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Chapter 11

Fluorescence Quenching Studies of γ-Butyrolactone-Binding Protein (CprB) from *Streptomyces coelicolor* A3(2)

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Abstract

Fluorescence spectroscopy is an important analytical tool which is widely employed to study biological systems. This technique can be applied to qualitatively and quantitatively probe protein-ligand interactions primarily because of its sensitivity, selectivity, nondestructive and rapid form of analysis. In this chapter we describe the utility of this technique to establish a label-free, universal screening protocol for putative γ-butyrolactone (GBL) receptors by exploiting the intrinsic fluorescence of a highly conserved tryptophan residue that constitutes the hydrophobic pocket for GBL binding, a unique feature possessed by this family of receptors. Here we demonstrate this technique using a combination of steady-state fluorescence quenching methods and fluorescence lifetime decay kinetics using CprB protein from *Streptomyces coelicolor* A3(2) as a model system. Interaction data between CprB and two chemically synthesized GBLs involved in quorum sensing, Cp1 and Cp2, have been used as example.

Key words Quorum sensing, γ-Butyrolactones, CprB, Fluorescence quenching, Potassium iodide quenching, Time-resolved fluorescence lifetime

1 Introduction

Quorum sensing is a networking mechanism in bacteria that enables synergistic regulation of gene expression [1, 2]. This community behavior involves cell-to-cell communication via production of small signaling molecules called autoinducers, followed by their release in the surrounding environment. When the concentration of the signaling molecule exceeds the threshold level (as a result of high cell density) a downstream response is elicited, activating pathways attuned to survival conditions [3]. Bacteria use this mechanism to control processes such as antibiotic and virulence factor production, sporulation, biofilm formation, and competence. *N*-acylhomoserine lactones (AHLs) [4], 4-hydroxy-2-alkyl

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quinolines (HAQs) [5], γ-butyrolactones (GBLs) [6], and cyclic and linear oligopeptides [7] are various chemical classes of signaling molecules. Since these signaling molecules trigger response at very low concentrations (pM–μM ranges) techniques that can accurately capture their binding profile in a similar range would be greatly beneficial. Moreover, the receptors that these molecules bind to are also difficult to produce and purify at high concentration. Hence, sensitive methods that can detect and probe into the mechanistic details of these interactions at the nano-micromolar scale are needed. Fluorescence spectroscopy, a robust complementary biophysical technique to investigate structure-function relationships, serves as an ideal platform to study these systems. Other techniques like isothermal titration calorimetry and crystallization are limited by the amount of reagents required, sensitivity, and scope of multiplexing.

Fluorescence spectroscopy is a trending technique in biological sciences that has been employed to unravel a plethora of cellular mechanisms and interactions [8, 9]. The timescale of bimolecular dynamics (nanoseconds) and heterogeneity observed in biological systems makes fluorescence spectroscopy an apt technique that can reveal a huge wealth of information. The utility of this analytical tool is augmented by the spectral selectivity that allows one to derive specific information in the biological structure. For instance, selective excitation at 295 nm in proteins reveals the conformational and microenvironment changes of the tryptophan residues [10, 11] and masks the signal arising from the fluorescent tyrosine residues. Another advantage of this technique is that even small spectral changes (fluorescence intensity or peak shifts) can be quantitated by employing sophisticated instrumentation like time-correlated single-photon counting (TCSPC), which can probe variations in spectral properties of excited states of both the ligand and protein of interest. Furthermore, the field of fluorescence spectroscopy can be expanded to encompass fluorescently silent molecules such as proteins and nucleic acids, which can be customized by site-specific labeling using various synthetic fluorophores [12, 13]. For example, cysteine and lysine reactive dyes [14] are commonly coupled to proteins to make them fluorescent. Similarly, fluorophores appended at the 5′ end of oligonucleotides like fluorescein and carboxytetramethylrhodamine (TAMRA) dyes as well as nucleic acid base-specific analogs like 2-aminopurine [15] and 3-methylisoxanthopterin [16] have been widely used to study binding properties and dynamics in these systems [16]. However, a drawback of introduction of foreign chemical groups is the potential alteration of structure. Therefore, wherever possible using natural tryptophan residues that serve as label-free probes is an excellent alternative.

