Video Article

Whole Mount RNA Fluorescent *in situ* Hybridization of *Drosophila* Embryos

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Abstract

Assessing the expression pattern of a gene, as well as the subcellular localization properties of its transcribed RNA, are key features for understanding its biological function during development. RNA *in situ* hybridization (RNA-ISH) is a powerful method used for visualizing RNA distribution properties, be it at the organismal, cellular or subcellular levels [1]. RNA-ISH is based on the hybridization of a labeled nucleic acid probe (*e.g.* antisense RNA, oligonucleotides) complementary to the sequence of an mRNA or a non-coding RNA target of interest [2]. As the procedure requires primary sequence information alone to generate sequence-specific probes, it can be universally applied to a broad range of organisms and tissue specimens [3]. Indeed, a number of large-scale ISH studies have been implemented to document gene expression and RNA localization dynamics in various model organisms, which has led to the establishment of important community resources [4-11]. While a variety of probe labeling and detection strategies have been developed over the years, the combined usage of fluorescently-labeled detection reagents and enzymatic signal amplification steps offer significant enhancements in the sensitivity and resolution of the procedure [12]. Here, we describe an optimized fluorescent *in situ* hybridization method (FISH) employing tyramide signal amplification (TSA) to visualize RNA expression and localization dynamics in staged *Drosophila* embryos. The procedure is carried out in 96-well PCR plate format, which greatly facilitates the simultaneous processing of large numbers of samples.

Video Link

The video component of this article can be found at https://www.jove.com/video/50057/

Protocol

1. RNA Probe Preparation

Overview: The following section describes the steps required to make Digoxigenin (Dig)-labeled RNA probes suitable for FISH. The first step involves cloning or PCR amplifying a sequence corresponding to the transcribed region of a gene of interest that will be used to generate a sequence-specific probe. This can be achieved by first cloning the gene segment into a plasmid in which the multiple cloning site is flanked by bacteriophage promoter elements (T7, T3 or Sp6), which can then serve as a template for PCR using flanking primers that incorporate the promoter sequences, thus generating a linear PCR product with different promoter sequences at each extremity (**Figure 1A**). Alternatively, to avoid the cloning step, one can also perform PCR amplification from genomic DNA or cDNA using oligonucleotides that include T7, T3 or Sp6 sequences at their 5' extremities (*e.g.* T7: 5'-TAATACGACTCACTATAGGGAGA-3'; T3: 5'-AATTAACCCTCACTAAAGGGAGA-3'; Sp6: 5'-ATTTAGGTGACACTATAGAAGAG-3'). The linear PCR products are then used as templates to make Dig-labeled sense or antisense RNA probes by run-off *in vitro* transcription with the appropriate polymerase (**Figure 1B**). When making probes for specific genes, we recommend preparing both sense and antisense RNA probes using distinct polymerases, which will be helpful for assessing FISH signal specificity (*i.e.* unless the gene of interest is transcribed in the antisense orientation, the sense RNA probe will reveal the level of background signal). In all of the following steps, one should take great care to avoid potential degradation by contaminating ribonucleases (RNAses) by first cleaning all bench areas and equipment with 70% ethanol or commercial RNAse decontamination solutions and by using RNAse-free supplies (*e.g.* water, tips, microcentrifuge tubes).

Materials

- 1.5 ml microcentrifuge tubes, (Abgene, Rochester, NY, USA Cat. No 0900)
- Gel-extraction kits (QIAGEN, Mississauga, ON, Canada; Cat. No. 28706).
- RNAse free water (Wisent, Inc. Cat. No. 809-115-CL)

- RNA polymerases (T7, T3, or SP6). (20 U/ µl, Fermentas Biosciences. Cat. No. EP0101, EP0111, EP0131).
- RNAse-OUT Ribonuclease inhibitors (40 U/ µl), (Invitrogen, Burlington ON. Canada.Cat. No. 10777-019).
- Digoxigenin (DIG) nucleotide mixes (DIG-11-UTP, Roche Applied Biosciences, Laval, QC, Canada. Cat. No.11209256910).

