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Single-molecule imaging revealed dynamic GPCR dimerization

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Single fluorescent-molecule video imaging and tracking in living cells are revolutionizing our understanding of molecular interactions in the plasma membrane and intracellular membrane systems. They have revealed that molecular interactions occur surprisingly dynamically on much shorter time scales ($\ll$1 s) than those expected from the results by conventional techniques, such as pull-down assays (minutes to hours). Single-molecule imaging has unequivocally showed that G-protein-coupled receptors (GPCRs) undergo dynamic equilibrium between monomers and dimers, by enabling the determination of the 2D monomer–dimer equilibrium constant, the dimer dissociation rate constant (typically $\sim$10 s^{-1}), and the formation rate constant. Within one second, GPCRs typically undergo several cycles of monomer and homo-dimer formation with different partners.

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Introduction

Despite the importance of G-protein-coupled receptors (GPCRs) in physiology, pharmacology, and medicine, GPCR researchers had been, and at certain levels still are, split into two camps; that is, the monomer camp and the dimer camp. Many GPCR homo-dimer and hetero-dimer descriptions have been published since the first report on this subject [1], thus forming the dimer camp [2–8]. However, the strengths of evidence for dimer formation in living cells vary greatly, depending on the employed method and experimental paradigm.

Meanwhile, many researchers reported that GPCR monomers are functional [6,8–11], forming the monomer camp. As pointed out by researchers in the monomer camp, many GPCR dimers reported in the literature might actually be artifacts due to overexpression, particularly in the case of the class-A GPCRs [12,13].

In recent years, the debate has focused on whether class-A GPCRs, which represent the majority of GPCRs ($\sim$85% in human genome), are organized into homo-dimers or even hetero-dimers or greater oligomers in the plasma membrane and whether class-A GPCR dimers, if they exist, play important roles in GPCR functions [6,8]. The important results reported since 2002 are summarized in Table 1a. Notably, Schertler and his colleagues were very careful about making any statements on GPCR monomers and dimers based on their crystallographic results [14••,15].

Single fluorescent-molecule video imaging is probably the most suitable method for determining whether GPCRs form dimers, and, if they do, how long they last before dissociating into monomers. With this technique, in ideal cases, we can track all fluorescent GPCR molecules in the view field as they jostle around in the plasma membrane, collide with each other, and perhaps bind to each other and diffuse together for some time until they become separated. In this article, we hope to show that the application of single fluorescent-molecule video imaging is now revolutionizing our views of the interactions of GPCRs with each other and with other signaling molecules.

Typical single fluorescent-molecule video imaging of a GPCR

To conduct single fluorescent-molecule imaging for detecting membrane protein dimer formation (and dissociation), ideally each and every molecule is labeled with a fluorescent dye molecule, precisely at a 1:1 molar ratio (not simply an average dye-to-protein ratio of 1). This has been accomplished previously [16••]. The use of mGFP and other fluorescent proteins will not achieve these conditions, because only some fractions of these tag proteins become fluorescent, although the tag protein is fused to the target protein at a precise 1:1 molar ratio [17••]. In these cases, the researcher must determine the actual percentages of the fluorescent fusion proteins [17••] (also see Figure 5 and its related text in [18]).

First, we present the results of single fluorescent-molecule imaging of a GPCR, *N*-formyl peptide receptor (FPR), a chemoattractant class-A GPCR, in living CHO cells, which do not express endogenous FPR, at

Table 1

Dimers and greater oligomers of GPCRs reported since 2002.

