CHAPTER TWELVE

Monitoring Recombination During Meiosis in Budding Yeast

Shannon Owens[*,†,‡], **Shangming Tang**[*,†,‡], **Neil Hunter**[*,†,1]
[*]Howard Hughes Medical Institute, University of California, Davis, Davis, CA, United States
[†]University of California, Davis, Davis, CA, United States
[‡]Biochemistry, Molecular, Cellular and Developmental Biology Graduate Group, University of California, Davis, Davis, CA, United States
[1]Corresponding author: e-mail address: nhunter@ucdavis.edu

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Abstract

Homologous recombination is fundamental to sexual reproduction, facilitating accurate segregation of homologous chromosomes at the first division of meiosis, and creating novel allele combinations that fuel evolution. Following initiation of meiotic

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recombination by programmed DNA double-strand breaks (DSBs), homologous pairing and DNA strand exchange form joint molecule (JM) intermediates that are ultimately resolved into crossover and noncrossover repair products. Physical monitoring of the DNA steps of meiotic recombination in *Saccharomyces cerevisiae* (budding yeast) cultures undergoing synchronous meiosis has provided seminal insights into the molecular basis of meiotic recombination and affords a powerful tool for dissecting the molecular roles of recombination factors. This chapter describes a suit of electrophoretic and Southern hybridization techniques used to detect and quantify the DNA intermediates of meiotic recombination at recombination hotspots in budding yeast. DSBs and recombination products (crossovers and noncrossovers) are resolved using one-dimensional electrophoresis and distinguished by restriction site polymorphisms between the parental chromosomes. Psoralen cross-linking is used to stabilize branched JMs, which are resolved from linear species by native/native two-dimensional electrophoresis. Native/denaturing two-dimensional electrophoresis is employed to determine the component DNA strands of JMs and to measure the processing of DSBs. These techniques are generally applicable to any locus where the frequency of recombination is high enough to detect intermediates by Southern hybridization.

1. INTRODUCTION

Meiosis is the specialized cell division that serves as the conduit for heredity, producing haploid gametes from diploid germline cells through two successive cell divisions (Hunter, 2015). Homologous chromosomes (homologs) segregate during the first division, and sister chromatids separate at the second division. Accurate segregation during meiosis-I requires that homologs become connected by chiasmata—the conjunction of interhomolog crossing over and sister chromatid cohesion (Fig. 1). These de novo chromosome connections enable stable bipolar attachment of homolog pairs to the spindle, which is the essential prelude to accurate disjunction. Crossover recombination also produces new combinations of gene alleles upon which natural selection can act. Thus, meiotic crossing-over underlies the fundamental laws of heredity.

Chiasma formation is the culmination of an elaborate series of interdependent chromosomal events that include programmed homologous recombination, and the intimate pairing and synapsis of homologs (Fig. 1). Meiotic recombination is initiated by programmed DNA double-strand breaks (DSBs), numbering ~200–300 per nucleus in budding yeast, mouse, and human (Lam & Keeney, 2014). Ensuing recombinational interactions promote the pairing of homologous chromosomes, and their intimate synapsis by zipper-like structures called synaptonemal complexes (SCs)

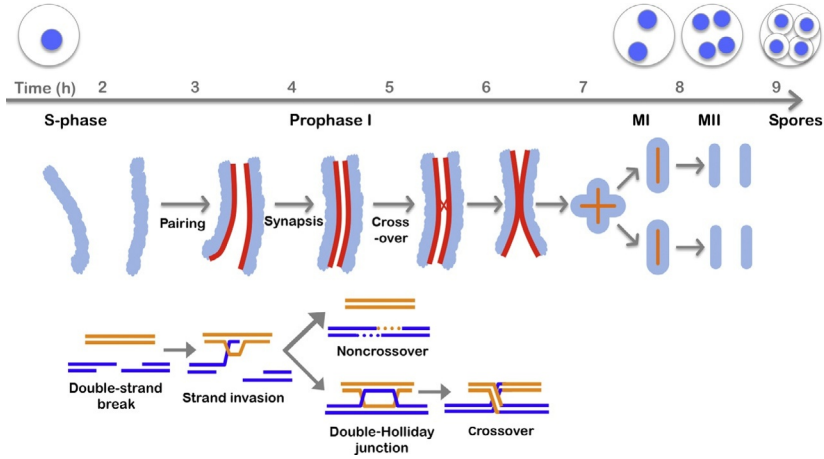

Fig. 1 Meiosis timeline in budding yeast illustrating the major nuclear, chromosomal, and DNA events of meiotic prophase.

(Fraune, Schramm, Alsheimer, & Benavente, 2012; von Wettstein, Rasmussen, & Holm, 1984; Zickler & Kleckner, 1999). Within the context of SCs, a few recombinational interactions mature into crossovers. After SCs are removed, homologs remain connected at their crossover points allowing them to condense into bivalent structures that can stably biorient on the spindle, and faithfully disjoin at the first meiotic division (Petronczki, Siomos, & Nasmyth, 2003; Watanabe, 2012).

The primary role of crossing-over in homolog disjunction dictates that each pair of homologs becomes connected by at least one exchange. Regulatory processes help ensure that each homolog pair obtains this obligatory crossover (crossover assurance), while total crossover levels remain low (on the order of one crossover per chromosome arm) (Jones, 1984). Crossover numbers appear to be minimized by a process called interference, which prevents two crossovers from forming in close proximity between the same chromosomes. Interference acts over huge physical distances. Consequently, on small chromosomes interference can preclude the formation of a second crossover, while on longer chromosomes interference causes adjacent crossovers to be widely and evenly spaced (Zickler & Kleckner, 2015).

Historically, fungal genetics has dominated studies of meiotic recombination because the four haploid products of individual meioses can be recovered in asci and subject to genetic analysis. Fungal genetics deduced the key features that define contemporary models of meiotic recombination including initiation by DSBs, strand exchange to form heteroduplex DNA and

joint molecule (JM) intermediates called double-Holliday junctions (dHJs) (Szostak, Orr-Weaver, Rothstein, & Stahl, 1983). These DNA intermediates were subsequently confirmed in budding yeast, *Saccharomyces cerevisiae*, using the gel electrophoresis and Southern hybridization methods described in this chapter (Allers & Lichten, 2001a, 2001b; Cao, Alani, & Kleckner, 1990; Hunter & Kleckner, 2001; Schwacha & Kleckner, 1994, 1995; Sun, Treco, Schultes, & Szostak, 1989).

A working model of meiotic recombination is shown in Fig. 2. DSB formation is catalyzed by the Spo11 transesterase (Lam & Keeney, 2014). DSB-ends undergo nucleolytic resection to remove ~800 nt of the 5′ strand yielding long single-stranded 3′-terminal tails (Mimitou, Yamada, & Keeney, 2017; Zakharyevich et al., 2010), which then assemble into nucleoprotein complexes comprising an oligomeric filament of a RecA-family protein (Dmc1 and/or Rad51) (Brown & Bishop, 2015). Dmc1 filaments catalyze pairing and strand exchange between the DSB-end and a homologous duplex to form a nascent JM called a displacement loop (D-loop). The D-loop structure provides a primer-template for the local DNA synthesis that is essential to repair the DSB and restore sequences removed during resection. Meiotic recombination ensues via one of two major pathways, distinguished by the fate of the nascent D-loop (Fig. 2). In the synthesis-dependent strand-annealing (SDSA) pathway, the extended strand is dissociated and anneals to complimentary sequences on the second DSB-end (McMahill, Sham, & Bishop, 2007; Nassif, Penney, Pal, Engels, & Gloor, 1994; Paques & Haber, 1999). SDSA always results in a noncrossover outcome. In the dHJ pathway, a metastable one-ended strand exchange intermediate called a Single-End Invasion (SEI) is formed, likely through extension of heteroduplex (Hunter & Kleckner, 2001). The second DSB-end is then engaged to form a dHJ intermediate in which recombining duplexes are connected by two exchange or "Holliday" junctions (Schwacha & Kleckner, 1995; Szostak et al., 1983). In theory, dHJ resolution can yield either a crossover or noncrossover, but in meiosis resolution is biased to yield crossovers (Allers & Lichten, 2001a, 2001b; Clyne et al., 2003; Zakharyevich, Tang, Ma, & Hunter, 2012).

