Engineering genetic circuits that compute and remember

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Memory and logic are central to complex state-dependent computing, and state-dependent behaviors are a feature of natural biological systems. Recently, we created a platform for integrated logic and memory by using synthetic gene circuits, and we demonstrated the implementation of all two-input logic gates with memory in living cells. Here we provide a detailed protocol for the construction of two-input Boolean logic functions with concomitant DNA-based memory. This technology platform allows for straightforward assembly of integrated logic-and-memory circuits that implement desired behaviors within a couple of weeks. It should enable the encoding of advanced computational operations in living cells, including sequential-logic and biological-state machines, for a broad range of applications in biotechnology, basic science and biosensing.

INTRODUCTION

In recent years, synthetic biologists have developed innovative approaches for biological computing based on artificial gene circuits and complex biomolecular systems[1–5]. Synthetic genetic systems have been previously described and adapted for a variety of applications, including logic circuits—for sensing and cellular control[3,6]—and feedback-based memory circuits[7–10], many of which can be reversibly toggled. However, many of these strategies have not integrated logic gates with concomitant memory, and they often require multigate cascades to achieve the desired computation. Recently, we described a recombinase-based platform for the efficient construction of synthetic genetic circuits in living *Escherichia coli* cells, which implement all two-input Boolean logic functions with integrated memory and do not require multilogic-gate cascades[11]. Memory and logic are important for building synthetic circuits that can process and record information and thus compute complex functions.

Our integrated logic and memory circuits leverage the ability of recombinases to invert or excise programmable stretches of DNA via the presence of recombinase-recognition sites. Bxb1 and phiC31 are unidirectional serine recombinases with nonidentical recognition sites known as *attB* (attachment site bacteria) and *attP* (attachment site phage). On the basis of the orientation of the recognition sites that surround a given stretch of DNA, these recombinases can irreversibly invert or excise the DNA[12]. By placing various gene-regulatory elements within the recombinase-recognition sites, including promoters, terminators and genes, one can program stable gene expression that exhibits desired logical behaviors with respect to specific inputs. In this setting, the inputs can be any regulatory signal, such as external inducers or internal events, which trigger the expression of recombinases. These inputs can be transient, as the expressed recombinases manipulate DNA and the resulting genetic information is passed down through cellular generations. As this platform encodes memory along with logic, it can perform sequential logic and implement state machines, which can address applications that stateless combinatorial logic cannot. For example, differentiation cascades can be viewed as biological-state machines in which the identity and order of cellular signals determine differentiation into distinct cellular states.

Here we expand on our previous work and describe in detail the stepwise construction of an AND logic gate with memory by using one-step Gibson assembly. We use an AND gate from our previous paper as an example[11], as various integrated logic-and-memory circuits can all be constructed in a similar manner. In this example, P_{Lux} and $P_{Ltet0-1}$ are used as input promoters and *gfp* is used as the output gene, as shown in **Figure 1**. However, any regulatory mechanisms that can control the expression of recombinases and any desired output gene can be adapted to this strategy. P_{Lux} and $P_{Ltet0-1}$, which are activated in the presence of *N*-acyl homoserine lactone (AHL) and anhydrotetracycline (aTc), respectively, control expression of Bxb1 and phiC31 via riboregulators[13] that enable tight inducible regulation. In the presence of the respective inputs, the phiC31 and Bxb1 recombinases find their recognition sites and flip the promoter and the *gfp* gene, respectively, resulting in GFP expression if the circuit has been exposed to both inputs. The behavior of the AND gate is quantified by using microscopy and flow cytometry, although techniques such as PCR and DNA sequencing can also be used.

Such a modular DNA assembly strategy enables straightforward plug-and-play implementation of integrated logic functions and memory in living cells. This approach takes advantage of the ability of recombinases to 'write' information in DNA[2,11,14–18], and it can be potentially applied to higher organisms. In our previous work[11], we focused on straightforward linear configurations of gene regulatory elements composed of recombinase-invertible upstream promoters, recombinase-invertible intervening unidirectional terminators and recombinase-invertible downstream genes, as illustrated in **Figure 1**. This configuration enables easy programmability of genetic logic based on the rule that the output (gene expression) is ON only if (>1 promoter is in the upright orientation) AND (no unidirectional terminators are in the upright orientation) AND (the output gene is in the upright orientation), where the promoters, terminators and genes are inverted from their original positions when the respective inputs are present (or ON).

