

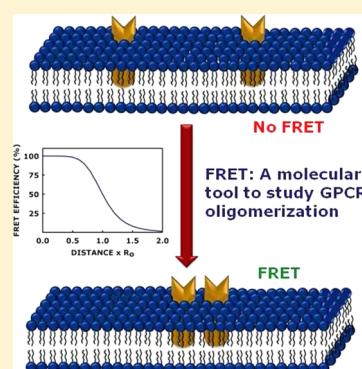
Excitements and Challenges in GPCR Oligomerization: Molecular Insight from FRET

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ABSTRACT: G protein-coupled receptors (GPCRs) are the largest family of proteins involved in signal transduction across cell membranes, and they represent major drug targets in all clinical areas. Oligomerization of GPCRs and its implications in drug discovery constitute an exciting area in contemporary biology. In this Review, we have highlighted the application of fluorescence resonance energy transfer (FRET) in exploring GPCR oligomerization, with special emphasis on possible pitfalls and experimental complications involved. Based on FRET photophysics, we discuss some of the possible complications, and recommend that FRET results in complex cellular environments should be interpreted with caution. Although both hetero- and homo-FRET are used in measurements of GPCR oligomerization, we suggest that homo-FRET enjoys certain advantages over hetero-FRET. Given the seminal role of GPCRs as current drug targets, we envision that methodological progress in studying GPCR oligomerization would result in better therapeutic strategies.

KEYWORDS: GPCR, oligomerization, hetero-FRET, homo-FRET, serotonin_{1A} receptor



G PROTEIN-COUPLED RECEPTORS

The G protein-coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, and is involved in information transfer (signal transduction) from outside the cell to the cellular interior.^{1–4} GPCRs are typically seven transmembrane domain proteins and include >800 members which are encoded by ~5% of human genes.⁵ They transmit the extracellular signal to the interior of the cell through concerted changes in the transmembrane domain structure.^{6,7} GPCRs exhibit remarkable evolutionary conservation, and it has been reported that GPCRs and G-protein signaling date back ~1.2 billion years.^{2,8} GPCRs dictate physiological responses to a diverse variety of stimuli that include endogenous ligands (such as biogenic amines, peptides, and glycoproteins) and exogenous ligands for sensory perception such as odorants, pheromones, and even photons. As a result, GPCRs mediate multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, entry of pathogens into host cells, and inflammatory and immune responses. It is therefore not surprising that GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas.^{9–12} It is estimated that ~50% of clinically prescribed drugs and 25 of the 100 top-selling drugs target GPCRs.^{13,14} In spite of this, only a small fraction of all GPCRs are presently targeted by drugs.¹⁵ This raises the interesting possibility that the receptors which are not recognized yet could be potential drug targets in future for diseases that are difficult to treat by currently available drugs.

MEMBRANE LIPID DEPENDENCE IN GPCR ORGANIZATION AND FUNCTION

GPCRs are transmembrane proteins with multiple passes across the membrane. A significant portion of any given transmembrane

receptor remains in contact with the membrane lipid environment. This raises the obvious possibility that the membrane could be an important modulator of receptor structure and function. Exploring such lipid–receptor interaction assumes relevance in light of the fact that a cell has the ability to vary its membrane lipid composition in response to a variety of stresses and stimuli, thereby changing the environment and the activity of the receptors in its membrane. Results from our laboratory and others have comprehensively demonstrated that the interaction of GPCRs with membrane lipids represents an important determinant in their structure and function.^{16–20} It has recently been reported that even the interaction between GPCRs and G-proteins could be modulated by membrane lipids.²¹ Interestingly, the membrane lipid environment of GPCRs has been implicated in disease progression during aging.²²

In particular, membrane cholesterol has been shown to modulate the function of a number of GPCRs.^{16–20,23–38} A specific GPCR that has received a lot of attention in terms of cholesterol sensitivity of its organization, dynamics, and function is the serotonin_{1A} receptor.^{17,18,20} The serotonin_{1A} receptor is localized in different regions of the brain receiving serotonergic input from the raphe nuclei. The serotonin_{1A} receptor is an important neurotransmitter receptor and is implicated in the generation and modulation of various cognitive, behavioral, and developmental functions.^{39–42} The agonists⁴³ and antagonists⁴⁴ of the serotonin_{1A} receptor represent major classes of molecules with potential therapeutic applications in anxiety- or stress-related disorders. As a result, the serotonin_{1A} receptor serves as an important drug target for neuropsychiatric disorders such as

