CHAPTER 9

In situ Hybridization of Embryos with Antisense RNA Probes

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Abstract

Detection of transcripts *in situ* is a rapid means by which gene expression can be characterized in many systems. In the nematode, *Caenorhabditis elegans*, the ease with which transgenics can be made and the general reliability of reporter fusion expression patterns, have made this technique comparatively less popular than in other systems. There are, however, still applications in which *in situ* hybridization is desired, such as for maternally expressed genes, or in related species without established transgene methods. The most frequently used method of *in situ* hybridization uses DNA probes and formaldehyde fixation. A newer approach that permits single-transcript detection has been reported and will not be described here (Raj and Tyagi, 2010). Rather, we describe an alternative protocol that uses RNA probes with a different fixative. This approach has been applied to *C. elegans* and related nematodes, providing reliable, sensitive detection of endogenous transcripts.

I. Introduction

Localization of transcripts by *in situ* hybridization is a desirable way to determine expression patterns, because it can detect endogenous mRNA in its natural context, and because it is a method that, once established, can be repeated on any number of genes by changing only the antisense probe used. In addition, regulatory mechanisms might also be identified, such as subcellular localization of the transcripts. Molecular approaches, such as quantitative PCR (qPCR), Northern blots, or genome-wide approaches such as microarrays or RNA-Seq require isolation of tissues, and in most cases, amplification of the endogenous material. In the nematode, *Caenorhabditis elegans*, *in situ* hybridization has historically not been the method of choice for assessing endogenous gene expression, due largely to the ease of construction of transgenic reporter strains and the general reliability of the expression patterns produced (see Chapter on Transgenesis in this volume). Nonetheless, there remain instances in which *in situ* detection of endogenous mRNA may be desired. Reporters may be difficult to construct for particular genes, or transgenes may not express, in particular those activated in the *C. elegans* germ line and very early embryo. Other nematode species remain refractory to transgene expression techniques, either because the DNA becomes silenced in the soma as well as germ line, or because the necessary reagents (e.g., specific mutant backgrounds in which to make transgenics) do not yet exist. Newer transgene protocols may overcome some of these limitations (Giordano-Santini *et al*., 2010; Praitis *et al*., 2001; Schlager *et al*., 2009; Semple *et al*., 2010); however, it remains to be seen whether transgenes in other nematode species will in general be as reliable as those seen in *C. elegans*.

Historically, *in situ* detection of mRNA has relied on detection of colorimetric or fluorescent signals from localized antisense DNA probes in whole-mount embryos (Seydoux and Fire, 1995; Tabara *et al*., 1996). For low-abundance transcripts, signal amplification can be used (Bobrow and Moen, 2001). Recently, a new approach

using multiple short, nonoverlapping fluorescent probes has been described (Raj and Tyagi, 2010; Raj *et al*., 2008). This approach permits detection of mRNAs as diffraction-limited spots, with the advantage that it is highly sensitive, yet specific, and permits quantification of individual transcripts. As this protocol has very recently been described in detail (Raj and Tyagi, 2010; Raj *et al*., 2008), we shall instead focus here on an alternative protocol that uses a nontoxic fixative (NTF) and less-expensive antisense RNA probes, which offers a qualitative assessment of gene expression. For the classic *C. elegans in situ* hybridization protocol, readers are referred to the original description (Seydoux and Fire, 1995), updated versions of which can be found on the WormMethods section of WormBook (http://www.wormbook.org/toc_wormmethods.html). The procedure described here can be performed in less than 3 days and offers good sensitivity with preservation of fine structure. It has been used to successfully detect embryonic transcripts in *C. elegans* and other nematode species (Broitman-Maduro *et al*., 2009; Coroian *et al*., 2005). Others have adapted the procedure, for example, for detection of transcripts in extruded *C. elegans* gonads (Sheth *et al*., 2010).

II. Rationale

In the method described here, whole embryos are mounted on coated glass microscope slides, fixed, and permeabilized. Antisense RNA probes are synthesized by *in vitro* transcription of PCR products, and include the use of Digoxigenin-tagged UTP. The use of RNA probes may improve sensitivity because RNA:RNA hybrids are more stable than DNA:RNA hybrids (Sugimoto *et al*., 1995). Following hybridization of the probe, the slides are rinsed and processed for detection of the DIG moiety using an anti-DIG antibody conjugated to alkaline phosphatase (AP). The bound antibodies are detected by the use of the standard AP substrates Nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Staining is observed with a microscope equipped with differential interference contrast (DIC), and color images are acquired with a digital camera. Positive controls are performed with known probes, or on transgene strains with known expression. Use of a sense RNA probe, or a mutant background known to result in the absence of the endogenous transcript, can each serve as negative controls.