As noted previously, the scalability of this approach can be enhanced by discovering additional orthogonal recombinases or by engineering artificial recombinases with programmable

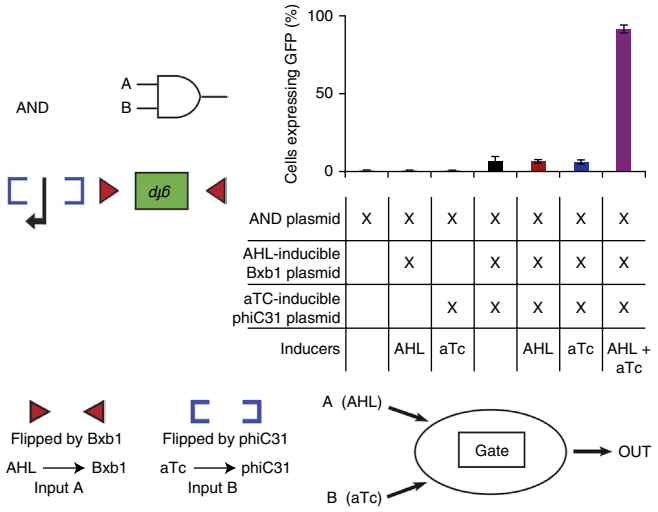

Figure 1 | Our recombinase-based platform for building integrated logic-and-memory devices, such as the AND gate shown here, consists of converting computational functions into (promoter(s))-(terminator(s))-(output gene) designs. AHL ('input A') induces expression of Bxb1 recombinase, whereas aTc ('input B') induces expression of phiC31 recombinase via orthogonal riboregulators in the *E. coli* cells used in this work. Flow cytometry is used to characterize the performance of this AND gate by measuring the percentage of cells that are GFP-positive after exposure to the indicated set of inputs. Controls include cells with the AND gate only, as well as cells containing the AND gate and only one of the two recombinase-expression plasmids. The error bars represent s.e.m., and measurements are from three independent experiments. Symbols used to describe the circuits are adapted from ref. 11. The arrow represents a promoter and the direction of transcription. The red triangles represent recombinase-recognition sites that are inverted by Bxb1, whereas the blue brackets represent recombinase-recognition sites that are inverted by phiC31.

DNA-binding specificities[19,20]. A combinatorial expansion in computational power can be achieved by nesting recombinase-recognition sites, allowing for arbitrary placement of gene regulatory elements and permitting recombinase-based excision in addition to inversion. The integration of these digital logic and memory circuits with analog computation circuits[21] has the potential to implement hybrid-state machines with both digital and analog features. Additional efforts to improve this technology may include minimization of leaky recombinase expression. This could be achieved through optimized riboregulators, tagging recombinases with degradation tags and reducing the copy number of the recombinase-expression cassettes. Furthermore, the role of fitness differences between different circuit states and the resulting stability of these states in cellular populations over time can be further mapped out, as has been recently described[10]. Ultimately, we believe that a wide range of complex circuit behaviors can be achieved with recombinase-based computation, including logic and memory, counters and digital-to-analog converters[2,14–16]. For example, one can envision constructing state machines in mammalian cells to guide the differentiation of stem cells into distinct cell fates on the basis of the timing and identity of external inputs and internal signals.

TABLE 1 | Primers used to construct an integrated logic-and-memory AND gate.

Name	Sequence (5′→3′)
PhiC31_attP_F	CAACGTCTCATTTTCGCCAGATATCGACGTC**GTGCCCCAACTGGGGTAACC**
PhiC31_attP_R	TTGTTTAACTTTATAATT**CCCCCAACTGAGAGAACTCAAA**
Inverted_Promoter_proD_F	CAGTTGGGGGAATTAT**AAAGTTAAACAAAATTATTTGTAGAGGG**
Inverted_Promoter_proD_R	CGCGTACTCC**TAAGAAACCATTATTATCATGACATTAACC**
phiC31_attB_F	CATGATAATAATGGTTTCTTA**GGAGTACGCGCCCGGGGA**
phiC31_attB_R	CGACAAGCCGGCCGTTATTATTA**TGCGGGTGCCAGGGCGT**
Bxb1_attB_F	GCACCCGCATAATAATAA**CGGCCGGCTTGTCGACG**
Bxb1_attB_R	CCTAGAATCGATGTTCACCTGC**GCCCGGATGATCCTGACGACG**
Inverted_*gfp*_F	CCGGGCGCAGGTGAACATCGAT**TCTAGGGCGGCGGATTTGTCCT**
Inverted_*gfp*_R	TACAAACCCCGACTAA**ATTAAAGAGGAGAAAGGTACCATG**
Bxb1_attP_F	TCTCCTCTTTAATTTA**GTCGGGGTTTGTACCGTACACCA**
Bxb1_attP_R	CGAGGAAGCGGAATATATCCCCTAGGTTA**GTCGTGGTTTGTCTGGTCAAC**
Sequencing_primer_F	ATACGCCCGGTAGTGATCTT
Sequencing_primer_R	TGGCATCTTCCAGGAAATCT

Bold letters represent the parts that anneal to the templates, and nonbold letters represent the overlap with the next DNA piece.

