BIOCHEMISTRY AND MOLECULAR BIOLOGY EDUCATION
Vol. 36, No. 6, pp. 417–427, 2008

Laboratory Exercises

Caenorhabditis elegans as an Undergraduate Educational Tool for Teaching RNAi*

Received for publication, July 8, 2008, and in revised form, July 29, 2008

Janet Andersen‡§¶, Alexander Krichevsky‡§, Joerg R. Leheste‖, and Daniel J. Moloney‡

From the ‡Department of Biochemistry and Cellular Biology, Center for Science and Mathematics Education, Stony Brook University, Stony Brook, New York 11794-5215 and ‖Department of Neuroscience, New York College of Osteopathic Medicine, New York Institute of Technology, Old Westbury, New York 11568-8000

Discovery of RNA-mediated interference (RNAi) is widely recognized as one of the most significant molecular biology breakthroughs in the past 10 years. There is a need for science educators to develop teaching tools and laboratory activities that demonstrate the power of this new technology and help students to better understand the RNAi process. *C. elegans* is an ideal model organism for the undergraduate laboratory because of the simplicity of worm maintenance, its well-studied genetic background, and the fact that it can be employed as a model organism in laboratory environments where vertebrate research is restricted. Certain unique features of *C. elegans* make it a very suitable organism for RNAi studies. Specifically, nematode strains highly sensitive to RNAi are readily available from public sources, and RNAi induction by a feeding method is an uncomplicated procedure that lends itself readily as an educational tool. In this article, we provide a detailed depiction of the use of *C. elegans* as an RNAi educational tool, describing two separate RNAi-based experiments. One is a qualitative experiment where students can examine the effects of knocking down the *unc-22* gene involved in the regulation of muscle contraction, which results in a "twitching" phenotype. The other experiment is a quantitative RNAi experiment, where students measure the effect of knocking down the *lsy-2* gene involved in neuronal development. Although these experiments are designed for a college-level study, nematode research projects can also be accomplished in secondary school facilities.

Keywords: RNAi, *C. elegans*, undergraduate education.

INTRODUCTION

RNA Interference

The RNA world opened up in the 1980s with the discovery that specific RNA structures housed enzymatic activity. The word "ribozyme" was coined by Dr. Thomas R. Cech after he discovered the catalytic self-splicing RNA from *Tetrahymena*, for which he won the Nobel prize in chemistry in 1989. During the 1980s, researchers also discovered a number of small RNAs in prokaryotes that regulated plasmid DNA replication and bacterial gene expression [1]. Many of these small regulatory RNAs were called "anti-sense" RNAs, since they were complementary to a targeted mRNA (sense) [1, 2]. In the 1990s, researchers developed various strategies to make appropriate "anti-sense" RNAs for gene inactivation in a number of eukaryotic organisms [3]. However, the mechanism of "anti-sense" RNA suppressive activity in eukaryotes remained obscured and enigmatic [2, 3]. Not until the discovery of the inhibitory effect of double-stranded RNA (dsRNA)[1] was RNA-mediated interference (RNAi) understood in eukaryotes. This article describes in detail the design and implementation of two laboratory activities that we are using at the college level to teach this new and exciting concept.

The mechanism of RNAi appears to be a highly conserved eukaryotic process (for review see ref. [4]), and RNAi activity has been found in yeast, protozoa, plants, and animals (including humans). RNAi is believed to be a cellular defense against infection by RNA-carrying viruses like influenza. The phenomenon is induced by production of a dsRNA with corresponding sequence to a target mRNA. Once taken up by a cell, dsRNA is cleaved into

*HHMI Grant 52003746 to Dr. R. David Bynum for continued support of BIO 311 "Techniques in Molecular and Cellular Biology" and for the Postdoctoral Teaching Fellowship to JRL.

§Equal contribution.

‡To whom correspondence should be addressed. Department of Biochemistry and Cellular Biology, 094 Life Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794-5215. Tel.: 631-632-1674; Fax: 631-632-9791. E-mail: jandersen@notes.cc.sunysb.edu.

[1]The abbreviations used are: dsRNA, double-stranded RNA; RNAi, RNA-mediated interference; siRNA, small interfering RNA; IPTG, isopropyl-β-D-thiogalactopyranoside; ASEL, ASE left; ASER, ASE right; MCS, multiple cloning site; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid.

 DOI 10.1002/bmb.20233

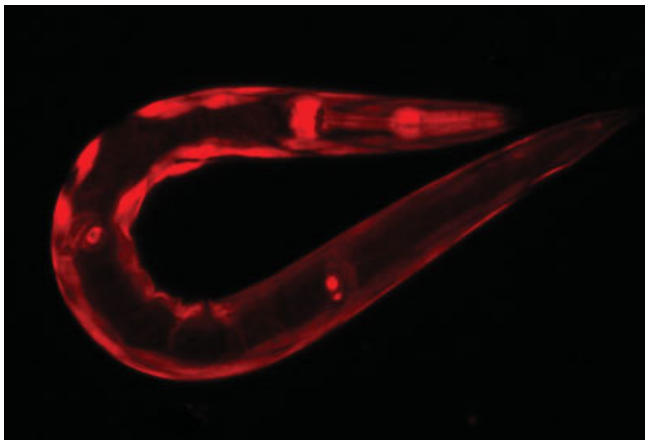

FIG. 1. **View of actin in *C. elegans* fixed with cold acetone and stained with fluorescein-conjugated phalloidin [5].**

small interfering RNA (siRNA) molecules (~23 bp long) by an enzyme called Dicer [4]. siRNAs bind to a complex known as RNA-induced silencing complex. Here, the double-stranded siRNA is unwound so that the single RNA strands are free to base-pair with complementary cellular mRNA molecules. The cellular mRNA molecules are then systematically degraded by another enzyme called Slicer, which obliterates the complementary RNA sequences.

RNAi is thus a biological mechanism that can silence the expression of a specific gene. RNAi gene "knockdown" acts via targeted and highly specific degradation of messenger RNAs, thus silencing the expression of a given gene at a posttranscriptional level. The specificity and potency of RNAi makes it ideal for investigating gene functions in many organisms, including the soil nematode *C. elegans*.

RNAi in Caenorhabditis elegans

C. elegans (Fig. 1) is a well-studied laboratory model organism that offers a great potential for genetic analysis, partly because of its rapid (3-day) life cycle, small size (1.5-mm-long adult), and ease of laboratory cultivation. Its name is derived from Latin: *Caeno* meaning "recent," *rhabditis* meaning "rod-like," and *elegans* meaning "nice or beautiful" [6]. Hundreds of these animals can be grown on a single Petri dish seeded with a lawn of *Escherichia coli* as the food source. Certain unique features of *C. elegans* also make it a suitable organism for RNAi studies. RNAi techniques include a variety of methods, from injecting *C. elegans* with double-stranded (ds) RNA to soaking *C. elegans* in a solution containing dsRNA. A relatively simple RNAi technique for knockdown of a targeted gene involves feeding the nematodes bacteria-expressing dsRNA corresponding to the gene's mRNA [7]. This feeding method is sufficient for successful RNAi induction in the worms and lends itself readily as an educational tool. Comprehensive procedures for "using RNAi in *C. elegans*" are well described on the Fire Lab website [8].

