Leakless Polymerase-Dependent Strand Displacement Systems

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Abstract

A grand challenge facing molecular programmers is the rational development of fast, robust, and isothermal architectures akin to "chemical central processing units" that can sense (bio-)chemical signals from their environment, perform complex computation, and orchestrate a physical response *in situ*. DNA strand displacement systems (DSDs) remain a compelling candidate, but are hampered by spurious reaction pathways that lead to incorrect output. DSDs that utilize the systematic *leakless* motif can be made arbitrarily robust at the cost of increasing redundancy and network size (scaling), and thus a degradation of kinetic performance. Another class of architectures utilize DNA hybridization, extension, and signal production of entirely sequestered outputs via strand-displacing polymerases (SDPs) that have resulted in impressive demonstrations; however, they face similar challenges of aberrant behavior such as mis-priming by incorrect signals. Our work introduces a unified polymerase-dependent toehold-mediated strand displacement (PD-TMSD) architecture that integrates the programmed specificity of DSDs with the unique advantages of SDPs. This unification enables systems that can be made arbitrarily robust, at any concentration range, without increasing network size. We propose a number of gate designs and composition rules to compute arbitrary Boolean functions, emulate arbitrary chemical reaction networks, and explore time-bounded probabilistic computation made possible by certain classes of SDPs. Our theoretical exploration is backed by preliminary experimental demonstrations. This contribution was inspired by the belief that molecular programming can meet or exceed the complexity exhibited in biology if we embrace its best understood molecular machinery and couple it with systematic design principles built upon a strong theoretical foundation.

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1 Introduction

DNA strand displacement (DSD) has emerged as a powerful and versatile mechanism for programming molecular interactions and constructing dynamic molecular circuits and nanoscale devices. In these systems, information is carried by DNA strands that interact through predictable and programmable Watson-Crick base pairing, enabling cascades of

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strand displacement reactions that form the basis of molecular computation [26]. The appeal of DNA strand displacement lies in its universality and scalability: any arbitrary reaction network can, in principle, be emulated by a suitable collection of strand displacement reactions, enabling the construction of dynamic reaction networks, logic circuits, oscillators, amplifiers, and neural networks [4, 20, 19, 23, 36, 30]. Despite the success of these systems, a key practical challenge has become evident. As these systems grow in complexity so-called *leak* reactions, in which outputs are spuriously produced in the absence of correct input, remain a persistent limitation. These typically result from unintended partial strand interactions or branch migration events, and can be exacerbated by poor DNA quality and fraying at the ends of DNA helices. Such leakage accumulates and severely degrades the reliability of large-scale DNA circuits. This not only limits circuit fidelity and scalability, but also poses major issues for applications like biosensing and diagnostics, where accurate circuit output remains paramount. The elimination or mitigation of leak has thus been recognized as a crucial component in scaling up DNA-based computation.

Strategies to mitigate leak include thresholding [23, 19], shadow cancellation circuits [28], and the systematic use of clamps at duplex ends [34]. The introduction of the "leakless" architecture marked a major step forward, offering the potential for arbitrarily low leak through the introduction of redundant domains [32, 35]. However, as the guarantee to leak suppression increases, so too does the required redundancy. This results in systems that require deeper reaction pathways and additional intermediates to propagate a signal. Moreover, without the use of systematic mismatches [11], sequence overlap between species introduces unproductive reactions via partial hybridization, leading to occlusion of necessary fuel complexes and propagating signals in the intended reaction pathway. This added complexity can complicate sequence design (to avoid crosstalk between partially overlapping domains) and slows productive reaction kinetics.

In parallel with the developments in enzyme-free DNA circuits, researchers have explored enzyme-assisted DNA computing architectures. Enzyme-assisted schemes bring catalytic speed-ups and active turnover of components, offering the potential for fast, biochemically efficient circuit operation. The PEN DNA toolbox, for example, combines a polymerase, an exonuclease, and a nicking enzyme to produce programmable chemical reaction networks (CRNs) [17, 1]. This strategy enabled the first practical realization of enzyme-assisted DNA dynamical circuits including synthetic DNA oscillators [17, 7] and bistable switchable memory circuits [16], demonstrating that DNA circuits could exhibit autonomous dynamic behaviors like those in biological networks. The versatility of the PEN toolbox has since been demonstrated in a wide range of applications, including isothermal digital detection of microRNAs [8], and spatial pattern formation in agent-based systems [9], establishing it as a robust and modular platform for dynamic molecular programming. More recently, synthetic molecular switches regulated by nicking endonucleases and polymerases have been built that enable enzymatically controlled ON/OFF switching of sticky end cohesion for structural reconfiguration of DNA-based materials [10]. However, reliance on multiple enzymatic components introduces complexity in design due to sequence constraints from enzyme recognition sites and reaction tuning, in addition to increased experimental complexity and potential for unintended reactions such as off-target nicking, incomplete degradation of DNA intermediates, and unintended cross-reactivity among circuit elements.

Simpler, enzyme-assisted architectures that rely on a single enzyme – most notably, strand-displacing DNA polymerases – to drive DNA circuits have also been explored. By harnessing the extension and strand-displacement activity of enzymes such as *Bst* or *Bsu* DNA polymerase, it is possible to design logic gates in which an input primer strand hybridizes to a gate and triggers polymerase-mediated extension, displacing a pre-annealed or previously

synthesized output strand. This displaced strand can then interact with downstream gates or reporter complexes. Notable implementations include polymerase-driven logic gates and catalytic amplifiers [27, 25, 31, 24]. Polymerase-mediated systems and primer exchange reactions have also been leveraged for programmable autonomous synthesis of single-stranded DNA and the construction of molecular event recorders that track the order in which distinct RNA inputs are detected [12]. Despite these advantages, such systems often suffer from reduced sequence discrimination and increased leak due to mispriming and nonspecific polymerase extension. Because polymerization can initiate from any hybridized strand with a free 3'-hydroxyl group, even partial hybridization or input mispairing – where an input is only partially complementary to an exposed domain – can trigger extension and result in spurious output. Furthermore, some architectures employing this motif are vulnerable to unproductive reaction pathways where the order in which inputs bind and are extended is critical to correct function – if correct inputs bind in an equally likely yet unintended order then the gate can deactivate thus preventing proper signal propagation.

