

Video Article

RNAi Screening to Identify Postembryonic Phenotypes in *C. elegans*

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Abstract

C. elegans has proven to be a valuable model system for the discovery and functional characterization of many genes and gene pathways¹. More sophisticated tools and resources for studies in this system are facilitating continued discovery of genes with more subtle phenotypes or roles.

Here we present a generalized protocol we adapted for identifying *C. elegans* genes with postembryonic phenotypes of interest using RNAi². This procedure is easily modified to assay the phenotype of choice, whether by light or fluorescence optics on a dissecting or compound microscope. This screening protocol capitalizes on the physical assets of the organism and molecular tools the *C. elegans* research community has produced. As an example, we demonstrate the use of an integrated transgene that expresses a fluorescent product in an RNAi screen to identify genes required for the normal localization of this product in late stage larvae and adults. First, we used a commercially available genomic RNAi library with full-length cDNA inserts. This library facilitates the rapid identification of multiple candidates by RNAi reduction of the candidate gene product. Second, we generated an integrated transgene that expresses our fluorescently tagged protein of interest in an RNAi-sensitive background. Third, by exposing hatched animals to RNAi, this screen permits identification of gene products that have a vital embryonic role that would otherwise mask a post-embryonic role in regulating the protein of interest. Lastly, this screen uses a compound microscope equipped for single cell resolution.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3442/>

Protocol

1. Screening strain construction

The careful design of the screening strain is critical for the success of the screen and has been described elsewhere³. For some researchers, using a strain that expresses a visible product from a transgene is needed for the experiment. Many strains harboring integrated transgenes are available from the CGC or individual researchers. If a transgenic strain is required for the screen but is not available, then it can be generated using a published method like bombardment⁴, UV/TMP⁵, or Mos transposon insertion⁶. In order to visualize our protein of interest, we inserted the *gfp*-coding sequence in frame with the mature cDNA sequence (we used *dbl-1* sequence). Because this GFP fusion protein is not visible by dissecting scope, we used a coinjection marker that was visible and did not affect the viewing of our protein of interest (*ttx-3p::rfp*, for a review of several other standard markers, see⁷). We then created an integrated transgene from the extrachromosomal array. We found that bombardment of the transgene yielded a low-copy number integrated line that neither rescued the dissecting microscope phenotype nor produced visible levels of GFP-tagged product (Beifuss and Gumienny, unpublished). UV/TMP integration of a multiple copy extrachromosomal array originally made by injection^{8,9} yielded visible, rescuing levels of transgene product (Figure 3A). Western blot with anti-GFP antibody confirmed that the transgene (allele name *texIs100*) is expressed and processed correctly (Beifuss and Gumienny, unpublished). Integrated transgenes should be outcrossed five times to remove extraneous background mutations regardless of source.

For our screen, we wanted the only protein of interest to be the transgenic, tagged form. Therefore, we removed the functional endogenous gene by introducing a loss-of-function mutant *dbl-1* allele.

Lastly, we sensitized the strain to the effects of RNAi. The RNAi from the library will, for many genes, produce a more severe reduction in gene product if animals contain a mutation that sensitizes the animals to the effects of RNAi^{10,11}. The tissue(s) of interest should be considered when choosing an appropriate RNAi sensitizing background¹¹. We used the canonical *rrf-3(pk1426)* allele to make our screening strain. RRF-3 is an RNA-directed RNA polymerase (RdRP) homolog that normally inhibits somatic RNAi¹⁰. Mutations in other RNAi hypersensitizing genes like *eri-1* or *eri-1 lin-15b* can be used instead to increase the effectiveness of the RNAi¹¹⁻¹³.

The screening strain we made has the genotype *rrf-3(pk1426); texIs100; dbl-1(nk3)*.

2. Choosing and preparing an RNAi library

Commercially available *C. elegans* cDNA libraries represent about 55% or 87% of the predicted genes in *C. elegans* individually (Vidal lab or Ahringer lab libraries, respectively). Individual clones are available for purchase (Open Biosystems, Geneservice, Ltd.). We chose the ORF-RNAi library constructed by the Vidal lab (Open Biosystems) because its clones are mostly full-length cDNAs Gateway cloned and ready for downstream applications (Invitrogen Corporation, Carlsbad, CA). The original Ahringer lab genomic cDNA library contains cDNA fragments that are not as widely useful for downstream characterization experiments^{14,15}. Both libraries use a vector that contains two T7 promoters flanking the insert (Figure 1). The constructs are grown in bacterial strain HT115, which expresses T7 polymerase upon induction by isopropyl- β -D-thiogalactopyranoside (IPTG). Bacteria containing library clones are induced with IPTG to produce dsRNA (see Step 3.4).

