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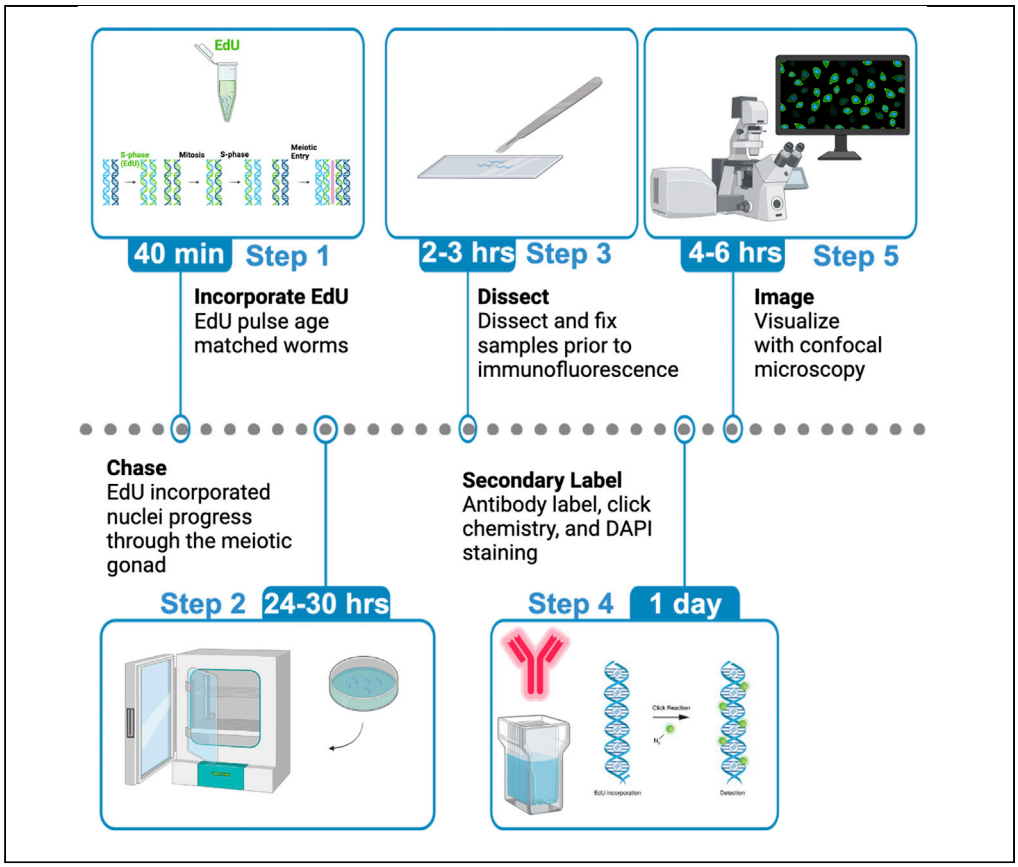
Single-sister labeling in the *C. elegans* germline using the nucleotide analog EdU



Reciprocal exchanges between genetically identical sister chromatids (sister chromatid exchanges or SCEs) have been challenging to study. Here, we describe a protocol that utilizes a pulse/chase of the thymidine analog 5-ethyl-3′-deoxyuridine (EdU) in combination with click chemistry and antibody labeling to selectively label sister chromatids in the *C. elegans* germline. Labeling has no discernable effects on meiosis, allowing for cytological quantification of SCEs. This protocol can be combined with a variety of imaging approaches, including STED, confocal and super-resolution.

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Highlights

Pulse/chase labeling of individual sister chromatids in the *C. elegans* germline

Can be readily combined with immunofluorescence or other labeling techniques

Wide fluorophore support suited for confocal, STED and super-resolution imaging ima

Variable chase times allows visualization of single sisters throughout meiosis

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Protocol

Single-sister labeling in the *C. elegans* germline using the nucleotide analog EdU

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SUMMARY

Reciprocal exchanges between genetically identical sister chromatids (sister chromatid exchanges or SCEs) have been challenging to study. Here, we describe a protocol that utilizes a pulse/chase of the thymidine analog 5-ethyl-3′-deoxyuridine (EdU) in combination with click chemistry and antibody labeling to selectively label sister chromatids in the *C. elegans* germline. Labeling has no discernable effects on meiosis, allowing for cytological quantification of SCEs. This protocol can be combined with a variety of imaging approaches, including STED, confocal and super-resolution.

For complete details on the use and execution of this protocol, please refer to Almanzar et al. (2021).

BEFORE YOU BEGIN

The protocol below uses wild-type (N2) *C. elegans* hermaphrodites. However, we have successfully applied this protocol to worms of different genotypes and combined it with various experimental perturbations, such as X-ray irradiation or auxin-mediated degradation (Zhang et al., 2015).

The principle of the protocol is incorporation of EdU during DNA replication of germline nuclei in the gonad - the only mitotic nuclei in the adult worm. During the chase, nuclei that incorporated EdU undergo pre-meiotic DNA replication without EdU and progress through meiotic prophase. EdU is visualized in oocytes immediately prior to fertilization (so-called diakinesis nuclei).

Preparation of Click-iT™ EdU kit

Timing: 2–3 h

1. Prepare the Click-iT™ EdU Cell Proliferation kit (Thermo Fisher Catalog# C10337) according to the manufacturer's instructions.

⚠ CRITICAL: Dissolve the EdU in solution using sterile deionized ("Millipore") water (dH_2O), NOT in DMSO.

Note: Final concentration of the EdU stock solution is 10 mM.

Note: DMSO should be used to dissolve the azide-conjugated fluorophore, per the kit's instructions.

⚠ CRITICAL: To achieve efficient incorporation into the germline, worms are soaked in 4 mM EdU - a concentration ~40× higher than what has been recommended by the manufacturer for cells in culture. However, the corresponding DMSO concentration – 40% – is lethal to *C. elegans* (0/30 worms alive after 40 min in 40% DMSO). We therefore recommend dissolving EdU in water, which we have found to work well.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Guinea Pig anti-HTP-3 (dilution 1:500)	Yumi Kim Lab	n/a
Cy3 AffiniPure Donkey anti-Guinea pig (dilution 1:500)	Jackson ImmunoResearch	Cat#706-165-148; RRID: AB_2340460
Chemicals, peptides, and recombinant proteins		
EdU	Thermo Fisher Scientific	Cat#A10044
Prolong Glass antifade agent	Thermo Fisher Scientific	Cat#P36980
HEPES	Fisher Bioreagents	Cat#7365-45-9
NaCl	VWR Life Science	Cat#7647-14-5
KCl	VWR Life Science	Cat#7447-40-7
Potassium phosphate monobasic	Sigma-Aldrich	Cat#7778-77-0
Sodium phosphate dibasic anhydrous	Sigma-Aldrich	Cat#7558-79-4
EDTA	Gentrax	Cat#30-012
EGTA	bioWORLD	Cat#40520008-1
n-Propyl gallate	MP Biomedicals	Cat#102747
Glycerol	Gentrax	Cat#60-020
Methanol	VWR Life Science	Cat#67-56-1
Tris base	Apex bioresearch	Cat#18-144
Tween20	VWR Life Science	Cat#0777-1L
Triton X-100	VWR Life Science	Cat#9002-93-1
Formaldehyde	Alfa Aesar	Cat#14835
Tetramisole	Sigma-Aldrich	Cat#5086-74-8
Roche Blocking Powder	Roche	Cat#11096176001
Sodium Hydroxide pellets	Supelco	Cat#1310-73-2
Maleic Acid	Alfa Aesar	Cat#110-15-7
Critical commercial assays		
Click-it EdU Cell Proliferation Kit	Thermo Fisher Scientific	Cat#C10337
FocalCheck Fluorescent Microspheres Kit, 6 µm, mounted on slides (OPTIONAL)	Thermo Fisher Scientific	Cat#F24633
Experimental models: Organisms/strains		
C. elegans: N2 Hermaphrodite	CGC	strain: N2
C. elegans: him-8(tm611) IV Hermaphrodite	CGC	strain: CA257
Software and algorithms		
Zen Blue 2.1	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software.html
Zen Black 2.3	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software.html
Fluorender	n/a	https://www.sci.utah.edu/software/fluorender.html
ImageJ	n/a	https://imagej.nih.gov/ij/
Other		
Zeiss Stemi 305 Dissection Scope	Zeiss	Cat#435063
KimWipes	Kimtech	Cat#34155
Ice bucket	Sigma-Aldrich	Cat#BAM168072003
Microwaveable Humid Chamber (Veggie Steamer)	Progressive	n/a
Metal cooling block	Benchmark Scientific	Cat#BSW01

