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Dual fluorescence detection of protein and RNA in *Drosophila* tissues

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Abstract

Detection of RNAs by *in situ* hybridization (ISH) is a well-established technique that permits the study of specific RNA expression patterns in tissues; however, not all tissues are equally amenable to staining using the same procedure. Here we describe a protocol that combines whole-mount immunofluorescence (IF) and fluorescence *in situ* hybridization (FISH) for the simultaneous detection of specific RNA transcripts and proteins, greatly enhancing the spatial resolution of RNA expression in complex, intact fly tissues. To date, we have successfully used this protocol in adult testis, larval male gonads, adult intestine and Malpighian tubules. IF is conducted in RNase-free solutions, prior to the harsh conditions of FISH, in order to preserve protein antigenicity within dissected tissues. Separate protocols are described for mRNA and miRNA detection, which are based on robust digoxigenin (DIG) RNA and locked nucleic acid (LNA) probes, respectively. The combined IF-FISH procedure can be completed in 2 d for miRNA detection and 4 d for mRNA detection. Although optimized for *Drosophila*, this IF-FISH protocol should be adaptable to a wide variety of organisms, tissues, antibodies and probes, thus providing a reliable and simple means to compare RNA and protein abundance and localization.

INTRODUCTION

Detection of RNAs by ISH is an invaluable tool that has advanced dramatically the study of specific RNA expression patterns in tissues^{1,2}. Historically, ISH was developed using radiolabeled probes that allowed high sensitivity but poor spatial resolution of signals and was accompanied by potential health risks associated with radioactivity^{3,4}. Therefore, nonradioactive ISH techniques gained popularity, including the use of alkaline phosphatase (AP)- or fluorophore-conjugated probes and fluorescent tyramide signal amplification (TSA)-based procedures. TSA, which is more sensitive, is based on the horseradish peroxidase (HRP)-catalyzed deposition of labeled tyramine molecules at sites of probe binding⁵. The improved resolution achieved by using TSA stems from the localized signal that is generated by a highly reactive oxidized intermediate, which binds rapidly and

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covalently near the HRP probe. In contrast, AP substrates precipitate diffusely at sites of AP enzyme activity⁶. As a result, the FISH-TSA approach greatly improves the spatial resolution of RNA detection, even allowing for the subcellular localization of specific RNA molecules.

Evaluation of gene expression patterns *in vivo* is informative; however, regulation of RNA stability, localization and translation often results in differences in patterns of RNA and protein expression. In addition, localizing the expression of an RNA of interest to a given cell type is often challenging and can be facilitated by the use of cell type-specific markers to confidently identify and locate diverse cell types in complex tissues. Our laboratory is particularly interested in the regulation of stem cell behavior by intrinsic and extrinsic factors. The ability to conclusively identify *Drosophila* germ-line stem cells and intestinal stem cells *in vivo*, without the need for dissociation of the tissue, makes them ideal model systems in which to study the interactions between stem cells and the surrounding microenvironment, called the niche. Therefore, we required a protocol to allow the visualization and localization of stem cell fate determinants within cells and throughout tissues.

Development of the protocol

Although established protocols for ISH exist, ISH does not work equally well for all *Drosophila* tissues. For example, embryos and imaginal discs have been quite amenable to ISH using standard protocols, whereas the cells within the testis have been quite difficult to analyze owing to the fact that the testis is surrounded by layers of muscle and pigment cells, which act as a barrier to probes. Therefore, the protocol described here for dual labeling of RNA and protein was developed initially to precisely determine RNA localization within cells comprising the *Drosophila* testis niche⁷.

Our protocol is based on several robust protocols previously described to detect RNA in *Drosophila* embryos^{8,9}, imaginal discs¹⁰ and testes^{11,12}, and miRNAs in zebrafish embryos¹³. However, as a result of the high annealing temperature of the RNA probe, testis tissue became fragile and antigens recognized by well-characterized antibodies were not detected, complicating simultaneous analysis of RNA and protein. To overcome this difficulty, we optimized this protocol to perform IF under RNase-free conditions before exposing tissues to the harsh conditions necessary for FISH (Fig. 1).

The modified protocol described below has been used to detect the expression of two important stem cell regulatory factors, *unpaired* (*upd*) and *escargot* (*esg*), in adult and larval testes⁷, the intestine and Malpighian tubules (Figs. 2–5). The mRNA detection, together with IF for well-characterized, tissue-specific markers or reporter proteins, facilitates the precise localization of stem cells within the tissue of interest. In addition, we have adapted the protocol for the detection of the miRNA *let-7* in adult testes using LNA probes⁷. Although the dual protocol for mRNA detection is performed on dissected tissues kept in a microcentrifuge tube throughout the protocol, miRNA detection required mounting and fixation of testes on slides to enable better probe penetration (Fig. 1). Despite the fact that the protocol is designed for analysis of *Drosophila* tissues, we predict that this approach (IF

first, followed by FISH) should be applicable to tissues in other organisms, provided that the fixation conditions are optimized for the sample being processed.

