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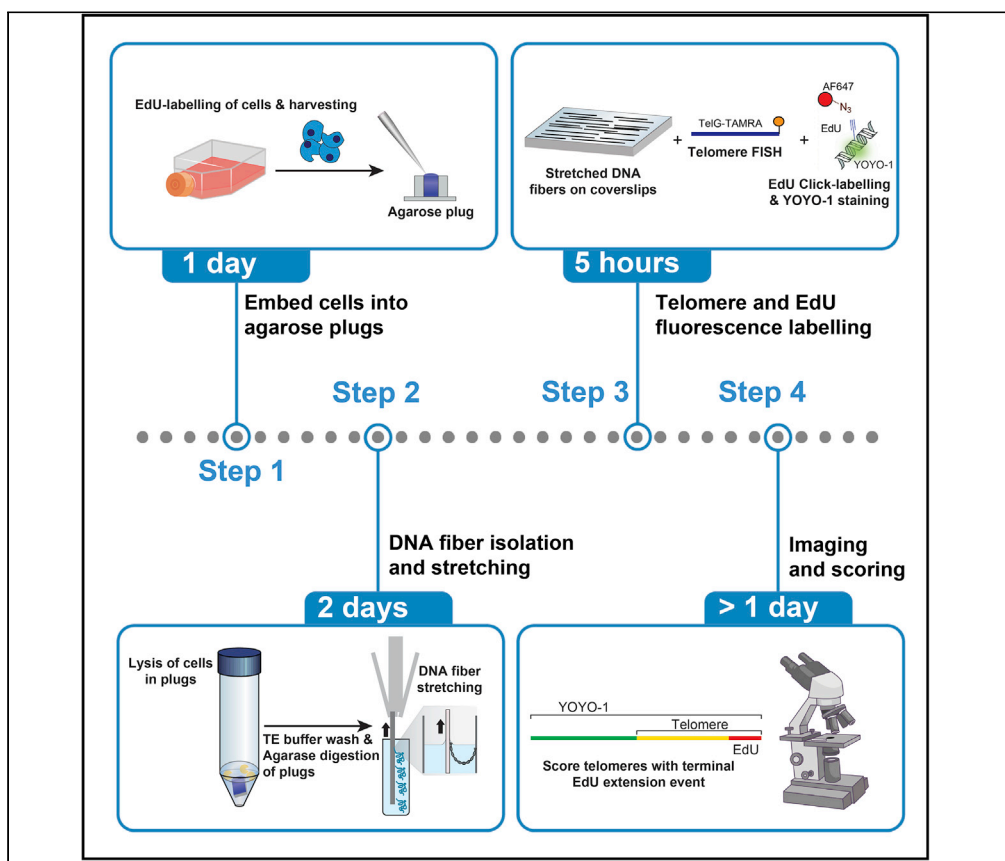
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Protocol

A DNA-fiber protocol for single molecule analysis of telomere (SMAT) length and extension events in cancer cells



Alternative lengthening of telomeres (ALT) is a homologous recombination-based telomere maintenance mechanism. It is active in approximately 10–15% of cancers. We present a DNA-fiber protocol, combining YOYO-1 staining of genomic DNA, telomere fluorescence *in situ* hybridization (FISH), and EdU labeling of nascent DNA, to measure telomere extension events in ALT cancer cells. The protocol can be used to delineate ALT-mediated telomere extension.

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Highlights
DNA fiber microscopy for measuring telomere extension events in ALT cancer cells

DNA fibers are stretched uniformly using a constant stretching factor

Delineates terminal from nonterminal EdU tracts at telomeres on DNA fibers

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Protocol

A DNA-fiber protocol for single molecule analysis of telomere (SMAT) length and extension events in cancer cells

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SUMMARY

Alternative lengthening of telomeres (ALT) is a homologous recombination-based telomere maintenance mechanism. It is active in approximately 10–15% of cancers. We present a DNA-fiber protocol, combining YOYO-1 staining of genomic DNA, telomere fluorescence *in situ* hybridization (FISH), and EdU labeling of nascent DNA, to measure telomere extension events in ALT cancer cells. The protocol can be used to delineate ALT-mediated telomere extension.

For complete details on the use and execution of this protocol, please refer to Barroso-Gonzalez et al. (2021).

BEFORE YOU BEGIN

⌚ Timing: 1 day

The protocol described here takes several days (including “before you begin”) to complete, and involves EdU labeling cells in culture, embedding harvested cells in agarose plugs, isolating and stretching DNA fibers onto coverslips, and detecting telomeres and nascent telomere synthesis at chromosome termini. This protocol can be used simultaneously to measure telomere length, the frequency of telomere extension events, and the length of telomere extension events, all at the single molecule level. Finally, we explain the steps required for imaging on a standard epi-fluorescence microscope, specifically the Zeiss Axio Imager.Z2, and image analysis using ZEN Lite imaging software.

Note: We use U-2 OS cells as a model cell line for telomere extension. The choice of cell line will have a major impact on ease of analysis. Cell lines with long telomeres are considerably easier to score, due to a minimum telomere size resolution of ~0.5 μm limited by fluorescence microscopy.

We recommend preparation of working solutions as well as “materials and equipment” in advance. All buffers are stored at 20°C–25°C, unless otherwise indicated. All enzymes used in this protocol are stored at –20°C for no longer than a year.

Preparation of equipment, solutions, and chemicals prior to day 1

1. Prepare a 10 mM stock solution of EdU in DMSO:
 - a. Dissolve EdU powder in prewarmed DMSO and incubate at 37°C in a water bath for 5 min in the dark, with occasional vortexing. Check it is fully dissolved before aliquoting.
 - b. Make ~50–100 μL aliquots and store all stocks at –20°C in the dark.
 - c. For use on the day of SMAT, quick thaw at 37°C.



Note: EdU preparation should be performed in a Class II biosafety cabinet with the light off to minimize exposure of EdU to light.

2. Prepare EDTA-Sarcosyl-Proteinase (ESP) buffer fresh from component stocks: 0.5 M sodium EDTA (pH 8), 10% (w/v) Sarcosyl/0.5 M EDTA, 20 mg/mL Proteinase K (see recipe in “[materials and equipment](#)” for preparation and concentration of components).

Note: Nuclease-free Eppendorf tubes and Milli-Q water (MQW) must be used for making all solutions.

3. Prepare a stock of 1.2% (w/v) low melting point (LMP) agarose.
 - a. Dissolve 1.2 g in 100 mL MQW and boil in a microwave until molten and agarose is completely dissolved.
 - b. Aliquot into separate 15 mL falcon tubes (~ 1–5 mL/tube).
 - c. Chill in fridge for 16 h after preparation until agarose is completely set.

Note: Care should be taken in the handling of superheated agarose. Follow safety procedures and use thermo-protective gloves.

4. Ensure there is sufficient Agarase (0.5 U/μL) for all samples. 2 μL is required for each sample, plus 20% extra to account for pipetting loss.
5. Sterilize 2 mL and 0.5 mL polypropylene microtubes (nuclease-free) by autoclaving.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
EdU (5-Ethynyl-2'-deoxyuridine) – store at –20°C	Thermo Fisher Scientific	Component of Click-iT™ Plus EdU Cell Proliferation Kit (Cat #C10640)
Tween-20	Sigma-Aldrich	Cat #P9416
Sodium azide	Sigma-Aldrich	Cat #S2002
NaCl	Sigma-Aldrich	Cat # S3014
Tris base	ChemSupply	Cat #TA034
1 M Na ₂ HPO ₄ dihydrate	Sigma-Aldrich	Cat #71643
Citric acid – store at 4°C	Sigma-Aldrich	Cat #791725
Maleic acid	Sigma-Aldrich	Cat # M0375
MgCl ₂	Sigma-Aldrich	Cat #M8266
NaOH (pellets)	Sigma-Aldrich	Cat #S5881
Sodium EDTA dihydrate	ChemSupply	Cat #EA023
Triton X-100	Sigma-Aldrich	Cat #T8787
Tween-20	Sigma-Aldrich	Cat #P9416
Formaldehyde solution about 37% (methanol-stabilized)	Sigma-Aldrich	Cat #104003
N-lauroylsarcosine sodium salt	Sigma-Aldrich	Cat #61743
Low-melting point (low-gelling temperature) agarose	Sigma-Aldrich	Cat #A9414
Fish gelatin (gelatin from cold water fish skin 40%–50% in H ₂ O) – store at 4°C	Sigma-Aldrich	Cat #G7765
Ethanol (absolute for analysis) >99.8%	Sigma-Aldrich - Supelco	Cat #1.00983
Bovine serum albumin (BSA) Fraction V – store at 4°C	Roche	Cat #10735086001
Proteinase K (20 mg/mL) – store at –20°C	Roche	Cat #03115852001
1 mM YOYO-1 iodide – store at –20°C	Invitrogen	Cat #Y3601
Agarase (0.5 U/μL) – store at –20°C	Thermo Fisher Scientific	Cat #EO0461
Formamide (deionized) – store at 4°C	Thermo Fisher Scientific	Cat #AM9342
Formamide (ACS reagent) – store at RT	Sigma-Aldrich	Cat #221198-1L

