

## Protocol 1

# Analysis of Replicating Yeast Chromosomes by DNA Combing

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Molecular combing of DNA fibers is a powerful technique to monitor origin usage and DNA replication fork progression in the budding yeast *Saccharomyces cerevisiae*. In contrast to traditional flow cytometry, microarray, or sequencing techniques, which provide population-level data, DNA combing provides DNA replication profiles of individual molecules. DNA combing uses yeast strains that express human thymidine kinase, which facilitates the incorporation of thymidine analogs into nascent DNA. First, DNA is isolated and stretched uniformly onto silanized glass coverslips. Following immunodetection with antibodies that recognize the thymidine analog and the DNA, the DNA fibers are imaged using a fluorescence microscope. Finally, the lengths of newly replicated DNA tracks are measured and converted to base pairs, allowing calculations of the speed of the replication fork and of interorigin distances. DNA combing can be applied to monitor replication defects caused by gene mutations or by chemical agents that induce replication stress. Here, we present a methodology for studying replicating yeast chromosomes by molecular DNA combing. We begin with procedures for the preparation of silanized coverslips and for assembly of a DNA combing machine (DCM) and conclude by presenting a detailed protocol for molecular DNA combing in yeast.

## MATERIALS

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It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

## Reagents

2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (7:3 [v:v] of MES hydrate:MES sodium salt [50 mM, pH 5.7])

Acetone

$\alpha$ -factor (5 mg/mL in 95% ethanol; stored at  $-20^{\circ}\text{C}$ )

Anhydrous ethanol

Anti-BrdU solution (BrdU antibody [AbD Serotec MCA2060], freshly diluted 1:40 in blocking buffer)

Anti-DNA solution (DNA antibody [Millipore MAB3034], freshly diluted 1:50 in blocking buffer)

Antisecondary solution <R>

Argon

$\beta$ -Agarase I (New England Biolabs M0392)

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Blocking buffer (PBS-T containing 10% [w/v] BSA; freshly prepared and sterilized with a 0.22- $\mu$ m syringe filter)  
BrdU (Sigma-Aldrich B5002; freshly prepared at 10 mg/mL in double-distilled H<sub>2</sub>O and filter-sterilized with a 0.22- $\mu$ m syringe filter)  
Chloroform  
Cyanoacrylate glue  
Double-distilled H<sub>2</sub>O (ddH<sub>2</sub>O), filtered  
EDTA (0.5 M) (optional; see Step 35)  
Heptane (anhydrous, 99%; Sigma-Aldrich)  
Lambda DNA (Sigma-Aldrich D9768-5U)  
Low-melting-point (LMP) agarose (Bioshop AGA101; freshly prepared at 1% [w/v] in 50 mM EDTA [pH 8.0])  
Methanol  
NaOH (1 M; filtered)  
Octenyltrichlorosilane (mixture of isomers, 96% purity; Sigma-Aldrich 539279)  
PBS (2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM NaHPO<sub>4</sub>, 2.7 mM KCl, 137 mM NaCl [pH 7.4])  
PBS-T (PBS containing 0.05% [v/v] Tween-20)  
Prolong Gold antifade reagent (Molecular Probes 36930)  
Pronase (10 mg/mL in double-distilled H<sub>2</sub>O; freshly prepared)  
Proteinase K solution <R>  
SCE buffer <R>  
Sodium azide (10% [w/v] in double-distilled H<sub>2</sub>O)  
TE<sub>50</sub> buffer (10 mM Tris-HCl [pH 7.0], 50 mM EDTA)  
TE buffer (10 mM Tris-HCl [pH 7.0], 1 mM EDTA)  
Yeast cultures (see Step 23)  
YOYO-1 solution (Molecular Probes Y3601, diluted 1:150 in TE<sub>50</sub> buffer)  
YPD <R>

