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***In vivo* imaging of DNA double-strand break induced telomere mobility during alternative lengthening of telomeres**

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Abstract

Repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) requires mobilization of chromatin for homology searches that allow interaction of the sequence to be repaired and its template DNA. Here we describe a system to rapidly induce DSBs at telomeres and track their movement, as well as a semi-automated workflow for quantitative analysis. We have successfully used this approach to show that DSBs targeted to telomeres in cells utilizing the alternative lengthening of telomeres (ALT) mechanism increase their diffusion and subsequent long-range directional movement to merge with telomeres on other chromosomes. These methods are simple to implement and are compatible with almost any cell line or *in vivo* microscopy setup. The magnitude of DSB-induced telomere mobility allows the investigator to easily test for factors regulating telomere mobility during ALT.

Keywords

ALT; Live cell imaging; Confocal microscopy; Chromatin mobility; Homologous recombination

1. Introduction

One of the hallmarks of alternative lengthening of telomeres (ALT) is coalescence of telomeres into large clusters known as ALT-associated PML Bodies (APBs). These structures contain telomeres from multiple chromosomes as well as extra-chromosomal telomere repeats (ECTR) [1–3]. APBs accumulate γ -H2AX and incorporate BrdU, suggesting that DSB-dependent telomere synthesis occurs within these recombination centers [4]. We hypothesized that homologous recombination could drive movement and

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clustering of telomeric DSBs into APBs to achieve net telomere synthesis and elongation during ALT. To this end, we generated a system to visualize the mobility of telomeres following a coordinated induction of DSBs.

DNA damage-dependent mobility increases have been described in both prokaryotes and eukaryotes, which suggests that increased movement of DSBs contribute to the repair of these foci [5–8]. These studies most frequently employed fluorescent molecular tags that allow tracking of DNA binding proteins such as 53BP1, while others have successfully used labeled dNTP analogs [9]. One of the challenges in employing these methods, however, has been the disconnection of modalities to generate the DSBs and subsequent tracking of the broken DNA, adding to the experimental complexity as well as requiring an interpolation that the surrogate DNA damage marker proteins accurately reflect the location of the DSBs.

Here we describe a simple approach that we have developed to rapidly induce DSBs at telomeres and simultaneously track their mobility *in vivo*. Our system consists of a fusion protein that contains domains for telomere localization (TRF1), DSB generation (FokI), fluorescence tracking (mCherry) and rapid protein induction (ER-DD). Transfection of mCherry-ER-DD-TRF1-FokI and subsequent induction using 4-OHT and Shield-1 leads to protein stabilization, nuclear translocation and accumulation onto telomeres within minutes, generating DSBs [10] (Fig. 1a). The trajectories of these mCherry foci are then followed in live cells using confocal microscopy. Importantly, we have also created a nuclease-inactive FokI D450A variant of our construct, which does not cleave the DNA [11]. This allows a controlled analysis of telomere movement that occurs as a result of DSB generation independent of ectopic protein overexpression. Our system can be employed in any cell type amenable to DNA transfection. It also allows visualization of a GFP-tagged second protein of interest via concurrent transfection.

2. Methods

2.1. Cloning of inducible TRF1-FokI

Coding sequences for estrogen receptor (ER), mCherry, TRF1 and FokI endonuclease domain [12] were cloned in frame downstream of the sequence for destabilization domain (DD) in the pLVX-pTuner N plasmid (Clontech). A complete sequence of the plasmid is provided (Supplementary file 1).

