A laboratory module that explores RNA interference and codon optimization through fluorescence microscopy using *Caenorhabditis elegans*

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Abstract

Authentic research experiences are beneficial to students allowing them to gain laboratory and problem-solving skills as well as foundational research skills in a team-based setting. We designed a laboratory module to provide an authentic research experience to stimulate curiosity, introduce students to experimental techniques, and promote higher-order thinking. In this laboratory module, students learn about RNA interference (RNAi) and codon optimization using the research organism *Caenorhabditis elegans* (*C. elegans*). Students are given the opportunity to perform a commonly used method of gene downregulation in *C. elegans* where they visualize gene depletion using fluorescence microscopy and quantify the efficacy of depletion using quantitative image analysis. The module presented here educates students on how to report their results and findings by generating publication quality figures and figure legends. The activities outlined exemplify ways by which students can acquire the critical thinking, data interpretation, and technical skills, which are beneficial for future laboratory classes, independent inquiry-based research projects and careers in the life sciences and beyond.

SCIENTIFIC TEACHING CONTENT

Learning Goals

- Gain experience working with *C. elegans*
- Understand the process of RNA interference and importance of codon optimization
- Learn basic microscopy techniques and image analysis
- Learn how to properly use the scientific method
- Enhance critical thinking skills

Learning Objectives

Students will be able to:

Lab 1 and 2:

- Identify specific larval stages of *C. elegans*
- Synchronize *C. elegans* larvae using alkaline hypochlorite treatment
- Understand codon usage
- Formulate hypotheses and design a controlled experiment

Lab 3 and 4:

- Acquire images using an epifluorescence microscope
- Effectively communicate results and formulate conclusions from data
- Describe what RNAi is and how it affects gene expression/activity
- Calculate mean fluorescent intensity from acquired fluorescence micrographs
- Perform statistical tests to determine the significance of results
- Generate publication quality figures and figure legends

INTRODUCTION

Inquiry-based learning is a form of active learning where students obtain the skills they need to problem solve and make unique discoveries about the natural world(1-3). In contrast to teacher-centered instruction, where facts are disseminated to students, inquiry-based learning encourages students to foster their own independent learning with the assistance of the instructor(1-3). In addition, inquiry-based learning puts emphasis on students developing science process skills, such as making observations, developing hypotheses, and formulating conclusions(1-3).

Course-based Undergraduate Research Experiences (CUREs) are a form of inquiry-based learning that provide students with a genuine research experience. Students enrolled in CUREs develop or are given a research question with an unknown outcome, use the scientific method to address the question, collect and analyze data, and communicate their results(3-5). Students that participate in a CURE learn the necessary skills and techniques they need to carry out the tasks required(6-8), and at the same time gain confidence in their ability to engage in the scientific process(9-11). Assessment of student learning gains reveal that CUREs improve student ability to think critically, interpret data, communicate results, and collaborate as a team, compared to traditional lab courses(12-17). A critical aspect of independent research is obtaining the foundational skills and introductory training needed for understanding a specific system and/or research topic of interest.

Here we describe a simple laboratory module employed in the first half of our upper division undergraduate CURE on developmental genetics, which is used to prepare students for independent inquiry-based group research projects that occur in the second half of the course. In this module,

students are introduced to the research organism, *Caenhorhabditis elegans* (*C. elegans*), to explore the concepts of RNA interference (RNAi) and codon optimization. *C. elegans* offers many advantages that make it an ideal research organism, such as a fast life cycle, large brood sizes, and easy access to genetic manipulation by forward and/or reverse genetic approaches(18-20). Additionally, they are transparent, which allows for visualization of all tissue types, and the real-time visualization of fluorescent-tagged reporter proteins expressed in various tissues of interest(21, 22). Using the protocols outlined in this paper, students will cultivate and utilize *C. elegans* to conduct an RNAi experiment where they will visualize first-hand how RNAi depletes a gene of interest and how codon optimization significantly impacts gene expression.

The importance of this module is that it not only teaches concepts in molecular genetics and introduces students to a model system, it also promotes higher-order thinking. With the instruction provided here, students will gain experience and expertise in working with *C. elegans*, basic microscopy, data analysis, and science communication. Students will have an intuition for probing a research question, developing hypotheses, planning experiments, thinking about possible results, and formulating conclusions. Therefore, modules like the one presented here have a positive impact on student development and at the same time provide the prerequisites needed for success in STEM-related fields and beyond.

Overall, this module can be used at various educational levels to enhance students' interest in science and provide the groundwork needed for independent scientific research. The module incorporates active learning styles of instruction that significantly enhance student performance and encourage engagement. Finally, this module can also be used as a "stepping-stone" or "bootcamp" exercise to provide students with a set of skills and tools for the inquiry-based module of a CURE using *C. elegans* as a model organism.

Intended Audience

This laboratory module was employed in the first half of upper-level undergraduate developmental genetics at Stony Brook University. Most students enrolled in the course were Juniors or Seniors; however, the module can be implemented at various educational levels (i.e. Freshman or Sophomores or first-year graduate students as a "bootcamp" exercise). It can also be adapted for use in a high school setting where students can be introduced to a model system commonly studied in the life sciences, learn concepts that pertain to gene expression and regulation, and reinforce their prior knowledge of the scientific method.

Required Learning Time

The module requires four lab sessions of approximately 3 hours each. We found this was ample time for students to become accustomed to working with *C. elegans* and proficient in the necessary skills needed to complete the module. However, the module timing can be adjusted as needed to any desired length of time.

Prerequisite Student Knowledge

Students enrolled in the course are required to have taken introductory biology laboratories and animal development. It is highly encouraged that students have familiarity with basic laboratory procedures, such as micropipetting and sterile techniques. Prior to the module, all necessary

materials and information needed to complete the assignments will be provided, and students will receive an introduction to *C. elegans*, RNAi, and basic microscopy.

