

Electronic Supplementary Information

Stacking Modular DNA Circuitry in Cascading Self-Assembly of Spherical Nucleic Acids

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S1 Simulation methods and additional experimental results

S1.1 Toehold Design of Two-Layer Cascaded Systems

In the two-layer cascaded assembly of SNAs, the invading (d) and incumbent (b) toeholds for the first toehold-exchange reaction in Machine-I are set to seven and five bases, respectively, referred as $7/5$. In the second toehold-exchange reaction, the lengths of toehold domains ($a+b$) and d_2 are set to $10/5$. For Machine-II, the toehold domains ($f+c_2$) and g are set to $7/3$, and the toehold domains ($i+g$) and f are set to $8/5$, for the first and second toehold-exchange reactions, respectively.

In the two-layer cascade system with Machine-I and machine-II being prepared using AuNPs and AuNRs, the toehold domains d and b are set to $5/4$, and ($a+b$) and d are set to $10/5$. In Machine-II, the toehold domains (c_2+f) and g are set to $7/5$, and ($i+g$) and f are set to $10/5$.

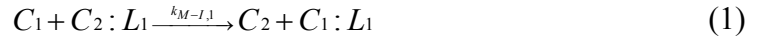
S1.2 Design Method of the Three-Layer Cascaded Assembly of SNAs

Given that leakage would conceal the real reaction signal, leakage is critical to its implementation in the design of multi-layer cascading of SNA assembly. For the single-layer circuit, the product concentration increases linearly with time. The situation is quite different for circuitry cascades, which potentially allow the polynomial and exponential amplification of input.¹ Therefore, to reduce the influence of leakage to ensure a perfect running of the three-layer cascaded assembly of the SNAs system, we purified the DNA complex-functionalized SNAs through centrifugation; the method can be found in detail in S1.5. On the other hand, the setup of toehold domains and the molar ratio of Machines-I, II, and III play an important role in leakage control. We adjusted the molar ratio of Machines-I, II, and III, and varied the length of domain a of the complex $C_2:L_1$ of SNA-2 in Machine-I to decrease the absolute circuit leakage of the three-layer cascaded circuitry of SNA assembly. The corresponding experimental results can be found in Fig. S21-S23, and the optimal result of the three-layer cascade is displayed in Fig. 5 of the main text.

S1.3 Theoretical models for two-layer cascaded modular circuitry of the SNA conjugate

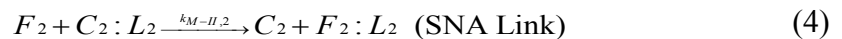
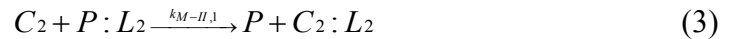
The spurious bindings and crosstalk among different species may slow down the effective rate of individual reactions as well as cause background leakage in the hybridization network.¹⁻⁴ In an integrated circuit, the increased complexity of interactions among molecules may introduce significant challenges to its robustness. Thus, a comprehensive understanding of its reaction mechanism is necessary to achieve an optimized system. We propose a model based on stochastic Monte Carlo method^{5, 6} to theoretically dissect the contribution of leak reactions to the kinetics of integrated circuit.

In our model, we suppose the presence of N_g single-stranded active oligonucleotides on SNA-1 (strand F_1) and N_g double-stranded active oligonucleotides on SNA-2 (duplex $C_2:L_1$). As shown in Fig. 1a in the main text, Machine-I is first initiated upon introducing strand C_1 , and runs continuously via two rounds of toehold-mediated strand-displacement reaction. Although strand displacement reactions are essentially a process of (re)breaking and (re)forming a series of individual base pairs, the overall kinetics still follow the bimolecular rate law. The net reactions are written as,



where $k_{M-I,1}$ and $k_{M-I,2}$ are biomolecular rate constants. The notation in the parenthesis indicates the crosslinking between SNA-1 and SNA-2 resulting from the formation of $F_1:L_1$.

Similarly, the mechanism of Machine-II can be expressed in following reactions,



where $k_{M-II,1}$ and $k_{M-II,2}$ are also biomolecular rate constants.

In this model, two SNAs, namely, SNA-1 and SNA-2, are considered in the same cluster once they are linked by at least one $F_1:L_1$ duplex. Intuitively, SNAs prefer to

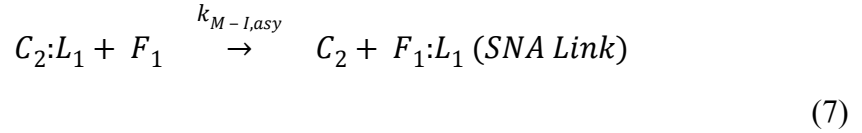
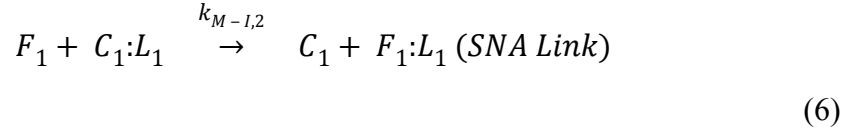
form small clusters consisting of a few particles at the early reaction stage. As reactions proceed to the later stage, these small clusters are preferentially crosslink into larger clusters containing more particles because of the multiple reactive oligonucleotides on each SNA (F_1 or $C_1:L_1$).

S1.4 Model and Monte Carlo algorithm for simulating the cascade of catalyst SNA circuitry

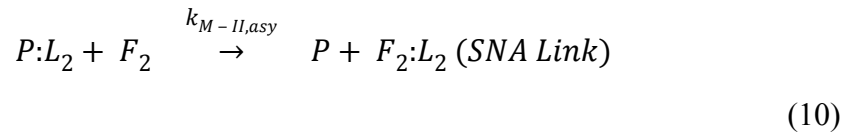
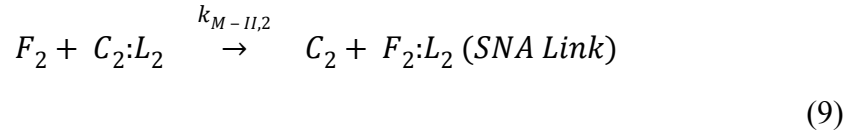
(1) Reaction scheme.

For the two-layer cascade of SNA circuitry, the net reactions are written as the following.

Machine-I:



Machine-II:



Equations (5)-(6) and (8)-(9) are the two rounds of toehold-mediated strand displacement reactions in Machines-I and II, respectively. Equations (7) and (10) are the asymptotic leakage reactions in these two machines. The typical values in the calculation are proximately estimated as^{1, 7, 8} $k_{M-I,1}$ and $k_{M-II,1}$, $3.47 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{M-I,2}$

and $k_{M-II,2}$, $2.59 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. The rate constants of asymptotic leakage, $k_{M-I,asy}$ and $k_{M-II,asy}$ are set to $6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$.

In our model, we assume that $N_g=5$ single-stranded active oligonucleotides on SNA-1 (strand F_1) and N_g double-stranded active oligonucleotides on SNA-2 (duplex $C_1:L_1$). The same setup is applied for SNA-3 and SNA-4 in Machine-II. As shown in Fig. 1a, in the main text, upon the introduction of single-stranded invader C_1 , Machine-I is first initiated and driven to run continuously *via* two rounds of toehold-mediated strand-displacement reaction, i.e., Equations (5) and (6). Although strand displacement reactions are essentially a process of (re)breaking and (re)forming many individual base pairs, the overall kinetics still follows the bimolecular rate law.

(2) Monte Carlo algorithm to simulate chemical reactions.

Gillespie's direct method of stochastic simulation, which is now called the stochastic simulation algorithm (SSA), was adopted herein for the kinetics simulation of chemical reactions.^{9, 10}

We assume that volume V contains a spatially homogenous mixture of N chemical species $(S_1, S_2, \dots, S_N)$, whose molecules can undergo M chemical reactions $(R_1, R_2, \dots, R_M)$ with their reaction constants $c_1, c_2, \dots, c_N$, that is, M reactions channels in total. Let $X_i(t)$ denotes the integer number of S_i molecules in the system at time t , we have $X(t) \equiv X_1(t), X_2(t), \dots, X_N(t)$. $P(x, t | x_0, t_0)$ represents the probability of $X(t) \equiv x = (x_1, x_2, \dots, x_N)$, given that the system was in state $X(t_0) \equiv x_0$ for $t_0 \leq t$.

The chemical master equation (CME) is the time evolution equation of $P(x, t | x_0, t_0)$,

$$\frac{\partial P(x, t | x_0, t_0)}{\partial t} = \sum_{j=1}^M [a_j(x - v_j)P(x - v_j, t | x_0, t_0) - a_j(x)P(x, t | x_0, t_0)] \quad (11)$$

Here, $v_j \equiv (v_{1j}, \dots, v_{Nj})$, where v_{ij} is the change in the S_i molecular population caused by one R_j event. a_j is the propensity function for reaction R_j , which is defined as,

$$a_j(x)dt \equiv \text{The probability, given } X(t) = x, \text{ that an } R_j \text{ event will occur somewhere in } [t, t + dt]$$

(12)

In the above mechanism of time evolution of the studied system, only the molecular populations are used to describe the system's state, without considering the position and velocities of individual molecules. That is, these considerations are based on the fundamental assumptions that the positions of the molecules become uniformly randomized throughout Ω , and the overwhelming majority of molecular collisions that take place in the system are elastic.

Since the CME is difficult to be solved for the probability density function $X(t)$, Gillespie proposed the SSA to calculate trajectories $X(t)$ versus t . The key to generating simulated trajectories of $X(t)$ is the new probability function $P(\tau, \mu | x, t)$, which is the mathematical basis for the stochastic simulation approach. $P(\tau, \mu | x, t)d\tau$ gives the probability that the next reaction event in the system with a state of $X(t)$ will occur in the time interval $[t + \tau, t + \tau + d\tau]$, and will be R_μ . To derive τ , μ and the analytical expression for $P(\tau, \mu | x, t)$, a function h_μ is defined for each reaction R_μ . h_μ is the number of distinct R_μ molecular reactant combinations available in the state $(X_1, X_2, \dots, X_N)$. For biomolecular reaction $S_1 + S_2 \rightarrow \dots$, $h_\mu = X_1 X_2$. Then, $a_\mu dt \equiv h_\mu c_\mu dt$ defines the probability that an R_μ reaction will occur in V in $(t, t + dt)$.

