Chapter 8

Gauging and Visualizing c-di-GMP Levels in *Pseudomonas aeruginosa* Using Fluorescence-Based Biosensors

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Abstract

Recent research has shown that the molecule c-di-GMP is an important second messenger regulating various functions in bacteria. In particular, the implication of c-di-GMP as a positive regulator of adhesion and biofilm formation has gained momentum as a highly relevant research topic, as detailed knowledge about the underlying regulatory mechanisms may enable the development of measures to control biofilms in both industrial and medical settings. Accordingly, it is in many cases of interest to measure the c-di-GMP level in bacteria under specific conditions or in specific mutant strains. We have developed a collection of fluorescence-based c-di-GMP biosensors capable of gauging the c-di-GMP level in *Pseudomonas aeruginosa* and closely related bacteria. Here, we describe protocols for the use of these biosensors in gauging and visualizing cellular c-di-GMP levels of *P. aeruginosa* both in in vitro setups such as continuous-culture flow-cell biofilms, and in in vivo settings such as a murine corneal infection model.

Key words c-di-GMP, Cyclic di-GMP, Biosensor, Fluorescence, Pseudomonas, Biofilm, Ocular infection

1 Introduction

Biofilms pose numerous problems both industrially and clinically due to their biofouling properties and their recalcitrance toward the immune system and antibiotics [1]. Over the past decade it has become clear that the bacterial second messenger c-di-GMP is a key signaling molecule in many bacteria, governing the transition from the planktonic motile life-form to the sessile life in biofilms [2]. Knowledge of c-di-GMP signaling pathways, from the environmental inputs to effector outputs, will ultimately allow us to develop measures to control biofilms in clinical and industrial settings.

The level of c-di-GMP in bacteria under specific physiologic conditions or in specific mutant strains is a key parameter for the study of c-di-GMP-mediated regulation. A golden standard for

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quantitating cellular c-di-GMP levels is analysis of bacterial extracts by LC/MS/MS [3]. Although precise and highly sensitive, there are drawbacks to the LC/MS/MS method, including end-point-only sampling as well as laborious and complex sample analysis. An alternative to the LC/MS/MS analysis is semiquantitative estimation of c-di-GMP levels using fluorescence-based biosensors that respond to fluctuations in the messenger level. These biosensors have the advantage of allowing nondestructive real-time measurements or visualizations of bacterial c-di-GMP levels in both in vitro and in vivo settings.

We have developed a range of fluorescence-based c-di-GMP biosensors for use in *P. aeruginosa* (Table 1) [4]. This bacterium is causing various persistent infections, and serves as a model organism for biofilm formation and c-di-GMP signaling. The biosensors are all based on a transcriptional fusion of the c-di-GMP-responsive *cdrA* promoter to *gfp*. The plasmid-based biosensors come in two different versions differentiated by a subscript C or S denoting their origin of creation (Copenhagen and Seattle, respectively). Furthermore, the C-set includes mini-Tn7-based integrative biosensors. Versions of the biosensors utilizing either stable Gfp(Mut3) or unstable Gfp(ASV) have been constructed. Transcription from the *cdrA* promoter is regulated by c-di-GMP through binding of the molecule to the transcriptional regulator FleQ. Utility of the biosensors may therefore be limited to *P. aeruginosa* and related bacterial species harboring close FleQ homologs.

Here, we describe protocols for the use of the biosensors in gauging and visualizing the cellular c-di-GMP levels in *P. aeruginosa* bacteria both in vitro using liquid batch cultures and continuous-culture flow-cell biofilms, and in vivo using a newly developed murine corneal infection model.

