antibody incubation solutions. No detergent is included in the washes with CMF-PBS, 1 M LiCl. After the final washes following incubation with the HRP-coupled secondary antibody, rinse the embryos twice briefly in 100 mM Tris-HCl, pH 7.4, without LiCl, replace with 0.5 mg/ml DAB in 100 mM Tris-HCl, add H<sub>2</sub>O<sub>2</sub> to a final concentration of 0.03% immediately, and develop the reaction in the dark. During these steps work quickly and stop the reaction using autoclaved water as soon as a dark brown color has developed. This minimizes incubation times and may help to avoid degradation of RNA. Postfix the embryos in 4% formaldehyde, 2 mM EGTA in CMF-PBS for 30 min and transfer to absolute methanol. At this point the embryos can be stored at -20°C before processing them further through the normal *in situ* hybridization protocol (presented above).

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## DISCUSSION OF POTENTIAL PROBLEMS

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Perhaps the most common problem encountered with the *in situ* hybridization and immunostaining protocol is unwanted background staining. Since there are many reasons for this problem, it is often very difficult to diagnose the reasons accurately. Some possibilities are addressed below.

### Unincorporated Hapten

This is one of the most common reasons for excessive background and seems to be particularly common when using short RNA probes. It is probably

caused because digoxigenin sticks to the chick tissue. After the synthesis reaction, we generally precipitate the probes twice in 2.5 vol of ethanol to remove unincorporated nucleotides which carry the digoxigenin hapten. A second method for removing free label is to use Sephadex G-25 spin columns prepared in RNase-free 20 mM Tris-HCl, 2 mM EDTA, pH 8.0.

### Cross-reactivity with Other Transcripts

This can be a problem especially when using short probes (less than 300 nt), where the stringency may need to be reduced, or when members of a large gene family are detected. In the former case, it is very difficult to obtain satisfactory results; however, it is probably worth spending some time to find the optimal hybridization temperature. In the latter case, compare the expression patterns using different nonoverlapping regions of the gene as probes. In particular, the 3' untranslated region is very likely to serve as a specific probe. Use of the 3' untranslated region as probe is also useful for analysis of quail/chick chimeras, since 3' probes are generally species specific.

### Trapping within Cavities

Internal cavities like the brain, heart, gut and eyes in 2- to 4-day embryos often accumulate an intensely colored precipitate, in particular when using NBT/BCIP. This could be due to trapping of probe, antibody, or color reaction product. Perforating the cavities with insect pins or a surgical blade improves the results considerably, probably by facilitating the exchange of solution during the wash steps.

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