Journal of Visualized Experiments

Cell cycle analysis in the C. elegans germline with the thymidine analog EdU

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58339R2
Full Title:	Cell cycle analysis in the C. elegans germline with the thymidine analog EdU
Keywords:	C. elegans, EdU, S-phase, Cell cycle, Germline, meiotic S-phase, M-phase, G2-phase
Corresponding Author:	Tim Schedl UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	ts@wustl.edu
Order of Authors:	Zuzana Kocsisova
	Ariz Mohammad
	Kerry Kornfeld
	Tim Schedl
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	4515 McKinley Ave St. Louis, MO 63110

TITLE:

Cell Cycle Analysis in the *C. elegans* Germline with the Thymidine Analog EdU

AUTHORS AND AFFILIATIONS:

Zuzana Kocsisova,[1, 2] Ariz Mohammad,[2] Kerry Kornfeld,[1] & Tim Schedl[2]

[1]Department of Developmental Biology, Washington University in St. Louis, Missouri, USA
[2]Department of Genetics, Washington University in St. Louis, Missouri, USA

Corresponding Author:

Tim Schedl
ts@wustl.edu
Tel: (314) 362-6162

Email Addresses of Co-authors:

Zuzana Kocsisova (zuzana@wustl.edu)
Ariz Mohammad (amohammad@wustl.edu)
Kerry Kornfeld (kornfeld@wustl.edu)

KEYWORDS:

C. elegans, EdU, S-phase, Cell cycle, Germline, meiotic S-phase, M-phase, G2-phase

SUMMARY:

This is an imaging-based method to identify S-phase and analyze cell cycle dynamics in the *C. elegans* hermaphrodite germline using the thymidine analog EdU. This method requires no transgenes and is compatible with immunofluorescent staining.

ABSTRACT:

Cell cycle analysis in eukaryotes frequently utilizes chromosome morphology, expression and/or localization of gene products required for various phases of the cell cycle, or the incorporation of nucleoside analogs. During S-phase, DNA polymerases incorporate thymidine analogs such as EdU or BrdU into chromosomal DNA, marking the cells for analysis. For *C. elegans*, the nucleoside analog EdU is fed to the worms during regular culture and is compatible with immunofluorescent techniques. The germline of *C. elegans* is a powerful model system for the studies of signaling pathways, stem cells, meiosis, and cell cycle because it is transparent, genetically facile, and meiotic prophase and cellular differentiation/gametogenesis occur in a linear assembly-like fashion. These features make EdU a great tool to study dynamic aspects of mitotically cycling cells and germline development. This protocol describes how to successfully prepare EdU bacteria, feed them to wild-type *C. elegans* hermaphrodites, dissect, stain for EdU incorporation into DNA, stain with antibodies to detect various cell cycle and developmental markers, image the gonad and analyze the results. The protocol describes the variations in the method and analysis for the measurement of S-phase, M-phase, G2 length, cell cycle length, rate of meiotic entry, and meiotic prophase progression. This method can be adapted to study the cell cycle or cell history in other tissues, stages, genetic backgrounds, and physiological

conditions.

INTRODUCTION:

In animal development, hundreds, thousands, millions, billions, or even trillions of cell divisions are required to form the adult organism. The cell cycle, the set of cellular events composed of G1 (gap), S (synthesis), G2 (gap), and M (mitosis) define the series of events that are executed each cell division. The cell cycle is dynamic and best appreciated in real time, which can be technically difficult. The techniques presented in this protocol allow one to make the measurements of the phases and timing of the cell cycle from still images.

Labeling with nucleoside analogs such as 5-ethynyl-2'-deoxyuridine (EdU) or 5-bromo-2'-deoxyuridine (BrdU) is the gold standard to identify S-phase in the studies of cell cycle dynamics in the *Caenorhabditis elegans* (*C. elegans*) adult hermaphrodite germline[1–5]. Both EdU and BrdU can be used in nearly any genetic background, as they do not rely on any genetic construct. Visualizing BrdU requires harsh chemical treatment to expose the antigen for anti-BrdU antibody staining, which is often incompatible with the assessment of other cellular markers using co-staining with additional antibodies. By contrast, visualizing EdU occurs by click chemistry under mild conditions and thus is compatible with antibody co-staining[6,7].

The specificity of the label is clear, since nuclei only incorporate the thymidine (5-ethynyl-2'-deoxyuridine) analogs into DNA during S-phase. Visualization takes place in fixed tissue. The EdU label is invisible by itself until an azide-containing dye or fluorophore reacts covalently with the alkyne in EdU by copper-catalyzed click chemistry[8]. EdU labeling can provide immediate information on which nuclei are in S-phase, using a short pulse of labeling. EdU can also provide dynamic information, using pulse-chase or continuous labeling; for example, in a pulse-chase experiment, the label is diluted at each cell division or propagated as nondividing cells progress through development.

The *C. elegans* hermaphrodite germline is a powerful model system for the studies of signaling pathways, stem cells, meiosis, and cell cycle. The adult germline is a polarized assembly-line with stem cells found at the distal end followed by entry and progression through meiotic prophase, coordinated with the stages of gametogenesis more proximally (**Figure 1**). At the proximal end, oocytes mature, are ovulated and fertilized and begin embryogenesis in the uterus[9–11]. The ~20 cell-diameter long region near the distal tip cell, which includes the mitotically cycling germline stem, progenitor cells and meiotic S-phase cells but not the cells in meiotic prophase, is called the progenitor zone[2,4,9,12]. The cell membranes provide incomplete separation between the nuclei in the distal germline, but the progenitor zone cells undergo mitotic cell cycling largely independently. The median mitotic cell cycle length of germline progenitor zone cells in young adult hermaphrodites is ~6.5 h; G1 phase is short or absent, and quiescence is not observed[1,2,13]. Germline stem cell differentiation occurs through essentially direct differentiation and thus lacks transit-amplifying divisions[4]. During differentiation in the pachytene stage, approximately 4 out of 5 nuclei will not form oocytes but instead undergo apoptosis, acting as nurse cells by donating their cytoplasmic contents to the developing oocyte[14,15].

In addition to labeling S-phase with nucleoside analogs, one can identify the cells in mitosis and meiosis using antibody staining. Nuclei in mitosis are immunoreactive to anti-phospho-histone H3 (Ser10) antibody (called pH3)[7,16]. Nuclei in meiosis are immunoreactive to anti-HIM-3 antibody (a meiotic chromosome axis protein)[17]. Nuclei in the progenitor zone can be identified by the absence of HIM-3, the presence of nucleoplasmic REC-8[18], or the presence of WAPL-1. WAPL-1 intensity is highest in the somatic gonad, high in the progenitor zone, and low during meiosis[19]. Several cell cycle measurements are possible with a few variations in the protocol: I) identify nuclei in S-phase (and M-phase); II) measure the S-phase index (and M-phase index); III) determine whether nuclei were in mitotic or meiotic S-phase; IV) measure the length of G2; V) measure the length of G2+M+G1 phases; VI) measure the rate of meiotic entry; VII) estimate the rate of meiotic progression.

One can make multiple cell cycle measurements from only a few types of wet-lab experiments. The protocol below describes a 30 min pulse labeling by feeding *C. elegans* adult hermaphrodites with EdU labeled bacteria and co-labeling M-phase cells by staining with anti-pH3 antibody and progenitor zone cells by staining with anti-WAPL-1 antibody. Only changes in the length of EdU feed (Step 2.5), antibodies (Step 5), and analyses (Step 8.3) are required for the additional measurements.

PROTOCOL:

1. Preparation of EdU-labeled Bacteria

1.1. Grow a starter culture of MG1693. *Escherichia coli* (*E. coli*) MG1693 carries a mutation in thyA.

1.1.1. Streak out *E. coli* MG1693 from a frozen glycerol stock onto a 120 mm lysogeny broth (LB) agar Petri dish. Grow at 37 °C overnight.

1.1.2. Inoculate from two individual *E. coli* MG1693 colonies into two duplicate 4 mL tubes of LB. Grow at 37 °C for ~16 h.

Note: MG1693 grows fine in LB without supplementing with thymine or thymidine.

1.2. Grow *E. coli* MG1693 in minimal media supplemented with EdU.

1.2.1. Autoclave a 500 mL conical flask.

1.2.2. Use sterile technique to add 5 mL of 20% glucose, 50 µL of 10 mg/mL of thiamine, 120 µL of 5 mM thymidine, 100 µL of 1 M $MgSO_4$, 200 µL of 10 mM EdU, 100 mL of M9 buffer, and 4 mL of freshly-grown overnight MG1693 culture.

Note: The final concentration of EdU is 20 µM in this culture[1,7]. This concentration leads to DNA

damage and cell cycle arrest if applied to mammalian cells in culture directly[20]. However, only a fraction of this EdU is incorporated into the *E. coli* and thus available to *C. elegans*. There is no evidence of cell cycle arrest and no change in the size of the progenitor zone or M-phase index after EdU treatment of young adult hermaphrodites.

1.2.3. Grow overnight, but no longer than 24 h at 37 °C with shaking at 200 rpm.

1.3. Concentrate EdU-labeled *E. coli* and apply to M9 agar Petri dishes.

1.3.1. Pre-cool a tabletop centrifuge to 4 °C

1.3.2. Use sterile technique to transfer the culture into 2-4 sterile 50 mL conical tubes.

1.3.3. Centrifuge the cultures at 3,000 x g at 4 °C for 30 min to pellet the EdU-labeled *E. coli*.

Note: Dispose of EdU-containing supernatant according to local and institutional guidelines.

1.3.4. Resuspend the pellets with 4 mL of fresh M9. Use a sterile 1 mL pipet tip or a sterile 5 mL serological pipet. Resuspending the pellets may take several minutes.

1.3.5. Use the same pipet to apply ~8 drops of resuspended EdU-labeled *E. coli* MG1693 to the center of room-temperature 60 mm M9 agar Petri dishes. One batch yields ~16 dishes.

1.3.6. Allow the dishes to dry for several hours or overnight at room temperature, then seal each dish with a strip of laboratory film. Dishes can be stored at 15 °C for ~2 weeks and at 4 °C for ~2 months. Use same batch of EdU dishes for each set of experiments.

2. Feeding EdU to *C. elegans.*

2.1. Synchronize population by timed egg lay, alkaline hypochlorite treatment followed by hatching into S-medium with cholesterol, or by picking the appropriate stage[21]. Grow the animals to desired stage (here, 24 h post-L4) on Nematode Growth Medium seeded with *E. coli* OP50 at 20 °C.

Note: Prepare 50-100 animals per experiment, as some may not dissect well, and others will be lost in the process of washing and transferring.

2.2. Allow the EdU dishes to warm to 20 °C (or the temperature required for the experiment).

2.3. Wash the animals from NGM dishes using M9 into a 1.5 mL tube. Allow the animals to settle briefly by gravity or a short spin in a microcentrifuge.

2.4. Remove the supernatant and wash the animals 1-2 times with ~1 mL of M9. Remove the

supernatant.

