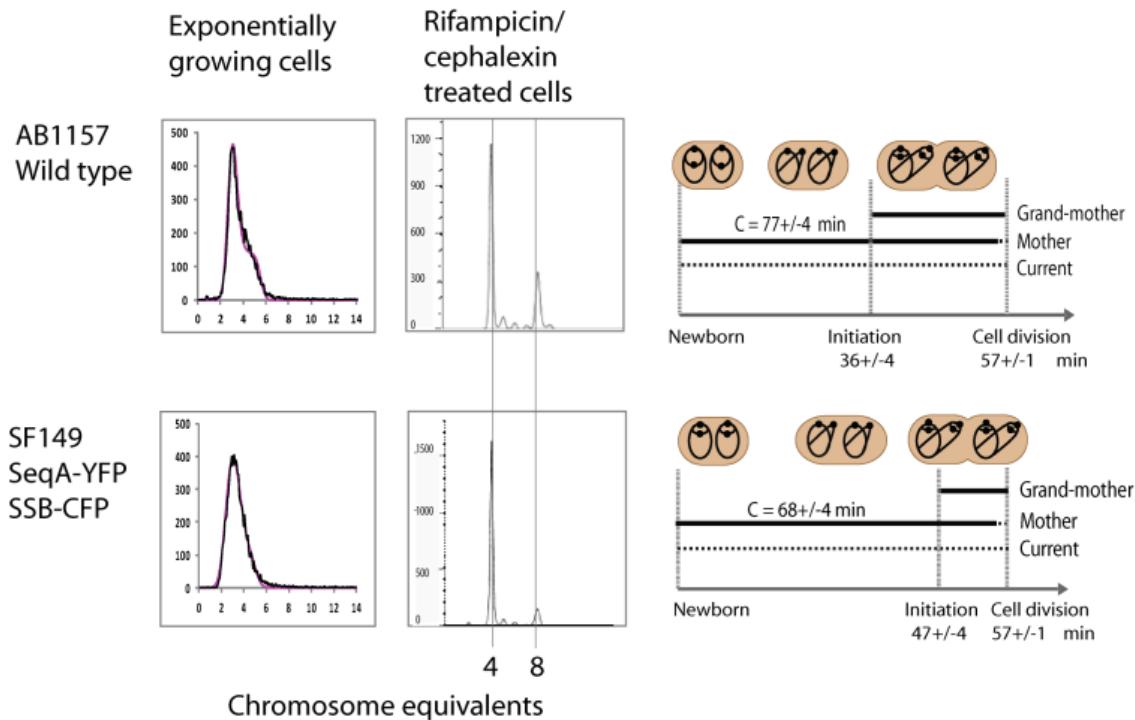


## SUPPLEMENTARY INFORMATION

**Figure S1**



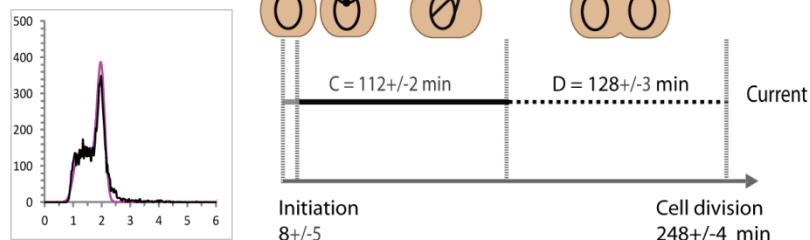
**Figure S1. Flow cytometry and cell cycle analysis of AB1157 and derivatives when grown in glucose-CAA medium at 28°C.**

Experimental DNA histograms (of one representative experiment) of exponentially growing cells are shown in left panels. The purple lines represent the theoretical simulation with best fit to the data obtained from exponentially growing cells. Cell cycle diagrams, based on flow cytometry data from three separate experiments, are shown in the right panels for all strains. The C-period is shown as a black line and the D-period as a stippled black line. Schematic drawings of cells at different stages of the cell cycle are shown above the diagram with chromosomes as black lines and origins as black dots.

