Frequently Asked Questions

Automatic DNA replication tract measurement to assess replication and repair dynamics at the single molecule level

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1. *What kind of questions in terms of replication or repair studies can be addressed using DNA stranding software?*

Studies using DNA fiber assay or molecular combing assay is a gold standard technique to address DNA replication fork dynamics and repair at single molecule level (1, 2). As described in the cartoon below (Fig.1), using the "DNA Stranding Software" we can address replication fork speed (1a), The ability to restart the replication fork in the presence of transient replication stress, measure by the perecentage of stalled replication forks (1b), The number of compensatory new origin firing in the presence of reduced fork speed or fork stalling (1c), The inter-origin distance (1d), Replication fork assymetry (e), the frequency of terminating replication forks (f) and finally the nascent DNA degradation phenomenon in a specific genetic background.

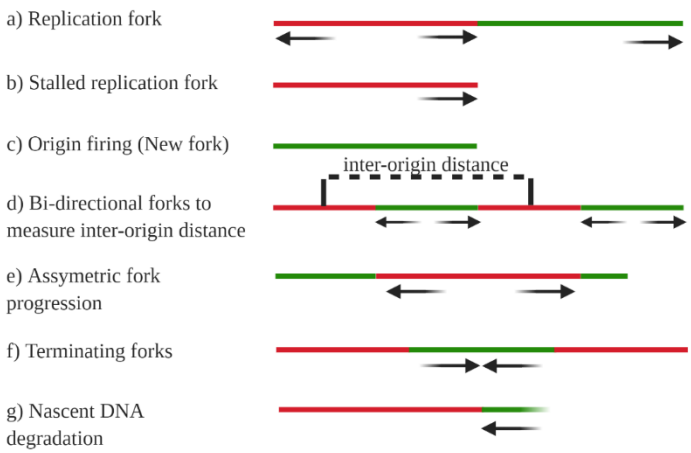
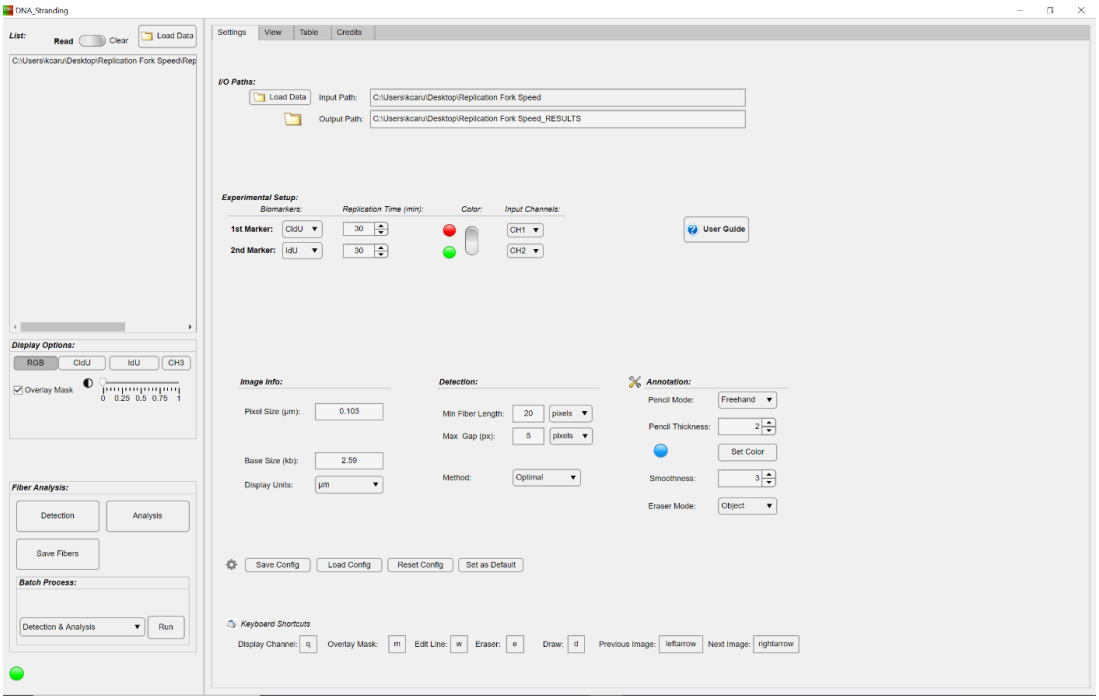


