

Labelling of Active Transcription Sites with Argonaute NRDE-3—Image Active Transcription Sites *in vivo* in *Caenorhabditis elegans*

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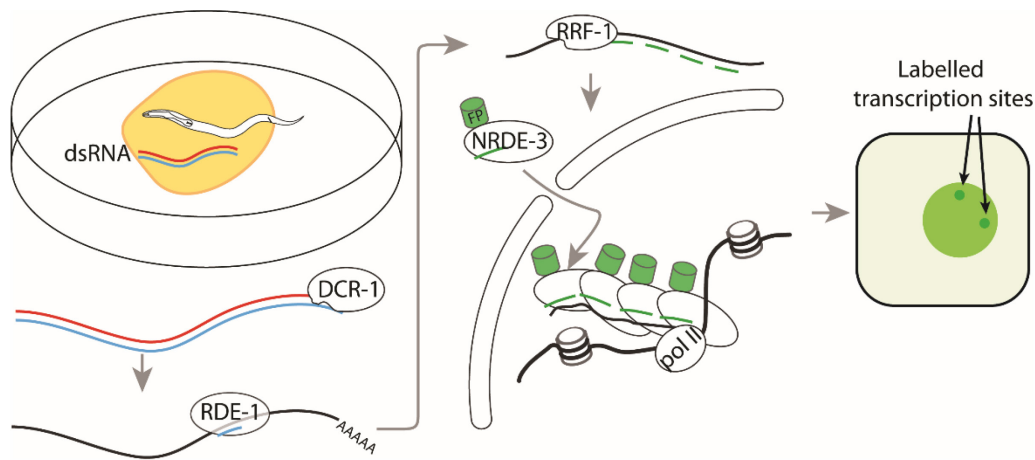
Abstract

Live labelling of active transcription sites is critical to our understanding of transcriptional dynamics. In the most widely used method, RNA sequence MS2 repeats are added to the transcript of interest, on which fluorescently tagged Major Coat Protein binds, and labels transcription sites and transcripts. Here we describe another strategy, using the Argonaute protein NRDE-3, repurposed as an RNA-programmable RNA binding protein. We label active transcription sites in *C. elegans* embryos and larvae, without editing the gene of interest. NRDE-3 is programmed by feeding nematodes with double-stranded RNA matching the target gene. This method does not require genome editing and is inexpensive and fast to apply to many different genes.

Keywords: Transcriptional labelling, mRNA labelling, *C. elegans*, Argonaute, *In vivo* imaging, Fluorescence microscopy

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Graphical abstract:



Background

We describe here a protocol to image transcription at transcription sites in *C. elegans* using an Argonaute protein NRDE-3, based on a method we recently published (Toudji-Zouaz *et al.*, 2021). In *C. elegans*, double-stranded RNA targeting the gene of interest is uptaken by the systemic RNAi mechanisms and distributed to all cells by the dsRNA transporter SID-1.

The exoRNAi pathway will process dsRNA and use them for recognition of target mRNAs for degradation. This recruits the RNA dependent RNA polymerase RRF-1 to the transcript, which will synthesise triphosphorylated, antisense, secondary small RNAs that are loaded into secondary Argonautes. The only somatic secondary Argonaute, NRDE-3, when unladen, is cytoplasmic. Once loaded with small RNA, NRDE-3 moves to the nucleus and binds to the nascent transcript, where it recruits other proteins to silence transcription.

In a wild-type background, the endogenous RNAi pathway will induce synthesis of secondary small RNAs, and constitutive nuclear localisation of NRDE-3 (Figure 1A, B). To prevent this, we introduce a mutation in *eri-1*. NRDE-3 is therefore unladen in most tissues (except for the germline, intestine, and early embryo), localises to the cytoplasm, and will move into the nucleus only if the gene of interest is expressed in the cell (Guang *et al.*, 2008) (Figure 1C, D). To prevent downstream transcriptional silencing, we also introduce a mutation in *nrde-2* (Guang *et al.*, 2010).