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Parafilm M	Genesee Scientific	Cat#16-101
Wheaton Coplin staining jars	Sigma-Aldrich	Cat#S5516-6EA
Xtreme Wear Nail Polish	Sally Hansen	(Color 109-Invisible)
Razor Blade	Personna Gem	Cat#62-0176
Vortex-Genie 2	Scientific Industries, Inc.	Cat#SI-0236
WormStuff Worm Pick	Genesee Scientific	Cat#59-AWP-B
Centrifuge- Eppendorf Centrifuge 5424-R	MilliporeSigma	Cat#EP405000042
VistaVision Histobond Microscope Slides	VWR Life Science	Cat#16004-406
Scalpel handle	Feather Brand No.3	Cat#72040-03
Surgical blade	Feather Brand No. 11	Cat#2976#11
Feather Surgical Blade Remover	Graham Field	Cat#2990
Nutator	Thermo Scientific	Cat#88882007
High Precision Microscope Cover Glasses	Deckglaser	Cat#0107052
Fisherbrand Offset Flat-Tip Forceps	Fisher Scientific	Cat#16-100-116
Flat-Top Graduated Microcentrifuge Tubes	Fisher Scientific	Cat#02-681-5

MATERIALS AND EQUIPMENT

Note: The following solutions can be made ahead of time and stored at 20°C–25°C unless otherwise noted. Solutions are good for one year at 20°C–25°C unless otherwise noted.

PBS 1% Triton X Solution (500 mL)		
Reagent	Final concentration	Amount
Phosphate Buffered Saline	1×	495 mL
Triton X-100	1% (v/v)	5 mL
Total	**n/a**	**500 mL**

Note: Triton X-100 may need to be gently nutated or rocked for up to 24 h at 20°C–25°C to fully dissolve. Store at 20°C–25°C.

M9 0.01% Triton X Solution (500 mL)		
Reagent	Final concentration	Amount
M9 Media (Stiernagle, 2006)	1×	500 mL
Triton X-100	0.01% (v/v)	50 µL
Total	**n/a**	**500 mL**

Note: Triton X-100 may need to be gently nutated or rocked for up to 24 h at 20°C–25°C to fully dissolve. Store at 20°C–25°C.

10× Egg Buffer (500 mL)		
Reagent	Final concentration	Amount
HEPES pH 7.4	250 mM	125 mL of 1M stock
NaCl	1.18 M	34.48 g
KCl	480 mM	17.89 g
EDTA	20 mM	20 mL of 0.5 M stock
EGTA	5 mM	5 mL of 0.5 M stock
dH_2O	n/a	to 500 mL
Total	**n/a**	**500 mL**

Note: Filter-sterilize and store at 20°C–25°C.

NPG-Glycerol (50 mL)	
Reagent	Amount
n-Propyl gallate	2 g
Glycerol	50 mL

Note: Place on nutator and dissolve by agitating at 40–80 rpm for at least 12 h or until fully dissolved, and store at 20°C–25°C.

2 M Tris base (not pH-adjusted) (250 mL)	
Reagent	Amount
Tris base	60.57 g
dH_2O	250 mL

Note: Store at 20°C–25°C.

10× Roche Blocking Solution (500 mL)	
Reagent	Amount
NaOH pellets/ 5 M solution	Variable
Maleic acid	0.1 M
NaCl	0.15 M
dH_2O	500 mL
Roche Blocking powder	50 g

Note: Add 250 mL dH_2O to the maleic acid powder and NaCl, stir until completely dissolved. Titrate the solution with NaOH pellets or solution until it reaches pH 7.5. Add the rest of the 500 mL dH_2O and Roche blocking powder. Autoclave with cap half unscrewed using "liquid cycle". Aliquot into 50 mL conical tubes near a flame. Store for 3 weeks at 4°C or indefinitely at −80°C freezer.

Additional Equipment and Materials: (Catalog numbers can be found in the key resources table).

Dissection stereomicroscope with oblique illumination and ~10–40× magnification, ice bucket capable of holding dry ice, humid chamber (×1), scalpel handle (×1), scalpel blade (one per slide), blade remover container, Coplin jars (at least 2; ideally 6–8), forceps, parafilm, Kimwipes, nail polish, worm pick, vortex, mini-centrifuge, nutator or rocker, vacuum trap, 1.5 mL snap-top tubes, pipettes (2 μL, 10 μL, 200 μL and 1,000 μL) and tips.

Note: The following solutions should be made on the day the solution is needed.

Dry ice (~200–500 g; obtain on day of dissection, for use in Dissection and Immunofluorescence step 7).

Egg Buffer/Tween20/tetramisole (EBT) (prepare on day of dissection, for use in step 8)	
Reagent	Amount
10× Egg Buffer	110 μL
10% Tween 20	10 μL

(*Continued on next page*)

Continued	
Reagent	Amount
10% tetramisole (stock prepared in dH_2O)	6.5 μL
dH_2O	850 μL
Total	**976.5 μL**

⚠ CRITICAL: Tetramisole is an alkaline phosphatase inhibitor and should be handled with caution using gloves.

Fix – 1% formaldehyde final (prepare on day of dissection, for use in step 8)	
Reagent	Amount
10× Egg Buffer	100 μL
37% formaldehyde	54 μL
dH_2O	846 μL
Total	**1 mL**

⚠ CRITICAL: Formaldehyde is a crosslinking agent and should be handled with caution using gloves.

1× Roche Block (prepare on day of dissection, for use in step 15)	
Reagent	Amount
10× Roche Blocking Solution	4 mL
PBST	36 mL
Total	**40 mL**

1× Click Buffer (100 μL per slide) (prepare on day of fluorescent labeling via click chemistry, step 26)	
Reagent	Amount
10× Buffer Additive	1 μL
dH_2O	9 μL
1× EdU Buffer	86 μL
$CuSO_4$	4 μL
Alexa Fluor azide	0.25 μL
Total:	**100 μL**

Note: Combine 10× Buffer Additive with dH_2O in a separate tube. Combine EdU Buffer, $CuSO_4$, and Alexa Fluor azide and mix well. Add 10× Buffer Additive to the combined tube, vortex to mix, and immediately pipette onto parafilm coverslips.

NPG-Glycerol Mounting Media (prepare day of mounting, for use in step 29)	
Reagent	Amount
2 M Tris (not PH-adjusted)	35 μL
dH_2O	15 μL
NPG-glycerol	450 μL
Total	**1 mL**

Note: Mix this solution using a P-1000 tip that has been cut with a razor blade, vortex and flick with your fingers until well-mixed, and then centrifuge for 1 min at top speed to remove air bubbles.

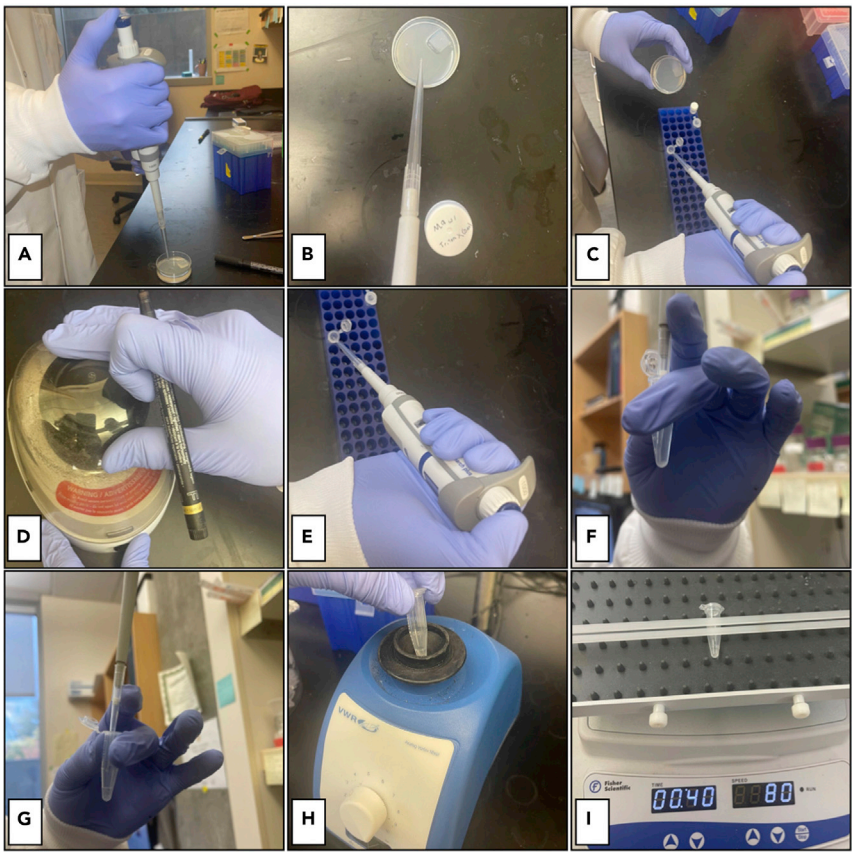

Figure 1. EdU incorporation into *C. elegans*

(A) Pipetting M9/Triton X onto a plate of L4 worms.
(B) Aspirating M9/Triton X with L4 worms.
(C) Transferring M9/Triton X and L4 worms to a fresh 1.5 mL tube.
(D) Spinning down worms.
(E) Washing once with M9/Triton X.
(F) Aspirating 40 µL of M9/Triton containing the worms into a fresh 1.5 mL tube.
(G) Adding 60 µL of 10 mM EdU.
(H) Vortexing gently to mix worms and EdU.
(I) Placing worms and EdU onto a nutator.