Table 1
Fluorescence-based c-di-GMP biosensors available (*see* Note 2)

Vector	Relevant genotype and characteristics	Reference
pCdrA-gfp^{C}	pUCP22Not-PcdrA-RBSII-gfp(Mut3)-T_0-T_1, Gm^r Amp^r	[4]
pCdrA-gfp^{S}	pUCP22Not-PcdrA-RBS-CDS-RNaseIII-gfp(Mut3)-T_0-T_1, Gm^r Amp^r	[4]
pCdrA-gfp(ASV)^{C}	pUCP22Not-PcdrA-RBSII-gfp(ASV)-T_0-T_1, Gm^r Amp^r	[4]
pCdrA-gfp(ASV)^{S}	pUCP22Not-PcdrA-RBS-CDS-RNaseIII-gfp(ASV)-T_0-T_1, Gm^r Amp^r	[4]
pTn7CdrA-gfp^{C}	miniTn7-PcdrA-RBSII-gfp(Mut3)-T_0-T_1 delivery vector, Gm^r (Amp^r)	[4]
pTn7CdrA-gfp (ASV)^{C}	miniTn7-PcdrA-RBSII-gfp(ASV)-T_0-T_1 delivery vector, Gm^r (Amp^r)	[4]

2 Materials

2.1 Gauging and Visualizing c-di-GMP Levels in P. aeruginosa in In Vitro Systems

1. Standard LB broth and LB agar for inoculum preparation.
2. A10 solution: 20 g/L $(NH_4)_2SO_4$, 60 g/L $Na_2HPO_4 \cdot 2H_2O$, 30 g/L KH_2PO_4, and 30 g/L NaCl in water. Upon preparation, pH of the solution should be 6.4. If the pH is not 6.4 the solution must be discarded. Do not adjust pH. Autoclave at 121 °C for 15 min.
3. $MgCl_2$ stock solution (1 M): 203.31 g/L $MgCl_2 \cdot 6H_2O$ in water.
4. $CaCl_2$ stock solution (1 M): 147.01 g/L $CaCl_2 \cdot 2H_2O$ in water.
5. Trace metal stock solution: 200 mg/L $CaSO_4 \cdot 2H_2O$, 200 mg/L $FeSO_4 \cdot 7H_2O$, 20 mg/L $MnSO_4 \cdot H_2O$, 20 mg/L $CuSO_4 \cdot 5H_2O$, 20 mg/L $ZnSO_4 \cdot 7H_2O$, 10 mg/L $CoSO_4 \cdot 7H_2O$, 12 mg/L $NaMoO_4 \cdot H_2O$, 5 mg/L H_3BO_3 in water.
6. BTrace solution: 1 mL $MgCl_2$ stock solution, 0.1 mL $CaCl_2$ stock solution and 0.1 μL trace metal stock solution per 900 mL of water. Autoclave at 121 °C for 15 min.
7. ABTrace medium: 100 mL A10 solution and 900 mL BTrace solution (*see* **Note 1**).
8. $FeCl_3$ stock solution (10 mM): 2.7 mg/mL $FeCl_3 \cdot 6H_2O$ in water. Filter sterilize through a 0.22 μm pore filter.
9. Glucose stock solution (10% w/v): 100 g/L glucose in water. Autoclave at 121 °C for 15 min.
10. Casamino acid stock solution (20% w/v): 200 g/L casamino acid in water. Autoclave at 121 °C for 15 min.
11. ABTraceFe growth medium supplemented with glucose and casamino acids for liquid batch culturing: 1 L ABTrace medium, 100 μL $FeCl_3$ stock solution, 20 mL glucose stock solution, and 25 mL casamino acid stock solution.
12. ABTrace growth medium supplemented with glucose for continuous culturing of biofilms in flow-cells: 1 L ABTrace medium supplemented with 100 μL trace metal stock solution, and 2.7 mL glucose stock solution.
13. NaCl solution (0.9% w/v): 9 g/L NaCl in water. Autoclave at 121 °C for 15 min.
14. A *cdrA-gfp* biosensor for gauging or visualizing c-di-GMP. *See* Table 1 and **Note 2** for a list and description, respectively, of the available biosensors.
15. A *P. aeruginosa* strain harboring the biosensor (*see* **Notes 3** and **4**).