Some strains of *C. elegans* are more sensitive to RNAi than others. Nematode strain NL2099 was used in our RNAi studies. A special strain of *E. coli*, HT115 (DE3), was used for RNAi studies in *C. elegans*; this strain lacks RNAse III, an enzyme that degrades dsRNA as it is being made. It also contains a T7 RNA polymerase gene under the control of the *lac* operon (DE3 designation) allowing initiation of RNAi production from a plasmid containing opposing T7 promoters following induction with IPTG. HT115 (DE3) can be transformed with RNAi-producing vectors containing parts or all of the sequence from an mRNA corresponding to the target gene to be silenced. The gene is placed between two T7 promoters juxtaposed to each other (Fig. 2*a*) and after induced expres-

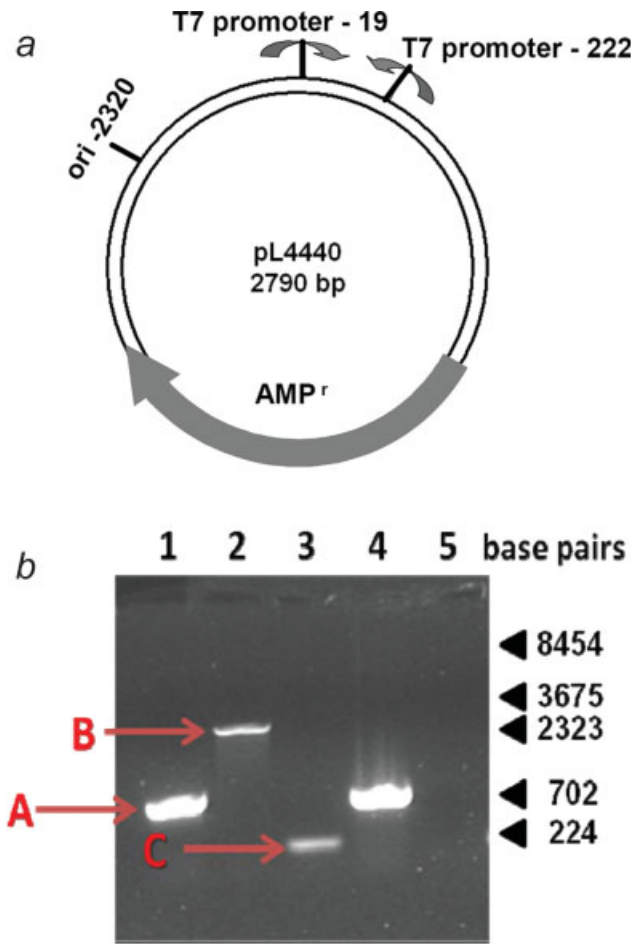

FIG. 2. **Map of the "empty" RNAi vector, L4440.** (*a*) T7 promoters are located about 200 base pairs (bp) apart flanking the multiple cloning site, where inserts—such as partial *lsy-2* or *unc-22* cDNAs—are cloned. The two arrows show the directions of transcription from these promoters. "AMP^r" and the solid arrow show the location and orientation of the ampicillin resistance gene, and "ori -2320" shows that location of the pBR322 origin of replication. (*b*) Verification of the cloned insert by PCR; lane 1) 698 bp amplicon for A culture containing RNAi plasmid for *unc-22;* lane 2) ~2300 bp amplicon for B culture containing RNAi plasmid for *lsy-2*; lane 3) 222 bp amplicon for C culture containing "empty" RNAi vector; 4) 698 bp amplicon for 10 ng of PCR positive-control RNAi plasmid for *unc-22*; lane 5) negative PCR result with no DNA template added; lane marked "base pairs") position of bands of the lambda BstEII DNA marker (New England Biolabs) corresponding to the number of base pairs shown.

sion of T7 RNA polymerase with isopropyl-β-D-thiogalactopyranoside (IPTG), simultaneous transcription from both promoters results in the production of complementary RNAs, eventually forming dsRNAs. Although the process is not completely understood, *C. elegans* ingests *E. coli*, but does not completely digest or break down the ingested dsRNA. The dsRNA is absorbed through the worm digestive tract, and the process of RNAi begins. RNAi is reported to be robust and may be transmitted to F1 offspring produced by the animal [4, 9].

The entire *C. elegans* genome was sequenced in 1998, making the *C. elegans* the first multicellular organism to have its genome fully sequenced [10]. Analysis of the genome sequence data has emphasized the considerable conservation of biological mechanisms and processes across the animal kingdom including humans. There are Internet sites such as Worm Base [11], Worm Atlas [12], and Worm Lab [13] that contain a wealth of information about *C. elegans*. This makes the nematode a useful tool for teaching students to use bioinformatics Internet resources that link genetic information with human diseases. *unc-22* and *lsy-2* are two *C. elegans* genes with mammalian homologues. In our course, we employ RNAi induced knockdown of both these genes in nematodes as educational tools for undergraduate students.

unc-22 Gene

There are several *C. elegans* genes with the designation "*unc*," which stands for "uncoordinated" (for more information see [14]). The *unc-22* gene [15] codes for the protein "twitchin," which affects muscle coordination in *C. elegans* and plays a role in the regulation of muscle contraction. According to Wormbase [11], the *unc-22* encoded protein functions in the actinomyosin-contraction relaxation cycle and is likely to interact with myosin to regulate its function. unc-22 protein localizes to myosin-containing A-bands [16] of the worm's striated body-wall muscle [15–17] and is suggested as a component of thick filaments [18]. Loss of function of *unc-22* in *C. elegans* leads to impaired movement and to the distinctive "twitcher" phenotype. It has been reported that the "twitcher" phenotype can be elicited by placing *unc-22* knockout worms on 1% nicotine or the nicotinic agonist levamisole, causing the mutants to twitch violently for many hours while wild-type animals become stiff and immobilized [18].

lsy-2 Gene

C. elegans are attracted to certain chemicals in search of food sources. As potential food sources, *C. elegans* moves toward water-soluble attractants such as ions, cyclic nucleotides, and amino acids. This directional movement toward specific chemicals is termed chemotaxis and is analogous to other animals' sensation of taste. In *C. elegans*, chemotaxis is mainly governed by two morphologically bilateral symmetric gustatory neurons, ASE left (ASEL) and ASE right (ASER) [19, 20]. The ions sodium, chloride, and potassium are sensed in a left/right asymmetric manner by two neuronal nodes ASEL and ASER. Specifically, ASEL is involved in sensing sodium ion, but not chloride and potassium ions [21]. If ASE L and R functions are ablated, only a weak residual response to water-soluble attractants is observed, which is distributed across alternative neuronal pathways [22, 23], suggesting a leading role of ASE neurons in chemotaxis [24].

