Analysis of ssDNA Gaps and DSBs in Genetically Unstable Yeast Cultures

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Abstract

DNA replication defects are an underlying cause of genome instability, which could stem from alterations in replication intermediates such as extensive single-stranded DNA (ssDNA). Under replication stress, ssDNA is a precursor of the ultimate double-strand breaks (DSBs). Indeed, mutations that render the cell incapable of mediating and protecting the replication forks produce ssDNA genome-wide at high frequency and cause lethality when encountering DNA damage or replication perturbation. Here we describe two related microarray-based methods to query genetically unstable yeast cultures, such as the *mec1* and *rad53* mutants. These mutants are defective in central protein kinases in the checkpoint pathway. To induce ssDNA and DSB formation in these mutants, we utilize hydroxyurea, a drug that causes nucleotide shortage in the cell.

Keywords

DNA replication checkpoint; Replication stress; Single-stranded DNA; Double-strand break; Hydroxyurea; Origins of replication; Chromosome fragile sites

1 Introduction

Eukaryotic chromosomal DNA replication is a complex process that involves the activation, coordination, and resolution of hundreds to thousands of origins of replication. These are the sites where replication initiates in a bidirectional manner, forming the so-called replication bubbles. At either end of the replication bubble are the junctions of replicated and unreplicated DNA, also called replication forks. The replication forks are particularly vulnerable structures that when unprotected could generate extensive single-stranded DNA (ssDNA) gaps [1, 2] and ultimately double-strand breaks (DSBs) [3].

Observation of ssDNA formation near the origins of replication during hydroxyurea-induced nucleotide shortage was first made visually by microscopy [1] and later confirmed by genome-wide approaches [2], which are facilitated by the development of a microarray-based ssDNA labeling method. It was then proposed that ssDNA formation at replication origins during nucleotide shortage caused by the treatment with hydroxyurea (HU, an inhibitor of the ribonucleotide reductase) is an evolutionarily conserved process and can be exploited in order to map origins of replication in diverse organisms. Indeed, HU treatment

has been used to identify origins of replication in diverse yeast species such as *Schizosaccharomyces pombe* [2], *Lachancea waltii* [4], and *Saccharomyces bayanus* (G. Alvino, M. Dunham, B. Brewer, and M.K. Raghuraman, unpublished). We have also observed that other forms of replication inhibition also induce ssDNA formation (Feng W. unpublished data), suggesting that it is a general response elicited by the cell under replication stress. In an attempt to examine the fate of ssDNA formation, particularly in mutant backgrounds where such an event brings about the cell's demise, it was then demonstrated that persistent ssDNA indeed ultimately led to DSBs at the replication forks as previously postulated [3]. Again, such experimental evidence was facilitated by the development of a similar microarray-based method to query persistent DSBs in the yeast genome.

Here we describe the details of two microarray-based protocols related to the abovementioned methods in order to interrogate the yeast genome for ssDNA and DSBs under replication stress caused by HU in a synchronous yeast culture (Fig. 1). However, we envisage that both methods can be applied to query ssDNA and DSBs produced by other replication stress or indeed agents (chemicals or genetic mutations) that may very well be unrelated to the replication process. We have mapped both in vitro restriction digestion-induced DSBs as well as in vivo HO (endonuclease)-induced irreparable DSBs in the yeast genome using the DSB labeling method [3]. We also note that the two methods described in this chapter are readily adaptable to use with the more superior next-generation sequencing platforms in place of the microarray platform in order to achieve higher sensitivity and better coverage of the genome. With this adaptation, it is then also feasible to apply these methods to the vastly more complex mammalian genomes in order to identify important chromosomal landmarks such as origins of replication and fragile sites.

2 Materials

All solutions are autoclaved or filter sterilized unless otherwise noted.

2.1 Cell Culture Sample Collection

1. AGD H_2O (autoclaved glass-distilled H_2O): All solutions are prepared in AGD H_2O unless otherwise noted.

2. YPD medium: 10 g/L Yeast extract, 20 g/L Bacto Peptone, 20 g/L D-glucose.

3. α-Factor (peptide sequence: NH_2–WHWLQLKPGQPMY–COOH, custom synthesized by ThermoFisher at >70 % purity), prepared as 200 μM or 3 mM stocks (1,000× stocks) for *bar1* and *BAR1* strains, respectively, and stored at −80 °C.

