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Svetlana V Harbaugh, Michael S. Goodson, Kateri Dillon, Sarah Zabarnick, and Nancy Kelley-Loughnane

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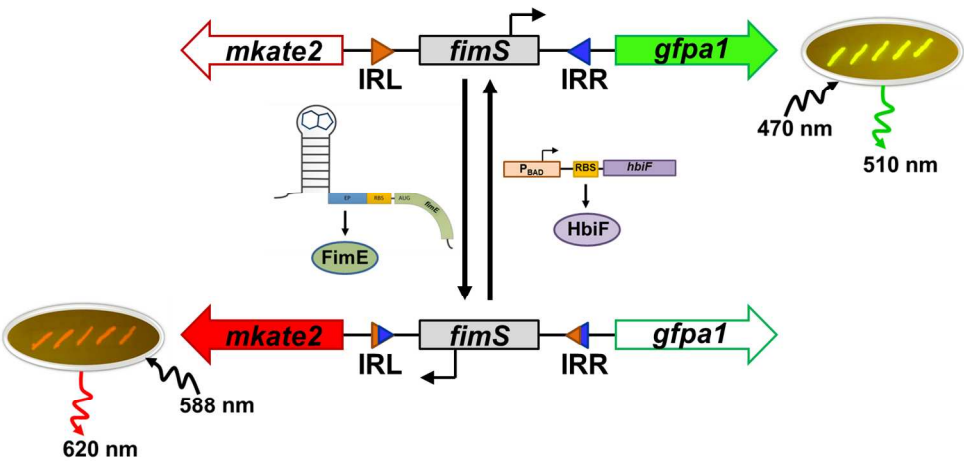
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Riboswitch-Based Reversible Dual Color Sensor

Svetlana V. Harbaugh,^{*,†} Michael S. Goodson,[‡] Kateri Dillon,[§] Sarah Zabarnick,[§]
and Nancy Kelley-Loughnane^{*}

711th Human Performance Wing, Airman Systems Directorate, Air Force Research Laboratory,
Wright-Patterson AFB, Ohio 45433, United States

[†]The Henry M. Jackson Foundation, 6720A Rockledge Drive, Bethesda, Maryland 20817,
United States

[‡]UES, Inc., 4401 Dayton-Xenia Road, Dayton, Ohio 45432, United States

[§]These authors contributed equally to this work.

^{*}Corresponding author: svetlana.harbaugh.1.ctr.ru@us.af.mil, nancy.kelley-loughnane.1@us.af.mil

Abstract

Riboswitches are RNA-based ‘sensors’ that utilize chemically-induced structural changes in the 5'-untranslated region of mRNA to regulate expression of downstream genes. Coupling a specific riboswitch with a reporter gene system translates chemical detection by the cell into a quantifiable reporter protein signal. For the majority of reporter gene systems the readout signal is only expressed in the presence of the target analyte. This makes it difficult to determine the viability and localization of the uninduced biosensor when it is used for ‘real-world’ applications. To address this problem, we developed a dual color reporter comprising elements of the *E. coli* fimbriae phase variation system: recombinase FimE controlled by a synthetic riboswitch; and an invertible DNA segment (*fimS*) containing a constitutively active promoter placed between two fluorescent protein genes. Without an analyte, the fluorescent reporter constitutively expressed green fluorescent protein (GFPa1). Addition of the analyte initiated translation of *fimE* causing unidirectional inversion of the *fimS* segment and constitutive expression of red fluorescent protein (mKate2). Thus, the sensor is always fluorescent, but its color is determined by detection of a specific analyte. We demonstrated that the recombinase-based dual color reporter can be successfully applied to monitor activation of a theophylline synthetic riboswitch that was used as our model system. To show the feasibility of the FimE recombinase-based system to serve as a

reporter for monitoring activation of multiple synthetic riboswitches and, therefore, expand the applicability of the system, we tested a number of previously developed synthetic riboswitches responsive to different analytes. We showed that the dual color reporter system can be successfully used to monitor activation of M6 and M6'' riboswitches responsive to ammeline and pyrimido[4,5-d]pyrimidine-2,4-diamine (PPDA), respectively, and a 2,4,6-trinitrotoluene (TNT)-responsive riboswitch developed in this study. We also demonstrated that the system can be reversed by HbiF recombinase-mediated *fimS* inversion to the initial state of the fluorescent reporter, creating a resettable and reusable cell-based sensor.

Key words: Biosensor, riboswitch, recombinase, dual color detection

Introduction

Synthetic biology combines engineering-driven approaches with fundamental biological components to design predictable functions in biological systems.^{1,2} This rapidly growing field utilizes the capabilities of cells by directing their natural functions and adding non-natural functions to complete specific tasks. Genetically engineered microorganisms have been widely applied for accurate and sensitive detection of target analytes in a diverse variety of fields such as medicine,³⁻⁶ environmental monitoring,⁷⁻¹⁰ defense,¹¹⁻¹⁴ food processing¹⁵ and safety.¹⁶⁻¹⁸ The sensing capability, i.e. the selectivity and specificity, of genetically engineered cell-based biosensors is usually determined by the nature of a sensing element. Though the vast majority of utilized sensing elements are based on transcriptional regulator and inducible promoter pairs,¹⁹ the use of RNA-based detection and regulation has been significantly growing over the last decade.^{20,21}

Riboswitches are a relatively new class of RNA-based ‘sensors’ that utilize chemically-induced structural changes in the 5'-untranslated region of mRNA to regulate the expression of associated genes. Riboswitches are composed of two functional domains: an aptamer domain that binds to a specific ligand; and an expression platform that controls the expression of a downstream gene via conformational changes that are induced by ligand binding to the aptamer domain.²² In addition to natural riboswitches found in living organisms,^{23, 24} synthetic riboswitches that respond to different analytes have been developed and used for sensing applications.^{19, 25-32}

Coupling a specific synthetic riboswitch with a reporter gene system translates chemical detection by the cell into a quantifiable reporter protein signal. However, for the majority of reporter gene systems the readout signal is only expressed in the presence of the target analyte. This makes it difficult to determine the viability and localization of the uninduced biosensor when it is used for ‘real-world’ applications. A dual color reporter system would be useful to address this problem. Recently, Süel and co-workers engineered a dual color nitric oxide-responsive *E. coli* cell-based sensor that utilizes a genetic circuit based on elements of the *E. coli* type 1 fimbriae (Fim) phase variation system.³³ The expression of type 1 fimbriae in *E. coli* is phase dependent, i.e. the bacteria shift periodically between a fimbriate and non-fimbriate states, termed “on” and “off”, respectively.^{34, 35} This phenomenon is due to the inversion of a 314-base pair (bp) DNA segment, known as the *fim* switch or *fimS*, containing the promoter for the fimbrial subunit gene, *fimA*. The inversion of *fimS* is a process of site-specific recombination catalyzed by specific recombinases, FimB and FimE.³⁶ The site-specific recombinases mediate the inversion of *fimS* by recognizing the 9-bp inverted repeats bordering *fimS*, named the left inverted repeat (IRL) and the right inverted repeat (IRR). The FimB recombinase inverts the *fim* switch in the “on”-to-“off” and the “off”-to-“on” directions with approximately equal efficiency while FimE, possessing different binding affinities for IRL and IRR, inverts it rapidly only in the “on”-to-“off” direction.^{37, 38} Particularly, the ability of FimE recombinase to perform unidirectional inversion is an attractive property which can be used to switch from the expression of one gene to another and change the output signal of the system. Recombinase-based systems require only brief induction to cause permanent and heritable changes in gene expression.^{33, 39} This property makes them useful as reporters to design cell-based sensors for long-term detection and monitoring. A variety of regulatory elements comprising DNA-protein complexes, such as inducible or regulated promoters, have been used to control recombinase production and trigger inversion recombination.^{33, 39-44}

Here, we demonstrate the development of a dual color reporter system comprising FimE recombinase controlled by a synthetic riboswitch, and an invertible DNA segment (*fimS*) containing a constitutively active promoter placed between two fluorescent protein genes. We used a number of synthetic riboswitches which function through translational regulation of gene expression by switching between different mRNA conformations that control the accessibility of

a ribosome binding site (RBS).^{26, 29-31} These riboswitches activated expression of FimE recombinase in *E. coli* cells upon binding of specific small-molecule analytes. In the absence of the analyte, translation of *fimE* mRNA was suppressed, and the fluorescent reporter switch constitutively expressed green fluorescent protein (GFPa1). Addition of the analyte initiated translation of *fimE* causing unidirectional inversion of the *fimS* segment and constitutive expression of red fluorescent protein (mKate2). Thus, the sensor is always fluorescent, but its color is determined by the presence of an analyte.

The advantage of using a recombinase system for cell-based detection is the possibility to create a resettable and reusable sensor by introducing a second recombinase into the system. Double inversion systems utilizing pairs of site specific recombinases were described previously, mostly for the design of complex synthetic memory circuits.^{40, 42-44} To construct a heritable sequential memory switch in *E. coli* cells, Arkin and co-workers used the FimB recombinase of the *fim* system from *E. coli* and the *hin* recombination system from *Salmonella*.⁴⁰ The two inversion systems were integrated in an overlapping manner creating a switch with multiple states determined by recombinase activation inputs. More recently, pairs of unidirectional serine integrases recognizing different recombination sites were applied to build memory circuits integrated with logic gates by flanking combinations of terminators, promoters, or reporter genes with pairs of recombinase target sites.⁴² However, due to the unidirectionality of recombinase action and different recognition sites, recombinase-mediated inversions of determined parts were irreversible. Recently a rewritable DNA inversion-based memory device, utilizing elements of the bacteriophage Bxb1 integrase excisionase system, was demonstrated in *E. coli* cells.^{43, 44} In this device, a DNA segment consisting of a promoter was flanked by two inversion sites, attB and attP, situated between two fluorescent protein genes. The inversion of the segment was driven by two transcription input signals, set and reset. A set signal drove expression of a Bxb1 gp35 integrase that inverted the DNA element between the attB and attP sites, converting the sequence of these sites to attL and attR sites. A reset signal induced coexpression of Bxb1 integrase and corresponding Bxb1 gp47 excisionase that flipped the DNA between attL and attR sites, restoring the attB and attP sites. Thus, depending on the orientation of the promoter, one of the fluorescent proteins was produced. Although the application of integrase excisionase system for development of rewritable DNA data storage in live cells was successful, this system

required careful tuning by controlling the excisionase-to-integrase ratio and the integrase-to-DNA target ratio, in order to avoid bidirectionality and to achieve complete recombination in both directions.

As a second enzyme for our riboswitch-based double inversion system we choose a site specific recombinase, called HbiF. HbiF recombinase is involved in the *E. coli* type 1 fimbriae phase variation and, along with FimB and FimE recombinases, regulates the inversion of the *fimS* segment.⁴⁵ Similar to FimE, HbiF-mediated *fimS* inversion is predominantly unidirectional however, in contrast to FimE, the switching occurs from an “off”-to-“on” orientation. As in the case with BxbI integrase and excisionase, in the FimE and HbiF-based system one recombinase generates inversion sites for another resulting in a reversible multi-state biological switch.

Results and Discussion

Construction of riboswitch-based dual color reporter system.

