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Multicolor Labeling in Developmental Gene Regulatory Network Analysis

Aditya J. Sethi,

National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

Robert C. Angerer, and

National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

Lynne M. Angerer

National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

Abstract

The sea urchin embryo is an important model system for developmental gene regulatory network (GRN) analysis. This chapter describes the use of multicolor fluorescent in situ hybridization (FISH) as well as a combination of FISH and immunohistochemistry in sea urchin embryonic GRN studies. The methods presented here can be applied to a variety of experimental settings where accurate spatial resolution of multiple gene products is required for constructing a developmental GRN.

Keywords

TSA Plus; Digoxigenin; Fluorescein; Lineage; Signaling; Linkages; Marker; Perturbations

1 Introduction

In recent years, considerable progress has been made towards elucidating gene regulatory networks (GRNs) underlying embryonic development [1–6]. Functional connections within and between GRNs are typically established by examining the responses of regulatory factor expression to experimental perturbations. For maximum accuracy, GRN responses need to be examined at several levels. These include quantitative measurements of transcript abundance in the entire embryo [7], cis-regulatory analyses of genes encoding key factors [8, 9], and evaluations of gene function in specific blastomeres at defined developmental stages [10, 11]. The last type of analysis requires precise assessment of the spatial distribution of a gene product(s) of interest.

Optimum spatial resolution is critical when characterizing developmental GRNs for several reasons. First, during early embryogenesis, many key regulatory factors have dynamic expression patterns that evolve rapidly. Thus, within a few cell divisions, the same factor may be expressed in different blastomeres that give rise to distinct embryonic lineages [10,

¹*Probe preparation:* A successful FISH experiment requires a pure antisense RNA probe of known sequence. It is essential to use a sequenced homogenous DNA template (either a completely sequenced highly purified plasmid preparation or a PCR amplicon generated from a cloned DNA fragment). We caution against the routine use of PCR products amplified from a heterogeneous source (e.g., cDNA synthesized from whole embryo RNA) unless the amplicon is sequenced to verify its identity. Also, a very important part of the probe preparation protocol is the column-based RNA purification protocol described above. Not only is it quicker than traditional RNA precipitation and isolation procedures, but, in our experience, it also yields in situ hybridization patterns with significantly superior signal intensity, specificity, and reproducibility.

²*Choice of labware:* We strongly recommend the use of 96-well flat-bottomed plates for all protocols described in this chapter. Compared to microcentrifuge tubes, 96-well plates offer superior visibility and retention of embryos through numerous buffer replacements and a more even distribution of embryos during hybridization and staining procedures. This facilitates consistent probe and buffer penetration and affords highly reproducible in situ hybridization patterns. Additionally, embryos can be inspected under fluorescence after completion of each staining step without the need for any intervening mounting or slide preparation and consequent loss of sample. Hybridization and staining reactions can be sealed as effectively in plates as in microcentrifuge tubes by using thermocycler-grade sealing films. These perform reliably through weeklong hybridization procedures at 50 °C. Finally, the use of plates makes handling of large numbers of samples much more efficient.

³*Probe conjugate selection:* We find that both digoxigenin and DNP-based labeling provide significantly superior sensitivity and signal specificity compared to fluorescein-based labeling. Furthermore, in our experience, digoxigenin and DNP-based detection give signals of equivalent intensity and specificity. Therefore, when performing dual-color FISH, detect transcripts of interest using digoxigenin and DNP-labeled antisense probes. When studying the distribution of three transcripts, rare/spatially diffuse transcripts should be detected using digoxigenin or DNP-coupled antisense probes, and the more abundant and/or highly localized mRNAs should be hybridized with a fluorescein-conjugated probe. Similarly, the relatively abundant transcripts synthesized from an exogenously introduced construct (e.g., a tracer sequence) are detected with a fluorescein-conjugated antisense probe and endogenous embryonic mRNAs with digoxigenin/DNP-coupled systems for optimum signals. If required, adjust the concentration of introduced DNA/RNA to maximize specific signals with the fluorescein-labeled probe and minimize embryo toxicity. Although our current FISH protocol uses digoxigenin, DNP, and fluorescein labeling to detect up to three mRNAs of interest, additional probe conjugates such as biotin have been used in other model systems [17, 22] for multicolor FISH.

