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# Monitoring receptor-mediated activation of heterotrimeric G-proteins by fluorescence resonance energy transfer

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**Abstract**

Green fluorescent protein (GFP)-centered fluorescence resonance energy transfer (FRET) relies on a distance-dependent transfer of energy from a donor fluorophore to an acceptor fluorophore and can be used to examine protein interactions in living cells. Here we describe a method to monitor the association and disassociation of heterotrimeric GTP-binding (G-proteins) from one another before and after stimulation of coupled receptors in living *Dictyostelium discoideum* cells. The $G\alpha_2$ and $G\beta\gamma$ proteins were tagged with cyan and yellow fluorescent proteins and used to observe the state of the G-protein heterotrimer. Data from emission spectra were used to detect the FRET fluorescence and to determine kinetics and dose–response curves of bound ligand and analogs. Extending G-protein FRET to mammalian G-proteins should enable direct in situ mechanistic studies and applications such as drug screening and identifying ligands of new G-protein-coupled receptors. © 2002 Elsevier Science (USA). All rights reserved.

*Keywords:* Resonance energy transfer; Green fluorescent protein; Fluorometer; Receptor activation

## 1. Introduction

While known for more than 50 years, fluorescence resonance energy transfer (FRET) is a property of fluorophores that has only recently been adapted for use in biology [1,2]. This process of energy transfer from a fluorescent donor molecule to a fluorescent acceptor occurs only when the emission spectrum of the donor fluorescence overlaps that of the acceptor excitation. This transfer of energy is distance dependent, with the efficiency of this process relying on the inverse sixth power of the distance between the donor and acceptor and was described by Förster as

$$E = 1/(1 + [r/(R_0)^6],$$

where $R_0$ equals the distance at which half the energy is transferred for a given donor and acceptor pair and is dependent on the extent of overlap between the donor emission and acceptor excitation spectra, the quantum yield of the donor, the absorption coefficient of the acceptor, and the relative orientation of the two fluorophores. The acceptor must be in close proximity (typically $< 10$ nm) for the excited state energy of the donor to be transferred. If FRET occurs, there will be a reduction in the donor's fluorescence intensity and an increase in the acceptor's emission intensity. FRET has become extremely useful since the emergence of genetically encoded fluorescent probes, such as the green fluorescent protein (GFP) [3]. Because FRET occurs over optimal distances for detecting protein–protein interactions, it has become an ideal tool for determining whether proteins are making specific contacts as opposed to being merely colocalized in the same compartments.

Sensory stimuli such as hormones, neurotransmitters, and chemoattractants exert their effect on cells through a family of serpentine G-protein-coupled receptors (GPCRs). On ligand binding, receptors catalyze the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) and the dissociation of the G-protein heterotrimer, allowing both the GTP-bound $\alpha$ subunit and free $\beta\gamma$ complexes to signal to downstream targets. The intrinsic guanosine triphosphatase (GTPase) activity of the $\alpha$ subunit hydrolyzes the bound GTP, allowing the heterotrimer to reassociate and completing the cycle [4,5]. The mechanistic studies that

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have resulted in our understanding of the G-protein cycle have been derived largely from in vitro studies using isolated membranes and purified proteins. The cycle has not yet been directly observed or tested in living cells. We determined, based on the known crystal structures of mammalian heterotrimers [6,7], that appropriately targeted GFP fusions would FRET when attached to the α and βγ subunits and should allow real-time measurements and imaging of G-protein signaling [8]. Such a breakthrough would shed light on the kinetics and the subcellular activation of G-proteins in living cells. Here we describe how G-protein FRET was accomplished and what instrumentation and techniques were used to monitor the state of the FRET fluorescence in living amoebae.

