Design and Characterization of DNA Strand-Displacement Circuits in Serum-Supplemented Cell Medium

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ABSTRACT: The functional stability and lifetimes of synthetic molecular circuits in biological environments are important for long-term, stable sensors or controllers of cell or tissue behavior. DNA-based molecular circuits, in particular DNA strand-displacement circuits, provide simple and effective biocompatible control mechanisms and sensors, but are vulnerable to digestion by nucleases present in living tissues and serum-supplemented cell culture. The stability of double-stranded and single-stranded DNA circuit components in serum-supplemented cell medium and the corresponding effect of nuclease-mediated degradation on circuit performance were characterized to determine the major routes of degradation and DNA strand-displacement circuit failure. Simple circuit design choices, such as the use of 5′ toeholds within the DNA complexes used as reactants in the strand-displacement reactions and the termination of single-stranded components with DNA hairpin domains at the 3′ termini, significantly increase the functional lifetime of the circuit components in the presence of nucleases. Simulations of multireaction circuits, guided by the experimentally measured operation of single-reaction circuits, enable predictive realization of multilayer and competitive-reaction circuit behavior. Together, these results provide a basic route to increased DNA circuit stability in cell culture environments.

KEYWORDS: molecular circuits, DNA strand displacement, nuclease, serum, degradation

Tasks across biotechnology, including drug targeting and release, in vitro cell culture, diagnostics, and tissue engineering, could benefit from the ability to use the concentration in time and space of several different biomarkers to make decisions such as directing release or collection processes. Ideally, such decision-making processes could be performed autonomously by molecular agents or circuits as they are accomplished in living systems. Developing such methods requires augmenting molecular detection techniques with molecular circuits that can reliably operate under physiological conditions.

One potential pathway to building these systems is through the use of DNA strand-displacement reactions. Circuits that are capable of signal amplification,† timing control,‡ complex logic functions,§ and directing the release of molecules or other material control processes have shown promising applications in cell culture or with cells,§,‖ or for protein and RNA detection due to the robust nature of DNA sequence specificity and innately high biocompatibility. However, current DNA-based technologies have limited applications and lifetimes in blood serum and serum-supplemented cell cultures due to the presence of nucleases that degrade the DNA components. Current approaches to using or protecting DNA complexes in serum or inside cells are generally of limited success because they require molecular components that are difficult or expensive to synthesize, or require medium additives that can damage cells or prevent their growth. New approaches to enable strand-displacement circuits to operate in serum are therefore still needed.

In this study, we investigate the usage of simple and inexpensive design principles of DNA strand-displacement circuits to enable these circuits to detect concentrations, operate signal cascades, and control timing in cell culture environments over as long as 70 h. The lifetimes of double-stranded fluorophore- and quencher-modified DNA complexes that perform these functions were measured for both 3′ and 5′ single-stranded overhangs (toeholds). Compared to 3′ toeholds, complexes with 5′ toeholds showed an increase in functional operation in the serum we tested. Additionally, single-stranded DNA species were shown to have an increased lifetime when a hairpin domain was added to the 3′ termini. DNA strand-displacement circuits designed with 5′ toeholds and 3′ hairpin domains were shown to have reaction rate constants dependent on toehold length in serum-supplemented cell media with the same rate of reaction rate constant slowdown with decreasing toehold length as in standard TAE/Mg2+ buffer. To investigate the operation of larger, more complex DNA circuits in the presence of nucleases, we developed a model using standard enzyme reaction dynamics.

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and DNA strand-displacement molecular reactions. The model was able to qualitatively predict the behavior of a multistage strand-displacement cascade and a timer circuit. While single-layer circuits can reliably operate for at least 12 h, more complex circuits are affected by nuclease degradation to a greater extent, and responses are most affected when the concentration of inputs is low.

