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Analysis of DNA double-strand break ends resection and single-strand annealing in *S. pombe*

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Abstract

DNA double strand break (DSB) end resection is an essential step for homologous recombination. It generates 3' single strand DNA needed for the loading of the strand exchange proteins and DNA damage checkpoint proteins. To study the mechanism of end resection in fission yeast, we apply a robust, quantitative and inducible assay. Resection is followed at a single per genome DSB synchronously generated by the tet-inducible *I-PpoI* endonuclease. An additional assay to follow resection involves recombination between two direct repeats by single-strand annealing (SSA), since SSA requires extensive resection to expose two single strand repeats for annealing. The kinetics of resection and SSA repair are then measured using Southern blots.

Keywords

DNA double-strand break; end resection; single-strand annealing; Southern blotting

1. Introduction

DNA double-strand breaks are repaired by either non-homologous end joining (NHEJ) or by homologous recombination (HR). The first step of HR called resection is the degradation of the 5' strands that generates 3' single strand DNA (ssDNA) [1]. The 3' ssDNA is essential for loading of the strand exchange protein Rad51 as well as DNA damage checkpoint proteins. DSB ends are initially resected by the MRN complex (MRX in budding yeast) assisted by Ctp1 (Sae2 in budding yeast) to generate short 3' ssDNA (initial resection) followed by long range resection by either Exo1 or Rhq1-Dna2 (Sgs1-Dna2 in budding yeast) to generate longer 3' ssDNA (extensive resection).

Here we describe a method to follow the resection kinetics at an inducible DSB in fission yeast (*Schizosaccharomyces pombe*). The gene encoding *I-PpoI* endonuclease controlled by the *tetO₇* promoter was inserted at the *leu1* locus [2]. The endogenous *I-PpoI* cleavage sites within rDNA repeats were eliminated by growing cells in the presence of constant *I-PpoI* expression where survivor cells emerged carrying altered sequences at *I-PpoI* cleavage sites [2] (see Note 1). Subsequently, new cleavage site of *I-PpoI* was inserted in the *lys1* locus on chromosome I or the *arg1* locus on chromosome III. Synchronous DSB induction is achieved by the addition of anhydrotetracycline (ahTET) to the growth medium. The

kinetics of resection is measured by Southern blotting with a series of probes detecting the restriction fragments generated at different distances from the DSB. An additional way to follow resection in fission yeast uses a repairable system where a DSB between two identical and direct lambda DNA repeats (755 bp long) is repaired by single-strand annealing (SSA) [3]. Extensive resection exposes single strand repeats that can anneal to each other forming an SSA product. Sequences between the repeats and one of the repeats are lost as a consequence of SSA. The kinetics of SSA product formation is monitored by Southern blotting. To eliminate alternative DSB repair pathways, SSA is followed in the absence of Rad51, which is dispensable for SSA. This SSA assay can only be used to monitor kinetics of repair, not viability (see Note 2).

These are robust and quantitative assays to study the mechanism of DSB end resection in fission yeast. These assays were used to define the function of helicases, nucleases, chromatin remodeling factors, checkpoint proteins and Rad52 in resection [3]. Materials and methods are nearly the same for assays designed to study resection kinetics or SSA kinetics except that different DNA probes, different restriction enzymes and different quantification methods are used.

2. Materials

Prepare all solutions using ultrapure water. Prepare and store reagents at room temperature unless indicated otherwise. Experiments using isotope should follow radiation safety regulations.

2.1 Yeast strains, cell culture and DSB induction

1. The genotype of the fission yeast strain used to study the kinetics of DSB ends resection is *leu1-32::pDUAL-TETp-I-PpoI ura4-D18 rDNA-I-Ppolmt ade6-216 lys1::I-PpoI-hph* (strain ySK113). The genotype of the fission yeast strain used to study the kinetics of SSA is *leu1-32::pDUAL-TETp-I-PpoI ura4-D18 rDNA-I-Ppolmt ade6-216 arg1::λ1-I-PpoI-λ2-hph* (strain yZY003). These strains are available upon request.
2. EMMG medium: For 1 L of liquid EMMG medium, dissolve 28.2 g EMMG powder (United States Biological, E2205-15) and six supplemental chemicals (Adenine, arginine, leucine, lysine, histidine and uracil, 250 mg each) in 1 L of H₂O. Medium should be filter sterilized (0.22 μm).
3. EMMG agar plates: Add 20 g agar to 1 L EMMG liquid medium, autoclave at 121°C for 20 min, and pour the medium into Petri dishes.
4. YES agar plates: For 1 L of YES agar medium, dissolve 5 g yeast extract, 30 g glucose and six supplemental chemicals (Adenine, arginine, leucine, lysine, histidine and uracil, 250 mg each) in 1 L of H₂O. Add 20 g agar, autoclave at 121°C for 20 min, and pour the medium into Petri dishes.
5. ahTET: Dilute anhydrotetracycline hydrochloride (Acros Organics, AC23313-1000) in H₂O at 10 mM concentration. Store 1 ml aliquots frozen at -80°C or -20°C. Cover the tubes with aluminum foil to protect from light.