## Equipment

Arduino Uno microcontroller board ([www.arduino.cc](http://www.arduino.cc))  
Beakers (500-mL and 100-mL)  
Bulldog clips  
Cardboard box  
Centrifuges (clinical centrifuge and microcentrifuge)  
Coplín jar  
Coverslip mini-racks (Molecular Probes C14784)  
Coverslip staining rack, stainless steel  
Desiccation chamber  
Drierite desiccant  
Drying oven  
Dual H-bridge motor driver chip (SN754410 or L293D)  
Enclosure (Nalgene 6740-1101 Acrylic Beta Box #3283-9aO)  
Filter, sterile 0.22- $\mu$ m  
Flow cytometer  
Fluorescence microscope, equipped with a 63 $\times$  oil-immersion objective, FITC and CY3 filter sets, and a charge-coupled device (CCD) camera  
Forceps  
Fume hood  
Gas regulator  
Glass microscope coverslips (22  $\times$  22-mm)

Glass microscope slides (76 × 26-mm, with frosted end)  
Heating block with fittings for 1.5- and 14-mL tubes  
Humidity chamber  
Hybridization oven  
Kimwipes  
Liquid water bath shaker  
M3 10-mm screws, nuts  
M3 25-mm supporting posts  
Mini-gel comb  
Motor and chassis (Sanyo Denki 103H548-0498 stepping motor)  
Pasteur pipette bulb  
Pasteur pipette, 9-inch (heated at the end and formed into a U-shaped scoop)  
Pencils and waterproof markers  
Phase-contrast microscope, equipped with a 40× air objective  
PLA filament (1.75-mm-diameter; Solidoodle)  
Plasma cleaner (Harrick Plasma, PDC-32G, 115V)  
Plug mold (Biorad 170-3713)  
Polycarbonate tubes (14-mL round bottom)  
Polypropylene centrifuge tubes (50-mL conical)  
Razor blade  
Retort stand  
Retort stand clamp  
Rotary cutting tools  
Screw cap tubes (2-mL)  
Snap action (travel-limit) switches (COM-00098; [www.sparkfun.com](http://www.sparkfun.com))  
Solidoodle 2 3D-printer  
Spectrophotometer  
SPST momentary normally open (N.O.) pushbutton (COM-11992; [www.sparkfun.com](http://www.sparkfun.com))  
Syringe, sterile  
Touch screen (Adafruit 2.8" TFT Touch Shield for Arduino; [www.adafruit.com/products/376](http://www.adafruit.com/products/376))  
Vacuum pump  
Water bath sonicator  
Whatman paper

## METHOD

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### Generating Glass Surfaces Suitable for DNA Combing

1. Place eight coverslips into a Teflon mini-rack and then, using forceps to hold the rack, completely submerge the coverslips in a 100-mL beaker filled with acetone to rinse them.

*It is important not to touch the coverslips with anything except forceps during all manipulations.*

2. Using forceps, transfer the racks to a 500-mL beaker with 250 mL of 50% methanol in double-distilled water (ddH<sub>2</sub>O).

*One 500-mL beaker can fit up to four coverslip racks.*

3. Secure the beaker in a retort stand clamp and position the retort stand beside the water bath sonicator. Lower the beaker into the bath until the total volume of liquid in the beaker is submerged. Sonicate for 20 min.

*The sonication steps must be carried out in a fume hood. It is important that coverslips remain separated during sonication to ensure uniform cleaning/coating.*

4. While in the fume hood, remove racks from the methanol beaker and rinse in a 100-mL beaker filled with chloroform. Place racks in a 500-mL beaker with 250 mL of chloroform and sonicate for 20 min.
5. Remove the racks from the chloroform and transfer the coverslips to a coverslip staining rack. Let residual chloroform evaporate in the fume hood at room temperature (RT).
6. While the coverslips are drying, prepare the plasma cleaner.
  - i. Turn on the vacuum pump and attach the front cover with needle valve fully closed.
  - ii. Set the RF level to MED and bleed in some air by slightly opening the needle valve for 3–4 sec; if successful, there will be a purple glow visible through the holes on top of the instrument.
  - iii. Run with no sample for 10 min. When finished, set RF to OFF and turn off vacuum.
  - iv. Fully open the needle valve to allow air into the chamber.
  - v. Once atmospheric pressure is reached, remove the front cover.
7. Remove the rack from the fume hood and place into the plasma cleaner. Run plasma cleaner with RF level set to LOW for 10 min.

