Chapter 4

Genome-Wide Detection of Meiotic DNA Double-Strand Break Hotspots Using Single-Stranded DNA

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Abstract

The controlled fragmentation of chromosomes by DNA double-strand breaks (DSBs) initiates meiotic recombination, which is essential for meiotic chromosome segregation in most eukaryotes. This chapter describes a straightforward microarray-based approach to measure the genome-wide distribution of meiotic DSBs by detecting the single-stranded DNA (ssDNA) that transiently accumulates at DSB sites during recombination. The protocol outlined here has been optimized to detect meiotic DSBs in *Saccharomyces cerevisiae*. However, because ssDNA is a universal intermediate of homologous recombination, this method can ostensibly be adapted to discover and analyze programmed or damage-induced DSB hotspots in other organisms whose genome sequence is available.

Key words: ssDNA, meiosis, double-strand breaks, hotspots, microarray.

1. Introduction

In most eukaryotes, the proper segregation of homologous chromosomes during meiosis I depends on their physical linkage by crossover recombination. The first step in the process of forming crossovers is the introduction of Spo11-dependent DNA double-strand breaks (DSBs) on every chromosome (1). Each DSB is processed via strand resection to expose ssDNA, which then serves as a template for homology search and recombinational repair (2). Because the number and location of DSBs influence where and how many crossovers can form, their distribution across chromosomes is important to ensure proper chromosome assortment and viability of the resulting gametes.

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Meiotic DSBs occur with high frequency in specific "hotspot" regions (3), and two methods have previously been described to measure DSB formation across chromosomes and genomes. First, Southern blot analysis has been used to detect DSB hotspots along restriction fragments and even whole chromosomes (4–6). This method can have very high spatial resolution; however, it is labor intensive and difficult to expand to a genome-wide scale. A second approach takes advantage of the fact that the Spo11 enzyme transiently forms a covalent bond with the DNA at a DSB site. Purification of Spo11-associated DNA enables the genome-wide detection of meiotic DSBs using microarrays (7) or high-throughput sequencing methods (8). However, this approach only detects Spo11-dependent DSBs and requires either epitope tags or antibodies against Spo11. Additionally, the *rad50S*-type mutations that improve Spo11 purification are known to change the distribution of DSB formation in budding yeast (9, 10).

As an alternative, we developed a microarray-based technique to detect the ssDNA that naturally accumulates at DSB hotspots. This method has the advantage that it can be used in wild-type, unperturbed cells, obviating the need for antibodies or epitope tags. Moreover, the repair mutations that trap ssDNA-containing DSBs, such as *dmc1Δ* or *rad52Δ*, have not been shown to affect DSB formation (9, 10). The analysis of mutants with persistent DSBs is useful because it enables cumulative DSB measurements and enhances the ssDNA signal of weaker or transient DSBs (9, 10). Finally, because ssDNA is a universal intermediate of homologous recombination, it should be straightforward to adapt this method to detect natural or induced DSB hotspots in other systems.

Our method utilizes the unique biochemical properties of ssDNA to specifically enrich and label DSB-associated sequences for microarray hybridization (**Fig. 4.1**). To detect meiotic DSB hotspots, cells are first synchronized in meiosis and total genomic DNA is carefully isolated and fragmented. At the strongest meiotic DSB hotspots, breaks are formed in only a small percentage of cells. Therefore, to gain sufficient signal for microarray detection, the ssDNA surrounding DSB sites must be enriched using benzoylated naphthoylated DEAE (BND) cellulose adsorption (11). Next, ssDNA regions are fluorescently labeled by carrying out a random priming reaction without denaturation of the template (12). Finally, enrichment of ssDNA is detected by comparative genomic hybridization (CGH) of the DSB-containing DNA with a control sample using high-density tiled microarrays. This approach allows for the specific and quantitative detection of meiotic DSB-associated ssDNA (9, 10).