This work introduces the first architecture that couples the robustness of leakless toehold-mediated DNA strand displacement (TMSD) with the enzymatic advantages afforded by polymerase-mediated strand displacement (PSD). Each gate complex integrates the specificity of TMSD – to discriminate, thermodynamically and kinetically, between correct and incorrect input – with the speed and strand displacing power of PSD – to produce sequence orthogonal output of arbitrary length, gated by a verified TMSD event. Leveraging PSD allows for the complete burial, in double-stranded form, of sequence unique to sequestered output. In contrast, every gate complex in an enzyme-free strand displacement system can bury at most a "toehold" length of each output sequence, relative to the displacing input(s).

By addressing leak both at the level of domain architecture and through enzymatic control, we circumvent common pitfalls leading to spurious reactions such as polymerase mispriming, branch migration-induced errors, and reaction breathing at elevated temperatures. In doing so, we also overcome key kinetic limitations of previous leakless systems caused by their required sequence redundancy – distributed across overlapping domains in multiple layers of related complexes – thereby eliminating an entire class of unproductive side-reactions and obviating the need to scale circuit size. Our system thus preserves the high specificity and robustness properties of prior (enzyme-free) leakless designs while leveraging the kinetic and scaling efficiencies made possible by strand displacing polymerases.

This contribution, arriving a decade after the original leakless architecture was proposed, extends the core design principles into an enzymatic domain, enabling a new class of leakless and modular molecular systems [32]. We believe this represents a significant advance in the design of molecularly programmable systems, enabling more scalable and reliable circuit construction and paving the way for larger and more efficient DNA-based CRNs.

Summary of contributions

This paper introduces a hybrid architecture, **polymerase-dependent toehold-mediated strand displacement (PD-TMSD)**, that integrates the precise, programmable control of DNA strand displacement systems (DSDs) with the kinetic advantages of strand-displacing polymerases (SDPs). The key contributions of this work are:

- We define a new class of molecular gates that leverage both toehold-mediated strand displacement and polymerase-mediated extension to achieve robust, leak-resistant signal propagation at arbitrary concentrations, without increasing network size.
- We formalize a set of design rules and polymerase extension constraints – including blocking via 3′-modifications, mismatch inhibition, and dNTP omission – to systematically control enzymatic activity within molecular circuits.

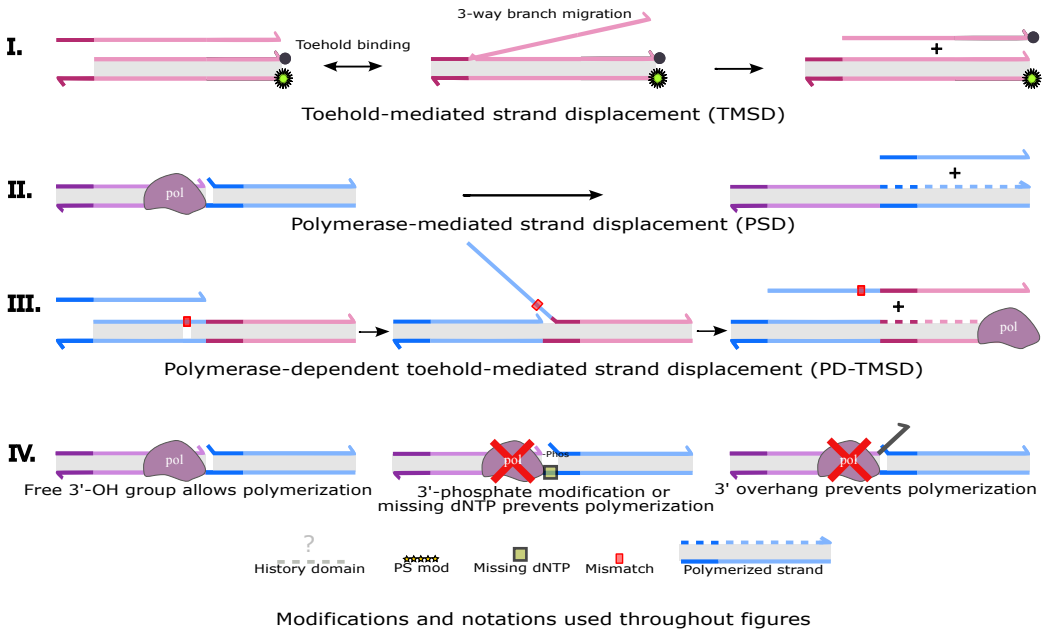


Figure 1 Overview of mechanisms and notations used throughout this work.

- We propose scalable composition strategies for PD-TMSD gates to compute arbitrary Boolean functions, emulate chemical reaction networks (CRNs), and implement time-bounded probabilistic computation.
- We provide a visual and theoretical framework for analyzing PD-TMSD systems, accompanied by preliminary experimental results (see Appendix).

Taken together, these contributions bridge the gap between two powerful yet previously disjoint paradigms – DSD and SDP – and lay the groundwork for molecular architectures that combine theoretical rigor with practical implementation.

2 Preliminaries

Figure 1 provides an overview of the key methodologies and modifications used throughout this work, with panels (I)–(IV) illustrating the core mechanisms and design principles used in our system.

I. Toehold-Mediated Strand Displacement (TMSD). Toehold-mediated strand displacement (TMSD) is a widely used mechanism in dynamic DNA nanotechnology. In TMSD, an incoming strand (the *invader*) binds to an exposed single-stranded region (the *toehold*). This interaction initiates branch migration, allowing the invader to displace an *incumbent* strand that is initially hybridized to the complementary domain.

II. Polymerase Strand Displacement (PSD). Polymerase-mediated strand displacement involves a strand-displacing DNA polymerase that extends a DNA primer by synthesizing a complementary strand along a template. During this process, the polymerase displaces downstream DNA strands hybridized to the same template, effectively replacing them with the newly synthesized strand.

III. Polymerase-Dependent Toehold-Mediated Strand Displacement (PD-TMSD). PD-TMSD is introduced in this work and combines the specificity of TMSD with the catalytic extension activity of a strand-displacing DNA polymerase. In this hybrid mechanism, toehold binding initiates a partial displacement into a complex and polymerase extension from a free 3′-OH group drives completion.

