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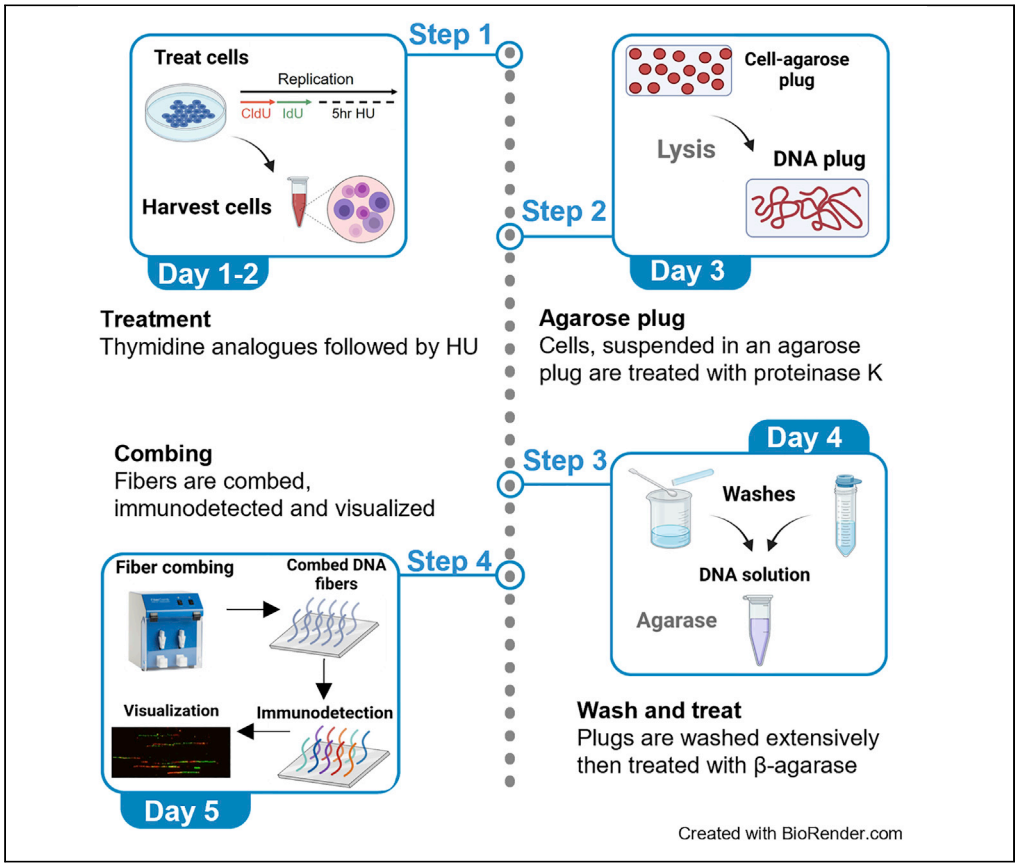
DNA fiber combing protocol using in-house reagents and coverslips to analyze replication fork dynamics in mammalian cells



DNA fiber combing is a versatile technique that provides insight into replication fork dynamics at single-molecule resolution. DNA fibers are bound to silanized coverslips and combed, which straightens and aligns the fibers along a single axis. Here, we present a DNA fiber combing protocol that does not use commercial kits; we detail the steps to prepare all materials, reagents, and silanized coverslips. We describe the use of DLD-1 cells, but the protocol is amenable to other cell types.

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Highlights

Single-molecule resolution and visualization of replication fork dynamics

Avoid commercial kits as details to prepare all materials, and reagents are included

Prepare high-grade hydrophobic silanized coverslips in-house avoiding purchase

Produce high-quality DNA fibers, aligned on a single axis and uniformly stretched

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DNA fiber combing protocol using in-house reagents and coverslips to analyze replication fork dynamics in mammalian cells

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SUMMARY

DNA fiber combing is a versatile technique that provides insight into replication fork dynamics at single-molecule resolution. DNA fibers are bound to silanized coverslips and combed, which straightens and aligns the fibers along a single axis. Here, we present a DNA fiber combing protocol that does not use commercial kits; we detail the steps to prepare all materials, reagents, and silanized coverslips. We describe the use of DLD-1 cells, but the protocol is amenable to other cell types.

BEFORE YOU BEGIN

DNA fiber combing is a highly valuable tool for the study of replication dynamics. DNA fibers were first visualized on glass slides tilted at a 45° angle allowing gravity to elongate and spread the DNA molecules (Parraa and Windle, 1993). DNA fiber spreading, also known as the "tilt method", can result in entangled, crossed fibers, in a condensed area on the coverslip which makes tract length analyses difficult. DNA fiber combing involves the DNA ends binding to a silanized coverslip which is slowly removed from the DNA solution. The meniscus of the solution straightens and aligns the DNA molecules. Conti et al., brilliantly described the process as like the long hair of a swimmer being pulled down their back as they emerge from the water (Conti et al., 2001). DNA fiber combing provides several advantages over fiber spreading as all the DNA molecules are stretched equally and aligned along a single axis. The uniformity, spacing, and alignment of DNA fibers facilitate reliability and confidence in tract length measurements. The method described here has the advantage of avoiding commercial kits as all materials and reagents can be prepared in an academic lab, including high quality hydrophobic silanized coverslips.

DNA fiber assays utilize halogenated thymidine analog incorporation into replicating DNA, allowing visualization of DNA replication at the single molecule level (Jackson and Pombo, 1998). A variety of replication fork dynamics can be investigated with this technique. Different aspects of fork progression can be measured by varying the duration and order of treatments and drug used to induce stress/damage. Many applications of DNA fiber combing have been described in detail previously (Quinet et al., 2017). These uses include analysis of replication of telomeres, centromeres, and specific loci (Gali et al., 2019; Kahl et al., 2020; Blin et al., 2021). Replication fork speed/progression is measured using a dual labeling system, i.e., use of two consecutive analogs. A progressing fork will present as a DNA tract with both analogs incorporated, Figure 1A (Maya-Mendoza et al., 2018). To test fork progression, a drug is added along with the second label, Figure 1B. A shortened tract indicates the fork is moving slower or has stalled. A longer tract would indicate the fork is moving faster. A pretreatment of drug before the analogs can be used, this approach is suited to long