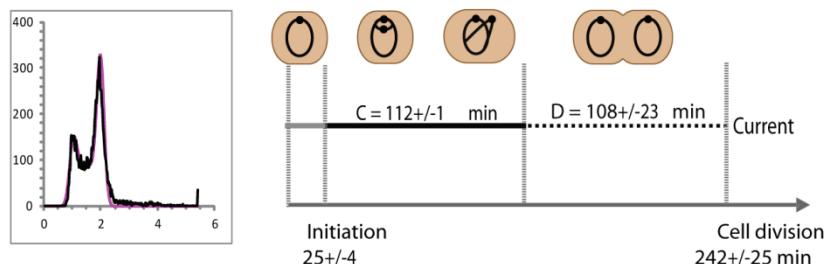
**Figure S2**

Exponentially  
growing cells

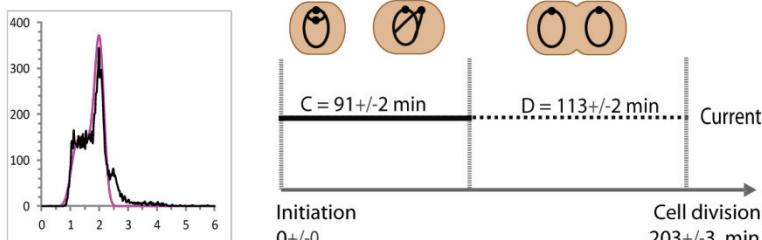
AB1157  
Wild type



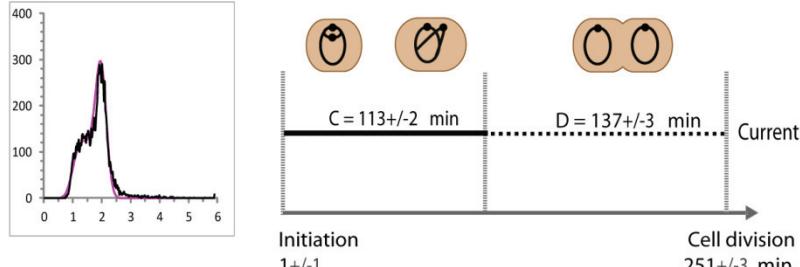
SF148  
SeqA-YFP  
*oriC*-mCherry



SF149  
SeqA-YFP  
SSB-CFP



SF171  
HolC-YPet  
SSB-CFP



Chromosome equivalents

**Figure S2. Flow cytometry and cell cycle analysis of AB1157 and derivatives when grown in acetate medium at 28°C.**

See Figure S2 for description of flow cytometry histograms and cell cycle diagrams. The B-period is shown as a grey line

## **Distance and colocalization analysis of cells with fluorescently tagged HolC and SSB proteins**

To perform a quality check of the script we analyzed cells (SF171), with fluorescently tagged HolC and SSB proteins. The HolC and SSB proteins are part of the replisome and the fluorescently tagged proteins were previously shown to colocalize within the resolution of widefield microscopy (1). Growth was performed in acetate medium at 28°C and exponentially growing cells were harvested and prepared for flow cytometry and snapshot fluorescence imaging (see below). Flow cytometry and cell cycle analysis showed that the replication pattern was about the same as for the AB1157 wild type cells (see Figures S2 and S3A). Snapshot imaging showed that the cells contained essentially the same numbers of SSB or HolC foci (one to two foci) or no foci at all (about 50% of the population) (Figures S3B and E). Cells without foci mainly represent cells in the D-period.

By looking at snap shot images and using Coli-Inspector as previously explained, it was clear that the trends of SSB/HolC movement and localization were essentially the same (Figures S3B, C and D).

