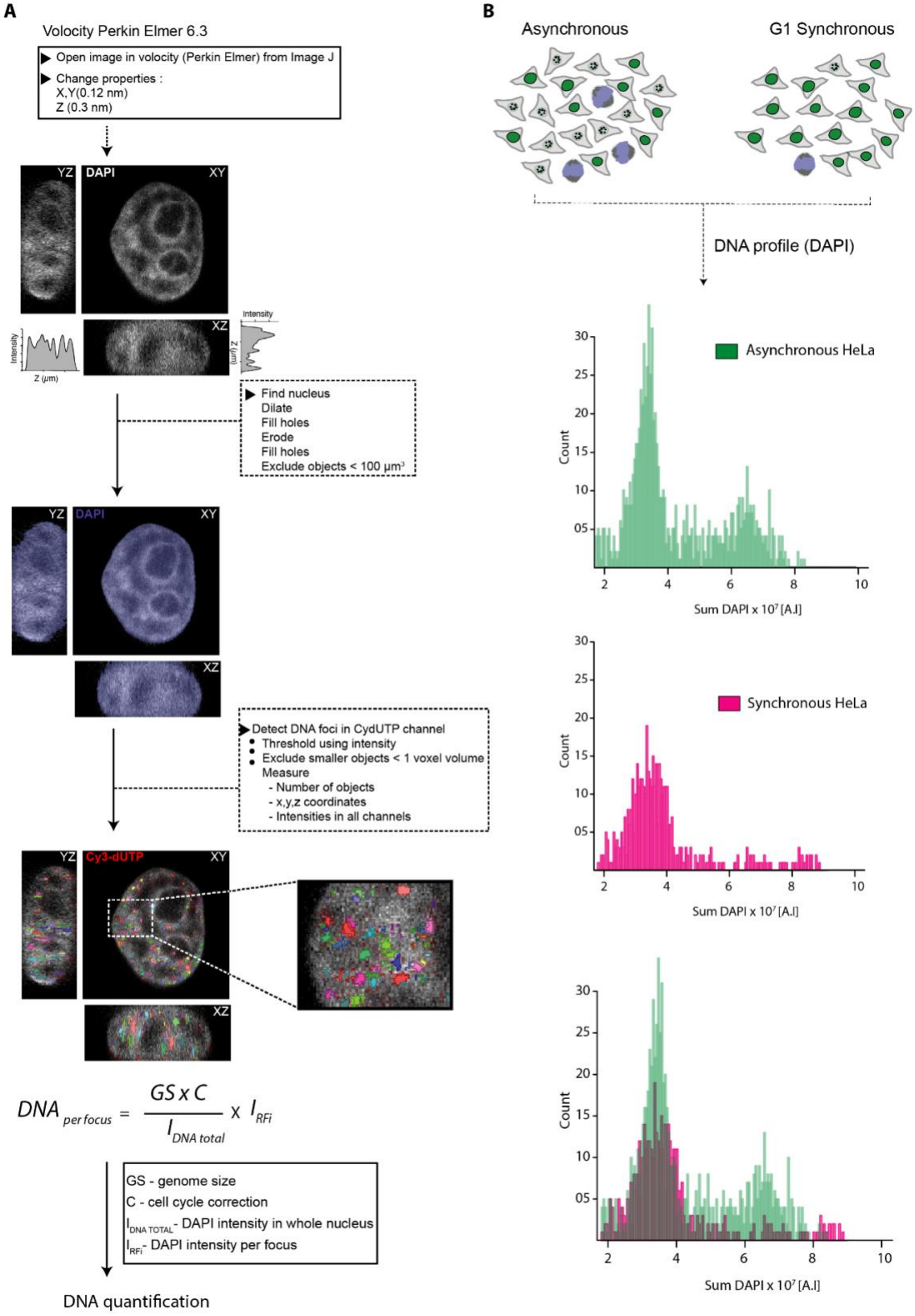
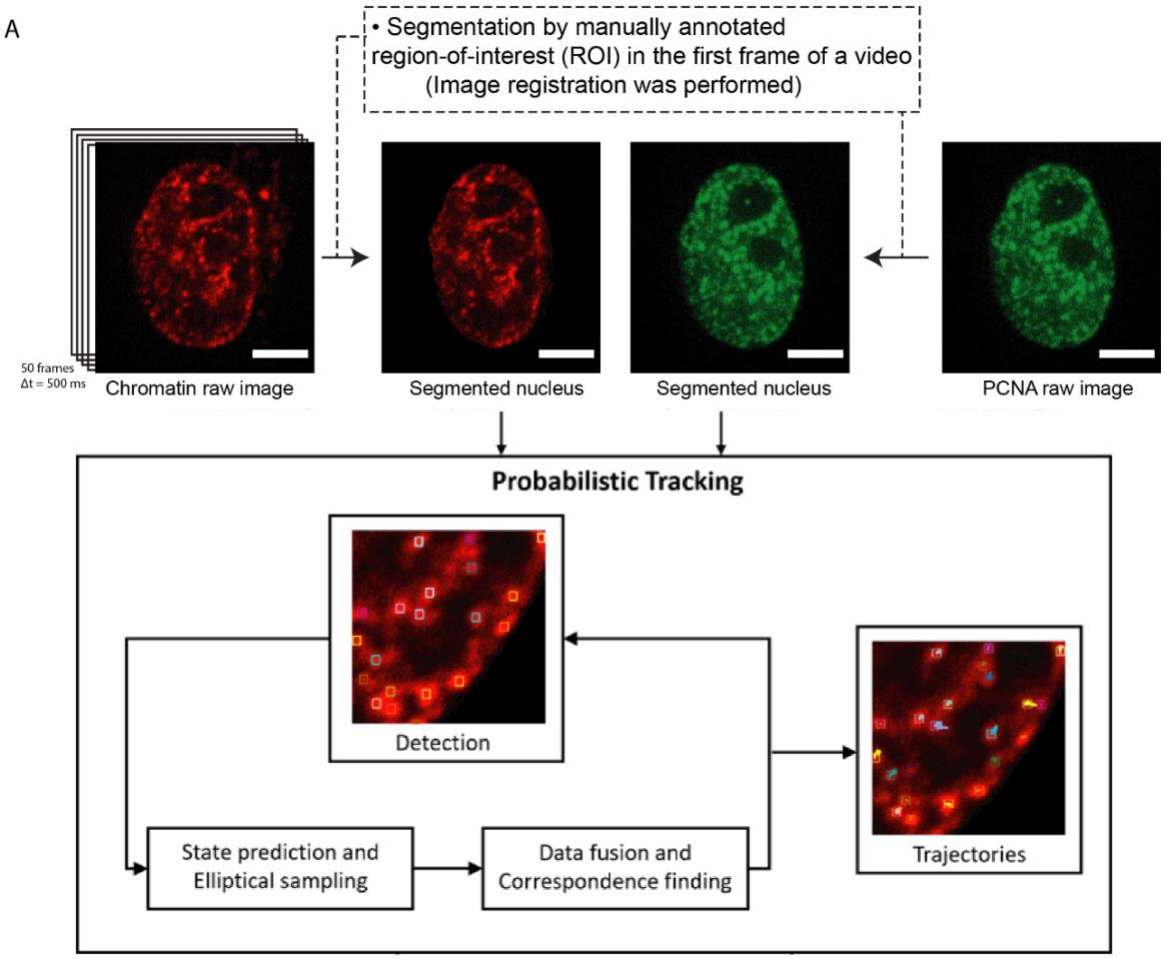

A
Volocity Perkin Elmer 6.3
Open image in volocity (Perkin Elmer) from Image J
Change properties :
X,Y(0.12 nm)
Z (0.3 nm)
YZ
DAPI
XY
XZ
Intensity
Z (µm)
Find nucleus
Dilate
Fill holes
Erode
Fill holes
Exclude objects < 100 µm³
Detect DNA foci in CydUTP channel
Threshold using intensity
Exclude smaller objects < 1 voxel volume
Measure
- Number of objects
- x,y,z coordinates
- Intensities in all channels
Cy3-dUTP
$DNA_{per\ focus} = \frac{GS \times C}{I_{DNA\ total}} \times I_{RFi}$
GS - genome size
C - cell cycle correction
$I_{DNA\ TOTAL}$ - DAPI intensity in whole nucleus
I_{RFi} - DAPI intensity per focus
DNA quantification
B
Asynchronous
G1 Synchronous
DNA profile (DAPI)
Asynchronous HeLa
Synchronous HeLa
Count
Sum DAPI x 10⁷ [A.I]

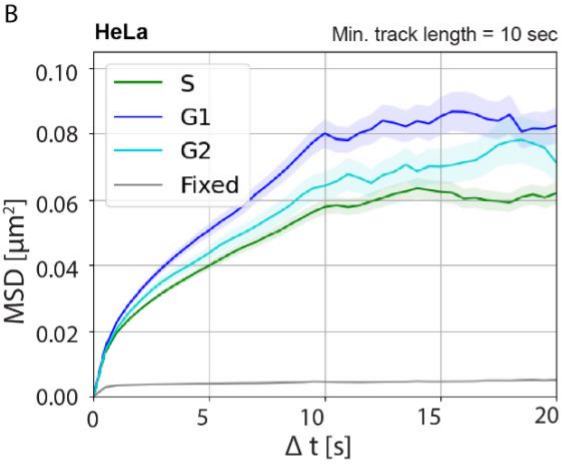
Figure 2-figure supplement 1: Pipeline and controls for DNA quantification of labeled replication foci using confocal data.