Experimental design

The protocol described here consists of three parts: (i) construction of the AND gate by PCR amplification and Gibson assembly; (ii) preparation of cells containing the logic-and-memory gate; and (iii) characterization of the integrated logic-and-memory gate.

Construction of the AND gate by PCR amplification and Gibson assembly. Here we describe PCR amplification of all the parts needed to build the AND gate shown in **Figure 1**. Subsequently, Gibson assembly[22] is used to join the following parts in a linear DNA sequence to construct the AND gate in a one-pot reaction: (i) phiC31 *attP*, (ii) inverted promoter (*proD*)[23], (iii) phiC31 *attB*, (iv) Bxb1 *attB*, (v) inverted output gene (*gfp*)[16] with terminator (T1)[16] and (vi) Bxb1 *attP*.

DNA fragments that overlap in sequence should be constructed by the design of PCR primers containing 'overhangs' that provide sequence overlap with adjacent fragments. The example primers used in this protocol are listed in **Table 1**.

Preparation of integrated logic-and-memory gate–containing cells. Competent *E. coli* DH5αPRO cells have been used to prepare and test our integrated logic-and-memory gates. The PRO cassette contributes high-level TetR expression, which is

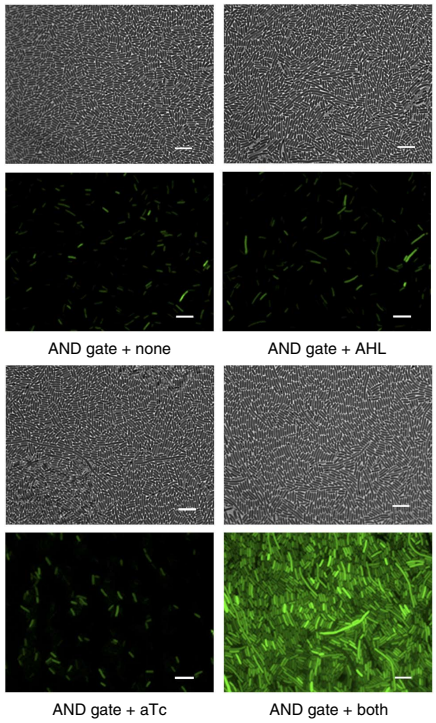

Figure 2 | Four states of the AND logic gate. Fluorescence microscopy was used to characterize the performance of the AND gate by imaging *gfp* expression of cells that contain the AND gate with both recombinase expression plasmids. These cells were uninduced (none) or induced with AHL, aTc or both AHL and aTc (both). Scale bars, 5 µm.

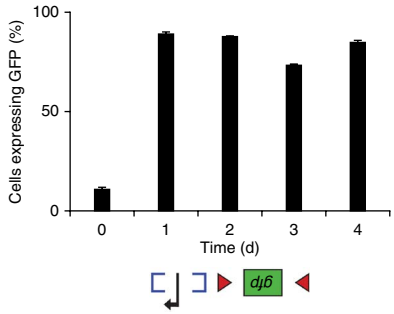

Figure 3 | Long-term stable memory maintenance over multiple cell generations. Cells containing the AND gate (shown at the bottom in the initial uninduced state) and both recombinase-expression plasmids were induced to the ON state after day 0. These cells were then repeatedly diluted and grown without inducers for 4 d. Flow cytometry was used to measure the percentage of cells that were GFP-positive (top). The error bars represent s.e.m., and measurements are from three independent experiments.

important for regulating expression from the $P_{Ltet0-1}$ promoter[24]. Other *E. coli* strains that express high levels of TetR should be usable as well. These strains should be made competent before use.

Characterizing the integrated logic-and-memory gate. Performance of the integrated logic-and-memory gate can be characterized through a variety of means, such as microscopy, flow cytometry, PCR and DNA sequencing. Here we will describe the use of flow cytometry and microscopy (**Figs. 1–3**) to characterize the behavior of the integrated logic-and-memory gate. Controls, such as cells that do not express GFP and cells that constitutively express GFP, can be used to set appropriate thresholds for determining which cells exhibit OFF versus ON outputs with flow cytometry. Additional controls can include cells containing the AND gate only and neither of the recombinase-expression vectors, cells containing the AND gate together with the riboregulated *bxb1* expression vector only and cells containing the AND gate together with the riboregulated *phiC31* expression vector only.

Microscopy can also be used to characterize the behavior of the integrated logic-and-memory gates. We recommend that microscopy be performed in combination with other quantitative techniques such as flow cytometry. If users would prefer to use microscopy as a quantitative technique, then we suggest using Matlab or the ImageJ image processing toolbox to quantify GFP expression in individual cells. The use of fluorescence microscopy to quantitatively measure gene expression in bacteria has been described[22,25,26], which can be applied here.

