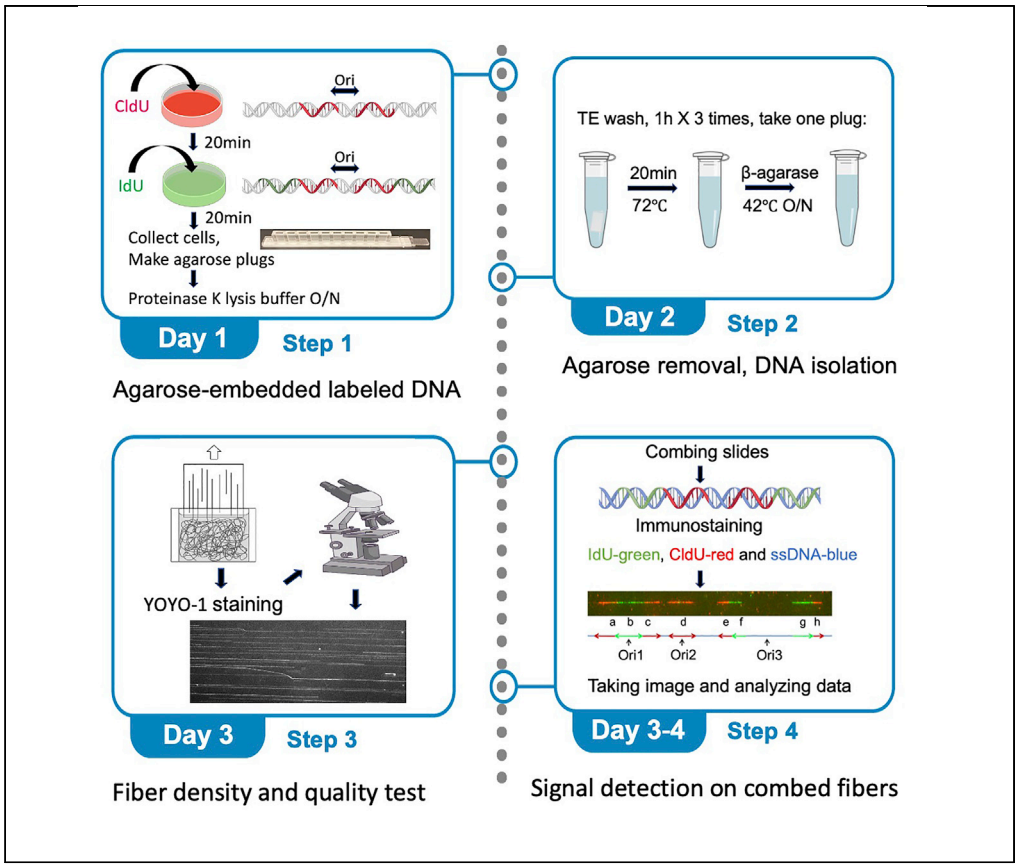
STAR Protocols

CellPress
OPEN ACCESS

Protocol

DNA replication profiling by molecular combing on single DNA fibers



In this protocol, the progression of DNA synthesis is profiled at a single-molecule resolution. DNA fibers are uniformly stretched on silanized coverslips, and replicating DNA can be traced with thymidine analogs using specific antibodies against distinct analogs. Single DNA fibers are visualized by an anti-single stranded DNA antibody. The protocol can be used to study DNA replication dynamics, the cellular response to replication stress, and replication fork progression at specific chromosomal regions when combined with fluorescent *in situ* hybridization.

Haiqing Fu, Mirit I. Aladjem

fuha@nih.gov (H.F.)
aladjemm@mail.nih.gov (M.I.A.)

Highlights

High-resolution profiling of the progression of DNA replication on single DNA fibers

Nucleotide analogs incorporated into DNA are traced using specific antibodies

The protocol measures DNA synthesis rates and distances between replication origins

DNA combing can characterize the cellular response to genotoxic stress

Fu & Aladjem, STAR Protocols *3*, 101290
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101290

STAR Protocols

CellPress
OPEN ACCESS

Protocol

DNA replication profiling by molecular combing on single DNA fibers

Haiqing Fu[1,2,*] and Mirit I. Aladjem[1,3,*]

[1]Developmental Therapeutics Branch, Center for Cancer Research, NCI, NIH, Bethesda, MD, USA

[2]Technical contact

[3]Lead contact

*Correspondence: fuha@nih.gov (H.F.), aladjemm@mail.nih.gov (M.I.A.)
https://doi.org/10.1016/j.xpro.2022.101290

SUMMARY

In this protocol, the progression of DNA synthesis is profiled at a single-molecule resolution. DNA fibers are uniformly stretched on silanized coverslips, and replicating DNA can be traced with thymidine analogs using specific antibodies against distinct analogs. Single DNA fibers are visualized by an anti-single stranded DNA antibody. The protocol can be used to study DNA replication dynamics, the cellular response to replication stress, and replication fork progression at specific chromosomal regions when combined with fluorescent *in situ* hybridization.

For complete details on the use and execution of this protocol, please refer to Conti et al. (2007), Fu et al. (2021), Kaykov et al. (2016), Redmond et al. (2018), and Schwob et al. (2009).

BEFORE YOU BEGIN

Note: The protocol below describes the specific steps using HCT116 cells. However, the same protocol is applicable to measuring replication dynamics in a variety of human and mouse cell lines. A notable variation for suspension cell lines: the medium changing steps between thymidine analogs can be skipped (sequentially add 5-Iodo-2′-deoxyuridine (IdU), 5-Chloro-2′-Deoxyuridine (CldU) and thymidine to the same medium, no centrifugation) to avoid disruptions in DNA replication pace due to temperature changes during centrifugation.