2.5. Use a glass Pasteur pipette to transfer the animals in a tiny drop of M9 onto the center of the EdU lawn. Wait a few minutes for the liquid to be absorbed, then incubate at 20 °C for 30 min (for direct S-phase measurement) or longer (to measure history of S-phase), as needed.

Note: EdU signal is detectable in germline nuclei after as little as 15 min of EdU feeding[1].

2.6. Wash the worms off the EdU plate with ~2 mL of phosphate-buffered saline (PBS) into a glass dissecting dish.

3. Dissection and Fixation of *C. elegans* Germline

Note: This protocol for the dissection, fixation, and antibody staining of the *C. elegans* hermaphrodite germline is nearly identical to that published by the Arur lab[22], except that the 1 mL glass tubes can be centrifuged to speed up washing steps and a drawn-out glass Pasteur pipette can be used to remove the liquid from 1 mL glass tubes more accurately.

3.1. Wash and dissect *C. elegans* germlines.

3.1.1. Allow the animals to settle to the bottom of the dissecting dish, swirl to collect the animals in the center, and use a long Pasteur pipette to remove PBS. Wash 1-2 times with ~2 mL of PBS.

3.1.2. Add 2 mL of PBS and 4 µL of 100 mM levamisole to immobilize the animals. Swirl the dish again to collect the animals in the center of the dish.

Note: Immobilization can take between a few seconds and a few minutes. Complete immobilization is not necessary for successful dissection. Some people have better success when dissecting incompletely immobilized animals.

3.1.3. Dissect the animals with a pair of 25G 5/8” needles by cutting at the head (approximately between the two pharyngeal bulbs) or the tail. Take care not to cut the loop of the germline. Intestine and germline should “pop out” of the body cavity due to internal pressure, but remain attached. This protocol is similar to previously published method[22].

Note: Keep the dissection time to ~5 min, certainly no more than 15 min. Longer dissection times may result in the loss of antibody staining signal (Sudhir Nayak, personal communication) and starvation in PBS may affect the animals’ physiology. Learning to dissect quickly and accurately may take some practice.

3.1.4. If needed, swirl to collect the dissected animals in the center, and use a long Pasteur pipette to remove as much PBS as possible.

3.2. Fix and dehydrate tissues

3.2.1. Add 2 mL of 3% paraformaldehyde (PFA) in PBS solution. Cover the dish loosely with laboratory film and store on a bench or in a drawer for 10 min.

Note: Thaw PFA solution in a 37 °C water bath and then cool to room temperature prior to dissecting germlines.

CAUTION: PFA solution is moderately toxic and a probable carcinogen and teratogen. Vapors emitting from paraformaldehyde solutions are flammable. Wear nitrile gloves. Dilute PFA from 16% to 3% in chemical fume hood. When working outside of fume hood, keep all containers covered.

3.2.2. Transfer the gonads carefully to a clean 5 mL glass centrifuge tube.

3.2.3. Add ~3 mL of PBSTw (PBS with 0.1% Tween-20) to the dish that contained the gonads to help retrieve remaining gonads and to dilute the PFA solution.

3.2.4. Spin down in a clinical centrifuge at 870 x g for ~1 min. Younger or smaller animals require longer spins than older or larger animals.

3.2.5. Using a long glass pipette, transfer the supernatant to unwanted material beaker for eventual discard in unwanted material bottle in the chemical hood.

3.2.6. Add 2 mL of high-grade methanol pre-chilled to -20 °C. Cover the centrifuge tube tightly with laboratory film.

Note: Use of fresh high-grade “gold label” methanol is essential for proper morphology with certain antibodies.

CAUTION: Methanol is a highly flammable liquid and vapor, toxic if swallowed, in contact with skin, or inhaled. Wear gloves and appropriate personal protective equipment. Use freezer appropriate for small volumes of flammables.

3.2.7. Store in -20 °C freezer for 1 h, even overnight or even several months.

Note: The protocol can be paused here.

4. **Rehydrate Germlines**

4.1. Fill the glass centrifuge tube to top with PBSTw to dilute the methanol and rehydrate the gonads. Spin down in a clinical centrifuge at 870 x g for ~1 min.

4.2. Using a long glass Pasteur pipette, transfer the supernatant to unwanted material

beaker for eventual discard in unwanted material bottle in the chemical hood.

4.3. Wash the gonads 3 times using ~5 mL of PBSTw, spinning down in a clinical centrifuge at 870 x g for ~1 min each time. Remove the supernatant.

4.4. Rinse a small 1 mL borosilicate glass tube and a long glass Pasteur pipette with PBSTw.

4.5. Add ~700 µL of PBSTw and use the long glass Pasteur pipette to transfer the gonads to the small tube. Use a few additional drops of PBSTw to ensure that all gonads are transferred.

4.6. Spin down in a clinical centrifuge at 870 x g for ~1 min. Using a drawn-out long glass Pasteur pipette, remove as much liquid as possible without disturbing the gonads. Leave no more than 50 µL.

Note: If antibody detection is not necessary, skip to Step 6.

5. Detect Antigens with Antibodies

5.1. Dilute the primary antibodies in 30% goat serum in PBS. In the present example, anti-WAPL-1 antibody is diluted 1:2000 and anti-pH3 antibody is diluted 1:500. Centrifuge freshly thawed serum for 10 min in a microfuge at 20,000 x g at 4 °C to remove the particulates. Use the supernatant, which can be stored at 4 °C for several days. Use the appropriate serum to match the host organism of secondary antibodies (goat serum is used here). An optional blocking step may be added prior to the addition of primary antibodies.

5.2. Apply 100 µL of diluted primary antibody to each small glass tube. Incubate at room temperature for 4 h.

Note: Incubation times vary by antibody. For some antibodies, 2 h at room temperature is sufficient. Longer incubations (*e.g.*, overnight) are possible, but may increase background.

5.3. Fill the tubes to top with PBSTw and spin down in a clinical centrifuge at 870 x g for ~1 min.

5.4. Wash the gonads 3 times using ~1 mL of PBSTw. Incubate for ~5 min per wash to allow excess primary antibody to diffuse into wash. Using a drawn-out long glass Pasteur pipette, remove as much liquid as possible without disturbing the gonads. Leave no more than 50 µL.

5.5. Dilute the secondary antibodies in 30% goat serum in PBS. In the present example, goat-anti-rabbit-594 and goat-anti-mouse-647 are each diluted at 1:400.

Note: Select secondary antibodies carefully to make sure dyes are distinct from the dye on the EdU kit. In the present example, the EdU kit contained a 488 nm excitation dye.

5.6. Apply 100 µL of diluted secondary antibody to each small glass tube. Incubate in the dark at room temperature 2 h.

Note: Incubation times vary by antibody. For some secondary antibodies, 1 h at room temperature is sufficient. Longer incubations (*e.g.*, overnight) are possible, but may increase background.

5.7. Wash the gonads 3 times using ~1 mL of PBSTw. Incubate for ~5 min per wash to allow excess secondary antibody to diffuse into wash. Using a drawn-out long glass Pasteur pipette, remove as much liquid as possible without disturbing the gonads. Leave no more than 50 µL.

Note: Gonads can be stored in 100 µL of PBS after this step, if necessary, although this may reduce signal. Remove PBS prior to proceeding.

6. Perform the EdU Click Reaction to Detect EdU

Note: Performing the EdU click reaction before the antibody staining steps (perform Step 6 before Step 5) is possible, depending on the antibodies used[7]. However, the click reagents may interfere with certain antigens (*e.g.*, REC-8 antibody is sensitive to fixation and permeabilization). The order presented here yields bright antibody staining with the REC-8, WAPL-1, HIM-3, pH3, FLAG, and CYE-1 antibodies used, among others.

6.1. Prepare the click EdU cocktail[8] fresh by adding the following to a clean 1.5 mL tube. The order of additions is important. Protect from light and work on ice. This recipe yields enough for one sample (100 µL); multiply the recipe as needed.

6.1.1. Add 2 mL of ultrapure water to the buffer additive. This makes 10x buffer additive, which must be diluted to 1x immediately prior to use.

6.1.2. Add 8.5 µL of 10x buffer to 76.5 µL of ultrapure water. Mix well.

6.1.3. Add 4 µL of 100 mM $CuSO_4$ (may be labeled as Component E). Mix well.

6.1.4. Add 0.25 µL of the 488 nm dye Azide. It must be thawed at room temperature, as its solvent, dimethyl sulfoxide, is solid at 4 °C. Mix well and protect from light.

6.1.5. Mix 9 µL of ultrapure water with 1 µL of the buffer additive in the cap of the tube. Pipet from the cap to add to the remaining cocktail and mix well by pipetting up and down.

6.2. Perform the EdU click reaction

6.2.1. Add ~100 µL of click EdU cocktail to the gonads in the small tube. Cover with laboratory film and incubate for 30-60 min at room temperature.

6.2.2. Wash once with 100 µL of reaction rinse buffer.

6.2.3. Wash the gonads 4 times using ~1 mL of PBSTw. Incubate for ~15 min per wash to allow excess EdU cocktail components to diffuse into wash. Using a drawn-out long glass Pasteur pipette, remove as much liquid as possible without disturbing the gonads. Leave no more than 50 µL.

7. Stain DNA and Prepare Slides

7.1. Add 1 drop (~25 µL) of antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI, used to visualize DNA) to the gonads. Wait a few minutes so it can settle and mix with the gonads.

Note: Alternately, a 1:1000 dilution of DAPI (from a 0.1 mg/mL stock) in PBS may be applied for 5 min, followed by 20 µL of 1,4-diazabicyclo[2.2.2]octane (DABCO) in 90% glycerol, or another antifade mounting medium.

7.2. Prepare a large 2.5% agarose pad on a standard glass microscope slide.

7.3. Use a new clean dust-free long glass Pasteur pipette to transfer the gonads to the agarose pad. Keep all liquid and gonads in the narrow bottom of the pipette to minimize the loss of gonads.

Note: Gonads stuck to small glass tube or in long glass Pasteur pipette can be "rescued" by rinsing with PBSTw, collecting the liquid in a dissecting dish, and picking individual animals onto the slide with an eyelash.

7.4. Use an eyelash (or a loop of thin hair) glued to a toothpick to distribute the gonads over the agarose pad and remove the dust particles.

7.5. Apply a rectangular glass coverslip. Lower slowly from one side to avoid air bubbles. Use a tissue to remove excess solution and prevent the coverslip from moving freely.

Note: Use the coverslips to match them to the microscope which will be used. #1 and #1.5 coverslips work well.

7.6. Allow the slides to settle and dry slightly overnight at room temperature or 4 °C. This helps to slightly flatten the gonads. Slides should be stored at 4 °C.

7.7. Optional: Seal the edges of the slide with nail polish, or another slide sealer. Sealing the corners first, then the sides, prevents the coverslip from shifting.

8. Confocal Imaging and Analysis

8.1. Image the distal gonad with a spinning disc confocal fluorescent microscope equipped with a high energy light source, plan-apochromatic objectives, and a high efficiency microscope camera. Capture the images with 1 µm or tighter spacing between z-stacks. Take note of laser power, sensitivity, and exposure time for all channels.