MATERIALS

REAGENTS

- *E. coli* strain DH5αPRO (F^-φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-, P_{N25}/tet^R, P_{laciq}/lacI, Sp^r), obtained from J. Collins (Boston University)[27]. The PRO cassette is also available from Expressys, and it can be integrated into the genomes of *E. coli* strains
- pZA31G vector[24]. This vector as well as the vectors containing other logic gates described in ref. 11 are available on request
- *phiC31* integrase gene in pACYC177 vector (Addgene, plasmid no. 18941)
- *bxb1* integrase gene, obtained from G. Hatfull (University of Pittsburgh)
- rrjc12y(rii)g riboregulator vector[13]. The rrjc12y(rii)g riboregulator vector (E118) expressing the *bxb1* recombinase gene is available upon request
- rrjt12(11)g riboregulator vector[13]. The rrjt12(11)g riboregulator vector (E238) expressing the *phiC31* recombinase gene is available upon request
- Primers, see **Table 1** (Integrated DNA Technologies)
- Restriction enzymes AatII and AvrII (New England Biolabs, cat. nos. R0117S and R0174S, respectively)
- Phusion high-fidelity PCR kit (New England Biolabs, cat. no. E0553S)
- QIAprep spin Miniprep kits (Qiagen, cat. no. 27106)
- QIAquick PCR purification kit (Qiagen, cat. no. 28104)
- QIAquick gel extraction kit (Qiagen, cat. no. 28704)
- LB medium (Fisher, cat. no. BP1426-2 for liquid)
- LB agar granules (Fisher, cat. no. BP9724-500)
- Agarose (Sigma-Aldrich, cat. no. A9414-50G)
- *N*-(3-Oxohexanoyl)-L-homoserine lactone (AHL; Sigma-Aldrich, cat. no. K3007)
- Carbenicillin (Teknova, cat. no. C2113)
- Kanamycin (Sigma-Aldrich, cat. no. K0254)
- Chloramphenicol (Sigma-Aldrich, cat. no. C3175)
- Gibson assembly master mix (New England Biolabs, cat. no. E2611L)
- Taq DNA ligase (New England Biolabs, cat. no. M0208L)
- Phusion high-fidelity polymerase (New England Biolabs, cat. no. M0530L)
- T5 exonuclease (New England Biolabs, cat. no. M0363L)
- Isothermal reaction buffer (ISO; https://www.addgene.org/plasmid_protocols/gibson_assembly/)

• 10× PBS (VWR, cat. no. 97064-158)
• S.O.C. medium (Invitrogen, cat. no. 15544-034)
• DNA loading dye (New England Biolabs, cat. no. B7021S)

EQUIPMENT

• Petri dishes, 100 × 15 mm
• Single-end frosted microscope slides (VWR, cat. no. 16005-106)
• Bio-Rad S1000 Thermal Cycler With dual 48/48 fast reaction modules (Bio-Rad)
• Microcentrifuge tubes, 1.7 ml (VWR, cat. no. 87003-294)
• Microcentrifuge 5424 (Eppendorf)
• Shaker incubator
• Multichannel pipette L12-200XLS (Rainin, cat. no. 17013810)
• Heating block
• Epifluorescence microscope (Zeiss)
• BD-FACS LSRFortessa-HTS cell analyzer (BD Biosciences)
• Nanodrop 2000 spectrophotometer (Thermo Scientific)
• Costar clear polystyrene 96-well plates (Fisher Scientific, cat. no. 3370)

REAGENT SETUP

pZA31G vector The pZA31G vector[24] is used as the backbone to build the AND circuit. AatII and AvrII restriction sites are used to cut the vector to remove the $P_{Ltet0-1}$ promoter and *gfp* gene and linearize the vector backbone in order to allow for insertion of AND gate DNA fragments assembled via Gibson assembly. The pZA31G vector contains a constitutively transcribed chloramphenicol resistance gene. The AND circuit built in this vector contains the recombinase-invertible components, which consist of the inverted proD promoter between the phiC31 *attP* and *attB* recognition sites, which is upstream of the inverted *gfp* gene located between the Bxb1 *attB* and *attP* recognition sites.

rrjc12y(rii)g riboregulator vector (E118) The rrjc12y(rii)g riboregulator vector (E118)[13] is engineered to express the *bxb1* recombinase gene[28]. The *bxb1* gene was a gift from members of the G.F. Hatfull laboratory, and it is cloned in the rrjc12y(rii)g riboregulator vector[13]. The *bxb1* gene is placed downstream of the P_{Lux} promoter, the crR12y *cis*-repressive sequence and the ribosome-binding sequence (RBS). This vector constitutively expresses the *luxR* gene, whose product regulates P_{Lux}. The rrjc12y(rii)g riboregulator vector (E118) also contains a constitutively transcribed kanamycin resistance gene and another P_{Lux} promoter that drives transcription of *trans*-activating RNA (taRNA) version taR12y, which is specific for the crR12y *cis*-repressive sequence. As noted below, degradation tags can be added to the *bxb1* gene to minimize leakage.

rrjt12(11)g riboregulator vector (E238) The rrjt12(11)g riboregulator vector (E238)[13] is engineered to contain the *phiC31* recombinase gene. The *phiC31* gene was obtained from Addgene plasmid 18941, and it is cloned into the rrjt12(11)g riboregulator vector[13]. The $P_{Ltet0-1}$ promoter drives the transcription of both taRNA version taR12 and *phiC31*. The crR12 *cis*-repressive sequence is located upstream of the RBS of *phiC31*, and it is specifically derepressed by taR12. This vector contains a constitutively transcribed carbenicillin resistance gene.