2.2. Cell preparation for imaging

2.2.1. Materials and reagents

- DMEM with GlutaMAX cell culture medium (ThermoFisher), supplemented with 10% bovine calf serum and 1% Penicillin/Streptomycin (Gibco)
- 6-well culture plates (Sarstedt)
- 22 mm × 22 mm No. 1.5 cover glass (Electron Microscopy Sciences)
- Incubator, 37 °C and 5% CO₂
- LipoD293 transfection reagent (Signagen)

2.2.2. Cell culture and transfection—One 22 mm × 22 mm square glass coverslip is placed into a 6-well plate and sterilized in the culture hood under UV light for 15 min. Cells are then trypsinized, counted and 200,000–250,000 cells are seeded in 2 ml culture media into the wells. The goal is to reach 60–90% confluence by the time imaging is performed, and this initial seeding number may change depending on additional treatments such as siRNA transfection. It is helpful to tap lightly on the coverslip using a pipette tip to remove any air trapped underneath in order to prevent the coverslip from floating in the media during incubation. Shaking the plate in perpendicular directions following plating allows optimal dispersion of cells throughout the well to prevent central clumping, which would be detrimental to imaging. After 24 h of incubation, 1 µg of mCherry-ER-DD-TRF1-FokI plasmid is transfected into cells using LipoD293 reagent and serum free DMEM. At this time, a plasmid coding for a GFP-tagged protein can be co-transfected if desired. Cells are further incubated for 16 h prior to protein induction, mounting and imaging (Fig. 1b).

2.3. Mounting, cell selection and image acquisition

2.3.1. Materials and reagents

- Leibovitz's L-15 medium with L-glutamine, without phenol red (ThermoFisher), supplemented with 10% bovine calf serum and 1% Penicillin/Streptomycin (Gibco)
- Shield-1 ligand (Clontech), stock concentration 0.5 mM
- 4-hydroxytamoxifen (Sigma), stock concentration 1 mM
- Inverted fluorescence microscope (DM6000, Leica Microsystems), equipped with a 100 × 1.4 NA objective, automated XYZ stage (Ludl Electronic Products), a charge-coupled device camera (QuantEM 512SC, Photometrics), an X-LIGHT Confocal Imager (Crisel Electrooptical Systems) and a SPECTRA X Light Engine (Lumencor)
- Magnetic coverslip mounting chamber (Chamlide CM-S22-1, LCI)
- Stagetop heating incubator (Tokai Hit)
- MetaMorph Software (Molecular Devices)

2.3.2. Procedure for mounting, nuclei selection and image acquisition—1 h prior to mounting, the culture media is changed with fresh supplemented DMEM containing 1 µM Shield-1 and 1 µM 4-OHT. After this incubation period, the coverslip is transferred to a magnetic coverslip mounting chamber and 1 ml of prewarmed (37 °C), supplemented L-15 media with 1 µM Shield-1 and 1 µM 4-OHT is added on top of the coverslip (Fig. 2a). It is important to pre-warm the L-15 media since imaging will proceed shortly after loading the coverslip and lower temperatures will alter whole cell dynamics as well as intranuclear particle mobility. The mounting chamber is loaded into a pre-equilibrated, humidified Tokai Hit stagetop incubator set to the following heating parameters: top heater 41 °C, stage heater 43 °C, lens heater 37 °C, leading to an intrachamber temperature of 37 °C (Fig. 2b and c).

Using the MetaMorph software, nuclei expressing mCherry are identified using the 100× objective. A total of 10–12 stage positions selected at random can be marked for imaging. If a GFP-tagged protein is co-expressed, cells expressing both mCherry and GFP are selected, and about 6 stage positions are selected in order to allow sufficient time for imaging both channels during the acquisition interval (2 min). Care should be taken to place the nucleus of interest at the center of the field of view, since during 60 min of image acquisition, some cells may occasionally migrate out of the stage view. The total time required from mounting the coverslip in the magnetic chamber to identifying the stage positions is approximately 15 min.

Once ready, images are collected as z-stacks at 0.6 μm intervals for a total of 8–10 μm in depth, which is enough to cover the entire nucleus from top to bottom. Each stage position is sequentially imaged in this manner at 2 min intervals, for a total of 60 min.