The ASEL versus ASER lateral developmental decision is governed by a complex gene regulatory network composed of transcription factors and microRNAs. It has been shown [24] that expression of *lsy-6* micoRNA is required for execution of the ASEL fate. A C2H2-type zinc finger transcription factor, *lsy-2*, related to the human protein ZNF404, is required for the expression of *lsy-6* and other ASEL-specifying factors [24]. In *lsy-2* null mutants [24], the ASEL neuron adopts the complete ASER gene-expression profile, resulting in two symmetric "right" ASE neurons [25]. The *lsy-2* knockout animals are insensitive to sodium ions because of improper ASEL development. Knocking down *lsy-2* using RNAi also results in improper ASEL development in the F1 generation and loss of chemotaxis toward sodium ion [25].

The ability of *C. elegans* to sense sodium ions and migrate toward them can be tested using chemotaxis plates. The chemical to be tested—in our case sodium ions in form of a NaCl solution—is placed at one end of a plate. A control chemical such as double-deionized water is placed at the opposite end. A group of worms starts out at the center of the plate at an equal distance between the attractant and control spot. Worms are allowed to move freely for about an hour and are thereafter immobilized by sodium azide. The numbers of animals that have migrated to the attractant versus control position are compared. The majority of the wild-type worms will migrate toward the attractant, while *lsy-2* knockdown *worms*, unable to sense sodium ion, will more or less equally disperse between the control (H_2O) and the attractant (NaCl).

MATERIALS

Solutions and Media (also see [20])

Standard NGM Agar Plates—Prepare 0.3% NaCl, 0.25% bactopeptone, 1.7% bactoagar. Autoclave the media, cool to 50°C, and then add the following sterile solutions to 5 μg/mL cholesterol; 1 mM $CaCl_2$ solution; 1 mM $MgSO_4$; 25 mM potassium phosphate buffer, pH = 6.0. Mix thoroughly before pipetting 5–8 mL of the solution into 6-cm Petri dishes.

Nematode RNAi Feeding Plates—Prepare standard NGM agar plates (see later) and add per liter: 50 μg/mL ampicillin, 12.5 μg/mL tetracycline, and 0.4 mM IPTG.

Chemotaxis Assay Plates—Prepare 2% bactoagar in double-deionized water. Autoclave the media, cool to 50°C, and then add the following sterile solutions to 5 mM potassium phosphate buffer, pH = 6.0; 1 mM $CaCl_2$; 1 mM $MgSO_4$. Pipette 10 mL into each 10-cm plate.

1 M Potassium Phosphate Buffer—Prepare 1 M solutions of each monobasic and diabasic potassium phos-

TABLE I
Week one

Week 1 materials	Per bench or student group	PCR analysis
Worms	1. For inoculation, *C. elegans* feed with OP50 bacteria on 60 mm nematode plates without AMP 2. Container for student's agar plates after inoculating 3. 16°C incubator	1. PCR Thermocycler (Amplitron II, Barnstead Thermolyne Corp.) 2. 100 0°C heat block 3. Filtered micropipette tips 4. Sterile ultrapure water
Bacteria	1. 1.5 mL induced culture of HT115 (DE3) + pLT61 1. 1.5 mL induced culture of HT115 (DE3) + pX-1P11 2. 1.5 mL induced culture of HT115 (DE3) + pL4440	5. Reaction tubes 6. 10X PCR buffer 7. 50 mM $MgCl_2$ 8. 10 mM dNTP mix 9. Taq Polymerase at5 U/μL 10. 2–10 ng each of the three control templates (pL4440, pLT61) 11. T7 promoter forward primer (20 μM) Forward Primer: T7 promoter primer (Invitrogen) sequence = TAATACGACTCACTATAGGG 12. PCR parameter, see Table II
Bench items	1. Kimwipes 2. ICE 3. Bins with 0.5 and 1.5 white tubes 4. Pipetteman 5. Tips for pipetteman 6. Sterile scalpel 7. Bifocal microscope with light 8. Sterile 1.5 mL and 0.5 mL centrifuge tube packets (3 per pack) 9. Bacterial cell spreader 10. Beaker for ethanol 11. Bunsen burner 12. Permanent markers and rulers 13. Three- 100 mm nematode plates with AMP/TET/IPTG 14. Three 10 cm chemotaxis plates with 10 mL "chemo" agar 15. 150 mL flask with 10 mL bleach 16. Parafilm 17. Container to freeze PCR tubes after reaction	
Shared equipment	1. 37°C bacterial incubator 2. Microcentrifuges	

phate solutions. Titrate KH_2PO_4 with K_2HPO_4 until a pH of 6.0 is reached. Sterilize by filtration or autoclaving.

LB Medium—Prepare 1% bactotryptone; 0.5% yeast extract; 0.5% NaCl. Sterilize through autoclaving. (see ref. [26] for protocols on bacterial growth and maintenance).

Additional Stock Solutions—Make sterile solutions of ampicillin 100 mg/mL; tetracycline 5 mg/mL; 2.5 M NaCl; 100 mM IPTG; and 2.5 M sodium azide.

Plasmids and Strains

Three RNAi plasmids were used in this study. L4440 (purchased from Addgene no. 1654) is an "empty" RNAi vector used in control worms (Fig. 2*a*; for additional information see refs. [27] and [8]). The "empty" RNAi vector (L4440) is a modified version of pBluescript with a T7 promoter on each side of the multiple cloning site (MCS) driving the transcription of dsRNA from both DNA strands. Plasmid pLT61 (purchased from Addgene no. 1690) was used to knockdown expression of *unc-22* (for more information on pLT61 please refer to Addgene [28]). Plasmid pX-1P11 was a kind gift from Dr. Oliver Hobert and was used to knockdown expression of *lsy-2* [24] (for more information on *lsy*-2 refer Hobert Lab Website [29]). Verification of each plasmid can be made through polymerase chain reaction (PCR) as shown in Fig. 2*b* and described Tables I and II.

The HT115(DE3) RNase III-deficient *E. coli* strain with IPTG-inducible T7 polymerase activity (available from the *Caenorhabditis* Genetics Center [30]; for further information please refer to [9]) was used for expression of the dsRNA and nematode feeding. *C. elegans* strain NL2099 with a homozygous deletion of the *rrf-3* gene *(genotype rrf-3[pk1426] II, homozygous rrf-3 deletion allele)* [31] encoding for an RNA-directed RNA polymerase homolog

TABLE II
PCR parameters

Step	Temperature (°C)	Time	Purpose
1	94	5 min	Denaturation
2	94	30 sec	Denaturation
3	55	30 sec	Annealing
4	72	3 min	Elongation
Repeat steps 2–4 above 30 times.			
5	72	6 min	End

that inhibits somatic RNAi and contributes to hypersensitivity to RNAi treatment in this strain comparative to the wild-type worms. This strain can also be purchased through the *Caenorhabditis* Genetics Center [30].