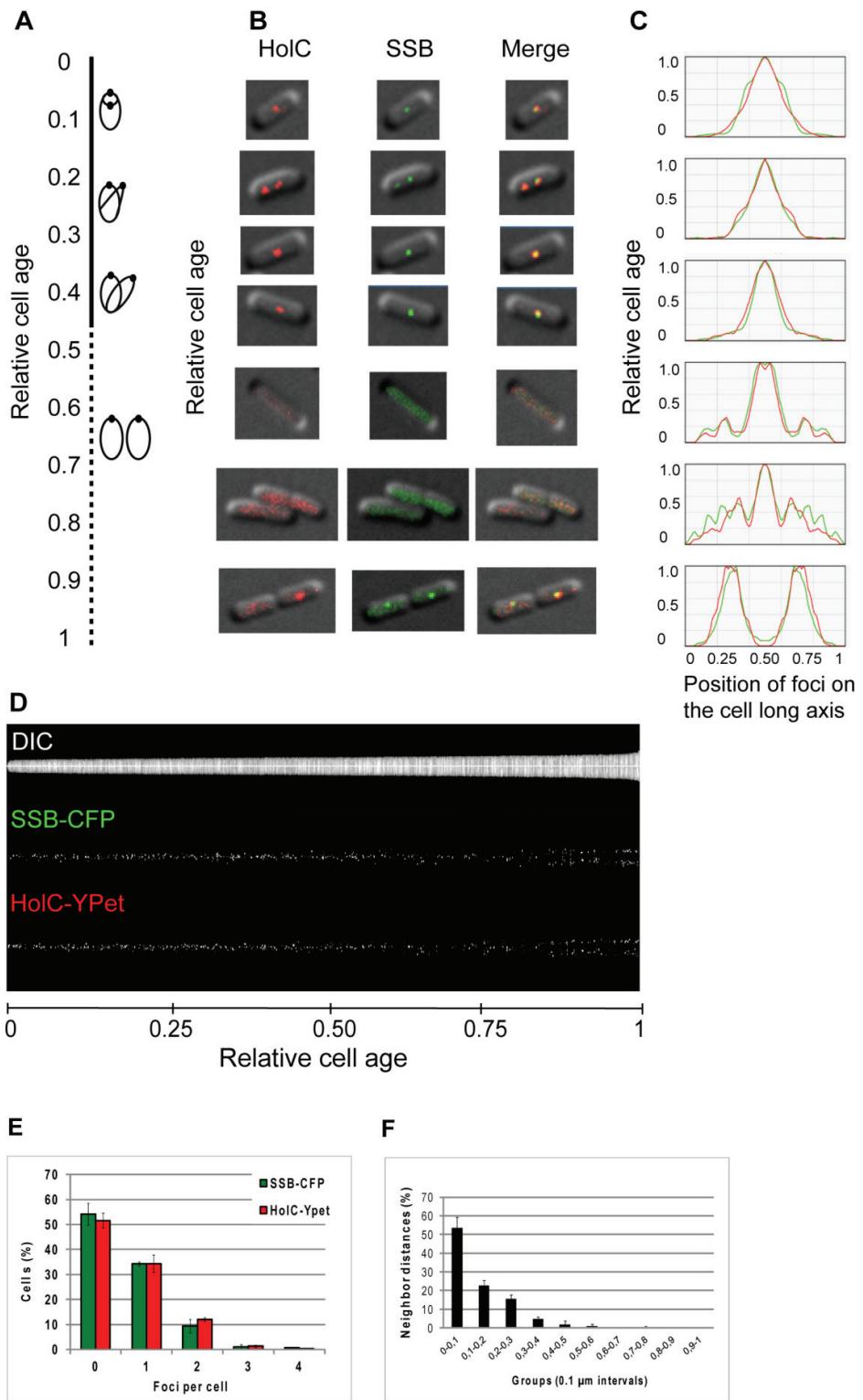
The analysis script was employed to investigate distances between the nearest neighboring HolC and SSB proteins. Initial image processing was performed as described below, and the average distance between neighboring foci was found to be about 135 nm (Table S1). More than 75% of the cells had HolC and SSB foci that were closer than 200 nm (Figure S3F). We also found that 85% of the foci were colocalized by the object-based method (see below for description of object-based method) (Table S1).

The percentage of colocalization found with the script was in accordance with previous results (1) indicating that image processing and running of the script worked well.

### **The quality of SSB foci**

The percentage of cells with SSB foci was slightly lower (8%) than expected from the duration of the C-period found with flow cytometry. This indicates that the SSB protein was not detected in absolutely all cells, which is in accordance with a previous report (2). The presence of SSB foci should therefore not be used as a measure of the C-period.

**Figure S3**



**Figure S3. HolC-Ypet and SSB-CFP both represent replisome tags and were found to be colocalized**

**(A)** Cell cycle diagram with parameters obtained by flow cytometry of cells (SF171), with tagged HolC (HolC-YPet) and replisome (SSB-CFP), exponentially grown in acetate medium at 28°C. See Figure 1A for description of cell cycle diagram. **(B)** Snapshot imaging showing the presence of discrete HolC-YPet (pseudo-colored red) and SSB-CFP (pseudo-colored green) foci or cells with no foci. **(C)** Local brightness of fluorescent SSB (green line) and HolC (red line) signals was plotted against foci positioning on the cell long axis (x-axis of histograms) using Coli-Inspector. The population was divided into six age groups based on relative cell length. The histograms of age groups are vertically plotted according to increasing cell age (youngest cells in top panel and oldest cells in bottom panel). **(D)** The integral fluorescence of each cell is plotted as a function of relative cell length (each cell is displayed as a vertical line) using the Coli-Inspector, where relative cell length corresponds to relative cell age (x-axis). The white horizontal line indicates midcell. **(E)** The percentages of cells with zero to four HolC or SSB foci per cell were plotted in a histogram, where error bars represent standard errors of the mean (SEM) of three independent experiments. A total of 722 cells were analyzed. **(F)** Distribution of HolC-SSB distances categorized into 0.1 μm intervals was plotted in a histogram (%). A total of 253 cells were analyzed.