*Transport rack covered in a plastic box outside of the fume hood to minimize exposure to dust particles.*
8. Remove rack from the plasma cleaner and bake in a drying oven for 1 h at 100°C.

*The stainless steel rack is hot after plasma cleaning and drying—use appropriate personal protection equipment.*
9. Return the coverslip staining rack to the fume hood and move the coverslips back to the Teflon mini-racks. Place the racks in a 500-mL beaker with 250 mL of heptane. Add 250 µL of octenyltrichlorosilane and swirl gently to mix.

*Once opened, store octenyltrichlorosilane under argon gas in a desiccator with drierite to minimize oxidation and polymerization. Discard opened containers of octenyltrichlorosilane after 3 mo.*
10. Place beaker into desiccation chamber with drierite in the fume hood and incubate overnight.
11. Transfer racks to a fresh 500-mL beaker with 250 mL of heptane and sonicate for 5 min.
12. Remove the racks from the heptane and rinse in a 100-mL beaker of ddH<sub>2</sub>O. Place racks in a 500-mL beaker with 250 mL of ddH<sub>2</sub>O. Sonicate for 5 min.

*Be careful when transferring racks to ddH<sub>2</sub>O because the nonpolar heptane can cause the coverslips to stick together.*
13. Transfer the racks to a 500-mL beaker with 250 mL of chloroform and sonicate for 5 min.
14. Remove the racks from chloroform and transfer the coverslips back to the coverslip staining rack. Allow excess chloroform to evaporate in the fume hood.

*See Troubleshooting.*
15. Store the coverslips in the coverslip staining rack, protected from light and dust, at RT.

### Building a Simple Machine Suitable for DNA Combing

16. Assemble the motor shield (Fig. 1C). (The 5-V voltage source and the ground connection are all provided by the Arduino Uno.)
17. Upload dcm\_firmware.ino (available as a supplementary file online at <http://cshprotocols.cshlp.org/>; also available at [http://bigten.med.utoronto.ca/tools/open-source\\_resources/dna-combing-machine](http://bigten.med.utoronto.ca/tools/open-source_resources/dna-combing-machine)) onto the Arduino Uno.
18. Use a rotary cutting tool with a cutting guide to make holes on the enclosure box, as indicated in 3D printing file enclosure.skp (available as a supplementary file online at <http://cshprotocols.cshlp.org/>; also available at [http://bigten.med.utoronto.ca/tools/open-source\\_resources/dna-combing-machine](http://bigten.med.utoronto.ca/tools/open-source_resources/dna-combing-machine)).



buttons. On selecting a parameter, it is highlighted on the screen and its value is instantly updated as it is modified by means of the “increase” or “decrease” buttons.