4. 10 % NaN_3.

5. HU powder.

6. Pronase.

7. 15- and 50-mL tubes.

2.2 Preparation of Cells Embedded in Agarose Plugs

1. Centrifuge with swing bucket rotors (e.g., TX-750 rotor, Thermo Scientific).

2. 0.5 M EDTA, pH 8.0.

3. 50 mM EDTA, pH 8.0.

4. 1 % Low-melt agarose: 1 % InCert or NuSieve GTG agarose (Lonza) in 50 mM EDTA pH 8.0, melted and then cooled to 42 °C before use.

5. 50-Well disposable plug molds (Bio-Rad); can be washed and reused.

6. Six-well sterile tissue culture-treated plates with flat bottoms.

7. TE: 10 mM Tris–HCl, 1 mM EDTA pH 8.0.

8. 1 M Sorbitol.

9. 1 M Tris–HCl pH 7.5.

10. Spheroplasting solution: 1 M Sorbitol, 20 mM EDTA pH 8.0, 10 mM Tris–HCl pH 7.5, 14 mM β-mercaptoethanol, 1.5 mg/mL Zymolyase 20-T. Prepare fresh.

11. 1× Sodium dodecyl sulfate (SDS) solution for plugs: 10 mM Tris–HCl pH 8.0, 1 % SDS, 100 mM EDTA. Prepare from a 10× stock.

12. 5× NDS: 10 mM Tris–base pH 8.0, 0.5 M EDTA, 1 % Sarkosyl.

13. 1× NDS: Diluted from 5× NDS.

2.3 In-Gel Labeling for ssDNA or DSBs

1. 10× ssDNA labeling reaction buffer: 500 mM Tris–HCl pH 6.8, 50 mM $MgCl_2$, 100 mM β-mercaptoethanol. Store at −20 °C.

2. 1× ssDNA labeling reaction buffer, prepared fresh from the frozen 10× stock, and used to equilibrate agarose plugs prior to the labeling reaction.

3. $TE_{0.1}$: 10 mM Tris–HCl pH 8.0, 0.1 mM EDTA pH 8.0.

4. 10× dNTP mix: 1.2 mM dATP, 1.2 mM dCTP, 1.2 mM dGTP, 1.6 mM dTTP, and 10 mM Tris–HCl pH 8.0, stored at −20 °C.

5. 2.5× ssDNA labeling reaction buffer stock: 125 mM Tris–HCl pH 6.8, 12.5 mM $MgCl_2$, 25 mM β-mercaptoethanol, 750 μg/mL random hexamers (custom synthesized). Store at −20 °C.

6. 1 mM Cy5- and Cy3-dUTP (GE Healthcare).

7. 50,000 units/mL Klenow Fragment (3′–5′ exo-) (NEB).

8. 10× End-repair labeling reaction buffer: 330 mM Tris–acetate pH 7.8, 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM dithiothreitol. Store at −20 °C.

9. 1× End-repair labeling reaction buffer, prepared fresh from the frozen 10× stock, and used to equilibrate agarose plugs prior to the labeling reaction.

10. End-It™ DNA End-repair kit (Epicentre).

11. $1 \times$ β-Agarase buffer: Diluted from $10\times$ β-Agarase buffer (NEB) with AGD H_2O.

12. 1,000 units/mL β-Agarase (NEB).

13. Bioruptor (Diagenode Standard Model).

14. QIAquick PCR Purifi cation Kit (Qiagen).

15. NanoDrop ND-2000 spectrophotometer (Thermo Scientifi c).

2.4 Microarray Hybridization

1. Agilent 4×44K ChIP-to-chip yeast microarrays.

2. Microarray hybridization facility and Agilent microarray scanner.

2.5 Generation of ssDNA/DSB Profiles

1. A file containing a list of genomic coordinates (chromosome number and coordinate) for the microarray probes that will be used in the analysis (e.g., excluding probes corresponding to mitochondrial DNA): For convenience, we shall call this file "ProbeCoordinates.txt." The list should be sorted by ProbeID (ascending order), one line per probe, and saved as a tab-delimited text file. Sorting by ProbeID should result in the list also being sorted by chromosome number and coordinate. To allow easy computer processing, chromosome numbers should be in Arabic numerals even though the convention for budding yeast is to use Roman numerals for chromosome numbers. This file needs to be prepared only once for each particular microarray platform and can subsequently be used for all experiments using that platform. Typically, we prepare this list by performing a batch BLAST search of the yeast genome using the vendor-provided list of probe sequences, discarding all sequences that either show more than one match to the genome or less than a perfect match. From the BLAST results, we extract the chromosome number and coordinate for the left end of each probe. The first row of the file should have column headers. The columns for chromosome number, coordinate (kb), and coordinate (bp) should be labeled "Chr," "Coord_kb," and "Coord_bp" (without the quotes) to match the file manipulations in Subheading 3.5, **step 12**.