Overview of technique

Here we describe two methods that we recently developed for combined IF and FISH on dissected tissues that allow simultaneous detection of proteins and mRNAs or miRNAs *in situ*, while maintaining tissue integrity⁷. Three changes over traditional IF-FISH protocols have led to the successful application of these protocols to *Drosophila* tissues. First, we avoid using the proteinase K treatment that is typically used to enhance probe penetration at the expense of antigen integrity. We perform IF prior to FISH, preventing protein denaturation by the high hybridization temperatures that are likely to affect protein antigenicity in whole-mount dissected tissues. Therefore, IF is performed under RNase-free conditions, including an excess of heparin and tRNA to block nonspecific binding of the antibody and to preserve the mRNA for subsequent detection. Similar conditions have been described previously for combined IF-FISH in embryonic gonads⁹. Second, samples are fixed with formaldehyde twice, immediately following dissection and again after the IF; cross-linking the secondary antibody to the tissue preserves the IF signal during the FISH. Third, in this method, we use TSA reagents to directly detect the peroxidase-dependent complex and generate a detectable product that is covalently linked at or near sites of probe binding⁵. The TSA reagent is suitable for RNA FISH detection and provides high signal resolution as well as markedly increased sensitivity.

Experimental design

Sample selection—We have applied the protocol to a number of *Drosophila* tissues, including larval and adult testes⁷, intestines and Malpighian tubules (M.L.-C., S. Landais and D.L.J., unpublished data). Thus, there should be no limitations to the choice of tissue. Also, note that, in addition to the genotype/conditions being tested, negative (loss-of-function) and/or positive (gain-of-function) samples should be analyzed in parallel to control for nonspecific staining of probes or antibodies.

Antibodies—Optimization of staining with desired antibodies (i.e., concentration) should be performed before starting the procedure. There should be no limitations on the choice of antibodies with respect to species of origin or conjugated fluorophores used. We have optimized the following primary antibodies for use in the IF-FISH protocol described here: the germ cell marker Vasa; the somatic cell (hub) marker Fascilin3 (Fas3); the cell-cell adhesion marker Armadillo (*Drosophila* homolog of β -catenin); and GFP. The antibodies, dilution and source of origin are listed in Table 1. In addition, we used secondary antibodies conjugated to three different Alexa Fluor fluorophores (Invitrogen). Note that this protocol is also amenable to simultaneous staining with two primary antibodies from different species (Fig. 4).

Probes—Obtaining sufficient amounts of a good-quality probe is crucial to the efficacy of the protocol. Preparation of a labeled probe is completed within 1 d, and it is recommended that the probe be generated at least 1 d before starting the protocol (day 0). As a template for the DIG-labeled antisense RNA probe, we use a linearized plasmid containing the full-

length cDNA sequence of the gene of interest followed by an RNA polymerase promoter (T7/T3/SP6). Alternatively, a DNA template can be prepared by PCR amplification of cDNA or exonic sequences from genomic DNA (above ~500 kb) with reverse primers that contain an RNA polymerase promoter sequence.

Biotin-labeled probes can be used as an alternative to DIG labeling (Fig. 2). The use of biotin-labeled probes reduces the length of the protocol by 1 d, as the labeled probe can be detected by streptavidin-HRP, bypassing the need for antibody detection. However, we found that results obtained with biotin-labeled probes were less consistent than those acquired using robust DIG-labeled riboprobes.

Specificity can be determined by using multiple, nonoverlapping antisense probes for the same RNA target, and sense probes (RNA polymerase promoter at the 5' end) can be used as negative controls to detect nonspecific staining. In addition, a probe for an RNA with a well-characterized expression pattern in the tissue of interest can be used to control for overall experimental conditions.

MATERIALS

REAGENTS

- Adult flies or wandering third-instar larvae of appropriate genotypes (Wild-type, control flies were Oregon R, obtained from the Bloomington *Drosophila* Stock Center at Indiana University.)
- Plasmid containing cDNA for the desired probe with either a *T7*, *T3* or *Sp6* promoter at the 3' end for generation of an antisense probe (see Box 1 for probe preparation)
- DIG RNA labeling mix (Roche, cat. no. 11 277 073 910)
- T7/T3/Sp6 RNA polymerase (Roche, cat. nos. 10 881 767 001, 11 031 163 001 and 10 810 274 001, respectively)
- Protector RNase inhibitor (Roche, cat. no. 03 335 399 001)
- Distilled water-DNase/RNase free (Gibco, cat. no. 10977-015) or diethyl pyrocarbonate (DEPC)-treated water (see REAGENT SETUP) ! **CAUTION** DEPC is a potent protein denaturant and a suspected carcinogen. Avoid contact and inhalation ▶ **CRITICAL** Prepare all solutions with DNase/RNase-free water.
- RQ1 RNase-free DNase (Promega, cat. no. M6101)
- RQ1 DNase stop solution (Promega, cat. no. M199A)
- DTT, 0.1 M (Invitrogen, cat. no. Y00147)
- Heparin sodium salt, grade I-A: from Porcine intestinal mucosa (Sigma-Aldrich, cat. no. H3393) ! **CAUTION** Avoid contact and inhalation.

