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

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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/RhlII 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)
to activate pLas (Table S1) (Fuqua et al., 1996; Meighen, 1994; Miller and Bassler, 2001; Ng and Bassler, 2009; Schuster et al., 2004; Stevens and Greenberg, 1997). However, the LuxR protein can also bind other HSLs, such as C7HSL and 3OC8HSL (Canton et al., 2008). When binding C12, LasR is able to activate pLux in addition to the naturally paired pLas promoter (Balagadde et al., 2008). Implications of such crosstalk on gene regulation and cell response remain largely unknown.

Here, we use rationally designed gene networks to probe crosstalk between the LuxR/I and LasR/I systems and investigate their elicited bistable behaviors from positive feedback topologies. By using a synthetic biology approach, all combinations of autoinducer, regulator, and promoter were tested to show that QS crosstalk can be dissected into signal crosstalk and promoter crosstalk. When studied in the context of a synthetic positive feedback gene network, our results indicate that QS crosstalk leads to distinct dynamic behaviors: signal crosstalk significantly decreases the circuit’s induction range for bistability, but promoter crosstalk causes transposon insertions into the regulator gene and yields trimodal responses due to a combination of mutagenesis and noise induced state transitions. To fully understand this complex response, we developed and experimentally verified a mathematical model that takes into account all of these factors to simulate and predict how varying the transposition rate can modulate this trimodality. This reveals a novel factor of host-circuit interactions in shaping complex responses of synthetic gene networks.

RESULTS

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

To characterize possible crosstalk between LuxR/I and LasR/I signaling systems, four synthetic circuits, CP (constitutive promoter)-LuxR-pLux (Figure 1A), CP-LasR-pLux (Figure 1B), CP-LasR-pLas (Figure S1A), and CP-LuxR-pLas (Figure S1B), were first built to test all autoinducer-regulator-promoter combinations’ impact on gene expression activation. C6 and C12 were applied independently to all constructs, and green fluorescent protein (GFP) expression under the regulation of pLux or pLas was measured as the readout.

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

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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
克服(Acar等，2005; Gardner等，2000)。

接下来，C12被用于诱导相同的元件来研究信号交叉影响对基因网络调节的冲击。类似的诱导实验被执行并且结果如图2C所示。可以被看作，这个电路也显示了迟滞性，但是与一个较小的双态区间(10^8到10^6 M C12)。流式细胞术的结果也显示没有双峰分布(Figure 2D，绿色和浅绿色; Figure S2B)。

为了量化由信号交叉影响导致的双态区间的缩小，构造了LuxR-pLux自激活的普通微分方程(ODE)模型。两个主要的事件，LuxR转录和翻译，被两个ODEs描述，所有化学物种的绑定被整合到模型中。通过使用现有文献和实验数据拟合参数，模型可以准确地捕获实验结果(图2B和2C)。对模型参数的观察揭示了由信号交叉影响导致的双态区间减小可以被归因于LuxR和C6和C12的差绑定亲和力。这为通过交叉感应的自动诱导剂来影响QS决策提供了一种新方式，这对于临床疗法是有用的。

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

通过建立一个正反馈回路，LasR被pLux的调节(图3A)。如图1B所示，LasR被C12诱导时可以激活pLux。因此，这个电路在存在C12时形成了一个正反馈回路。GFP被pLux的调节下再次被作为读数。实验对迟滞的探索进行并且结果显示在图3B。可以被观察到，初始的OFF细胞(蓝色)对C12诱导表现出非单调响应：GFP表达随C12浓度增加，但开始均匀降低当C12诱导超过10^8 M (Figure 3B，以及Figure S3A和3B)。用10^4 M C12诱导的细胞被收集并稀释到新鲜介质中，其诱导浓度与初始OFF细胞相同。流式细胞术数据表明所有样品都显示出单峰最小荧光信号，比基线GFP信号还低。
expression of initial OFF cells (Figure 3B and 3C green, and Figure S3B).

Considering that both C12 and exogenous gene overexpression may be toxic to cells, as well as the fact that initial OFF cells can be turned on with lower induction dosages, cells induced with lower than $10^{-4}$ M but higher than $10^{-10}$ M C12 were collected as new initial ON cells to further explore possible hysteresis of this circuit. Collected cells were diluted into fresh medium with the same concentrations of C12. These new initial ON cells demonstrate the same expression pattern as the initial OFF cells when grown in inducer concentrations from 0 to $10^{-9}$ M, but they show much lower fluorescence values at higher concentrations. For example, the red points in Figure 3B illustrate the GFP average of $10^{-9}$ M induced initial ON cells when collected and re-diluted into a range of C12 concentrations (See Figure S3C for results with other initial induction dosages). Examination of the flow cytometry measurements of these ON cells reveals that bimodal distributions emerge within the concentration range of $10^{-8}$ M to $10^{-4}$ M C12. Interestingly, one peak of the distribution is at the high state and the other is at the minimal expression state, even lower than basal expression (Figure 3C, red). So unlike classic bimodal responses due to bistability, LasR-pLux positive feedback exhibits bimodality with the lower peak’s expression even weaker than the OFF state. To exclude the possibility that this bimodality is triggered by inherent properties of the LasR-C12 complex, similar hysteresis experiments were carried out for the linear CP-LasR-pLux circuit (Figure 1B). Results show that the initial OFF and ON cells both exhibit unimodal expression without hysteresis (Figure S3D). The bimodality is, therefore, unique to the initial ON cells with LasR-pLux positive feedback.

Bimodality results from circuit-host interactions.

