Supporting Information

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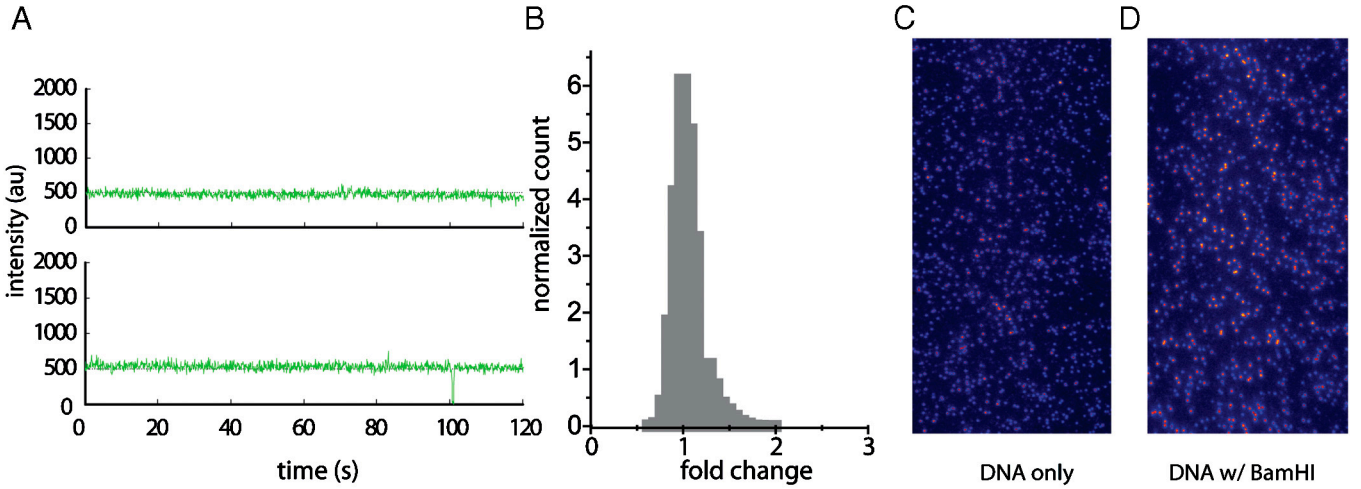


Fig. S1. (*A*) A representative DNA-only trace. More than 95% of molecules display a constant intensity without fluctuations as represented by the traces shown. (*B*) Histogram of cumulative intensity of 30 molecules (Xc = 1.04). Intensity histograms are normalized to the unbound (lowest) peak intensity from each trace. In this case, there is only one peak. Refer to Fig. S2 for a more in-depth description of our analysis. Normalized count (the y-axis) is a count value that takes into account the length of the trace, so that every trace is weighted equally. (*C*) Image of the DNA construct with binding site 1 bp away from Cy3 end. (*D*) Image of the same DNA after addition of BamHI. Cy3 intensities become brighter.