## Results and Discussion

### Inhibition of Nuclease Using Competitor Molecules.

In order to protect the DNA strand-displacement circuit components from potential nuclease-mediated degradation, we first investigated the use of additional biocompatible medium supplements that may act as competitive inhibitors to screen the nuclease from circuit components. In a DNA strand-displacement reaction, an invading strand binds a substrate complex and displaces an incumbent strand. The invading strand binds to a single-stranded “toehold” region of the substrate complex and undergoes branch migration with the incumbent strand of the substrate until the incumbent strand is fully displaced (Figure 1A). In our studies, a double-stranded DNA complex modified with a fluorophore and quencher on complementary strands, called a Reporter, was used as a basis for measuring the functional stability of the circuit components in the presence of nuclease. A 5 base pair (bp) toehold on the 5’ end enabled strand displacement to occur upon the addition of the invading strand, termed the Output, which in a more complex circuit would be the released species whose concentration would be measured by interaction with the Reporter complex (Figure 1A). When the Output interacts with a Reporter complex, the fluorescence increases due to the Output displacing the quencher-modified strand. Upon degradation of the Reporter complex by nuclease, the two strands of the Reporter complex may dehybridize, also causing an increase in fluorescence (Figure 1B). Thus, a change in the measured fluorescence of a sample containing nucleases can be due to either the reaction of the Reporter complex with an Output strand or digestion of the Reporter complex by nucleases. In DMEM with 10% fetal bovine serum, nuclease induced degradation of the Reporter complexes causes complex dehybridization (Figure 1C).

Actin has previously been shown to inhibit the activity of DNases.24-26 When actin was added to a final concentration of 200 nM to serum-supplemented DMEM, the increase in fluorescence over 18 h from 100 nM Reporter complexes was reduced by half (Figure 1C). To further reduce the degradation of Reporter complexes, a second DNA complex designed to act as a competitive inhibitor for nucleases was added to the medium at 10 μM. This mimic DNA contained a double-stranded region of the same length as the region in the Reporter complex and 5 bp long single-stranded overhangs on the 3’ end of each of the sequences (see Supplemental Table 10 for sequences). The inclusion of both this mimic and actin in the serum-supplemented DMEM further reduced the rate of degradation of the Reporter complex (Figure 1C). Finally, 12 μM poly(T)20 single-stranded DNA and 0.1 mg/mL single-stranded DNA from salmon testes were added to act as competitors for nucleases that bind single-stranded DNA. Together, the inclusion of these four additives in serum-supplemented medium reduced the rate of fluorescence increase of the Reporter complex in the absence of Output (which is presumably due to nuclease degradation) by about an order of magnitude. Unless otherwise specified, all DNA strand-displacement reactions in the experiments that follow were performed in serum-supplemented DMEM containing actin, poly(T)20 mimic DNA, and single-stranded DNA from salmon testes at the concentrations given above, which we term nuclease-screened medium or NS medium.

### Design of DNA Circuit Components with Increased Nuclease Resistance.

In order to build DNA strand-displacement circuits that can reliably process signals using reaction cascades in serum-supplemented medium, we next investigated how changes to the complexes themselves could reduce the rate of nuclease digestion without eliminating the ability to use the complexes to design molecular circuits with additional...
controlled rates. The Reporter complex was again chosen as a model for the investigation of DNA circuit component design. In addition to the degradation of double-stranded domains by exonucleases (Figure 1C), exonucleases can digest any single-stranded region (Figure 1B). Since Reporter toeholds are generally placed at either the 5′ or 3′ termini of complexes (Figure 1A), the toehold is susceptible to degradation from exonucleases, leading to the loss of the toehold domain and reactivity to Output strand input over time (Figure 1B). For each base removed from the toehold, the reaction rate constant between the Reporter and Output species is expected to decrease by an order of magnitude, based on measurements in standard buffers.27

To characterize the functional operation of Reporter complexes, the maximum fluorescence of the mixtures was measured after Output strands were added to 5′ or 3′ toehold Reporter complexes incubated for different periods of time at 37 °C in NS medium (Figure 2). Reporters with 3′ toeholds showed a marked decrease in the change in fluorescence upon Output addition for Reporter incubation times as short as 6 h, indicating that the incubation of the Reporter significantly reduced its ability to interact with the Output. In contrast, Reporters with 5′ toeholds showed only a 25% drop in fluorescence when the Reporter complexes were incubated in NS medium for 70 h prior to addition of the Output strands. Further, the time to reach reaction equilibrium upon the addition of the Output strand was much slower for the Reporter with the 3′ toehold as compared to the Reporter with a 5′ toehold, suggesting that the kinetics of strand displacement was affected more by incubation for the Reporter with the 3′ toehold (Supplemental Figure 1). The difference between the responses of the two types of complexes that was observed is consistent with a significantly larger concentration or activity of 3′ exonucleases than 5′ exonucleases present in the serum.28,29