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anxiety, depression, and Alzheimer's disease.^{45,46} Another important role of the serotonin_{1A} receptor is in neural development.⁴⁷

■ OLIGOMERIZATION OF GPCRS: NEW VISTAS IN THERAPEUTICS

Oligomerization of membrane proteins and receptors is often necessary for their functionality. Yet, monitoring oligomerization of membrane proteins poses considerable experimental challenge. An emerging and exciting area in GPCR research is oligomerization of GPCRs and the possible role of oligomerization in GPCR function and signaling.^{48–52} Such oligomerization is implicated in proper folding of receptors, thereby providing the framework for efficient and controlled signal transduction. The potential implications of GPCR oligomerization are tremendous, keeping in mind the role of GPCRs as major drug targets.^{53,54} GPCR oligomerization facilitates an increased cross-talk between receptors via homo- and/or heterodimers as well as higher-order oligomers.^{51,54–56} Importantly, membrane lipids (particularly cholesterol) and the actin cytoskeletal network have recently been implicated in the modulation of GPCR oligomerization.^{55–57} Interestingly, oligomerization of certain GPCRs has been shown to be constitutive.^{55,58}

■ HOW TO DETECT GPCR DIMERS AND OLIGOMERS IN THE CELLULAR MILIEU: CHALLENGES AND PITFALLS

Co-immunoprecipitation is the most common biochemical approach to detect GPCR oligomers. Although commonly used, this approach suffers from a number of drawbacks such as cross-reactivity of antibodies. More importantly, this approach requires solubilization of the cell membrane and therefore is unsuitable to monitor oligomerization in live cells. Also, solubilization of very hydrophobic GPCRs poses considerable challenge⁵⁹ and could lead to GPCR aggregation during solubilization, resulting in promiscuous (nonselective) dimers.⁶⁰

Fluorescence Resonance Energy Transfer: A Popular Tool in Monitoring GPCR Oligomerization. Fluorescence resonance energy transfer (FRET) methods such as hetero-FRET (FRET between two different fluorophores, termed donor and acceptor) represents an extensively used approach for studying GPCR oligomerization in live cell membranes.⁴⁹ The advantages of hetero-FRET include measurement in live cells and a high spatial resolution. Due to these reasons and the easy availability of commercial fluorescence microscopes with FRET accessory and software, hetero-FRET enjoys a high level of popularity in GPCR oligomerization studies.

However, hetero-FRET measurements are often associated with a number of inherent complications and unless special care is taken to avoid these problems, the results from such studies could be misleading. FRET takes place between two fluorophores *only* when their distance of separation is typically $\sim 2\text{--}10\text{ nm}$.^{61,62} Representative values of Förster distances (i.e., the distance between the donor and acceptor that results in 50% energy transfer efficiency, R_0) for biologically relevant donor–acceptor pairs are shown in Table 1. Given this range of R_0 , what would be typical distances ideally measured by FRET? FRET efficiency varies as the sixth power of the distance between two fluorophores (donor and acceptor), and this dependence is illustrated in Figure 1. The figure shows the efficiency of energy transfer as a function of distance. FRET efficiency is high for donor–acceptor distances $< R_0$. Due to the sixth power

Table 1. Representative Förster Distances for FRET between Fluorophore Pairs Commonly Used in Biology

donor	acceptor	Förster distance ($R_0, \text{ Å}$) ^a
tryptophan	dansyl	21 ⁶²
tryptophan	dehydroergosterol	16 ⁶³
Cy3	Cy5	54 ⁶⁴
BODIPY	BODIPY	57 ⁶⁵
ECFP	EGFP	48 ⁶⁶
ECFP	EYFP	49 ⁶⁶
EGFP	EYFP	56 ⁶⁶
EGFP	EGFP	47 ⁶⁶
EYFP	EYFP	51 ⁶⁶
GFP	Cy3	63 ⁶⁷
YFP	BODIPY	52 ⁶⁸

^aValues from refs 62–68.