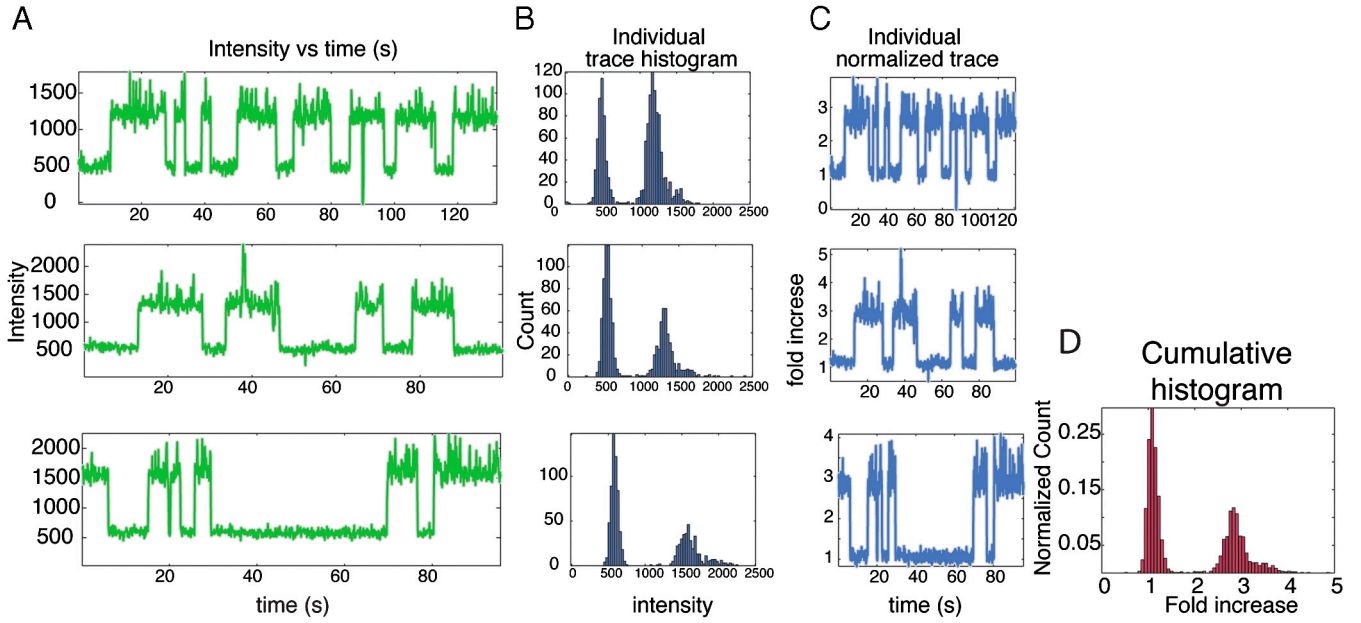
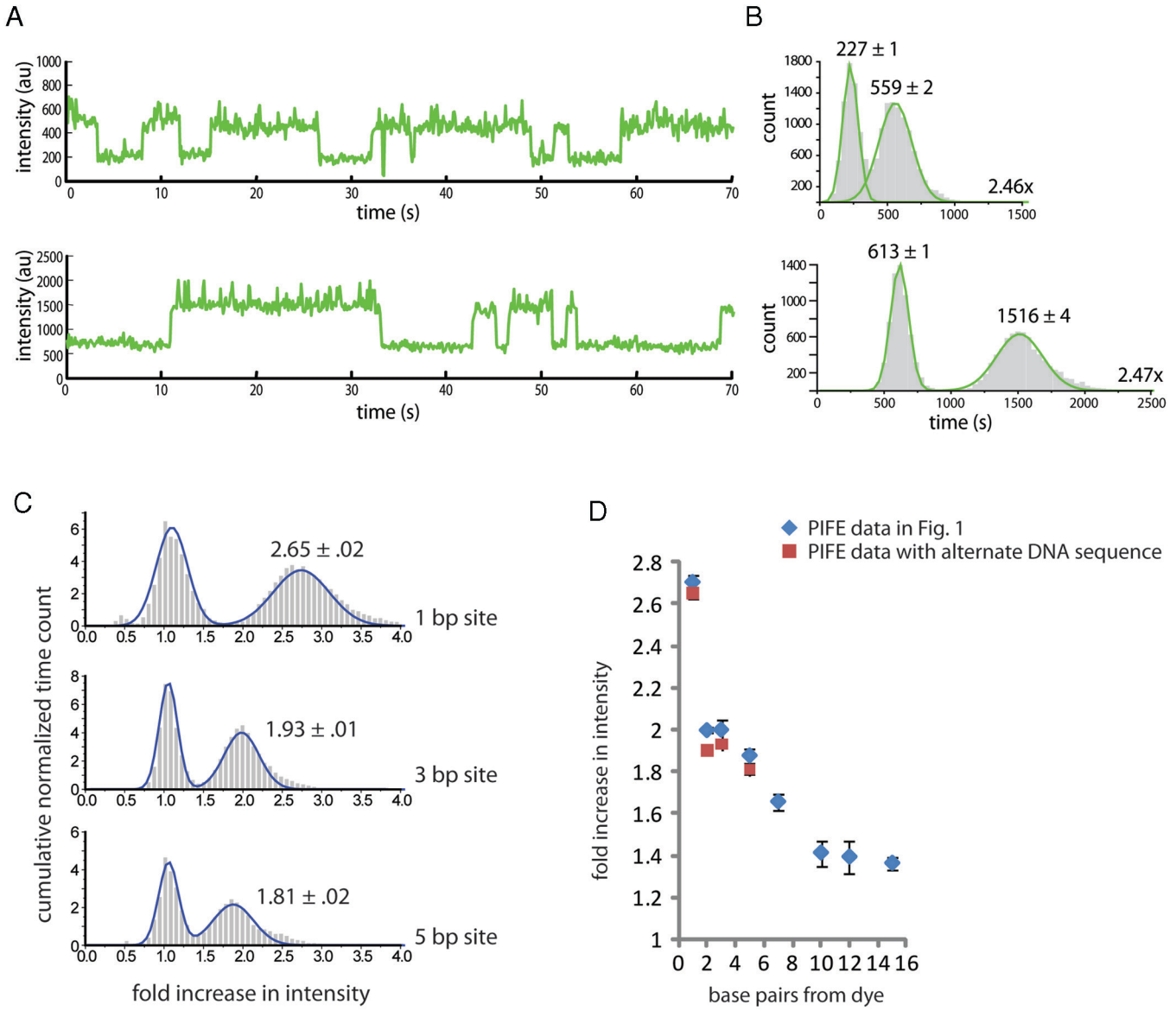