For strand-displacement circuits to operate reliably in serum, DNA complexes and strands that interact with or are released by the complexes (e.g., Output strands) must remain intact when they are in single-stranded form rather than being degraded by nucleases. Two possible DNA modifications, phosphorothioate backbones23,30,31 and an inverted thymine modified base30 placed at strand termini (Supplemental Figure 2A), have previously been shown to increase the stability of single-stranded DNA molecules against nuclease digestion. To test whether strands containing phosphorothioate bonds (PS-bonds) or an inverted dT (Inv-dT) modification would remain capable of participating in strand-displacement reactions in medium for longer periods of time than unmodified strands, we designed a series of Output strands with these modifications at different sites and measured the fraction of the strands that were able to react with the Reporter to produce a change in fluorescence as a function of its initial incubation time in NS medium. Output strands containing 7 nucleotides with PS-bonds at both termini and strands terminated with an Inv-dT at the 3′ end showed about a 25% decrease in the concentration of strands capable of strand-displacement reactions over 7 h of incubation in NS medium (Supplemental Figure 2). The concentration of reactive unmodified Output strands decreased 85% in the same amount of time. Additionally, DNA strands with PS-bonds demonstrated a significant decrease in reaction rate compared to unmodified or Inv-dT modified DNA, indicating a decrease in strand invasion capabilities for DNA with PS-bonds.23 This was further demonstrated using DNA strands fully composed of PS-bonds (Supplemental Figure 2). Thus, the use of modified DNA backbones designed to resist nuclease degradation did not sufficiently increase the Output strand’s lifetime over 7 h of incubation in nuclease-screened medium while maintaining high reactivity with the Reporter complex.

Since the majority of exonuclease attack single-stranded DNA at their 3′ termini,28,29 DNA strands containing inert, double-stranded DNA regions on the 3′ ends of the resulting complex/strand would be expected to have longer lifetimes in exonuclease-containing serum-supplemented DMEM than unmodified strands.28,29 We thus designed a modified Output strand with a hairpin added to its 3′ end (Figure 3), providing a terminal double-stranded region to inhibit exonuclease interactions. When we used Output strands with this additional domain at the 3′ end, the stability of the Output in NS medium was greatly improved over 7 h of incubation (Figure 3, Supplemental Figure 3). However, degradation did occur at longer times, with very little of the Output being able to react with the Reporter complex after 55 h. In contrast, 85% of the unmodified strands were no longer able to react with the Reporter after just 7 h. This modification therefore enables single-stranded species to operate over extended time periods without chemical modification. Thus, modifying 3′ termini with

**Figure 2.** Comparison of the functional stability of Reporter complexes with either 3′ or 5′ toeholds in nuclease-screened serum-supplemented DMEM. Output strands (250 nM) were added to 200 nM Reporter complexes at 0.5, 2, 6, 24, 32, 48, 56, 66, and 72 h post Reporter addition to medium at 37 °C. Reporter complexes with a 3′ toehold showed a marked decrease in fluorescence intensity change when the Output was added at subsequent time points compared to the sustained reactivity of the Reporter with a 5′ toehold. Both complexes show an increase in fluorescence in the absence of Output over time due to the presence of nucleases, potentially leading to the decrease in 5′ Reporter reactivity that was observed (Supplemental Figure 1). Points are relative to the intensity change upon Output addition at 0.5 h of incubation (Supplemental Note 1) and are the average value of 2–3 replicates. Error bars are one standard deviation of replicate results.
hairpins, the use of nuclease-screened medium, and the use of 5’ toehold Reporter complexes together suggested a way by which we could design strand-displacement circuits that can operate in cell medium.

To further demonstrate the versatility of using 3’ terminal hairpins to protect 3’ termini from exonuclease attack, we redesigned the Reporter with 3’ toeholds to include a 3’ hairpin adjacent to the toehold region (Supplemental Figure 4). We measured the Output strand activity in experiments analogous to those shown in Figure 2 for this modified Reporter. The addition of the 3’ hairpin increased the functional lifetime of the 3’ toehold Reporter to slightly less than a 5’ toehold Reporter over 32 h. Thus, DNA strand-displacement architectures that depend on free 3’ termini for toehold domains and reversible reactions could operate as reliably as those with only 5’ toeholds if a hairpin motif is included at the 3’ end.