Equipment

- Table top micro-centrifuge
- Gel-electrophoresis apparatus

1. PCR amplify gene-specific sequence from genomic DNA, cDNA, or plasmid DNA to generate a linear PCR product flanked by bacteriophage T7, T3 or Sp6 promoter sequences. Follow the manufacturer's recommendations for PCR conditions.
2. Verify the quality and size of the PCR product by agarose gel electrophoresis. Excise and purify PCR product using a standard gel-extraction kit according to the manufactures recommendations and elute the product in 50 µl of buffer.
3. Precipitate the PCR product by adding 5 µl of 3M sodium acetate (pH 5.2) and 150 µl of ice cold 100% ethanol, and place the tubes at -70 °C overnight. Centrifuge tubes at 13,000 x g for 10 min at 4 °C and wash the pellet with 70% ethanol. Spin again, remove all traces of ethanol and air dry the pellet. Resuspend in 25-50 µl of RNAse-free water.
4. Transcribe Dig-labeled probe using 200-500 ng purified PCR product in a 20 µl reaction as follows: 2 µl of 10X T7 transcription buffer (Invitrogen), 1 µl (20 U/ µl) of RNAse inhibitor, 2 µl Dig-NTP mix, and 2 µl T7 RNA polymerase (20 U/ µl). Bring the final volume to 20 µl with RNAse-free water. Incubate the transcription reaction at 37 °C for 3-4 hr.
5. Adjust transcription mix to 50 µl with RNAse-free water and precipitate the Dig-labeled RNA probe as described in Step 4. Resuspend the washed RNA pellet in 100 µl of RNAse-free water. Store the resuspended samples at -70 °C.
6. Quantify the probe using a nanodrop spectrophotometer. To verify the probe quality, run a 1-2 µl sample on a 1-2% agarose gel stained with ethidium bromide.

2. Collection and Fixation of *Drosophila* Embryos

Overview: This section describes steps for harvesting and processing staged *Drosophila* embryo. Depending on the number of embryos needed, flies can be maintained in population cages of various sizes. The following steps are for collections performed using 900 cm^3 cylindrical cages using 100mm apple juice collection plates. Proper fixation requires the removal of two protective layers surrounding the embryo: the outer chorion and the inner vitelline membrane [13]. Once harvested, the embryos are first bathed in a 50% bleach solution to remove the chorion, then they are mixed in a biphasic solution containing heptane (permeabilizes the vitelline membrane), and PBS containing 3.7% formaldehyde. The vitelline membrane is then cracked and removed by transferring the embryos into a biphasic mixture of heptane and methanol. Proper embryo fixation and removal of the vitelline membrane is critical for the success of the downstream FISH procedure.

Materials

- Apple-juice agar plates (40% apple juice, 4% sucrose, 3.5% agar, 0.3% methyl paraben) with dime-sized yeast paste (baker's yeast mixed with water to butter consistency)
- Powdered paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA; Cat. No. 19208)
- Heptane
- Methanol
- 3% Bleach solution
- 20 ml glass scintillation vials (Thermo Fisher Scientific, Ottawa, ON, Canada; Cat. No. 03-337-15).

Equipment

- Population cages
- Collection baskets (made using a Nitex mesh and the top portion of a cut 50 ml polypropylene tube in which a hole has been cut in the lid).
- Small paint brush
- Table top vortex

1. Pre-clear well-fed population cages with a fresh apple juice/yeast plate for 1 hr at 25 °C.
2. Add a new apple juice/yeast plate to the cage to harvest early stage embryos. Incubate the cage for 4 hr at 25 °C (note that collection times can be adjusted depending on the desired stages).
3. Prepare a fresh 37% formaldehyde solution by combining 3.7 g paraformaldehyde powder, 70 µl of 2N KOH, and 7.3 ml of milliQ water in a glass scintillation vial under a chemical hood. Boil the solution until the paraformaldehyde powder is completely dissolved, then cool to room temperature and filter into new scintillation vial.
4. Prepare a biphasic fixation solution by combining 2.25 ml of 1X PBS with 250 ml of 37% formaldehyde in a scintillation vial, then adding 7.5 ml of heptane.
5. Harvest the apple juice plate from the cage and collect the embryos by adding a bit of room temperature tap water and delicately brushing the embryos off the plate with the paint brush. Transfer the resuspended embryos into a basket and rinse with lukewarm tap water.
6. Dechorionate embryos for 90 sec by bathing the collection baskets in bleach solution, then rinse thoroughly with lukewarm tap water (until bleach smell is gone).
7. Disassemble the collection basket, collect embryos gently using a paintbrush and transfer them into the biphasic fixation solution. The embryos will accumulate at the interface between the PBS and heptane layers.
8. Seal scintillation vials and fasten to a vortex mixer. Shake for 20 min on lowest setting.
9. Remove and discard most of the lower PBS and upper heptane phases using a Pasteur pipette. Aspirate the remaining PBS/heptane/embryos mixture into the pipette. In a dropwise fashion, discard the lower PBS phase from the pipette, taking care not to eliminate the embryos. Deposit the embryos into a 1.5 ml tube containing a biphasic solution of 500 µl heptane (upper phase) and 500 µl methanol (lower phase).