Duplicate the entire library upon receipt and use the duplicate for all experiments. The original and duplicate libraries should be stored in different -80°C freezers that are connected to separate electrical lines.

3. Preparation of bacteria with library clones

1. (Within two months of the experiment) Streak bacteria from selected feeding library clones, as well as positive and negative controls, on labeled LB- carbenicillin/ tetracycline plates (we grow 8 cultures per 100 mm plate) and incubate at 37°C overnight (>16 hours) (Figure 1). For each 24-well plate, we use two controls. The positive control, *bli-4* (K04F10.4), produces a dose-dependent post-embryonic phenotype^{16,17}. The negative control, C06C3.5, is a predicted pseudogene (WormBase.org) that causes no observed phenotypes (Beifuss and Gumienny, unpublished).
2. (Within two months of the experiment, preferably sooner) Prepare 24-well Nematode Growth Medium (NGM) plates containing 25 – 50 μ g/ml carbenicillin and 1mM IPTG. Store at 4°C.
3. (Day 1) For each clone, inoculate 2 ml LB media containing 50 μ g/ml carbenicillin with a single colony from the streaked plate(s) (in Step 3.1) using standard sterile technique. Incubate at 37°C overnight (>16 hours) in a shaker set to 220 – 240 rpm (Figure 1). The solution should be cloudy after 16 hours.
4. (Day 2) The next morning, induce double stranded RNA (dsRNA) production by adding IPTG to the cultures to a final concentration of 1mM. Incubate cultures at 37°C, 220 – 240 rpm, for an additional 4 – 5 hours. This step ensures a more consistent and robust RNAi-induced knockdown of gene product than relying solely on the IPTG in the agar of the NGM plate (in Step 3.5).
5. Spot 30 μ l of each induced bacterial culture into separate wells of a 24-well NGM RNAi plate containing 25 – 50 μ g/ml carbenicillin and 1mM IPTG (Figure 1, see Figure 4 for template used to track RNAi experiments). Put plates, uncovered, in a sterile flow hood for 20 minutes or until the bacterial spots are dry. Do not overdry. Overdrying causes the edges of the agar to pull away from the plate and animals will be irretrievable if they crawl into the resulting crevices.

4. Preparation of nematodes

Start with a staged population of nematodes. Staging animals decreases the chance that differences observed between the control and the experimental RNAi trials are simply due to differences in developmental stage of the animals (however, if the RNAi causes a developmental delay, this should be noted by the screener (Figure 2)). Furthermore, starting the RNAi experiment with L1 larvae obviates potential confounding effects of embryonic lethality by RNAi of genes that may also play postembryonic roles of interest.

Staging is accomplished by first bleaching a mixed-stage population of the screening strain, which only eggshell-protected embryos survive. This stages animals to within about 12 hours at 20°C or about 18 hours at 16°C¹⁸. To more tightly stage animals, arrest animals in the first larval stage (L1) by letting embryos hatch in M9 medium without food. Starved L1 animals placed on food resume growth from the same starting age¹⁹.