The remaining question is: what is the cause of the minimal expression state? To resolve this problem, new initial ON samples at concentrations of $10^{-11}$ M to $10^{-4}$ M C12 (Figure 3B, red triangles) were collected. Their plasmids were extracted and digested for genotyping. The agarose gel electrophoresis results show that a new band (~3.2 kb) replaces the original fragment band (wild type, ~1.9 kb) for samples in $10^{-8}$ to $10^{-4}$ M C12, and that a faint original-fragment band can also be seen for samples with $10^{-8}$ and $10^{-7}$ M C12 inductions (Figure 3D). Further sequencing analyses verify that an IS10 transposase is inserted into the
LasR gene at the 682 bp site and this insertion is flanked by two 9 bp direct repeats
5’-CGCGTAGCG-3’ (Figure 3D and Supplementary Information), which is consistent with
reported hotspots for IS10 insertion (Kovarík et al., 2001).

The insertion abolishes LasR’s ability to activate downstream GFP expression, which in
turn causes the cells’ fluorescence signal to be even weaker than basal expression when LasR
is intact. Cells with this type of mutation form the low GFP peak in the bimodal distributions
in Figure 3C. On the other hand, cells that do not mutate are able to maintain a high GFP
expression due to positive feedback, forming the GFP ON peak of the bimodal distributions.

Taken together, the combination of gene network activated GFP expression and mutation
caused GFP inhibition drive the emergence of a bimodal distribution.

Trimodality predicted by expanded model.

In light of the verified mutation in the LasR-pLux positive feedback system, the mathematical
model was expanded to take into account crosstalk triggered genetic changes to better
describe the circuit. To enable comparison with flow cytometry results, the ODEs were
transformed into corresponding biochemical reactions and simulated stochastically (Gillespie,
1977). In addition, each cell was assigned a probability of mutation throughout the simulation
(Figure 4C inset), which is dependent on the cell’s current LasR/GFP level and the
transposition rate. Once mutated, the cells had only minimal GFP expression strength and
remained mutated until the end of the simulation. Finally, growth rate differences between
wild type and mutated cells were computed from experiments (Figure S4A) and taken into
consideration in the simulation. Results of stochastic simulations of this expanded model are
shown in Figure 4A, exhibiting the bimodal distribution observed experimentally (red curves
in Figure 4A, simulation; and 4B, experiment).

To further investigate the impact of this mutation on the circuit’s functions, simulations
were carried out with perturbed parameters to mimic various scenarios. First, the transposition
rate was artificially set to zero, and the simulations show that the system can also exhibit a
bimodal distribution (Figure 4A, blue), with the OFF peak exhibiting basal GFP expression.

Bimodality has been reported to arise from stochastic state switching of a bistable system
without any genetic changes (Acar et al., 2005; Gardner et al., 2000; Tan et al., 2009). The
same mechanism leads to simulated bimodality of this LasR-pLux circuit when there is no mutation. While it is almost impossible to eliminate mutation, it is possible to decrease the transposition rate experimentally. To explore the impacts of mutation in a more realistic scenario, simulations were carried out with positive but smaller transposition rates. Interestingly, the system demonstrates a trimodal distribution (Figure 4A, green). In this distribution, there are three groups of cells: ON, OFF, and Mutated. Those cells initialized at the ON state freely transition to and from the OFF state, due to the system’s bistability. Meanwhile, all cells have the chance to mutate and stay mutated (Figure 4C). Given enough time and the right measurement window, all three groups of cells would be visible. Within this window, the portion of ON and OFF cells will gradually decrease and the number of mutated cells will increase because the mutation is irreversible. The effect of a decreased transposition rate is essentially slowing down the ON to Mutation transition rate and giving enough time for ON to OFF transitions and hence the emergence of the OFF peak. Time courses of the simulations demonstrate gradual emergence and evolution of these three populations of cells (Figure 4D).

Experimental validation of trimodal responses by lowering growth temperature. Previous reports indicated that transposition frequency can be perturbed by growth temperatures (McClintock, 1984; Ohtsubo et al., 2005; Sousa et al., 2013). To tune the transposition rate, experiments were carried out with cells cultured at a lower 34 °C temperature, which was shown to slow down crosstalk triggered mutation of this circuit (Figure S4B). Consistent with model predictions, initial ON cells induced with 10⁻⁸ M C12 exhibited a trimodal response when the growth temperature was tuned from 37 °C to 34 °C (Figure 4B, green). Moreover, temporal evolution of the proportion of each subpopulation was consistent with model predictions: the portion of ON cells gradually decreased, the Mutation portion increased, and the OFF portion increased first and then decreased as time went on (Figure 4E). Growth rates of cells at Mutated, ON, or OFF states were also measured and show no difference when cultured at these two different temperatures (Figure S4A). The 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 8^{th} 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 8^{th} 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
LuxR/LuxI and LasR/LasI, have been used in synthetic biology for a wide range of applications (Balagadde et al., 2008; Basu et al., 2004, 2005; Brenner et al., 2007; Chen et al., 2014; Chuang et al., 2009; Danino et al., 2010; Hong et al., 2012; Kobayashi et al., 2004; Payne et al., 2013; Prindle et al., 2012; Tabor et al., 2009; Tamsir et al., 2011; Pai et al., 2012).

However, evolutionary pressures from limited resources in a competitive environment promote promiscuous bacterial communication, which takes the form of either different genera of bacteria producing the same types of autoinducers or non-specific regulator-autoinducer binding (Balagadde et al., 2008; Gray et al., 1994; Hong et al., 2012; Miller and Bassler, 2001; Pérez et al., 2011; Winzer et al., 2000). As a result, QS regulator-autoinducer pairs are not orthogonal, and there is crosstalk between them.

