Whole mount *in situ* hybridization techniques for analysis of the spatial distribution of mRNAs in sea urchin embryos and early larvae

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Chapter outline

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Abstract

A critical process in embryonic development is the activation and spatial localization of mRNAs to specific cells and territories of the embryo. Revealing the spatial distribution of mRNAs and how it changes during development is a vital piece of information that aids in understanding the signaling and regulatory genes driving specific gene regulatory networks. In the laboratory, a cost-efficient, reliable method to determine the spatial distribution of mRNAs in embryos is *in situ* hybridization. This sensitive and straightforward method employs exogenous antisense RNA probes to find specific and complementary sequences in fixed embryos. Antigenic moieties conjugated to the ribonucleotides incorporated in the probe cross-react with antibodies, and numerous staining methods can be subsequently employed to reveal the spatial distribution of the targeted mRNA. The quality of the data produced by this method is equivalent to the experience of the researcher, and thus a thorough understanding of the numerous steps comprising this method is important for obtaining high quality data. Here we compile and summarize several protocols that have been employed chiefly on five sea urchin species in numerous laboratories around the world. Whereas the protocols can vary for the different species, the overarching steps are similar and can be readily mastered. When properly and carefully undertaken, *in situ* hybridization is a powerful tool providing unambiguous data for which there currently is no comparable substitute and will continue to be an important method in the era of big data and beyond.

1 Introduction

Understanding how signaling pathways and transcription factors are deployed in time and space is a critical component of embryonic gene regulatory analysis. One of the most sensitive, straightforward, and relatively inexpensive tools to observe spatiotemporal gene expression is whole mount *in situ* hybridization (WMISH). This technique employs a small amount of exogenous antisense, gene-specific RNA that has been synthesized *in vitro* with modified ribonucleotides to detect endogenous mRNA expression of single or multiple genes within cells of whole, fixed embryos. RNA probe-based *in situ* hybridization methods using antisense RNA probes were originally developed by the Angerer Lab and they first used this technique on sea urchin embryos in the 1980s (Angerer, Cox, & Angerer, 1987; Cox, Angerer, Lee, Davidson, & Angerer, 1984; Cox, DeLeon, Angerer, & Angerer, 1984). In contrast to the DNA probes previously employed to monitor mRNA expression, single-stranded RNA probes provided much greater sensitivity. This technique uses radiolabeled RNA probes applied to sectioned tissue from embryos, providing the ability to analyze spatiotemporal gene expression as

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well as the relative level of gene expression by measuring radioactive grain counts. While this was a common method for regulatory analysis, non-radioactive chromogenic WMISH methods began to replace this method in the early 1990s (Harkey, Whiteley, & Whiteley, 1992; Lepage, Sardet, & Gache, 1992) and are now more widely used for detecting the spatial distribution of RNAs. Although this approach lacks the ability to quantify the level of RNA transcripts, it has several advantages over the radioactive method insofar as it is safer, faster and can be used on whole embryos allowing analyses of spatial patterns in three dimensions.

Each of the techniques used to determine the molecular mechanisms that govern embryonic development has strengths and weaknesses. In the case of chromogenic or fluorescent WMISH, the nature of the chemical reactions precludes quantitative analyses. To overcome this limitation, researchers often complement WMISH with quantitative polymerase chain reaction (qPCR) and/or nanostring (see chapter "Whole mount *in situ* hybridization techniques for analysis of the spatial distribution of mRNAs in sea urchin embryos and early larvae" by Erkenbrack et al.). However, these quantitative techniques are inherently limited insofar as they do not provide spatial information unless paired with cell isolation (see chapter "Identifying gene expression from single cells to single genes" by Oulhen et al.). Likewise, another flaw of WMISH is that it only provides information on the localization of mRNA, and not where proteins are functional. Whole mount immunohistochemistry resolves this limitation, revealing where and when a protein is likely functioning. However, it is difficult to find commercial antibodies that cross-react with sea urchin proteins, and the benefits gained by generating antibodies in the sea urchin system are often outweighed by the time and costs spent to generate them (see chapter "Generation, expression and utilization of single-domain antibodies for *in vivo* protein localization and manipulation in sea urchin embryos" by Schrankel et al.). Taken together, each of these techniques complements one another and rounds out a research program. If they are used in concert they provide a powerful combination for understanding developmental processes at the mechanistic level. Later in this chapter we will also provide information on how to combine WMISH and whole mount immunohistochemistry.

While WMISH is a straightforward technique once mastered, there are many steps in any given protocol that require special attention to ensure that embryos with high signal-to-noise ratios are consistently generated. It is important to note that in many instances some RNA probes produce more background than other probes targeting the same gene. This is often the case with probes that recognize mRNA that is not expressed at high copy numbers, *e.g.,* transcription factors. In addition, researchers have empirically discovered that different solutions, reagents, incubation times, and temperatures are necessary to provide consistent results, depending on the species used for developmental studies. Here we provide a comprehensive list of WMISH protocols for five sea urchin species, supplemented with helpful suggestions to consider for each sea urchin species routinely used to study development around the world. These protocols are for chromogenic, digoxygenin (DIG)-based WMISH with alkaline phosphatase (AP) staining, though they may also be modified to generate staining for multicolor fluorescent WMISH (Croce & McClay, 2010; Sethi, Angerer, & Angerer, 2014).

2 Whole mount *in situ* hybridization

Here we provide variations of WMISH protocols, adapted from those deployed all over the world in several developmental biology laboratories, for the following five sea urchin species: *Strongylocentrotus purpuratus* (Arenas-Mena, Cameron, & Davidson, 2000; Range, Martinez-Bartolomé, & Burr, 2017; Sethi et al., 2014), *Hemicentrotus pulcherrimus*, *Lytechinus variegatus* (Sethi et al., 2014), *Paracentrotus lividus* (Croce, Lhomond, & Gache, 2003; Lepage et al., 1992), and *Eucidaris tribuloides* (Erkenbrack, 2016; Erkenbrack et al., 2016; Erkenbrack & Davidson, 2015). We describe in detail the stages, materials, and durations of WMISH (Fig. 1). Fixation, hybridization, and wash buffers are complex mixtures of reagents that differ among the various protocols and are critical for consistent results. These buffers have been created to preserve species-specific morphologies, limit background from nonspecific probe or antibody binding, and increase signal-to-noise ratio. Buffer recipes for all stages of WMISH can be found at