## 2. The green fluorescent proteins

The advent of genetically encoded dyes began with the discovery of GFP and culminated with the cloning of the GFP from the jellyfish *Aequorea victoria* [9,10]. GFP can be used as a reporter by itself or when genetically engineered as a fusion with other proteins, permitting the localization of proteins in both living and fixed specimens. GFP has been targeted to nearly every major organelle in the cell, revolutionizing modern cell biology [11]. GFP is a single-chain protein of 238 amino acids, normally adopting an 11-strand β-barrel structure that protects the fluorophore from the surrounding environment by burying it within its core [12]. The GFP can be fused in-frame with the gene of interest and is expressed in the cell or organism under investigation. Proteins can usually be tagged on their amino or carboxyl terminus; however, it is highly dependent on the protein. Due to certain modifications or structural constraints, some proteins have been found to be functional only by insertion of GFP into the middle of the protein [13]. Functionality of the protein fusion can normally be assessed by proper localization and, when possible, by determining the biochemical activity of the molecule either in vitro or in vivo. Although not always possible, the best way to determine functionality of the GFP fusion is by inserting a chimeric protein containing GFP into a cell line or organism that contains no endogenous protein and rescuing the aberrant phenotype.

The spectral properties of *Aequorea* GFP have been altered by extensive mutagenesis and have produced a number of GFP variants with differing excitation and emission peaks and increased fluorescence quantum yield [11,14–18]. A serine-65-to-threonine mutant resulted in a severalfold improvement of green light emission due to stabilization of the hydrogen-bonding network in the chromophore [14,19]. The more photostable and enhanced version of GFP is referred to as eGFP, which excites at 488 nm, emits at 509 nm, and is commercially available [16,20]. Improvements have also been obtained by changing the codon usage to optimize translation in both plants and mammalian cells [21,22]. Spectral variants were obtained when the tyrosine-66 residue was changed to histidine, emitting a blue color on excitation [11,17]. This blue fluorescent protein (BFP) has a very low quantum yield and photobleaches rapidly. It does have significant spectral overlap with the longer-wavelength GFPs, permitting studies of BFP as a donor for FRET [23]. BFP has been replaced considerably since the cyan (blue-green) fluorescence protein eCFP has been identified [15,17]. The eCFP variant, which is brighter and more resistant to photobleaching, resulted from a change of the tyrosine-66 to tryptophan along with a number of residue changes within the β-barrel structure. The maximal excitation of this mutant is at 476 nm, although it is unique in that it has a second peak at 505 nm. Although the origin of this double peak is not fully understood, the emission does overlap the spectral excitation wavelengths of another color variant, the yellow fluorescent protein (eYFP), making the two useful for FRET studies [11]. The eYFP has a very high quantum yield with an excitation peak at 514 nm and a peak emission at 527 nm. It is also less resistant to photobleaching; this bleaching property has been taken advantage of in FRET studies [24,25].

## 3. Description of FRET

FRET is a quantum mechanical phenomenon that involves the radiationless transfer of energy that occurs when two fluorophores are in very close molecular proximity (<10 nm) and the emission spectrum of one fluorophore, the donor, overlaps the excitation spectrum of the second, acceptor fluorophore. The efficiency of this process depends on the inverse sixth power of the distance between donor and acceptor, where $R_0$ is the distance at which half of the energy is transferred (see earlier Förster equation). In theory, FRET can be used as a spectroscopic ruler [26]. However, FRET is not generally used for this purpose since $E$ is dependent on the orientation of the probes, which is unstable and difficult to determine. The development of fluorescent protein tags has made FRET a very powerful tool for detecting changes in conformation, rather than absolute changes in distance. There are two main criteria that must be satisfied for FRET to occur between fluorescent probes: (1) The donor emission spectrum must overlap the excitation spectrum of the acceptor molecule, and (2) the distance between the donor and acceptor fluorophores must lie between 0.2 and 10 nm. Although it is also important for the fluorophores to be in a favorable orientation to one another, this is difficult to

control. Finding probes where the donor emission and acceptor excitation spectra overlap significantly can improve FRET efficiency. Improving the FRET efficiency, however, will significantly increase your background signal due to spectral cross-talk. Spectral cross-talk is also contributed by emission of the acceptor fluorophore by excitation wavelengths used to excite the donor. Greater separation of donor and acceptor emission peaks allows the increase in acceptor emission from FRET to be determined more readily since donor emission cross-talk is minimized. Because cross-talk is a significant problem, steps must be taken to determine the amount of spectral cross-talk and to isolate the small acceptor signal resulting only from FRET. While the spectral overlap of BFP with GFP and YFP is adequate for FRET to occur [27], this review focuses on protein fusions using CFP and YFP, due to the larger overlap of the CFP emission with the absorption spectrum of YFP (Fig. 1) [28].