16. 50 mL Erlenmeyer flasks.
17. Black 96-well microplates suitable for fluorescence measurements.
18. A plate reader (e.g., Victor X4, Perkin Elmer) equipped with a filter set for measuring green fluorescence (Ex 485 nm/Em 535 nm) and a filter for measuring light absorbance at 590 nm.
19. SYTO®62: 5 μM Red Fluorescent Nucleic Acid Stain (Molecular Probes, USA) dissolved in 0.9% (w/v) NaCl (working solution).
20. A flow-cell setup identical to the one described by Crusz et al. [5].
21. A confocal laser scanning microscope (e.g., Zeiss LSM710) with lasers equipped for visualizing Gfp (biosensor output) and Syto®62 (total biofilm biomass).
22. IMARIS image processing software (Bitplane AG, Switzerland).

2.2 Visualization of c-di-GMP Levels in *P. aeruginosa* in a Murine Ocular Infection Model

1. Standard LB broth and LB agar for inoculum preparation and CFU counts.
2. NaCl solution (0.9% w/v) for serial dilutions: 9 g/L NaCl in water. Autoclave at 121 °C for 15 min.
3. *P. aeruginosa* PAO1.
4. *P. aeruginosa* PAO1/pCdrA-gfp^{C}.
5. *P. aeruginosa* PAO1 Tn7::P_{lac}-gfp (constitutive *gfp* expression).
6. Female C57BL/6 mice (Invivos, Singapore) 7–8 weeks old (*see* **Note 5**). Maintain on water and standard mouse chow ad libitum.
7. Miniblade (BD-Beaver, Cat. No. 376400).
8. Tweezers.
9. Surgical scissors.
10. Pellet pestle (autoclavable).
11. Glass beads (5 mm diameter).
12. SYTO®62 Red Fluorescent Nucleic Acid Stain (Molecular Probes, USA). 5 μM in 0.9% (w/v) NaCl (working solution).
13. μ-Slide 8 Well glass bottom chamber slide.
14. A confocal laser scanning microscope (e.g., Zeiss LSM780) with lasers equipped for visualizing Gfp (biosensor output) and Syto®62 (total biofilm biomass).
15. A Zeiss Stemi-2000C dissecting microscope.
16. IMARIS image processing software (Bitplane AG, Switzerland).

3 Methods

3.1 Gauging c-di-GMP Levels in P. aeruginosa in In Vitro Systems During Liquid Batch Culturing

1. Prepare a starter culture of the *P. aeruginosa* PAO1 ΔwspF ΔpelA ΔpslBCD/pCdrA-gfp^{C} biosensor strain (15 mL ABTraceFe medium supplemented with glucose and casamino acids in a 50 mL Erlenmeyer flask). Use a couple of freshly grown colonies from an LB plate as inoculum and incubate the culture at 37 °C under well-shaken conditions (e.g., orbital shaking with a diameter of 30 mm and a speed of 200 rpm) for 16–18 h (*see* **Note 6**).
2. Prepare the experimental culture of the biosensor strain (15 mL ABTraceFe medium supplemented with glucose and casamino acids in a 50 mL Erlenmeyer flask). Measure OD_{600} of the outgrown starter culture (should be 3–4) and dilute it into the experimental culture to a final OD_{600} of 0.01.
3. Incubate the culture at 37 °C under well-shaken conditions (e.g., orbital shaking with a diameter of 30 mm and a speed of 200 rpm) for 14 h.
4. Sample 100 μL of the culture every hour and transfer the sample to an empty well of a black 96-well microplate suitable for fluorescence measurements in a plate reader.
5. Read the growth level (Abs590nm) and the biosensor output (Gfp fluorescence) of the most recent sample in the plate reader after each sampling.
6. Once the experiment is concluded, plot growth curves (Abs590 over time), absolute fluorescence curves (Gfp fluorescence over time) and relative fluorescence curves (Abs590-normalized Gfp fluorescence over time) of the culture and evaluate the relative c-di-GMP level of the culture (*see* **Note 7**). A typical result is shown in Fig. 1. In addition to the scatter plot data representation displaying relative monitor activity and growth as a function of time, data from a single representative time-point may be extracted and displayed in a column chart. This is particularly useful when comparing multiple different strains, growth conditions, treatments etc.