Central to the physical monitoring of recombination intermediates are synchronous meiotic time course experiments that allow detection of transient events (Fig. 1). Genomic DNA is extracted from cells sampled at various time throughout meiosis and subject to gel electrophoresis and Southern analysis to detect and quantify DNA events at defined recombination/DSB hotspots, such as the *HIS4::LEU2* and *ERG1* loci (Hunter &

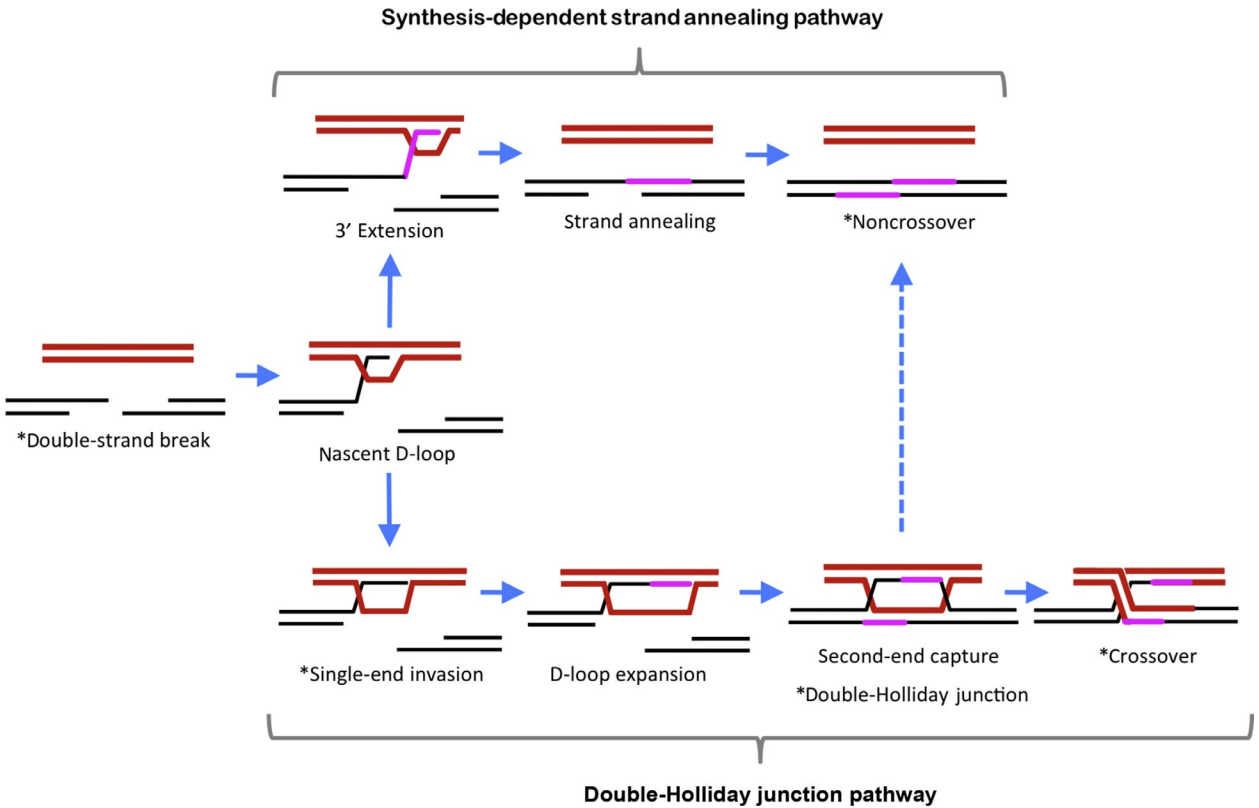

Fig. 2 Model of meiotic recombination (see text for details). *Asterisks* indicate DNA species detectable using the assays described here. Recombination-associated DNA synthesis is highlighted in *magenta*.

Kleckner, 2001; Lao, Cloud, et al., 2013; Thacker, Mohibullah, Zhu, & Keeney, 2014). Restriction enzyme site polymorphisms produce DNA fragments that are diagnostic of parental chromosomes, JMs formed between homologs or sister chromatids, crossovers, and noncrossovers (Fig. 3). JM intermediates may be lost through spontaneous branch migration, which is exacerbated by DNA purification treatments that employ heat and EDTA to inhibit nucleases (Panyutin & Hsieh, 1994). To preserve JMs, cells sampled from meiotic time courses are treated with psoralen and UV light to induce interstrand DNA cross-links (Frobel, Reiffers, Torres

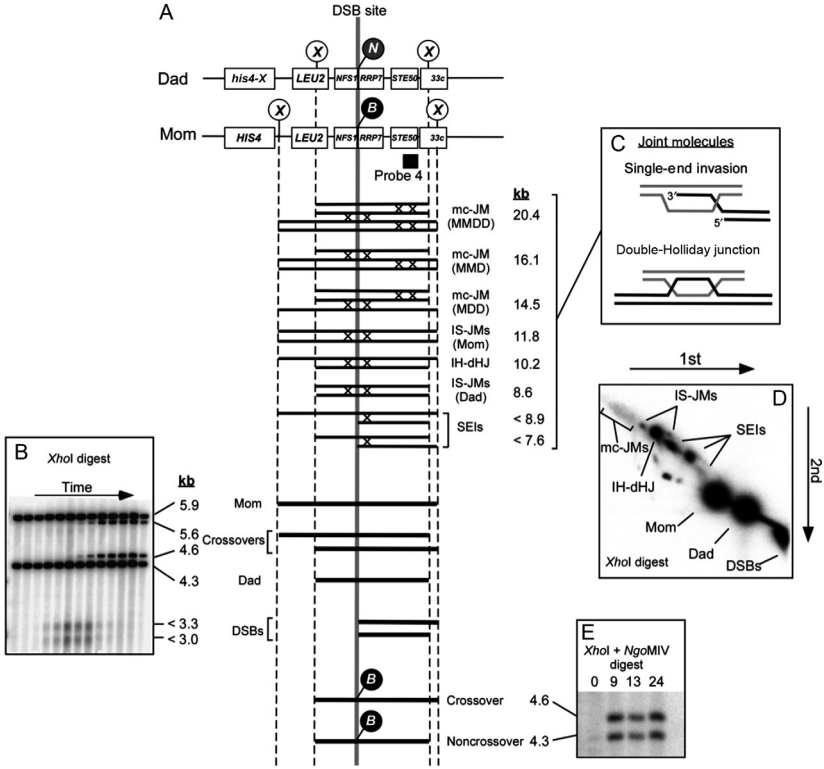

Fig. 3 Physical assay system for monitoring meiotic recombination. (A) Map of the *HIS4::LEU4* locus showing diagnostic restriction sites and position of the probe. DNA species detected are shown. *B*, *Bam*HI; *DSB*, double-strand break; *IH-JM*, interhomolog joint molecule; *IS-JM*, intersister joint molecule; *mc-JM*, multichromatid joint molecule; *N*, *Ngo*MIV; *SEI*, single-end Invasion; *X*, *Xho*I. (B) Southern image of one-dimensional gel analysis of crossovers and DSBs. (C) Inferred structures of SEI and dHJ intermediates. (D) Southern image of native/native 2D gel analysis of JMs. Species detailed in (A) are highlighted. (E) Noncrossover analysis. Southern image of *Xho*I + *Ngo*MIV double-digested genomic DNA showing diagnostic bands (see Fig. 4). Note that the noncrossover assay detects a representative subset of total recombinants.

Ziegenbein, & Gilch, 2015). One- and two-dimensional (1D and 2D) electrophoresis is applied to resolve DNA species of interest. Conditions optimized for 2D analysis resolve DNA molecules according to molecular weight in the first dimension but exaggerate any shape component in the second dimension such that branched DNA molecules, such as JMs, migrate more slowly than linear molecules of the same molecular weight (Bell & Byers, 1983) (Fig. 3).

The suit of assays described here allow the following steps to be monitored: DSB formation and resection, strand exchange to form SEIs, second-end capture to form dHJs, and resolution to form crossovers and noncrossover products (Cao et al., 1990; Hunter & Kleckner, 2001; Schwacha & Kleckner, 1995; Zakharyevich et al., 2010). In addition, interhomolog bias (template choice), at both SEI (Kim et al., 2010) and dHJ (Lao, Cloud, et al., 2013; Schwacha & Kleckner, 1994) stages, can be determined by measuring the ratio of interhomolog to intersister JMs. Nonallelic (ectopic) crossing over can also be monitored between the *HIS4::LEU2* locus and homologous sequences located ~25 kb away (Grushcow et al., 1999).

Native/denaturing 2D gel electrophoresis allows the strand composition of DSBs and JMs to be determined (Lao, Tang, & Hunter, 2013; Oh et al., 2009; Schwacha & Kleckner, 1995). In this case, DNA molecules are denatured prior to running the second-dimension gel under denaturing conditions. This method has been utilized to measure the extent of DSB resection (Zakharyevich et al., 2010) and to infer the structures of JMs including SEIs, dHJs, recombinant JMs (containing crossover and parental length strands), and aberrant JMs containing three and four interacting duplexes (multichromatid JMs) (Allers & Lichten, 2001a, 2001b; Hunter & Kleckner, 2001; Oh et al., 2007; Oh, Lao, Taylor, Smith, & Hunter, 2008; Schwacha & Kleckner, 1995). For JM analysis by native/denaturing 2D gels, psoralen cross-links must be removed in situ, within gel slices, before running samples in the second dimension under denaturing conditions.

2. SYNCHRONOUS MEIOTIC CULTURES, PSORALEN CROSS-LINKING, AND PREPARATION OF GENOMIC DNA

2.1 Meiotic Time Course—Pregrowth, Sporulation, and Psoralen Cross-Linking

S. cerevisiae strains that sporulate efficiently, such as SK1 (Kane & Roth, 1974), can be induced to undergo meiosis with reasonable synchrony

enabling temporal analysis of meiotic events. For a full temporal analysis of the DNA events of meiosis, cultures are sampled at 0, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, and 10 h after transfer to sporulation medium. These times may need to be adjusted for mutants that delay the progression of meiosis. Standard culture growth and sporulation methods are detailed later. Sharper synchrony may be achieved using a recently developed method that triggers meiosis by inducing expression of *IME1*, the master transcriptional regulator of meiosis (Chia & van Werven, 2016).