Then, the reaction probability density function is given by,

$$P(\tau, \mu | x, t) = e^{-a_0(x)\tau} a_\mu(x) \quad (13)$$

where, $a_0(x) \equiv \sum_{k=1}^M a_k(x)$. In Gillespie's procedure, the pair (τ, μ) is generated from the set of random pairs whose probability density function is $P(\tau, \mu | x, t)$. If we generate two random numbers r_1 and r_2 using the unit-interval uniform random

number generator, the value of τ and μ can be calculated using,

$$\tau = 1/a_0 \ln(1/r_1) \quad (14)$$

$$\sum_{v=1}^{\mu-1} a_v < r_2 a_0 \leq \sum_{v=1}^{\mu+1} a_v \quad (15)$$

The relationship between stochastic reaction constant c_μ and the deterministic reaction rate constant k_μ . For the molecules S_1 and S_2 in V undergoing the reaction,



Then, we have relationship $k_1 = V c_1 \langle X_1 X_2 \rangle / \langle X_1 \rangle \langle X_2 \rangle$. The angular brackets denote an average over an ensemble of identical systems. Given that $\langle X_1 X_2 \rangle = \langle X_1 \rangle \langle X_2 \rangle$, the relationship can be simplified as,

$$k_1 = V c_1 \quad (17)$$

For the reaction,



then the reaction constant

$$k_2 = V c_2 \langle X_1 X_2 \rangle / \langle X_1 \rangle \langle X_2 \rangle = V c_2 \langle X_1(X_1 - 1)/2 \rangle / \langle X_1 \rangle \langle X_1 \rangle = \dots$$

(3) Algorithm for computer implementation.

To obtain full information of the cascade of catalyst SNA circuitry operation process, the states (including the reaction groups, connection between SNAs and others) of each SNA are recorded during the simulations. For the first

toehold-exchange reaction in Machine-I, the hybridization between $C_2:L_1$ and C_1 strands occurs on the surface of AuNPs, and results in the formation $C_1:L_1$ and the release of C_2 for the operation of Machine-II. In our system setup, two types of functional group are set on AuNP-1. To facilitate the description of our methodology for simulations, the chemical species are defined as *unit* and the functional groups are defined as *type*. Note that, C_1 and other single-stranded oligomers are also set to a *unit* containing only one *type*. That is, in the program implementation, each chemical species (SNA-1, SNA-2, $C_1, \dots$, and so on) are described using the variable *unit*, which contains:

- Name of the unit (SNA-1, SNA-2, $C_1, \dots$);
- Species type index of the unit in our system. Such as, SNA-1 is set to 0, SNA-2 is 1, catalyst C_1 is 2, ...;
- Vector *UnitFg*, each element of which describing the name ($C_1, C_2:L_1, C_1:L_1, F_1, F_1:L_1, \dots$), state (reactive or unreactive), functional group type index, and number of each type of functional group ($N_g = 5$ for all SNAs and 1 for oligomers in our system) in one chemical species *unit*.

The variable *type* represents the information for each type of functional groups, containing:

- Name ($C_1, C_2:L_1, C_1:L_1, F_1, F_1:L_1, \dots$);
- Number of functional group.
- The name of *unit* which the functional group belongs to.

A map *cluster* to save the each cluster and the index of its *units*.

A map *clusterList* to save the size of cluster and amount of clusters which own the same cluster size.

Initialization

(1) Define the reactions $R_1 \sim R_M$ as equations (1) to (6), that is $M=6$; and

input the stochastic reaction constants $c_1 \sim c_M$. In our simulations, the concentrations of each chemical species are set the same as that in the

experiments. Moreover, the volume (V) used in our simulations is set to $0.545 \times 10^{-4} \mu\text{L}$.

- (2) Define the numbers of units for our studied system. We set $[N_1, N_2, N_3 \dots N_7]$ for the species [SNA-1, SNA-2, C_1 , C_2 , SNA-3, SNA-4, and C_3] in the two-layer circuit. Note that, $N_1=N_2$, $N_3=N_4$. Moreover, the ratio of N_1 and N_2 is set to the molar ratio of Machine-I and Machine-II.
- (3) The setup of functional groups.
 Each SNA-1 contains five reactive $C_2:L_1$, and five unreactive $C_1:L_1$.
 Each catalyst C_1 contains only one reactive group C_1 .
 A similar setup exists for the other chemical species.
- (4) The time of our investigated system is set at $t = 0$.

Simulation running

- (1) Calculate and store $(a_1, \dots, a_M)$ for reactions $(R_1, \dots, R_M)$. Herein,

$$a_k(x) = c_k * \prod x_i, \quad x_i \text{ is the reactive amount of functional groups involved in reaction } R_k. \text{ Then, we derive}$$

$$a_0(x) \equiv \sum_{k=1}^M a_k(x)$$

- (2) We generate two random numbers r_1 and r_2 using the unit-interval uniform random number generator, the value of τ and μ can be calculated using,

$$\tau = 1/a_0 \ln(1/r_1)$$

$$\sum_{v=1}^{\mu-1} a_v < r_2 a_0 \leq \sum_{v=1}^{\mu+1} a_v$$

- (3) Using the τ and μ values obtained in step (2), increase time t by τ . Pick the reaction R_μ for molecular population adjustment. It should be noted that the reactants in R_μ are the functional groups, rather than the chemical species. So we need to choose which functional groups will be changed from the reactive state to the unreactive state, and more importantly, if the reaction R_μ results in the linkage between two SNAs; these two SNAs are recorded in one

cluster, and *cluster* and *clusterList* should be updated.

For example, if reaction R_2 ($F_1 + C_1:L_1 \longrightarrow C_1 + F_1:L_1$ (SNA Link)) is the chosen reaction, the states of randomly selected functional groups (*type*) F_1 and $C_1:F_1$ will be changed from reactive state to unreactive state, and the states of randomly selected functional groups C_1 and one of the remaining unreactive $F_1:L_1$ groups of the SNA-4 (*unit*) which the chosen reactant (*type*) $C_1:F_1$ belongs to will be changed to reactive. Thereafter, the two SNAs (*units*) that F_1 and $C_1:F_1$ belong to will be recorded in the same cluster. In some situations, these two SNAs may belong to two different clusters. In such cases, all the SNAs in these two clusters are recorded in one cluster after the reaction, and *cluster* and *clusterList* should be updated.

After step (3), the program will return to step (1). It should be noted that $(a_1, \dots, a_M)$ will be updated after one (1)-(2)-(3) loop. The time evolution of the proposed circuit will be halted at time $t = 12.0 h$.

S1.5 Kinetics of the two-layer cascade of SNA circuitry

The UV/Vis absorbance decline originates from the aggregation of crosslinked SNAs, crosslinking of SNA-1 and SNA-2 driven by Machine-I together with crosslinking of SNA-3 and SNA-4 by Machine-II. In the model, each SNA is supposed to bear $N_g = 5$ oligonucleotides on its surface. The typical biomolecular reaction constants are estimated as^{1, 7, 8}, $k_{M-I,1}$ and $k_{M-II,1}$ of $3.47 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, as well as $k_{M-I,2}$ and $k_{M-II,2}$ of $2.59 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. The parameter f_{init} was experimentally estimated to be less than 0.1¹. By considering the destruction of $C_2:L_1$ as a lesser event, we take an even smaller value of 0.01 for the initial leakage (f_{init}). Thus, $f_{init} = 0.01$ and $k_{asy} = 6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$ were first adopted in the modeling. Supposing that the performance of integrated circuitry is relevant to the molar ratio of SNA-1 (or SNA-2) in Machine-I to SNA-3 (or SNA-4) in Machine-II (termed as RMM), we considered two cases: (1) a small upstream Machine-I and a large downstream Machine-II by assigning RMM 1:3; (2) a large upstream Machine-I and a small downstream

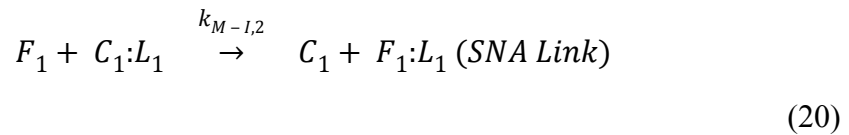
Machine-II with RMM 3:1. Obviously, the cascaded circuit with architecture of RMM 1:3 exhibits slower kinetics relative to RMM 3:1 (Fig. S7a and c). For instance, the value of *Abs* declines only 5% in the system of RMM 1:3 for $[C_1] = 0.05 \times 62 = 3.1$ nM after 12 h of reaction (Fig. S7a), compared with a decline of $\sim 20\%$ for RMM 3:1 (Fig. S7c). In the system with RMM 1:3, a small quantity of C_1 such as 3.1 nM seems neither able to drive the upstream Machine-I operating energetically for crosslinking SNA-1 and SNA-2 nor able to release sufficient C_2 for downstream Machine-II to efficiently crosslink SNA-3 and SNA-4. Consequently, a very small drop of *Abs* was observed in Machine-I and Machine-II (black curves in Fig. S7b). By contrast, with the architecture of RMM 3:1, such a small amount of C_1 (3.1 nM) is still unable to drive upstream Machine-I (black curve of upper panel in Fig. S7d). However, a sufficient amount of C_2 can be released to drive Machine-II, resulting in a large decrease of *Abs* itself (black curve of lower panel in Fig. S7d) and contributing to the 20% drop of *Abs* in the cascaded circuit (black curve in Fig. S7d).