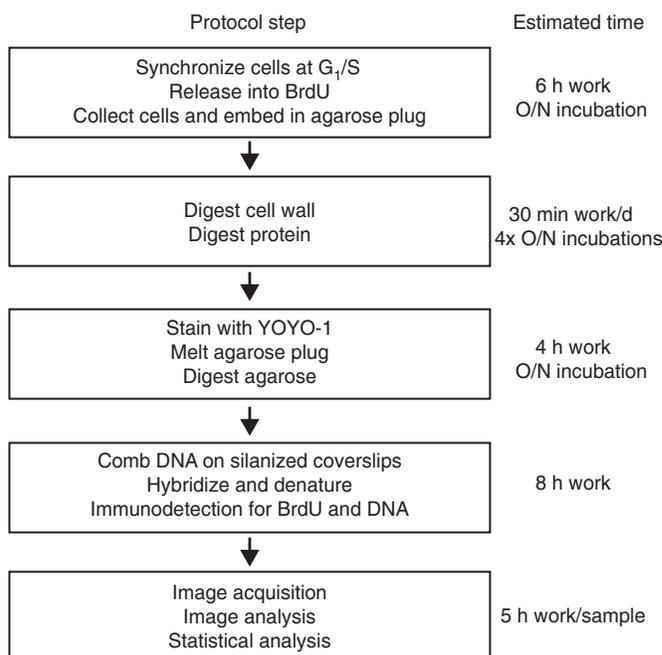
- i. Position. The position of the combing stage (in  $\mu\text{m}$ ) is determined from the stepper motor by the microcontroller. As such, the position is constantly updated on the display during motion. With the “position” button selected, push the “increase” button to raise the DCM stage to a user-defined maximum height (see Step 22.v); conversely, push the “decrease” button to lower the DCM stage to its minimum position (0  $\mu\text{m}$ ).
- ii. Speed. The combing speed can be set to between 0 and 1 cm/sec. We use 710  $\mu\text{m}/\text{sec}$ .
- iii. Type of travel. The travel button switches between “one-way” and “two-way” travel for the stage; two-way travel returns the stage to the starting position after a user-defined incubation time. The motor can be stopped midway by either the pushbutton or travel-limit switches located above and below the moving stage.
- iv. Incubation timer. For programmed sample incubation, a user can define a time period for which the machine, on reaching its bottom-most position, will pause before initiating withdrawal of the slide.
- v. Range and calibration. The “Range” button allows a user to change the maximum stage height (in  $\mu\text{m}$ ). This sets the upper travel-limit relative to 0  $\mu\text{m}$ , which is the lower travel-limit. The “Calibration” button enables users to modify the current position thus shifting the entire travel range up or down to accommodate different coverslip dimensions or reservoir depths.

## Molecular Combing of DNA Fibers

An overview of the procedure is shown in Figure 2.

### Cell Synchronization and BrdU Labeling

23. Grow yeast cultures in YPD liquid broth at 30°C to early logarithmic phase ( $\text{OD}_{600} = 0.20\text{--}0.30$ ) in a water bath shaker. Allow at least two cell doublings when diluting from a saturated culture. Remove an aliquot for flow cytometry.



**FIGURE 2.** The DNA combing procedure, with estimated times for each stage. BrdU, bromodeoxyuridine; O/N, overnight.

*S. cerevisiae* cells are unable to incorporate BrdU because they lack a thymidine kinase. Strains used for molecular combing must ectopically express the herpes simplex virus thymidine kinase (HSV-TK). Ectopic expression of the human equilibrative nucleoside transporter 1 (hENT1) improves BrdU uptake from the media but is not mandatory for incorporation into DNA.

24. To arrest cells in G<sub>1</sub> phase, add 5 mg/mL  $\alpha$ -factor to 2.5  $\mu$ M final (0.83  $\mu$ L/mL) and continue growth for 75 min. Add an additional 0.33  $\mu$ L/mL  $\alpha$ -factor (1  $\mu$ M final) and continue growth for 45 min.
25. Add 40  $\mu$ L/mL BrdU (400  $\mu$ g/mL final) and continue growing for 30 min.  
*Addition of BrdU while cells are arrested in G<sub>1</sub> phase facilitates uptake into the cells.*
26. Inspect culture under a phase-contrast microscope. Approximately >90% of cells should have the “shmoo” morphology—if not, grow culture for an additional 30 min and check again. Remove an aliquot for flow cytometry before proceeding.
27. To release cells into S phase, add pronase to a final concentration of 100  $\mu$ g/mL (10  $\mu$ L of stock solution per milliliter of culture) directly to the culture and continue growth. The duration of S phase labeling can be varied, but 30 min is typical.
28. To harvest cells, transfer 20 mL of culture to a precooled 50 mL conical polypropylene centrifuge tube containing 10  $\mu$ L sodium azide stock per milliliter of culture (0.1% w/v final), mix by inverting several times and incubate on ice for 15 min. If multiple samples are being collected, samples can be left on ice for up to 2 h. Remove an aliquot for flow cytometry.  
*If desired, the cell cycle arrest and release can be confirmed by analyzing cellular DNA contents by flow cytometry, using the aliquots collected at Steps 23, 26, and 28.*