2. A file containing a list of probes to exclude from the final analysis (e.g., probes corresponding to Ty element sequences), formatted as above: Again, this file needs to be prepared only once for each microarray platform.

3. The statistical software package R, available online at <http://cran.r-project.org/>.

4. The following set of commands saved as a plain-text file to be run in R (Subheading 3.5, **step 12**); name the file (for example)

   "ssDNA_smoothing.R" (hanging indentations in the code text below indicate text that all belongs on one line):

   # R script for loess smoothing of ssDNA/DSB data

```
files <- list.files(path = "path_to_ssDNA_or_DSB_files", full. names = TRUE)

for(i in seq(along=files)) { dataIn <- read.delim(files[i]) attach(dataIn)

Sphase_ratio_raw <- Sphase_raw/G1_raw

Sratio.loess <- loess(Sphase_ratio ~ Coord_kb, dataIn, span = winSize/
tail(Coord_kb, n=1))

Sratio.predict <- predict(Sratio.loess, Coord_kb)

dataOut <- data.frame(Chr, Coord_kb, Coord_bp, Sphase_ raw, G1_raw,
Sphase_ratio_raw, Sphase_ratio_loess=Sratio. predict)

write.table(dataOut, files[i], quote=FALSE, sep="\t", eol="\r", row.names =
FALSE)

detach(dataIn)

}
```

3 Methods

3.1 Cell Collection

1. Grow yeast cells in YPD medium at the appropriate temperature to log phase (at $OD_{660} = 0.25$) (*see* **Note 1**).

2. Add α-factor to the log-phase culture (at $OD_{660} = 0.25$) at 200 nM or 3 μM for *bar1* or *BAR1* strains, respectively.

3. Continue growing the cells until the percentage of unbudded cells reaches 90 % (*see* **Note 2**).

4. Collect an appropriate volume of cells as the G1 control sample by pouring the cells into an appropriate container, e.g., a 50-mL tube (for a small sample) or a 250-mL graduated cylinder (for a large sample), with 0.01 volumes of 10 % NaN_3 already added such that its final concentration would be 0.1 %. Store the G1 control sample on ice until further processing.

5. Add HU powder to the culture at 200 mM (*see* **Note 3**).

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[1]Inoculate the cells in 5 mL of YPD medium and culture over-night. The culture may or may not reach saturation depending on the generation time of the strain at the chosen culturing temperature. Then, inoculate cells from the primary culture into 25 mL of YPD medium and measure the generation time. Based on the generation time and the cell density of the 25 mL culture, inoculate the cells from the 25 mL culture into fresh YPD medium with a volume that is appropriate for sample collection. Determine culture volume based on the number of agarose plugs per sample desired. We typically obtain three agarose plugs per 50 mL of culture at ~10^7 cells/mL. Bear in mind that each S-phase sample is to be paired with a G1 control sample for co-hybridization onto the microarray; therefore, one must collect a larger sample for the G1 control accordingly. The amount of ssDNA or DSBs in a given strain is also an important point for consideration, which might require experimental trials to determine the optimal amount of cells to utilize in the microarray analysis.

[2]Transfer 1 mL of the culture to a 1.5-mL microcentrifuge tube. Sonicate the culture by Bioruptor ("low" setting, 30 s ON and 30 s OFF, two cycles). Pipet 2 μL of the culture onto a microscope slide, and cover it with a cover glass. Then count the number of budded cell in a minimum of total 200 cells counted.

[3]Weigh HU and deliver into the culture via a weighing boat or a piece of folded weighing paper while tilting the culturing flask to prevent the powder from sticking to the neck of the vessel. Mix well by swirling the culture vigorously. HU should dissolve in 1–2 min.

