Supporting Information for:

A single-molecule view of the assembly pathway, subunit stoichiometry and unwinding activity of the bacteriophage T4 primosome (helicase-primase) complex

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Table S1. *Nomenclature and sequences for the DNA constructs used in this study.*

<table>
<tr><th>Construct</th><th>DNA Sequence</th></tr>
<tr><td>smFRET experiment</td><td>5′-Biotin-CAGTCATAATATGCGAiCy5GCGATTATATATGCTTTTACCACTTTCACTCACGTGCTTAC-3′
|||||||||||||||| ||||||||||
3′-GTCAGTATTATACGCTiCy3CGCTAATATACCACG(T)$_{29}$-5′</td></tr>
<tr><td>Helicase unwinding assay</td><td>5′-CAGTCATAATATGCGAGCGATTATATATGCTTTTACCACTTTCACTCACGTGCTTAC-3′
||||||||||||||||||||||||||
3′-GTCAGTATTATACGCTCGCTAATATACCACG(T)$_{29}$-5′</td></tr>
<tr><td>ATPase assay</td><td>5′-dT$_{22}$-dG-dT$_{22}$-3′</td></tr>
</table>

- iCy3 and iCy5 refer to internally labeled Cy3 and Cy5 donor-acceptor chromophores.

Bulk unwinding assays to determine the effect on the apparent unwinding activity of the T4 primosome helicase of the incorporation of iCy3 / iCy5 fluorophores

into the backbones of the DNA fork assay substrates. We carried out unwinding experiments with unlabeled and iCy3 / iCy5 backbone-labeled DNA constructs to determine the effect of the presence of the iCy3 / iCy5 chromophore complex on the activity of the T4 primosome helicase. The lagging (5'→ 3') strand was labeled with γ^{32}P at the 5′-end and was annealed to the leading strand. The DNA fork substrates (5 nM) were added to a reaction buffer containing 33 mM Tris-OAc (pH 7.8), 150 mM KOAc, and 6 mM $Mg(OAc)_2$, in addition to 3.5 mM ATP. The reaction was initiated by the sequential addition of T4 gp41 and gp61 (300 nM and 50 nM, respectively). 5 μL were taken at the indicated intervals. The 5 μL time points were quenched with an equal volume of a stop solution containing 1 mg/ml proteinase k, 0.2% SDS, 20 mM Tris-OAc (pH 7.5) and 2 mM $Ca(OAc)_2$. The quenched reactions were then incubated at 45˚C for 5 minutes, after which 6 μL of native gel loading dye was added to the samples. The samples were then subjected to non-denaturing gel electrophoresis on 10% polyacrylamide TBE gels (19:1 acrylamide / bis) at 20 mA constant current for 1.5 hours.

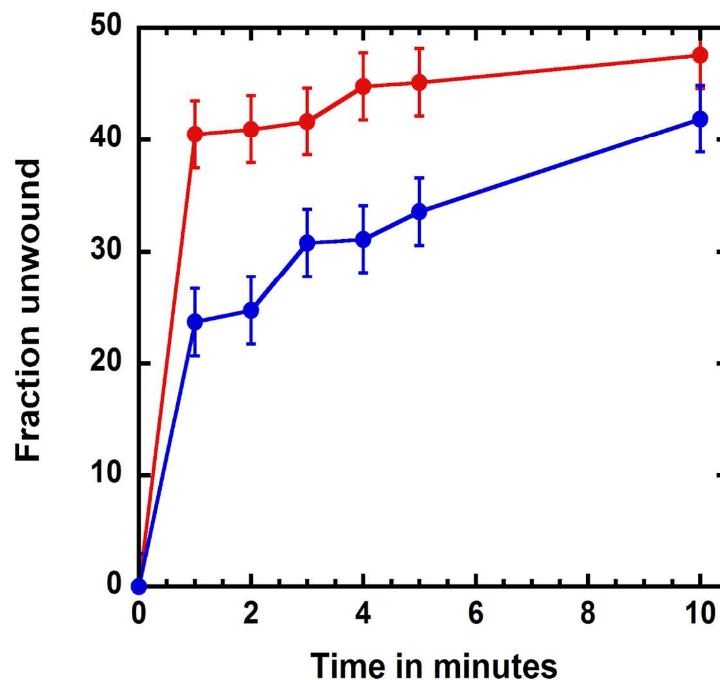

Figure S1. Bulk helicase unwinding assays catalyzed by the T4 primosome helicase using either unlabeled or iCy3 / iCy5-labeled DNA fork constructs. Helicase unwinding assay with DNA fork constructs containing no iCy3 / iCy5 (red circles)

and with iCy3 / iCy5 chromophores incorporated into the DNA backbones of the constructs (blue circles). The DNA constructs used are shown in Table S1.