2.2 DNA processing

1. Glass disruptor beads, 0.5 mm (Sigma, acid washed).
2. STES buffer: 0.2 M Tris-HCl [pH 7.6], 0.5 M NaCl, 0.1% SDS, 0.01 M EDTA.
3. Phenol/Chloroform/Isoamyl Alcohol (25:24:1, v/v).
4. Chloroform.
5. TE buffer: 10 mM Tris-HCl [pH 8.0], 1 mM EDTA.
6. RNase, 10 mg/ml.
7. NaAc, 3 M.
8. *EcoRI* and *EcoRI* buffer from New England Biolabs (NEB)
9. *HpaI*, *StuI*, *BspHI* and CutSmart buffer from NEB

2.3 DNA electrophoresis

1. TBE buffer: For 4 L of 5x TBE buffer, add 242.2 g Tris base, 123.6 g boric acid and 13.4 g Na₂-EDTA to 3 L of H₂O. When dissolved completely, bring up to a final volume of 4 L with H₂O. Dilute with H₂O to 1x TBE before use.
2. 0.8% Agarose gel: Add 3.2 g of agarose to 200 ml 1x TBE buffer, and microwave for ~3 min. Add another 200 ml 1x TBE buffer and swirl to mix. Let sit at room temperature (RT) for 5 min to cool. Add 8 µl 1% ethidium bromide and swirl to mix. Pour the liquid into a gel box to cast the gel and leave it at RT for 30 min (small gel) or 60 min (large gel) before use.

2.4 Southern blotting

1. Denaturing buffer/transfer buffer: 0.4 M NaOH.
2. Filter paper (46 × 57 cm, Whatman, 3030–917).
3. Blotting pad (20 × 20 cm, VWR, North American 28298–032).
4. Transfer membrane: Positively charged nylon hybridization membrane (PerkinElmer, NEF988001PK)
5. 20% SDS: Prepare in a chemical fume hood. For 2 L 20% SDS, add 400 g SDS to 1.5 L of warm H₂O (warmed for ~4 min in microwave). When dissolved completely, bring up to a final volume of 2 L with H₂O.
6. 20x SSC: For 4L 20x SSC, add 689.3g NaCl and 353g Na-Citrate to 3 L of H₂O. When dissolved completely, bring up to final volume of 4 L with H₂O.
7. Wash buffer: 2x SSC, 0.2% SDS.
8. Hybridization buffer: For 500 ml hybridization buffer, add 250 ml 0.5 M Na₂HPO₄ [pH 7.2], 175 ml 20% SDS, 1 ml 0.5 M EDTA [pH 8.0] and 74 ml of H₂O.

9. RadPrime DNA labeling system (Invitrogen, 18428–011). Mix the 0.5 mM dCTP, dGTP, and dTTP together in a 1:1:1 ratio to make the “CGT mix”.
10. [α - ^{32}P] dATP, 3000Ci/mmol 10mCi/ml (PerkinElmer, BLU512H250UC).
11. Isotope purification column: Micro Bio-Spin Chromatography Column, P-30 (Bio-Rad, 732–6223).
12. Strip buffer: 1% SDS, 0.1% SSC.

10. Methods

3.1 Cell culture

1. Grow fission yeast strain at 30°C on YES or EMMG agar plates.
2. Day 1: inoculate cells in 50 ml EMMG medium and culture overnight to saturation on a shaker at 30°C. Day 2: inoculate with 5–10 ml of the saturated culture into 1000 ml EMMG in a 2 L flask and culture overnight on a shaker at 30°C (usually 12 to 16 hr). For mutant strains that grow slower than wild-type, inoculate with 10–20 ml of the saturated preculture.

3.2 Induction of DSBs and collection of cells

1. Day 3: when the cell density reaches $2\text{--}4 \times 10^6/\text{ml}$, collect 200 ml of cells and mark as the “time 0” sample. Then add ahTET to the rest of the culture to a final concentration of 4 μM . Turn the light off or cover the flask with aluminum foil to protect the ahTET from degradation (see Note 2).
2. Spin down the cells at $>3,500\text{ g}$ for 2 min. Transfer the cell pellet to a 1.5 ml EP tube, spin down at $>4,000\text{ g}$ for 30 sec., remove the remaining liquid and store the pellets at -20°C .
3. Collect and store the cells at time points indicated in Table 1.

