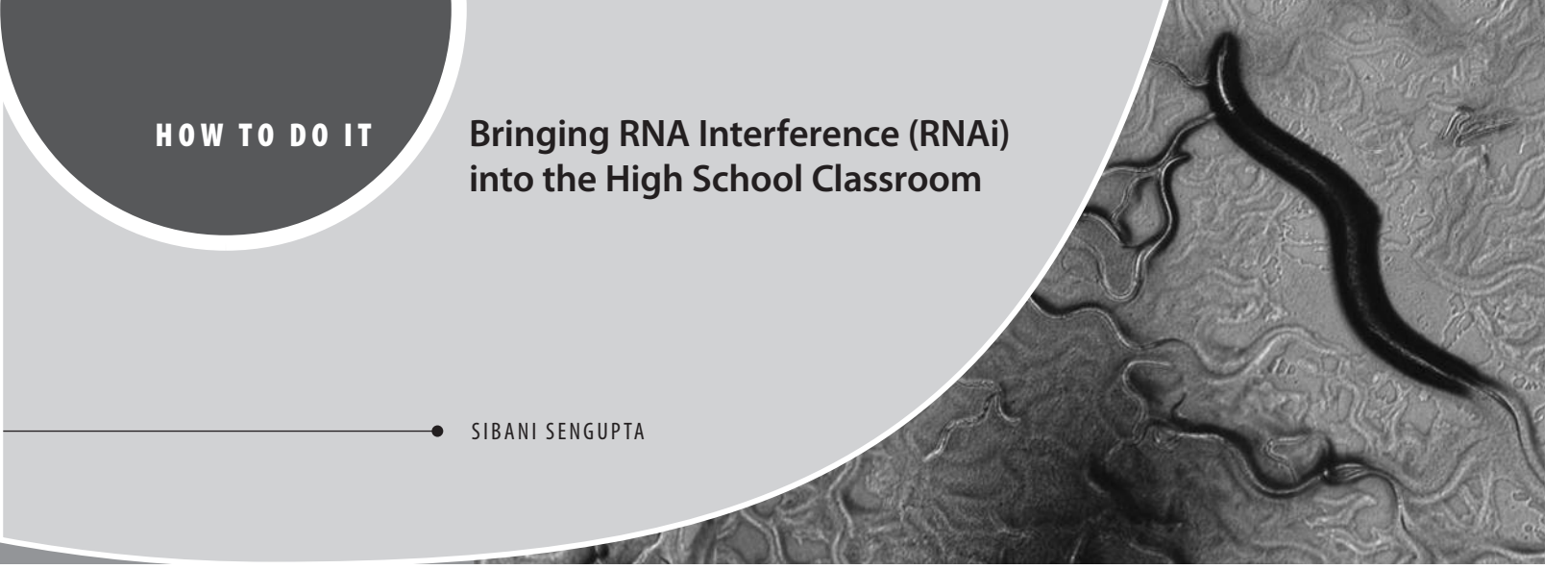
HOW TO DO IT

Bringing RNA Interference (RNAi) into the High School Classroom



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Abstract

RNA interference (abbreviated RNAi) is a relatively new discovery in the field of mechanisms that serve to regulate gene expression (a.k.a. protein synthesis). Gene expression can be regulated at the transcriptional level (mRNA production, processing, or stability) and at the translational level (protein synthesis). RNAi acts in a gene-specific manner and degrades the specific message (mRNA) to lower mRNA stability and, in the process, decreases protein production. The RNAi mechanism thus acts as a negative regulator of gene expression and undoubtedly has been one of the most significant developments in genetics and molecular biology in recent years. I present a teaching module that can help high school students experience this unique post-transcriptional regulatory mechanism.

