

# First passage time study of DNA strand displacement

D. W. Bo Broadwater, Jr.,<sup>1</sup> Alexander W. Cook,<sup>1</sup> and Harold D. Kim<sup>1,\*</sup>

<sup>1</sup>School of Physics, Georgia Institute of Technology, Atlanta, Georgia

**ABSTRACT** DNA strand displacement, in which a single-stranded nucleic acid invades a DNA duplex, is pervasive in genomic processes and DNA engineering applications. The kinetics of strand displacement have been studied in bulk; however, the kinetics of the underlying strand exchange were obfuscated by a slow bimolecular association step. Here, we use a novel single-molecule fluorescence resonance energy transfer approach termed the “fission” assay to obtain the full distribution of first passage times of unimolecular strand displacement. At a frame time of 4.4 ms, the first passage time distribution for a 14-nucleotide displacement domain exhibited a nearly monotonic decay with little delay. Among the eight different sequences we tested, the mean displacement time was on average 35 ms and varied by up to a factor of 13. The measured displacement kinetics also varied between complementary invaders and between RNA and DNA invaders of the same base sequence, except for T → U substitution. However, displacement times were largely insensitive to the monovalent salt concentration in the range of 0.25–1 M. Using a one-dimensional random walk model, we infer that the single-step displacement time is in the range of ~30–300  $\mu$ s, depending on the base identity. The framework presented here is broadly applicable to the kinetic analysis of multistep processes investigated at the single-molecule level.

**SIGNIFICANCE** DNA strand displacement occurs when a single nucleic acid strand invades and replaces another nearly identical strand in a duplex. This process is ubiquitous in biology and is fundamental to the field of DNA nanotechnology. Previous kinetic studies of strand displacement either used DNA strands much longer than those found in practical applications or were obscured by a rate-limiting bimolecular step known as toehold formation. In this study, we introduce a new, single-molecule scheme that enables direct measurement of the strand displacement first passage time. Our observed kinetics demonstrate highly nontrivial sequence dependence as well as surprising differences between RNA and DNA invaders.

## INTRODUCTION

Nucleic acids’ ability to form hydrogen bonds between complementary Watson-Crick bases allows for a rich set of complicated, multistep kinetic behaviors such as duplex hybridization (1) and dehybridization (2), Holliday junction structural dynamics (3,4), and strand invasion (5). In particular, strand displacement, which is the exchange of bases between two competing nucleic acid strands of identical sequence, occurs in homologous recombination (6,7), DNA replication (8), and RNA transcription (9), as well as

CRISPR/Cas (10) and the related Cascade complex (11). In addition to fundamental genomic processes, DNA nanotechnology exploits strand displacement to create nanoscale gadgets (12–14) and computational circuits (15–18). Strand displacement also aids in the development of quantitative assays for detection of nucleic acid (19–21) and enzymatic activity (22,23) with improved probe specificity (17,24,25).

For practical applications, strand displacement is implemented with the “invader” strand and a partial duplex composed of the “incumbent” strand and the “substrate” strand (Fig. 1; (18)). The partial duplex has two distinct domains: 1) the single-stranded overhang called the toehold, which is critical to the speed and efficiency of the reaction (26), and 2) the duplex region called the displacement domain. Toehold-mediated strand displacement is initiated when the invader strand anneals to the toehold in a bimolecular reaction. Once a stable toehold interaction is formed,

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\*Correspondence: [harold.kim@physics.gatech.edu](mailto:harold.kim@physics.gatech.edu)

D. W. B. Broadwater and Alexander W. Cook contributed equally to this work.

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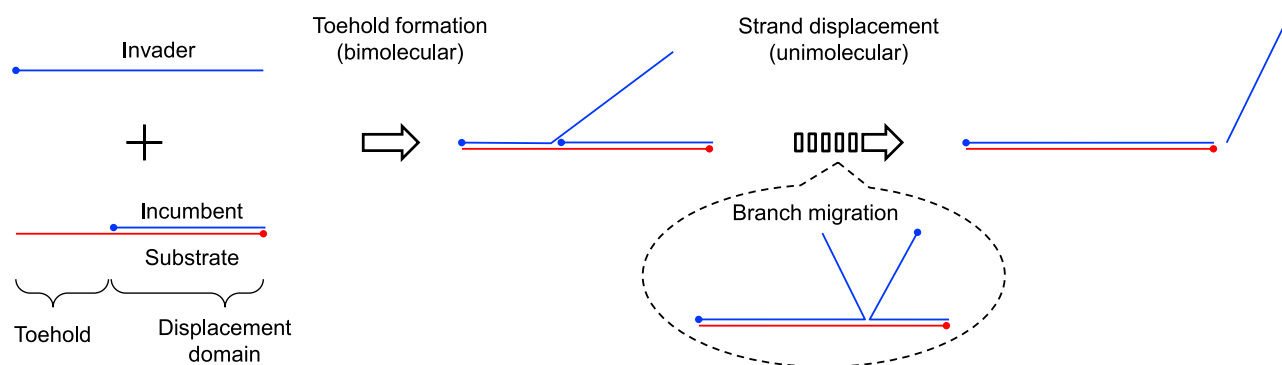


FIGURE 1 Toehold-mediated DNA strand displacement. Each line represents a single-stranded DNA with the 5' end marked by a dot. Blue and red colors represent complementary sequences. In bulk studies, the apparent reaction kinetics are dominated by the slow bimolecular step. In this study, we focus on the unimolecular strand displacement, which is thought to occur through branch migration. To see this figure in color, go online.

the incumbent can be displaced by the dangling strand of the invader through spontaneous opening of a basepair between substrate and incumbent and closing of a basepair between substrate and invader. This unimolecular strand displacement is also called branch migration (27–29).

Great attention has been paid to the kinetics of toehold-mediated strand displacement (26,29–32). However, these kinetics have been measured mostly in bulk, in which the reaction kinetics are limited by bimolecular toehold association. Therefore, the measured kinetics do not shed light on the unimolecular branch migration. Other studies using long ( $\sim 1$  kbp) DNA estimated the branch migration time per basepair step to be  $\sim 10 \mu\text{s}$  (27,28), but strand displacement in those studies took place in a D-loop geometry that restricted the movement of dangling strands during branch migration, unlike the geometry of current interest. Also,  $\sim 1$  kbp scale extends far beyond the length scales of interest for DNA nanotechnology. To understand whether and how sequence can be used to control displacement kinetics, we require experimental studies on unimolecular displacement of short oligos that can be modeled at the single base level.

In this study, we introduce a DNA “fission” assay to study toehold-mediated DNA strand displacement kinetics. The fission assay employs single-molecule fluorescence resonance energy transfer (smFRET) to directly measure the first passage displacement time (33,34) for the unimolecular reaction that occurs between toehold formation and incumbent dissociation. Using a wide-field total internal reflection fluorescence microscope, we measured the displacement kinetics for a 14-nucleotide displacement domain of eight different sequences. The mean displacement time varied by more than 10-fold between the slowest and fastest sequence and was on average  $\sim 35$  ms, and the histograms of displacement times obtained at 4.4-ms resolution showed a monotonic decay with little to no lag. We found that the displacement kinetics depend on the base sequence and the nucleic acid type (DNA versus RNA) of the invader but not on monovalent salt concentration. We analyzed the first passage time histograms of strand

displacement using a symmetric random walk model to extract single basepair step times. The best fit to the histograms was obtained with  $\sim 33$ ,  $\sim 200$ ,  $\sim 250$ , and  $\leq 33 \mu\text{s}$  for A, C, T, and G, respectively. Our study reports the displacement rates of short DNA oligos and reveals biophysical mechanisms that govern DNA strand displacement kinetics.

