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Live flow cytometry analysis of c-di-GMP levels in single cell populations

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Abstract

Second-generation RNA-based fluorescent biosensors have been developed that enable flow cytometry experiments to monitor the population dynamics of c-di-GMP signaling in live bacteria. These experiments are high-throughput, provide information at the single-cell level, and can be performed on cells grown in complex media and/or under anaerobic conditions. Here we describe flow cytometry methods for three applications: (1) high-throughput screening for diguanylate cyclase activity, (2) analyzing c-di-GMP levels under anaerobic conditions, and (3) monitoring cell population dynamics of c-di-GMP levels upon environmental changes. These methods showcase RNA-based fluorescent biosensors as versatile tools for studying c-di-GMP signaling in bacteria.

Keywords

Cyclic dinucleotide; Cyclic di-GMP; Flow cytometry; RNA-based fluorescent biosensor; Spinach aptamer; Anaerobic growth; c-di-GMP signaling

1. Introduction

Cyclic di-GMP (c-di-GMP) is a near-universal bacterial signal that controls the transition from a free-living state to a surface-attached biofilm, a lifestyle decision that requires coordinated changes in motility, exopolysaccharide production, quorum sensing, and other behaviors [1]. To date, many analytical methods have been developed to measure cellular c-di-GMP levels. Direct *in vitro* methods include thin-layer chromatography (TLC) [2], high-performance liquid chromatography (HPLC) followed by mass spectrometry (MS) [3], detection of c-di-GMP G-quadruplexes [4], and detection based on binding to effector protein domains or riboswitch aptamers [5,6]. Indirect methods include phenotypic assays for motility [7] and biofilm formation [8]. More recently, *in vivo* methods for screening and live cell imaging of c-di-GMP have been developed based on genetic reporters whose expression is controlled by c-di-GMP binding effectors [9], or engineered fluorescent biosensors whose fluorescence or Forster resonance energy transfer (FRET) efficiency is changed upon c-di-GMP binding [10–12].

Our lab has focused on the design and development of RNA-based fluorescent biosensors for different bacterial signals and cofactors, including c-di-GMP [13,14]. These biosensors are genetically encodable RNA sequences incorporating a riboswitch aptamer as the sensing domain that changes conformation upon binding the target ligand, e.g. c-di-GMP. This ligand-dependent conformational change is engineered to stabilize the second domain of the biosensor, the dye-binding Spinach aptamer, which binds and enhances the fluorescence of the dye molecule DFHBI (3,5-difluoro-4-hydroxybenzylidene imidazolinone) [15]. Thus, the biosensor system is comprised of a genetically encodable RNA sequence that is a riboswitch-Spinach fusion and an exogenously added dye compound [16]. DFHBI and related dyes are cell permeable and have sufficiently low background fluorescence that they can be kept in the media during imaging experiments.

Our first-generation c-di-GMP biosensor was the Vc2 GEMM-I riboswitch aptamer fused to the Spinach aptamer in a tRNA scaffold [13,12]. Recently, we developed second-generation c-di-GMP biosensors with a broader range of binding affinities, including sensitivity down to a few c-di-GMP molecules per cell, higher fold fluorescence turn-on and overall brightness, and faster turn-on kinetics [17]. These biosensors, called Ct, Dp, and Pl-B [17], are comprised of different GEMM-I riboswitch aptamers fused to the Spinach2 aptamer, a second-generation dye-binding domain that works with DFHBI and DFHBI-1T (4-(3,5-difluoro-4-hydroxybenzylidene)-2-methyl-1-(2,2,2-trifluoroethyl) imidazolone) [18], in a tRNA scaffold (Table 1). With the improvements to biosensor properties stated above, these RNA-based fluorescent biosensors are optimized for use in live cell flow cytometry experiments to measure c-di-GMP in single cell populations. In this chapter, we describe flow cytometry methods for three applications: (1) high-throughput screening for diguanylate cyclase activity, (2) analyzing c-di-GMP levels under anaerobic conditions, and (3) monitoring cell population dynamics of c-di-GMP upon environmental changes. We encourage the reader to consider these methods as a starting foundation for other biosensor applications using flow cytometry, including screening for phosphodiesterase activity and analyzing c-di-GMP levels or dynamics in other conditions or in other organisms.

2. Materials

2.1. Equipment

1. Micropipettor.
2. Vortex mixer.
3. Microcentrifuge.
4. PCR thermocycler.
5. PCR thermocyclere quipped with 96 -well platform.
6. Incubator shaker set to 37 °C(Maximum shaking speed of 325 rpm or higher).
7. Benchtop orbital shaker.
8. Timer.
9. Flow cytometer equipped with 488 nm laser and autosampler.

10. Hand-operated crimper for aluminum seals.

2.2. Supplies

1. Micropipettor tips.
2. 1.5 mL microcentrifuge tubes.
3. Polyethersulfone membrane sterile syringe filter units (25 mm diameter, 0.2 μ m pore size).
4. 14 mL Culture tubes and petri dishes.
5. 12-Well suspension culture plate, sterile, with lid.
6. Plating glass beads, sterile.
7. Anaerobic culture tubes.
8. Chlorobutyl rubber stoppers.
9. Aluminum seals for culture tubes.
10. Argon gas cylinder.
11. Plastic tubing (inner diameter = 1/4 inch) and tubing connectors (straight and Y-shaped).
12. 1 mL syringes.
13. Syringe needles (18G, 1.5 inch).
14. Round bottom, non-treated, sterile polypropylene 96-well plates.
15. 96-well PCR plates, clear.
16. 96-well deep well microplates(well volume ~ 2.2 mL).
17. Gas-permeable sealing membrane for microtiter plates, sterile.

