

# Getting swept off your toe(hold)s: Single-molecule DNA fission analysis offers glimpse into kinetics of branch migration

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The predictable, straightforward nature of basepairing and the well-understood physical properties of the double helix make nucleic acids (DNA and RNA) versatile tools for assembly of complex nanostructures as well as nonequilibrium nanodevices. By varying sequences of building blocks, one can assemble virtually any imaginable geometric shape and pattern. At physiological salt concentrations and temperatures, double helices of B-form DNA and A-form RNA are inherently thermodynamically stable. When, however, a partial duplex is mixed with an invading single strand complementary to the longer strand in the duplex, an exchange of the strands can take place, thus allowing construction of active devices where nucleic acids are used as both structural materials and as a fuel (1). Such strand displacement reactions involving complementary or partially complementary sequences are referred to as “toehold-mediated” strand displacement, as the reaction is initiated by pairing of the invading strand to a short single-stranded overhang region (a “toehold”) on the substrate strand. The toehold pairing is then followed

by eviction of the existing complement to the substrate, known as the “incumbent” strand (Fig. 1). Strand displacement is a cornerstone of many nanotechnology applications including self-assembly of complex nanostructures, nanoscale circuits, autonomous walkers, tunable nanodevices, diagnostics for the presence of genetic mutations and polymorphisms, and programmable switches in synthetic biology applications (see (3) for a recent review). In nature, strand displacement, and branch migration in general, is a feature common to many processes of DNA and RNA metabolism such as formation of D-loops during homologous genetic recombination, rearrangements of broken DNA replication forks, branch migration of Holliday junctions, formation and processing of R-loops in RNA transcription, and sequence recognition by the CRISPR-Cas system, among many others.

In toehold-mediated strand displacement reactions, the toehold formation is a rate-limiting step. It is also the best-understood step, as one can easily control the reaction rate by changing the toehold length (usually between two and eight nucleotides), toehold sequence, and buffer conditions. Increasing the toehold length generally speeds up the reaction by increasing the association rate and also drives the reaction forward by making the

products more thermodynamically favorable than the substrates. One can also control the toehold-mediated strand displacement by deliberately “hiding” the toehold domain. This can be achieved by hybridization of the dangling toehold with a removable oligonucleotide, positioning it within a hairpin or triplex forming structure, or by nucleobase caging (reviewed in (3)). The overall reaction rate displays an exponential dependence on the toehold length suggesting that the initial pairing is the rate-limiting step (4), and the overall toehold-mediated strand displacement reaction is commonly modeled as a bimolecular association. Further, a three-step model has been used to quantitatively predict strand displacement kinetics from thermodynamics of DNA hybridization (5). This model takes into account two parameters, which are the rate constants for DNA hybridization and for branch migration, and works reasonably well for sequences devoid of the potential to form secondary structures (5). The proposed energy landscapes for the toehold-mediated strand displacement assume that there is a slight penalty to initiate branch migration ( $\sim 2$  kcal/mol at 25°C) (6), after which branch migration proceeds in single basepair steps by a random one-dimensional walk (Fig. 1; (6,7)). Basepair mismatches between the invading and the target strands

Submitted March 5, 2021, and accepted for publication April 16, 2021.

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Editor: Jason Kahn.

