



The DNA fibre technique – tracking helicases at work



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ABSTRACT

Faithful duplication of genetic material during every cell division is essential to ensure accurate transmission of genetic information to daughter cells. DNA helicases play a crucial role in promoting this process by facilitating almost all transactions occurring on DNA, including DNA replication and repair. They are responsible not only for DNA double helix unwinding ahead of progressing replication forks but also for resolution of secondary structures like G4 quadruplexes, HJ branch migration, double HJ dissolution, protein displacement, strand annealing and many more. Their importance in maintaining genome stability is underscored by the fact that many human disorders, including cancer, are associated with mutations in helicase genes. Here we outline how DNA fibre fluorography, a straightforward and inexpensive approach, can be employed to study the *in vivo* function of helicases in DNA replication and the maintenance of genome stability at a single molecule level. This approach directly visualizes the progression of individual replication forks within living cells and hence provides quantitative information on various aspects of DNA synthesis, such as replication fork processivity (replication speed), fork stalling, origin usage and fork termination.

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1. Introduction

The human genome contains 3 billion base pairs of double stranded DNA divided into 23 chromosomes. During multiple cellular processes, including DNA replication and repair, this double-stranded DNA needs to be separated (unwound). This activity is carried out by a class of enzymes called DNA helicases. These are motor proteins that utilize energy derived from hydrolysis of nucleoside triphosphates to separate DNA strands. The human genome codes for almost 31 DNA helicases [1,2], and their importance in maintaining genome stability is underscored by the fact that various cancers and human genetic disorders (e.g. Werner, Bloom, Rothmund-Thomson, Baller-Gerold, Rapadilino syndromes, Fanconi Anaemia, Xeroderma pigmentosum, Cockayne syndrome, Dyskeratosis congenita, Trichothiodystrophy or Warsaw breakage syndrome) are associated with mutations in helicase genes. During the S-phase of the cell cycle the entire genome of the cell is duplicated. This process relies on the precisely controlled activation of thousands of replication origins distributed along the chromosomes. However, replication through chromosomal regions that are inherently difficult to duplicate (e.g. highly repetitive sequences such as rDNA and telomeres, G4 quadruplex-forming motifs, DNA:RNA hybrids or widely spaced origins) challenges replication fork stability [3–5]. If cells cannot properly stabilise slowed down and/or stalled replication forks, they accumulate DNA damage, including DNA double-strand breaks (DSBs), adversely impacting on genome integrity. The challenges imposed on replication machinery during genome duplication are collectively defined as “replicative stress”. In order to counteract the adverse effect of endogenous and exogenous agents on faithful DNA replication cells activate the replicative stress response pathways. These allow for a coordinated and highly regulated response enabling the replication machinery to preserve the stability and functionality of perturbed replication forks. Fundamental to this response are the DNA helicases that promote remodelling of challenged replication forks and/or resolve DNA secondary structures that could impede fork progression. Since replication stress is a major driver of tumour progression [6,7] it is important from a therapeutic point of view to determine how various factors, including helicases, counter the adverse effect of perturbed replication fork progression. Such knowledge may lead to the development of novel anti-cancer therapeutic approaches.

Until recently, the dynamics of DNA replication under unperturbed conditions, as well as in response to replicative stress remained poorly defined. This was mainly due to the lack of experimental tools to quantitatively analyse replication in cells. The main approach to determine the complex replication programme relied on measuring the overall rates of DNA synthesis in populations of BrdU pulse-labelled cells by flow cytometry or immunofluorescence analysis. However, this approach does not allow to qualitatively analyse the impact a gene of interest (and the protein it encodes) has on the spatio-temporal programme of DNA replication.

While the DNA fibre fluorography also utilizes incorporation of labelled nucleotides into newly synthesised DNA, a crucial difference is that this technique allows for direct visualization of the incorporated nucleotides on the level of single DNA molecules as opposed to the cellular level. The development of this technique has allowed researchers to gain new insight into the dynamics of DNA replication in eukaryotes on a genome-wide basis in both a quantitative and qualitative manner [8].

