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Regulable toehold lock for the effective control of strand displacement reaction sequence and circuit leakage

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ABSTRACT

Strand displacement reaction enables the construction of enzyme-free DNA reaction networks, thus has been widely applied to DNA circuit and nanotechnology. It has the characteristics of high efficiency, universality and regulatability. However, the existing regulation tools cannot enable effective control of the reaction sequence, which undoubtedly limits the construction of complex nucleic acid circuits. Herein, we developed a regulation tool, toehold lock, and achieved strict control of reaction sequence without loss of the main reaction signal output. Furthermore, we applied the tool to scenarios such as seesaw circuits, AND/OR logic gates, and entropy-driven circuits, and respectively demonstrated its significant superiority compared to the original method. We believe that the proposed toehold lock has greatly optimized the efficiency of DNA strand displacement-based networks, and we anticipate that the tool will be widely used in multiple fields.

Deoxyribonucleotides form the basis for constructing DNA circuits, capitalizing on their abundant availability within living organisms and unique biological properties that are not present in conventional silicon circuits [1,2]. Various methods have been employed to develop DNA circuits, such as utilizing hybridization chain reaction (HCR) [3], DNAzyme [4–6], protease-catalyzing [7–9], and more. Notably, toehold-mediated strand displacement reaction [10–12] has been proven to be a promising approach towards achieving automatic hybridization without the need for enzyme mediation or catalysis. This method has exhibited high efficiency, convenience, and universality [13], hence rendering it suitable for a wide range of applications such as molecular assembly [14–17], hydrogels [18–22], biometrics [23–28], and information storage [29–35].

However, one issue that has intermittently hindered the development of toehold-mediated DNA circuit is the deficiency of current methods to exert optimal control over the reaction sequences [36]. In the realm of DNA circuits, the general approach is to establish kinetic disparities between two toehold reactions through creating the dissimilarities in the length of their respective toehold domains. As illustrated in Fig. 1a, Gate L possesses a longer toehold domain than Gate S, which will thus have greater probability to react with Input according to kinetic analysis. Nonetheless, this approach, which hinges mainly on kinetic attributions, exhibits significant limitations since it fails to provide enough control over the reaction sequences. The rate constant of the toehold reaction displays a sigmoidal profile with the length of toehold domain, growing very slowly beyond 5 nt [10,13]. Consequently, a mere manipulation of toehold region length differences does not confer adequate kinetic advantage to achieve strict regulation of the reaction sequences.

The above problem undoubtedly limits the effective construction of complex nucleic acid circuits, as stringent control of leakage is prominent in multilayer cascaded circuits. To solve this problem, we have developed a novel component, “toehold lock”, which enables precise regulation of the sequential response and significantly surpasses the efficacy of conventional approach. Theoretically, this element holds the potential to govern the sequence of multiple strand displacement reactions.

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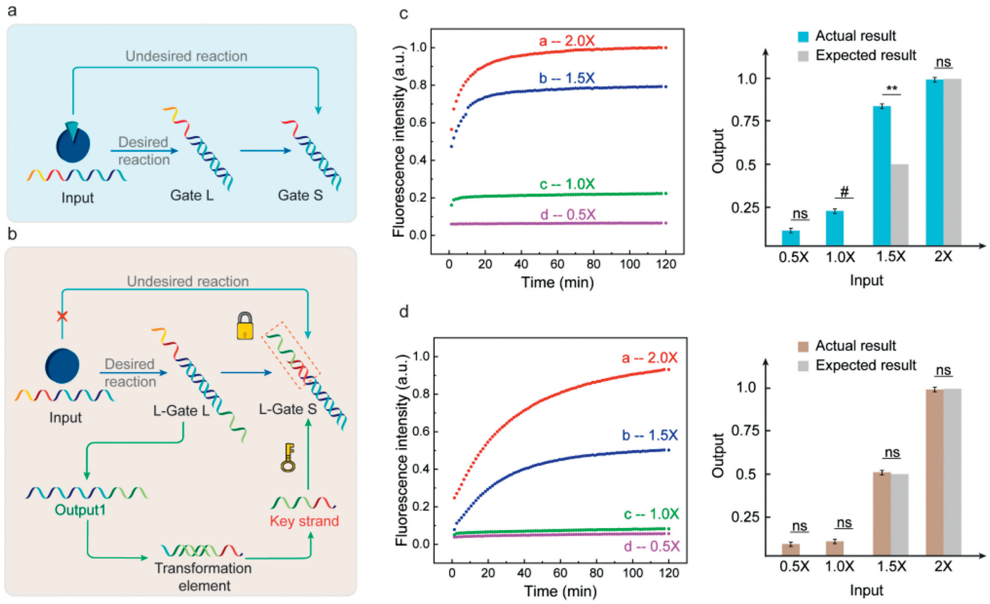