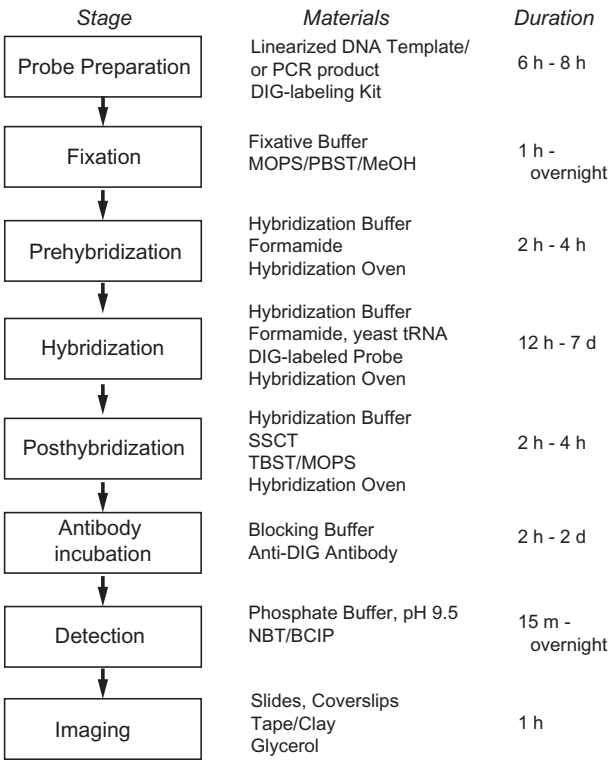

FIG. 1

A flow chart showing each stage of the WMISH procedure, important materials and reagents for each stage, and approximate duration of each stage.

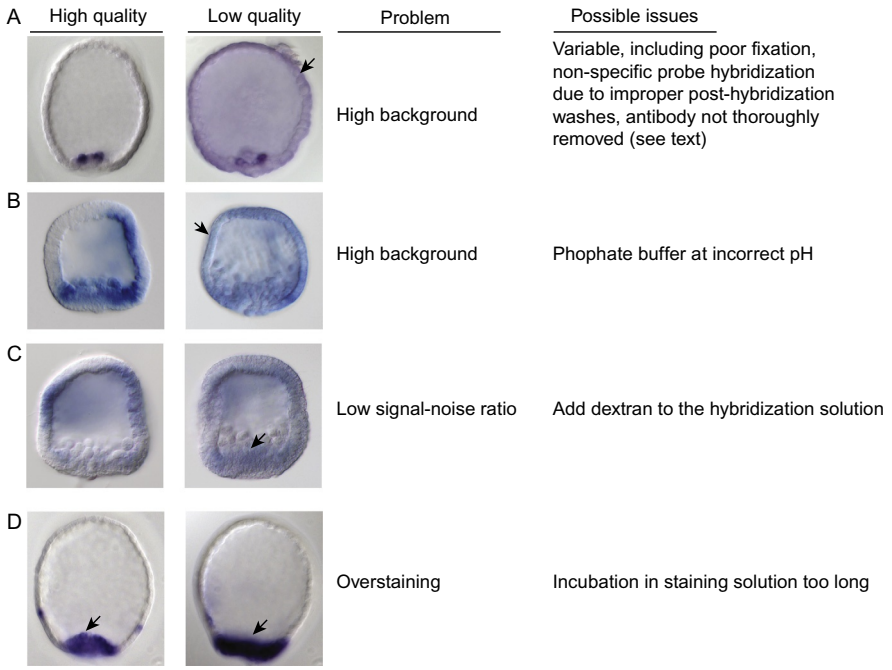

FIG. 2

Examples of stained sea urchin embryos when WMISH has been successful (high quality) and when some aspect of the WMISH procedure has gone wrong (low quality). (A) Generally, when high background is seen (purple/blue color throughout the embryo), the low signal-noise ratio can be attributed to various aspects of the procedure. This makes troubleshooting WMISH difficult. (B) An example of the background observed when the incorrect pH is used at the staining step. Arrow shows background signal. (C) An example increased signal-noise ratio when adding dextran to the hybridization buffer solution. Arrow shows background signal. (D) An example of overstaining, which occurs when the embryos have been allowed to incubate in the staining solution for too long. This results in a very intense signal in each cell and staining in the embryo looks exaggerated (arrow, low quality), making it difficult to obtain high quality images. Optimally, nuclei can be observed in each cell (arrow, high quality).

the end of the chapter. Examples of WMISH embryos when some aspect of these procedures has gone awry are provided in Fig. 2.

One important point regarding all these protocols is that for every solution change, from fixation until the final wash steps, two precautions should always be taken: (1) leave enough buffer to keep the embryos submerged at all times; (2) always resuspend embryos when adding new solutions either by gently pipetting up and down (for *S. purpuratus*, *H. pulcherrimus*, *E. tribuloides*) or by creating a swirl in the well by gently blowing with a mouth pipette (for *P. lividus* and *L. variegatus*), this second step being especially critical to enhance the signal-to-noise ratio.

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2.1 Probe preparation

Antisense RNA probes are sequences of single-stranded RNA complementary to the coding sequence of the desired target mRNA. Antisense RNA is synthesized by in-vitro transcription using highly specific DNA-dependent RNA polymerases derived from bacteriophages such as SP6, T7, and T3 polymerases. DIG-UTP ribonucleotides are incorporated into the antisense RNA, allowing detection with an anti-DIG antibody. It is important to generate probes that are at least 800 bps in length, when possible, to increase probe specificity. Many labs generate full-length clones that are more than a thousand base pairs in length and have found that these produce better results.

A. Template preparation. DIG-labeled antisense RNA probes are classically prepared by *in vitro* transcription from linearized DNA template cloned into a vector and flanked by two distinct polymerase promoters on each side of the cloning site (*e.g.*, T7, SP6, or T3). Vector linearization should be done appropriately depending on DNA orientation to later generate antisense (or sense) RNA probes. For the linearization, restriction enzymes generating 5′ overhangs should be favored over restriction enzymes leaving blunt ends. Restriction enzymes resulting in 3′ overhangs should be avoided because they may generate wrap-around products that can reduce sensitivity and increase background. To confirm the complete linearization of the vector after the digestion reaction, run 5 µL of digested DNA versus uncut DNA used as a reference on an agarose gel. *Note: do not utilize the entire digested DNA for this confirmation step since it will be purified in subsequent steps.* Additional endonuclease digestion will be necessary if there is incomplete digestion of the plasmid.