Figure.1. DNA replication patterns used to study comprehensively DNA replication and repair dynamics at single molecule level. a) A typical two labelled replication fork pattern predominantly observed upon fibre spread which is used for calculating replication fork speed. b) Stalled replication fork indicates the inability of a replication fork to restart upon high dose of transient replication fork stalling. c) New replication fork, or origin firing denotes the origin that remained dormant are fired. d) Multiple origin firing indicates the number of replication forks from a same fibre and this is useful to calculate the inter-origin distance (IOD). e) Bidirectional Replication fork gives rise to symmetric and Asymmetric replication forks. Alterations in repliation fork speed increases the asymmetric fork progression. f) Replication fork termination is a frequently observed pattern indicating a segment of DNA replication is complete at converging fork. g) Dual labelling of nucleotide analogue and stalling of forks with high dose of genotoxic replication fork stalling agents degrade nascent DNA in the absence of replication fork protection factors.

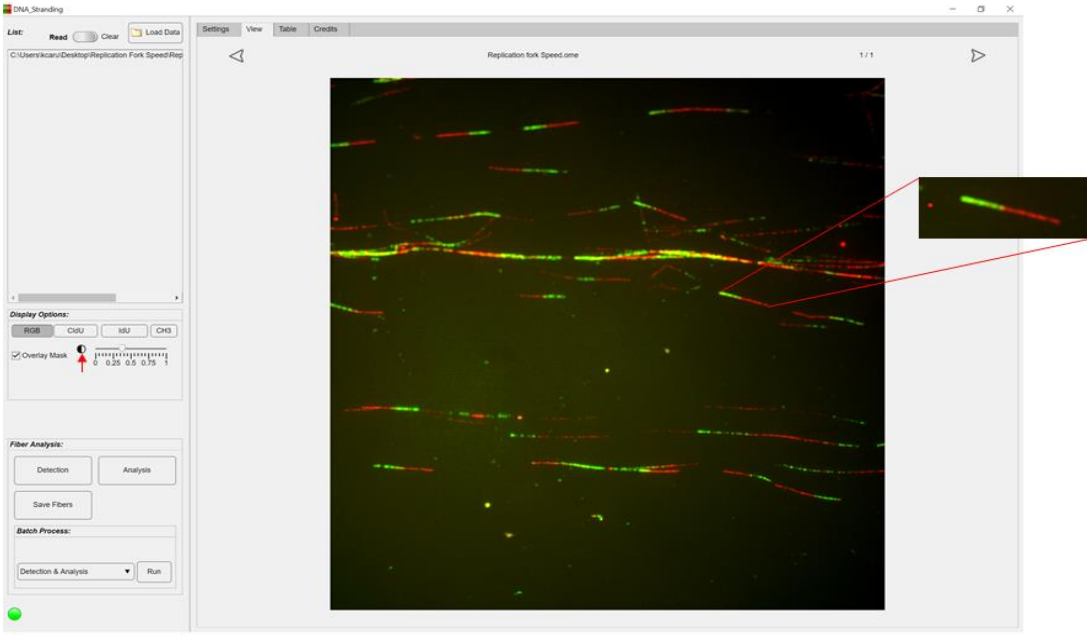
2. *How to begin with the DNA Stranding Software ?*

Firstly, the user should input the experimental set up in the settings page (Fig. 2a) of the "DNA Stranding Software". In general, labelling of cells for 10, 15 or 30 min's with nucleotide analogs such as CIdU and IdU are enough to visualise and quantify replication fork length. Secondly, the user can upload their ome.tif format fibre images. Here, the user can choose the CIdU, IdU labelling pattern and its timing as they fancy based on their respective secondary antibody (Fig.2a,1). Thirdly, the user can choose whether the analysed image should be respresented in terms of **µm** or in terms of **kb** (Fig.2a,2). The software uses the conversion of 1 µm = 2.59 kb (2). Finally, once the parameters are fixed, the user can move to **"View"** the fibre image from the software (Fig.2b). The user can increase or decrease image contrast under display options (Fig.2b,1). Next, the fiber detection can be done with just one click on the **"Detection"** icon. The detected fibres are optionally overlayed with a line (Fig.2c).

a)



b)



c)

