In Situ Hybridization: Fruit Fly Embryos and Tissues

Ronit Wilk,[1,2] Sreenivasa U.M. Murthy,[1,2] Haixu Yan,[1,2] and Henry M. Krause[1,2]

[1]Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada
[2]The Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario, Canada

ABSTRACT

It is well known that transcript localization controls important biological processes, including cell fate determination, cell polarity, cell migration, morphogenesis, neuronal function, and embryonic axis specification. Thus, the sub-cellular visualization of transcripts in 'their original place' (in situ) is an important tool to infer and understand their trafficking, stability, translation, and biological functions. This has been made possible through the use of labeled 'anti-sense' probes that can be readily detected after hybridization to their 'sense' counterparts. The following is a series of protocols for conducting in situ hybridization in *Drosophila* embryos or tissues. These methods include standard alkaline phosphatase methods, as well as higher resolution and throughput variations using fluorescence-based probe detection. New modifications that enhance probe penetration and detection in various tissues are also provided. *Curr. Protoc. Essential Lab. Tech.* 4:9.3.1-9.3.24. © 2010 by John Wiley & Sons, Inc.

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OVERVIEW AND PRINCIPLES

As with northern blotting, in situ hybridization is based on the fact that DNA or RNA antisense sequences will undergo hydrogen bonding to complementary RNA sequences. By labeling the 'anti-sense' probe appropriately, its bound counterpart mRNA can be visualized (see Fig. 9.3.1). As the name implies, in situ hybridization allows one to analyze the spatial distributions of mRNAs in situ (i.e., in place) within appropriately fixed cells and tissues, thereby providing a two- or three-dimensional view of where genes are transcribed, and how those transcripts are subsequently processed.

In situ hybridization was first introduced in 1969 to study gene expression at the cellular level (Buongiorno-Nardelli and Amaldi, 1969; John et al., 1969). It was later adapted to tissue sections, and finally, with the help of better fixation and solubilization techniques, to intact tissues and embryos (reviewed by Levsky and Singer, 2003). The first probes were radioactively labeled, and detected over periods of days to months using photographic emulsions (Buongiorno-Nardelli and Amaldi, 1969; reviewed by Brady and Finlan, 1990). The first fluorescent in situ hybridization (FISH) approach was conducted in the early 1980s, using an antisense RNA probe labeled with a fluorescent molecule covalently attached to its 3′ end (Bauman et al., 1980). However, this approach lacked sensitivity. Better sensitivity was achieved by incorporating hapten-modified nucleotides (e.g., biotin-UTP) into the probes, which could then be detected using appropriate antibodies. Further sensitivity was achieved by incorporating secondary antibodies and additional enzymatic amplification steps. The latest approaches incorporate both sensitivity and resolution, using the enzymatic production of fluorescent molecules that do not diffuse away from the specifically hybridized probe (Raap et al., 1995; Kosman et al., 2004; Figs. 9.3.1 and 9.3.3).

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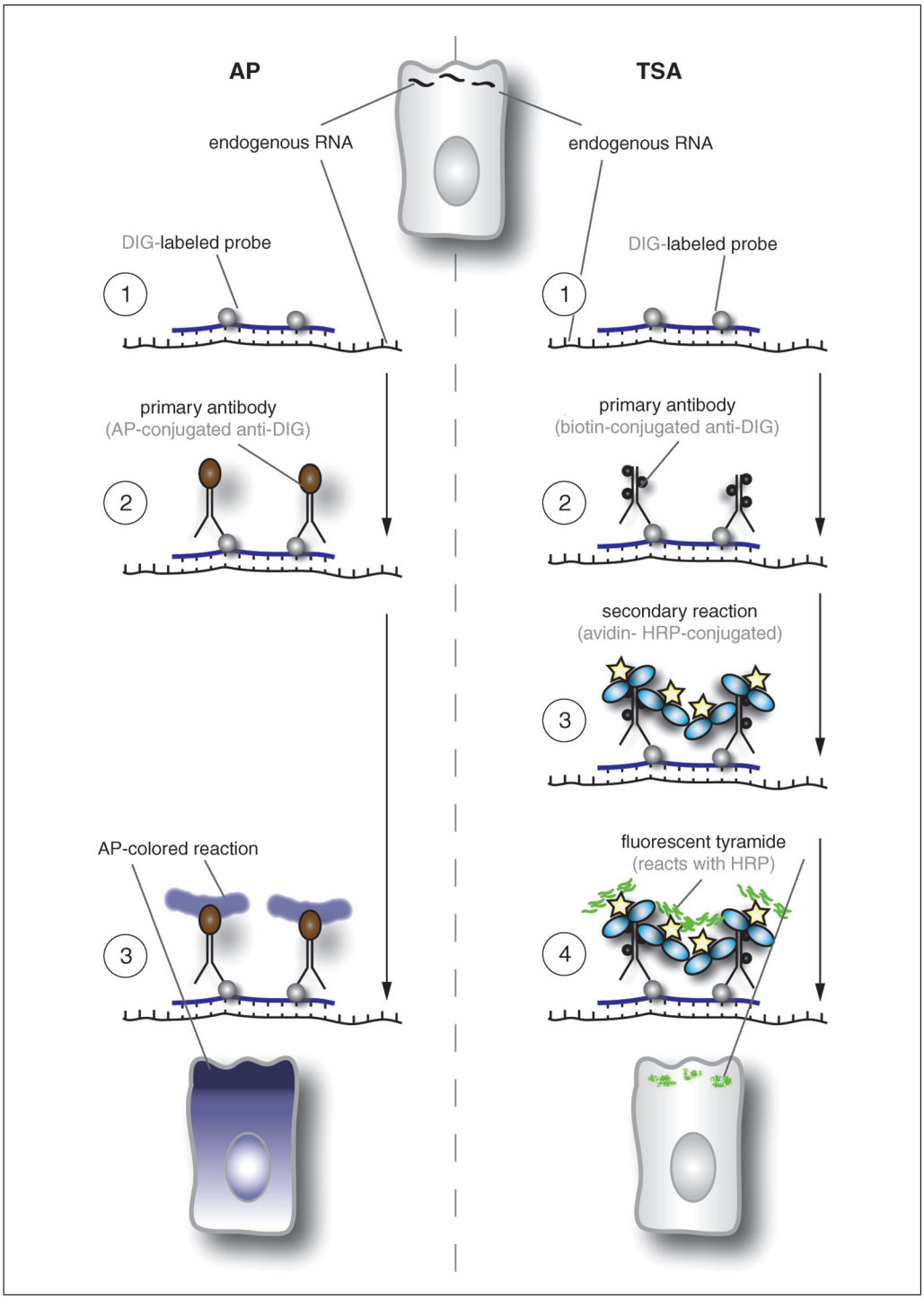

Figure 9.3.1 The basic idea behind RNA in situ hybridization. Schematic representation of the in situ hybridization technique to detect endogenous mRNAs at the cellular level. The steps on the left of the figure represent a cell analyzed by a DIG-labeled probe (1) that recognizes its endogenous complementary RNA. This probe is detected by an anti-DIG antibody conjugated to alkaline phosphatase (AP) (2), followed by an AP-colored reaction in purple (3). The steps on the left start from the same DIG-labeled probe (1′), detected by an anti-DIG-biotin-conjugated primary antibody (2′), that is in turn amplified by a streptavidin-HRP conjugated complex (3′) detected by fluorescent tyramide (TSA, green) (4′). Note that the alkaline phosphatase method is simpler, less expensive, and requires a less sophisticated microscope system. On the other hand, TSA provides a much higher resolution signal, and can be used together with other fluorescent signals without obscuring them.

These more recent enhancements to the procedure have revealed that the majority of RNAs are differentially localized within cells, and that this sub-cellular localization plays a major role in subsequent protein localization and function (Lécuyer et al., 2007). Knowing where a transcript localizes, and which other RNAs and proteins co-localize, has enormous diagnostic potential for the elucidation of gene function and possible avenues for manipulation. One clear example of the importance of this approach is in neuroscience, where mRNAs that encode neurotransmitters and receptors can be mapped to specific regions of the brain, cell types, and neuronal processes. Other commonly used medical applications include diagnostics to assess chromosomal integrity, cancer diagnosis, and tumor analyses.