This technique allows for the labelling of actively progressing replication forks *in vivo* and the sequential incorporation of the two different halogenated nucleotides IdU (5-Iodo-2'-deoxyuridine) and CldU (5-Chloro-2'-deoxyuridine) into the nascent DNA strands

provides information about replication directionality. Once the different sets of nucleotides are incorporated, cells are lysed and individual DNA fibres are stretched on a microscope slide. Subsequently, the labelled DNA replication tracts are stained with antibodies directed against the halogenated nucleotides and visualized by fluorescence microscopy [8,9] (Fig. 1). Consequently, an ongoing fork is represented as adjacent red and green signals. Importantly, the consecutive use of two different nucleotide analogues not only provides information on replication fork directionality but also shows a snapshot of how DNA is replicated. Different modes of replication can be observed by quantifying the frequency of four replication structures [10], namely: elongating forks (ongoing); new initiation events (origin firing); termination events and stalled/collapsed forks (Fig. 2) [11,12]. These parameters provide powerful information allowing to assess, in detail, the cellular responses to replicative stress aiming to ensure efficient DNA replication and the role of individual proteins in this process. Accordingly, this approach has been successfully used to monitor DNA replication, origin usage and replication fork recovery in a variety of organisms, including chicken DT40 cells, *Xenopus* and mammals (e.g. human and mouse cells) [10,13–16].

Here, we describe in detail the DNA fibre fluorography, a useful assay for the analysis of the DNA replication programme.

2. Description of the method

2.1. Materials

5-Iodo-2'-deoxyuridine (IdU) (Sigma-Aldrich, I7125) – stock solution: 5 mM in cell culture medium, briefly heated to 60 °C and vortexed to dissolve.

5-Chloro-2'-deoxyuridine (CldU) (Sigma-Aldrich, C6891) – stock solution: 2.5 mM in cell culture medium, briefly heated to 60 °C and vortexed to dissolve.

Cell lysis solution – 200 mM Tris-HCl, pH 7.5, 50 mM EDTA and 0.5% SDS.

Blocking solution – 5% BSA in PBS.

Primary antibodies: Anti-BrdU antibody (mouse) (BD Biosciences, 347580) and Anti-BrdU antibody (rat) (Abcam, ab6326).

Secondary antibodies: sheep anti-mouse Cy3 (Sigma-Aldrich, C2181) and goat anti-rat Alexa Fluor 488 (LifeTechnologies, A110060).

Vectashield mounting medium (Vector lab, H-1000).

Microscope slides SuperFrost (VWR international, 631-0910).

Coverslips 24 × 50 mm thickness No1 (Scientific Laboratory Supplies LTD, MIC3234).

2.2. Method

The protocol described in this section is used in the following publications [10,13,17] and presented in [16].

2.2.1. *In vivo* nascent DNA labelling

Exponentially growing cells are initially pulse labelled with the first nucleotide analogue. IdU is added to the cell culture to a final concentration of 25 µM. Cells are incubated for 20 min. After labelling with the first nucleotide cells are pulse labelled with the second label. CldU is added to a final concentration of 250 µM, mixed and cells incubated for 20 min. After double labelling cells are washed with ice cold PBS, harvested and resuspended in ice cold PBS to the concentration optimal for the particular cell line (e.g. for DT40 – 7.5×10^5 cells/ml, for HeLa – 5×10^5 cells/ml). Labelled cell suspension is kept on ice.