Key Words: *RNAi; RNA interference; siRNA; small interfering RNA;* Caenorhabditis elegans*; wild type;* egl-1*; egg laying defect;* pos-1*; posterior segregation.*

RNA interference (RNAi) is a great tool for studying gene interactions. Scientists can use RNAi to "turn off" a gene in an organism, study the mutant phenotypes, and gain a better understanding of gene function (Sengupta, 2010). Since RNAi was first discovered in petunias (see http://www.pbs.org/wgbh/nova/sciencenow/3210/02.html), this technology has come a long way and has been tried in many experimental models, including the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. The latter is a great genetic model because it has transparent skin, simple anatomy, and fast reproduction time (Figure 1) and is easy to manipulate (see http://www.wormatlas.org). *Caenorhabditis elegans* is a free-living worm that exists predominantly as a hermaphrodite and rarely as a male. The hermaphrodite can self-fertilize; hence, it has been used to propagate lab strains over many generations, thus enabling creation of progeny that are genetically identical to the parent worm (see http://avery.rutgers.edu/WSSP/StudentScholars/project/introduction/worms.html). *Caenorhabditis elegans* can be grown easily in labs, including high school labs, on bacterial lawns because they feed on bacteria and take ~3 days to go from egg laying to adulthood (Figure 1). The fertilized eggs are at the 40-cell stage when they are laid by the hermaphrodite and, upon hatching (post utero development), go through four larval stages: L1 → L2 → L3 → L4. As shown in Figure 1, each transition (L1 → L2, L2 → L3, etc.) involves molting and can be referred to as markers of worm development. The L4 larvae take another ~18 hours to become a mature adult with egg-laying capabilities. Under unfavorable conditions such as crowding and high temperature, the L1 larvae, instead of moving on to L2, will enter a dormant state called the "Dauer form." The worm can stay in this condition for months and then, upon restoration of a favorable environment, return to the L4 stage. In the time since Brenner's studies, *C. elegans* genomics has come a long way, especially now that the Worm project has successfully sequenced the 19,735 protein-coding genes in the worm genome and >90% of these are backed by experimental evidence as well (Hillier et al., 2010). This has provided a major impetus to scientists and educators alike to design and adapt studies using *C. elegans* aimed at understanding how genes regulate form and function. According to the *National Science Education Standards*, in grades 9–12, students should be actively engaged in a wide variety of hands-on activities so that they can appreciate science as a dynamic process (National Research Council, 1996). The RNAi module offered here serves as a great foundation to get students excited about RNAi and follows up with an inquiry-style activity in which students can choose the gene-pair interactions that they would like to investigate using RNAi. This module is appropriate for Honors or AP Biology classes but can certainly be modified for other classes as well. This was planned out as a class project and was introduced in early spring after the class had covered units on genetics and cell biology. Our classes were run on a block schedule and were ~80 minutes in length. Depending on workload, until we moved the worms to the RNAi plate, we were usually able to accommodate the lab-related activities in ~30 minutes.

