Control of DNA Strand Displacement Kinetics using Toehold Exchange
Supporting Materials

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S1. Quasi-steady state (QSS) derivation of the BM rate constant

Validity of QSS

Many systems of chemical reactions obey QSSA in all but the initial moments of the reaction [1]. QSSA treats the rates of change of the intermediates' concentrations ($\frac{d[\mathrm{I}]}{dt}$ and $\frac{d[\mathrm{J}]}{dt}$ in our system) as small enough to be approximated as 0. The validity of QSSA is ensured when the timescale of the overall reaction is slower than the timescale at which I and J reach their quasi-steady state values. For all experiments presented in this paper except for those in Fig. 7, the timescale of the overall reaction is at least 15 minutes, while the timescale of intermediate equilibration is estimated to be on the order of 20 seconds:

The value of parameter k_b was fitted to be 1.0 s^{-1}, so I and J equilibrate with each other on a time scale faster than 1 s. The time constant τ of the initial rise of [I] from 0 to its quasi-steady state value $[\mathrm{I}]_{qss}$ is estimated by $\tau \approx \frac{[\mathrm{I}]_{qss}}{k_f[\mathrm{X}(m,n)][\mathrm{S}]}$. In time τ, the concentration of [I] rises to $[\mathrm{I}]_{qss}(1-\frac{1}{e}) \approx 0.6[\mathrm{I}]_{qss}$.

For convenience, define $x = k_{r(\beta^m)}$ and $y = k_{r(\gamma^n)}$. In equation (7) in the next section, the expression for $[\mathrm{I}]_{qss}$ is seen to be $\frac{k_f(k_b+k_{r(\beta^m)})[\mathrm{X}(m,n)][\mathrm{S}]}{k_{r(\gamma^n)}k_{r(\beta^m)}+k_{r(\gamma^n)}k_b+k_{r(\beta^m)}k_b}$.

$$\begin{aligned}
\tau &\approx \frac{[\mathrm{I}]_{qss}}{k_f[\mathrm{X}(m,n)][\mathrm{S}]} \\
&= \frac{k_f(k_b+x)[\mathrm{X}(m,n)][\mathrm{S}]}{xy+xk_b+yk_b} \cdot \frac{1}{k_f[\mathrm{X}(m,n)][\mathrm{S}]} \\
&= \frac{k_b+x}{xy+xk_b+yk_b}
\end{aligned}$$

If $x > k_b$, $\tau = \frac{k_b+x}{xy+xk_b+yk_b} < \frac{2x}{xy+xk_b+yk_b} < \frac{2x}{xk_b} = \frac{2}{k_b}$. Since the value of k_b was numerically fitted to be 1.0 s^{-1}, this corresponds to a time constant of less than 2 seconds.

If $x < k_b$, $\tau = \frac{k_b+x}{xy+xk_b+yk_b} < \frac{2k_b}{xy+xk_b+yk_b} < \frac{2k_b}{xk_b} = \frac{2}{x}$. From our calculated binding energy of $\beta^7 = -10.17$ kcal/mol and $k_f \approx 3 \cdot 10^6$ M^{-1} s^{-1}, $x \equiv k_{r(\beta^7)} \approx 0.106$ s^{-1}, and the time scale is less than $\frac{2}{0.106} \approx 20$ s.

Thus, QSS is valid for the conditions presented in this paper as well as in similar laboratory circumstances.

Derivation of the BM rate constant

$$\mathrm{X}(m,n)+\mathrm{S} \underset{k_{r(\gamma^n)}}{\overset{k_f}{\rightleftharpoons}} \mathrm{I} \underset{k_b}{\overset{k_b}{\rightleftharpoons}} \mathrm{J} \underset{k_f}{\overset{k_{r(\beta^m)}}{\rightleftharpoons}} \mathrm{Y}+\mathrm{L}(m,n)$$

We aim to use the three-step model of toehold exchange to derive forward and reverse BM rate constants:

$$\mathrm{X}(m,n)+\mathrm{S} \underset{k_{(\gamma^n,\beta_m,\beta^m)}}{\overset{k_{(\beta^m,\beta_m,\gamma^n)}}{\rightleftharpoons}} \mathrm{Y}+\mathrm{L}(m,n)$$

$$\frac{d[\mathrm{Y}]}{dt} = \frac{d[\mathrm{L}(m,n)]}{dt} = k_{(\beta^m,\beta_m,\gamma^n)}[\mathrm{X}(m,n)][\mathrm{S}] \quad (1)$$

$$\frac{d[\mathrm{X}(m,n)]}{dt} = \frac{d[\mathrm{S}]}{dt} = k_{(\gamma^n,\beta_m,\beta^m)}[\mathrm{Y}][\mathrm{L}(m,n)] \quad (2)$$

To derive the expression for $k_{(\beta^m,\beta_m,\gamma^n)}$, we use QSS to analyze the production rate of Y in the absence of the reverse reaction. We first set up the steady state conditions on the intermediates I and J:

$$\frac{d[\mathrm{I}]}{dt} = k_f[\mathrm{X}(m,n)][\mathrm{S}] + k_b[\mathrm{J}] - k_b[\mathrm{I}] - k_{r(\gamma^n)}[\mathrm{I}] \approx 0 \quad (3)$$

$$\frac{d[\mathrm{J}]}{dt} = k_b[\mathrm{I}] + k_f[\mathrm{Y}][\mathrm{L}(m,n)] - k_b[\mathrm{J}] - k_{r(\beta^m)}[\mathrm{J}] \approx 0 \quad (4)$$

