Supporting Information for

Single-Molecule Clocks Controlled by Serial Chemical Reactions

Alexander Johnson-Buck[1,2,3,4] *and William M. Shih*[1,2,3]*

[1] Department of Cancer Biology, Dana Farber Cancer Institute, Boston, Massachusetts 02215, United States

[2] Wyss Institute for Biologically Inspired Engineering and [3]Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, United States

[4] Present Address: Department of Internal Medicine, The University of Michigan, Ann Arbor, Michigan, 48109, United States

*E-mail: William_Shih@dfci.harvard.edu; Phone: 617-632-5143; Fax: 617-394-2897

Materials and Methods

Materials. All oligonucleotides were purchased from Integrated DNA Technologies with HPLC (fluorophore-labeled strands) or PAGE purification (all other strands). Many strands were modified with 3′-overhangs and/or phosphates to block unwanted polymerization. Oligonucleotide sequences are shown in Table S1. Bst DNA polymerase large fragment (M0275M) and dNTPs (N0447S) were purchased from New England Biolabs. Protocatechuate-3,4-dioxygenase (PCD, ICN15197595), 3,4-dihydroxybenzoic acid (PCA, 507009365), hexane (AC411550025), ammonium hydroxide (AC205840010), and hydrogen peroxide (AC202460010) were purchased from Fisher. Dichlorodimethylsilane (DDS, 440272-100ML) was obtained from Sigma-Aldrich.

TIRF microscopy. Single-molecule measurements were performed at room temperature using the TIRF module of a Zeiss LSM 710 confocal/TIRF combination microscope. For immobilization of biotinylated samples, glass coverslips and microscope slides were functionalized with dichlorodimethylsilane and sandwiched together with double-sided tape to form a flow cell, followed deposition of biotinyl-BSA, passivation with 0.2% Tween-20 in T50 buffer (50mM Tris-HCl, pH 8.0, 50mM NaCl and 1mM EDTA), and incubation with streptavidin as described(1). Following streptavidin incubation, the channel was flushed with 2 volumes of ThermoPol buffer (New England Biolabs), and biotinylated samples were immobilized for imaging (see below). Excitation at 640 nm was used for detection of Cy5 and Alexa Fluor 647, with an acquisition rate of 1 Hz.

After preparation of flow cells and coating with streptavidin, flow cells were incubated with 25 pM Initiator (*I*) for 5 min. The surface was then imaged in TIRF mode in the presence of an imaging buffer containing ThermoPol supplemented with 40 mM NaCl, 25 nM Primer (*P*), 200 nM Template (*T),* 100 μM dNTPs (unless otherwise specified), 1.65 μM (≡12,000 units/mL) *Bst* DNA polymerase, Large Fragment, and an oxygen scavenger system consisting of 50 nM PCD and 5 mM PCA(2). We have found it critical to use fresh PCA that is stored in frozen aliquots at pH no higher than 8.0-8.5; higher pH and/or repeated freeze-thaw cycles result in a darkly colored degradation product that strongly inhibits *Bst* DNA polymerase. The surface was imaged under TIRF illumination at 640 nm for 10 min.

DPAGE assay of initiator-dependent polymerization. Reaction mixtures were prepared with 100 nM fluorescent Primer, 0 or 100 nM of the 41-nt Template, 0 or 100 nM Initiator, 0 or 1.65 μM *Bst* DNAP, 100 μM dNTPs, 5 mM PCA, and 50 nM PCD in 1X ThermoPol buffer + 40 mM NaCl. After incubating for 5 min at room temperature, reactions were quenched with a tenfold volumetric excess of formamide loading buffer (90% formamide in 0.5X TBE buffer) + 2% SDS, then run on a 20% denaturing polyacrylamide gel. The gel was scanned for Cy5 fluorescence on a Typhoon FLA 9500 Imager (Fig. 2a).

DPAGE time course of primer extension by *Bst* DNA polymerase. A template mixture was prepared by combining 1 μM Initiator, 1.5 μM Template, and 1 μM Primer in 1X ThermoPol buffer, and incubating for 5 min at room temperature. The template mixture was diluted in 1X ThermoPol (final template concentration 5 nM) and combined with dNTPs (final concentration 0.8 or 1.6 μM). The reactions were initiated by adding *Bst* DNAP (final concentration 1,600 U/mL). At specified time points, 1 μL of each reaction was removed and quenched with 4 μL of formamide loading buffer + 2% SDS.