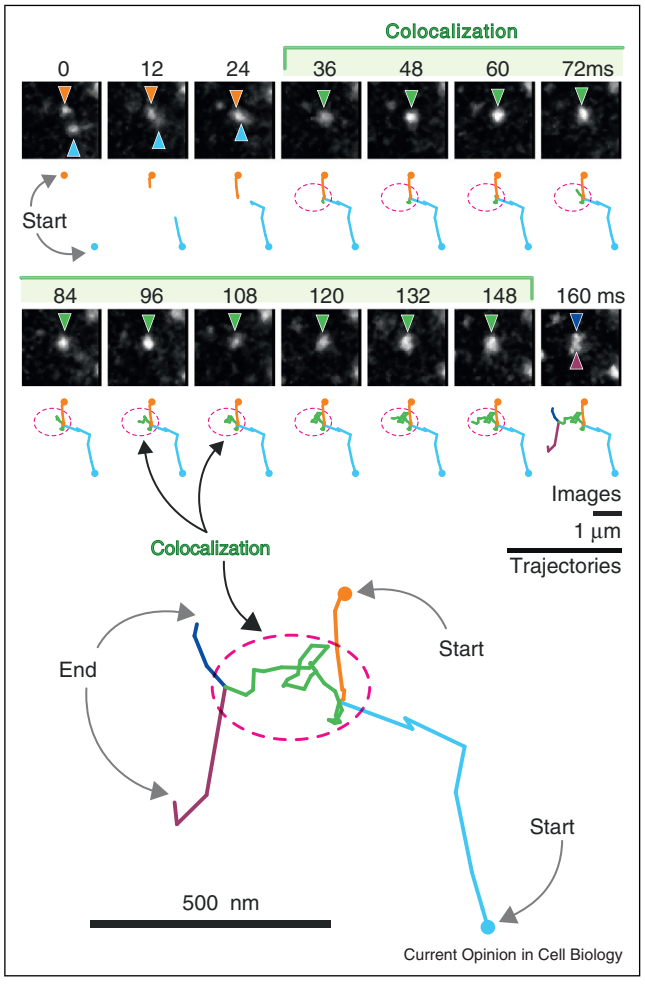
Molecule	Monomer	Homo-dimer	Hetero-dimer	*T* (°C)	Environment	Observation methods	Remarks	Ref.
(a) Results obtained by conventional many-molecule experiments								
Class A								
β1 adrenergic aeceptor	No	Yes	DU	NA	Crystal	Crystallography	Possibly linear aggregates. Crystal but in a lipid membrane-like environment consisting of synthetic lipids	[38•]
	Yes	Yes	Yes (β2-AR)	DU	Live cell	BRET	At 1-100x higher concentrations than physiological levels	[48]
β2 adrenergic aeceptor	Yes	Yes	DU	25	Reconstituted membrane	FRET	Predominant homo-tetramers found.	[42]
	Yes	Never at any expression levels	DU	DU	Live cell	BRET	No homo-tetramers found	[12]
	Yes	Yes	Yes (β1-AR)	DU	Live cell	BRET		[48]
	Yes	Yes	DU	DU	Live cell	Time-resolved FRET		[7]
	No	Yes (very long)	DU	23	Live cell	FCS	No homo-tetramers found	[45]
α1b adrenergic aeceptor	No	Yes (very long)	DU	23	Live cell	FCS	No homo-tetramers found	[45]
5-HT_{2C} serotonin receptor	No	Yes (constitutive)	DU	23	Live cell	FCS	No homo-tetramers found	[46]
M1 muscarinic receptor	No	Yes (very long)	DU	23	Live cell	FCS	No homo-tetramers found	[45]
M2 muscarinic receptor	No	Yes (very long)	DU	23	Live cell	FCS	No homo-tetramers found	[45]
CXCR4	DU	Yes (constitutive)	Yes (CCR2) (constitutive)	DU	Live cell	BRET		[49]
CCR2	DU	Yes (constitutive)	Yes (CXCR4) (constitutive)	DU	Live cell	BRET		[49]
A1 Adenosine receptor	Yes	Yes	DU	DU	Live cell	Time-resolved FRET		[7]
A2A Adenosine receptor	DU	DU	Yes (D2R) (probably constitutive)	DU	Fixed cell for FRET, Live cell for BRET	BRET and FRET		[40]
	Yes	Yes (dominant, probably constitutive)	Yes (D2R)	DU	Fixed cell for FRET, Live cell for BRET	BRET, FRET and Time-resolved FRET		[39]
D1 dopamine receptor	No	Yes (very long)	DU	23	Live cell	FCS	No homo-tetramers	[45]
D2 dopamine receptor	DU	Yes (constitutive)	DU	NA	Electrophoresis	Crosslinking and electrophoresis	At least dimers	[43]
	DU	Yes	No	DU	Live cell	BiFC, bimolecular luminescence complementation, and chemical crosslinking		[44]
	DU	DU	Yes (adenosine A2A) (probably constitutive)	DU	Fixed cell for FRET, Live cell for BRET	BRET and FRET		[40]
MT1 melatonin receptor	DU	Yes	Yes (MT2R)	23-24	Live cell	IP and BRET		[47]
Rhodopsin	Yes	Yes	DU	NA	Detergent complex	Negative staining EM with single particle analysis	A dimer interacts with a single transducin molecule	[25•]
V2 vasopressin receptor	Yes	Yes	DU	DU	Live cell	Time-resolved FRET		[7]
V1a vasopressin receptor	Yes	Yes	DU	DU	Live cell	Time-resolved FRET		[7]

