Improved Methods for Single-Molecule Fluorescence *In Situ* Hybridization and Immunofluorescence in *Caenorhabditis elegans* Embryos

Dylan M. Parker,[1] Lindsay P. Winkenbach,[1] Annemarie Parker,[1] Sam Boyson,[1] and Erin Osborne Nishimura[1,2]

[1]Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado
[2]Corresponding author: *erin.nishimura@colostate.edu*

Visualization of gene products in *Caenorhabditis elegans* has provided insights into the molecular and biological functions of many novel genes in their native contexts. Single-molecule fluorescence *in situ* hybridization (smFISH) and immunofluorescence (IF) enable the visualization of the abundance and localization of mRNAs and proteins, respectively, allowing researchers to ultimately elucidate the localization, dynamics, and functions of the corresponding genes. Whereas both smFISH and immunofluorescence have been foundational techniques in molecular biology, each protocol poses challenges for use in the *C. elegans* embryo. smFISH protocols suffer from high initial costs and can photobleach rapidly, and immunofluorescence requires technically challenging permeabilization steps and slide preparation. Most importantly, published smFISH and IF protocols have predominantly been mutually exclusive, preventing the exploration of relationships between an mRNA and a relevant protein in the same sample. Here, we describe protocols to perform immunofluorescence and smFISH in *C. elegans* embryos either in sequence or simultaneously. We also outline the steps to perform smFISH or immunofluorescence alone, including several improvements and optimizations to existing approaches. These protocols feature improved fixation and permeabilization steps to preserve cellular morphology while maintaining probe and antibody accessibility in the embryo, a streamlined, in-tube approach for antibody staining that negates freeze-cracking, a validated method to perform the cost-reducing single molecule inexpensive FISH (smiFISH) adaptation, slide preparation using empirically determined optimal antifade products, and straightforward quantification and data analysis methods. Finally, we discuss tricks and tips to help the reader optimize and troubleshoot individual steps in each protocol. Together, these protocols simplify existing workflows for single-molecule RNA and protein detection. Moreover, simultaneous, high-resolution imaging of proteins and RNAs of interest will permit analysis, quantification, and comparison of protein and RNA distributions, furthering our understanding of the relationship between RNAs and their protein products or cellular markers in early development. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Sequential immunofluorescence and single-molecule fluorescence *in situ* hybridization
Alternate Protocol: Abbreviated protocol for simultaneous immunofluorescence and single-molecule fluorescence *in situ* hybridization
Basic Protocol 2: Simplified immunofluorescence in *C. elegans* embryos
Basic Protocol 3: Single-molecule fluorescence *in situ* hybridization or single-molecule inexpensive fluorescence *in situ* hybridization

Keywords: *Caenorhabditis elegans* • immunofluorescence • smFISH • smiFISH

CURRENT PROTOCOLS
A Wiley Brand

Current Protocols e299, Volume 1
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.299

How to cite this article:
Parker, D. M., Winkenbach, L. P., Parker, A., Boyson, S., & Nishimura, E. O. (2021). Improved methods for single-molecule fluorescence *in situ* hybridization and immunofluorescence in *Caenorhabditis elegans* embryos. *Current Protocols*, *1*, e299. doi: 10.1002/cpz1.299

INTRODUCTION

The study of spatial and temporal expression patterns can provide fundamental insights into gene function and importance. By querying the abundance and spatial patterning of mRNAs and their protein products in whole animals, it is possible to gain an understanding of their transcription, translation, mRNA stability, protein dynamics, developmental regulation, and their functional roles (Levesque & Raj, 2013; Parker et al., 2020; Perez-Burgos et al., 2003; Toki, Cecchi, Hembrough, Syrigos, & Rimm, 2017; Trcek, Rahman, & Zenklusen, 2017). Visualizing RNAs and proteins in the same intact animal requires methods that are sensitive, minimally disruptive, and most importantly, compatible with one another. Typically, researchers label target RNAs and proteins *in situ*, using single-molecule fluorescence *in situ* hybridization (smFISH) and immunofluorescence (IF), respectively. In smFISH, hybridization of fluorescent antisense probes labels RNAs, and in IF, fluorescent antibodies stain proteins (Femino, Fay, Fogarty, & Singer, 1998; Orjalo, Johansson, & Ruth, 2011; Raj, van den Bogaard, Rifkin, van Oudenaarden, & Tyagi, 2008). Thus, researchers can study the relationships between RNA and proteins by performing smFISH and IF protocols on the same sample.

Standard smFISH and IF protocols, however, do have challenges. For instance, low signal plagues standard smFISH protocols, due to the poor photostability of some fluorophores. High background exacerbates this low signal as a result of the relatively thick *z*-dimension of *Caenorhabditis elegans* embryos (Ji & Oudenaarden, 2012). These issues ultimately lead to low signal-to-noise ratios (SNR) and difficulty in detecting RNA spots. Further, the probes used in the standard smFISH protocol are costly, preventing many research groups from adopting this technique (Tsanov et al., 2016). Meanwhile, standard IF protocols pose problems in *C. elegans* embryos due to the embryo's strong eggshell and robust permeability barrier (Duerr, 2006; Olson, Greenan, Desai, Müller-Reichert, & Oegema, 2012). Ultimately, this has resulted in the use of harsh fixatives (aldehydes, picric acid), reducing reagents (β-mercaptoethanol, DTT), and enzymatic treatments (collagenase), which are capable of damaging nucleic acids (Bonin, Petrera, Rosai, & Stanta, 2005; Duerr, 2013; Hoetelmans et al., 2001; Masuda, Ohnishi, Kawamoto, Monden, & Okubo, 1999; Tyrrell, Elias, & Longley, 1995; Williams et al., 1999), and require technically demanding slide preparations for freeze-crack permeabilization (Duerr, 2006; Duerr, 2013). Perhaps most importantly, most smFISH and IF protocols are mutually exclusive in the *C. elegans* embryo due to incompatibilities in the reagents and protocols used.

By optimizing, simplifying, and combining smFISH and IF protocols, we have remedied several of the issues in existing protocols. In Basic Protocol 1, we present a one-tube protocol to perform IF and smFISH sequentially on the same sample. In this protocol, embryos are first harvested, fixed, and permeabilized using mild conditions, thus mitigating issues of nucleic acid damage and challenging slide preparations. IF is then performed on fixed embryos using reagents that protect the integrity of RNA in the sample, allowing smFISH to be performed subsequently. Finally, slides are prepared using antifade solutions that protect the smFISH signal from photobleaching and reduce the background signal to raise the SNR of RNA spots. In the Alternate Protocol, an abbreviated protocol

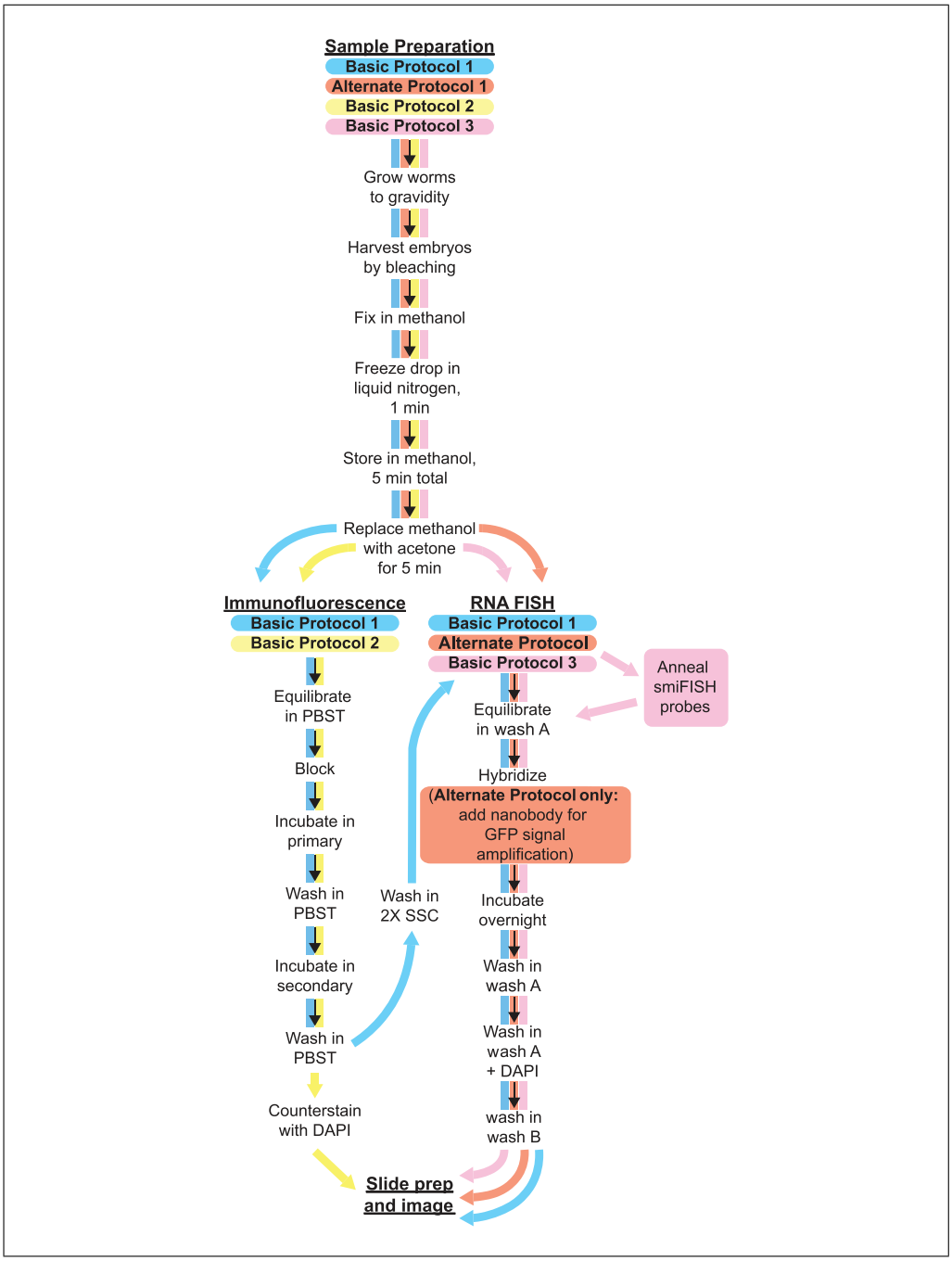

Figure 1 Schematic illustration of the IF, FISH, and IF/FISH protocols described in this article. An overview illustrating the workflow of the sequential IF/FISH (Basic Protocol 1), simultaneous IF/FISH (Alternate Protocol), simplified IF (Basic Protocol 2), and smFISH or smiFISH (Basic Protocol 3), from sample preparation to slide preparation. IF, immunofluorescence; FISH, fluorescence *in situ* hybridization; smFISH, single-molecule FISH; smiFISH, single molecule inexpensive FISH.

for performing IF and smFISH is described, where embryos are harvested, fixed, and a standard smFISH protocol is performed with the inclusion of nanobodies to stain protein. For those interested in performing the techniques individually, we provide two protocols. Basic Protocol 2 describes a simplified immunofluorescence protocol and Basic Protocol 3 describes the steps to perform smFISH, including steps to perform the low-cost adaptation, single-molecule inexpensive fluorescence *in situ* hybridization (smiFISH). An overview of the entire workflow is shown in Figure 1.

It is worth noting that while we will discuss some data analysis tools and best practices, we will not describe here how to perform data analysis in detail.

BASIC PROTOCOL 1

SEQUENTIAL IMMUNOFLUORESCENCE AND SINGLE-MOLECULE FLUORESCENCE *IN SITU* HYBRIDIZATION

This protocol describes methods for isolating *C. elegans* embryos and fixing them in a manner compatible with both immunofluorescence and RNA FISH. Steps for performing immunofluorescence subsequently followed by smFISH are then outlined. Finally, slide preparation is described. This approach can be used for simultaneous visualization of RNA transcripts and a protein of interest, provided the fluorescence *in situ* hybridization (FISH) probes and fluorescent antibody are selected in distinct channels.

CAUTION: To avoid RNA degradation, use RNA-appropriate sterile technique and RNAse-free reagents where possible.

CAUTION: Many reagents in this protocol are hazardous. Follow all appropriate guidelines and regulations for the use and disposal of each reagent.

Materials

C. elegans strain or genotype of interest (Caenorhabditis Genetics Center, CGC)
Nematode Growth Medium Plates (see recipe)
OP50 *Escherichia coli* (Caenorhabditis Genetics Center, cat. no. OP50)
100% Reagent Grade Acetone (Thermo Fisher Scientific, cat. no. A18-500), cooled to -20°C
100% Reagent Grade Methanol (Thermo Fisher Scientific, cat. no. A412-500), cooled to -20°C
Bleaching Solution (see recipe)
M9 buffer (see recipe)
10× Tween 20 in PBS (PBST; see recipe)
BSA (MilliporeSigma, cat. no. A9418-5G)
Primary Antibody or Fluorescently Labeled Nanobody/ScFv, e.g., K76, anti-PGL-1 (DHSB, cat. no. K76, RRID:AB_531836)
Fluorescent Secondary Antibody, e.g., Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch, cat. no. 115-545-03, RRID: AB_2338840)
DAPI (Invitrogen brand, Thermo Fisher Scientific, cat. no. D1306)
RNasin® Ribonuclease Inhibitor (Promega, cat. no. N2111)
2× SSC (see recipe)
Wash Buffer A (see recipe)
Wash Buffer B (see recipe)
Hybridization Buffer (see recipe)
Mounting Medium (see recipe)
smFISH probes, 25 µM stocks in 10 mM Tris, pH 8.0, e.g., *nos-2* probe set (Biosearch Technologies, Custom Probes made using Stellaris Probe Designer)
RNAse-free Water (Invitrogen brand, Thermo Fisher Scientific, cat. no. AM9922)
VECTASHIELD Mounting Medium (Vector Laboratories, cat. no. H-1000-10)
Liquid Nitrogen

150-mm Petri Dishes (Genesee Scientific, cat. no. 32-106 or equivalent; other sizes are acceptable)
Fume Hood (Hamilton Safeaire II, cat. no. 54L2769 or equivalent)
Stereomicroscope (Nikon, cat. no. SMZ800N or equivalent)
10-ml Serological Pipets (Genesee Scientific, cat. no. 12-104 or equivalent)
25-ml Serological Pipets (Genesee Scientific, cat. no. 12-106 or equivalent)

Pipet Controller (Drummond, cat. no. 4-000-101 or equivalent)
2,000-ml Erlenmeyer Flask (MilliporeSigma, cat. no. CLS51002L or equivalent)
1,000-ml Glass Bottles (VWR, cat. no. 10754-820 or equivalent)
250-ml Glass Bottles (VWR, cat. no. 89000-236 or equivalent)
15-ml Centrifuge Tubes (Olympus Plastics, cat. no. 28-103 or equivalent)
1.5-ml Microcentrifuge Tubes (Olympus Plastics, cat. no. 14-125 or equivalent)
1.5-ml Amber Microcentrifuge Tubes (Olympus Plastics, cat. no. 22-282AM or equivalent)
Nutating Mixer (Labnet, cat. no. S0500 or equivalent)
Centrifuge (Eppendorf, cat. no. 5810 or equivalent)
Microcentrifuge (Eppendorf, cat. no. 5424 or equivalent)
Mini Centrifuge (Genesee, cat. no. 27-523 or equivalent)
Vortex Mixer (VWR, cat. no. 12620-838 or equivalent)
Liquid Nitrogen Container (Thermo Fisher Scientific, cat. no. 2122 or equivalent)
Thermal Cycler (Bio-Rad, cat. no. T100 or equivalent)
ThermoMixer (Eppendorf, cat. no. 05-412-503)
8-mm, #1$\frac{1}{2}$ thickness, round cover glass (Electron Microscopy Sciences, cat. no. 72296-08)
Glass microscope slides (VWR, cat. no. 48312-401)
No. 1$\frac{1}{2}$ thickness, 22- × 22-mm cover glass (VWR, cat. no. 48366-227)
Grace Bio-Lab Press-To-Seal silicon isolator (MilliporeSigma, cat. no. GBL664504-25ea)
Widefield Fluorescent Microscope (DeltaVision Elite or equivalent)
-20°C freezer
4°C refrigerator
15°C to 25°C incubator
10-, 200-, and 1,000-µl micropipets and tips

Embryo preparation and fixation

1. Grow worms to gravidity on OP50-seeded NGM plates. Synchronize by bleaching if necessary.

   Typically, one or two 150-mm NGM plates seeded with ∼2 ml of OP50, chunked with the strain of interest and grown until worms are gravid, is sufficient. Other bacterial stocks, such as inducible RNA interference (RNAi) vector-containing E. coli, can be used if desired.