6. Add pronase to the culture at 0.02 mg/mL or 0.3 mg/mL for *bar1* or *BAR1* strains, respectively, to release cells from α-factor arrest (*see* **Note 4**).

7. Collect an appropriate volume of S-phase cells, typically after 1-h exposure to HU, similarly as described in **step 4** above. Multiple samples collected at other time intervals may be desired. Store the collected samples on ice until further processing.

3.2 Preparation of Genomic DNA in Agarose Plugs

3.2.1 Preparation of Cells Embedded in Agarose Plugs

1. Centrifuge the collected samples in swing bucket rotors (e.g., TX-750 rotor) at $2,500 \times g$ for 10 min at 4 °C.

2. Wash the cell pellets with 10 mL of 50 mM EDTA and centrifuge again. Repeat once.

3. Resuspend the cell pellets with 1 mL of 50 mM EDTA and transfer to a 1.5-mL microcentrifuge tube.

4. Centrifuge in a tabletop microcentrifuge at ~$2,500 \times g$ for 5 min at 4 °C. Aspirate the liquid thoroughly, and resuspend the cells in 50 mM EDTA to a final concentration of 2×10^9 cells/mL. Measure the volume of cell suspension with a pipette.

5. Add equal volume of 1 % low-melt InCert agarose pre-warmed to 42 °C (*see* **Note 5**). Mix thoroughly, and quickly pipet 100 μL aliquots into each well of the plug molds to harden at room temperature (*see* **Note 6**).

6. Extrude each plug from the plug molds into a 6-well plate containing 6 mL of TE with a maximum of three plugs per 6 mL TE. The plugs can be stored at 4 °C for up to 48 h before spheroplasting.

3.2.2 Spheroplasting in Agarose Plugs

1. Make fresh spheroplasting solution.

2. Aspirate the TE buffer from the 6-well plates containing plugs, and add 6 mL of fresh spheroplasting solution to each well.

3. Incubate with gentle shaking for 4 h at 37 °C.

4. Aspirate off the spheroplasting solution, and add 6 mL of 1× SDS solution for plugs.

5. Incubate with gentle shaking for at least 15 min at 37 °C.

6. Exchange the 1× SDS solution with 6 mL of fresh 1× SDS solution.

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[4]Weigh the pronase powder and dissolve in AGD H_2O before use. It can be temporally stored on ice.
[5]Melt the 1 % low-melt InCert or NuSieve agarose by heating in the microwave, taking precautions not to let the solution boil over. Equilibrate to 42 °C in a water bath for at least 1 h.
[6]Hold the microcentrifuge tube containing the mix tightly to keep the mix warm, quickly pipet to mix, and then transfer into the plug mold. Avoid bubbles during pipetting.

7. Incubate with gentle shaking overnight at 37 °C.

8. Aspirate the 1× SDS solution.

9. Wash the plugs twice with 6 mL of 1× NDS solution with 30 min with gentle shaking at room temperature.

10. Wash the plugs three times with 6 mL of TE for 30 min with gentle shaking at room temperature.

11. Add 6 mL of fresh TE to each well. Cover the plate with plastic wrap. The samples can be stored at 4 °C for up to 6 months. Exchange with fresh TE periodically.

3.3 In-Gel Labeling of ssDNA or DSBs and DNA Extraction and Purification

3.3.1 In-Gel Random-Primed Labeling of ssDNA by Klenow Fragment (3′–5′ Exo-)

1. Make fresh 1× ssDNA labeling reaction buffer from 10× stock.

2. Transfer one plug from each sample collected at a specific time point to a well in a new 6-well plate, with one plug per well. Transfer the same number of plugs from the G1 control sample to the plate as well in order to pair each S-phase sample with a G1 control sample.

3. Wash the plugs twice with 5 mL of $TE_{0.1}$ per well for 15 min at room temperature with gentle shaking.

4. Wash the plugs twice with 5 mL of 1× ssDNA labeling reaction buffer per well for 30 min at room temperature with gentle shaking.

5. Assemble a small water chamber (to prevent desiccation of the agarose plugs) using an emptied 1-mL pipette tip box with approximately 50 mL AGD H_2O added to the bottom. Cut a piece of parafilm, and place it on the tip rack, marking the position/sample name on the edge of the parafilm to indicate where the plugs would be positioned.