EXPERIMENTAL DESIGN AND METHODS FOR TEACHING

Maintenance of Worm Cultures

C. elegans are extremely easy to grow as they feed on plates of *E. coli* and multiply rapidly. Furthermore, they can be cryogenically preserved like tissue cultures, which is critical during periods of intermittent research. Cultures of *C. elegans* were maintained on NGM plates as described [6]. An auxotrophic, untransformed strain of *E. coli* (OP50) was used to routinely feed the worms. One week before the laboratory exercise, cultures of *C. elegans* were synchronized by starvation, which is important for the subsequent RNAi feeding. The chemotaxis assay plates were prepared at the same time.

Week 1 (see Table I for Preparation Details)

RNAi Induction—The instructor needs to perform the RNAi induction before each laboratory session. The HT115(DE3) bacterial cells containing either *unc-22* (pLT61) or *lsy-2* (pX-1Pl1) RNAi constructs, or control ("empty") L4440 RNAi vector, were grown overnight at 37°C in LB culture medium in the presence of 100 μg/mL of ampicillin and 12.5 μg/mL of tetracycline. The overnight cultures are diluted 20-fold with fresh LB media supplemented with antibiotics and grown additionally for 3–4 hours at 37°C until an OD_{600} of 0.6 has been reached. RNAi is produced after induction with 0.4 mM IPTG followed by incubation for 4–5 hours (see Fig. 3*b*) (Note: the last step of RNAi induction with IPTG can be alternatively carried out at room temperature overnight). The induced cultures are concentrated by centrifugation by the students before they are spread on the RNAi feeder plates (see later).

Verification of Plasmids Identity Using PCR—Our students start out by sizing the inserts of the RNAi plasmids through PCR to confirm that the bacteria are transformed with empty, *unc*-22 or *lsy*-2 RNAi expression vectors (Fig. 2*b*). Five microliters of each bacterial culture are boiled for 5 minutes in 50 μL of ultrapure water and centrifuged to sediment cellular debris. The clear supernatants are used in a PCR reaction using a primer specific for the T7 RNA polymerase promoter (see Table I). Only one primer is needed because the RNAi vector backbone contains two identical T7 promoter sequences flanking the gene insertion site. The PCR conditions can be found in Table II. The PCR amplification mixture was prepared according to the manufacturer instructions (Invitrogen, Carlsbad, CA).

After the reaction, the reaction mix and a DNA molecular weight standard are loaded on a 2% agarose gel containing 1× Tris, acetate, EDTA buffer (BioRad Laboratories, Inc., Hercules, CA), electrophoresed and stained with SYBR-Green (Invitrogen). Upon completion, the gel was illuminated under UV and photographed (Fig. 2*b*).

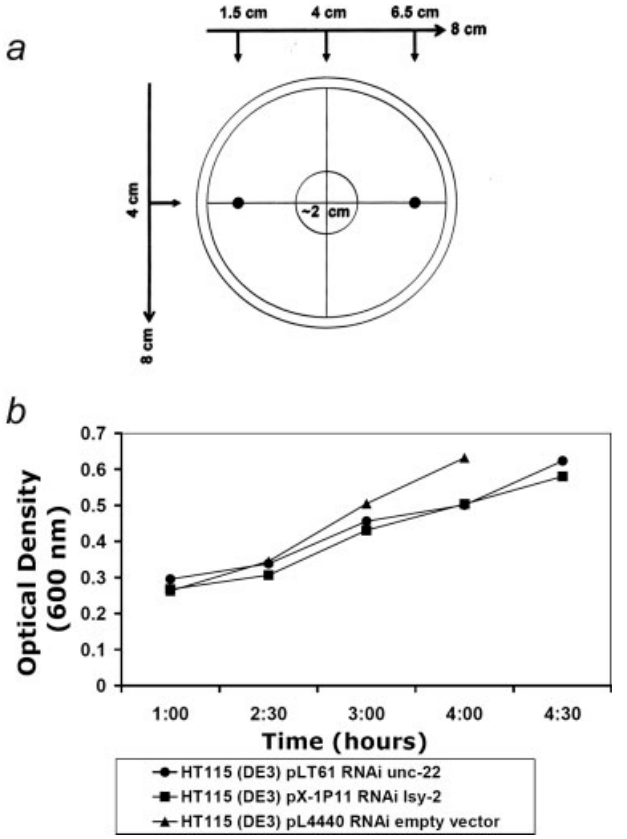

FIG. 3. (*a*) Schematic representation of the markings on a 10-cm Petri dish for the chemotaxis experiment; dots on the grid show locations of attractant (NaCl) and control (sterile water). The central circle is where the harvested worms are placed. (*b*) Representative results of optical density of bacterial cultures prior to dsRNA expression induction with IPTG for one of the sections.

Feeding protocols and RNAi through ingestion of dsRNA have been described in detail [27]. First, the students have to prepare their own *E. coli* HT115 (DE3) feeder plates. Each student or student group prepares three feeder plates as follows:

1. *E. coli* containing the pLT61 plasmid, which contains a gene coding for dsRNA targeted to the *unc-22* gene (hereafter denoted as "A"),
2. *E. coli* containing the pX-1P11 plasmid, which contains a gene coding for dsRNA targeted to the *lsy-2* gene (hereafter denoted as "B"),
3. *E. coli* containing the empty L4440 plasmid, which is used as a control (hereafter denoted as "C").

For each nematode RNAi feeding plate, 1.5 mL of the induced HT115 (DE3) cultures were concentrated by centrifugation in a microcentrifuge tube, resuspended in 100 μL of LB medium, transferred to and spread on an NGM agar plate containing 50 μg/mL of ampicillin, 12.5 μg/mL of tetracycline, and 0.4 mM IPTG, and incubated at 37°C for 3 hours. This concentration step is necessary to provide enough food for the worms.