In assuming no reverse reaction, we remove the $k_f[\mathrm{Y}][\mathrm{L}(m,n)]$ term in the expression for $\frac{d[\mathrm{J}]}{dt}$. Experimentally, this is achieved by quickly removing Y from the system (using the fluorescence reporter complex), so that the reverse reaction in negligible. Rearranging the simplified equation (4),

$$[\mathrm{J}] = \frac{k_b[\mathrm{I}]}{k_b + k_{r(\beta^m)}} \quad (5)$$

Substituting this expression for [J] back into equation (3) and rearranging,

$$k_f[\mathrm{X}(m,n)][\mathrm{S}] = [\mathrm{I}]\left(\frac{k_{r(\gamma^n)}k_{r(\beta^m)} + k_{r(\gamma^n)}k_b + k_{r(\beta^m)}k_b}{k_b + k_{r(\beta^m)}}\right) \quad (6)$$

$$[\mathrm{I}] = \frac{k_f(k_b + k_{r(\beta^m)})[\mathrm{X}(m,n)][\mathrm{S}]}{k_{r(\gamma^n)}k_{r(\beta^m)} + k_{r(\gamma^n)}k_b + k_{r(\beta^m)}k_b} \quad (7)$$

$$[\mathrm{J}] = \frac{k_f k_b[\mathrm{X}(m,n)][\mathrm{S}]}{k_{r(\gamma^n)}k_{r(\beta^m)} + k_{r(\gamma^n)}k_b + k_{r(\beta^m)}k_b} \quad (8)$$

Finally, the rate of production of Y is calculated:

$$\frac{d[Y]}{dt} = k_{r(\beta^m)}[J]$$

$$= \frac{k_{r(\beta^m)}k_f k_b}{k_{r(\gamma^n)}k_{r(\beta^m)} + k_{r(\gamma^n)}k_b + k_{r(\beta^m)}k_b}[X(m,n)][S]$$

S2. Calculations of the critical concentration for the accuracy of the BM rate constant

In the BM, every species that is not product (Y or L(m,n)) is still a reactant; thus, $[\mathrm{X}(m,n)]_{BM} = [\mathrm{X}(m,n)]_0 - [\mathrm{Y}]$ and $[\mathrm{S}]_{BM} = [\mathrm{S}]_0 - [\mathrm{Y}]$, where $[\mathrm{X}(m,n)]_0$ and $[\mathrm{S}]_0$ denote the initial concentrations of X(m,n) and S, and [Y] denotes the measured amount of product Y. In the three-step model $[\mathrm{X}(m,n)] = [\mathrm{X}(m,n)]_0 - [\mathrm{Y}] - [\mathrm{I}] - [\mathrm{J}]$ and $[\mathrm{S}] = [\mathrm{S}]_0 - [\mathrm{Y}] - [\mathrm{I}] - [\mathrm{J}]$.

When conditions are such that [I] and [J] are low (i.e. $[\mathrm{X}(m,n)]_{BM} = [\mathrm{X}(m,n)] + [\mathrm{I}] + [\mathrm{J}] \approx [\mathrm{X}(m,n)]$ and $[\mathrm{S}]_{BM} = [\mathrm{S}] + [\mathrm{I}] + [\mathrm{J}] \approx [\mathrm{S}]$), the kinetics of toehold exchange is well-modeled by a bimolecular reaction with the following BM rate constant:

$$k_{(\beta^m,\beta_m,\gamma^n)} \;=\; \frac{k_{r(\beta^m)} k_f k_b}{k_{r(\gamma^n)} k_{r(\beta^m)} + k_{r(\gamma^n)} k_b + k_{r(\beta^m)} k_b} \tag{9}$$

In contrast, when the concentrations of I and J are high, the above BM rate constant will overestimate the kinetics of the toehold exchange reaction.

To evaluate the conditions under which the BM rate constant grossly overestimates the kinetics of toehold exchange, we analyze the concentration of [I]: We arbitrarily define the condition $[\mathrm{X}(m,n)] \approx [\mathrm{X}(m,n)]_{BM} = [\mathrm{X}(m,n)] + [\mathrm{I}] + [\mathrm{J}]$ to be satisfied when $\frac{[\mathrm{I}]}{[\mathrm{X}(m,n)]} \leq 0.1$. Because $[\mathrm{J}] = [\mathrm{I}]\frac{k_b}{k_b + k_{r(\beta^m)}} < [\mathrm{I}]$, $[\mathrm{X}(m,n)]_{BM} < 1.2 \cdot [\mathrm{X}(m,n)]$. Similarly, $[\mathrm{S}] \approx [\mathrm{S}]_{BM} = [\mathrm{S}] + [\mathrm{I}] + [\mathrm{J}] < 1.2 \cdot [\mathrm{S}]$ when $\frac{[\mathrm{I}]}{[\mathrm{S}]} \leq 0.1$. The expression for the quasi-steady steady production rate of Y depends on product of $[\mathrm{X}(m,n)]$ and [S]:

$$\begin{aligned} \frac{d[\mathrm{Y}]}{dt} \;&=\; k_{(\beta^m,\beta_m,\gamma^n)} [\mathrm{X}(m,n)][\mathrm{S}] \\ &>\; k_{(\beta^m,\beta_m,\gamma^n)} (\frac{1}{1.2}[\mathrm{X}(m,n)]_{BM})(\frac{1}{1.2}[\mathrm{S}]_{BM}) \end{aligned}$$

$$k_{(\beta^m,\beta_m,\gamma^n)} [\mathrm{X}(m,n)]_{BM} [\mathrm{S}]_{BM} < 1.44 \frac{d[\mathrm{Y}]}{dt}$$

Thus when $\frac{[\mathrm{I}]}{[\mathrm{X}(m,n)]}, \frac{[\mathrm{I}]}{[\mathrm{S}]} \leq 0.1$, the rate of production of Y predicted by the derived BM rate constant is overestimated by no more than 44%. Rearranging (4), we solve for the critical concentration of S below which $\frac{[\mathrm{I}]}{[\mathrm{X}(m,n)]} \leq 0.1$:

$$[\mathrm{S}] \;\leq\; \frac{0.1}{k_f} \cdot \frac{k_{r(\gamma^n)} k_{r(\beta^m)} + k_{r(\gamma^n)} k_b + k_{r(\beta^m)} k_b}{k_b + k_{r(\beta^m)}} \tag{10}$$

Similar derivation on the condition $\frac{[\mathrm{I}]}{[\mathrm{S}]} \leq 0.1$ leads to the same critical concentration for X(m,n). The critical concentration varies monotonically with the figure of merit; for example, the BM predicted kinetics are off by no more than an order of magnitude when $[\mathrm{X}(m,n)]$ and [S] are below $\frac{1}{k_f} \cdot \frac{k_{r(\gamma^n)} k_{r(\beta^m)} + k_{r(\gamma^n)} k_b + k_{r(\beta^m)} k_b}{k_b + k_{r(\beta^m)}}$ (when $\frac{[\mathrm{I}]}{[\mathrm{X}(m,n)]}, \frac{[\mathrm{I}]}{[\mathrm{S}]} \leq 1$).

