Chapter 8

Live Cell Imaging Using Riboswitch-Spinach tRNA Fusions as Metabolite-Sensing Fluorescent Biosensors

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Abstract

The development of fluorescent biosensors is motivated by the desire to monitor cellular metabolite levels in real time. Most genetically encodable fluorescent biosensors are based on receptor proteins fused to fluorescent protein domains. More recently, small molecule-binding riboswitches have been adapted for use as fluorescent biosensors through fusion to the in vitro selected Spinach aptamer, which binds a pro-fluorescent, cell-permeable small molecule mimic of the GFP chromophore, DFHBI. Here we describe methods to prepare and analyze riboswitch-Spinach tRNA fusions for ligand-dependent activation of fluorescence in vivo. Example procedures describe the use of the Vc2-Spinach tRNA biosensor to monitor perturbations in cellular levels of cyclic di-GMP using either fluorescence microscopy or flow cytometry. The relative ease of cloning and imaging of these biosensors, as well as their modular nature, should make this method appealing to other researchers interested in utilizing riboswitch-based biosensors for metabolite sensing.

Key words Fluorescence imaging, Fluorescence microscopy, Flow cytometry, RNA biosensor, Cyclic dinucleotide, Cyclic di-GMP, Vc2 riboswitch, Spinach aptamer

1 Introduction

Fluorescent biosensors provide a measureable output that changes in real time in response to binding of a specific small molecule, making them ideal tools for quantifying and tracking cellular signals in vivo. Most biosensors employed in live cell imaging utilize Förster resonance energy transfer (FRET) between two fluorescent proteins fused to a receptor protein [1]. However, many small molecule metabolites and signals exist for which no corresponding protein receptor is known. Furthermore, engineering a biosensor from a natural protein receptor that produces a large change in FRET signal remains difficult.

Riboswitches are natural RNA-based receptors for metabolites and signaling molecules that bind cognate ligands with high selectivity and affinity, making them strong candidates as biosensor scaffolds [2]. Previously, reporter systems have been developed

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using riboswitches to control the expression of a downstream reporter gene such as β-galactosidase or GFP [3]. These systems have the advantage of providing signal amplification, but the read-out of metabolite concentrations is indirect and not responsive in real-time.

The recent development of the Spinach aptamer [4] has enabled the generation of RNA-based fluorescent biosensors that function in vivo. Spinach is an in vitro selected RNA aptamer that binds to 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), a pro-fluorescent small molecule analog of the GFP chromophore. The Spinach aptamer forms a G-quadruplex motif structure that binds DFHBI and shields the chromophore from solvent- and rotation-mediated forms of energetic decay, causing a 1,000-fold increase in fluorescence [5, 6]. Furthermore, it was shown that DFHBI binding and fluorescence activation by Spinach can be modulated by controlling the formation of the P2 stem with a small molecule-binding aptamer fused to this stem [7]. Following this strategy, we have used a natural riboswitch aptamer to develop a fluorescent biosensor for cyclic di-GMP [8, 9], which is a key second messenger in bacteria that regulates processes including biofilm formation, cell cycle progression, and virulence [10].

Here we describe the application of an RNA-based biosensor for live cell imaging of the bacterial second messenger cyclic di-GMP. We demonstrate two methods, fluorescence microscopy and flow cytometry, to analyze changes in cyclic di-GMP levels in *Escherichia coli* upon co-expression of the biosensor and diguanylate cyclase enzymes. These procedures are expected to be generalizable for other Spinach-based biosensors developed for different cellular metabolites.

2 Materials

2.1 Equipment and Supplies

1. Micropipettor.
2. Vortex mixer.
3. Microcentrifuge.
4. Millipore water filter with a BioPak unit.
5. Micropipettor tips.
6. 1.5 mL microcentrifuge tubes.
7. Sterile filter units.
8. PCR thermocycler.
9. 14 mL Culture tubes and petri dishes.
10. Incubator shaker set to 37 °C.
11. VWR VistaVision Cover Glasses No. 1½.
12. VWR VistaVision Microscope Slides.

13. Zeiss 200M AxioVert microscope (Zeiss, Jena, Germany) equipped with a mercury light source X-Cite 120 Series (Exfo Life Science Divisions, Ontario, Canada), a 63×/1.4 Plan-Apochromat oil DIC objective lens and a 1.6× tube lens. For monitoring fluorescence, a GFP filter set with an excitation 470/40 BP, FT 495 beamsplitter, and emission 525/50 BP was used (Filter Set 38 HE).
14. ImageJ data analysis software.
15. 5 mL Falcon polypropylene tubes with cell-strainer cap.
16. BD Influx v7 cell sorter with BD FACS Sortware (Version 1.0.0.650).