Dissecting the crosstalk is critical for unraveling the underlying principles of bacterial decision-making and survival strategies for both natural and synthetic systems.

In this work, we used synthetic biology approaches to dissect QS crosstalk between LuxR/I and LasR/I. By applying engineering principles to construct modular gene networks, we were able to characterize and categorize QS crosstalk into signal crosstalk, where LuxR binds with the non-naturally paired C12 to activate pLux, and promoter crosstalk, where LasR binds with C12 to activate non-naturally paired pLux. However, regulator crosstalk, in which the naturally paired autoinducer and promoter function through a cross-talking regulator protein, was not detected in this work.

When signal crosstalk is constructed and tested in the context of positive feedback, our results showed a significant shrinkage of the bistable region. Because of this topology’s bistable capability and wide presence in most bacterial QS decision-making circuits, such a decrease in bistability robustness due to QS crosstalk suggests a new strategy for developing anti-infection therapeutics. Namely, we might exploit “artificial” crosstalk to disrupt intercellular communication specificity and collapse the group’s coordination, which could be an efficient and economic approach in medical treatments, especially for QS-dependent bacterial infection.

On the other hand, promoter crosstalk caused complex trimodal responses when embedded within a positive feedback circuit. This can only be explained when network bistability, gene expression stochasticity, and genetic mutations are all taken into consideration. These results
highlight the potential for engineering gene networks to express complex behaviors due to host-circuit interactions. We computationally predicted and experimentally verified that the C12-LasR-pLux positive feedback circuit could drive the formation of three subpopulations from an isogenic initial culture: one population expressing high GFP expression, the second showing basal GFP expression, and the third population with no GFP expression. The high and low GFP states are the result of positive feedback enabled bistability and gene expression stochasticity-induced random state transitions: commonly reported as a hallmark of many bistable systems (Acar et al., 2005; Gardner et al., 2000; Tan et al., 2009; Guido et al., 2006; Isaacs et al., 2003). This population heterogeneity is not caused by genetic factors.

The third non-GFP population is the result of genetic mutation from IS10 insertion. The mutation only happened in the C12-LasR-pLux positive feedback circuit but not in CP-LasR-pLux-C12 (Figure S 3D) or the C12-LuxR-pLux positive feedback circuit (Figure S 2B). It is, therefore, possible that the special sequence arrangements of the positive feedback circuit (for example, the symmetric pLux promoters flanking the LasR gene) on the plasmid coupled with the stress of exogenous protein overexpression led to transposon activation and gene network destruction. Given that many current synthetic gene circuits are constructed with a similar symmetric structure in a plasmid (such as Promoter-RBS-Gene1-RBS-Gene2-, or Promoter-RBS-Gene1-Terminator-Promoter-RBS-Gene2-Terminator), the mutation may occur for a wide range of engineered gene circuits. On the other hand, from an engineer’s perspective, the mutation stands in contrast to previously reported host-circuit interactions, which are primarily related to resource limitation and resulting growth defects (Brophy and Voigt, 2014). Here we were able to illustrate that both the components used and the topology of the network constructed could contribute to resource independent host-circuit interactions.

This concept of combining nonlinear dynamics and host-circuit interactions to enrich population diversity expands our understanding of mechanisms contributing to cell-cell variability, and suggests new directions in engineering gene networks to utilize hybrid factors. Taken together, our studies not only showcase living cells’ amazing complexity and the difficulty in the refining of engineered biological systems, but also reveal an overlooked mechanism by which multimodality arises from the combination of an engineered gene circuit and host-circuit interactions (Ellis et al., 2009; Hussain et al., 2014; Litcofsky et al., 2012;
SIGNIFICANCE

Widespread quorum-sensing (QS) enables bacteria to communicate and plays a critical role in controlling bacterial virulence. QS components have also been widely used in synthetic biology applications. However, effects of promiscuous QS crosstalk remain unexplored. Here we systematically studied the crosstalk between LuxR/I and LasR/I systems. Combining synthetic biology and mathematical modeling, this work reveals the complexity of QS crosstalk, which is critical for unraveling the underlying principles of bacterial decision-making and survival strategies for both natural and synthetic systems.

Furthermore, the unusual hybrid multimodality arising from the combination of engineered gene circuits and circuit-host interactions could be utilized in biotechnology.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions and Media

All cloning experiments were performed in E.coli DH10B (Invitrogen, USA), and measurements of positive feedback response were conducted in DH10B and MG1655. Cells were grown at 37 °C (unless specified) in liquid and solid Luria-Bertani (LB) broth medium with 100 µg/mL ampicillin. Chemical 3OC6HSL and 3OC12HSL (Sigma-Aldrich, USA) were dissolved in ddH₂O and DMSO, respectively. Cultures were shaken in 5 mL or 15 mL tubes at 220 rotations per minute (r.p.m), and inducers were added at OD₆₀₀~0.1.