Kanamycin Prepare a 1,000× stock solution of kanamycin at a concentration of 30 mg/ml by dissolving it in water. Divide the solution into aliquots and store them at −20 °C for up to 3 months, taking care to minimize freeze-thaw cycles. The working concentration of kanamycin in all experiments is 30 μg/ml.

Chloramphenicol Prepare a 1,000× stock solution of chloramphenicol at a concentration of 25 mg/ml by dissolving it in ethanol. Divide the solution into aliquots and store them at −20 °C for up to 3 months. The working concentration of chloramphenicol in all experiments is 25 μg/ml.

Carbenicillin Prepare a 1,000× stock solution of carbenicillin at a concentration of 50 mg/ml by dissolving it in water. Divide the solution into aliquots and store them at −20 °C for up to 3 months, taking care to minimize freeze-thaw cycles. The working concentration of carbenicillin in all experiments is 50 μg/ml.

LB medium LB medium can be prepared with commercial premixed LB medium components according to the manufacturer's instructions, and it should be autoclaved before use. Additional components such as antibiotics and inducers should be added after the autoclaved medium has cooled to room temperature (~25 °C).

LB agar plates LB agar plates can be prepared by using commercial premixed LB agar components according to the manufacturer's instructions, and they should be autoclaved before use. Additional components such as antibiotics should be added after the autoclaved LB agar has cooled below ~55 °C.

***N*-(3-Oxohexanoyl)-L-homoserine lactone** Prepare a 100 mM stock solution by dissolving 21.323 mg of AHL in 1 ml of water. Filter-sterilize it and store it at −20 °C for up to several months.

Anhydrotetracycline (aTc) Prepare a 1,000× stock aTc solution at a concentration of 250 μg/ml by dissolving it in 50% (vol/vol) ethanol. Store it at −20 °C in a foil-covered tube to avoid excess light exposure.

1.1× Phusion premix stock Prepare 1 ml of 1.1× Phusion premix by adding 10 μl of Phusion polymerase (2 U/μl), 22 μl of dNTPs (10 mM), 220 μl of HF or GC buffer (5×) and 748 μl of ddH_2O, which are all supplied with the polymerase. Divide the 1.1× Phusion premix into aliquots and store them at –20 °C for 1 month, taking care to minimize freeze-thaw cycles.

Gibson assembly master mix Add 320 μl of 5× ISO buffer, 0.64 μl of 10 U/μl T5 exonuclease, 20 μl of 2 U/μl Phusion polymerase, 160 μl of 40 U/μl Taq ligase and enough water to bring the volume to 1.2 ml. Prepare 15-μl aliquots; use each aliquot for a single Gibson reaction. Alternatively, ready-to-use Gibson Assembly master mix can also be purchased from New England Biolabs.

Microscope slide with 1% agarose Prepare 1% (wt/vol) liquid agarose and add 100 μl of it on the microscope slide. The microscope slide with the freshly added agarose should be covered with a coverslip and left at room temperature for the liquid agarose to cool and solidify.

PROCEDURE

Gibson assembly ● TIMING 3–5 d

1| To construct the parts to be used in the Gibson assembly of the AND gate (phiC31 *attP*, inverted promoter proD, phiC31 *attB*, Bxb1 *attB*, inverted *gfp* with terminator T1 and Bxb1 *attP*), assemble the following templates and primers: template phiC31 *attP* plus primers phiC31_attP_F and phiC31_attP_R; template promoter proD plus primers Inverted_Promoter_proD_F and Inverted_Promoter_proD_R; template phiC31 *attB* plus primers phiC31_attB_F and phiC31_attB_R; template Bxb1 *attB* plus primers Bxb1_attB_F and Bxb1_attB_R; template *gfp* with terminator T1 plus primers Inverted_*gfp*_F and Inverted_*gfp*_R; and template Bxb1 *attP* plus primers Bxb1_attP_F and Bxb1_attP_R.

