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Review

# Single-Molecule Imaging of GPCR Interactions

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**G protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors and are of great interest as pharmacological targets. Although the occurrence of GPCR signaling nanodomains has long been hypothesized based on indirect evidence, this and other fundamental aspects of GPCR signaling have been difficult to prove. The advent of single-molecule microscopy methods, which allow direct visualization of individual membrane proteins with unprecedented spatiotemporal resolution, provides unique opportunities to address several of these open questions. Indeed, recent single-molecule studies have revealed that GPCRs and G proteins transiently interact with each other as well as with structural components of the plasma membrane, leading to the formation of dynamic complexes and 'hot spots' for GPCR signaling. Whereas we are only beginning to understand the implications of this unexpected level of complexity, single-molecule approaches are likely to play a crucial role to further dissect the protein–protein interactions that are at the heart of GPCR signaling.**

## Trends

Single-molecule microscopy is emerging as a powerful tool to analyze dynamic processes in living cells such as those involved in GPCR signaling.

These methods can achieve a temporal resolution of a few milliseconds and a spatial resolution of 10–30 nm.

Single-molecule methods have important advantages compared with ensemble measurements. Above all, they allow the direct investigation of protein–protein interactions, such as those involved in GPCR dimerization or G-protein activation.

Recent single-molecule studies have revealed an unexpected complexity in GPCR organization, whereby receptors undergo transient interactions among themselves, with G proteins and with the cytoskeleton to form dynamic signaling nanodomains at the plasma membrane.

## Emerging Concepts in GPCR Signaling

G protein-coupled receptors (GPCRs) mediate the effects of a large number of hormones and neurotransmitters, are implicated in several human diseases, and represent major pharmacological targets [1–3]. Given their fundamental role in physiology and disease, GPCRs have been intensively investigated, above all *in vitro* [1]. Recent years have seen a major advance in this field with the crystallization of GPCRs in different functional states [4–6]. Moreover, our views are rapidly changing with the advent of new optical methods that allow us to directly investigate the dynamics of GPCR signaling in living cells. These studies have surprisingly demonstrated that GPCRs do not signal exclusively at the plasma membrane but also at intracellular sites [7], such as early endosomes [8] and the Golgi/trans-Golgi network [9,10]. Furthermore, they are beginning to reveal a much more complex scenario than previously thought, whereby GPCRs form nanodomains on biological membranes, where they dynamically interact with each other, with their signaling partners, and with the surrounding structural proteins.

In particular, innovative single-molecule microscopy methods allow investigating GPCR signaling in living cells with single-molecule sensitivity and unprecedented spatiotemporal resolution. These methods are providing new important insights into the mechanisms of GPCR signaling and are likely to play a crucial role in future studies aimed at unraveling the nanoscopic organization of GPCR signaling cascades. In this review, we will introduce single-molecule microscopy methods and review the latest exciting results that have been obtained with this approach. For a comprehensive review of fluorescence correlation spectroscopy methods, which are complementary to single-particle tracking (SPT) and enable precise measurements

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of average diffusion and interaction kinetics, we refer the reader to the review by Briddon and Hill in this special issue [11].

## Protein–Protein Interactions at the Heart of GPCR Signaling

The ternary complex model [12,13] occupies a central position in GPCR signaling. At the heart of this model lies the idea that receptors and G proteins interact via random collisions in the absence of agonists and that agonists act via stabilizing the so-called ternary complex, consisting of an agonist, an active receptor, and a G protein [12,13]. However, depending on the stability of receptor–G-protein interactions, this model allows several possible scenarios, ranging from very transient interactions to the formation of long-lived, precoupled receptor–G-protein complexes.

Recent structural and biophysical data have provided fundamental insights into the mechanisms that lead to the formation of the ternary complex and, thus, to GPCR signaling (see [14,15] for an update based on recent results). This has been shown to proceed through a series of rearrangements, which culminate with large conformational changes in the fully active complex. These include a 7–14 Å outward movement of transmembrane domain 6 (TM6) of the receptor, which creates a binding pocket for the G protein, as well as an approximately 120° rotation of the α-helical domain of the Gα subunit relative to its ras-like domain, so that the α-helical domain can stabilize the interaction with the receptor [6]. Although this guanine nucleotide-free ternary complex is thought to represent the fully active receptor complex and can be purified *in vitro* in the absence of guanine nucleotides, it is probably extremely short lived – if not virtually absent – in living cells, where guanine nucleotides (GDP and GTP) are abundant. Thus, it remains unclear for how long receptors and G proteins interact in living cells.

Another interesting aspect is related to receptor dimerization. Whereas GPCRs have long been viewed as monomeric receptors, accumulating biochemical evidence has suggested that these receptors can associate into dimers or higher-order oligomers at the plasma membrane [16,17]. This phenomenon has been implicated in different aspects of GPCR biology, including trafficking, cell surface expression, and signaling [16,17]. Probably the best case for a role of GPCR dimerization in signaling comes from class C receptors, such as γ-aminobutyric acid ($GABA_B$), metabotropic glutamate (mGlu), and calcium sensing (CaS) receptors. Indeed, mGlu and CaS receptors have been shown to form homodimers linked by disulfide bridges, which suggests that, at least for these receptors, dimerization is constitutive [18–20]. Interestingly, it has been shown that $GABA_B$ receptors are functional heterodimers between $GABA_{B1}$, which alone cannot reach the cell surface and is not capable of signaling, and $GABA_{B2}$, which is signaling competent but cannot bind GABA [21,22]. Receptor dimerization also explains the presence of a **dominant negative effect** (see Glossary) of heterozygous GPCR mutations responsible for diseases such as retinitis pigmentosa [23] or thyroid-stimulating hormone resistance [24,25]. In most of these cases, it has been shown that the mutant receptors are retained in the endoplasmic reticulum (ER), where they interact with the remaining wild-type receptors, causing their intracellular entrapment [26,27]. These observations are consistent with the view that GPCR dimerization occurs early during GPCR biosynthesis and might play a role in the ER quality control system that allows only correctly folded receptors to exit the ER and reach the plasma membrane [26,27]. In addition, the formation of **receptor heterodimers** might confer unique signaling properties, which could be exploited pharmacologically [28–30].

## The Complexity of the Plasma Membrane and Its Role in Signaling

All the initial and crucial steps in GPCR signaling, from agonist binding to the activation of effectors and production of second messengers, take place at the plasma membrane. Thus, interactions between components of GPCR signaling and both intrinsic and extrinsic elements of the plasma membrane, including lipids, membrane proteins, and the associated

### Glossary

**Dominant negative effect:** effect of a heterozygous gene mutation, whereby the resulting mutated protein affects the function of the wild-type protein in the same cell.
**Efficacy:** maximal effect that a drug acting on a receptor is capable of producing; it allows distinguishing full agonists (which produce the maximal possible response) from partial agonists (which produce only a partial response).
**Ensemble methods:** methods that measure the average behavior of entire populations of molecules, in contrast to single-molecule ones.
**Receptor heterodimers:** dimers formed by two receptors of different type.
**Total internal reflection fluorescence:** method used to excite only the fluorophores that are within 100–200 nm from the interface between the glass coverslip and the cells adhering to it, thus eliminating fluorescence from deeper inside the cell.