Alternatively, PCR product can be used as template for RNA transcription. For this method, add a promoter sequence (*e.g.*, SP6) to the reverse PCR primer for the subsequent *in vitro* transcription reaction. Standard PCR reactions can be performed from total cDNA or cDNA gene clones. If amplifying from cDNA clones it is important to perform around 20 PCR cycles or less, so as to avoid nonspecific amplification and sequence errors. Also, run the PCR product on an agarose gel to confirm the correct size of the product.

Once the linearized vector or PCR product is generated, it should be subsequently purified using either standard PCR purification kits, gel extraction, or phenol-chloroform extraction, to ensure that the product is free of RNases. PCR purification kits are preferred over gel or phenol-chloroform extractions, as the last two methods classically lead to more important product loss. Gel purification may nonetheless be required if the reaction produces multiple PCR products. In this case, the researcher should rerun the PCR under more stringent conditions to obtain fewer products or gel purify the product of interest. Please note that at least 100 ng (ideally 500–2000 ng) of linearized vector or PCR product is required to proceed with probe synthesis. At the end of the purification or extraction protocol, the DNA template should be resuspended in 10–12 µL of nuclease free distilled water (dH_2O) to maximize its concentration. The purified linear DNA is now ready to use for *in vitro* transcription and can be stored at −20°C for later use.

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B. Probe synthesis. Antisense probe synthesis is performed by *in vitro* transcription reaction using a DIG RNA labeling mix, an appropriate transcription buffer, and the appropriate RNA polymerase (an RNase inhibitor can also be added). Many labs classically set up a 20 µL reaction containing at least 0.5 µg (although routinely 2 µg) of total DNA template (*e.g.*, 4 µL 5× OPTIZYME™ Transcription buffer [Fisher BioReagents®], 1 µL OPTIZYME™ SP6, T7, or T3 RNA Polymerase [20 U/µL, Fisher BioReagents®], optional 0.5 µL RNase inhibitor [40 U/µL, Promega], and 2 µL 10× DIG RNA labeling mix [Roche Diagnostics]). When preparing the reaction mix, never vortex the enzymes and always make sure that the transcription buffer is completely thawed and well resuspended before use. For this, incubate the tube at 37°C for a few minutes and then vigorously vortex the tube until any precipitate is fully dissolved. Repeat this step if necessary. Once the reaction mix is prepared, incubate it at 37°C for 2 h. This incubation step should be performed in a convection incubator rather than in a dry block heater to prevent evaporation in the tubes. For SP6 polymerase reactions, a longer incubation time often leads to better yield. After the reaction, add 1 µL of Turbo DNAse™ (2 U/µL, Thermo Fischer Scientific) into the mix and incubate at 37°C for 15 min to digest the DNA template.

Purification of the probe should then be performed by running the sample through a bead or column-based RNA purification kit for removal of unincorporated nucleotides from RNA labeling reaction (*e.g.*, illustra™ ProbeQuant™ G-50 Micro Column protocol [GE Healthcare]). Then, precipitate the RNA probe by adding 1 µL of glycogen (stock 20 mg/µL; Roche Diagnostics), 7.5 µL of lithium chloride precipitation solution (Ambion), and 190 µL of 100% RNAse-free ethanol. After incorporation of the ethanol, gently mix the solution by tube inversion and incubate at −20°C for at least 30 min to allow RNA precipitation. Centrifuge at 14,000 rpm for 30 min at 4°C to pellet RNA. Wash the pellet once with 70% RNase-free ethanol, air-dry the sample (no more than 5 min) and resuspend in an appropriate volume of DEPC-water. Alternatively, the RNA synthesis reaction can be cleaned up using a commercially available RNA purification kit (*e.g.*, RNeasy Kit [Qiagen]). Store the samples on ice and determine the concentration.

It is important to examine the integrity of RNA probes before using them for WMISH. A straightforward method is to add 2–3 µL of the probe to 10 µL of formamide, which prevents degradation in the gel. Incubate the solution at 65°C for 5 min, and then place it on ice for 2 min. Directly run the probe/formamide solution on an agarose gel for at least 20 min. Do not add a running buffer to the probe/formamide solution since the solution is viscous enough to sink to the bottom of the wells. The probe is considered free of degradation if a tight band or bands are observed.

Stock aliquots of antisense RNA probe should be stored at −80°C. Working stocks can be generated from the −80°C stocks, diluted either in DEPC-water or in hybridization buffer, and stored at −20°C for several freeze/thaw cycles without significant degradation. WMISH experiments can be very sensitive to probe concentration. Therefore, when using a new RNA probe, test and optimize probe concentration using the same working stock to identify the most suitable final probe concentration.

2.2 Embryo fixation

Proper fixation is critical to prevent degradation of endogenous mRNA and maintain the morphological integrity of embryos during the WMISH procedure. All steps of the various protocols starting with fixation can be conveniently performed in 96-well flat-bottom plastic tissue culture plates (Fig. 3A). These plates allow one to easily remove and add solutions to embryos with an appropriate pipettor while observing embryos with a stereoscope. Similarly, fixation can also be conducted in single or 9-well glass plates (Fig. 3B and C). Alternatively, if large numbers of embryos need to be fixed, fixation can be carried out in microcentrifuge tubes or conical tubes.

A. *Embryo handling (applicable to all five species).* The protocols here are for WMISH on embryonic or larval stages from before fertilization up to several hours or days post-fertilization (note that older larvae can also be fixed as described below although their arms will often be rumpled upon the WMISH procedure). For cleavage stage embryos (*i.e.*, after fertilization but before hatching), fertilization envelopes must be damaged or removed prior to fixation, as this embryonic structure impedes probe penetration. For all species described here, with the exception of *P. lividus*, hardening of the fertilization envelope can be inhibited by fertilizing eggs in the presence of either 1 mM 3-aminotriazole