The FRET fluorescence results in a number of quantifiable changes that can be measured. These include sensitized emission of the acceptor fluorophore, quenching of the donor fluorophore, and changes in lifetimes of both the donor and acceptor. The use of fluorescence microscopy to measure these changes has been reported with varying degrees of difficulty and success [29–32]. These methods often involve extensive mathematical calculations based on imaging using multiple filter sets and requiring numerous images and exposures of the same cells over time. The movement of cells between image acquisitions and capturing for equivalent exposure times convey just two problems one may encounter using multiple filter sets. Microscopic techniques using photobleaching and measuring fluorescence lifetimes are also available [27,33–35]; these procedures normally require fixing cells and are therefore not useful for live cell imaging. Other approaches have been developed to look at protein interactions on the surface of cells [36]. The method described here used conventional scanning fluorometry and allowed us to monitor G-protein FRET and the loss of this fluorescence signal on receptor stimulation in living cells.

## 4. Description of method

Described below are the experimental protocols for performing G-protein FRET analysis of living cells in a conventional fluorometer. For further discussions on detection of FRET by fluorometric analysis see [37–39].

## 5. Engineering constructs

There are a variety of vectors for expressing genetically engineered fusion proteins in *Dictyostelium discoideum* cells [40–42]. Fusions described here were cloned into the CV5 vector, an integrating and extrachromosomal plasmid. CV5 was derived from p88 by the addition of an actin 15 expression cassette from pMC34 [43]. Guided by the crystal structures of several mammalian heterotrimers [6,7], the gene encoding the full-length enhanced CFP; a class 5 (F64L, S65T, Y66W, N146I, M153T, V163A) variant was inserted into a *Spe*I restriction site after residue 90, in the loop between the αA and the αB helices of the $G\alpha_2$ cDNA. The *Spe*I site, engineered in by polymerase chain reaction (PCR) amplification, also added a threonine residue and serine residue at the N- and C-terminal boundaries of the insertion. A similar fusion construct has been reported using the mammalian G-protein $\alpha_q$ subunit [44]. Previous attempts to GFP tag G-protein α subunits at the N and C termini resulted in nonfunctional proteins. Many G-protein α subunits, including the *D. dis-*

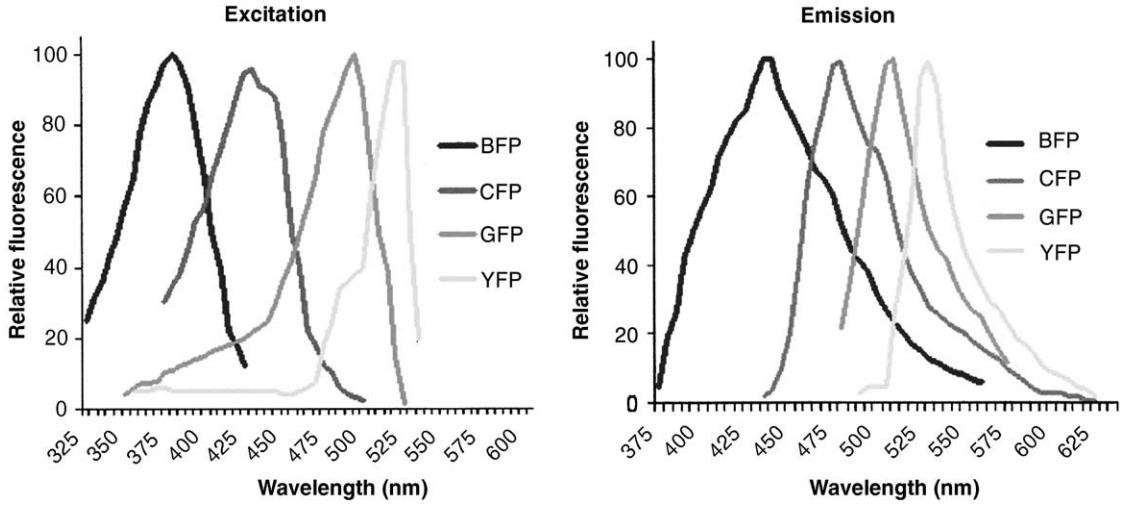


Fig. 1. Excitation and emission spectra for GFP variants. Note the overlap of the CFP emission with the YFP excitation absorption.

