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

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Abstract

Scientific 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 a guided research experience to stimulate curiosity, introduce students to experimental techniques, and provide students with foundational skills needed for higher levels of guided inquiry. 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 improve their critical thinking, data interpretation, and technical skills, all of 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

General knowledge:

- Gain experience working with *C. elegans*
- Understand the process of RNA interference and importance of codon optimization

Technical skills:

- Develop mastery in fluorescence microscopy techniques and image analysis

Communication skills:

- Enhance their writing skills

Learning Objectives

Students will be able to:

General knowledge:

- Demonstrate the ability to synchronize *C. elegans* nematodes and perform an RNAi experiment
- Describe what RNAi is and how it affects gene expression/activity
- Explain what codon usage means

Technical skills:

- Acquire images using an epifluorescence microscope
- Calculate mean fluorescence intensity from acquired fluorescence micrographs
- Perform statistical tests to determine the significance of results

Communication skills:

- Generate publication quality figures and figure legends
- Effectively formulate conclusions from data and logically present results

INTRODUCTION

Inquiry-based learning is a form of active learning where students can gain problem solving skills that can help better facilitate inquisitive thinking while simultaneously allowing them to 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 scientific skills, such as making observations, developing hypotheses, analyzing data, 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, apply 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 students' abilities to think critically, interpret data, communicate results, and collaborate as a team, when compared to traditional lab courses(12-17). A critical aspect of CUREs, as well as independent research, is obtaining the foundational skills and introductory training needed for understanding a specific system and/or research topic of interest. Several inquiry-based learning models have been developed to provide students with these foundational skills prior to their independent research projects(18-20).

Here we describe a level 1 guided-inquiry laboratory module(21) that prepares students for higher levels of guided inquiry and CUREs. This module is 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, *Caenorhabditis 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(22-24). Additionally, they are transparent, which allows for visualization of all tissue types, and the real-time visualization of fluorescently-tagged reporter proteins expressed in various tissues of interest(25, 26). Using the protocols outlined in this paper, students will conduct an RNAi experiment using *C. elegans* where they will visualize first-hand how RNAi depletes a GFP-tagged transgene and how codon optimization significantly impacts gene expression.

Similar to other laboratory modules involving *C. elegans* and RNAi (27, 28), this module allows students to make connections between the concepts they learn about in molecular and developmental genetics with the observations they make while conducting the RNAi experiment in the laboratory. At the same time, students gain experience working with an organism commonly used in the research setting. Our goal is that the experiences gained from this module will prepare students for higher levels of guided inquiry by enhancing their scientific and communication skills. 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. Finally, 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 CUREs.

Intended Audience

This laboratory module was employed in the first half of upper-level undergraduate developmental genetics laboratory course (BIO327) at Stony Brook University. Most students enrolled in the course were Juniors or Seniors; however, the module can be implemented as a

122 "bootcamp" exercise for first-year graduate students to gain hands-on bench experience working
123 with *C. elegans*.
124
125 *Required Learning Time*
126 The module requires a minimum of four lab sessions of approximately 3 hours each. We
127 found this was ample time for students to become accustomed to working with *C. elegans* and
128 proficient in the necessary skills needed to complete the module. Instructors can adjust the timing of
129 the module to any desired length of time they feel is appropriate.
130
131 *Prerequisite Student Knowledge*
132 To complete this module, students should have taken introductory biology and introductory
133 biology laboratory that exposes students to core biological principles, such as gene expression, and
134 basic organismal biology. It is highly encouraged that students have familiarity with basic laboratory
135 procedures, such as micropipetting and sterile techniques. Prior to the module, all necessary
136 materials and information needed to complete the assignments are provided, and students receive
137 an introduction to RNAi, codon optimization, and basic microscopy. We highly recommend this
138 module be implemented after students have gained a basic understanding of how to work with and
139 manipulate *C. elegans* (29).
140
141 *Prerequisite Teacher Knowledge*
142 Instructors implementing this course should have experience working with *C. elegans*. Ideally, there
143 should be access to temperature controlled incubators and other equipment needed for *C. elegans*
144 maintenance(29). Importantly, a good understanding of concepts involving RNA interference(30)
145 and codon optimization(31, 32) is essential for this module. We have provided a PowerPoint
146 presentation with an accompanying script for instructors to use when teaching students about RNAi

147 and codon optimization (Supporting file S1. A Laboratory Module-GFP RNAi *C. elegans* Lecture). In
148 addition, we have provided instructors with a list of common misconceptions and questions from
149 students when conducting the module (Supporting file S14. A Laboratory Module-Common Student
150 Misconceptions and Questions). Lastly, instructors should know how to operate stereomicroscopes,
151 compound light microscopes, epifluorescence light microscopes, and image processing software,
152 such as Fiji/ImageJ(33).
153
154
155 **SCIENTIFIC TEACHING THEMES**
156
157 *Active Learning*
158 Several active learning strategies that are implemented throughout this module include a
159 modified think-pair-share exercise, clicker polling questions, and a peer review activity. Students are
160 asked a series of clicker polling questions during the RNAi lecture that focus on students' conceptual
161 understanding of RNAi(Supporting file S1: A Laboratory Module-GFP RNAi *C. elegans* Lecture). For
162 the modified think-pair-share exercise, prior to the GFP RNAi experiment, students are assigned a
163 GFP RNAi worksheet to work on independently at home (Think component) (Supporting file S5. A
164 Laboratory Module-Student GFP RNAi Worksheet). In brief, the worksheet contains a series of
165 questions, which promotes independent thinking about the RNAi experiment, and guides the
166 students in formulating their hypothesis (see below). After completing the worksheet at home,
167 students form into groups during their next lab session (Pair component), and while preparing for
168 the GFP RNAi experiment, they are encouraged to discuss amongst themselves their findings and
169 share their hypotheses. When conducting the experiment in class, instructors and teaching assistants
170 approach each group and ask them to share their findings from the worksheet (Modified share
171 component). This is followed by a series of additional questions asked by the instructor(s) to further

172 test their understanding of RNAi(Supporting file S3-A Laboratory Module-GFP RNAi Module
173 Worksheet Discussion Questions & Answers). This modified share component of the think-pair-share
174 activity provides an equitable opportunity for all groups to validate their understanding rather than
175 a select few groups sharing in front of the entire class(34).

