Chapter 5

Analysis of Meiotic Sister Chromatid Cohesion in *Caenorhabditis elegans*

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Abstract

In sexually reproducing organisms, the formation of healthy gametes (sperm and eggs) requires the proper establishment and release of meiotic sister chromatid cohesion (SCC). SCC tethers replicated sisters from their formation in premeiotic S phase until the stepwise removal of cohesion in anaphase of meiosis I and II allows the separation of homologs and then sisters. Defects in the establishment or release of meiotic cohesion cause chromosome segregation errors that lead to the formation of aneuploid gametes and inviable embryos. The nematode *Caenorhabditis elegans* is an attractive model for studies of meiotic sister chromatid cohesion due to its genetic tractability and the excellent cytological properties of the hermaphrodite gonad. Moreover, mutants defective in the establishment or maintenance of meiotic SCC nevertheless produce abundant gametes, allowing analysis of the pattern of chromosome segregation. Here I describe two approaches for analysis of meiotic cohesion in *C. elegans*. The first approach relies on cytology to detect and quantify defects in SCC. The second approach relies on PCR and restriction digests to identify embryos that inherited an incorrect complement of chromosomes due to aberrant meiotic chromosome segregation. Both approaches are sensitive enough to identify rare errors and precise enough to reveal distinctive phenotypes resulting from mutations that perturb meiotic SCC in different ways. The robust, quantitative nature of these assays should strengthen phenotypic comparisons of different meiotic mutants and enhance the reproducibility of data generated by different investigators.

Key words Cohesin, Meiosis, Gametogenesis, Kleisin, Chromosome segregation, Aneuploidy, rec8, coh-3, coh-4, Rad21L

1 Introduction

During mitosis and meiosis, sister chromatid cohesion (SCC) tethers replicated sister chromatids and is critical for accurate chromosome segregation. Sister chromatid cohesion is mediated by a widely conserved protein complex called cohesin. Pioneering work in yeast demonstrated that mitotic and meiotic cohesins differ in a key subunit, the α-kleisin, and that changing this one subunit is critical to establishing the distinct mitotic and meiotic patterns of chromosome segregation [1]. Meiotic cohesin complexes associate with the α-kleisin Rec8, and Rec8 is cleaved in two steps by the

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cysteine protease separase to allow the separation of homologous chromosomes in anaphase of meiosis I and of sister chromatids in anaphase of meiosis II [2–4]. Mitotic cohesin complexes associate with the α-kleisin Scc1 (also called Mcd1 and Rad21) [5–7]. During mitosis, a single round of separase-dependent cleavage removes Scc1 cohesin, allowing sisters to separate [8].

Our work in *C. elegans* demonstrated that kleisins other than REC-8 are essential for normal gametogenesis. Two nearly identical and functionally redundant α-kleisins, called COH-3 and COH-4 (hereafter, COH-3/4) act together with REC-8 to mediate meiotic SCC [9]. Remarkably, the kleisin subunit influences nearly every facet of meiotic cohesin function [10]. The kleisin specifies the mechanisms that promote the stable binding of cohesin to chromosomes and that trigger cohesin to establish SCC. The kleisin determines whether a complex promotes sister chromatid co-orientation, the process that ensures the attachment of sister chromatids to microtubules from the same spindle pole in meiosis I. Finally, the kleisin determines whether a cohesin complex may persist on chromosomes after anaphase of meiosis I to hold sisters together until anaphase of meiosis II [10]. It has since been shown that multiple, functionally specialized kleisins are required for meiosis in mammals and plants, indicating that the involvement of multiple kleisins in gametogenesis is widely conserved [11–16].

Given the complex nature of meiotic sister chromatid cohesion in higher eukaryotes, it is critical to develop reliable and quantitative methods to characterize defects in the establishment or maintenance of SCC and to determine the consequences of cohesin misregulation on the reduction of ploidy. Here, I describe two such methods. The first is a cytological method that allows direct visualization of SCC defects. The second is a molecular approach to identify embryos with abnormal chromosomal content formed as a consequence of errors in meiotic chromosome segregation. Because this technique allows the analysis of a large number of embryos, the nature of the chromosome segregation defect can often be inferred from the population averaged pattern of chromosomal inheritance. In the remainder of this section, I introduce these methods and discuss their utility for studies of meiotic SCC.

The ability to uniquely mark a single chromosome with a fluorescently labeled, sequence-specific DNA binding protein revolutionized studies of nuclear architecture and chromosome segregation [17]. The most widely used implementation of this technique relies on the binding of a Green Fluorescent Protein—Lac Repressor fusion protein (GFP-LacI) to a chromosomally integrated array of 256 tandem repeats of *lac operator (lacO)* DNA [18–20]. The high affinity of GFP-LacI for *lacO* DNA results in the specific accumulation of GFP fluorescence at the *lacO* array. A number of researchers have implemented the GFP-LacI/*lacO* system in *C. elegans* (*see* ref. 21 for a thorough review). GFP-LacI can

be expressed from a transgene that is integrated either together with the *lacO* array or into a different chromosomal site [22–24]. Both methods allow visualization of *lacO*-tagged chromosomes in living and fixed samples. Alternatively, purified LacI or GFP-LacI can be used to stain fixed tissues using standard protocols developed for immunofluorescence [10, 25]. This approach may yield a better signal to noise ratio than can be achieved with in vivo expression of GFP-LacI because there is no background fluorescence from nucleoplasmic GFP-LacI that has not bound the *lacO* array. Additionally, many of the transgenes used for expression of GFP-LacI in nematodes are transcriptionally silenced in the germ line, precluding their use for studies of meiotic cohesion [21]. For these reasons, I prefer to stain *lacO* integrants with bacterially expressed, purified GFP-LacI.

The GFP-LacI/*lacO* system offers significant advantages over other methods used for the analysis of meiotic SCC in *C. elegans*. The simplest and perhaps most commonly used technique for quantifying SCC defects is counting the number of chromosomal structures in meiotic nuclei stained with a DNA dye like DAPI or Hoechst. Counting DAPI-stained bodies requires that chromosomes be far enough apart that they can be resolved. This condition is often met in late diakinesis nuclei of wild-type worms, which have six bivalents, and in mutants defective for meiotic crossover recombination, which have 12 univalents. In contrast, accurate quantification of DAPI-stained bodies is very difficult when 12–24 are present, as occurs in worms with severe SCC defects. Moreover, reliance on this technique limits analysis of SCC to diakinesis and prometaphase, since nuclei are smaller and chromosomes much less compact in earlier stages of meiosis.

Analysis of SCC by fluorescence in situ hybridization (FISH) allows more accurate quantification of SCC defects than does counting DAPI-stained bodies because FISH utilizes a sequence specific probe to fluorescently mark a single chromosome. Thus, FISH can reliably detect detached sister chromatids in crowded nuclei and in all stages of meiosis and mitosis. However, because FISH relies on a nucleic acid probe, chromosomal DNA must be denatured to allow the probe to bind. Denaturation degrades chromosomal morphology, an effect that is particularly severe in cohesin mutants. Moreover, because FISH probes are usually designed to hybridize to endogenous sequences that are present in both homologs of the targeted chromosome, an increased number of FISH foci can result from defects in synapsis and/or crossover recombination as well as defects in SCC establishment or maintenance. Because cohesin mutations often impair synapsis and crossover formation, relating the number of FISH signals in a nucleus to the frequency of sister separation as a result of defective meiotic cohesion can be challenging.

The GFP-LacI/*lacO* system circumvents many of the shortcomings of FISH and counting DAPI-stained bodies. Like FISH, the GFP-LacI/*lacO* system fluorescently tags a single chromosome, allowing sister separation to be detected even in crowded nuclei. However, unlike FISH probes, binding of GFP-LacI to DNA does not require denaturation. Additionally, because the *lacO* sequence to which GFP-LacI binds is derived from the bacterial *lac* operon rather than an endogenous *C. elegans* chromosomal sequence, SCC can be analyzed in animals heterozygous for the *lacO* array (*see* **Note 1**). In *lacO* heterozygotes, GFP-LacI binding labels the two sisters of a single homolog; therefore, the presence of two discrete GFP foci is a clear indication of an SCC defect (*see* **Note 2**). Moreover, because the GFP foci mark the two sisters of a single homolog, the average distance between sisters can be used as a measure of the severity of the SCC defect in animals of a given genotype. A mutation that eliminates meiotic SCC is expected to result in random positioning of the two sisters within the nucleus, allowing their separation by distances as large as the nucleus is wide. In contrast, a mutation that only weakens SCC may slightly increase sister separation but still allow the sisters to maintain their close proximity.

Although the GFP-LacI/*lacO* system has several major advantages over FISH and counts of DAPI-stained bodies for analysis of SCC, it also has some minor disadvantages. The GFP-LacI/*lacO* approach requires the construction of specialized strains that will likely only be used for analysis of SCC. However, the time needed to build the required strains is minimal, so this should not daunt a stalwart *C. elegans* geneticist. Two other potential concerns should be kept in mind. First, the GFP-LacI/*lacO* system allows analysis of only one chromosome in any given experiment. Other chromosomes may behave differently. For example, some mutations that disrupt meiosis have disparate effects on X chromosomes and autosomes [26–28]. Second, the chromosomally integrated *lacO* array could somehow alter the behavior of meiotic chromosomes. However, we have seen no evidence that this occurs to date.