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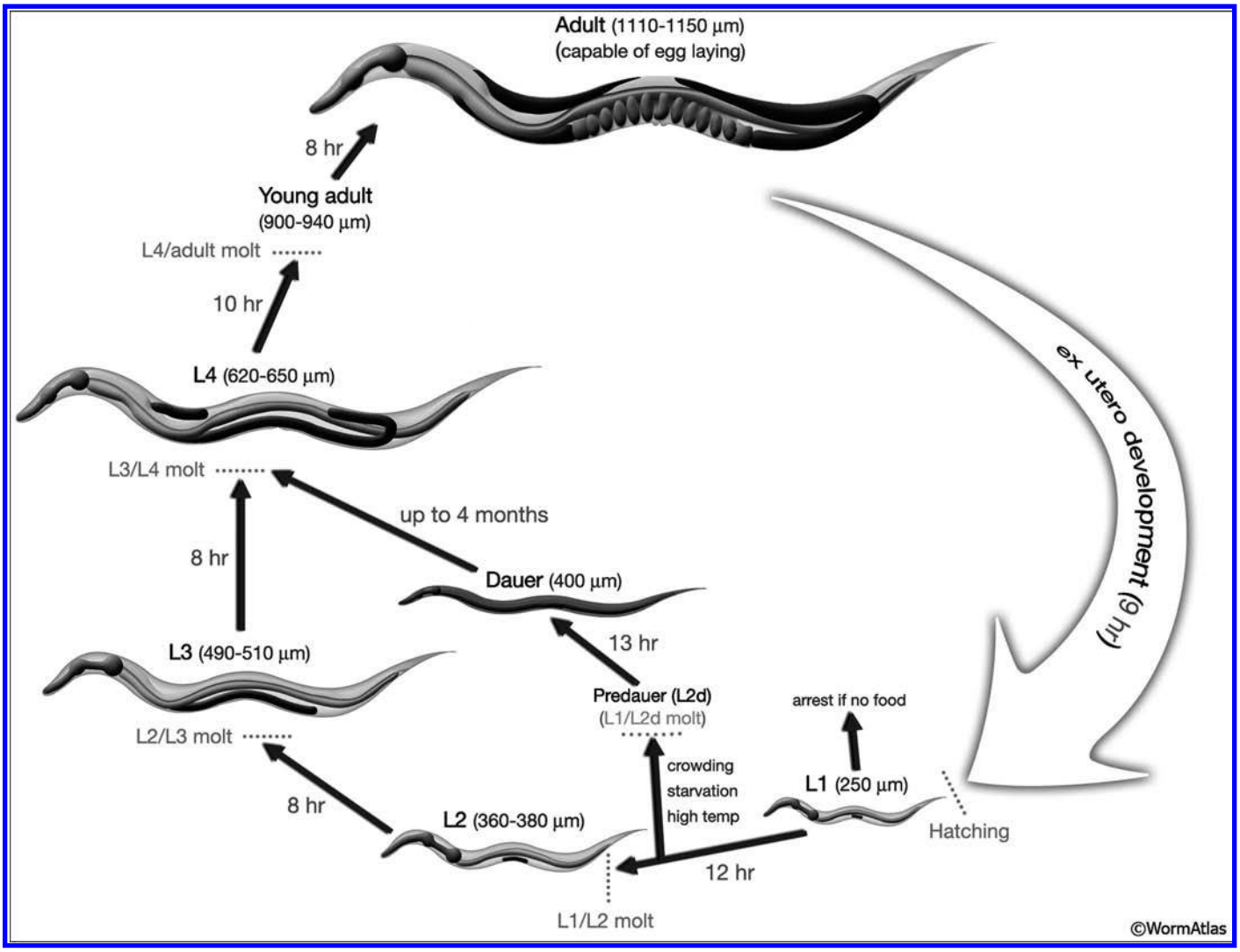

Figure 1. Life cycle of the nematode *Caenorhabditis elegans* (reprinted with permission from http://www.wormatlas.org).

○ Introduction

The project is introduced to the class by showing them their study model, *C. elegans*, and discussing the advantages of this genetic model. The nematode is easily viewed (adults are ~1 mm long) with the stereomicroscopes and light microscopes commonly found in high school science laboratories. Although it needs ~3 days to go from egg laying to adulthood at 25°C, its growth rate can easily be regulated by manipulating the growth temperature (at 15°C, the life cycle takes ~6 days). For the most part, we have grown the nematodes at room temperature, about 20–22°C. Next, the class is given a brief overview of RNAi (Sengupta, 2010; http://www.pbs.org/wgbh/nova/sciencenow/3210/02.html) and the potential that it holds as a tool to study gene function, by turning off genes in a targeted fashion and studying resultant phenotypes. The class is then assigned their first worksheet (Figure 1), based on a 15-minute Web tutorial. The information from the worksheet is eventually incorporated into the project report.