⁴*Fluor selection:* In our experience, both Cy3- and fluorescein-TSA Plus detection systems give signals of equivalent intensity and specificity. With our current microscopy platform, the Cy5-TSA Plus kit gives signals of equivalent specificity to those of Cy3 and fluorescein, but significantly lower intensity. Nevertheless, Cy5-TSA Plus is a viable option with abundant, highly localized transcripts if Cy3 and fluorescein have already been allocated to other gene products in the same experiment. In addition to these, other TSA Plus fluors are available from PerkinElmer.

⁵*Order of probe detection:* When performing three-color FISH, the fluorescein-conjugated probe must be detected before those labeled with DNP and digoxigenin. This specific sequence avoids the anti-fluorescein-HRP antibody binding with previously deposited TSA-fluorescein staining reagent and amplifying artifactual signals in successive fluor deposition steps. Subsequent detection of DNP- and digoxigenin-labeled transcripts can then be executed in any order with appropriately selected TSA Plus fluors. When detecting two transcripts of interest, fluorescein-conjugated probes should not routinely be used and hence digoxigenin and DNP-labeled transcripts can be detected in any order.

⁶*Dynamic range:* Single and multicolor FISH have a narrower dynamic range of specific signal compared to traditional alkaline phosphatase-based whole-mount in situ hybridization. We believe this to be a function of the kinetics of the final staining reaction in each procedure. In the case of the FISH protocol described in this chapter, the final fluor deposition is extremely rapid (2–8 min to saturation) with limited user control. Conversely, alkaline phosphatase-based colorimetric deposition proceeds more slowly (1–24 h to saturation) and can be terminated whenever desired signal intensity has been achieved. This difference becomes significant when attempting to assess subtle changes in the levels of gene expression across a set of experimental conditions. Standard colorimetric detection would therefore be a superior choice in such situations. The restricted dynamic range of detection seen in FISH experiments is less significant when larger differences in transcript abundance are seen across perturbations. Both approaches are at best, semiquantitative, and ideally should be supplemented with more direct quantitative measurements of transcript abundance (Q-PCR etc.).

⁷*Signal specificity and background issues:* We have occasionally observed punctate background staining throughout the embryo when performing single or multicolor FISH. This is particularly evident when the target transcript is expressed at very low levels or has a broadly distributed expression pattern. Furthermore, sea urchin embryos at late mesenchyme blastula and gastrula stages tend to exhibit such nonspecific staining more frequently than at other developmental stages. This does not appear to be related to the choice of fluor or probe conjugate, since the incidence of nonspecific staining is comparable across different fluors and with digoxigenin-, DNP-, as well as fluorescein-coupled antisense RNA probes. A modest improvement in background staining can sometimes be achieved by lowering the concentration of the specific antisense probe. We have however, not encountered this issue with colorimetric in situ hybridization. Therefore, when characterizing a novel gene expression pattern, we strongly recommend an initial developmental series using both alkaline phosphatase and fluorescence-based detection. For mRNAs expressed ubiquitously or at very low levels, FISH may not be a viable option.