3.3 DNA extraction

1. Add glass beads on top of the cell pellets to a total volume of 500 μl . Add 120 μl TE buffer, 300 μl STES buffer, and 350 μl chloroform/phenol/isoamyl alcohol.
2. Break the cells by vortexing the cells for 6 min at 4°C (this will break the chromosomes into ~20 kb DNA fragments). Centrifuge the mixture at $>16,000\text{ g}$ for 5 min., then transfer the aqueous top layer (~400 μl) to a new 1.5 ml EP tube.
3. Add 350 μl chloroform/phenol/isoamyl alcohol, mix and centrifuge the samples at $>16,000\text{ g}$ for 5 min, then transfer the top aqueous layer (~400 μl) to a new 1.5 ml EP tube.
4. Add 350 μl chloroform (do this in a chemical fume hood), mix and centrifuge the samples at $>16,000\text{ g}$ for 5 min, then transfer the top aqueous layer (~400 μl) to a new 1.5 ml EP tube.

5. Precipitate the DNA by adding 40 μ l 3M NaAC and 1 ml ethanol (100%) and mixing.
6. Centrifuge the mixture at $>16,000$ g for 20 min. Remove the supernatant by aspiration (avoid disrupting the pellet). Then wash the pellet with 600 μ l 70% ethanol, centrifuge for 5 min at $>16,000$ g and remove the supernatant by aspiration. Spin down the tube again briefly and remove the remaining ethanol by aspiration. Dry the pellet at RT for 30–60 min.
7. Re-suspend the pellet in 300 μ l of TE. Add 2 μ l RNase (10 mg/ml) and incubate at 37°C for 2 hrs to overnight, then precipitate the DNA by adding 30 μ l NaAC and 900 μ l ethanol. Centrifuge and wash as in chapter 3.3 (6). Then resuspend the pellet in 150 μ l H₂O for ~30 min at RT.

3.4 Restriction enzyme digestion and DNA separation

1. Digest all DNA (150 μ l) by adding 30 μ l 10x reaction buffer and 2 to 5 μ l restriction enzyme(s) and H₂O to a total reaction volume of 300 μ l. Digest genomic DNA overnight (16–20 hr) at the temperature indicated by the vendor. After digestion, precipitate the DNA by adding and mixing 30 μ l NaAc and 900 μ l ethanol. Centrifuge and wash as in chapter 3.3 (6). Then resuspend the pellet in 30–40 μ l of TE buffer. Restriction enzymes specific for monitoring resection at nonrepairable breaks and for monitoring SSA kinetics are specified in Table 2.
2. Check the quality and completeness of DNA digestion by loading and separating 2 μ l of each sample in a small 0.8% agarose gel for ~1 hour at 80–120 V.
3. Take a picture of the gel using the UV gel imaging system to make sure that the DNA is fully digested. Measure the amount of DNA in each sample using a fluorometer (e.g. Qubit) and equalize the amount of DNA in samples with TE buffer.
4. Cast a large 0.8% agarose gel in 1x TBE buffer with a comb appropriate for the sample number and volume. For a large gel box, a 300 ml gel is used.
5. Add 4 μ l DNA gel loading dye (6x) to 20 μ l DNA, mix and centrifuge for a few seconds. Load 18 to 24 μ l of each sample.
6. Run the DNA gel at 47–60 V for 14–19 hr until the DNA fragments between 2 kb and 10 kb are well separated.
7. Take a picture of the gel using a UV gel-imaging system to make sure samples are well equalized and separated.

3.5 Alkaline gel transfer

1. Cut the DNA gel to the size of the blotting paper (20 $\times$ 20 cm) and place in a glass tray on an orbital shaker at ~30 rpm. Denature the DNA with denaturing buffer for 20 min (buffer should cover the gel), then replace with new denaturing buffer, and continue to shake for another 20 min.