S1.6 Theoretical models for three-layer cascade of SNA circuitry

For Machine-I, the added initial catalyst strand C_1 first invades the $C_2:L_1$ of SNA-2, and results in the intermediate $C_1:L_1$,

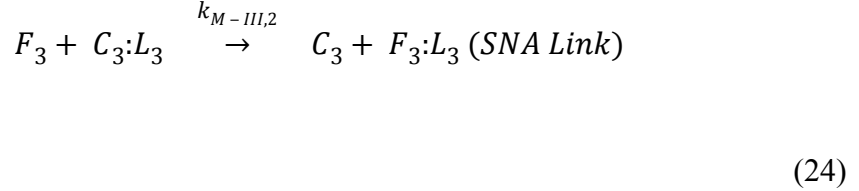
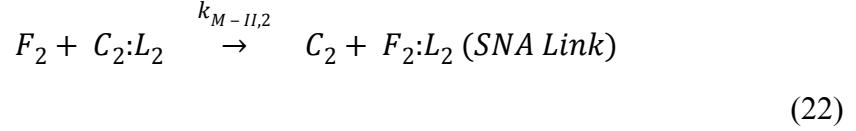


Then, a toehold exchange reaction occurs between $C_1:L_1$ and F_1 of SNA-1, and achieves the formation of $F_1:L_1$,

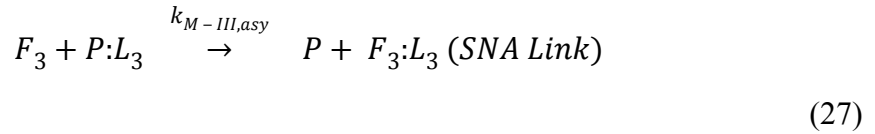
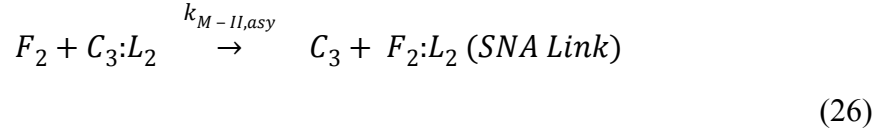
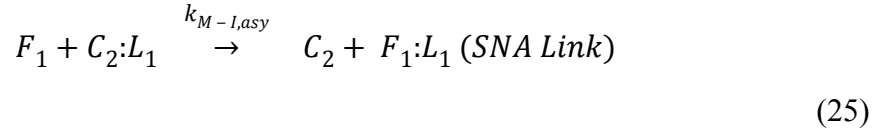


Similarly, the mechanisms of in Machine-II and mechanism-III can be written as,





The asymptotic leakage for the three-layer cascade of SNA circuitry can be expressed as,



For simplicity, the values of $k_{M-I,1}$, $k_{M-II,1}$, and $k_{M-III,1}$, are set to $3.47 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, the values of $k_{M-I,2}$, $k_{M-II,2}$, and $k_{M-III,2}$ are set to $2.59 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Moreover, the rate constant of asymptotic leakage is set to $k_{M-I,asy} = k_{M-II,asy} = k_{M-III,asy} = 6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$.

S1.7 Kinetics of the three-layer cascade of SNA circuitry

We proposed a Monte Carlo model to understand the behavior of this three-layer integrated circuitry. For simplicity, equivalent values of f_{init} and k_{asy} as used in the two-layer circuit were employed even though the setup of the toehold domains differs slightly. Keeping of the amount of SNA constant, two systems with RMM values of 1:1:1 and 1:1:3 were considered for comparison with experiments illustrated in the following section. The system with a larger downstream Machine-III (RMM 1:1:3)

exhibited a deceleration behavior in driving SNA assembly (Fig. S9a and c). The end-layer (Machine-III) in both systems of RMM 1:1:1 and 1:1:3 operates with faster kinetics compared with upstream machines (Machine-I or Machine-II) when adding a small amount of C_1 such as 0.05×62 nM (Fig. S10). Thereafter, it exhibits slower kinetics relative to upstream machines once more C_1 ($\geq 0.2 \times 62$ nM) is added (see red curves in Fig. S9b and d). This indicates that the ability of each individual machine for driving SNA assembly is related to the quantities of catassembler C_1 to some extent.

According to its reaction mechanism, both desired outputs and leakage produced from the integrated circuitry could be understood via following the kinetics of output strand C_2 from the first layer (Machine-I) and C_3 from the second layer (Machine-II). Under ideal conditions, $[C_1]$ that is not consumed during reactions will sustain at a constant concentration in the reaction courses, then $[C_2]$ should increase linearly with time, which further induces a quadratic increase of $[C_3]$.³ As predicted, we observed linear kinetics behavior for $[C_2]$ at the lower concentration limitation of input C_1 , such as less than 0.2×62 nM (Fig. S15). Because of the accumulation of leakages from the above two-layer circuits, severe deviation from the quadratic behavior of C_3 is observed, even at very low concentrations limitation of C_1 (Fig. S16).

S2 Supporting Figures and Additional Results

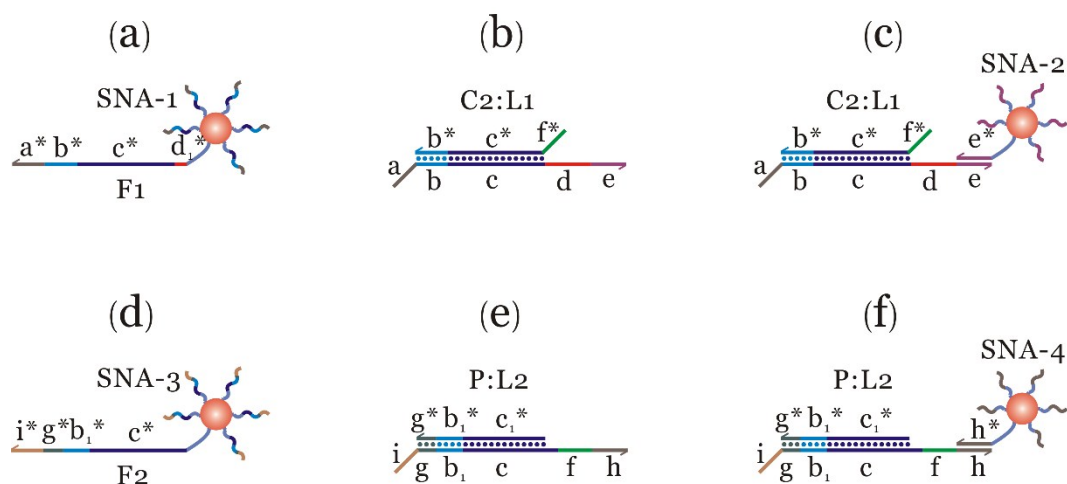


Fig. S1 Graphical representation of the structures of the molecules necessary for the construction of the two-layer cascaded assembly of SNAs.

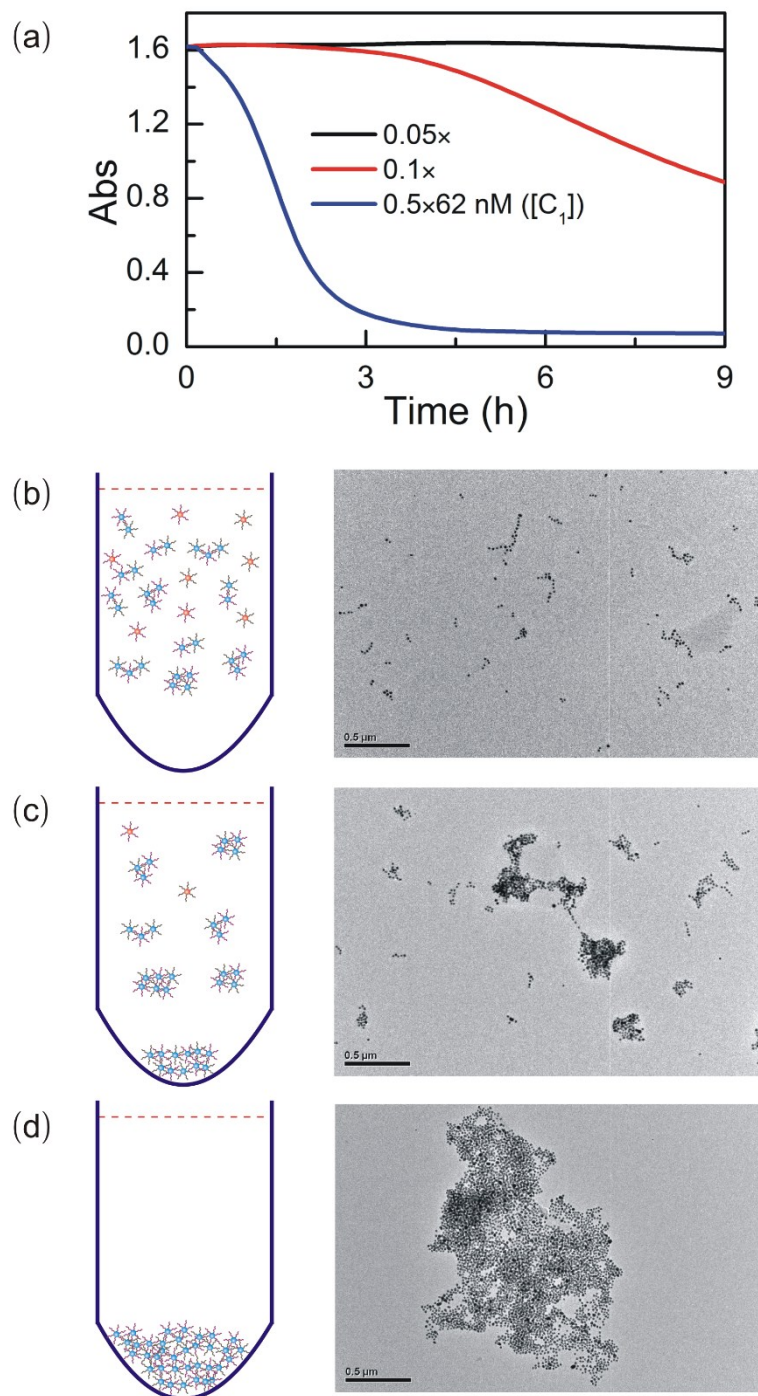


Fig. S2 (a) The UV/Vis kinetic curves of the one-layer Machine-I in the two-layer cascade of SNA circuitry with initial C_1 concentrations of 3.1, 6.2, and 62 nM. The corresponding graphical representations and TEM images of the one-layer Machine-I after 9 h of reaction using C_1 concentrations of (b) 3.1 nM, (c) 6.2 nM, and (d) 62 nM, respectively. $[SNA-1] = [SNA-2] = 3.0$ nM.