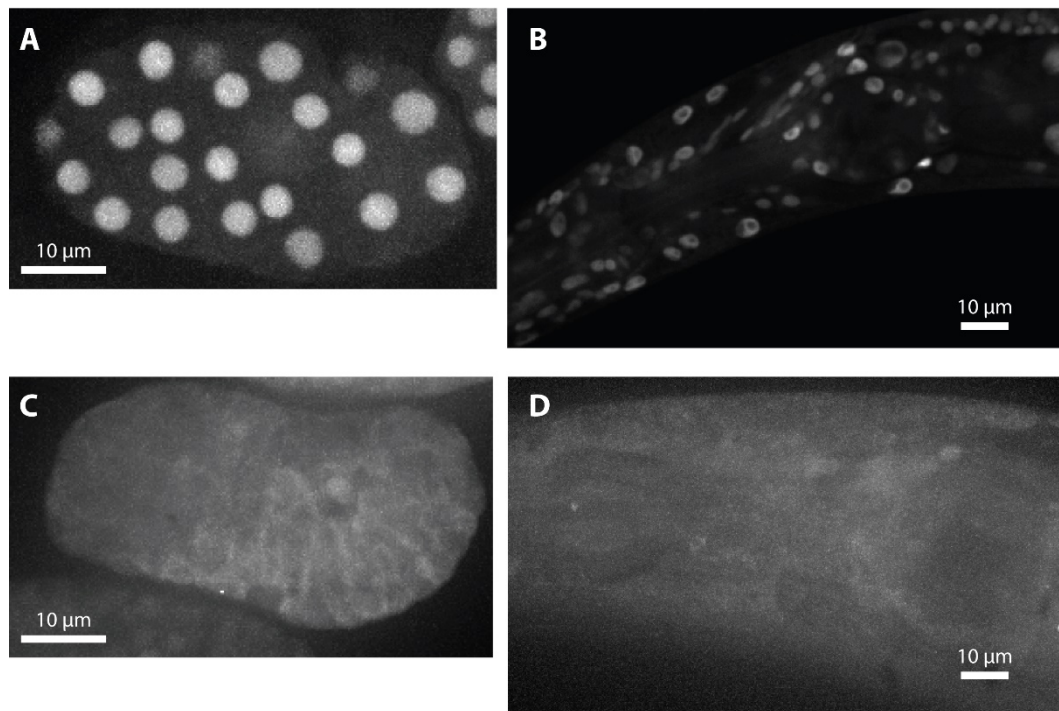


Figure 1. NRDE-3 nuclear localisation is dependent on small RNAs.

Nuclear localisation of fluorescently labelled NRDE-3 in the embryo (A) and adult somatic tissues (head) (B) in a *eri-1(+)* genetic background. Cytoplasmic localisation of NRDE-3 in a *eri-1(-)* background, in the embryo (C) and adult (D). Note the nuclear localisation in the germline primordium in the embryo (C).

Materials and Reagents

1. Petri dishes, 60 mm diameter (Falcon, catalog number: 353004)
2. Microscopy slides (Ghääsel, catalog number: 29-201-307)
3. Coverslips (Knittel, catalog number: 100037)
4. Sodium chloride (Roth, CAS: 7647-14-5)
5. Bacto agar (BD, CAS: 9002-18-0)
6. Bacto peptone (BD, CAS: 51142-18-8)
7. 5 mg/mL cholesterol in ethanol, filter sterilised (Sigma, CAS: 57-88-5)
8. KH_2PO_4 (Roth, CAS 7778-77-0)
9. K_2HPO_4 (VWR, CAS: 16788-57-1)
10. 1 M MgSO_4 (Roth, CAS: 7487-88-9)
11. 1 M CaCl_2 (VWR, CAS: 10035-04-8)
12. Ampicillin 1,000× (Sigma, CAS: 69-53-4) or carbenicillin 1,000× (Roth, CAS: 4697-36-3) 25 mg/mL (store at -20°C)
13. IPTG (Sigma, CAS: 367-93-1), 1 M (store at -20°C)
14. Na_2HPO_4 (VWR, CAS: 7558-79-4)
15. Feeding RNAi clones targeting the gene of interest, either from libraries (Ahringer library, Source Bioscience; Vidal library, Horizon discovery, catalog number: RCE1181; stored at -80°C) or homemade
16. Serotonin (Sigma, catalog number: H7752-5G), stock solution 25 mM in M9, keep at -20°C
17. 0.1 µm diameter polystyrene microspheres (Polysciences, catalog number: 00876-15, 2.5% w/v suspension in M9)
18. Nematode strains from *Caenorhabditis* Genetics Center (University of Minnesota): VBS662, VBS663, VBS664, VBS668