176 For the peer review activity, after completing their lab report assignment (Supporting file S2.
177 A Laboratory Module-Grading Rubric and Example Lab Report), students are randomly assigned to
178 review and constructively critique another fellow student's laboratory report. Students are first
179 instructed to upload their lab reports into their designated Google Drive folder as a Google document
180 (.docx file), which allows their peer reviewer to easily comment on the reports in real-time and create
181 editable suggestions. Each peer reviewer is instructed to provide feedback and suggestions on the
182 required components of their lab report (i.e. Nucleotide alignment figure, data table of quantification,
183 etc.; See Supporting file S2. A Laboratory Module-Grading Rubric and Example Lab Report).
184 Specifically, each student must review each other's work with specific criteria in mind, such as the
185 clarity of writing (Is a hypothesis clearly stated and is there enough detail to understand the results?),
186 statistical tests performed (Are appropriate statistical tests performed on the data?), and
187 organization of data (Is the data organized in such a way that results can be clearly interpreted?). We
188 emphasized to the students that all critiques should be professional and constructive and should
189 avoid any condescending language. The purpose of this assignment is to get students to become
190 familiar with the scientific process of peer review, appreciate the importance of quality work in
191 delivering a clear message, and encourage the exchange of ideas. Most importantly, peer review as
192 an active learning strategy stimulates students to reflect on their own written work, and results in
193 improvements on their own writing(35, 36).

194

195 *Assessment*

196 Student assessments are conducted at multiple levels throughout the module. During the short
197 introductory lectures given, students are asked a series of clicker polling questions incorporated into
198 the lecture (Supporting file S1: A Laboratory Module-GFP RNAi *C. elegans* Lecture) and are informally
199 assessed based on whether their answers are correct or incorrect. We also informally assessed
200 students on their ability to provide constructive feedback during the peer-mediated review activity
201 (see above), which counted as part of their participation grade, as well as their ability to answer
202 questions asked by instructors during the modified think-pair-share activity (Supporting file S3-A
203 Laboratory Module-GFP RNAi Module Worksheet Discussion Questions & Answers). Although we did
204 not require students to submit a lab notebook for the course, we did create a Google Drive folder
205 organized by class section, where students were encouraged to upload their quantified data and any
206 observations made into their individualized sub-folders. They were also asked to submit their
207 completed lab report as a Google doc for grading by instructors and teaching assistants into their
208 individualized sub-folder. Along these lines, students are formally graded based on the quality of
209 their lab report assignment, which includes a graph and table of their results, a "publication quality"
210 figure using acquired fluorescence micrographs along with an accompanying figure legend, and a
211 results text write-up (Supporting file S2. A Laboratory Module-Grading Rubric and Example Lab
212 Report).
213
214 *Inclusive Teaching*
215 We have designed this module to be all-inclusive by differentiating content and lesson material to
216 reach all types of learners. The hands-on activities of this module capture the attention and
217 engagement of kinesthetic and tactile learners. Our short lectures that contain images, provide
218 written instruction, and facilitate discussion amongst the class are accommodating to both visual and
219 auditory learners. Given that Stony Brook University consists of a highly diverse population of
220 students, during group activities, we can easily divide our class into diversified groups at random

using a freely available random name picking software called wheeldecide.com. We highly recommend that instructors utilize this tool given that it avoids any self-selection or instructor selection biases.

To ensure that students feel welcomed, we establish classroom "etiquette", similar to that suggested by Tanner 2013, where we emphasize that all students are expected to support one another and share their ideas in a judgement free manner (37). On the very first day of class, we implemented an ice-breaker activity, called "catch the ball", where all students and faculty "threw" around an imaginary ball to one another, and those who "caught" the ball on a turn introduced themselves, shared their interests, hobbies, and goals. We suggest a similar activity be implemented during the start of the course so that instructors can familiarize themselves with their students. To further create an inclusive learning environment, we ensure that all students have the means to be successful in the module. We ensure class material for the lesson is posted on Blackboard and/or in Google Drive in a timely fashion so that students can access it prior to the start of class and after. For students that may not have equal access to technology, hard copies, as well as digital copies, of assignments and lab protocols were provided to students. We also hold office hours on request and have discussion boards available so everybody can benefit from each other's questions and/or discussions. Moreover, based on information obtained from class assessments (see above) and observations, students who have difficulties with any of the class content receive extra support and guidance as needed. Thus, the module ensures equity and inclusivity by reaching all types of learners and ensuring students receive the support they need to succeed in the module.

LESSON PLAN

Overview of the module

245 In this module, students will use *C. elegans* as a model organism to understand how codon
246 optimization significantly impacts gene expression and how RNAi interference can precisely
247 downregulate gene activity.

248 Specifically, students will work with two GFP-expressing *C. elegans* strains, where one strain
249 expresses a non-codon optimized (NCO) GFP fusion protein (GFP_{NCO}), while the other strain
250 expresses a codon optimized (CO) GFP fusion protein (GFP_{CO}). The GFP_{NCO} and GFP_{CO} tags are each
251 fused to the histone protein, *his-58* (H2B), and are each expressed under the control of a ubiquitous
252 promoter, *eft-3,* which promotes expression in all cells. Students will treat each strain with an empty
253 vector (control) RNAi bacterial clone or an RNAi bacterial clone that produces double stranded RNA
254 (dsRNA) specific to only the non-codon optimized GFP variant (GFP_{NCO}) (Review Timmons and Fire,
255 1998 for a detailed description on how RNAi works in *C. elegans*). Through fluorescence microscopy,
256 students will observe differences in GFP expression in each strain due to codon optimization, and
257 they will observe that significant depletion occurs only in the strain expressing *eft-3>H2B::*GFP_{NCO}.
258 From their understanding of RNAi and codon optimization, we anticipate that students will be able
259 to accurately predict these results and explain why depletion occurs only in the strain expressing *eft-*
260 *3>H2B::*GFP_{NCO}.

261 Prior to the module, we present students with a lecture on gene regulation (Supporting file
262 S1: A Laboratory Module-GFP RNAi *C. elegans* Lecture). We have provided instructors with a script
263 that accompanies the lecture (Supporting file S1: A Laboratory Module-GFP RNAi *C. elegans* Lecture).
264 We recommend that instructors review Corsi et al., 2015 for a comprehensive overview of *C. elegans*
265 as a research organism. The lecture discusses the topic of RNAi, which is a biological process where
266 in the presence of exogenous dsRNA results in post-transcriptional gene silencing(23, 38-41). One
267 method used to administer *C. elegans* with dsRNA is to feed them with *E. coli* expressing a vector
268 capable of producing dsRNA, that is complementary to a target gene of interest(42-44). *C. elegans* are
269 unique in that they have a systemic RNAi response, meaning that dsRNA spreads throughout all

270 tissues, with the exception of most neurons(45, 46). Thus, loss-of-function phenotypes for genes of
271 interest can be assessed in almost any tissue of interest using RNAi.

272 We also provide our students with a brief overview of codon optimization when discussing
273 the GFP RNAi worksheet (Supporting file S5. A Laboratory Module-Student GFP RNAi Worksheet).
274 For a detailed overview of codon optimization, we highly recommend instructors review Hanson and
275 Coller, 2018. Codon optimization is the modification of a DNA sequence such that the frequency of
276 codons used by a particular organism, for a specific amino acid, is taken into consideration when
277 designing gene fusions or introducing exogenous DNA(47-49). Codon optimization significantly
278 enhances the expression level of a particular protein due to the correlation between codon usage and
279 tRNA abundance, and mRNA stability(50-53). Thus, the expression levels of codon optimized genes
280 will be more robust than those of non-codon optimized genes.