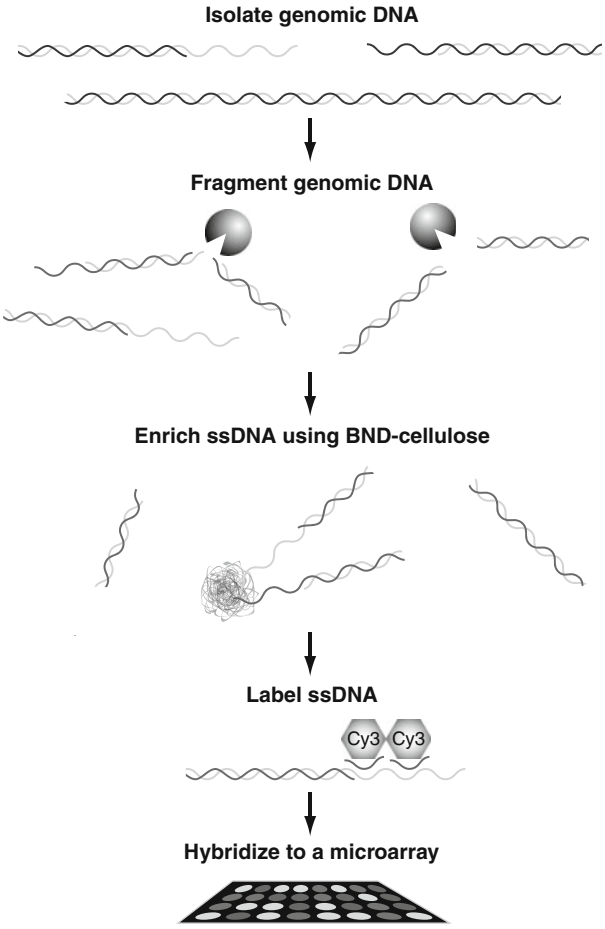

Fig. 4.1. Overview of the procedure used to detect DSB hotspots by measuring ssDNA enrichment. Genomic DNA is carefully isolated and then fragmented by restriction enzyme digestion. Next, the population of molecules containing ssDNA is enriched by batch adsorption to BND cellulose. Subsequently, the ssDNA regions are fluorescently labeled by carrying out a random priming reaction without denaturation of the template DNA in the presence of Cy3- or Cy5-dUTP. Finally, labeled probes are denatured and hybridized to a microarray to detect regions of ssDNA enrichment.

2. Materials

2.1. Cell Synchronization

1. YPG plates: 1% yeast extract, 2% peptone, 3% glycerol, 2% agar, 0.03 mg/ml adenine.
2. 4% YPD plates: 1% yeast extract, 2% peptone, 4% glucose, 2% agar, 0.03 mg/ml adenine.
3. Liquid YPD medium: 1% yeast extract, 2% peptone, 2% glucose, 0.03 mg/ml adenine.

4. Buffered YTA (BYTA) medium: 1% yeast extract, 2% bactotryptone, 1% potassium acetate, 50 mM potassium phthalate. Store at room temperature in the dark for several weeks.
5. Sporulation (SPO) medium: 0.3% potassium acetate, pH 7.0 with 250 μl of 5% acetic acid (v/v) per liter.

2.2. ssDNA Isolation

1. Ethanol, 70% (v/v)
2. Sorbitol buffer: 1 M sorbitol, 0.1 M EDTA, 20 mM Tris-HCl, pH 7.4
3. β-Mercaptoethanol
4. Zymolyase 100T (Associates of Cape Cod, Inc.): 10 mg/ml stock in 1 M sorbitol, store at –20°C
5. 10 mM Tris-HCl, pH 9.5
6. NDS: 0.5 M EDTA, 1% SDS, 10 mM Tris-HCl, pH 9.5 (*see* **Note 1**)
7. TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA
8. Proteinase K: 14 mg/ml
9. Phenol:chloroform:isoamylalcohol (25:24:1)
10. Chloroform
11. RNase A solution: 30 mg/ml (Sigma), store at −20°C
12. 3 M sodium acetate, pH 5.2 with acetic acid
13. Ethanol, absolute

2.3. Genomic DNA Fragmentation

1. *Eco*RI restriction enzyme and 10X EcoRI reaction buffer (New England Biolabs)
2. Spermidine, >98% (Sigma)

2.4. ssDNA Enrichment Using BND Cellulose Adsorption

1. NET buffer: 1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA
2. 5 M NaCl
3. Caffeine elution buffer: NET + 1.8% caffeine (w/v). Put solution at 50°C to dissolve caffeine and then equilibrate to room temperature. This solution should be prepared fresh for each experiment.
4. Benzoylated naphthoylated DEAE–cellulose (Sigma, B-6385), 50% slurry in NET buffer, prepared as follows:
   (a) Weigh out 10 g BND cellulose into a 50 ml tube.
   (b) Wash BND cellulose five times by resuspending the resin in 5 M NaCl in a 50 ml total volume, spinning down for 2 min at 1,350×*g* in a bench top centrifuge and pouring off the supernatant.
   (c) Wash once with water in a 50 ml total volume.

(d) Wash twice with NET buffer in a 50 ml total volume.

(e) Adjust to 50% (v/v) BND cellulose in NET buffer.

(f) Store at 4°C for up to 1 year.

5. 2 ml round bottom microcentrifuge tubes
6. 15 ml conical tubes
7. 14 ml round bottom polypropylene tubes (*see* **Note 2**)