Table 1 (*Continued*)

Molecule	Monomer	Homo-dimer	Hetero-dimer	*T* (°C)	Environment	Observation methods	Remarks	Ref.
Thrombin receptor (protease activated receptor 1)	Yes	Yes	DU	DU	Live cell	Time-resolved FRET		[7]
Class B/C								
Pituitary adenylyl cyclase activating polypeptide receptor (class B)	DU	Yes	DU	DU	Live cell	Time-resolved FRET		[7]
$GABA_{B1}$ receptor (Class C)	DU	DU	Yes ($GABA_{B2}R$)	DU	Live cell	Time-resolved FRET	Hetero-tetramers ($GABA_{B1/2}R$ + $GABA_{B1/2}R$) found	[7]
	DU	DU	Yes ($GABA_{B2}R$) (stable)	DU	Live cell	Time-resolved FRET	Hetero-tetramers ($GABA_{B1/2}R$ + $GABA_{B1/2}R$) found	[41]
$GABA_{B2}$ receptor (Class C)	DU	Yes	Yes ($GABA_{B1}R$)	DU	Live cell	Time-resolved FRET	Hetero-tetramers ($GABA_{B1/2}R$ + $GABA_{B1/2}R$) found	[7]
	DU	DU	Yes ($GABA_{B1}R$) (stable)	DU	Live cell	Time-resolved FRET	Hetero-tetramers ($GABA_{B1/2}R$ + $GABA_{B1/2}R$) found	[41]
metabotropic glutamate receptor 1 (mGluR1) (class C)	DU	Yes (constitutive)	DU	DU	Live cell	Time-resolved FRET	No higher-order oligomers found	[7]
(b) Results obtained by single fluorescent-molecule video imaging								
Class A								
β1 adrenergic receptor	Yes	Yes (5.1 s)	DU	20.5	Live cell	SFMI	Homo-tetramers found	[20••]
β2 adrenergic receptor	Yes	Yes (4.6 s)	DU	20.5	Live cell	SFMI	Homo-tetramers found	[20••]
M1 muscarinic receptor	Yes	Yes (0.7 s)	No	23	Live cell	SFMI		[19]
N-formyl peptide receptor	Yes	Yes (0.1 s)	NA	37	Live cell	SFMI		[17••]
Class B/C								
$GABA_{B1}$ receptor (Class C)	No	Yes	Yes ($GABA_{B2}R$)	20.5	Live cell	SFMI	Predominantly hetero 2–8mers found	[20••]
$GABA_{B2}$ receptor (Class C)	Yes	Yes	Yes ($GABA_{B1}R$)	20.5	Live cell	SFMI	Monomers ~ homo-tetramers found	[20••]

