Supplementary information

Engineering a dual-loop molecular circuit with buffering capability to solve molecular information tasks

Xin Liu,†[a] Shuang Cui,†[a] Xun Zhang,[a] Yao Yao,[a] Bang Zhang,[b] Bin Wang,[b] and Qiang Zhang*[a]

a. School of Computer Science and Technology, Dalian University of Technology, Dalian 116024, China.
b. Key Laboratory of Advanced Design and Intelligent Computing, Ministry of Education, School of Software Engineering, Dalian University, Dalian 116622, China.
† These authors contributed equally.
Email: zhangq@dlut.edu.cn

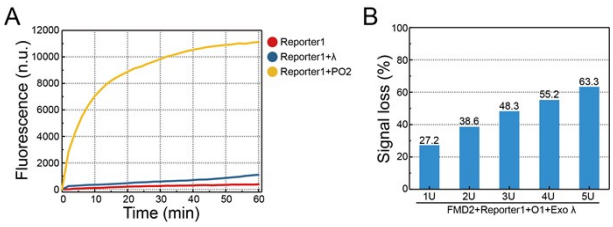

Figure S1. Fluorescence response of Reporter1. (A) The positive and negative curves of Reporter1. The maximum fluorescence intensity of fully triggered Reporter1 is used as the standard positive signal ($F_{standard}$). (B) Signal loss under the enzyme gradients. The calculation formula for the signal loss is $F_{loss} = (F_{standard} - F) / F_{standard}$. F is the fluorescence intensity measured under the current conditions.

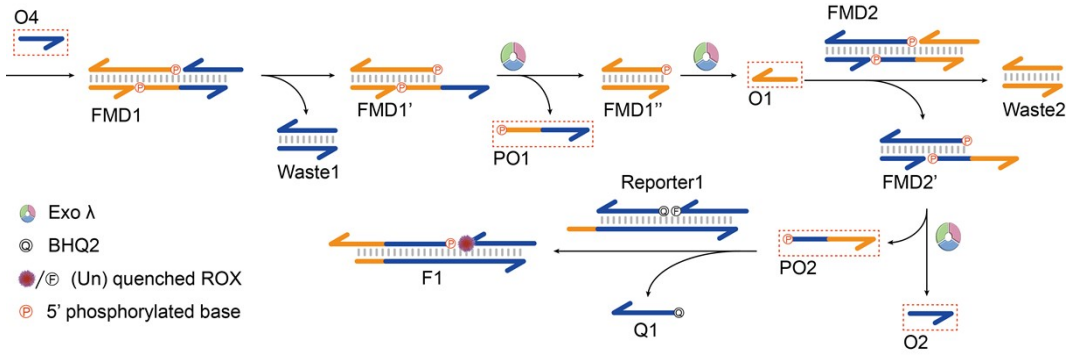

Figure S2. Schematic illustration of the cascaded FMD1–FMD2. In the cascaded FMD1–FMD2, O2 produced by FMD1 is the upstream signal of FMD2.

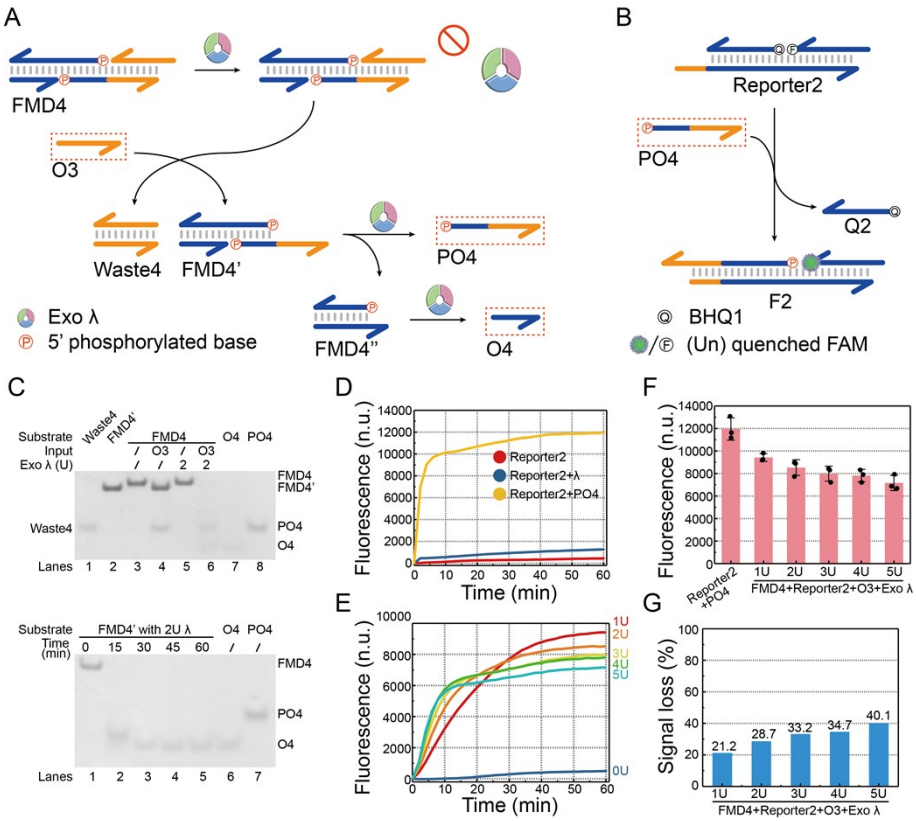

Figure S3. The verification of replaced sequences for FMD. (A) Schematic illustration of FMD4 triggered by O3. (B) Principle of signal response of Reporter2. (C) Gel verification for the hydrolysis of FMD4. Verification of FMD4 triggered by O3 for the first gel, and verification of FMD4' hydrolysis under a time gradient for the second gel. (D) The positive and negative intensities of Reporter2. The maximum fluorescence intensity of fully triggered Reporter2 is used as the standard positive signal ($F_{standard}$). (E) Fluorescence output of FMD4 and Reporter1 triggered by O3. Maximum fluorescence output (F) and signal loss (G) under an enzyme gradient. The calculation formula for the signal loss is $F_{loss} = (F_{standard} - F) / F_{standard}$. F is the fluorescence intensity measured under the current conditions. In both Native PAGE and fluorescence experiments, the substrate or input is 20 pmol, and the fluorescence data represent the average of three replicate experiments.

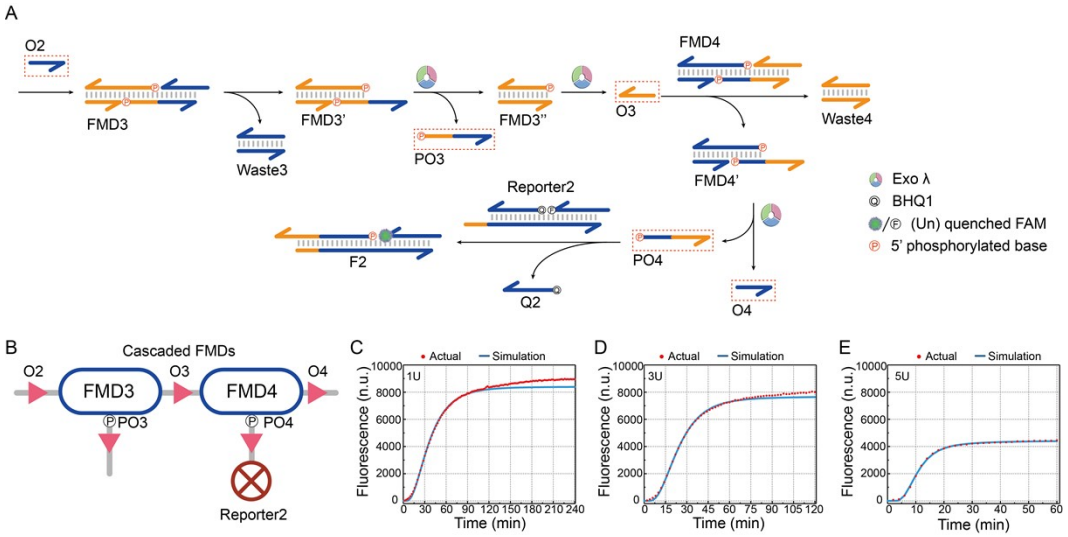

Figure S4. Verification of cascaded FMD3–FMD4. (A) Schematic illustration of the cascaded FMD3–FMD4. In the cascaded FMD3–FMD4, O3 produced by FMD3 is the upstream signal of FMD4. (B) Schematic diagram of cascaded FMD3–FMD4. Actual fluorescence output and its simulation of cascaded FMDs triggered by O2 at enzyme of 1 U (C), 3 U (D), and 5 U (E).