Operation of Simple DNA Strand-Displacement Circuits. To investigate how well the NS medium and 3’ hairpin modifications work together to protect the DNA components of strand-displacement circuits from nuclease-mediated degradation, we developed a simple DNA strand-displacement circuit, termed a Release module, that is designed to release an Output strand at a programmed rate upon the displacement circuit, termed a Release module, that is designed mediated degradation, we developed a simple DNA strand-displacement process after incubation in serum was tested using the Reporter complex shown. (B) The relative concentrations of 100 nM Output strands, with or without the hairpin, were measured using a 5’ toehold Reporter (200 nM) that was added after different periods of Output incubation in NS medium. Output strands with the hairpin domain showed sustained stability over 7 h of incubation. Without the hairpin domain, Output strands were largely degraded within 7 h. Degradation is measured as the average value of relative Output concentration over 55 h of incubation.

To verify that the kinetics of strand-displacement reactions could also be controlled by varying the length of the toehold when the reactions occurred in NS medium at 37 °C, we measured the kinetics of Output release using Source complexes with toeholds of length 0, 2, and 5 bases in length presented at the 5’ end. Green colored toeholds (t) are 5 base pairs, and numbered domains are 13 base pairs. The 1 domain is split into a and b parts where b’ serves as a “clamp,” or energy barrier that reduces the rate of undesired strand-displacement reactions. The black hairpin domains on the Output and Initiator strands decrease the propensity for exonuclease-mediated degradation at the 3’ ends of single-stranded DNA regions without significantly affecting the rates of hybridization or strand displacement.

is critical for building strand-displacement cascades with reliable responses to different inputs and timing.

To verify that the kinetics of strand-displacement reactions could also be controlled by varying the length of the toehold when the reactions occurred in NS medium at 37 °C, we measured the kinetics of Output release using Source complexes with toeholds of length 0, 2, and 5 bases. Each Source complex was combined with Initiator, and the Output strand concentration was measured as a function of time by measuring the fluorescence produced by its reaction with a 5’
toehold Reporter (Supplemental Note 2, Supplemental Figure S). The kinetics were measured using a Source concentration of 200 nM for toehold lengths of 2 and 5 bases with Initiator concentrations ranging from 0 to 300 nM. Since the kinetics were slower for 0 nucleotide toeholds, higher reactant concentrations were used. The Source and Initiator concentrations were kept equal and ranged from 0.5 to 1.5 μM. In each case, the Reporter concentration was at 200 nM. In all cases, some increase in fluorescence was observed when Reporter and Source complexes were mixed together before Initiator was added (Figure S). This initial increase was higher than when the Reporter was incubated alone in NS medium (Supplemental Figure S). One possible reason for the increase is the degradation of Source complexes by nuclease into partially degraded, but still “active”, Output strands. Since a strand-displacement reaction is expected to occur between an Output and Reporter complex so long as the Output has a toehold and a free 1 domain (Figure 4), some excision fragments of the Source after nuclease digestion could lead to the release of partial Output strands that are “active” because they have a contiguous toehold and 1 domain. While this reaction occurs, it is generally not the dominant pathway for Output release. For each reaction studied, the rate of Output released from strand-displacement reactions was faster than the rate of release without Initiator present, i.e., through nuclease-mediated release alone.

To test whether 3’ hairpins and 5’ toehold design choices alone enable reliable operation of strand-displacement circuits in cell medium without the addition of the actin and DNA that functioned as a screen for nucleases, we conducted the same Release reactions using Source complexes with 5 bp or with 2 bp toeholds without nuclease-screening components (Supplemental Figure 6). We found that, without the nuclease-screening components, the rate of digestion of Reporter and Source complexes in the absence of Initiator increased 2-fold over 40 h of incubation. Additionally, for Source complexes with a 2 bp toehold, only a minimal difference in [Disassembled Reporter] was observed for Initiator concentrations between 50 and 300 nM.

Modeling DNA Strand-Displacement Circuits under Nuclease-Degrading Conditions. To design strand displacement systems with reliable kinetics and dose–response behavior in serum, we will need to be able to predict the kinetics of both strand-displacement and degradation-mediated release of oligonucleotides. To work toward such a capacity for prediction, we developed a model of circuit kinetics (section SI 2) that incorporates both standard strand-displacement reactions3,4 and reactions for nuclease–substrate binding.25,35 Our goal was to build the simplest possible model that is capable of predicting the kinetics we expected to observe, so our model does not necessarily completely capture the many potential degradation pathways and interactions between intermediates in the system.

