Chapter 3

FRET-Based Biosensors for the Detection and Quantification of AI-2 Class of Quorum Sensing Compounds

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Abstract

Intercellular small molecular weight signaling molecules modulate a variety of biological functions in bacteria. One of the more complex behaviors mediated by intercellular signaling molecules is the suite of activities regulated by quorum sensing molecules. These molecules mediate a variety of population-dependent responses, including the expression of genes that regulate bioluminescence, type III secretion, siderophore production, colony morphology, biofilm formation, and metalloprotease production. Given their central role in regulating these responses, the detection and quantification of QS molecules has important practical implications. Until recently, the detection of QS molecules from Gram-negative bacteria has relied primarily on bacterial reporter systems. These bioassays though immensely useful are subject to interference by compounds that affect bacterial growth and metabolism. In addition, the reporter response is highly dependent on culture age and cell population density. To overcome such limitations, we developed an in vitro protein-based assay system for the rapid detection and quantification of the furanosyl borate diester (BAI-2) subclass of autoinducer-2 (AI-2) QS molecules. The biosensor is based on the interaction of BAI-2 with the *Vibrio harveyi* QS receptor LuxP. Conformation changes associated with BAI-2 binding to the LuxP receptor change the orientation of cyan and yellow variants of GFP (CFP and YFP) fused the N- and C-termini, respectively, of the LuxP receptor. LuxP-BAI2 binding induces changes in fluorescence resonance energy transfer (FRET) between CFP and YFP, whose magnitude of change is ligand concentration dependent. A set of ligand-insensitive LuxP-mutant FRET protein sensor was also developed for use as control biosensors. The FRET-based BAI-2 biosensor responds selectively to both synthetic and biologically derived BAI-2compounds. This report describes the use of the LuxP-FRET biosensor for the detection and quantification of BAI-2.

Key words: Autoinducer, Quorum sensing, LuxP, Ligand, BAI-2, DPD, FRET, Biosensor, GFP, CFP, YFP, Dissociation constant, Quantification, Fluorescence

1. Introduction

Vibrio harveyi bioassays for the autoinducer 2 (AI-2) class of quorum sensing (QS) compounds have been used for over a decade to monitor QS signals in biological samples. *V. harveyi*

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uses a two-component sensor kinase system to detect autoinducer 1 [AI-1, *N*-(3-hydroxybutanoyl)-l-homoserine lactone] and the boron derivative of autoinducer 2 [BAI-2, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate]. These QS compounds regulate the expression of genes involved in bioluminescence, type III secretion, siderophore production, colony morphology, and metalloprotease production (1–4). Until recently, the detection of the BAI-2 class of QS compounds was based on BAI-2-induced bioluminescence using *V. harveyi* bioassays. The BAI-2 bioassay system, however, was notoriously difficult to standardize, takes several hours to complete, and is subject to substantial environmental and biological perturbations (5, 6). Fluctuations in culture pH, metabolites, and growth inhibitor concentrations can all affect BAI-2 bioassays. These shortcomings necessitated the need for the development of a more rapid, ligand-specific assay for the detection and quantification of BAI-2. With this knowledge, we developed an in vitro LuxP-FRET-based biosensor (CLPY) consisting of a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) fused to the surface-exposed N- and C-termini of the BAI-2 receptor protein LuxP, devoid of its N-terminal periplasmic targeting peptide (23 amino acids).

LuxP belongs to a large family of bacterial periplasmic-binding proteins (bPBP) (7, 8). The LuxP class of bPBPs is highly conserved among many *Vibrio* species including several potential human pathogens (9). The bPBPs are ideally suited for the development of FRET-based biosensors with their N- to C-termini distances between 10 and 100Å (10, 11). They typically have two globular protein domains tethered by a flexible hinge region that encompasses the ligand-binding site (8). Structure–function analyses of bPBPs have demonstrated that the binding of the ligand induces substantial conformational changes in the receptor protein (12–14) including changes in the protein radius of gyration and the distance between the N- and C-termini of the protein (7, 8, 12–14).

The CLPY biosensor (MW: 98 kDa, Fig. 1) is conveniently expressed in *Escherichia coli* using an inducible T5-promoter/lac operator vector construct and purified using an N-terminal 6x Histidine tag. Herein, we describe a rapid, highly sensitive, BAI-2 biosensor, CLPY, and accompanying control biosensors (M2CLPY and M3CLPY) for the FRET-based detection and quantification of BAI-2 from biological samples.

2. Materials

2.1. Bacterial Strains and Plasmids

1. *E. coli* BL21 (*luxS*$^-$) and *V. harveyi* strains BB120 (wild type), MM30 (*luxS*$^-$), and MM32 (*luxS*$^-$, *luxN*$^-$) (generously provided by Dr. Bonnie L. Bassler – Princeton University).