cytoskeleton, are likely to play an important role in GPCR signaling [31]. These interactions have been suggested to contribute to the formation of signaling nanodomains at the plasma membrane where GPCRs can induce local signals, thus providing a mechanism to explain both signaling efficiency and specificity. Biochemical studies provided first, indirect evidence supporting the existence of such nanodomains [32,33]. Several of these classical studies focused on the lipid composition of the plasma membrane, which can be manipulated pharmacologically. The results suggested that GPCR signaling preferentially occurs in so-called lipid rafts, that is, small (10–200 nm) cholesterol- and sphingolipid-rich membrane domains that are resistant to detergent extraction at low temperature [32]. However, due to their small size, these domains have been difficult to investigate directly and their existence, size, and stability in living cells have proven controversial [34,35]. Moreover, other studies have suggested that caveolae, that is, small invaginations of the plasma membrane with lipid composition similar to rafts, but with unique protein composition (including caveolins), might also provide specialized platforms for GPCR signaling [36,37]. Furthermore, there is some biochemical evidence that receptors, G proteins, and effectors can interact, most likely indirectly, with the cytoskeleton underneath the plasma membrane [31]. Although the anatomical nature of the nanodomains potentially resulting from interactions with lipid domains and cytoskeletal proteins could not be directly addressed using biochemical methods, these studies were conceptually important. Above all, they suggested a mechanism, that is, the local enrichment of signaling molecules, for the high speed and efficiency observed in GPCR signaling. Indeed, the latter would be hard to explain if receptors, G proteins, and effectors, which are usually present at low densities, were to interact by pure random collisions [32]. This concept is further supported by a number of theoretical studies and mathematical simulations, which suggest that the local composition of the plasma membrane might be crucial for regulating the protein–protein interactions involved in GPCR dimerization and signaling, and, thus, for attaining fast and specific signal transduction [38–40].

## Lessons from Ensemble Biophysical Methods in Living Cells

The introduction of **ensemble methods** based on fluorescence and bioluminescence resonance energy transfer (FRET and BRET, respectively), which allow monitoring GPCR signaling in living cells, represented an important step forward in the field (see [41,42] for a comprehensive review).

Studies based on FRET have revealed that the kinetics of GPCR activation is faster than previously thought, with apparent rate constants of approximately 20–30 $s^{-1}$ [43]. In addition, experiments comparing full, partial, and inverse agonists have provided experimental support for the hypothesis that different compounds may induce different receptor conformations [43–45]. In addition, the results of ensemble measurements have suggested that G-protein activation might occur without dissociation into $\alpha$ and $\beta\gamma$ subunits [46] or even without dissociation from the receptor [47].

Furthermore, results obtained with FRET and BRET have provided evidence for the existence of signaling microdomains/nanodomains both at the plasma membrane and at intracellular compartments. This seems to be true even for soluble second messengers like cAMP, which have been shown to exert local effects via the formation of microdomains/nanodomains where cAMP concentrations are high [48–51]. This phenomenon assumes particular relevance in light of the recent demonstration that GPCRs induce cAMP production and protein kinase A activation not only at the plasma membrane, but also at intracellular sites [7–10].

Another important contribution of FRET and BRET has been the demonstration of homodimerization and heterodimerization of GPCRs in living cells (reviewed in [41]). This represented a

major step forward, as classical biochemical methods (e.g., coimmunoprecipitation) require cell disruption and membrane solubilization, which render them more prone to artifacts.

Despite these major advances, ensemble measurements based on FRET, BRET, or fluorescence recovery after photobleaching have given contrasting results regarding the stability of the protein–protein interactions involved in GPCR signaling, such as those implicated in receptor dimerization or in the interactions between receptors and G proteins [52]. This is probably mostly due to technical limitations, especially the fact that FRET and BRET cannot readily distinguish between conformational rearrangements within a protein complex and its association/dissociation [52].

## Single-Molecule Methods

The idea of visualizing and manipulating individual molecules or atoms is not new. Inspired by the revolutionary ideas of Feynman and Gilbert [53] and by the first measurement of the absorption spectrum of a single pentacene molecule by Moerner and Kador in 1989 [54], generations of scientist have worked to make this once a dream reality (see [55] for a historical overview). With the advent of new microscopy methods with improved signal-to-noise ratio, more sensitive cameras, and brighter fluorophores, it soon became possible to measure individual fluorescent molecules in solution, in reconstituted systems, or even in living cells [55].

One of first applications of single-molecule microscopy to investigate GPCRs in living cells was the pioneering work performed in Akihiro Kusumi's laboratory, in which μ opioid receptors labeled with gold nanoparticles were imaged and tracked at the impressive temporal resolution of 25 μs [56]. This study revealed that GPCRs are hopping among adjacent compartments of approximately 200 nm size, which provided important support to the fence-and-picket model of the plasma membrane (see the following discussion).

In a typical SPT experiment, fluorescently labeled molecules, for example, of a receptor, are imaged on a fast, highly sensitive camera. In the case of membrane proteins, this is usually achieved via **total internal reflection fluorescence** (TIRF) illumination, which produces a higher signal-to-noise ratio compared with epifluorescence. The resulting diffraction-limited spots (particles) are then automatically detected in each frame of the acquired image sequence. Tracking algorithms are finally used to link the detected particles in each frame, and, thus, reconstruct their trajectories (Figure 1). Although these diffraction-limited spots appear much larger (approximately 200–300 nm) than the real size of a typical fluorescently labeled membrane protein (diameter of the GPCR 7TM bundle approximately 3 nm), their position can be determined with high precision via fitting to a two-dimensional Gaussian distribution (Box 1). Indeed, the localization precision of single-molecule microscopy, which is mostly dictated by the number of collected photons, is typically in the range of 10–30 nm, that is, about ten times higher than the resolution limit of fluorescence microscopy and much closer to the size of a GPCR. For both detection and tracking to work, the fluorescently labeled single molecules must be well separated from each other. This poses a limit, as only particles with densities below about 1 molecule/$\mu m^2$ can be analyzed, which for several membrane signaling proteins lies within or just below their physiological range.

Single-molecule methods have a number of important advantages compared to ensemble ones, which make them very attractive to investigate the organization of GPCR signaling cascades at the plasma membrane. First, they enable the precise localization (with few nanometer and millisecond resolution) and direct counting of molecules (Box 2), thus providing detailed information on their spatial distribution as well as on the stoichiometry of supramolecular complexes. Second, they do not require synchronization of the examined molecules (e.g., via agonist stimulation), which is often used to analyze kinetics in ensemble measurements, and can

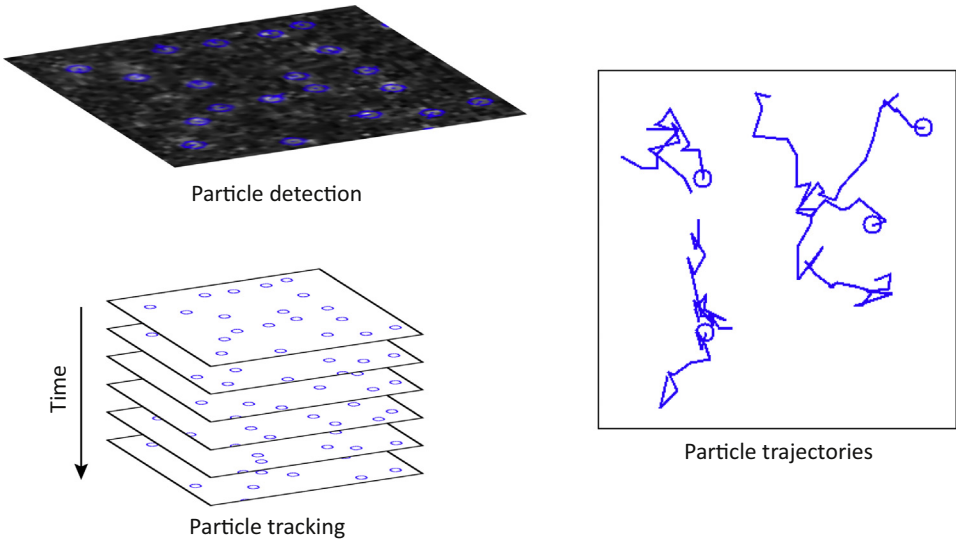


Figure 1. Single-Particle Tracking (SPT). Single-molecule microscopy data are typically analyzed by SPT. First, all diffraction-limited spots (particles) in each frame of the acquired image sequence are detected via an automated computer algorithm. This process gives information about the intensity and position of each particle in each frame. An automated tracking algorithm is then used to link the spots detected between two consecutive frames and, ultimately, reconstruct the trajectory of each particle over time.

investigate heterogeneous molecular populations, thereby separately analyzing their constituting components. Third, they allow to directly estimate the association and dissociation rates of protein complexes, thus providing a full quantitative characterization of dynamic processes, such as those involved in receptor dimerization (Box 3). Fourth, they reveal rare and transient events, which are usually hidden in ensemble measurements because of averaging.