Fig. 1. (a) Schematic illustration of the method of controlling the reaction sequence by controlling the length of the toehold domain. (b) Schematic illustration of the toehold lock element to control the reaction sequence. (c) The output of the DNA circuit constructed using the principle of controlling the length of the toehold domain. The histogram visualizes the difference between the expected output and the actual result. The expected output is 0 at Input concentrations of 0.5X and 1.0X, half of the maximum signal at Input concentration of 1.5X, and the maximum signal at Input concentration of 2.0X. (d) The output of the DNA circuit constructed using the toehold lock element, and histogram shows that the difference between the actual result and the expected output of this system is significantly smaller. 1X = 1 μmol/L. The histogram data was demonstrated in mean ± standard deviation (SD), $n = 3$ independent experiments. Statistical significance was calculated by two-tailed Student's *t*-test; ns, nonsignificant; $^{\#}P < 0.05$; $^{**}P < 0.01$.

The "toehold lock" element is operated according to the principles depicted in Fig. 1b, wherein the toehold domain of Gate S is "blocked" (referred to as L-Gate S), rendering it incapable of reacting with Input just added. This design enables Input to react only with L-Gate L, thereby generating Output1, which subsequently reacts with the transformation element to produce a key chain. The key strand serves to unlock the toehold previously "locked" and then initiates the reaction between Input and L-Gate S. By contrast to the conventional approach of controlling toehold domain length, the "toehold lock" element directly inhibits the undesired reaction at the outset, thereby creating a greater kinetic difference and achieving tighter regulation of the reaction sequence. Consequently, the "toehold lock" element has the potential to comprehensively address the issue of toehold reaction sequence.

First, we aimed to demonstrate that controlling the length of toehold domain cannot effectively regulate the reaction sequence. Specifically, we designed two chains, Gate S and Gate L, featuring toehold domain lengths of 5 and 10 nt, and then added Input at different concentrations into the system containing both Gates S and L (Table S1-1 in Supporting information). As shown in Fig. 1c, a significant leakage signal at Gate S was observed for both Input concentrations of 1X and 1.5X, which proved that controlling the toehold domain length could not strictly control the reaction sequence. Subsequently, to validate the function of the toehold lock element, we incorporated it into the experimental setup following the above trials (Table S1-2 in Supporting information). As depicted in Fig. 1d, negligible leakage signals were detected when Input concentrations of 0.5X, 1X, and 1.5X were introduced, indicating that Input interacts initially with L-Gate L, followed by reaction with L-Gate S. These results demonstrate the ability of the toehold lock element to thoroughly regulate the sequence of toehold reactions.