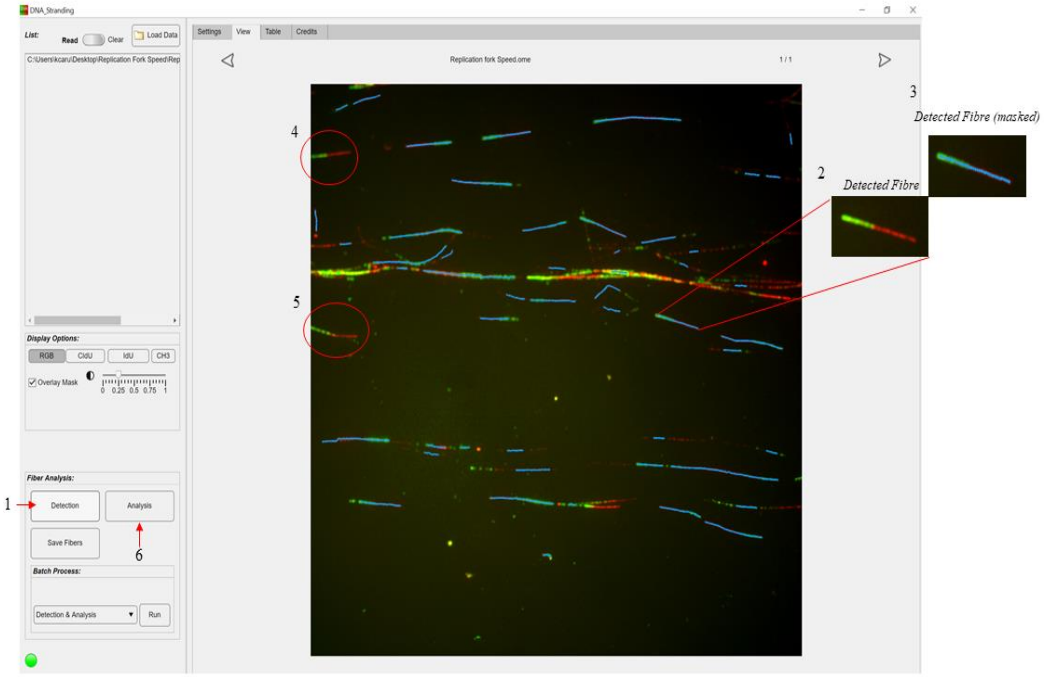


Figure.2. DNA stranding experimental set up, image viewing and detection of DNA fibres is shown. a) An example of settings page is shown. Once the image is loaded, the user can set the type of nucleotide labelling, colour and the timing of the assay, which are critical. b) Clicking "view" tab will help to see the uploaded image and the red arrow indicates the possibility of adjusting contrast for the image. Right inset: Zoomed in representative image of intact fibre. c) 1. Clicking onto detection will, detect all the possible fibre patterns. 2 and 3 shows the example of the fibres that is unmasked and masked. 4 and 5 are the examples that show the accuracy of the software that excludes the fibres that are not intact or at the edges. 6. Once the fibres are detected, the user can click for "Analyses" (Analyses table will be discussed in next figure).

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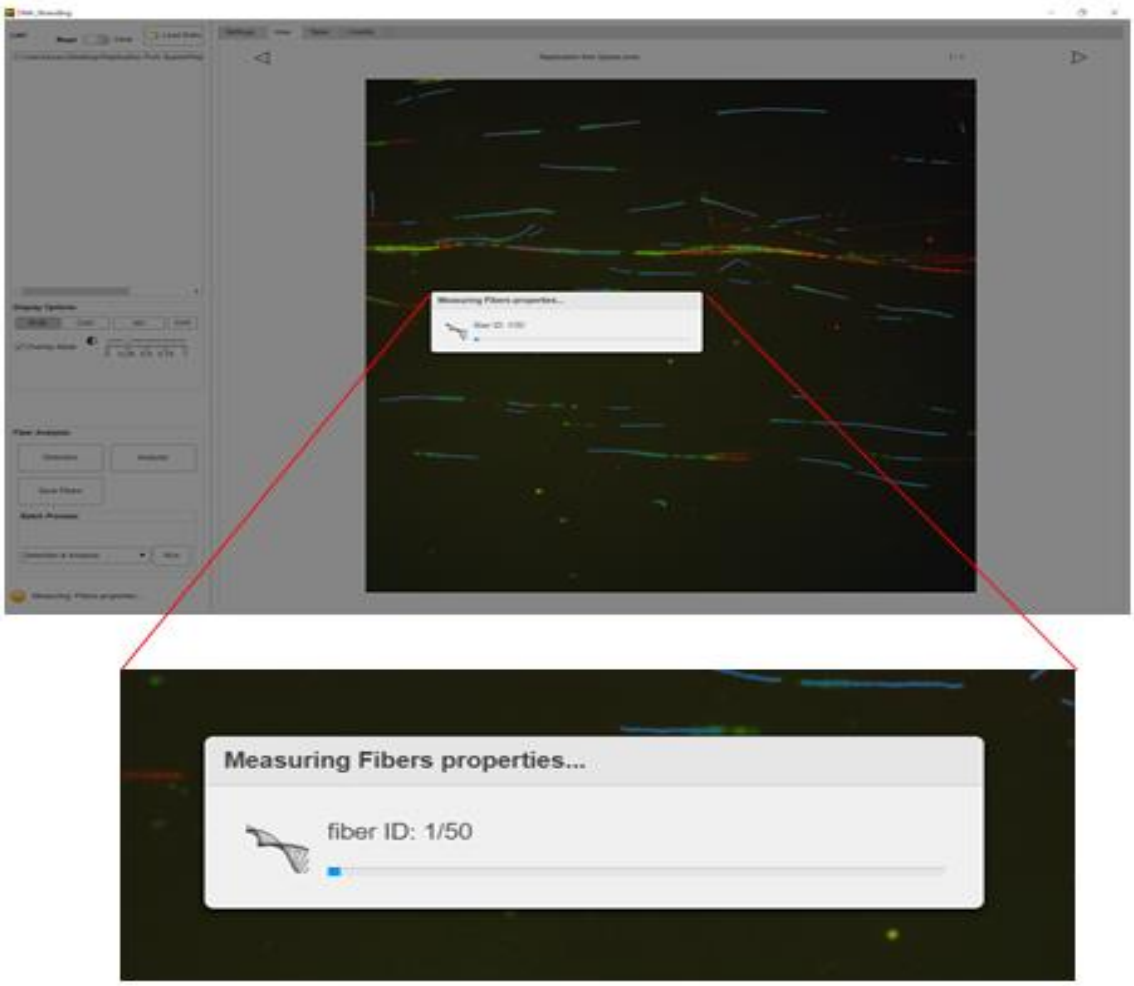
3. *How to identify different replication or repair patterns using the software?*