To achieve this, the investigated molecule(s) must be labeled with a suitable fluorophore, which is typically achieved by means of a fluorescent ligand or a fluorescently labeled antibody, via fusion with a fluorescent protein or by insertion of a protein tag that can be labeled with small organic fluorophores (Box 4). The labeling method should be chosen with care, as any modification of the molecule of interest can potentially alter its function or subcellular localization, which should be tested experimentally. In this respect, direct labeling with small organic fluorophores might be better tolerated than the insertion of medium-sized fluorescent protein tags or the binding of large fluorescently labeled antibodies.

Disadvantages of single-molecule methods compared to ensemble ones include the fact that they can analyze only low densities/concentrations, which might be limiting in case of abundant proteins, require complex and time-consuming analyses, and are so far usually limited to simple cell models.

## Lessons from Single-Molecule Studies *In Vitro*

Since the first demonstration of single-molecule FRET (smFRET) on a DNA template [57], this technique has become the main single-molecule tool to investigate the function of biological molecules *in vitro* [55]. smFRET experiments can be performed on diffusing, confined, or tethered molecules, although immobilization, for example, via biotin–streptavidin linkage, offers several advantages.

## Box 1. Precision in Single-Molecule Localization

The position of a diffraction-limited object can be determined to much greater precision than its apparent size through a far-field microscope by finding its center of mass (centroid). This is achieved by fitting the pixel intensities of the diffracted spot with a two-dimensional (2D) Gaussian distribution plus a constant to account for background (Figure I). The lateral localization precision is commonly expressed in terms of the localization error standard deviation ($\sigma_x$), which is given by the following equation [78]:

$$\sigma_x = \sqrt{\frac{s^2 + \frac{a^2}{12}}{N}\left(\frac{16}{9} + \frac{8\pi s^2 b^2}{a^2 N}\right)} \quad [I]$$

where $N$ is the number of collected photons, $a^2$ is the pixel area of the imaging detector, $b^2$ is the average number of background photons per pixel, and $s$ corresponds to the standard deviation of the point spread function. With some approximations, this equation implies that the localization precision mainly depends on the number of collected photons and the signal-to-background ratio (SBR):

$$\sigma_x \sim \sqrt{\frac{s^2}{N \cdot SBR}} \quad [II]$$

Notice that there is no absolute theoretical limit to localization precision. Increasing the number of collected photons or the SBR both increase the localization precision. However, with the currently available fluorophores and technology, this typically lies within 10–30 nm.

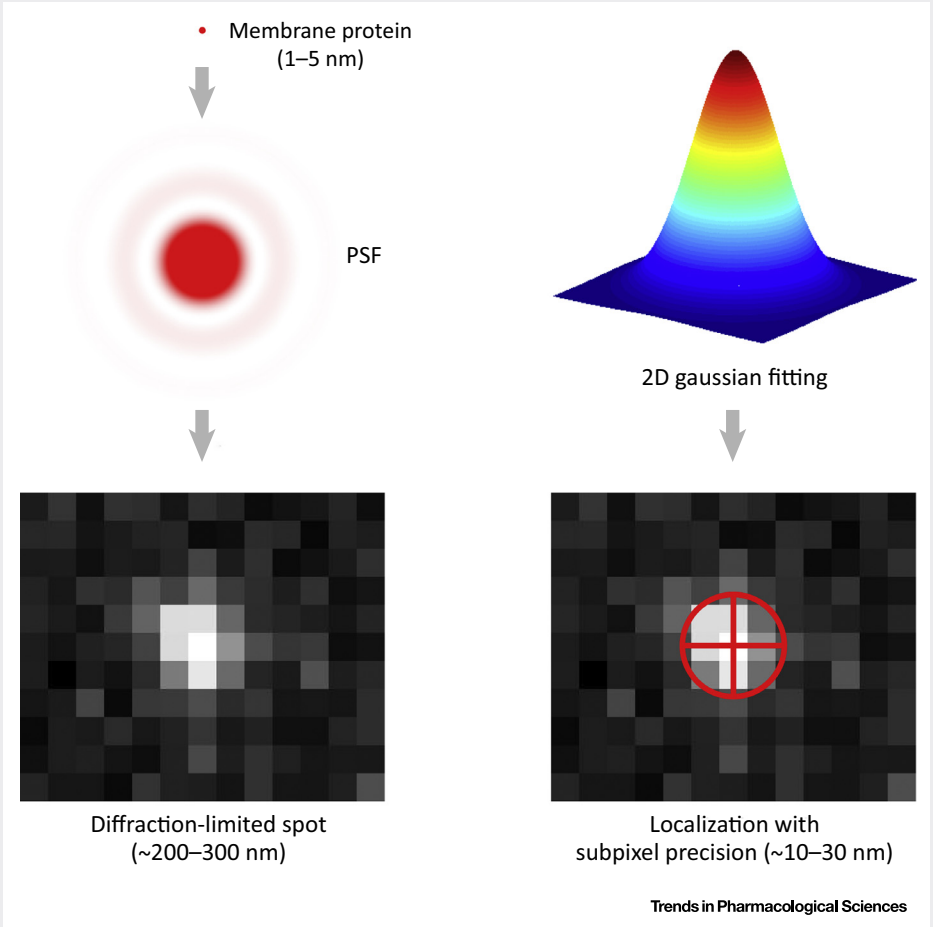


Figure I. Localization Precision in Single-Molecule Microscopy. A typical membrane protein with a diameter of 1–5 nm appears through the lenses of a microscope as a diffraction-limited spot of approximately 200–300 nm, corresponding to the point spread function (PSF) of the microscope. However, the center of mass of the diffracted spot can be determined with higher precision via fitting its pixel intensities to a two-dimensional (2D) Gaussian distribution. This results in a subpixel precision of approximately 10–30 nm.

## Box 2. Subunit Counting by Single-Molecule Microscopy

Single-molecule microscopy allows investigators to directly count the number of fluorescent molecules present in the identified diffraction-limited spots, provided that both the stoichiometry and efficiency of labeling are known. Whenever possible, a stoichiometry of 1:1 (i.e., one fluorophore per protein) and high labeling efficiency should be aimed for and verified. For integral membrane proteins, this can be achieved, for instance, via insertion of a SNAP-tag at the N terminus and covalent labeling with small organic fluorophores, which can reach efficiencies as high as 90% [67–69].

Two main methods are used to calculate protein stoichiometry based on single-molecule data. A first method consists in fitting the distribution of particle intensities with a mixed Gaussian model (Figure IA) [67–69]. This approach can reveal complex mixtures of monomers, dimers, and higher-order oligomers, the relative abundance of which can be estimated from the area under the curve of each corresponding component.