Before preparing IdU and CldU labeled genomic DNA in agarose plugs – day 1

Timing: 2–3 h

1. Prepare stock solutions of 100 mM IdU, CldU in DMSO, and 200 mM of thymidine in PBS. Store stock solutions at −20°C in aliquots to avoid repeated freeze-thaw cycles. The stock solutions are stable at −20°C for at least 1 year.

   Note: IdU and CldU are sensitive to light. Work under yellow light or under dimmed lights whenever handling IdU, CldU, as well as IdU and CldU incorporated DNA and cells.

2. 3 × 10^5 exponentially growing HCT116 cells in 2 mL DMEM with 10% FBS are plated in 6-well plates for about 24 h (about 60%–70% confluency) are used for optimal IdU and CldU incorporation.
3. Pre-warm medium to 37°C for IdU, CldU and thymidine incubations.

Note: It is recommended to recoaliquot the culture medium (2 mL/well times N+1 wells) in 3 tubes for the IdU, CldU and thymidine labeling steps. Leave the tubes in a tissue culture incubator with the caps loose for 12–18 h. This step will help acclimate both temperature and CO2 level to avoid a disruption or slowing of DNA synthesis during labeling.

4. Prepare cold PBS on ice. It is suggested to always keep a chilled bottle of PBS in refrigerator.
5. Set up a water bath to 50°C.
6. Prepare 1.5% of SeaPlaque low melting point (LMP) agarose in PBS (tissue culture grade). It can be stored at 4°C for 6 months. Melt it with boiling water or microwave and keep it in the water bath at 50°C for at least 30 min before use.
7. Prepare cell lysis buffer with proteinase K: add cell lysis buffer (50 mL of cell lysis buffer for agarose plugs in the materials and equipment section) to a 15 mL centrifuge tube, 2 mL/sample (prepare a master mix). Just before use, add proteinase K to the lysis buffer at a final concentration of 1 mg/mL.

Before the release of purified DNA from agarose plug - day 2

8. Set up a water bath to 42°C and a heat block to 70°C 30 min before use to ensure stable temperatures in the next steps.
9. Make 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES), 1.6 mL/sample, by diluting 0.5 M of MES in DNase free water.

Before DNA combing and immunodetection-day 3 - day 4

10. Prepare a YOYO-1 staining solution: 2 μL YOYO-1 to 10 mL of 0.1 M MES. The working solution is stable for at least 2 months at 20°C–25°C in dark.
11. Prepare 200 mL of 0.5 N NaOH solution from 10 N of NaOH. 0.5 N NaOH working solution is good for at least 2 months if the bottle is sealed well.
12. Prepare 5% BSA in PBS by dissolving 2.5 mg Bovine Serum Albumin (BSA) in 50 mL PBS.
13. Prepare 1,000 mL PBS/T (0.05% TritonX-100 in PBS) by adding 0.5 mL of Triton X-100 to 1,000 mL PBS, mix well with a magnetic stir bar.
14. Prepare 200 mL of 70%, 90% and 100% ethanol.

KEY RESOURCES TABLE

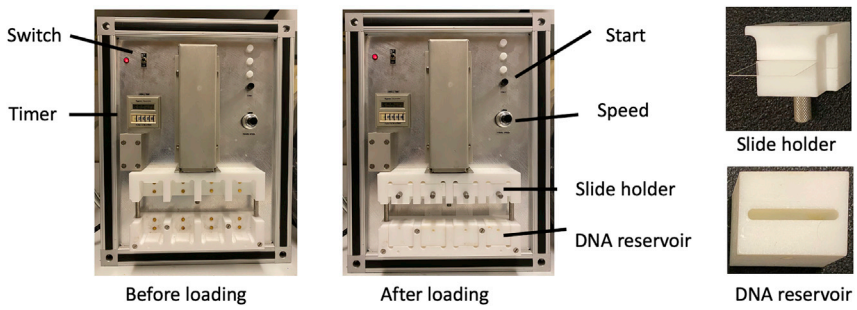
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-BrdU antibody for IdU, IgG1 (1:20 dilution)	BD	#347580 RRID: AB_10015219
Rat anti-BrdU antibody for CldU, IgG2a (1:100 dilution)	Abcam	#Ab6326 RRID: AB_305426
Mouse anti-single strand DNA, IgG2a (1:200 dilution)	Millipore	#MAB3034 RRID: AB_11212688
Goat anti-mouse (Cy3), IgG (H+L) (1:100 dilution)	Abcam	#AB97035 RRID: AB_10680176
Goat anti-rat (Cy5), IgG (H+L) (1:100 dilution)	Abcam	#AB6565 RRID: AB_955063
Goat anti-mouse (Brilliant Violet 480), IgG (H+L) (1:50 dilution)	Jackson ImmunoResearch	#115-685-166 RRID: AB_2651095
Chemicals, peptides, and recombinant proteins		
5-Iodo-2′-deoxyuridine (IdU)	Sigma-Aldrich	#I-7125
5-Chloro-2′-Deoxyuridine (CldU)	MP biomedical	#105478
Thymidine	Sigma-Aldrich	#T1895