After clicking analysis (Fig.2c, 6), the software will analyse different replication and repair intermediates (Fig.3a). All the analysed fibre patterns that has been picked up by the sofware will be displayed as results in "Table" tab (Fig.3b). In the table, the "Class" section will display all the different replication pattern names such as Replication fork, Bi-directional Replication fork, Stalled Replication fork, New Replication fork, Terminating fork, Multiple Origin firing (Fig.3b 1 and 2).

a)



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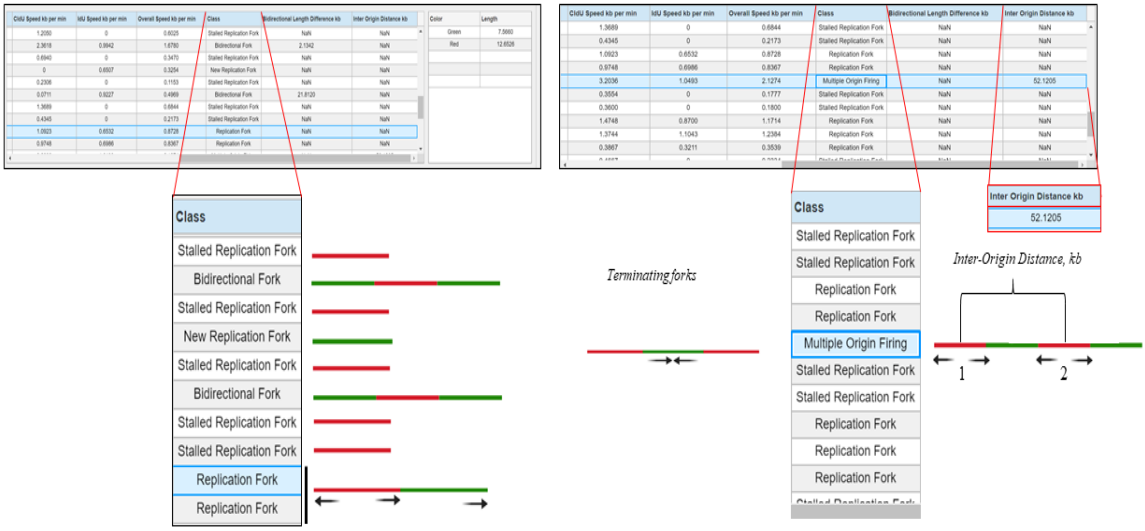

Figure.3. Fibre patterns identification and representation. a) Once the user clicks Analysis (2c, 6), the software begins to meaure the fiber properties. b) The measure of fibre properties are presented comprehensively in the table section and the each replication or repair patterns are presented according to the pattern discussed in FAQ, 1.