Fig. S2. Intensity normalization analysis. (*A*) Single-molecule traces that show binding and unbinding events are selected. (*B*) The intensities from the selected traces are used to build an intensity histogram for each trace. (*C*) The trace is normalized to the lower peak value, which represents the DNA-only intensity. (*D*) A cumulative histogram is formed based on all the selected traces in that folder. All data presented here are taken from the 1-bp-site data mentioned in Fig. 1*B*.



E Alternate DNA sequence

2 bp	/5Cy3/ TAG GAT CCA TAT AGC GTA GCG TAG CGT AGC GTA GCG TAG G
3 bp	/5Cy3/ TAT GGA TCC ATA AGC GTA GCG TAG CGT AGC GTA GCG TAG G

Fig. S3. PIFE effect is independent of laser intensity and DNA sequence. (*A*) Sample traces from 1-bp-site DNA after addition of BamHI from movies of low (top) and high (bottom) laser excitation levels. As expected, the overall intensity is substantially higher under the higher excitation (bottom). (*B*) Raw intensities are used to build histogram distributions. The intensity fold increase is about 2.5-fold regardless of the level of the excitation intensity. (*C*) Histograms built from PIFE experiment on DNAs that vary in sequence composition in the DNA regions outside of the recognition sequence as shown in (*E*). (*D*) The overlay of the PIFE data on Fig. 1*D* and the PIFE data on the alternate sequence shown in *E*. (*E*) The alternate sequence designed for 2-bp and 3-bp DNA construct.