Component	Amount per sample (μl)	Final concentration
DNA template (~50 ng/μl)	1	1 ng/μl
Forward primer (20 μM)	1.25	0.5 μM
Reverse primer (20 μM)	1.25	0.5 μM
Phusion premix	up to 50	

2| Perform PCR by using the following conditions:

Cycle number	Denature	Anneal	Extend	Hold
1	98 °C, 30 s			
2–40	98 °C, 10 s	2–3 °C above primer's Tm	72 °C, 30 s/1 kb	
41			72 °C, 6 min	
42				4 °C

3| Purify the PCR products by using the QIAquick PCR purification kit, according to the manufacturer's instructions.

4| Add one volume of DNA loading dye to five volumes of purified DNA, and run the samples on a 1–2% (wt/vol) agarose gel to verify the size of the PCR products.
? TROUBLESHOOTING

5| Measure the DNA concentration (ng/μl) of each DNA fragment to be assembled (made in Step 2) by using a Nanodrop 2000 spectrophotometer or any other technique for measuring DNA concentration.

6| Linearize the pZA31G vector by digesting it with AatII and AvrII restriction enzymes, as described in the Reagent Setup section. Use a 1% (wt/vol) agarose gel to verify the size of the linearized vector backbone.

7| Extract the vector DNA from the gel by using the QIAquick gel extraction kit, according to the manufacturer's instructions.

8| Thaw a 15-μl aliquot of Gibson assembly master mix on ice, and keep it on ice until use.

9| While keeping the thawed 15-μl aliquot of Gibson assembly master mix on ice, add to it 50–100 ng of the linearized vector backbone and equimolar amounts of all the other DNA fragments in a 20 μl total volume of assembly reaction mixture.

10| Incubate the assembly reaction at 50 °C for 60 min.

11| Place the tube on ice or store it at 4 °C.
■ **PAUSE POINT** Assembly reactions can be stored at 4 °C overnight.

12| Transformation of the assembly reaction into chemically competent *E. coli* cells: thaw two tubes of 100 μl each of competent *E. coli* DH5αPRO cells on ice for 10 min.
▲ **CRITICAL STEP** Any transformation protocol may be used at this step depending on user preference. Transformation efficiency varies on the basis of the efficiency of the batch of competent cells used and on the number of Gibson-assembled parts involved in the reaction.

13| Add 5–10 μl of the assembly reaction into one of the tubes of the thawed competent cells. Flick the tube 5–6 times to mix the DNA and cells. For a negative control, add 1–5 μl containing the digested pZA31G vector to the second tube of 100 μl of competent *E. coli* DH5αPRO cells.
▲ **CRITICAL STEP** Do not vortex the transformation mix.

14| Put the tubes on ice for 30 min, heat-shock the cells for 30 s at 42 °C and place the tubes on ice for 5 min. Throughout this process, do not mix the samples.

15| Pipette 900 μl of S.O.C. medium into the mixture and place it at 37 °C for 60 min, and shake it vigorously at 250–300 r.p.m.

16| While the cells are shaking, warm the agar plates containing chloramphenicol at 37 °C.

17| Spread 100 µl of each of the transformation and control samples (Step 15) onto chloramphenicol selection plates and incubate them overnight at 37 °C. Alternatively, the plates can be incubated at 25 °C for 48 h or at 30 °C for 24–36 h.
▲ **CRITICAL STEP** We usually expect that the transformation plate to produce at least 50 colonies and the negative control plate to produce at most only a few colonies.

18| To test colonies to ensure that the Gibson assembly was successful, select 5–10 colonies from the transformation plate and inoculate each colony into 5 ml of LB medium supplemented with chloramphenicol (25 µg/ml final concentration), and allow the cultures to grow overnight at 37 °C.

19| Extract plasmid DNA from the overnight-grown cultures by using the QIAprep spin miniprep kit according to the manufacturer's instructions.

20| Sequence the regions of interest in the plasmids by using the vector-specific Sequencing_primer_F and Sequencing_primer_R (**Table 1**), which anneal upstream and downstream of the circuit design, respectively. Select a plasmid that has the desired sequence of the AND gate.
▲ **CRITICAL STEP** Additional, internal AND gate–specific primers may be required to obtain the full sequence coverage of the AND gate.
? TROUBLESHOOTING
■ **PAUSE POINT** The selected plasmid can be stored at –20 °C until needed.

Preparation of logic gate–containing cells ● TIMING 4–7 d
21| Make or purchase competent *E. coli* DH5αPRO cells or any other competent *E. coli* strain with high-level expression of the TetR transcriptional repressor (see Reagent Setup).

22| Transform competent DH5αPRO cells as described in Steps 12–15 with 1 µl of ~100 ng/µl pZA31G-based plasmid containing the AND gate (Step 20), 1 µl of ~100 ng/µl of the rrjc12y(rii)g-based riboregulator vector (E118) containing the *bxb1* recombinase gene and 1 µl of ~100 ng/µl of the rrjt12(11)g-based riboregulator vector (E238) containing the *phiC31* recombinase gene.