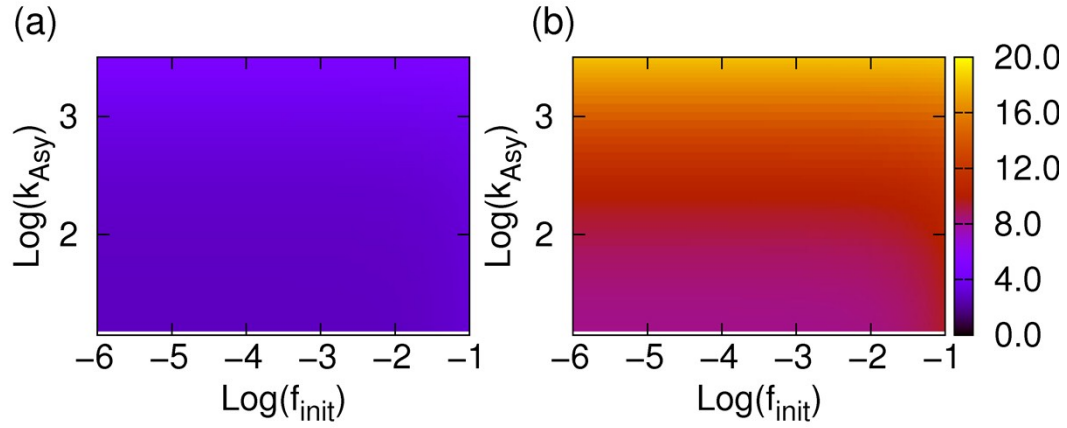


Fig. S3 Simulated amount of C_2 (in nM) at 12.0 h as a function of f_{init} and k_{asy} (shown in the x and y axes, respectively) for the two-layer cascaded circuit with Machine-I : Machine-II being set to 1:3 (a) and 3:1 (b). The concentration of initial catalyst C_1 is $0.2 \times c_0$, and c_0 is 62.0 nM.

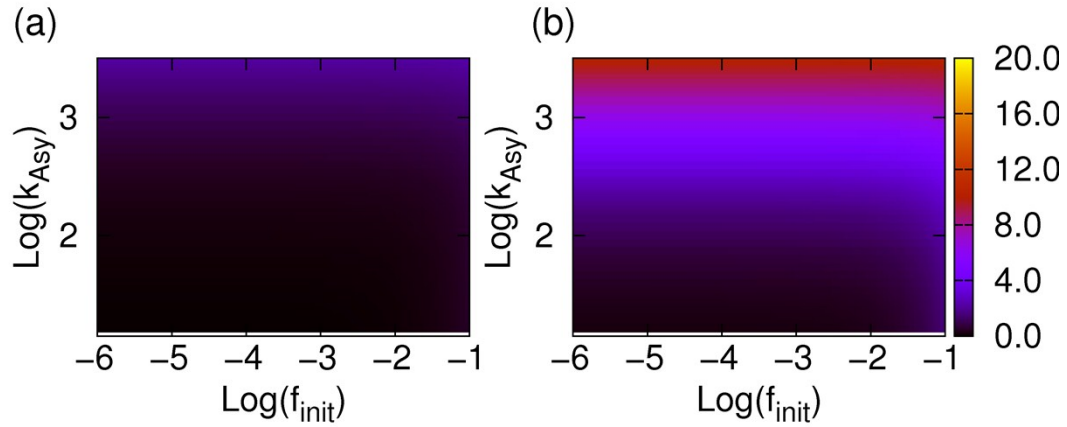


Fig. S4 Simulated amount of C_2 (in nM) contributed by leakage (including initial and asymptotic) at 12.0 h, as a function of f_{init} and k_{asy} (shown in the x and y axes, respectively) for the two-layer cascaded circuit with Machine-I : Machine-II being set to 1:3 (a) and 3:1 (b). The concentration of initial catalyst C_1 is $0.2 \times c_0$, and c_0 is 62.0 nM.

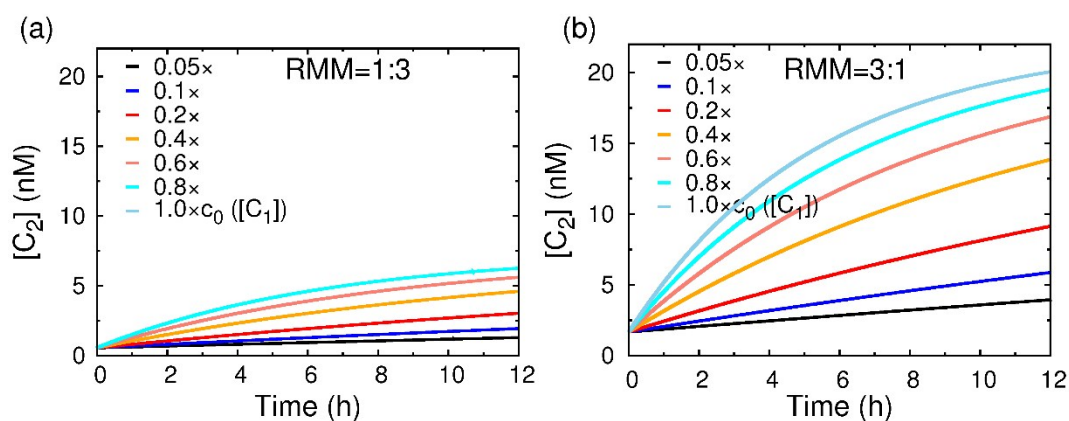


Fig. S5 Calculated kinetics of C_2 in the two-layer cascaded circuit of SNA assembly triggered with varied C_1 concentration, for the systems with RMM being set to 1:3 (a) and 3:1 (b), respectively. c_0 is 62.0 nM, and the rate constant of asymptotic leakage (k_{asy}) is set to $6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$, and the initial leakage f_{init} is set to 0.01.

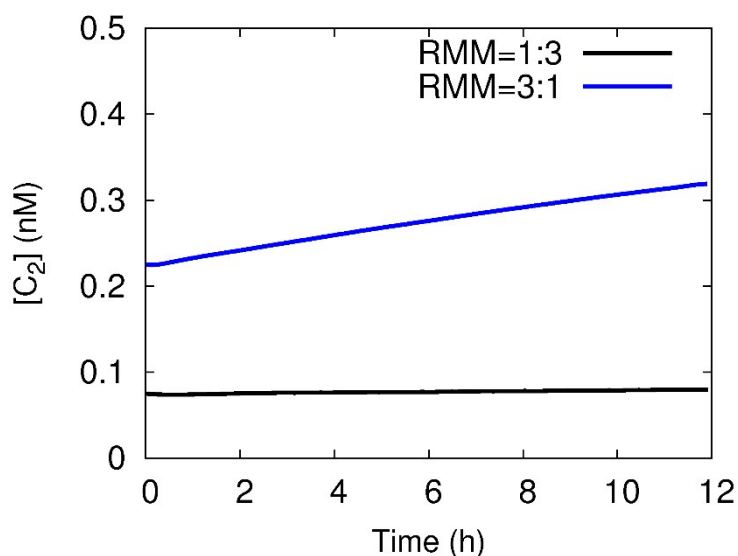


Fig. S6 Calculated kinetics of C_2 contributed by leakage in the two-layer cascade of SNA catalyst circuitry triggered with C_1 concentration of $0.05 \times 62.0 \text{ nM}$ using stochastic simulations, for the systems with RMM being set to 1:3 and 3:1. The rate constant of asymptotic leakage (k_{asy}) is set to $6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$, and the initial leakage f_{init} is set to 0.01.

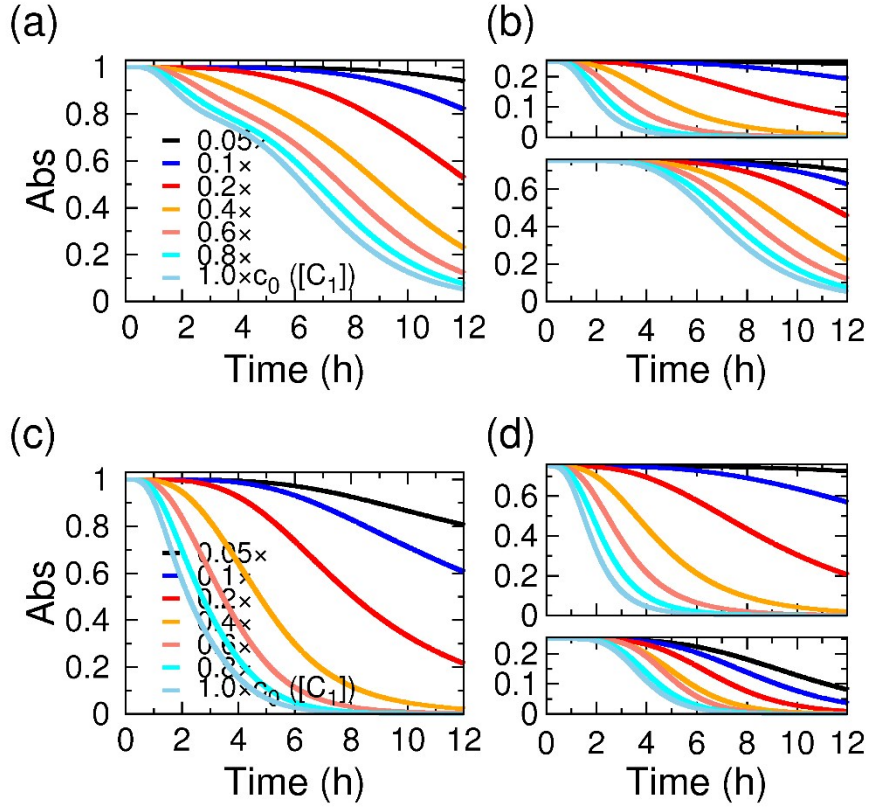


Fig. S7 Stochastic simulation of the two-layer integrated circuitry for SNA assembly using standard Gillespie algorithm. (a) and (c) $[C_1]$ dependence of kinetics of systems with RMM 1:3 and 3:1, respectively, in keeping initial leakage f_{init} at 0.01 and asymptotic leakage k_{asy} at $6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$. (b,d) Kinetics of SNA assembly contributed by Machine-I (upper) and Machine-II (lower).