STEP-BY-STEP METHOD DETAILS

EdU incorporation in *C. elegans*

Timing: 1.5 h

This brief pulse of EdU is sufficient to achieve single-sister incorporation of EdU in germline nuclei. Figure 1 illustrates key steps.

1. Collect 50–100 age matched L4 or young adult (12–24 h post-L4) *C. elegans* hermaphrodites for EdU incorporation.

   Note: Chase times vary depending on the animals' age when EdU incorporation occurs and on the temperature at which the chase is conducted. The slowdown of nuclei movement in the gonad with aging means that chase times for worms incorporating EdU as L4s will be shorter

than those that incorporate EdU as young adults (~24 h post L4). Since the rate nuclei move through the gonad slows dramatically as the animal ages (Tolkin and Hubbard, 2021), an age-matched population facilitates consistent and repeatable results for the chase. Animals could be synchronized by picking L4 animals onto a new seeded plate and incubating for 12–24 h, or by bleaching gravid adults to obtain eggs (Porta-de-la-Riva et al., 2012). Further synchronization could be achieved by starving embryos to obtained synchronized L1s (Hibshman et al., 2021); however, we have found this additional step is not necessary to achieve sufficient synchronization for this protocol.

Note: In the examples shown here we incorporated EdU into N2 and *him-8* worms at the L4 stage and at 24 h post L4 (young adult), and chased them for 24, 27 and 30 h to obtain single-sister labeling at diakinesis (See expected outcome).

2. Transfer population of worms to a tube for EdU labeling.
   a. Add 1 mL M9/Triton X solution to the plate (Figure 1A), swirl gently 3–5 times and aspirate the liquid with the worms into a clean 1.5 mL tube (Figures 1B and 1C).

   Note: The small concentration of Triton X prevents worms sticking to plastic surfaces on the tube and the pipette tip with minimal effects on the worms (Peter Boag, personal communication).

   b. Spin down worms using a tabletop centrifuge for 30 s at 2,500 g (Figure 1D). Aspirate as much of the supernatant as possible without disturbing the loose worm pellet and wash an additional time with 1 mL M9/Triton X to remove excess bacteria (Figure 1E; it is not necessary to completely remove bacteria).
   c. Transfer the bottom 60 μL of M9/Triton X which contains the age-matched worms to a clean 1.5 mL tube (Figure 1F).

   Note: This ensures precise volume of liquid.

   d. Add 40 μL of 10 mM EdU in water to the worms (final concentration: 4 mM EdU in 100 μL total volume; Figure 1G).

3. Vortex gently (Figure 1H), and then transfer tube with worms to a nutator for 40 min at 20°C–25°C at 80 RPM (Figure 1I).
   a. After 40 min, wash worms 2× with 1 mL M9/Triton X (as in step 2b above).
   b. Spin down as above, and aspirate all but ~50–100 μL of solution without disturbing the loose worm pellet.
4. Transfer the ~50–100 μL of the solution containing the worms to a fresh NGM plate and let dry for 10–30 min or until all liquid has been absorbed. Plates can be left open in a chemical hood to facilitate drying.
5. Transfer plates to an incubator set to the desired temperature and note time as the beginning of the chase. Troubleshooting 1.

⚠ CRITICAL: Chase times throughout this protocol begin AFTER EdU incorporation is complete (i.e., if worms incorporate EdU for 40 min starting at 10:00 AM, then the chase time starts at 10:40 AM).

Chase times are highly dependent on age and rearing temperature. Visualization of labeled single sister at diakinesis is generally possible with ~27 h chase for wild type (N2) worms that incorporate EdU as L4s and chased at 20°C. Longer chase time are required when EdU is incorporated to adults compared to L4s, and when worms are grown at 15°C compared to 20°C. Troubleshooting 2.

Dissection, fixation, and immunofluorescence of *C. elegans* gonads

Timing: 5–6 h

Adapted from (Phillips et al., 2009). *Consult the protocol for additional explanations and usage notes.*

6. Make EBT, Fix and Roche Blocking solutions fresh.

⚠ CRITICAL: Formaldehyde is a crosslinking agent. Prepare Fix solution using gloves in a well-ventilated area or a fume hood.

7. Place a Coplin jar with methanol in a −20°C freezer and place a metal block on dry ice. Prepare a humid chamber (see step 20 below).

⚠ CRITICAL: Methanol is a crosslinking agent. Use gloves when handling methanol.

Note: Label all HistoBond slides with pencil, as methanol will dissolve ink-based marks.

8. Dissect age-matched adults.
   a. Place a coverslip on top of a glass slide under a dissection microscope (Figure 2A). Fit a scalpel blade into a scalpel holder (Figure 2B).

⚠ CRITICAL: Scalpel blades are very sharp and should be handled with caution.

   b. Pipette 30 μL of EBT onto the coverslip (Figure 2C). Pick 10–30 worms into the drop of EBT (Figure 2D) and allow 30–60 s for worms to anesthetize.
   c. Cut the heads and/or tails off the worms to extrude the gonads (Figure 2E).
   d. After all animals are dissected, pipette 30 μL of Fix solution into the drop of dissected worms (Figure 2F).
   e. Pipette up and down 3–5 times to mix the solutions and help release more gonads.
   f. Leave the Fix solution on for 1 min.

9. Aspirate excess liquid, leaving as little as possible remaining (~15 μL) while trying to keep the worm carcasses separated from one another (Figure 2G).
10. Pick up the drop by lightly touching a HistoBond (75 × 25 × 1 mm) microscope slide on top of it (Figure 2H).

Note: Try to get as many worm carcasses as possible in the middle of the slide, avoiding the edges.

11. Freeze the sample by placing it on the ice block for >1 min (Figure 2I). Ice crystals will form between the slide and the coverslip. Slides can be kept on ice until all samples are dissected (~30–60 min).
    a. Repeat steps 8–11 until all samples are dissected.
12. Freeze-crack: Carefully flick off the cover slip by inserting a razor blade under the edge of the coverslip (Figure 2J).

⚠ CRITICAL: Coverslips can shatter during freeze crack. Glass shards are very sharp and should be handled with care. Collect all broken coverslips, and carefully place in the glass waste.

