Quorum-sensing crosstalk driven synthetic circuits: from unimodality to trimodality

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SUMMARY

Widespread quorum-sensing (QS) enables bacteria to communicate and plays a critical role in controlling bacterial virulence. However, effects of promiscuous QS crosstalk and its implications for gene regulation and cell decision-making remain largely unknown. Here we systematically studied the crosstalk between LuxR/I and LasR/I systems and found that QS crosstalk can be dissected into signal crosstalk and promoter crosstalk. Further investigations using synthetic positive feedback circuits revealed that signal crosstalk significantly decreases circuit's bistable potential while maintaining unimodality. Promoter crosstalk, however, reproducibly generates complex trimodal responses resulting from noise-induced state transitions and host-circuit interactions. A mathematical model that integrates the circuit's nonlinearity, stochasticity, and host-circuit interactions was developed, and its predictions of conditions for trimodality were verified experimentally. Combining synthetic biology and mathematical modeling, this work sheds light on the complex behaviors emerging from QS crosstalk, which could be exploited for therapeutics and biotechnology.

INTRODUCTION

Quorum-sensing (QS) is a widespread mechanism bacteria use to regulate gene expression and coordinate population behavior based on local cell density (Ng and Bassler, 2009). It is achieved through the binding of QS regulators with their cognate signal molecules (autoinducers) to regulate downstream QS pathways. Autoinducers are produced inside the cell and diffuse into and out of bacterial cells. Therefore, an autoinducer's intracellular concentration correlates with local cell density (Ng and Bassler, 2009). There are diverse QS mechanisms allowing for bacterial communication: gram-positive bacteria generally use two-component systems mediated by peptides, while gram-negative bacteria primarily use LuxR/LuxI-type systems mediated by acylated homoserine lactones (AHL) (Miller and Bassler, 2001; Ng and Bassler, 2009). Many bacterial activities are controlled or regulated by QS, such as antibiotic production, biofilm development, bioluminescence, colonization, sporulation, symbiosis, and virulence (Jayaraman and Wood, 2008; LaSarre and Federle, 2013; Miller and Bassler, 2001; Ng and Bassler, 2009; Solano et al., 2014).

With well-defined and characterized biological properties, several QS regulators and corresponding autoinducers have also been used for synthetic gene networks. For example, LuxR/LuxI and/or LasR/LasI pairs were used to generate programmed patterns (Basu et al., 2005; Payne et al., 2013), trigger biofilm formation (Hong et al., 2012; Kobayashi et al., 2004), develop synthetic ecosystems and program population dynamics (Balagadde et al., 2008; Brenner et al., 2007), and construct synchronized oscillators (Danino et al., 2010; Prindle et al., 2012), edge detectors (Tabor et al., 2009), and pulse generators (Basu et al., 2004). RhlR/RhlI has also been used in the study of generic mechanisms of natural selection (Chuang et al., 2009) as well as for carrying out biological computations as chemical 'wires' (Tamsir et al., 2011).

However, effects of QS crosstalk, functional interactions between QS components that are not naturally paired, remain unexplored. For example, widely used LuxR-family regulators share extensive homologies and structural similarities in their corresponding autoinducers. LuxR binds its natural ligand 3-oxo-C6-HSL (3OC6HSL, hereafter denoted as C6) to activate the pLux promoter, while LasR bind 3-oxo-C12-HSL (3OC12HSL, hereafter denoted as C12)

1   to activate pLas (Table S1) (Fuqua et al., 1996; Meighen, 1994; Miller and Bassler, 2001; Ng

2   and Bassler, 2009; Schuster et al., 2004; Stevens and Greenberg, 1997). However, the LuxR

3   protein can also bind other HSLs, such as C7HSL and 3OC8HSL (Canton et al., 2008). When

4   binding C12, LasR is able to activate pLux in addition to the naturally paired pLas promoter

5   (Balagadde et al., 2008). Implications of such crosstalk on gene regulation and cell response

6   remain largely unknown.

7   Here, we use rationally designed gene networks to probe crosstalk between the LuxR/I and

8   LasR/I systems and investigate their elicited bistable behaviors from positive feedback

9   topologies. By using a synthetic biology approach, all combinations of autoinducer, regulator,

10 and promoter were tested to show that QS crosstalk can be dissected into signal crosstalk and

11 promoter crosstalk. When studied in the context of a synthetic positive feedback gene network,

12 our results indicate that QS crosstalk leads to distinct dynamic behaviors: signal crosstalk

13 significantly decreases the circuit's induction range for bistability, but promoter crosstalk

14 causes transposon insertions into the regulator gene and yields trimodal responses due to a

15 combination of mutagenesis and noise induced state transitions. To fully understand this

16 complex response, we developed and experimentally verified a mathematical model that takes

17 into account all of these factors to simulate and predict how varying the transposition rate can

18 modulate this trimodality. This reveals a novel factor of host-circuit interactions in shaping

19 complex responses of synthetic gene networks.

20

21 **RESULTS**

22 **Dissecting the crosstalk between LuxR/I and LasR/I using synthetic circuits.**

23 To characterize possible crosstalk between LuxR/I and LasR/I signaling systems, four

24 synthetic circuits, CP (constitutive promoter)-LuxR-pLux (Figure 1A), CP-LasR-pLux

25 (Figure 1B), CP-LasR-pLas (Figure S1A), and CP-LuxR-pLas (Figure S1B), were first built

26 to test all autoinducer-regulator-promoter combinations' impact on gene expression activation.

27 C6 and C12 were applied independently to all constructs, and green fluorescent protein (GFP)

28 expression under the regulation of pLux or pLas was measured as the readout.

29   It can be seen in Figure 1A that in addition to its natural partner C6, LuxR can also bind

4

with C12 molecules to activate pLux, which suggests that the binding with C6 or C12 results in a similar conformational change of LuxR and therefore its activating functions remain uninterrupted. Such an activation of a natural QS regulator-promoter pair by a cross-talking autoinducer is here termed signal crosstalk. It can be seen that this signal crosstalk can fully activate the system with comparable induction dosages. However, similar tests of signal crosstalk of C6 with the Las regulator-promoter pair (Figure S1A) only show comparable induction when the autoinducer concentration is as high as 10^{-3} M. This suggests that the efficacy of signal crosstalk is QS system specific.

In addition to promiscuous autoinducer binding resulting in signal crosstalk, the systems studied also displayed crosstalk between regulators and promoters, here termed promoter crosstalk. It is shown in Figure 1B that, in addition to being able to activate pLas, LasR significantly activates pLux when induced with its natural cognate ligand C12, though not with C6, which suggests that LasR's DNA binding domain can recognize both pLas and pLux when bound with its natural partner. This promoter crosstalk is robust over a wide range of autoinducer concentrations. Similar tests of promoter crosstalk of C6-LuxR to pLas (Figure S1B) show only weak induction. This suggests that the efficacy of promoter crosstalk is also QS system specific. It should also be noted that a third type of crosstalk, regulator crosstalk, in which naturally paired autoinducer and promoter function through a cross-talking regulator protein, only exhibited minimal levels of activation (gray bar in Figure 1B and black bar in Figure S1B).

To further verify the crosstalk under physiologically relevant dosages of autoinducers, synthase genes LuxI and LasI were introduced to replace commercial chemicals in eight different circuits (Figure S1C and 1D). The results further confirm that pLux can be activated by LuxR with LuxI or LasI, as well as by LasR with LasI. This is consistent with the results using commercial chemicals, indicating the crosstalk categorization is also applicable *in vivo*. All combinatorial activations between LuxR/I and LasR/I systems are summarized in Figure 1C, with crosstalk highlighted in red. Taken together, detectable crosstalk between LuxR/I and LasR/I systems can be categorized into two types: LasI (C12) can crosstalk with the LuxR protein to induce pLux transcription (signal crosstalk), and the LasR-LasI (C12) complex can also crosstalk with and activate the pLux promoter (promoter crosstalk).

Signal crosstalk induces distinct responses from positive feedback circuits.

Next, synthetic positive feedback circuits were constructed to investigate the impact of QS crosstalk in the context of gene regulatory networks. It is shown that the core of many bacteria's QS decision-making circuits is a positive feedback motif (Ji et al., 1995; Kaplan and Greenberg, 1985; de Kievit and Iglewski, 2000; Pestova et al., 1996; Piper et al., 1993; Seed et al., 1995). Because of its potential bistability, such a topology enables the bacteria to make appropriate binary decisions in response to changing environments (Ozbudak et al., 2004; Xiong and Ferrell, 2003; Guido et al., 2006; Isaacs et al., 2003). Synthetic positive feedback circuits serve as suitable platforms to probe the effects of signal and promoter crosstalk within the framework of gene regulatory networks.

The design shown in Figure 2A was first constructed to study signal crosstalk. In this circuit, expression of LuxR is regulated by the promoter pLux, which can be activated by LuxR when induced, forming a positive feedback loop. pLux driven GFP expression serves as the readout for LuxR levels. Robustness of history-dependent responses (hysteresis), a hallmark of many positive feedback topologies, is used as the main measure of signal crosstalk impacts as it captures the effectiveness of the circuit's decision-making functionality (Acar et al., 2005; Gardner et al., 2000; Wu et al., 2013).

As a benchmark, uninduced (Initial OFF) cells with the circuit were first induced with different concentrations of LuxR's natural inducer C6 and measured using flow cytometry (Figure 2B, blue). It can be seen that GFP is only turned on with 10^{-8} M or higher C6 induction. The cells treated with 10^{-4} M C6 (Initial ON) were then collected and diluted into new medium with the same concentrations of C6 (Figure 2B, red). These cells keep high GFP expression even with low C6 inductions (below 10^{-9} M) due to the self-sustaining nature of positive feedback loops. Taken together, these results illustrate this circuit's hysteretic response with C6 inducer concentrations between 0 and 10^{-8} M. This indicates that under C6 induction the positive feedback circuit is bistable between 0 and 10^{-8} M C6 induction. However, no bimodal distribution was observed within the bistable region based on flow cytometry measurements (Figure 2D, purple and light purple; and Figure S2A), suggesting that the barrier between the two states is too high for inherent gene expression stochasticity to

1   overcome (Acar et al., 2005; Gardner et al., 2000).

2   Next, C12 was used to induce the same construct to investigate the impact of signal

3   crosstalk on gene network regulation. Similar induction experiments were carried out and the

4   results are shown in Figure 2C. It can be seen that this circuit also displays hysteresis, but

5   with a much smaller bistable region between 10^{-8} and 10^{-6} M C12. Flow cytometry results

6   within the bistable region also show no bimodal distributions (Figure 2D, cyan and light cyan;

7   and Figure S2B).

8   To quantitatively understand the signal crosstalk caused shrinkage of the bistable region, an

9   ordinary differential equation (ODE) model of LuxR-pLux auto-activation was developed.

10   Two major kinetic events, LuxR transcription and translation, are described by two ODEs

11   with all binding between chemical species incorporated into model terms. After fitting the

12   parameters using existing literature and experimental measurements (Table S2), the model can

13   capture the experimental results (lines in Figure 2B and 2C) with accuracy. Inspection of

14   model parameters reveals that the bistable region decrease caused by signal crosstalk can be

15   largely accounted for by differential binding affinities between LuxR and C6 and C12. This

16   suggests a new way to perturb QS decision-making through utilization of crosstalking

17   autoinducers, which could be useful for clinical therapies.

18

19   **Promoter crosstalk induces unexpected and complex bimodal responses.**

20   To study the impacts of promoter crosstalk, a positive feedback circuit was constructed with

21   LasR under the regulation of pLux (Figure 3A). It is shown in Figure 1B that LasR can

22   activate pLux when induced by C12. Therefore this circuit also forms a positive feedback

23   loop in the presence of C12. GFP under regulation of pLux is again included as a readout for

24   LasR. Experimental explorations of hysteresis were carried out and the results are shown in

25   Figure 3B. It can be seen that initial OFF cells (blue) exhibit a non-monotonic response to

26   C12 induction: GFP expression increases with C12 concentration, but begins to uniformly

27   decrease when C12 induction exceeds 10^{-8} M (Figure 3B, and Figure S3A and 3B). Cells

28   induced with 10^{-4} M C12 were then collected and diluted into fresh medium with the same

29   inducer concentrations as the initial OFF cells. Flow cytometry data show that all samples

30   exhibit unimodal minimal fluorescence signals that are even lower than the basal GFP

1 expression of initial OFF cells (Figure 3B and 3C green, and Figure S3B).

2 Considering that both C12 and exogenous gene overexpression may be toxic to cells, as

3 well as the fact that initial OFF cells can be turned on with lower induction dosages, cells

4 induced with lower than 10^{-4} M but higher than 10^{-10} M C12 were collected as new initial ON

5 cells to further explore possible hysteresis of this circuit. Collected cells were diluted into

6 fresh medium with the same concentrations of C12. These new initial ON cells demonstrate

7 the same expression pattern as the initial OFF cells when grown in inducer concentrations

8 from 0 to 10^{-9} M, but they show much lower fluorescence values at higher concentrations. For

9 example, the red points in Figure 3B illustrate the GFP average of 10^{-9} M induced initial ON

10 cells when collected and re-diluted into a range of C12 concentrations (See Figure S3C for

11 results with other initial induction dosages). Examination of the flow cytometry

12 measurements of these ON cells reveals that bimodal distributions emerge within the

13 concentration range of 10^{-8} M to 10^{-4} M C12. Interestingly, one peak of the distribution is at

14 the high state and the other is at the minimal expression state, even lower than basal

15 expression (Figure 3C, red). So unlike classic bimodal responses due to bistability,

16 LasR-pLux positive feedback exhibits bimodality with the lower peak's expression even

17 weaker than the OFF state. To exclude the possibility that this bimodality is triggered by

18 inherent properties of the LasR-C12 complex, similar hysteresis experiments were carried out

19 for the linear CP-LasR-pLux circuit (Figure 1B). Results show that the initial OFF and ON

20 cells both exhibit unimodal expression without hysteresis (Figure S3D). The bimodality is,

21 therefore, unique to the initial ON cells with LasR-pLux positive feedback.

22

23 **Bimodality results from circuit-host interactions.**

24 The remaining question is: what is the cause of the minimal expression state? To resolve this

25 problem, new initial ON samples at concentrations of 10^{-11} M to 10^{-4} M C12 (Figure 3B, red

26 triangles) were collected. Their plasmids were extracted and digested for genotyping. The

27 agarose gel electrophoresis results show that a new band (~3.2 kb) replaces the original

28 fragment band (wild type, ~1.9 kb) for samples in 10^{-8} ~10^{-4} M C12, and that a faint

29 original-fragment band can also be seen for samples with 10^{-8} and 10^{-7} M C12 inductions

30 (Figure 3D). Further sequencing analyses verify that an IS10 transposase is inserted into the

1   LasR gene at the 682 bp site and this insertion is flanked by two 9 bp direct repeats

2   5'-CGCGTAGCG-3' (Figure 3D and Supplementary Information), which is consistent with

3   reported hotspots for IS10 insertion (Kovarík et al., 2001).