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

287

288 *GFP RNAi Module:*

289 Student and instructor preparation

290 To carry out the GFP RNAi module, both instructors and students should have a general
291 understanding of *C. elegans* development(26). To prepare the students for the experiment, we
292 presented a short lecture on RNAi and codon bias (Supporting file S1: A Laboratory Module-GFP RNAi
293 *C. elegans* Lecture) and devised a "GFP RNAi worksheet" (Supporting file S5. A Laboratory Module-
294 Student GFP RNAi Worksheet). The goal of this worksheet is to drive students to formulate

295 hypotheses as to whether the GFP_{NCO} RNAi clone will efficiently knock down GFP intensity levels in
296 the strain expressing H2B::GFP_{CO} or H2B::GFP_{NCO}. In this worksheet, the students are provided with
297 the nucleotide and amino acid sequences for the codon and non-codon optimized H2B::GFP fusion
298 proteins, as well as the dsRNA nucleotide targeting sequence (in DNA form) for the GFP_{NCO} RNAi clone
299 (Supporting file S5. A Laboratory Module-Student GFP RNAi Worksheet). Using the sequences
300 provided, students will make a pairwise sequence alignment using EMBOSS Needle
301 (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). They will then compare the percent
302 similarities between the different sequences and determine whether the dsRNA targeting sequence
303 for GFP_{NCO} RNAi is most similar to H2B::GFP_{CO} or H2B::GFP_{NCO}. Through this process, students will see
304 that the dsRNA targeting sequence encoded by the GFP_{NCO} RNAi clone is 100% identical to the GFP_{NCO}
305 sequence and not the GFP_{CO} sequence, and therefore should hypothesize that the GFP_{NCO} RNAi clone
306 will significantly deplete the H2B::GFP_{NCO} strain. Students will also appreciate that the control RNAi
307 clone is called "empty vector" because it does not produce a dsRNA product.

308 To conduct the RNAi experiment, the students should grow up both the *eft-3>H2B::*GFP_{CO} and
309 *eft-3>H2B::*GFP_{NCO} strains (DQM583 and DQM594, respectively) initially on NGM plates containing an
310 *E. coli* diet (*E. coli* variant OP50)(29) (Supporting file S4. A Laboratory Module-Detailed Protocols,
311 Section II). Please note that worms are initially grown on OP50-seeded NGM plates prior to treatment
312 with a different variant of *E. coli* (variant HT115(DE3)) that expresses dsRNA-producing vectors.
313 Along these lines, RNAi plates utilize the HT115 variants of *E. coli* that can produce dsRNA rather
314 than OP50 (44). Prior to the experiment, instructors should have RNAi plates made that contain *E.*
315 *coli* specific to empty vector control (*T444T*) and GFP_{NCO} (Supporting file S4. A Laboratory Module-
316 Detailed Protocols, Section IV). Moreover, we recommend that instructors have additional RNAi
317 plates as students do tend to make occasional errors, such as accidentally contaminating plates. To
318 acquire a sufficient number of L1 larvae for the experiment, we recommend that instructors ensure

319 that students have at least six NGM plates containing ~250 gravid adults for bleach
320 synchronization(29, 54).

321 When NGM plates are full of gravid adults (~250 adults on each plate), students should
322 treat each strain with alkaline hypochlorite solution(55) (Figure 1, Step 1) (Supporting file S4. A
323 Laboratory Module-Detailed Protocols, Section V) to create synchronized L1s. Approximately 50-100
324 L1 animals should be pipetted onto control and GFP_{NCO}-specific RNAi plates (Figure 1, Step 2).
325 Individual RNAi plates should have no more than ~50-100 worms to prevent overcrowding and
326 depletion of the *E. coli* food source (Figure 1, Step 2) (Please note that instructors may need to do the
327 bleaching and plating steps for students to allow for efficient completion of the RNAi experiment).
328 The L1s are then cultured on the RNAi plates at the desired temperature until the L3 or L4 stage is
329 reached (Figure 1, Step 3). Once the desired stage is reached, students can mount the animals on
330 microscope slides for imaging. To immobilize the worms for image analysis, worms can be added to
331 a droplet of M9 buffer (5μL) in the center of the slide, surrounded by Nemagel solution (InVivo
332 Biosystems) or ~1 μl of M9 containing 5mM levamisole (Figure 1, Step 3). We recommend that
333 students pick ~10 animals for imaging at a time. (Supporting file S4. A Laboratory Module-Detailed
334 Protocols, Section VI).

335

336 Student Experimental Results

337 Students quantified H2B::GFP fluorescence depletion using two wide-field epifluorescence
338 microscopes, the Accu-Scope or Leica DMLB (Figure 1, Step 4, Figure 2 A and B). For imaging
339 consistency, instructors should predetermine the imaging settings (exposure time, magnification,
340 camera gain and binning) using the *eft-3>H2B::GFP*$_{CO}$ strain (DQM583) as a baseline due to it having
341 the highest expression level. Both *eft-3>H2B::GFP*$_{CO}$ and *eft-3>H2B::GFP*$_{NCO}$ strains were imaged for
342 each RNAi treatment (control and GFP_{NCO}). From the data acquired by the students, several
343 qualitative observations were made (Figure 2 A and B). First, the overall fluorescence intensity of the

344 GFP_{CO} strain was visually much brighter than the GFP_{NCO} strain. Second, treating the GFP_{NCO} strain
345 with GFP_{NCO} RNAi strongly reduced the fluorescence intensity of GFP, whereas treating the GFP_{CO}
346 strain with GFP_{NCO} RNAi did not (Figure 2 A and B, *eft-3>H2B::GFP* column). Third, in the GFP_{NCO} strain
347 treated with GFP_{NCO} RNAi, although the fluorescence intensity of GFP was strongly reduced, some
348 nuclei still showed high levels of GFP, which correspond to the cells that are insensitive to RNAi, most
349 notably neurons (Figure 2A , *eft-3>H2B::GFP*$_{NCO}$; GFP_{NCO} RNAi).