3.2 Visualizing c-di-GMP Levels in P. aeruginosa in In Vitro Continuous-Culture Flow-Cell Biofilms

1. Set up the flow-cell system according to the guidelines described by Crusz et al. [5].
2. Prepare a starter culture of *P. aeruginosa* PAO1 containing the Tn7-integrative biosensor encoding unstable Gfp(ASV) (Table 1) using 5 mL of LB broth in a standard test tube. Use a couple of freshly grown colonies from an LB plate as inoculum and incubate the culture at 37 °C with 200 rpm shaking for 16–18 h.

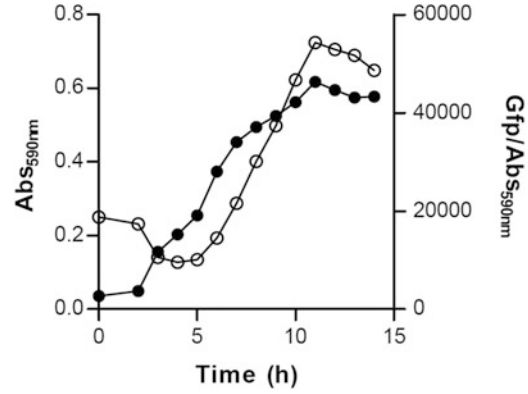

Fig. 1 Example of the biosensor output from *P. aeruginosa* during planktonic culturing. Growth (Abs590nm, closed circles) and relative fluorescence (Gfp/Abs590nm, closed circles) of a planktonic *P. aeruginosa Δ wspF Δ pelA Δ pslBCD*/pCdrA-gfp^{C} culture are plotted as a function of incubation time. The graph shows that the biosensor output is low during the early and mid-exponential phases and increases in the late exponential phase. The decrease in biosensor output during the early exponential growth phase is due to residual stable Gfp being present in cells from the outgrown culture used as inoculum. The high *PcdrA-gfp* gene dose from the plasmid-based reporter allows monitoring of c-di-GMP fluctuations in a growing culture of the *Δ wspF Δ pelA Δ pslBCD*/pCdrA-gfpC strain

3. Dilute the starter culture to an OD_{600} of 0.001 using 0.9% saline and inoculate each flow-cell chamber with 300 μL of the dilution according to Crusz et al. [5].
4. Incubate the system at 37 °C for the biofilm to develop. For *P. aeruginosa PAO1*, the flow-cell biofilm development during a standard experiment running for 96 h normally progresses through stages of substratum attachment, microcolony formation and maturation of the microcolonies (e.g., mushroom-type microcolony formation). A defined dispersal stage is not reached due to the continuous feed of nutrients. Experiments may last beyond the typical 96 h.
5. Remove the system from incubation at the desired time points to investigate the biosensor output and spatial location in the flow-cell-grown biofilms using confocal laser scanning microscopy.
6. Stain the total biofilm biomass immediately before conducting the microscopy by very gentle injection of 300 μL Syto®62 solution into the chamber under investigation in a manner similar to the initial inoculation of the flow-cell. Take great care not to disrupt the biofilm. Leave the chamber protected from light for 10 min before image acquisition for the stain to have effect. Once the biofilm has been stained with Syto®62 it should be excluded from the remainder of the experiment due to the nucleic acid binding properties of the molecule (*see* **Note 8**).