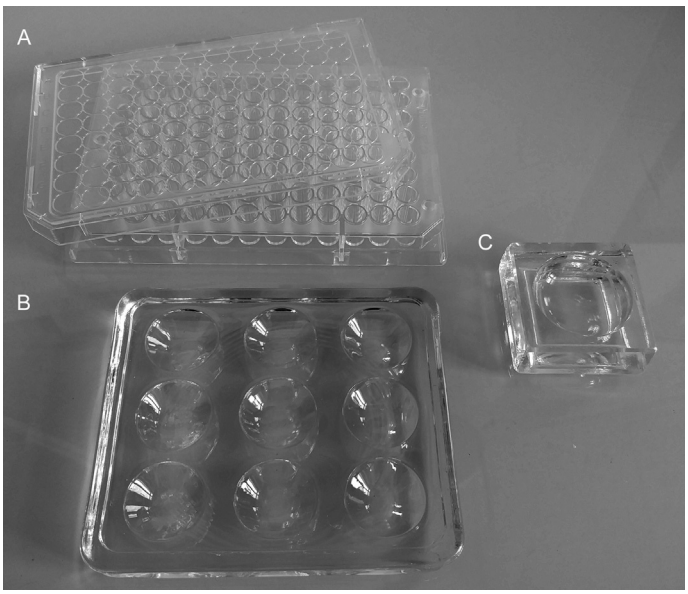

FIG. 3

Representative material in which to conduct WMISH for sea urchin embryos. (A) A 96-well plate. (B) A 9-well glass plate. (C) A single well glass dish.

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(3AT) or 1–3 mM *para*-aminobenzoic acid (PABA), the most effective concentration of these small molecules depending on the species. For *P. lividus* PABA and 3AT must be used simultaneously at a concentration of 2 mM each. Following fertilization, these chemicals and excess sperm should be removed by washing the zygotes twice with fresh artificial sea water (ASW). Breaking up the fertilization envelopes can then be done mechanically at the desired stage, although fewer problems arise when performed on 1-cell stage zygotes. For most species, a convenient method is to fertilize eggs attached to protamine sulfate-coated injection plates (see chapter "Microinjection methods for sea urchin eggs and blastomeres" by Yaguchi, Vol. 150) and subsequently dislodge the embryos from the fertilization envelopes with mechanical force generated by back-and-forth water movement with a mouth pipette or a pipettor. Alternatively, a second method consists of passing the embryos through a fine Nitex mesh (*e.g.*, 70 µm for *P. lividus*, 80 µm for *S. purpuratus* and *H. pulcherrimus*, 100 µm for *L. variegatus*) two to three times. For *E. tribuloides,* passing cleavage stage embryos through a Nitex mesh results in loss of the morphological integrity of the embryos. In this species, the fertilization envelope of cleavage stage embryos simply needs to be ruptured or damaged by forcefully removing them from a protamine-sulfate coated petri dish. Subsequently, to eliminate all broken fertilization envelopes the embryos should be washed two times with fresh ASW before fixing them or putting them under appropriate conditions for further development.

When working with swimming embryos (*i.e.*, after hatching), note that the swimming behavior can be stopped by adding two to three drops of freshly prepared room temperature (RT) fixative to petri dishes or injection plates containing embryos in ASW. Mix gently by pipetting with a 200 µL pipettor, allowing them to settle (~1–2 min) before proceeding to transfer. *Note: a gentle circular movement of the dish or plate will efficiently concentrate the embryos in the center.*

To transfer embryos into individual wells prior to fixation (*e.g.*, of 96-well plates or of glass plates), and especially when handling a small number of embryos (*e.g.*, injected embryos), a mouth pipette should be used (for instructions on how to build a mouth pipette, see chapter "Microinjection methods for sea urchin eggs and blastomeres" by Yaguchi, Vol. 150). Alternatively, Pasteur pipettes or micropipettors can also be used, and this will primarily be the case when handling large numbers of cultured embryos.

B. *Fixation and washes for S. purpuratus, H. pulcherrimus, and E. tribuloides.*
To fix swimming embryos, add two to three drops of freshly prepared RT fixative to wells containing embryos in ASW, mix gently by pipetting with a 200 µL pipettor, and allow them to settle (~1–2 min). Remove the fixative solution and conduct two additional rounds of washes with fixative solution (180 µL), leaving embryos in the second fixative wash for 1 h at RT. After the 1-h fixation step, wash embryos five times at RT with 180 µL of MOPS wash buffer, waiting 5 min between washes. As mentioned earlier, make sure to thoroughly mix the embryos

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into the wash buffer by gently pipetting them up and down several times after each wash step. Fixed embryos can as such be stored at 4°C for 2 days, but if stored longer, 0.2% sodium azide should be added to the MOPS wash buffer to prevent bacterial growth.

C. *Fixation and washes for P. lividus and L. variegatus*. At the desired stage, embryos are transferred to an appropriate volume of ASW in either wells or tubes. For instance, if fixing in 96-well plates, embryos should be placed in 100 µL of ASW; if fixing in recessed glass plates, 400–800 µL of ASW; if fixing in 15 mL conical tubes, 8 mL of ASW; and if fixing in 50 mL conical tubes, 26 mL of ASW. Add freshly-prepared fixative solution twice-concentrated to embryos using the same volumes than ASW. For fixation in 96-well plates and glass plates, due to the small volumes being used, the fixative solution is added to the embryos at once and mixed by gentle up and down pipetting. When fixing in conical tubes, however, the fixative solution is added gradually, and in between each addition the solution containing the embryos is mixed by gently flipping of the tube up and down. *Note: The trick here is to avoid creating bubbles at any step even after closing the tube, which should hence be completely full of liquid*. Once in fixative, embryos are stored for 1 h at RT either directly on the bench for those in wells and plates or onto a benchtop tube rotator for those in tubes.

After fixation, embryos are washed once at RT in TBST for 1–2 min with 180 µL if in 96-well plate or 1 mL if in a glass-plate, or in ASW with 10 mL if in 15 mL conical tube or 35 mL if in 50 mL conical tube. Before storage, the embryos are further washed one to two times at RT for 1 to 2 min with about the same volumes of cold −20°C 100% MeOH. Subsequently, embryos are stored in fresh cold −20°C 100% MeOH at −20°C for up to 1 week, if fixed in small volumes, and for much longer (*e.g.*, years) if fixed in tubes. *Note: to ensure proper permeabilization of the embryos, this −20°C storage step must be conducted for at least 2 h before moving on to the prehybridization step*.