4. *How do I measure replication fork speed or velocity using DNA Stranding Software?*

Average replication fork speed differs from cell line to cell line. Once the user enter precisely in the settings, the labelling pattern of nucleotide analogue, its respective colour and the timing of each incorporation, the images can be uploaded, viewed, detected and clicked for analyses. In the analyses table, the first three columns are the fibre number, number of fibre segments and the total fibre length (in terms of kb or µm). The fourth and the fifth column individually gives the length CldU and IdU length in terms of kb (FAQ - 1). In this example table, the labelling was performed for 30 min each (Green and Red). Accordingly, the CIdU and IdU speed per min is calculated in terms of kb. In the next column the "**overall fork speed**" has been calculated by the software that displays the replication fork speed for a specific genetic background or on a treatment condition.

a) **b)**

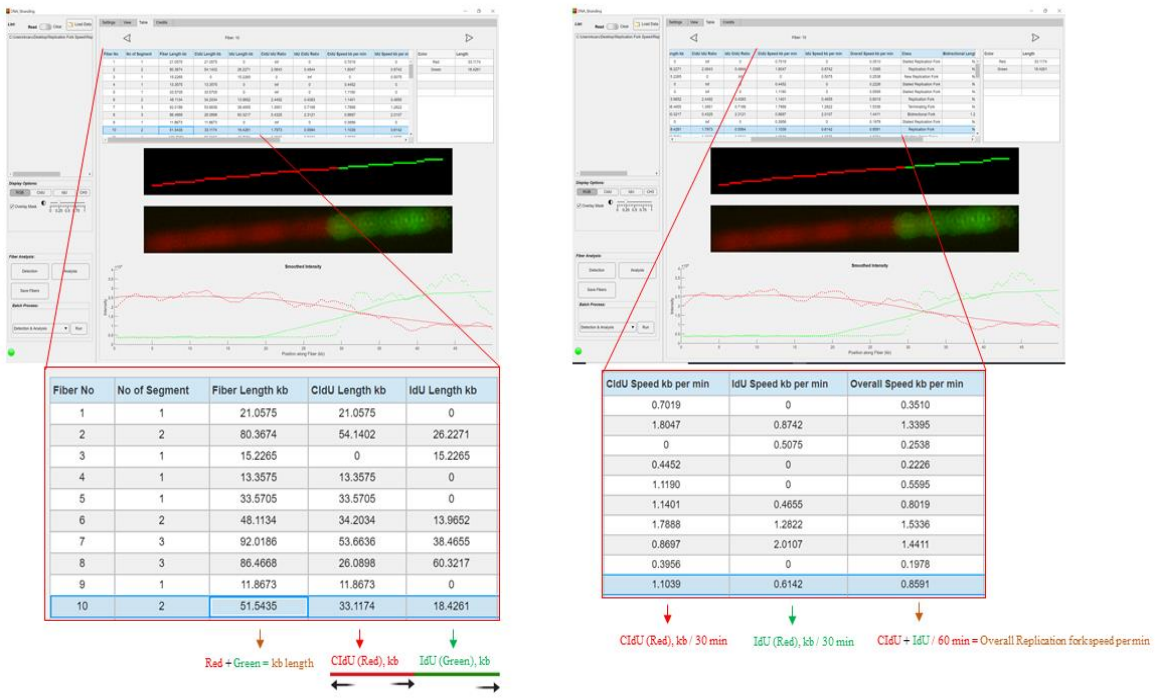

Figure. 4. Measuring replication fork speed. a) In the analyses table, number of fiber segments, total fibre length and individual length of CIdU and IdU are represented in terms of kb. b) Here replication fork speed per minute considering each label as 30 min and 30 min each is calculated for CIdU, IdU labels seperately and total fork length per minute is also calculated and represented.

5.Altered replication fork speed are accompanied with assymetric fork progression, how do I measure the assymetric fork progression?

"Assymetric forks" are basically "Bi-directional Forks". The software captures a bi-directional fork, and presents the difference between the length's of the rightward and leftward moving fork. The user determines the "**severity of replication fork assymetry**" by comparing the leftward and rightward moving forks in comparison with their controls.