Reactions were heated to 80 °C for 2 min and then run on a 20% denaturing polyacrylamide gel, and visualized with an Amersham Imager 600 fluorescent gel scanner (Figure S2).

Kinetic analysis of single-molecule data. Custom MATLAB code was used to identify regions of interest within the field of view that contained fluorescent primer or probe binding events. Hidden Markov modeling (HMM) with the third-party extension vbFRET(3) was used to identify and measure time intervals with high fluorescence (probe bound) or low fluorescence (no probe bound) in an automated fashion. Except for imposing a two-state model, the default settings of vbFRET were used in all analyses. Dwell times in the bound (high-fluorescence) and unbound (low-fluorescence) states were pooled from all molecules observed and plotted in histograms for analysis by least-squares fitting with single-exponential and gamma distribution functions in MATLAB.

Monte Carlo Simulations. Dwell times of single-molecule clock reactions were simulated in MATLAB as a sum of exponentially distributed random variables whose expectation values depend on the length and sequence of the template. Simulations were carried out for template sequences T_{41}, T_{57}, T_{97}, and T_{153}; the 14 nucleotides at the 3′-end of each template constitute the primer-binding footprint and were thus omitted from simulations. For each condition tested, 10^4 replicates were performed. N_{app} was determined as $1/c_v^2$ (where c_v is the coefficient of variation). For simplicity and due to the high concentration of Bst DNAP used, binding of the polymerase was assumed to be fast; however, pseudo-first order binding rate constants faster than ~0.5 s^{-1} had little effect on results for the templates examined.

Table S1 | Oligonucleotide sequences

Name	Sequence
Primer (*P*)	/5Cy5/TAAGTTAGGGAGTA
Primer 2 (P_2)	/5Cy5/TAAGTTAGGGAGTAGTG
Initiator (*I*)	TTCCTCACATCTCAAGCTTTTTTTTTT/3BioTEG/
Initiator 2 (I_2)	GCACTACTCCCTAACATCTCAAGCTTTTTTTTTT/3BioTEG/
Templates (*T*)	
T_{41}	AGCTTGAGATGTGAGGAAGTAGTCACCTACTCCCTAACATT/3Phos/
T_{57}	AGCTTGAGATGTGAGGAAGTAGTCACCTTATAACCATACTATCTACTCCCTAACATT/3Phos/
T_{62}	GCTTGAGATGTTAGGGAGTAGTGCCTAGAGTGCCGCTTTCAGCCGCACTACTCCCTAACAAT/3Phos/
T_{97}	AGCTTGAGATGTGAGGAAGTAGTAATCGCTAATGCGTAATCGGTAATCCGTAATCGCTAATGCGTAATCGGTAATCCGTAATCTACTCCCTAACATT/3Phos/
T_{153}	AGCTTGAGATGTGAGGAAGTAGTAATCGGTAAACGGTAATCCGTAAACCGTAATCGCTTAACGCTAATGCGTATAGCGTAATCGGTAAACGGTAATCCGTAAACCGTAATCGCTTAACGCTAATGCGTATAGCGTAATCTACTCCCTAACATT/3Phos/