S3. Calculation of Toehold Binding Energies

The binding energies of the toehold are calculated as:

$$\begin{aligned}\Delta G^\circ(\gamma^n) &= \Delta G^\circ(\mathrm{I}(0,n)) - \Delta G^\circ(\mathrm{S}) \\ \Delta G^\circ(\beta^m) &= \Delta G^\circ(\mathrm{J}(m,0)) - \Delta G^\circ(\mathrm{L}(m,0))\end{aligned}$$

Default calculation method

Here, we show a step-by-step method for calculating ΔG° of two complexes, S, and I(0,3), which is in turn used to infer the binding energy of the toehold γ^3. Calculation of ΔG° values for other complexes are analogous.

$\Delta G^\circ(\mathbf{S})$

We start by calculating the ΔG° of S:

$$\Delta G^\circ(\mathrm{S}) = 1 * \Delta G^\circ_{\mathrm{init}} + \Delta G^\circ_{\alpha\ \mathrm{dangle}} + \Delta G^\circ_{\gamma\ \mathrm{dangle}} + \Delta G^\circ_{\mathrm{stacks}}$$

For each of the ΔG° terms, $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$.

Hybridization initiation. The $\Delta G^\circ_{\mathrm{init}}$ refers to the energetic cost of initiating a helix. SantaLucia et al. [2] report that $\Delta H^\circ_{\mathrm{init}} = +0.2$ kcal/mol and $\Delta S^\circ_{\mathrm{init}} = -5.7$ cal/mol·K, leading to $\Delta G^\circ_{\mathrm{init}} = +1.90$ kcal/mol at 25 °C.

Terminal dangles. For S, the terminal dangle on the α domain is a 5' T dangle with a C nearest neighbor. Bommarito et al. [3] report $\Delta H^\circ_{\alpha\ \mathrm{dangle}} = -4.0$ kcal/mol and $\Delta S^\circ_{\alpha\ \mathrm{dangle}} = -10.9$ cal/mol·K, leading to $\Delta G^\circ_{\alpha\ \mathrm{dangle}} = -0.75$ kcal/mol at 25 °C.

For S, the terminal dangle on the γ domain is a 5' A dangle with a C nearest neighbor. Bommarito et al. [3] report $\Delta H^\circ_{\gamma\ \mathrm{dangle}} = -6.3$ kcal/mol and $\Delta S^\circ_{\gamma\ \mathrm{dangle}} = -17.1$ cal/mol K, leading to $\Delta G^\circ_{\gamma\ \mathrm{dangle}} = -1.20$ kcal/mol at 25 °C. For Ss and Sw, the γ dangle would be a 5' G and a 5' T, respectively.

Stacks. There are 20 base pairs in the β domain of S, leading to 19 total stack terms. Summing these using the values reported by SantaLucia et al. [2] yield a total $\Delta H^\circ_{\mathrm{stacks}} = -152.4$ kcal/mol and $\Delta S^\circ_{\mathrm{stacks}} = -409.4$ cal/mol·K, leading to $\Delta G^\circ_{\mathrm{stacks}} = -30.40$ kcal/mol at 25 °C.

Total standard free energy. Summing all the previous terms, $\Delta G^\circ(\mathrm{S}) = -30.45$ kcal/mol.

$\Delta G^\circ(\mathbf{I(0,3)})$

The binding energy of I(0,3) is calculated as:

$$\begin{aligned}\Delta G^\circ(\mathrm{I}(0,3)) = {} & 2 * \Delta G^\circ_{\mathrm{init}} + \Delta G^\circ_{\alpha\ \mathrm{dangle}} + \Delta G^\circ_{\gamma\ \mathrm{dangle}} + \Delta G^\circ_{\mathrm{stacks}} \\ & + \Delta G^\circ_{\mathrm{AT\ termination}} + \Delta G^\circ_{\mathrm{nick}} + \Delta G^\circ_{\mathrm{coaxial\ dangle}}\end{aligned}$$

Initiation. $\Delta G^\circ_{\text{init}} = +1.90$ kcal/mol as calculated before, but we multiply it by 2 in this calculation because I(0,3) is composed of three strands of DNA.

Terminal dangles. The α dangle is exactly the same, so $\Delta G^\circ_{\alpha\text{ dangle}} = -0.75$ kcal/mol. The γ dangle in this case is a 5' G with a A nearest neighbor, which is reported to contribute $\Delta H^\circ_{\gamma\text{ dangle}} = -1.1$ kcal/mol and $\Delta S^\circ_{\gamma\text{ dangle}} = -1.6$ cal/mol·K, leading to $\Delta G^\circ_{\gamma\text{ dangle}} = -0.62$ kcal/mol.

Stacks. I(0,3) has 23 total base pairs, and consequently 22 stacks, but one of these stacks is the coaxial stacking term at the nick. Summing the remaining 21 stacks yields $\Delta H^\circ_{\text{stacks}} = -168.4$ kcal/mol and $\Delta S^\circ_{\text{stacks}} = -452.6$ cal/mol·K, leading to $\Delta G^\circ_{\text{stacks}} = -33.53$ kcal/mol.

AT termination. The $\Delta G^\circ_{\text{AT termination}}$ is a special energetic penalty term that is added for helices terminating in an A-T base pair. This term is part of the commonly accepted energy parameters presented by SantaLucia et al. [2]. $\Delta H^\circ_{\text{AT termination}} = +2.2$ kcal/mol and $\Delta S^\circ_{\text{AT termination}} = +6.9$ cal/mol·K, leading to $\Delta G^\circ_{\text{AT termination}} = +0.14$ kcal/mol.

