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# Electronic Supplementary Information

# DNA logic circuits based on FokI enzyme regulation

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## Contents

## 1. DNA Sequences

For the purpose of developing higher-order logic gates with available DNA sequences, all of the sequences used in this study were designed using Nupack[1]. A DNA single-stranded is a random sequence consisting of A, T, C, and G on the basis of additional specific conditions. Then, some bases of the problematic subsequences were altered by hand, for example, 4 or more G consecutive occurrences on the DNA strand, more than 4 A's in a row causing synthesis difficulties, multiple recognition domain, etc. So this the selection method reduces potential secondary structure[2]. The above procedures were repeated until the available DNA sequences were obtained. Next, the designs of the DNA structures were verified by the NUPACK software to ensure minimal spurious interactions[3], so as to ensure that the desired DNA complexes were well-formed.

**Table S1**. DNA sequences

| Strands | Sequence (from 5'-terminal to 3'terminal) |
|---|---|
| A' | ACTGAGGATGCTCGTGCACGTGCTCAGTCTCGTTGCTAGAACG |
| B' | CGTTCTAGCAACGAGACTGAGCACGTGCACGAGCATCCTCAGT |
| a1 | GTGCTCAGTCTCGTTGCTAGAACG |
| b1 | CGTTCTAGCAACGAGACTGA |
| c1 | CGTTCTAGCAACGAGACTGAGCAC |
| A-0 | CTCGTGCACGTGCTCAGTCTCGTTGCTAGAACG |
| A-3 | GTGCACGTGCTCAGTCTCGTTGCTAGAACG |
| A-6 | CACGTGCTCAGTCTCGTTGCTAGAACG |
| A-9 | GTGCTCAGTCTCGTTGCTAGAACG |
| IN-0 | CGTAAGTCTACTGAGGATG |
| IN-3 | CGTAAGTCTACTGAGGATGCTC |
| IN-6 | CGTAAGTCTACTGAGGATGCTCGTG |
| IN-9 | CGTAAGTCTACTGAGGATGCTCGTGCAC |
| IN-12 | CGTAAGTCTACTGAGGATGCTCGTGCACGTG |

| | |
|---|---|
| B | CGTTCTAGCAACGAGACTGAGCACGTGCACGAGCATCCTCAGTAGACTTACG |
| A1 | CAGCACGACGTGCTCAGTCTCGTTGCTAGAACG |
| B1 | CGTTCTAGCAACGAGACTGAGCACGTCGTGCTGCATCCTCAGTAG |
| D | CTCGTGCACCTTCGACACGCTAGTCATCTTGCTAGAACG |
| e1 | CGTGTCGAAGGTG |
| e2 | CGTTCTAGCAAGATGACTAG |
| c2 | CGTTCTAGCAAGATGACTAGCGTGTCGA |
| d2 | CTTCGACACGCTAGTCATCTTGCTAGAACG |
| Demux-IN1 | GCATCACTGCTCGTAAGTCTACTGAGGATG |
| Demux-IN2 | CGTGTCGAAGGTGCACGAGCATCCTCAGTAGACTTACGAGCAGTGATGC |
| Demux-IN3 | GCATCACTGCTCGTAAGTCTACTGAGGATGCTCGTG |
| Mux-IN1 | CGTGTCGAAGGTGCACGAGCATCCTCTCGACGAC |
| Mux-IN2 | GACCATCCTCTCGACGACACAGC |
| Mux-IN3 | GCTGTGTCGTCGAGAGGATGGTCGACC |
| A2 | TCGTCGAGAGGATGGTAGACGACGTGCTCAGTCTCGTTGCTAGAACG |
| B2 | CGTTCTAGCAACGAGACTGAGCACGTCGTCTAC |

