

***In situ* Hybridization Using Antibody-Based Methods**

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Introduction

In situ hybridization (ISH) is a term that collectively refers to histochemical techniques that reveal the location of cells that contain specific nucleic acid sequences. In its broadest applications, ISH has been used to detect all types of DNA and RNA, including chromosomal DNA, intronic RNA, ribosomal RNA, and mRNA. Fundamentally, all ISH techniques involve the hybridization of single nucleic acid strands that incorporate a label with complementary strands in cells. The hybridization events are made visible using various histochemical techniques, including, most often, autoradiography, enzyme cytochemistry, or fluorescent molecules. The essential challenge for ISH protocols is to obtain a robust hybridization signal that can be interpreted as specific rather than nonspecific or spurious.

The literature is rich with descriptions of ISH protocols and their applications to almost every conceivable tissue and cell type (Knoll et al., 2007). Many of the common ISH protocols were developed almost 20 years ago and are still reliable; they can be found in older literature and monographs. In most instances, ISH protocols take one of two basic approaches: radioactive (isotopic) or nonradioactive (nonisotopic). Radioactive techniques require the use of ISH probes that are labeled with a radioactive atom, most often ^{35}S or ^{33}P , but sometimes ^{125}I or ^3H . Radioactive ISH procedures use liquid autoradiographic emulsion or autoradiographic film to localize the ISH events. Nonradioactive ISH techniques use standard histochemical and immunocytochemical methods in order to identify the cellular location of ISH events, which are most often detected by bright-field or fluorescence microscopy.

For this chapter, it is assumed that readers are familiar with the elements of autoradiography, histochemistry, and immunocytochemistry, including the appropriate reagents and controls for each. Our discussion will focus mainly on the application of immunocytochemical methods to ISH, and place some emphasis on multiple labeling techniques. Because the protocols presented here are based principally on the practices in our laboratory, the recommendations are founded largely on anecdotal and empirical experience; we have found these methods to work well with the tissues, probes, reagents, and antibodies used in our laboratory. However, nothing presented here should be considered dogmatic or definitive. As in most labs, we have worked with these protocols until we have obtained usable data for our particular investigations; rarely have we spent undue time and resources to optimize each reagent, pH, temperature, time, or dilution. It is our usual practice

to use standard protocols and adapt them to our particular needs, so there is nothing particularly mysterious or exotic about the procedures or reagents. That being said, there is no need to abandon or modify the protocols other labs employ if they are working. The protocols presented here can be considered starting points if you are new to nonradioactive ISH or as possible new approaches to consider if you have not tried them yet. We highly recommend the protocols and information about ISH by Scott Young and Éva Mezey, found at <http://intramural.nimh.nih.gov/lcmr/snge/Protocols/ISHH/ISHH.html> (Young and Mezey, 2004).

In our experience, performing ISH with antibodies is simple and reproducible, provided that good laboratory techniques are followed, the tissues are properly prepared, and the reagents (especially the probes) are specific. Simply stated, nonradioactive ISH with antibodies is no more difficult than routine immunocytochemistry: all the admonitions, precautions, controls, and techniques that one uses for routine immunocytochemistry apply equally to nonradioactive ISH.

Tissue Preparation

Ideally, for ISH, it is desirable to optimize both tissue morphology and preservation of mRNA. The key question here is whether to use fixed tissue or unfixed tissue. The methods adopted should be determined empirically, for no hard and fast rules apply equally to all tissues and mRNAs. We find that it is usually best to begin with slide-mounted cryostat sections of tissue that have been initially frozen without fixation. Doing so also allows the tissue to be used for other purposes, such as receptor binding and PCR analysis. Fresh tissues are frozen in isopentane cooled with dry ice, placed in small plastic bags, and stored at -80°C . If tissues are to be embedded in OCT compound, they should be immersed in OCT in a mold before freezing (the same holds for fixed tissues). Avoid putting frozen tissues directly into OCT compound. We have not found the OCT compound to interfere with ISH.