IV. Polymerase Extension Rules and Diagram Notation. Polymerase extension proceeds only from strands that terminate in a free 3′-OH group. Polymerization is blocked in the following cases:

- if the 3′ end is modified with a phosphate group
- if a missing dNTP is required to complement a position of the template downstream of the primer (denoted by a light green box)
- if the 3′ end is a mismatched overhang with the template

The following notational conventions are used throughout:

- **Dotted light gray lines with question marks** mark *history domains*.
- **Solid black regions** denote *clamps*. Each clamp domain is unique to its respective gate.
- Each signal is color-coded, with the *toehold domain* shown in a darker shade and the *long domain* in a lighter shade of the same color.
- **Yellow stars** denote phosphorothioate-modified backbone linkages, which resist exonuclease degradation (see Section 6.2).
- **Red boxes** indicate *mismatches*, which are used to help inputs "lock in" to their target complex and slow the rate that the incumbent can displace them back off, allowing time for the polymerase to find the free 3′ end.
- **Colored dotted lines** represent strands synthesized via polymerization.

These definitions and visual conventions serve as the foundation for interpreting the molecular designs and experimental results presented throughout this paper.

3 A simple motif for polymerase-dependent strand displacement

The main contribution of this work is the synthesis of two successful molecular computing architectures via the simple motif depicted as a translator gate, from input X to output Y, in Figure 2. The motif is composed of two logical steps:

(i) Input discrimination via TMSD

As in normal TMSD, incorrect inputs quickly fail during their invasion attempts due to the thermodynamic *and kinetic* penalty incurred when replacing a complementary sequence with one that is not. Input discrimination via displacement is typically absent in architectures that utilize strand-displacing polymerases. The reliance on thermodynamic discrimination alone among all possible hybridization events can limit the scalability in these other architectures, since a lack of kinetic sequence discrimination in the face of a growing number of sequences will lead to increased rates of mis-priming, subsequent polymerization extension, and thus spurious output production. In this section and in Section 4, we adopt a standardized structure for signal strands, each strand consisting of a unique identity sequence, with the toehold domain shown in a darker shade and the long domain in a lighter shade, followed by a universal domain depicted in yellow. This universal domain is identical across all signal strands. Although this domain could be made unique for each signal, logical correctness is still preserved when the same sequence is used across strands.

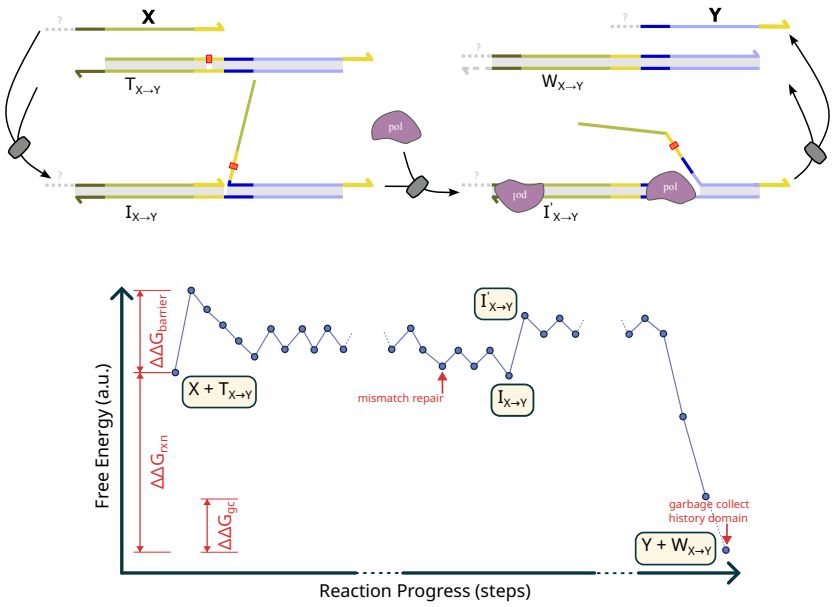


Figure 2 A translator gate converting an input signal X to a sequence orthogonal output signal Y using a single instance of the polymerase-dependent toehold-mediated strand displacement (PD-TMSD) motif. (a) The intended reaction pathway. (b) The intuitive-energy landscape [29].

(ii) Polymerase-mediated strand displacement

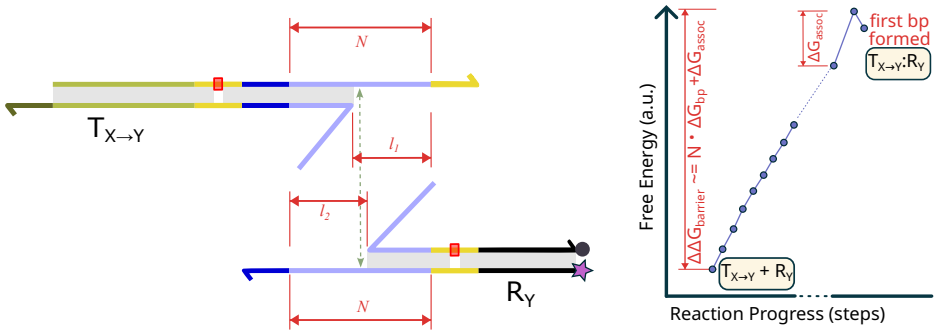
Only after successful consumption of an input via TMSD, and only if its 3′ end is paired with the template and contains a free hydroxyl group can polymerase extension and polymerase-mediated strand displacement of the output signal occur.

3.1 An arbitrarily high barrier to "leak" between gates

Remarkably, a single complex can produce an output signal that is sequence orthogonal to its input while guaranteeing an arbitrarily high energy barrier to leak. The intuition for this claim is depicted in Figure 3a: if a signal domain has not yet been produced then it is only found sequestered in double-stranded regions of gates thus necessitating an energy barrier to leak proportional to fraying open an entire long domain (see Figure 3b).

The robustness of this architecture is possible due to the complete sequestration of output signal domains in double stranded regions of gates. This point can be subtle. It is the systematic composition of this motif, into larger gates and then into entire circuits or reaction networks, that maintain an invariant on the barrier to leak. While our focus in this paper is on the description of the architecture, in the complete absence of input, it is a straightforward exercise to arrive at the following result. (We introduce `Join` and `Fork` gates in Section 4.)

▶ **Theorem 1.** *Given an arbitrary composition of PD-TMSD (activatable) translator,* `Join` *and* `Fork` *gates, with long domains of length N, then in the absence of input the production of a* first *output must overcome an energy barrier proportional to $\Theta(N)$.*



(a) (b)

Figure 3 Leak between two fuel complexes. (a) Initiation requires that a long-domain worth of base pairs fray open prior to the formation of the first intermolecular base pair. (b) The energy barrier to leak scales with N, the chosen length for long-domains. ΔG_{assoc} is the entropic cost of combining two complexes into one [5] and ΔG_{bp} is the average free energy of a base pair.

