Chapter 2

In Situ Hybridization Methods for RNA Visualization in *C. elegans*

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Abstract

A protocol is described for the detection of endogenous mRNA in whole-mount tissue using antisense RNA probes of at least 400 bases in length. The protocol takes a few days and is inexpensive, yet sensitive enough to detect low-abundance transcripts. Although optimized for the nematode *C. elegans*, the protocol should work on similar tissue.

Key words Whole-mount in situ hybridization, Transcript detection, Fixation, Antisense RNA probe

1 Introduction

Detection of mRNA in situ offers a number of advantages for the identification of transcripts in fixed tissue samples. For example, endogenous messages can be detected in specimens that lack an efficient transgene technology. Even in species for which such technology is available, in situ hybridization can be used to confirm transgene expression patterns. Limitations on in situ hybridization include possible difficulty in identifying cells, preserving structure during fixation, and obtaining reproducible permeabilization.

A significant development in detection of individual mRNAs in situ is single-molecule Fluorescent In Situ Hybridization (smFISH), which can be used to quantify the number of individual mRNAs expressed at cellular resolution [1]. Though smFISH is also achievable on a short time scale, it requires robust fluorescence microscopy, high-resolution image acquisition/processing, and much more costly probe sets [2].

The method described here uses antisense RNA probes generated in vitro, and may be useful as an alternative to smFISH when highly quantitative information is not needed. The protocol has been customized for the nematode *C. elegans*, but which should work with any transparent animal tissue that can be fixed on microscope

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slides. Sample embryonic expression patterns are shown for two embryonically expressed genes. The method is relatively inexpensive and can be accomplished in approximately 3 days' time.

2 Materials

Most solutions for RNA-based in situ hybridization (RNA-ISH) can be made in advance and stored frozen at –20 °C in single-use aliquots, or kept at room temperature (20–25 °C) after autoclaving. Due to the sensitivity of the assay to contaminants that can degrade RNA, it is best to make enough reagents for repeat experiments and troubleshooting. All recipes for premade reagents have been scaled for multiple experiments, and some like Sheared Salmon Sperm are not practical to make on a smaller scale. For these, it may be advantageous to purchase ready-made components. The solutions that can be premade will be addressed first; these can be scaled according to experimental need. It is best to use new or RNA-designated glassware for reagents that can be stored at room temperature and use them only for their designated buffers. All others can be made conveniently in sterile, disposable Falcon tubes (or equivalent).

2.1 Solutions that Are Stable at Room Temperature

2.1.1 Ready-Made

1 M Tris, pH 7.5 (Fisher, 1757-500)

20× SSC (Fisher BP1325-1)

0.5 M EDTA, pH 8.0 (Fisher M4055)

10× PBS (Life Technologies, AM9625, RNase-free)

2.1.2 Researcher-Prepared

DEPC-treated ddH_2O (0.1 % v/v) (500 mL)

Millipore-quality ddH_2O 500 mL

DEPC (MP Biomedical 150902) 0.5 mL

Suspend DEPC in 500 mL ddH_2O and stir overnight in 500 mL glass bottles. Autoclave for 20 min at 121 °C. Make at least five 500 mL bottles, as all reagents require DEPC-treated ddH_2O to protect the integrity of RNA targets and probes.

2 M NaCl (500 mL)

NaCl 58.44 g

DEPC-treated ddH_2O up to 500 mL

Mix and autoclave. Store at room temperature.

1 M MgCl (500 mL)

MgCl 47.6 g

DEPC-treated ddH_2O up to 500 mL

Mix and autoclave. Store at room temperature.

Tris-NaCl Buffer (TN, 500 mL)

1 M Tris, pH 7.5 50 mL

2 M NaCl (above) 37.5 mL

DEPC-treated ddH_2O up to 500 mL

Make from sterile components (above) in an RNA-designated, autoclaved bottle and store at room temperature. Make two bottles, as TN is needed to make TN-EDTA (Stop Solution).

TN-EDTA (TNE, 500 mL)

TN (above) 490 mL

0.5 M EDTA, pH 8.0 10 mL

Add EDTA to autoclaved TN (Above) and make note that this is now a Stop Solution. Store at room temperature (20–25 °C).

2× Polyvinyl Alcohol (PVA, 500 mL)

PVA (Fluka 40-88, Sigma # 81386) 50 g

DEPC-ddH_2O 300 mL (up to 500 mL total)

Prepare in a wide-mouthed bottle. Add PVA to freshly autoclaved ddH_2O that is still hot. Mix to get all PVA into the hot ddH_2O. Place jar into a 55 °C water bath. Stir every few hours with a sterile, disposable plastic pipette (25 or 50 mL works best because of its strength). Do not invert the bottle to mix, as reagent may seal the lid permanently shut. This solution will take several days of occasional stirring to fully suspend. Once suspended, it will resemble glycerol. Do not use a stir bar to mix solution and do not autoclave. This recipe is used to make 20 portions of developer, but is not practical to make in smaller quantities. Store at room temperature (20–25 °C).