2. Harvest gravid worms by washing them off plates using M9 and collecting them in a 15-ml conical tube in an ∼15 ml total volume.

   Aggressive pipetting will increase yield by releasing more worms from the plates. Be sure not to pierce the plate's surface, as agar carried into the sample will persist.

3. Spin conical tube at 2,000 × *g* for 1 min at room temperature to pellet gravid worms. Alternatively, allow gravid worms to settle over time.

4. Remove supernatant using a pipet or aspirator, being careful not to disturb the worm pellet.

5. Resuspend worm pellet in 15 ml M9.

6. Repeat step 3.

7. Repeat steps 4-6 until the supernatant is clear, discarding supernatant after the final wash.

8. Add ∼15 ml bleaching solution to the worms and nutate or hand-shake for 6-8 min until embryos are released from the mothers.

Check on the condition of the worms periodically throughout bleaching. The gravid adults should break into about two pieces before continuing. If worms are bleached for too long, some early-stage embryos may be damaged. For tips on harvesting embryos, see Porta-de-la-Riva, Fontrodona, Villanueva, and Cerón, 2012.

9. Centrifuge conical tube at 2,000 × *g* for 1 min at room temperature to pellet. Immediately remove supernatant and quench bleaching with 15 ml M9.

   At this point, embryos typically stick to the tube and the supernatant can be carefully decanted to decrease the time before quenching.

10. After adding M9, vortex pellet to release remaining worm fragments. Centrifuge at 2,000 × *g* for 1 min at room temperature.

11. Discard M9 and wash with 15 ml M9 two more times (for a total of three washes), vortexing pellet after the addition of M9 each time.

    The aroma of bleach should be completely gone by the end of washing.

12. Transfer remaining embryos to a 1.5-ml microcentrifuge tube and pellet in a tabletop microcentrifuge for 30 s at 2,000 × *g* at room temperature. Turn tube 180° and repeat until a pellet has formed. Typically, between two and four spins is sufficient. Remove any remaining M9.

    C. elegans embryos will stick to the side of microcentrifuge tubes during centrifugation, leading to a smear of embryos and poor pelleting if tubes are not rotated and spun repeatedly.

13. Add 1 ml pure methanol cooled to -20°C, vortex to break up the pellet, and immediately submerge in liquid nitrogen for 1 min to crack the eggshell and promote permeabilization.

14. Remove tube from the liquid nitrogen and immediately begin pelleting at 2,000 × *g* in 30 s intervals at room temperature, rotating tube 180° between each spin.

    The sample will still be partially frozen for the first spins but it is best to get the sample pelleting early to prevent over fixation.

15. Repeat step 14 until the embryos are pelleted or until the embryos have been in methanol for a total of 5 min.

16. Once the sample has been in methanol for a total of 5 min, including the time spent pelleting, remove methanol and replace it with 1 ml pure acetone cooled to -20°C. Store sample at −20°C for ∼3 min.

17. Pellet embryos by centrifugation as in step 14 until a pellet forms or the embryos have been in acetone for a total of 5 min.

18. After embryos have been fixed in acetone for a total of 5 min, remove acetone and immediately continue to immunofluorescence.

Immunofluorescence

19. Add 1 ml 1× PBST to the sample from step 18 and nutate for 5 min to wash.

20. Pellet embryos by centrifuging at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until a pellet forms.

21. Pipet out or aspirate as much of the supernatant PBST as possible without disrupting the pellet.

22. Repeat steps 19-21 two more times (three washes total).

23. Add 250 µl 1× PBST containing 1% (w/v) BSA. Incubate 30 min at 37°C with nutation, to block.

    IMPORTANT: If RNA FISH will be performed subsequently, it is essential to add 1 unit/µl RNasin® (Promega) during steps where BSA is included, to prevent RNA degradation. RNAse-free BSA can be used if issues with RNA degradation occur with sequential IF/smFISH protocols; however, it is much more expensive.

24. Centrifuge embryos at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.

25. Pipet or aspirate as much of the supernatant as possible without disrupting the pellet.

26. Add a maximum of 100 µl primary antibody diluted in 1× PBST with 1% (w/v) BSA (and 1 unit/µl RNasin® if FISH will be performed subsequently). Nutate at room temperature for at least 1 hr or overnight at 4°C.

    Example: For the data shown in Figure 2, we used 40 µl of a 1:20 dilution of K76 antibody and 100 µl of a 1:1,000 dilution of 2A4 antibody.

    Overnight incubations provide better IF signals but can increase RNA degradation. Optimal antibody concentrations must be determined for each antibody. The volume of primary antibody solution added should be minimized to reduce the consumption of antibodies, where possible.

27. Add 1 ml 1× PBST directly to the sample and nutate 5 min to wash out free antibody.

28. Centrifuge embryos at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until a pellet forms.

29. Pipet out or aspirate as much of the supernatant PBST as possible without disrupting the pellet.

30. Repeat steps 27-29 two more times (three washes total).

31. Add a maximum of 250 µl fluorescently labeled secondary antibody diluted in 1× PBST and incubate for at least 1 hr in the dark at room temperature with nutation.

    Example: For the images in Figure 2, we used 125 µl of a 1:250 dilution of Alexa Fluor 488 goat anti-mouse secondary antibody.

    Optimal antibody concentrations must be determined for each antibody. The volume of secondary antibody solution added should be minimized to reduce the consumption of antibodies, where possible.

32. Add 1 ml 1× PBST and nutate 5 min to wash out excess antibody.

33. Centrifuge embryos at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.

34. Pipet out or aspirate as much of the supernatant PBST as possible without disrupting the pellet.

35. Repeat steps 32-34.

36. Add 1 ml 2× SSC and nutate 5 min.

    2× SSC facilitates smFISH probe hybridization. Washing in 2× SSC is used to remove PBST and equilibrate embryos in an smFISH-compatible solution.

37. Centrifuge embryos at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.

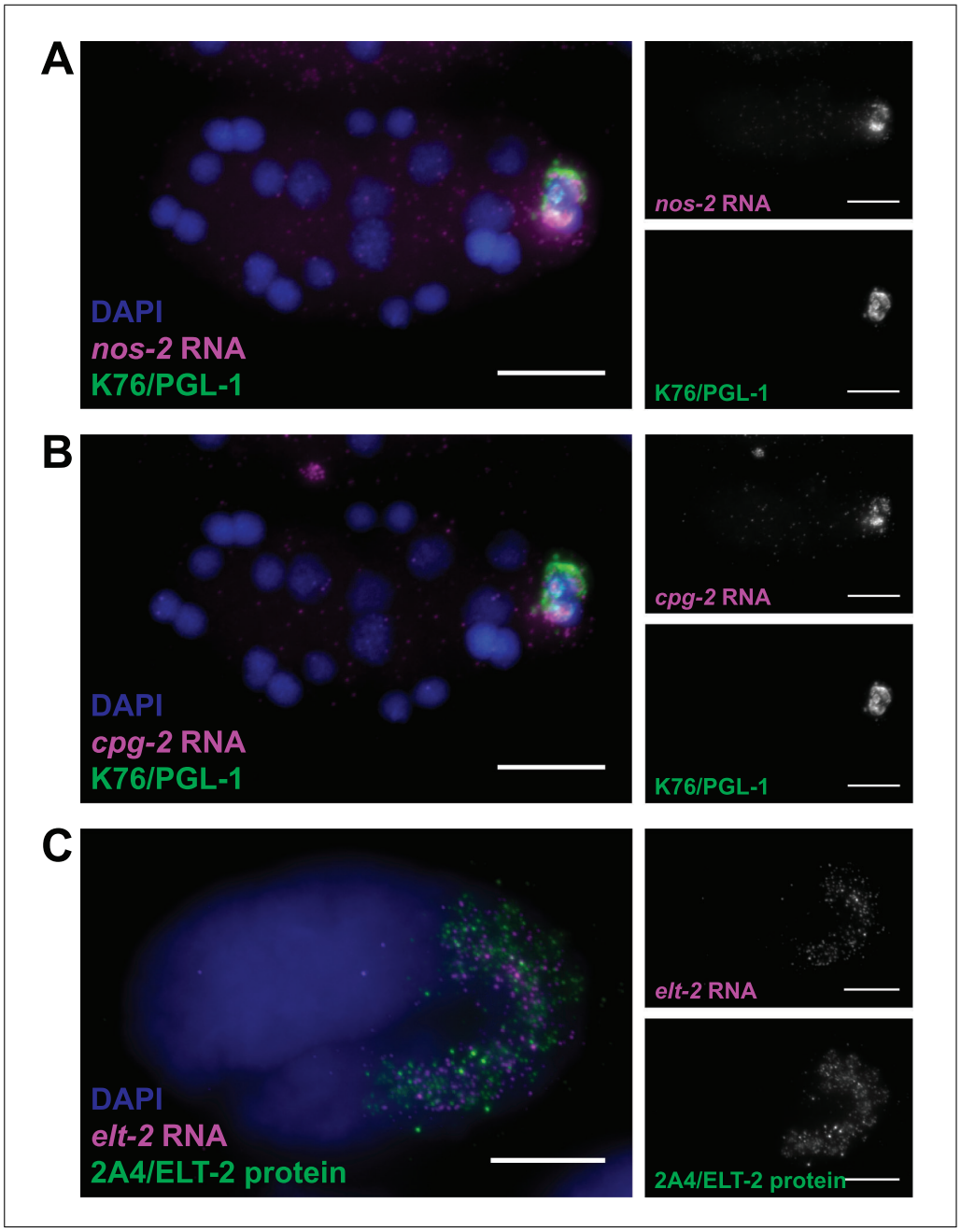

Figure 2 Sequential IF/FISH. Immunofluorescence followed by smFISH was performed on N2 embryos. IF was performed using K76 (**A** and **B**) or 2A4 (**C**) primary antibodies to identify PGL-1-containing P granules and ELT-2 protein (magenta), respectively. smFISH was used to simultaneously detect the P granule constituent RNAs *nos-2* (**A**) and *cpg-2* (**B**), or *elt-2* mRNA (**C**), all in magenta. Embryos were counterstained with DAPI (blue). Three biological replicates were performed for each experiment. Scale bars represent 10 μm. IF, immunofluorescence; FISH, fluorescence *in situ* hybridization; smFISH, single-molecule fluorescence *in situ* hybridization.

38. Pipet out or aspirate as much of the supernatant 2× SSC as possible without disrupting the pellet.
39. Repeat steps 36-38.
40. Continue to smFISH.

41. Prepare fresh buffers by adding 10% formamide to stocks of wash buffer A and hybridization buffer. For each sample, prepare separate tubes with 3 ml wash buffer A and 110 µl hybridization buffer, and add formamide to each to a final concentration of 10%.

    Wash buffer A and hybridization buffer should always have formamide added immediately preceding the experiment. Formamide can decompose over time, particularly at higher temperatures, leading to less stringent probe binding. It can also acidify when exposed to air resulting in fluorophore quenching. Formamide stocks should be stored frozen and their pH monitored periodically (pH 7-8 is ideal).

42. Prepare a 1:20 dilution of the 25 µM smFISH probes in RNAse-free water (i.e., 1.25 µM final concentration). Add 2 µl of the 1.25 µM dilution to 110 µl hybridization buffer (with formamide). If performing experiments using multiple probe sets with different fluorophores, add 2 µl of each diluted probe set. Mix well; hybridization buffer is viscous.

    Example: To obtain the data in Figure 2, we used 2 µl of 1.25 µM nos-2 (Quasar 670), cpg-2 (Cal Fluor 610), and elt-2 (Cal Fluor 610) probe dilutions.

    Although 2 µl of 1.25 µM probe solution has worked well for most of the probe sets we have used, it is helpful to perform a titration over ∼1 order of magnitude of concentrations (∼0.25-2.5 µM) to identify optimal probe concentrations on an individual-probe-set basis.

43. Centrifuge embryos at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.

44. Pipet out or aspirate as much supernatant as possible without disturbing the pellet.

45. Add 1 ml wash buffer A (with formamide) to prehybridize sample and incubate at room temperature for ∼5 min.

    Nutation is optional during this wash step.

46. Centrifuge embryos at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.

47. Pipet out or aspirate as much supernatant as possible without disturbing the pellet.

48. Add 100 µl hybridization buffer with probes (from step 42) to the pelleted embryos and hybridize at 37°C in the dark for a minimum of 8 hr, shaking at 450 rpm to ensure even probe penetration.