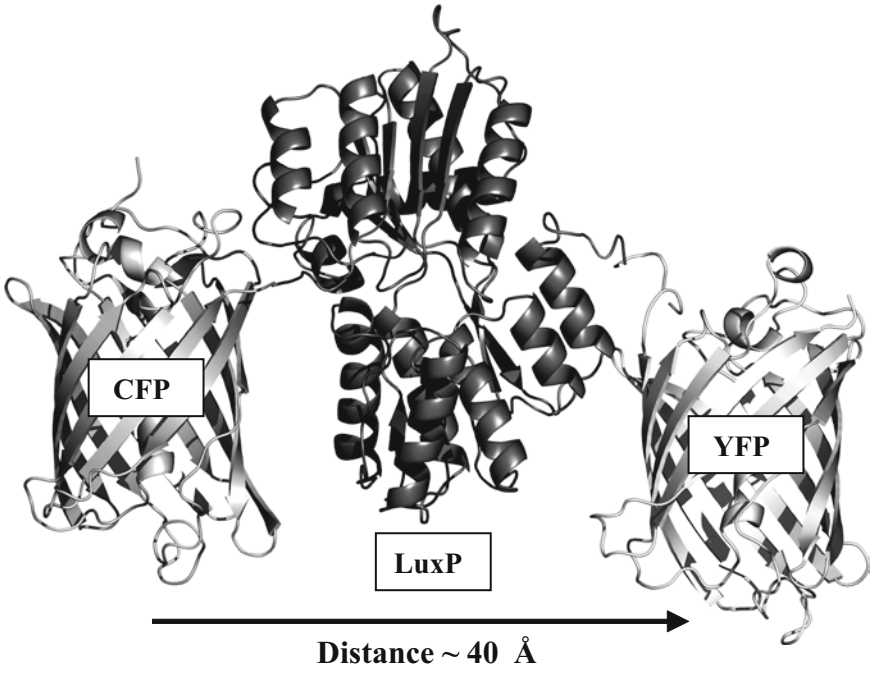

Fig. 1. LuxP-protein biosensor (CLPY). Schematic representation of LuxP-protein biosensor with CFP and YFP proteins attached to the N- and C-termini of LuxP. The calculated distance between the flurophores of ~40 Å is optimal for measuring conformational changes within LuxP. The ensemble measure of LuxP-BAI-2 binding induced distance and conformational changes in LuxP is observed by proportional decrease in the CLPY FRET ratio (YFP/CFP fluorescence ratio) changes.

2. Strain BL21 (*luxS*$^-$) carrying plasmid constructs pQE30-CLPY (wild type LuxP biosensor), LuxP-mutant biosensors: pQE30-M2CLPY (Q77A and S79A) and pQE30-M3CLPY (Q77A, S79A, and W82F) (9).

2.2. Bacterial Culture Media

1. Unless otherwise stated, media, stocks, and solutions are made with deionized distilled water prepared using Milli-Q® water purification system (Millipore).
2. Luria-Bertani (LB) medium: 5.0 g/l yeast extract, 10.0 g/l bactotryptone, and 10.0 g/l sodium chloride are dissolved in water and autoclave.
3. Luria-Marine (LM) medium (15): 5.0 g/l yeast extract, 10.0 g/l bactotryptone, and 20.0 g/l sodium chloride are dissolved in water and autoclave.
4. Autoinducer Bioassay (AB) medium (2): Prepare solutions A, B, and C first as detailed below.

   Solution A: 0.3 M NaCl (17.53 g/l), 0.05 M $MgSO_4 \cdot 7H_2O$ (12.32 g/l), 0.2% Casamino acids (2.0 g/l) in 960 ml water. Adjust pH to 7.5 with 10.0 M KOH, autoclave, and cool to room temperature.

Solution B: 1.0 M NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) is prepared by mixing autoclaved stocks of 1.0 M NaH_2PO_4 (39 ml) and Na_2HPO_4 (61 ml).

Solution C: 50% glycerol, autoclave.

Solution D: 0.1 M arginine, filter sterilize.

To 960 ml of Solution A, add 10 ml of Solution B, 20 ml of Solution C, and 10 ml of Solution D to make 1.0 l of AB media.

5. Antibiotics: ampicillin (100 mg/l) and kanamycin (100 mg/l) are used with growth media to needed final concentrations by diluting a 1,000× stock prepared in water. Before use, stocks are filtered with 0.22 μm PVDF membrane syringe filter disk and sterilized. Aliquots of 1.0–5.0 ml are stored at –20°C.
6. Solid agar media plates: Prepare using 15 g/l of select agar in desired liquid media and autoclave 20 min.
7. Incubator shaker fitted with desired adaptors for bacterial culture growth.