10. Shake tubes vigorously by hand for 30-45 sec to crack the vitelline membranes. Once cracked, the embryos will sink into the methanol.
11. Discard the upper heptane phase and uncracked embryos at the heptane/methanol interphase, and add 500 µl of methanol. Shake for 5 sec.
12. Wash 3 times with methanol and store the embryos at -20 °C (at this point embryos can be stored for weeks/months).

3. Post-fixation Processing, Hybridization and Post-hybridization Washes

Overview: This section details the processing steps for embryo permeabilization prior to FISH, pre-hybridization, hybridization with the RNA probes and post-hybridization washes. For the initial permeabilization steps the embryos are handled as a pool in a single tube (steps 1-9 below, aiming for 10-15 µl of settled embryos/sample), but they are aliquoted into individual wells of a 96-well PCR plate before proceeding with the pre-hybridization step (step 10 below). This helps to ensure even treatment of all embryos in the initial phases of the experiment. When setting up a FISH experiment, it is important to include negative controls to determine the level of background signal in individual experiments. For this, we recommend including a 'no-probe' sample and, as noted in section 1, a sample hybridized with the 'sense' RNA probe, which will typically give a signal comparable to the 'no-probe' condition.

Materials:

- Methanol
- Phosphate buffered saline with 0.1% Tween-20 (PBT)
- Proteinase-K 1 mg/ml stock solution (Sigma Aldrich, Oakville, ON, Canada. Catalog No. P2308)
- Glycine (20 mg/ml stock solution)
- Hybridization solution: 50% Formamide, 5X SSC, 0.1% Tween-20, 100 g/ml of sheared salmon sperm DNA, 100 µg/ml Heparin.
- 0.2 ml halfskirted 96-well PCR plates, Abgene, Rochester, NY, USA Cat. No 0900)

Equipment

- Hybridization oven, digital heating block or water bath adjustable to 56 °C, 80 °C or 100 °C, or a PCR machine.
- Multichannel pipettors (recommended to simplify washing steps)
- Nutating mixer

1. Rinse the embryos in methanol, then in a 1:1 mixture of methanol:PBT, and then twice in PBT.
2. Fix the embryos for 20 min in 1 ml of PBT containing 3.7% formaldehyde (freshly prepared as described in step 11) with constant rocking on a nutator.
3. Wash embryos for 3 times for 2 min in PBT while rocking.
4. Prepare a solution of 3 µg/ml of proteinase K (PK) diluted in PBT and add 500 µl of solution to samples. Incubate samples for 10 min at room temperature without shaking. Mix embryos every 2 min by jetting with a pipette or by gently inverting the tubes.
5. Transfer embryos samples on ice for 1 hr.
6. Move samples to room temperature and remove PK solution. Wash 2 times for 2 min with 1 ml of PBT + 2 mg/ml glycine.
7. Fix samples 20 min in 1 ml of PBT + 3.7% formaldehyde with rocking. Rinse embryos 5 times in PBT.
8. Rinse embryos with 1 ml of a 1:1 mixture of PBT and Hyb solution. Replace with 0.5-1 ml of 100% Hyb solution (at this step the embryos can be stored for several days/weeks at -20 °C).
9. Aliquot embryos into individual wells of a 96-well plate (aim for ~10-15 µl of settled embryos/well, take care to use wide aperture tips to avoid damaging the embryos).
10. Boil 100 µl/sample of Hyb solution for 5 min and then cool on ice for 5 min. This solution is used for sample pre-hybridization (Pre-Hyb).
11. Add 100 µl/sample of Pre-Hyb solution and incubate for at least 2 hr at 56 °C.
12. For each sample, dilute ~100 ng of probe in 100 µl of Hyb solution. Heat at 80 °C for 3 min, then cool on ice for 5 min (heated probes can be kept on ice for several hours).
13. Remove the Pre-Hyb solution from the embryos and replace with the RNA probe solution.
14. Hybridize at 56 °C for 12-16 hr.
15. Pre-warm the following wash solutions at 56 °C: (A) 200 µl/sample of Hyb solution; (B) 100 µl/sample of a 3:1 mix of Hyb:PBT; (C) 100 µl/sample of a 1:1 mix of Hyb:PBT; (D) 100 µl/sample of a 1:3 mix of Hyb:PBT; (E) 400 µl/sample of PBT.
16. Rinse samples with 100 µl of wash solution A, then perform the wash step with 100 µl/sample of solutions A-D at 56 °C for 15 min each. Then, wash samples 4 times for 5 min with 100 µl/sample of solution E at 56 °C.
17. Cool samples to room temperature.