6. During the last step of washing, prepare the ssDNA labeling reaction mix. For each plug (~50 μL), mix the following (*see* **Note 7**): 10 μL of 10× dNTP mix, 20 μL of 2.5× ssDNA labeling reaction buffer stock, 6 μL of Cy5- or Cy3-dUTP, 3 μL of 50,000 units/mL Klenow Fragment (3′–5′ exo-), and 11 μL of AGD H_2O.

7. Transfer the plugs onto the parafilm according to the arranged position with a clean spatula.

8. Pipet 50 μL of the ssDNA labeling reaction mix onto each agarose plug (generally Cy5 for G1-phase sample and Cy3 for S-phase sample).

9. Close the tip box, and incubate the reactions for 2–3 h in the dark at 37 °C.

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[7]The apparent final concentration of dNTP mix is 2×, but the final concentration is 1× considering further dilution from the gel volume (~50 μL) as the dNTP mix is not included in the equilibration step.

3.3.2 In-Gel End Repair by the End-It™ Kit (T4 DNA Polymerase)

1. Prepare fresh $1\times$ end-repair labeling reaction buffer from $10\times$ stock.

2. Transfer one plug from each sample collected at a specific time point to a well in a new 6-well plate, with one plug per well. Transfer the same number of plugs from the G1 control sample to the plate as well in order to pair each S-phase sample with a G1 control sample.

3. Wash the plugs twice with 5 mL of $TE_{0.1}$ per well for 15 min at room temperature with gentle shaking.

4. Wash the plugs twice with 5 mL of $1\times$ end-repair labeling reaction buffer per well for 15 min at room temperature with gentle shaking.

5. Assemble a small water chamber (to prevent desiccation of the agarose plugs) using an emptied 1-mL pipette tip box with approximately 50 mL AGD H_2O added to the bottom. Cut a piece of parafilm, and place it on the tip rack, marking the position/sample name on the edge of the parafilm to indicate where the plugs would be positioned.

6. During the last step of washing, prepare the end-repair labeling reaction mix. For each plug (~50 μL), mix the following (*see* **Note 7**): 5 μL of $10\times$ end-repair buffer (from the End-It™ end-repair kit), 10 μL of 10 mM ATP (from the End-It™ end-repair kit), 10 μL of $10\times$ dNTP mix, 6 μL of Cy5- or Cy3-dUTP, 3 μL of End-It™ Enzyme mix, and 16 μL of AGD H_2O.

7. Transfer the plugs onto the parafilm according to the arranged position with a clean spatula.

8. Pipet 50 μL of the end-repair labeling reaction mix onto each agarose plug (generally Cy5 for G1-phase sample and Cy3 for S-phase sample).

9. Close the tip box, and incubate the reactions for 1–1.5 h in the dark at 25 °C.

3.3.3 Extract and Purify DNA from Agarose Plug—The following steps should be performed in dim light to prevent inactivation of the Cy dyes.

1. Transfer plugs from the parafilm into a new 6-well plate with one plug per well. Wrap the plate in aluminum foil to prevent light exposure; do likewise for the subsequent steps.

2. Wash the plugs twice with 5 mL of $TE_{0.1}$ for 15 min at room temperature with gentle shaking.

3. Equilibrate the plugs twice with 200 μL of $1\times$ β-Agarase buffer for 30 min at 0–4 °C (by placing the 6-well plate on ice) with gentle shaking.

4. Transfer one G1- and one S-phase plug into the same 1.5-mL microcentrifuge tube with a clean spatula (*see* **Note 8**).

5. Melt the plugs for 10 min in a 65 °C water bath with occasional mixing to encourage melting (*see* **Note 9**).

6. Equilibrate the melted plugs for 10 min in a 42 °C water bath.

7. Add 2 μL of β-Agarase to each tube, and incubate for 1 h at 42 °C.

8. Centrifuge the mix in a tabletop microcentrifuge at >17,000 × *g* for 20 min at 4 °C.

9. Transfer the supernatant to a fresh 1.5-mL microcentrifuge tube.

10. Sonicate the mix to shear the DNA by Bioruptor ("low" setting, 30 s ON and 30 s OFF, 30 cycles, 4 °C).

11. Purify the DNA by the QIAquick PCR Purification Kit following the manufacturer's instructions.

12. Elute the DNA from each pair of pooled samples with 45 μL of EB buffer.

13. Measure the DNA concentration using a NanoDrop ND-2000 spectrophotometer (*see* **Note 10**). The labeled DNA is ready for hybridization or can be stored in the dark at 4 °C overnight.