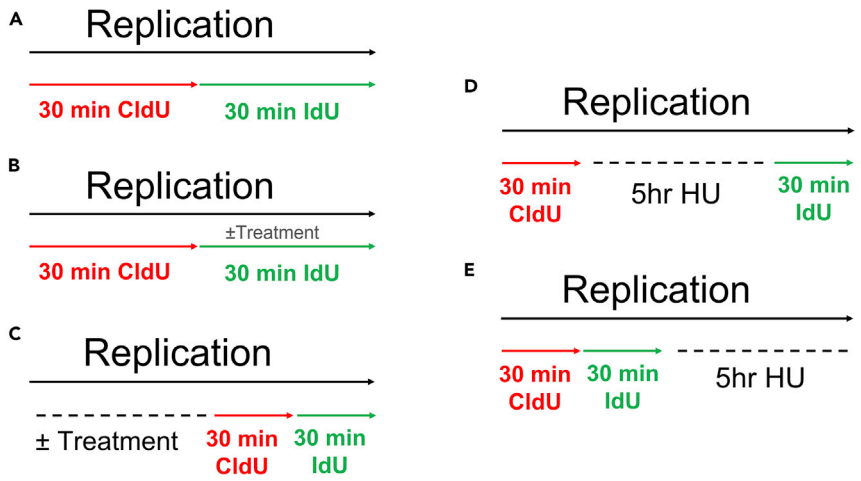

Figure 1. Example labeling schemes to evaluate replication dynamics

(A–E) Example labeling schemes to investigate (A) replication speed/progression unperturbed, (B) perturbed replication speed/progression, (C) perturbed replication speed/progression with pretreatment, (D) fork restart and (E) fork protection after treatment with hydroxyurea, using the thymidine analogs, CldU (Red) and IdU (Green).

treatment periods (e.g., 24 h), Figure 1C. Pretreatment is also used to investigate origin firing and interorigin distance (Dhar et al., 2019). Fork restart investigations require a drug treatment (e.g., hydroxyurea), following the first label, before the second label, Figure 1D. Hydroxyurea (HU) treatment causes the replication fork to stall through depletion of the dNTP pool in the cell. HU is a well-studied inhibitor of ribonucleotide reductase (RNR), which prevents the reduction of ribonucleotides to deoxynucleotides (Elford, 1968). A dual-colored tract indicates fork restart, Figure 1D.

Replication fork protection functions to protect newly replicated DNA from degradation. Nascent DNA formed at stalled replication forks is susceptible to nucleolytic degradation. Degradation can be blocked by proteins binding to the nascent DNA thereby protecting the fork (Ying et al., 2012; Costanzo et al., 2010). A compound is utilized to induce replicative stress or DNA damage after dual thymidine analog treatment, Figure 1E. A decrease in DNA tract length is indicative of nucleolytic degradation, and thus, a lack of fork protection. The sequential dual label approach ensures broken/ damaged forks and origins fired during the second pulse are excluded from the analysis. The protocol described here uses the specific example of monitoring fork protection but can be modified by the user to analyze other aspects of replication fork dynamics with the appropriate labeling schemes. In our example, we compare the role of BRCA2 in an isogenic human DLD-1 cell line. However, many cell types can be processed and analyzed with careful consideration of replication timing and cell density.

The following buffers/reagents can be prepared before the experiment, in large volumes, and will maintain activity long-term with the correct storage.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat Anti-Rat Cy5	Abcam	ab6565
Mouse Anti-BrdU	BD Biosciences	347580
Rat Anti-BrdU	Abcam	ab6326

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sheep Anti-Mouse Cy3	Sigma	C2181
Bacterial and virus strains		
Lambda DNA	NEB	N3011
Chemicals, peptides, and recombinant proteins		
Agarose, low gelling temperature	Sigma	A9045
β-Agarase	NEB	M0392
5-Chloro-2′-deoxyuridine	Sigma	C6891
Hydroxyurea	Sigma	H8627
5-Iodo-2′-deoxyuridine	Sigma	I7125
MES	Sigma	M5287
N-Heptane	Millipore	104390
Octenyltrichlorosilane	Sigma	539279
Prolong Anti-Fade	Thermo Fisher Scientific	P36970
Proteinase K	NEB	P8107
Sodium Chloride	American Bioanalytical	ab01915
Sodium Hydroxide	VWR	MK7708-10
Trypsin-EDTA	Sigma	T3924
YOYO-1	Thermo Fisher Scientific	Y3601
Experimental models: Cell lines		
DLD-1 BRCA2$^{-/-}$	Horizon	HD 105-007
Software and algorithms		
ImageJ	ImageJ	https://imagej.nih.gov/ij/
Other		
Combing Reservoirs	Genomic Vision	RES-001
Coverslips	Fisher Scientific	12-540-B
Fiber Comb	Genomic Vision	MCS-001
Gel plug mold	Bio-Rad	1703713
Microscope slides	Fisher Scientific	12-550-15
Orbi-shaker	Benchmark	BT3000
Rotating Mixer	Benchmark	R5010
UVO Cleaner	Jelight	Model 42
Wash-N-Dry Rack	Sigma	Z688568
Water bath sonicator	Branson	M2800

MATERIALS AND EQUIPMENT

ESP Buffer	
Reagent	Amount
EDTA pH=8 (0.5 M)	200 μL
10% (w/v) Sarkosyl/EDTA pH8 (0.5 M)	25 μL
Proteinase K (20 mg/mL)	50 μL
Total	**275 μL**
Do not store, use fresh.	

- CldU and IdU: 10 mM in 1 N NaOH – store at −20°C, protected from light and avoid freeze-thaw cycles. IdU does not easily dissolve, if needed briefly heat to 60°C to completely dissolve.
- 1.2% low melting Agarose in PBS – store at 4°C.
- 10% (w/v) Sarcosyl in 0.5 M pH=8 EDTA – store at 20°C–25°C.
- TE Buffer – 10 mM Tris-HCL, 1 mM pH=8 EDTA in 1 L – store at 20°C–25°C.

STEP-BY-STEP METHOD DETAILS

Day 1 – Seed cells

Timing: 30 min

We use DLD-1 BRCA2$^{-/-}$ and BRCA2 WT complemented cells, a derivative of the colorectal adenocarcinoma cell line, DLD-1, a BRCA2 heterozygous cell line. BRCA2 was knocked out in DLD1, using a construct targeting exon 11 with flanking LoxP sites (Hucl et al., 2008). The BRCA2 WT complemented cell line was described previously (Chatterjee et al., 2016).