⁸*FISH ± immunostaining:* Immunostaining following a FISH procedure is used to detect antigens whose epitopes persist through the FISH process. SoxB1, Myc, phospho-Smad1/5/8, Sm30, and Sm50 can all be detected reliably using the protocol described above after completion of a single or dual-color FISH procedure. Typically, epitopes that require methanol-based fixation are not amenable to a combination of FISH and immunostaining as described herein. Furthermore, compared to immunofluorescent detection performed in isolation, there is a significant loss of signal intensity of the protein epitope-antibody complex when a complete FISH procedure is followed by immunostaining. Modest improvements in staining intensity can sometimes be achieved with a higher concentration of primary antibody/longer incubations in the presence of the primary antibody but must be weighed against a concomitant increase in background labeling.

lineages at a single developmental stage, and the same gene can have very different roles in each of these lineages [10, 14]. Also, experimental perturbations targeting a specific gene can have both localized and general developmental consequences. In such situations, whole-embryo measurements of gene product abundance are of limited value unless supplemented by accurate spatial data. Second, at early stages, embryos show relatively few morphological features that permit reliable identification of specific blastomeres expressing a particular gene product(s). Consequently, they must be monitored at the molecular level. Third, during initial embryonic specification, more than one lineage-specific GRN can operate in a group of progenitors [2]. Understanding GRN function during lineage segregation at these early stages therefore requires accurate spatial information about multiple gene products in the same blastomeres [2, 17]. In each of these situations, the distribution of a gene product(s) of interest needs to be resolved with reference to expression of a well-characterized molecular “landmark.”

Specific instances of GRN analysis where spatial assays are necessary for establishing accurate network connections include:

(a) *Identifying targets of transcription factors*: Direct targets of key GRN transcription factors are expressed/repressed within several hours of their upstream regulators in the same cells. Indirect targets, on the other hand, can be regulated by transcription factor activities or intermediate signaling events within or outside an upstream factor’s expression domain. Spatial assays are therefore critical to locate both the upstream GRN factor and its putative target in order to establish GRN linkages. (b) *Lineage-specific regulation of target genes*: GRN factors frequently regulate distinct sets of target genes in precursors of multiple embryonic lineages. In these cases, whole-embryo assays of target abundance, by themselves, are incapable of resolving GRN responses within a specific lineage. This requires spatial assays of target gene responses relative to an appropriate lineage marker. (c) *Defining feedback loops*: GRN factors commonly autoregulate themselves or respond to inputs from their targets through feedback loops. To distinguish between cell-autonomous and non-autonomous feedback loops, it is essential to locate the upstream GRN factor, the target it initially regulates, and the site of the “feedback” response. Thus, responses to experimental perturbations that test the loop must be resolved in space.

In this chapter we describe two methods that permit accurate localization of a gene product relative to a known molecular “landmark” in sea urchin embryos. The first employs multicolor fluorescent in situ hybridization (FISH) to examine the spatial distribution of transcripts encoded by up to three genes of interest. The second method combines FISH with immunohistochemistry in whole embryos. In contrast to conventional alkaline phosphatase-mediated detection, fluorescent labeling allows for unambiguous resolution of multiple gene products, which is especially critical when they are expressed in overlapping territories [2, 17, 18]. Furthermore, fluorescence-based approaches permit precise optical sectioning and volume projections of image stacks, thereby providing 3-dimensional descriptions of spatial expression patterns. By incorporating nuclear counterstaining, fluorescent labeling can easily be used to assess the numbers and distributions of cells expressing a gene product of interest.

The techniques described herein have been used to investigate a number of aspects of sea urchin GRN function. These include characterizing the expression of a regulatory factor during embryogenesis [18, 19] as well as understanding the effects of an experimental perturbation on the expression of a gene in specific blastomeres marked by a second gene product [20]. Additionally, these methods have been used to identify regulatory targets in blastomeres adjacent to cells that selectively receive a reagent to disrupt a signaling process [20, 21]. They can also be used to distinguish between a localized response to an experimental perturbation and a general developmental consequence such as toxicity caused by disruption of basic cellular function throughout the embryo [20].