**Table S2**. Strands combination for YES, OR, AND, INHIBIT and SWITCH circuit.

| Circuit | Strands |
|---|---|
| FokI | A’,B’, c1 |
| JPM | A-0,A-3,A-6,A-9,B,IN-0,IN-3,IN-6,IN-9,c1 |
| RBM | A1,B1, IN-0,IN-3,IN-6,IN-9,IN-12, c1 |
| YES | A-3,B,IN-3,c1 |
| AND-1 | D,e1,e2,Demux-IN1, Demux-IN2, c2 |
| AND-2 | D,e1,e2,Mux-IN1, Mux-IN3, c2 |
| INHIBIT-1 | A1,B1, Demux-IN1, Demux-IN2, c1 |
| INHIBIT-2 | A2,B2, Mux-IN2, Mux-IN3, c1 |
| OR | A1,B1, Demux-IN1, Demux-IN3, c1 |

| | |
|---|---|
| Demultiplexer | D,e1,e2, A1,B1, Demux-IN1,Demux-IN2, c1, c2 |
| Multiplexer | D,e1,e2,A2,B2,Mux-IN1,Mux-IN2,Mux-IN3, c1, c2 |

**2. FokI enzyme**

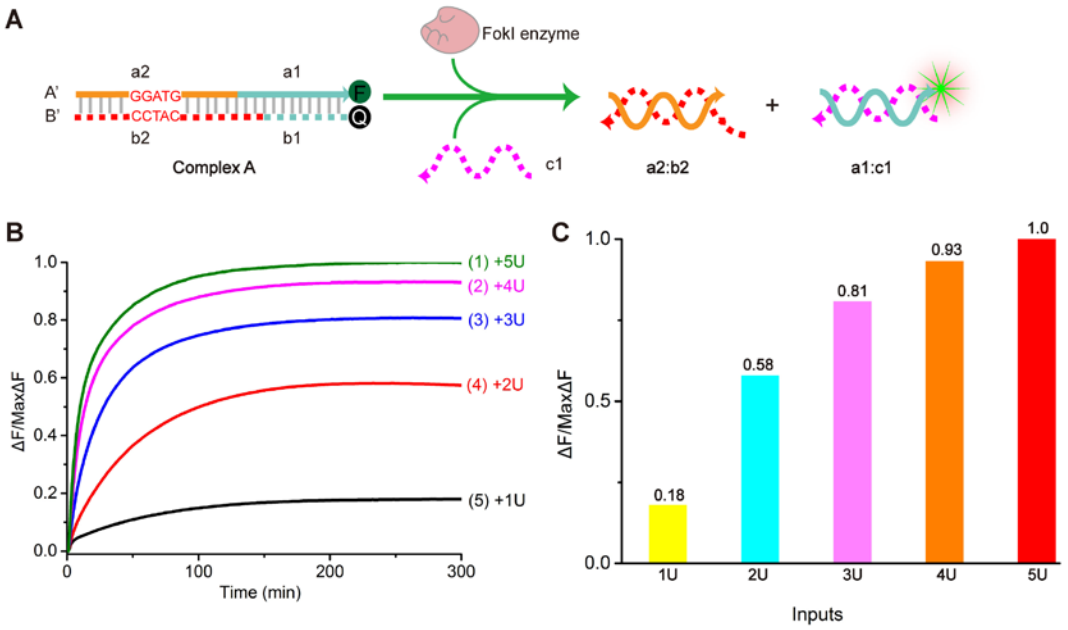


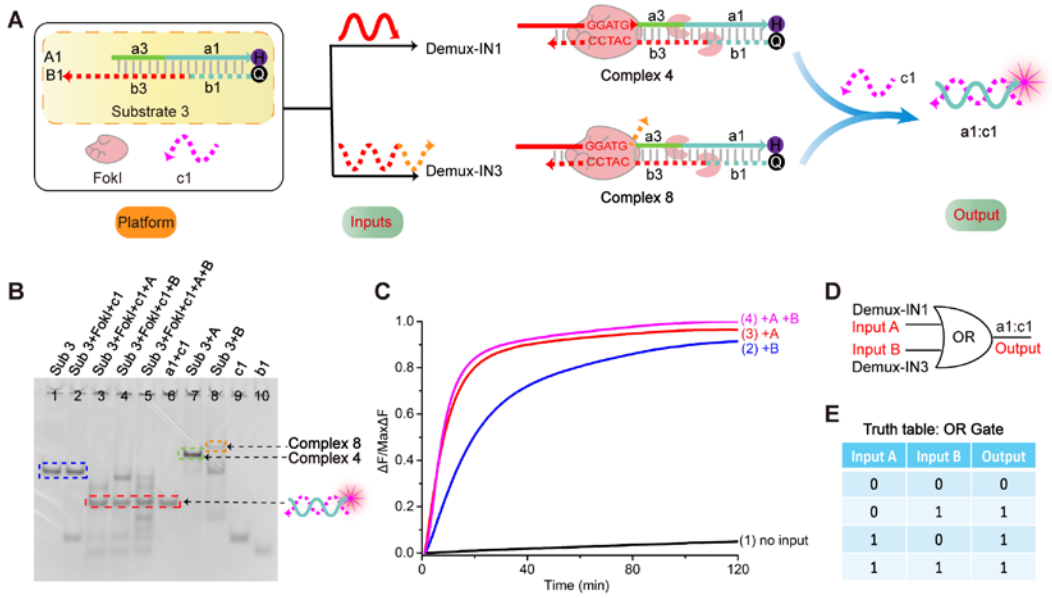

**Figure S1**. (A) The working principle of the enzyme digestion of FokI enzyme. (B) Time-dependent normalized fluorescence changes at different concentrations of FokI enzyme levels. The curves (1) to (5) indicate that the enzyme cleavage responses at different concentrations of FokI enzyme were 1 U, 2 U, 3 U, 4 U and 5 U, respectively. (C) Normalized fluorescence histogram of different concentrations of FokI enzyme. The sampling interval was 1 min. All data represent the average of three replicates. Error bars represent one standard deviation of three replicate analyses.

As can be seen from Figure S2 (A), the principle of enzymatic cleavage of the FokI enzyme. The single strands A' and B' respectively containing a partial recognition domain are annealed to form Complex A. In order to fluorescence assays, the quencher BHQ1 and the fluorophore FAM were modified at the 5 and 3 ends of Complex A, respectively. The FokI enzyme was added to a solution containing Complex A and single-stranded c1, and when the FokI enzyme recognized and bound to a specific DNA sequence (5'-GGATG-3': 5'-CATCC-3'). 9 to 13 nucleotides downstream of the binding site will be cleaved to produce a double-stranded a2:b2 and a double-