For many samples and transcripts, optimal morphology and excellent ISH signals can be achieved using tissue that has been perfused by a buffered aldehyde fixative, generally formalin (or paraformaldehyde), followed by immersion in a buffered sucrose solution and freezing. In our experience (which is predominately with rodent brains), ISH is “less sensitive” with tissue that has been perfused in paraformaldehyde, compared with fresh-frozen tissue. We see this trait manifest in lower ISH signals when using nonradioactive ISH methods, especially when probing

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for receptor mRNA, although this is less of a problem for abundant transcripts such as pro-opiomelanocortin (POMC) and NPY in the hypothalamus.

The tissue preparation for nonradioactive ISH is conventional. All reagents are prepared with RNAase-free reagents, and slides are handled with gloves. Slides are treated with a formamide solution and then with a paraformaldehyde solution before beginning the hybridization procedure. Fundamentally, there are no basic differences in the procedures for the hybridization protocols for radioactive and nonradioactive ISH, except as may be dictated empirically by probe concentrations, probe labeling, probe types (i.e., riboprobes vs oligonucleotides), and controls (which are similar to controls for immunocytochemistry).

Here is a basic ISH tissue prep protocol that we use with labeled oligonucleotides and riboprobes. All steps are done at room temperature (unless noted otherwise):

- (1) Remove slides from freezer and thaw to room temperature.
- (2) Immerse in fresh 4% paraformaldehyde at 4°C.
- (3) PBS (0.1 M Na₂HPO₄/0.15 M NaCl, pH 7.4), 4°C.
- (4) 0.1 M triethanolamine (TEA), pH 8.0, fresh, rinse.
- (5) TEA containing acetic anhydride (875 µL in 350 ml TEA, mix well), 10 min.
- (6) 2 × SSC rinse.
- (7) 70%, 95%, 100% ethanol (2 min each).
- (8) Chloroform, 5 min (in hood).
- (9) 100%, 95% ethanol (2 min each).
- (10) Air-dry slides if they are to be stored in a freezer. Place in PBS if they are to be used immediately for ISH.

Probes and Probe Labeling

The investigator must first decide whether to use oligonucleotide probes or riboprobes, each of which has advantages and disadvantages. Oligonucleotides (short antisense DNA strands) are easy to obtain and label, and can be very specific. The downside is their relatively low label-to-probe ratios. Since they are generally end-labeled, relatively few haptens can be attached to each oligonucleotide, making their detection by immunocytochemical methods more challenging if they are directed against relatively scarce mRNAs. In contrast, riboprobes (long RNA antisense strands, often hundreds of bases long) can be labeled with many haptens to a very highly specific activity (Flanagan et al., 2000; Darby et al., 2006).

However, the preparation and labeling of riboprobes from cDNA templates involve a certain degree of technical skill and more exacting preparation conditions so as to avoid artifacts. For nonradioactive ISH, probes (either oligonucleotides or riboprobes) are usually labeled with a hapten for which antibodies are readily available from commercial sources. The most common haptens are digoxigenin, biotin, and fluorescein. Standard protocols for labeling oligonucleotides and riboprobes with these haptens can be found in numerous articles and will not be detailed here. An excellent discussion on biotin and digoxigenin labels for ISH can be found in the literature (Niedobitek et al., 1989; Arnold et al., 1992; Chevalier et al., 1997).

The question of whether to use oligonucleotide probes or riboprobes for nonradioactive ISH with antibodies is often settled by what is available to the investigator. When cDNAs are available and can be easily transcribed into suitable antisense riboprobes (cRNA strands), they are (in our opinion) ideal for ISH because of the strong signals they tend to produce. A 400-base riboprobe would have the potential for labeling at approximately 100 sites, using labeled UTP in the synthesis, although the optimal labeling ratios are generally in the range of 1:1 to 1:3 of labeled-to-unlabeled UTP in the synthesized probe. Thus, approximately 30-50 hapten-labeled UTP molecules would be incorporated into the 400-base probe. In this way, a single ISH event potentially can produce many haptens for detection by immunocytochemistry. In contrast, oligonucleotides, which normally have a range of 30-60 bases, can be readily synthesized and obtained commercially. Oligonucleotides are labeled by enzymatic attachment of a hapten to one end of the molecule (although methods are available for adding more), thereby limiting the sensitivity of detecting an ISH event. In our experience, riboprobes have the advantages of potentially generating stronger signals and producing superior results when performing fluorescence *in situ* hybridization (FISH). The reason that high-density probe labeling is important in nonradioactive ISH (and why we prefer riboprobes) is because the greater abundance of immunogenic haptens in the hybrids produced by labeled riboprobes significantly increases the ability to detect the hybridization with immunocytochemical techniques.

