Beyond allostery: Catalytic regulation of a deoxyribozyme through an entropy-driven DNA amplifier

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Additional file 1: Supplementary text

Section 1: Materials and methods

1.1 Oligonucleotides

All oligonucleotides were ordered from Integrated DNA Technologies (IDT, Coralville, IA) and resuspended in double distilled H_2O (ddH_2O). The resuspended DNA oligonucleotides were ethanol-precipitated to remove residual organics that might interfere with absorbance readings at 260 nm, resuspended again in ddH_2O, and then quantified based on their absorbance at 260 nm. All DNA oligonucleotides were stored in 1x TE (10 mM Tris, pH 7.5, 1 mM EDTA) at 10 to 100 µM concentration. Similar to the observations from earlier studies [1-3], we observed non-specific adsorption of DNA onto the surface of plastic tubes, plate wells, and pipette tips. Therefore, all DNA samples at concentrations less than 10 µM were stored in 1x TE supplemented with 1 µM $(dT)_{20}$ (to occupy non-specific binding sites, as suggested by Zhang et al. [4]). All experiments were performed in the presence of 1 µM $(dT)_{20}$.

The sequences of the DNA strands used in this work are listed below. Modifications are noted according to the sequence code used by IDT: /3IAblkFQ/ represents a 3' Iowa Black Quencher modification; /56-FAM/ represents a 5' 6-carboxyfluorescein modification.

Trigger:	5' ATTCAATACCCTACGTCTCCA
Mutant **Trigger**:	5' ATTCAATACCCTACGTATCCA
Dock:	5' TGGAGACGTAGGGTATTGAATGAGACG
Invader:	5' GAGGGACGTAAATATTGGCGCGTCTCATTCAATACCCTACG
Fuel:	5' CGTCTCATTCAATACCCTACG
Blocker:	5' GAGACGCGCCAATATTTACGTCCCTC
DNAzyme:	5' CTGGGAGGGAGGGAGGGACGTAAATATTGGCG
qBlocker:	5' GAGACGCGCCAATATTTACGTCCCTC/3IAblkFQ/
fDNAzyme:	5' /56-FAM/AGGGACGTAAATATTGGCG
$(dT)_{20}$:	5' TTTTTTTTTTTTTTTTTTTT

1.2 Preparation of **Gate**, **fReporter**, and **Reporter** duplexes

Double-stranded constructs (**Gate**, **fReporter** and **Reporter**) were formed by mixing the two single stranded oligonucleotides in 1x TE, heating to 90° C for 1 min, and then cooling to 25° C at 0.1^o C/min. Although native PAGE purification has been established as the most rigorous method to isolate duplexes in order to ensure the 1:1 stoichiometry of the two strands and to eliminate at least a portion of the mis-synthesized and mis-hybridized gates, we reasoned that slight excesses of the 'non-carrier strands' (**Dock**, **qBlocker** and **Blocker** strands for the **Gate**, **fReporter** and **Reporter** duplexes, respectively) should not qualitatively change the performance of the circuits, even though, quantitatively, excess **qBlocker** and **Blocker** would create a threshold for the **Invader**. In contrast, we anticipated that an excess of 'carrier strands' (**Invader**, **fDNAzyme**, and **DNAzyme**, for **Gate**, **fReporter**, and **Reporter** duplexes, respectively) might lead to non-specific signaling. Therefore, we used a 1.2:1 ratio of 'non-carrier' to 'carrier' strands when preparing the duplexes and omitted native PAGE purification. The impact of this sample preparation method on the performance of the circuit is discussed in **Section 2** of this document.