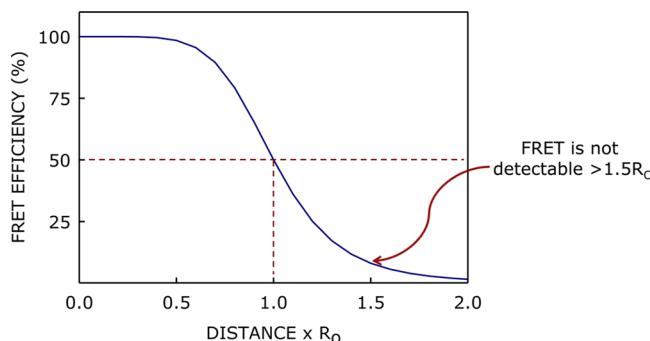


Figure 1. Dependence of FRET efficiency on distance between donor and acceptor. FRET varies with the sixth power of distance between donor and acceptor. As a result, FRET efficiency drops steeply with increasing distance. The Förster distance (R_0) is defined as the distance where FRET efficiency is 50%. FRET is negligible and hardly detectable when distance between the fluorophores is $>1.5R_0$.

dependence, FRET efficiency sharply drops with increasing distance. FRET therefore becomes increasingly negligible when distance between the fluorophores is increased beyond $1.5R_0$. This means that if the R_0 value for a given donor–acceptor pair is 40 Å, the limit of distance measurable using FRET would be at most 60 Å. This point is often not appreciated in measurements of GPCR oligomerization using FRET, especially keeping in mind the size of the GFP (green fluorescent protein, MW $\sim 27\text{ kDa}$)⁶⁹ tag itself. A practical issue in hetero-FRET measurements is the so-called “bleed-through” problem.⁶¹ This is usually manifested by the emission of one fluorophore being detected in the photomultiplier channel for the second fluorophore (due to the very broad bandwidths and asymmetrical spectral profiles). An important aspect is the orientation and local motion of the bulky GFP tags. This assumes importance in situations where the GFP tag is attached to the carboxy-terminal of GPCRs with varying lengths of the C-terminal. If the C-terminal end is very long for a GPCR (as in the case of the members of the serotonin₂ receptor family), the possibility of FRET could be reduced due to conformational flexibility of the C-terminal end. In cases like these, GFP probes attached to a pair of GPCRs may not detect FRET, although the GPCRs could actually oligomerize in the membrane. It should therefore be noted that absence of FRET in such cases does not rule out receptor oligomerization. Another concern arises from the definition of distance between the acceptor and donor. It is not clear whether distances between the van der Waals boundaries or transition dipoles will apply for

calculation of distance separating fluorophores. These considerations should be kept in mind while interpreting FRET measurements using fluorescently tagged GPCRs in the cellular environment.

Hetero-FRET measurements are performed utilizing two different fluorophores with sufficient spectral overlap. A major factor in hetero-FRET measurements in cells is the concentration of fluorophores (donors and acceptors). This problem arises due to the use of receptors conjugated to two different fluorophores, and the lack of control in their relative expression levels. In the case of heterologously expressed proteins, the expression levels of the tagged proteins may vary, making intensity-based hetero-FRET measurements difficult to interpret. The effect of overexpression of fluorescently tagged receptors on FRET efficiency was addressed by Meyer et al.⁷⁰ and is shown in Figure 2. The figure shows that, at physiological