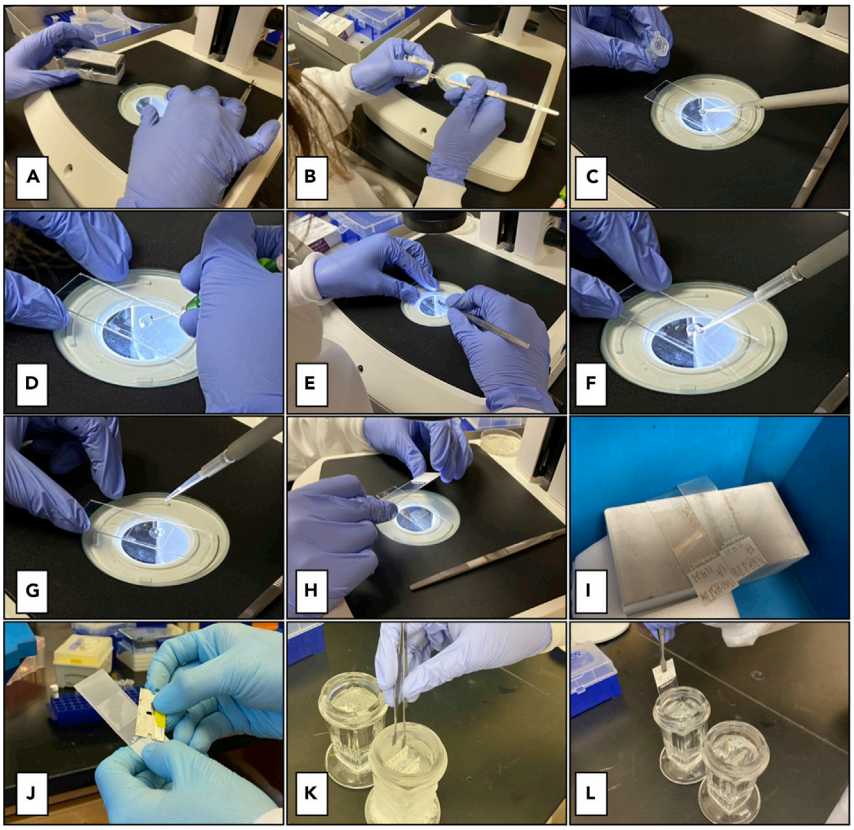

Figure 2. Dissection and Fixation of *C. elegans* gonads

(A) Placing a 22 × 22 mm coverslip onto a transparent glass slide on a dissecting stereo microscope.
(B) Attaching a fresh blade to a scalpel handle (Caution - blades are very sharp!).
(C) Adding EBT solution to the center of the coverslip.
(D) Transferring worms by picking into the EBT solution.
(E) Dissecting *C. elegans* gonads by using the scalpel to remove the head or tail of the animal.
(F) Adding fix solution to the worms in EBT.
(G) Aspirating as much liquid as possible (~45 μL), being careful not to aspirate the worms.
(H) Gently touching the bonded side of the slide to the coverslip - the coverslip and worms should stick to the Histobond slide.
(I) Transferring the slide, coverslip facing up, onto an ice block cooled on dry ice.
(J) Using a razor blade, flicking off the coverslip by inserting the blade under a corner and gently prying.
(K) Transferring the slide into 100% methanol cooled to −20°C.
(L) Transferring slides to a fresh Coplin jar of PBST.

13. Place the slide immediately in −20°C methanol (Figure 2K), incubate for one minute, and then transfer the slides to a fresh Coplin jar of PBST at 20°C–25°C using forceps (Figure 2L).
    a. Repeat steps 12–13 until all samples are in PBST.

Note: Multiple slides could be placed in a single Coplin jar. However, if the slides touch each other, the worm carcasses might be washed off. Make sure each slide uses separate railings in the Coplin jar.

14. Wash slides three times in PBST (5–10-min per wash; Figure 2L).
15. Block slides in 1× Roche Block in a Coplin jar for 30 min to 2 h at 20°C–25°C, or 12–16 h at 4°C. Save 100 μL/slide for the 1° antibody solution buffer (e.g., 3 slides = 300 μL; Figure 3A).

Alternatives: Blocking can also be done in Normal Goat Serum (NGS 1:10 in PBST) or BSA (0.5% in PBST); however, these solutions should be assessed for compatibility with each antibody.

16. Primary Antibody (1°): Prepare antibody solution in Roche block (from step 15).
    a. Mix by flicking the tube or by gently pipetting up and down. DO NOT VORTEX.

Note: For the examples shown here, we have used a guinea pig antibody against HTP-3 at 1:500 concentration. For conventional applications, up to two different antibodies could be used, with one channel used for EdU labeling (in the examples shown here, 488), and 405 for DAPI.

17. Prepare parafilm squares slightly thinner than the width of the slides. Apply 100 μL of 1° antibody solution to the parafilm; avoid forming bubbles (Figure 3B).
18. Carefully wipe the slides (avoid touching the worm carcasses; Figure 3C). Make sure the area containing the carcasses never gets dry.
19. Touch the slide face down to the parafilm slip with antibody, ensuring all carcasses are immersed in liquid (Figure 3E).
20. Incubate in a humid chamber for 2 h at 20°C–25°C, or 12–16 h at 4°C (Figure 3D and 3F).
21. Remove the parafilm squares by carefully dipping each slide in a Coplin jar of PBST and letting the parafilm square float off (Figure 3G). Pick the parafilm squares with forceps and discard.

Note: Do not drag the parafilm squares on the railings while you place the slide in the Coplin jar. This might release the gonads from the slide.

22. Wash slides three times (10 min/wash) by moving slides to fresh Coplin jars of PBST.

23. Secondary Antibody (2°): Prepare secondary antibody solution in PBST.

Note: For the images shown here, we used a Cy3 anti-guinea pig secondary at 1:500 in PBST. For conventional applications, two antibodies could be used, with the EdU occupying one channel (in our case, 488), and the DAPI utilizing the 405 channel. Mix by gently flicking the tube or pipetting up and down. DO NOT VORTEX.

    a. Repeat steps 17–19 with secondary antibody in PBST.

24. Remove the parafilm by floating, and wash in a fresh Coplin jar of PBST for 10 min at 20°C–25°C.

Note: It is typical for some worm carcasses to be washed off during wash steps. To reduce the number of worms lost, be sure to aspirate as much liquid as possible between dissection and freezing (step 9). This can help the worms stick better to the slides. In addition, make sure the slides do not rub against each other or against the sides of the Coplin jar, and that the parafilm does not drag on the rails of the Coplin jar (steps 21 and 24). Troubleshooting 3.

Fluorescent labeling of EdU via click chemistry

Timing: 1–2 h

Reaction with an azide-containing fluorophore and $CuSO_4$ allows for rapid and specific labeling of DNA strands containing EdU by covalently linking the fluorophore to the DNA. A crucial advantage compared to using 5-bromo-2′-deoxyuridine (BrdU) is that access to the alkene group on EdU does not require denaturing of the DNA.

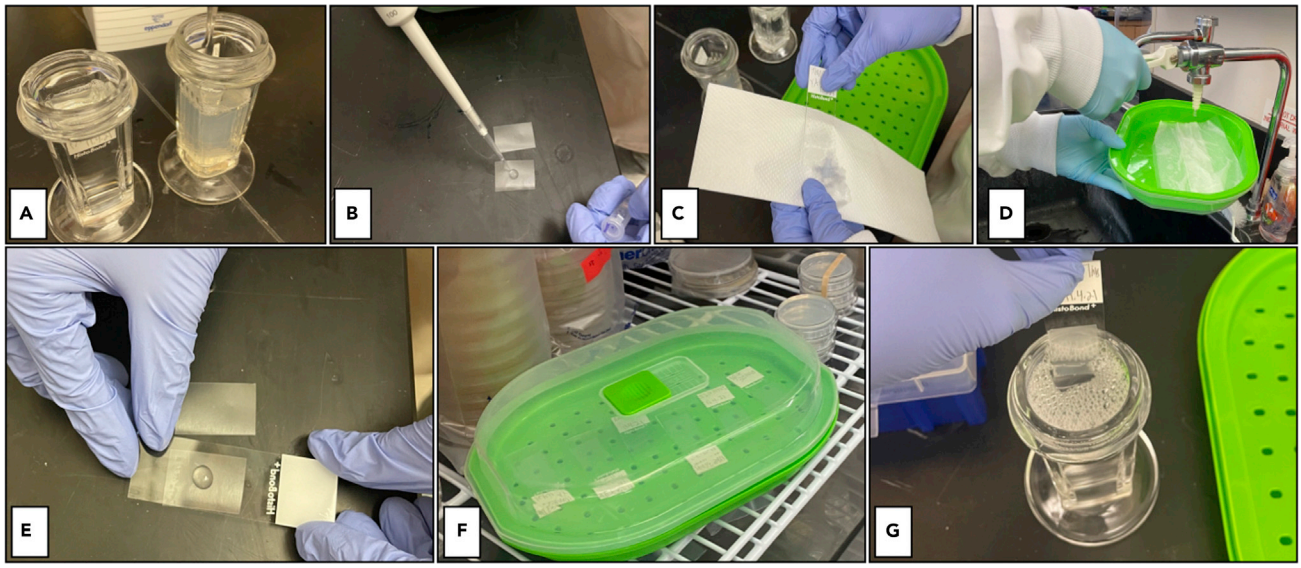

Figure 3. Antibody staining of *C. elegans* gonads

(A) Blocking slides in 1× Roche Block.

(B) Adding 100 μL of primary antibody solution to a parafilm square (one square per slide).