8.1.1. Use the following: 405 nm laser line excitation with a 485 nm (W60) emission filter for DAPI, 488 nm laser line excitation with a 527 nm (W55) emission filter for EdU, 561 nm laser line excitation with a 615 nm (W70) emission filter for WAPL-1, and a 640 nm laser line excitation with a 705 nm (W90) emission filter for pH3.

Note: Signal intensity and background intensity will vary. Likewise, the required exposure times will vary, possibly up to 10-fold.

8.2. Use the Cell Counter plug-in[23] in Fiji[24,25] to manually count each nucleus. Label each individual nucleus according to the presence and absence of pH3, EdU, and WAPL-1. Use the classes of nuclei described in **Table 1** and **Figure 2**, as these will facilitate all of the calculations outlined below.

Note: Skilled counters can accurately count all nuclei in a 3D image without double-counting or missing any nuclei. Alternately, one may count every nucleus in every z-plane, and the Marks-to-Cells R script[3] may be used to remove multiply-counted nuclei.

8.3. Calculate cell numbers and frequencies from the above counts depending on the type of cell cycle measurement needed. The types of nuclei are defined in **Table** 1 and **Figure 2**. The calculations are summarized in **Table 2**.

8.3.1. **(variation I)** To identify the nuclei in S-phase, feed the animals EdU for 30 min. Any nuclei showing EdU label are S-phase nuclei. To calculate, take the sum A and C nuclei, see **Table 1** and **Figure 2**.

Note: In a 30 min EdU pulse in wild-type adult hermaphrodites, all EdU labeled nuclei are co-labeled with progenitor zone markers[1].

8.3.2. To identify the nuclei in M-phase, stain with pH3 antibody. Any pH3 immunoreactive nuclei are M-phase nuclei. This works regardless of the length of the EdU feed. To calculate, take the sum of A and B nuclei, see **Table 1** and **Figure 2**.

8.3.3. **(variation II)** To measure the progenitor zone, stain with a progenitor zone marker such as REC-8 or WAPL-1 antibody. The progenitor zone is defined here as all nucleoplasmic REC-8[18] or WAPL-1 immunoreactive germline nuclei. To calculate, sum all WAPL-1 immunoreactive nuclei (A+B+C+D, see **Table 1** and **Figure 2**).

Note: WAPL-1 also labels the DTC and somatic gonad nuclei which should not be counted. Somatic nuclei are easy to identify by extremely intense WAPL-1 signal, position slightly outside

the germline, and a “fried-egg” morphology of the nuclei.

8.3.4. To measure the S-phase index, perform a 30 min EdU experiment and co-label with REC-8 or WAPL-1 antibody. The S-phase index is defined as the proportion of the progenitor zone that is in S-phase. To calculate, count all S-phase nuclei, and then divide by the total number of progenitor zone nuclei (A+C / A+B+C+D, see **Table 1** and **Figure 2**).

8.3.5. To measure the M-phase index, co-label with pH3 and REC-8 or WAPL-1 antibodies. The M-phase index is defined as the proportion of the progenitor zone that is in M-phase. To calculate, count all M-phase nuclei, and then divide by the total number of progenitor zone nuclei (A+B / A+B+C+D, see **Table 1** and **Figure 2**).

8.3.6. **(variation III)** Nuclei in mitotic and meiotic S-phase both label with EdU. In order to tell the two populations apart, ask whether the S-phase was followed by mitosis or by meiosis. To determine whether nuclei are in mitotic or meiotic S-phase, feed EdU for 4 h and co-label for pH3 (an M-phase marker) and HIM-3 (a meiotic chromosome axis protein) by antibody staining. Record the nuclei which show both EdU and pH3 (type A, see **Table 1** and **Figure 2**) as mitotic S-phase while nuclei which show both EdU and HIM-3 (type E, see **Table 1** and **Figure 2**) as meiotic S-phase.

8.3.7. **(variation IV)** Calculate the length of G2 phase.

Note: G2-phase separates S-phase from M-phase. Although no marker currently labels G2 in the *C. elegans* germline, one can calculate the length of G2 phase by combining data from several experiments that label M-phase (at the time of dissection) and S-phase (starting at several h before dissection). A cell which shows both M-phase and S-phase markers completed G2-phase during the course of the experiment. A cell which shows only the M-phase marker and not the S-phase marker was not in S-phase during the experiment.

8.3.7.1. To calculate the length of G2-phase, feed EdU for 2 h and co-label with pH3 antibody. Examine only nuclei which label with pH3 (these are in M-phase at the time of dissection) for the presence of EdU (these were in S-phase during the 2 h EdU label prior to dissection). Calculate the fraction of M-phase nuclei which completed G2-phase (A / A+B, see **Table 1** and **Figure 2**).

8.3.7.2. Repeat this experiment with a 3 h EdU label, and again with a 4 h EdU label (and optionally a 5 h EdU label). Plot the percent of pH3 positive nuclei that are EdU positive on the y-axis and the length of EdU label on the x-axis, as shown in **Figure 3A**.

8.3.7.3. Calculate the median length of G2-phase by connecting the points on the graph and finding where the line crosses 50%, as shown in **Figure 3A**.

8.3.7.4. Calculate the maximum length of G2-phase by connecting the points on the graph and finding where the line crosses 99%, as shown in **Figure 3A**.

8.3.8. **(variation V)** Calculate the length of G2+M+G1.

Note: In the *C. elegans* germline, G1 phase is unusually short. Although no marker currently labels G1 in the *C. elegans* germline, one can estimate the sum length of G2, M, and G1 phase, and then compare this estimate with the G2-phase estimate described above.

8.3.8.1. To calculate the length of G2+M+G1, feed EdU for 2 h and co-label with REC-8 or WAPL-1 antibody. Determine the fraction of the progenitor zone that underwent S-phase during this time (A+C / A+B+C+D, see **Table 1** and **Figure 2**). That fraction corresponds to the fraction of nuclei that completed G2+M+G1 phases in that time.

8.3.8.2. Repeat this experiment with a 3 h EdU label, and again with a 4 h EdU label (and optionally a 5 h EdU label). Plot the percent of REC-8 or WAPL-1 positive nuclei that are EdU positive on the y-axis and the length of EdU label on the x-axis, as shown in **Figure 3B**.

8.3.8.3. Calculate the maximum length of G2+M+G1 by connecting the points on the graph and finding where the line crosses 99%, as shown in **Figure 3B**.

Note: It is possible to perform the experiments for the length of G2 and G2+M+G1 as a single set of 2, 3, 4, and 5-h EdU experiments when one co-labels with both rabbit-anti-WAPL-1 and mouse-anti-pH3 antibodies.

8.3.9. **(variation VI)** To identify the nuclei which replicated in the progenitor zone but have since entered meiosis, feed the animals EdU for 10 h and co-label with REC-8 or WAPL-1 antibodies. Any nuclei showing EdU label were in S-phase during those 10 h. Any nuclei which do not show nucleoplasmic REC-8 or WAPL-1 staining were in meiosis. Simply count the nuclei with EdU labeling which do not show labeling with the progenitor zone marker (E, see **Table 1**).

Note: Conversely, if gonads are stained for the meiotic prophase marker HIM-3 with anti-HIM-3 antibodies, count the number of nuclei with EdU labeling that are also positive for HIM-3.

8.3.10. To calculate the rate of meiotic entry, perform the above experiment with a 5 h, 10 h, and 15 h label of EdU. Plot the number of nuclei which entered meiosis on the y-axis and the length of the EdU label on the x-axis, as shown in **Figure 3C**. Then use a simple linear regression to calculate the slope (nuclei entered meiosis per h) from y=mx+b.

Note: It is critical to use a linear regression to calculate the rate of meiotic entry. It would be incorrect to simply divide the number of nuclei which entered meiosis by the length of the EdU label, because the y-intercept is not zero.

8.3.11. **(variation VII)** Measure the rate of meiotic progression.

Note: Since EdU is covalently incorporated into DNA, it can be used to track a population of cells through differentiation. The cells which underwent S-phase in the progenitor zone retain the EdU label as they enter into meiosis, progress through meiosis, and undergo oogenesis. A pulse-chase experiment with EdU can be used to measure the rate of meiotic progression.

8.3.11.1. Feed EdU-labeled bacteria to the animals for 4 h (the "pulse"). Transfer the animals to unlabeled OP50 bacteria for 48 h (the "chase"), then dissect and co-label with a progenitor zone marker such as REC-8 or WAPL-1 (or a meiotic prophase marker such as HIM-3) if desired.

8.3.11.2. When imaging, look for the position of the most proximal EdU-labeled nucleus. The rate of meiotic progression is the distance (in cell diameters from the end of the progenitor zone) traveled by the most proximal EdU labeled nucleus during the 48 h chase.

REPRESENTATIVE RESULTS:

Since DNA synthesis is required to incorporate EdU, one can safely assume that EdU-labeled nuclei underwent S-phase during the EdU-labeling time window. One may interpret the nuclei that label in a 30 min feeding with EdU labeled bacteria as nuclei in S-phase at the time of dissection. Nuclei which label in a longer continuous EdU feeding experiment may have labeled early in the time window and since left S-phase, or may have labeled in the late part of the EdU time window. EdU signal co-localizes with DAPI signal. In some nuclei, EdU signal covers all chromosomes, while in other nuclei EdU signal localizes to 1-2 bright puncta (**Figure 4**). These puncta are likely the X-chromosome, which replicates late in S-phase[13].

Here, the animals were fed with EdU continuously for 30 min and dissected, as described above and in **Figure 5**. One example of successful EdU staining in a young adult animal and one example of unsuccessful EdU staining in an older adult animal (see below) are shown in **Figure 4**. EdU signal from a 30 min labeling localizes to approximately half of the nuclei in the progenitor zone (defined by WAPL-1 antibody labeling but approximated by DAPI morphology[26–28]). S-phase index, the proportion of the progenitor zone which is EdU positive, was previously reported at 57 +/- 5% and as high as 70% in young adults[1–3]. M-phase index is approximately 2% - 3%[1,29]. In continuous feeding for 4 h or longer, all nuclei in the progenitor zone label with EdU, and some nuclei which labeled in the progenitor zone have since entered meiotic prophase[1].

While the technique works consistently in wild-type young adult animals, a significant fraction of mated 5 day old hermaphrodites (even those containing sperm) failed to label in a 30 min EdU pulse (**Figure 4E**). However, with a 4 h EdU feeding, nearly all these animals label. Other labs have observed sporadic failure to label in genetic female animals with short pulses of EdU[30]. There may be other situations which result in sporadic failure to label.

