in situ hybridization of embryos with antisense RNA probes

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1. Introduction

Localization of endogenous transcripts is a desirable approach for confirming expression patterns. Here we present an *in situ* hybridization protocol that uses a non-toxic fixative with RNA probes, and which can be performed in less than three days. Combined with other steps used to optimize signal/noise ratio, this approach may offer increased sensitivity, perhaps because RNA:RNA hybrids are more stable than DNA:RNA hybrids (Sugimoto et al., 1995). The procedure has been used successfully to detect embryonic transcripts in *C. elegans* and other nematode species (Broitman-Maduro et al., 2006; Coroian et al., 2005). A summary and timetable of the procedure is given as the last figure (Fig. 6).

2. Probe design and synthesis

Antisense RNA probes are created from *in vitro* transcription of short (200bp – 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 (Figure 1). Alternatively, a cloned cDNA fragment can be used. Some may choose to also synthesize the complementary (sense) strand as a control probe, or use one of the suggested probes (section 5) for specificity.

(Figure 1 would go here)

2.1. PCR to generate probe synthesis template

Assemble the following in a PCR tube:

Forward primer (25pmol/µL)	1µL
Reverse primer (25pmol/µL)	1µL
dNTPs (10mM)	0.5µL
PCR buffer (10x stock)	2.5µL
Genomic DNA (200ng/µL)*	1µL
Taq Polymerase**	0.5µL
ddH_2O	18.5µL (total volume: 25µL)

*Alternatively, use 1µL of a solution carrying 10ng of plasmid template.
**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 with repetition of [95°C for 30 sec, 72°C for 30 sec, 55°C for 30 sec] for 30 cycles, then 72°C for 10 min. Check an aliquot (1µL) on an agarose gel to make sure the PCR product is of the expected size.

2.2. DIG-labeled probe synthesis:

Use DIG RNA labeling kit (Roche, #1175025)

PCR reaction generated above (not purified) 3µL

10xNTP mixture with DIG-11UTP	1µL
10xTranscription Buffer	1µL
RNase Inhibitor	0.5µL
T7 RNA Polymerase	1µL
DEPC-ddH_2O	3.5µL (total volume: 10 µL)

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

3. Animal Preparation and Fixation

3.1. Synchronization of strain 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 (10µg/mL) at 20°C. Embryos are collected by centrifugation and plated on large NGM plates for three days at 20°C. After approximately three days the plates should contain gravid hermaphrodites with early embryos. If later-staged embryos are required, incubation may be extended a further five hours. The appropriate time will depend on incubation temperature, abundance of food, general health of the strain, and tendency of the worms to retain embryos.

3.2. Fixation of gravid hermaphrodites and embryos

Harvest the worms by washing each large plate with 3mL 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 1mL of Hypaque Meglumine (60%, available from http://www.nanric.com as Reno-60). Invert the tube several times and centrifuge for 30 seconds at 2000rpm. After centrifugation, the worms will float to the top of the eppendorf tube (Figure 2A). Remove the worm layer and rinse 2-3x in M9. Examine an ~80µL 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 (Figure 2B). If a many bacterial clumps are still present, repeat the meglumine flotation until worms appear clean. Resuspend worms in 750µL of M9.

(Figure 2 would go here)

3.3. 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 4h at at 180°C. Alternatively, glassware may be cleaned thoroughly with RNase away (MBP, #7003, 1L). Where indicated, disposable plastics may be used for convenience.

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 50mL of non-toxic fixative (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 five 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 them.

(Figure 3 would go here)

Resuspend the worms in M9 by inversion. Pipette 80µL of worm suspension onto the center of a poly-L-lysine coated slide (Fisher, #12-550-19) and spread droplet with a clean razor blade so that the worms contact the surface of the slide. Begin cutting worms with the razor in an up-down motion while observing 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 22x40mm 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 five minutes before proceeding to the hydration series.