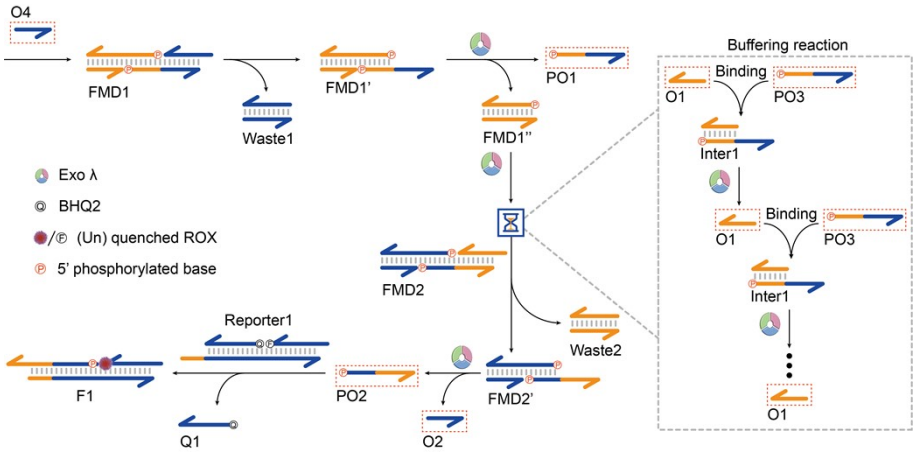

Figure S5. Schematic illustration of the cascaded FMD1–FMD2 with buffering. O1 is being produced during the hydrolysis of FMD1'', which is then buffered by PO3 to flow into FMD2 with a delayed time.

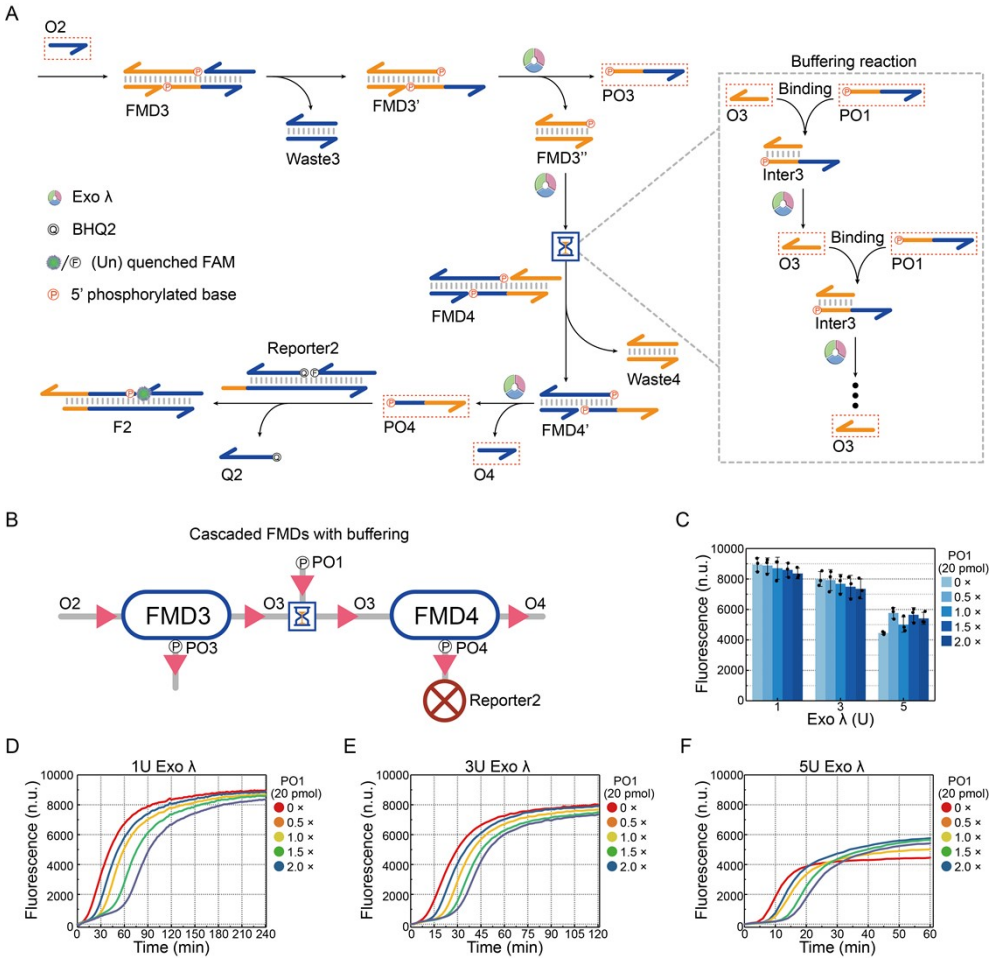

Figure S6. Verification of the cascaded FMD3–FMD4 with buffering. (A) Schematic illustration of the cascaded FMDs with buffering. O3 is being produced during the hydrolysis of FMD3'', which is then buffered by PO1 to flow into FMD4 with a delayed time. (B) Schematic diagram of cascaded FMD3–FMD4 with buffering. (C) Maximum fluorescence values of cascaded FMD3–FMD4 with gradient concentration of buffering sequence. Fluorescence curves of cascaded FMD3–FMD4 with gradient concentration of buffering sequence at enzyme of 1 U (D), 3 U (E), and 5 U (F). All the substrates are 20 pmol, input gradient is 0-1.0 × 20 pmol, the gradient of buffering sequence PO1 is 0-2.0 × 20 pmol, and fluorescence data is the average of three repeated experiments.

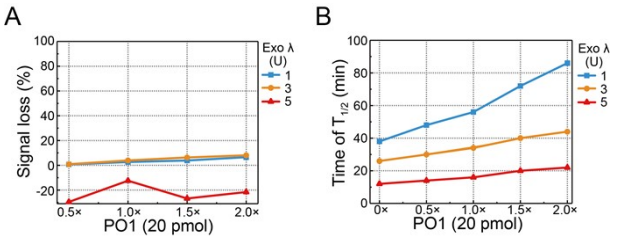

Figure S7. Signal transmission analysis of cascaded FMD3–FMD4 with buffering. (A) Signal loss of cascaded FMD3-FMD4 with gradient concentration of buffering sequence and enzyme. (B) Time delay characteristics of cascaded FMD3-FMD4 with gradient concentration of buffering sequence and enzyme.

