Published in final edited form as:

Methods Mol Biol. 2013 ; 962: 147–155. doi:10.1007/978-1-62703-236-0_12.

Use of the DNA Fiber Spreading Technique to Detect the Effects of Mutant p53 on DNA Replication

Rebecca A. Frum, Sumitra Deb, and Swati Palit Deb

Abstract

DNA replication involves a coordinated progression through S phase, and disruption of these regulated steps may cause gene abnormalities, which may lead to cancer. Different stages of DNA replication can be detected immunofluorescently that would indicate how replication is progressing in a cell population or under specific conditions. We describe a method for labeling replicating DNA with two nucleotide analogs, and then detecting the sequential patterns of incorporation using fluorescently labeled antibodies on DNA spread onto a glass slide.

Quantification of the different types of replication patterns produced by this method reveals how replication is achieved under different conditions by the predominance and lengths of elongating

¹IdU and CldU are light sensitive, so it is recommended that labeling is performed without the light on in the hood.

²Plating 374,000 cells per 60 mm plate is recommended to ensure proper availability of nucleotide analogs during pulse labeling.

³Aphidicolin can be used to increase the number of cells synchronously entering S phase after 24 h; however, this may result in checkpoint activation. Confluence arrest alone generally creates a sufficient population of cells entering S phase synchronously. It generally takes about 13–14 h for confluence-arrested cells to reach early S phase following replating and approximately 8–10 h for cells to progress through S phase.

⁴To save time during labeling, mixtures of IdU/media or CldU/media can be made in advance on the day of labeling and added directly to plates to save time with multiple additions.

⁵While creating DNA fiber spreads, keeping the cell sample at room temperature just before streaking the cells onto the slide creates an even spread of cells and prevents the cell sample from forming droplets when streaked across the slide.

⁶After streaking the cell sample on the slide, the streak appears rounded on the surface. Add the lysis buffer when the surface of the cell sample is flat to the surface of the slide but before it is dry.

⁷After tilting the slide to create the fiber spreads, use a p20 pipette tip to create a small “point” on the edge of the droplet. This encourages the drop to proceed down the slide at a good rate (usually 5–10 min).

⁸To save time, the antibody mixtures can be made up during the initial blocking step in BSA and stored in dark at 4°C.

⁹Bubbles generated when placing the plastic strips over the slides during antibody addition steps can be removed by sliding the plastic a little off the slide and then returning it.

¹⁰When mounting coverslips with antifade, bubbles can also be generated. To prevent bubbles, place the glass coverslip on the bench and place three drops of antifade (about 100 µl total per slide) onto the coverslip. Dry the back surface of the slide and place it face down onto the antifade drops. Usually, keeping a hold of the slide while allowing it to just barely touch the surface of the drops at the same time creates an even spreading of the antifade with minimal bubbles. When bubbles are present, they can be removed by tapping one side of the bubble with tweezers.

¹¹When focusing the confocal microscope to find the fibers, they are on the same plane as the surface of the slide. Focusing on the surface of the slide first (tiny dots of fluorescence or any label on the slide) will enable the detection of the DNA fibers.

¹²Images can be analyzed using the Image J software program. To ensure that all fibers in an image are scored, create an RGB image and after measuring the track length or counting the number of fibers, erase the scored fiber in the RGB image using the eraser tool. Since there are always fibers present in the images that are unscorable (overlapping fibers, overly stretched fibers, undistinguishable labeled tracks), this helps ensure that all scorable fibers are counted in the image.

¹³Slides can usually be analyzed for up to a month after staining.

¹⁴This type of analysis can give both quantitative (length of fibers) and qualitative (percent of type of replication patterns) present in the samples. If checking for both parameters, it is recommended that the length of the fibers be measured and the number of these scored fiber lengths be used to generate the percent of each replication pattern out of the total number of scored patterns. This ensures that the same fibers are used for both analyses.

replication forks progressing from single or clustered origins, as well as the sites of termination from two converging forks.

Keywords

DNA replication; Fiber spreading; Origins; Clusters; Fork elongations and terminating forks

1. Introduction

The replication of DNA has been studied on the level of individual fibers for almost five decades, and the techniques for studying it have developed over the years to detect and address different aspects of the replication process. Early replication studies used autoradiography to show that replication proceeds in distinct patterns and made use of a pulse-chase with tritiated thymidine to show the direction of fork movement from origins as indicated by the different intensities of the incorporated radioactivity (1–3). In the 1990s, several groups were able to use fluorescence in situ hybridization (FISH) to map decondensed DNA in nuclei or to detect single fibers of DNA extended onto slides (4–7). Other groups have subsequently combined the incorporation of single (8) or double (9) nucleotide analogs such as iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) and their detection by fluorescently labeled antibodies to show different patterns of replication by color instead of radioactivity intensity. The double labeling method has been used for analyzing replication dynamics in subsequent studies (10–12).