23| Plate the transformed cells on LB agar supplemented with chloramphenicol (25 µg/ml), carbenicillin (50 µg/ml) and kanamycin (30 µg/ml), and incubate the transformed cells overnight at 37 °C. We suggest negative controls that consist of DH5αPRO cells alone (untransformed), DH5αPRO cells containing the AND gate only, DH5αPRO cells containing the AND gate together with the riboregulated *bxb1* expression vector and DH5αPRO cells containing the AND gate together with the riboregulated *phiC31* expression vector. Plate the transformed negative controls on LB agar supplemented with the appropriate combinations of chloramphenicol (25 µg/ml), carbenicillin (50 µg/ml) and/or kanamycin (30 µg/ml), and incubate the plates overnight at 37 °C.

24| Pick colonies from the transformation plates and grow them overnight in a shaker at 37 °C in 5 ml of LB medium supplemented with chloramphenicol (25 µg/ml), carbenicillin (50 µg/ml) and/or kanamycin (30 µg/ml), as appropriate for the plasmids under selection.
▲ **CRITICAL STEP** Note that the cells may grow slower because of the presence of multiple transformed plasmids. Therefore, the cells may need to be left in the shaker at 37 °C longer until the stationary phase is reached.

Microscopy-based characterization of the integrated logic-and-memory gate ● TIMING 2–3 d
25| Dilute the cells from Step 24 to an OD_{600} of 0.2–0.3 in separate tubes with 5 ml of fresh medium with the appropriate antibiotics and with no inducer (both inputs OFF), with inducer AHL at a final concentration of 1 µM (input AHL ON, input aTc OFF), with inducer aTc at a final concentration of 200 ng/ml (input AHL OFF, input aTc ON) or with inducer AHL at a final concentration of 1 µM and inducer aTc at a final concentration of 200 ng/ml (both inputs ON). Prepare three samples or tubes for each condition.

26| Grow the cells overnight in a shaker incubator at 37 °C and 300 r.p.m.

27| The next day, centrifuge 1 ml of overnight cells for 2.5 min at 16,000*g* at room temperature, and resuspend them in 30 µl of PBS.
▲ **CRITICAL STEP** Note that the cells may grow slower because of the presence and size of the three plasmids. Therefore, the cells may need to be left in the shaker at 37 °C longer until stationary phase is reached.

28| Remove the coverslip from the microscope slide treated with 1% (wt/vol) agarose and prepared as described in Reagent Setup. Add 5–10 µl of the resuspended cells to the microscope slide. Cover the cells with the coverslip and prepare to image them.

29| Image the cells for the expression of GFP by using an inverted Zeiss fluorescence microscope with a ×100 oil-immersion objective. Save the images in TIFF or JPEG format. Other fluorescence microscopes may be used at this step to measure GFP expression, and the images can be saved in other formats as well, depending on the user's preference. Characterize the performance of the AND gate (**Fig. 2**) by quantifying the proportion of cells that do or do not express GFP under conditions where they have been uninduced, induced with AHL only, induced with aTc only and induced with both AHL and aTc.
? TROUBLESHOOTING

Flow cytometry–based characterization of the integrated logic-and-memory gate ● TIMING 6–9 d
30| Repeat Steps 25 and 26.

31| The next day, centrifuge 1 ml of overnight cells for 2.5 min at 16,000*g* at room temperature and wash them in medium without the inducer.
▲ **CRITICAL STEP** Note that the cells may grow slower because of the presence of multiple transformed plasmids. Therefore, the cells may need to be left in the shaker at 37 °C longer until the stationary phase is reached.

32| For each sample to be tested, dilute the cells by 1:100 into a new 96-well plate containing fresh PBS, and immediately assay them by using a BD-FACS LSRFortessa-HTS cell analyzer. We recommend using a multichannel pipette and mixing well when diluting the cells. Other flow cytometers may be used for measuring *gfp* expression.

33| Measure fluorescence with a 488-nm laser and a 515-nm to 545-nm emission filter. We typically collect 50,000 cells for each sample and gate these by forward scatter (FSC) and side scatter (SSC). A consistent fluorescence threshold should be used on the data to determine the percentage of cells deemed GFP-positive (ON state) or GFP-negative (OFF state). Characterize the performance of the AND gate (**Fig. 1**) by quantifying the proportion of cells that do or do not express GFP under conditions where they have been uninduced, induced with AHL only, induced with aTc only and induced with both AHL and aTc.
▲ **CRITICAL STEP** To determine the optimal fluorescence threshold, use cells that do not express GFP (OFF state) as negative controls, and use the GFP-expressing cells (ON state) as positive controls.
? TROUBLESHOOTING

34| Repeat Steps 30–33 twice or more in order to have at least three independent experiments.

Stable memory maintenance ● TIMING 4–5 d
35| Repeat Steps 30–33.