Plasmids Construction

Plasmids were constructed according to standard molecular cloning protocols and the genetic circuits were assembled using standardized BioBricks methods based on primary modules (Table S4) from the iGEM Registry (www.parts.igem.org). The receiver CP-LuxR-pLux was constructed from six BioBrick standard biological parts: BBa_K176009 (Constitutive promoter, CP), BBa_B0034 (Ribosome binding site, RBS), BBa_C0062 (luxR gene), BBa_B0015 (transcriptional terminator), BBa_R0062 (lux promoter), and BBa_E0240 (GFP...
As an example, to produce the RBS-LuxR part, LuxR plasmid was digested by XbaI and PstI to produce a fragment while the RBS plasmid was digested by Spel and PstI as the vector. The fragment and vector were purified by gel electrophoresis (1% TAE agarose gel) and extracted using a PureLink gel extraction kit (Invitrogen). Then, the fragment and vector were ligated together using T4 DNA ligase, the ligation products were transformed into E.coli DH10B and clones were screened by plating on 100 µg/mL ampicillin LB agar plates. Finally their plasmids were extracted and verified by double restriction digest (EcoRI and PstI) and DNA sequencing (Biodesign sequencing lab in ASU). After confirming that the newly assembled RBS-LuxR was correct, subsequent rounds to produce the RBS-LuxR-Terminator were performed similarly until completing the entire receiver CP-LuxR-pLux construction. All the other receivers and positive feedback circuits were assembled similarly. Restriction enzymes and T4 DNA ligase were from New England Biolabs. All the constructs were verified by sequencing step by step. To keep all the constructs’ expression consistent in the cell, we transferred all the fragments into the pSB1A3 vector before test.

**Flow Cytometry.**

All the samples were analyzed at the time points indicated on an Accuri C6 flow cytometer (Becton Dickinson, USA) with 488 nm excitation and 530±15 nm emission detection (GFP). The data were collected in a linear scale and noncellular low-scatter noise was removed by thresholding. All measurements of gene expression were obtained from at least three independent experiments. For each culture, 20,000 events were collected at a slow flow rate. Data files were analyzed using MATLAB (MathWorks).

**Hysteresis Experiment**

For OFF→ON experiments, initially uninduced overnight culture was diluted into fresh media, grown at 37 °C and 220 r.p.m for about 1.5 h (OD₆₀₀~0.1), then distributed evenly into new tubes and induced with various amounts of C6 or C12. Flow cytometry analyses were performed at 6, 12, and 21 hours to monitor the fluorescence levels, which generally became stable after 6 hours induction according to our experience. For ON→OFF experiments,
initially uninduced cells were induced with $10^{-4}$ M (or $10^{-9}$ M) autoinducer and tested by flow cytometry to ensure they were fully induced. Cells were then collected with low-speed centrifugation, washed twice, resuspended with fresh medium, and at last inoculated into fresh medium with varying inducer concentrations at a 1:80 ratio. For the LasR-pLux positive feedback system, we only diluted once and grew them for 6, 12, 18, 24, or 32 hours, but for the other hysteresis experiments, the ON cells were collected and diluted twice into new medium with the same concentrations of C6 or C12 at 12 h and 24 h.

**Growth Curve Assay**

First, different initial states cells were collected: initial OFF cells were cells grown overnight without inducers, initial ON cells were initial OFF cells induced with $10^{-9}$ M C12 for 12 hours, and the Mutated cells were cells induced with $10^{-4}$ M C12 for 12 hours, diluted into fresh media with $10^{-4}$ M C12, and grown at 37 °C for another 12 h. Before the growth rate assay, all the cells’ fluorescence was tested by flow cytometry to verify their states. Growth rate was measured by using absorbance at 600 nm with a plate reader (BioTek, USA). Cells from each state were then diluted into fresh LB media (1000 µL, O.D. ~0.06) with $10^{-8}$ M C12 and grown at 37 or 34 °C. For each sample, OD was measured by using 200 µL cultures in a 96-well plate and tested over 24 hours. The experiments were independently replicated three times.

**Microfluidics, Fluorescence Microscopy, and Image Processing**

The use of microfluidic devices coupled with fluorescence measurement allowed us to measure gene network dynamics in single cells. Media flow direction and speed was controlled through hydrostatic pressure. A detailed description of the chip can be found in the work of Ferry MS, et al (Ferry et al, 2011). Once the cell was loaded into the trap, the flow was reversed and its rate was slowed to ~120 µm/min to ensure that the cells would not be washed away and would receive enough nutrients. Furthermore, care was taken to avoid introducing bubbles to any part of the chip as they considerably disrupt flow. The chip temperature was maintained at 34 °C with an external microscope stage (Tokai Hit, Japan). Inducer concentrations were controlled by adjusting the heights of the inducer-containing
media syringes relative to one another.

Images were taken using a Nikon Eclipse Ti inverted microscope (Nikon, Japan) equipped with an LED-based Lumencor SOLA SE Light Engine with the appropriate filter sets. The excitation wavelength for GFP was 472 nm, and fluorescence emission was detected with a Semrock 520/35 nm band pass filter. Phase and fluorescent images were taken under a magnification of 40X, and perfect focus was maintained automatically using Nikon Elements software.

Initially OFF cells (K-12 MG1655) induced with $10^{-9}$ M C12 (6 hours) were collected as the initial ON cells, washed, resuspended with fresh media and then loaded into the trap. 100 µg/mL ampicillin was added into media 1 and 2, but only media 2 was augmented with the corresponding inducer ($10^{-8}$ M C12). The microfluidic device was used to control the chemical concentration by switching between media 1 and 2. For initial ON cells, media 2 was provided to the cells for the duration of the experiment. To prevent photobleaching and phototoxicity to the cells in the trap, exposure time was limited to 100 ms for GFP.

Images were taken every 5 minutes for about 28 hours in total. The pixels in all images are normalized to 0 – 1 range before analysis. One image was chosen for quantification every 15 minutes (i.e. three images). For each cell, the intensity was calculated by averaging three selected points (left, middle, and right) in the cell and then subtracting the background. Since all the cells are offspring of the first initial ON cell, each branch in Figure 5b stands for one progeny. The cells that were washed away or had less than three generations were not analyzed.

