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Optimized RNA ISH, RNA FISH and protein-RNA double labeling (IF/FISH) in *Drosophila* ovaries

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Abstract

In situ hybridization (ISH) is a powerful technique for detecting nucleic acids in cells and tissues. Here we describe three ISH procedures that are optimized for *Drosophila* ovaries: whole-mount, digoxigenin-labeled RNA ISH; RNA fluorescent ISH (FISH); and protein immunofluorescence (IF)–RNA FISH double labeling (IF/FISH). Each procedure balances conflicting requirements for permeabilization, fixation and preservation of antigenicity to detect RNA and protein expression with high resolution and sensitivity. The ISH protocol uses alkaline phosphatase–conjugated digoxigenin antibodies followed by a color reaction, whereas FISH detection involves tyramide signal amplification (TSA). To simultaneously preserve antigens for protein detection and enable RNA probe penetration for IF/FISH, we perform IF before FISH and use xylenes and detergents to permeabilize the tissue rather than proteinase K, which can damage the antigens. ISH and FISH take 3 d to perform, whereas IF/FISH takes 5 d. Probe generation takes 1 or 2 d to perform.

INTRODUCTION

Overview of ISH, FISH and IF/FISH

Since the development of ISH as a means of detecting specific RNA or DNA sequences within cytological preparations^{1,2}, empirical efforts and technological breakthroughs have facilitated adaptation of ISH to a broad range of other applications. Analysis of the spatial and temporal distribution of transcripts within tissues³, quantitative determination of gene copy number or transcript levels^{4–6} and ascertainment of the physical location of mRNAs or chromosomal segments within the nucleus⁷ all extend the power of the initial technique and make ISH a key component in the biologist's toolkit.

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS C.A.B. supervised the project. C.A.B., N.C.P., A.E.A. and S.G.Z. designed the experiments. N.C.P., A.E.A. and S.G.Z. performed the experiments. C.A.B. and A.E.A. optimized the ISH methods; N.C.P., A.E.A. and S.G.Z. optimized the FISH methods; and S.G.Z. optimized the dual IF/FISH methods. N.C.P. performed dissections for Supplementary Video 1. S.G.Z. filmed and edited Supplementary Video 1. C.A.B. and S.G.Z. wrote the paper and N.C.P. and A.E.A. commented on drafts of the manuscript.

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An important breakthrough in ISH technology occurred in 1989, when Tautz and Pfeifle³ developed a nonradioactive method for whole-mount ISH of *Drosophila* embryos. This method, which relies on digoxigenin-labeled probes and an alkaline phosphatase-based colorimetric reaction for probe detection, yields a product that is easily visualized using bright-field or differential interference contrast (DIC) microscopy. Although the protocol is highly sensitive, diffusion of the colorimetric-reaction products hampers resolution^{8–10}. Another limitation is the difficulty in resolving colocalized or overlapping expression patterns of multiple transcripts¹¹. Nevertheless, colorimetric ISH continues to be an important and widely used technique; for example, in a recent seminal paper, Yakoby *et al.*¹² used ISH to define a combinatorial code of gene expression patterns in the follicular epithelium of the *Drosophila* ovary.

Fluorescent ISH (FISH) of RNA offers several advantages over alkaline phosphatase-based methods. Conjugated fluorescent molecules do not diffuse¹⁰ and they allow the use of laser confocal microscopy, providing better resolution (e.g., subcellular localization of mRNA¹³, including intranuclear distribution of actively transcribed genes¹⁴), detection of signals in internal sections of the tissue, optical sectioning, 3D reconstruction of optical planes and simultaneous analysis of two different transcripts^{15,16}. Furthermore, TSA can markedly enhance the sensitivity compared with conventional IF and FISH methods¹⁷.

Combining protein IF with FISH allows simultaneous detection of multiple proteins and mRNAs. For each method, the investigator seeks to maximize detection sensitivity while preserving morphology. Achieving these goals depends on several factors: the copy number of the endogenous molecules, the length and GC content of the RNA probe, the structural features of the tissues and cells and the sensitivity of complexes to denaturing chemicals. Moreover, when analyzing multiple genes simultaneously, one needs to take into account the differential stability and range of detection of the various molecules of interest. Here we evaluate these issues using the *Drosophila* ovary, which has emerged as a premier model system for analyzing DNA replication; cell signaling; epithelial morphogenesis; cytoskeletal architecture; and chromosomal, RNA and protein dynamics¹⁸.

Development of the protocol and comparison with other procedures

Many investigators have optimized protocols for ISH and FISH to various tissues, including *Drosophila* embryos^{13,19}, imaginal discs^{13,20}, salivary glands¹³ and testes^{21,22}, as well as for tissues from vertebrates such as *Xenopus*^{23,24}, zebrafish²⁵, mouse²⁶ and chick²⁷. Structural features of the *Drosophila* ovary, however, render it less amenable to protocols optimized for other tissues. Tissue thickness and a surrounding muscle layer interfere with penetration of probes. These features require balancing conflicting needs during tissue fixation and permeabilization. Simultaneous detection of protein and RNA adds a third competing requirement: preservation of antigens for antibody binding. We therefore set out to develop a method that would optimize ISH, FISH and dual protein-RNA detection specifically for ovaries. The workflow diagram in Figure 1 outlines the steps in this protocol.

By using the alkaline phosphatase-based ISH technology³ as a foundation, our group and others developed protocols for visualizing transcripts in the *Drosophila* ovary^{28–31} using double-stranded DNA probes and making only modest changes to accommodate work with

dissected ovaries rather than laid eggs and embryos. The present protocol uses instead more sensitive RNA probes (reviewed in Lehmann and Tautz³²) and as a result uses higher temperatures for prehybridization, hybridization and subsequent washes—temperatures that are optimal for RNA probes³³.

Fixation and permeabilization—One of the keys to successful ISH, FISH and IF/FISH in *Drosophila* ovaries is appropriate tissue preparation. We optimized fixation and postfixation steps for *Drosophila* ovaries to preserve tissue morphology during the permeabilization steps and high-temperature washes, as shown for ISH in Figure 2. After ovary dissection, the ISH and FISH protocols include a 1-h fixation step in 4% (wt/vol) paraformaldehyde with 1% (vol/vol) DMSO, a process that allows adequate penetration of the fixative throughout the thick tissue to preserve its morphology. We then treat the tissue with increasing concentrations of ethanol; this procedure introduces a pause point, which allows the indefinite storage of samples. We have found that the morphology of samples is superior when dehydrated and rehydrated in ethanol, even when not storing for long periods²⁸. After rehydration, we include a permeabilization step using proteinase K that is more aggressive compared with that used in most ISH and FISH protocols for other *Drosophila* tissues^{13,19–21,34} and for tissues of other species^{25,26,35}. Permeabilization methods for *Drosophila* tissues vary; some alternative methods use detergents (e.g., RIPA for ovaries)³⁶ or organic solvents (e.g., xylenes and acetone for ovaries, embryos, larvae and imaginal discs)^{15,16,20} (N.H. Patel and M. Ronshaugen, personal communication). Others omit a permeabilization step altogether (e.g., for embryos, testes, Malpighian tubules or posterior midguts)^{19,22}. Most protocols for ISH and FISH in *Drosophila* ovaries use proteinase K and encompass a range of concentrations from 25 to 100 $\mu\text{g ml}^{-1}$ (refs. 29–31,37) and treatment time from a few minutes to 1 h. To aid in minimizing the effect of variation in activity of the proteinase K and to achieve more reproducible results, we use a modest concentration (50 $\mu\text{g ml}^{-1}$) for a relatively long time (1 h), followed by a 30-min postfixation step.

Dual protein-RNA labeling (IF/FISH) must preserve protein epitopes yet dissociate the tissue and reduce cellular barriers to allow entrance of the probe. We conduct the entire protein IF staining procedure before FISH, followed by a second fixation step that cross-links the antibodies to the tissue and thereby preserves these complexes during the FISH steps that follow. In contrast, traditional methods for IF/FISH perform ISH before IF staining. Other groups independently developed IF/FISH methods for *Drosophila* embryos or testes that also reverse the order of the FISH and IF and include a postfixation step before FISH^{22,38}. This reversal markedly improves detection of protein while maintaining a strong FISH signal (Fig. 3).

Our protocol uses a conventional IF staining method optimized for *Drosophila* ovaries, with an initial 20-min fixation step. Fixing for too long at this stage can interfere with access of the antibody into the tissue. For the FISH portion of the IF/FISH protocol, we leave out the proteinase K treatment, which is detrimental to protein IF and results in a weak or no signal. Instead, we include two alternative permeabilization steps based on combining methods previously described for FISH and IF/FISH in other tissues and for ISH in ovaries. We first permeabilize using xylenes and ethanol just before rehydration of the tissue (based on FISH

and IF/FISH protocols for *Drosophila* embryos, larvae and imaginal discs^{16,20}). After rehydration of the tissue, we further permeabilize using detergents (RIPA; according to an ISH protocol for *Drosophila* ovaries³⁶).

To develop the IF/FISH protocol, we tested RIPA, xylenes and acetone separately and in combination using two different probes (against germline transcripts encoded by *gurken* and follicle cell transcripts encoded by *broad*) and compared these results with proteinase K permeabilization or no permeabilization for ISH (Fig. 2) and IF/FISH (Fig. 3c–f, and data not shown). For ISH, proteinase K permeabilization resulted in a strong signal relative to background within 15 min for *gurken* (Fig. 2a) and 45 min for *broad* (Fig. 2h). Neither *gurken* nor *broad* expression was detectable at 15 min and 45 min, respectively, using any of the other permeabilization methods (Fig. 2c–g, j–n, left). After allowing the color reaction to proceed for 2 h, we could detect *gurken* expression in the oocyte with all of the treatments (Fig. 2c–g, right), surprisingly, even with no permeabilization (Fig. 2g, right). After 5.5 h, *broad* expression in follicle cells was detectable but was extremely weak and variable after permeabilization with xylenes and acetone, xylenes alone or with no permeabilization (Fig. 2l–n, right). With RIPA alone, the staining was slightly stronger (Fig. 2k, right). Of the alternative permeabilization methods, using both RIPA and xylenes resulted in the strongest and most consistent signal (Fig. 2j, right). For IF/FISH, proteinase K treatment resulted in little or no protein signal for the antibodies we tested (not shown), even with a reduced concentration of proteinase K (20 versus 50 $\mu\text{g ml}^{-1}$). When the ovaries were permeabilized with xylenes alone, the protein signal was strong and specific, but the FISH staining was variable. Sporadic egg chambers had a specific *broad* FISH signal, but most remained unstained (Fig. 3c,d). The result was similar but moderately better using RIPA alone (data not shown). Of the methods we tested, we found that the best permeabilization method for simultaneously detecting protein and RNA is a combination of permeabilization in xylenes just before rehydration of the tissue with a second permeabilization in RIPA after rehydration (Fig. 3f).

Others have developed protocols for IF/FISH in *Drosophila* ovaries and other tissues^{20,39–41}. Schotman *et al.*⁴¹ and Lerner *et al.*⁴⁰ used a detergent-based permeabilization method to detect transcripts and proteins in follicle cells, whereas Vanzo and Ephrussi⁴² modified a proteinase K–based protocol developed by Hughes and Krause⁴³ for embryos to simultaneously detect mRNA and protein in the germline. Lécuyer *et al.*³⁹ modified their method for dual RNA-protein detection in embryos for use with the fly ovary to detect *gurken* RNA and Gurken protein; they permeabilized the tissue on ice in a dilute proteinase K solution (3 $\mu\text{g ml}^{-1}$) and then used successive TSA steps to detect both the protein and RNA. By using xylenes and RIPA rather than proteinase K, and by reversing the order of the FISH and IF steps, we did not find it necessary to amplify the protein signals, but amplification could be an option for particularly weak antibody signals.

RNase inactivation—On the basis of a protocol for ISH in rat tissue sections⁴⁴, we inactivate RNases in the tissue by including washes in 0.1% (vol/vol) active diethyl pyrocarbonate (DEPC). This step increases the sensitivity of the mRNA detection for ISH, FISH and IF/FISH (for ISH, compare Fig. 2a with Fig. 2b and Fig. 2h with Fig. 2i). For IF/FISH, the signal-to-background improvement is greater if the DEPC treatment is performed

on the first day, during the protein IF staining procedure, rather than later during the FISH portion of the protocol (compare Fig. 3e with 3f). Toledano *et al.*²² do not include an active-DEPC treatment in their protocol, but they do add RNase inhibitor to antibody solutions, which can contain RNases. With the exception of the images shown in Figure 3a,b, we did not add RNase inhibitor to the antibody solutions for any of the images presented here, but we did subsequently test its effectiveness when included in addition to the active DEPC treatment. We compared *broad* FISH signals in ovaries treated with active DEPC alone with ovaries treated with RNase inhibitor and DTT during antibody incubation in addition to the active DEPC treatment. The FISH signal was slightly brighter in the egg chambers treated with both DEPC and RNase inhibitor and DTT, and thus we have added this step to our protocol.