    The standard Stellaris RNA FISH protocol suggests hybridization should occur for anywhere between 4-16 hr. We have found that hybridizations shorter than 8 hr lead to variable smFISH signals. Additionally, we have had successful experiments using hybridizations as long as 48 hr. It is worth noting that longer hybridizations can increase the risk of RNA degradation if the sample was contaminated with RNase during the immunofluorescence steps. Store prepared wash buffer A at room temperature or 37°C during this incubation. Warm buffer will increase the stringency of probe binding and decrease background and non-specific binding. If available, use a thermomixer to shake the hybridization solution, although standard incubators are also acceptable.

49. Add 1 ml warm wash buffer A (with formamide) directly to the embryos in the hybridization solution.

50. Incubate at 37°C in the dark for 30 min, shaking at 450 rpm in a thermomixer or incubator.

51. Centrifuge embryos at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.

52. Pipet out or aspirate as much supernatant as possible without disturbing the pellet.

53. Prepare 1 ml wash buffer A (with formamide) containing 1 ng/µl DAPI for each sample to be imaged. Add 1 ml of the mix to the sample.

    If 3 ml of wash buffer A were prepared, as described in step 41, this should be the remaining volume of wash buffer A. No additional buffer should need to be prepared.

54. Incubate at 37°C in the dark for 30 min, shaking at 450 rpm in a thermomixer or incubator.

55. Centrifuge embryos at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.

56. Pipet out or aspirate as much supernatant as possible without disturbing the pellet.

57. Add 1 ml wash buffer B and incubate for ∼5 min.

58. Repeat step 55 and 56.

59. Resuspend in 50 µl mounting medium and incubate at 4°C for 30 min to ensure antifade penetrance.

    Less mounting medium can be added if the embryo yield is low. The ideal volume of mounting medium to add is based on slide preparation. When a second cover glass is added to the sample in step 63, 2-6 µl of embryos in mounting medium mixed 1:1 with VECTASHIELD should be dense enough that they will be easy to find on a microscope but sparse enough that they do not clump together.

60. Move to slide preparation.

Slide preparation

61. Working at a dissecting microscope, drop 2-6 µl of embryos suspended in mounting medium (from step 59) onto a single 8-mm, 1.5 thickness round cover glass resting on a glass slide.

    The volume of embryos in mounting medium to add can be tuned to optimize slide preparation. Add enough so that the embryos will be abundant but not clumped together after adding an equal volume of VECTASHIELD in step 62. Do not add more than 6 µl, as most of the sample will be squeezed out when the second cover glass is added

    Always wear gloves when handling slides and cover glass to prevent smudging and contamination.

62. Add an equal volume of VECTASHIELD antifade to the embryos from step 61 in mounting medium on the cover glass and pipet up and down to mix thoroughly. Try to keep the final volume on the cover glass to ∼4-6 µl by removing some of the mixture.

    This is a good time to break up any large clumps of embryos by pipetting.

63. Place a 1.5 thickness, 22- × 22-mm square cover glass on top, trying to avoid bubbles.

    Do not let the cover glass touch the slide. The sample solution will pour over the edge of the round cover glass and seal it to the slide beneath through surface tension. Having the round cover glass close to the edge of the slide can provide some extra working height. Additionally, gently lowering the square cover glass from front to back over the round cover glass until surface tension pulls the round cover glass up will help prevent spillover.

64. Flip the cover glass sandwich so the square cover glass is on the bottom. Remove as much liquid as possible from between the two cover glasses using a torn Kimwipe placed against the round one.

    The aim is to flatten the embryos as much as possible without damaging them. Samples can be firmly pressed on with a pipet tip as long as the cover glass does not slide from side to side. The ideal depth of an embryo on the slide is ∼12-20 µm. The signal-to-noise ratio will decrease and photobleaching will increase with increasing thickness due to out-of-focus light and more image acquisitions, respectively.

65. Affix cover glass sandwich to a microscope slide using a Grace Bio-Lab press-to-seal silicon isolator such that the embryos will be imaged through the square cover glass.

66. Head off to the microscope.

    Acquisition settings will vary depending on the molecules being imaged and the microscope setup. Using our GE DeltaVision Elite microscope with an Olympus PLAN APO 60×, 1.42 NA objective, an Insight SSI Solid State Light Engine, and the standard DeltaVision DAPI, FITC, mCherry, Cy5 polychroic filter set, our image acquisition settings are as follows for the sample data shown in Figure 2A and 2B: Cy5 (imaging nos-2 RNA labeled with Quasar 670), % transmittance = 100%, exposure = 0.8 s; mCherry (imaging cpg-2 RNA labeled with Cal Fluor 610), % transmittance = 100%, exposure = 1.0 s; FITC (imaging PGL-1 protein stained with K76 primary antibody and Alexa Fluor 488 secondary antibody), % transmittance = 10%, exposure = 0.025 s; DAPI (imaging DNA), % transmittance = 2%, exposure = 0.025 s. We always image through the entire z-stack at the longest wavelength before moving to the next longest wavelength, to prevent photobleaching of the more labile fluorophores at the red end of the spectrum. We use 0.2 µm z-spacing between images. Typically, imaging ten embryos at a particular developmental stage over three biological replicates is sufficient to detect statistically significant changes in RNA and protein abundances or distributions. It is often worthwhile, however, to perform a power analysis using the measurements from a pilot experiment to determine requisite sample sizes empirically before performing true experimental replicates. It is essential to tightly bin embryos of different cell stages or use equal numbers of embryos from each stage for all conditions where statistical analysis will be performed. The RNA and protein content of embryos changes rapidly and can lead to skewed statistics if stages are not carefully monitored. For example, the abundance of the cpg-2 RNA changes from an average of ∼12,500 molecules at the two-cell stage to ∼5,000 molecules at the four-cell stage (Parker et al., 2020).

ABBREVIATED PROTOCOL FOR SIMULTANEOUS IMMUNOFLUORESCENCE AND SINGLE-MOLECULE FLUORESCENCE *IN SITU* HYBRIDIZATION

ALTERNATE PROTOCOL

In some instances, an abbreviated protocol allows the user to perform IF and smFISH simultaneously, negating the two-step approach described in Basic Protocol 1. This is the case, for instance, when using the anti-GFP nanobody (Chromotek, cat. no. gt-250). Though not all antibodies work with this streamlined approach, we have had success with high-affinity antibody derivatives such as nanobodies, single-chain variable fragments, and fragmented antibodies (Doshi et al., 2014). This Alternate Protocol has multiple advantages over Basic Protocol 1, namely, it is faster, requires fewer reagents, and avoids some reagents utilized in IF that contain RNases. In the Alternate Protocol, IF and smFISH are performed simultaneously using an adapted smFISH protocol. Embryos are first harvested and fixed. An adapted smFISH protocol is then performed, including high-affinity antibody derivatives (e.g., nanobodies, single-chain variable fragments, or fragmented antibodies; Doshi et al., 2014) in the RNA FISH buffers. This adapted smFISH protocol circumvents the need to perform a separate immunofluorescence protocol and reduces the time from sample preparation to imaging from 2 to 3 days to roughly 16 hr. Samples are then prepared on slides and are ready for imaging.

In our hands, small, high-affinity antibody derivatives, such as nanobodies, have improved the success of this simplified protocol. Moreover, we have only had success with primary staining using this protocol. Immunofluorescence using secondary antibody amplification during wash steps has not succeeded in our hands; however, a recent study suggests that some primary and secondary antibody combinations are likely compatible with this protocol (Tocchini, Rohner, Stetina, & Mango, 2021). It is essential to verify that antibody staining is effective, by comparing protein localization to known distributions or using Basic Protocol 2. If a desired antibody or antibody pair does not work with the Alternate Protocol, it is more likely to succeed using Basic Protocol 1. Regardless, it is worth trying Alternate Protocol when testing new antibodies due to the protocol's comparative simplicity.

Additional Materials *(also see Basic Protocol 1)*

C. elegans strain or genotype of interest (Caenorhabditis Genetics Center, CGC)
Nematode Growth Medium Plates (see recipe)
OP50 *E. coli* (Caenorhabditis Genetics Center, cat. no. OP50)
100% Reagent Grade Acetone (Thermo Fisher Scientific, cat. no. A18-500), cooled to -20°C
100% Reagent Grade Methanol (Thermo Fisher Scientific, cat. no. A412-500), cooled to -20°C
Bleaching Solution (see recipe)
M9 buffer (see recipe)
Fluorescently Labeled Nanobody/ScFv, e.g., anti-GFP nanobody (Chromotek, cat. no. GT-250)
DAPI (Invitrogen brand, Thermo Fisher Scientific, cat. no. D1306)
Wash Buffer A (see recipe)
Wash Buffer B (see recipe)
Hybridization Buffer (see recipe)
Mounting Medium (see recipe)
smFISH probes, 25 µM stocks in 10 mM Tris, pH 8.0, e.g., *nos-2* probe set (Biosearch Technologies, Custom Probes made using Stellaris Probe Designer)
RNAse-free water (Invitrogen brand, Thermo Fisher Scientific, cat. no. AM9922)
VECTASHIELD Mounting Medium (Vector Laboratories, cat. no. H-1000-10)
Liquid Nitrogen

1. Perform embryo preparation and fixation as in Basic Protocol 1, steps 1-18.

   Do not perform any immunofluorescence steps. Move directly from embryo preparation and fixation to the smFISH protocol.

2. Perform smFISH as in Basic Protocol 1, steps 41-60, with the following exceptions:

   a. At step 42, add the appropriate concentration of primary antibody or antibody derivative to the hybridization buffer.

      Example: To generate the data in Figure 3, we used a 2.37 µg/ml final concentration of anti-GFP nanobody (conjugated to Janelia Fluor 549) and 2 µl of a 1.25 µM dilution of nos-2 (Quasar 670) smFISH probes.

   b. In step 50, increase the length of the second wash A incubation from 30 min to 1 hr.

      If attempting a primary- and secondary-based antibody pair for this protocol, add the appropriate concentration of secondary antibody during the second wash A incubation, at step 49.

   c. Perform imaging and analysis as in Basic Protocol 1, steps 61-66.

      See sample data in Figure 3.

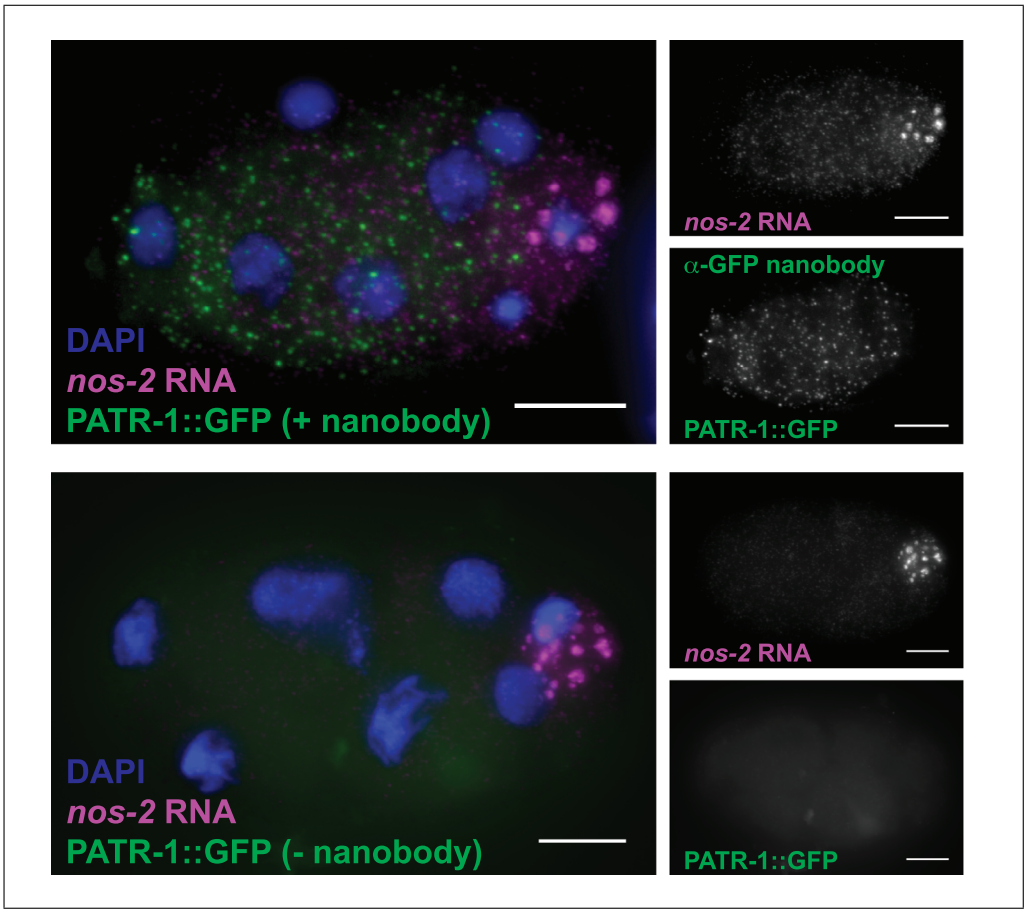

Figure 3 Simultaneous IF/FISH. smFISH was performed on N2 embryos with the addition of anti-GFP nanobody to hybridization buffer. *nos-2* mRNA (magenta) was probed using smFISH probes conjugated to Quasar 670. PATR-1::GFP (green) signal was visualized using 2.37 μg/ml Janelia Fluor 549 (Tocris 6147) conjugated anti-GFP nanobody (Chromotek, gt-250), top. A no nanobody control is also shown (bottom). DNA was counterstained with DAPI (blue). Three biological replicates were performed for each experiment. Scale bars represent 10 μm. IF, immunofluorescence; FISH, fluorescence *in situ* hybridization; smFISH, single-molecule FISH; GFP, green fluorescent protein.

SIMPLIFIED IMMUNOFLUORESCENCE IN *C. ELEGANS* EMBRYOS

BASIC PROTOCOL 2

This protocol presents a simplified protocol for performing IF alone in *C. elegans* embryos. It can be desirable to image protein distributions without visualizing RNA in the same sample to simplify the procedures. Here, embryos will be harvested before being concurrently fixed and permeabilized using a combination of methanol and acetone. IF is then performed to stain a protein of interest. Finally, due to the optimized fixation steps, a simple slide preparation protocol can be used in place of the traditional freeze-cracking method. Ultimately, the user will then visualize the protein of interest on a fluorescent microscope.