Mathematical Modeling. Ordinary differential equation models were solved and analyzed by MATLAB. Stochastic simulations were written in MATLAB and run on a standard personal computer (details are provided in Supplemental Information).

SUPPLEMENTAL INFORMATION

Supplemental Information contains full details about the mathematical modeling construction and parameter fittings, five figures and four tables and can be found with this article online.
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.

ACKNOWLEDGMENTS

We thank Jeff Hasty for the microfluidic setup and chip design. We also thank Riqi Su and Philippe Faucon for helpful discussions and suggestions. D.J.M is partially supported by ASU IRA Fulton School of Engineering’s Dean’s fellowship. This study was financially supported by National Science Foundation Grant DMS-1100309, American Heart Association Grant 11BGIA7440101, and National Institutes of Health Grant GM106081 (to X.W.).

REFERENCES


Xiong, W., and Ferrell, J.E. (2003). A positive-feedback-based bistable “memory module” that

Figure legends

Figure 1. QS crosstalk dissected using synthetic gene circuits. (A) LuxR can crosstalk with C12 to activate pLux. Top panel: schematic diagram of a synthetic gene circuit where a constitutive promoter (gray arrow) regulates LuxR (purple rectangle) expression. LuxR protein (purple bars), when dimerized and bound with C6 or C12, can activate pLux (purple arrow) to induce GFP (green rectangle) expression. The autoinducers, genes, and promoters are color coded so that naturally paired partners are in the same color. Bottom panel: dose response of the circuit when induced with C6 (gray) or C12 (black). (B) LasR can crosstalk with pLux when bound with C12. Top panel: schematic diagram of a circuit similar to that in (A), where a constitutive promoter regulates LasR (cyan rectangle) expression. LasR protein, when bound with C6 or C12, can activate pLux to induce GFP expression. Bottom panel: Dose response of this circuit when induced with C6 (gray) or C12 (black). Bar heights are averages of three independent flow cytometry measurements shown as mean ± SD. (C) Summary of crosstalk induction of all 16 different combinations, including inductions by both chemicals and corresponding synthase genes. The four combinations shown in (A) and (B) are highlighted with a gray background. Quantified results for other combinations are included in Figure S1.

Figure 2. Signal crosstalk causes shrinkage of bistable region. (A) Schematic diagram of a synthetic gene circuit where the pLux promoter regulates expression of LuxR, which in turn can bind with C6 or C12 to further activate pLux, forming a positive feedback loop (shown as simplified diagram). GFP under the regulation of pLux serves as the readout for LuxR levels. All components are color coded similarly as in Figure 1. (B) The average of three replicate flow cytometry measurements is plotted as a square with error bars for each dose of C6 induction, where red indicates Initial ON cells while blue denotes Initial OFF cells. Solid lines 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 agarose 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,
k3=3.6e-6), low (green, k3=4e-7), and none (blue, k3=0). Histograms were constructed from 8000 single cell stochastic simulations at 1000 (k3=3.6e-6) and 1900 (k3=0 and k3=4e-7) minutes. (B) Experimental validation of the model predictions in (A). Red and green curves correspond to the high and low transposition rates from (A), and they exhibit similar bi- and trimodal responses, respectively. No blue curve is included because mutation could not be eliminated entirely experimentally. (C) Representative stochastic simulations of single cell fluorescence starting from the ON state. All possible transitions are shown. Inset diagram illustrates all possible state transitions in the simulation. (D) Model predictions of GFP expression with low transposition rate showing temporal evolution of the population from primarily ON cells at an early time (green), to trimodal distributions at intermediate time (blue), eventually falling into a primarily Mutated state at late time (red). (E) Flow cytometry measurements taken at 12 hours (green), 24 hours (blue), and 32 hours (green). Populations show similar dynamics to those predicted by the model in (D), starting with a large ON peak, transitioning to a trimodal distribution, then into primarily Mutated or OFF cells.

Figure 5. Fluorescence microscopy validation of mathematical model predictions. (A) GFP fluorescence (top) and phase contrast (bottom) images of cells growing in the microfluidic chamber at 0, 8, 16, and 24 hours. Magnification: 40x. (B) Normalized fluorescence expression of representative cells from (A), showing similar behavior to that predicted by the model from Figure 4C. Four cells are colored corresponding to the scenarios in Figure 4C, and the other 11 cells are grey. Each trajectory follows one cell, with the trajectory branching as the cells divide. One frame equals five minutes. (C) Diagram of the mechanism for trimodality. Each “valley” represents one state. The blue curve represents the landscape at 37 °C, and the dotted grey curve is the landscape at 34 °C. At 37 °C, ON state cells can more easily transition to the Mutated state because of the low barrier; while at 34 °C, the barrier between ON and Mutated states increases, resulting in more ON cells transitioning to OFF state and promoting the emergence of trimodality.
Summarization of the crosstalk between LuxR/I and LasR/I signal systems.

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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

C

D

Concentration (log10, Molar)

Normalized Fluorescence

Cell Count

GFP Fluorescence (a.u.)

LasR

GFP

IS10 Transposase

pLux

LasR

C12

pLux

C

D

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

4K

3K

2K

1.5K

pLux

pLux

GFP
Figure A: Fluorescence and transmitted images of cells at different time points (0 h, 8 h, 16 h, 24 h).

Figure B: Intensity graph showing ON-OFF, ON-Mu, ON-OFF-ON-OFF, and ON-OFF-ON-Mu conditions.