2.4. Image processing, telomere tracking and MSD analysis

2.4.1. Materials and reagents

- ImageJ Fiji (NIH, <http://fiji.sc/>)
 - StackReg plugin (Dr. Philippe Thévenaz, EPFL)
 - TrackMate plugin (<http://imagej.net/TrackMate>)
- MATLAB software (MathWorks)
- MSD analyzer scripts (Dr. Jean-Yves Tinevez, Institut Pasteur)
[importTrackMateTracks](https://github.com/fiji/TrackMate/blob/master/scripts/importTrackMateTracks) (<https://github.com/fiji/TrackMate/blob/master/scripts/importTrackMateTracks.m>)
- MSD Analyzer (<https://github.com/tinevez/msdanalyzer>)

2.4.2. Procedure for image processing and telomere tracking—In ImageJ, the Image Sequence function under the Import menu is used to load all z-stacks for a given stage position. The Grouped Z Project function is then used to produce a series of projected single images for each z-stack set. “Max Intensity” is used as the projection method (Fig. 3a). Using a projected 2D image of the 3D stacks discards information about the particles’ mobility in the z plane. Despite this, U2OS nuclei are relatively flat and particles travel predominantly in the x–y plane which makes 2D analysis sufficient for quantitation of telomere mobility. This also greatly simplifies the process for telomere identification and tracking.

The nuclei often move across the stage, change their shape or rotate significantly during the 60 min of imaging. Therefore, it is critical to employ a process of image registration, which is an algorithmic transformation that allows alignment of the images in the series to the reference image (the first image in the series). For this purpose, the StackReg plugin is used [13]. The option, “scaled rotation”, is used.

Telomere tracking is performed using the TrackMate plugin in Fiji [14]. Once the image sequence is loaded in TrackMate, the scale is set to μm based on the pixel- μm conversion

ratio for the 100× objective on the microscope. All telomere foci are annotated using the following settings: Log detector, estimated blob diameter 0.7 μm, filter by contrast and quality. While the majority of spots are automatically identified in this manner, some errors need to be corrected manually. For instance, one of the most common issues is failure of the algorithm to identify separate telomere foci that overlap temporarily over the course of imaging due to the fact that the analyzed images are 2D flat projections of the 3D nucleus. Following this manual correction, track analysis can be performed.

Each “track” representing the movement of a telomere spot over the 60 min of imaging is established using TrackMate. The “simple LAP tracker” setting is used, with a linking max distance of 2 μm, gap-closing max distance of 2 μm, and gap-closing max frame gap of 2. This allows the plugin to generate a collection of individual tracks that each represents the movement of a telomere focus over time (Fig. 3b). TrackMate is capable of handling particle merging and division. Two tracks that have merged will be considered a single track for the rest of the sequence. Finally, these tracks containing time and positional data are exported to an XML file for MATLAB analysis using a pulldown function available when reaching the last menu options page in TrackMate.

2.4.3. MSD analysis—The track XML files are imported into MATLAB using a script, importTrackMateTracks. A mean square displacement (MSD) analysis is performed with a second script, MSD Analyzer [15]. The MSD is a measure of the average distance that a particle travels. For a particle, i , traveling in a 2D plane, it is given by the Eq. (1).

$$MSDi_{\Delta t} = \frac{1}{N} \times \sum_{t=0}^N \sqrt{(x_{t+\Delta t} - x_t)^2 + (y_{t+\Delta t} - y_t)^2} \quad (1)$$

In other words, for particle i , its squared displacements during all possible Δt intervals are summed and averaged to generate $MSDi_{\Delta t}$. This means that for tracking telomere mobility over 60 min with 30 time points at 2 min intervals, calculation of MSD at $\Delta t = 2$ min involves averaging 29 interval squared displacements (between $t = 0$ and $t = 2$ min, between $t = 2$ min and $t = 4$ min etc). For $\Delta t = 4$ min, there would be 28 averaged values, and so on until $\Delta t = 60$ where there is just one value (between $t = 0$ and $t = 60$ min). Finally, the MSD values at each Δt are averaged for all particles in the experiment and plotted on a graph with Δt on the x-axis and MSD on the y-axis.