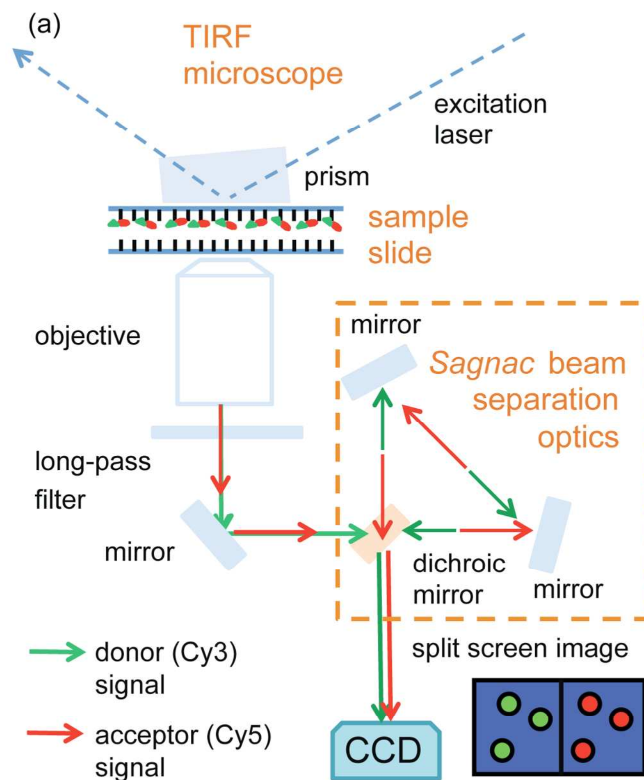

Figure S2. Schematic diagram of the single molecule (SM)-FRET instrument used in these studies. (a) Molecules tethered to the quartz slide sample surface via biotin-neutravidin linkages were monitored using a prism-based total internal reflection

fluorescence (TIRF) microscope. Donor and acceptor fluorescence were collected using a microscope objective and directed through a long-pass filter. The paths of the donor and acceptor fluorescence were separated using a *Sagnac* interferometer, and their images were projected side-by-side onto the active area of an electron-multiplied charge-coupled-device camera (EMCCD). (b) Photographic detail of the *Sagnac* interferometer. The fluorescence image was directed onto a dichroic mirror, which was placed at the input beam-splitter position of the interferometer. We used broadband dielectric mirrors ($R_{\mathbf{eflectivity,\ avg}}$: > 99%, 400 – 750 nm).

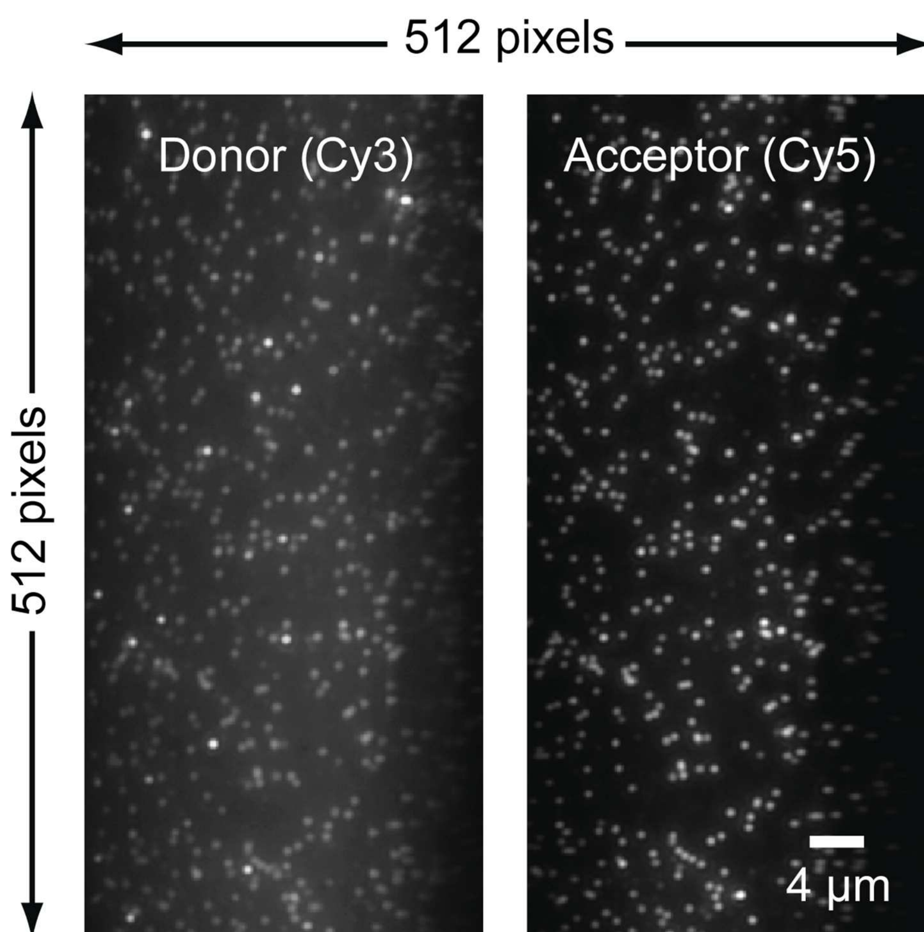

Figure S3. Representative single molecule images obtained with our instrument. The image is split into donor (left side) and acceptor (right side) channels, each 30 × 60 μm^2. The images of a donor molecule and its paired acceptor are spatially separated on the camera by 255 pixels.