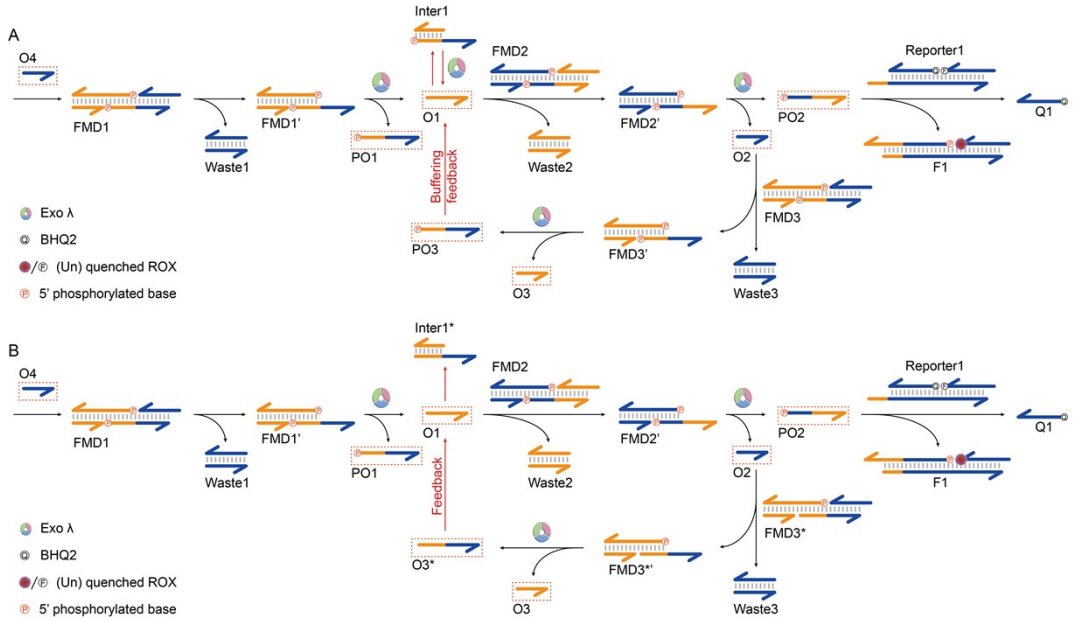

Figure S8. Feedback principle of cascaded FMD1–FMD2–FMD3/FMD3*. Schematic illustration of the buffering feedback (A) and the feedback without buffering (B). The phosphorylated PO3 is the buffering feedback signal, while the unphosphorylated O3* is the feedback signal.

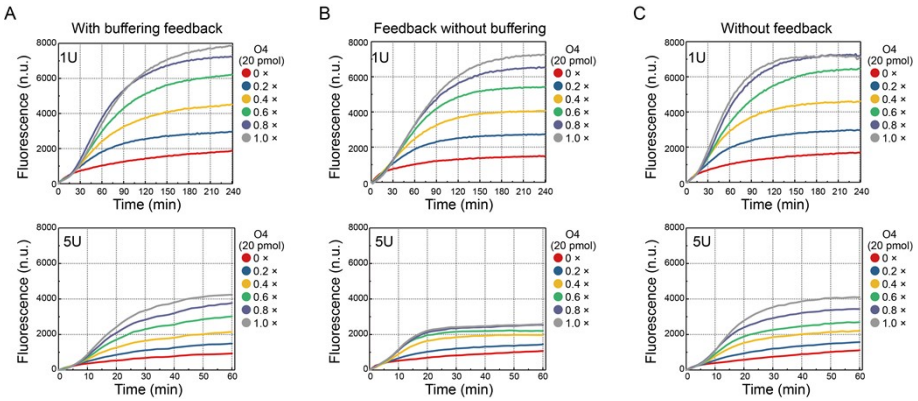

Figure S9. Fluorescence response of the molecular circuits in Figs. 3A, B, and C under gradient concentration of enzyme and input. Fluorescence response of FMD1–FMD2–FMD3 (A), FMD1–FMD2 (B), and FMD1–FMD2–FMD3* (C) under gradient concentration of enzyme and input. All the substrates are 20 pmol, the input gradient ranged from 0 to 1.0 × 20 pmol, and Exo λ are 1U and 5U. Fluorescence data represents the average of three repeated experiments.

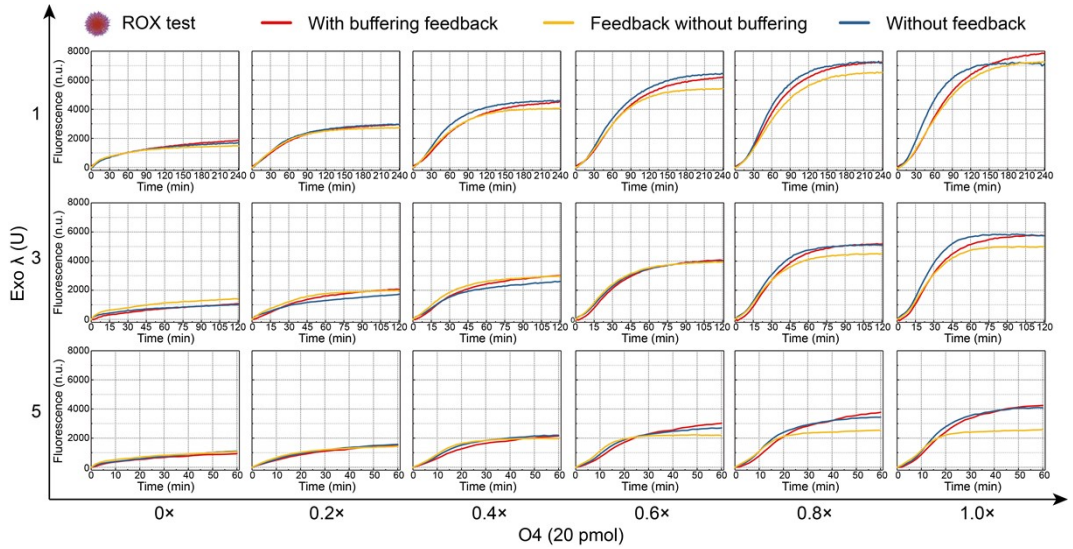

Figure S10. Comparison of fluorescence characteristics of the molecular circuits in Figs. 3A, B, and C under the combination of enzyme gradient and input concentration gradient.

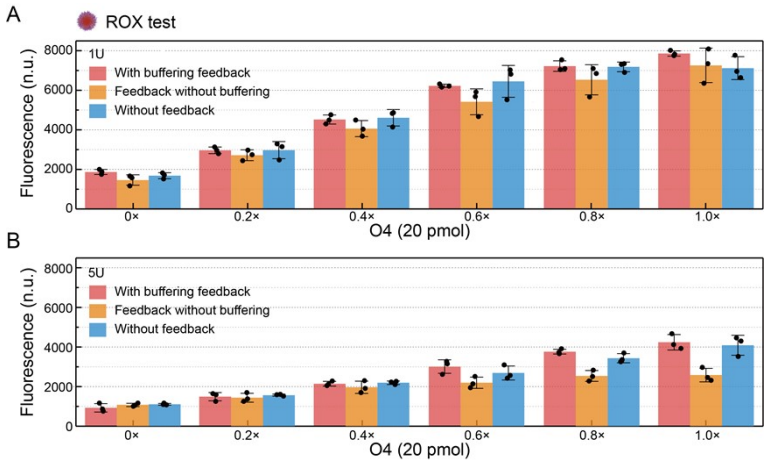

Figure S11. Maximum fluorescence values of the molecular circuits in Figs. 3A, B, and C at enzyme of 1 U (A) and 5 U (B).