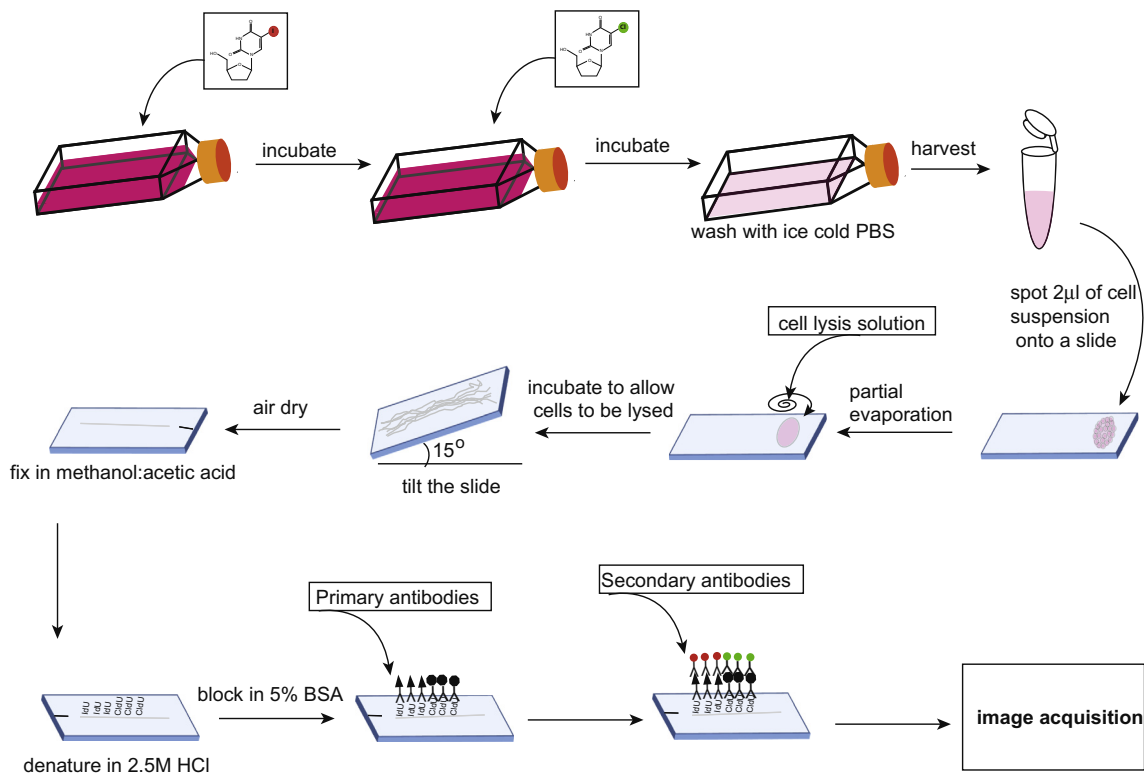


Fig. 1. Schematic showing DNA fibre technique protocol (see text for details).

2.2.2. Preparation of DNA spreads

2 µl of the cell suspension are spotted at the end of the microscope slide and air-dried for 5 min. Drop evaporation time may vary between different laboratory environments and may require adjustment. To obtain optimal results the volume of the cell suspension drop should be significantly reduced but equally, the drop should not have dried completely. Subsequently, 7 µl of the lysis solution are applied on top of the cell suspension, then mixed by gently stirring with a pipette tip and incubated for 2 min. Following cell lysis, slides are tilted to 15° to allow the DNA fibres spreading along the slide. Once the spread fibres have dried, the stretched DNA is visible at this point as a thin opaque line along the slide and the beginning can be marked with a pencil to aid in fibre localisation during staining and visualization.

2.2.3. Immunofluorescent DNA staining

Slides are fixed in methanol/acetic acid (3:1) solution in a staining jar and incubated for 10 min. Subsequently, slides are washed in distilled H₂O and immersed in 2.5 M HCl for 80 min. After DNA denaturation, slides are washed in PBS for 5 min. This step is repeated two more times. After washes, excess PBS is removed with a paper towel and slides are placed horizontally in a humidified chamber. Blocking solution is applied on each slide and the slides are gently covered with a coverslip to spread the blocking solution evenly. During the blocking step, the primary antibody solution is prepared (1:25 anti-BrdU (mouse) and 1:400 anti-BrdU (rat) in blocking solution). After blocking for 20 min, the coverslips are removed by gently moving down the slide without applying force. Should the coverslip stick to the slide, rehydration in PBS will help to loosen the coverslip. Excess blocking solution is removed with a paper towel and slides placed horizontally in a humidified chamber. 50 µl of the primary antibody solution are pipetted onto each slide, slides are covered again with a coverslip to spread the antibody solution evenly over the slide and incubated

for 2 h at room temperature. Subsequently, coverslips are removed as before and slides washed three times with PBS for 5 min. The secondary antibodies are diluted in blocking solution (1:500 sheep anti-mouse Cy3 and 1:400 goat anti-rat Alexa Fluor 488) and 50 µl of the secondary antibody solution are applied on each slide and slides are covered again with coverslips. Protected from light, slides are incubated in a humidified chamber at room temperature for 1 h. After removing the coverslips, slides are washed three times with PBS as before and subsequently a drop of mounting medium is spotted onto each slide, coverslips are applied by gently pressing down. Slides are sealed with transparent nail polish, let to dry and stored at −20 °C until being analysed.