Next class, the process of RNAi is discussed using the worksheet as the backdrop. At this point, the teacher can use simple sketches – some self-drawn and some figures from the PBS Nova Web tutorial (http://www.pbs.org/wgbh/nova/sciencenow/3210/02.html) as well to illustrate the mechanism of RNAi (http://www.hhmi.org/biointeractive/rna/rna_interference/01.html), and students can take notes on the second worksheet (Figure 2). The following day, the class discusses the answers to the worksheet questions as a group and is then assigned the article "Decoding the riddle: the dawn of RNAi for the study of gene–gene and gene–environment interactions" (Stribinskis & Ramos, 2004) for homework.

○ Rationale

In this lab the class will try to suppress the *egl-1* phenotype (and rescue the worms) by turning off another gene called *pos-1* with the help of RNAi. The first *C. elegans* mutation is *egl-1*, which stands for "egg-laying defect," and worms that have this defect are unable to release their eggs (Trent et al., 1983). Even when the eggs have been fertilized, they are still not released and instead are hatched while inside the worm. This creates a "bag of worms" (see Figure 3). These worm embryos will continue to develop inside the adult worm, and

eventually the worms are released, but the adult worm dies from having too many other worms inside it. *Pos-1*, which stands for "posterior segregation," encodes a protein that regulates embryonic development (Tabara et al., 1999), and *pos-1* mutant worms produce eggs that are arrested in embryonic development. So if *pos-1* is turned off by RNAi in the *egl-1* mutant worms, the eggs inside the *egl-1* worm will not hatch and, thus, the *egl-1* worms will now display a "bag of eggs" phenotype (see Figure 4).

RNAi Self-study Worksheet 1

Play the video [http://www.pbs.org/wgbh/nova/sciencenow/3210/02.html] and answer the following questions in your own words.

1. Name any five diseases that could be cured by RNAi. For each disease, briefly indicate how RNAi could provide a treatment option.
2. Explain how proteins are made, using the castle-and-cookbook analogy. Where are these recipes sent? What does the chef do with these protein recipes?
3. How was RNAi discovered? In which organism was the discovery made?
4. (Explain the experiment in detail: what was their aim → how did they start → what was their plan → what was the result?)
5. Why were petunias picked for their experiment? Name the scientist who was trying to make the purple petunia. What was the surprising finding?
6. What does "RNAi"stand for? Write a simple explanation that outlines your understanding of RNAi.

Figure 2. RNAi self-study worksheet 1.

RNAi Self-study Worksheet 2

1. What aspect of viral infection signals the cell about a possible invasion? How does RNAi help the cell to counteract the viral attack?
2. Briefly describe the functions of siRNA, DICER, and RISC.
3. Discuss the importance of the RISC/RNA complex in the RNAi pathway. Then, in a diagram, describe the roles played by double stranded RNA (dsRNA), DICER, siRNA, and RISC in the RNAi mechanism.

Figure 3. RNAi self-study worksheet 2.

○ Materials & Methods

- *C. elegans N2* (wild type) and *egl-1* mutants, obtained from http://www.silencinggenomes.org/ (Tabara et al., 1999)
- *Escherichia coli* OP50 (wild type *E. coli B* strain used to feed *C. elegans*) and *pos-1* RNAi feeding strain, now called "HT115(DE3)/pL4440(pos-1)," obtained from http://www.silencinggenomes.org/. The *pos-1* strain of *E. coli* is engineered so that it will make *pos-1* dsRNA (double stranded RNA) required to induce *pos-1* gene silencing via RNAi (Tabara et al., 1999).
- NGM (nematode growth medium) lite media powder (catalog no. N1005), ampicillin (no. A2262), and IPTG (no. I8500-05) were purchased from US Biologicals, MA.
- Petri dishes (100 × 15 mm; can be purchased from any common vendor, e.g., Ward's Science or Carolina Biological Supply)
- Dissecting microscopes, compound light microscopes, Bunsen burners, scalpels, permanent markers, pipettes, alcohol

Media preparation and culture methods used were either as outlined at http://www.silencinggenomes.org/ or were modified as indicated below to suit the target audience.