## MATERIALS AND METHODS

### Sample preparation

Custom DNA oligomers were purchased from Integrated DNA Technologies (Coralville, IA). The 26-nt substrate was internally labeled near the end distal to the toehold with a Cy3 fluorophore. The 24-nt invader molecule was labeled with a Biotin-TEG linker at the end proximal to the toehold for surface immobilization. The 14-nt incumbent sequences were labeled with a Cy5 fluorophore at the end distal to the toehold. All oligos were purified by the manufacturer with high-performance liquid chromatography. The specific sequences are in Tables S1–S3. Partial duplexes were constructed by combining substrate and incumbent at a 1:10 ratio ( $0.5 \mu\text{M}$  substrate,  $5.0 \mu\text{M}$  incumbent) in buffer at pH 7 containing 1 M NaCl, 10 mM Tris, and 1 mM EDTA. The excess of incumbent strands was meant to minimize the number of single-stranded substrates in solution; unpaired substrates can compete with the partial duplexes for binding with the surface-bound invaders, whereas lone incumbent strands do not bind to the invaders and will not fluoresce on their own. The mixture was heated to  $95^\circ\text{C}$  and slowly cooled for 3 h to  $4^\circ\text{C}$  to ensure the partial duplex was fully annealed.

### Experimental setup

Molecules were observed with an objective-type total internal reflection fluorescence microscope assembled on a commercial microscope body (IX81; Olympus, Tokyo, Japan). Fluorophores were excited by a 532 nm laser (BWN-532-50E; B&W Tek, Newark, DE). Images were  $2 \times 2$  binned and captured with an EMCCD (DU-897ECS0-#BV; Andor Technology, Belfast, UK), and images were recorded at 228 fps with 3.96-ms exposure time using Micro-Manager software (35). This high frame rate was achieved by cropping the image height to 64 superpixels. Experiments were performed on flow cells constructed as previously described in Le and Kim (36), whereas flow volume and flow rate ( $900 \mu\text{L min}^{-1}$ ) were controlled by a syringe pump (NE-1000; New Era Pump Systems, East Farmingdale, NY).

The surface was passivated with polyethylene glycol to minimize nonspecific binding (36). After NeutrAvidin coating, the biotin-containing invader molecules were immobilized by flowing in at a concentration of 1 nM. Next, 20  $\mu$ L of partial duplexes were pumped into the flow cell at 2.5 nM in an oxygen-scavenging imaging buffer (37), which contained 1 nM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 5 mM protocatechuic acid, 100 nM protocatechuate 3,4-dioxygenase, and 100 mM Tris-HCl (pH 7).

An appearance of a high-FRET signal marked the formation of the toehold. A low-FRET signal appeared as strand displacement concluded. The FRET signal time series was recorded and analyzed using in-house MATLAB software (The MathWorks, Natick, MA). The lifetime of the high-FRET state was observed for many molecules to collect a distribution of displacement times.

## Statistics of displacement times

Here, we provide an analytical expression we used to fit the histograms of displacement times. We model strand displacement as a one-dimensional random walk:

$$0 \xrightleftharpoons[r_1]{f_0} 1 \xrightleftharpoons[r_2]{f_1} 2 \xrightleftharpoons[r_3]{f_2} \dots \xrightleftharpoons[r_n]{f_{n-1}} n. \quad (1)$$

In this model, each state is denoted by  $i$ , the number of displaced bases, and the measured displacement time corresponds to the first passage time from the reflecting state 0 on the left boundary to the absorbing state  $n$  on the right boundary. The forward rate and reverse rate from state  $i$  are denoted as  $f_i$  and  $r_i$ , respectively. Because state  $n$  is the absorbing state,  $r_n = 0$ . The time dependence of the system is governed by the master equation:

$$\frac{\partial |\psi(t)\rangle}{\partial t} = \mathbf{L} |\psi(t)\rangle, \quad (2)$$

where  $\mathbf{L}$  is the transition matrix operator, and the ket vector  $|\psi(t)\rangle$  represents the system state. The probability amplitude to be in the absorbing state  $|n\rangle$  at some time  $t$  after starting in  $|0\rangle$  is then given by (38)

$$P(n, t | 0) = \langle n | e^{\mathbf{L}t} | 0 \rangle. \quad (3)$$

The experimentally accessible datapoints in single-molecule experiments are the number of displacement events  $\Delta N$  detected during a short time interval or bin time  $\Delta t$ . These numbers form the so-called dwell time or survival-time histogram. For a sufficiently large number of total events  $N_0$ ,  $\Delta N$  in the  $i$ -th bin is related to the probability amplitudes according to

$$\begin{aligned} \frac{\Delta N(i)}{N_0} &= P(n, i\Delta t | 0) - P(n, (i-1)\Delta t | 0) \\ &= \langle n | e^{\mathbf{L}i\Delta t} (1 - e^{-\mathbf{L}\Delta t}) | 0 \rangle. \end{aligned} \quad (4)$$

This can be expanded using the left and right eigenvectors of  $\mathbf{L}$ ,  $\langle \phi_k^L |$  and  $|\phi_k^R\rangle$  that satisfy  $\langle \phi_k^L | \phi_m^R \rangle = \delta_{km}$ , and their corresponding eigenvalue  $-\mu_k$ :

$$\frac{\Delta N(i)}{N_0} = \sum_k \langle n | \phi_k^R \rangle \langle \phi_k^L | 0 \rangle e^{-\mu_k i \Delta t} (1 - e^{\mu_k \Delta t}). \quad (5)$$

In the limit of  $\Delta t \rightarrow 0$ , Eq. 5 yields the first passage time density  $f(t)$ :

$$\begin{aligned} f(t) &\equiv \lim_{\Delta t \rightarrow 0} \frac{1}{N_0} \frac{\Delta N(i)}{\Delta t} = P(n, t | 0) \\ &= \sum_k \langle n | \phi_k^R \rangle \langle \phi_k^L | 0 \rangle (-\mu_k) e^{-\mu_k t}. \end{aligned} \quad (6)$$

We used Eq. 5 to fit the measured histograms of displacement times with a fixed  $\Delta t$  that corresponds to the frame time of 4.4 ms. In the representation of  $|0\rangle \rightarrow (1, 0, 0, \dots, 0)^T$ ,  $\mathbf{L}$  is an asymmetric tridiagonal matrix:

$$\mathbf{L} = \begin{bmatrix} -f_0 & r_1 & 0 & \cdots & 0 & 0 \\ f_0 & -(f_1 + r_1) & r_2 & \cdots & 0 & 0 \\ 0 & f_1 & -(f_2 + r_2) & \cdots & 0 & 0 \\ \vdots & \vdots & \vdots & \ddots & \vdots & \vdots \\ 0 & 0 & 0 & \cdots & -(f_{n-1} + r_{n-1}) & 0 \\ 0 & 0 & 0 & \cdots & f_{n-1} & 0 \end{bmatrix}, \quad (7)$$

whose left and right eigenvectors and eigenvalues can be obtained using MATLAB.