- Formaldehyde, 16% (vol/vol) (methanol free) ultrapure EM grade (Polysciences, cat. no. 18814) ! **CAUTION** Formaldehyde is a carcinogen, highly toxic, an irritant and a mutagen.
- Tween-20 (Sigma-Aldrich, cat. no. P-7949)
- tRNA from baker's yeast (Roche, cat. no. 10109495001)
- Formamide, deionized minimum (99.5%,GC) (Sigma-Aldrich, cat. no. F9037) ! **CAUTION** Avoid contact and inhalation.
- EDTA (Sigma-Aldrich, cat. no. E5134)
- NaH₂PO₄ (Merck, cat. no. 1,06346,1000)
- NaCl (Sigma-Aldrich, cat. no. S3014)
- Citric acid trisodium salt (Sigma-Aldrich, cat. no. C3674)
- PBS stock solution, 10× (80 g of NaCl, 2 g of KCl, 14.4 g of Na₂HPO₄ and 2.4 g of KH₂PO₄ in 1 liter of water)
- SSC (20×) stock solution (dissolve 175.3 g of NaCl and 88.2 g of citric acid trisodium salt in 1 liter of DEPC-treated water; keep overnight and autoclave)
- Sheared salmon sperm DNA (10 mg ml⁻¹; Ambion, cat. no. AM9680)
- HCl 1:1 (Sigma-Aldrich, cat. no. H1758), 50% (vol/vol) in water
! **CAUTION** HCl is poisonous, can be explosive, might be fatal by inhalation, and is also harmful by ingestion and destructive to the skin.
- Sheep DIG-POD specific antibody (anti-DIG-POD; Roche, cat. no. 11 207 733 910) diluted 1:500 (vol/vol) in Tris-NaCl-blocking (TNB) buffer. The use of this antibody is crucial to the success of the experiment; substitution with a similar product reduced the FISH signal
- Tyramide signal amplification (TSA) fluorescence kit with Cyanine 3 (PerkinElmer, cat. no. NEL704A)
- Blocking reagent (PerkinElmer TSA kit) needed to prepare TNB buffer
- Vectashield mounting medium with DAPI (Vector Laboratories, cat. no. H-1200)
! **CAUTION** DAPI is a possible carcinogen, which might be harmful by inhalation, ingestion or skin absorption, and might also cause irritation.
- *let-7* LNA-DIG miRNA probe (miRCURY LNA microRNA detection probe: dme-*let-7* (Exiqon, cat. no. 330001-15))
- miRNA ISH buffer set (Exiqon, cat. no. 9000)
- Ethanol
- Nitrogen tank

Box 1**DIG-labeled RNA probe preparation ● TIMING 6 h****DNA template preparation using plasmid linearization ● TIMING 3 h**

- 1** For each antisense probe, linearize 5 µg of plasmid (containing cDNA or an exon of interest), using a restriction enzyme that has a unique site located 5' of the insert. The plasmid should contain a T7 (or T3 or SP6) RNA polymerase promoter sequence at the 3' end to the insert. Incubate the restriction reaction for 2 h at the recommended temperature.
- 2** Gel-purify the linearized DNA template with the Qiagen gel extraction kit according to the manufacturer's protocol. Measure DNA concentration (expect ~3 µg of DNA to be recovered).

Preparation of DIG RNA-labeled probe ● TIMING 3 h

- 3** For each probe, add the components listed below to a 20-µl reaction in a 1.5-ml tube. Adjust with nuclease-free water according to the volume of the eluted linearized DNA template. Mix, briefly spin down and incubate for 2 h at 37 °C.

Component	Amount per reaction (µl)	Final concentration
Eluted linearized DNA template	up to 12 µl	1 µg
Transcription buffer, 10×	2 µl	1×
DIG RNA labeling mix, 10×	2 µl	1×
T7 or T3 or SP6 RNA polymerase	2 µl	40 U
RNase inhibitor	2 µl	80 U
Nuclease-free water	To a final volume of 20 µl	

▲ **CRITICAL STEP** Alternatively, probes can be labeled with a biotin labeling mix (Fig. 2d), but the signal is less robust from experiment to experiment when compared with DIG-labeled riboprobes; please see Experimental design for details.

- 4** Spin down and add 2 µl of RNase-free DNase. Mix and incubate for 15 min at 37 °C.
 ▲ **CRITICAL STEP** Use a DNA control to verify DNase activity when preparing each probe, as DNase activity is temperature sensitive.
- 5** Stop the reaction by adding 2 µl of DNase stop solution and 100 µl of DEPC-treated water; mix and spin down the mixture.
- 6** Resuspend RNA micro-column resin. Remove the top cap and then snap off the bottom tip. Place the micro-column into a sterile 1.5-ml tube. Spin for 1 min at 1,000g at room temperature to remove the storage buffer and to pack the resin. Discard the tube.

- 7 Place the column into a new sterile 1.5-ml tube. Load the RNA probe solution onto the center of the resin and centrifuge for 4 min at 1,000*g* at room temperature. Discard the column and retain the flow-through, which contains the DIG-labeled RNA.
- 8 Run 2 µl of probe on an agarose gel for ~10 min at 100 V. The probe should appear as one discrete strong band and not as a smear, which would indicate degradation.
- 9 Measure the probe concentration (expect ~50 ng µl⁻¹).

? TROUBLESHOOTING

- **PAUSE POINT** Divide RNA probe into 1-µg aliquots to prevent repeated thawing and freezing, and then store them at – 80 °C (can be kept for months).

EQUIPMENT

RNase-free 1.5-ml tubes (Axygen Scientific)

Agarose gel (Agarose A, Biotechnology grade, BioPioneer) and DNA gel apparatus (Galileo Bioscience)

Gel extraction kit (Qiagen, cat. no. 28704)

Fine forceps (Roboz Surgical Instrument Company, cat. no. RS5045)

Nine-well depression glass spot plates (Pyrex, cat. no. 7220-85)

Mini quick spin RNA columns (Roche, cat. no. 11 814 427 001)

Dissecting microscope (Leica Microsystems, Leica S6E)

Fluorescence microscope (Zeiss, Axio Observer Z1 with Apotome slider) or confocal microscope (Zeiss LSM 780)

Heat block, 80 °C (Labnet International, AccuBlock Digital dry bath)

Water baths, 37 and 65 °C (covered; Fisher Scientific, Isotemp 215)

Aluminum foil (Reynolds Wrap)

Parafilm (Parafilm, PM-996)

SuperFrost Plus slides (VWR Scientific, cat. no. 48311-703)

Coverslips 22 × 22 mm, no. 1.5 (VWR micro cover glass, cat. no. 48366-227)

Small, fine-haired artist's paintbrush

Coplin jars (Wheaton Science Products)

Kimwipes

Humid chamber (Place a few wet Kimwipes underneath the tip holder rack of an empty tip box, replace the rack on top and place slides on top of the rack. Seal the box with Parafilm.)