One can calculate the length of the cell cycle by performing several EdU-labeling experiments with pH3 labeling in each. The length of G2 was estimated by analyzing the percent of nuclei in M-phase (pH3 immunoreactive) that were EdU positive during the time course (**Figure 6**). This approach gives median and maximum length of G2 (**Figure 3A**). The median time was

interpolated, showing an approximate G2 length of 2.5 h in young adult hermaphrodites. The length of G2+M+G1 was estimated from the percentage of all progenitor zone nuclei (WAPL-1 immunoreactive) that were EdU positive (**Figure 6**). The G2+M+G1 method provides a maximum length measure for the combined phases (**Figure 3B**). The 99th percentile time was interpolated, showing an approximate G2+M+G1 length of 3.4 h in young adult hermaphrodites. Data from the same experiments were used to calculate the rate of meiotic entry (nuclei per h). The rate is the slope of the linear regression of the number of nuclei which entered meiosis (EdU positive, WAPL-1 negative or HIM-3 positive) over the length of the EdU label (**Figure 3C**). The values for wild-type 1 day old adult hermaphrodites are shown in **Table 2**.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of *C. elegans* germline and cell cycle. (**A**) The cell cycle of germ cells in the young adult hermaphrodite germline. (**B**) *C. elegans* hermaphrodites have two U-shaped germlines. (**C**) Diagram of an unfolded *C. elegans* germline. The distal progenitor zone (highlighted in red based on WAPL-1 antibody staining) contains mitotically cycling stem, progenitor cells and cells in meiotic S-phase. Cells in mitotic and meiotic S-phase label with a 30 min EdU pulse and are indicated in green. The distal tip cell (DTC) provides the GLP-1/Notch ligand to maintain the stem cell fate of these cells. As the cells migrate away from the DTC, they exit the progenitor zone and enter meiotic prophase.

Figure 2: Venn diagram of the classes of nuclei. Nuclei are grouped by the presence and absence of three cell fate and cell cycle markers: WAPL-1 indicates progenitor zone cells (red), EdU indicates S-phase cells (green), and pH3 indicates M-phase cells (blue). Cell types are identified as **A-G**. Note that cells of type G are not found in wild-type young adult hermaphrodites and cells do not co-label with EdU and pH3 outside of the (WAPL-1 positive) progenitor zone. See **Table 1** for more detail.

Figure 3: Graphical presentation of cell cycle length and rate of meiotic entry experimental data. The length of G2 phase is interpolated from pH3 and EdU co-labeling following varied-length EdU pulses (**A**). The length of G2+M+G1 phase is interpolated from EdU and REC-8 co-labeling following varied-length EdU pulses (**B**). The rate of meiotic entry (in nuclei per h – see **Table 2**) is calculated from the slope of the regression line (**C**). Figures reprinted with permission from Fox *et al.* 2011[1].

Figure 4: Example of successful and unsuccessful 30 min EdU staining. Confocal microscope images of a 1 day old (**A-D**) and a 5 day old (**E-H**) hermaphrodite gonad (not sperm depleted) after a 30 min EdU labeling experiment. The dashed white line marks the end of the progenitor zone. The asterisk marks the position of the distal tip. Green marks EdU staining visualized by click chemistry (**A**). Unsuccessful EdU labeling results in low-level background staining but no bright EdU+ nuclei (**E**). Red marks WAPL-1 immunofluorescence (**B,F**). Yellow indicates overlap (**C, G**). Blue marks DAPI staining for DNA (**D, H**). Single arrowheads indicate a nucleus with EdU staining throughout the chromatin. Double arrowheads indicate a nucleus with EdU puncta on only one pair of chromosomes. Images were obtained with a 63X objective. A 10 μm scale bar is shown (**D, H**).

Figure 5: Experimental Workflow. A summary of the experimental protocol to grow (**A**), EdU label (**B**), dissect (**C**), antibody stain (**D**), perform the click reaction to attach a dye to EdU (**E**), stain DNA (**F**), image germlines (**G**), and quantify EdU labeled and antibody stained nuclei (**H**).

Figure 6: Example of successful 4 h EdU staining. Confocal microscope images of a 1 day old adult hermaphrodite gonad after a 4 h EdU labeling experiment. The dashed white line marks the end of the progenitor zone. The asterisk marks the position of the distal tip. Magenta marks pH3 immunofluorescence (**A, C**). Green marks EdU staining visualized by click chemistry (**B,C**). Red marks WAPL-1 immunofluorescence (**D**). Yellow indicates the overlap of EdU and WAPL-1 (**E**). Blue marks DAPI staining for DNA (**F**). Single arrowheads indicate nuclei co-labeled with EdU and pH3. Double arrowhead marks a pH3+ EdU- nucleus – a rare occurrence in a 4 h EdU labeling. Arrows mark EdU+ WAPL-1 – nuclei which have entered meiosis. Images were obtained with a 63X objective. A 10 µm scale bar is shown (**F**).

Table 1: Classes of nuclei. *See text (Introduction and **Step 8)** for the length of EdU labeling for relevant experiment.

Table 2: Cell cycle calculations. *Letters represent the classes of nuclei defined in **Table 1** and **Figure 3**. Calculations are modified from Fox *et al.* 2011[1]. **Values for wild-type hermaphrodites raised at 20 °C aged to 24 h post mid-L4 stage. ***Note that since the y-intercept intercept is not zero, a regression is necessary for an accurate calculation of the rate of meiotic entry.

DISCUSSION:

Preparation of EdU-labeled bacteria (part 1) is critical for this protocol, and the first point for troubleshooting. Wild-type young adult hermaphrodites label very reliably in a 4 h EdU-pulse, making this a useful control for every new batch of EdU-labeled bacteria. Additionally, intact EdU-labeled bacteria which enter the intestine (in older animals or certain pharynx/grinder defective mutants) will label with click chemistry and show up as bright oblong puncta in the gut. An alternate technique for labeling hermaphrodites uses a "soak" in a high concentration (1 mM) of EdU[3]. This technique starves the animals for the duration of labeling, but provides a useful way to bypass making EdU-labeled bacteria when troubleshooting fixation and click chemistry. If an EdU "soak" experiment is successful while an EdU feed is not, prepare fresh EdU-labeled bacteria. To reach a sufficient bacterial density while also achieving a high EdU content, the concentrations of EdU and thymidine may need to be adjusted.

The main limitation of this technique for labeling of S-phase is in the need to feed EdU-labeled bacteria to animals. The animals which cannot feed (due to genotype or stage) may not be labeled with this technique. Nevertheless, nucleoside analogs are currently the only method to identify S-phase nuclei in the *C. elegans* germline, and their use does not require that any transgenes be crossed into animals. Additionally, once incorporated, EdU remains in nuclei even as they exit S-phase, progress through the cell cycle, divide, or differentiate. The signal weakens by half with every cell division. This makes EdU perfect for tracking a cell's history

even through a few cell divisions.

The stability of EdU makes pulse-chase experiments straightforward; simply rinse excess EdU bacteria from the animals after the desired length pulse is finished and transfer the animals to unlabeled bacteria. EdU remains in DNA and remains visible even after multiple cell divisions. However, the experiments are limited to a single type of S-phase label (a single pulse of EdU). Co-labeling with EdU and BrdU is possible in mammalian cells[31] but has not been reported in *C. elegans*. Co-labeling of IdU and CldU is used in mammals[32] but also has not been reported in *C. elegans*.

The main advantages of EdU labeling are that the method requires no transgenes, EdU can be fed to *C. elegans* during regular culture, the chemistry is compatible with immunofluorescent techniques, and EdU persists in DNA for a long time after feeding has stopped. These features make EdU a great tool to study many aspects of the cell cycle and germ cell dynamics.

Cell cycle and germ cell dynamics analysis with EdU can be applied to a variety of research questions. Just a few examples of further applications of this method: How do the dynamics of the cell cycle change in animals with cell cycle gene mutations? How do physiological conditions affect the cell cycle in stem cells, the rate of germ cell entry into meiotic prophase, and the rate of germ cell progression through meiotic prophase? How does the cell cycle change during larval development? How do major signaling pathway disruptions affect the cell cycle, in addition to changes in cell fate (such as ectopic proliferation)? This system can be modified to study what the cells are doing under many different conditions.

ACKNOWLEDGMENTS:
We are grateful to the *E. coli* stock center for MG1693; Wormbase; the *Caenorhabditis* Genetics Center which is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40OD010440) for strains; Zach Pincus for statistical advice; Aiping Feng for reagents, Luke Schneider, Andrea Scharf, Sandeep Kumar, and John Brenner for training, advice, support, and helpful discussion; and the Kornfeld and Schedl labs for feedback on this manuscript. This work was supported in part by National Institutes of Health [R01 AG02656106A1 to KK, R01 GM100756 to TS] and a National Science Foundation predoctoral fellowship [DGE-1143954 and DGE-1745038 to ZK]. Neither the National Institutes of Health nor the National Science Foundation had any role in the design of the study, collection, analysis, and interpretation of data, nor in writing the manuscript.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Fox, P. M., Vought, V. E., Hanazawa, M., Lee, M.-H., Maine, E. M. & Schedl, T. Cyclin E and CDK-2 regulate proliferative cell fate and cell cycle progression in the *C. elegans* germline. *Development (Cambridge, England)* **138** (11), 2223–2234 (2011).
2. Crittenden, S. L., Leonhard, K. A., Byrd, D. T. & Kimble, J. Cellular analyses of the mitotic

region in the *Caenorhabditis elegans* adult germ line. *Molecular biology of the cell* **17** (7), 3051–61 (2006).
3. Seidel, H. S. & Kimble, J. Cell-cycle quiescence maintains *Caenorhabditis elegans* germline stem cells independent of GLP-1/Notch. *eLife* **4** (2015).
4. Fox, P. M. & Schedl, T. Analysis of Germline Stem Cell Differentiation Following Loss of GLP-1 Notch Activity in *Caenorhabditis elegans*. *Genetics* **201** (9), 167–184 (2015).
5. Kocsisova, Z., Kornfeld, K. & Schedl, T. Cell cycle accumulation of the proliferating cell nuclear antigen PCN-1 transitions from continuous in the adult germline to intermittent in the early embryo of *C. elegans*. *BMC Developmental Biology* **18** (1), 12 (2018).
6. Salic, A., & Mitchison, T. J. A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proceedings of the National Academy of Sciences 105 (7), 2415-2420 (2008).
7. van den Heuvel, S. & Kipreos, E. T. *C. elegans* Cell Cycle Analysis. *Methods in Cell Biology* **107**, 265–294 (2012).
8. ThermoFisher *Click-iT EdU Imaging Kits*. (2011). at <https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp10338.pdf>
9. Pazdernik, N. & Schedl, T. Germ Cell Development in *C. elegans.* **757**, 1–16 (2013).
10. Hirsh, D., Oppenheim, D. & Klass, M. Development of the reproductive system of *Caenorhabditis elegans. Developmental Biology* **49** (1), 200–219 (1976).
11. Brenner, S. The genetics of *Caenorhabditis elegans. Genetics* **77** (1), 71–94 (1974).
12. Hansen, D. & Schedl, T. Stem cell proliferation versus meiotic fate decision in *Caenorhabditis elegans. Advances in Experimental Medicine and Biology* **757**, 71–99 (2013).
13. Jaramillo-Lambert, A., Ellefson, M., Villeneuve, A. M. & Engebrecht, J. Differential timing of S phases, X chromosome replication, and meiotic prophase in the *C. elegans* germ line. *Developmental Biology* **308** (1), 206–221 (2007).
14. Agarwal, I., Farnow, C., *et al.* HOP-1 presenilin deficiency causes a late-onset notch signaling phenotype that affects adult germline function in *Caenorhabditis elegans*. *Genetics* **208** (2) 745–762 (2018).
15. Gumienny, T. L., Lambie, E., Hartwieg, E., Horvitz, H. R. & Hengartner, M. O. Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. *Development (Cambridge, England)* **126** (5), 1011–1022 (1999).
16. Hendzel, M. J., Wei, Y., *et al.* Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106** (6), 348–360 (1997).
17. Zetka, M. C., Kawasaki, I., Strome, S. & Mü Ller, F. Synapsis and chiasma formation in *Caenorhabditis elegans* require HIM-3, a meiotic chromosome core component that functions in chromosome segregation. at <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC317003/pdf/x8.pdf>
18. Hansen, D., Hubbard, E. J. A. & Schedl, T. Multi-pathway control of the proliferation versus meiotic development decision in the *Caenorhabditis elegans* germline. *Developmental Biology* **268** (2), 342–357 (2004).
19. Crawley, O., Barroso, C., *et al.* Cohesin-interacting protein WAPL-1 regulates meiotic