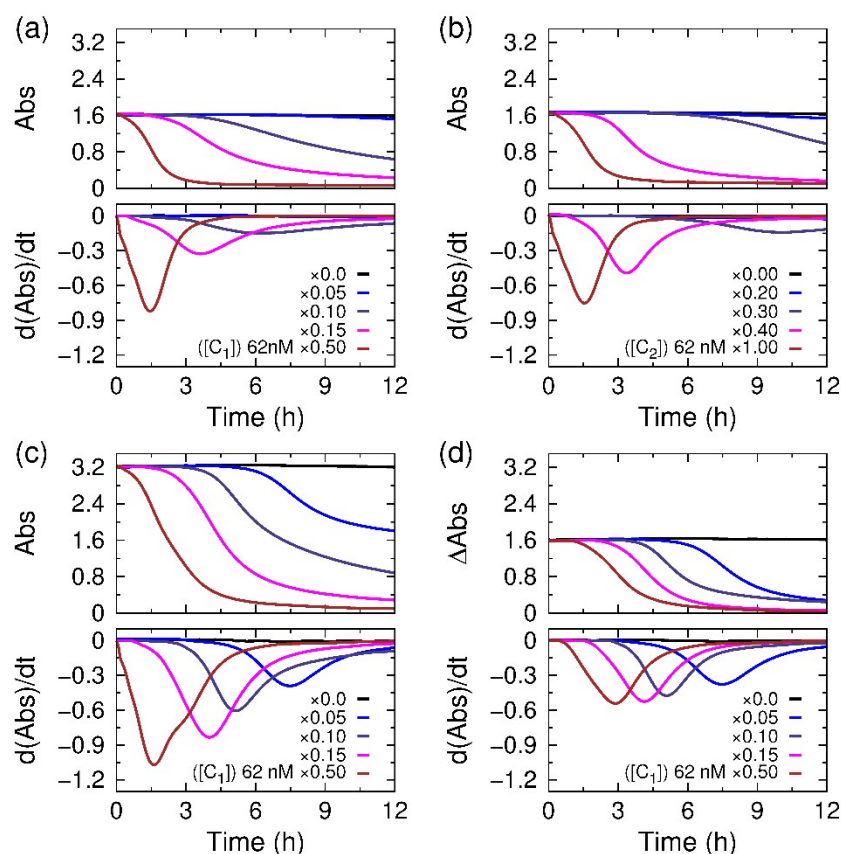


Fig. S8 Performance of one-layer Machine-I (a), one-layer Machine-II (b), and the two-layer cascaded circuit (c), showing the response of the circuit to different concentrations of initial catalyst C_1 . (d) presents the difference of UV/Vis signal between the two-layer cascaded circuit and the one-layer Machine-I. Each profile in (d) is derived using these two systems with the same initial C_1 concentration. Note that the time derivative of the signal was calculated for each studied system, displaying at the lower panel of individual sub-image. $[\text{SNA-1}] = [\text{SNA-2}] = [\text{SNA-3}] = [\text{SNA-4}] = 3.0 \text{ nM}$. The molar ratio of Machine-I and machine-II is 1:1.

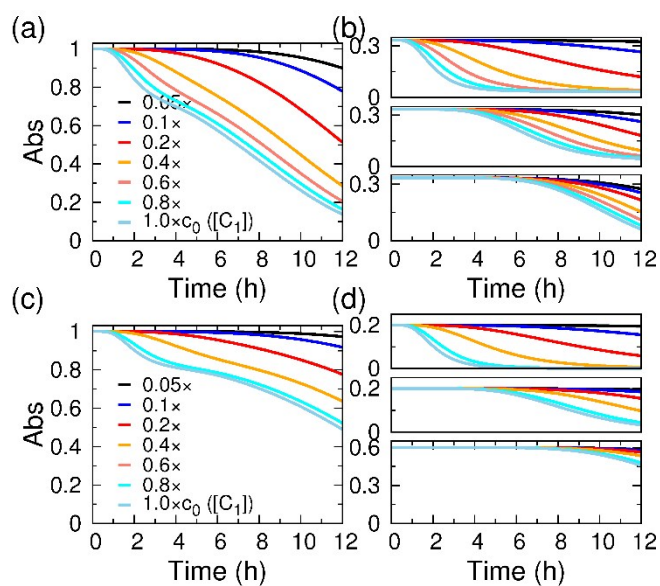


Fig. S9 The performance of three-layer circuitry of SNA derived using simulations. (a,c) $[C_1]$ dependence of kinetics of the integrated circuitry for RMM 1:1:1 and 1:1:3, respectively, with initial leakage $f_{init} = 0.01$ and asymptotic leakage $k_{asy} = 6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$. (b,d) Kinetics of SNA assembly contributed by Machine-I (top), Machine-II (middle), and Machine-III (bottom).

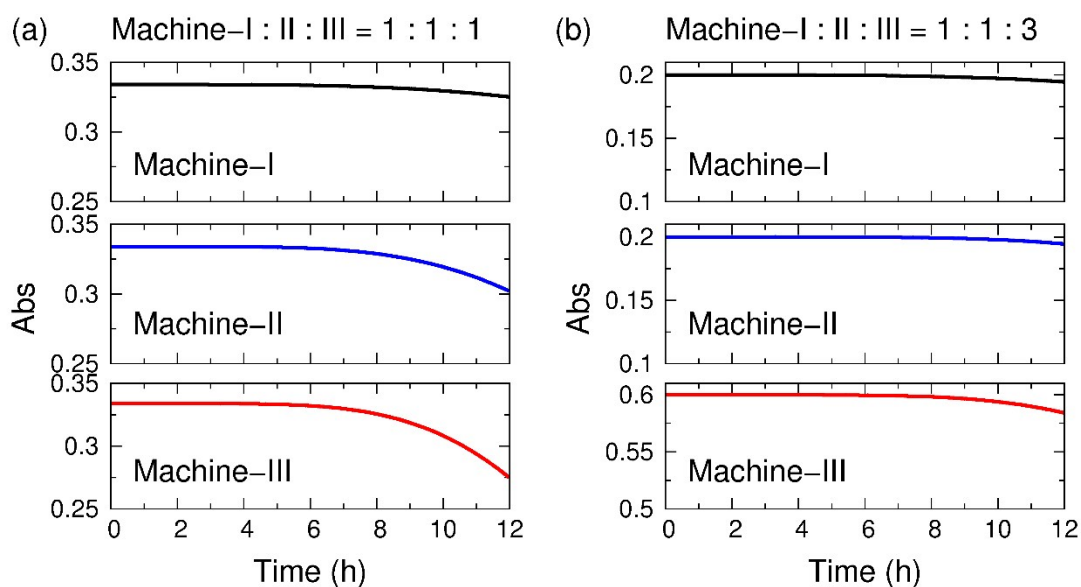


Fig. S10 Calculated kinetics of each constituent machine of the three-layer cascade of SNA circuitry triggered with C_1 having a concentration of $0.05 \times c_0$ ($c_0 = 62.0 \text{ nM}$) using the stochastic simulations. The molar ratios of Machines-I, II, and III for the systems are set to 1:1:1 (a) and 1:1:3 (b), respectively.

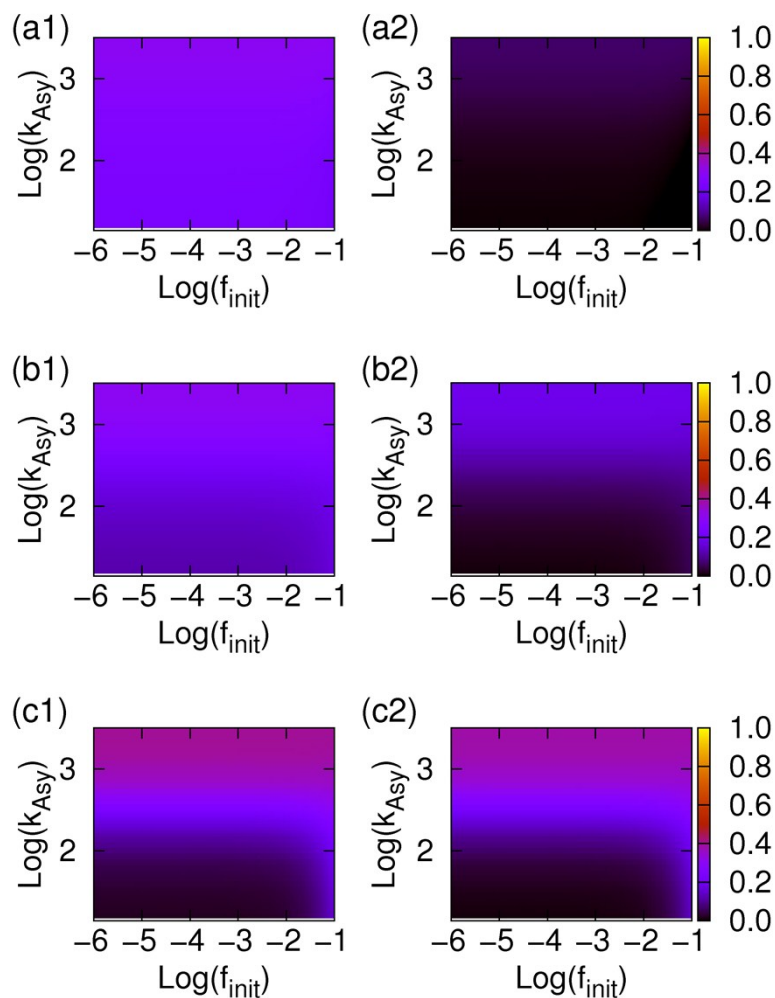


Fig. S11 Simulated performance of each constituent machine of the three-layer cascaded SNA-based circuit with architecture of RMM 1:1:1 as a function of f_{init} and k_{asy} . (a1) represents the total variation of UV/Vis absorbance in Machine-I at 12.0 h; (a2) represents the contribution of leakage reaction to the total absorbance variance. (b1)-(b2) represent the counterparts of (a1)-(a2) occurring in Machine-II; (c1)-(c2) are those in Machine-III. The concentration of initial C_1 is $0.2 \times c_0$, and c_0 is 62.0 nM.