2.3. Reagents

1. Sterile water: sterile filter-sterilized pyrogen-, nuclease-, protease- and bacteria-free water, 18.2 M Ω
2. 1 $\times$ TAE buffer: 40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.4.
3. Carbenicillin: 50 mg/mL stock concentration, filtered through a 0.2 μ m nitrocellulose filter.
4. Kanamycin: 50 mg/mL stock concentration, filtered through a 0.2 μ m nitrocellulose filter.
5. Luria Broth(1% tryptone, 0.5% yeast extract, 1% sodium chloride), autoclave-sterilized.
6. Luria Broth containing 50 μ g/mL carbenicillin(referred to as LB/Carb), autoclave-sterilized.

7. Luria Broth containing 50 µg/mL carbenicillin and 50 µg/mL kanamycin (referred to as LB/Carb/Kan), autoclave-sterilized.
8. Petri plate LB/Carb agar (Pour warm LB/Carb solution containing 1.5% agar into 100 mm petri dishes and cool the plates to solidify).
9. Petri plate LB/Carb/Kan agar (Pour warm LB/Carb/Kan solution containing 1.5% agar into 100 mm petri dishes and cool the plates to solidify).
10. 12-well plate LB/Carb/Kan agar (Pour warm LB/Carb/Kan containing 1.5% agar solution into a 12-well suspension culture plate and let it cool to solidify).
11. IPTG (isopropyl β-D-1-thiogalactopyranoside, dissolved in sterile water to the final concentration of 0.5 M), filtered through a 0.2 µm nitrocellulose filter.
12. ZYP-5052 autoinduction media (50 mM KH₂PO₄, 50 mM Na₂HPO₄, 25 mM (NH₄)₂SO₄, 1 mM MgSO₄, 1% trypton, 0.5% yeast extract, 0.05% g glucose, 0.2% g α-lactose, 0.5% glycerol) supplemented with 50 µg/mL carbenicillin and 50 µg/mL kanamycin (referred to as AI/Carb/Kan), autoclave-sterilized.
13. ZYP-5052 autoinduction media supplemented with 50 µg/mL carbenicillin (referred to as AI/Carb)
14. S.O.C. media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose), autoclave-sterilized.
15. DFHBI-1T (4-(3,5-difluoro-4-hydroxybenzylidene)-2-methyl-1-(2,2,2-trifluoroethyl) imidazolone): stock concentration of 20 mM DFHBI-1T in 100% DMSO, stored in 50 µL aliquots at −20 °C.
16. 1 M ZnCl₂ in sterile water, filtered through a 0.2 µm nitrocellulose filter (freshly prepared stock).
17. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4 in sterile water, filtered through a 0.2 µm nitrocellulose filter,.
18. *E. coli* BL21(DE3) Star chemically competent cells
19. Biosensor constructs in pET31b plasmid (see Table 1 for construct sequences).
 - a. Ct biosensor (Addgene #79158, pET31b-T7-Spinach2-Ct, expresses Spinach2-Ct biosensor under a T7 promoter).
 - b. Dp biosensor (Addgene #79159, pET31b-T7-Spinach2-Dp, expresses Spinach2-Dp biosensor under a T7 promoter)
 - c. Pl-B biosensor (Addgene #79161, pET31b-T7-Spinach2-Pl-B, expresses Spinach2-Pl-B biosensor under a T7 promoter).
 - d. Spinach2 control (Addgene #79783, pET31b-T7-Spinach2, expresses Spinach2 biosensor under a T7 promoter).
20. Enzyme constructs in pCOLA plasmid.

- a. Diguanylate cyclase WspR WT (Addgene #79162, pCOLA-T7-WspR, expresses WspR WT under a T7 promoter).
- b. Inactive mutant WspR G249A (Addgene #79163, pCOLA-T7-WspR:G249A, expresses WspR G249A under a T7 promoter).
- c. Diguanylate cyclase WspR D70E (Addgene #79164, pCOLA-T7-WspR:D70E, expresses constitutively active WspR D70E under a T7 promoter).
- d. Phosphodiesterase YhjH (Addgene #79165, pCOLA-T7-YhjH, Expresses YhjH under a T7 promoter).
- e. pCOLA empty vector.

3. Methods

3.1. Screening for diguanylate cyclase activity

We have used RNA-based fluorescent biosensors to screen 29 candidate GGDEF enzyme genes for c-di-GMP and/or c-AMP-GMP synthase activity using flow cytometry [19]. Fig. 1 shows the results of the screen for diguanylate cyclase (c-di-GMP synthesis) activity. Enzymatic activity of these signaling enzymes can be assayed *in vivo* even in the absence of activating signal because overexpression often drives dimerization to the active state. In this section, we describe the procedure for performing the high-throughput screen in a 96-well format.