2.3. Protein Overexpression and Purification

1. 1.0 M isopropyl thiogalactoside (IPTG) is dissolved in distilled water and filter sterilized with 0.22 μm PVDF membrane syringe filter disk.
2. Lysozyme (50 mg) in 1.0 ml of distilled water, stored in 25 μl aliquots at –20°C.
3. 0.1 M phenylmethyl sulfonyl fluoride (PMSF) in isopropanol, stored at –20°C (see Note 6).
4. Following stocks in water are prepared and autoclaved: 1.0 M NaH_2PO_4, 1.0 M Na_2HPO_4, and 3.0 M NaCl.
5. A buffer stock of 1.0 M NaH_2PO_4–Na_2HPO_4 (pH 8.0) is prepared by mixing appropriate volumes of 1.0 M NaH_2PO_4 and 1.0 M Na_2HPO_4. For 100 ml of stock, add 94.7 ml of Na_2HPO_4 and 5.3 ml of NaH_2PO_4 and check the final pH using pH electrode.
6. 2.5 M imidazole in water, adjust pH to 7.5 with HCl and filter sterilize. Store at 4°C.
7. Buffer A (Column equilibration/wash buffer): 25 mM NaH_2PO_4–Na_2HPO_4 (pH 8.0), 35 mM NaCl, and 10 mM imidazole.
8. Buffer B (Lysis buffer): To buffer A solution, add 15 mM 2-mercaptoethanol (2-ME), 1 mM PMSF, and 0.2 mg/ml lysozyme (see Note 6).
9. Buffer C (Elution buffer): 25 mM NaH_2PO_4–Na_2HPO_4 (pH 8.0), 35 mM NaCl, and 50 mM imidazole.
10. HIS-Select™ nickel affinity gel (Sigma) (see Note 1) and empty 2.5 × 30 cm Econo-Column (BioRad) for affinity gel packing.

11. Cary Eclipse spectrofluorometer (Varian, Inc.) integrated with Cary Eclipse software.
12. Cell homogenizor/sonicator (Biologics, Inc., Model: 300 V/T, Ultrasonic homogenizer) fitted with a microtip.
13. Refrigerated floor centrifuge with rotors, centrifuge tubes, bottle, and adaptors.

2.4. Protein Estimation, Denaturing Gel Electrophoresis, and Fluorescence Detection

1. Bradford protein assay kit (BioRad) – store at 4°C.
2. Other components needed for protein assay: clean 13×100 mm test tubes, test tube racks, vortex mixer, Whatman no. 1 filter paper, 1 cm path length cuvettes.
3. Bovine serum albumin (BSA) standard stock solution at 2 mg/ml in water – store at 4°C for up to a month or at –20°C for long-time storage.
4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) procedure is described assuming the use of BioRad protein mini gel apparatus setup.
5. BioRad precast 10% SDS–PAGE (see Note 7), electrode, see blue protein standard (Invitrogen), pipettes, and 1–200 µl gel loading tips.
6. SDS–PAGE Tris–glycine electrophoresis buffer (1×): 25 mM Tris, 250 mM glycine, 0.1% SDS (pH 8.3) in water. A 5× stock of the above buffer can be prepared by dissolving 15.1 g/l of Tris base and 94.0 g/l of glycine in 950 ml water and 50 ml of 10% (w/v) of SDS. This can be stored at 4°C until use.
7. In water, 1.0 M Tris–HCl (pH 6.8), 1.0 M dithiothreitol is prepared for making 2× protein gel loading dye: 100 mM Tris–HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol. 100 µl aliquots can be made and stored at –20°C.
8. For preparing protein samples for gel electrophoresis, boiling water bath or heat block capable of reaching temperatures of 100°C.
9. For SDS–PAGE Coomassie brilliant blue staining (detection range 100–1,000 ng):
    a. Coomassie brilliant blue stain solution: 40% methanol/50% water/10% acetic acid/0.025% Coomassie brilliant blue R-250. This solution can be stored at room temperature.
    b. Destain solution: 40% methanol/50% water/10% acetic acid, store at room temperature.
    c. Other requirements include a rocking shaker and a gel imager.

10. Cary eclipse spectrofluorometer (Varian, Inc.) integrated with Cary Eclipse software, four-side clear quartz cuvette or disposable plastic cuvettes for fluorescence measurements.

2.5. Boron-Depleted Media and Conditioned Media Preparation

1. Amberlite® IRA743 borate-specific chelating resin (Sigma).
2. Column 5 × 10 cm Econo-Column (BioRad).
3. Following stocks in water: 3.0 M ammonium hydroxide, 1.0 M hydrochloric acid, 0.16 M nitric acid, and 10.0 M potassium hydroxide for pH adjustment.
4. 3.0 kDa membrane cut-off filter with omega membrane – 3K Microsep™ centrifugal device (Pall Life Science) and 0.2 μm HT Tuffryn® membrane syringe filter (Pall Life Science).

3. Methods

For best results, the use of borosilicate glassware should be avoided for the entire procedure. BAI-2 is formed from the cyclization of DPD in the presence of borate. It has been previously determined that a DPD:borate ratio of 1:4 leads to a yield of 10% BAI-2 and potentially other borate derivatives of AI-2 (not LuxP ligand) (16). All the buffer and media preparations should be stored in clean polystyrene/polypropylene containers. Bacterial culturing is done using sterile polystyrene 14 ml culture tubes (BD Biosciences). To avoid changes in the DPD:borate ratio that potentially alter the detected BAI-2 concentrations, the media should be cleaned of any contaminating borate prior to use, following the procedure described in Subheading 3.4.