19. Wizard Plus SV miniprep kit (Promega, catalog number: A1460)
20. LB agar plates with 50 µg/mL ampicillin and 10 µg/mL tetracycline
21. Liquid LB with 50 µg/mL ampicillin
22. 1 M KPO₄ buffer (see Recipes)
23. M9 buffer (see Recipes)

Equipment

1. Peristaltic pump (Wheaton Omnisense plus)
2. Confocal microscope, Spinning disk Roper on Nikon Eclipse Ti, with argon laser for illumination at 515 nm
3. Incubator (Pol Eko apartura), set at 20°C
4. Centrifuge (Eppendorf 5804R)
5. Worm pick, prepared according to (Stiernagle, 2006)

Software

1. Fiji (<https://fiji.sc/>)

Procedure

This procedure is relatively simple and can be implemented in a few days (Figure 2). It includes preparation of RNAi plates for bacteria expressing dsRNA, exposure of nematodes, and imaging.

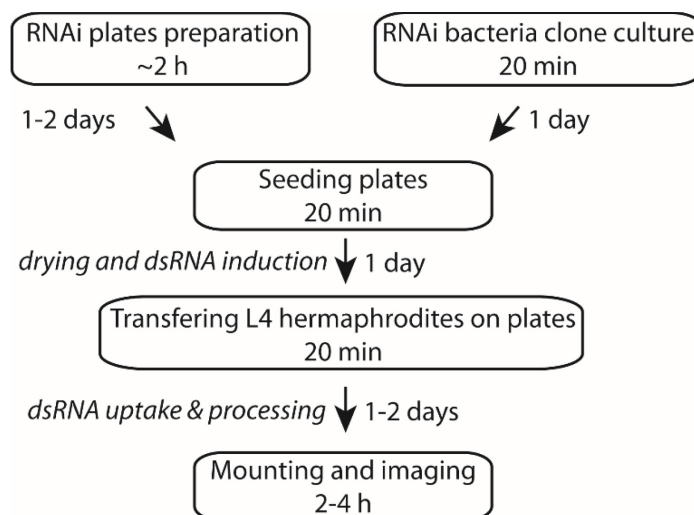


Figure 2. Procedure flowchart.

Preparing plates

Prepare plates with standard Nematode Growth Media (Stiernagle, 2006), supplemented with ampicillin or carbenicillin (to select bacteria carrying the plasmid of interest) and IPTG (to induce transcription of double-stranded RNA). Follow the standard guidelines for RNAi by feeding (Ahringer, 2006). Incorporating antibiotics during plate preparation, rather than afterwards, increases dsRNA expression consistency.

A. Preparation of NGM RNAi plates

1. Mix 3 g of NaCl, 17 g of agar, and 2.5 g of peptone in a bottle; add 975 mL of ddH₂O. Autoclave for 50 min.
2. Let bottle cool down to ~55°C.
3. Add 1 mL of 1 M CaCl₂, 1 mL of 5 mg/mL cholesterol in ethanol, 1 mL of 1 M MgSO₄, 25 mL of 1 M KPO₄ buffer, 1 mL of IPTG, and 1 mL of carbenicillin or ampicillin.
4. Pour plates with the peristaltic pump, under sterile conditions next to a flame (7 mL for 60 mm diameter plates).
5. Plates should be left to dry for at least 24 h in the dark before seeding; if too wet, the bacteria will not dry properly after seeding. For best practice, plates should be used within two weeks, although we have observed labelling with month-old plates.

Choosing RNAi clones

Two large libraries of RNAi clones in the *E. coli* strain HT115 bacteria are available: the Ahringer library (Kamath *et al.*, 2003), no longer commercially distributed by Source Bioscience, but widely available, covering genomic sequences with ~1 kb long clones; and the Vidal ORFeome library (Rual *et al.*, 2004), still commercially distributed by Horizon Discovery, containing full reading frames. If your gene of interest is not available in these libraries, make your own vector by cloning the ORF of interest in the L4440 empty vector (Ahringer, 2006).

Labelling of transcription sites relies on the tiling of fluorescently tagged NRDE-3 on transcripts; it derives that, the longer the dsRNA fed to the worms, the more fluorescent proteins will accumulate on each transcript. When we tested RNAi clones targeting only one exon of *hlh-1*, we were able to observe nuclear localisation of NRDE-3 in muscle cells, but failed to observe active transcription sites. To maximise signal, it is therefore preferable to use long RNAi clones. The RdRP RRF-1 processes the target RNA 3'→5'; therefore, a RNAi clone in 3' of the target transcript will induce secondary siRNAs covering more of the transcript.