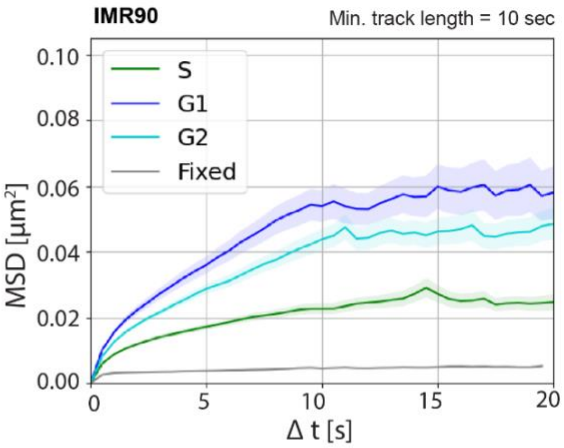
(A) All image sequences were acquired using spinning disk microscopy (60x objective, 1.45 numerical aperture). For analysis, the (16 bit) images were imported from ImageJ into PerkinElmer Volocity 6.3 software. Using the DAPI channel, nuclear segmentation (object recognition) was performed to obtain a mask for the whole nuclear volume and determine the total DNA intensity (mask highlighted with blue). The labeled chromatin foci (Cy3-dUTP) were detected using the intensity and segmented in 3D (highlighted in color palette). The sum DAPI intensity within the segmented foci (I_{RFi}) were also obtained. The ratio of DNA amount per focus and total DNA in the nucleus corrected for cell cycle stage gives the DNA amount per focus. For quantification see Figure 2. The intensity values of the total DAPI intensity within each segmented replication foci (I_{RFi}) are plotted as a histogram. **(B)** HeLa Kyoto cells, either synchronously (magenta) (Methods) or asynchronously growing (green), were seeded on coverslips. The sum DAPI (DNA profile) of cells was plotted individual and in an overlap histogram (brown).

A



B





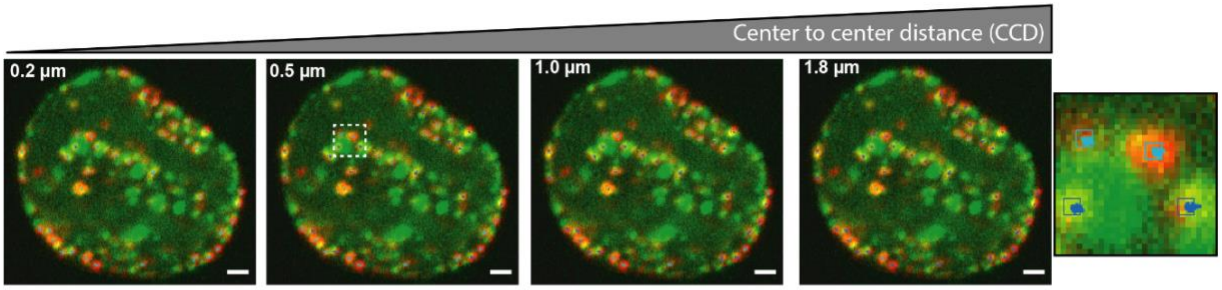
	α	D [μm²/s x 10⁻⁵]
G1 (930 trajectories)	0.41	143.5
G2 (829 trajectories)	0.42	123.3
S-phase (2364 trajectories)	0.36	105.6
Fixed cells (1689 trajectories)	0.15	8.5

	α	D [μm²/s x 10⁻⁵]
G1 (470 trajectories)	0.40	99.8
G2 (873 trajectories)	0.40	79.6
S-phase (717 trajectories)	0.33	43.7
Fixed cells (624 trajectories)	0.19	8.8

Figure 3-figure supplement 1: Workflow of chromatin motion and proximity analysis for confocal data.

(A) Raw images of chromatin and PCNA were first segmented to remove the background noise outside the nucleus. In case of IMR90 cells, affine image registration was performed to address the stronger cell movement compared to HeLa cells (Methods). The probabilistic tracking method was then performed which includes detection of chromatin particles and tracking over the time to obtain trajectories. The tracks were then used to plot MSD curves or perform proximity analysis with PCNA. (**B)** Result of motion analysis of computed chromatin tracks for G1, G2, S-phase cell cycle stages (Figure 3) for HeLa and IMR90 cells. Mean Square Displacement (MSD, µm2) curves were plotted over time (s). Mean square displacement curves for G1/G2, S-phase, fixed cells with a minimum track length of 10 seconds and a total time of 20 seconds were plotted with error bars (SEM - standard error of the mean) representing the deviations between the MSD curves for an image sequence in transparent color around the curve. Scale bar: 10 µm.

A


Center to center distance (CCD)
0.2 µm
0.5 µm
1.0 µm
1.8 µm

B

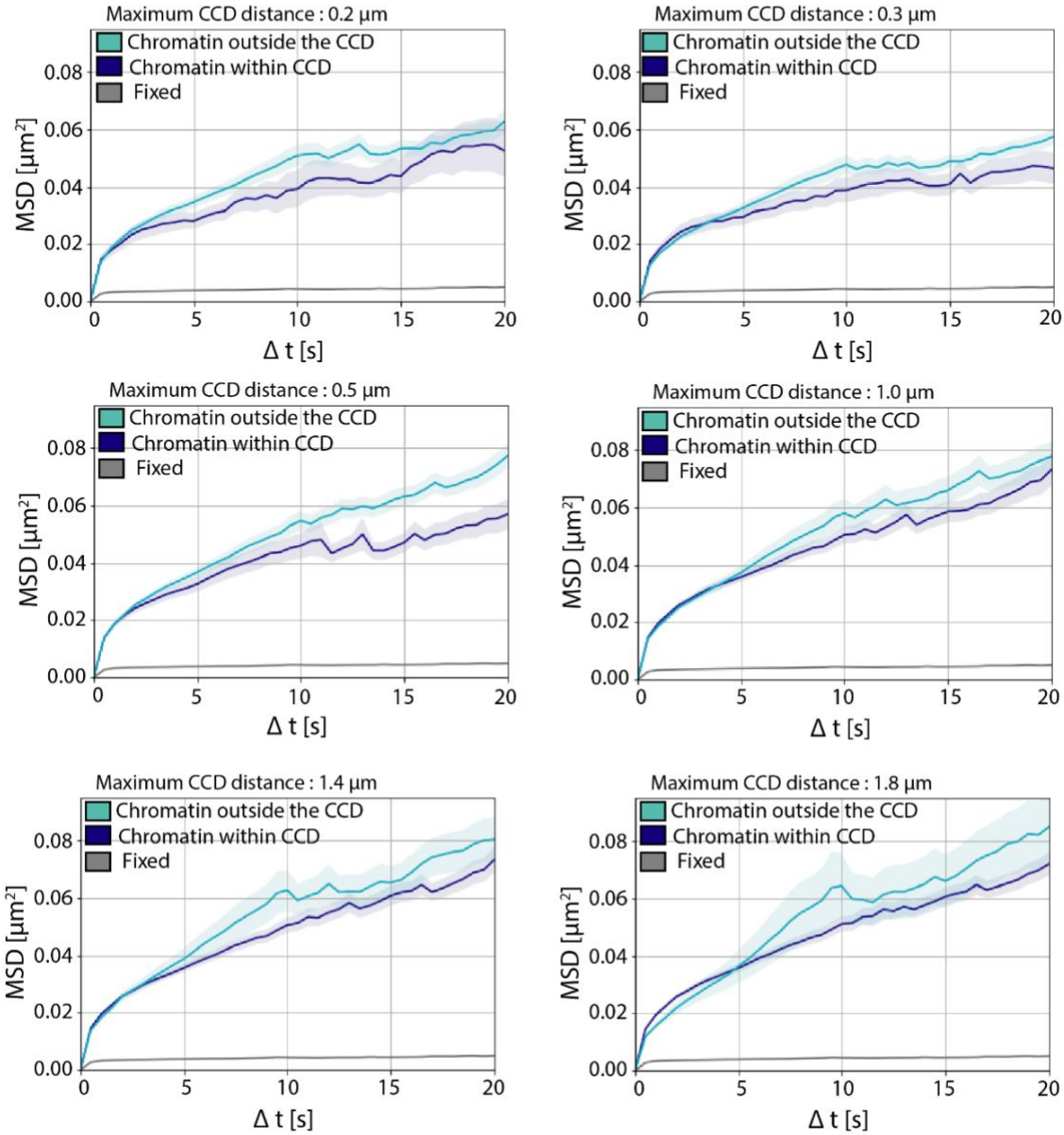

Maximum CCD distance : 0.2 µm
Maximum CCD distance : 0.3 µm
Maximum CCD distance : 0.5 µm
Maximum CCD distance : 1.0 µm
Maximum CCD distance : 1.4 µm
Maximum CCD distance : 1.8 µm
Chromatin outside the CCD
Chromatin within CCD
Fixed
MSD [µm²]
Δ t [s]
0.00
0.02
0.04
0.06
0.08
0
5
10
15
20

Figure 4-figure supplement 1: Mean square displacement analysis of labeled chromatin in the proximity of active replication sites at varying center to center distances.