The second approach described here utilizes PCR and restriction digests to identify and characterize embryonic aneuploidy in the progeny of mutant animals in which the two homologs of chromosome II are differentially marked by Snip-SNPs: single nucleotide polymorphisms (SNPs) that create restriction fragment length polymorphisms (RFLPs) (Fig. 1) [9]. Because this method will detect aneuploidy that occurs as a consequence of any defect in chromosome segregation during meiosis or the mitotic divisions of germ line stem cells, it is best used in combination with other methods, such as the GFP-LacI/*lacO* method described above and quantification of the number of polar bodies (see below) [9]. The power of this approach lies in the fact that large numbers of embryos can be analyzed. This allows the identification of rare aneuploidies in mutants with infrequent meiotic errors and reveals patterns of aberrant chromosome segregation that can differentiate between mutations that cause

premature, equational separation of sister chromatids at anaphase I (as occurs in *rec-8* single mutants) and those that cause random segregation of homologs at anaphase I (as occurs in *coh-4 coh-3* double mutants) [9]. As with the GFP-LacI/*lacO* analysis described above, this technique requires building specialized strains, and it only analyzes the segregation of a single autosome. However, the sensitivity and robustness of this approach offer advantages that far outweigh these minor shortcomings.

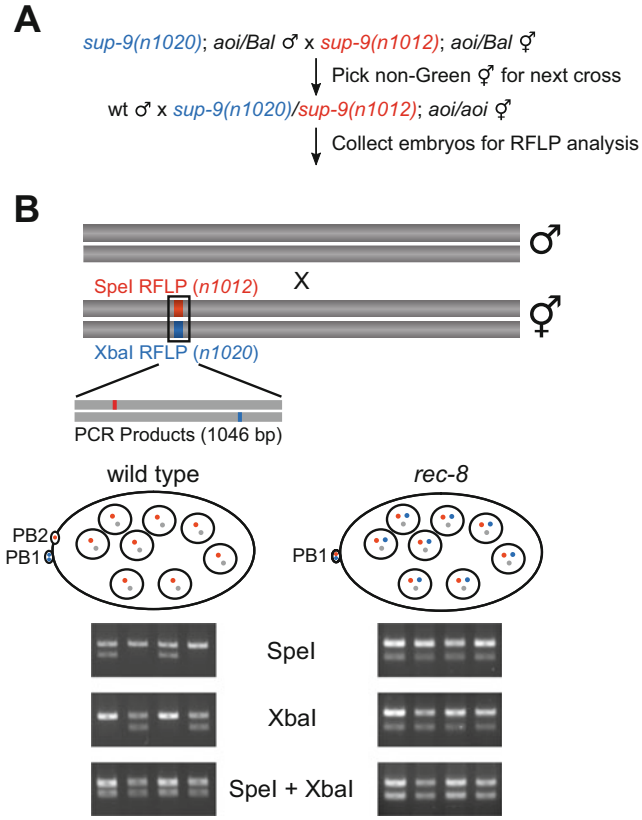

Fig. 1 A method to detect meiotic chromosome segregation errors using restriction fragment length polymorphisms. (**a**) A series of two crosses is used to generate embryos in which the patroclinous copy of chromosome II and both potential matroclinous copies can be molecularly identified by restriction fragment length polymorphisms (RFLPs). The RFLPs result from single nucleotide polymorphisms (SNPs) in the *sup-9* gene. *aoi* the mutant *a*llele *o*f *i*nterest being tested for effects on meiotic chromosome segregation. *Bal* the balancer chromosome used to allow the stable propagation of *aoi* heterozygotes. (**b**) In the second cross of the series, male worms that are homozygous for the wild-type *sup-9* allele are mated with heteroallelic *sup-9(n1020)/sup-9(n1012)* hermaphrodites that were generated in the first cross. The mutation in *sup-9(n1012)* creates an SpeI restriction site, while the mutation in *sup-9(n1020)* creates an XbaI site. To genotype embryos and larvae for the wild-type, *n1012*, and *n1020* alleles of *sup-9*, a PCR product that spans both SNPs is cut in three restriction digests: an SpeI single digest, an XbaI single digest, and an SpeI + XbaI double digest. Analysis of the restriction fragments by agarose gel electrophoresis can yield information regarding the pattern of meiotic chromosome segregation.

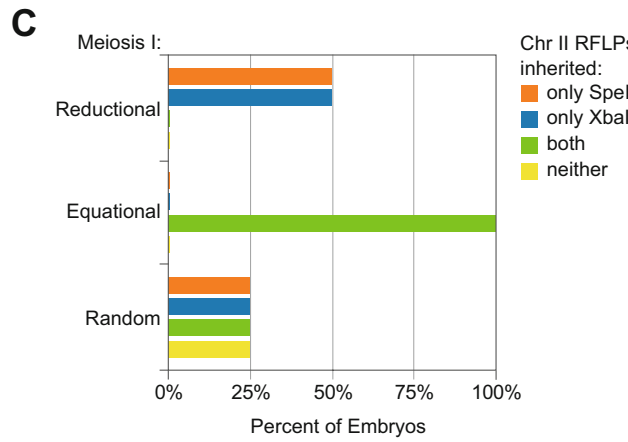

Fig. 1 (continued) (**b**, **c**) During normal meiosis, homologs segregate away from one another in meiosis I (reductional division). One set of homologs is inherited by the zygote, and the other is extruded into the first polar body (PB1). Sisters separate in meiosis II. One set of sisters is inherited by the zygote, and the other is extruded into the second polar body (PB2). The zygote is equally likely to inherit the SpeI RFLP or the XbaI RFLP. In meiosis I of mutants like *rec-8*, sister chromatids segregate prematurely away from one another (equational division). The zygote therefore inherits both the SpeI and the XbaI RFLPs. Polar body extrusion fails in meiosis II. In other mutants, like *coh-4 coh-3* double mutants, homologs segregate randomly in meiosis I, and the zygote is equally likely to inherit the SpeI RFLP, the XbaI RFLP, neither RFLP, or both RFLPs

2 Materials

The protocols in this chapter were written under the assumption that the reader will have access to most equipment typically found in a *C. elegans* lab, such as dissecting scopes equipped with light sources for transmitted light and epifluorescence, NG agar plates with a large lawn of OP50 *E. coli* for strain maintenance, mating plates seeded with a small drop of OP50 for conducting crosses, etc. A basic understanding of the fundamental techniques in nematode genetics, including methods to distinguish between self-progeny and cross-progeny, are also assumed.

Mutations that disrupt meiotic SCC usually result in the production of inviable, aneuploid embryos. Such mutations are therefore typically maintained in a heterozygous state by a dominantly marked, homozygous lethal balancer chromosome, and the protocols described here are written with this expectation in mind. We will use the terms "AOI" to refer to the mutant *a*llele *o*f *i*nterest that is being tested for effects on meiotic SCC and "balanced AOI" to refer to the allele of interest maintained over a balancer chromosome. With minor modifications, the methods described here can be used in RNAi experiments to study the meiotic roles of genes for which no mutation exists or genes that are also required during mitosis.

All reagents should be prepared using Milli-Q/Nanopure water or equivalent.

2.1 Construction of lacO-Tagged Strains

1. Standard equipment and reagents for PCR amplification and agarose gel electrophoresis, including 0.2 mL thin wall PCR tubes (either eight tube strips or multiwell plates), *Taq* DNA polymerase (5 U/μL) and 10× PCR buffer, dNTP mix (25 mM each dNTP) and sterile, nuclease free water.
2. Access to a compound microscope equipped with epifluorescence optics and at least a 40× oil immersion objective lens.
3. A *C. elegans* strain harboring your mutant allele of interest balanced by a dominantly marked, homozygous lethal balancer chromosome (hereafter referred to as the "balanced AOI").
4. A *C. elegans* strain carrying a chromosomally integrated array of *lacO* sequences. The *syIs44* array is an excellent choice for analysis of meiotic SCC (strain TY5434, full genotype *syIs44[dpy-20(+)*, *hsp-16^{promoter}::gfp-lacI*, *lacO(256)]* V). *syIs44* contains multiple, tandem copies of a 256× *lacO* repeat, a gene encoding a GFP-LacI fusion under control of the heat shock promoter, and a wild-type copy of the *dpy-20* locus. The array is integrated into chromosome V (*see* **Note 3**) [10, 24, 29]. The large number of *lacO* sequences within the array yields very robust staining of germ line nuclei with purified LacI-His_6-GFP (*see* Subheadings 3.2 and 3.4) [10]. Other strains with integrated *lacO* arrays are available [21], and the methods described here can be adapted to analyze meiotic SCC using these arrays (*see* **Note 4**) [30–32].
5. *C. elegans* lysis buffer: 0.15 mg/mL proteinase K in 1× PCR buffer (*see* **Note 5**).
6. 100 μM stocks of oligonucleotide primers for following *syIs44* by PCR. AFS366 (GCCATGTGTAATCCCAGCA) and AFS369 (GGTGAAACCAGTAACGTTA) amplify an approximately 1100 bp product from homozygous *syIs44/syIs44* and heterozygous *syIs44/+* worms (*see* **Note 6**).
7. Oligonucleotide primers for following the AOI. If the allele cannot be followed by PCR, other strategies will be required to demonstrate its presence or absence.
8. A 33 °C incubator or waterbath for heat shock.
9. Multiwell slides (*see* **Note 7**).
10. Cover glass, 22 mm × 50 mm, #1.
11. Phosphate buffered saline (PBS): In 800 mL of water, dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4. Adjust pH to 7.4 with HCl if necessary. Adjust volume to 1 L with additional water. Sterilize by autoclaving.
12. PBS + 0.2 % sodium azide: Add 1 μL 20 % (w/v) sodium azide to 100 μL 1× PBS.