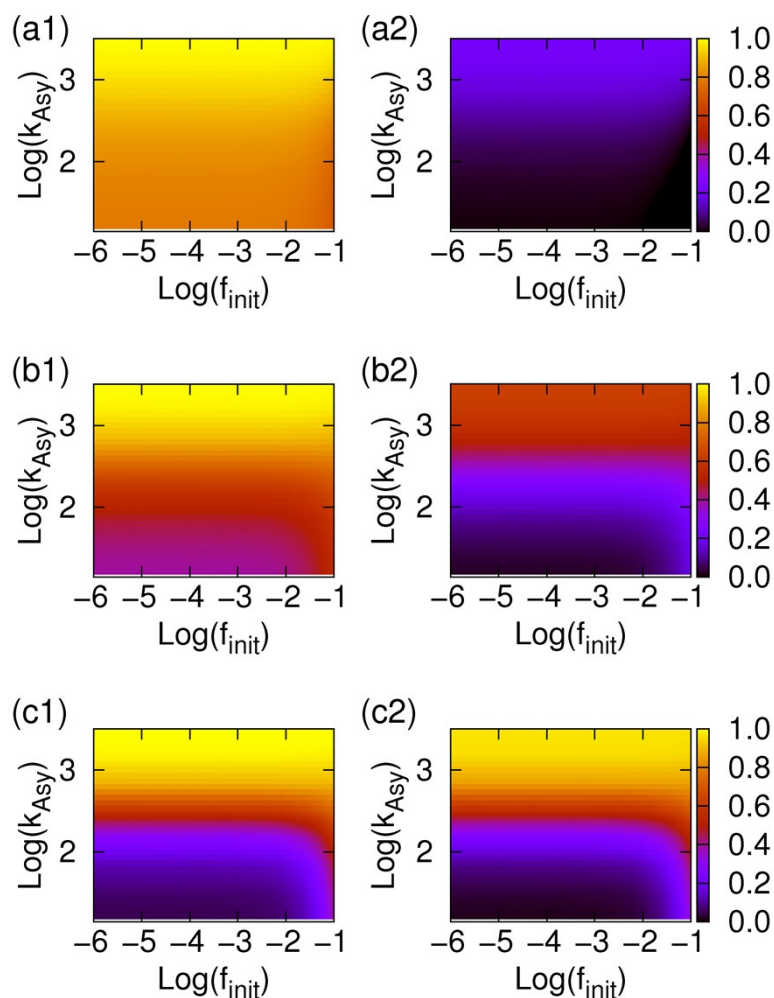


Fig. S12 Normalized variance of UV/Vis absorbance (left column) and its corresponding leakage contribution (right column) for each constituent machine of the three-layer cascaded SNA-based circuit with an architecture of RMM 1:1:1 as a function of f_{init} and k_{asy} . Considering that the molar concentration of each constituent machine may vary considerably in different systems, we here presented the normalized variance of UV/Vis and its corresponding leakage contribution. Note that the presented data herein is derived from that of Fig. S11, that is, the absolute variance of UV/Vis absorbance and the corresponding leakage contribution for each constituent machine in Fig. S11 are normalized to its respective maxima. The displayed normalized values herein are derived using the absolute values divided by 1/3. Such normalization treatment helps to understand and compare the leakage contributions of circuit layers without considering its molar concentration. Similar to Fig. S11, (a1)-(a2): Machine-I, (b1)-(b2): Machine-II, (c1)-(c2): Machine-III.

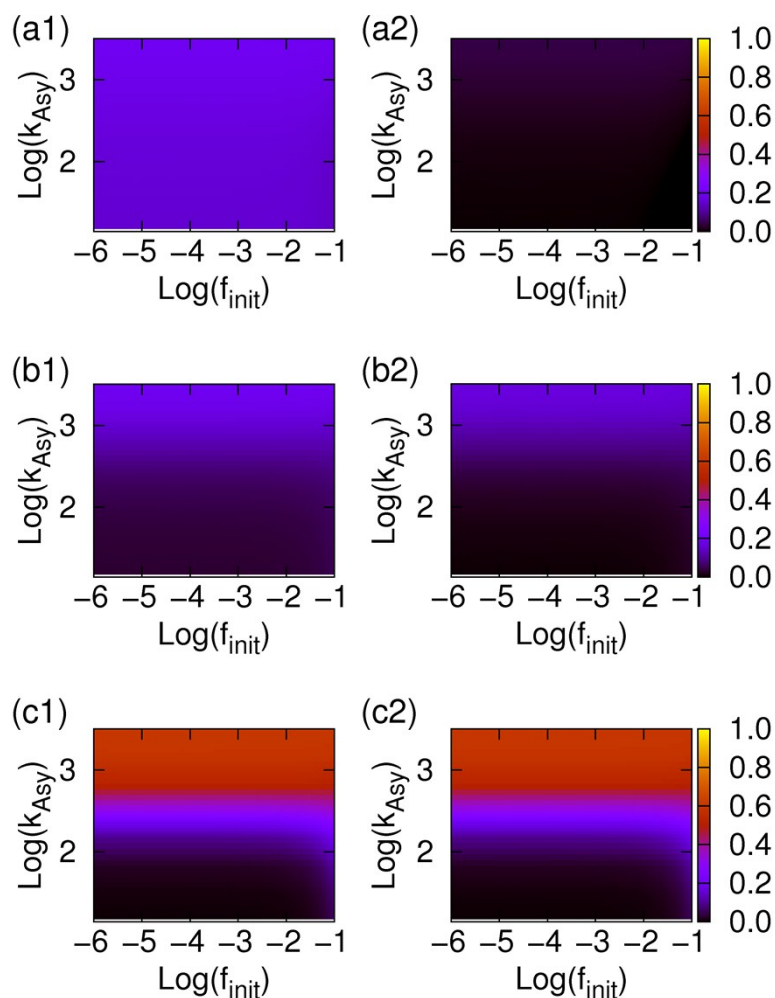


Fig. S13 Simulated performance of each constituent machine of the three-layer cascaded SNA-based circuit with an architecture of RMM 1:1:3 as a function of f_{init} and k_{asy} . (a1) represents the total variation of UV/Vis absorbance in Machine-I at 12.0 h; (a2) represents the contribution of leakage reaction to the absorbance variance. (b1)-(b2) represent the counterparts of (a1)-(a2) occurring in Machine-II; (c1)-(c2) are those in Machine-III. The concentration of initial C_1 is $0.2 \times c_0$, and c_0 is 62.0 nM.

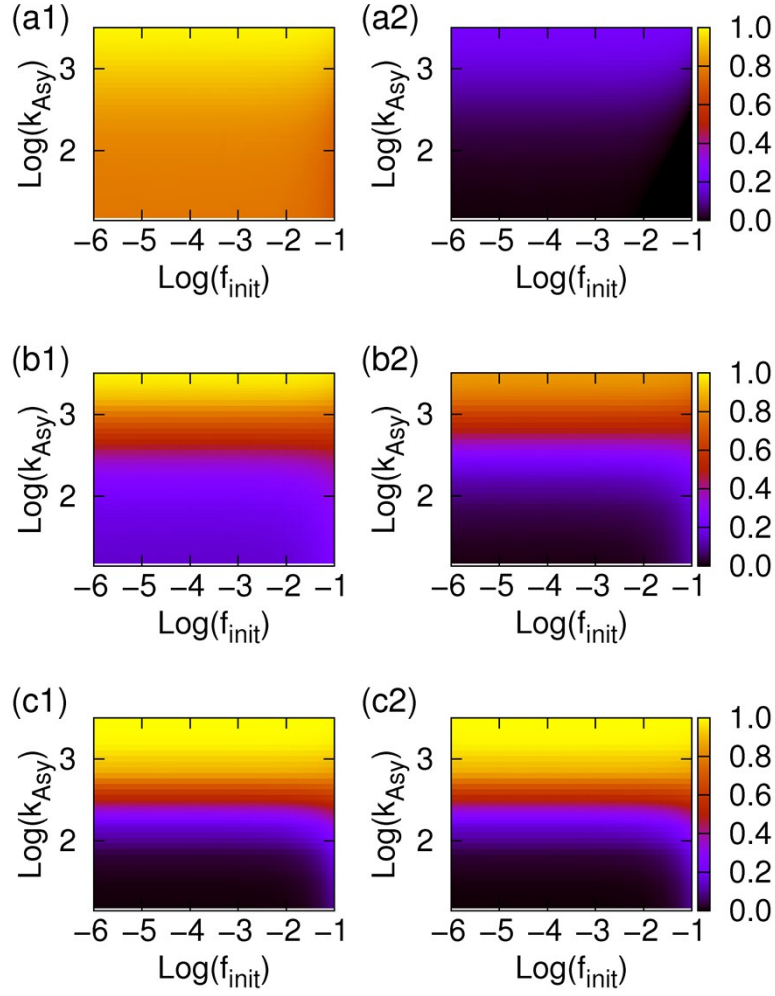


Fig. S14 Normalized variance of UV/Vis absorbance (left column) and its corresponding leakage contribution (right column) for each constituent machines of the three-layer cascaded SNA-based circuit with an architecture of RMM 1:1:3 as a function of f_{init} and k_{asy} . The displayed normalized values herein are derived using the absolute values presented in Fig. S13 divided by 0.2, 0.2, and 0.6 for Machines-I, -II, and -III, respectively. Similar to Fig. S13a, (a1)-(a2): Machine-I, (b1)-(b2): Machine-II, (c1)-(c2): Machine-III.