**Table S1. Distance measurement between neighboring HolC and SSB foci using the script**

Strain	Fluorescence marker	Distance (nm)	Colocalization (%)*
SF171	HolC and SSB	135±13	85±4

\*Colocalization is defined when neighboring HolC-YPet and SSB-CFP foci are closer than the resolution limit for YPet (234 nm).

Numbers are average±standard error of the means (SEM) of three independent experiments

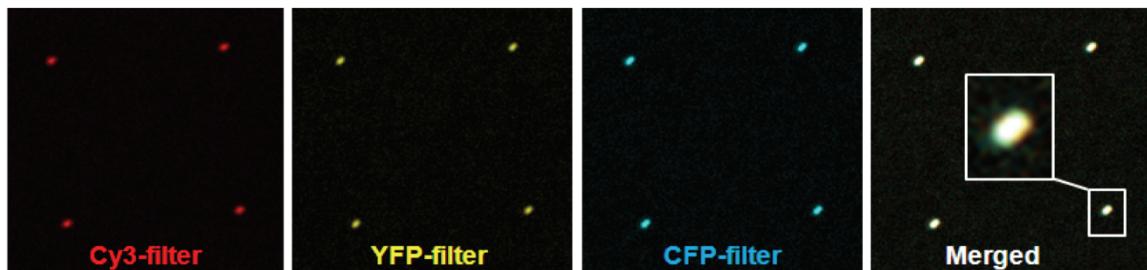
A total of 208 cells were analyzed.

### Microscopic alignment of fluorescence channels

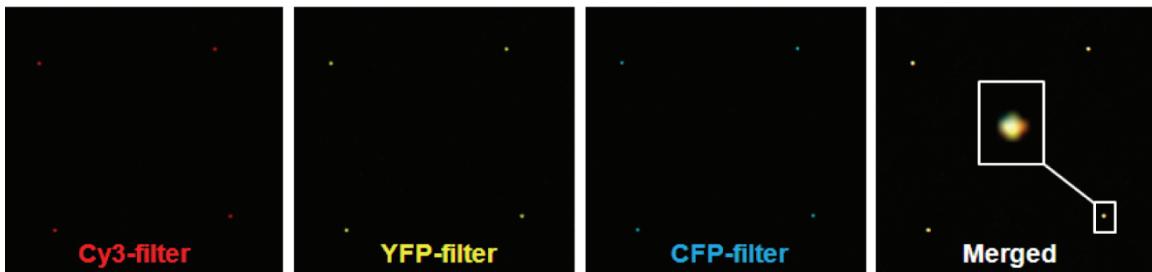
Calibration and instrumentation adjustment are required for high-precision imaging of fluorescent probes, particularly in multicolor applications. To investigate aberrations and distortions in the image plane, an Image Registration Target Slide (Applied Precision, a GE Healthcare company, Issaquah, WA) was employed. The slide consists of a symmetric grid with ~100 nm holes. These appear as diffraction-limited spots when the slide is illuminated with transmitted white light and behave analogous to a bead sample. Images were acquired for relevant channels/filter sets (Cy3, YFP and CFP). Since all spots from each channel overlapped completely (colocalized), it was clear that the lens system was proper for colocalization studies (Figure S4).

**Figure S4**

A



B



**Figure S4. Grid alignment of fluorescence channels**

Images of the Image Registration Target Slide were acquired with the HCX PL APO 100x/ 1.46 NA objective (A) or the HCX PLAPO 100x/ 1.42 NA objective (B) using filter sets for Cy3, YFP and CFP. Images were then deconvolved with the Richardson-Lucy algorithm (100 iterations).

## **Automated analysis of foci using Fiji**

We have developed a Python-based script for the open source software Fiji for automated measurements of distances between fluorescent spots that are detected in two different fluorescent channels. The script utilizes the “Find maxima” function to detect spots and then measures the distance to the nearest neighbouring spot. The resulting output can give distances that are below the resolution limit. This is rational since the neighbouring spots are located in different channels (3-5).

The input to the script is a composite image with two fluorescence channels, a list of cell outlines (regions-of-interest) and noise-levels for the “Find Maxima” function applied by the script to detect centres-of-mass. The script then measures the distance from the peak of every spot detected in the first channel to the nearest peak of spots detected in the second channel, per cell outline. It also provides a per-cell count of number of spots, as well as their position relative to the cell centre.