(Figure 4 would go here)

3.4. Hydration series

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

1) 5 minutes- 100% methanol (at -20°C)
2) 5 minutes- 90% methanol (room temperature)
3) 5 minutes- 70% methanol (room temperature)
4) 5 minutes- 50% methanol (room temperature)
5) 5 minutes- DEPC-ddH_2O (room temperature)
6) 1 hour- NTF (pre-warmed to 37°C)

Place Coplin jar, with NTF and slides, into a 37°C incubator during the one hour incubation. During incubation, prepare 10mL Prehybridization Buffer (PB) and incubate at 65°C with occasional vortexing to resuspend components. PB may require up to 30 minutes at 65°C to become fully resuspended.

4. Hybridization and Signal Development

4.1. Prehybridization and Probe Hybridization

Rinse slides in the following buffers:

1) 5 minutes – DEPC-ddH_2O
2) 5 minutes – DEPC-ddH_2O
3) 5 minutes – 2xSSC
4) 5 minutes – 2xSSC

Have a humid chamber ready. It is convenient to use empty pipette tip boxes, with an unfilled pipette tip rack inserted, and with a 3cm depth of 42°C water underneath. Place slides face up in the chamber and add 300µL PB onto fixed worms. Be sure that the samples are completely covered with PB. Place the humid chambers into a hybridization oven (or other incubator) pre-warmed to 42°C and incubate 1 hour.

Towards the end of the incubation, thaw frozen probe(s) and dilute 1:500 to 1:1000 in PB (typically, 1-2µL probe in 1mL PB) and heat to 65°C for 5 minutes. Add the diluted probe to the top of the slides. Hybridize at 42°C overnight. It is convenient to prepare two 50mL aliquots each of 2xSSC and Formamide Buffer (FB) and incubate them at 42°C overnight.

4.2. Rinse and Antibody Incubation

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

1) 5 minutes – 2xSSC (42°C)
2) 5 minutes – 2xSSC (42°C)
3) 5 minutes – FB (42°C)
4) 5 minutes – FB (42°C)

Rinse slides in the following buffers at room temperature:

1) 5 minutes – 2xSSC (room temperature)
2) 5 minutes – 2xSSC (room temperature)
3) 5 minutes – Tris-NaCl (TN) (room temperature)
4) 5 minutes – TN (room temperature)

Block slides prior to antibody incubation at 37°C in 30mL Blocking Buffer (BB) for 30 minutes.

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

4.3. Rinsing and Signal Development

Rinse slides in the following buffers:

1) 10 minutes – TN (room temperature)
2) 10 minutes – TN (room temperature)
3) 10 minutes – TNM pH 9.5 (room temperature)
4) Developer – Incubate 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 minutes each, and add mounting medium (e.g. Vectashield, Vector Labs). Cover with 22x40mm 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.

5. Reagents

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

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

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. Non-Toxic Fixative (NTF)

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

Add DEPC-ddH_2O up to 1L. Make and store in an autoclaved glass container at room temperature. Heat 30-50mL 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 re-use.)

3. 50xDenhardt's Solution

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

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

4. Salmon Sperm DNA

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

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

5. Prehybridization Buffer

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

Add DEPC-ddH_2O up to10mL. Vortex the suspension and heat to 65°C to get into solution. This may take 30 minutes or more.

6. Formamide Buffer (FB)

Formamide	30mL
20xSSC	0.5mL

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

7. Tris pH 7.5, NaCl (TN)

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

Add up to 500 mL DEPC-ddH_2O and autoclave.

8. Blocking Buffer

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

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

9. 1M Tris, pH 9.5

1M Tris, pH 7.5 (Fisher, #1757-500)	500mL
10M NaOH	43.5mL

Dispense aliquots into sterile 50-mL falcon tubes and freeze at -20°C. May be thawed in 37°C incubator prior to use.

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

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

Autoclave for 15 min at 121°C,15 lb/in^2. Dispense aliquots into 50mL Falcon tubes. Store at -20°C. Thaw the morning that it is needed.