350 To analyze the data quantitatively, we instructed students to quantify whole-body GFP
351 fluorescence intensity for 10 animals from each strain grown on control and GFP_{NCO} RNAi, using
352 Fiji/ImageJ2(33). Briefly, the entire body of each worm was outlined and the mean fluorescence
353 intensity (MFI) was then measured for both GFP and an area of background. The background MFI
354 measurement was then subtracted from the GFP MFI measurement to reduce background noise and
355 obtain a mean gray value (MGV). Mean gray values were normalized by dividing the MFI in RNAi-
356 treated animals by the average MFI in control-treated animals (Supporting file S4. A Laboratory
357 Module-Detailed Protocols, Section VII; Supporting file S6. A Laboratory Module-Student Instructions
358 for GFP RNAi Module; Supporting file S7. A Laboratory Module-Student Transcripts for Tutorial
359 Videos 1-5; Supporting file S8. A Laboratory Module-Opening Images in Fiji/Image J Tutorial Video;
360 Supporting file S9. A Laboratory Module-Measuring Mean Fluorescence Intensity for Single Z data
361 Tutorial Video; Supporting file S10. A Laboratory Module-Measuring Mean Fluorescence Intensity for
362 Confocal Z-stack data Tutorial Video; and Supporting file S11. A Laboratory Module-Compiling Data
363 Tutorial Video). The mean gray values obtained from each imaging system (microscope) are plotted
364 next to their respective micrographs (Figure 2 A' and B').

365 By plotting the normalized MGV, students were able to clearly see that treating the GFP_{NCO}
366 strain with GFP_{NCO} RNAi significantly reduced the expression of GFP compared to control-treated
367 animals (Figure 2A, 2A', and 2B, 2B', NCO strain; control RNAi vs. GFP_{NCO} RNAi). Moreover, the
368 students noted that treatment with GFP_{NCO} RNAi had no effect on GFP expression levels in the codon-

369 optimized strain (Figure 2A, 2A', and 2B, 2B', CO strain; control RNAi vs. GFP_{NCO} RNAi). To determine
370 the statistical significance of their results, students performed a Student's t-test comparing control
371 MGV's to the MGV's for the GFP_{NCO} and GFP_{CO} strains. To assess whether the students successfully
372 carried out the experiment, we instructed them to document their results as part of their lab report
373 assignment by creating a publication quality figure. Their figures included representative images of
374 their fluorescent micrographs, along with a dot plot of their quantified data, table of their raw data
375 values, and written description of their results (Supporting file S2. A Laboratory Module-Grading
376 Rubric and Example Lab Report and Supporting file S6. A Laboratory Module-Student Instructions
377 for GFP RNAi Module). From these results, and the results obtained from the GFP RNAi worksheet, it
378 should become evident to the students that RNAi specificity is largely dependent on the sequence
379 homology/similarity between the target gene sequence and the sequence of the dsRNA produced by
380 the RNAi clone itself.
381

382 Extended Results (Optional)

383 Compared to wide-field epifluorescence microscopy, confocal microscopy improves
384 resolution such that unwanted out-of-focus light is significantly reduced, and the detail of cellular
385 objects is greatly enhanced(56). Thus, to show students high quality images of nuclear DNA labeled
386 with H2B::GFP, we acquired spinning-disk confocal images for both the *eft-3>H2B::GFP*$_{CO}$ and *eft-*
387 *3>H2B::GFP*$_{NCO}$ strains (Figure 2C, 2C', 3, and 4). Importantly, these spinning-disk confocal images
388 served to better illustrate some of the key concepts discussed in the lab module, such as codon
389 optimization and lineage specific differences in RNAi susceptibility.

390 From the confocal fluorescence micrographs, it becomes more apparent that treatment with
391 GFP_{NCO} RNAi significantly reduces GFP fluorescence intensity in the GFP_{NCO} strain, but not in the GFP_{CO}
392 strain (Figure 2C, 2C'; CO strain vs. NCO strain; GFP_{NCO} RNAi vs. control RNAi). To highlight the
393 differences in expression levels between codon optimized and non-codon optimized H2B::GFP fusion

394 proteins, we took spinning disk confocal images of the *C. elegans* germline. In general, codon
395 optimized transgenes are more robustly expressed in the germline than non-codon optimized
396 transgenes(57, 58). In line with this, H2B::GFP fluorescence expression was more robust in germ cells
397 when GFP is codon-optimized as opposed to when it is non-codon optimized the transgene is
398 silenced, likely due to piRNA sequences present in the GFP_{NCO} nucleotide sequence ((59)) (Figure 3;
399 CO strain vs. NCO strain).

400 In *C. elegans*, certain cell lineages show different sensitivities to exogenous dsRNA. For
401 example, neurons and pharyngeal cells are less sensitive to RNAi compared to other somatic
402 tissues(45, 60-62). To emphasize to students that certain lineages are more resistant to RNAi, we
403 acquired spinning-disk confocal images of nuclei from various cell lineages commonly studied in *C.*
404 *elegans* (Figure 4A), such as pharyngeal cells (Figure 4B), intestinal cells (Figure 4C), somatic gonadal
405 cells (Figure 4D), and vulval precursor cells (figure 4E). For each of the cell lineages examined, once
406 again treatment with GFP_{NCO} RNAi significantly reduced GFP fluorescence intensity levels in the
407 GFP_{NCO} strain, but not in the GFP_{CO} strain (Figure 4B-E). However, with respect to the GFP_{NCO} strain
408 treated with GFP_{NCO} RNAi, the percent decrease in GFP intensity levels in the pharyngeal cells was
409 much less than the decrease found in the other cell types examined (Figure 4B compared to Figures
410 4C-E). Thus, these observations can be used in the classroom to clearly illustrate to students that
411 certain cell types show different sensitivities to exogenous dsRNA.

412

413 **TEACHING DISCUSSION**

414 The laboratory module presented here teaches a variety of common techniques employed by
415 *C. elegans* researchers and exposes students to various concepts in molecular genetics and
416 microscopy. During this module, students will become proficient at working with a widely used
417 research organism, be able to conduct controlled experiments, analyze data, produce publication
418 quality images, and have a basic understanding of microscopy. In addition, students will have a solid

419 foundation as to how RNAi works, how it can be used to study gene function, and the importance of
420 codon optimization on proper gene expression

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

430 Upon completing this module, students will acquire the basic foundational skills needed for
431 independent inquiry-based research projects involving *C. elegans*. Some examples of inquiry-based
432 research projects that can follow this module, as part of a laboratory course such as our
433 developmental genetics course, include a reverse genetics screen to identify genes important for
434 specific processes of interest, such as longevity. In this example, with the assistance of their
435 instructor, students can design a simple research question, such as "Do fat metabolism genes play a
436 role in regulating lifespan?". Students can search the literature for fat metabolism genes of interest,
437 use either the Ahringer or Vidal RNAi libraries (Source Bioscience) to isolate clones specific for those
438 genes, and determine if their depletion reduces or enhances longevity. The search for genes can be
439 conducted individually or as a group. If the instructor decides to have students work together in a
440 group, each student can select a gene they are interested in and then collectively decide on one gene
441 to limit their focus on. The instructor could then have groups present in front of the class, where each
442 student in a group explains why they chose their gene of interest, and then further explain why as a
443 group they decided to follow up on their agreed upon gene. Working in groups is highly encouraged

444 given that it promotes inclusivity, encourages the sharing of opinions, gives each student a sense of
445 responsibility, and enhances student learning as a whole(63, 64).