Antibodies for Nonradioactive ISH

Digoxigenin is an ideal molecule to serve as an immunogenic hapten for nonradioactive ISH with animal tissues because it is a sterol found exclusively in *Digitalis* leaves and flowers. Likewise, biotin (vitamin B7) works well as a hapten for nonradioactive

ISH, although it occurs naturally in many cells, and appropriate controls are necessary to prevent non-specific ISH. Provided that the ISH protocol has been performed with optimal conditions for probe hybridization, valid detection of the hapten-labeled transcripts depends on performing the proper immunocytochemical technique with the customary controls. Basically, the sections are treated with a standard immunocytochemical staining protocol with antisera against the hapten label. Thus, for an ISH probe labeled with digoxigenin, following a blocking step in PBS containing 5% normal serum from the species used to generate the second antibody (usually sheep or goat), the sections are incubated with antibody to digoxigenin in PBS (overnight at 6°C). In our experience, working with digoxigenin-labeled probes is the ideal way to initiate a FISH study because high-affinity anti-digoxigenin antibodies are readily available commercially and work very reliably. Anti-digoxigenin antibodies that are directly conjugated to alkaline phosphatase, peroxidase, or fluorescent molecules can be purchased from several vendors and work well if the labeled hybrids are abundant.

We typically use fluorescently labeled anti-digoxigenin antibodies as primary antibodies (mainly because we have them on hand) in a two-step procedure. In it, the anti-digoxigenin antibodies are followed by second antibodies that are directed against the anti-digoxigenin IgG and are conjugated with alkaline phosphatase, peroxidase, or a fluorescent molecule. We have found that the amplification resulting from this two-step procedure reliably produces exceedingly intense ISH signals. We also have had good success with antibodies directed against biotin for nonradioactive ISH and FISH. However, in our hands, biotin is less optimal because of the potential nonspecific binding of anti-biotin antibodies to endogenous biotin (although this is seldom a problem in practice) as well as seeming less sensitive than the digoxigenin ISH methods. Likewise, fluorescein antibodies can be used for nonradioactive ISH in order to detect fluorescein-labeled probes. However, we have found fluorescein methods even less sensitive than the use of biotin.

Here are the key steps in the ISH protocol prior to applying antibodies for immunocytochemical detection. Consult ISH manuals for specific details on proper handling of slides and applying hybridization mixtures; in many cases, paying attention these details can be critical to achieving quality results. Initially work with small numbers of slides, and note that good technique is essential. The procedures for ISH are basically the same as for oligonucleotides and riboprobes, except for the conditions for incubation

and posthybridization washes, which will depend on the T_m of the probe being used for hybridization.

- (1) Estimate the amount of probe needed for the number of slides to be hybridized, and prepare the hybridization mixture following established protocols.
- (2) Apply probe mixture to the slides and keep them in a covered, moist container.
- (3) Transfer slides to oven at desired temperature for desired time.
- (4) At end of hybridization period, rinse slides in SSC and perform the posthybridization washes. The steps of the posthybridization washes differ for oligonucleotides and riboprobes; consult standard manuals for details.