REAGENT SETUP

DEPC-treated water and 1× PBS-DEPC—Add 1 ml of fresh DEPC to 1 liter of distilled water or 1× PBS. Shake well to disperse the DEPC through the solution and leave for 12 h or more at room temperature (RT, 22–25 °C). Autoclave at 15 p.s.i. on liquid cycle for 20 min to inactivate the remaining DEPC. The solution is stable for many months in RNase-free conditions at RT. Note that the DEPC is not active after being autoclaved.

Tween-20, 10% (vol/vol)—Tween-20 is diluted in DEPC-treated water. Prepare in advance, as the viscosity of the detergent requires overnight solubilization. Store at room temperature for up to 1 month.

Fixative 1—Prepare fresh for each experiment, 1 ml per sample. Combine 4% (vol/vol) formaldehyde in 1× PBS-DEPC and 0.1% (vol/vol) Tween-20.

Fixative 2—Prepare fresh for each experiment, 1 ml per sample. Combine 10% (vol/vol) formaldehyde in 1× PBS-DEPC and 0.1% (vol/vol) Tween-20.

PBS-Tween-heparin (PBTH)—Mix 1× PBS-DEPC, 0.1% (vol/vol) Tween-20, 50 µg ml⁻¹ heparin and 250 µg ml⁻¹ tRNA. Prepare approximately 10 ml per sample. PBTH may be stored at 4 °C for up to 2 weeks.

PBTHR—Prepare fresh for each experiment, 100 µl per sample. PBTHR contains PBTH with 0.2 U ml⁻¹ RNase inhibitor (Roche, 1:200) and 1 mM DTT (1:100).

Hybridization buffer (HB)—For 50 ml of HB, mix 25 ml of deionized formamide, 12.5 ml of 20× SSC, 0.125 ml of heparin (100 mg ml⁻¹), 0.5 ml of 10% (vol/vol) Tween-20, 0.5 ml of 10 mg ml⁻¹ heat-denatured salmon sperm DNA and 1 ml of 1:1 HCl (see REAGENTS) to adjust the pH to 5.0. Add to 50 ml with DEPC water. The buffer is stable for a couple of months at – 20 °C (remains as liquid).

Prehybridization buffer (PHB)—Prepare fresh, 1 ml per sample. Mix HB with tRNA (10 mg ml⁻¹).

PBT—Mix 1× PBS-DEPC and 0.1% (vol/vol) Tween-20. Prepare 6 ml per sample. PBS is stable for many months in RNase-free conditions at RT.

Proteinase K buffer—Mix 5 mM Tris-HCl (pH 7.4), 1 mM EDTA and 1 mM NaCl. The buffer is stable for many months in RNase-free conditions at RT.

TNB buffer—Mix 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl and 0.5% (wt/vol) blocking reagent (blocking powder supplied in the TSA kit, PerkinElmer). Aliquot and store at – 20 °C. The buffer is stable for many months.

PROCEDURE

Tissue preparation and fixation ● TIMING 2 h

- 1| Adult or larval testes, or midguts of the desired age and genotype, can be dissected using options A, B or C, respectively.

(A) Dissections of adult testes

- i. Collect adult flies by anesthesia on a CO₂ pad. Use fine forceps to dissect testes from an anesthetized male placed in a drop (~300 µl) of 1× PBS-DEPC on a glass microscope slide, coated to avoid liquid dispersion.
▲ **CRITICAL STEP** Tissues from animals of the same age should be used, as tissue morphology and gene expression can change during development and with age^{7,14}.
- ii. Dissect testes away from seminal vesicles and discard damaged tissue.
- iii. Use forceps to immediately transfer the testes to a 1.5-ml tube containing 1 ml of fixative 1, placed on ice. Dissect additional flies and add testes to the ice-cold fix, but for no longer than 1 h. Tissue is often damaged during FISH/IF, and thus it is advised to be conservative at this step and dissect as much tissue as possible. For more details on how to dissect adult testes, see refs. 11 and 15.
- iv. To analyze miRNA expression, proceed to Box 2. Otherwise, proceed directly to Step 2.

Box 2

IF with FISH for *let-7* miRNA in *Drosophila* testes ● TIMING 8.5 h + overnight

Tissue preparation and fixation ● TIMING 2 h

▲ **CRITICAL** During this protocol, samples are squashed on the slide and all washes are done in Coplin jars. Incubations with fixative solutions, antibodies and probes are done by dropping the solution (100 µl of volume per slide) on the sample and covering with 'Parafilm coverslips' that are used to prevent solution evaporation.

- 1 Transfer the dissected testes to 10 µl of 1× PBS-DEPC on a coverslip, and spread out the testes. Squash the samples by carefully placing it on a SuperFrost Plus slide.
- 2 Immediately freeze the covered slide by soaking it in liquid nitrogen.
■ **PAUSE POINT** Samples can be kept indefinitely in a sealed liquid nitrogen tank.
- 3 Crack off the coverslip with a razor blade; the samples will remain adhered to the slide.
- 4 Immediately immerse the slide in a Coplin jar containing cold 95% (vol/vol) ethanol for 10 min at – 20 °C.