(C) Removing slide from PBST and gently drying the back of the slide, taking care not to disturb the worms stuck to the front of the slide.

(D) Preparing the humid chamber by wetting a paper towel and placing it on the bottom of a perforated chamber with a lid.

(E) Gently touching the slide (worms down) to the primary antibody. The parafilm should stick to the slide.

(F) Incubating the slides, parafilm side up, in a humid chamber with the lid closed.

(G) Transferring slides to a fresh, fully filled Coplin jar of PBST, allowing the parafilm squares to float to the top. Steps depicted in panels (B–G) are repeated for the secondary antibody labeling and click chemistry steps.

⚠ CRITICAL: Always perform click reaction after primary and secondary antibody labeling. Reaction with $CuSO_4$ has the potential to damage epitopes and inhibit antibody labeling. Troubleshooting 4.

25. Transfer slides to a fresh Coplin jar of PBS with 1% Triton X (v/v) for 30 min at 20°C–25°C.

Note: We found that this permeabilization step helps with the click reaction, likely by facilitating the entry of the click chemistry reagents into the dissected gonad.

26. Prepare the Click-iT™ reagents fresh for each experiment according to the kit's specifications.
27. Apply 100 μL of Click-iT™ solution onto fresh parafilm squares and gently touch the slides to the liquid as shown in Figure 3E. Troubleshooting 5.
    a. Place in humid chamber for 30 min at 20°C–25°C.
    b. Float parafilm squares off and then wash slides twice for 5 min in PBST.
28. DNA labeling with DAPI. Add DAPI (Stock: 5 mg/mL in H_2O; 1:10,000 dilution for 0.5 μg/mL final concentration) to a Coplin jar of PBST and mix gently.
    a. Place slides in the DAPI-containing Coplin jar and incubate for 20 min at 20°C–25°C.
    b. Wash slides for 5 min in PBST.
29. Mount slides using either NPG-glycerol (see Solutions, above) or Prolong Glass Antifade Mounting Media.
    a. If using NPG-glycerol:
        i. Pipette ~11 μL of NPG-glycerol (you may have to cut the pipette tip - the NPG-glycerol is viscous) onto a high-Performance coverslip (Figure 4A).
        ii. Dry the slide as much as possible using a paper towel to aspirate liquid around the worms (do not touch the worm carcasses!), and then gently touch the slide to the mounting media (Figure 4B).

iii. Remove excess mounting media using a pipette tip connected to a vacuum trap (Figure 4C).
iv. Seal with nail polish (Figure 4D).

b. If using Prolong Glass:
i. Place a small drop of mounting media onto a high-Performance coverslip (Figure 4A).
ii. Dry the slide as much as possible using a paper towel to aspirate liquid around the worms (do not touch the worm carcasses!), and then gently touch the slide to the mounting media (Figure 4B).
iii. Cure for 24 h at 20°C–25°C by placing in a dark box or drawer before sealing with nail polish (Figure 4E).

30. Store slides in a covered container at 4°C or −20°C (Figure 4E).

Pause point: Generally, slides can be stored in PBST at any point during the IF or click reaction for up to 48 h (essentially extending any of the PBST washes, e.g., steps 14, 22, 24 or 27B). After mounting and sealing, slides can typically be stored covered for 60 days at 4°C or −20°C with minimal loss of signal.

Visualization of labeled *C. elegans* gonads

Timing: 1–2 h

Imaging was done using a Zeiss LSM880 confocal microscope equipped with AiryScan and running ZEN Black 2.3, as described below. However, various other microscopes or imaging modalities such as widefield microscopes are also compatible with this protocol.

31. Using a standard dissection microscope, place the slide coverslip down by propping it on two slides. Locate the gonads (since the gonads are transparent, tilting the oblique illumination will help) and mark each gonad with a dot or a circle using a black pen.

Note: This will help locating the sparse gonads when placed under a high-magnification objective on the confocal microscope (Figures 5A and 5B).

32. Mount slides on the microscope and locate samples using the black dots. Locate the diakinesis stage, which is located on the proximal end of the gonad. In the DAPI channel, this region of the gonad is occupied by large oocytes that appear as mostly dark ovals with a few bright bodies clustered together (see expected outcome; Figure 8).

Note: It is useful to also check the EdU channel by eye to ensure that your samples are correctly labeled (Figure 5C).

33. Image the diakinesis nuclei by creating a z-stack, covering a volume of about 6–7 μm in depth.

Note: Diffraction-limited microscopy will be required to observe single sister chromatids. We have used a confocal microscope, Zeiss LSM880 in an AiryScan mode. However, similar results could be obtained with other confocal or widefield microscopes capable of capturing diffraction-limited images. Troubleshooting 6.

Note: For images taken with the LSM880 in AiryScan mode, typical acquisition parameters are:

a. Pixel size = 0.04 μm.
b. Pixel dwell = 0.75–1.00 μs.
c. Z-spacing = 0.159 μm.

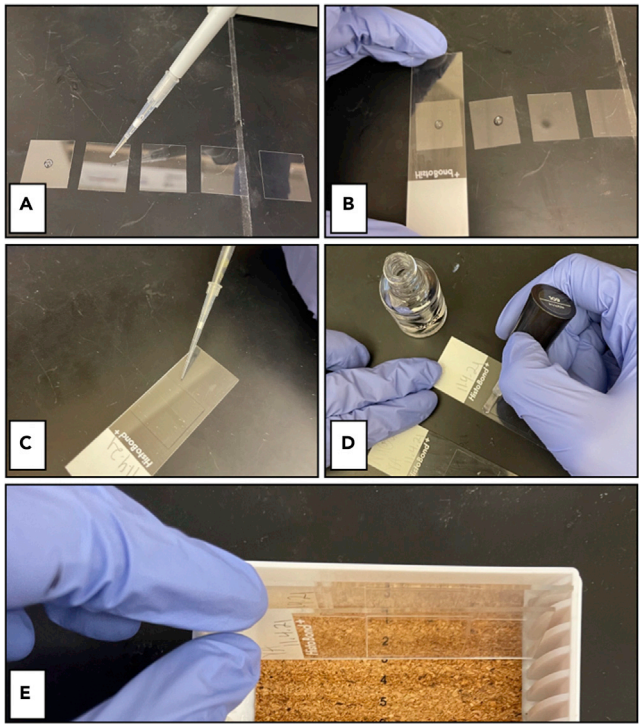

Figure 4. Mounting and sealing

(A) Adding 1 drop (~12 μL) of Prolong Glass or NPG-Glycerol to a fresh coverslip.

(B) Gently touching the samples to the mounting media on the coverslip.

(C) If using NPG-Glycerol, aspirating excess mounting media using a pipette tip connected to a vacuum trap.

(D) Sealing the slides with nail polish.

(E) Storing slides covered at 4°C or −20°C before imaging.

34. Repeat steps 31–33 until all gonads are imaged.
35. Process the images per the recommended practices.
    a. On the LSM880 this includes 'Airy Processing' with default parameters.
    b. For widefield microscopy processing will likely involve deconvolution.

EXPECTED OUTCOMES

Successful labeling of a single sister is easily apparent in the diakinesis stage of meiosis, where chromosomes are condensed in preparation to the meiotic divisions (roughly equivalent to mitotic metaphase chromosomes). Homologous chromosomes that have undergone a crossover appear as bivalents: elongated DAPI bodies, with the axis marker HTP-3 forming a cruciform (Figure 6A); each sister manifests cytologically as quadrant of the structure. If a chromosome has not undergone a crossover, it appears as a univalent: an oval body, with a single line of HTP-3 staining running through its middle (Figure 6B); each sister occupies half of the oval.