To 25 mL of 2xTNM, add 25 mL DEPC-ddH_2O. This is used as the last wash prior to development. The other 25mL 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. 2x Polyvinyl Alcohol (PVA)

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

This solution takes very long to go into solution. It is best to start with freshly autoclaved DEPC-ddH_2O and add PVA to a sterile container. Incubate at 65°C for several days, stirring often with a 25mL plastic serological pipet; 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

2xTNM	25mL
2xPVA	25mL
NBT	200µL
BCIP	200µL
Levamisole	7.2mg

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

6. Notes

6.1. Suggested positive controls

Gene	Expression Pattern	Primers (forward primer, T7-tagged reverse primer)
pgl-1	Germline and embryos	gtt caa gga atc aac tcg aag act c, TAA TAC GAC TCA CTA TAG GGA CTt ggc aga gct act gat ttc gtt gga
end-1	Early E lineage (E, Ea/Ep)	ttc aat cgt acg atc cag cac aac aat cg, TAA TAC GAC TCA CTA TAG GGA CTT GCA CTc aat agc tcc tga atc agt t
hlh-1	Mid to late embryogenesis, muscle lineages	aaa cca gcc agc tta cta cct ccc gtc cta, TAA TAC GAC TCA CTA TAG GGA CTc gtt ccc gag ctt atg atg atc tct atc
opt-2	Mid to late embryogenesis, intestine	gta atg gcg att gga ctc tca cat atg acc, TAA TAC GAC TCA CTA TAG GGA CTt ctg ctg ccg tga tga caa cga ttt gtg
myo-2	Late stage embryos, pharynx muscle	gga tgt tgt cca aga gat gaa tcc acc a, TAA TAC GAC TCA CTA TAG GGA CTt gtt caa tat cgc aag aag cga cac gtc
myo-3	Late stage embryos, body muscle	tct cgt gtc gtc cgt cag gca cca gga gag, TAA TAC GAC TCA CTA TAG GGA CTc ctg gtg atg atc cac ttg aac ata cgg
cup-4	4 coelomocytes in embryos, 6 in adults	gta gta gca tct cta ata tcc atg acg ttc, TAA TAC GAC TCA CTA TAG GGA CTt cct tga acg tat tag gaa tgt att ctt

6.2. 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).

6.3. Non-Toxic Fixative

Although formaldehyde is a widely-used fixative, NTF is an alternative to formaldehyde that is easily made, stable and non-toxic. More importantly, we and 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), but it has been discontinued. NTF is based on the composition of STF (documented in United States Patent 5460797) and in our hands is equally effective.

6.4. Purchase of ready-made reagents

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

6.5. Labeling of nuclei

We have found that use of fluorescence (e.g. to visualize DAPI) causes purple color to develop very rapidly. 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.

6.6. Staining small quantities of embryos or other stages

A larger number of animals is good 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.

6.7. Staining of other species

We and others have been successful in staining other nematode species, including *C. remanei* and *C. briggsae* (e.g. Coroian *et al*., 2005) and *Pristionchus pacificus* (George Hsu, 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 less 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 *dpy* mutant of *C. briggsae* with good results); 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.

7. Troubleshooting guide

Problem	Cause	Remedy
Lack of signal	Lack of template for	Confirm amplification of PCR product with gel

	transcription	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 a (+)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.)
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 1µL of probe, diluted in 1mL 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 cover-slip and the specimens do no 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 less animals. Make sure there is no debris or worm clumps that would hold up the coverslip in parts.

8. Sample images

For imaging of stained embryos, we use an Olympus BX51 equipped with DIC optics and a 60x/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 differential interference microscope images. As the *in situ* staining appears brown or purple, even faint signal can more readily be seen if a color camera is used. We have had good results with a consumer grade Canon 350D digital SLR camera with a C-mount converter from LMscope (http://www.lmscope.com/index_e.html), a platform that together costs approximately $1500, and which is also suitable for fluorescence microscopy.

(Figures 5 and 6 would go here)

9. Acknowledgments

We thank others who have helped us test and develop the protocol and provide advice about reagents, in particular George Hsu, Andre Pires da Silva, Lenore Price, David Fitch, and Andy Fire. The protocol was developed through funding from the NSF (grants #0416922, #0643325) to M.M.