2.2 Solutions that Are Stored at 4 °C

2.2.1 Ready-Made

Formamide (Fisher, BP227-500)

Anti-DIG-AP (Roche, 11093274910)

2.3 Solutions that Are Stored at –20 °C

2.3.1 Ready-Made

Reagents for PCR, (*Taq* PCR Core Kit, Qiagen-201223)

Custom-designed oligos for probe synthesis (Integrated DNA Technologies, or equivalent)

RNA DIG Labelling Kit (Roche, 1175025)

RNase Inhibitor (rRNAsin, Promega N2511)

NBT/BCIP (Roche 11383213001 and 11383221001)

2.3.2 Researcher-Prepared

50× Denhardt's Solution (10 mL)

Can be made and stored in 200 µL frozen aliquots or purchased as a stock solution and stored.

Ficoll (Type 400; Fisher BP525-5) 0.1 g

Polyvinylpyrrolidone (PVP; Fisher BP431-100) 0.1 g

Bovine Serum Albumin (BSA; Fisher BP9703-100) 0.1 g

DEPC-treated ddH_2O up to 10 mL

Mix components in sterile, 10 mL Falcon tube. Filter though a 0.45 µm syringe filter and store frozen in 200 µL aliquots (–20 °C). This recipe makes fifty 200 µL aliquots.

Sheared Salmon Sperm DNA (50 mL)

Can be made and stored frozen in 1 mL aliquots. If only a few experiments are needed, purchasing pre-sheared DNA is recommended.

Salmon Sperm DNA (Fisher NC0448132) 0.5 g

Tris-EDTA pH 8.0 (TE, Fisher BP2473) 50 mL

Suspend DNA in TE in an autoclavable glass bottle. Autoclave suspension for 15 min in order to shear the high molecular weight DNA into 200 bp–5 kbp fragments. Run an aliquot (1–2 µL) on a standard agarose gel (0.7 %) to ensure that correct fragmentation has occurred. DNA can be sonicated to decrease size further, if needed. Aliquot sheared DNA into 1 mL (10 mg) aliquots and freeze. Smaller volumes should be avoided due to loss of liquid levels during the autoclaving process. This recipe makes fifty 1 mL aliquots.

Hybridization Buffer (HB-10 mL)

Can be made ahead and stored, after suspension, in –20 °C. Since buffer does not freeze at –20 °C, aliquots are not necessary.

20× SSC (Fisher, BP1325-1) 2 mL

Dextran Sulfate (Fisher, BP1585-100) 1 g

Formamide (Fisher, BP228-100) 5 mL

0.5 M EDTA, pH 8.0 40 µL

50× Denhardt's Solution (above) 200 µL

Sheared Salmon Sperm DNA (above) 1 mL

DEPC-ddH_2O (above) up to 10 mL

Add 20× SSC to dextran sulfate and 1 mL DEPC-ddH_2O in a 50 mL conical tube and vortex to mix. Add remaining components and vortex. Add DEPC-treated ddH_2O to obtain a final volume of 10 mL. Heat in 55–60 °C water bath for at least 30 min, or until the components are all in solution. Use, or freeze in –20 °C. This recipe makes enough for 5–10 experiments depending on the number of probes and amount of slides.

Tris Buffer, pH 9.5 10× 50 mL aliquots (TB, pH 9.5)

1 M Tris pH 7.5 (Fisher, 1757-500) 500 mL

10 M NaOH 43.5 mL

Add NaOH directly to 1 M Tris, pH 7.5 bottle and invert to mix. Dispense into aliquots into 50 mL Falcon tubes and freeze at –20 °C. 100 mL are needed to make 500 mL of 2× TNM (see below).

Tris Buffer (pH 9.5), NaCl, $MgCl_2$ (2× TNM, 500 mL)

1 M Tris (pH 9.5) 100 mL

2 M NaCl 50 mL

1 M $MgCl_2$ 50 mL

DEPC-ddH_2O 300 mL

Add sterile components together and filter sterilize (if desired) as autoclaving solution may result in a precipitate. Dispense into 50 mL aliquots and freeze. Make enough for ten experiments.

2.4 Solutions that Are Prepared Fresh, as Needed

Researchers are given the choice between two fixatives, the traditional formaldehyde fixative and the nematode tissue fixative (NTF). Although formaldehyde is toxic and must be handled with gloves, it is readily available in many molecular laboratories. NTF is less toxic; however, the components are not generally available on hand. In our hands, NTF improves the signal clarity after development and is therefore recommended for low-abundance messages; however, formaldehyde fixation is likely to be sufficient for most applications.