A second, complementary approach consists in counting the number of photobleaching steps associated with each fluorescent particle, which corresponds to the number of fluorophores (Figure IB) [67–69,79]. This is because the light-induced destruction (photobleaching) of a single fluorophore results in its instantaneous disappearance. Thus, particles containing *n* fluorophores progressively bleach, producing a characteristic intensity profile with *n* steps. This method has the advantage of directly estimating the number of fluorophores in each particle and does not suffer from ambiguities associated with the partial overlap usually seen between the intensity distributions of monomers, dimers, and higher-order oligomers. However, the method based on bleaching steps can underestimate the size of protein complexes, because two nearly simultaneous photobleaching steps can be detected as a single event due to insufficient temporal resolution.

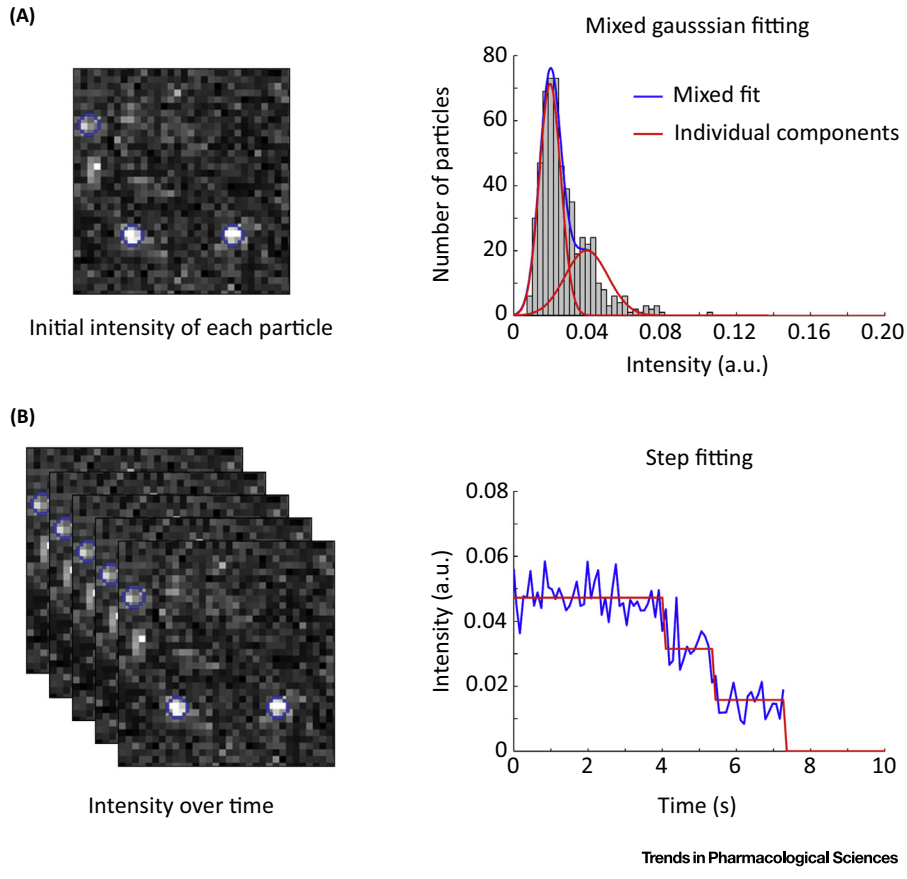


Figure I. Single-Molecule Analysis of the Size of Supramolecular Complexes. The intensities of each diffraction-limited spot over time (obtained via single-particle tracking) can be used to determine the size of protein complexes. A first approach (A) is based on the analysis of particle intensity at the initial frame of an image sequence. The intensity distribution of all detected particles is fitted with a mixed Gaussian model. The relative abundance of the underlying subpopulations (in this example monomers and dimers) can then be estimated from the area under the curve of each component of the fitting. This method requires knowledge of the intensity distribution of single fluorophores, which can be measured by spreading the fluorophores on a clean glass coverslip. A second approach (B) is based on the analysis of particle intensity over time. Since fluorophores undergo light-induced destruction (photobleaching) over time, the size of protein complexes can be determined by counting the photobleaching steps associated with each diffraction-limited spot.

### Box 3. Analysis of Protein–Protein Interactions by Single-Molecule Microscopy

The frequency and duration of protein–protein interactions can be estimated based on the position of the individual particles over time, which can be measured with high precision [65,67–69,74]. Once two particles fall below the resolution limit, they are considered to colocalize. This allows to measure the frequency and duration of protein–protein interactions. Since the density of the labeled proteins can also be easily derived from the data, this also permits to estimate the association and dissociation rate constants ($k_{on}$ and $k_{off}$) of the examined interactions and, from them, to derive equilibrium dissociation constants ($K_d = k_{off}/k_{on}$) [65,67–69,74]. A potential problem with this approach is that the spatial resolution is still insufficient to distinguish true interactions from random colocalizations. Thus, appropriate controls must be included to estimate the frequency and duration of random colocalizations and to correct the measured $k_{on}$ and $k_{off}$ for these factors. Controls typically include synthetic data obtained via computer simulations [67,74] or, in case of two-color acquisition, data in which one channel has been flipped to decorrelate the localizations of the two channels [64]. In addition, the use of a monomeric integral membrane protein as negative control is recommended [67–69,74]. Finally, the results should be corrected for the rate of fluorophore photobleaching or particle loss at tracking [65,67,74].

Recently, new exciting results have been obtained by applying smFRET to purified GPCRs. Vafabakhsh *et al.* [58] have investigated the conformational changes induced by agonist activation in mGlu receptors (mGlu2 and mGlu3) via smFRET on immobilized full-length receptors. Their results suggest that the large ligand-binding domain (LBD) of these receptors is in equilibrium among a resting, an activated, and a short-lived intermediate state. Interestingly, they found that the occupancy of the active conformation correlates with agonist **efficacy**. In addition, mGlu3 showed a unique behavior in that it fluctuated among the three states in the absence of agonist, consistent with the fact that mGlu3, but not mGlu2, has $Ca^{2+}$-dependent basal activity. This study also provided an estimate of the residence time in the fully closed (active) or fully open (inactive) states, which were found to be in the order of tens of milliseconds to seconds. This is much longer than the sub-millisecond fluctuations measured for isolated LBDs in solution by pulsed interleaved excitation fluorescence cross-correlation spectroscopy [59]. It remains to be elucidated whether such differences depend on the use of the full-length receptor instead of the isolated LBD or, rather, on the different temporal resolution of the employed methods.

The same group used single-molecule subunit counting and smFRET to investigate the homodimerization and heterodimerization of purified mGluRs [60]. They found that both homodimerization and heterodimerization depend on interactions between the upper lobes

### Box 4. Methods Used to Label GPCRs for Single-Molecule Microscopy

A variety of approaches have been used to fluorescently label GPCRs in single-molecule microscopy experiments (for a recent review see Tian *et al.* [80]). A first method consists in labeling the receptors via the use of a specific antibody, often conjugated with a bright fluorescent probe such as a quantum dot. Alternatively, GPCRs can be labeled using fluorescently modified agonists or antagonists. Another approach consists in generating chimeric DNA constructs that, once introduced into cells, are translated into tagged receptors. The employed tags can be fluorescent *per se* (e.g., GFP) or can be conjugated in a second step with a small organic fluorophore. The latter is often achieved via self-labeling protein tags, that is, modified enzymes that interact irreversibly with fluorescent substrates. The SNAP-tag, a widely used self-labeling protein tag derived from the human DNA repair enzyme $O^6$-alkylguanine-DNA alkyltransferase, forms covalent adducts with fluorescent benzylguanine substrates [66]. This method is versatile, as several SNAP fluorescent substrates with different excitation and emission spectra are available. Orthogonal labeling (i.e., the simultaneous labeling of two proteins with two different fluorophores) can be achieved via combination with a CLIP-tag, which is a modified SNAP-tag reacting selectively with fluorescent benzylcytosine substrates [81]. Another example of self-labeling protein tag is represented by the HaloTag, derived from the bacterial enzyme haloalkane dehalogenase, which forms a covalent bond with fluorescent chloroalkane substrates [82]. Although labeling of intracellular proteins via self-labeling protein tags was limited by the poor permeability of most available fluorescent substrates, this limitation has been recently circumvented by the development of a series of new substrates with improved cell permeability [83,84]. Finally, labeling via unnatural amino acids is a novel promising method, which relies on the site-specific introduction of a single modified amino acid that is subsequently chemically conjugated with a small organic fluorophore [85]. This method utilizes specific orthogonal aminoacyl-tRNA synthetase–tRNA pairs to suppress the amber codon and introduce an unnatural amino acid into the newly synthesized protein. Efforts are underway to develop orthogonal labeling with two distinct unnatural amino acids in living cells [86].

of the LBDs, with modest contribution of an intersubunit disulfide bridge and of interactions between the TM domains. Based on smFRET results, they also concluded that these LBD interactions prevent spontaneous LBD closure. Moreover, by using photoswitchable tethered ligands they found that the LBD–LBD interactions lead to cooperativity in the receptor activation process.