chromosome structure and cohesion by antagonizing specific cohesin complexes. *eLife* **5** (2), 1–26 (2016).
20. Zhao, H., Halicka, H. D., *et al.* DNA damage signaling, impairment of cell cycle progression, and apoptosis triggered by 5-ethynyl-2'-deoxyuridine incorporated into DNA. *Cytometry Part A* **83** (11), 979–988 (2013).
21. Stiernagle, T. Maintenance of *C. elegans. WormBook* 1–11 (2006). doi:10.1895/wormbook.1.101.1
22. Gervaise, A. L. & Arur, S. Spatial and Temporal Analysis of Active ERK in the *C. elegans* Germline. *Journal of Visualized Experiments* (117), e54901–e54901 (2016). doi:10.3791/54901
23. Vos, K. De Cell Counter Plugin. (2015).
24. Schindelin, J., Arganda-Carreras, I., *et al.* Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9** (7), 676–682 (2012).
25. Rasband, W. . ImageJ. (2016). at <http://imagej.nih.gov/ij/>
26. Michaelson, D., Korta, D. Z., Capua, Y. & Hubbard, E. J. A. Insulin signaling promotes germline proliferation in *C. elegans. Development (Cambridge, England)* **137** (4) 671–80 (2010).
27. Qin, Z., Jane, E., Hubbard, A. & Hubbard, E. J. A. Non-autonomous DAF-16/FOXO activity antagonizes age-related loss of *C. elegans* germline stem/progenitor cells. *Nature communications* **6** (5), 7107 (2015).
28. Luo, S., Kleemann, G. A., Ashraf, J. M., Shaw, W. M. & Murphy, C. T. TGFB and Insulin Signaling Regulate Reproductive Aging via Oocyte and Germline Quality Maintenance. *Cell* **143** (2), 299–312 (2010).
29. Narbonne, P., Maddox, P. S. & Labbe, J.-C. daf-18/PTEN locally antagonizes insulin signalling to couple germline stem cell proliferation to oocyte needs in *C. elegans*. *Development* 4230–4241 (2015). doi:10.1242/dev.130252
30. Cinquin, A., Chiang, M., *et al.* Intermittent Stem Cell Cycling Balances Self-Renewal and Senescence of the *C. elegans* Germ Line. *PLoS Genetics* **12** (4), e1005985 (2016).
31. Invitrogen EdU (5-ethynyl-2'-deoxyuridine). 1–7 (2010). at <https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp10044.pdf>
32. Tuttle, A. H., Rankin, M. M., *et al.* Immunofluorescent Detection of Two Thymidine Analogues (CldU and IdU) in Primary Tissue. *Journal of Visualized Experiments* (46), e2166–e2166 (2010).doi:10.3791/2166

Figure 1

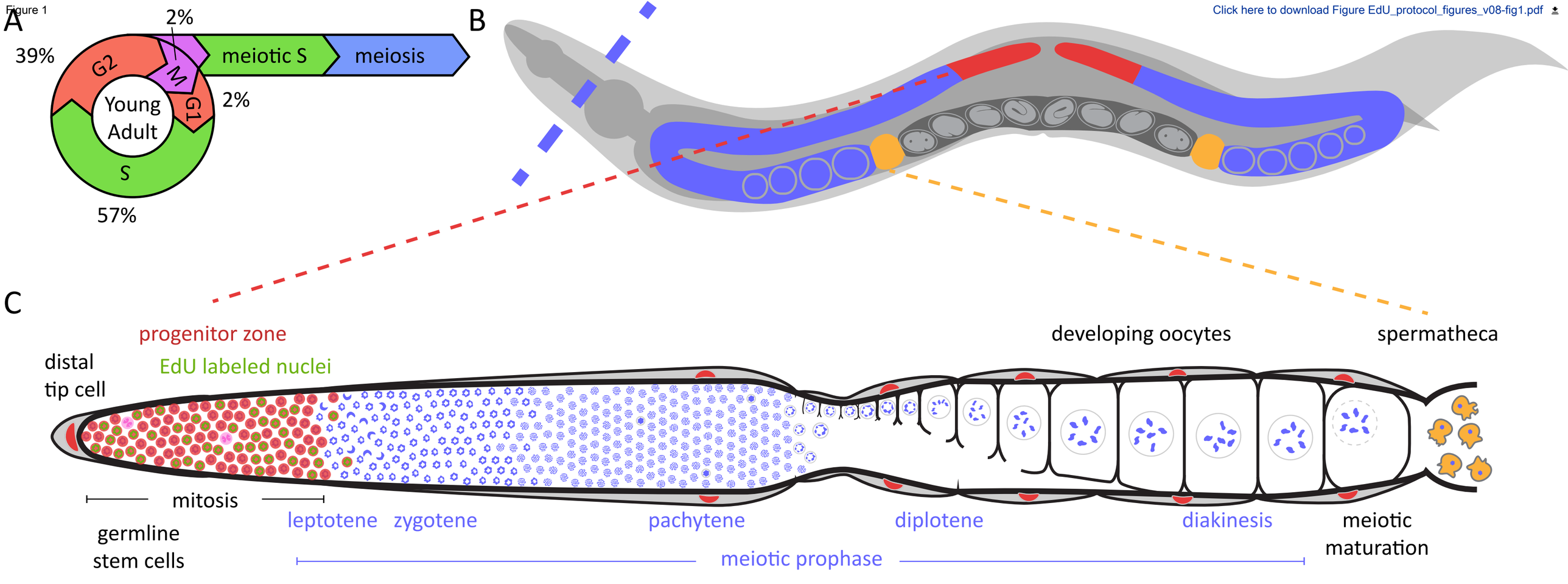
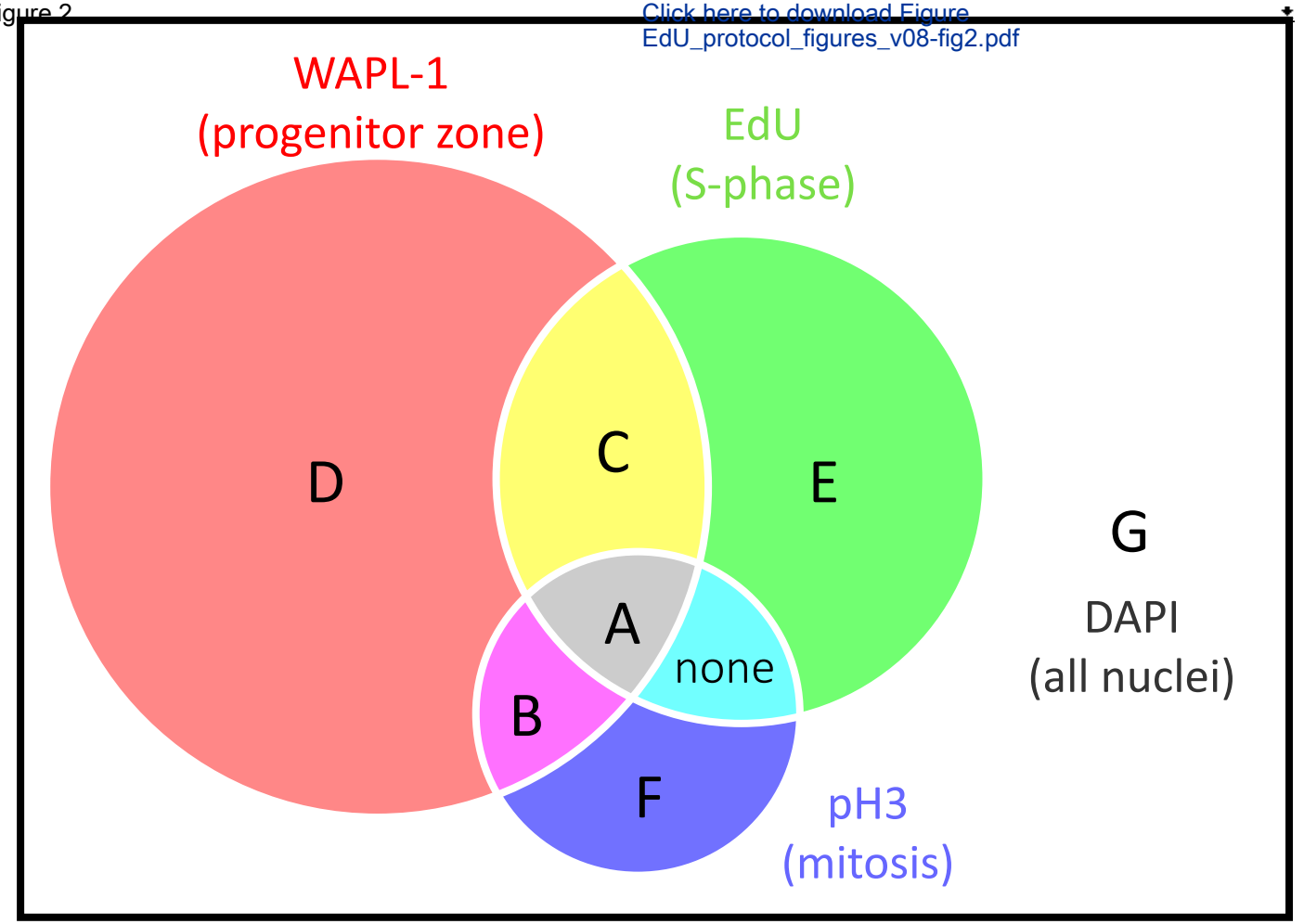