We also present here the expression we use to analyze the mean first passage time  $\tau$  (39).  $\tau$  can be computed using Eq. 6 as

$$\tau = \int_0^\infty t f(t) dt. \quad (8)$$

A more useful expression can be obtained in terms of an invertible sub-matrix of  $\mathbf{L}$ , which we term  $\mathbf{A}$ :

$$\mathbf{L} = \begin{bmatrix} [\mathbf{A}] & 0 \\ \vdots & 0 \\ 0 \cdots f_{n-1} & 0 \end{bmatrix}. \quad (9)$$

Using the normalization of probability amplitude

$$P(n, t | 0) = 1 - \sum_{i=0}^{n-1} P(i, t | 0), \quad (10)$$

we can express  $\tau$  in terms of the inverse of  $\mathbf{A}$

$$\begin{aligned} \int_0^\infty t f(t) dt &= - \int_0^\infty t d \left[ \sum_{i=0}^{n-1} \langle i | e^{-\mathbf{A}t} | 0 \rangle \right] \\ &= \int_0^\infty \sum_{i=0}^{n-1} \langle i | e^{-\mathbf{A}t} | 0 \rangle dt \end{aligned}$$

$$= \sum_{i=0}^{n-1} \langle i | \mathbf{A}^{-1} | 0 \rangle. \quad (11)$$

In the matrix presentation, the inverse matrix  $\mathbf{A}^{-1}$  is related to matrix cofactors by

$$\mathbf{A}^{-1} = \frac{1}{\det(\mathbf{A})} \mathbf{C}^T. \quad (12)$$

Plugging Eq. 12 into Eq. 11,

$$\tau = \frac{1}{\det(\mathbf{A})} \sum_{j=1}^n C_{1j}. \quad (13)$$

This sum of cofactors can be equated to the determinant of matrix  $\mathbf{A}'$ , which replaces the first row of  $\mathbf{A}$  with 1. Hence, the mean first passage time is given by the ratio of two matrix determinants (32):

$$\tau = \frac{\begin{vmatrix} 1 & 1 & \cdots & 1 \\ a_{21} & a_{22} & \cdots & a_{2n} \\ \vdots & \vdots & & \vdots \\ a_{n1} & a_{n2} & \cdots & a_{nn} \end{vmatrix}}{\begin{vmatrix} a_{11} & a_{12} & \cdots & a_{1n} \\ a_{21} & a_{22} & \cdots & a_{2n} \\ \vdots & \vdots & & \vdots \\ a_{n1} & a_{n2} & \cdots & a_{nn} \end{vmatrix}}, \quad (14)$$

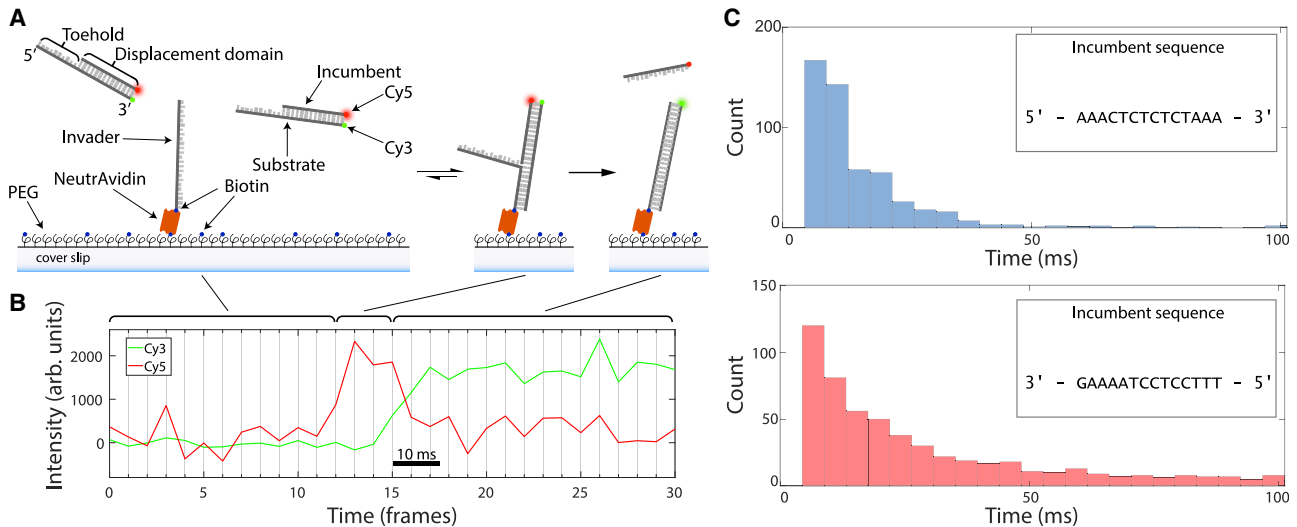
where  $a_{ij}$  represents the matrix elements of  $\mathbf{A}$ . Eq. 14 can also be expressed in terms of the bias factor  $\alpha_i = f_{i-1}/r_i$  as (32)

$$\tau = \frac{1}{f_0} + \frac{1 + \alpha_1}{\alpha_1} \cdot \frac{1}{f_1} + \frac{1 + \alpha_1 + \alpha_2 \alpha_1}{\alpha_2 \alpha_1} \cdot \frac{1}{f_2} + \cdots \quad (15)$$

## RESULTS

To focus on the unimolecular kinetics of strand displacement, we took a surface-based smFRET approach (Fig. 2). In this approach, the invader is immobilized on the glass surface of a flow chamber, and the partial duplex between the donor (Cy3)-labeled substrate and the acceptor (Cy5)-labeled incumbent are perfused into the chamber. The toehold length (10-bp) is chosen so that toehold formation is practically irreversible throughout the experiment. Upon toehold formation, a diffraction-limited spot emerges out of the diffusive background in the Cy5 channel. Upon incumbent dissociation, the spot changes fluorescence emission from the Cy5 channel to the Cy3 channel. We termed this experimental scheme “fission” because the duplex labeled with the FRET pair is split as a result of strand displacement.

Partial duplexes were constructed by annealing Cy3-labeled substrate molecules and Cy5-labeled incumbent molecules. Invader molecules were biotinylated near the end containing the toehold sequence and immobilized onto the surface (see Fig. 2 A). As shown in Figs. 2 B and S10, high-FRET signals started to appear in the field of view after partial duplexes were flowed into the chamber.



**FIGURE 2** An overview of strand displacement data acquisition. (A) Experimental scheme of fission assay. Invader strands were immobilized on a polyethylene glycol-passivated coverslip surface via a biotin-NeutrAvidin linker. Partial duplexes were labeled with a FRET pair (Cy5, incumbent; Cy3, substrate) and were drawn into the flow cell. After a diffusive search process, the partial duplex binds to the toehold, and the incumbent strand is displaced. (B) Sample acceptor and donor time traces. Acceptor (Cy5) signal increases upon toehold binding, and high-FRET signal is sustained until displacement occurs, which is indicated by a low-FRET signal. The displacement time is identified as the high-FRET lifetime. (C) Displacement time distributions for different sequences. Displacement time distributions show sequence dependence in the displacement domain (incumbent sequence shown). Displacement lifetimes are collected for many traces and assembled into a distribution. The binning size is the single frame time (4.4 ms). To see this figure in color, go online.

The average time at which spots appeared became shorter at a higher concentration of partial duplexes, and the transition of FRET from high to low only occurred in the presence of the matching displacement domain. Without the matching displacement domain, the high-FRET spots remained until they photobleached, which confirms that dissociation of the 10-bp toehold is much slower than the typical minute-long observation period. The red signal jumped to a high level in one or two frames, which suggests that toehold formation is much faster than our time resolution, and can therefore be considered instantaneous for analysis purposes. This high-level red signal lasted for variable periods of time from trace to trace, but the eventual transition back to low-FRET always occurred in one or two frames. Simultaneously with the disappearance of the signal from the Cy5 channel, a new signal appeared in the Cy3 channel, consistent with the fission scheme (Fig. 2 A). Based on these observations, the first arrival of a high-FRET spot was attributed to toehold formation, and the transition from high- to low-FRET was attributed to completion of strand displacement. Hence, the dwell time in the high-FRET state (Fig. 2 B) represents the displacement time.