2.2 Reagents

1. Phusion DNA polymerase with 5× HF and 5× GC buffers (NEB).
2. 10× dNTPs (2 mM each ATP, CTP, GTP, TTP).
3. 1× TAE buffer: 40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.4.
4. 1 % agarose solution: 1 g agarose in 100 mL 1× TAE buffer.
5. 2-log DNA ladder (NEB).
6. Gel extraction or PCR cleanup kits.
7. Restriction enzymes: BglII & XhoI with NEBuffer 3.1 or EagI-HF & SacII with NEB CutSmart buffer along with NdeI & XhoI with NEB CutSmart buffer.
8. Calf intestinal alkaline phosphatase (Fisher).
9. T4 DNA ligase with 10× ligase buffer (NEB).
10. Luria Broth (LB, Fisher Scientific).
11. *E. coli* TOP10 chemically competent cells (Life Technologies).
12. LB/agar.
13. Carbenicillin: 50 mg/mL stock concentration, filtered through a 0.2 μm nitrocellulose filter.
14. Kanamycin: 50 mg/mL stock concentration, filtered through a 0.2 μm nitrocellulose filter.
15. pET31b(+) plasmid.
16. pET24a or pCOLADuet-1 plasmid.
17. 40 μM stock concentration of the following DNA oligonucleotide primers (**BOLD** = T7 promoter; UNDERLINED = tRNA scaffold; CAPS = Spinach sequence; **BOLD UNDERLINED** = T7 terminator; *ITALICS* = restriction enzyme recognition site; lower case = other):
    (a) 5′-cgatcccgcgaaat**TAATACGACTCACTATAGGG** GCCCGGATAGCTCAG TCGGTAGAGCAGCGGCCG GACGCGACTGAATGAAATGGTGAAGGACGGG.

(b) 5′-**AGAGGCCCCAAGGGGTTATGCTA**TGGCGCCCGAACAGGGACTTG AACCCTGGACCCGCGGCCGGACGCGACTAGTTACGGAGCTCACACTCTACTC.

(c) 5′-cagtca*AGATCT*cgatcccgcgaaat**TAATACGACTCACTATAGGG.**

(d) 5′-catcag*CTCGAG***CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCA AGGGGTTATGCTA.**

(e) 5′-gatc*CGGCCG*GACGCGACTGAATGAAATGGTG.

(f) 5′-catg*CCGCGG*CCGGACGCGACTAGTTACGGAGCTC.

(g) 5′-cttg*CATATG*cacaaccctcatgagagcaag.

(h) 5′-catg*CTCGAG*tcagcccgccggggc.

18. *E. coli* BL21 (DE3) Star chemically competent cells (Life Technologies).
19. Borate buffer: 2.1 g/L boric acid, 9 g/L sodium tetraborate decahydrate; pH to 8.5. Filter through a 0.2 μm nitrocellulose filter and store at ambient temperature.
20. 1 M HCl solution.
21. 24× Poly-D-Lysine solution: 2 mg/mL poly-D-Lysine hydrobromide (Sigma, CAS: 27964-9904). Filter through a 0.2 μm nitrocellulose filter and store at −20 °C.
22. Ethanol.
23. M9 minimal media, pH 7.0: 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 20 mM NH_4Cl, 5 mM $MgSO_4$, 0.4 % glucose, 100 μM $CaCl_2$.
24. DFHBI (3,5-difluoro-4-hydroxybenzylidene imidazolinone): stock concentration of 20 mM DFHBI in DMSO, stored in 50 μL aliquots at 20 °C.
25. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.

3 Method

3.1 Construction and Transformation of Biosensor and Enzyme Constructs

In these experiments, two separate plasmids are used to separately express the biosensor or the enzyme (*see* **Note 1**). The Vc2-Spinach tRNA biosensor is cloned into pET31b and alleles of the WspR diguanylate cyclase from *Pseudomonas fluorescens* [11] are cloned into pET24a or pCOLADuet-1. Previous reports have shown that plasmids containing the same origin of replication (e.g., pET vectors) and different antibiotic resistances are incompatible for co-expression as both are not stably maintained in the cell due to competition for the same replication factors [12]. On the contrary, recent reports have shown that the degree of incompatibility

depends on the expression system and that plasmids with the same origin can stably persist for several overnight growth cycles [13]. We have found that both incompatible plasmids (pET31b and pET24a) as well as compatible plasmids (pET31b and pCOLA-Duet-1) function in these experiments to detect altered levels of cdiG, but it is advised to use compatible expression vectors for highly sensitive experiments in order to minimize plasmid loss during bacterial growth.

Though the expression of both the biosensor and enzyme are driven by IPTG-induction in the following experiments, use of the RNA-based biosensor is highly adaptable for many experimental systems. For instance, various promoters can be cloned directly upstream of the Vc2-Spinach tRNA construct to achieve either constitutive or inducible biosensor expression in *E. coli* or other bacterial species. Various enzyme alleles, gene homologs, or mutant enzyme libraries can be coexpressed to assess in vivo enzymatic activity. Furthermore, the biosensor can be expressed alone to study cyclic dinucleotide levels in different strains or in bacterial transposon libraries.