The measured distances can also be used to estimate the level of colocalization (object-based colocalization). Objects (or foci) will be considered colocalized if the distance separating them is within a reference distance defined by the optical resolution of the system. The reference distance in our analyses was set to the resolution limit based on longest emission wavelength of the two fluorophores investigated.

Cell length was estimated through Fit Ellipse. Fit Ellipse is a function that replaces a cell outline with a best fit elliptical shape, where the major axis-length of this shape corresponds to cell length.

Detailed information about the script code can be found in the script file as comments in green text, prefixed with hash tags (#).

### **i)      Image processing in Image J**

Before running the script, microscopy images were optimized in ImageJ/Fiji, by applying a set of built in functions along with imported plug-ins. Background and uneven illumination were corrected for with a rolling ball background subtraction procedure and out-of-focus light was reverted by iterative deconvolution. Noise was removed with a median filter and finally objects were isolated from the background by applying a Max Entropy threshold.

The distributions of foci per cell, during growth in acetate medium, were found to be essentially the same by manual counting and script analysis (data not shown). However, during growth in glucose-CAA medium, the numbers of foci per cell were found to be about 10% higher by manual counting than by script analysis. The reason for this was that images for the script were acquired

and processed to preserve a full dynamic range, so in a rapid growth situation with numerous foci, it was more demanding to detect all of them. Therefore, during growth in glucose-CAA medium, the numbers of foci per cell were found by manual counting.

Image processing was performed in Image J in the following order:

**Fluorescence images:**

- The procedure “Subtract background” was applied to remove background and correct for uneven illumination. This procedure uses the Rolling Ball Background Subtraction algorithm where a local background value is estimated from averaging over a large “ball” around each pixel. The only user input is the radius of the ball, which in our images was set to 10 pixels in general.
- Deconvolution was applied using a theoretical point spread function generated through the Diffraction PSF 3D plug-in (OptiNav, Inc., Copyright (c) 2005) and the Deconvolution Lab plug-in with the Richardson-Lucy algorithm (100 iterations) (6).
- Outliers were removed by filtering with the Median Filter using minimum radius. The Median Filter is a ranking filter which removes isolated noise pixels while preserving details.
- Diffuse fluorescent labelling was separated from candidate spots by Max Entropy thresholding, and subsequently removed. In some occasions a manual adjustment of the threshold was needed to retain weaker spots. The Max Entropy procedure determines a threshold by histogram partitioning, where the inter-class *entropy* is maximized.
- Finally, the noise-level for the Find Maxima function was estimated, and later fed into the script. The Find Maxima algorithm determines the local maxima that protrude over the surroundings by more than a given noise-value.

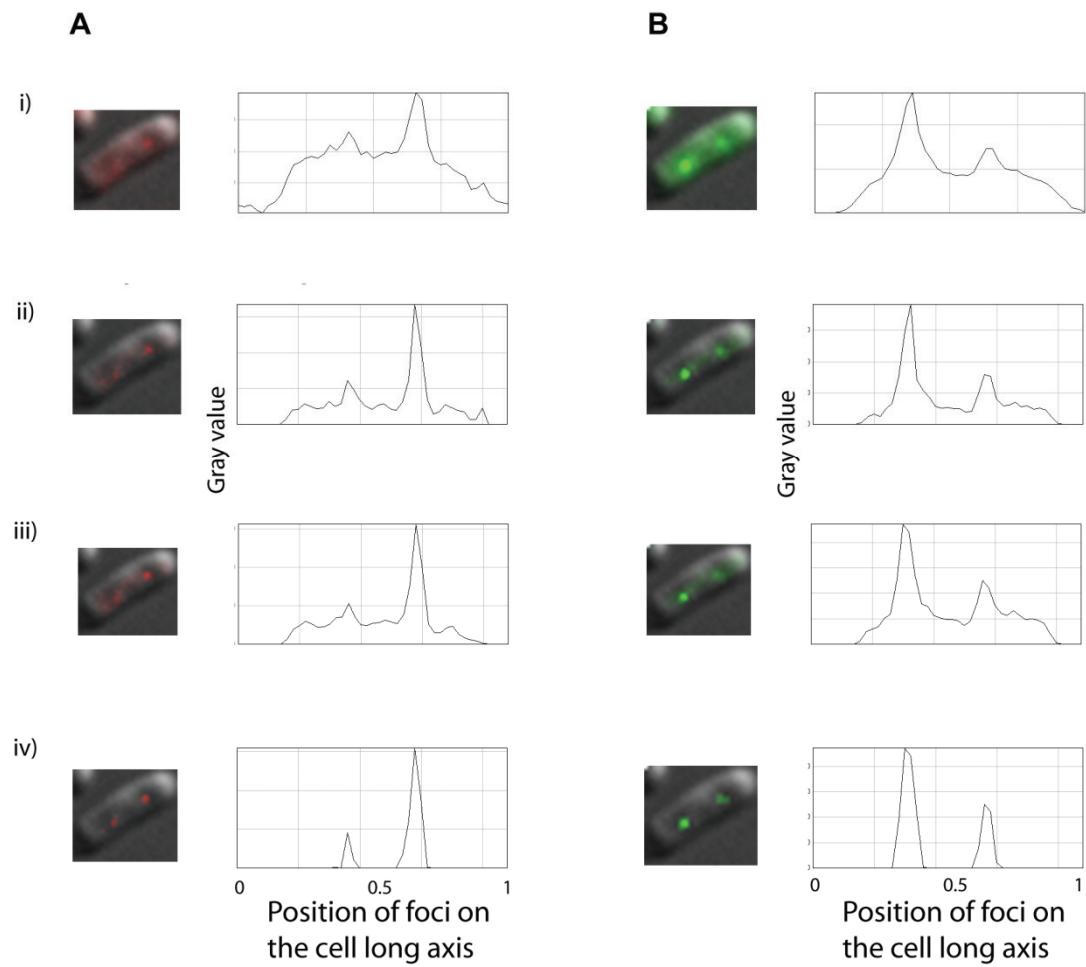
**Differential interference contrast images (DIC):**

