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Direct Quantitative Monitoring of Homology-Directed DNA Repair of Damaged Telomeres

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Abstract

Homology-directed DNA repair (HDR) is an evolutionary conserved mechanism that is required for genome integrity and organismal fitness across species. While a myriad of different factors and mechanisms are able to execute HDR, all forms necessitate common steps of DNA damage recognition, homology search and capture, and assembly of a DNA polymerase complex to conduct templated DNA synthesis. The central question of what determines HDR mechanism utilization in mammalian cells has been limited by an inability to directly monitor the DNA damage response and products of repair as they arise from a defined genomic lesion. In this chapter, we describe several methodologies to delineate major steps of HDR during alternative lengthening of telomeres in human cells. This includes procedures to visualize interchromosomal telomere homology searches in real time and quantitatively detect HDR synthesis of nascent telomeres emanating from synchronous activation of telomere DNA double-strand breaks. We highlight the critical details of these methods and their applicability to monitoring HDR at telomeres in a broad variety of mammalian cell types.

1. INTRODUCTION

Homology-directed DNA repair (HDR) is an evolutionary conserved DNA repair mechanism and critical determinant of genome integrity and organismal fitness. Indeed, inherited loss-of-function mutations within genes that mediate HDR are responsible for human syndromes that encompass developmental abnormalities, bone marrow failure, premature aging, and cancer predisposition (Prakash, Zhang, Feng, & Jasin, 2015). Conversely, aberrant gain of function recombination is tumor sustaining in ~15% of human cancers that utilize a poorly understood HDR mechanism for telomere maintenance known as alternative lengthening of telomeres (ALT) (Bryan, Englezou, Dalla-Pozza, Dunham, & Reddel, 1997; Bryan, Englezou, Gupta, Bacchetti, & Reddel, 1995; Dilley & Greenberg, 2015).

Despite clear differences in repair factor requirements and substrate preferences, all HDR variants necessitate several common steps. Recombination mediator proteins nucleate single-stranded overhangs for homology capture, followed by assembly of DNA polymerase

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complexes for templated synthesis. Canonical BRCA-Rad51 recombination involves strand invasion into duplex DNA to capture homology, while alternative HDR anneals single-stranded regions harboring as little as a few bases of homology (Bhowmick, Minocherhomji, & Hickson, 2016; Sfeir & Symington, 2015; Verma & Greenberg, 2016). Finally, a process that has been largely defined in yeast called break-induced replication (BIR) involves up to 100kb of DNA synthesis following homology capture (Anand, Lovett, & Haber, 2013; Costantino et al., 2014; Lydeard, Jain, Yamaguchi, & Haber, 2007; Saini et al., 2013; Wilson et al., 2013). Interestingly, yeast and human cells can utilize a BIR-like mechanism of HDR for telomere maintenance (Chen, Ijpma, & Greider, 2001; Dilley et al., 2016; Lundblad & Blackburn, 1993; Lydeard et al., 2007; Roumelioti et al., 2016).

The central question of what determines HDR mechanism utilization in mammalian cells has been severely limited by an inability to directly monitor the DNA damage response and products of repair as they arise from a defined genomic lesion. While elegant reporter assays have been devised to monitor the products of recombination days after repair has been completed (Moynahan, Pierce, & Jasin, 2001; Pierce, Johnson, Thompson, & Jasin, 1999; Willis et al., 2014), none allow the dynamic quantification of HDR in real time. We have overcome this barrier using break-induced telomere synthesis as a model system to delineate major steps of noncanonical HDR during ALT in human cells. The methodologies described later allow for direct visualization of telomere damage recognition, homology search and capture, and the products of HDR synthesis at an endogenous genomic location in mammalian cells.

2. DIRECT DETECTION OF BREAK-INDUCED TELOMERE SYNTHESIS

Direct detection of DNA synthesis in vivo relies on the incorporation of synthetic nucleoside analogs into replicating DNA. The methods described in this section utilize the analogs 5-bromo-2′-deoxyuridine (BrdU, Fig. 1A), 5-iodo-2′-deoxyuridine (IdU), 5-chloro-2′-deoxyuridine (CldU), and 5-ethynyl-2′-deoxyuridine (EdU), which can be detected using specific antibodies or click chemistry in the case of EdU. After pulsing cells with the analogs, the samples are processed by fluorescence microscopy or immunoprecipitation (IP) in order to study the nascent DNA generated during the experiment.

When studying DNA repair synthesis, it is necessary to have a well-defined method of creating DNA damage. Examples include nucleases that directly cut DNA such as FokI or Cas9, as well as chemical compounds that create replication stress or DNA breaks. We use the telomere-specific nuclease (TRF1–FokI), which entails fusion of the telomere-binding protein TRF1 with the FokI endonuclease, for a multitude of reasons, not least of which is to gain insights into the mechanisms of break-induced telomere synthesis and ALT (Cho, Dilley, Lampson, & Greenberg, 2014; Dilley et al., 2016; Fig. 1B). Ectopic expression of TRF1–FokI results in robust induction of HDR-driven telomere synthesis in cells that utilize ALT or telomerase for telomere maintenance, making it broadly applicable to nearly any cell type of interest. Additionally, it is relatively easy to detect break-induced synthesis emanating from the telomere due to the high number of defined telomeric repeats throughout the genome. We suggest that the protocols described here can be adapted to study break-induced synthesis using different damaging agents at other genomic regions.

It is important to note that DNA synthesis due to S-phase replication of the genome may impact the ability to detect break-induced synthesis. As such, the signal to noise of the break-induced synthesis is critical. We have found several parameters to be important when planning experiments: the length and timing of the analog pulse, the cell cycle phase of the cell population, the length and synchrony of DNA damage, and the type of detection method used. We will present optimized conditions for detection of break-induced telomere synthesis after TRF1–FokI damage.

In this section, we focus on the novel assays adapted by our lab, namely BrdU IP and single-molecule fiber labeling to directly detect break-induced telomere synthesis. We also commonly employ routine immunofluorescence (IF) microscopy of intact cells labeled with BrdU. In our experience, results from BrdU IF experiments closely match those seen with other assays and therefore can serve as a validation. For a review of these methods, see Jackson and Cook (2008a, 2008b, 2008c). Recent studies have utilized cell microscopy, flow cytometry, and fiber labeling of thymidine analogs to detect break-induced synthesis after replication stress (Bhowmick et al., 2016; Costantino et al., 2014; Minocherhomji et al., 2015; Sotiriou et al., 2016) and at telomeres (Dilley et al., 2016; Min, Wright, & Shay, 2017; Roumelioti et al., 2016).

In addition to the direct measurement of analog incorporation during break-induced synthesis, assays designed to detect the repaired DNA products resulting from break-induced synthesis are also available. Developed in yeast, these assays have provided invaluable mechanistic insights into DNA repair pathways (Anand et al., 2013; Malkova & Ira, 2013). Since analogous systems in mammalian cells are lacking, the protocols described here provide new avenues of investigation into mammalian break-induced synthesis.

2.1 BrdU Immunoprecipitation of Nascent Telomeres

Isolation of DNA allows for many downstream applications such as southern blot, PCR, sequencing, electron microscopy, or in vitro reactions. BrdU IP has previously been used to study S-phase replication of human and yeast cells (Hansen et al., 2010; Viggiani, Knott, & Aparicio, 2010). We adapted these methods for the detection of nascent telomere sequences generated after TRF1–FokI breaks (Dilley et al., 2016; Fig. 1B). We prefer to visualize the nascent telomeres using a dot blot probed with radioactive telomeric repeat oligonucleotides (oligos), as described elsewhere for telomere ChIP and C-circle assays (Henson et al., 2009, 2017; Liu, Feng, & Ma, 2017). However, nonradioactive visualization using digoxigenin-labeled probes can also be used. Additionally, isolated nascent telomeres can be analyzed using PCR and next-generation sequencing methods (Cawthon, 2002; Lee et al., 2017). It is important to note that the DNA isolated after BrdU IP will be single-stranded, which may affect downstream applications.