Detection—Probes for ISH are commonly labeled with nucleotides conjugated to haptens (e.g., biotin⁴⁵ and digoxigenin³). If the probe is labeled with fluorophores, detection is direct but is limited by low sensitivity because the signal is not amplified. Alternatively, fluorescently tagged secondary antibodies recognize primary antibodies directed against the modified nucleotides, but low sensitivity is still a problem. For our ISH protocol, we use digoxigenin-labeled probes that are recognized by antibodies conjugated to alkaline phosphatase; the enzymatic reaction amplifies the signal while producing a colored product at the site of the hybrid. For FISH, TSA¹⁷ greatly enhances the sensitivity with high resolution. In this case, the digoxigenin antibody is biotinylated, and horseradish peroxidase (HRP) bound to streptavidin catalyzes the tyramide reaction. We based the TSA portion of our protocol on the Invitrogen TSA kit protocol and the work of Lécuyer *et al.*³⁹, with minor modifications. Others have found it advantageous to generate their own tyramide substrates³⁵.

Applications of the methods

The methods described here are appropriate for examining a wide range of processes, including patterning in follicle cells, polarity determination in the oocyte, transport of maternal mRNAs into early oocytes, cell-cycle changes during egg chamber maturation and mosaic analyses in germline and soma. We have used the ISH, FISH and IF/FISH protocols to investigate differential gene expression and regulatory pathways in late stages of oogenesis⁴⁶. For example, Figure 4 shows results using ISH and FISH for somatic and germline transcripts. Geisbrecht *et al.*⁴⁷ used our ISH method to evaluate signaling components in border cells, and Kucherenko *et al.*⁴⁸ adapted our FISH protocol for use in both developing and in adult brain tissue. We used the IF/FISH protocol to simultaneously detect nuclear-localized proteins (a *bunched-lacZ* reporter and a transcription factor, Bullwinkle (not shown)), membrane-localized proteins (α -Spectrin and E-Cadherin) and *broad* or *gurken* mRNA (Fig. 5). McLean and Cooley⁴⁹ have also used our dual IF/FISH protocol to analyze follicle cell clones.

Advantages and limitations

As RNA ISH is a relatively labor-intensive procedure requiring 3 to 5 d of effort, any improvement in ease, timing, sensitivity or cost will facilitate analysis. Our ISH protocol enables more rapid detection of transcripts than that achieved with previous methods,

primarily due to adding an active-DEPC treatment (Fig. 2a,b,h,i). To obtain a modest but uniform signal, the color reaction is often 5–8 times faster with our optimized protocol, depending on the probe and the expression level of the transcript. Two additional variations (FISH and IF/FISH) provide still greater sensitivity and tissue resolution or allow simultaneous analysis of proteins and RNA (Figs. 1,4 and 5). The IF/FISH protocol also offers a tremendous advantage when comparing wild-type with mutant expression patterns: with IF/FISH, one can analyze mRNA levels in mosaic tissue within a single egg chamber by using a GFP antibody to define either the wild-type or mutant cells. Thus, IF/FISH allows more accurate comparisons by circumventing problems with variability between samples. Overall, these protocols allow the analysis of a wide variety of transcripts in *Drosophila* ovarian tissue, which is often not amenable to methods that work in other tissues. Our protocol should be adaptable to other tissues and organisms as well.

One limitation of this protocol is the inability to detect expression in the oocyte in Stages 11–14 because the vitelline membrane blocks access of the RNA probes to this cell. For FISH in the oocyte at Stage 11 and later stages, see protocols developed by Dernburg^{50–52} for fixed tissues and by Gavis and colleagues^{53,54} for live imaging of single molecules.

As stochastically labeled probes can vary in their ability to detect different sequences^{55,56}, particularly short transcripts such as microRNAs, our labeling and detection approach has limitations. Kucherenko *et al.*⁴⁸ modified our FISH protocol to detect microRNAs using locked nucleic acid (LNA) probes. For investigations that require exquisite spatial or temporal resolution, or those that seek to distinguish homolog-specific expression or those that involve quantitating the amount of small RNAs or single molecules, alternative methods are available that label defined positions along the probe^{57,58}, use peptide nucleic acids that distinguish genotype^{59,60}, or that amplify the probe *in situ*^{61,62}.

Our protocol is suitable for a low-throughput format. Others have implemented systematic high-throughput ISH and FISH in *Drosophila* embryos^{19,63–66} and in other experimental systems^{25,67–69}.

Experimental design

Overview of the procedure—This protocol describes three options (ISH, FISH and IF/FISH) for whole-mount ISH as outlined in the workflow diagram (Fig. 1). Initially (Steps 1–19), the single ISH and FISH protocols are identical, involving ovary dissection, fixation in paraformaldehyde and a series of ethanol dehydration steps. After rehydration, permeabilization with proteinase K allows penetration of the RNA probe, and a postfixation step (postfix) stabilizes tissue structures after the proteinase K treatment. Incubation in active DEPC (freshly added and unautoclaved) inactivates RNases and enhances the signal relative to background. Optimized prehybridization, hybridization, posthybridization and blocking steps provide a high signal-to-background ratio in ovaries. At this point, the protocol provides two options for detecting the digoxigenin-labeled probe. The first option uses an alkaline phosphatase-conjugated digoxigenin antibody followed by a reaction using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) for colorimetric ISH (Step 20A, Figs. 2 and 4). The second option uses a TSA-based detection method for FISH (Step 20B, Fig. 4). The third method described in this protocol, double-

label protein-RNA detection (IF/FISH, Fig. 5), follows Steps 1 and 2 of the PROCEDURE and then continues in Box 1. For IF/FISH, the entire protein immunofluorescent antibody staining process, including an RNase inactivation step in active DEPC, precedes FISH. A postfixation step immediately following the antibody staining preserves the fluorescent labeling of the antigens throughout the subsequent FISH protocol (Fig. 3a,b)^{22,38}. The protocol then describes the steps for an alternative permeabilization method that is effective for simultaneous protein and RNA detection. These steps include tissue dehydration in ethanol, permeabilization using xylenes, rehydration, a second permeabilization step using detergents (RIPA) and then a second postfixation step in paraformaldehyde. After this point, the IF/FISH protocol is identical to the single FISH protocol (from Step 13 onward). Boxes 2 and 3 provide protocols for digoxigenin-labeled RNA probe generation and for performing a dot blot to estimate probe concentration and labeling efficiency.

Box 1

IF/FISH ● TIMING 5 d

Tissue preparation and primary antibody incubation ● TIMING 5 h + overnight

- 1 After dissecting ovaries (Step 2 of the PROCEDURE), remove the EBR (leave the ovaries slightly covered so they do not dry out) and fix them in 500 μ l (or enough liquid to ensure coverage and mixing) of 4% (wt/vol) paraformaldehyde in 1 $\times$ PBT with 1% (vol/vol) DMSO for 20 min on a nutator at room temperature. Freshly prepare the fixative on the day of use.

! CAUTION Paraformaldehyde depolymerizes in solution to formaldehyde, which is mutagenic and carcinogenic. Wear gloves and use a fume hood while working with it. Dispose of the excess solution and soiled gloves appropriately.
- 2 Remove fixative, and rinse the ovaries three times for 5 min each in 1 ml of PBT.
- 3 Permeabilize the samples for 1 h in 1 ml of 1% (vol/vol) Triton X-100 in PBT and break up the ovaries by very gently pipetting up and down with a large (P-1000) pipettor.
- 4 Wash the ovaries three times for 5 min each in 1 ml of PBT.
- 5 Rinse the ovaries twice for 15 min each in 1 ml of PBT with 0.1% (vol/vol) active DEPC.

! CAUTION DEPC is toxic and is hazardous when inhaled. Wear gloves and use a fume hood while working with this reagent.
- 6 Rinse the samples three times for 5 min each in 1 ml of PBT.
- 7 Block the samples for 1 h in 1 ml of PBT:WBR.
- 8 Incubate the ovaries in primary antibodies diluted in PBT:WBR overnight at 4° C on a nutator. Use at least 250 μ l in a 1.5-ml tube so that ovaries remain covered with the antibody solution. Optimal antibody dilutions depend on the

particular antibody and the type of tissue being analyzed and should therefore be determined empirically. See Table 1 for the dilutions we use for egg chambers. A solution of 10% NaN₃ can be added at a ratio of 1:500 if the primary antibody solution is to be saved and reused. RNase inhibitor (0.2 U μl^{-1}) and DTT (1 mM) may be added (optional) to the antibody solution²².

Secondary antibody incubation, postfixation and dehydration of tissue ● TIMING 5–6 h

- 9** Remove primary antibody and save if desired.
- 10** Wash the samples four times for 10 min each in 1 ml of PBT:WBR.
- 11** Remove the last wash and incubate in secondary antibodies diluted 1:500 in 1 ml of PBT:WBR at room temperature for 2–3 h on a nutator, protected from light. RNase inhibitor (0.2 U μl^{-1}) and DTT (1 mM) may be added (optional) to the antibody solution.

■ **PAUSE POINT** Alternatively, the ovaries can be incubated in secondary antibody overnight at 4 °C.

- 12** Remove the secondary antibody and wash the samples four times for 10 min each in 1 ml of PBT:WBR (protect from light).
- 13** Remove the PBT:WBR and wash the samples once for 10 min in 1 ml of PBT.
- 14** Remove the PBT. Fix the tissue in 500 μl of 4% (vol/vol) paraformaldehyde diluted in PBT (no DMSO) for 30 min at room temperature on a nutator.
- 15** Remove the fixative and rinse the samples three times for 5 min each in 1 ml of PBT.
- 16** Dehydrate the ovaries in a volume of 1 ml through a series of ethanol solutions diluted in PBT on a nutator at room temperature: 5 min in 25% (vol/vol) ethanol; 5 min in 50% ethanol; 5 min in 75% ethanol; and three washes of 5 min each in 100% ethanol.

■ **PAUSE POINT** The dehydrated ovaries can be stored at –20 °C overnight or until you are ready for the next step. Antibody stocks are stored for years at –20 °C in glycerol. The dyes are often solubilized in ethanol without adverse effects and dehydrated ovaries can be stored for years at –20 °C. Although we presume the dyes would last as long as those on comparably IF-stained tissues in glycerol, we have not tested this assumption.

Permeabilization and rehydration for FISH ● TIMING 4.5 h

- 17** Permeabilize tissue in xylenes by incubating in 1 ml of a 9:1 ratio of xylenes:ethanol for 1 h on a nutator at room temperature. Cover with foil to protect from light.

! **CAUTION** Use a fume hood when handling xylenes.

- 18** Remove the xylenes and rinse the samples by adding 1 ml of 100% ethanol, letting the tissue settle, followed by removing the ethanol, and then rinse the samples for 5 min in 1 ml of 100% ethanol.
- 19** Rehydrate the samples in a volume of 1 ml through a series of ethanol solutions diluted in PBT on a nutator at room temperature: 5 min in 75% (vol/vol) ethanol; 5 min in 50% ethanol; 5 min in 25% ethanol; and three washes of 5 min each in PBT.
- 20** Prepare RIPA by making 3 ml of RIPA buffer per tube of tissue (freshly prepare this buffer on the day of use).
- 21** Permeabilize tissue again by washing three times for 30 min each in 1 ml of RIPA buffer on a nutator at room temperature (protect from light).
- 22** Rinse the tissue twice for 5 min each in 1 ml of PBT. Fix in 500 μ l of 4% (wt/vol) paraformaldehyde in PBT for 30 min on a nutator at room temperature.
- 23** Rinse the tissue three times for 5 min each in 1 ml of PBT and proceed to Step 13 of the PROCEDURE to perform the FISH protocol.

▲ CRITICAL STEP The fluorescent antibodies are sensitive to light. Protect the tissue from light in all subsequent steps.

Box 2

Generation of DIG-labeled RNA probes ● TIMING 2 d

Preparation of template DNA ● TIMING 4–6 h

- 1** Linearize at least 3 μ g of the plasmid DNA containing the cDNA or sequence of interest in a 30- μ l volume by digesting with an appropriate restriction enzyme. Use an enzyme that cuts 5' of the insert to produce an antisense probe or 3' of the insert to produce a sense probe (i.e., cut the plasmid on the side opposite to the polymerase promoter to be used). Incubate the samples for 2 h at 37 °C (or at the recommended temperature for the enzyme), and run cut and uncut DNA samples (1 μ l) on a gel to verify complete linearization. If linearization is incomplete, continue the digestion and reanalyze the samples by running another aliquot on a gel.

▲ CRITICAL STEP If unlinearized plasmid is present, RNA polymerase will processively synthesize the transcript, circling the plasmid, using up reagents and making RNA from plasmid sequences as well as the insert. This process will significantly decrease the yield of usable probe and could give high background in tissue.