- 5 Take out the slide and fix by dropping 100 μ l of fixative 1 on the sample and incubate for 7 min at RT. Drain the fixative by tapping the slide on its side against a Kimwipe.
- 6 Rinse the slide once and wash it for 10 min in a Coplin jar containing PBTH.

Immunofluorescence ● TIMING 1.5 h

- 7 Block in PBTHR, and then incubate with primary and secondary antibodies as described in Steps 4–6 of the main PROCEDURE. The incubation with the primary and secondary antibodies is done for 30 min each in a humid chamber at RT.

Postfixation and probe hybridization ● TIMING 2.5 h

- 8 Wash twice quickly with PBTH (emptying the Coplin jars by inversion and refilling).
- 9 Rinse once in proteinase K buffer and incubate in 1 μ l of proteinase K (20 mg ml^{-1}) per 1 ml of proteinase K buffer (1:1,000) for 3 min.
- 10 Rinse quickly with 100 μ l of PBTH on the slide and wash in PBTH for 5 min in a Coplin jar.
- 11 Fix again with fixative 1 for 20 min.
- 12 Rinse twice with PBTH and then wash for 10 min in PBTH.
- 13 Briefly dip the slide 10 times into 70% (vol/vol) ethanol in a Coplin jar, and then wash for 1 min in 70% (vol/vol) ethanol.
- 14 Repeat step 13 with 96% (vol/vol) ethanol, and then with 99.9% (vol/vol) ethanol.
- 15 Air-dry the slides.
- 16 Dilute the *let-7*LNA-DIG probe 1:500 (40 nM final conc.) in 1 $\times$ HB (this HB is part of the Exiqon kit).
- 17 Heat up the probe mix to 80 $^{\circ}\text{C}$ for 10 min, briefly spin down and cool to $\sim$ 52 $^{\circ}\text{C}$.
- 18 Apply 25 μ l of probe to the slide to evenly cover the specimen and hybridize for 1 h in a humid chamber at 52 $^{\circ}\text{C}$ (in a dry oven with a Parafilm coverslip).
- 19 Remove the Parafilm coverslip and wash the slide in 5 $\times$ SSC for 5 min at 52 $^{\circ}\text{C}$ in Coplin jars.
- 20 Wash twice in 1 $\times$ SSC for 5 min at 52 $^{\circ}\text{C}$.
- 21 Wash twice in 0.2 $\times$ SSC for 5 min at 52 $^{\circ}\text{C}$.
- 22 Wash once in 0.2 $\times$ SSC for 5 min at RT.

Signal detection ● TIMING overnight + 2.5 h

- 23 Wash with PBS for 5 min.

24	Block in TNB buffer for 30 min.
25	Incubate with anti-DIG-POD (1:500 in TNB buffer) overnight in a humid chamber at 4 °C.
26	Detect the signal with TSA as described in Steps 20–23 of the main PROCEDURE.
27	Remove the last wash, add 8 µl of VectaShield and place a glass coverslip over the sample. After 1 h, the samples can be visualized using a fluorescence microscope.

(B) Dissections of larval testes

- i. Collect third-instar (L3) wandering larvae and wash them in a nine-well depression plate containing 1× PBS-DEPC.
- ii. Identify male larvae by visualizing their gonads under a dissecting microscope. Testes are spherical organs surrounded by an opaque tissue known as the fat body, which gives them a translucent appearance in a dorsolateral view of a male larva. In contrast, the posterior third of female larvae appears to contain a tiny hole embedded in the fat body (location of smaller larval ovaries), which facilitates determining the sex of the larvae.
- iii. To begin dissections, pick one male larva onto a new well containing 1× PBS-DEPC (~400 µl). With a pair of fine forceps in one hand, hold the larva near the anterior end; with forceps in the other hand, open and tear in the middle of the body. Discard the anterior half of the larva and pull out the gut from the posterior half. Invert the posterior half of the larva, exposing organs including the testes embedded in the fat body. To minimize tissue loss during subsequent washes, keep the testes attached to the fat body and body wall.
- iv. Use forceps to immediately transfer the inverted posterior half body and attached testes into a 1.5-ml tube containing 1 ml of fixative, placed on ice.
- v. To analyze miRNA expression, proceed to Box 2. Otherwise, proceed directly to Step 2.

(C) Dissections of adult posterior midguts and Malpighian tubules

- i. Sort anesthetized adults according to their gender—it is preferable not to pool males and females, as there often are noticeable differences in the staining efficiency of male and female guts that could skew downstream analyses.
- ii. Transfer a fly onto a well containing 1× PBS-DEPC (~400 µl) to start dissections. Use a pair of fine forceps to gently hold the fly between the abdomen and thorax, preferably underneath the wings. If you are interested in dissecting the entire gut out, we recommend that the head of the fly be pulled off just before the next step. With another pair of forceps, grab firmly the dorsal half of the anal plate and pull it out slowly but steadily. Keep pulling until the distal hairpin is out of the abdominal cavity (the distal hairpin is easily recognizable in well-fed flies as a thinning of the

midgut). If done correctly, only the gut and its associated Malpighian tubules are dissected out of the abdominal cavity using this approach. If the gonads are pulled out as well, dissect them away from the gut. If you are interested only in the posterior midgut and hindgut, pinch and pull at the distal hairpin to free both from the rest of the midgut and foregut. Discard each adult fly after removing the gut to minimize clutter in the dissection well.