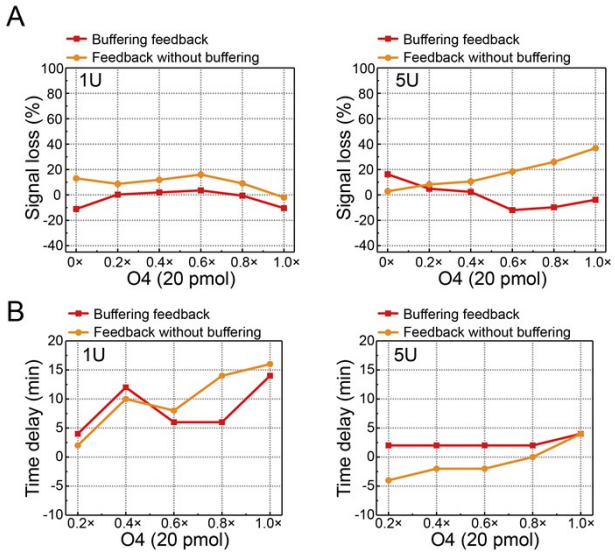

Figure S12. Signal loss (A) and time delay (B) of buffering feedback (Fig. 3A) and feedback without buffering (Fig. 3B) relative to the circuit without feedback (Fig. 3C).

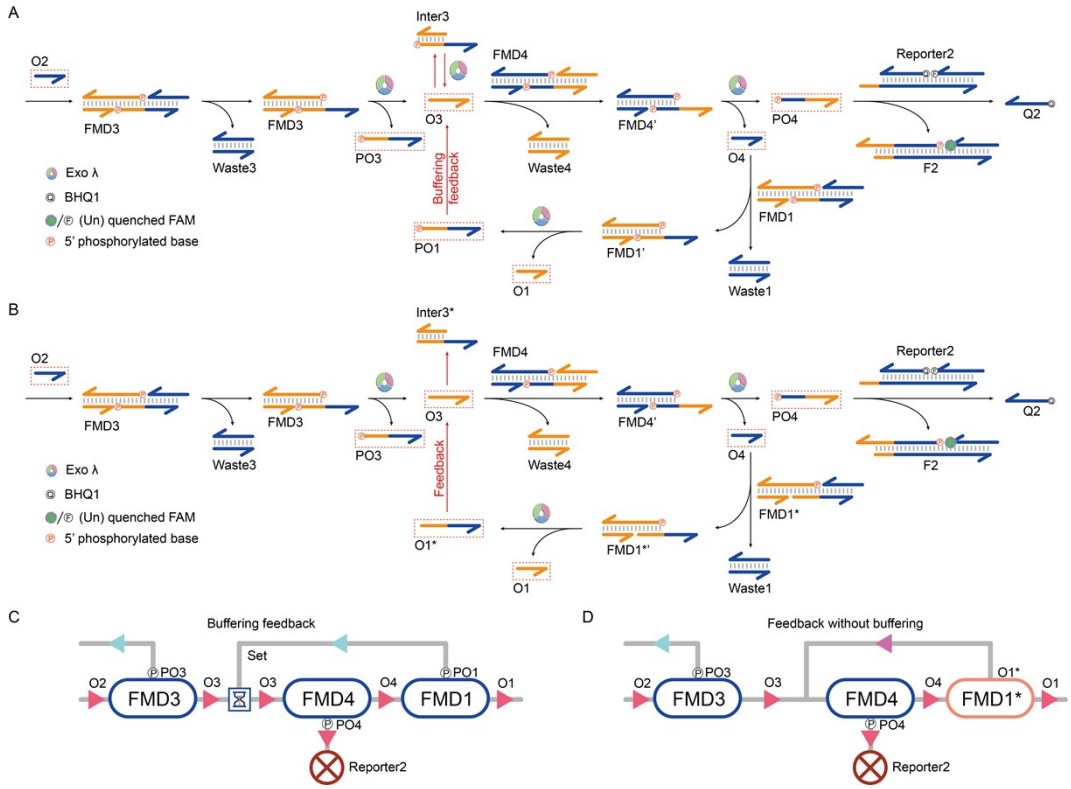

Figure S13. The feedback principle of cascaded FMD3–FMD4–FMD1/FMD1*. Schematic illustration of FMD3–FMD4–FMD1 (A) and FMD3–FMD4–FMD1* (B). Schematic diagram of FMD3–FMD4–FMD1 (C) and FMD3–FMD4–FMD1* (D).

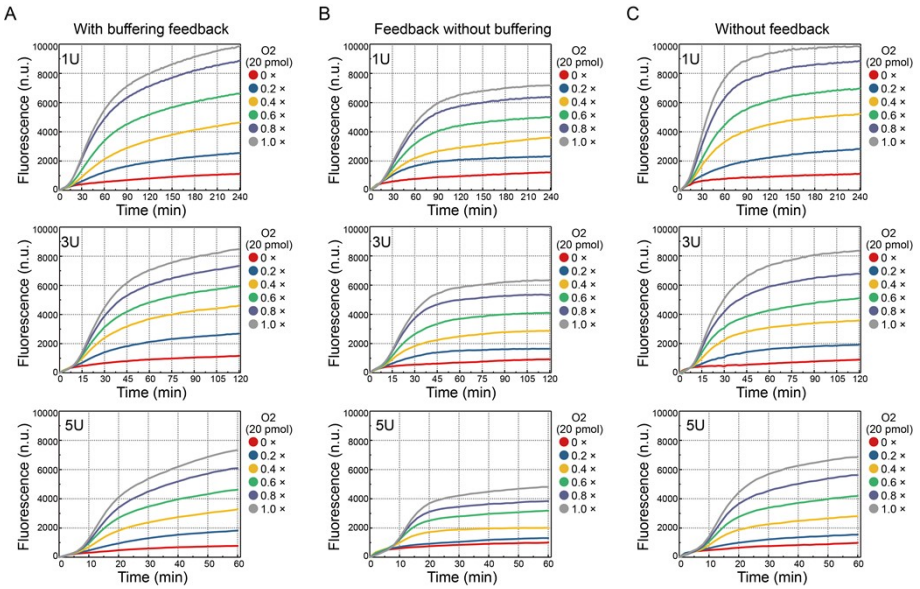

Figure S14. Fluorescence response of the molecular circuits in Figs. S13C, D, and Fig. S4B under the combination of enzyme gradient and input concentration gradient. Fluorescence response of FMD3–FMD4–FMD1 (A), FMD3–FMD4 (B), and FMD3–FMD4–FMD1* (C). All the substrates are 20 pmol, the input gradient ranged from 0 to 1.0 × 20 pmol, and Exo λ are 1U, 3 U and 5 U. Fluorescence data represents the average of three repeated experiments.

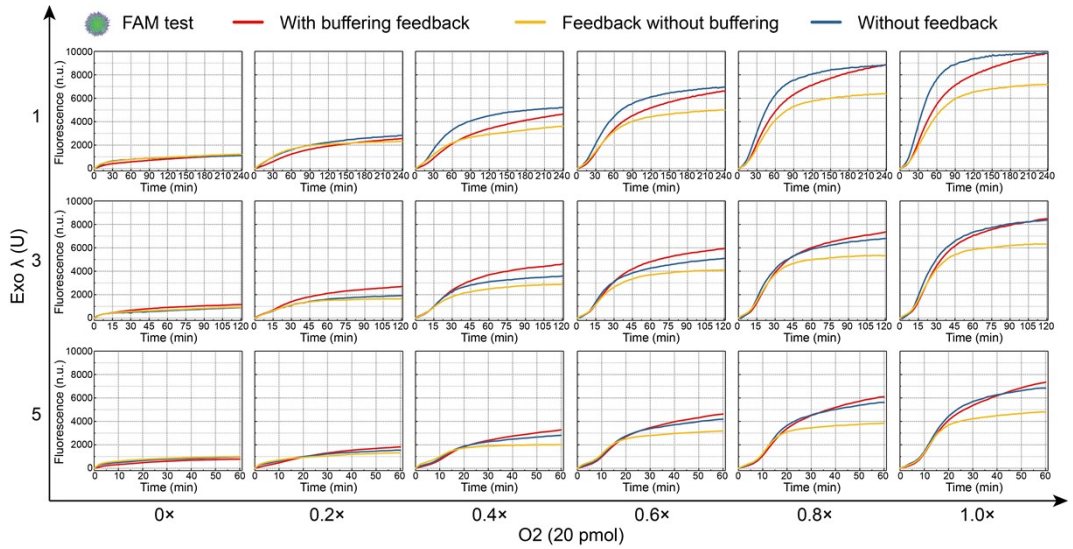

Figure S15. Comparison of fluorescence characteristics of the molecular circuits in Figs. S13C, D, and Fig. S4B under the combination of enzyme gradient and input concentration gradient.