3.1.1 Cloning of Biosensor Expression Vector

For stable expression in vivo, the riboswitch-Spinach fusion, WHICH IS the construct used for in vitro experiments, is inserted within a $tRNA^{Lys}_3$ scaffold to reduce susceptibility to RNases and to ensure homogenous end processing in cells (Fig. 1a) [14]. In vitro analysis has confirmed that placement of the Vc2-Spinach construct within the tRNA scaffold has little to no effect on the ligand binding affinity [8]. For initiation and termination of transcription, respectively, the Vc2-Spinach tRNA construct is flanked

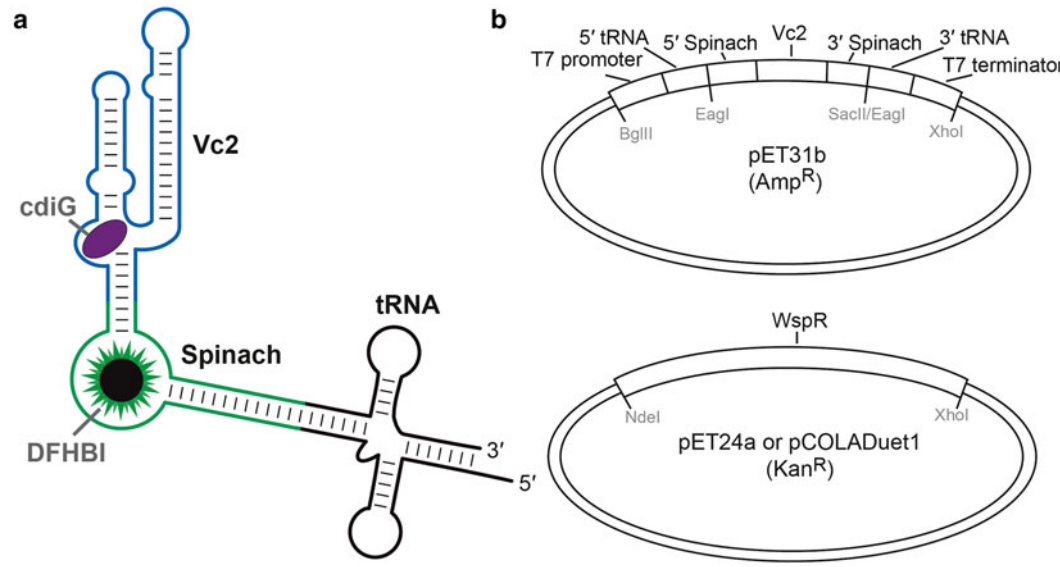

Fig. 1 Design of RNA-based fluorescent biosensor and enzyme constructs. (**a**) Model of Vc2-Spinach tRNA construct bound to cdiG and DFHBI. The tRNA scaffold serves to stabilize the biosensor fold in vivo. (**b**) Diagram of biosensor and enzyme expression plasmid constructs

by T7 promoter and T7 terminator sequences. The existing T7 promoter sequence in the expression plasmid pET31b is removed during the restriction digest to ensure homogenous expression of the RNA from a single promoter site.

Two procedures for constructing the biosensor expression plasmid are described below. In the first case, the Vc2-Spinach tRNA insert is generated through PCR and cloned into the original pET31b vector (*see* **Note 2**). Alternatively, the Vc2-Spinach biosensor can be cloned into a pET31b plasmid already containing the tRNA scaffold (Fig. 1b). The second strategy is recommended when the user already has a biosensor expression plasmid and is interested in swapping out the specific biosensor construct for another. Shown below is the sequence of the Vc2-Spinach tRNA biosensor construct (*italics* = restriction enzyme recognition site; **BOLD** = T7 promoter; UNDERLINED = tRNA scaffold; CAPS = Spinach sequence (*see* **Note 3**); lowercase = Vc2 sequence; BOLD ITALICS = T7 terminator):

*agatct*CGATCCCGCGAAAT**TAATACGACTCACTA**
TAGGGGCCCGGATAGCTCAGTCGGTAGAGCAG*CG*
*GCCG*GACGCGACTGAATGAAATGGTGAAGGACGGGTCC
Acacgcacagggcaaaccattcgaaagagtgggacgcaaagcctccggcctaaaccagaa
gacatggtaggtagcggggttaccgatgTTGTTGAGTAGAGTGTGAGCT
CCGTAACTAGTCGCGTC*CGG*CCGCGG*GTCCAGGGTTCA*
AGTCCCTGTTCGGGCGCCATAGCATAACCCCTTGGGGC
CTCTAAACGGGTCTTGAGGGGTTTTTTG*ctcgag*

Procedure Starting from Original pET31b Vector

Step 1a: Generation of Vc2-Spinach tRNA construct (Estimated time: 2 h).

1. To prepare the tRNA-scaffold biosensor insert, combine the following in a PCR tube:

   10 µL 5× Phusion HF buffer.

   5 µL 10× dNTPs.

   0.8 µL 40 µM primer a.

   0.8 µL 40 µM primer b.

   1 µL Vc2-Spinach DNA template (10–50 ng) (*see* **Note 4**).

   31.4 µL ddH_2O.

   1 µL Phusion DNA polymerase.

2. Amplify the construct using the following standard thermocycler protocol: initial denaturation 98 °C for 1 min; 35 cycles of 98 °C for 5–10 s, 66 °C for 20 s, 72 °C for 15 s, final extension 72 °C for 5 min.