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MES, free acid, ULTROL® Grade	Millipore	Cat #475893
Blocking Reagent – store at –20°C	Roche	Cat #11096176001
Prolong Gold antifade mountant	Thermo Fisher Scientific	Cat #P10144
Critical commercial assays		
Click-iT™ Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 dye	Thermo Fisher Scientific	Cat #C10640 https://www.thermofisher.com/order/catalog/product/C10640?SID=srch-hj-C10640
FiberComb® molecular combing starter pack – includes machine, 10x reservoirs and reservoir bench holder	Genomic Vision	Cat #MCS-001-S https://store.genomicvision.com/equipment/22-fibercomb-molecular-combing-system.html
Experimental models: Cell lines		
U-2 OS cells	ATCC	HTB-96 RRID: CVCL_0042
Oligonucleotides		
TelG-TAMRA probe: TAMRA-00-(TTAGGG) ₃ PNA probe – store at –20°C	Panagene	Cat #F2002
Software and algorithms		
ZEN Blue lite v3.1	Zeiss	RRID:SCR_013672 https://www.zeiss.com/microscopy/int/products/microscope-software/ZEN-lite.html
Microsoft Office Excel	Microsoft	RRID:SCR_016137 https://www.microsoft.com/en-au/
Other		
Box of engraved vinyl silanized coverslips (x50)	Genomic Vision	Cat #COV-002-RUO https://store.genomicvision.com/consumables/23-box-of-combicoverslips-50-coverslips.html
Wash-N-Dry™ coverslip rack (holds 10 coverslips)	Sigma-Aldrich	Cat #7688568
Dark humidifying chamber (hand-made with foil wrapping)	N/A	N/A
Disposable plug mold (10-well)	Bio-Rad	Cat #170-3713
Round-headed tweezers	Sigma-Aldrich	Cat #Z680214
Micro spatula	Sigma-Aldrich	Cat #Z193216
Bottle top filter 295–4545 500 mL 0.45 μm GL45	Nalgene	Cat #1-7350-08
0.45 μm syringe filter	Agilent	Cat #5190-5274
Fan-forced oven that can go up to 60°C. If it has glass windows, cover them with aluminum foil.	N/A	N/A
Heating block	N/A	N/A
Heated water bath	N/A	N/A
Benchtop minicentrifuge for spinning microtubes	N/A	N/A
Beckman Coulter Counter Z1 or any other cell counter	Beckman Coulter	N/A
Aluminum foil	N/A	N/A
Gauze swabs	BSN Medical	Code No. 36361350
Orbital shaker	N/A	N/A
Microscopy slide folder	N/A	N/A
Crystal violet (powder)	Sigma-Aldrich	Cat #C6158

MATERIALS AND EQUIPMENT

Alternatives: Critical to this protocol is that we stretch DNA fibers with a constant stretching factor (for instance 2 kb/μm). We recommend using the FiberComb® Molecular Combing System (Genomic Vision). The FiberComb® immerses coverslips in reservoirs containing the DNA fiber solution for ~2 min, then steadily draws them out over ~3 min to achieve a constant stretching factor of 2 kb/μm, for accurate quantitation of fiber length measurements. Other mechanical systems can also be assembled to create a constant stretching factor. DNA fiber stretching from a harvested cell pellet can be outsourced to Genomic Vision. An alternative to

mechanical stretching of DNA fibers is to utilize the common method of gravity-assisted DNA spreading of lysed cells. This is used for single-molecule analysis or replicating DNA (SMARD) and can be performed directly on cells attached to slides, requiring fewer cells (2,000 cells) (Bai et al., 2020; Jackson and Pombo, 1998). The DNA spreading method is less robust and more user dependent. As a result, we do not recommend comparing telomere extension quantitation scored from separate experiments using the DNA spreading method.

Note: Reservoirs can be re-used by discarding old fiber solution and washing with water to remove any dried salts. Gently agitate reservoirs and their rubber lids in 1% sodium hypochlorite or RelyOn™Virkon™ (Med-con #500607) for at least 2 h to destroy any DNA, rinse thoroughly to remove any residue, then wash finally with deionized water. Reservoirs can then be air-dried in a fan-forced oven for at least 24 h prior to use (do not autoclave).

Alternatives: This protocol uses 22×22 mm² Combicoverslips, which are vinyl silane coated coverslips for stretching DNA fibers. Combicoverslips are commercially available from Genomic Vision (key resources table) and are engraved with an alpha-numeric sequence on one face to distinguish samples and orientation. As a cost-effective alternative, glass coverslips could be treated with vinyl silane solution (Sigma, Cat #T5051) in-house and etched with a diamond tipped pencil to indicate identity and orientation.

Alternatives: This protocol uses the Click-iT Plus EdU labeling kit (Thermo Fisher Scientific) for convenient and robust labeling of EdU incorporation into DNA fibers. Kit components (including sodium ascorbate, Alexa Fluor 647 picolyl azide and copper protectant) can be purchased separately from standard suppliers e.g., Sigma, ThermoFisher Scientific or Jena Bioscience (<https://www.jenabioscience.com/click-chemistry/click-reagents-by-chemistry/auxiliary-cu-i-click-reagents/clk-067-bttaa> and <https://www.jenabioscience.com/images/PDF/CLK-071.0001.pdf>).

ZEN Blue lite v3.1 microscopy imaging software: This protocol uses ZEN Blue lite software for analysis of Zeiss microscopy files (".czi" format). It can be downloaded free after email registration and allows for viewing, toggling fluorescence channels on and off, adjusting images while viewing, and editing measurements or marking images during analysis, that other freeware software cannot do as well. Additionally, the software uses an image pyramid to allow zooming in and out on a large, tiled image. For further information on how to use ZEN - https://asset-downloads.zeiss.com/catalogs/download/mic/8db1eb8d-7b2a-46e8-8427-f259dcfd1fb3/EN_quick-guide_ZEN-blue-edition_first-steps.pdf.

10% (w/v) sarcosyl/0.5 M EDTA solution

Reagent	Final concentration	Amount
sodium EDTA pH 8 (0.5 M)	0.5 M	100 mL
N-lauroylsarcosine sodium salt	10% (w/v)	10 g
Total		100 mL

Filter, using vacuum, through a 0.45 μm bottle top filter after making buffer to remove insoluble components.

EDTA-Sarcosyl-proteinase (ESP) buffer

Reagent	Final concentration	Amount
sodium EDTA pH 8 (0.5 M)	0.36 M	200 μL
10% (w/v) sarcosyl/0.5 M EDTA solution	0.9% (v/v)	25 μL
Proteinase K (20 mg/mL)	3.6 mg/mL	50 μL
Total		275 μL

- Dissolve Proteinase K in nuclease-free water from lyophilized powder at RT for 10 min. Aliquot into 500 μ L volumes and store at -20°C .
- Prepare 0.5 M EDTA from sodium EDTA salt. 0.5 M EDTA is difficult to dissolve. Add concentrated NaOH to pH 8 for dissolution.
- Prepare 10% (w/v) Sarcosyl/0.5 M EDTA by dissolving 10 g of sodium N-lauryl-sarcosine in 100 mL of 0.5 M EDTA.
- 0.5 M EDTA and 10% Sarcosyl/EDTA solution can be stored at RT.