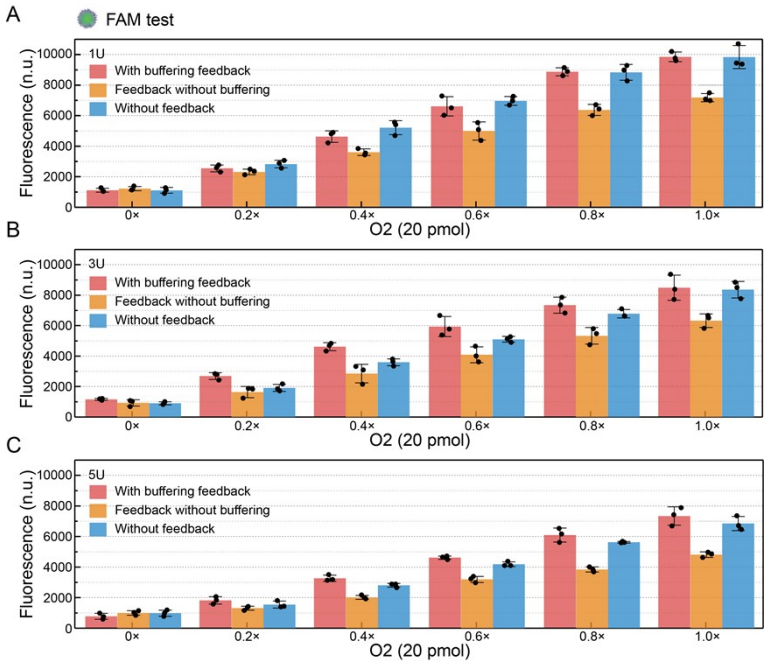

Figure S16. Maximum fluorescence values of the molecular circuits in Figs. S13C, D, and Fig. S4B at enzyme of 1 U (A), 3 U (B), and 5 U (C).

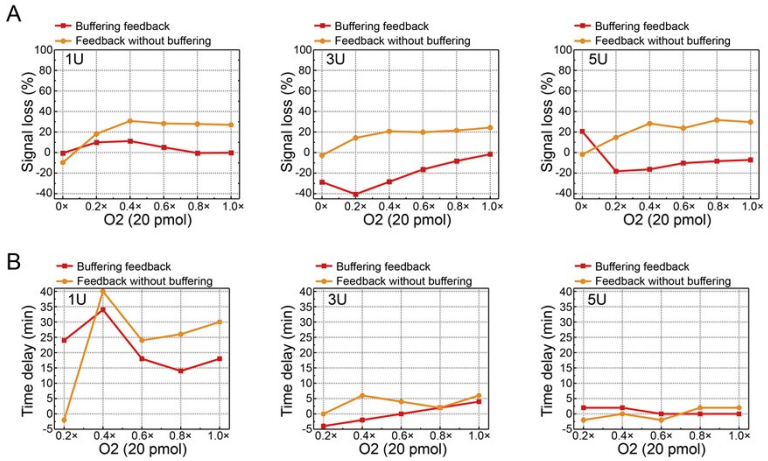

Figure S17. Signal loss (A) and time delay (B) of buffering feedback (Fig. S13C) and feedback without buffering (Fig. S13D) relative to the circuit without feedback (Fig. S4B).

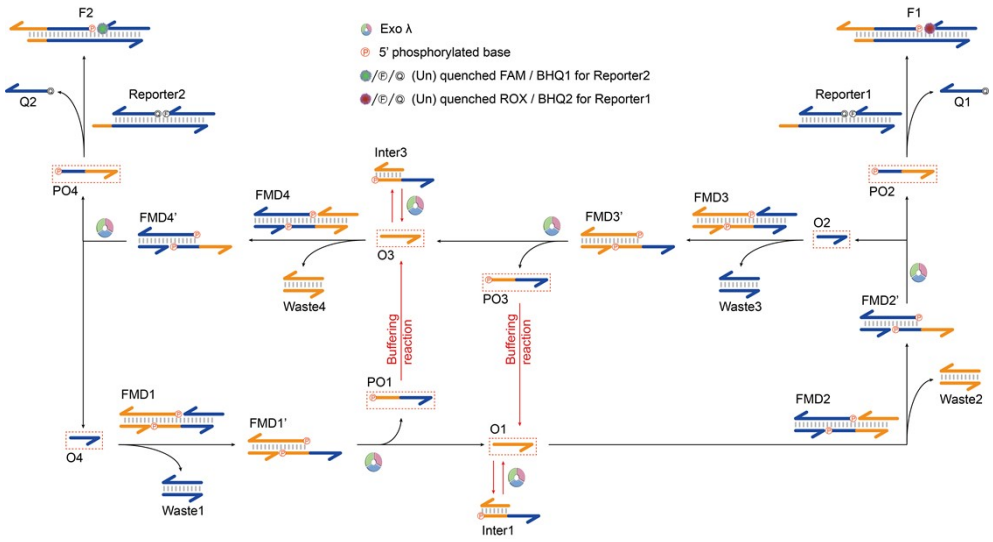

Figure S18. Schematic illustration of dual-loop molecular circuit in Fig. 4A with buffering.

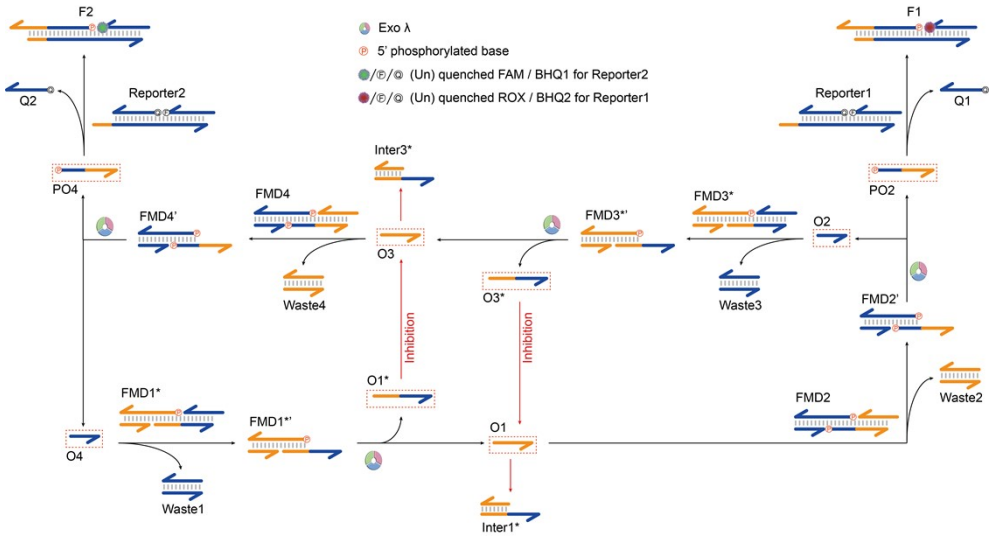

Figure S19. Schematic illustration of dual-loop molecular circuit in Fig. 4B without buffering.