Our system was based on expression of FimE recombinase and an invertible constitutive promoter controlling fluorescence in the same cell. As our model sensing element we used a synthetic riboswitch responsive to the anti-asthma drug, theophylline.²⁶ The theophylline synthetic riboswitch was placed upstream of the FimE recombinase encoding sequence in one plasmid (Supporting Information Figure S1A). A second plasmid carried a fluorescent reporter composed of two fluorescent protein genes (mKate2 and GFPa1) and an invertible DNA segment (*fimS*) containing a constitutively active promoter (Supporting Information Figure S1B). In the *E. coli* chromosome, *fimS* represents a 314 bp segment of DNA which is bordered by the 9 bp inverted repeats, Inverted Repeat Left (IRL) and Inverted Repeat Right (IRR), and harbors the *fimA* promoter that directs transcription of the *fim* subunit genes.³⁴⁻³⁸ Each inverted repeat is flanked by overlapping recombinase binding sites; the occupancy of these sites by recombinases leads to inversion of the *fimS* segment.³⁶⁻³⁸ The *fimS* element also contains binding sites for accessory proteins, integration host factor (IHF) and the leucine-responsive regulatory protein (Lrp).⁴⁶⁻⁴⁸ One IHF binding site is located just outside the IRL sequence at one end of *fimS*. There is still some uncertainty regarding the role and importance of accessory proteins in

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2
3 recombinaise-mediated *fimS* inversion. Indeed, *fim* recombinases (FimE and FimB) were able to
4 act on a substrate in which most of the switch was replaced with non-*fim* DNA, leaving only *fim*
5 recombinaise binding sites and Inverted Repeats.⁴⁹ Therefore, *fim* recombinases can be active in
6 the absence of Lrp and IHF binding. To construct our fluorescent reporter switch, we used the
7 480 bp DNA sequence originally located between the *fimE* and *fimA* genes in the *E. coli*
8 chromosome containing the binding sites for all proteins involved in *fimS* recombination
9 (Supporting Information Figure S2). This choice was based on the hypothesis that the maximum
10 performance of FimE recombinase can be achieved only when all described components of the
11 system are present. The 480 bp DNA segment containing the invertible promoter element was
12 placed between two sequences encoding fluorescent proteins: highly bright monomeric far-red
13 mKate2, derived from DsRed;⁵⁰ and extremely bright with perfect (100%) quantum efficiency
14 GFPa1, identified in amphioxus *Branchiostoma floridae*.⁵¹ mKate2 and GFPa1 are good
15 candidates for dual color sensing since both fluorophores emit colors which are easily
16 distinguishable to the human eye and have a low degree of spectral overlap (Supporting
17 Information Figure S3). Thus, detection is made more accurate by minimizing bleed-through or
18 crossover of fluorescence emission.

19
20
21 The schematic of the performance of the constructed riboswitch-based reporter system is shown
22 in Figure 1. In the absence of an analyte, the extensive base pairing of the riboswitch with the
23 ribosome binding site (RBS) prevents translation of FimE recombinase. Without production of
24 FimE, the invertible *fimS* segment is oriented toward IRR; the fluorescent reporter switch
25 constitutively expresses GFPa1. Thus, cells emit green light at 510 nm upon excitation at 470
26 nm. Addition of the analyte changes the conformation of the riboswitch, unpairing the RBS and
27 initiating translation of *fimE* mRNA. The produced FimE recombinase facilitates the
28 unidirectional inversion of the switch, resulting in reorientation of *fimS* toward IRL and
29 constitutive expression of mKate2. Thus, without an analyte cells emit green light at 510 nm
30 upon excitation at 470 nm; addition of an analyte results in color switch and cells emit red light
31 at 620 nm upon excitation at 588 nm.

Characterization of theophylline riboswitch-based sensor.

Time course study. In order to evaluate the performance of the created biosensor, riboswitch activation in *E. coli* cells was measured over time. Previously it was shown that theophylline concentrations greater than 5 mM are toxic for *E. coli*, leading to cell death.⁵² We used 2.5 mM theophylline (Thy) for riboswitch activation experiments. To confirm a theophylline-dependent increase in FimE recombinase activity resulting in FimE-mediated switching of the fluorescent reporter, we compared three types of *E. coli* cells. The first type harbors the riboswitch construct and the fluorescent reporter switch. These cells should constitutively express GFPa1 without an analyte and switch to the expression of mKate2 in the presence of theophylline. The second type represents positive control cells harboring the fluorescent reporter switch and FimE recombinase encoding sequence without the riboswitch and under the control of the constitutive promoter (Supporting Information Figure S1C); the cells should switch to constitutive expression of mKate2. The third type of cells is a negative control containing the fluorescent reporter switch and an empty plasmid lacking the *fimE* gene. These cells should constitutively express GFPa1. As expected, the fluorescence intensity of GFPa1 measured in riboswitch cultures decreased over time in the presence of theophylline (Figure 2A). In contrast, the fluorescence intensity of mKate2 measured in the same cultures increased over time (Figure 2B). Moreover, the curves corresponding to riboswitch cell cultures grown in the presence of theophylline were similar to the positive control where *fimE* was expressed directly and recombinase-mediated inversion resulted in constitutive expression of mKate2. When riboswitch harboring cells were not treated with theophylline, the fluorescence intensity of GFPa1 increased over time. Though changes in the fluorescence intensity of riboswitch cell cultures without the analyte were similar to those of negative control lacking the *fimE* gene and constitutively expressing GFPa1, we observed a small increase in the fluorescence of mKate2 due to a low level of FimE recombinase expression even without the analyte. These results demonstrated that changes in spectral profiles of riboswitch harboring cell cultures were attributed to riboswitch-based induction of FimE recombinase expression and FimE-mediated recombination of the invertible segment in the fluorescent reporter switch. Riboswitch activation of FimE recombinase gene expression followed by irreversible inversion and color switch from green (GFPa1) to red (mKate2) resulted in an 11.3-

fold increase in the fluorescence intensity of mKate2 in response to its analyte, theophylline, after 18 hours of cell growth.

A large visual difference was observed between riboswitch cell cultures in the absence and in the presence of the analyte. Images of cell cultures collected at indicated time points (Figure 2C) showed a gradual increase in the red fluorescence signal produced from mKate2 due to FimE-mediated inversion of *fimS* in riboswitch-activated cells. Cell cultures that were not treated with theophylline showed a gradual increase in the green fluorescence signal from expressed GFPa1. The color change from green to red was detected in cell cultures at 4-6 hours post-theophylline addition. Figure 2D shows the performance of the sensor at a single cell level. Cells grown in the presence of theophylline had a reduced GFPa1 fluorescence and increasing mKate2 fluorescence over time. After 8 hours of theophylline exposure, the majority of the cells switched to mKate2 expression.

When cells are grown in medium, the medium can possess some fluorescent background that may interfere with “true” fluorescence coming from expressed fluorescent reporter proteins. To eliminate the background fluorescence of the medium we measured the fluorescence intensity in cell pellets (Supporting Information Figure S4A, B). Although the values of fluorescence intensity in cell pellets were higher compared to those measured in cell cultures, there was not a significant difference between changes in the fluorescence intensity in cell pellets and in cell cultures. However, the higher fluorescence enabled easier visual distinction of the changes in the intensity and color in cell pellets. Indeed, we were able to observe the color switch in daylight by eye (Supporting Information Figure S4C, D).

Verification of FimE-catalyzed inversion. In order to confirm that color switching of the sensor in the presence of the analyte occurs due to flipping of the DNA segment containing a constitutive promoter, and that the orientation of the promoter determines which fluorescent protein will be produced, the inversion states corresponding to controls and riboswitch harboring cells were assayed using “culture PCR” with specific primers. A culture PCR is a common technique which was previously used to verify the states of recombinase-based devices.⁴⁰ We used three different primers in two sets to examine the orientation of the promoter sequence in a fluorescent reporter switch (Figure 3A). The primers were designed to amplify DNA fragments

of a certain orientation. Since the orientation of the DNA fragments was changing due to FimE-mediated inversion, the chosen set of primers either resulted in a PCR product or generated no product at all. As shown in Figure 3B, when a set of primers designed to amplify DNA in the direct orientation was used to probe inversion states in negative control cell cultures, the PCR resulted in a well-defined 1.1 kb DNA band after gel electrophoresis. In contrast, the set of primers for the inverted orientation of the *fimS* segment did not generate PCR amplification products from the same cultures. In positive control cell cultures, both set of primers resulted in PCR products visualized as 1.1 kb DNA bands (Figure 3C). However, the intensity of the band corresponding to the *fimS* segment in the direct orientation decreased over time and the intensity of the band corresponding to inverted segment increased over time due to FimE-mediated inversion. When cell cultures harboring a riboswitch without an analyte were assayed, we observed clear PCR product bands corresponding to the direct orientation of *fimS* segment (Figure 3D). We also detected a weak DNA band related to the inverted state at 18 hours post-analyte addition. As mentioned above, the appearance of the inverted product in riboswitch cell cultures without the analyte is caused by a low expression of *fimE* due to the inability of the riboswitch to completely suppress the expression of a downstream gene when the analyte is not present. The results of PCR in riboswitch cell cultures in the presence of the analyte demonstrated the occurrence of direct and, at a later time points, inverted states of fluorescent reporter switch (Figure 3D). Similar to the positive control, the intensity of the band related to the inverted *fimS* orientation increased over time in riboswitch cell cultures due to FimE-catalyzed recombination upon riboswitch activation in the presence of the analyte. However, we were able to detect the PCR product bands corresponding to the direct state of the reporter switch in riboswitch cell cultures treated with theophylline even at the later time points. Perhaps, the inability to achieve a 100% inversion of the reporter switch in riboswitch activated cells is a result of low expression and low efficiency of FimE recombinase. Previously, Gallivan and co-workers showed that riboswitch incorporation can significantly reduce the expression of its downstream gene due to conformational restrictions in the 5'-untranslated region of mRNA. For example, in their theophylline synthetic riboswitch from *E. coli* clone 8.1, the analyte induces translation of only ~ 16% of the transcripts while ~ 84% remain inactive.⁵² The benefit for this conformational limitation is that it confers more translational control, yielding fewer false positives and lower background. However, the presence of the PCR product band corresponding

to the direct state of the reporter switch in positive control cells harboring FimE encoding gene under control of a strong constitutive promoter (even after 18 hours of expression) gives evidence of low catalytic efficiency of FimE recombinase (Figure 3C). Nevertheless, even without full inversion, the cellular system produced a much greater and a more easily detectable output signal in the presence of the analyte compared to that of the uninduced sensor.

Dose-response study. In order to investigate thresholds of the detection, we examined the fluorescence signal response in terms of analyte concentration. As depicted in Figure 4A, B, the changes in the fluorescence intensity of either GFPa1 or mKate2 in response to different concentrations of theophylline demonstrate typical dose-response curves. The decrease in the fluorescence intensity of GFPa1 was detected at theophylline concentrations down to 100 μ M, and the increase in the fluorescence intensity of mKate2 was detected at theophylline concentrations down to 250 μ M. Figure 4C shows the switch from green to red fluorescence upon increase of analyte concentration.

Riboswitch activation in a system with a degradation-tagged reporter. We attempted to improve the sensor performance by increasing the rate of GFPa1 degradation. The nucleotide sequence encoding the LVA protein degradation tag (partsregistry.org) was added at the 3'-end of GFPa1 coding sequence in a fluorescent reporter switch (Supporting Information Figure S1D). Since uninduced sensor (before an analyte addition) always fluoresces green, we expected that GFPa1 degradation will result in increasing red color in riboswitch cultures in the presence of the analyte by decreasing residual green fluorescence. A time course study was then performed (Figure 5). To eliminate the background of the culture medium, we also monitored changes of the fluorescence intensity in resuspended cell pellets (Supporting Information Figure S5). Similar to the system without a degradation tag, the riboswitch behavior was compared to negative control and positive control cells. Negative control cells harbored an empty plasmid lacking FimE gene and a fluorescent reporter switch with the degradation tagged GFPa1. Positive control cells contained the same fluorescent reporter switch and FimE recombinase encoding sequence without a riboswitch and under the control of constitutive promoter. As shown in Figures 5A, B and S5A, B, we observed a decrease in fluorescence intensity of GFPa1 and an increase in fluorescence intensity of mKate2 in the positive control and riboswitch cell cultures treated with theophylline over time. Degradation of GFPa1 resulted in a steady state level of protein

expression in negative control and riboswitch cell cultures without theophylline after 6 hours. The fluorescence intensity of GFPa1 in all cell cultures was reduced when compared to that for the system with non-tagged GFPa1. Surprisingly, coupling GFPa1 with a protein degradation tag also reduced the level of background mKate2 expression in riboswitch harboring cells in the absence of theophylline, resulting in an increased riboswitch activation ratio of 37-fold (estimated as the ratio of fluorescence intensity of mKate2 in cell cultures grown in the presence of theophylline for 18 hours to those grown without the analyte for the same time period). We were able to observe a visual difference between riboswitch in the absence and in the presence of the analyte (Figure 5C and Supporting Information Figure S5C, D), however the green colors of the negative control and riboswitch cell cultures without theophylline were less bright compared to those for the system with non-tagged GFPa1. Thus, although coupling GFPa1 with a protein degradation tag allowed us to improve the riboswitch activation ratio, the color contrast between riboswitch harboring cells in the absence and in the presence of theophylline was reduced due to GFPa1 degradation, making this system less applicable for visual analyte detection. When riboswitch activation was observed at a single cell level, the appearance of “red” cells expressing mKate2 was detected after 4 hours post-theophylline exposure, and after 6 hours the majority of cells harboring a reporter with LVA-tagged GFPa1 were switched to mKate2 expression (Figure 5D). In this case, degradation of GFPa1 decreased the time required for analyte detection (see Figure 2D for the system with non-tagged GFPa1). As in the system with non-tagged GFPa1, we performed a dose-response study. Due to GFPa1 degradation, changes in the fluorescence intensity of GFPa1 upon increase of the analyte concentration were not as drastic as for the system without the degradation tag (Supporting Information Figure S6A). For example, when the system with non-tagged GFPa1 was used, the increase in theophylline concentration from 0 to 5000 μ M resulted in 5-fold decrease in the fluorescence intensity of GFPa1 (Figure 4A). For the system with a fluorescent reporter containing GFPa1 coupled with a degradation tag, the same changes in theophylline concentration caused only 2-fold decrease in the fluorescence intensity of GFPa1 (Supporting Information Figure S6A). The increase in the fluorescence intensity of mKate2 was detected at theophylline concentrations down to 250 μ M (Supporting Information Figure S6B, C) similar to the system with non-tagged GFPa1.