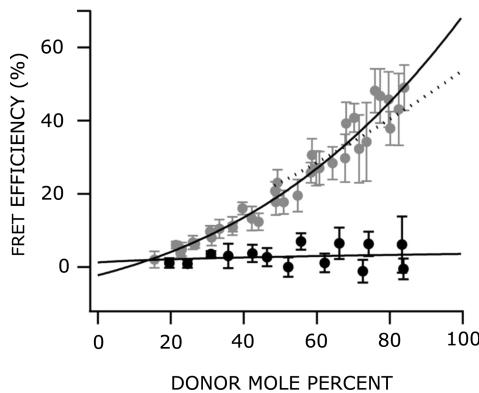


Figure 2. Effect of receptor overexpression on FRET efficiency. FRET efficiency as a function of donor concentration in HEK293 cells expressing neurokinin-1 receptors fluorescently labeled with Cy3 and Cy5 fluorophores is shown. The apparent FRET efficiency was plotted against donor concentration for cells expressing different receptor concentrations. At higher level of the receptor ($\sim 63\,000$ receptors/cell, shown in gray), donor concentration-dependent FRET was detected indicating “apparent” oligomerization of the receptor. The lower plot shows that there is no significant FRET at any donor concentrations for cells expressing lower level of the receptor ($\sim 25\,000$ receptors/cell, shown in black), indicating that the receptor is monomeric under these conditions. The figure clearly brings out the point that control of expression level is crucial for deriving physiologically meaningful conclusions about receptor oligomerization. Adapted and modified from ref 70 with permission (copyright (2006) National Academy of Sciences, U.S.A.).

level of expression ($\sim 25\,000$ neurokinin-1 receptors/cell), no FRET was detected even at high donor concentration. This indicates that the receptor is monomeric under these conditions. In contrast, considerable FRET was detected and FRET efficiency displayed an increase with increasing donor concentration for cells with higher expression level of the same receptor ($\sim 63\,000$ receptors/cell). A fit of this data yielded an apparent aggregation number of ~ 4 (indicating tetrameric receptors).⁷⁰ Such concentration-dependent oligomerization of tyrosine kinase receptors has also been reported.⁷¹ It is therefore important to carry out receptor oligomerization hetero-FRET experiments under physiological conditions with lower levels of receptor expression, since higher levels of expression could lead to misleading conclusions as shown in the above examples.

The origin of this type of anomalous FRET has recently been attributed to the phenomenon of “bystander” FRET.⁷² Bystander

FRET arises from membrane proteins that do not interact, but still give rise to FRET since they happen to be within the required distance for energy transfer (see Figure 3). This issue assumes relevance in view of the highly crowded nature of the cell membrane⁷³ and high levels of expression of fluorescently tagged receptors. FRET from such noninteracting (bystander) pairs complicates the interpretation of FRET results. Recently, an experimentally verified theoretical framework has been reported for correcting such bystander FRET.⁷⁴ According to this framework, a 20% bystander FRET efficiency corresponds to an acceptor density of $2000\text{ molecules}/\mu\text{m}^2$. Given that the surface area of cells is between 1000 and $5000\ \mu\text{m}^2$, this density corresponds to an expression level of $2\text{--}10 \times 10^6$ receptors/cell. Such high levels of expression would lead to complications due to bystander FRET and should be avoided. In general, cells expressing $\sim 10\,000\text{--}50\,000$ receptors/cell would be physiologically relevant to monitor constitutive oligomerization of GPCRs,⁷⁵ considering the typical cellular surface area to be $\sim 2500\ \mu\text{m}^2$.⁷⁶ It is possible to differentiate “bystander” FRET from FRET due to receptor oligomerization by measuring FRET signal as a function of donor/acceptor ratio without changing the receptor expression level.⁷⁷ This could be achieved by photo-bleaching the acceptor, without changing the expression of the donor. For a nonrandom distribution (i.e., where receptors are present as oligomers), FRET efficiency would depend on the donor/acceptor ratio, while the expression of receptor is constant. Photo-bleaching of acceptor is a better way to modulate donor/acceptor ratio rather than increasing the cellular expression of donor containing receptor.

Homo-FRET: A Convenient Tool for Detecting GPCR Oligomerization.

The above limitations have restricted the usefulness of hetero-FRET in monitoring GPCR oligomerization. In contrast to hetero-FRET, homo-FRET represents a superior approach. Homo-FRET is a simpler variant of energy transfer, since it takes place between identical fluorophores and therefore requires only a single type of fluorophore. Homo-FRET, like hetero-FRET, depends on the inverse sixth power of separation between interacting fluorophores on the nanometer scale and is therefore sensitive to receptor oligomerization. The excitation and emission spectra of fluorophores exhibiting homo-FRET should have considerable overlap. Fluorophores with relatively small Stokes’ shift therefore have a greater probability of homo-FRET. Homo-FRET gets manifested by reduction in fluorescence anisotropy, a parameter largely independent of the concentration of fluorophores.⁷⁸ The probability of homo-FRET in a membrane is related to the surface density (molecules/unit area) of the fluorophore and the square of Förster distance.⁷⁹ Another disadvantage of hetero-FRET measurements is the lack of ability to distinguish dimers from higher-order oligomers. This is important, particularly in a microheterogeneous environment such as the membrane, where multiple types of oligomeric clusters can coexist. Fortunately, homo-FRET measurements can provide an estimate of higher-order oligomerization.^{55,80,81}