Using a sterile scalpel, a cube of agar (~0.2–0.5 cm) containing starved *C. elegans* was cut from a freshly prepared NGM master plate and transferred to the center of a richly seeded NGM RNAi feeder plate. The transferred

TABLE III
Week 2

Week 2 materials	Per bench or student group	Analysis of amplicons
For RNAi analysis	1. 2 NGM agar plates (6 cm) 2. Three Chemotaxis test plates with 10 μL on each spot a. Spot "W"= dd H_2O b. Spot "Na" = 2.5 M NaCl	1. Power supply 2. Gel electrophoresis apparatus 3. Photography equipment for gel 4. Flask for preparation of agarose gel 5. Agarose 6. TAE 10X buffer 7. Ultrapure water 8. 100 and 500 mL cylinders 9. SYBR green 10. 10 μL lambda BstEI1 marker 11. 10 μL 100 bp marker 12. 45 μL 6X TD
Bench items	1. Kimwipes 2. ICE 3. Ultrapure water on ice 4. Three 15 mL conical tubes 5. Bins with 0.5 and 1.5 white tubes 6. Pipetteman 7. Tips for pipetteman 8. Bifocal microscope with light 9. 0.25 M Na Azide 10. Set of four colored marker pens 11. 5 mL pipettes 12. Pipette Aid 13. Flask with 10 mL of bleach	

worms were allowed to reproduce for 5–7 days (at least one generation time) at 16°C. This system has been adjusted to yield results after 7 days, when the students return for the subsequent laboratory section. It is however possible to grow worms at slightly higher temperatures (20–25°C) requiring shorter feeding times (~3–4 days) to induce the same phenotype.

Week 2 (see Table III for Preparation Details)

Preparation of the Salt Gradient on Chemotaxis Plates—The chemotaxis assay plates need to be prepared ahead of time by the instructor (see solutions and media). Additionally, the students can mark the bottom part of their chemotaxis plates as shown in Fig. 3*a* during the first week of the laboratory exercise. A day before the chemotaxis assay, a Na^+ gradient is set up on one side of the plate by placing 10 μL of a 2.5 M NaCl solution on the agar by the Na "dot." (Please note that the Cl^- ion is evenly dispersed through NGM agar due to the addition of $MgCl_2$). On the opposite side of the Na^+ "dot," the negative control is placed in the form of 10 μL of sterilized double-deionized water on the agar by the W "dot." Additional control plates should have water added to the agar by both dots. The substances are allowed to diffuse through the agar for 14–16 hours or overnight at room temperature to establish a sodium ion gradient.

Harvesting C. elegans for Analysis—The students wash the worms off each RNAi feeder plate with 5 mL of ice-cold sterile water three times, and transfer each rinse to a separate 15 mL tube kept on ice. Then the tubes are covered and inverted several times. The worms are allowed to settle for 10 minutes in the conical tube on ice before the water is removed, leaving a residual 1 mL volume containing the worms. The wash is repeated a second time by adding ice-cold water to 15 mL, inverting, and allowing the worms to resettle. After removing 14 mL of wash water, the worm suspension is transferred to a sterile 1.5 mL microcentrifuge tube, centrifuged for 1 minute at 10,000 rpm, and the pelleted worms are gently resuspended in 50 μL of the residual liquid. The nematodes are then transferred to the appropriate assay plate. Pipette tips may have to be cut to prevent damage to the worms.

Chemotaxis Assays—On the day of the experiment, while the worms are being washed off the RNAi feeder plates, the students place 2–3 μL of 0.25 M sodium azide solution on the agar by the NaCl and water spots to immobilize worms approaching those spots. Please note that residual bacteria, salts, and other potential food materials that may attach to the worms can interfere with the chemotaxis assay. It is therefore very important to thoroughly wash nematodes used for the chemotaxis assay. The harvested worms are then transferred to the 2 cm circle located in the middle of the plate (see Fig. 3*a*). While under the microscope, excess water is gently removed using the twisted end of a Kimwipe.

The worms are allowed to move on the plate for 1 hour. Results are obtained by counting worms located near the NaCl attractant spot (Na), the negative control spot (W), or the center circle of the plate. Worms are easily counted by looking at the inverted plates under a dissecting microscope while methodically dotting the position of each worm with a permanent ink marker. The ink dots can be later counted without using the microscope (see Fig. 4).

unc-22 Assay—Nematodes are washed, placed on NGM plates, and observed under a dissecting microscope. The "twitcher" phenotype of *unc-22* silenced worms can be observed in direct comparison with the control worms, treated with an "empty" RNAi vector. It may be helpful to touch the worms with the end of a hair accentuating the phenotype of the *unc-22* knockdown worms.

RESULTS AND DISCUSSION

Expected Results Student Data Analysis

As previously described, the laboratory RNAi exercise started with inducing growing cultures of HT115(DE3),

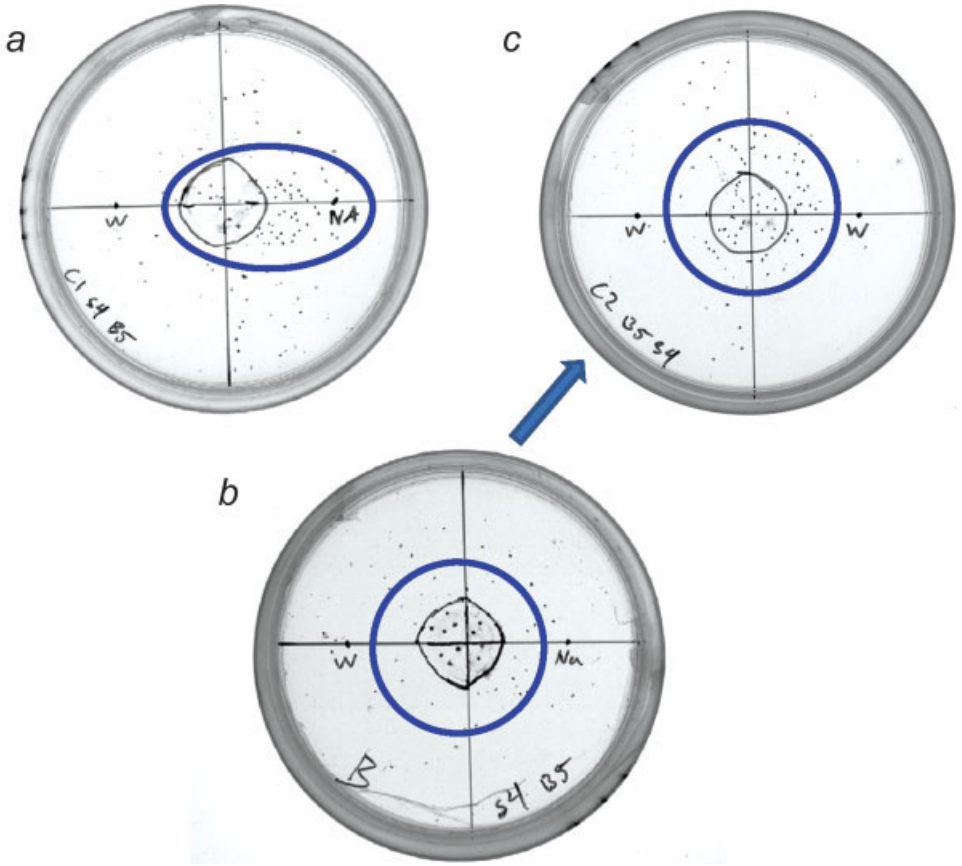

FIG. 4. **Representative chemotaxis experiment results obtained by one of the student groups**. Circles mark the areas of the agar where most worms were found. Plates demonstrate (*a*) migration of control ("empty" RNAi vector) nematodes toward the NaCl attractant (marked "NA" on the plate); (*b*) *lsy-2* RNAi nematodes failed to sense the attractant and dispersed evenly on the plate; (*c*) the control nematodes ("empty" RNAi vector) did not prefer either side of the plate when the attractant was absent, and both spots treated with sterile water (marked "W" on the plate). The arrow points to the similarity of plate C with plate B. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