Using this property of toehold lock, we aimed to effectively address the issue of leakage in seesaw circuits. Specifically, three main sorts of leakage have been identified in seesaw circuits: I, leakage between fuel and gate; II, leakage between gates in two layers; III, leakage at the threshold [37]. The leakage III exists primarily because the reaction sequence is loosely controlled. As shown in Fig. 2a, threshold only has a longer toehold domain than the Gate: output, which leads to the fact that some of the Input can cross the threshold and react directly with the Gate: output. In contrast, circuit utilizing the toehold lock element effectively blocks the toehold domain of the Gate: output (referred to as the locked gate, as depicted in Fig. 2b), thereby enabling the newly added Input to only react with the L-threshold. The activator displaced by Input subsequently reacts with transformation element downstream, generating the key strand required to unlock the locked gate. Transformation element has two key strands to ensure fully unlocking the locked gate when the L-threshold is entirely reacted. In fact, toehold lock element not only resolves the issue of leakage III resulting from improper control of the reaction order but also eliminates the occurrence of leakage I and II ($\Delta G > 0$ for both reactions after toehold being blocked). The circuit designed in accordance with Figs. 2a and b was evaluated by adding different concentrations of Input to both systems. The concentrations of gate, threshold, fuel, and reporter in the system are detailed in Table S2 (Supporting information). The results of the evaluation are presented in Fig. 2c and Fig. S1 (Supporting information). The original seesaw circuit exhibited significant leakage signals at Input concentrations of 0.4X and 0X, fully reflecting the impact of the three sorts of leakage in the seesaw circuit system. In contrast, our system exhibited no significant leakage signal at Input concentrations of 0.4X and 0X, providing evidence that the toehold lock element effectively resolves the issue of leakage in seesaw circuits.

During our experiments, we discovered that the unlocking domain's length of key strand could affect the unlocking effect. This is because the strand displacement reaction may encounter a certain degree of steric hindrance when Input combines with the toehold domain of unlocked gate. Consequently, unlocking effect is not optimal when the length of the key strand unlocking domain is equivalent to the length of the toehold domain. To address this

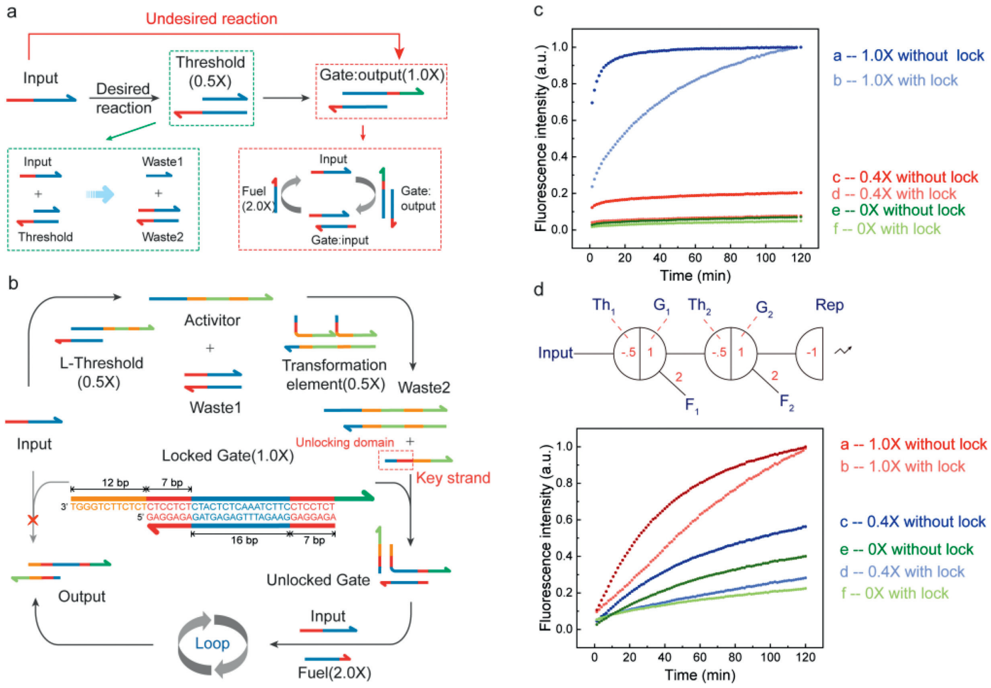

Fig. 2. (a) Schematic illustration of the original seesaw circuit structure, where part of the Input can directly cross the Threshold and react with Gate: output. (b) Seesaw circuit constructed with toehold lock element. (c) Comparison between the original seesaw circuit and the seesaw circuit with the addition of the toehold lock element, where the same color indicates the same concentration of Input and the lighter color indicates the circuit with toehold lock element. (d) Abstract circuit structure of two-layer seesaw circuit and the results of corresponding comparison experiment. 1X = 200 nmol/L.