3.1.1. High-throughput generation of *E. coli* strains

- 1 To prepare cells co-expressing biosensor and enzyme library members, aliquot 10 μ L of BL21(DE3) Star *E. coli* chemically competent cells into 96-well PCR plate on ice and add ~50 ng of each plasmid DNA (Dp biosensor in pET31b and enzyme genes of interest in pCOLA) to each well. Also, prepare a negative control (Dp biosensor in pET31b and pCOLA empty vector) and positive control (Dp biosensor in pET31b and Diguanylate cyclase WspR WT in pCOLA). Incubate the plate of cells with DNA on ice for 30 minutes.
- 2 Turn on the PCR thermocycler equipped with 96-well platform. Create and run a program to keep the sample holder temperature at 42 °C.
- 3 Transfer the 96-well PCR plate from ice to the heated sample holder for 1 minute to apply heat shock.
- 4 Place the 96-well PCR plate back on ice for 3 minutes.
- 5 In the meantime, prepare recovery plate by adding 250 μ L of S.O.C. media to each well of a 96-well deep well microplates.
- 6 Gently add transformed cells to each well of the recovery plate containing S.O.C. media.
- 7 Cover the plate with a gas-permeable sealing membrane and shake for one hour at 37 °C and 225 rpm to recover.

- 8 In the meantime, prepare 12-well plate LB/Carb/Kan agar plates.
- 9 Transfer 30 μ L of recovered cells onto LB/Carb/Kan agar in each well of 12-well plates. Spread cells by adding two or three Plating Beads in each well and rolling the beads around in a circular motion for about 30 seconds.
- 7 Cover plates with covers and incubate inverted at 37 °C for 18 hours

3.1.2. Preparation of samples for flow cytometry

1. Pick single colonies from the 12-well plate and inoculate in 500 μ L of LB/Carb/Kan in a high-wall 96-well plate, including positive and negative controls. Grow cells by shaking at 325 rpm 37 °C, 18 hours until cells reached an $OD_{600} > 3$ (see Note 1).
2. Transfer 1 μ L of cell cultures to 500 μ L of AI/Carb/Kan in another high-wall 96-well plate and grow by shaking at 325 rpm for 16 hours at 37 °C. At this step, cells will induce expression of biosensor and enzyme.
3. Prepare 1 $\times$ PBS containing 50 μ M DFHBI-1T and protect from light (see Note 2). Aliquot 70 μ L of the solution to each well of a round bottom 96-well polypropylene plate (the flow plate). Protect the plate from light by wrapping the plate with aluminum foil before use.

3.1.3. Set up of flow cytometry parameters

1. Start up the flow cytometer equipped with 488 nm laser and associated software. Run performance test following manufacturer's protocol if necessary.
2. Transfer 1 μ L of the negative control and positive control cell cultures from the induction plate in step 3.1.2 step 2 into wells of the flow plate containing PBS-DFHBI-1T prepared in step 3.1.2 step3. Incubate for 5 min to allow the DFHBI-1T dye to diffuse into cells.
3. Do test runs with negative control and positive control cells in PBS-DFHBI-1T solution in the flow plate to establish the forward scatter (FSC) and side scatter (SSC) regions and to optimize the voltage gain settings. In this experiment, negative controls are cells expressing Dp biosensor in pET31b but harbor the pCOLA empty vector while positive controls are cells expressing Dp biosensor in pET31b and wild type WspR in pCOLA.
 - a. On the software, create a 96-well plate experiment (see Note 3).
 - b. On the workspace window, load three dot plots and one histogram. Set up the axes for these plots – FSC-Area/SSC-Area (both log axes), FSC-

¹To test independent biological replicates, pick different single colonies to inoculate. It is recommended to analyze at least 3 biological replicates.

²The PBS buffer stock should be filter sterilized before use. It is highly recommended to prepare fresh PBS-DFHBI-1T solution each time. The approximate total volume of the solution needed is ~70 μ L for each independent sample in the experiment. DFHBI can be used in place of DFHBI-1T.

³The steps described here are based on the software specific to Attune instrument. The software for different flow cytometry instrument may have different instructions for set-up.

Height/FSC-Area (both log axes), and Time (linear axis)/FSC-Area (log axis) for the three dot plots, and count/GFP(530/30-A) for the histogram, respectively(Fig. 2).

- c. Set up the flow rate, draw volumes and stop settings. Recommended settings are 12.5 $\mu\text{L}/\text{min}$ flow rate, 65 μL draw volumes (for sample volumes of 70 μL), and at least 10,000 readings before stop.
- d. Place the negative control sample in the sample holder.
- e. Load the negative control sample and run the flow cytometry analysis to obtain data for the four plots in step b.
- f. On the FSC/SSC dot plot, generate a gate to exclude reading any signals from debris (Fig. 2A).
- g. On the FSC-A/FSC-H dot plot, display readings only from the gate in step f. You should see a diagonal pattern. Generate a narrow diagonal gate to exclude readings off the diagonal. This gate excludes multiplet readings frequently generated from cell clumps (Fig. 2B).
- h. Check the Time/FSC-A dot plot to check that the flow of cells is stable. If any areas show a poor flow rate, generate a gate to exclude readings from that area (Fig. 2C).
- i. On the histogram, display readings only from the gates in steps f to h (Fig. 2D).
- j. Load the positive control sample and run the flow cytometry analysis. While running the sample, the four plots described in step f to i are displayed in real time by the software.
- k. Adjust the voltage gain settings and repeat analysis of positive and negative controls so that the readings from negative control and positive control appear around 10^2 and 10^4 of the fluorescence intensity, respectively.
- l. Save all settings as a template file to use for analysis of samples from the same experiment.