By performing the fission assay multiple times, we could record hundreds of strand displacement events for one particular displacement system and build a histogram of displacement times. To investigate the sequence dependence of strand displacement kinetics, we tested eight unique strand displacement systems, each with a different sequence in the displacement domain. We obtained these histograms at the finest bin width of 4.4 ms, two of which are shown

in Fig. 2 C. Note that displacement events faster than the exposure time do not produce a clear signal in the acceptor channel, and therefore, the first bin of the histogram starts from 4.4 ms. For comparison of the histogram across all eight different sequences, we also present the histograms as a normalized heat map in Fig. 3. The salient feature of these histograms is that they decay monotonically with little or no delay. Six out of eight sequences show decay from the first bin; only two sequences show more events in the second bin than in the first bin. Nonetheless, we find a significant difference in the characteristic decay time among the tested sequences (*black dots*, Fig. 3). The fastest mean displacement time is 8 ms, whereas the slowest is 107 ms. The average over all sequences is 35 ms.

To ensure that the observed difference between different sequences is not due to the uncertainties of the histograms, we need to establish the baseline uncertainties in the empirical histograms. As explained above, each histogram is obtained by combining displacement events taken from multiple runs of the fission assay in 1 day using the same reagents and flow cell. Hence, each histogram possesses statistical uncertainty because of the finite number of events and empirical uncertainty because of fluctuations in the experimental conditions. To estimate these uncertainties, we randomly sampled 80% of the events collected on the same day and re-evaluated the mean displacement time (Fig. S11). The spread of the mean values is nonuniform among different sequences. For example, sequences 4 and 6 have a similar total mean but show different uncertainties. Nonetheless, the uncertainty in the mean for each sequence

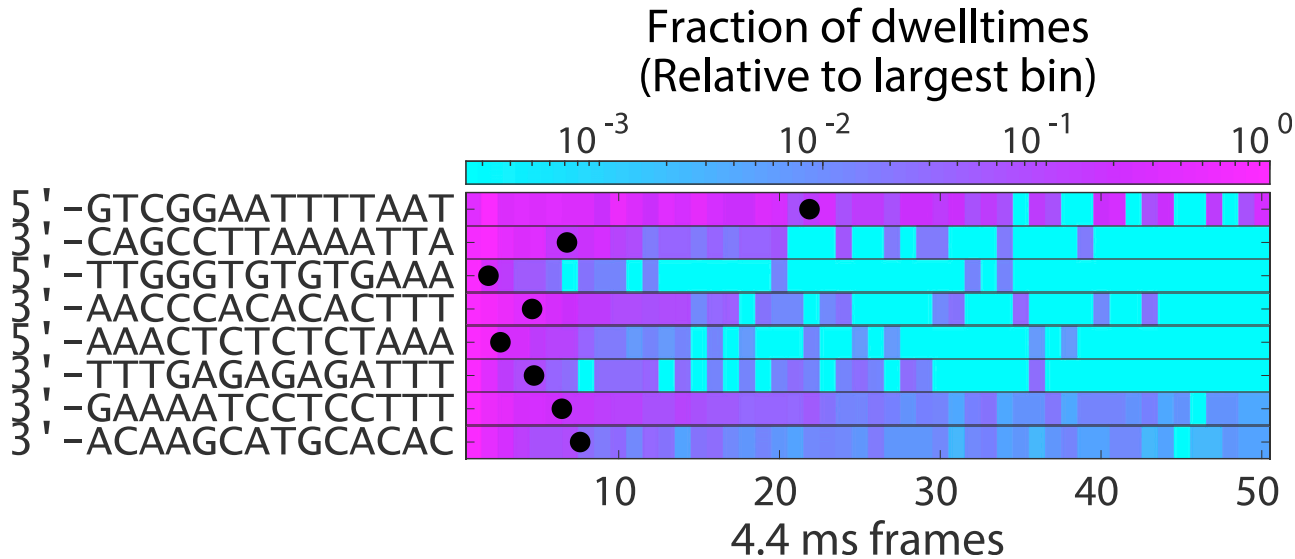


FIGURE 3 A heat map demonstrating the distribution of displacement times within the first 50 frames of a typical experiment for each of the eight featured sequences. The sequences of the incumbent strands are listed to the left. The color map is set to a logarithmic scale to better represent the exponentially decaying distributions. Here, each experiment is scaled such that the most populous bin is set to unity. The second bin is the most populous in experiments with incumbent strands 5'-GTCGGAATTTTAAT and 3'-CAGCCTTAAAATTA; in all other experiments, the first bin is the most populous. The mean of all recorded displacement times is marked by a black dot for each experiment. From top to bottom, the number of events recorded for each experiment is 273, 751, 143, 890, 522, 169, 627, and 609. To see this figure in color, go online.

is much narrower than the variation among different sequences. We also documented the variability of the histogram means obtained at different times over a 4-year span by two users (Fig. S12). This empirical variability is much higher than the statistical variability because of reasons that are not completely clear. For transparency, we present these individual mean values in Fig. 4.

In addition to the basepair sequence of the displacement domain, the base sequence of the invader can also affect the displacement kinetics. As shown in Fig. 4 A, the same displacement domain can be invaded using a toehold extended from either the 5' end or the 3' end of the displacement domain. We refer to these complementary invasions as top and bottom invasions. The mean displacement times of top and bottom invasions are clearly different for all three displacement domains we tested. No particular invasion side was consistently faster; for sequence 1, bottom invasion is faster, but for sequence 2, top invasion is. Interestingly, when RNA with an identical sequence except for T → U substitution was used as an invader in place of DNA, the faster side was switched (Fig. 5). All of these results suggest that the displacement kinetics are not completely determined by the basepair sequence or the thermodynamic stability of the displacement domain, but rather that the measured displacement kinetics are sensitive down to the chemical makeup of invading bases.

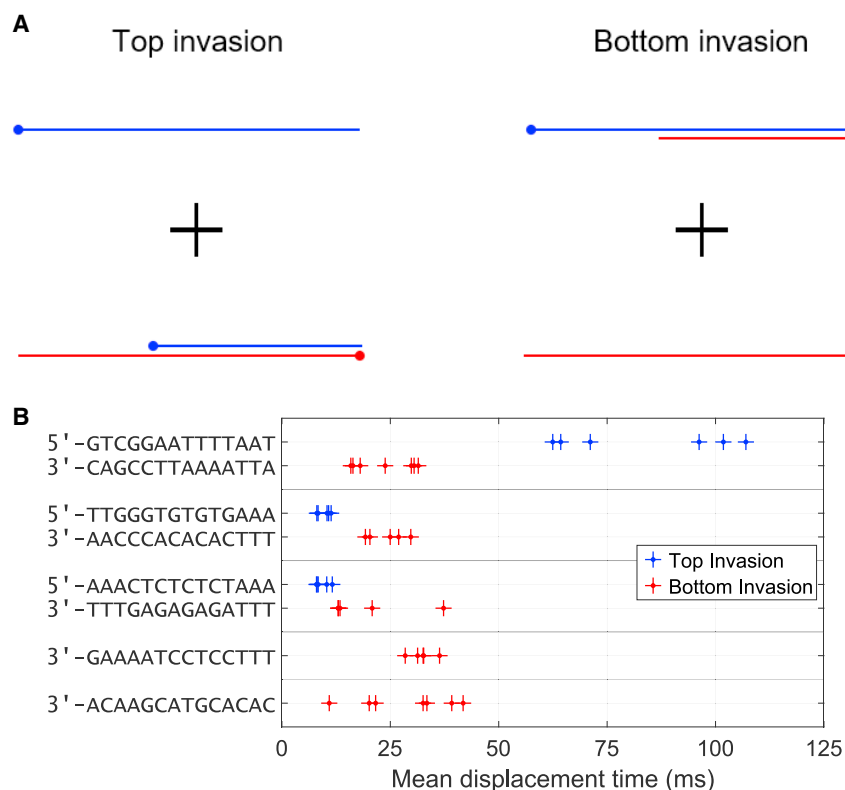
Last, we investigated the salt dependence of displacement kinetics. Monovalent salt can screen the negative charges on

the phosphate backbone and alter the thermodynamics and kinetics of basepairing (40). However, its effect on the kinetics of branch migration is less clear because branch migration involves the basepairing dynamics of two competing strands. As shown in Fig. 6, the mean displacement time shows little change from 250 mM to 1 M [NaCl].