containing an "empty" RNAi vector (L4440), used in the control worms (C), and either pLT61 RNAi *unc-22* (A) or pX-1P11 RNAi *lsy-2* (B), the experimental vectors used to knockdown expression of *unc-22* and *lsy-2*, respectively. Fig. 3*b* shows the growth of all three bacterial cultures prior to IPTG induction for one section. In order to successfully induce bacterial culture in the time-constrained student laboratory, the dilution of the overnight starter culture has to be precise, and best determined empirically by the instructors. The diluted culture has to be in the logarithmic growth phase, which translates into a minimum of one to two cell divisions. Dilution of the starter culture below $OD_{600} = 0.1$ may result in a significantly lengthened growth time until the target induction of $OD_{600} = 0.6$. An insufficient dilution above $OD_{600} = 0.4$ will not allow the bacteria to enter the log phase, and thus induction with IPTG will not be effective, resulting in an inefficient RNAi experiment. In this particular assay system, we have found that a 20× dilution of HT115 (DE3) fresh overnight culture works best, allowing to reach target optical density within the time allotted.

The three groups of *C. elegans*, used in the chemotaxis and *unc-22* analyses, were first observed on their NGA plates after 1 week of feeding on their respective *E. coli* RNAi feeder bacteria. Table IV summarizes student observations about the *C. elegans* with respect to general behavior, appearance, and abundance (density on plate). Comparative worm abundance was estimated

TABLE IV
C. elegans phenotype after RNAi

Characteristic	A: pLT61	B: pX-1P11	C: L4440
Average size and behavior	Comparatively small. Extremely lethargic. Many individuals static.	Comparatively small. Extremely lethargic. Many individuals static.	Comparatively large. Extremely lively and motile.
Estimated relative abundance (order of magnitude per viewing field)	10^1	10^1	10^2

TABLE V
RNAi unc-22 student observations

Characteristic	A: *RNAi unc-22* (pLT61)	C: empty vector (L4440)
Particulars in movement	Some worms move erratically, exhibiting jerky movement rather than smooth movement.	Worms appear to move smoothly and easily.
Other particulars	Average worm size is small compared to C. More worms are dead than on plate C.	Average worm size is large compared to A. Fewer worms are dead than on plate A.

by counting the number of worms in one average viewing field under identical magnification. In the experimental groups (A, *unc-22* RNAi and B, RNAi *lsy-2*), no distinction was observed between large and small worms with respect to lethargy—both exhibited the characteristic. Among worms in the control group (C), larger worms generally exhibited more movement.

After general observations, the worms were washed and redistributed onto new plates. A more detailed observation was conducted of the *unc-22* silenced worms (group A). Student observations about the *unc-22* silenced worms are recorded (Table V). Chemotaxis trials were performed over a period of 1 hour with the *lsy-2* silenced worms (group B) and were compared to the control group (C).

Chemotaxis Assay

For the chemotaxis assay, plates were evenly divided into halves, one containing sodium ion attractant and the other sterile water as control (for marking scheme see Fig. 3*a*). Representative results of a successful chemotaxis experiment, obtained by a group of 67 students, are shown in Fig. 4. Control worms, bearing "empty" RNAi vector and thus having wild-type *C. elegans* phenotype, predominantly migrate to the Na^+ ion gradient on half of the plate as expected (Fig. 4*a*). The nematodes with *lsy-2* RNAi inhibited expression, on the other hand, disperse evenly on the plate as expected due to lose sensitivity to the attractant sodium ion due to faulty neuronal development of the left node (Fig. 4*b*). As an additional control, chemotaxis plates with water spots on both halves of the plate (no Na ion or other attractant used) were employed and seeded with "wild type" (empty RNAi construct treated) nematodes. A random migration and even dispersion was observed (Fig. 4*c*). Worms that had failed to exit the central circle at the end of one-hour chemotaxis experiment were assumed dead or otherwise damaged and were counted as separate group. The chemotaxis assay itself without RNAi feeding has great potential as an educational tool.

Statistically, chemotaxis results of RNAi fed worms that were obtained by the students could be divided into three groups, as shown in Table VI: Group A—expected results had been obtained, Group B—*lsy-2* RNAi inhibited nematodes behaved similarly to control animals and, Group C—overall number of nematodes was insufficient to establish statistical significance. Three representative chemotaxis plates from group A were selected to demonstrate a successful RNAi experiment. Figure 5*c* shows that the control "wild type" nematodes indeed dispersed evenly on "no attractant" plates where water was used at both spots. When Na^+ ion was used as an attractant (Fig. 5*a*), the "wild type" *C. elegans*, treated with "empty" RNAi vector, strongly preferred the half of the chemotaxis plate containing the Na^+ ion gradient. In contrast, as shown in Fig. 5*b*, *lsy-2* RNAi inhibited worms dispersed evenly on the Na^+ ion containing plate. This behavior is similar to the control group on plates without attractant (Fig. 5*c*) and suggests to the inability to discriminate between either half of the plate due to RNAi-impaired attractant sensing.

Other student results, which failed to produce what was expected, were divided according to potential reasons for their failure to Groups B and C (Table VI). Group C, representing 23% of all student sections, failed due to insufficiently low numbers of nematodes or a very low number of nematodes capable to leave the 2 cm circle in the center of the plate. This most probably reflects poor animal harvesting techniques, which may be improved through better *C. elegans* washing. Although group B, representing ~40% of all student sections, successfully maintained large numbers of live nematodes, the students failed to produce the expected results as their *lsy-2* RNAi inhibited worms behaved similarly to the control nematodes. This most probably results from failure to inhibit *lsy-2* by RNAi, and may be addressed by improving the induction of HT115 (DE3) cultures and/or using higher concentrations of induced bacteria on the RNAi feeder plates. Notably, in most cases, the total number of *lsy-2* silenced nematodes has been similar to the total number of the control nematodes.