2.2.4. Image acquisition and data analysis

Stained DNA fibres are visualized using a fluorescence microscope equipped with a camera. Pencil marks will help to localize the fibres. Pictures for analysis should be taken in regions where fibres are well separated and not entangled. It is important to capture images in different areas along the slide as only one region may not provide representative data. To avoid bias only one channel is used to select regions for taking pictures.

Pictures are then analysed using an appropriate image analysis software e.g. ImageJ (<http://rsbweb.nih.gov/ij/>). This software allows to measure the lengths of the fibre tracts and thus to estimate speed of replication (1 µM roughly corresponds to 2.59 kb [8]) as well as the relative frequency of different replication structures determined as percentage of all structures scored.

3. Applications

3.1. Replication analysis in unperturbed cells

The labelling protocol presented in the method section allows for analysis of the replication programme in unchallenged cells

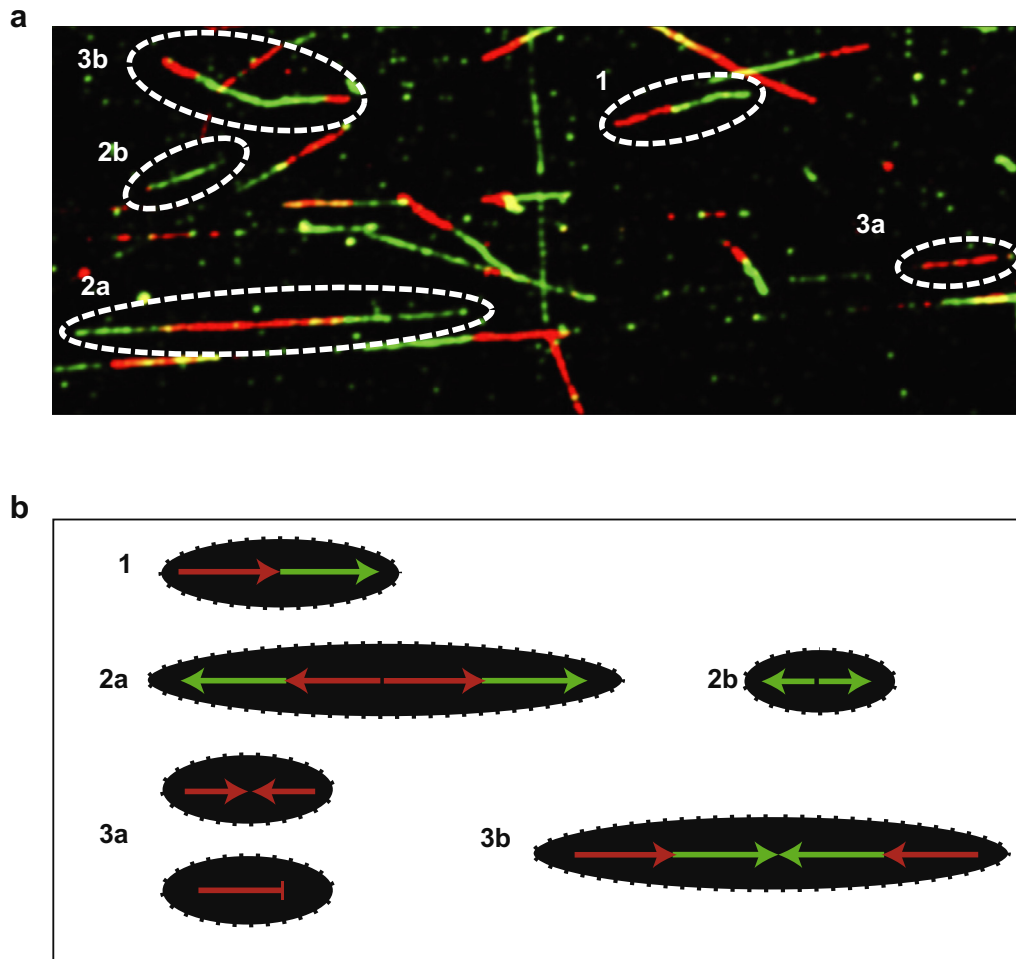


Fig. 2. Different replication structures. a) Representative image of DNA fibres with selected replication structures marked and b) schematic showing marked replication structures: 1 – ongoing replication; 2a – origin of replication fired during first pulse of labelling; 2b – origin of replication fired during second pulse of labelling; 3a – stalled fork or termination during first pulse; 3b – termination.

at the single molecule level (Fig. 3a). This approach is usually employed as a first step to quantitatively analyse the function of a gene in DNA replication. Examples of successful implementation of this strategy include the identification of the role of the Fanconi Anemia proteins, including the DNA translocase FANCM, in promoting global replication fork dynamics [3,10], the analysis of PIF1's helicase function in DNA replication in oncogene transformed cells [18] or the characterisation of the RECQ4 – MCM10 interaction in promoting efficient origin firing [19].