2.2 Expression and Purification of LacI-His$_6$-GFP

1. pLacI-His$_6$-GFP plasmid [33], available from the author on request.
2. Competent BL21 bacteria (transformation efficiencies obtained with the $CaCl_2$ method [34] should be sufficient).
3. LB Agar plates containing 50 μg/mL ampicillin.
4. LB medium, Miller formulation: to 800 mL water, add 10 g tryptone, 5 g yeast extract, and 10 g NaCl. Adjust volume to 1 L with additional water. Sterilize by autoclaving.
5. 50 mg/mL (1000×) ampicillin stock, dissolved in water and sterile filtered.
6. Sonicator equipped with a microtip.
7. Cobalt IMAC resin (e.g., HisPur Resin, Thermo Scientific, Waltham, MA).
8. Disposable polypropylene column (e.g., Poly-Prep Chromatography Columns, Bio-Rad, Inc., Hercules, CA).
9. Equilibration/Wash Buffer (pH 7.0): 50 mM sodium phosphate, 300 mM NaCl.
10. Elution Buffer (pH 7.0): 50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole.
11. Wash Buffer with 5 mM imidazole: Add 0.3 mL Elution Buffer to 9.7 mL Equilibration/Wash Buffer.
12. PBS (*see* Subheading 2.1).
13. 80% glycerol: Add 20 mL water to a 100 mL graduated cylinder. Slowly add glycerol until the total volume equals 100 mL. Mix thoroughly by sealing the graduated cylinder with Parafilm and inverting periodically over the course of several hours or by adding a stir bar and mixing on a stir plate. Sterilize by autoclaving.

2.3 Generating syIs44 Heterozygotes

It is often desirable to examine LacI-His$_6$-GFP in meiotic nuclei of animals heterozygous for syIs44. For example, pachytene nuclei of syIs44 homozygotes could have two LacI-His$_6$-GFP foci as a consequence of asynapsis or defective sister chromatid cohesion, but the presence of two foci in syIs44 heterozygotes is strong evidence of an SCC defect (*see* **Note 2**). Moreover, when two LacI-His$_6$-GFP foci are detected in meiotic nuclei of syIs44/+ heterozygotes, measuring the average distance between foci can provide information about the severity of the SCC defect (*see* Subheading 3.5) [10]. This is not true when analyzing syIs44 homozygotes, because it is not possible to know which foci mark sister chromatids and which mark homologous chromosomes.

1. Fertile males carrying the balanced AOI and the *syIs44* array (*see* Subheading 3.1 and **Note 8**). Depending on the genotype of the strain being tested, *syIs44* may be heterozygous (if covered by the balancer) or homozygous.

2. Fertile hermaphrodites. This strain should have the same genotype as the males, except that it should lack *syIs44*.

2.4 Staining Gonads with LacI-His$_6$-GFP

1. Glass slides (75 × 25 × 1 mm; *see* **Note 9**).
2. Subbing solution [35]: Bring 200 mL of water to 60 °C, then add 0.4 g gelatin (from porcine skin). Cool to 40 °C, then add 0.04 g chrome alum (chromium potassium sulfate), 200 mg poly-L-lysine (*see* **Note 10**), and sodium azide to 1 mM final concentration (65 µL of 20% stock).
3. A slide drying rack.
4. PAP pen (e.g., Electron Microscopy Sciences 71312 or equivalent).
5. A scalpel handle (#3, Stainless Steel) and blades (#15) (*see* **Note 11**).
6. Cover glass, 18 mm × 18 mm.
7. Cover glass, 22 mm × 22 mm.
8. Humid chamber (*see* **Note 12**).
9. Liquid nitrogen (*see* **Note 13**).
10. 10× Dernburg's Modified Egg Buffer (10× EB): 250 mM HEPES-NaOH, pH 7.4, 1.18 M NaCl, 480 mM KCl, 20 mM EDTA, 5 mM EGTA (*see* **Note 14**).
11. 250 mg/mL levamisole: Add 0.5 g levamisole (also called tetramisole hydrochloride) to 2 mL of water. Store in 100 µL aliquots at −20 °C for up to a year (*see* **Note 15**).
12. 20% Tween 20. To prepare 200 mL, add 160 mL of water to a 250 mL graduated cylinder. Slowly add Tween 20 until the total volume is 200 mL. Mix thoroughly by sealing the graduated cylinder with Parafilm and inverting periodically over the course of several hours or by adding a stir bar and mixing on a stir plate.
13. Paraformaldehyde, 16% solution, electron microscopy grade (Electron Microscopy Sciences, Hatfield, PA). After opening, ampules should be sealed with Parafilm and stored at 4 °C for 2 weeks to 1 month.
14. Methanol, chilled to −20 °C in a Coplin jar.
15. 10× PBS: To 800 mL of water, add 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4. Adjust pH to 7.4 with HCl if necessary. Bring volume to 1 L by addition of water. Sterilize by autoclaving.
16. 0.5 M EDTA, pH 8.0: Add 93.06 g disodium EDTA dihydrate (fw 372.24) to 400 mL water. Gradually add approximately 9 g of NaOH pellets. As the pH approaches 8.0, the solution will begin to clear. At this point, add 5 N NaOH (approximately 5–10 mL) very patiently until the pH stabilizes

at 8.0. Because each addition of base drives more EDTA into solution, it can take a while to reach equilibrium. Add water to bring the total volume to 500 mL. Sterilize by autoclaving.

17. Blocking solution: To 89 mL water, add 10 mL 10× PBS, 1 mL 20% Tween 20, 0.1 mL 20% sodium azide, and 1 g bovine serum albumin (BSA). Rock on a nutator until the BSA has dissolved completely. Store in 5 mL aliquots at −20 °C.
18. PBST wash buffer: To 888 mL water, add 100 mL 10× PBS, 10 mL 20% Tween 20, and 2 mL 0.5 M EDTA.
19. Purified LacI-His_6-GFP (*see* Subheading 3.2).
20. Anti-GFP primary antibody (I use a chicken anti-GFP antibody from Life Technologies (Grand Island, NY).
21. Fluorescently conjugated secondary antibody that recognizes the anti-GFP primary antibody (*see* **Note 16**).
22. DAPI (4′,6-diamidino-2-phenylindole dihydrochloride): Resu spend at a 1 mg/mL concentration in water and store at −20 °C.
23. Mounting medium: Prolong Gold anti-fade reagent (Life Technologies, Grand Island, NY). Prolong must cure for 24 h before imaging to acquire the best optical properties. For faster results, Vectashield (Vector Labs, Burlingame, CA) can be used. To label chromosomes, DAPI is added directly to the mounting medium (1 μg/mL final concentration).
24. Clear fingernail polish for sealing coverslips.

2.5 Analysis of LacI-GFP Focus Number and Distribution

1. A high quality system for collection of digital microscope images. Either a confocal or widefield microscope will work, but because of the large number of images that must be acquired, high speed acquisition is desirable. A wide variety of suitable microscopes and imaging systems are available, and I make no specific recommendations here. However, some minimal requirements must be met for the method described here to give reliable results. Most importantly, the microscope must have an accurate and precise computer-controlled focusing mechanism and a high numerical aperture, oil immersion 60× or 100× objective. My lab uses a DeltaVision microscope (GE Life Sciences, Pittsburgh, PA) equipped with a 100×/1.4 NA objective lens.
2. Fiji image processing and analysis software [36]. Fiji is based on ImageJ [37], but Fiji is installed with plugins and addins that enable it to handle a large number of image formats out of the box. It is therefore recommended for users without significant experience with ImageJ. However, the methods described here can also be performed with ImageJ.
3. Microsoft Excel, Apple Numbers, LibreOffice Calc, or equivalent spreadsheet software.