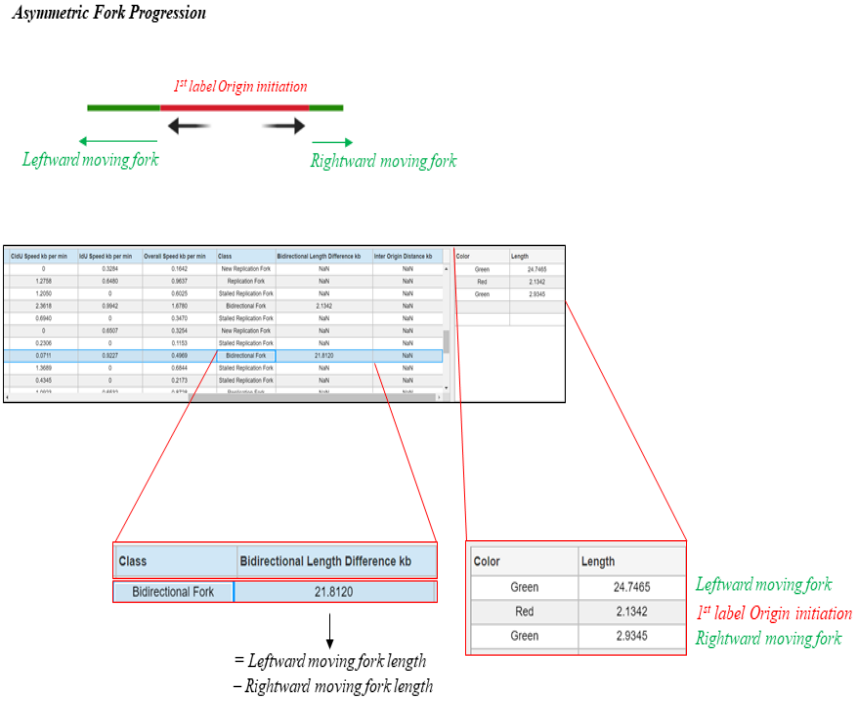

Figure.5. Measuring assymetric fork progression. Above: Representative Assymetric Fork progression has been shown, where rightward moving sister-fork is significantly slower compared to the leftward moving fork. Below: As mentioned earlier, the software will give both the length of the leftward, rightward and first label origin. The user can represent the way they fancy, the severity of the assymetric fork progression. However, the software gives a calculation of bidirectional fork length difference.

6). How to measure the frequency of new origin firing ?

DNA replication fork speed and origin firing are closely inter-connected. According to the replication pattern explained in question.1. If the labelling pattern is Red and Green, the "second labelled" or green only fibres are the new origins or new replication fork. To measure the new origin the user can scroll the table to find the **"class"** column, the number of **"new replication fork"** will directly reflect the new origin firing under a specific genetic background. Please see figure.3b.

7). How do I measure the frequency of repliation termination events ?

In the **"Analyses table",** if the first labelling is Red and the second labelling is Green. The Red, Green and Red labels are the terminating events. In the "**Class**"section the"**Terminating forks**"are catergorised. Counting the number of "**Terminating forks**" will help identify the percentage of terminating forks in the presence of replication stress or under fork stalling.

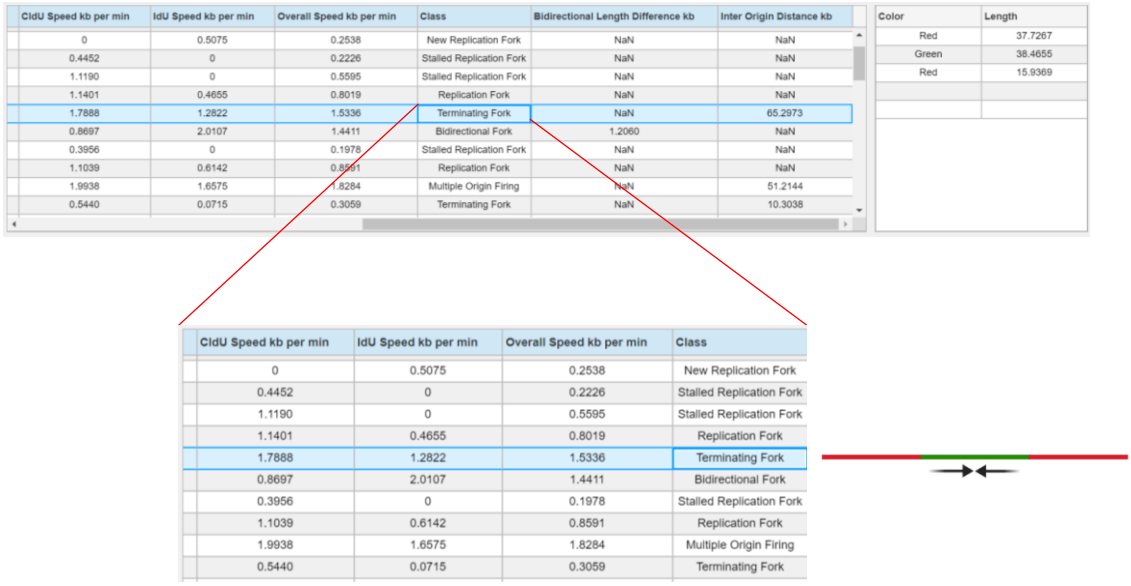

Figure.7. Measuring terminating replication forks. In the "class" section terminating replication forks are categorised, their individual label length and fork speed are measured in analyses section.