Filter, using vacuum, through a 0.45 μm bottle top filter after making buffer to remove insoluble components.

△ CRITICAL: Add the ingredients in the order listed in the table.

TE wash buffer, pH 8		
Reagent	Final concentration	Amount
1 M Tris HCl pH 8	10 mM	10 mL
0.5 M EDTA pH 8	1 mM	2 mL
Milli-Q water	N/A	988 mL
Total	N/A	1 L

Store at RT

Antibody dilution buffer (ABDIL)		
Reagent	Final concentration	Amount
1 M Tris pH 7.5	20 mM	5 mL
BSA	2% (w/v) BSA	5 g
45% Fish gelatin from cold water fish skin	0.2% (v/v)	1.11 mL
5 M NaCl	150 mM	7.5 mL
Triton-X 100	0.1% (v/v)	250 μ L
2% (w/v) Sodium azide	0.1% (v/v)	12.5 mL
Milli-Q water	N/A	up to 250 mL
Total		250 mL

Filter, using vacuum, through a 0.45 μm bottle top filter after making buffer to remove insoluble components. Store at 4°C for up to one year.

△ CRITICAL: do not use metallic objects to handle sodium azide. Prepare 2% from powder using a plastic spoon.

0.5 M MES, pH 5.5		
Reagent	Final concentration	Amount
MES, free acid, ULTROL® Grade	0.5 M	9.762 g
Milli-Q water	10% (w/v)	Initially 80 mL, then adjust to 100 mL after adding NaOH.
10 M NaOH and 1 M NaOH	N/A	Starting pH for 0.5 M MES solution is 3.23, adjust to pH 5.5. Start with 10 M NaOH then use 1 M NaOH dropwise to adjust.
Total		100 mL

Filter, using vacuum, through a 0.45 μm bottle top filter after making buffer to remove insoluble components. Store at 4°C for up to a month.

10 M NaOH stock is prepared by dissolving 200 g of NaOH pellets in 0.5 L of Milli-Q water. 1 M NaOH solution is then prepared by diluting the 10 M stock 1:10 with Milli-Q water.

Formamide hybridization buffer

Reagent	Final concentration	Amount
Formamide (deionized)	70% (v/v)	175 mL
10% Blocking Reagent	0.25%	6.25 mL
1 M Tris pH 7.5	10 mM	2.5 mL
1 M MgCl ₂	25 mM	6.25 mL
1 M Na ₂ HPO ₄ pH 7.5	82 mM	20.5 mL
Citric acid	9 mM	432.279 mg
Milli-Q water	N/A	39.5 mL
Total		250 mL

Dissolve Blocking Reagent powder in 0.1 M maleic acid buffer, pH 7.5. Prepare 0.1 M maleic acid buffer by adding maleic acid powder to Milli-Q water then adjusting with 1 M NaOH to pH 7.5. Prepare 10% (w/v) of Blocking Reagent by dissolving 1 g Blocking Reagent powder in 10 mL 0.1 M maleic acid buffer, pH 7.5, and vortex until completely dissolved.

Working stock of TAMRA-00-(TTAGGG)3 (TelG-TAMRA)

Reagent	Final concentration	Amount
TelG-TAMRA (25 ng/μL)	0.3 ng/μL	12 μL
Formamide hybridization buffer	N/A	988 μL
Total		1 mL

Filter through 0.45 μm syringe filter after making buffer to remove insoluble components. Store at 4°C for up to one year.

20× SSC buffer, pH 7

Reagent	Final concentration	Amount
NaCl	N/A	175.32 g
Sodium citrate dihydrate	N/A	88.23 g
Milli-Q water	N/A	Up to 1 L
Total		1 L

Add ~900 mL of MQW water. pH with a few drops of HCl to adjust to pH 7.

Filter, using vacuum, through a 0.45 μm bottle top filter after making buffer.

1× SSC/50% formamide wash buffer

Reagent	Final concentration	Amount
20× SSC	1× SSC	10 mL
Milli-Q water	N/A	90 mL
Formamide (ACS reagent, Sigma)	50% (v/v)	100 mL
Total	N/A	200 mL

Note: keep solution in the dark or wrapped in aluminum foil. Discard after 1 month at RT.

2× SSC/0.1% Tween wash buffer

Reagent	Final concentration	Amount
20× SSC	2× SSC	20 mL
Milli-Q water	N/A	179.6 mL
Tween-20 (Molecular biology grade, Sigma)	0.1% (v/v)	0.4 mL
Total	N/A	200 mL

Click-It & YOYO-1 staining master mix (CYMM)

Reagent	Number of coverslips		
	1× (μL)	4× (μL)	10× (μL)
1× Click-iT reaction buffer	132	528	1320
Copper protectant (Component E)	3	12	30
Alexa Fluor 647 (AF647) picolyl azide	0.36	1.44	3.6
Reaction buffer additive	15	60	150
YOYO-1 (1:100 dilution of 1 mM YOYO-1 stock in ABDIL)	1.5	6	15
Total volume	150	600	1500

Follow the manufacturer's instructions for preparation of Click-iT reaction buffer and reaction buffer additive components. Prepare master mix fresh.

△ CRITICAL: Add the ingredients in the order listed in the table. Use within 15 min of adding buffer additive.

Reaction buffer additive and YOYO-1 are stored at -20°C . All other reagents are stored at 4°C .

STEP-BY-STEP METHOD DETAILS

A schematic of this protocol is shown in the Graphical Abstract.

Cell harvesting and embedding in agarose plugs

⌚ Timing: 1–6 h

These steps describe the method of harvesting cells for SMAT such that each treatment replicate yields an agarose plug embedded with $1-2 \times 10^6$ cells. It then describes the process of embedding cells in agarose, followed by lysis and digestion with Proteinase K.

Note: Due to potential processing of labeled DNA by various endo/exo-nucleases, work quickly, avoid light exposure, and keep cells on ice prior to embedding in agarose when harvesting multiple samples.

EdU pulsing/labeling of cells

The number of cells required per treatment replicate ranges from $1-2 \times 10^6$. This protocol has been optimized for the U-2 OS adherent ALT cancer cell line but can also be used on non-adherent cells.

1. Culture asynchronous cells such that they are 50%–80% confluent on the day of EdU pulsing. This protocol can also be applied to treated cells, e.g., drug-treated, siRNA transfected, or synchronized cells, when pathway analysis or cell-cycle effects are being investigated.
2. Add 1:1,000 dilution of 10 mM EdU stock directly to the culture media, for a final concentration of $10 \mu\text{M}$, gently agitate flask to mix, then return the flask to the 37°C incubator for 1–5 h. The length of the EdU pulse is dependent on cell cycle duration and the temporal window of analysis in the case of synchronized cells. For U-2 OS, we pulse synchronized cells for 2 h and asynchronous cells for 5 h.

Note: Due to reports of EdU-induced toxicity in the literature, we performed an experiment whereby we compared untreated U-2 OS cells, U-2 OS cells labeled with EdU for 5 h or mock-treated with DMSO vehicle control, washed out and allowed for recover for 24 h or one cell-cycle. We report no significant impact of EdU labeling as shown in [Figure S1](#).

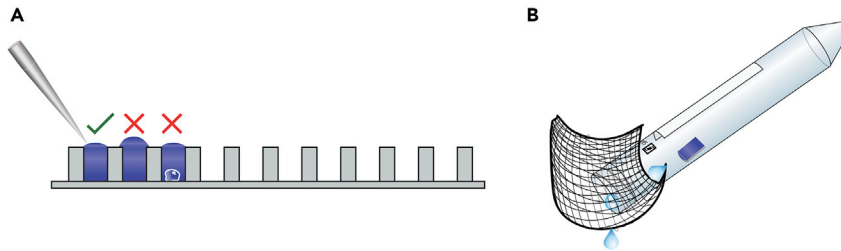


Figure 1. Embedding cells into agarose plugs and washing

(A) Preparation of agarose plugs. Pipette the cell suspension & LMP agarose mix into the plug mold as indicated. Do not overfill the wells or create bubbles.