In our experience, the success of this assay hinges on a tightly controlled damage system that is delivered to the majority of cells in synchrony. We use cell lines with a doxycycline (Dox)-inducible TRF1–FokI fused to the estrogen receptor to allow for control of expression and entry into the nucleus. Overnight addition of Dox, followed by a 2 h pulse of 4-hydroxytamoxifen (4-OHT) along with BrdU, generates robust telomere damage and

nascent telomere synthesis (Dilley et al., 2016). The length of damage in this system is flexible since later time points yield similar results.

2.1.1 Equipment

- Covaris S220 sonicator and tubes or equivalent
- Standard agarose gel equipment
- Heat block
- End-over-end rotator
- Magnetic bead rack
- Bio-Dot Microfiltration Apparatus (Bio-Rad) or equivalent
- Amersham Hybond-N^+ nylon membrane (GE Healthcare)
- Stratagene UV Stratalinker 1800
- Radioactive workspace
- Standard hybridization oven and tubes for radioactive material
- Storage phosphor screen and cassette (GE Healthcare)
- STORM 860 Imager (Molecular Dynamics) or equivalent
- ImageQuant and Fiji for visualization and analysis

2.1.2 Buffers and Reagents

- IP buffer: 0.0625% (v/v) Triton X-100 in phosphate-buffered saline (PBS)
- Elution buffer: 1% (w/v) SDS in Tris–EDTA (TE) buffer
- Denaturation buffer: 0.5 N NaOH, 1.5 *M* NaCl
- Neutralization buffer: 0.5 *M* Tris, 1.5 *M* NaCl, pH adjusted to 7.0
- 20× Saline sodium citrate (SSC) pH adjusted to 7.0
- MasterPure DNA Purification Kit (Epicenter)
- PerfectHyb Plus Hybridization Buffer (Sigma)
- Anti-BrdU antibody (mouse B44, BD 347580)
- 10m*M* BrdU stock dissolved in water
- ChIP DNA Clean & Concentrator Kit (Zymo)
- Protein G Magnetic Beads (Pierce)
- Bridging antibody (Active Motif)
- $(TTAGGG)_6$ or $(CCCTAA)_6$ telomere oligos resuspended at 10 μ*M*
- T4 polynucleotide kinase (PNK) (NEB)
- ATP, [γ–^{32}P] at 10mCi/mL

- Illustra MicroSpin G-25 Columns (GE Healthcare)

2.1.3 Procedure

1. Induce damage: for our TRF1–FokI-inducible cells we add 40ng/mL Dox for 16–24h followed by 1μ*M* 4-OHT for 2 h.
2. Pulse cells with 100μ*M* BrdU for 2h prior to collection. The pulse time can be adjusted from 30min to 4h if desired.
3. Collect cells and isolate genomic DNA (gDNA) using MasterPure DNA isolation kit. Resuspend gDNA in TE.
4. Shear gDNA into 100–300bp fragments using a Covaris S220 sonicator. Check concentration and purity using NanoDrop. Fragment size should be checked by agarose gel electrophoresis (Fig. 1C).
5. Put 1–4μg sheared gDNA in a safe-lock tube, add 5μL of 10× PBS, and add sterile water to get 50μL final volume. Denature for 10 min at 95°C and then cool in an ice-water bath. Save 10% inputs from original sheared gDNA.
6. Add 80μL of anti-BrdU antibody (2μg) and 120μL of IP buffer to each denatured sample. Rotate overnight at 4°C. Samples incubated with anti-IgG serve as a control.
7. Put 30μL per sample of protein G magnetic beads in a safe-lock tube (e.g., 300μL beads for 10 samples). Wash once and resuspend in original volume with 1× PBS. Add 5μL of bridging antibody per 30μL beads. Mix by flicking and rotate for 1h at 4°C.
8. Add 30μL of prebound bead mixture to each IP sample. Rotate for 1h at 4°C.
9. Apply sample tubes to magnetic rack for at least 10s. Aspirate supernatant. Wash 3× with 1mL of IP buffer, followed by one wash with 1 mL of TE. Only remove supernatant with pipet after TE wash. Be careful not to aspirate beads.
10. Incubate beads with 50μL of elution buffer for 15 min at 65°C. Remove eluate to new tube. Repeat elution step and pool eluate.
11. Clean eluate with ChIP DNA Clean & Concentrator Kit. Elute in 50μL.
12. Dilute eluate, along with 10% inputs, to 100μL with freshly made 2× SSC. Boil samples for 5 min at 95°C. Place on ice until ready to add to dot blot.
13. Blot samples onto an Amersham Hybond-N^+ nylon membrane using Bio-Dot Microfiltration Apparatus according to manufacturer protocol.
14. Place membrane face-up on a piece of filter paper soaked in denaturation buffer for 10min. Move to a piece of filter paper soaked in neutralization buffer for 10min. Place membrane on a dry piece of filter paper.
15. UV cross link the membrane with 454nm UV-C irradiation at 1200 J, rotate the membrane 90 degrees, and repeat.

16. Place membrane in hybridization tube with 10 mL of Perfect-Hyb Plus hybridization buffer and prehybridize by rotating for 1h at 37°C.

17. While membrane is prehybridizing, prepare telomere probe. Combine 2μL of PNK buffer, 1μL of 10μ*M* telomere or control oligo, and 1μL of T4 PNK. Move to radioactive workspace and add 3μL of 10mCi/mL ATP, [γ−^{32}P] and 13μL of sterile water. Incubate reaction for 45 min at 37°C.

18. Add 80μL of TE to labeling reaction. Purify 100μL of labeled oligo using an illustra MicroSpin G-25 Column. Add all 100μL of labeled oligo to hybridization tube. Rotate overnight at 37°C.

19. Wash membrane 2× with ~30 mL of 2× SSC, rotating for 20 min at 37°C both times.

20. In radioactive workspace, remove membrane using forceps, briefly place on filter paper, and then wrap tightly in polyvinyl wrapping film. Fold the edges over to make sure no remaining liquid leaks out. Place wrapped membrane in exposure cassette, add clean storage phosphor screen, and expose overnight.

21. Scan storage phosphor screen using STORM 860 with ImageQuant. Analyze image using Fiji or other image processing program (Fig. 1D).

2.1.4 Notes

- As a control, we usually use uninduced cells (no Dox or 4-OHT treatment). Alternatively, induction of a nuclease-defective mutant of TRF1–FokI (D450A) can be used. Other controls to include at least once are a no BrdU control as well as IgG IPs for all samples.
- To confirm telomere damage we prefer to perform IF staining of PCNA, 53BP1, or γH2AX on fixed cells after TRF1–FokI induction. Colocalization of these factors with telomeres/TRF1–FokI should be clearly seen compared to uninduced cells. Damage can also be assessed by whole-cell western blot of γH2AX or p-KAP1. Telomere pulsed-field gel electrophoresis will show a downward shift in the telomere smear.
- $(TTAGGG)_6$ oligos detect the C-rich telomere strand, whereas $(CCCTAA)_6$ oligos detect the G-rich telomere strand. We have not found any differences between results from these two probes. Although we mainly use 6× oligos, 4× oligos also yield the same results. For a control, we use a probe to Alu repeat sequences. α-Satellite probes can also be used. These control probes should reveal constant levels of nascent DNA compared to the increases seen with telomere probes.
- After initial hybridization, membranes can be stripped and reprobed with another labeled oligo. To do this, incubate the membrane with boiling hot 0.1% SDS buffer with gentle rocking. Once the buffer cools to room temp, repeat 2–3 more times. Then proceed to prehybridization.