The use of more than one marker such as different intensities of incorporation of radioactivity, or the use of more than one nucleotide analog sequentially incorporated into replicating DNA, enables the detection of different types of replication events occurring in the cell based on the directionality of fork movement. In the method we describe here, asynchronous cells are first pulsed with IdU for 10 min, which is then washed out and is followed by a pulse with CldU for 20 min. The IdU and CldU are incorporated into replicating DNA sequentially in the direction of fork movement. The cells are then collected, and a defined small number of cells is streaked across the top of a silane-coated slide and is then lysed. The slides are then tilted to a 45° angle, and as the droplet of lysis buffer is pulled down the slide by gravity, one end of the DNA sticks to the slide and the fiber is straightened by the force of the movement of the buffer down the slide. The slides are then air-dried and fixed, and the incorporated IdU and CldU are detected with fluorescent-coupled antibodies that identify IdU in red and CldU in green to reveal the direction of fork movement.

There are four readily distinguishable patterns of red and green that can be produced by this labeling strategy that indicate sites where replication is initiating at single or clustered origins bidirectionally, elongating or extending at forks, or where replication is merging between two adjacent replicons (terminations). As shown in Fig. 1a, a single red track at the center of two green tracks indicates a single bidirectional origin, with the origin of replication initiating during the first pulse with IdU (detected in red) and extending in both directions from the point of initiation as determined by the subsequent bidirectional

incorporation with CldU (detected in green). Clusters of replicons are defined by the merging of two or more red and two or more green tracks that indicate two or more adjacent replicons that complete replication within the defined total pulse time (in this case, 30 min). If a red track within a cluster is surrounded by a green track on both sides, this center red track likely contains an origin. Elongating forks are represented by red and green tracks, while a red track flanked by a green track indicates fork movement from a different replicon towards the other origin in the cluster. Merging of two forks from two adjacent replicons indicates the site of termination by the pattern of a green track at the center of two red tracks, where replication forks are extending in the direction from red to green, since IdU (red) was incorporated first followed by CldU (green). Single tracks of red or green can also be detected in this type of analysis as well, with a red track likely indicating a site where replication terminated or stalled before the nucleotide from the second pulse could be incorporated, while a single green track is likely indicative of a site where replication initiated after the first pulse with IdU had already been washed out. Asynchronous or synchronized cells at different hours of S phase could be used to study replication (11). Also, different pulse times with nucleotide analogs can be employed to reveal how replication extends from newly fired origins (12). In addition to these studies, analyses of replication on individual DNA fibers have been used to show the rate of fork elongation (11, 13), origin interference (14), the use of latent origins caused by a slower rate of replication (15), and differences in replication between normal and cancer cells (11, 12, 16).

Here, we describe a method for fluorescently detecting the different patterns of replication using DNA from H1299 cells either expressing mutant p53 or stably transfected with vector. The method involves the incorporation of IdU and CldU into replicating DNA, the creation of DNA fiber spreads by extending DNA linearly onto glass slides, and the immunofluorescent detection of the incorporated nucleotide analogs by confocal microscopy. We also describe strategies for quantifying and analyzing the replication patterns after images are obtained and discuss the different types of information that can be obtained from this type of analysis.

2. Materials

2.1. Nucleotide Analogs (Sigma)

1. Iododeoxyuridine (IdU, 100 mM).
2. Chlorodeoxyuridine (CldU, 100 mM).

2.2. Reagents

1. Hanks balanced salt solution (HBSS), phosphate-buffered saline (PBS), or Dulbecco's phosphate-buffered saline (DPBS).
2. DNA lysis buffer: 0.5% SDS, 200 mM Tris-HCl pH 7.4, 50 mM EDTA.
3. Methanol/Acetic acid (3:1).
4. 2.5 N HCl.
5. PBS/0.1% Tween 20.

6. 2% BSA. Make fresh. Dilute in PBS.
7. Rat anti-Bromodeoxyuridine.
8. Mouse anti-Bromodeoxyuridine.
9. Stringency buffer: 10 mM Tris-HCl pH 7.4, 400 mM NaCl, 0.2% Nonidet P40 (NP40).
10. Alexafluor 488-conjugated chicken anti-rat (Molecular Probes) (1:250).
11. Alexafluor 594-conjugated rabbit anti-mouse (Molecular Probes) (1:333).
12. 2% Normal goat serum (NGS). Make fresh. Dilute in PBS.
13. Alexafluor 488-conjugated goat anti-chicken (Molecular Probes) (1:250).
14. Alexafluor 594-conjugated goat anti-rabbit (Molecular Probes) (1:333).
15. Antifade without DAPI (Invitrogen).