The model uses mass action kinetics and makes the standard assumption that enzymes initially reversibly bind to their substrate to form an intermediate. An irreversible reaction involving the enzyme–substrate complex produces a product species and a newly freed enzyme. To reduce the number of species and potential reactions, actin and the other screening components were grouped into a general class of molecules termed “Inhibitors”, which reacted with nucleases through the following composite reactions:

\[
E \text{ + Inh} \xrightarrow{k_{E\text{Irev}}} E\text{:Inh} \xrightarrow{k_{E\text{Ideg}}} E + \text{waste}
\]

where E represents the combined pool of endonuclease and exonuclease enzymes, Inh are inhibitors, and E:Inh is an enzyme–inhibitor complex. While we assume in eq 1 that actin is degraded by nucleases for simplicity, this has not been shown to be the case thus far experimentally.

DNA complexes that were strand-displacement circuit components were assumed to interact with and be degraded by nucleases via a similar set of composite reactions. Each DNA complex was assumed to first be degraded by nucleases into a partial complex, with partial complexes then being degraded
into waste/product molecules. This model produces the delayed degradation dynamics observed in experiments involving Reporter alone or with Source but no Initiator molecule (Supplemental Figures 7 and 9–12). To model the increase in fluorescence that is caused by the separation of the FAM fluorophore and quencher molecule when the Reporter complexes are degraded, we used the reactions

\[
E + \text{Rep} \xrightarrow{k_{ER\text{rev}}} E:\text{Rep} \xrightarrow{k_{	ext{E}R \text{deg}}} E + \text{Rep}_{\text{partial}}
\]

\[
E + \text{Rep}_{\text{partial}} \xrightarrow{k_{ER\text{rev}}} E:\text{Rep}_{\text{partial}} \xrightarrow{k_{	ext{E}R \text{deg}}} E + \text{FAM} + \text{Quencher}
\]

(2)

(3)

where \text{Rep} and \text{Rep}_{\text{partial}} represent the complete and partially degraded Reporter complex, and FAM is a species containing a FAM fluorophore, which, because of the extent of degradation, now does not react significantly with other species. The degradation of other double-stranded DNA molecules, such as the Source complex, is modeled in the same manner as the degradation of the Reporter, except that the degradation products were either an “active” Output strand or waste DNA molecules:

\[
E + \text{Source} \xrightarrow{k_{ES\text{rev}}} E:\text{Source} \xrightarrow{k_{	ext{E}S \text{deg}}} E + \text{Source}_{\text{partial}}
\]

\[
E + \text{Source}_{\text{partial}} \xrightarrow{k_{ES\text{rev}}} E:\text{Source}_{\text{partial}} \xrightarrow{k_{	ext{E}S \text{deg}}} E + \text{waste}
\]

\[
E + \text{Source}_{\text{partial}} \xrightarrow{k_{ES\text{rev}}} E:\text{Source}_{\text{partial}} \xrightarrow{k_{	ext{E}S \text{deg}}} E + \text{Output}
\]

(4)

(5)

(6)

An “active” Output strand contains at least part of the toehold domain and is capable of conducting toehold-mediated strand-displacement reactions with downstream complexes (e.g., a Reporter) to completion. These equations account for the observed fluorescence intensity increase of the Source and Reporter mixtures in the absence of Initiator strands over that of Reporter complexes alone (Figure 5).

To model the degradation of the single-stranded DNA components of strand-displacement circuits (e.g., Initiator and Output strands), we used a similar model, except that only one round of degradation was assumed to be sufficient for complete inactivation of the strand as this simpler model was sufficient to capture most of the kinetics observed in Figure 3:

\[
E + \text{ssDNA} \xrightarrow{k_{	ext{E}ss\text{DNA}\text{rev}}} E:\text{ssDNA} \xrightarrow{k_{	ext{E}ss\text{DNA}\text{deg}}} E + \text{Waste}
\]

(7)

where ssDNA is a single-stranded DNA component (e.g., Initiator or Output).

Finally, the DNA strand-displacement reactions were modeled as bimolecular reactions following earlier methods.3,4 Both intact and partially degraded complexes (e.g., Source_{\text{partial}} and Reporter_{\text{partial}}) were assumed to react with their respective input species:

\[
\text{Output} + \text{Rep}_{\text{report}} \xrightarrow{k_{E\text{FAM}}} \text{FAM} + \text{Quencher}
\]

(8)

\[
\text{Output} + \text{Rep}_{\text{partial}} \xrightarrow{k_{E\text{FAM}}} \text{FAM} + \text{Quencher}
\]

(9)

\[
\text{Source} + \text{Initiator} \xrightarrow{k_{\text{Output}}} \text{Output} + \text{Waste}
\]