○ Procedure

Petri dishes with *C. elegans N2* and the mutant *egl-1* strain are introduced to the class, and students check their understanding of the mutant worms by simply studying them under the microscope (Figure 4). At this time, students also learn how to differentiate between the adult worm and the ones

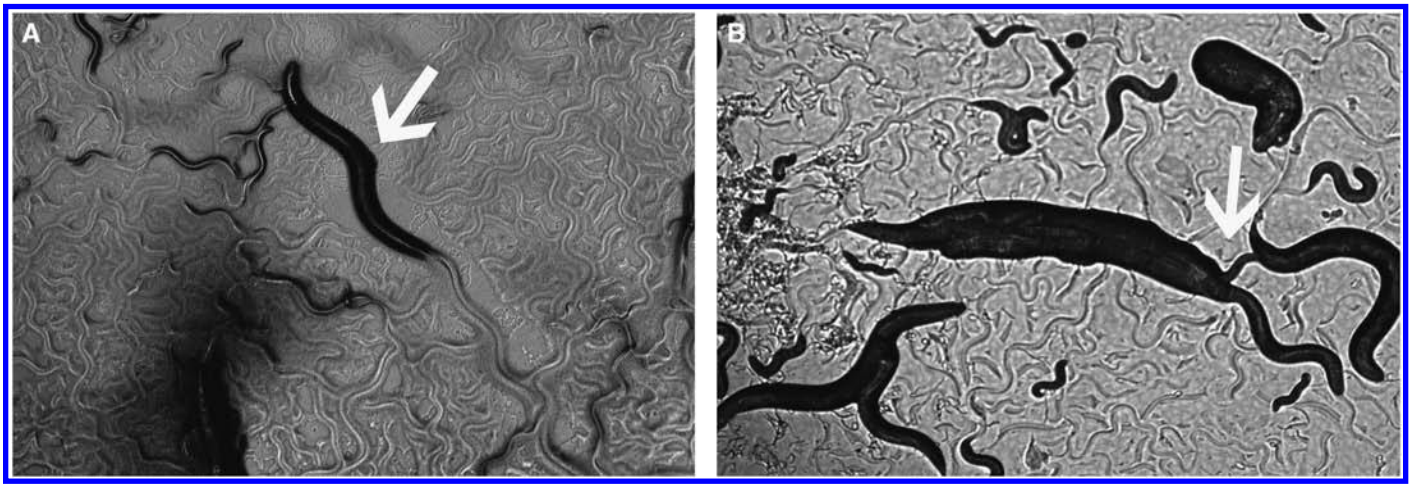

Figure 4. Wild type *Caenorhabditis elegans N2* (wild type). (A) An adult worm (white arrow) in the center and L2, L3, and L4 worms (in the background) and the *egl-1* strain. (B) Center: an adult worm (white arrow) with larvae coming out of it, displaying the "bag of worms phenotype," with L2, L3, and L4 mutant worms in the background.

in L1–L4 stages. As outlined in Figure 1, the worm continually grows as it moves through L1, L2, L3, and L4 stages but nearly doubles in length when it goes from L4 to adult.

First, each group makes starter plates. They receive two Petri dishes with NGM lite media that are labeled *N2* and *egl-1*, respectively. The Petri dishes are seeded with *E. coli OP50* and incubated overnight at 37°C to allow bacterial growth. The next day, student groups transfer agar chunks (see Laboratory 2 at http://www.silencinggenomes.org/) with either *N2* or *egl-1* worms in L4 stage from the master plates to the group starter plates. To perform the chunking method, first the scalpel needs to be sterilized by dipping it in alcohol and then holding it in the flame of the Bunsen burner. Then, using the dissecting microscope, a place is located on the original plate where there are only L4 larvae. Next, using the scalpel, a 1-cm chunk is cut out of the NGM where there are no adults and transferred to the starter plates. These plates are incubated for ~48 hours at room temperature.