Coaxial stacking. The $\Delta G^\circ_{\text{nick}}$ is the coaxial stacking term at the boundary of the β and γ domains. The bases flanking the nick are 5'-G and T-3'. Protozanova et al. [4] report the ΔG° of this coaxial stack to be -2.04 kcal/mol at 37 °C [4]. Unfortunately, this work did not provide explicit ΔH° and ΔS° values, so the ΔG° value at 25 °C cannot be directly calculated.

Protozanova et al. [4] suggested the approximation that $\Delta S^\circ \approx -25$ cal/mol·K for all coaxial stacks in order to estimate ΔG° at other temperatures. This would imply that $\Delta G^\circ_{25} = \Delta G^\circ_{37} - 0.3$ kcal/mol for all coaxial stacks.

Observing the distribution of ΔS° and ΔH° values for standard stacks [2, 5, 6], we felt that $\Delta S^\circ \approx a\Delta H^\circ$ was a better approximation than $\Delta S^\circ \approx 25$ cal/mol·K, where a is a fitted constant (see Fig. S1). A standard least squares fit yielded $a = 0.0027$ K^{-1}.

Because $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, $\Delta G^\circ_{37} \approx (1 - 310 * 0.0027)\Delta H^\circ = 0.163\Delta H^\circ$ and $\Delta G^\circ_{25} \approx (1 - 298 * 0.0027)\Delta H^\circ = 0.195\Delta H^\circ$. Dividing the two equations and rearranging, $\Delta G^\circ_{25} = 1.2 \cdot \Delta G^\circ_{37}$. For the coaxial stack in question, $\Delta G^\circ_{\text{nick}} \approx 1.2 \cdot -2.04 = -2.45$ kcal/mol.

Protozanova et al.'s reported values for the ΔG°_{37} of coaxial stacks [4] range from -0.12 kcal/mol to -2.70 kcal/mol. Using our approximation leads to ΔG°_{25} ranging from -0.144 to -3.24 kcal/mol, while using $\Delta S^\circ \approx 25$ cal/mol·K leads to ΔG°_{25} ranging from -0.42 to -3.00 kcal/mol. Thus, the two approximations should differ by no more than 0.3 kcal/mol in all cases.

Coaxial stack dangles. The $\Delta G^\circ_{\text{coaxial dangle}}$ term refers to the energy contribution of the last G on the β domain of input X(m, n), when the latter is bound to S by only the toehold. As explained in the main paper, the value of this parameter has not been characterized, and is assumed to be 0.

Total standard free energy. Summing the terms above, ΔG°(I(0,3)) $= -33.41$ kcal/mol. The binding strength of toehold γ^3 is inferred to be ΔG°(I(0,3)) $- \Delta G^\circ$(S) $= -2.95$ kcal/mol. This value is then

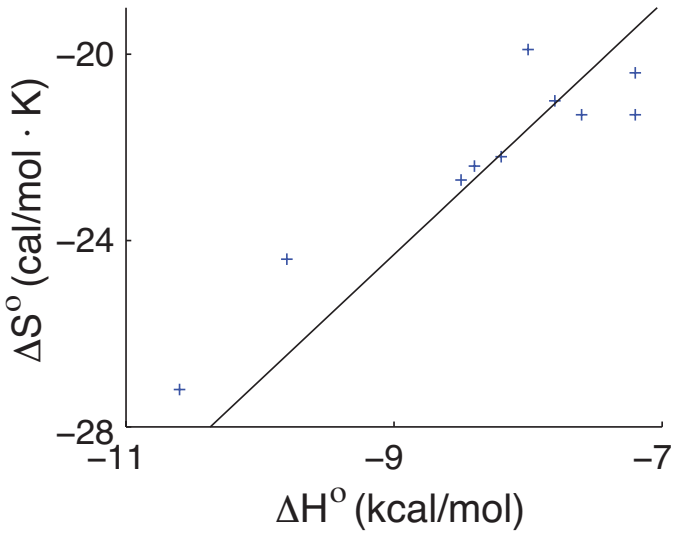

FIG. S1: Distribution of ΔH° and ΔS° values for standard nearest-neighbor stacks, as reported by ref. [2]. The black line indicates the approximation $\Delta S^\circ = 0.0027\mathrm{K}^{-1}\Delta H^\circ$.

rounded to 1 decimal point and are shown in Table 2 and Table S1.

Pyshnyi's coaxial stacking terms

For this energetics model, calculations were performed completely analogously to the default method, except using the coaxial stacking parameters reported by Pyshnyi et al. [7]. Pyshnyi et al. did report ΔH° and ΔS° values for all of their coaxial stacking terms, so ΔG°_{25} was directly calculated for the coaxial stack. The infered toehold binding energies are shown in column 3 of Table S1.

NUPACK + Protozanova

For energetics calculations using the NUPACK folding software [8], we used the "energy" program in the downloadable NUPACK 2.1 software. This program computes the standard free energy of a particular microstate entered by the user, entered in dot-paren notation. For example, our input file for I(0,3) was the following:

```
CCACATACATCATATTCCCTCATTCAATACCCTACG
CCCTCATTCAATACCCTACGTCT
GAAGTGACATGGAGACGTAGGGTATTGAATGAGGG
1 2 3
..............((((((((((((((((((((+.................(((+...........))))))))))))))))))))))
```

According to the user's manual for NUPACK, calculations using the "dangles = some" option (the recommended default) calculates dangle energies "for each unpaired base flanking a duplex (a base flanking two duplexes contributes only the minimum of the two possible energies)." Thus, the "dangles = some" option does not include any coaxial stacking energetics term, but does include the coaxial stacking dangle term (with energy equivalent to an analogous terminal dangle). We thus manually add our modified

Protozanova et al. coaxial stacking terms to the standard free energy of complexes predicted by NUPACK, where applicable.

Additionally, as of this writing, NUPACK calculates the standard free energy of complexes to satisfy the thermodynamic equilibria of the molecules in mole fraction, rather than concentration (in molar). This means that an additional corrective term needs to be manually added for calculating the toehold binding energy.