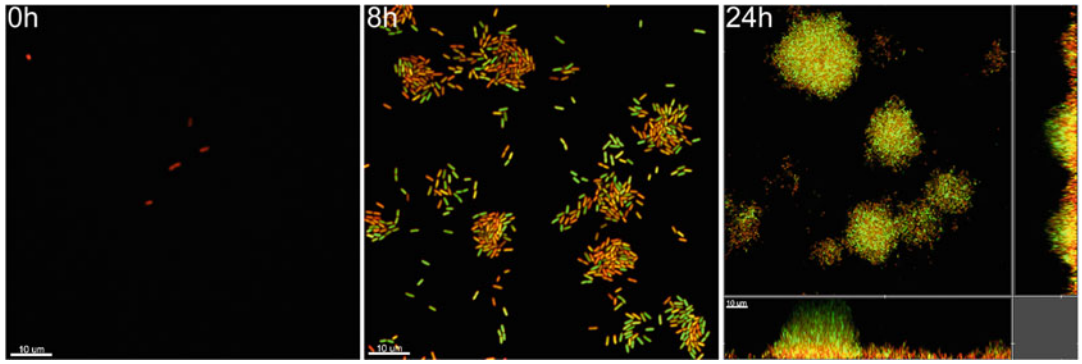

Fig. 2 Example of biosensor visualization during *P. aeruginosa* flow-cell biofilm development. Flow-cell grown biofilms of *P. aeruginosa* PAO1 Tn7::cdrA-gfp(ASV)^{C} were stained red with SYTO®62 and visualized by confocal laser scanning microscopy as described in Subheading 3.2. The biofilms shown are from 0, 8, and 24 h post attachment to the substratum. *Green* coloring depicts the biosensor output while *red* coloring depicts the total biofilm biomass. The 0 h and 8 h images display a top-down view while the 24 h image displays a central top-down view with flanking vertical cross sections. Scale bar equals 10 μm. The pictures show that biosensor output is absent in planktonic cells when first attaching to the substratum (0 h) indicating low cellular c-di-GMP levels. When the biofilm develops the monitor output from the bacteria increases indicating that the cellular levels of c-di-GMP increase (8 h and 24 h). Notice also the heterogeneity of the biosensor output in the biofilm cells. The single-gene dose of the chromosomally integrated *PcdrA-gfp* reporter is sufficient to render biofilm-grown PAO1 wild-type bacteria fluorescent, but does not give rise to fluorescence from PAO1 wild-type bacteria grown in planktonic culture, indicating that biofilm-grown *P. aeruginosa* bacteria have much higher c-di-GMP content than planktonically grown bacteria

7. Acquire suitable image stacks visualizing the biosensor output (Gfp fluorescence) and the total biofilm biomass (Syto®62 fluorescence) using the Zeiss LSM710 confocal laser scanning microscope. Take care to avoid pixel saturation (loss of fluorescence level information) for the detection of biosensor-derived Gfp fluorescence in order to be able to qualitatively analyze the spatio-temporal fluctuations of c-di-GMP levels within the cells of the biofilm. Use the Imaris image processing software (or similar) for the qualitative analysis of the different image stacks and to prepare publication-ready images. An example is shown in Fig. 2.

3.3 Gauging c-di-GMP Levels in P. aeruginosa *Bacteria in a Murine Ocular Infection Model*

3.3.1 Inoculum Preparation

1. Streak *P. aeruginosa* PAO1 or *P. aeruginosa* PAO1/pCdrA-gfp^{C} or P. *aeruginosa* PAO1 Tn7::P_{lac}-gfp on LB agar and grow at 37 °C for 16 h.
2. Pick a colony and grow in 2 mL LB broth at 37 °C and 200 rpm for 16 h. The CFU/mL of the overnight culture is estimated to be between 1×10^9 and 1×10^{10}.
3. Transfer 1 mL of culture into a 1.5 mL microcentrifuge tube and centrifuge at 12,000 × *g* for 3 min.
4. Remove the supernatant and resuspend the cell pellet in 1 mL 0.9% NaCl.