Single sister labeling occurs when premeiotic nuclei undergo a single round of DNA replication with EdU present, followed by two or more rounds of DNA replication without EdU before entry into meiosis (see Figure 9 for details). Our protocol is designed to expose the worms to EdU for a single S phase, after which all replicating sister chromatids contain an EdU-incorporated strand. Visualization of single sister labeling requires at least one additional round of DNA replication, which could be pre-meiotic DNA replication. Due to the nature of DNA replication, the EdU containing strand is not lost (Meselson and Stahl, 1958); therefore, additional rounds of mitotic DNA replication prior to

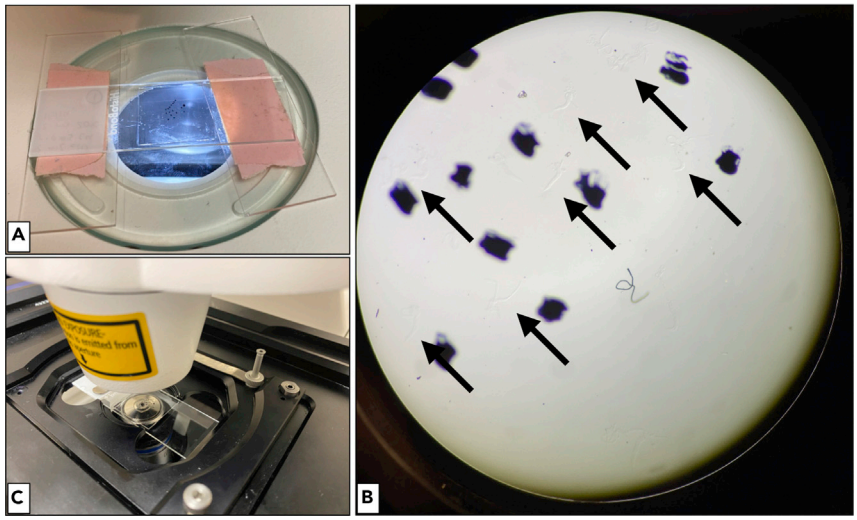

Figure 5. Imaging *C. elegans* gonads stained for EdU

(A) Placing slide coverslip down onto a dissecting scope, between two clear slides with tape.

(B) Locating the worm carcasses (black arrows) and marking them with a nearby dot.

(C) Mounting slide coverslip down onto your imaging platform. Use the black dots to help locate the sample and image.

meiotic entry will still yield single sister labeling, although the number of EdU-containing DNA strands in a nucleus will stochastically diminish.

Nuclei that undergo EdU incorporation in pre-meiotic S phase will have EdU signal on all four sisters of each bivalent (Figure 9, top). Nuclei that incorporated EdU during a mitotic S phase prior to meiotic entry will have two of the four sisters with EdU signal (Figure 9, middle). Nuclei that undergo two or more S phases after EdU incorporation will have single sisters labeled with EdU on variable number of chromosomes (Figure 9, bottom), since EdU labeled chromatids segregate randomly during the mitotic divisions. Therefore, a bivalent might have only one out of four sisters labeled (see example in Figure 6C).

To visualize single sisters at diakinesis, chase times must be optimized such that diakinesis nuclei in the -3, -2 and -1 positions have undergone at least one additional S phase without EdU after EdU incorporation (e.g., a mitotic S-phase in the presence of EdU and pre-meiotic S-phase during the chase in the absence of EdU). In cases where there are no properly labeled chromosomes in diakinesis, chase time should be increased one hour at a time until EdU labeling is present in the -3, -2, and -1 nuclei of the gonad. Similar approach should be taken if in diakinesis all four sister chromatids are labeled. Notably, since meiotic progression in the *C. elegans* gonad is semi-synchronous (Crittenden et al., 2006; Fox et al., 2011), it is expected that even when using an ideal chase time, only a subset of nuclei in a subset of gonads will show single sister labeling. We have noticed a strong age-related slowing of the progression of EdU containing nuclei in both wild type and *him-8* animals, which do not pair the X chromosome (Figures 10 and 11). Therefore, careful age matching is necessary to ensure reliable and replicable results.

Another reason for incomplete synchrony between nuclei is that the X chromosome replicates later than the autosomes in the gonad (Jaramillo-Lambert et al., 2007; Mlynarczyk-Evans and Villeneuve, 2017). Therefore, when labeling with a pulse of EdU, multiple outcomes are possible when visualizing diakinesis nuclei. There will be a population of nuclei in which the X chromosome is the only chromosome labeled (two of 24 sister chromatids; Figure 7, yellow circles). This occurs when a nucleus is late in S phase at the time of the EdU pulse and the autosomes have finished replicating

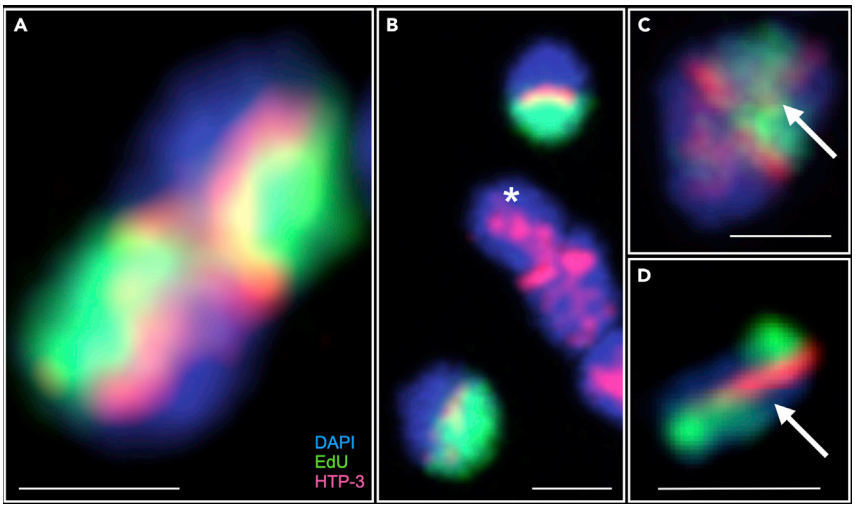

Figure 6. Single sister labeling in *C. elegans* chromosomes

Chromosome axis (HTP-3) is labeled in red, EdU is in green and DAPI in blue.

(A) A bivalent chromosome with two EdU containing strands (one per sister pair).

(B) Two univalent chromosome with two EdU containing sisters (one per sister pair). Unlabeled autosome is marked with an asterisk.

(C) An EdU containing sister that underwent an SCE (white arrow) in a bivalent chromosome.

(D) A univalent chromosome with an EdU containing sister that underwent an SCE (white arrow).

Scale bars = 1 μm. Reprinted with permission from (Almanzar et al., 2021).

before the EdU is added. These nuclei are found most proximally in the gonad. In contrast, when a nucleus just entered S phase at the time of the EdU pulse, only the autosomes will incorporate EdU (10 of 24 sister chromatids; Figure 7, red circles), as the EdU is no longer present when the X replicates. These nuclei are found most distally among labeled nuclei. The population of nuclei in which 12 of 24 chromatids (all six chromosomes are labeled) are found in between these two populations (Figure 7, green circles). The mitotic cell cycle in the gonad is estimated to last between 6.5–8 h, with S phase occupying 50%–57% of the total length of the cell cycle (Crittenden et al., 2006; Fox et al.,

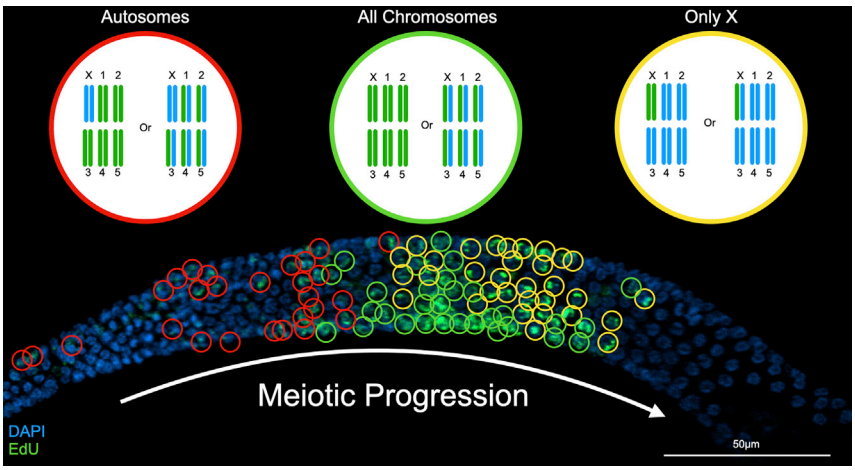

Figure 7. EdU incorporation patterns in the *C. elegans* gonad

EdU can be incorporated into only the X chromosome (yellow circles), all six chromosomes (green circles), or only the autosomes (red circles), depending on the time when EdU is present during S phase. The nuclei are circled using the same color scheme to indicate their relative location in the gonad. Bottom, a gonad dissected after five hours of chase at 20°C from an N2 animal labeled as a young adult.