3.1.4. Analysis of enzyme library by flow cytometry

1. Transfer 1 μL of sample cell cultures from the induction plate in step 3.1.2 step 2 into wells of the flow plate containing PBS-DFHBI-1T prepared in step 3.1.2 step 3. Incubate for 5 min to allow the DFHBI-1T dye to diffuse into cells.
2. Load the flow plate onto the autosampler of the flow cytometer.
3. On the heat map, highlight the wells you are analyzing and create experiments. Typically, two technical replicates are performed by analyzing each well twice.
4. Run flow cytometry analysis of the samples using parameter settings from 3.1.3 step 3.

3.1.5. Determination of mean fluorescence intensity (MFI)

1. Export all the results to create FCS files for analysis.
2. Open the FCS files with a flow cytometry analysis software (e.g. FlowJo).
3. Double-click on a sample to open FSC-Area/SSC-Area plot and repeat generating gates as described in 3.1.3. As the first step, change both axis settings to log, and generate a gate to exclude any signals from debris as in 3.1.3 step 2.f.
4. Double-click on the new created subset file. Change axis settings to FSC-Height and FSC-Area (both log axes). Generate a thin diagonal gate to exclude multiplet readings as in 3.1.3 step 2.g.
5. Double-click on the new created subset file. Change axis settings to histogram and GFP(530/30)-A(log axis) as in 3.1.3 step 2. b.
6. Repeat steps 3.1.5 steps 3 to 5 for all samples. Replicate all gates and settings to all samples by clicking on the first subset containing the FSC-Height and FSC-Area plot (usually named as “Lymphocytes” by default) and dragging it onto the other samples.
7. To analyze the mean fluorescence intensity (MFI) values, open histograms in the layout editor.
8. Normalize the y-axis of histograms to mode.
9. Perform statistical analysis for “Σ Mean: BL1-A” to display the MFI results on the layout window.
10. Calculate the standard deviation for MFI values of at least three independent biological replicates (see Note 1).

3.2. Analysis of c-di-GMP levels under anaerobic conditions

Some diguanylate cyclases and phosphodiesterases harbor oxygen-sensing domains, suggesting that oxygen regulates c-di-GMP levels [20–22]. In these experiments, we describe using an RNA-based fluorescent biosensor to analyze c-di-GMP levels in *E. coli* grown under anaerobic conditions and after oxygen recovery. When Ct biosensor is co-expressed with either inactive (G249A) or constitutively active (D70E) diguanylate cyclase WspR, clear differences in fluorescence intensities are observed that correspond to the expected low and high c-di-GMP levels, respectively (Fig. 3). These results demonstrate that the biosensor functions similarly with or without oxygen and that DFHBI and related dyes remain fluorescent under anaerobic conditions [17]. In contrast, fluorescent proteins in the GFP family that are commonly employed in protein biosensor construction require oxygen for chromophore maturation [23,24], and are therefore not suitable for anaerobic analysis.

3.2.1. Generation of *E. coli* strains

1. To prepare cells co-expressing biosensor and enzyme, add ~50 ng of each plasmid (e.g. Ct biosensor in pET31b and diguanylate cyclase WspR D70E in pCOLA) to BL21(DE3) Star *E. coli* chemically competent cells and transform the plasmids following the manufacturer’s protocol. Also, prepare positive

control (diguanylate cyclase WspR G249A in pCOLA and Spinach2 in pET31b) and negative control (diguanylate cyclase WspR G249A in pCOLA and empty pET31b) cells for setting up flow cytometry parameters. The positive control is the constitutively fluorescent Spinach2 and the negative control is the non-fluorescent empty vector. The controls also co-express diguanylate cyclase WspR G249A, which is an inactive mutant, so that the controls have similar expression levels and growth media conditions as the experimental samples.

2. Plate cells on LB/Carb/Kan agar plates and incubate for 12–16 h at 37 °C. The plates can be stored at 4 °C for 2 weeks (see Note 4).

3.2.2. Preparation and growth of anaerobic cultures

1. Autoclave anaerobic culture tubes (Balch tubes) and chlorobutyl rubber stoppers separately before use.
2. Add 3 mL AI/Carb/Kanto each anaerobic culture tube.
3. Inoculate the AI/Carb/Kan media with a single colony. (see Note 1, 5, and 6)
4. Close the culture tubes with chlorobutyl rubber stoppers. It is important to completely push the stoppers all the way down before proceeding to next step (see Note 7).
5. Cap the top of the culture tubes with aluminum seals and crimp using a hand-operated crimper (see Note 8). It is very important to ensure that all the tubes are completely sealed before proceeding to next step.
6. To prepare argon apparatus to sparge the headspace, connect one side of plastic tubing to the argon gas cylinder, and the other side of tubing to a Y-shaped tubing connector. Use additional tubing and Y-shaped connectors to set up multi-channel sparging system (see Fig. 4 for the illustrations of the set-up).
7. To create a connection between the rubber tubing and needle, use a syringe head, which are obtained by cutting 1 mL syringes, at each end of channel. Seal all connections firmly with parafilm.
8. Stick two syringe needles into each culture tube through the rubber stopper so that the tips of syringe needles are in the head-space of the culture inside tubes. To make the later step 10 easier, try to place two needles parallel each other.

⁴Do not use colonies from old plates. Old colonies from high overexpression strains such as BL21(DE3) Star *E. coli* tend to result in flow cytometry histogram with multiple humps and exhibit low fluorescence intensity in flow cytometry analysis. Similarly, we have seen variable results from frozen glycerol stocks. Preparing fresh transformants on agar plates also helps to obtain biological replicates as described in Note 1.

⁵This procedure is suitable for facultative anaerobic bacteria, or may be adapted for obligate anaerobic bacteria using a glove box.