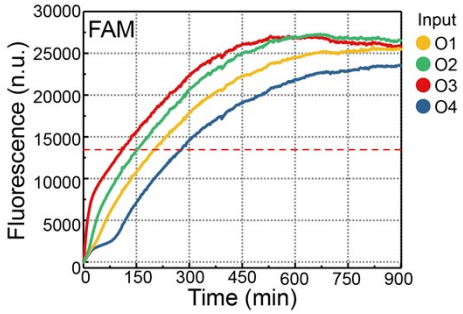

Figure S20. Fluorescence response of the dual-loop buffering circuit in Fig. 4A at different input positions. The red dashed line represents the fluorescence intensity of a 20 pmol reporter, and the fluorescence data represents the average of three repeated experiments.

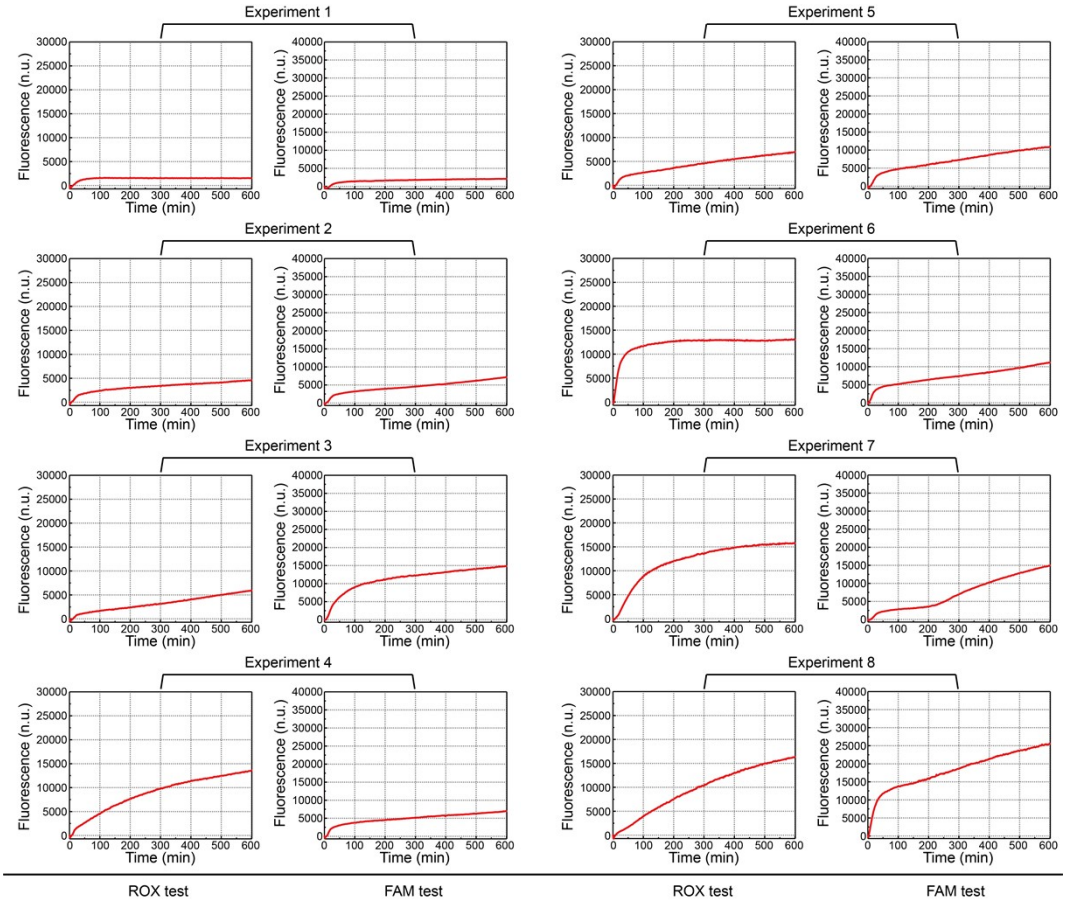

Figure S21. Experiments 1–8 in L-16 orthogonal array of Table S1.

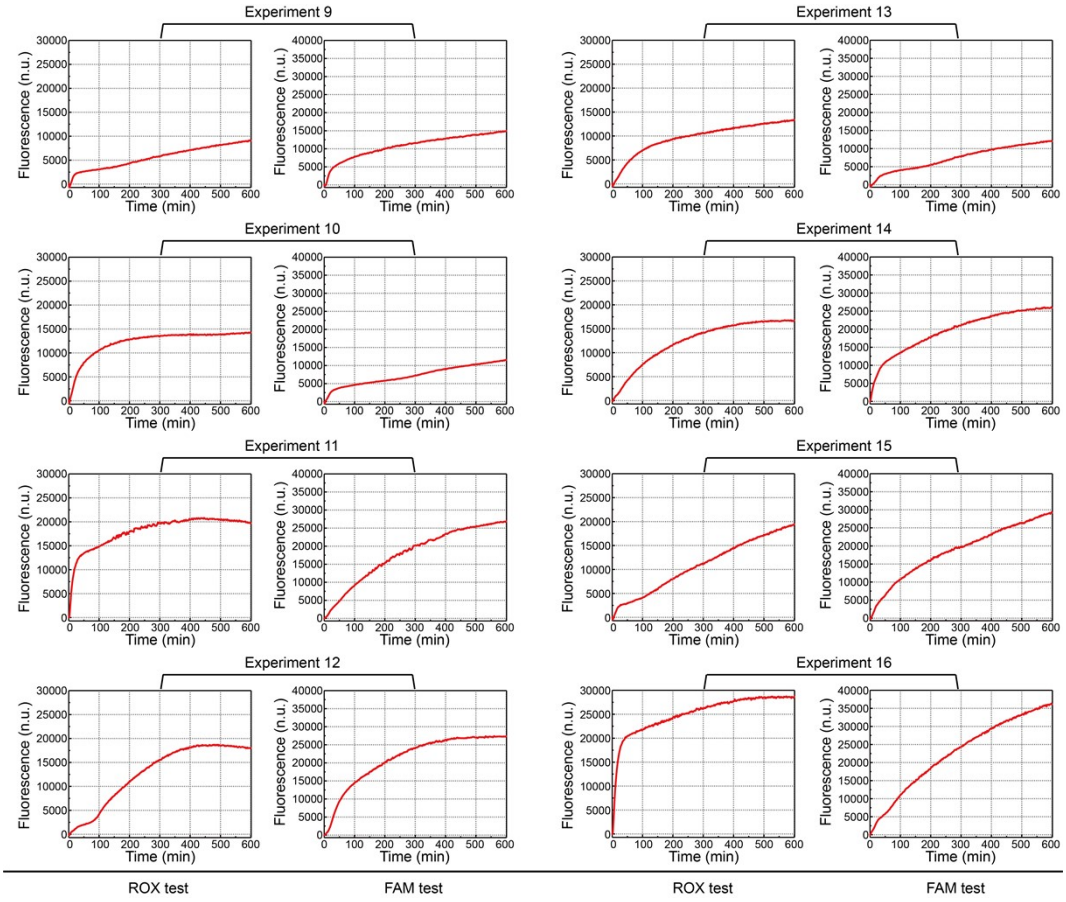

Figure S22. Experiments 9–16 in L-16 orthogonal array of Table S1.

