DNA Strand-Displacement Timer Circuits
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Supporting Information

ABSTRACT: Chemical circuits can coordinate elaborate sequences of events in cells and tissues, from the self-assembly of biological complexes to the sequence of embryonic development. However, autonomously directing the timing of events in synthetic systems using chemical signals remains challenging. Here we demonstrate that a simple synthetic DNA strand-displacement circuit can release target sequences of DNA into solution at a constant rate after a tunable delay that can range from hours to days. The rates of DNA release can be tuned to the order of 1–100 nM per day. Multiple timer circuits can release different DNA strands at different rates and times in the same solution. This circuit can thus facilitate precise coordination of chemical events in vitro without external stimulation.

While gene networks in cells can orchestrate intricate processes by modulating gene expression to release a series of target molecules at specified times,1,2,3 synthetic in vitro biochemical protocols commonly involve manual steps performed by an experimenter, in which reagents are added, filtered, heated or otherwise altered. Artificial mechanisms to automate the temporal release of trigger molecules would make it possible to direct sequential events without the need for external stimulation. Further, the timed release of molecules using such a process could act as a trigger to control acellular external stimulation.  

The timer circuit consists of two simultaneous abstract chemical processes: production (eq 1) and delay (eq 2)

\[
\text{source} \xrightarrow{k_{\text{prod}}} O
\]  

(1)

\[
O + D \xrightarrow{k_{\text{delay}}} O_{\text{waste}}
\]  

(2)

In the production process, the output O is released by a zero-order reaction at a constant rate \( k_{\text{prod}} \). In the delay reaction, O is rapidly converted into inert waste when it reacts with a delay species D. If \( k_{\text{delay}}[O][D] \gg k_{\text{prod}} \), O cannot accumulate until all of D has been depleted (Figure 1).

We call the time during which O cannot accumulate the delay time, \( t_{\text{delay}} \), which is the time needed to produce enough O to consume all of the D that is initially present:

\[
t_{\text{delay}} = \frac{[D]_0}{k_{\text{prod}}}
\]  

(3)

This time can be easily tuned by changing the initial concentration of D.

During the delay period, \([O]\) remains very small because any molecules of O that are produced are rapidly removed. After D
is depleted, however, [O] increases linearly with time. The approximate concentration of free O is therefore

\[
[O] \approx \begin{cases} 
  \frac{k_{\text{prod}}}{k_{\text{delay}}(D)} & \text{if } t < t_{\text{delay}} \\
  k_{\text{prod}}(t - t_{\text{delay}}), & \text{otherwise}
\end{cases}
\]

(4)

where [O] and [D] are functions of time.

To construct a timer circuit that controls the release of a DNA strand, we built a set of DNA strand-displacement reactions that emulate the abstract reactions in eqs 1 and 2 (Figure 2). The domain level structure of our complexes follows a DNA architecture previously used for Boolean logic circuit evaluation. Within this implementation, strand O is initially partially bound within a complex. The production process frees O from this complex, making O available in its full single-stranded form (Figure 2a). The delay process likewise sequesters O in a waste complex in which the toehold domain of O is covered (Figure 2b). Because an exposed toehold domain is generally required to initiate downstream strand-displacement reactions, the delay circuit will control when O is available in a functional form.

**Production.** The production reaction releases an output molecule O when a source complex S and an initiator strand I react (Figure 2a). This strand-displacement process is facilitated by the spontaneous pairing and unpairing of the bases on the ends of the source complex, i.e., fraying. During the short time periods when these bases are frayed, I can bind and compete with O until one or the other is displaced.

Reactions initiated by fraying alone have an exceptionally small reaction rate constant, on the order of 0.5 M⁻¹ s⁻¹, which we denote by \( k_{\text{prod}}^{\text{op}} \). On the order of days, very little S and I react, allowing us to assume \([S]\) and \([I]\) remain effectively constant when considering shorter time scales. To release an appreciable concentration of O, we use a large amount of S and I in a reaction. The rate at which O is released into solution can therefore be approximated as a constant we term \( k_{\text{prod}} \):

\[
\frac{d[O]}{dt} = k_{\text{op}}[S][I] \approx k_{\text{prod}}
\]

(5)

Eq 5 shows that \( k_{\text{prod}} \) can be easily tuned by changing the initial concentrations of S and I. For simplicity, and to maximize the time during which the approximation of constant concentrations is reasonable, we keep their initial concentrations equal, i.e., \([S]_0 = [I]_0\). At longer time scales, the approximation of a constant \( k_{\text{prod}} \) is violated and [O] increases according to second order reaction kinetics.

**Delay.** To keep [O] low while the delay species is present, the delay reaction must sequester O at a rate much faster than \( k_{\text{prod}} \). The delay complex D (Figure 2b), which has a 7 base pair (bp) single-stranded toehold domain that binds to O and colocalizes it with D, acts as a concentration thresholding device. Reactions mediated by 7 bp toeholds proceed at approximately 6 orders of magnitude faster than reactions without a mediating toehold.24,25 A 2 bp clamp (green in Figure 2b) inhibits some undesired interactions between S and D while ensuring that the reaction between O and D remains strongly forward-biased. Clamps with only 1 bp may not reliably prevent interactions at that end and clamps with greater numbers of bases (e.g., 5 bp) are expected to strongly decrease the sequestering ability of the thresholding device due to reaction reversibility.11,17

To monitor the reaction’s progress, we also include a reporter complex modified with a fluorophore and an associated quencher to track the concentration of free O over time. This complex reacts reversibly with the output strand on a time scale much faster than the production reaction, but slower than the delay process, and produces fluorescence as a function of [O] at a given time (Figure 2c). The concentration of O is related to the fluorescence levels using a calibration curve (SI 1).26,27 To build a timer circuit, the source complex S and the delay complex D are initially combined and the timer is triggered upon the addition of the initiator strand I.