Formaldehyde Fixative (50 mL) (toxic—wear gloves and dispose in toxic waste)

Formaldehyde (37 % solution) 5 mL

10× PBS 5 mL

DEPC-treated ddH_2O 40 mL

Make in sterile, disposable 50 mL conical tubes and heat in RNaseAWAY treated Coplin jar at 37 °C, 20 min prior to use. Fixative is not stable and must be made fresh each time. Do not allow fixation to proceed longer than 30 min at 37 °C.

Or:

Nematode Tissue Fixative (NTF, 50 mL)

2-bromo-2-nitro-1,3-propanediol (Fisher, #AC15882-1000) 1.5 g

Diazolidinyl Urea (MP Biomedical, #ICN19019183) 1.5 g

Zinc Sulfate Heptahydrate, $ZnSO_4 \cdot 7H_2O$ 0.6 g

Sodium Citrate 0.15 g

Sodium Citrate (originally in recipe) may be omitted with no deleterious effect.

Add up to 50 mL DEPC-treated ddH_2O, heat to 50 °C to dissolve all components, filter sterilize, and place in RNase-AWAY treated Coplin jar at 37 °C, 20 min prior to use.

Do not allow fixation to proceed longer than 30 min at 37 °C.

Methanol Hydration Series (50 mL each, in four clean, RNase-free Coplin jar)

1. 100 % methanol (chilled to –20 °C)
2. 70 % methanol (room temperature)
3. 50 % methanol (room temperature)
4. DEPC-treated ddH_2O (room temperature)

2× SSC (50 mL, make 2–50 mL portions in clean, RNase-free Coplin jars). Make fresh prior to use. Do not store.

20× SSC (Fisher, BP1325-1) 5 mL

DEPC-ddH_2O 45 mL

Formamide Buffer (FB-50 mL, make 2–50 mL portions). Make fresh prior to use. Do not store.

Formamide (Fisher, BP228-100) 30 mL

20× SSC (Fisher, BP1325-1) 0.5 mL

DEPC-treated ddH_2O 19.5 mL

Mix components in a 50 mL Falcon tube and transfer to RNase-free Coplin jar. Heat to 37 °C just prior to use.

2×Blocking Buffer (50 mL). Make fresh just prior to use. Do not store.

Milk Blocker (Bio-Rad #170-6404) 2.5 g

BSA 2.5 g

TN (above) up to 50 mL

Mix milk blocker and BSA in TN in a 50 mL Falcon tube and shake/vortex to resuspend. Do not use until blocking buffer is well suspended. Add 25 mL TN to 25 mL 20×Blocking Buffer. The other aliquot will be used to incubate the antibody with the slides.

TNM Rinse (50 mL)

Thaw 50 mL 2× TNM and divide into two 25 mL aliquots. Add 25 mL DEPC-ddH_2O to one of the aliquots. This will be used as the final rinse before developing the signal.

Developer (50 mL) (make fresh just prior to use, keep away from light)

To the second TNM (25 mL) aliquot add:

2× PVA (below) 25 mL
NBT 50 µL
BCIP 50 µL
Levamisole 7.2 mg

Mix contents by inverting the tube and pour into aluminum foil-covered Coplin jar.

3 Methods

3.1 Probe Design

Antisense RNA probes for in situ hybridization are generated by in vitro transcription of double-stranded PCR products containing the recognition sequence for the T7 (or T3) RNA polymerase at one end (Fig. 1). Either genomic DNA or cDNA may be used as a probe template, however, some care should be given when choosing a region to amplify in order to maximize probe hybridization and minimize background. For example, repetitive sequences (including