2.1.1 Equipment

Suppliers are listed, but equivalent products can be substituted.

1. 2.8-L triple-baffled Fernbach flasks (Bellco, Part # 2551-02800).
2. Orbital platform shaker in 30°C warm room (New Brunswick Innova or similar)
3. 500-mL centrifuge bottles (Corning, Manufacturer # 431123).
4. 50-mL disposable conical plastic tubes (Falcon, Manufacturer # 352070).
5. 60 × 15-mm Petri dishes (Corning, Manufacturer # 430166).
6. Long-wave UV transilluminator (UVP High Performance UV Transilluminator, Variable Intensity Model, 365 nm, or similar model).
7. Benchtop centrifuge (Sorvall Legend RT or similar)

2.1.2 Culture Media, Buffers, and Other Reagents

Suppliers are given where deemed important; in the absence of an indication, any high-quality source will do.

1. YPG agar: 1% (w/v) Bacto™ yeast extract (BD, Franklin Lakes, NJ), 2% (w/v) AMRESCO peptone (VWR), 1.5% agar (molecular biology grade, Teknova), 2% glycerol.
2. YPD broth: 1% (w/v) Bacto™ yeast extract, 2% (w/v) AMRESCO peptone, 2% D-glucose, 0.005% adenine hemisulfate (Sigma-Aldrich, A9126). Prepare broth without glucose or adenine as a 1.05 × solution and autoclave, mix with sterile 40% (w/v) D-glucose in a 19:1 ratio, and sterile 0.5% adenine hemisulfate in a 99:1 ratio before use. YPD agar is identical but contains 2% agar.
3. Presporulation broth (SPS): 1% (w/v) AMRESCO peptone (VWR), 0.5% (w/v) Bacto™ yeast extract, 1% (w/v) potassium acetate, 0.17% (w/v) Difco™ yeast nitrogen base without amino acids, 0.5% (w/v) ammonium sulfate, 1.5% (w/v) potassium hydrogen phthalate. Adjust pH to 5.5 with 10 N KOH, as sodium inhibits sporulation.

Make fresh and autoclave. Add two drops of Antifoam 204 (Sigma-Aldrich, A6426) per liter of media with a P200 pipette.

4. Sporulation medium (KAc): 1% (w/v) potassium acetate, 0.02% (w/v) D-(+)-raffinose pentahydrate (low glucose; US Biological). Add two drops of Antifoam 204 per liter of media from a P200 pipette. Additional nutrients (amino acids, purines, pyrimidines) required to supplement auxotrophic requirements of the strain being sporulated are added at 1/5th the levels used in vegetative growth medium.
5. DAPI stock: 1 μg/mL 4′,6-diamidino-2-phenylindole.
6. Fixation solution: 70% ethanol.
7. 10% (w/v) sodium azide.
8. 5 × psoralen stock solution: 0.5 mg/mL Trioxsalen dissolved in ethanol (200 proof). Store at 4°C wrapped in aluminum foil. To completely dissolve prior to use, shake at room temperature overnight.
9. 1 × psoralen working stock: diluted in 50 m*M* Tris–HCl pH 8.0, 50 m*M* EDTA pH 8.0. Keep on ice, wrapped in aluminum foil. Make fresh on the day of the experiment.
10. 50 m*M* EDTA, 50 m*M* Tris–HCl pH 8.0.

2.1.3 Procedure

Day 1 (5 days prior to the time course experiment)

1. Patch desired diploid strain from −80°C glycerol stock onto YPG plate.
2. Incubate at 30°C overnight for <17 h to select against petite colonies.

Day 2

3. Streak for single colonies on YPD plates.

Day 4

4. Inoculate single diploid colonies into 5 mL YPD broth. Grow to saturation at 30°C overnight.
5. Prepare and autoclave SPS (500 mL for each strain to be sporulated, plus a few mL extra to use as a blank in the spectrophotometer), and 1% SPM (1 L per strain). Autoclave three 2.8-L baffled flasks, one 1-L graduated cylinder, and two 500-mL graduated cylinders per strain to be sporulated.

Day 5

6. Measure 250 mL of SPS into each of two 2.8-L baffled flask.
7. 17 h before the start of the time course experiment, dilute cells 1/800 and 1/1000 from YPD overnight cultures into 250 mL SPS.
8. Incubate with vigorous shaking (350 RPM) at 30°C for 17 h. Make sure that the flasks are tightly clamped.

9. Measure 500 mL SPM into a 2.8-L baffled flask (one for each strain to be sporulated). Prewarm flasks at 30°C and keep remaining 1% SPM at 30°C. Note that volumes up to 1 L can be sporulated in 2.8-L baffled flasks, but may not aerate properly causing inefficient/asynchronous meiosis. 500 mL maintains ideal aeration.

Day 6

10. After 17 h of growth, measure the OD_{600} of each culture. Select cultures with an OD_{600} ~1.0 (but not higher than 1.1). Transfer 0.5 mL of selected cultures into a 1.5-mL microfuge tube, harvest the cells by centrifugation, and resuspend in 0.5 mL ice-cold 70% ethanol. Stain cells with DAPI by mixing 5 μL cells with 5 μL DAPI stock. Place 4–5 μL of stained cells onto a slide, cover with a coverslip, and view cells by fluorescence microscopy. Choose cultures with ≥80% of G1/G0 cells (defined as cells with no bud and with a single DAPI-staining body).
11. Transfer selected cultures into 500-mL centrifuge bottles and harvest cells at 3200 × *g*, at 28°C, for 3 min. Wash cells by resuspending in an equal volume of 1% SPM. Harvest once more and resuspend in the 500 mL prewarmed SPM in the 2.8-L baffled flask. Take the zero time-point sample (see next step) and incubate the culture with vigorous shaking (350 RPM) at 30°C.
12. Sampling volumes and times will depend on the goal of the experiment and the specific strain being analyzed. For each sample, collect at least 25 mL of culture in a 50-mL conical tube containing a 1:100 dilution of 10% sodium azide. 50 mL of culture should be collected for the zero time point, as DNA replication has not yet occurred and DNA content is half that of subsequent samples. Also transfer 0.5 mL of culture into a 1.5-mL microfuge tube, harvest the cells by centrifugation, and resuspend in 0.5 mL ice-cold 70% ethanol. Freeze at −20°C or process immediately for DAPI staining to monitor the progression of meiotic divisions.
13. Centrifuge the larger samples in the 50-mL tubes, at 3200 × *g* for 3 min. Pour off the supernatant and resuspend cell pellets in 2.5 mL ice-cold 1 × psoralen solution.
14. Transfer the cell/psoralen mixture to a 60-mm Petri dish and place on the long-wave UV box on the "high" setting. Allow cells to expose for 10 min, swirling the cells twice during this period.
15. Transfer the cells back into the original 50-mL tube. Recover any remaining cells in the Petri dish by washing with 2 mL ice-cold 50 m*M* EDTA/50 m*M* Tris–HCl solution and add to the original tube.

16. Harvest the cells at 3200 × *g* for 3 min and drain well. Spin again briefly and pull off last traces of supernatant with a P200 and freeze at −20°C.

2.2 DNA Extraction and Purification—Guanidine/Sarkosyl Preparation of Psoralen Cross-Linked DNA

2.2.1 Equipment

1. 1.5-mL microfuge tubes.
2. 37°C water bath; 65°C water bath.
3. Wide-orifice 1-mL micropipette tips (Rainin, RT-L1000W).

2.2.2 Buffers and Reagents

1. Spheroplasting buffer: 1 *M* sorbitol, 50 m*M* potassium phosphate pH 7.0, 10 m*M* EDTA pH 7.5. Filter sterilize and store at 4°C.
2. β-Mercaptoethanol.
3. Zymolyase 100T (US Biological).
4. Guanidine lysis solution pH 8.0: 4.5 *M* guanidine-HCl, 0.1 *M* EDTA, 0.15 *M* NaCl, 0.05% sodium lauroyl sarcosinate (Sarkosyl). Adjust to pH 8.0 with 50% NaOH.
5. RNase stock solution: 10 × TE pH 8.0 (100 m*M* Tris–HCl, 10 m*M* EDTA pH 8.0), 50 μg/mL RNase (DNase-free). Store at −20°C.
6. Proteinase-K solution: 20 mg/mL proteinase-K, 20 m*M* $CaCl_2$, 10 m*M* Tris–HCl pH 7.5, 50% glycerol. Store at −20°C.
7. Phenol pH 8.0/chloroform/isoamyl alcohol mixed in a 25:24:1 ratio.
8. 200 Proof ethanol.
9. 3 *M* sodium acetate pH 5.2.
10. 1 × TE pH 8.0 (10 m*M* Tris–HCl, 1 m*M* EDTA pH 8.0).