⁶For facultative anaerobic bacteria, an alternative to argon sparging as described below to establish an anaerobic culture is to shake the sealed cultures at 37 °C for 16 hours or longer so that residual oxygen gets rapidly used up by the bacteria. To assist with this, more media can be added to reduce the headspace in the tube.

⁷Usually, it is difficult to cap the tubes properly just by pushing the stoppers down with fingers. Instead, first push down the stopper gently and then rotate the stopper as if screwing closed the lid of a water bottle.

⁸If the aluminum seal crimps loosely, remove the seal and repeat crimping using a new aluminum seal. If the problem persists, uncap/recap the rubber stopper and retry crimping.

9. Connect one of two needles on each tube to the syringe heads from step 8 and flow argon gas to sparge culture tubes with argon for >15 minutes. While sparging, the air is effused out through the other needle.
10. Keeping the argon gas flow on, quickly remove the two needles simultaneously to minimize the introduction of external air. Never leave argon flowing into the sealed tube without an outlet, as that could cause the pressurized tube to break.
11. Place all culture tubes in a tube rack and grow in an incubator shaker at 37 °C for 18 – 24 hours until cells reach an OD₆₀₀ > 3 (see Note 9).

3.2.3. Analysis of anaerobic cultures by flow cytometry

1. Prepare the 96-well flow plate containing PBS-DFHBI-1T as in 3.1.2. step 3.
2. Start up the flow cytometer.
3. Take out anaerobic culture tubes of positive and negative controls from the incubator shaker and place on ice.
4. While keeping the culture tubes on ice, break the aluminum seal of negative control culture (see Note 10).
5. Open the rubber stopper and immediately take out 1 µL of the negative control culture to dilute into the PBS-DFHBI-1T in the 96-well flow plate.
6. Load the flow plate onto the autosampler of the flow cytometer and analyze the sample on the flow plate.
7. Repeat 3.2.3 step 4–6 for the positive control culture.
8. Establish the FSC and SSC regions to optimize the voltage gain settings as described in 3.1.3.
9. Take out anaerobic culture tubes of sample cultures from the incubator shaker and place on ice.
10. On the heat map, highlight the well you are analyzing and create an experiment. Typically, two technical replicates are performed by analyzing each well twice.
11. Repeat 3.2.3 step 4–6 for each sample in turn.

3.2.4. Analysis of oxygen recovery cultures by flow cytometry

1. For oxygen recovery, agitate the opened cultures from 3.2.3 steps 3 and 9 by tapping the side of tubes and place them at 4 °C for 2 hours. The temperature is kept low to ensure that the enzyme and biosensor expression levels in cells do

⁹Since *E. coli* cells tend to grow slower in anaerobic conditions, longer incubation time than 18 hours may be needed to reach proper culture density for induction of expression. In trial experiments, control cultures can be prepared in Balch tubes and opened to check the incubation time required to reach the proper OD₆₀₀.

¹⁰Because it is important to rapidly read the fluorescence before bacteria are exposed to oxygen, the seals must be broken individually (in other words, one tube at a time) before flow cytometry analysis. Do not break seals of two or more tubes at the same time. Alternatively, it may be possible to sample the culture through the stopper with a gas-tight syringe equipped with a very long needle.

not change throughout the oxygen recovery step. If possible, gently shake the tubes on an orbital shaker(< 100 rpm).

- 2 On the heat map, highlight the well you are analyzing and create an experiment. Typically, two technical replicates are performed by analyzing each well twice.
- 3 Take out 1 μ L of each oxygen-recovered culture and dilute into the PBS-DFHBI-1T in the 96-well flow plate.
- 4 Load the flow plate onto the autosampler of the flow cytometer and analyze the samples on the flow plate using the settings described in 3.2.3step 8 (see Note 11).
- 3 Once all samples have been analyzed, export all your results to create FCS files and analyze the MFI values for each sample using FlowJo software as described in 3.1.5.

3.3. Monitoring cell population dynamics of c-di-GMP upon environmental changes

Flow cytometry can be a powerful method to monitor the cell population dynamics of c-di-GMP upon environmental changes. One advantage is that the experiments are high-throughput and provide information at the single-cell level. In addition, experiments monitoring c-di-GMP dynamics can be performed on cells grown in complex media (e.g. LB or autoinduction media), which cannot be done using fluorescence microscopy due to the high autofluorescence of media components. It should be noted that flow cytometry does not track c-di-GMP levels of an individual cell over time, because cells are discarded after they are flowed through the analysis chamber. However, this can also be considered an advantage, because cells are not repeatedly subjected to light excitation and media changes. Instead, single-cell fluorescence of a cell population is analyzed by flow cytometry over time. When used in combination with our fluorescent biosensors, analysis over time enables monitoring the dynamics of c-di-GMP levels.

In this section, we describe a representative experiment in which the PI-B biosensor was used to monitor cellular c-di-GMP increase upon Zn(II) depletion (Fig. 5) [25], which is dependent on the zinc-sensing diguanylate cyclase DgcZ in *E. coli* [26]. One critical aspect to performing the depletion experiment is the preparation of spent media, which is required to avoid changing media composition when cells are necessarily diluted for flow cytometry analysis. The preparation instructions for spent media are provided below (see Fig. 5 for schematic illustration). This method can be applied to monitor the effect of other input compounds added to the media.