(A) Overlay images of GFP-PCNA (active replication sites, green) and labeled chromatin (red). Chromatin outside the center-to-center distance (CCD) is highlighted with cyan dots and chromatin within center-to-center distance (CCD) is highlighted with dark blue dots within the magnified inset (demarcated by a white box). **(B)** Mean square displacement (MSD) curves of chromatin outside the center-to-center distance (CCD) and chromatin within center-to-center distance (CCD) along with fixed cell control with increasing distance from 0.2 µm to 1.8 µm. This allows us to analyze the effect of replication factors on chromatin mobility at increasing distances. The MSD curves were plotted with error bars (standard deviation) represented in transparent color around the curve. Scale bar: 1 µm.

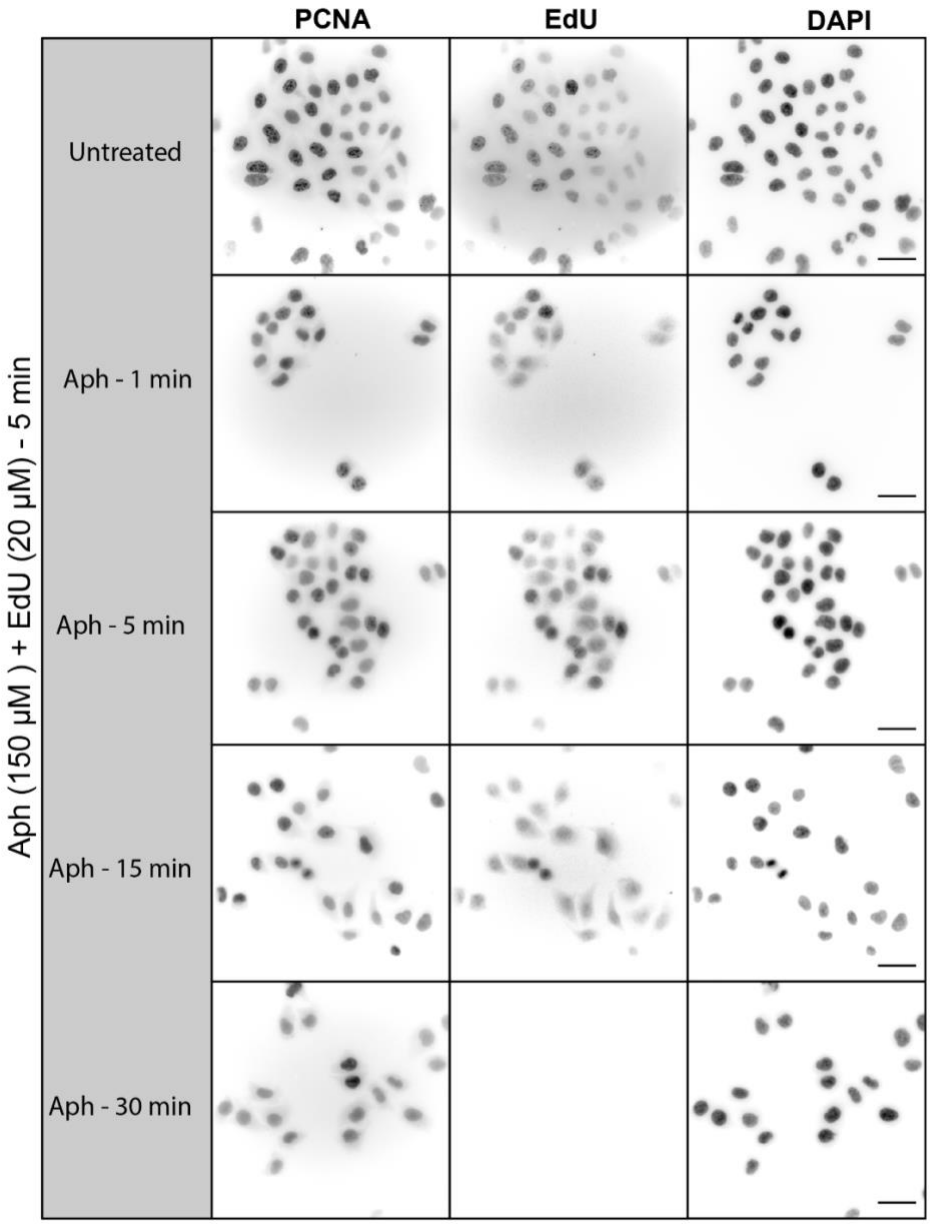
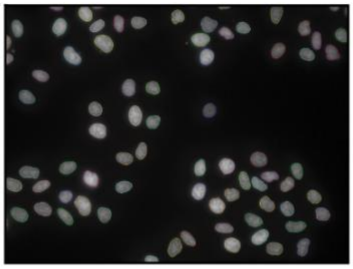


Figure 5-figure supplement 1: Time course analysis of inhibition of DNA synthesis upon aphidicolin treatment.

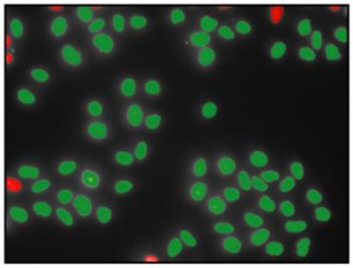
(A) HeLa cells stably expressing GFP-proliferating cell nuclear antigen (PCNA) were treated with aphidicolin (150 µm). Time course of treatments depicting the distribution of PCNA as well as the incorporation of the thymidine analog EdU is shown. EdU was used to detect DNA synthesis. For quantification see Figure 5A. Scale bar: 100 µm.

High content microscopy

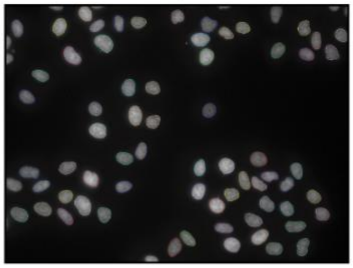
High-troughput
Analysis



Find nuclei using parameters
diameter : 13 µm
splitting coefficient : 0.07
common threshold : 0.4



Select population
- exclude border touching objects



Measure DAPI properties

Measure POI properties - EdU
- within the selected nuclei
- Filter population with Mean > 50 A.U
- Measure signal in EdU positive cells