- We use Fiji to quantify images. Intensity values can be measured using a constant area circle with background subtraction. The dot blot macro can also be used. Quantifications should always be normalized to 10% input. An example dot blot is shown in Fig. 1D.

2.2 Single-Molecule Analysis of Nascent Telomeres

This approach, referred to as single-molecule analysis of replicating DNA (SMARD), was first developed to understand the duplication of the Epstein–Barr virus genome (Norio & Schildkraut, 2001) and is now commonly used to study the features of individual replication forks by labeling replicating DNA with two different thymidine analogs, CIdU and IdU. The technique involves pulsing cells with CIdU and IdU to allow for the differentiation of replication tracts generated at two different time points. CIdU and IdU can be distinguished using their corresponding antibodies generated in two different hosts. The combination of the two analogs has been extensively used to identify factors required for the protection and restart of the replication fork during S-phase replication (Breslin et al., 2006; Merrick, Jackson, & Diffley, 2004; Nieminuszczy, Schwab, & Niedzwiedz, 2016). An elegant study combined the use of CIdU and IdU with a third thymidine analog, EdU, which allowed the authors to demonstrate that replication can occur on either side of an adduct crosslinking two parental strands (Huang et al., 2013). Recently, the combination of the two different analogs was coupled with fluorescence in situ hybridization (FISH) to study the replication features of human and mouse telomeres (Drosopoulos, Kosiyatrakul, Yan, Calderano, & Schildkraut, 2012; Sfeir et al., 2009). We adapted this strategy to examine the nature of HDR DNA synthesis that occurs postinduction of DSBs at the telomeres (Dilley et al., 2016; Fig. 2).

2.2.1 Labeling of Nascent Telomeres Formed Postinduction of DSBs (Day 0–1)

2.2.1.1 Reagents

- TRF1–FokI engineered cell lines and its respective media
- Doxycycline
- Thymidine analogues: IdU and CIdU

2.2.1.2 Equipment

- Cell culture hood
- 37°C Cell incubator

2.2.1.3 Procedure

1. Day 0: As discussed in the previous section, add doxycycline to a final concentration of 40ng/mL to a 10-cm dish of 70% confluent cells for 16–20h. For every cell line, include an uninduced control plate.
2. Day 1: After 16 h of doxycycline treatment, add 1 μ*M* 4-OHT and 30 μ*M* IdU to each plate and incubate the cells at 37°C for 2h. After 2h, remove the media,

wash all plates twice with 10 mL PBS, and subsequently add medium containing 30μ*M* CIdU. Incubate at 37°C for two additional hours.

2.2.2 Isolation of Telomeric DNA Formed Postinduction of DSBs (Day 1–4)

2.2.2.1 Reagents

- 0.25% Trypsin
- Phosphate buffer saline (PBS)
- 2% Agarose in PBS
- Tris–ethylenediaminetetraacetic acid (EDTA) (TE) buffer
- Disposable plug mold
- Solution for cell lysis: 100m*M* EDTA +0.2% sodium deoxycholate (SDS) +1% sodium lauroyl sarcosine. Add 0.5 mL of proteinase K stock solution (20mg/mL) to 50mL of lysis buffer. Add proteinase K to lysis solution immediately before use
- Restriction enzymes: *Mbo*I and *Alu*I and their respective buffer. If using *Alu*I and *Mbo*I from New England Biolabs (NEB), use the CutSmart Buffer

2.2.2.2 Equipment

- Cell culture hood
- Cell counter
- Benchtop centrifuge
- Heat block and 55°C water bath
- Tube rotator

2.2.2.3 Procedure

1. Day 1: Prior to harvesting the cells, prepare 2% agarose in 1× PBS and place in a 55°C water bath to maintain the agarose solution in a liquid state.
2. When ready to trypsinize, make 1 mL aliquots of the melted agarose in eppendorf tubes and place in a 70°C heat block.
3. Four hours after addition of 4-OHT, harvest the cells by trypsinization. Spin down the trypsinized cells at 200×*g* and resuspend the cell pellet in 1 mL PBS. Proceed to cell counting.
4. Transfer 1 million cells to 1.5mL eppendorf tubes and pellet down the cells at 200×*g*.
5. Resuspend the cells in 50 μL of PBS. Incubate one sample at a time in the 70°C heat block for 30 s and then add 50 μL of 2% agarose to the cells, while the tube is still in the 70°C heat block. Immediately pipette up and down to mix.

6. Transfer the cell suspension to a plug holder by pipetting on one side until it is filled to the brim.
7. Once all the samples have been transferred to the plug holder, leave the plug holder at 4°C for ~20 min to allow the agarose to solidify.
8. Meanwhile, aliquot 1mL of lysis solution into 1.5mL amber-colored tubes.
9. Transfer the plugs to the lysis solution and incubate the tubes containing the cell plugs at 50°C overnight.
10. Day 2: Change the lysis solution and incubate the cell plugs at 50°C for an additional 24 h.
11. Day 3: Aspirate the lysis solution from the plugs and add 1 mL of TE buffer. This volume ensures that the plug is uniformly washed. Transfer the plug-containing tubes to a tube rotator. Rotate for 30min at 30rpm. Perform all subsequent washes on the tube rotator.
12. Next, perform one wash with 1 mL TE buffer containing 1m*M* PMSF at 4°C for 60min.
13. Perform two washes at room temperature (RT) with 1mL TE buffer for 30min each.
14. Wash once with 1 mL deionized water for 30min at RT.
15. Equilibrate the plugs with 500 μL of the 1× CutSmart Buffer for 30 min at RT.
16. Replace with 500 μL of fresh 1× CutSmart Buffer and add *Mbo*I (100 units) and *Alu*I (100 units) to each plug. Digest the samples at 37°C for 16h.

Notes

1. The thymidine analogs are light sensitive. Avoid exposing the plugs to light.
2. After cell lysis, the plugs can be stored in TE buffer at 4°C for a couple of weeks. The buffer should be changed once or twice a week.

2.2.3 Purification of Telomeric DNA (Day 4–5)

2.2.3.1 Reagents

- Ultrapure low melting point (LMP) agarose
- Agarose
- 0.5× TBE (Tris/borate/EDTA buffer)
- β Agarase-I
- YOYO-1 Iodide (491/509)
- Beta-mercaptoethanol (βME)
- 5 *M* Sodium chloride (NaCl)

2.2.3.2 Equipment

- Gel caster with a 1.5-mm comb and a powerpack
- Water bath at 55°C

2.2.3.3 Steps

1. Day 4: In a cold room, cast a 0.7% LMP agarose gel in 0.5× TBE containing ethidium bromide. Ensure that the comb is wide enough to hold the agarose plugs. A 1.5-mm wide comb is good enough for this purpose. The gel usually takes ~2 h in the cold room to solidify.
2. Melt 1% agarose in 0.5× TBE and place in 55°C water bath. This agarose will eventually be used to seal the wells of the gel.
3. Once the gel is ready to load, aspirate the digestion mix off the plugs and add 500 μL of 0.5× TBE. Equilibrate for 5 min.
4. Add 0.5× TBE on the polymerized gel to help remove the comb. Try to be gentle as the gel is very fragile.
5. Gently wash the wells with 0.5× TBE and then aspirate the TBE.
6. Using a blunt end forcep, insert the gel plugs into the wells.
7. Use the melted 1% agarose to seal the wells.
8. Cover the gel with aluminum foil and leave it for 5–10 min to allow the agarose to solidify.
9. Carefully transfer the loaded gel to a running tank containing 0.5× TBE and run at 20 V for 20–24 h at 4°C. Cover the lid with aluminum foil.
10. Day 5: Prepare agarase digestion buffer: 4.9mL TE+ 100μL 5 *M* NaCl+5 μL βME.
11. Due to the GC-rich nature of the telomeric region, telomeres are resistant to digestion by *Alu*I and *Mbo*I restriction enzymes. Only nontelomeric gDNA is digested by these enzymes and will therefore run as a smear. On the other hand, undigested telomeres will run as an intact band (Fig. 3). Excise the telomere band, mince the gel piece, and remove any extra gel pieces. Transfer the gel piece to an amber-colored tube. Since this is a single-molecule assay, a small but brightly stained gel piece is ample. Additionally, the small size of the gel sample ensures complete digestion of the agarose in the subsequent step. The remaining gel pieces of the excised telomere band can be stored for weeks in agarase digestion buffer at 4°C.
12. Add 100 μL of the agarase digestion buffer to the gel piece. Make sure the gel piece is completely covered, else scale up the buffer volume.
13. Incubate the eppendorf tubes containing the gel slice at 45°C for 5min.
14. Next, move the tubes to a 69°C heat block and incubate for 18min (69°C is close to the melting point of the LMP agarose).