3.3.1. Preparation of initial cultures

1. To prepare cells expressing biosensor, add ~50 ng of PI-B in pET31b plasmid to BL21(DE3) Star *E. coli* chemically competent cells and transform the plasmids following the manufacturer's protocol. Also, prepare positive control (Spinach2

¹¹You can also monitor dynamics during oxygen recovery by taking 1 μ L of each sample at various time points and analyzing with the flow cytometer.

in pET31b vector) and negative control (empty pET31b vector) cells for setting up flow cytometry parameters.

2. Plate cells on LB/Carb agar plates and incubate for 12–16 h at 37 °C. Plates can be stored at 4 °C up to two weeks (*see* Note 4).
3. Inoculate 3 mL AI/Carb with a single colony of the transformed cells (*see* Note 1). Shake cell cultures at 37 °C for 2 hours.
4. Split each cell cultures into two tubes (1.5 mL each). Add 1.5 µL of freshly prepared 1 M ZnCl₂ stock solution to one of the tubes to a final concentration of 1 mM. Shake cell cultures for additional 18 – 24 hours until cultures reach an OD₆₀₀ > 3.

3.3.2. Preparation of spent media

1. Take 1.2 mL of each cell culture (from 3.3.1 step 4) and centrifuge at 5,000 rpm for 5 minutes at room temperature. Make sure to save remaining 0.3 mL cell culture containing Zn(II) at 37 °C. The cell culture will be further used in 3.3.3 steps 3–4.
2. Carefully take the supernatant and filter through a 0.22 µm sterile filter to obtain the spent media with and without Zn(II).
3. Supplement each spent media with DFHBI-1T to the final concentration of 25 µM. Keep the media away from light.

3.3.3. Flow cytometry experiments

1. Start up the flow cytometer and do test runs with PBS-DFHBI-1T solution with negative control and positive control cells to establish the forward scatter (FSC) and side scatter (SSC) regions and to optimize the voltage gain settings as described in 3.1.3. In this experiment, the negative control refers to cells expressing empty pET31b vector and the positive control to cells expressing Spinach2 in pET31b vector.
2. For Zn(II)-depletion experiment, add 1 µL of cells grown with Zn(II) from cell culture saved from 3.3.2 step 1 to 500 µL spent media-DFHBI-1T solution prepared in 3.3.2 step 3 without Zn(II) and start timer (*see* Note 12).
3. For no-depletion control experiment, add 1 µL of cells grown with Zn(II) saved from 3.3.2 step 1 to 500 µL spent media-DFHBI-1T solution with Zn(II), which was obtained by sterile filtration of the same culture in 3.3.2 step 3 (*see* Note 13).
4. Shake the diluted cultures at 37 °C for 5–9 min to allow equilibration with DFHBI-1T.

¹²With the 1:500 dilution into autoinduction media without Zn(II) supplementation, cells change from a growth environment with 1 mM Zn(II) to less than or equal 2 µM Zn(II). Media components may contribute additional trace amount of Zn(II).

¹³To monitor c-di-GMP dynamics upon addition of an input compound instead of depletion, add the compound after dilution into spent media then start the timer. For example, this can be achieved by adding cells grown without Zn(II) saved from 3.3.2 step 1 to 500 µL spent media-DFHBI-1T solution with Zn(II). This dilution procedure avoids needing to centrifuge the cells to be analyzed, and is necessary to obtain sample cell density in the proper range (~10⁶ cells/mL) for flow cytometry.

5. Take 70 μ L aliquots from each sample and analyze by flow cytometry, This samples will represent the first time-point(=10 min).
6. Take 70 μ L aliquots every 5 min and repeat the analysis for up to 40 minutes.
7. Obtain MFI values at each time point using FlowJo software as described in 3.1.5.

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References

1. Romling U, Galperin MY, Gomelsky M. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiology and molecular biology reviews: MMBR*. 2013; 77(1):1–52. DOI: 10.1128/MMBR.00043-12 [PubMed: 23471616]
2. Tischler AD, Camilli A. Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. *Molecular microbiology*. 2004; 53(3):857–869. DOI: 10.1111/j.1365-2958.2004.04155.x [PubMed: 15255898]
3. Spangler C, Bohm A, Jenal U, Seifert R, Kaever V. A liquid chromatography-coupled tandem mass spectrometry method for quantitation of cyclic di-guanosine monophosphate. *Journal of microbiological methods*. 2010; 81(3):226–231. DOI: 10.1016/j.mimet.2010.03.020 [PubMed: 20385176]
4. Stelitano V, Brandt A, Fernicola S, Franceschini S, Giardina G, Pica A, Rinaldo S, Sica F, Cutruzzola F. Probing the activity of diguanylate cyclases and c-di-GMP phosphodiesterases in real-time by CD spectroscopy. *Nucleic acids research*. 2013; 41(7):e79.doi: 10.1093/nar/gkt028 [PubMed: 23358823]
5. Kellenberger CA, Sales-Lee J, Pan Y, Gassaway MM, Herr AE, Hammond MC. A minimalist biosensor: Quantitation of cyclic di-GMP using the conformational change of a riboswitch aptamer. *RNA biology*. 2015; 12(11):1189–1197. DOI: 10.1080/15476286.2015.1062970 [PubMed: 26114964]
6. Roelofs KG, Wang J, Sintim HO, Lee VT. Differential radial capillary action of ligand assay for high-throughput detection of protein-metabolite interactions. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108(37):15528–15533. DOI: 10.1073/pnas.1018949108 [PubMed: 21876132]
7. Wolfe AJ, Berg HC. Migration of bacteria in semisolid agar. *Proceedings of the National Academy of Sciences of the United States of America*. 1989; 86(18):6973–6977. [PubMed: 2674941]
8. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic approaches to study of biofilms. *Methods in enzymology*. 1999; 310:91–109. [PubMed: 10547784]
9. Koestler BJ, Waters CM. Bile acids and bicarbonate inversely regulate intracellular cyclic di-GMP in *Vibrio cholerae*. *Infection and immunity*. 2014; 82(7):3002–3014. DOI: 10.1128/IAI.01664-14 [PubMed: 24799624]
10. Ho CL, Chong KS, Oppong JA, Chuah ML, Tan SM, Liang ZX. Visualizing the perturbation of cellular cyclic di-GMP levels in bacterial cells. *Journal of the American Chemical Society*. 2013; 135(2):566–569. DOI: 10.1021/ja310497x [PubMed: 23289502]
11. Christen M, Kulasekara HD, Christen B, Kulasekara BR, Hoffman LR, Miller SI. Asymmetrical distribution of the second messenger c-di-GMP upon bacterial cell division. *Science*. 2010; 328(5983):1295–1297. DOI: 10.1126/science.1188658 [PubMed: 20522779]
12. Kellenberger CA, Wilson SC, Sales-Lee J, Hammond MC. RNA-based fluorescent biosensors for live cell imaging of second messengers cyclic di-GMP and cyclic AMP-GMP. *Journal of the American Chemical Society*. 2013; 135(13):4906–4909. DOI: 10.1021/ja311960g [PubMed: 23488798]

