

Chapter 2

Quantitative Genome-Wide Measurements of Meiotic DNA Double-Strand Breaks and Protein Binding in *S. pombe*

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Abstract

The fission yeast *Schizosaccharomyces pombe* is especially well suited for studying meiosis in molecular detail. Experiments with *S. pombe* strains that undergo a nearly synchronous meiosis—at variable temperatures—have elucidated the mechanisms of meiotic progression and the proteins that are involved. For example, studies focused on the initiation of meiotic recombination by programmed DNA double-strand breaks (DSBs) have proven exceptionally informative. In meiosis, some regions of DNA have more frequent DSBs than the surrounding regions. These DSB hotspots can be visualized by Southern blot hybridization of restriction fragments ranging from kilobases (kb) to megabases (Mb) in size. More recently, the benefits of genome-wide analysis to map the distribution and frequency of meiotic DSBs have been attained, with resolution down to the nucleotide level. Infrequent, non-hotspot DSBs previously not detectable have been observed, creating a better understanding of how recombination is regulated. Additional genome-wide analyses have shown proteins that bind specifically to DSB hotspots, providing insight into how the DSB initiation complex functions. We describe here detailed methods for achieving these results.

Key words Fission yeast, *Schizosaccharomyces pombe*, Meiotic induction, DNA double-strand breaks (DSBs), Rec12 (Spo11), Pulsed-field gel electrophoresis (PFGE), Chromatin immunoprecipitation (ChIP), DNA microarray hybridization, Massive parallel sequencing

1 Introduction

Meiosis with high synchrony is necessary to determine the temporal order of events at the molecular level, such as formation and repair of DNA double-strand breaks (DSBs). In the fission yeast *Schizosaccharomyces pombe* high synchrony can be achieved using a mutant with an altered protein kinase Pat1. The Pat1 protein represses meiosis; its inactivation allows meiosis to be initiated and to proceed at least through DSB formation and repair in either haploid or diploid cells [1]. There are two ways to inactivate Pat1 quickly to achieve high synchrony—by raising the temperature of a mutant with the *pat1-114* allele [2], or by adding a small molecule inhibitor at any temperature to a mutant (*pat1-as*) altered in the Pat1 ATP-binding pocket that makes the protein sensitive to the

inhibitor [3, 4]. In each case, an *S. pombe* strain is grown to mid-log phase in minimal medium and then shifted to minimal medium without a nitrogen source. This starvation causes most of the cells to arrest in the G1 phase of the cell cycle. After nitrogen is added back, either the culture is shifted to high temperature (*pat1-114*) or the small molecule inhibitor is added (*pat1-as*); each method inactivates Pat1 repression and allows the cells to proceed synchronously into meiosis.

After meiosis is initiated at 34 °C, the cells go through DNA replication between ~2 and 3 h later. After completion of replication, at ~3.5 h, DSBs are made by the topoisomerase-related protein Rec12 (Spo11 ortholog); the DSBs are repaired by ~5 h [1]. At 25 °C these events occur about 1 h later. Each strand of DNA is broken by one Rec12 molecule (presumably acting as a dimer), which is covalently bound to each of the 5' ends of the DSB by a phosphodiester bond [5–7]. Rec12 remains bound to the DNA until it is removed by endonucleolytic cleavage ~10–30 nucleotides distant [5, 8]. This cleavage depends on the Mre11-Rad50-Nbs1 (MRN) complex and Ctp1 and produces a Rec12-DNA oligonucleotide complex [5]; the 5' end of the oligonucleotide is the precise point of DSB formation. These characteristics of the DNA cleavage by Rec12 are very important, as they allow for the isolation, enrichment, and detection of the locations in the genome where DSBs are made.

Meiotic DSBs have been visualized using restriction enzyme-digested DNA, followed by Southern blot hybridization with a radioactive DNA probe: a DSB results in two fragments that are smaller than the native restriction fragment [9]. When pulsed-field gel electrophoresis is used, large regions (Mb) of the genome can be analyzed. However, whole-genome analysis of DSBs is much more informative and more efficient, especially for analyzing many mutant strains. By taking advantage of the Rec12 protein covalently bound to sites of DSBs, the genomic distribution of Rec12 can be assayed by chromatin immunoprecipitation, followed by microarray hybridization (ChIP-chip) [10–13]. For this analysis, a mutation in *rad50* (*rad50S*) is used to block enzymatic removal of Rec12 from the DNA and allow accumulation of broken DNA [9]; sonication is used to produce ~0.5 kb-long DNA attached to Rec12, which is important for efficient hybridization to the microarray. Epitope-tagged Rec12 is immunoprecipitated with antibodies specific for the tag. The antibodies bound to magnetic beads permit isolation of the Rec12-bound chromatin from the cell lysates, allowing the chromatin to be purified and the DNA to be amplified and labeled with a fluorescent dye. A whole-cell extract (WCE) containing total DNA is prepared the same way, but this DNA is labeled with a second fluorescent dye, and both sets are mixed and hybridized to a DNA microarray. The intensity of each dye is measured at DNA probes spaced throughout the genome and

assembled onto a microarray (chip); the ratio of immunoprecipitate (IP) signal to WCE signal is measured and median normalized. Regions with DSBs are enriched for DNA-bound Rec12 in the IP compared to the input whole-cell lysate. When all the DNA probes are aligned to the *S. pombe* genome, the distribution of DSBs formed during meiosis and their relative intensities can be measured.

This ChIP-chip approach can also be used to analyze the chromosomal localization of proteins, including any epitope-tagged protein, with an additional step to chemically crosslink the protein to the DNA [10, 11]. These protein analyses have been instrumental in discovering how structural proteins involved in sister chromatid cohesion and homolog alignment regulate the formation of meiotic DSBs [14]. The synaptonemal complex-related proteins of *S. pombe*, known as linear elements or LinEs, form microscopically visible foci dependent on the meiosis-specific cohesin subunits Rec8 and Rec11; LinEs include Rec10, Rec25, Rec27, and Mug20 [15–17]. ChIP-chip analysis of each of these proteins has demonstrated highly specific localization of Rec25, Rec27, and Mug20 to sites of future DSB hotspots independent of Rec12 action; therefore, LinE protein binding likely occurs before DSB formation. Rec10 binds with lesser specificity to DSB hotspots and may bind to DSB-cold regions as well [14]. Rec8 and Rec11, though necessary for formation of most DSBs at hotspots, are nearly uniformly distributed along the genome [14].

The resolution of the DSBs mapped by ChIP-chip is limited by the distance between probes on the array, usually ~300 bp. In order to achieve resolution of the DSBs down to a single nucleotide, a different approach is taken. The short DNA oligonucleotide that remains bound to Rec12 after MRN-Ctp1-dependent cleavage from the end of the DSB is isolated, amplified, and sequenced *en masse* [8]. These sequences can then be directly aligned to the *S. pombe* genome to give a detailed, high-resolution map of meiotic DSBs. This method also has far greater sensitivity than either Southern blots or ChIP-chip DSB assays and thus allows assaying less-frequent DSB events along with DSB hotspots. The results have greatly enhanced our understanding of how meiotic recombination is regulated.

2 Materials

2.1 *S. pombe* Culture Media for Meiotic Induction

1. Yeast extract liquid (YEL) medium: 5 g of yeast extract, 30 g of glucose. Make to 1 L with water and autoclave. For yeast extract agar (YEA) add 20 g of agar before autoclaving.
2. EMM2* (modified Edinburgh minimal medium 2): 50 mL of 20× EMM2 salts, 25 mL of 20% NH₄Cl, 25 mL of 0.40 M Na₂HPO₄, 12.5 mL of 40% glucose, 1 mL of 1000× vitamins, 0.1 mL of 10,000× trace elements. Make to 1 L with water and filter-sterilize.