(B) Graphic showing TE wash using a mesh gauze to prevent plugs from falling out.

- During EdU labeling, melt the aliquot of LMP agarose at 68°C in a water bath for 20–30 min. Transfer the molten LMP agarose to a 50°C water bath to hold in a molten state.

Note: Addition of EdU to cells should be performed in a Class II biosafety cabinet. Leftover EdU solution can be re-frozen. Discard EdU aliquot after 10 freeze-thaws.

- Harvesting cells:
 - Trypsinize or scrape the cells. Pellet the cells at 300 g and at RT for 5 min in a 15 mL falcon tube. This can be increased to 400 g for mitotic cells.
 - Resuspend cells in 10 mL of PBS and count cell number using a hemocytometer, or an automated cell or particle counter e.g., Beckman Coulter Counter Z1.
 - Distribute sufficient cells (in fresh falcon tubes if necessary) such that $1\text{--}2 \times 10^6$ cells are allocated to each plug. Extra tubes can be prepared if backup plugs are desired.
 - Pellet the cells at 300 g and at RT for 5 min then aspirate PBS from each sample, leaving behind $\sim 45 \mu\text{L}$ in each tube for each agarose plug. As a guide, this can be achieved by leaving $\sim 2 \text{ mm}$ of PBS above the cell pellet.

Note: Typically for U-2 OS cells, 80% confluency in a T75 flask yields three plugs.

△ CRITICAL: Loading too many cells in a plug can result in excessive fiber density, making it difficult to distinguish the telomere ends. Too few cells can lead to insufficient telomere ends being available for scoring.

- Immediately place the cell pellet on ice in the dark for up to 30 min, while harvesting the rest of the samples.
- Resuspend cell pellets using a P200 pipette on ice, then quickly warm the samples in the 50°C water bath for 10 s (5 s for mitotic cells). Immediately add $\sim 45 \mu\text{L}$ molten 1.2% LMP agarose to the sample, quickly pipette to mix.
- Immediately pipette each $\sim 90 \mu\text{L}$ cell and agarose mix into a plug mold (Figure 1A).
- Allow the plug to set for 30–40 min at 4°C before commencing the next step. For long-term storage of plugs, wrap plugs tightly in parafilm to avoid dehydration.

Note: Avoid overfilling plug molds with the cell-agarose mix. The tops of overfilled plugs can easily flake off and become lost during the TE wash steps (they are small enough to pass through gaps in the gauze) (Figure 1B).

Protein digestion in plugs and stretching DNA onto coverslips

⌚ Timing: 2 days 6 h

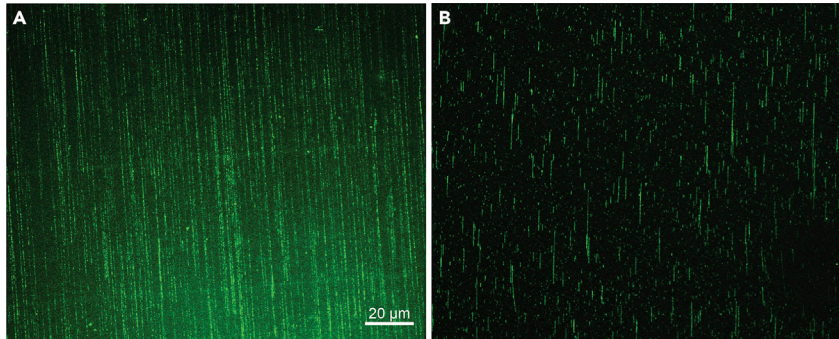


Figure 2. Example of YOYO-stained DNA fibers

(A) intact good-quality fibers and (B) sheared low-quality fibers. Scale bar 20 μm .

Cells embedded in plugs are lysed in a Proteinase K lysis buffer for 16 h and washed with TE buffer to remove proteins. Agarase digestion is then used to liberate the deproteinated DNA fibers from the plugs. Digestion is performed in 0.5 M MES pH 5.5, which enables strong binding of DNA to vinyl silane coated coverslips. DNA fibers are stretched onto vinyl silanized coverslips using a Molecular Combing System, and fixed by baking (Gallo et al., 2016).

9. Add 275 μL of ESP solution (made fresh on the day) to a fresh 15 mL falcon tube for each sample/plug. For each plug, add in the following order, mix, and centrifuge for 1 min at 300 g:
 - a. 200 μL of 0.5 M EDTA pH 8.0
 - b. 25 μL 10% sarcosyl/0.5 M EDTA
 - c. 50 μL 20 mg/mL Proteinase K
10. Remove the plastic seal backing on the plug mold. The plugs should remain in the molds and not fall out.
11. Using round-headed tweezers, gently push the solidified agarose plugs into each falcon tube containing ESP solution.
12. Gently flick the tube down such that all the plugs are completely immersed in ESP solution.
13. Incubate the tubes in a 50°C water bath, without agitation, in the dark for 16 h.

△ CRITICAL: After lysis of the cells followed by protein digestion, the DNA in the agarose plugs is vulnerable to shearing. Handle plugs carefully after Proteinase K digestion and do not shake or vortex. Sheared DNA fibers will negatively impact quantitation (Figure 2B).

14. The next day, check that the plugs are intact.
15. Discard the ESP buffer by carefully tilting the tube while ensuring the plug does not come out. It does not matter if some of the ESP buffer remains in the tube.
16. Wash plugs three times for 1 h each with TE buffer:
 - a. Fill each 15 mL tube (including plug) with TE buffer up to the top, wrap the tube in aluminum foil, and incubate for 1 h at 20°C–25°C in the dark with slow rotation on an orbital shaker. Do not use darkened tubes as you will need to check for the presence of the plug.
 - b. Discard TE wash, using a mesh gauze to prevent plugs from falling out (Figure 1B).
 - c. During each TE wash, examine each tube for the presence of the agarose plug. Plugs may be difficult to see but can be best visualized in natural light next to a window.

Note: Plugs are fragile. If they get stuck on the gauze, gently tap them off using the rim of the falcon tube. Alternatively, use fresh TE buffer to wash plugs off the gauze and back into the tube.

- d. Repeat TE buffer washes two more times incubating with rotation for 1 h at 20°C–25°C each time.

17. Wash for a fourth and final time with TE buffer for 3–4 h, then discard the wash.

▣ **Pause point:** Plugs can then be stored in TE buffer in the dark in a 4°C cold room for several months.

△ **CRITICAL:** Do not store plugs in a fridge, as vibrations may shear the DNA.

18. Melt agarose plugs to release the DNA:

- a. Set a heat block to 68°C.
- b. Add 1 mL of 0.5 M MES pH 5.5 solution to fresh 2 mL microtubes (nuclease-free).
- c. Use a micro sampling spatula with a bent end to gently transfer each plug from the falcon tube to the 2 mL microtube.
- d. Check that the plug is fully immersed in MES solution and incubate at 68°C for 20 min to melt the plug (covered with aluminum foil).
- e. Adjust the heat block temperature to 42°C and allow to equilibrate for 10 min.

19. In meantime, aliquot Agarase such that you have enough for 2 µL per sample plus 20% extra.

20. After the 10 min is up, warm the aliquoted Agarase for ~10 s on the heat block, then add 2 µL of Agarase to each sample and gently invert the microtube to mix (do not vortex).

21. Incubate on the heat block at 42°C for 16 h (covered with aluminum foil).

△ **CRITICAL:** The agarose plug must be completely melted into the MES solution or DNA fibers will clump on the coverslip when stretching the DNA fibers.