As an example, consider ΔG°(S):

$$\begin{aligned} \Delta G^\circ_{NUPACK} &= -RT\ln(K_{NUPACK}) \\ K_{NUPACK} &= \frac{\chi_{\mathrm{S}}}{\chi_{\mathrm{Y}}\chi_{\mathrm{V}}} \end{aligned}$$

where V is the heretofore unnamed bottom strand of the S complex (containing the domains $\bar{\gamma}$ and $\bar{\beta}$. To convert the equilibrium constant K to expressed in terms of M^{-1}, a correction $RT\ln(c)$ term needs to be added, where c is the total concentration of all species in solution. The total concentration of all species is solution is dominated by the solvent water molecules, which exist at 55 M, implying that the correction is roughly $RT\ln(55) \approx 2.38$ kcal/mol. Note that this correction is to be multiplied by the $N-1$, where N is the total number of strands in the complex. For the energies listed in column 4 in Table S1, we manually added the correction where applicable.

NUPACK, dangles = all

According to the user's manual for NUPACK, calculations using the "dangles = all" option calculates dangle energies "for each base flanking a duplex regardless of whether it is paired." Thus, the "dangles = all" option includes not only the coaxial stacking dangle term as before, but also approximates the coaxial stack energy as the sum of the two dangles. For example, in I(0,n), the nick is "G / T"; the energetics of this nick is approximated by NUPACK as the sum of that of 5' A dangle with nearest neighbor C, and a 3' C dangle with nearest neighbor A. This method of approximation is not scientifically justified, but rather exists as a placeholder until coaxial stacking energetics are better understood.

As in the previous method, manual corrective terms of 2.38 kcal/mol per extra strand was added to convert ΔG° for mole fractions to ΔG° for molar units. The corrected inferred toehold binding energies are shown in column 5 of Table S1.

Owczarzy's Mg^{2+} correction

Owczarzy et al.'s correction formula [9] modifies ΔS° based on the ΔH° of the structure in 1 M Na^+. The formula depended on the length of the helix involved; for our calculations, we assumed that the nick does not disrupt the helix (e.g. I(0,3) would contain a helix of length 23, rather than 2 helices of lengths 20 and 3). Furthermore, since this study did not characterize the salt corrections for energy contributions from dangles, we used Bommarito et al.'s dangle values (for 1 M Na^+) [3].

Mathematically, the formula we used was:

$$\begin{aligned}\Delta H^{\circ}_{\mathrm{Mg^{2+}}} &= \Delta H^{\circ}_{\mathrm{Na^{+}}} \\ \Delta S^{\circ}_{\mathrm{Mg^{2+}}} &= \Delta S^{\circ}_{\mathrm{Na^{+}}} + \Delta H^{\circ}_{stacks,\mathrm{Na^{+}}} \cdot (a + bx + f_{\mathrm{GC}}(c + dx) + \frac{e + fx + gx^2}{2(N_{\mathrm{bp}} - 1)})\end{aligned}$$

where $x = \ln[\mathrm{Mg^{2+}}] = -4.465$, and f_{GC} is the fraction of the bases in the helix that are purines. Parameters $a = 3.92 \cdot 10^{-5}$ K^{-1}, $b = -9.11 \cdot 10^{-5}$ K^{-1}, $c = 6.26 \cdot 10^{-5}$ K^{-1}, $d = 1.42 \cdot 10^{-5}$ K^{-1}, $e = -4.82 \cdot 10^{-4}$ K^{-1}, $f = 5.25 \cdot 10^{-4}$ K^{-1}, and $g = 8.31 \cdot 10^{-5}$ K^{-1} are fitted values reported by Owczarzy et al. [9]. The toehold binding energies calculated using this model are shown in column 6 of Table S1.

Toehold	Default	Pyshnyi coaxial	NUPACK, dangles=some	NUPACK, dangles=all	Owczarzy Mg^{2+}
γ^0	+1.9	+1.9	+1.9	+2.5	+1.9
γ^1	+0.2	+0.2	+0.5	+1.5	+0.3
γ^2	-1.7	-1.6	-1.2	-0.3	-1.4
γ^3	-3.0	-2.9	-2.6	-1.6	-2.5
γ^4	-4.7	-4.7	-4.3	-3.4	-4.1
γ^5	-6.9	-6.9	-6.5	-5.5	-6.1
γ^6	-8.3	-8.3	-7.9	-6.9	-7.3
γ^7	-9.2	-9.2	-8.8	-7.9	-8.0
γ^8	-11.9	-11.9	-11.4	-10.5	-10.5
γ^9	-12.9	-12.9	-12.5	-11.5	-11.3
γ^{10}	-14.8	-14.8	-14.3	-13.4	-13.0
γ^{15}	-21.8	-21.8	-21.4	-20.4	-19.1
γs^0	+1.9	+1.9	+1.9	+2.5	+1.9
γs^1	-1.1	-1.0	-2.0	+0.2	-1.0
γs^2	-3.2	-3.1	-4.1	-1.9	-2.9
γs^3	-5.0	-5.0	-6.0	-3.8	-4.6
γs^4	-8.0	-7.9	-8.9	-6.7	-7.3
γs^5	-10.3	-10.2	-11.2	-9.0	-9.4
γs^6	-12.1	-12.0	-13.1	-10.9	-11.0
γs^7	-15.1	-15.0	-16.0	-13.8	-13.8
γs^8	-17.3	-17.3	-18.3	-16.1	-15.8
γs^9	-19.2	-19.1	-20.2	-18.0	-17.5
γs^{10}	-21.2	-21.2	-22.2	-20.0	-19.3
γw^0	+1.9	+1.9	+1.9	+2.5	+1.9
γw^1	+0.2	-0.6	-0.4	+1.2	+0.2
γw^2	-0.8	-1.5	-1.4	+0.2	-0.6
γw^3	-2.1	-2.8	-2.6	-1.1	-1.6
γw^4	-3.8	-4.5	-4.4	-2.8	-3.2
γw^5	-4.3	-5.0	-4.9	-3.3	-3.5
γw^6	-5.3	-6.0	-5.9	-4.3	-4.3
γw^7	-7.0	-7.7	-7.6	-6.0	-5.9
γw^8	-7.5	-8.2	-8.1	-6.5	-6.2
γw^9	-8.9	-9.7	-9.6	-8.0	-7.5
γw^{10}	-8.9	-9.6	-9.4	-7.9	-7.2