In this chapter, we describe a simple, rapid, label-free approach using fluorescence spectroscopy to screen ligands of the γ-

butyrolactone (GBL) receptor proteins such as CprB. GBLs are small diffusible quorum-sensing molecules used by *Streptomyces* and its related genera for triggering secondary metabolism. The mode of binding of GBLs to their cognate receptor family of proteins remains elusive as they are difficult to purify and there is a general paucity of X-ray structural information available in this regard. However, the structure of the apo [17] and DNA-bound forms of CprB [18] from *S. coelicolor* A3(2) provided information about the GBL pocket and aided in design of the screening assay presented here. Similar to all tetracycline receptors, CprB is a Ω-shaped molecule possessing two domains: an *N*-terminal DNA-binding domain, and a divergent *C*-terminal ligand-binding domain (the regulatory domain) that is proposed to bind the cognate quorum-sensing molecule. The protein exists in a dimeric state with the two units being related by a pseudo-2-fold axis. The regulatory domain is composed of an antiparallel bundle of five helices (α5–α10) with helix α6 forming the base of the cavity. The large cavity has a depth of approximately 20 Å and a diameter of 5 Å that is lined by hydrophobic residues. This cavity contains a tryptophan residue (W127) (Fig. 1a) that is known to be conserved among the members (Fig. 2). Docking studies of CprB with γ-butyrolactones have shown that the indole ring of tryptophan interacts with the γ-butyrolactone ring via hydrogen bonding and hydrophobic base stacking interactions [19] (Fig. 1b). Therefore, here the intrinsic fluorescence of this conserved tryptophan was exploited to screen GBLs. The beauty of this method lies in exploring the structure in its innate form, without having to attach

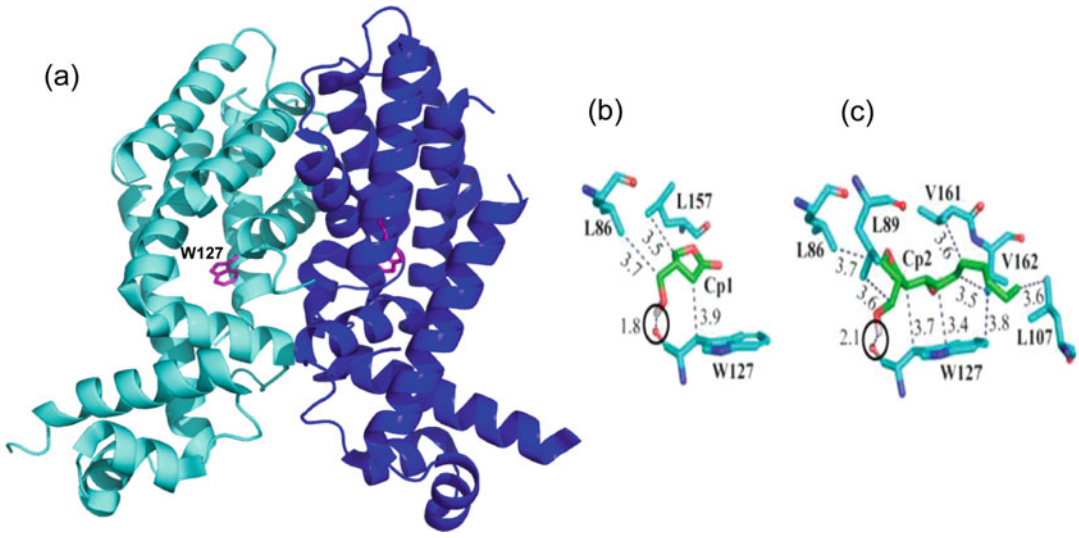

Fig. 1 (**a**) Structure of the dimeric protein CprB (PDB ID: 1UI5) depicting the hydrophobic pocket containing the conserved tryptophan, W127, (**b**) and (**c**) docking of Cp1 and Cp2 with CprB, respectively, revealing the interaction of tryptophan with the butyrolactone ring. Adapted from [19]

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CprB   GVPVRPPLPHPFTEWREIATSRLLDAVRQSDVHQDIDVDSVAHTLVCSVVGTRVVGGTLE
ArpA   G--VFLGGPHPWGDWIDATARMLELGQERGEVFPQIDPMVSAKIIVASFTGIQLVSEADS
BarA   QGAVDFSDANPFGEWGDICAQLLAEAQERGEVLPHVNPKKTGDFIVGCFTGLQAVSRVTS
ScbR   QQAHGLDRRGPFRRWHETLLKLLNQAKENGELLPHVVTTDSADLYVGTFAGIQVVSQTVS
FarA   VNAGGLDRSAPFRNWVDKFTDLLEKAQAQGELLPHVVPAETADVITGAYGGVQSMSQALT
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Fig. 2 Sequence alignment of GBL family of receptor proteins. The conserved tryptophan is shown in *red*