#### **Agarose Plug Preparation and Digestion**

29. Determine the cell density (expressed as cells/mL) and centrifuge  $1.2 \times 10^8$  cells in prechilled centrifuge tubes at 800g for 3 min at 4°C.  
*The density of cells is an important step in plug preparation. If the density is too low, the concentration of DNA fibers will be too low, making the microscopy difficult and time consuming; conversely, if the density is too high, the subsequent plug digestion and melting steps will not be efficient, resulting in clumped and tangled DNA fibers. The cell density used here is a midpoint, and optimal density can vary from this by as much as 10-fold. Density should be optimized for each strain and experimental setup. We analyze samples as indicated by flow cytometry to ensure that the G<sub>1</sub> arrest and release into S phase are as expected.*
30. Aspirate the supernatant, wash the pellet in ice-cold TE<sub>50</sub> buffer, transfer to a prechilled microcentrifuge tube, and centrifuge at 16,000g for 1 min at 4°C.  
*Steps 31–33 should be carried out in succession for each sample so the low-melting-point (LMP) agarose does not solidify. Ensure you have enough dissolved 1% LMP agarose solution at 68°C and SCE buffer prepared before continuing.*
31. Resuspend pellet in SCE buffer at RT such that the total volume of cells plus buffer is 200  $\mu$ L. For the density used here, 160  $\mu$ L of SCE is sufficient.
32. Add 200  $\mu$ L of 1% LMP agarose and mix by gentle pipetting. Avoid air bubbles.
33. Transfer 100  $\mu$ L into the plug mold by pipetting down the side to avoid air bubbles or voids. Cast three plugs per sample.  
*BrdU is light sensitive, and precautions should be taken from this step forward to minimize light exposure.*
34. Incubate plugs in molds for 45 min at 4°C to allow agarose to solidify.
35. Using a Pasteur pipette bulb, eject plugs into 14-mL round-bottom polycarbonate tubes and add 0.5 mL of SCE buffer per plug. Incubate overnight at 37°C.  
*Alternatively, plugs can be stored in 0.5 M EDTA in 2-mL screw cap tubes until digestion. This is not optimal but can be done if the samples need to be prepared and then shipped to another location.*
36. Remove old SCE buffer and replace with the same volume of fresh SCE buffer. Incubate again at 37°C overnight.  
*A 15-well SDS-PAGE mini-gel comb can be placed over the top of the tube, allowing the old solution to be poured out while retaining the plugs.*

37. Remove SCE buffer and rinse plugs three times in 1 mL of TE<sub>50</sub> buffer. Add 0.5 mL of prewarmed proteinase K solution per plug and incubate overnight at 50°C.
38. Remove old proteinase K solution and replace with fresh prewarmed proteinase K solution. Incubate overnight at 50°C. Repeat this step once more—making a total of three overnight incubations with proteinase K solution.
39. Remove the last proteinase K solution and wash plugs five times for 10 min in TE<sub>50</sub> at RT.
  - i. Transfer plugs, using a 9-inch Pasteur pipette formed into a U-shaped scoop, to 2-mL screwcap tubes with 1 mL TE<sub>50</sub>.
  - ii. Store at 4°C, protected from light.