<https://doi.org/10.1016/j.bpj.2021.04.014>

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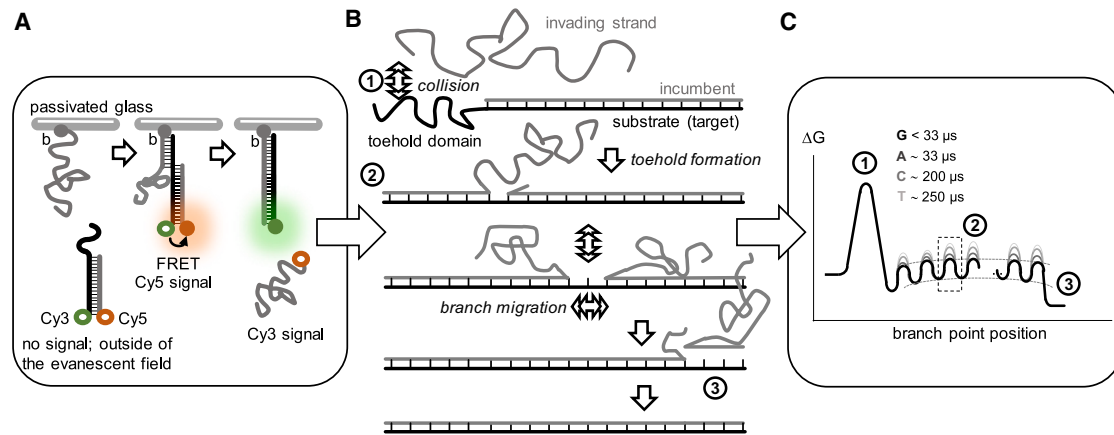


FIGURE 1 Schematic of the toehold-mediated strand displacement reaction. (A) Experimental scheme for the single-molecule fission experiment. The biotinylated invading strand (gray line) is surface tethered through a neutravidin-biotin (gray circle) interaction. Formation of the toehold brings the Cy3/Cy5-labeled duplex into the evanescent field, which is observed as an appearance of the Cy5 signal. Competition of the strand displacement reaction releases the Cy5-labeled incumbent strand, resulting in an appearance of the Cy3 signal. Time between appearances of the Cy5 and Cy3 signals is designated as the strand displacement first-passage time. (B) There are three steps in the reaction: 1) toehold formation, 2) branch migration via one-dimensional random walk, and 3) dissociation of incumbent strand. (C) The work by Broadwater and colleagues (2) provided an important update to the free energy landscape of the strand displacement by quantifying the exchange rates for different nucleotides, suggesting that the barriers for each individual step depend on the nucleotide being replaced. To see this figure in color, go online.

introduce an energetic penalty, which slows down branch migration by enhancing the backstepping rate at the mismatch position (6,8,9). It has been well appreciated that strategic placement of the mismatch in the invading or incumbent strand can alter the kinetics of the reaction without affecting its thermodynamics. It is not known, however, how sequence influences the kinetics of branch migration.

A number of studies used biophysical analyses and Markov chain modeling to develop thermodynamic and kinetic models for the toehold-mediated DNA strand displacement reaction. In this issue of *Biophysical Journal*, Broadwater and colleagues (2) applied single-molecule Förster resonance energy transfer (smFRET) to further improve these models. To directly measure the strand displacement kinetics and to evaluate its sequence dependence, Broadwater and colleagues applied an elegant DNA “fission” assay, in which the biotinylated “invader” strands are tethered to the surface of the total internal fluorescence microscopy flow cell. Partial duplex molecules containing Cy3 (FRET donor)-labeled “target” and Cy5 (FRET acceptor)-

labeled “incumbent” strands are freely diffusing in solution (Fig. 1 A). Neither the invader strand nor the partial duplex are visible until the toehold hybridizes with the complementary sequence on the invading strand. Formation of the toehold brings the partial duplex within the evanescent field, resulting in Cy3 direct excitation, as well as Cy5 excitation via FRET from Cy3. Because of dyes’ proximity and corresponding high FRET, the Cy3 dye is very dim, and the toehold formation is observed as an appearance of the fluorescence signal in the Cy5 channel. The Cy5 fluorescence persists in the location on the slide where the invader strand is tethered until strand displacement is completed. Dissociation of the incumbent strand results in disappearance of the Cy5 fluorescence and concomitant increase in the Cy3 fluorescence. Clearly defined steps of toehold formation (start of the Cy5 signal) and incumbent dissociation (stepwise increase in the Cy3 signal) bracket the beginning and the end of the reaction cycle, allowing the authors to measure the strand displacement first-passage time. Although an impressive 4.4 ms time resolution is still too long to observe the individual