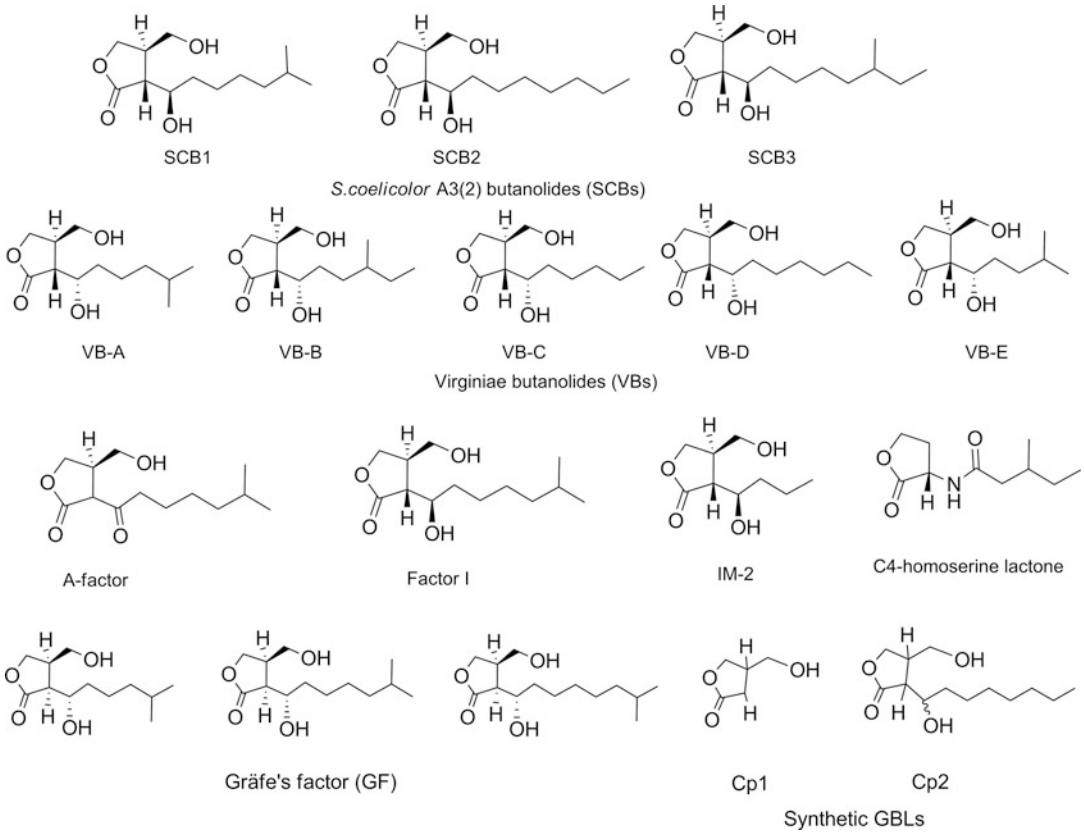

Fig. 3 γ-Butyrolactones and their variants

extrinsic fluorophores that could potentially lead to conformational changes in the protein.

The ligand binding studies in this protocol are performed with two chemically synthesized GBLs, Cp1 and Cp2 (Fig. 3). Cp1 (3-hydroxymethylbutanolide) is a basic butyrolactone moiety whereas Cp2 (2-(1′-hydroxyoctyl)-3-hydroxymethylbutanolide) has an additional aliphatic chain with eight carbon atoms. If a library of γ-butyrolactones and their variants is available, then an array of these ligands (Fig. 3) and various γ-butyrolactone receptors can be screened rapidly in a fluorescence microplate reader setup.

Thus large-scale screening with small quantities of ligands can be undertaken efficiently with microplate reader systems.