2 Materials

2.1 Materials for In Situ Probe Synthesis

1. *Probe labeling*: 10× digoxigenin labeling mix (Roche) and 10× fluorescein labeling mix (Roche); ATP, CTP, GTP, and UTP (lithium salts, Roche); DNP-11-UTP (PerkinElmer); 10× DNP labeling mix consists of 10 mM each of ATP, CTP, and GTP, 6.5 mM UTP, and 3.5 mM DNP-11-UTP.
2. *In vitro transcription*: SP6 and T7 RNA polymerases (New England Biolabs and others) with included 10× RNA transcription buffers. Templates are discussed in Subheading 3.1, **step 2** and 4 **step 1**.
3. *Probe purification*: Turbo DNase I (Ambion); ethylenediaminetetraacetic acid (EDTA); Qiaquick PCR purification kit (Qiagen) or size-fractionation columns (Illustra ProbeQuant G-50 Micro Columns, GE Healthcare); diethylpyrocarbonate (DEPC)-treated (nuclease-free) water.

2.2 Materials for Multicolor FISH and Immunostaining

1. *Labware*: 96-well flat-bottomed plates; thermocycler-grade sealing film; rubber tubing; glass Pasteur pipettes; standard suction line terminating in a 200 µL disposable pipette tip.
2. *Fixative*: 4 % paraformaldehyde (v/v, use 16 % EM grade paraformaldehyde solution, EM Sciences); 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS); 0.1 % Tween-20 in Artificial Sea Water (ASW: 0.461 M NaCl, 0.0024 M NaHCO₃, 0.009 M KCl, 0.026 M MgCl₂·6H₂O, 0.032 M MgSO₄, 0.0097 M CaCl₂, pH 8.1).
3. *Wash and hybridization buffers*: Use nuclease-free molecular biology grade water. “MOPS wash buffer”: 0.1 M MOPS, pH 7.0, 0.5 M NaCl, and 0.1 % Tween-20. “Hybridization buffer 1”: 70 % formamide (v/v, use stock solution of commercial molecular-grade formamide, pH 7–9, Sigma-Aldrich), 0.5 M NaCl, 0.1 M MOPS, pH 7.0, 1 mg/mL bovine serum albumin (BSA), and 0.1 % Tween-20. “Hybridization buffer 2”: 50 % formamide (v/v), 0.5 M NaCl, 0.1 M MOPS, pH 7.0, 1 mg/mL BSA, and 0.1 % Tween-20 (add formamide only after all other buffer components have mixed to allow BSA dissolution or use a stock solution of BSA at 100 mg/mL, stored at –20 °C).

4. *Probe detection*: Anti-fluorescein-HRP (horseradish peroxidase) antibody (PerkinElmer); anti-digoxigenin-POD (peroxidase) Fab fragments (Roche, reconstituted to give 150 U/mL stock), both antibodies stored at 4 °C; anti-DNP-HRP stored at 4 °C (part of TSA DNP-HRP kit, PerkinElmer); lamb serum/normal goat serum (stored at –20 °C); BSA; “blocking buffer 1”: 10 % lamb/goat serum, 2.5 mg/mL BSA in MOPS wash buffer; “blocking buffer 2”: 5 % lamb/goat serum in MOPS wash buffer; Tyramide Signal Amplification Plus (TSA Plus) system (PerkinElmer); 30 % hydrogen peroxide solution (v/v, Sigma-Aldrich, use protective labwear when handling hydrogen peroxide, store stock at RT away from light).
5. *Immunostaining and nuclear counterstaining*: “PBST wash buffer”: 1× phosphate buffered saline (PBS) (use commercial 10× PBS) and 0.1 % Tween-20; Alexa series of secondary antibodies (Invitrogen), stored at 4 °C; 4', 6-diamidino-2-phenylindole, dilactate (DAPI).
6. *Mounting and slide preparation*: 25 × 75 × 1 mm glass slides; 22 × 22 mm #1 coverslips; 13 or 20 mm inner diameter custom imaging spacers (Grace Bio-Labs, catalog #3181209 or 654006, respectively); custom MatTek 96-well flat-bottomed plate with 1.0 coverslip thickness (MatTek Corporation, catalog # P96G-1.0-5-F); glycerol.