13. Kellenberger CA, Hallberg ZF, Hammond MC. Live Cell Imaging Using Riboswitch-Spinach tRNA Fusions as Metabolite-Sensing Fluorescent Biosensors. *Methods in molecular biology*. 2015; 1316:87–103. DOI: 10.1007/978-1-4939-2730-2_8 [PubMed: 25967055]
14. Kellenberger CA, Hammond MC. In vitro analysis of riboswitch-Spinach aptamer fusions as metabolite-sensing fluorescent biosensors. *Methods in enzymology*. 2015; 550:147–172. DOI: 10.1016/bs.mie.2014.10.045 [PubMed: 25605385]
15. Paige JS, Wu KY, Jaffrey SR. RNA mimics of green fluorescent protein. *Science*. 2011; 333(6042): 642–646. DOI: 10.1126/science.1207339 [PubMed: 21798953]
16. Paige JS, Nguyen-Duc T, Song W, Jaffrey SR. Fluorescence imaging of cellular metabolites with RNA. *Science*. 2012; 335(6073):1194.doi: 10.1126/science.1218298 [PubMed: 22403384]
17. Wang XC, Wilson SC, Hammond MC. Next-generation RNA-based fluorescent biosensors enable anaerobic detection of cyclic di-GMP. *Nucleic acids research*. 2016; doi: 10.1093/nar/gkw580
18. Song W, Strack RL, Svensen N, Jaffrey SR. Plug-and-play fluorophores extend the spectral properties of Spinach. *Journal of the American Chemical Society*. 2014; 136(4):1198–1201. DOI: 10.1021/ja410819x [PubMed: 24393009]
19. Hallberg ZF, Wang XC, Wright TA, Nan B, Ad O, Yeo J, Hammond MC. Hybrid promiscuous (Hypr) GGDEF enzymes produce cyclic AMP-GMP (3', 3'-cGAMP). *Proceedings of the National Academy of Sciences of the United States of America*. 2016; 113(7):1790–1795. DOI: 10.1073/pnas.1515287113 [PubMed: 26839412]
20. Tuckerman JR, Gonzalez G, Sousa EH, Wan X, Saito JA, Alam M, Gilles-Gonzalez MA. An oxygen-sensing diguanylate cyclase and phosphodiesterase couple for c-di-GMP control. *Biochemistry*. 2009; 48(41):9764–9774. DOI: 10.1021/bi901409g [PubMed: 19764732]
21. Lacey MM, Partridge JD, Green J. Escherichia coli K-12 YfgF is an anaerobic cyclic di-GMP phosphodiesterase with roles in cell surface remodelling and the oxidative stress response. *Microbiology*. 2010; 156(Pt 9):2873–2886. DOI: 10.1099/mic.0.037887-0 [PubMed: 20522491]
22. An S, Wu J, Zhang LH. Modulation of Pseudomonas aeruginosa biofilm dispersal by a cyclic-Di-GMP phosphodiesterase with a putative hypoxia-sensing domain. *Applied and environmental microbiology*. 2010; 76(24):8160–8173. DOI: 10.1128/AEM.01233-10 [PubMed: 20971871]
23. Reid BG, Flynn GC. Chromophore formation in green fluorescent protein. *Biochemistry*. 1997; 36(22):6786–6791. DOI: 10.1021/bi970281w [PubMed: 9184161]
24. Craggs TD. Green fluorescent protein: structure, folding and chromophore maturation. *Chemical Society reviews*. 2009; 38(10):2865–2875. DOI: 10.1039/b903641p [PubMed: 19771333]
25. Jongchan Yeo XCW, Ming C. Hammond Imaging Single-Cell Dynamics of Cyclic di-GMP in E. coli.
26. Zahringer F, Lacanna E, Jenal U, Schirmer T, Boehm A. Structure and signaling mechanism of a zinc-sensory diguanylate cyclase. *Structure*. 2013; 21(7):1149–1157. DOI: 10.1016/j.str.2013.04.026 [PubMed: 23769666]

Figure 1.