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Trypsin-EDTA (0.05%)	Thermo Fisher Scientific	#25-300-120
SeaPlaque agarose	Lonza	#50100
PBS	Thermo Fisher Scientific	#10010031
N-Lauroylsarcosine sodium salt	Sigma-Aldrich	#L9150
Proteinase K solution (20 mg/mL)	Thermo Fisher Scientific	#25530049
TE pH 8.0	Quality Biology	#351-011-131
Ultra pure water	KD Medical	#RGC3410
Bovine Serum Albumin (BSA)	Sigma-Aldrich	#A9647
2-(N-morpholino)ethanesulfonic acid (MES)	Sigma-Aldrich	#M3671
β-agarase	New England Biolabs	#M0392S
Triton X-100	Sigma-Aldrich	#T9284
YOYO-1 Iodide	Thermo Fisher Scientific	# Y3601
Experimental models: Cell lines		
HCT116	ATCC	#CCL-247
Software and algorithms		
FiberStudio	Genomic Vision	http://www.genomicvision.com
ImageJ software	Schneider et al., 2012	https://imagej.nih.gov/ij/
Other		
Agarose plug mold	Bio-Rad	#170-3713
Eppendorf Safe-Lock Tubes, 2.0 mL	Eppendorf	#022363433
Water bath	N/A	N/A
Heat block	N/A	N/A
Rocker/shaker	N/A	N/A
Teflon reservoir	Made in-house	N/A
Greiner Bio-One Sample Cup for Coulter Counter™ Analyzers	Thermo Fisher Scientific	#07-000-694
Engraved Combicoverslips	Genomic Vision	#COV-002-RUO
Sample Holders for Scanner HLD-001	Genomic Vision	#HLD-001
Thomas cover glass staining outfits	Thomas Scientific	#8542E40

MATERIALS AND EQUIPMENT

We use an in-house constructed DNA combing machine to stretch DNA onto silanized combing coverslips. The combing machine consists of a motor, Teflon slide holders and Teflon DNA solution reservoirs (see the picture below). The motor slowly (300 μm/s) inserts coverslips into the DNA solutions in the reservoir, incubates for a few minutes and slowly (300 μm/s) pulls the coverslips from the reservoirs.



As an alternative, commercial DNA combing machines are available by Genomic Vision (http://www.genomicvision.com).

We currently use the molecular combing platform from Genomic Vision (http://www.genomicvision.com/products/molecular-combing-platform) to acquire images of DNA fibers. This can be substituted by other fluorescence microscopes that can combine images to create montages that can follow the entire lengths of DNA fibers.

50 mL of cell lysis buffer for agarose plugs		
Reagent	Final concentration	Amount
0.5 M EDTA, PH 8.0	100 mM	10 mL
10% N-lauroyl-sarcosine	1%	5 mL
1 M Tris-Cl, PH 8.0.	10 mM	0.5 mL
ddH_2O	n/a	34.5 mL
Total	**n/a**	**50 mL**
Store at 20°C–25°C for up to one year.		

50 mL of 0.5 M [2-(N-morpholino) ethanesulfonic acid, pH 6.5] (MES)		
Reagent	Final concentration	Amount
MES powder	0.5 M	4.88 g
ddH_2O	n/a	To 50 mL
Total	**n/a**	**50 mL**
Adjust pH to 6.5 by a pH meter, need about 1.5 mL of 10 N NaOH. Autoclave and store at 4°C for up to one year.		

STEP-BY-STEP METHOD DETAILS

Preparing IdU and CldU labeled genomic DNA in agarose plugs – day 1

Timing: 2–3 h

In this major step, 2 thymidine analogs, CldU and IdU, are added to cells sequentially to label replicating DNA. Then genomic DNA is purified in agarose plugs in order to prevent DNA breakage and obtain very long DNA fibers.

1. Replace medium of the HCT116 cells in 6 well-plates with 2 mL pre-warmed fresh medium containing 25 μM of CldU (add 100 mM CldU stock solution to the pre-warmed medium before adding to cells). Incubate for 20 min in a tissue culture incubator at 37°C and 5% CO2.

   Note: Pre-warmed medium avoids perturbing or slowing of DNA replication. Minimize the time cells spend outside of the incubator. With this protocol, there is no need to wash with PBS during medium changes.

   Note: HCT116 cells are small and do not spread sparsely on the culture surface. Therefore, 6-well plates are sufficient to obtain enough cells for combing. For some larger cells and/or cells that are spreading very sparsely on the plate surface, for example, fibroblast cells, use larger containers such as 10 cm dishes or flasks.

2. Completely aspirate the CldU-containing medium and add 2 mL pre-warmed fresh medium containing 100 μM of IdU (add 100 mM IdU stock solution to the pre-warmed medium before adding to cells). Incubate for 20 min in a tissue culture incubator at 37°C and 5% CO2.

Optional: Completely aspirate the medium and add 2 mL pre-warmed fresh medium containing 200 μM of thymidine. Incubate for 60 min in a tissue culture incubator at 37°C and 5% CO2. This step prevents the formation of single strands of ongoing forks on the IdU labeled DNA, which can significantly reduce mechanical breaks at IdU-labeled DNA fibers.

3. Harvest cells.
   a. Trypsinize cells at 20°C–37°C, monitoring the cells periodically under a microscope. Once cells round up, stop the trypsin digestion immediately with 2 mL of cold DMEM containing 10% FBS to keep cells healthy.
   b. Transfer cells to 15 mL centrifuge tubes and spin at approximately 220 × *g* for 5 min at 4°C.
   c. Wash cells once with 10 mL cold PBS. Resuspend the cells in cold PBS at 1 × 10^6/mL.

⚠ CRITICAL: To avoid DNA degradation, it is critical to keep cells cold at all times after the trypsin digestion till the cells embedded in plugs are in lysis buffer.

⚠ CRITICAL: Make sure to count cells and use an accurate and consistent cell number for all the samples to obtain similar DNA densities on all slides.

Note: We normally use 5 × 10^4 cells per plug. It could be adjusted to have the best fiber density using 2.5–10 × 10^4 cells per plug (Resuspend the cells in cold PBS at 0.5 to 2 × 10^6/mL). If cell number is limited, the minimal number of cells that yield interpretable results could be as low as 1 × 10^4 cells/plug.