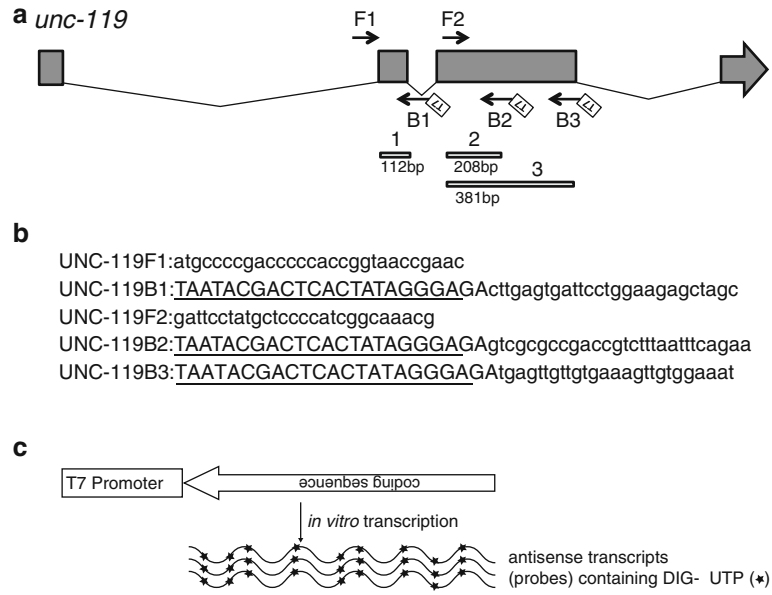

Fig. 1 Structure of the *unc-119* locus of *C. elegans*, primers used to amplify PCR templates for in vitro transcription, and primer sequences. (**a**) The coding exons (*gray boxes*) of *unc-119* are shown. The genomic region is 2,285 bp and the exonic regions total 675 bp. (**b**) Sequences of primers used to amplify antisense probes. The reverse-facing primers B1, B2, and B3 contain the sequence of the T7 RNA polymerase promoter (capital letters; core sequence underlined). The sizes and locations of the PCR products are shown beneath the middle exons. (**c**) General structure of antisense in vitro transcription template, representation of the transcripts generated, and the sequences of the primers are shown 5′–3′

the poly-A sequence in a cDNA, or any other homopolymeric runs of A, if present) should be avoided. For a genomic template, it is best to target the largest exons that are most unique in sequence. Genes that have multiple homologues or share common domains may have ambiguous staining results. If the genome sequence of the species is known, candidate probe sequences could be used to search the genome sequence for possible off-target homology. Whenever possible, it is best practice to use at least two non-overlapping probes for a given gene and to include controls that predictably abrogate or alter the expression pattern. It is also advantageous to synthesize a sense probe as a negative control, and for a positive control, to use a GFP probe in a GFP-expressing transgenic strain that is transcriptionally active at a similar time point during development. The advantage with a GFP probe is that it can theoretically stain any GFP, YFP, or CFP transgenic strain, since these sequences are largely similar. When using this strategy as a positive control, attention should be paid to which coding sequence is used in the organism of choice: In *C. elegans*, GFP and related sequences are typically derived from the original *A. victoria* GFP sequence [3]. In other systems, the synthetic "EGFP" sequence, which has been recoded for codon usage optimization, is often used instead [4].

Probe size can vary between 200 base pairs (bp) to several kilobase pairs (kbp), although around 500 bp–1 kbp are desired to balance probe specificity, access to the target mRNA, and ease of amplification. Oligonucleotide primers for PCR can be synthesized at a 25 nM scale and no special purification is necessary. The forward primer (collinear with the direction of transcription) should be around 30 nucleotides long, while the reverse primer contains the T7 recognition sequence (23mer):TAA TAC GAC TCA CTA TAG GGA GA, followed by ~25 nucleotides of homology to the gene of interest totaling a 50mer oligo primer. If a sense control is desired, the T7 recognition sequence will be on the forward facing primer. In either the sense or antisense experiment, the T7 sequence must be on only one of the two primers.

3.2 Probe Template Preparation

A standard PCR reaction is used to generate a T7-tagged PCR product. A 25 or 50 μL volume can be used, the former is described below.

Assemble the following in a PCR tube:

PCR buffer (10×) 2.5 μL

dNTPs (2.5 mM each) 2.5 μL

Forward Primer (25 pmol/μL) 1 μL

Reverse Primer (25 pmol/μL) 1 μL

Genomic DNA (200 ng/μL) 1 μL

Taq polymerase 0.1–1 μL

ddH_2O up to 25 μL total

Standard thermocycling conditions are used, such as 95 °C denaturation for 5 min, followed by 30 cycles of 95, 72, and 55 °C for 30 s each, then 10 min at 72 °C. The PCR product, which will be used to generate the RNA probe, can be purified by gel electrophoresis, followed by column purification (QIAquick Gel Extraction Kit, Qiagen, 28704), or the PCR-amplified product can be used directly. If the PCR product is not purified by gel electrophoresis, a small aliquot should nonetheless be run on a gel to confirm amplification of the expected product. The DNA in the entire PCR reaction is generally not needed for probe synthesis, which allows the remainder to be saved and used as a template for future use.

3.3 RNA Probe Preparation

The T7-tagged DNA template generated above is used as a template for DIG-labeled RNA synthesis described below. The DIG-labeling reagents can be purchased as a kit from Roche (1#175025) or separately. It is critical to note the use of UTP rather than dUTP, as the probe is made of RNA. The 10× transcription buffer should be thawed completely and vortexed before use. Thawed components should be stored on ice and RNase-free plastics and reagents should be used for RNA synthesis.