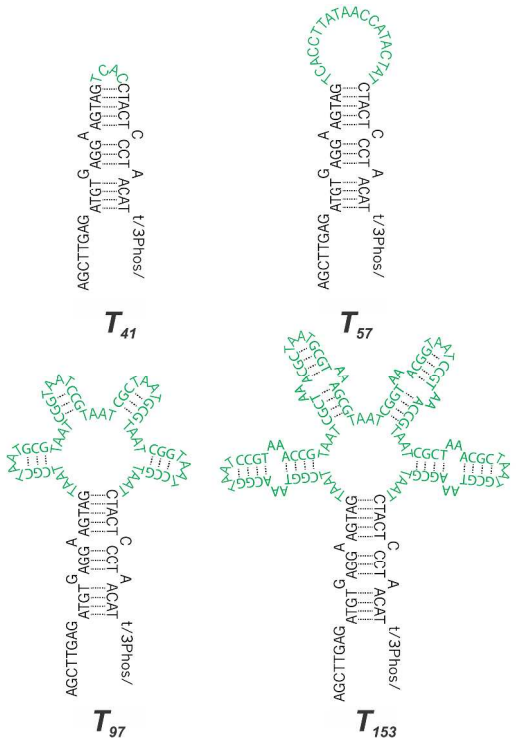

Figure S1. Detailed secondary structure designs of linear clock templates. Templates were designed to provide (i) accessibility of the 5′-terminus to invasion by initiator ***I***; (ii) efficient closing of the main helical stem (black), resulting in sequestration of the primer-binding sequence at the 3′-terminus in the absence of initiator; (iii) absence of undesired secondary structure that might compete with the closing stem; and (iv) strategic introduction of mismatches to minimize unnecessarily stable secondary structure and undesired interactions between sequences (*e.g.*, between the initiator and primer).

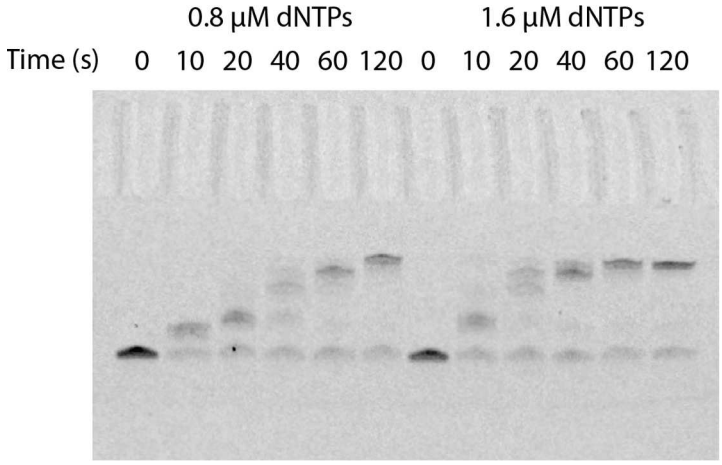

Figure S2. Ensemble time courses of primer extension. Denaturing polyacrylamide gel electrophoresis showing time-dependent extension of fluorescent primer (***P*$_2$**) by Bst DNA polymerase, large fragment, in the presence of initiator (***I*$_2$**) and template (***T*$_{61}$**).

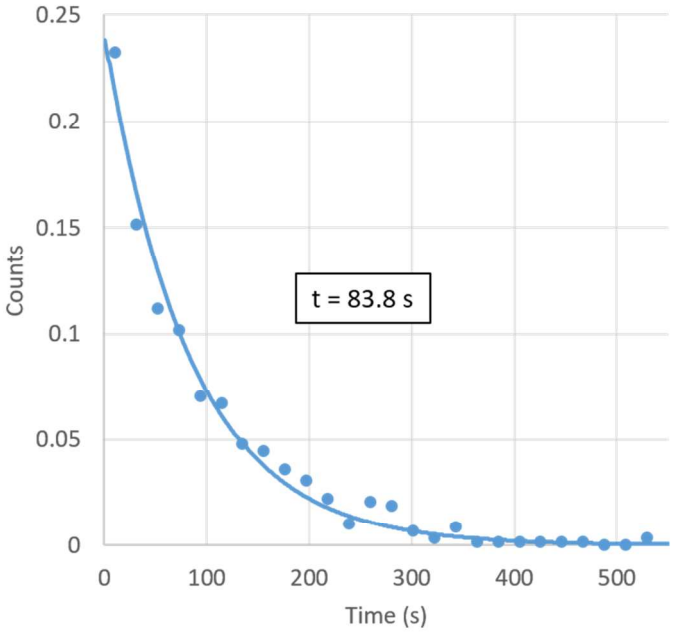

Figure S3. Photobleaching Kinetics. Dwell time distribution for primer (*P*) and 41-nucleotide template (T_{41}) binding to initiator in absence of DNA polymerase or dNTPs. A single-exponential fit (solid line) yields a characteristic lifetime of 83.8 s.