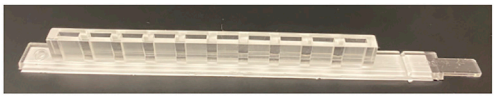
Note: Cell number is critical for optimal fiber density. The number above is optimized for HCT116 cells, but we have used similar densities in all diploid or pseudo-diploid cells. Confluence depends on the tissue culture conditions and cell size – cells should not be too crowded to facilitate a larger S-phase fraction. For polyploid cells, fewer cells should be used in the protocol and the exact number should be determined empirically for cancer cells with unstable karyotypes.

4. Add 0.35 mL of 1.5% of LMP agarose, which is kept in a 50°C water bath before use, to an Eppendorf tube. Mix cells completely by pipetting, then transfer 0.35 mL cells to the Eppendorf tube with the same volume agarose and mix cells completely by pipetting again. Fill plug molds with the cell-agarose mixture, about 90–100 μL/plug, 6 plugs per sample.

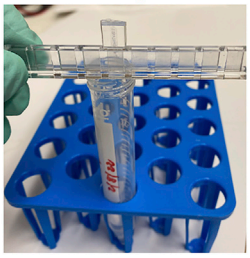
Note: Typically, only one plug is needed for the following steps. We usually prepare 3-6 plugs/samples as backups. One plug per sample is fine for limited material.

⚠ CRITICAL: In this step, genomic DNA is prepared in low melting point (LMP) agarose plugs to preserve the long DNA fibers and prevent mechanical shearing. Mix cells with agarose thoroughly to ensure that cells are distributed homogeneously in the plugs.

5. Let agarose plugs solidify for 20–30 min at 4°C. Transfer all the 6 plugs from one biological sample directly into one 15 mL tube containing 2 mL of plug lysis buffer (1 mg/mL proteinase K is added to lysis buffer right before use) (see the picture below for the procedure required to make and transfer plugs to 15 mL tubes) Incubate 16–18 h at 50°C in a water bath. Gentle rocking is helpful, but not essential.



Fill the agarose plug mold with agarose: cells mixture, leave at 4°C for 20-30min.



Remove the tape under plug mold and use the attached insert of the plug mold to push plugs to a 15ml tube containing lysis buffer

Note: Transfer the agarose plugs to the agarose plug lysis buffer ASAP once agarose is solid. It's not necessary to let the plugs to solidify for more than 30 min. Too long time could lead death and DNA degradation.

Release purified DNA from agarose plug-day 2

Timing: 8–20 h

In this major step, the day 1 prepared agarose plugs are melted, and the melted agarose is degraded by β-agarase to release the purified long genomic fibers into MES solution.

6. Wash plugs.
   a. Discard plug lysis buffer.
   b. Rinse plugs once with 10 mL of TE buffer per tube.
   c. Wash plugs 3 times on a rocker with a low speed (about 80 rpm) at room temperature using 10 mL of TE per tube. 1 h per wash.
   d. Store plugs in TE buffer at 4°C. DNA in plugs are stable for at least 1 year at 4°C.

⚠ CRITICAL: Make sure all buffers and containers are DNase free from this step until the completion of the immunostaining steps. Even residual DNase can shorten the DNA fiber length before combing.

7. Transfer a single plug to a 2 mL Eppendorf tube containing 1.6 mL of 0.1 M MES, pH 6.5. Melt the agarose plugs at 70°C by incubating them for 20 min in a heating block.

Note: Agarose plugs are melted, and DNA is released to the MES solution. After the 20-min 70°C heating step, the samples should be manipulated very gently to avoid mechanical DNA shearing.

8. Let the solution cool to 42°C by leaving the tubes for 10 min in a 42°C water bath, then add 2 μL of β-agarase to each sample and incubate 16–18 h to remove the melted agarose in the MES solution.

Note: The β-agarase digestion time can be as short as 4 h.

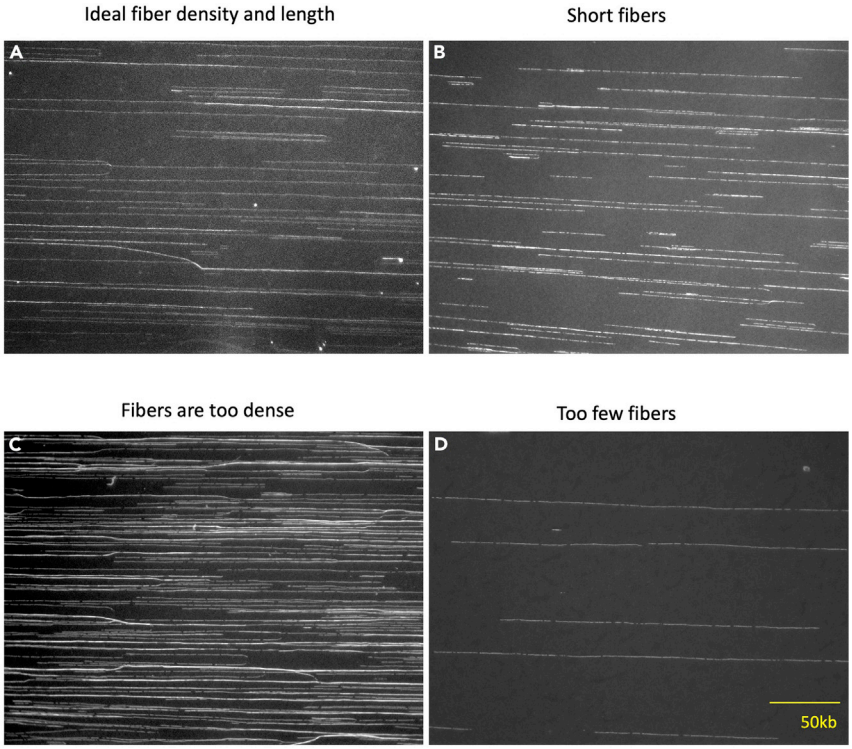


Figure 1. DNA fibers stained by YOYO-1

DNA purified in agarose plugs was stretched on silanized coverslips, stained with YOYO-1 for 1 min. DNA was visualized by an Olympus fluorescent microscope with a 60× objective lens to evaluate fiber density.
(A–D) Representative images with appropriate and unsatisfactory fibers are shown.