III. Methods

A. Probe Design and Synthesis

Antisense RNA probes are created from *in vitro* transcription of short (200 bp–1.5 kbp) PCR products carrying the T7 RNA polymerase recognition sequence at one end. The most convenient template is genomic DNA, using primers that will amplify as high a proportion of exon-containing sequence as possible (Fig. 1). Alternatively, a cloned cDNA fragment can be used. Some may choose to also synthesize the

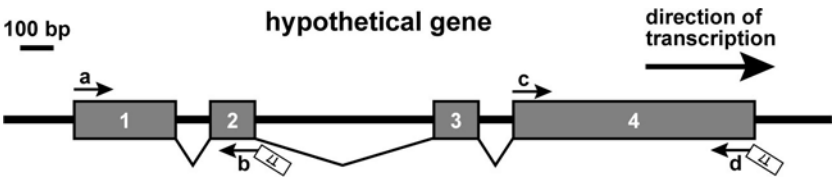

Fig. 1 Typical primer design considerations for a hypothetical four-exon gene. A good set of primers might be the pairs indicated by a/b or c/d. The 3′ primer carries the recognition sequence for T7 RNA polymerase, 5′-TAATACGACTCACTATAGGG-3′, followed by 25–30 bases of target homology; the forward primer has 25–30 bases of identity but lacks the T7 tag.

complementary (sense) strand as a control probe, or use one of the suggested probes (Section 4) for specificity.

B. PCR to Generate Probe Synthesis Template

Assemble the following in a 0.6-mL (PCR) tube:

Forward primer (25 pmol/μL)	1 μL
Reverse primer (25 pmol/μL)	1 μL
dNTPs (10 mM)	0.5 μL
PCR buffer (10× stock)	2.5 μL
Genomic DNA (200 ng/μL)[a]	1 μL
Taq polymerase[b]	0.5 μL
ddH_2O	18.5 μL (total volume: 25 μL)

[a] Alternatively, use 1 μL of a solution carrying 10 ng of plasmid template.
[b] We routinely use a crude Taq preparation with good results (Engelke *et al*., 1990).

Perform a standard PCR reaction, for example 95°C for 3 min and then repeat [95°C for 30 s, 72°C for 30 s, 55°C for 30 s] for 30 cycles, 72°C for 10 min and ending at 4°C. Check an aliquot (5 μL) on an agarose gel to make sure the PCR product is of the expected size.

C. DIG-Labeled Probe Synthesis

Use DIG-RNA labeling kit (Roche, #1175025)

PCR product generated above (not purified)	3 μL
10× NTP mixture with DIG-11-UTP	1 μL
10× Transcription Buffer	1 μL
RNase Inhibitor	0.5 μL

T7 RNA Polymerase	1 μL
DEPC-ddH_2O	3.5 μL (total volume: 10 μL)

Incubate in a thermocycler with a heated lid at 37°C, overnight. In the morning add 30 μL DEPC-ddH_2O and 0.5 μL RNase Inhibitor to the incubations and store at −20°C.

IV. Animal Preparation and Fixation

A. Synchronization to Produce Gravid Hermaphrodites

Hermaphrodites must be synchronized for *in situ* experiments because the fixation and freeze-crack techniques work best on animals that are similarly sized, rather than a mixture of adults and larvae. In order to synchronize worms, two to three 10-cm plates containing gravid hermaphrodites are bleached and the embryos are hatched overnight in M9 + cholesterol [10 μg/mL] at 20°C. Synchronized L1 larvae are collected by centrifugation and plated on 10-cm NGM plates for 3 days at 20°C. After approximately 3 days the plates should contain gravid hermaphrodites with early embryos. If later-staged embryos are required incubation may be extended a further 5 h, but will depend on incubation temperature, abundance of food, general health of the strain, and tendency of the worms to retain embryos.