4   The insertion abolishes LasR's ability to activate downstream GFP expression, which in

5   turn causes the cells' fluorescence signal to be even weaker than basal expression when LasR

6   is intact. Cells with this type of mutation form the low GFP peak in the bimodal distributions

7   in Figure 3C. On the other hand, cells that do not mutate are able to maintain a high GFP

8   expression due to positive feedback, forming the GFP ON peak of the bimodal distributions.

9   Taken together, the combination of gene network activated GFP expression and mutation

10   caused GFP inhibition drive the emergence of a bimodal distribution.

11

12 Trimodality predicted by expanded model.

13   In light of the verified mutation in the LasR-pLux positive feedback system, the mathematical

14   model was expanded to take into account crosstalk triggered genetic changes to better

15   describe the circuit. To enable comparison with flow cytometry results, the ODEs were

16   transformed into corresponding biochemical reactions and simulated stochastically (Gillespie,

17   1977). In addition, each cell was assigned a probability of mutation throughout the simulation

18   (Figure 4C inset), which is dependent on the cell's current LasR/GFP level and the

19   transposition rate. Once mutated, the cells had only minimal GFP expression strength and

20   remained mutated until the end of the simulation. Finally, growth rate differences between

21   wild type and mutated cells were computed from experiments (Figure S4A) and taken into

22   consideration in the simulation. Results of stochastic simulations of this expanded model are

23   shown in Figure 4A, exhibiting the bimodal distribution observed experimentally (red curves

24   in Figure. 4A, simulation; and 4B, experiment).

25   To further investigate the impact of this mutation on the circuit's functions, simulations

26   were carried out with perturbed parameters to mimic various scenarios. First, the transposition

27   rate was artificially set to zero, and the simulations show that the system can also exhibit a

28   bimodal distribution (Figure 4A, blue), with the OFF peak exhibiting basal GFP expression.

29   Bimodality has been reported to arise from stochastic state switching of a bistable system

30   without any genetic changes (Acar et al., 2005; Gardner et al., 2000; Tan et al., 2009). The

1   same mechanism leads to simulated bimodality of this LasR-pLux circuit when there is no

2   mutation.

3   While it is almost impossible to eliminate mutation, it is possible to decrease the

4   transposition rate experimentally. To explore the impacts of mutation in a more realistic

5   scenario, simulations were carried out with positive but smaller transposition rates.

6   Interestingly, the system demonstrates a trimodal distribution (Figure 4A, green). In this

7   distribution, there are three groups of cells: ON, OFF, and Mutated. Those cells initialized at

8   the ON state freely transition to and from the OFF state, due to the system's bistability.

9   Meanwhile, all cells have the chance to mutate and stay mutated (Figure 4C). Given enough

10   time and the right measurement window, all three groups of cells would be visible. Within this

11   window, the portion of ON and OFF cells will gradually decrease and the number of mutated

12   cells will increase because the mutation is irreversible. The effect of a decreased transposition

13   rate is essentially slowing down the ON to Mutation transition rate and giving enough time

14   for ON to OFF transitions and hence the emergence of the OFF peak. Time courses of the

15   simulations demonstrate gradual emergence and evolution of these three populations of cells

16   (Figure 4D).

17

18   **Experimental validation of trimodal responses by lowering growth temperature.**

19   Previous reports indicated that transposition frequency can be perturbed by growth

20   temperatures (McClintock, 1984; Ohtsubo et al., 2005; Sousa et al., 2013). To tune the

21   transposition rate, experiments were carried out with cells cultured at a lower 34 °C

22   temperature, which was shown to slow down crosstalk triggered mutation of this circuit

23   (Figure S4B). Consistent with model predictions, initial ON cells induced with 10^{-8} M C12

24   exhibited a trimodal response when the growth temperature was tuned from 37 °C to 34 °C

25   (Figure 4B, green). Moreover, temporal evolution of the proportion of each subpopulation

26   was consistent with model predictions: the portion of ON cells gradually decreased, the

27   Mutation portion increased, and the OFF portion increased first and then decreased as time

28   went on (Figure 4E). Growth rates of cells at Mutated, ON, or OFF states were also measured

29   and show no difference when cultured at these two different temperatures (Figure S4A). The

30   emergence of the OFF peak, therefore, is fully accounted for by the decrease of transposition

rate, which slows down the direct transitions from ON to Mutation and therefore gives the cells time to layover at the OFF state. This is also evidenced by the smaller portion of Mutated cells when grown at 34 °C compared with 37 °C (Figure S4B).

Furthermore, a microfluidic platform coupled with time-lapse imaging was also employed to verify model predictions (Ferry et al., 2011). Cells were pretreated with 10^{-9} M C12 until steady state as the initial ON cells before being loaded into the device and induced with 10^{-8} M C12 at 34 °C to mimic the experimental protocols used in Figure 4E. Initially, there was only one ON cell loaded into the trap (Figure 5A and Movie S1). At the 8th hour, it can be seen that two populations began to emerge: some cells became OFF and some stayed ON. Mutations started to occur shortly after the 8th hour, and the OFF and Mutation cells accounted for around 90 percent of the population after 16 hours. Eventually mutation state cells took up the majority of the population. There also existed several OFF cells which became ON again, owing to stochastic gene expression noise, but they eventually exhibit a similar evolving process: ON to OFF or Mutation (Figure 5B and Movie S1), which is consistent with the stochastic model simulations shown in Figure 4C.

Altogether, the flow cytometry and microfluidic data confirmed the model's predicted trimodality, which arises from bistability of the positive feedback circuit and host-circuit interactions. In the context of positive feedback, cells transition freely between the ON and OFF states, but it is easier for ON state cells to transition to the OFF state because of the asymmetric energy barrier (Figure S4C). However, the ON cells can also transition to the Mutated state, which carries an advantage of growth rate (Figure S4A). Compared to OFF state cells, those in the ON state would transition more frequently to the Mutated state at 37 °C, leading to the bimodal distribution (Figure 3). When the growth temperature is reduced to 34 °C, the transposition frequency also decreases, meaning that the barrier between ON and Mutated state increases. Therefore, more ON cells would transition to the OFF state, which promotes the emergence of trimodality (Figure 5C).

DISCUSSION

QS is a ubiquitous mechanism in nature, and its regulator-autoinducer pairs, such as

1 LuxR/LuxI and LasR/LasI, have been used in synthetic biology for a wide range of

2 applications (Balagadde et al., 2008; Basu et al., 2004, 2005; Brenner et al., 2007; Chen et al.,

3 2014; Chuang et al., 2009; Danino et al., 2010; Hong et al., 2012; Kobayashi et al., 2004;

4 Payne et al., 2013; Prindle et al., 2012; Tabor et al., 2009; Tamsir et al., 2011; Pai et al., 2012).

5 However, evolutionary pressures from limited resources in a competitive environment

6 promote promiscuous bacterial communication, which takes the form of either different

7 genera of bacteria producing the same types of autoinducers or non-specific

8 regulator-autoinducer binding (Balagadde et al., 2008; Gray et al., 1994; Hong et al., 2012;

9 Miller and Bassler, 2001; Pérez et al., 2011; Winzer et al., 2000). As a result, QS

10 regulator-autoinducer pairs are not orthogonal, and there is crosstalk between them.

11 Dissecting the crosstalk is critical for unraveling the underlying principles of bacterial

12 decision-making and survival strategies for both natural and synthetic systems.

13 In this work, we used synthetic biology approaches to dissect QS crosstalk between LuxR/I

14 and LasR/I. By applying engineering principles to construct modular gene networks, we were

15 able to characterize and categorize QS crosstalk into signal crosstalk, where LuxR binds with

16 the non-naturally paired C12 to activate pLux, and promoter crosstalk, where LasR binds with

17 C12 to activate non-naturally paired pLux. However, regulator crosstalk, in which the

18 naturally paired autoinducer and promoter function through a cross-talking regulator protein,

19 was not detected in this work.

20 When signal crosstalk is constructed and tested in the context of positive feedback, our

21 results showed a significant shrinkage of the bistable region. Because of this topology's

22 bistable capability and wide presence in most bacterial QS decision-making circuits, such a

23 decrease in bistability robustness due to QS crosstalk suggests a new strategy for developing

24 anti-infection therapeutics. Namely, we might exploit "artificial" crosstalk to disrupt

25 intercellular communication specificity and collapse the group's coordination, which could be

26 an efficient and economic approach in medical treatments, especially for QS-dependent

27 bacterial infection.

28 On the other hand, promoter crosstalk caused complex trimodal responses when embedded

29 within a positive feedback circuit. This can only be explained when network bistability, gene

30 expression stochasticity, and genetic mutations are all taken into consideration. These results

1 highlight the potential for engineering gene networks to express complex behaviors due to

2 host-circuit interactions. We computationally predicted and experimentally verified that the

3 C12-LasR-pLux positive feedback circuit could drive the formation of three subpopulations

4 from an isogenic initial culture: one population expressing high GFP expression, the second

5 showing basal GFP expression, and the third population with no GFP expression. The high

6 and low GFP states are the result of positive feedback enabled bistability and gene expression

7 stochasticity-induced random state transitions: commonly reported as a hallmark of many

8 bistable systems (Acar et al., 2005; Gardner et al., 2000; Tan et al., 2009; Guido et al., 2006;

9 Isaacs et al., 2003). This population heterogeneity is not caused by genetic factors.

10 The third non-GFP population is the result of genetic mutation from IS10 insertion. The

11 mutation only happened in the C12-LasR-pLux positive feedback circuit but not in

12 CP-LasR-pLux-C12 (Figure S 3D) or the C12-LuxR-pLux positive feedback circuit (Figure S

13 2B). It is, therefore, possible that the special sequence arrangements of the positive feedback

14 circuit (for example, the symmetric pLux promoters flanking the LasR gene) on the plasmid

15 coupled with the stress of exogenous protein overexpression led to transposon activation and

16 gene network destruction. Given that many current synthetic gene circuits are constructed

17 with a similar symmetric structure in a plasmid (such as Promoter-RBS-Gene1-RBS-Gene2-,

18 or Promoter-RBS-Gene1-Terminator-Promoter-RBS-Gene2-Terminator), the mutation may

19 occur for a wide range of engineered gene circuits. On the other hand, from an engineer's

20 perspective, the mutation stands in contrast to previously reported host-circuit interactions,

21 which are primarily related to resource limitation and resulting growth defects (Brophy and

22 Voigt, 2014). Here we were able to illustrate that both the components used and the topology

23 of the network constructed could contribute to resource independent host-circuit interactions.

24 This concept of combining nonlinear dynamics and host-circuit interactions to enrich

25 population diversity expands our understanding of mechanisms contributing to cell-cell

26 variability, and suggests new directions in engineering gene networks to utilize hybrid factors.

27 Taken together, our studies not only showcase living cells' amazing complexity and the

28 difficulty in the refining of engineered biological systems, but also reveal an overlooked

29 mechanism by which multimodality arises from the combination of an engineered gene circuit

30 and host-circuit interactions (Ellis et al., 2009; Hussain et al., 2014; Litcofsky et al., 2012;

1   Nevozhay et al., 2013; Prindle et al., 2014).

2

3   **SIGNIFICANCE**

4   **Widespread quorum-sensing (QS) enables bacteria to communicate and plays a critical**

5   **role in controlling bacterial virulence. QS components have also been widely used in**

6   **synthetic biology applications. However, effects of promiscuous QS crosstalk remain**

7   **unexplored. Here we systematically studied the crosstalk between LuxR/I and LasR/I**

8   **systems. Combining synthetic biology and mathematical modeling, this work reveals the**

9   **complexity of QS crosstalk, which is critical for unraveling the underlying principles of**

10   **bacterial decision-making and survival strategies for both natural and synthetic systems.**

11   **Furthermore, the unusual hybrid multimodality arising from the combination of**

12   **engineered gene circuits and circuit-host interactions could be utilized in biotechnology.**

13

14   **EXPERIMENTAL PROCEDURES**

15   **Strains, Growth Conditions and Media**

16   All cloning experiments were performed in *E.coli* DH10B (Invitrogen, USA), and

17   measurements of positive feedback response were conducted in DH10B and MG1655. Cells

18   were grown at 37 °C (unless specified) in liquid and solid Luria-Bertani (LB) broth medium

19   with 100 μg/mL ampicillin. Chemical 3OC6HSL and 3OC12HSL (Sigma-Aldrich, USA)

20   were dissolved in ddH$_2$O and DMSO, respectively. Cultures were shaken in 5 mL or 15 mL

21   tubes at 220 rotations per minute (r.p.m), and inducers were added at OD$_{600}$~0.1.

22

23   **Plasmids Construction**

24   Plasmids were constructed according to standard molecular cloning protocols and the genetic

25   circuits were assembled using standardized BioBricks methods based on primary modules

26   (Table S4) from the iGEM Registry (www.parts.igem.org). The receiver CP-LuxR-pLux was

27   constructed from six BioBrick standard biological parts: BBa_K176009 (Constitutive

28   promoter, CP), BBa_B0034 (Ribosome binding site, RBS), BBa_C0062 (luxR gene),

29   BBa_B0015 (transcriptional terminator), BBa_R0062 (lux promoter), and BBa_E0240 (GFP

1 generator, RBS-GFP-T). As an example, to produce the RBS-LuxR part, LuxR plasmid was

2 digested by *XbaI* and *PstI* to produce a fragment while the RBS plasmid was digested by *SpeI*

3 and *PstI* as the vector. The fragment and vector were purified by gel electrophoresis (1% TAE

4 agarose gel) and extracted using a PureLink gel extraction kit (Invitrogen). Then, the fragment

5 and vector were ligated together using T4 DNA ligase, the ligation products were transformed

6 into *E.coli* DH10B and clones were screened by plating on 100 μg/mL ampicillin LB agar

7 plates. Finally their plasmids were extracted and verified by double restriction digest (*EcoRI*

8 and *PstI*) and DNA sequencing (Biodesign sequencing lab in ASU). After confirming that the

9 newly assembled RBS-LuxR was correct, subsequent rounds to produce the

10 RBS-LuxR-Terminator were performed similarly until completing the entire receiver

11 CP-LuxR-pLux construction. All the other receivers and positive feedback circuits were

12 assembled similarly. Restriction enzymes and T4 DNA ligase were from New England

13 Biolabs. All the constructs were verified by sequencing step by step. To keep all the

14 constructs' expression consistent in the cell, we transferred all the fragments into the pSB1A3

15 vector before test.

16

17 **Flow Cytometry.**

18 All the samples were analyzed at the time points indicated on an Accuri C6 flow cytometer

19 (Becton Dickinson, USA) with 488 nm excitation and 530±15 nm emission detection (GFP).