Prerequisite Teacher Knowledge

It is encouraged that instructors have some experience with *C. elegans*; however, the information presented in this manuscript should be sufficient for an instructor with no experience in *C. elegans* biology to learn the techniques required. Instructors should have a good understanding of concepts involving RNA interference(23) and codon optimization(24, 25). Additionally, instructors should know how to operate compound light microscopes, epifluorescence light microscopes, and image processing software, such as Fiji/ImageJ(26).

SCIENTIFIC TEACHING THEMES

Active Learning

Several active learning strategies that were implemented throughout this module include group activities, polling questions, and independent-learning activities. To complete the life history stages assignment and GFP RNAi experiment discussed below, students were arranged into groups, which fostered peer-to-peer communication, teamwork, and promoted student engagement. Moreover, prior to the GFP RNAi experiment, students were assigned a GFP RNAi worksheet (see below) which prompted independent thinking about the experiment. By completing the GFP RNAi worksheet, students had an opportunity to complete a task on their own without supervision and troubleshoot through any obstacles they may have encountered.

Assessment

Student assessments were conducted at multiple levels throughout the module. During the short introductory lectures given, students were asked a series of polling questions incorporated into the lecture (Supplement 1) and were assessed based on their answers. Students were evaluated on their ability to perform lab tasks and follow directions given by teaching assistants and instructors. Additionally, students were graded based on the quality of their lab assignment, which included data analysis, figure generation and figure legend writing (Supplement 2).

Inclusive Teaching

We have designed this module to be all-inclusive by differentiating content and lesson material to reach various types of learners. The hands-on activities of this module capture the attention and engagement of kinesthetic and tactile learners. Our short lectures that contain images, provide written instruction, and facilitate discussion amongst the class are accommodating to both visual and auditory learners. In addition, we encourage splitting the class into groups to include both men and women, as well as students of different ethnic backgrounds to foster an inclusive instructional environment. Thus, the module ensures equity by reaching all types of learners and encourages diversity through the formation of disparate groups.

LESSON PLAN

Overview of the module

In the module discussed in detail below, various concepts that are central to the understanding of gene expression and gene regulation will be explored. Using *C. elegans* as a model organism, students will understand how codon optimization of a nucleotide sequence significantly impacts the effectiveness of RNAi-mediated gene depletion.

Specifically, students will work with two GFP-expressing *C. elegans* strains, where one strain expresses a non-codon optimized (NCO) GFP tag (GFP_{NCO}), while the other strain expresses a codon optimized (CO) GFP tag (GFP_{CO}). The GFP_{NCO} and GFP_{CO} tags are each fused to the histone protein, *his-58* (H2B), that is driven by a ubiquitous promoter, *eft-3,* which promotes expression in all cells. Students will treat each strain with an empty vector (control) RNAi bacterial clone or an RNAi bacterial clone that produces dsRNA specific to only the non-codon optimized GFP variant (GFP_{NCO}) (Review Timmons and Fire, 1998 for a detailed description on how RNAi works in *C. elegans*). Through fluorescence microscopy, students will visually see differences in GFP expression in each strain due to codon optimization, and they will observe that significant depletion occurs only in the strain expressing *eft-3>H2B::*GFP_{NCO}. From their understanding of RNAi and codon optimization, we anticipate that students will be able to accurately predict these results and explain why these outcomes occur.

Prior to the module, we present students with a series of lectures that include an introduction to *C. elegans* and discussion about gene regulation (Supplement 1) (Review Corsi et al., 2015 for a comprehensive overview of *C. elegans*). We discuss the topic of RNAi, which is a biological process that in the presence of exogenous double stranded RNA (dsRNA) results in post-transcriptional gene silencing(19, 27-29). One method used to administer *C. elegans* with dsRNA is to feed them with *E. coli* expressing a vector capable of producing dsRNA, that is complementary to a target gene of interest(30, 31). *C. elegans* are unique in that they have a systemic RNAi response, meaning that dsRNA spreads throughout all tissues, with the exception of most neurons(32, 33). Thus, loss-of-function phenotypes for genes of interest can be assessed in almost any tissue of interest using RNAi.

We also engage our students by having a discussion on codon optimization, or the modification of a sequence of DNA such that the frequency of codons used by a particular organism for a specific amino acid is taken into account(34-36). Codon optimization significantly enhances the expression level of a particular protein due to the correlation between codon usage and tRNA abundance, and mRNA stability(37-40). Thus, the expression levels of codon optimized genes will be more robust than those of non-codon optimized.

In all, we anticipate this module will fulfill several goals, which include student proficiency in using the scientific method and development of critical thinking skills. After completing this module, students will be able to conduct controlled experiments using a model organism. In addition, they will be able to explain what RNAi is and how it can be used to assess loss-of-function phenotypes for any gene of interest. Lastly, students will be able to state the importance of codon optimization as it pertains to gene expression.

Labs 1 and 2: Introduction to C. elegans and microscopy

To carry out the GFP RNAi module, both instructors and students should have a general understanding of the life cycle and larval stages of *C. elegans* development(22). Briefly, *C. elegans* are free-living soil nematodes (worms) that have two sexes, hermaphrodites and males(18). After the completion of embryogenesis, *C. elegans* develop through four larval stages (L1-L4) prior to reaching adulthood. L1 larvae are the smallest of the larval stages, while adult hermaphrodites are characterized by the formation of eggs within the midline of the animal(18, 41). The life cycle of *C. elegans* varies by temperature (15°C–25°C), but typically, development from egg to egg-laying adult is approximately 2 days at 25°C, 3.5 days at 20°C, or 5 days at 15°C(18, 41). We recommend instructors prepare a lecture that is devoted to giving an overview of the life cycle of *C. elegans*(22).