8). How do I measure the Inter- Origin distance ?

Fibres containing more than two origins are categorised as "**Multiple Origin Firing**"in the "**Class**" section. The software automatically measures the distance between two origins and represents as Inter-Origin Distance (IOD) (Fig. 8.1). The IOD is also measured in the presence of two origins in case of "terminating forks" (Fig.7 and 8.2).

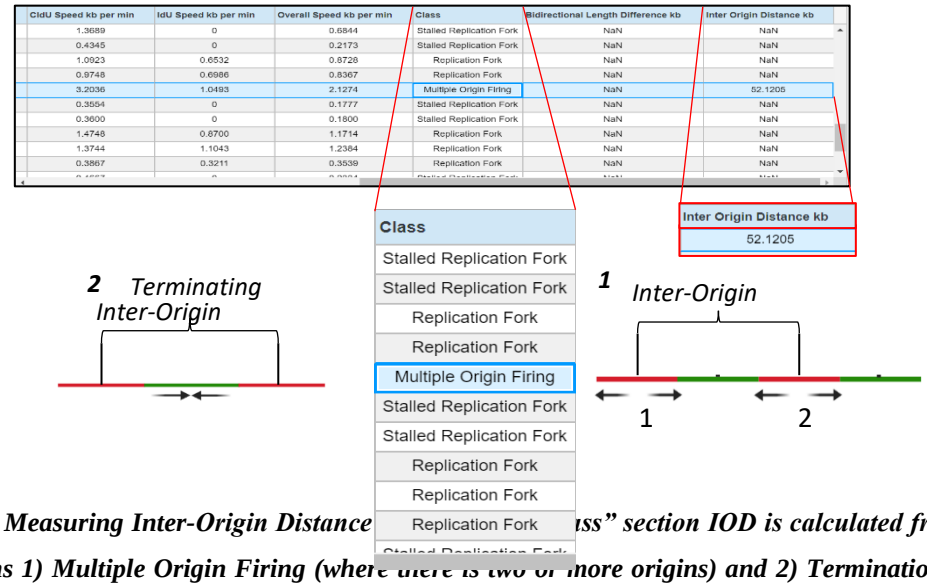

Figure.8. Measuring Inter-Origin Distance ***ss" section IOD is calculated from two kinds of patterns 1) Multiple Origin Firing (where there is two or more origins) and 2) Termination events. The inter-origin distance is shown in term of kb's in the inset.***

9. *How do I check the percentage of stalled replication fork in my experiment that is stalled transiently with genotoxic agents?*

Replication fork restart experiments performed, when forks are labelled with Red (CIdU) for defined timing and treated with high dose of replication fork stalling drugs for 2 to 5 hours. Next, the drug is removed and labelled with Green (IdU) for specfic duration. The efficiency of the second labelling, Green (IdU) is directly proportional to the ability of the cells to restart or repair the stalled replication forks. When the efficiency of replication fork restart is severly affected, the number of "Red only" fibres tend to increase significantly. These "Red only" fibres are called as "**stalled replication forks**"(Fig.3b). Hence, in "**class**" section, the software categorises "Red only" fibres as "**stalled replication forks**" when the first labelling is Red and Second labelling is Green (Fig.3b). Quantifying the percentage of "**stalled replication forks**" will help to undertand the efficiency of fork restart under specific genetic background.

10. How do I measure the nascent DNA stability upon prolonged replication fork stalling?

Sequential labelling of Red (CIdU) and Green (IdU) 30 min each (timing depends on the each lab's protocol) followed by prolonged stalling of replication forks by Hydroxy Urea (HU) trigger nascent DNA degradation in the absence of factors that protect fork from nascent DNA degradation (3-7). With this experimental condition, the ratio of Green (IdU)/ Red (CIdU) will help to address the stability of newly replicated DNA. If the ratio of Green (IdU)/ Red (CIdU) is ~ 1 then the replication forks are stable. For example in the specific genetic background that lacks replication fork protecction factors, the ratio of Green (IdU)/ Red (CIdU) is < 1 or comparitively less with cells that contains replication fork protection factors. This comparison helps to ascertain under specific genetic background forks are stable or not.