- Obtain intensity measurements
- Plot data in R

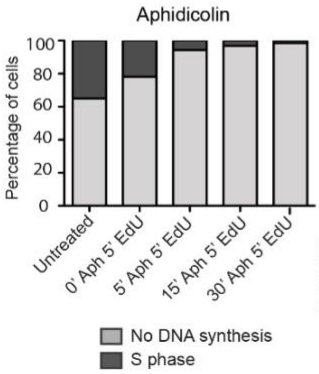
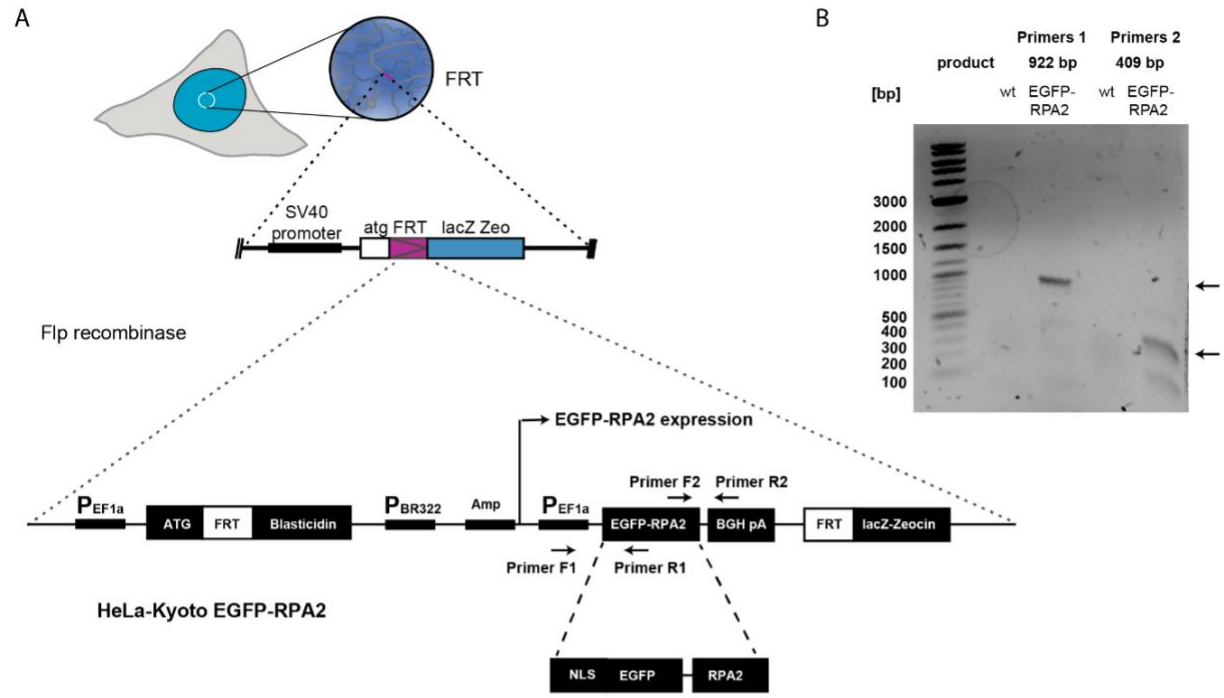
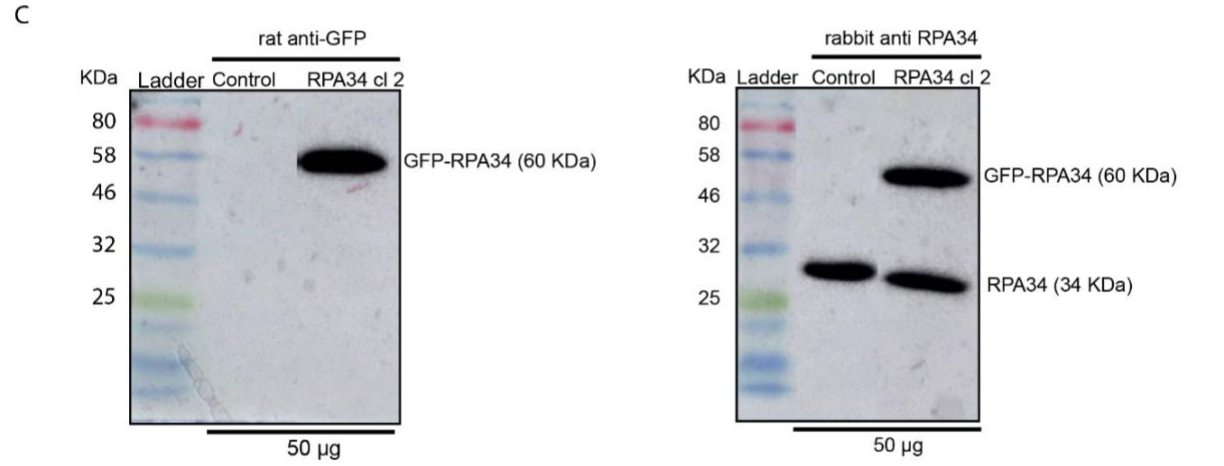


Figure 5-figure supplement 2: Schematic overview of the high throughput analysis pipeline of DNA synthesis inhibition by aphidicolin.

Images were obtained using the 40x objective (numerical aperture 0.95) in the DAPI and EdU channels. Once the images were acquired, they were analyzed using the Harmony software from PerkinElmer. Briefly, the DAPI channel was used to segment nuclei using properties including diameter, splitting coefficient and threshold. Once the nuclei were segmented, the population cells were then selected for analysis by removing the objects either touching the border or fused. In the population of cells selected, the properties including mean, median, standard deviation and sum intensities were measured in the DAPI and EdU channels. The data measurements were then imported to R and visualized for the EdU positive population. Scale bar: 500 µm.


A
FRT
SV40 promoter
atg FRT
lacZ Zeo
Flp recombinase
EGFP-RPA2 expression
Primer F2
Primer R2
PEF1a
ATG
FRT
Blasticidin
PBR322
Amp
PEF1a
EGFP-RPA2
BGH pA
FRT
lacZ-Zeocin
Primer F1
Primer R1
HeLa-Kyoto EGFP-RPA2
NLS
EGFP
RPA2
B
Primers 1
Primers 2
product
922 bp
409 bp
[bp]
wt
EGFP-RPA2
wt
EGFP-RPA2
3000
2000
1500
1000
500
400
300
200
100


C
rat anti-GFP
KDa
Ladder
Control
RPA34 cl 2
80
58
46
32
25
GFP-RPA34 (60 KDa)
50 µg
rabbit anti RPA34
KDa
Ladder
Control
RPA34 cl 2
80
58
46
32
25
GFP-RPA34 (60 KDa)
RPA34 (34 KDa)
50 µg

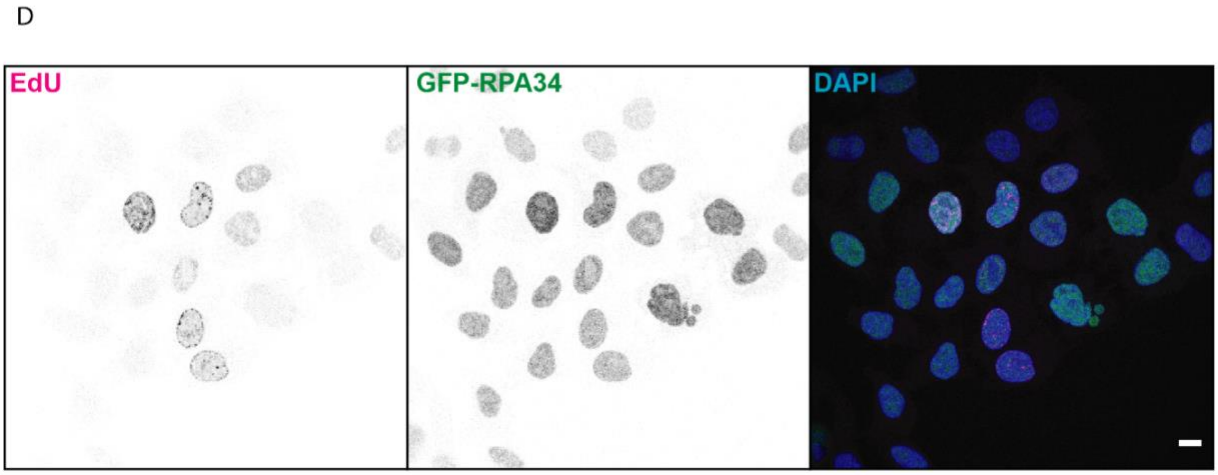

D
EdU
GFP-RPA34
DAPI

Figure 5-figure supplement 3: HeLa GFP-RPA cell line characterization after generation using the Flp-recombinase system.

(A) Schematic representation of the insertion of fluorescently tagged RPA34 (aka RPA2) encoding plasmid DNA using the chromosomally inserted FRT site in the previously generated HeLa Kyoto FRTLacZ (Methods). Positive clones were selected using 1 µg/ml blasticidin in media. **(B)** PCR was performed on genomic DNA isolated from wild type and GFP-RPA34 clones using different primer pairs as indicated. **(C)** Cells from the positive clones were collected to perform a western blot using the anti-GFP antibody to test the level of GFP-RPA34 and an anti-RPA34 antibody was used to detect and compare both endogenous and fusion protein levels in control versus the positive clone. **(D)** The cells were seeded on glass coverslips, fixed and imaged to validate the protein localization relative to active DNA replication sites labeled EdU as well as DNA labeled with DAPI. Scale bar: 5 µm.