*coideum* $G\alpha_2$, are modified at their N terminus by the attachment of saturated fatty acids [45,46]. It is likely these failed because GFP inhibits the membrane association that is facilitated by the fatty acid attachments [47]. Similarly, nonfunctional fusions at the C terminus of $G\alpha_2$ likely disrupted the integrity of the heterotrimer.

The gene encoding the full-length eYFP was fused to the N terminal of the Gβ gene, similar to the Gβ–GFP fusion previously reported [48]. This is a class 4 (S65G, V68L, S72A, T203Y) GFP variant. In this review we refer to the eYFP and eCFP simply as YFP and CFP. The $g\beta^-$ cells were transformed by electroporation [49] with 5.0 μg of plasmid containing the Gβ–YFP fusion. Similarly, the $g\alpha_2^-$ cells were transformed with $G\alpha_2$–CFP. Equal amounts of plasmids (10.0 μg of total DNA) carrying the $G\alpha_2$–CFP and Gβ–YFP fusions were mixed, and also transformed into $g\alpha_2^-$ cells. Cell lines were designated Gβ–YFP/$g\beta^-$, $G\alpha_2$–CFP/$g\alpha_2^-$, and $\alpha_2$-CFP:β-YFP cells, respectively. G418-resistant clones were selected in 2–3 weeks.

## 6. Analyzing function

The amoeba, with its attractive genetic system, permits targeted gene disruptions, allowing the activities of the fusion proteins to be assessed by phenotypic rescue of $G\alpha_2$- and Gβ-null cell lines [50,51]. Stable clones that aggregated and formed fruiting bodies on bacterial lawns of *Klebsiella aerogenes* were isolated. Fusion of YFP to the $NH_2$ terminus of Gβ rescued the chemotactic defects of Gβ-null mutants ($g\beta^-$ cells) [48]. Stable expression of $G\alpha_2$–CFP rescued the chemotactic and developmental defects of the $g\alpha_2^-$ cells. Similarly, cotransformation with $G\alpha_2$–CFP and Gβ–YFP rescued the $g\alpha_2^-$ cells. All cell lines were subjected to immunoblot analyses, confirming that the protein fusions were the correct molecular weights. $G\alpha_2$, Gβ, and GFP (Clontech) antisera detected appropriate bands in wild-type cells and appropriately shifted bands in cells transformed with the CFP and YFP fusions, respectively. When visualized under a fluorescence microscope, the fluorescently tagged G-proteins targeted to the membrane and cytosol as expected based on biochemical assays. Also, we found that greater than 90% of the cotransformed cells exhibited fluorescence of both colors when examined using the proper filter sets. Fluorescence levels and presumably expression levels differed markedly between individual cells. We found that chemoattractants triggered in vivo actin polymerization (assayed by fixation and phalloidin staining) and chemoattractant-filled micropipets induced chemotactic responses in these cells. The $g\beta^-$ cells cotransformed with $G\alpha_2$–CFP and Gβ–YFP also had their chemotactic defects rescued; the cotransformants in the $g\alpha_2^-$ background ($\alpha_2$-CFP:β-YFP cells) appeared more vigorous and, therefore, we focused on them for our studies (Fig. 2). We recently reversed the GFP variants tagged to the G-proteins so that $G\alpha_2$ was fused to YFP and Gβ to CFP. This FRET pair should theoretically be better since we are using the $g\alpha_2^-$ background for our studies. All Gβ–CFP molecules will be paired up with $G\alpha_2$–YFP molecules in a $g\alpha_2^-$ background, and thus all the Gβ–CFP should be quenched. In our current configuration detailed here, some $G\alpha_2$–CFP molecules may be paired up with endogenously expressed, untagged β. We are currently testing whether this new arrangement results in a better FRET signal.