1. Seed cells in 6 cm^2 plates, exact cell numbers will depend on the cell line used. Aim for cells that are sparse and actively growing at time of treatment (the following day). The cells should be in exponential growth and must not be over-confluent at the time of treatment. The number of plates seeded is determined by the number of experimental conditions being tested. Optimize cell seeding number to have cells at ~30% confluency at time of treatment (note: correct cell density is crucial). Troubleshooting 1.

Day 2 – Treatment with thymidine analogs (CldU and IdU) and fork stalling agent

Timing: 8 h

Nascent DNA in replicating cells is sequentially labeled with two different halogenated thymidine analogs, 5-chloro-2′deoxyuridine (CldU) followed by 5-iodo-2′deoxyuridine (IdU), for 30 min each. Cells are treated with 100 μM CldU in cell culture media containing FBS, for 30 min, washed three times with PBS, then treated with 100 μM IdU for 30 min, followed by three washes with PBS. The nascent DNA synthesized during the sequential treatments will incorporate CldU and IdU facilitating the visualization of DNA fibers at later steps. Immediately following CldU and IdU treatment, cells are washed to remove all traces of CldU/IdU and treated with 4 mM HU in the media for 5 h resulting in replication forks to stall (Schlacher et al., 2011). Silanized coverslips are prepared which provide a positively charged, clean glass surface to which the DNA fibers will adhere. This method uses a combination of the liquid-phase silanization and UV-ozone cleaning (Labit et al., 2008; Marheineke et al., 2009). Troubleshooting 2.

2. Treat with thymidine analogs (time sensitive).
   a. Prepare CldU in cell culture media at a 100 μM concentration, prepare fresh. Stock is 10 mM in NaOH. Very light sensitive, prepare and use in the dark.
   b. Remove cell culture media from cells, add media containing CldU to cells, incubate cells for 30 min exactly, at 37°C and 5% CO_2 (or standard tissue culture incubator conditions).
   c. Prepare IdU in cell culture media at a 100 μM concentration, prepare fresh. Stock is 10 mM in NaOH. Very light sensitive, prepare and use in the dark.
   d. After 30 min, remove CldU media and wash cells quickly 3× with PBS.
   e. Remove last PBS wash from cells and add media with IdU, place cells back in incubator for 30 min exactly.
   f. After 30 min, remove IdU media. Wash cells quickly 3× with PBS.

⚠ CRITICAL: Thymidine analogs are light sensitive; all analog treatments should be performed in the dark. Protect cells from light throughout treatments. The timing of analog incubations needs to be exact as just small variations will influence the results. Add and remove analogs/washes quickly as replication is ongoing during these intermissions.

3. Induce fork stalling using hydroxyurea.
   a. Make a 1 M stock of hydroxyurea (HU) using water.

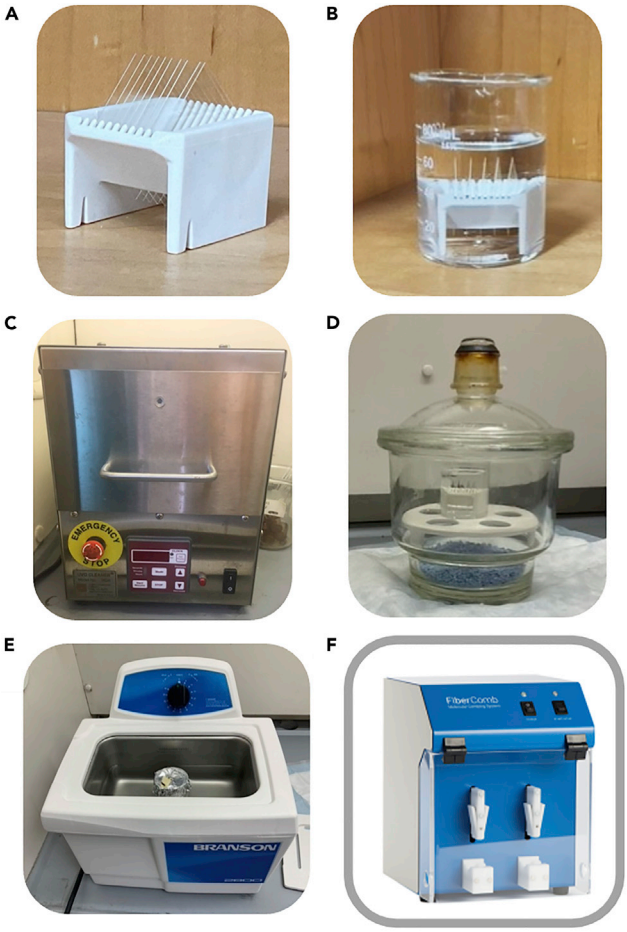

Figure 2. Example equipment used for this protocol

(A–F) (A) Wash-N-Dry coverslip rack, (B) submerged coverslips, (C), Jelight UV Ozone cleaner, (D) glass desiccator jar, (E) water bath sonicator, (F) Fiber Comb machine.

b. Dilute stock to 4 mM using cell culture media.
c. Remove last PBS wash from cells and add HU media for 5 h.

⚠ CRITICAL: Hydroxyurea 1 M stock needs to be made fresh each time from powder (Schwob et al., 2009).

4. Coverslip preparation – perform in the fume hood (Labit et al., 2008; Gallo et al., 2016). Silanized coverslips perform best when prepared and used fresh. Preparation should be done in parallel with the DNA fiber combing experiment, the coverslips will be fully prepared the evening before combing. The preparation is a two-step process: cleaning followed by the formation of the organosilane monolayer.
   a. Place 10 coverslips in a Wash-N-Dry rack (Figure 2A) using a fine point tweezers to handle the coverslips.
   b. Submerge the Wash-N-Dry rack in 200 proof Ethanol for 5–10 min, Figure 2B.
   c. Allow coverslips to dry uncovered, in the fumehood for 30 min.
   d. Treat each side of the coverslips using a UV Ozone cleaner, 30 min, seen in Figure 2C.
      i. Line the UV Ozone cleaner with aluminum foil.
      ii. Place coverslips on foil and treat for 30 min.
      iii. Flip coverslips and treat the other side for 30 min.

e. Dehydrate the coverslips in the rack, for 1 h, in a 70°C incubator.
f. Submerge coverslips in 70 mL N-Heptane + 1 mL Silane in a glass desiccator jar with drierite in the fumehood for 16–18 h, as seen in Figure 2D.