3.2 *Activatable* translator gates

The simple translator motif can be extended into a multi-armed complex that remains inactive until a specific signal is present to "deprotect" the gate, at which point it becomes functionally identical to the original translator. This conditional gating strategy enables the system to remain dormant, sensing and collecting input signals without triggering downstream reactions until the final readout step. As shown in Figure 4, this approach is particularly well-suited for long-term applications such as environmental monitoring – where inputs may accumulate over days or weeks – and fuel strands, dNTPs and polymerase can be withheld from the reaction mixture until readout.

3.3 Activatable reactions and modulating rate constants

The PD-TMSD architecture supports engineered systems that require control of relative rate constants. As with TMSD, PD-TMSD reaction rate constants can be modulated by the careful design of toehold strengths and and the introduction of secondary structure features into the branch migration domain such as mismatch introduction and mismatch replacement [15].

Additionally, the rate of the polymerase extension portion of a PD-TMSD reaction can also be modulated via sequence design and choice of dNTP concentrations. It is common to design signal strands over a three-letter code, reserving a fourth nucleotide that could be used as a rare or missing base to introduce conditional behavior. For example, one or more complements to a rare dNTP could be incorporated into the template strand of a gate, as illustrated in Figure 1 part IV. By changing the available concentration of the rare dNTP, the rate at which the polymerase is able to progress through that region can be tuned.

To prevent any polymerization through gates containing the rare dNTP complement, the corresponding dNTP could be omitted entirely from the reaction mixture, effectively disabling the associated reaction pathways. If desired, these pathways can later be activated by simply spiking in the missing dNTP, restoring gate function and enabling polymerase extension. The use of a missing dNTP to precisely halt polymerization has been previously demonstrated as an effective control mechanism in other contexts [12, 10].

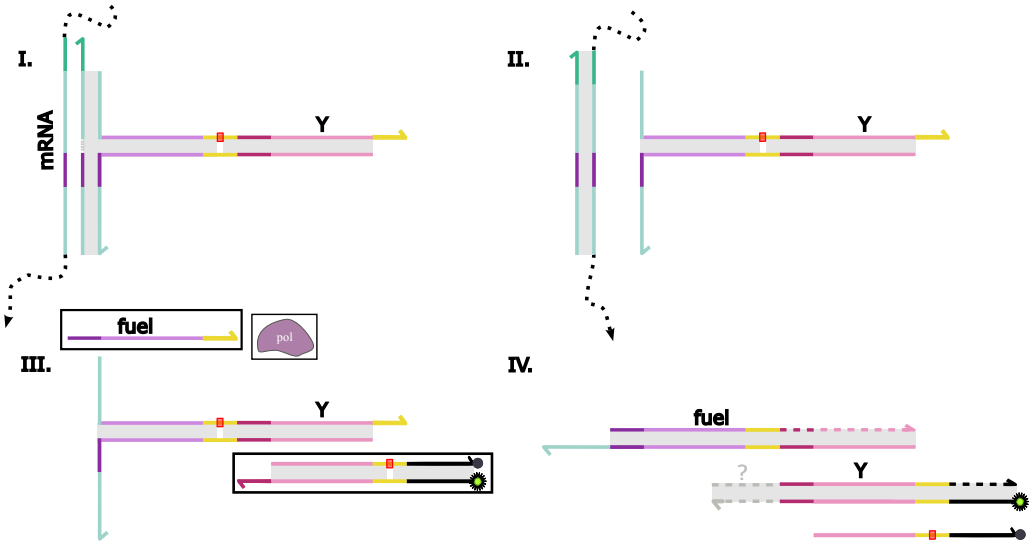


Figure 4 An activatable translator for long term environmental monitoring or detection of mRNA. Gates consume input mRNAs before fuel, reporter, and polymerase is added for quick readout.

4 Boolean circuits from leakless `Join` and `Fork` gates

In this section we introduce a leakless `Join` gate, capable of AND logic, and a leakless `Fork` gate capable of fanout. By design these gates naturally compose via the common PD-TMSD motif to realize arbitrary Boolean circuits that utilize dual-rail inputs and common gate outputs to emulate OR logic in the usual manner.[2] The signals in this section are compatible with the translator gates from Section 3 as they utilize the same domain structure for signal strands: a unique toehold domain, a unique long domain, and a common universal domain.

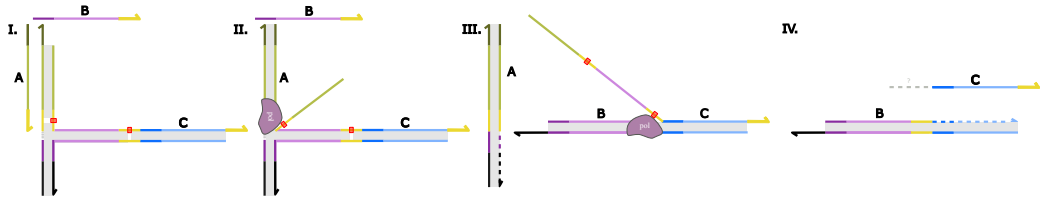
4.1 Leakless `Join`

Figure 5a introduces a three-stranded `Join` gate, a natural extension of the translator architecture and similar in style to the activatable translator. This design implements a conditional logic where the output C is only produced when both inputs A and B are present, and avoids sequence overlap between the two inputs – a desirable property for scalable systems.

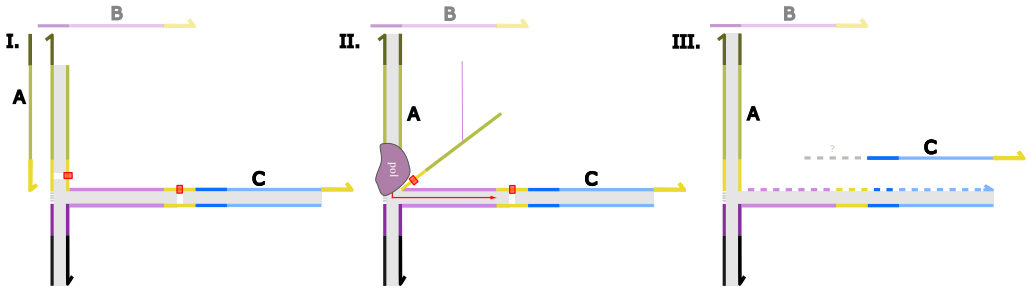
The gate operates as follows: input A first binds via TMSD to the exposed arm of the gate. This triggers polymerase-mediated extension, which displaces the *protector* strand. This displacement reveals a previously sequestered internal toehold, enabling input B to bind via TMSD, be extended by polymerase, and displace output C. In theory, this architecture realizes a leakless join operation, where both inputs are required sequentially for productive output.