DNA combing and immunodetection-day 3 - day 4

Timing: about 8–24 h

In this major step, we first comb the genomic DNA in MES solution onto a silanized coverslip by a combing machine, then check DNA fiber quality by YOYO-1 staining. If DNA fibers are sufficiently long, we continue the immunostaining procedure to detect CldU, IdU and single-strand DNA to trace replication signals and DNA fibers.

9. Carefully pour the DNA solution from the 2 mL Eppendorf tube into a Teflon reservoir, which can hold about 1.7 mL solution. A little residual solution in the tube that is hard to pour out is OK.

   Note: To avoid DNA shearing, don't use pipetting to transfer DNA.

10. DNA is combed onto a silanized coverslip by a combing machine (see the combing machine picture in the materials and equipment section). Silanized coverslips held by the coverslip holders slowly dip into the Teflon reservoirs containing the DNA solution and incubate for 1–2 min, then slowly pull it out.
11. Stain the coverslip with YOYO-1 by dropping the coverslips into YOYO-1 solution and incubate for 1 min. Check the length of DNA fibers under a fluorescent microscope (most fibers should be >300 kb) and verify that DNA fiber density is appropriate (see Figure 1).

    Note: We use a Coulter counter analyzer sample cup to hold the 10 mL YOYO-1 solution. The coverslip can be dropped in vertically (standing, to facilitate removal), and just submerged (see picture below; the red tape on the coverslip is a label for easy visualization). Other small cups/bottles with similar diameter are fine.

YOYO-1 staining: a coverslip combed with DNA is dropped into YOYO-1 solution.

Note: Since the DNA solutions for all samples in the same experiment are prepared concomitantly under the same conditions from plating the cells to preparing the DNA solutions, and the same number of cells are used to make plugs, the DNA concentration of all samples should be very similar. It's not necessary to perform YOYO-1 staining for all the samples. You may randomly choose one sample's DNA solution to comb a coverslip and stain with YOYO-1 to check the DNA density and length. This will save time and coverslips.

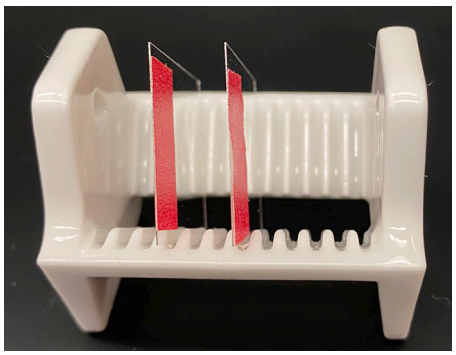
Note: Optimizing the length of coverslip incubation with the DNA solution (step 10) somewhat helps to improve DNA fiber density. Increase the incubation time to increase density and vice versa. Repeating step 10 also helps to increase fiber density.

Note: If needed, it is possible to increase fiber density by combining up to two plugs in step 7 or to decrease fiber density by using half a plug or diluting the DNA with 0.1 M MES. The best way to control DNA fiber density on the coverslip is to use a consistent and accurate number of cells in step 3.

12. If fiber density and length are both appropriate, comb a new silanized coverslip for each sample, put the coverslips in a glass staining rack, and bake for 2 h at 60°C to crosslink DNA to the coverslips.

Note: Repeat the combing step to obtain additional combed coverslips per sample as needed.

13. DNA denaturation.
    a. Pour about 150 of 0.5 N NaOH to a 200 mL glass beaker.
    b. Put the cover glass staining rack holding the combed coverslips (see picture below, the red tape on coverslip is just for easy visualization) into the beaker with NaOH. The coverslips should be submerged in the 0.5 N NaOH solution and incubate for 20 min with gentle rocking.



14. Wash the coverslips with PBS to neutralize the NaOH for 1 min, 5 times by transferring the glass staining rack with coverslips to a new beaker containing fresh PBS.

Note: Never put the rack with coverslips into a beaker before pouring PBS over the coverslips.

15. For blocking, incubate coverslips in a wet chamber with 25 μL of 5% BSA in PBS for 10 min at 20°C–25°C.

Note: All the blocking and antibody incubations are performed by adding 25 μL of blocking agents/antibodies on the glass slides, then putting coverslips, DNA side down, on the glass slides to form a sandwich. One glass slide can host 2 coverslips.

⚠ CRITICAL: To avoid a high staining background, like all the immunofluorescent staining experiments, never let the coverslips dry before completing the last wash at the end of the immunostaining.

16. Prepare the primary antibody solution: mouse anti-BrdU antibody for IdU, 1:20; rat anti-BrdU antibody for CldU, 1:100 in PBS with 5% BSA. Incubate 16–18 h at 4°C in a wet chamber.

Note: As for the blocking step, use 25 μl the above diluted antibodies per coverslip to form sandwiches between coverslips and glass slides.

Pause point: This step is considered as a pause point. The primary antibody step can be performed for 1–2 h at 20°C–25°C if you plan to complete your experiment from combing to immunodetection in one day.