(10)

\[
\text{Source}_{\text{partial}} + \text{Initiator} \xrightarrow{k_{\text{Output}}} \text{Output} + \text{Waste}
\]

(11)

Using the described model as a foundation, we next sought to fit the rate constants for each reaction by constraining the model using the experimental data presented in Figures 2, 3, and 5 and section SI 2. We used the experiments for the reaction involving the Reporter and Output alone (Figure 2, Supplemental Figure 7) to fit reaction rate constants for eqs 1–3, 8, and 9. We were able to find rate constants for nuclease-directed degradation and strand-displacement reactions that together closely recapitulated the reaction kinetics observed in Figure 3. In NS medium at 37 °C, we fitted a reaction rate constant of 8.5(±0.2) × 10^3 1/M-s for the strand-displacement reaction involving the Reporter and the Output, slightly less than 10-fold slower than the rate measured in TAE/Mg^2+ at 25 °C, 5 × 10^4 1/M-s.27 A slower reaction rate constant in NS medium could be due to transient binding between Reporter complexes and nucleases and the lower concentration of divalent cations, but higher concentration of monovalent cations, in DMEM (1.8 mM Ca^2+, 0.8 mM Mg^2+, 5.4 mM K+, 154.5 mM Na+) compared to the 12.5 mM Mg^2+ present in TAE/Mg^2+.13,36,37

To avoid fitting a large number of parameters simultaneously, we used the fitted reaction rate constants for the Reporter reaction to next fit a model involving Release reactions. We used data from Figure 5 to fit reaction rate constants for reactions between nucleases and Source complexes with 0, 2, and 5 base pair toeholds and found that the model was sufficient to capture most of the kinetics that were observed in experiments. In contrast to the fitted reaction rate constant between the Output and Reporter complexes, the fits to the rate constants between the Source and Output were comparable to the rate constants for the same reactions in TAE/Mg^2+ at 25 °C (section SI 2.3).27 In general, the model and parameter fits demonstrate that interactions with nucleases in serum-supplemented medium are the primary contributors to deviations from established behavior of these circuits in TAE/Mg^2+, as compared to changes in temperature (e.g., 25 vs 37 °C) or ion concentrations.

Due to the assumptions in our model, the reaction rate parameters for nuclease-dependent reactions should be considered qualitative in nature. The unknown concentrations and relative activities of the individual nuclease subtypes and their respective interactions with each inhibitor molecule mean that the model will have to be recalibrated for different serum concentrations or formulations.
Additionally, because the measurements of changes in fluorescence due to Reporter complex digestion or interaction with the Output only indirectly capture the kinetics of nuclelease-driven digestion, and the rates of these processes vary widely, the confidence intervals for some fits to reaction rates were very wide. Despite these limitations, the accuracy with which this simple model is able to capture the dynamics of most of the reactions occurring in NS medium across a large variation in strand-displacement reaction rate constants demonstrates the potential for this or similar models to be used as predictors of DNA strand-displacement circuit behavior in serum.

Predicting and Verifying the Operation of Complex DNA Strand-Displacement Circuits in Medium. To understand how well the methods for reducing the effect of nucleases on DNA strand-displacement circuits scaled with complexity of DNA circuit systems, we used the model described in eqs 1–11 to design and build two additional circuit systems, a multistage cascade and a timer, and investigate their function. These reactions were chosen because they illustrate the use of two design principles for scaling: the multistage cascade uses multiple reactions in parallel, while the timer circuit uses multiple reactions that occur in series.

In the multistage cascade, an Initiator signal interacts with a first complex to release a second signal, which then interacts with a second complex to release another signal, and so on, until within the final layer the signal reacts with a final complex to release an output. We designed and characterized the kinetics of a two-layer cascade, where an Initiator interacts with a Source at the second layer to release a strand that is the Initiator for the first layer. This Initiator then interacts with another Source to release the Output (Figure 6A). The kinetics of this process can then be measured by recording the change in fluorescence of a Reporter complex that the Output strand can react with.