▲ CRITICAL STEP An excess (>3 μ g) of purified plasmid DNA for each probe is recommended at the beginning of this step to allow for loss during the purification process.

- 2** Purify the linearized plasmid using the QIAquick PCR purification kit according to the manufacturer's instructions (alternatively, use phenol:chloroform extraction and ethanol precipitation). Measure the DNA concentration. The concentration should be at least 1 µg in 13 µl. From a digestion of 3 µg of plasmid DNA, purified and eluted in 30 µl, expect to obtain a concentration of ~100–150 ng µl⁻¹. If the concentration is too low, vacuum-dry the sample to concentrate it.

▲ CRITICAL STEP Use DEPC-treated water for the elution step. From this point on, prepare all solutions with DEPC-treated H₂O, or DEPC-treat the solution after preparation unless indicated otherwise.

■ PAUSE POINT Store the DNA at –20 °C until ready for probe generation.

DIG-labeled RNA probe generation ● TIMING 4.5 h

- 3** For each antisense and sense probe, choose an RNA polymerase (T7, T3 or SP6) to be used on the basis of the orientation of the cDNA and the strand you wish to transcribe. For each probe, set up the following 20-µl reaction mix on ice in a sterile, RNase-free 1.5-ml tube using reagents from the DIG RNA labeling kit. Mix gently by flicking the tube, centrifuge it briefly and incubate the reaction for 2 h at 37 °C:

Component	Volume per reaction	Final (in 20 µl)
1 µg of purified, linearized plasmid DNA	× µl	1 µg
Add DEPC-treated water, so DNA + water = 13 µl	× µl	NA
10× NTP labeling mixture (with DIG-UTPs)	2 µl	1×
10× transcription buffer	2 µl	1×
RNase inhibitor	1 µl	20 U
RNA polymerase (T3, T7 or SP6, as appropriate)	2 µl	40 U
Total volume	20 µl	

▲ CRITICAL STEP For each probe, use a polymerase that begins transcription at the end of the insert, opposite to the cut end for 'run off' transcription.

- 4** Add 2 µl of RNase-free DNase1 from the DIG RNA labeling kit to the reaction tube, and incubate the samples for 15 min at 37 °C to degrade the template DNA.
- 5** Stop the reaction by adding 2 µl of 0.2 M EDTA and mix well. Save 1 µl of this reaction to run on a formaldehyde denaturing gel (steps 7–14) to check the quality of the probe (e.g., that it is not degraded by RNases) and to test if the probe is of the expected size (Fig. 6).

- 6** Precipitate the probe by adding the following to each reaction, mixing and freezing the sample for at least 2 h or overnight at -80°C :

3 μl of 3 M sodium acetate

10 μg of carrier tRNA

90 μl of ethanol

While the probe is precipitating, run a denaturing agarose/MOPS/formaldehyde gel (below) to verify the probe quality.

■ **PAUSE POINT** Instead of precipitating for 2 h, the probe can be precipitated overnight or indefinitely at -80°C .

Check RNA probe quality on a denaturing agarose/MOPS/formaldehyde gel ●
TIMING 2 h (while probe is precipitating)

- 7** For each 25 ml of gel, heat 0.25 g of agarose in 18 ml of DEPC-treated H_2O , and then cool it to 60°C .
- 8** For each 25 ml of gel, add 2.5 ml of $10\times$ MOPS running buffer and 4.5 ml of 37% (wt/vol) formaldehyde; mix well and pour the gel.
- ! **CAUTION** Formaldehyde is toxic upon contact with skin and inhalation of vapors. Perform this step under a fume hood.
- 9** Add 2 μl of formaldehyde load dye to each 1 μl of RNA sample. Ethidium bromide can be added to the formaldehyde load dye at a final concentration of $10\text{ }\mu\text{g ml}^{-1}$.
- ! **CAUTION** Ethidium bromide is a mutagen. Wear gloves while working with this dye.
- ▲ **CRITICAL STEP** Add the same volume of load dye to each sample and to the ladder. For example, add 2 μl of load dye to a 1- μl RNA sample and add 2 μl of load dye to 4 μl of ladder.
- ▲ **CRITICAL STEP** It is important to use the same amount of ethidium bromide in all the samples (including the size marker) because ethidium bromide affects RNA migration in agarose gels.
- 10** Immediately before loading the gel, heat samples at $65\text{--}70^{\circ}\text{C}$ for 10 min (to denature the RNA probes), and then cool them on ice.
- 11** Once the gel has cooled, remove the comb, pour enough MOPS running buffer to cover the gel and load samples onto the gel. Use an appropriate RNA ladder, such as the RNA Millenium marker.
- 12** Run the gel at $5\text{--}6\text{ V cm}^{-1}$ until the bromophenol blue (the faster-migrating dye) has migrated at least 2–3 cm into the gel or as far as two-thirds the length of the gel.
- 13** If you have added ethidium bromide to the formaldehyde load dye, proceed to step 14. If you have not added ethidium bromide to the formaldehyde load

dye, poststain and destain the gel as follows: transfer the gel to a tray and rinse it twice for 10 min each in DEPC-treated H₂O on a shaker at room temperature to remove the formaldehyde, stain in 50 ml of 1× MOPS buffer combined with 5 µl of 10 mg ml⁻¹ ethidium bromide (1 µg ml⁻¹ final concentration) for 10 min on the shaker, and then destain by rinsing in DEPC-treated H₂O for 30 min on the shaker.

! CAUTION Ethidium bromide is a mutagen. Wear gloves while working with this reagent. Formaldehyde is toxic upon contact with the skin and inhalation of vapors. Dispose of the excess solution and soiled gloves appropriately.

- 14** Visualize the gel on a UV transilluminator. Verify that the probe appears as a distinct band of the correct size (see Fig. 6 for examples of successful and unsuccessful probe generation).

! CAUTION UV light can damage eyes. Make sure that UV shielding is in place. Wear gloves when handling the gel.

? TROUBLESHOOTING

Resuspension and hydrolysis of probe ● TIMING 1 h

- 15** For this step, perform either option A or option B depending on whether the probe is to be hydrolyzed to reduce the length of the probe to 200–400 bases.

(A) Resuspension of precipitated probe without hydrolysis

- i.** Remove the tube with the probe from the freezer and centrifuge it at 13,000g for 15 min at 4 °C to pellet the nucleic acids.
- ii.** Remove the supernatant, wash the pellet with 70% (vol/vol) ethanol and air-dry the pellet for ~10 min.
- iii.** Resuspend the pellet in 20 µl of HYB and estimate the concentration of probe with a dot blot (Box 3). A dot blot will also give additional information on labeling efficiency and verify the antibody and developing reagent activity.
- iv.** After estimating the probe concentration, dilute the probe with HYB to the desired probe concentration. The Invitrogen TSA kit manual (MP 20912) recommends a range of 5–50 ng µl⁻¹. We estimate our probe stocks are in a range of ~10–30 ng µl⁻¹ after diluting the stock with ~100 µl of HYB. This solution is your probe stock. When stored at –20 or –80 °C, the probe will last for years⁷¹. We have reused DNA probes several times and others have reused RNA probes at least once or twice with potential improvement of signal-to-noise ratios⁷⁸. A dilution of 1:500 or 1:1,000 of this stock probe solution is a good starting point for a working concentration for ISH, but the optimal dilution depends on the expression of the transcript and may need to be optimized further.

! CAUTION HYB contains formamide, which is hazardous if inhaled or on contact with the skin or eyes. Use gloves and a fume hood while working with this solution.

B) Resuspension and hydrolysis of the probe

- i. Remove the tube with the probe from the freezer, and centrifuge it at 13,000g for 15 min at 4 °C.
- ii. Remove the supernatant, wash the pellet with 70% (vol/vol) ethanol and air-dry the pellet for ~10 min.
- iii. Resuspend the pellet in 20 µl of hydrolysis buffer.
- iv. Hydrolyze the probe down to ~200–400 base fragments by incubating at 60 °C for *X* min according to the following equation:

$$X = (L_o - L_f) / (0.11 \times L_o \times L_f)$$

where *L_o* = the original length in kilobases and *L_f* = the desired length in kilobases.

- v. Stop hydrolysis by adding 10 µl of 1 M Tris-HCl (pH 7.5), and then estimate probe concentration by using a dot blot (Box 3). A dot blot will also give additional information on labeling efficiency and verify the antibody and developing reagent activity.
- vi. After estimating the probe concentration, dilute the probe with HYB to the desired probe concentration. The Invitrogen TSA kit manual (MP 20912) recommends a range of 5–50 ng µl⁻¹. We estimate our probe stocks are in a range of ~10–30 ng µl⁻¹ after diluting the stock with ~100 µl of HYB. This solution is your probe stock. Store the probe at –20 °C or at –80 °C (indefinitely). A dilution of 1:500 or 1:1,000 of this stock probe solution is a good starting point for a working concentration for ISH, but the optimal dilution depends on the expression of the transcript and may need to be optimized further.

! CAUTION HYB contains formamide, which is hazardous upon inhalation or contact with the skin or eyes. Use gloves and a fume hood working with this reagent.

Box 3

Dot blot to estimate RNA-probe concentration and DIG-labeling efficiency

● TIMING 4 h

1. Prepare 10 ml of sterile RNA dilution buffer by mixing RNase-free, DEPC-treated water:20× SSC:37% formaldehyde in the ratio 5:3:2 (always freshly prepare the buffer).

! CAUTION Formaldehyde is toxic through contact with skin and inhalation of vapors. Perform this step under a fume hood.

2. For each cm² of nylon membrane, prepare 0.5 ml of 1× blocking solution by diluting 10× WBR at a ratio of 1:10 with maleic acid buffer (add the WBR fresh on the day of use). That is, for a 10- × 10-cm membrane, prepare 50 ml of 1× blocking solution. Use half of this solution in step 3 to prepare the antibody solution used in step 10, and use the other half to block in step 9. Larger volumes may be appropriate depending on the size of container used to hold the membrane, as long as the membrane is completely covered by solution during all the incubation steps.
3. Prepare antibody solution by centrifuging the antibody (digoxigenin–alkaline phosphatase) in its original vial at 8,000g for 5 min at 4 °C before each use, and pipet the necessary amount carefully from the surface. Dilute the antibody in the ratio of 1:5,000 (150 mU ml⁻¹ final concentration) in blocking solution (freshly prepare on the day of use).
4. Dilute the DIG-labeled control RNA from the DIG RNA labeling kit (100 ng µl⁻¹ starting concentration) at a ratio of 1:10 in the RNA dilution buffer to a starting concentration of 10 ng µl⁻¹ (e.g., dilute 5 µl of control RNA in a total of 50 µl of RNA dilution buffer). If the concentration of your newly synthesized labeled RNA is known, dilute in RNA dilution buffer to a starting concentration of 10 ng µl⁻¹. If the concentration is unknown, assume the yield of the labeling reaction was 10 µg of labeled RNA per 1 µg of template in a volume of 20 µl (Box 2, step 14A(iii)) or 30 µl (Box 2, step 14B(v)). Thereafter, dilute your labeled probe accordingly (e.g., if the yield of the reaction is 10 µg in a 20-µl volume after precipitating and resuspending the probe (Box 2, step 14A(iii)), dilute 2.0 µl in a total volume of 100 µl of RNA dilution buffer).
5. Dilute control labeled RNA and newly synthesized probes in RNA dilution buffer as follows:

Tube number	RNA (µl)	From tube number	RNA dilution buffer (µl)	Dilution	Final concentration
1	5	Diluted probe or control RNA	20	1:5	2 ng µl ⁻¹
2	5	1	15	1:4	500 pg µl ⁻¹
3	10	2	40	1:5	100 pg µl ⁻¹
4	25	3	25	1:2	50 pg µl ⁻¹
5	25	4	25	1:2	25 pg µl ⁻¹
6	20	5	30	1:2.5	10 pg µl ⁻¹
7	25	6	25	1:2	5 pg µl ⁻¹
8	25	7	25	1:2	2.5 pg µl ⁻¹
9	20	8	30	1:2.5	1 pg µl ⁻¹
10	5	9	45	1:10	0.1 pg µl ⁻¹

Tube number	RNA (μl)	From tube number	RNA dilution buffer (μl)	Dilution	Final concentration
11	0	–	50	–	0

▲ CRITICAL STEP Diluted RNA is not stable. Perform the dot blot immediately.