B. Fixation of Gravid Hermaphrodites and Embryos

Harvest the worms by washing each 10-cm plate with 3 mL of M9 into several Eppendorf tubes and centrifuging at 2000 rpm. This procedure often carries bacterial contamination along with gravid hermaphrodites, depending on how much bacteria remains on the plates. Excess bacteria will result in slides with high background after development. We routinely resuspend the worm pellet in 1 mL of Hypaque meglumine (60%, available from http://www.nanric.com as Reno-60). Invert the tube several times and centrifuge for 30 s at 2000 rpm. After centrifugation, the worms will float to the top of the Eppendorf tube (Fig. 2A). Remove the worm layer and rinse 2–3× in M9. Examine an ∼80 μL droplet of worms on a microscope slide under a dissecting microscope. If there are no bacterial clumps, then the worms are clean and ready for freeze-cracking and fixation (Fig. 2B). If many bacterial clumps are still present, repeat the meglumine flotation until worms appear clean. Resuspend worms in 750 μL of M9.

C. Freeze-Cracking and Fixation

The following steps are carried out in RNase-free glassware, which should be designated for RNA use and be rinsed with DEPC-ddH_2O and baked for 4 h at 180°C. Alternatively, glassware may be cleaned thoroughly with RNase away (MBP, #7003, 1L). Where indicated, disposable plastics may be used for convenience.

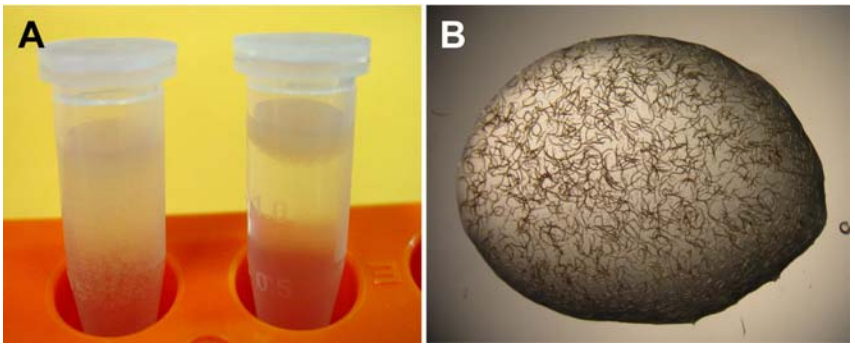

Fig. 2 (A) Appearance of worms in Hypaque meglumine in 1.5-mL Eppendorf tubes. The tube on the left has been inverted and worms are suspended throughout. After 5 min at room temperature, or after a brief 2000 rpm centrifugation, the worms will float to the top (right tube) and can be removed by pipetting the top layer. (B) 80 μL droplet of worms on a glass slide. (For color version of this figure, the reader is referred to the web version of this book.)

Prepare a hydration series of methanol:DEPC-ddH_2O dilutions in five clean 50-mL Coplin jars as follows:

#1 – methanol pre-chilled at −20°C
#2 – 90% methanol at room temperature
#3 – 70% methanol at room temperature
#4 – 50% methanol at room temperature
#5 – DEPC-ddH_2O at room temperature

Prepare 50 mL of NTF in a Coplin jar and warm it to 37°C in an incubator.

Place an aluminum disc (Fig. 3) onto crushed dry ice for at least 5 min and bring the disc, still on dry ice, near a dissecting microscope. Make sure the block is smooth and free from surface irregularities, to allow microscope slides to make good contact with it.

Fig. 3 Aluminum disc, approximately 125 mm in diameter ×15 mm, on dry ice inside a Styrofoam shipping carton.

Resuspend worms in the M9 by inversion. Pipette 80 μL of worm suspension onto the center of a poly-L-lysine coated slide (Fisher, #12-550-19) and spread the droplet with a clean razor blade so that the worms come in contact with the surface of the slide. Begin cutting worms with the razor in an up–down motion while observing the worms through the dissecting microscope. Stop when the majority of worms are cut in half and the embryos are liberated. Starting at one side, cover the worms with a 22 × 40 mm cover glass (Gold Seal #3316), taking care not to introduce air bubbles. While looking at the worms through the dissecting microscope, wick away excess liquid by placing the edge of a Kimwipe or paper towel in contact with the edge of the coverslip. This should be stopped just as adult worm carcasses cease to move but before embryos burst open (Fig. 4). As this step is critical for proper permeabilization, it may need to be practiced until the researcher is confident that worms and embryos have adhered properly to the slide. When a slide is ready, place it coverslip side up on the aluminum disc and press down on the side that has no coverslip to ensure complete contact. Incubate slides at least 5 min before proceeding to the hydration series.