4. A mouse with a scroll wheel. Although not essential, it is extremely helpful.

2.6 Analysis of Meiotic Chromosome Segregation Using Chromosome II RFLPs

1. Standard equipment and reagents for PCR amplification (*see* Subheading 2.1).
2. A 37 °C incubator.
3. An aspirator tube assembly (Sigma-Aldrich, St. Louis, MO).
4. Capillary tubes for mouth pipets (e.g., Kimble-Chase 51 Expansion Borosilicate Glass Melting Point Capillaries, 100 mm length, approximately 1.5 mm outside diameter, 0.25 mm wall thickness, open on both ends).
5. Butane lighter.
6. Worm strains: TY4236 *him-8(e1489)* IV; *mIs10[myo-2::gfp]* V, TY4851 *sup-9(n1012)* II, and TY4852 *sup-9(n1020)* II.
7. Two strains carrying the balanced AOI and a polymorphic allele of *sup-9*. One strain should have the *sup-9(n1012)* allele, which creates an SpeI cleavage site not present in wild-type, and the other should have the *sup-9(n1020)* allele, which creates an XbaI cleavage site not present in wild-type (*see* **Note 17**). These strains can be constructed using the strains TY4851 *sup-9(n1012)* II and TY4852 *sup-9(n1020)* II and techniques similar to those outlined for building strains with *syIs44* (*see* Subheading 2.1), with the following exceptions: First, the oligonucleotides AFS155 and AFS156 must be substituted for the AFS366 and AFS369 oligonucleotides used to genotype for *syIs44*. Second, a small amount of PCR product should be digested with SpeI or XbaI to genotype for the *n1012* and *n1020* alleles, respectively (*see* Subheading 3.6).
8. *C. elegans* lysis buffer (*see* Subheading 2.1).
9. Oligonucleotide primers for amplifying the *sup-9* polymorphisms (100 µM each): AFS155 (GACGGAGAATGAGATT CTGCAGG) and AFS156 (CGGCTCGTCTTATGAA ACGGA).
10. *sup-9* PCR mastermix (without *Taq* polymerase) sufficient for one hundred 30 µL reactions: To 1.725 mL of water, add 200 µL of 10× PCR buffer (*see* **Note 18**), 30 µL of dNTP mix (25 mM each dNTP), and 15 µL each of AFS155 and AFS156 oligos (100 µM stock). I typically scale this mastermix up tenfold, then freeze 100 reaction mixes at −20 °C until needed.
11. Restriction endonucleases: SpeI (10 U/µL) and XbaI (20 U/µL), along with stocks of the recommended buffers and BSA if not included in the buffer mix.
12. A large gel box for agarose gel electrophoresis; for example, the Owl D3-14 "Centipede" system.

3 Methods

3.1 Construction of lacO-Tagged Strains

1. Using standard genetic methods, perform crosses to introduce *syIs44* or another integrated *lacO* array into your mutant background. Crossing *syIs44* into a strain with a balanced AOI requires two sequential crosses. In the first cross, *syIs44* homozygotes are mated with a strain carrying the balanced AOI (P0 generation). Next, a cross is set up between male cross progeny that inherited the balancer and their hermaphrodite siblings that did not (F1 generation; *see* **Note 19**). In most cases, either 1/4 or 1/8 of the F2 progeny that inherit the balancer will be the desired genotype, depending on whether *syIs44* is covered by the balancer (for example, *nT1*) or needs to be maintained in a homozygous state (*see* **Note 20**).
2. Pick a sufficient number of L4 hermaphrodites carrying the balancer to ensure a high probability of finding at least one worm of the desired genotype. If the fraction of worms expected to have the correct genotype is 1/4, 16 L4s should be sufficient to ensure that one will obtain the desired strain 99% of the time. If the fraction is 1/8, pick 32 L4s. Every worm should be picked onto a separate plate.
3. Allow hermaphrodites to self-fertilize overnight at 25 °C. When the singled worms have laid at least 40–50 eggs, genotype them by single-worm PCR. Thaw aliquots of *C. elegans* lysis buffer and dispense into PCR tubes, 10 µL per tube (if genotyping for *syIs44* and a single mutant allele; *see* **Note 21**). It is often convenient to use 8-well strips or multiwell plates. Briefly centrifuge to collect the buffer in the bottom of the tube. Pick the mom from each plate into the drop of lysis buffer.
4. Incubate at 60 °C for 60 min, then 95 °C for 20 min. Visually examine the tubes on a dissecting scope to confirm that the lysis reactions worked. Some embryos and a bit of cuticle might remain, but most of the worm should have been digested.
5. Vortex the PCR tubes to break up any remaining tissues, then briefly centrifuge the PCR tubes to collect the buffer in the bottom of the tube.
6. Move 5 µL of lysate into a new tube or well for each genotyping PCR. If available, a multichannel pipettor greatly speeds up this process.
7. On ice, make a PCR mastermix sufficient to genotype each worm for *syIs44*. For each reaction, use 13 µL water, 1.5 µL of 10× PCR buffer, 0.2 µL of dNTP mix (25 mM each dNTP), 0.1 µL each of AFS366 and AFS369 oligos (100 µM stock). Also make a PCR mastermix to genotype for your AOI. When both mixes are ready, add *Taq* polymerase. For the *syIs44* PCR,

use 0.1 μL of *Taq* (5 U/μL) per reaction. Mix well by gently pipetting up and down (*see* **Note 22**)

8. On ice, add 15 μL of mastermix to each PCR tube. Centrifuge briefly to collect liquid in the bottom of tubes.
9. Perform PCR. AFS366 and AFS369 work well with 35 cycles, a 54 °C annealing temperature, and a 1 min extension time.
10. Separate your PCR products on a 1–2 % agarose gel. A band of approximately 1100 bp indicates that the lysed worm was either heterozygous or homozygous for *syIs44*. No band will be detected if the lysed worm had not inherited the *syIs44* array.
11. If the balancer used to maintain the AOI also covers *syIs44* (for example, the *nT1* balancer), PCR genotyping for *syIs44* is sufficient to determine whether the lysed worm was *syIs44/Balancer* or *+/Balancer*. However, if *syIs44* is not covered by the balancer, it will be necessary to identify *syIs44* homozygous strains by fluorescence microscopy. Once the progeny of the lysed worms are young adults, heat shock the *syIs44* positive plates at 33 °C for 1 h. Two hours later, pipet 2 μL of PBS + 0.2 % sodium azide into four wells in the middle of a gasketed slide. Pick 20 L4 or young adult hermaphrodites from a heat-shocked plate into the first well, then repeat for three additional plates. Work quickly to add the worms before the drops evaporate. If necessary, the slide can be stored in a humid chamber while picking worms (*see* Subheading 2.4). Gently lower a 22 mm × 50 mm coverslip onto the slide. Examine the worms in each well for expression of LacI-GFP. The sodium azide will paralyze the worms, simplifying the visualization of GFP foci, but GFP will bleach very quickly. If the mom was homozygous for *syIs44*, GFP should be visible in somatic nuclei of every worm, and most nuclei should have two bright GFP foci (*see* **Note 23**). In contrast, if the mom was heterozygous for *syIs44*, only 75 % of the progeny will express LacI-GFP, and 2/3 of these will have a single GFP focus in each somatic nucleus.

3.2 Expression and Purification of LacI-His_6-GFP

1. Transform the pLacI-His_6-GFP plasmid into competent BL21 bacteria. Plate transformed bacteria on LB plates containing 50 μg/mL ampicillin. Grow overnight at 37 °C.
2. Inoculate a 5 mL LB culture containing 50 μg/mL ampicillin with a single colony. Shake the culture in a 30 °C incubator overnight.
3. Inoculate a 500 mL LB culture containing 50 μg/mL ampicillin with 50 μL of the overnight culture. Shake the culture in a 30 °C incubator overnight.
4. Pellet bacteria by centrifugation at 3000 × *g* for 10 min. Discard the supernatant and freeze the pellet at −80 °C.

5. Perform all subsequent steps at 4 °C. All solutions should be chilled to 4 °C prior to thawing the bacterial pellet.
6. Resuspend pellet in 40 mL Equilibration/Wash buffer.
7. Lyse bacteria by sonicating on ice. The following conditions work well with our sonicator: At maximum microtip power, pulse 1 s on, then 1 s off. After 15 bursts, pause for 1 min. Repeat this cycle three more times.
8. Pellet insoluble debris by centrifugation at 10,000 × *g* for 20 min at 4 °C.
9. While centrifuging the bacterial lysate, resuspend the cobalt IMAC resin and transfer a sufficient quantity to yield a 2 mL bed volume to a 50 mL conical tube. Centrifuge at 700 × *g* for 2 min, then remove the supernatant. Wash the resin with 20 mL Equilibration/Wash Buffer. Centrifuge, remove the supernatant, and repeat.
10. Mix the lysate supernatant with the resin and rock 20 min. Centrifuge at 700 × *g* for 5 min. Remove the supernatant.
11. Add 20 mL 1× Equilibration/Wash Buffer to resin. Rock 10 min, then centrifuge and remove the supernatant. Repeat 2×.
12. Add 2 mL Equilibration/Wash Buffer to resin and vortex to resuspend. Pour into column, let settle, then drain until liquid is at level of top of bed. Wash with 10 mL Equilibration/Wash Buffer.
13. Wash with 10 mL Wash Buffer with 5 mM imidazole.
14. Elute in 10 mL Elution Buffer. Collect 0.5 mL fractions.
15. Fractions containing LacI-His_6-GFP will be noticeably yellow. Pool these fractions and concentrate in an Amicon Ultra-15 Centrifugal Filter Unit (30 kDa MWCO) by centrifuging at 4000 × *g* for 15 min. This should be sufficient to reduce the volume to approximately 250–500 μL.
16. Discard the flow-through, then add 10 mL of 1× PBS to the filter unit. Centrifuge at 4000 × *g* for 15 min or until retentate volume is less than 500 μL. Repeat 2×.
17. Gently pipet retentate up and down to resuspend any LacI-His_6-GFP precipitate that may have collected on the membrane. Transfer to a microcentrifuge tube and adjust the total volume to 500 μL by addition of 1× PBS.
18. Add 835 μL of 80% glycerol and mix thoroughly by pipetting gently up and down. Centrifuge at top speed in a refrigerated microcentrifuge to pellet any aggregates.
19. Freeze 100 μL aliquots in liquid N2. Store one working aliquot at −20 °C. The remaining tubes can be stored for several years at −80 °C.
20. Determine ideal concentration for staining: Dissect gonads, then fix and stain with LacI-His_6-GFP diluted 1:250, 1:500, 1:1000, and 1:2000 in block (*see* Subheading 3.4).