2.5. ssDNA Labeling and Microarray Hybridization

1. DNA Polymerase I, Large (Klenow) Fragment (50,000 units/ml) and 10X NEBuffer 2 (New England Biolabs)
2. Filter-sterilized TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA
3. Random nonamer oligonucleotides: 867 μg/ml in filter-sterilized TE (25% of each nucleotide, IDT)
4. LowT dNTP mix: 2.4 mM each dATP, dCTP, dGTP, 1.2 mM dTTP, diluted in filter-sterilized TE
5. Cy3-dUTP and Cy5-dUTP (GE Healthcare), supplied as 1 mM stock solutions
6. 30,000 MWCO Amicon Ultra filter columns (Millipore)
7. 4x44K yeast whole genome tiled oligonucleotide microarrays (Agilent) or equivalent
8. 2X Hi-RPM hybridization buffer (Agilent) or equivalent
9. Slide hybridization chambers and gasket slides (Agilent)
10. 20X SSPE: 3 M NaCl, 200 mM NaH_2PO_4, 200 mM EDTA, pH 7.4 using NaOH. Filter sterilize and store at room temperature.
11. 20% *N*-lauroylsarcosine, sodium salt solution (Sigma)
12. Wash 1: 6X SSPE, 0.005% *N*-lauroylsarcosine. Filter sterilize and store at room temperature.
13. Wash 2: 0.6X SSPE, 0.005% *N*-lauroylsarcosine. Filter sterilize and store at room temperature.
14. Wash 3: Agilent stabilization and drying solution (*see* **Note 3**)
15. Agilent microarray scanner (or equivalent)

2.6. Microarray Detection of DSBs Using ssDNA Enrichment

1. Feature Extraction software (Agilent) or an equivalent program that can calculate Cy3 and Cy5 intensities from scanned microarray TIFF images.
2. R, a computer language and environment for statistical computing (v2.1.0, http://www.r-project.org), or an equivalent program that can be used to perform statistical analyses and visualize data and results.

3. A program for normalizing microarray data, such as the limma package (www.bioconductor.org) (13) for R (in the sample data set we used a similar but now obsolete R package, Statistics for Microarray Analysis (SMA) (http://www.stat.berkeley.edu/~terry/zarray/Software/smacode.html) (14)).

3. Methods

The protocol outlined below provides a step-by-step procedure for the isolation and labeling of meiotic ssDNA. When performing the initial experimental design, it is important to consider two key parameters that influence the ability to detect DSB-associated ssDNA: the relative abundance of DSBs (*see* **Note 4**) and the presence of non-break-associated ssDNA in the sample (*see* **Note 5**). Both should be taken into account when choosing the strain background and culture conditions. Moreover, preparing a proper control sample in parallel is critical for the quantitative detection of hotspots (*see* **Note 6**). We have provided a sample experiment in which we demonstrate one method to calculate ssDNA enrichment and identify DSB hotspots. In this experiment, we used a *dmc1Δ* strain, which accumulates meiotic DSBs, and an *spo11-Y135F* strain, which does not make meiotic DSBs. Samples were collected from each strain at 0 h before DSBs were formed and at 5 h after meiotic induction when the *dmc1Δ* cells had completed break formation. Biological replicate experiments were performed for each strain.

3.1. Synchronous Meiotic Time Course

Synchronization procedures vary between strain backgrounds. Below is a procedure that provides high synchrony for meiotic cultures of the SK1 background.

1. Remove cells from the –80°C stock onto a YPG plate and grow them overnight at 30°C.
2. Transfer cells onto a 4% YPD plate and grow them overnight at 30°C.
3. Inoculate a 12–15 ml liquid YPD culture for each strain and grow on a shaker for 24 h at room temperature to reach saturation.
4. Dilute the saturated cultures to $OD_{600} = 0.3$ in BYTA presporulation medium and grow on a shaker for 16 h at 30°C.
5. Collect cells from the BYTA cultures by centrifugation for 3 min at 1,350×*g* in a bench top centrifuge. Wash cells with 2 vol. of sterile water.
6. To induce sporulation, resuspend the cells in SPO at $OD_{600} = 1.9$ in highly aerated flasks (maximum SPO culture

volume = 10% of flask volume). Incubate cultures at 30°C on a shaker.

7. At the appropriate time points (e.g., 0 and 5 h for the *dmc1Δ* and *spo11-Y135F* strains), harvest 25–50 ml of SPO culture by centrifugation using 50 ml conical tubes.
8. Resuspend the cell pellet in 25 ml of 70% ethanol and store in ethanol at –20°C.

3.2. Genomic DNA Extraction

Care must be taken when isolating genomic DNA to avoid the artificial creation of ssDNA during the purification procedure. Improper handling can both increase background and/or create artifacts. Two rules of thumb should preserve the original ssDNA content. First, samples should never be exposed to temperatures higher than 50°C to avoid heat denaturation of DNA duplexes. Second, random DNA shearing should be avoided. Therefore, samples should never be vortexed, but rather mixed thoroughly by inversion. Furthermore, wide-orifice pipette tips (which can be purchased or made by cutting off the end of regular pipette tips with a razor blade) should be utilized for **Sections 3.2**, **3.3**, and **3.4**.