For the purposes of this module, animals can be evaluated at the L3 or L4 larval stages; however, any stage can be evaluated as desired. The L3 or L4 stages can be reached by plating synchronized L1 larvae onto RNAi plates for ~34 or 48 hours at 20°C, respectively(41). Alternatively, the L3 or L4 stages can be reached by growing synchronized L1s for ~60 or 80 hours at 15°C(41). If analysis of earlier larval stages is desired, an instructor or lab technician can simply place synchronized L4 animals on RNAi plates and collect L1 or L2 progeny from those animals for analysis.

To help students get accustomed to working with *C. elegans*, we devoted one lab session (Lab 1) to introduce basic worm techniques. These techniques include worm picking, slide making and alkaline hypochlorite treatment (bleaching) for animal synchronization(42, 43) (see Supplement 3 and 4). An additional lab session was devoted to an introduction to compound light microscopy and a tutorial on microscopy. After the conclusion of these introductory sessions, students demonstrate their ability to work with *C. elegans* and operate a compound light microscope available in class, by imaging the life history stages (different larval stages) of the worms. Additionally, students compile a figure containing images of the life stages along with a descriptive figure legend (Supplement 2 and 3). We find this exercise extremely valuable for students to master important worm husbandry techniques (*i.e.* worm picking), identify larval stages, and become familiar with microscopy techniques, all of which will be necessary to successfully perform the GFP RNAi experiment.

Lab 3 and 4: GFP RNAi Module

Student and instructor preparation

To prepare the students for the experiment, we presented a short lecture on RNAi and codon bias (Supplement 1) and devised a "GFP RNAi worksheet" (Supplement 5). The goal of this worksheet is to drive students to formulate hypotheses as to whether the GFP_{NCO} RNAi clone will efficiently knock down GFP intensity levels in the strain expressing H2B::GFP_{CO} or H2B::GFP_{NCO}. In this worksheet, the students are provided with the nucleotide and amino acid sequences for the codon and non-codon optimized H2B::GFP tags, as well as the short-interfering nucleotide sequence from the GFP_{NCO} RNAi clone (Supplement 5). Using the sequences provided, students will make a pairwise sequence alignment, using EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). They will then compare the percent similarities between the different sequences and determine whether the short-interfering nucleotide sequence for GFP_{NCO} RNAi is most similar to H2B::GFP_{CO} or H2B::GFP_{NCO}. Through this process, students will see that the siRNA sequence (in DNA form) encoded by the GFP_{NCO} RNAi clone is 100% identical to the GFP_{NCO} sequence and not the GFP_{CO} sequence. Students will also appreciate that the control RNAi clone is called "empty vector" because it does not produce a dsRNA product.

To conduct the RNAi experiment, the students should grow up both the *eft-3>H2B::GFP$_{CO}$* and *eft-3>H2B::GFP$_{NCO}$* strains (DQM583 and DQM594, respectively) initially on NGM plates containing an *E. coli* diet (OP50)(43)(Supplement 4 Section II). Please note that worms are initially grown on OP50-seeded NGM plates prior to treatment with *E. coli* expressing dsRNA-producing vectors, and that RNAi plates utilize an *E. coli* food source different from OP50. Prior to the experiment, instructors should have RNAi plates made that contain *E. coli* specific to empty vector control (*T444T*) and GFP$_{NCO}$ (Supplement 4 SectionIV). To acquire a sufficient number of L1 larvae for the experiment, we recommend that instructors ensure that students have an adequate number of NGM plates containing gravid adults for bleach synchronization(42, 43).

When NGM plates are full of gravid adults (>100 adults on each plate or food source is near depletion), students should treat each strain with alkaline hypochlorite solution **(Figure 1 Step 1)**(Supplement 3 and Supplement 4 Section V) to create synchronized L1s. Approximately 50-100 L1 animals should be pipetted onto control and GFP$_{NCO}$-specific RNAi plates **(Figure 1 Step 2)**. Individual RNAi plates should have no more than ~50-100 worms to prevent overcrowding and depletion of the *E. coli* food source **(Figure 1 Step 2)**. The L1's are then cultured on the RNAi plates at the desired temperature until the L3 or L4 stage is reached **(Figure 1 Step 3)**. Once the desired stage is reached, students mount the animals on 5% agarose slides containing a droplet of M9 buffer and sodium azide to anesthetize the animals **(Figure 1 Step 3)**. We recommend that students pick ~10 animals for imaging at a time. We also encourage instructors to ensure students handle sodium azide with care as it is toxic(44)(Supplement 3 and Supplement 4 Section VI).

Student Experimental Results

Students quantified H2B::GFP fluorescence depletion using two wide-field epifluorescence microscopes, the Accu-Scope or Leica DMLB fluorescence microscopes (**Figure 1 Step 4, Figure 2 A and B**). For imaging consistency, instructors should predetermine the imaging settings (exposure time, magnification, camera gain and binning) using the *eft-3>H2B::GFP$_{CO}$* strain (DQM583) as a baseline due to it fluorescing the brightest. Both *eft-3>H2B::GFP$_{CO}$* and *eft-3>H2B::GFP$_{NCO}$* strains were imaged for each RNAi treatment (control and GFP$_{NCO}$). From the data acquired by the students, several qualitative observations were made **(Figure 2 A and B)**. First, the overall fluorescence intensity of the GFP$_{CO}$ strain was visually much brighter than the GFP$_{NCO}$ strain. Second, treating the GFP$_{NCO}$ strain with GFP$_{NCO}$ RNAi strongly reduced the fluorescence intensity of GFP, whereas treating the GFP$_{CO}$ strain with GFP$_{NCO}$ RNAi did not **(Figure 2 A and B, *eft-3>H2B::GFP* column)**. Third, in the GFP$_{NCO}$ strain treated with GFP$_{NCO}$ RNAi, although the fluorescence intensity of GFP was strongly reduced, some nuclei still showed high levels of GFP, these correspond to the cells that are insensitive to RNAi, most notably neurons **(Figure 2A , *eft-3>H2B::GFP$_{NCO}$*; GFP$_{NCO}$ RNAi)**