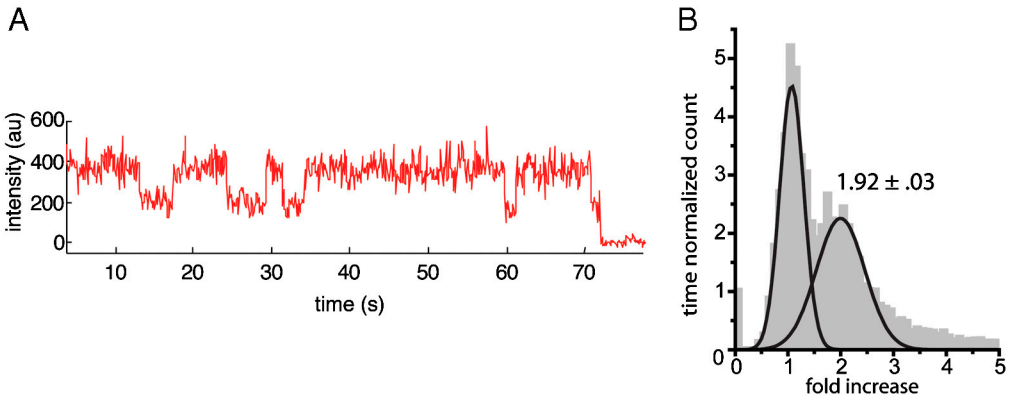


Fig. S4. (*A*) Cy5-labeled DNA (1-bp-away site) shows fluctuations that represent binding and unbinding of BamHI to DNA, similar to Cy3-conjugated 1-bp-site DNA. (*B*) The cumulative histogram from 60 selected traces yields about 1.9-fold increase in intensity, which is lower than the case of Cy3.

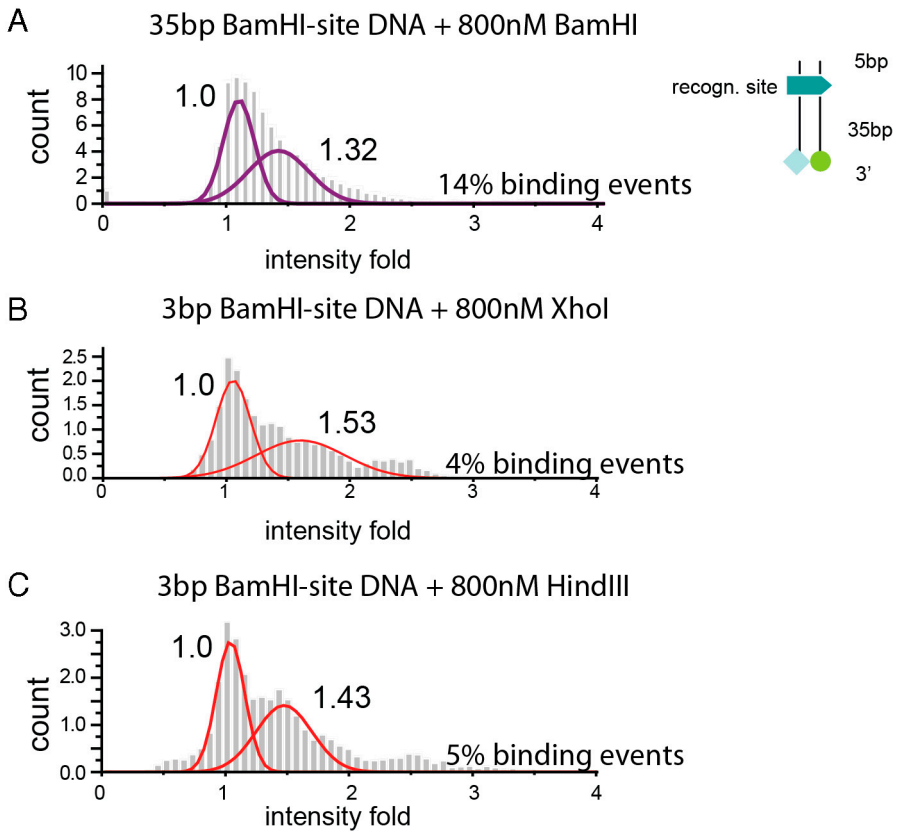


Fig. S5. PIFE effect on DNA with distant recognition site and with nonspecific enzymes. (*A*) DNA-bearing BamHI recognition site 35 bps away from Cy3 yields about 1.32-fold increase in intensity after the addition of BamHI (800 nM). The level of increase seen here is similar to that of 12-bp- and 15-bp-away sites shown in Fig. 1*D*, implying that this represents a basal level of PIFE on account of protein binding outside the PIFE sensitive distance. The sequence used here is the following: 5′-CGT ATG GAT CCA TAC GTA GCG TAG CGT AGC GTA GCG TAG G/Cy3-3′ and 5′-CCT ACG CTA CGC TAC GCT ACG CTA CGT ATG GAT CCA TAC G/3BiosG. (*B* and *C*) Intensity fold increase histograms of XhoI and HindIII binding to 3-bp-BamHI site DNA. Less than 5% of the molecules show intensity increase.