2.3. DNA Fiber Spreading

1. Silane-coated slides (Sigma).
2. Coplin jars.
3. Plastic strips cut into the size of the glass slide surface.
4. Nail polish.
5. Confocal microscope.
6. Image J: This software can be downloaded from the NIH Web site.

2.4. Optional Reagents for Synchronization or S Phase Checkpoint Analysis

1. Aphidicolin.
2. Caffeine (50 mM).

3. Methods

3.1. Labeling of Replicating DNA

1. Add 2 μ l of 100 mM IdU to 4 ml of media in the plate. Incubate for 10 min.
2. Wash off IdU two times with HBSS. Work quickly since cells are still actively replicating to ensure continuity of labeled tracks.
3. Add 4 μ l of 100 mM CldU to 4 ml of media in the plate. Incubate for 20 min.
4. Wash two times with HBSS and trypsinized cells. Collect into a tube.
5. Spin cells at $500 \times g$ for 6 min.
6. Wash with 4 ml of DPBS.
7. Repeat steps 5 and 6 once.

8. Count the number of cells present in the sample. Add DPBS to the sample so that the cells are suspended at 200–300 cells per μl .

3.2. Creating Spreads of DNA Fibers

1. Label slides in pencil. Make enough slide duplicates so that enough fibers will be obtained for analysis, which is usually three to ten depending on the experiment.
2. Vortex cells. Immediately streak 2 μl of the cell suspension across the glass near the top of the slide.
3. Allow most of the liquid to evaporate but do not let the streak dry.
4. Add 9 μl of DNA lysis buffer across the initial streak of cells. Allow cells to lyse for 10 min.
5. Tilt the slide to an approximately 45° angle to allow the buffer to run down the slide. Add 2 μl of extra buffer to the droplet if it starts to dry out.
6. Leave slides at the same tilted angle and allow to air-dry for 2 h.
7. Fix in 3:1 methanol/acetic acid. Dip in slide fixing chamber for 2 min. Place slides on an absorbent surface and allow them to dry overnight in a fume hood.
8. The next day, place the cells in a freezer at -20°C for at least 24 h.

3.3. Immunostaining

1. Remove slides from freezer and allow them to return to room temperature.
2. Fill Coplin jar(s) with 50 ml of 2.5 N HCl to cover the slides. Place slides into the jar and mix up and down a few times. Incubate for 30 min.
3. Using additional Coplin jars, wash once in PBS/0.1% Tween 20, followed by two washes in PBS for 3 min each.
4. Fill unused pipette tip boxes with hot water almost to the top to create a humid chamber. Remove the slides from the last wash and dry the back of the slide with a paper towel. Place onto dry surface of the tip box filled with hot water. Add 2 ml of 2% BSA onto the glass surface of the slide to block nonspecific binding of the antibodies. Spread 2% BSA solution evenly over the surface of the slide by creating surface tension between the solution on the slide and the wide opening of a 1 ml pipette tip. Incubate 2 h.
5. Discard the blocking solution on the slides into a beaker. Dry the back of the slide and add 100 μl of the primary antibody solution (1:250 rat anti-bromodeoxyuridine (detects CldU) plus 1:250 mouse anti-bromodeoxyuridine (detects IdU) in 0.2% BSA in PBS). Tilt the slide to make sure the antibody solution covers the entire glass surface and cover with a piece of plastic. Place onto dried surface of humid chamber and incubate for 1 h.
6. Wash slides for 10 min in stringency buffer in Coplin jar.
7. Wash slides two times in PBS in Coplin jar.

8. Dry the back surface of the slides and add 100 μ l of the secondary antibody solution to the slide (1:250 Alexafluor 488-conjugated chicken anti-rat plus 1:333 Alexafluor 594-conjugated chicken anti-rat in 0.2% BSA in PBS). Tilt the slide to make sure the antibody solution covers the entire glass surface and cover with a piece of plastic. Place onto dried surface of humid chamber and incubate for 30 min in the dark.
9. Wash slides once in PBS/0.1% Tween 20, and then twice in PBS for 3 min each in a Coplin jar.
10. Dry the back surface of the slide and replace slide onto dried surface of the humid chamber. Add 1–2 ml of 2% NGS and spread the solution evenly over the slide as in step 3. Incubate for 15 min.
11. Discard the NGS blocking solution into a beaker and dry the back surface of the slide. Add 100 μ l of the third antibody solution to the slide (1:250 Alexafluor 388-conjugated goat anti-chicken plus 1:333 Alexafluor 594-conjugated goat anti-rabbit in 0.2% NGS in PBS). Tilt the slide to make sure the antibody solution covers the entire glass surface and cover with a piece of plastic. Place onto dried surface of humid chamber and incubate for 30 min in the dark.
12. Wash slides once in PBS/0.1% BSA, then twice in PBS for 3 min each in Coplin jar.
13. Mount coverslips onto slides using antifade mounting solution. Seal coverslips onto slide with nail polish if necessary.
14. Collect images using a confocal microscope. Analyze images using Image J software program.