To analyze the data quantitatively, we instructed students to quantify whole-body GFP fluorescence intensity for 10 animals from each strain grown on control and GFP$_{NCO}$ RNAi, using Fiji/ImageJ2(26). Briefly, the entire body of each worm was outlined and the mean fluorescence intensity (MFI) was then measured for both GFP and an area of background. The background MFI measurement was then subtracted from the GFP MFI measurement to reduce background noise and obtain a mean gray value (MGV). Mean gray values were normalized by dividing the MFI in RNAi-treated animals by the average MFI in control-treated animals (Supplement 6, Supplement 4 Section

329 VII, Tutorial Videos 1-4, Supplement 7). The mean gray values obtained from each imaging system
330 (microscope) are plotted next to their respective micrographs **(Figure 2 A' and B')**.
331 By plotting the normalized MGV, students were able to clearly see that treating the GFP_{NCO}
332 strain with GFP_{NCO} RNAi significantly reduced the expression of GFP compared to control-treated
333 animals **(Figure 2A, 2A', and 2B, 2B', NCO strain; control RNAi vs. GFP_{NCO} RNAi)**. Moreover, the
334 students noted that treatment with GFP_{NCO} RNAi had no effect on GFP expression levels in the codon-
335 optimized strain **(Figure 2A, 2A', and 2B, 2B', CO strain; control RNAi vs. GFP_{NCO} RNAi)**. To
336 determine the statistical significance of their results, students performed a Student's t-test
337 comparing control MGV's to the MGV's for the GFP_{NCO} and GFP_{CO} strains. To assess whether the
338 students successfully carried out the experiment, we instructed them to document their results by
339 creating a publication quality figure similar to that described for the life history stages, which also
340 included a table of their raw data and description of their results (Supplement 2 and 6). From these
341 results, and the results obtained from the GFP RNAi worksheet, it becomes evident that RNAi
342 specificity is largely dependent on the sequence homology/similarity between the target gene
343 sequence and the sequence of the siRNA produced by the RNAi clone itself.
344
345 Extended Results (Optional)
346 Compared to wide-field epifluorescence microscopy, high resolution fluorescence
347 microscopy greatly improves the detail and resolving power of fluorescence micrographs, such that
348 unwanted out-of-focus light is significantly reduced, and the detail of cellular objects is greatly
349 enhanced(45). Thus, to show students high quality images of nuclear DNA labeled with H2B::GFP, we
350 acquired spinning-disk confocal images for both the *eft-3>H2B::GFP*$_{CO}$ and *eft-3>H2B::GFP*$_{NCO}$ strains
351 **(Figure 2C, 2C', 3, and 4)**. Importantly, these spinning-disk confocal images served to better
352 illustrate some of the key concepts discussed in the lab module, such as codon optimization and
353 lineage specific differences in RNAi susceptibility.
354 From the confocal fluorescence micrographs, it becomes more apparent that treatment with
355 GFP_{NCO} RNAi significantly reduces GFP fluorescence intensity in the GFP_{NCO} strain, but not in the GFP_{CO}
356 strain **(Figure 2C, 2C'; CO strain vs. NCO strain; GFP_{NCO} RNAi vs. control RNAi)**. To highlight the
357 differences in expression levels between codon optimized and non-codon optimized H2B::GFP fusion
358 proteins, we took spinning disk confocal images of the *C. elegans* germline. In general, codon
359 optimized transgenes are more robustly expressed in the germline than non-codon optimized
360 transgenes(46, 47). In line with this, H2B::GFP fluorescence expression was more robust in germ cells
361 when GFP is codon-optimized as opposed to when it is non-codon optimized **(Figure 3; CO strain
362 vs. NCO strain)**.
363 In *C. elegans*, certain cell lineages show different sensitivities to exogenous dsRNA. For
364 example, neurons are less sensitive to RNAi compared to other somatic tissues(32, 48). This is
365 because neurons lack the dsRNA-gated channel, *sid-1*, which promotes the uptake of dsRNA into cells
366 that express it(49). To emphasize this concept to students, we acquired spinning-disk confocal
367 images of nuclei from various cell lineages commonly studied in *C. elegans* (**Figure 4A)**, such as
368 pharyngeal neurons **(Figure 4B)**, epidermal cells **(Figure 4C)**, somatic gonadal cells **(Figure 4D)**,
369 and vulval precursor cells **(figure 4E)**. For each of the cell lineages examined, once again treatment
370 with GFP_{NCO} RNAi significantly reduced GFP fluorescence intensity levels in the GFP_{NCO} strain, but not
371 in the GFP_{CO} strain **(Figure 4B-E)**. However, with respect to the GFP_{NCO} strain treated with GFP_{NCO}

RNAi, the percent decrease in GFP intensity levels in the pharyngeal neurons was much less than the decrease found in the other cell types examined **(Figure 4B compared to Figures 4C-E)**. Thus, these observations can be used in the classroom to clearly illustrate to students that certain cell types are more or less sensitive to exogenous dsRNA, and this sensitivity is in part dictated by the surface proteins these cells express(49).

TEACHING DISCUSSION

The laboratory module presented here teaches a variety of common techniques employed by *C. elegans* researchers and exposes students to various concepts in molecular genetics and microscopy. During this module, students will become proficient at working with a widely used research organism, be able to conduct controlled experiments, analyze data, produce publication quality images, and have a basic understanding of microscopy. In addition, students will have a solid foundation as to how RNAi works, how it can be used to study gene function, and the importance of codon optimization on proper gene expression

This module clearly illustrates that certain cell types are less or more prone to the effects of dsRNA treatment, and that codon optimization results in improved gene expression in tissues (i.e. the germline). The advantage of using a strain that drives ubiquitous expression of H2B::GFP is that it is extremely bright and nuclear localized, and therefore easily visible on widefield epifluorescence microscopes, which are commonly available in most laboratory classrooms. For classrooms that have access to a high resolution microscope, such as a spinning-disk confocal, laser-scanning confocal microscope, or structured illumination microscopes, this module can be easily adapted for use on those types of microscopes as shown in Figures 2C, 3, and 4. The additional benefit of the strains used in this module is that students can immediately see differences in depletion between H2B::GFP_{CO} and H2B::GFP_{NCO} upon GFP_{NCO} RNAi treatment. Such is a bonus when attempting to keep students intrigued and engaged.