15. Equilibrate two tubes at a time on a 45°C heat block for 2min and add 3 μL of agarase by pipetting along the side of the tube. The tubes can be held in hand while adding agarase. Incubate the tubes at 45°C for 3h. Once the agarose has melted, DNA is sensitive to shearing. Be gentle with the sample while handling and pipetting. Do not vortex.

16. After 3h, place the tubes at RT. Gradually add 10 μL of βME and 0.4 μL of 1m*M* YOYO-1 stain along the sides of the tube and incubate overnight at RT in the dark.

2.2.3.4 Notes

1. Avoid exposing the samples to light.

2. It is possible that gel pieces may remain after the 3 h digestion. In that situation, either extend the duration of the digestion or take the DNA from the top of the solution in the subsequent steps as the gel pieces will settle down.

3. To identify the location of the telomeric band on the gel, southern blot analysis using a telomeric probe can be performed.

4. We have tested the quality of the YOYO-1-stained DNA after a week of storage at RT and they look fine.

An autoradiogram of an agarose gel, hybridized with a ^{32}P-labeled telomere probe, is shown in Fig. 3. It is a representative example of *Alu*I- and *Mbo*I-digested gDNA sample from U2OS cells. Note that the gDNA runs as a smear and telomeric DNA runs as an intact band.

2.2.4 Slide Preparation (Day 5)

2.2.4.1 Reagents

- 50 mm Cover glasses
- 1% SDS
- >98% Aminosilane solution (APTES)
- Conc. nitric acid (HNO_3)
- Conc. hydrochloric acid (HCl)
- Methanol
- Ethanol
- Saturated solution of sodium bicarbonate ($NaHCO_3$)

2.2.4.2 Equipment

- Fume hood
- Digital orbital plate shaker
- Vacuum-sealed desiccator

2.2.4.3 Procedure

1. Day 4: Soak the cover glasses in 1% SDS for an hour in a 10-cm glass petridish (3–4 cover glasses per dish).
2. Wash the cover glasses in the following order: 1% SDS solution, hot water, and 2× with RT water. Now transfer the slides to fresh glass petridishes.
3. Place a sheet of aluminum foil in a fume hood. Top the foil with saran wrap. Place the petridish containing the washed slides onto the saran wrap.
4. Combine HNO_3 +HCl (2:1) in the hood and immediately add to the slides. Carefully seal the petridish with saran wrap and leave the slides overnight in the fume hood.
5. Day 5: Using acid-resistant forceps, transfer the slides to a fresh petridish. Discard the acidic solution by neutralizing it with a saturated $NaHCO_3$ solution.
6. Wash the coverslips thoroughly with distilled water three times for 3min each by keeping the petridishes on an orbital shaker at 70rpm.
7. After the third wash with water, perform two washes with methanol. Wipe any water on the cover or sides of the petridish by using a kimwipe. It is critical to ensure that there is no water during the aminosilanization step.
8. After the second methanol wash, add 20mL of methanol to each dish and then add 440 μL APTES dropwise to the cover glasses, while they are shaking in the petridish at 70rpm. Incubate for exactly 1h.
9. After an hour, wash once with methanol, three times with water, and then rinse with 95% ethanol. Each wash is at 70rpm for 3min.
10. Allow the cover glasses to dry by placing them in dark on a kimwipe with the aminosilated surface facing upward.
11. Transfer the cover glasses to a clean slide box and store them in the dark in a vacuum-sealed desiccator.

2.3.4.4 Notes

1. It is highly recommended to make small aliquots of APTES and store in a vacuum-sealed desiccator. Do not reuse aliquots and discard APTES after 3 months of opening.
2. Aminosilzation is sensitive to water. Ensure to remove any residual water before adding APTES.
3. Mixing at 70rpm and the 1 h incubation are both crucial for proper aminosilzation of the cover glasses.
4. It is always preferable to store the slides for at least a day before stretching the DNA fibers. Do not use slides that are more than 2 days old.

2.2.5 DNA Combing and Telomere FISH (Day 6)

2.2.5.1 Reagents

- Frosted glass slides
- PAP pen for immunostaining
- Methanol+0.1% βME
- Ethanol
- Formamide
- Blocking reagent for nucleic acids (Roche11096176001) dissolved in 100 m*M* maleic acid buffer pH 7.5
- 1 *M* Tris pH 7.5
- 100μ*M* Biotin-OO-$(CCCTAA)_4$ locked nucleic acid (LNA) TelC probe (Exiqon)
- Denaturation solution: 0.1N NaOH in 70% ethanol and 0.1% βME
- Fixation solution: 0.5% glutaraldehyde in the denaturation buffer

2.2.5.2 Equipment

- Wide-field microscope with a 60×/100× objective
- 37°C incubator

2.2.5.3 Procedure

1. Day 6: Take frosted slides and make hydrophobic boundaries along the edges using a PAP pen and allow to dry.
2. Take another set of frosted slides and flip the silanated cover glasses using forceps such that the aminosilated surface faces the slides. Also take care that the edge of these cover glasses aligns well with the slides.
3. Using a blunt-ended tip, pipette ~10 μL from the central part of the YOYO-stained digestion tube and place the drop on the side of the coverslip by letting the drop touch the slide. Tilt the slide to approximately 45 degrees and allow the liquid to flow by capillary action. Take the slide to the scope and image at 60× or 100× to visualize the quality of the stretching. Fig. 4 shows examples of well (Fig. 4A) and poorly stretched (Fig. 4B) DNA fibers. The quality of DNA stretching mainly depends on the how well the cover glasses are silanized. If the cover glasses are not uniformly silanized and are too sticky, DNA tends to aggregate and does not spread well.
4. If the fibers look well stretched, flip back the coverslip on a kimwipe using a razor so that the side with the DNA faces upward. Next, clean the immersion oil by using an ethanol-soaked kimwipe and place the coverslip in a moisturized hybridization chamber. Cover the coverslip with methanol containing 0.1% βME. Perform these steps for the rest of the samples.

5. Once all DNA samples are stretched, aspirate the methanol and add the denaturation buffer.

6. Two minutes before the end of the denaturation time, add glutaraldehyde to the denaturation buffer to a final concentration of 0.5%.

7. Remove the denaturation buffer and add the fixation buffer for 5 min.

8. Now perform a series of washes with 70%, 90%, and 100% ethanol. To perform these washes, incubate the cover glasses for 2 min with each ethanol solution, then aspirate and dry the coverslips before adding a more concentrated ethanol solution.