D. *Fixation with glutaraldehyde (applicable to all five species)*. PFA-based fixation protocols on the whole provide excellent morphological preservation. In certain cases, though, embryos that possess a large blastocoel, *e.g.*, cidaroid sea urchins at late blastula stages and onward, or old sea urchin larvae, pose a challenge. Under these circumstances, fixation with glutaraldehyde, a larger aldehyde molecule that provides exceptional, if not better, preservation (Ransick & Davidson, 2012) can be attempted. In general, glutaraldehyde fixation requires more time than PFA, and thus overnight incubation at 4°C is highly recommended. Upon transfer into wells (or tubes), remove as much seawater as possible from embryos. Add to each well 200 µL of Fixation Solution I, mix gently by up and down pipetting, and incubate on ice for 15–30 min. Replace Fixative Solution I by Fixative Solution II, which will also be mixed gently by up and down pipetting and incubate overnight at 4°C. Upon glutaraldehyde fixation, embryos are washed three times in TBST buffer.

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At this stage, they can be directed either to the proteinase K step (see below) or directly to the prehybridization stage depending on the species.

E. *Proteinase K treatment (applicable to all five species).* An optional proteinase K treatment can be supplemented to enhance the signal-to-noise ratio of paraformaldehyde and glutaraldehyde-fixed embryos. For this, embryos are washed at RT for 5 min in 5 μg/mL freshly prepared proteinase K solution (2 mg proteinase K in 400 μL dH_2O) made in TBST. This solution can be carefully removed as the embryos sink to the bottom of the well. Immediately thereafter to stop the proteinase K reaction embryos are rinsed in 25 mM glycine in TBST for 5 min at RT, and they are then washed two times for 5 min at RT in TBST. Post-fixation is achieved by a 30-min incubation at RT in 50 mM MOPS (pH 7.0), 150 mM NaCl, and 4% PFA. While this optional treatment may significantly enhance signal-to-noise ratio, it should be noted that adverse effects have been observed when the duration of proteinase K incubation is too long. Therefore, this step requires species-specific calibration and should be undertaken with great care.

2.3 General hybridization and washing protocols

The goals of the following protocols are to hybridize the exogenous RNA probes, remove all nonspecifically bound RNA probes, and prepare the embryos for the color reaction step. Here, it is important to note that the probe concentrations vary depending on the species as do the composition of the buffers and their use.

A. *Prehybridization for S. purpuratus and H. pulcherrimus.* After the fixation and wash steps, remove the last washing buffer. Add 180 μL of a 2:1 ratio of MOPS wash buffer to hybridization buffer and incubate for at least 20 min at RT. Remove the buffer and add 180 μL of a 1:2 ratio of MOPS wash buffer to hybridization buffer, mix embryos into the solution, and incubate for at least 20 min at RT. Finally, wash embryos into 100–150 μL of 100% hybridization buffer and incubate in a 50°C oven for 1–3 h. Importantly, seal the wells with an adhesive sheet (BioRad microseal film) to prevent evaporation. Incubation temperatures ranging from 58 to 65°C have also been successfully utilized. An overnight incubation is acceptable if the researcher is familiar with the RNA probe.

B. *Prehybridization for P. lividus* and *L. variegatus*. Following fixation and −20°C MeOH storage, embryos will be present, or can be transferred, into individual wells of a 96-well-plate. The embryos will be in a final volume of about 60 μL of cold MeOH. To gradually rehydrate the embryos, add into the wells 60 μL of 1:1 ratio RT MeOH to TBST and incubate for 5 min at RT. Add again 60 μL of TBST and incubate once more 5 min at RT. Repeat this last step one additional time. Remove approximately 200 μL from the wells, wash embryos with 180 μL of TBST and incubate 5 min at RT. These progressive rehydration steps are crucial both for the signal-to-noise ratio as well as to

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promote recovery of normal embryo morphology. For prehybridization, replace the TBST buffer with 180 µL of hybridization buffer and incubate the embryos for 1 h in a 65°C oven. For this incubation, the 96-well plate is placed in the oven in a humidity chamber to avoid evaporation.

C. *Prehybridization for E. tribuloides*. A modified Tris-based hybridization protocol at higher incubation temperatures produced excellent results in *E. tribuloides* embryos. Prehybridize embryos in hybridization buffer for 1 h at 58–62°C. Refer to the materials section for the hybridization buffer recipe.

D. *Hybridization and washes for S. purpuratus and H. pulcherrimus*. From a working stock, add antisense RNA probe to a new microcentrifuge tube containing a fresh aliquot of hybridization buffer and 500 µg/mL yeast tRNA to a final concentration of 0.1–0.3 ng/µL of probe. Yeast tRNA is added to the probe solution to decrease nonspecific binding of the anti-sense probe. The concentration of the probe will vary for each target. Concentrations ranging from 0.1 to 1 ng/µL have been successfully utilized. Vortex the probe solution gently and preheat it to 50°C. While preheating the probe solution, aspirate the prehybridization buffer from the embryos, then add 100 µL of the pre-heated probe solution to the prehybridized embryos, seal the 96-well plate with an adhesive sheet, and hybridize at 50°C for 4–7 days. Incubation times can vary depending on the probe. Embryos can be incubated in small boxes fitted with a wet paper towel to protect against evaporation in the case the adhesive sheet fails to seal properly. After hybridization, wash embryos five times with 180 µL of freshly made MOPS wash buffer at 50°C for a total of 3 h. Then, wash embryos three more times for at least 15 min between washes with 180 µL of MOPS wash buffer at RT.

E. *Hybridization and washes for P. lividus and L. variegatus*. To make the probe solution, dilute the antisense RNA probe into a fresh aliquot of hybridization buffer maintained at RT to a typical final concentration of 1 ng/µL. Depending on the gene analyzed the final concentration of RNA probe could vary from 0.5 to 1.5 ng/µL. After prehybridization, replace the hybridization buffer with 180 µL of probe solution and incubate overnight at 65°C, maintaining the 96-well plate in a humidity chamber. The hybridization incubation may vary between 12 and 24 h. For low-copy number genes, the prehybridization and hybridization incubations could also be performed at 60°C, which may improve the signal-to-noise ratio.

Following hybridization, embryos are washed several times with 65°C pre-heated solutions. The goal of these increasingly stringent post-hybridization washes is to eliminate all nonspecifically bound probes. For each wash the 96-well plate is incubated in a humidity chamber at 65°C for 15 min. First, wash embryos with 180 µL of pre-heated hybridization buffer. Then, wash embryos twice with 180 µL of pre-heated 1:1 ratio solution of hybridization buffer and 2× SSCT. Note: embryos are initially transparent in the hybridization solution and progressively become more visible during these two washes. The following three washes are carried out using 180 µL of the following pre-heated solutions: 2× SSCT, 0.2× SSCT, and finally 0.1× SSCT. The last step

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of this wash series prepares embryos for blocking and antibody incubation solutions. It is carried out at RT with 180 µL of RT 1× TBST for 5 min.