20 The data were collected in a linear scale and noncellular low-scatter noise was removed by

21 thresholding. All measurements of gene expression were obtained from at least three

22 independent experiments. For each culture, 20,000 events were collected at a slow flow rate.

23 Data files were analyzed using MATLAB (MathWorks).

24

25 **Hysteresis Experiment**

26 For OFF→ON experiments, initially uninduced overnight culture was diluted into fresh media,

27 grown at 37 °C and 220 r.p.m for about 1.5 h (OD$_{600}$~0.1), then distributed evenly into new

28 tubes and induced with various amounts of C6 or C12. Flow cytometry analyses were

29 performed at 6, 12, and 21 hours to monitor the fluorescence levels, which generally became

30 stable after 6 hours induction according to our experience. For ON→OFF experiments,

1 initially uninduced cells were induced with 10^{-4} M (or 10^{-9} M) autoinducer and tested by flow

2 cytometry to ensure they were fully induced. Cells were then collected with low-speed

3 centrifugation, washed twice, resuspended with fresh medium, and at last inoculated into

4 fresh medium with varying inducer concentrations at a 1:80 ratio. For the LasR-pLux positive

5 feedback system, we only diluted once and grew them for 6, 12, 18, 24, or 32 hours, but for

6 the other hysteresis experiments, the ON cells were collected and diluted twice into new

7 medium with the same concentrations of C6 or C12 at 12 h and 24 h.

8

9 **Growth Curve Assay**

10 First, different initial states cells were collected: initial OFF cells were cells grown overnight

11 without inducers, initial ON cells were initial OFF cells induced with 10^{-9} M C12 for 12 hours,

12 and the Mutated cells were cells induced with 10^{-4} M C12 for 12 hours, diluted into fresh

13 media with 10^{-4} M C12, and grown at 37 °C for another 12 h. Before the growth rate assay, all

14 the cells' fluorescence was tested by flow cytometry to verify their states. Growth rate was

15 measured by using absorbance at 600 nm with a plate reader (BioTek, USA). Cells from each

16 state were then diluted into fresh LB media (1000 µL, O.D. ~0.06) with 10^{-8} M C12 and

17 grown at 37 or 34 °C. For each sample, OD was measured by using 200 µL cultures in a

18 96-well plate and tested over 24 hours. The experiments were independently replicated three

19 times.

20

21 **Microfludics, Fluorescence Microscopy, and Image Processing**

22 The use of microfluidic devices coupled with fluorescence measurement allowed us to

23 measure gene network dynamics in single cells. Media flow direction and speed was

24 controlled through hydrostatic pressure. A detailed description of the chip can be found in the

25 work of *Ferry MS, et al* (Ferry et al, 2011). Once the cell was loaded into the trap, the flow

26 was reversed and its rate was slowed to ~120 µm/min to ensure that the cells would not be

27 washed away and would receive enough nutrients. Furthermore, care was taken to avoid

28 introducing bubbles to any part of the chip as they considerably disrupt flow. The chip

29 temperature was maintained at 34 °C with an external microscope stage (Tokai Hit, Japan).

30 Inducer concentrations were controlled by adjusting the heights of the inducer-containing

1 media syringes relative to one another.

2 Images were taken using a Nikon Eclipse Ti inverted microscope (Nikon, Japan) equipped

3 with an LED-based Lumencor SOLA SE Light Engine with the appropriate filter sets. The

4 excitation wavelength for GFP was 472 nm, and fluorescence emission was detected with a

5 Semrock 520/35 nm band pass filter. Phase and fluorescent images were taken under a

6 magnification of 40X, and perfect focus was maintained automatically using Nikon Elements

7 software.

8 Initially OFF cells (K-12 MG1655) induced with 10^{-9} M C12 (6 hours) were collected as

9 the initial ON cells, washed, resuspended with fresh media and then loaded into the trap. 100

10 μg/mL ampicillin was added into media 1 and 2, but only media 2 was augmented with the

11 corresponding inducer (10^{-8} M C12). The microfluidic device was used to control the

12 chemical concentration by switching between media 1 and 2. For initial ON cells, media 2

13 was provided to the cells for the duration of the experiment. To prevent photobleaching and

14 phototoxicity to the cells in the trap, exposure time was limited to 100 ms for GFP.

15 Images were taken every 5 minutes for about 28 hours in total. The pixels in all images are

16 normalized to 0 − 1 range before analysis. One image was chosen for quantification every 15

17 minutes (i.e. three images). For each cell, the intensity was calculated by averaging three

18 selected points (left, middle, and right) in the cell and then subtracting the background. Since

19 all the cells are offspring of the first initial ON cell, each branch in Figure 5b stands for one

20 progeny. The cells that were washed away or had less than three generations were not

21 analyzed.

22

23 **Mathematical Modeling.** Ordinary differential equation models were solved and analyzed by

24 MATLAB. Stochastic simulations were written in MATLAB and run on a standard personal

25 computer (details are provided in Supplemental Information).

26

27 **SUPPLEMENTAL INFORMATION**

28 Supplemental Information contains full details about the mathematical modeling construction

29 and parameter fittings, five figures and four tables and can be found with this article online.

30

AUTHOR CONTRIBUTIONS

X.W. and F.W. designed the study; F.W. performed the experiments and carried out the mathematical modeling; X.W. and F.W. analyzed the data; D.J.M. and F.W. made the microfluidic chips; F.W, D.J.M., and X.W. wrote the manuscript.

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2

3 **Figure legends**

4

5 **Figure 1. QS crosstalk dissected using synthetic gene circuits.** (**A**) LuxR can crosstalk with

6 C12 to activate pLux. Top panel: schematic diagram of a synthetic gene circuit where a

7 constitutive promoter (gray arrow) regulates LuxR (purple rectangle) expression. LuxR

8 protein (purple bars), when dimerized and bound with C6 or C12, can activate pLux (purple

9 arrow) to induce GFP (green rectangle) expression. The autoinducers, genes, and promoters

10 are color coded so that naturally paired partners are in the same color. Bottom panel: dose

11 response of the circuit when induced with C6 (gray) or C12 (black). (**B**) LasR can crosstalk

12 with pLux when bound with C12. Top panel: schematic diagram of a circuit similar to that in

13 (**A**), where a constitutive promoter regulates LasR (cyan rectangle) expression. LasR protein,

14 when bound with C6 or C12, can activate pLux to induce GFP expression. Bottom panel:

15 Dose response of this circuit when induced with C6 (gray) or C12 (black). Bar heights are

16 averages of three independent flow cytometry measurements shown as mean ± SD. (**C**)

17 Summary of crosstalk induction of all 16 different combinations, including inductions by both

18 chemicals and corresponding synthase genes. The four combinations shown in (**A**) and (**B**) are

19 highlighted with a gray background. Quantified results for other combinations are included in

20 Figure S1.

21

22 **Figure 2. Signal crosstalk causes shrinkage of bistable region.** (**A**) Schematic diagram of a

23 synthetic gene circuit where the pLux promoter regulates expression of LuxR, which in turn

24 can bind with C6 or C12 to further activate pLux, forming a positive feedback loop (shown as

25 simplified diagram). GFP under the regulation of pLux serves as the readout for LuxR levels.

26 All components are color coded similarly as in Figure 1. (**B**) The average of three replicate

27 flow cytometry measurements is plotted as a square with error bars for each dose of C6

28 induction, where red indicates Initial ON cells while blue denotes Initial OFF cells. Solid lines

29 represent results calculated from model fittings. The bistable region ranges from 0 to 10^{-9} M

C6. Labels 1 and 2 indicate representative experiments within the region to be shown as histograms in (**D**). (**C**) Similar experiments as in (**B**) but with C12 inductions. The bistable region ranges from 10^{-8} to 10^{-6} M C12. Labels 3 and 4 indicate representative experiments within the bistable region to be shown as histograms in (**D**). (**D**) Histograms of flow cytometry measurements labeled in (**B**) and (**C**). One representative measurement from each point is shown. No bimodal distributions are observed.

Figure 3. Promoter crosstalk induces mutation and leads to population heterogeneity. (**A**) Schematic diagram of a synthetic LasR-pLux positive feedback circuit. GFP under the regulation of pLux serves as the readout for LuxR levels. All components are color coded similarly to Figure 1. (**B**) The average of three replicate flow cytometry measurements is plotted as a square with error bars for each dose of C12 induction. Blue denotes Initial OFF cells, while green and red indicate the Initial ON cells induced with 10^{-4} M C12 and 10^{-9} M C12 before being re-diluted into concentrations of C12, respectively. Labels 1, 2, 3, and 4 indicate experiments to be shown in detail as histograms in (**C**). (**C**) Histograms of flow cytometry measurements labeled in (**B**). One representative measurement from each point is shown. A bimodal distribution is only observed for label 3: which is Initial ON cells (induced with 10^{-9} M C12 before redilution) at 10^{-8} M C12. (**D**) DNA analysis for the Initial ON samples shown as red in (**B**). Top: Plasmid DNA was extracted and digested with *EcoRI* and *PstI*, and argarose gel electrophoresis results indicated gene mutation happened in samples with 10^{-8} M and higher doses of C12. Lane 1 is the wild-type plasmid as the control, lanes 2 to 9 are samples in 10^{-11} to 10^{-4} M C12, and Lane 10 is the 1kb DNA marker. V: vector; F: wild-type DNA fragment (the LasR-pLux positive feedback circuit); M: mutated fragment. Bottom: Schematic representation of the mutation and the features of IS10 transposase insertion: the target site (first CGCGTAGCG) in the LasR gene, its duplication (second CGCGTAGCG) due to insertion of IS10 transposase, and the IS10 sequence (red box and shown in *italics*).

Figure 4. Model predictions and experimental validations of mutation induced trimodality. (**A**) Model predictions of GFP expression at several transposition rates: high (red,

1   k3=3.6e-6), low (green, k3=4e-7), and none (blue, k3=0). Histograms were constructed from

2   8000 single cell stochastic simulations at 1000 (k3=3.6e-6) and 1900 (k3=0 and k3=4e-7)

3   minutes. (**B**) Experimental validation of the model predictions in (**A**). Red and green curves

4   correspond to the high and low transposition rates from (**A**), and they exhibit similar bi- and

5   trimodal responses, respectively. No blue curve is included because mutation could not be

6   eliminated entirely experimentally. (**C**) Representative stochastic simulations of single cell

7   fluorescence starting from the ON state. All possible transitions are shown. Inset diagram

8   illustrates all possible state transitions in the simulation. (**D**) Model predictions of GFP

9   expression with low transposition rate showing temporal evolution of the population from

10   primarily ON cells at an early time (green), to trimodal distributions at intermediate time

11   (blue), eventually falling into a primarily Mutated state at late time (red). (**E**) Flow cytometry

12   measurements taken at 12 hours (green), 24 hours (blue), and 32 hours (green). Populations

13   show similar dynamics to those predicted by the model in (**D**), starting with a large ON peak,

14   transitioning to a trimodal distribution, then into primarily Mutated or OFF cells.

15

16   **Figure 5. Fluorescence microscopy validation of mathematical model predictions.** (**A**)

17   GFP fluorescence (top) and phase contrast (bottom) images of cells growing in the

18   microfluidic chamber at 0, 8, 16, and 24 hours. Magnification: 40x. (**B**) Normalized

19   fluorescence expression of representative cells from (**A**), showing similar behavior to that

20   predicted by the model from Figure 4C. Four cells are colored corresponding to the scenarios

21   in Figure 4C, and the other 11 cells are grey. Each trajectory follows one cell, with the

22   trajectory branching as the cells divide. One frame equals five minutes. (**C**) Diagram of the

23   mechanism for trimodality. Each "valley" represents one state. The blue curve represents the

24   landscape at 37 °C, and the dotted grey curve is the landscape at 34 °C. At 37 °C, ON state

25   cells can more easily transition to the Mutated state because of the low barrier; while at 34 °C,

26   the barrier between ON and Mutated states increases, resulting in more ON cells transitioning

27   to OFF state and promoting the emergence of trimodality.

28

29

30

A

C6/C12 → LuxR --→ pLux → GFP

Mean Fluorescence (a.u.)
x 10⁶
C6
C12
Concentration (log10, Molar)

B

C6/C12 → LasR --→ pLux → GFP

x 10⁵
C6
C12
Concentration (log10, Molar)
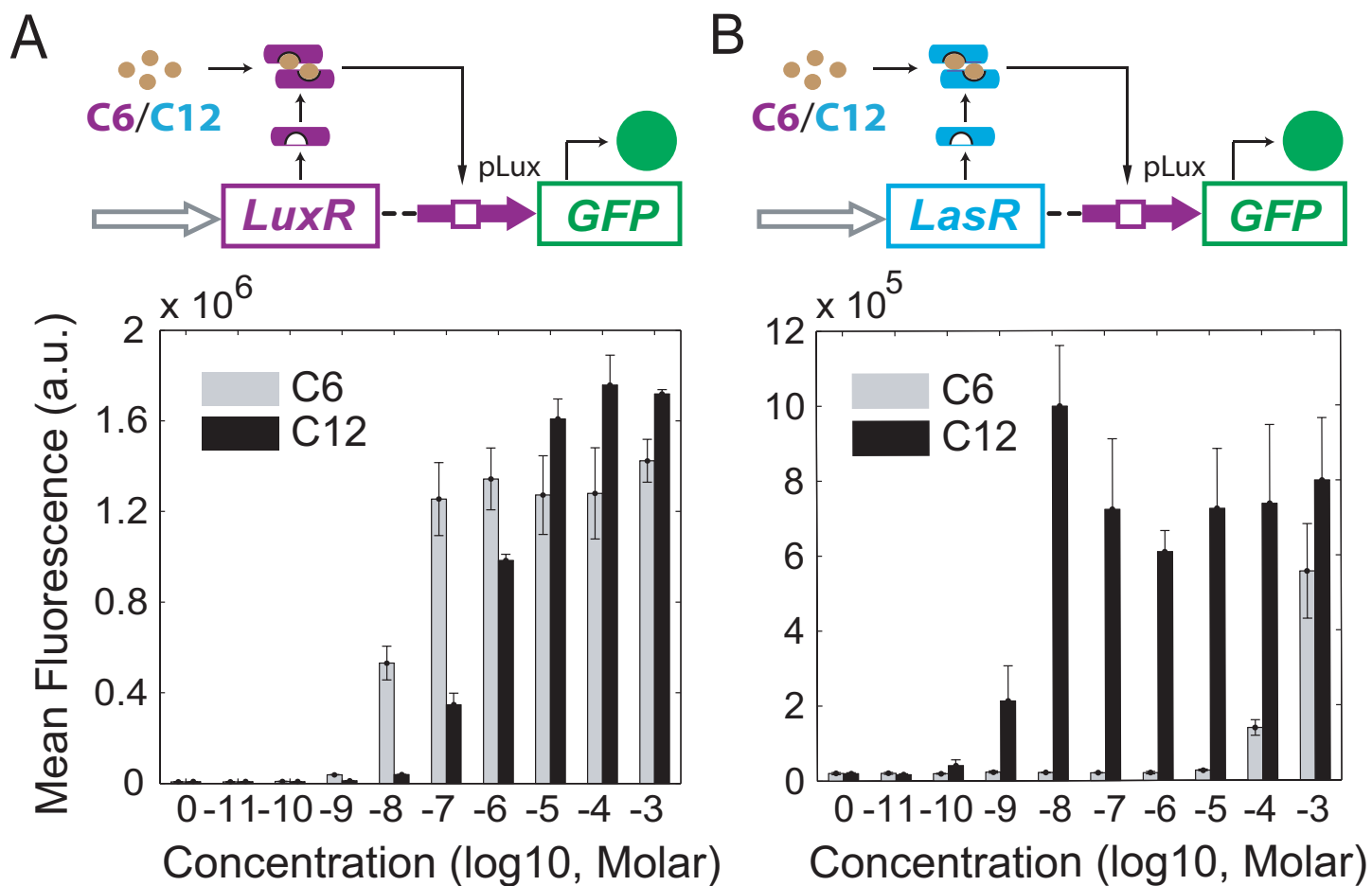
C

Summarization of the crosstalk between LuxR/I and LasR/I signal systems.