⚠ CRITICAL: Extreme caution must be taken in use of N-Heptane and silane, and they must be used in the fumehood. Do not cover the beaker containing silanization solution with parafilm or aluminum foil as the vapors will dissolve these coverings, the glass desiccator jar will provide an appropriate environment for the vapors. Other cleaning methods can be used if a UV Ozone cleaner is not available such as piranha solution or ion plasma cleaning (Marheineke et al., 2009).

Day 2 – Preparation of agarose plugs and proteinase K treatment

Timing: 2 h

The labeled cells are harvested and embedded in an agarose plug. This plug provides a scaffold which protects the cells from mechanical stress. Proteinase K treatment of the plug digests the cell membrane and proteins present in the labeled cells. The high concentration of EDTA present throughout the proteinase K treatment inhibits DNase activity protecting the genomic DNA.

5. Agarose plug preparation.
   a. Set one water bath at 68°C and a second water bath at 50°C.
   b. Place 1.2% low melting agarose aliquot at 68°C for 10 min, once melted, move agarose to 50°C water bath. 45 μL of agarose is needed per sample.
   c. Trypsinize and count cells. For DLD-1 cells, approximately 1.5×10^5 cell number is ideal for future steps. The cell number required is dependent on the replication aspect being investigated. Between 1×10^5 to 3×10^5 cells per plug is recommended as starting material for global replication analysis. 1–2 million cells per plug are needed for replication analysis of specific loci or telomeres.
   d. Pellet appropriate cell suspension volume for required cell number by centrifuging at 200 g for 5 min.
   e. Wash cell pellet with 1 mL PBS by gently pipetting up and down.
   f. Pellet cells by centrifuging at 200 g for 5 min.
   g. Resuspend cells in 45 μL of trypsin by pipetting up and down.
   h. Warm cell suspension in 50°C water bath for 10 s. Add 45 μL of agarose and mix by pipetting up and down with a P200 pipette, while holding tube in the water bath.
   i. Add trypsin-agarose solution to a well in a sealed plug mold (sealed using tape, Figures 3A and 3B), avoid bubbles.
   j. Place mold upright in a box, cover, and incubate at 4°C for 1 h to solidify.
6. Proteinase K treatment – prepare fresh.
   a. Prepare ESP buffer – 275 μL per plug.
      i. 200 μL 0.5 M EDTA pH=8.
      ii. 25 μL 10% (w/v) Sarkosyl/0.5 M EDTA pH=8.
      iii. 50 μL Proteinase K (20 mg/mL).
   b. Place 275 μL of ESP buffer in a 15 mL tube for each sample.
   c. Push each plug out using a plastic plunger into the ESP solution ensuring plug is immersed in ESP buffer.
   d. Incubate tubes at 50°C for 16–18 h.

Note: Multiple plugs can be prepared for each sample. If more fibers are needed, multiple plugs per sample should be prepared to ensure an individual plug is not overloaded with DNA. See step 5 (c) in relation to appropriate cell number per plug. The optimal cell number per plug will be determined by the purpose of the study, and the cell type used, this number

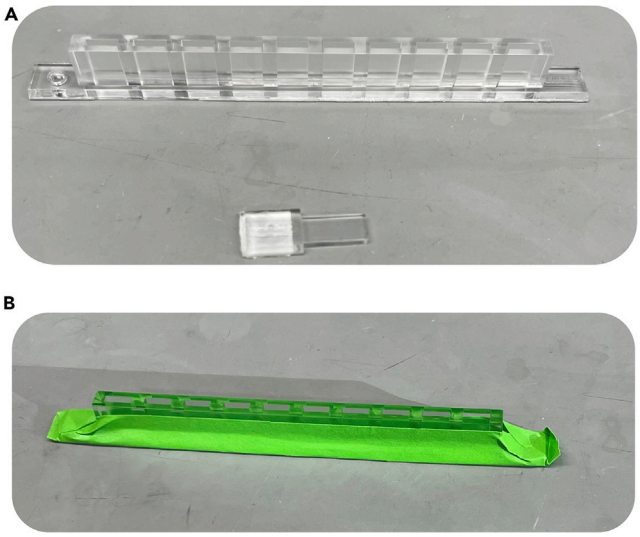

Figure 3. Gel plug mold setup
(A and B) (A) Bio-Rad plug mold with plunger, (B) plug mold sealed with tape.

should be determined by each user based on the unique details of their study. Multiple plugs for the same sample can be washed (step 7) together.

⚠ CRITICAL: If plug isn't fully solidified (step 6 (c)), do not move forward. Incubate at 4°C for another hour.

⚠ CRITICAL: Plugs are translucent and fragile after treatment, caution is needed during the following wash steps.

Day 3 – Plug washing and β-Agarase treatment

Timing: 9 h

Plugs are extensively washed to remove any debris from the previous proteinase K treatment. The agarose is then melted at 68°C and the plug treated with β-Agarase to digest the agarose, releasing the DNA fibers into solution.

7. Wash plugs with TE buffer (10 mM Tris-HCL, 1 mM EDTA pH=8).
   a. Fill (pipette gently) 15 mL tubes with buffer to the very top of the tube to avoid air/bubbles which can damage the plug.
   b. Place 15 mL tubes on rotating mixer set at 15 rpm for 1 h.
   c. Using a spatula and large glass beaker or a cell strainer, gently remove each wash. Tilt the tube, pouring off the liquid using the spatula to retain the plug. Gently pour off the wash buffer, do not aspirate. Plugs can be easily damaged.
   d. Perform 3× 1 h washes on rotating mixer set at 15 rpm, removing the wash buffer after each wash.
   e. Perform final 1× 3.5 h wash for extensive washing.
8. β-Agarase treatment.
   a. Set two water baths at 68°C and 42°C.
   b. Add 1 mL of 0.5 M MES (pH=5.5) into a 2 mL round bottom tube. A round bottom tube is necessary to avoid damaging the plug.

c. Using a spatula transfer each plug to a round bottom tube ensuring immersion in MES solution.
d. Incubate tubes at 68°C for 20 min, ensure the plug is melted before proceeding.
e. Quickly transfer tubes into 42°C water bath for 10 min.
f. Add 1.5 μL of β-Agarase to each tube while in the water bath.
g. Incubate at 42°C for 16–18 h, making sure the tube is in a fixed position avoiding agitation.