5. Repeat the wash step twice.
6. Measure the optical density at 600 nm of the bacterial suspension and adjust it to 0.58 (5×10^8 CFU/mL) in 1 mL 0.9% NaCl.

3.3.2 Establishing the Experimental Keratitis

1. Examine the eyes of the mice under the slit lamp for potential corneal defects and abnormalities. If any defects or abnormalities are observed the mouse is excluded from the experiment.
2. Anaesthetize the mice with 120 μL of premixed xylene (10 mg/kg) and ketamine (40 mg/kg).
3. Using the dissecting microscope, scratch each corneal epithelia with a sterile miniblade creating four superficial scratches of 1–2 mm in length.
4. Apply 10 μL of *P. aeruginosa* suspension (from Subheading 3.3.1) topically to each scratched cornea.
5. Place the mice in labeled cages and allow the corneal infection to develop. Examine the corneas on a daily basis using the dissecting microscope.

3.3.3 Quantification of Colony Forming Units in Biofilms

1. After 2, 5, and 7 days of infection (*see* **Note 9**), anaesthetize the mice with 120 μL of premixed xylene (10 mg/kg) and ketamine (40 mg/kg). Kill the mice by cervical dislocation. Use the tweezers and surgical scissors to excise and extract the corneas.
2. Place the cornea in a 1.5 mL microcentrifuge tube and crush it using the pellet pestle.
3. Resuspend the cornea in 500 μL 0.9% NaCl and add in 10 glass beads. Disrupt the biofilm cells in the crushed cornea by vortexing at maximum speed for 5 min.
4. Perform serial dilutions on the bacterial cells by adding 100 μL of cell suspension to 900 μL 0.9% NaCl sequentially from dilution factor 10^0–10^{-7} (*see* **Note 10**). Spread 100 μL of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions on LB agar plates. Incubate the plates at 37 °C for 16 h.
5. Count the number of colonies formed on each LB agar plate. Choose plates with a minimum of 30 and a maximum of 300 colonies for calculating the CFU/mL.

3.3.4 Imaging Biofilms on Cornea

1. Use *P. aeruginosa* PAO1/pCdrA-gfp^C and *P. aeruginosa* PAO1 Tn7::P_{lac}-gfp to establish the experimental keratitis as described above. *P. aeruginosa* Tn7::P_{lac}-gfp acts as a positive control for Gfp fluorescence.
2. After 2, 5, and 7 days of infection, anaesthetize the mice with 120 μL of premixed xylene (10 mg/kg) and ketamine

(40 mg/kg). Kill the mice by cervical dislocation. Use the tweezers and surgical scissors to excise and extract the corneas.

3. Place the excised cornea in 200 μL SYTO®62 solution on the μ-Slide 8-well glass bottom chamber slide.
4. Incubate the samples in the dark for 10 min at room temperature, to stain both bacterial and murine cells with SYTO®62.
5. Capture image stacks of biofilm on the cornea using the Zeiss LSM780 confocal laser scanning microscope equipped with a 63× oil objective. Visualize the biosensor output as Gfp fluorescence and visualize the total biofilm and murine cell biomass as Syto®62 fluorescence. Take care to avoid pixel saturation (loss of fluorescence level information) for the detection of biosensor-derived Gfp fluorescence in order to be able to qualitatively analyze the spatio-temporal fluctuations of c-di-GMP levels within the cells of the biofilm.
6. Process the images with the IMARIS software. Using the Section 2D display, present the Z-stack images as a central horizontal cross-section (X and Y axes) with flanking vertical cross-sections below (X and Z axes) and to the right (Y and Z axes) of the central section. Create images with both single (Gfp or SYTO®62) and merged channels (both Gfp and SYTO®62). An example is shown in Fig. 3.