However, in practice, we observed complications that undermine the theoretical leaklessness. Prior work [33] has shown that strand-displacing polymerases can exhibit template switching or "track jumping" in the absence of single-stranded binding proteins (SSBs), particularly when polymerizing through regions of secondary structure. In our gate, this

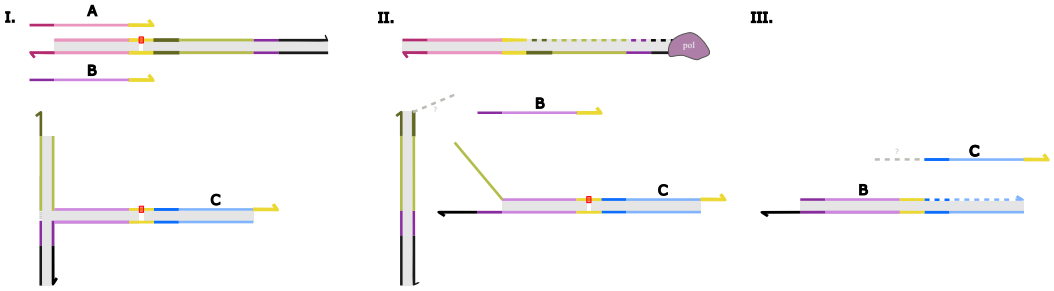
[2] For example, $\mathsf{C} := \mathsf{OR}(\mathsf{A}, \mathsf{B})$ can be realized by the parallel reactions $\mathsf{A} \to \mathsf{C}$ and $\mathsf{B} \to \mathsf{C}$.



(a) A multi-arm join gate that is *leakless* in theory.

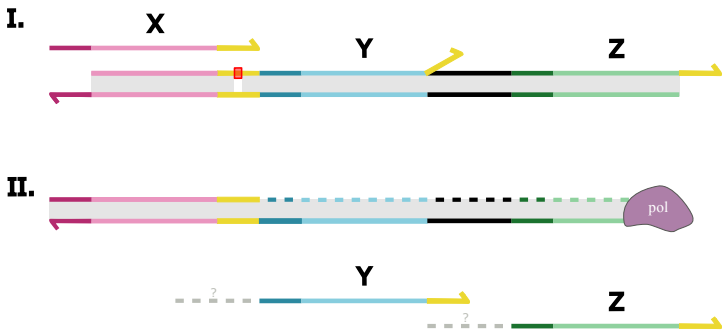


(b) Off-target "track jumping" activity of polymerase can lead to spurious output of C, absent input B. Here input B is faded out to denote its absence.

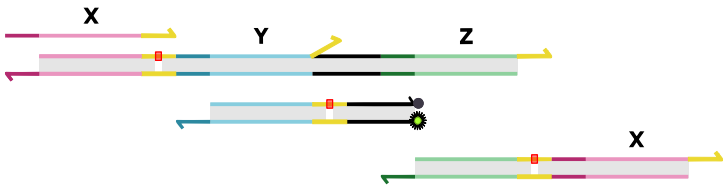


(c) Leakless `Join` that eliminates potential for "track jumping".

Figure 5 `Join` gates that are leakless in theory and practice.



(a) A leakless `Fork` gate.



(b) Composing the same `Fork` gate with a translator gate, that converts Z back into X, results in a linear amplifier.

Figure 6 Signal fanout and amplification using leakless `Fork` and translator gates.

behavior results in polymerase switching from the intended path to the adjacent duplex arm, producing spurious output C even without input A (Figure 5b). Thus, despite its appealing design, the gate is not functionally leakless under experimental conditions (data not shown).

To address the issue of track jumping, we added a specialized PD-TMSD translator gate (Figure 5c) for correct computation. In this design, input A binds the translator gate that produces an output strand which can remove the entire *protecting* strand on the three-stranded complex through TMSD. This eliminates the possibility of the polymerase choosing to follow an incorrect strand as the template. Following this deprotection event, an internal toehold is revealed that allows input B to bind and extend, resulting in the production of output C. Functionally, this gate resembles the activatable translator introduced in Section 3.2, where signal-dependent deprotection enables conditional activation, here utilized to implement join logic. This structural similarity highlights the modularity of the PD-TMSD motif and its adaptability to different computational tasks.

4.2 Leakless `Fork`

Figure 6a presents a leakless `Fork` gate that converts a single input X into two outputs, Y and Z. When input X binds to the fork gate via TMSD, it is extended by a polymerase, subsequently displacing two signal strands Y and Z. The overhang of the universal domain following signal Y has a free 3′ end; however, it is sufficiently long and a designed to mismatch with the template in order to prevent spurious initiation of polymerase extension. The `Fork` gate can be generalized to produce an arbitrary number of outputs by extending the template strand to accommodate additional bound signals to be released via PD-TMSD initiated by a single input.