3. 20× EMM2 salts: 30.6 g of K phthalate (monobasic), 10 g of KCl, 5 g of MgCl₂, 0.1 g of Na₂SO₄, 0.1 g of CaCl₂. Make to 500 mL with water and autoclave.
4. 1000× vitamins: 1 mg of biotin, 10 mg of calcium pantothenate, 1 g of nicotinic acid, 1 g of myoinositol. Make to 100 mL with water and autoclave.
5. 10,000× trace elements: 0.5 g of H₃BO₃, 0.4 g of MnSO₄, 0.4 g of ZnSO₄·7H₂O, 0.2 g of FeCl₃·6H₂O, 0.15 g of Na₂MoO₄, 0.1 g of KI, 0.04 g of CuSO₄·5H₂O, 1 g of citric acid. Make to 100 mL with water and filter-sterilize.
6. Pombe minimal (PM) medium: prepared as EMM2* but with 20 mL of 0.40 M Na₂HPO₄ and 50 mL of 40 % glucose per liter.
7. Shaking water baths at 25 and 34 °C.
8. ATP-analog inhibitors: for *pat1-as2* (L95A), 1-NM-PP1 (4-amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine; Toronto Research Chemicals, Inc.); for *pat1-as1* (L95G), 3-MB-PP1 (4-amino-1-tert-butyl-3-(3-methylbenzyl)pyrazolo[3,4-d]pyrimidine; Toronto Research Chemicals, Inc.).

2.2 Chromatin Immunoprecipitation

1. 500 mL centrifuge bottles, autoclaved.
2. 30 mL centrifuge tubes, autoclaved.
3. Tris buffered saline (TBS): 20 mM Tris-HCl pH 7.6, 150 mM NaCl, sterilized.
4. 2.5 M glycine.
5. Fix Solution (made fresh daily): 11 % formaldehyde, 100 mM NaCl, 1 mM EDTA pH 8.0, 50 mM HEPES pH 7.6.
6. Bead beating buffer: 100 mM Tris-HCl pH 8.0, 20 % glycerol, sterilized.
7. 2 mL flat bottom microcentrifuge tubes with screw caps, autoclaved.
8. 100 mM phenylmethanesulfonyl fluoride (PMSF), in isopropanol or ethanol.
9. Acid-washed glass beads (500 μm).
10. BeadBeater (BioSpec Products or equivalent).
11. Syringe needle, 22 or 25 G.
12. 5 mL polystyrene round-bottom tubes, 12×75 mm style.
13. FA Buffer: 50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 % Triton X-100, 0.1 % Na deoxycholate, filter-sterilized.
14. Complete ULTRA protease inhibitor mini tablets (Roche).
15. Sonicator, with microtip.
16. Magnetic Protein A or G Dynabeads.

17. 1.5 mL low adhesion microcentrifuge tubes, autoclaved.
18. Anti-FLAG antibody, clone M2 from mouse.
19. Magnetic particle concentrator (MPC) (ThermoFisher MagnaRack or equivalent).
20. Bovine serum albumin (BSA; immunoglobulin IgG-free).
21. Phosphate buffered saline (PBS): 137 mM NaCl, 12 mM Na₂HPO₄, 2.7 mM KCl, pH 7.4, autoclaved.
22. Microcentrifuge tube rotator.
23. UltraFree-MC filter, 0.45 µm.
24. FA-HS Buffer: 50 mM HEPES pH 7.6, 500 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100, 0.1% Na deoxycholate, filter-sterilized.
25. RIPA Buffer: 10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA pH 8.0, 0.5% NP-40, 0.5% Na deoxycholate, filter-sterilized.
26. 3× FLAG peptide, 5 mg/mL in TBS.
27. 2× Stop Buffer: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 20 mM EDTA pH 8.0, 1% SDS.
28. Glycogen, 20 mg/mL.
29. Proteinase K, fungal: 20 mg/mL in 10 mM Tris-HCl pH 7.5, 20 mM CaCl₂, 50% glycerol. Store at -20 °C.
30. Phenol-chloroform-isoamyl alcohol (25:24:1 v/v).
31. 3.0 M Na-acetate pH 5.2.
32. 100% ethanol.
33. 70% ethanol.
34. Tris-EDTA (TE): 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, autoclaved.
35. RNase A, 10 mg/mL.
36. DNA PCR purification column kit.

**2.3 DNA
Amplification
and Labeling:
Microarray
Hybridization**

1. 0.5 mL PCR tubes, sterile.
2. Sequenase Version 2.0 DNA polymerase (Affymetrix).
3. 5× Sequenase Buffer: 200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl.
4. 3 mM dNTP mix (3 mM each of dATP, dCTP, dGTP, and dTTP).
5. 0.1 M dithiothreitol (DTT).
6. Bovine serum albumin (BSA), 0.5 mg/mL.
7. Primer A (5' GTT TCC CAG TCA CGA TCN NNN NNN NN 3'), 40 µM in dH₂O.

8. Sequenase Dilution Buffer: 10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.1 mM EDTA.
9. Thermal cycler.
10. 1.5 mL microcentrifuge tubes, autoclaved.
11. Amicon Ultra 0.5 mL 30K centrifugal filter.
12. Taq DNA polymerase.
13. 10× Taq PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl).
14. 50 mM MgCl₂.
15. aminoallyl-dUTP (aa-dUTP, 50 mM).
16. aa-dUTP-dNTP mix: 5 mM aa-dUTP, 5 mM dTTP, 10 mM each dATP, dCTP, dGTP.
17. Primer B (5' GTT TCC CAG TCA CGA TC 3'), 20 μM in dH₂O.
18. DNA PCR purification column kit.
19. UV spectrophotometer.
20. 0.2 M NaHCO₃, pH 8.9.
21. Cy3 and Cy5 (N-hydroxysuccinimide (NHS) ester) Post-Labeling Reactive Dye packs (GE Healthcare).
22. Dimethyl sulfoxide (DMSO), treated with 100 g of activated molecular sieves per liter of DMSO.
23. 4 Å activated molecular sieves.
24. 4.0 M hydroxylamine.
25. 10× oligo aCGH/ChIP-on-Chip Blocking Agent (Agilent).
26. 2× Hi-RPM Hybridization Buffer (Agilent).
27. 95 °C heat block.
28. 37 °C water bath.
29. *S. pombe* genome 4× 44K microarray (Agilent).
30. Agilent SureHyb chamber (or equivalent).
31. Oligo aCGH/ChIP-on-chip wash Buffer 1 (Agilent).
32. Oligo aCGH/ChIP-on-chip wash Buffer 2 (Agilent).
33. Agilent Microarray Scanner System C.
34. Agilent Feature Extraction software version 10.7.3.1.

2.4 Microarray Data Analysis

1. Spreadsheet software (e.g., Microsoft Excel), optional.
2. Peak-calling software (e.g., ChIPOTle [18]), optional.
3. Statistical and sequence analysis software (e.g., R [<https://www.r-project.org/>], Bioconductor [<https://www.bioconductor.org/>])

2.5 *Rec12-Oligo* *Isolation*

1. 250 mL centrifuge bottles, autoclaved.
2. 2 mL flat bottom microcentrifuge tubes with screw caps, autoclaved.
3. Magnetic Protein A or G Dynabeads.
4. 1.5 mL low adhesion microcentrifuge tubes, autoclaved.
5. Anti-FLAG antibody, clone M2 from mouse.
6. Microcentrifuge tube rotator.
7. Magnetic particle concentrator (MPC) (ThermoFisher MagnaRack or equivalent).
8. 10 % ice-cold trichloroacetic acid (TCA).
9. Acid-washed glass beads (500 μ m).
10. BeadBeater (BioSpec Products or equivalent).
11. Syringe needle, 22 or 25 G.
12. 5 mL polystyrene round-bottom tubes, 12 \times 75 mm style.
13. SDS solubilization buffer: 0.5 M Tris-HCl pH 8.1, 2 % SDS, 1 mM EDTA pH 8.0.
14. β -mercaptoethanol.
15. 2 \times IP buffer: 167 mM NaCl, 16.7 mM Tris-HCl pH 8.1, 1.1 mM EDTA, 1.1 % Triton X-100, 0.01 % Na dodecylsulfate (SDS), filter-sterilized.
16. 10 \times TdT reaction buffer: 500 mM K-acetate, 200 mM Tris-acetate, 100 mM Mg-acetate, pH 7.9.
17. 2.5 mM CoCl₂.
18. Terminal deoxynucleotidyl transferase (TdT).
19. [α -³²P] dCTP.
20. Proteinase K buffer: 100 mM Tris-HCl pH 7.5, 0.5 % SDS, 1 mM EDTA pH 8.0, 1 mM CaCl₂.
21. Proteinase K, DNA- and RNA-free, purified to remove any residual DNA and RNA. *See* Lam et al. (Chapter 3, this volume, Subheadings 2.3.1 and 3.3.1 for procedure).
22. 6 \times gel loading buffer: 30 % glycerol, 0.25 % bromophenol blue in dH₂O.
23. 15 % native polyacrylamide gel (acrylamide:bis-acrylamide in the ratio 19:1) in 100 mM Tris-acetate buffer, pH 7.5.
24. 10 mM Tris-HCl pH 8.0.
25. Glycogen, DNA- and RNA-free, treated with Benzonase to remove any residual DNA and RNA. *See* Lam et al. (Chapter 3, this volume, Subheadings 2.3.2 and 3.3.2 for procedure).
26. 3.0 M Na-acetate pH 5.2, autoclaved.
27. 100 % ethanol.