- Flat field correction was used to correct for uneven illumination.
- Cell outlines were created in a step-wise fashion due to the asymmetrical shading of cells in DIC-images:
  - 1) Cells were made uniform in intensity by first adding shadows in the opposite direction of the inherent ones and then applying a maxima filter.
  - 2) A threshold was set to make a binary representation which was optimized by applying functions like Close, Dilate and Erode to finally correctly represent silhouettes of the cells.
  - 3) The binary representation was then used to create cell outlines, or region-of-interests (ROI), by applying the procedure Analyse Particles. This procedure outputs

selections by their coordinates to the Region-of-Interest-manager, and from this a list of ROI's can be stored as a separate file.

We found that image processing, with the setup described above, worked well in order to reduce background and retain florescent spots (Figure S5).

**Figure S5**



**Figure S5. Fluorescence intensity profiles after image processing**

**(A)** One representative cell with two HolC-YPet foci and **(B)** two SSB-CFP foci with the corresponding fluorescence intensity profiles obtained using the line tool in Image J (right panels in A and B). Results from image processing after (i) Background Subtraction of raw image, (ii) deconvolution using the Richardson-Lucy algorithm of (i), (iii) Median Filtering of (ii) and (iv) Max Entropy of (iii).

**ii) Protocol for colocalization and fluorescent spot distance analysis - the script**

- Prepare a composite image of the two fluorescence channels and save it in 16-bit format.
- Open the script file in Fiji, along with the composite image and the saved ROI zip file containing cell outlines.
- Fill in the following variables in the script: noise level in channel 1 and 2 for the Find Maxima function, and the pixel size.
- Click on Run
- The output from the script is in the form of two tables. The first table reports the number of spots in the two channels and distance to cell centre. The second table reports the distance measurements between spots, cell length and area of the cell.

Other options in the script:

- The script can perform Max Entropy threshold automatically before further analysis.
- Pearson's correlation coefficient can be estimated for both the cell in whole and for a 5x5 pixel matrix around each spot. This option should not be used in conjunction with filtering, Max Entropy thresholding or any other modification affecting the intensity distribution.
- The mean intensity of foci can be estimated for a 5x5 pixel matrix around each spot. As for Pearson's analysis, intensity measurements should not be performed on processed/modified images.
- There is an option to measure all distances from each spot in the first channel to each spot in the second channel instead of only the nearest neighbor.
- The script can also generate a gallery-view of the cells and this can be filtered by cell length, cell area, the number of spots and by spot distances.

### **iii) Investigation of the robustness of the automated analysis script**

#### **TetraSpeck Beads**

The accuracy of localizing the centre-of mass was tested using 0.1  $\mu\text{m}$  TetraSpeck fluorescent microspheres (Molecular Probes). These microspheres are uniform and multiply stained with dyes that display well-separated excitation/emission peaks. The beads were imaged in two separate channels for fluorescent dyes with excitation/emission peaks of 560/580 nm (YFP filter), and 660/680 nm (Cy3 filter). The TetraSpeck microspheres were used to verify the ability of the instrumentation, image processing and the script to colocalize and resolve objects emitting different wavelengths of light in the same optical plane. Colocalization is defined when signal originating from the same beads in the YFP and Cy3 channel are closer than the resolution limit of Cy3 (255 nm).