⚠ CRITICAL: β-Agarase is stored at −20°C, to avoid local solidification when added to plug-MES solution, hold the β-Agarase in the pipette tip for 10 s before adding. Do not mix the DNA solution when adding the β-Agarase, this can damage the DNA which is now unprotected and easily suffers mechanical stress. Allow the β-Agarase to spontaneously diffuse in the DNA solution.

⚠ CRITICAL: The pH of the MES solution is critical and should be accurately determined in freshly made MES on the day of use. The MES pH dictates the density, degree of stretching and the specific binding of the DNA to various surfaces (Allemand et al., 1997). It can be varied and optimized in a range of pH 5.2–6.5 depending on the fibers required (Iyer et al., 2018).

Alternatives: Heat blocks can be used instead of water baths, be aware of dehydration of small liquid volumes during long incubations and samples will take longer to reach required temperature compared to a water bath, keep this in mind during short incubations.

9. Coverslip preparation continued – perform in the fumehood (Gallo et al., 2016; Labit et al., 2008).
   a. After overnight silane treatment, submerge coverslip rack in 70 mL of N-Heptane and place in a water bath sonicator for 5 min, as seen in Figure 2E.
   b. Submerge coverslip rack in distilled H_2O for 5 min in a water bath sonicator.
   c. Allow coverslips to dry uncovered, in the fumehood.
   d. Submerge coverslip rack in fresh chloroform for 5 min in a water bath sonicator.
   e. Dry the coverslips, in the rack using air gas. Stream air over the coverslips until all liquid has been removed and coverslips appear dry.
   f. Store protected from dust until use, at 20°C–25°C. We suggest a clean, plastic box, stored within a cabinet. See below note for length of storage.

Optional: Quality control (QC) of fully prepared coverslips can be performed using lambda DNA and YOYO-1 staining. Most problems with DNA combing are a result of poor quality silanized coverslips, QC at this step is key to troubleshooting. Troubleshooting 2.

Note: Coverslips are most effective when prepared fresh for each experiment. The positively charged coating degrades over time and can lead to inconsistent fiber binding (Demczuk and Norio, 2009).

Day 4 – Combing and immunodetection

Timing: 9 h

A silanized glass coverslip is submerged into the DNA fibers solution and removed at a slow constant rate. These fibers are stretched and aligned while binding the silanized coverslip. The meniscus of the solution with the slow, constant rate of movement (300 μm/s) straightens and aligns each individual DNA fiber along the same axis (fibers are elongated at a constant rate of 2 kb/μm) (Michalet et al., 1997). Subsequent immunodetection steps with primary and secondary antibodies culminate in visualization of the fibers using a fluorescent microscope with one slide containing hundreds of individual DNA molecules.

10. DNA fiber combing.
    a. Add 1 mL of 0.5 M (pH=5.5) MES solution to a Teflon reservoir for each sample.
    b. Pour the DNA solution into the reservoir, do not pipette!
    c. Use a DNA combing machine, see Figure 2F, to submerge a coverslip into the DNA solution.
        i. Use a fine point tweezers to handle silanized coverslips so as not to damage the coating.
        ii. Clamp a silanized coverslip above a Teflon reservoir.
        iii. Submerge the coverslip in the DNA fiber solution.
        iv. Remove the coated coverslip at a slow, constant rate. The Fiber Comb combing machine removes coverslips at a rate of 300 μm/s.
    d. Return each combed coverslip to a rack.
    e. Dehydrate coverslips in a 65°C incubator for 2 h. This ensures the DNA fibers are fully attached to the glass coverslip.

⚠ CRITICAL: Do not pipette the DNA solution into the reservoir, gently pouring will avoid damaging the delicate DNA fibers.

Note: Ensure each reservoir is full of liquid, top up with 0.5 M MES if necessary. After combing, both sides of the coverslip are coated in DNA.

Optional: Quality control of combed coverslips can be performed using YOYO-1 staining. This will confirm DNA fibers have bound to the coverslip and show the quality of the DNA fibers. The Fiber Comb machine can comb two coverslips at once into the same sample allowing one slide to be used for quality control YOYO-1 staining.

Note: We use disposable Teflon reservoirs and the Fiber Comb machine purchased from Genomic Vision (www.genomicvision.com), these can be built, or 3D printed in-house. How to build a combing machine is described in-depth by Gallo et al. A motorized platform with clamps to hold coverslips can be used as a non-commercial alternative, ensure the clamps remove the coverslips at a constant rate of 300 μm/s.

11. Immunodetection.
    a. Denature combed coverslips using 0.5 M NaOH + 1 M NaCl (make fresh in water) for 8 min by submerging rack into solution into a glass beaker, as seen in Figure 2B, and shaking on an orbital shaker, set at 75 rpm (use this setting for all future shaking steps).
    b. Wash 3× by submerging rack in PBS – 2 min and shaking as above.
    c. Dehydrate coverslips in 70% ethanol (freshly prepared) by submerging rack for 5 min and shaking as above.
    d. Further dehydrate the coverslips in 100% ethanol for 5 min with shaking.
    e. Dry coverslips on the bench for 30 min or until completely dry, meaning no droplets or liquid can be seen on the glass surface.
    f. Blocking: 5% BSA in PBS – submerge coverslips in rack into 5% BSA solution in a glass beaker and incubate at 37°C for 30 min.
    g. Prepare a moist box, shown in Figure 4A as follows:
        i. Use a plastic box. If transparent, cover with foil (the secondary antibodies need protection from light).
        ii. Place parafilm on the bottom of the box.
        iii. Surround the edge of the box with tissue paper, wet this tissue paper to maintain humidity in the box during incubations.
    h. Prepare primary antibody in 5% BSA, per sample: 40 μL BSA, 4 μL mouse anti-BrdU, 1 μL rat anti-BrdU (total= 45 μL per sample).
    i. In this moist box, add 45 μL to parafilm (Figure 4B) and place coverslip on top (Figure 4C). Keep track of which side has antibody.
    j. Incubate at 37°C for 1 h.

Figure 4. Setup of humid chamber for antibody incubations

(A–C) (A) Setup of a moist, plastic box, protected from light for antibody incubation of coverslips, (B) addition of antibody solution, (C) addition of coverslips on top of antibody solution. Purple color used for contrast and visualization in this image.

k. Return coverslips to rack, keep track of orientation. Wash 3× in PBS – 5 min and shaking.
l. Prepare secondary antibody in 5% BSA, per sample: 44 µL BSA, 0.5 µL anti-mouse Cy3, 0.5 µL anti-rat Cy5.
m. Add 45 µL to clean parafilm and place coverslip on top. Keep track of which side has antibody.
n. Incubate at 37°C for 30 min.
o. Return coverslips to rack, keep track of orientation. Wash 3× in PBS – 5 min and shaking, protect from light while washing.
p. Mount coverslips on microscope slides using 15 µL of Prolong Diamond Antifade.
q. Store slides at 4°C for 24 h before imaging.