2.2.3 Procedure

1. Prepare sufficient spheroplasting buffer to allow 0.5 mL per cell pellet sample. Add 0.25 mg zymolyase 100T and 1% β-mercaptoethanol per sample. To process 12 samples (25 mL of SPM culture) from a typical time course requires 6 mL spheroplasting buffer with 3 mg zymolyase and 60 μL β-mercaptoethanol. For samples from larger volumes of culture, increase the spheroplasting buffer volume (for example, 0.45 mL of culture will require 0.9 mL spheroplasting buffer).
2. Thoroughly resuspend the cell pellets from Section 2.1.3 (step 15) in 0.5 mL spheroplasting buffer. Incubate at 37°C for 15 min. Gently flick

the tubes with fingers to mix the sample (finger-vortexing) twice during the incubation. See Note 1.

3. Harvest spheroplasted cells by centrifugation for 4 min, at 1500 × *g*. Using a P1000 pipette, carefully remove as much of the supernatant as possible without disturbing the loose cell pellet.
4. Add 1.5 mL guanidine lysis solution and resuspend the stringy spheroplast pellet by gentle finger-vortexing. Place at 65°C for 20 min. Finger vortex several times during this incubation to completely disperse the pellet and lyse the cells (incubation time can be extended 10–20 min to ensure the pellet is completely lysed).
5. Cool tubes on ice. Add 1.5 mL ethanol. Mix tubes well by inversion and store at −20°C overnight (alternatively, incubate at −20°C for at least 20 min before proceeding).
6. Pellet the precipitated material by centrifugation for 15 min at 3200 × *g* in a benchtop centrifuge. Drain well, centrifuge briefly, and carefully remove the last traces of supernatant with a pipette.
7. Add 0.5 mL RNase solution. Dislodge the pellet from the bottom of the tube by finger vortexing and allow the pellet to sit in the RNase solution for 5 min. Once the pellet has loosened, gently break up the pellet by finger vortexing (do not use a pipette at this stage). Incubate in a 37°C water bath for 1 h. Finger vortex several times during this incubation to completely disperse the pellet.
8. Add 15 μL proteinase-K solution and incubate at 65°C for 1 h. Finger vortex several times during this incubation. Spin tubes briefly to collect all solution to the bottom, and then transfer to a microfuge tube using wide-orifice pipette tips. At this point, cell lysates may be frozen at −20°C overnight before proceeding to the next step.
9. Add 0.5 mL phenol/chloroform/isoamyl alcohol (25:24:1). Shake and invert for ~30 s to mix thoroughly, let stand for 3 min, and shake again (do not vortex). Centrifuge at full speed (15,000 RPM) for 10 min in a microcentrifuge. *Carefully* remove the upper aqueous layer using a wide-orifice pipette tip and dispense into a new microcentrifuge tube, avoiding the white interface.
10. Extract a second time with 0.5 mL phenol/chloroform/isoamyl alcohol. Centrifuge at full speed in a microcentrifuge for 10 min. Carefully remove the upper, aqueous layer to a fresh microcentrifuge tube. See Note 2.
11. Ethanol precipitate the DNA: add 1/20th volume of 3 *M* sodium acetate pH 5.2 and two volumes of 100% ethanol. Mix well by

inversion, and let the samples stand for 20 min. DNA should form a visible precipitate. Centrifuge briefly and decant the supernatant. If no spool is visible or the spool is very small, centrifuge for 5 min at full speed in a microcentrifuge.

12. Rinse the DNA pellets by adding 1 mL 70% ethanol, centrifuge briefly, decant the supernatant, and drain the tubes. Pulse-spin in the centrifuge and remove the last traces of ethanol from the DNA pellets with a pipette.
13. Air dry the DNA pellets for at least 10 min (or until pellet looks dry). See Note 3. Add 50 μL 1 × TE and allow the DNA to hydrate in the refrigerator overnight. Mix well by flicking the tube. Do not vortex.
14. Determine DNA concentration of sample and adjust as required (see Section 2.3).
15. Store samples at −20°C.

2.3 Quantification of DNA Concentration

2.3.1 Equipment

1. Promega GLOMAX multidetection system (or equivalent 96-well plate reader).
2. 96-Well assay plate (Flat bottom black polystyrene; COSTAR®).

2.3.2 Buffers and Reagents

1. 10 × TNE pH 8: 0.5 *M* Tris–HCl, 1 *M* NaCl, 10 m*M* EDTA.
2. Hoechst 33342 stock solution: 1 mg/mL in water. Store in the dark at 4°C.

2.3.3 Procedure

1. Prepare Hoechst working solution. For 50 mL: 5 mL 10 × TNE buffer, 5 μL Hoechst 33342 stock solution and 45 mL Milli-Q water. Mix well and wrap in aluminum foil at room temperature. Prepare fresh each day.
2. Add 199 μL Hoechst working solution to all wells that will be used. To the first 8–10 wells, add 1 μL each from a dilution series of DNA standards ranging from 50 to 600 ng/μL. We dilute DNA standards from a 10-mg/mL stock of salmon sperm DNA (Sigma-Aldrich, D1626) and measure their concentrations on a NanoDrop spectrophotometer. Fluorescence readings from these samples are used to prepare a standard curve in Microsoft Excel by plotting concentration vs absorbance, and adding a trendline (best fit). The equation from this trendline is used

to calculate the concentrations of all DNA samples from their fluorescence readings.

3. Starting in the next row, add 1 μL of each meiotic DNA sample to the Hoechst working solution using a P2 pipette and mix all samples by pipetting up and down with a P200 pipette.
4. Read the fluorescence intensity of each sample using the Hoechst settings in the Promega GLOMAX multidetection system. Average the fluorescence readings for all blanks wells (wells that only contain Hoechst working solution) to obtain an average background signal. Subtract this signal from the fluorescence readings of all DNA samples.
5. Use Microsoft Excel to calculate the DNA concentration according to the standard curve. Adjust DNA concentrations to 100–500 ng/μL with TE buffer (we typically adjust to 200 ng/μL).

3. ONE-DIMENSIONAL NATIVE GEL ELECTROPHORESIS

3.1 DNA Digestion and One-Dimensional Gel Electrophoresis

3.1.1 Equipment

1. Buffer Puffer™ gel box, Model A5 (Owl Separation Systems) with a 36-well comb (Fig. 5).
2. Long-wave UV transilluminator (UVP High Performance UV Transilluminator, Variable Intensity Model, 365 nm, or similar model).

3.1.2 Buffers and Reagents

1. *Xho*I (20,000 U/mL, #R0146L) and *Ngo*MIV (10,000 U/mL, #R0564L) restriction enzymes and CutSmart buffer (New England Biolabs)
2. SeaKem LE agarose (Lonza).
3. TBE: 90 m*M* Tris base, 90 m*M* boric acid, 2 m*M* EDTA. Prepare as 10× stock (900 m*M* Tris base, 900 m*M* boric acid, 20 m*M* EDTA) and dilute to 1× as needed.
4. Loading buffer: 100 μL 6× loading dye +60 μL 10× NEB3 restriction enzyme buffer (New England Biolabs; the additional salt prevents samples from drifting out of the wells).
5. 6× loading dye: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll in water.

6. λ*Bst*EII and λ*Hin*dIII DNA molecular weight markers (New England Biolabs).
7. 10 mg/mL ethidium bromide in dH_20. Store at room temperature wrapped in aluminum foil.

3.1.3 Procedure

Day 1

1. For *DSB and crossover* analysis, digest 2 μg of DNA in 80 μL total volume with 5 μL *Xho*I, and 8 μL CutSmart buffer. Incubate at 37°C for 4 h. This releases parental, crossover, and double-strand break fragments (Fig. 3).
2. For noncrossover analysis, digest 2 μg of DNA in 120 μL total volume with 5 μL *Xho*I, 5 μL *Ngo*MIV, and 12 μL CutSmart buffer. Incubate at 37°C for 4 h. This combination of enzymes produces a fragment that is diagnostic for a subset of noncrossovers formed via conversion of a *Bam*HI/*Ngo*MIV polymorphism located directly at the DSB site (Fig. 4).
3. Precipitate digested DNA by adding 190 μL ethanol and 5 μL 3 *M* sodium acetate pH 5.2; for the noncrossover digest with 120 μL volume, add 285 μL ethanol and 7.5 μL 3 *M* sodium acetate pH 5.2. Let sit for 20 min at room temperature.
4. Centrifuge for 10 min at full speed in a microcentrifuge.
5. Decant the supernatant and wash the pellet by adding 100 μL 70% ethanol.
6. Centrifuge for 5 min at full speed in a microcentrifuge.
7. Decant, pulse spin tubes and pipette off residual ethanol.
8. Air dry pellets for ~10 min at room temperature.
9. Resuspend pellets in 15 μL 1 × TE. Allow DNA to hydrate in the refrigerator overnight. Mix well by flicking the tube.

Day 2

1. Prepare a 0.6% agarose gel: add 2.1 g SeaKem LE agarose to 350 mL 1 × TBE (*without* ethidium bromide). Dissolve completely by heating in the microwave and boiling steadily for 30 s (approximately 4 min total). Cool to 55°C and pour into a clean, level gel tray in the cold room. Allow gel to harden for at least 30 min.
2. Add 5 μL loading buffer to each digested DNA sample, mix gently.
3. Carefully load gel and run in ~2 L 1 × TBE (*without* ethidium bromide) at 70 V (~2 V/cm) for 26 h at room temperature.