In order to ascertain the entry of the ligand in the pocket of the regulatory domain and the ligand binding capacity of CprB, we have adopted fluorescence quenching and lifetime decay kinetics for ligand screening that would be universally applicable to the family of GBL receptors. Fluorescence quenching can be described as a process that decreases the fluorescence intensity of a sample. Quenching can be either dynamic or static or a combination of both. Dynamic, i.e., collisional quenching, occurs when excited fluorophore is deactivated upon contact with the quencher molecule in solution. In this case, the fluorophore returns to the ground state during a diffusive encounter with the quencher. Static quenching on the other hand occurs due to the formation of a nonfluorescent ground-state complex between the fluorophore and the quencher. Dynamic and static quenching can be distinguished by their differing dependence on temperature. Higher temperature results in faster diffusion and larger amounts of collisional quenching. The quenching constant increases with increasing temperature for dynamic quenching; however, it decreases with increasing temperature for static quenching [20]. Steady-state intrinsic tryptophan fluorescence is an excellent indicator of conformational changes in proteins and interaction with ligands that can act as quenchers. Time-resolved fluorescence lifetime studies provide complementary information in addition to the excited-state lifetimes which is determined from the slope of the decay curve. Dynamic or static mechanism of quenching can be distinguished by estimating τ_o/τ, where τ_o and τ are the average fluorescence lifetimes in the absence and presence of the quencher, respectively. For static quenching $\tau_o/\tau = 1$. In contrast, for dynamic quenching there is a decrease in lifetime as depopulation of the excited state occurs. Apart from dynamic and static mechanism, quenching could be a result of other processes such as energy transfer and molecular rearrangements.

In the protocol described here changes in the fluorescence emission characteristics of the conserved tryptophan (W127) are employed to assess the ligand binding in both the native and W185L mutant (single-tryptophan system) forms of the protein. Furthermore, to confirm whether quenching is static or dynamic in nature and to gauge the surface accessibility of the tryptophan residues, fluorescence lifetime and potassium iodide (KI) quenching studies are performed, respectively. KI is a bulky molecule that is used as a marker to determine accessibility of the intrinsic tryptophan. This protocol can be extended for the development and study of quorum-sensing molecule inhibitors against pathogenic virulent strains, which can have a profound pharmacological relevance and impact.

2 Materials (*See* Notes 1 and 2)

Milli-Q water is used throughout. Buffers are stored at 4 °C and protein stocks at −80 °C.

1. Luria-Bertani (LB) broth: 1% (wt/vol) casein enzymic hydrolysate, 0.5% (wt/vol) yeast extract, 1% (wt/vol) sodium chloride in distilled water. Sterilize by autoclaving.
2. Antibiotic stock solutions: 100 mg/ml Chloramphenicol and 100 mg/ml kanamycin dissolved in ethanol or water, respectively. Filter sterilize and store at −20 °C.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution: 1 M IPTG in sterile Milli-Q water. Store at −20 °C.
4. Binding buffer: 50 mM Sodium phosphate, pH 7.
5. Elution buffer 1: 50 mM Sodium phosphate, 0.1 M NaCl, pH 7.
6. Elution buffer 2: 50 mM Sodium phosphate, 0.2 M NaCl, pH 7.
7. Elution buffer 3: 50 mM Sodium phosphate, 0.3 M NaCl, pH 7.
8. Elution buffer 4: 50 mM Sodium phosphate, 0.4 M NaCl, pH 7.
9. Desalting buffer: 50 mM Sodium phosphate, 100 mM NaCl, pH 7.
10. Ligands Cp1 and Cp2 [19].
11. Potassium iodide (KI) solution: Dissolve KI in desalting buffer to 1 M concentration (*see* **Note 3**).
12. SP-Sepharose beads.
13. Desalting columns.
14. Fluorescence cuvettes (*see* **Note 4**).
15. Cary Eclipse spectrofluorimeter.
16. Time-correlated single-photon counting system, IBH Horiba-JY fluorocube.
17. Spectrophotometer.
18. Two liter conical flasks.
19. Shaking incubator.