issue, we found it necessary to extend the unlocking domain of the key strand by a small complementary domain which could match the migration domain of output. In order to found the optimal length of this structural domain, we designed a series of experiments (Fig. S2 in Supporting information). Our results indicate that the optimal length of this complementary domain is 2 bases. We also briefly investigated the impact of nucleic acid sequences on the effectiveness of the system (Fig. S3 in Supporting information).

Next, we aimed to implement our approach to the two-layer structure of the seesaw circuit. In this regard, toehold lock elements were added to both two layers of the circuit, as demonstrated in Fig. S4 (Supporting information). We designed the original seesaw two-layer circuit and the two-layer circuit with toehold lock while keeping the concentrations of threshold, fuel and gate same in two layers (Table S3 in Supporting information). The results of our experimentation are presented in Fig. 2D, where the toehold lock method shows a lower leakage signal for Input concentrations of 0X and 0.4X (Fig. S5 in Supporting information). Additionally, we observed that the length of the locked gate free single strand domain (Fig. 2b) plays a pivotal role in this system. Specifically, too short a length will lead to difficult attachment of key strand, while too long single strand domain will introduce unnecessary crosstalk (mainly referring to the reaction with single-stranded domain of key strand in the transformation element). Therefore, we designed gradient experiments, varying the number of bases in this domain, and the results indicated that the optimal number of bases is 12 (Fig. S6 in Supporting information).

Based on the ability of the toehold lock element to control the reaction sequence, we can extend its application to the AND and OR logic gates. As shown in Fig. 3a, by changing the concentration of the threshold in seesaw researchers can implement the switching of AND/OR gates in a same circuit. By incorporating the toehold lock element, we can further enhance the performance of threshold. It is important to note that accurately predicting the output value of the integrated gate is not feasible, as the ΔG of reaction between Input and the integrated gate is 0. To explore the optimum concentration of Threshold in the AND gate, we conducted experiments, and the results are presented in Fig. S7 (Supporting information). These results indicate that the optimum concentration of threshold in the AND gate is 1.0X, implying that the reaction degree of Input with the integrated gate is approximately 50%. Thereby we determined that the optimum concentration of threshold in the OR gate is 0.5X. Additionally, while constructing the logic gate, threshold reaction and the unlocking of the downstream Gate should complete simultaneously in order to maximize the efficiency of the system. However, the concentration of transformation element and key strand combined with it can only be N times ($N = 1, 2\ldots$), which limits the concentration setting of threshold (it can only be the concentration of the downstream Gate divided by N). Therefore, to address this limitation, we introduced "offset element" which did not possess an unlocking function and solved this problem by assigning each element the proper concentrations (Fig. S8 in Supporting information). Subsequently, we constructed AND logic gates by respectively using the original seesaw circuit and the toehold lock element. We utilized the optimal Threshold concentration obtained in the previous experiment, while the concentrations of the other elements are presented in Table S4 (Supporting information). The efficacy of the two systems in handling different Input signals is demonstrated in Fig. 3b. Our method produced a full signal at Input of (1, 1), while the leakage signal at (1, 0), (0, 1), (0, 0) was significantly lower than the control group. Moreover, OR gates were constructed using the same principles for comparison, and the results are shown in Fig. 3c. Notably the comparison between the two methods at (0, 0) was significant. Further, we also built a cascade circuit with two upstream OR gate outputs used as inputs for one AND gate and the dual-track half adder, and the results were also as expected (Fig. 3d and Fig. S9 in Supporting information). All these experiments showed that it is feasible to control the leakage in AND gates and OR gates with toehold lock elements.