D. Hydration Series

Wedge a clean razor blade under one corner of the coverslip, and with a twisting motion quickly pop off the cover glass in one motion. There should be an audible "cracking" sound as the coverslip is popped off. Incubate the slide as follows:

1) 5 min – 100% methanol (at −20°C)
2) 5 min – 90% methanol (room temperature)
3) 5 min – 70% methanol (room temperature)
4) 5 min – 50% methanol (room temperature)
5) 5 min – DEPC-ddH_2O (room temperature)
6) 1 h – NTF (prewarmed to 37°C)

Fig. 4 Appearance of desired density of worms and embryos under coverslip, prior to freezing, as viewed through a dissecting microscope.

Place Coplin jar, with NTF and slides, into a 37°C incubator during the 1 h incubation.

During incubation, prepare 10 mL prehybridization buffer (PB) and incubate at 65°C with occasional vortexing to resuspend components. PB may require up to 30 min at 65°C to become fully resuspended.

V. Hybridization and Signal Development

A. Prehybridization and Probe Hybridization

Rinse slides in the following buffers:

1) 5 min – DEPC-ddH_2O
2) 5 min – DEPC-ddH_2O
3) 5 min – 2× SSC
4) 5 min – 2× SSC

Have a humid chamber ready. It is convenient to use empty pipette tip boxes, with the rack in place, with water in the bottom to a depth of a few centimeters. Place slides face up in the chamber and add 300 μL PB onto fixed worms. Be sure that samples are completely covered with PB. Place humid chambers into a hybridization oven prewarmed to 42°C and incubate 1 h. There is no need to cover the slides with a coverslip as the hybridization buffer will not evaporate under these conditions.

Towards the end of the incubation, thaw frozen probe(s) and dilute 1:500 to 1:1000 in PB (typically, 1–2 μL probe in 1 mL PB) and heat to 65°C for 5 min. Add 100 μL of the diluted probe to the PB already on the top of the slides. Alternatively, tilt the slides so that the PB runs off, and add 300 μL of the PB+Probe to the slides to cover the worms. Hybridize at 42°C overnight. It is convenient to prepare two 50 mL aliquots each of 2× SSC and formamide buffer (FB) and incubate them at 42°C overnight. These will be used for rinses on the following day.

B. Rinsing and Antibody Incubation

Place slides back into Coplin jars with the following buffers at 42°C:

1) 5 min – 2× SSC (42°C)
2) 5 min – 2× SSC (42°C)
3) 5 min – FB (42°C)
4) 5 min – FB (42°C)

Rinse slides in the following buffers at room temperature:

1) 5 min – 2× SSC (room temperature)
2) 5 min – 2× SSC (room temperature)
3) 5 min – Tris-NaCl (TN) (room temperature)
4) 5 min – TN (room temperature)

Following the TN rise, block slides prior to antibody incubation at 37°C in 30 mL blocking buffer (BB) for 30 min.

Add 10 μL of anti-Digoxigenin-AP Fab fragments (Roche, #11093274910) to 30 mL fresh BB and incubate for 3 h at 37°C.

C. Rinsing and Signal Development

Rinse slides in the following buffers:

1) 10 min – TN (room temperature)
2) 10 min – TN (room temperature)
3) 10 min – TNM pH 9.5 (room temperature)
4) Developer – incubate with slides overnight in the dark (e.g., by covering entire Coplin jar in aluminum foil) at room temperature.

The next day, rinse twice in TN-EDTA for 10 min each, and add mounting medium (e.g., Vectashield, Vector Labs). Cover with 22×40 mm coverslip, wipe off excess mounting medium, and seal the coverslip with clear nail polish. Slides can be kept up to 6 months at 4°C, but for best image quality observe within a week. After several weeks, a colored precipitate will begin to form.

VI. Materials

1. DEPC-ddH_2O (0.1% v/v)

Millipore water or equivalent	500 mL
DEPC (Sigma)	0.5 mL

Suspend the DEPC by shaking vigorously, leave in a fume hood over night, and autoclave (121°C at 15 lb/in^2 for 15 min).