4. Probe Detection

Overview: This section describes the antibodies and detection reagents used to detect and visualize the distribution of the RNA probe signal following hybridization. These steps are outlined schematically in **Figure 2**. Here we only present the standard detection reagents that we use for robust signal amplification, but there exists a variety commercially available reagents that can be used as alternatives, especially when performing double-FISH experiments to co-detect different RNAs simultaneously for protein-RNA double staining. Examples of results obtained with this protocol are displayed in the mRNA expression/localization pattern mosaic in **Figure 3**.

Materials

- HRP-conjugated mouse monoclonal anti-DIG (Jackson ImmunoResearch Laboratories Inc. Cat. No. 200-032-156)
- Biotin-conjugated mouse monoclonal anti-DIG (Jackson ImmunoResearch Laboratories Inc. Cat. No. 200-062-156)
- Streptavidin-HRP conjugate (Molecular Probes, Eugene OR, USA, Cat. No.S991)
- Cy3-tyramide conjugates (Perkin Elmer Life Sciences, Boston, MA, USA, Cat. No. SAT704A)

- DABCO mounting solution: In a 50 ml tube, dilute 1.25 grams of DABCO (1,4-diazabicyclo [2.2.2] octane; Sigma D-2522) in 15 ml of 1X PBS, then add 35 ml of glycerol and mix until fully homogeneous. By premixing with PBS, the DABCO powder is dissolved very quickly. Store mounting solution at -20 °C.
- Glass slides, coverslips

Equipment

- Nutating mixer
- Multichannel pipettor
- Fluorescence microscope

1. Prepare PBTB solution, containing PBT + 1% non-fat dry milk (usually 50-100 ml is sufficient for all the detection reagent incubation and washing steps).
2. Block samples for 10 min at room temp in 100 µl PBTB/sample while rocking on nutator.
3. Remove blocking solution from embryos and incubate samples for 1.5-2 hr at room temperature with 100 µl/sample the monoclonal biotin anti-DIG antibody solution (diluted 1/400 from stock into PBTB) with rocking.
4. Wash samples 6 times for 5 min each with 100 µl PBTB/sample with rocking.
5. Incubate 1.5 hr at room temperature with 75 ml/sample of streptavidin-HRP solution (diluted 1/100 from stock in PBTB, 10 µg/ml final concentration) with rocking.
6. Wash samples 6 times for 5 min each 100 µl PBTB/sample with rocking (for the DNA counterstaining, dilute DAPI to 1X working concentration in PBTB and use this solution in the first wash step).
7. Rinse embryos with PBT, then wash 2 times for 5 min with PBS.
8. Incubate samples for 2 hr at room temperature on the nutator with 50 µl/sample of Cy3-tyramide solution (diluted 1/50 in Tyramide amplification buffer). From this step on, make sure to keep samples in a light shielded receptacle.
9. Wash samples 6 times for 5 min each with 100 µl PBS/sample on nutator.
10. Remove PBS and add 100-125 µl/sample of mounting solution containing 70% glycerol and 2.5% (DABCO). Store samples at 4 °C. These samples can be stored for several years.
11. Using a wide-bore tip, resuspend the embryos by gently pipetting the samples and transfer 25 µl of embryos onto a glass slide, then place a coverslip over the embryos and seal onto the slide with fingernail polish.
12. Analyze embryo samples using a fluorescence microscope.

Representative Results

When performed successfully, this procedure offers a strikingly enhanced level of detail in the spatio-temporal analysis of gene expression and mRNA localization dynamics during early *Drosophila* embryogenesis. Indeed, as illustrated in **Figure 3A** for the classical pair-rule gene *runt* (*run*), one can use this protocol to observe gene expression events via the detection of nascent transcript foci in groups of expressing nuclei. In addition, as shown in the embryo mosaic in **Figure 3B**, the method enables the visualization of mRNA localization features in high-resolution.