Clearly, understanding the cellular and sub-cellular distributions of RNAs within all cells and tissues of an organism will be an essential next step in this genomics-based scientific era. This unit focuses on the steps required to achieve whole mount in situ

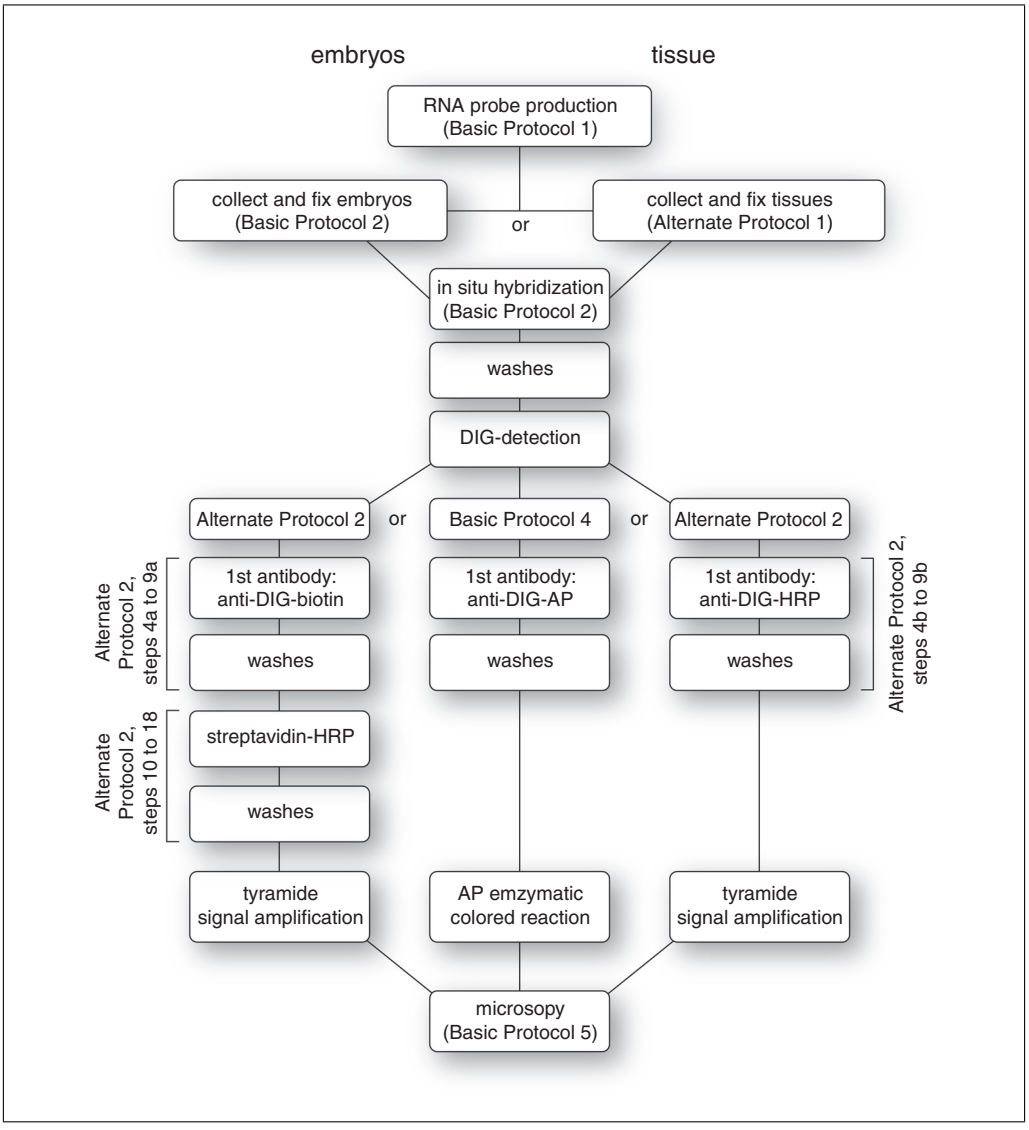

Figure 9.3.2 A general flowchart of protocols described. The flowchart shows a guideline to illustrate the order of the protocols in this unit, with protocol numbers in parenthesis. The left side should be followed for embryonic samples. Similarly, the right side is for tissues. The center shows protocols common to all procedures. To detect the DIG-labeled probe use either: an AP-conjugated antibody developed with a colored reaction (see Basic Protocol 4); a biotin-conjugated antibody followed by avidin-HRP and tyramide amplification (see Alternate Protocol 2); or, an HRP-conjugated anti-DIG antibody followed by tyramide amplification (see Alternate Protocol 2).

hybridization to messenger RNA (mRNA) molecules within *Drosophila* tissues and embryos, but is applicable to many other tissue and embryo types. In general, there are four main steps to in situ hybridization: (1) probe preparation, (2) tissue preparation, (3) hybridization, and (4) signal development. The following sections will deal with each of these steps, providing first the aims and considerations, then critical parameters, and finally, optimized protocols. In some cases, such as tissue preparation and signal development, alternate protocols are provided. For example, for signal development, both a relatively quick and simple colorimetric detection procedure, as well as a more sensitive and high-resolution fluorescent-based approach are included. A flow chart indicating these steps and options is shown in Figure 9.3.2. Also included in each section and at the end are numerous tips, details, recommendations, and troubleshooting guides to help avoid common difficulties and pitfalls.

STRATEGIC PLANNING

Table 9.3.1 summarizes the critical parameters that should be considered to plan strategically the appropriate type, scale, and management of the whole in situ hybridization protocol to be conducted. More details are included in each described protocol. Refer to Figure 9.3.2 for a general summary of all the protocols described.

Table 9.3.1 Critical Parameters

Critical parameters	Significance
RNA probe length, concentration, and sequence	Affects signal intensity and specificity
Sample permeabilization	Facilitates probe and antibody accessibility to target mRNAs
Pre-hybridization	Reduces nonspecific binding
Hybridization temperature	Controls nonspecific background
Post-hybridization washes	Multiple washes at elevated temperature help avoid nonspecific signal
Antibody concentrations	Intensity of signal and background are in part determined by antibody concentration
Tyramide dilution	Determines the intensity of the final signal
Blocking	Helps reduce nonspecific binding of antibodies

PROTOCOLS

Basic Protocol 1: RNA Probe Production

The choice, production, and application of probes are some of the most critical aspects of performing a successful hybridization. Initial protocols made use of DNA probes, the most recent of which include synthetic oligonucleotides and *Taq*-amplified probes (reviewed by Morrison et al., 2003). While easier to make and use, due to their stability, DNA probes do not hybridize to RNAs as strongly and specifically as RNA probes. Hence, RNA probes, if made and used properly, provide added sensitivity and specificity, and are used in current state-of-the-art protocols. However, care must be taken to ensure an RNase-free environment, vessels, and reagents for their production and storage.

The length of a probe is also critical for optimal success. Optimal probe length is between 700 and 1000 base pairs (bp). Longer probes decrease tissue penetration, increase nonspecific binding, and reduce clearance of unbound probe. Shorter probes can reduce signal and increase off-target binding. If the probe template includes a larger-than-optimal sequence, the DNA template can either be cleaved using a restriction enzyme that cuts the

DNA at an appropriate distance from the RNA transcription start site, or an appropriate restriction fragment can be released and purified from an agarose gel. Template DNAs can also be generated by PCR-amplification.

T7, T3, or SP6 polymerases are all suitable for making RNA probes. However, T7 polymerase tends to be the most consistently active and easy to use. While RNA probes can be labeled in a number of ways, including the incorporation of radioactive or fluorescent nucleotides, the simplest, safest, and most sensitive approach is to incorporate antibody-detectable hapten-tagged nucleotides such as digoxigenin (DIG)- or biotin-UTP (see Fig. 9.3.3A). Other small tags with available antibodies, such as fluorescein or Alexa dyes, can also be used with similar results. Using different tags/labels for different probes provides the ability to monitor multiple RNAs within a single sample. A good source for protocols using multiple RNA detection probes ("multiplex" in situ hybridization) can be found in Kosman et al. (2004).

The ratio of hapten-labeled UTP to unlabeled UTP is fixed to produce an optimally sensitive probe for hybridization. For most applications, this is one tagged residue per 20 to 25 nucleotides. This density permits optimal interactions between the hapten-labeled residues and bound antibody complexes (see the online non-radioactive in situ hybridization manual from Roche; *http://www.roche-applied-science.com/PROD_INF/MANUALS/InSitu/InSi_toc.htm*).

The following protocol describes the production of a DIG-labeled RNA probe.

IMPORTANT NOTE: Throughout the protocol, work on a clean bench, wear gloves, and use RNase/DNase-free tips and tubes to avoid probe degradation.