Materials

C. elegans strain or genotype of interest (Caenorhabditis Genetics Center, CGC)
Nematode Growth Medium Plates (see recipe)
OP50 *E. coli* (Caenorhabditis Genetics Center, cat. no. OP50)
100% Reagent Grade Acetone (Thermo Fisher Scientific, cat. no. A18-500)
100% Reagent Grade Methanol (Thermo Fisher Scientific, cat. no. A412-500)
Bleaching Solution (see recipe)
M9 buffer (see recipe)
10× PBST (see recipe)

BSA (MilliporeSigma, cat. no. A9418-5G)
Primary Antibody or Fluorescently Labeled Nanobody/ScFv, e.g., K76, anti-PGL-1 (DHSB, cat. no. K76, RRID:AB_531836)
Fluorescent Secondary Antibody, e.g., Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch Laboratories, cat. no. 115-545-003, RRID:AB_2338840)
DAPI (Invitrogen brand, Thermo Fisher Scientific, cat. no. D1306)
RNasin® Ribonuclease Inhibitor (Promega, cat. no. N2111)
Mounting Medium (see recipe)
VECTASHIELD Mounting Medium (Vector Laboratories, cat. no. H-1000-10)
Liquid Nitrogen

150-mm Petri Dishes (Genesee Scientific, cat. no. 32-106 or equivalent; other sizes are acceptable)
Fume Hood (Hamilton Safeaire II, cat. no. 54L2769 or equivalent)
Stereomicroscope (Nikon, cat. no. SMZ800N or equivalent)
10-ml Serological Pipets (Genesee Scientific, cat. no. 12-104 or equivalent)
25-ml Serological Pipets (Genesee Scientific, cat. no. 12-106 or equivalent)
Pipet Controller (Drummond, cat. no. 4-000-101 or equivalent)
1,000-ml Glass Bottles (VWR, cat. no. 10754-820 or equivalent)
250-ml Glass Bottles (VWR, cat. no. 89000-236 or equivalent)
15-ml Centrifuge Tubes (Olympus Plastics, cat. no. 28-103 or equivalent)
1.5-ml microcentrifuge tubes (Olympus Plastics, cat. no. 14-125 or equivalent)
1.5-ml amber microcentrifuge tubes (Olympus Plastics, cat. no. 22-282AM or equivalent)
Nutating Mixer (Labnet, cat. no. S0500 or equivalent)
Centrifuge (Eppendorf, cat. no. 5810 or equivalent)
Microcentrifuge (Eppendorf, cat. no. 5424 or equivalent)
Mini Centrifuge (Genesee, cat. no. 27-523 or equivalent)
Vortex Mixer (VWR, cat. no. 12620-838 or equivalent)
Liquid Nitrogen Container (Thermo Fisher Scientific, cat. no. 2122 or equivalent)
ThermoMixer (Eppendorf, cat. no. 05-412-503)
8-mm, #1$\frac{1}{2}$ thickness round cover glass (Electron Microscopy Sciences, cat. no. 72296-08)
Glass microscope slides (VWR, cat. no. 48312-401)
No. 1$\frac{1}{2}$ thickness, 22- × 22-mm cover glass (VWR, cat. no. 48366-227)
Grace Bio-Lab Press-To-Seal silicon isolator (MilliporeSigma, cat. no. GBL664504-25ea)
Widefield Epifluorescent Microscope (DeltaVision Elite or equivalent)
-20°C freezer
4°C refrigerator
10-, 200-, and 1,000-µl micropipets and tips

1. Perform embryo preparation and fixation as in Basic Protocol 1, steps 1-18.
2. Perform immunofluorescence as in Basic Protocol 1, steps 19-39, with the following exceptions and additional steps:
   a. Do not perform any smFISH protocol steps.
   b. At step 39, nutate sample in 1× PBST for 10 min (instead of 5 min).
3. Pellet embryos by centrifuging at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.
4. Pipet or aspirate as much of the supernatant PBST as possible without disrupting the pellet.

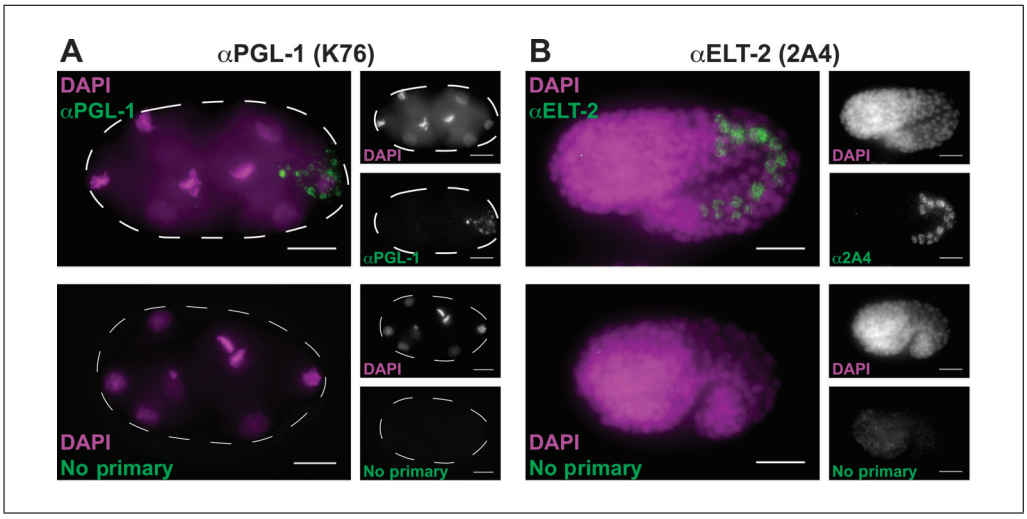

Figure 4 Simplified immunofluorescence in *C. elegans* embryos. Immunofluorescence was performed on N2 embryos as described (Basic Protocol 2). Embryos were incubated with 1:20 dilutions of K76 (DHSB, AB_531836) (**A**) or 1:1,000 dilutions 2A4 (DHSB, AB_2618114) (**B**) primary antibodies, followed by incubation with 1:250 dilutions of Alexa Fluor 488 goat anti-mouse secondary antibody (Jackson ImmunoResearch, AB_2338840, green). In the presence of K76 (anti-PGL-1), P granules are observed (A, top), while 2A4 (anti-ELT-2) stained the intestine-specific ELT-2 transcription factor (B, top). Non-specific binding of the secondary antibody was not observed in either instance (A and B, bottom). Three biological replicates were performed for each experiment. Scale bars represent 10 μm. smFISH, single-molecule fluorescence *in situ* hybridization; smiFISH, single molecule inexpensive FISH.

5. Counterstain with 1 ml 1× PBST containing 2 μl of 500 ng/ml DAPI for 10 min.
6. Pellet embryos by centrifuging at 2,000 × *g* in 30 s intervals at room temperature, rotating the tube 180° between each spin until pellet forms.
7. Pipet or aspirate as much of the supernatant PBST as possible without disrupting the pellet.
8. Add 1 ml 1× PBST directly to sample and nutate 10 min to wash out excess DAPI.
9. Repeat steps 6-8, followed by steps 6 and 7 (for two 1× PBST washes).
10. Resuspend in 50 μl mounting medium (or less if the sample is small, see note from Basic Protocol 1, step 59) and incubate at 4°C for 30 min to ensure antifade penetrance.
11. Prepare slides as in Basic Protocol 1, steps 61-66.

See sample data in Figure 4.

SINGLE-MOLECULE FLUORESCENCE *IN SITU* HYBRIDIZATION OR SINGLE-MOLECULE INEXPENSIVE FLUORESCENCE *IN SITU* HYBRIDIZATION

BASIC PROTOCOL 3

This protocol describes the workflow for performing smFISH or smiFISH in embryos. It is often desirable to visualize RNA distributions and abundance without the complication of performing immunofluorescence. Performing the smFISH protocol alone simplifies the workflow. Additionally, the fluorescence of some GFP fusion proteins persists through fixation, allowing RNA and protein to be visualized simultaneously without using an immunofluorescence protocol. Moreover, we have also validated the recently developed single-molecule inexpensive smiFISH protocol to reduce the cost of visualizing RNA in *C. elegans* embryos.

Here, users will harvest embryos before fixing and permeabilizing them as in Basic Protocol 1. Primary and secondary smiFISH probes can then be annealed to make low-cost smFISH probe analogs. Embryos are then hybridized with either smFISH or smiFISH probes and placed on slides. The user should then be able to visualize single molecules of RNA *in situ* as discrete fluorescent puncta.

Materials

C. elegans strain or genotype of interest (Caenorhabditis Genetics Center, CGC)
Nematode Growth Medium Plates (see recipe)
OP50 *E. coli* (Caenorhabditis Genetics Center, cat. no. OP50)
100% Reagent Grade Acetone (Thermo Fisher Scientific, cat. no. A18-500)
100% Reagent Grade Methanol (Thermo Fisher Scientific, cat. no. A412-500)
Bleaching Solution (see recipe)
M9 buffer (see recipe)
DAPI (Invitrogen brand, Thermo Fisher Scientific, cat. no. D1306)
Wash Buffer A (see recipe)
Wash Buffer B (see recipe)
Hybridization Buffer (see recipe)
Mounting Medium (see recipe)
Probes:
  smFISH probes, 25 µM stocks in 10 mM Tris, pH 8.0, e.g., *nos-2* probe set (Biosearch Technologies, Custom Probes made using Stellaris Probe Designer)
  Primary smiFISH probes, 100 µM stocks in 10 mM IDTE buffer, e.g., *nos-2* probe set (IDT, custom order made using the OLIGOSTAN probe designer)
  Secondary fluorescently labeled smiFISH probes, e.g., 5′ and 3′ Quasar 670 modified FLAP Y probes (Biosearch Technologies, Custom Probes ordered using the custom oligo designer)
RNAse-free water (Invitrogen brand, Thermo Fisher Scientific, cat. no. AM9922)
VECTASHIELD Mounting Medium (Vector Laboratories, cat. no. H-1000-10)
Liquid Nitrogen

New England Bio Labs Buffer 3 (or 3.1; NEB, cat. no. B7203S)
150-mm Petri Dishes (Genesee Scientific, cat. no. 32-106 or equivalent; other sizes are acceptable)
Fume Hood (Hamilton Safeaire II, cat. no. 54L2769 or equivalent)
Stereomicroscope (Nikon, cat. no. SMZ800N or equivalent.)
10-ml Serological Pipets (Genesee Scientific, cat. no. 12-104 or equivalent)
25-ml Serological Pipets (Genesee Scientific, cat. no. 12-106 or equivalent)
Pipet Controller (Drummond, cat. no. 4-000-101 or equivalent)
1,000-ml Glass Bottles (VWR, cat. no. 10754-820 or equivalent)
250-ml Glass Bottles (VWR, cat. no. 89000-236 or equivalent)
15-ml centrifuge tubes (Olympus Plastics, cat. no. 28-103 or equivalent)
1.5-ml microcentrifuge tubes (Olympus Plastics, cat. no. 14-125 or equivalent)
1.5-ml amber microcentrifuge tubes (Olympus Plastice, cat. no. 22-282AM or equivalent)
Nutating Mixer (Labnet, cat. no. S0500 or equivalent)
Centrifuge (Eppendorf, cat. no. 5810 or equivalent)
Microcentrifuge (Eppendorf, cat. no. 5424 or equivalent)
Mini Centrifuge (Genesee, cat. no. 27-523 or equivalent)
Vortex Mixer (VWR, cat. no. 12620-838 or equivalent)
Liquid Nitrogen Container (Thermo Fisher Scientific, cat. no. 2122 or equivalent)
Thermal Cycler (Bio-Rad, cat. no. T100 or equivalent)
ThermoMixer (Eppendorf, cat. no. 05-412-503)

8-mm #1$\frac{1}{2}$ thickness round cover glass (Electron Microscopy Sciences, cat. no. 72296-08)
Glass microscope slides (VWR, cat. no. 48312-401)
No. 1$\frac{1}{2}$ thickness, 22- × 22-mm cover glass (VWR, cat. no. 48366-227)
Grace Bio-Lab Press-To-Seal silicon isolator (MilliporeSigma, cat. no. GBL664504-25ea)
Widefield Epifluorescent Microscope (DeltaVision Elite or equivalent)
−20°C freezer
4°C refrigerator
10-, 200, and 1,000-µl micropipets and tips

1. Perform embryo preparation and fixation as in Basic Protocol 1, steps 1-18.

   Do not perform any immunofluorescence steps. Proceed directly from embryo preparation and fixation to the smFISH protocol.

2. Perform smFISH as in Basic Protocol 1, steps 41-60, with the following exception at step 42: If using smiFISH probes, the following protocol is required to generate annealed primary plus secondary smiFISH probes in place of the simple smFISH probe dilution at step 42.

   a. Combine 8-24 smiFISH primary probes at equimolar ratio and dilute to 0.833 µM in Tris, pH 8.0.

      This primary probe mixture is stable at −20°C indefinitely.

   b. In a PCR tube, prepare a solution of:

      2 µl primary probe set;
      1 µl 50 µM FLAP secondary probe;
      1 µl NEB Buffer 3 (or 3.1);
      6 µl RNAse free water.

   c. Anneal primary probe set to fluorophore-labeled secondary probes using the following thermocycling conditions:

      1 cycle at 85°C for 3 min;
      1 cycle at 65°C for 3 min;
      1 cycle at 25°C for 5 min.

      Annealed smiFISH probes are viable at -20°C for at least a week. Treat annealed smiFISH probes as diluted smFISH probes; 2 µl annealed smiFISH probe works well for most hybridizations. smiFISH probes can be used simultaneously with traditional smFISH probes if their fluorophores have distinct spectral profiles. If large fluorescent aggregates are seen when imaging, they may be aggregates of secondary smiFISH probes (see Critical Parameters). If these are observed, an alternate annealing protocol has helped reduce their prevalence in our samples. For this, the smiFISH mixture from step 2b is first heated to 95°C for 3 min before decreasing the temperature by 10°C increments at 0.1°C/min to 45°C, incubating for 2 min at each 10°C interval.

3. Prepare slides as in Basic Protocol 1, step 61-66.
   See sample data in Figure 5.

REAGENTS AND SOLUTIONS

Bleaching solution

For 50 ml:
- 40 ml deionized, distilled water
- 7.2 ml 5 M NaOH (Thermo Fisher Scientific, cat. no. S318-400)
- 4.5 ml 5% NaOCl (Ricca, cat. no. 7495.5-32)
- Make fresh and store for no more than 1 week at room temperature; precipitates can form in embryo pellets when using old bleaching solution.