3.4 Microarray Hybridization and Data Extraction

1. Hybridize the labeled DNA onto Agilent ChIP-to-chip yeast microarray slides following the manufacturer's instructions.

2. Extract data with the Feature Extraction software (Agilent).

3.5 Generating an ssDNA or a DSB Profile for Each Chromosome in the Yeast Genome

1. Duplicate the data file from Subheading 3.4, **step 2**. Leave the original file untouched as an archival copy; give the duplicate file a distinctive name, and perform all further manipulations on this duplicate.

2. Open the file in Microsoft Excel (or equivalent), and delete the first rows of descriptors, if any, so that the row of column headers (ProbeID, ProbeName, etc.) becomes the first row.

3. Select all the data in the file and sort by ProbeID (ascending sort).

4. Delete the rows corresponding to hybridization control spots (rows 2–331 for Agilent 4 × 44 ChIP arrays).

5. Delete the rows corresponding to mitochondrial DNA probes (the rows at the end of the data table). Important: Perform this step only if you have eliminated mitochondrial DNA probes from the list of probe coordinates in file ProbeCoordinates.txt (see Subheading 2.5, **item 1**). If you have retained mitochondrial DNA probe information in Subheading 2.5, **item 1**, you must retain the corresponding rows in the data file from **step 4** above. At this point, the number

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[8]As the G1- and S-phase sample are subjected to co-hybridization onto the microarray, pooling them at this step is acceptable. However, we recommend assessing the efficiency of DNA extraction from the gel from each independent sample first for quality control.

[9]Longer incubation time might be necessary. Up to 20 min has been tested and proven acceptable.

[10]The incorporation of Cy-dUTP using the ssDNA labeling method typically yields ~1.5 pmol/μL, while the incorporation of Cy-dUTP using the DSB labeling method is often tenfold lower. Scaling up the samples for each microarray labeling is recommended.

of rows of data in this file should correspond exactly to the number of rows of probe coordinates in file ProbeCoordinates.txt.

6. Optional step: Delete columns that you will not be using, so as to reduce the file size and speed subsequent manipulations. Typically, we only retain the following columns: ProbeName, gProcessedSignal, rProcessedSignal, gMedianSignal, rMedian-Signal, gBGMedianSignal, and rBGMedianSignal.

7. With the data still sorted by ProbeID, insert the list of genomic locations (chromosome and coordinates, file ProbeCoordinates from Subheading 2.5, **item 1**) as the leftmost columns in the table (i.e., after this step, the chromosome number will be in column 1 and coordinates as kb and bp will be in columns 2 and 3, respectively). Because the data file (*see* **step 6** above) and the list of coordinates (ProbeCoordinates.txt) were both sorted in ascending order by ProbeID, each row of microarray data is now identified by its genomic location. Save the file as tab-delimited text, and continue.

8. Identify the "ProcessedSignal" columns corresponding to the G1-DNA and ssDNA or DSB-DNA channels. For example, if G1-DNA was labeled with Cy5, the column corresponding to the G1-DNA signal will be the column labeled "rProcessedSignal." For convenience and for future manipulations, re-label these two columns in the file as "G1_raw" and "Sphase_raw."

9. Use the exclusion list (Subheading 2.5, **item 2**) to eliminate the rows corresponding to genomic locations you wish to exclude from the final output (*see* **Note 11**).

10. Split the file by chromosome; that is, create a set of files, each containing the data for just one chromosome but retaining the column headers (Chr, Coord_kb, etc.) from the source file. Create a sub-folder, and save each file as tab-delimited text within that folder. For clarity, save each file with a common base name, differing only in a numerical index indicating the chromosome corresponding to that file (e.g., chr01_ssDNA. txt, chr02_ssDNA.txt) (*see* **Note 12**).