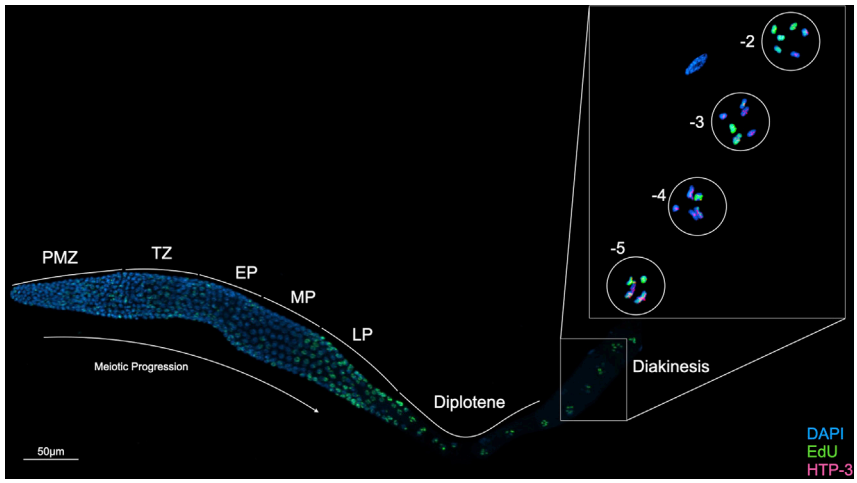

Figure 8. Single sister labeling in diakinesis

Gonad zones are labeled: PMZ, premeiotic zone; TZ, transition zone; EP, Early Pachytene, MP, Mid-Pachytene, LP, Late Pachytene, Diplotene, and Diakinesis (square). Inset, magnification of the -2, -3, -4, and -5 nuclei indicate all are successfully labeled with EdU. Numbering is indicative of the position relative to the fertilized oocyte, i.e., -3 is two nuclei before the terminal diakinesis nucleus. Note that each nucleus contains a different number of EdU labeled chromosomes. Axis (HTP-3) is in red (omitted in the zoomed-out image for clarity), EdU is in green and DAPI in blue.

2011). We have determined that 40 min of EdU exposure is sufficient to produce a population of nuclei in which many DNA strands have incorporated EdU, but short enough to ensure EdU is not present for more than one S phase.

Single sister EdU labeling of diakinesis nuclei allows for quantification of reciprocal exchanges between sister chromatids (sister-chromatid exchanges, or SCEs). To visualize SCEs, both sisters must be oriented perpendicular to the angle of imaging, and there must be a robust marker delineating the individual sisters (i.e., an axis marker such as HTP-3). Reciprocal exchanges between labeled and unlabeled chromatin (see examples in Figure 6) are scored as SCEs. This system is advantageous in that it can be used to visualize SCEs on chromosomes with or without crossovers. In our analysis, we have found meiotic SCEs to be rare in wild-type worms, with only ~4% chromatids undergoing an SCE. Therefore, worms that make abundant SCEs, such as *him-6* mutant worms, may be used as a positive control for experiments quantifying SCEs (Almanzar et al., 2021). Finally, by utilizing mutants that fail to make programmed double strand breaks in meiosis (e.g., *spo-11;* (Dernburg et al., 1998)), it is possible to quantify mitotic SCEs relative to the number of double strand breaks by introducing exogenous breaks with carefully timed ionizing radiation (Almanzar et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

Images were analyzed using the ZEN Blue software package version 2.1. Distance of EdU traveled was quantified in animals grown at 20°C and dissected at either 24-, 27-, or 30-h post EdU introduction. Distance traveled was quantified by observing the furthest meiotically progressed nucleus that contained EdU signal. SCEs can be scored when the EdU labeled chromatid crosses the inter-sister interface (labeled with anti-HTP-3), creating a reciprocal exchange of EdU-containing chromatin. Exchanges are scored only between chromatids that could be clearly distinguished in the XY plane. See (Almanzar et al., 2021) for additional details on quantification and analysis of SCEs.

LIMITATIONS

Successful visualization of single sister chromatids depends on both the orientation and morphology of the chromatid in question, and an effective chase (see expected outcomes above). If the

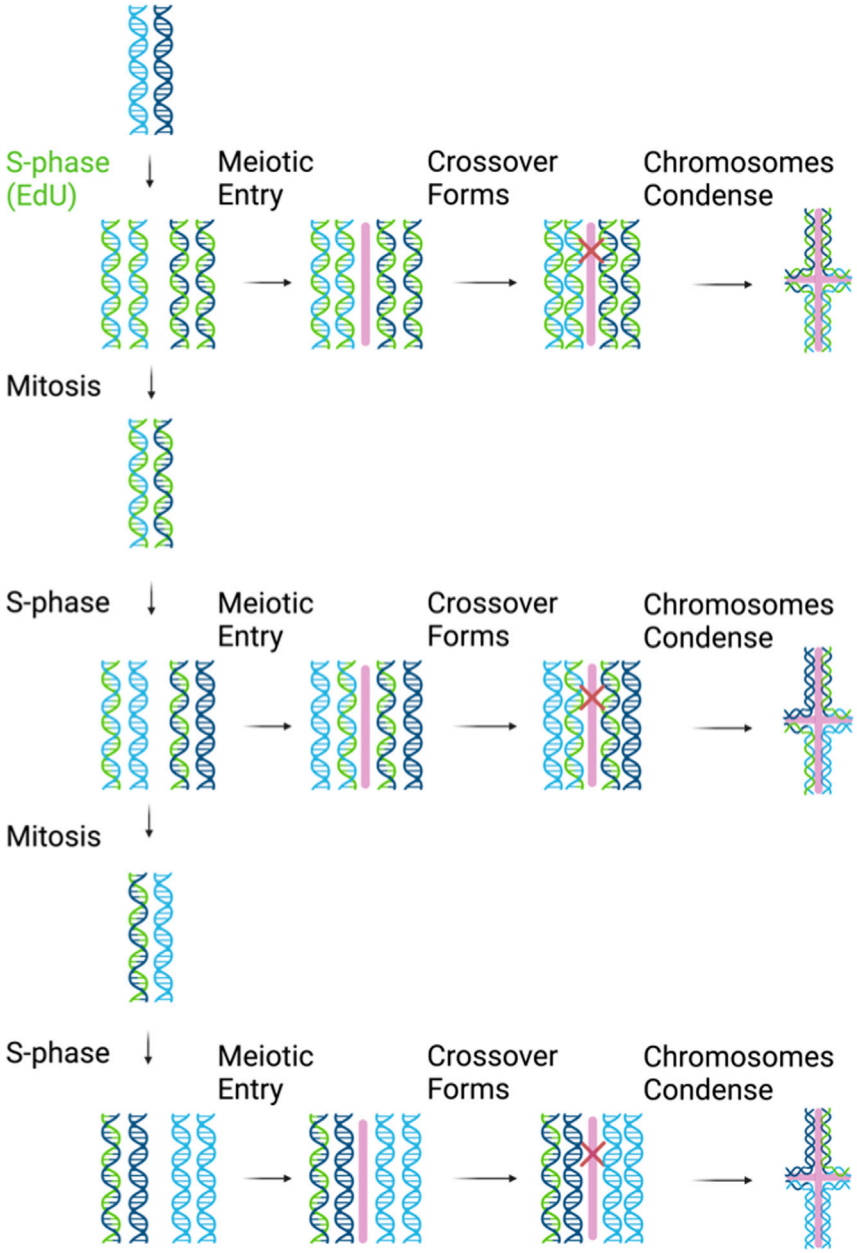

Figure 9. Schematic of pulse/chase experiment

Each vertical section represents an additional S phase (mitotic or pre-meiotic). Top, EdU incorporation results in all sisters containing EdU. Middle, following an additional S phase without EdU reduces the number of EdU containing sisters to two out of four per chromosome, allowing for quantification of reciprocal exchanges between labeled and unlabeled chromatin. Bottom, an additional S phase without EdU before meiosis further reduce the number of EdU containing sisters.

chromatid is oriented such that the axis is not perpendicular to the microscope objective, quantification will usually not possible. However, utilizing software that can produce a 3D render of the imaged volume such as Imaris (https://imaris.oxinst.com) or FluoRender (https://www.sci.utah.edu/software/fluorender.html) can help with scoring chromosomes that are slightly off axis or resolve chromosome that are near each other. The semi-synchronous passage through the mitotic cell cycles and entry to meiosis entails that only a subset of nuclei is labeled. As noted above,