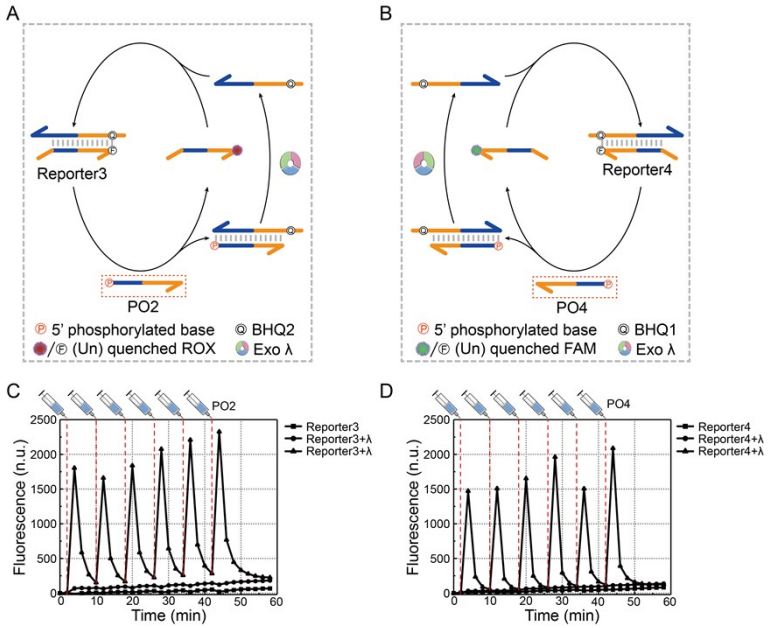

Figure S23. The redesigned reporter with signal self-restoration. Schematic illustration of Reporter3 (A) and Reporter4 (B). Six consecutive input tests on Reporter3 (C) and Reporter4 (D). The inputs and reporters are 20 pmol, and each cycle for the addition of corresponding input is 8 min.

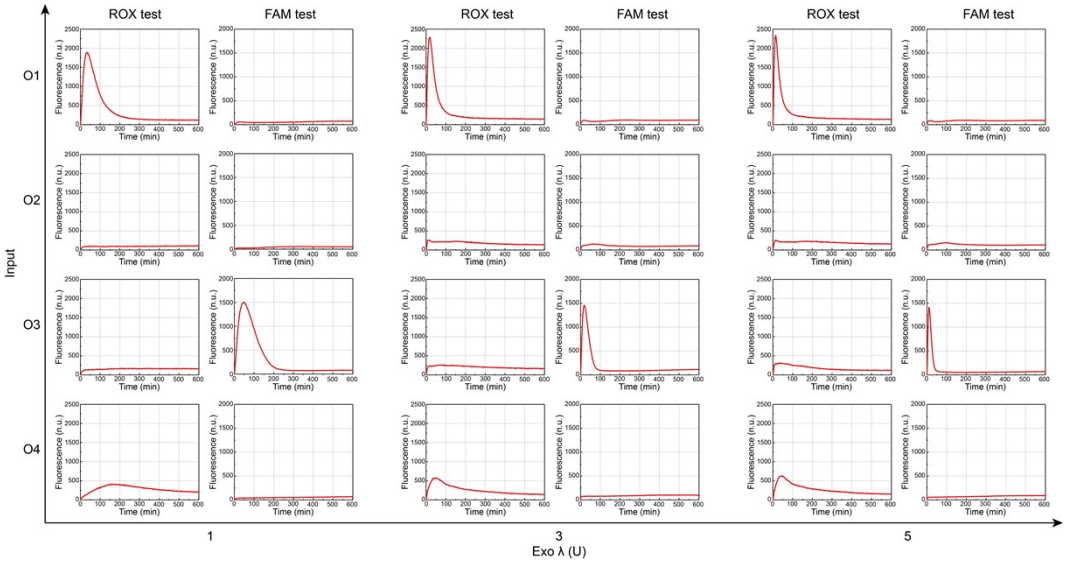

Figure S24. Pulse signal response of dual-loop molecular circuit in Fig. 5A with buffering.

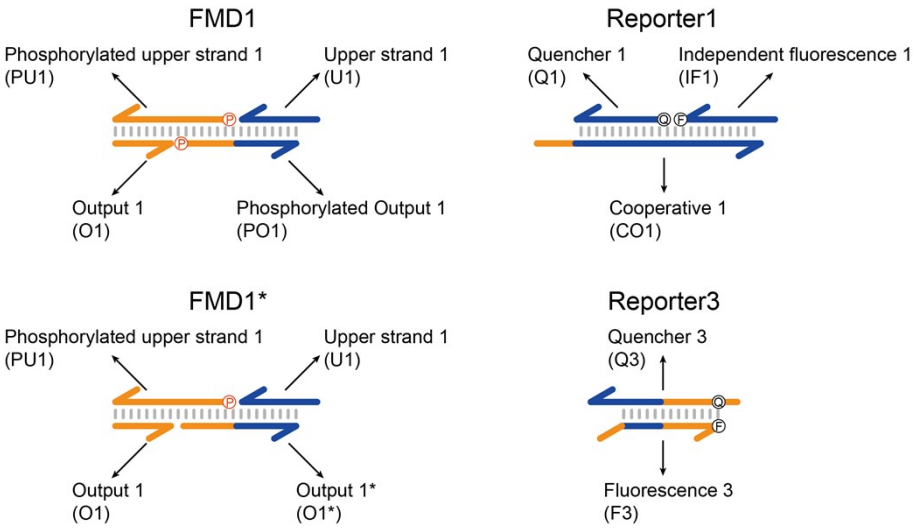

Figure S25. The naming rules for all sequence in this article. Taking FMD1 and FMD1* as examples in FMD, the naming for other FMDs only needs to change the numbering. According to the structure of the reporter, Reporter1 and Reporter2 have the same naming rules, while Reporter3 and Reporter4 have the same naming rules.

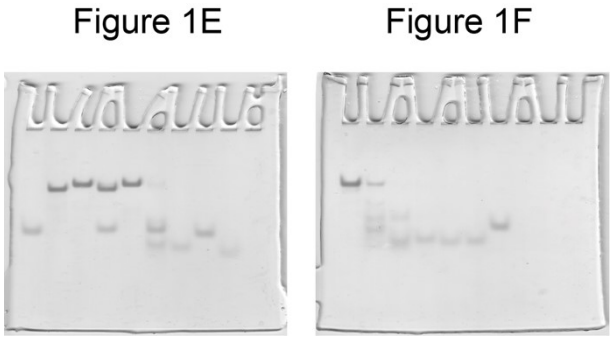

Figure S26. Uncropped and unedited gel image for Figures 1E and 1F.

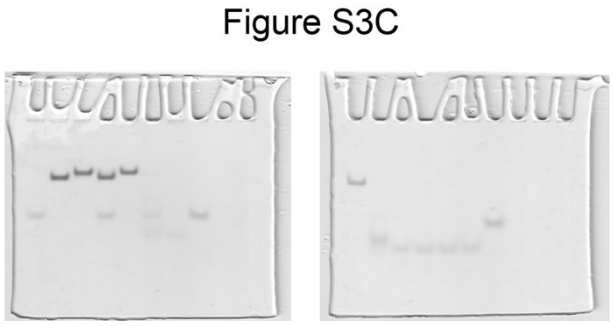

Figure S27. Uncropped and unedited gel image for Figure S3C.