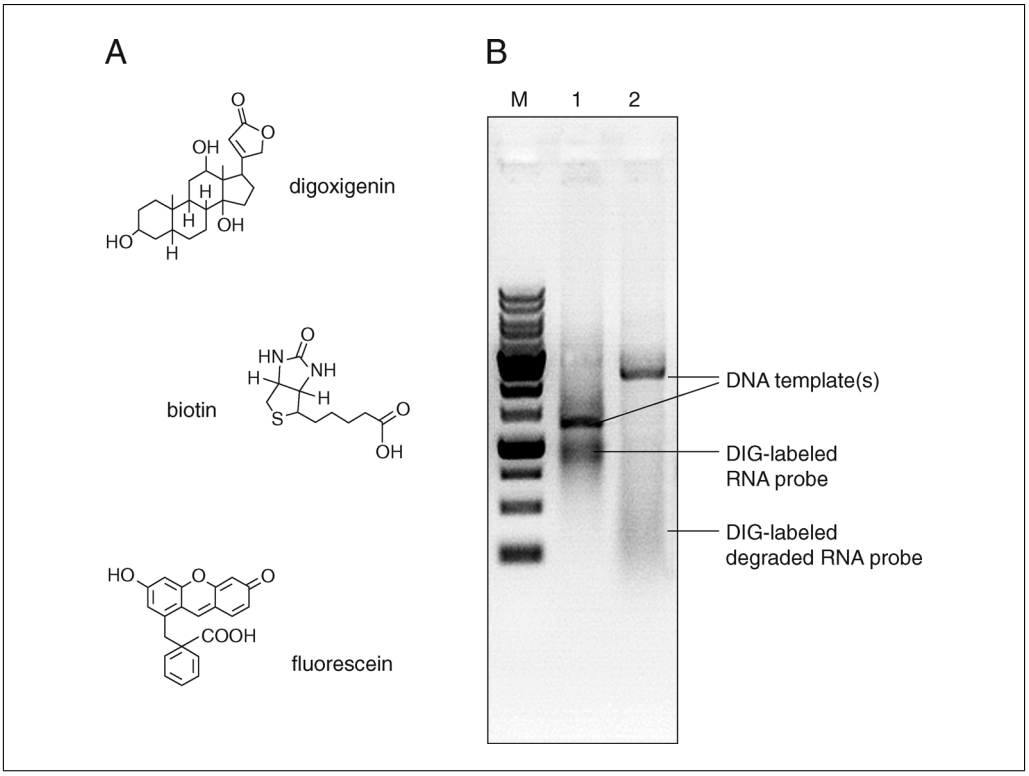

Figure 9.3.3 Probe labels and labeling. (**A**) Schematic representation of the structure of the most commonly used haptens. Haptens are small molecules that can induce an immune response when fused to a larger protein. Excellent antibodies that recognize each of these haptens are readily available. (**B**) Example of a good DIG-labeled RNA probe (lane 1) alongside a degraded RNA probe (lane 2) on a 1% agarose gel stained with ethidium bromide. Note that the DNA template migrates slower, and the RNA probe is relatively abundant. M, standard DNA size markers.

Materials

Template DNA
DIG-RNA labeling mix (Roche Applied Science, cat. no. 11 277 073 910)
10× transcription buffer (supplied with polymerase or 0.4 M Tris·Cl, pH 8.0; 60 mM $MgCl_2$; 100 mM dithiothreitol; 20 mM spermidine)
RNAguard RNase inhibitor (Amersham Biosciences, cat. no. 27 0816 01)
T3, T7, or SP6 RNA polymerase (Fermentas Life Sciences, cat. no. EP0101, EP0111, or EP0131, respectively)
Diethylpyrocarbonate (DEPC)-treated water (*UNIT 5.2*; autoclaved)
1% agarose gel
RNase-free glycogen (Fermentas, cat. no. R0551)
3 M sodium acetate, pH 5.2 (*UNIT 3.3*)
70% and 100% ethanol, cold

0.5- or 1.5-ml microcentrifuge tube(s) (RNase/DNase-free)
RNase/DNase-free tips
37°C incubator
Agarose gel electrophoresis equipment
Microcentrifuge

1. Place an RNase-free microcentrifuge tube on ice and mix the following: 0.5 to 1 μg template DNA, 2 μl DIG-biotin mix, 2 μl of 10× transcription buffer, 1 μl RNase inhibitor, 2 μl appropriate RNA polymerase, and make up the final volume to 20 μl with RNase-free DEPC-treated water.

   The template can be obtained from a linearized plasmid, purified restriction fragment, or PCR-amplified DNA (see UNITS 8.4 & 10.1). Make sure the probe that is produced is the antisense probe for the transcript. The sense probe of the same transcript can be used as a negative control.

2. Incubate 2 to 4 hr at 37°C.

3. Once probe synthesis is complete, add 30 μl of RNase-free water for a total volume of 50 μl. At this point, run a small aliquot (3 to 5 μl) on a 1% agarose gel.

   A visible ethidium bromide–stained RNA band that runs faster than the original DNA template should be observed (see example in Fig. 9.3.3B).

4. Precipitate probe(s) by adding 1 μg of RNase-free glycogen, 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol of cold 100% ethanol.

   The glycogen acts as a carrier that helps assure RNA precipitation and pellet recovery.

5. Mix well and store at −70°C for a minimum of 30 min or overnight.

6. Microcentrifuge 20 min at maximum speed, 4°C, and pour out supernatant.

   The inverted tube can be tapped gently against an absorbant Kimwipe-covered surface to remove most of the remaining alcohol. Careful aspiration or pipetting with RNase-free tips are alternative ways to remove supernatant.

   The precipitated RNA/DNA pellet should be visible at this point.

7. Gently add 100 μl of cold 70% ethanol, and microcentrifuge 10 min at maximum speed, 4°C.

8. Remove supernatant as in step 6, and allow to air dry.

   The pellet should not smell like ethanol if properly dried.

9. Resuspend pellet in 25 μl of RNase-free water. Pipet up and down a few times or vortex briefly to dissolve pellet.

10. Run 5 μl of probe on a 1% agarose gel with ethidium bromide staining.

Figure 9.3.3B shows an example of how the probe migration and intensity should appear.

11. Store probes up to 1 year at −70°C until needed.

Basic Protocol 2: Collection, Fixation, and Preparation of Embryos

Samples for in situ hybridization can range from whole embryos to tissues or sections on slides. In the case of embryos and some tissues, specific treatments may be required to remove protective structures or membranes (e.g., embryonic chorion). Subsequent steps may include permeabilization via organic solvents, detergents, and/or proteinase treatment to allow free movement of probes and antibodies. Sometimes, at more than one step, tissue fixation is necessary to keep structures intact and prevent mRNA breakdown or disbursement. Once properly prepared, samples can be stored in methanol or ethanol for extended periods (weeks to months at −20°C) prior to hybridization.

There are two major methods for fixation. These include the use of organic solvents or cross-linking agents. Organic solvents such as acetone or alcohol precipitate proteins and nucleic acids within their native cellular architecture, whereas formaldehyde and other cross-linkers bond proteins and nucleic acids to one another within their normal cellular context. Usually cross-linking provides superior results, but some epitopes can be obscured or altered during this process. In such cases, fixation with organic solvents is preferred. For example, the observation of many cellular cytoskeletal components is best detected using alcohol fixation (Kaku et al., 1983).

The following protocol describes the preparation of *Drosophila* embryos for hybridization. For tissues, the reader should proceed to Alternate Protocol 1. With embryos, the hard chorion is first removed, followed by fixation and removal of the secondary vitelline membrane. The embryos are then treated with proteinase K to increase mRNA accessibility, and fixed a second time to re-establish tissue integrity and protease destruction.

NOTE: Filter all final solutions through 0.2-μm filters.

Materials

Drosophila flies contained in cylinders/cages
Agar-fruit juice plates of appropriate size (see recipe)
1:1 chlorine bleach solution diluted in water (final concentration of ∼3% sodium hypochlorite; prepare fresh)
Heptane (HPLC-grade; do not use commercial grade or old bottles)
10× (40%) formaldehyde solution (see recipe)
1× PBS solution (see recipe)
Methanol (HPLC-grade)
PBT solution (see recipe)
20 mg/ml proteinase K stock solution (see recipe)
2 mg/ml glycine solution (see recipe)
RNA hybridization solution (see recipe)

Soft brushes
Collecting meshes/screens: 250- and 150-μm mesh (60 and 100 Mesh, Fisher Scientific, respectively)
20-ml glass scintillation vials or glass bottles with water-tight lids
Mechanical shaker
15- and 50-ml conical tubes (Falcon) or 0.5- or 1.5-ml microcentrifuge RNase-free tubes
96-well microtiter plates, optional

Wide-mouth or cut tips to transfer embryos
Nutator mixer or rocking platform
RNase-free tips

Collect and fix embryos

1. Set up healthy fly cylinders/cages. Use appropriately sized agar-fruit juice plates for collecting embryos.

   Refer to Campos-Ortega and Hartenstein (1997) or Hartenstein (1993) to determine collection times appropriate for observing developmental stages of interest.

2. Pre-collect embryos for 1 to 2 hr using a fresh agar-fruit juice collection plate.

   This step is especially important if collecting a particular stage of embryos. It clears the females of developing eggs that can be retained if food is old. Do not use this collection for experiments.

3. Collect embryos according to the embryonic stages that are needed.

   If all stages of embryogenesis are needed, use an overnight collection. However, early stages may be underrepresented.

4. Harvest the embryos by gently using tap water and a soft brush. Rinse into an appropriately sized mesh container. Repeat as necessary.

   A screen with a 150-μm mesh will retain embryos and allow water and solubilized food to pass through. A pre-screen mesh of 250-μm can be used to filter out adult flies, wings, chunks of food, etc. (see Fig. 9.3.4A).