28. 70 % ethanol.
29. ~20 nt DNA oligo, of known concentration for use as standard.
30. Illustra Microspin G-25 column (GE Healthcare).
31. 20 mM EDTA pH 8.0.
32. 2× Laemmli buffer: 4 % SDS, 20 % glycerol, 125 mM Tris-HCl pH 6.8, 10 % β-mercaptoethanol, 0.004 % (w/v) bromophenol blue.
33. 5× TBE concentrated stock: 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA pH 8.0, made to 1 L in water.
34. 20 % denaturing polyacrylamide gel (acrylamide:bis-acrylamide in the ratio 19:1), 1× TBE, 7.0 M urea.
35. Phosphor-image screens and scanner (GE Healthcare Typhoon or equivalent).

2.6 Analysis of Rec12-Oligo Sequences

1. Statistical and sequence analysis software (e.g., R [<https://www.r-project.org/>], Bioconductor [<https://www.bioconductor.org/>]).
2. Sequence mapping software (e.g., Bowtie [bowtie-bio.sourceforge.net/], SHRiMP [compbio.cs.toronto.edu/shrimp/]).

3 Methods

Genome-wide analysis in *S. pombe* benefits from a reproducibly synchronous meiosis. Any volume of culture from a *pat1* strain can be induced for meiosis, and the timing of events is consistent: replication occurs 2–3 h after the induction of meiosis; DSBs, including release of the Rec12-oligo for sequencing, at 3–5 h; first meiotic division at 4.5–5 h; and second meiotic division at 5.5–7 h. This progression is used to determine at which time points to collect cells, depending on the goal of the experiment. These times are for 34 °C; add approximately 1 h for 25 °C. A *rad50S* strain with the *rec12-FLAG* (or another epitope) allele is desirable to obtain the greatest amount of DNA-bound Rec12 for ChIP-chip mapping of DSBs, though a *rad50⁺* strain will work and gives the same result, with less enrichment of DNA-bound Rec12 due to ongoing DSB repair [19].

3.1 Cell Culture Conditions for Meiosis

1. Streak an *S. pombe pat1-114* or *pat1-as* strain from a –80 °C glycerol stock onto a YEA plate with appropriate supplements (100 µg/mL). Incubate the plate at 25 °C.
2. When colonies are grown (~4–5 days), pick an isolated colony to 5 mL of YEL with appropriate supplements (100 µg/mL; see **Note 1**) and incubate on a roller drum or with shaking at 25 °C until the culture is saturated (2–3 days).

3. Use the starter culture to inoculate (1:100) a 50 mL EMM2* (*see Note 2*) culture with any required nutritional supplements (amino acids, purines, or pyrimidines at 75 $\mu\text{g}/\text{mL}$). Incubate at 25 °C with moderate shaking (100–200 rpm) until the OD₆₀₀ is between 1.5 and 2.0 (2–3 days). Do not let the OD₆₀₀ become much higher than this, as subsequent growth will be affected.
4. Approximately 14–16 h before starvation is to start (*see Note 3*), dilute the EMM2* culture into 500 mL of EMM2* to an OD₆₀₀ of 0.1 in a 2-L flask. Incubate at 25 °C with moderate shaking (100–200 rpm).
5. When the culture reaches an OD₆₀₀ of 0.3–0.4, centrifuge the cells for 5 min at 4000 rpm (2800 $\times g$) and wash once in 1 volume of sterile water. Resuspend the cells in 500 mL of EMM2* without NH₄Cl and with supplements limited (10 $\mu\text{g}/\text{mL}$) to starve the cells. Incubate at 25 °C with moderate shaking for 16–18 h; the OD₆₀₀ will approximately double after starvation, to $\sim 1 \times 10^7$ cells/mL.
6. Add 12.5 mL of 20% NH₄Cl and supplements to 75 $\mu\text{g}/\text{mL}$ and either 1) place the culture in a 34 °C water bath with moderate shaking (for *pat1-114*) or 2) add 1-NM-PP1 (for *pat1-as2*) or 3-MB-PP1 (for *pat1-as1*) to 25 μM and incubate at the desired temperature with moderate shaking. This time marks the initiation of meiotic induction. To relate the timing of replication to that of other events, such as DSB formation, follow DNA replication by flow cytometry of small samples taken at hourly or half-hourly intervals (*see Note 3*).

3.2 Chromatin Crosslinking, Extraction, Sonication, and Immunoprecipitation

The chromatin can be extracted and purified (**steps 1–8**) for immediate use, or stored at –80 °C for later use. If the chromatin is to be immunoprecipitated immediately, the Protein G beads must be prepared and incubated with the antibody 1 day in advance (**steps 9–15**).

1. At the desired time (at 34 °C, 5 h for *rec12-FLAG rad50S* DSB analysis; 3.5 h for *rad50**), spin down 250 mL of a meiotically induced culture in a 500 mL centrifuge bottle at 5000 $\times g$. Wash twice with 25 mL of ice-cold TBS in a 30 mL tube. The cell pellets can be stored at –20 °C for later use, or the procedure can be continued.
2. For localization of proteins other than Rec12, it is necessary to first crosslink the protein to DNA before harvesting cells. To the 250 mL of culture, add 25 mL of freshly prepared Fix Solution, and shake slowly at room temperature for ~5 min (*see Note 4*). Add 18 mL of 2.5 M glycine and shake an additional 5 min; then centrifuge the cells at 5000 $\times g$. Wash the cells twice in 25 mL of ice-cold TBS. The cell pellets can

be frozen at -20°C for later use, or the procedure can be continued.

3. Resuspend the cells in 0.5 mL Bead Beating Buffer with 1 mM PMSF freshly added, and transfer to a 2 mL flat bottom screw-cap microcentrifuge tube. To break open the cells, add 0.5 mL acid-washed glass beads, and place the tubes in a BeadBeater at 4°C . Shake on high speed for 30 s; remove tubes and cool on ice for 2 min. Repeat for a total of five cycles (*see Note 5*).
4. Collect the lysate by puncturing the bottom of the tube with a small gauge syringe needle and placing the punctured tube in a 5 mL round-bottom tube (the 2 mL tube should sit at the top of the 5 mL tube and not slide completely in). Spin at $1000\times g$ to separate the lysate from the beads, and transfer to a new 2 mL microcentrifuge tube.
5. Prepare 5 mL FA Buffer for each cell lysate sample by dissolving one tablet of protease inhibitors in every 10 mL of FA buffer needed.
6. Add 1 mL of FA buffer with added protease inhibitors to each lysate. Mix by inversion and spin at $16,000\times g$ for 1 min at 4°C to pellet the chromatin and cell debris. Discard the supernatant, and repeat two more times, gently pipetting to resuspend the chromatin and cell debris. Do not vortex.
7. Resuspend the pellet in 300 μL of FA Buffer with protease inhibitors. To solubilize the chromatin and break the DNA into roughly 0.5-kb pieces, sonicate on a low setting (keep foaming at a minimum) with a microtip for 10 s, and cool on ice for 2 min. Repeat a total of 8–12 times, wiping the microtip with ethanol between samples.
8. Add 1.2 mL of FA Buffer with protease inhibitors, mix by inversion, and centrifuge at $16,000\text{--}20,000\times g$ for 30 min at 4°C to pellet cellular debris, leaving the broken chromatin in the soluble fraction. Turn each tube 180° , and repeat the spin. Remove the supernatant with a pipet, taking care to avoid cell debris, and transfer to a new 2 mL microcentrifuge tube. The procedure can be continued, or the chromatin can be frozen in liquid nitrogen and store at -80°C for later use.
9. The bead preparations (**steps 9–15**) must incubate overnight and thus need to be started THE DAY BEFORE the IP. Make fresh IgG-free BSA solution (5 mg/mL) in PBS. Each IP sample will require ~ 4 mL of BSA; make an appropriate volume and keep on ice.
10. Vortex the suspension of magnetic Dynabeads Protein G beads for 30 s to mix well (*see Note 6*). Pipet 20 μL of magnetic Protein G beads into each 1.5 mL low adhesion microcentrifuge tube and concentrate the beads with a magnetic particle

concentrator (MPC) and remove supernatant. Do not leave beads in the MPC for more than a couple of min, to avoid persistent aggregation of the beads.