3. Analyze the product on a 1 % agarose gel (expected size: 290 bp). Purify the product via commercial gel extraction or PCR cleanup kits following the manufacturer's protocol and elute the product in ddH_2O or the provided elution buffer.

Step 2a: Generation of Vc2-Spinach tRNA construct with T7 promoter and restriction sites (Estimated time: 2 h).

1. Complete construction of the biosensor construct containing restriction enzyme recognition sites by combining the following in a PCR tube:

   10 μL 5× Phusion HF buffer.

   5 μL 10× dNTPs.

   0.8 μL 40 μM primer c.

   0.8 μL 40 μM primer d.

   1 μL Vc2-Spinach-tRNA DNA template (10–50 ng).

   31.4 μL ddH_2O.

   1 μL Phusion DNA polymerase.
2. Amplify the construct using the same protocol as in **step 1.2** above, but with an annealing temperature of 68 °C.
3. Analyze and purify the product (expected size: 338 bp) as in **step 1.3** above.

Step 3a: Cloning of biosensor insert into expression vector (Estimated time: 2 days)

1. To insert the Vc2-Spinach tRNA construct into the expression plasmid pET31b, perform a double digest on both the insert and the vector using BglII and XhoI restriction enzymes. Prepare 50 μL digestion reactions by combining either 43 μL of the complete Vc2-Spinach-tRNA construct or pET31b plasmid with 5 μL NEBuffer 3.1, 1 μL BglII, and 1 μL XhoI.
2. Incubate the reaction at 37 °C for 2 h.
3. To reduce plasmid re-ligation, dephosphorylate the digested pET31b vector by adding 1 μL Calf Intestinal Alkaline Phosphatase to the digested reaction and incubate at 55 °C for 30 min.
4. Check the digested and dephosphorylated products on a 1 % agarose gel to ensure the integrity of the products and to check that the plasmid was linearized. Purify the products via commercial PCR cleanup kit following the manufacturer's protocol and elute the DNA in ddH_2O or the provided elution buffer.
5. Ligate the digested products by combining the following in a PCR tube: 1 μL 10× T4 DNA ligase buffer, 25 ng digested/dephosphorylated plasmid, 20 ng digested insert, ddH_2O to 9.5 μL, and 0.5 μL T4 DNA ligase. Incubate the reaction for 10 min at room temperature or at 16 °C overnight.
6. Deactivate the ligase by heating it to 65 °C for 10 min.

7. Use 1–5 μL of the ligation reaction to transform 50 μL *E. coli* TOP10 chemically competent cells following the manufacturer's protocol. Plate the cells on LB/Carb (50 μg/mL Carb) plates and incubate 12–16 h at 37 °C.
8. Check transformed colonies for clones with the correct sequence by streaking single colonies on a LB/Carb master plate. Inoculate overnight LB/Carb cultures from the master plate, then isolate the plasmids using a commercial kit and submit the plasmids for sequencing. Use only a plasmid containing the desired sequence for future experiments.

Alternative Procedure Starting from Existing Biosensor Construct

Step 1b: Generation of biosensor-Spinach insert

1. Amplify the Vc2-Spinach insert with EagI and SacII restriction sites by combining the following in a PCR tube:

   10 μL 5× Phusion HF buffer.

   5 μL 10× dNTPs.

   0.8 μL 40 μM primer e.

   0.8 μL 40 μM primer f.

   1 μL Vc2-Spinach-tRNA DNA template (10–50 ng).

   31.4 μL ddH_2O.

   1 μL Phusion DNA polymerase.
2. Amplify the construct using the following thermocycler protocol: Initial denaturation 98 °C for 1 min; 35 cycles of 98 °C 5–10 s, 66 °C for 20 s, 72 °C for 15 s, final extension 72 °C for 5 min.
3. Check the product on a 1 % agarose gel (expected size: 175 bp) and purify the product via commercial PCR cleanup or gel extraction kits following the manufacturer's protocol and elute the DNA in ddH_2O or the provided elution buffer.

Step 2b: Cloning of biosensor insert into expression vector (Estimated time: 1 day)

1. Digest the pET31b-Spinach-containing plasmid and the Vc2-Spinach insert by combining either 43 μL of the Vc2-Spinach construct or pET31b-Spinach plasmid with 5 μL NEB CutSmart buffer, and 1 μL SacII. Incubate the reaction at 37 °C for 1 h, then add 1 μL EagI-HF and digest at 37 °C for another hour. It is necessary to do a sequential digest since first cutting with SacII eliminates the second EagI restriction enzyme recognition site.
2. Follow **steps 3a.3–3a.8** above to ligate, transform, and sequence to confirm the pET31b Vc2-Spinach-tRNA biosensor construct.

3.1.2 Construction of Enzyme Expression Vector

Step 4: Generation of WspR diguanylate cyclase insert (Estimated time: 3 h)

1. Amplify the WspR diguanylate cyclase insert by combining the following in a PCR tube:

   10 μL 5× Phusion GC buffer.