Here 5 $\mu\text{l}$  TetraSpeck suspension was mixed with 25 $\mu\text{l}$  1x PBS and added to a microscope slide containing a 17x28 mm agarose pad (1% agarose in phosphate-buffered saline) and covered with a #1.5 coverslip. Fluorescent images were acquired and processed as described above, and distance measurements between fluorescent spots detected in the YFP/Cy3 fluorescent channels were performed with the script.

We found that the average distance between fluorescent signals in YFP/Cy3 channels was 60 nm and that object-based colocalization was 100%. These results show that the accuracy of localizing the centre-of mass is 0-1 pixel (pixel size 0.092  $\mu\text{m}$ ). A total of 213 beads were analyzed.

### **3D-SIM imaging of SeqA-YFP indicate a more dynamic behaviour of newly replicated DNA at about halfway through the replication period in rapidly growing cells**

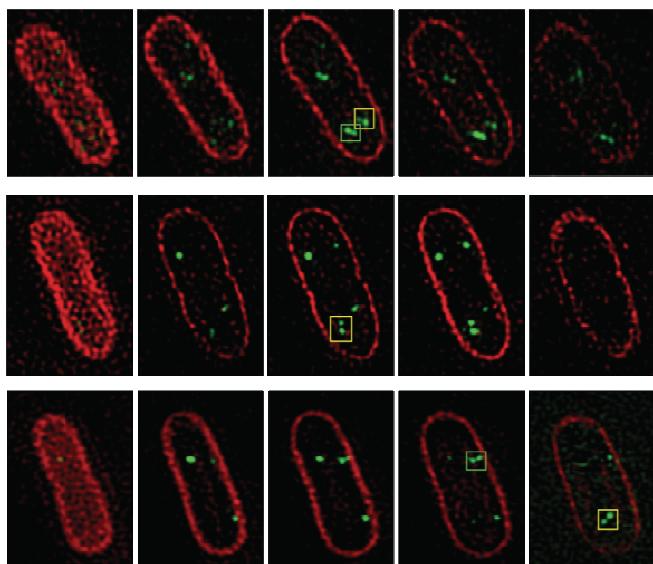
We performed three-dimensional structured illumination (3D-SIM) microscopy with a resolution of about 120 nm of the SeqA protein in cells containing SeqA-YFP (strain SF128). The cells were grown at 28°C in glucose-CAA medium ( $\tau=63$  min). Samples for flow cytometry were prepared as explained previously. For visualization of cell outlines, the membrane dye *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64) was added to a final concentration of 30  $\mu$ M approximately 15 min before imaging.

Imaging was performed using a Deltavision OMXV4 system (Applied Precision, a GE Healthcare company, Issaquah, WA) equipped with an Olympus 60x NA 1.42 objective, cooled sCMOS cameras and a 488 nm diode laser. We acquired Z-stacks covering the whole cell, usually consisting of about 15 Z planes. For each Z-plane, 15 raw images (3 rotations and 5 phases) were collected. Final super-resolution images were reconstructed using Softworx software (Applied Precision, a GE Healthcare company, Issaquah, WA).

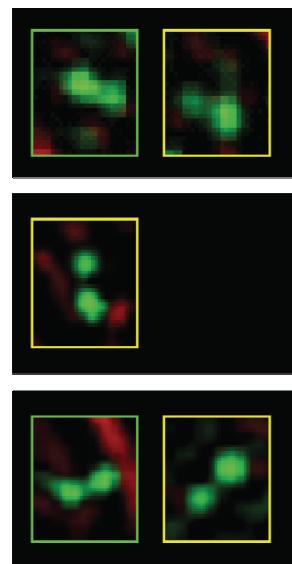
For image analysis we categorized the cells according to relative cell length to evaluate the number of SeqA foci and foci positioning in the cells with respect to cell cycle events obtained from flow cytometry analysis (see (7); data not shown). We found that a few cells presented numbers and positioning of SeqA foci representative of two foci behind single replication forks (about 6%). Figure S6A shows 3D-SIM Z-stack images of three such cells, where the green and yellow squares indicates closely spaced SeqA foci likely bound behind one replication fork. For a better view of these foci magnified images are presented in Figure S6B. The length/age of these cells indicated that they contain two replicating chromosomes/ four replication forks and that they have replicated more than 25-50% of the chromosome (Figure S6C). Thus, this period of the cell cycle can be compared to that of the SF149 cells in which the paired replication forks separate and the cells contain four distinct SeqA foci (see Figure 1 in main article). The result indicates that the newly replicated sister strands of DNA are more mobile relative to each other in this period, but that they are kept closer than 120 nm through most of the cell cycle.