1. Pellet the cells for 3 min at 1,350×*g* in a bench top centrifuge and discard the ethanol.
2. Wash the cells once with 10 ml of sorbitol buffer.
3. Resuspend the cells in 10 ml of sorbitol buffer containing 100 µl β-mercaptoethanol and 200 µl of Zymolyase stock by gently pipetting. Incubate at 37°C for 25 min to digest the cell walls.
4. Collect the spheroplasts by spinning for 4 min at 500×*g* in a bench top centrifuge and discard the supernatant.
5. Carefully resuspend the cells in 2 ml of 10 mM Tris-HCl, pH 9.5, by pipetting up and down using a 5 ml pipette. Transfer cells to a 15 ml conical tube.
6. Add 3 ml of NDS and 100 µl of proteinase K and incubate at 50°C for 1 h to digest proteins.
7. Add 2 ml of TE to increase the sample volume for phenol extraction.
8. Extract the DNA three times with 5 ml of phenol:chloroform:isoamyl alcohol. Invert tubes approximately 60 times per extraction to ensure thorough mixing. Centrifuge at 2,800×*g* for 10 min in a bench top centrifuge to separate the phases. It is normal for the aqueous phase to remain cloudy throughout these extractions. It should become clear during the next step (*see* **Note 7**).
9. Extract the DNA once with 5 ml of chloroform to remove traces of phenol and transfer the top phase to a new 15 ml tube.

10. Precipitate the DNA by adding 9 ml of absolute ethanol and pellet by centrifugation at 2,800×*g* for 10 min at room temperature in a bench top centrifuge.
11. Wash the pellet once with 5 ml of 70% ethanol.
12. Drain the ethanol and dissolve the pellet in 3 ml of water by careful tapping.
13. Add 3.5 μl of RNase A and mix by inversion. Incubate samples at 37°C for 30 min to eliminate co-purified RNA (*see* **Note 8**).
14. Add 300 μl of sodium acetate and 7 ml of absolute ethanol to precipitate the DNA. Place tube at –20°C for 10 min. Pellet the DNA by centrifugation at 2,800×*g* for 10 min in a bench top centrifuge.
15. Wash the pellet once with 5 ml of 70% ethanol.
16. Drain the ethanol, spin briefly, and remove any residual ethanol by aspiration. Dissolve the pellet immediately in 1 ml of TE. Store the sample at 4°C for up to several months (*see* **Note 9**).
17. Measure the concentration of genomic DNA using a spectrophotometer. The expected yield from 50 ml of cells is approximately 0.5–1 mg of genomic DNA.

3.3. Genomic DNA Fragmentation

1. Digest approximately 250 μg of DNA with 200 U of *Eco*RI restriction enzyme plus 2 μl of spermidine in a 2.5 ml reaction with 1X EcoRI buffer for 3–4 h at 37°C (*see* **Note 10**).
2. To precipitate the DNA, add 250 μl of sodium acetate and 5.5 ml of absolute ethanol to the digestion reaction. Place at –20°C for 10 min and then collect the precipitated DNA by centrifugation at 2,800×*g* for 10 min in a bench top centrifuge.
3. Discard the supernatant and eliminate traces of ethanol with a pipette. Resuspend the pellet in 500 μl of TE and store the sample at 4°C.
4. Confirm the completion of the digest by analyzing 20 μl of the digestion reaction on a 0.7% agarose gel. Incompletely digested samples usually contain a bright band above the 12 kb band of the ladder.

3.4. ssDNA Enrichment Using BND Cellulose Adsorption

1. Prepare 3 ml of fresh caffeine elution buffer per sample.
2. Adjust *Eco*RI-digested samples to a final concentration of 1 M NaCl by adding 125 μl of 5 M NaCl.
3. For each sample, prepare a 500 μl bed volume of BND cellulose by placing 1 ml 50% BND cellulose slurry in a 2 ml round bottom tube using a wide-orifice pipette tip.

Briefly pellet the resin at full speed in a microcentrifuge and remove the NET buffer with a pipette.

4. Apply the entire *Eco*RI-digested genomic DNA sample to the prepared tube of BND cellulose and resuspend the resin by inverting and flicking the tube. Bind the ssDNA to the BND cellulose by rotating the suspension at room temperature for 5 min.
5. Pellet the BND cellulose for 30 s at full speed in a microcentrifuge. Remove the supernatant with a pipette and discard it.
6. Wash the resin five times with one bed volume (500 μl) NET buffer by inverting and flicking the tube to resuspend the resin. Pellet the resin and remove the supernatant as in the previous step. Discard the washes.
7. Elute the ssDNA by washing the BND cellulose five times with 600 μl of caffeine elution buffer and saving each elution. The five elutions (3 ml total) should be combined into a single 15 ml conical tube.
8. Spin each sample for 10 min at 1,350×*g* in a bench top centrifuge to remove any excess BND cellulose.
9. Carefully pour the supernatant into a 14 ml round bottom tube, which has been labeled on the side of the tube.
10. Add 6 ml of absolute ethanol and incubate at –20°C overnight to precipitate the eluted DNA.
11. Pellet the DNA by spinning for 10 min at 9,800×*g* in a Beckman JA25.50 or comparable rotor. Caps must be removed for the tubes to fit in the JA25.50 rotor adapters.
12. Wash the pellet once with 3 ml of 70% ethanol by spinning as described above. Dry the pellet completely.
13. Resuspend the pellet in 100 μl of TE and transfer the sample to a 1.5 ml microcentrifuge tube.
14. Spin the sample briefly at full speed in a microcentrifuge to remove the excess BND cellulose. Transfer the supernatant to a new 1.5 ml microcentrifuge tube for storage at 4°C.
15. Measure the OD_{260} to estimate the yield of enriched ssDNA. Typically, a total of about 20–25 μg of genomic DNA is recovered after BND cellulose adsorption for both 0 and 5 h samples.