Figure C: Temperature changes at 34 °C and 37 °C with ON and Mutated states.
Figure S2

A

LuxR

Initial OFF
12 h

Initial ON
(1E-4) 24 h

3OC6HSL (M)

Mean Fluorescence (log10, a.u.)

B

LuxR

Initial OFF
12 h

Initial ON
(1E-4) 24 h

3OC12HSL (M)

Mean Fluorescence (log10, a.u.)
Figure S3

A

LasR → C12 → LasR → GFP

B

Initial OFF
37 °C 6 h

Initial ON (1E-4)
37 °C 12 h

Initial ON (1E-8)
37 °C 12 h

Initial ON (1E-9)
37 °C 12 h

C

Mean Fluorescence (log10, a.u.)

D

C12 → LasR → GFP

Mean Fluorescence (log10, a.u.)
Figure S4

A

Cell growth (OD 600)

Time (hour)

OFF-37 °C
Mu-37 °C
ON-37 °C
OFF-34 °C
Mu-34 °C
ON-34 °C

B

37 °C

6 h
26.35%

12 h
73.41%

24 h
84.82%

34 °C

6 h
3.2%

12 h
37.22%

24 h
43.57%

GFP Fluorescence (a.u.)
Figure S5

A

\[ \text{LuxR} \rightarrow \text{pLux} \rightarrow \text{GFP} \]

\[ \text{LasR} \rightarrow \text{pLux} \rightarrow \text{GFP} \]

Mean Fluorescence (a.u.)

LuxR+pLux LasR+pLux

0 0.3 0.6 0.9 1.2 1.5 1.8 2.1

B

\[ \text{LuxR} \rightarrow \text{pLux} \rightarrow \text{GFP} \]

\[ \text{LasR} \rightarrow \text{pLux} \rightarrow \text{GFP} \]

Mean Fluorescence (a.u.)

LuxR+pLux LasR+pLux

0 0.3 0.6 0.9 1.2 1.5 1.8 2.1

C

\[ \text{LuxR} \rightarrow \text{pLux} \rightarrow \text{GFP} \]

\[ \text{C6} \rightarrow \text{pLux} \rightarrow \text{GFP} \]

Normalized Fluorescence

[3OC6HSL] (log10, Molar)

Qi = 1.65e-8
ni = 1.10

D

\[ \text{LasR} \rightarrow \text{pLas} \rightarrow \text{GFP} \]

\[ \text{C12} \rightarrow \text{pLas} \rightarrow \text{GFP} \]

Normalized Fluorescence

[3OC12HSL] (log10, Molar)

Ki = 6.46e-9
ni = 1.14

E

\[ \text{LuxR} \rightarrow \text{pLux} \rightarrow \text{GFP} \]

\[ \text{C12} \rightarrow \text{pLux} \rightarrow \text{GFP} \]

Normalized Fluorescence

[3OC12HSL] (log10, Molar)

Ki = 6.6e-7
ni = 1.1

F

\[ \text{LasR} \rightarrow \text{pLas} \rightarrow \text{GFP} \]

\[ \text{C12} \rightarrow \text{pLas} \rightarrow \text{GFP} \]

Normalized Fluorescence

[3OC12HSL] (log10, Molar)

Ki = 1.27e-9
ni = 6.41
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 \( \Delta U_{\text{OFF} \to \text{ON}} \) is
much greater than \( \Delta U_{\text{ON} \to \text{OFF}} \), 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 \( (n_i) \), 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

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

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<td>Leakage without LasR protein after gene mutation (min⁻¹)</td>
<td>0.005</td>
<td>Estimated and experiment indicated</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Biobrick number</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBa_R0062</td>
<td>pLux</td>
<td>Promoter activated by LuxR in concert with 3OC6HSL</td>
</tr>
<tr>
<td>BBa_R0079</td>
<td>pLas</td>
<td>Promoter activated by LasR in concert with 3OC12HSL</td>
</tr>
<tr>
<td>BBa_K176009</td>
<td>CP</td>
<td>Constitutive promoter family member J23107 actual sequence (pCon 0.36)</td>
</tr>
<tr>
<td>BBa_B0034</td>
<td>RBS</td>
<td>Ribosome binding site</td>
</tr>
<tr>
<td>BBa_B0015</td>
<td>T</td>
<td>Transcriptional terminator (double)</td>
</tr>
<tr>
<td>BBa_C0062</td>
<td>LuxR</td>
<td>LuxR repressor/activator</td>
</tr>
<tr>
<td>BBa_C0079</td>
<td>LasR</td>
<td>LasR activator</td>
</tr>
<tr>
<td>BBa_C0161</td>
<td>LuxI</td>
<td>Autoinducer synthetase for AI from <em>Aliivibrio fischeri</em></td>
</tr>
<tr>
<td>BBa_C0178</td>
<td>LasI</td>
<td>Autoinducer synthetase for PAI from <em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>BBa_E0240</td>
<td>GFP</td>
<td>GFP generator</td>
</tr>
<tr>
<td>pSB1A3</td>
<td>pSB1A3</td>
<td>High copy BioBrick assembly plasmid</td>
</tr>
</tbody>
</table>
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:

\[
\begin{align*}
\text{LuxR} + \text{HSL} & \rightarrow (\text{LuxR-HSL}) \\
2 \ (\text{LuxR-HSL}) & \rightarrow (\text{LuxR-HSL})_2 \\
(\text{LuxR-HSL})_2 + \text{DNA} & \rightarrow (\text{LuxR-HSL})_2-\text{DNA} \\
(\text{LuxR-HSL})_2-\text{DNA} & \xrightarrow{k_1} \text{mRNA} + 2\text{LuxR} + 2\text{HSL} + \text{DNA} \\
\text{mRNA} & \rightarrow \text{LuxR} + \text{mRNA} \\
\text{mRNA} & \rightarrow \emptyset \\
\text{LuxR} & \rightarrow \emptyset 
\end{align*}
\]

where \( \text{LuxR} \) is the monomer form of LuxR protein; \( \text{HSL} \) is the autoinducer 3OC6HSL; \( (\text{LuxR-HSL}) \) is the complex of LuxR bound with \( \text{HSL} \); \( (\text{LuxR-HSL})_2 \) is the dimer of \( (\text{LuxR-HSL}) \); \( (\text{LuxR-HSL})_2-\text{DNA} \) represents \( (\text{LuxR-HSL})_2 \) binding to the pLux promoter; \( \text{mRNA} \) 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 \( (\text{LuxR-HSL}) \) (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 ($f$):

$$ f = \frac{[\text{HSL}]}{([\text{HSL}]+K_i)} $$

[Eq1]

where $n_i$ 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 $\text{LuxR-HSL}$ 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:

$$ [\text{LuxR}_2] = \frac{[\text{LuxR}]}{K_d} $$

[Eq2]

where $K_d$ is the dissociation constant for LuxR dimerization. According to reaction (2), two ($\text{LuxR-HSL}$) molecules bind together to form a dimer and activate transcription. Additionally, it is necessary to point out that even without autoinducer $\text{LuxR}_2$ 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:

$$ C = (c_0 + f^*)[\text{LuxR}]^2/K_d $$

[Eq3]

Here $C$ represents the concentration of functional LuxR dimer ($\text{(LuxR-HSL)}_2$ and $\text{LuxR}_2$); $c_0$ is the fraction of $\text{LuxR}_2$ that can recognize and bind pLux in the absence of autoinducers; $K_d$ is the dissociation constant for dimerization.

($\text{LuxR-HSL})_2$ then recognizes and binds to the pLux promoter to form the ($\text{LuxR-HSL})_2$-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:

$$ S_m = c_i + k_1C/(C + K_i) $$

[Eq4]

where $S_m$ represents the production of mRNA; $c_i$ represents the basal mRNA expression
without LuxR protein; \( k_j \) 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:

\[
S_p = k_2 \cdot [\text{mRNA}]
\]  
[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:

\[
D_m = d_1 \cdot [\text{mRNA}]
\]  
[Eq6]

where \( d_1 \) is the degradation rate of mRNA.

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

\[
D_p = d_2 \cdot [\text{LuxR}]
\]  
[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:

\[
\begin{align*}
\frac{d[M]}{dt} &= S_m - D_m \\
\frac{d[R]}{dt} &= S_p - D_p
\end{align*}
\]  
[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:

\[
\begin{align*}
\frac{d[M]}{dt} &= c_1 + \frac{k_1 C}{C + K_n} - d_1 [M] \\
\frac{d[R]}{dt} &= k_2 [M] - d_2 [R].
\end{align*}
\]

where \( C = \frac{(c_0 + f^2) \cdot [R]^2}{K_d} \)

Where \( f = \frac{[\text{HSL}]^n}{[\text{HSL}]^n + K_i^n} \)  
[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):

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

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:

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

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 $\Delta U$ (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:

$$P_m = \frac{[\text{LasR}]}{K + [\text{LasR}]^n}$$

[Eq 11]

where $P_m$ 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:

$$M_u = k_3 * P_m * [\text{LasR}]$$

[Eq 12]

where $k_3$ is the transposition rate; $[\text{LasR}]$ is the amount of LasR in the cell at a given time, and 
$P_m$ 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 ($k_3$) 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 \( \text{OD}_{\text{Mutation}}/\text{OD}_{\text{ON}} \), \( \text{OD}_{\text{OFF}}/\text{OD}_{\text{ON}} \), and \( \text{OD}_{\text{ON}}/\text{OD}_{\text{ON}} \): 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 (\( F_{\text{mu}} \)), OFF cells (\( F_{\text{off}} \)) and ON cells (\( F_{\text{on}} \)) are:

\[
F_{\text{mu}} = S_{\text{mu}} \times (\text{OD}_{\text{Mutation}}/\text{OD}_{\text{ON}});
\]
\[
F_{\text{off}} = S_{\text{off}} \times (\text{OD}_{\text{OFF}}/\text{OD}_{\text{ON}});
\]
\[
F_{\text{on}} = S_{\text{on}} \times 1;
\]

where \( S_{\text{mu}}, S_{\text{off}}, \) and \( S_{\text{on}} \) are the primary number of cells which finished the simulation in the Mutation, OFF, and ON states, respectively. Therefore, the proportions of Mutation cells (\( P_{\text{mu}} \)), OFF cells (\( P_{\text{off}} \)), and ON cells (\( P_{\text{on}} \)) are:

\[
P_{\text{mu}} = F_{\text{mu}}/(F_{\text{mu}} + F_{\text{off}} + F_{\text{on}});
\]
\[
P_{\text{off}} = F_{\text{off}}/(F_{\text{mu}} + F_{\text{off}} + F_{\text{on}});
\]
\[
P_{\text{on}} = F_{\text{on}}/(F_{\text{mu}} + F_{\text{off}} + F_{\text{on}});
\]

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 \((n_i)\) 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: yellow; ribosomal binding site: blue; LasR: cyan; IS10 transposase: pink; Terminator: red; GFP generator: green; pSB1A3 vector: grey.