## DISCUSSION

Using the fission assay, we measured the unimolecular branch migration kinetics in toehold-mediated DNA strand displacement. Using wide-field total internal reflection fluorescence microscopy and subregion readout of an electron-multiplying charge-coupled device camera, we were able to record thousands of strand displacement events at a 4.4 ms frame rate. Our fission assay begins in a dark field of view with unlabeled invader strands immobilized on the surface and monitors displacement events through the appearance and disappearance of the FRET signal on the surface. The experimental design permits us to use high-excitation intensity to detect fast displacement events at high signal/noise; strong excitation of fluorescent molecules begins only at the start of branch migration. Hence, the undesirable effect of photobleaching is eliminated.

Our fission assay produces data that could not be obtained to date. It separates out the bimolecular toehold formation step from the rest so that the apparent displacement time truly reflects a unimolecular process. In the language of



**FIGURE 4** Kinetics of various displacement systems. (A) Schematic of invasion designs. Top invasion is defined by an invader with a 5' toehold. In bottom invasion, all strands are replaced by their reverse complement. Bottom invasion is defined by an invader with a 3' toehold. These systems are highly related as duplex basepairing is identical in both systems. (B) Mean displacement times. The mean displacement time is calculated from data below the 95th percentile. The data collection process was entirely repeated several times for each invasion system. The mean times can change by an order of magnitude depending on the sequence. The sequences are the invader nucleotides that are beyond the toehold with the nt most proximal to the toehold written first. The top-invasion sequences therefore correspond to the conventional 5' to 3' direction, whereas the bottom invasion sequences are listed 3' to 5'. To see this figure in color, go online.

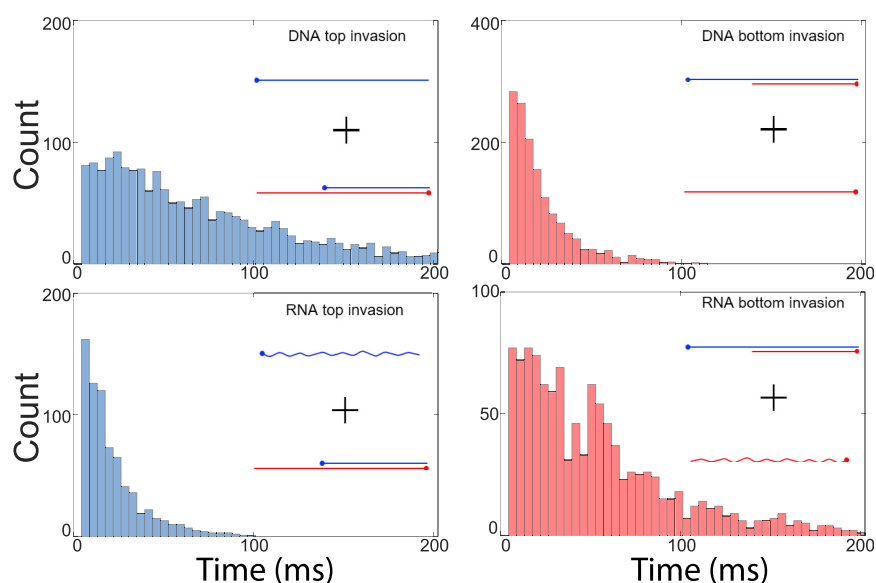


FIGURE 5 Comparison between RNA and DNA invasion. We measured displacement times for a pair of complementary DNA invaders that exhibited the largest difference between sides of invasion (*blue*, top invasion; *red*, bottom invasion) in comparison to all other pairs of invaders we observed (*top row*, straight invader). We measured displacement times for RNA versions of the invaders that were identical in sequence except for a T  $\rightarrow$  U substitution (*bottom row*, wavy invader). DNA partial duplexes were employed in all cases. Again, we found a difference in passage time depending on the side of invasion. Further, we noticed that the relative times switched with respect to the side of invasion. To see this figure in color, go online.

stochastic processes, the displacement time represents the first passage time: the time taken for the branch point to start from the first position and reach the last for the first time. The fission scheme allows access to the full distribution of individual displacement times, which is more informative than just the average values. Below, we use the first passage time analysis to extract single-step migration rates from the measured histograms and discuss potential microscopic mechanisms that may control these rates.

The most elementary model to describe DNA strand displacement is a one-dimensional random walk among

states defined by the number of displaced basepairs (Eq. 1). Displacement is initiated after the invader hybridizes to the toehold and continues until the incumbent loses all basepairs with the substrate to the invader. Any intermediate state during this process can be envisioned as two dangling strands branching off from the duplex stem (Fig. 7). At the junction or the branch point, an incumbent (invader) base can spontaneously break away from the substrate base, and the most adjacent invader (incumbent) base can base-pair with the substrate base. As a result, the branch point can move by one base in either direction. The branch point,

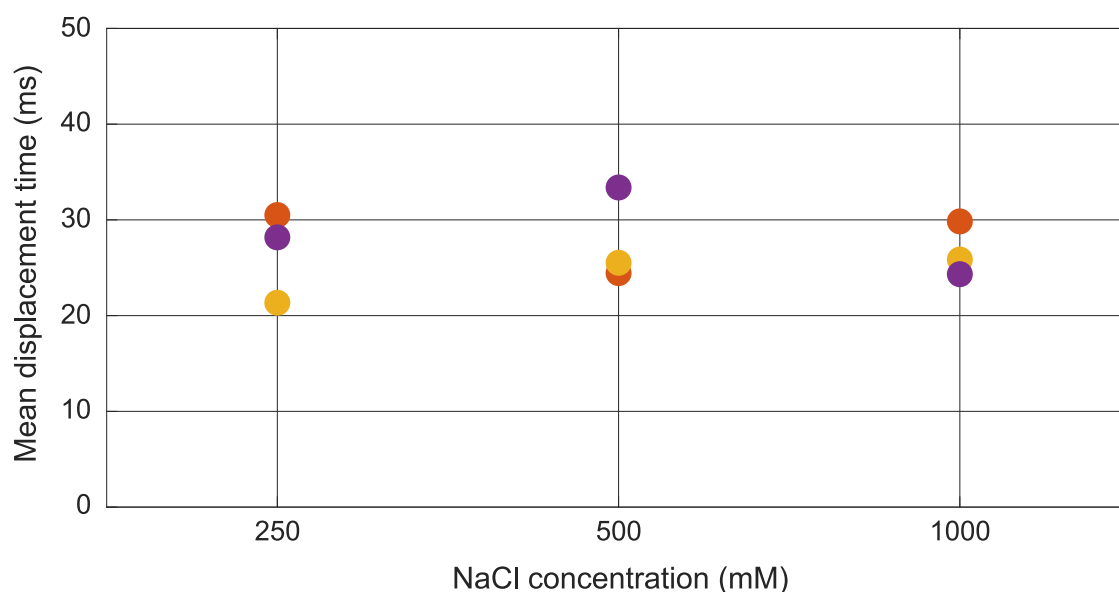


FIGURE 6 Salt dependence of mean displacement time. Data points sharing the same color were collected on the same day using the same reagents, aside from partial duplex solutions containing different concentrations of NaCl. The mean displacement time does not appear to depend strongly on the NaCl concentration. The incumbent sequence used for these measurements is 5'-ATTAAATTCCGAC-3'. To see this figure in color, go online.

