Simultaneous single-molecule measurements of phage T7 replisome composition and function reveal the mechanism of polymerase exchange

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Supporting Information

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SI Methods

Coverslip Functionalization. To reduce surface sticking of labeled protein and to tether the DNA construct, glass coverslips were functionalized with high-molecular weight polyethylene glycol (PEG) and biotin-PEG, respectively. The details of the surface functionalization have been described previously (1). Briefly the glass surface is first cleaned with alternating sonications in ethanol and 1 M KOH and then coupled to an aminosilane, creating a surface of reactive amine groups. These amino groups are subsequently reacted with a 50:1 mixture of nonbiotinylated and biotinylated succinimidyl propionate-PEG, creating a PEG-coated surface displaying a layer comprised of biotin and non-reactive methyl groups. The functionalized coverslips are stored under vacuum. Immediately prior to use, the biotinylated coverslip is incubated for approximately 30 min with 0.2 mg/mL solution of streptavidin (Sigma) in PBS (pH 7.3) buffer.

Flow Cell Construction. Flow cell construction was carried as described previously (1). Briefly, a 1.5–2 mm channel was cut out of double-sided adhesive tape (Grace BioLabs) and sandwiched between a quartz slide and a functionalized coverslip. The quartz slide has three predrilled holes allowing for the insertion of two polyethylene inlet tubes (0.76 mm inlet diameter and 1.22 mm outer diameter; Becton Dickinson) and one outlet tube. Quick-dry epoxy was used to seal the flow cell. The surface of the flow cell was blocked further by incubating the chamber with 20 mM Tris pH 7.5, 2 mM EDTA, 50 mM NaCl, 200 μg/mL bovine serum albumin (BSA) and 0.005% Tween.

DNA Substrate and Quantum Dot Labeling. The surface-tethered DNA construct with replication fork and primer was made by annealing and ligating the following PAGE-purified oligodeoxynucleotides (Integrated DNA Technologies) with λ-phage DNA (New England Biolabs): biotinylated fork arm (A: 5′-biotin AAA AAAAAAAAAAAAAGAGTACTGTACGATCTAGCAT CAA TCACAGGGTCAGGTTCGTTATTGTCCAACTTGCTGTCC-3′); λ-complementary fork arm (B: 5′-GGGCGGCGACCTGGA CAGCAAGTTGGACAATC TCGTTCTATCACT AATTCACT AATGCAGGGAGGATTTCAGATATGGCA-3′); fork primer (C: 5′-TGCCATATCTGAAATCCTCCCTGC-3′); and the λ-complementary filler end (D: 5′-AGGTCGCCGCCC-3′). See ref. 2 for further details.

The forked λ-phage DNA construct described above was site-specifically labeled with a quantum dot ∼15 kilobases from the replication fork by using a protocol based on the work of Kuhn and Frank-Kamenetskii (3, 4). Treatment of the replication fork DNA construct with the nicking endonuclease Nt.Bst.NBI results in sequence-specific, single-strand breaks along the backbone. Fortuitously, some of these nicks are close together and on the same strand. In this case, the gap can be melted out and replaced with a digoxigenin-modified oligo of the same sequence.

Nicking reactions with Nt.Bst.NBI (NEB, 20 units) were performed on the forked DNA substrate (2 μg) at 50 °C for 2 hours in Buffer 3 (NEB). The nicked DNA was mixed with a 100-fold excess of 4 oligonucleotides (IDT): (5′- GCCGTGGCCCGTGG TGAGTCGCTC-3), (5′-ATCATCGGGCTTTTTGGCGAATGA AATTTAGCTACGCTTTCGAGTCTCAT-3′), (5′-ATCGTGAA GAGTCGGCG-3′), and the digoxigenin-modified sequence (5′-digoxigenin-TTCAGAGTCTGAC-3′) for quantum dot capture. This mixture was heated at 50 °C for 10 min and then allowed to slowly cool to room temperature, resulting in the highly efficient replacement of the native sequence with the digoxigenin-modified oligonucleotide. Prior to ligation the DNA mixture was diluted 5-fold and an 8× equivalent of 10× T4 ligase buffer (NEB) was added. Ligation proceeded for 2 hours at room temperature upon the addition of T4 ligase (NEB, 800 units). Any residual nicking or ligase activity was quenched by adding 20 mM EDTA. Quantum dots (QDs) (Invitrogen, 605 nm) were functionalized with sheep antidigoxigenin Fab fragments (Roche) to bind to the DNA using the Invitrogen QDot Antibody Conjugation Kit and following manufacturer's instructions.