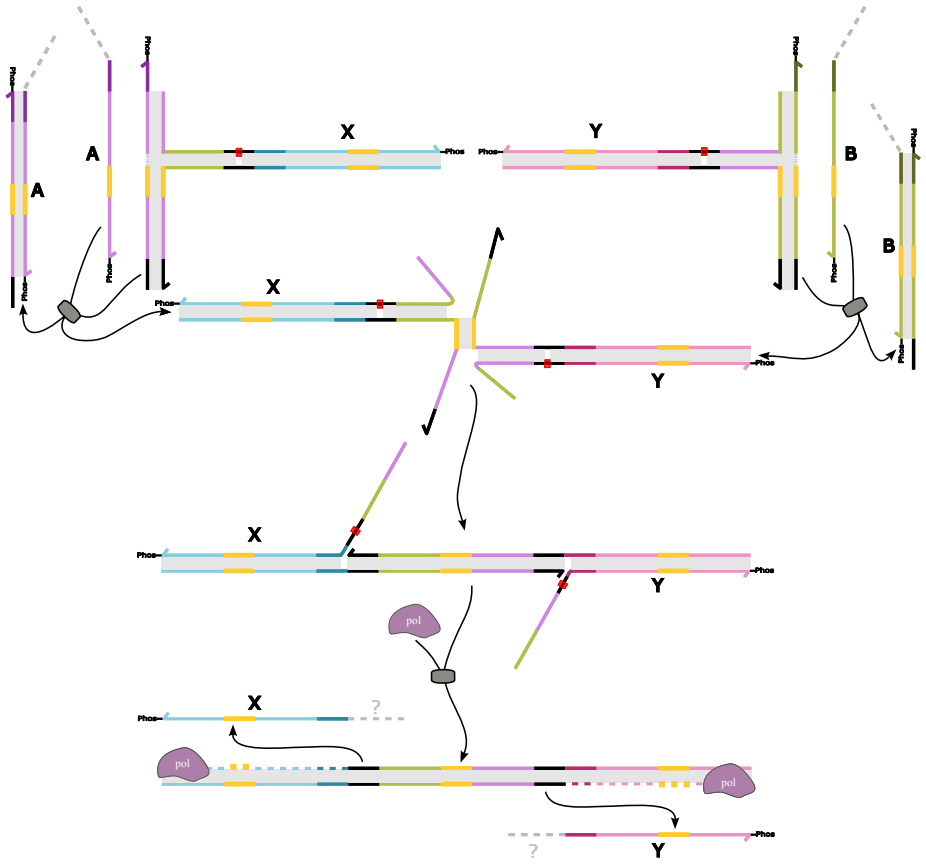


Figure 7 *Pas de deux* (PDD) bimolecular reaction gate pathway.

By adding a single additional translator gate that converts output Z back into input X, signal fanout can be transformed into a linear signal amplifier (Figure 6b). This implementation enables signal amplification using only one polymerase step per gate, and two per cycle, using a minimal set of components.

5 Emulating arbitrary chemical reaction networks

While our previously proposed `Join` gate and `Fork` gate seemingly compose to implement the bimolecular reaction $A + B \rightarrow X + Y$ that is not the case. In particular, the `Join` gate can irreversibly consume its first input in the complete absence of the second. While this behavior is fine for feedforward networks, dynamical systems such as oscillators require reactants to be (irreversibly) consumed, and then products produced, only when all reactants are present. In the remainder of this section we focus on a gate that implements *rateless* bimolecular reactions, but point the reader to Section 3.3 for a discussion of how rate constants can be programmed with these gates.

Pas de deux bimolecular reaction gate

We introduce the "*pas de deux*" (PDD) gate.[3] Like its namesake, it exhibits symmetry and elegant coordination of a pair as they act alone and then in a choreographed sequence to achieve their collective aim. Although the sequence of events in our system unfolds slightly out of order compared to a *grand pas de deux*, the underlying motifs remain the same. Each input first interacts with its respective gate, stripping off the *protector* strand via toehold-mediated strand exchange (TMSE) – this step mirrors the solo variations of male and female dancers, each showcasing their individual abilities. The combination of 3′ phosphate modifications on inputs and toeholds to prevent polymerization along with TMSE at this stage, render these reactions reversible. Only when both inputs are present can the two deprotected components of the gates come together – analogous to the adagio when dancers join in a coordinated, slow duet. In the final stage of the reaction, the polymerase is able to find and extend from the two free 3′ hydroxyl groups, displacing both output strands in a final, coordinated motion – this mirrors the coda where both dancers re-emerge with renewed energy, and the performance culminates in a dazzling, synchronized flourish.

6 Practical considerations and exploitations

6.1 Improving PSD kinetics with mismatches

To facilitate more efficient strand displacement by Klenow fragment (exo-) polymerase, we introduced intentional mismatches into the duplex regions downstream of input binding. Unlike more processive polymerases or those operating at elevated temperatures – where decreased duplex stability can aid in duplex unwinding – Klenow operates at relatively low temperatures (25–37°C) and lacks intrinsic helicase activity, making polymerase-mediated strand displacement more challenging. In natural systems, single-stranded binding proteins (SSBs) have been shown to enhance strand displacement activity by binding and stabilizing the displaced strand, preventing its rehybridization to the template, thus enabling continuous polymerase progression [18]. However, because our system uses single-stranded DNA as signal strands, the inclusion of SSBs is incompatible, as they would bind these functional signals and interfere with downstream reactions. In the absence of such stabilizing factors, the displaced strand can readily rehybridize to the template, creating a kinetic barrier to polymerase progression. By incorporating mismatches or bulges into the displaced strand, we reduce its hybridization stability and effectively slow the reverse reaction, lowering the energy barrier for forward strand displacement synthesis. A similar strategy has been observed in biological systems; for example, HIV-1 reverse transcriptase leverages single unpaired nucleotides or bulged structures within its RNA genome to promote strand displacement and enhance polymerase processivity through otherwise inhibitory duplex regions [13]. Consistent with this, Srinivas *et al.* (2013) showed that branch migration initiation can be a key rate-limiting step in strand displacement and that tuning the stability of the incumbent strand's interactions with the template can modulate these interactions to favor forward displacement [29]. This design strategy provides a simple and effective means to improve polymerization kinetics in systems where traditional accessory proteins cannot be used.

[3] In classical ballet, "*pas de deux*" (French for "step of two") is a duet typically performed by a male and female dancer in concert. A *grand pas de deux* follows the structure of an entrée, a slow partnered adagio, individual solos for each dancer, and culminates in a fast virtuosic coda danced in unison.

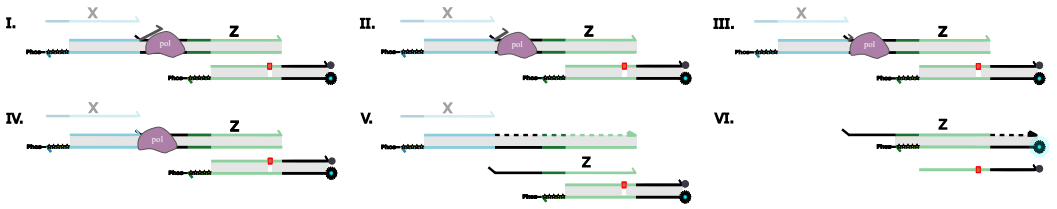


Figure 8 A molecular fuse gate utilizing the $3' \to 5'$ exonuclease activity of Klenow fragment polymerase. Signal X is shown faded out to denote its absence.

6.2 Exploitations of proof-reading polymerases

While the majority of this paper focuses on systems built with the exonuclease-deficient Klenow fragment (Klenow exo−), our architecture is readily adaptable to polymerases that retain $3' \to 5'$ exonuclease activity, such as the full Klenow fragment polymerase. Before exploring the new behaviors this enables, we first address several important design considerations necessary for working with proofreading polymerases.