In another study, Lamichhane *et al.* [61] investigated by single-molecule microscopy $\beta_2$-adrenoceptors ($\beta_2$-ARs) labeled with Cy3 near the cytoplasmic end of TM6 and reconstituted in phospholipid nanodiscs. This study exploited the changes in Cy3 fluorescence caused by the changes in the environment surrounding the fluorophores that are associated with receptor activation. The authors observed spontaneous transitions between two distinct states, which they assigned to an active and an inactive conformation. The resulting equilibrium was shifted toward the active or the inactive conformation by treatment with a full agonist or an inverse agonist, respectively [61].

The conformational changes occurring in purified $\beta_2$-ARs have been further investigated in a very recent smFRET study by Gregorio *et al.* [62]. In this study, purified $\beta_2$-ARs were labeled with Cy3B and Cy7 at the cytoplasmic ends of TM6 and TM4, respectively. Stimulation with ligands characterized by increasing efficacy caused a progressive reduction of the mean FRET values, consistent with an average outward movement of TM6 of approximately 4 Å in the presence of adrenaline. When the experiment was repeated in the presence of $G_s$ protein, a lower FRET state appeared. This was consistent with a further outward movement of TM6 that brought the two fluorophores at a distance of approximately 55 Å, in agreement with molecular dynamics simulations. Interestingly, the proportion of receptors that occupied this low-FRET state correlated with agonist efficacy. About 20% of the receptors were in the low-FRET state already in the absence of agonist, consistent with the known constitutive activity of $\beta_2$-AR. Interestingly, the estimated apparent rates of $G_s$ protein binding to the $\beta_2$-AR were much lower than expected for protein–protein interactions in solution, which suggested that $G_s$ coupling might be rate limited by conformational rearrangements. In addition, the authors observed transient receptor–G-protein complexes in the presence of GDP or GTP, suggesting that these complexes might not be stable under physiological conditions.

## Lessons from Single-Molecule Studies in Intact Cells

Pioneering studies by Akihiro Kusumi's group used single-molecule microscopy to investigate the mobility of GPCRs and other membrane proteins in living cells [63]. These studies demonstrated that membrane proteins and even lipids do not diffuse on the plasma membrane according to pure Brownian motion, but rather jump between adjacent compartments, with an apparent size of 40–300 nm, in which they are partially confined [63]. These observations lead to the formulation of the fence-and-picket model, according to which membrane-associated cytoskeletal filaments and tubules (fences) and integral membrane proteins bound to the cytoskeleton (pickets) form barriers that limit the diffusion of membrane molecules and cause their confinement in small nanodomains [63].

Subsequently, single-molecule/SPT methods have been applied to investigate GPCR dimerization. A first study by Hern *et al.* [64] investigated $M_1$ muscarinic receptors expressed in CHO cells. To visualize the receptors, the authors employed a fluorescently labeled antagonist (telenzepine). They found that $M_1$ receptors form transient dimers with an apparent lifetime of 0.5 s at 23 °C. At equilibrium, about 30% of the receptors were in dimeric form. The authors also confirmed these results using two-color SPT.

The following year, Kasai *et al.* [65] published a study in which they used SPT to investigate formyl peptide (FP) receptors, which they visualized using a fluorescently labeled FP that

retained agonistic activity. Importantly, this study was the first one to provide a detailed quantitative characterization of a GPCR monomer–dimer equilibrium. The results were indicative of fast association and dissociation: on average, at 37°C and physiological densities of two receptors/$\mu m^2$ (corresponding to approximately 6000 molecules/cell), FP receptors formed a dimer every 150 ms and dissociated after approximately 90 ms.

Subsequently, our group developed a method based on direct receptor labeling with small organic fluorophores via SNAP-tags [66] and SPT, which allows to directly compare the dimerization/oligomerization of different GPCRs [67–69]. Since labeling efficiency is higher than 90%, this method is superior to labeling with fluorescent proteins, a relevant fraction of which does not become fluorescent [67–69]. In addition, small organic fluorophores are more resistant to photobleaching, which allows longer observation times. We used this method to compare three prototypical GPCRs, that is, $\beta_1$-adrenoceptor, $\beta_2$-adrenoceptor, and $GABA_B$ receptors. We found that these GPCRs had different tendencies to form dimer/oligomers as well as different spatial arrangements at the plasma membrane. Both $\beta_1$- and $\beta_2$-ARs were in dynamic equilibrium among monomers, dimers, and small fractions of trimers/tetramers. Although the lifetimes of $\beta_1$- and $\beta_2$-AR dimers were similar (approximately 4 s at 20.5 °C), $\beta_2$-ARs had a higher tendency of forming dimers at comparable densities. By contrast, we found $GABA_B$ receptors to assemble into larger oligomers formed by B1–B2 subunits, which we estimated to mainly contain two subunits (i.e., one heterodimer) at low densities and a mixture of four and eight subunits at the highest densities analyzed [67]. Importantly, the direct labeling of the receptors, instead of using fluorescent ligands, also allowed us to investigate the effect of agonist stimulation on receptor dimerization/oligomerization. We found no effects of agonist stimulation, consistent with several but not all previous studies based on biochemical and ensemble biophysical methods [67]. Remarkably, while $\beta_1$- and $\beta_2$-ARs were grossly homogeneously distributed on the plasma membrane, $GABA_B$ receptors were arranged in rows through interactions (presumably indirect) with the actin cytoskeleton, which might be important for the spatial organization of $GABA_B$ receptors at synapses [67].

The equilibrium between monomers and dimers seems to be established already in the ER, as indicated by a recent study on the corticotropin-releasing factor receptor type 1 [70]. In addition, single-molecule studies have revealed a high heterogeneity in the diffusion of GPCRs at the plasma membrane, with a relevant fraction of confined as well as of immobile receptors, which might at least partially represent receptors associated with clathrin-dependent structures [71].

Single-molecule approaches have also been used to investigate receptor dimerization in fixed cells, confirming that GPCRs form dimers/oligomers with different sizes and spatial arrangements [72]. Although fixation might introduce artifacts, it simplifies the analysis and could achieve an even higher spatial resolution. For instance, Tabor *et al.* [73] recently investigated dopamine D2 receptors in fixed CHO cells at a resolution surpassing 10 nm, obtained by imaging the sample at liquid helium temperature ($T$ = 4.3 K). This allowed them to directly measure the distance between two D2 receptors in a dimer, which was approximately 9 nm. Such a short distance is consistent with a direct physical interaction between D2 receptors.