Figure 2

Click here to download Figure
EdU_protocol_figures_v08-fig2.pdf



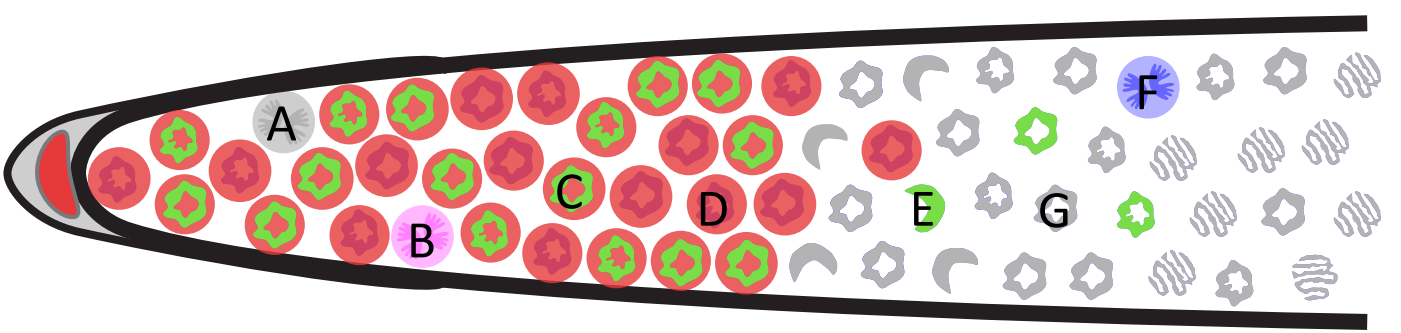
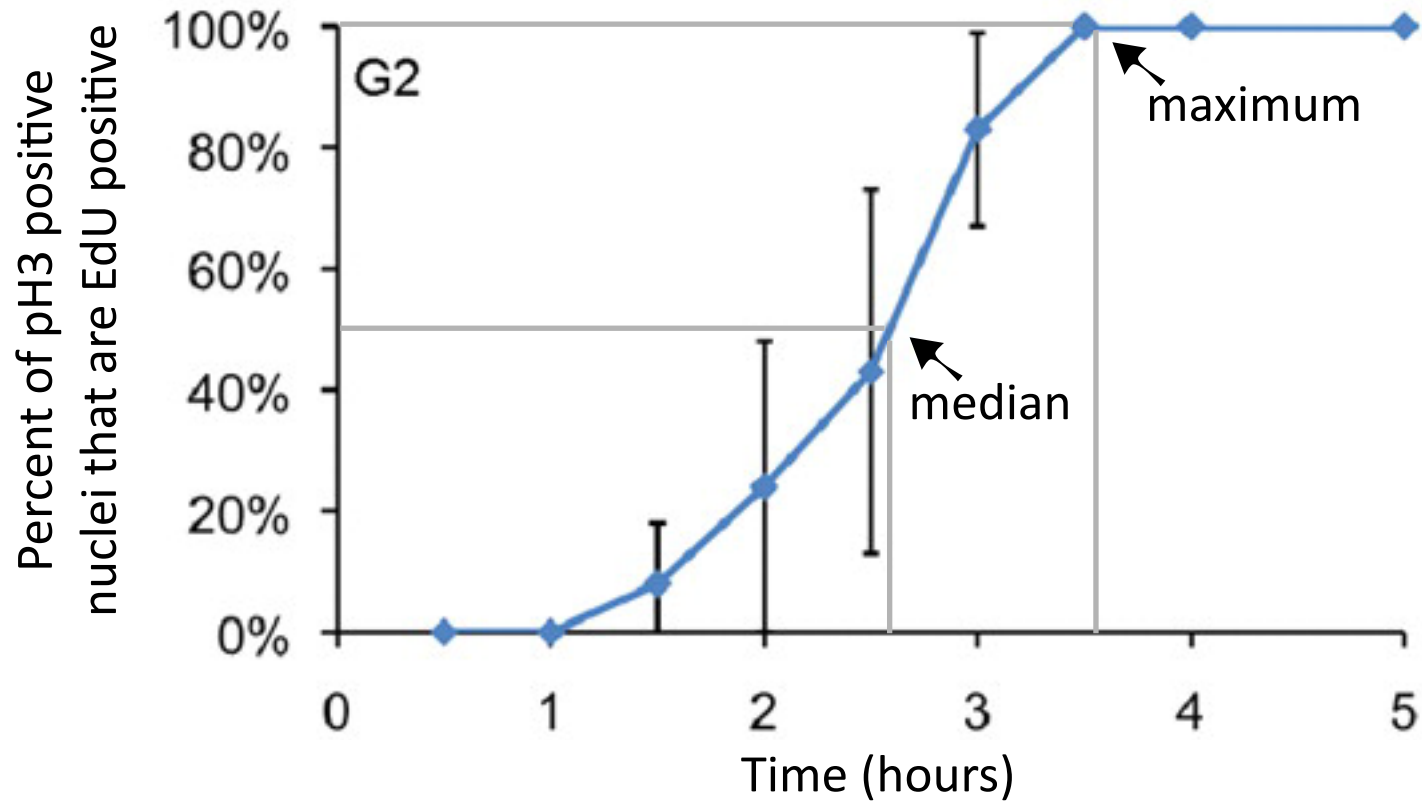
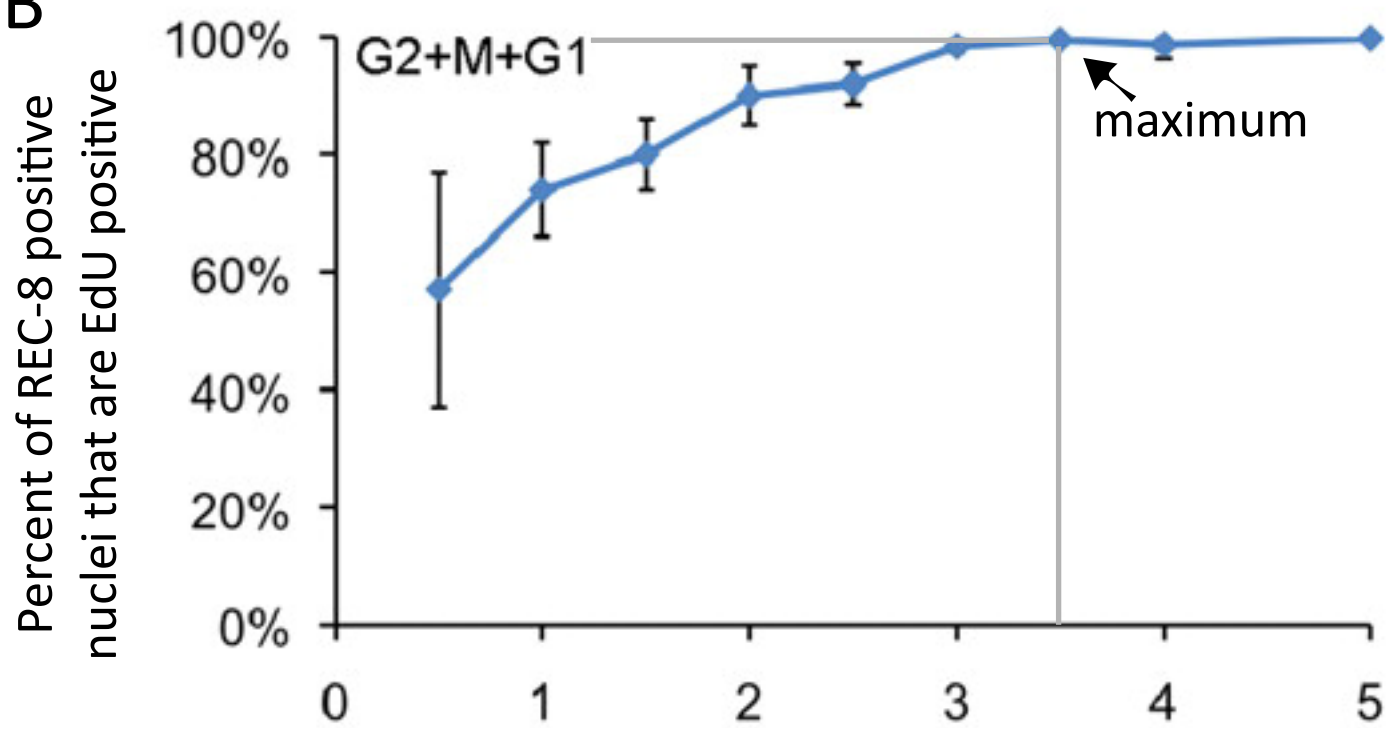