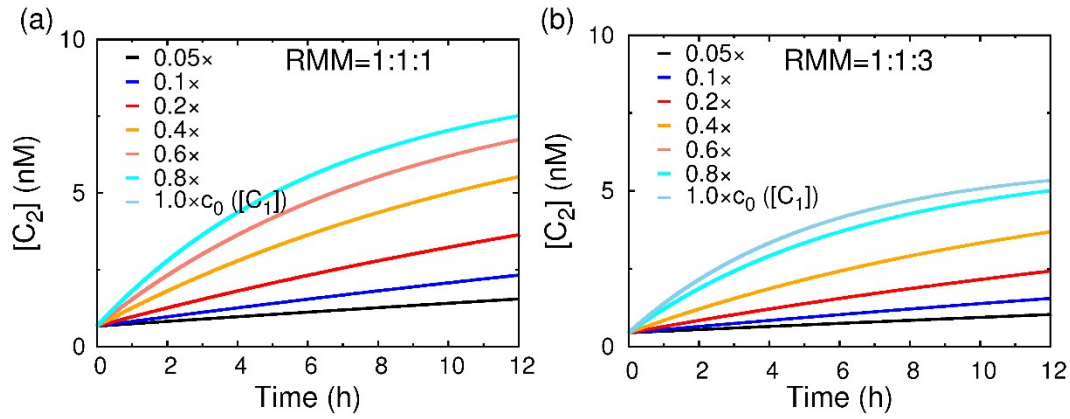


Fig. S15 Simulated kinetics of C_2 in the three-layer cascade of SNA circuitry triggered using varied C_1 concentrations using the stochastic simulations; for the systems with molar ratios of Machines-I, II, and III being set to 1:1:1 (a) and 1:1:3 (b), respectively. In these simulations, the initial leakage f_{init} is 0.01, and the asymptotic leakage k_{asy} is $6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$, and c_0 is 62.0 nM.

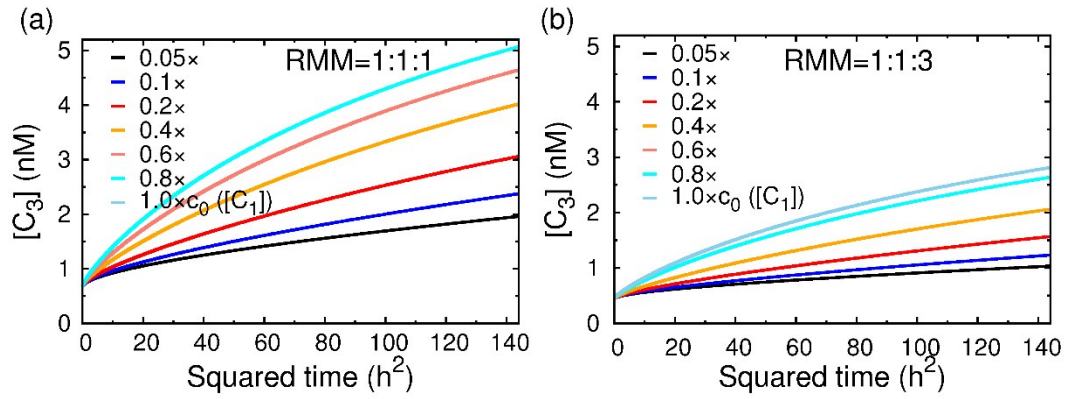


Fig. S16 Simulated kinetics of C_3 in the three-layer cascade of SNA assembly triggered using varied C_1 concentrations using the stochastic simulations, for the systems with molar ratios of Machines-I, II, and III being set to 1:1:1 (a) and 1:1:3 (b), respectively. In these simulations, the initial leakage f_{init} is 0.01, and the asymptotic leakage k_{asy} is $6.85 \text{ M}^{-1} \cdot \text{s}^{-1}$, and c_0 is 62.0 nM.

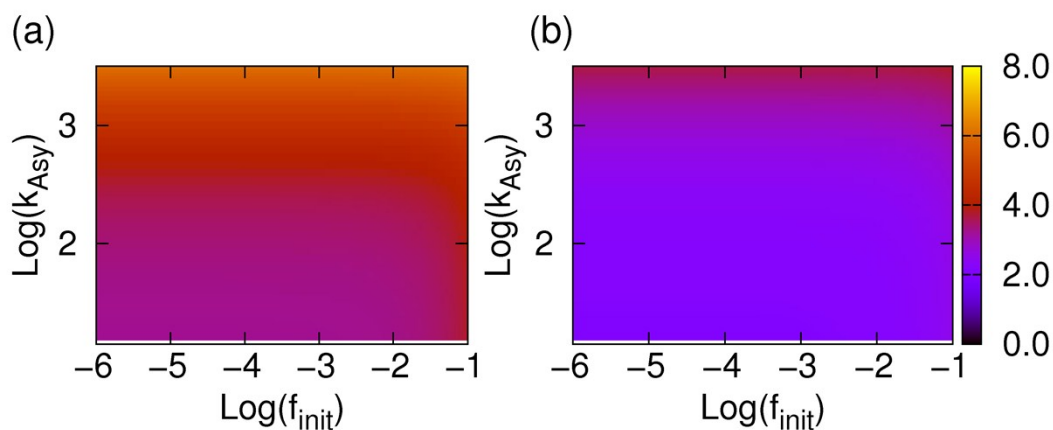


Fig. S17 Simulated amount of C_2 (in nM) produced at 12.0 h, as a function of f_{init} and k_{asy} (shown in the x and y axes, respectively) for the three-layer cascaded circuit with Machine-I : Machine-II : Machine-III being set to 1:1:1 (a) and 1:1:3 (b), respectively. The concentration of initial catalyst C_1 is $0.2 \times c_0$, and c_0 is 62.0 nM.

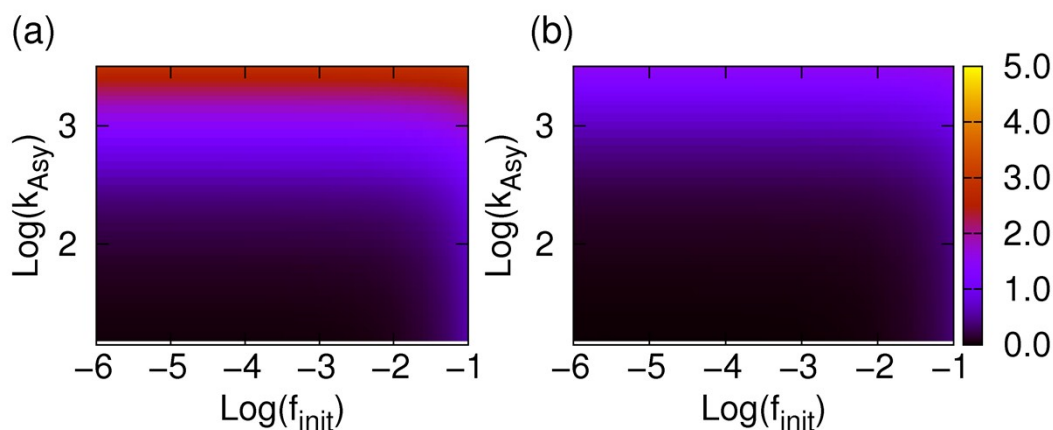


Fig. S18 Simulated amount of C_2 (in nM) contributed by leakage (including initial and asymptotic) at 12.0 h, as a function of f_{init} and k_{asy} (shown in the x and y axes, respectively) for the three-layer cascaded circuit with Machine-I : Machine-II : Machine-III being set to 1:1:1 (a) and 1:1:3 (b). The concentration of initial catalyst C_1 is $0.2 \times c_0$, and c_0 is 62.0 nM.

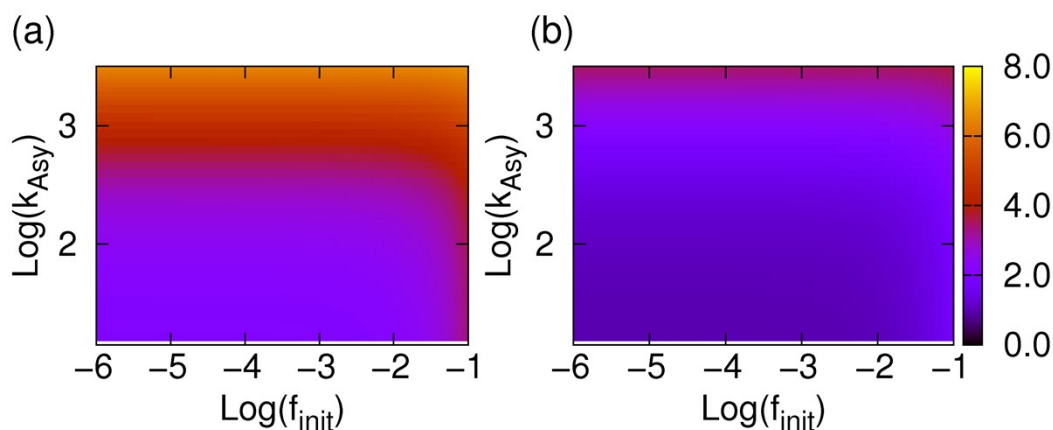


Fig. S19 Simulated amount of C_3 (in nM) produced at 12.0 h, as a function of f_{init} and k_{asy} (shown in the x and y axes, respectively) for the three-layer cascaded circuit with Machine-I : Machine-II : Machine-III being set to 1:1:1 (a) and 1:1:3 (b). The concentration of initial catalyst C_1 is $0.2 \times c_0$, and c_0 is 62.0 nM.