3.4. Optional Treatments

1. Normal cells can be synchronized by confluence arrest. Change the media as required by the cell line.
2. Replate cells at 374,000 cells on a 60 mm plate the day before labeling. Optionally, aphidicolin can be added to the plate for 24 h at a final concentration of 2 μ g/ml for synchronization of cells.
3. To knockdown the intra-S phase checkpoint, treat cells with 2 mM caffeine for 30 min prior to labeling. During labeling with IdU and CldU, keep the same concentration of caffeine in the media to ensure that the checkpoint remains inactivated.

Acknowledgments

This study was initiated by pilot project funds from the VCU Massey Cancer Center (P30CA016059) and was supported by funds from NCI (CA121144) to Sumitra Deb. We thank Catherine Vaughan and Shilpa Singh for technical assistance and editing the manuscript.

References

1. Cairns J. Autoradiography of HeLa cell DNA. *J Mol Biol.* 1966; 15:372–373. [PubMed: 5912048]
2. Huberman J, Riggs AD. Autoradiography of chromosomal DNA fibers from Chinese hamster cells. *Proc Natl Acad Sci USA.* 1966; 55:599–606. [PubMed: 5221245]
3. Huberman J, Riggs AD. On the mechanism of DNA replication in mammalian chromosomes. *J Mol Biol.* 1968; 32:327–341. [PubMed: 5689363]
4. Heng HHQ, Squire J, Tsui L-C. High-resolution mapping of mammalian genes by in situ hybridization to free chromatin. *Proc Natl Acad Sci USA.* 1992; 89:9509–9513. [PubMed: 1384055]
5. Wiegant J, et al. High-resolution in situ hybridization using DNA halo preparations. *Hum Mol Genet.* 1992; 1:587–591. [PubMed: 1301167]
6. Lawrence JB, Carter KC, Gerdes MJ. Extending the capabilities of interphase chromatin mapping. *Nat Genet.* 1992; 2:171–172. [PubMed: 1345163]
7. Parra I, Windle B. High resolution visual mapping of stretched DNA by fluorescent hybridization. *Nat Genet.* 1993; 5:17–21. [PubMed: 8106079]
8. Jackson DA, Pombo A. Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J Cell Biol.* 1998; 140:1285–1295. [PubMed: 9508763]
9. Windle, B.; Parra, I.; Silvas, E. Analysis of structure and function by high resolution visual mapping of extended DNA. In: Andreeff, M.; Pinkel, D., editors. *Introduction to fluorescence in situ hybridization: principles and clinical applications.* Wiley-Liss; New York: 1999. p. 119-132.
10. Merrick CJ, Jackson D, Diffley JF. Visualization of altered replication dynamics after DNA damage in human cells. *J Biol Chem.* 2004; 279:20067–20075. [PubMed: 14982920]
11. Frum RA, Khondker ZS, Kaufman DG. Temporal differences in DNA replication during the S phase using single fiber analysis of normal human fibroblasts and glioblastoma T98G cells. *Cell Cycle.* 2009; 8:3133–3148. [PubMed: 19738421]
12. Frum RA, Chastain PD II, Qu P, et al. DNA replication in early S phase pauses near newly activated origins. *Cell Cycle.* 2008; 7:1440–1448. [PubMed: 18418075]
13. Daboussi R, Courbet S, Benhamou S, et al. A homologous recombination defect affects replication-fork progression in mammalian cells. *J Cell Sci.* 2008; 121:162–166. [PubMed: 18089650]
14. Lebofsky R, Heilig R, Sonnleitner M, et al. DNA replication origin interference increases the spacing between initiation events in human cells. *Mol Cell Biol.* 2006; 17:5337–5345.
15. Courbet S, Gay S, Arnoult N, et al. Replication fork movement sets chromatin loop size and origin choice in mammalian cells. *Nature.* 2008; 455:557–560. [PubMed: 18716622]
16. Martin R, Oppenheim A. Initiation points for DNA replication in nontransformed and simian virus 40-transformed Chinese hamster lung cells. *Cell.* 1977; 11:859–869. [PubMed: 196764]

Fig. 1.

The different types of replication patterns produced by the fluorescent detection of the sequential incorporation of IdU followed by CldU into replicating DNA are shown in (a). Since cells are labeled first with IdU (detected in *red*) followed by CldU (detected in *green*), the direction of fork movement can be determined in merged *red-green* tracks to reveal bidirectional replication from origins, elongating forks, as well as merged tracks of two or more adjacent replicons (*clusters*) and sites where two converging unidirectional forks from adjacent replicons merge (*terminations*). The bar graph in (b) shows higher percentage of origin clusters in H1299 cells expressing a tumor-derived mutant of p53 (273H).