**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→
CAGGGAACCCTTAACTATACAAATAGGGATACGTACAGGCAAGAATTGTTTCT
TATAGTCAATTTTACTAGACAAAGGGAGAAACTAGATGGCTGGTGTTGACGGGTTTTCTTGAGCTGGGAACGCTAGTGGAAATTGGAGTGGAGCGCCATCCTCCAGAAGATGGCGAGCGACCTTGGATTCTGAAGATCCTGTTCGGCCTGTTGCCTAAGGACAGCCAGACTACGAGAACGCCTTCATCGTCGGCAACTACCCGGCCGCCTGGCGCGAGCATTACGACCGGGCTGGCTACGCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAGCGTACTGCCGATTTTCTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAGGAAGCCTCGGCCGCCGGCCTGCTATGGGCTGACCATGCCGCTGCATGGTGCTCGCGGCGAACTCGGCGCGCTGAGCCTCAGCGTGGAAGCGGAAAACC
```
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→
CGGGGGCTCAATAAAAAACGAAAGGCTCAAGTCGAAAAGACTGGGCCCTTTCGT
TTATCTCTTGTTTGTCTCTGGTGAACGCTCTCTACTAGAGTCACACTGGGCTC
ACCCTGCGGTGGGCCTTTCTGCCTTTGATACTAGAGCACTAGCTGGGCTC
TACAGGTTTACGCAAAGAAATGTGTTTGTATAGTCGAAATAAATACTAGAG
TCACACAGGAAGTACTAGATGGCTAAGGGAAGAAACTTTTACTGAG
TTGTCCCCATTCTCTGTTGAAATTAGATGGGTATGTTATGTTGGCAAAATT
TCGTCGAGAGGAGGTGAAGGTGACATGGCAAATACGGGAAACTTACCCT
TAAATTTATTTCTAGAAGTAGGGAAAACTTACCTGTTCCATGGCAAAACTTG
tCACACTTTTCGGTTATGGTGTTCAATGCTTTTGGCGAGATACCAGATCAT
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
GGGGGGGGGTTGGAATACTACCTTACCTCTCTTCTTTACCCATCATCTTACTTTCC
2 GTCTAGCATCTCAGTTCTCTTCTTTACCCATCATCTTACTTTCC
3 ACCTGTACGATCCTACAGGTCTCTAGTATATAAACGCAGAAAGGCCCACC
4 CGAAGGTGAGCCAGTGTGACTCTAGTAGAGAGCGTTCACCGACAAACAAC
5 AGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGA
6 TGCCTGGCTCTAGTATTATTAGAGAGTAATAAGACCCAAATTAACGGCCA
7 AAAATCCGGGGGGATAAACACCCGGGAAAAAAAACTTGGTGGAAACC
8 AAAAGGGGCCCGCGCGGTGTTGTTCTGGTGGTGGGGGTTTTTTTT
```

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

BB-C-Reverse
Sequence: AATACGCTTGTGAGTGAGC
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→
GGGCCTGAGTACGCTCAGGCAGCGCAACGACTACGCAAGCTGTCGTAACCTGACAACCTAAGTGAGATCAAAACATAACATCAA
  2
  3
  4
  5
  6
  7
  8
  9
 10
 11
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 45
 46
 47
 48
 49
 50
 51
 52
 53
 54
 55
 56
 57
 58
 59
 60
 61
 62
 63
 64
 65
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→
AGGAGCCAGGGCATCAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCT

Note:
Highlight (green, 69-944): E0240, GFP generator;
Highlight (yellow, 953-1007): R0062, pLux promoter;
TTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACT
GGCTCACCTTCGAGGCTCTTTCTCTGCGTTTATATACTAGTAGCGGCCGC
TGCAGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGGCC
CGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC
AGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCA
GGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCACAGGCTCCCCCCC
CCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCC
GACAGGACTATAAAAGATACCAAGCCGTTTCCCTGGAAGCTCTCTGTCG
TTCGAGCCGACCCTGGCTGGCCTTTATGCGTAACCTGCTCTTGGATCAAAC
CCGCTAAGCACGACTTTATCGCCACTGGCAGCAGCCACTGGTAACAGGAT
TAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGC
CTAACTACGGCTACACTAGAAGAACATTTGTTATCTGCGCTCTGCTGA
AAGCCAGTTACCTTGGGAAAAAAGATGGTGATAGCTTTGATCCGAAAACA
AACCACGCTGCTGATAGGCTGTTTTTTTGTGTTGGAAACAGCAGATAACGC
GCAGAAAAAAAGGTATCATGAAAGAGATCCTTTTGATCTCTGCTCTGCTG
AAGCCGACTGGAAGAAAACACTGACTTAAGGGTATTGGTCTAGTGAAGAT
ATCAAAAAAGATCTTTCACCTAGATCCTTTTAAAATAAAATAAGGTTTTA
AAATCAATCTAAAGTATAATATGGAATGAAACCTTTGCTGACGTTACAAATG
GCTTAATCAAGGAGGCGACCTTAATCATAAGGGATCTGCTCTATTTCGTTCA
TCCCTAATATGGCCTGATCCTCCCGCTGCGTGAAAATTTACAAAA
CCGGGGGGGGGGCTTTAATCGGGGCGGCCCCAGGGGGGGTGCCAAG
GGAGAAACCCCGCAGAAAAACCCCCCGCCCCCAGGGGGGCTC
CCAAAAAATTTTTTTATTATTTAACACCCAAAA

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