*Plugs are extremely fragile and should be handled with care; they are stable in TE<sub>50</sub> at 4°C for many months.*

### **Plug Melting and DNA Combing**

40. Remove one plug and transfer to a round-bottom polycarbonate tube. Add 150 µL of YOYO-1 solution and incubate at RT for 30 min.
41. Remove YOYO-1 solution and wash plugs three times for 5 min in 10 mL of TE buffer.
42. Remove last TE wash and incubate in 2 mL of MES buffer for 5 min at RT.
43. Remove MES buffer and replace with 2 mL of fresh MES buffer. Incubate for 10–15 min at 72°C. Gently rock tube horizontally once to disperse agarose and incubate for an additional 10 min at 72°C.

*It is crucial that the agarose plug is completely melted into the MES solution or DNA fibers will appear clumped during analysis. From this point on, the DNA fibers in solution are extremely fragile and must be handled gently to avoid mechanical shearing, which will result in short fibers.*
44. Transfer the DNA fiber solution to 42°C and equilibrate for 15 min. Add 3 units of β-agarase I and incubate overnight at 42°C.

*Do not mix the fiber solution.*
45. Heat the DNA fiber solution for 10 min to 72°C and cool to RT.
46. Carefully pour the fiber solution into the reservoir of the combing machine (see Steps 16–22).
47. Mount the silanized coverslip (prepared in Steps 1–15), lower it into the solution, and incubate for 5 min.

*The incubation time can be increased to up to 20 min to facilitate more DNA fiber binding to the coverslip.*
48. Pull the coverslip out of the solution at a constant speed of 710 µm/sec.
49. Place coverslips on Whatman paper in a cardboard box and bake in a hybridization oven for 90 min at 60°C.

*Be careful to note the orientation of DNA fibers on the coverslip and maintain the direction of the fibers.*
50. Mount the coverslip on a glass slide by placing a small drop of cyanoacrylate glue ~1 cm from the clear end. Carefully mount the combed coverslip, centered on the drop of glue, with the end of the coverslip that was clamped pointing toward the frosted end. This orients the DNA fibers parallel to the long side of the glass slide. Label the frosted end of the slide with pencil. Leave to dry in a cardboard box for 5 min at RT.

*See Troubleshooting.*  
*For optimal immunodetection of BrdU, proceed directly to immunostaining, but, if necessary, mounted coverslips can be stored overnight at –20°C.*

### **Immunodetection**

51. Place slides in Coplin jars and dehydrate by incubating sequentially in 70%, 90%, and anhydrous ethanol for 5 min each at RT.

*Make sure there is enough volume to completely submerge the coverslip during incubations. Dilute anhydrous ethanol in filtered ddH<sub>2</sub>O.*

52. Remove slides and wipe excess ethanol with a Kimwipe, being careful not to touch the coverslip. Place slides in covered cardboard box and let air dry for 5 min at RT.

*Slides can now be labeled with a waterproof marker.*

53. Place slides into a clean Coplin jar and denature DNA in 1 M NaOH for 25 min at RT.

54. Remove NaOH and wash five times for 1 min in PBS and then incubate for 5 min in PBS-T in the Coplin jar.

55. Remove slides from jar, wipe excess PBS-T from around the edges and place in a humidity chamber. Add 21  $\mu$ L of blocking buffer on the coverslip and place a loose coverslip on top to evenly disperse the liquid. Incubate in the humidity chamber for 30 min at 37°C.

*See Troubleshooting.*

56. Dip slide into Coplin jar containing PBS-T to remove coverslip and place back into humid chamber. Add 21  $\mu$ L of anti-BrdU solution and incubate in humidity chamber for 1 h at 37°C.

57. Remove coverslips and wash three times for 5 min in PBS-T. Add 21  $\mu$ L of anti-DNA solution and incubate in humidity chamber for 1 h at 37°C.

58. Remove coverslip and wash three times for 5 min in PBS-T. Add 21  $\mu$ L of antisecondary solution and incubate in humidity chamber for 1 h at 37°C.

59. Remove coverslip and wash three times for 5 min in PBS-T.

60. Wipe excess PBS-T from slide. Add 10  $\mu$ L of ProLong Gold antifade reagent, cover with a fresh coverslip, and leave to dry in cardboard box overnight at RT.