stranded a1:b1 with 4-nt toehold. Subsequently, the double-stranded a1:b1 and the single-stranded c1 undergo a strand displacement reaction, resulting in a double-stranded a1:c1 with a fluorescent FAM modification. We first tested the persistence of the FokI enzyme and set the total enzyme digestion reaction time to 5 hours. The experimental results of Figure S2 (B) indicate that the enzyme cleavage of the FokI enzyme is persistent, which ensures that the length of time of using FokI enzyme is used during subsequent logic gate construction does not affect the enzymatic cleavage effect of the system. In addition, we also tried the enzyme cleavage of substrates at different temperatures, respectively, to verify the enzymatic cleavage effect of FokI enzyme at 25 ° C, 30 ° C, 35 ° C, 37 ° C. The experimental results showed that the temperature had no significant effect on the enzymatic cleavage effect. At the same time, according to the ideal enzyme cleavage temperature provided by Takara Bio Inc, we chose 37 °C for further experiments.

As shown in Figure S2 (B), the FAM fluorescence intensity of double-stranded a1:c1 increased with the increase in the concentration of FokI, and reached a platform with relatively high efficiency and stability at a concentration of up to 4 U. Thus, 4 U was selected for the ensuing experiments. Among them, 1U is equal to 10x FokI of 0.1ul in the 20uL system. In order to display the experimental results more intuitively, we normalized the different concentrations of FokI enzyme to obtain a fluorescence histogram, Figure S2 (C).