3.5. ssDNA Labeling and Microarray Hybridization

The ssDNA regions are specifically labeled by carrying out a random priming reaction, without denaturing the genomic DNA. Because the 0 and 5 h BND-enriched ssDNA samples from each culture will be co-hybridized to a single microarray, one is labeled with Cy3 and the other with Cy5. Biological replicates are labeled

as dye swaps; the 0 h sample is labeled with Cy3 for one experiment and Cy5 for the replicate.

1. For each sample, combine 20 μl (approximately 5 μg) of enriched ssDNA, 5 μl of random nonamer oligonucleotides, 3.5 μl of 10X NEBuffer 2, and 6.5 μl of water in a thermocycler tube or plate.
2. Heat the samples to 50°C in a thermocycler for 5 min to remove secondary structure in the ssDNA. Cool the samples to 4°C to allow annealing of the primers to the ssDNA.
3. For each sample, prepare 5 μl of extension mix containing 1.25 μl of water, 0.5 μl of 10X NEBuffer 2, 1 μl of lowT dNTP mix, 2 μl of Cy3- or Cy5-dUTP, and 0.25 μl (12.5 U) of Klenow DNA polymerase. Add the extension mix to the samples while they incubate at 4°C and mix well by pipetting.
4. Heat the samples to 37°C at a rate of increase of 0.1°C/s to allow extension of the primers. Incubate at 37°C for 1 h to complete the extension/labeling reaction. Store samples at 4°C in the dark until proceeding to the next step.
5. Remove unincorporated Cy3- and Cy5-dUTP by applying the sample to a Amicon Ultra column, as per manufacturer's instructions. Preload each column with 450 μl of filter-sterilized TE. Add the entire volume of the 0 and 5 h samples for each experimental array to a single column.
6. Spin the column at 14,000×*g* in a microcentrifuge for approximately 8 min to reduce volume to <100 μl.
7. Wash the sample two more times with 450 μl of filter-sterilized TE, followed by centrifugation as described in **Section 3.5**, step 6.
8. Make sure the final volume is reduced to a volume appropriate for microarray hybridization. For a 4x44K Agilent format, this is less than 56.5 μl.
9. Recover the labeled sample by flipping the column into a clean 1.5 ml microcentrifuge tube (provided) and spinning at 1,000×g for 3 min.
10. Adjust the volume to 56.5 μl with filter-sterilized TE (55 μl for hybridization and 1.5 μl for quality control).
11. Measure the Cy3- and Cy5-dUTP incorporation of 1.5 μl of each sample on a NanoDrop spectrophotometer using the microarray application and DNA setting. A typical labeling reaction should yield a total of 20–30 pmol each of Cy3 and Cy5 in each sample pair (*see* **Note 11**).

12. Boil the samples at 95°C for 5 min.
13. Immediately add 55 μl of 2X Hi-RPM hybridization buffer and mix each sample carefully by pipetting without creating bubbles.
14. Spin the samples at full speed for 1 min in a microcentrifuge to remove large bubbles and particulate matter.
15. Apply the entire sample to a single microarray and hybridize according to manufacturer's instructions. Agilent microarrays are hybridized at 65°C for 16–24 h in an Agilent rotating hybridization oven.
16. Set up slide washing chambers containing wash 1, wash 2 and wash 3. An additional open container of wash 1 is needed for opening the slide assembly.
17. Remove the array and gasket slide assembly from the hybridization chamber and submerge the slides under wash 1 in the open container. Immediately remove the array slide from the gasket slide by inserting forceps between the slides to release them.
18. Transfer the microarray slide to the chamber containing wash 1 for 1–5 min, then to wash 2 for 5 min, and finally to wash 3 for 30 s. Use either a stir bar or gentle manual agitation to fully clean the slides in each wash step. Remove the slide from wash 2 carefully so that the solution does not carry over to wash 3. Remove the slide from wash 3 very slowly, allowing the surface tension to gently remove all particulate matter from the surface of the microarray. If particulate matter is visible on the surface of the slide, repeat the wash 2 and wash 3 steps. Let the slides dry completely.
19. Scan the microarrays using an Agilent or equivalent scanner and appropriate laser power such that no microarray features have saturated signals in the Cy3 or Cy5 channel. The resulting data are stored in a split TIFF file that contains the Cy3 and Cy5 images for each slide.

3.6. Microarray Detection of DSBs Using ssDNA Enrichment

Following microarray hybridization and scanning, the raw image data are extracted to calculate ssDNA enrichment for all features ("spots") on the array, and DSB hotspots are identified. Reliable measurements require a number of controls and normalizations that are outlined below. For the sample data set, we performed the extraction and the subsequent calculations using the Agilent Feature Extraction program and R, although equivalent alternatives exist (*see* **Note 12**). The biological replicate experiments for the *dmc1Δ* and *spo11-Y135F* strains were hybridized to independent microarrays, so four total microarrays were analyzed.