9. Remove ethanol as best as possible and then add methanol containing 0.1% βME for 15min.

10. Toward the end of this incubation period, make the hybridization buffer.

    For 2 mL of hybridization buffer:

    700 μL Formamide

    100 μL Blocker

    200 μL 1 *M* Tris pH 7.5

    2 μL 100 μ*M* Biotinylated LNA TelC probe

    998 μL H_2O

11. Pick each cover glass with forceps and vacuum it dry. Take the frosted slide with marked hydrophobic edges and spread 110 μL of the hybridization buffer. Take the dried cover glass with the stretched DNA and flip it on the slide so that the DNA faces the hybridization mix. Bubbles may form but they will dissolve during the overnight incubation. Keep the slide in a humidified hybridization chamber. Repeat these steps for the other cover glasses and then leave the hybridization chamber over-night in a 37°C incubator.

2.2.5.4 Notes

1. DNA is very sensitive to shearing and hence one needs to pipette and stretch the DNA fragments gently. We often cut the pointed ends of the micropipette tips to prevent DNA shearing during pipetting.

2. The time of denaturation is critical and needs to be optimized based on the length of the telomere. If the telomeres are long, extend the denaturation time. For telomeres isolated from U2OS and HeLa1.3 cells, a 9-min incubation time works well.

3. Glutaraldehyde is light sensitive and rapidly polymerizes in the presence of light. Turn off all lights before making the fixation solution and keep the lights off until the 70% ethanol wash.

4. We discard the glutaraldehyde bottle after 3–4 freeze thaw cycles.

2.2.6 Immunofluorescence Staining (Day 7)

2.2.6.1 Reagents

- 3% BSA in PBS
- Alexa 405-conjugated streptavidin
- Alexa 568-conjugated antimouse and Alexa 488-conjugated antirat
- Anti-CIdU antibody (AbD Serotec)
- Anti-IdU antibody (Becton Dickinson)

2.2.6.2 Procedure

1. Day 7: Next morning, flip open the coverslip using a razor such that the DNA containing side faces up.
2. Cover the cover glasses with a solution of 4× SSC containing 40% formamide for 3 min.
3. Wash the cover glasses with 1× PBS containing 0.03% IGEPAL. Incubate for ~1–2 min.
4. Wash the cover glasses with 1× PBS.
5. Remove PBS and add blocking buffer (3% BSA in PBS). Incubate for 30 min.
6. Aspirate the blocking buffer and add 200μL of 1:250 dilution of Alexa 405 streptavidin antibody in blocking buffer and incubate for 30 min at RT.
7. Wash three times (5 min each) with 1× PBS+0.03% IGEPAL.
8. Add 200 μL of 1:45 dilution of antistreptavidin antibody in blocking buffer and incubate for 30min at RT.
9. Wash three times (5 min each) with 1× PBS+ 0.03% IGEPAL.
10. To amplify the telomere FISH signal, repeat steps 6–9.
11. Add 200 μL of 1:45 diluted antistreptavidin, anti-IdU, and anti-CIdU and incubate for 1h.
12. After three washes with 1× PBS+ 0.03% IGEPAL, add 1:250 dilution of Alexa 405 streptavidin, Alexa 568 antimouse, and Alexa 488 antirat and incubate for 1h at RT.
13. Wash three times (5min each) with 1× PBS+0.03% IGEPAL, followed by a wash with PBS.
14. To mount the cover glasses, aspirate any liquid from the cover glass to dry. Pipette 100 μL of Prolong Gold antifade mounting media on a glass slide and mount the cover glass. Press the cover glass with a kimwipe gently to remove excess mounting media. Incubate the slide overnight at RT in the dark and allow the mounting media to harden.

15. The following morning, seal the edges of the coverslip with nail polish and allow the nail polish to dry at RT (~30 min). The slides can be then stored at 4°C until imaging.

2.2.7 Image Acquisition, Analysis, and Interpretation

2.2.7.1 Equipment

- Wide-field fluorescence microscope with 60×/100× objective
- ImageJ
- GraphPad Prism 7 or other data analysis program

2.2.7.2 Procedure

1. Acquire images using a wide-field fluorescence microscope, which is set up for three-color imaging. Images can be acquired using either a 60× or 100× objective.
2. Open the image file with ImageJ.
3. *Quantification of the percent of nascent telomere fibers and interpretation of the differentially labeled telomere fibers*: In ImageJ, use the Image>Color>Channel tool to generate a composite image of the three colors. Percent of fiber labeled=(blue fibers labeled in green or red)/(total number of fibers in blue). The different types of labeling observed are depicted in Fig. 5A (schematic) and B (representative images).
4. *Measurement telomere fiber length:* In ImageJ, use the Straight-Line tool to measure the fiber length. Select Analyze>Plot Profile to obtain the length of the fibers in microns. Measure the length of blue fibers to retrieve the length of telomeres. Measure the length of red/green label overlapping with blue to obtain the length of nascent fibers. For converting microns to kilobases, 10bp (equals one turn of the helix) has a linear length of 3.4nm. Hence, 0.26μm corresponds to 1kb of DNA.

2.2.7.3 Notes

1. For accuracy in length measurements, we do not quantify the length of fibers that are not straight.

3. IN VIVO IMAGING OF DNA DOUBLE-STRAND BREAK-INDUCED TELOMERE MOBILITY

Mobility increases following DNA damage have been previously described for both prokaryotes and eukaryotes, suggesting that such increases contribute to the repair of damage foci (Aten et al., 2004; Lesterlin, Ball, Schermelleh, & Sherratt, 2014). Mobility seems to play a particularly prominent role in DNA damage responses at telomeres (Cho et al., 2014; Dimitrova, Chen, Spector, & de Lange, 2008; Lottersberger, Karssemeijer,

Dimitrova, & de Lange, 2015). Here, we describe an in vivo system to rapidly induce double-strand breaks at telomeres and simultaneously track their mobility.

Our system involves the use of a fusion protein containing domains TRF1, FokI, mCherry, and ER-DD for telomere localization, DSB generation, fluorescence tracking, and rapid protein induction, respectively. The mCherry–ER-DD–TRF1–FokI construct is transfected into cells, and its expression is induced using 4-OHT and Shield-1. The stabilized protein construct moves to the nucleus and accumulates onto telomeres within minutes, where it generates DSBs. The trajectories of the resulting mCherry foci can be followed over time using confocal microscopy. The nuclease-inactive FokI D450A variant of our construct can be used as a negative control since it does not cleave DNA and can therefore eliminate the possibility of telomere movement resulting from ectopic protein over-expression rather than DSB generation (Cho et al., 2014; Shanbhag, Rafalska-Metcalf, Balane-Bolivar, Janicki, & Greenberg, 2010; Tang et al., 2013).

3.1 Materials

- DMEM cell culture medium with GlutaMAX (ThermoFisher), supplemented with 10% bovine calf serum and 1% Penicillin/Streptomycin (Gibco)
- 6-Well culture plates (Sarstedt)
- LipoD293 transfection reagent (Signagen)
- 22×22mm No 1.5 cover glass (Electron Microscopy Sciences)
- Incubator, 37°C and 5% CO_2
- Leibovitz's L-15 medium with L-glutamine, without phenol red (ThermoFisher)
- 4-Hydroxytamoxifen (Sigma) at 1mM stock concentration
- Shield-1 ligand (Clontech) at 0.5mM stock concentration
- Inverted fluorescence microscope (DM6000, Leica Microsystems), equipped with a charge-coupled device camera (QuantEM 512C, Photometrics), a 100×1.4 NA objective, automated XYZ stage (Ludl Electronic Products), an X-LIGHT Confocal Imager (Crisel Electrooptical Systems), and a SPECTRA X Light Engine (Lumencor)
- Stagetop heating incubator (Tokai Hit)
- Magnetic coverslip mounting chamber (Chamlide CM-S22–1, LCI)
- MetaMorph software (Molecular Devices)
- ImageJ Fiji (NIH, http://fiji.sc/)
  - TrackMate plugin (http://imagej.net/TrackMate)
  - StackReg plugin (Dr. Philippe Théevenaz, EPFL)
- MATLAB software (MathWorks)