17. Wash.
    a. Put the coverslip staining into a beaker containing about 150 mL PBS/T.
    b. Transfer the coverslips to the coverslip staining rack quickly once removed from the glass slides to avoid coverslip drying. Leave the beaker on a shaker with gentle shaking for 5 min.
    c. Transfer the coverslips on the staining rack to a new beaker with 150 mL PBS/T and leave the beaker on a shaker with gentle shaking for 5 min.
    d. Repeat one more time.
18. Prepare second antibody solution: goat anti-rat cy5, 1:100; goat anti-mouse cy3, 1:100 in PBS with 5% BSA. Incubate coverslips with the secondary antibodies for 45 min to 1 h at 20°C–25°C in a wet chamber.

⚠ CRITICAL: The fluorophores for the secondary antibodies here are selected for the Genomic Vision scanner. You may choose the fluorescent dyes for all the second antibodies according to the filters on your microscope.

19. Wash with PBS/T for 5 min × 3 times as above for primary antibodies (step 17).
20. Prepare mouse anti-single stranded DNA antibody solution by 1:200 dilution of the antibody in PBS with 5% BSA. Incubate coverslips with the diluted mouse anti-single stranded DNA antibody in a wet chamber for 1–2 h at 20°C–25°C.

Note: It is advantageous to use 2 secondary antibodies prepared from 2 different anti-mouse IgG subclasses and conjugated with 2 distinct fluorescent dyes matching your microscope (for example, Alexa Fluor 488 conjugated goat anti-mouse IgG1 for IdU (# A-21121, Invitrogen) and Alexa Fluor 647 conjugated goat oat anti-mouse IgG2a for anti-single-stranded DNA (# A-21241, Invitrogen)) This will allow the user to perform 2 rounds antibody incubations with coverslips instead of 4 rounds as above. With secondary antibodies from distinct subclasses, all the primary antibodies (anti-IdU, CldU, and ssDNA) can be mixed in one tube and

incubated with coverslips. After the PBS/T wash, all the secondary antibodies can be mixed in one tube and incubated with coverslips.

21. Wash for 5 min × 3 times with PBS/T.
22. Dilute goat anti-mouse (Brilliant Violet 480) in PBS with 5% BSA, 1:50, incubate for 45–60 min at 20°C–25°C in a wet chamber.
23. Wash for 5 min × 3 times with PBS/T.
24. Rinse slide with water and dehydrate the coverslips sequentially in 70%, 90% and 100% ethanol, 2 min each concentration and air dry.
25. Attach coverslips to coverslip carriers and scan the coverslips.

Note: The last 2 steps are for the Genomic Vision scanner only. For other microscopes, add mounting medium to mount the slide and visualize using fluorescent imaging after step 23.

EXPECTED OUTCOMES

DNA fibers visualized by YOYO-1 staining is an important step to check DNA fiber quality and density. Figure 1 shows examples of an appropriate DNA fiber density and length, where most visible fibers are single DNA fibers (not DNA bundles) and are longer than 300 kb (A). B-D are examples of inappropriate quality: too short (B), density too high (C), or too low (D). Short DNA fibers (B) make it hard to find intact replication fork signals and detect multiple replication origin signals on the same fiber. An excessively high DNA fiber density (C) will result in an increased prevalence of DNA fiber bundles, which render it hard to determine whether multiple replication signals reside on the same fiber. Sparse DNA fibers (D) make it hard or impossible to obtain sufficient replication signals to draw conclusions from the experiment.

As shown in Figure 2, a representative image with DNA replication signals by DNA combing, a good combing image should have: 1, many, but not too dense, long, straight, individual DNA fibers (blue). 2, replication signals that are sufficiently bright for CldU (red) and IdU (green), allowing the user to trace replication forks.

QUANTIFICATION AND STATISTICAL ANALYSIS

We use a commercial software named FiberStudio that came with the molecular combing platform from Genomic Vision (http://www.genomicvision.com/products/molecular-combing-platform) to

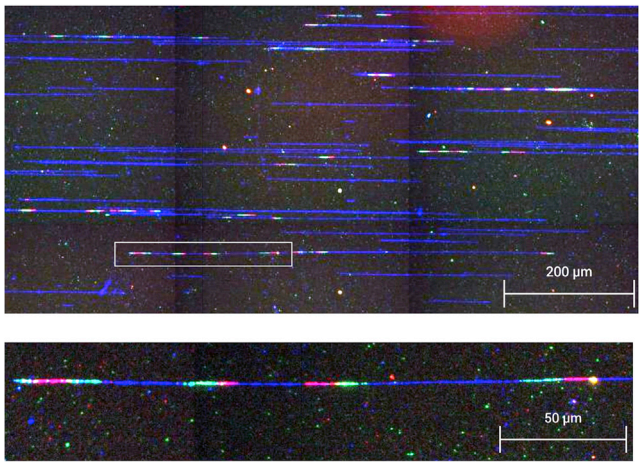


Figure 2. A representative DNA combing image showing replication signals

HCT116 cells were labeled with CldU (red) for 20 min, then IdU (green) for 20 min. DNA fibers were detected by anti-single strand DNA antibody (blue). A screenshot of a small part of one big combing image generated by the FiberStudio. 1 µm=2 kb.

measure fork rates, inter-origin distances and fork stability. FiberStudio has a detailed manufacturer manual that is designed to be followed in front of a running FiberStudio program, hence we don't describe the details here. Users can operate the program on their own personal computer, but only one person can access it at a time for each account. The program locates fibers, identify replication signals (origins, terminations, ongoing forks, etc., but because of frequently inaccuracies users need to validate every signal. The validated signals (Region of interest, ROI) can be exported as excel file for statistical analysis. We usually perform statistical analyses and render graphs from combing data in Prism (GraphPad, https://www.graphpad.com/scientific-software/prism/). To determine statistical significance, since the distributions of fork rate and origin distance are not normal, we use the Mann-Whitney test.