- iii. Use forceps to immediately transfer the gut to a 1.5-ml tube containing 1 ml of fixative, placed on ice. Make sure to hold the hindgut next to the anal plate to minimize damage to the areas of interest. Avoid grabbing the posterior midgut, as it is typically much more fragile. Proceed according to Step 2 in the PROCEDURE.

- 2| Fix at RT in fixative 1 for 20 min with gentle rotation. Do not overfix, as overfixation often results in low signal.

? TROUBLESHOOTING

- 3| Wash and block three times, for 5 min each, in 1 ml of PBTH with gentle rotation.

▲ **CRITICAL STEP** For these and all subsequent washes in the tubes, allow the tissue to settle to the bottom of the tube (~2 min) before removing the wash solution.

▲ **CRITICAL STEP** The relatively high concentration of tRNA ($250 \mu\text{g ml}^{-1}$) and heparin ($50 \mu\text{g ml}^{-1}$) in PBTH saturates non-specific binding sites for the antibody as efficiently as would serum or BSA, commonly used for IF. However, the PBTH does not contain RNases to keep the mRNA intact for further detection.

▲ **CRITICAL STEP** Even though proteinase K has been used to permeabilize the tissue for some ISH protocols¹¹, it is not required during this protocol, thus preserving the protein antigenicity for subsequent detection by IF.

IF detection of protein expression ● TIMING overnight + 2.25 h

- 4| Remove as much of the last wash as possible, but do not allow the tissue to dry completely. Add 100 μl of PBTHR with primary antibody at the recommended concentration. Incubate the tubes overnight at 4 °C (gentle rocking is not required, but it is recommended) or at RT for 4 h.

▲ **CRITICAL STEP** The reducing agent DTT and the RNase inhibitor enzyme in PBTHR are essential to inactivate RNases commonly found in antibody solutions. If you plan to double stain with another antibody, dilute both of them (from different animal origins) in PBTHR (Fig. 4).

? TROUBLESHOOTING

- 5| Wash three times, for 5 min each time, at RT in 1 ml PBTH with gentle rotation.

? TROUBLESHOOTING

- 6| Incubate with a fluorescent secondary antibody (1:100) in PBTHR for 2 h at RT; wrap tubes in aluminum foil, as the fluorescent secondary antibodies are light sensitive.

? TROUBLESHOOTING

- **PAUSE POINT** Alternatively, incubate the tissues in secondary antibody overnight at 4 °C.

Postfixation and probe hybridization ● TIMING 2.5 h + overnight

- 7| Wash three times, for 5 min each time, at RT in 1 ml PBTH with gentle rotation.
- 8| Remove the last wash and fix in 1 ml of fixative 2 for 20 min with a gentle rotation.
- ▲ **CRITICAL STEP** The postfixation step is necessary to cross-link the secondary antibody to its site during the harsh ISH conditions without affecting the fluorescence signal.
- 9| Wash three times, for 5 min each time, in PBTH.
- ▲ **CRITICAL STEP** During these washes, heat up 0.5 ml of salmon sperm DNA (10 mg ml⁻¹) at 80–100 °C for 10 min and make the HB.
- 10| Allow tissues to settle to the bottom of the tube and remove 0.5 ml of the PBTH wash and rinse with 0.5 ml of prewarmed HB for 10 min in a 65 °C water bath.
- 11| Remove HB and block with 0.5 ml of PHB for 1 h in a 65 °C water bath.
- 12| Approximately 15 min before prehybridization is complete, prepare the RNA probe. Add the RNA probe to 0.5 ml of PHB in a 1.5-ml tube, to a final concentration of 2 µg ml⁻¹ (this is typically ~20 µl per tube for probes at 50 ng µl⁻¹). Heat the mix to 80 °C for 10 min, briefly spin down and cool to ~65 °C.
- ▲ **CRITICAL STEP** Do not cool the probe solution on ice before addition of the samples, as this may cause the formation of secondary structures in long probes, which inhibits their binding to target RNAs.
- 13| When prehybridization is complete, remove PHB from the samples and add 0.5 ml of probe solution immediately. Hybridize the tissues with the probe overnight (ideally 18 h) at 65 °C.
- ▲ **CRITICAL STEP** The high hybridization temperature (65 °C) and the high formamide concentration in HB decrease the occurrence of cross-hybridization.

Washes and FISH detection ● TIMING ~ 2 d (3 h + overnight + 1.5 h)

- ▲ **CRITICAL** After probe hybridization, the RNA of interest is double stranded (less sensitive to RNases). Thus, washes with PBT are sufficient; DEPC-treated PBT is not required.

- 14| Prepare three dilutions of PBT in HB (vol/vol) as follows: 1:4 (solution A), 1:1 (solution B) and 4:1 (solution C) (2 ml per sample). Prewarm at 65 °C.
 ▲ **CRITICAL STEP** These high-stringency washes prevent nonspecific hybridization of the probe and have been found to be particularly important when working with adult testis tissue¹⁶.
- 15| Remove the probe, rinse once quickly in solution A and then wash twice in 0.5 ml of solution A for 20 min at 65 °C.
- 16| Repeat Step 15 for solution B, and then for solution C.
- 17| Remove the last wash of solution C and bring the tissues back to RT. Wash the tissues twice in 0.5 ml of PBT, for 15 min each time, with gentle agitation on a rocking platform.
- 18| Block the tissues in TNB buffer for 30 min at RT.
- 19| Remove the TNB buffer and incubate the samples with DIG-POD-specific antibody in TNB buffer (1:500) overnight at 4 °C.