3 Methods

3.1 In Situ Probe Synthesis

Take necessary precautions at each step of probe synthesis to maintain an RNase-free environment (gloves, dedicated labware for RNA work, and nuclease-free water as indicated). Also, refer to notes for comments on probe preparation.

1. Set up a 10 µL reaction for in vitro transcription of each probe.
2. Use 250–500 ng (plasmid) or 100–250 ng (PCR product) of template DNA in 3–5 µL of water. Templates can be appropriately restricted plasmids or PCR products containing full-length or partial cDNA sequence of the gene of interest flanked by a suitable bacteriophage promoter sequence. Aim for sequence lengths between 300 and 1,000 nt. For rare targets, use as long a template sequence as possible. Alternatively, two PCR templates spanning different regions of the mRNA can be mixed either at the synthesis step, if they use the same RNA polymerase, or after synthesis and before hybridization. Use of two probes also allows one to hybridize both and each separately, providing confidence in the results.
3. Prior to use, template DNA should be purified using a column-based method (Qiaquick Gel Extraction or PCR purification kit, Qiagen) and eluted in nuclease-free water.
4. Add to the DNA 1 µL of 10× RNA transcription buffer, 1 µL (this can be reduced to as low as 0.3 µL per 10 µL reaction to save reagent) each of the 10× digoxigenin, DNP, or fluorescein labeling mix, nuclease-free water to bring the final volume to 10 µL and 0.5–1.0 U of RNA polymerase (SP6 or T7, usually 0.5–1.0 µL).

5. Incubate at 37 °C for 2 h.
6. Add 1–2 U (0.5–1.0 µL) of Turbo DNase I directly to the transcription reaction (Turbo DNase I is engineered to be highly tolerant of the salt concentration in transcription buffers) and incubate at 37 °C for 15 min to remove template DNA.
7. Add 1 µL of a 100 mM EDTA, pH 8.0 stock solution (make stock in nuclease-free water), incubate at room temperature (RT) for 5 min to stop enzymatic reactions. Addition of EDTA significantly improves binding of RNA to, and elution from, Qiagen columns (*see step 8*), resulting in substantially higher recovery of product yields.
8. Dilute reaction to 100 µL with DEPC-treated water.
9. Purify synthesized probe using the Qiagen Qiaquick PCR purification kit as per the manufacturer's instructions for purification of standard PCR products using a microcentrifuge (Qiagen). Alternatively, purification of synthesized RNA through size-fractionation columns (Illustra ProbeQuant G-50 Micro Columns, GE Healthcare; follow manufacturer's instructions) achieves comparable yields [15].
10. Elute RNA in 30–50 µL of DEPC-treated water if using the Qiagen PCR purification kit. Follow manufacturer's instructions for the Illustra ProbeQuant G-50 columns for elution. Quantitate product by spectrophotometry. Store probe at –20 °C between uses. For long-term use, store probes as ethanol precipitates or in hybridization buffer 1 containing 70 % formamide and yeast tRNA, both of which inhibit RNase.

3.2 Multicolor Fluorescent In Situ Hybridization (FISH) to Detect Endogenous Transcripts

1. Embryos are visualized with a dissecting microscope at 15–30× magnification and typically carried in 96-well flat-bottomed plates making solution transfers by mouth suction and pulled glass pipettes attached to rubber tubing. All steps from fixation through hybridization and staining are done in the wells of these plates (*see notes for comment on choice of plates for this procedure*). Add 50 µL of fixative and allow embryos to settle for 5 min at RT. Remove supernatant from well with suction line and replace with 150–200 µL of fixative; incubate at RT for 1 h. Embryos may also be fixed overnight at 4 °C, although this results in significantly lower signal. Fixative should be freshly made on the day of embryo collection and can be used for up to 24 h at RT.
2. Wash embryos five times with 200 µL of MOPS wash buffer at RT. If required, embryos can be stored in MOPS wash buffer at 4 °C for up to 7 days prior to further processing. Seal 96-well plates when storing embryos to minimize evaporation. If storing embryos longer than 2 days, add 0.01 % sodium azide (w/v) to the MOPS wash buffer to prevent bacterial contamination. For optimum signals and minimal background staining, MOPS wash buffer should be freshly made and used on the day of preparation if stored at RT. For long-term use, store buffer at –20 °C.