F. *Hybridization and washes for E. tribuloides*. Prepare hybridization buffer with antisense probes at desired concentration (usually 0.5–1 ng/µL). Hybridize probes overnight at 58–62°C. The following day, post-hybridization washes are carried out to strip unbound probes from targets in the embryo. These washes all take place at 58–62°C, and consist of one wash each in the following buffers for the indicated times: hybridization buffer for 15 min; 1 wash in 1:1 ratio of hybridization buffer to 2× SSCT for 15 min; 1 wash in 2× SSCT for 15 min; 1 wash in 0.5× SSCT for 20 min; and finally 1 wash in 0.2× SSCT for 20 min. Embryos are subsequently washed into TBST and are ready for blocking and antibody incubation.

G. *Optional hybridization with 5% dextran (applicable to all five species)*. As mentioned in Section 1 some RNA probes produce more background than other probes or exhibit faint signal when the target is not expressed in high copy number. In this case, try performing the probe hybridization step in hybridization buffer containing 5% dextran sulfate sodium salt. However, *do not use Dextran Sulfate Hybridization buffer for prehybridization*. The addition of dextran to hybridization buffers produces a viscous mix, but often increases the signal-to-noise ratio. When making a 5% dextran hybridization solution, add dextran sulfate to dH_2O and salt solution before introducing detergents and formamide. Incubate this mixture at 55°C and vortex intermittently until the dextran sulfate completely dissolves. Subsequently add the detergents and formamide and proceed with the hybridization recipe or store at −20°C.

2.4 Antibody incubation and color reaction

After the final washes of hybridization buffer from embryos and larvae, the DIG-labeled probe is ready for detection. There are different methods for the various species and optional steps that may enhance the alkaline phosphatase (AP) signal.

A. *Antibody incubation for S. purpuratus, H. pulcherrimus, and E. tribuloides*. After the final probe wash, aspirate the MOPS or TBST wash buffer and add 180 µL of blocking buffer to the embryos, mix gently, and incubate for at least 45 min at RT or overnight at 4°C. Then, remove the blocking buffer and add a solution of blocking buffer containing a 1:1500 dilution of AP-conjugated anti-DIG antibody, mix gently and incubate overnight at RT in a sealed plate to avoid evaporation. Allowing the embryos to incubate in the antibody for longer durations can increase nonspecific background staining. Alternatively, the antibody incubation step can be conducted for 1–2 h at RT with well characterized probes. After antibody incubation, wash embryos with 180 µL MOPS wash buffer at RT six times for 5 min each (or until embryos sink to the bottom of the wells). Embryos can be stored *overnight* at 4°C after this step.

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B. *Antibody incubation for P. lividus and L. variegatus.* To reduce background due to nonspecific binding of the anti-DIG-antibody, embryos are first incubated at RT for 45 min to 1 h in 180 μL of blocking buffer. Then, the embryos are placed in 180 μL of a freshly prepared blocking buffer solution containing a dilution of 1:2000 (for *P. lividus*) or 1:1000 (for *L. variegatus*) of anti-DIG antibody (Roche Diagnostics). Embryos are incubated in the presence of the antibody at RT for 1.5–2 h. Alternatively, this step can also be conducted at 4°C overnight. Once the incubation is over, wash embryos twice at RT for 15 min and then four times at RT for 10 min with 180 μL of 1× TBST. *Note: in contrast to the protocol for S. purpuratus embryos, P. lividus embryos should not be stored at this point in TBST, as this significantly weakens the signal. Furthermore, it is crucial at this step to use TBST and not PBST, as the phosphate present in the PBST buffer will cross-react with the color reaction buffer that will subsequently be used.*

C. *Alkaline phosphatase color reaction (applicable to all five species).* Aspirate the antibody wash out of the wells and add 180 μL of freshly made pH 9.5 buffer. Wash embryos in pH 9.5 buffer or a total of three times over the course of 30 min (for *P. lividus* 2 washes for 5 min is sufficient, longer may impair the color reaction). This step is essential to bring the embryos to the correct pH for the color reaction to work efficiently. After the pH 9.5 buffer incubation step, remove the solution and add the color reaction solution that contains the nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) reagents. *Note: the NBT and BCIP reagents have a tendency to precipitate. When pipetting the bottom of the vial/tube should be avoided and should be briefly spun down so as to avoid precipitants at the bottom of the tube, which if present in the color reaction will increase background signal in the embryos. Furthermore, the color reaction buffer is light sensitive; thus the plate should be wrapped in aluminum foil to protect it from light.* The color reaction can proceed at different temperatures and at different rates in different species. For instance, the color reaction in *S. purpuratus*, *P. lividus* and *L. variegatus* can be very rapid, with color being observed within 15 min of the start of the reaction with a highly abundant target and an excellent probe. In these species, as well as in others, however, the color reaction can also proceed for hours at RT and may be facilitated by an overnight incubation at 4°C. This is frequently the case in *E. tribuloides*. Under these circumstances, embryos are generally left for several hours at RT before being incubated overnight at 4°C, replacing the color reaction solution after lengthy durations at RT. Typically, many labs initially incubate embryos at RT when working with a new probe, checking the reaction every 15–30 min. Once the reaction rate of a probe is determined, embryos can be placed at 37–50°C to accelerate the reaction. To slow down the reaction embryos can be placed at 4°C and left overnight.

Once the AP color reaction has reached an appropriate signal-to-noise ratio (this ratio must be empirically determined), stop the reaction by washing embryos three to five times in MOPS wash buffer (for *S. purpuratus*, *H. pulcherrimus*, and *E. tribuloides*) or in 50 mM EDTA in PBST two times for 10 min and then in

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PBST five times for 5 min (for *P. lividus* and *L. variegatus*). For *P. lividus* and *L. variegatus*, while EDTA is used to rapidly quench the color reaction, washing it out afterward is essential to avoid its crystallization over time in the wells. For all species, embryos can then be stored in 30% glycerol in MOPS wash buffer or in PBST for months at 4°C.

D. *Optional alkaline phosphatase color reaction with polyvinyl alcohol (PVA) (applicable to all five species).* Often for some probes it can take over 8 h for the AP color reaction to produce enough signal for detection. To shorten these incubation times PVA can be added to the NBT/BCIP phosphate buffer detection solution to a final concentration of 5%. See recipe for making this solution at the end of the chapter.