3.3 Generating syIs44 Heterozygotes

1. Set up 4–8 crosses, each with 6–10 *syIs44* positive males and a single *syIs44* negative hermaphrodite. Males and hermaphrodites should both be balanced heterozygotes if your AOI is lethal. Label each mating plate with the date and a unique identifier (cross A, B, etc.).
2. Transfer the males and hermaphrodites to fresh mating plates every day. Label each plate with the date and the unique identifier.
3. When transferring crosses, check plates from previous days for the presence of males. Once a hermaphrodite has mated with a male, most of her offspring will be cross progeny, and approximately 50% will be male. The first plate from each cross to have male progeny should be discarded, because this plate will have a mixture of self progeny produced before the hermaphrodite mated and self-progeny produced after she mated. Worms from the second day of male production or from any subsequent day can be used for staining, provided that there are still approximately 50% male worms on the plate.

3.4 Staining Gonads with LacI-His$_6$-GFP

1. Isolate L4 hermaphrodites that are homozygous for the AOI from working crosses (*see* Subheading 3.3). These worms should be heterozygous for *syIs44*. Grow the animals for 24 h at 20 °C. Isolating L4s and then allowing them to mature for a defined time ensures that all animals analyzed are similar in age.
2. While waiting for the L4s to mature, prepare adherent "subbed" slides: Immerse slides in a Coplin jar filled with subbing solution for ~30 s, then move slides to a drying rack. Dry slides in a drying oven (approximately 30 min at 80 °C or 3 h at 60 °C should be sufficient) or overnight at room temperature. If drying overnight, cover slides with aluminum foil to protect them from dust and light. Subbed slides can be stored at room temperature in the dark for up to a week, but freshly prepared slides work best.
3. Prepare 1× Egg Buffer + Tween 20 and Levamisole (EBTL): To 885 µL water, add 100 µL 10× EB, 10 µL 250 mg/mL levamisole, and 5 µL 20% Tween 20. Make fresh on day of staining.
4. Prepare 1× Egg Buffer + 2% Paraformaldehyde (EB-PFA): To 775 µL water, add 100 µL 10× EB and 125 µL 16% PFA. Make fresh on day of staining.
5. Using a PAP pen, draw a 1 cm × 1 cm grease square on the front surface of a slide (Fig. 2a).
6. Pipette 6.5 µL of EBTL into the middle of the grease square.
7. Pick ten hermaphrodites and coax them off the end of your pick by swirling it around in the drop of buffer. The levamisole should rapidly anesthetize the worms; if they continue to thrash, it is likely that the anesthetic has gone bad.

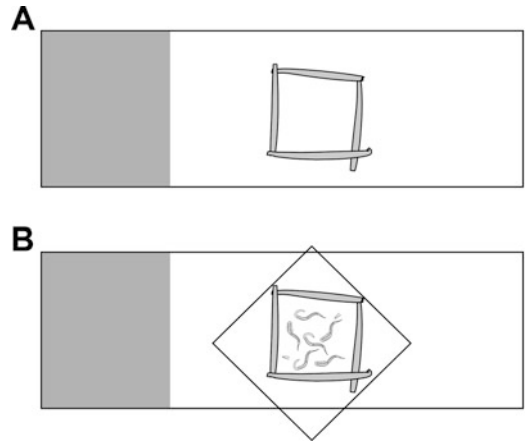

Fig. 2 Freeze-crack fixation of dissected gonads for LacI-GFP staining. (**a**) An approximately 1 cm square grease barrier is drawn on the slide surface using a PAP pen. The hydrophobic barrier allows staining with as little as 5 μL of antibody solution. (**b**) After gonads have been microsurgically extruded from the body of the worm and fixed in PFA, a coverslip is lowered onto the slide at a 45° angle. The overhanging lip allows easy removal of the coverslip by flicking it off with the tip of a finger

8. Using your scalpel, lop off the head of each worm between the pharynx and the gonad (*see* **Note 24**).
9. Once all worms have been dissected, add 6.5 μL of EB-PFA.
10. Gently lower a 22 mm × 22 mm coverslip at a 45° angle to the slide, such that the coverslip entirely covers the grease square but still hangs over one edge of the slide (Fig. 2b).
11. Examine the slide. It should be clear whether the dissected worms stuck to the slide and if the coverslip has compressed the gonadal tissue. Ideally, the coverslip should provide sufficient pressure to slightly flatten the gonad. If this is not the case, press the torn edge of a paper towel gently against the side of the coverslip where it meets the slide to wick out a small amount of liquid. Continue until the coverslip is lightly pressing down on the gonad.
12. Incubate the slide in a humid chamber for 5 min, then gently lower the slide into a beaker filled with liquid nitrogen.
13. Slides should be left in liquid nitrogen for at least 2 min, but may be kept in liquid nitrogen for much longer. If preparing more than one slide, repeat **steps 5–12**, collecting slides in liquid nitrogen until all animals have been dissected and fixed.
14. Remove the slide from liquid nitrogen with a pair of forceps. Hold the slide with the coverslipped side pointed away from your body, and flick the overhanging corner of the coverslip to remove the coverslip from the slide.

15. Immerse the slide in a Coplin jar filled with −20 °C methanol for 1 min, then move the slide to a Coplin jar filled with PBST wash buffer. Wash for 5 min. Repeat 1×.
16. Wipe off the front and back surfaces of the slide (except for the area inside the grease square). Add 20 μL blocking solution and incubate for 30 min in a humid chamber.
17. Wick off the blocking solution with a folded-up Kimwipes or paper towel. Add 5 μL of LacI-His_6-GFP diluted in PBS + 1% BSA + 0.2% Tween 20. Place the slide in a humid chamber. Incubate at least 1 h at room temperature or overnight at 4 °C.
18. Wash 3× in PBST, 10 min each.
19. Wipe off the front and back surfaces of the slide. Add 5 μL of anti-GFP primary antibody diluted in PBS + 1% BSA + 0.2% Tween 20. Place the slide in a humid chamber. Incubate at least 1 h at room temperature or overnight at 4 °C.
20. Wash 3× in PBST, 10 min each.
21. Wipe off the front and back surfaces of the slide. Add 5 μL of fluorescently labeled secondary antibody diluted in PBS + 1% BSA + 0.2% Tween 20. Place the slide in a humid chamber. Incubate at least 1 h at room temperature or overnight at 4 °C.
22. Wash 3× in PBST, 10 min each.
23. Wipe off the front and back surfaces of the slide. Add 7 μL of Prolong Gold or Vectashield mounting medium containing 1 μg/mL DAPI.
24. Apply an 18 mm × 18 mm cover glass to the slide. Seal the edges with fingernail polish. Let nail polish dry for at least 1 h before examining the slide on a microscope.

3.5 Analysis of LacI-GFP Focus Number and Distribution

Because of the wide variety of microscopes and imaging systems available, the specific methods required to collect image datasets are beyond the scope of this paper. However, a few best practices should be followed to ensure the best results. Images should be collected at less than or equal to 0.2 μm axial spacing. Avoid binning, which sacrifices XY resolution to increase the signal to noise ratio. Because images of nuclei on the side of the gonad closest to the coverslip will be of much higher quality than images of nuclei on the opposite side of the rachis, it is advisable to limit the analysis to nuclei adjacent to the coverslip.

Once images have been collected, the number of nuclei with intact sister chromatid cohesion (one spot per nucleus) or defective sister chromatid cohesion (two spots per nucleus) can be quantified [10]. With this information, one can determine whether SCC is normal or defective. However, not all SCC defects are equal, and measuring the distance between LacI-GFP foci can reveal differences in the severity of SCC disruption in different mutants [10].

Thus, it is informative to measure the distances between LacI-GFP foci in addition to tabulating the number of nuclei in which one or two foci could be detected.