11. Add 1 mL of freshly prepared BSA (5 mg/mL, IgG-free). Rotate at room temperature for 5 min. Concentrate the beads with an MPC and remove supernatant. Repeat the wash two more times, for a total of three washes.
12. Add 100 μ L of BSA (5 mg/mL, IgG-free) to the beads from each sample, and resuspend well. Add 1 μ L of anti-FLAG M2 antibody (4 μ g/ μ L) to each sample. Rotate overnight at 4 $^{\circ}$ C to allow beads to bind the antibody.
13. Prepare fresh BSA (5 mg/mL, IgG-free) in PBS and keep on ice. Pulse-spin the tubes in a microcentrifuge to collect the antibody-bead suspension in the bottom of the tube.
14. Put the tubes in the MPC and remove the supernatant. Resuspend the beads in 1 mL of BSA (5 mg/mL, IgG-free) in PBS and wash for 5 min on a rotator at room temperature. Repeat the wash one time.
15. Suspend each bead sample in 30 μ L of BSA (5 mg/mL, IgG-free). Keep the beads on ice.
16. If proceeding with frozen chromatin from **step 8**, thaw the samples in room-temperature water. Remove 250 μ L of chromatin and filter through a Millipore Ultrafree-MC 0.45 μ m unit by spinning at 16,000 $\times g$ for 1 min at 4 $^{\circ}$ C. This step removes cellular debris from the chromatin sample. Aliquot 40 μ L of filtrate, which contains broken chromatin, into whole-cell extract (WCE) tubes, and set aside on ice until **step 22**. The WCE contains all of the protein and DNA present in the cells, to which the specific enrichment of the IP can be compared.
17. Add the remaining 200 μ L of filtered chromatin to 30 μ L of Protein G beads from **step 15** (*see Note 7*). Rotate at room temperature for 90 min to allow bead-bound anti-FLAG antibody to bind Rec12-FLAG-DNA complexes. Meanwhile, freeze the remaining chromatin samples in liquid nitrogen and store at -80° C.
18. Pulse-spin the 1.5 mL tubes in a microcentrifuge to collect the antibody-bead suspension in the bottom of the tube. Concentrate the beads with an MPC. Remove the supernatant and set aside for use as the “unbound fraction” control if desired. Resuspend the beads, containing the anti-FLAG-protein-DNA complexes, in 1 mL of FA buffer with protease inhibitors by gently pipetting, and rotate at room temperature for 5 min. Concentrate the beads with an MPC, then remove and discard the supernatant.

19. Repeat the wash two more times with FA buffer (protease inhibitors are not necessary at this step), two times with FA-HS buffer and 1 time with RIPA buffer, for a total of five washes.
20. Elute the chromatin from the beads by adding 10 μL of 3 \times FLAG peptide (5 mg/mL in TBS) and briefly vortex to resuspend, followed by adding 40 μL of RIPA buffer and vortex again to mix. Incubate the beads for 30 min at room temperature on the rotator, briefly vortexing every 10 min. Concentrate with an MPC and save the supernatant in a new 1.5 mL microcentrifuge tube. Repeat the elution once more, and then combine both eluates (100 μL total). Alternatively, or if using any antibody other than anti-FLAG, elution can be done by resuspending the beads in 50 μL of 2 \times stop buffer and incubating at 65 $^{\circ}\text{C}$ for 15 min. Concentrate with an MPC and save the supernatant in a new 1.5 mL microcentrifuge tube. Repeat the elution once more, and then combine both eluates (100 μL total). Pool any duplicate sample eluates at this step.
21. Add either 1 volume of 2 \times stop buffer (after 3 \times FLAG peptide elution) or 1 volume of dH_2O (after heat elution). To the WCE samples set aside in **step 16**, add 60 μL of dH_2O and 100 μL of 2 \times stop buffer. Add 1 μL of glycogen (20 mg/mL in H_2O) to each sample.
22. Add 2 μL of proteinase K (20 mg/mL). Incubate at 55 $^{\circ}\text{C}$ for several hr or overnight.
23. Extract with 1 volume of phenol–chloroform–isoamyl alcohol; repeat once more. Add 0.1 volume of 3.0 M Na-acetate, pH 5.2, and precipitate the DNA with 2 volumes of ethanol. Wash once with 70% ethanol, and allow the pellets to air dry.
24. Resuspend each pellet in 50 μL of TE. Add 1 μL of RNase A (10 mg/mL in H_2O) and incubate at 37 $^{\circ}\text{C}$ for 1 h.
25. Purify the DNA using a PCR purification column kit, and elute in 50 μL of the supplied elution buffer or water.
26. The quality of the IP can be checked at this stage by PCR, using 1 μL of DNA from each of the IP and the WCE samples as template. A primer pair specific to a known DSB hotspot, such as *mbs1* or *ade6-3049* where there should be abundant amounts of bound Rec12, will give more PCR product from the IP than from the WCE. Primers specific to a cold region, such as the *ura1* gene, will generate approximately equal amounts of PCR product in each (*see Note 8*).

3.3 DNA Amplification, Labeling, and Hybridization to Microarrays

1. Concentrate the 50 μL of IP DNA (Subheading 3.2 step 25) to 7 μL in a spinning vacuum or vacuum desiccator at room temperature, and pipet into a PCR tube. Aliquot 7 μL of WCE DNA into a PCR tube. To each tube, add 2 μL of 5 \times Sequenase buffer and 1 μL of Primer A (40 μM). Prepare a separate master

enzyme mix of the following (volumes given are per reaction): 1 μL of 5 \times Sequenase buffer, 1.5 μL of dNTP mix, 0.75 μL of 0.1 M DTT, 1.5 μL of BSA (0.5 mg/mL in H_2O), 0.3 μL of Sequenase DNA polymerase. Keep on ice.

2. The primer extension reactions are run in a thermal cycler as follows: 94 $^{\circ}\text{C}$ for 2 min; 10 $^{\circ}\text{C}$ for 5 min; 37 $^{\circ}\text{C}$ for 16 min; 94 $^{\circ}\text{C}$ for 2 min; 10 $^{\circ}\text{C}$ for 5 min; 37 $^{\circ}\text{C}$ for 8 min; hold at 4 $^{\circ}\text{C}$. When the temperature drops to 10 $^{\circ}\text{C}$ at the second step, add 5.05 μL of the enzyme mix to each tube, pipetting gently. Next, prepare a second diluted enzyme master mix: 0.24 μL of Sequenase polymerase and 0.96 μL of Sequenase dilution buffer. When the reactions reach 10 $^{\circ}\text{C}$ at the fifth step, add 1.2 μL of the diluted enzyme mix.
3. Once the PCR reactions are complete, transfer the reactions to a 1.5 mL microcentrifuge tube containing 585 μL of dH_2O , and briefly vortex to mix.
4. Load the sample into an Amicon Ultra 0.5 mL 30K centrifugal filter, and spin at 14,000 $\times g$ for 10 min; discard the flow through. Recover the sample by flipping the filter over into the collection tube provided and spin at 1000 $\times g$ for 3 min.
5. Add water to each sample so that the total volume is 39 μL . If the volume is more than 39 μL , concentrate in a spinning vacuum or vacuum desiccator. Pipet the 39 μL into a new PCR tube.
6. Set up the following PCR master mix (volumes given are for each single reaction): 5 μL of 10 \times Taq buffer, 2.5 μL of Primer B (20 μM), 1.25 μL of 50 mM MgCl_2 , 1.25 μL of aa-dUTP:dTTP dNTP mix (*see* **Note 9**), 1 μL of Taq DNA polymerase (5 units/ μL). Add 11 μL of PCR mix to each 39 μL sample, and pipet gently to mix. Run the reactions in a thermal cycler as follows: 94 $^{\circ}\text{C}$ for 2 min; 94 $^{\circ}\text{C}$ for 30 s; 40 $^{\circ}\text{C}$ for 30 s; 50 $^{\circ}\text{C}$ for 30 s; 72 $^{\circ}\text{C}$ for 1 min; repeat **steps 2–5** for 32 cycles; 72 $^{\circ}\text{C}$ for 7 min; hold at 4 $^{\circ}\text{C}$.
7. Purify the PCR reactions with a PCR purification column kit. Elute the DNA into 50 μL of dH_2O in a 1.5 mL microcentrifuge tube, and repeat elution into the same tube to produce 100 μL total. Use dH_2O for elution and DO NOT use any Tris-containing elution buffer, as this will interfere with the subsequent dye-coupling reaction.
8. Determine the DNA concentration by absorbance at 260 nm in a spectrophotometer. Check the specificity of the enrichment again by PCR as in Subheading **3.2 step 26**, except this time use 1 μL of each amplified DNA sample after diluting each to 1 ng/ μL . Starting with a normalized DNA template, the enrichment at DSB hotspots of IP compared to WCE will be more apparent, as will the lack of enrichment at the DSB

coldspot control. If IP enrichment is not apparent, do not proceed and repeat the amplification.