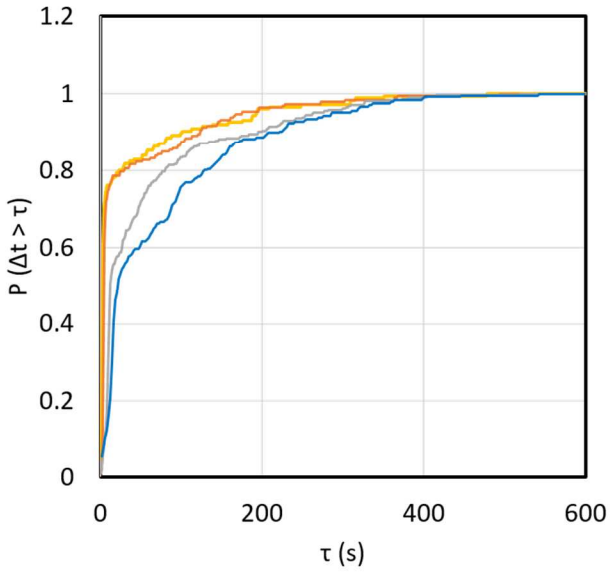

Figure S4. Cumulative dwell time distributions for linear Bst DNAP clocks. Cumulative distributions for templates with 41 (yellow), 57 (orange), 97 (gray), and 153 (blue) nucleotides are shown. In addition to the rapid gamma-distributed phase, which accounts for the majority of events, a second exponential distribution becomes increasingly prominent with increasing template length. The lifetime of the exponential phase (60-120 s) is comparable to the timescale of photobleaching (Figure S3), suggesting that the dwell times of some very long events are not quantifiable.

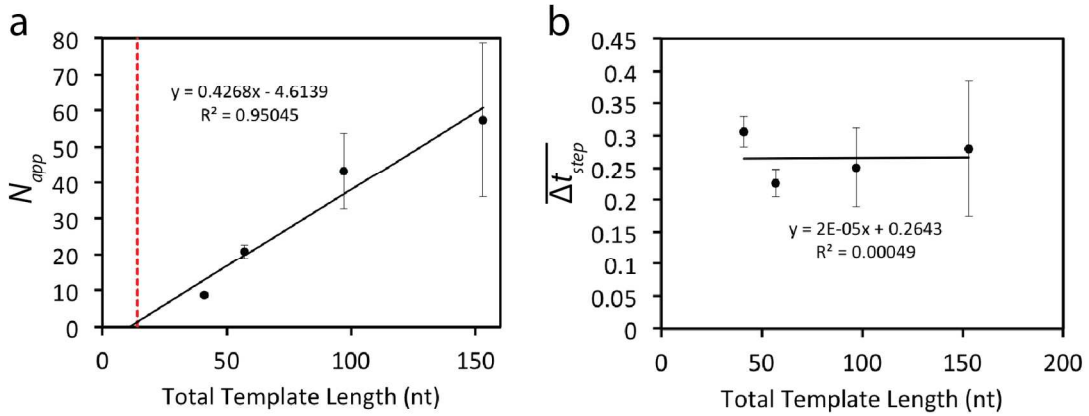

Figure S5. Parameters from gamma distribution fit to dwell time histograms as a function of template length. (**a**) The apparent number of steps N_{app} increases linearly with template length, albeit with a proportionality constant significantly lower than 1. (**b**) The apparent time per step is independent of template length. Error bars are 95% confidence bounds from nonlinear least squares fitting of dwell time histograms.