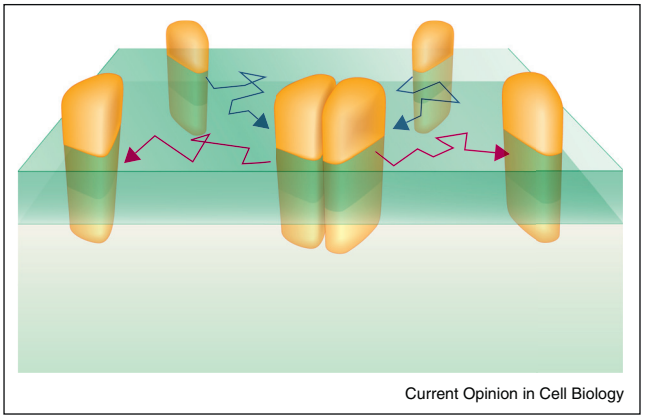
Abbreviations: Yes, detected; No, not detected; DU, description unfound in the paper; NA, not applicable; BRET, bioluminescence resonance energy transfer; FRET, Förster (fluorescence) resonance energy transfer; FCS, fluorescence correlation spectroscopy; BiFC, bimolecular fluorescence complementation; IP, immunoprecipitation.

Figure 1



FPR dimers continually form and disintegrate dynamically. A typical image sequence (recorded at a 4-ms resolution, shown every 12 ms [every third frame]), showing two diffusing D71A-FPR (bound by Alexa488-formyl peptide) molecules and their trajectories. Two molecules (orange and blue arrowheads and trajectories) first became colocalized in the fourth frame (36 ms), diffused together for ~120 ms (indicated as colocalization; see green trajectories), and then separated into monomers (dark blue and purple arrowheads and trajectories).

Figure 2



Schematic model, showing dynamic equilibrium of the class-A GPCR between monomers and dimers. Class-A GPCRs continually form dimers and disintegrate into monomers. In the case of FPR, the equilibrium has been fully characterized: a 2D equilibrium constant of 3.6 copies/μm^2; dissociation and 2D association rate constants of 11.0 s^{-1} and 3.1 [copies/μm^2]$^{-1}s^{-1}$, respectively. Ligand binding did not affect the dynamic monomer–dimer equilibrium.

37 °C [17••]. FPR was fluorescently labeled with the formyl peptide (agonist) conjugated with the fluorescent dye Alexa594 at a precise 1:1 ratio, and the ligands bound to FPR molecules located on the bottom cell surface were observed at the level of single molecules, using TIRF illumination. Under standard conditions with 6 nM fluorescent formyl peptide in the medium, 73% of the cell surface FPR molecules were bound by the fluorescent ligand, as determined by a 2D–3D Scatchard plot, based on single-molecule images. To avoid agonist-induced concentration of FPR in the clathrin-coated structures, a non-internalizable D71A-FPR mutant was employed (D71A; separate experiments confirmed that the dimerization properties are not affected by this mutation or by agonist binding).

The still images in Figure 1 display a typical image sequence, recorded at a rate of every 4 ms (250 Hz, ~8 times faster than normal video rate), showing that D71A molecules undergo diffusion and frequent colocalization and codiffusion with other D71A molecules. The trajectories are also shown in Figure 1. Each colocalization–codiffusion event often lasts longer than the incidental approaches, followed by separation into monomers. Virtually all of the D71A molecules underwent diffusion as well as frequent transient colocalization and dissociation throughout the plasma membrane.

These results clearly illustrated that the monomer and dimer camps were both right and wrong. The GPCRs appear to rapidly interconvert between monomers and dimers (Figure 2). Whether monomers and/or dimers are related to GPCR functions, and how they are linked to their functions, will be addressed in the next stage of the research.

Each time a colocalization event was found, its duration was measured at a time resolution of 4 ms, and the distribution of colocalization durations was obtained. The distribution could be fitted by a single exponential decaying function, providing a lifetime of 91 ms, after correction for the photobleaching lifetime and subtraction of the incidental colocalization lifetime. These results clearly showed that FPR exists in very dynamic equilibrium between monomers and dimers, with a dimer lifetime of 91 ms.

Single fluorescent-molecule video imaging studies of class-A GPCR dimers

Historically, the single GPCR-molecule imaging result was first published by Hern *et al.* [19] with M1 muscarinic acetylcholine receptor, a class-A GPCR, as the experimental paradigm. Using two-color single molecule imaging, where the receptor molecules were labeled with a fluorescent antagonist covalently conjugated with green and orange dyes, they clearly showed that the M1 muscarinic receptor molecules form dimers. More importantly, they found that the receptor dimers quickly dissociate into monomers, in a matter of 0.7 s, at 23 °C. This result suggested that GPCR monomers and dimers are in equilibrium in live cells, although the temperature was lower and dimer formation must have been enhanced. Whether the M1 receptor forms dimers in the absence of the antagonist and/or after the antagonist binds is unknown.