B. Preparation of RNAi cultures

1. Streak clones from the frozen library onto LB plates containing 50 µg/mL ampicillin (to select for presence of the dsRNA coding plasmid), and 10 µg/mL tetracycline (to select for a mutation in a dsRNase in HT115), using a sterile pipette tip or inoculation loop, and grow overnight at 37°C.
2. (Optional) Miniprep and sequence the clone. A minority of clones in these libraries are erroneously annotated; it is therefore good practice to confirm their identity.
3. Grow RNAi clone in 5 mL of liquid LB with 50 µg/mL ampicillin, between 8 h and overnight at 37°C and 200 rpm.
4. Pellet down the culture, pour out the supernatant, resuspend the pellet in 200–300 µL and seed 100 µL on plates. IPTG should induce transcription of dsRNA within a few hours. We typically transfer worms on plates the next day or when plates are sufficiently dry.

Despite the mutation in *nrde-2* abrogating secondary silencing at the transcriptional level (Guang *et al.*, 2010; Toudji-Zouaz *et al.*, 2021), with some dsRNA clones (*e.g.*, *ant-1.1*, *ama-1*), we observed deleterious effects (sterility, lethality), possibly due to the primary RNAi pathway being sufficient for a significant level of knockdown. By diluting the RNAi culture 1/2 to 1/5 with bacteria carrying the empty vector L4440, it is possible to bypass these deleterious effects.

Choosing which strain to use

Four strains are available from the CGC; all transgenes were integrated as single copy by CRISPR, at loci ttTi5605 (chromosome II) or ttTi4348 (chromosome I).

VBS662 *eri-1(mg366) IV; nrde-2(gg95) p_{eef-1A.1}::YFP::nrde-3 II*

In the absence of RNAi treatment, the YFP signal is nicely homogenous in the cytoplasm at all stages; expression under the control of the strong *eef-1A.1* promoter is high; therefore, the background signal in the

nucleus will be strong, and weak transcription signals will be difficult to observe. YFP also has a low photostability, making it less appropriate for timelapse imaging.

VBS663 *eri-1(mg366) IV; nrde-2(gg95) p_{rps-27}::mNeonGreen::flag::nrde-3 II*

mNeonGreen tends to form aggregates in the cytoplasm of embryos, and is therefore less optimal for imaging at this stage. In larval stages, signal is nicely homogenous. The expression level under the control of the weaker *rps-27* promoter ensures a higher signal-to-noise ratio. The higher photostability makes it better for long timelapses.

VBS668 *eri-1(mg366) IV; nrde-2(gg95) p_{eeef-1A.1}::YFP::nrde-3::SL2::sid-1 II*

By expressing the dsRNA transporter *sid-1* in all tissues, this strain improves efficiency in neurons (Calixto *et al.*, 2010). However, it does not reach the efficiency of other somatic tissues; several components of the RNAi pathway appear to be sparsely expressed in neurons (Cao *et al.*, 2017). In worms fed on dsRNA targeting GFP, we observe nuclear localisation in neurons in adults, but this is less efficient in larvae. The lower efficiency means that one has to look at multiple individuals to see proper nuclear localisation and transcriptional labelling.

VBS664 *eri-1(mg366) IV; nrde-2(gg95) p_{eeef-1A.1}::VenusC::nrde-3 II; p_{eeef-1A.1}::VenusN::nrde-3 I*

Bipartite Venus:NRDE-3 allows reconstitution of fluorescence when multiple NRDE-3 accumulate on the same transcript; background fluorescence in nucleus and cytoplasm is therefore reduced. In those cells expressing the gene of interest, in addition to active transcription sites, the nucleoplasm is still visible: once Venus is reconstituted, it does not dissociate again; after unloading from the transcript, NRDE-3 will stay or translocate back to the nucleus, leading to some background nucleoplasm signal. Some spontaneous reconstitution is observed associated to the cytoskeleton in some cells. The reduced background allows observation of cytoplasmic transcripts as faint spots that bleach rapidly (Figure 3). Occasionally, more than two nuclear spots are observed; they might indicate transcripts in processing and export.