	LuxR-3OC6HSL	LuxR-3OC12HSL	LasR-3OC6HSL	LasR-3OC12HSL	LuxR-LuxI	LuxR-LasI	LasR-LuxI	LasR-LasI
pLux	+	+	−*	+	+	+	−	+
pLas	−	−	−*	+	−	−	−	+

1: Black "+" indicates the original pairs; red "+" signifies pairs showing crosstalk.

2: "*" indicates LasR could only activate the promoter at high 3OC6HSL concentration.

Figure

A

B LuxR-pLux-C6

C LuxR-pLux-C12

D

Figure

A

C12

pLux   *LasR*   pLux   *GFP*

LasR

B

- Initial OFF
- Initial ON (1e-4)
- Initial ON (1e-9)

Normalized Fluorescence

1.2

0.8

0.4

0

1

4   3

2

Concentration (log10, Molar)

0   −12   −10   −8   −6   −4

C

Cell Count

1200

800

400

0

- 1
- 2
- 3
- 4 (basal)

GFP Fluorescence (a.u.)

10^2 10^3 10^4 10^5 10^6

D

1   2   3   4   5   6   7   8   9   10

4K

3K

M ▶

V ▶
F ▶

2K

1.5K

LasR   IS10 Transposase

...CGCGTAGCG *ctgagagatcccc.................ggggatcatcag* CGCGTAGCG...

pLux   pLux   GFP

Figure

Figure S1

Figure S2
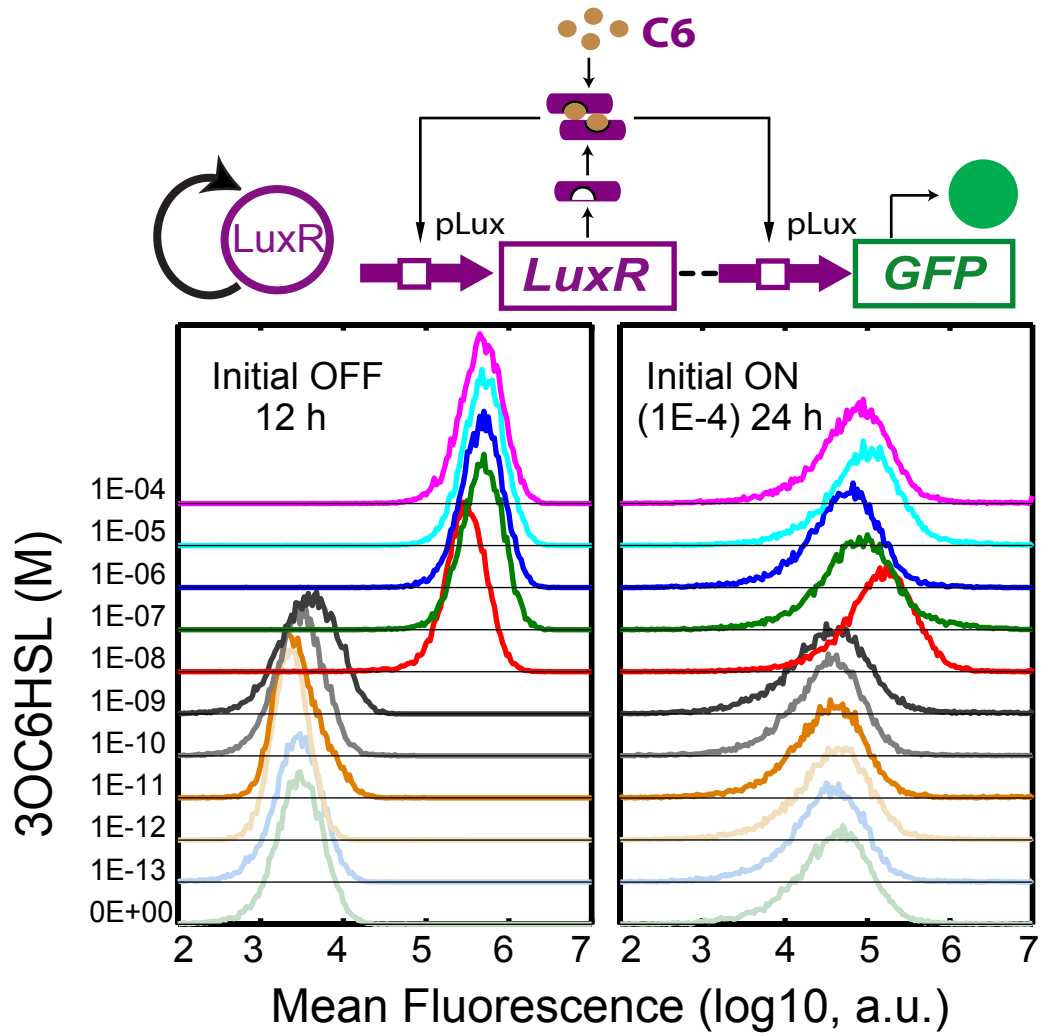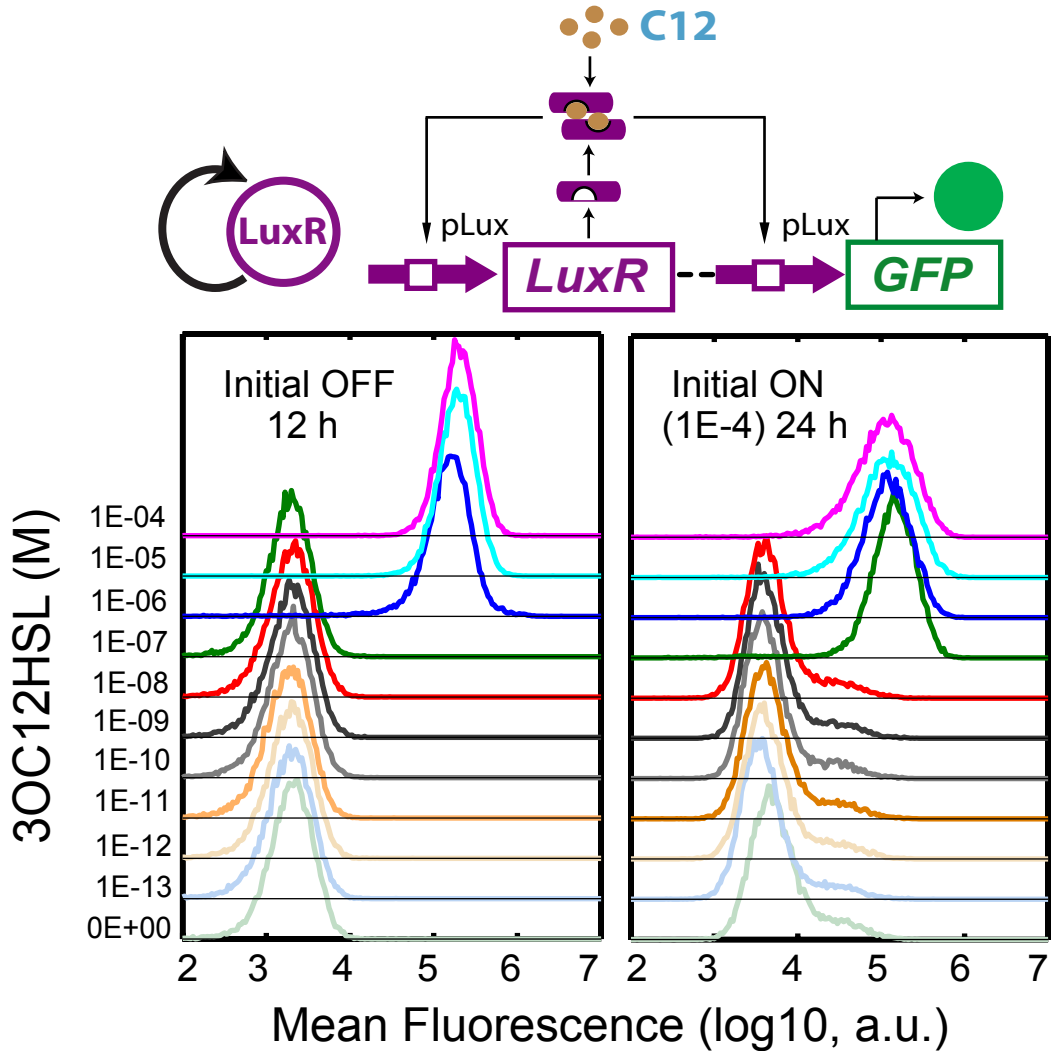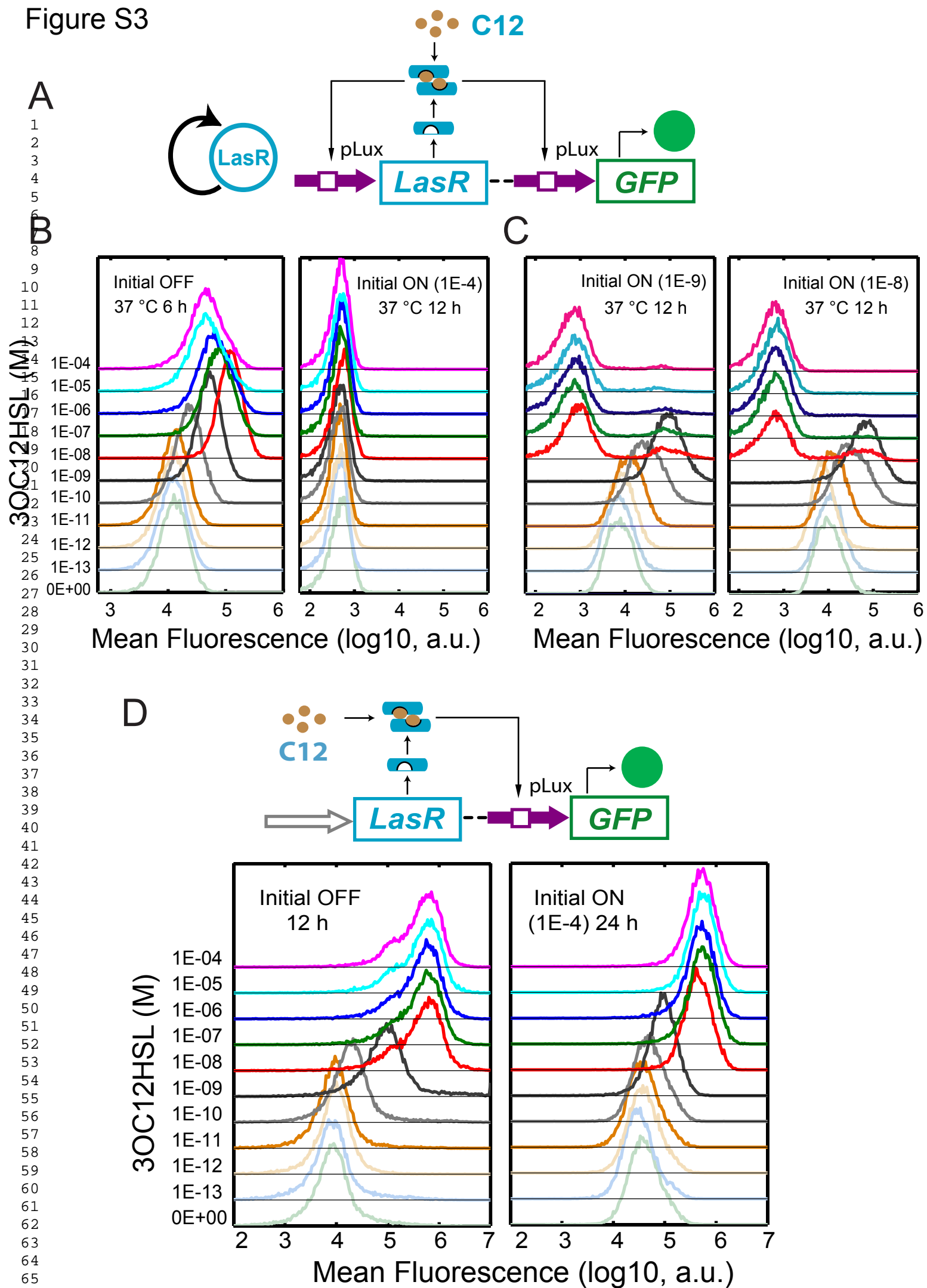
Figure S3