Kasai *et al.* [17••] were the first to fully characterize the monomer–dimer dynamic equilibrium of a GPCR (in fact, the first for any membrane molecules), providing the 2D equilibrium constant as well as the association and dissociation rate constants. For this study, they developed a single molecule methodology, termed superquantification, to determine these values. FPR was employed as an experimental paradigm (Figure 1) and observations were performed at 37 °C.

Both before and after ligation, the dimer–monomer 2D equilibrium remained unchanged, giving an equilibrium constant of 3.6 copies/μm^2, with dissociation and 2D association rate constants of 11.0 s^{-1} and 3.1 [copies/μm^2]$^{-1}s^{-1}$, respectively. At physiological expression levels of ~2.1 FPR copies/μm^2 (in neutrophils, ~6000 copies/cell), these parameters revealed that monomers continually convert into dimers every 150 ms, dimers dissociate into monomers in 91 ms (exponential lifetime), and at any moment, 2500 and 3500 receptor molecules participate in transient dimers and monomers, respectively. Within one second, FPR molecules undergo 4 cycles of repeated monomer and homo-dimer formation with different partners.

Although obtaining the monomer–dimer equilibrium constant is difficult, it is important because firstly, the very acquisition of the equilibrium constant represents the eventual proof for the dynamic dimer–monomer equilibrium in biologically meaningful time scales, secondly, different GPCR species can be compared in terms of the monomer–dimer interconversion without the influence of the expression levels, and thirdly the actual numbers (or number densities) of monomers and dimers (at any given instance) can be calculated from the number density of the GPCR expressed in the plasma membrane. As such, the equilibrium and expression levels can be separated, with the knowledge of the equilibrium constant. Note that even under conditions (of higher GPCR expression levels) where the dimer population dominates, the dimer lifetime will remain the same as that under the conditions where dimers occur rarely (low expression conditions). Under high expression conditions, only the monomer lifetime will be shortened.

Longer dimer lifetimes of adrenergic receptors?

These single-molecule studies were followed by an investigation by Calebiro *et al.* [20••], using other class-A GPCRs, β1-adrenergic and β2-adrenergic receptors (SNAP-tagged), in live cells at the rather low temperature of 20.5 °C. They found that both β1-receptors and β2-receptors form transient homo-dimers (hetero-dimers were not examined) with lifetimes on the order of ~4 s. This value is about 40 times longer than that of FPR (37 °C) and about 6 times longer than that of M1 receptor (23 °C). This might be caused by the lower temperature employed in this study and/or induced by the quite different molecular interactions in the dimeric state of adrenergic receptors.

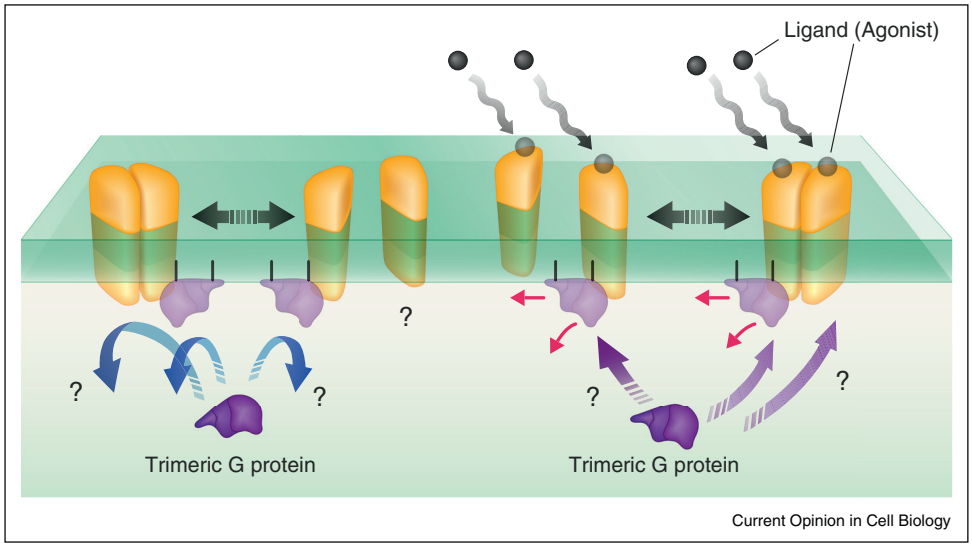
Interestingly, β2 receptors exhibited an apparently greater tendency to form dimers than β1 receptors. Since the dimer lifetimes for these receptors are similar to each other, the authors argued that this difference in the dimerization tendencies might result from factors such as different efficiencies in converting a 'collision' (collision frequency is simply determined by the diffusion coefficient and local concentration of molecules) into an interaction, distinct interactions with other proteins capable of interfering with dimerization, or localizations in different microdomains in the plasma membrane (such as those found in cardiomyocytes [21], in addition to the molecular differences between these two receptors. Agonist stimulation did not alter receptor dimerization.