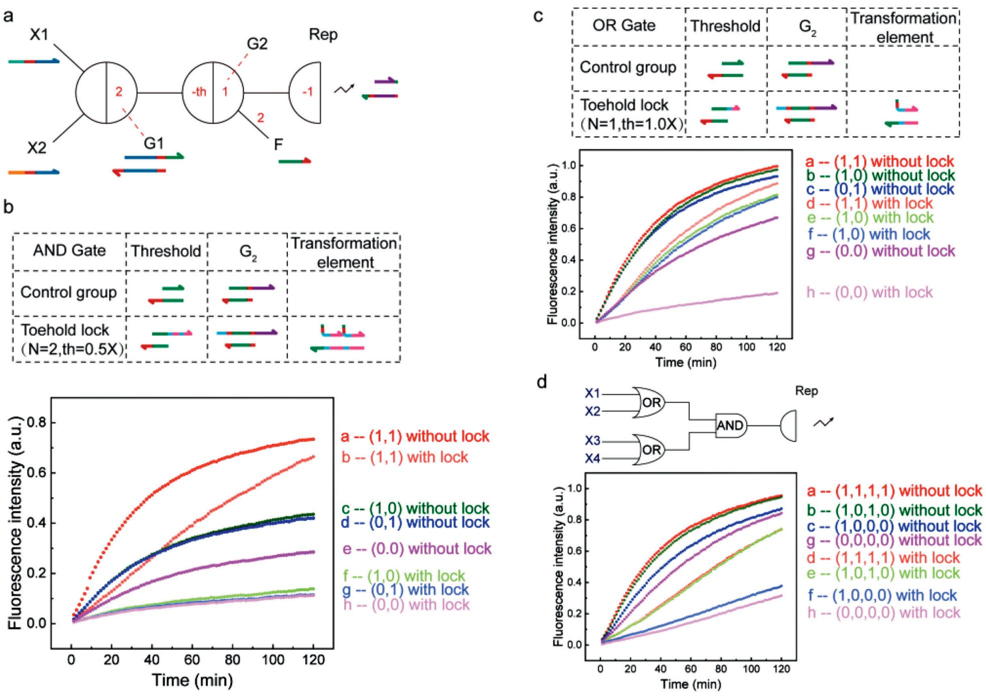

Fig. 3. (a) Abstract structure of AND gate and OR gate, red numbers indicate the concentration, Threshold's concentration is variable. (b) Concrete circuit structure of AND gate and experimental results. The table shows circuit design of the two methods to construct the AND gate, and the control group indicates the toehold domain length-control method. (c) The specific circuit structure of the OR gate and the experimental results. (d) The two upstream OR gates are connected in parallel and together form a series connection with the downstream AND gate. Four different Input concentrations are designed in the experiment, and theoretically only (1, 1, 1, 1) and (1, 0, 1, 0) output signals. 1X = 200 nmol/L.

To further demonstrate the stability and compatibility of the toehold lock element at different temperatures and in different components, we first investigated the effect of different temperatures on the control of reaction sequence. The reaction systems previously described were performed at 37 °C, so we set the reaction temperature to 32 °C and kept the other reaction conditions constant (Fig. S10 in Supporting information). The results show that changing the temperature does not significantly affect the function of the toehold lock element. We then applied the toehold lock element to the entropy-driven circuit [38]. Entropy-driven circuits are characterized by high rates and modularity, but have little ability to distinguish Input concentrations [39]. So, we first designed a fundamental entropy-driven circuit architecture, integrated Threshold to confer a rectification function to the system