*Slides can be stored long term at -20°C.*

### **Image Acquisition and Analysis**

61. Perform fiber visualization with an appropriate fluorescence microscope equipped with a CCD camera for image acquisition. Acquire images under a 63 $\times$  oil-immersion objective lens with CY3 and FITC filter sets for ssDNA and BrdU, respectively.

*An image of combed DNA fibers is shown in Figure 3.*

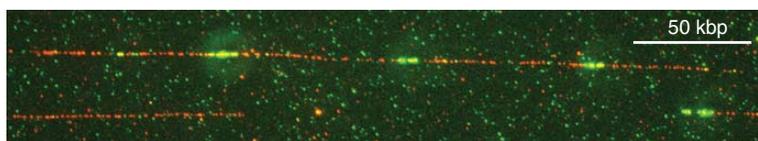
62. Analyze images using the open-source software ImageJ (<http://rsb.info.nih.gov/ij>).

63. Measure lengths in pixels and convert to base pairs using a conversion factor. This factor depends on the magnification of the objective, the pixel size of the CCD camera, and the stretching of DNA fibers. DNA fibers of known length, such as bacteriophage  $\lambda$  DNA, can be combed to determine the conversion factor, as summarized below.

- i. Prepare a 2-mL solution of  $\lambda$  DNA at 250 ng/mL in MES buffer.

*Avoid pipetting or vortexing, which can shear the DNA.*

- ii. Heat for 10 min at 65°C (to increase the fraction of monomeric DNA molecules) and then transfer to ice for 10 min.



**FIGURE 3.** Raw merged image of combed DNA fibers. AlexaFluor 546 (red) marks the DNA and AlexaFluor 488 (green) marks the BrdU incorporated into replicating DNA.

- iii. Pour the DNA solution into the reservoir of the combing machine (Step 46) and complete Steps 47 through 62 (omitting the steps and reagents used to detect BrdU) to detect the DNA fibers.
- iv. Measure the lengths of the DNA fibers (expressed as pixels) and plot a histogram to determine the mode of the main peak (which is the 48502-bp monomer).
- v. Use the mode to calculate a pixel per base-pair conversion factor.

*It is routine to observe a sharp peak of very short DNA molecules that represent molecules that have been sheared before the combing steps; smaller peaks at multiples of the mode are concatamers of lambda DNA. As the length of the lambda DNA in pixels depends on the imaging system used, we recommend calibrating the microscope using a micrometer. We find that combed  $\lambda$  DNA is 20–22  $\mu\text{m}$  in length.*

64. Depict track lengths and interorigin distance values graphically as box plots. The distributions of these values are non-normal and thus a Mann–Whitney *U*-test should be performed to determine the statistical significance of differences between sample distributions.

*BrdU track lengths represent bidirectional replication forks progressing from a single origin.*

65. To provide an estimate of replication fork rate, divide the median track length in half and then divide by the labeling time.
66. Calculate the median distance between labeled tracks on the same fiber (interorigin distance) to provide a measure of origin firing efficiency. Alternatively, express origin usage as the number of active origins per mega-base-pair of total DNA.

*Note that, for accurate interorigin distance (IOD) comparisons between samples, it is important that DNA fibers be consistently of similar lengths and approximately four times longer than the average IOD (Tuduri et al. 2010).*

## TROUBLESHOOTING

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**Problem (Step 14):** There is a white residue present after the chloroform evaporates on the coverslips.

**Solution:** The coverslips should be clear with no residue—if the white residue persists, discard the coverslips.

**Problem (Step 50):** Excess glue seeps from the edge and dries on the coverslip.

**Solution:** Do not put too much glue on the slide when mounting as it will interfere with subsequent immunodetection steps—if some glue does seep out, use a razor blade to carefully scrape the coverslip clean.