3.1. Biosensor Overexpression and Purification

1. An overnight starter culture of BL21 (*luxS*$^-$) cells transformed with pQE30-CLPY or LuxP-mutant constructs are started as single colony inoculum in 14 ml sterile tubes containing 3.0 ml of LB broth supplemented with 100 mg/l ampicillin. The cultures are grown in a roller drum or shaker for overnight (O/N) at 37°C (see Note 8).
2. A 500 ml conical flask with 75.0 ml LB broth supplemented with 100 mg/l ampicillin is inoculated with 1% of above O/N culture (750 μl) to begin second overnight shake cultures (250 rpm) at 28°C for 16 h.
3. The O/N culture is then transferred to 1.5 l of LB in 4.0 l volume Erlenmeyer flask (5% innoculum) supplemented with 100 mg/l ampicillin.
4. The culture is grown at 28°C, shaking at 200 rpm. At about 3–4 h, the optical density at 600 nm (OD_{600}) is measured using spectrophotometer. A small volume of culture is then

aliquoted into 1 cm path length cuvette and OD_{600} is measured with fresh LB media as blank control. When the OD_{600} is 0.6, 1 ml of culture is removed and cell pellet collected by centrifugation (benchtop centrifuge – 13,000 rpm/3 min) and stored at –20°C for subsequent SDS–PAGE analysis. The remaining culture is induced for CLPY expression by adding 0.3 mM IPTG and grown for an additional 6 h.

5. After 6.0 h growth, 300 µl of culture is removed to collect the cell pellet by centrifugation for use with SDS–PAGE analysis. The rest of the bacterial cells are then harvested by centrifugation using a floortop centrifuge at 8,000 × *g* for 10 min and protein purifications carried out at 4°C (see Notes 9 and 10).
6. Cell pellet is resuspended in 35 ml of 25 mM NaH_2PO_4–Na_2HPO_4 (pH 8.0), 35 mM NaCl, 10 mM imidazole, 15 mM 2-mercaptoethanol, and 1.0 mM phenylmethyl sulfonyl fluoride (buffer B), placed in an ice bath and lysed by sonication as follows. A sonicator fitted with microtip is used for six rounds of sonication with power set at 40 and pulsed ten times with a 1 min pause between each round to allow for cooling.
7. Cell lysate supernatant is separated from cell debris and unlysed cells by centrifugation at 12,000 × *g* for 20 min. About 200 µl of clarified lysate is removed and stored at –20°C for SDS–PAGE analysis (Subheading 3.2). The rest of the clarified cell lysate is then loaded onto a 2.5 × 30 cm column containing 7.5 ml bed volume of His-Select™-HC Nickel affinity gel (Sigma) equilibrated with five column volumes of buffer A.
8. The protein bound resin is washed with five column volumes of buffer A and eluted by adding three column volumes of 25 mM NaH_2PO_4–Na_2HPO_4 (pH 8.0), 35 mM NaCl, and 50 mM imidazole (buffer B). In clean tubes, eluate containing biosensor protein (characteristics pale to brighter yellow color) is collected as 3.0 ml fractions.
9. The same column is now ready to be equilibrated and reused or can be stored in 70:30 ethanol:water (v/v) for later use (see Note 1).

3.2. Biosensor Quantification and SDS–PAGE Analysis

1. The purified CLPY biosensor fractions are quantified using BioRad protein assay method using BSA as standard. The assay is carried out as detailed in the user's manual with some modifications.
2. The dye reagent is prepared by diluting one part dye reagent concentrate with four parts Millipore™ water. The solution is filtered through Whatman no. 1 filter (or equivalent) to remove particulates. This diluted dye reagent can be used for approximately 2 weeks when stored at room temperature.

3. Prepare six dilutions of a protein BSA standard (0.2, 0.4, 0.5, 0.6, 0.8, and 0.9 mg/ml) – store at 4°C or –20°C for long-term storage.
4. Add 1.0 ml of diluted dye reagent to clean-dry test tubes; add 20 µl of each standard and CLPY fraction solution and vortex for 5 s.
5. Incubate at room temperature for 5 min and measure absorbance at 595 nm using spectrophotometer (see Note 11).
6. The protein concentration is determined using the standard curve. Typically the protein yields in visibly colored fractions range from 0.1 to 0.3 mg/ml.
7. For SDS–PAGE analysis, a precast 10% acrylamide gel is assembled in the minigel apparatus as per the user's manual. Immediately add 1× Tris–glycine electrophoresis buffer to fill the gel reservoir to limit idling and drying of the gel. Using both hands carefully remove the comb in a single vertical motion (see Note 16).
8. The samples for SDS–PAGE are prepared as follows. Bacterial cell pellet, cell lysate, and purified protein (Subheading 3.1) are left on ice to provide sufficient time for frozen samples to thaw entirely.
9. Resuspend the cell pellets in 50 µl of water. 50 µl of clarified cell lysate and purified protein (say concentration ~50 ng/µl) are aliquoted in 1.5 ml centrifuge tubes. To all the samples, add 50 µl of 2× SDS–PAGE loading dye and gently mix with the pipette tip. Place the tubes in boiling water bath for 10 min. Remove the tubes and leave at RT to cool. In a benchtop centrifuge spin down (13,000 rpm/1 min), the sample tubes that contain bacterial cells. Carefully remove 30 µl of sample using the gel loading tip and load onto designated wells. Add 10–15 µl of protein standard for use as molecular weight marker.
10. Once the sample is loaded, connect the apparatus to the power supply and initially run at 20 mA (2+ hours to allow sample through stacking gel) and then increase to 40 mA until the bromophenol blue starts running out of the gel. At this point, remove the assembly and carefully remove the gel for staining.
11. Place the gel in Coomassie staining solution so it immerses the gel fully. The gel is left on a rocking shaker for 2 h to overnight in the staining solution.
12. The staining solution is transferred to a new container and can be reused later. Rinse the gel with water and destain by adding sufficient volume of destain solution and let incubate for additional 2–3 h. Following destaining remove two-thirds