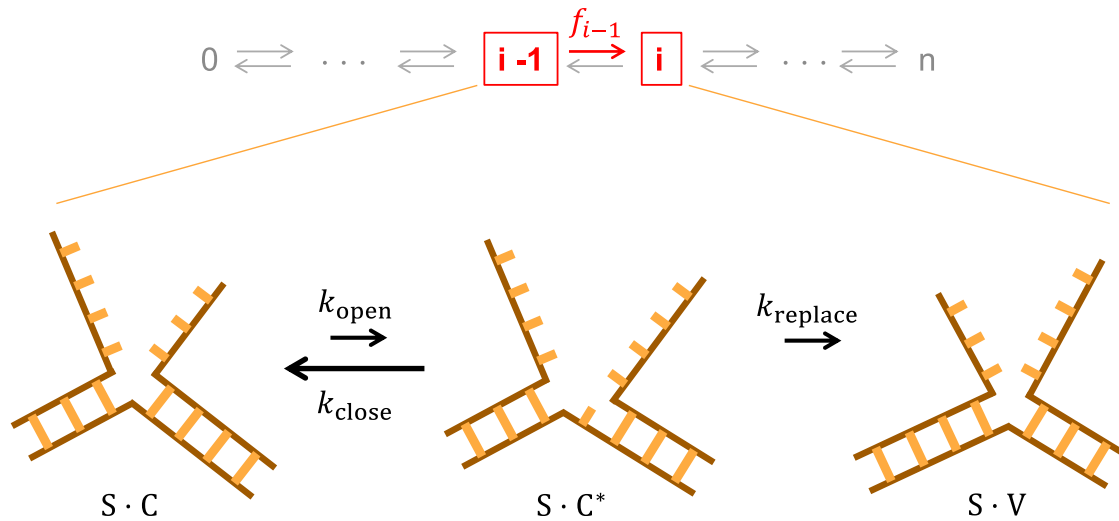


FIGURE 7 A closer look at the single basepair step transition. Any forward arrow in the one-dimensional Markov chain involves opening of a basepair between the substrate (S) and the incumbent (C) and closing of a basepair between the substrate and the invader (V). For a reverse arrow, the roles of incumbent and invading bases are simply flipped. Hence, each step can be modeled as a first passage from an initial state  $S \cdot C$  to a final state  $S \cdot V$  through an intermediate state  $S \cdot C^*$  in which a single basepair is transiently open. The transiently unbound incumbent base can rebound the substrate base at a rate of  $k_{\text{close}}$  or be replaced with the invading base at a rate of  $k_{\text{replace}}$ . To see this figure in color, go online.

however, cannot recede into the toehold region because the incumbent is shorter than the substrate. Therefore, branch migration can be modeled as a one-dimensional random walk with single base steps from a reflecting boundary on one end (state 0) to an absorbing boundary on the other (state  $n$ ).

It is straightforward to derive the first passage time statistics from a Markov chain like Eq. 1. The simplest model is a uniform random walk in which all transition rates are equal ( $\{f_i, r_i\} = k$ ). Such a model can be represented by a free energy landscape shown in Fig. 8 with troughs separated by equal height barriers. Based on Eq. 15, the mean first passage time ( $\tau$ ) is given by

$$\tau = \frac{1}{2k} n(n+1). \quad (16)$$

Using Eq. 16,  $n = 14$ , and the measured mean first passage time of 30 ms, we can estimate the single-step migration time ( $k^{-1}$ ) to be  $\sim 286 \mu\text{s}$ . This estimate is also consistent with the measured histogram of displacement times. If single-step migration occurs more slowly than the time resolution, the histogram of displacement times must exhibit a strong delay or lag in the early times (Fig. S13). However, our measured histograms at 4.4 ms bin width show little or no lag, which points to a single-step migration time much shorter than 4.4 ms.

However, this estimated time of  $\sim 286 \mu\text{s}$  per step is likely to be longer than the true value because displacement events faster than the  $\sim 4$ -ms exposure time are not included in our measurement. To extract the single-step migration rates in a more accurate, unbiased way despite this missing fraction of

events, we fit the analytical solution Eq. 5 to all eight histograms with four shared parameters representing rates for A, G, C, and T. This global fitting procedure looks for the best set of rates that describe all eight histograms in the least-squares sense, excluding the missing first bin. It also implies a nonuniform symmetric random walk (Fig. 8) in which the single-step migration rate depends only on the identity of the base to be displaced. Therefore, each step has the same forward and reverse rates ( $f_{i-1} = r_i$ ). The extracted step times for A, C, and T bases are  $\sim 33$ ,  $\sim 200$ , and  $\sim 250 \mu\text{s}$ , respectively. The step time for the G base did not converge, but the goodness of fit increased with faster values. Thus, we estimate the step time for G to be  $\leq 33 \mu\text{s}$ . As predicted, these times obtained by fitting histograms in their entirety are all faster than  $\sim 286 \mu\text{s}$  obtained from the mean values that omit fast events.

Our estimated single-step migration rates ( $0.003 \mu\text{s}^{-1}$  to  $0.03 \mu\text{s}^{-1}$ ) appear to be much slower than the rate of basepair fraying or base flipping ( $\geq 1 \mu\text{s}^{-1}$ ) (41–44). Similarly, a previous study by Srivinas et al. (29) also inferred the single-step migration rate to be much slower than the fraying rate. This discrepancy suggests that a single basepair opening event does not always lead to single-step branch migration. As shown in Fig. 7, a basepair between the substrate and the incumbent can transiently open and close with rate constants of  $k_{\text{open}}$  and  $k_{\text{close}}$ , respectively. When the substrate base is transiently unbound ( $S \cdot C^*$ ), the invading base can basepair with the substrate base and replace the incumbent base at a rate of  $k_{\text{replace}}$ . We can safely assume that  $k_{\text{close}}$  is much faster than  $k_{\text{open}}$  based on the known basepair stability (45). Coarse-grained molecular dynamics simulations (29) show that the branch migration intermediate frequently

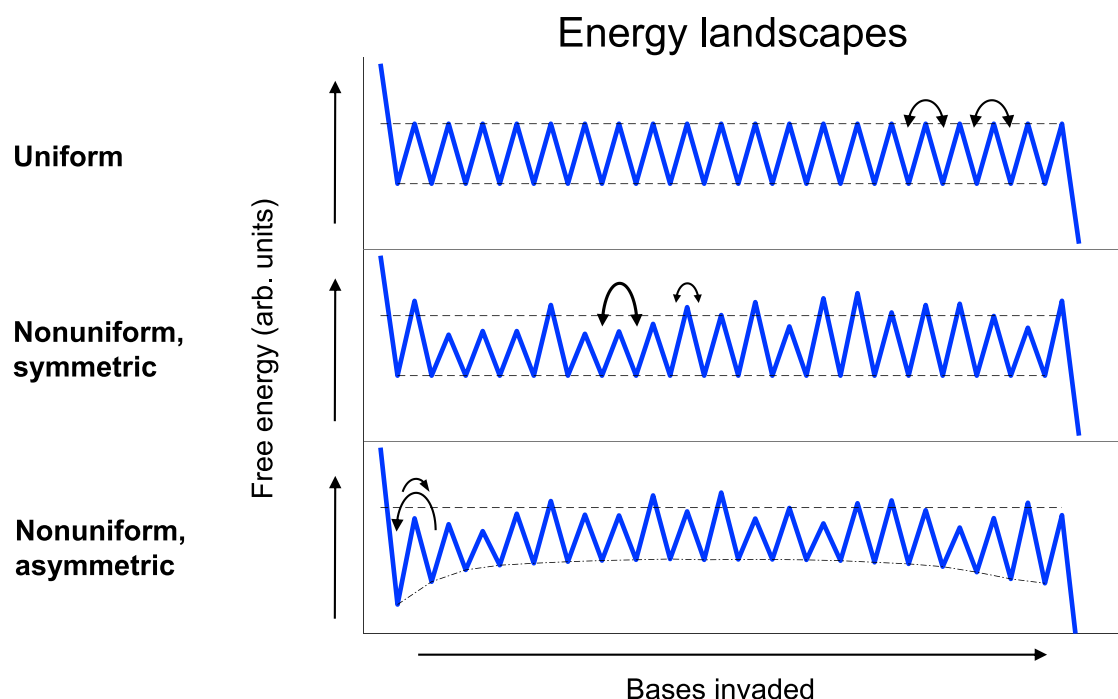


FIGURE 8 Free energy landscapes. All landscapes contain a reflecting boundary to the left and an absorbing boundary to the right. The uniform landscape is characterized by one uniform rate for transitions in any direction. The nonuniform, symmetric landscape allows for variation in rates so long as the forward rate from one state to another is set equal to the corresponding reverse rate. The nonuniform, asymmetric landscape additionally allows forward and reverse rates to differ. The relative free energies of the states in the nonuniform, asymmetric model are drawn from Srinivas et al. (29). To see this figure in color, go online.