3 Methods

3.1 Protein Expression and Purification

1. The clone of native CprB in the pET26b(+) expression vector was a gift to us by Ryo Natsume (JBIC, Japan) [19]. These plasmids are transformed in *Escherichia coli* BL21(DE3) pLysS cells, after which the cultures are grown at 37 °C with 250 rpm shaking in 1 l of LB broth supplemented with the 30 μg/ml chloramphenicol and 35 μg/ml kanamycin, in 2 l conical flasks.
2. Measure the optical density of the cultures by spectrophotometry at 600 nm wavelength (OD_{600}). When the OD_{600} of the cultures reaches ~0.6, the expression of the proteins is induced by adding 1 mM IPTG.
3. The induced cells are cultured for 3 h at 37 °C, and then for additional 3 h at 25 °C. Harvest the cells by centrifugation.
4. The harvested cells are resuspended in 10 ml of binding buffer and lysed by sonication.
5. Cell debris is removed by centrifugation at 17,500 × *g* at 4 °C, and the supernatant is added to 500 μl of SP-Sepharose slurry previously equilibrated with 15 ml of binding buffer.
6. The bead suspensions are gently stirred for 1.5 h. The beads are subsequently separated by centrifugation at 200 × *g* and mounted on columns followed by a slow wash for 6–8 h with 200 ml of binding buffer.
7. Proteins are gradient eluted with 1 ml each of elution buffer 1, 2, and 3 and final elution is performed with elution buffer 4 till all the protein is eluted out from the beads.
8. The eluted proteins are buffer exchanged in columns with 5 ml of desalting buffer, and used for fluorescence studies.
9. The purity of the protein is verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with 15% polyacrylamide gel followed by Coomassie blue staining. Protein concentrations are quantified in a spectrophotometer by measuring the absorbance at 280 nm.

3.2 Steady-State Fluorescence Measurements

Fluorescence quenching is an efficient method to probe interactions between biomolecules. The most common method to quantitate the information obtained from quenching data is to plot changes in fluorescence intensity versus quencher concentration in the form of a Stern-Volmer plot that allows one to determine the relative accessibility of the fluorophore (K_{SV}) which in turn can be used to derive binding constants. In the system described here, the conserved active-site tryptophan residue of CprB is the fluorophore and the γ-butyrolactone ligands Cp1 and Cp2 are quenchers. Comparison of quenching constants K_{SV} is used as a measure of quencher strength where high K_{SV} signifies stronger quenching (plausibly enhanced binding) and similar K_{SV} values are reflective of comparable binding affinities of the screened ligands.

3.2.1 Instrument Setup

1. Turn on the spectrofluorimeter. Depending on the instrument, several minutes could be required in order to stabilize the light source before using it.
2. To probe the intrinsic fluorescence of tryptophan in the protein, set the excitation wavelength to 295 nm. Set the emission scan range from 305 to 450 nm.
3. Set the excitation and emission slit widths to 10 nm (*see* **Notes 5** and **6**).
4. The voltage is set to medium, i.e., 600 V.

3.2.2 Recording the Spectrum

1. Record the baseline fluorescence by placing the desalting buffer in a 1 cm pathlength cuvette. This spectrum is later used to subtract from the sample emission spectrum.
2. All measurements are carried out at 24 °C (*see* **Note 7**).
3. Record the emission spectrum of 1 ml of CprB solution which is at a final concentration of 3.75 μM (*see* **Notes 8** and **9**).
4. Dissolve the ligands Cp1 and Cp2 to a concentration of 200 μM in desalting buffer supplemented with 2% (vol/vol) dimethyl sulfoxide (DMSO).
5. Cp1 titration is performed by successive additions of 5 μl of the Cp1 solution prepared in **step 4** into the cuvette already containing the protein. Incubate for 5 min. After each titration, thoroughly mix the solution and record the fluorescence spectrum (Fig. 4).
6. Repeat the above titration **step 5** till the fluorescence intensity saturates.
7. Note down the intensity values at the peak emission wavelength. The values are corrected for dilution (*see* **Note 10**).
8. Plot a graph of F_0/F versus quencher concentration, [Q] where F_0/F are the fluorescence intensities in the absence and presence of the quencher (Fig. 4).
9. In accordance to the Stern-Volmer equation (Eq. 1), the slope of this graph represents K_{SV}, the Stern-Volmer quenching constant (*see* **Note 11**):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \tag{1}$$

10. The K_{SV} value is an average of at least three independent experiments.
11. The same procedure is followed with ligand Cp2 and the K_{SV} values are estimated.
12. Similarly the entire protocol is repeated with the mutant version of CprB, W185L. This tryptophan residue is located away