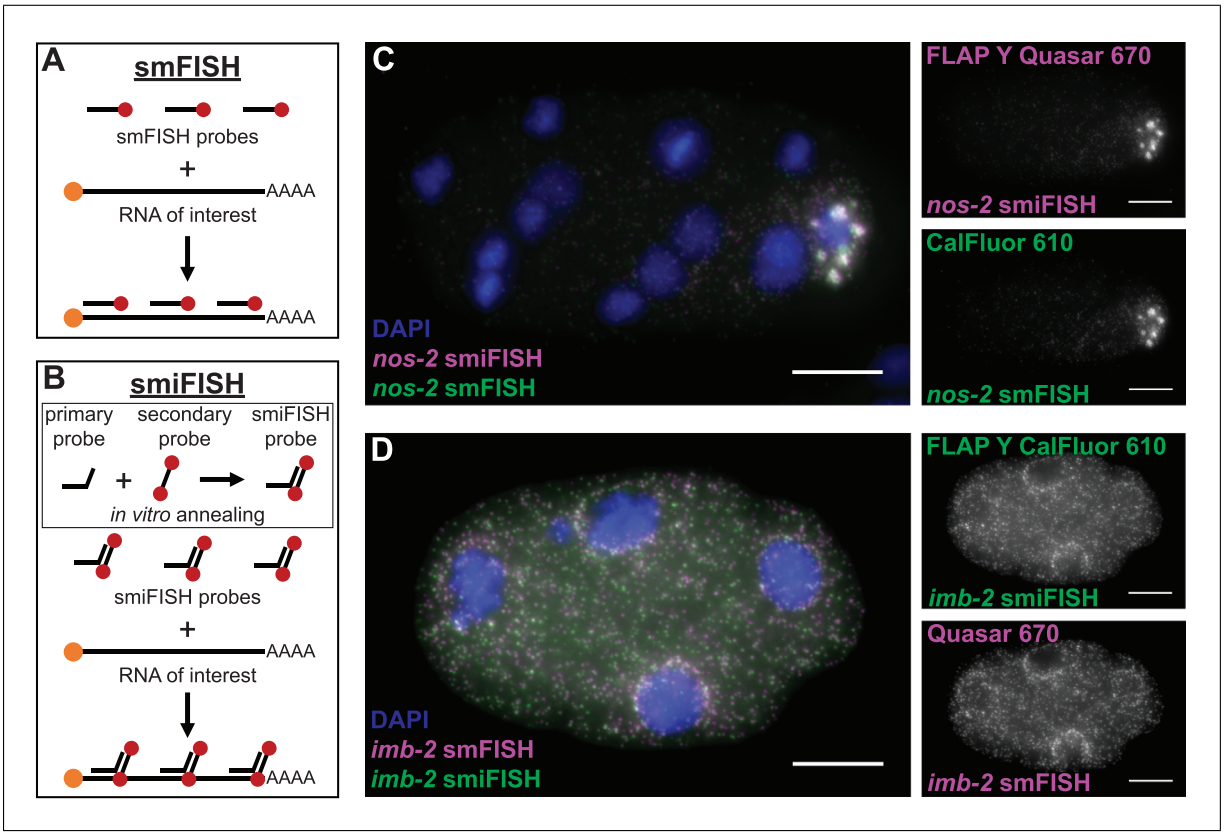

Figure 5 smFISH and smiFISH in *C. elegans* embryos. (**A**) Schematic illustration of smFISH probes. (**B**) Schematic illustration of smiFISH probes. (**C**) *nos-2* RNA was visualized using smiFISH (magenta) and smFISH (green). *nos-2* smiFISH primary probes used FLAP-Y sequences, and the secondary FLAP-Y probe was 5′ and 3′ dual-conjugated with Quasar 670 fluorophores. *nos-2* smFISH probes were 3′ single-conjugated with Cal Fluor 610. (**D**) *imb-2* RNA was visualized using smFISH (magenta) and smiFISH (green). *imb-2* smFISH probes were 3′ single-conjugated with Quasar 670 fluorophores. *imb-2* smiFISH primary probes used FLAP-Y sequences, and the secondary FLAP-Y probe was 5′ and 3′ dual-conjugated with Cal Fluor 610. Embryos were counterstained with DAPI, in blue (**C** and **D**). A representative image is shown from three biological replicates, performed using newly annealed smiFISH probes for each replicate. Scale bars represent 10 μm. smFISH, single-molecule fluorescence *in situ* hybridization; smiFISH, single molecule inexpensive FISH.

Hybridization buffer

- Prepare 110 µl for each sample in an experiment: Mix 99 µl Stellaris Hybridization Buffer (Biosearch Technologies, cat. no. SMF-HB1-10/0) with 11 µl of deionized formamide (MilliporeSigma, cat. no. S4117; final concentration formamide: 10%, v/v). Prepare hybridization buffer fresh for each experiment.
- Store hybridization buffer without formamide at 4°C for up to several months.

M9 buffer

- 3 g KH_2PO_4 (MilliporeSigma, cat. no. P0662-500G)
- 6 g Na_2HPO_4 (MilliporeSigma, cat. no. RDD022-500G)
- 5 g NaCl (Thermo Fisher Scientific, cat. no. S271-500)
- Add deionized, distilled water (ddH_2O) to 1 L final volume.
- Sterilize by autoclaving.
- Add 1 ml 1 M $MgSO_4$ (MilliporeSigma, cat. no. MX0075-1) using sterile technique after solution cools, to prevent precipitation
- Store at room temperature until precipitates form.

Mounting medium

For 5 ml:

- 2.5 ml 100% glycerol (MilliporeSigma, cat. no. G5516-100ML)

- 100 mg *n*-propyl gallate (MilliporeSigma, cat. no. 02370-100G)
- 400 µl 1 M Tris, pH 8.0 (MilliporeSigma, cat. no. 10708976001)
- Vortex until *n*-propyl gallate has dissolved.
- Store in amber tubes or covered in foil (solution is light sensitive) at either 4°C or −20°C until solution begins to yellow or crystallize; typically, this takes several months.

CAUTION: n-propyl gallate is toxic.

Nematode growth medium plates

- 3 g NaCl (Thermo Fisher Scientific, cat. no. S271-1)
- 17 g agar (Thermo Fisher Scientific, cat. no. AAJ10654P2)
- 2.5 g peptone (Thermo Fisher Scientific, cat. no. BP1420-500)
- Mix in a 2-L Erlenmeyer flask.
- Add 975 ml ddH_2O.
- Autoclave for 50 min.
- Add 1 ml of sterile 1 M $CaCl_2$ (MilliporeSigma, cat. no. C4901).
- Add 1 ml of 5 mg/ml cholesterol in 100% ethanol (MilliporeSigma, cat. no. C8667).
- Add 1 ml of sterile 1 M $MgSO_4$ (MilliporeSigma, cat. no. MX0075-1).
- Add 25 ml of sterile 1 M potassium phosphate buffer (see recipe).
- Swirl to mix.
- Add to 150-mm Petri dishes using sterile technique.
- Once cool and solidified, seed with 1.5-2 ml OP50 *E. coli* (Caenorhabditis Genetics Center, cat. no. OP50).
- Once dry, store for up to 1 month at 4°C.

Potassium phosphate buffer, 1 M, pH 6.0

- 108.3 g KH_2PO_4 (MilliporeSigma, cat. no. P0662)
- 35.6 g K_2HPO_4 (MilliporeSigma, cat. no. P3786)
- Add ddH_2O to 1 L.
- pH should not need to be adjusted but should be checked.
- Autoclave to sterilize.
- Store at room temperature until precipitates form.

SSC, 2×

Dilute 20× SSC (see recipe) 1:10 in deionized, distilled water to make the working concentration of 2× SSC.

Store at room temperature until precipitates form.

SSC, 20×

- 800 ml deionized, distilled water
- 175.2 g NaCl (Thermo Fisher Scientific, cat. no. S271-500)
- 88.2 g sodium citrate tribasic dihydrate (MilliporeSigma, cat. no. S4641-500G)
- pH to 7.0 with 1 M HCl
- Add deionized, distilled water to 1 L and autoclave.
- Store at room temperature until precipitates form.

Tween 20 in PBS (PBST), 1×

Dilute 10× PBST (see recipe) 1:10 in deionized, distilled water to make the working concentration of 1× PBST.

Store at room temperature until precipitates form.

Tween 20 in PBS (PBST), 10×

- 80 g NaCl (Thermo Fisher Scientific, cat. no. S271-500)
- 2 g KCl (MilliporeSigma, cat. no. P3911-500G)

- 14.2 g Na_2HPO_4 (MilliporeSigma, cat. no. RDD022-500G)
- 2.4 g KH_2PO_4 (MilliporeSigma, cat. no. P0662-500G)
- 1% Tween® 20 detergent, w/v (MilliporeSigma, cat. no. P1379-500ML)
- Add deionized, distilled water to 1 L final volume.
- Store at room temperature until precipitates form.

Wash buffer A

- 600 µl Stellaris Wash Buffer A (Biosearch Technologies, cat. no. SMF-WA1-60)
- 2.1 ml DEPC-treated RNAse-free water (Invitrogen brand, Thermo Fisher Scientific, cat. no. AM9922)
- Store wash buffer A *without* formamide at 4°C for up to several months.
- When needed, prepare 3 ml for each sample to be hybridized and add 300 µl of deionized formamide (MilliporeSigma, cat. no. S4117).
- Prepare wash buffer A with formamide fresh for each experiment.

Final concentration formamide: 10% (v/v).

Wash buffer B

- Stellaris Wash Buffer B (Biosearch Technologies, cat. no. SMF-WB1-20)
- Add 88 ml RNAse-free water (Invitrogen brand, Thermo Fisher Scientific, cat. no. AM9922) to wash buffer B stock before use.
- Store wash buffer B with water at 4°C for up to several months.

COMMENTARY

Background Information

Visualization of proteins and RNAs (Femino et al., 1998; Orjalo et al., 2011; Raj et al., 2008) has been a staple of cell biology for decades. As a result, many methods have been developed for sample preparation, fixation and permeabilization, IF, and smFISH. These methods can be highly divergent in different model systems or even at different stages of development in a single model organism. In the preceding protocols, we present simplified and optimized methods to fix *C. elegans* embryos before staining for protein and probing for RNA, in a sequential (Basic Protocol 1), simultaneous (Alternate Protocol), or independent (Basic Protocols 2 and 3) manner.

In situ visualization of biological molecules requires that samples be preserved to mirror living cells as closely as possible. Modern fixation was developed in the sixteenth century primarily to preserve tissue samples for histological studies, first using solutions of alcohols, waxes, acetic acid, and arsenic (Cole, 1951). As the mechanisms of fixation were characterized, researchers identified countless reagents for preserving samples using diverse mechanisms. Common fixatives include formaldehyde/formalin, picric acid, or organic solvents such as methanol, ethanol, and acetone. Formaldehyde/formalin acts by creating crosslinked, covalent chemical bonds in the sample, primarily at lysine residues. Notably, formalin can also cause C-T and G-A mutations on DNA sequences, as characterized by PCR (Williams et al., 1999). Moreover, formaldehyde/formalin fixation affects tertiary amines in RNA sequences, resulting in modification of up to nearly 40% of A and C residues in formalin-fixed tissues (Masuda et al., 1999). Due to the high degree of alteration that occurs on nucleic acids, formaldehyde/formalin fixation is not an ideal fixative for nucleic acid visualization. Similarly, picric acid is a problematic fixative for smFISH experiments, as its low pH catalyzes nucleic acid degradation and leads to poor hybridization (Bonin et al., 2005; Tyrrell et al., 1995). As an alternative to crosslinking fixatives, alcohols and other organic solvents have been identified as superior nucleic acid fixatives (Srinivasan, Sedmak, & Jewell, 2002). Alcohols and organic solvents, such as ethanol, methanol, and acetone, function by dehydrating clathrate water molecules around proteins and nucleic acids, thus precipitating biological molecules into a fixed state without significant chemical alteration. As with crosslinking fixatives however, alcohols and organic solvents have their detriments. These fixatives can disrupt cell membrane structures, cytoplasmic organelles, and soluble cell components such as microtubules (Hoetelmans et al., 2001; Vielkind & Swierenga, 1989). However, due to their preservation of nucleic acid composition, alcohols and organic solvents are ideal fixatives for single-molecule

RNA detection assays. Further, we have found that liquid nitrogen freezing combined with short fixations alcohol/organic solvent fixation allows efficient antibody penetration through the embryonic permeability barrier and without disrupting the protein epitopes we have targeted through IF, as some previous studies have shown (Levitt & King, 1987). Ultimately, the use of methanol and acetone fixation greatly simplifies slide preparation for IF samples by removing the need to mechanically permeabilize the embryos on a slide while circumventing many of the challenges that standard fixation protocols using formaldehyde/formalin and picric acid pose. By permitting an in-tube fixation and permeabilization compatible with protein and RNA detection, samples fixed in this manner will be ready for both IF and smFISH protocols.

Immunofluorescence is a standard protocol in cell biology for the visualization of proteins in fixed cells. By targeting a protein of interest with fluorescently labeled primary and/or secondary antibody pairs, the distribution, abundance, timing, and other features of protein expression can be visualized (Duerr, 2006). Since the adoption of *C. elegans* as a model organism, various adaptations have been implemented to overcome the specific challenges of visualizing protein in this organism. These adaptations provide information for antigen production, peptide coupling, antibody purification, fixation conditions, and protocols related to IF in *C. elegans* (Duerr, 2006; Strome & Wood, 1982; Ward & Klass, 1982). However, most have focused on larval stages of development and are not optimized for embryos. Most protocols use some combination of reducing reagents, enzymatic treatments, formaldehyde fixation, and "freeze-cracking" mechanical disruption. The standard immunofluorescence freeze-cracking procedure is the compression of samples between slides, followed by their rapid separation, not to be confused with freeze cracking of the eggshell in liquid nitrogen (Duerr, 2013). The single-tube fixation protocol described here mitigates some issues with traditional IF protocols by removing reducing reagents and enzymatic treatments while permeabilizing the eggshell, thereby simplifying the entire protocol, from fixation to slide preparation. This adapted IF protocol stains proteins of interest while preserving the RNA content of the embryo. Users can then visualize the protein immediately or continue to perform an smFISH protocol subsequently (Strome & Wood, 1982; Wiesenfahrt et al., 2015).

The current gold standard for *in situ* single-molecule RNA detection is single-molecule fluorescence *in situ* hybridization (smFISH). smFISH allows researchers to characterize the developmental dynamics of RNA expression, including abundance, localization, decay, and the onset of transcription. RNA *in situ* hybridization was initially developed using radiolabeled and enzymatically driven colorimetric assays with low spatial resolution. It then evolved to use antisense probes conjugated to multimeric fluorophores, leading to high background signal and low specificity (Young, Jackson, & Wyeth, 2020). In current smFISH protocols, single-molecule RNA visualization occurs by annealing a series of ∼24 to 48 fluorescently labeled short antisense oligonucleotide probes to a transcript of interest in fixed cells and/or animals (Femino et al., 1998; Orjalo et al., 2011; Raj et al., 2008). Annealing multiple fluorescent probes to an RNA produces a discrete, punctate signal for individual RNA molecules *in situ*. smFISH probes can be designed and synthesized in the laboratory (Ji & Oudenaarden, 2012; Raj et al., 2008) or ordered as a set from Biosearch Technologies. Some typical fluorophores include Cy5, Quasar 670, Alexa 594, Cal Fluor 610, and fluorescein, among many others. In RNA FISH experiments, it is crucial to obtain the highest possible signal-to-noise ratio (SNR) to ensure reliable interpretation of the data.