446 To experimentally determine if depletion of their gene of interest affects longevity, a lifespan
447 analysis can be conducted(65). Here, students can take ~100 synchronized adult worms and feed
448 them with an RNAi bacterial clone that produces dsRNA specific to their gene of interest or empty
449 vector (as a control). The students can then monitor the worm's survival over time until their death
450 (defined as the inability to respond to prodding)(65). Students can plot their data in the form of a
451 Kaplan-Meier survival curve, and their results can then be documented and written up in research
452 paper format or as a lab report. Additionally, students can practice their communication and
453 presentation skills by presenting their findings to the class. The independent inquiry-based research
454 projects that follow this module are limitless and can focus on a wide range of cellular processes,
455 such as cell cycle regulation, cellular invasion, stress-resistance pathways, vesicle trafficking, and
456 much more.

457 Although most lecture and laboratory-based classrooms use expository styles of instruction,
458 classrooms that utilize active learning styles of instruction significantly enhance student
459 performance and learning outcomes(15, 66, 67). Examples of active learning strategies that have
460 been implemented throughout this module include a variation of Think-Pair-Share(68) and a Peer
461 Review activity (see section on "Active Learning"). Our modified Think-Pair-Share activity gives
462 students an opportunity to independently test their understanding of a concept(s), facilitates
463 dialogue and the exchange of ideas between individuals, and allows students to verify their
464 understanding with an instructor by sharing their findings and results. In contrast to the traditional
465 share component, discussing their findings privately with instructors is a modification of the think-
466 pair-share activity that gives all groups an opportunity to share their understanding of class content,
467 as opposed to only a few representative groups sharing their knowledge to the entire class(34). One
468 large advantage of the peer review activity implemented in this module is that it allows students to

469 become familiar with the scientific process of peer review. Additionally, it prepares students to
470 become accustomed to giving and receiving feedback in the workforce(69), and stimulates students
471 to reflect on their own written work, which results in improvements on their own writing(35, 36,
472 70).

473 One additional active learning strategy that can be utilized in this module is the Jigsaw
474 method(71). The jigsaw method is a two-phase activity where students are responsible for learning
475 course content and teaching it to their peers(72). Although this active learning strategy was not
476 implemented in this specific module of our course, we have designed a jigsaw activity that can be
477 administered while introducing students to codon bias and optimization (Supporting file S13. A
478 Laboratory Module-Jigsaw Active Learning Activity & Post-Module Assessment (Optional)). In the
479 first phase of the activity, students are divided into several groups or teams, where each team focuses
480 on three activities: 1. Transcription and translation, 2. Codon bias, and 3. Sequence alignment.
481 Although group sizes will vary depending on class size, we recommend that groups consist of three
482 students. For each activity, a learning goal and learning objective is provided so that students have a
483 broad understanding of the purpose of the activity and know what they should be able to complete
484 at the end of the activity (Supporting file S13. A Laboratory Module-Jigsaw Active Learning Activity
485 & Post-Module Assessment (Optional)). Additionally, instructions for each activity are provided for
486 the students to follow to become "experts" in each activity (Supporting file S13. A Laboratory Module-
487 Jigsaw Active Learning Activity & Post-Module Assessment (Optional)). To assess their
488 understanding and mastery of the activities, we have developed a series of questions that are
489 associated with the learning levels of Blooms Taxonomy (Supporting file S13. A Laboratory Module-
490 Jigsaw Active Learning Activity & Post-Module Assessment (Optional)). We estimate that 30 minutes
491 to 1 hour is sufficient to complete all three activities simultaneously; however, instructors may have
492 to adjust their time needs accordingly. Once each group has "mastered" their activity, the second
493 phase begins where new groups are created that consist of one student from each original group. In

494 these new groups, each student or "expert" teaches the other about their expertise or the subject
495 matter from the first phase of the activity. To determine if students are adequately taught the subject
496 matter by their peers, we have developed a post-assessment activity that consists of 15 questions
497 along with an answer key for instructors. The advantage of this activity is that it promotes
498 cooperation between peers in a team-based setting and greatly improves student learning and
499 retention(73). In all, there are various active learning strategies that can be implemented in this
500 module, which foster peer-to-peer communication, promote student engagement, and stimulate
501 higher-order thinking.

502 An additional advantage of this module is that it can be adapted for remote teaching and
503 online learning. The RNAi lecture and imaging tutorials on various aspects of the module (i.e.
504 measuring mean fluorescence intensity) can be held synchronously during the scheduled time of
505 class by utilizing the share screen option in video conferencing apps, such as Zoom or Google Meet,
506 or asynchronously by uploading the image analysis video tutorials supplied onto Blackboard, Google
507 Drive (Supporting file S8. A Laboratory Module-Opening Images in Fiji/Image J Tutorial Video;
508 Supporting file S9. A Laboratory Module-Measuring Mean Fluorescence Intensity for Single Z data
509 Tutorial Video; Supporting file S10. A Laboratory Module-Measuring Mean Fluorescence Intensity for
510 Confocal Z-stack Data Tutorial Video; and Supporting file S11. A Laboratory Module-Compiling Data
511 Tutorial Video; and Supporting file S12. A Laboratory Module-Formatting Images for Figure
512 Generation Tutorial Video). Depending on the instructor and/or institution, the module can be
513 implemented in a fully remote, or in a hybrid fashion, with in-person and online components. If fully
514 remote, instructors can teach image analysis alongside with their lectures over Zoom and provide
515 students with access to previously acquired raw data sets from epifluorescence and/or confocal
516 microscopes through Blackboard or Google Drive. The students can then take those images and
517 quantify the data in front of their instructor over Zoom or some other platform (or at home if more
518 time is needed). For the GFP RNAi worksheet, after working on it independently at home, the

519 instructor could create groups using breakout rooms, allowing each group to discuss their findings
520 in a team-based setting. After an allotted amount of time (i.e. 20-30 minutes), the instructor can then
521 join each breakout room to hear their discussion. Alternatively, a hybrid setting approach could be
522 implemented where students could come into class on specific days to acquire their data and then
523 perform the quantifications and other components of the module (lab report generation, peer-review
524 activities, etc.) online or at home on other days. We adapted this distance learning technique for the
525 second half of our course during the SARS-CoV2 pandemic in the Spring of 2020 and 2021 and
526 received positive feedback from our students about the adaptability of the course.

527 Whether fully in class, or online/hybrid, based on the knowledge gained from the tutorials,
528 compiled raw data, and the GFP RNAi worksheet, students will be able to formulate their hypothesis,
529 test it by analyzing the supplied data, and present their findings by generating a publication quality
530 figure. One additional advantage of this module is that at the graduate level, it can be particularly
531 useful for graduate student rotations and can serve as an introductory "bootcamp" or "stepping-
532 stone" to introduce the experimental techniques used in *C. elegans* research. Here, entry-level
533 graduate students who have not previously worked with *C. elegans* will have the opportunity to do
534 so and can immediately start acquiring data by conducting a reverse genetics screen devised by the
535 principal investigator and/or themselves. Over time, these students can become confident enough to
536 develop and plan their own projects.