Assemble the following in a PCR tube in the following order:

T7-tagged template (PCR reaction)* 6.5 μL

10× NTP with DIG-11-UTP 1 μL

10× Transcription Buffer 1 μL

RNase Inhibitor 0.5 μL

T7 RNA polymerase 1 μL

*Template concentration will typically be between 200 and 500 ng/μL from a standard PCR reaction.

Incubate at least 8 h to overnight in a thermocycler or incubator at 37 °C. After the incubation, add an additional 0.5 μL RNase inhibitor and 30 μL DEPC-treated ddH_2O to the reaction. No further cleanup is needed. Once diluted, the probe is ready for use, or it can be stored at −20 °C until needed.

3.4 Animal Preparation

Worms can be stained at any stage, although we do not have experience with the dauer stage. *C. elegans* can be synchronized by dilute bleach treatment of a culture that includes many gravid hermaphrodites [5]. The isolated early embryos are allowed to hatch overnight in M9 with cholesterol (10 μg/mL). These starved L1s can then be plated onto seeded 10 cm NGM plates and grown to the desired stage. If L1s are desired, eggs should be allowed to hatch on unseeded plates and the L1s collected. If embryonic stages are required, animals are grown until the adults first become gravid, for early embryos. For older embryos, gravid adults can be

transferred to plates without *E. coli*, so that eggs will be retained by hermaphrodites at later stages. Alternatively, a mutant strain (e.g., Egl) that retains eggs can be used. If possible, embryos should be isolated by cutting gravid hermaphrodites open or herniating them as described below. Obtaining embryos from plates is more difficult, as they tend to stick to the bacterial lawn, and are more difficult to clean.

Animals are harvested by washing in 3–5 mL M9 followed by centrifugation at 2,000 rpm. Repeat the M9 washes until the worms are clean and free of bacterial contamination or clumps. Three washes are usually sufficient, however, if further cleaning is required, a sucrose flotation or equivalent can be used [6]. The final suspension volume will depend on the starting quantity of animals; worms from one crowded 10 cm plate can usually be suspended in a final volume of 750 µL. The final animal concentration should be several hundred per 50 µL droplet. Once animals are sufficiently cleaned, they can be set aside until ready to proceed. Do not leave animals for longer than 30 min in liquid, as they will become hypoxic, which could affect gene expression.

3.5 Reagent Preparation

Prepare the freeze-cracking chamber by placing a clean, dry aluminum block on top of crushed dry ice in a Styrofoam ice bucket. This will serve as the contact surface for the slides. The aluminum block facilitates instant and uniform contact with the cold surface and instantly freezes the specimen.

Prepare the hydration series by diluting methanol with DEPC-treated ddH_2O as described in Sect. 2. Place the 100 % methanol at –20 °C to chill.

Next, prepare the fixative of choice and place in a 55 °C water bath to dissolve, followed by 37 °C to equilibrate (NTF) or directly into a 37 °C bath (formaldehyde). The choice of fixative may be determined by numerous factors, including access to reagents on hand, or desired sensitivity. Although, NTF is our fixative of choice because of enhanced sensitivity and less toxicity than formaldehyde (carcinogen), we recognize that formaldehyde is the fixative of choice in many laboratories, and thus offer the option of using it in this protocol.

Once the aluminum block, hydration series and fixative are prepared, the worms can be adhered to slides for RNA-ISH.

Previously, we have described a worm cutting protocol that uses a clean razor blade to cut worms in order to liberate embryos [6]. Here, we describe an alternate method that results in more reproducible permeabilization for those less experienced with freeze-cracking.

3.6 Animal Fixation

The steps for fixation are shown in Fig. 2. A 50–80 µL droplet, containing several hundred animals suspended in M9, is placed onto the center of an adherent microscope slide (Fisherbrand

