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Single-molecule analysis of DNA replication by molecular combing (PROT36)

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Introduction

Efficient duplication of eukaryotic genomes relies on the sequential activation of thousands of replication origins distributed along the chromosomes. This process follows a complex spatial and temporal program, which is under the control of epigenetic mechanisms and is tightly linked to the functional organization of the nucleus (Méchali, 2001). Recent evidence indicates that this replication program is also controlled by checkpoint kinases that monitor the correct execution of S phase, such as ATR in human and Mec1 in budding yeast (Tourriere and Pasero, 2007). Over the last twenty years, a wide variety of methods has been developed to map replication origins in eukaryotic cells (DePamphilis, 1997). These techniques have shown that eukaryotic genomes contain an excess of potential replication origins and that only a fraction of these origins fire within a given S phase. However, the dynamics of DNA replication remains poorly defined at the level of individual chromosomes, essentially because biochemical assays provide averaged replication profiles for a population of cells.

The recent development of single-molecule assays has shed new light on the dynamics of DNA replication at the level of individual chromosomes. These techniques have been successfully used to monitor DNA replication in a variety of organisms, including bacteria, yeasts, xenopus and mammals (Anglana et al., 2003; Breier et al., 2005; Herrick et al., 2000; Jackson and Pombo, 1998; Lemaitre et al., 2005; Pasero et al., 2002; Patel et al., 2006). Here, we describe the analysis of DNA replication in *S. cerevisiae*
by DNA combing, one of the most widely used single-molecule assay (Bensimon et al., 1994; Michalet et al., 1997).

In this assay, replication origins are first labeled with bromodeoxyuridine (BrdU) in early S phase. Chromosomal DNA is then purified in agarose plugs and stretched on silanized coverslips. This procedure generates long, parallel DNA fibers, with a uniform extension of 2 kb/µm. Newly-replicated DNA is then detected with a monoclonal antibody directed against BrdU and DNA fibers are counterstained with an antibody against single-stranded DNA. Replicating DNA fibers are revealed with fluorescent secondary antibodies and are imaged with an epifluorescence microscope coupled to a CCD camera. Representative examples of large DNA fibers (>400 kb) are shown in Figure 1. From these images, a large number of replication parameters can be derived, such as the rates of initiation and elongation and the percentage of substitution for individual DNA fibers. When combined with fluorescence *in situ* hybridization, this technique allows a precise mapping of active origins along a chromosome of interest (Anglana et al., 2003; Lebofsky et al., 2006; Pasero et al., 2002). DNA combing can also be used to monitor fork recovery after a replication stress, using a combination of halogenated nucleotides (CldU/IdU) that can be distinguished with specific anti-BrdU antibodies (Luke et al., 2006; Tourriere et al., 2005).

The protocol described below is meant to detect BrdU incorporation in yeast cells arrested in early S phase with hydroxyurea (HU). It can be easily adapted to monitor ongoing DNA replication in asynchronous yeast cultures or to analyze DNA replication in mammalian cells. Specific details regarding the preparation of BrdU-labeled genomic DNA from mammalian cells are available upon request.

Procedure

BrdU labeling of HU-arrested cells

Yeast cells are normally unable to incorporate BrdU because they lack thymidine kinase activity and synthesize dTMP *de novo*. We therefore use genetically-modified strains containing seven copies of the *Herpes simplex* TK gene (Lengronne et al., 2001). Since BrdU accumulates in yeast cells by passive diffusion, we also transform cells with a *CEN*-based plasmid bearing the human nucleoside transporter hENT1 (gift of Grant Brown, Toronto) to accelerate BrdU uptake. Alternative strategies to facilitate BrdU incorporation in yeast have been developed (Vernis et al., 2003; Viggiani and Aparicio, 2006) but the combination of 7 integrated copies of HSV-TK plus hENT1 on a plasmid remains the most efficient option in our hands.

1. Grow cells overnight at 25°C to a density of 5.10^6 cells/ml in 100 ml of complete synthetic medium.
2. Add α-factor (1 µg/ml) for at least 2.5 hours to arrest cells in G_1. Add a second dose of α-factor part way through the incubation to ensure that cells do not escape the G_1 block.
3. Add BrdU to a final concentration of 400 µg/ml (or 40 µg/ml if cells express hENT1), at least 15 minutes before releasing cells into S phase (see note 1).
4. Add HU to a final concentration of 200 mM (15 mg/ml) and release cells from α-factor with the addition of 50 µg/ml Pronase (Calbiochem). Adjust the pH of the medium to 7.0 with phosphate buffer (see note 2).
5. After 90 minutes, add sodium azide in the culture to a final concentration of 0.1% and collect cells by centrifugation.
6. Check cells under the microscope. More than 90% of the cells should display small buds, which are indicative of entry into S phase.