adopts a coaxially unstacked state in which a transiently open incumbent base would be closer to the substrate base than the invading base ( $S \cdot C^*$ , Fig. 7). Therefore, we reason that  $k_{\text{close}}$  is also much faster than  $k_{\text{replace}}$ . Given  $k_{\text{close}} \gg k_{\text{open}}, k_{\text{replace}}$ ,  $S \cdot V$  will appear at the rate of

$$k \approx \frac{1}{[S \cdot V]} \frac{d[S \cdot V]}{dt} \approx \frac{k_{\text{open}}}{k_{\text{close}}} \cdot k_{\text{replace}} \ll k_{\text{open}}. \quad (17)$$

Hence, the single-step migration rate  $k$  is expected to be much slower than the single basepair opening rate.

We stress that a symmetric random walk is an oversimplification of strand displacement. As shown in Fig. S14, the symmetric random walk model significantly underrepresents the range of observed displacement times; the fastest observed histogram and the slowest observed histogram are outside the range represented by the fitted curves. Therefore, the observed sequence dependence calls for a more complicated model. We list below several microscopic mechanisms that indicate strand displacement is more properly described as an asymmetric random walk ( $f_{i-1} \neq r_i$ ).

First, displacement of the first basepair is energetically less favorable than the rest because it creates steric exclusion between dangling bases (29,46). Srinivas et al. (29) measured the thermodynamic penalty for the steric exclusion to be 2.0 kcal/mol at 25°C, which corresponds to  $\sim 30$ -fold slower  $f_0$  than all other rates ( $k$ ). According to

Eq. 15, a bias in the first step ( $\alpha_1 = f_0/r_1$ ) alters the mean first passage time to

$$\tau = \frac{1}{2k} n \left( n + \frac{2}{\alpha_1} - 1 \right). \quad (18)$$

With a strong reverse bias ( $\alpha_1 = 1/30$ ) in the first step, the single-step time ( $k^{-1}$ ) is estimated to be 69  $\mu\text{s}$ , faster than our previous estimate of 286  $\mu\text{s}$  based on a completely symmetric random walk. 69  $\mu\text{s}$  per step also falls well within the range (53–103  $\mu\text{s}$ ) inferred by Srinivas et al. (29). Second, the stability of a basepair is highly influenced by its nearest neighboring basepair, which would render the basepair opening rate direction dependent. For example, let us consider an A base in two adjacent branch migration intermediates  $G^{\vee}AC$  and  $GA^{\vee}C$ , where “ $\vee$ ” refers to the branch point. In  $G^{\vee}AC$ , A is stacked more closely on C, whereas in  $GA^{\vee}C$ , A is stacked more closely on G. Therefore, the rate of A flipping out would be different between forward and reverse transitions. Third, the incumbent and the invader base at the branch point carry dangling strands of variable lengths depending on the state. These dangling strands will inevitably affect the diffusion rates of the bases at the branch point. To demonstrate this idea, we performed the fission assay with an invader extended by five nucleotides at the 3' end. As shown in Fig. 9, the displacement kinetics become significantly slower even with the same

displacement domain. This result is consistent with the idea that a base with a longer dangling strand invades more slowly. As strand displacement progresses, the dangling part of the invader becomes shorter, and the dangling part of the incumbent becomes longer. Hence, the forward rate should become faster ( $f_{i-1} < f_i$ ) and the reverse rate slower ( $r_{i-1} > r_i$ ). These dangling strand-dependent rates produce asymmetric barriers in the free energy landscape, causing the basins to follow a concave curve (Figs. 8 and S15). Previous oxDNA simulations also predicted a concave free energy landscape (29,46). In the asymmetric random walk model, single-step rates are not only base dependent but also position dependent. Determining these rates would require measurements at a much larger scale, which is beyond the scope of this study.

We assumed that the number of steps is equal to the number of basepairs for modeling purposes. This could raise concern that this number may not accurately reflect the number of actual branch migration steps taken because the incumbent can spontaneously dissociate near the end of migration. In our previous work (32), we estimated spontaneous dissociation of a 2-bp incumbent to be  $\sim 10 \mu\text{s}$ , which would be comparable to the branch migration step rate we measured in this study. This means that the last few steps can occur either via branch migration or spontaneous dissociation, and the rate would be dominated by the faster of the two. Regardless, our proposed asymmetric branch migration model, in which forward rates become faster and reverse rates become slower, would effectively account for such an effect.

We made an interesting observation that RNA invasion and DNA invasion occur at very different rates even with the same invader sequence (except for T to U substitution). For the one sequence we tested, RNA invaded faster than

DNA (Fig. 5) from one side but more slowly from the other side. Several factors may contribute to this finding. Structurally, an RNA-DNA hybrid duplex adopts an A-form helix (47–49), whereas a DNA-DNA duplex adopts a B-form helix. The thermodynamic stability difference between RNA-DNA and DNA-DNA duplexes depends on the sequence (50), with purine (AG)-rich substrate favoring RNA-DNA hybrid duplexes (51). Directional differences in stacking between DNA and RNA are known to persist even in the single-stranded form (52) and could contribute to this inversion of side dependence. In a similar vein, a recent study shows that coaxial stacking between an RNA-DNA hybrid duplex and a DNA-DNA homoduplex is stronger when the interhelical junction contains a 5' RNA end than when it contains a 3' RNA end (53). This effect may partially contribute to the faster top invasion by RNA shown in Fig. 5. However, another RNA sequence we tested exhibited faster bottom invasion than top invasion, suggesting that the base sequence is a stronger determinant of the displacement rate than the invasion polarity. RNA invasion of a DNA duplex in particular is a fundamental feature of the CRISPR-Cas system (10,54,55). R-loop formation appears to be the rate-limiting step for DNA cleavage (56,57) and is highly sequence dependent (56,58,59) but proceeds much more slowly ( $\sim 1$  s) than the spontaneous displacement rate we measured in this study. It will be thus interesting to investigate whether the sequence dependence is preserved between spontaneous and enzyme-mediated displacement reactions in the future.