Signal detection (TSA amplification) ● TIMING 1.5 h

- 20| Wash three times, for 10 min each time, in PBT.
- 21| Prepare a TSA dilution of 1:50 (vol/vol) in amplification diluent (both from the TSA kit) in a tube. Remove the wash solution and incubate the samples with TSA for 30 min at RT; wrap the tubes in aluminum foil, as this step is light sensitive.
- 22| Wash three times, for 10 min each time, in PBT.
- 23| Remove the last wash. If an RNA probe is being used for the first time, mount only a small sample and use the microscope to check for background. If the fluorescence background is too high to observe a specific signal, wash for 10 min in PBT at 65 °C, followed by another 5-min wash in PBT at RT, and then mount the tissues (Steps 24–28).

? TROUBLESHOOTING

Mounting tissues ● TIMING 0.5 h

- 24| Remove the tissues from tubes with a minimum volume of the last PBT wash by using a precut transfer pipette (prewet with PBT), and lay them on a coated slide to prevent liquid dispersion.
- 25| Use fine forceps to separate clusters of joined tissues and to remove any debris. Debris can appear as nonspecific fluorescence signals.
- 26| Put a drop (10 µl) of Vectashield mounting medium with DAPI on a coverslip and place the clean tissues in the drop. Leave for 5 min.
 ■ **PAUSE POINT** Alternatively, store the tissues in Vectashield in a sealed microcentrifuge tube overnight at 4 °C.

- 27| If you are working with the testis, use fine forceps to straighten adult testes or to gently push away the fat body around the larval gonad to expose the apical tip. When mounting guts or Malpighian tubules, use fine forceps to anchor tissue by the hindgut, posterior to the pylorus (Fig. 3a). While holding the hindgut, gently drag the tissue over the coverslip in order to straighten the guts and align them in parallel, with the same anterior-posterior orientation.
- 28| Slowly lower a coverslip onto the drop of Vectashield to avoid bubbles. Press gently to squash the tissue to facilitate visualization using a fluorescence microscope.

? TROUBLESHOOTING

● TIMING

For mRNA detection

Box 1, preparation of DIG RNA-labeled RNA probe: 6 h. The probe can be prepared before starting the procedure (recommended)

Steps 1–3, tissue preparation and fixation: 2 h (For an inexperienced user, dissections may take longer, in which case we recommend overnight antibody incubation.)

Steps 4–6, IF detection of protein expression: 2.25 h + overnight

Steps 7–13, postfixation and probe hybridization: 2.5 h + overnight

Steps 14–19, washes and FISH detection: ~2 d

Steps 20–23, signal detection: 1.5 h

Steps 24–28, mounting tissues: 0.5 h

For miRNA detection

Box 2, IF with FISH for *let-7* miRNA in *Drosophila* testes: 8.5 h + overnight

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

ANTICIPATED RESULTS

Examples of results obtained with this protocol for adult testes (Figs. 2, 4 and 5), larval testes (Fig. 2), intestine (Fig. 3) and Malpighian tubules (Fig. 3) are shown. We anticipate that the protocol will be suitable for additional *Drosophila* tissues, as well as adaptable for complex multicellular tissues of other organisms. By using this method, the dual expression pattern of proteins and transcripts can be analyzed simultaneously. At present, the technical limiting factors are maintaining an RNase-free environment and handling the tissues, from dissections to the final staining step, without drying, losing or damaging the exposed tissue. When comparing expression of the same probe in different conditions or within different genetic backgrounds, all controls should be tested within the same experiment, as the intensity of the signal may vary from one experiment to another.

A low signal may be masked by background fluorescence from uncleared debris or nonspecific staining of adjacent tissue (e.g., the fat body surrounding larval gonads (Fig. 2f) and midgut tracheae). To adjust exposure in order to visualize your specific signal, ensure that such sources of background fluorescence are out of the field of view, which allows increasing camera exposure times to capture the signal of interest. High background can also occur from insufficient washing or prolonged incubation with the TSA fluorophore. This background can be distinguished from a real, but weak, signal by looking for the punctate pattern, characteristic of mRNA. IF with a known marker expressed in the same cell type also facilitates discriminating true signal from high background noise. For our purposes, the high spatial resolution of the combined IF-FISH signals enabled the visualization of intrinsic and extrinsic factors that regulate stem cell behavior. However, this technique will be useful whenever it is necessary to examine colocalization of RNA and proteins, particularly when there is evidence that the RNA of interest may undergo post-transcriptional regulation.

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Figure 1.

Summary of steps involved in dual fluorescence detection of protein and mRNA/miRNA and the approximate time needed. Dashed boxes indicate that probe preparation is recommended at least 1 d before beginning the protocol. Gray boxes indicate the timing for miRNA detection.

Figure 2.