To demonstrate that the individual reactions performed as desired and determine how the production rate varied with initial concentrations of S and I, we tested each reaction in isolation at 25 °C (Figure 3a,b, SI 2). To test the production reaction, we varied \([S]_0 = [I]_0\) from 0.25 μM to 2 μM (Figure 3a).
Figure 3. Production, Delay and Timer circuit reactions. (a) Production kinetics in the absence of D. Release rate is dependent upon initial [S] and [I]. (b) [O] decreases proportionally to added [D]. (c) Delay time was tuned by changing the initial concentration of the delay species D. [S]0 = [I]0 = 1 μM. (d) Plot of delay time vs initial concentration of D, showing an approximately linear relationship after an initial offset.

3a) and determined the average $k_{\text{prod}}$ to be 0.49 ± 0.13 M$^{-1}$ s$^{-1}$ (SI Table S2) which is in good agreement with previous estimates.24,25 We calculated, using $[S]_0$ and $[I]_0$, that $k_{\text{prod}}$ varied from 0.15 to 4 nM/hour over the range of concentrations tested (SI Table S2). When the delay reaction was tested in isolation, the delay complex sequestered free O, resulting in a sudden decrease in output detected by the reporter (SI Figure S7). The decrease in [O] matched the concentration of D added to the reaction solution (Figure 3b).

To characterize the delay time before S begins accumulating as a function of $[D]_0$ (eqs 3 and 4), we varied $[D]_0$ while keeping the production rate (determined by $[S]_0$ and $[I]_0$) constant. O remained low for a delay period that increased with $[D]_0$ (Figure 3c). For each trial, we used linear least-squares fitting to identify the portion of the production regime with the steepest slope (SI 3) and used the slope and y-intercept of this fit to calculate the delay time. This method allowed us to measure the delay time without being affected by the ramp up in release that occurs because small amounts of D are still present when release noticeably begins. We observed that the delay time varied linearly with respect to the initial concentration of D (Figure 3d). We also tested two other timer circuits with lower initial concentrations of S, I and D, and obtained systems with similar delay times but slower rates of output release (SI 3).

Because the delay circuit is based on DNA strand-displacement events involving a particular DNA sequence, it is possible to create multiple circuits that use different sequences and can trigger the delayed release of two different DNA strands (Figure 4). To characterize the operation of two timer circuits in a single solution, we prepared a second timer circuit and reporter complex with different sequences and fluorophore/quencher than our original system. The second system had the same qualitative behavior, and it was possible to programatically tune both delay times and release rates. Differences in rates and delay times were observed between the two systems, possibly due to differences in toehold sequence that affect reaction rates (SI 4–5). The two systems were able to operate together in the same solution with virtually identical kinetics to those observed when the systems were operated apart (Figure 4).

The timer circuit developed here successfully releases target strands of DNA into solution at a constant rate after a delay period. The sequence, delay time and production rate were easily tuned without needing to redesign the release system. We demonstrated delay times on the order of hours to days and production rates from a few nM/day to a hundred nM/day, which for volumes of 100 μL are approximately 0.1–10 nmol/day.

While the release of O observed (Figure 3, SI Figures S8–S10) qualitatively followed our simple model described in eqs 1–5, there were differences between experiments and reaction curves predicted by the model. Notably we found that the release rate of O decreased faster and to a greater degree than the predicted reaction curves, suggesting the existence of uncharacterized reactions (i.e., leak reactions) between S and D or the reporter. On the basis of the experiments, we hypothesize that DNA synthesis errors (e.g., base-mismatches, truncations, additions or deletions) in the bottom strand of the S complex led to leak reactions and pathways that explained most of the deviation from the simple model. By accounting for these and other more minor leak reactions, a model was developed that provided a close fit with the experimental observations (SI 6), indicating that models can be used to program the rate and timing of output release.

By designing a circuit in which the output DNA sequence is an aptamer,25 i.e., sequences of DNA that bind specifically to non-DNA species such as proteins29 and other small molecules,30 the circuit described here could also be used to control the dynamics of a wide range of other chemical systems beyond DNA strand-displacement.

Timer devices that can be programmed to release a particular species with a prespecified delay could be important for designing cascades for therapeutics or for self-assembly5,32 in which different species are activated at different times. The constant low-rate of production we have shown could be used to design therapeutic hydrogels with novel, linear release profiles or within a reliable pulsed delivery system by combining it with a threshold amplifier system such as those used for signal restoration in molecular logic circuits.14,31
**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00170.

Materials and Methods; Reporter calibration and data processing; Production and Delay reaction characterization; Timer experiments; Multiplexing experiments; Modeling and Simulations (PDF)

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