Dynamic monomer–dimer interconversion is probably a general property of class-A GPCRs

As described, four class-A GPCRs, M1 receptor, FPR, and β1-adrenergic and β2-adrenergic receptors, were found to undergo dynamic equilibrium between monomers and homo-dimers in live cells (Table 1b). In addition, the class-A GPCR neurokinin-1 receptor is likely to undergo monomer–dimer equilibrium [13]. Taken together, all of the class-A GPCRs probably undergo dynamic equilibrium between monomers and homo-dimers. It follows then that, although GPCRs changed almost all of their amino acids through evolution, they maintained their physical property of forming transient homo-dimers, in addition to the seven membrane spanning structure. Therefore, dynamic homo-dimers must be crucial for some GPCR functions.

However, the downstream signaling by the binding of trimeric G proteins under resting and activated conditions (Figure 3), and the downregulation of activated GPCRs

Figure 3



Schematic model, showing possible downstream signaling by (transient) interactions of the trimeric G protein with transient monomers and dimers of GPCRs in dynamic equilibrium, both before and after agonist binding. The activation of trimeric G proteins by the GPCR may occur by way of GPCR monomers and/or dimers, under resting (left) and/or activated (right) conditions. The interaction of trimeric G proteins with GPCRs might occur transiently, on time scales shorter than the lifetimes of GPCR monomers and/or dimers. It may proceed with or without G-protein dissociation into α and $\beta\gamma$ subunits (arc-like blue arrows and purple and red arrows, respectively). The latter event may happen even with non-activated GPCRs, because the GPCRs' downstream signals occur constitutively, although their intensities are low. How the downstream signaling is linked to the dynamic GPCR monomer–dimer equilibrium will be the next key issue to resolve in the near future. Similarly, it will be extremely interesting to elucidate the down-regulation of activated GPCRs by GPCR kinases and the subsequent binding of β-arrestin, which might be differentially induced or even blocked by GPCR monomers or dimers. These interesting issues, which are difficult to clarify by conventional methods, will be effectively addressed by single fluorescent-molecule video imaging.

by GPCR kinases and the subsequent binding of β-arrestin, might be differentially induced or even blocked by monomers or dimers [22–24,25•,26] (note that these signaling events might also occur in endosomes [27••,28]). These questions should be answered, and could be addressed well by single fluorescent-molecule imaging of GPCRs and their downstream molecules.

Five single fluorescent-molecule imaging methods for detecting molecular binding and interaction

The main problem with the single-molecule imaging method is the signal-to-noise (background) ratio of the observed spots of single molecules. It is generally less than 3 (but greater than 2). Most of the background comes from cellular autofluorescence or the fluorescent molecules added to the cell (the concentration of non-bound fluorescent molecules should generally be lower than 20 nM). Another problem is the fluctuation of the background signal, which often appears like flickering weak-intensity (or somewhat broadened) single-molecule spots. Thus, single fluorescent molecules are generally on the verge of detection. Therefore, if molecular binding is found by more than one method, the result would become more reliable. Furthermore, depending on the expression levels, background, and molecules, the suitable methods will differ.