22. The next day, add 700 µL of MES buffer to fresh disposable reservoirs.

23. Remove the Agarase-digested samples from the 42°C heating block and allow to cool to 20°C–25°C.

24. Transfer Agarase-digested DNA fiber solution to disposable reservoirs by tipping.

25. Gently invert the disposable reservoirs to homogenize the DNA fiber solution.

26. Place the reservoirs in the reservoir bench holder.

▣ **Pause point:** DNA fiber solutions in MES buffer can be stored in the dark for up to a week at 20°C–25°C.

27. Stretch the DNA fibers onto coverslips (see [Video S1](#)):

- a. Place a reservoir into the FiberComb® machine receptacle. Load the vinyl silanized coverslip.
- b. Stretch the DNA fibers onto the coverslips (~ 5 min) using the FiberComb® machine.
- c. Carefully place the freshly prepared coverslips onto a coverslip rack holder

Note: Avoid vibrations or bumping the bench while the FiberComb® machine is operating.

Note: The FiberComb® machine should be regularly calibrated according to the manufacturer's instructions to ensure a constant stretching factor of 2 kb/µm is maintained. Perform a no-sample operating cycle (see [Video S1](#)) and check that the machine's soaking time is 5 min ± 10 s and the combing time is 1 min and 40 s ± 5 s. If the times differ from required, perform instrument maintenance as described in the instruction manual from Genomic Vision - https://store.genomicvision.com/index.php?controller=attachment&id_attachment=2.

28. Bake coverslips at 60°C in a fan-forced oven:

- a. For "[DNA-fiber quality check](#)", stretch DNA fibers from one reservoir onto an extra coverslip and bake for 1–2 h. We routinely perform a DNA-fiber quality check on one slide per experimental batch.
- b. For "[Telomere FISH probing, Click-iT EdU-labeling and YOYO-1 staining](#)", bake coverslips for 3–4 h.

29. Let coverslips cool to 20°C–25°C protected from light.

▮▮ **Pause point:** Baked and dried coverslips can be stored for up to a year at 20°C–25°C in the dark.

Note: Coverslips are stored on a coverslip rack holder for convenience. Washes can be performed on the rack holder, minimizing handling of the coverslips and saving time.

DNA-fiber quality check

⌚ **Timing:** 1 h

Stretched DNA is stained with YOYO-1, which is a sensitive dsDNA binding fluorophore, to check that the Proteinase K treatment and Agarase digestion of plugs has been performed correctly. YOYO-1 staining also enables fiber density and quality to be checked, to ensure that fibers are not short or sheared (Figure 2), prior to progressing with the protocol: [Telomere FISH probing, Click-iT EdU-labeling and YOYO-1 staining](#).

Note: The YOYO-1 staining intensity from the DNA-fiber quality check will be more intense than after the Click-iT EdU-labeling and YOYO-1 staining process. Additionally, the fiber density will be slightly higher. This should be considered when deciding whether to proceed (Figure 2A).

Note: If the DNA fibers are too dense after checking the YOYO-stain (i.e., you cannot readily distinguish YOYO-1 stained DNA ends), dilute the fiber solution in the reservoir with more 0.5 M MES solution and re-stretch on fresh coverslips. If the fibers are too sparse, stretching DNA onto the same coverslip again after initial stretching can help increase fiber density for imaging.

30. Dilute the 1 mM YOYO-1 stock 1:4,000 in ABDIL or equivalent blocking solution.
31. Incubate coverslip with YOYO-1/ABDIL solution for 15 min at 20°C–25°C.
 - a. Remove coverslip and wash twice in 1 × PBS for 5 min each.
 - b. Rinse with deionized water to remove salt traces from your coverslips
32. Air dry coverslips and visualize on the microscope

Telomere FISH probing, Click-iT EdU-labeling, and YOYO-1 staining

⌚ **Timing:** 6 h up to 22 h

DNA fibers that have been stretched and baked onto coverslips are subject to non-denaturing FISH with G-rich telomere probe conjugated to TAMRA fluorophore (TelG-TAMRA). We then describe the washing steps and subsequent labeling of incorporated EdU (on the DNA fiber), followed by detection of DNA fibers with YOYO-1. Non-denaturing FISH is required for YOYO-1 staining to work. YOYO-1 and EdU-labeling is performed in a single step to save time. Stained slides are then mounted with antifade solution for microscopy imaging.

33. Subject coverslips to an ice-cold (pre-chilled at –20°C) ethanol dehydration series: 70% ethanol for 3 min, 90% ethanol for 2 min, 100% ethanol for 2 min.
 - a. Immerse coverslips, held in a coverslip rack holder, in a small beaker containing ~80 mL ice-cold ethanol solution.
 - b. Replace ethanol solution according to ethanol series then repeat from previous sub-step.
34. Air dry coverslips at 37°C for 10 min.
35. While the coverslips dry, prepare slides in a dark humidifying chamber by adding 50 µL TelG-TAMRA PNA probe to each slide. Gently place each coverslip on the PNA probe, label side up (or down if desired).
36. Incubate at 20°C–25°C in the dark humidifying chamber for 3 h at 37°C.

△ **CRITICAL:** While handling coverslips, it is important to label the front of the coverslip rack holder and record the order and orientation of coverslips in the holder e.g., label face up. Incubations should always be performed with consistent positioning of the label to ensure correct staining of the same side of each coverslip.

△ **CRITICAL:** Non-denaturing FISH is required for subsequent YOYO-1 staining to work. It is important to use the TelG (TTAGGG)₃ PNA probe. For reasons that are unclear, TelC (CCCAAT)₃ produces a poor signal.

Note: Bubbles trapped underneath the coverslip can be released by gently moving the coverslip. Do not push down on the coverslip as this will scrape off the DNA fibers.

37. Transfer the coverslips back to the coverslip rack holder and commence washes (~80 mL each) in a small beaker in the dark:
 - a. Wash coverslips for 5 min in prewarmed 37°C 1 × SSC/50% formamide (use Formamide (ACS reagent) from Sigma – see “[materials and equipment](#)”).
 - b. Wash twice for 5 min with prewarmed 37°C 2 × SSC.
 - c. Wash once for 5 min with 2 × SSC/0.1% Tween at 20°C–25°C.
 - d. Rinse once with deionized water.
38. Fix coverslips with 4% formaldehyde solution in PBS for 10 min at 20°C–25°C.

△ **CRITICAL:** Fixation with 4% formaldehyde in PBS is essential to stop denaturation of DNA fibers. Denaturation will result in reduced YOYO-1 signal, which will inhibit fiber detection.

39. Discard fixative then wash twice for 5 min with PBS.
40. Prepare a 1:100 dilution of 1 mM YOYO-1 iodide solution (Invitrogen) in ABDIL. Store at –20°C in the dark.
41. Prepare the Click-It & YOYO-1 staining master mix (CYMM) as listed in “[materials and equipment](#)”. It is important to add the components in order, otherwise the reaction will not proceed optimally. Use the Click-iT® reaction cocktail within 15 min of preparation.
 - a. Prepare 1 × Click-iT® EdU buffer additive by diluting the 10 × solution (Click-iT™ Plus EdU Cell Proliferation Kit for Imaging) 1:10 in MQW. Prepare this additive solution fresh and discard any unused 1 × solution. Other components can be returned to storage temperature.
 - b. Add 150 μL of CYMM to each slide then overlay with the coverslip.
 - c. Incubate for 30 min at 20°C–25°C in a dark humidifying chamber.

Note: We combine EdU Click-iT labeling and YOYO-1 staining into a single step.

42. Transfer coverslips back to the coverslip rack holder and wash twice with PBS for 5 min each.
43. Air dry the slides at 37°C for 30 min in a fan-forced oven or for 16 h at 20°C–25°C in the dark.
44. Mount the coverslips onto microscope slides using Prolong gold antifade.
45. Allow slides to cure for 30 min at 20°C–25°C in the dark. Store slides in the fridge in a slide folder (in the dark).

Note: Prolong Gold® is a curing mounting agent and as such needs to harden through drying and storage in the fridge prior to imaging. Other commercial or homemade antifade mounting reagents are also likely to be suitable.

▣ **Pause point:** Mounted coverslips can be stored for up to 6 months in the fridge.