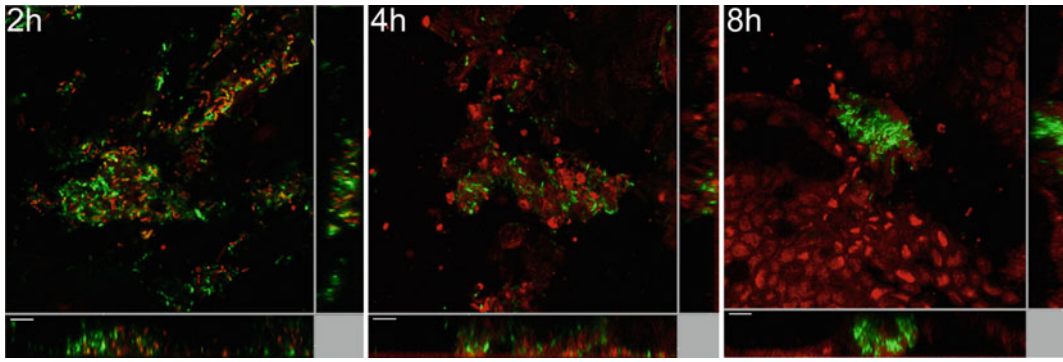

Fig. 3 An example of the biosensor output in corneal biofilms of *P. aeruginosa* obtained using a murine ocular infection*see also Pathogenicity Assay* model. The *P. aeruginosa* PAO1/pCdrA-gfp^C biofilms shown are from corneas removed 2, 4, and 8 h post infection. *Green* coloring depicts the biosensor output while *red* coloring depicts the total biofilm biomass in combination with host immune cells. The images were acquired using confocal laser scanning microscopy as described in Subheading 3.3.4 and displayed as central top-down views with flanking vertical cross sections. Scale bar equals 10 μm. At the early infection stage (2 h) with little or no biofilm formation, there is a high degree of heterogeneity of the monitor output indicating fluctuations in the c-di-GMP level across the population. At the late infection stage (8 h) a biofilm has developed displaying a high biosensor output with less heterogeneity and hence high cellular levels of c-di-GMP throughout the biofilm population

4 Notes

1. The A and BTrace parts of the ABTrace medium are prepared and sterilized separately to avoid the formation of undesirable precipitates that will form if the two solutions are mixed and autoclaved together.
2. The biosensors available are either plasmid-based or integrative. The plasmid-based (pUCP22Not) biosensors have the highest basal output and signal-to-noise ratio making them useful when working with plate readers or other equipment where the strength of the excitation source (such as a UV lamp with a mounted band pass filter) is modest. The single-copy integrative Tn7-based biosensors have a much lower basal output compared to the plasmid-based biosensors but are especially suitable for use in flow-cell biofilm experiments where the strong excitation and sensitive detection of fluorescence emission offered by confocal laser scanning microscopy is used as the visualization method. In addition, the biosensors come in two variants employing either stable Gfp or unstable Gfp(ASV). Using the biosensors with stable Gfp leads to the accumulation of the fluorescent signal which improves the output and the signal-to-noise ratio. However, employing unstable Gfp has the advantage of recording fluctuations in cellular c-di-GMP levels over time.
3. For gauging c-di-GMP levels in planktonic cultures of *P. aeruginosa* PAO1 we use a mutant strain with deletions of *wspF*, *pelA*, and *pslBCD* harboring the pCdrA-gfp^{C} biosensor. The *wspF* mutation results in a high basal level of c-di-GMP due to increased activation of the c-di-GMP synthesizing diguanylate cyclase WspR leading to a strong basal fluorescent output from the biosensor which increases the signal-to-noise ratio. The *pelA* and *pslBCD* mutations render the strain exopolysaccharide deficient thereby avoiding formation of aggregates during culturing, which would otherwise occur in the *wspF* deletion background and obscure growth measurements. In addition, we use the plasmid-based biosensor with stable Gfp to further improve the output and the signal-to-noise ratio. For visualization of c-di-GMP levels in flow-cell biofilms using confocal laser scanning microscopy, there are no restrictions on the *P. aeruginosa* strain type or biosensor type due to the strength of the laser-based excitation source and the eminent sensitivity of fluorescence emission detection offered by the microscope setup. We prefer, however, to use the integrative Tn7CdrA-gfp(ASV)^{C} biosensor with unstable Gfp to be able to monitor the fluctuations in reporter activity over time within the biofilm without the need for proper selection during the prolonged incubation of the experiment.