9. Take 1 μg of the purified DNA from **step 7** (not the locus-specific control PCR) and dry the DNA into a pellet in a spinning vacuum or vacuum desiccator at room temperature (*see Note 10*).
10. Equilibrate the Cy3 and Cy5 NHS ester dyes to room temperature for 20–30 min before use. Dissolve each dye in 11.5 μL of DMSO that has been treated with molecular sieves (*see Note 11*). Each dye tube is enough for two reactions.
11. Resuspend the amplified DNA in 4.5 μL of dH_2O , add 4.5 μL of 0.2 M NaHCO_3 pH 8.9, briefly vortex the samples and pulse spin. Add 4.5 μL of Cy3 to WCE DNA and 4.5 μL of Cy5 to IP DNA (*see Note 12*), vortex samples and pulse-spin in a microcentrifuge. Incubate in the dark at room temperature for 1 h.
12. Add 4.5 μL of 4.0 M hydroxylamine to quench each reaction. Combine the Cy3 WCE and Cy5 IP DNA for each sample. Remove the unincorporated dye on a DNA PCR purification column, and elute with 50 μL of the supplied elution buffer. The clear eluate should be slightly purple in color, a result of equal presence of the red and blue dyes.
13. Check the incorporation of the Cy3 and Cy5 dyes with a UV spectrophotometer. Determine the DNA yield at 260 nm, and measure dye incorporation of Cy3 by absorbance at 550 nm and of Cy5 by absorbance at 650 nm. Optimal labeling yields one dye molecule per ~ 30 nucleotides.
14. From **step 12**, 44 μL of the Cy3- and Cy5-labeled DNA samples are processed according to Agilent's Yeast ChIP-on-chip Protocol version 9.2. Specifically, to 44 μL of the combined Cy3- and Cy5-labeled DNAs, add 11 μL of 10 \times Oligo aCGH/ChIP-on-Chip Blocking Agent and 55 μL of 2 \times Hi-RPM Hybridization Buffer, for a final volume of 110 μL .
15. Heat the samples to 95 $^\circ\text{C}$ for 3 min and then immediately transfer to a 37 $^\circ\text{C}$ water bath, and incubate for 30 min. Load 100 μL of the sample onto the microarray and assemble into an Agilent SureHyb chamber (*see Note 13*). Place the assembled chamber in the rotisserie of the hybridization oven set at 65 $^\circ\text{C}$ and hybridize for 24 h, with a rotation speed of 20 RPM.
16. After hybridization disassemble the chamber and wash the slide for 5 min at room temperature in Oligo aCGH/ChIP-on-chip wash Buffer 1, followed by a wash for 5 min at 31 $^\circ\text{C}$ in Oligo aCGH/ChIP-on-chip wash Buffer 2.
17. Immediately after washing, scan the slide using the Agilent Microarray Scanner System C set at 5 μm resolution with 100% Green and Red photomultiplier tube (PMT). Extract the images with Feature Extraction version 10.7.3.1, using protocol Chip_105_Dec08.

3.4 Microarray Data Analysis

1. For microarray data analysis, the Agilent Feature Extraction output (a tab-delimited text file) can be directly opened and manipulated using Microsoft Excel. Alternatively, use the R software environment, which can more efficiently handle these large data tables and also helps to streamline analyses.
2. Remove header rows containing global, array-wide statistics (typically the first nine rows); keep the row containing the column titles and subsequent rows (each representing a probe). Control probes (which are not from the *S. pombe* genome) indicated by a non-zero value in the 'ControlType' column should be sorted and removed. The remaining data should all be ControlType=0.
3. Aberrant *S. pombe* probes should be removed based on specific criteria: low or near-zero signal in either or both channels; exceptionally high signal in both channels, suggesting the probe is represented multiple times in the genome; or individual probes with an IP/WCE ratio significantly higher than closely neighboring probes (given the DNA shear length, true protein-DNA associations should cover multiple probes).
4. Generate an "MA plot". For each probe, calculate the log of the ratio of the Cy5 intensity/Cy3 intensity ($\log[\text{Cy5}/\text{Cy3}] = M$); also calculate the mean of these two log values ($\frac{1}{2}\log[\text{Cy5} \cdot \text{Cy3}] = A$). Plot M vs A for each probe. This analysis should reveal many data points tightly clustered around $M=0$ (most probes have a ratio near 1) and a broad distribution in the A -dimension. Large positive M values due to IP enrichment should be seen only at probes with large positive A values (high overall intensity); these data suggest valid peaks of protein-DNA associations. The variation of M vs. A reflects the degree of IP enrichment; plots exhibiting tightly clustered probes symmetric around the A -axis indicate poor enrichment. Probes significantly outside the distribution are suspicious and should be flagged or removed (for example, probes with a high M value but a low A value reflect poor hybridization, and probes with a low M value but a high A value reflect exceptionally strong hybridization but little enrichment).
5. Trim irrelevant data (columns) from the filtered probe list to make the data table more manageable (if necessary). For example, keep the 'LogRatio' and 'GeneName' columns (the latter contains the chromosome and probe start/end positions) but remove the 'SubTypeMask' and 'SubTypeName' columns (among others).
6. Graph the dye ratios (as IP/WCE) along chromosomes, or across sub-regions, by plotting the 'LogRatio' column value against the probe's genomic position. Alternatively, plot the transformed (linear) values rather than their logs (Fig. 1a). The visual inspection of chromosomal regions can often reveal