1. Open Fiji. The instructions below have been tested on Fiji 2.0.0-rc-30/1.49 t with 64-bit Java 1.6.0_24 installed on Windows 7. Although the exact menu formats and keyboard shortcuts may differ based on operating system or Fiji version, every function that is essential for the analysis described below should be present in all versions and on all platforms.
2. Open your dataset in Fiji. This can be done in several ways. For data types that are supported by the built-in Bio-Formats Image Importer plugin, one can click on File/Open, then navigate through the file system to select your file. Alternatively, one can drag-and-drop the file icon onto the main Fiji window. If your file type is recognized by Fiji, a dialog box entitled "Bio-Formats Import Options" will open. For the analysis of DeltaVision files, I recommend setting the drop-down menu entitled, "Stack viewing/View stack with:" to "Hyperstack," and the drop-down menu entitled, "Color options/Color mode" to "Colorized." Additionally, make sure that only the Autoscale checkbox is selected.
3. Set your preferred display colors (lookup table, or LUT) for each channel. Many investigators prefer to show DNA in red and LacI-GFP in green, although deuteranopes may prefer a different palette. In the Image/Color/menu, click on Channels Tool. Select Channel 2, then click on the "More" button and choose your preferred color for that channel. Repeat for Channel 1. Choose "Composite" from the drop-down menu. You should now see an RGB image with Channels 1 and 2 pseudocolored using the selected LUTs.
4. At the bottom of the image window there are two scroll bars, labeled "c" and "z." The "z" scrollbar changes the focal plane. The focal plane can also be changed with the mouse scroll wheel, although this requires holding down a modifier key—currently the Alt key on Windows. In past versions of Fiji, the Ctrl key was used. Scroll through the image stack to find a focal plane that allows visualization of the entire gonad, if possible.
5. Zoom in on the image: Click the "Magnifying glass" tool in the main Fiji toolbar, then click on the image window. I find that 150–200% zoom works well for point picking using our image acquisition settings. Once the image magnification has been increased, the image region being viewed can be changed by selecting the "Scrolling" tool (hand icon in the main Fiji toolbar) and dragging the image to a new location.
6. Locate the "Point Tool" in the row of buttons in the main Fiji window. The button looks like a cross with a yellow point in

the center. Right-clicking on the button will toggle between the "Point Tool" and the "Multi-point Tool." The "Point Tool" is the best choice for the analysis described here because it allows recording of the *X*, *Y*, and *Z* coordinates of a point, while the "Multi-point Tool" currently assigns the same *Z* coordinate to every point.

7. Double-click on the "Point Tool" icon. Make sure that the "Auto-measure" and "Label points" checkboxes are selected, and the other checkboxes are empty. Click the "OK" button.
8. Scroll through the *Z* stacks and identify nuclei with a single GFP focus (*see* **Note 25**). For each nucleus in this category, click on the centroid of the LacI-GFP focus. Each mouse click should create a new row in the Results window and log the image name, the *X* and *Y* coordinates of the point selected, and the *Z* section, or slice, that was visible when the point was selected.
9. Once the position of the LacI-GFP focus in each nucleus with a single spot has been recorded, copy the data from the Results window: From the Edit menu, choose "Select All," then "Copy." Paste into a spreadsheet program. There should be six columns of data, corresponding to the Measurement ID Number, the Slice Label, the *X* and *Y* coordinates of the selected point, and the Channel and *Z* Slice that were active when the selection was made. In most spreadsheet programs, this data will be in columns A-F.
10. Paste a second copy of the data immediately to the right of the first copy (i.e., in columns G-L).
11. Clear the data from the Fiji Results window: From the Edit menu, choose "Select All," then "Clear."
12. Scroll through the *Z*-stacks and identify nuclei with two LacI-GFP foci. Work through the dataset systematically, successively clicking on the two foci within each nucleus.
13. Once all the nuclei have been analyzed, copy and paste the data into columns A–F of the spreadsheet, underneath the existing data. Next, edit the spreadsheet such that the measurements for the first focus in a nucleus are in columns A–F and the measurements for the second focus in the nucleus are in columns G–L of the same row. Clean up the spreadsheet by deleting any empty or duplicate rows created while cutting and pasting (*see* **Note 26**).
14. For each row, subtract the *X*, *Y*, and *Z* coordinates in columns in G–L from the *X*, *Y*, and *Z* coordinates in columns A–F, respectively, to calculate the distance between the two LacI-GFP spots in each dimension. I refer to these distances as *d(x)*, *d(y)* and *d(z)*. For nuclei in which two foci could not be resolved, all three distances should equal zero. For nuclei in which two foci could be resolved, some values will be positive

and others will be negative; this is expected, and will not affect the calculation of the shortest distance separating the two foci.

15. Ensure that the distance measurements $d(x)$, $d(y)$, and $d(z)$ all have the same units (e.g., microns). $d(x)$ and $d(y)$ may be converted from number of image planes to microns by multiplying by the XY pixel size, in microns. $d(z)$ may be converted from pixels to microns by multiplying $d(z)$ by the step size, in microns, used during image acquisition. If the XY pixel dimensions are not known, they can be measured using a stage micrometer; however, the methods used to determine pixel size are beyond the scope of this protocol.
16. Once $d(x)$, $d(y)$ and $d(z)$ have been calculated and converted to the same units, the shortest distance between each pair of foci can be calculated using the equation $D = [d(x)^2 + d(y)^2 + d(z)^2]^{1/2}$.

3.6 Analysis of Meiotic Chromosome Segregation Using Chromosome II RFLPs

1. Cross 1: To generate heteroallelic *sup-9(1020)/sup-9(1012)* hermaphrodites that are homozygous for your AOI, cross *sup-9(n1020)* males with *sup-9(n1012)* hermaphrodites. The males and hermaphrodites should both carry the balanced AOI. Ensuring that the same *sup-9* allele is always contributed by the male simplifies a data validation step that will be performed later.
2. Cross 2: When Cross 1 is working, pick 8–12 L4 hermaphrodites that are homozygous for your AOI individually onto mating plates. Add 6–10 young adult *him-8(e1489)* IV; *mIs10[myo-2::GFP]* V males to each plate (*see* **Notes 27** and **28**).
3. Collect embryos for analysis: When Cross 2 is working, transfer the mated hermaphrodites and males to fresh mating plates. Check the plates frequently to observe the number of eggs laid. When most worms have laid approximately 12–15 embryos, remove the mothers from the plates (*see* **Note 29**).
4. Allow the collected embryos to develop on the plate for approximately 16 h at 20 °C. This amount of time should be sufficient to allow all embryos to develop until they have either hatched or ceased divisions. However, it should be short enough that the embryos will not decay to the point that their DNA is unsuitable for PCR analysis.
5. Prepare a mouth pipet: Hold a capillary tube by both ends and heat the middle over a gas burner until the glass begins to melt. Remove the capillary from the flame and quickly pull the ends in opposite directions. Ideally, this will draw out the molten glass such that a thin, relatively flexible central region connects the two ends. Carefully bend the thin central region near one end to break the glass, creating a mouth pipet with an

approximately 1.5 in. long, narrow tip. Repeat for the other side. The inside diameter of the tip should be approximately 100 μm, or slightly larger than an embryo.

6. Create an approximately 45° bend in the tip of the capillary tube by holding it near the flame of a lit butane lighter. The angled tip will be easier to insert into the wells of a 96-well plate than a straight tip. Place the thick end of the capillary tube into the opening of an aspirator tube assembly.
7. Thaw a 500 μL aliquot of *C. elegans* lysis buffer and dispense 5 μL into each well of a 96-well plate. Centrifuge the plate briefly to collect the liquid in the bottom of the wells. Keep the plate on ice or in a 96-well plate cooling block between embryo additions.
8. Flood one plate of embryos with 1–2 mL of water. Place the tip of the mouth pipette next to an embryo or larva and apply gentle suction to the aspirator tube assembly to draw the specimen into the capillary tube (*see* **Note 30**). It may be necessary to gently dislodge embryos from the surface of the plate using the tip of the capillary tube. Place the 96-well plate of lysis buffer on the dissecting scope and focus on the first well. If possible, use low enough magnification that both the top of the well and the drop of lysis buffer are in focus. Carefully insert the tip of the capillary into the tube until it is immersed in lysis buffer. It may be necessary to adjust the focus or magnification during this process. Once the capillary has broken the surface of the lysis buffer, increase the magnification and focus on the tip of the capillary. Gently blow the embryo or larva out of the mouth pipet. As long as no air bubbles are created, the specimen should be visible as it exits the tip. Repeat this process with the remaining embryos and larvae on the mating plate, then repeat for the remaining mating plates until samples have been added to 90 wells. Make sure that each well receives only one embryo or larva. If there is any doubt that a well contains a single specimen, mark the questionable well for exclusion from subsequent analysis. Keep track of which mating plate each specimen came from and whether it was a larva or arrested embryo. To the remaining six wells, add three L1 larvae from *sup-9(1012)* homozygous worms and three from *sup-9(n1020)* homozygous worms as controls.
9. Seal the plate and freeze at −80 °C for 1 h to overnight.
10. Thaw the 96-well plate and gently remove the seal. Thaw a 500 μL aliquot of *C. elegans* lysis buffer and dispense 5 μL into each well. Centrifuge the plate briefly to collect the liquid in the bottom of the wells.
11. Seal the plate and incubate at 60 °C for 60 min, then 95 °C for 20 min to lyse the embryos.