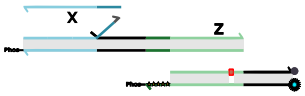
In systems using strand displacing polymerases with an exonuclease domain, all exposed $3'$ overhangs – including toeholds – must be protected from degradation. This is typically achieved using phosphorothioate (PS) backbone modifications, which render the strand resistant to exonuclease activity. Once these protective modifications are in place, the exonuclease activity of the polymerase can be harnessed as a feature rather than a threat. In particular, we can exploit the gradual degradation of single-stranded overhangs by exonuclease activity.

As the polymerase chews back mismatched or unpaired regions from the $3'$ end of a strand, it will eventually reveal a perfectly matched primer-template duplex. At this point, the enzyme can initiate extension. This delayed onset of polymerization effectively functions as a molecular timer, with the length of the single-stranded region acting as a programmable delay: the longer the overhang, the longer the delay before polymerization can proceed.

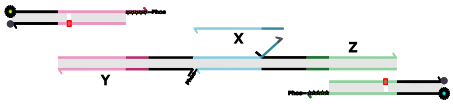
Timed delay elements are increasingly important in molecular systems that require sequential, transient, or conditional behavior. Prior work has demonstrated timer circuits and sequential release mechanisms using DNA strand displacement [6, 22, 14], as well as enzymatic timers for pulse generation and orthogonal delays [2, 3, 21]. However, many of these designs depend on auxiliary structures, multi-step cascades, or reaction-specific configurations, which can limit composability and generalizability. Our approach offers a simple and scalable alternative: delay elements based on a single enzyme and tunable overhangs. We use this principle to build a new class of timing-enabled molecular gates, that highlight how proofreading polymerases, when used strategically, can unlock compact and programmable temporal control in DNA-based molecular computation.

A molecular "*fuse*"

Here we introduce the molecular fuse gate (Figure 8), where polymerization is triggered after a tunable delay set by the length of the gray single-stranded overhang. In the absence of input X, the proofreading polymerase begins degrading the overhang from the $3'$ end via its exonuclease activity. If sufficient time passes and the overhang is completely digested, the polymerase will reach a fully paired primer-template junction and initiate extension. This results in the production of an output that can be routed into another reaction gate or reporter complex, so fluorescent output can be observed.



(a) A time-bounded probabilistic implementation of `NOT` X.



(b) A time-bounded probabilistic implementation of `if` X `then` Y `else` Z.

Figure 9 Time-bounded probabilistic gates.

In contrast, when input X is present, it binds to the overhang and displaces the primer strand via standard strand displacement, allowing for immediate polymerase extension – functionally behaving like a conventional translator gate. The fuse gate thus acts as a *timed checkpoint*: if a particular input is not detected within a programmable time window, determined by the overhang length, a default output is produced. This behavior enables built-in responses in molecular circuits that require temporal decision-making in the absence of expected inputs.

Time-bounded probabilistic `NOT` gates and thresholds

Figure 9a shows a probabilistic `NOT` gate that operates within a bounded time window. In the absence of input X, the proofreading polymerase gradually degrades the long single-stranded overhang from the $3'$ end. Once fully degraded, polymerase extension proceeds, displacing an output strand that can trigger a fluorescent reporter or other downstream gate. In the presence of input X, the overhang serves as a toehold, enabling X to bind and displace the primer strand before it can be extended. This inhibits production of output Z, acting as a logical `NOT` gate.

However, this behavior is temporally constrained: the $3' \to 5'$ exonuclease activity will eventually begin degrading not just the spacer domain (gray), but also the toehold for X. As the toehold shortens, the probability that X can successfully bind and displace the primer decreases. Thus, the likelihood of output production increases over time – even in the presence of X – leading to a time-bounded probabilistic inversion. Early in the time course, X has a high chance of binding and blocking signal propagation; later, as its binding site erodes, the system effectively "commits" to the `NOT` operation being bypassed.

Figure 9b extends the probabilistic `NOT` gate architecture by introducing a second output arm, resulting in a time-bounded `If-Then-Else` gate. In this system, the polymerase can generate one of two outputs – Y or Z – depending on the presence or absence of input X. In the absence of X, the exonuclease slowly degrades the long single-stranded overhang. Once fully processed, polymerase extension proceeds, leading to the production of output Z, which can be routed to a specific reporter (*e.g.*, a fluorescent channel) or downstream gate. This mirrors the behavior of the fuse and `NOT` gates described earlier. In the presence of X, the overhang instead serves as a toehold, allowing X to bind and displace the primer strand. This both prevents the generation of Z and triggers the release of output Y through strand displacement, enabling an alternate reporter or reaction pathway. The resulting behavior can be logically described as: If X, then output Y; else output Z. As with the `NOT` gate, this decision is time-sensitive – if X fails to bind before its toehold is degraded then the system defaults to producing Z.

7 Discussion

We presented a versatile molecular programming framework (PD-TMSD) that integrates toehold-mediated strand displacement with the enzymatic activity of strand-displacing polymerases to realize an unprecedented level of robustness in a compact, programmable molecular implementation. This system is capable of implementing arbitrary Boolean circuits and emulating arbitrary chemical reaction networks. While polymerase-based systems expand the design space for molecular logic and signal processing, they also introduce practical constraints and benefits that must be considered for real-world applications. We explored these trade-offs in novel uses of time-dependent probabilistic gates. In practice, unlike enzyme-free systems, these enzyme-driven architectures depend on the activity and stability of the polymerase. Many commonly used polymerases are not stable at ambient temperatures and have limited functional lifetimes. Although some thermostable variants exist, most require refrigeration or freezing to preserve activity, and not all polymerases are amenable to lyophilization. As a result, systems that rely on polymerase function – particularly for biosensing or diagnostic applications – may require a cold-chain infrastructure, which can limit deployment in resource-limited or field-based environments. Despite these limitations, the modularity, composability, and kinetic control offered by PD-TMSD systems open new possibilities for building robust, programmable molecular systems.