Stability of riboswitch-based system. A key feature of recombinase-based devices is the ability to maintain stable output memory after the input withdrawal.⁴² To test if riboswitch-based system could hold the inverted state even after analyte removal, we activated the riboswitch by theophylline addition and then repeatedly subdiluted and grew these cells for 6 days (or 8 days for the system harboring fluorescent reporter containing GFPa1 with a degradation tag) without any further analyte addition (Figure 6 and Supporting Information Figure S7). The new generations of cells were able to maintain the output signal (mKate2 fluorescence) throughout the entire period after riboswitch activation. Moreover, the multiple generations of uninduced cells maintained the direct state of the fluorescent reporter over the same time period. This property should enable the development of cell-based sensors where instantaneous response is not required and the signal output detection can be performed after some period of time.

Creation of the reversible switch.

The advantage of using a reversible detection system as a part of a biosensor is that it can be reused a number of times for detection of the same type of target. In order to build a reversible dual color cell-based sensor, we supplemented a theophylline riboswitch-FimE recombinase-based system with a second recombinase, HbiF. An HbiF recombinase encoding sequence was placed under control of the arabinose inducible promoter, P_{BAD}. Thus, the expression of the two recombinases could be induced independently and HbiF-mediated inversion could take place only after the *fimS* DNA segment has been flipped by FimE recombinase (Figure 7A). Next, we determined if successive expression of FimE and HbiF recombinases could mediate DNA inversion from one orientation to the other and result in switching color from green to red and from red to green due to expression of the appropriate protein in cell cultures. The cells were grown in LB media and exposed to either theophylline or arabinose. The activity of one recombinase in the presence of the other was minimized by reducing its concentration and the concentration of the analyte inducing its expression: cell cultures were diluted and a new analyte was added to activate expression of an appropriate recombinase. As depicted in Figure 7, induction of translation of FimE recombinase by addition of theophylline in cells constitutively expressing GFPa1 resulted in color switch from green to red due to inversion of *fimS* segment and expression of mKate2. When diluted cell cultures were treated with arabinose, the system changed the color from red to green due to HbiF-mediated inversion of *fimS* segment and

constitutive expression of GFPa1. Thus, the cell-based sensor was reversed to its initial state. We demonstrated that the riboswitch-based sensor can be activated and reversed reliably at least four times. This was reflected by an increase of the fluorescence intensity of mKate2 and, in the same time, decrease of the fluorescence of GFPa1 when cells were grown in the presence of theophylline, and an increase of the fluorescence intensity of GFPa1 and decrease of the fluorescence of mKate2 in the presence of arabinose. It should be noted, that the fluorescence intensity of GFPa1 originally produced in cells without any inducer was higher (~ 3.5-fold) than the green fluorescence of the reversed system (Figure 7B), possibly due to an inability to achieve a 100% inversion of the reporter switch. We observed an easily distinguishable color difference in collected cell cultures and cell pellets depending on the presence of a specific analyte, either theophylline or arabinose (Figure 7C). Cells were fluorescing red in the presence of theophylline and green in the presence of arabinose.

It should be noted that riboswitch performance study was performed in *E. coli* TOP10 cells. Previously, this strain was successfully used for engineering FimE recombinase-based nitrogen oxide cell-based sensor.³³ Although this strain is not specified as *fim⁻* in its genotype, the authors did not notice any activity coming from endogenous FimE that could affect the performance of the sensor. Similar to Archer et al.³³ we did not observe any interference of endogenous FimE with plasmid expressed recombinase. As was mentioned above and shown in Figure 3B, PCR analysis of negative control cell cultures (harboring the fluorescent reporter switch and an empty plasmid lacking the *fimE* gene) did not result in generation of DNA segments corresponding to the inverted orientation of *fimS* segment. Thus, there was no detectable switching without expression of plasmid *fimE*. *E. coli* TOP10 cells also still contain *hbiF* in the genome that may cause reverse switching after FimE-mediated inversion of *fimS* segment. However, we did not observe reverse switching in positive control cells (harboring the fluorescent reporter switch and constitutively expressing *fimE*) as it was demonstrated by culture PCR and shown in Figure 3C.

Testing synthetic riboswitches with FimE recombinase reporter system.

Synthetic riboswitches can be useful tools for the development of cell-based biosensors. To demonstrate the feasibility of the FimE recombinase-based system to serve as a reporter for monitoring activation of multiple synthetic riboswitches and, therefore, expand the applicability

of the system, we chose three previously developed synthetic riboswitches responsive to particular analytes (Table 1). These riboswitches were developed using different techniques and demonstrated the ability to up-regulate expression of downstream reporter genes in an analyte-dependent manner in *E. coli* cells. M6 and M6'' riboswitches responsive to ammeline (Amm) and pyrimido[4,5-d]pyrimidine-2,4-diamine (PPDA), respectively, were identified by reengineering natural adenine-sensing *add* A-riboswitch by using a combination of chemical genetics and genetic selection.²⁹⁻³¹ 2A2 riboswitch, responsive to 2,4-dinitrotoluene (DNT), was developed by coupling an *in vitro* selected aptamer with a randomized expression platform followed by screening a created riboswitch library *in vivo*.²⁷ Riboswitch-recombinase systems were constructed as described earlier: each riboswitch was placed upstream of the FimE recombinase coding sequence in one plasmid, and a second plasmid carried a fluorescent reporter composed of two fluorescent protein genes (mKate2 and GFPa1) flanking an invertible DNA segment (*fimS*) containing a constitutively active promoter. If the riboswitch prevents translation of *fimE* without a specific analyte, the fluorescent reporter constitutively expresses green fluorescent protein (GFPa1). Addition of the analyte initiates translation of *fimE* causing unidirectional inversion of the *fimS* segment and constitutive expression of red fluorescent protein (mKate2). Previously, it was noticed that in some cases switching a reporter system causes changes in riboswitch functionality, and functionality can be restored by insertion of a linker consisting of a 5' fragment of the reporter gene that was used for riboswitch selection upstream of a new reporter.^{29, 30} Since the ammeline (AMM)-responsive M6 riboswitch^{29, 30} and pyrimido[4,5-d]pyrimidine-2,4-diamine (PPDA)-responsive M6'' riboswitch³¹ were originally reengineered from natural adenine-sensing *add* A-riboswitch using enhanced green fluorescent protein (eGFP) as a reporter, the first 105 bp of the original *eGFP* sequence were fused in frame to the *fimE* gene to prevent the secondary structure within *fimE* coding sequence from interfering with riboswitch function.^{30, 31} To preserve the functionality of 2,4-dinitrotoluene (DNT)-responsive 2A2 riboswitch²⁷, the first 99 bp of the Tobacco Etch Virus (TEV) protease sequence were fused in frame to the *fimE* gene. Addition of these linkers at the 5'-end of *fimE* coding sequence did not affect the ability of FimE recombinase to perform the inversion of the *fimS* DNA segment with or without the intended riboswitch analyte (Supporting Information Figure S8). It should be noted that earlier described theophylline synthetic riboswitch was successfully used to control expression of a variety of different reporter systems^{26, 53-56} and did not require insertion of an

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3 additional linker between an expression platform and a reporter gene sequence. Relying on
4 structural studies of theophylline synthetic riboswitch²⁶, we assumed that the expression platform
5 prevents riboswitch interaction with the downstream reporter gene sequence suggesting the
6 existence of universal expression platform that can be coupled with analyte binding aptamers
7 possessing structural similarities and result in functional riboswitches. To test the modularity of
8 the expression platform and create a new riboswitch, we replaced the theophylline aptamer in the
9 original theophylline synthetic riboswitch coupled with *fimE* gene, with a previously developed
10 2,4,6-trinitrotoluene (TNT) binding aptamer.⁵⁷
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18 Activation of the created riboswitch constructs was tested in *E. coli* cells by measuring
19 fluorescence intensity of GFPa1 and mKate2 after 18 hours of cell growth in the presence of a
20 specific analyte (Table 1 and Figure 8). Compared to riboswitch cell cultures without the
21 analytes the fluorescence intensity of GFPa1 was either increased (2A2 riboswitch) or did not
22 significantly change (M6, M6'', TNT riboswitches) when cells were treated with appropriate
23 analytes (Figure 8A). The fluorescence intensity of mKate2 measured in the cell cultures
24 harboring M6, M6'' or TNT riboswitch constructs was increased in the presence of appropriate
25 analytes (Figure 8B). In contrast, fluorescence of mKate2 was decreased when cells harboring
26 2A2 riboswitch construct were treated with the analyte (DNT). Thus, riboswitch-recombinase
27 systems showed analyte-dependent performance resulting in different fluorescent outputs of
28 created biosensors. However, only M6, M6'' and TNT riboswitches acted similar to our model
29 system, theophylline synthetic riboswitch, i.e., promoting expression of mKate2 in the presence
30 of a specific analyte.
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42 **Characterization of riboswitch-recombinase systems.**

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45 We chose to further optimize and characterize the systems for the M6 and TNT riboswitches,
46 since M6 and M6'' riboswitches were developed from the same origin.²⁹ To improve the
47 performance of the created riboswitch-recombinase systems we attempted to reduce the
48 fluorescence intensity of mKate2 in riboswitch cell cultures in the absence of the analyte by
49 addition of the nucleotide sequence encoding the LVA protein degradation tag (partsregistry.org)
50 to *mKate2* and increase of the mKate2 degradation rate. Degradation of mKate2 did not affect
51 changes in the fluorescence intensity of GFPa1, however, it reduced fluorescence of mKate2 in
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cell cultures harboring M6 and TNT riboswitches, compared to the system containing mKate2 without a degradation tag (Figure 8 and Supporting Information Figure S9). This resulted in an increased riboswitch activation ratio of 3.4-fold for M6 riboswitch and of 4.6-fold for TNT riboswitch.

Time course study. In order to evaluate the performance of the created biosensors, activation of TNT and M6 riboswitches in *E. coli* cells was measured over time (Figure 9 and Supporting Information Figure S10). Similar to the theophylline synthetic riboswitch, the behavior of TNT and M6 riboswitches was compared to negative control and positive control cells. Negative control cells harbored an empty plasmid, lacking the *fimE* gene, and the fluorescent reporter switch plasmid with the degradation tagged mKate2. Positive control cells contained the same fluorescent reporter switch plasmid and a plasmid containing the FimE recombinase encoding sequence without a riboswitch and under the control of constitutive promoter. As shown in Figure 9A, B and Supporting Information Figure S10A, B, we observed an overtime decrease in fluorescence intensity of GFPa1 and an increase in fluorescence intensity of mKate2 in the positive control and riboswitch cell cultures treated with the analyte. In contrast to negative control cell cultures, which exhibited an increase in the fluorescence intensity of GFPa1 over time, the green fluorescence of riboswitch cell cultures in the absence of the analyte was decreased (similar to riboswitch cell cultures treated with the analyte) due to a background FimE recombinase expression resulting in *fimS* fragment inversion even without the analyte. Coupling mKate2 with a protein degradation tag resulted in decreased red fluorescence and a steady state level of protein expression in riboswitch cell cultures without the analyte at 8 hours' time point. We were able to observe a visual difference between riboswitch cell cultures in the absence and in the presence of the analyte (Figure 9C and Supporting Information Figure S10C), however the fluorescence color switched from yellow (without the analyte) to orange (in the presence of the analyte) instead of green to red, likely due to a background *fimE* expression and mKate2 degradation.