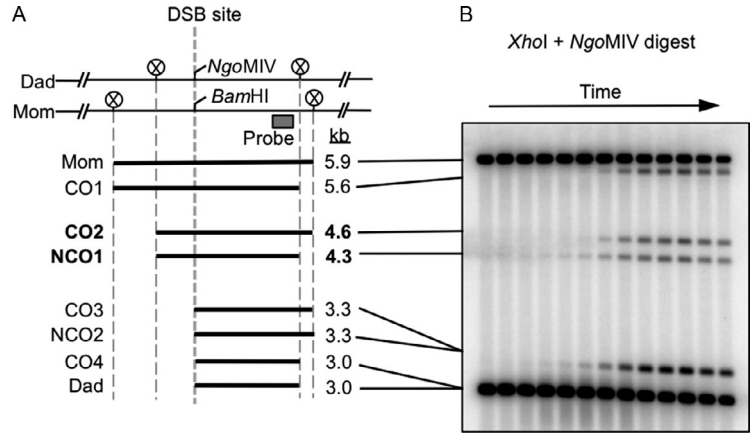

Fig. 4 Noncrossover assay. Map of the *HIS4::LEU2* hotspot highlighting the DSB site, diagnostic restriction sites, and position of the probe used in Southern analysis. Sizes of diagnostic fragments are shown. *Circled Xs* indicate *Xho*I sites. Note that NCO1 (in *bold*) is the only band that is unambiguously diagnostic of a noncrossover product. NCO1 is quantified as percentage of total hybridization signal. NCO1 and CO2 are compared to assess changes in the CO/NCO ratio.

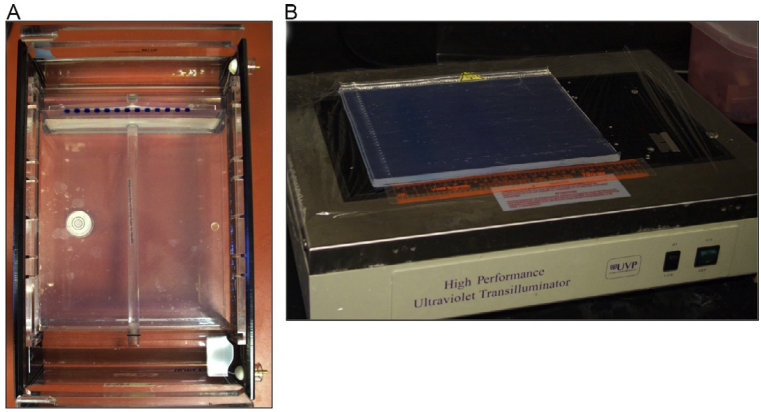

Fig. 5 First dimension of 2D gel electrophoresis. (A) Digested DNA samples loaded onto a 0.4% gel in the Buffer Puffer™ gel apparatus. (B) After electrophoresis, the gel is stained with ethidium bromide and placed on a long-wave UV transilluminator to visualize DNA, check for even loading and efficient digestion, and excise gel slices for second-dimension gels.

Day 3

1. Stain gel in 1 L dH_2O with 0.5 μg/mL (50 μL of 10 mg/mL stock) ethidium bromide in a clean Pyrex dish with gentle shaking for 30 min.
2. View gel on UV box to check that DNA digestion is complete and that loading is even (Fig. 5).
3. Blot the gel by alkaline transfer overnight (see Section 6).

4. TWO-DIMENSIONAL GEL ELECTROPHORESIS (NATIVE/NATIVE)

4.1 Equipment

1. Buffer Puffer™ gel box, Model A5 (Owl Separation Systems) with a 36-well comb.
2. Large pyrex dishes (39 × 45 cm) for staining gels.
3. Platform shaker.
4. Long-wave UV transilluminator (UVP High Performance UV Transilluminator, Variable Intensity Model, 365 nm, or similar model).
5. Clean razor blades.
6. Fluorescent ruler.

4.2 Buffers and Reagents

1. SeaKem Gold Agarose (Lonza); SeaKem LE Agarose (Lonza).
2. 1 × TBE: 90 m*M* Tris base, 90 m*M* boric acid, 2 m*M* EDTA. Prepare as 10 × stock (900 m*M* Tris base, 900 m*M* boric acid, 20 m*M* EDTA) and dilute to 1 × as needed.
3. λ*Bst*EII and λ*Hin*dIII DNA molecular weight markers (New England Biolabs).
4. 10 mg/mL ethidium bromide in dH_20. Store at room temperature wrapped in aluminum foil.

4.3 Procedure

Day 1

1. Prepare a 0.4% agarose gel: add 1.4 g SeaKem Gold agarose to 350 mL 1 × TBE (*without* ethidium bromide). Dissolve completely by heating in the microwave and boiling steadily for 30 s (approximately 4 min). Cool to 55°C and pour into a clean, leveled gel tray in the cold room. Allow gel to harden for at least 30 min.
2. Add 5 μL loading buffer to each digested DNA sample, mix gently (for sample digestion, see Section 3.1.3).
3. Carefully load the gel, leaving at least one lane space between samples (Fig. 5). Also load λ*Bst*EII and λ*Hin*dIII DNA ladder on either side of the gel. Run the first dimension gel for 21 h at 35 V (1 V/cm) at room temperature.
4. Prepare two 5-L batches of 1 × TBE containing 0.5 μg/mL (250 μL of 10 mg/mL stock solution) ethidium bromide and put in the cold room to chill overnight.

Day 2

5. Stain gel in 1 L prechilled 1 × TBE + 0.5 μg/mL ethidium bromide for 30 min at room temperature with gentle shaking. To analyze 12 samples (a standard time course) prepare two 400 mL 0.8% agarose gels: add 3.2 g SeaKem LE agarose to 400 mL 1 × TBE. Dissolve completely by heating in the microwave and boil steadily for 30 s. Add ethidium bromide to 0.5 μg/mL, mix and place to cool in a 55°C water bath.
6. Lay a piece of Saran Wrap on top of a *long wave* UV box and gently slide the gel on top. Visualize the DNA and carefully cut ~9.5-cm slices from the lanes to cover the size range of interest, typically ~0.5 cm from the wells down to the 2.2-kb band of the λ*Bst*EII ladder (Fig. 6). Start by cutting off the top and bottom of the gel and then excise each lane as cleanly and as quickly as possible.
7. Using a flexible plastic ruler, place the excised lanes horizontally in three rows of two in a 20 × 25-cm gel tray (rows spaced ~8 cm apart) (Fig. 6). In the cold room, carefully pour the 0.8% agarose around the slices to *just* cover them. Allow gel to harden for 30 min. Do the same for the second gel (12 samples for a full time course analysis).

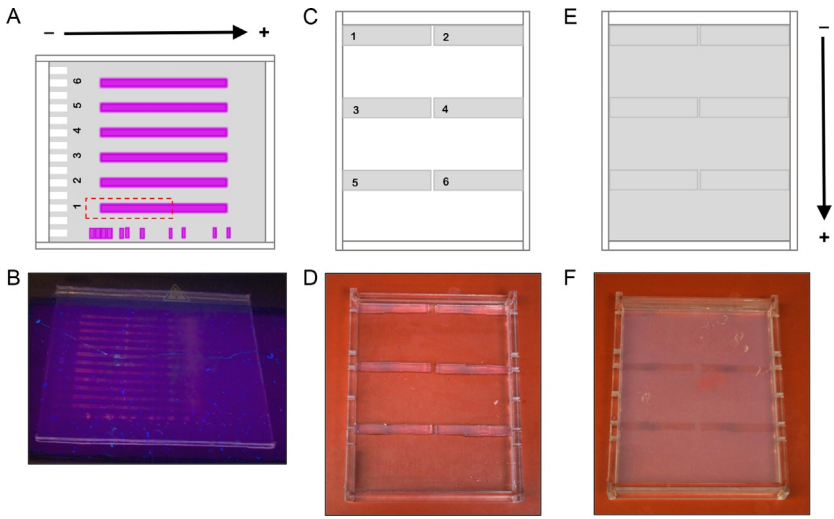

Fig. 6 2D Gel Setup. (A and B) Digested DNA electrophoresed in the first dimension is visualized using long-wave UV light. (C and D) Each lane slice is cut to the appropriate size and arranged perpendicularly in a clean gel tray, with the higher molecular weight DNA oriented to the *left*. (E and F) Molten agarose gel is poured around the lane slices to just cover them.

8. Run the gels in the 4°C cold room using the prechilled 1 × TBE with 0.5 μg/mL ethidium bromide at 170 V (6 V/cm) for 6 h.
9. Blot gels by alkaline transfer overnight (see Section 6).

5. TWO-DIMENSIONAL GEL ELECTROPHORESIS (NATIVE/DENATURING)

This procedure is used to analyze the strand composition of recombination intermediates, including JMs and DSBs (Oh et al., 2008; Zakharyevich et al., 2010) (Fig. 7). For JMs, psoralen cross-links must be reversed prior to running the second-dimension gel under denaturing conditions (Oh et al., 2009). Steps unique to this procedure are highlighted in *italics*. Strand analysis of DSBs (e.g., to measure 5′ resection) is typically performed on DNA samples that were not cross-linked with psoralen (Lao, Tang, et al., 2013).