One common question surrounding smFISH is whether commercial reagents (i.e., Stellaris) are superior to homemade ones (Ji & Oudenaarden, 2012; Orjalo et al., 2011). By comparing the signal-to-noise ratio of transcripts imaged by smFISH, we have found Stellaris buffers, in general, to perform better than homemade buffers (for example, see Fig. 6). Another common concern with smFISH experiments is photolability. Due to the relatively low signal, high laser powers, and the small number of fluorophores (∼24 to 48) utilized in smFISH experiments, photobleaching can occur rapidly. Photobleaching is of particular concern with thick samples that must be imaged through many z-stacks, as is the case with *C. elegans* embryos (∼12 to 20 μm thickness as prepared in Basic Protocol 1 or ∼60 to 100 stacks per embryo at 0.2 μm spacing between z-stacks). One of the primary causes of photobleaching is the degradation of fluorophore molecules by oxygen radicals produced upon laser excitation (Greenbaum,

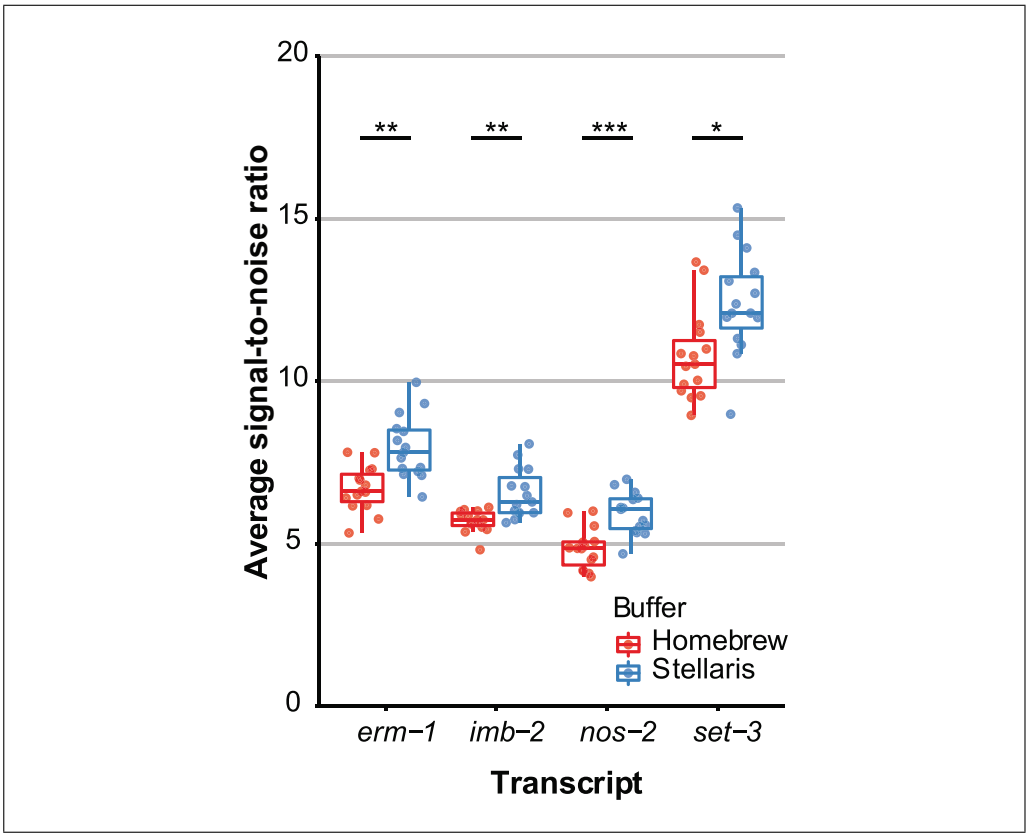

Figure 6 Stellaris buffers provide higher signal-to-noise ratios than homebrew buffers. Signal-to-noise ratios were calculated for each RNA puncta identified when smFISH was performed using homebrew (red) or commercial Stellaris (blue) buffers. The signal-to-noise ratio was calculated by identifying RNA spots using FISHquant (Mueller et al., 2013) before using the ImJoy SNR plugin (Imbert et al., 2021). In short, the SNR plugin compares the intensity at the coordinates of RNA puncta identified by FISHquant to the average intensity of a sphere surrounding the spot to calculate SNR. Four Stellaris smFISH probe sets were used, *erm-1* conjugated to Cal Fluor 610, *imb-2* conjugated to Quasar 670, *nos-2* conjugated to Quasar 670, and *set-3* conjugated to Cal Fluor 610. Individual dots represent the average SNR in one embryo. Three biological replicates were performed for each experiment, and 15 embryos were quantified for each condition over the three replicates. *p* values from Benjamini-Hochberg corrected *t*-tests are shown (.05 >*, .005 >**, .0005 >***).

Rothmann, Lavie, & Malik, 2000). Therefore, free-radical scavenging antifade solutions are commonly used to reduce the degree of experimentally induced photobleaching. We have observed that the optimal antifade solution can vary depending on the probe set or fluorophore (Fig. 7). In our hands, VECTASHIELD, *n*-propyl gallate, or a mixture of the two, provide the best signal stability for Cal Fluor 610 and Quasar 670 labeled RNAs in *C. elegans* embryos. We generally avoid probes conjugated with fluorescein, as they tend to have very low signal-to-noise ratios.

Because each probe in a standard smFISH probe set requires chemical conjugation with fluorophores and is specific to each transcript, smFISH probe sets are relatively expensive (Femino et al., 1998; Orjalo et al., 2011; Tsanov et al., 2016). Targeting a single RNA typically costs in the range of ~$500 USD. Recently, Tsanov et al. (2016) outlined a straightforward, flexible method for reducing the cost of single-molecule RNA detection: Single-molecule inexpensive fluorescence *in situ* hybridization (smiFISH). smiFISH brings down the cost of single-molecule RNA detection by taking advantage of a single, universal fluorophore-labeled secondary probe annealed *in vitro* to gene-specific primary probes (Fig. 5A). Primary smiFISH probes contain two main parts facilitating efficacy and cost reduction: The gene-specific region complementary to the transcript of interest and the FLAP region complementary to the fluorescently labeled secondary probe. *In situ*, the complementary region of the primary probes binds to the target RNA, while its FLAP region is annealed to a fluorophore-labeled

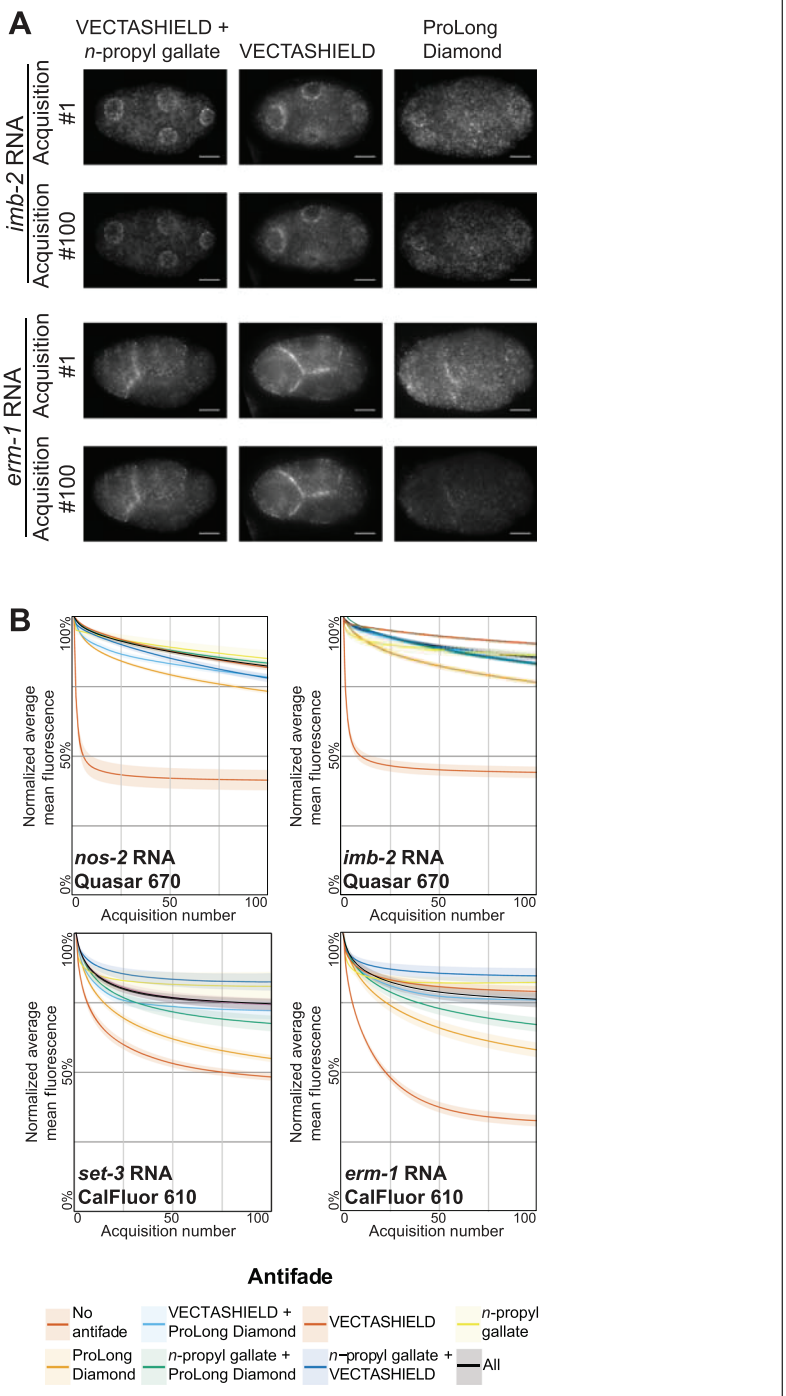

Figure 7 Effect of antifade composition on smFISH signal intensity. The mean fluorescence intensity of smFISH signal over 100 exposures was measured in embryos using various antifade solutions and their combinations. Experiments were performed using four different smFISH probe sets: *erm-1* conjugated to Cal Fluor 610, *imb-2* conjugated to Quasar 670, *nos-2* conjugated to Quasar 670, and *set-3* conjugated to Cal Fluor 610). (**A**) Representative images of the first and final acquisitions for *imb-2* (top) and *erm-1* (bottom) RNAs using VECTASHIELD and *n*-propyl gallate (left), VECTASHIELD only (middle), and ProLong Diamond (right) antifades. (**B**) The average mean intensity throughout imaging was normalized to the intensity of first acquisition for each embryo. The shaded region represents the standard error of the mean for each exposure. Three biological replicates were performed for each experiment and no less than nine embryos were quantified for each condition. smFISH, single-molecule fluorescence *in situ* hybridization.

secondary FLAP probe. This regime significantly reduces the cost of single-molecule RNA visualization by eliminating the need to create chemically conjugated probe sets for each specific target RNA. smiFISH primary probes can be designed as described using the R script Oligostan (Tsanov et al., 2016). Primary probes can be ordered in 96-well plates from IDT on the 25 nmol scale prediluted to 100 μM in IDTE buffer, pH 8.0. Alternatively, if ordering 96 or more individual probes, oligos can be ordered on the 500 pm scale, providing ample primary probes for hundreds of experiments. For most experiments, approximately twelve to sixteen primary probes per transcript are sufficient, although testing as few as eight primary probes has produced discernable single-molecule spots in *C. elegans* embryos (Parker et al., 2020). An increased number of primary probes typically increases the signal-to-noise ratio for any given transcript. Secondary FLAP probes can also be ordered as 5′ and/or 3′ single- or dual-fluorophore-labeled oligos from either Biosearch Technologies or IDT. We have previously demonstrated that smiFISH performs as well as traditional smFISH in *C. elegans* embryos (Parker et al., 2020). We found that smiFISH faithfully reproduces the sensitivity, spatial resolution, and reliability of smFISH probes in embryos. Notably, smiFISH is less effective in larval stages than smFISH using our protocols, possibly due to lower larval permeability, preventing smiFISH probe entry. Here, we provide an example, comparing *nos-2* or *imb*-2 smFISH and smiFISH probes on the same sample (Fig. 5). Using the smFISH or smiFISH protocols presented here facilitates visualization of single RNA molecules in fixed samples. Additionally, if the smFISH protocol is performed after IF as described in Basic Protocol 1, both protein and RNA can be imaged in the same sample.

Traditional approaches to visualizing mRNA and protein simultaneously in *C. elegans* have relied on the visibility of a fluorescently tagged protein persisting under RNA labeling conditions. However, these fluorescent protein tags can often bleach during fixation. The combination of IF and smFISH protocols facilitates the simultaneous visualization of protein and RNA without requiring the genetic manipulation of fluorescently tagging a protein. Yoon, Pendergrass, and Lee (2016) developed a protocol to perform IF and smFISH concurrently in *C. elegans* but it requires manual dissection of animals to isolate the antibody-permeable gonad. We provide a protocol that combines both IF and smFISH in *C. elegans* embryos with a single-tube approach, requiring no genetic manipulations and using techniques accessible to novices. These protocols permit a broader range of *C. elegans* experimentalists to compare protein and RNA distributions in the same sample.

Critical Parameters

Permeabilization and fixation

C. elegans embryos are well isolated from environmental contaminants. This is partly due to the permeability barrier, a membranous barrier that prevents fluid exchange between the embryo and the environment (Olson et al., 2012). The fixative reagent and incubation time are important for permeabilizing the embryo to antibodies, which are roughly twenty times the mass and radius of smFISH probes (Ab ∼150 kDa and ∼60 Å, 20mer oligo ∼7.5 kDa and ∼3 Å; Fernandes, Ortega, Martínez, & de la Torre, 2002; Hawe, Hulse, Jiskoot, & Forbes, 2011). In our experience, a brief methanol fixation and liquid nitrogen freeze cracking followed by a quick acetone fixation was most effective at allowing antibodies to pass through the eggshell and permeability barrier, while maintaining antigen recognition and FISH probe accessibility. We found that the use of acetone was necessary for antibody staining. We interpret this result as acetone solubilizing permeability barrier components, thus increasing the size of molecules that can enter the embryo, although we have not rigorously examined the effective pore size under different fixation conditions. Our experiments with longer fixation times with both methanol and acetone reduced antigen recognition by antibodies (as well as GFP fluorescence for protein fusions). Moreover, the use of formalin/formaldehyde reduces the binding and photostability of FISH probes. Some antigens are likely more compatible with different fixatives and Duerr (2006) describes alternative fixation strategies if the fixation conditions presented here are incompatible with an antigen of interest. If alternative fixation strategies must be pursued, it is crucial to keep in mind their effect on the permeability of the eggshell and permeability barrier.