Before experimentally characterizing the kinetics of the 2-layer cascade reactions, we used our model of strand displacement and nuclease degradation and the fitted reaction rate constants to predict the kinetics of the cascade (Figure 6B). In the absence of nuclease-driven degradation, the concentration of disassembled Reporter would be expected to converge to the initial concentration of the Initiator, provided that the concentration of Source is at least as high at each step in the cascade. We observed that, in the 1-layer cascade in NS medium, the disassembled Reporter concentration was generally higher than this value, and this trend was enhanced in the 2-layer cascade circuits. Because the concentration of disassembled Reporter is also limited by the total concentration of Reporter, the 2-layer cascade circuits had reduced sensitivity in its output fluorescence to different concentrations of input Initiator concentration compared to the single layer circuit. This is expected in the 2-layer cascade because there are two sets of Source complexes that can be degraded to trigger an output in the absence of inputs (section SI 3.1). Cascades with slower kinetics of strand displacement, i.e., those with a lower number of nucleotides in the toehold domain, had a narrow range of disassembled Reporter concentrations in response to the range of tested input concentrations, presumably because there was more time for nuclease-mediated degradation to occur before the strand-displacement cascade could reach its final state. The simulations therefore predict that, for circuits with slow release of Output strand over time, better schemes for preventing DNA degradation will be necessary to ensure that the dynamics of the circuit are not dominated by nonspecific nuclease degradation.

To verify the simulations’ predictions, we experimentally implemented a two-layer cascade reaction (along with a reporter reaction) using 5 bp toeholds as initiation domains (Figure 6C). Overall, the model predicted the trend of Output release, but there were quantitative differences between the model predictions and the experimental measurements. While the model predicted that there would be significantly different rates of Reporter disassembly depending on whether 0 or 50 nM Initiator2 was provided as input, there was no difference in these rates in experiments. This discrepancy suggests that the model underestimated the rate of degradation of Initiator strands, Output strands, or Source complexes.

We also developed a model for the behavior of a timer circuit that controls the delay before the release of an output begins and its rate of release (section SI 3.2). The circuit consists of a 0 bp Release reaction (Figure 4) and a delay reaction (Supplemental Figure 17). The Release reaction slowly releases Output, which is quickly resequestered by Delay complexes until all Delay complexes have been reacted. We used our model to predict the release kinetics of the Output for a timer circuit as a function of the concentration of the Delay complexes, which should control the length of the delay before the Output begins to be released. The simulations predicted that increasing the Delay concentration decreased the rate of Output release in the absence of Initiator, because “active” Output released from degraded Source complexes can become

Figure 6. Operation of multilayer cascade circuits in cell medium. (A) Schematic of the DNA complexes used in a two-layer cascade reaction. Initiator2 reacts with a Source2 complex to produce Initiator1, Initiator1 then reacts with Source1 complexes to release an Output. Reporter complexes (Figure 4) are used to detect the rate of Output release over time. (B) Simulation of a 2-layer cascade with 5 bp toeholds using the fitted parameters as described in the text. [I]0 is the concentration of Initiator2. (C) Experimental operation of the 2-layer cascade circuit. Both Source complexes are at 200 nM and contained 5 bp toeholds in both the simulation and experiments. Reporter complexes are at 200 nM.
ressequistered by Delay complexes. Further, we observed that changing the initial Delay concentration appeared to have only a minor effect on the timing of Output release from the circuit in the presence of Initiator. Experimental measurements of the timer circuit (Supplemental Figures 19 and 20) showed that the model overpredicted the rate of Source and Delay complex degradation. Ultimately, both the experiments and model agreed that the timer circuit did not function well in serum because the Delay complex is incapable of efficiently ressequistering Output strands after they are released from the Source complex. While, in buffers without serum, the Output is captured by Delay complexes until they are fully depleted at a reliable rate, the increased rate of release from the Source complexes, which are very abundant, and the degradation of some Delay complexes due to interactions with nucleases mean that the rate at which these parallel reactions occur is very difficult to control in serum.

CONCLUSION

In this paper, we developed methods to allow DNA strand-displacement circuits to operate in cell medium supplemented with 10% FBS serum, with the goal of designing molecular systems that can detect and respond to specific concentrations and combinations of molecules by releasing different concentrations of output species. Nucleases present in the serum degraded single-stranded DNA via their 3’ ends and double-stranded DNA complexes via endonuclease activity. By inhibiting nuclease activity using actin protein, and by modifying DNA complexes with hairpin extensions on the 3’ ends of DNA strands, the half-life of DNA strands increased 10-fold.

Using these modifications to the system, we were able to build a circuit that releases a desired Output strand with kinetics controlled by the length of the toehold domain. Fits to a model of the system showed that reaction rate constants between Initiator and Source complexes were comparable to reactions conducted in TAE/Mg2+ at 25 °C despite the additional DNA-binding enzymes in serum that could reduce the effective reaction rate constant and the difference in temperature. By including interactions between nucleases and DNA circuit components, the model was able to predict the dynamic behavior of Output strand release in one- and twolayer circuits. The use of models lays the groundwork for the design of more complex circuits going forward.