12. Briefly centrifuge the 96-well plate to collect the lysate in the bottom of the wells. Place the plate on ice or in a chilled 96-well plate cooling block.
13. Thaw an aliquot of *sup-9* PCR mastermix (or prepare fresh). Add 15 μL *Taq* polymerase (5 U/μL). Mix well by gently pipetting up and down. Dispense 20 μL into each well of the PCR plate. Seal plate and centrifuge briefly to collect liquid at the bottom of each well. Return plate to ice or 96-well plate cooling block.
14. Run the PCR program on a thermocycler. Amplification with the AFS155 and AFS156 oligonucleotides works well with 35 cycles, a 50 °C annealing temperature, and a 75 s extension time (*see* **Note 31**).
15. Once the PCR program is complete, visually inspect the wells of the PCR plate. If any wells lost some volume due to evaporation, add a little water to equalize their volume with the other wells.
16. Run 2 μL of the PCR reactions in column 1 of the PCR plate on a 1.5% agarose gel. If the reactions worked, a band of approximately 1 kb in size should be visible. From this gel, estimate the volume of PCR product needed to see bands after restriction digests. Enough PCR product should be cut that a band equivalent to approximately 1/3 of the total digested DNA will be visible. Cutting more DNA than necessary increases the likelihood of incomplete digestion. A maximum of 7 μL of PCR product can be included in each digest.
17. Replicate the PCR plate to three new 96-well plates. Use a multichannel pipet to transfer the volume estimated in the previous step. Once the PCR product has been added, adjust the total volume in each well to 7 μL by addition of water, if necessary.
18. Prepare three 2× restriction digestion mastermixes (*see* **Note 32**). Keep the mastermixes on ice and add the enzymes just before dispensing into 96-well plates. After adding the enzymes, mix the reaction well by pipetting up and down. SpeI single digest: To 540 μL water, add 140 μL 10× CutSmart buffer and 20 μL of SpeI. XbaI single digest: To 520 μL water, add 140 μL 10× CutSmart buffer and 40 μL of XbaI. SpeI + XbaI double digest: To 500 μL water, add 140 μL 10× CutSmart buffer, 20 μL of SpeI, and 40 μL of XbaI.
19. Add 7 μL of mastermix to each well of a 96 well plate. If available, a repeat pipettor greatly simplifies this process.
20. Seal each plate and centrifuge briefly to collect liquid in the bottom of the wells. Incubate plates overnight in a 37 °C incubator (*see* **Note 33**).

21. Analyze the digests by gel electrophoresis on a 1.5% agarose gel. The Owl D3-14 Centipede system we use can be configured to have three rows of 50 wells each, which allows the gel to be loaded in such a way that all three digests from a single PCR reaction are aligned in a single column, which greatly simplifies data analysis.
22. Determine whether the control PCR products from the *sup-9(n1012)* and *sup-9(n1020)* homozygous worms were digested to completion. If so, the SpeI and SpeI + XbaI digested DNA from *sup-9(n1012)* and the XbaI and SpeI + XbaI digested DNA from *sup-9(n1020)* mutants should have a prominent band of approximately 800 bp but no detectible 1046 bp band. If a 1046 bp band is visible, the control reactions did not cut to completion and all data from the plate are unreliable.
23. Determine whether any individuals analyzed were self-progeny of the *sup-9(n1020)/sup-9(n1012)* hermaphrodite used in Cross 2 rather than cross progeny of the hermaphrodite and the *him-8*; *mIs10* males. All cross progeny will have a *sup-9(+)* allele inherited from the father, resulting in an uncut 1046 bp band in the SpeI + XbaI double digest. Individuals lacking this band should be excluded from the analysis.
24. Identify lanes with nonspecific PCR or digestion products. All digests other than those in the six control lanes should have either one band of 1046 bp or two bands, one of 1046 bp and one of approximately 800 bp. An approximately 200 bp digestion product may also be visible, but this band is often faint or undetectable. If additional bands are present in a digest, data from that individual should be excluded from the analysis.
25. Determine whether each specimen remaining in the analysis inherited only the *sup-9(n1012)* allele, only the *sup-9(n1020)* allele, both *sup-9* alleles, or neither *sup-9* allele. Detection of an approximately 800 bp band in the SpeI single digest indicates that the embryo inherited the *sup-9(n1012)* allele, while detection of an approximately 800 bp band in the XbaI single digest indicates that the embryo inherited the *sup-9(n1020)* allele.
26. Compare the data for all embryos and larvae collected from the same mother. Did any inherit the *sup-9(n1020)* allele? If not, the mother was likely a *sup-9(n1012)* homozygote rather than a *sup-9(n1020)/sup-9(n1012)* heterozygote, and the data from all of the siblings should be excluded from the analysis.
27. For all individuals that passed the three quality control checks (control PCR products from the *sup-9(n1012)* and *sup-9(n1020)* homozygotes were digested to completion, an uncut band was present in the SpeI + XbaI double digest, and the *sup-9(n1020)* allele was detected in at least one sibling), tabulate how many inherited only the *sup-9(n1012)* allele, only the *sup-9(n1020)* allele, both *sup-9* alleles, and neither *sup-9* allele.

28. From the tabulated data, determine the likely pattern of chromosome segregation. If most individuals completed meiosis normally, approximately 50% of individuals should have inherited only the *sup-9(n1012)* allele, and the other 50% should have inherited only the *sup-9(n1020)* allele (Fig. 1c, Reductional pattern). If crossover formation failed but sisters remained together during meiosis I, as occurs in *coh-4 coh-3* double mutants [9], it is expected that 25% of individuals would inherit only the *sup-9(n1012)* allele, 25% would inherit only the *sup-9(n1020)* allele, 25% would inherit both *sup-9* alleles, and 25% would not inherit either *sup-9* allele (Fig. 1c, Random pattern). If meiotic recombination failed and premature, equational sister separation occurred in meiosis I (as occurs in *rec-8* mutants) [9], most individuals analyzed should test positive for both *sup-9(n1012)* and *sup-9(n1020)* (Fig. 1c, Equational pattern) (*see* **Note 34**).

4 Notes

1. Because *lacO* sequences are common in plasmids used for routine cloning, many transgenic worm strains have *lacO* sequences in their genome. While GFP-LacI binding to a single *lacO* site is usually not detectable, GFP-LacI binding to simple extrachromosomal and integrated arrays made with plasmids harboring single copy *lacO* sequences often is [21].
2. The presence two GFP-LacI foci in meiotic nuclei of a *lacO* heterozygote could also result from mitotic chromosome segregation defects in germ line progenitor cells. However, such defects would also lead to the formation of nuclei with zero foci and more than two foci. Such nuclei cannot result from a defect in meiotic SCC.
3. I mapped *syIs44* to chromosome V using a panel of 48 single nucleotide polymorphisms that introduce or remove cleavage sites for the restriction endonuclease DraI [38]. The exact map position of *syIs44* is unknown. However, analysis of recombinant strains generated while linking *syIs44* to *coh-3(gk112)* and *coh-4(tm1857)* positioned *syIs44* between *coh-4* (LG V:-6.34) and *coh-3* (LG V:6.41), perhaps somewhere around –4 cM.
4. Many of the available strains with *lacO*-tagged chromosomes created by the miniMos method [30] also carry *syIs46*, an integrated extrachromosomal array that contains a GFP-LacI transgene under control of the heat shock promoter. Because the plasmids in this array also harbor *lacO* sequences [21], it is advisable to outcross these strains to eliminate *syIs46* before utilizing them to analyze meiotic SCC.

5. I typically make lysis buffer in 10 mL batches, then freeze 50 µL and 500 µL aliquots in liquid nitrogen. These aliquots are sufficient to set up 5 µL lysis reactions in 8 tube strips and 96-well plates, respectively. Aliquots can be stored at −80 °C for up to 1 year.
6. Because *syIs44* is a repetitive, integrated array and the exact structure and chromosomal location of the array are unknown, designing primers to follow the array is challenging. Primers AFS366 and AFS369, which bind to the GFP sequences and LacI sequences in the array, respectively, reliably amplify a product of approximately 1100 bp from *syIs44* and have not given false positive results in strains carrying other integrated GFP transgenes, such as those used to dominantly mark many balancers.
7. The multi-well slides that we use are manufactured by Thermo Fisher Scientific Slides & Specialty Glass (Portsmouth, NH) and can be ordered through Fisher Scientific (Pittsburgh, PA) as a non-catalog/special-order item. Reference part number 30-943A, Epoxy Blue Slide with 18 wells. Similar products from other manufacturers will likely work equally well.
8. If the *syIs44*-tagged chromosome is contributed by the male, then any worms with *lacO* arrays must be cross progeny and therefore heterozygous for *syIs44*. Any hermaphrodite self-progeny would not inherit *syIs44*. In contrast, if *syIs44* were contributed by the hermaphrodite, both cross-progeny and self-progeny would inherit *syIs44*. Because self-progeny would be homozygous for *syIs44*, their inclusion could confound the analysis.
9. *C. elegans* gonads do not adhere well to standard glass microscope slides. To increase the adhesion of gonadal tissues, one can use commercially available charged slides like Fisherbrand SuperFrost Plus slides. Alternatively, one can coat standard slides with "subbing solution." In our experience, each method has distinct advantages and disadvantages. Worm gonads adhere very strongly to subbed slides, making tissue loss less likely but also decreasing the chances that the gonad will spread out completely and lay flat on the slide. Worm gonads adhere less strongly to charged slides, making it easier to get a nicely spread gonad at the expense of increased likelihood of tissue loss.
10. Making 200 mL of subbing solution requires 200 mg of poly-L-lysine. For convenience, I order 2 × 100 mg vials of poly-L-lysine (Sigma P1524-100MG). Alternatively, one can order larger vials to minimize costs; however, due to the sticky, cotton candy-like nature of poly-L-lysine, it is difficult to weigh out small amounts. When larger volumes are ordered, I recommend dissolving the entire volume to a 100 mg/mL concentration in water. The aqueous solution can be stored at −20 °C for up to a year.