- Mean square displacement (MSD) analyzer scripts (Dr. Jean-Yves Tinevez, Institut Pasteur)
  - importTrackMateTracks (https://github.com/fiji/TrackMate/blob/master/scripts/importTrackMateTracks.m)
  - MSD analyzer (https://github.com/tinevez/msdanalyzer)

3.2 Procedure

1. Place 22×22mm square glass coverslips into 6-well plates and sterilize under UV light for 15min. Seed 200,000–250,000 cells in 2mL culture media in the wells. Cells should be at 60%–90% confluence on the day of imaging.
2. Following 24h of incubation, use the LipoD293 reagent and serum-free DMEM to transfect 1μg of mCherry–ER-DD–TRF1–FokI plasmid into cells. Incubate cells for 16 h prior to protein induction.
3. Replace the culture media with fresh DMEM containing 1μ*M* Shield-1 and 1μ*M* 4-OHT 1h prior to mounting. Then, transfer the coverslips to a magnetic coverslip mounting chamber. Place 1mL of prewarmed (37°C) L-15 media with 1μ*M* Shield-1 and 1μ*M* 4-OHT on the coverslip. The mounting chamber should be loaded into a preequilibrated, humidified Tokai Hit stagetop incubator with the following settings: top heater 41°C, stage heater 43°C, lens heater 37°C, and an intrachamber temperature of 37°C.
4. Use the 100× objective to visualize mCherry-expressing nuclei. Using the MetaMorph software, select 10–12 stage positions for imaging. It is important to put the nucleus of interest at the center of the field of view as it is possible that cells migrate out of the stage view during imaging.
5. Acquire images as *z*-stacks of 0.6μm interval for a total depth of 8–10μm which should be enough to cover the entire nucleus. Each stage position should be sequentially imaged for 2 min adding up to a total imaging time of 60 min.

3.3 Quantitative Analysis

The resulting images can be processed using the ImageJ software.

1. Load all *z*-stacks for a given stage position and obtain a maximum intensity projection of the *z*-stack set. U2OS cells are relatively flat, and therefore, the information contained in the *x*–*y* plane is sufficient for quantitation of telomere mobility. Moreover, since the nuclei move and change shape during live-cell imaging, it is important to register the images (Thenevaz, Ruttimann, & Unser, 1998). Image registration is an algorithmic transformation which allows the alignment of the images in the series to the reference image. This can be achieved using the “scaled rotation” option of the StackReg plugin.
2. Telomere tracking is achieved using the TrackMate plugin in Fiji (Schindelin et al., 2012). For each image, the scale should be set to μm according to the pixel—μm conversion ratio for the 100× objective on the microscope. The following

settings should be used for the annotation of telomere foci: log detector, estimated blob diameter 0.7μm, filter by contrast and quality. Manual corrections are often necessary when the algorithm fails to separate telomere foci that may overlap temporarily during imaging. Each track represents the movement of a telomere spot over the 60min of imaging. Use the "simple LAP tracker" setting to establish the tracks in TrackMate with the following settings: linking max distance of 2μm, gap-closing max distance of 2μm, and gap-closing max frame gap of 2. Then, export the time and positional data generated from telomere tracking to an XML file for MATLAB analysis.

3. Import the track XML files into MATLAB using the importTrackMateTracks script.

4. Use the script, MSD analyzer, to perform a MSD analysis, which is a measure of the average distance that a particle travels (see equation in Fig. 6A) (Tarantino et al., 2014). In MSD analysis, MSD values are generated for each time interval. The MSD values at each time interval are averaged for all particles involved in the experiment and plotted on a graph of MSD on the *y*-axis and time interval on the *x*-axis (Fig. 6B). For example, for a 60-min experiment with 30 time points, where the time interval is 2min, 29 interval square displacements are averaged.

3.4 Notes

Alternative analyses can also be used to quantify telomere mobility. For instance, counting the number of telomere merging events occurring per nucleus per hour is a metric of the magnitude of telomere movement. A simpler approach would be to count the number of telomere foci at time 0 and compare it to the number of foci at the end of the sequence (Cho et al., 2014).

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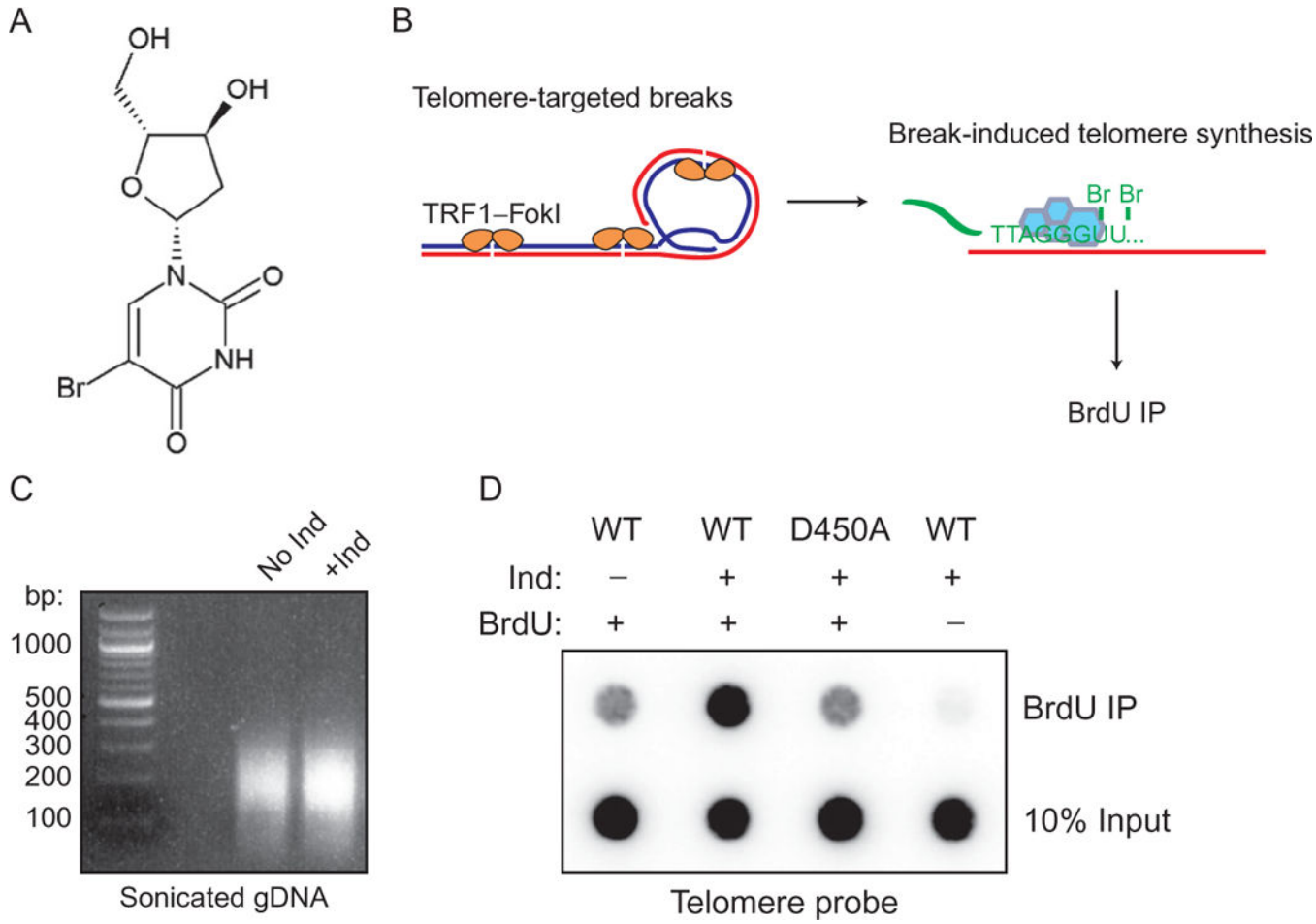

Fig. 1.