The next day, the class seeds two NGM lite plates with *E. coli OP50* and two NGM lite + IPTG plates with *pos-1* (see Laboratory 6 at http://www.silencinggenomes.org/; the *pos-1* dsRNA production in this strain is IPTG inducible, which means that *pos-1* RNA production is turned on by adding IPTG). Next day, these four NGM lite plates that were seeded the previous day with either *OP50* strain or *pos-1* strain are marked:

1. *N2/OP50* 2. *egl-1/OP50*
3. *N2/pos-1* 4. *egl-1/pos-1*

The class then transfers the worms onto these plates using the chunking method (see Laboratory 6 at http://www.silencinggenomes.org/). Here, I have slightly modified the protocol. The original protocol stipulates picking five L4 worms and transferring them to the new plates, using a simple tool, appropriately called the "worm pick," that resembles the classic platinum loop used for inoculating microbial cultures. Initial trials revealed that using this worm pick as a transfer tool, besides being expensive, was time consuming and quite exhausting for high school students. So I modified the protocol as follows. The class identifies a part of the plate with five nonadult worms (in any of the L1–L4 stages), cuts out this chunk using a sterile scalpel, and transfers it to the newly seeded plate. The reasoning behind the modification is that because only the adult worm has egg-laying and reproductive capabilities, inducing RNAi in any of the earlier stages should still work to suppress the *egl-1* mutant phenotype. The plates are then incubated upside down at 20°C (see Laboratory 6 at http://www.silencinggenomes.org/). The students start making observations 48 hours later, and then check the plates daily. A class divided into five groups will have five sets of experimental data that are highly similar.

Results

My class carried out the activity described above, with the following results. The goal of the project was to suppress the *egl-1* mutation by turning off *pos-1* via the RNAi technique. The *N2* strain of *C. elegans* growing on *OP50* was the control to ensure that the *N2* strain was growing normally so that worms in different stages of the life cycle were visible (Figure 5A). The second plate had the *egl-1* strain of *C. elegans* growing on *OP50*; because of the egg-laying defect, the adult worms in this plate displayed the "bag of worms" phenotype, as expected (Figure 5B).

The next set of two Petri dishes had *C. elegans* that had been subjected to RNAi. The wild type strain *N2* growing on *pos-1* RNAi feeding strain was expected to show "embryonic lethality" because *pos-1* regulates embryonic development. In these plates, therefore, the eggs did not hatch and the Petri dish was filled with eggs (Figure 5C). This plate was our control to ensure that *pos-1* RNAi was working as expected. The final set of Petri dishes had *egl-1* worms growing on the *pos-1* RNAi feeding strain. We expected that if *pos-1* RNAi was successful, then, in the absence of *pos-1*, the adult worms should display the "bag of eggs" phenotype (Figure 5D). Also as expected, the adult worms survived and, hence, the *egl-1* mutation was successfully suppressed (data summarized in Table 1). The data from the various student groups were highly comparable (Figure 6). Even though the module was time consuming, the sheer excitement in the