1. Measure the fluorescence values and monitor the quality of each array hybridization using Feature Extraction. For each slide to be analyzed, select the TIFF image to be extracted and 'CGH' from the pull-down 'Protocols' menu. The program will automatically find and analyze the fluorescent levels in and around each feature on the array for both channels. Subsequently, several output files are produced, including quality control measures (*see* **Note 13**), a picture of each array, and a text file containing the results of the extraction. The text results file is used as the input for **Section 3.6**, step 2.
2. Calculate the adjusted log ratio of ssDNA enrichment for the 5 h sample versus 0 h sample for each array feature (*see* **Note 14**). Feature Extraction performs a log ratio calculation, which can be used directly from the imported results text file, or the limma function normalizeWithinArrays can be applied. For the sample data sets, the log ratio was calculated using the SMA package for R (13). The mean signal and mean background intensities of Cy3 and Cy5 for each array feature were imported into an R data file from the text results file. The SMA function stat.ma was applied to the data file to calculate log ratios for each feature on the array.
3. Perform scale normalizations for each set of biological replicate experiments. The sample data were normalized using the SMA function stat.norm.exp (the limma equivalent is the function normalizeBetweenArrays). This step normalizes the median absolute deviation of the log ratios for the individual experiments. The resulting scaled data sets are used for steps 4 and 5 of **Section 3.6**.
4. To visualize the results, plot the ssDNA enrichment for each array feature with respect to its chromosomal location. For the sample experiment, we plotted the ssDNA enrichment at 5 h versus the 0 h control for all points along chromosome 3 for the *dmc1Δ* and *spo11-Y135F* strains (**Fig. 4.2a**, black dots). To reduce the contribution of background noise, the results of the replicate experiments were averaged, and subsequently the log ratios were transformed into linear ratios to show fold enrichment. Consistent with the finding that >1 kb of ssDNA can be exposed at each DSB site (15), we observed clusters of adjacent features exhibiting specific ssDNA enrichment that were absent in the *spo11-Y135F* strain. These peaks of ssDNA enrichment in the *dmc1Δ* strain were confirmed by comparing the ssDNA profile from the microarray experiment to a Southern blot for full-length chromosome 3, which exhibited strong DSB hotspots at the same locations (**Fig. 4.2b**).

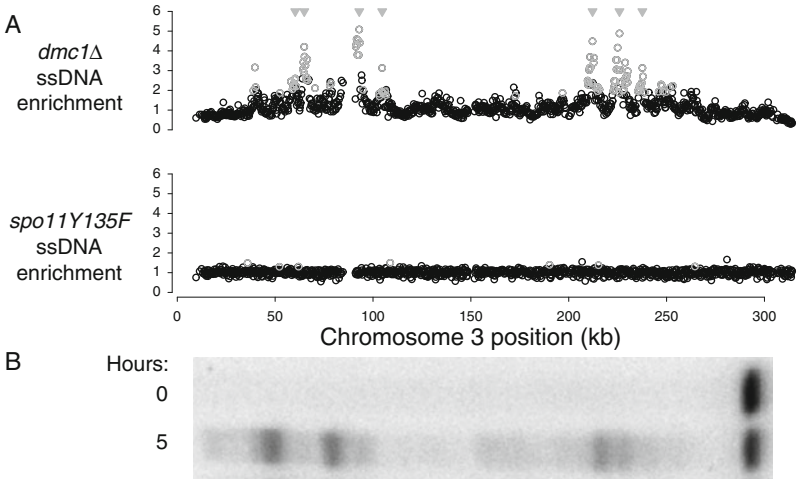

Fig. 4.2. Meiotic DSB hotspots predicted from the site-specific enrichment of ssDNA across the budding yeast genome. **a** DSB hotspot distribution on chromosome 3, as measured by ssDNA enrichment analysis. The mean ssDNA enrichment for biological replicate experiments is plotted with respect to position along chromosome 3 for the *dmc1Δ* (*top*) and *spo11-Y135F* (*bottom*) strains. Features that exhibited significant ssDNA enrichment ($p<0.125$) in both biological replicate experiments are indicated in *gray*, whereas all other features are drawn in *black*. *Inverted gray triangles* represent the positions of clusters of greater than three significantly enriched features, which denote the position of strong DSB hotspots predicted from the ssDNA enrichment. **b** The DSB hotspot distribution of chromosome 3 by Southern blot analysis. Samples were collected from the *dmc1Δ* strain at the indicated time points and DNA was separated by pulsed-field gel electrophoresis. A Southern blot for full-length chromosome 3 was carried out using a probe close to the left telomere.