**Figure S6**

**A**

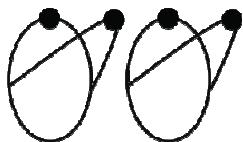


**B**



Z-planes --->

**C**



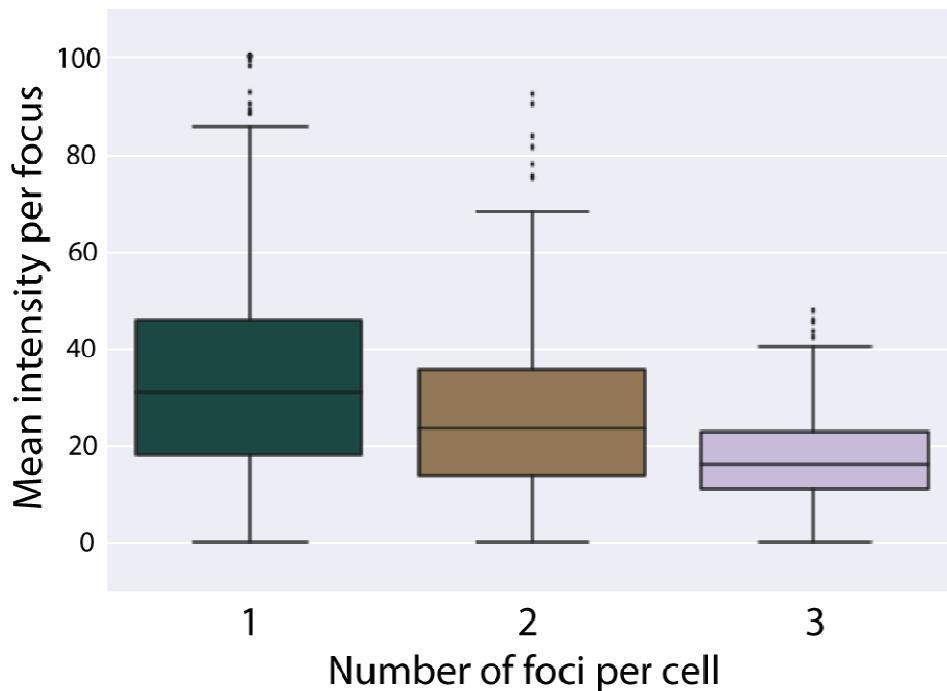
**Figure S6. 3D-SIM imaging of SeqA-YFP reveals that SeqA structures on sister DNA strands can be resolved in a small percentage of the population**

**(A)** Z-stack images of three cells that have two SeqA foci likely bound behind one replication fork at one or several locations. The distances between closely spaced SeqA foci in these cells were measured manually and found to be 140 and 160 nm (green and yellow boxes, respectively). Single foci, such as those seen in the upper part of the cell in panel 2 represent single replication forks (a pair of non-resolvable SeqA structures). **(B)** Magnified images of closely situated SeqA foci from green and yellow boxes in (A). **(C)** Schematic drawing of the approximated replication stage of cells shown in snap shot images (A).

### The number of SeqA foci per cell correlates inversely with focus intensity

As described in the main article, the two pairs of SeqA structures trailing two replication forks could sometimes, but most often not, be resolved by widefield microscopy in the replication period. We therefore wished to investigate whether the SeqA-YFP focus intensity was inversely correlated with the number of foci per cell and thereby strengthen the argument that SeqA structures behind two forks are colocalized in cells containing one SeqA focus. In order to do this we employed the previously described image analysis script, which also outputs foci intensities (estimated for a 5x5 pixel matrix around each focus) on images of SeqA-YFP in slowly growing cells (SF149 in acetate medium). We further post-processed (sorted) the SeqA intensity data and plotted it according to the number of foci per cell (Figure S7). Note that the entire population of cells is included in the plot, which means that cells containing 3 foci (and some of the cells containing one and two foci) originate from cells in the D-period. From the box plot it can be seen that the mean intensity per SeqA focus correlates inversely with the number of foci per cell. The analysis thus highlights that several SeqA complexes are localized within one SeqA-YFP focus in widefield images.

**Figure S7**



**Figure S7. The number of SeqA foci per cell correlates inversely with focus intensity**

Box plot showing mean SeqA-YFP intensity per focus (y-axis) versus the number of foci per cell (x-axis). Approximately 700 cells were analyzed

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