Verification of FimE-catalyzed inversion. In order to confirm that the color switch of the riboswitch-based sensors in the presence of specific analytes occurs due to inversion of the DNA segment containing a constitutive promoter, and that the orientation of the promoter determines which fluorescent protein is produced, the inversion states in riboswitch harboring cells were

assayed using culture PCR with specific primers (Supporting Information Figure S11A). The results of culture PCR for M6 and TNT riboswitches are presented in Supporting Information Figure S11B, C. When a set of primers designed to amplify DNA in the direct orientation was used to probe inversion states in riboswitch cell cultures without the analyte and riboswitch cell cultures in the presence of the analyte, the PCR resulted in a 1.1 kb DNA band after gel electrophoresis. When the same cultures were assayed with a set of primers for the inverted orientation of the *fimS* segment, the PCR also resulted in a 1.1 kb DNA band even at 0 hours' time point. The appearance of the inverted product in riboswitch cell cultures in the absence of the analyte and at 0 hours' time point in cell cultures treated with the analyte was caused by a background expression of *fimE* due to the inability of the riboswitch to completely suppress the expression of the reporter gene when the analyte was not present. However, the intensity of the band related to the inverted *fimS* orientation increased over time in riboswitch cell cultures in the presence of the analyte due to FimE-catalyzed recombination upon riboswitch activation.

Although it was demonstrated that both riboswitches, M6 and TNTs, can induce expression of FimE recombinase in the presence of a specific analyte, we further characterized only TNT riboswitch due to its potential practical application for cell-based biosensing. TNT is one of the most commonly used explosives for military and industrial applications; its detection is important from a security perspective and due to its high toxicity.

Dose-response study. In order to investigate thresholds of the detection for TNT-responsive riboswitch, we examined the fluorescence signal in response to varying concentrations of TNT. As can be observed in Figure 10, the increase in the fluorescence intensity of mKate2 was detected at TNT concentrations down to 25 μ M, which is comparable with threshold of detection previously reported whole-cell biosensors for explosives.^{13, 14} We were not able to use concentrations of TNT higher than 100 μ M due to the toxic effects on cell growth and viability.

Stability of TNT riboswitch-based system. To test if, similar to the theophylline riboswitch, the TNT riboswitch-based system could hold the inverted state of the fluorescent reporter even after the analyte removal, we activated the riboswitch by addition of TNT and then repeatedly subdiluted and grew these cells for 6 days without any further analyte addition (Supporting

Information Figure S12). Since we used a system with a fluorescent reporter containing mKate2 coupled with a protein degradation tag, we observed a decrease in the fluorescence intensity of mKate2 from a new generation of cells (day 2). However, the next new generations of cells were able to maintain this output signal (mKate2 fluorescence) throughout the entire period of growth. We also observed that, similar to theophylline riboswitch-based system, the multiple generations of uninduced cells harboring TNT riboswitch construct maintained the direct state of the fluorescent reporter over the whole time period of cell growth.

Creation of the reversible TNT riboswitch-based sensor. To demonstrate that, similar to the theophylline riboswitch-FimE recombinase system, the TNT riboswitch-based sensor can be reversible, we introduced an HbiF recombinase under control of the arabinose inducible promoter, P_{BAD} . As depicted in Supporting Information Figure S13, induction of translation of FimE recombinase by addition of TNT resulted in increase of fluorescence intensity of mKate2 and, in the same time, decrease of the fluorescence of GFPa1. When diluted cell cultures were treated with arabinose, this promoted an increase of the fluorescence intensity of GFPa1 and decrease of the fluorescence of mKate2. Thus, the cell-based sensor was reversed to its initial state. We also observed a color difference in collected cell cultures depending on the presence of a specific analyte, either TNT or arabinose (Supporting Information Figure S13B).

Conclusions

This work demonstrated a resettable and reusable cell-based sensor that can be reliably activated and reversed many times. Specifically, we have developed a dual color riboswitch-based sensor comprising elements of the *E. coli* fimbriae phase variation system: recombinase FimE controlled by a synthetic riboswitch and an invertible DNA segment (*fimS*) containing a constitutively active promoter placed between two fluorescent protein encoding sequences, green fluorescent protein (GFPa1) and red fluorescent protein (mKate2). Without an analyte, the fluorescent reporter switch constitutively expresses GFPa1. Addition of the analyte activates the riboswitch initiating translation of *fimE* causing unidirectional inversion of the *fimS* segment and constitutive expression of mKate2. In our model system we used a theophylline synthetic riboswitch. We have demonstrated the performance of the created riboswitch-recombinase-based biosensor by monitoring riboswitch activation and fluorescence color changes over time in *E.*

coli cell cultures in the presence of theophylline. Riboswitch activation of FimE recombinase gene expression followed by irreversible inversion and color switch from green (GFPa1) to red (mKate2) resulted in an 11.3-fold increase in the fluorescence intensity of mKate2 in response to theophylline compared to the system without an analyte. Coupling GFPa1 with a protein degradation tag allowed us to improve the performance of the biosensor by increasing riboswitch activation ratio to 37-fold. We have also demonstrated the feasibility of the FimE recombinase-based system to serve as a reporter for monitoring activation of two synthetic riboswitches, M6 and M6", and constructed a functional synthetic riboswitch coupled with a FimE recombinase encoding sequence by replacing theophylline aptamer in original theophylline riboswitch with a 2,4,6-trinitrotoluene (TNT) binding aptamer. Although a new TNT riboswitch may still require some optimization, it can find a practical application for cell-based biosensing due to a high importance of TNT detection.

We have shown that the riboswitch-based dual color system can maintain a stable output memory over many generations of cells after the input is withdrawn. This useful property can enable the construction of sentinel cell-based sensors where instantaneous response is not required and the detection of a specific target (analyte) can be performed after some period of time. We have also demonstrated that the riboswitch-based system can be reversed by introduction of HbiF recombinase. In contrast to FimE, HbiF recombinase catalyzes *fimS* inversion from an "off"-to-"on" orientation. Thus, one recombinase generates inversion sites for another resulting in a reversible multi-state biological switch. The riboswitch-based system can be activated and reversed reliably many times allowing us to build a resettable and reusable cell-based sensor.

In a previously developed reversible integrase excisionase system⁴³, inversion of DNA by integrase alone is unidirectional. However, for a reversible inversion, excisionase should be complexed with an appropriate integrase; this can cause biased directionality of excisionase-mediated recombination resulting in incomplete DNA flipping. Unidirectionality of excisionase-mediated inversion and complete reversibility of an integrase excisionase system can only be achieved by controlling the excisionase-to-integrase ratio and dynamics, and also the integrase-to-DNA target ratio. In our system, FimE and HbiF recombinases function independently catalyzing a unidirectional DNA inversion. For successful performance, the system does not require extensive tuning. FimE and HbiF-based system can be considered as an alternative

simple way to create reversibility in cells. This property adds reusability to the beneficial attributes of cell-based sensors.

In addition, utilizing the recombinase-based system as a mean of riboswitch selection will negate the requirement to re-optimize the riboswitch when a different reporter is necessary, since a new reporter can be placed on either side of *fimS* keeping the riboswitch-FimE construct intact. This will expand the applicability of synthetic riboswitches.

Methods

Reagents, bacterial strains and plasmids. Ampicilline, chloramphenicol, kanamycin, theophylline, 2-aminopurine, 2,4-dinitrotoluene, DMSO, acetonitrile and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO). 4,6-Diamino-2-hydroxy-1,3,5-triazine (ammeline) solution (1,000 mg/L) was purchased from o2si smart solutions (Charleston, SC) and 2,4,6-trinitrotoluene was purchased from Cerilliant (Round Rock, TX). Pyrimido[4,5-d]pyrimidine-2,4-diamine was obtained from 3B Scientific Corporation (Libersville, IL). Phusion DNA polymerase, restriction enzymes and NEBuilder HiFi DNA assembly master mix were purchased from New England Biolabs (Ipswich, MA). The plasmid pHWG640 and the plasmid pSAL8.1 were the generous gifts from Dr. Josef Altenbuchner from the Institute of Industrial Genetics at the University of Stuttgart, Germany, and Dr. Justin Gallivan from Emory University, Atlanta, GA, respectively. The plasmid pHIS8:GFPa1 was kindly provided by Dr. Dimitri Deheyn from Scripps Institution of Oceanography, University of California, San Diego, CA. Theophylline riboswitch sequence upstream of FimE recombinase coding sequence, mKate2 encoding sequence, and *fimS* DNA segment sequence were purchased from DNA2.0 (Menlo Park, CA). PCR primers and gBLOCK DNA fragments were obtained from Integrated DNA Technologies (Coralville, IA).

Plasmid manipulations were performed using MAX Efficiency DH5α chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA). Full descriptions of primer sequences and plasmids construction techniques are available in the Supporting Information. The sequences of all constructs have been verified by DNA sequencing at the Plant-Microbe Genomics facility at The Ohio state University. Confirmed plasmids were transformed into One Shot TOP10 chemically

competent *E. coli* cells (Invitrogen) for expression. Bacteria were maintained at 37 °C in LB broth (Becton, Dickinson and Company, Sparks, MD) and on LB-agar (Becton, Dickinson and Company) plates supplemented with appropriate antibiotics (ampicillin 100 µg/mL, chloramphenicol 25 µg/mL, kanamycin 25 µg/mL).

Time course experiments. For time course studies of theophylline synthetic riboswitch activation, one of the plasmids containing a bidirectional reporter (pHWG640:mKate2-*fimS*-GFPa1 or pHWG640:mKate2-*fimS*-GFPa1LVA) and a FimE protease expressing plasmid (either pSAL:FimE or pSAL:RSFimE) were transformed into chemically competent *E. coli* TOP10 cells. To create a negative control, pHWG640:mKate2-*fimS*-GFPa1 or pHWG640:mKate2-*fimS*-GFPa1LVA and empty pSAL plasmid were transformed into chemically competent *E. coli* TOP10 cells. Three separate colonies of *E. coli* TOP10 cells harboring either the positive control (pHWG640:mKate2-*fimS*-GFPa1 and pSAL:FimE or pHWG640:mKate2-*fimS*-GFPa1LVA and pSAL:FimE), the riboswitch (pHWG640:mKate2-*fimS*-GFPa1 and pSAL:RSFimE or pHWG640:mKate2-*fimS*-GFPa1LVA and pSAL:RSFimE), or the negative control (pHWG640:mKate2-*fimS*-GFPa1 and pSAL or pHWG640:mKate2-*fimS*-GFPa1LVA and pSAL) were picked from LB agar plates containing ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL) and separately grown overnight at 37 °C in 5 mL LB media supplemented with ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL). A 250 µL aliquot of the overnight cultures was used to inoculate 25 mL LB supplemented with ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL). Cultures harboring positive control, riboswitch, or negative control were treated with 2.5 mM theophylline in DMSO, or equivalent volume of DMSO for riboswitch without the analyte. Cultures were grown at 37 °C. OD₆₀₀ and fluorescence levels of mKate2 and GFPa1 were assayed at indicated time points (time zero corresponded to theophylline or DMSO addition) on a SpectraMax M5 Plate Reader (Molecular Devices) set to the appropriate excitation (mKate2, 588 nm; GFPa1, 470 nm) and emission (mKate2, 620 nm; GFPa1, 510 nm) wavelength. To measure changes in the fluorescence intensity of cell pellets, 1 mL aliquots of cell cultures were collected at indicated time points. Cells were harvested by centrifugation at 11000 x g for 3 min, washed with 1 mL 10 mM phosphate-buffered saline, pH 7.4, and resuspended in 200 µL 10 mM phosphate-buffered saline, pH 7.4. Measurements of fluorescence intensity of resuspended cell pellets were performed as described above for the cell cultures.