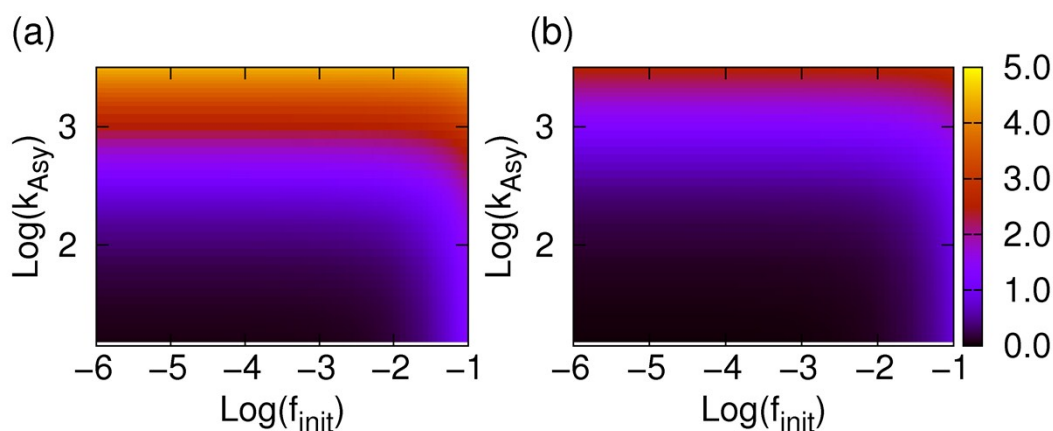


Fig. S20 Simulated amount of C_3 (in nM) contributed by leakage (including initial and asymptotic) at 12.0 h, as a function of f_{init} and k_{asy} (shown in the x and y axes, respectively) for the three-layer cascaded circuit with Machine-I : Machine-II : Machine-III being set to 1:1:1 (a) and 1:1:3 (b), respectively. The concentration of initial catalyst C_1 is $0.2 \times c_0$, and c_0 is 62.0 nM.

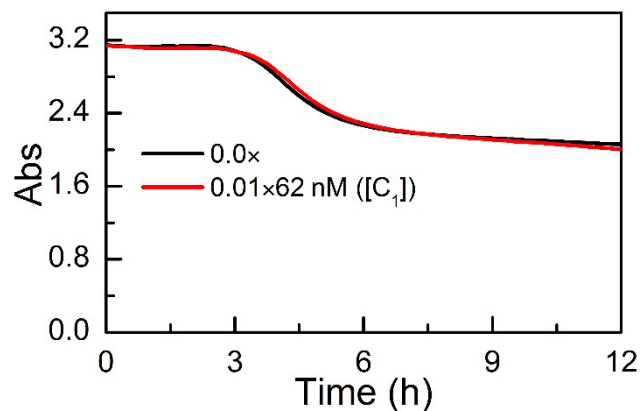


Fig. S21 Responses of the three-layer cascaded circuit to different molar concentrations of C_1 , with the molar ratio of Machine-I, II, and III being set to 1:1:1. In this experiment, length of domain a in Machine-I is four bases. In Machine-I, the toehold domains d and b are set $5/4$, and toehold domains $(a+b)$ and d are set to $8/5$. In Machine-II, the toehold domains f and g are set to $5/4$, and $(i+g)$ and f are set to $10/5$. In Machine-III, the toehold domains (c_2+l) and j are set to $7/5$, and $(n+j)$ and l are set to $10/5$. $[SNA-1] = [SNA-2] = [SNA-3] = [SNA-4] = [SNA-5] = [SNA-6] = 2.0$ nM.

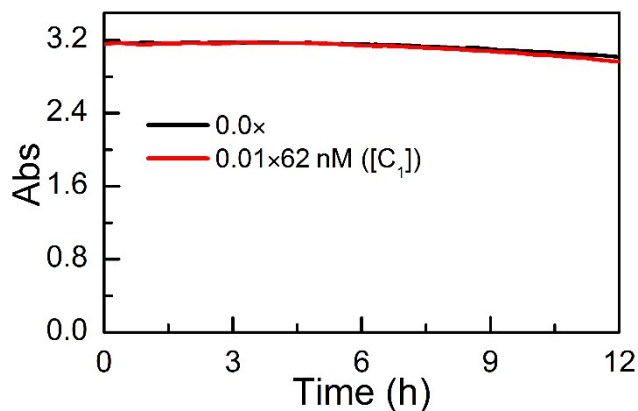


Fig. S22 Responses of the three-layer cascaded circuit to different molar concentrations of C_1 , with the molar ratio of Machine-I, II, and III being set to 1:1:5.5. In this experiment, the length of domain a in Machine-I is four bases. In Machine-I, toehold domains d and b are set to $5/4$, and $(a+b)$ and d are set to $8/5$. In Machine-II, toehold domains f and g are set to $5/4$, and $(i+g)$ and f are set to $10/5$. In Machine-III, toehold domains (c_2+l) and j are set to $7/5$, and $(n+j)$ and l are set to $10/5$. $[SNA-1] = [SNA-2] = [SNA-3] = [SNA-4] = 0.8$ nM, $[SNA-5] = [SNA-6] = 4.4$ nM.

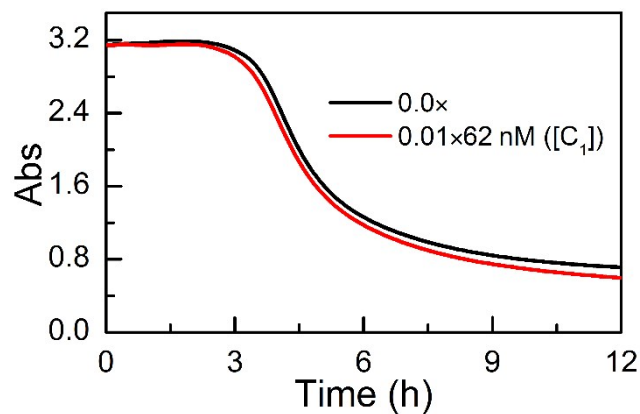


Fig. S23 Responses of the three-layer circuit to different molar concentrations of C_1 , with the molar ratio of Machine-I, II, and III being set to 1:1:3. In this experiment, length of domain a in Machine-I is six bases. In Machine-I, toehold domains d and b are set to $5/4$, and $(a+b)$ and d are set to $10/5$. In Machine-II, toehold domains f and g are set to $5/4$, and $(i+g)$ and f are set to $10/5$. In Machine-III, toehold domains (c_2+l) and j are set $7/5$, and $(n+j)$ and l are set to $10/5$. $[SNA-1] = [SNA-2] = [SNA-3] = [SNA-4] = 1.2$ nM, $[SNA-5] = [SNA-6] = 3.6$ nM.

S3 Sequences of DNA Oligonucleotides.

Name	Sequences (5' to 3')
Linker-1	CCCTCACACCTTCATCTCACTACCTACTCGCACACCTTCTCTCCC TAT
Linker-2	TCCCACTCCACCTTCATCTCACTACCTCTGACTTCACTCTCACCC TAC
Catassembler-1	TGCGAGTAGGTAGTGAGATGAA
Catassembler-2	GTCAGAGGTAGTGAGATGAAGGTGT
Protector	GTAGTGAGATGAAGGTGGAG
SNA-1	SH-TTTTTTTTTTTGTAGGTAGTGAGATGAAGGTGTGAGGG
SNA-2	SH-TTTTTTTTTTTTTTTATAGGGAGAGAAGGTG
SNA-3	SH-TTTTTTTTTTTAGGTAGTGAGATGAAGGTGGAGTGGA
SNA-4	SH-TTTTTTTTTTTTTTTGTAGGGTGAGAGTGAA

Table S1: DNA oligonucleotides sequences used in the two-layer cascaded assembly of SNAs.

Name	Sequences (5' to 3')
Linker-1	CATCCCTCCATTTCATCTCACTACCTACCTGACTTCACTCTCACCC TAC
Linker-2	CCCTCACACCTTCATCTCACTACCTACTCGCACACCTTCTCTCCC TAT
Catassembler-1	GTCAGGTAGGTAGTGAGATGAA
Catassembler-2	TGCGAGTAGGTAGTGAGATGAATGGA
Protector	AGGTAGTGAGATGAAGGTGT
SNA-1	SH-TTTTTTTTTTTGTAGGTAGTGAGATGAATGGAGGGATG
SNA-2	SH-TTTTTTTTTTTTTTTGTAGGGTGAGAGTGAA
CNA-1	SH-TTTTTTTTTTTGTAGGTAGTGAGATGAAGGTGTGAGGG
CNA-2	SH-TTTTTTTTTTTTTTTATAGGGAGAGAAGGTG

Table S2: The DNA oligonucleotides sequences used in the two-layer cascaded assembly of SNAs and CNAs (Machine-I and Machine-II were prepared using AuNPs and AuNRs, respectively).

Name	Sequences (5' to 3')
Linker-1(4 bases in domain <i>a</i>)	CCACCTACTTCATCTCACTACCTACTAGCCCACCAATACTCCTC AC
Linker-1(6 bases in domain <i>a</i>)	TCCCACCTACTTCATCTCACTACCTACTAGCCCACCAATACTCC TCAC
Linker-2	CATCCCTCCATTTCATCTCACTACCTACCTGACTTCACTCTCACC CTAC
Linker-3	CCCTCACACCTTCATCTCACTACCTACTCGCACACCTTCTCTCC CTAT
Catassembler-1	GGCTAGTAGGTAGTGAGATGAA
Catassembler-2	GTCAGGTAGGTAGTGAGATGAAGTAG
Catassembler-3	TGCGAGTAGGTAGTGAGATGAATGGA
Protector	AGGTAGTGAGATGAAGGTGT
SNA-1	SH-TTTTTTTTTTTGTAGGTAGTGAGATGAAGTAGGTGGGA
SNA-2	SH-TTTTTTTTTTTTTTTTTGTGAGGAGTATTGGTG
SNA-3	SH-TTTTTTTTTTTGTAGGTAGTGAGATGAATGGAGGGATG
SNA-4	SH-TTTTTTTTTTTTTTTTTGTAGGGTGAGAGTGAA
SNA-5	SHTTTTTTTTTTTGTAGGTAGTGAGATGAAGGTGTGAGGG
SNA-6	SHTTTTTTTTTTTTTTTTTATAGGGAGAGAAGGTG

Table S3: DNA oligonucleotides sequences used in the three-layer cascaded assembly of SNAs.

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