Supporting Information

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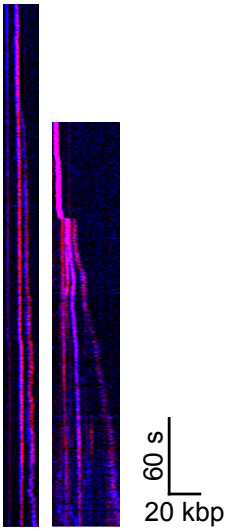

Fig. S1. Kymographs of reaction products with Alexa Fluor 488-labeled gp5/trx (blue) and Cy5-labeled gp2.5 (red). The overlap between Alexa Fluor 488-gp5/trx and Cy5-gp2.5 suggest that polymerases preferentially bind to gp2.5 coated ssDNA. The initial recruitment of DNA polymerases to the helicase is mediated by the acidic C terminus of gp4 (37). The similarity between the gp4 C-terminal domain and the C-terminal region of the ssDNA-binding proteins causes DNA polymerases to interact with gp2.5-coated ssDNA, rendering it challenging to determine within the diffraction-limited spot of the replisome how many polymerases are tethered to the helicase and how many are bound to the transiently exposed nearby ssDNA. To selectively displace the polymerases non-specifically bound to ssDNA, we included *E. coli* SSB, which has a 10 times higher K_d for ssDNA than gp2.5 (16, 37). Addition of *E. coli* SSB resulted in the binding of 1 labeled polymerase per ~1,500 bp of ssDNA instead of 1 per ~500 bp, which reduced the binding of fluorescent polymerases within the replication loop to less than 1. Hence, with the addition of *E. coli* SSB, the replisome intensity more accurately represents the amount of labeled polymerases bound.

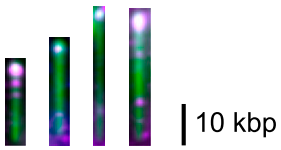

Fig. S2. Fluorescence images of replication intermediates on M13 DNA rolling-circle templates, with green representing the DNA products (labeled with Sytox Orange) and purple representing the labeled polymerases. The DNA products have a length of 19, 23, 32, and 31 kb from left to right.