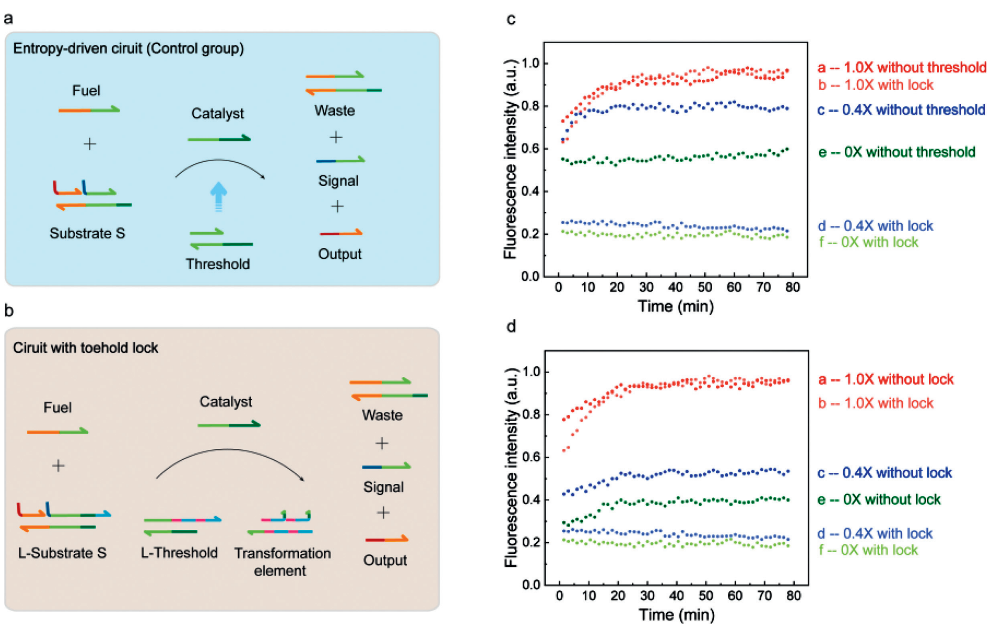

Fig. 4. (a) Basic structure of the entropy-driven circuit, here showing the incorporation of threshold. (b) Schematic illustration of the entropy-driven circuit with toehold lock element. (c) Comparison results of the original entropy-driven circuit and that with toehold lock element. (d) Comparison results of the toehold domain length-control method and the "toehold lock" method. 1X = 100 nmol/L.

(Fig. 4a), and subsequently incorporated toehold lock elements to mitigate leakage (Fig. 4b). The results of the native entropy-driven circuit and the toehold lock system under varied Inputs is presented in Fig. 4c, wherein the former displayed no discernment of input concentration. Moreover, we devised a comparative study between the toehold lock component and the toehold-length-control method, and the results (Fig. 4d) demonstrate the superior efficacy of the toehold lock element within the entropic circuit. Another experiment is the optimization for one kind of DNA biocomputing circuits emulating the metal-oxide-semiconductor (MOS) transistors [40]. We successfully improved its signal-to-noise ratio and interference resistance against low-level input signal by applying toehold lock elements, while maintaining the original structure and sequence in the paper (Fig. S11 in Supporting information.).

Through experiments above, we have demonstrated that toehold lock element enables precise regulation of reaction sequence in diverse circuit configurations. However, the efficiency of this method may diminish with increasing circuit complexity, especially when upstream reactions have not yet finished but adequate downstream reactants are already unlocked. Nonetheless, the introduction of the Transformation element prevents the leakage reaction between Input and downstream reactants from occurring directly, and exerts a necessary intermediate unlocking step, making the occurrence of leakage reaction very difficult. Thus, the toehold lock element retains its ability to exert strong control over reaction sequence. We also used NUPACK to investigate the degree of binding and stability of the nucleic acid strand in system (Fig. S12 in Supporting information).

In conclusion, by blocking the toehold domain of the Undesired reaction and designing the corresponding “unblocked” components, we made the product generated from the first reaction a prerequisite for the later reaction to occur, thus achieving strict control of the reaction sequence. We successfully implemented this method to circumvent the leakage issue in scenarios such as seesaw circuits, entropy-driven circuits, and beyond. During these experiments, “offset element” was proposed for mismatch problem of two-layer reactants’ concentrations. It can eliminate the restriction on concentrations of elements, thus expanding the scope of application of toehold lock. Furthermore, we investigated the impact of structural modifications on the outcome and found optimum scheme. This novel technique enables strict regulation of the order of two DNA strand displacement reactions and has the potential to be extended to the control of multiple DNA strand displacement reactions’ sequence, with prospective applications in fields such as neural networks [41–43], molecular machines [44], and nanomaterials [45].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.109104.

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