5.1 Equipment

1. Buffer Puffer™ gel box, Model A5 (Owl Separation Systems) with a 36-well comb.
2. Large pyrex dishes (39 × 45 cm) for staining and equilibrating gels.
3. Platform shaker.
4. Long-wave (350 nm) UV transilluminator box.
5. Clean razorblades.
6. Flexible plastic ruler.
7. A two-toothed comb to create wells for the molecular weight standards, see Note 4.

5.2 Buffers and Reagents

1. SeaKem Gold Agarose; SeaKem LE Agarose (Lonza).
2. TBE: 90 m*M* Tris base, 90 m*M* boric acid, 2 m*M* EDTA. Prepare as 10 × stock.
3. TE: 10 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA. Prepare as 10 × stock.
4. DNA molecular weight standards covering the size range of interest.
5. 10 mg/mL ethidium bromide in dH_2O. Store at room temperature wrapped in aluminum foil.
6. 6 × Gel Loading Dye: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll (Type 400, Pharmacia) in dH_2O. Store at room temperature.

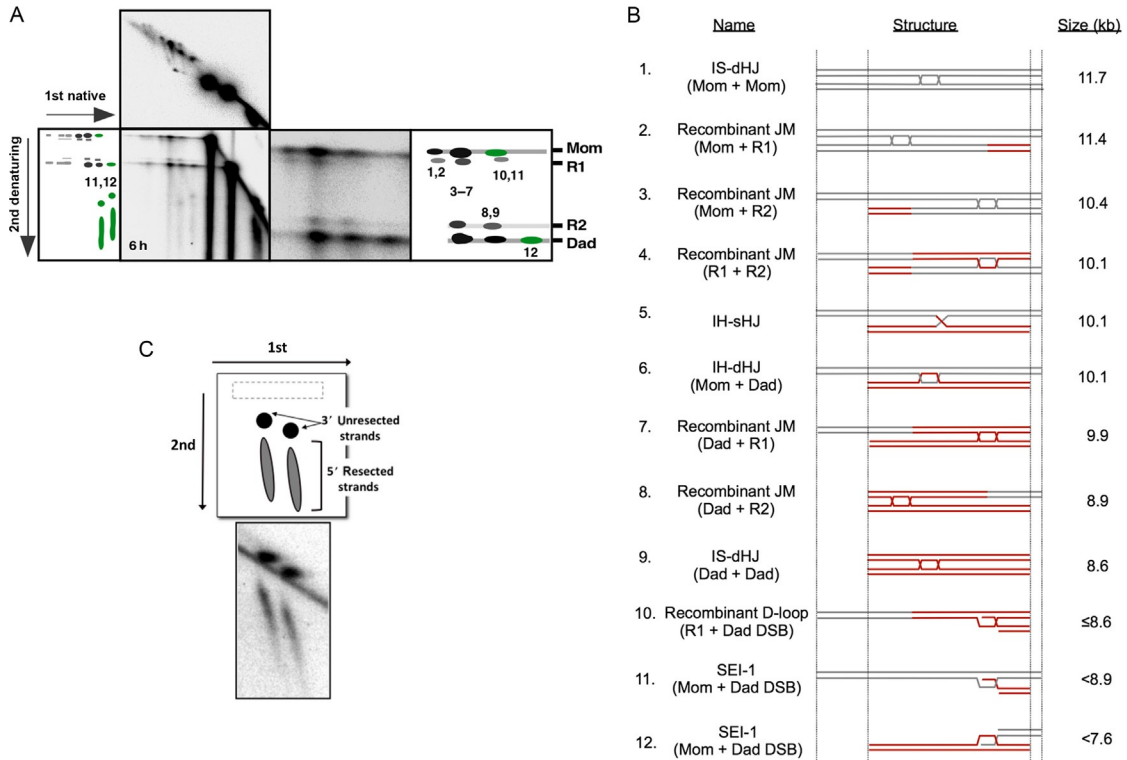

Fig. 7 Native/denaturing two-dimensional gel electrophoresis. (A) A full image of the native/denaturing 2D blot is shown together with a blowup of the region of interest, to the *right*. The corresponding native/native 2D panel is shown to orient native JM species with their component strands. Interpretative diagrams highlight signals of interest, and their corresponding JMs are shown in (B) (Oh et al., 2008). Signals colored *green* correspond to the component strands of SEIs (Hunter & Kleckner, 2001). (C) Native/denaturing 2D gel analysis of DSBs revealing discreet 3′-terminal strand and heterogeneously resected 5′-terminal strands (Zakharyevich et al., 2010).

7. Native Gel Loading Buffer: 100 µL 6× loading dye + 60 µL 10× NEB3 restriction enzyme buffer (New England Biolabs)
8. Equilibration Buffer: 5 m*M* Na_3PO_4. Warm solution to 65°C and adjust to pH 12 using 50% NaOH.
9. Cross-link Reversal Buffer: 1 m*M* Na_3PO_4, 3 *M* urea. Warm solution to 65°C and adjust to pH 12 using 50% NaOH.
10. 5× Alkaline Running Buffer: 250 m*M* NaOH, 5 m*M* EDTA in dH_2O. Make fresh for each use.
11. 1× Alkaline Running Buffer: 50 m*M* NaOH, 1 m*M* EDTA in dH_2O. Make fresh for each use.
12. 6× Alkaline Gel Loading Buffer: 300 m*M* NaOH, 6 m*M* EDTA, 18% (w/v) Ficoll (Type 400, Pharmacia), 0.15% (w/v) bromocresol green, 0.25% (w/v) xylene cyanol in dH_2O. Store in plastic tubes at 4°C.

5.3 Procedure

Day 1

1. To release DNA fragments of interest, digest an appropriate amount of sample genomic DNA to completion. For analysis of DSB processing in budding yeast, we digest 4–10 µg of genomic DNA at a concentration of 1 µg per 20 µL, with a fourfold excess of restriction enzyme. *For analysis of JM species, we digest ≥10 µg of DNA.*
2. Ethanol precipitate, air dry, and resuspend DNA in 15 µL 1× TE. Add 5 µL native loading buffer and load the samples into a 0.6% SeaKem *Gold* agarose gel (*0.4% for JM analysis*) prepared as described in Section 4.3. Leave one lane space between different samples and also between the molecular weight standards and the samples. Each second-dimension gel can fit six samples total, with two columns of samples, and three rows.
3. Run the first dimension at 70 V (1 V/cm) for 24 h.
4. Chill at least 7 L dH_2O in the cold room (for preparation of the alkaline running buffer). Also chill 1 L dH_2O + 0.5 µg/mL ethidium bromide.

Day 2

5. Prewarm a shaking water bath to 65°C. *Prepare 500 mL Equilibration Buffer and 500 mL Cross-link Reversal Buffer for JM analysis.*
6. Stain gel in 1 L prechilled dH_2O + 0.5 µg/mL ethidium bromide for 30 min at room temperature with gentle shaking.
7. Prepare a 1.2% alkaline agarose gel (*1% gel for JM analysis)*: Dissolve 5.4 g (*4.5 g*) SeaKem LE agarose in 427.5 mL 1 m*M* EDTA and cool

in a 50°C bath (keep a stir bar in the flask). See Note 5 regarding running conditions. Separately, prepare 50 mL of 1 *M* NaOH (fresh, using solid NaOH).

8. Lay a piece of Saran Wrap on top of the long wave UV box and carefully slide the gel on top. Visualize the DNA and carefully cut slices from the lanes to cover the size range of interest. Cut off one corner of the gel slice to keep track of the orientation. Weigh the gel slices to estimate their volume.
9. In a small plastic container (one per gel slice), rinse the gel slices in ≥10 volumes cold dH_2O with gentle shaking for 15 min (≥10 volumes are used in all subsequent rinses and incubations, steps 6–14). Very carefully decant the wash and repeat.
10. *Incubate as above with prewarmed Equilibration Buffer in the 65°C water bath. Repeat this wash. During steps 5–7, the gel slices become fragile and slippery, and great care must be taken when decanting buffers.*
11. *Incubate in prewarmed Cross-link Reversal Buffer for 2–4 h at 65°C. Repeat this incubation.*
12. *Briefly rinse gel slice in 500 mL dH_2O at room temperature.*
13. *Wash twice with ≥ 10 volumes ice-cold dH_2O for 15 min to remove remaining urea.*
14. Wash twice, for 15 min, with ice-cold 5 × alkaline running buffer to denature the DNA.
15. Wash twice, for 15 min, with ice-cold 1 × alkaline running buffer to equilibrate gel slices.
16. Place gel slices in a gel tray perpendicular to first dimension. Add 25 mL of the 1 *M* NaOH solution to the cooled gel (for a final concentration of 50 m*M* NaOH) while stirring.
17. Promptly pour the agarose to just cover the gel slice. Allow the gel to harden in the cold room for 30 min.
18. Add 1 × alkaline running buffer to just cover the gel. Load molecular weight standard in denaturing loading buffer, see Note 6. Lay saran wrap on top of the buffer to exclude air. Run at 55 V (~1.7 V/cm) for 30 h with a change of buffer after 15 h, see Note 7. *For JM analysis, run at 55 V (~1.7 V/cm) for 50 h with a change of buffer after ~24 h.* Run times may need to be optimized to resolve molecules of interest.
19. Rinse the gel twice in 1 L of dH_2O with gentle shaking.
20. Blot gel by alkaline transfer overnight (see Section 6).