## 3. OR Gate




| Input A | Input B | Output |
|---|---|---|
| 0 | 0 | 0 |
| 0 | 1 | 1 |
| 1 | 0 | 1 |
| 1 | 1 | 1 |

**Figure S2**. (A) Simulate the reaction schematic of the OR gate. The fluorophore HEX and the quencher BHQ1 were modified at the 3' and 5' of the double-stranded substrate 3, respectively, and the enzymatic cleavage products reacted with single-stranded c1 to trigger the fluorescence signal. (B) Native PAGE analysis of OR gate products. Each lane number is marked with a different reaction of the input strand and substrate. Lane 2: input reaction (0,0); lane 3: input reaction (1,0); lane 4: input reaction (0,1); lane 5: input reaction (1,1). (C) Time-dependent normalized fluorescence changes of the OR gate during the 2 hour reaction. A indicates the input strand Demux-IN1, and B indicates the input strand Demux-IN3. The sampling interval is set to 1 minute. Fluorescence results show that the fluorescence intensity was significantly enhanced regardless of whether the input strand A, B are present simultaneously or separately in the solution (curves 2-4). All data represent the average of three replicates. Error bars represent one standard deviation of three replicate analyses. (D) The logic gate symbol corresponding to the OR gate, which was activated by strand Demux-IN1 or Demux-IN3 to generate an output signal a1:c1. (E) Truth table corresponding to the OR gate.

In order to further test the feasibility of the enzyme-assisted cleavage regulation

system, an OR gate can be easily constructed using the reaction principle of the JPM and the reaction principle of the RBM, Figure S2 (A). In this logic gate, both input strands Demux-IN1 and Demux-IN3 can specifically hybridized with double-stranded Substrate 3. Both Complex 4 and Complex 8 are formed with the recognition domain of the FokI enzyme. Then, the activity of the FokI enzyme was initiated to perform the enzymatic cleavage reaction, and the displacement reaction between the enzyme cleavage fragment and strand c1 occurred to form a fully complementary double-strand a1:c1 with a HEX modification.

Figure S2 (B) shows the polyacrylamide gel analysis of the interaction between different DNA strands used in OR logic operation. The results of the gel electropherogram show that the Substrate 3 bands (blue dotted box in lanes 1-2) in the solution did not change without any input strands. When the input strand Demux-IN1 was added, it specifically bound to Substrate 3 to generate Complex 4 (green dotted box in lane 7). When the input strand Demux-IN3 was added, it specifically bound to Substrate 3 to generate Complex 8 (yellow dotted box in lane 8). When the FokI enzyme was added, the corresponding Complex 4 and Complex 8 bands basically disappeared, and double-strand a1:c1 (red dotted box in lanes 3-5) was generated, with lane 6 is a comparison track. This micrograph demonstrated the feasibility of the OR gate.

To analyse the OR gate in quantity, a fluorescence assay was also employed. The fluorescence analysis results of OR gate are shown in Figure S2 (C). It is easy to observe that when adding either or both of the triggers Demux-IN1 and Demux-IN3, significant fluorescence intensity increases were obtained (curves 2, 3 and 4). In the absence of an input strands, no significant strong fluorescence signal was produced (curve 1). A logic gate symbol and truth table corresponding to OR gate is shown in Figure S3(D), (E).

## 4. AND Gate

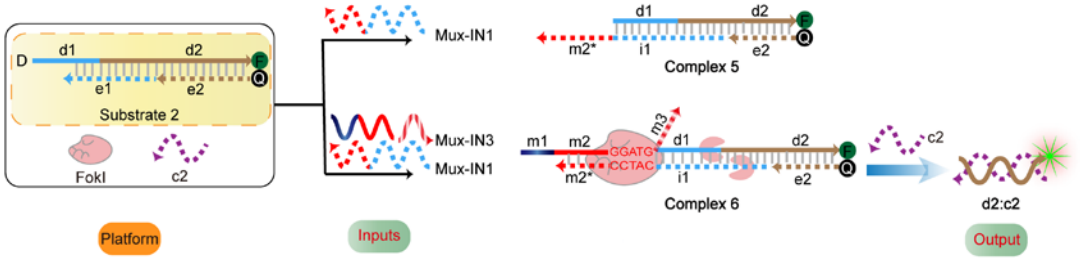



**Figure S3**. Simulate the reaction schematic of the AND gate. The fluorophore FAM and the quencher BHQ1 were modified at the 3' and 5' of the double-stranded Substrate 2, respectively, and the enzymatic cleavage products reacted with single-stranded c2 to trigger the fluorescence signal.