Microscopy imaging

46. Remove slides from the fridge and allow to equilibrate to 20°C–25°C for about 10 min.

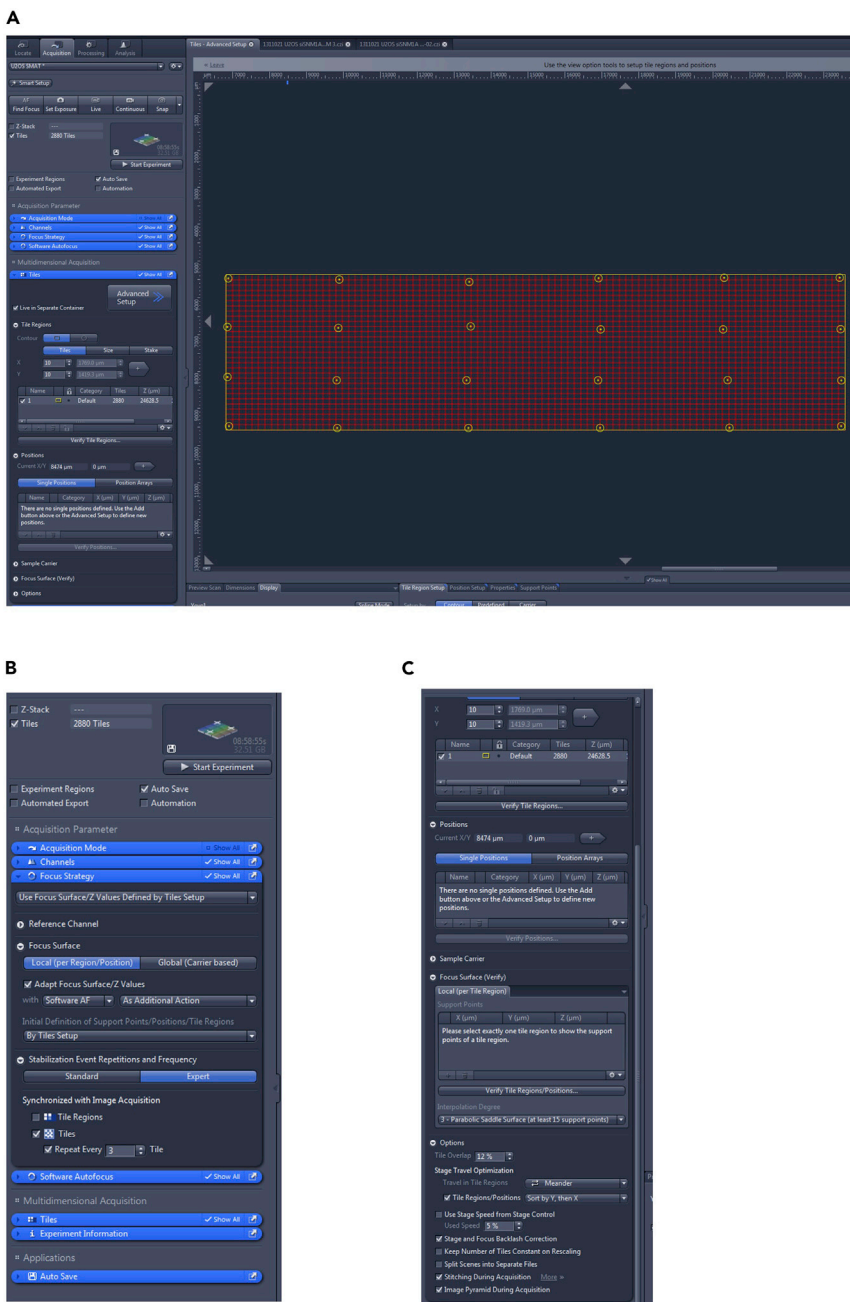


Figure 3. ZEN Blue microscope imaging interface and key settings used for imaging

- (A) Screenshot of tiling setup (red grid) and support points (yellow markers).
 (B) Settings used for adaptive autofocus under the “Focus Strategy” tab on the left side panel.
 (C) Settings used under the “Tiles” tab on the left side panel.

47. Perform automated imaging of 2,500–3,500 tiles on the Axio Imager Z.2 microscope or any other epifluorescence microscope with semi-automated tiling capability. For U-2 OS cells, ~2,800 tiles are sufficient; however, if fiber density is lower the number of tiles imaged may need to be increased (Figure 3A). We perform imaging using the Plan-APO 63X/1.40-Oil objective.

- a. First, select the FITC, DsRed, and Cy5 excitation and emission filters to detect YOYO-1 (DNA), TelG-TAMRA (Telomere G-probe) and AF647 (EdU Click-label) fluorescence, respectively. Guideline exposure settings are in [Methods S1](#)
- b. Enable “Tiles” on the ZEN software and change settings in “Focus Strategy” to enable the adaptive focus imaging method on ZEN ([Figure 3B](#)). This uses the initial Z-coordinates defined by the support points generated by the user to create a dynamic focal plane, which updates in real-time to compensate for drift.
- c. For convenience, we have attached an example imaging method file in “[Methods S1](#)”. The settings file that must be opened in ZEN software. After importing into ZEN, check that the following settings are correct:
 - i. Select YOYO-1 as the reference channel. This will be used for the adaptive focusing.
 - ii. Set up approximately 20–30 support points on the ZEN Blue microscope imaging software ([Figure 3A](#)).
 - iii. Set up tiling according to the settings shown in [Figure 3B](#). Enable stitching to allow analysis of telomere tracts that traverse separate tiles ([Figure 3C](#)).
 - iv. Verify the support points by manually adjusting the focus until the YOYO-1 DNA fiber staining becomes focused.
 - v. Select “Start Experiment” and allow imaging to perform for 12–16 h, depending on tile imaging area.

Note: We advise supervising the imaging for several tiles to check that the autofocus is working correctly. If the autofocus does not work, switching to TAMRA as the reference channel may help.

EXPECTED OUTCOMES

An ALT cell line like U-2 OS has very long telomeres with an average telomere length of 30–50 kb. Notably, the telomere length distribution of ALT cells in general is heterogeneous with very short to very long telomeres ([Reddel et al., 2001](#)). A recent publication has shown that telomere length measurement on DNA fibers is consistent with terminal restriction fragment (TRF) analysis, and translates to 15–25 μm average telomere length tract on DNA fibers from U-2 OS cells ([Kahl et al., 2020](#)).

Telomere extension has been shown to occur specifically in ALT cells outside of S-phase, at roughly 2%–4% of telomeres after scoring ~ 150 telomeres in an asynchronous cell population following a 5 h EdU pulse. It is important to note that previous DNA fiber methods involving telomere FISH have not combined the use of YOYO-1 staining during imaging, and so may have overestimated the true number of extension events ([Lu et al., 2019](#); [Sobinoff et al., 2017](#)).

After imaging each coverslip, telomere extension events and lengths need to be quantitated (for details see “[quantification and statistical analysis](#)”). Initial examination of the tiled image would expect a YOYO-1 staining pattern shown in [Figure 2A](#). Telomere or EdU tracts should align with YOYO-1 stained DNA fibers. Telomere signals may not be immediately apparent and it may be necessary to pseudo-color the channels ([Figure 4B](#)) to improve ease-of-visibility when scoring. Examples of telomere extension events, defined by terminal EdU tracts at telomeres are shown in [Figure 5A](#). Non-terminal EdU tracts are not considered to be extension events ([Figure 5B](#)).

QUANTIFICATION AND STATISTICAL ANALYSIS

Scoring of telomeres

Analysis of microscopy images is performed using Zeiss ZEN blue lite 2.1 software (<https://www.zeiss.com/microscopy/int/products/microscope-software/ZEN-lite.html>). First, telomeres are identified. We aim to quantify at least 150 telomere fibers per coverslip (per replicate).