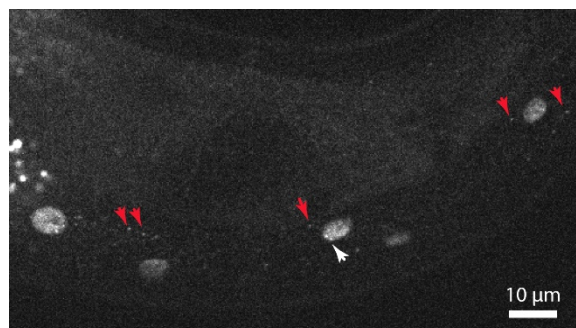


Figure 3. Labelling of cytoplasmic transcripts with bipartite Venus.

Labelling of *hlh-1* transcription sites (white arrowheads) and cytoplasmic transcripts (red arrowheads) with split Venus complementation on transcripts.

C. Exposure to dsRNA and imaging

1. Transfer well-fed L4 hermaphrodites to RNAi plates. To reduce bacteria contamination, first pick the worms to an unseeded plate, and then transfer them to the RNAi plate. We recommend to transfer ~20 hermaphrodites.
2. Leave worms on the plate for at least 24 h, and up to 4 days, at 20°C. Optimal exposure can vary depending on the gene and the developmental stage of interest; try out a few exposure times.
3. For embryos, dissect gravid hermaphrodites and mount embryos, on a 2–5% agar pad in water on a slide, taking care to limit bacteria transfer as much as possible; add a coverslip and seal the edges to prevent evaporation (Walston and Hardin, 2010).

4. For larvae and adults, mount on a 5% agar pad, with 1 to 2 μ L polystyrene beads, and 2 μ L of serotonin in M9 for immobilisation (Lee *et al.*, 2019), taking care to avoid bacteria transfer; add a coverslip and seal the edges to prevent evaporation.
5. Image on confocal microscope, preferably a spinning disk confocal microscope to reduce photobleaching. YFP, mNeonGreen, and Venus, the fluorescent proteins used in these strains, are compatible with excitation at 515 nm. Laser intensity and exposure should be adjusted to limit photobleaching; we try to stay around 0.3 mW. We perform image acquisition with a 60 \times /1.20 NA objective, which gives sufficient resolution and a long enough working distance, and a Z step of 0.5 μ m.
6. You should observe nuclear localisation in the cells expressing the gene of interest, and in some of these cells, one or two dots, corresponding to active transcription sites. If working with a gene with multiple paralogs, or in a polyploid tissue (*e.g.*, gut or hypodermis), more than two dots can be observed. The transcription size spots should have a diameter <1 μ m; the size should be diffraction limited. Theoretically, spots should become observable as soon as NRDE-3 moves to the nucleus.

After exposure to dsRNA over multiple generations, transcription sites are less often observed. We therefore recommend limiting exposure to one or two generations.

Alternative immobilisation methods are discouraged: sodium azide, being a general metabolic poison, is best avoided; while transcription spots can be observed, we have generally failed to observe dynamics with azide. Levamisole can work, provided concentrations are low enough.

While an epifluorescence microscope is sufficient to observe nuclear localisation, it is unlikely to successfully image transcription spots. A confocal microscope is necessary to successfully differentiate the weak transcription site from the background. Imaging over multiple hours is taxing on the worms, due to phototoxicity and oxygen deprivation; with mNeonGreen::NRDE-3, after multiple hours, non-specific spots can appear in the cytoplasm. It is therefore preferable to limit laser power (<0.4 mW) and limit the amount of bacteria transferred to the slide.

Positive and negative controls

As a positive control, we recommend targeting the gene *hlh-1*, coding for the MyoD orthologue, expressed in all muscle cells at a moderate level. As a negative control, we use the empty vector clone L4440.

D. Imaging controls

1. Prepare plates with the *hlh-1* RNAi clone B0304.1 (coordinates II-3J04 in the Ahringer library) and the empty vector clone L4440, as detailed above.
2. Transfer L4 larvae from strain VBS662 or VBS663 to RNAi plates.
3. Wait 24 to 48 h for uptake and processing of dsRNA through the RNAi pathway, then mount and image the progeny larvae. In worms exposed to *hlh-1* dsRNA, you should observe strong and consistent nuclear localisation of fluorescently labelled NRDE-3 in all muscle cells. In a subset (~5%), you should observe one or two nuclear spots (Figure 4A). Nuclear localisation can be visible on a fluorescence dissecting scope. In worms exposed to the empty vector, you should observe constitutive nuclear localisation in the germline, early embryo, and intestine (see section “Nuclear localisation of NRDE-3”). All other tissues should show cytoplasmic localisation. In the early embryo, bright perinuclear spots are also visible.
4. If observing the parental (P0) individuals that were transferred to the positive control plate, you should be able to observe nuclear localisation of NRDE-3 in muscle cells. However, double-stranded RNA will be unevenly distributed throughout the worm, and nuclear localisation will be weaker, and not observed in every muscle cell (Figure 4B).