Detection of colocalization of two single molecules for two frames or longer

(Co-diffusion of two molecules would be better: if two molecules are diffusing and are still colocalized in several or many consecutive image frames, the results become more trustworthy [16••,17••,29]). For determination of single-color colocalization, see Kasai *et al.* [17••]. For two-color single-molecule colocalization, see [16••,17••,19,30]. Both the single-color and two-color methods have similar accuracies [17••].

FRET detection at the level of single molecules

If FRET is detected between two single molecules for several frames, with decreased (increased) signal intensity of the donor (acceptor) spot (called anticorrelation), it is likely that these two molecules are located within the Förster distance, which is typically ~5 nm; that is, they are bound to each other [16••,31]. However, the failure of FRET detection does not mean the absence of protein binding. FRET is extremely sensitive to the donor–acceptor distance, and depending on the locations of the fluorescent molecules on the proteins, even if proteins

are bound to each other, the fluorophores might be located farther than the Förster distance.

Detection of bimolecular fluorescence complementation (BiFC) at the level of single molecules

In BiFC, two potentially interacting proteins are fused to N-terminal and C-terminal half-molecules of YFP (YN and YC), respectively. If these fusion proteins interact, YFP may be reconstituted [32,33]. Thus, if BiFC is detected at the level of single molecules (appearing as a single-molecule spot in the dark background), it would lend strong support for protein binding [16••,17••]. These BiFC spots turn on and off, indicating that the BiFC dimers, formed by interactions of the target proteins and the YN–YC interactions, are in dynamic equilibrium between dimers and monomers (YFP formed by YN and YC is unstable, and dissociates into YN and YC in ~160 ms), which is consistent with the reversible YN/YC binding as described previously [34,35]. Many researchers are confused about this point: they believe that the YN–YC pair forms a stable complex. However, if this were true, all of the target proteins would appear to interact because the BiFC occurrence would be dominated by extremely strong YN–YC interactions.

Observation of the distribution of signal intensities of individual spots

In the histogram showing the distribution of signal intensity of individual spots, a second peak might appear, which is a strong indication of the presence of dimers and greater oligomers [16••,17••].

Detecting two-step photobleaching of individual spots

The time-dependent changes in the fluorescent signal intensity of each individual spot can reveal whether the spot represents a monomer or a dimer (single-step or double-step photobleaching, respectively) [36••]. This method is quite suitable for immobile molecules, because tracking their signal intensity is easier.

Conclusions

Single fluorescent-molecule video imaging is particularly suited to observe molecular interactions in live cells, and provides an unprecedented method for viewing dynamic molecular interactions. It has revealed the dynamic equilibrium between monomers and homo-dimers of GPCRs (Table 1b). Furthermore, it enables superquantification of the GPCR monomer–dimer equilibrium, determining the equilibrium constant, and the dimer formation and dissociation rate constants (Figures 1 and 2). It is an extremely powerful method for examining whether the molecular interactions suggested by *in vitro* experiments actually occur in living cells and, if so, for determining their dynamics.

The application of single fluorescent-molecule video imaging is not limited to GPCRs. It will become one of the most critical methods for studying the interactions of GPCRs with downstream signaling molecules, including trimeric G proteins, GPCR kinases, and arrestins (Figure 3). Thus far, it has revealed transient homo-dimers of three glycosylphosphatidylinositol (GPI)-anchored receptors, which are stabilized by raft-lipid interactions [16••] (also see [37•]), and homo-dimers of the ABC-A1 transporter when it is ready to transfer cholesterol to the Apo-AI protein for nascent HDL formation [36••]. It was also used to observe fluorescent GTP binding to small G proteins, H-Ras and K-Ras, and thus can detect not only the location and movement but also the activation of single molecules [31].

The application of single fluorescent-molecule video imaging to molecular behavior analyses in living cells will make important contributions in various fields of cell biology, and will revolutionize our view of molecular interactions in living cells.

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- •• of outstanding interest

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