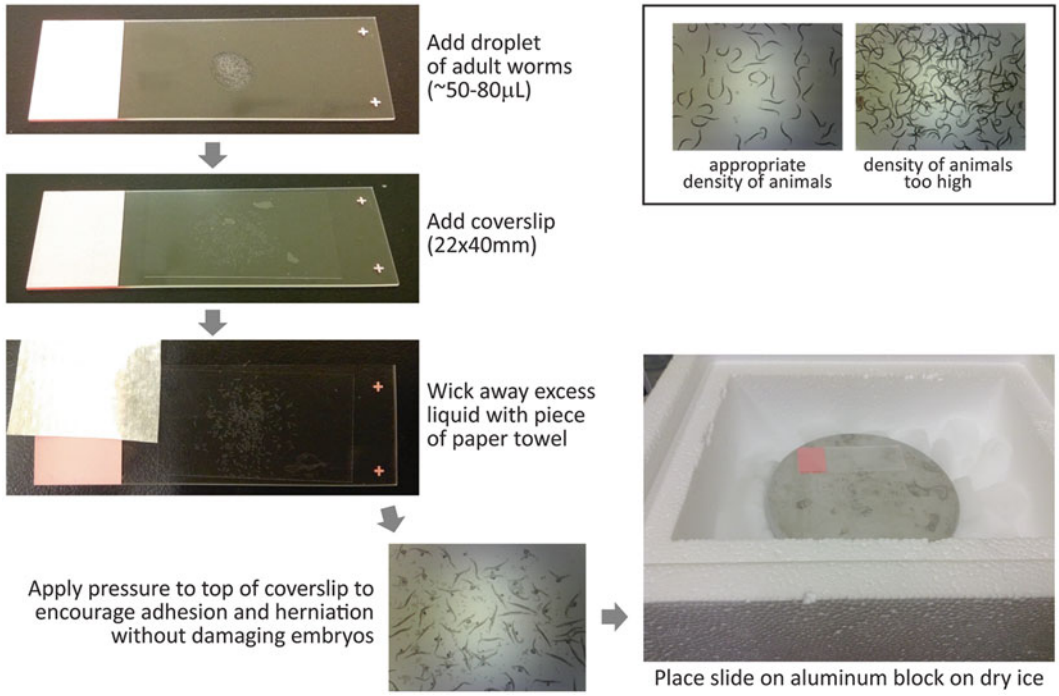

Fig. 2 Preparation of animals on microscope slides. The images shown apply to staining of adults and/or embryos. For other stages, synchronized animals (i.e., of similar size) will be mounted to assure even pressure from the coverslip across the slide, which is important for good freeze-cracking. In this case, rupture of adults can be taken as an indication of appropriate wicking away of liquid. *Inset*: Assessment of density of specimens. After the coverslip is added, there should be very little overlap of animals. An adult *C. elegans* is approximately 1 mm long

Colorfrost #12-550-19) and covered with a 22×40 mm coverslip. This should result in a uniform distribution of animals. Care should be taken not to introduce excess air bubbles as these will impede staining. The density of animals should also not be so high that most adults overlap one another. Once the animals are covered, excess liquid is wicked away by applying Whatman paper, paper towel or Kimwipe around the perimeter of the slide. If the majority of animals are not adhered (evidenced by movement), a pipet tip can be used to apply gentle pressure to the top of the slide, while keeping the Whatman paper or Kimwipe at the edge of the coverslip. As the liquid is reduced, the worms will stop floating above the surface of the slide and some will herniate at the vulva, liberating embryos. Care should be taken not to wick away too much liquid, as this will compromise fixation. If done correctly, the majority of worms should be properly adhered between the coverslip and the slide. It may be difficult to get the entire slide to behave uniformly, however, there should be some areas that are well adhered and will give good staining, as well as some sections where the embryos and adults are damaged, and thus will not stain.

It is worth noting that the presence of adults under the coverslip helps prevent damage to the embryos if too much liquid is wicked away.

Once a slide is ready, it is placed directly onto the cold aluminum block. As the aluminum generally gets covered by a layer of frost, it is helpful to push down on the sides (around the coverslip) in order to improve contact of the slide with the cold surface. The liquid under the coverslip should very quickly be seen to freeze as the slide contacts the block. Prepare all slides as described and add to aluminum block until all samples are finished. Slides should be incubated for a minimum of 15 min up to an hour while additional slides are prepared.

Remove 100 % methanol from freezer and place at beginning of hydration series. Pick up slide with forceps. Wedge a clean, one-sided razor blade, under one corner of the coverslip, and remove the coverslip with a swift twisting motion. The slide should make an audible "cracking" sound and have resistance as the coverslip is removed from the frozen sample. Place slides into the following:

1. 100 % cold methanol for 3 min
2. 70 % methanol for 3 min
3. 50 % methanol for 3 min
4. DEPC-treated ddH_2O for 3 min
5. 37 °C fixative (in water bath or incubator) for 30 min
6. Two rinses in DEPC-treated ddH_2O for 3 min each

3.7 Probe Hybridization

Cold hybridization buffer (without probe) is removed from storage at −20 °C and placed in a 65 °C water bath to equilibrate. Rinsed slides are prepared for hybridization by incubating in 2× SSC in 3 min intervals. This is repeated for a total of two 3-min rinses. A humid chamber is prepared by using an empty pipette tip box, with lid and rack, filled with water in the bottom half. Slides are placed face up in the humid chamber on top of the plastic rack, and covered with 300–500 μL of warm hybridization buffer. Make sure the buffer completely covers the fixed animals but does not run off the sides of the slide. The buffer is viscous and will not dehydrate, so there is no need to cover the slides with a coverslip or Parafilm. The pipette tip box lid is closed and incubated in an air incubator or hybridization oven at 42 °C for 30 min.