Optional: Denaturation step can be adapted and optimized. NaOH denaturation is optimal for IdU visualization but is moderate for CldU. HCL can be used for denaturing and provides best visualization of CldU but does not work well for IdU. If NaOH denaturing results in limited CldU visualization, consider increasing the CldU concentration used (Iyer et al., 2018).

Optional: All ssDNA can be fluorescently visualized alongside the nascent DNA fibers. This can be done using a layered approach, meaning the primary and secondary antibody incubations to detect IdU and CldU are completed and followed by a 30 min primary antibody incubation for ssDNA (Millipore MAB3868) and a 30 min secondary antibody incubation (BD Biosciences #746352) (Bianco et al., 2012). This is necessary as all ssDNA antibodies available are either of mouse or rat origin. To ensure no cross-reactivity, the mouse ssDNA primary antibody is incubated separately with the secondary being a different isotype. After mounting, all ssDNA on the coverslip can be visualized using a blue-fluorescence lens. If available, other species of BrdU antibodies can be used along with mouse ssDNA antibodies to avoid this layered approach.

⚠ CRITICAL: The use of a moist box for incubations at 37°C is important to maintain humidity which prevents the DNA fibers from desiccation.

Day 5 – Imaging and analysis

DNA fibers are viewed and imaged using an epifluorescent microscope with a 60× objective. Using ImageJ software, the length of each tract is measured and a ratio of IdU/CldU represents the level of fork protection/degradation. A reduction in the IdU/CldU ratio indicates the IdU tract is shorter in length, implying degradation of the nascent DNA, and therefore, a deficit in fork protection.

12. Imaging - troubleshooting 1.
    a. Visualize fibers at 60× magnification using an oil immersion lens in an inverted epifluorescent microscope e.g., Keyence BZ-X800E.
    b. Visualize Cy3 labeled tracts with a TRITC filter and Cy5 labeled tracts with a Cy5 filter.

c. Overlay both filters to produce images of entire DNA tracts, meaning both the CldU and IdU tracts.
d. Take multiple images per sample, ensuring each field of view is separate, not overlapping. The number of images taken is determined by the density of fibers in each field of view, an average of 10–20 fields of view has proven sufficient in our studies.
e. A minimum of 100 fibers per sample is needed for future analysis to ensure reliable estimation of fiber length accounting for the variability between fibers within a sample.
f. Include a scale bar for later quantification, 1 μm = 2 kilobases.

13. Analysis.
a. Analyze images using the Java-based image analysis program ImageJ, developed by the NIH.
b. Set the global scale using a scale bar in μm.
i. Draw a straight line across the scale bar.
ii. Select Analyze, Set Scale.
iii. Set length of scale bar as Known Distance and the unit of the scale bar as the Unit of Length.
iv. Select global.
c. Using the line drawing tool measure the length of each tract (red/green) of each fiber, only include bicolored fibers i.e., fibers with both tracts.
d. Calculate the ratio of IdU/CldU, with a minimum of 100 fibers measured for each sample.

⚠ CRITICAL: Avoid measuring fibers that are too short/long, non-consecutive or fiber bundles. These fibers are unreliable, and this can skew the data. See troubleshooting 3 and troubleshooting 4.

Optional: DNA fibers can be analyzed in many different ways using the length of the DNA tracts. Independent of the replication parameter being investigated, the data acquired are based on the lengths of the fluorescent DNA tracts. Length of individual tracts (red or green) can be analyzed, also the length of the entire DNA tract in total can be measured. Differences in tract length can provide information on many replication aspects such as the progression of the fork, stalling of the fork, the speed of the fork. To analyze the speed of the replication fork, the length of each tract, once measured, is converted from μm to kilobases, with the conversion factor of 2 kb/μm (Michalet et al., 1997). This conversion rate is specific for DNA combing method. The fork speed is then calculated by dividing the tract length by the labeling time (kb/min). Other dynamics such as replication fork restart, fork symmetry, new origin firing, interorigin distance, and ssDNA gaps can be investigated using DNA fiber combing. An example of origin firing in a DNA fiber is shown in Figure 5A. These approaches and analyses are reviewed in-depth by Quinet et al., in DNA Fiber Analysis: Mind the Gap!

EXPECTED OUTCOMES

This protocol should result in high quality uniformly stretched fibers that are easy to visualize and measure. An example of a DNA fiber image and individual fibers produced using this protocol is shown in Figure 5. Regarding fork protection analysis described above (Figure 6A), length measurements of fiber tracts are used to calculate the ratio of IdU/CldU for each sample, a ratio of 1 is indicative of functional fork protection (e.g., BRCA2 WT cells). At a stalled replication fork, nascent DNA is susceptible to nucleolytic degradation by nucleases. Degradation can be blocked by proteins binding to the nascent DNA, thereby protecting the fork from degradation. If protected, we expect both tracts to be of similar length as each analog is incorporated for 30 min, therefore, a ratio of 1 indicates functional fork protection. A reduction in the IdU/CldU ratio is indicative of nucleolytic degradation of the nascent DNA, and therefore, a deficit in fork protection functionality. A reduction in this ratio could also indicate (Figures 6B and 6C) the BRCA2$^{-/-}$ (ratio 0.72) cells have a defect in fork protection in comparison to the BRCA2 WT (ratio of 1) cells. BRCA2 loads RAD51 onto nascent ssDNA

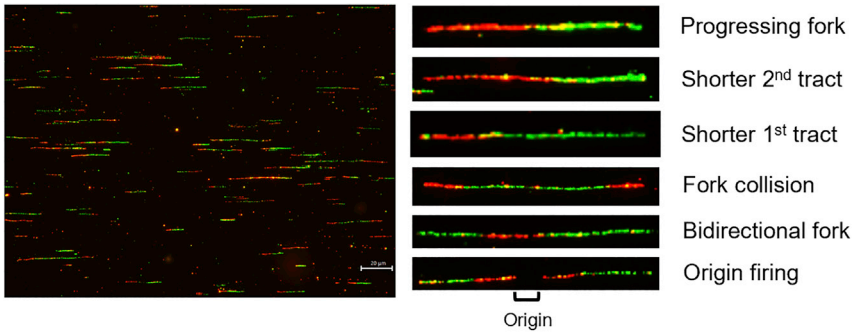

Figure 5. Example image of DNA fibers prepared using this protocol

Representative image of cells pulse-labeled with CldU and IdU followed by a 5 h hydroxyurea (HU; 4 mM) treatment with the representative image showing CldU (Red) and IdU (Green) replication tracks of DLD-1 cells after HU treatment. Scale bar, 20μm.