2.5 Combination of *in situ* hybridization with immunohistochemistry (developed for *S. purpuratus* and *H. pulcherrimus)*

Immunostaining can be performed after the completion of the WMISH procedure (Yaguchi, Yaguchi, & Burke, 2006). Combining these two techniques affords greater resolution of spatiotemporal overlap among proteins and mRNAs.

A. *Protocol for antigens that are stable after WMISH.* Antigens must be assessed to determine if they are stable after exposure to formaldehyde fixation and hybridization buffer at high temperature. If so, then after the final wash step of the chromogenic or fluorescent WMISH protocol, block specimens with 2.5% skim milk in MOPS wash buffer for 10–60 min at RT. Remove the blocking buffer and incubate embryos/larvae in 100 µL/well of MaxBlot I (MBL, Nagoya, Japan) with diluted primary antibody for overnight at 4°C. The concentration of the primary antibody must be determined empirically. Upon incubation, wash samples five times with MOPS wash buffer at RT for 5–7 min per wash, and subsequently incubate them in fluorescently-labeled secondary antibody at a concentration of 1:2000 in MaxBlot II (MBL) for 2 h at RT. Wash samples with MOPS buffer at RT for 5–7 min per wash.

B. *Protocol for antigens that are unstable after WMISH.* Many antigens are difficult to detect after WMISH, likely due to protein denaturation. However, antibody signal can be amplified with tyramide signal amplification (TSA)-based fluorescent signal (Yaguchi & Katow, 2003). The protocol is the same as above except that in place of a fluorescently-labeled secondary antibody, embryos/larvae should be incubated with horse radish peroxidase (HRP)-conjugated secondary antibody at a concentration of 1:1000 in MaxBlot II (MBL) for 2 h at RT. Then, samples should be washed five times with MOPS wash buffer at RT allowing 5–7 min between washes. For TSA treatment, remove as much MOPS wash buffer from the well as possible without exposing embryos and then add TSA diluent, allowing 5–10 min for the reaction to proceed. Wash the sample five times with MOPS wash buffer at RT with 5–7 min between washes. Importantly, all TSA steps should be performed to minimize exposure to light.

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2.6 Mounting embryos for image capturing (applicable to all five species)

Mix embryos into the same solution used for the final wash solution (MOPS wash buffer or PBST) containing 30% glycerol. The glycerol provides a refractive index necessary for microscopy. *Note: embryos can be stored in this solution at 4°C for several weeks. Simply seal the plate with plastic paraffin to prevent evaporation.* Classically, embryo mounting consists of depositing the embryos between a standard slide and a standard coverslip of 1 mm thickness (Fig. 4). To avoid compressing the embryos with the coverslip, spacers can be used consisting of strips of thin double-sided adhesive tape of 100–200 μm thickness. Arrange two strips in parallel or three into a triangle with small gaps among the strips onto the slide. Position the embryos between the strips in a small volume of approximately 40 μL of mounting solution. Then, gently place a coverslip onto the slide with forceps. Apply gentle pressure to the coverslip at the level of the strips to adhere. *Note: embryos can also be "rolled" into the desired orientation using a fine tipped glass injection needle (see chapter "Microinjection methods for sea urchin eggs and blastomeres" by Yaguchi, Vol. 150) before overlaying with a coverslip.*

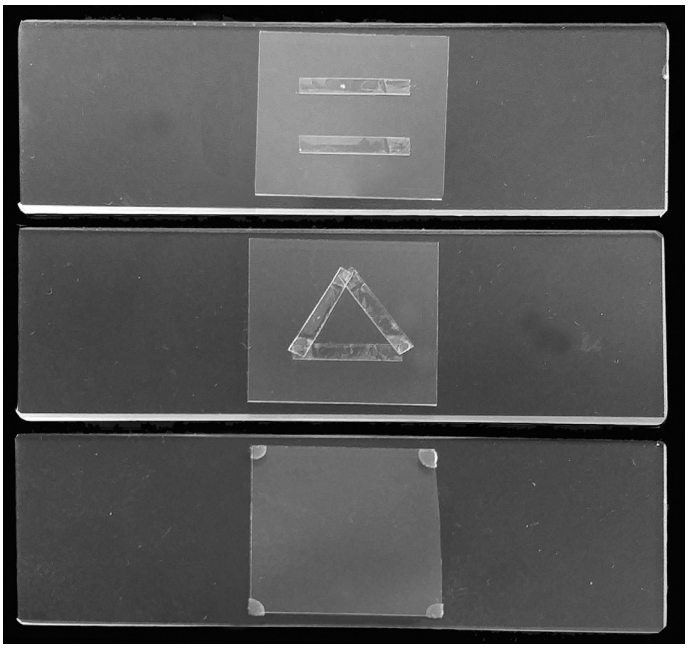

FIG. 4

Examples of different slide preparations for imaging sea urching embryos after WMISH. Top two slides: Adhesive tape can be utilized in either parallel strips or a triangular shape. Bottom slide: Clay or adhesive tape can be applied to each corner of the coverslip, allowing space between the embryos and the coverslip and slide to roll the embryos.

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Alternatively, to acquire distinct images of the same embryo in different orientations, a second mounting approach can be employed whereby the embryos are deposited on an untreated slide in a final volume of about 40 µL mounting solution, allowing free movement to orient the embryos. Small deposits of clay are placed on the four corners of the coverslip. Observe the embryos under the microscope as you begin to depress the clay feet corners. As the clay becomes more and more compressed, embryos will be caught between slide and coverslip. At this point the embryos can be rolled into specific orientations by gently pushing on the coverslip. Once mounting is achieved, by either way, slides can be sealed with nail polish to prevent evaporation for long-term storage.