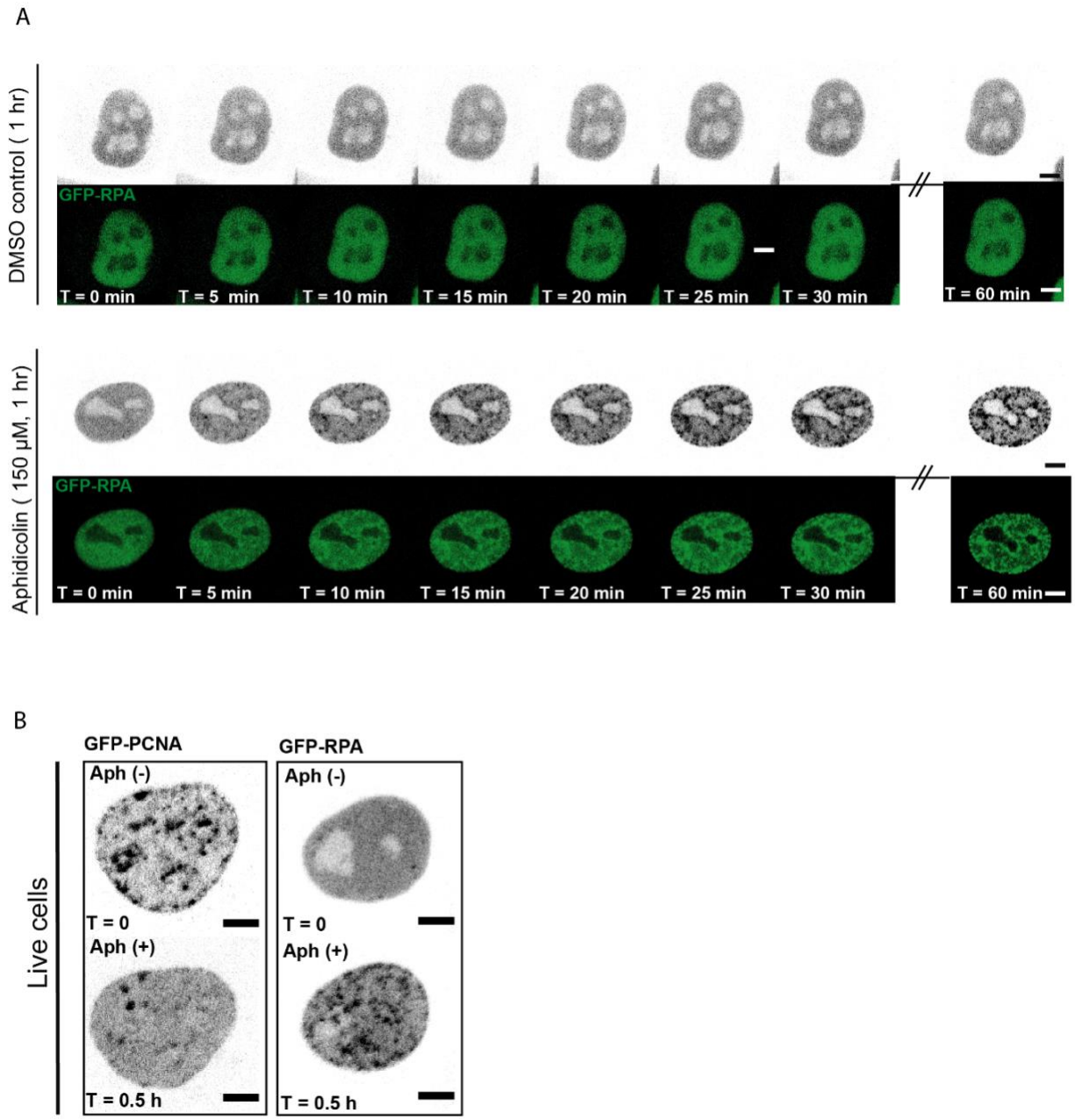


Figure 5-figure supplement 4: Live-cell time-lapse microscopy of HeLa GFP-RPA34 cells to determine RPA accumulation at replication sites upon aphidicolin treatment.

(A) Confocal time-lapse microscopy of HeLa cells stably expressing GFP-RPA34 treated with DMSO (control), and aphidicolin. Videos were acquired at a frame rate of 5 minutes and at every time a Z stack was acquired. A single Z-plane at different timepoints was shown as a representative image for all three conditions. The time-lapse images were imported to PerkinElmer Volocity 6.3 for analysis and the accumulation of GFP-RPA34 at replication foci was measured over time. **(B)** Representative images of live HeLa cells stably expressing GFP-PCNA or GFP-RPA34 are shown in gray scale before and after aphidicolin treatment. Scale bar: 5 µm.

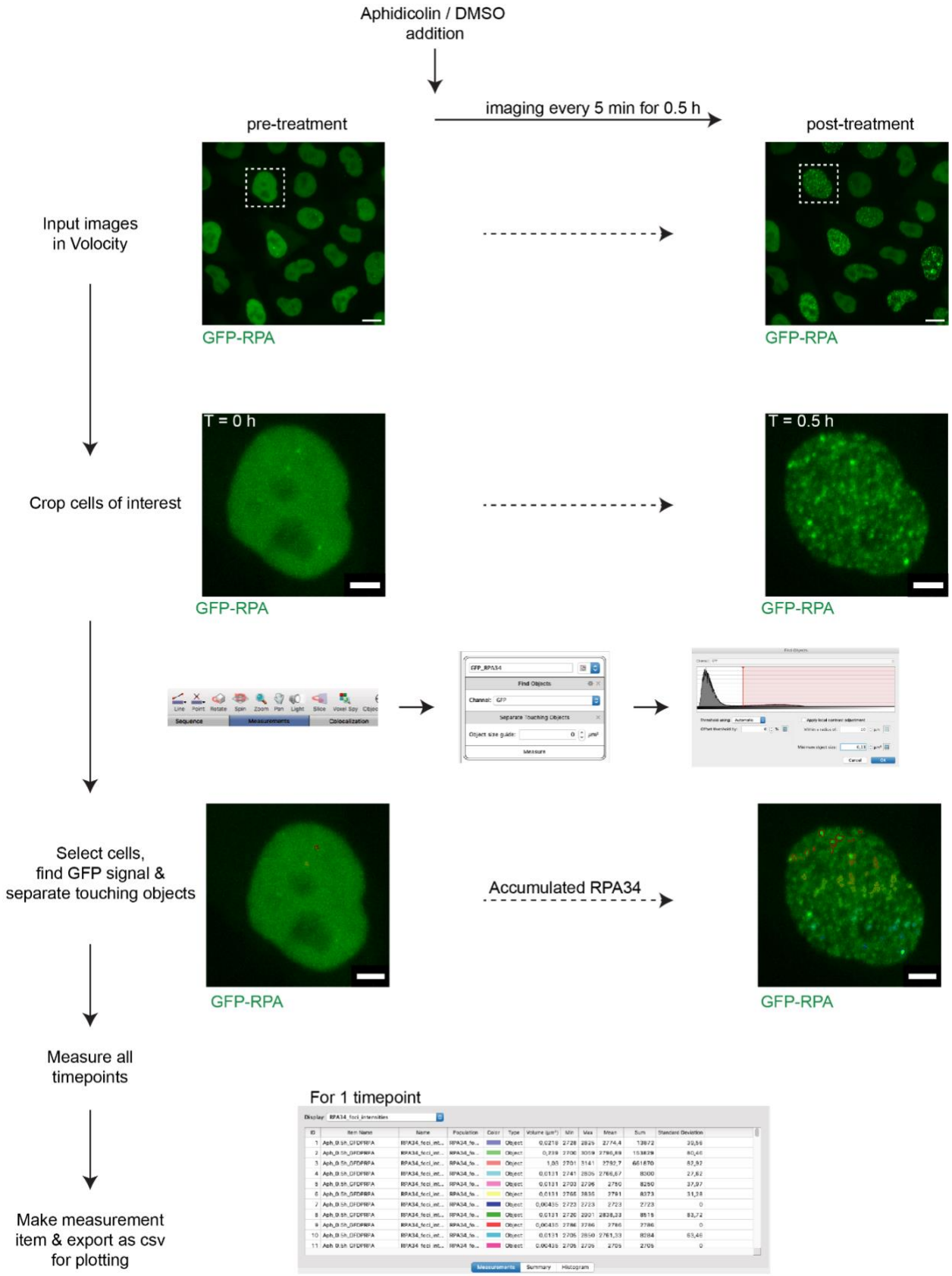
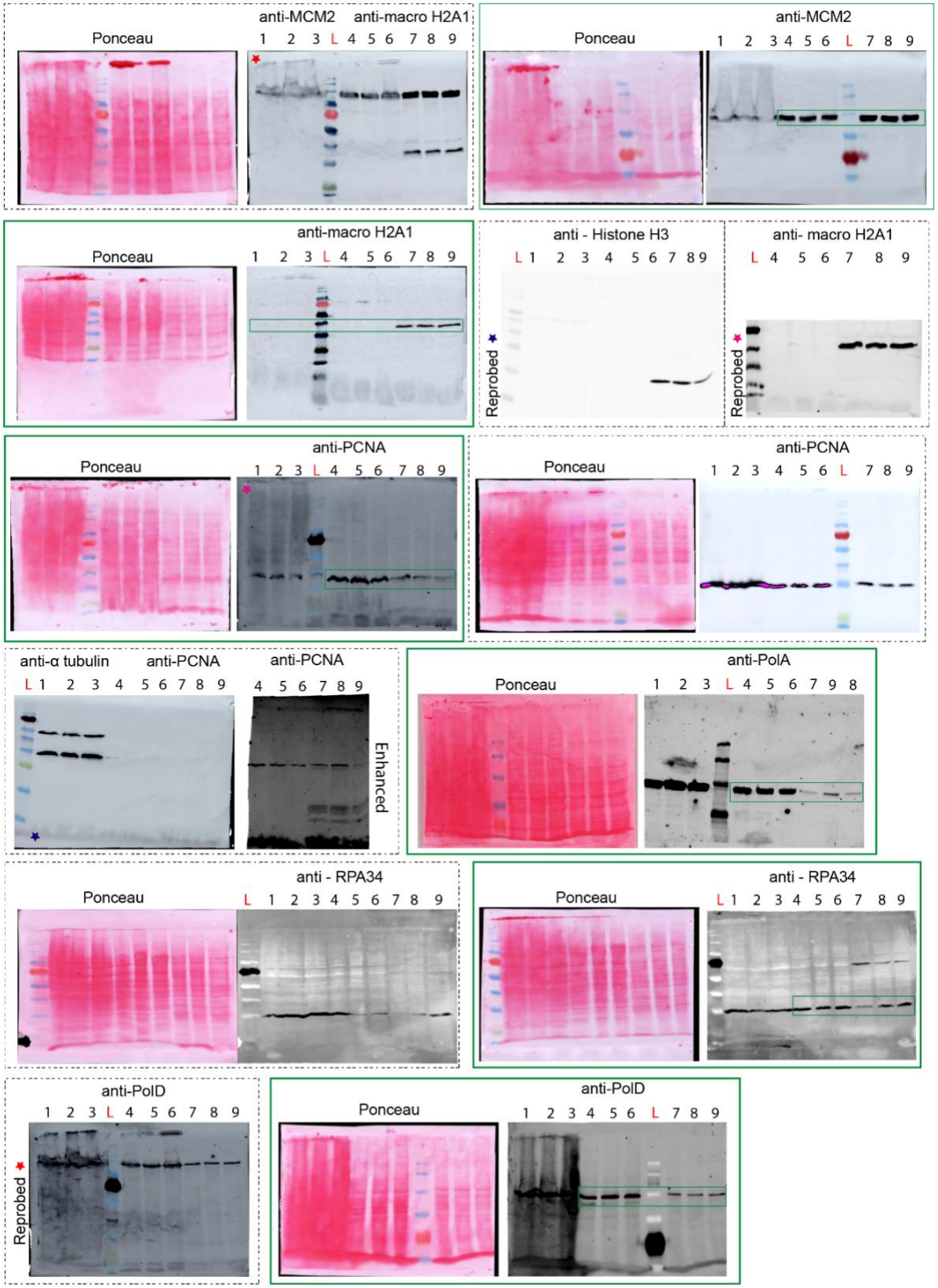