Dual labeling of the stem cell niche in adult and larval *Drosophila* testes. (a) Schematic showing the apical tip of the *Drosophila* testis. Germline stem cells (GSCs) and cyst stem cells (CySCs) surround and are in contact with hub cells, which express *upd*. (b,c) Hybridization of an adult testis (from a 1-d-old male fly) with an RNA probe for the self-renewal factor *upd*. (b) Detection of DIG-labeled *upd* riboprobe with the AP-based procedure. The AP substrate precipitates diffusely at sites of AP enzyme activity as shown by a homogenous stain (black) of hub cells¹¹. (c) Detection of DIG-*upd* riboprobe (red) with tyramide signal amplification (TSA) procedure. The peroxidase at the HRP-linked probe reacts rapidly with Tyramine, which results in a much increased spatial resolution of *upd* transcript detection. DAPI (blue) marks the nuclei of the cells at the apical tip of the testis. Note that the testis appears larger in c because of reduced tissue dehydration during processing compared with b. (d-f) Dual labeling with antibodies to the germ cell-specific marker Vasa (d) and FISH for *upd* using a biotin-labeled riboprobe (e); a merged image of Vasa (green) and *upd* (red) is shown in panel f. (g,h) Dual labeling with antibodies to the hub cell marker Fas3 (g) and FISH for DIG-*upd* (h). (i) Merged panel showing Fas3 (green), DIG-*upd* (red) and DAPI (blue). (j,k) Dual labeling for Fas3 (j) and FISH for DIG-*upd* (k) of third-instar larval (L3) male gonads. (l) Merged panel showing Fas3 (green), DIG-*upd* (red) and DAPI (blue); arrows point to background signal from the fat body surrounding the larval gonad. Genotype of all imaged flies is OregonR. All panels are single sections. Scale bars, 20 μm.

Figure 3.

Dual labeling of the *Drosophila* midgut and Malpighian tubules. **(a)** Shown are (left) an adult *Drosophila* gut viewed with a $\times 10$ objective and stained with DAPI showing the posterior midgut (PM), Malpighian tubules (MT), pylorus (Pyl) and hindgut (H), and (right) a higher-magnification view of the posterior midgut showing an intestinal stem cell (ISC)/enteroblast (EB) nest (bracket), a polyploid enterocyte (*) and a Prospero⁺ enteroendocrine cell (arrowhead). **(b)** Dual labeling with FISH for *upd* (red) and IF for GFP expression in ISCs/EBs (green) of an adult midgut from a fly of genotype *esgGAL4*, *UAS-gfp*. Note *upd* expression in rows of circular muscle. **(c,d)** Dual labeling with FISH for *esg* (red) and IF for GFP (green) of an adult midgut **(c)** or Malpighian tubules **(d)**. Note colocalization of *esg* mRNA and the GFP reporter in both tissues **(b,c)**. Scale bars, 10 μ m.

Figure 4. FISH in combination with multiple antibodies during IF. (a–d) Apical tip of the testis (genotype: *esgGAL4*, *UAS-gfp*) showing *upd* mRNA expression in the hub (a) coimmunolabeled for the cell-cell adhesion marker Armadillo (Arm; b) and for GFP (c); merged image showing *upd* (red), Arm (blue) and GFP (green) is shown in d. Note *upd* and Arm colocalization in the hub. (e–h) Apical tip of testis showing *esg* mRNA (e) coimmunolabeled with Arm (f) and GFP (g); merged image showing *esg* (red), Arm (blue) and GFP (green) is shown in h. Note the difference in *esg* mRNA expression and expression of GFP driven from the *esgGAL4* reporter line. Scale bars, 20 μm.

Figure 5.

Dual labeling of *let-7* miRNA and Fas3 protein in the testis. An LNA-*let-7*-DIG-labeled probe was used to detect *let-7* miRNA expression in adult testes from 1-d-old male flies (genotype: *updGAL4*; UAS-*let7^{701.12.9}*). (a–c) Fas3 (a), *let-7* (b) and a merged panel, showing Fas3 (green), *let-7* (red) and DAPI (blue) (c). Scale bar, 10 μ m.

TABLE 1

Optimized antibody conditions.

Antibody (source)	Dilution in PBTHR	Source
Vasa (rabbit)	1:3,000	Paul Lasko laboratory (Fig. 1)
GFP (rabbit)	1:5,000	Invitrogen (A11122; Figs. 2–4)
Fascin-3 (mouse)	1:20	Developmental Studies Hybridoma Bank (7G10; Figs. 2 and 5)
Armadillo (mouse)	1:20	Developmental Studies Hybridoma Bank (N2 7A1; Figs. 3 and 4)
Prospero (mouse)	1:50	Developmental Studies Hybridoma Bank (MR1A; Fig. 3)

TABLE 2

Troubleshooting table.

Step	Problem	Possible cause	Solution
Box 1	Poor RNA synthesis	Unstable RNA fragment or inactive polymerase	Use a longer DNA template for transcription, preferably a full-length cDNA
	Degraded RNA on gel	RNases	Test polymerase activity Use gloves and prepare fresh solutions
2, 4–6	No detectable IF staining	Incompatibility between primary antibodies	Use antibodies separately and in combination
		Poor fixation of tissue after dissection	Make fresh formaldehyde and Tween-20 stocks; fix in 4% (vol/vol) formaldehyde in DEPC-PBS (without Tween-20)
23, 28	High FISH background	Excess probe and/or hybridization and washes are not stringent enough	Decrease the quantity of probe used; increase heparin concentration to 75 $\mu\text{g ml}^{-1}$. Wash for 10 min in PBT at 65 °C, and rinse once in PBT before mounting in Vectashield
	Nonhomogenous staining	Part of the samples dried out or were not exposed to the probe	Do not invert the tubes during washes to keep the tissue in the solution. Gently swirl the tubes
	No FISH staining	DNase is not working	Test DNase activity
		No DIG labeling	Test a drop of probe by a dot-blot with DIG-specific antibody
	Bright fluorescent background signal	Excessive debris on sample (Inherently fluorescent tissue (e.g., fat body surrounding larval gonads or tracheae in adult tissues))	Remove debris before mounting When imaging, exclude highly fluorescent back-ground from field of view