Immunocytochemical Detection of a Single ISH Probe

Once the ISH protocol is complete, the hybridized transcripts can be detected using conventional immunocytochemical procedures. A sample protocol for detecting POMC mRNA with a hybridized riboprobe labeled with digoxigenin-UTP is as follows (Baskin et al., 1999a,b):

- (1) Immerse slides in Tris/NaCl buffer (0.1 mole/Tris/150 mmol/l NaCl, pH 7.4).
- (2) Apply mouse anti-digoxigenin monoclonal IgG diluted 1:5000 in Tris/NaCl buffer containing 0.05% Triton X-100 (Tris/NaCl/TX/Triton) and 1% normal goat serum (NGS) for 3 h at 37°C.
- (3) Wash slides three times in Tris/NaCl/TX/Triton buffer for 5 min each.
- (4) Apply goat anti-mouse IgG conjugated with Cy3, diluted 1:200 in Tris/NaCl/TX/Triton X-100 buffer for 1 h at 37°C.
- (5) Wash in buffer, as above.

This protocol can be adapted to enzymatic antibody detection methods. In this case, the second antibody in step 4, above, is replaced with a second antibody conjugated with HRP or alkaline phosphatase, and the appropriate enzymatic detection system is used to produce a colored reaction product that is visible with brightfield microscopy.

Immunocytochemical Simultaneous Detection of Two FISH Probes

Antibodies can be used to detect simultaneously two different ISH probes in the same section. The protocol that we have used employs a cocktail of two different labeled riboprobes for the ISH step, followed by a cocktail of two different antibodies in order to discriminate between the respective haptens. The

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procedure we used for detecting separate mRNAs for POMC and NPY in the arcuate nucleus using hapten-labeled riboprobes is as follows (Hahn et al., 1998; Baskin et al., 1999b):

- (1) Prepare a riboprobe to POMC mRNA using biotin-UTP and a riboprobe to NPY using digoxigenin-UTP.
- (2) Combine the two riboprobes into a single hybridization mixture, making sure that the final concentrations of the respective probes are optimal (based on previous FISH with each probe used alone). Note that the times and temperatures for a cocktail hybridization should be adjusted for the minimal stringency required.
- (3) Following the hybridization, wash sections as usual and place slides in 0.1 mole/l Tris/NaCl/TX/Triton buffer, pH 7.4.
- (4) The immunocytochemical detection system employs an antibody cocktail consisting of a mixture of primary antibodies to the haptens that were used to label the riboprobes (such as rabbit polyclonal anti-digoxigenin IgG and mouse monoclonal anti-biotin IgG). Each primary antibody is diluted to its optimal working concentration, as determined by single hybridizations. The immunocytochemical cocktail is applied for 3 h at 37°C or overnight in a refrigerator, followed by rigorous washes.
- (5) The antibodies bound to the haptens are made visible with fluorescent-conjugated second antibodies applied in a single cocktail. As above, prepare a mixture of goat anti-rabbit IgG-Cy3 conjugate and goat anti-mouse Cy2 conjugate (Alexa dyes and other fluorochromes can be substituted for these particular fluorochromes), each at their final working dilution. Apply the second antibody cocktail for 1 h at 37°C (Note: 1 h at room temperature is often adequate).
- (6) Rinse and mount coverslip with a slow-fading mounting medium.

The resulting preparation is viewed with a fluorescence microscope outfitted with appropriate filters for the fluorochromes used in FISH. This is a critical aspect of FISH that is often overlooked, because many investigators select fluorochromes without regard to the characteristics of the filters in their microscope. Ideally, the fluorescence filter sets (“blocks”) should be optimized for the fluorochromes used in the immunocytochemical detection system. Filters that are not well matched to the specific fluorochromes used for immunocytochemistry can result in low fluorescence brightness (i.e., a weak FISH signal)

when the proper filter set can produce very bright images. Likewise, inappropriate filters that are not well matched to the particular fluorophors employed in the procedure can result in spurious and nonspecific fluorescence resulting from the overlapping of excitation and emission spectra (sometimes called “fluorescence bleedthrough”).

Rigorous controls are needed to ensure the specificity of the respective FISH signals. In addition to the usual ISH specificity controls (e.g., use of sense probes, RNAase digestion), the customary immunocytochemical controls must be used. The specificity of the antibodies for detecting the haptens is generally very high, and thus, nonspecific binding of these antibodies is generally not a problem. However, care must be exercised with anti-biotin antibodies in order to avoid spurious binding to endogenous biotin that may be in the tissue.