In vivo fluorescent biosensor screen of GGDEF genes from *Geobacter sulfurreducens* for diguanylate cyclase activity. Mean fluorescence intensity (MFI) values were measured by flow cytometry analysis of *E.coli* BL21 (DE3) Star cells co-expressing the Dp biosensor and GGDEF enzymes from *G. sulfurreducens* strain PCA, empty pCOLA vector, or diguanylate cyclase WspR. Error bars represent the standard deviation from three independent biological replicates.

Figure 2.

Analysis for setting up flow cytometry gate parameters. Examples of (a) FSC-A/SSC-A (both log axes) (b) FSC-H/FSC-A (both log axes), (c) time (linear axis)/FSC-A (log axis) and (d) histogram for GFP(530/30)-A (log axis), along with two gates (labeled as R1 and R2) are shown.

Figure 3.

Flow cytometry histograms of *E. coli* BL21(DE3) Star cells co-expressing Ct biosensor and indicated enzymes (inactive WspR G249A or constitutively active WspR D70E) under aerobic, anaerobic, and oxygen recovery conditions.

Figure 4.

Schematic for the set-up of multi-channel argon gas sparging system. The main gas flow is split into four or more paths using Y-shaped tubing connectors, and syringe heads are connected to each end. All connections should be sealed with parafilm. Two needles are stuck through rubber stoppers and each channel is connected to one of two needles to allow argon gas to flow inside and the air is expelled out.

Figure 5.

Flow cytometry analysis of cell population dynamics of c-di-GMP. (a) Representative flow cytometry histograms for wild-type BL21(DE3) Star cells expressing Pl-B biosensor upon Zn(II) depletion. (b) Dynamic changes in single-cell fluorescence of BL21(DE3) Star cells expressing c-di-GMP biosensor in the presence of Zn(II) (+ Zn(II), closed symbols) and upon Zn(II) depletion at time 0 (– Zn(II), open symbols). MFIs were obtained at indicated time points with flow cytometry. Error bars represent the standard deviations for the results from three independent biological replicates.

Figure 6.

Experimental schematic for the Zn(II) depletion experiment. After overnight growth in autoinduction media for biosensor expression, cells need to be diluted for flow cytometry experiments. To avoid changing media composition and centrifugation, cell cultures are simply diluted into spent media, which is prepared by sterile filtration of the culture supernatant from the same overnight cultures, with the addition of DFHBI-1T, the dye used by the biosensor.

Table 1

Sequences of c-di-GMP biosensors (*ITALICS* = T7 promoter; UNDERLINED = tRNA scaffold; CAPS = Spinach2 sequence; **BOLD** = c-di-GMP riboswitch aptamer sequence (Dp, Ct or Pl-B); **BOLD ITALICS**= T7 terminator)

Biosensor	Sequence
Dp	TAATACGACTCATAAGGGGCCCGGATAGCTCAGTCGGTAGAGCAGCGGCCG GATGTAAGTGAATGAAATGGTGAAGGACGGGTCCA CUUCUGACAAAGGCAAAACCCUCCGGAGGGGGGACGCAAGCCCAACGAAACUCCGCGUGCUCCGUCUUCUUCUACAGGGCAGCAGGAAAGUUGCCGGGGCCACCGAAAGAAAG TTGTTGAGTAGAGTGTGAGCTCCGTAAGTACATCCGGCCGGGTCCAGGGTTCAAGTCCCTGTTCTGGGGCGCCA TAGCATAACCCCTTGGGGCCCTAAACGGGGTCTTGAGGGGTTTTTG
Ct	TAATACGACTCATAAGGGGCCCGGATAGCTCAGTCGGTAGAGCAGCGGCCG GATGTAAGTGAATGAAATGGTGAAGGACGGGTCCA ATGAAACAGGGCAAAATACCGAAAGGTGATGACGCAAAAGCCATGGGTCTACTGTTTAAACAATGTTTAAAGCTATGATCGCCAGGCTGCCAT TTGTTGAGTAGAGTGTGAGCTCCGTAAGTACATCCGGCCGGGTCCAGGGTTCAAGTCCCTGTTCTGGGGCGCCA TAGCATAACCCCTTGGGGCCCTAAACGGGGTCTTGAGGGGTTTTTG
Pl-B	TAATACGACTCATAAGGGGCCCGGATAGCTCAGTCGGTAGAGCAGCGGCCG GATGTAAGTGAATGAAATGGTGAAGGACGGGTCCA CTTCGATAACGGCAAACTTCTCGAAAGATAAGGACGCAAAAGCCACAGGGGCCCTTCTTGATGAACCCGTCAATGGCAGCCTGGCTACCGAAG TTGTTGAGTAGAGTGTGAGCTCCGTAAGTACATCCGGCCGGGTCCAGGGTTCAAGTCCCTGTTCTGGGGCGCCA TAGCATAACCCCTTGGGGCCCTAAACGGGGTCTTGAGGGGTTTTTG
Spinach2	TAATACGACTCATAAGGGGCCCGGATAGCTCAGTCGGTAGAGCAGCGGCCG GATGTAAGTGAATGAAATGGTGAAGGACGGGTCCA TTGTTGAGTAGAGTGTGAGCTCCGTAAGTACATCCGGCCGGGTCCAGGGTTCAAGTCCCTGTTCTGGGGCGCCA TAGCATAACCCCTTGGGGCCCTAAACGGGGTCTTGAGGGGTTTTTG