   5 μL 10× dNTPs.

   0.8 μL 40 μM primer g.

   0.8 μL 40 μM primer h.

   1 μL WspR DNA template (10–50 ng).

   3 μL DMSO.

   28.4 μL ddH_2O.

   1 μL Phusion DNA polymerase.

2. Amplify the construct using the following thermocycler protocol: Initial denaturation 98 °C for 1 min; 35 cycles of 98 °C 5–10 s, 68 °C for 20 s, 72 °C for 45 s, final extension 72 °C for 10 min (*see* **Note 5**).

3. Check the amplified product on a 1 % agarose gel (expected size: 1,061 bp) and purify the product by commercial PCR cleanup or gel extraction kits following the manufacturer's protocol and elute the DNA in ddH_2O or the provided elution buffer.

Step 5: Cloning of WspR diguanylate cyclase into expression vector (Estimated time: 2 days)

1. Digest the amplified WspR insert along with pET24a or pCOLADuet-1 plasmid by combining the following: 43 μL of either the WspR insert or the plasmid with 5 μL NEB CutSmart buffer, 1 μL NdeI, and 1 μL XhoI. Incubate the reaction at 37 °C for 2 h.

2. Follow **steps 3a.3–3a.8** in Subheading 3.1.1, but with cultures grown in LB/Kan (50 μg/mL) in order to obtain sequence-confirmed pET24a or pCOLADuet-1 WspR plasmid.

3.1.3 Transformation of Expression Vectors for Live Cell Imaging

Step 6: Generation of strains containing both biosensor and enzyme constructs (Estimated time: 1 day)

1. To prepare cells co-expressing the biosensor and enzyme, transform BL21 (DE3) Star *E. coli* cells with ~60 ng each of the pET31b and pET24a constructs following the manufacturer's protocol.

2. Plate the cells on LB/Carb/Kan (Carb: 50 μg/mL, Kan: 50 μg/mL) plates and incubate at 37 °C for 12–16 h. Colonies should contain both plasmids and the plates can be stored at 4 °C for ~3 weeks.

3.2 Live Cell Imaging of the RNA-Based Biosensor

In general, cellular imaging with Spinach-based biosensors requires that several conditions be met, including that DFHBI can diffuse into the cell and that the RNA biosensor is expressed at sufficient levels for visualization. The permeability of DFHBI depends upon the composition of the cell membrane or cell wall and thus may fluctuate between different bacterial species and strains or under different growth conditions. Similarly, RNA expression levels may vary between different conditions or stressors on the cell (e.g., co-expression of a heterologous enzyme). Thus, to ensure that observed fluorescence changes are due to changes in metabolite or signaling molecule levels rather than changes in intracellular DFHBI and RNA concentrations, it is recommended that the Spinach aptamer alone be used as a control. If cellular fluorescence of the Spinach-tRNA construct with DFHBI and RNA expression levels remain constant under the experimental conditions, then differences in biosensor fluorescence under these conditions should provide an accurate readout of changing metabolite levels.

Two distinctly advantageous techniques for live cell imaging of the fluorescent biosensor include fluorescence microscopy and flow cytometry. For the former, microscopy allows for direct visualization of cell morphology, for tracking cellular fluorescence over time, and for monitoring spatial dynamics of fluorescence (Fig. 2a). Generally, any fluorescence microscope with power to resolve individual bacterial cells can be used, but analysis of results with the