MSD Analyzer also allows calculation of a weighted standard error of the mean and displays this as error bars for each time delay [1] (in referenced paper, see Fig. 3D). This serves as a measure of statistical confidence of the MSD value at each Δt . Weights are taken to be the number of averaged data points at each time interval, which means that MSDs at shorter time intervals carry a smaller s.e.m. and a higher confidence level than MSDs at longer time intervals where fewer data points are available. To better achieve statistical significance at a desired Δt , one should ideally obtain images for a longer period of time than Δt such that more data points are available for averaging. Alternatively, the number of telomere tracks included in the analysis could simply be increased to gain statistical confidence since in this

mode of averaged MSD analysis, we assume that all particles are sampling the same process; i.e. they have the same diffusion property.

In order to describe the mobility characteristics of telomere particles, the MSD trajectories are fitted using a single exponential time dependence diffusion model described by $MSD = \Gamma t^\alpha$, where Γ is a generalized coefficient indicating the magnitude of travel, and α is a time dependence coefficient that can describe type of diffusion exhibited by the particle [16]. Of note, α is of particular importance in characterizing particle diffusion. An α value of 1, which represents an MSD trajectory that increases linearly, indicates that the particles are undergoing normal Brownian motion. This occurs for particles that travel unhindered through a constant medium in a random walk, such as pollen grains diffusing in water as originally described by Robert Brown. Trajectories where α is less than 1 demonstrate sub-diffusion, also known as anomalous diffusion, that occurs as a result of molecular crowding as seen in a dense environment such as the nucleus that constrains normal diffusion [17]. Finally, $\alpha > 1$ where the slope of the MSD trajectory increases as t increases indicates that the particle is traveling in a directed manner, such as dynein-driven molecular motors [18] (Fig. 4a–c).

Lastly, the time-dependent diffusion coefficient, $D(t)$, at a given time point can be calculated using the equation $D(t) = MSD/t = \Gamma t^{\alpha-1}$ [19]. These values were used to compare the diffusion coefficient at matched time points with previous studies.

2.4.4. Alternative modes of analysis—While the MSD analysis requires a labor intensive tracking process, telomere mobility can be quantified more expediently using alternative analyses. A useful method is to count the number of telomere merging events that occur per nucleus per hour, a metric that correlates with the magnitude of telomere movement. Even more simply, the number of telomere foci at time 0 can be compared to the number of foci at the end of the sequence, although this method is limited by the fact that some of the least fluorescent foci may no longer be visualized at the end due to photobleaching.

3. Discussion

Here we have described a streamlined system for evaluation of DSB-induced telomere mobility. While there are limitations and challenges to the system, there are several distinct advantages for assessing telomere movement. First, using mCherry-ER-DD-TRF1-FokI is exceedingly simple and experiments can be done rapidly. Seeding cells, transfection of the plasmid and visualization of telomeres can be accomplished within 48 h. This straightforward workflow allows the investigator to survey factors of interest that may affect telomere mobility using siRNA or small molecule inhibitors. Many different cell types can be used, even those with low transfection efficiencies since cells expressing mCherry can be easily identified and individually imaged.

Furthermore, using 4-OHT and Shield-1 leads to a coordinated induction of the protein and generation of DSBs. mCherry foci begin to uniformly appear within the nuclei by 15 min, increasing intensity to a steady level by about 1 h. γ -H2AX levels by western blot and

immunofluorescence on fixed cells also rise with similar kinetics during this period, indicating that DSBs also accumulate rapidly upon induction of TRF1-FokI. Simultaneous induction of DSBs at telomeres is critical for visualizing ALT telomere mobility, since many clustering events occur expediently following recruitment of TRF1-FokI to telomeres.

The D450A catalytic domain mutant serves as a critical control for evaluation of telomere mobility. Telomeres in U2OS cells demonstrate a baseline level of diffusive mobility as well as rare clustering events [20]. Using D450A allows the investigator to assess increases in telomere mobility and clustering that are due to DSB generation, independent of potential complicating factors such as mCherry expression and TRF1 overexpression.