Of note, in this approach a mild phenotype may be missed due to the significant variability in replication kinetics between individual forks [20]. Therefore, it is advisable to re-evaluate the initial phenotype with subsequent analyses employing more complex experimental designs (examples for helicases will be discussed in the following sections of this review).

3.2. Replication under conditions of replicative stress

Addressing specific questions related to the role of a particular protein in maintaining genome stability frequently requires monitoring replication dynamics under conditions of replicative stress. This is usually achieved by treating the cells with known replication inhibitors such as camptothecin (CPT), hydroxyurea (HU) or aphidicolin (APH). Cells can be subjected to treatment during both labelling pulses or even, if required treatment may precede and continue during both pulses of labelling (Fig. 3b). Examples of this

approach include characterisation of the role of the FANCF helicase in promoting replication past G4 quadruplexes, indicating an important role for this protein in aiding replication over sequence dependent replication barriers [17]. A similar experimental design was also successfully implemented to show that depletion of various components of the MCM2-7 helicase complex in human and mouse cells causes a decrease in dormant origin usage under conditions of HU induced replicative stress [21,22].

Alternatively, cells may be subjected to replicative stress only during the second label (Fig. 3c). In this setup the first label serves as an internal control of replication speed in unchallenged cells. Therefore, this experimental design not only allows to measure replication tract lengths but it also allows to determine the ratio between unperturbed/perturbed replication tracts – 1st/2nd label (Fig. 3c).

This approach was used recently by Clynes et al. [23] to monitor replication fork progression in mouse fibroblasts deficient for the ATRX helicase under conditions of HU-induced replication stress. In this example the average replication speed was not statistically different between WT and ATRX^{-/-} cells, however, a significant increase in the proportion of fibres with high 1st/2nd label ratio was observed. Based on these observations the authors concluded that ATRX has a role in maintaining processive DNA synthesis under conditions of replicative stress in a subset of genomic loci [23]. Slower replication in cells exposed to HU-induced replication stress was also observed in the absence of the FANCM and FANCF