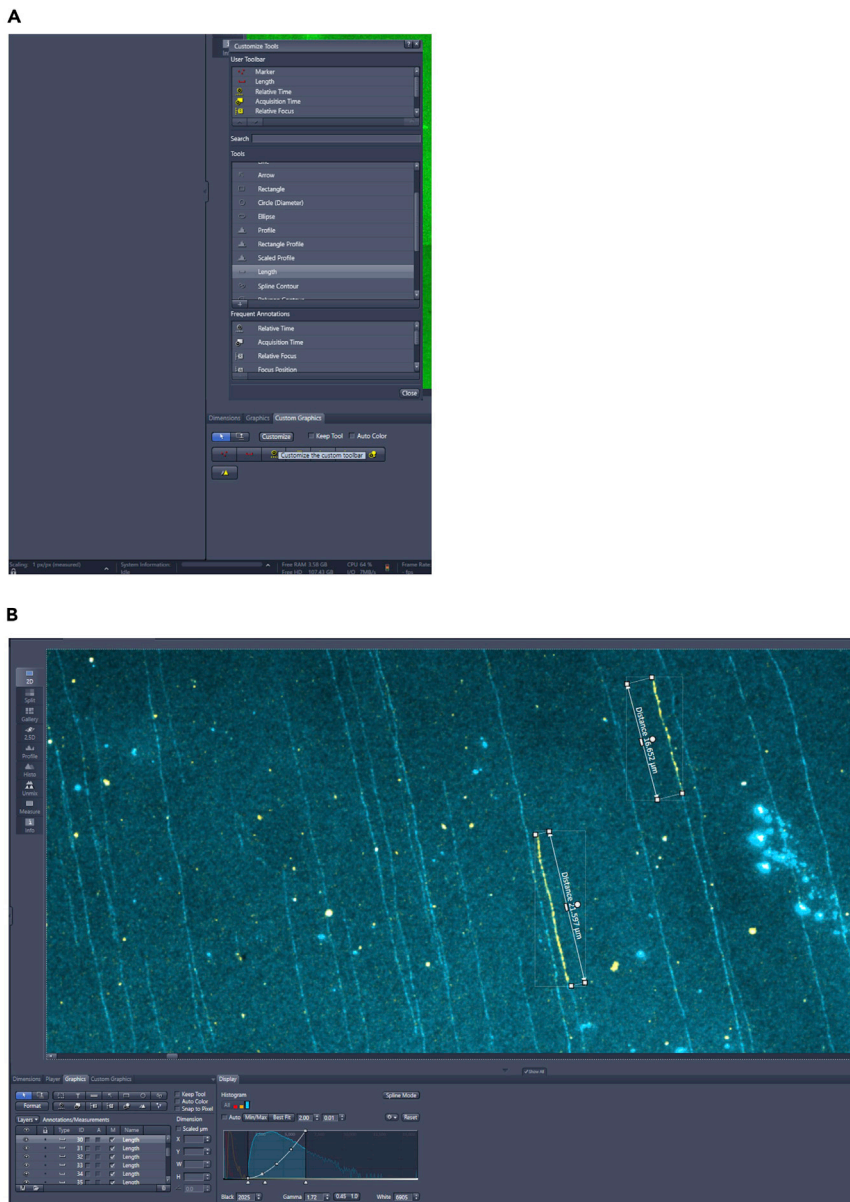


Figure 4. How to perform measurements of telomeres in ZEN Blue lite

(A) Where to find the measurement tool on ZEN Blue lite.

(B) Example of telomere length measurement of a telomere tract (yellow) at the terminus of a YOYO-1 stained DNA fiber (light blue). Note the measurements on the “Graphics” tab in the bottom left corner. Record measurements according to [Table 1](#) and the example dataset shown in [Data S2](#).

Note: The optimal number of telomeres scored was determined based on calculations of statistical power using an example dataset ([Data S2](#)). The example dataset provided average telomere extension frequencies of 0.02 and 0.027 from asynchronous and G2/M synchronized U-2 OS cells, respectively. Binomial distribution analysis determined that 150 telomeres is an acceptable number of telomeres to be scored to reliably obtain 2–5 extension events. Details of the power calculation are included in [supplemental information - Methods S2](#).

Telomere lengths are scored manually using the software’s “Measurement” tool, and lengths recorded in Microsoft Excel. When you first open the program, the Measurement tool can be added

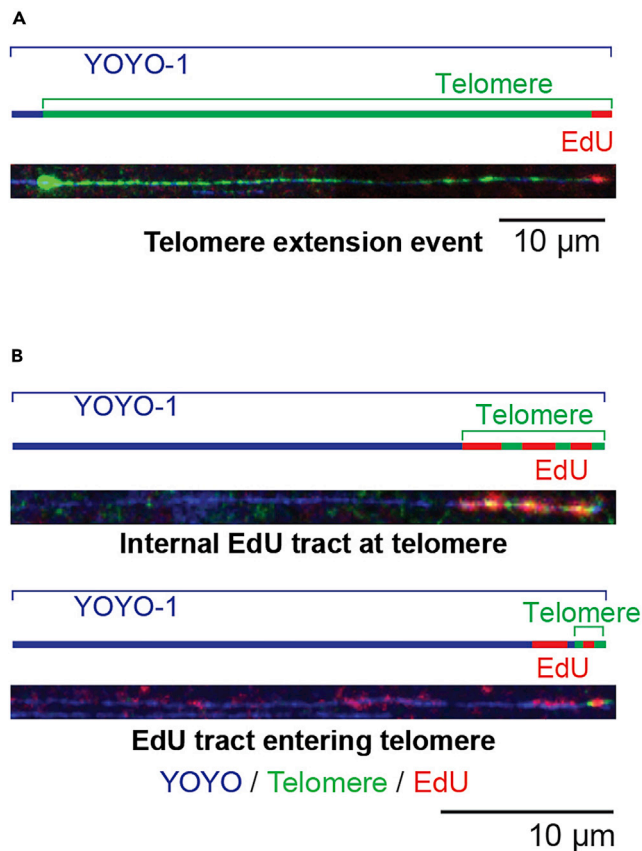


Figure 5. Schematic and representative examples of telomere synthesis on DNA fibers

(A) telomere extension events and (B) non-terminal EdU tracts. Genomic DNA is stained with YOYO-1 (pseudo-colored blue), telomere (pseudo-colored green) with Tel-G-TAMRA probe, EdU labeled with Alexa Fluor 647 (red). Note the terminal position of the EdU tract in (A) relative to the telomere and YOYO-1 tracts.

to the “Custom Graphics” tab (Figure 4A), then select “Keep Tool”. Scored telomeres or measurements appear as Graphics under the “Graphics” tab in ZEN. These measurements can then be reviewed or edited if desired.

At each measured telomere, identify whether there is an EdU tract, and whether it is an extension event (i.e., whether the EdU tract is terminal). Telomere extension events are defined as terminal telomeric EdU tracts (Figure 5A). This is because telomere extension is a continuous conservative DNA synthesis mode that originates at the ends of telomere to extend the repeat tract (Sobinoff et al., 2017). Mark each telomere extension event using the “Measurement tool”, then measure and record both the extension tract length (EdU tract length) and the pre-extension tract length (total telomere tract length minus the EdU tract length). Non-terminal EdU tracts are not measured. An example of a scoring template is shown in Table 1.

The four major parameters that can be quantitated include:

1. Telomere length (which can be measured by omitting or ignoring the EdU labeling step).
2. The frequency of telomere extension events, calculated by the number of telomeres with a terminal EdU signal divided by the total number of telomeres scored.
3. The length of telomere extension events, which corresponds to the length of the terminal telomeric EdU tract.
4. The frequency of non-terminal EdU tracts at telomeres. This can occur when:

Table 1. Template used to record measurements in Microsoft Excel

Telomere length (μm)	EdU Y/N	EdU extension length (μm)	Pre-extension length (telomere length - extension length) (μm)	Internal EdU tract	EdU starts in YOYO-1
15	Y			Y	
9.8	Y	3.1	6.7		
17.6					

Note, if the EdU tract at the telomere is not an extension event, the length in the 3rd column is left blank. Measurement length values are in μm . See [Data S2](#), which is an example dataset of telomere extension in U-2 OS cells.