In order to further demonstrate the flexibility of the dual-input initiators in an enzyme-assisted cleavage regulation mechanism, another AND gate was constructed using the reaction principle of the RBM (Figure S3). The recognition domain of the FokI enzyme was designed to be simultaneously regulated by both input strands Mux-IN1 and Mux-IN3. D: e2 was a double-stranded with a reporter function. The m3 domain on the input strands Mux-IN3 are designed to be of different lengths. Take into account the best effects of the reaction principle of the RBM, as well as the leakage of inhibit and multiplexer. Finally, we chose the base length of m3 as 7-nt toehold for the experiment. Only when Mux-IN1 and Mux-IN3 were simultaneously input did specific hybridization occur with Substrate 2 to form Complex 6 with the FokI recognition domain. Then, the activity of the FokI enzyme was initiated to perform the enzymatic cleavage reaction, and the displacement reaction between the enzyme cleavage fragment and strand c2 occurred to form a fully complementary double-strand d2:c2 with a FAM modification.

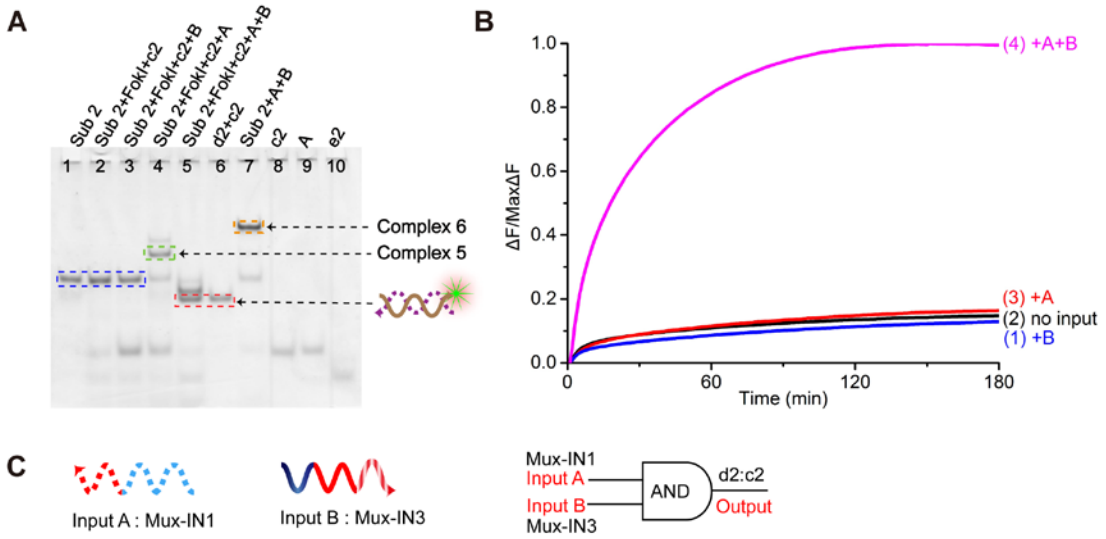



**Figure S4**. (A) Native PAGE analysis of AND gate products. Each lane number is marked with different reactions between input strand and substrate. Lane 2: input reaction (0,0); lane 3: input reaction (1,0); lane 4: input reaction (0,1); lane 5: input reaction (1,1). (B) Time-dependent normalized fluorescence changes of the AND gate during the 3 hour reaction. A indicates the input strand Mux-IN1, and B indicates the input strand Mux-IN3. The sampling interval is set to 1 minute. The fluorescence assay results showed that only when the input strand A and B were in solution at the same time could the fluorescence intensity be significantly enhanced (curve 4). All data represent the average of three replicates. Error bars represent one standard deviation of three replicate analyses. (C) Logic gate symbol corresponding to an AND gate, which was activated by strand Mux-IN1 and Mux-IN3 to produce the output signal d2:c2.