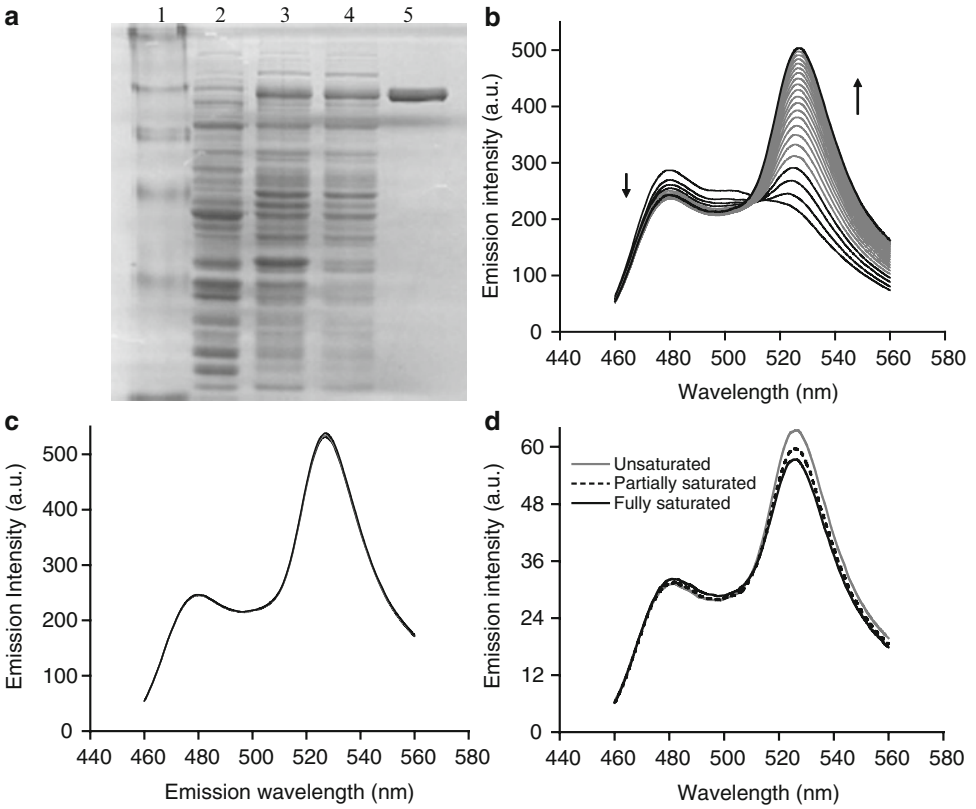

Fig. 2. Characterization of biosensor. (**a**) SDS–PAGE analysis of CLPY purification. Lanes: (1) see blue protein standard, (2) uninduced BL21(*luxS*$^{-}$)-pQE30-CLPY, (3) 6 h induced BL21(*luxS*$^{-}$)-pQE30-CLPY, (4) cell-lysate supernatant, (5) purified CLPY (~98 kDa). (**b**) Time-dependent change in FRET associated with YFP maturation. CLPY protein immediately after purification when monitored at room temperature [λ_{ex} 440 nm (slit 5 nm) and λ_{em} 460–560 nm (slit 5 nm) shows time-dependent FRET increase]. (**c**) Mature FRET sensor. The purified CLPY stored at room temperature for 5 h or at 4°C for 48 h showed no time-dependent FRET changes. Shown here is an overlay of several emission spectra scans recorded for mature CLPY protein over 30 min. (**d**) CLPY responses to BAI-2 ligand. Concentrations: unsaturating (*gray line*), partially saturating (*dotted black line*), and fully saturating (*black line*) concentrations of BAI-2 ligand.

of the destain solution and replace the volume with water, which allows the shrunk gel (during staining and destaining) to swell back to its original size.

13. The gel image can be captured using a gel imager or document scanner layered with saran™ wrap (Fig. 2a).

3.3. Biosensor Characterization and Flurophore Maturation

Fluorescence measurements are carried out at room temperature using Cary Eclipse spectrofluorometer (Varian, Inc.) set in a scanning mode. CLPY and LuxP mutants are monitored by CFP excitation (λ_{ex} 440 nm/slit 5 nm) and the emission spectrum (λ_{em}) is measured from 460 to 560 nm using a 5 nm slit width. Alternatively, the FRET ratio (527/485 nm) is recorded using an excitation wavelength of 440 nm.