537 In summary, this module is an excellent resource for instructors interested in conveying a
538 real-life science experience to their students and serves as an excellent opportunity for students to
539 gain the hands-on experience they need in order to pursue a career in biology.

540

541 **SUPPORTING MATERIALS**

542 Supporting file S1: A Laboratory Module-GFP RNAi *C. elegans* Lecture

543 Supporting file S2. A Laboratory Module-Grading Rubric and Example Lab Report

544 Supporting file S3-A Laboratory Module-GFP RNAi Module Worksheet Discussion Questions &
545 Answers
546 Supporting file S4. A Laboratory Module-Detailed Protocols
547 Supporting file S5. A Laboratory Module-Student GFP RNAi Worksheet
548 Supporting file S6. A Laboratory Module-Student Instructions for GFP RNAi Module
549 Supporting file S7. A Laboratory Module-Student Transcripts for Tutorial Videos 1-5
550 Supporting file S8. A Laboratory Module-Opening Images in Fiji/Image J Tutorial Video
551 Supporting file S9. A Laboratory Module-Measuring Mean Fluorescence Intensity for Single Z data
552 Tutorial Video
553 Supporting file S10. A Laboratory Module-Measuring Mean Fluorescence Intensity for Confocal Z-
554 stack Data Tutorial Video
555 Supporting file S11. A Laboratory Module-Compiling Data Tutorial Video
556 Supporting file S12. A Laboratory Module-Formatting Images for Figure Generation Tutorial Video
557 Supporting file S13. A Laboratory Module-Jigsaw Active Learning Activity & Post-Module Assessment
558 (Optional)
559 Supporting file S14. A Laboratory Module-Common Student Misconceptions and Questions
560
561
562

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574
575
576 **Figure Legends**

577 **Figure 1: Workflow diagram of the GFP RNAi module. (Step 1)** For both codon optimized (*eft-*
578 *3>H2B::GFP*$_{CO}$) and non-codon optimized (*eft-3>H2B::GFP*$_{NCO}$) strains, 10 OP50-seeded NGM plates
579 each containing ~50-100 *C. elegans* gravid adults were treated with alkaline hypochlorite solution to
580 obtain synchronized larvae. **(Step 2)** After 24 hours in M9 solution (and up to 72 hours), hatched L1
581 larvae are ready for plating onto RNAi plates (control or *T444T* RNAi and GFP$_{NCO}$ RNAi). For optimal
582 RNAi efficiency and to avoid overcrowding/starvation, ~50 worms per plate will suffice. **(Step 3)** L1
583 larvae are grown until the L3/L4 larval stage and then mounted on 5% agarose pad slides (containing
584 levamisole (anesthetic) and a drop of M9 buffer) for image acquisition. *Growth times will vary based
585 on temperature (see text for more details). **(Step 4)** Images are acquired and then analyzed using
586 Fiji/ImageJ to determine the mean fluorescence intensity. Results are briefly explained in the lab
587 report and submitted along with a publication quality figure with figure legend.

588

589 **Figure 2. RNAi specificity between codon optimized genes and non-codon optimized H2B::GFP**
590 **strains. (A-C)** Representative DIC and fluorescence micrographs of *eft-3*>H2B::GFP$_{CO}$ (CO strain) and
591 *eft-3*>H2B::GFP$_{NCO}$ (NCO strain) strains treated with RNAi against empty vector (control) or GFP$_{NCO}$.
592 Micrographs were collected by students using AccuScope (A) or Leica (B) epifluorescence compound

593 microscopes or were collected by instructors using a custom modified upright spinning disk confocal
594 microscope (C). Images represent either the whole-body (A and B) or the midsection (C) of the
595 animal. **(A'-C')** Quantification of the normalized mean gray value (Normalized M.G.V) of H2B::GFP
596 expression, shown as a percentage, in CO and NCO strains. Mean fluorescence intensity for either
597 whole-body (A and B) or midsection nuclei (C) are shown. N≥8 animals per treatment (A' and B') or
598 N≥6 animals per treatment and n>100 midsection nuclei (C'). Error bars denote mean with SD. Scale
599 bars: 50 µm (A), 25 µm (A), 5 µm (C). Arrow heads (A) denote neurons in the *eft-3*>H2B::GFP_{NCO} not
600 affected by GFP_{NCO} RNAi; inset shows the same image inverted. Statistical analysis was performed
601 using an unpaired, two-tailed, Student's *t-test* with Welch's correction or Mann-Whitney U test. n.s.:
602 not significant. p-value ****≤0.0001.

603

604 **Figure 3. Codon optimization results in improved gene expression in the germline. (A)**
605 Representative DIC and fluorescence micrographs of the *C. elegans* germline for *eft-3*>H2B::GFP_{CO}
606 (CO strain) and *eft-3*>H2B::GFP_{NCO} (NCO strain) strains. Insets represent increased magnification of
607 the germline to emphasize expression differences between the CO and NCO strains. **(B)**
608 Quantification of the Normalized M.G.V of H2B::GFP expression in individual nuclei of the midsection,
609 shown as a percentage, in CO and NCO strains. Colored lines represent the mean M.G.V for individual
610 lineages. N≥6 animals per strain and n>100 midsection nuclei. Scale bars: 50 µm (insets: 25 µm).
611 Error bars denote mean with SD. Statistical analysis was performed using an unpaired, two-tailed,
612 Mann-Whitney U test. p-value ****≤0.0001

613

614 **Figure 4. Lineage specific differences in RNAi susceptibility. (A)** Cartoon schematic of a single *C.*
615 *elegans* nematode with different cell lineages outlined. The cell lineages shown and quantified

include pharyngeal cells (B), intestinal cells (C), somatic gonadal cells (D), and vulval precursor cells (VPCs) (E). **(B-E)** For each lineage, the Normalized M.G.V for individual nuclei was quantified in CO and NCO strains treated with control or GFP_{NCO} dsRNA. For each lineage, N≥6 animals/RNAi clone and n≥30 nuclei. Scale bars: 5 µm. Statistical analysis was performed using an unpaired, two-tailed, Student's *t-test* with Welch's correction or Mann-Whitney U test. n.s.: not significant. p-value **≤0.01, ****≤0.0001.