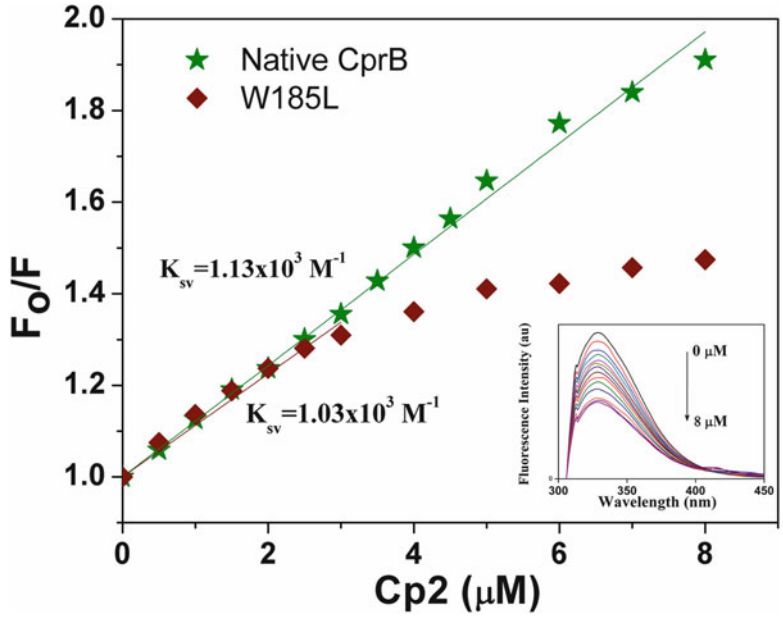

Fig. 4 Stern-Volmer plot for native CprB protein and mutant W185L in the presence of quencher Cp2 (inset: Steady-state emission spectra of CprB (3.75 μM) in the presence of increasing concentrations (0–8 μM) of Cp2)

from the binding pocket and will enable to delineate the contribution of fluorescence quenching by W185 from that of the tryptophan residue of interest W127.

3.3 KI Quenching Studies

Fluorescence quenching experiment using KI is an excellent indicator of solvent accessibility of tryptophans. Iodide ions being bulky are unable to penetrate into the protein core and hence K_{SV} values obtained using iodide as a quencher are indicative of the location of tryptophan residues, i.e., surface or buried. Here, very low K_{SV} values obtained via KI quenching indicated that the intrinsic tryptophan residues are deeply buried in the core of the protein, which the smaller and specific γ-butyrolactones could selectively access.

1. Record the emission spectrum of 1.5 μM CprB as described in Subheading 3.2.
2. Perform a series of titration of KI solution into the CprB solution, such that the final concentration of KI varies from 0 to 50 μM.
3. Record the fluorescence spectrum after each titration.
4. Determine the K_{SV} values as described in Subheading 3.2.

3.4 Time-Resolved Fluorescence Lifetime Measurements

Time-resolved fluorescence lifetime measurements are performed in the same timescale as the lifetime of the fluorophore. Hence conformational changes of the fluorophore and its plausible interactions with the neighboring environment can be additionally

deduced. In static quenching, formation of a nonfluorescent ground-state complex occurs between the fluorophore and the quencher. Thus the lifetime of the free (τ_o) or complexed fluorophore (τ) remains approximately the same. Contrastingly, in dynamic quenching, collisions between the fluorophore and the quencher occur during the lifetime of the excited state and a decrease in lifetime will be observed upon increasing quencher concentrations.

3.4.1 Instrument Setup

1. Time-resolved fluorescence experiments are performed on an IBH Horiba-JY fluorocube.
2. Attach the NanoLED 295 nm and switch on the instrument. Keep it on for 30 min to stabilize the source before using.
3. To avoid polarization artifacts decays are recorded under magic angle conditions. Emission polarizer is kept at 54.7° and excitation polarizer is kept in vertical position, i.e., 0°.
4. Emission slit width is set to 12 nm.
5. Time per channel is set to 7 ps.
6. The instrument response function (IRF) viz. prompt is done at the excitation wavelength 295 nm using a glass light scatterer. The FWHM is found to be 700 ps.

3.4.2 Recording the Lifetime Decay

1. 5 μM CprB is placed in a 1 cm pathlength quartz cuvette and excited at 295 nm and the emission is collected at 328 nm.
2. Decay is recorded till the number of counts in the peak channel is at least 3000.
3. Lifetime decay kinetics is then collected for native CprB in the presence of varying concentrations of Cp2, ranging from 1 to 10 μM (Fig. 5).