1. A ligand-binding-independent increase in FRET is observed with recently purified biosensor (Fig. 2b). This is due to the delayed maturation of YFP over CFP, which is characterized by faster fluorophore maturation.
2. To eliminate this problem, immediately after purification, the biosensor should be left for maturation at RT (5 h) or 4°C (2 days) before beginning ligand-binding studies.
3. Following this step, the FRET change does not happen in the absence of the ligand (Fig. 2c). At this time, the biosensor is ready to use for BAI-2 ligand-binding studies.
4. The biosensor response to the detection of BAI-2 is characterized by a decrease in the FRET ratio (YFP/CFP ratio). This decrease is proportional to the amount of BAI-2 ligand concentration present in a given test sample. As reported elsewhere (9), Fig. 2d shows a representative spectrum of biosensor responses to unsaturating, partially saturating, and fully saturating BAI-2 concentrations in test sample.

3.4. Boron-Depleted Media Preparation

1. The protocol is adapted from Bennett et al. (17) with some modifications.
2. 30 ml of Amberlite® IRA743 resin is used per liter of media. Here we use AB media as an example for describing the procedure.
3. The column is treated with 150 ml 3.0 M ammonium hydroxide, 600 ml distilled water, 180 ml of 1.0 M hydrochloric acid, 150 ml distilled water, 180 ml of 0.16 M nitric acid, 300 ml distilled water.
4. Following these treatments, a liter of AB medium is passed through the column and the pH adjusted to 6.75 using 10 M potassium hydroxide.

3.5. BAI-2 Ligand Detection and Quantification from V. harveyi Cultures

1. As an example, we show here the determination of *V. harveyi* BAI-2 ligand concentrations as a function of culture age. This procedure can be suitably followed for the determination of BAI-2 from other Vibrio strains and other bacteria that synthesize and use BAI-2 as a signal molecule.
2. *V. harveyi* BB120 (wild-type) grown overnight at 28°C for 16 h is used to make 2% (v/v) inoculum in fresh AB media (2.0 ml, 0.5 mM borate) in round bottom polystyrene tubes (BD Biosciences, USA). Set up 36 tubes of 2.0 ml cultures for using three tubes at a given time point.
3. For this experiment, every 2.5 h (including the 0 h time point) the cell density is determined by plating serial dilutions of *V. harveyi* cultures on LM agar plates. Before removing cultures for plating, the culture tubes are vortexed for 10–15 s to eliminate any visible cell clumps. The dilutions are made in

fresh LM media and different volumes are plated out on LM agar (see Note 12). The agar plates are left at 30°C for 24 h before counting the colonies.

4. At the same time, the BB120 cell-free supernatant is collected using a refrigerated benchtop centrifuge – 13,200 rpm/5 min.
5. The supernatant is collected and to remove proteins (proteases) and cell debris, it is passed through a 3 kDa membrane cut-off filter (3 K Microsep™ centrifugal device, Pall Life Science) pretreated with 2.0 ml water by centrifugation at 5,000 × *g* for 20 min (see Notes 4 and 14).
6. The flow through of the culture supernatant containing the BAI-2 signal is collected after centrifugation at 5,000 × *g* for 30 min.
7. Working stock of 0.015 mg/ml of CLPY is made in buffer C. For triplicate measurements, three different batches of CLPY preparations are used.
8. In a 1.5 ml centrifuge tube aliquot 1.0 ml of 0.015 mg/ml of CLPY and leave at room temperature for 15 min to equilibrate.
9. The YFP/CFP fluorescence or FRET ratio (527/485 nm) response of CLPY for the given sample is measured at room temperature after 5 min incubation of the biosensor with the sample. The sample is mixed with 1.0 ml of CLPY by gently inverting the tube 4–5 times and incubating before transferring the contents to a cuvette for FRET measurements (see Note 13).
10. For instance, 150 µl of *V. harveyi* BB120 culture filtrate is mixed with 1.0 ml of CLPY and used for generating a graph representative of FRET response versus time and cell number. This assay provides an indication of the BAI-2 concentration in the media as a function of culture age (Fig. 3a).