1. (Day 1) Use three 100 mm plates of fed, clean screening strain animals that contain gravid adults and embryos. Wash animals from plates using sterile M9 by pipeting liquid across the plate surface gently to loosen animals and embryos. Transfer the wash into a sterile 15 ml tube with screw cap.
2. If the wash is greater than 3.5 ml, spin down the animals (~3000 rpm for 30 seconds) and remove liquid to 3.5 ml. If the wash is less than 3.5 ml, add H₂O or M9 to the tube to total 3.5 ml.
3. Wear appropriate protective gear (lab coat, gloves, and goggles) while handling chemicals. Mix 0.5 ml 5 N NaOH with 1 ml fresh bleach (5% sodium hypochlorite, less than 1 year old). Add this mixture to the 3.5 ml nematode wash. Start a timer counting up. This process should not take more than 10 minutes. If it does, then embryos will be endangered and yield may be reduced, or the bleach is old and should be replaced.
4. Vortex the tube for about 10 seconds. Repeat the vortexing every two minutes for 4 to 10 minutes. After each vortexing, observe the solution under a dissecting scope for animal carcasses.
5. When embryos are released and adults are fragmented (6 – 8 minutes), pellet the embryos by spinning the tube in a table top centrifuge (~3000 rpm, 30 seconds).
6. Aspirate as much of the supernatant as possible without disturbing the pellet. Be careful, not greedy.
7. Add sterile M9 to ~ 10ml. Vortex briefly or shake well to resuspend the pellet.
8. Spin and remove supernatant again. If the smell of bleach can be detected in the tube, repeat rinse as necessary.
9. Resuspend embryos in 10 – 15 ml sterile M9 and transfer to a small (25 ml) Erlenmeyer flask (for aeration). Shake at the proper temperature for the strain and experiment (15 – 25°C) overnight (> 16 hours at 20°C). In this time, the animals will all hatch and arrest in the L1 diapause.
10. (Day 2) Spin down L1 stage animals in a 15 ml tube and resuspend in 0.5 – 1 ml M9 (~3000 rpm, 30 sec.). Place 5 μ L solution on a separate plate and count the number of animals. Make adjustments to the volume, if needed, to yield 30 worms/ 3 – 10 μ L solution.
11. Spot approximately 30 hypochlorite synchronized L1 stage animals onto the bacteria in each well of the prepared 24-well plate. Too many more than 30 animals per well may result in the population starving before reaching maturity. Let the plate dry with the lids askew. Replace the lid and secure it with a rubber band. Invert the plate and place it at the appropriate temperature for the time needed to score at the

desired stage. Because *rff-3(pk1426)* is a temperature sensitive allele, our screening strain animals were grown at 15 – 17°C for three to four days to observe L4 to adult stage animals (Figure 1).

5. Observation of nematodes

(Day 5) Once the nematodes have grown to the desired stage (Figure 2A), confirm that the positive and negative controls produce the expected phenotypes by using a dissecting microscope. We use *bli-4* as a control for RNAi efficacy, because it produces dose-dependent post-embryonic defects that range from blistered adults (mild RNAi phenotype, not shown) to developmentally arrested, tiny larvae (strong, expected RNAi phenotype) (Figure 2B). Then observe the animals in each experimental well using a dissecting microscope and note obvious abnormal phenotypes induced by RNAi (Figure 2C). After the controls are confirmed and gross phenotypes noted, screen the RNAi experiments for phenotypes of interest. We use a compound microscope equipped with fluorescence and a 63x objective to observe at least five animals from each RNAi experiment, beginning with controls (Figure 3).

1. Prepare a slide (standard 75 x 25 mm) by making a 4% agar pad (4% agar in H₂O). To ensure a uniform thickness of agar pad, place a clean glass slide between two spacers (each spacer is made of a microscope slide with two layers of lab tape on it). Pipette about 100 µL (two to three drops from a glass Pasteur pipet) of molten 4% agar onto the center of the clean glass slide. Form an agar pad by covering the molten agar using an additional slide placed quickly but gently on top of the spacers and the molten agar. Expose the agar pad by gently sliding the glass slides apart, leaving the pad adhering to one slide.
2. Place a ~5 µL drop of anesthetic (levamisole or sodium azide, for example) on the agar pad. Mount 8 – 12 animals in the anesthetic. Cover with a 22 x 22 mm #1.5 coverslip (or the thickness of coverslip best suited for the compound microscope) and observe animals using a compound microscope.
3. Record gene, number of animals observed, and phenotype results for each RNAi experiment. For a screen, make and use a standard template (see example, Figure 5).
4. Verify the RNAi-produced phenotype by another, secondary phenotype, if possible, and by repeating the experiment. (For example, the example screen shown used alteration of GFP-tagged DBL-1 localization as a primary screen and body size as a secondary screen²⁰.) Bacteria can be used from the same streak (stored at 4°C) for up to two months. Older streaks may result in poor growth in liquid overnight cultures and should be restreaked first.
5. Sequence at least one end of the cDNA insert to confirm that the insert matches the library label (the Ahinger library has had a reliability analysis performed; see <http://biocompute.bmi.ac.cn/CeIRNAi/>)²¹. Recommended primers (for inserts from either library) anneal on sites in the L4440-derived vector and flank the insertion site: 5'-AGCGAGTCAGTGAGCGAG-3' and 5'-GTAAACGACGCCAGT-3' (M13f20 primer).