2. NTF

2-bromo-2-nitro-1,3-propanediol (Fisher, # AC15882-1000)	30 g
Diazolidinyl Urea (MP Biomedical, # ICN19019183)	30 g
Zinc Sulfate Heptahydrate, $ZnSO_4 \cdot 7H_2O$	12 g
Sodium Citrate (anhydrous), $Na_3C_6H_5O_7$	2.9 g

Add DEPC-ddH_2O up to 1 L. Make and store in an autoclaved glass container at room temperature. Heat 30–50 mL to 37°C in a Coplin jar prior to use (Of this heated aliquot, do not add unused fixative back to stock, and do not reuse.).

3. 50× Denhardt's solution

Ficoll (Type 400)	0.1 g
Polyvinylpyrrolidone (PVP)	0.1 g
Bovine Serum Albumin (BSA)	0.1 g
DEPC-ddH_2O	to 10 mL

Dissolve components, pass through a 0.45 μm filter, and store in 200 μL aliquots at −20°C.

4. Salmon sperm DNA

Lyophilized DNA	1 g
Tris-EDTA pH 8.0 (TE, Fisher #BP2473-1)	100 mL

Add TE to DNA in an autoclavable glass Wheaton bottle. Autoclave (121°C at 15 lb/in^2 for 15 min), cool to room temperature, dispense into Eppendorf tubes in 1 mL aliquots, and freeze at −20°C. As a result of autoclaving, the DNA will have been sheared to the 200 bp–5 kbp range, which can be checked by running an aliquot on an agarose gel.

5. Prehybridization buffer

20× SSC (Fisher, #BP1325-1)	2 mL
Dextran Sulfate	1 g
Formamide	5 mL
0.5 M EDTA	40 μL
50× Denhardt's Solution	200 μL
Sheared Salmon Sperm DNA	1 mL

Add DEPC-ddH_2O up to 10 mL. Vortex the suspension and heat to 65°C to get into solution. This may take 30 min or more.

6. Formamide buffer (FB)

Formamide	30 mL
20× SSC	0.5 mL

Add up to 50 mL with DEPC-ddH_2O in sterile plastic Falcon tube or equivalent.

7. Tris pH 7.5, NaCl (TN), and TN-EDTA

1M Tris, pH 7.5 (Fisher, #1757-500)	50 mL
2M NaCl	37.5 mL

Add up to 500 mL DEPC-ddH_2O and autoclave.

For TN-EDTA, add 1 mL of 0.5M EDTA to 50 mL of TN.

8. Blocking buffer

Milk Blocker (Bio-Rad # 170-6404)	2.5 g
TN	to 50 mL

Make in sterile plastic 50 mL Falcon tubes. Suspend by vortexing.

9. 0.93M Tris, pH 9.5

1M Tris, pH 7.5 (Fisher, BP1757500)	500 mL
10M NaOH	43.5 mL

Dispense aliquots into sterile 50-mL Falcon tubes and freeze at −20°C. May be thawed in 37°C incubator prior to use. (We have found pH adjustment of the RNase-free Fisher stock to be a more convenient way to make this buffer than to make 1M Tris pH 9.5 by other means.)

10. Tris, NaCl, $MgCl_2$, (pH 9.5) (2× TNM),

0.93M Tris (pH 9.5) (see above)	100 mL
2M NaCl	50 mL
1M $MgCl_2$	50 mL
DEPC-ddH_2O	to 500 mL

Autoclave for 15 min at 121°C, 15 lb/in^2. Dispense aliquots into 50 mL Falcon tubes. Store at −20°C. Thaw the morning that it is needed. If a white precipitate occurs, heat in a 65°C waterbath and vortex, then cool before use.

To 25 mL of 2× TNM, add 25 mL DEPC-ddH_2O. This is used as the last wash prior to development. The other 25 mL aliquots are used to make the Developer solution.

11. Nitro Blue Tetrazolium (NBT)

NBT is resuspended to 100 mg/mL in 70% dimethyl formamide and stored in aliquots at −20°C.

12. 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)
BCIP is resuspended to 50 mg/mL in anhydrous dimethyl formamide and stored in aliquots at −20°C.

13. 2× Polyvinyl alcohol (PVA)

PVA (Fluka 40-88, Sigma #81386)	50 g
DEPC-ddH_2O	to 500 mL

This solution takes very long to go into solution. It is best to start with freshly autoclaved (warm) DEPC-ddH_2O and add PVA to a sterile container. Incubate at 65°C for several days, stirring often with a 25 mL plastic serological pipette; A stir bar is not recommended. When it is fully in suspension, the solution will have the consistency of glycerol. Store at room temperature.