Dose-response experiments. Dose-response experiments for theophylline and TNT riboswitch-based whole cell biosensors were performed as described above for time course experiments. Theophylline was added to the media at concentrations of 10, 50, 100, 250, 500, 1000, 2500, and 5000 μ M. TNT was added to the media at concentrations of 10, 25, 50, and 100 μ M. OD₆₀₀ and fluorescence levels of mKate2 and GFPa1 were assayed at time 18 h on a SpectraMax M5 Plate Reader (Molecular Devices) set to the appropriate excitation (mKate2, 588 nm; GFPa1, 470 nm) and emission (mKate2, 620 nm; GFPa1, 510 nm) wavelength.

Fluorescence microscopy. For imaging, cultures of *E. coli* TOP10 cells, harboring theophylline riboswitch construct (pHWG640:mKate2-*fimS*-GFPa1 and pSAL:RSFimE or pHWG640:mKate2-*fimS*-GFPa1LVA and pSAL:RSFimE) were grown in the presence of 2.5 mM theophylline, and 1 mL aliquots were collected at indicated time points. Cells were harvested by centrifugation at 11000 x g for 3 min, washed with 1 mL nuclease free water, and resuspended in 200 μ L nuclease free water. OD₆₀₀ of resulted cell suspensions were measured and adjusted to OD₆₀₀ of cells collected at time zero (corresponded to theophylline addition) by appropriate dilutions with water. 100 μ L aliquots of obtained cell suspensions were placed on glass slides and dried overnight; 10 μ L nuclease free water were added to the dried cells, and cover slips were placed over water drops. Fluorescence microscopy images were taken using an Olympus IX71 inverted microscope.

Culture PCR. An orientation of the DNA segment containing a constitutive promoter in a fluorescent reporter switch plasmid was determined by PCR amplification using three primers mKate2rv (5'- GTGTCGACTTATTAGCGGTGGCCCAGCTTG -3'), FimSrv (5'- CTGTCCATATCATAAATAAGTTACGTATTTTTTCTCAAGC -3'), and FimSfw (5'- TGACTCATAGAGGAAAGCACGCGAACA-3') in two sets (Figure 3A and Supporting Information Figure S11). For DNA templates, 50 μ L aliquots were taken from negative control, positive control, or riboswitch cell cultures at indicated time points; the cells were harvested by centrifugation at 11000 x g for 3 min, resuspended in water to achieve OD₆₀₀ of 0.05. The culture PCR was performed as follows. A PCR master mix (containing 5 μ L 5X Phusion HF buffer, 2 μ L 2.5 mM dNTPs, 0.25 μ L Phusion High-Fidelity DNA polymerase, 1 μ L 5 μ M primers in 10 μ L nuclease free water) was mixed with 5 μ L cell suspension. The PCR cycling parameters were

98 °C for 30 seconds, 20 cycles of (98 °C for 10 seconds, 68 °C for 50 seconds, and 72 °C for 36 seconds), 72 °C for 10 minutes, then hold at 4 °C.

Maintenance of stable output memory in riboswitch-based system. To test the stability of theophylline riboswitch-based systems over multiple cell generations, overnight cultures of *E. coli* TOP10 cells harboring the riboswitch (pHWG640:mKate2-*fimS*-GFPa1 and pSAL:RSFimE or pHWG640:mKate2-*fimS*-GFPa1LVA and pSAL:RSFimE) were inoculated into fresh medium (50 µL of culture into 5 mL LB) supplemented with ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL). Cell cultures harboring pHWG640:mKate2-*fimS*-GFPa1 and pSAL:RSFimE or pHWG640:mKate2-*fimS*-GFPa1LVA and pSAL:RSFimE were treated with 2.5 mM theophylline in DMSO or equivalent volume of DMSO for riboswitch without the analyte. Then, cell cultures were repeatedly grown and diluted 1:100 every day in medium without the analyte for 6 days (or 8 days for cells containing pHWG640:mKate2-*fimS*-GFPa1LVA and pSAL:RSFimE). The ability of the cells to maintain their state was monitored by measuring fluorescence intensity of mKate2 in the cell cultures as described for time course experiments.

Testing of the reversible switch. To create a theophylline riboswitch-based reversible switch, three plasmids containing a bidirectional reporter, pHWG640:mKate2-*fimS*-GFPa1, a riboswitch construct, pSAL:RSFimE, and a HbiF recombinase encoding sequence under control of arabinose inducible promoter, pBAD:HbiF, were co-transformed into chemically competent *E. coli* TOP10 cells. Three separate colonies of *E. coli* TOP10 cells were picked from LB agar plates containing ampicillin (100 µg/mL), kanamycin (25 µg/mL) and chloramphenicol (25 µg/mL) and separately grown overnight at 37 °C in 5 mL LB media supplemented with the same antibiotics. A 50 µL aliquot of the overnight cultures was used to inoculate 5 mL LB supplemented with appropriate antibiotics. Cultures were treated with 2.5 mM theophylline in DMSO, or equivalent volume of DMSO for control with riboswitch without the analyte. Cultures were grown at 37 °C with shaking for 16 hours. Theophylline treated cultures were inoculated into fresh medium (50 µL of culture into 5 mL LB) supplemented with antibiotics, and expression of HbiF recombinase was induced with 0.2% arabinose. The cultures were grown at 37 °C with shaking for 16 hours. Control cultures were grown without the inducer. Arabinose treated cultures were inoculated into fresh medium (50 µL of culture into 5 mL LB)

supplemented with appropriate antibiotics, and FimE expression was induced with 2.5 mM theophylline. Cell cultures were grown for 16 hours; dilutions and treatments with appropriate inducers were repeated. In total, cell cultures were consecutively treated four times with theophylline (for riboswitch activation and expression of FimE recombinase) and four times with arabinose (for induction of an HbiF recombinase expression). OD₆₀₀ and fluorescence levels of mKate2 and GFPa1 were assayed after 16 hours of cell growth with an appropriate inducer on a SpectraMax M5 Plate Reader (Molecular Devices) set to the appropriate excitation (mKate2, 588 nm; GFPa1, 470 nm) and emission (mKate2, 620 nm; GFPa1, 510 nm) wavelength. Measuring changes in the fluorescence intensity of cell pellets was performed as described for time course experiments.

Supporting Information

Full descriptions of primer sequences, plasmids construction techniques, and supporting information figures (S1 – S13). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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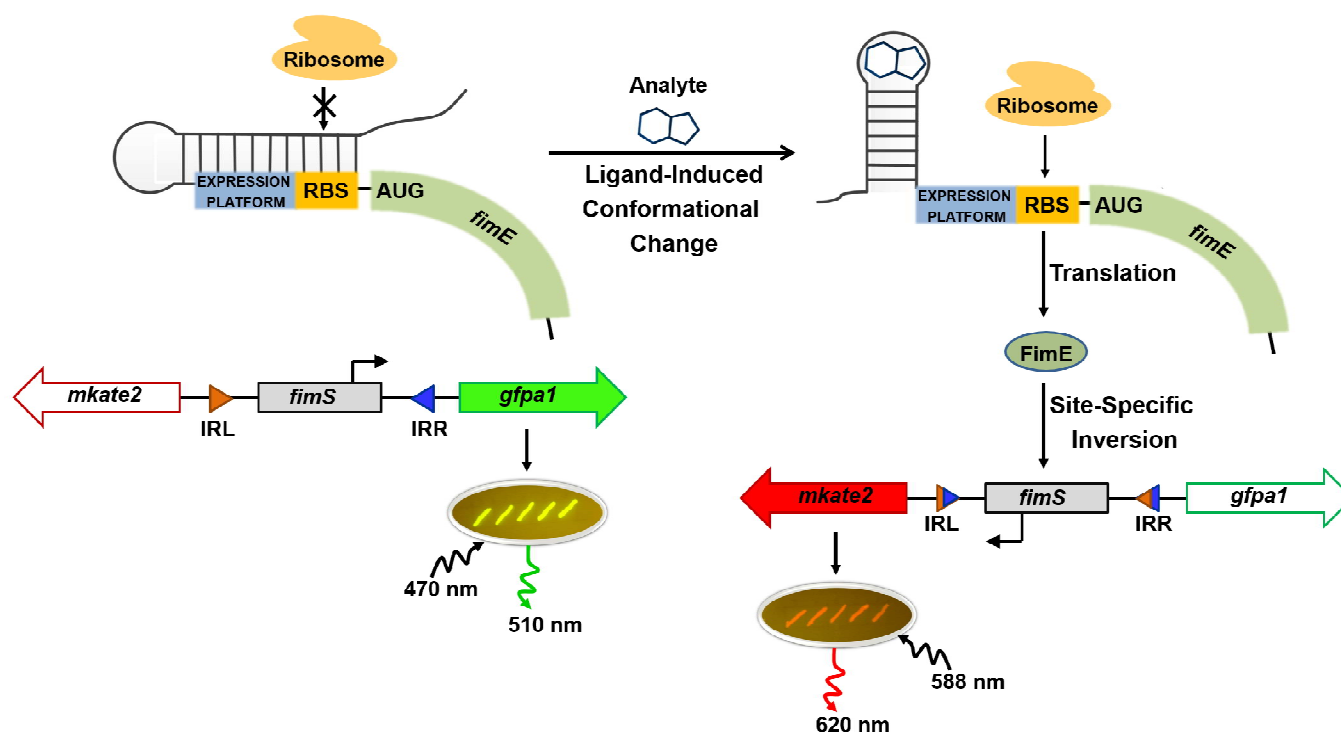


Figure 1. Riboswitch-based reporter system for dual-color detection of a small molecule analyte. A RNA-based 'sensor', a synthetic riboswitch, is placed upstream of the FimE recombinase encoding sequence in one plasmid. The second plasmid encodes a fluorescent reporter switch composed of two fluorescent protein genes (*mKate2* and *GFPa1*) and an invertible DNA segment (*fimS*) containing a constitutively active promoter. The *fimS* segment is flanked with two FimE recombinase binding sites, Inverted Repeat Left and Inverted Repeat Right, (IRL and IRR, respectively) and placed between two fluorescent protein genes. Without an analyte, extensive pairing of the riboswitch with the ribosome binding site (RBS) prevents translation of FimE recombinase; *fimS* is oriented toward IRR, and the fluorescent reporter switch constitutively expresses green fluorescent protein (GFPa1). Addition of theophylline reveals the RBS and initiates translation of FimE recombinase causing unidirectional inversion of the *fimS* segment toward IRL and constitutive expression of red fluorescent protein (mKate2).