5. To identify meiotic DSB hotspots using ssDNA enrichment, we applied several criteria to our ssDNA enrichment data.
   (a) A *p*-value cutoff was applied to determine all features significantly enriched above background in the 5 h sample versus the 0 h sample for each individual experiment. In this example, a cutoff of $p<0.125$ was applied to *p*-values that were determined using the pnorm function in R and assuming a single-tailed normal distribution of the data.
   (b) Only features that were reproducibly enriched in both of the individual replicate data sets were considered for further analysis (**Fig. 4.2a**, gray dots).
   (c) Because strong DSB hotspots should be surrounded by 1–2 kb of ssDNA, only peaks of ssDNA enrichment that contained greater than three contiguous features with a significant ssDNA enrichment signal were counted (**Fig. 4.2a**, inverted gray triangles).

This method identified a set of the strongest meiotic DSB hotspots that could be predicted with the highest confidence (*see* **Note 15**). No DSB hotspots were detected in the *spo11-Y135F* strain (**Fig. 4.2a**), indicating that this method does not detect specific ssDNA enrichment from DNA replication, telomeres, or spontaneous DNA damage repair (9).

4. Notes

1. The SDS in the NDS buffer precipitates after storage at room temperature. To resuspend the SDS, heat the buffer to 50°C immediately before use.
2. These tubes will be spun at high speed during the protocol. Polystyrene tubes cannot be used, as they can shatter in the centrifuge.
3. Wash 3 contains acetonitrile, which is highly toxic. Caution should be exercised and acetonitrile gloves should be used when working with this solution.
4. Choice of genetic background plays an important role in detecting DSB-associated ssDNA. As ssDNA is a transient intermediate in the repair process, a high level of synchrony in the experimental culture is crucial for ssDNA detection in wild-type cells. This can be achieved in efficiently sporulating backgrounds, such as SK1. Even in SK1, however, the signal-to-noise ratio was consistently higher in mutants that fail to complete the strand invasion step of homologous recombination, and thus prevent turnover and repair of ssDNA. The use of these mutants may be essential to detect ssDNA-associated DSB hotspots in other strains or organisms.
5. Cellular processes other than DSB formation, most notably DNA replication, can lead to the production of large amounts of ssDNA on all chromosomes (12). Under normal circumstances, replication-associated ssDNA occurs transiently and the synchrony between cells is insufficient to lead to local accumulation of signal. However, certain circumstances, such as the use of replication inhibitors or specific mutants that accumulate excess ssDNA at replication forks, may create abnormally high levels of ssDNA that could obscure the ssDNA signal at DSB hotspots.
6. The quantitative detection of ssDNA requires experimental samples to be compared to a control sample to normalize the data for biases generated by the method of sample preparation or microarray hybridization. The CGH method is a powerful tool for the quantitative analysis of DSB-associated ssDNA, because it enables the reliable measurement of small differences (less than twofold) in the enrichment of sequences relative to each other (16). For every ssDNA microarray experiment, we co-hybridize the experimental sample with a control DNA sample from the same strain collected at 0 h of the experimental time course, prior to meiotic DSB initiation. Alternatively, DNA from an isogenic but DSB-defective mutant (e.g., *spo11Δ*),

cultured in parallel to the experimental strain, can be used. It is critical that the control sample is treated identically to the experimental sample at every step of the protocol, to control for biases introduced by the ssDNA purification and labeling method, especially since other ssDNA is likely present in cells (*see* **Note 5**). Indeed, we consistently observe robust recovery and labeling of ssDNA from 0 h control samples.

7. When transferring the aqueous phase (top) to a new tube, strictly avoid the white interface. This can be aided by removing the aqueous phase with a disposable 5 ml plastic pipette.
8. Completion of this step is critical, because RNA can compete with ssDNA for binding to BND cellulose.
9. As with other applications sensitive to the intact nature of the DNA, purified DNA samples should never be frozen.
10. Do not let the digest proceed for longer or DNA degradation can occur.
11. Incorporation rates greater than 75 pmol of dye in either channel often lead to saturated signals on Agilent arrays. If this occurs, an appropriate portion of the labeled sample can be removed for hybridization, and the volume readjusted to 55 μl with filter-sterilized TE.
12. We used R to calculate ssDNA enrichment, identify hotspots, and visualize results, due to the ease of manipulation and comparison of large data sets in a Unix- and R-based environment. Additionally, the SMA or limma packages for R contain specific microarray normalization functions used to compare samples within or across separate microarray experiments. However, all of the calculations and graphs produced in **Section 3.6** could be performed using other available database and spreadsheet programs, such as Microsoft Excel. Because the Feature Extraction text results file is large and contains information that is not necessary for downstream processing, manipulation of these files is cumbersome in Excel. Therefore, a smaller file can be created for each microarray that contains only the relevant columns of data for every array feature (i.e., chromosome, position, description, log ratios, and significance), which can be used to perform simple calculations or visualize the data.
13. The Agilent Feature Extraction program provides multiple measures of quality control. The original TIFF can be visualized to monitor the quality of hybridization. During the extraction step, irregularities such as saturated or non-hybridizing features are detected. The appearance of a large number of irregularities often indicates insufficient

or saturated hybridization signals, dirt on the surface of the slide, or other hybridization problems that can interfere with data analysis. Because spike-in control samples are not used in the hybridization, error messages referring to the negative signal of control spots should be ignored.