Aphidicolin / DMSO addition
imaging every 5 min for 0.5 h
pre-treatment
post-treatment
Input images in Volocity
GFP-RPA
GFP-RPA
Crop cells of interest
T = 0 h
T = 0.5 h
GFP-RPA
GFP-RPA
Select cells, find GFP signal & separate touching objects
Accumulated RPA34
GFP-RPA
GFP-RPA
Measure all timepoints
For 1 timepoint
Make measurement item & export as csv for plotting

Figure 5-figure supplement 5: Pipeline for the analysis of RPA enrichment at replication sites in living cells.

The enrichment or accumulation analysis was performed using PerkinElmer Volocity 6.3 software. Briefly cells of interest (white inserts) were cropped and Z-stacks along with different timepoints were imported together. The GFP-RPA34 intensities at different timepoints for aphidicolin (every 5 min / 0.5 h) were measured and the coefficient of variation c_V was calculated (Methods, Accumulation analysis). The intensity values were exported as CSV files for further analysis. The plots were generated in R studio. Scale bar: 5 µm. Cropped cells scale bar: 1 µm.


Ponceau
anti-MCM2
anti-macro H2A1
1 2 3 L 4 5 6 7 8 9
Ponceau
anti-MCM2
1 2 3 4 5 6 L 7 8 9
Ponceau
anti-macro H2A1
1 2 3 L 4 5 6 7 8 9
anti - Histone H3
L 1 2 3 4 5 6 7 8 9
Reprobed
anti- macro H2A1
L 4 5 6 7 8 9
Reprobed
Ponceau
anti-PCNA
1 2 3 L 4 5 6 7 8 9
Ponceau
anti-PCNA
1 2 3 4 5 6 L 7 8 9
anti-α tubulin
anti-PCNA
L 1 2 3 4 5 6 7 8 9
anti-PCNA
4 5 6 7 8 9
Enhanced
Ponceau
anti-PolA
1 2 3 L 4 5 6 7 9 8
Ponceau
anti - RPA34
L 1 2 3 4 5 6 7 8 9
Ponceau
anti - RPA34
L 1 2 3 4 5 6 7 8 9
anti-PolD
1 2 3 L 4 5 6 7 8 9
Reprobed
Ponceau
anti-PolD
1 2 3 4 5 6 L 7 8 9

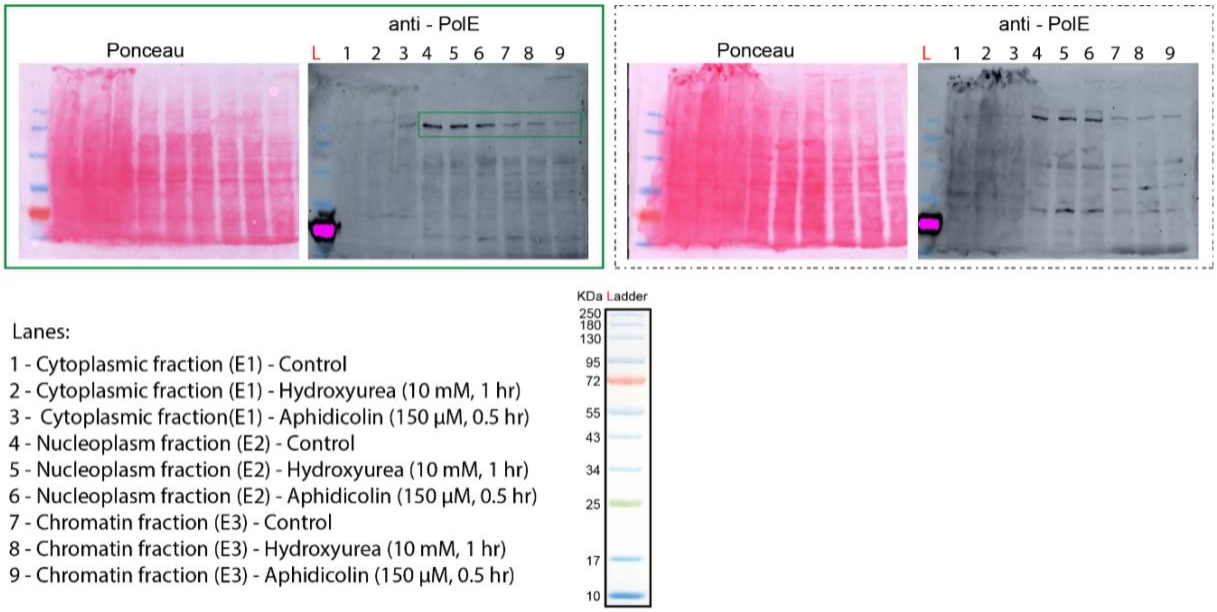


Figure 5-figure supplement 6: Full length western blots probed for different replication factors with cytoplasm, nucleoplasm and chromatin fractions.

The blots that are reprobed with a different antibody are highlighted with color stars. The blots highlighted with green inserts are the full length and single contrast blots that are cropped for easier visualization as the loading order of samples and ladder is different and are summarized in Figure 5C.