Preparation of genomic DNA plugs

Genomic DNA is prepared in low melting point (LMP) agarose plugs in order to prevent its mechanical shearing. Plugs are stable for several months at 4°C.

1. Wash cells with 10 mM Tris-HCl, 50 mM EDTA, pH 8.0 and determine cell concentration with a cell counter.
2. Resuspended cells in prewarmed Zymolyase buffer (42°C) to a final concentration of 1.10^9 cells/ml.
3. Add an equal volume of molten LMP agarose (42°C) and prepare agarose plugs using a plug mold (GE Amersham). Each plug (90 µl) should contain 5.10^7 cells or approximately 850 ng of genomic DNA.
4. Let agarose plugs solidify for 30 minutes at 4°C.
5. Use a Pasteur pipette rubber bulb to eject plugs directly into 12 ml round-bottom tubes containing Zymolyase buffer (0.4 ml per plug). Incubate overnight at 37°C.
6. Replace Zymolyase buffer with Proteinase K buffer (0.4 ml per plug) and incubate for 48 hours at 50°C.
7. Wash 5 x 10 min in 10 ml of 10 mM Tris, 50 mM EDTA and store plugs at 4°C.

DNA combing

DNA combing is performed on silanized coverslips essentially as described previously by the Bensimon lab (Michalet et al., 1997).

1. Stain DNA plugs for 30 min with 1.5 µl YOYO-1 (Molecular Probes) in 100 µl TE.
2. Wash thoroughly in 10 ml TE (3 x 5 min) and transfer each plug in a round-bottom polycarbonate tube containing 5 ml of 50 mM MES pH 5.7
3. Melt the agarose plugs for 15 min at 67°C with a heating block (see note 3).
4. Let the solution cool down to 42°C before adding β-agarase (3 units, New England Biolabs) and incubate overnight.
5. Incubate at 65°C for 10 minutes and store at room temperature until use.
6. Carefully pour the DNA solution into a 2 ml Teflon reservoir. Save the rest for further use.
7. Insert a silanized coverslip into the DNA solution and incubate for 5 minutes at room temperature. Remove carefully the coverslip from the reservoir at the speed of 300 µm/s (see note 4). Repeat with another coverslip as many times as needed.
8. Check DNA fibers under the microscope using a 40x objective and a FITC filter block. To this aim, attach the coverslip to a metal holder with a hole in the middle, put a drop of immersion oil directly on the coverslip and visualize DNA fibers on the bottom side of the coverslip.
9. Place the coverslip on a sheet of Whatman paper and bake for 2 h at 60°C to crosslink DNA to coverslips.
10. Stick coverslips on glass slides with cyanoacrylate glue. Label slides with a diamond tip engraving pen and store at -20°C until use.

Immunodetection

The following protocol is for the immunodetection of BrdU-substituted nascent DNA and parental DNA fibers, as illustrated in figure 1a. Other combinations of primary antibodies can be used to visualize pulses of CldU and IdU or to detect CldU, IdU and DNA fibers simultaneously. Fluorescence *in situ* hybridization (FISH) can also be performed if needed (see optional protocol below).

1. Dehydrate slides for 5 min in successive baths of 70%, 90% and 100% EtOH.
2. Incubate for 25 min in 1M NaOH.
3. Wash extensively with PBS pH 7.4 to neutralize NaOH (5 washes of 1 minute each).
4. Perform FISH at this step (see next section), otherwise proceed with step 5.
5. Incubate for 15 min in PBS/T containing 1% BSA.
6. Add 18 µl of PBS/T containing primary antibodies and cover with a coverslip. Incubate for 45 min at 37°C in a humid chamber.
7. Wash 5 x 2 minutes times with PBS/T (see note 5).
8. Detect with secondary antibodies (30 min at 37°C in humid chamber)
9. Wash 5 x 3 minutes with PBS/T.
10. Dry slides and mount with 20 µl of Prolong Gold Antifade reagent (Molecular Probes). Let mounting reagent polymerase for 2 hours at room temperature before proceeding with microscopy. Mounted coverslips are stable for months at -20°C.