Figure S4
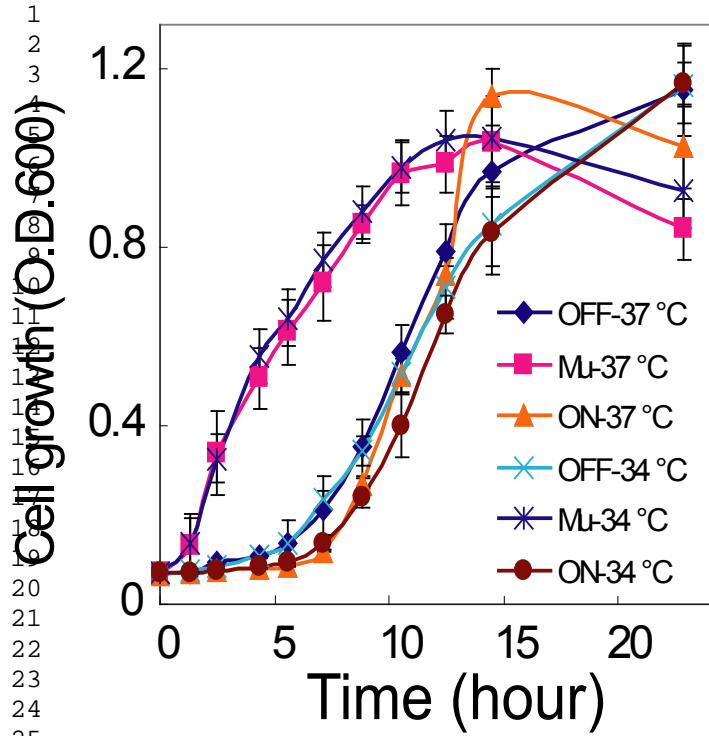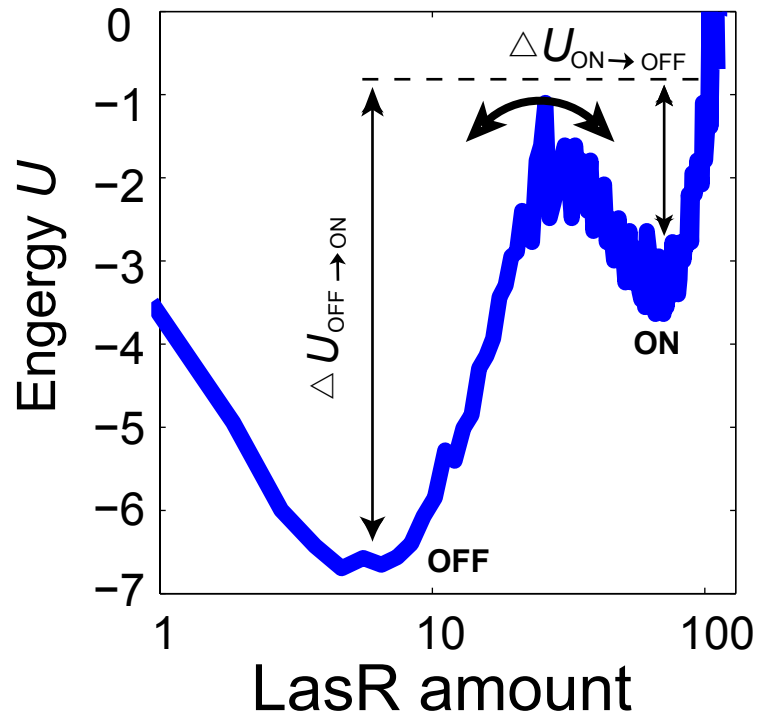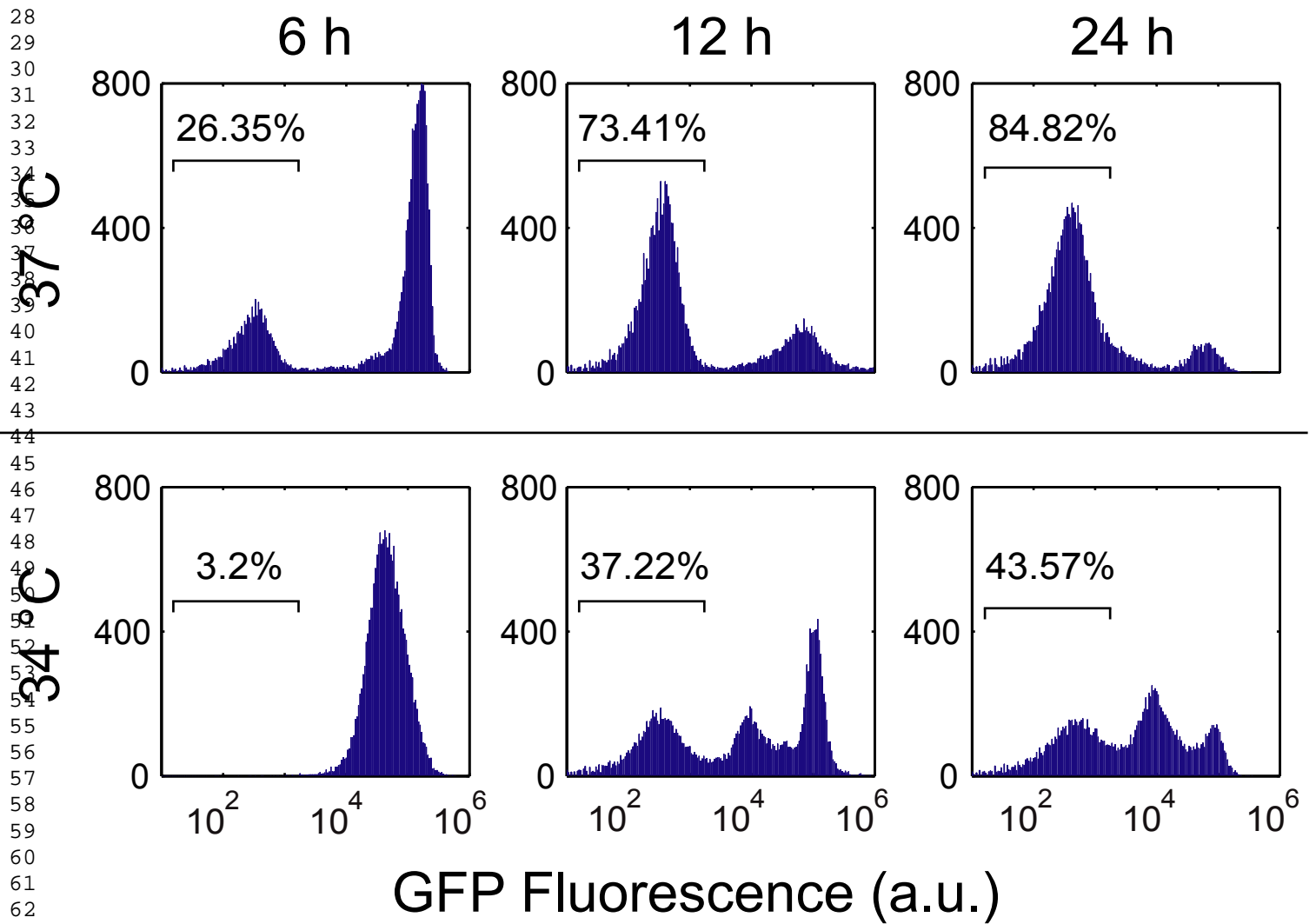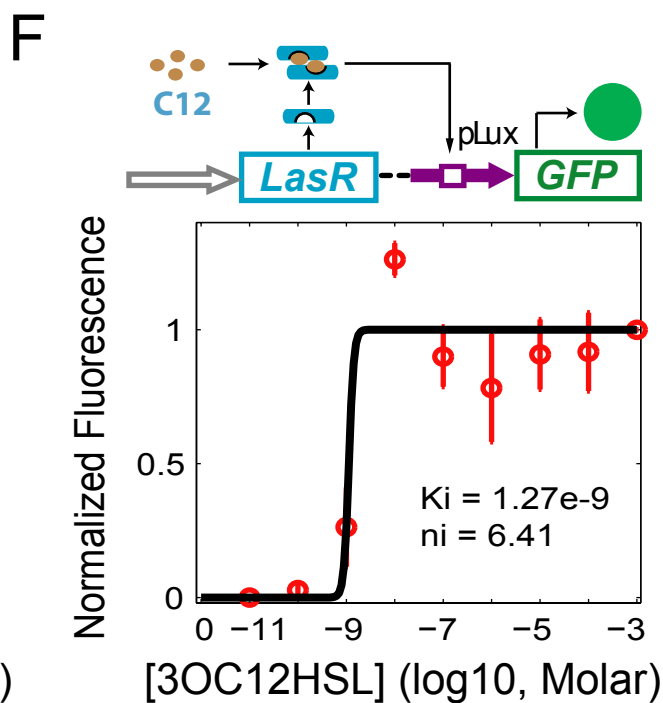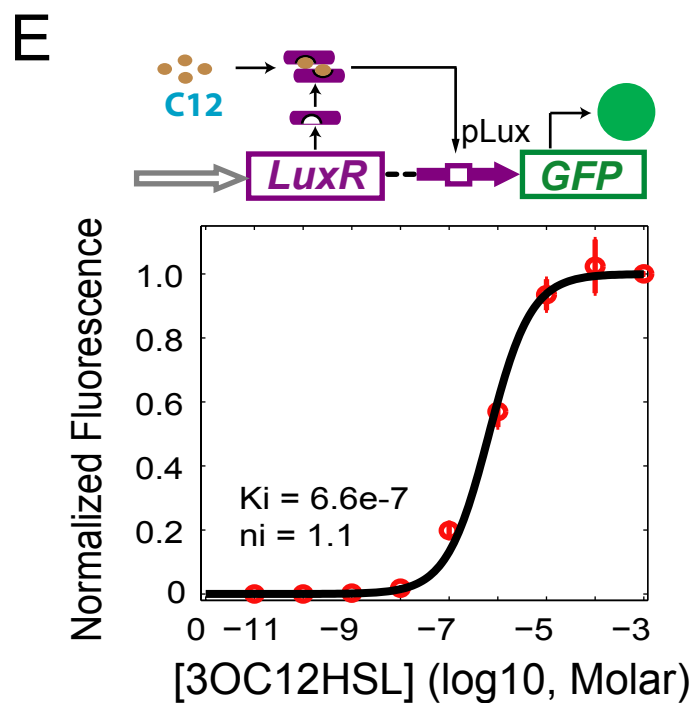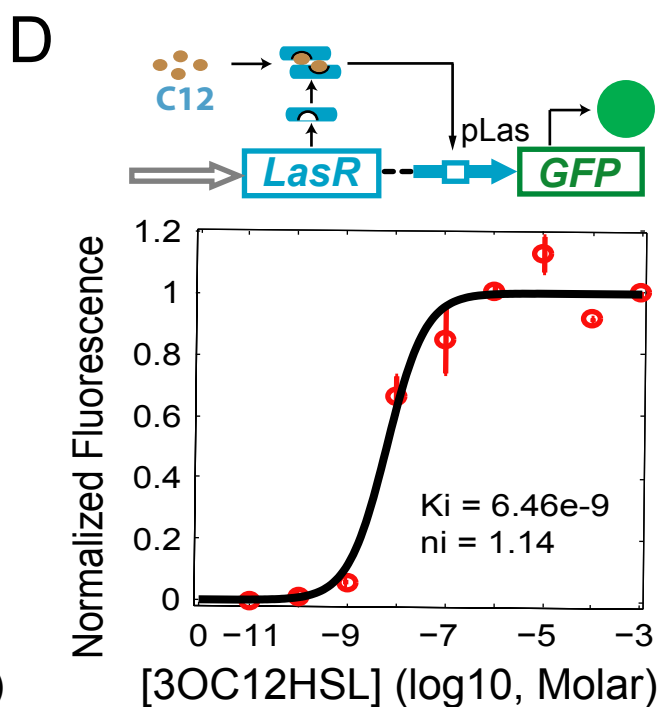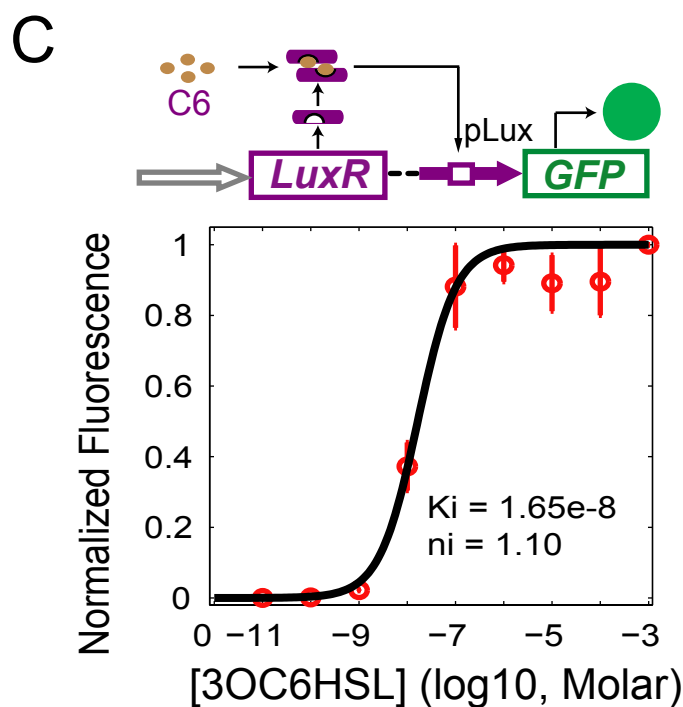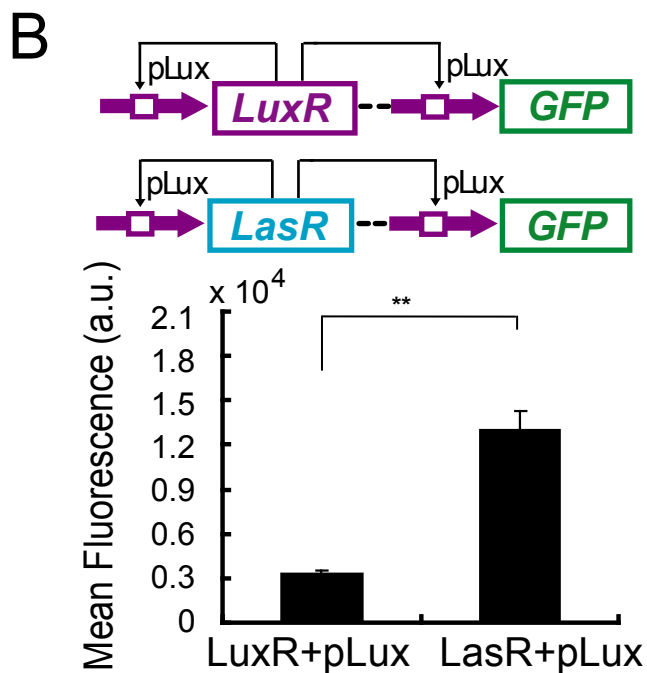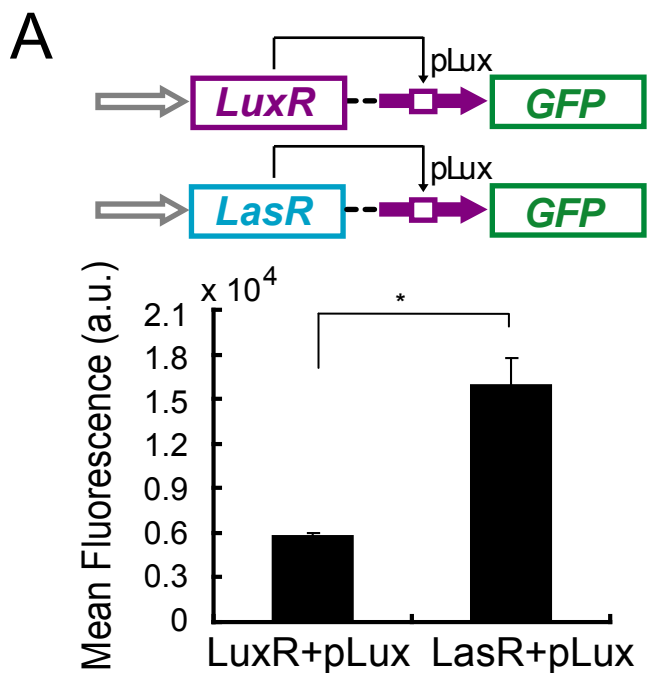
Figure S5