Table S1: Comparison of toehold binding energies using various methods (in kcal/mol)

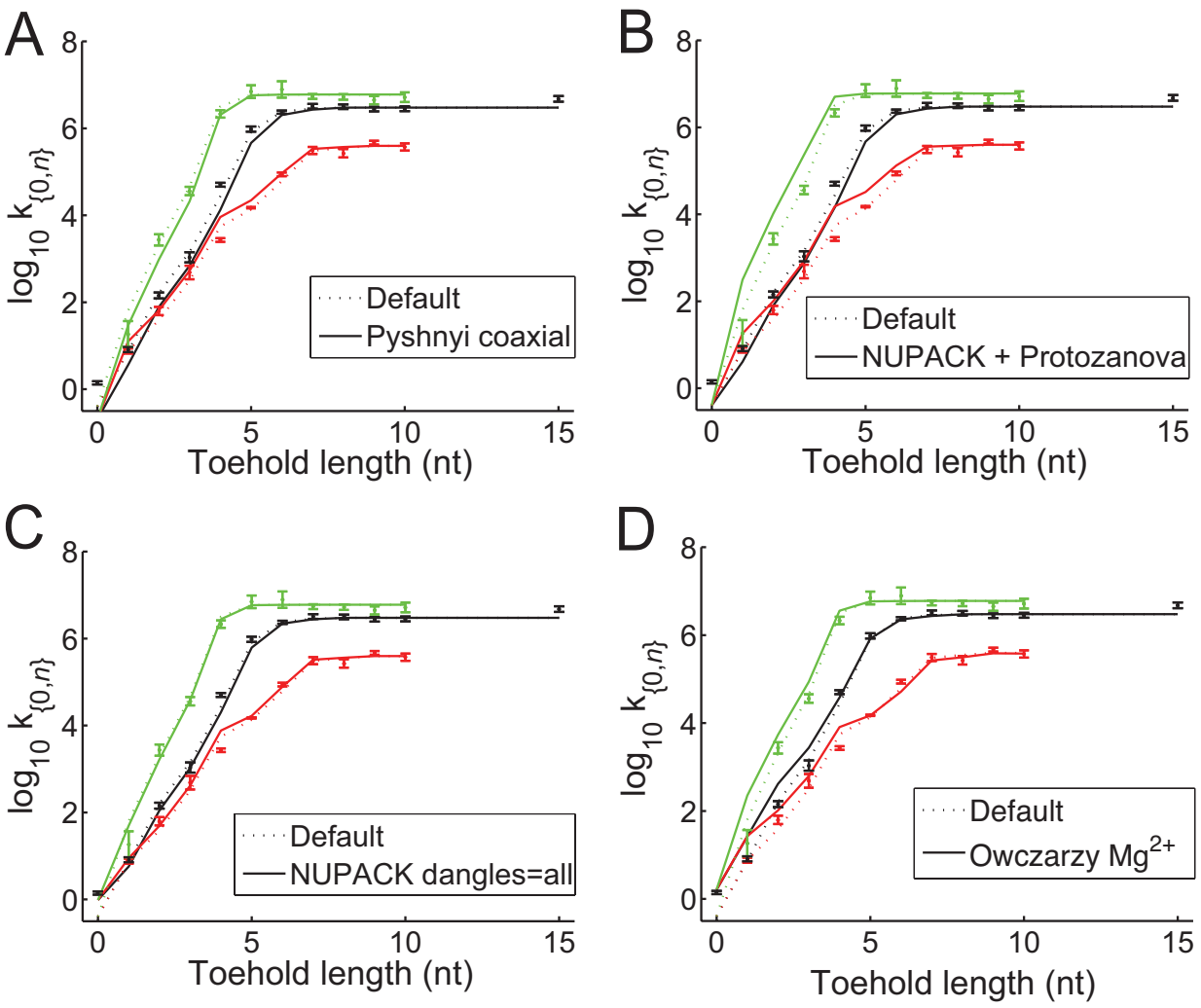

FIG. S2: Comparison of different energy models and their effects on the model-predicted BM rate constant. The dots represent the best-fit rate constants from data in the main paper. The dotted lines show the model-predicted BM rate constants based on the toehold binding energies listed in the main paper. **(A)** The solid lines show the BM rate constants predicted using coaxial stacking terms reported by Pyshnyi et al. [7], but otherwise identical to the default model. For this set of energy parameters, $k_b = 0.5\ \mathrm{s}^{-1}$. **(B)** The solid lines show the BM rate constants predicted using energies predicted by NUPACK [8], using "dangles = some," so that the coaxial stack dangle are calculated as terminal dangles. The modified Protozanova et al. [4] coaxial stacking parameter was manually added, as well as a +2.377 kcal/mol conversion term to convert the standard free energy to molar units (from mole fraction). See Text S3 for details on how NUPACK was used. For this set of energy parameters, $k_b = 1\ \mathrm{s}^{-1}$. **(C)** The solid lines show the BM rate constants predicted using energies predicted by NUPACK, using "dangles = all." The coaxial stack dangles are calculated as terminal dangles, and the coaxial stacking parameter is calculated as two overlapping dangles. A +2.377 kcal/mol conversion term to convert the standard free energy to molar units (from mole fraction). For this set of energy parameters, $k_b = 7\ \mathrm{s}^{-1}$. **(D)** The solid lines show the BM rate constants predicted using toehold binding energies as calculated by the default model, corrected for magnesium concentration using the method by Owczarzy et al. [9]. For this set of energy parameters, $k_b = 4\ \mathrm{s}^{-1}$.