14. To calculate a log ratio, most protocols first subtract local background, then mean normalize the Cy3 and Cy5 channels, and correct for dye bias at different intensities. This step is important to remove biases that are either inherent to the fluorescent dyes at different intensities or that can be introduced by differences in the amount of input DNA, the efficiency of labeling with Cy3 and Cy5, and cross-experiment variation. There are several programs that can be used to perform these calculations. We have used both the Agilent Feature Extraction program with 'CGH' or 'ChIP' settings and the SMA package in R to calculate the log ratios for ssDNA enrichment. The absolute values of the log ratios differ in each case, due to the specific data normalization methods used to calculate the log ratios. In spite of the different absolute values produced, all three of these methods enabled the detection of DSB hotspots and other prominent trends in the data. If an alternative method is used to calculate log ratios, the user should ensure that all of these normalization steps are performed. A good measure of the quality of data normalization is to plot the log ratio versus the log of the average intensity for each array feature (an M versus A plot), to ensure that the average log ratio is 0 across the range of all intensities.
15. Weaker hotspots can also be identified by using a less stringent *p*-value or by demanding that fewer contiguous features are enriched at a given site.

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References

1. Keeney, S., Giroux, C.N., and 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.
2. Bishop, D.K., and Zickler, D. (2004) Early decision; meiotic crossover interference prior to stable strand exchange and synapsis. *Cell* **117**, 9–15.

3. Petes, T.D. (2001) Meiotic recombination hot spots and cold spots. *Nat Rev Genet* **2**, 360–369.
4. Baudat, F., and Nicolas, A. (1997) Clustering of meiotic double-strand breaks on yeast chromosome III. *Proc Natl Acad Sci USA* **94**, 5213–5218.
5. Game, J.C. (1992) Pulsed-field gel analysis of the pattern of DNA double-strand breaks in the Saccharomyces genome during meiosis. *Dev Genet* **13**, 485–497.
6. Zenvirth, D., Arbel, T., Sherman, A., Goldway, M., Klein, S., and Simchen, G. (1992) Multiple sites for double-strand breaks in whole meiotic chromosomes of Saccharomyces cerevisiae. *EMBO J* **11**, 3441–3447.
7. Gerton, J.L., DeRisi, J., Shroff, R., Lichten, M., Brown, P.O., and Petes, T.D. (2000) Inaugural article: global mapping of meiotic recombination hotspots and coldspots in the yeast Saccharomyces cerevisiae. *Proc Natl Acad Sci USA* **97**, 11383–11390.
8. Pan, J., Sasaki, M., Kniewel, R., Murakami, H., Blitzblau, H.G., Tischfield, S.E., Zhu, X., Neale, M.J., Jasin, M., Socci, N.D., Hochwagen, A., and Keeney, S. (2011) A hierarchical combination of factors shapes the genome-wide topography of yeast meiotic recombination initiation. *Cell* **144**, 719–731.
9. Blitzblau, H.G., Bell, G.W., Rodriguez, J., Bell, S.P., and Hochwagen, A. (2007) Mapping of meiotic single-stranded DNA reveals double-strand-break hotspots near centromeres and telomeres. *Curr Biol* **17**, 2003–2012.
10. Buhler, C., Borde, V., and Lichten, M. (2007) Mapping meiotic single-strand DNA reveals a new landscape of DNA double strand breaks in Saccharomyces cerevisiae. *PLoS Biol* **5**, 2797–2808.
11. Huberman, J.A., Spotila, L.D., Nawotka, K.A., el-Assouli, S.M., and Davis, L.R. (1987) The in vivo replication origin of the yeast 2 microns plasmid. *Cell* **51**, 473–481.
12. Feng, W., Collingwood, D., Boeck, M.E., Fox, L.A., Alvino, G.M., Fangman, W.L., Raghuraman, M.K., and Brewer, B.J. (2006) Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication. *Nat Cell Biol* **8**, 148–155.
13. Smyth, G.K. (2005) Limma: linear models for microarray data. In Bioinformatics and computational biology solutions using R and bioconductor, R.C. Gentleman, V.J. Carey, S. Dudoit, R. Irizarry, W. Huber, eds. (New York, NY: Springer), pp. 397–420.
14. Yang, Y.H., Dudoit, S., Luu, P., and Speed, T.P. (2001) Normalization of cDNA microarray data. In SPIE BiOS 2001. San Jose, CA.
15. Bishop, D.K., Park, D., Xu, L., and Kleckner, N. (1992) DMC1: a meiosis-specific yeast homolog of E. coli recA required for recombination, synaptonemal complex formation, and cell cycle progression. *Cell* **69**, 439–456.
16. Yabuki, N., Terashima, H., and Kitada, K. (2002) Mapping of early firing origins on a replication profile of budding yeast. *Genes Cells* **7**, 781–789.