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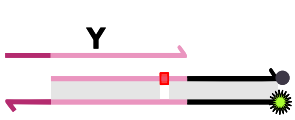
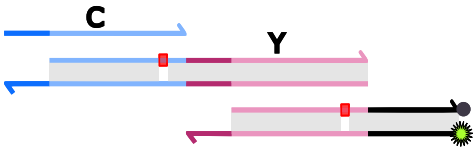
A Supplementary Information

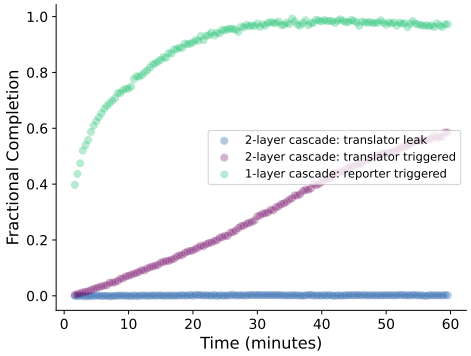
A.1 Preliminary experimental demonstration



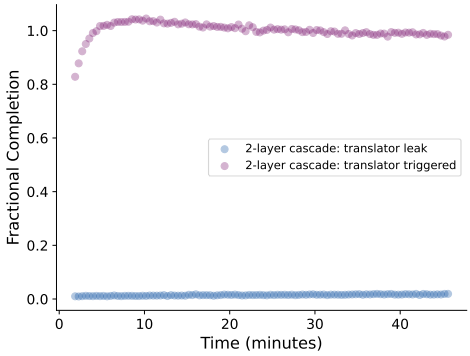
(a) 1-layer cascade.



(b) 2-layer cascade.



(c) $= 0.3\mathbf{U}$ polymerase.



(d) $= 2\mathbf{U}$ polymerase.

Figure 10 Shallow cascades with varying concentrations of polymerase. Translator, reporter, and inputs were always at $1\times = 100\,\text{nM}$ and polymerase concentration was at $0.03\,\text{U}/\mu\text{L}$ or $0.2\,\text{U}/\mu\text{L}$. $10\,\mu\text{L}$ reactions were performed in NEB r2.1 buffer at 37°C.

We performed two preliminary experiments with 1-layer and 2-layer cascades to validate the feasibility of our PD-TMSD architecture in achieving fast, programmable signal propagation while maintaining minimal leak – properties critical for building scalable molecular computing architectures. Note that this experimental architecture is modified from the proposed motif in the main paper, with signal strands lacking the universal domain following a unique toehold and long domain. In the single-layer cascade, fluorescent output is produced through the toehold-mediated strand displacement of trigger Y into the reporter complex, followed by the polymerase-mediated strand displacement from the $3'$ end of the trigger through the clamp region, resulting in the quencher strand being displaced and observed fluorescence (Figure 10a). The two-layer cascade introduces an additional translator upstream of the reporter (Figure 10b). Here, the initial trigger C goes through the translator via the same PD-TMSD mechanism, producing trigger Y which can then interact with the reporter.

Fractional completion is used as a normalized measure of reporter signal in our experiments. A value of 1.0 corresponds to the maximum fluorescence from a fully triggered reporter, while the value of 0.0 corresponds to a fully quenched reporter.

We first evaluated the systems at low polymerase concentration (0.3 units of Klenow fragment exo-). The 1-layer cascade exhibited a reaction halftime of approximately 2.5 mins, reaching completion within 30 minutes. As expected, the 2-layer cascade experienced a kinetic slowdown with a halftime of approximately 50 minutes, reflecting the additional step introduced by the translator (Figure 10c). When the polymerase concentration was increased to 2 units, we observed a dramatic acceleration in the 2-layer cascade with the reaction completing in less than 5 minutes and a half-time that was complete prior to first fluorescence measurement (Figure 10d). This tunable speed demonstrates a key advantage of PD-TMSD, reaction kinetics – up to a certain point – can be tuned through enzyme concentration, enabling rapid signal propagation without need for architectural redesign.

Importantly, the improvement in kinetics of the triggered cases did not come at the cost of increased leak in the cases lacking input. Even at elevated polymerase concentrations, the background signal in the absence of input remained minimal over the observed time period. This confirms the compatibility of toehold-mediated and polymerase-driven strand displacement, enabling the construction of molecular systems that are both fast and robust. These preliminary experiments demonstrate that our underlying theoretical framework provides a strong foundation for the construction of larger and more sophisticated architectures.

A.2 DNA sequences

Strand ID	Sequence
Signal-Y	CCTATCCACTCTCACCT
Quencher-top	CCACTCTCATCTTACACATTCCAAA/3BHQ_1/
Reporter-bottom	/5$ATTO488N$/TTTGGAATGTGTAAGGTGAGAGTGGATAGG
Signal-C	CCACACTTCTCTTCTCC
C-Y-translator-top	CTTCTCTTCACCCCTATCCACTCTCACCT
C-Y-translator-bottom	AGGTGAGTGTGGAGAGGGGAGAAGAGAAGTGTGG

A.3 DNA sequence design and gate preparation

DNA sequences were designed using NUPACK `dna04` model at 25°C, 50mM Na^+, and 12.5mM Mg^{2+}. All signal strands were designed to be unstructured, over the A, T, C alphabet, and orthogonal to each other in order to minimize occlusion. Strands were ordered from Integrated

DNA Technologies, dried and unpurified at 100 nmole scale. Single strands were dPAGE purified in a 15% polyacrylamide gel with a 5% stacking gel using a Hoefer SE6000X Chroma Deluxe Electrophoresis unit heated to 55°C.

To prepare gates, strands were annealed with 1.2× excess top strand in 1× TEM buffer(12.5 mM $MgCl_2$, 10 mM Tris, 1 mM EDTA, pH 8.0). Annealing protocol was executed in an Eppendorf Mastercycler Nexus thermocycler, heating to 90°C over two minutes then cooling by 1°C/min to 20°C.

Complexes were PAGE purified in a 12% polyacrylamide gel with 6% stacking gel in 1× TAE/$MgCl_2$ buffer (40 mM Tris-acetate, 1mM EDTA, 12.5 mM $MgCl_2$, pH 8.0) using a Hoefer SE6000X Chroma Deluxe Electrophoresis unit. Following purification, desired band was excised from gel, cut into strips, and eluted into 1× TEMT (12.5 mM $MgCl_2$, 10 mM Tris, 1 mM EDTA, 0.01% Tween, pH 8.0) buffer overnight. Following elution, stock concentrations were estimated from A260 measurements on a Nanodrop One.

A.4 Fluorescence spectroscopy

All experiments were conducted on a Biotek Cytation 5 microplate reader with a bottom read and 100 gain. The excitation and emission wavelengths for used for ATTO488 were 500 nm and 525 nm, respectively.

Individual components for each experiment were combined using an ECHO 525 Acoustic Liquid Handler (Beckman Coulter) into Corning® 384 well plates. Each experimental condition presented was prepared in duplicate or triplicate. After transfers from ECHO were complete, the plates were vortexed and centrifuged. Plates were read within 2 minutes of coming out of ECHO.