In our hands, PVA in the developer greatly improves signal quality, and is worth the trouble to make.

14. Developer Solution

2× TNM	25 mL
2× PVA	25 mL
NBT	200 μL
BCIP	200 μL
Levamisole	7.2 mg

Mix reagents in a Falcon tube and mix by inversion. Prepare immediately before use.

VII. Notes

A. Suggested Positive Controls

Gene	Expression Pattern	Primers (forward primer, T7-tagged reverse primer)
pgl-1	Germ line and embryos	5′-gtt caaggaatcaactcgaag act c-3′, 5′-TAA TAC GAC TCA CTA TAG GGA CTtggcagagct act gat ttcgtt gga-3′
end-1	Early E lineage (E, Ea/Ep)	5′-ttc aatcgtacgatc cag cacaacaat cg-3′, 5′-TAA TAC GAC TCA CTA TAG GGA CTT GCA CT caatagctcctgaatcagt t-3′
hlh-1	Mid to late embryogenesis, muscle lineages	5′-aaaccagccagcttactacctcccgtccta-3′, 5′-TAATACGACTCACTATAGGGACTcgttcccga gcttatgatgatctctatc-3′
opt-2	Mid to late embryogenesis, intestine	5′-gtaatggcgattggactctcacatatgacc-3′, 5′-TAATACGACTCACTATAGGGACTtctgctgccg tgatgacaacgatttgtg-3′
myo-2	Late stage embryos, pharynx muscle	5′-gga tgttgtccaaga gat gaatccacc a-3′, 5′-TAA TAC GAC TCA CTA TAG GGA CTtgttcaa tat cgcaagaagcgacac gtc-3′
myo-3	Late stage embryos, body muscle	5′-tct cgtgtcgtccgt cag gcaccagga gag-3, 5′-TAA TAC GAC TCA CTA TAG GGA CTcctggtgatg atccacttgaacata cgg-3′
cup-4	4 coelomocytes in embryos, 6 in adults	5′-gtagtagcatctctaatatccatgacgttc-3′, 5′-TAATACGACTCACTATAGGGACTtccttgaac gtattaggaatgtattctt-3′

B. Hypaque meglumine

Bacterial contamination (usually *E. coli* OP50 from plates) often forms clumps around worms and deposits onto slides, attracting probe and contributing to high background. In our hands, cleaning worms with meglumine gives the most consistent results. It may be possible to bypass this step if the source plates are clean enough (David Fitch, personal communication). Alternatively, sucrose flotation may be tried (suspend animals in 30% sucrose at 4°C and centrifuge at 4000 rpm).

C. Nontoxic Fixative

Although formaldehyde is a widely used fixative, NTF is an alternative to formaldehyde that is easily made, stable, and nontoxic. More importantly, we have found it to greatly improve sensitivity and preservation of fine structure, and it appears to be less prone to overfixation. This protocol was originally developed using STF (Streck Tissue Fixative; Streck Laboratories; Montgomery *et al*., 1998), but this reagent has been discontinued. NTF is based on the composition of STF (documented in United States Patent 5460797) and in our hands appears to be equally effective.

D. Purchase of Ready-Made Reagents

Many of the reagents in this protocol, such as 2× SSC and 1 M Tris-HCl, can be easily made. The ready-made forms are purchased only to make it less likely that RNase contamination might be introduced.

E. Labeling of Nuclei

We have found that use of fluorescence (e.g., to visualize DAPI) causes the purple color to develop very rapidly in a nonspecific manner. If the slides are dehydrated and mounted in a permanent mounting medium (e.g., Permount with DAPI) the nuclei can be visualized, although the morphology is adversely affected.

F. Staining Small Quantities of Embryos or Other Stages

A larger number of animals is necessary for consistent freeze-cracking. When the number of specimens is limiting, sterile adults of recognizable body morphology could be added (e.g., Dpy; Glp). If it is desired to stain larvae or adults, they should be synchronized so that they are all the same size prior to mounting on the slides.