Single-molecule microscopy can be used to investigate interactions not only among receptors but also between receptors and other signaling molecules. For instance, our group recently succeeded in directly visualizing and investigating the interactions between individual receptors ($\alpha_{2A}$- and $\beta_2$-ARs) and G proteins ($G_i$ and $G_s$, respectively) on the surface of living cells [74]. This was achieved via labeling the receptors and G proteins with two different organic fluorophores via SNAP-tags and CLIP-tags, which were then simultaneously imaged by fast two-color TIRF microscopy (Figure 2A, Key Figure). We found that receptors and G proteins undergo transient

## Key Figure

### Complex G Protein-Coupled Receptor (GPCR) Interactions at the Plasma Membrane as Revealed by Single-Molecule Microscopy

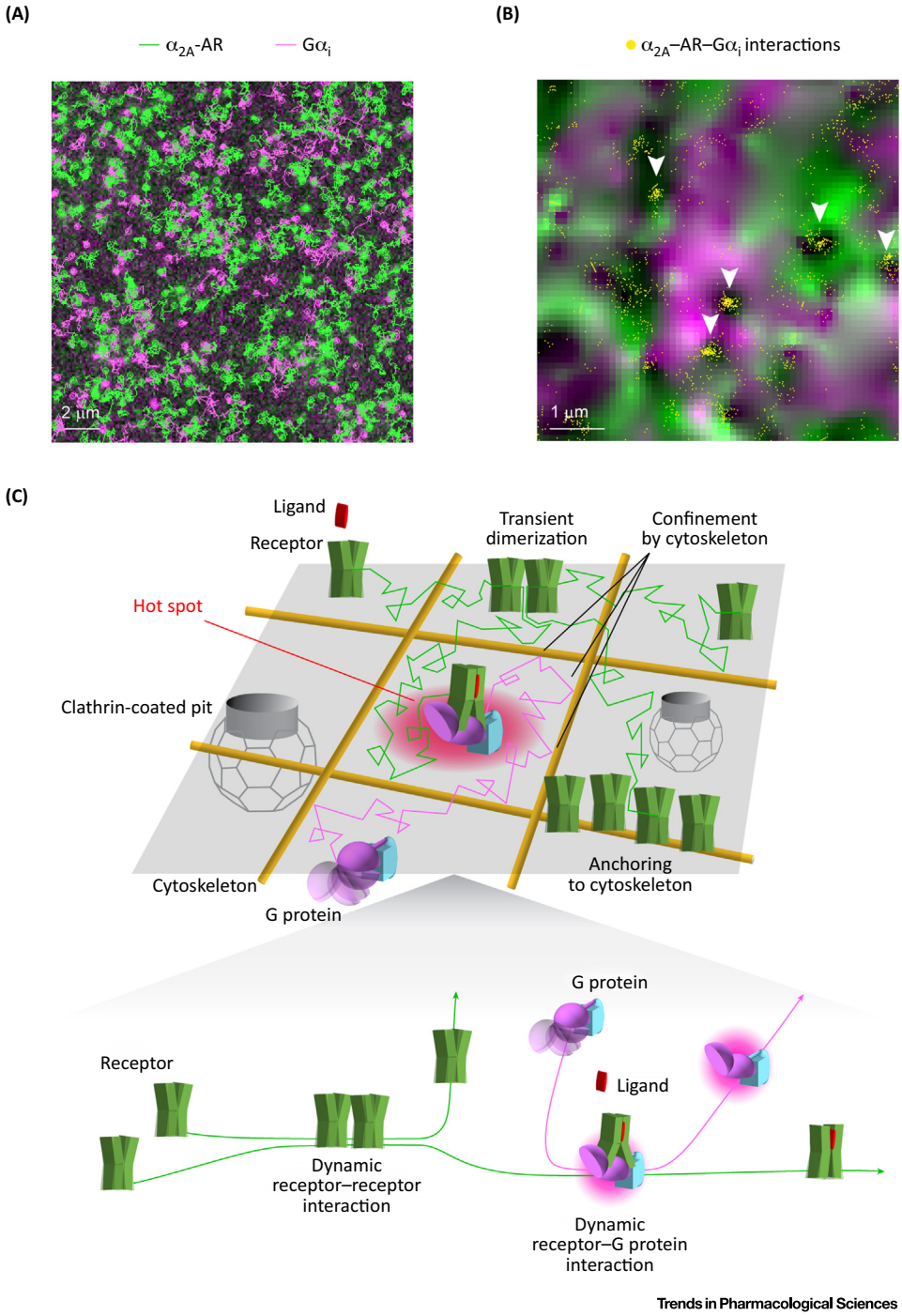


Figure 2. (A) Simultaneous single-molecule visualization and tracking of individual $\alpha_{2A}$-adrenoceptor ($\alpha_{2A}$-AR) and $G_i$ proteins as they diffuse on the surface of a living cell. (B) 'Hot spots' for receptor–G-protein interactions on the plasma

*(Figure legend continued on the bottom of the next page.)*

interactions that last approximately 1 s and that are already occurring in the absence of agonist due to constitutive receptor activity [74]. Intriguingly, agonist stimulation caused up to a tenfold increase in the association rate constant ($k_{on}$) for receptor–G-protein interactions, while causing only minor changes in the dissociation rate constant ($k_{off}$) [74]. These findings suggest that the interactions between receptors and G proteins are mainly controlled by the large conformational changes that occur during the formation of the ternary complex [14,15]. Moreover, we found that receptor–G-protein interactions and G-protein activation do not occur randomly at the cell surface, but rather at preferential sites, which we term 'hot spots' [74] (Figure 2B). These 'hot spots' are apparently generated by complex interactions of the receptors and G proteins with the cytoskeleton and other structural components (e.g., clathrin-coated pits) of the plasma membrane [74] (Figure 2C). We hypothesize that these hot spots play an important role in GPCR signaling by increasing the speed and efficiency of receptor–G-protein interactions, while allowing G-protein activation and downstream signaling to occur locally.

## Concluding Remarks

Studies based on single-molecule microscopy have revealed a complex picture at the plasma membrane, whereby receptors undergo dynamic interactions with each other and with G proteins, as well as with the surrounding cytoskeleton and other structural components. This results in transient receptor dimerization, discrete spatial arrangements (e.g., along actin fibers in the case of $GABA_B$ receptors), and 'hot spots' for receptor–G-protein interactions and signaling (Figure 2C). Future studies are thus required to further investigate the functional consequences of this complex organization as well as to attempt its manipulation for pharmacological purposes (see Outstanding Questions).

These studies also demonstrate the power of single-molecule methods, which allow to precisely analyze protein–protein interactions in living cells. We expect single-molecule methods, which are becoming each day more accessible to nonspecialists, to play a crucial role in future studies investigating the organization of GPCR signaling nanodomains at the plasma membrane as well as at intracellular locations. In this context, smFRET is likely to play an important role, as it might be used to directly investigate receptor conformational changes in living cells. Moreover, SPT could be combined with other super-resolution methods to image more abundant proteins, for example, cytoskeletal elements, or to visualize local signaling events [75]. Finally, recent exciting advances in cryo-electron microscopy have permitted the reconstruction of near-atomic-resolution three-dimensional structures of GPCR complexes based on averaging thousands of single-molecule images [76,77]. In the future, we expect these methods to be increasingly used as part of a multidisciplinary effort, whereby data obtained with different experimental approaches, including structural biology, pharmacology, and advanced imaging, will be combined to understand the complex biology of GPCRs and develop innovative pharmacological treatments.

The day when the life of a single GPCR in living cells is ultimately revealed is looming on the horizon.

membrane as revealed by single-molecule microscopy. Receptors and G proteins tend to be confined within 'hot spots' (dark areas), where they preferentially interact (arrowheads) and signal. Receptor–G-protein interactions are dynamic, lasting approximately 1 s. Data in (A) and (B) are modified from [74]. (C) Updated model of GPCR interactions at the plasma membrane. Dynamic interactions between receptors lead to the formation of transient receptor dimers. The cytoskeleton provides barriers to receptor and G-protein diffusion as well as anchors for certain receptors [e.g., γ-aminobutyric acid ($GABA_B$)]. Clathrin-coated pits and other structural elements provide additional obstacles and anchoring points. This complex organization of the plasma membrane results in the formation of 'hot spots' where receptors and G proteins preferentially interact and signal.