Figure 3

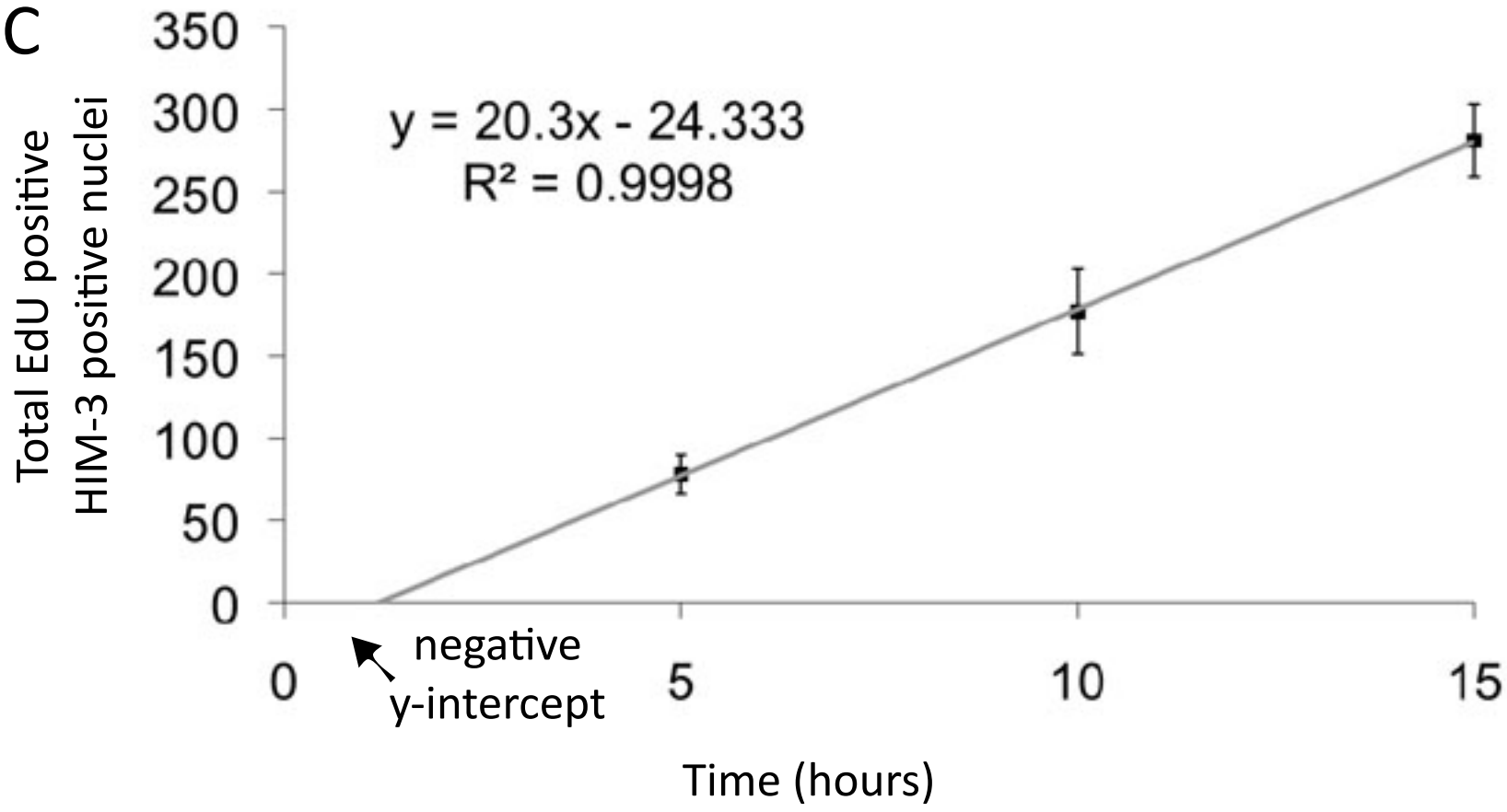
A

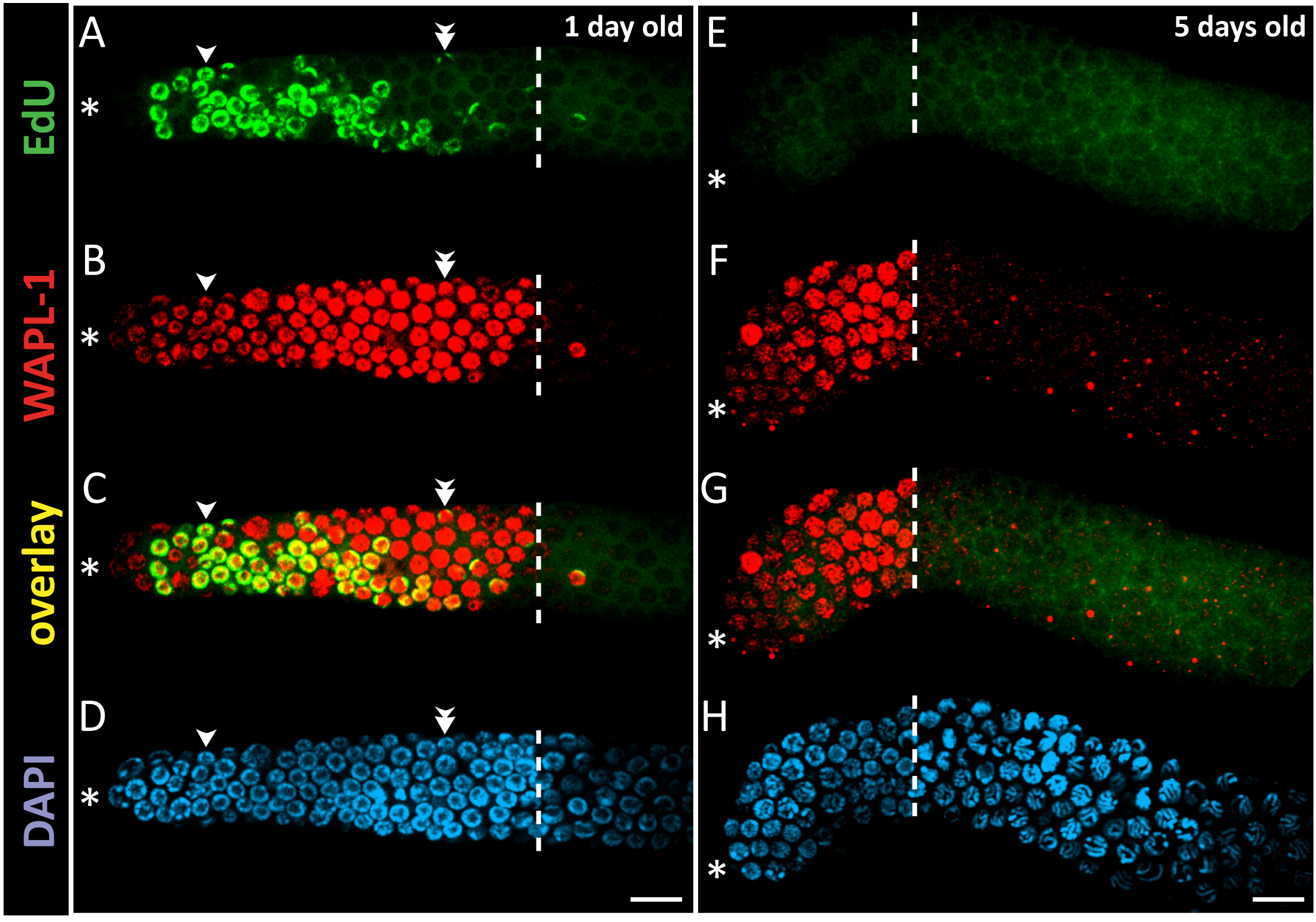


B

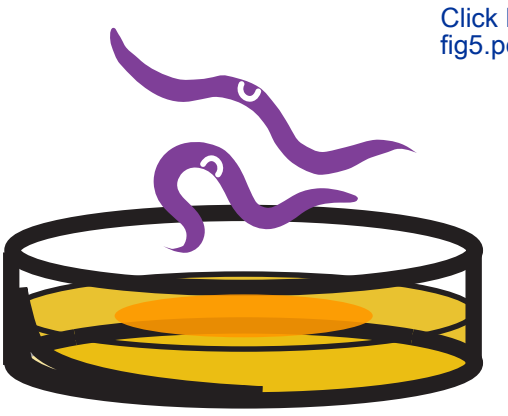


C



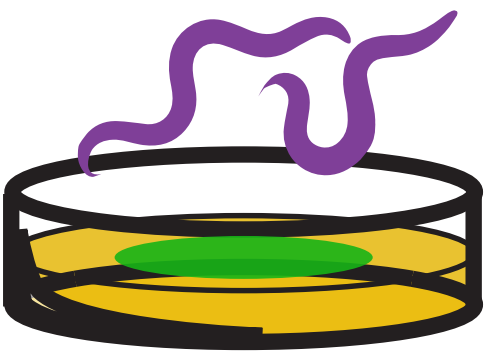


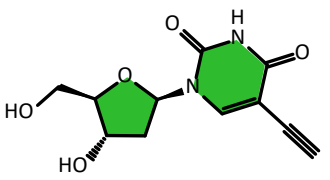
A



Culture synchronized population with *E. coli*

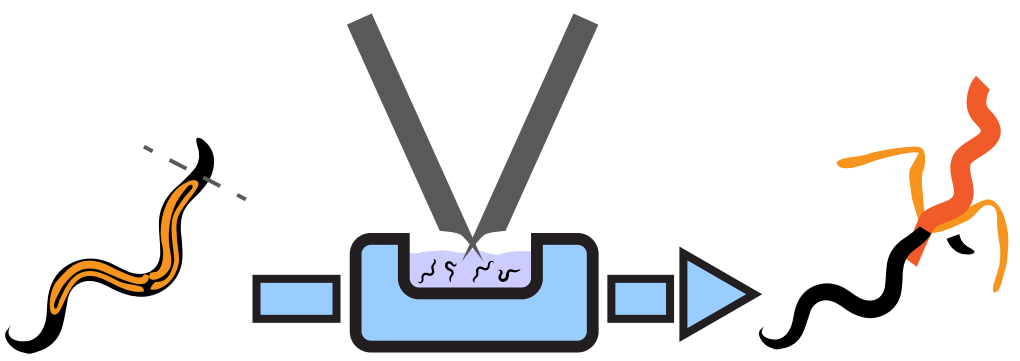
B





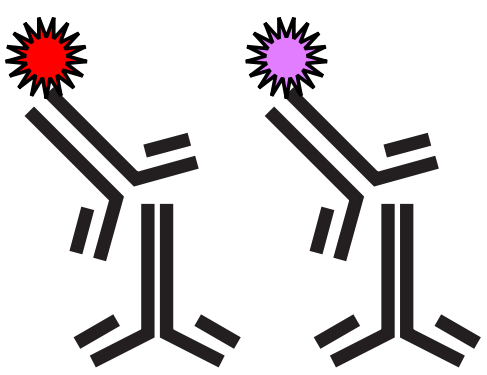
Culture with EdU labeled bacteria for 0.5, 4, 7, or 10 hours