3.6. BAI-2 Quantification from V. harveyi Cultures

1. For a given culture age, a fixed volume of diluted culture filtrate is added to the CLPY biosensor (0.015 mg/ml).
2. For instance to determine the concentration of BAI-2 from 7.5 h old 2% *V. harveyi* in AB media (0.5 mM Borate), 200 µl of various dilutions of culture supernatant (prepared as detailed in the previous section) is made in borate-free AB media (Subheading 3.3) and added to CLPY (1.0 ml, 0.015 mg/ml) to generate a biosensor response saturation curve. The following volumes 5, 10, 20, 40, 60, 80, 100, 120, 150, and 200 µl are made to 200 µl with borate-free AB media and used for generating the CLPY biosensor saturation curve.
3. The biosensor response curve is plotted as a function of dilution of cell supernatant versus the FRET ratio response (Fig. 3b, *x*-axis shown in log10 scale). The dilution volume for each data point is determined by dividing the volume

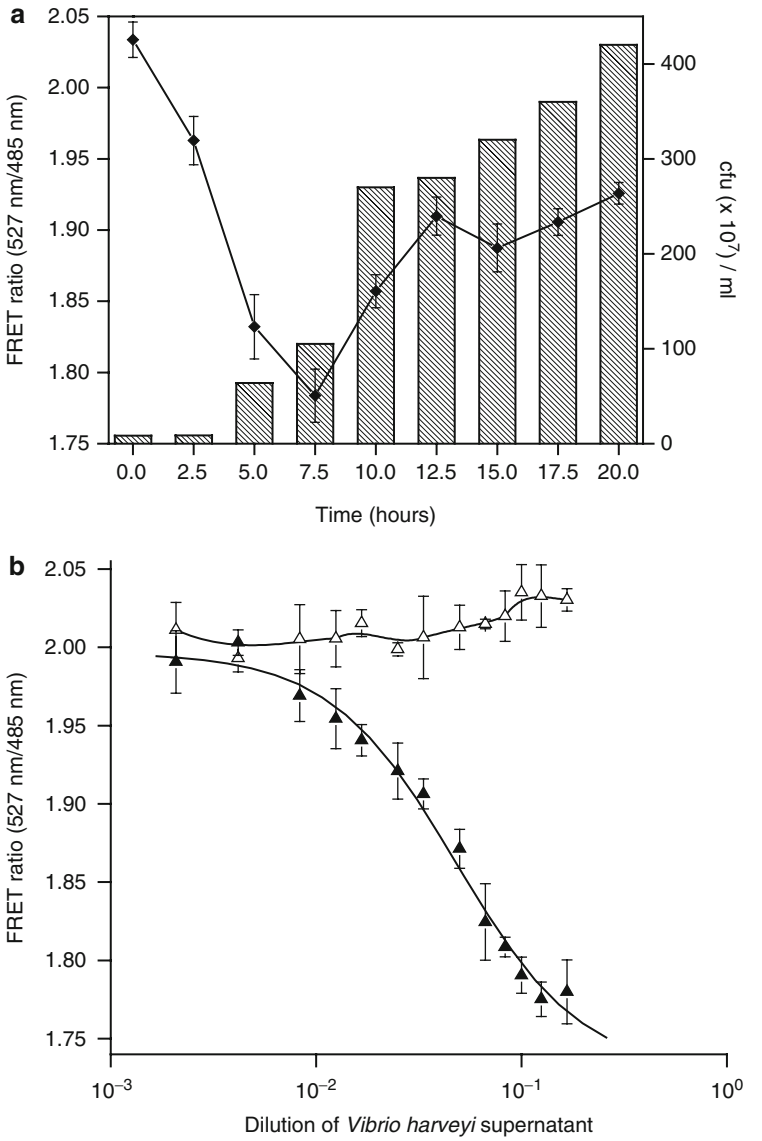

Fig. 3. Quantification of BAI-2 from *Vibrio harveyi* cultures using the CLPY biosensor. (**a**) Wild-type *V. harveyi* BAI-2 levels monitored as a function of time and culture density. Cell-free culture filtrates were prepared by passing the bacterial cell culture media through a 3 kDa MW cut-off filter. The filtrate (150 μl) was added to CLPY (0.015 mg/ml). The YFP/CFP FRET ratio (*closed diamonds*) is plotted as a function of cell density (CFU $\times 10^7$/ml; bars). The error bars represent the standard deviations from three independent experiments. (**b**) CLPY fluorescence response to culture filtrate from wild-type (BB120) *V. harveyi* (*closed triangles*) and *luxS*$^-$ mutant (MM30, *open triangles*) culture filtrate. The error bars represent the standard deviations from three independent experiments. (**a**) and (**b**) Adapted with permission from ref. (9). Copyright© 2007 American Chemical Society.

of conditioned media used (do not include the volume of fresh borate-free AB media used for diluting) by the total volume of the FRET assay mix (this volume will remain the same when calculating all the dilutions).

4. Dilutions in borate-free AB media help maintain the ligand DPD and boron ratios the same in the bacterial growth media. This is important since changes in DPD and boron ratio might affect the equilibrium of BAI-2 ligand.
5. Cell-free supernatant from 7.5 h old *V. harveyi* MM32 (a *luxS*$^{-}$ mutant devoid of BAI-2 synthesis) culture is used as a control (Fig. 3b) (see Note 15).
6. Using a software program (such as "OriginLab") capable of fitting a nonlinear regression relationship shown in Eq. 1, the CLPY response to various dilutions of *V. harveyi* supernatant "*v*" can be used for determining half saturation volume "*h*". Where "*R*" is the observed CLPY FRET ratio response for different dilutions of *V. harveyi* supernatant "*v*," with "R_{max}" and "R_{min}" represent the ligand-free and ligand-saturated FRET ratios, respectively.