Thioredoxin Labeling and Characterization. Purified thioredoxin was labeled nonspecifically with an amine reactive dye, Alexa Fluor 555 Succinimidyl Ester (Invitrogen). One can bias the labeling to the N-terminus by running the coupling reaction at near neutral pH.

Thioredoxin was first dialyzed into labeling buffer (PBS, pH 7.3, 1 mM DTT) at 4 °C. To the recovered protein was added 1/10 of the protein volume of 100 mM sodium bicabonate (pH 8.2) that acts to raise the pH to ∼7.5. A 1 mM solution of the dye dissolved in anhydrous DMSO was added to the protein solution in a ratio of 3.75:1. The reaction was allowed to proceed for 2 hours at room temperature. Finally, the reaction solution was diluted by a factor of three with a solution of PBS and 5 mM DTT and free dye was removed by immediately dialyzing against thioredoxin storage buffer (20 mM potassium phosphate, pH 7.5, 1 mM DTT, 0.1 mM EDTA, 50% glycerol) at 4 °C. The degree of labeling was determined to be ∼2 dyes per thioredoxin by UV-visible spectrophotometry. Varying the dye to protein ratio resulted in a reproducible degree of labeling curve (see Fig. S1). To form the fluorescently labeled DNA polymerase, labeled thioredoxin was mixed with gp5 in a 1:1 mixture and stored at −20 °C.

The activity of the labeled thioredoxin was measured by comparing its activity to the wild type in strand displacement synthesis on M13 dsDNA with a replication fork (5). Reactions were run with 3.5 nM dsDNA at 37 °C in replication buffer (50 mM tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM potassium glutamate, 5 mM DTT, BSA 0.1 mg/mL, and 500 μM each of dATP, dCTP, dGTP, and dTTP). DNA synthesis began with the addition of 7.5 nM gp5, 5 nM gp4 and thioredoxin and subsequently quenched after 15 min with 150 mM EDTA. Reactions were run with 0, 5, 20, 80, 240, and 600 nM thioredoxin. DNA synthesis was detected by the amount of [^{3}H] deoxythymidine monophosphate incorporated into the DNA template. No significant difference in activity was observed between the labeled and WT thioredoxin activity as shown in Fig. S2. Attempts to label the polymerase (gp5 and thioredoxin) using a similar labeling procedure resulted in a significant decrease in leading strand synthesis.

Stroboscopic Imaging of the Replisome. A 488 nm solid-state laser (Coherent, Sapphire 488-50) was used to selectively excite 605 nm quantum dots whereas the Alexa Fluor 555 labeled polymerase was imaged with a 532 nm laser (Compass 215M-75, Coherent). Both laser beams were expanded and overlapped on a dichroic mirror (Chroma, z488bcm). The collimated excitation beams were focused through the rear port of an inverted microscope (Olympus, IX71) and onto the back aperture of a high NA objective (Olympus, NA 1.45). Vertical translation of the focusing lens resulted in total internal reflection. Experiments were performed under 60× magnification that corresponds to a field of view of approximately 1.9×10^4 μm^2. Quantum dot imaging was performed with a 488 nm laser flux of 3 W/cm^2 whereas the

labeled polymerase imaging was performed using 14 W/cm^2 of 532 nm laser light. A custom filter set was used to filter the excitation beams (Chroma, z488/532dbx), reflect the excitation light to the sample (Chroma, z532rdc-xr) and filter the emission of the quantum dots and the Alexa Fluor dye (Chroma, HQ600/75m). The filtered fluorescence emission was imaged onto an electron multiplying charge coupled device camera (Hamamatsu, EM-CCD 9100-13). Laser shutters (Uniblitz) synced to the camera were controlled by custom Labview (National Instruments) code. Camera exposures were 0.2 s with the labeled polymerase imaged every 7 s and the QD imaged continuously. Fig. S3 shows how the stroboscopic imaging greatly extends the lifetime of the fluorescent polymerase.

Single-Molecule Fluorescence Measurements of Polymerase Exchange. Preassembled leading-strand synthesis reactions were carried out in the flow cell by incubating DNA with 10 nM gp4 (hexameric) and 20 nM unlabeled gp5/trx in buffer A (50 mM tris-HCl, pH 7.5, 50 mM potassium glutamate, 1 mM DTT, BSA 0.1 mg/mL, and 700 μM each of dATP, dCTP, dGTP, and dTTP) for 15 min. The flow cell was washed with ∼15 flow cell volumes of protein-free buffer A to remove excess protein. Replication was then initiated by flowing in buffer B (50 mM tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM potassium glutamate, 1 mM DTT, BSA 0.1 mg/mL, and 700 μM each of dATP, dCTP, dGTP, and dTTP) along with 1 nM of fluorescently labeled gp5/trx. Replication reactions showing the exchange of Y526F and labeled polymerases were performed by continuously flowing the two polymerases each at 1 nM in buffer B along with 2 nM of gp4.