steps of branch migration, it was sufficient to allow the authors to directly measure the strand displacement first-passage time for eight different 14-basepair sequences. Notably, the invasion by the DNA and RNA strands with identical sequences occurred at different rates. Also unexpectedly, no dependence on the salt concentration was observed. Fitting the passage time distribution data from hundreds of strand displacement events and applying a nonuniform symmetric random mechanism to model the strand displacement steps yielded a quite unexpected sequence dependence of branch migration kinetics. The fastest rate for the base to be replaced (not more than 33  $\mu$ s per step) was determined for Gs, whereas replacement rates of ~33, 200, and 250  $\mu$ s were determined for A, C, and T bases, respectively, which are all slower than the basepair fraying rate. The basepair at the branch point may open and close multiple times or remain open for an extended period of time before the branch migration step results in a new pairing between the substrate and incumbent strands.

This work represents an important step toward the development of a

theoretical framework and methodologies to understand DNA and RNA strand displacement phenomena. Further studies will be needed to determine the exact basis of the observed differences between displacement of different basepairs and whether there is a more complicated sequence dependence. This will likely require systematic analysis of more DNA sequences and substrate lengths. Other important questions to address are how to factor in the entropy of the invading and displaced strands and whether the geometry, rigidity, and flexibility of complex nanostructures affect the exchange rates. In nanostructures with topological constraints, as well as in many biological systems, the displacement kinetics may be further complicated by whether a more stable plectonemic or a less stable paranemic joint is formed. High temporal resolution of smFRET analyses can prove instrumental in addressing these challenges. A similar type of smFRET-based assay has been previously used to monitor a

DNA strand exchange reaction by bacterial recombinase RecA, yielding a wealth of information on the mechanism of the protein-mediated strand displacement reaction (10). These studies, along with the analysis of CRISPR-mediated R-loop formation, may need to be revisited with an eye on sequence dependence.

## ACKNOWLEDGMENTS

Work in the Spies lab is financially supported by the National Institutes of Health (NIH) (NIH/NIGMS R35GM131704, NIH/NCI R01 CA23 2425), DOD/CDMRP (BC180227P1), and National Science Foundation (1836351 EAGER).

## REFERENCES

1. Yurke, B., A. J. Turberfield, ..., J. L. Neumann. 2000. A DNA-fuelled molecular machine made of DNA. *Nature*. 406:605–608.
2. Broadwater, D. W. B., Jr., A. W. Cook, and H. D. Kim. 2021. First passage time study of DNA strand displacement. *Biophys. J.* 120:2400–2412.
3. Guo, Y., B. Wei, ..., H. Liang. 2017. Recent advances in molecular machines based on toehold-mediated strand displacement reaction. *Quant. Biol.* 5:25–41.
4. Yurke, B., and A. P. Mills. 2003. Using DNA to power nanostructures. *Genet. Program. Evolvable Mach.* 4:111–122.
5. Zhang, D. Y., and E. Winfree. 2009. Control of DNA strand displacement kinetics using toehold exchange. *J. Am. Chem. Soc.* 131:17303–17314.
6. Irmisch, P., T. E. Ouldridge, and R. Seidel. 2020. Modeling DNA-strand displacement reactions in the presence of base-pair mismatches. *J. Am. Chem. Soc.* 142:11451–11463.
7. Srinivas, N., T. E. Ouldridge, ..., E. Winfree. 2013. On the biophysics and kinetics of toehold-mediated DNA strand displacement. *Nucleic Acids Res.* 41:10641–10658.
8. Broadwater, D. W. B., Jr., and H. D. Kim. 2016. The effect of basepair mismatch on DNA strand displacement. *Biophys. J.* 110:1476–1484.
9. Haley, N. E. C., T. E. Ouldridge, ..., A. J. Turberfield. 2020. Design of hidden thermodynamic driving for non-equilibrium systems via mismatch elimination during DNA strand displacement. *Nat. Commun.* 11:2562.
10. Rangunathan, K., C. Joo, and T. Ha. 2011. Real-time observation of strand exchange reaction with high spatiotemporal resolution. *Structure*. 19:1064–1073.