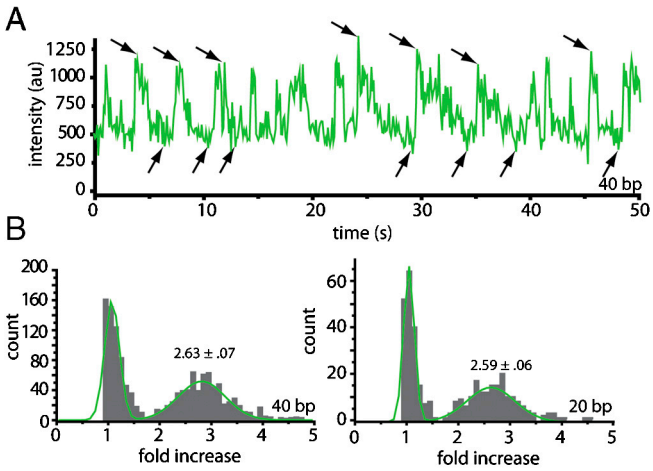


Fig. S6. (*A*) A representative single-molecule trace of RIGh translocation on 40-bp duplex. The minimum and maximum intensities (arrows) were used to generate normalized histograms. (*B*) Cumulative histograms for 40-bp (left) and 20-bp (right) DNA constructs both indicate approximately 2.6-fold increase in intensity.

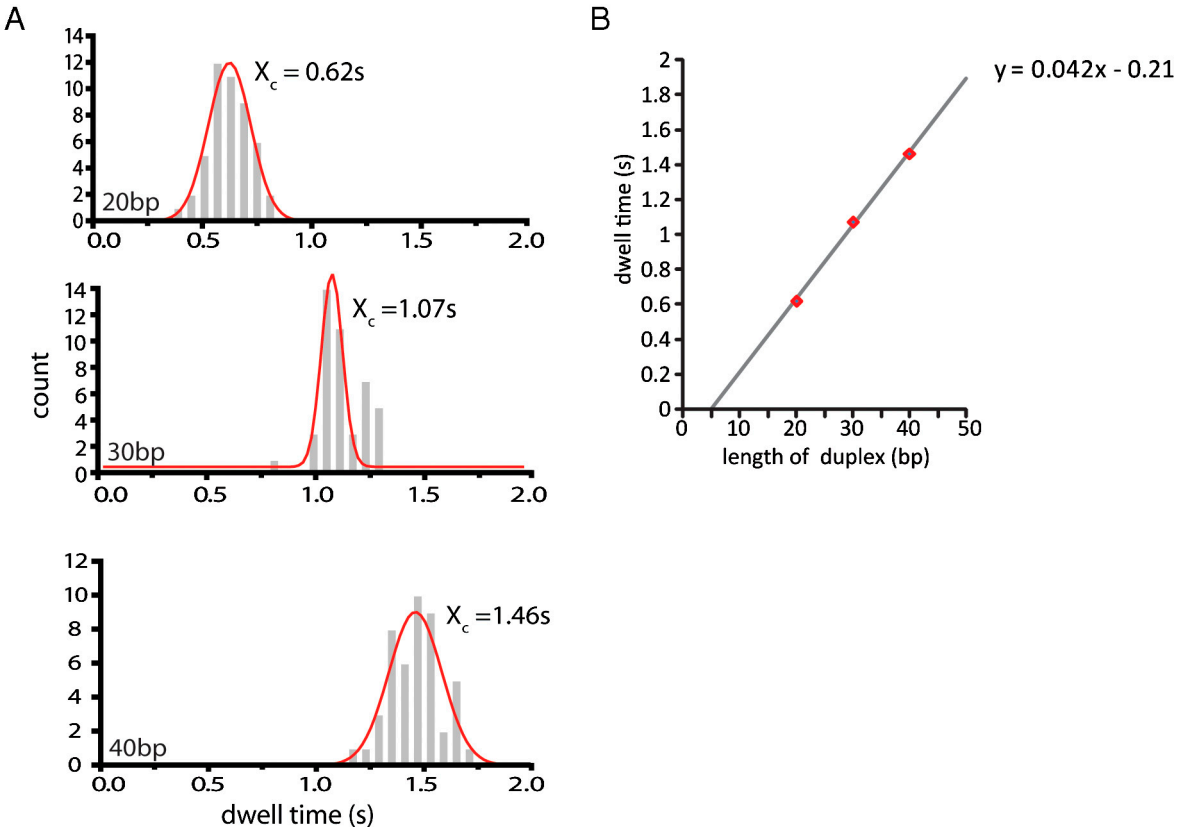
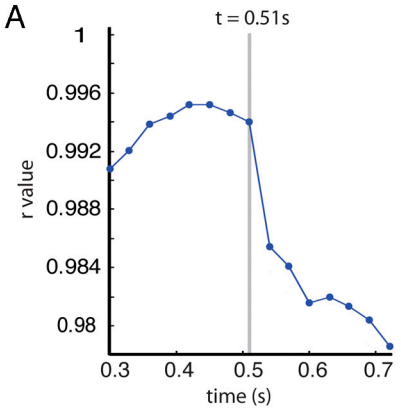