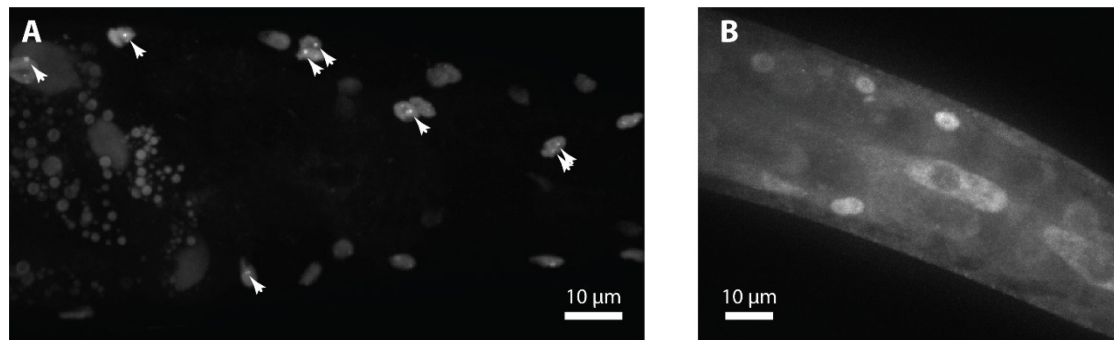


Figure 4. Labelling of *hhh-1* active transcription sites.

(A) Nuclear localisation of fluorescently labelled NRDE-3 in muscle cells and labelling of active *hhh-1* transcription sites (arrowheads) in the progeny of individuals exposed to *hhh-1* dsRNA. (B) Weak nuclear localisation of NRDE-3 in P0s exposed to *hhh-1* dsRNA.

Crossing in mutations

It might be desirable to cross mutations or transgenes of interest in NRDE-3 strains. *eri-1* (*mg366*) is located on chromosome IV; *nrde-2*(*gg95*) and YFP:NRDE-3 (at location ttTi5605) on chromosome II, 1.6 cM apart.

1. Cross spontaneous males from VBS662, VBS663, VBS664, or VBS668 (*eri-1*(-) has a mild him phenotype) to hermaphrodites from the strain of interest.
2. Isolate fluorescent F1s, which should be showing nuclear localisation of YFP or mNeonGreen::NRDE-3 in all tissues due to the *eri-1*(+).
3. Let F1 hermaphrodites self-fertilise, and pick fluorescent F2.
4. To homozygous in F2 and F3, pick hermaphrodites with cytoplasmic localisation of fluorescent signal in most tissues. It should be noted that the maternal effect of *eri-1*(+) can extend up to the F3 generation (Zhuang and Hunter, 2011).
5. To homozygous *nrde-2*(*gg95*) and the fluorescent transgene, select lines that transmit 100%. Because *nrde-2* and ttTi5605, the insertion site of fluorescent transgenes, are just 1.6 centiMorgans apart, it is easy to simply select 100% transmission of the fluorescent transgene. To select directly for *nrde-2*(-) homozygosity, select by resistance to lethal RNAi: RNAi targeting *lir-1* (F18A1.3; clone II-5B14 from the Ahringer library), as in the original *nrde* screen (Guang *et al.*, 2010), causes arrest in L1 in *nrde-2*(+). *lir-1* is in an operon with *lin-26*; if nuclear RNAi is active, RNAi targeting *lir-1* will also silence *lin-26*, causing L1 lethality (Bosher *et al.*, 1999). Alternatively, primers CCTTCAAGTATCTATCCAGCTGCTCC/GATCCAGTAGCCGAAGCTCTAGTTC can be used to track the *gg95* mutation by PCR. The wild-type PCR product is 585 nucleotides long, while the mutant is 330 nucleotides long.