Fluorescence in situ hybridization (optional)

1. Amplify probe DNA (1 to 3 kb) by PCR and purify products on Qiagen columns.
2. Label probe with DIG-dUTP by random priming and eliminate free label on a G50 column.
3. Add a 20-fold excess of competitor DNA (salmon sperm DNA) and EtOH precipitate.
4. Resuspend probe in Probe mix to a final concentration of 10 to 50 ng/µl and denature for 5 min at 65°C.
5. Dilute Probe mix in Hybridization mix to a final concentration of 2 ng/µl.
6. Add 20 to 50 µl of hybridization mixture per slide, seal with an adhesive hybridization chamber and incubate at 37°C for at least 5 hours.
7. Wash 3 x 5 minutes in 2xSSC, 50% formamide.
8. Wash 3 x 5 minutes in 2xSSC.
9. Wash 3 x 5 minutes in PBS/T.
10. Proceed with step 5 of "*Immunodetection*" section. Use a mouse anti-DIG antibody (Roche) instead of the anti-DNA antibody to visualize FISH probes.

Image acquisition and analysis

Image acquisition is performed with a fully motorized Leica DM6000B microscope equipped with a CoolSNAP HQ CCD camera and controlled with MetaMorph (Roper Scientific). On images acquired with this CCD camera and a 40x objective, 1 pixel = 340 bp. BrdU tracks and DNA fibers are measured manually with an offline version of MetaMorph and data are transferred to an Excel spreadsheet (Microsoft). Statistical analysis of BrdU track length and inter-origin distances is performed with Prism 5.0 (GraphPad), as described in figure 1.

Materials & Reagents

Zymolyase buffer	50 mM phosphate buffer, pH 7.0 50 mM EDTA, pH 8.0 10 mM DTT 0.4 mg/ml Zymolyase 20T (Seikagaku)
Proteinase K buffer	10 mM Tris pH 7.5 50 mM EDTA 1% Sarkosyl 2 mg/ml proteinase K
10x MES buffer pH 5.7	Mix 70 ml of 500 mM MES hydrate (Sigma) with 30 ml of 500 mM MES sodium salt (Sigma) and adjust to pH 5.7 with a small volume of 500 mM of MES sodium salt.
PBS/T	1x PBS pH 7.4 0.1% TritonX100
Probe mix	2x SSC 60% deionized formamide 50 mM phosphate buffer (Na2HPO4/NaH2PO4), pH 7.0

Hybridization mix	2x SSC 50% deionized formamide 10% dextran sulfate 50 mM phosphate buffer (Na2HPO4/NaH2PO4), pH 7.0
Primary antibody mix	Rat anti-BrdU (1/20, Sera Lab, clone BU1/75) Mouse anti-DNA (1/300, Chemicon MAB3034) PBS/T
Secondary antibody mix	Goat anti-rat Alexa 488 (1/50, Molecular Probes, A11006) Goat anti-mouse Alexa 546 (1/50, Molecular Probes, A11030) PBS/T

Author Notes

1. BrdU is dissolved in 10% DMSO at a concentration of 5 mg/ml and stored at -20°C.
2. We usually save 20 ml of the culture to monitor cell cycle progression by FACS in the absence of HU. Cells should enter S phase 20-30 min after the addition of Pronase.
3. From this step, the DNA solution should be manipulated very gently.
4. Combing devices are no longer distributed by Pasteur Instruments. Contact Aaron Bensimon (a.bensimon@genomicvision.com) for combing machines and silanized coverslips. Protocols for gas-phase silanisation of coverslips and instructions for the assembly of custom combing devices are available upon request.
5. Dip the slide in a beaker containing PBS/T in order to remove the upper coverslip without damaging the DNA fibers.