Upon completing this module, students will acquire the basic foundational skills needed for independent inquiry-based research projects involving *C. elegans*. Some examples of inquiry-based research projects that can follow this module include a reverse genetics screen to identify genes important for specific processes of interest, such as longevity. In this example, with the assistance of their instructor, students can design a simple research question, such as "Do fat metabolism genes play a role in regulating lifespan?". Students can search the literature for fat metabolism genes of interest, use either the Ahringer or Vidal RNAi libraries (Source Bioscience) to isolate clones specific for those genes, and determine if their depletion reduces or enhances longevity. Results can then be documented, written up in research paper format, and presented to the class. The independent inquiry-based research projects that follow this module are limitless and can focus on key areas of research implicated in a wide range of cellular processes, such as cell cycle regulation, cellular invasion, stress-resistance pathways, vesicle trafficking, and much more.

Whereas most lecture and laboratory-based classrooms use expository styles of instruction, classrooms that utilize active learning styles of instruction, such as inquiry-based learning strategies, significantly enhance student performance and learning outcomes(15, 50, 51). Several active learning strategies that can be implemented throughout this module include variations of the Jigsaw technique(52) and Think-Pair-Share(53). With respect to the Jigsaw technique, students can be divided into several teams, with each team focusing on identifying a specific life history stage as

415 described above. When those teams complete the assignment, the class is divided once again into
416 new groups. Each new group consists of one member from the original teams, with each member
417 being responsible for teaching the group how to identify their originally assigned life history stage.
418 We performed a variation of the Jigsaw technique and found the students to be engaged and excited
419 with the task provided. To further promote student thinking and awareness, an instructor can also
420 take advantage of Think-Pair-Share. Here, an instructor can pose a question, allow the students to
421 think independently about the question, then form pairs or groups to discuss their ideas collectively,
422 and share with the class. This strategy can be easily administered for the GFP RNAi worksheet. Here
423 students can be given the worksheet as a homework or in-class assignment to think independently
424 about which strain the GFP_{NCO} RNAi clone will be more efficient against. Later in the lab session or
425 during the next lab session, the students can form pairs or groups, discuss their opinions with one
426 another, and then present them to the class. In all, there are various active learning strategies that
427 can be implemented in this module, which fosters peer-to-peer communication, promotes student
428 engagement, and stimulates higher-order thinking.
429 This module can be further adapted for remote teaching and online learning. Instructors can
430 teach image analysis alongside with their lectures online and provide students with previously
431 acquired raw data sets from epifluorescence and/or confocal microscopes. Lessons can be held
432 synchronously by utilizing the share screen option in video conferencing apps, such as Zoom or
433 Google Meet, or asynchronously by recording lectures and lessons ahead of time, and uploading them
434 onto Blackboard, Microsoft Cloud, or Google Drive along with our image analysis video tutorials
435 (Supplemental Tutorial Videos 1-5). Based on the knowledge gained from the lectures, compiled raw
436 data, and the GFP RNAi worksheet, students will be able to formulate their hypothesis and test it by
437 analyzing the supplied data. We adapted this distance learning technique for the second half of our
438 course during the SARS-CoV2 pandemic in the Spring of 2020 and received positive feedback from
439 our students about the adaptability of the course.
440 An additional advantage of this module is that it not only applies to the undergraduate setting,
441 it can be adapted to a variety of educational levels, such as the high school or graduate level. At the
442 high school level, this module can enhance critical thinking, promote independence at an early stage
443 of a student's career, and instill awareness by introducing the field of genetics and organismal
444 biology. Moreover, it inherently promotes student engagement, by allowing students to work with
445 an organism that most are unaware exists, work in groups to share ideas, and visualize cellular
446 processes live. Additionally, we provide simplified protocols and instructions to make it easy for
447 instructors who have little experience with *C. elegans* to facilitate this module in their classroom. At
448 the graduate level, this module can be particularly useful for graduate student rotations and can serve
449 as an introductory "boot camp" or "stepping-stone" to introduce the *C. elegans* and experimental
450 techniques used in *C. elegans* research. Here, entry-level graduate students who have not previously
451 worked with *C. elegans* will have the opportunity to do so, and can immediately start acquiring data
452 by conducting a reverse genetics screen devised by the principal investigator and/or themselves.
453 Over time, these students can become confident enough to develop and plan their own projects.
454 In summary, this module is an excellent resource for instructors interested in conveying a
455 real-life science experience to their students and serves as an excellent opportunity for students to
456 gain the hands-on experience they need in order to pursue a career in biology.
457

SUPPORTING MATERIALS

Supplemental Files:
Supplement 1: Lecture – GFP RNAi *C. elegans*
Supplement 2: Grading Rubric and Example lab report
Supplement 3: Student Instructions for Lab 1
Supplement 4: Detailed Protocols
Supplement 5: Student GFP RNAi worksheet
Supplement 6: Student Instructions for Lab 2/3
Supplement 7: Transcripts for Tutorial Videos 1-5

Supplemental Movies:
Tutorial Video 1: Opening Images in Fiji/Image J – Brightness and Contrast
Tutorial Video 2: Measuring Mean Fluorescence Intensity in Fiji/Image J: Single Z data
Tutorial Video 3: Measuring Mean Fluorescence Intensity in Fiji/Image J: Confocal Z-stack data
Tutorial Video 4: Compiling Data
Tutorial Video 5: Formatting images for figure generation