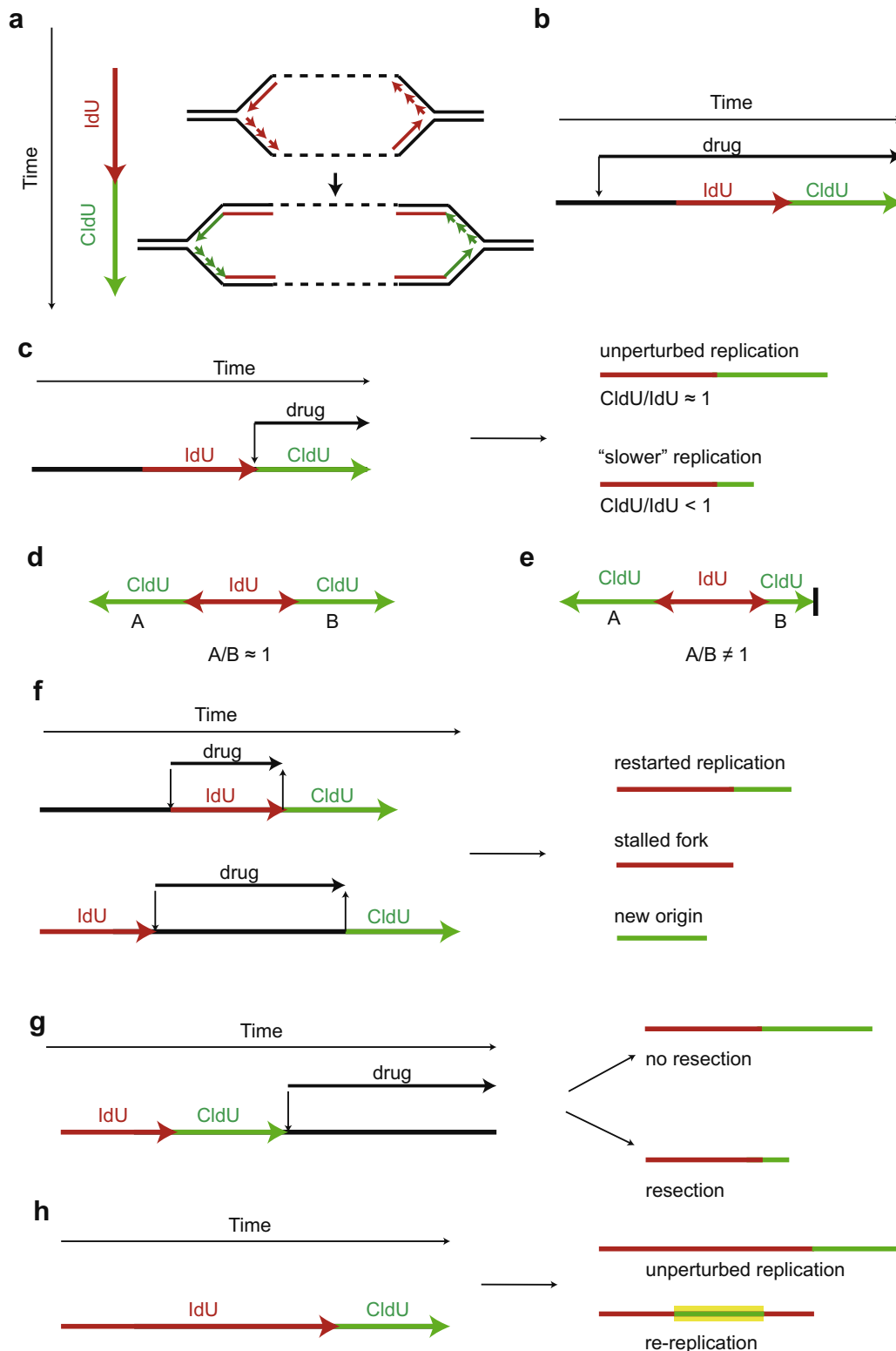


Fig. 3. Schematic representation of different labelling variants as discussed in the text. a) unchallenged replication with schematic showing replication forks; b) replication under condition of replicative stress; c) replication under condition of replicative stress induced only during the second label pulse; right panel illustrates principles of tract length ratio calculation; d) symmetric and e) asymmetric fork analysis; f) replication recovery analysis; right panel illustrates examples of main structures observed; g) analysis of DNA resection at replication fork; h) analysis of re-replication events.

helicases [13,17]. An analogous approach was used to show the requirement for PIF1, a G4-quadruplex processing helicase, in promoting global replication fork dynamics in cancerous cells under conditions of replication stress induced by G4 stabilizing ligands

[18]. Moreover, an elegant work combining biochemical analysis with the DNA fibre technique identified a requirement for RECQ1 in restarting replication after fork reversal induced by TOP1 inhibition by low doses of CPT [24].

3.3. Sister fork symmetry analysis

Slower replication (shorter tracts) may be an outcome of two possible scenarios: a result of slower overall DNA polymerization rate or increased frequency of fork stalling. Taking advantage of the double labelling strategy it is possible to distinguish between these two possibilities by analysing fates of two forks emanating in opposite directions from the same origin. These sister forks are likely to travel with similar speed [11,20] resulting in their tract lengths ratio being close to 1 (Fig. 3d). Decrease in the general polymerization speed would affect both of them equally (ratio remaining close to 1) whereas fork stalling would affect them independently resulting in sister fork asymmetry (ratio \neq 1) (Fig. 3e). This type of analysis allowed us to show a role for FANCM [13] and FANCI [17] in maintaining replication processivity upon HU induced replication stress, and a requirement for FANCI to efficiently replicate over G4 quadruplex containing sequences [17]. A similar approach was utilized by Kawabata et al. to monitor increased replication fork stalling in unchallenged *Mcm4^{Chaos3/Chaos3}* mouse fibroblasts [25].