Figures

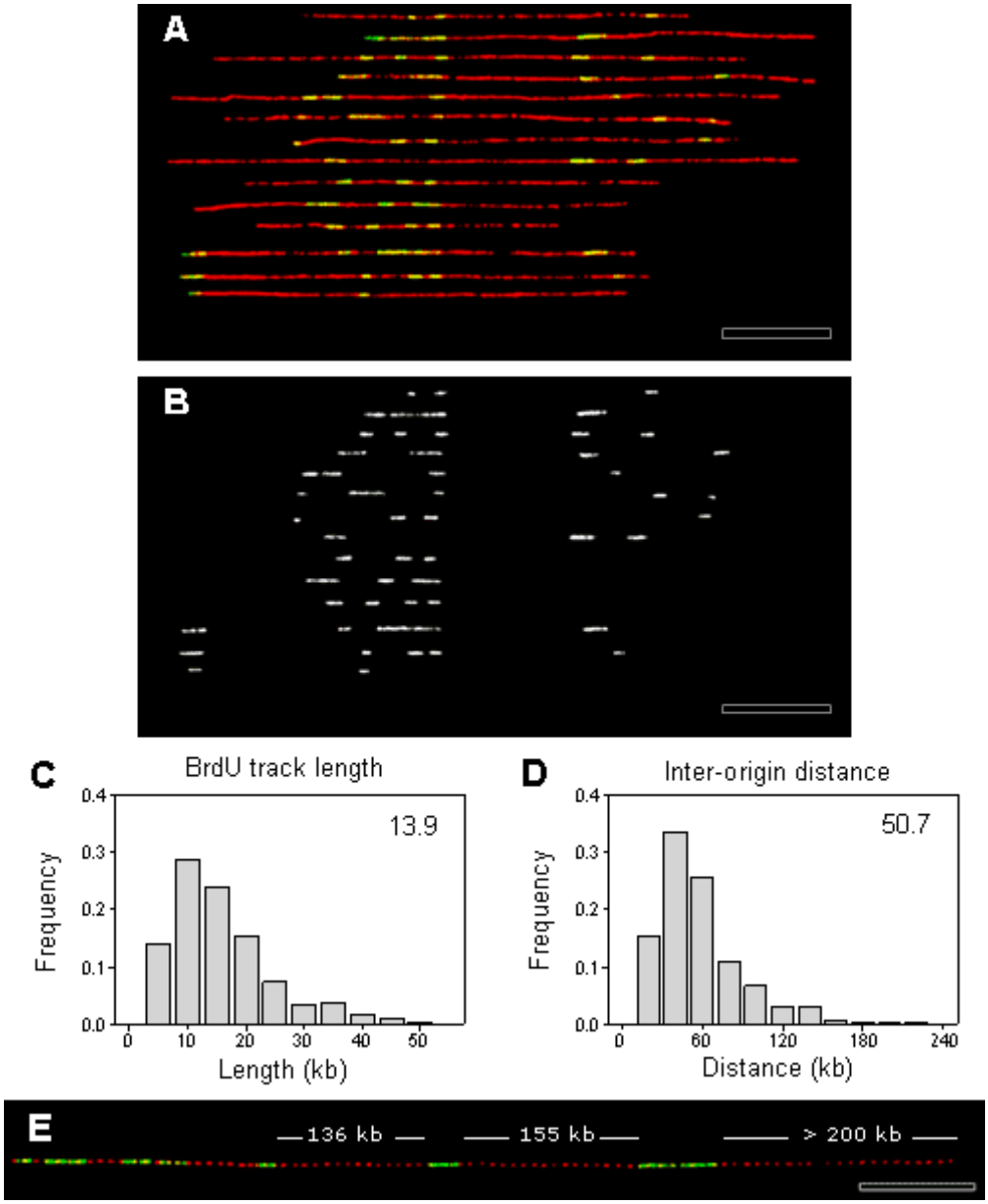

Figure 1: Analysis of DNA replication profiles in budding yeast by DNA combing. **(A)** Images of representative DNA fibers from different fields of view were processed with Adobe Photoshop to eliminate background signals and arbitrarily aligned to stress the clustering of early-firing origins. Green: BrdU. Red: DNA. Scale bar is 100 kb. **(B)** BrdU tracks (green channel only). **(C)** Distribution of BrdU track length in HU-arrested wild type cells. **(D)** Distribution of inter-origin distances (IODs) in HU-arrested wild type cells. BrdU track length and IODs were measured with MetaMorph (Roper Scientific) and frequency distributions were calculated with Prism 5.0 (GraphPad). Median values are indicated in kb. **(E)** Distribution of active origins along the yeast rDNA array. Each rDNA unit contains a potential replication origin but only 20% of these origins fire every cell cycle. Combined FISH (red dots) and BrdU detection (green tracks) show that active origins form clusters of ~3 adjacent units separated from each other with large silent regions. This replication pattern is imposed at least in part by the histone deacetylase Sir2 (Pasero et al, 2002).

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