6. Spot 1 μl of each dilution onto a nylon membrane.
7. Fix the RNA to the membrane by cross-linking in a UV Stratalinker for 30 s.
8. Rinse the membrane briefly (~30 s) in washing buffer.
9. Incubate the membrane for 30 min in blocking solution.
10. Incubate the membrane for 30 min in antibody solution.
11. Wash the membrane twice for 15 min each with washing buffer.
12. Equilibrate the membrane for 2–5 min in detection buffer.
13. Prepare fresh color substrate by diluting NBT/BCIP stock solution at a ratio of 1:50 in the detection buffer. Prepare an appropriate volume to cover the membrane. Incubate the membrane at room temperature in an appropriate container in the dark. Do not shake during the color development. The color precipitate starts to form within a few minutes and is complete after 16 h. Monitor the development periodically until the desired spot intensities are achieved.
14. Stop the reaction by rinsing with 50 ml of deionized water. Photograph the blot, and estimate the probe concentration by comparing it with the control RNA spots (see example in Fig. 6). The intensity of the DIG-labeled probe spots compared with the control DIG-labeled RNA spots will depend on the yield of synthesized labeled probe from the labeling reaction, which depends on the amount, size and purity of the DNA template. Under the standard conditions of the DIG RNA labeling kit, the spot intensities of the synthesized probe should be similar to the control RNA spots. Detection of the control DIG-labeled RNA will also verify that the antibody and developing agent are working.

? TROUBLESHOOTING

Preparation of *Drosophila* and dissection of ovaries—Select 1-d-old females along with some males (to ensure continued egg production), and age them in vials or bottles with wet yeast paste for 24–48 h. If female flies are selected on the day of eclosion, fatten them for at least 48 h. Otherwise, if the females are too young at the time of dissection, the ovaries will be small and contain excess fat body, and the desired stages may not exist in sufficient number. Virgin female flies and females of some mutant strains will retain their eggs; thus, aging the females for too long will result in ovaries that consist of mainly late-stage egg chambers. Avoid overcrowding the fly stocks during larval development, as overcrowding can affect the quality of the ovaries in the adults. Gently comb apart the ovarioles with

forceps to improve access of the fixative and subsequent reagents but take care that you do not damage the tissue (Supplementary Video 1). Variability in staining can occur on the basis of whether individual egg chambers are fully exposed to the antibody or probe solutions. Dissect a sufficient number of ovaries to score potentially variable staining, to account for loss during the washing steps and to ensure a sufficient number of egg chambers of the desired stages.

Tissue preparation—Optimal detection of proteins and mRNA in tissue requires balancing the competing needs for preservation of tissue morphology, antigenicity and permeability. For ISH and FISH, we use a 1-h initial fixation step, and we add DMSO to help the fixative penetrate the thick tissue. Fixing for too long can damage antigenicity of the tissue, and thus we use a shorter initial fixation (20 min) for IF/FISH, consistent with the fixation time for traditional IF alone. We include 0.1% (vol/vol) Tween 20 in the fixative and all other washing and incubation buffers throughout the protein IF staining protocol to help prevent binding of antibodies to nonspecific sites. The fixation times and the inclusion of Tween 20 or another surfactant can be tailored to the requirements of the particular antibodies and tissue under analysis. We did not find it necessary to amplify the protein signal for the antibodies we tested during the development of this protocol. For antibodies with weaker signals, however, this protocol could be modified to include successive application of TSA to amplify both protein and FISH signals (as described in Lécuyer *et al.*³⁹).

Antibodies—This protocol should be amenable to a wide range of antibodies. We tested primary antibodies against α -Spectrin (cytoplasmic side of plasma membrane), E-Cadherin (adherens junctions), Broad (nuclear), Bullwinkle (nuclear) and β -Galactosidase in egg chambers expressing a *bunched-lacZ* (nuclear) or *rhomboid-lacZ* (cytoplasmic) reporter. Table 1 lists each antibody, its source and the dilution we used. For secondary antibodies, we used AlexaFluor 488, 546, 555, 568 and 647 (Invitrogen) at a ratio of 1:500. See Peters *et al.*⁴⁶ for antibody information and results from experiments using *rhomboid-lacZ* or Broad antibody. The fluorescent signal may be weakened after being subjected to the FISH protocol, but the extent of this reduction will vary with the antigen and the choice of antibodies. When using an antibody for the first time for IF/FISH, we recommend testing a dilution series to determine the optimal antibody concentration to achieve a strong signal and minimal background.

To determine the optimal antibody dilution before proceeding with the FISH steps (after the secondary incubation and postfixation (Box 1, after Step 15)), equilibrate a small aliquot of ovaries in 80% (vol/vol) glycerol for each dilution, mount the egg chambers on a slide and examine the slides using a fluorescent microscope. For the antibodies we tested (Table 1), we were able to acquire good quality four-color images (two proteins, FISH and DAPI) simply by adjusting the parameters during laser confocal imaging (Fig. 5). A potential problem with cross-activation between channels can occur during multichannel imaging when the signal from a fluorophore in one channel (e.g., using a fluorophore that excites at 546 nm) is relatively weak and requires much higher laser transmission or laser power relative to a fluorophore with a shorter-wavelength excitation (e.g., 488 nm) and relatively

stronger signal. Because of the overlap between the two excitation spectra, a small amount of emission from the fluorophore with the shorter-wavelength excitation may be intense enough to be detectable in the portion of the longer-wavelength channel emission being acquired. A remedy for this signal overlap is to use a short-wavelength excitation for the weakest signal (e.g., use AlexaFluor 488 for a relatively weak antibody signal) and a longer wavelength for a stronger signal (e.g., use AlexaFluor 546 or 647 for a relatively strong FISH, amplified signal). Alternatively, reduce the FISH signal strength by decreasing the tyramide concentration and/or by decreasing the incubation time in the tyramide solution (Step 20B(x, xi); Fig. 3g–i).

Probe design and synthesis—One consideration in designing probes for visualizing transcripts in the fly ovary is that cells in this tissue have vastly different ploidy levels and expression programs⁷⁰. To facilitate transcript analysis in all cell types, our protocol is designed to detect transcripts over a large dynamic range of expression. For most genes, we maximize base pairing by generating probes from full-length cDNAs. To distinguish splice variants or members of gene families, however, we create unique probes from specific exons and/or UTRs.

In our protocol, we use bacteriophage RNA polymerases to synthesize RNA probes from a template that consists of a linearized plasmid^{5,44} (e.g., pOT2a, pOTB7, TOPO, pBluescript SK[−] or pFlc-1). The plasmid should have T7, T3 or SP6 promoter sequences flanking the insertion site to allow RNA polymerases to bind and initiate transcription, with different binding sites flanking the cloned cDNA so that the choice of RNA polymerase determines which strand is synthesized. Alternatively, one can generate a template by PCR of genomic DNA using primers that include appropriate bacterial promoters^{5,19,71,72}. The same plasmid or PCR product can produce both anti-sense (experimental) and sense (control) probes. As RNA probes are subject to degradation by RNases and autolysis, it is important to maintain RNase-free conditions when generating and handling RNA probes.

Whether or not to hydrolyze a probe depends on the particular tissue, the size of the full-length probe and potentially on the fixation and permeabilization conditions. Under the conditions of this protocol, we have used full-length probes up to 1.7 kb in length and found them to be comparable to hydrolyzed probes for detecting expression in ovarian follicle cells and germline cells, although hydrolyzing the probe may increase penetration and improve signal to noise for some probes. We have not tested probes >1.7-kb long.

Controls—Performing positive and negative controls will determine whether the buffers and probes were generated properly and whether the protocol and reagents are being applied correctly. For a negative control, using a sense probe produced from the same template as the antisense probe will determine the level of non-specific binding. For a positive control, use a probe for a transcript with a well-known expression pattern in the tissue of interest. For example, a *broad* probe should produce a modest signal in the columnar follicle cells during middle stages of oogenesis and a strong signal in the follicle cells of the dorsal-appendage primordia in Stage 10B egg chambers. A *gurken* probe gives a strong signal localized in the posterior of the egg chamber during early stages of oogenesis and then later localizes to the dorsal anterior of the oocyte. To control for concentration and labeling efficiency of a newly

generated probe, perform a dot blot (Fig. 6) to compare it with a similarly labeled RNA with a known concentration. Before using a probe for FISH or IF/FISH, we recommend first testing it and then troubleshooting, if necessary, using the ISH protocol, especially for genes whose expression level or pattern are unknown. Although ISH does not provide the cellular resolution of FISH, it is less laborious and less expensive, allows more flexible temporal control of the staining reaction and is more easily visualized (by simply looking in the dish using a stereoscopic microscope).

Hybridization conditions—The probe hybridization temperature is crucial for achieving appropriate stringency and preventing excessive background. Lower temperatures allow non-specific binding of the probe and produce more background. The temperatures given in this protocol and hybridization times of 12–20 h (refs. 33,73) produce a strong signal with low background for the probes we have tested. If adjustment of the hybridization conditions is warranted, consider several criteria. The upper limit for the hybridization temperature is in the mid-to-high 60s (Celsius), depending on the probe length and GC content: longer probes and higher GC content require an increase in the hybridization temperature^{32,33,73,74}. Increasing the probe concentration will decrease the lengthy hybridization time needed for longer probes⁷³.

Blocking reagent—The choice of blocking reagent is important. We have had good success with Western blocking reagent (WBR) from Roche, whereas normal goat serum can cause unacceptably high background in the FISH signal. We have not tested powdered milk or BSA as blocking reagents in this protocol.

Microscopy and image processing—We acquire fluorescent images on a Zeiss 510 scanning confocal microscope. To process the images, we use Photoshop CS (Adobe), ImageJ (available from the US National Institutes of Health (NIH)) and FIJI (ImageJ-based, NIH). For colorimetric ISH, we acquire images using DIC microscopy. Because of a limited depth of field on most microscopes, DIC works best if the tissue is as flat as possible, as flattening brings the desired features into a single optical plane. Such flattening, however, limits the number of egg chambers that can be mounted on a single slide and risks the destruction of the egg chambers during mounting. To circumvent these limitations, we use an imaging and processing method that involves mounting egg chambers under lighter pressure, acquiring DIC images of multiple, partially focused z-planes for a given egg chamber, and then processing these images into a single, focused image with the photography program Helicon Focus (Helicon Soft).

MATERIALS

REAGENTS

- Yeast (Bulkfoods.com, cat. no. 40034)
- Young adult female *Drosophila melanogaster* of desired genotype, fattened on wet yeast paste along with male flies (see <http://www.fruitfly.org> for a list of stock centers)
- NaCl (Fisher Scientific, cat. no. S271-3)

- KCl (Fisher Scientific, cat. no. P217-500)
- CaCl_2 (Sigma-Aldrich, cat. no. C5670)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Deionized water (highly purified deionized or double-distilled H_2O)
- DEPC (Sigma-Aldrich, cat. no. D5758) ! **CAUTION** DEPC is a suspected carcinogen. Use a fume hood and wear gloves while working with it.
- Paraformaldehyde, 16% (wt/vol), EM grade, methanol free, 10 × 10 ml (Ted Pella, cat. no. 18505) ! **CAUTION** Paraformaldehyde fumes are toxic. Wear gloves and use a fume hood while working with it.
- NaH_2PO_4 (Sigma-Aldrich, cat. no. S8282)
- Na_2HPO_4 (Sigma-Aldrich, cat. no. S7907)
- DMSO (Sigma-Aldrich, cat. no. D8418)
- Ethanol (Decon Labs, cat. no. 2716GEA)
- RNase Zap (Ambion, cat. no. AM9780)
- Proteinase K (Sigma-Aldrich, cat. no. P2308)
- Glycine (Sigma-Aldrich, cat. no. 50046)
- Tween 20 (Affymetrix, cat. no. T1003)
- Formamide (Sigma-Aldrich, cat. no. F9037) ! **CAUTION** Formamide is a skin, eye, nose and throat irritant and is a possible teratogen. Wear gloves and use a fume hood while working with it.
- Sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$; Fisher Scientific, cat. no. S279)
- Heparin (Sigma-Aldrich, cat. no. H3393)
- tRNA (Invitrogen, cat. no. 15401029)
- Salmon sperm DNA (Sigma-Aldrich, cat. no. D1626)
- Digoxigenin-alkaline phosphatase antibody (Roche, cat. no. 11093274910)
- NBT/BCIP stock solution (Roche, cat. no. 11681451001)
! **CAUTION** NBT/BCIP is toxic.
- MgCl_2 (Sigma-Aldrich, cat. no. M8266)
- Tris base (Avantor, cat. no. 4109)
- Glycerol (Fisher Scientific, cat. no. G33-500)
- Nail polish, fast drying (Sally Hansen)
- Biotin-conjugated digoxigenin antibody (Jackson ImmunoResearch Laboratories, cat. no. 200-062-156)