1.3 Fluorescence assays

For fluorescence assays, a '*master mix*' that contained 200 nM **fReporter** and 25 mM $MgCl_2$ in TEdT buffer (1x TE supplemented with 1μM $(dT)_{20}$) was prepared. A series of 10 μL '*reaction mixtures*' was then prepared in separate tubes. Each reaction contained 200 nM **Gate**, 600 nM **Fuel**, and various concentration of the **Trigger** strands in TEdT buffer. Either the **Gate** or the **Trigger** strand was not added until directly before the addition of the '*master mix*'. To synchronize the initiation of the reactions, 10 μL of '*master mix*' was simultaneously added to the tubes containing the '*reaction mixture*' with a multichannel pipette, resulting in a 20 μL volume where the final concentrations of **Gate**, **Fuel**, and **fReporter** were 100 nM, 300 nM, and 100 nM, respectively. 16 μL of each 20 μL reaction was then added to a 384-well (NUNC, black polypropylene, shallow well) plate and the fluorescence signal of FAM was immediately read at room temperature for 1.5 hours using a SAFIRE plate reader (TECAN).

1.4 Colorimetric peroxidase assays

The amplification reactions in 10 μL volume were set up similar to those described above, except that the final concentration of **Gate**, **Fuel**, and **Reporter** were 2 μM, 5 μM, and 2 μM, respectively. The amplification reactions were incubated for 30 min at room temperature followed by addition of 10 μL of 2 μM hemin dissolved in 1x GQH buffer (25 mM Tris-HCl, pH 4.5, 150 mM NaCl, 20 mM KCl, 0.03% Triton X-100, 1% DMSO) and additional incubation for 20 min at room temperature. Then 80 μL of substrate solution containing H_2O_2 and ABTS dissolved in 1x GQH buffer was added to give a final volume of 100 μL. The final concentrations of **Gate**, **Fuel**, and **Reporter** were 200 nM, 500 nM, and 200 nM, respectively, while the final concentrations of hemin, H_2O_2, and ABTS were 200 nM, 2 mM and 2 mM, respectively. Peroxidase activity was measured at room temperature by taking absorbance readings every 30 s at 414 nm in a 384-well plate (NUNC, optically clear bottom, white wall,

rounded square wells) in a SAFIRE plate reader. In addition, a picture of the plate was taken 15 min after the addition of the substrate solution with a digital camera.

It should be noted that although the circuits (including all DNA species) and small molecule reactants (including hemin, H_2O_2 and ABTS) were added sequentially in our experiment, the experiment could also have been carried out by mixing all components together at the same time. However, additional background might be caused by the latter method due to uncatalyzed oxidation of ABTS. Nonetheless, this source of leakage should be less prominent than uncatalyzed displacement of **Invader** by **Fuel** (see discussion below), and thus the circuit is expected to have similar performance regardless of the order of mixing.

Section 2: Circuit leakage and detection limits

The preparation methods for DNA strands and complexes have been shown to have a significant impact on circuit performance, especially on the speed and extent of undesired side reactions (i.e., circuit leakage). The most rigorous methods involve purging faulty gate duplexes by incubating the gate duplexes with input molecules (with or without toeholds) and / or fuel strands, followed by native PAGE purification to isolate the unreacted gate duplexes. This method is hereafter referred to as the 'purge-n-PAGE' method. However, these more rigorous methods usually require longer and more labor-intensive purification procedures that could increase the complexity or cost of eventual assays.

Therefore, we initially chose not to PAGE purify any strand or duplex, but to simply ethanol-precipitate the oligonucleotides and use a 1:1.2 ratio of 'carrier' to 'non-carrier' strands when assembling the duplexes (see **Section 1.1** and **1.2**). This method resulted in greater circuit leakage than in previous reports where the 'purge-n-PAGE' method was applied [1-2], and these more rigorous purification methods may be required for sensitive detection or more complex circuits.

A closer inspection of the circuit using the fluorescent assay provided insights into the source of leakage. As shown in **Figure 2B** (blue line) and **Figure S1** (blue dots), when 100 nM **Gate** and 100 nM **fReporter** were mixed a biphasic increase in fluorescent signal was observed. The presence of early exponential growth with a decreasing rate, followed by slow, quasi-linear growth indicated the likely presence of at least two major side reactions. The kinetics of signal increase can best be fitted to the equation $y = A(1-\exp(-k_E \cdot t)) + r_L \cdot t + C$ (**Figure S1**, blue line), where A and k_E are amplitude and first-order rate constants for fast exponential growth, respectively, r_L is the rate of quasi-linear growth, and C is the initial background fluorescent signal. The amplitude of fast exponential growth (A) was fitted to be 0.1 RFU, corresponding to ~7 nM of released **fDNAzyme**. Given that there should have been excess **qBlocker**, the amplitude of ~7 nM suggests that the total concentration of faulty duplexes (including