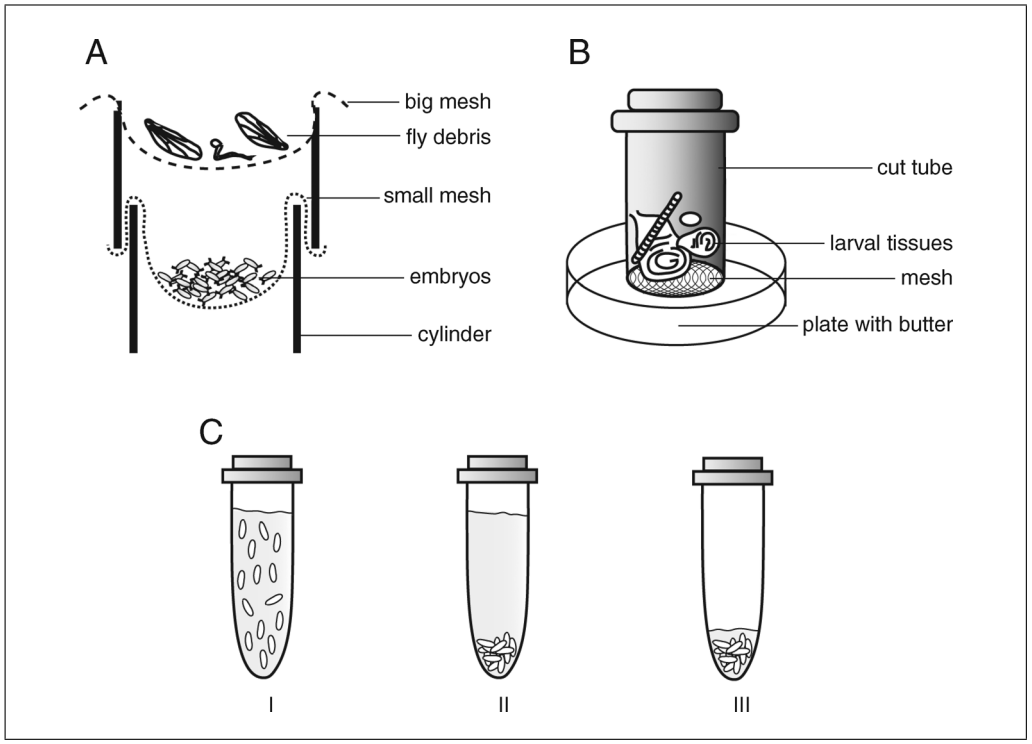

Figure 9.3.4 Technical tips. (**A**) Two different size meshes should be used to collect large amounts of embryos. The first one catches flies and debris, and the second catches the embryos. Water flows freely through both meshes. Dechorionate embryos on the smaller mesh, and rinse with excess water. (**B**) Tissues can be taken through the in situ protocol in a regular microcentrifuge tube, or in a tube cut at the bottom with a sealed mesh on the bottom. For every wash, or change of liquid, move the tube to a small plate with wash or buffer on it. (**C**)Throughout the protocols with embryos, make sure that embryos move around when new liquid is added and that the volume used for washes or rinses is appropriate for the tube of choice (I). Embryos should be at the bottom of the tube (II) before any liquid is removed. Always leave some liquid (III) to avoid drying.

5. Dechorionate the embryos in a 1:1 bleach/water solution for ~90 sec and rinse generously with ample water.

   This step can be done while the embryos are still on the smaller mesh, by dipping the screen container into the bleach/water solution, and gently shaking with circular motions. The majority of embryos should move to the surface once dechorionation is complete. A vigorous rinse with tap water will help to remove residual chorions as well as bleach. Check for chorion loss under a dissecting microscope. Embryos should look shiny and smooth with no dorsal appendages.

6. Transfer embryos to heptane by dipping the embryo-covered screen into the heptane solution (e.g., 20-ml glass scintillation vial containing 5 ml heptane).

   The heptane volume and container size is determined by the amount of embryos collected. Typically, for one or two small cylinder cages, a 20-ml glass scintillation vial with 5 ml heptane is ideal. For larger collections, a glass bottle with a tight sealing lid should be used. Many plastic tubes contain material or residue that interferes with fixation, and should therefore be avoided. A soft paintbrush can be used to transfer small numbers of embryos from screens to heptane.

7. Add an equal volume of freshly prepared 4% formaldehyde solution in PBS into the container with embryos and heptane (1:1 heptane/4% formaldehyde in PBS).

   Formaldehyde quality and freshness is crucial. Most commercially available solutions are of insufficient purity and activity for these purposes. Hence, it is best to prepare it using paraformaldehyde shortly before fixation.

8. Shake for 20 min to ensure uniform exposure of all embryo surfaces to both heptane and formaldehyde.

   Shaking does not have to be vigorous. However, embryos must move continuously within the heptane-fixative interphase to be exposed uniformly to the solvent and fixative. A rocking platform, rotator, or shaker can be used to automate this.

9. Swirl mixture and allow embryos to settle at the interphase. Remove the majority of heptane and PBS, leaving the embryos at the interphase. Add fresh heptane (same volume as removed) and an equal volume of methanol. Shake vigorously for 30 sec. Remove most of the heptane and methanol and repeat one additional time with fresh heptane and methanol.

   Embryos will start at the interphase of the heptane (upper) and PBT-formaldehyde (lower) layers. After shaking with methanol, most of the embryos will settle to the bottom as vitelline membranes are removed. The second heptane/methanol treatment helps to remove residual aqueous solution, which interferes with vitelline membrane removal.

   If fixing large amounts of embryos; embryos can be transferred to a new bottle containing fresh heptane and methanol.

10. Again, remove all of the liquid possible, replace with methanol (same volume as removed) and shake for 15 sec. Repeat two additional times.

    All embryos should now be at the bottom of the tube. Embryos can be stored in methanol for up to 1 year at −20°C.

Prepare embryos for hybridization

11. Transfer ~50 µl of settled fixed embryos for each hybridization planned, and prepare the embryos for all planned hybridizations together within a single appropriately sized tube.

    For example, for three genes/probes, use ~150 µl of settled embryos in a 1.5-ml microcentrifuge tube.

    For a full 96-well plate, use 2 ml of settled embryos in a 15-ml centrifuge tube.

Use the methanol in the container and a micropipettor with a wide (cut) tip to transfer embryos. Remove excess methanol after transferring embryos.

12. Wash embryos in fresh methanol once for 5 to 7 min.

    All washes can be done on a Nutator or rocking platform with the tube positioned horizontally. Allow all embryos to settle to the bottom of the tube before removing the methanol (see schematic in Fig. 9.3.4C). Do not remove all liquid; embryos should always be moist (see schematic in Fig. 9.3.4C).

13. Rehydrate embryos starting with a 1:1 mixture of methanol/PBT for 5 to 7 min.

    Make sure that there is excess liquid to rehydrate the embryos (as in Fig. 9.3.4C); for example, for a 1.5-ml tube, add ~1 ml of liquid and rock for at least 5 min; for a 15-ml tube, add ~10 ml of liquid and rock for 7 min.

14. Hydrate in 1× PBT for 5 to 7 min two times.

    Use the same volumes as in step 13.

15. Post-fix embryos 20 min in PBT plus 4% formaldehyde.

    Use 500 to 1000 μl for a 1.5-ml microcentrifuge tube; use 5 to 10 ml for a 15-ml tube.

16. Wash with PBT for 2 min three times.

17. Dilute the stock 20 mg/ml proteinase K in PBT to a final concentration of 2.67 μg/ml.

    Remove the proteinase K stock solution from −20°C freezer, place on ice, and thaw it slowly while preparing the embryos. It requires time to thaw, so thaw well in advance.

18. Add the 2.67 μg/ml proteinase K solution in PBT to the embryos.

    Add 500 to 700 μl of diluted proteinase K for a 1.5-ml microcentrifuge tube and 10 ml for a 15-ml tube.

19. Incubate 13 min at room temperature. Gently mix two or three times during this period by drawing up a small amount of the solution and jetting it below the embryos.

    This step is very important and may need optimization with every new batch of proteinase K to prevent excessive or poor digestion. If fractured embryos are observed at the end of the procedure, too much protease is being added to the embryos (and possible mishandling of the embryos may be occurring). If too little proteinase K is being used, signal strength will be suboptimal. In general, too little is better than too much.

20. Gently transfer embryos to ice and incubate for an additional 1 hr in proteinase K solution.

    This decelerated treatment allows the protease to penetrate and function more uniformly in all embryos and tissues.

21. Remove the proteinase K solution by carefully pipetting out the solution.

22. Add 2 mg/ml glycine solution and rock for 2 min. Repeat two times.

    Add 500 to 700 μl of glycine solution for a 1.5-ml microcentrifuge tube, and 10 ml for a 15-ml tube.

    The glycine acts to divert residual proteinase K activity.

23. Rinse embryos two times in PBT to remove the glycine.

    For this and subsequent steps, use ~1 ml of solution for a 1.5-ml tube and ~12 ml of solution for a 15-ml tube.

24. Post-fix embryos in PBT plus 4% formaldehyde, rocking for 20 min.

    Add 500 to 1000 μl PBT plus 4% formaldehyde for a 1.5-ml microcentrifuge tube and 5 to 10 ml for a 15-ml tube.