- TSA kit, with HRP-streptavidin (Invitrogen, e.g., AlexaFluor 488, cat. no. T-20932)
- DAPI (Roche, cat. no. 10236276001) ! **CAUTION** DAPI is a potential carcinogen. Wear gloves while working with it, and dispose of it properly.
- DABCO mounting medium (1,4-diazabicyclo[2.2.2]octane) (Sigma-Aldrich, cat. no. 290734; or any other appropriate mounting medium with an antifade reagent for preserving fluorescence) ! **CAUTION** It is a skin and eye irritant. It is also an inhalation irritant in the powder form.
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- Primary antibodies for protein IF (many vendors, see Table 1 for sources of antibodies used in the development of this protocol)
- Secondary antibodies for protein IF (Invitrogen, many available, e.g., AlexaFluor546 goat anti-rabbit, cat. no. A-11035)
- RNase inhibitor (Protector RNase inhibitor, Roche, cat. no. 03335399001)
- DTT (Sigma-Aldrich, cat. no. D9779)
- Sodium azide (NaN_3 ; Sigma-Aldrich, cat. no. S2002) ! **CAUTION** Sodium azide is toxic and reacts violently with some metals. Wear gloves while working with it and dispose of it appropriately.
- Xylenes (Sigma-Aldrich, cat. no. 247642) ! **CAUTION** Xylenes affect the CNS. Vapors of xylenes are flammable. Use a fume hood while working with this reagent.
- Nonidet P-40 (NP-40; Sigma-Aldrich, cat. no. 74385)
- Sodium deoxycholate (Sigma-Aldrich, cat. no. 30970)
- SDS (Sigma-Aldrich, cat. no. L3771)
- cDNAs: a large inventory of directional cDNA clones is available through the *Drosophila* Genomics Resource Center (DGRC) (<https://dgrc.cgb.indiana.edu/>). A list of labs and distributors for cDNAs is also available on the Berkeley *Drosophila* Genome Project (BDGP) website (<http://www.fruitfly.org>)
- RNA polymerases: T3 (Roche, cat. no. 11031163001), T7 (New England Biolabs, cat. no. M0251S) or SP6 (New England Biolabs, cat. no. M0207S)
- Primers for amplifying genomic DNA (many suppliers, we use Operon)
- TOPO TA dual promoter kit (Invitrogen, cat. no. K4600-01)
- QIAprep spin miniprep kit (Qiagen, cat. no. 27104)
- Restriction enzymes (New England Biolabs)
- Agarose (Invitrogen, cat. no. 16500500)
- QIAquick PCR purification kit (Qiagen, cat. no. 28104)

- DNA ladder, 1 kb plus (Invitrogen, cat. no. 10787-018)
- Gel-loading dye (e.g., Blue (6×) NEB, cat. no. B7021S)
- DIG RNA labeling kit (Roche, cat. no. 11175025910)
- EDTA (J.T. Baker, cat. no. 8993)
- NaOH (Fisher Scientific, cat. no. S318-3)
- Sodium acetate trihydrate (NaOAc·3H₂O; J.T. Baker, cat. no. 3460-01)
- Glacial acetic acid (Fisher Scientific, cat. no. A38SI-212)
- MOPS (Sigma-Aldrich, cat. no. M1254)
- Formaldehyde, 37% ACS grade, for gels (VWR MK501602)

! CAUTION Formaldehyde is toxic if swallowed, inhaled or absorbed through the skin. Wear gloves and use a fume hood while working with it.

- Formaldehyde load dye (Ambion, cat. no. AM8552) **! CAUTION** Formaldehyde load dye is toxic, as it contains both formaldehyde and formamide. Wear gloves and use a fume hood while working with this reagent.
- Ethidium bromide (Sigma-Aldrich, cat. no. E7637) **! CAUTION** Ethidium bromide is a mutagen. Wear gloves while working with it and dispose of it appropriately.
- RNA Millennium Marker (Applied Biosystems, cat. no. AM7150)
- HCl (Sigma-Aldrich, cat. no. 320331)
- NaHCO₃ (J.T. Baker, cat. no. 3506-01)
- Na₂CO₃ (Sigma-Aldrich, cat. no. S7795)
- Nylon membrane, positively charged, 10 × 15 cm (Roche, cat. no. 11209299001)
- Western blocking reagent (WBR) (Roche, cat. no. 11921673001)
- Maleic acid (Sigma-Aldrich, cat. no. M0375)

EQUIPMENT

- CO₂ pad (FlyStuff.com, cat. no. 59-119)
- Sharpening stone (Fine Science Tools, cat. no. 29008-22)
- Black Plexiglas plate no. 2025, 1/4 inch × 6 inches × 12 inches (eStreetPlastics, cat. no. B012500612)
- Dissecting microscope
- Dissecting dishes
- Fine forceps for dissecting (Dumont no. 5ST Inox, Fisher Scientific, cat. no. NC9473431)
- RNase-free microcentrifuge tubes, 1.5 ml (Genesee, cat. no. 22-281)

- Nutator or rocker
- Heat block set at 68–70 °C
- Water baths set at 60 °C and 65 °C
- Tabletop centrifuge
- Costar 24-well plates (Corning, cat. no. 3526)
- Microscope slides (Thermo Scientific, cat. no. 12-518-101)
- Coverslips, no. 1.5, 22 × 22 mm (Fisher Scientific, cat. no. 12-553-454) or 22 × 50 mm (Fisher Scientific, cat. no. 12-553-471)
- Microscope equipped with DIC optics and camera (for DIC images, we use a Nikon Microphot FXA equipped with a Nikon Coolpix 995 digital camera)
- Laser confocal microscope (we acquire the images for fluorescently labeled samples on a Zeiss 510 scanning confocal microscope)
- Gel imaging equipment (we use an Alpha Innotech Alphaimager HP system with Multi-image II)
- UV Stratalinker
- P-2, P-20, P-200, and P-1000 pipettors

REAGENT SETUP

Wet yeast paste—In a 400- or 600-ml plastic beaker, weigh out 50 g of baker's yeast. Add 50 ml of deionized H₂O and stir slowly to make a thick paste. Add another 50 ml of H₂O, and do not stir. Microwave for 20 s at 50% power, stir and then microwave again for 20 s at 50% power. Do not overheat the mixture or it will kill the yeast. Stir thoroughly until a smooth and creamy, butter frosting-like texture appears. Yeast paste can be used immediately or can be stored covered with Parafilm or aluminum foil at 4 °C for 1–2 weeks.

HEPES, 1 M—Dissolve 23.83 g of HEPES in 80 ml of deionized H₂O. Adjust the pH of the solution to 6.9 using NaOH pellets. Make up the total volume to 100 ml using deionized H₂O. Filter-sterilize the solution (do not autoclave HEPES). This solution can be stored at 4 °C for up to 1 year.

EBR, 10× (modified Ephrussi-Beadle Ringer's solution)—EBR (10×) is 1.3 M NaCl, 47 mM KCl, 19 mM CaCl₂ and 100 mM HEPES (pH 6.9). To prepare 200 ml of solution, dissolve 15 g of NaCl, 0.7 g of KCl, 0.42 g of CaCl₂ (or 1.16 g of CaCl₂·2H₂O) in 160 ml of deionized H₂O. Add 20 ml of 1 M HEPES (pH 6.9). Bring the total volume to 200 ml with deionized H₂O. Filter-sterilize the solution (do not autoclave HEPES). This solution can be stored at 4 °C or frozen at –20 °C.

DEPC-treated H₂O—Incubate 0.1% (vol/vol) active DEPC in deionized H₂O overnight at room temperature (21–23 °C). Autoclave the water for 20 min to inactivate DEPC. The

DEPC-treated water can be stored at room temperature indefinitely. ▲ **CRITICAL** Maintain RNase-free conditions when preparing all solutions.

PBS, 10×—PBS (10×) is 1.5 M NaCl and 100 mM NaPO₄ (pH 6.8). For each liter of 10× PBS, mix 300 ml of 5 M NaCl, 46.3 ml of 1 M Na₂HPO₄ and 53.7 ml of 1 M NaH₂PO₄. Make up the total volume to 1 liter with deionized H₂O. Treat the solution with 1 ml of active DEPC overnight at room temperature, and then autoclave the solution to deactivate DEPC. When diluted to 1×, the pH of the solution will be 7.4.

PBS, 1×—Dilute 10× PBS into DEPC-treated, sterile water. This solution can be stored indefinitely at room temperature.

Paraformaldehyde, 4% (wt/vol), in 1× PBS—Dilute 16% (wt/vol) paraformaldehyde in 1× PBS at a ratio of 1:4. Freshly prepare this solution on the day of use.

Tris-HCl buffer, 1 M (pH 7.5, 8.0 or 9.5)—Dissolve 121.1 g of Tris base in 800 ml of DEPC-treated H₂O. Adjust the pH of the solution to the desired value using HCl. Bring the volume to 1 liter. Sterilize the solution by autoclaving. This solution can be stored at room temperature indefinitely. (Note: Tris solutions cannot be treated with DEPC but can be made with DEPC-treated H₂O.)

EDTA, 0.5 M (pH 8.0)—Dissolve 93.5 g of EDTA in 400 ml of water. Adjust the pH to 8.0 using NaOH pellets, and then add deionized H₂O to a volume of 500 ml. Add 0.5 ml of DEPC, incubate the mixture overnight and then autoclave the solution and store it at room temperature indefinitely.

Proteinase K solution—Proteinase K solution is 50 µg ml⁻¹ proteinase K, 50 mM Tris-HCl (pH 7.5) and 50 mM EDTA. Prepare a stock solution of 20 mg ml⁻¹ proteinase K in DEPC-treated H₂O, divide the stock solution into aliquots and store the solution at -20 °C. To make 5 ml of working solution, add 12.5 µl of proteinase K stock solution, 250 µl of 1 M Tris-HCl (pH 7.5) and 500 µl of 0.5 M EDTA to 4.24 ml of DEPC-treated water. Freshly prepare the solution on the day of use. ▲ **CRITICAL** The activity of proteinase K can vary from lot to lot, so test the activity with each new lot and adjust the working concentration if necessary.

PBT, 1×—Prepare 1× PBT by diluting 0.1% (vol/vol) Tween 20 in 1× PBS.

Active DEPC solution—Add DEPC to PBT to obtain a concentration of 0.1% (vol/vol). ▲ **CRITICAL** DEPC is unstable once it is in aqueous solution. Always prepare it fresh immediately before using it. DEPC hydrolyzes when it is exposed to moisture, so observe the correct storage conditions and expiration date. Improperly handled or expired DEPC may no longer be effective.

Sheared salmon sperm DNA stock—To make a 10 mg ml⁻¹ stock solution, dissolve 1 g of salmon sperm DNA in 100 ml of DEPC-treated water, autoclave the solution for 20 min

to shear the DNA and divide the solution into aliquots of 5 ml per tube. This stock solution can be stored indefinitely at -20°C .

tRNA stock—For each bottle of 50 mg of lyophilized tRNA, add 500 μl of DEPC-treated water to dissolve the tRNA and make a 100 mg ml^{-1} stock. Divide the solution into aliquots of 100 μl per tube. This stock solution can be stored indefinitely at -20°C .

Saline-sodium citrate (SSC), 20 $\times$ —SSC (20 $\times$) is 3 M NaCl and 300 mM sodium citrate. To prepare 1 liter of 20 $\times$ SSC, dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of DEPC-treated water. Adjust the pH of the solution to 7.2 using HCl. Bring the total volume to 1 liter. (As an alternative to using DEPC-treated water, DEPC-treat the solution by adding 1 ml of active DEPC and incubating it overnight at room temperature). Autoclave the solution and store it at room temperature indefinitely.

Hybridization (HYB) solution—HYB solution is 50% (vol/vol) formamide (molecular biology grade or deionized), 5 $\times$ SSC, 50 $\mu\text{g ml}^{-1}$ heparin, 0.1% (vol/vol) Tween 20, 100 $\mu\text{g ml}^{-1}$ tRNA and 100 $\mu\text{g ml}^{-1}$ sheared, boiled salmon sperm DNA (denatured just before adding). For each 50 ml of HYB, combine 25 ml of formamide, 12.5 ml of 20 $\times$ SSC, 50 μl of heparin (50 mg ml^{-1} stock), 50 μl of Tween 20, 50 μl of tRNA (100 mg ml^{-1} stock) and 500 μl of sheared, salmon sperm DNA (10 mg ml^{-1} stock, boiled for 5 min just before adding). Add 11.85 ml of DEPC-treated water to a total volume of 50 ml. This solution can be stored at 4°C for at least 1 year.

PBT:WBR—Prepare 1 $\times$ PBT:WBR by diluting 10 $\times$ WBR in PBT. Freshly prepare this reagent on the day of use. To facilitate handling of WBR, divide the solution into aliquots and freeze 10 $\times$ WBR at -20°C .

NaCl, 1 M—Dissolve 29.22 g of NaCl in 350 ml of deionized H_2O . Bring the total volume to 500 ml with deionized H_2O . DEPC-treat the solution by adding 500 μl of active DEPC and by incubating it overnight at room temperature. Autoclave the solution. This solution can be stored indefinitely at room temperature.

Buffer 3—To make 20 ml of buffer 3, combine 2 ml of 1 M NaCl, 2 ml of 0.5 M MgCl_2 , 2 ml of 1 M Tris-HCl (pH 9.5) and 14 ml of DEPC-treated H_2O . Freshly prepare the buffer on the day of use.