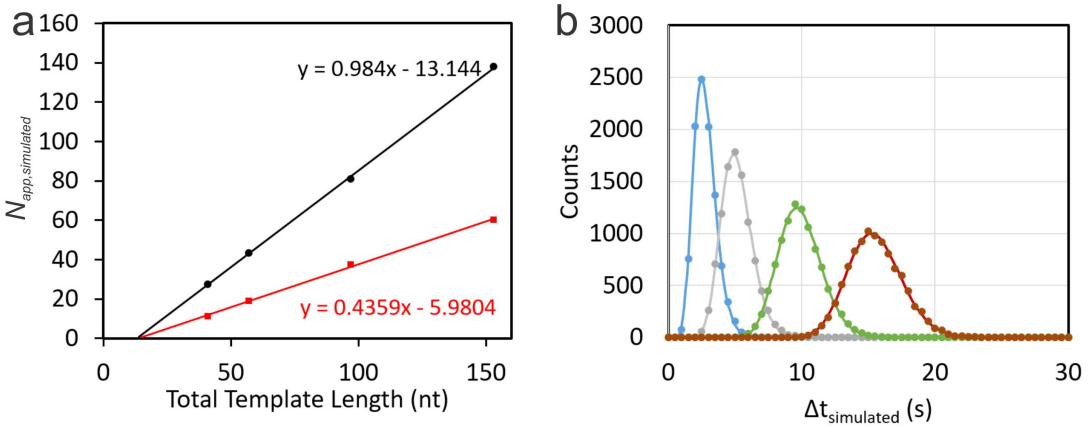

Figure S6. Monte Carlo simulations of linear clock kinetics. (**a**) N_{app} as a function of length assuming identical incorporation kinetics for the four dNTPs (black circles) or 9-fold slower incorporation kinetics for one dNTP than the other three (red squares). (**b**) Simulated histograms of dwell times in the bound state for templates with 41 (blue), 57 (gray), 97 (green), and 153 (red) nucleotides, assuming 9-fold slower kinetics of incorporation for one of the four nucleotides. Each histogram is modeled by a gamma probability density function.

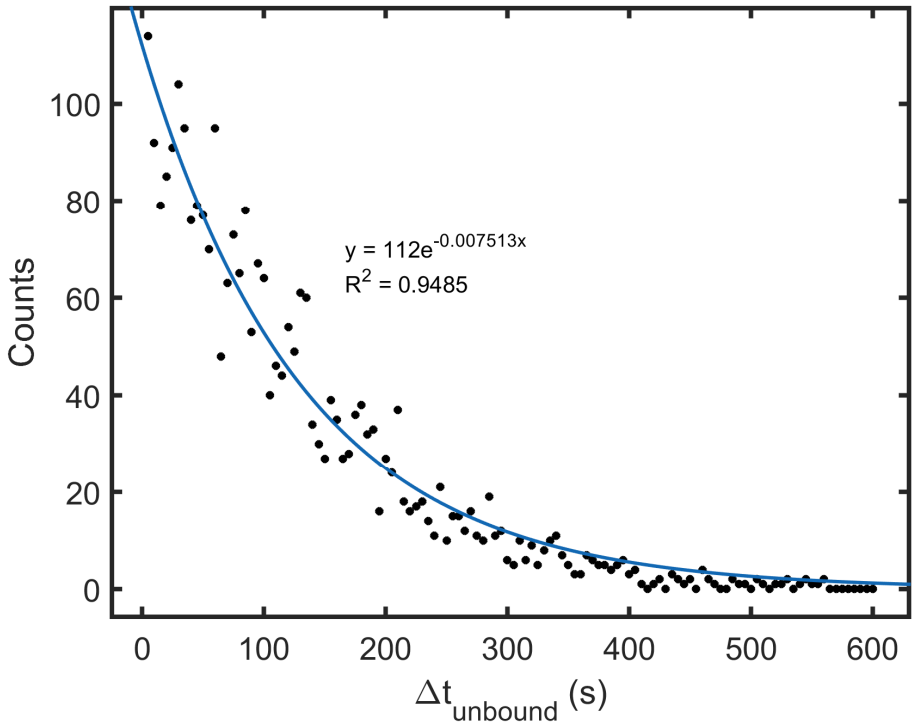

Figure S7. Distribution of dwell times in the unbound state for the 41-nucleotide linear single-molecule clock. A single exponential decay function is fit to the dwell time histogram (solid line).

Supporting References

(1) Hua, B.; Han, K. Y.; Zhou, R.; Kim, H.; Shi, X.; Abeysirigunawardena, S. C.; Jain, A.; Singh, D.; Aggarwal, V.; Woodson, S. A.; *et al. Nat. Methods* **2014**, 11, 1233–1236.

(2) Aitken, C. E.; Marshall, R. A.; Puglisi, J. D. *Biophys. J.* **2008**, 94, 1826–1835.

(3) Bronson, J. E.; Fei, J.; Hofman, J. M.; Gonzalez Jr., R. L.; Wiggins, C. H. *Biophys. J.* **2009**, 97, 3196–3205.