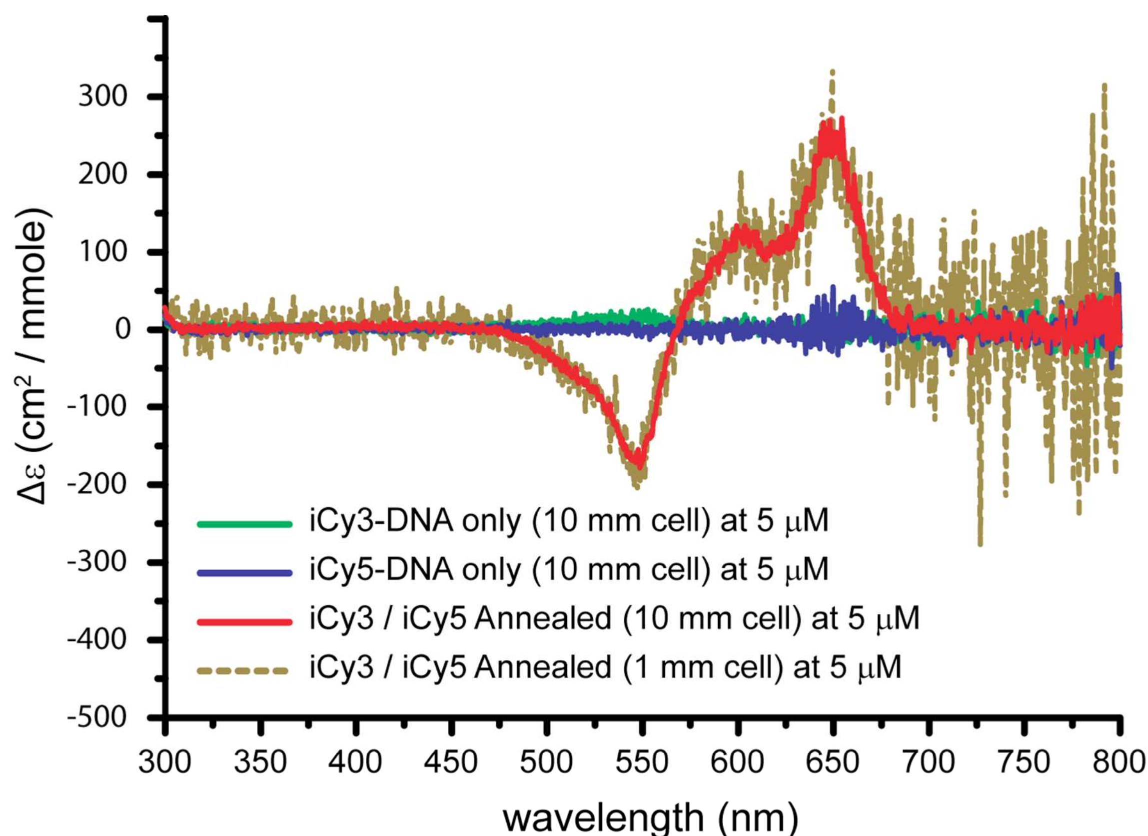

Figure S4. CD spectra at λ>300 nm of the fully annealed dsDNA replication fork constructs and the constituent ssDNA strands with and without the iCy3 / iCy5 chromophores of the internally labeled FRET complex. The concentrations of the DNA construct molecules were 5 μM, and the measurements were made at room temperature and in standard imaging buffer (10 mM Tris at pH 8.0, 100 mM NaCl, and 6 mM $MgCl_2$). CD spectra were measured between λ = 300 and 800 nm on a Jasco model J-720 CD spectrometer. We note that the strong CD signal over the 475 to 680 nm wavelength range (red curve) indicates that the iCy3 / iCy5 chromophores are rigidly positioned within the duplex sugar-phosphate backbone. This chiral CD signal is not present in the CD spectra of the separated iCy3 and iCy5-labeled ssDNA strands (green and blue curves, respectively). We include both the 1 mm and the 10 mm cell scans of the annealed duplex to show that, while the 1 mm scan data are very noisy, the 1 and 10 mm cell scans are the same, thus demonstrating that the significant features of the CD scan of the duplex are independent of cell length.