(A) Chemical structure of BrdU. (B) Schematic of BrdU incorporation into nascent telomeres after TRF1–FokI breaks. (C) Sample ethidium bromide-stained agarose gel of sonicated gDNA from U2OS cells induced with TRF1–FokI for 2 h. (D) Sample BrdU IP dot blot for telomere content using a ^{32}P-labeled telomere oligonucleotide from U2OS cells induced with TRF1–FokI for 2 h. *BrdU*, 5-bromo-2′-deoxyuridine; *IP*, immunoprecipitation; *gDNA*, genomic DNA; *Ind*, induction of TRF1–FokI; *WT*, wild-type TRF1–FokI; *D450A*, nuclease-deficient TRF1–FokI.

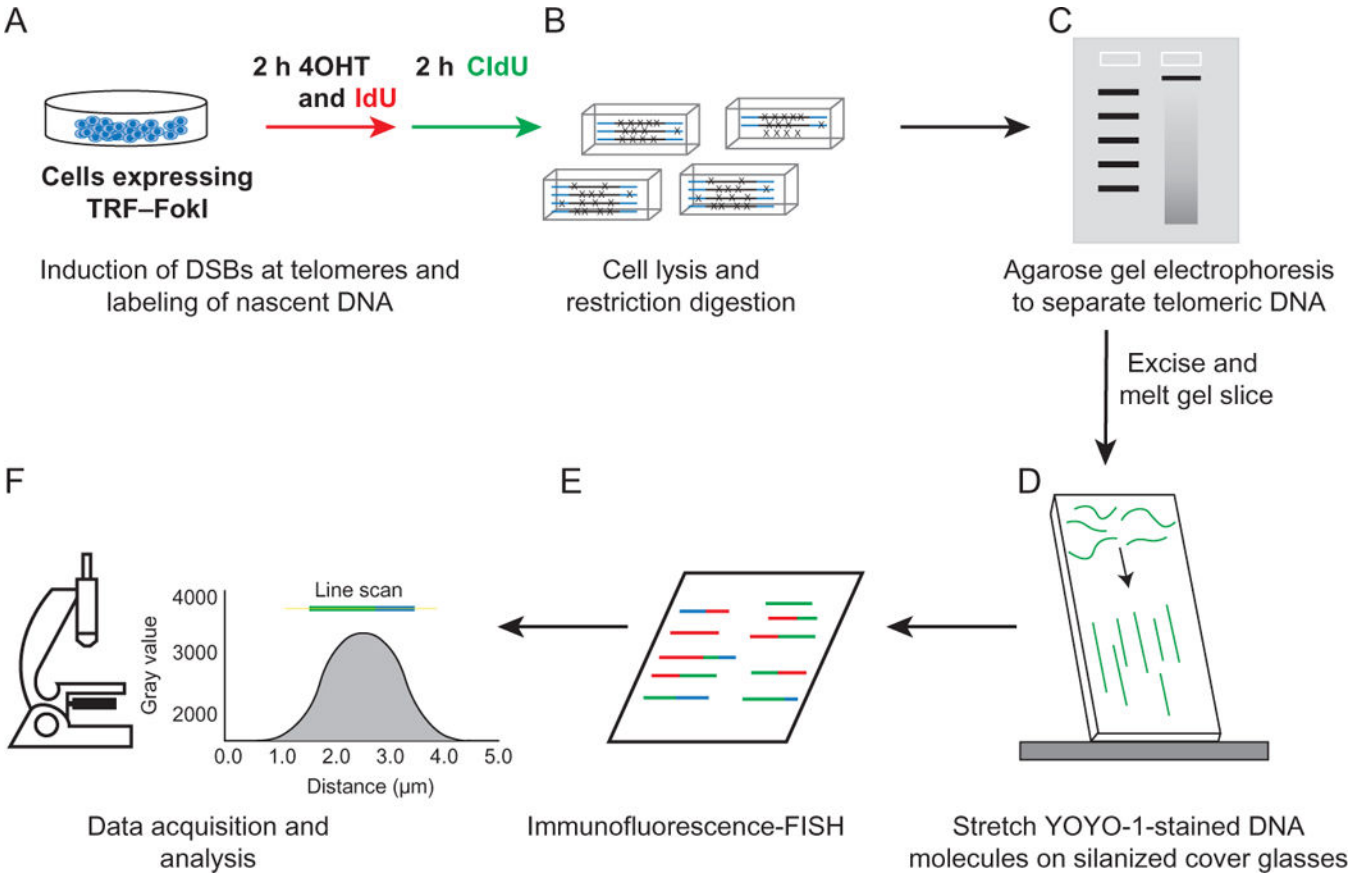

Fig. 2.

Overview of the SMARD assay to study break-induced telomere synthesis. (A) Induction of DSBs at the telomeres and labeling of nascent DNA by thymidine analogues: IdU and CIdU. (B) Cell lysis and digestion of genomic DNA by *Alu*I and *Mbo*I. The × sign represents multiple restriction sites for the two enzymes in nontelomeric DNA. However, due to the GC-rich nature of telomeric DNA, most of the telomeres remain uncut by these two frequent cutters. (C) Agarose gel electrophoresis to separate the digested nontelomeric genomic DNA from the telomeres. (D) The isolated DNA is YOYO-1 stained and stretched onto silanized cover glasses. (E) Immunofluorescence-FISH to detect nascent telomeres. (F) Image acquisition and analysis.

Fig. 3.

Autoradiogram of an agarose gel hybridized with a ^{32}P-telomeric probe. Lane 1: Undigested DNA from a U2OS cell line. Lane 2: DNA from U2OS cells digested with *Alu*I and *Mbo*I. Lane 3: DNA from U2OS cells labeled with 30μ*M* IdU and 30μ*M* CIdU and digested with *Alu*I and *Mbo*I. This lane shows that the incorporation of the thymidine analogue does not affect the digestion efficiency of the restriction enzymes.