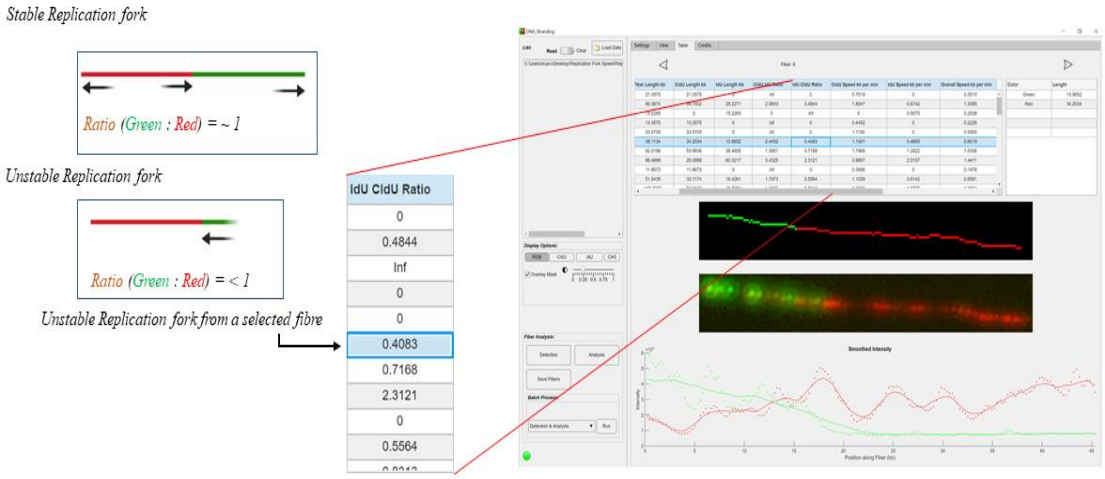

Fig. 9. ***Nascent DNA degradation experiment.*** *Left: Example of Stable and Unstable forks are shown. When cells were treated with CIdU follwed by IdU and stalled for a significant amount of time with genotoxic agents. In the absence of fork protection factors such as BRCA1, BRCA2, FANCD2 and Rad51, leads to unstable replication forks due to degradation of nascent DNA. In such experimental conditions, ratio of second label (IdU): first label (CIdU) can be used to determine the severity of nascent DNA degradation.*

11. Can I run this software in a semi-automated fashion?

Once the automatic detection is performed using the software, using pencil tool, the user (experienced in interpreting replication repair patterns) can draw the fibres that are not picked by the software. The manually drawn and automatically detected fibres are analysed together as mentioned above for different experimental conditions.

12. Can the DNA stranding sofware be used manually to detect all the fibre patterns?

The software is created to avoid user bias and to reduce the time for analyses that is frequently encountered. However, DNA stranding software gives an option to manually detect the fibres with a drawing pencil tool. Once fibres are manually drawn by an expert in DNA replication and repair field, the analyses can be performed with the software.

References:

1. Lebofsky R, Bensimon A. Single DNA molecule analysis: applications of molecular combing. Brief Funct Genomic Proteomic. 2003;1(4):385-96.

2. Herrick J, Bensimon A. Single molecule analysis of DNA replication. Biochimie. 1999;81(8-9):859-71.

3. Kolinjivadi AM, Sannino V, De Antoni A, Zadorozhny K, Kilkenny M, Techer H, et al. Smarcal1-Mediated Fork Reversal Triggers Mre11-Dependent Degradation of Nascent DNA in the Absence of Brca2 and Stable Rad51 Nucleofilaments. Mol Cell. 2017;67(5):867-81 e7.

4. Liu W, Krishnamoorthy A, Zhao R, Cortez D. Two replication fork remodeling pathways generate nuclease substrates for distinct fork protection factors. Sci Adv. 2020;6(46).

5. Schlacher K, Christ N, Siaud N, Egashira A, Wu H, Jasin M. Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. Cell. 2011;145(4):529-42.

6. Somyajit K, Saxena S, Babu S, Mishra A, Nagaraju G. Mammalian RAD51 paralogs protect nascent DNA at stalled forks and mediate replication restart. Nucleic Acids Res. 2015;43(20):9835-55.

7. Somyajit K, Subramanya S, Nagaraju G. Distinct roles of FANCO/RAD51C protein in DNA damage signaling and repair: implications for Fanconi anemia and breast cancer susceptibility. J Biol Chem. 2012;287(5):3366-80.