Supplemental Figure and Movie Legends:

Figure S1, related to Figure 1. QS crosstalk dissected using synthetic gene circuits. (A) High concentrations of C6 can crosstalk with LasR-pLas. Top panel: schematic diagram of the synthetic gene circuit (CP-LasR-pLas). Bottom panel: dose response of the circuit when induced with C6 or C12. Compared to original pair of LasR-C12, the pLas promoter can only be activated by LasR with extremely high C6 concentration (signal crosstalk). **(B)** Promoter crosstalk of C6-LuxR with pLas is observed under high concentrations of autoinducer. Top panel: schematic diagram of the circuit (CP-LuxR-pLas). Bottom panel: Dose response of this circuit when induced with C6 or C12. LuxR can bind with C6 to activate pLas starting from 10^{-6} M (promoter crosstalk), while it cannot with C12. **(C)** Characterizing the crosstalk with the pLux promoter using synthase genes. LuxR, with either LuxI or with LasI, can activate pLux, while LasR with LasI can activate pLux. Left: schematic diagram of the synthetic gene circuits constructed to test crosstalk. LasI (cyan) and LuxI (purple) synthesize C12 and C6 molecules in cells, respectively. Right: GFP fluorescence in cells carrying the circuits was measured by flow cytometry at 12 h. LasI with LuxR, and LasI with LasR can significantly activate pLux (signal crosstalk, and promoter crosstalk, respectively). **(D)** Characterizing the crosstalk to the pLas promoter using synthase genes. No significant crosstalk was observed for LuxR- or LasR-pLas combinations. Left: schematic diagram of the synthetic gene circuits constructed. Right: GFP fluorescence in cells carrying the circuits was measured at 12 h. Both LasI-LuxR and LuxI-LuxR cannot activate pLas, and the latter shows ~ two-fold inhibition, and no signal crosstalk is observed for LasR-pLas. All the data are averages of three independent measurements shown as mean ± SD (*p<0.05, and **p<0.01).

Figure S2, related to Figure 2. Hysteresis of the LuxR-pLux positive feedback circuit. (A) C6 induced hysteresis of the LuxR-pLux positive feedback circuit. Flow cytometry measurements of GFP expression for initial OFF cells (left) at 12 h and initial ON cells (right) at 24 h and 37 °C under different concentrations of C6 induction. Initial ON cells were collected from the cells induced with 10^{-4} M C6 for 6 hours and diluted twice into fresh media with the same concentrations of C6 at 12 h and 24 h. The positive feedback circuit displays

hysteresis with a bistable region from 0 to 10^{-8} M C6. No bimodal distribution was observed. (**B**) C12 induced hysteresis of the LuxR-pLux positive feedback circuit. Flow cytometry measurements of GFP expression for initial OFF cells (left) at 12 h and initial ON cells (right, induced with 10^{-4} M C12 for 6 hours before redilution) at 24 h and 37 °C under C12 induction. The initial ON cells were collected and diluted twice into new medium with the same concentrations of C12 at 12 h and 24 h. The positive feedback circuit displays hysteresis with a bistable region from 10^{-8} to 10^{-6} M C6. No bimodal distribution was observed.

Figure S3, related to Figure 3. Hysteresis of the LasR-pLux circuits. (**A**) Schematic representation of the LasR-pLux positive feedback loop induced with C12. (**B**) Flow cytometry measurements of GFP expression for initial OFF cells (left) at 6 h and initial ON cells (right) at 12 h and 37 °C under different concentrations of C12 induction. For initial OFF cells, GFP expression increases with C12 concentration, but begins to decrease uniformly when C12 induction exceeds 10^{-8} M. For initial ON cells (induced with 10^{-4} M C12 before redilution), all the samples exhibit unimodal minimal fluorescence signals that are even lower than the basal GFP expression of initial OFF cells. (**C**) Initial OFF cells were first induced with 10^{-9} or 10^{-8} M at 37 °C for 6 hours to become the new Initial ON cells, which were then collected and rediluted into fresh media with different doses of C12. These two Initial ON groups show a similar GFP expression pattern: unimodal distributions similar to the initial OFF cells for samples in the lower inducer concentrations of 0 to 10^{-9} M, and bimodal distributions within the higher concentration range of 10^{-8} to 10^{-4} M C12. GFP fluorescence was measured by flow cytometry at 12 h. (**D**) C12 induced hysteresis of the CP-LasR-pLux circuit. Flow cytometry measurements of GFP expression in initial OFF cells (left) at 12 h and initial ON cells (right, induced with 10^{-4} M C12 for 6 hours before redilution) at 24 h and 37 °C under C12 induction. Results show that the initial OFF and ON cells show a similar distribution pattern, and both exhibit unimodal expression without hysteresis.

Figure S4, related to Figure 4. (**A**) Growth curves for initial ON, OFF and Mutated cells in 10^{-8} M C12 at 37 °C and 34 °C. The initial ON and OFF cells' growth curves were similar, with a long lag phase in 10^{-8} M C12, while the Mutated cells directly entered exponential

growth phase. All populations reached stationary phase after about 15 hours. The three cell types show similar growth curves at 37 °C and 34 °C, indicating that growth temperature does not significantly influence their growth rate. (**B**) Temperature changes the transposition rate. Top: temporal evolution of the initial ON cells grown in 10^{-8} M C12 at 37 °C. Bottom: time course of the same initial ON cells grown in 10^{-8} M C12 but at 34 °C. Flow cytometry was used to measure the GFP fluorescence at 6 h, 12 h, and 24 h. For each measurement, the percentage of Mutated state cells was calculated. Data shows that higher temperature increases the transposition rate and IS10 transposase insertion, which promotes the transition from the ON state to the Mutated state. (**C**) Quasi-potential U and the transition dynamics between stable steady states in the LasR-pLux positive feedback system (without genetic mutation). The lower 'valley' (with lower potential U) is the stable OFF state and the higher is the stable ON state. According to the stochastic simulation, the energy barrier $\Delta U_{\mathrm{OFF}\rightarrow\mathrm{ON}}$ is much greater than $\Delta U_{\mathrm{ON}\rightarrow\mathrm{OFF},}$ which suggests it is easier for ON state cells to transition to the OFF state. The energy function is calculated according to the probability density distribution of steady state LasR concentrations in each cell.

Figure S5, related to Figures 2 and 4. Model parameter determination. (**A**) Comparison of the basal GFP expression from the pLux promoter between the two linear CP-LuxR-pLux and CP-LasR-pLux circuits. (**B**) Comparison of basal GFP expression from the pLux promoter between the two LuxR-pLux and LasR-pLux positive feedback circuits. All the data shows that the leakage from the pLux promoter in LasR-pLux circuits is greater than in LuxR-pLux circuits. All the data were averages of three independent measurements shown as mean ± SD (*$p<0.05$, and **$p<0.01$). Parameters determination from experimental tests: (**C**) the CP-LuxR-pLux circuit induced with C6; (**D**) the CP-LasR-pLas circuit induced with C12; (**E**) the CP-LuxR-pLux circuit induced with C12; (**F**) the CP-LasR-pLux circuit induced with C12. (**C**) and (**D**) are the original pairs used to test the functionality of all modules, while (**E**) and (**F**) were used to characterize the signal and promoter crosstalk. All of the red data points represent the mean of three independent measurements shown as mean ± SD. The solid black curves, corresponding Hill coefficients (*ni*), and dissociation constants (K_i) between LuxR/LasR and C6/C12 were fitted from the dose response curves by the same fitting method

used in our previous work (Ellis et al., 2009; Wu et al., 2013).

Movie S1, related to Figure 5. A time lapse movie corresponding to Fig. 5A, for about 28 hours at 34 °C.

Table S1, related to Figure 1. **Autoinducer information**
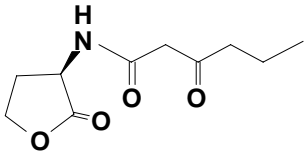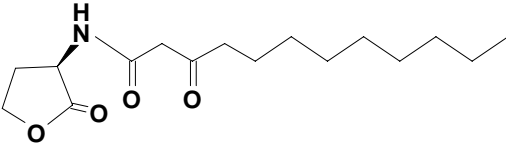
Full name	Molecular abbreviation	Molecular structure	Coding gene	Original binding regulator and promoter	Organism
N-(3-Oxohexanoyl)-L -homoserine lactone	3OC6HSL		LuxI (coding the enzyme, which synthesizes 3OC6HSL)	LuxR, pLux	*Aliivibrio fischeri*
N-(3-Oxododecanoyl)- L-homoserine lactone	3OC12HSL		LasI (coding the enzyme, which synthesizes 3OC12HSL)	LasR, pLas	*Pseudomonas aeruginosa*

Table S2, related to Figures 2 and 4. Parameters for the three positive feedback models

Parameters	Description	LuxR-pLux -3OC6HSL	LuxR-pLux -3OC12HSL	LasR-pLux -3OC12HSL	Source
k1	Transcription rate (min^{-1})	1.8	1.8	1.8	Ref.(Milo et al., 2010)
k2	Translation rate (min^{-1})	1.6	1.6	1.6	Ref.(Milo et al., 2010)
d1	LuxR/LasR degradation rate (min^{-1})	0.01	0.01	0.01	Ref.(Basu et al., 2005; Sayut and Sun, 2010)
d2	mRNA degradation rate (min^{-1})	0.33	0.33	0.33	Ref. (Bakshi et al., 2012; Milo et al., 2010)
c1	Leakage without LuxR or LasR protein (min^{-1})	0.08	0.08	0.08	Approximated according to Ref.(Danino et al., 2010)
c0	Leakage without AHL	0.007	0.007	0.03	Estimated and experiment indicated
Kd	Dissociation constant of LuxR-HSL dimerization	600	180	720	Estimated
Kn	Dissociation constant of $[LuxR-HSL]_2$ binding DNA	2.6	14.7	177	Estimated
Ki	HSL concentration producing half occupation of pLux promoter	1.6e-8	6.6e-7	6.9e-9	Measured by experiments
ni	Hill coefficient	1.3	1.1	6.4	Measured by experiments

Table S3, related to Figure 4. Parameters for the genetic mutation event in the stochastic simulation of LasR-pLux positive feedback system

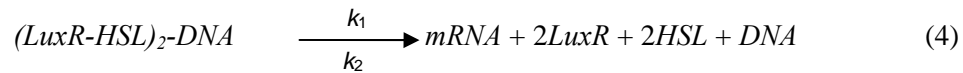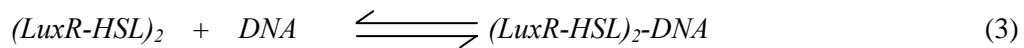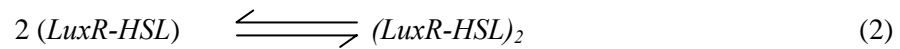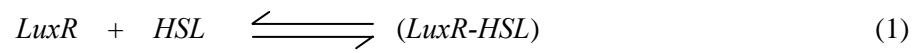| Parameters	Description	Value	Source
n	Cooperativity of IS10 transposase binding to the plasmid of LasR	5	Estimated and experiment indicated
K	Dissociation constant between transposase and the plasmid DNA	400	Estimated and experiment indicated
k3 (37 °C)	Transposition rate at 37 °C (min^{-1})	3.6e-6	Approximated according to experimental results and Ref.(Craig, 2002; Sousa et al., 2013)
k3 (34 °C)	Transposition rate at 34 °C (min^{-1})	4.0e-7	Approximated according to experimental results
c1	Transcription rate after gene mutation (min^{-1})	0.01	Estimated and experiment indicated
k1	Leakage without LasR protein after gene mutation (min^{-1})	0.005	Estimated and experiment indicated

**Table S4, related to Figure 1, 2, 3 and 4. Plasmids used in the circuits'
construction. All materials are from the Registry of standard biological parts**

Biobrick number	Abbreviation in the paper	Description
BBa_R0062	pLux	Promoter activated by LuxR in concert with 3OC6HSL
BBa_R0079	pLas	Promoter activated by LasR in concert with 3OC12HSL
BBa_K176009	CP	Constitutive promoter family member J23107 actual sequence (pCon 0.36)
BBa_B0034	RBS	Ribosome binding site
BBa_B0015	T	Transcriptional terminator (double)
BBa_C0062	LuxR	LuxR repressor/activator
BBa_C0079	LasR	LasR activator
BBa_C0161	LuxI	Autoinducer synthetase for AI from *Aliivibrio fischeri*
BBa_ C0178	LasI	Autoinducer synthetase for PAI from *Pseudomonas aeruginosa*
BBa_E0240	GFP	GFP generator
pSB1A3	pSB1A3	High copy BioBrick assembly plasmid

Supplemental Experimental Procedures:

Deterministic Model Construction

   In the positive feedback loop circuit, LuxR production is controlled by the pLux promoter with only one LuxR-HSL binding site, which is bound and activated by the complex of LuxR and the autoinducer (3OC6HSL or 3OC12HSL, hereafter denoted as C6 and C12, respectively). GFP expression, as a reporter of the system, is regulated by the same pLux promoter and therefore follows the dynamics of LuxR. Therefore, we can directly analyze the LuxR dynamics in the model, comparing the output to the cells' fluorescence, without any loss of explanatory power. Since the LuxR-pLux and LasR-pLux positive feedback systems are characterized similarly and described by the same mathematical equations, we explain only the technical details for the LuxR-pLux positive feedback loop. Our model is based on the following biochemical reactions:

$$LuxR \; + \; HSL \; \rightleftharpoons \; (LuxR\text{-}HSL) \tag{1}$$

$$2\,(LuxR\text{-}HSL) \; \rightleftharpoons \; (LuxR\text{-}HSL)_2 \tag{2}$$

$$(LuxR\text{-}HSL)_2 \; + \; DNA \; \rightleftharpoons \; (LuxR\text{-}HSL)_2\text{-}DNA \tag{3}$$

$$(LuxR\text{-}HSL)_2\text{-}DNA \; \xrightarrow[k_2]{k_1} \; mRNA + 2LuxR + 2HSL + DNA \tag{4}$$

$$mRNA \; \longrightarrow \; LuxR + mRNA \tag{5}$$

$$mRNA \; \xrightarrow{d_1} \; \varnothing \tag{6}$$

$$LuxR \; \xrightarrow{d_2} \; \varnothing \tag{7}$$

where *LuxR* is the monomer form of LuxR protein; *HSL* is the autoinducer 3OC6HSL; *(LuxR-HSL)* is the complex of LuxR bound with *HSL*; *(LuxR-HSL)$_2$* is the dimer of *(LuxR-HSL)*; *(LuxR-HSL)$_2$-DNA* represents *(LuxR-HSL)$_2$* binding to the pLux promoter; *mRNA* is the messenger RNA of the LuxR gene; k_1 and k_2 are the transcription and translation rates, respectively; d_1 and d_2 are the degradation rates of mRNA and LuxR, respectively.