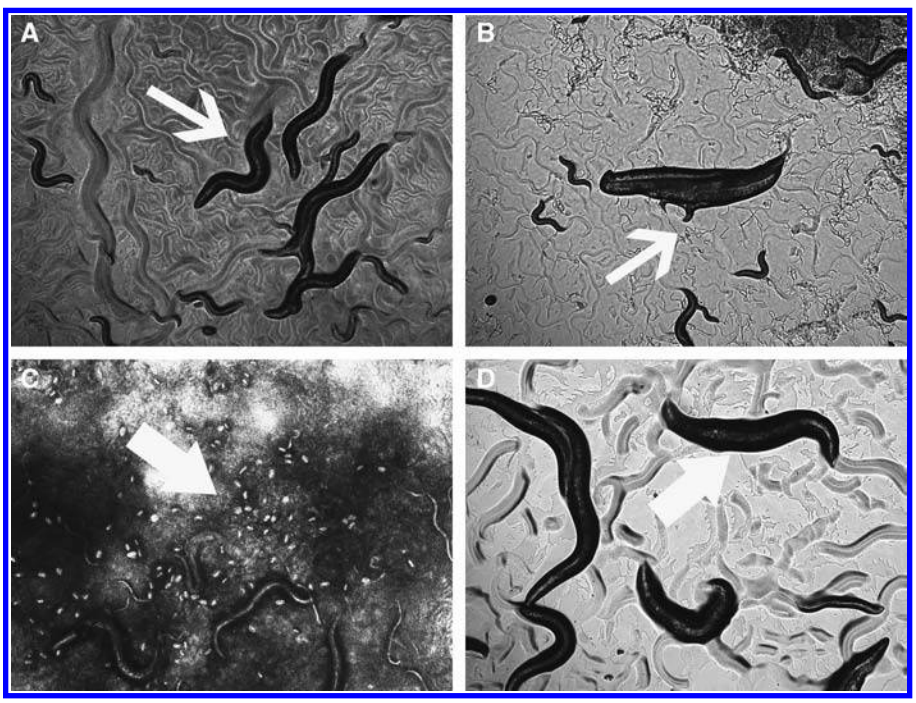

Figure 5. *Caenorhabditis elegans N2* (wild type) and *egl-1* growing on *OP50* and *pos-1* RNAi feeding strain of *Escherichia coli*. (A) *N2* on *OP50*: adults and various larval stages. (B) *Egl-1* on *OP50*: adults showing the "bag of worms" phenotype – larvae emerging from the adult (arrow). (C) *N2* on *pos-1* RNAi feeding strain: eggs are unhatched (thick arrow) because embryonic development is blocked. (D) *Egl-1* on *pos-1*: adults are bloated ("bag of eggs" phenotype, dashed arrow) because embryonic development is blocked in these egg-laying defective worms.

Table 1. Summary of RNAi results.

C. elegans strain / *E. coli* strain	***N2* (wild type)**	***Egl-1* (egg-laying defect) mutant**
OP50 (regular feeding bacteria)	Normal adults + L1–L4	"Bag of worms" phenotype – worms emerging from the adults
***Pos-1* (pos-1 RNAi inducing strain)**	Unhatched eggs	"Bag of eggs" phenotype – bloated worms

class during experimentation and upon the successful completion of the RNAi project was incredible.

Conclusion

This module offers high school students an opportunity to learn and appreciate RNAi. From the geneticist's standpoint, the *egl-1*/*pos-1* interaction would be more meaningful if the second mutation restored the egg-laying