Glycerol, 50% (vol/vol)—Combine 25 ml of glycerol (31.5 g) with 25 ml of 1 $\times$ PBS in a 50-ml tube. Glycerol is viscous; it is easier to weigh out the correct volume and add it to the PBS than to pipette the correct volume. This solution can be stored at room temperature indefinitely.

Glycerol, 80% (vol/vol)—Combine 40 ml of glycerol (50.4 g) with 10 ml of 1 $\times$ PBS in a 50-ml tube. This solution can be stored at room temperature indefinitely.

Sodium deoxycholate, 5% (wt/vol)—To prepare 50 ml of 5% (wt/vol) sodium deoxycholate, dissolve 2.5 g of sodium deoxycholate in 40 ml of DEPC-treated water. Make

up the total volume to 50 ml with DEPC-treated water. Filter-sterilize the solution. This reagent can be stored at room temperature for 5 years, protected from light.

Paraformaldehyde, 4% (wt/vol), in PBT—Dilute 16% paraformaldehyde in 1× PBT at a ratio of 1:4. Freshly prepare the solution on the day of use.

NaN₃, 10% (wt/vol)—Dissolve 0.1 g of NaN₃ per ml of DEPC-treated H₂O. Filter-sterilize the solution (do not autoclave). Divide the solution into aliquots and store it at 4 °C for several months. **! CAUTION** This reagent is highly toxic. Wear gloves while working with it and dispose of it appropriately. Do not heat this reagent.

SDS, 20% (wt/vol)—To prepare 50 ml of 20% (wt/vol) SDS, weigh 10 g of SDS and add it to 40 ml of DEPC-treated H₂O. Mix and heat it to 68 °C to dissolve. Make up the total volume to 50 ml with DEPC-treated H₂O. Filter-sterilize the solution (do not autoclave). This reagent can be stored at room temperature for several months. **! CAUTION** SDS is a skin and eye irritant. Wear a mask or prepare SDS in a fume hood to avoid inhalation of the SDS dust.

RIPA buffer—RIPA buffer is 150 mM NaCl, 1% (vol/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 1 mM EDTA and 50 mM Tris-HCl (pH 8.0). For every ml of RIPA buffer, combine the following (make 3 ml per sample and freshly prepare the buffer on the day of use):

Component	Volume (ml) to add per ml	Final molarity or %
NaCl, 1 M	150	150 mM
NP-40, 100%	10	1% (vol/vol)
Sodium deoxycholate, 5% (wt/vol)	100	0.5% (wt/vol)
SDS, 20% (wt/vol)	5	0.1% (wt/vol)
EDTA, 0.5 M	2	1 mM
Tris-HCl, 1 M, pH 8.0	50	50 mM
DEPC-treated water	683	Not applicable

DAPI stock solution—This solution is 100 µg ml⁻¹ (100×) DAPI in 1× PBS. This solution can be stored indefinitely in aliquots at -20 °C.

DABCO mounting medium—Dissolve DABCO to a final concentration of 2.5% (wt/vol) in 80% (vol/vol) glycerol and 20% (vol/vol) 1× PBS. Heat the medium to 70 °C to dissolve. Divide the medium into aliquots. This solution can be stored at -20 °C indefinitely.

Sodium acetate, 3 M (pH 5.2)—Dissolve 40.8 g of sodium acetate trihydrate in 70 ml of water. Adjust the pH of the solution to 5.2 with glacial acetic acid. Bring the total volume to 100 ml. Add 100 µl of DEPC, incubate the solution overnight and then autoclave it. This solution can be stored at room temperature indefinitely.

MOPS buffer, 10× (for running formaldehyde gels)—Prepare 10× MOPS buffer consists of 0.4 M MOPS (pH 7.0), 0.1 M sodium acetate and 0.01 M EDTA. To prepare 500 ml of 10× MOPS, dissolve 41.9 g of MOPS in 16.67 ml of 3 M sodium acetate and 350 ml of DEPC-treated water, adjust the pH to 7.0 using NaOH, add 10 ml of 0.5 M EDTA and then bring the volume to 500 ml with DEPC-treated water. Filter-sterilize the solution. This solution can be stored in the dark at room temperature and used until the solution becomes pale yellow (several months). Do not use the solution if it turns dark yellow.

Hydrolysis buffer—Hydrolysis buffer is 40 mM NaHCO₃ and 60 mM Na₂CO₃. To prepare 100 ml of hydrolysis buffer, dissolve 336 mg of NaHCO₃ and 636 mg of Na₂CO₃ in 80 ml of deionized H₂O. Bring the total volume to 100 ml with deionized H₂O. Adjust the pH of the solution to 10.0. DEPC-treat the solution or use DEPC-treated water and RNase-free components. This solution can be stored at room temperature indefinitely.

RNA dilution buffer (for dot blots)—Use a 5:3:2 ratio of DEPC-treated water: 20× SSC:37% (wt/vol) formaldehyde. To prepare 10 ml of the buffer, combine 5 ml of DEPC-treated water, 3 ml of 20× SSC and 2 ml of 37% (wt/vol) formaldehyde. Freshly prepare the buffer on the day of use.

Maleic acid buffer (for dot blots)—Maleic acid buffer is 0.1 M maleic acid and 0.15 M NaCl. To prepare 1 liter of the buffer, dissolve 11.6 g of maleic acid and 8.77 g of NaCl in 800 ml of deionized water. Adjust the pH to 7.5 using NaOH pellets. Bring the total volume to 1 liter with deionized H₂O. Autoclave the buffer and store it at 15–25 °C indefinitely.

Washing buffer (for dot blots)—Washing buffer is maleic acid buffer and 0.3% (vol/vol) Tween 20. To prepare 500 ml of the buffer, add 1.5 ml of Tween 20 to 500 ml of maleic acid buffer. This buffer can be stored at 15–25 °C indefinitely.

Detection buffer (for dot blots)—Detection buffer is 0.1 M Tris-HCl and 0.1 M NaCl. To prepare 250 ml of the detection buffer, add 25 ml of 1 M Tris-HCl buffer (pH 9.5) and 25 ml of 1 M NaCl to 175 ml of deionized H₂O. Adjust the pH of the buffer to 9.5. Bring the total volume to 250 ml with deionized H₂O. This buffer can be stored at 15–25 °C indefinitely.

PROCEDURE

Setup of fly cultures for ovary dissection ● TIMING 1–2 d

▲ **CRITICAL** For IF/FISH, perform Steps 1 and 2 followed by Box 1; then continue with the single FISH protocol from Step 13 onward (Fig. 1).

▲ **CRITICAL** Maintain RNase-free conditions when preparing solutions and performing this protocol.

- 1| Fatten 1- to 2-d-old adult female flies by placing the desired number of female flies, along with some males, in vials or bottles with a generous dab of wet yeast paste for 24–48 h at 25 °C. If the female flies are less than 1-d old, fatten them for at least 48 h.

▲ CRITICAL STEP The time needed to fatten the flies on wet yeast paste should be optimized to account for different temperatures, to account for different genetic backgrounds and to maximize the number of egg chambers at the desired stage. Start with stocks of the correct genotype that have been expanded to ensure sufficient tissue for each sample. Keep in mind that mutants often have smaller ovaries compared with wild type, and thus more ovary pairs may be needed.

Dissection of ovaries for ISH, FISH and IF/FISH (day 1) ● TIMING 1 h per sample

▲ CRITICAL For IF/FISH, perform Step 2 followed by the procedure described in Box 1, and then continue from Step 13 of the PROCEDURE.

- 2| Dissect 25–50 ovary pairs per sample in cold 1× EBR. Anesthetize the flies on a CO₂ pad. Use forceps to select a fly and submerge it in the EBR. Gently grasp the lower part of the thorax and upper abdomen with one pair of forceps. With the other pair of forceps, tear off the posterior tip of the abdomen and pull out the ovaries, separating them from the intestine and Malpighian tubules. Sometimes it helps to squeeze gently on the abdomen with the forceps to help push out the ovaries. To avoid interaction with spilled gut material, transfer the ovary pair to a dish with fresh EBR. Comb the forceps gently through the germarium end of the ovaries to break up the ovarioles. This action improves access of the fixative, permeabilization solutions, antibodies and DIG-labeled probe at later steps and avoids a gradient of staining. See Supplementary Video 1 for an example of the ovary dissection procedure. Place the dissected ovaries in EBR in a 1.5-ml tube, and store the tube on ice until you are ready for the fixation step (ideally, no longer than 1 h).

▲ CRITICAL STEP Dissect a sufficient number of ovaries to account for loss during the washing steps, to account for variability in staining and to ensure a sufficient number of egg chambers at the desired stages. We recommend 25–50 ovary pairs, but fewer (on the order of 10) ovary pairs could suffice if one is analyzing abundant stages and care is taken to avoid loss in subsequent washing steps. Never allow the ovaries to become dry.

? TROUBLESHOOTING

Preparation of tissue for ISH and FISH ● TIMING 3 h

- 3| Remove most of the EBR (keep the ovaries covered so they do not dry out), and fix the ovaries in 500 µl of 4% (wt/vol) paraformaldehyde in 1× PBS with 1% (vol/vol) DMSO for 1 h on a nutator at room temperature.

! CAUTION Paraformaldehyde depolymerizes in solution to formaldehyde, which is mutagenic and carcinogenic. Wear gloves and use a fume hood while working with this reagent. Dispose of the excess solution and soiled gloves appropriately.

▲ CRITICAL STEP Prepare the solution fresh on the day of dissection.

- 4| Rinse the sample three times for 5 min each on a nutator at room temperature in 1× PBS.
▲ CRITICAL STEP To avoid the loss of sample at this and all subsequent steps, allow the tissue to settle to the bottom of the tube after shaking and before removing solutions.
- 5| Dehydrate the ovaries in a volume of 1 ml through a series of ethanol solutions diluted in 1× PBS on a nutator at room temperature: 5 min in 25% (vol/vol) ethanol followed by 5 min in 50% ethanol, 5 min in 75% ethanol and three times for 5 min each in 100% ethanol.
■ PAUSE POINT The dehydrated ovaries can be stored at −20 °C indefinitely.

Rehydration, permeabilization, RNase inactivation and probe hybridization (day 2) ●

TIMING 5–7 h + overnight

- 6| Rehydrate the ovaries in a volume of 1 ml through a series of ethanol solutions diluted in 1× PBS on a nutator at room temperature: 5 min in 75% (vol/vol) ethanol followed by 5 min in 50% ethanol, 5 min in 25% ethanol and three times for 5 min each in 1× PBS.
- 7| Permeabilize the rehydrated egg chambers from Step 6 in 1 ml of proteinase K for 1 h on a nutator at room temperature.
▲ CRITICAL STEP Proteinase K treatment facilitates probe penetration but can damage the tissue if the activity of the proteinase K is too high or the tissue is left in this solution too long.
- 8| Inactivate proteinase K by rinsing twice for 5 min in 1 ml of 1× PBS + 0.2% (wt/vol) glycine on a nutator at room temperature.
- 9| Rinse the ovaries on a nutator at room temperature for 5 min in 1 ml of 1× PBS and then twice for 5 min each in 1 ml of 1× PBT (note transition to using PBT).
- 10| Postfix the samples in 500 µl of 4% (wt/vol) paraformaldehyde in 1× PBT (prepared on the day it is used) for 30 min on a nutator at room temperature.
! CAUTION Paraformaldehyde depolymerizes in solution to formaldehyde, which is mutagenic and carcinogenic. Wear gloves and use a fume hood while working with this reagent. Dispose of the used solution and soiled gloves appropriately.
- 11| Remove the fixative. Rinse the ovaries twice for 5 min each in 1 ml of PBT, and then incubate them twice for 15 min on a nutator at room temperature in 1 ml of PBT with 0.1% (vol/vol) active DEPC.
! CAUTION DEPC is toxic and is a hazard upon inhalation or contact to the skin. Wear gloves and use a fume hood while working with DEPC.
- 12| Wash the samples three times for 5 min each on a nutator at room temperature in 1 ml of 1× PBT.

- 13|** Equilibrate the samples by incubating once for 5 min in 1 ml of 50% PBT/50% HYB (vol/vol) and then once for 5 min in 1 ml of 100% HYB on a nutator at room temperature.
- 14 P** e-hybridize the samples for 1 h at 60 °C in 1 ml of HYB solution.
- **PAUSE POINT** Prehybridization can be extended to overnight (optional). On the basis of our experience, overnight prehybridization does not affect the success of subsequent steps.
- 15|** Dilute the DIG-labeled RNA probe (for instructions on how to prepare RNA probes, see Box 2) by diluting the RNA probe's stock solution at a ratio of 1:500 or 1:1,000 (assuming the concentration of the stock solution is 10–30 ng μl^{-1} , based on the dot blot estimate, see Box 3) into 0.5 ml (or enough volume to cover the tissue) of HYB solution in a 1.5-ml tube. These dilutions should be a good starting point. The optimal dilution for a particular probe should be determined empirically.
- 16|** Denature the diluted RNA probe for 10 min at 68 °C. Chill the probe on ice for 5 min to prevent formation of secondary structures, and briefly spin down the condensate to collect the entire probe.
- 17|** Remove as much HYB solution from the ovaries (Step 14) as possible, add the probe and incubate the samples overnight at 60 °C.
- ▲ **CRITICAL STEP** The temperature is crucial for achieving appropriate stringency and preventing excessive background. Lower temperatures allow nonspecific binding of probe and produce more background.