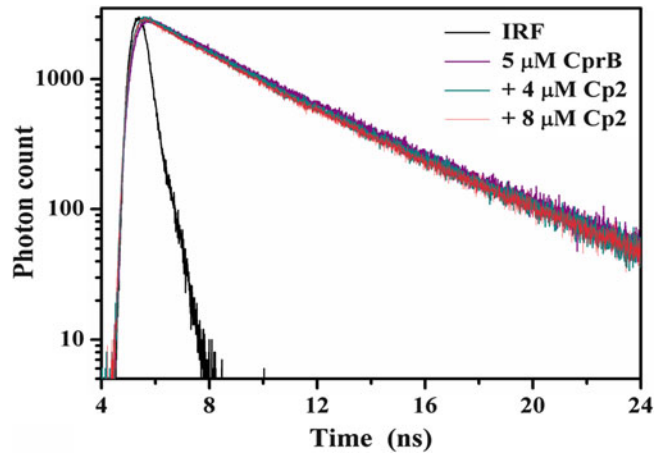

Fig. 5 Time-resolved fluorescence decay curve of CprB (5 μM) and CprB complexed with Cp2. Adapted from [19]

3.4.3 Data Analysis

1. The decay obtained is fitted by the global reconvolution fitting technique using the software FluoFit (Global Fluorescence Decay Data Analysis Software, Pico Quant).
2. The analysis is a nonlinear least-squares iterative reconvolution based on the Levenberg-Marquardt algorithm and expressed as a sum of exponentials with Eq. 2:

$$I(t) = \Sigma\alpha_i \exp(-t/\tau_i) \tag{2}$$

where α_i represents the amplitude of the ith component associated with fluorescence lifetime τ_i such that $\Sigma\alpha_i = 1$. $\Sigma\ \alpha_i\ \tau_i$ gives the mean lifetime τ_m of the system.

3. The goodness of fits is determined from the reduced χ^2 values (~1) as well as from the randomness of the residuals.

4 Notes

1. Solvents and chemicals must be devoid of fluorescent contaminants that can act as a quencher or alter the background signal.
2. Buffers and solutions must be free of particles/tissue fibers or aggregates that can scatter light and produce artifacts in the readings. In such situations it is ideal to filter all the solutions.
3. KI solutions should be freshly prepared as they are sensitive to light degradation.
4. All the faces of the fluorescence cuvette should be completely clean. Avoid cleaning of cuvettes with detergent solutions as they could potentially contain fluorescent compounds. Clean the cuvettes thoroughly with chromic acid on a regular basis. Before placing the cuvette in the cell holder, wipe the surface of the cuvette with a fiber-free tissue and be careful to not touch the sides of the cuvette. Position the cuvette reproducibly in the cell holder each time. During the titration process avoid spillage of sample on the sides of the cuvette.
5. Initial optimization of the instrument parameter settings such as slit widths for your sample will have to be done in order to obtain a good signal-to-noise ratio. Slit widths are chosen based on fluorescence yield of the sample. Ideally larger slit widths are not selected due to interference of noise which may result in loss of data resolution. In such cases it is preferable to increase the concentration of the fluorophore and maintain narrow slit widths.
6. Once the optimal instrument parameter settings are known all the steady-state fluorescence measurements are performed at the same settings for all the samples. Do not compare results that have different parameter settings. This can lead to a wrong interpretation of the results.

7. As fluorescence measurements are sensitive to temperature it is ideal to use thermostatted cell holders.
8. Ideally measure absorbance of each sample before taking fluorescence measurements to reduce inner filter effects. It is also crucial to measure the absorbance of the quencher sample alone. If the quencher has an absorption peak at the excitation wavelength, the decrease in fluorescence intensity could be wrongly interpreted as quenching. Under such circumstances appropriate correction factors need to be applied and then interpret the data carefully.
9. Monitor if the protein chosen for the study is stable under the given experimental conditions and the time course of the experiment. It should not form aggregates or be sensitive to photodegradation.
10. Make correction for the concentrations of the fluorophore at each point of titration. At this point interpret the data carefully. The changes in fluorescence intensity should not be a result of dilution. If small amounts of titrant are added the changes in concentration of fluorophore can be considered to be negligible.
11. K_{SV} values signify the extent of accessibility of the quencher to tryptophan. While determining the slope from the Stern-Volmer plot fix the intercept at 1. If the plot deviates from linearity then calculate K_{SV} only from the linear part of the plot. Deviation from linearity could represent various conditions such as fractional accessibility to quencher and conformational changes that lead to exposure of previously shielded tryptophan residues. Hence deviations in Stern-Volmer plot must be carefully interpreted.

Acknowledgements

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