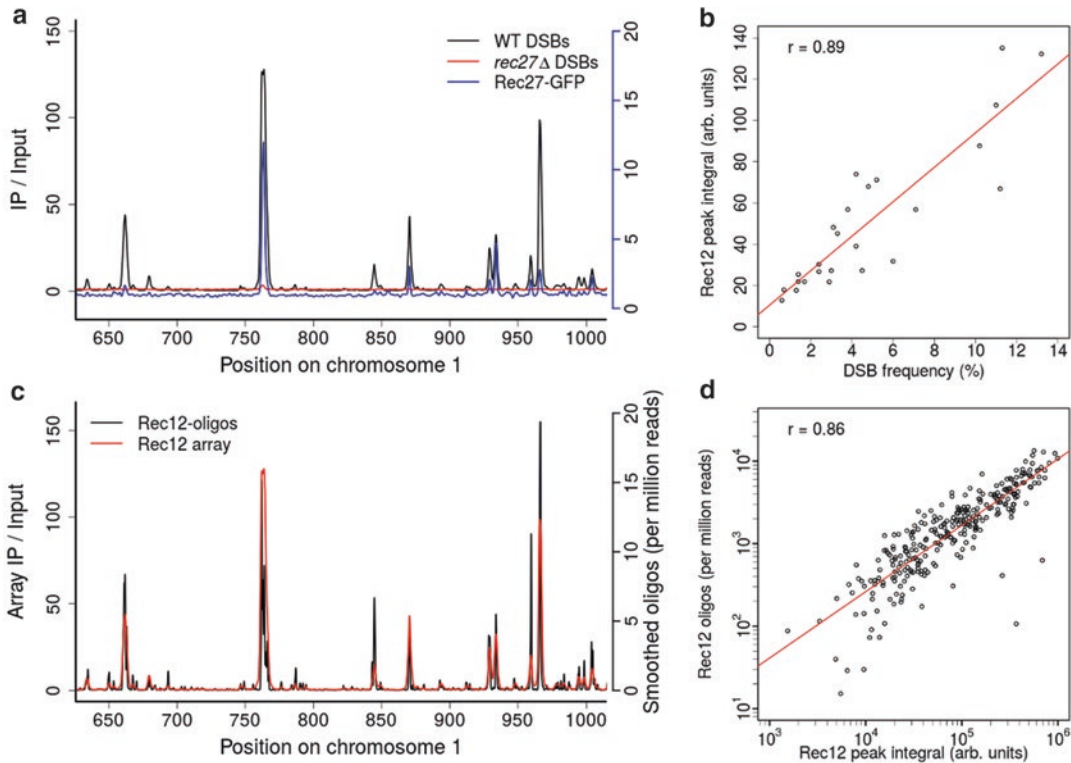


Fig. 1 (a) Comparison of DSB and Rec27-binding distributions determined by ChIP-chip analyses. The ratio of DNA hybridization signal of the Rec12-FLAG IP to that of the WCE extract (input) was median-normalized and plotted (left scale) vs. genome position (bottom scale, in kb) [14]. The region shown is for part of the 0.5 Mb *NotI* fragment J on chromosome 1. In wild-type cells (black line) DSBs at hotspots, such as *mbs1* at 765 kb, are >100-times more frequent than in DSB-cold regions, such as that between 770 and 840 kb. In *rec27Δ* (red line) DSB hotspots are essentially eliminated. The Rec27-GFP fusion protein (blue line, median-normalized IP/WCE; right scale) binds with high specificity to DSB hotspots. (b) ChIP-chip analysis accurately reflects DSB distribution. The integrated signals for Rec12-FLAG IP at hotspots (left scale, in arbitrary units) are plotted against the amount of broken DNA determined by Southern blot hybridization at each of 25 hotspots in a 1.9 Mb region of the left arm of chromosome 1 (Spearman's correlation coefficient $r = 0.89$) [6]. (c) DSB distributions determined by ChIP-chip and ChIP-seq are equivalent. Median-normalized ChIP-chip signals for Rec12-FLAG (red line; left scale) and smoothed oligo counts from ChIP-seq (black line; right scale) are nearly coincident across the part of *NotI* fragment J shown in panel (a) [8]. (d) Data, such as those in panel (c), for 288 hotspots across the genome show a high correlation ($r = 0.86$) [8]

problematic sites or systematic problems. To generate a meaningful scale, the dye log ratios for each probe should be divided by the median of all log values across the genome (“median normalization”).

7. Sites of significant enrichment (peaks) can be determined in an automated fashion using either a stand-alone peak-calling program (e.g., ChIPOTle [18]) or a custom algorithm using a defined significance threshold and peak parameters. ChIP-chip of Rec12 covalently bound (self-linked) to DNA typically

yields low background signal and high dynamic range with peaks that do not depend greatly on the cutoff choice; IP of formaldehyde cross-linked proteins have generally greater background, requiring more tailored cutoffs that depend on the protein of interest (see below). In addition to peak height (degree of enrichment), other parameters are important in distinguishing true binding events: peaks <500 bp in width (the expected mean length of the sheared DNA) or spanning too few probes should be set aside as possible artifacts.

8. Assess the remaining sites using the above chromosomal graphs to determine the peak correctness. Correlate these data with DSB hotspots or protein-binding sites determined independently; for example, DSBs by Southern blot hybridizations or proteins by ChIP-PCR assays (*see* Subheading 3.6 step 7 for references). This is often an iterative process to properly assess peaks: alter threshold parameters as necessary and reassess peak-calling.
9. Spatial correlations with other protein factors can be made apparent by averaging across multiple sites. Take all peak positions and determine the mean enrichment of other proteins using published binding data (ChIP-chip or ChIP-seq). Compare this with the mean of multiple random sites (equal to the number of peaks), which reflects the expected mean background.
10. Data visualization and interpretation may be improved by smoothing the DSB or protein-binding profile. This is accomplished using a “sliding window” that applies a mathematical function to a set of chromosomally adjacent probes. Basic functions that use a uniform distribution, such as an averaging window, tend to broaden DSB peaks. Instead, functions that weight central probes more than probes at the edges, such as a Hann window, are better suited to smooth robust, distantly spaced peaks.

3.5 *Rec12-Oligo* Isolation

1. Grow 1–2 L of meiotic culture (as in Subheading 3.1 steps 1–6) of a *rec12-FLAG rad50⁺* strain and spin down into six aliquots per liter in 250 mL centrifuge bottles. This yields ~0.5 g of wet cell pellet per aliquot (*see* Note 14). Resuspend each aliquot of cells in 1 mL of ice-cold dH₂O and transfer to a 2 mL flat bottom screw-cap microcentrifuge tube. Repeat the wash once, decant the supernatant, and store cell pellet at –20 °C.
2. Prepare the Protein G beads as above (THE DAY BEFORE; Subheading 3.2 steps 9–15), except that 50 µL of Protein G beads are incubated with 10 µg of anti-FLAG antibody per g of cells. Keep on ice until use.
3. Thaw the cells on ice and add 0.5 mL of ice-cold 10% TCA to inactivate proteases and nucleases by denaturation and

precipitate the Rec12-DNA oligos. Add 1 mL of acid-washed glass beads.

4. Place the tubes in a BeadBeater at 4 °C and shake on high setting for 30 s; then place on ice for 2 min. Repeat four more times. Check for cell breakage (*see* **Note 5**).
5. Collect the lysate by puncturing the bottom of the tube with a small gauge syringe needle and placing the punctured tube in a 5 mL round-bottom tube (the 2 mL microcentrifuge tube should sit at the top of the 5 mL tube and not slide completely in). Spin at $1000\times g$ to separate the lysate from the beads, and transfer to a new 2 mL microcentrifuge tube.
6. To pellet the protein and nucleic acid, centrifuge the lysate at $16,000\times g$ for 10 min at 4 °C. Decant the supernatant and resuspend the pellet in SDS solubilization buffer, 1 mL per g of cells (*see* **Notes 15** and **16**). Add 18 μ L of β -mercaptoethanol, vortex briefly to mix, and place in a boiling water bath for 5 min to dissolve most of the protein and nucleic acid.
7. Centrifuge at $16,000\times g$ at 4 °C for 10 min to pellet cell debris and insoluble proteins. To the supernatants, add an equal volume of 2 \times IP buffer. Add to the prepared Protein G anti-FLAG beads (**step 2**), and incubate overnight with rotation at 4 °C.
8. Concentrate the beads with an MPC and remove the supernatant. Wash the beads three times in 300 μ L of 1 \times IP buffer for 5 min; concentrate the beads with an MPC, and pipet off all the supernatant each time. Set the washed beads on ice. At this point, a second immunoprecipitation can be used in place of **steps 9–14**; *see* **Note 17**.
9. When purifying the oligos by polyacrylamide gel electrophoresis (*see* **step 12**), a small radioactive “tracer” reaction is prepared to find the position of the oligos on the gel and to aid their excision. To prepare a “tracer” amount of labeled Rec12-oligos, pipet into a new 1.5 mL microcentrifuge tube 10% of the bead suspension. (Set the remainder aside on ice for **step 10**.) Wash the beads, as in **step 8**, twice in 250 μ L of 1 \times TdT buffer. Concentrate the beads with an MPC, remove the supernatant, and add 25 μ L of TdT labeling mix: 1 \times TdT reaction buffer, 0.25 mM CoCl₂, 5 units of terminal transferase, and 8 μ Ci of [α -³²P] dCTP (3000 Ci/mmol). Incubate at 37 °C for 1 h. Remove the reaction buffer and wash three times with 150 μ L of 1 \times IP buffer, properly disposing of the radioactive supernatant.
10. Resuspend the beads—both the unlabeled and the “tracer” reaction—in each tube in 30 μ L of proteinase K buffer and place in a boiling water bath for 5 min to elute the Rec12-DNA from the beads. Pulse-spin the tubes briefly, and concentrate the beads with an MPC. Remove the supernatant

containing the eluted Rec12-oligos and pipet into a new 1.5 mL low-adhesion microcentrifuge tube.