3. To equilibrate embryos in hybridization buffer 1, incubate them in 150–200 μL of each the following solutions for 30 min: 2:1 and 1:2 mix (v/v) of MOPS wash buffer and hybridization buffer, followed by hybridization buffer 1 alone. Store excess hybridization buffer at $-20\text{ }^{\circ}\text{C}$.
4. Prior to hybridization, place embryos in 200 μL of hybridization buffer 1 for at least 1 h at $50\text{ }^{\circ}\text{C}$.
5. Replace hybridization buffer 1 with 150–200 μL of preheated hybridization buffer 1 (at $50\text{ }^{\circ}\text{C}$) containing 0.05–0.3 ng/ μL of digoxigenin – and/or DNP – and/or fluorescein-labeled antisense RNA, as well as 500 $\mu\text{g}/\text{mL}$ yeast tRNA to minimize nonspecific probe binding. Seal plates and hybridize at $50\text{ }^{\circ}\text{C}$ for 3–7 days. Refer to Subheading 4 below for guidelines on probe conjugate selection.
6. Wash embryos five times with 200 μL of MOPS wash buffer at $50\text{ }^{\circ}\text{C}$ for 3 h, followed by three washes with 200 μL of MOPS wash buffer at RT over a total period of 1 h.
7. Block embryos in 150–200 μL of blocking buffer 1 for 30–60 min at RT.
8. Incubate embryos overnight at either RT or $4\text{ }^{\circ}\text{C}$ in 150 μL of a 1:750 dilution (v/v) of anti-fluorescein-HRP in blocking buffer to label fluorescein-containing hybrid RNA duplexes. Seal 96-well plate to minimize evaporation.
9. Wash embryos six times with 200 μL of MOPS wash buffer over 1.5–2 h at RT to remove unbound antibody.
10. Detect bound anti-fluorescein-HRP by adding 50 μL of freshly made Tyramide Amplification working solution (a 1:150 dilution of the TSA stock solution in the included $1\times$ plus amplification diluent) for 2–8 min at RT (a 6-min incubation usually yields an optimum balance of signal intensity and minimal background staining). Ensure that all TSA kit solutions are at RT prior to use, and place embryos in the dark for all steps after addition of TSA Amplification working solution. Refer to the guidelines on fluor selection in Subheading 4.
11. Wash embryos six times each with 200 μL of MOPS wash buffer at RT for 5–10 min per wash to remove excess TSA detection reagent.
12. Quench the peroxidase activity of peroxidase-conjugated antibody by incubating embryos in 200 μL of a solution of MOPS wash buffer containing 3 % hydrogen peroxide (v/v, diluted from a 30 % stock solution) at RT for 1 h.
13. Remove excess hydrogen peroxide by washing embryos six times, each with 200 μL of MOPS wash buffer at RT over a total of 90 min.
14. Incubate embryos overnight at $4\text{ }^{\circ}\text{C}$ in 150 μL of a 1:1,000 dilution (v/v using reconstituted antibody stock) of anti-digoxigenin-POD Fab fragments in blocking buffer 2 to label digoxigenin-containing hybrid duplexes. Seal 96-well plate to minimize evaporation.
15. Wash embryos six times, each with 200 μL of MOPS wash buffer over 1.5–2 h at RT to remove unbound antibody.