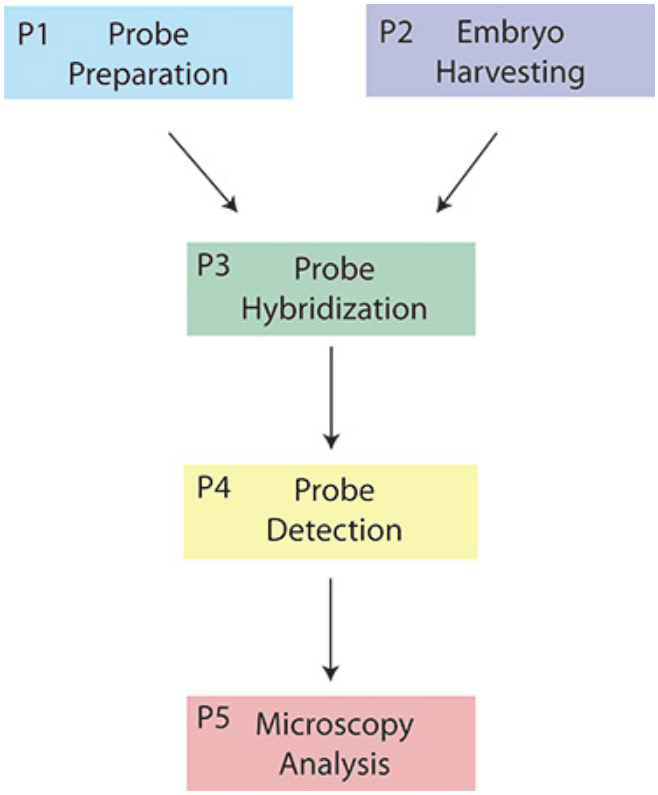

Figure 1. Outline of RNA probe preparation procedure. (A) Schematic representation of our strategy for synthesizing Dig-labelled RNA probes by *in vitro* transcription using a linear DNA template flanked with bacteriophage RNA polymerase promoter elements. (B) Picture of standard 1% agarose gel showing examples of *in vitro* transcribed RNA probes for the histone h2a, run and doc transposon RNAs. We usually perform a side-by-side electrophoresis of both the PCR template and RNA probe. The RNA typically appears as an intense discrete band with higher mobility compared to the template

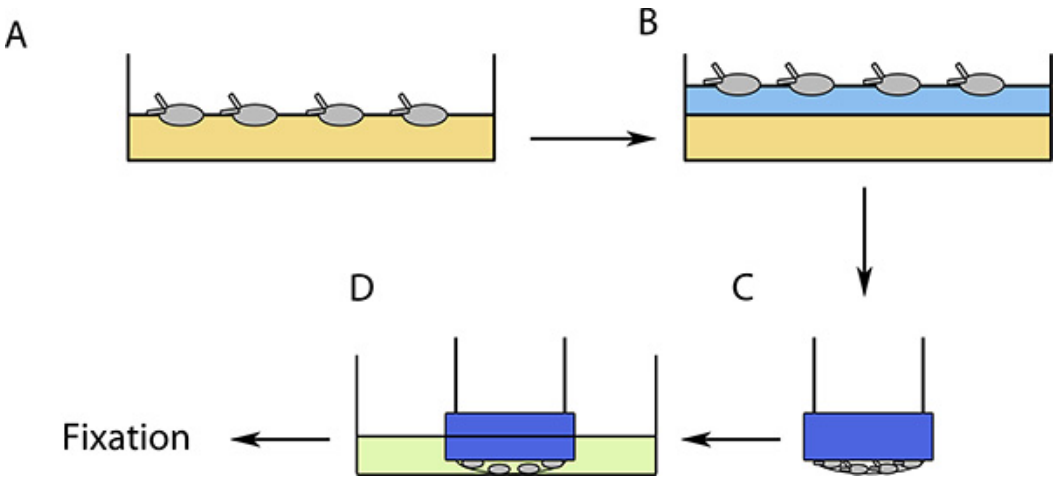

Figure 2. Schematic representation of the probe detection steps in the FISH method. Following probe hybridization to processed embryos, the Dig-labelled probes are recognized using a biotinylated α-Dig antibody, which is then complexed to HRP-conjugated streptavidin. Tyramide signal amplification (TSA) is then performed resulting in the transformation of the tyramide reagent into free radical form through the activity of HRP. Transiently reactive tyramide free radicals covalently bind to aromatic residues of nearby proteins, preventing their diffusion away from the probe location. The tyramide substrate is complexed to fluorescent dyes, which can be detected by fluorescence microscopy.