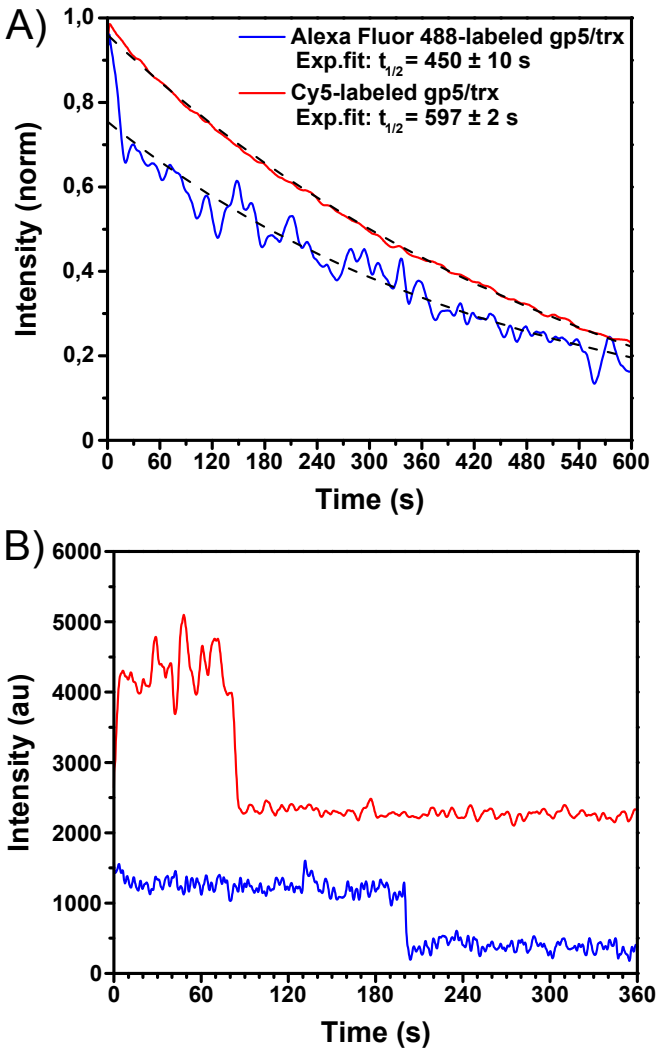

Fig. S3. Bleaching of labeled polymerases. (*A*) Intensity over time of Cy5- (red) and Alexa Fluor 488- (blue) labeled gp5/trx dissolved in agarose over the whole field of view. Exponential fits (Exp.fit) gave a $t_{1/2}$ of 450 ± 10 s for Alexa Fluor 488-gp5/trx and 597 ± 2 s for Cy5-gp5/trx. These time scales are an order of magnitude higher than the decay constants of the autocorrelation function (Fig. 2*C*) and therefore have a minor contribution to the autocorrelation function. (*B*) Representative fluorescent bleaching traces of a single Cy5-labeled (red) and Alexa Fluor 488-labeled (blue) polymerase positioned at the end of the DNA product. Note that the data for both Cy5 and Alexa Fluor 488, measured at the same experimental conditions as our previous experiments, show no photophysical fluctuations.

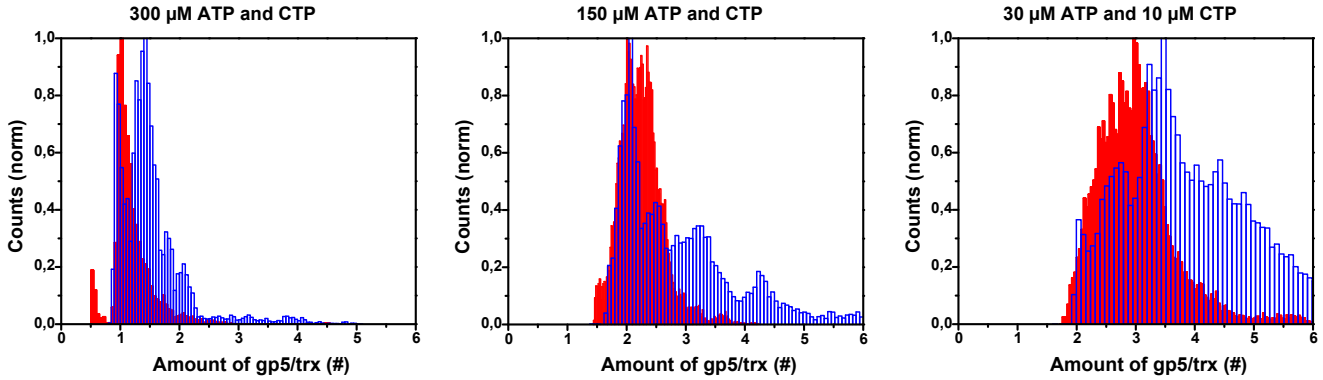

Fig. S4. Intensity distributions of the Cy5-labeled (red) and Alexa Fluor 488-labeled (blue) polymerases at the replisomes for the different NTP concentrations used. The amount of gp5/trx was determined by dividing the total fluorescent intensity of the fluorophores at the replisome by the fluorescent intensity of a single fluorophore determined by dissolving single labeled polymerases in agarose. Median values of the distributions were found to be 1.1 ± 0.4 and 1.4 ± 0.6 for 300 µM ATP and CTP, 2.2 ± 0.4 and 2.8 ± 1.7 for 150 µM ATP and CTP, and 3.0 ± 0.8 and 4.0 ± 2.0 gp5/trx per replisome for 30 µM ATP and 10 µM CTP (values for Cy5- and Alexa Fluor 488-labeled polymerases, respectively). The structurally slightly higher values of the Alexa Fluor 488-labeled polymerases may indicate photophysical effects rendering the observed Cy5 intensity a bit lower. However, both the Cy5- and Alexa Fluor 488-labeled polymerases show similar dependence on ribonucleotide concentration.