Posthybridization washes and blocking (day 3) ● TIMING 2 h

- 18|** Preheat the wash solutions detailed below to 65 °C. Remove the probe from the samples and, if desired, store the solution at –20 °C for reuse. Wash the samples at 65 °C in the prewarmed solutions as follows: 20 min in 1 ml of 100% HYB followed by 20 min in 50% PBT/50% HYB (vol/vol) and five times for 5 min each in 1× PBT.
- ▲ **CRITICAL STEP** It is important to add prewarmed (65 °C) solutions to each tube and then maintain this temperature in a water bath for the duration of the wash. The proper temperature is crucial for removing nonspecifically bound probe and minimizing background signal. Tip: prepare sufficient (or even excess) wash solution for all the washes 1 d in advance, and leave the solutions in a 65 °C water bath overnight to equilibrate.
- 19|** Block the samples for 1 h in 1 ml of PBT:WBR at room temperature on a nutator.

DIG antibody incubation and signal detection

- 20|** To detect mRNA transcripts, use option A for ISH or option B for FISH and IF/FISH.

(A) DIG antibody incubation and colorimetric signal detection for ISH ●**TIMING overnight + at least 2 h**

- i. Remove the blocking solution, and add a 1:2,000 dilution of the digoxigenin-alkaline phosphatase antibody (Roche) (before each use, centrifuge the antibody in its original vial at 8,000g for 5 min at 4 °C and pipette carefully from the surface) in PBT:WBR. Incubate the samples on a nutator overnight at 4 °C.
- ii. Wash the samples three times for 5 min each in 1× PBT.
- iii. Wash the samples three times for 20 min each in 1× PBT.
- iv. Transfer the samples to a 24-well plate or dissecting dishes, and wash them twice for 5 min each in buffer 3 (freshly prepared).

▲ CRITICAL STEP Ovaries and egg chambers become clumpy and sticky at this point because the washes no longer contain any detergent. To prevent samples from sticking all over the well, tilt one end of the 24-well plate on an Eppendorf rack to allow the egg chambers to settle at the near side of each well. To ensure that the egg chambers remain hydrated, rinse them gently with buffer 3 and avoid washing the egg chambers up on the sides of the wells.

- v. Prepare fresh NBT/BCIP solution by adding 20 µl of NBT/BCIP stock solution to 1 ml of buffer 3. Incubate the ovaries in 200 µl of the NBT/BCIP solution.

▲ CRITICAL STEP The duration of this color reaction will vary depending on the sample, probe sequence and probe concentration. Watch carefully for several minutes in case the color develops immediately (within minutes). Some probes give a strong signal within 15 min and others within several hours. Keep in mind that the color will appear fainter under Nomarski (DIC) optics. Let the reaction with the control (sense) probe continue for the same length of time as the reaction with the experimental (antisense) probe.

- vi. Stop the reaction by removing the NBT/BCIP solution and adding 400 µl of 50% (vol/vol) glycerol in 1× PBS. After 5 min, replace the 50% glycerol with 80% glycerol in 1× PBS, and let the ovaries equilibrate for 1 h to overnight.
- vii. Transfer a small aliquot of ovaries onto a clean coverslip (e.g., 15–20 µl on a 22- × 22-mm coverslip or 50 µl on a 22- × 50-mm coverslip). Dissect the ovaries to separate the egg chambers. Remove the Stage 14 egg chambers, which prevent flattening, and then pick up the coverslip with a clean slide. For the best images, mount fewer egg chambers to achieve a flatter preparation. Seal the edges with transparent nail polish.

? TROUBLESHOOTING

- viii. Examine the samples promptly using a DIC microscope or freeze the slides at –20 °C to limit color diffusion. The glycerol-suspended ovaries that were not mounted on slides can be stored at –20 °C indefinitely.

? TROUBLESHOOTING

(B) DIG antibody incubation and TSA for FISH and IF/FISH ● TIMING
overnight + 6 h

- i. Remove the blocking solution, and add a 1:500 dilution of biotin-conjugated digoxigenin antibody (Jackson ImmunoResearch) in PBT:WBR (or another appropriate antibody if you are performing multiple tyramide amplifications⁴³). Incubate the samples on a nutator overnight at 4 °C.
- ii. Wash the samples six times for 10 min each in 1 ml of PBT:WBR.
- iii. Incubate the samples for 1 h in a 1:100 dilution of streptavidin-HRP (from the TSA kit) in PBT:WBR at room temperature with constant mixing. Use at least 250 µl when rocking on a nutator to ensure that the tissue remains covered in the solution.
- iv. (Optional) To perform nuclear counterstaining, rinse the ovaries once with PBT:WBR and incubate them for 10 min with 1 µg ml⁻¹ (final concentration) DAPI in PBT:WBR. To save time, the DAPI may be added directly to the streptavidin-HRP solution during the last 10 min of Step 20B(iii).

! CAUTION DAPI may cause eye, skin and respiratory irritation. Wear gloves while working with this reagent.

- v. Wash the samples six times for 10 min each in 1 ml of PBT:WBR.
- vi. Wash the samples once for 5 min in 1× PBT.
- vii. Wash the samples twice for 5 min each in 1× PBS.
- viii. At the same time as performing the washes, prepare hydrogen peroxide stock solution by diluting 1 µl of hydrogen peroxide in 200 µl of amplification buffer (both provided with the TSA kit).
- ix. Dilute the stock solution at a ratio of 1:100 in amplification buffer.
- x. Add tyramide (in the TSA kit) to diluted stock solution to make a 1:50 to 1:100 dilution of tyramide. The concentration of tyramide may be adjusted to suit individual conditions. Prepare 250 µl of tyramide solution for each sample so that the tubes can be rocked on a nutator.

▲ CRITICAL STEP You must pay close attention to this step. Tyramide amplification will not work without the right concentration of hydrogen peroxide.

- xi. Remove the last PBS wash from the samples (Step 20B(vii)), add the 250 µl of tyramide solution and incubate the samples at room temperature in the dark with constant mixing for 15 min to 2 h.

▲ CRITICAL STEP Determine empirically what combination of dilution and incubation time results in the strongest signal and lowest background for a particular probe and mRNA expression level. For a weak probe or low- abundance transcript, especially for FISH without IF, start with 2 h. For a strong probe or high-abundance transcript, or for IF/FISH with the FISH signal detected at a shorter wavelength than protein signals (e.g., FISH detected at 488 nm and proteins detected at 546 and 647 nm), start with 30 min.

- xii.** Wash the samples six times for 10 min each with 1 ml of PBS, protecting them from light.
- xiii.** Resuspend the samples in 100–200 μ l of an appropriate anti-bleaching mountant (e.g., DABCO). Allow the egg chambers to equilibrate for 1–3 h or overnight at 4 °C. Protect the samples from light at all times. Samples can be stored for months in microcentrifuge tubes or covered tissue culture plates at 4 °C or for years at –20 °C in light-shielded receptacles¹³.
- xiv.** Transfer a small aliquot of ovaries onto a clean coverslip (e.g., 15–20 μ l on a 22- $\times$ 22-mm coverslip or 50–80 μ l on a 22- $\times$ 50-mm coverslip). Dissect the ovaries to separate the egg chambers of the desired stage, and then pick up the coverslip with a clean slide. Seal the edges with transparent nail polish. Slides can be stored in the dark for a few weeks at 4 °C. When stored at –20 °C, the AlexaFluor fluorescent dyes will last for years, but the DAPI may diffuse from the tissue after a few weeks on slides¹³. Therefore, a fresh aliquot of the sample should be mounted if tissue stained with DAPI is to be analyzed at a later date.

? TROUBLESHOOTING

- xv.** Analyze the ovaries by conventional fluorescence or confocal microscopy.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

● TIMING

The flowchart provided in Figure 1 outlines the steps of the protocol.

Step 1, setup of fly cultures for ovary dissection: 1–2 d

Step 2, dissection of ovaries for ISH, FISH and IF/FISH: 1 h per sample

Steps 3–5, preparation of tissue for ISH and FISH: 3 h

Steps 6–17, rehydration, permeabilization, RNase inactivation and probe hybridization: 5–7 h + overnight

Steps 18 and 19, posthybridization washes and blocking: 2 h

Step 20A, DIG antibody incubation and colorimetric signal detection for ISH: overnight + at least 2 h

Step 20B, DIG antibody incubation and TSA for FISH and IF/FISH: overnight + 6 h

Box 1, IF/FISH: 5 d

Box 2, generation of DIG-labeled RNA probes: 2 d

Box 3, dot blot to estimate RNA-probe concentration and DIG-labeling efficiency: 4 h

ANTICIPATED RESULTS

Performing ISH, FISH and IF/FISH as described in this protocol results in successful detection of mRNA or simultaneous detection of protein and mRNA in whole-mount *Drosophila* ovaries (Figs. 2–5 and Peters *et al.*⁴⁶). We have achieved high signal-to-background detection with a wide range of hydrolyzed and nonhydrolyzed probes (~50 different probes) in both somatic (Figs. 2h, 4a–i and 5a–e) and germline (Figs. 2a, 4j–l and 5f) cells. Although ploidy levels vary within egg chambers and increase during the temporal progression of oogenesis, this single protocol can detect rare, moderately abundant and abundant transcripts (Fig. 4 and ref. 46). The FISH and IF/FISH methods provide highly sensitive mRNA detection with subcellular resolution and allow laser confocal imaging and optical sectioning (Figs. 4 (arrowheads) and 5).

Potential difficulties with ISH, FISH and IF/FISH are high background and low or no detection. To avoid these problems, stringent hybridization and washing temperatures, adequate permeabilization of the tissue, maintenance of RNase-free conditions and controls are important. One of the key improvements in this protocol for achieving a strong signal and minimal background is a step to inactivate RNases in the tissue by incubating the samples in active DEPC (Figs. 2a,b,h,i and 3e,f). Another important control is to evaluate probe quality. Figure 6a,b shows examples of RNA probes run in gels that illustrate the appearance of successfully generated probes and those that had technical problems. An RNA product that is too short (Fig. 6a, arrows) result from the transcription template forming secondary structure (causing the polymerase to fall off), or due to a failure to maintain RNase-free conditions, causing probe degradation. An RNA product that is longer than expected (Fig. 6b, arrows) usually results from incomplete linearization of the template DNA. Adhering to the manufacturer's instructions for enzymatic reaction conditions, maintaining RNase-free conditions and running a gel to assess the complete linearization of the DNA template before proceeding to probe synthesis will help to avoid these problems. Finally, performing a dot blot (Box 3 and Fig. 6c) will provide information on probe concentration and labeling efficiency.

Although these protocols were optimized for *Drosophila* ovaries, key improvements such as active DEPC treatment of the tissue, optimized permeabilization methods and reversal of order for IF/FISH could improve analyses in other *Drosophila* tissues and other organisms as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

Comparison of permeabilization methods using ISH. Shown are Stage 10B egg chambers. Anterior is to the left. Dashed lines indicate dorsal midlines. (a–g) *gurken* mRNA localizes to the dorsal anterior region of the oocyte, between the nucleus and the oocyte membrane. (h–n) *broad* mRNA localizes to two patches of somatic, dorsal, follicle cells that make up the dorsal-appendage primordia. Proteinase K permeabilization and DEPC treatment (a and h) compared with no DEPC treatment (b and i). (c–g and j–n) Comparison of alternative permeabilization methods, all using DEPC treatment, as indicated for detection of *gurken* mRNA expression: 15 min (c–g, left images), 2 h (c–g, right images); and *broad* mRNA expression: 45 min (j–n, left images), and 5.5 h (j–n, right images). Xyl., xylene; RIPA, detergents; Acet., acetone; No perm., no permeabilization. Scale bars, 50 μ m.

Effect of reversing the order of FISH and IF protocol steps:

Figure 3.

Optimization of dual protein and RNA analyses. Shown are Stage 10B egg chambers.