   After C6 concentration reaches a certain threshold, LuxR binds to HSL molecules and forms the active LuxR monomers in the form of (*LuxR-HSL*) (Reaction 1). To quantitatively capture the relationship between the autoinducer concentration and the active LuxR

monomers, a Hill function is employed to represent the fraction of LuxR monomers bound by HSL (*f*):

$$f = [HSL]^{ni}/([HSL]^{ni} + K_i^{ni}) \qquad [Eq1]$$

where *ni* is the binding cooperativity (Hill coefficient) between LuxR and HSL, and K_i represents the dissociation constant between LuxR and HSL (the HSL concentration producing half conversion of LuxR monomers into *LuxR-HSL* complexes). It should be noted that different autoinducers will have different K_i values. Here we assume that the activator LuxR is abundant, and the fraction of active LuxR is independent from LuxR abundance in the cell.

LuxR needs to form a dimer to bind the promoter and activate transcription. We describe the relationship between the dimer and the monomer as the following expression:

$$[LuxR_2] = [LuxR]^2/K_d \qquad [Eq2]$$

where K_d is the dissociation constant for LuxR dimerization. According to reaction (2), two (*LuxR-HSL*) molecules bind together to form a dimer and activate transcription. Additionally, it is necessary to point out that even without autoinducer *LuxR₂* can still bind the pLux promoter and initiate leaky transcription of downstream genes. Taken together, the concentration of the functional LuxR dimer that will bind to pLux and activate its transcription is:

$$C = (c_0 + f^2)*[LuxR]^2/K_d \qquad [Eq3]$$

Here *C* represents the concentration of functional LuxR dimer (*(LuxR-HSL)₂* and *LuxR₂*); c_0 is the fraction of *LuxR₂* that can recognize and bind pLux in the absence of autoinducers; K_d is the dissociation constant for dimerization.

(LuxR-HSL)₂ then recognizes and binds to the pLux promoter to form the *(LuxR-HSL)₂-DNA* complex together with RNA polymerase and other transcription factors to initiate transcription and produce mRNA (Reactions 3 and 4). So the expression of mRNA can be modeled as:

$$S_m = c_1 + k_1 C/(C + K_n) \qquad [Eq4]$$

where S_m represents the production of mRNA; c_1 represents the basal mRNA expression

without LuxR protein; k_1 is the transcription rate; K_n is the dissociation constant between C and pLux promoter.

After transcription, mRNA is translated into LuxR protein (Reaction 5). Here we simplify the whole translation process and capture the production of LuxR protein in the form of:

$$S_p = k_2 * [\text{mRNA}] \qquad \text{[Eq5]}$$

where S_p represents the synthesis of LuxR and k_2 is the translation rate.

Next, we take the constitutive degradation of mRNA in the cell into account (Reaction 6) with the equation:

$$D_m = d_1 * [\text{mRNA}] \qquad \text{[Eq6]}$$

where d_1 is the degradation rate of mRNA.

Similarly, the degradation of LuxR protein (Reaction 7) is:

$$D_p = d_2 * [\text{LuxR}] \qquad \text{[Eq7]}$$

where d_2 is the degradation rate of LuxR.

Finally, we combine the synthesis and degradation (Eq4, 5, 6, and 7) to find the rates of change of the concentrations of mRNA and LuxR:

$$\begin{cases} d[M]/dt = S_m - D_m \\ d[R]/dt = S_p - D_p \end{cases} \qquad \text{[Eq8]}$$

where M and R represents mRNA of LuxR and LuxR monomers, respectively. Combining all the parameters, the two ODE equations can be rewritten as follows:

$$\begin{cases} \dfrac{d[M]}{dt} = c_1 + \dfrac{k_1 C}{C + K_n} - d_1[M]; \\ \dfrac{d[R]}{dt} = k_2[M] - d_2[R]. \end{cases} \quad \text{Where} \quad \begin{aligned} C &= \dfrac{(c_0 + f^2) \cdot [R]^2}{K_d} \\ f &= \dfrac{[\text{HSL}]^{ni}}{[\text{HSL}]^{ni} + K_i^{ni}} \end{aligned} \qquad \text{[Eq9]}$$

These two ordinary differential equations were used to model the three positive feedback loops: LuxR-pLux-C6, LuxR-pLux-C12, and LasR-pLux-C12. Owing to the signal and promoter crosstalk, the dissociation constants K_i, K_d, and K_n may be different, as may also be the case with the Hill coefficients and leaky expression without autoinducer. Setting of parameter values is introduced below.

Stochastic Simulation Coupled with Genetic Mutation

The Gillespie algorithm was employed to perform stochastic simulations of the positive feedback loops(Gillespie, 1977). According to our deterministic model (Eq9), two equations capture the time evolution of the biochemical reactions. In this model, there are four independent events in total – mRNA production, mRNA decay, LuxR production, and LuxR decay – which are translated directly to the stochastic model. Simulation data was collected for 8000 cells, and each simulation was run for 40000 steps.

The energy-like function $U(x)$, which denotes the probability and direction of transitions between attractors in a noisy environment, can also be used to interpret state transitions(Zhou et al., 2012). After finishing all simulations, we first calculated the amount of LasR present in each cell (assuming the cells had reached steady state), then divided by the total number of cells. This yielded a probability density distribution of steady state LasR concentrations, which was used to calculate the energy function $U(LasR)$ by the following approach(Zhou et al., 2012):

$$U(LasR, t) \quad \sim \quad -\ln(P(LasR), t) \hspace{2cm} [Eq10]$$

where $P(LasR, t)$ is the steady-state probability for each LasR concentration at a given time t. In practice, the $P(LasR, t)$ was derived from the following equation:

$$P(LasR) = \text{hist}(LasR)/Cellnum \hspace{2cm} [Eq11]$$

where hist($LasR$) is a histogram of the amount of LasR in each cell and *Cellnum* is the total number of simulated cells. The energy-like function U gave us a more vivid and direct understanding of the quasi-potential landscape and the transition dynamics between stable steady states in this positive feedback system. The transition rates between ON and OFF states are decided by the energy barrier ΔU (Figure S4C). Unlike the typical bimodality emerged from bistable systems, C12-LasR-pLux positive feedback loop displayed an asymmetric bimodal distribution at a population level, which only happened from ON state to OFF state. The model suggests that this asymmetry comes from the different energy barrier of switching between ON and OFF states (Figure S4C).

To take the genetic mutation in the LasR-pLux positive feedback circuit into account, we

added another event in addition to mRNA and LasR production and degradation. Since the genetic mutation only happened in initial ON cells, and because it is easier for cells in high C12 concentration to mutate, we inferred that more LasR in the cell resulted in a higher mutation probability. Moreover, the mutation occurred in the LasR open reading frame, so theoretically the mutation probability is positive as long as the LasR gene is present. Here, we used a Hill function to describe the probability of mutation:

$$Pm = [\text{LasR}]^n/(K^n + [\text{LasR}]^n) \qquad\qquad [\text{Eq11}]$$

where Pm represents the probability of mutation; n is the Hill coefficient indicating the cooperativity of mutation causing factors related to LasR concentration; and K represents the dissociation constant in the complicated biochemical reactions. In the Gillespie simulation, the mutation event, independent of the other four events, was described mathematically as:

$$Mu = k_3 * Pm * [\text{LasR}] \qquad\qquad [\text{Eq12}]$$

where k_3 is the transposition rate; [LasR] is the amount of LasR in the cell at a given time, and Pm is the probability of mutation as described above. Generally, once the mutation has happened, the LasR gene is broken into two parts and the functional mRNA of LasR cannot be produced any more. Mutated cells theoretically retain the ability to switch state. However, the probability of this occurring is small. In practice, for each cell, when the mutation event had occurred, the transcription rate (k_1) and leaky expression from pLux (c_1) were reduced to very low values, the cell would remain mutated, and the simulation was ended. By tuning the transposition rate, we fit the parameters according to experimental data, which we then used to make predictions.

Next, since the ON, OFF, and Mutation cells have different growth curves under the same experimental conditions, growth rate differences between the three populations were added into the model. From the growth curves, it can be seen that the initial ON and OFF cells' growth curves were similar, with a long lag phase in 1e-8 M C12, while the Mutation cells directly entered exponential growth. All three populations went to stationary phase after about 15 hours (Figure S4A). Instead of using a population balance model, we employed a simple and efficient method to combine the stochastic model with population dynamics. The cells

with greater growth rate would acquire an extra advantage in their final quantity: each of the three original populations was multiplied by its relative growth rate and then its ratio in the three populations was adjusted.

To simplify the case, we chose three time points (2.5 h, 7.1 h and 12.5 h) and compared their O.D. values (by $OD^{Mutation}/OD^{ON}$, OD^{OFF}/OD^{ON}, and OD^{ON}/OD^{ON}: ON cells grew slowest) and then made an average to get an averaged relative growth rate, which then was taken into the simulation results. So the final amount of Mutation cells (*Fmu*), OFF cells (*Foff*) and ON cells (*Fon*) are:

$$Fmu = Smu * (OD^{Mutation}/OD^{ON});$$

$$Foff = Soff * (OD^{OFF}/OD^{ON});$$

$$Fon = Son * 1;$$

where *Smu*, *Soff*, and *Son* are the primary number of cells which finished the simulation in the Mutation, OFF, and ON states, respectively. Therefore, the proportions of Mutation cells (*Pmu*), OFF cells (*Poff*), and ON cells (*Pon*) are:

$$Pmu = Fmu/(Fmu + Foff + Fon);$$

$$Poff = Foff/(Fmu + Foff + Fon);$$

$$Pon = Fon/(Fmu + Foff + Fon);$$

In this way, the population with a greater growth rate acquired an advantage in its quantity under identical conditions.

Determinations of parameter values

In the *E.coli* cells, even though the transformed plasmid is high-copy, there is also a maximum expression value. According to the B10NUMB3R5 database(Milo et al., 2010), each protein generally has no more than 1000 copies. Therefore, we chose 1000 molecules per cell to be the maximum expression value of LuxR and LasR. All other parameters were adjusted under this assumption.

Specifically, the transcription rate (k_1), translation rate (k_2), and degradation rates of mRNA and LuxR (d_1 and d_2, respectively) were estimated from previous reports and the B10NUMB3R5 database (Table S2). Since pLux was the only promoter used in the positive feedback circuits, the leaky expression without LuxR or LasR (c_1) did not change between

simulations, and it was estimated to be 0.08 min^{-1}. In addition, according to experimental results, basal GFP expression in the absence of autoinducers (c_0) in the LasR-pLux positive feedback circuit is about three times larger than in its LuxR-pLux counterpart (Figure S5A and S5B). Therefore c_0 was set to 0.03 and 0.007 for LasR-pLux and LuxR-pLux, respectively. The Hill coefficients (ni) and dissociation constants (K_i) between LuxR/LasR and the C6/C12 were fitted from the dose response curves (Figure S5C-S5F) by the same fitting method used in our previous work(Ellis et al., 2009; Wu et al., 2013). Considering experimental variations, parameters were adjusted within 10% relative error.

The generic parameters K_d and K_n are constant and fit to make the model consistent with with experimental results (Figure 2B and 2C). With these fitted parameters, our model captured the experimental hysteresis results and provided insights to understand the difference between the three positive feedback loop variants induced by QS crosstalk. For example, K_d in LuxR-pLux-C12 positive feedback was smaller than in LuxR-pLux-C6, while K_n was larger for LuxR-pLux-C12. This suggests that C12 might bind more easily to LuxR (relative to C6), but the original LuxR-C6 pair has higher affinity for the pLux promoter. Additionally, K_n in the LasR-pLux positive feedback loop is much bigger for LasR-C12 than for either LuxR-C6 or LuxR-C12, which indicates that the LasR-C12 dimer has less affinity for pLux, and therefore it is more difficult for the system to reach saturation. The parameter combination for the LasR-pLux positive feedback loop was used in the stochastic simulation and for predicting trimodality.

To fit the probability of the LasR gene's mutation against experimental results at 37°C, we first approximated the Hill coefficient (n) and the dissociation constant (K) based on the difference between fluorescence values at the ON and OFF states. Different n and K combinations were generated, and it was discovered that $n = 5$ and $K = 400$ best fit the experimental data (Figure 4A and 4B). In addition, previous reports indicated that transposition rates of IS elements in *E.coli* usually range from 1e-3 to 1e-7 min^{-1} (Craig, 2002; Sousa et al., 2013). So the transposition rate in our model was estimated ($k_3 = 3.6e-6$ min^{-1}) according to the final experimental data (Figure 4B). To predict the trimodal response, k_3 was adjusted but all the other parameters were held constant. With $k_3 = 4.0e-7$ min^{-1}, the

simulation exhibited trimodality, which was validated by the experimental results at 34 °C (Figure 4D and 4E).

All the parameter values are listed in Table S2 and S3.

Mutation verification by DNA sequencing

For the initial ON cells growing in 10^{-10} to 10^{-4} M C12, plasmids were extracted, digested for genotyping, and sequenced. Several primers were used to check for mutation. Following the order of assembly shown in Supplementary Fig. 16b, these primers were: BB-N-Forward, LasR-C-Forward, GFP-N-Reverse, BB-C-Reverse, and GFP-C-Forward. Descriptions and sequencing results for each primer are below. Combining these results, we concluded that the mutation happened within the LasR gene, and the other fragments and backbone were correct.

For convenience, all fragments are highlighted: pLux promoter: <mark style="background:yellow">yellow</mark>; ribosomal binding site: <mark style="background:blue">blue</mark>; LasR: <mark style="background:cyan">cyan</mark>; IS10 transposase: <mark style="background:magenta">pink</mark>; Terminator: <mark style="background:red">red</mark>; GFP generator: <mark style="background:lime">green</mark>; pSB1A3 vector: <mark style="background:grey">grey</mark>.