The lack of salt dependence of the measured displacement kinetics was at first surprising to us because salt has a substantial effect on basepairing thermodynamics (60). Experimental measurements of salt-dependent opening and closing rates of a single basepair are scarce, but we can still infer their salt dependence from molecular

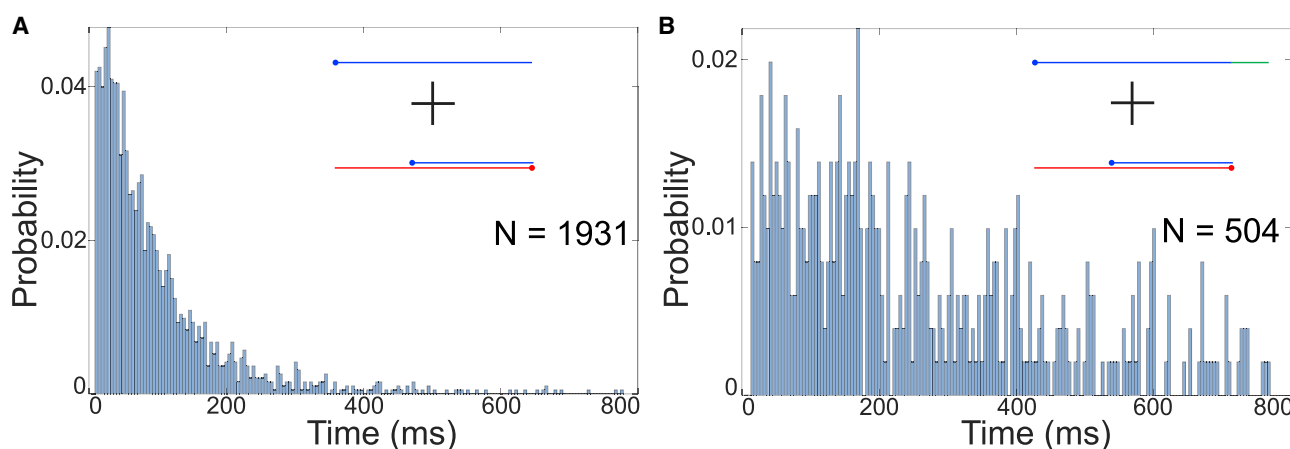


FIGURE 9 An overhang on the invader strand increases displacement times. (A) A top-invasion experiment with incumbent sequence 5'-GTCGGAATTT-TAAT-3'. The distribution of displacement times forms an exponential decay with little lag. After invasion has completed, every base on the invader strand is bound to the substrate. (B) An experiment conducted with a modified invader possessing a 5T overhang on the toehold-distal end (green). These additional bases affect the kinetics of displacement but should not bind to the substrate. The invader overhang increases overall displacement times and introduces a clear lag to the distribution. To see this figure in color, go online.

dynamics study (61) and hybridization and dissociation measurements of short oligos (62,63). These studies show that monovalent cations stabilize basepairing mainly by increasing the rate of basepair closing instead of decreasing the rate of basepair opening. Despite the strong salt dependence of basepair closing ( $k_{\text{close}}$  and  $k_{\text{replace}}$ ), our proposed three-state model for branch migration (Eq. 17; Fig. 7) predicts that salt dependences of  $k_{\text{close}}$  and  $k_{\text{replace}}$  will cancel each other out and render step migration rates,  $f$  and  $r$ , largely salt independent.

Even in the case in which  $f$ s and  $r$ s all carry a weak salt dependence through  $k_{\text{open}}$ , we can show that the overall salt dependence of the mean displacement time remains weak. Based on an experimental study (63), we assume a simple power law dependence of  $k_{\text{open}}$  on  $[\text{Na}^+]$  ( $k_{\text{open}} \sim [\text{Na}^+]^\alpha$ ) so that all  $f$ s and  $r$ s change by the same factor  $c$  upon changing  $[\text{Na}^+]$ . According to Eq. 14, the mean first passage time is equal to the ratio of two matrix determinants:

$$\tau = \frac{\det(\mathbf{A}')}{\det(\mathbf{A})}. \quad (19)$$

Because  $\det(\mathbf{A}') \sim c^{n-1}$  and  $\det(\mathbf{A}) \sim c^n$ ,  $\tau \sim c^{-1}$ . Hence, the overall displacement of the  $n$  basepair domain follows the weak salt dependence of  $k_{\text{open}}$ . Either way, we are able to rationalize the weak salt dependence of the mean displacement time (Fig. 6).

We hope that our results will be beneficial to the field of DNA nanotechnology. Our work has provided sequence-specific branch migration step times that could be used to rationally design sequences with desired kinetics. For example, our results could aid in the design of complex interaction networks between competing reactions with specifically tuned kinetics. Further, our fission assay opens the door to understanding branch migration kinetics in more reaction conditions than we studied here (e.g., buffers, pH, and temperature).

In this study, we assumed that strand displacement proceeds through one-dimensional branch migration, but it is possible that other mechanisms are at play. The invader might invade through the end distal to the toehold when terminal basepairs fray or through internal basepairs that spontaneously open up. Although internal invasion is highly unlikely for the short displacement domain we used here, it would be more probable for longer displacement domains. We also cannot rule out direct swapping between segments of invader and incumbent (64), invasion through triplex formation (65,66), or concurrent dissociation of a weakly bound incumbent strand (31,32). All these processes can occur in parallel, which makes it difficult to predict the strand displacement rate for any given sequence. In this regard, a future study on a much larger set of displacement domain sequences would help us to attain more accurate

phenomenological models for explaining the sequence dependence of strand displacement kinetics.

## CONCLUSIONS

We developed a novel smFRET assay that we call fission to study the timing of the unimolecular reaction that occurs during toehold-mediated strand displacement. Our fission assay separates the timescales between the slower toehold formation step and the faster displacement step and enabled us to tally displacement first passage times distributions for 11 separate invasion schemes. We found nontrivial sequence dependence in the distributions, whereas the mean first passage times varied by an order of magnitude. Further, we highlighted significant differences between the “side” of invasion, which suggest the kinetics are not completely determined by basepair sequence alone. Curiously, we showed that DNA and RNA invaders can behave drastically differently despite having identical sequences (apart from a  $T \rightarrow U$  substitution). Finally, we demonstrated that displacement times were relatively unchanged over a wide range of salt concentrations. Motivated by these results, we developed a one-dimensional random walk model described above to estimate single base displacement times. This model is widely relevant to multistep processes, and we anticipate our analysis to be highly important to an array of biological reactions.

## APPENDIX: ASYMMETRIC RANDOM WALK MODEL

We attempt to expand the random walk model to explain the 3.3-fold slower mean strand displacement time with the longer invader. This result suggests that the forward transition barrier would decrease with position as the overhang length of the invader becomes shorter. Likewise, the reverse transition barrier should increase with position. Although the exact position dependence of the barrier height is not known, we assume a simple analytical form (Hill equation or exponential function) for forward and reverse barrier heights (Eqs. SS.1 and SS.2).

$$\Delta G_f^*(i) = \frac{8.5RT}{1 + i/K} \quad (\text{S.1})$$

$$\Delta G_r^*(i) = \frac{8.5RT}{1 + (14 - i)/K}. \quad (\text{S.2})$$

Here,  $i$  represents the position along the energy landscape ( $i \in \{0, 1, \dots, 14\}$ ), the numerator comes from a previous estimate (29), and the factor  $K$  in the denominator determines the concavity of the landscape. For example, by substituting  $K \approx 240$ , we obtain a concave energy landscape as shown in Fig. S15, whose midpoint (position 7) of the landscape is about 1 kcal/mol higher than the end states (position 0 or 14). This concavity is similar to that seen in the landscape constructed from simulations (29). If the invader is lengthened by five extra bases, all forward barriers become higher:

$$\Delta G_f^*(i) = \frac{8.5RT}{1 + (i - 5)/K}, \quad (\text{S.3})$$

whereas the reverse barriers are unchanged (Eq. S5.3). Hence, the energy landscape becomes tilted (*dotted line*, Fig. S6), and the corresponding mean first passage time is predicted to be slower by 3.3-fold based on Eq. 14 of the main text. This crude exercise shows that our observed overhang effect is semiquantitatively consistent with a simple asymmetric random walk model.

## SUPPORTING MATERIAL

Supporting material can be found online at <https://doi.org/10.1016/j.bpj.2021.01.043>.

## AUTHOR CONTRIBUTIONS

D.W.B.B. and H.D.K. designed research. D.W.B.B. and A.W.C. performed experiments. D.W.B.B., A.W.C., and H.D.K. contributed analytical tools. D.W.B.B. and A.W.C. analyzed data. D.W.B.B., A.W.C., and H.D.K. wrote the manuscript.

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