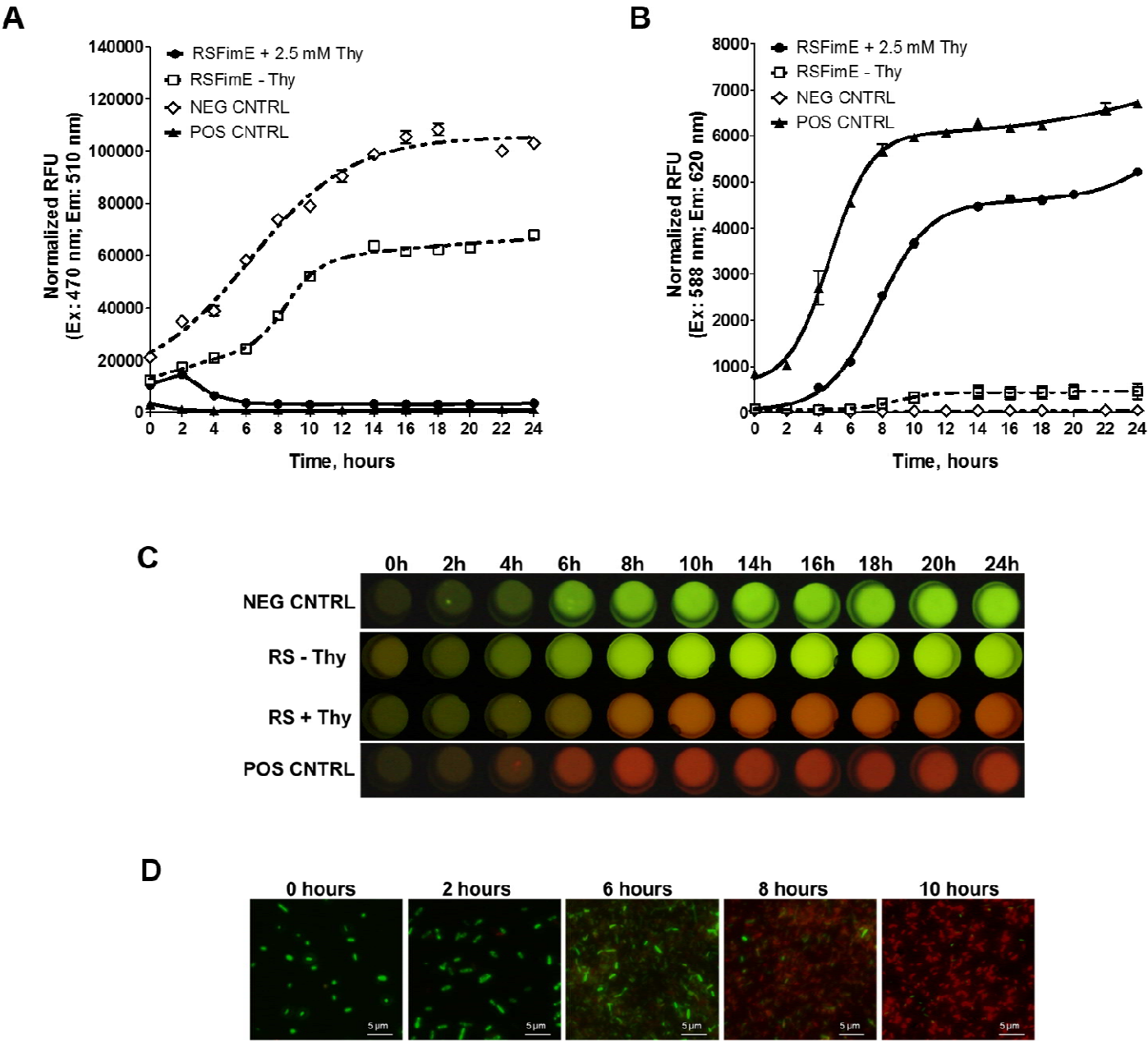


Figure 2. Riboswitch activation and fluorescence changes over time in response to theophylline addition. (A) Emission at 510 nm (emission of GFPa1) or (B) emission at 620 nm (emission of mKate2) were monitored in cell cultures of TOP10 *E. coli* cells harboring a fluorescent reporter switch, pHWG640:mKate2-*fimS*-GFPa1, and one of the following constructs: pSAL:FimE – FimE under control of constitutive promoter (POS CNTRL) (black triangles); empty pSAL plasmid (NEG CNTRL) (white diamonds); pSAL:RSFimE – riboswitch-FimE recombinase (RSFimE - Thy) (white squares); riboswitch-FimE recombinase in the presence of theophylline (RSFimE + 2.5 mM Thy) (black circles). Theophylline

(Thy) was added at time 0, and samples were collected at indicated time points. The fluorescence data were normalized relative to OD₆₀₀ values at the time of harvesting. The studies were performed as three experiments conducted in triplicates, and data are presented as the mean $\pm$ SEM. (C) To visualize fluorescence changes due to theophylline dependent riboswitch activation, images of cell cultures without theophylline treatment (RS - Thy) and cells exposed to theophylline (RS + Thy) were taken using a FUJIFILM digital camera and Dark Reader transilluminator (Clare Chemical Research). For visual comparisons, images of negative (NEG CNTRL) and positive (POS CNTRL) control cell cultures were also taken. (D) Fluorescence microscopy images of TOP10 *E. coli* cells harboring fluorescent reporter and riboswitch-FimE encoding plasmids and treated with 2.5 mM theophylline were taken. Increase in the population of 'red cells' and decrease in the population of 'green cells' occurs over time.

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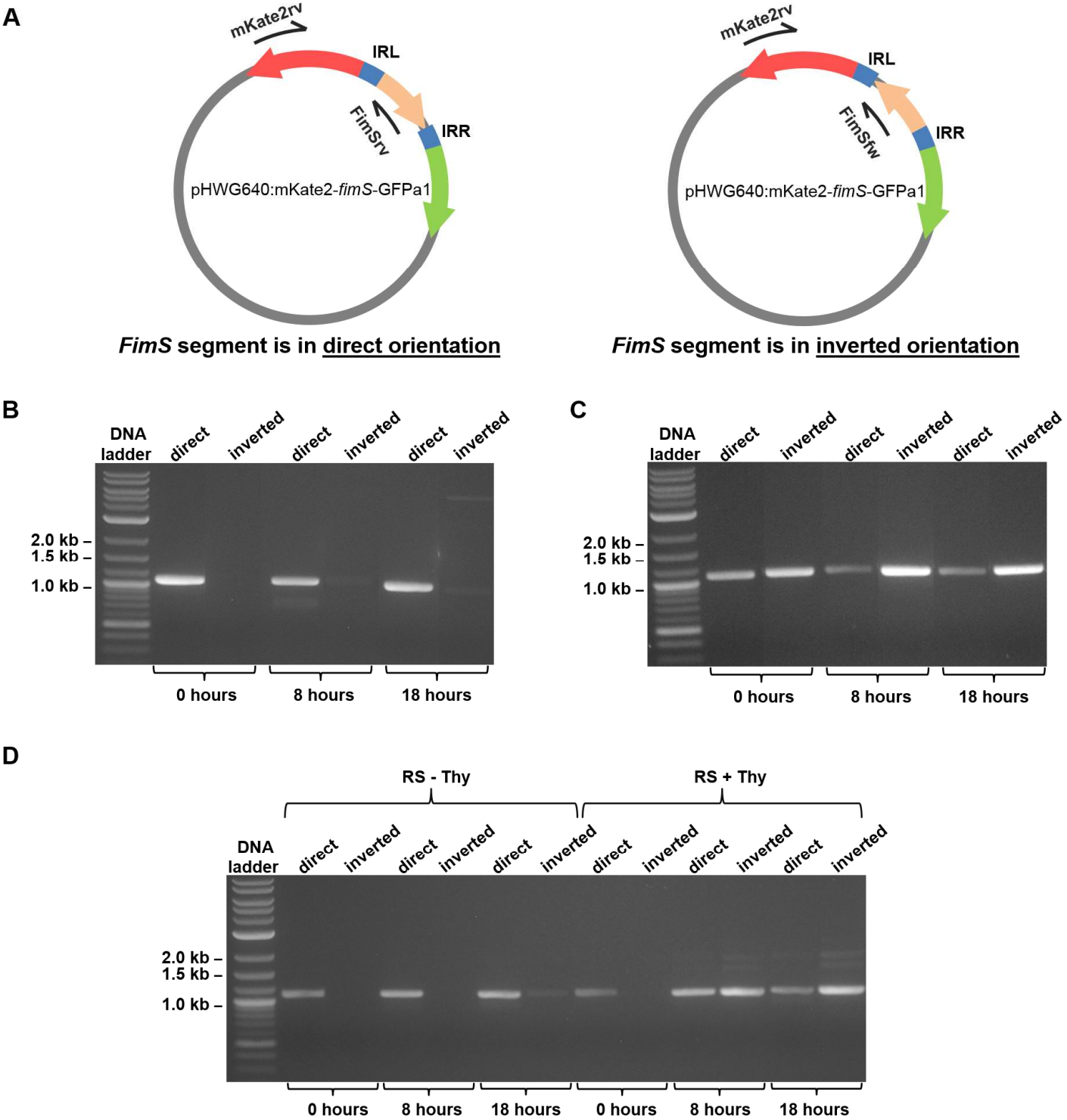


Figure 3. Culture PCR results for verification of FimE-catalyzed *fimS* segment inversion. Two sets of primers were used to examine an orientation of the promoter sequence in a fluorescent reporter switch (A). A 1% agarose gel shows PCR amplification products of negative control (B), positive control (C) or

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3 riboswitch (D) cell cultures collected at the indicated time points. Riboswitch was activated with 2.5 mM
4 theophylline. To determine the orientation of the promoter, two PCRs with two sets of specific primers
5 were performed for each culture.
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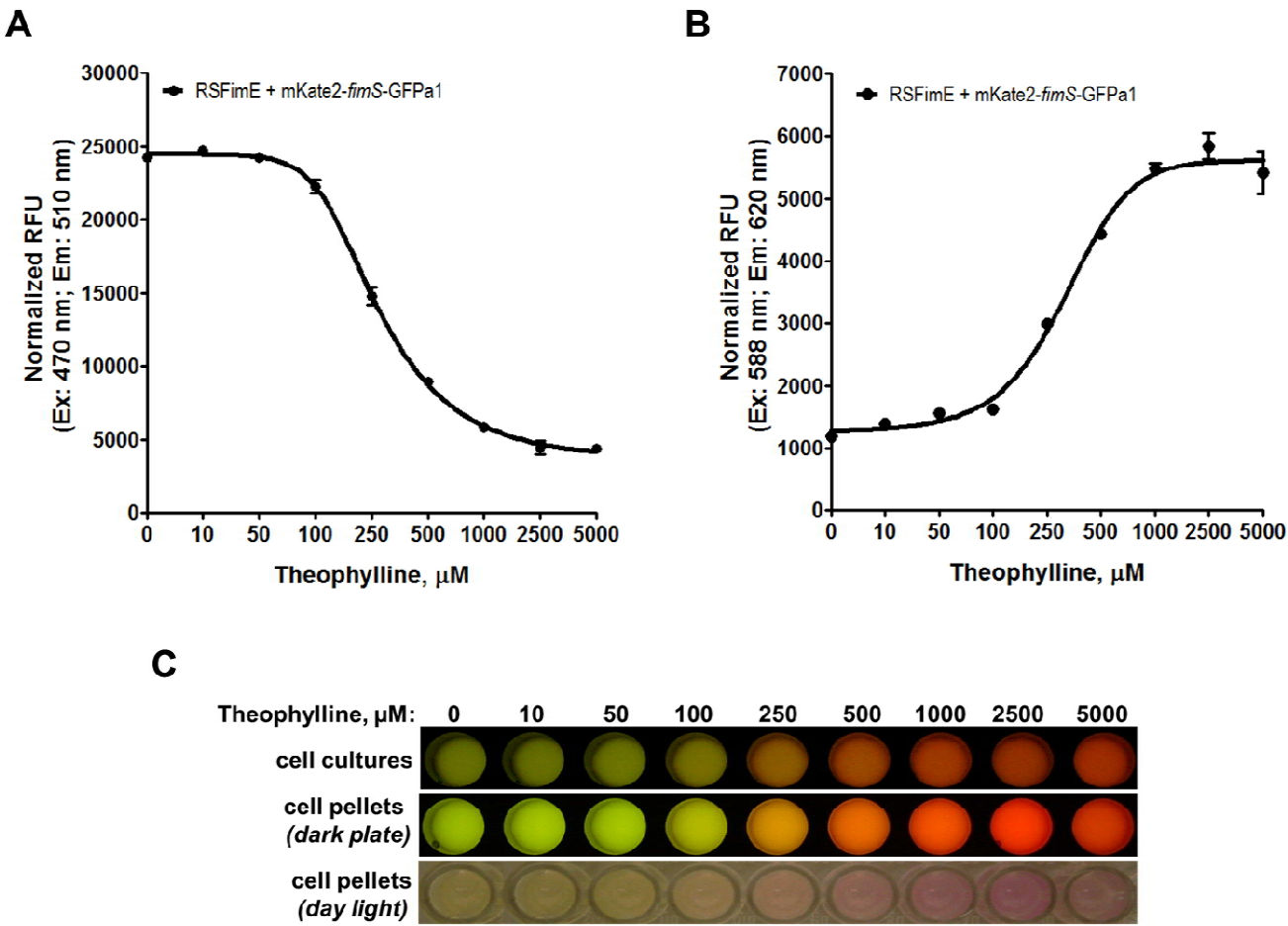


Figure 4. Dose-response study of theophylline riboswitch activation and fluorescence changes. (A) Emission at 510 nm (emission of GFPa1) or (B) emission at 620 nm (emission of mKate2) were monitored in cell cultures after activation of riboswitch with theophylline concentrations ranging from 10 to 5000 μM . The fluorescence data were normalized relative to OD_{600} values at the time of harvesting. The studies were performed as three experiments conducted in triplicates, and data are presented as the mean $\pm$ SEM. (C) To visualize fluorescence changes in response to varying concentrations of theophylline, images of cell cultures and cell pellets were taken using a FUJIFILM digital camera and a Dark Reader transilluminator (Clare Chemical Research).