Application of Homo-FRET to Monitor Oligomerization of the Serotonin_{1A} Receptor. In view of the advantages enjoyed by the homo-FRET approach, this approach was utilized to explore the oligomerization of the serotonin_{1A} receptor, a representative GPCR which acts as a drug target for disorders related to anxiety and depression.⁵⁵ Homo-FRET was quantitated from the increase in fluorescence anisotropy upon progressive photo-bleaching of the receptor, in which fluorescence depolarization due to energy transfer was prevented by photo-bleaching of fluorophores.⁸² Photo-bleaching results in

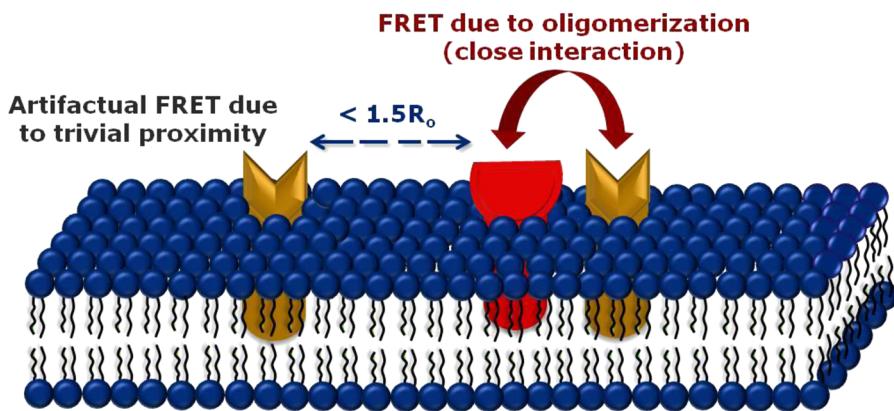


Figure 3. Schematic representation of bystander artifactual FRET in crowded cellular membranes. A bystander (noninteracting but closely located) protein, whose distance of separation is $< 1.5R_0$ could participate in FRET with the protein of interest in membranes with high receptor concentration. The FRET signal arising in such a scenario does not exclusively contain information on oligomerization and therefore is misleading. The contribution of such artifactual FRET would be as high as $\sim 30\%$ when, for example, receptors are expressed at a concentration $> 500\,000$ receptors/cell.⁷¹ Adapted and modified from ref 72.

depletion of fluorophore concentration, thereby reducing FRET, resulting in an increase in anisotropy. Overall, anisotropy displays an increase with progressive photobleaching of the fluorophore. The initial anisotropy of serotonin_{1A}R-EYFP (serotonin_{1A} receptor tagged to EYFP) in control cells was significantly low (~ 0.22) compared to the fundamental anisotropy (r_0) of EYFP (0.38)⁸³ (see Figure 4a). The observed depolarization of emission is attributed to energy transfer (homo-FRET) between receptor oligomers. Figure 4a shows that there is a steady increase in fluorescence anisotropy of serotonin_{1A}R-EYFP with progressive photobleaching, typical for a system undergoing homo-FRET.