10. References

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Figure Legends

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 5' 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.

Fig. 2. 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 minutes at room temperature, or after a brief 2000rpm centrifugation, the worms will float to the top (right tube) and can be removed by pipetting the top layer.

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

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

Fig. 5. Antisense RNA probes detect endogenous mRNAs consistent with published reports. (A) Maternal transcripts of *pgl-1* at the 2-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 50μm long.

Fig. 6. Suggested timetable of steps in the procedure.

Figure 1

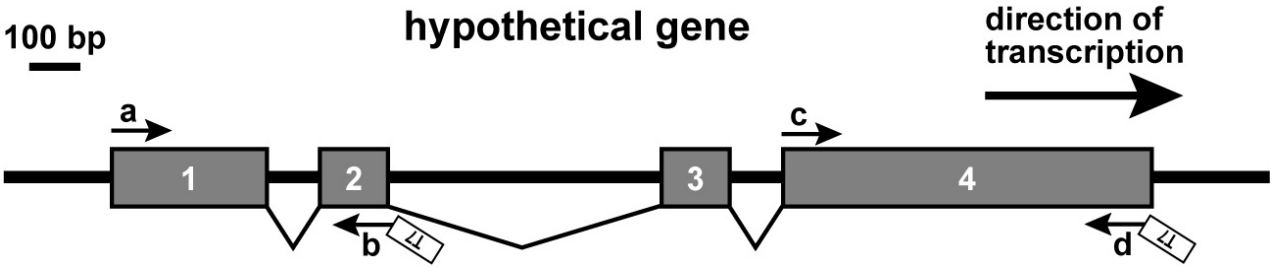


Figure 2

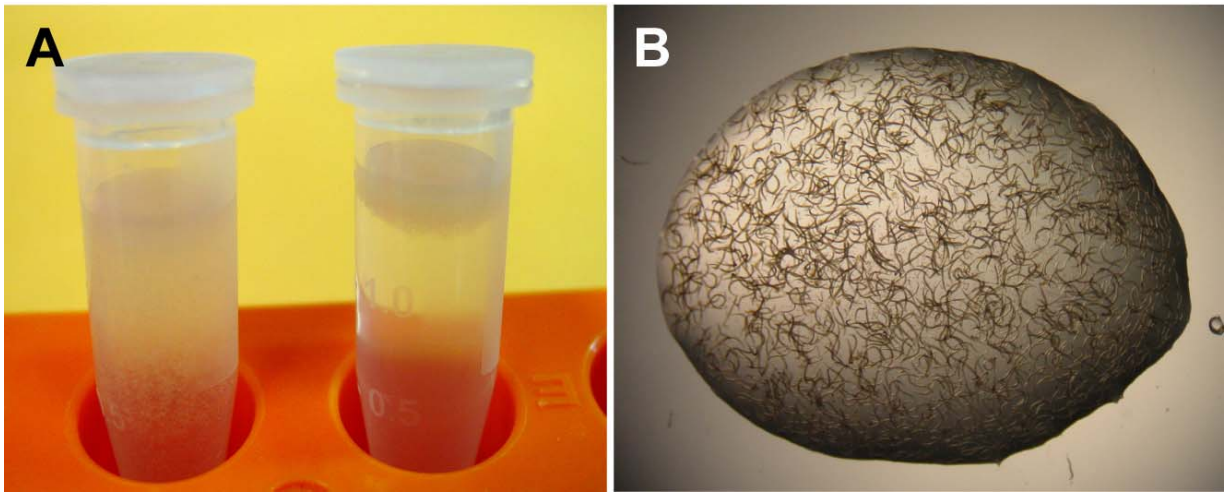
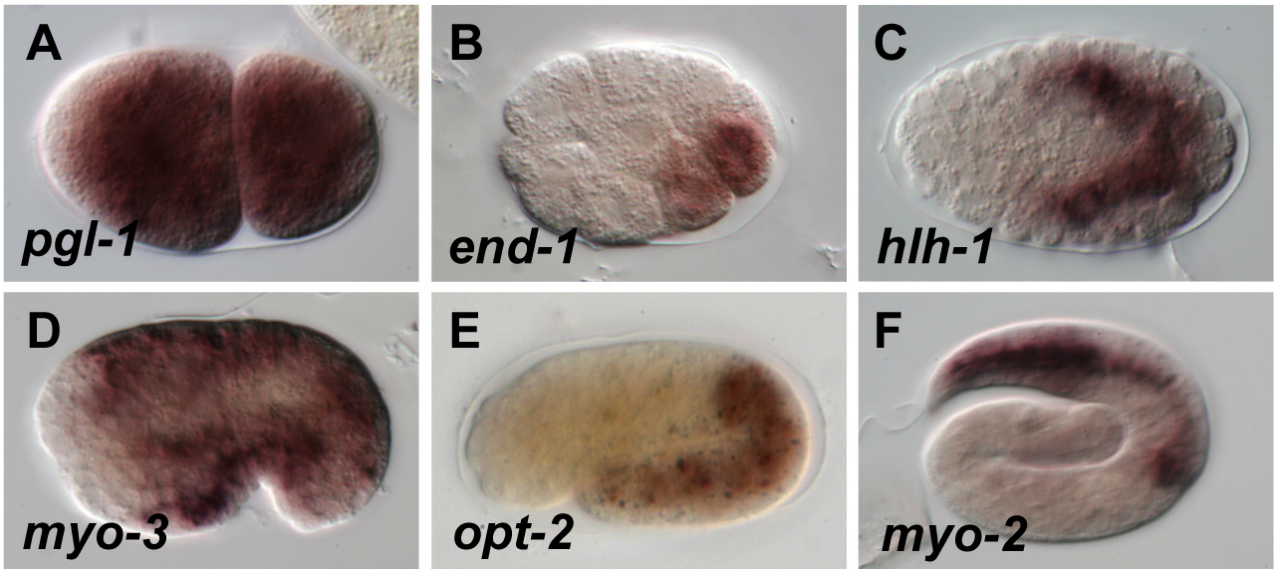