The locations of replication forks were determined by monitoring the position of QDs as the direction of buffer flow was varied from forward to reverse. The replication fork sits at the midpoint between these two extremes in QD position. QD positions were accurately determined by using custom Matlab code to fit two-dimensional Gaussian functions to QD point-spread functions. Under experimental conditions, QD positions indicated between 75–80% relative extension (see Fig. S4). The worm-like chain model for DNA predicts ∼1 pN of force at the QD position. Referring to established force-extension curves for double- and single-stranded DNA one can calculate the expected length conversion for leading-strand synthesis (2, 6, 7).

The number of labeled polymerases at the replication fork was determined by tracking the intensity of a 3×3 pixel box placed at the replication fork. Photobleaching trajectories of labeled polymerases were used to calculate the average intensity of a polymerase labeled with a single fluorophore (see Fig. S5).

Photobleaching of a field of view of labeled polymerases revealed a distribution in the number of labels resulting from the nonspecific labeling scheme. Polymerases were found to be predominately labeled with a single fluorophore (74%) with 24% labeled with two labels and only ∼2% labeled with three fluorophores (see Fig. S6). Association of a labeled polymerase, as measured by changes in polymerase fluorescence, were found to occur in jumps consistent with the average fluorescence of a singly labeled polymerase (see Figs. 2*B* and 3 of the main text).

Single-Molecule Measurements Using Unlabeled Proteins. Single-molecule DNA flow-stretching experiments were performed as previously described (1, 6). Briefly, DNA molecules similar to those used in the fluorescence experiments were tethered to the surface of a flow cell. To the untethered 3′ end was attached a 2.8-μm diameter paramagnetic bead (Dynal). To prevent nonspecific surface interactions a magnet was positioned above the flow cell to apply ∼1 pN of force to lift the beads off of the surface. The flow of buffer through the flow cell resulted in a total force on the bead of ∼3 pN. Beads were imaged with a 10× objective through an inverted IX51 microscope (Olympus) and onto a CCD camera. Trajectories of the bead position were determined by tracking software (Semasopht).

Preassembled leading-strand synthesis reactions were carried out in the flow cell by incubating DNA with 10 nM gp4 (hexameric) and 20 nM unlabeled gp5/trx in buffer A for 15 min. The flow cell was washed with ∼15 flow cell volumes of protein-free buffer A to remove excess protein. Replication was then initiated by flowing in buffer B along with varying amounts of Y526F polymerase. The processivity of the preassembled WT polymerase was found to be dependent on the concentration of the challenging Y526F polymerase as shown in the processivity histograms of Fig. S7.

Leading-strand synthesis complexes with 10 nM gp4-ΔC17 (hexameric) and 20 nM unlabeled gp5/trx were preassembled as described above. Replication was then initiated by flowing in buffer B along with 20 nm Y526F polymerase. The processivity of the preassembled WT polymerase (see Fig. S8) was found to decrease to 5800 ± 1400 bp and trajectories showing a change in DNA synthesis rate consistent with exchange dropped dramatically to below ∼10%.

1. Tanner NA, van Oijen AM (2009) Single-molecule observation of prokaryotic DNA replication. *Methods Mol Biol* 521:397–410.
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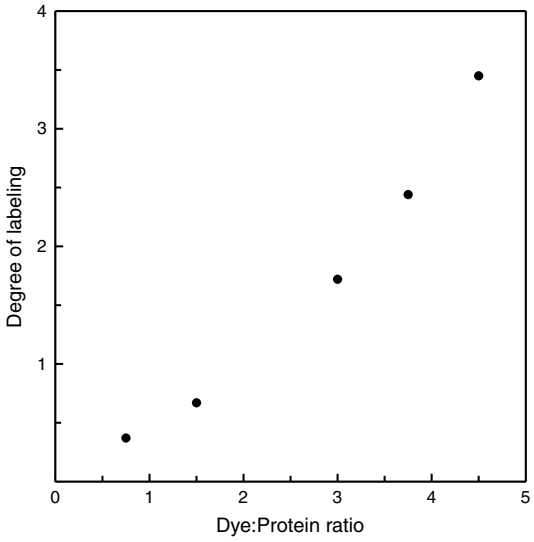

Fig. S1. The degree of labeling of thioredoxin as a function of the dye to protein ratio used in the labeling reaction.