### Outstanding Questions

What is the nature, size, and composition of GPCR nanodomains at the plasma membrane?

For how long do receptors, G proteins, and effectors interact within such nanodomains?

Do GPCRs form nanodomains also on intracellular membranes?

How stable/dynamic are these nanodomains?

Which mechanisms (interactions with proteins, structural elements, lipids, etc.) are involved in their formation?

Do they differ in composition among different cell types?

What is their impact on signaling?

What is their contribution to signaling efficiency and specificity?

What is their role in physiology?

Are they altered under pathological conditions and what is their contribution to disease mechanisms?

Can they be manipulated for pharmacological purposes?

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## References

1. Pierce, K.L. *et al.* (2002) Seven-transmembrane receptors. *Nat. Rev. Mol. Cell. Biol.* 3, 639–650
2. Vassart, G. and Costagliola, S. (2011) G protein-coupled receptors: mutations and endocrine diseases. *Nat. Rev. Endocrinol.* 7, 362–372
3. Calebiro, D. *et al.* (2014) PKA catalytic subunit mutations in adrenocortical Cushing's adenoma impair association with the regulatory subunit. *Nat. Commun.* 5, 5680
4. Cherezov, V. *et al.* (2007) High-resolution crystal structure of an engineered human $\beta_2$-adrenergic G protein-coupled receptor. *Science* 318, 1258–1265
5. Rasmussen, S.G. *et al.* (2007) Crystal structure of the human $\beta_2$ adrenergic G-protein-coupled receptor. *Nature* 450, 383–387
6. Rasmussen, S.G. *et al.* (2011) Crystal structure of the $\beta_2$ adrenergic receptor-$G_s$ protein complex. *Nature* 477, 549–555
7. Calebiro, D. *et al.* (2010) Signaling by internalized G-protein-coupled receptors. *Trends Pharmacol. Sci.* 31, 221–228
8. Irannejad, R. *et al.* (2013) Conformational biosensors reveal GPCR signalling from endosomes. *Nature* 495, 534–538
9. Irannejad, R. *et al.* (2017) Functional selectivity of GPCR-directed drug action through location bias. *Nat. Chem. Biol.* 13, 799–806
10. Godbole, A. *et al.* (2017) Internalized TSH receptors en route to the TGN induce local $G_s$-protein signaling and gene transcription. *Nat. Commun.* 8, 443
11. Briddon, S.J. and Hill, S.J. (2018) Studying GCPR pharmacology in membrane microdomains: fluorescence correlation spectroscopy comes of age. *Trends Pharmacol. Sci.* 39, 158–174
12. De Lean, A. *et al.* (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J. Biol. Chem.* 255, 7108–7117
13. Samama, P. *et al.* (1993) A mutation-induced activated state of the $\beta_2$-adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.* 268, 4625–4636
14. Manglik, A. and Kobilka, B. (2014) The role of protein dynamics in GPCR function: insights from the $\beta_2$AR and rhodopsin. *Curr. Opin. Cell. Biol.* 27, 136–143
15. Manglik, A. *et al.* (2015) Structural insights into the dynamic process of $\beta_2$-adrenergic receptor signaling. *Cell* 161, 1101–1111
16. Milligan, G. (2004) G protein-coupled receptor dimerization: function and ligand pharmacology. *Mol. Pharmacol.* 66, 1–7
17. Terrillon, S. and Bouvier, M. (2004) Roles of G-protein-coupled receptor dimerization. *EMBO Rep.* 5, 30–34
18. Romano, C. *et al.* (1996) Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J. Biol. Chem.* 271, 28612–28616
19. Ray, K. *et al.* (1999) Identification of the cysteine residues in the amino-terminal extracellular domain of the human $Ca^{2+}$ receptor critical for dimerization. Implications for function of monomeric $Ca^{2+}$ receptor. *J. Biol. Chem.* 274, 27642–27650
20. Pin, J.P. *et al.* (2005) Allosteric functioning of dimeric class C G-protein-coupled receptors. *FEBS J.* 272, 2947–2955
21. Jones, K.A. (1998) $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_BR1$ and $GABA_BR2$. *Nature* 396, 674–679
22. White, J.H. (1998) Heterodimerization is required for the formation of a functional $GABA_B$ receptor. *Nature* 396, 679–682
23. Rajan, R.S. and Kopito, R.R. (2005) Suppression of wild-type rhodopsin maturation by mutants linked to autosomal dominant retinitis pigmentosa. *J. Biol. Chem.* 280, 1284–1291
24. Calebiro, D. *et al.* (2005) Intracellular entrapment of wild-type TSH receptor by oligomerization with mutants linked to dominant TSH resistance. *Hum. Mol. Genet.* 14, 2991–3002
25. Persani, L. *et al.* (2010) Genetics and phenomics of hypothyroidism due to TSH resistance. *Mol. Cell. Endocrinol.* 322, 72–82
26. Bulenger, S. *et al.* (2005) Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol. Sci.* 26, 131–137
27. Persani, L. *et al.* (2007) Technology Insight: modern methods to monitor protein-protein interactions reveal functional TSH receptor oligomerization. *Nat. Clin. Pract. Endocrinol. Metab.* 3, 180–190
28. Pin, J.P. *et al.* (2007) International Union of Basic and Clinical Pharmacology. LXVII. Recommendations for the recognition and nomenclature of G protein-coupled receptor heteromultimers. *Pharmacol. Rev.* 59, 5–13
29. Milligan, G. (2009) G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. *Br. J. Pharmacol.* 158, 5–14
30. Ferré, S. *et al.* (2009) Building a new conceptual framework for receptor heteromers. *Nat. Chem. Biol.* 5, 131–134
31. Neubig, R.R. (1994) Membrane organization in G-protein mechanisms. *FASEB J.* 8, 939–946
32. Insel, P.A. *et al.* (2005) Compartmentation of G-protein-coupled receptors and their signalling components in lipid rafts and caveolae. *Biochem. Soc. Trans.* 33, 1131–1134
33. Pontier, S.M. *et al.* (2008) Cholesterol-dependent separation of the $\beta_2$-adrenergic receptor from its partners determines signaling efficacy: insight into nanoscale organization of signal transduction. *J. Biol. Chem.* 283, 24659–24672
34. Munro, S. (2003) Lipid rafts. *Cell* 115, 377–388
35. Eggeling, C. *et al.* (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* 457, 1159–1162
36. MacDougall, D.A. *et al.* (2012) Caveolae compartmentalise $\beta_2$-adrenoceptor signals by curtailing cAMP production and maintaining phosphatase activity in the sarcoplasmic reticulum of the adult ventricular myocyte. *J. Mol. Cell. Cardiol.* 52, 388–400
37. Wright, P.T. *et al.* (2014) Caveolin-3 regulates compartmentation of cardiomyocyte: $\beta_2$-adrenergic receptor-mediated cAMP signaling. *J. Mol. Cell. Cardiol.* 67, 38–48
38. Gross, W. and Lohse, M.J. (1991) Mechanism of activation of A2 adenosine receptors. II. A restricted collision-coupling model of receptor-effector interaction. *Mol. Pharmacol.* 39, 524–530
39. Kalay, Z. *et al.* (2012) Confining domains lead to reaction bursts: reaction kinetics in the plasma membrane. *PLoS One* 7, e32948
40. Marino, K.A. *et al.* (2016) Impact of lipid composition and receptor conformation on the spatio-temporal organization of mu-opioid receptors in a multi-component plasma membrane model. *PLoS Comput. Biol.* 12, e1005240
41. Milligan, G. and Bouvier, M. (2005) Methods to monitor the quaternary structure of G protein-coupled receptors. *FEBS J.* 272, 2914–2925
42. Lohse, M.J. *et al.* (2012) Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. *Pharmacol. Rev.* 64, 299–336
43. Vilardaga, J.P. *et al.* (2003) Measurement of the millisecond activation switch of G protein-coupled receptors in living cells. *Nat. Biotechnol.* 21, 807–812
44. Vilardaga, J.P. *et al.* (2005) Molecular basis of inverse agonism in a G protein-coupled receptor. *Nat. Chem. Biol.* 1, 25–28
45. Zürn, A. *et al.* (2009) Fluorescence resonance energy transfer analysis of $\alpha_{2A}$-adrenergic receptor activation reveals distinct agonist-specific conformational changes. *Mol. Pharmacol.* 75, 534–541