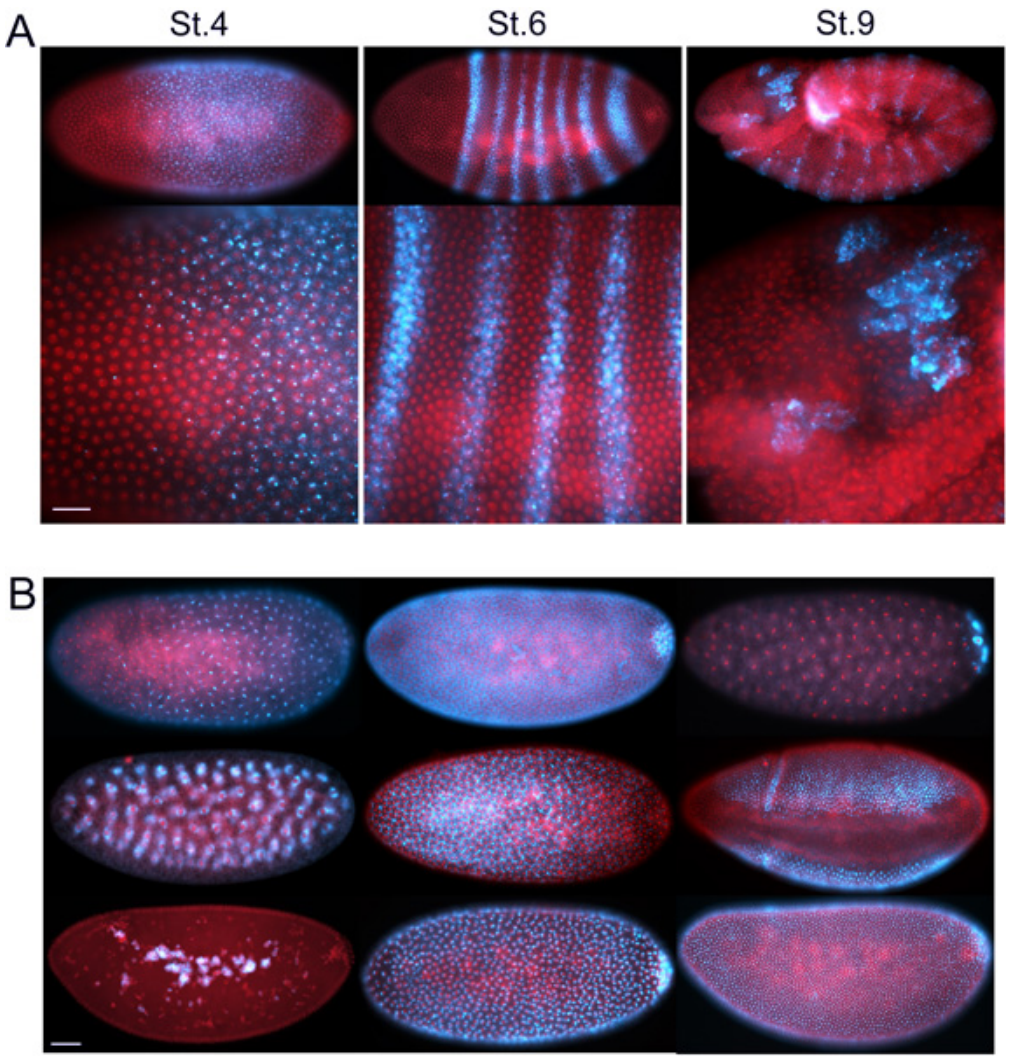

Figure 3. Examples of results obtained using this FISH procedure highlighting the diversity of mRNA expression and subcellular localization patterns in *Drosophila* embryos. (A) FISH analysis of the pair-rule gene *run* at distinct stages of embryogenesis reveals how the initial broad expression in the mid-portion of the embryo at embryonic stage 4 is refined into a segmented stripe pattern in later stages. **(B)** Mosaic depiction of FISH results showing different varieties of mRNA localization patterns in stage 4-7 embryos. FISH was performed using Dig-labeled antisense probes that were hybridized and detected by sequential incubations with the biotinylated anti-Dig antibody, streptavidin-HRP, and Cy3 tyramide. RNA = blue, DNA = red. Scale bar in (A) = 25 μM, while that in (B) = 50 μM.