There are some limitations to the system. Since the directional component of ALT telomere movement occurs over minutes, a 60 min imaging window was sufficient for surveillance of these events. However, continuous imaging over 90–120 min leads to significant attenuation of the mCherry signal such that the data is no longer usable for telomere tracking. A second issue is that often there can be cell-to-cell variability in terms of starting number of telomeres, telomere size, and the nucleus size. This may relate to the cell cycle variation in the population, which was not tracked in this system. As a result, data gathered from 20 to 30 cells, representing thousands of mobility tracks, were pooled to generate the MSD curves and this is labor intensive.

Ways to improve the system could be to incorporate a way to switch off the expression of TRF1-FokI. While clustering of telomeres was the predominant reaction, foci could be sometimes seen splitting into separate signals, perhaps representing a resolution event following recombination. Turning on TRF1-FokI and subsequent inactivation or degradation of the protein may allow increased frequency of these dissolution events and allow us to study this process. It would also be interesting to devise a way to induce a DSB at just one or a few telomeres, instead of all telomeres within the nucleus. This would enable a more precise evaluation of the relationship between the damaged chromatin and the intact template. Finally, employing light-sheet microscopy to image telomere clustering instead of confocal imaging described here could be very useful given that it will allow longer imaging on the order of days to potentially capture clustering and resolution of telomeres in sequence following inactivation of TRF1-FokI. It could also provide better 3D tracking of telomeres, an advantage over our 2D analysis.

In summary, the TRF1-FokI system allows visualization of DSB-dependent telomere mobility in an easy to use protocol that combines DSB generation and telomere tracking. This system can be adopted in a number of different cell lines to assess telomere mobility and the factors that regulate chromatin dynamics in this setting. DNA repair by HR requires a complex manipulation of DNA strands involved, and the TRF1-FokI system offers a convenient viewing window into this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALT	alternative lengthening of telomeres
HR	homologous recombination
DSB	double-strand break
MSD	mean square displacement
ER	modified estrogen receptor domain
DD	destabilization domain
4-OHT	4-hydroxytamoxifen

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymeth.2016.07.010>.

Fig. 1. Illustration of mCherry-ER-DD-TRF1-FokI and experimental workflow. (A) Once transfected, mCherry-ER-DD-TRF1-FokI is sequestered in the cytoplasm by the ER domain and is also continually degraded by the DD domain. Upon administration of 4-OHT and Shield-1, the protein is stabilized and rapidly translocated to the nucleus where it binds telomeres and generates DSBs. (B) Standard workflow for telomere imaging is illustrated.

Fig. 2. Mounting coverslips in preparation for live cell imaging. (A) The square coverslip is placed in a magnetic coverslip chamber, and pre-warmed L-15 media is pipetted directly on top of the coverslip inside the chamber. (B) The magnetic chamber is placed into a pre-temperature equilibrated Tokai Hit stage with water in the surrounding moat to ensure humidification. The objective heater sleeve is placed around the objective lens. (C) Temperature settings are set as indicated in the text.

Fig. 3. Example of images obtained from live imaging. (A) Representative z-projection of cells transfected with mCherry-ER-DD-TRF1-FokI D450A (left) or WT (right) and imaged for one hour. (B) Representative telomere tracks for cells transfected with mCherry-ER-DD-TRF1-FokI D450A (left) or WT (right), generated using TrackMate.

Fig. 4.

Illustration of MSD plots that represent different classes of mobility. The MSD trajectories are fitted using a model described by $MSD = \Gamma t^\alpha$, where Γ is a generalized coefficient indicating the magnitude of travel, and α is a time dependence coefficient indicating the type of movement exhibited by the particle. (A) $\alpha = 1$ describes a particle undergoing normal Brownian diffusion. (B) α values of less than 1 indicates sub-diffusion, also known as anomalous diffusion, that occurs as a result of restriction to diffusion such as molecular crowding within the nucleus. (C) α values of greater than 1 indicates that the mobility is super-diffusive. In particular, $\alpha = 2$ is characteristic of particles traveling in a directed manner.