m	n	[S]	[X(m,n)]	[R]
0	0-1	100 nM	2 μM, 4 μM, and 6 μM	300 nM
0	2	100 nM	200 nM, 400 nM, and 600 nM	300 nM
0	3	10 nM	20 nM, 40 nM, and 60 nM	30 nM
0	4	10 nM	2 nM, 4 nM, and 6 nM	30 nM
0	5-10, 15	1 nM	0.2 nM, 0.4 nM, and 0.6 nM	3 nM
1-7	10	1 nM	0.4 nM	3 nM
5-7	9	1 nM	0.4 nM	3 nM
4-7	8	1 nM	0.4 nM	3 nM
4-7	7	1 nM	0.4 nM	3 nM
2-5	6	1 nM	0.4 nM	3 nM
6-7	6	10 nM	4 nM	30 nM
1-4	5	1 nM	0.4 nM	3 nM
5-6	5	10 nM	4 nM	30 nM
7	5	10 nM	40 nM	30 nM
1-7	4	10 nM	40 nM	30 nM
1-3	3	10 nM	40 nM	30 nM
4-5	3	10 nM	400 nM	30 nM
6	3	10 nM	40 nM	30 nM
1-5	2	10 nM	400 nM	30 nM
1-3	1	10 nM	4 μM	30 nM
4	1	10 nM	400 nM	30 nM

Table S2: Concentrations used for toehold exchange reactions using X(m,n)

m	n	[Sw]	[Xw(m,n)]	[R]
0	1	10 nM	2 μM, 4 μM, and 6 μM	30 nM
0	2	10 nM	200 nM, 400 nM, and 600 nM	30 nM
0	3-5	10 nM	20 nM, 40 nM, and 60 nM	30 nM
0	6	1 nM	2 nM, 4 nM, and 6 nM	3 nM
0	7-10	1 nM	0.2 nM, 0.4 nM, and 0.6 nM	3 nM

Table S3: Concentrations used for toehold mediated strand displacement using Xw(m,n)

m	n	[Ss]	[Xs(m,n)]	[R]
0	1	10 nM	2 μM, 4 μM, and 6 μM	30 nM
0	2	10 nM	20 nM, 40 nM, and 60 nM	30 nM
0	3	10 nM	2 nM, 4 nM, and 6 nM	30 nM
0	4-10	1 nM	0.2 nM, 0.4 nM, and 0.6 nM	3 nM

Table S4: Concentrations used for toehold mediated strand displacement using Xs(m,n)

S4. Sample Matlab code used for fitting rate constants.

The main program for fitting the BM rate constant of toehold exchange using input X(4,8) is as follows:

```
k0 = log(1E6);
scale0 = log(4e13);

[k, fval] = fminunc(@TE_4_8, [k0, scale0]);
```

The variable $k0$ shows an initial "guess" of the rate constant (set to 10^6 M^{-1} s^{-1} here), while $scale0$ shows an initial guess of the scaling constant (set to $4 \cdot 10^{13}$ counts per mole of unquenched fluorophore). Both variables are fitted during runtime.

The error function TE_4_8 for this is as follows:

```
function err_func = TE_4_8(input)

data = load('/Users/daveyzhang/Desktop/work/expt/Fluorescence/20080229/004m.txt');

k = exp(input(1));
scalingconst = exp(input(2));

err_func = 0;
options = odeset('RelTol', 1e-4, 'AbsTol', 1e-30);

datasize = size(data, 1);

%(4,8) data
t = data(6:datasize,1)-300;
y0 = [1e-9, 4e-10, 0, 3e-9, 0];
[t, y2] = ode23s(@rdy, t, [k, y0], options);

ye = y2(:,6) * scalingconst + data(5,2);
for i = 7:size(data, 1)
    err_func = err_func + (ye(i-5) - data(i,2))^2/ye(i-5);
end
```

The variable $y0$ sets the initial conditions of the simulation, and the Matlab function $ode23s$ is used to simulated the reactions defined in the function rdy. Note that we use $ode23s$ rather than $ode45$ because the system is "stiff," containing reactions with very different time scales.

The rdy function is as follows:

```
function dy = rdy(t, y)
%S + X -> OB
%OB + OF_OQ -> OF
krep = 1.30e6; %fitted earlier
```

```
dy = zeros(6,1);
dy(2) = -y(1) * y(2) * y(3);
dy(3) = -y(1) * y(2) * y(3);
dy(4) = y(1) * y(2) * y(3) - krep * y(4) * y(5);
dy(5) = -krep * y(4) * y(5);
dy(6) = krep * y(4) * y(5);
```

The parameter *krep* denotes the rate constant of the reaction between the output product Y and the reporter complex R (previously fitted to be $1.3 \cdot 10^6$ M^{-1} s^{-1}.

S5. Approximation of the BM rate constants and critical concentrations.

In Fig. 8 of the main text, we show a simplified approximation flowchart for estimating the BM rate constant and the critical concentration below which the BM rate constant is a valid predictor of kinetics. Here, we justify the results in those figures.

Approximating the BM rate constant

Recall equation (7), the expression for the BM rate constant:

$$\begin{aligned} k_{\{m,n\}} &\equiv k_{(\beta^m,\beta_m,\gamma^n)} \\ &= \frac{k_f k_{r(\beta^m)} k_b}{k_{r(\gamma^n)} k_{r(\beta^m)} + k_{r(\gamma^n)} k_b + k_{r(\beta^m)} k_b} \end{aligned}$$

At room temperature of 25 °C, with 11.5 mM Mg^{2+}, and average distribution of base pairs for toeholds β^m and γ^n, each base contributes on average approximately 1.4 kcal/mol to the binding energy of the toehold. Numerical substitution yields:

$$\begin{aligned} \Delta G^\circ(\beta^m) &\approx 1.9 - 1.4m[\mathrm{kcal/mol}] \\ k_{r(\beta^m)} &= k_f \frac{2}{b-m} e^{\Delta G^\circ(\beta^m)/RT} \\ &= 3 \cdot 10^6 \frac{2}{20-m} (e^{-2.36})^{(m-1.35)} [\mathrm{M}^{-1}\ \mathrm{s}^{-1}] \\ &= 3 \cdot 10^6 \frac{2}{20-m} (10.6)^{1.35-m} [\mathrm{M}^{-1}\ \mathrm{s}^{-1}] \\ &\approx 6 \cdot 10^{6-m} [\mathrm{M}^{-1}\ \mathrm{s}^{-1}] \end{aligned}$$

Similarly, $k_{r(\gamma^n)} \approx 6 \cdot 10^{6-n} [\mathrm{M}^{-1}\ \mathrm{s}^{-1}]$. Compared to the empirically measured value of $k_b = 1.0\ \mathrm{s}^{-1}$, $k_{r(\beta^m)}$ and $k_{r(\gamma^n)}$ are smaller than k_b when m and n are greater than 6, respectively.