Combining Radioactive ISH with Nonradioactive ISH Using Immunocytochemistry

Nonradioactive ISH such as FISH can be combined with radioactive ISH. We have used this approach to colocalize neuropeptide mRNAs with the long isoform of the leptin receptor ($LepR_b$) mRNA, which is present at low concentrations in neurons but difficult to detect by conventional nonradioactive ISH methods (Baskin et al., 1999a). Here is a simple protocol that works well on cryostat sections of rodent brain mounted on RNase-free slides and prepped as described above:

- (1) Transcribe antisense riboprobes for NPY mRNA and $LepR_b$ mRNA from cDNA templates using appropriate polymerases. The $LepR_b$ riboprobe is prepared with ^{33}P -UTP. The NPY riboprobe is transcribed with digoxigenin-UTP.
- (2) The hybridization procedure uses a cocktail mixture of the two labeled probes (as described in the previous section).
- (3) Localize the NPY FISH using a rabbit polyclonal anti-digoxigenin IgG followed by goat anti-rabbit IgG-Cy3 conjugate, as described above. Examine representative slides to confirm the presence of FISH.
- (4) Coat slides with NTB2 nuclear track emulsion (Kodak, Rochester, NY) and dry them.
- (5) Expose for 14 days (this period is variable).
- (6) Develop slides for autoradiography and coverslip them using a slow-fading glycerol-based mounting medium containing 1 μ g/ml Hoechst 33342 nuclear stain (visualized with a DAPI filter set).

Examine the preparation with fluorescence optics optimized for Cy3 in order to localize the NPY FISH. The autoradiographic grains produced by the LepR_b ISH in the same field can be seen by switching to brightfield optics, although we prefer to use darkfield optics, which allows easier detection and quantification of the grains. The images of each ISH result can be captured digitally and merged to observe colocalization (or the lack of it).

We have successfully combined the above radioactive ISH protocol with dual FISH in order to obtain triple ISH in which FISH for both NPY and POMC mRNAs was visualized with mRNA for LepR_b. Here are the basic steps in the protocol (Baskin et al., 1999a), which can be adapted to other probes:

- (1) Prepare the NPY riboprobe with digoxigenin-UTP and the POMC riboprobe with biotin-UTP. Prepare the LepR_b riboprobe with ³³P-UTP.
- (2) Prepare a hybridization cocktail mixture of all three riboprobes, with each at its respective desired concentration.
- (3) Perform the hybridization using times and temperatures that are adjusted for the minimal stringency required. Optimum stringency conditions must be empirically determined through trial and error. It helps if the probes are selected for maximum compatibility of stringency conditions.
- (4) Detect the NPY FISH using a rabbit polyclonal anti-digoxigenin IgG followed by goat anti-rabbit IgG-Cy3 conjugate, 1 h, at 37°C. Examine representative slides to confirm the presence of NPY FISH.
- (5) Detect POMC FISH with a tyramide signal amplification (TSA) kit (PerkinElmer, Waltham, MA) to amplify the Cy2 fluorescence signal from hybrids with the biotinylated POMC riboprobe. Incubate slides with streptavidin-HRP, followed by biotinyl tyramide and streptavidin-Cy2 conjugate, following the manufacturer's protocol. Examine representative slides to confirm the presence of POMC FISH.
- (6) Perform the liquid emulsion autoradiography, as described above, to produce silver grains over sites of LepR_b hybrids.
- (7) To visualize nuclei, coverslip with a slow-fading mounting medium containing Hoechst.
- (8) Visualize and digitally record the images produced by each probe, and merge the images to observe the distribution of ISH for the different transcripts.

This protocol sounds complicated but is actually very simple in practice if the individual steps have been worked out satisfactorily. The use of the TSA amplification (Zaidi et al., 2000) is not required, but we find that it is helpful for FISH with biotinylated riboprobes (even for transcripts as abundant and POMC and NPY in the arcuate nucleus) when Cy2 is used to detect FISH. We have not tried this protocol with the Alexa dyes, but expect they should work equally as well. We avoid the use of FITC, rhodamine, and Texas red because of rapid photo bleaching and generally lower signal intensity.