The difference between the extrapolated and predicted (0.38) anisotropy value at 100% photobleaching could be used for predicting the oligomeric state.⁸¹ The larger the difference, greater the fraction of higher-order oligomers. Figure 4b shows the theoretically predicted fluorescence anisotropy under progressive photobleaching for an unbiased distribution of monomers, dimers, trimers, and tetramers. However, achieving very high degree of photobleaching is experimentally difficult (due to low signal/noise ratio under high photobleaching conditions). The linearly extrapolated anisotropy was therefore compared with the predicted anisotropy to comment on the presence of higher-order oligomers (Figure 4b). The presence of constitutive oligomers of the serotonin_{1A} receptor was proposed based on the difference between predicted and extrapolated anisotropy (Figure 4c) upon progressive photobleaching of serotonin_{1A}R-EYFP.⁵⁵ This approach was utilized to monitor the effect of membrane cholesterol, actin cytoskeleton destabilization, and ligand stimulation on the oligomerization of the serotonin_{1A} receptor. The initial anisotropy was reduced in the presence of serotonin (agonist) and cytochalasin D (CD, actin destabilizing agent) (Figure 4a), and led to an increase in the difference between extrapolated and predicted anisotropy relative to control (Figure 4c). These results imply the presence of higher-order oligomers under these conditions. On the other hand, acute cholesterol depletion (by methyl- β -cyclodextrin (M β CD)) and treatment with antagonist (*p*-MPPI) resulted in an increase in initial anisotropy, leading to reduction in the difference between extrapolated and predicted anisotropy. In other words, antagonist treatment and cholesterol depletion led to a reduction in the population of higher-order oligomers. These results bring out the power of homo-FRET, combined with

photobleaching, in assessing oligomerization of GPCRs such as the serotonin_{1A} receptor, and could be extended to other GPCRs.

CONCLUSIONS AND THE ROAD AHEAD

A central theme of this Review is that although FRET is a well established technique for *in vitro* applications and soluble proteins, certain inherent complexities are encountered in its application to study GPCR oligomerization in cell membranes. Based on FRET photophysics, we have described some of the caveats that could complicate interpretation of FRET results. We recommend that appropriate caution should be exercised during execution and interpretation of FRET experiments designed to determine GPCR oligomerization. Homo-FRET appears to possess distinct advantages in this respect.

GPCRs are key signaling molecules in higher eukaryotes. Interestingly, although GPCRs represent the most predominant therapeutic targets, a large fraction of the GPCR receptorome is still unexplored from both basic biology and drug discovery perspectives.⁸⁴ It is estimated that close to 150 GPCRs are orphan receptors whose endogenous ligands and functions are not yet known. These orphan receptors would be very useful in future drug discovery efforts. GPCR oligomerization and cross-talk add yet another dimension to this process. The exciting possibility of homo- and hetero-oligomerization of GPCRs provides tremendous diversity and potential to future drug discovery. The recent report on the modulation of the dimeric interface in GPCR oligomers by membrane cholesterol offers a new perspective in this context.⁵⁷ In this overall context, the need for developing novel approaches for monitoring GPCR oligomerization under physiological conditions assumes relevance. Knowledge of receptor oligomerization state under various pathophysiological conditions is of greater significance in the pharmacology of GPCRs since oligomerization gives rise to pharmacological diversity,⁸⁵ opening new avenues for therapeutics. We envision that, with progress in knowledge on receptor oligomerization using better tools, our overall understanding of GPCR function and pharmacology would improve significantly, thereby enhancing our ability to design better therapeutic strategies.