Fig. S7. (*A*) Dwell times of RIGh translocation on 20-, 30-, and 40-bp duplexes were collected and represented as histograms. (*B*) The average dwell times were plotted against the duplex length and fitted to a line. The x-intercept (5 bp) indicates the binding occupancy of RIGh.



B

Duplex length	bp translocated	Dwell time (s)	linear portion (%)	PIFE sensitivity (bp)
20	15	0.62	82.2%	12.3
30	25	1.07	47.7%	11.9
40	35	1.46	34.9%	12.2
average				*12.1 ± .1*

Fig. S8. (*A*) The linear regression value, "*r*" was calculated from the averaged intensities of 20-, 30-, and 40-bp constructs. The r-value 1 indicates a straight line. Because the *r*-value decreases drastically at 0.51 s, we can use 0.51 s as a cutoff to calculate the percentage of linear region of the translocation event. Assuming that RIGh translocate at a constant rate, 0.51 s can be translated to approximately 12-bp distance. (*B*) PIFE sensitivity for each construct is calculated as the product of distance traveled and the proportion of linearity.

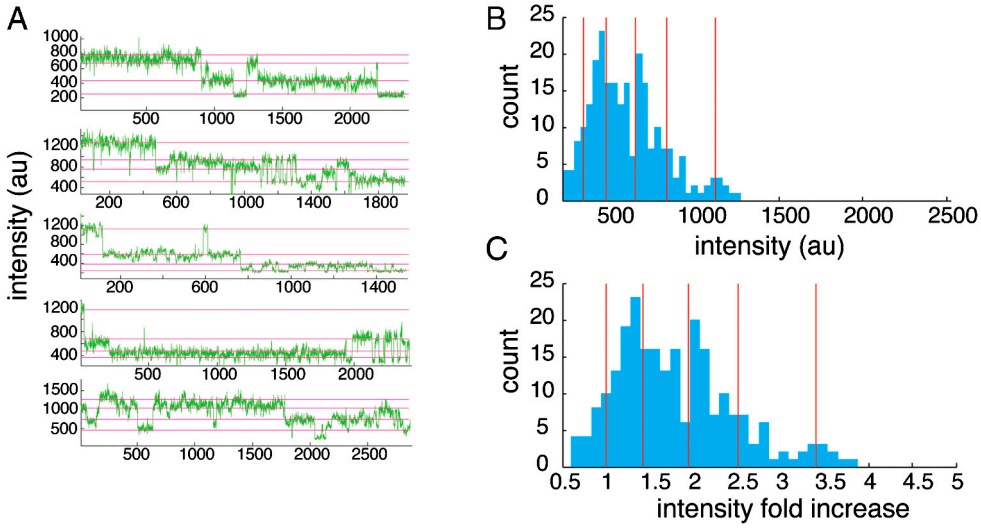


Fig. S9. PIFE clustering analysis. (*A*) Traces that show at least four states are selected. K- means clustering is done to identify the states in each trace (red lines). (*B*) These states are plotted as a histogram. (*C*) The states are then clustered again using k-means. The number of clusters are set to the expected number of states +1 (M_0, DNA-only state) of monomers binding. In pdT13, we expect five states. Cluster centers that contain less than 10% of the total states are disregarded.