25. Rinse the embryos in PBT five times.

26. Rinse once with a 1:1 mixture of PBT and RNA hybridization solution.

27. Replace with 100% RNA hybridization solution.

    At this point, embryos can be stored for several days at −20°C. Embryos may appear to be relatively translucent and somewhat swollen at this stage. Handle carefully while in hybridization solution.

28. Carefully transfer embryos with a wide-mouth pipet tip or a cut-tip, to 0.5-ml micro-centrifuge tubes or a 96-well microtiter plate, and keep on ice or at 4°C and use immediately (<1 hr). Each tube/well will be used for a different probe.

    Embryos can be processed together up until this point, unless different genotypes are being analyzed. At this point, divide the embryos for use with individual probes. Subsequent protocols provide volumes of solutions to use with various sizes of micro-centrifuge tubes and/or microtiter plates.

    Note that microtiter plates can be cut into strips containing the necessary number of wells. These can be covered during hybridization with tape. For higher throughput, 96-well plates with filter-covered bottoms are also available for use with vacuum manifolds (Millipore, cat. no. MADV N65).

Alternate Protocol 1: Dissection, Fixation, and Preparation of Tissues

In this protocol, the dissection, permeabilization, and fixation of *Drosophila* larval tissues for subsequent hybridization are described. Similarly sized tissues from other developmental stages or organisms can be prepared in the same manner. Tissues from larvae and adults must be dissected appropriately to expose them to fixative and reagents. Alternatively, section the animals and mount the sections onto slides.

When HRP-based detection is to be employed (as in TSA staining), consideration must be given to the possible presence of endogenous HRP enzyme. While less of a problem with embryos, larval and adult tissues can have significant HRP activity, which is dealt with by pretreatment of fixed tissues with H_2O_2 before initiating the in situ protocol.

Additional Materials *(also see Basic Protocol 2)*

Well-fed larvae
PBTT plus 4% formaldehyde (from fresh 40% formaldehyde stock; see recipe)
PBTT (see recipe)
3% H_2O_2 in PBS, made fresh
Acetone, –20°C
Dissecting microscope and tools

1. Collect healthy, well-fed larvae (staged if appropriate) and dissect in cold PBS. Transfer dissected tissues into appropriately sized tubes containing cold PBS.

   For a general larval dissection, remove the posterior end of the larvae and invert the larva by pushing the head through the body and out the opening created in the posterior (similar to inverting a sock). This dissection method facilitates probe penetration to all tissues, and retains brain, CNS (central nervous system), ring gland, salivary glands, most imaginal discs, and much of the gut, trachea, musculature and fat bodies. If specific tissues must be analyzed, these can be dissected away at this time. However, fine dissection and orientation can also be done at the end of the in situ hybridization procedure before mounting.

   Although the dissected tissue can be placed into 1.5-ml microcentrifuge tubes for subsequent steps, mesh-bottomed tubes can also be used. These can be made within the laboratory for smaller studies or purchased in a microtiter plate format (Millipore). Tubes that are made in the laboratory are made by cutting off the bottom of a 1.5-ml microcentrifuge tube, melting the cut surface, and quickly adhering 150-μm mesh to the bottom, making

sure the whole perimeter is sealed (Fig. 9.3.4B). The mesh-bottomed tubes or plates are then transferred from one solution to the next (with blotting) at each step (except hybridization). This approach tends to retain and keep intact smaller tissues that could be removed or damaged by pipetting. Commercially available 96-well plates with meshes or filter-covered bottoms also exist for either manual washes (Millipore MultiScreen-Mesh filter plates) as described above, or for use in vacuum manifolds (Millipore, cat. no. MADV N65). These are more suitable for use in high-throughput applications.

2. Fix tissues for 20 min rocking in PBTT plus 4% formaldehyde.
3. Wash three times, 5 min each wash, with PBT on a rocking platform.

   For this and subsequent steps, use ~1 ml of solution for a 1.5-ml tube and ~400 µl of solution for a 0.5-ml tube. Remove all solutions by carefully pipetting off the solution.

4. Rinse two times with PBS to remove detergent and prevent excess bubble formation in the next step.
5. Quench endogenous HRP using 350 µl (enough to cover all tissue) of 3% H_2O_2 in PBS. Incubate 15 min at room temperature and keep the tube open to prevent gas buildup.
6. Rinse two times with PBT.
7. Wash two times with PBT, 10 min per wash.
8. Incubate 10 min in 500 µl cold 80% acetone at –20°C.

   This provides additional tissue permeability making protease treatment unnecessary.

9. Wash two times, 5 min per wash, with PBT on a rocking platform.
10. Post-fix for 10 min in PBT plus 4% formaldehyde.

    This step may not always be necessary, but if performed, renders tissues more firm and resilient.

11. Wash five times, 2 min per wash, with PBT rocking on a platform.
12. Rinse with 1:1 mixture of PBT and RNA hybridization solution.
13. Rinse in RNA hybridization solution.
14. Store 1 to 4 weeks at −20°C until needed in hybridization solution.

Basic Protocol 3: Hybridization

This protocol includes introduction of the fixed samples into hybridization buffer, heat denaturation of both the target and probe mRNAs, and then an overnight hybridization at an optimal temperature, which for RNA probes is ~56°C. The most critical parameters here are probe quality, concentration, and mixing. Positive and negative controls should be included. Controls to be considered, along with their purpose, are summarized in Table 9.3.2.

Materials

RNA hybridization solution (see recipe)
Embryos or tissues
DIG-labeled RNA probes (see Basic Protocol 2)
PBT (see recipe)

Boiling heating block or water bath
56°C stable sand bath, water bath, or oven

Table 9.3.2 Important Controls

Control	Significance
No probe	Negative control: indicates levels of general background (see Fig. 9.3.6A)
A probe that has been successfully tested for that tissue or stage	Positive control: validates quality and functionality of the protocol and reagents
Sense strand control	Negative control: demonstrates specificity of the antisense probe
Use of multiple probes for same sequence	Positive control: confirms probe specificity

Prehybridize

1. Boil RNA hybridization solution for 5 min.

   Use ~200 to 300 μl per 0.5-ml tube; use ~800 μl per 1.5-ml tube; use ~100 μl per well of a 96-well PCR-plate (10 ml total for a full plate).

2. Quickly cool the tube on ice.
3. Leave on ice for 5 min.
4. Remove liquid from embryos or tissues by carefully pipetting off the liquid without disturbing the embryos or tissues.
5. Add cooled hybridization solution (see step 1 for volumes) to each sample at room temperature.
6. Incubate sample for at least 2 hr at 56°C in a sand or water bath.

   IMPORTANT NOTE: *This is a critical step. Pre-hybridization with single-stranded DNA prevents nonspecific binding of probe. Prehybridized samples should be hybridized with probe on the same day.*

Hybridize

7. Prepare a probe-specific solution by diluting ~50 ng of DIG-labeled RNA probe in 100 μl of RNA hybridization solution (for embryos) or 100 ng of DIG-labeled RNA probe in 200 μl of RNA hybridization solution (for larval tissues).

   Approximate probe concentration can be determined by comparing the intensity of staining on a gel to known amounts of RNA controls.

8. Approximately 10 to 15 min prior to the end of the pre-hybridization, heat the diluted probe to 80°C for 3 min.
9. Cool on ice for 5 min.

   IMPORTANT NOTE: *Denaturation of probe is a critical step. Single-stranded RNA probes tend to have extensive secondary structure, which can interfere with hybridization to their cellular targets. Heat denaturation and snap-cooling renders linear conformations for more effective hybridization.*

10. Remove pre-hybridization solution from embryos or tissues.
11. Quickly, add the probe solution, making sure that the embryos or tissues move around when the solution is added.

    Using a pipetman to jet the solution under the embryos or tissue can help to ensure uniform access of probes to samples.

12. Hybridize 12 to 16 hr (overnight) at 56°C in a sand or water bath.

Wash

Nonspecific hybrids are less stable than specific hybrids. They can be dissociated and removed by proper washing of tissues or embryos.

13. Pre-warm the following solutions at 56°C:

    RNA hybridization solution (enough for two washes)
    3:1 RNA hybridization solution/PBT (for one wash)
    1:1 RNA hybridization solution/PBT (for one wash)
    1:3 RNA hybridization solution/PBT (for one wash)
    PBT (enough for four washes).

    Prepare 400 μl of solution per each 0.5-ml tube, or ~800 μl for each 1.5-ml tube. For a full 96-well plate, prepare 10 ml per wash; add 100 μl per well using a multichannel pipettor and a sterile dish.

14. Carefully remove the probe from the embryos or tissue.

    The used probe(s) can be stored at −80°C and used again (up to two additional times) with equivalent, if not better, results.

15. Wash with pre-warmed hybridization solution for 15 min at 56°C two times.

    This and subsequent washing steps must be done quickly to keep the embryos or tissues from cooling-down to room temperature. Keep all solutions at 56°C until ready to use.