11. Other investigators prefer #11 blades, while still others prefer to use a pair of 25 GA syringe needles. The best tool for the job is the one that gets the head off the worm most quickly in your hands!
12. The humid chamber prevents evaporation of the small amounts of solution applied to slides during prolonged incubations. Any airtight container lined with a layer of an absorbent material soaked with water can serve as a humid chamber. I prefer Snapware brand 4.5 cup food storage containers, which have a tightly sealing, gasketed lid. In each Snapware container, we place a single layer of open-weave tool drawer liner (e.g., Cleverbrand liner, ASIN B003XNWD1U, or Craftsman 65514), several layers of paper towels, and then another layer of drawer liner. The top layer of drawer liner elevates the slides slightly above the damp paper towels and prevents them from shifting when the chamber is moved, while the layer of paper towels and the bottom layer of drawer liner retain enough water to ensure that the box remains humid for the duration of the experiment.
13. The freeze-crack permeabilization protocol we use relies on rapid freezing of the fixed sample, which is immobilized under a cover glass, followed by abrupt removal of the cover glass. To freeze slides in liquid nitrogen, I place a 250 mL Nalgene beaker in a Styrofoam shipping box, then add liquid nitrogen until the beaker is full and the bottom of the box is covered by about an inch. After each slide is fixed, it is lowered into the beaker of liquid nitrogen. The beaker keeps the slides in an upright position, which simplifies their later retrieval. Others prefer to freeze slides by pressing them against a flat aluminum block in a bed of dry ice [39]. In my hands, the two methods produce equivalent results.
14. The original *C. elegans* egg buffer was developed by Lois Edgar to sustain development of embryonic blastomeres that have been removed from their eggshells [40]. This formulation was adopted and subsequently modified by Abby Dernburg and colleagues to preserve chromosomal morphology during fixation of gonadal tissues [39]. The revised formulation has a higher concentration of HEPES buffer and replaces the sources of divalent cations ($CaCl_2$ and $MgCl_2$) with chelators (EDTA and EGTA), which may reduce nuclease activity. I have used both formulations for gonadal staining with equivalent results. I usually make 100 mL batches and store 5 mL aliquots at –20 °C.
15. Levamisole is an acetylcholine receptor agonist and triggers muscle contraction and paralysis in *C. elegans*. Paralysis aids in the dissection of gonadal tissues, while the contraction of body wall muscles may facilitate extrusion of the gonad. Once thawed, aliquots of levamisole can be stored at 4 °C for 1–2

months, although the effectiveness will diminish over time. Other investigators prefer to immobilize worms with sodium azide (15–30 mM final concentration) [39, 41].

16. Staining with LacI-His_6-GFP is compatible with most standard immunofluorescence protocols used for analysis of *C. elegans* meiosis. GFP foci are usually bright enough to image without use of additional antibodies, at least when analyzing *syIs44* strains. Nevertheless, I typically stain with an anti-GFP primary antibody and a secondary labeled with Alexa 488 or an equivalent fluor. I have not determined whether LacI-His_6-GFP is compatible with fluorescent *in situ* hybridization (FISH). However, others have shown that 256× *lacO* repeats can be reliably detected with a fluorescently labeled oligonucleotide FISH probe [32].
17. The *sup-9* alleles used here were identified in screens for suppressors of a semidominant, gain-of-function *unc-93* allele [42]. Neither allele causes a detectible phenotype other than suppression of *unc-93*.
18. Although the total volume of each PCR reaction will be 30 μL, only 2 μL of 10× PCR buffer per reaction is needed because the 10 μL of worm lysate used as template already includes 1× PCR buffer.
19. In principle, the sexes can be reversed and the cross can be set up with hermaphrodites that inherited the balancer and males that did not. However, since many balancers are dominantly marked by GFP expression, passing the balancer through the male allows the identification of cross progeny in the next generation.
20. Other strategies are necessary when working with unmarked or homozygous viable balancers or when using meiotic recombination to link *syIs44* to a mutation on the same chromosome. These approaches are beyond the scope of this review.
21. If genotyping for more than two loci, use 5 μL of lysis buffer per locus. With well-designed oligos, the lysate from a single adult worm is sufficient for at least five PCR reactions.
22. In my experience teaching newbies to PCR genotype worms, inconsistent amplification (i.e., a few reactions work very well but most fail miserably) is almost always a consequence of insufficient mixing, which leaves most of the *Taq* polymerase on the bottom of the tube of mastermix.
23. The GFP signal is often strongest in intestinal nuclei, but since these nuclei are polyploid, the number of GFP foci cannot be used to determine homozygosity. Be careful to analyze only healthy adult worms, since heat shock often does not induce robust GFP-LacI expression in elderly worms.

24. It is also possible to extrude the gonad by nicking the tail of the worm. However, in my hands the best extrusion occurs when the incision is made just posterior to the pharyngeal bulb.
25. In regions where two nuclei contact one another, it may not be possible to determine which nucleus a LacI-GFP focus belongs to. In such cases, both nuclei should be excluded from the analysis.
26. The following process simplifies pasting the coordinates of two subnuclear foci into one row of the spreadsheet: After copying the data from the Fiji Results window, paste the data into columns A–F of the spreadsheet, leaving a single empty row between any existing data and the data you are pasting. Next, paste the same data into columns G–L, but this time, do not leave an empty row above the pasted data. Delete the first row (the row with empty cells in columns A–F and the coordinates of the first focus analyzed in columns G–L) and every second row after that.
27. Many meiotic mutants produce aneuploid embryos that die during embryogenesis. Thus, one cannot use the presence of male progeny to determine when a cross has started working. For this reason, it is helpful to use males that are homozygous for a transgene that drives robust GFP expression, such as *mIs10[Pmyo-2::gfp]*. Because many aneuploid embryos undergo cellular differentiation before arresting, the pharyngeal GFP expressed from the *mIs10* transgene can be detected in arrested embryos once the cross is working.
28. This cross will allow analysis of defects in chromosome segregation during oocyte meiosis. To analyze defects during spermatogenesis, mate mutant males produced in Cross 1 with wild-type hermaphrodites [9]. If the mutation of interest results in high levels of aneuploidy in sperm, successful crosses can be identified by the presence of dead embryos.
29. We collect 12–15 embryos from each of 8–12 crosses for three reasons. First, if some embryos produced by a hermaphrodite hatch and others arrest during embryonic development, it is easy to introduce experimental bias by selecting a disproportionate number of dead embryos or hatched larvae for analysis. This can be avoided by collecting a relatively small number of progeny from each hermaphrodite and genotyping them all for the *sup-9* alleles. Second, at least six embryos or larvae should be analyzed from each worm. If six or more embryos all have the *sup-9(n1012)* allele derived from the grandmother but lack the *sup-9(n1020)* allele derived from the grandfather, the grandmother was almost certainly a *sup-9(n1012)* homozygote rather than the desired *sup-9(n1012)/sup-9(n1020)* transheterozygote. Third, embryos from at least six worms should be included in the analysis in case there is brood-to-brood variability.

30. Avoid sucking the embryo or larva very far into the tip. Ideally, one should stop applying suction as soon as the specimen disappears into the tip. If a large volume of liquid is drawn into the capillary, a large volume of liquid will also need to be expelled from the capillary to eject the embryo. This will change the volume and composition of the lysis reaction.
31. Commercial hot-start products are not necessary for successful amplification of the *sup-9* amplicon. However, this single-embryo PCR is somewhat touchy, and I recommend keeping the PCR plate on ice or in a 96-well plate cooling block until the thermocycler has reached the denaturation temperature. The program can then be paused temporarily while the PCR plate is placed in the machine.
32. The recipes described here were developed using enzymes and buffers from New England Biolabs, but they should work equally well with enzymes from other manufacturers with minimal changes.
33. I usually place the three 96-well plates in covered 96-well plate racks, then place these in a single layer on the bottom of a covered plastic bin that I have pre-warmed to 37 °C. I put several layers of wet paper towels both underneath and on top of the plates to minimize any chance of evaporation. However, this may not be necessary.
34. It is formally possible that patterns of chromosome segregation other than those described here could give rise to similar patterns of RFLP inheritance. For example, the reason that the progeny of *rec-8* mutant hermaphrodites test positive for both RFLPs is that premature, equational sister separation occurs during meiosis I, then polar body extrusion fails during meiosis II. The zygote therefore inherits one sister from each homolog. Consistent with this, DAPI staining of fixed 1 and 2 cell-stage embryos shows that most embryos have a single polar body [9]. The same pattern of RFLP inheritance would be observed if polar body extrusion failed during both rounds of meiosis and the zygote inherited every chromatid from every homolog. However, in such cases, zygotes would not have any polar bodies. Thus, it is desirable to examine DAPI stained early embryos (1–4 cell stage) and also LacI-GFP/*lacO* in pronuclear migration-stage embryos to validate the conclusions drawn through RFLP analysis [9].

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