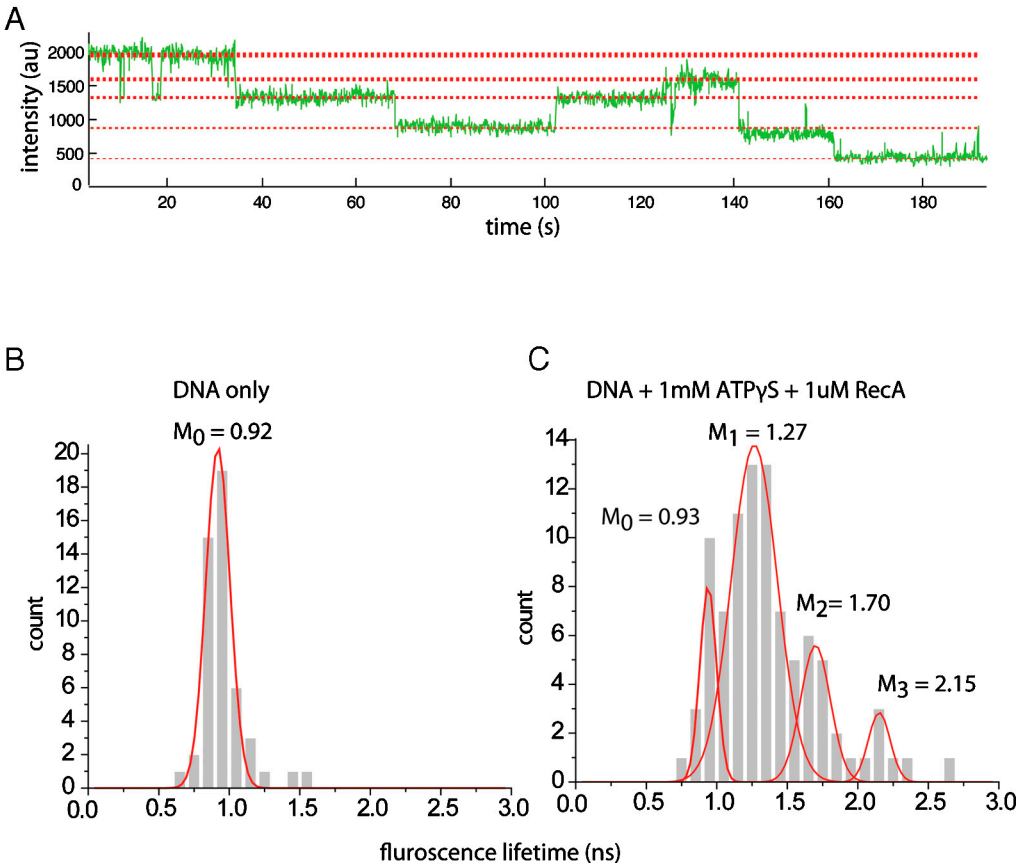


Fig. S10. (*A*) Sample trace from pdT13-DNA after addition of RecA and ATPγS. Like with ATP, RecA monomers with ATPγS seem to bind and disassociate on pdT13, giving multiple intensity states. (*B*) Histogram of lifetime measurements from TCSPC of pdT13-DNA before the addition of RecA. (*C*) Fluorscence lifetime (ns) after 10 min of incubation with the addition of ATPγS and 1uM RecA to pdT13. The dynamic binding and disassociation of RecA on pdT13 is observed with both PIFE and lifetime measurements.