Figure 3

Figure 4

Figure 5



in situ hybridization timetable

Advance preparation

Prepare and autoclave:

DEPC-ddH_2O (5L in 500ml bottles)[1]
1M Tris-HCl, pH 7.5 (2L)[1]
1M $MgCl_2$ (1L)[1]
1M NaCl (1L)[1]
20xSSC (1L)[1]
1M Tris-HCl, pH 9.5 (1L)[2]
10mg/mL Salmon Sperm in TE[2]
0.5M EDTA[1]

Prepare:

10% PVA[1]
NBT[2]
BCIP[2]
Probe(s) (order oligos, PCR, synthesis)[2]
Denhardt's[2]
2xdeveloper[2]

Worm Culture

Grow two 10cm plates of worms until gravid and bleach. Allow to hatch in two falcon tubes with 5mL M9 + cholesterol in each. Spin down 1mL aliquots and plate 1mL large plate. Allow 3 days for worms to become gravid (e.g. Friday-Monday at 20°C).

Day 1

Clean Glassware

Rinse Coplin jars with RNase-away and DEPC-ddH_2O

Make working solutions

NTF[1]
Hydration series[3]
2xSSC[3]
Prehybe buffer[2]
FB[3]

Experimental Technique

Rinse, clean worms
Freeze crack worms
Hydration Series
Fixation
Rinse
Pre-hybridization
Hybridization

Day 2

Make working solutions

2xSSC[3]
TN[3]
Blocker[3]
TNM (thaw 2x developer)[3]
1xdeveloper[3]

Experimental Technique

Rinse
Antibody incubation
Rinse
Development (overnight)

Day 3

Experimental Technique

Rinse;
Mount with Vectashield or mounting medium of choice;
Add cover slip and seal with nail polish;
Analyze signal by light microscopy.

Slides will keep at 4°C for several weeks, but are best within first week.

1-stable at room temperature; 2-store at -20°C in aliquots, 3-make fresh from stock components