$$\mathrm{R} = \mathrm{R}_{max} - \left(\frac{(\mathrm{R}_{max} - \mathrm{R}_{min}) \times \mathrm{n} \times \mathrm{v}}{(\mathrm{h} + \mathrm{v})} \right). \quad (1)$$

7. By fitting the above equation, the "*h*" value is determined and used for calculating the unknown BAI-2 concentration "*M*" using the following relationship given in Eq. 2. As reported previously, the BAI-2 and LuxP binding affinity "K_d" – 270 nM (9) is used for determining "*M*" in a given culture supernatant.

$$\mathrm{M} = \mathrm{Kd}/\mathrm{h} \quad (2)$$

8. Other controls for these experiments include using M2CLPY and M3CLPY biosensors to confirm if the CLPY FRET response is specifically due to LuxP-BAI-2 binding (see Note 2).

4. Notes

1. There are other commercial suppliers for the same Ni-NTA affinity resin (e.g., Qiagen, Fisher Sci., etc.). Once obtained these gels can be reused 3–4 times depending on the column condition. Once the column looks faded (initially bright nickel blue), the resin can be cleaned and recharged with nickel for reuse. Following is a modified procedure from Qiagen that is used regularly for obtaining good protein

purification (refer to their procedure for more stringent treatment). Our modified procedure includes the following steps: wash resin with one column volume of water, followed by five column volumes of 100 mM EDTA (pH 8.0) to remove nickel and bound impurities and three column volumes of water and recharge the resin with two column volumes of 100 mM $NiSO_4$, wash with two column volumes of water and equilibrate with three column volumes of buffer A before use. Always store the column in small volumes of buffer for short-time storage or with 70% ethanol for long-time storage.

2. Chloride ions (Cl^-) can affect YFP fluorescence (18). You will note that the FRET ratios are lower in the presence of chloride due to YFP fluorescence (CFP remains unaffected). So care must be taken to minimize buffer and media concentrations between samples.
3. FRET ratio of mature CLPY with the choice of bacterial media can be determined to use as initial FRET ratio for your culture supernatant experiments and remember to maintain the same chloride ion concentrations (by diluting your used media in fresh media).
4. Pall centrifugal devices fitted with Omega™ membrane were found to have less interference with BAI-2 ligand sticking to membrane. So care must be taken with the choice of filtration device and the accompanying membrane.
5. In case the *E. coli* strain BL21(*luxS*$^-$) is not available, DH5α can be used. However, appropriate protease inhibitors should be added as DH5α is not protease deficient strain unlike BL21 derivatives.
6. PMSF has a shore half-life in aqueous solutions (~30 min at pH 8.0), PMSF should be added to the lysis buffer immediately before initiation of cell lysis to avoid loss of potency.
7. The precast gels are recommended to be used within certain period of time. Also, in case of need for reducing expenses, SDS–PAGE gels can be conveniently prepared in the lab following published procedures. We recommend you refer to standard laboratory manuals such as molecular cloning: A laboratory manual by E.F. Fritsch, J. Sambrook, T. Maniatis (1989) for detailed procedure.
8. A single colony culture can be used to make 15% glycerol stock and stored at –80°C. For starting the cultures, scrape out small amount of frozen stock into fresh media. Care must be taken not to freeze–thaw stock vials that may result in loss of cell viability.
9. At this point, the culture flasks can be moved to 4°C and left standing O/N before harvesting. Next day, the cells can be noticed to have settled to the bottom. The culture flask can

be gently removed and ~500 ml of the culture can be decanted. The rest of the culture can be resuspended in the remaining media and the cells harvested using a refrigerated centrifuge set at 4°C.

10. The cell pellet can be stored at −80°C for prolonged periods of time without thawing. To thaw the cells, remove the cell pellet from −80°C and leave it O/N at −20°C. Transfer to ice next day to enable slow thawing of cells.
11. It is important to make the protein assay reads within minutes of each other. Over time the response intensifies, so care must be taken not to incubate for prolonged time. It is also recommended to go through user's manual for troubleshooting.
12. Under the given growth conditions for *V. harveyi* BB120, dilutions ranged from 10^6 at 0 h to 5×10^7 at 10 h and later. It is recommended that a pilot experiment is conducted to determine the dilution series required for the strain of bacteria you are working with before you take up the biosensor quantification.
13. Care must be taken not to vortex the contents; this may result in CLPY protein denaturation and loss of biosensor functionality.
14. If later time point cultures have cell debris that clogs the 3 kDa membrane, try including an additional step of passing the cell supernatant through 0.2 μm HT Tuffryn® membrane syringe filter (Pall Life Science) before 3 kDa membrane filtration.
15. In case the bacterial strain you are working with has no *luxS* deletion derivative, try using borate-free AB media grown culture filtrate as control.
16. To have good sample runs clean the wells carefully. Using a pipette set at 100 μl and fitted with 1–200 μl gel loading tip, pipette in electrophoresis buffer into wells (3–5 times) to remove any residual polymerized acrylamide on the loading well walls that might stick to sample and create a drag during sample run.

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