If an RNA probe has been stored at −20 °C, it is thawed briefly in a 65 °C water bath. 1 μL of RNA probe is added to 1 mL of hybridization buffer and mixed thoroughly. Incubate for a further 5 min at 65 °C to denature the probe. Each 1 mL aliquot is sufficient for about five slides. Aliquots that are not used may be saved for future use at −20 °C if desired. 200 μL of diluted probe are added directly to the top of each slide containing hybridization buffer. The buffer should remain within the side boundaries and not flow off the side. If too much hybridization buffer is present, some may be removed with a pipettor prior to adding diluted probe. The probe is allowed to hybridize at 42 °C overnight in the humid chamber.

3.8 Antibody Incubation

Prepare four Coplin jars containing 2× SSC and two containing Formamide Buffer (see above). Wash slides as follows in 5 min increments to remove excess unbound probe:

1. 2× SSC
2. 2× SSC
3. Formamide Buffer
4. Formamide Buffer
5. 2× SSC
6. 2× SSC

Prepare slides for antibody incubation by rinsing in Tris-NaCl (TN) buffer in 5 min increments and prepare Blocking Buffer (BB) while slides are incubating:

1. TN
2. TN
3. BB—15 min

To a fresh 30 mL aliquot of BB in a 50 mL Falcon tube, add 10 μl anti-DIG AP and invert to mix. Add to fresh Coplin jar and incubate slides in antibody-BB mixture for at least 2 h and up to 6 h at 37 °C. While slides are incubating, remove 1–50 mL aliquot of 2× TNM from freezer to thaw. Prepare 1× TNM and Developer (as described above).

3.9 Signal Development

Rinse slides in the following to remove excess antibody for 10 min each:

1. TN
2. TN
3. TNM

Add Developer to clean, aluminum foil-covered Coplin jar and incubate slides overnight at room temperature. The following morning, rinse twice with TN and then twice in TN-EDTA (TNE) for 5 min each. Slides are now ready to mount in glycerol-based mounting medium such as Vectashield (Vector, H-1000). Cover with 22 × 40 mm coverslip and seal the sides with nail polish. Slides may be kept at 4 °C for several days; however, a precipitate may form with prolonged storage.

3.10 Microscopy and Imaging of Staining Patterns

The dark purple color that results from probe signal can be observed using conventional bright field or Differential Interference Contrast (DIC) microscopy. In our experience, imaging is best accomplished using a color camera, as contrast from the tissue itself may resemble staining if a monochrome camera is used.

Sample images are shown in Fig. 3. Three probes of approximately 100 bases, 200 bases, and 400 bases were constructed for