Sequential IF/FISH protocol

Simultaneous detection of an RNA and its cognate protein can reveal a wealth of information regarding the expression patterns, regulation, and functions of genes. However, the combination of IF and FISH is often

challenging due to slight incompatibilities in traditional protocols. When immunofluorescence is performed in series with smFISH, all reagents must be RNAse free, where possible. It is absolutely essential that solutions that contain BSA be treated with an RNAse inhibitor to prevent RNA degradation. If RNAse-free reagents (not including BSA) are not available or if degradation is occurring, the addition of RNAse inhibitors can slow RNA degradation. Though most reagents listed do not cause apparent RNA degradation, we typically try to only use RNAse inhibitor in steps containing BSA because it is expensive to include with every reagent.

Simultaneous IF/FISH protocol

A simplified protocol can often be utilized if performing IF with a high-affinity nanobody or single-chain variable fragments (ScFvs). Under these circumstances, simultaneous smFISH/IF can be achieved by performing the smFISH protocol (Basic Protocol 3) with the simple addition of fluorescently labeled nanobody or ScFv to the hybridization buffer before overnight incubation with the FISH probes and sample. It is unclear, however, why some nanobodies and ScFv work with this simplified protocol. Perhaps their smaller size allows better permeation during hybridization or their higher affinity is more compatible with RNA FISH hybridization temperatures.

Validation of antibodies

It is essential to validate antibody function and specificity in any IF experiment. Primary antibodies can be validated using null strains or RNAi to ensure that the antibody is binding specifically to the target antigen. Secondary antibodies can be tested for specificity by incubating them in the absence of primary antibodies to ensure no staining of endogenous antigens. Should an antibody have some non-specific binding, it may be possible to increase specificity by depleting the antibody using a null allele (Duerr, 2006). It is also necessary to optimize antibody concentrations over one or two orders of magnitude (starting with manufacturer recommendations) to determine optimum conditions for antigen detection without non-specific binding. Note that optimum antibody concentrations can change with experimental conditions where protein concentrations vary significantly. Because altering antibody concentrations can make downstream quantification inaccurate, it is beneficial to use identical staining conditions when possible.

Validating new smFISH/smiFISH probe sets

There are several ways to validate new smFISH and smiFISH probe sets for target specificity and labeling efficiency. Testing a probe set in a wild-type and deletion strain for the target of interest ensures the probe set is specifically binding only when the RNA is present. If a deletion allele is not available, RNAi can be utilized to a similar end. However, it is important to note that residual fluorescent signal may be present after RNAi due to incomplete knockdown or partial degradation of the targets. Target specificity can also be determined by targeting a transcript with two separate probe sets in different colors, which should colocalize if the probes are specific. The labeling efficiency of a probe set can be determined by comparing transcript abundance found using smFISH data to other sources, such as quantitative reverse transcription PCR (RT-qPCR), digital-droplet PCR, or quantitative sequencing data.

Positive controls

Positive control smFISH probe sets should be consistently employed. These probe sets have the added benefit of marking specific cell lineages or developmental stages, and thereby identify the embryo's orientation or stage. By comparing the performance across replicates, researchers can identify outliers or problems in protocol execution. When troubleshooting, smFISH probe sets that anneal to highly abundant RNAs (polyA) or previously validated targets can ensure success and aid in diagnosing issues.

Low signal-to-noise ratio (SNR)

Because *C. elegans* embryos are relatively thick (∼20 to 30 μm), the use of widefield microscopy will capture out-of-focus signal from non-focal z-planes, thereby decreasing the SNR. Embryos can be flattened during slide preparation to improve SNR. We have found that samples from ∼12 to 20 μm thick have an optimal SNR without perturbing sample morphology. While pressing down on embryos does not seem to affect their morphology, any lateral motion during slide preparation will shear embryos, so pressing directly down when making slides is essential.

Crosstalk of smiFISH secondary probes

It is possible to use two independent primary probe sets containing the same FLAP sequence without crosstalk. Tsanov et al. (2016) demonstrated that multiple primary probe sets containing the same FLAP sequence could be

utilized in the same sample without observable mislabeling of their target transcripts by annealing them to secondary probes labeled with distinct fluorophores (i.e., probeset-1 FLAP-Y-Cal Fluor 610, probeset-2 FLAP-Y-Quasar 670). We have previously confirmed this in the *C. elegans* embryo (Parker et al., 2020).

Troubleshooting

Please see Table 1 for a list of common problems with the protocols, their causes, and potential solutions.

Understanding Results

Performing the RNA FISH or immunofluorescence protocols described in this article should result in dual-labeled, stacked images reporting fluorescently stained protein targets and clear punctate spots for RNA targets either in combination or independently (Figures 2 to 5). For instance, in Figure 2A and 2B, our sequential immunofluorescence and smFISH protocol demonstrates that the PGL-1 antibody stains large fluorescent condensates known as P granules, while probing for *nos-2* and *cpg-2* RNAs showed overlapping clusters of these RNAs localized within P granules. Overall, these results demonstrate a robust series of protocols for producing high-quality data examining the distributions of proteins and RNAs of interest in the *C. elegans* embryo.

smFISH and smiFISH data analysis

There are several routes for interpreting smFISH data, depending on the biological questions at hand. These analyses range from characterizing the quality of the data, counting the number of RNAs in the samples, or identifying spatial distributions of RNA within cells of interest.

The most common method for quantification of smFISH data is counting the number of RNAs within the sample. For this purpose, some commonly used tools are FISH-quant (Mueller et al., 2013) and StarSearch (Raj et al., 2008). These algorithms function by enhancing spot signals through various filtering methods, setting a threshold for RNA spot detection, and identifying individual spots. Thresholds are often set manually by testing a range of intensity values. Therefore, when plotting these values against the number of detected spots, a plateau can often be seen corresponding to threshold values separating RNA spots from lower intensity noise. When performing spot detection analysis of smFISH data, it is imperative to ensure that the SNR of the data is sufficient to identify spots unambiguously. SNR can be calculated using an ImJoy plugin, which compares the intensity of a detected spot to the surrounding background intensities (*https://github.com/fish-quant*). In our experience, if SNR values are below ∼3 to 4, spot detection becomes less reliable. When analyzing smFISH data using FISH-quant or StarSearch, if there is no apparent plateau of RNA counts over various threshold values, the SNR is likely too low for accurate RNA spot detection.

As smFISH has become more widely utilized, novel methods of analysis beyond spot counting are continuously being developed. For instance, FISH-quant has been ported from Matlab to an open-source implementation in Python and successfully applied to two large-scale screen projects (Chouaib et al., 2020; Safieddine et al., 2021). This package includes methods for detecting RNAs, deconvolving overlapping RNAs to increase the counting accuracy of highly abundant or clustered RNAs (Chouaib et al., 2020; Parker et al., 2020), measuring the signal-to-noise ratio of an image (*https://github.com/fish-quant*), and identifying diverse subcellular localization patterns of RNA (Chouaib et al., 2020; Samacoits et al., 2018). Further, to facilitate its usage by non-specialists, several plugins providing user interfaces for the data analysis platform ImJoy (Ouyang, Mueller, Hjelmare, Lundberg, & Zimmer, 2019) were developed. As more laboratories adopt smFISH methodologies and more high-throughput methods of *in situ* RNA detection develop (Eng et al., 2019; Lubeck, Coskun, Zhiyentayev, Ahmad, & Cai, 2014; Moffitt et al., 2016; Querido, Dekakra-Bellili, & Chartrand, 2017; Xia, Babcock, Moffitt, & Zhuang, 2019), more sophisticated analysis methods are likely to arise. An exciting initiative is Starfish, an open-source software suite with the goal of building a unified data-analysis tool and file format for several spatial transcriptomic techniques (Perkel, 2019).

IF data analysis

Standard methods of analysis for IF experiments include measuring the total internal fluorescence and measuring colocalization between different markers. These methods require imaging conditions, such as laser intensity and exposure times, to be held constant across samples and replicates. We will highlight publicly available tools for analysis here; however, most microscopes ship with instrument-specific software packages capable of performing these analyses. Total

Table 1 Troubleshooting

Problem	Possible cause	Solution
No antibody staining	Antibody did not bind its target	Use validated primary and secondary antibody pair as positive control
	Overfixed sample	Do not fix for more than a total of 10 min between methanol and acetone
	Poor permeabilization	Check embryo permeability with antibody sized fluorescent dextran (~150 kDa)
	Wrong concentration of antibody	Titrate antibodies
	Primary antibody does not bind	Validate primary antibody using a previously validated secondary antibody
	Secondary antibody does not bind	Validate secondary antibody using a previously validated primary antibody
No antibody staining (Alternate Protocol only)	Antibody is not compatible with FISH buffers	Use Basic Protocol 1 or 2
Protein or RNA looks abnormal	Sample was damaged	Use an RNA or protein marker to ensure cell morphology is in tact
No RNA spots	smFISH probe did not bind its target	Use validated smFISH probes as a positive control
	RNA was degraded	Ensure reagents are RNAse free or use RNAse inhibitor at all steps
No protein/RNA spots	Sample was stored too long	Reduce length of incubations during IF and/or smFISH steps
Rapid RNA spot photobleaching	Wrong antifade mixture	Check antifade specs to make sure they are compatible with the FISH fluorophore
	Antifade has not permeated the embryo	Give the embryos time to incubate after slide preparation to ensure the antifade penetrates the sample
	Not imaging from high wavelength to low wavelength	Image the entire z-volume using the highest wavelength before moving to the next highest and so on
Low SNR	Sample is too thick	Press more firmly when preparing slides, aiming for a thickness of ~12-14 μm
	Low number of probes	Increase number of probes
Low SNR (smiFISH only)	Single fluorophore-labeled secondary probes	Order dual 5′ and 3′ labeled secondary smiFISH probes
Impossible to design more than 8 probes for target transcript	Short transcript	Consider amplification-based RNA FISH methods (Choi et al., 2016, 2018; Marras, Bushkin, & Tyagi, 2019; Wang et al., 2012; Xia et al., 2019)
Bright aggregates when using smiFISH	Secondary probe aggregation	Vortex smiFISH probes aggressively after annealing and pellet aggregates using minifuge spin and/or use the alternate slow annealing program
Low embryo yield (Basic Protocol 1)	No detergent in PBS	Use PBST; embryos will stick to the tube without it
Clumpy embryos (Basic Protocol 1)	Undetermined	Vortex aggressively during all IF steps and break up clumps with pipet when preparing slides

Abbreviations: IF, immunofluorescence; FISH, fluorescence *in situ* hybridization; smFISH, single-molecule FISH; smiFISH, single molecule inexpensive FISH; SNR, signal-to-noise ratio.

internal fluorescence compares the intensity of a protein visualized by IF in a control sample and an experimental condition, such as an RNAi knockdown or protein knockout. Total internal fluorescence can be measured over the total volume of the embryo or, if specific regions must be analyzed, regions of interest can be masked automatically or manually. Regardless of whether particular segmentations are required, these analyses can be performed relatively quickly in FIJI Is Just ImageJ (FIJI; Schindelin et al., 2012; Schneider, Rasband, & Eliceiri, 2012). Additionally, FIJI plugins are available to analyze a protein of interest's colocalization with another fluorescent marker. When performing colocalization analyses, it is crucial to consider optimal uses for any given colocalization metric, as there are well-documented circumstances where these metrics can be misleading (Dunn, Kamocka, & McDonald, 2011). Helpful instructions, for example, for segmentation and colocalization analysis, can be found at *https://imagej.net/*.

Combined IF/FISH data analysis

As with the IF data analysis, colocalization analyses may be performed on combined IF/FISH data. However, due to the punctate nature of FISH signals, RNA spots may not overlap with a colocalization marker as well as expected, resulting in deceptively low colocalization coefficients. This can occur for several reasons. First, the small total volume of RNA puncta can lead to high variability in colocalization. This variability is compounded by the low temporal resolution of fixed cell experiments and the stochastic movements of RNA in the cell, even for tightly localized transcripts. For these reasons, several groups are developing novel metrics for comparing RNA and protein data and analyzing the spatial relationships between them. For instance, by spatially modeling the coordinates of each RNA puncta and comparing their distributions to other RNAs or organelles, it is possible to identify RNA patterning at various cellular features such as cortical membranes, nuclear membranes, condensates/puncta, cellular protrusions, centrosomes, and more (Chouaib et al., 2020; Parker et al., 2020; Safieddine et al., 2021; Samacoits et al., 2018).

Time Considerations

The entire procedure described in Basic Protocol 1 can be completed in 2 to 3 days. On the first day, embryos are harvested from gravid worms, fixed, and stained overnight with primary antibodies. On the second day, fluorescently labeled secondary antibodies are added to complete the immunofluorescence protocol before performing smFISH. smFISH probes can then be incubated for as few as 4 hr before washing; however, overnight smFISH probe hybridization vastly improves the RNA signal. Thus, smFISH probes are washed from the sample on the evening of the second day or the beginning of the third day before imaging. Alternate Protocol, Basic Protocol 2, and Basic Protocol 3 can be performed in 2 days. Once again, the embryos are harvested on the first day before overnight incubation with both antibody and smFISH probes (Alternate Protocol), primary antibody alone (Basic Protocol 2), or smFISH/smiFISH probes alone (Basic Protocol 3). In both Alternate Protocol and Basic Protocol 3, unbound antibodies and/or probes are washed on the second day before imaging. In Basic Protocol 2, immunofluorescence is first completed by adding secondary antibodies prior to washing and imaging.

Acknowledgments

The authors would like to thank Rob Williams and Wyatt Beyers for fruitful discussions about fixation methods and quantification strategies. We also thank Dustin Updike and James McGhee for reagents. A great thanks goes out to the entire ImJoy development team for making an invaluable platform for data analysis. In particular, the authors extend our appreciation to Florian Mueller for help designing analysis pipelines, providing helpful insights, and feedback on the manuscript. Some strains were provided by the Caenorhabditis Genetics Center, which is funded by National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440).