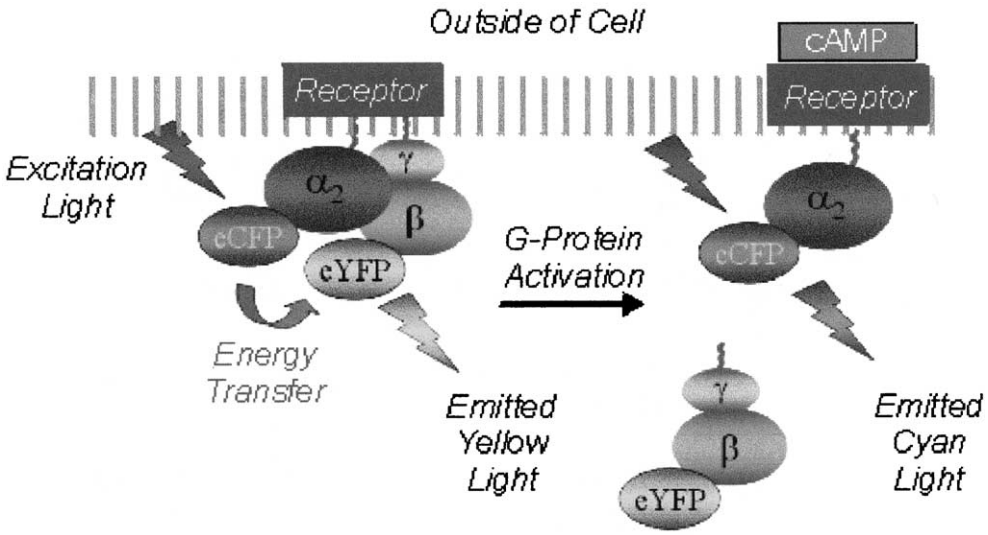


Fig. 2. Diagram of G-protein FRET in *D. discoideum*. CFP was inserted into the helical domain of $G\alpha_2$ in a position optimal for FRET with YFP fused to the $NH_2$ terminus of Gβ. On receptor stimulation with cAMP, the heterotrimer disassociates and FRET fluorescence decreases, resulting in a loss of the yellow fluorescent signal.

### 6.1. Measuring FRET fluorescence

When *D. discoideum* amoebae are starved, they enter a developmental program and cells aggregate into a multicellular organism. At aggregation centers, cyclic adenosine 3′, 5′-monophosphate (cAMP) is periodically secreted at 6-min intervals. These oscillations are required for early gene expression of many signaling proteins, including that of the cAMP receptor cAR1. This receptor is coupled to the $G\alpha_2$ subunit and is essential for our experiments. Cells were first grown in HL-5 medium as previously described [52]. To ensure proper gene expression, cells were washed in DB buffer and differentiated at $2 \times 10^7$ cells/ml while shaking at 200 rpm with repeated 50 nM cAMP pulses for 6 h. Cells were bathed in 3 mM caffeine for 20 min to prevent the activation of adenylyl cyclases and production of cAMP. In all experiments, cells were kept shaking continuously until 1-ml aliquots were procured and pipetted into a quartz cuvette. To ensure that cells did not settle to the bottom of the cuvette, cells were pipetted up and down rapidly and immediately scanned. It was essential here that production of large air bubbles be avoided, as they can significantly add to the background scattering. Temperature was also an important component in these experiments. It only takes a short time (minutes) for *D. discoideum* cells to expire when exposed to temperatures above 28 °C. The temperature in the room was lowered since the fluorometer generates a significant amount of heat. Most fluorometers come with a water-cooled jacket that should be more than adequate for keeping the fluorometer from overheating the cells. Working with mammalian cells may require the use of a heated-water jacket to keep the cells at 37 °C. All scans were performed on a Spex Fluoromax-2 fluorometer. We found this fluorometer easy to use and reliable. Slits were set at 3 nm and each wavelength was scanned for 0.4 s.