Combining FISH with Protein Immunocytochemistry

The literature contains many published protocols for simultaneously combining ISH for mRNA with immunocytochemistry for proteins. Some protocols require ISH to be performed before the protein immunocytochemistry, and others specify just the opposite. It is therefore difficult to generalize about the "best" protocol. Instead, investigators must empirically determine the approach that best meets their needs. The need for combining protein immunocytochemistry with mRNA ISH seems to have been eclipsed in part by laser capture microdissection (LCM) techniques for picking cells that are subsequently analyzed for mRNA expression by real-time quantitative PCR. Note, however, that paraformaldehyde fixation must usually be avoided to obtain high-quality RNA for PCR. We have found that many antibodies work well with cryostat sections of rat brain dipped in 70% ethanol before initiating the immunocytochemical staining protocol (Williams et al., 2008). The immunostained cells, visualized by Cy3 fluorescence, are picked by LCM and extracted for RNA. These samples are enriched in mRNA from the immunostained cell type, and PCR of the samples has the added advantage that expression of potentially thousands of genes from these immunostained cells can be performed using chip analysis techniques.

Here is a protocol that we have used for combined immunocytochemistry of LepR_b and NPY mRNA ISH (Baskin et al., 1999b). In this case, we used sections of rat brains that were perfused in 4% paraformaldehyde and prepared by cryostat sectioning.

- (1) Place slides in fresh 0.1 M TEA buffer, pH 8.0, for 10 min, followed by acetylation in TEA, as described in Tissue Preparation, above.
- (2) Dehydrate with ethanol and treat with chloroform.

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- (3) Add 100% and 95% ethanol (2 min each).
- (4) Place slides in 0.05 M PBS at 4°C.
- (5) Immerse in blocking buffer containing PBS and 5% normal donkey serum, 40-60 min, followed by rinsing in PBS.
- (6) Apply primary antibody (in this case, we used an rabbit polyclonal antibody to LepR_b) in PBS containing 0.25% Tween 20 at appropriate dilution, overnight, 4°C, followed by three 5-min washes in PBS without Tween.
- (7) Detect LepR_b immunoreactive sites with donkey anti-rabbit IgG-Cy3, at room temperature, 1 h. Observe representative slides to verify the presence of positive immunostaining.
- (8) Perform FISH for NPY as described above and detect with Cy2 second antibody.
- (9) Mount under coverslip with glycerol-based antifading mounting medium containing Hoechst dye.

Although this protocol has worked well for us, we make no claims that it is the best. It also works well with HRP and alkaline phosphatase detection systems for brightfield microscopy.

Antibody Detection of ISH with Fluorescent Alkaline Phosphatase Reaction Products

In situations where conventional FISH protocols are not sensitive enough to detect low abundance mRNAs, the investigator may wish to consider using TSA and other means such as fluorescent alkaline phosphatase substrates for amplifying the ISH signal. We have found that combining FISH with TSA is sometimes insufficient to detect LepR_b transcripts. One alternative is to use a method that produces fluorescent substrate from the enzymatic action of alkaline phosphatase. We have adopted the so-called Enzyme-Labeled-Fluorescent (ELF) method, which uses a fluorescent alkaline phosphatase substrate reaction product to amplify the ISH signal (Larison et al., 1995; Paragas et al., 1997; Telford et al., 2001; Paragas et al., 2002). We found that while either TSA or ELF methods were ineffective for FISH detection of LepR_b mRNA, combining the two methods into a single protocol resulted in an extraordinarily bright FISH signal for LepR_b mRNA. The TSA reaction is performed first to amplify the amount of biotin at the hybridization sites, followed by the ELF reaction to generate a very bright fluorescent reaction product.

We have used the ELF 97 mRNA *In Situ* Hybridization Kit #2 that was originally developed by Molecu-

lar Probes (now a part of Invitrogen, Carlsbad, CA). The reaction product of the ELF-97 alkaline phosphatase substrate shows bright green fluorescence and thus complements the red Cy3 fluorochrome in double-labeling protocols. TSA amplification is performed with reagents obtained from Perkin Elmer.