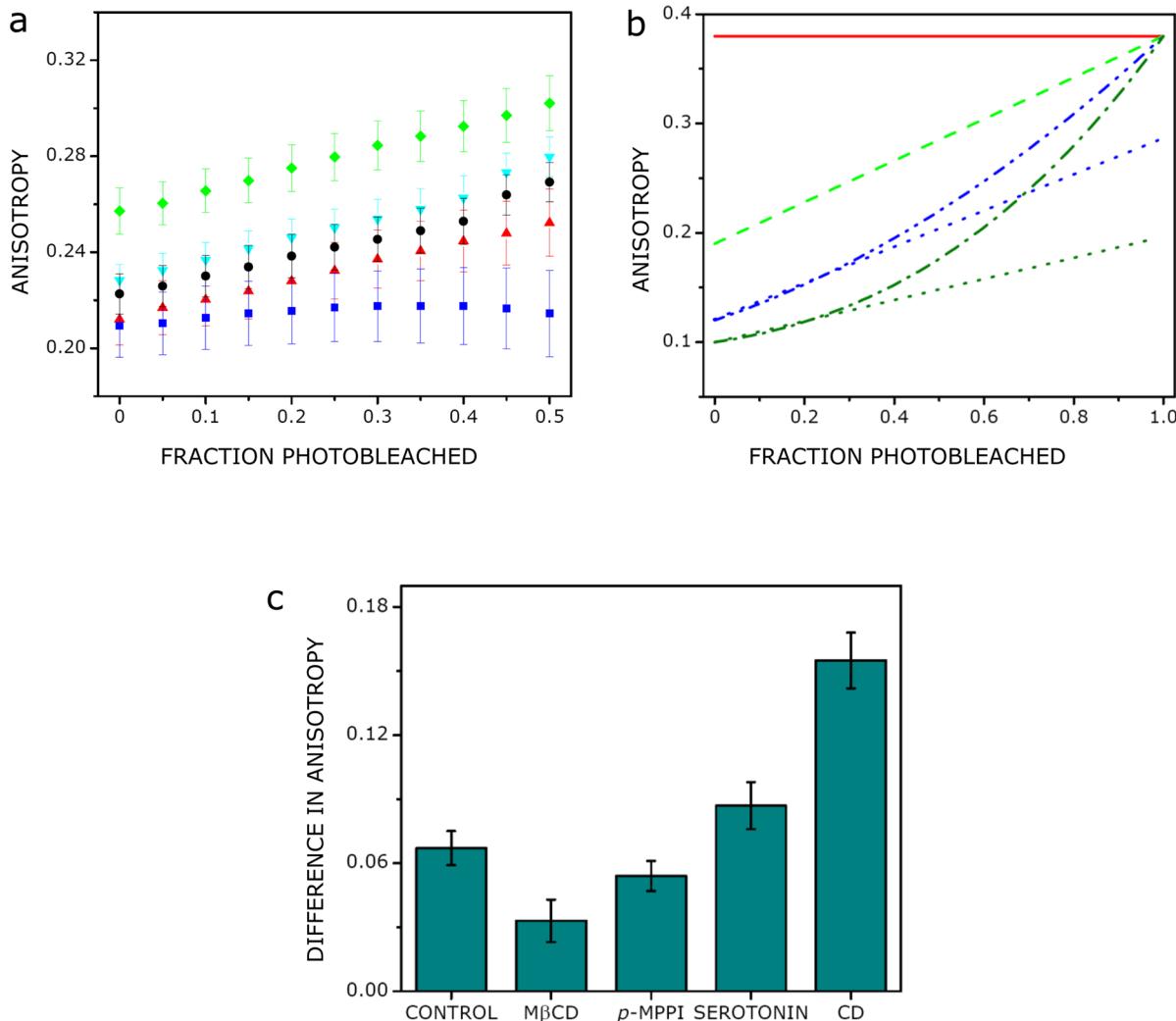


Figure 4. GPCR oligomerization utilizing homo-FRET. (a) Oligomerization of the serotonin_{1A} receptor was assessed by enhancement in fluorescence anisotropy upon photobleaching serotonin_{1A} receptors tagged to EYFP (serotonin_{1A}R-EYFP) in various conditions. The anisotropies of serotonin_{1A}R-EYFP are plotted for control cells (black, ●), cells treated with the agonist serotonin (red, ▲), the antagonist p-MPPI (cyan, ▼), cytochalasin D (blue, ■), and M β CD (green, ◆) upon photobleaching. M β CD and cytochalasin D (CD) were used to deplete membrane cholesterol and destabilize actin cytoskeleton, respectively. (b) Simulated enhancement of fluorescence anisotropy upon progressive photobleaching for a population of unbiased distribution of oligomers containing N subunits. N values correspond to 1 (monomer, red solid line), 2 (dimer, light green dashed line), 3 (trimer, blue dash-dot-dotted line), and 4 (tetramer, deep green dash-dotted line). Simulation was carried out using a formalism previously developed by Yeow and Clayton.⁸¹ In addition, the extrapolated anisotropies derived from initial points corresponding to fractional bleaching (<0.3) in the case of trimer and tetramer are shown (green dotted line). (c) Difference between the extrapolated anisotropy (extrapolated to complete photobleaching) and the predicted anisotropy of serotonin_{1A}R-EYFP. The extrapolated anisotropy is estimated from a linear fit of the photobleaching data shown in panel (a). The magnitude of deviation offers a measure of the level of oligomerization. Adapted and modified from ref 55. See text for other details.

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Notes

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