3 Materials

3.1 General solutions

Artificial sea water (ASW): 516 mM NaCl, 10 mM KCl, 11 mM $CaCl_2 \cdot 2H_2O$, 34 mM $MgCl_2 \cdot 6H_2O$, 22 mM $MgSO_4 \cdot 7H_2O$, 10 mM TrisBase, diluted up to 0.8 volume with H_2O, adjust to pH 8.2 with HCl, adjust to 1 volume with H_2O.
10% Polyvinyl alcohol (PVA) stock solution: Add 10 g of PVA into 100 mL of dH_2O, heat low on a stir plate while stirring until solution becomes clear, and then remove from heat immediately. PVA is a synthetic polymer so it is important to dissolve it slowly because excessive heating can disrupt the integrity of the polymer. This 10% PVA solution can be stored at room temperature for up to 1 year.
5% PVA color reaction buffer: Make a 2× phosphate buffer (see below), dilute it 1:1 with the 10% PVA stock solution and then add 4.5 µL of nitro blue tetrazolium (NBT) (75 mg/mL) and 3.5 µL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/mL) (Roche Diagnostics) per mL of color reaction buffer in preparation.

3.2 Buffers for *S. purpuratus, H. pulcherrimus* and *E. tribuloides*

Fixative solution: Dilute 16% electron microscopy grade paraformaldehyde (PFA) (VWR, Cat. 100503) in 10 mM MOPS pH 7.0, 0.1% Tween-20, and artificial seawater (ASW) to a final concentration of 4%. For 20 mL fixative use 5 mL 16% PFA, 15 mL ASW, 200 µL 1 M MOPS pH 7.0, and 20 µL Tween-20.
MOPS wash buffer: 0.1 M MOPS pH 7.0, 0.5 M NaCl and 0.1% Tween-20 in dH_2O. For 40 mL MOPS wash buffer use 4 mL 1 M MOPS pH 7.0, 4 mL 5 M NaCl, 32 mL dH_2O, and 40 µL Tween-20.
Hybridization buffer: 70% formamide, 0.1 M MOPS pH 7.0, 0.5 M NaCl, 1 mg/mL BSA and 0.1% Tween-20. For 40 mL hybridization buffer use 4 mL 1 M MOPS pH 7.0, 4 mL 5 M NaCl, 4 mL dH_2O, 0.04 g BSA, and 40 µL Tween-20.

Mix well by vortexing, add 28 mL formamide, and vortex again. This buffer can be stored at −20°C for several months.
Alkaline phosphatase (pH 9.5) buffer: 0.1 M Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1 mM Levamisole, and 0.1% Tween-20 to the embryos. For 20 mL pH 9.5 buffer contains 2 mL 1 M Tris pH 9.5, 400 µL 5 M NaCl, 1 mL 1 M $MgCl_2$, 20 µL Tween-20, 16.6 mL dH_2O, and 5 mg Levamisole.

3.3 Buffers for *P. lividus* and *L. variegatus*

All buffers are prepared and stored at room temperature, unless otherwise stated.

Fixative solution: 8% reagent grade, crystalline paraformaldehyde (Sigma) diluted up to 0.7 volume with ASW. Heat at 65 °C and vortex periodically every 30 min. When dissolution is complete, let cool at room temperature, add 20 mM EPPS pH 8.0 and adjust to 1 volume with ASW. Aliquot if needed and keep at room temperature for immediate use. For usage within 24 h, store at 4°C. For usage in longer-term store at −20°C. Frozen fixative solution is good for months. When stored at −20°C, before usage, let the fixative solution come to room temperature. You can thaw it overnight at 4°C or for several hours at room temperature or a couple of hours at 65°C. Prior to use vortex to ensure that everything is well dissolved and make sure to use at room temperature.
10× TBS: 1.5 M NaCl, 100 mM Tris-HCl pH 8.0, in H_2O.
1× TBST: 0.1 volume of 10× TBS diluted up to 0.7 volume with dH_2O, add 0.1% Tween-20, mix, wait for the foam to disappear, adjust to 1 volume with dH_2O, mix (stored at 4°C).
10× PBS: 1.37 M NaCl, 0.027 M KCl, 0.1 M Na_2HPO_4, 0.018 M KH_2PO_4, diluted up to 0.8 volume with dH_2O, adjust to pH 7.4 with HCl, adjust to 1 volume with dH_2O
1× PBST: 0.1 volume of 10 × PBS diluted up to 0.7 volume with dH_2O, add 0.1% Tween-20, mix, wait for the foam to disappear, adjust to 1 volume with dH_2O, mix (store at 4°C).
20× SSC: 3 M NaCl, 0.3 M $CiNa_3$ diluted up to 0.8 volume with dH_2O, adjust to pH 7.0 for washing solutions or pH 5.0 for hybridization buffer with HCl, adjust to 1 volume with dH_2O.
2× SSCT/0.2× SSCT/0.1× SSCT: 0.1/0.01/0.05 volume of 20× SSC diluted up to 0.7 volume with dH_2O, add 0.1% Tween-20, mix, wait for the foam to disappear, adjust to 1 volume with dH_2O, mix (store at 4°C).
Hybridization buffer: 25 mL ultrapure, deionized formamide, 12.5 mL 20× SSC pH 5.0, 50 µL heparin 50 mg/mL, 50 µL yeast RNA 50 mg/mL, 50 µL Tween-20, adjust volume to 50 mL with dH_2O (stored at -20°C).
Heat-inactivated sheep serum: plain sheep serum incubated 30 min at 60°C, cooled down at room temperature, aliquoted, and stored at −80°C.

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Blocking solution: 2% heat inactivated sheep serum, 5 mg/mL BSA, in 1× TBST. Should be prepared right before use.
Alkaline phosphatase buffer: 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, in dH_2O.
This pre-preparation can be kept for 1–6 months at room temperature. Right before use add to it 5 mM Levamisole and 0.1% Tween-20.
Color reaction buffer: *Add 4.5 μL of nitro blue tetrazolium (NBT) (75 mg/mL) and 3.5 μL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/mL) (Roche) to 1 mL of phosphate buffer.*

3.4 Alternative buffers for *E. tribuloides* and *S. purpuratus*

Fixative solution I: 32.5% filtered seawater or ASW, 32.5 mM MOPS (pH 7.0), 162.5 mM NaCl, and 0.66% glutaraldehyde (freshly opened ampule, Sigma).
Fixative solution II: 32.5% filtered seawater or ASW, 32.5 mM MOPS (pH 7.0), 162.5 mM NaCl, and 1.32% glutaraldehyde (freshly opened ampule, Sigma).
MOPS wash buffer: 32.5% filtered seawater, 32.5 mM MOPS (pH 7.0), and 162.5 mM NaCl.
Hybridization buffer: 50% formamide, 5× SSC, 20 mM Tris pH 7.5, 5 mM EDTA, 0.5 μg/μL yeast tRNA, 2× Denhardt's solution, and 0.05 μg/L Heparin.

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