EdU
10 min
Control / Aph
30 min
Preextraction
Fixation
Immunostaining
High throughput
imaging and analysis

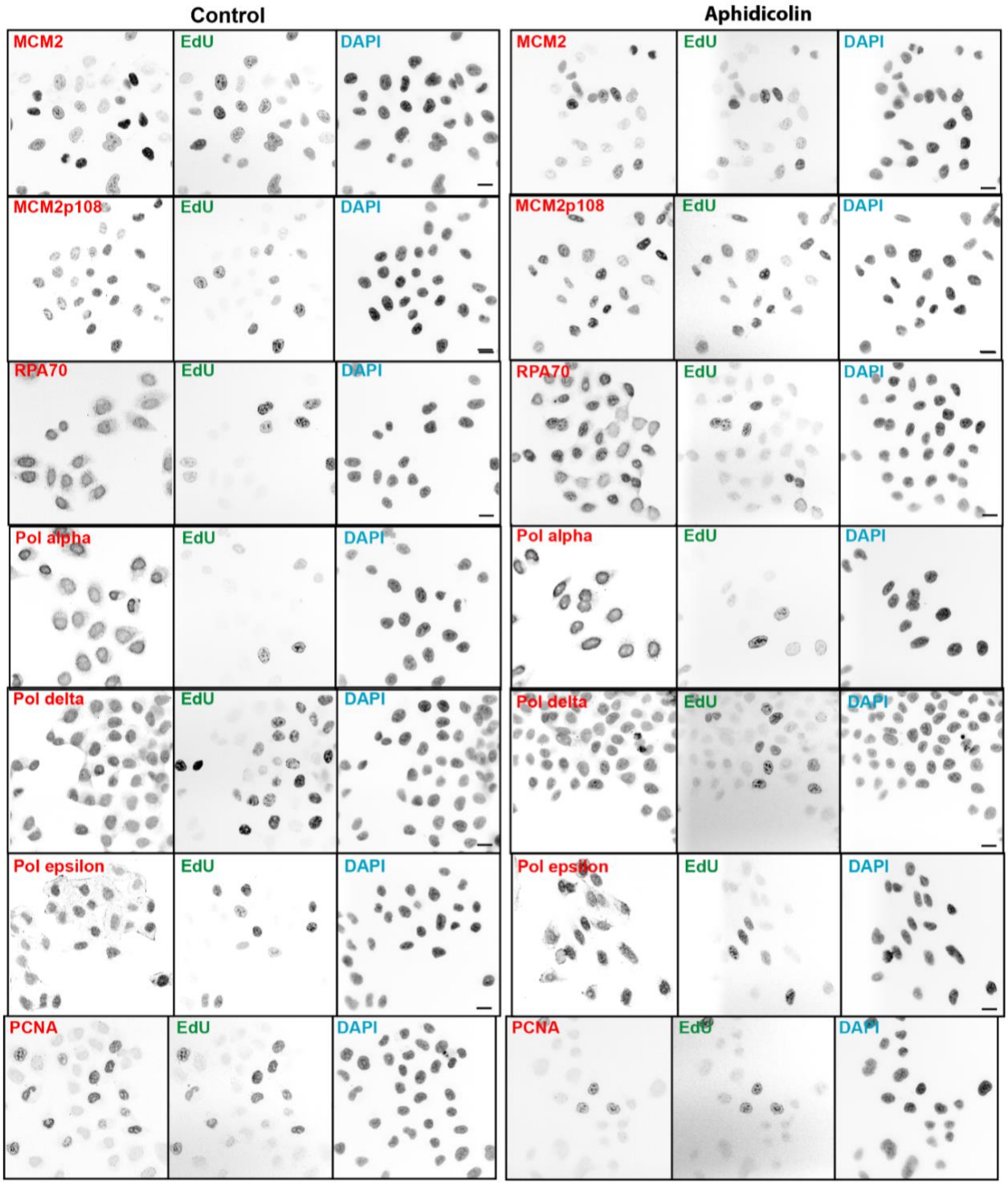
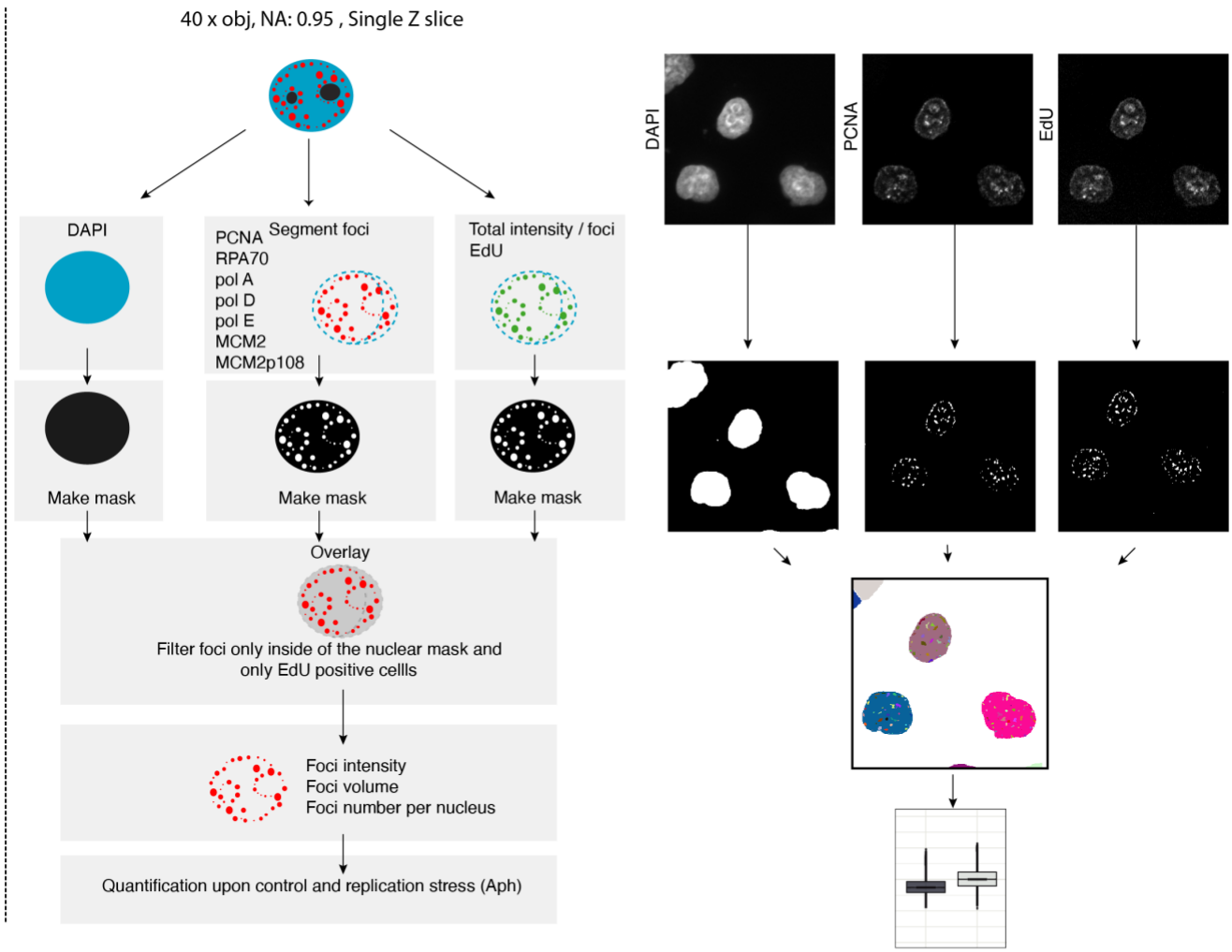

Control
Aphidicolin
MCM2
EdU
DAPI
MCM2p108
RPA70
Pol alpha
Pol delta
Pol epsilon
PCNA
pre-extracted

Figure 5-figure supplement 7: Analysis of replisome component enrichment at replication sites upon replication stress.

Spatial localization and accumulation analysis of DNA replication factors such as DNA helicase subunit MCM2 and active MCM2 phosphorylated at S108, polymerase clamp protein (PCNA), single stranded binding protein (RPA) and DNA polymerases (α, δ, ε) catalytic subunits using immunostainings. The cells in S-phase were identified using an EdU pulse before the treatment. Representative low magnification images of protein localization in control and aphidicolin treated HeLa cells. For quantification see Figure 5D. Scale bar: 10 µm.