C



Dissect and fix gonad

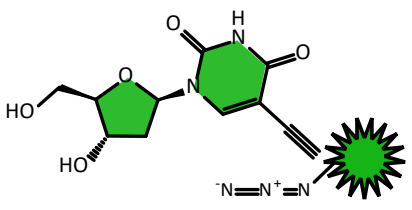
D



Stain proteins with antibodies

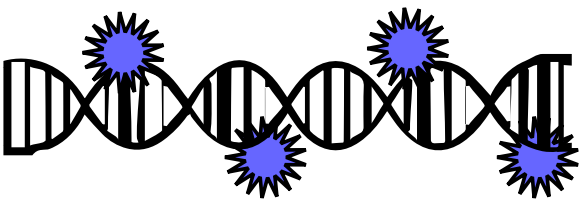
WAPL pH3

E



Stain EdU with Click reaction

F

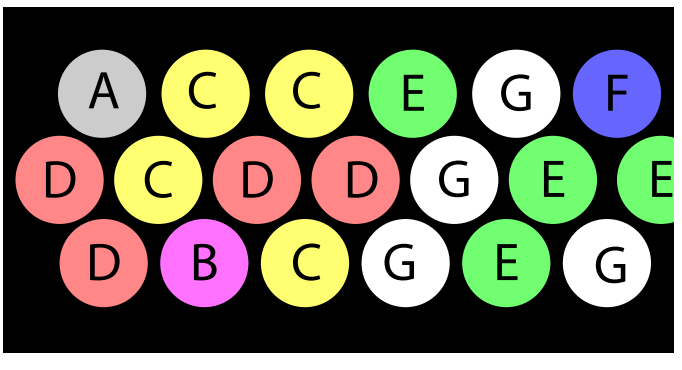


Stain DNA with DAPI

G

Confocal microscopy

H



Count nuclei

Figure 6

Click here to download Figure
EdU_protocol_Figures_top.pdf

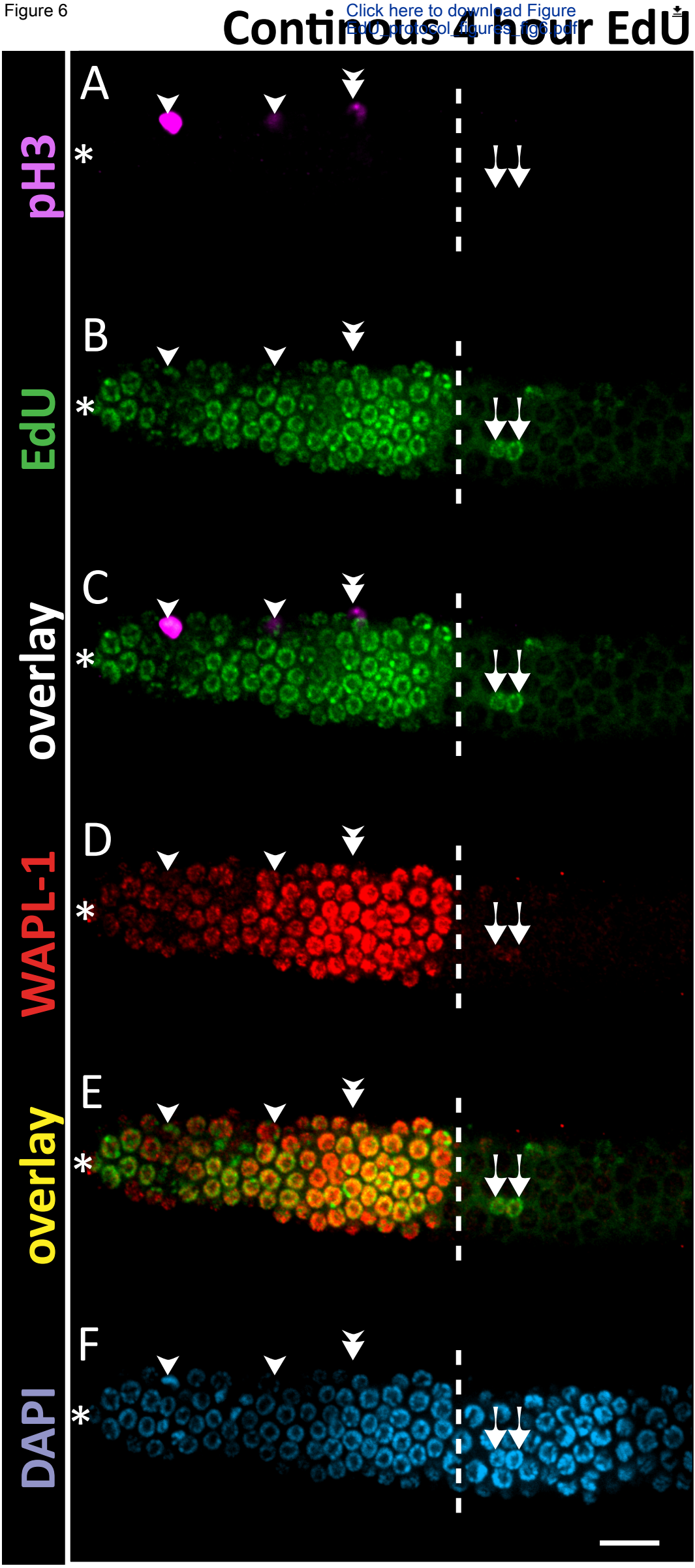


Table 1

Class	Mitosis pH3	S-phase* EdU*	Progenitor Zone WAPL-1 or REC-8	Meiosis HIM-3	Interpretation
A	+	+	+	-	in M-phase, in progenitor zone, were in mitotic S-phase during EdU label (completed G2)
B	+	-	+	-	in M-phase, in progenitor zone, were not in S-phase during EdU label
C	-	+	+	-	in Interphase, in progenitor zone, were in S-phase during EdU label
D	-	-	+	-	in Interphase, in progenitor zone, were not in S-phase during EdU label
E	-	+	-	+	in meiosis, were in meiotic S-phase during EdU label (meiotic entry nuclei)
F	+	-	-	-	return to mitosis (found in some mutants) or meiotic divisions (in spermatogenesis)
G	-	-	-	+	in meiosis, were not in S-phase during EdU label
	sum total pH3 positive; all cells in M-phase	sum total EdU positive; all cells in S-phase	sum total WAPL-1 positive; all cells in progenitor zone	sum total HIM-3 positive; all cells in meiotic prophase	

Table 2

	Operational Definition	**Calculation***	**Value****
Progenitor Zone Cells	sum of all WAPL-1 (or REC-8) positive, HIM-3 negative nuclei	A+B+C+D	231 ± 23
S-phase nuclei	sum of all nuclei EdU positive after 30 minute EdU label	A+C	133 ± 20
M-phase nuclei	sum of all pH3 positive nuclei	A+B	5.2 ± 2.3
M-phase index	M-phase / Progenitor Zone	A+B/ A+B+C+D	2%
S-phase index	S-phase / Progenitor Zone	A+C/ A+B+C+D	57%
Meiotic Entry cells	EdU labeled nuclei in meiosis	E	varies by length of EdU label
Meiotic Entry rate	Meiotic entry nuclei per hour of EdU label	Slope from Figure 6C***	20.3 nuclei/hour
G2 length (median)	50% intercept from Figure 5A		2.5 hours
G2 length (maximum)	99% intercept from Figure 5A		3.5 hours
G2+M+G1 length (maximum)	99% intercept from Figure 5B		3.5 hours
Cell cycle length (median)	median G2 length / G2-index		6.5 hours
Cell cycle length (maximum)	maximum G2 length / G2-index		8.1 hours

Table of Materials

Click here to download Table of Materials JOVE_Materials_EdU_v08.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
E. coli MG1693	Coli Genetic Stock Center	6411	grows fine in standard unsupplemented LB
E. coli OP50	Caenorhabditis Genetics Center	OP50	
Click-iT EdU Alexa Fluor 488 Imaging Kit	Thermo Fisher Scientific	C10337	
5-Ethynyl-2'-deoxyuridine	Sigma	900584-50MG	or use EdU provided in kit
Glucose	Sigma	D9434-500G	D-(+)-Dextrose
Thiamine (Vitamin B1)	Sigma	T4625-5G	Reagent Grade
Thymidine	Sigma	T1895-1G	BioReagent
Magnesium sulfate heptahydrate	Sigma	M1880-1KG	MgSO4, Reagent Grade
Sodium Phosphate, dibasic, anhydrous	Fisher	BP332-500G	Na2HPO4
Potassium Phosphate, monobasic	Sigma	P5379-500G	KH2PO4
Ammonium Chloride	Sigma	A4514-500G	NH4Cl, Reagent Plus
Bacteriological Agar	US Biological	C13071058	
Calcium Chloride dihydrate	Sigma	C3881-500G	CaCl
LB Broth (Miller)	Sigma	L3522-1KG	Used at 25g/L
Levamisole	Sigma	L9756-5G	0.241g/10ml
Phosphate buffered saline	Calbiochem Omnipur	6506	hommemade PBS works just as well
Tween-20	Sigma	P1379-500ML	
16% Paraformaldehyde, EM-grade ampules	Electron Microscopy Sciences	15710	10ml ampules
100% methanol	Thermo Fisher Scientific	A454-1L	Gold-label methanol is critical for proper morphology with certain antibodies
Goat Serum	Gibco	16210-072	Lot 1671330
rabbit-anti-WAPL-1	Novus biologicals	49300002	Lot G3048-179A02, used at 1:2000
mouse-anti-pH3 clone 3H10	Millipore	05-806	Lot#2680533, used at 1:500
goat-anti-rabbit IgG-conjugated Alexa Fluor 594	Invitrogen	A11012	Lot 1256147, used at 1:400
goat-anti-mouse IgG-conjugated Alexa Fluor 647	Invitrogen	A21236	Lot 1511347, used at 1:400
Vectashield antifade mounting medium containing 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI)	Vector Laboratories	H-1200	mounting medium without DAPI can be used instead, following a separate DAPI incubation
nail polish	Wet n Wild	DTC450B	any clear nail polish should work
S-medium	various		see wormbook.org for protocol

M9 buffer	various		see wormbook.org for protocol
M9 agar	various		same recipe as M9 buffer, but add 1.7% agar
Nematode Growth Medium	various		see wormbook.org for protocol
dissecting watchglass	Carolina Biological	42300	
Parafilm laboratory film	Pechiney Plastic Packaging	PM-996	4 inch wide laboratory film
petri dishes			60 mm diameter
Long glass Pasteur pipettes			
1ml centrifuge tubes	MidSci Avant	2926	
Tips			
Serological pipettes			
500 mL Erlenmyer flask			
Aluminium foil			
25G 5/8” needles	BD PrecisionGlide	305122	
5ml glass centrifuge tube	Pyrex		
Borosilicate glass tubes 1ml			
glass slides			
no 1 coverslips 22 x 40 mm			no 1.5 may work, also
37 °C Shaker incubator			
Tabletop Centrifuge			
Clinical Centrifuge	IEC	428	with 6 swinging bucket rotor
Mini Centrifuge			
20 °C incubator			
4 °C refrigerator			
-20 °C freezer			
Observer Z1 microscope	Zeiss		
Plan Apo 63X 1.4 oil-immersion			
objective lens	Zeiss		
Ultraview Vox spinning disc confocal system	PerkinElmer		Nikon spinning disc confocal system works very well, also, as described here: http://wucci.wustl.edu/Facilities/Light-Microscopy

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Cell cycle analysis in the C.elegans germline with the thymidine analog EdU

Author(s): Zuzana Kocsisova, Ariz Mohammad, Kerry Kornfeld & Tim Schedl

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

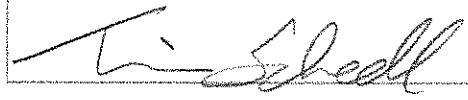
CORRESPONDING AUTHOR:

Name: Tim Schedl

Department: Department of Genetics

Institution: Washington University School of Medicine

Article Title: Cell cycle analysis in the C. elegans germline with the thymidine analog EdU

Signature: 

Date: 4/23/2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dr. Tim Schedl
Department of Genetics
Washington University School of Medicine
4566 Scott Ave. Campus Box #8232
St. Louis, MO 63110
(314)362-6164
ts@wustl.edu

Bing Wu, Ph.D.
Review Editor, JoVE

June 28, 2018

Dear Dr. Wu:

Thank you for arranging an insightful and constructive review of our manuscript and allowing us the opportunity to submit a revised version.

We modified the manuscript to address the specific points, and a list of point-by-point responses to the comments is provided below.

Sincerely,

Tim Schedl
Professor of Genetics

Response to Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

2. For in-text referencing, please put the reference number before a period or comma.

Done.

3. Please use h, min, s for time units.

Done.

4. Please define all abbreviations before use.

Done.

5. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include Click-iT EdU, Vectashield, etc.

Done.

6. Please do not highlight notes for filming.

Done.

7. Step 1.3.5: What is used to add the drops?

We use a 5 mL pipet. We added this information to step 1.3.5

8. 2.1: What condition is used to grow the animal?

We grew the animals at 20 °C. We added this information to step 2.1.

9. 2.5: What's the temperature for incubation?

The temperature is 20 °C. We added this information to step 2.5.

10. Step 3: Please do not highlight a step without highlighting any of the sub-steps.

Done.

11. 6.1.3: What's the concentration of $CuSO_4$?

The concentration is 100 mM. We added this information to step 6.1.3.

12. 8.3.4, 8.3.5, 8.3.6, 8.3.9: Please ensure that all text is written in imperative tense.

Done.

13. Figure 2: Please add a short description to the Figure title in the Figure Legend.

Done.