Anterior is to the left. Dashed lines mark the dorsal midline. **(a,b)** Performing the protein IF protocol before **(a)** or after **(b)** the FISH protocol affects the ability to detect *broad* mRNA (left images), β -galactosidase protein (middle images) and E-cadherin protein (right images). Permeabilizations were performed with xylenes and RIPA. Tyramide dilution was 1:50. Z-projections were rendered using ImageJ (**a**, left and middle, 5 μ m; **a**, right, 1 μ m; **b**, 6 μ m). Identical adjustments were made in Photoshop for each pair of images being compared. **(c-i)** *broad* mRNA expression in dorsal-appendage primordia. Identical adjustments were made to all images in Photoshop. **(c-f)** Tyramide dilution was 1:50. **(c,d)** Permeabilization only

with xylenes results in variable FISH detection: absence of signal in most egg chambers (**c**) and sporadic detection in a few samples (**d**). (**e,f**) Effect of timing of active-DEPC treatment on *broad* FISH signal strength and background. (**e**) Late DEPC: active-DEPC treatment on day 3 of the IF/FISH protocol. Laser transmission was increased to 20% to improve the signal strength. (**f**) Early DEPC: DEPC treatment on day 1 of the IF/FISH protocol. Laser transmission was 10%. (**g–i**) Effect of tyramide incubation time on *broad* FISH signal. Permeabilizations were performed with xylenes and RIPA. Samples were incubated in a 1:100 dilution of tyramide for 15 min (**g**), 30 min (**h**) and 1 h (**i**). Scale bars, 50 μ m.

Figure 4.

Detection of rare, moderate and abundant transcripts with ISH and FISH. Anterior is to the left. Dashed lines indicate dorsal midlines. (a–c) Low-abundance somatic transcript (*mirror*). (d–f) Medium-abundance somatic transcript (*Paxillin*). (g–i) High-abundance somatic transcript (*Rab40*). (j–l) Germline transcript (*katanin 80*). (a,d,g,j) ISH DIC images (projections were rendered using Helicon Focus software); (b,e,h,k) FISH laser confocal images, 25-μm projections of *xy* optical planes; (c,f,i,l) FISH, orthogonal views of egg chambers in b, e, h and k with 1-μm *xy* projections and 25-μm *xz* and *yz* projections. Note higher resolution and sensitivity of FISH compared with ISH as demonstrated by subcellular localization of nascent transcripts at genomic loci in germline cells (red arrowheads in l) and somatic cells (white arrowheads in c, f and i; inset in l shows a zoomed-in view of the follicle-cell nucleus boxed in white). Probes were generated for ISH and FISH in Peters *et al.*⁴⁶. Information on tissue expression level is from modENCODE mRNA-seq data available on FlyBase. Scale bars, 50 μm.

Figure 5.

Optimized protein IF/FISH of Stage 10B egg chambers. Anterior is to the left. Dashed white lines mark the dorsal midline. **(a)** Merge of **b–d**: *broad* mRNA (green), α -Spectrin (magenta), *bunched-lacZ* reporter (blue). **(b)** *broad* mRNA expression in dorsal-appendage primordia detected by FISH. **(c)** Gradient of *bunched-lacZ* transcriptional reporter activity detected by immunofluorescent localization of β -galactosidase. **(d)** α -Spectrin immunofluorescence localization. **(e)** IF/FISH reveals distributions of *broad* mRNA (green) and E-cadherin protein (magenta). **(f)** IF/FISH detects the germline expression of *gurken* mRNA (magenta) and follicle cell expression of α -Spectrin. DAPI (blue) marks nuclei. Scale bars, 50 μ m.

Figure 6.

Probe verification and quantification. All bands are at the expected size except where indicated. (a) Denaturing formaldehyde RNA gel. Lane 1: RNA Millenium marker. Lanes 2–8: probes used for ISH in Peters *et al.*⁴⁶. Of the 20- μl transcription reactions, 1 μl is loaded in each lane. Lanes 2 and 3, *nervana 3* antisense and sense (1.7 kb); lanes 4 and 5, *shibire* antisense (~1 kb) and sense (difference in length due to position of restriction cuts); lane 6, *shibire* antisense, longer template (2.3 kb); lanes 7–8, *huckebein* antisense and sense (1.6 kb). Note the bands running faster than expected (arrows, aberrant bands; arrowheads, expected bands), possibly due to nuclease degradation of template DNA or RNA product or because of secondary structure in the template causing the polymerase to fall off prematurely. (b) Denaturing formaldehyde RNA gel. Lane 1: RNA Millenium marker. Lanes 2–3: 1 μl of 20 μl transcription reactions loaded in each lane for *tigrin* antisense and sense probes (~0.6 kb). Note the bands running slower than expected in lane 2 (arrows, aberrant bands; arrowhead, expected band), possibly due to incompletely linearized template DNA, which allows the RNA polymerase to make multiple processive cycles around the plasmid, yielding longer transcripts. (c) Dot blot. DIG-labeled antisense and sense probes against *Drosophila shark* comparing hydrolyzed and nonhydrolyzed (full-length) probes with DIG-labeled control RNA.

TABLE 1

Primary antibodies.

Antibody	Dilution	Source
α -Spectrin (mouse)	1:100	Developmental Studies Hybridoma Bank (DSHB) (3A9 [concentrate]; Dubreuil <i>et al.</i> ⁷⁵) (Fig. 5)
β -Galactosidase (rabbit)	1:5,000 for <i>bun-lacZ</i> (pre-adsorbed to fixed, DEPC-treated ovaries)	MP Biomedicals ⁴⁶ Cappel (Figs. 3 and 5)
Bullwinkle (rabbit)	1:2,000	Berg Laboratory (RCB1.4 II; Kim <i>et al.</i> ⁷⁶) (not shown)
E-Cadherin (rat)	1:50	DSHB (DCAD2-c [concentrate]; Oda <i>et al.</i> ⁷⁷) (Figs. 3 and 5)

TABLE 2

Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Few egg chambers of correct stage	Females are too young, too old, or are unfed or unmated	Select day-old females and age them on wet yeast with males for 1–2 d
20A(vii), 20B(xiv)	Too few egg chambers to mount	Insufficient number of ovaries dissected	Dissect 25–50 ovary pairs per sample
		Failure to allow tissue to settle completely before removing solutions	Allow tissue to settle completely before removing solutions
		Exuberant washing splashed samples out of tubes or pipetting caused loss	Use caution when adding solutions to tubes and look directly at tube contents when removing solutions
20A(viii), 20B(xv)	High background	Low hybridization temperature and/or low washing temperature	Verify and maintain correct hybridization (60 °C) and washing temperatures. For long probes or those with a high GC content, increase the temperature of the hybridization and wash steps to 68 °C
		Probe template DNA was not completely linearized	Digest the template longer and run it on a gel to verify linearization. Completely linearized templates will appear as a single band of the expected size. Incompletely linearized templates will appear as multiple bands, some of which are the wrong size
		DEPC is degraded	Use proper storage conditions and observe expiration dates
		Inadequate blocking	Use PBT:WBR and treat tissue for 1 h or more
	Low or no ISH or FISH signal	Hydrogen peroxide was not added to TSA reaction	Pay close attention to dilution and addition of reagents in TSA protocol
		Poor probe quality or labeling efficiency	Perform quality-control verifications during probe generation, as described in Boxes 2 and 3. Test probes by ISH first. Run hybridization experiments in parallel, using a positive control probe to verify proper application of the protocol
20A(viii), 20B(xv)		Probe concentration not high enough	Increase the probe concentration during the hybridization step up to tenfold. Increase hybridization time up to several days; 20 h should suffice for moderately expressed transcripts but rare transcripts might benefit from longer hybridization times. We have not tested whether hybridization for longer than 20 h will improve the signal
		Inadequate permeabilization	Verify that the permeabilization reagents are prepared correctly. Adhere to the incubation times specified in the protocol. Proteinase K enzyme activity may vary from lot to lot, so test each new lot on the tissue of interest and adjust the concentration to achieve adequate tissue permeabilization. Proteinase K concentrations can range between 25 and 100 µg ml ⁻¹ and treatment periods can vary from a few minutes to 1 h. Lower concentrations combined with longer (up to 1 h) incubation times give more easily reproducible results
		Probe is degraded	Maintain RNase-free conditions
		Problem with digoxigenin antibody binding to labeled probe	Check proper dilution of antibody. Dilute in fresh blocking solution. Incubate overnight at 4 °C. Use proper antibody storage conditions
	Poor tissue morphology	Inadequate fixation	Prepare fixative on the day of use. Follow the optimized fixation times and paraformaldehyde and DMSO concentrations given in the protocol. If tissues other than <i>Drosophila</i> ovaries are being analyzed, adjust the timing of fixation and/or concentration of fixative to tissue morphology by testing a series of times and concentrations during

Step	Problem	Possible reason	Solution
			initial fixation or post fixation following tissue permeabilization
		Tissue becomes dry	Leave a small amount of solution on the ovaries at each rinsing step. Use sufficient volume while rocking on a nutator to ensure that egg chambers do not become beached in the upper part of the tube
		Permeabilization reagent (proteinase K, xylenes or RIPA) concentration is too high or incubation time is too long	Verify that the permeabilization reagents are prepared correctly. Adhere to the incubation times specified in the protocol. Proteinase K enzyme activity may vary from lot to lot. Test each new lot on the tissue of interest and adjust the concentration to achieve adequate tissue preservation. Proteinase K concentrations can range between 25 and 100 $\mu\text{g ml}^{-1}$ and for a few minutes to 1 h. Lower concentrations combined with longer (up to 1 h) incubation times give more easily reproducible results. Omit rocking or nutating during permeabilization
20B(xv)	Weak antibody signal in IF/FISH	Excessive fixation	Verify correct paraformaldehyde concentration. Adhere to the specified fixation time for IF (20 min). Examine the antibody signal with IF alone to ascertain whether the paraformaldehyde fixation is disrupting the epitope
20B(xv)		Antibody concentration is too low	Verify that the optimal antibody dilution (if known) is being used. Perform a titration series of the antibody dilutions before proceeding with FISH to determine the optimal concentration (Box 1, after step 15)
		Primary antibody inherently gives a weak signal or protein being detected is not abundantly expressed	Amplify the protein signal using TSA
	High IF staining background in IF/FISH	Antibody concentration is too high or incubation is too long	Pre-adsorb the primary antibody: before using antibodies on experimental tissue, treat fixed ovaries or embryos, preferably lacking the antigen, with the antisera to remove antibodies that bind to epitopes on other proteins Dilute the antibody to the lowest concentration necessary for detecting an adequate signal. Perform a titration series of the antibody dilutions before proceeding with FISH to determine the optimal concentration. Perform the incubation at 4 °C (Box 1, after step 15)
	During laser confocal microscopy, emission from the fluorophore with the shorter wavelength excitation is detectable in the longer wavelength channel emission being acquired	A weak signal in the longer wavelength channel requires high laser power, allowing transactivation of a relatively strong signal in a shorter wavelength channel	Use a short wavelength excitation for the weaker signal (e.g., AlexaFluor 488) and a longer wavelength for the stronger signal (e.g., AlexaFluor 546 or 647). Reduce the FISH signal strength by decreasing the tyramide concentration and/or incubation time in the tyramide solution (Step 20B(x,xi))
Box 2, step 14	A smear appears on the gel, running at a molecular weight higher than expected	The template DNA was not completely linearized and the RNA polymerase circumnavigated the plasmid for multiple, processive cycles to produce long transcripts	Digest the template longer. Run an aliquot of the digest on a gel to verify complete linearization. Completely linearized templates will appear on the gel as a single band of the expected size. Incompletely linearized templates will appear as multiple bands, some of which are the wrong size
	A smear appears on the gel, running at a molecular weight that is lower than expected	RNase in one or more solution is degrading the product	Maintain RNase-free conditions
		The DNA template is degraded or forms a secondary structure that causes the RNA polymerase to fall off	Run the template on a gel to verify its quality and size before proceeding to probe synthesis

Step	Problem	Possible reason	Solution
	Samples appear to run at the wrong size on the gel	Unequal amounts of ethidium bromide were added to the samples and the size marker	Use the same amount of ethidium bromide in all samples (including the size marker). Ethidium bromide affects RNA migration in agarose gels
		The samples have secondary structures that alter their migration on the gel	Heat-denature the samples immediately before loading on the gel and run the gel at high voltage
Box 3, step 14	Dot blot fails to produce a color reaction, even for the control sample	Preparation of the solutions for processing the dot blot was inaccurate	Remake the solutions, prepare a new dot blot and repeat the procedure
Box 3, step 14		Samples were not cross-linked to the nylon	Prepare a new dot blot and crosslink the RNA probes to the nylon membrane according to the manufacturer's instructions
	Dot blot gives a color reaction for the control sample but not for the experimental sample	The concentration of the experimental probe is too low due to inadequate probe preparation or degradation	Run the probe on a gel to examine its quality and evaluate its concentration as described in Box 2, steps 7–14. Prepare a new probe if necessary
		Preparation of the dilutions for the dot blot was incorrect	Prepare new dilutions and a new dot blot, and repeat the procedure
		The probe RNA was lost during the pelleting and washing steps	Make a new probe and take care when pelleting and washing the probe RNA