Next we describe in brief an ISH procedure we used successfully to increase the intensity of the FISH signal of LepR_b (Breininger and Baskin, 2000), combining ELF with TSA, although it can be adapted to other applications (Baskin et al., 2000). Reactions are carried out in a moist chamber at room temperature unless otherwise noted.

- (1) Prepare riboprobe with biotin-UTP label.
- (2) Perform a standard ISH procedure on sections that have been prepped for ISH.
- (3) streptavidin-HRP diluted 1:100 in TNT buffer Apply (Tris/NaCl/Tween) for 30 min.
- (4) Wash 3 times in TNT buffer, 5 min each.
- (5) Apply biotinyl tyramide diluted 1:50 in the amplification buffer (provided with the reagents).
- (6) Wash 3 times in TNT buffer, 5 min each.
- (7) Place slides in blocking buffer containing 30 mM Tris (pH 7.4), 150 mM NaCl, 1% BSA, 0.5% Triton X-100, and 1 mM levamisole (Sigma, St. Louis, MO), 30 min.
- (8) Apply the streptavidin-alkaline phosphatase conjugate (from the kit) at 1:50 in TNT buffer, 30 min.
- (9) Wash exhaustively 3-5 times (5 min each) in a pre-reaction buffer containing 30 mM Tris and 150 mM NaCl to remove residual BSA and detergent that inhibit the ELF reaction.
- (10) Apply the ELF substrate diluted 1:20 in the buffer supplied with the kit and then filter with a 0.2 μM spin filter.
- (11) Add the A and B additives from the kit to the filtrate at a dilution of 1:500.
- (12) Apply the final substrate solution to each slide for exactly 10 min. The timing is critical!
- (13) Immediately stop the reaction by plunging slides into a large volume of a buffer containing 100 mM Tris (pH7.4), 25 mM EDTA, 0.5% Triton X-100, and 1 mM levamisole.
- (14) Wash exhaustively 3-5 times in the above stop buffer.
- (15) Blot slides to remove excess liquid; coverslip with mounting medium supplied by the kit.

We cannot overemphasize the importance of paying careful attention to detail and closely adhering to the manufacturer's instructions when using the

ELF method, in order to avoid failures and artifacts and if one wishes to have successful and reproducible results. Although the ELF method is not very forgiving of carelessness, it can produce amazingly precise and beautiful results when properly performed. Some hints and cautions with the ELF method are as follows:

- The ELF-97 reaction product is visualized as yellowish-green fluorescence with a 320-390 nm excitation filter, a 400 nm dichroic longpass filter, and a 535 nm barrier filter (available from Chroma Technology Corp. Rockingham, VT).
- The ELF reaction product precipitate appears as fine, brightly fluorescent grains localized at the sites of ISH, quite unlike the results usually seen with FISH. The grains bear a superficial resemblance to those produced by liquid emulsion autoradiography. The ELF method is predisposed to the widespread, excessive formation of brightly fluorescent, needle-shaped crystals that detract from the specific labeling (which normally is not needle-shaped). We found that the formation of these crystals can be minimized by tightly adhering to the length of the ELF reaction time. The reaction is very fast and can occur in seconds. Adjust conditions in order to slow the reaction. We find that 10 min is just right for our particular protocol, but 11 min produces unwanted crystals and 9 min yields lower signals. The optimal reaction times for the particular conditions of each hybridization system must be determined empirically by the investigator; following the time suggested in the instructions is a good starting point.
- You may return to your slides after a week or two to find them overgrown with crystals. Exhaustive washing is critical to avoid this problem, especially after the ELF reaction. Any residual ELF substrate remaining in the tissue will continue to produce reaction product over time. You may return to your slides after a week or two to find them overgrown with crystals.
- Use the mounting medium provided in the kit. Apparently this contains chemicals that retard crystal formation.
- Use an inhibitor of alkaline phosphatase activity (levamisole) in the ELF blocking buffer to inhibit endogenous alkaline phosphatase activity.

- Avoid mounting coverslips with organic solvents such as xylene or toluene, which can dissolve the specific reaction product precipitate.

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