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Figure Legends

Figure 1: Workflow diagram of the GFP RNAi module. (Step 1) For both codon optimized (*eft-3>H2B::GFP*$_{CO}$) and non-codon optimized (*eft-3>H2B::GFP*$_{NCO}$) strains, 10 OP50-seeded NGM plates each containing ~50-100 *C. elegans* gravid adults were treated with alkaline hypochlorite solution to obtain synchronized larvae. **(Step 2)** After 24 hours in M9 solution (and up to 72 hours), hatched L1 larvae are ready for plating onto RNAi plates (control or *T444T* RNAi and GFP$_{NCO}$ RNAi). For optimal RNAi efficiency and to avoid overcrowding/starvation, ~50 worms per plate will suffice. **(Step 3)** L1 larvae are grown until the L3/L4 larval stage and then mounted on 5% agarose pad slides (containing sodium azide (anesthetic) and a drop of M9 buffer) for image acquisition. *Growth times will vary based on temperature (see text for more details). **(Step 4)** Images are acquired and then analyzed using Fiji/ImageJ to determine the mean fluorescence intensity. Results are briefly explained in the lab report and submitted along with a publication quality figure with figure legend.

500

501 **Figure 2. RNAi specificity between codon optimized genes and non-codon optimized H2B::GFP**
502 **strains. (A-C)** Representative DIC and fluorescence micrographs of *eft-3*>H2B::GFP_{CO} (CO strain) and
503 *eft-3*>H2B::GFP_{NCO} (NCO strain) strains treated with RNAi against empty vector (control) or GFP_{NCO}.
504 Micrographs were collected by students using AccuScope (A) or Leica (B) epifluorescence compound
505 microscopes, or were collected by instructors using a custom modified upright spinning disk confocal
506 microscope (C). Images represent either the whole-body (A and B) or the midsection (C) of the
507 animal. **(A'-C')** Quantification of the normalized mean gray value (Normalized M.G.V) of H2B::GFP
508 expression, shown as a percentage, in CO and NCO strains. Mean fluorescence intensity for either
509 whole-body (A and B) or midsection nuclei (C) are shown. N≥8 animals per treatment (A' and B') or
510 N≥6 animals per treatment and n>100 midsection nuclei (C'). Error bars denote mean with SD. Scale
511 bars: 25 µm (A&B), 5 µm (C). Arrow heads (A) denote neurons in the *eft-3*>H2B::GFP_{NCO} not affected
512 by GFP_{NCO} RNAi; inset shows the same image inverted. Statistical analysis was performed using an
513 unpaired, two-tailed, Student's t-test with Welch's correction or Mann-Whitney U test. n.s.: not
514 significant. p-value ****≤0.0001.

515

516 **Figure 3. Codon optimization results in improved gene expression in the germline. (A)**
517 Representative DIC and fluorescence micrographs of the *C. elegans* germline for *eft-3*>H2B::GFP_{CO}
518 (CO strain) and *eft-3*>H2B::GFP_{NCO} (NCO strain) strains. Insets zoom into the germline to emphasize
519 expression differences between the CO and NCO strains. **(B)** Quantification of the Normalized M.G.V
520 of H2B::GFP expression in individual nuclei of the midsection, shown as a percentage, in CO and NCO
521 strains. Colored lines represent the mean M.G.V for individual lineages. N≥6 animals per strain and
522 n>100 midsection nuclei. Scale bars: 25 µm. Error bars denote mean with SD. Statistical analysis was
523 performed using an unpaired, two-tailed, Mann-Whitney U test. p-value ****≤0.0001

524

525 **Figure 4. Lineage specific differences in RNAi susceptibility. (A)** Cartoon schematic of a single *C.*
526 *elegans* nematode with different cell lineages outlined. The cell lineages shown and quantified
527 include pharyngeal neurons (B), epidermal/hypodermal cells (C), somatic gonadal cells (D), and
528 vulval precursor cells (VPCs) (E). **(B-E)** For each lineage, the Normalized M.G.V for individual nuclei
529 was quantified in CO and NCO strains treated with control or GFP_{NCO} dsRNA. For each lineage, N≥6
530 animals/RNAi clone and n≥30 nuclei. Scale bars: 5 µm. Statistical analysis was performed using an
531 unpaired, two-tailed, Student's t-test with Welch's correction or Mann-Whitney U test. n.s.: not
532 significant. p-value **≤0.01, ****≤0.0001.