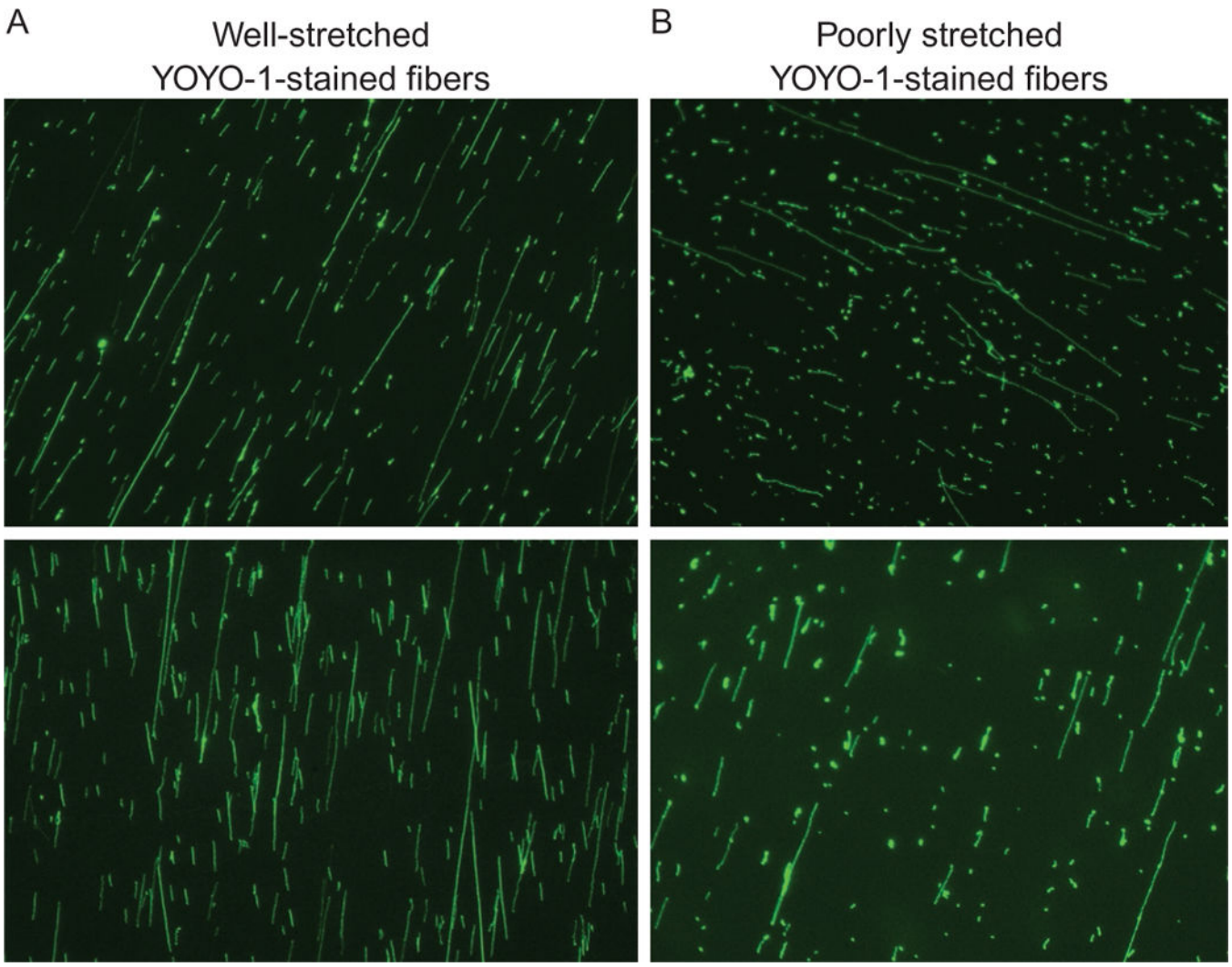

Fig. 4.

YOYO-1-stained DNA fibers stretched onto silanized coverslips. (A) Well-stretched DNA fibers. (B) Poorly stretched DNA fibers.

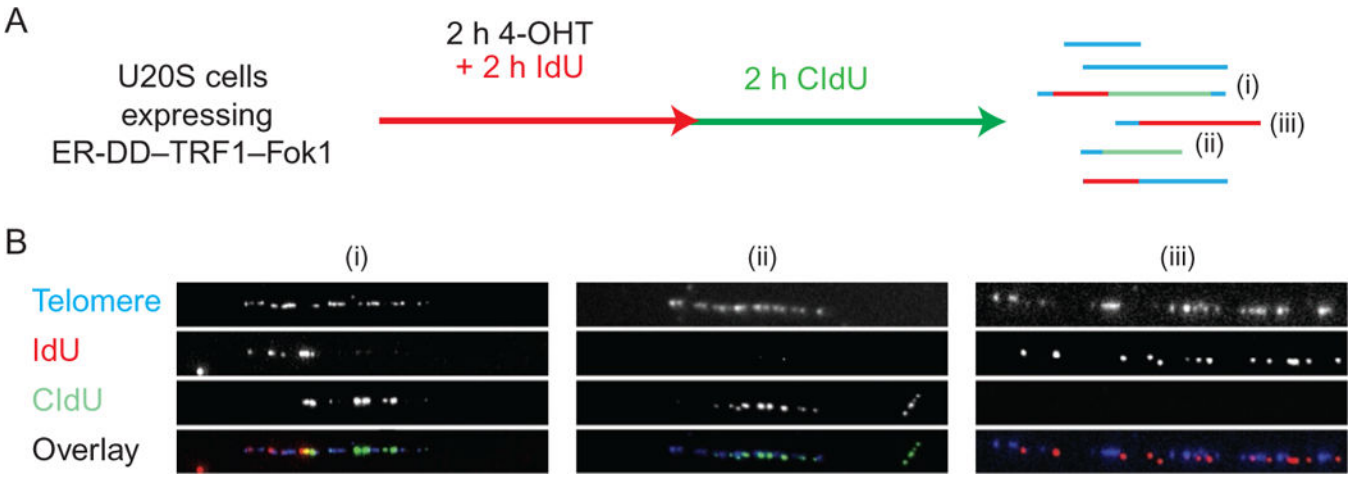

Fig. 5.

(A) Schematic of the labeling pattern observed on telomeres (*blue*) post labeling with IdU (*red*) and CIdU (*green*). (B) Representative examples of telomeres (*blue*) tracts labeled with (i) both IdU and CIdU (ii) CIdU only (iii) IdU only.

A

$$\mathrm{MSD}i_{\Delta t} = \frac{1}{N} x \sum_{t=0}^{N} \sqrt{(x_{t+\Delta t} - xt)^2 + (y_{t+\Delta t} - yt)^2}$$

B

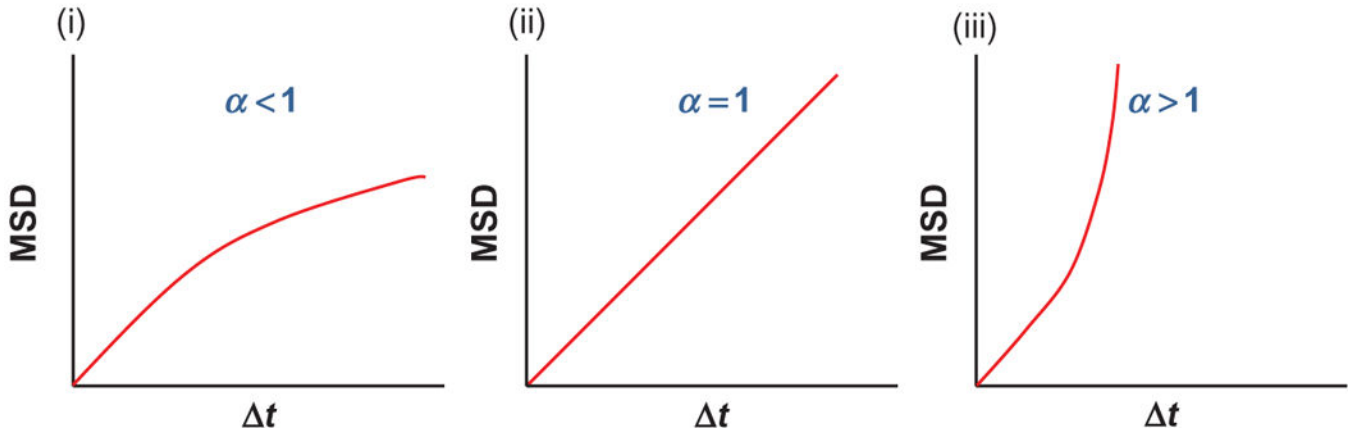

Fig. 6.

(A) Equation of mean square displacement (MSD) during Δt intervals for a particle i traveling in a 2D plane. (B) MSD plots of different classes of mobility. The equation, MSD= Γt^{α}, is used to provide a best fit curve for the MSD trajectories. Coefficient Γ indicates the magnitude of travel, while α is a time-dependence coefficient which indicates the type of movement the particle exhibits. (i) α Values of less than 1 indicate subdiffusion resulting from restriction to diffusion which would occur during molecular crowding in the nucleus. (ii) α Values of 1 indicate Brownian diffusion. (iii) α Values of greater than 1 indicate superdiffusive mobility. An α value of 2 would be characteristic of a particle traveling in a directed manner (Guigas & Weiss, 2008; Marshall et al., 1997; Zajac, Goldman, Holzbaur, & Ostap, 2013).