**Problem (Step 55):** Air bubbles appear under the coverslip.

**Solution:** To avoid air bubbles, place one edge of the coverslip down and use a pipette tip to help lower the other edge down—all antibody stages (Steps 56–60) are performed in the same manner.

## DISCUSSION

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In this protocol, we began with procedures for preparation of silanized coverslips (Steps 1–15), then described the assembly of a machine suitable for DNA combing (Steps 16–22), and concluded by presenting a method for molecular DNA combing in yeast (Steps 23–66; Fig. 2). Steps 1–15 featured an adaptation of the liquid-phase silanization procedure described previously by Labit et al. (2008). Alternative procedures for silanization in the vapor phase can also be considered (Schwob et al. 2009). Note too that suitable surfaces are also available commercially (<http://www.genomicvision.com>).

We next presented a methodology (Steps 16–22) for a robust, easy-to-use, and cost-efficient combing machine to pull coverslips from a reservoir of DNA solution at a constant speed. In this machine (Fig. 1), which can be built for ~\$150, a touch screen and a pushbutton act as input devices for a microcontroller, which communicates with a motor shield to drive a stepper motor to control the DCM stage. Note too that suitable combing machines are also available commercially (<http://www.genomicvision.com>).

In the final part of this protocol, we have outlined our standard DNA combing protocol for detecting incorporation of the halogenated thymidine analog BrdU into newly replicated DNA isolated from budding yeast. It is optimized for use with the E1670 yeast strain that lacks an endogenous thymidine kinase but expresses seven copies of the human thymidine kinase to allow incorporation of halogenated thymidine analogs into nascent DNA (Lengronne et al. 2001). Following pulse-labeling with BrdU, the cells are then embedded into agarose, where the cell wall and protein components are digested. The plug is melted and the DNA is combed onto silanized coverslips, where it is denatured and subjected to immunodetection for BrdU and DNA. The coverslips are then imaged using fluorescence microscopy (Fig. 3) and the images are analyzed using computer software to measure nascent DNA track lengths and distances between replication origins. This procedure is suitable for measuring replication fork rates and replication origin usage. The protocol (Steps 23–66) is adapted from the procedures of the Schwob and Pasero laboratories (Lengronne et al. 2001; Versini et al. 2003; Schwob et al. 2009; Bianco et al. 2012). It is also amenable to more complicated double-labeling procedures involving the sequential addition of IdU and CldU to measure replication fork stalling and fork asymmetry.

For an overview of replication analysis techniques suitable for yeasts, see Introduction: Single-Molecule Analysis of Replicating Yeast Chromosomes (Gallo et al. 2015).

## RECIPES

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### *Antisecondary Solution*

Prepare blocking buffer by adding bovine serum albumin (BSA) to 10% (w/v) in PBS-T (phosphate-buffered saline with 0.05% [v/v] Tween-20). Then, add Alexa Fluor anti-rat 488 (Molecular probes A11006) at a 1:75 dilution and Alexa Fluor anti-mouse 546 (Molecular probes A11030) at a 1:50 dilution into blocking buffer. Prepare fresh before use.

### *Proteinase K Solution*

1 mg/mL proteinase K  
1% (w/v) sarkosyl  
10 mM Tris-HCl (pH 7.0)  
50 mM EDTA

Prepare fresh. Preheat to 50°C for 30 min before use.

### *SCE Buffer*

1 M sorbitol  
100 mM sodium citrate  
10 mM EDTA (pH 8.0)  
0.125% (v/v)  $\beta$ -mercaptoethanol  
10 U/mL zymolyase (Bioshop ZYM001.1)

Add  $\beta$ -mercaptoethanol and zymolyase fresh before use.

### YPD

Peptone, 20 g  
Glucose, 20 g  
Yeast extract, 10 g  
H<sub>2</sub>O to 1000 mL

YPD (YEPD medium) is a complex medium for routine growth of yeast. To prepare plates, add 20 g of Bacto Agar (2%) before autoclaving.

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