G. Staining of Other Species

We and others have been successful in staining other nematode species, including *C. remanei* and *C. briggsae* (Coroian *et al*., 2005; Lin *et al*., 2009) and

Pristionchus pacificus (George Hsu, Heather Roberson, G.B.-M. and M.M., unpublished observations) with species-specific probes. One consideration for embryonic staging is the degree to which embryos are retained by adults. In our hands, *C. remanei* (strain PB4641) and *C. briggsae* (AF16) retain fewer embryos than *C. elegans*. Possible solutions are to limit the amount of food to promote egg retention; use of a mutant background (e.g., Egl) that retains eggs (we have used *ir12*, an uncharacterized recessive *dpy* mutant of *C. briggsae* with good results); to bleach gravid adults and let the isolated embryos develop for several hours; or to bleach to isolate embryos from a plate. In our experience, bleaching solution negatively affects staining quality, so it is recommended to use a diluted bleach solution and rinse animals thoroughly. For consistent freeze-cracking, adults can be added back as suggested above.

VIII. Troubleshooting Guide

Problem	Cause	Remedy
Lack of signal	Lack of template for transcription	Confirm amplification of PCR product with gel electrophoresis.
	Error in T7 sequence	Confirm that primer sequence was correct as ordered; subclone PCR product into T/A vector and sequence it; be certain correct (reverse) primer had the T7 sequence, not the sense primer
	Wrong synthesis kit or components	Confirm use of all components from a DIG-UTP kit, not DIG-dUTP (used for DNA probes)
	Poor transcription	Run side-by-side aliquots of (+)T7 and (−)T7 transcription reactions to confirm synthesis of RNA
	Developer problem	Spot the probe onto a blotting membrane (e.g., nitrocellulose or Hybond-N+) and test for color turnover. Try a previously successful probe. Remake developer components and solutions. (It is recommended to include a positive control in all experiments to rule out problems with the reagents or solutions.)

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Problem	Cause	Remedy
Slides have very high background or are all purple	Probe excess (signal throughout embryos and adults; developer may turn purple overnight)	Dilute probe further. Typically 1 μL of probe, diluted in 1 mL of hybridization buffer, is sufficient
	Inconsistent freeze-cracking technique (nonspecific signal on "top" of animals)	1. When wicking liquid away from the animals, avoid having floating worm carcasses (excess liquid left) or burst embryos (too little liquid). If slight pressure is applied to the coverslip and the specimens do not float around but swell slightly in response to the pressure, then it is likely a good mount. 2. When "cracking" the cover slip off, make sure you feel pressure as the cover slip is removed. If it comes off too easily, then the outer cuticle or egg shell of the animals will likely not be permeabilized properly
	Choice of probe	Try a different or longer probe to your gene of interest
Uneven signal (some staining that looks real, but some areas with nothing)	Overcrowding of animals	This is usually the result of inconsistent freeze-cracking. Make sure animals are evenly dispersed on the slide prior to adding the coverslip. Try using fewer animals. Make sure there is no debris or worm clumps that would hold up the coverslip in parts

IX. Imaging of Stained Embryos

For imaging of stained embryos, we use an Olympus BX51 equipped with DIC optics and a 60×/1.4 oil immersion lens. It is highly recommended that a color camera be used to document *in situ* staining. DIC images of embryos can contain regions that are darker simply due to the nature of DIC itself. As the *in situ* staining appears brown or purple, faint signal can more readily be seen and distinguished from the surrounding tissue if a color camera is used. We have had good results with a consumer grade Canon APS-C-size sensor Digital SLR camera with a C-mount converter from LMscope (http://www.lmscope.com/