Performing mutagenesis or transgenesis by CRISPR in those strains is possible. However, given that Cas9 is optimally active at 25°C, a temperature at which *eri-1*(-) induces sterility, it is best done in a wild-type background, before crossing it in.

Extrachromosomal arrays, due to their repetitive nature, can cause simultaneous sense and antisense transcription. As a result, even in the absence of exposure to dsRNA, we can observe nuclear localisation and active transcription sites (Figure 5); they should be avoided if possible.

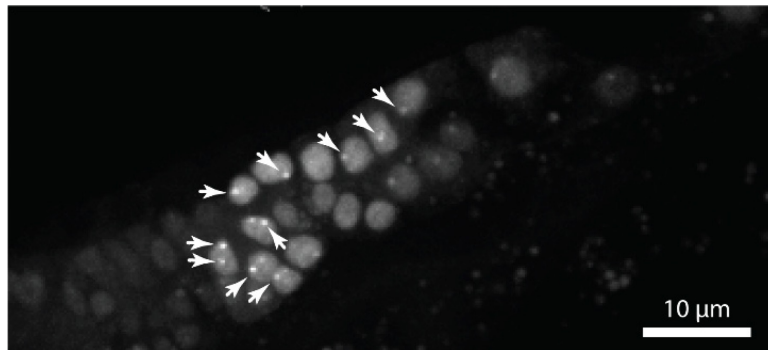


Figure 5. Extrachromosomal arrays induce spurious labelling of transcription sites.

Nuclear localisation and transcription site labelling (arrowheads) in individuals carrying a repetitive extrachromosomal array, without exposure to dsRNA.

Data analysis

No dedicated software package currently exists for this method. We use FIJI and the Trackmate software package (Tinevez *et al.*, 2017) to segment active transcription sites and measure the fluorescence intensity of active transcription sites and the nuclear background.

Nuclear localisation of NRDE-3

Nuclear localisation of NRDE-3 signals that the gene of interest was expressed in this cell; however, it cannot tell how recently it was expressed. Once NRDE-3 has loaded a small RNA, it should not dissociate: the affinity of Argonautes to their guide RNA is rather high, with K_d on the order of 15 nM to 32 μ M (MacRae *et al.*, 2008; Wang *et al.*, 2009). Once translocated to the nucleus, NRDE-3 stays nuclear even if no active transcription is ongoing. At this point, we would advise against using nuclear/cytoplasmic localisation to assess whether transcription is actively ongoing.

We observe nuclear localisation in the gut, germline, and early embryo in the absence of double-stranded RNA exposure. We have nonetheless managed to observe active transcription sites in the gut after exposure to dsRNA targeting *elt-2*; in the germline and early embryo, we have so far failed.

Other factors might affect nuclear localisation: low temperatures (15°C or 4°C) or UV irradiation can induce synthesis of risiRNAs, a class of triphosphorylated small RNAs loaded by NRDE-3 (Zhou *et al.*, 2017), and cause nuclear localisation of NRDE-3 and formation of spots at nucleoli. It is possible that other undescribed stressors could induce risiRNA synthesis; therefore, unexpected nuclear localisation of NRDE-3 should be carefully interpreted.

Limitations

Several limitations of this method need to be highlighted. NRDE-3 based labelling does not work equally well in all tissues. The germline is so far refractory, and while labelling is possible in neurons, the efficiency is reduced. Labelling has not been successful for all genes (see Supplementary Table 1 in Toudji-Zouaz *et al.*, 2021).

Because the synthesis of secondary small RNAs is dependent on the presence of the target mRNA in the cytoplasm, this method is not able to label the first initiation of transcription. The length of this delay has not been estimated. While the *nrde-2* mutation is sufficient to block transcriptional silencing, some downregulation by the primary RNAi pathway, necessary for synthesis of secondary small RNAs, is unavoidable. This can be mitigated by titrating down the concentration of dsRNA-expressing bacteria. Finally, while NRDE-3-bound transcripts are able to be exported to the cytoplasm (Figure 3), it is possible that they are affected in their processing, transport, or stability.

Recipes

1. 1 M KPO₄ buffer

108.3 g KH₂PO₄
35.6 g K₂HPO₄
ddH₂O to 1 L

2. M9 buffer

3 g KH₂PO₄
6 g Na₂HPO₄
5 g NaCl
1 mL of 1 M MgSO₄
ddH₂O to 1 L

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Competing interests

The authors declare no competing interests.

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