16. Detect bound anti-digoxigenin-POD by adding 50 μ L of freshly made Tyramide Amplification working solution (a 1:150 dilution of the TSA stock solution in the included 1 $\times$ plus amplification diluent) for 2–8 min at RT (6 min usually is optimal).
17. Repeat **steps 11** through **13**.
18. Incubate embryos overnight at 4 $^{\circ}$ C in 150 μ L of a 1:250 dilution (v/v) of anti-DNP-HRP in blocking buffer 2 to label DNP-containing hybrid duplexes. Seal 96-well plate to minimize evaporation.
19. Wash embryos six times, each with 200 μ L of MOPS wash buffer over 1.5–2 h at RT to remove unbound antibody.
20. Detect bound anti-digoxigenin-POD by adding 50 μ L of freshly made Tyramide Amplification working solution (a 1:150 dilution of the TSA stock solution in the included 1 $\times$ plus amplification diluent) for 2–8 min at RT (6 min usually is optimal).
21. Wash embryos six times, each with 200 μ L of MOPS wash buffer at RT for 5–10 min to remove excess detection reagent. Include an appropriate dilution of DAPI (based on specific stock purchased) in the first MOPS wash if performing nuclear counterstaining.
22. Equilibrate embryos in 50 % glycerol by successive washes through 15, 30, and 50 % glycerol made by diluting glycerol with MOPS wash buffer (v/v). Allow embryos to equilibrate at RT for 15 min between steps to minimize osmotic damage. Glycerol aids in embryo handling during slide preparation, and also provides a refractive index appropriate for microscopy. Alternatively, embryos can be transferred in MOPS wash buffer to 1.0 coverslip-thick flat-bottomed 96-well plates and photographed directly (*see* custom MatTek plates in **item 6** of Subheading 2.2).

An example of three-color FISH is shown in Fig. 1.

3.3 Multicolor FISH to Detect Transcripts Synthesized from an Exogenously Introduced DNA as Well as Endogenous Embryonic Transcripts

For this application, the stringency of the hybridization conditions is reduced to prevent denaturation of the introduced double-stranded DNA, thereby excluding formation of RNA/DNA hybrids. Post-hybridization high stringency washes are used to maximize the signal-to-noise ratio. Modifications to the multicolor FISH protocol from Subheading 3.1 follow (only modified steps with corresponding step numbers are described below; the rest of the protocol is identical to the one described for standard multi-color FISH):

1. To equilibrate embryos in hybridization buffer 2, incubate them in 150–200 μ L of each of the following solutions for 30 min: 2:1 and 1:2 mix (v/v) of MOPS wash buffer and hybridization buffer 2, followed by hybridization buffer 2 alone. Store excess hybridization buffer 2 at -20° C.

2. Prior to hybridization, place embryos in 200 μ L of preheated hybridization buffer 2 for at least 1 h at 45 °C.
3. Replace prehybridization buffer with 150–200 μ L of preheated hybridization buffer 2 (at 45 °C) containing 0.05–0.3 ng/ μ L of digoxigenin-labeled antisense RNA, DNP-labeled antisense RNA, and/or fluorescein-labeled antisense RNA and 500 μ g/mL yeast tRNA. Seal plates and hybridize at 45 °C for 3–7 days. Refer to Subheading 4 below for guidelines on probe conjugate selection.
4. Wash embryos five times, each with 200 μ L of MOPS wash buffer at 50 °C for a total of 3 h, followed by two washes with 200 μ L of hybridization buffer 1 (containing 70 % formamide) at 50 °C over an hour. Wash three times with 200 μ L of MOPS wash buffer at RT over an hour (Fig. 2).

An example of two-color detection of transcripts synthesized from an exogenous template and an endogenous transcript is shown in Fig. 2.

3.4 FISH Followed by Immunostaining

To combine FISH with immunostaining, use the following protocol after completing the in situ hybridization procedure in Subheading 3.1 (i.e., after final MOPS wash buffer replacements in **step 18**). For a list of antibodies that have been found to give acceptable signals in sea urchin embryos using this approach, refer to Subheading 4 below.