While we demonstrate the predictability of the DNA strand-displacement circuits using the presented model, the behavior of the circuits is still far from ideal for quantitative applications due to the strong interactions with nucleases that causes a significant loss of circuit material over time. One potential solution to enable applicability in in vitro cell culture experiments is to use serum-free medium, or KnockOut Serum Replacement or heat-inactivated serum as a medium-supplement. However, the feasibility of both those methods largely depends on the cell type of interest and the deviation of cell behavior due to variation from the more commonly used FBS-supplemented conditions.

Nucleases are expected to be present in both in vitro and in vivo settings due to nucleases secreted from dying cells or those present in blood serum, supporting greater relevancy of testing DNA strand-displacement circuits under nuclease-present conditions. It is important to note, however, that the strategy developed here of using a nuclease-screened medium is not applicable for in vivo devices. Implementing strand-displace-

MATERIALS AND METHODS

Reagents. Fetal bovine serum (FBS, cat. no. 10437010) and penicillin/streptomycin (P/S, cat. no. 15140148) were purchased from Thermo Fisher Scientific. Dulbecco’s modified Eagle’s medium (DMEM, cat. no. D6429) and single-stranded DNA from salmon testes (STssDNA, cat. no. D7656) were purchased from Sigma-Aldrich. Chicken gizzard muscle actin protein (cat. no. AS99) was purchased from Cytoskeleton, Inc. Standard cell culture medium was prepared as DMEM supplemented with 10% FBS and 1% P/S and stored up to one month. All DNA for strand-displacement circuits was purchased from Integrated DNA Technologies, Inc., with standard desalting purification except for quencher-modified strands, which were ordered HPLC purified. Sequences of all DNA strands used here are listed in Supplemental Table 10.

Preparation of DNA Complexes. Source, Delay, and Reporter complexes were annealed in 1× TAE buffer supplemented with 12.5 mM magnesium acetate (TAE/Mg2+) from 90 to 20 °C using an Eppendorf PCR at 1°C/min. Zero base pair toehold Source complexes were incubated 1:1 with their Initiator strand without a hairpin domain for ∼16 h prior to PAGE purification to remove some poorly formed or synthesized DNA complexes. After annealing, Source and Delay complexes were PAGE purified at 150 V for 3 or 1.5 h using 15% or 10% polyacrylamide gels. The complexes were eluted from the excised gel band using TAE/Mg2+ and overnight incubation. Reporter complexes were not PAGE purified.

qPCR Measurements of DNA Strand-Displacement Reactions in Cell Medium. Agilent Stratagene Mx3000 or Mx3005 qPCRs were used to test the stability and lifetime of DNA strands and complexes in serum-supplemented DMEM at 37 °C. The final volume of liquid in each well was fixed at 150 μL. Of this volume, 120–140 μL was serum-supplemented DMEM and the remaining volume consisted of nuclease-screening components and DNA complexes and strands. Additional components were added to the medium to screen for additional DNA-binding enzymes in serum that could reduce the effective reaction rate constant and the difference in temperature. By including interactions between nucleases and DNA circuit components, the model was able to predict the dynamic behavior of Output strand release in one- and twolayer circuits. The use of models lays the groundwork for the design of more complex circuits going forward.

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the nucleases for increased protection of DNA strand-displacement circuit components. Actin protein (200 nM), 12 μM poly(T)10 single-stranded DNA, 0.1 mg/mL STssDNA, and 10 μM double-stranded DNA complex with two 3′ single-stranded DNA tails (mimic DNA) were mixed with the serum-supplemented DMEM immediately prior to the addition of stranded DNA tails (mimic DNA) were mixed with the serum-supplemented DMEM immediately prior to the addition of circuit components. This mixture is referred to as nuclease-screened medium (NS medium) in the text. The fluorescence intensity of Reporter complexes alone (prior to nuclease degradation) was subtracted from all measurements (Supplemental Note 2). For Release, cascade, and timer experiments, the Reporter was initially added at 200 nM, followed by the Source and/or Delay complexes. Initiator strands were added about 1–4 h post Source addition.

**ASSOCIATED CONTENT**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00105.

Characterization of DNA circuit component degradation and modeling and fitting DNA strand-displacement reactions in serum (PDF)

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**Notes**

The authors declare no competing financial interest.

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