11. Add 200 μg of purified DNA- and RNA-free proteinase K (*see* Lam et al. Chapter 3 Subheadings 2.3.1 and 3.3.1 for the procedure) to each 30 μL of eluate, and incubate at 50 °C for 2 h.
12. Mix each sample with a 1/5 volume of 6 \times gel loading buffer and load into a 15% Tris-acetate polyacrylamide [19:1] gel, loading $\frac{1}{2}$ of the labeled “tracer” reaction on each side of the unlabeled DNA oligos to mark the oligos’ position on the gel. The expected size of the DNA is ~10–30 nt. Electrophorese until the bromophenol blue in the loading buffer has migrated $\frac{2}{3}$ of the length of the gel. In a 15% native gel, this dye runs with 15-nt DNA.
13. Expose the gel to a film or on a phosphor-imager plate overnight.
14. Cut out the section of the gel containing the unlabeled DNA oligos (using the ^{32}P -labeled flanking tracer reactions as a guide). Soak the gel slices in 600 μL of 10 μM Tris-HCl pH 8.0 at 4 °C overnight.
15. The next day, remove the gel slices and add 1 μL of purified glycogen (10 $\mu\text{g}/\mu\text{L}$, *see* Lam et al. Subheadings 2.3.2 and 3.3.2 for the procedure), 55 μL of 3.0 M Na-acetate pH 5.2, and 1.5 mL of 100% ethanol. Mix by inversion and incubate on dry ice for 15 min. Centrifuge at 16,000–20,000 $\times g$ (maximum speed) for 30 min at 4 °C. Decant and wash the pellet once with 70% ethanol, and briefly dry the pellet. Resuspend in 50 μL of 10 mM Tris-HCl pH 8.0, and pool all the samples.
16. Quantify the oligos by TdT labeling and comparison with a known oligo standard. Pipet 3 μL of purified Rec12-oligos into a 1.5 mL microcentrifuge tube, add 2 μL of filter-purified water and 3.5 μL of TdT reaction mix (*see* step 9). Pipet gently to mix. Also label 1, 2, and 4 fmol of a known 20-mer oligo with 3.5 μL of TdT reaction mix, adding filter-purified water to make a total volume of 8.5 μL . Incubate the reactions at 37 °C for 3 h.
17. Add 42.5 μL of 20 μM EDTA pH 8.0 to each reaction, briefly mix, and spin reaction in a Microspin G-25 column to remove unincorporated ^{32}P at 800 $\times g$ for 2 min. Pipet 25 μL of each reaction into a 1.5 mL microcentrifuge tube, add 25 μL of 2 \times Laemmli buffer and load samples on a 20% denaturing polyacrylamide [19:1] gel with 7.0 M urea. Electrophorese in 1 \times TBE until the bromophenol blue has migrated $\frac{2}{3}$ the length of the gel. In a 20% denaturing gel, this dye runs with 8-nt DNA.
18. Expose the gel on a phosphorimager plate overnight. Use quantification software (such as ImageQuant) to measure the

relative signal of the meiotic oligos and the oligo standards, and determine the concentration of oligos.

19. Use a minimum of 50 fmol of purified oligos for subsequent sequencing analysis, as described in the Chapter 3 of this volume “Sequencing Spo11 oligonucleotides for mapping meiotic DNA double-strand breaks in yeast” by Lam et al.

3.6 Analysis of Rec12-Oligo Sequences

1. Reads with sequence similarity to PCR primers or adapters used in preparing material should be removed. See the Chapter 3 by Lam et al. for details.
2. Trim sequence reads of 5' terminal C's and 3' terminal G's to account for the TdT labeling of Rec12-oligo ends. This trimming introduces some ambiguity, as *bone fide* Rec12-oligos with 5' terminal C's will now be mapped one to several bases from their true position. Once mapped (see below), sequence-reads mapping to a position with an upstream C or downstream G can be computationally shifted to attempt to mitigate this uncertainty. For example, a read with three consecutive C-residues at the 5' end would be trimmed such that the fourth base is the new 5' end; following mapping, if the map position of the new 5' end has a C immediately upstream (toward the 5' end of the genome sequence), the sequence can be extended to include this base (assuming that the Rec12-oligo sequence prior to TdT labeling included this C).
3. Sequence-read mapping will partition the data into sequences that map to a single site in the genome (those whose locus of origin can be precisely determined, or “unique-mappers”) and those that map to multiple sites (ambiguous “multi-mappers”). For all analyses described below, multi-mappers can be set aside and only unique-mappers used (as is typical in many ChIP-seq analyses). However, the short nature of Rec12-oligos (~10–30 nucleotides) results in a substantial number of *bone fide* reads not being mapped uniquely. To prevent the loss of this information, multi-mappers can be handled in several ways, such as random assignment among the n genomic sites to which they map. Alternatively, one can use the unique reads to fractionally assign multi-mappers based upon the inferred likelihood of DSB formation at each of the n sites. For each multi-mapper, count the number of unique-mappers within ± 250 bp of each map site and assign each site a value proportional to this sum. For example, a read that maps to two regions, one with 100 neighboring oligos and the other with 10, would result in 0.9 of the multi-mapper being assigned to the first site and 0.1 to the latter.
4. Sequence-read coverage (i.e., the frequency each base in the genome was sequenced) represents a general DSB map and can be used to quickly determine the DSB profile.

5. To generate a more precise map, the 5' end of each read should be used to represent discrete break events. Generate a tabulated list of 5' end positions across all positions in the genome, such that each read is counted at a single base position. The resulting list will be sparse; most positions in the genome will lack a mapped 5' end and be absent. This list should be populated with zeroes at these positions to reflect the actual break frequency base-to-base. The resulting map should reflect the frequency of 5' Rec12-oligo ends at each base in the genome.
6. Smooth the high-resolution break map using a sliding window approach (*see* Subheading 3.4 step 10) with various window sizes. For hotspot level analyses, a 1 kb Hann window may sufficiently emphasize hotspots over cold-regions; for finer detail, use a 500 bp Hann window.
7. Use known DSB hotspots to validate the spatial precision of the Rec12-oligo map by comparing the smoothed oligo map to autoradiographic traces of Southern blots during meiotic induction (e.g., the commonly used *NotI* fragments J [9], K or D [19] on chromosome 1) or to previously published microarray maps across the genome [6, 14]. Using a wide smoothing window (≥ 1 kb), individual hotspot sites should be apparent and align well with previous maps; high-resolution Southern blots that reveal hotspot substructure [i.e., having close positions broken at variable frequencies; e.g., those at *ade6* DSB hotspots [20] or at the *mbs1* hotspot [21] should be compared with oligo maps smoothed using a narrow (~ 500 bp) window. Fine resolution comparisons can be made using previously published Rec12-oligo maps [8] by comparing the number of oligos found at each base or across defined regions.
8. Determine the number of oligos within each previously defined hotspot and compare this with known break frequencies (determined either by Southern blot or microarray hybridization as noted in step 7). Hotspot sites that do not exhibit a linear relationship between oligo count and break frequency should be investigated for allele differences between experiments (e.g., if a deletion or insertion is present in the strain used for sequencing or microarray analysis; see [6] for such an example). Furthermore, these sites should be searched for repetitive DNA (resulting in artificially low oligo counts) or DNA with similarity to sequencing adapters or primers (spurious reads that artificially inflate oligo counts). As in step 7, published Rec12-oligo maps [8] can be used to quantitatively compare oligo frequencies across the genome (e.g., bin the genome and compare oligo counts on a bin-by-bin basis).