6. SOUTHERN TRANSFER AND HYBRIDIZATION

6.1 Southern Transfer

6.1.1 Equipment

1. Large pyrex dishes (39 × 45 cm) for staining and equilibrating gels, and to use as a reservoir for the Southern transfer.
2. Platform shaker.
3. Glass plate (22.5 × 43 cm) to span the large pyrex dish and serve as a platform for the transfer.
4. Whatman 3MM paper (46 × 57-cm sheets).
5. GeneScreen Plus (from PerkinElmer) or ZetaProbe nylon hybridization transfer membrane—20 × 25 cm for each gel.
6. Single-fold paper towels.
7. Plastic/plexiglass plate (22 × 28 cm) to place on top of the transfer stack.

6.1.2 Buffers and Reagents

1. 0.25 *M* HCl (1 L per gel). Make immediately before use.
2. 0.4 *M* NaOH (3 L per gel). Make immediately before use.
3. 1 *M* sodium phosphate buffer pH 7.2.

6.1.3 Procedure

Day 1

1. Soak the gel in 1 L dH_2O for 10 min. All subsequent steps are in 1 L volumes with gentle shaking. Prepare 0.25 *M* HCl and 0.4 *M* NaOH solutions. You will need sufficient NaOH to soak the gel and do the transfer, i.e., 3 L per gel.
2. Flip the gel so that the bottom, flat side is facing up. To do this, gently sandwich the gel between two flat, gel-sized plastic plates, quickly flip, and slide the gel gently back into the dish containing dH_2O.
3. Soak gel in 0.25 *M* HCl for 20 min with gentle shaking.
4. Pour the HCl into a large beaker and rinse the gel briefly with 1 L dH_2O. Soak the gel in 0.4 *M* NaOH for 30 min with gentle shaking.
5. During steps 3–4, prepare the following: four pieces of Whatman blotting paper the same size as the gel (25 × 20 cm), one piece of Whatman paper suitable for a wick (see Note 8), and one piece of nylon membrane the same size as the gel (see Fig. 8). Use gloves and a clean dry surface when measuring and cutting the membrane (do not remove the protective paper; keep the membrane free of dust; avoid any

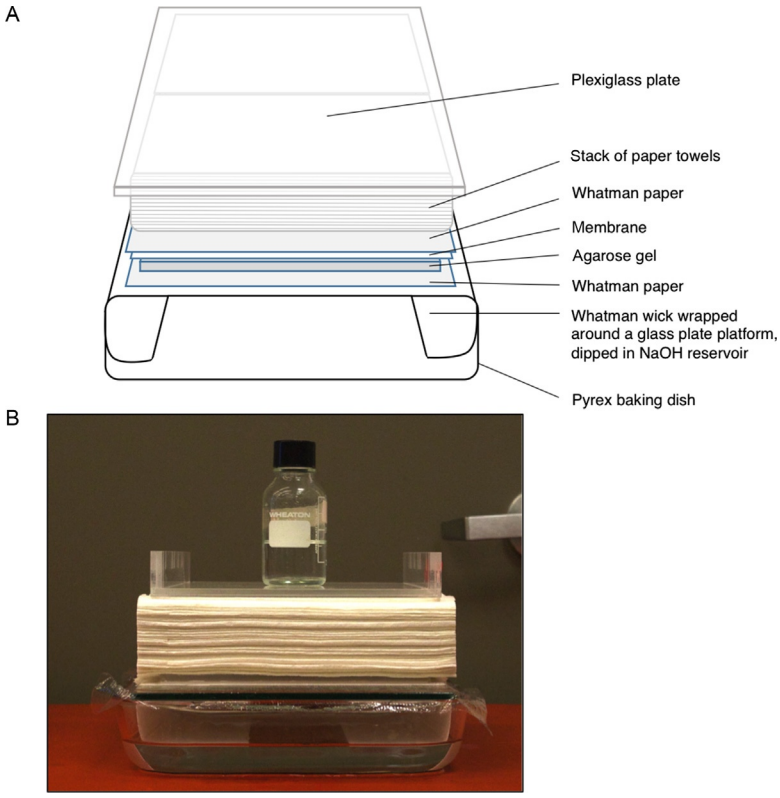

Fig. 8 Southern transfer. (A) Schematic of Southern transfer setup. (B) Photo of transfer setup. See text for details.

crumpling or other mechanical stress on the membrane; all will increase background in subsequent hybridization). Use a fine-tipped sharpie or pencil to write the date and identifier on the top right hand side of the membrane.

6. Set up the blot. Fold the wick evenly across a clean glass plate (see Note 9). Place the glass plate and wick on top of a clean pyrex dish with both ends of the wick in the tray. Wet the surface of the wick with 0.4 *M* NaOH and flatten by rolling with a 25-mL glass pipette. Add more 0.4 *M* NaOH to the dish to create a reservoir. Place two gel-size pieces of Whatman in the center of the wick, wet well with NaOH and roll flat (see Fig. 8). You will use ~1 L total NaOH solution in this step.
7. Carefully slide the gel from the tray to the wick. Gently push any trapped air bubbles from under the gel with gloved fingers. Empty the NaOH used for gel soaking into the beaker containing the used HCl (see Note 10). Rinse the dish and fill with dH_2O.

8. Wet the nylon membrane in the dH_2O for 5 min (see Note 11). Minimize handling and hold only at the edges. Carefully align the top edges of the membrane and the gel and lower the membrane onto the gel. Do not move the membrane around once it contacts with gel.
9. Soak the two additional gel-sized pieces of Whatman in the dH_2O and carefully place on top of the membrane. Take a 25-mL pipette and carefully, but firmly, roll out any bubbles working out from the center.
10. Place a large piece of plastic wrap across the whole construction and cut around the gel with a sharp razor blade. The plastic wrap should seal around the edges of the gel so that capillary action occurs only via the gel and membrane.
11. Take a pack of single-fold paper towels; unfold several towels and lay them flat on top of the Whatman paper. Evenly split the rest of the stack into two and arrange on top of the gel stack horizontally in a side-by-side arrangement. Place a plexiglass plate (not glass) and a *small* weight (small bottle containing less than 50 mL of liquid) on top of the blot (see Fig. 8).
12. Blot for at least 6 h, generally overnight.

Day 2

13. Prepare 1 L, 50 m*M* sodium phosphate pH 7.2 and pour into a clean pyrex dish that can accommodate the nylon membrane. Deconstruct the blot: remove the weight, plate, paper towels, and top two pieces of Whatman paper. Immediately peel off the membrane and place into the sodium phosphate solution to neutralize. Do not allow the membrane to dry out prior to neutralization as this will result in high background. Gently shake membrane for 10 min with one change of buffer.
14. Move directly to the prehybridization step (Section 6.2.3, step 1), or briefly drain the membrane on a piece of Whatman paper and store the damp membrane in plastic wrap at 4°C (short term) or −20°C (longer than 2 days).

6.2 Hybridization—See Note 12

6.2.1 Equipment

1. Radioactive workstation with appropriate protective screening, Geiger counter, microcentrifuge, 37°C and 95°C heating blocks and waste containers for dry and liquid radioactive waste.
2. Large pyrex dish (39 × 45 cm) to accommodate the Southern blot membrane.
3. Hybridization oven and bottles (Autoblot mini hybridization oven, BELLCO glass, Inc. or equivalent).

4. ProbeQuant G-50 Micro Columns (GE Healthcare).
5. Large shaking water bath at 65°C.
6. Large plastic container (Tupperware or similar) with a sealing lid to accommodate the Southern blot membrane and 1 L of wash buffer.
7. Long round-nosed forceps to remove membrane from hybridization bottle.
8. Whatman paper.
9. Plastic wrap.

6.2.2 Buffers and Reagents

1. 1 *M* sodium phosphate pH 7.2.
2. Hybridization Buffer: 0.25 *M* sodium phosphate pH 7.2, 0.25 *M* NaCl, 1 m*M* EDTA, 7% SDS, 5% Dextran Sulfate. For 500 mL: 125 mL 1 *M* Na-phosphate pH 7.2, 25 mL 5 *M* NaCl, 1 mL 0.5 *M* EDTA, 35 g SDS, 25 g Dextran Sulfate. Add liquids to a 1-L beaker. Make up to ~300 mL with sterile deionized water. Slowly add SDS and Dextran Sulfate while stirring. Heat to 65°C and stir until fully dissolved. Aliquot into 50-mL plastic tubes. Store at room temperature. SDS will come out of solution so heat to 65°C and mix well before use.
3. Sheared Salmon sperm DNA (10 mg/mL, Sigma) in 0.2 mL aliquots. Store at −20°C.
4. Prime-It® RmT Random Primer Labeling Kit (Agilent Technologies).
5. Radiolabeled nucleotide: ^{32}P αdCTP (PerkinElmer BLU013H250UC or equivalent). Buy only as needed and store at −20°C.
6. 20× Saline Sodium Citrate (SSC) stock solution (3 *M* NaCl, 300 m*M* sodium citrate, adjust to pH 7.0).
7. 20% SDS solution.
8. Low Stringency Wash: 2× SSC, 0.1% SDS. For 1 L, place 100 mL 20× SSC in a 1-L measuring cylinder and make up to 995 mL. Add 5 mL 20% SDS.
9. High Stringency Wash: 0.1× SSC, 0.1% SDS. For 1 L, place 990 mL water in 1-L measuring cylinder. Add 5 mL 20% SDS and 5 mL 20× SSC.