BB-N-Forward

Sequence: TGCCACCTGACGTCTAAGAA

Description: Forward, starting from the N terminal of the multiple cloning site (MCS)

Sequencing with BB-N-Forward on the vector verified the promoter pLux (yellow), the ribosomal binding site (blue), part of LasR (cyan, 681 bp), and a new inserted sequence (pink). This new sequence was determined to be part of an IS10 transposase gene according to BLASTn results from NCBI. The transposon target site is also marked (Bold black, highlighted pink). Sequencing results are as follows:

>LasR-pLux-PF-BBF   1360   ABI

1→

CACGGAACTTAACCTATACAAATAGGCGTATCACGAGGCAGAATTTCAGA

TAAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCC

GCTTCTAGAG<mark style="background:yellow">ACCTGTAGGATCGTACAGGTTTACGCAAGAAAATGGTTTG</mark>

<mark style="background:yellow">TTATAGTCGAATAAA</mark>TACTAGAG<mark style="background:blue">AAAGAGGAGAAA</mark>TACTAG<mark style="background:cyan">ATGGCCTTG</mark>

<mark style="background:cyan">GTTGACGGTTTTCTTGAGCTGGAACGCTCAAGTGGAAAATTGGAGTGGAG</mark>

<mark style="background:cyan">CGCCATCCTCCAGAAGATGGCGAGCGACCTTGGATTCTCGAAGATCCTGT</mark>

<mark style="background:cyan">TCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTC</mark>

<mark style="background:cyan">GGCAACTACCCGGCCGCCTGGCGCGAGCATTACGACCGGGCTGGCTACGC</mark>

<mark style="background:cyan">GCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAGCGTACTGCCGATTT</mark>

<mark style="background:cyan">TCTGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAG</mark>

<mark style="background:cyan">GAAGCCTCGGCCGCCGGCCTGGTGTATGGGCTGACCATGCCGCTGCATGG</mark>

<mark style="background:cyan">TGCTCGCGGCGAACTCGGCGCGCTGAGCCTCAGCGTGGAAGCGGAAAACC</mark>

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GGGCCGAGGCCAACCGTTTCATAGAGTCGGTCCTGCCGACCCTGTGGATG
CTCAAGGACTACGCACTGCAAAGCGGTGCCGGACTGGCCTTCGAACATCC
GGTCAGCAAACCGGTGGTTCTGACCAGCCGGGAGAAGGAAGTGTTGCAGT
GGTGCGCCATCGGCAAGACCAGTTGGGAGATATCGGTTATCNTGCACTGC
TCGGAAGCCAATGTGAACTTCCATATGGGAAATATTCGGCGGAAGTTCGG
TGTGACCTCCCGCCGCGTAGCGCTGAGAGATCCCCTCATAATTCCCCCAA
GCGTAACCATGTGTGAATAAATTTTGAGCTAATAGGGTTGCAGCCACGAG
TAAGTCTTCCCTTTGTATTGTGTAACCAGAATGCCGCAAAACTTCCATGC
CTAAGCGAACTGTTGAAAGTACGTTTCGATTTCTGACTGTGTTAACCTGA
AAGTGCTTGGTCCCACCTTGTTTCTGAACATGAACGCCCCGCAAGCCAAC
ATGTTAGTTTGAAACTTCAGGGGGAATTACCAACAGGAAATCATAAACGC
TCTGAACCTTGCTCGTTTGGGTTTGGGGGAAGGGCCTAATTTCCGGAGGG
CAGGAACTTTTTTCAGGTTTCGGGAAAGGGGGTTTTTTTTCAATTCTTTC
ATTTTTCCCTTCTTCAAAAAAAAAATATTATAAAAAAAAAGTTTTGGTG
TGGGGGGGGGGTTTGTTTAAAATATTTTTTCTAACCAACGCGGGGAAAGA
AAATATTTTT
```

Note:
Highlight (yellow, 111-165): R0062, pLux promoter;
Highlight (blue, 174-185): B0034, Ribosome binding site;
Highlight (cyan, 192-872): C0079, LasR (part);
Highlight (pink, 873-1211): IS10 transposase (part);
CGCGTAGCG: target site for transposition.

LasR-C-Forward
Sequence: TGGGTCTTATTACTCTCTAA
Description: Forward, starting from the C terminal of LasR

   Sequencing with LasR-C-Forward showed that the sequence remained as expected – terminator (red), pLux promoter (yellow), and GFP generator (green) from left to right – which shows the absence of mutation. Sequencing results are as follows:

> LasR-pLux-PF-LasRC-F   1345   ABI
1→
```
CGGGGGCTCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGT
TTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTC
ACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGACCTGTAGGATCG
TACAGGTTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATACTAGAG
TCACACAGGAAAGTACTAGATGCGTAAAGGAGAAGAACTTTTCACTGGAG
TTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTT
TCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCT
TAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTG
TCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGATACCCAGATCAT
```

```
ATGAAACAGCATGACTTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACA
GGAAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTG
AAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGT
ATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAA
CTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAA
TCAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAA
CTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCT
TTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATC
CCAACGAANAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCT
GGGATTACACATGGCATGGATGAACTATACAAATAATAATACTAGAGCCA
GGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTT
ATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACC
TTCGGGTGGGCCTTTTCTGCGTTTATATACTATTAGCGGGCGCTGCAGGC
TTCCCTCGCTCACTGACTCCCCTGCGCCTCGGTCGTTCGGGCTGGCGGGA
AGCGGGTATCAGCTTCACTCCAAAGGGGGGGTAATACGGTTTTTTCCCCCC
AAAAATCCGGGGGGATAAACACCCGGGAAAAAAAAAACTTGGTGGAAACC
AAAAAGGGGCCCCCACAAAAAAGGGGCCCCGGGAAAACCCGGTAAAAAAA
AAGGGGCCCCCCGGCGGTGTTGTTCTGGTGGTGGGGGTTTTTTTT
```

Note:
Highlight (red, 8-129): B0015, Terminator;
Highlight (yellow, 138-192): R0062, pLux promoter;
Highlight (green, 193-1077): E0240, GFP generator;

GFP-N-Reverse
Sequence: GTGCCCATTAACATCACCATC
Description: Reverse, starting at 55th bp from the N terminal of GFP

   Sequencing with GFP-N-Reverse showed that the sequence was not the same as expected. From left to right, these results showed: part of GFP (green), pLux promoter (yellow), terminator (red), C terminal of LasR (cyan), and a new inserted sequence (pink). This new sequence was demonstrated to be part of an IS10 transposase gene according to BLASTn results from NCBI. The transposon target site is also marked (Bold black, highlighted pink). Sequencing results are as follows:

>LasR-pLux-PF-GFPN-R   1403   ABI
1→

```
GGGGGGGGGGGTTGGAAAAGTTGCTTCTCCTTTACGCATCTAGTACTTTCC
TGTGTGACTCTAGTATTTTATTCCCTTTTTAGCAAACCATTTTCTTGCGTAA
ACCTGTACGATCCTACAGGTCTCTAGTATATAAACGCAGAAAGGCCCACC
CGAAGGTGAGCCAGTGTGACTCTAGTAGAGAGCGTTCACCGACAAACAAC
AGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGA
TGCCTGGCTCTAGTATTATTAGAGAGTAATAAGACCCAAATTAACGGCCA
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TAATGGCCGCTACGCGCTGATGAATCCCCTCATGATTTCGGCAAAAATCA
TTAATGTGAGGTGGATACTTGTCTTGCCAGATGATCAAATGGTTTCGCGT
AAACTCTTGAATCAGACCACATGATGTGCGATCTCGATATTTTACATCAC
TCTCTTTAAGAATTCTGCCCTGAATTACAGTTAGAACGACTCAACAGCTG
AACGTTGCGCTTGTCACGCCTTACTTGAGTGTAACACTCTCACTCTTACC
GAAATTGGTCGTAACCTGACAACCTAAGTGAGATCAAAACATAACATCAA
ACGACTCGACGGATTGGTATGTAATCGTCACCTCCGCAAAGAGCGACTCG
CTGTATACCGTTGGCCTGCTATCTATATCTGTTCGGGCAATACGATGCCC
ATTGTAGTTGGTGACTGGTCTGATATTCGAGAGCCTAAACAACTTATCGT
GGTTGCGAGCTTCAGTCGCACTACCTGGTCGTTCTGATACTCTTTATGAG
AAAGCGTTCCCATCTTCCGATCTTATGCTCAAGAAAGCTCATGATCATTT
TCTAGCCGATCTTGTCGATAGGTCATCGGAGAACTCCACTCTCGCTCATT
GTCAGGGATGTGGGCTTTTAAGGGACGTGGTATAATCCCTTGGAAAATTT
GGGTAGGTACTGGGTTACTCGAAGAGGAAGGAAAATTTCATATGCGGGAC
CTTAGGAAGGGAAAACCGGGAAACCATTACAGCACTTAACTGTTTTTTGT
TCTCTATACCCTCCAAAAATTTTGGTGTTTAAAAGTGTTTAATTAAAAAA
AATTCCAATTTCTAGTGCAAATGTCGTTTTAAAAAATTCTTTTGTTCTAA
GGGCCTGAAGAAATTTGACCTTTCTGCAGGGGAAACCTTTTGTCTCACCG
CCCAGCAATTTAAAAACTTCTTTCTGCACGGGTGGGGCAGGGGGAGACCC
GGGAGGTCCCTTTTTCCACCTAACTTTTTCGCCTGTTGTTAAAAAATTAC
AAAACACACCCCTACCAAATCTCTTTTTTTATAAAATTTACCTCCTTCTTA
GGAATAGCCCGAGAGTAGGCCAAAAAATATATTAAAAAAAAATTAAACAC
TCT
```

Note:

Highlight (green, 14-57): E0240, GFP generator (N terminal);

Highlight (yellow, 66-120): R0062, pLux promoter;

Highlight (red, 129-257): B0015, Terminator;

Highlight (cyan, 266-307): C0079, LasR (C terminal);

CGCTACGCG: target site for transposition (duplication).

Highlight (pink, 317-1038): IS10 transposase (part);

BB-C-Reverse

Sequence: AATACCGCCTTTGAGTGAGC

Description: Reverse, starting from the C terminal of MCS on the plasmid

Sequencing with BB-C-Reverse verified the GFP generator (green) and pLux promoter (yellow), which indicate a lack of mutation. Sequencing results are as follows:

> LasR-pLux-PF-BBR   1382   ABI

1→

GGGCGCCATCGTACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCCTG

CAGCGGCCGCTACTAGTA==TATAAACGCAGAAAGGCCCACCCGAAGGTGAG==
==CCAGTGTGACTCTAGTAGAGAGCGTTCACCGACAAACAACAGATAAAACG==
==AAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCTC==
==TAGTATTATTATTTGTATAGTTCATCCATGCCATGTGTAATCCCAGCAGC==
==TGTTACAAACTCAAGAAGGACCATGTGGTCTCTCTTTTCGTTGGGATCTT==
==TCGAAAGGGCAGATTGTGTGGACAGGTAATGGTTGTCTGGTAAAAGGACA==
==GGGCCATCGCCAATTGGAGTATTTTGTTGATAATGGTCTGCTAGTTGAAC==
==GCTTCCATCTTCAATGTTGTGTCTAATTTTGAAGTTAACTTTGATTCCAT==
==TCTTTTGTTTGTCTGCCATGATGTATACATTGTGTGAGTTATAGTTGTAT==
==TCCAATTTGTGTCCAAGAATGTTTCCATCTTCTTTAAAATCAATACCTTT==
==TAACTCGATTCTATTAACAAGGGTATCACCTTCAAACTTGACTTCAGCAC==
==GTGTCTTGTAGTTCCCGTCATCTTTGAAAAATATAGTTCTTTCCTGTACA==
==TAACCTTCGGGCATGGCACTCTTGAAAAAGTCATGCTGTTTCATATGATC==
==TGGGTATCTCGCAAAGCATTGAACACCATAACCGAAAGTAGTGACAAGTG==
==TTGGCCATGGAACAGGTAGTTTTCCAGTAGTGCAAATAAATTTAAGGGTA==
==AGTTTTCCGTATGTTGCATCACCTTCACCCTCTCCACTGACAGAAAATTT==
==GTGCCCATTAACATCACCATCTAATTCAACAAGAATTGGGACAACTCCAG==
==TGAAAAGTTCTTCTCCTTTACGCATCTAGTACTTTCCTGTGTGA==CTCTAG
TA==TTTATTCGACTATAACAAACCATTTTCTTGCGTAAACCTGTACGATCC==
==TACAGGT==CTCTAGTATATAAACGCAAAAAGGGCCACCCCGAAGGGTGAGC
CAGTGTGACTCTAATAGAGAGCGGTCACCGACAAACAACAGAAAAAAACG
AAAGGGCCCAGTCTTTCGAACGGAACCTTTTCGTTTTAATTTGAATGCCT
GGGTTCTTAATATTTATTTAAAAAAGAAAAAAGAAACCAAAATTTTACGG
GCCCATAAGGGGGCCGCCCTCCCCCCGCGGAAGAAAACCCCCCCCAAGAG
AAATTTTGGGTAAAAAAAAAACATTTTAAAGTTTAAAGGGGGGGGGAAAA
CACACCCCTCCTTGTTGTTCTCAAATAAGATATATAAAAAAGGGGGGGTT
TTTTTCGCCCGCAAAAAAAAAAATAAAAAAAAA

Note:
Highlight (green, 69-944): E0240, GFP generator;
Highlight (yellow, 953-1007): R0062, pLux promoter;

GFP-C-Forward
Sequence: GGCATGGATGAACTATACAAATAA
Description: Forward, starting from the C terminal of GFP

   Sequencing with GFP-C-Forward showed that the sequence was the same as expected – terminator (red), and pSB1A3 backbone (light grey) – which indicated a lack of mutation. Sequencing results are as follows:

> LasR-pLux-PF-GFPC-F   1334   ABI
1→
AGGAG==CCAGGGCATCAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCT==

TTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACT
GGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGC
TGCAGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGG
CGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC
AGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCA
GGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCACAGGCTCCGCCCC
CCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCC
GACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGC
GCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTC
CCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAG
TTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCG
TTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAAC
CCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGAT
TAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGC
CTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACA
AACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGC
GCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCT
GACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATT
ATCAAAAAGGATCTTCACCTAGATCCTTTTAAATAAAAAATGAAGTTTTA
AATCAATCTAAAGTATATATGAAGTAAACTTGGTCTGACAGTTACCAATG
GCTTAATCAGTGAGGCACCTTATCTCAACGGATCTGTCCTATTTCGTTCA
TCCCATAATTGGCCTGAACTCCCCCGTCCGTGGAAAAAAAACTTACAAAA
CCGGGGGGGGGGCTTTACCCATTCGGGGGCCCCCCAGGGGGGGGTGCCAAG
GGAGAAACCCCGGCGAAAAAACCCCCCCGCCCCCCCCCCCGGGGGGCCTC
CCCAAAAAAAATTTTTATTATTTAACACCCAAAA

Note:
Highlight (red, 6-134): B0015, Terminator;
Highlight (light grey, 151-1182): pSB1A3, vector backbone (part).

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