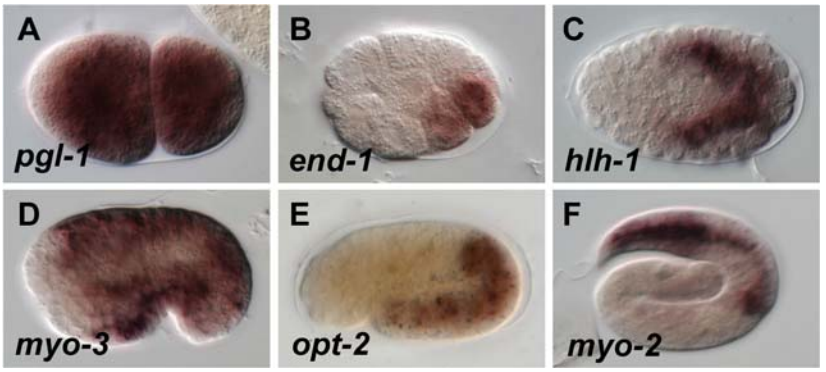

Fig. 5 Antisense RNA probes detect endogenous mRNAs consistent with published reports. (A) Maternal transcripts of *pgl-1* at the two-cell stage (Kawasaki *et al*., 1998). (B) Expression of *end-1* in the E daughter cells (Zhu *et al*., 1997). (C) Expression of *hlh-1* in muscle precursors (Krause *et al*., 1990). (D) Activation of *myo-3* in body muscle cells (Okkema *et al*., 1993). (E) Expression of *opt-2* in intestine cells (Nehrke, 2003). (F) Expression of *myo-2* in pharynx muscle cells (Okkema *et al*., 1993). Anterior is left, and dorsal is up. Embryos are approximately 50 μm long. (For color version of this figure, the reader is referred to the web version of this book.)

index_e.html), a platform that together costs approximately $1500, and which is also suitable for fluorescence microscopy. It is also recommended that many animals (>25) of specific stages be examined for staining, and that the number of animals with good staining be quantified. Under optimal conditions, we routinely observe that at least 75%, and usually greater than 80%, of embryos will show detectable signal (Lin *et al*., 2009). The sensitivity of the approach has been confirmed by quantification of transcripts by single molecule detection (Raj *et al*., 2010). Quantification of endogenous transcripts of zygotic genes expressed in endoderm specification has shown that there are at most some 400 transcripts of *end-3* in the early E lineage (Raj *et al*., 2010). We have observed very strong expression of *end-3* in the E cell of early embryos (Fig. 5B) (Maduro *et al*., 2007), suggesting that this procedure is sensitive enough to detect several hundred transcripts. Given that the signals observed for *end-3* are fairly strong, it is likely that smaller numbers of transcripts (e.g., around 100) could be detected with this approach.

X. Summary

The detection of mRNA *in situ* provides a rapid means by which to determine the expression pattern of endogenous genes. A timetable is provided to assist in planning (Fig. 6). We have described a protocol that, in our hands, results in reproducible staining of endogenous mRNAs with a lower limit of at most several hundred

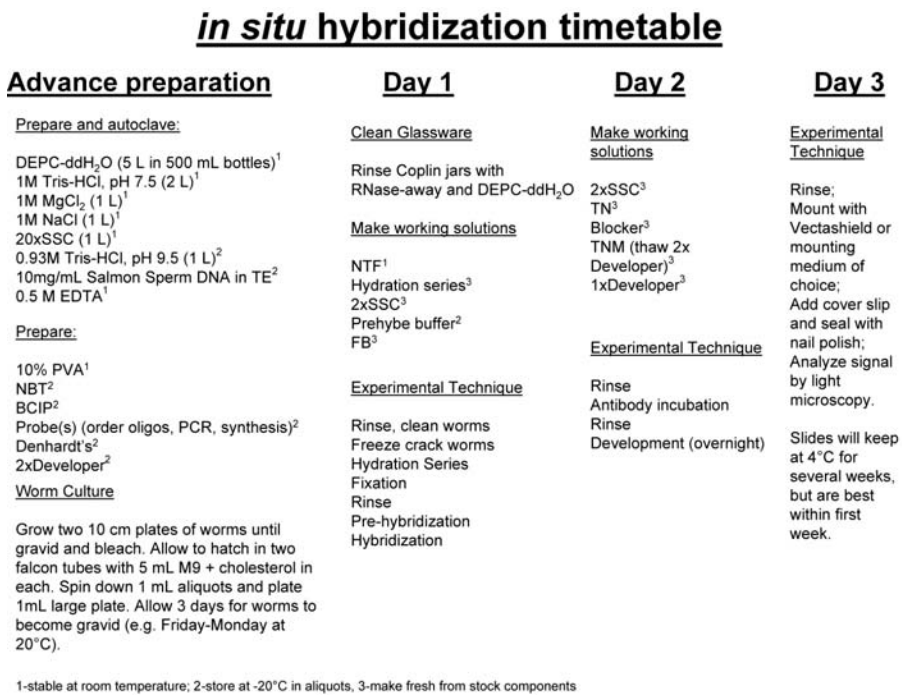

Fig. 6 Suggested timetable of steps in the procedure.

transcripts per cell. This approach should be useful for laboratories that wish to make a semiquantitative determination of gene expression at a modest cost in *C. elegans* or other related species.

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