Alternatively, fibers can be quantified and analyzed using ImageJ, a free software that can measure replication tracks and determine fork rates, inter-origin distances (involving some additional manual calculations) and replication fork stability. ImageJ can also measure the total length of DNA fibers to calculate replication forks/Mb DNA ratios.

When you open an image in ImageJ, change the image by pressing "+" or "-" key to render the real size of the image (100%). Change image brightness and contrast as needed. Select the "Point" tool (if "multi-Point" tool is shown, just click and hold it, an option to switch to "Point" tool will appear), then click the left side of a replication signal/DNA fiber to mark the start point. Hold the "Shift" key and click the right of the same signal to mark end of the signal. You may hold the "Shift" key and keep clicking to mark many signals. After marking, click "Measure" under the "Analyze" Menu (short key on Mac: ⌘M). You should see the result window with all the numbers, and copy all to Excel, and label "G" for green (IdU) or "R" for red (CldU). If many green and red signal on the same single fiber, label gaps between the green and red replicating signals as "-" or "gap". Leave an empty roll in excel between fibers to indicate the signals are from different fibers, which is important for inter-origin distance calculation. The important parameters are the X and Y for signal coordinates (which can help you find the originals signals later) and the length of you signals (=X(n+1)-Xn). Signal intensity is also shown but it is not useful for this analysis. After collecting enough signals, group all the valid green and red signals (see Figure 3) to calculate replication fork speed. Also, according to the pattern of "G", "R" and "-", calculate origin distance (see example below). The unit of the signal length here is pixel. You need the information from your microscope to convert it to Micrometer. 1 Micrometer=2 kb. As stated above, we transfer data to Prism for the statistical analysis and graph generation.

LIMITATIONS

Due to the weak signals of the single-molecule DNA fibers, computer software-based automatic identification of replication signals can be imprecise. In many cases, manual analysis or validation

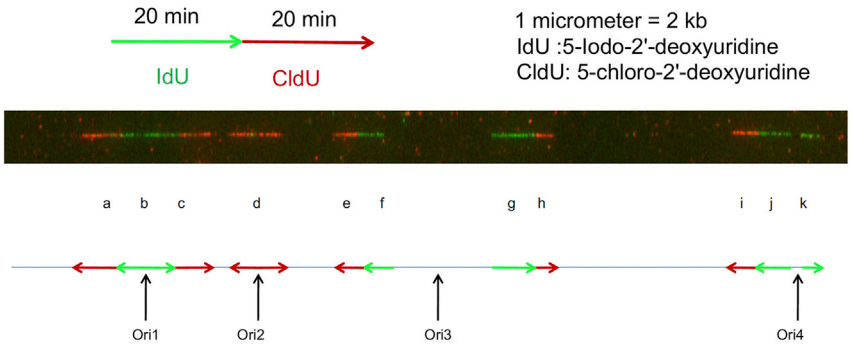


Figure 3. DNA replication analysis for fork speed and inter-origin distance by DNA combing

Replication track patterns for typical origins and replication tracks that can (a, c, e, f, g, h, I and j) and cannot (b and d due to replication timing is not known and k, a broken fork at the fiber end) be used for replication fork calculation are shown.

is required for each signal identified by computer programs, making this technique unsuitable for high throughput applications. Due to the limited number of events analyzed, elimination of subjective signal selection is the key to obtaining reliable and reproducible results. The FiberStudio program from Genomic Vision can help prevent subjective bias by employing random pop-up signals, and double-blind quantification and validation should ideally be performed.

The minimum fluorescent track length required to be identified and measured is about 4 μm (8 kb) and the Median range DNA fiber length stretched by this protocol is around 400–500 kb. Based on this resolution and fiber length limit, this protocol can detect the inter-origin distance (distances between 2 adjacent origins) between 20 kb and 500 kb. It should be noted, however, that fibers that are longer than 500 kb are rare, and therefore, inter-origin distances that are longer than 500 kb could inherently be under-counted. Because fiber lengths vary among experiments and the distribution of inter-origin distances can vary with the average fiber length, we suggest comparing inter-origin distance among samples prepared and visualized within the same experiment. It is also important to measure and record the distribution of fiber lengths among samples. This precaution is especially critical when comparing fibers from cells undergoing treatments that could cause DNA damage and cell death. If variations in fiber lengths are detected within a single experiment, the frequency of replication fork on measured per Mb of fibers is a good alternative to evaluate replication initiation frequency.

TROUBLESHOOTING

Problem 1

Short DNA fibers (step 11 and Figure 1B).

Potential solution

Short DNA fibers could result either from mechanical shearing or from chemical/enzymatic DNA degradation. Mechanical shearing can be avoided by handing samples gently as mentioned in steps 7–9. To prevent enzymatic DNA degradation, it is essential to ensure that all reagents, solutions and containers that come in contact with plugs and DNA solutions are DNase free (for example, by autoclaving). Incomplete proteinase K digestion in the lysis buffer and TE wash could also cause DNA degradation because residual DNase is left. If necessary, repeat the proteinase lysis step. Healthy cells are the key to longer DNA fiber. Because prolonged exposure of cells to trypsin can lead to cell death and subsequent DNA degradation, it's critical to stop the trypsinization with a cold medium containing FBS as soon as possible once cells are detached.

Problem 2

Dense DNA fibers (step 11 and Figure 1C).

Potential solution

Dense DNA leads to multiple DNA bundles on slides, and/or cause DNA fibers to be too close to each other and allow the user to identify individual molecules. When DNA fibers are bundled or too dense, it's hard or impossible to evaluate if multiple replication signals reside on a single DNA fiber or on 2 or more DNA fibers that bundle together. Identifying individual fibers is critical for obtaining reliable results, especially for measuring inter-origin distances. Using the recommended optimal cell number in each plug is a good solution to this problem. For polyploid cancer cells with increased DNA content, decrease the cell number used for each plug. Diluting recovered DNA with MES can also reduce fiber density but may result in shorter DNA fibers. Leaving the recovered DNA in MES solution at 4°C 16–18 h can help resolve some bundle problems.