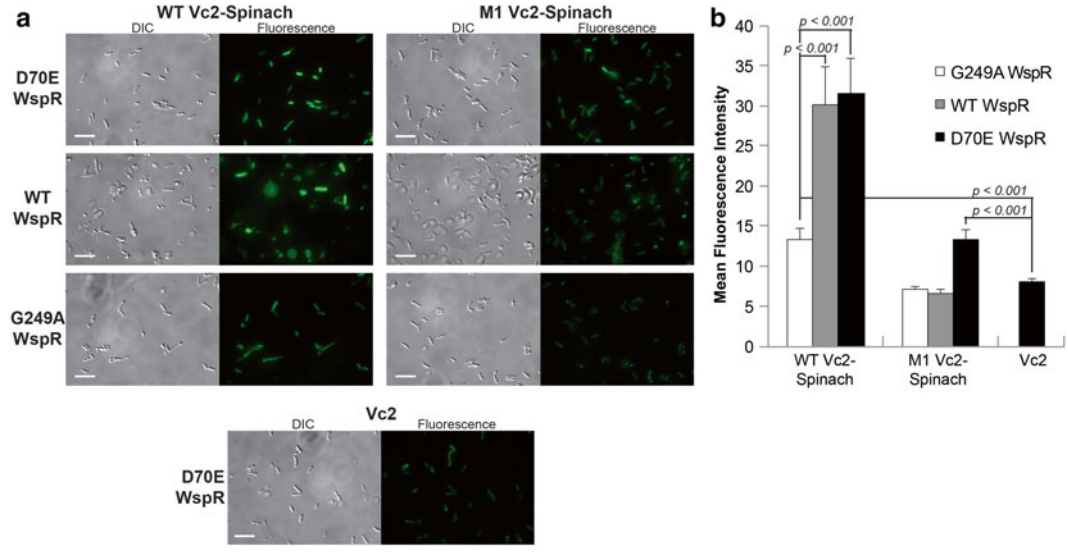

Fig. 2 The Vc2-Spinach biosensor detects cdiG through fluorescence microscopy of live *E. coli*. (**a**) Differential interference contrast (DIC) and fluorescence microscopy images are shown of representative *E. coli* expressing variants of the Vc2-Spinach biosensor and the WspR cdiG synthase. Scale bars represent 10 μm. (**b**) Quantitation of mean fluorescence intensity from fluorescence microscopy experiments. Error bars represent the SEM for at least 50 quantified cells. Indicated *p* values were calculated using a student's *t*-test. Figure used with permission since data is from JACS paper (reference)

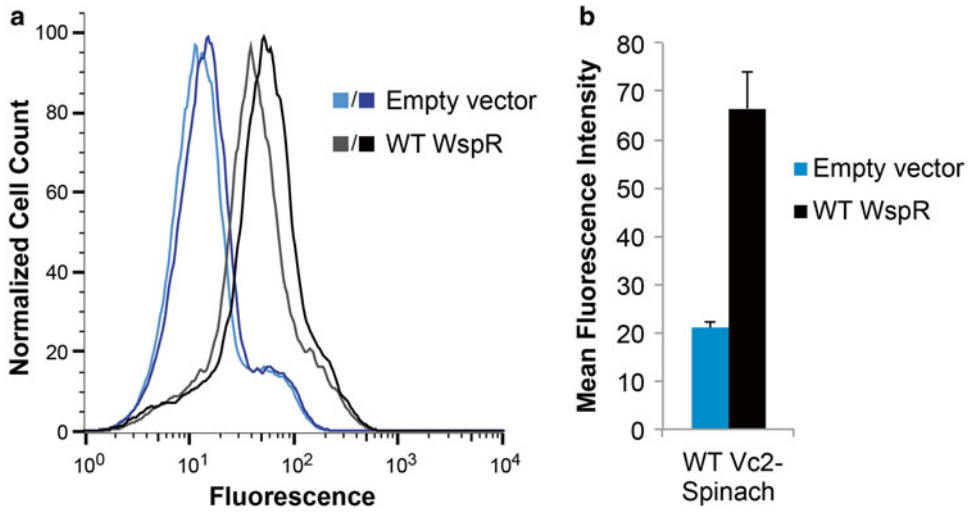

Fig. 3 The Vc2-Spinach biosensor detects cdiG through flow cytometry of live *E. coli*. (**a**) Flow cytometry data of cells coexpressing the WT Vc2-Spinach biosensor along with empty vector or WT WspR. (**b**) Quantitation of mean fluorescence intensity from flow cytometry experiments. Error bars represent the standard deviation of independent biological replicates of at least 35,000 cells

currently available software is time-consuming. On the contrary, flow cytometry or fluorescence activated cell sorting (FACS) allow for rapid and high-throughput analysis of thousands of cells (Fig. 3a). These flow-based methods provide a snapshot of bacterial characteristics of a large population at a specific time, and the ability to sort cells exhibiting differential fluorescent outputs. However, flow techniques are not suited for tracking the behavior of individual cells over time. Nevertheless, both experimental techniques have proven robust and reproducible using RNA-based biosensors.

3.2.1 Preparation of Poly-D-Lysine Coverslips for Fluorescence Microscopy

In performing live cell fluorescence microscopy experiments, the coverslips used for adhering the cells are first prepared and can be stored at room temperature for several months. Cells will adhere to the poly-lysine coated coverslips through electrostatic interactions between the positive charge of the lysine residues on the coverslips and the negative charge of the bacterial cell. Specially designed plates and other poly-lysine coated materials can alternatively be ordered from commercial vendors such as MatTek.

Step 7: Acid rinse of coverslips (Estimated time: 11 h)

1. To coat the coverslips with a negative charge, soak an adequate number of coverslips in 1 M HCl and heat the solution to ~50 °C for 1 h. Let the solution cool to ambient temperature for 2–10 h with occasional mixing.

2. Carefully remove the coverslips from the acid solution, then rinse the coverslips twice with ddH_2O and once with 100 % ethanol. Let the coverslips dry at ambient temperature for at least 1 h. Take care to neutralize the acid solution and to follow proper waste disposal guidelines.

Step 8: Poly-D-Lysine rinse of coverslips (Estimated time: 11 h)

1. To coat the coverslips with poly-D-Lysine, dilute 100 µL 4× poly-D-Lysine solution with 300 µL ddH_2O. Let the coverslips soak in this solution in a petri dish for 1–10 h on a rocker at ambient temperature.
2. Rinse the coverslips carefully twice with ddH_2O water and once with 100 % ethanol. Remove the coverslips from the petri dish and dry them on filter paper for at least 1 h at ambient temperature. Coverslips can be stored at room temperature for ~3 months.