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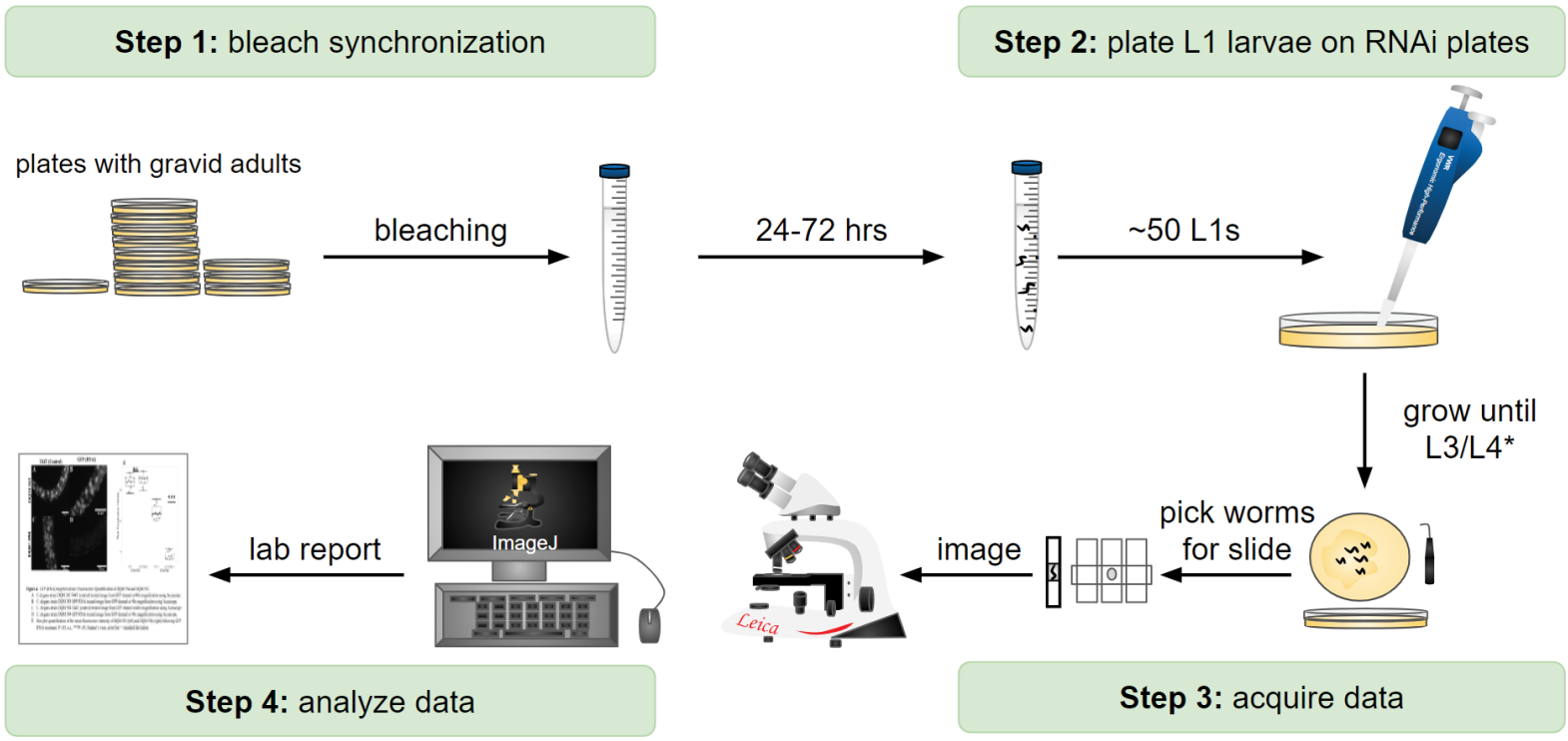
Step 1: bleach synchronization
Step 2: plate L1 larvae on RNAi plates
plates with gravid adults
bleaching
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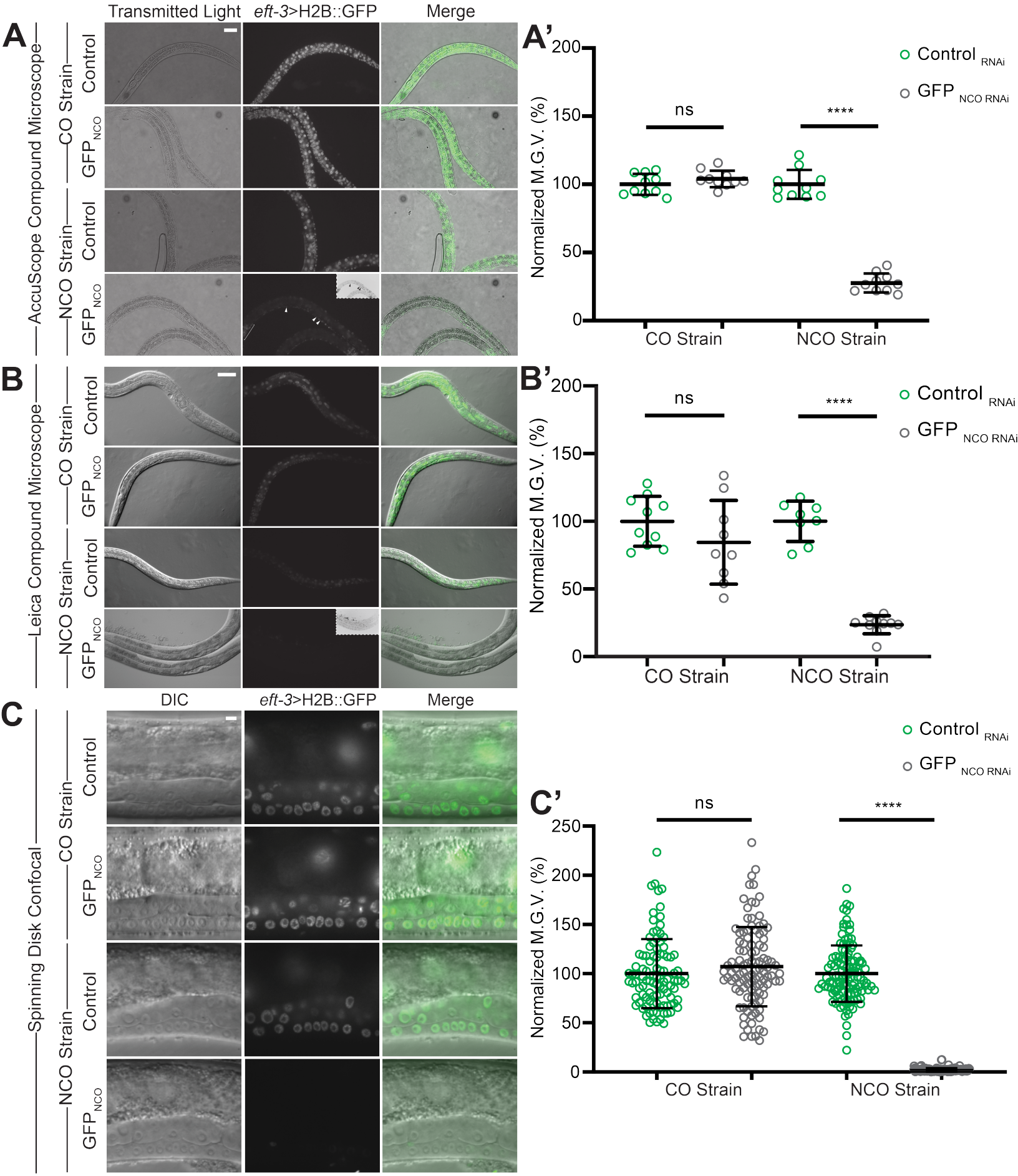
image
ImageJ
lab report
Step 4: analyze data
Step 3: acquire data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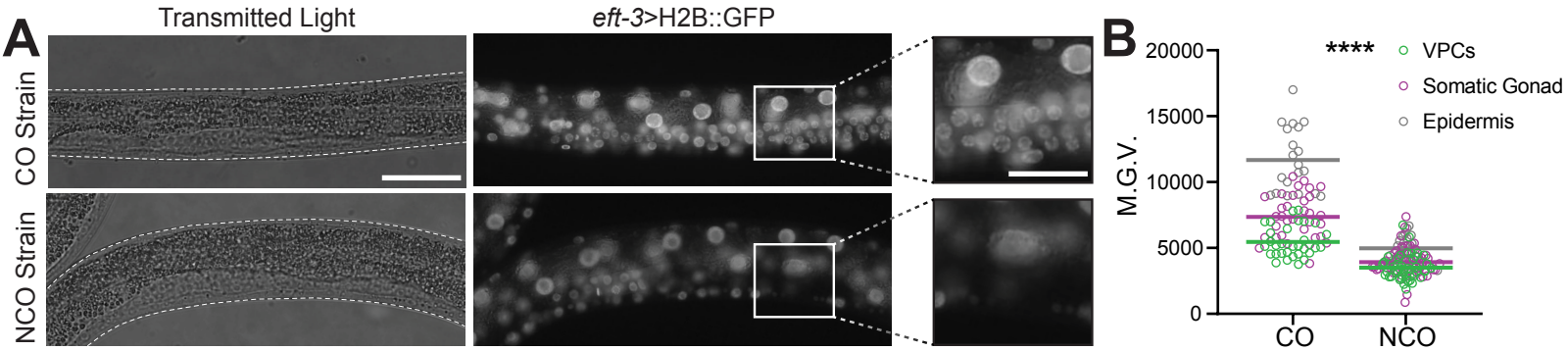