36| Dilute the cells to an OD_{600} of 0.2–0.3 in a tube with 5 ml of fresh medium with the appropriate antibiotics and no inducers. Grow the cells overnight in a shaker incubator at 37 °C and 300 r.p.m. Assay the cells with flow cytometry and/or microscopy as described above.

37| Repeat Steps 35 and 36 for as many days as desired (**Fig. 3**).

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
4	Failure to obtain the right PCR product	Preparing the genetic fragments for Gibson assembly via PCR can occasionally pose a challenge, especially with long primers that contain sequences that self-anneal, such as terminator sequences	Decreasing primer lengths and using shorter overlap regions can solve this problem. We usually perform successful Gibson assembly with fragments with a minimum total overlap of 20 bp, and we recommend avoiding overlaps that are shorter than 20 bp Note that the primer Inverted_Promoter_proD_R does not anneal directly on the promoter but rather anneals to a sequence upstream of the promoter. This is done to increase the size of the DNA piece that will be flipped by the recombinase. Therefore, care needs to be taken when performing PCR for the promoter piece to include the upstream sequences on the DNA template
20	Failure to obtain colonies containing the AND gate via Gibson assembly	Gibson assembly of a large number of parts can be challenging and the efficiency of successful Gibson assembly can decrease with the number of parts used in a single reaction	We recommend testing multiple colonies to identify cells containing constructs where Gibson assembly was completed successfully. An alternate strategy to the one described here is to decrease the number of parts used in any given Gibson assembly reaction. For example, one can build the logic gate construct in two steps. In the first step, one can first construct: (i) phiC31 *attP*-inverted proD-phiC31 *attB* and (ii) Bxb1 *attB*-inverted *gfp*-Bxb1 *attP* via Gibson assembly in two separate plasmids. In the second step, the two aforementioned parts can be combined via Gibson assembly
29, 33	Leaky recombinase expression	Leaky expression of recombinases can lead to unwanted flipping of components in the integrated logic-and-memory gate owing to the high efficiency of recombinases	We recommend the use of riboregulators, such as those from Siuti *et al.*[11] and Callura *et al.*[13], for tight regulation of recombinase expression. Sequence verification of these constructs is important. For example, a point mutation in the loop formation sequence of the *cis*-repressive sequence in the riboregulators can dramatically decrease the strength of repression and result in undesirable leakage of recombinase expression Other strategies for controlling leaky protein expression could include using tightly regulated promoters, engineering ribosome-binding sequences, adding degradation tags to the recombinases to decrease their half-lives and placing recombinase-expression cassettes on lower-copy constructs
	Recombinase efficiency is low	Recombinase efficiency can be correlated with the length of the DNA that is being targeted. For example, very short DNA sequences can be challenging to invert with recombinases because of the inability to form DNA loops that bring the recognition sites close to each other for flipping to occur	A general relationship between formation of a DNA loop and recombinase activity has been described by Ringrose *et al.*[29]. Our integrated logic-and-memory platform enables many different circuit designs, which should implement the same logical behavior. Thus, if specific performance characteristics are required, we recommend the construction of several circuit variants with different recombinase-invertible DNA sizes to achieve the desired behaviors. For example, DNA fragments can be padded with extra DNA sequences to increase their size

● TIMING

Steps 1–20, Gibson assembly: 3–5 d
Steps 21–24, preparation of logic gate–containing cells: 4–7 d
Steps 25–29, microscopy-based characterization of the integrated logic-and-memory gate: 2–3 d
Steps 30–34, flow cytometry–based characterization of the integrated logic-and-memory gate: 6–9 d
Steps 35–37, stable memory maintenance: 4–5 d

ANTICIPATED RESULTS

The AND gate should have a low percentage of GFP-positive cells in the uninduced state. Upon the addition of any one of the inputs by itself (AHL alone or aTc alone), the AND gate should continue to have a low percentage of GFP-positive cells.

Upon addition of both of the inputs (AHL and aTc), the AND gate should result in an ON state where the majority of cells express GFP. We recommend the use of negative and positive controls, as described above, in order to set the threshold for determining GFP-positive and GFP cells with flow cytometry and to calibrate optimal exposure times for microscopy. The anticipated behavior of this circuit under flow cytometry and microscopy is illustrated in **Figures 1–3**.

Flow cytometry and microscopy can help determine the performance of the integrated logic-and-memory gate, as well as optimization strategies. For example, the kinetics of the synthetic circuits can be determined by time-course assays in which the cells are induced for different lengths of time. This information can help determine whether the synthetic circuits will be suitable for specific applications with required dynamics. In addition, if in the OFF states there are too many cells expressing the output gene or if in the ON states there are too few cells that are expressing the output gene, additional optimization can be performed, such as reducing recombinase leakage or increasing recombinase expression, respectively.

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