16. Wash with pre-warmed 3:1 hybridization solution/PBT for 15 min at 56°C.

17. Wash with pre-warmed 1:1 hybridization solution/PBT for 15 min at 56°C.

18. Wash with pre-warmed 1:3 hybridization solution/PBT for 15 min at 56°C.

19. Wash with pre-warmed PBT for 5 min at 56°C four times.

20. Cool embryos or tissues to room temperature. For longer storage, keep at 4°C for no longer than 1 day.

Basic Protocol 4: Probe Detection Using Alkaline Phosphatase (AP)

This protocol describes a relatively simple, quick, and inexpensive visible color-based detection system for DIG-labeled probes. The alkaline phosphatase (AP)–based system produces a strong and easily observed blue reaction product (Figs. 9.3.1 and 9.3.5A,C). However, since the AP reaction product is diffusible, resolution of the signal within cells can be poor. These signals can also obscure tissues below or other signals produced when using multiple labeling. Hence, it should not be used when high levels of resolution or overlapping expression patterns are of interest.

A protocol for tyramide signal amplification (TSA) detection of hybridized DIG-labeled probes in *Drosophila* embryos, together with DAPI staining to visualize nuclei, is provided in Alternate Protocol 2.

Materials

PBTB (see recipe)
Embryos or tissues in PBT
Anti-digoxigenin-AP Fab fragments (Roche Applied Science), store at 4°C
Alkaline phosphatase buffer (AP buffer; see recipe)
PBT (see recipe)
AP developing solution (see recipe)
100% ethanol
Rocking platform

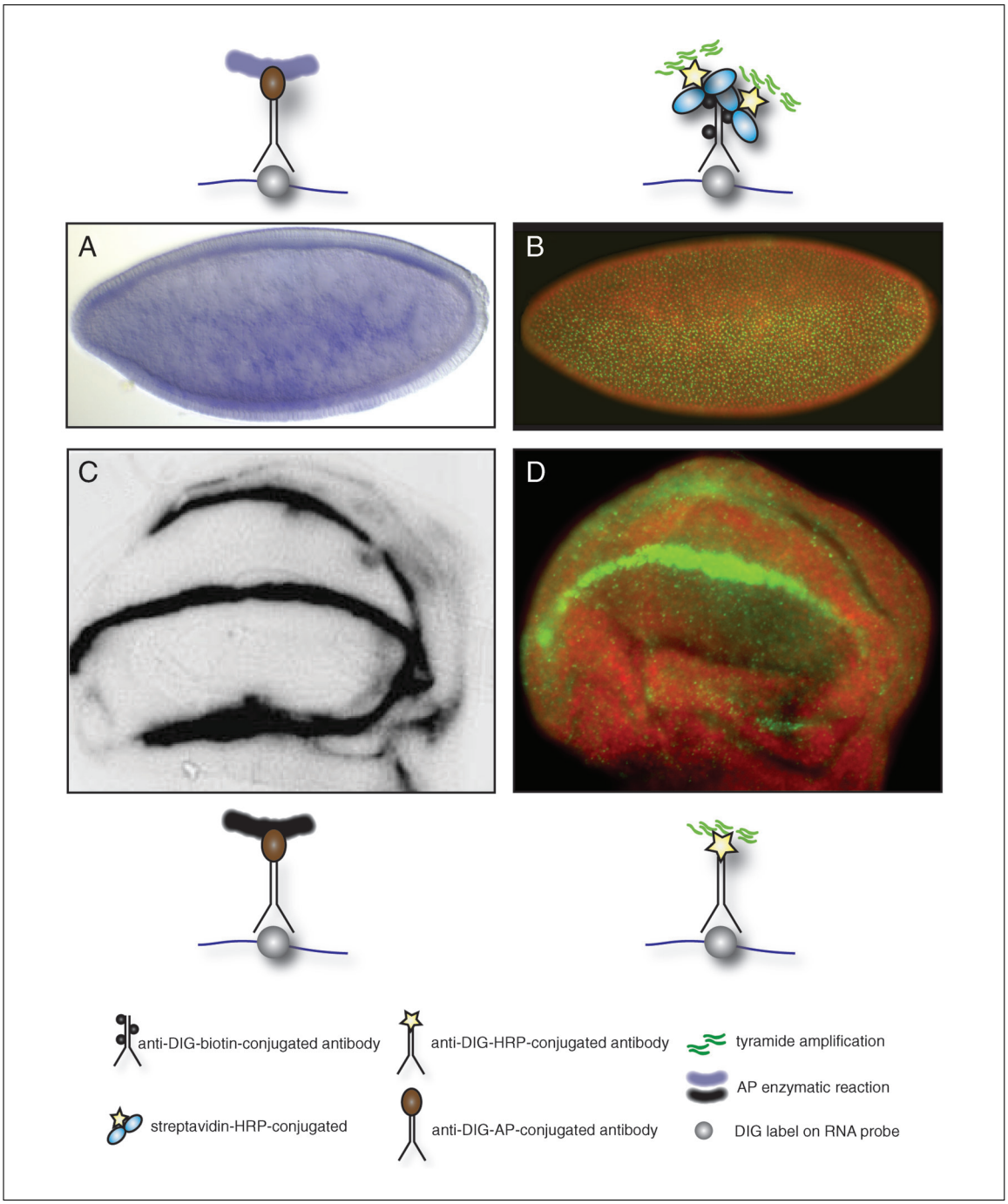

Figure 9.3.5 Alternative methods to detect DIG-labeled probes. (**A,B**) In situ hybridization of stage 5 *Drosophila* embryos. In situ hybridization was performed with the same DIG-labeled probe (CG9461; FBX011 homolog). In the first case (A; Tomancak et al., 2002, 2007; reproduced with permission), DIG was detected with an anti-DIG antibody conjugated with AP, followed by a colored enzymatic reaction. The signal appears diffuse and was annotated as ubiquitous (*http://www.fruitfly.org/cgi-bin/ex/bquery.pl?qtype=report&find=CG9461&searchfield=CG*) by the BDGP public database. In contrast, the embryo in panel B was developed using TSA, along with DAPI (pseudo-colored red) to visualize nuclei. This embryo shows specific zygotic transcription in the ventral half of the embryo; very little transcript is detected in the dorsal part and nothing in the pole cells. (**C,D**) In situ hybridization performed on a third instar larval wing disc for the *wingless* (*wg*) transcript. The wing disc shown in panel C (Nagaso et al., 2001; reproduced with permission) was developed with an anti-DIG-AP conjugated antibody, followed by a colored reaction against the phosphatase. The wing shown in panel D was developed with an anti-DIG-HRP-conjugated antibody, followed by tyramide amplification.

Block prior to DIG detection

All antibody incubations and washes are done in PBS buffer containing PBTB, which helps to reduce nonspecific protein interactions, and therefore background. All incubations and washes are done at room temperature with rocking.

1. Prepare PBTB.
2. Remove PBT solution from embryos or tissues.
3. Block embryos or tissues for 10 min in 1% PBTB.

   Volumes used are proportional to tube size; use 400 μl of solution per 0.5-ml tube, or ~800 μl for a 1.5-ml tube. For a full 96-well plate, prepare 10 ml per wash, add 100 to 120 μl per well using a multichannel pipettor and a sterile dish.

Incubate with primary antibody

4. Incubate embryos or larval tissues with anti-DIG antibody coupled to alkaline phosphatase—diluted to a final concentration of 1:1000 in PBTB (enough volume to cover tissues two times) and incubate 2 hr at room temperature while rocking.

   This incubation can also be done overnight at 4°C.

5. Wash three times with PBTB, 5 min per wash.
6. Wash four times with PBT, 20 to 30 min per wash.

Develop AP enzymatic colored reaction

7. Rinse embryos or tissues with PBT at room temperature once.
8. Wash two times in alkaline phosphatase buffer, 5 to 10 min each time at room temperature with rocking.
9. Remove alkaline phosphatase buffer.
10. Add AP developing solution (same volumes as in step 3), mix occasionally.

    The time of the reaction varies depending on the RNA that is being detected. Samples must be observed occasionally with a dissecting microscope (about every 10 min) to determine when staining is complete. Note that the stain will appear somewhat weaker after subsequent steps and higher magnification.

11. Stop the reaction with three changes of PBT.
12. Rinse six times in 100% ethanol.
13. Rinse in PBT.

Alternate Protocol 2: Probe Detection Using TSA

By using primary or secondary antibodies directly labeled with fluorochromes, resolution can be greatly enhanced, but at a detriment to sensitivity. However, this lack of sensitivity can be countered by signal amplification, which is best done by tyramide signal amplification (TSA). Tyramide, a substrate for the enzyme HRP, binds covalently to the tyrosine residues of nearby proteins upon processing by the enzyme. When the tyramide is conjugated to fluorescent dyes, a highly amplified and localized signal is produced (see Figs. 9.3.1, 9.3.5, and 9.3.6). Different levels of amplification can be provided using biotin- and streptavidin-conjugated antibodies and enzyme, and alternative wavelength signals can be produced by using different fluorescently conjugated tyramide reagents. This provides the potential for multiple mRNA detection. Other cellular organelles, such as nuclei, mitochondria, and membranes, can also be visualized at the same time by including appropriate fluorescent markers during the last steps of the procedure.