3.2.2 Fluorescent Microscopy Experiments

Step 9: Bacterial growth and induction conditions (Estimated time: 16–20 h)

1. Inoculate a 3 mL LB/Carb/Kan culture with a single colony of the transformed BL21 star cells. Incubate the culture at 37 °C in an incubator/shaker for 12–16 h or overnight.
2. In the morning, inoculate a fresh 3 mL LB/Carb/Kan culture with 200 µL of overnight culture. Grow the cells in a 37 °C incubator shaker and monitor growth by measuring the OD_{600}.
3. Once the cells have reached an OD_{600} 0.4–0.6 (approx. 2 h), induce the expression of the biosensor and plasmid with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Continue growing the cells in a 37 °C incubator shaker for 2.5 h.

Step 10: Harvesting cells and preparation of slides (Estimated Time: 3.5 h)

1. To collect cells and remove LB, pellet 100 µL cells at 4,500 rcf for 3 min at ambient temperature. Remove the supernatant and resuspend the cell pellet in 500 µL M9 minimal media, pH 7.0. Wash the cells of LB by centrifuging again, removing the supernatant, and resuspending in another 200 µL M9 minimal media, pH 7.0 (*see* **Note 6**).
2. Adhere the suspended cells to the prepared poly D-Lysine coverslips by pipetting the cells onto each coverslip and allow the cells to attach for ~30 min at 37 °C.
3. To remove any unadhered cells, gently rinse the coverslips with 3 mL M9 minimal and aspirate off the solution.
4. Add 200 µL of 200 µM DFHBI in M9 minimal media, pH 7.0 to the cells and incubate the cells at 37 °C for ~1–1.5 h to

allow DFHBI to permeate the membrane and to bind the biosensor RNA. Keep the cells in the dark from this point on as DFHBI is light-sensitive.

5. Remove excess liquid by aspiration and place the coverslip on top of a microscope slide. Seal the edges of the coverslip with nail polish to prevent drying of the cells.

Step 11: Imaging cells using fluorescence microscopy (Estimated time: 1 h)

1. To image cells via microscopy, first place the brightest expected sample on the microscope platform and focus on the cells using bright-field imaging. Determine the fluorescence exposure to be used by maximizing the exposure time to increase fluorescence signal without overexposing any cells (*see* **Note 7**). Note this fluorescence exposure setting and revert to bright-field exposure while fluorescence imaging is not active.
2. Image all samples by first taking a snapshot of cells in the bright field, then switch to green fluorescence filters using the previously determined exposure time and take a snapshot of the same field visualized by fluorescence. For each sample, repeat this procedure for at least three different viewing sections, where at least a total of 50 individual cells can be visualized. Follow this procedure for all samples of interest and save all images as files that can be opened in ImageJ (e.g., tif files).

Step 12: Analysis of fluorescence microscopy data (Estimated time: 3 h)

1. To normalize all microscope images to the brightest sample of the set, open the fluorescent tif file in ImageJ of the brightest sample tested. In the adjustment category of the image menu, select Brightness/Contrast and set it to Auto. Note the maximum displayed value of the 8-bit image as this will be used to set the maximum brightness of all images.
2. To determine the background fluorescence of each sample, which varies based upon the amount of DFHBI left on the coverslip, outline at least four areas of the image that contain no cells and use the measurement tool to determine the median fluorescence for each area. The average median fluorescence of the four areas (typically ± 2 units within the minimum fluorescence set by the Auto function) is the background fluorescence of the image.
3. Adjust each image individually by setting the minimum displayed value to the background fluorescence found in **step 2**, and the maximum displayed value to the maximum brightness found in **step 1**. Click ok and then apply within the Brightness and Contrast tool bar. Complete this process for all images to be analyzed.

4. To determine the mean fluorescence of each cell, individual cells will be manually outlined in the bright-field image and then overlaid on the corresponding fluorescence image. Open the bright-field image in ImageJ then manually outline each cell using the heart-shaped freehand selection tool. Hold down the Shift key while outlining to keep all previously outlined cells selected and periodically save the outlined cells as regions of interest (ROIs). If at any point an error is made during this process, click on the background of the image to clear the mistake, then select the last object in the ROI manager to redo that step.
5. Once all cells in the bright-field image have been outlined, delete all ROI entries except for the last object containing all outlined cells. Open the fluorescence image, then select the object in the ROI manager to have the outlines appear on this image. Some adjustment may be necessary if the camera shifted between taking snapshots of the bright-field and fluorescence images. If so, simply move the ROI selections using the arrow keys. Save the image as a separate file by overlaying the ROI selections from the ROI manager.
6. Obtain the median fluorescence for each outlined cell by selecting to Split the combined object in the ROI manager. This separates each outlined object, and the fluorescence statistics can be obtained by highlighting the individual cells in the ROI manager and selecting Measure.
7. Record the obtained cell statistics and also measure the background fluorescence of four areas of the image that have no cells. Determine the mean fluorescence intensity of each cell by subtracting this background from the mean fluorescence of each cell. For each sample tested, determine the fluorescence of at least 50 cells to calculate the average mean fluorescence intensity for this sample. Assess the statistical significance of the differences in sample fluorescence using a student's *t* test (Fig. 2b).