Discussion

For probe synthesis steps, we typically generate run-off antisense RNA probes by *in vitro* transcription from full-length *Drosophila* cDNAs amplified from plasmids found in the *Drosophila* Gene Collection (DGC), a resource detailed at the following website: http://www.fruitfly.org/DGC/index.html [14,15]. This approach has been used extensively in large scale ISH studies aimed at mapping gene expression and mRNA localization patterns in fly embryos [9,16]. DGC plasmids can be ordered from the *Drosophila* Genomic Resource Center (https://dgrc.cgb.indiana.edu/vectors/). The flexibility in starting materials and designing custom primers with T7, SP6, or T3 overhangs allows one to easily generate probes to detect specific mRNA isoforms (*e.g.* alternatively spliced variants) or non-coding RNAs that are not represented in the standard cDNA collections. When considering designing isoform-specific probes, we have found that templates ranging from 250-1,000 bases can yield excellent FISH results.

For FISH in fly embryos, we do not fragment our probes by carbonate treatment; rather we use full-length run-off transcripts, which can range in size between 500-5,000 bases. When performing FISH on other tissues that are less permeable to large probes, fragmentation may indeed favor probe penetration. However, we prefer the option of preparing probes using smaller PCR-amplified DNA templates, or pooling multiple individually-synthesized small probes together, rather than performing chemical fragmentation of large probes which may give variable results and reduce overall probe quality.

As described in the embryo collection steps, we usually add yeast paste to the embryo collection plates. Yeast paste will greatly increase the number of eggs laid by the flies, as egg production depends on the nutritional environment[17]. In addition, *Drosophila* flies will frequently lay their eggs directly into the yeast paste. These embryos can easily be collected by dissolving the yeast paste in water using a small paint brush and passing the mixture through a collection basket. For the dechorionation step, a 3% bleach solution, prepared by diluting commercial bleach

(typically 6% concentration) 1:1 with water, is used in most protocols [13,18,19]. In our experience, we have found that incubating embryos with a 3% bleach solution for 90 sec offers efficient dechorionation. These parameters may need to be adjusted depending on the commercial bleach solution that is available, but one should take care not to overexpose the embryos to bleach since this can damage the samples. To monitor the dechorionation procedure more closely, it is possible to look at the embryos under a microscope to make sure the dechorionation reaction is complete, *i.e.* the embryos lose their dorsal appendages when they are dechorionated. Finally, to ensure proper embryo fixation, we use freshly prepared formaldehyde solutions, as old solutions tend to precipitate. For further reference, this procedure has been described in previous methods articles [13,18,19].

For steps pertaining to permeabilization of embryonic tissues with proteinase K, or detection reagents such as the antibodies and Cy3-Tyramide, we have found optimal results using the dilutions described in this protocol. However, due to variations in lab environments and reagent stocks, we recommend that each laboratory empirically test their own reagents to determine the best working concentrations for their specific needs. In addition, we have found the optimal reagent concentrations may vary depending on the tissues analyzed.

To ensure that the FISH procedure is giving consistently reproducible results, we recommend always adding several positive controls to the assay, which could include probes for transcripts with well-defined expression/localization patterns (*e.g. run*). Transcripts with striking mRNA localization patterns during early *Drosophila* embryogenesis can be found on the Fly-FISH web site (http://fly-fish.ccbr.utoronto.ca/). Conversely, to control for background signal detection, as mentioned above, one should also include 'no-probe' and/or sense RNA probe samples.

In addition to the Cy3 tyramide conjugate described in this protocol, there are a variety of other commercially available fluorochrome-conjugated tyramide reagents from Perkin Elmer Life Sciences or Molecular Probes (Invitrogen, Canada, Inc), which can provide additional flexibility for multicolor imaging experiments. While this method describes our basic procedure for single RNA FISH, variations of this method can be implemented for more complex experiments, such as double-RNA FISH or RNA-protein co-detection. For details pertaining to these procedures, we recommend following the procedures described in existing FISH methods papers [18-20].

Disclosures

No conflicts of interest declared.

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