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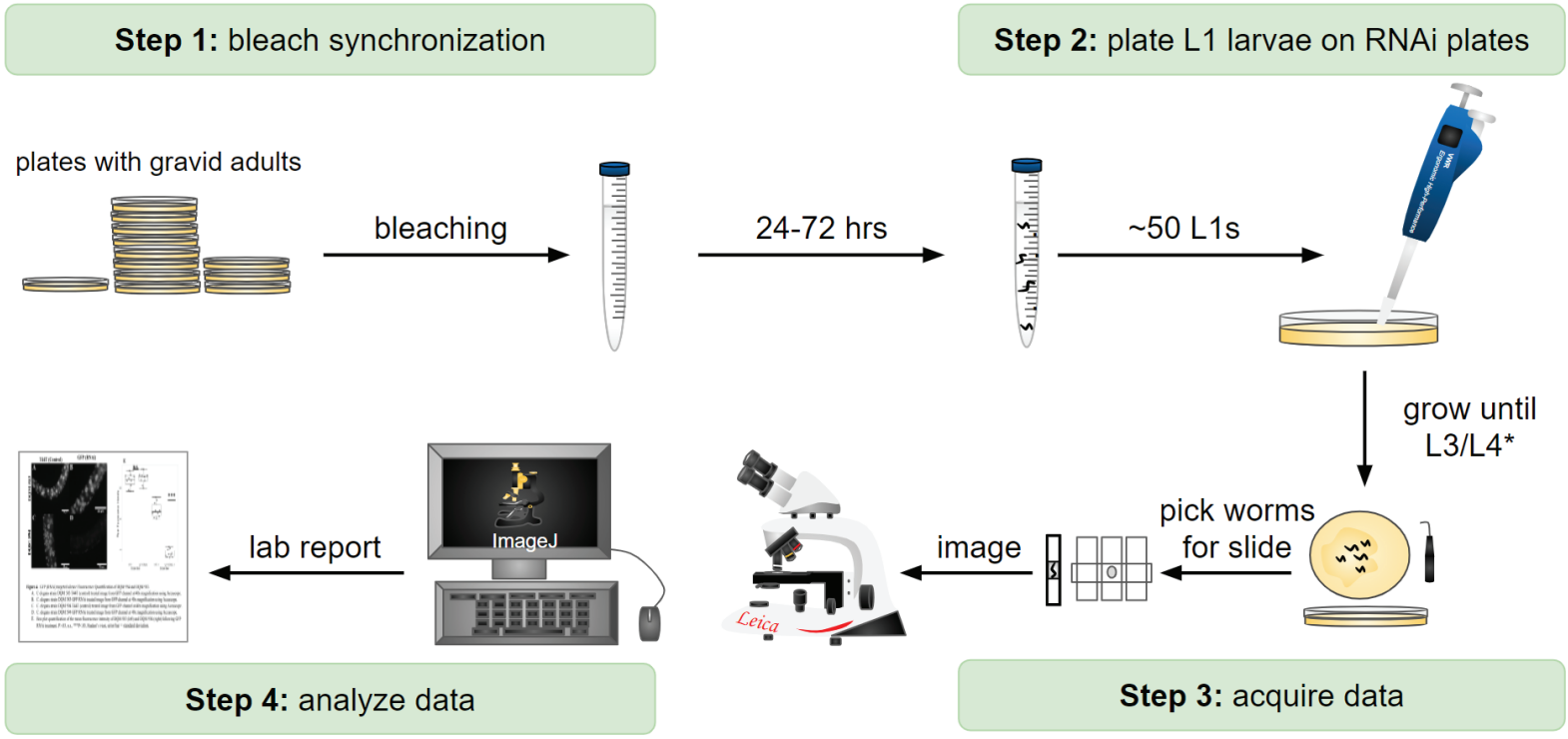
Figure 1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Step 2: plate L1 larvae on RNAi plates
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grow until L3/L4*
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ImageJ
lab report
Step 4: analyze data
Step 3: acquire data

Figure 2

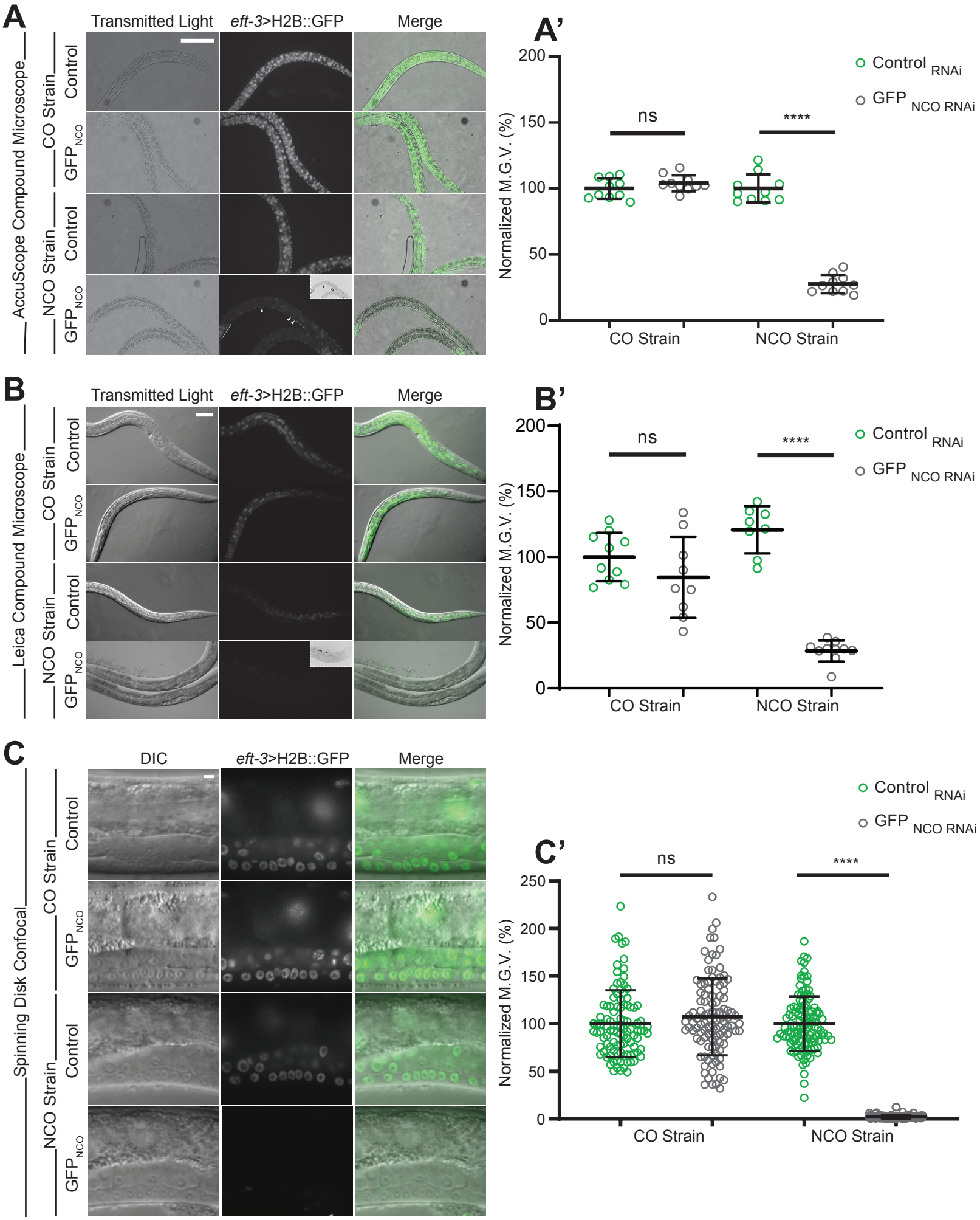
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Figure 3