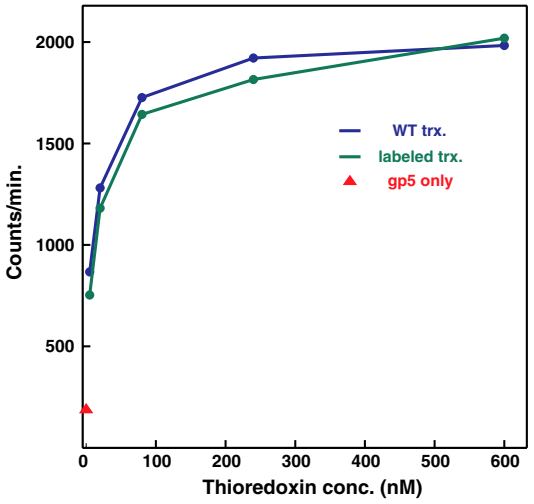

Fig. S2. Leading-strand activity assay as a function of thioredoxin concentration.

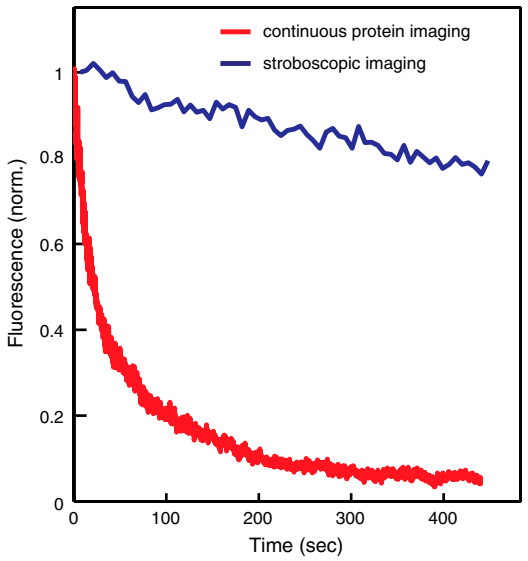

Fig. S3. Photobleaching of the labeled gp5/trx complex. Continuous excitation of the labeled protein with 532 nm excitation (red line) results in a photobleaching lifetime of ~35 s. Using the experimental stroboscopic imaging (blue line) greatly extends the lifetime of the labeled protein while allowing for continuous observation of QDs attached to DNA substrates.

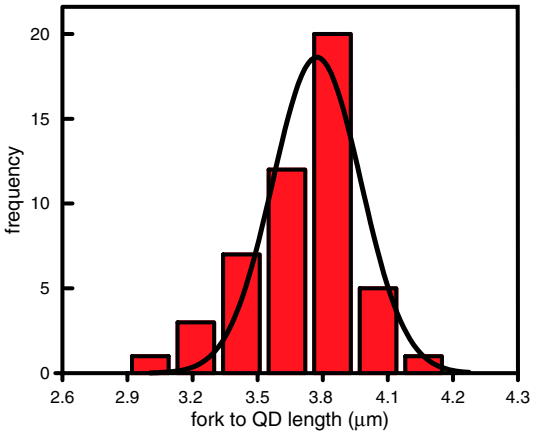

Fig. S4. Distance from the replication fork to QD. The mean distance corresponds to 78% relative extension of the DNA as compared to the contour length.

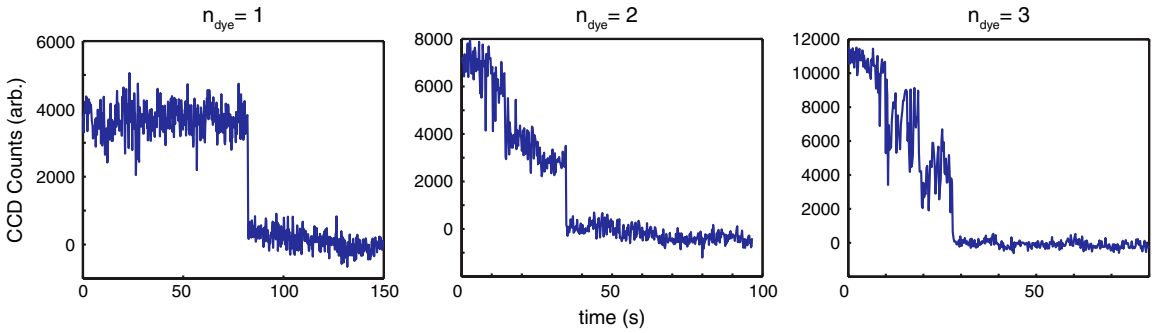

Fig. S5. Representative photobleaching trajectories of a polymerase labeled with 1, 2, and 3 dyes.