unhybridized **Invader**, mis-hybridized/mis-synthesized **Gate**, and mis-hybridized **fReporter**) that could undergo fast strand displacement was at least 7 nM. The rate of quasi-linear growth (r_L) was fitted to be ~0.002 RFU/min, corresponding to ~2 pM·s^{-1}. Since we do not know the amplitude of this quasi-linear growth, this rate implies either that: (i) the **Gate** and the **fReporter** could interact to form unquenched reporter with a second-order rate constant of ~ (2 pM·s^{-1}) / (100 nM) / (100 nM) = ~ 200 M^{-1}s^{-1} (assuming the amplitude of quasi-linear growth was ~100 nM); or (ii) a fraction of faulty **Gate** and/or **fReporter** could interact with their partners with a rate constant even higher than 200 M^{-1}s^{-1} (assuming the amplitude of quasi-linear growth was significantly smaller than 100 nM).

When 300 nM of **Fuel** was mixed with to 100 nM **Gate** and 100 nM **fReporter**, even more leakage was observed (**Figure 2B**, green line and **Figure S1**, green dots). After subtracting the ~7 nM rapidly produced product (as revealed by the fast phase of 100 nM **Gate** + 100 nM **fReporter** mixing), the remainder of the fluorescent signal increase can be fitted to a single exponential equation with the amplitude of ~ 0.2 RFU, corresponding to ~ 13 nM, and a rate constant of ~ 7×10^{-4} s^{-1} (**Figure S1**, green line). These results imply that ~13 nM of the **Gate** duplex is faulty such that it can react with **Fuel** to produce the **Invader** with a second order rate constant of ~ (7×10^{-4} s^{-1}) / (300 nM) = ~ 2300 M^{-1}s^{-1}.

Overall, the multiphasic kinetics of leakage strongly suggests the presence of faulty, fast-reacting complexes, and again suggests both that the current method has inherent sensitivity limitations and that 'purge-n-PAGE' or other purification methods will likely be required for further assay development.

Supplementary Figures:

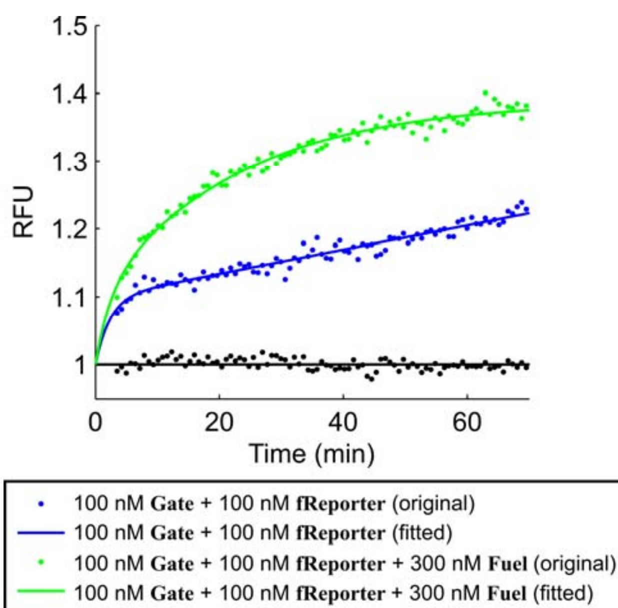

Figure S1. The kinetics of circuit leakage. The data and color coding are the same as in **Figure 1B**, except the y-axis is scaled for better visualization. The original data and fitted data are shown in dots and lines, respectively.

References

1. Seelig G, Soloveichik D, Zhang DY, Winfree E: **Enzyme-free nucleic acid logic circuits.** *Science* 2006, **314:**1585-1588.
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3. Zhang DY, Winfree E: **Dynamic allosteric control of noncovalent DNA catalysis reactions.** *J Am Chem Soc* 2008, **130:**13921-13926.
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