PAGE experiments were also performed to further identify the DNA interaction in AND gate logic operation, Figure S4(A). When there is no input strand or only input strand demux-in3 was added, the substrate 2 band (blue dotted box in lanes 1–3) in the solution did not change. When the input strand Demux-IN1 was added, it specifically bound to Substrate 2 to generate Complex 5 (green dotted box in lane 4). However, when both single-stranded Demux-IN1 and Demux-IN3 were added and no FokI enzyme was present, Complex 6 (yellow dotted box in lane 7) was generated. When the FokI enzyme was added, the corresponding Complex 6 band basically disappeared, and double-strand d2: c2 (red dotted box in lane 5) was generated, with

lane 6 being a contrast channel. This micrograph demonstrated the feasibility of our AND gate.

The fluorescence analysis results of our AND Gate are shown in Figure S4(B). Curves 1-3 indicate that no significant strong fluorescence signal was produced in the absence of an input strands or in the presence of only one of them. In contrast, curve 4 indicates that a significant increase in fluorescence intensity was observed when two input strands were added in the presence of both the FokI enzyme and the strand c2. The logic gate symbol corresponding to the AND gate, Figure S4(C). Among them, Input A represents the input chain Mux-IN1, and Input B represents the input chain Mux-IN3.

**5. INHIBIT Gate**

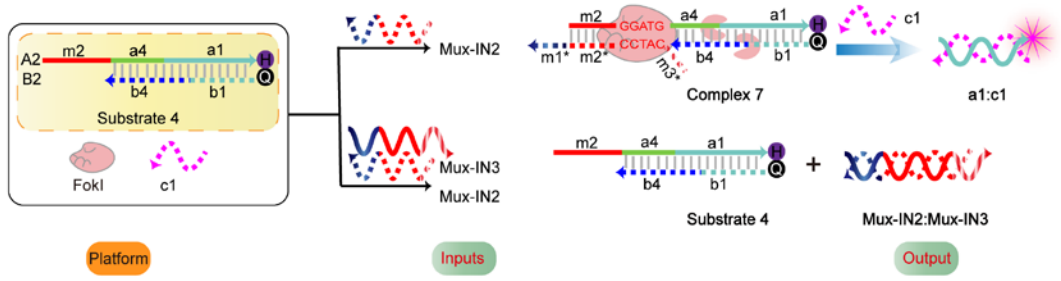



**Figure S5**. Simulate the reaction schematic of the INHIBIT gate. The fluorophore HEX and the quencher BHQ1 were modified at the 3' and 5' of the double-stranded Substrate 4, respectively, and the enzymatic cleavage products reacted with single-stranded c1 to trigger the fluorescence signal.

We also constructed another INHIBIT gate (Figure S5) using the reaction principle of the RBM. The INHIBIT gate includes a data input (Mux-IN2) and a control input (Mux-IN3). If the Mux-IN3 input is true, the logic gate fails and the output is always false. If the Mux-IN3 input is false, the output depends on Mux-IN2. In addition, the best effects of the reaction principle of the RBM and the leakage of inhibit are considered. Finally, we chose the base length of m3* as 3-nt toehold for the experiment. When there were no input strands or only the input strand Demux-IN3 exists, the entire logic system was not react with each other. In the case where Demux-IN2 and Demux-IN3 coexisted, which the priority of hybridization between Demux-IN2

and Demux-IN3 over their respective interactions with Substrate 4. So inhibit the enzyme digestion reaction, and the output signal was 0. The logic output was true if and only if the input strand Demux-IN2 was present, with the HEX fluorescence modified final product a1:c1 as the output signal that implements the inhibit logic operation.