Antibody dilution is a critical factor in this protocol. In TSA-based methods, it is often the secondary antibody dilution that determines the amount of nonspecific signal or background. An optimal concentration of HRP conjugates also needs to used to ensure that background levels are not high. The amounts used below are a good starting point, but reagents may vary.

Washing extensively after each antibody incubation reduces nonspecific background significantly.

This protocol describes tyramide signal amplification (TSA) detection of hybridized DIG-labeled probes in *Drosophila* embryos, together with DAPI staining to visualize nuclei.

***Additional Materials** (also see Basic Protocol 4)*

Anti-DIG antibody:
- For embryos: biotin-conjugated mouse monoclonal anti-DIG (1/400 dilution of a 1 mg/ml stock solution in PBTB; Jackson ImmunoResearch Laboratories, cat. no. 200-062-156) *or*
- For tissues: HRP-conjugated mouse monoclonal anti-DIG (1/500 dilution of a 1 mg/ml stock solution in PBTB (Jackson Immuno-Research Laboratories, cat. no. 200-032-156)

100× DAPI (4′,6-diamidino-2-phenylindole) solution (0.1 mg/ml; Sigma, cat. no. D-9542)

Streptavidin-HRP conjugate (1/1000 dilution from a 1 mg/ml stock solution in PBTB (Molecular Probes, cat. no. S991) (for embryos)

Fluorescent conjugate:
- Cy3 tyramide conjugate (1/50 dilution of stock solution in amplification buffer; Perkin Elmer Life Sciences, cat. no. SAT704A) *or*
- Alexa Fluor 488 tyramide conjugate (1/50 dilution of stock solution in amplification buffer; Molecular Probes, cat. no. T-20932)

Block prior to DIG detection

1. Prepare 1% PBTB.
2. Remove PBT solution from embryos or tissues.
3. Block embryos or tissues for 10 min in 1% PBTB.

   Volumes are proportional to tube size; use 400 μl of solution per 0.5-ml tube, or ~800 μl for a 1.5-ml tube. For a full 96-well plate, prepare 10 ml total per wash and add 100 to 120 μl per well using a multichannel pipettor and a sterile dish.

For embryos, primary antibody incubation using biotin-avidin-HRP

4a. Prepare a fresh dilution of the stock anti-DIG-biotin antibody in 1% PBTB for a final concentration of 2.5 μg/ml.

5a. Remove most of the blocking solution from embryos and add 100 μl of diluted antibody.

6a. Incubate 1.5 to 2 hr at room temperature with rocking.

   Optionally, the primary antibody incubation can be done overnight at 4°C.

7a. Remove used antibody solution from embryos.

8a. Rinse embryos two times with PBTB.

   Use 400 μl per 0.5-ml tube per wash/rinse. For a full 96-well plate, prepare 11 ml per wash and add 100 to 120 μl per well with a multichannel pipettor and sterile dish.

9a. Wash with PBTB at least six times, 10 to 15 min per wash (~1.5 hr in total). Proceed to step 10.

For tissues, primary antibody incubation using HRP

4b. Remove the majority of blocking solution from tissues.

5b. Add 200 to 300 µl of 1/500 dilution of HRP-conjugated anti-DIG antibody in 1% PBTB for a final concentration of 2 µg/ml and incubate 1.5 to 2 hr at room temperature with rocking.

6b. Remove used antibody from tissue and rinse with PBTB two times (use ~700 to 900 µl for a 1.5-ml tube or use ~400 µl for a 0.5-ml tube).

7b. Wash at least five times with PBTB, 10 to 15 min per wash.

For nuclei counterstaining, dilute DAPI or propidium iodide in 1% PBTB and use this for the second wash. Make sure to keep these tissues in the dark for the remaining steps of the protocol.

8b. Wash with PBT for 5 min.

9b. Wash with PBS two times, for 5 min each wash. Proceed to step 19.

Secondary antibody incubation (for embryos only)

10. Dilute the streptavidin-HRP stock to 1 µg/ml in PBTB just prior to use.

11. Carefully remove most of the PBTB from embryos.

12. Add 75 µl of diluted 1 µg/ml streptavidin-HRP to embryos.

13. Incubate 1.5 to 2 hr at room temperature with rocking.

14. Remove used antibody solution from embryos.

15. Rinse embryos two times with PBTB.

Use 400 µl per 0.5-ml tube per wash/rinse. For a full 96-well plate, prepare 11 ml per wash and add 100 to 120 µl per well with a multichannel pipettor and a sterile dish.

16. Wash six times with PBTB as in step 9a over a ~1.5-hr period, 10 min per wash rocking.

For nucleic acid counterstaining, dilute DAPI or propidium iodide in 1% PBTB and use this for the second wash. If used, then embryos should then be kept in the dark for all subsequent steps.

17. Wash 15 min with PBT.

18. Rinse with PBS two times.

Embryos can be stored overnight at 4°C at this point.

Amplify tyramide signal

IMPORTANT NOTE: Perform all washes and incubations in the dark. Tyramide dilutions may need to be optimized.

19. Wash embryos or tissues in PBS for 5 min at room temperature three times.

Use 400 µl of solution per each 0.5-ml tube per wash or rinse. For a full 96-well plate, prepare 11 ml per wash and add 100 to 120 µl per well with a multichannel pipettor and a sterile dish.

20. Dilute tyramide solution 1:50 in amplification buffer (supplied with tyramide reagent).

21. Remove PBS from embryos or tissues and add 50 µl of the tyramide solution.

    Use 50 µl per tube. For a full 96-well plate, prepare 5 ml of diluted tyramide solution and add 50 µl per well with a multichannel pipettor.

22. Rock for 2 hr at room temperature.

23. Remove tyramide solution and rinse embryos three times with PBS.

24. Wash ten to twelve times in PBS, 10 min per wash.

    Many washes are necessary to remove the excess tyramide from the embryonic tissues.

    Ideally, the last PBS wash is removed and anti-fade solution is added immediately. The embryos will slowly start sinking. They can be mounted after they sink to the bottom, or store up to 1 week at 4°C.

Basic Protocol 5: Mounting Samples and Microscopy

Fine dissection will be required for this protocol if whole larvae were used. Carefully remove unwanted tissue, and orient discs, etc., properly with sharp needles before covering with the coverslip. Avoid damaging tissues and avoid placing tissues on top of one another. Time and patience is necessary to produce better images and data.

Materials

Embryo or tissue samples
Anti-fade mounting solution (see recipe) or 70% glycerol
Clear nail polish

Wide-mouth or cut tips to transfer embryos or tissue
Microscope slides
Microscope coverslips (22 × 22–mm)
Fluorescence or confocal microscope equipped with all the required filters and a sensitive digital camera

1. Remove the last solution in which the samples were stored after probe detection.

2. Add ~100 to 200 µl of anti-fade mounting solution and store at 4°C until embryos or tissues fall to the bottom of the tube.

   The embryos or tissues will float initially when mounting solution is added and slowly clear and fall to the bottom of the tube. Avoid moving the tube for 5 to 10 min after the addition of the mounting solution.

   If a non-fluorescent reaction was done, mount in 70% glycerol (no need to use the anti-fading reagent).

   Embryos or tissues can be stored at 4°C in the dark in this solution for as much as 1 month or longer.

3. Using a wide-bore pipet, mount embryos or tissues on a microscope slide, using minimum mounting fluid to transfer, and spreading them evenly on the slide on the area where the coverslip will be placed. Add appropriately sized coverslip and seal with clear nail polish. When properly sealed, slides can be kept for long periods (months) in the dark at 4°C.

   Make sure to use wide bored tips or cut tips for transfer to avoid any damage to tissues or embryos. Fine dissection of tissues can still be done at this point.

4. Analyze on a microscope using appropriate filters and objectives.

   On the first day, fluorescent background will be higher. Waiting a couple of days will improve photo images. Analyze positive and negative controls to ensure everything worked as expected, and to detect nonspecific background.