regions present at the fork protecting the DNA from nucleolytic degradation, however, in the absence of BRCA2 and RAD51, nucleases such as MRE11 can degrade DNA at the fork leading to collapse and eventual cell death (Schlacher et al., 2011). Representative images of individual fibers as well as a dot plot analysis of the IdU/CldU ratio confirm this result (Figures 6B and 6C).

LIMITATIONS

To date, the biggest limitation to DNA fiber combing has been the lack of reliability in the quality of the fibers, including fibers that are too short, too long, or entanglement. Other limitations include the labor-intensive data analysis, the expense of kits, silanized coverslips, and equipment required. The DNA fiber combing method described here overcomes both the reliability issues and the need for commercial kits. This method will produce high quality fibers, aligned on a single axis, and equally elongated with reagents and coverslips generated by the user. The time required for data analysis is the largest limitation of the experiment. The time required per sample varies based on fiber density, condition, and quality. Examples of poor conditions and low-quality fibers can be seen here, troubleshooting 3.

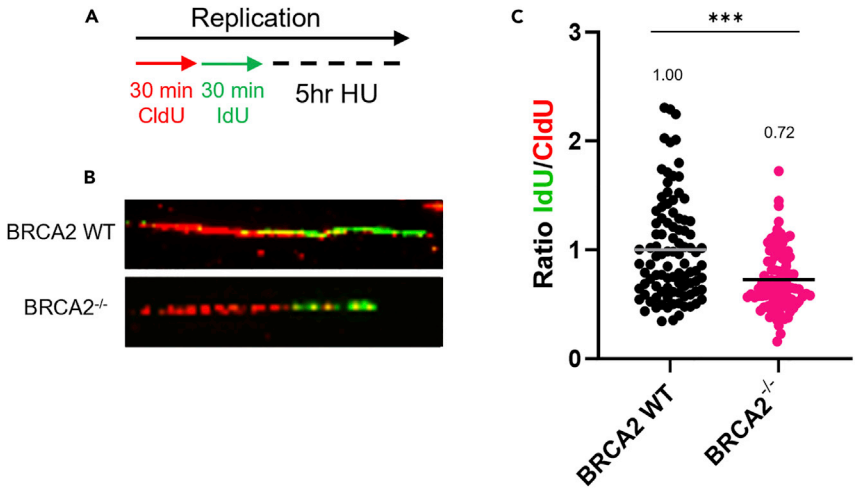

Figure 6. DNA fiber analysis of BRCA2 WT complemented and BRCA2$^{-/-}$ cells in response to HU treatment

(A and B) (A) Schematic of CldU/IdU pulse-labeling followed by a 5 h hydroxyurea (HU; 4 mM) treatment with representative images (B) of CldU (Red) and IdU (Green) replication tracks after HU treatment.

(C) Dot plot of IdU to CldU tract length ratios for individual replication forks in HU-treated cells for BRCA2$^{-/-}$ and BRCA2 WT complemented cells. The median value of 100 or more IdU and CldU tracts per experimental condition is indicated. ***p value<0.001.

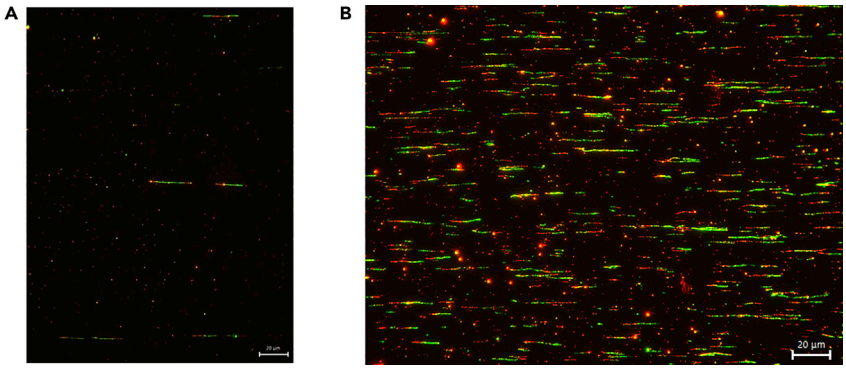

Figure 7. Examples of low-density and high-density DNA fiber images prepared using this protocol

(A and B) Images of DNA fibers at (A) low density and (B) high density in representative images of DLD-1 cells pulse-labeled with CldU and IdU followed by a 5 h hydroxyurea (HU; 4 mM) treatment with the representative image showing CldU (Red) and IdU (Green) replication tracks. Scale bar, 20µm.

ImageJ provides an open-source software for manual analysis of DNA fibers. Analysis of DNA fiber images is quite simple but can be time consuming. Unfortunately, no reproducible, robust, automated software is currently widely available. Vigilance is required when analyzing DNA fibers to avoid introducing biases. Bias can arise from experimental aspects such as sample size, minimum and maximum fiber length measured, and presence/absence of ssDNA counter staining. A study on the bias in DNA fiber analysis found biological variability introduces an inherent variability of 15% therefore to overcome this, any result showing a difference of less than 15% needs to be reproduced several times to be deemed significant (Técher et al., 2013). A free automated analysis approach would significantly reduce both the time required and the possibility of bias in the protocol.

Cells cultured in media supplemented with nucleotides (especially thymidine) may not be suitable for this method as the addition of nucleotides in the culture media may affect the incorporation of the thymidine analogs (Demczuk and Norio, 2009).

> *Alternatives:* Genomic Vision provides a high throughput service (Easy Scan), scanning the entire coverslip surface and analyzing the fibers through Fiber Studio software. The Easy Scan service requires shipping the coverslips to Genomic Vision (in France) for analysis which can incur delays. Customers can purchase access to the Fiber Studio software to quantify fiber images taken without the Easy Scan. The Fiber Studio is manual and requires significant time for analysis. Ghesquière et al. have published an automated, free software for DNA fiber analysis. Using this software, the results produced did not match manual analysis or well-established control experiments (Ghesquière et al., 2019). There are several other automated analysis software packages available such as Metamorph but these all require a purchase.