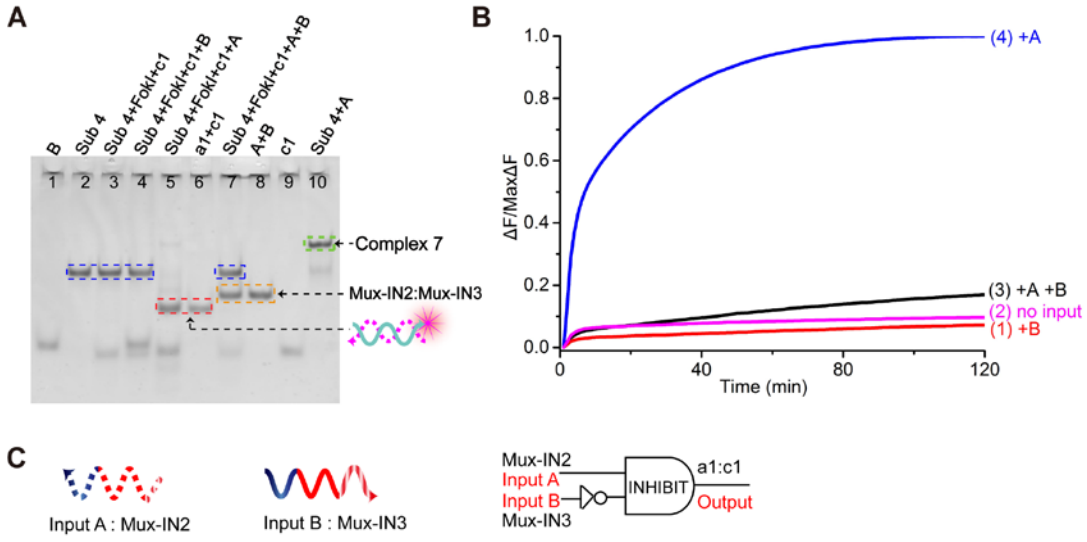



**Figure S6**. (A) Native PAGE analysis of INHIBIT gate products. Each lane number is marked with different reactions of input strand and substrate, lane 3: input reaction (0,0); lane 4: input reaction (0,1); lane 5: input reaction (1,0); and lane 7: input reaction (1,1). (B) Time-dependent normalized fluorescence changes of the INHIBIT gate during the 2 hour reaction. A indicates the input strand Mux-IN2, and B indicates the input strand Mux-IN3. The sampling interval is set to 1 minute. The fluorescence results showed that only when the input strand A was added to the solution could the fluorescence intensity be significantly enhanced (curve 4). All data represents the average of three replicates. (C) Logic gate symbol corresponding to an INHIBIT gate, which was activated by strand Mux-IN2 to generate the output signal a1:c1.

Figure S6 (A). Native polyacrylamide gel analysis of the interactions among Substrate 4, Mux-IN2, and Mux-IN3. DNA strands Substrate 4, Mux-IN2, and Mux-IN3 are abbreviated as Sub 4, A and B, respectively. The sample in each lane and the identities of the main bands are indicated above and at the sides of the gel image, respectively. Lane 1 band represents a single strand B. The blue dotted box in lanes 2-

4 indicate that the Sub 4 strips in the solution did not change in any of the input strands or only in the input strand B. In the coexistence of input strands A, B and the double-stranded Sub 4, two bands were found from lane 7. One band appeared at the position similar to that of Sub 4, another appeared at a position similar to that of Mux-IN2/Mux-IN3. However, when the input strand A and the double-stranded Sub 4 coexist, a new band appears at the position of the green dotted box in the lane 10, indicating that Sub 4/A forms Complex 7. Then, under the action of the FokI enzyme, the corresponding Complex 7 band basically disappeared. In addition, a double-stranded a1:c1 (red dotted box in lane 5) with a HEX modification was generated, and lane 6 was the corresponding contrast channel.

Figure S6 (B) shows that the results of fluorescence experiments and PAGE were consistent with each other. In this logic operation, when the input was 0 or only the single-stranded B was input, no significant strong fluorescent signal was produced (curve 1-2). A small amount of leakage can be observed in curve 3. The explanation for this phenomenon may be that when two input strands were added at the same time, there are still a few input strand A combined with the Sub 4 resulting in a slight increase in the HEX fluorescence signal. A strong fluorescent signal was generated when only the input strand A was added (curve 4). The logic gate symbol corresponding to the INHIBIT gate can be found in Figure S6(C). The fluorescence assay result indicates that the DNA interactions occurred as expected.

## 6. References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