3.4. Analysing the ability to recover DNA synthesis after replication stalling

Finally, to determine the role of a DNA repair factor/helicase of interest on the recovery of stalled or collapsed replication forks, treatment with a drug that impedes replication may be performed during the first pulse of labelling, or alternatively immediately after, but before adding the second halogenated nucleotide analogue (Fig. 3f). The drug has to be washed out before the second label is applied. This experimental design enables assessing not only efficiency of fork recovery but also detection of any changes in origin usage and/or dormant origin firing frequency. Examples include the characterisation of the role of BLM [26], WRN [27], ATRX [23,28] and two members of SNF2 family ATP-dependent molecular motor proteins SMARCA1 (HARP) and AH2 [29,30] in maintenance of replication fork stability and DNA synthesis recovery after impeding replication with HU [23,27,28,30] or APH [26,29]. A similar experimental strategy was employed to monitor the fate of stalled forks in FANCI deficient cells. In this case the replication recovery after HU-mediated replication block was delayed but eventually equally efficient as observed in WT cells indicating that stalled forks remain stable and primed to resume DNA synthesis in the absence of FANCI [17]. Likewise PIF1 seemed to be dispensable for maintaining replication fork stability; however, prolonged exposure to HU resulted in significant decrease in new origin firing in oncogene transformed PIF1 depleted cells [18].

Finally this approach was also used to analyse the cellular consequences of FBH1 mediated RAD51 ubiquitination [31]. The authors observed an increase in replication fork recovery in cells expressing mutant RAD51 protein resistant to ubiquitination [31].

It is important to note that a significant increase in dormant origin firing may result in a labelling pattern visually indistinguishable from a *bone fide* fork restart, as DNA synthesis may be recovered by a fork arriving from an adjacent origin of replication. This was observed to be the case for FANCM deficient DT40 cells whereby increased frequency of replication recovery after CPT induced replication block was caused by unscheduled firing of dormant origins of replication [10]. This example highlights the potential of DNA fibre technique in assessing the role of a protein of interest in maintaining origin/dormant origin firing schedule.

3.5. Analysing resection at the replication fork

Resection of the nascent DNA upon replicative stress is another aspect of replication dynamics that can be assessed using the DNA

fibre technique. In order to perform this type of analysis double labelling is followed by treatment with a replication inhibitor and shortening of the 2nd label tract is monitored [32] (Fig. 3g). This approach was utilized to show a stabilizing function of the WRN RecQ helicase on nascent DNA in cells exposed to CPT [33].

3.6. Analysing re-replication

Strict control of genome duplication ensures that DNA is replicated only once within the same cell cycle. Re-replication of even small portion of the genome can result in DNA damage and genome instability. Although re-replication has not been yet linked with dysfunction of helicases, the DNA fibre technique allows to detect these type of events with significant sensitivity as shown in cells depleted for Cdt1, Geminin or Eme1 [34,35]. In this type of analysis longer pulse of first label is followed by shorter 2nd label pulse. Re-replication events are visualized as 2nd label tracts embedded within the 1st label tracts resulting in yellow tracts visible on merged images (Fig. 3h).

4. Summary

In recent years, various techniques employing the basic principles of DNA fibre have been developed. They employ microfluidic platforms or DNA combing to capture stretched DNA fibres on a microscope slide [36–41]. When combined with fluorescence in situ hybridization (FISH) [42], these techniques have the potential to provide more comprehensive insights into the DNA replication programme at certain genomic loci. However, they are technically more challenging and require very specialised and relatively expensive equipment.

The DNA fibre fluorography technique outlined in this review can be successfully implemented in most laboratories with access to microscopy facilities. A key element in ensuring data reproducibility is to obtain the best possible stretching/spreading of intact DNA fibres on a microscopic slide. The critical step to achieve this is the incubation time of the cell suspension on the slide and time of the lysis. These parameters are variable depending on the temperature and humidity in every lab environment and may require adjustment. The number of cells may also require optimization for each cell line. Thus, the examples suggested in this review should be used only as guidelines for protocol optimization.

Different experimental designs combining DNA dual labelling with replication inhibition have been presented here as independent experiments. However, it is important to note that in the majority of cases multiple approaches are required to gain in depth understanding of the impact a protein of interest has on the global replication programme [10,13,17,18,23].

In conclusion, the fibre technique outlined here allows visualization of the progression of individual replication forks within living cells, thus providing quantitative and qualitative information on various aspects of DNA synthesis. These include fork processivity, stalling, and replication origin usage that are of importance for functional studies of proteins engaged in transactions occurring on DNA.

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