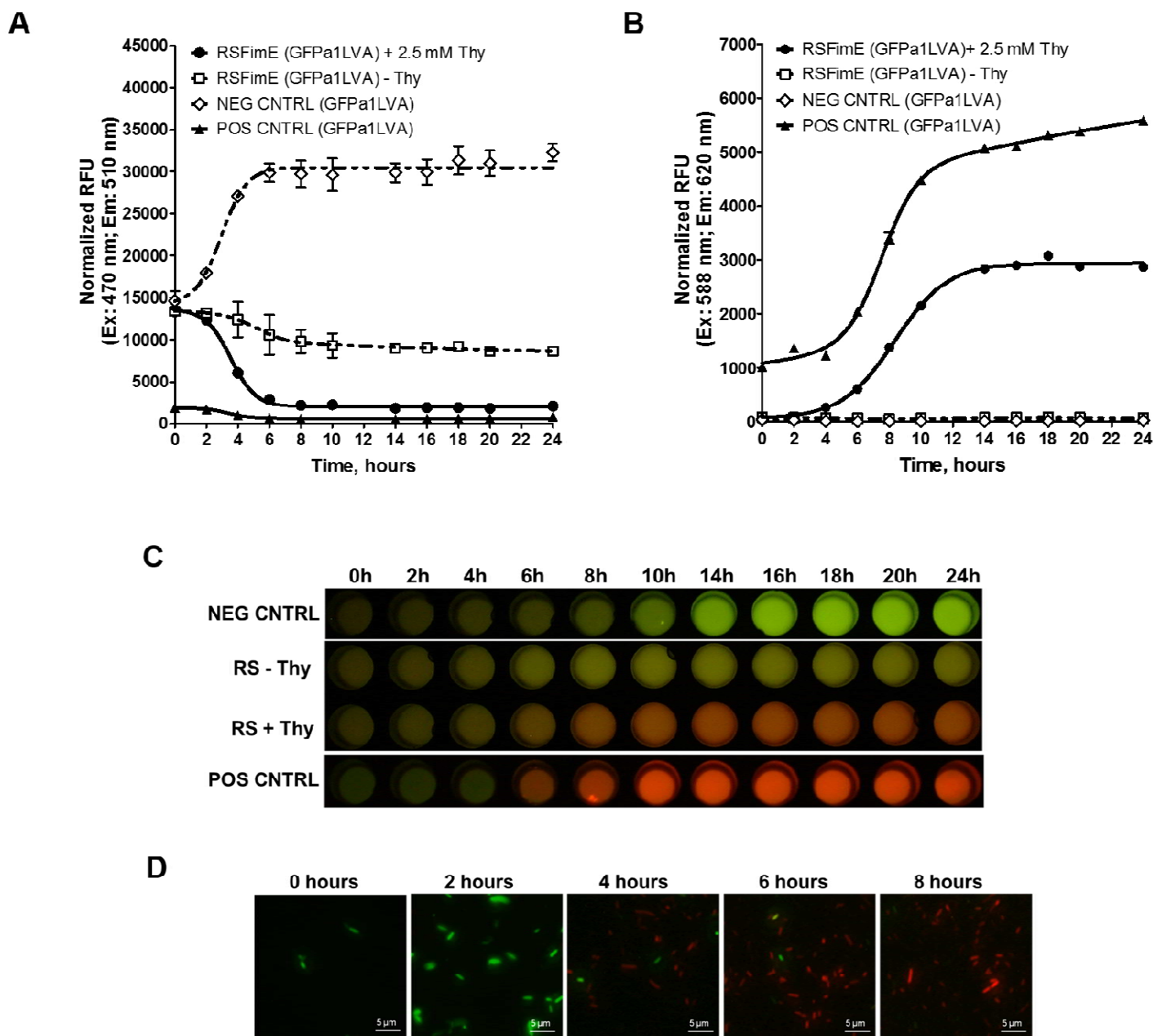


Figure 5. Time course study of riboswitch-activated changes in fluorescence intensity and color in response to theophylline in cells containing a degradation-tagged reporter. (A) Emission at 510 nm (emission of GFPa1) or (B) emission at 620 nm (emission of mKate2) were monitored in cell cultures of TOP10 *E. coli* cells harboring a fluorescent reporter switch, pHWG640:mKate2-*fimS*-GFPa1LVA, and one of the following constructs: pSAL:FimE – FimE under control of constitutive promoter (POS CNTRL) (black triangles); empty pSAL plasmid (NEG CNTRL) (white rhombs); pSAL:RSFimE – riboswitch-FimE recombinase (RSFimE - Thy) (white squares); riboswitch-FimE recombinase in the presence of theophylline (RSFimE + 2.5 mM Thy) (black circles). Theophylline (Thy) was added at time

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0, and samples were collected at indicated time points. The fluorescence data were normalized relative to OD₆₀₀ values at the time of harvesting. The studies were performed as three experiments conducted in triplicates, and data are presented as the mean ± SEM. (C) To visualize fluorescence changes, images of cell cultures without theophylline treatment (RS - Thy) and cells exposed to theophylline (RS + Thy) were taken using a FUJIFILM digital camera and a Dark Reader transilluminator (Clare Chemical Research). For visual comparisons, images of negative (NEG CNTRL) and positive (POS CNTRL) control cell cultures were also taken. (D) Fluorescence microscopy images of TOP10 *E. coli* cells harboring fluorescent reporter and riboswitch-FimE encoding plasmids and treated with 2.5 mM theophylline were taken. An increase in the population of ‘red cells’ and a decrease in the population of ‘green cells’ occurs over time.

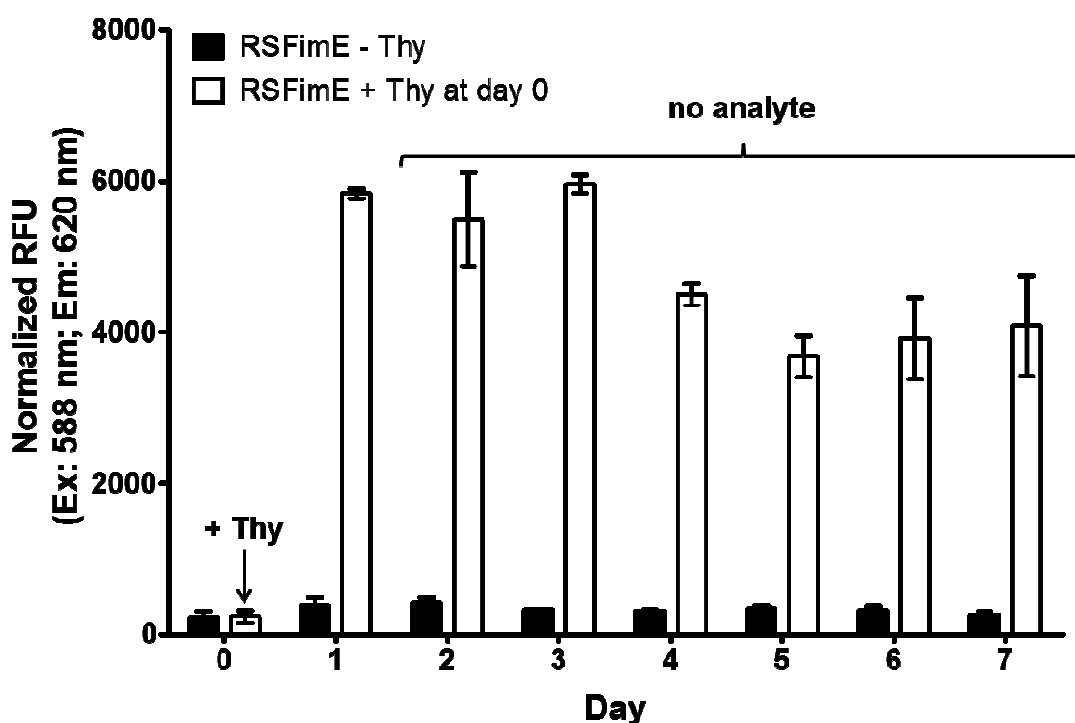


Figure 6. Maintenance of stable output memory in theophylline riboswitch-based system over multiple cell generations. Emission at 620 nm (emission of mKate2) was monitored in cell cultures of TOP10 *E. coli* cells harboring a fluorescent reporter switch, pHWG640:mKate2-*fimS*-GFPa1, and a riboswitch construct, pSAL:RSFimE. Riboswitch was activated at day 0 by addition of 2.5 mM theophylline (Thy), and cells were grown for 1 day. After day 1, the cells were continuously diluted and grown without an analyte for 6 more days. Uninduced cells (RS - Thy) were also repeatedly subdiluted and grown for the same period of time. The fluorescence data were normalized relative to OD₆₀₀ values at the time of harvesting. The studies were performed as two repeat experiments conducted in triplicates, and data are presented as the mean $\pm$ SEM.

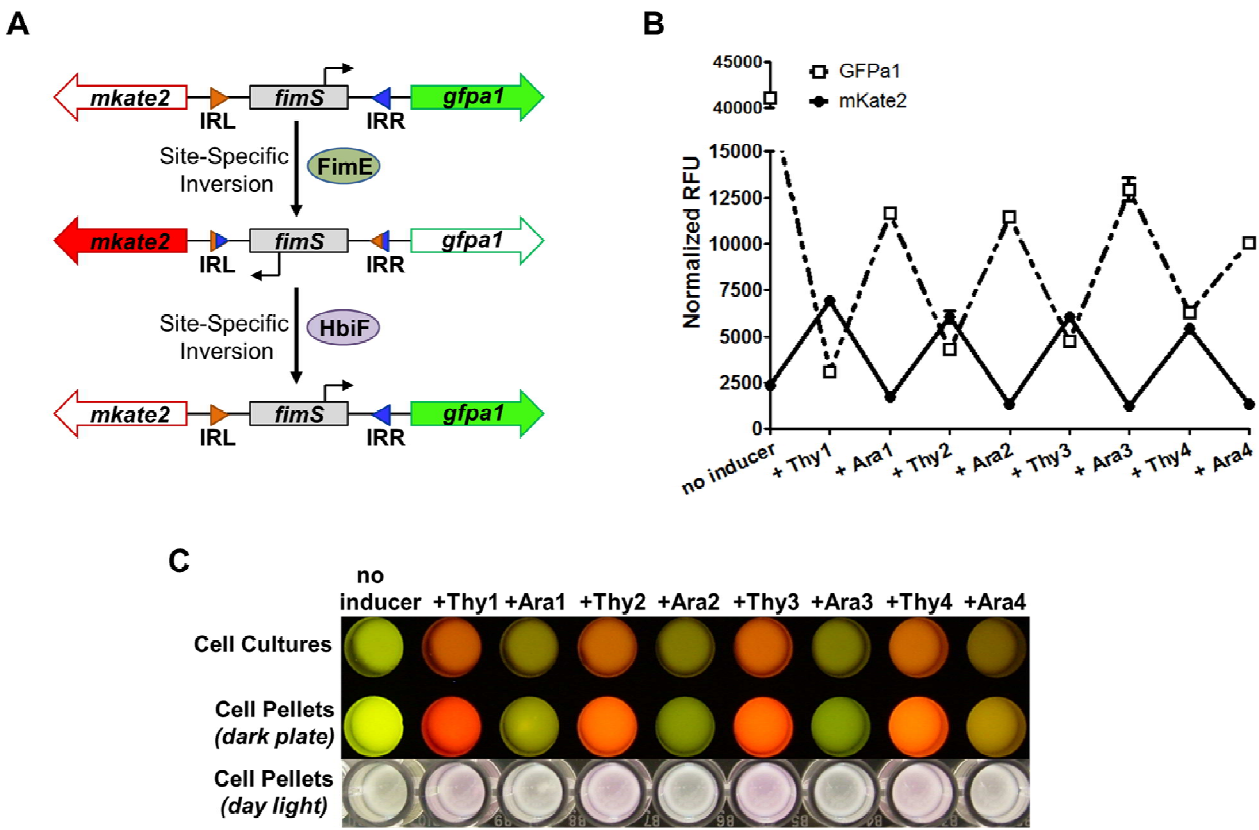


Figure 7. Construction and characterization of reversible dual color switch. (A) Schematic representation of FimE- and HbiF-mediated inversions of *fimS* DNA segment. Initially, the invertible *fimS* segment is oriented to IRR constitutively driving transcription of *gfpa1*. When FimE recombinase is produced, it facilitates the inversion of *fimS* toward IRL resulting in loss of *gfpa1* transcription and constitutive expression of *mkate2*. Production of HbiF recombinase results in site-specific inversion of *fimS* toward IRR preventing transcription of *mkate2* mRNA and restoring expression of *gfpa1*. (B) Activation and reversibility of the riboswitch-based sensor. Emission at 510 nm (emission of GFPa1) and emission at 620 nm (emission of mKate2) were monitored in cell cultures of TOP10 *E. coli* cells harboring fluorescent reporter switch (pHWG640:mKate2-*fimS*-GFPa1), riboswitch construct (pSAL:RSFimE), and plasmid containing *hbif* encoding sequence under control of arabinose inducible promoter (pBAD:HbiF), after continuous addition of a specific analyte, 2.5 mM theophylline (Thy) or 0.2% arabinose (Ara). Cell cultures were diluted before the addition of each analyte and then grown for 16 h. The fluorescence data were normalized relative to OD₆₀₀ values at the time of harvesting. The studies were performed as two repeat experiments conducted in triplicates, and data are presented as the mean $\pm$ SEM. (C) Images of cell cultures and resuspended cell pellets alternately treated with theophylline and arabinose. Images were taken using a FUJIFILM digital camera and a Dark Reader transilluminator (Clare Chemical Research).