It is essential in FRET experiments to show that the FRET fluorescence is the result of a true transfer of energy from the donor fluorophore to the acceptor molecule. Studies of $\alpha_2$-CFP : β-YFP cell lines expressing both fluorescent subunits showed direct, specific transfer of resonance energy from $G\alpha_2$–CFP to Gβ–YFP. To demonstrate this, cells were excited at 440 nm and their emission spectrum was recorded between 460 and 600 nm. We found that the cotransformed cells showed an extra emission peak near 527 nm, corresponding to the FRET fluorescence. While this peak was readily observable when viewing the raw data scan, this may not always be the case as the shape of the curve is dependent on the background autofluorescence of the cells carrying the FRET pairs. Our cell lines expressing $G\alpha_2$–CFP alone or mixtures of cells containing either $G\alpha_2$–CFP or Gβ–YFP alone did not display this additional peak. Addition of the chemoattractant cAMP to $\alpha_2$-CFP:β-YFP cells triggers a rapid loss of FRET fluorescence at 527 nm reflecting receptor-mediated activation and dissociation of the G-protein heterotrimer; (Fig. 3). As expected, cells containing both constructs were the only cells that showed a change in FRET fluorescence on cAMP stimulation.

The magnitude of the FRET fluorescence compared with that of Gβ–YFP directly excited at 490 nm and not relayed through $G\alpha_2$–CFP can be calculated. In our experiments it varied from 7 to 12%. This variation is probably due to differences in expression levels of the fused proteins, and could also be attributed to the endogenously expressed Gβ subunit that would be expected to compete with the Gβ–YFP and would influence the FRET fluorescence. Minor differences in the background autofluorescence of cell lines on a day-to-day basis also likely contribute variability. This makes it important to treat cells identically in all experiments. To determine the magnitude of the FRET fluorescence in a population of cells, the excitation wavelength was set at 440 nm and emission spectra were collected between 460 and 600 nm of the following lines: $\alpha_2$-CFP:β-YFP, a mixture of $G\alpha_2$–CFP/$g\alpha_2^-$ and Gβ–YFP/$g\beta^-$, and wild-type AX3. All data can be exported from the Specs software program and calculations can be performed in a spreadsheet program such as Microsoft Excel. Spectra were normalized to the fluorescence at 600 nm of the $\alpha_2$-CFP:β-YFP cells and the wild-type spectrum was subtracted from each of the others. These corrected spectra were then normalized to the fluorescence at 490 nm of the $\alpha_2$-CFP:β-YFP cells.

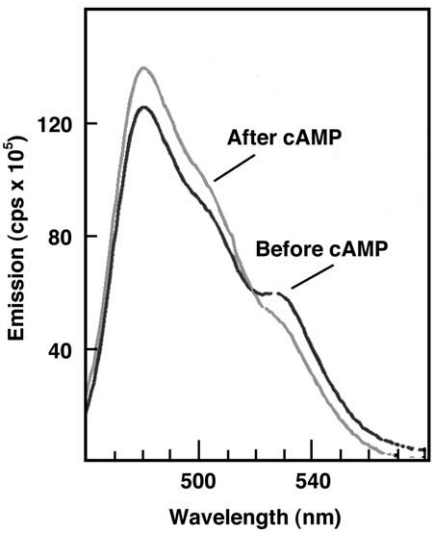


Fig. 3. Emission spectra of $\alpha_2$-CFP:β-YFP before and after treatment with 100 μM cAMP. These spectra were normalized at 600 nm and then the "before" and "after cAMP" spectra had the wild-type spectrum subtracted from them, leaving the fluorescent signal. Reprinted, with permission from Janetopoulos et al. *Science* 291, 2408–2411 (2001). Copyright © 2001 American Association for the Advancement of Science.

The amount of FRET fluorescence was determined by subtracting from the spectrum of the $\alpha_2$-CFP:β-YFP cells either the spectrum of the mixed cells or those of the $G\alpha_2$-CFP/$g\alpha_2^-$ cells. In the latter case, a further correction was made for the fluorescence emitted from Gβ-YFP/$g\beta^-$ directly excited at 440 nm. Subtractions using data from the mixed cells or those of the $G\alpha_2$-CFP/$g\alpha_2^-$ cells gave similar results. These calculations allowed us to subtract the spectral cross-talk components of both the donor and acceptor and extract the FRET fluorescence. This resulting FRET fluorescence can then be compared with the amount of fluorescence of Gβ–YFP directly excited at 490 nm and not relayed through $G\alpha_2$–CFP, giving an indication of the FRET efficiency.