2. Cut the filter paper and the transfer membrane to the size of the gel. Cut four filter papers to 20×20 cm, 4 filter paper strips to 46×4 cm, and one filter paper to 16×18 cm. Cut the transfer membrane to 21×21 cm (1 cm longer and 1 cm wider than the gel). Mark the corner of the membrane with the date and experiment number.
3. Transfer the gel to the transfer membrane by assembling a “transfer sandwich” as shown in Fig. 1 (also known as a capillary blotting apparatus). In detail, place two packs of paper towels on the table top (5–10 cm high for each pack), then place 5–6 blotting pads on top of the paper towels. Wet two layers of Whatman filter paper (20×20 cm) and place on top of the blotting pad avoiding any bubbles. Wet the transfer membrane and put it beneath the gel. Hold the bottom of the membrane and put the membrane and gel together on top of the filter/blotting paper stack. Wet another two filter papers and put them on top of the gel. Roll out all the bubbles between each layer of the “sandwich” using a long plastic pipette. Put two bi-layer “buffer bridge” wide strips on top of the stack, put the two ends of each bridge into the transfer buffer (the bridge acts as a wick for buffer transfer). Put another filter paper (16×18 cm) on top of the bridges to connect them together. Cover the stack with a piece of plastic wrap and put a weight (about 0.5 kg) on top of the stack. Cover the transfer sandwich with plastic wrap to prevent buffer evaporation.
4. Let the transfer of DNA to the membrane occur for ~20 hr. Disassemble the “transfer sandwich”, place the membrane in a clean glass tray and wash the membrane with wash buffer for 20 min on an orbital shaker at ~30 rpm. Dry the membrane on a paper towel at RT for at least 20 min.

3.6 Probe labeling and hybridization

1. DNA probes (500–800 bp) for Southern blotting are prepared by PCR, purified by regular gel-extraction (NucleoSpin Gel and PCR Clean-up, MACHEREY-NAGEL, 740609.250) and diluted to a concentration of ~50 ng/ μ l. Probes next to the DSB are used to test initial DSB end resection and probes at over 10 kb away from the DSB are used to test extensive resection. DNA probes specific for monitoring resection at nonrepairable breaks and for monitoring the kinetics of SSA are specified in Table 2.
2. For one Southern blot, add 30–50 ng of the desired probe in a 1.5 ml EP tube, and H_2O to a total volume of 20 μ l. Denature the DNA probe in a 100°C Dri-Bath block for 5 min (hold the cap by a CapLock tube clip to keep the cap closed), then cool it down on ice for 3 min.
3. Add 20 μ l 2.5x RadPrime buffer (Invitrogen), 3 μ l CGT mix, 1 μ l Klenow Fragment and 3 μ l [α - ^{32}P] dATP to the tube, mix gently, and centrifuge for 15 sec. Incubate at 37°C for 0.5–2 hr.
4. Purify the probe using a Micro Bio-Spin Chromatography Column according to the manufacturer’s instructions (Bio-Rad). Heat denature the purified sample at 100°C for 5 min and cool it down on ice for 5 min.

5. Put the membrane in the hybridization tube with 50 ml hybridization buffer. Put the hybridization tube in the hybridization incubator. Remove any bubbles between the membrane and hybridization glass tube. Set the temperature to 65°C. Incubate for at least 30 min for prehybridization.
6. Pipette ~40 µl of the purified probe mix into the hybridization tube and hybridize overnight.
7. Discard the hybridization buffer, add 50 ml wash buffer, and let rotate in the hybridization incubator for 30 min. Repeat the washing two additional times.
8. Remove the membrane from the hybridization tube, place on a paper towel to remove the remaining buffer and quickly wrap with plastic wrap (do not let it dry). Place the membrane face up inside the exposure cassette and put the phosphor screen on top of the membrane, phosphor side down. Close the cassette and leave it at room temperature for several hours up to overnight depending on the strength of the signal. (Note: Bleach the phosphor screen on a white light transilluminator for 30 min every time before use.)
9. Put the phosphor screen on the glass surface of the Typhoon Trio scanner. Align the screen to the top left.
10. Open the Typhoon Trio Imager software, select the region to be scanned and follow the instructions to scan the screen at the desired resolution. After scanning, wrapped blots can be stored at 4°C for a few months.
11. To reprobe the same membrane remove the plastic wrap and put the blot in a plastic tray. Heat 400 ml strip buffer in the microwave for 3 min and pour the hot buffer on top of the blot. Gently shake the tray for 3 min then discard the strip buffer. Repeat the wash 3 times to remove hybridized probe. Dry the blot by leaving it on paper towels at RT. Rehybridize the blot with additional probes as needed.

3.7 Data analysis

1. Use ImageQuant TL (GE Healthcare) or ImageJ (NIH) software to quantify the intensity of the bands on a Southern blot. Export the band intensity values to Excel for analysis.
2. For resection kinetics, normalize the intensity of the bands by the intensity of the loading control. Then divide the normalized intensity of each band by the normalized band intensity at time 0 to get the percentage of unresected DSB ends (Fig. 2). For single-strand annealing, normalize the intensity of the SSA product band by the intensity of the loading control. Then divide the normalized intensity of the SSA product band by the normalized intensity of the 1.6 kb parental band at time 0 to get the percentage of SSA product formation (Fig. 3).