46. Bünemann, M. *et al.* (2003) $G_i$ protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc. Natl. Acad. Sci. U. S. A.* 100, 16077–16082
47. Galés, C. *et al.* (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. *Nat. Struct. Mol. Biol.* 13, 778–786
48. Lefkimmiatis, K. and Zaccolo, M. (2014) cAMP signaling in subcellular compartments. *Pharmacol. Ther.* 143, 295–304
49. Calebiro, D. and Maiellaro, I. (2014) cAMP signaling microdomains and their observation by optical methods. *Front. Cell. Neurosci.* 8, 350
50. Maiellaro, I. *et al.* (2016) cAMP signals in Drosophila motor neurons are confined to single synaptic boutons. *Cell Rep.* 17, 1238–1246
51. Surdo, N.C. *et al.* (2017) FRET biosensor uncovers cAMP nanodomains at β-adrenergic targets that dictate precise tuning of cardiac contractility. *Nat. Commun.* 8, 15031
52. Qin, K. *et al.* (2008) Abundance and stability of complexes containing inactive G protein-coupled receptors and G proteins. *FASEB J.* 22, 2920–2927
53. Feynman, R. and Gilbert, D. (1961) *Miniaturization,* pp. 282–296, Reinhold
54. Moerner, W.E. and Kador, L. (1989) Optical detection and spectroscopy of single molecules in a solid. *Phys. Rev. Lett.* 62, 2535–2538
55. Walter, N.G. *et al.* (2008) Do-it-yourself guide: how to use the modern single-molecule toolkit. *Nat. Methods* 5, 475–489
56. Suzuki, K. *et al.* (2005) Rapid hop diffusion of a G-protein-coupled receptor in the plasma membrane as revealed by single-molecule techniques. *Biophys. J.* 88, 3659–3680
57. Ha, T. *et al.* (1996) Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor. *Proc. Natl. Acad. Sci. U. S. A.* 93, 6264–6268
58. Vafabakhsh, R. *et al.* (2015) Conformational dynamics of a class C G-protein-coupled receptor. *Nature* 524, 497–501
59. Olofsson, L. *et al.* (2014) Fine tuning of sub-millisecond conformational dynamics controls metabotropic glutamate receptors agonist efficacy. *Nat. Commun.* 5, 5206
60. Levitz, J. *et al.* (2016) Mechanism of assembly and cooperativity of homomeric and heteromeric metabotropic glutamate receptors. *Neuron* 92, 143–159
61. Lamichhane, R. *et al.* (2015) Single-molecule view of basal activity and activation mechanisms of the G protein-coupled receptor $\beta_2$AR. *Proc. Natl. Acad. Sci. U. S. A.* 112, 14254–14259
62. Gregorio, G.G. *et al.* (2017) Single-molecule analysis of ligand efficacy in β2AR–G-protein activation. *Nature* 547, 68–73
63. Kusumi, A. *et al.* (2011) Hierarchical mesoscale domain organization of the plasma membrane. *Trends Biochem. Sci.* 36, 604–615
64. Hern, J.A. *et al.* (2010) Formation and dissociation of M1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. *Proc. Natl. Acad. Sci. U. S. A.* 107, 2693–2698
65. Kasai, R.S. *et al.* (2011) Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. *J. Cell. Biol.* 192, 463–480
66. Keppler, A. *et al.* (2003) A general method for the covalent labeling of fusion proteins with small molecules *in vivo*. *Nat. Biotechnol.* 21, 86–89
67. Calebiro, D. *et al.* (2013) Single-molecule analysis of fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics and organization. *Proc. Natl. Acad. Sci. U. S. A.* 110, 743–748
68. Calebiro, D. *et al.* (2014) Real-time monitoring of GPCR/cAMP signalling by FRET and single-molecule microscopy. *Horm. Met. Res.* 46, 827–832
69. Sungkaworn, T. *et al.* (2014) High-resolution spatiotemporal analysis of receptor dynamics by single-molecule fluorescence microscopy. *J. Vis. Exp.* e51784
70. Teichmann, A. *et al.* (2014) The specific monomer/dimer equilibrium of the corticotropin-releasing factor receptor type 1 is established in the endoplasmic reticulum. *J. Biol. Chem.* 289, 24250–24262
71. Veya, L. *et al.* (2015) Single molecule imaging deciphers the relation between mobility and signaling of a prototypical G protein-coupled receptor in living cells. *J. Biol. Chem.* 290, 27723–27735
72. Jonas, K.C. *et al.* (2015) Single molecule analysis of functionally asymmetric G protein-coupled receptor (GPCR) oligomers reveals diverse spatial and structural assemblies. *J. Biol. Chem.* 290, 3875–3892
73. Tabor, A. *et al.* (2016) Visualization and ligand-induced modulation of dopamine receptor dimerization at the single molecule level. *Sci. Rep.* 6, 33233
74. Sungkaworn, T. *et al.* (2017) Single-molecule imaging reveals receptor-G protein interactions at cell surface hot spots. *Nature* 550, 543–547
75. Mo, G.C. *et al.* (2017) Genetically encoded biosensors for visualizing live-cell biochemical activity at super-resolution. *Nat. Methods* 14, 427–434
76. Liang, Y.L. *et al.* (2017) Phase-plate cryo-EM structure of a class B GPCR-G-protein complex. *Nature* 546, 118–123
77. Zhang, Y. *et al.* (2017) Cryo-EM structure of the activated GLP-1 receptor in complex with a G protein. *Nature* 546, 248–253
78. Mortensen, K.I. *et al.* (2010) Optimized localization analysis for single-molecule tracking and super-resolution microscopy. *Nat. Methods* 7, 377–381
79. Ulbrich, M.H. and Isacoff, E.Y. (2007) Subunit counting in membrane-bound proteins. *Nat. Methods* 4, 319–321
80. Tian, H. *et al.* (2017) Labeling and single-molecule methods to monitor G protein-coupled receptor dynamics. *Chem. Rev.* 117, 186–245
81. Gautier, A. *et al.* (2008) An engineered protein tag for multiprotein labeling in living cells. *Chem. Biol.* 15, 128–136
82. Los, G.V. *et al.* (2008) HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* 3, 373–382
83. Lukinavičius, G. *et al.* (2013) A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nat. Chem.* 5, 132–139
84. Lavis, L.D. (2017) Teaching old dyes new tricks: biological probes built from fluoresceins and rhodamines. *Annu. Rev. Biochem.* 86, 825–843
85. Liu, C.C. and Schultz, P.G. (2010) Adding new chemistries to the genetic code. *Annu. Rev. Biochem.* 79, 413–444
86. Tian, H. *et al.* (2014) Bioorthogonal fluorescent labeling of functional G-protein-coupled receptors. *Chembiochem* 15, 1820–1829