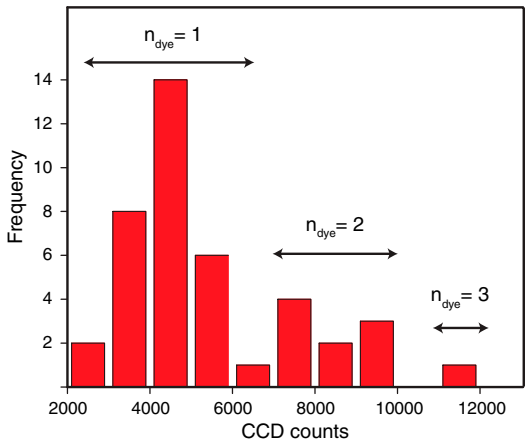

Fig. S6. Histogram of polymerase photobleaching events for a single field of view.

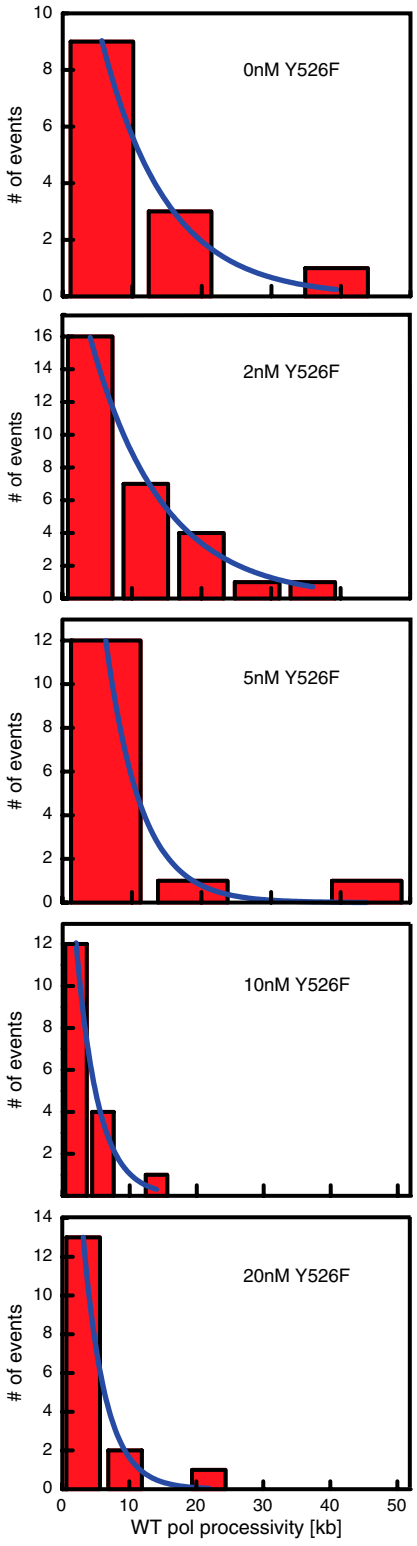

Fig. S7. Processivity histograms of the preassembled WT polymerase as a function of the challenging Y526F concentration.

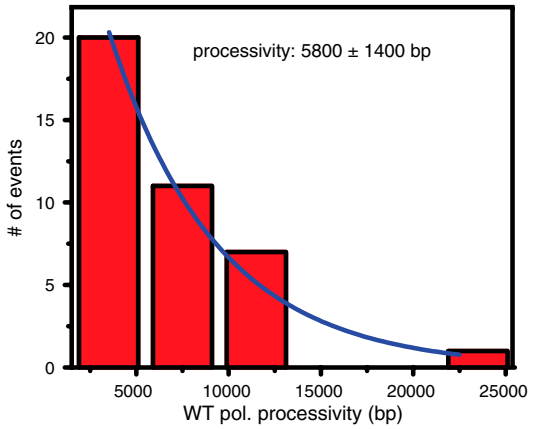

Fig. S8. The distribution of processivity of a preassembled WT polymerase on gp4-ΔC17, challenged with 20 nm Y526F polymerase.