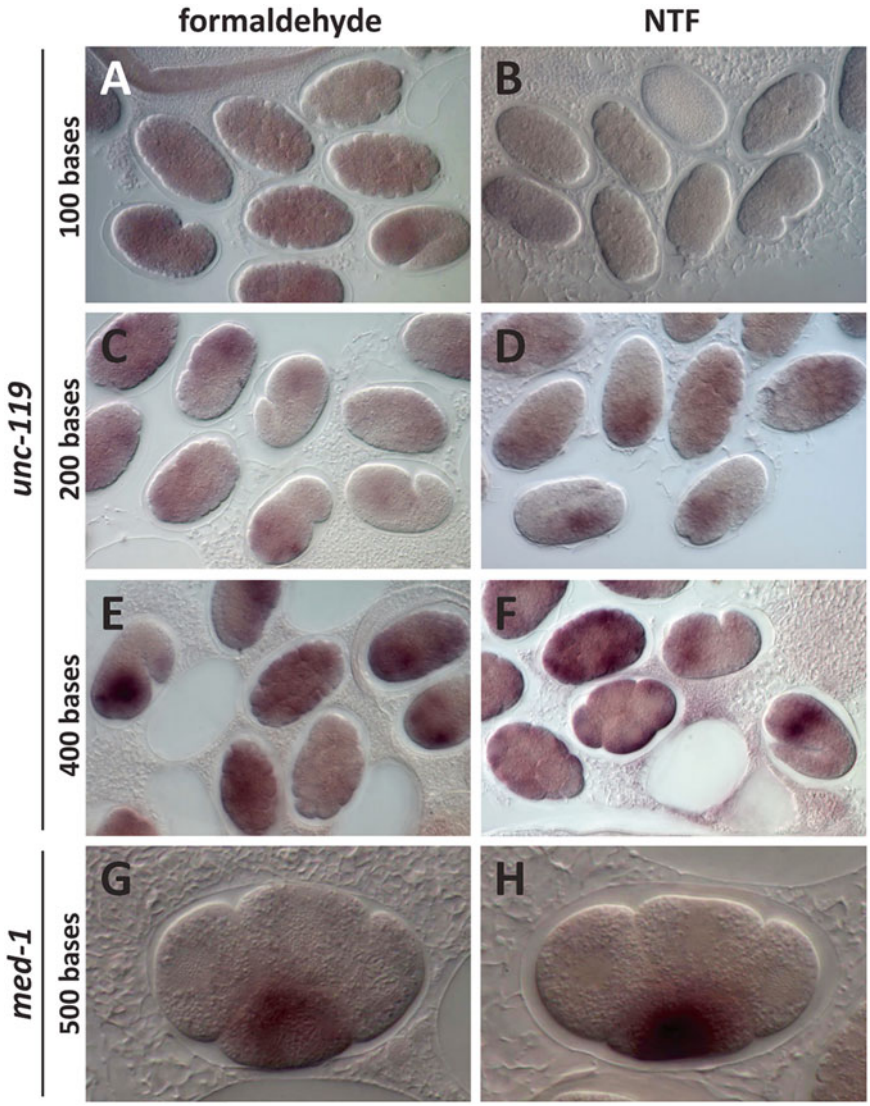

Fig. 3 Comparison of fixation methods and probe size. (**a**–**f**) Expression of *unc-119* detected by antisense probes of 100–400 bases in size. (**g** and **h**) expression of early embryonic transcripts of *med-1* (which also detects mRNA for the nearly identical *med-2* gene) present in a single cell at the 6-cell stage. Images representative of multiple embryos at the 4–6 cell stage in each slide ($n = 30$ formaldehyde, $n = 26$ NTF). Color images through an Olympus BX51 DIC microscope were acquired by a Canon T1 DSLR connected to an LM Microscope adapter (Micro Tech Lab, Graz, Austria). A *C. elegans* embryo is approximately 50 μm long

unc-119, a primarily neural gene that is moderately expressed throughout development [7] (Figs. 1 and 3a–f). The smallest probe demonstrated little to no hybridization, likely due to the stringency of the washes. The second probe, some expression could be discerned, but with suboptimal staining. The largest probe showed consistent staining throughout the slide with very little background. In general, it is best to construct multiple probes in a

range of sizes (with 400 being the smallest) up to around 1 kb. If a transiently expressed or extremely low-abundance gene product is being detected, greater care must be taken to insure that all steps are optimized. While the formaldehyde fixative works well for a moderately abundant gene such as *unc-119*, its limitations may be apparent when probing for transiently expressed low-level transcripts such as *med-1*,*2*, for which NTF produced slightly stronger staining (Fig. 3g, h).

4 Notes

This protocol, when executed optimally should result in even, consistent purple staining that is intense, sharp, and reproducible among similarly staged animals. The background is low and easily distinguishable from signal (Fig. 3). Although an extensive troubleshooting guide has been published previously [6], we draw attention to two common problems with RNA-ISH, namely excess staining (all tissues appear purple) or lack of signal. When excess signal is observed, in our hands it is almost always due to either an excess of probe, or an inconsistency with freeze cracking. When attempting this protocol initially, it is best to do so with a positive control such as an antisense GFP probe on a transgenic strain. In other instances, unexpected staining may be obtained with a highly cross-reactive probe. If this is suspected, multiple, non-overlapping probes to the same gene should be tested. A lack of signal can be due to a failure of probe synthesis, which can have multiple causes. All reagents should be checked to make sure proper kit components were used, especially for the use of DIG-11-UTP rather than DIG-11-dUTP. Primers for template synthesis should be checked that they were ordered correctly for the proper T7 sequence on the reverse primer. Both the PCR and in vitro transcription reactions can be run side by side on a standard agarose gel, for which the RNA should be suspended in formamide loading buffer (Ambion). If both the DNA and RNA look to be intact, then the problem may be that the endogenous signal is too weak to be detected. In our experience, we can routinely detect embryonic *C. elegans* transcripts, such as for *med-1* or *end-3*, that by other methods have as few as 300 copies per cell [8]. For additional troubleshooting, readers are referred to our prior guide [6].

5 General Conclusion

We have described a protocol that can be used to detect endogenous mRNA for genes in the nematode, *C. elegans*. The protocol works well in *C. elegans* and related nematodes [9–13] and should be adaptable to other "soft" specimens that can be mounted on

microscope slides. In our hands, we have found it to be more sensitive than other protocols that use DNA probes [14]. Compared with a prior version of our protocol [6], we have modified the steps for use with formaldehyde; used herniation of adult animals by pressure from the coverslip in order to release embryos; and assessed probes of different size for the same gene. Detection remains slightly stronger with "Nematode Tissue Fixative" but results are still acceptable with formaldehyde. We also find that antisense RNA probes of size 400 bases are sufficient for robust detection of mRNAs, while shorter probes produce weaker signal. Compared with protocols that can detect mRNAs individually (and therefore quantitatively), our method is less expensive overall and can give useful qualitative information about gene expression.

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