Problem 3

Very few, or no, DNA fibers (step 11 and Figure 1D).

Potential solution

It is critical to count cells before mixing with the agarose. Also, ensure that the cells are mixed thoroughly with agarose to obtain homogeneous plugs. Use a thermometer to double check the temperature used to melt plugs, especially when using a heat block. Ensure that the agarose plugs are completely melted to let all DNA be released into the MES solution.

Problem 4

IdU, CldU, or ssDNA staining produces weak or no signals (see Figure 4A, for missing green replication tracks due to a bad IdU antibody, Figure 4B for very weak ssDNA staining due to a bad second antibody).

Potential solution

Check that IdU, CldU can incorporate into cells and that all 3 antibodies can produce detectable signals when used in immunofluorescence staining on fixed cells.

Silanized coverslips quality and age of the coverslips may affect the results. The coverslips baking and denaturing conditions in this protocol are based on the engraved Combicoverslips from Genomic Vision. For other sources of coverslip, the 60°C baking time and/or denaturation conditions might need to be optimized.

For secondary antibodies conjugated with Alexa 647, we find that the mounting medium Prolong antifade mountant (#Cat P36934, ThermoFisher) results in brighter signals.

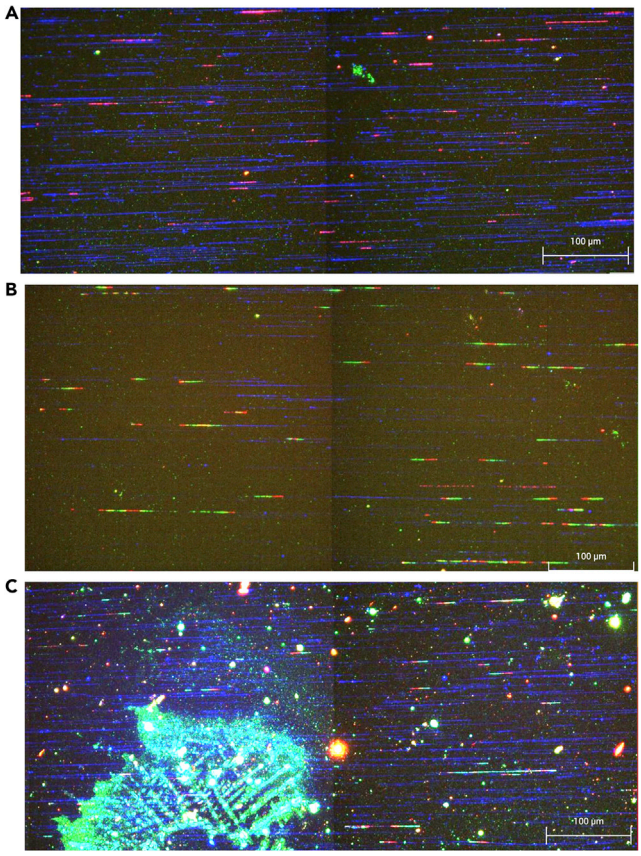


Figure 4. Examples of unsatisfactory combing and immnostaining

(A–C) Illustrative images showing missing IdU-green replication tracks (A), very weak single stranded DNA (ssDNA) staining (B) and high background (C).

Problem 5

Dirty/high background images (steps 15–23 and Figure 4C).

Potential solution

Following the wash steps detailed above is usually sufficient for obtaining low background, clean slides. However, high background can result from dried coverslips. Because there is only a very thin layer of buffer on each coverslip, it is critical to ensure that all incubation steps are performed in a well-sealed wet box. When transferring coverslips during incubations and washes, especially when a coverslip is removed from a slide, transfer it as quickly as possible to the PBS/T wash buffer.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, [Mirit I. Aladjem] (aladjemm@mail.nih.gov).

Materials availability

This study did not generate new unique reagents

Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

This study was supported by the Intramural Research Program of the NIH, Center for Cancer Research, National Cancer Institute, United States (project ZIA BC010411 to M.I.A.).

AUTHOR CONTRIBUTIONS

H.F. developed the protocol and wrote the original draft. M.I.A. commented on the protocol and edited the draft.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Conti, C., Sacca, B., Herrick, J., Lalou, C., Pommier, Y., and Bensimon, A. (2007). Replication fork velocities at adjacent replication origins are coordinately modified during DNA replication in human cells. Mol. Biol. Cell *18*, 3059–3067.

Fu, H., Redon, C.E., Thakur, B.L., Utani, K., Sebastian, R., Jang, S.M., Gross, J.M., Mosavarpour, S., Marks, A.B., Zhuang, S.Z., et al. (2021). Dynamics of replication origin over-activation. Nat. Commun. *12*, 3448.

Kaykov, A., Taillefumier, T., Bensimon, A., and Nurse, P. (2016). Molecular combing of single DNA molecules on the 10 megabase scale. Sci. Rep. *6*, 19636.

Redmond, C.J., Fu, H., Aladjem, M.I., and Mcbride, A.A. (2018). Human papillomavirus integration: analysis by molecular combing and fiber-FISH. Curr. Protoc. Microbiol. *51*, e61.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods *9*, 671–675.

Schwob, E., De Renty, C., Coulon, V., Gostan, T., Boyer, C., Camet-Gabut, L., and Amato, C. (2009). Use of DNA combing for studying DNA replication in vivo in yeast and mammalian cells. Methods Mol. Biol. *521*, 673–687.