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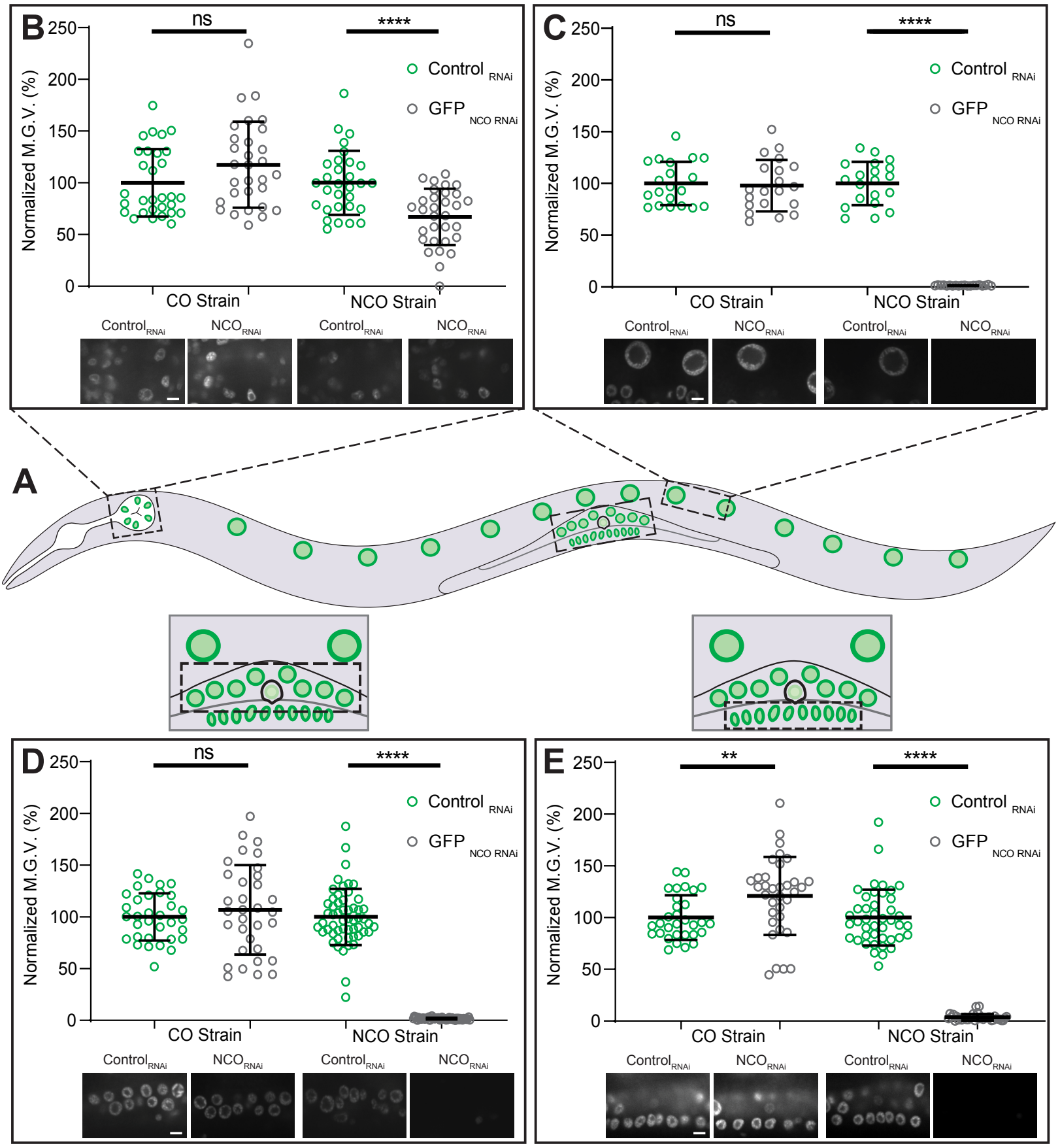
Transmitted Light
eft-3>H2B::GFP
CO Strain
NCO Strain
B
****
VPCs
Somatic Gonad
Epidermis
M.G.V.
20000
15000
10000
5000
0
CO
NCO


B
C
D
E
A
ns
****
**
Normalized M.G.V. (%)
250
200
150
100
50
0
Control RNAi
GFP NCO RNAi
CO Strain
NCO Strain
Control RNAi
NCO RNAi