- EdU starts in the upstream YOYO-1 and extends into the telomere, or EdU encompasses the entire telomere tract.
- There is an internal EdU tract within the telomere. This includes multiple EdU tracts present on same telomere.

Note: What to score:

- Telomere ends are defined by co-termination of both YOYO-1 counterstain and telomere FISH signal, without continuation of YOYO-1 staining beyond the telomere tract.
- Due to the stickiness of DNA fibers, telomeres may sometimes contact adjacent fibers. As long as the telomere tract can be distinguished and the end determined, it can still be analyzed.

Note: What not to score:

- Branched or Y-shaped i.e., non-linear telomeres
- Telomeres without upstream YOYO-1 staining
- "Internal" telomeres. Telomere tracts that sit flush in a YOYO-1 tract. This is due to fibers sticking to each other.

After scoring and recording telomere length, extension and non-terminal EdU tract measurements as shown in [Table 1](#), convert measured lengths in " μm " to "kb". We apply a $1 \mu\text{m} = 2 \text{ kb}$ stretching factor conversion to determine the absolute telomere length. The same is applied to the telomere extension lengths. The frequency of extension events can then be expressed as a percentage of telomeres scored ([Figure 6](#)). Telomere lengths, length of telomere extension events and frequency of telomere extension events can then be graphed for $n=3$ replicates. Statistical analysis for frequency data is typically via Student's t-test as data points are expected to be normally distributed, while for length measurements comparisons are via Mann-Whitney test. In the event where there is a lack of telomere extension events, we do not perform statistical comparisons between extension lengths. An example tabular dataset, for [Figure 6](#), is shown in [Data S2](#).

LIMITATIONS

A key caveat to this technique is that it cannot distinguish homology-directed repair synthesis from bulk genomic replication, since it relies on EdU as the sole nucleoside analogue ([Jackson and Pombo, 1998](#)). The scoring criteria excludes telomere extension events at telomeres if there is an internal or upstream EdU tract in the DNA fiber (see "[quantification and statistical analysis](#)"). Also, because of this reason and the abundance of EdU incorporation in S-phase, SMAT cannot be applied to cells synchronized in S-phase, and telomere extension events in S-phase cannot be accurately measured.

Another limitation of this technique is the time-consuming nature of imaging the large number of tiles required to obtain sufficient telomeres for SMAT analysis. While several samples can be combed onto coverslips for imaging in parallel, each sample or coverslip can only be imaged one at a time and takes 14–20 h of tiled imaging (routinely performed overnight). There is a current lack of adequate automated analysis methods, due to the complex scoring criteria required to select

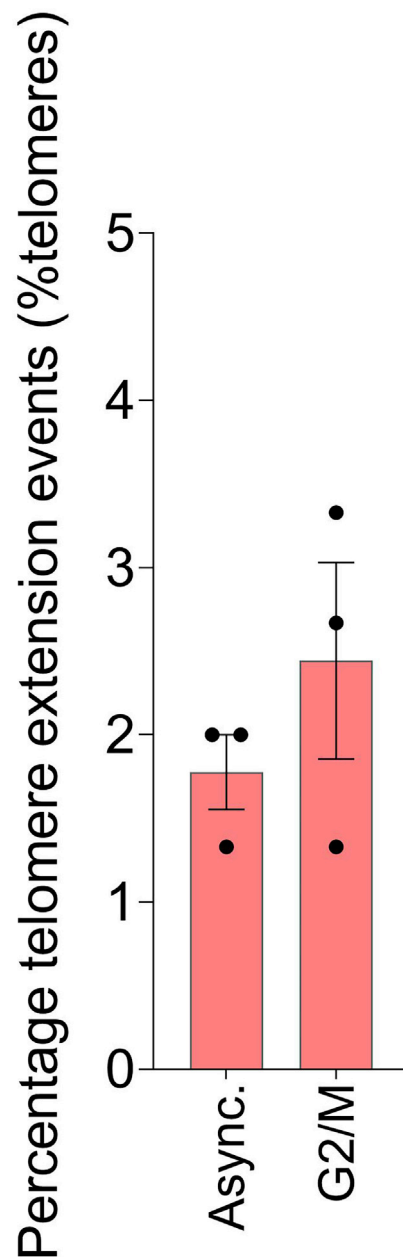


Figure 6. Example SMAT analysis graph showing the frequency of telomere extension events (% telomeres)

Bar graph represents the mean \pm SEM from $n = 3$ biological replicates (150 telomeres scored per replicate) from asynchronous or G2/M synchronized U-2 OS cells (via RO-3306-mediated arrest and thymidine block). For both treatments EdU was labeled for 2 h. Related to [Data S2](#).

only “true” telomere extension events (see “[scoring of telomeres](#)”) that uses positional comparison across three label channels, YOYO-1, TelG-TAMRA, EdU-647. The fluorescent labels occupy the FITC, Cy3 and Cy5 filter sets, respectively, and are commonly used with standard epifluorescence microscopes. Additionally, while an automated method to measure telomeres on DNA fibers exists, it does not account for the added complexity of EdU-tract labeling and scoring criteria ([Kahl et al., 2020](#)). Finally, data storage is another potential limitation. The file sizes of typical DNA fiber tiled images are large, requiring 8–20 Gb of storage per coverslip.

TROUBLESHOOTING

Problem 1

YOYO-1 staining is weak and/or white film is observed on coverslips after baking.

Potential solution

Agarase digestion of plugs is not complete or the concentration of MES buffer in the reservoir is too high (happens if water evaporates from the reservoirs). Discard sample and restart the experiment. We recommend preparing backup samples if this happens frequently, but we have observed this problem to be random, even within an experiment.

Problem 2

YOYO-1 staining is very faint or undetectable and/or telomere FISH signal is weak, despite good fiber-quality check.

Potential solution

Decrease coverslip baking time. Alternatively, alter the stringency of the SSC wash steps by changing the temperature or salt concentration.

Problem 3

DNA fibers are not aligned, appear branched, or are unevenly distributed across the coverslip.

Potential solution

Incomplete mixing of the DNA fiber solution prior to stretching the DNA fibers, since fibers may sink to the bottom of the reservoir over time, or incomplete Proteinase K or Agarase digestion. Incomplete digestion causes fibers to become trapped together, which can lead to uneven distribution and stretching on the coverslips. Reheat DNA fiber solution (in 0.5 M MES) to 68°C to melt any agarose, add an additional 2 μ L of Agarase and digest again for 16 h (Proteinase K lysis of cells in plugs and stretching onto coverslips).

Problem 4

YOYO-1 stained DNA fibers are sticking to each other excessively or look bunched up.

Potential solution

DNA fiber solution too concentrated. Using a blunted P1000 tip, remove some DNA fiber solution and dilute with 0.5 M MES pH 5.5 solution. Invert the reservoir gently to mix. Restretch the DNA fiber solution onto coverslips (Proteinase K lysis of cells in plugs and stretching onto coverslips). Alternatively, create new plugs with a lower number of cells.

Problem 5

EdU staining is absent on DNA fibers.

Note: There should be rare EdU tracts throughout genomic YOYO-1 staining.

Potential solution

Coverslip incorrectly orientated during Telomere FISH probing, Click-iT EdU-labeling and YOYO-1 staining steps. Ensure that the same side of the coverslip is stained throughout the protocol.

CYMM solution excessively diluted. Remove as much residual PBS after washes (prior to EdU-labeling) with a kimwipe through gentle flicking and dabbing of the corners of the coverslip.

CYMM solution not prepared correctly or left too long prior to use (15 min maximum).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Hilda Pickett (hpickett@cmri.org.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101212>.

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AUTHOR CONTRIBUTIONS

Conceptualization and Methodology, R.L., J.A.M.A., and H.A.P.; Investigation, R.L. and J.A.M.A.; Statistical Analysis, P.G.; Writing, R.L., J.A.M.A., and H.A.P.; Funding Acquisition and Supervision, H.A.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPORTING CITATIONS

The following references appear in the supplemental information: [Casella and Berger \(2021\)](#) and [Devore \(2016\)](#).

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