This work was supported by a MIRA (R35) Award overseen by the National Institute of General Medical Sciences (National Institutes of Health, R35GM124877 to E.O.N.). D.M.P and L.P.W. were supported in part by a National Science Foundation training grant (DGE-1450032).

Author Contributions

Dylan M. Parker: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing original draft, writing review and editing; **Lindsay P. Winkenbach:** Conceptualization, data curation, funding acquisition, investigation, methodology, validation, visualization,

writing review and editing; **Annemarie Parker:** Data curation, formal analysis, investigation, writing review and editing; **Sam Boyson:** Conceptualization, data curation, investigation, methodology, validation, visualization, writing review and editing; **Erin Osborne Nishimura:** Conceptualization, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing original draft, writing review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available at the Colorado State University repository at *https://doi.org/10.25675/10217/233940*.

Supplementary data: Supplementary information and datasets used to generate figures in this article are available at *https://github.com/erinosb/smFISH_IF_methods.git*.

Literature Cited

Bayer, L. V., Batish, M., Formel, S. K., & Bratu, D. P. (2015). Drosophila oogenesis, methods and protocols. *Methods in Molecular Biology*, *1328*, 125–136. doi: 10.1007/978-1-4939-2851-4_9.

Bonin, S., Petrera, F., Rosai, J., & Stanta, G. (2005). DNA and RNA obtained from Bouin's fixed tissues. *Journal of Clinical Pathology*, *58*(3), 313. doi: 10.1136/jcp.2004.016477.

Calvo, L., Ronshaugen, M., & Pettini, T. (2021). smiFISH and embryo segmentation for single-cell multi-gene RNA quantification in arthropods. *Communications Biology*, *4*(1), 352. doi: 10.1038/s42003-021-01803-0.

Choi, H. M. T., Calvert, C. R., Husain, N., Huss, D., Barsi, J. C., Deverman, B. E., … Pierce, N. A. (2016). Mapping a multiplexed zoo of mRNA expression. *Development*, *143*(19), 3632–3637. doi: 10.1242/dev.140137.

Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., … Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: Multiplexed, quantitative, sensitive, versatile, robust. *Development*, *145*(12), dev165753. doi: 10.1242/dev.165753.

Chouaib, R., Safieddine, A., Pichon, X., Imbert, A., Kwon, O. S., Samacoits, A., … Bertrand, E. (2020). A dual protein-mRNA localization screen reveals compartmentalized translation and widespread co-translational RNA targeting. *Developmental Cell*, *54*(6), 773–791.e5. doi: 10.1016/j.devcel.2020.07.010.

Cole, F. J. (1951). Wilkins lecture–History of micro-dissection. *Proceedings of the Royal Society of London. Series B - Biological Sciences*, *138*(891), 159–187. doi: 10.1098/rspb.1951.0012.

Doshi, R., Chen, B. R., Vibat, C. R. T., Huang, N., Lee, C.-W., & Chang, G. (2014). In vitro nanobody discovery for integral membrane protein targets. *Scientific Reports*, *4*(1), 6760. doi: 10.1038/srep06760.

Duerr, J. (2006). Immunohistochemistry. *WormBook*. doi: 10.1895/wormbook.1.105.1.

Duerr, J. S. (2013). Antibody staining in *C. elegans* using "freeze-cracking". *Journal of Visualized Experiments*, *80*, e50664. doi: 10.3791/50664.

Dunn, K. W., Kamocka, M. M., & McDonald, J. H. (2011). A practical guide to evaluating colocalization in biological microscopy. *American Journal of Physiology-Cell Physiology*, *300*(4), C723–C742. doi: 10.1152/ajpcell.00462.2010.

Eng, C.-H. L., Lawson, M., Zhu, Q., Dries, R., Koulena, N., Takei, Y., … Cai, L. (2019). Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH+. *Nature*, *568*(7751), 235–239. doi: 10.1038/s41586-019-1049-y.

Femino, A. M., Fay, F. S., Fogarty, K., & Singer, R. H. (1998). Visualization of single RNA transcripts in situ. *Science*, *280*(5363), 585–590. doi: 10.1126/science.280.5363.585.

Fernandes, M. X., Ortega, A., Martínez, M. C. L., & de la Torre, J. G. (2002). Calculation of hydrodynamic properties of small nucleic acids from their atomic structure. *Nucleic Acids Research*, *30*(8), 1782–1788. doi: 10.1093/nar/30.8.1782.

Greenbaum, L., Rothmann, C., Lavie, R., & Malik, Z. (2000). Green fluorescent protein photobleaching: A model for protein damage by endogenous and exogenous singlet oxygen. *Biological Chemistry*, *381*(12), 1251–1258. doi: 10.1515/bc.2000.153.

Hawe, A., Hulse, W. L., Jiskoot, W., & Forbes, R. T. (2011). Taylor dispersion analysis compared to dynamic light scattering for the size analysis of therapeutic peptides and proteins and their aggregates. *Pharmaceutical Research*, *28*(9), 2302–2310. doi: 10.1007/s11095-011-0460-3.

Hoetelmans, R. W. M., Prins, F. A., Velde, I. C., van der Meer, J., van de Velde, C. J. H., & van Dierendonck, J. H. (2001). Effects of acetone, methanol, or paraformaldehyde on cellular structure, visualized by reflection contrast microscopy and transmission and scanning electron microscopy. *Applied Immunohistochemistry & Molecular Morphology*, *9*(4), 346–351. doi: 10.1097/00022744-200112000-00010.

Imbert, A., Ouyang, W., Safieddine, A., Coleno, E., Zimmer, C., Bertrand, E., … Mueller, F. (2021). FISH-quant v2: A scalable and modular analysis tool for smFISH image analysis. *BioRxiv*, 2021.07.20.453024. doi: 10.1101/2021.07.20.453024.

Ji, N., & van Oudenaarden, A. (2012). Single molecule fluorescent in situ hybridization (smFISH) of *C. elegans* worms and embryos. *WormBook : The Online Review of C. elegans Biology*, 1–16. doi: 10.1895/wormbook.1.153.1.

Levesque, M. J., & Raj, A. (2013). Single-chromosome transcriptional profiling reveals chromosomal gene expression regulation. *Nature Methods*, *10*(3), 246–248. doi: 10.1038/nmeth.2372.

Levitt, D., & King, M. (1987). Methanol fixation permits flow cytometric analysis of immunofluorescent stained intracellular antigens. *Journal of Immunological Methods*, *96*(2), 233–237. doi: 10.1016/0022-1759(87)90319-x.

Lubeck, E., Coskun, A. F., Zhiyentayev, T., Ahmad, M., & Cai, L. (2014). Single-cell in situ RNA profiling by sequential hybridization. *Nature Methods*, *11*(4), 360–361. doi: 10.1038/nmeth.2892.

Marras, S. A. E., Bushkin, Y., & Tyagi, S. (2019). High-fidelity amplified FISH for the detection and allelic discrimination of single mRNA molecules. *Proceedings of the National Academy of Sciences*, *116*(28), 13921–13926. doi: 10.1073/pnas.1814463116.

Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M., & Okubo, K. (1999). Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Research*, *27*(22), 4436–4443. doi: 10.1093/nar/27.22.4436.

Moffitt, J. R., Hao, J., Wang, G., Chen, K. H., Babcock, H. P., & Zhuang, X. (2016). High-throughput single-cell gene-expression profiling with multiplexed error-robust fluorescence in situ hybridization. *Proceedings of the National Academy of Sciences*, *113*(39), 11046–11051. doi: 10.1073/pnas.1612826113.

Mueller, F., Senecal, A., Tantale, K., Marie-Nelly, H., Ly, N., Collin, O., … Zimmer, C. (2013). FISH-quant: Automatic counting of transcripts in 3D FISH images. *Nature Methods*, *10*(4), 277–278. doi: 10.1038/nmeth.2406.

Okamoto, I. (2018). X-chromosome inactivation, methods and protocols. *Methods in Molecular Biology*, *1861*, 149–159. doi: 10.1007/978-1-4939-8766-5_12.

Olson, S. K., Greenan, G., Desai, A., Müller-Reichert, T., & Oegema, K. (2012). Hierarchical assembly of the eggshell and permeability barrier in *C. elegans*. *Journal of Cell Biology*, *198*(4), 731–748. doi: 10.1083/jcb.201206008.

Orjalo, A., Johansson, H. E., & Ruth, J. L. (2011). Stellaris™ fluorescence in situ hybridization (FISH) probes: A powerful tool for mRNA detection. *Nature Methods*, *8*(10), i–ii. doi: 10.1038/nmeth.f.349.

Ouyang, W., Mueller, F., Hjelmare, M., Lundberg, E., & Zimmer, C. (2019). ImJoy: An open-source computational platform for the deep learning era. *Nature Methods*, *16*(12), 1199–1200. doi: 10.1038/s41592-019-0627-0.

Parker, D. M., Winkenbach, L. P., Boyson, S., Saxton, M. N., Daidone, C., Al-Mazaydeh, Z. A., … Nishimura, E. O. (2020). mRNA localization is linked to translation regulation in the *Caenorhabditis elegans* germ lineage. *Development*, *147*(13), dev186817. doi: 10.1242/dev.186817.

Perez-Burgos, L., Peters, A. H. F. M., Opravil, S., Kauer, M., Mechtler, K., & Jenuwein, T. (2003). Generation and characterization of methyl-lysine histone antibodies. *Methods in Enzymology*, *376*, 234–254. doi: 10.1016/s0076-6879(03)76016-9.

Perkel, J. M. (2019). Starfish enterprise: Finding RNA patterns in single cells. *Nature*, *572*(7770), 549–551. doi: 10.1038/d41586-019-02477-9.

Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A., & Cerón, J. (2012). Basic *Caenorhabditis elegans* methods: Synchronization and observation. *Journal of Visualized Experiments*, *64*, e4019. doi: 10.3791/4019.

Querido, E., Dekakra-Bellili, L., & Chartrand, P. (2017). RNA fluorescence in situ hybridization for high-content screening. *Methods*, *126*, 149–155. doi: 10.1016/j.ymeth.2017.07.005.

Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A., & Tyagi, S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. *Nature Methods*, *5*(10), 877–879. doi: 10.1038/nmeth.1253.

Safieddine, A., Coleno, E., Salloum, S., Imbert, A., Traboulsi, A.-M., Kwon, O. S., … Bertrand, E. (2021). A choreography of centrosomal mRNAs reveals a conserved localization mechanism involving active polysome transport. *Nature Communications*, *12*(1), 1352. doi: 10.1038/s41467-021-21585-7.

Samacoits, A., Chouaib, R., Safieddine, A., Traboulsi, A. M., Ouyang, W., Zimmer, C., … Mueller, F. (2018). A computational framework to study sub-cellular RNA localization. *Nature Communications*, *9*(1), 4584. doi: 10.1038/s41467-018-06868-w.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., … Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. doi: 10.1038/nmeth.2019.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. doi: 10.1038/nmeth.2089.

Srinivasan, M., Sedmak, D., & Jewell, S. (2002). Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *The American Journal of Pathology*, *161*(6), 1961–1971. doi: 10.1016/s0002-9440(10)64472-0.

Strome, S., & Wood, W. B. (1982). Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences*, *79*(5), 1558–1562. doi: 10.1073/pnas.79.5.1558.

Tocchini, C., Rohner, M., Stetina, S. E. V., & Mango, S. E. (2021). *Translation-dependent mRNA localization to Caenorhabditis elegans adherens junctions*. bioRxiv, 2021.05.20.444977. doi: 10.1101/2021.05.20.444977.

Toki, M. I., Cecchi, F., Hembrough, T., Syrigos, K. N., & Rimm, D. L. (2017). Proof of the quantitative potential of immunofluorescence by mass spectrometry. *Laboratory Investigation*, *97*(3), 329–334. doi: 10.1038/labinvest.2016.148.

Trcek, T., Rahman, S., & Zenklusen, D. (2017). mRNA Decay, methods and protocols. *Methods in Molecular Biology*, *1720*, 35–54. doi: 10.1007/978-1-4939-7540-2_4.

Tsanov, N., Samacoits, A., Chouaib, R., Traboulsi, A. M., Gostan, T., Weber, C., … Mueller, F. (2016). SmiFISH and FISH-quant - A flexible single RNA detection approach with super-resolution capability. *Nucleic Acids Research*, *44*(22), e165–e165. doi: 10.1093/nar/gkw784.

Tyrrell, L., Elias, J., & Longley, J. (1995). Detection of specific mRNAs in routinely processed dermatopathology specimens. *The American Journal of Dermatopathology*, *17*(5), 476–483. doi: 10.1097/00000372-199510000-00008.

Vielkind, U., & Swierenga, S. H. (1989). A simple fixation procedure for immunofluorescent detection of different cytoskeletal components within the same cell. *Histochemistry*, *91*(1), 81–88. doi: 10.1007/bf00501916.

Wang, F., Flanagan, J., Su, N., Wang, L.-C., Bui, S., Nielson, A., … Luo, Y. (2012). RNAscope A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *The Journal of Molecular Diagnostics*, *14*(1), 22–29. doi: 10.1016/j.jmoldx.2011.08.002.

Ward, S., & Klass, M. (1982). The location of the major protein in *Caenorhabditis elegans* sperm and spermatocytes. *Developmental Biology*, *92*(1), 203–208. doi: 10.1016/0012-1606(82)90164-6.

Wiesenfahrt, T., Berg, J. Y., Nishimura, E. O., Robinson, A. G., Goszczynski, B., Lieb, J. D., & McGhee, J. D. (2015). The function and regulation of the GATA factor ELT-2 in the *C. elegans* endoderm. *Development*, *143*(3), 483–491. doi: 10.1242/dev.130914.

Williams, C., Pontén, F., Moberg, C., Söderkvist, P., Uhlén, M., Pontén, J., … Lundeberg, J. (1999). A high frequency of sequence alterations is due to formalin fixation of archival specimens. *The American Journal of Pathology*, *155*(5), 1467–1471. doi: 10.1016/s0002-9440(10)65461-2.

Xia, C., Babcock, H. P., Moffitt, J. R., & Zhuang, X. (2019). Multiplexed detection of RNA using MERFISH and branched DNA amplification. *Scientific Reports*, *9*(1), 7721. doi: 10.1038/s41598-019-43943-8.

Yoon, D. S., Pendergrass, D. L., & Lee, M.-H. (2016). A simple and rapid method for combining fluorescent in situ RNA hybridization (FISH) and immunofluorescence in the *C. elegans* germline. *MethodsX*, *3*, 378–385. doi: 10.1016/j.mex.2016.05.001.

Young, A. P., Jackson, D. J., & Wyeth, R. C. (2020). A technical review and guide to RNA fluorescence in situ hybridization. *PeerJ*, *8*, e8806. doi: 10.7717/peerj.8806.