11. Using a text editor, open the text file "ssDNA_smoothing.R" created in Subheading 2.5, **item 4**. In this file, replace "path_ to_ssDNA_or_DSB_files" with the actual path to the folder containing the files saved after splitting the data by chromosome; retain the quotes surrounding the folder path. Replace "winSize" with the desired smoothing window in kb (but "win-Size" should be replaced just by the numerical

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[11]This step can be accomplished using a scripting language such as Perl or Python, or Microsoft Office VBA for Applications, depending on the investigator's skill/comfort with such languages. Alternatively, it can be accomplished using Microsoft Excel and Microsoft Word without any scripting. For example, each open reading frame (ORF) in the exclusion list can be converted to a pair of rows in the file: one row containing the chromosome number and start coordinate for that ORF and containing the word "START" as a third field in that row, and one row with the chromosome number and the end coordinate and containing the word "END" as the third field for that row. This list of start and end coordinates can be appended to the data file and whole file sorted by chromosome and coordinate and saved as a tab-delimited text file. Now, each set of probes to be excluded will be preceded by a row containing the word "START" and followed immediately by a row containing the word "END." Next, using the "Advanced Find and Replace" command with wild card searching enabled in Microsoft Word, the rows between each START…END pair can be deleted. Using Microsoft Excel again to sort the data, the lines containing START and END can be grouped and deleted, leaving just the desired microarray data.

[12]The next step (the smoothing step) will result in alteration of the input file, so we recommend making an archival copy of the files before performing the smoothing operation.

value of the desired window and should not contain the text "kb"). Typically, for ssDNA and DSB DNA profiles, we use a smoothing target window of 6 kb. Save the text file (*see* **Notes 13** and **14**).

12. Smooth the ssDNA or DSB-DNA data for each chromosome by running the following batch command in the unix shell after navigating to the folder/directory containing the file "ssDNA_smoothing.R" (*see* **Note 15**):

R CMD BATCH ssDNA_smoothing.R

The command will invoke the R script and apply loess smoothing to the data, appending a column of raw S-phase/G1 ratios and a column of smoothed data values to the file for each chromosome. The columns with raw and smoothed S-phase/G1 ratios will have the labels, "Sphase_ratio_raw" and "Sphase_ratio_loess," respectively.

13. Using the graphing application of your choice, plot the data for each chromosome (Sphase_ratio_loess as a function of Coord_kb or Coord_bp). Set the *x*-axis dimension to some constant scaling factor (e.g., 1 cm = 100 kb) such that profiles for all chromosomes are on the same scale.

14. *See* Figs. 2 and 3 for a sample plot of ssDNA and DSB, respectively.

Acknowledgements

References

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[13] Although the R script is called ssDNA_smoothing.R, the same script will work for smoothing DSB-DNA data also.

[14] Be aware that unix, Microsoft Windows, and Apple Macintosh files typically use different line delimiters. For example, Macintosh files typically use carriage returns (ASCII character 13) to signify line endings, while unix files use the "linefeed" character (ASCII character 10). So, some experimentation with line delimiters within the R script file and in the output files defined in the R script file may be necessary to find the appropriate format for the system being used. For example, the "end-of-line" (eol) definition in the R script (*see* **item 4** of Subheading 2.5) can be changed from "\r" (carriage-return) to "\n" (linefeed).

[15] The R batch command syntax is appropriate for unix systems, e.g., to be run in the Terminal application in Mac OS X. The same batch file (ssDNA_smoothing.R) will also work in Windows systems, but the syntax to invoke the file will have to be modified appropriately.
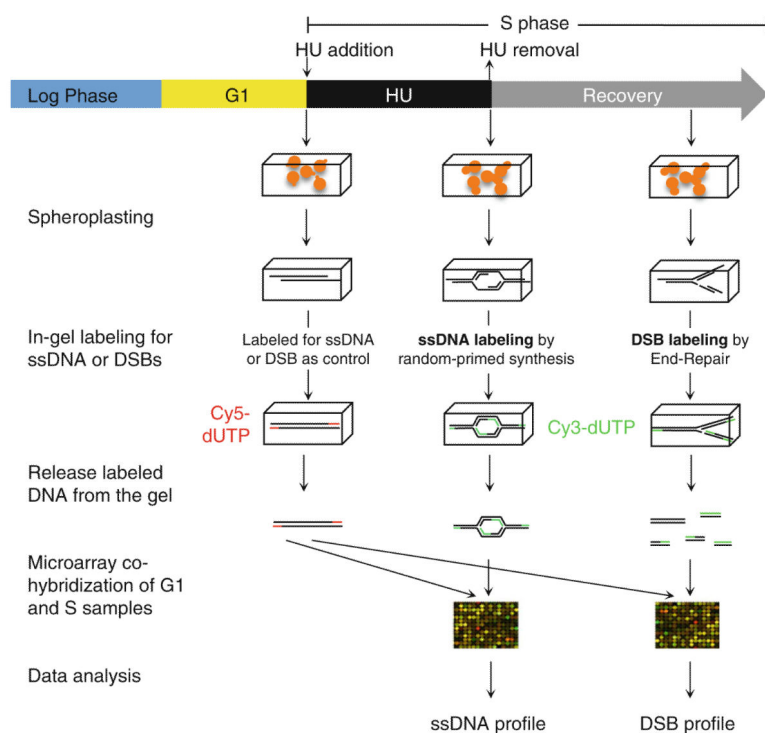
Fig. 1.