For situations where $n \geq m$, $k_{r(\beta^m)} \geq k_{r(\gamma^n)}$, and the expression for $k_{\{m,n\}}$ can be approximated as:

$$k_{\{m,n\}} \approx \frac{k_f k_{r(\beta^m)} k_b}{(k_{r(\gamma^n)} + k_b) k_{r(\beta^m)}} = \frac{k_f k_b}{k_{r(\gamma^n)} + k_b}$$

When $n > 6$, $k_{r(\gamma^n)} < k_b$, and the expression for $k_{\{m,n\}}$ is approximated as $k_f \approx 3 \cdot 10^6$ M^{-1} s^{-1}. When $n \leq 6$, $k_{r(\gamma^n)} > k_b$, and the expression for $k_{\{m,n\}}$ is approximated as $\frac{k_f k_b}{k_{r(\gamma^n)}} = 5 \cdot 10^{n-1}$ M^{-1} s^{-1}.

For situations where $n < m$, $k_{r(\beta^m)} < k_{r(\gamma^n)}$, and the expression for $k_{\{m,n\}}$ can be approximated as:

$$k_{\{m,n\}} \approx \frac{k_f k_{r(\beta^m)} k_b}{(k_{r(\beta^m)} + k_b) k_{r(\gamma^n)}}$$

When $m > 6$, $k_{r(\beta^m)} < k_b$, and the expression for $k_{\{m,n\}}$ can be approximated as $\frac{k_f k_{r(\beta^m)}}{k_{r(\gamma^n)}} = k_f \cdot 10^{n-m} \approx 3 \cdot 10^{6+n-m}$ M^{-1} s^{-1}. When $m \leq 6$, $k_{r(\beta^m)} > k_b$, and the expression for $k_{\{m,n\}}$ can be approximated as $\frac{k_f k_b}{k_{r(\gamma^n)}} = 5 \cdot 10^{n-1}$ M^{-1} s^{-1}.

Approximating the critical concentration

For estimating the critical concentrations below which the BM rate constant accurately predicts kinetics, recall the expression for the critical concentration:

$$[\mathrm{X}(m,n)], [\mathrm{S}] \leq \frac{0.1}{k_f} \cdot \frac{k_{r(\gamma^n)} k_{r(\beta^m)} + k_{r(\gamma^n)} k_b + k_{r(\beta^m)} k_b}{k_b + k_{r(\beta^m)}}$$

For situations where $m > 6$, $k_{r(\beta^m)} < k_b$, and the critical concentration can be approximated as $\frac{0.1 \cdot (k_{r(\gamma^n)} + k_{r(\beta^m)})}{k_f}$. The numerator can be approximated as $0.1 \cdot \max(k_{r(\gamma^n)}, k_{r(\beta^m)}) = 6 \cdot 10^{5-\min(m,n)}$ s^{-1}, and the critical concentration is approximated as: $2 \cdot 10^{-1-\min(m,n)}$ M.

For situations where $m \leq 6$, $k_{r(\beta^m)} > k_b$, and the critical concentration can be approximated as $\frac{0.1 \cdot (k_{r(\gamma^n)} + k_b)}{k_f}$. When $n > 6$, the critical concentration is approximated as $\frac{0.1 k_b}{k_f} \approx 3 \cdot 10^{-8}$ M. When $n \leq 6$, the critical concentration is approximated as $\frac{0.1 k_{r(\gamma^n)}}{k_f} = 2 \cdot 10^{-1-n}$ M.

S6. Matlab script for generating BM rate constant based on toehold energies

The follows shows a script for calculating the BM rate constant and the c_{crit} values for a toehold exchange reaction, taking the two toehold energies, the branch migration length, the temperature, and the energy model as inputs.

```
function output = BM_rate(input)

%input format:  [(invading toehold energy), (incumbent toehold energy),
%               (branch migration length), (temperature),  (energy model)]
%
%output format: [(BM rate constant), (critical concentration)]
%
%Assumes toeholds composition has all 4 bases (i.e. kf = 3.5e6)
```

```
%
%Energy model: 1 = default, 2 = Pyshnyi, 3 = Nupack + Protozanova,
%              4 = Nupack(dangles = all), 5 = Owczarzy
%
% NOTE:  User must manually add 2.38 kcal/mol for the input energies to NUPACK's
%        mole fraction energies

invading_energy = input(1);
incumbent_energy = input(2);
BM_length = input(3);
temperature = input(4)+273.15;
energy_model = input(5);

kf = 3.5e6;

if (energy_model == 1)
    kb = 1 * 400 / (BM_length * BM_length);
end
if (energy_model == 2)
    kb = 0.5 * 400 / (BM_length * BM_length);
end
if (energy_model == 3)
    kb = 1 * 400 / (BM_length * BM_length);
end
if (energy_model == 4)
    kb = 7 * 400 / (BM_length * BM_length);
end
if (energy_model == 5)
    kb = 4 * 400 / (BM_length * BM_length);
end

invading_offrate = kf * exp(invading_energy * 4180 / temperature / 8.314) * (2 / BM_length);
incumbent_offrate = kf * exp(incumbent_energy * 4180 / temperature / 8.314) * (2 / BM_length);

%BM rate constant
BM_rate = kf * kb * incumbent_offrate / (invading_offrate * incumbent_offrate ...
    + kb * invading_offrate + kb * incumbent_offrate)

%critical concentration
c_crit = (0.1 / kf) * (invading_offrate * incumbent_offrate + kb * invading_offrate ...
    + kb * incumbent_offrate) / (incumbent_offrate + kb)

output = [BM_rate, c_crit];
```

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