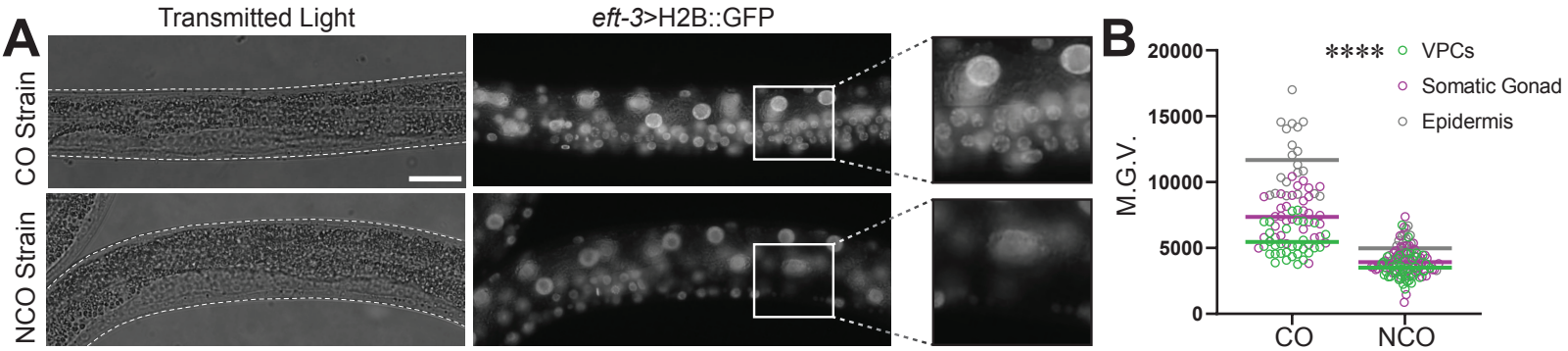
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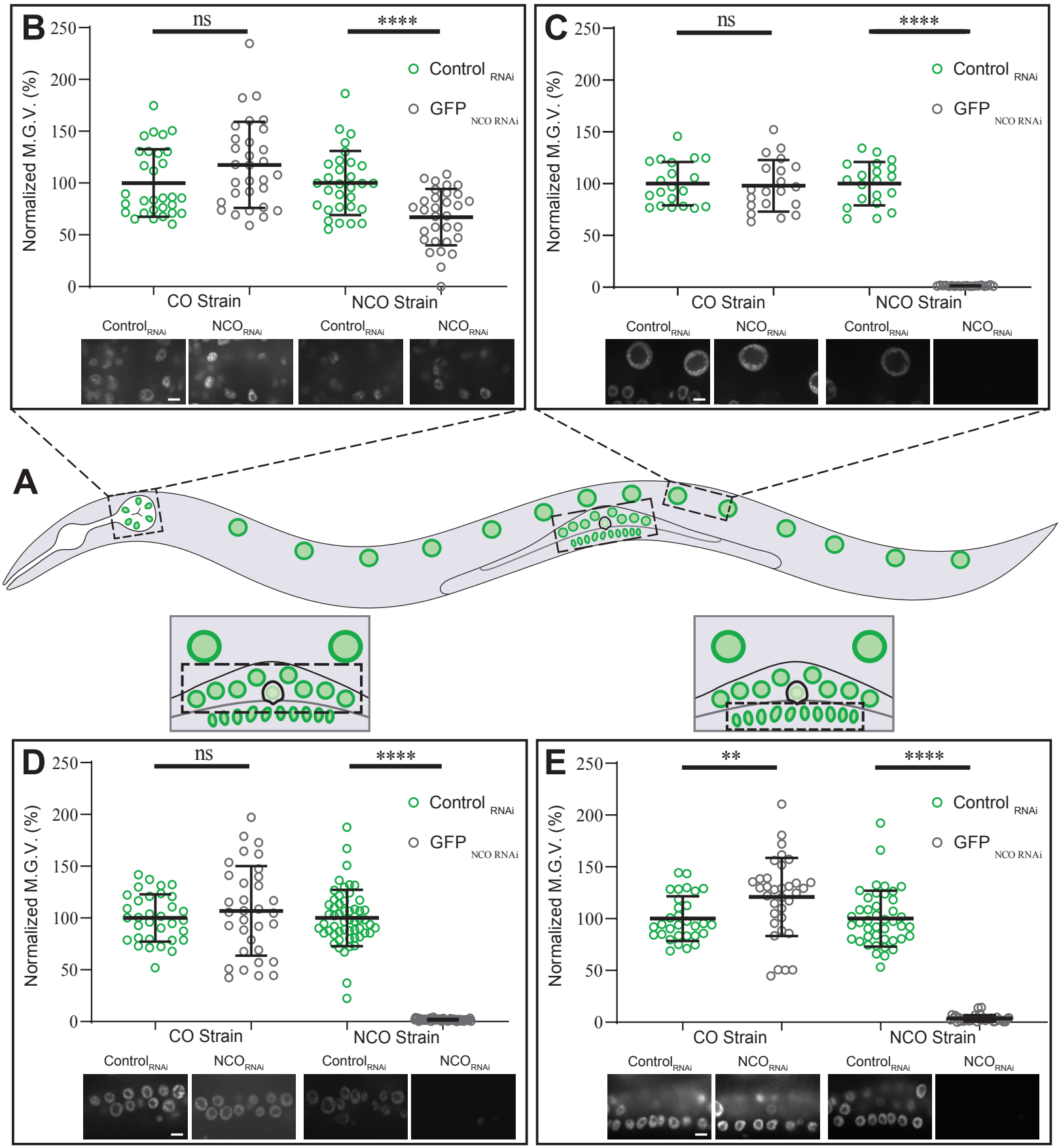
Figure 4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