Using this method, a single person can reasonably stage and observe two to four sets of experiments every week. For instance, animals staged on Monday and Tuesday can be observed Thursday and Friday, respectively, and can again be staged on Friday and Saturday for Monday and Tuesday observation. Depending on the phenotype(s) screened each day, a set may comprise one 24-well plate by compound microscopy or more if the phenotype is quickly identified. Thus, at least 88 different RNAi experiments can be observed in one week, taking into account positive and negative controls and rate of screening. A dissecting scope screen could be performed much faster, without the need for mounting animals on slides for viewing. Multiple people could also increase a lab's throughput by staggering schedules and/or using multiple microscopes. An alternative method for growing animals on a thin film of agar and transferring a slice of the agar containing animals directly to a slide for viewing may save time. This variation was successfully used for screening male tail abnormalities at 400x magnification²².

6. Representative Results

Examples of normal and altered localization of GFP-tagged DBL-1 are shown in Figure 3. Normal expression of GFP-tagged DBL-1 includes ventral nerve cord cell bodies and a row of punctae (Figure 3A). DBL-1 is severely attenuated when animals are fed RNA that prevents *dbl-1* mRNA translation (Figure 3B). *dbl-1(RNAi)* also produces small animals, the secondary screen in this example (data not shown). Genes that affect DBL-1 localization are readily identified by RNAi using a strain designed for this screen (Figure 3C).

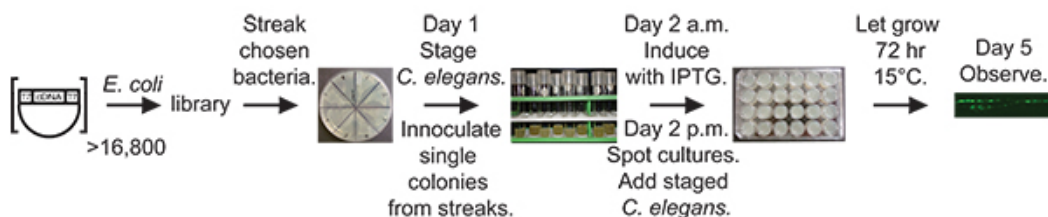


Figure 1. Screen scheme to identify extracellular regulators of DBL-1 signaling. Bacteria from the feeding library are grown overnight, induced with IPTG, and incubated at 37°C for an additional 4 hours to allow the bacteria to produce double stranded RNA (dsRNA). 30 µl of the induced bacterial culture is spotted per well onto a 24-well NGM (nematode growth medium) plate containing 25 µg/ml carbenicillin and 1mM IPTG and allowed to dry in a sterile flow hood. We stage first larval stage (L1) larvae by letting hypochlorite-treated embryos hatch in media without food, which induces an L1 diapause (arrested development). Approximately 30 synchronized L1 stage animals are plated onto each well containing bacteria from the RNAi library, which allows staged animals to resume growth and to consume the dsRNA created by the bacteria²³. After 72 hr at 15°C, the young adult worms are screened for a visible phenotype. At this age, body size defects are evident and fluorescence is bright from *tex1s100* transgene expression.

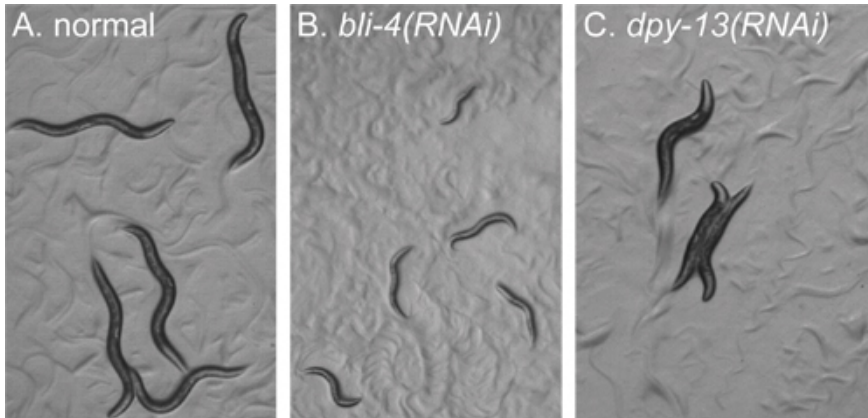


Figure 2. Examples of phenotypes to identify before screening. All images of animals in a plate well were taken at the same magnification using a dissecting microscope. Animals were all imaged about 72 hours after plating as starved L1 larvae. All images were treated identically. A) RNAi of a gene that gives no gross morphological defects. Animals appear wild type. B) RNAi of *bli-4*. Animals display arrested development, and are tiny compared to the animals in panel A. C) RNAi of *dpy-13*. Animals are at the same stage of development as animals in panel A, but display a "dumpy" body morphology.