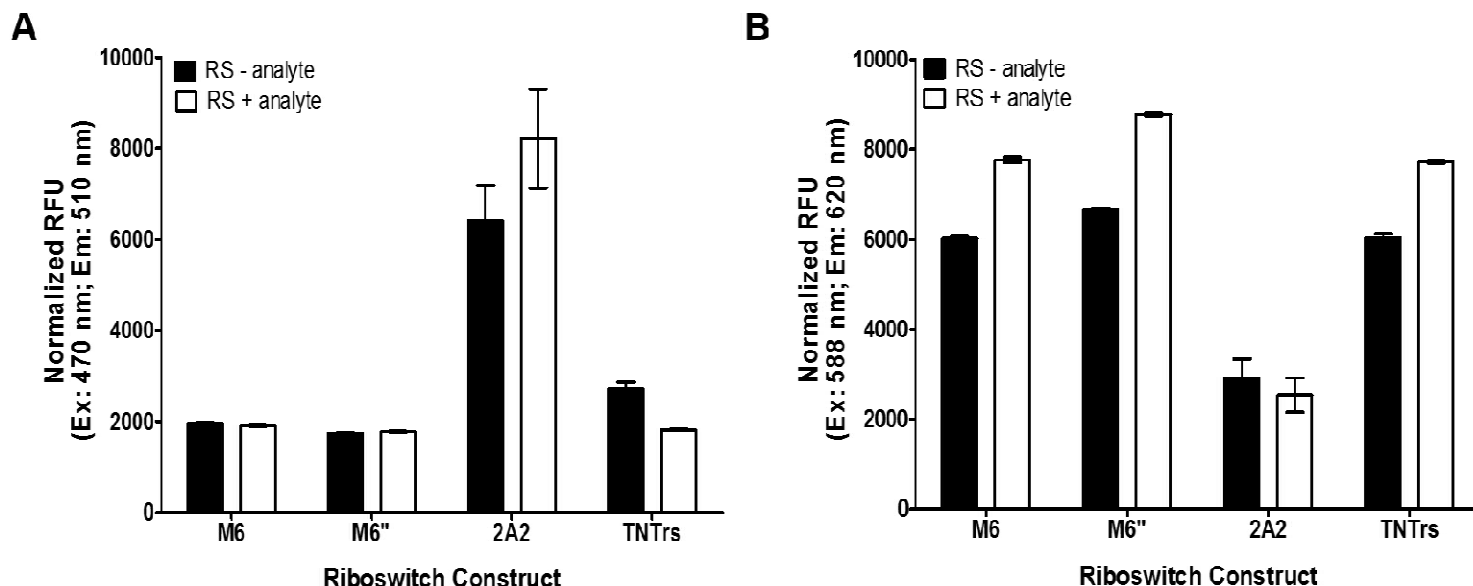


Figure 8. Testing synthetic riboswitches with FimE recombinase reporter system. (A) Emission at 510 nm (emission of GFPa1) or (B) emission at 620 nm (emission of mKate2) were monitored in cell cultures of TOP10 *E. coli* cells harboring a fluorescent reporter switch, pHWG640:mKate2-*fimS*-GFPa1, and one of the following constructs: pSAL:M6FimE – ammeline (Amm) riboswitch (M6); pSAL:M6''FimE – pyrimido[4,5-d]pyrimidine-2,4-diamine (PPDA) riboswitch (M6''); pSAL:2A2FimE, – 2,4-dinitrotoluene (DNT) riboswitch (2A2); and pSAL:TNTrsFimE – 2,4,6-trinitrotoluene (TNT) riboswitch. *E. coli* cells were grown for 18 hours without an analyte (RS - analyte) (black bars) or in the presence of appropriate analyte (RS + analyte) (white bars). Added analyte concentrations were 0.5 mM Amm, 0.5 mM PPDA, 0.5 mM DNT, and 0.1 mM TNT. The fluorescence data were normalized relative to OD₆₀₀ values at the time of harvesting. The studies were performed as six experiments conducted in duplicates, and data are presented as the mean $\pm$ SEM.

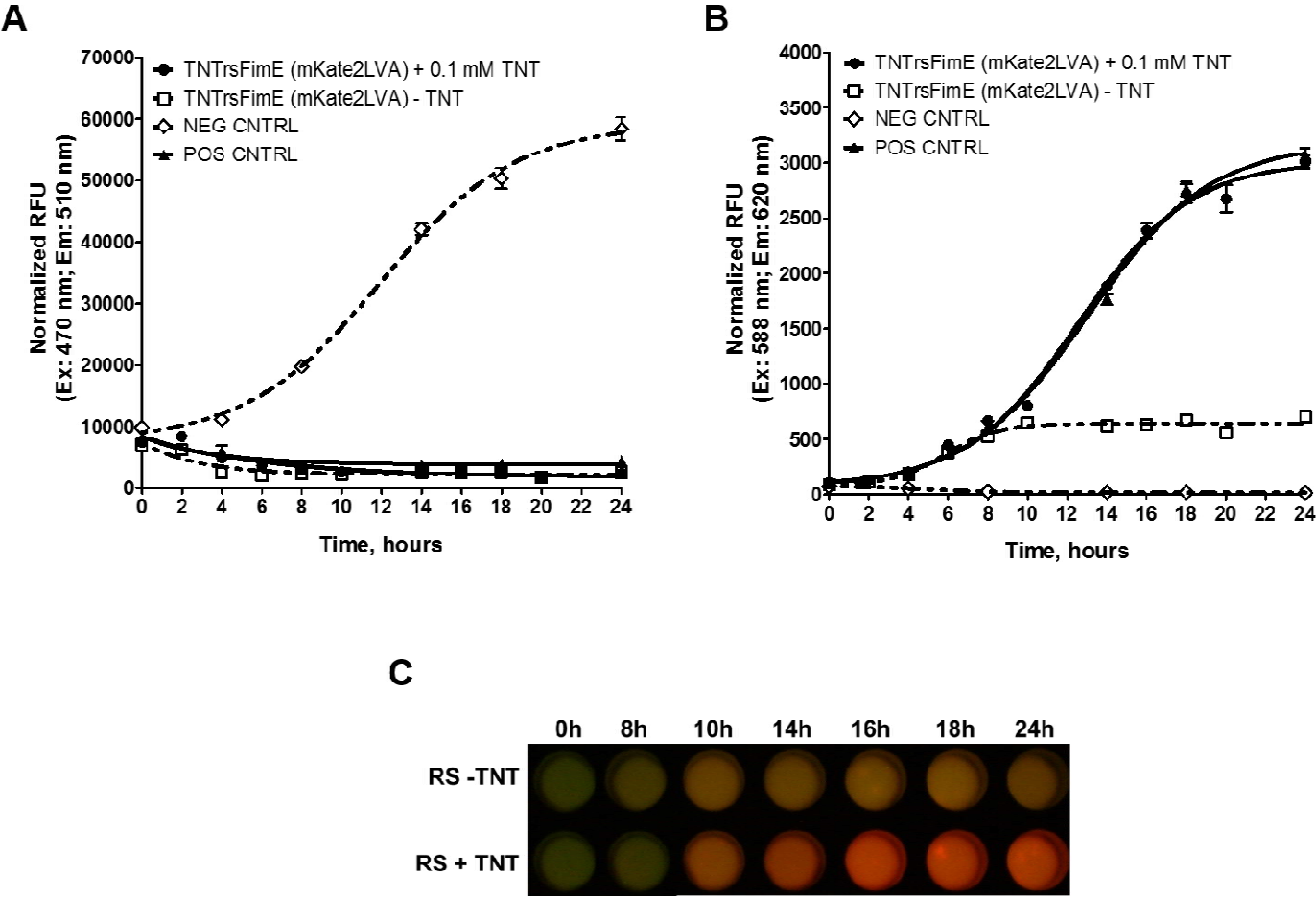


Figure 9. Time course study of TNT riboswitch activation and fluorescence changes in response to addition of 2,4,6-trinitrotoluene. (A) Emission at 510 nm (emission of GFPa1) or (B) emission at 620 nm (emission of mKate2) were monitored in cell cultures of TOP10 *E. coli* cells harboring a fluorescent reporter switch, pHWG640:mKate2LVA-*fimS*-GFPa1, and one of the following constructs: pSAL:FimE – FimE under control of constitutive promoter (POS CNTRL) (black triangles); empty pSAL plasmid (NEG CNTRL) (white rhombs); pSAL:TNTrsFimE – riboswitch in the absence of 2,4,6-trinitrotoluene (TNT) (TNTrsFimE - TNT) (white squares); riboswitch in the presence of TNT (TNTrsFimE + 0.1 mM TNT) (black circles). TNT was added at time 0, and samples were collected at indicated time points. The fluorescence data were normalized relative to OD₆₀₀ values at the time of harvesting. The studies were performed as three experiments conducted in triplicates, and data are presented as the mean ± SEM. (C) To visualize fluorescence changes, images of cell cultures without TNT treatment (RS - TNT) and cells exposed to TNT (RS + TNT) were taken using a FUJIFILM digital camera and a Dark Reader transilluminator (Clare Chemical Research).

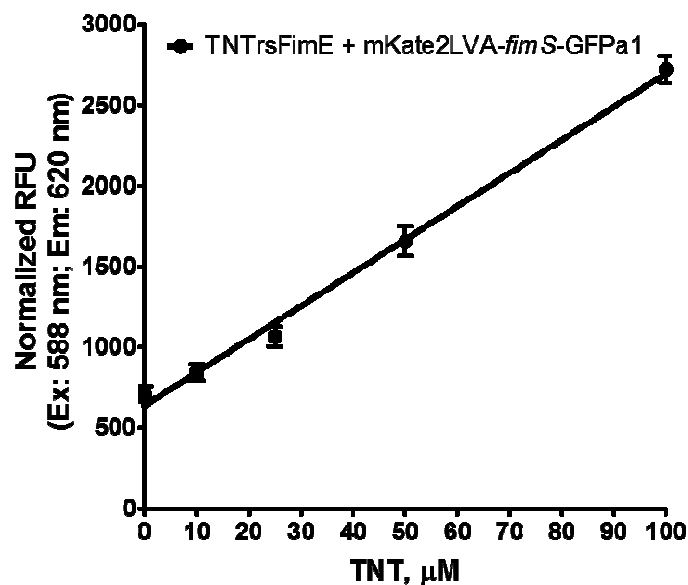


Figure 10. Dose-response study of TNT riboswitch activation. Emission at 620 nm (emission of mKate2) was monitored in cell cultures after activation of riboswitch with TNT concentrations ranging from 0 to 100 μM . The fluorescence data were normalized relative to OD_{600} values at the time of harvesting. The studies were performed as two repeat experiments conducted in triplicates, and data are presented as the mean $\pm$ SEM.

Riboswitch	Analyte	Reference
M6	Ammeline (Amm)	49, 50
M6"	pyrimido[4,5-d] pyrimidine-2,4-diamine (PPDA)	49, 51
2A2	2,4-dinitrotoluene (DNT)	27
TNTrs	2,4,6-trinitrotoluene (TNT)	this study

Table 1. Synthetic riboswitches tested with FimE recombinase reporter system.