G-protein FRET allowed us to examine the kinetics of transient loss of FRET on addition and removal of cAMP. We have made a number of attempts to obtain very rapid kinetic data (<5 s) by using stop-flow systems or by pipetting in cAMP through a special portal hole in the top of the fluorometer. Unfortunately these procedures have resulted in significant noise. During these experiments incomplete mixing of cAMP occurred and the addition of air bubbles into the samples was problematic. In addition, the cells often began settling during the course of the experiment. The Spec fluorometer was equipped with a stirrer, but this was insufficient to keep the heavy *D. discoideum* from settling. Manually pipetting in cAMP, rapidly pipetting up and down, and scanning quickly obtained very consistent results. It takes about 1 min to obtain a full emission profile at 0.4 s per $\lambda$. To obtain rapid kinetics, scans were limited to an emission profile ranging from 515 to 535 nm and emission was collected for only 0.1 s per $\lambda$. These preliminary experiments showed a loss of FRET in less than 5 s; however, this technique is still being developed. Collaborations are being constructed with groups that have better stop-flow systems and more sensitive fluorometers so that we can look at kinetics at subsecond intervals. We are also working to observe the changes in FRET under a fluorescence microscope. FRET microscopy at the current time will actually only get us down to a resolution of a few seconds; long exposure times and the necessity to use multiple filter sets and acquire three images impede this process. In addition, three images of the cells must be taken prior to stimulation. To excite the CFP fluorophores, four exposures must be acquired at the shorter wavelengths, often damaging the cells in the process. We hope to somewhat circumvent this problem by paraformaldehyde-fixing cells prior to and after cAMP stimulation. Cells then can be assessed for FRET fluorescence by more reliable photobleaching experiments. Ultimately, it is possible that we may try fluorescence lifetime imaging (FLIM), which normally focuses on the donor fluorescence lifetime and would detect the nanosecond decay kinetics of the CFP molecule. One setback for performing FLIM experiments is that the equipment is rather expensive.

### 6.2. Determination of dose–response curves

G-Protein FRET can be used to establish dose–response curves. We treated cells with increasing concentrations of cAMP and two of its analogs, 2′-deoxyadenosine 3′, 5′-monophosphate (2′-dcAMP) and 8-bromoadenosine 3′, 5′-monophosphate (8-Br-cAMP) and showed that the FRET fluorescence decreased in a dose-dependent manner (Fig. 4A). To quantify the response for each cAMP analog, spectra were first normalized to the integral from 475 to 550 nm and we then subtracted the spectrum of the highest concentration from that of each of the lower concentrations. The areas under the differences curves were integrated from 475 to 520 nm and from 520 to 550 nm and