A


40 x obj, NA: 0.95 , Single Z slice
DAPI
PCNA
RPA70
pol A
pol D
pol E
MCM2
MCM2p108
Segment foci
Total intensity / foci
EdU
Make mask
Make mask
Make mask
Overlay
Filter foci only inside of the nuclear mask and only EdU positive cellls
Foci intensity
Foci volume
Foci number per nucleus
Quantification upon control and replication stress (Aph)
DAPI
PCNA
EdU

B

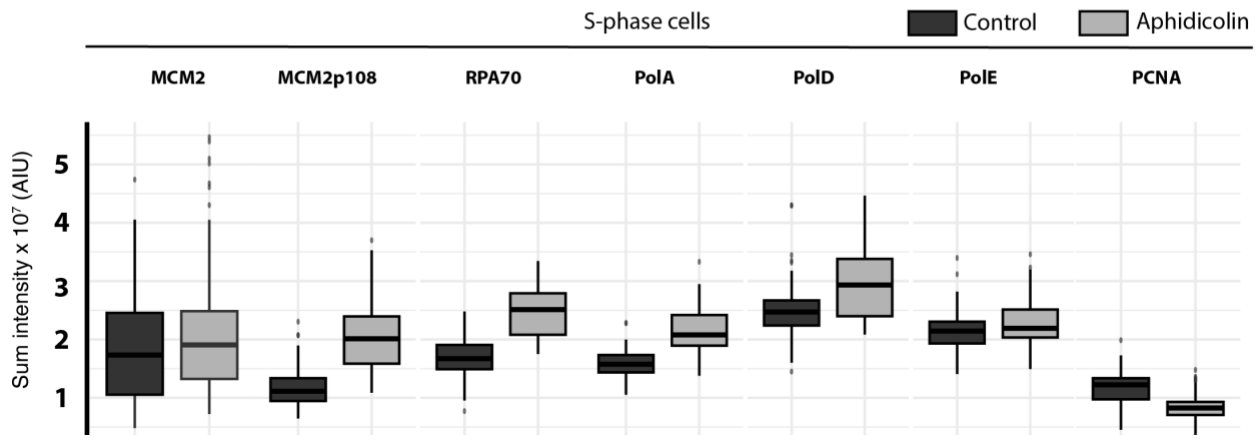

S-phase cells
Control
Aphidicolin
MCM2
MCM2p108
RPA70
PolA
PolD
PolE
PCNA
Sum intensity x 10⁷ (AIU)
5
4
3
2
1

Figure 5-figure supplement 8: Pipeline for the analysis of replisome components on chromatin upon stress.

(A) The accumulation analysis was performed. The channels were separated, the DAPI channel was used for creation of nuclear mask, the EdU and replication proteins channels were used for replication foci segmentation. The nuclear mask was overlaid with the replication foci mask, so that only foci inside of the nuclear area were selected. The replication foci parameters were measured as follows: focus intensity parameters, focus area, foci number per nucleus (Methods, High throughput image analysis of replisome component). The replication foci values per nucleus together with the DAPI nuclear intensity were exported for further analysis. The plots were generated in R studio. **(B)** Box plots from Figure 5D are shown here at the same Y axis scale.

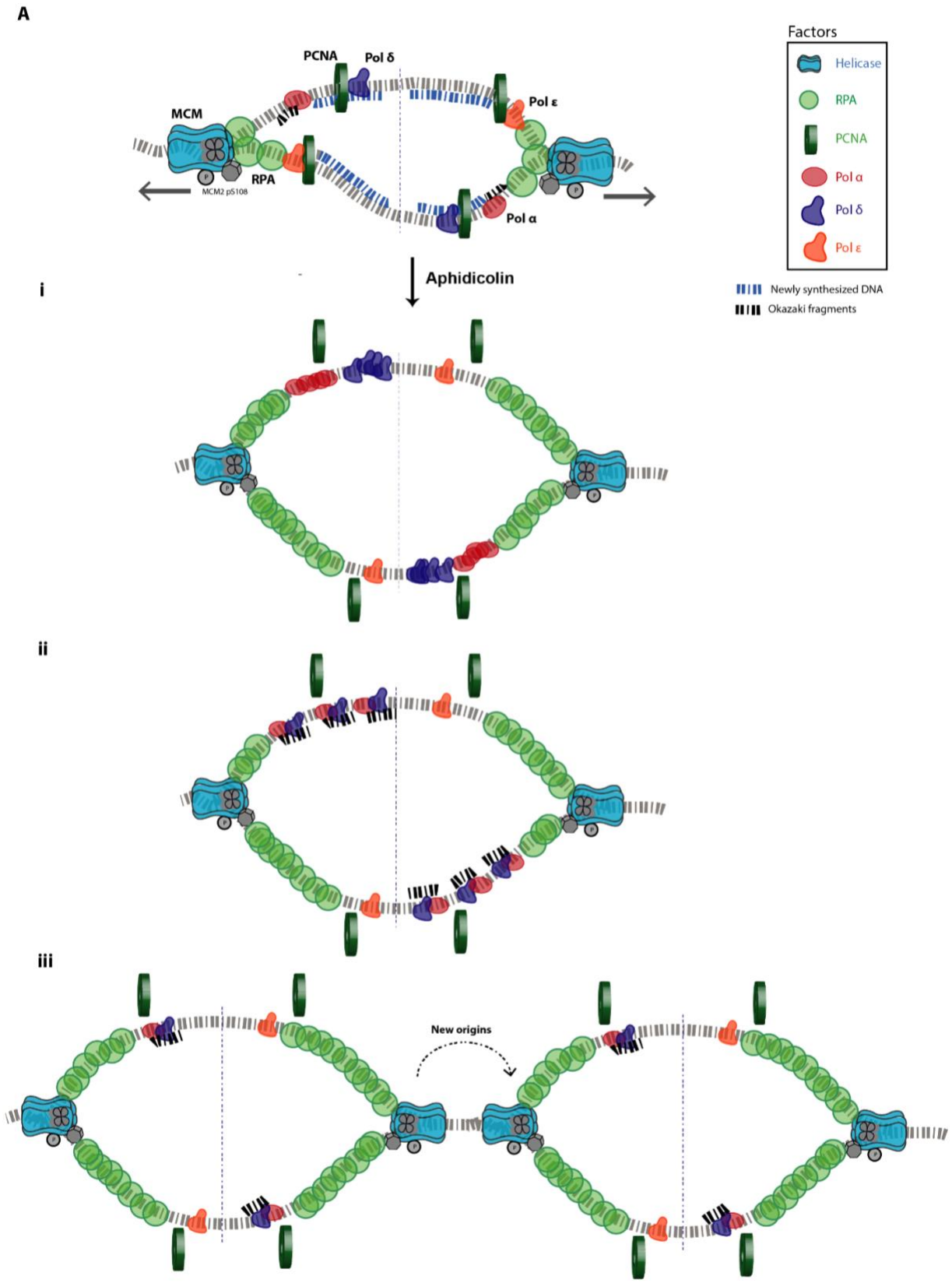


Figure 6-figure supplement 1: Model describing the different scenarios of replisome response to stress.

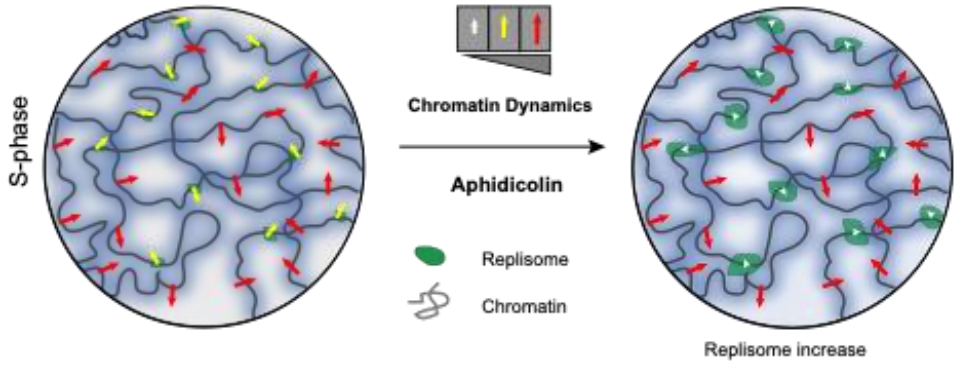


Figure 6-figure supplement 2: Graphical abstract showing chromatin dynamics during S-phase and replication stress.

Video captions

Video 1: Time lapse microscopy of HeLa K cells in G1 phase expressing fluorescent PCNA (green) and labeled chromatin (red). Scale bar: 5 µm.

Video 2: Time lapse microscopy of HeLa K cells in G2 phase expressing fluorescent PCNA (green) and labeled chromatin (red). Scale bar: 5 µm.

Video 3: Time lapse microscopy of HeLa K cells in S phase expressing fluorescent PCNA (green) and labeled chromatin (red). Scale bar: 5 µm.

Video 4: Time lapse microscopy of IMR90 cells in G1 phase expressing fluorescent PCNA (green) and labeled chromatin (red). Scale bar: 5 µm.

Video 5: Time lapse microscopy of IMR90 cells in S phase expressing fluorescent PCNA (green) and labeled chromatin (red). Scale bar: 5 µm.

Video 6: Time lapse microscopy of HeLa K cells pre and post aphidicolin treatment in G1/G2 phase expressing fluorescent PCNA and RPA and labeled chromatin (red). Scale bar: 5 µm.

Video 7: Time lapse microscopy of HeLa K cells pre and post aphidicolin treatment in S phase expressing fluorescent PCNA and RPA and labeled chromatin (red). Scale bar: 5 µm.