6.2.3 Procedure

1. Prewarm the Hybridization Solution to 65°C and mix well by inverting the bottle several times. Turn on the hybridization oven and set to 65°C. Rinse the hybridization bottles, lids, and O-rings (if used) with dH_2O. Prewarm bottles in the oven.

2. Add 10 mL Hybridization Solution per small bottle or 20 mL per large bottle. Place bottles back into the oven.
3. Denature a 0.2-mL aliquot of sheared Salmon sperm DNA by heating in a 95°C dry block for 5 min. Immediately quench on ice/water and then store on ice.
4. Submerge the membrane to be hybridized, with the DNA-side facing up, in 50 m*M* sodium phosphate pH 7.2 in a clean pyrex dish. Carefully roll up the membrane with DNA face on the inside (avoid touching the DNA-side during the rolling process) and drain out excess buffer by touching one edge of the rolled-membrane on paper towel. Place membrane into prewarmed hybridization bottle and slowly unroll by turning the bottle, keeping the opening face wet with hybridization solution. Avoid bubbles. Add the Salmon sperm DNA, promptly return to the oven and prehybridize for >30 min, preferably several hours.
5. Remove radiolabeled nucleotide from the freezer and allow it to thaw (this can be done during the prehybridization step). Sign out the amount of radiolabel to be used in the logbook. Sign into the radioactive workstation and monitor the area using a Geiger counter.
6. Label 25 ng of probe DNA using a Prime-It® RmT Random Primer Labeling Kit (or equivalent) and ^{32}P αdCTP according to the manufacturers protocol: Add appropriate amounts of probe and water to total 42 μL of probe mix with 25 ng of probe. Add 5 μL ^{32}P αdCTP, 3 μL polymerase, and pipette up and down briefly. Incubate at 37°C for at least 1 h (see Note 13).
7. Separate the probe from unincorporated nucleotides using a ProbeQuant G-50 Micro Column. Remove storage liquid from column by centrifuging at 3000 RPM for 1 min. Add probe-labeling reaction mix to column, spin at 3000 RPM for 2 min, and collect flow-through in a new tube.
8. Denature the probe in a 95°C heating block or boiling water bath for 2 min. Quench on ice for 2 min (see Note 14).
9. Add the denatured probe to the hybridization bottle and promptly return to the oven. Monitor the workstation with a Geiger counter, clean up any contamination immediately, and sign out.
10. Hybridize for >6 h, typically overnight.
11. Turn on a shaking water bath and prewarm to 65°C. Sign into the radioactive workstation.
12. Make up 1 L Low Stringency Wash and add to a 1-L flask. Heat to 65°C (for 1 L this takes ~3.5 min in a microwave on full power).

Check the temperature carefully—do NOT heat above 65°C or you will strip the probe.

13. Carefully pour off the hybridization mix into the radioactive liquid waste (record all volumes of liquid waste added). Fill the bottle ~1/2 full with preheated wash and return promptly to the oven. Incubate for 10 min.
14. Pour the wash into the radioactive liquid waste and record the volume. Repeat the low-stringency wash. Prepare 2 L High-Stringency wash and heat to 65°C.
15. Pour the second low stringency wash into liquid waste and record the volume. Fill the bottle ~1/2 full with preheated High Stringency wash and return promptly to the oven. Incubate for 30 min.
16. Pour the wash into the radioactive liquid waste and record the volume. Carefully and quickly remove the membrane from the tube with long round-nosed forceps and place into a clean plastic container with 1 L prewarmed High Stringency wash. Cover the container and incubate in a 65°C shaking water bath for 30 min with gentle shaking. To prevent the container from floating, you will need to add a weight (we use a piece of lead flashing).
17. Repeat the high-stringency wash.
18. Monitor the blot—the edges should be essentially free of counts (~2 × background with the Geiger counter on its most sensitive setting). If there are significant background counts, wash again. Distinct signals corresponding to specific hybridizing species should be detectable.
19. Drain excess wash from the blot onto a wad of paper towels. Carefully wrap the damp blot in plastic wrap. Using small pieces of tape, mount onto a piece of Whatman paper with the DNA side facing out.

6.3 Imaging and Analysis

6.3.1 Equipment

1. Phosphorimager (Typhoon™, Storm™, or PhosphorImager™; GE Healthcare)
2. Phosphorimager screens and cassettes (GE Healthcare)
3. ImageQuant™ (GE Healthcare) and Excel™ (Microsoft) software packages. Open-source software such as ImageJ (http://rsb.info.nih.gov/ij/) can also be used for image analysis.

6.3.2 Procedure

1. Expose the hybridized Southern blots to storage phosphor screens for >30 min to overnight and scan the screens on a Phosphorimager.

To obtain the optimum exposure for quantification, we aim to have the strongest signals in the upper third of the dynamic range of the phosphorimager.

2. Open the Southern image in ImageQuant. To quantify the relative levels of hybridizing species, integrated signal intensities are measured in by drawing shapes around individual bands/spots and subtracting background signal from shapes of equivalent size. The level of a given species is expressed as a fraction of the total hybridization signal (the sum of all analyzed bands/spots minus background) or "% DNA."
3. For the analysis of DSB processing during recombination, we calculate a "resection profile," which describes the distribution of resection lengths for the 5-strands of DNA double-strand breaks. To do this, first draw a line tracing through the lane containing the DNA molecular weight standards.
4. Create a graph using the analysis tool. The graph will show distance in mm vs radioactive signal counts. Distinct peaks correspond to the individual fragments of the DNA standards. The position of the wells is the reference point for migration distance and is indicated by a tiny peak located above the largest signal peak for the DNA standards.
5. Import the ImageQuant graph data into Excel. Assign a size (bp) to the migration distance (mm) of each band of the DNA standards.
6. Obtain a linear regression equation by graphing migration distances (x) vs *log* (size in bp) (y).
7. For each signal of interest, calculate the migration distance (x). Enter this value into the regression equation and solve for y. To determine the fragment size in bp, take the inverse *log* of y.
8. To create the "resection profile," the signal intensities for 5′ strands within successive length bins of 100 nt are calculated and plotted in a bar graph as percentages of the total hybridizing signal (Zakharyevich et al., 2010). To correct for background signal, the same process is repeated for an adjacent region of the Southern blot without DSB signals.

7. NOTES

1. Time this step carefully. Over-spheroplasted cells will not form a tight pellet and may require repeated centrifugation. Under-spheroplasted cells will form a dense pellet (resembling intact cells) and should be gently resuspended and incubated for an additional 2–5 min.

2. If a distinct white interface is visible in any of the extracted solutions, perform another extraction.
3. DNA pellets should not be over dried. DNA can be rehydrated for several hours to overnight at 4°C.
4. A makeshift comb for DNA standards can be made by turning a regular gel comb over in its holder (comb side facing up) and attaching well-sized plastic sticks using adhesive tape. Alternatively, teeth can be broken from an existing comb to leave wells at desired positions (typically at the two ends). When running two rows of samples, marker lanes should be staggered.
5. Note that agarose percentage may need to be optimized to resolve the size range of interest. Stated concentrations are optimal for analysis of component DNA strands at the *HIS4::LEU2* locus
6. To accurately estimate the size of DNA strands, take special care to align the gel slices with the marker wells. The migration distance of the marker will be used to calculate the sizes of component strands being analyzed.
7. NaOH buffer is rapidly depleted, which will slow electrophoresis and lead to overheating. Buffer should be replaced at least once.
8. For a wick, cut a large sheet of Whatman paper in half.
9. The wick will drape into the reservoir filled with NaOH, ensuring the entire blot setup will remain alkaline for transfer.
10. Check the pH of the final acid/base mixture and neutralize for disposal.
11. Float the membrane on top of the water and then tilt the tray up and down to completely submerge.
12. Southern hybridization with ^{32}P–radiolabeled DNA probes is performed using routine methods (Green & Sambrook, 2012; Ruven, Seelen, Lohman, Mullenders, & van Zeeland, 1994). Probes should hybridize to the ends of the DNA fragments being analyzed to allow unambiguous estimation of DNA strand lengths. To detect the molecular weight standards, we add 0.05 ng of the DNA standard to probe-labeling reactions. If strand-specific probes are required, we amplify and radiolabel probes using linear PCR, as described (Ruven et al., 1994).
13. To maximize specificity, DNA probes should be highly purified. Cloned probes should be gel purified from the vector backbone. PCR amplified probes should be prepared by nested PCR and gel purified.
14. Lid locks may be used to boil the labeled probe to prevent aerosol radioactive contamination from tubes popping open.

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