TROUBLESHOOTING

Problem 1

Density of DNA fibers is either (A) too low or (B) too high as seen in Figures 7A and 7B.

Potential solution

A. Low DNA fiber density can be due to a low cell count, which can be resolved by increasing the cell density of cells seeded in step 1, increasing number of cells per plug without going over the limit (see step 5(c) and the following note) or increasing the number of plugs prepared per sample. Insufficient Proteinase K activity can cause low fiber density. To rectify, use a fresh aliquot of Proteinase K and check the storage conditions. Low fiber density can also be due to the MES pH being too high,

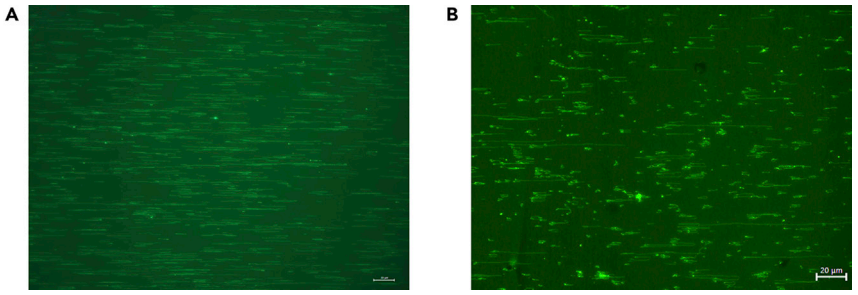

Figure 8. Examples of high-quality and low-quality silanized coverslips
(A and B) Representative images of a (A) high-quality and (B) low-quality silanized coating using Lambda DNA (Green) and YOYO-1 staining. Scale bar, 20µm.

double check the pH and prepare fresh (Marheineke et al., 2009). Tourriere et al., suggest storing the DNA fiber solution at 4°C for several days to increase DNA resuspension in MES buffer before combing, this can resolve low fiber density (Tourrière et al., 2017).

B. High DNA fiber density can be due to a high cell count and can be resolved by decreasing the cell density of cells seeded in step 1 or reducing the number of cells per plug. Diluting the DNA fiber solution further using MES solution before combing can also help rectify the problem.

Problem 2

Unsuccessful combing of Lambda DNA, indicative of low quality silanization of coverslip, as seen in Figure 8B. Successful combing of Lambda DNA is shown in Figure 8A.

Potential solution

Silanize a fresh batch of coverslips. Use fresh reagents to ensure no degradation or contamination. Use the gas-phase silanization procedure instead of the liquid-phase and the plasma cleaning opposed to the UV-ozone cleaning (Marheineke et al., 2009).

Problem 3

DNA fibers are not individualized – presence of very bright fibers, known as bundles (A), or long fiber strings (B), shown in Figure 9.

Potential solution

These problems can be caused by incomplete digestion of proteins during Proteinase K treatment, use a fresh aliquot of Proteinase K, and check the storage conditions. Bundles may be due to cell aggregates present in the plug, ensure homogenous suspension of cells in agarose. Insufficient

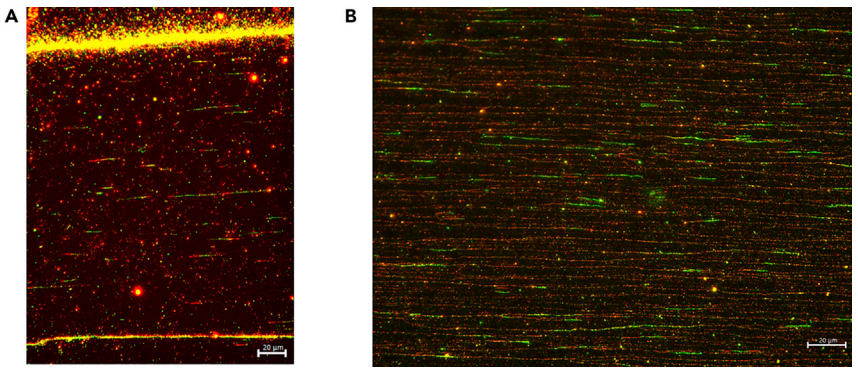

Figure 9. Examples of non-individualized fibers
(A and B) Representative images of a (A) fiber bundles and (B) long fiber strings and non-continuous fibers. Scale bar, 20µm.

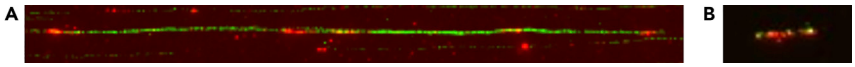

Figure 10. Example image of an extremely long and extremely short DNA fiber

(A and B) Representative images of fibers that are (A) too long and (B) too short. These lengths are specific to this cell line and this analog incubation time.

activity of trypsin can induce these issues, use a fresh aliquot of trypsin, and check the storage conditions. This may also be caused by the agarose plug not fully melting, ensure the plug is dissolved after 68°C incubation. Also ensure complete degradation of agarose during β-Agarase treatment by using a fresh aliquot of β-Agarase and checking the storage conditions.

Problem 4

Fibers too long (Figure 10A) or too short (Figure 10B) to analyze.

Potential solution

Fibers that are too long or too short should be avoided during analysis. It would be inappropriate to give an exact length as this varies based on incubation time of analog as well as replication speed of the cell type used. For each cell type, when treated with the analog for a given time there will be an average fiber length observed. Extreme outliers of this average, either too long or too short, should be avoided in the analysis.

Problem 5

Most problems with DNA combing are a result of issues with the silanization of the coverslips. The steps in coverslip preparation should be adhered to diligently including the timing of each step. Poor quality silanized coverslips will not yield high quality DNA fibers. Quality control of individual batches of coverslips, using YOYO-1 staining is key to troubleshooting. YOYO-1 staining of the DNA after combing before immunodetection is another key troubleshooting step. This will indicate the quality of the DNA, the fiber density, and will indicate if the fiber quality is sufficient to progress to immunodetection.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ryan Jensen (ryan.jensen@yale.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any datasets.

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AUTHOR CONTRIBUTIONS

G.M. performed experiments, data analysis, and wrote the manuscript. G.M. and J.J.S. optimized and troubleshooted the protocol. All authors edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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