3.2.3 Flow Cytometry Experiments

Step 13: Growth of cells for flow cytometry (Estimated: 16 h)

1. To prepare cells for flow cytometry, follow **step 9** as discussed in Subheading 3.2.2 (*see* **Note 8**).

Step 14: Preparation of cells for flow cytometry (Estimated time: 1 h)

1. To collect cells and remove LB, pellet 250 µL cells at 4,500 rcf for 3 min at ambient temperature. Remove the supernatant and resuspend the pellet in 500 µL 1× PBS (*see* **Notes 6** and **9**).
2. Add 1–5 µL of the suspended cells to 250 µL of the 25 µM DFHBI in 1× PBS. Assuming that $OD_{600} = 1$ corresponds with approximately 1×10^9 cells/mL, this should give a final concentration of between 3,000 and 15,000 cells/µL.
3. Filter the suspension through the cell-strainer caps into polypropylene tubes for analysis.

Step 15: Analysis of cells by flow cytometry (Estimated time: 1 h)

1. For future analysis of cells via flow cytometry, establish the appropriate gating settings using both positive and negative fluorescence controls. To do this, first establish the forward scatter (FSC) and side scatter (SSC) regions corresponding to cells by first analyzing DFHBI-PBS solution alone, followed by a solution containing cells in DFHBI-PBS solution.
2. To optimize the voltage gain settings, pass the negative control—either cells expressing no dinucleotide cyclase or a non-functional biosensor—through the analyzer, and compare to the positive control with cells coexpressing the Vc2-Spinach biosensor with WT WspR.
3. Analyze all samples by collecting data for at least 10,000 cells within the gating window.

Step 16: Analysis of flow cytometry data (Estimated time: 1 h)

1. Using FlowJo software, generate a gate from the FSC/SSC dot plot to analyze data points solely corresponding to cells and limiting the amount of debris analyzed. Apply this same gate to all samples tested.
2. From this gate, use the FlowJo statistical analysis to determine the mean fluorescence intensity of each sample. Calculate the standard deviation from the mean fluorescence intensity of at least two independent biological replicates (*see* Fig. 3b).

4 Notes

1. To use the biosensor to analyze the effect of an enzyme on metabolite levels, it is advisable to compare cells harboring both biosensor and enzyme versus cells harboring both biosensor and empty vector. The latter control eliminates variation in expression caused by cells grown under different antibiotic conditions and with different plasmid loads.
2. As described in Subheading 3.1, other pET vectors may be used, but it is recommended to use compatible plasmids if coexpressing the biosensor with an enzyme.
3. This protocol describes the use of the Spinach system with the original Spinach aptamer and fluorophore DFHBI. An improved version of Spinach, termed Spinach2, and alternative fluorophores, DFHBI-1T and DFHBI-2T, have recently been developed for in vivo analysis [15, 16]. The Spinach2 aptamer has increased folding stability and displays 2.1-fold and 3.2-fold increases in fluorescence in bacterial and mammalian cells, respectively.
4. The Vc2-Spinach DNA template can either be ordered as a DNA oligo from a commercial vendor or can be generated through overlapping PCR [9].

5. Amplification of genes with high GC-content such as WspR can be difficult. If necessary, 1.5–3 μL of DMSO can be added to the PCR amplification. Alternatively, consider using a different enzyme gene that would suit the experiment.
6. One of the most important parameters with both microscopy and flow cytometry analyses is to ensure that the DFHBI solution is the same concentration across all samples. We generally make the 1× PBS or M9 Minimal Media solution containing DFHBI fresh for each set of experiments.
7. If no fluorescence is observed, yet the biosensor has been confirmed to function in vitro, examine the effect of the tRNA scaffold on fluorescence in vitro. Alternatively, test whether the Spinach-tRNA construct alone functions in vivo to assess if the problem is due to RNA expression, DFHBI permeability, or the microscopy protocol. If Spinach expression can be visualized, consider that ligand concentration might be too low for fluorescence activation or other effects of ligand on fluorescence visualization. If Spinach expression cannot be visualized, check that the RNA is being expressed via Northern blot or RT-PCR analysis.
8. Flow cytometry experiments can also be carried out by growing and analyzing cells in 96-well plate format. In a 1,000 μL deep-well plate, inoculate 200 μL of LB/Carb/Kan media with 5 μL of overnight culture and incubate the cells in a 37 °C shaker for ~1.5 h. Induce the cells with 1 mM IPTG, then harvest the cells after ~3 h incubation by centrifuging the entire plate at 4,500 rcf for 3 min at ambient temperature. Decant the supernatant and resuspend the cells in 500 μL 1× PBS. Add 1 μL resuspended cells to 150 μL of the DFHBI-PBS solution in a 96 well plate and read using a BD High Throughput Sampler.
9. We have found that both M9 minimal media, pH 7.0 and 1× PBS are suitable for preparing cells for flow cytometry. Due to the longer incubation for adhering cells in the microscopy procedure though, we recommend using M9 minimal media for microscope experiments.

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