4. The plasmid-based biosensors are favorably introduced into *P. aeruginosa* by electroporation using the sucrose-based protocol described by Choi et al. [6]. To reduce the incidents of detrimental sample arching during electroporation, we use a slightly modified version of the protocol by using only 2 mL of outgrown liquid culture which is finally resuspended in 500 μL sucrose. Introduce the integrative Tn7-based biosensors into *P. aeruginosa* by four-parental mating conjugation as described by Klausen et al. [7].
5. Research involving animals such as mice requires permission from the proper authorities prior to being conducted. We encourage researchers to obtain such permissions and to perform the experiments according to any ethical guidelines that exist within the field of research.
6. The biosensors are sensitive to the growth conditions and require sufficient aeration for an optimal fluorescent output to be achieved.
7. Calculation of the relative fluorescence allows comparison of biosensor output between cultures differing in their growth characteristics. Still, care should be taken only to compare cultures that are in the same growth phase.
8. An alternative to invasive nucleic acid staining of the total biofilm biomass using Syto®62 is the use of a constitutively expressed fluorescent marker gene compatible with Gfp. An example of such a marker is the red-fluorescent protein mCherry. Using such a noninvasive genetic marker would allow for repeated visualization of the same biofilm at different time-points, thereby following the temporal fluctuations of cellular c-di-GMP levels down to the single-cell level in real time.
9. The corneas were observed on Days 2, 5, and 7 for the formation and development of biofilms. As previously described [8], bacteria adhered onto the corneal surface on Day 1, while bacteria were embedded in extracellular polymeric substances (EPS) on Day 2. By Days 5 and 7, biofilms of *P. aeruginosa* cells densely packed in EPS were observed.
10. As a viscous slurry of bacteria and cornea will be used for serial diluting, extreme caution must be taken to ensure that the correct volume will be transferred from one dilution to another with a pipette, so as to reduce errors in liquid transfer.

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References

1. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284 (5418):1318–1322
2. Romling U, Galperin MY, Gomelsky M (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. Microbiol Mol Biol Rev 77(1):1–52. doi:10.1128/MMBR.00043-12
3. Irie Y, Parsek MR (2014) LC/MS/MS-based quantitative assay for the secondary messenger molecule, c-di-GMP. Methods Mol Biol 1149:271–279. doi:10.1007/978-1-4939-0473-0_22
4. Rybtke MT, Borlee BR, Murakami K, Irie Y, Hentzer M, Nielsen TE, Givskov M, Parsek MR, Tolker-Nielsen T (2012) Fluorescence-based reporter for gauging cyclic di-GMP levels in Pseudomonas aeruginosa. Appl Environ Microbiol 78(15):5060–5069. doi:10.1128/AEM.00414-12
5. Crusz SA, Popat R, Rybtke MT, Camara M, Givskov M, Tolker-Nielsen T, Diggle SP, Williams P (2012) Bursting the bubble on bacterial biofilms: a flow cell methodology. Biofouling 28(8):835–842. doi:10.1080/08927014.2012.716044
6. Choi KH, Kumar A, Schweizer HP (2006) A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods 64(3):391–397. doi:10.1016/j.mimet.2005.06.001. S0167-7012(05)00158-2 [pii]
7. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T (2003) Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol Microbiol 48(6):1511–1524
8. Saraswathi P, Beuerman RW (2015) Corneal biofilms: from planktonic to Microcolony formation in an experimental keratitis infection with Pseudomonas aeruginosa. Ocul Surf 13 (4): 331–345. doi: 10.1016/j.jtos.2015.07.001 [pii]