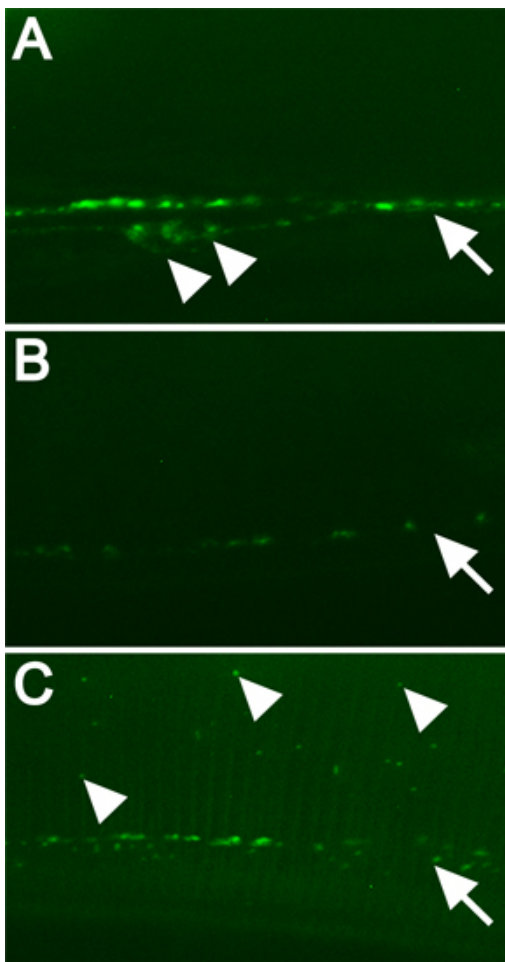


Figure 3. Examples of fluorescently tagged protein and how RNAi of specific genes alters localization pattern. All images were taken at 630x magnification with spinning disk confocal microscopy, 5 sec. exposure. Scale bar = 10 μ m. All images were treated identically. Open arrowheads indicate cell bodies. Arrows mark line of punctae. Filled arrowheads indicate some aberrantly localized GFP-tagged DBL-1. A) RNAi-fed pseudogene C06C3.5 ("wild-type"). B) *dbl-1(RNAi)* control. C) RNAi of a gene required for normal localization of GFP-tagged DBL-1.

Date:

	1	2	3	4	5	6
A						
B						
C						
D						

Figure 4. Template example for tracking RNAi experiments (library clones) in 24-well plates. This template can be used to create a permanent record of the experiments, unlike directly labeling plates.

Date

C. elegans RNAi Screen

Well; Gene	phenotype 1	tissue affected 1	cell affected 1	Other observations
Animal # 1				
2				
3				
4				
Well; Gene				
Animal # 1				
2				
3				
4				

Figure 5. Template example for recording RNAi phenotypes. Expand as needed.

Discussion

The RNAi screening method presented here enables a sensitive and rapid analysis of gene products required for a normal (or transgenic) postembryonic phenotype. The example shown is a screen for genes involved in the subcellular localization of a fluorescently tagged protein. However, this protocol can be modified to identify genes affecting other postembryonic phenotypes of interest.

This method takes advantage of a candidate gene approach by using an RNAi library. Forward genetic screens using mutagenesis techniques identify randomly induced novel alleles, but those alleles require substantial time, effort, and/or funds to identify²⁴. With the cDNA library, on the other hand, sequencing of the cDNA insert that caused an RNAi phenotype of interest rapidly confirms the candidate gene. Also, cloning-ready cDNA inserts facilitate downstream analyses of the identified candidates. Furthermore, genes that are lethal when mutated may be identified and analyzed using RNAi. By placing hatched animals on the induced bacteria, this method permits the observation of postembryonic defects for genes that also have vital embryonic roles.

Take care to confirm that observed animals are not starved. Starvation can affect the phenotype of the animal. If novel RNAi phenotypes are identified in hungry animals, repeat to confirm that the phenotype is a result of the RNAi and not starvation. To prevent starvation, use fresh bacteria that have been grown to stationary phase (the growth medium is very "cloudy"), and use only ~ 30 animals per well.

Disclosures

We have nothing to disclose.

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