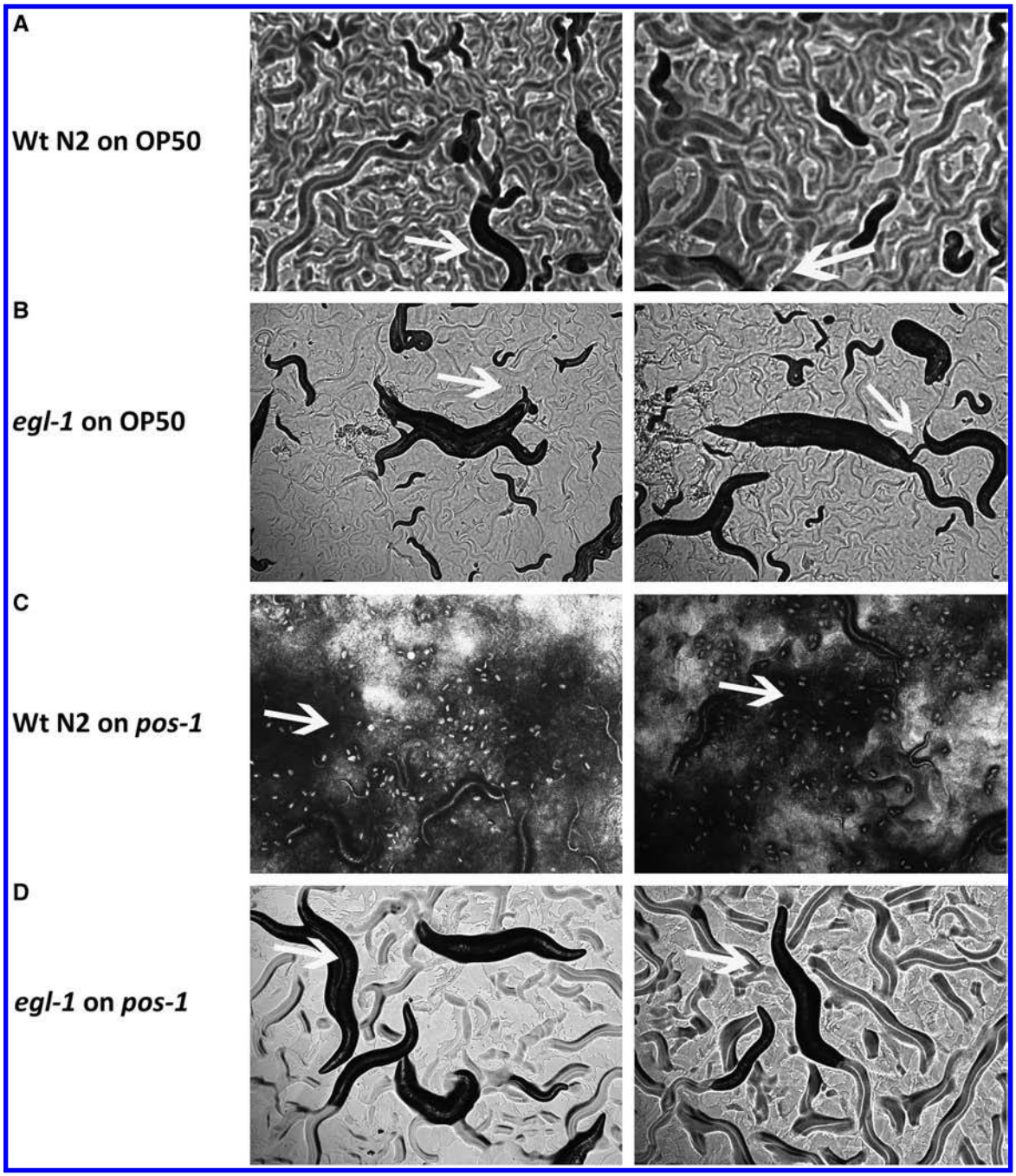

Figure 6. Sample data from various student groups. (A) *Caenorhabditis elegans N2* (wild type) on *Escherichia coli OP50*: adults and various larval stages. (B) *C. elegans egl-1* on *OP50*: adults showing "bag of worms" phenotype – larvae emerging from the adult. (C) *N2* on *pos-1* RNAi feeding strain shows unhatched eggs. (D) *Egl-1* on *pos-1* RNAi feeding strain shows bloated adult worms ("bag of eggs" phenotype).

capability instead of allowing the adult worms to survive by causing embryonic lethality (Tabara et al., 1999). In this case, the teacher can explain gene interactions and how a defect in one gene (*egl-1* mutation) can be suppressed by knocking down a second, related gene such as *pos-1*. Besides allowing the teacher to discuss genetic principles like suppression and rescue of gene function, the activity helps introduce a complex post-transcriptional regulatory mechanism, in a way that captures student attention and interest.

○ Acknowledgments

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