Outline of procedures for cell culture collection followed by in-gel ssDNA and DSB labeling by random-primed synthesis via Klenow and by end repair via T4 DNA polymerase, respectively. These procedures were followed by microarray analysis. *HU* hydroxyurea. Note that significant DSBs were observed only during the "recovery" phase after transient exposure to HU in the *mec1* mutant [3]
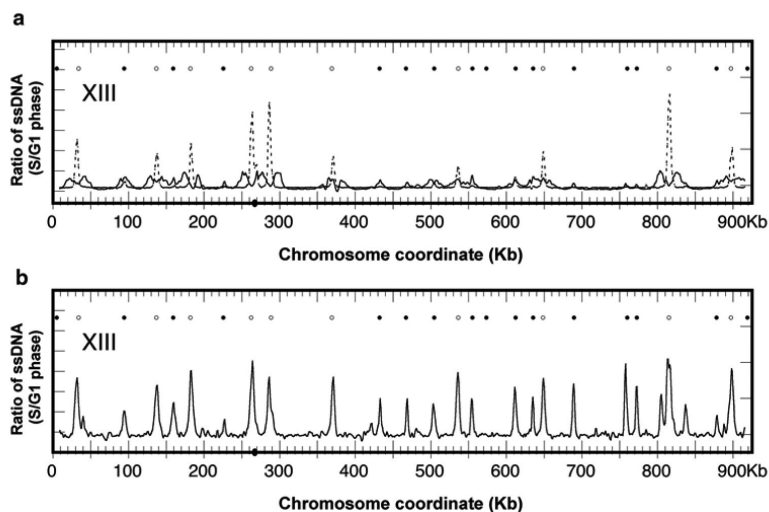
Fig. 2.
ssDNA profiles of chromosome XIII. (**a**) ssDNA profiles for WT (W303) cells synchronously released into S phase in YPD containing 200 mM HU for 1 h (*dashed line*) and 3 h (*solid line*). ssDNA migrates away from the origins bidirectionally during the 2-h period between sample collections. (**b**) ssDNA profile for isogenic *rad53K227A* mutant cells [1] after synchronously released into S phase in YPD containing 200 mM HU for 1 h. In both plots, *solid* and *open circles* represent previously defined Rad53-checked and Rad53-unchecked replication origins, respectively [2]. The *dot* on the *x*-axis denotes the centromere
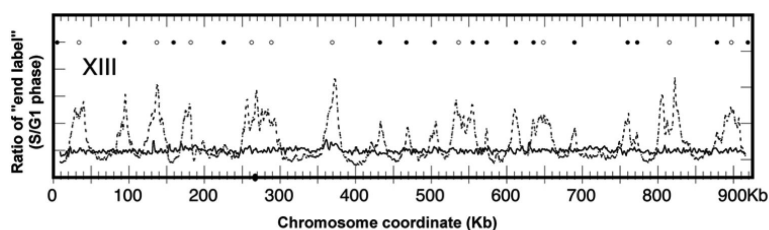
Fig. 3.
DSB profiles of chromosome XIII for WT (A364a, *solid line*) and an isogenic *mec1-1 sml1-1* mutant (*dashed line*) after transient exposure to 200 mM HU for 1 h and recovery for 2 h, respectively. In both plots, *solid* and *open circles* represent previously defined Rad53-checked and Rad53-unchecked replication origins, respectively [2]. The *dot* on the *x*-axis denotes the centromere