5. Refer to Figures 9.3.5 and 9.3.6 to help with the interpretation of the results.

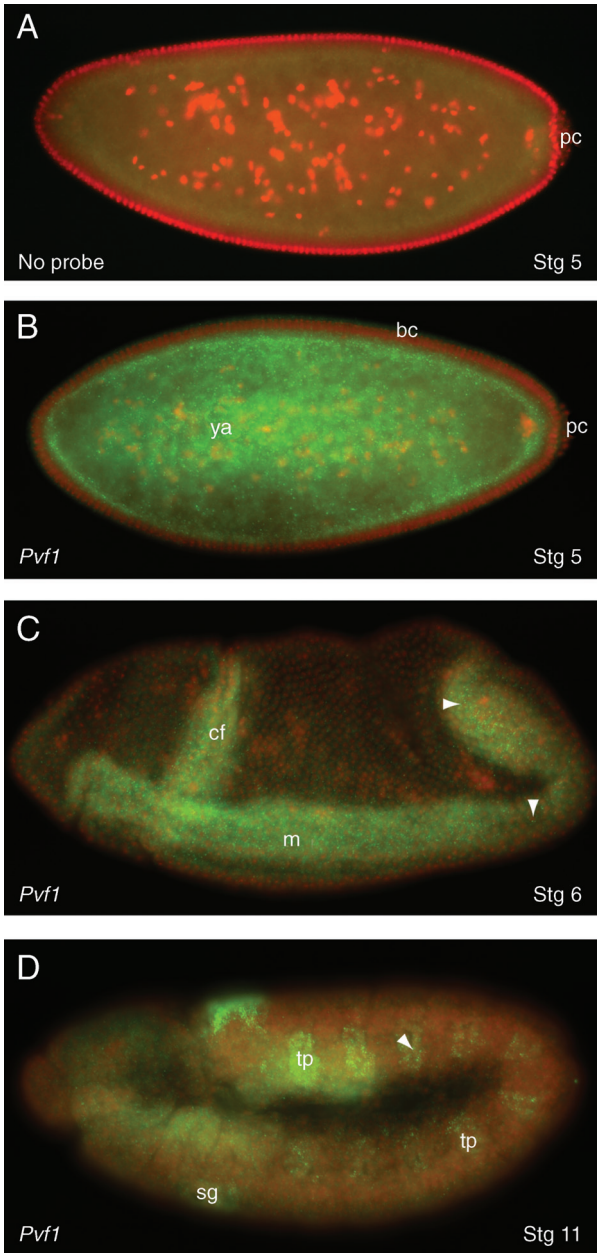

Figure 9.3.6 Interpreting results. This figure shows an example of the *Pvf1* transcript at various stages of embryonic development, to help with interpretation of results. The DIG-labeled probe was detected as described in Figure 9.3.5B. (**A**) A no-probe control showing a stage 5 embryo co-stained with DAPI (pseudo-colored red). This is what is normally used as a negative control. (**B,C,D**) Stages 5, 6, and 11 embryos, respectively, that show DAPI (red) and a DIG-labeled RNA antisense signals for the *Pvf1* gene transcript (green). At stage 5 (B), the RNA is detected as 'dots' inside the yolk-area (ya) of the embryo and it is absent everywhere else (pole cells (pc) and blastoderm cells (bc). At stage 6 (C) during gastrulation, *Pvf1* RNA is found in the mesoderm (m) and the cephalic furrow (cf). Zygotic transcription is detected as two bright dots inside some of the nuclei (arrowheads). By stage 11 (D) this transcript is seen in invaginating tracheal pits (tp), the salivary gland primordium (sg), and other tissues. Some zygotic transcription is detected in expressing cells (arrowhead).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Agar-fruit juice plates

700 ml water
25 to 30 g agar
300 ml juice concentrate (apple or grape)
0.5 g methyl paraben (*p*-hydroxymethylbenzoate)
20 ml 95% ethanol

Autoclave agar and water for 40 min to make up solution A. Add 20 ml of 95% ethanol to the methyl paraben vial. Add this solution to the 300 ml juice concentrate to make up solution B. Mix solutions A and B together and pour into the appropriately sized vessel. This must be done fast, before the agar solidifies.

Alkaline phosphatase buffer (AP buffer)

0.1 M NaCl
0.05 M $MgCl_2$
0.1 M Tris·Cl, pH 9.5 (*UNIT 3.3*)
0.1% Tween-20
Prepare fresh before use

Anti-fade mounting solution

70% glycerol
2.5% DABCO (1,4-diazabicyclo [2.2.2.] octane; Sigma-Aldrich, cat. no. D-2522)
1× PBS (see recipe)

In a light-shielded tube, add 1.25 g of DABCO crystals, 15 ml of 1× PBS, and 35 ml of glycerol and mix on rocking platform until the solution is homogeneous. Store up to 1 year at –20°C in the dark.

AP developing solution

For 10 ml developing solution, add:
45 µl 75 mg/ml nitroblue tetrazolium (NBT; Roche Applied Science) in 70% dimethylforamide (30% distilled H_2O)
35 µl 50 mg/ml bromochloro indoyl phosphate (BCIP; Roche Applied Science) in 100% dimethylformamide
10 ml AP buffer (see recipe)
Prepare just before use

Formaldehyde solution (40% and 4%)

For 40% stock solution:

Prepare fresh on the day of use from paraformaldehyde. In scintillation vial, mix 0.92 g of paraformaldehyde in 2.5 ml of distilled water containing 35 µl of 1 N KOH or NaOH. In a fume-hood, dissolve the paraformaldehyde by carefully heating to 70° to 80°C on a hot plate (setting 6 to 7), with gentle swirling. Note that fumes and solution are caustic and potentially carcinogenic. The solution will become clear once temperature is reached and the paraformaldehyde de-polymerizes into formaldehyde. Once the solution cools down, filter through a 0.45-µm filter and store at 4°C until ready for use. Scale up the recipe if a larger volume is required.

For 4% fixation solutions, dilute the 40% stock solution 1:10 in PBS, PBT, or PBTT (see recipes) as prescribed.

CAUTION: *Formaldehyde should be handled and disposed of according to manufacturer's instructions.*

Glycine solution (2 mg/ml)

Dissolve glycine in PBT (see recipe) to a concentration of 2 mg/ml. Store indefinitely at room temperature.

PBS, 1×

8 g NaCl
0.2 g KCl
0.24 g KH_2PO_4
2.72 g $Na_2HPO_4 \cdot 7H_2O$
800 ml DEPC-treated water (*UNIT 5.2*)
Adjust pH to 7.4 with HCl
Adjust volume to 1 liter
Sterilize by autoclaving
Alternatively, prepare a 10× stock and dilute as needed with DEPC-treated sterile water (keep indefinitely at room temperature)

PBT solution

1× PBS (see recipe)
0.1% Tween-20
Use DEPC-treated sterile water (*UNIT 5.2*)
Store up to 2 months at room temperature

PBTB solution

1× PBS (see recipe)
0.1% Tween-20
1% milk powder
Prepare fresh

PBTT solution

PBT (see recipe)
0.1% picric acid
0.3% Triton-X 100
Prepare fresh

Proteinase K (20 mg/ml)

Dissolve proteinase K (Sigma Aldrich, cat. no. P2308) in double distilled water to a concentration of 20 mg/ml and store 25-µl aliquots up to 1 year at –20°C (do not freeze and thaw more than two times).

RNA hybridization solution

50% formamide
5× SSC (see recipe)
100 µg/ml heparin
100 µg/ml sonicated salmon sperm DNA (Sigma-Aldrich, cat. no. D9156)
0.1% Tween-20
Filter through a 0.2-µm filter
Store up to 1 year at –20°C

SSC, 20×

For 1 liter, dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of distilled RNAse-free water. Adjust the pH to 7.0 with a few drops of 1 M HCl. Adjust the volume to 1 liter with additional distilled RNAse-free water. Sterilize by autoclaving.

Table 9.3.3 List of Potential Problems and Solutions

Problem	Possible solutions
No signal in in situ reaction	Check probe integrity on a gel (Fig. 9.3.3B, lane 1). Expression of the gene may be low. Confirm expression at a given stage by other methods. Use different or multiple probes that recognize different parts of the transcript.
High background	Concentrations of antibodies or tyramide may be too high; optimize concentrations using a dilution series to obtain best signal to noise ratio. Increase number of washes/washing time.
Minimal background in control but the samples have high background	Check the probe, as degraded probes usually give higher background. Reduce probe concentration.
Positive controls work, but other probes do not	Expression of the gene is low. Confirm expression at a given stage by other methods. Use different or multiple probes that recognize different parts of the transcript. Ensure probe integrity on a gel.
Quenching of the signal	Use freshly made anti-fade reagent in the glycerol-based mount. Work and store samples in dark.
Uneven staining of cells or tissues	Embryos were not shaken while fixing with heptane. Protease digestion was incomplete. Check concentration/dilution and titrate protease before use. Probe and tissues were not mixed thoroughly before/during hybridization.
Embryos or tissues are fragmented or misshapen	Reduce amount of protease used. Improve handling/manipulation skills. When transferring specimens, make sure pipet tips have been cut to allow multiple embryos/tissues to easily pass through. Check buffer concentrations/composition. Ensure that all fixation steps have been carried out and that formaldehyde is freshly prepared and diluted.

COMMENTARY

Understanding Results

Refer to Figures 9.3.5 and 9.3.6 for detailed examples. Figure 9.3.6 shows a more detailed example of patterns of RNA localization in whole embryos at different stages of development, where tyramide amplification was used (Lécuyer et al., 2007, 2008).

To achieve better contrast, in all the figures shown, the signal from DAPI has been pseudo-colored in red (normally blue), and the signal from the Cy3-Tyramide (mRNA transcript) in green (normally red).

Troubleshooting

Table 9.3.3 summarizes some of the most common problems and troubleshooting tips.

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Internet Resources

http://www.fruitfly.org/about/methods/RNAinsitu.html

Berkeley Drosophila Genome Project—BDGP Resources—96-well RNA in situ hybridization protocol.

http://www.sdbonline.org/fly/atlas/00atlas.htm

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http://www.roche-applied-science.com/PROD_INF/MANUALS/InSitu/InSi_toc.htm

Nonradioactive in situ hybridization application manual—Roche Diagnostics.