Chemical reaction formula for FMD3–FMD4–FMD1:

$$FMD3 + O2 \xrightarrow{k_{10}} Waste3\,(W3) + FMD3' \quad \text{* MERGEFORMAT (1)}$$

$$FMD3' \xrightarrow{k_{11}} PO3 + FMD3'' \quad \text{* MERGEFORMAT (2)}$$

$$FMD3'' \xrightarrow{k_{12}} O3 \quad \text{* MERGEFORMAT (3)}$$

$$PO3 \xrightarrow{k_{13}} \varnothing \quad \text{* MERGEFORMAT (4)}$$

$$PO1 + O3 \xrightarrow{k_{b4}} Inter3 \quad \text{* MERGEFORMAT (5)}$$

$$Inter3 \xrightarrow{k_{b5}} O3 \quad \text{* MERGEFORMAT (6)}$$

$$PO1 \xrightarrow{k_{b6}} \varnothing \quad \text{* MERGEFORMAT (7)}$$

$$FMD4 + O3 \xrightarrow{k_{14}} Waste4\,(W4) + FMD4' \quad \text{*MERGEFORMAT(8)}$$

$$FMD4' \xrightarrow{k_{15}} PO4 + FMD4'' \quad \text{* MERGEFORMAT (9)}$$

$$FMD4'' \xrightarrow{k_{16}} O4 \quad \text{* MERGEFORMAT (10)}$$

$$PO4 \xrightarrow{k_{17}} \varnothing \quad \text{* MERGEFORMAT (11)}$$

$$PO4 + Reporter2 \xrightarrow{k_{18}} F2 + Q2 \quad \text{* MERGEFORMAT (12)}$$

Table S1. L-16 orthogonal array of Taguchi design

Experiment number	Cn (0.01U/μl)	Ci (0.2μM)	Om	Cs (0.2μM)	Empty
1	1 ×	0.5 ×	O1	3 ×	1
2	1 ×	1.0 ×	O2	4 ×	2
3	1 ×	1.5 ×	O3	5 ×	3
4	1 ×	2.0 ×	O4	6 ×	4
5	3 ×	0.5 ×	O2	5 ×	4
6	3 ×	1.0 ×	O1	6 ×	3
7	3 ×	1.5 ×	O4	3 ×	2
8	3 ×	2.0 ×	O3	4 ×	1
9	5 ×	0.5 ×	O3	6 ×	2
10	5 ×	1.0 ×	O4	5 ×	1
11	5 ×	1.5 ×	O1	4 ×	4
12	5 ×	2.0 ×	O2	3 ×	3
13	7 ×	0.5 ×	O4	4 ×	3
14	7 ×	1.0 ×	O3	3 ×	4
15	7 ×	1.5 ×	O2	6 ×	1
16	7 ×	2.0 ×	O1	5 ×	2

As a supplement to the fifth variable, the empty group with 4 values is used to complete the L-16 orthogonal array of Taguchi design, and the empty group will not affect the experimental results.

Table S2. Fluorescence data of the classification testing of samples 1-9 in dual loop buffering circuit.

Sample number	1	2	3	4	5	6	7	8	9
True class	O1	O1	O1	O1	O1	O1	O1	O1	O1
ROX_{Max} (a.u.)	1363	1507	1543	2336	1573	1624	1497	1426	1694
FMA_{Max} (a.u.)	64	62	109	94	104	124	58	59	114
r	1.33	1.39	1.15	1.40	1.18	1.12	1.41	1.38	1.17

Table S3. Fluorescence data of the classification testing of samples 10-18 in dual loop buffering circuit.

Sample number	10	11	12	13	14	15	16	17	18
True class	O2	O2	O2	O2	O2	O2	O2	O2	O2
ROX_{Max} (a.u.)	190	206	224	254	225	220	233	201	192
FMA_{Max} (a.u.)	155	156	338	156	332	307	178	152	325
r	0.09	0.12	-0.18	0.21	-0.17	-0.15	0.12	0.12	-0.23

Table S4. Fluorescence data of the classification testing of samples 19-27 in dual loop buffering circuit.

Sample number	19	20	21	22	23	24	25	26	27
True class	O3	O3	O3	O3	O3	O3	O3	O3	O3
ROX_{Max} (a.u.)	230	231	178	313	190	188	222	230	178
FMA_{Max} (a.u.)	1218	1182	1346	1416	1246	1159	1191	1139	1287
r	-0.72	-0.71	-0.88	-0.66	-0.82	-0.79	-0.73	-0.69	-0.86

Table S5. Fluorescence data of the classification testing of samples 28-36 in dual loop buffering circuit.

Sample number	28	29	30	31	32	33	34	35	36
True class	O4	O4	O4	O4	O4	O4	O4	O4	O4
ROX_{Max} (a.u.)	544	544	490	632	549	531	560	571	532
FMA_{Max} (a.u.)	67	61	52	93	62	58	84	66	59
r	0.91	0.95	0.97	0.83	0.95	0.96	0.82	0.94	0.96

Table S6. DNA sequences in this work.

Hybrids	Oligos	Sequences (from 5’ to 3’)
FMD1	U1	TAAGAGTATGATAGATTGAGGA
	PU1	PO_4-TTGGATGGTTATTGAAGTAGTGTATGTAGTATGAGTAGGTTG
	PO1	PO_4-CTACTTCAATAACCATCCAATCCTCAATCTATCATA
	O1	CAACCTACTCATACTACATACA
FMD2	U2	TGTATGTAGTATGAGTAGGTTG
	PU2	PO_4-TAACTCTTCCATCATTCTATCCTAATACTCCCTAACACTTCA
	PO2	PO_4-ATAGAATGATGGAAGAGTTACAACCTACTCATACTA
	O2	TGAAGTGTTAGGGAGTATTAGG
FMD3	U3	CCTAATACTCCCTAACACTTCA
	PU3	PO_4-ACCTACTCATACTACATACACTACTTCAATAACCATCCAATC
	PO3	PO_4-TGTATGTAGTATGAGTAGGTTGAAGTGTTAGGGAGT
	O3	GATTGGATGGTTATTGAAGTAG
FMD4	U4	CTACTTCAATAACCATCCAATC
	PU4	PO_4-ACTCACTCCAGGTTGTAACTTAAGAGTATGATAGATTGAGGA
	PO4	PO_4-AGTTACAACCTGGAGTGAGTGATTGGATGGTTATTG
	O4	TCCTCAATCTATCATACTCTTA
FMD1*	U1	TAAGAGTATGATAGATTGAGGA
	PU1	PO_4-TTGGATGGTTATTGAAGTAGTGTATGTAGTATGAGTAGGTTG
	O1*	CTACTTCAATAACCATCCAATCCTCAATCTATCATA
	O1	CAACCTACTCATACTACATACA
FMD3*	U3	CCTAATACTCCCTAACACTTCA
	PU3	PO_4-ACCTACTCATACTACATACACTACTTCAATAACCATCCAATC
	O3*	TGTATGTAGTATGAGTAGGTTGAAGTGTTAGGGAGT
	O3	GATTGGATGGTTATTGAAGTAG
Reporter 1	IF1	TCACTTCTCTACCAAT-ROX

	Q1	**BHQ2**-ATAGAATGATGGAAGA
	CO1	TGTAACTCTTCCATCATTCTATATTGGTAGAGAAGTGA

Hybrids	Oligos	Sequences (from 5’ to 3’)
Reporter 2	IF2	TTTTTTCACTTCTCTACCAAT-FAM
	Q2	**BHQ1**-AGTTACAACCTGGAGT
	CO2	TTTTTTCACTCACTCCAGGTTGTAACTATTGGTAGAGAAGTGA
Reporter 3	F3	TTTTTTAGTTACAACCAGTGTG-ROX
	Q3	TTTTTT/**iBHQ2dT**/CACACTGGTTGTAACTCTTCCATCATTCTAT
Reporter 4	F4	TTTTTTTGAGTGATTGTGAGAG-FAM
	Q4	TTTTTT/**iBHQ1dT**/CTCTCACAATCACTCACTCCAGGTTGTAACT

Modifications:
PO_4: Phosphorylated modification
FAM: FAM modification
BHQ1: Black Hole Quencher 1
/**iBHQ1dT**/: Black Hole Quencher 1 modified thymine base
ROX: ROX modification
BHQ2: Black Hole Quencher 2
/**iBHQ2dT**/: Black Hole Quencher 2 modified thymine base