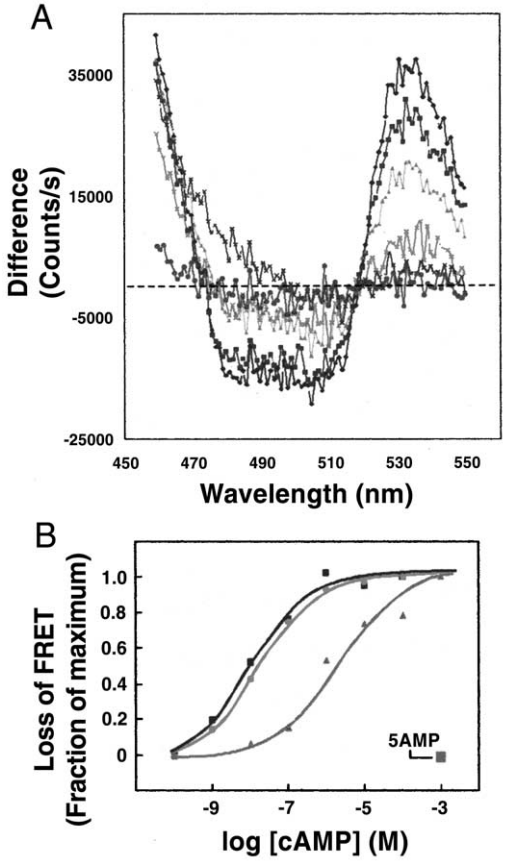


Fig. 4. Dose–response curves for cAMP, 2′-dcAMP, 8-Br-cAMP, and 5′-AMP. (A) Difference fluorescence spectra of $\alpha_2$-CFP:β-YFP cells treated with increasing concentrations of cAMP. Cells were treated with caffeine and stimulated with 0, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM cAMP (dashed line) in the presence of 10 mM dithiothreitol, and analyzed in the fluorometer 15 s after mixing. Similar spectra were obtained for the other two cAMP analogues (13). (B) Dose–response curves for cAMP (squares), 2′-dcAMP (circles), 8-Br-cAMP (triangles), and 5′-AMP (no response).

the absolute values were summed. The negative values from 475 to 520 nm reflected the gain in cyan fluorescence while the positive values from 520 to 550 nm reflected the loss in yellow fluorescence. These data provided us with dose curves that mirrored their known binding affinities [53] and also further demonstrated that G-protein FRET was receptor mediated (Fig. 4B).

## 7. Concluding remarks and future directions

We have described a technique that uses FRET to monitor the interactions between the two G-proteins, $G\alpha_2$ and $G\beta$, fused to GFP variants. We have shown that this is a valuable tool for indicating receptor occupancy and the state of the G-protein heterotrimer. When the receptor is unbound by ligand, the G-protein heterotrimer is in its resting (trimeric) state and the labeled proteins will FRET. On binding of ligand, the receptor catalyzes the exchange of GTP for GDP, the heterotrimer disassociates, and FRET fluorescence decreases. We found that the FRET fluorescence in living cells and the change in the FRET fluorescence on receptor stimulation can be readily monitored using a conventional fluorometer. This technique can be used to measure the kinetics of G-protein activation and to create a dose–response curve of cAMP and its analogs that bind cAR1.

When using FRET to determine if two proteins interact, controls must be performed to demonstrate that the extra fluorescence peak seen at 427 nm is really due to FRET. In cases where one is trying to prove that there is an interaction between two proteins that are always in close proximity, this becomes much more difficult. One control that should be carried out is to express (preferably using the same expression construct in all experiments) two noninteracting fusion proteins in the same cells that carry CFP and YFP and show that there was no FRET fluorescence after normalizing and making corrections for the cross-talk. With G-protein FRET, there was a change in FRET fluorescence on receptor activation, due to the disassociation of the two proteins. Transforming protein fusions of YFP–$G\gamma$ and CFP-$G\beta$ into the same cells was one additional control we performed. These two proteins were tagged on their respective N termini, showed FRET, and did not show a change in FRET fluorescence when the cells were stimulated with cAMP. The $G\beta\gamma$ complex should remain together after stimulation with cAMP and release from $G\alpha_2$. Recent experiments tagging both the G-proteins $\beta$ and $\gamma$ with YFP and then coexpressing with a third fusion, $G\alpha_2$–CFP, resulted in an increase in the FRET fluorescence signal. This increase in signal should be useful for FRET microscopy. Experiments engineering multiple YFPs (up to four with short linkers) on the N terminus of the same $G\beta$ protein did not lead to significant increases in the FRET signal, although the YFP signal did go up dramatically.

The assay presented here describes a powerful method for monitoring the state of heterotrimeric G-proteins and allows detection of FRET fluorescence in intact living cells. G-Protein FRET provides a unique tool for analyzing the G-protein cycle while the proteins are in their native environment, reducing artifacts that would be found by working on fractionated cells and membrane preparations. Membrane preparations, however, also appear to FRET and can be stimulated by cAMP and GTP$\gamma$S. This may provide an important tool for in vitro experiments where we can add back or take away signaling components and see how they influence the G-protein cycle. Importantly, we are working to adapt this technique to mammalian cells. Since we used the mammalian crystal structure to strategically target our fluorescent tags, we expect that this method can be applied to mammalian cells. This should enable the analysis of G-protein-coupled receptor systems in higher organisms and may be developed for use in high-throughput screening assays to detect agonists and antagonists that bind unknown and known receptors.

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