4. Notes

1. A drawback of this system is that each of the rDNA sequences contains a cleavage site of I-*PpoI*, so only the I-*PpoI*-resistant rDNA mutation strains can be used. Other fission yeast systems using HO endonuclease or the I-*Scel* endonuclease to induce site specific recombination were developed [4, 5]. However, I-*PpoI* provides the most synchronous induction of DSBs.
2. I-*PpoI* is leaky, leading to some DSB induction even before the addition of anhydrotetracycline to the media. To eliminate cells carrying the SSA assay that induced DSBs prior to anhydrotetracycline addition, SSA was designed to have two essential genes between the lambda repeats. Thus, cells that induced I-*PpoI* enzyme and SSA repair before anhydrotetracycline addition die and are eliminated from the population due to loss of essential genes (Fig. 2).

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Fig. 1.

Scheme of the Southern blot “transfer sandwich”. (1) Two packs of paper towels. (2) Six layers of blotting pads, 20×20 cm. (3) Two layers of Whatman filter paper, 20×20 cm. (4) Positively charged membrane. (5) Agarose gel with DNA. (6) Two layers of Whatman filter paper cut to the size of the gel. (7) Four cups with denature/transfer buffer, ~250 ml per cup. (8) Two strips of bridge filter papers, 46×4 cm. (9) One layer of Whatman filter paper, 16×18 cm.

Fig. 2.

Analysis of resection by Southern blots and the resection kinetics. **(a)** Southern blots showing resection at DSB ends and 3.2 kb from DSB ends. Loading control is shown. **(b)** Plots showing initial resection kinetics. The 0 kb plot shows the relative band signal intensities compared to the band at 30 min. The 3.2 kb plot shows the relative band intensities compared to the band at 0 min. **(c)** Southern blot showing extensive resection at 17 and 27 kb from DSB ends. **(d)** Plots showing extensive resection kinetics. The plots show the relative band intensities compared to the band at 0 hr. Band signal intensities were normalized by the intensities of the loading control bands. Error bars denote standard deviation (n=3).

Fig. 3. SSA assay. (a) Schematic of the SSA assay between λ sequences. λ DNA sequence harboring a I-*PpoI* cleavage site in the middle was inserted at the *arg1* locus on chromosome III and an identical λ sequence was inserted 21 kb upstream of the I-*PpoI* cleavage site. Boxes marked “EG” represent two essential genes. (b) Southern blot analysis of the kinetics of SSA product formation in *rad51Δ* cells. (c) Plots showing SSA kinetics. The intensity of each band was normalized to the loading control.

Table 1.

Time and volume of cell culture collected.

Extensive resection	Time (hr)	0	1	2	4	6	8	10	12
	Volume (ml)	200	180	160	135	120	90	80	70
Initial resection	Time (min)	0	30	60	90	120	150	-	-
	Volume (ml)	200	200	180	160	135	135	-	-

Table 2.

Primers for preparing Southern blot probes by PCR.

Primer name	Restriction enzyme (s)	Sequence
0kb	<i>EcoRI</i>	5'- CAGAACAGCGGCAGTTCGG-3' 5'- GGTTGTAACGAGACTTGGGGTAAGG- 3'
3.2kb	<i>EcoRI</i>	5' - CCATGTTTAGAATGTGAACGAGATGC-3' 5'- GGACGTGGCATGAAGAATGTC-3'
17.3kb	<i>HpaI/StuI</i>	5' - GTATACGTTACTTGACATGTGCACACC-3' 5' - CGAGCTAAAATACTTAGTGCCTCTGGAC-3'
27.0kb	<i>HpaI/StuI</i>	5'- CCTACGCAACTGAGTCTAATAAGTC-3' 5'- CCTTGCTCCAGTTTGTTAAGC-3'
lys1 load control <i>HpaI/StuI</i>	<i>HpaI/StuI</i>	5' - CGTGAGGTTGCTAGGAGAATCAAAC TTC-3' 5'- CTCTTCAAGCTCACC ACTCCCG-3'
lys1 load control <i>EcoRI</i>	<i>EcoRI</i>	5'- GGACTTCAAGGGCCCGTTAAC-3' 5' - CGTGGCAGCTTATAGTTGTGAAGGAG-3'
SSA lambda	<i>BspHI</i>	5'- CATTGAGCAGTGCAGCGAAC-3' 5'- CCCGCCAGATGATAAGCATC-3'
SSA load control	<i>BspHI</i>	Same as lys1_load_control_ <i>HpaI/StuI</i>