1. Wash embryos twice, each with 200 μ L PBST wash buffer at RT over 20 min.
2. Introduce an appropriate dilution of primary antibody(s) of interest in 150 μ L of PBST blocking buffer (consisting of 5 % lamb or goat serum in PBST wash buffer). Incubate embryos in this blocking buffer containing primary antibody(s) overnight at 4 °C.
3. Wash embryos four times with PBST wash buffer at RT for a total of 1 h to remove unbound primary antibody(s).
4. Detect bound primary antibody(s) by adding 150 μ L of a working dilution (determined separately for each secondary antibody used, usually 1:750 or 1:1,000, v/v) of an appropriate secondary antibody (i.e., specific for the species that each primary antibody was raised in and conjugated to a fluor different from the one(s) used in the FISH procedure).
5. Wash embryos three to four times, each with 200 μ L of PBST wash buffer at RT for 5–10 min to remove unbound secondary antibody(s). Include an appropriate dilution of DAPI (based on the specific stock purchased) in the first PBST wash if performing nuclear counterstaining.
6. Equilibrate embryos in 50 % glycerol and PBST wash buffer as in **step 19** above.

An example of two-color detection of an RNA by FISH and a protein by immunostaining is shown in Fig. 3.

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Fig. 1.

Multicolor FISH to characterize the expression patterns of three endogenous mRNAs: *z13* (red, **a, d**), *foxq2* (green, **b, d**), and *gcm* (blue, **c, d**) mRNAs were hybridized with DNP-, digoxigenin-, and fluorescein-conjugated antisense probes, respectively, and detected sequentially as described in Subheading 3.2. Wild-type embryos were fixed at the mesenchyme blastula stage (22 h postfertilization) (shown in DIC in **e**). At this stage, *z13* mRNA is expressed in endoderm progenitors (*E*) in the vegetal region of the embryo, *foxq2* transcripts are restricted to the ectoderm near the animal (*An*) pole (**e**), and *gcm* mRNA accumulates in pigment cell precursors within the secondary mesoderm (*M*) (**e**). Scale bar in (**d**) represents approximately 20 μ m

Fig. 2.

Dual-color FISH to detect a secondary mesoderm GRN response to a transcription factor introduced exogenously in clones of cells in blastula-stage embryos: This experiment was designed to show induction of the secondary mesoderm specification gene, *gcm*, in cells adjacent to those expressing the transcription factor, Pmar1 [20]. DNA constructs were used to misexpress Pmar1 and GFP under control of the hatching enzyme (HE) promoter. When these constructs are co-injected, Pmar1 and GFP are expressed in the same blastomeres, traced here with a fluorescein-conjugated probe hybridized with *gfp* mRNA (red, **a**, **c**). A digoxigenin-coupled probe was used to detect ectopic induction of *gcm* (green, **b**, **c**) in cells adjacent to Pmar1 (and GFP)-misexpressing cells at the late hatching blastula stage (DIC in **d**) [20]. Endogenous *gcm* transcripts (arrowheads in **b**, **c**) accumulate in specific blastomeres (non-skeletogenic mesenchyme precursors) not immediately adjacent to Pmar1 (and GFP)-misexpressing cells. Hybridization conditions were adjusted as per the protocol in Subheading 3.3. Scale bar in (**d**) represents approximately 20 μ m

Fig. 3.

FISH + immunostaining: Multicolor labeling was used to show the distribution of cells containing SoxB1 protein (*green*, **b–e**) compared to secondary mesoderm progenitors expressing the pigment cell gene, *gcm* (*red*, **a, c, e**). In this experiment, *gcm* transcripts were detected using a digoxigenin-conjugated antisense RNA probe and TSA-red, followed by immunohistochemical localization of endogenous SoxB1 protein and nuclear counterstaining with DAPI (*blue*, **d, e**) [20]. A stack of optical sections acquired with an axial tomography device (Zeiss) was used to generate a volume projection using Imaris 5.7.2 (Bitplane). Scale bar in (**e**) represents approximately 20 μm