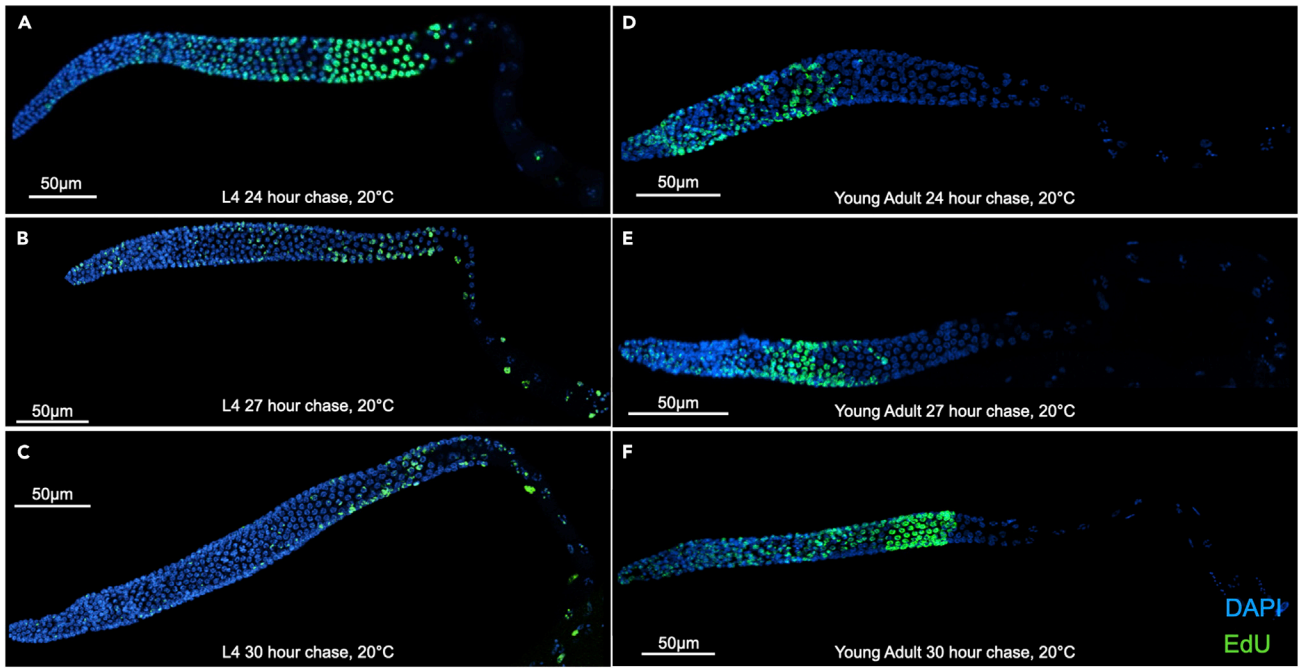

Figure 10. EdU progression after various of chase times

(A–C) N2 worms incorporated EdU as L4 and dissected at 24-, 27-, and 30-h after EdU incorporation.

(D–F) Worms incorporated EdU as young adults (24 h post-L4) and dissected at 24-, 27- and 30-h after EdU incorporation.

Note the progression of EdU signal in the gonad, and the dramatically more proximal signal in worms incorporating EdU as L4s versus young adults.

many times only the X chromosome or only the autosomes are labeled (see expected outcomes). In a typical experiment, we were able to score 20%–30% of the chromosomes that were single sister labeled. While there is obviously a minimum size of reciprocal exchange that could be scored, we have successfully scored SCEs along >80% of chromosome lengths (Almanzar et al., 2021). Finally, if a mutant strain results in severe defects in chromosome morphology – i.e., meiotic axis mis-localization, severe chromosome fragmentation, or incomplete chromosome condensation – visualization of single sisters might prove too difficult.

TROUBLESHOOTING

Problem 1

Worms starve before chase time is complete.

Potential solution

For long chase times, large numbers of EdU-incorporated animals could consume all the bacteria on the growth plate before chase is complete. One potential solution is to split the EdU-incorporated worms onto multiple plates or onto a larger plate. If splitting between plates, mix the worms by gently pipetting up and down, and then alternate drops between two (or more) plates. An alternative solution is to add concentrated bacteria to the plate after 1–2 days.

Problem 2

Single sister labeled chromosomes are not located at the correct stage of meiosis (examples in Figures 10A and 10D–10F; for equivalent successful outcomes see Figures 10B and 10C).

Potential solution

Optimal chase times vary with age and temperature, but for incorporation of EdU in L4 worms, 27–30 h at 20°C or 24–27 h at 25°C is generally a good starting point for labeling. If diakinesis nuclei are

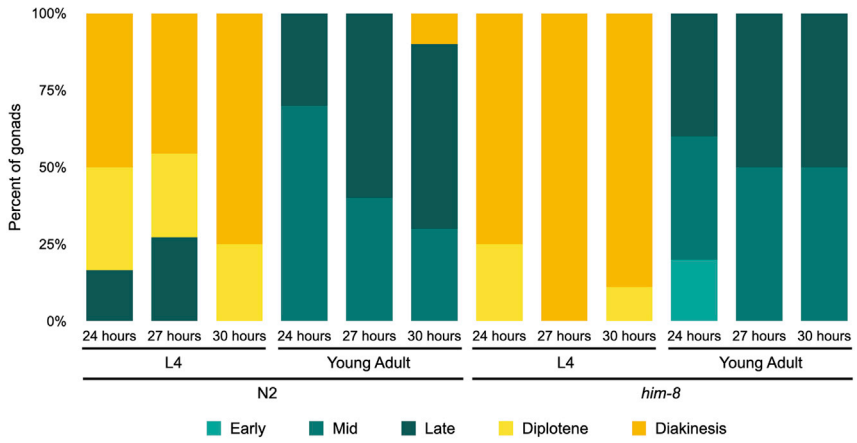

Figure 11. Quantification of EdU progression after various of chase times

Distribution of EdU containing nuclei in L4 and young adult N2 and *him-8* worms at different times of dissection. The furthest EdU labeled nucleus in each gonad was used to define the stage in meiotic prophase reached by nuclei. The y axis represents percentage of total gonads per experimental group that were labeled in that region. Note the more proximal movement in L4 relative to the young adults in both the N2 and *him-8* backgrounds. At least 6 gonads were analyzed in each timepoint.

fully labeled (i.e., all four sister chromatids have EdU signal), or if only the X chromosomes are labeled, then chase time should be increased one hour at a time until single sister labeling is achieved. For temperature-sensitive mutants that need to be grown at 15°C, chase times of 48 h or more may be required to achieve single sister labeling in diakinesis.

Problem 3

Many worms are dissected, but only a few gonads remain after IF and click chemistry.

Potential solution

After dissection and fixation and right before placing on dry ice, be sure to aspirate as much liquid as possible. Minimize storage of the slides in PBST outside of normal wash steps as excessive storage in PBST can cause the gonads to detach from the slides. Take care when floating parafilm coverslips off and when transferring slides between Coplin jars so as not to perturb the gonads.

Problem 4

IF signal is diminished when combined with EdU labeling.

Potential solution

The click reaction solution contains components that have the potential to affect or alter epitopes, thereby reducing the affinity of certain antibodies. To avoid this, click reaction should always be carried out AFTER all immunofluorescence steps have been completed. We have not noticed any negative effects of the click reaction on the distribution or signal strength when all immunofluorescence steps are carried out before click reaction.

Problem 5

EdU signal is weak or not present after click reaction.

Potential solution

EdU penetrance into the gonad should not be a problem when dissolved to 4 mM in H_2O and incubated for >40 min at 20°C–25°C. Repeating the click chemistry step an additional time before DAPI labeling can sometimes increase label strength if signal is faint. According to the protocol, the click reaction terminates after 30 min, so leaving the click reagents on for additional time is unlikely to improve labeling.

To rule out problems stemming from the microscope (e.g., weak illumination), it is recommended to attempt imaging the samples on a different microscope or use the same fluorophore to label a known entity (e.g., if Alexa 488 azide fails to label EdU, use a secondary antibody conjugated to Alexa 488 in conjunction with a well-characterized antibody, like the one against HTP-3).

Problem 6

EdU and DAPI signal do not completely overlap.

Potential solution

The expected result is for the EdU label to be completely included within the DAPI signal. Misalignment between the visible and invisible channels can sometime occur. This can be corrected for by utilizing alignment beads (e.g., FocalCheck Fluorescent Microspheres Kit). We have had success in mixing the beads in with our mounting media and using the beads as a fiducial to correct for misalignment. Obvious image misalignment could also be corrected using automatic channel alignment in Zen Blue or ImageJ.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ofer Rog (ofer.rog@utah.edu), or by the technical contact, David Almanzar (david.almanzar@utah.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

D.E.A. developed and optimized the protocol. D.E.A. and A.H. performed additional experiments. D.E.A., A.H., and O.R. wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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