RNAi knockdown in nematodes, a multistep and complex assay, is not easy to establish and properly execute—even for seasoned scientists. In the original study where each worm was singularly subjected to the chemotaxis assay, only 44% of the RNAi *lsy-2* feed worms lost sensitivity to sodium ion compared to 0% of the

TABLE VI
Analysis of students' RNAi lsy-2 results

	No. of lab teams	Percentage (%)
Group A (expected results)	11	37
Group B (*lsy-2* same as control)	12	40
Group C (insignificant numbers)	7	23
Total plates	30	100

control worms [24]. A 37% success rate among our students was therefore considered satisfactory. Potential failures when using RNAi as a teaching tool can be reduced, but probably not totally eliminated. Explaining why expected results are not obtained can become important discussion topics in the students' laboratory reports and better prepares them for the research environment.

unc-22 Assay

The *unc-22* suppressed worms were placed on NGM plates and observed under a dissecting microscope. The *unc-22* RNAi treated nematodes were compared with "wild type" animals treated with an "empty" RNAi vector for the "twitcher" phenotype. We assume that the unc-22 assay is subject to the similar potential pitfalls described for the chemotaxis assay, and thus rarely yields a 95–100% *unc-22* knockdown. However, most students (~75%) are able to see some worms exhibit the "twitcher" phenotype after *unc-22* RNAi treatment. A summary of observations from successful students are recorded in Table V.

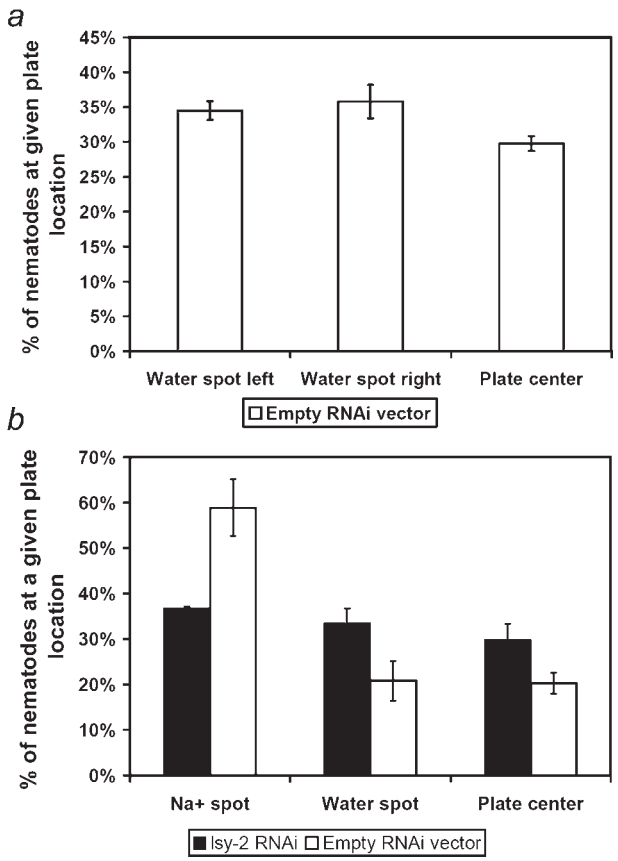

FIG. 5. **Statistical dissection of the chemotaxis experimental results obtained by the students in group A, where expected results have been achieved.** (*a*) Migration of control nematodes on plate with no attractant (water/water) (*b*) Chemotaxis of nematodes towards attractants (Na^+ spot/water spot) after treatment with *lsy-2* inhibited (black bars) or control "empty" RNAi vector (white bars). Standard deviations are calculated based on independent results obtained from three study sections.

This assay is attractive for a college setting because of its simplicity and the fact that the *unc-22* homolog (Titin) is implicated in human disease. Titin functions in a similar manner in humans by aiding muscle contraction and relaxation [26]. Deficiencies in Titin are related to a variety of myopathies and cardiomyopathies, including muscular dystrophy.

Placing the *unc-22* knockdown worms on nicotine or levamisole plates caused a phenotype similarly observed in wild-type worms (data not shown). However, knockdown of *unc-22* resulted in a transient uncoordinated phenotype and the worms displayed some degree of erratic movement and jerkiness when transferred to fresh NGM agar plates without nicotine or levamisole.

Assessment

At the end of the 2 weeks, each student writes an extensive lab report that contains summary, introduction, results, and discussion sections. The summary is a short paragraph where the students address what they set out to accomplish and the outcomes they expected. Background information is provided in the introduction that aids the reader in understanding the experiments and their significance. The students place all their data (gel pictures, graphs and tables) in the results section and write a meaningful descriptive narrative of the data. In the discussion section, the students explain what they expected to find, and how their results compare with their expectations. They also give plausible, scientific explanations for unexpected results, explain how they could test the explanation to determine if it is correct and based on their results, propose questions they would next address for new insights about the study. In addition, the students are guided in writing the discussion section by a series of questions found in the laboratory protocol for the two lab sessions. For example, for this laboratory exercise, the students had to answer the following questions: "What is the purpose of feeding the worms *E. coli* with the RNAi empty vector? What other *E. coli* might be feed the worms? Explain. What additional information would be gained by doing this?" The discussion section also allows room for personal expression from the students about the laboratory exercise. Many students think the lab is "cool," because it allows them to work on a live organism and see genetic manipulations in a timely manner (former students, personal communication). The writing of the laboratory reports in the scientific format prepares students for reading and writing research papers.

CONCLUSIONS

The discovery of the process of RNAi of gene expression is widely recognized as one of the most significant molecular biology breakthroughs in the past 10 years. A challenge science educators now face is the development of teaching tools and lab activities that demonstrate the power of this new technology that will help students who are training to become future researchers and medical professionals to understand the process.

Why choose *C. elegans* to study the RNAi process? Much of the RNAi pathway was elucidated in *C. elegans*. Andrew Fire and Craig Mello's RNAi research in *C. elegans* resulted in both being awarded the 2006 Nobel prize in medicine. *C. elegans* is an ideal model organism for a teaching laboratory setting because of the simplicity of maintenance. Furthermore, materials required for *C. elegans* research are inexpensive and found in most basically equipped biology laboratories. In addition, *C. elegans* is an invertebrate and can be used as a model organism in laboratory environments where vertebrate research is restricted.

Most students in our experience are initially hesitant to perform experiments on worms. However, after examining the worms under a microscope and observing how beautiful and graceful they move, feed, lay eggs, and multiply, most students become emotionally attached to their plate of nematodes as if the worms were pets. The students are often excited to see the worms' progression from week to week.

This article describes two separate RNAi-based experiments. One is a qualitative experiment where students can examine the effects of knocking down the *unc-22* gene, involved in muscle contraction resulting in a "twitch" phenotype. The other experiment is a more sophisticated and quantitative RNAi experiment where students measure the effect of knocking down *lsy-2*—a gene involved in neuronal development.