4 Notes

1. A completely prototrophic strain or a strain with one auxotrophy is ideal. Strains with more than one auxotrophy can fail to arrest in G1 and not induce completely or at all, likely due to added nutrients serving as nitrogen sources. Also, when inducing stable h^+/h^+ or h^-/h^- diploids selected and maintained by heterozygous auxotrophies, always grow in minimal medium to maintain diploidy. The complementing alleles *ade6-210* and *ade6-216* are useful for making such diploids.
2. Pombe Minimal (PM) medium is routinely used in place of EMM2*; we have not observed any differences in culturing.
3. This is for well-growing strains. Mutant strains with growth defects (*rad51*, *mus81*, *rad32*, etc.) will need to grow an additional 4–6 h to reach the appropriate density. They will still, however, undergo synchronous meiosis. DNA replication can be followed by flow cytometry of 1 mL samples [22].
4. The optimal fixation time can vary (typically 1–10 min) and will need to be determined for each epitope-tagged protein. We found that 5 min gave good results for the experiments we performed [14].
5. If a BeadBeater is not available, vortexing at the highest speed for 45 min at 4 °C is acceptable. Check for cell breakage using a light microscope, comparing intact cells with broken cell walls; 50–70% breakage can be obtained with a BeadBeater.
6. Protein A and Protein G have different affinities for the different species of Ig's and their subclasses, and the appropriate beads should be chosen based on the antibody to be used for the IP. Consult the manufacturer's instructions for more information.
7. If the DNA yield from the IP is low, it will be necessary to perform multiple IP reactions for each sample. Spin an extra 200 μ L of chromatin through the filter for each additional IP reaction. IP replicates can be pooled after elution from the beads (Subheading 3.2 step 21).
8. This is not to be used as a quantitative measure of IP enrichment; rather, it is a quick quality-check of the IP procedure. See [6, 19] for examples and specific oligos used. A more rigorous qPCR can be done if desired.
9. The aa-dUTP is incorporated for the post-amplification labeling reaction with the Cy3 and Cy5 NHS ester dyes. If the dye reaction does not give sufficient Cy3 and Cy5 labeling, increase the ratio of aa-dUTP:dTTP in the DNA amplification.
10. The Agilent *S. pombe* microarray slide has four arrays, each with 44,000 DNA probes. Therefore, it is best to have four IP

and parallel WCE DNA samples ready before proceeding to minimize the expense of a microarray assay. Custom arrays can be manufactured, which is helpful if regions with repetitive DNA are to be probed. See [23] for useful oligos in *S. pombe* repetitive DNA regions, including pericentric regions and Tf transposon-related elements.

11. The DMSO should be dried using 100 g of 4 Å molecular sieve pellets per L of DMSO. Prior to use, activate the pellets by baking at 300–350 °C for 3 h, or at 200 °C under a vacuum for 2 days, followed by cooling in a desiccator. Add the pellets directly to the container of DMSO, followed by occasional shaking.
12. The IP and WCE DNA samples can be labeled conversely with the Cy3 and Cy5 dyes as a control (“dye swap”), to eliminate any potential dye-specific interactions with the microarray.
13. The hybridization can take place in any incubator that can accommodate the secure rotation of the slide at the desired temperature.
14. In order to generate enough Rec12-oligos for sequencing, it may be necessary to grow and extract from multiple liters of culture, depending on the yield. A typical yield is 50–250 fmol/L.
15. It is easiest to resuspend the pellet by first adding 0.1 mL of the buffer and placing a small glass rod or capillary pipette into the tube; vortex at half-speed until the material is in a uniform suspension, and then add the remaining 0.4 mL of buffer
16. In all steps during the Rec12-oligo isolation, filter-purified water (Nanopure, for example) should be used to avoid contaminating the reactions with even minute amounts of nucleic acid. Additionally, the Proteinase K and glycogen must be purified to remove contaminating DNA (*see* Lam et al. Chapter 3 of this volume, Subheadings 2.3.1, 3.3.1, 2.3.2, and 3.3.2 for the procedures).
17. In later experiments, a second immunoprecipitation gave a higher yield of Rec12-oligos (~250 fmol/L of culture) than gel electrophoretic purification as in Subheading 3.5 steps 9–14 (~30–40 fmol/L of culture), presumably at the expense of oligo purity. For a second immunoprecipitation, resuspend the beads in 350 µL of 2× SDS solubilization buffer and boil for 5 min. Concentrate the beads with an MPC, remove the supernatant and transfer it to a new 2 mL microcentrifuge tube. Resuspend the beads in 350 µL of 0.5× SDS solubilization buffer and boil for 5 min. Concentrate the beads with an MPC, remove the supernatant and combine with 350 µL from the first boiling. Add 700 µL of 2× IP buffer, invert to mix, and add to 100 µL of Protein G Dynabeads prebound with 20 µg of anti-FLAG antibody (Subheading 3.2 steps 9–15).

Incubate with rotation for 2 h at 4 °C. Wash the beads as in Subheading 3.5 step 8, then resuspend the beads in 200 µL of proteinase K buffer containing 200 µg of purified DNA- and RNA-free proteinase K, and incubate at 50 °C overnight. Concentrate beads with an MPC, remove the supernatant and transfer to a new 2 mL microcentrifuge tube. Proceed with the ethanol precipitation in Subheading 3.5 step 15.

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References

1. Cervantes MD, Farah JA, Smith GR (2000) Meiotic DNA breaks associated with recombination in *S. pombe*. *Mol Cell* 5:883–888
2. Iino Y, Yamamoto M (1985) Mutants of *Schizosaccharomyces pombe* which sporulate in the haploid state. *Mol Gen Genet* 198:416–421
3. Guerra-Moreno A, Alves-Rodrigues I, Hidalgo E, Ayte J (2012) Chemical genetic induction of meiosis in *Schizosaccharomyces pombe*. *Cell Cycle* 11:1621–1625
4. Cipak L, Hyppa RW, Smith GR, Gregan J (2012) ATP analog-sensitive Pat1 protein kinase for synchronous fission yeast meiosis at physiological temperature. *Cell Cycle* 11:1626–1633
5. Milman N, Higuchi E, Smith GR (2009) Meiotic DNA double-strand break repair requires two nucleases, MRN and Ctp1, to produce a single size class of Rec12 (Spo11)-oligonucleotide complexes. *Mol Cell Biol* 29:5998–6005
6. Cromie GA, Hyppa RW, Cam HE, Farah JA, Grewal SHIS, Smith GR (2007) A discrete class of intergenic DNA dictates meiotic DNA break hotspots in fission yeast. *PLoS Genet* 3:e141
7. Keeney S, Giroux CN, Kleckner N (1997) Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* 88:375–384
8. Fowler KR, Sasaki M, Milman N, Keeney S, Smith GR (2014) Evolutionarily diverse determinants of meiotic DNA break and recombination landscapes across the genome. *Genome Res* 24:1650–1664
9. Young JA, Schreckhise RW, Steiner WW, Smith GR (2002) Meiotic recombination remote from prominent DNA break sites in *S. pombe*. *Mol. Cell* 9:253–263
10. Ren B, Robert F, Wyrick JJ, Aparicio O, Jennings EG, Simon I, Zeitlinger J, Schreiber J, Hannett N, Kanin E et al (2000) Genome-wide location and function of DNA binding proteins. *Science* 290:2306–2309
11. Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M, Brown PO (2001) Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. *Nature* 409:533–538
12. Strahl-Bolsinger S, Hecht A, Luo K, Grunstein M (1997) SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev* 11:83–93
13. Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467–470
14. Fowler KR, Gutiérrez-Velasco S, Martín-Castellanos C, Smith GR (2013) Protein determinants of meiotic DNA break hotspots. *Mol Cell* 49:983–996

15. Estreicher A, Lorenz A, Loidl J (2012) Mug20, a novel protein associated with linear elements in fission yeast meiosis. *Curr Genet* 58:119–127
16. Spirek M, Estreicher A, Cszaszar E, Wells JL, McFarlane RJ, Watts FZ, Loidl J (2010) SUMOylation is required for normal development of linear elements and wild-type meiotic recombination in *Schizosaccharomyces pombe*. *Chromosoma* 119:59–72
17. Davis L, Rozalén AE, Moreno S, Smith GR, Martin-Castellanos C (2008) Rec25 and Rec27, novel components of meiotic linear elements, link cohesin to DNA breakage and recombination in fission yeast. *Curr Biol* 18:849–854
18. Buck MJ, Nobel AB, Lieb JD (2005) ChIPOTle: a user-friendly tool for the analysis of ChIP-chip data. *Genome Biol* 6:R97
19. Hyppa RW, Cromie GA, Smith GR (2008) Indistinguishable landscapes of meiotic DNA breaks in *rad50⁺* and *rad50S* strains of fission yeast revealed by a novel *rad50⁺* recombination intermediate. *PLoS Genet* 4:e1000267
20. Steiner WW, Schreckhise RW, Smith GR (2002) Meiotic DNA breaks at the *S. pombe* recombination hotspot *M26*. *Mol Cell* 9:847–855
21. Cromie GA, Rubio CA, Hyppa RW, Smith GR (2005) A natural meiotic DNA break site in *Schizosaccharomyces pombe* is a hotspot of gene conversion, highly associated with crossing over. *Genetics* 169:595–605
22. Li YF, Smith GR (1997) The *Schizosaccharomyces pombe rec16* gene product regulates multiple meiotic events. *Genetics* 146:57–67
23. Cam HP, Sugiyama T, Chen ES, Chen X, FitzGerald PC, Grewal SI (2005) Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. *Nat Genet* 37:809–819

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