The *unc-22* twitching or uncoordinated phenotype may be a subtle phenotype and requires close examination of the worms under binocular or microscope. By proper comparison of the *unc-22* knockdown worms versus controls ("empty" RNAi vector), students should be able to distinguish worms that twitch from worms that do not. To add a quantitative element to the activity, students may count the number of worms on the plate and determine the percentage that exhibit a phenotype. Students can be introduced to the idea of evaluating the plates "blindly"—not knowing whether they are examining *unc-22* knockdown worms or control knockdown worms. As part of their assignment, students are required to search for the function of *unc-22* online, and to report if there is a homolog of *unc-22* in humans. This experiment helps to emphasize the two important concepts: 1) scientists can sometimes determine the function of a gene by blocking its expression; and 2) through understanding the function of genes in animal model systems, scientists may be able to determine the role of a gene in humans.

The RNAi *lsy-2* experiment takes a quantitative approach to demonstrating and analyzing RNAi. Successful knockdown of the *lsy-2* gene results in *C. elegans* that exhibit impaired chemotaxis to sodium ion. Although the preparation time for this laboratory experience is much greater, and the complexity of the analysis much higher than the *unc-22* analysis, students can measure, count, record data, photograph the phenotype results, and accomplish a statistical analysis of their RNAi results. This experiment teaches not only about the process of RNAi, but also about chemotaxis, neurobiology, and the proper use of various controls in an experiment. It also extends itself to a discussion of the role of microRNAs in the regulation of gene expression since knocking down *lsy-2* results in the silencing of *lsy-6* microRNA, which is important to proper ASEL development [24].

RNAi is the latest breakthrough allowing scientists to study gene function. Researchers have already begun to exploit the process of RNAi in attempting to combat virus infections and knocking down genes causing cancer and other pathologies. It is important to introduce this topic to the next generation of scientists and medical professionals, and even to the public.

Acknowledgments— We thank Dr. Oliver Hobert for his kind gift of pX-1P11 RNAi *lsy-2.* We also thank the undergraduate students and graduate teaching assistants of BIO 311 for their work and help with the RNAi laboratory exercises.

REFERENCES

[1] M. Inouye, N. Delihas (1988) Small RNAs in the prokaryotes: A growing list of diverse roles, *Cell* **53**, 5–7.
[2] W. Nellen, C. Lichtenstein (1993) What makes an mRNA anti-sensitive? *Trends Biochem. Sci.* **18**, 419–423.
[3] A. Coleman (1990) Antisense strategies in cell and developmental biology, *J. Cell Sci.* **97**, 399–409.
[4] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, C. C. Mello (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature* **391**, 806–811.
[5] M. Koelle, B. Bucher, A. Chisholm (revised 2008) Phalloidin-stained worms, available at: http://cobweb.darmouth.edu/.
[6] E. B. I. A. Hope (1999) *C. elegans*: A Practical Approach, Oxford University Press Inc, New York.
[7] L. Timmons, A. Fire (1998) Specific interference by ingested dsRNA, *Nature* **395**, 854.
[8] Fire lab web site (revised 2008) http://www.ciwemb.edu/.
[9] L. Timmons, D. L. Court, A. Fire (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*, *Gene* **263**, 103–112.
[10] S. Walsh, M. Anderson, S. W. Cartinhour (1998) ACEDB: A database for genome information. *Methods Biochem. Anal.* **39**, 299–318.
[11] Worm Base (revised 2008) http://www.wormbase.org/.
[12] Worm Atlas (revised 2008) http://www.wormatlas.org/.
[13] Worm Lab (revised 2008) http://130.15.90.245/wormlab_recipe_book.htm.
[14] M. Blaxter (revised 2008) An introduction to the biology and genetics of *C. elegans*, available at: http://www.nematodes.org/Caenorhabditis/index.shtml.
[15] D. G. Moerman, G. M. Benian, R. H. Waterston (1986) Molecular cloning of the muscle gene *unc-22* in *Caenorhabditis elegans* by Tc1 transposon tagging, *Proc. Natl. Acad. Sci. USA* **83**, 2579–2583.
[16] D. G. Moerman, G. M. Benian, R. J. Barstead, L. A. Schriefer, R. H. Waterston (1988) Identification and intracellular localization of the *unc-22* gene product of *Caenorhabditis elegans*, *Genes Dev.* **2**, 93–105.
[17] R. H. Waterston, J. N. Thomson, S. Brenner (1980) Mutants with altered muscle structure of *Caenorhabditis elegans*, *Dev. Biol.* **77**, 271–302.
[18] J. E. Kiff, D. G. Moerman, L. A. Schriefer, R. H. Waterston (1988) Transposon-induced deletions in *unc-22* of *C. elegans* associated with almost normal gene activity, *Nature* **331**, 631–633.
[19] S. Ward (1973) Chemotaxis by the nematode *Caenorhabditis elegans*: Identification of attractants and analysis of the response by use of mutants. *Proc. Natl. Acad. Sci. USA* **70**, 817–821.
[20] C. I. Bargmann (revised 2008) Chemosensation in *C. elegans*. WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/ wormbook.1.123.1, available at: http://www.wormbook.org.
[21] J. T. Pierce-Shimomura, S. Faumont, M. R. Gaston, B. J. Pearson, S. R. Lockery (2001) The homeobox gene lim-6 is required for distinct chemosensory representations in *C. elegans*, *Nature* **410**, 694–698.
[22] C. I. Bargmann, H. R. Horvitz (1991) Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in *C. elegans*, *Neuron* **7**, 729–742.
[23] A. Kaufman, A. Keinan, I. Meilijson, M. Kupiec, E. Ruppin (2005) Quantitative analysis of genetic and neuronal multi-perturbation experiments. *PLoS Comput. Biol*. **1**, e64.

[24] R. J. J. Johnston, O. Hobert (2005) A novel *C. elegans* zinc finger transcription factor, lsy-2, required for the cell type-specific expression of the lsy-6 microRNA. *Development* **132**, 5451–5460.
[25] C. O. Ortiz, J. F. Etchberger, S. L. Posy, C. Frøkjaer-Jensen, S. Lockery, B. Honig, O. Hobert (2006) Searching for neuronal left/right asymmetry: Genomewide analysis of nematode receptor-type guanylyl cyclases, *Genetics* **173**, 131–149.
[26] Sambrook Molecular Cloning, OMIM (revised 2008) available at: http://www.ncbi.nlm.nih.gov/sites/entrez.
[27] L. Timmons, A. Fire (1998) Specific interference by ingested dsRNA, *Nature* **395**, 854.
[28] Addgene (revised 2008) available at: http://www.addgene.org/.
[29] Hobert Lab Webpage (revised 2008) available at: http://www.cumc.columbia.edu/dept/gsas/biochem/labs/hobert/whoweare.html.
[30] *Caenorhabditis* Genetics Center (revised 2008) available at: http://biosci.cbs.umn.edu/CGC/CGChomepage.htm.
[31] T. Sijen, J. Fleenor, F. Simmer, K. L. Thijssen, S. Parrish, L. Timmons, R. H. Plasterk, A. Fire (2001) On the role of RNA amplification in dsRNA-triggered gene silencing, *Cell* **107**, 465–476.