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REVIEW ARTICLE

Revealing G-protein-coupled receptor oligomerization at the single-molecule level through a nanoscopic lens: methods, dynamics and biological function

Marco Scarselli[1], Paolo Annibale[2], Peter J. McCormick[3], Shivakumar Kolachalam[1], Stefano Aringhieri[1], Aleksandra Radenovic[2], Giovanni U. Corsini[1] and Roberto Maggio[4]

1 Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Italy
2 Laboratory of Nanoscale Biology, Institute of Bioengineering, School of Engineering, EPFL, Lausanne, Switzerland
3 School of Pharmacy, University of East Anglia, Norwich, UK
4 Biotechnological and Applied Clinical Sciences Department, University of L'Aquila, Italy

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Correspondence
M. Scarselli, Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Via Roma 55, Pisa 56126, Italy
Fax: +39-050-2218717
Tel: +39-050-2218716
E-mail: marco.scarselli@med.unipi.it

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The introduction of super-resolution fluorescence microscopy has allowed the visualization of single proteins in their biological environment. Recently, these techniques have been applied to determine the organization of class A G-protein-coupled receptors (GPCRs), and to determine whether they exist as monomers, dimers and/or higher-order oligomers. On this subject, this review highlights recent evidence from photoactivated localization microscopy (PALM), which allows the visualization of single molecules in dense samples, and single-molecule tracking (SMT), which determines how GPCRs move and interact in living cells in the presence of different ligands. PALM has demonstrated that GPCR oligomerization depends on the receptor subtype, the cell type, the actin cytoskeleton, and other proteins. Conversely, SMT has revealed the transient dynamics of dimer formation, whereby receptors show a monomer–dimer equilibrium characterized by rapid association and dissociation. At steady state, depending on the subtype, approximately 30–50% of receptors are part of dimeric complexes. Notably, the existence of many GPCR dimers/oligomers is also supported by well-known techniques, such as resonance energy transfer methodologies, and by approaches that exploit fluorescence fluctuations, such as fluorescence correlation spectroscopy (FCS). Future research using single-molecule methods will deepen our knowledge related to the function and druggability of homo-oligomers and hetero-oligomers.

Introduction

Fluorescence microscopy investigation of G-protein-coupled receptor (GPCR) oligomerization should enable the visualization of individual molecules and their relative proximity. Although individual fluorescent

Abbreviations
AR, adrenergic receptor; BRET, bioluminescence resonance energy transfer; FCCS, fluorescence cross-correlation spectroscopy; FCS, fluorescence correlation spectroscopy; FPR, formyl peptide receptor; FRET, fluorescence resonance energy transfer; GPCR, G-protein-coupled receptor; ICS, image correlation spectroscopy; LHR, luteinizing hormone receptor; PALM, photoactivated localization microscopy; PCH, photon-counting histogram; PD, photocontrollable dye; PTH1, parathyroid hormone 1; RET, resonance energy transfer; SMT, single-molecule tracking; sptPALM, single-particle tracking photoactivated localization microscopy; SPT, single-particle tracking; SSTR, somatostatin receptor; tICS, temporal image correlation spectroscopy; TIRF, total internal reflection fluorescence; TR-FRET, time-resolved fluorescence resonance energy transfer; WT, wild-type.

molecules are easily resolved when isolated, diffraction-limited methods are not able to determine their relative positions when many of them are densely packed in close proximity, as is often the case for GPCRs expressed on the plasma membrane. The concentration of GPCRs on the plasma membrane is highly variable, and depends on the receptor and cell type, covering the range from a few receptors per μm^2 up to hundreds of receptors per μm^2 [1,2]. Traditionally, fluorescence microscopy approaches try to avoid this problem. One of these approaches is based on an indicator of proximity such as resonance energy transfer (RET) between fluorescent or bioluminescent probes labelling two different receptors instead of visualizing their positions as individual receptors in a complex. An alternative method is based on fluorescence correlation spectroscopy (FCS), providing information on the diffusivity and aggregation state of oligomeric complexes.

However, the recent introduction of super-resolution fluorescence microscopy techniques [i.e. photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy, and stimulated emission depletion microscopy], recognized by the 2014 Nobel Prize in Chemistry, has provided an extraordinary tool with which to visualize biological structures in the nanometre range, and to characterize protein behaviour at the single-molecule level, independently of local density [3–5]. These new techniques permit: (a) counting the number of molecules in a protein cluster; (b) probing spatial interactions between different protein species; (c) determining the precise protein stoichiometry in signalling complexes; (d) visualizing interactions between receptors and their ligands; and (e) observing how single molecules move, interact and collide in living cells [6,7]. However, these techniques also present experimental challenges, and, in order to avoid artefacts, certain issues need to be taken into consideration before these methodologies can be applied correctly [8]. Traditional fluorescence microscopy has a diffraction-limited ability to resolve cellular structures, and the best resolution that can be achieved is two orders of magnitude larger than the actual molecule size (~ 250 nm). In contrast, the new super-resolution microscopy techniques, in particular those exploiting the stochastic activation of photocontrollable fluorophores, allow extension of the resolving power of conventional optics, and the localization of single molecules can therefore be determined with a precision up to 5–10 nm, which is much closer to their molecular size [9,10]. The advantages that can be gained by employing these novel approaches in the field of receptor signalling and molecular pharmacology are significant, particularly considering that the existence of receptor aggregates such as dimers and oligomers is a central topic in modern biology and biophysics.

This is relevant for GPCRs, for which the presence of receptor dimers and higher-order oligomers has been demonstrated [11–13]. On the one hand, evidence points to the fact that GPCR monomers are functional, but, on the other hand, many data support the existence of receptor dimers and oligomers [14–17].

The relevance of these studies is based on the premise that GPCR dimers (homodimers and heterodimers) and oligomers (homo-oligomers and hetero-oligomers) might be promising novel targets for developing more selective drugs that have fewer side effects [18,19]. To test the druggability of many GPCR oligomers that have been discovered to date, certain important questions related to receptor oligomerization need to be properly answered, such as: what are the sizes of such oligomers, what kinds of interactions are responsible for their formation, what functions do they serve, what are the factors controlling their formation, and do they exist *in vivo*?

This review addresses most of these questions by examining the findings obtained with PALM and single-molecule tracking (SMT) approaches. PALM uses photocontrollable fluorescent proteins, and SMT allows tracking of isolated single proteins in live cells. PALM is probably the most powerful method for the visualization of single molecules in dense samples where many receptors are localized within a few micrometres with a density similar to their physiological concentration [7,20–22]. In live cells, and at lower molecular concentrations, SMT allows visualization of receptors as single molecules, making it an ultimate tool with which to investigate their behaviour and interactions on the plasma membrane in the presence of different ligands [23]. This review also attempts to compare the results of super-resolution microscopy with those of classic approaches such as RET and FCS methodologies. Most of the experiments performed with fluorescence microscopy methods, including those using super-resolution imaging, support the existence of receptor oligomers of various sizes, depending on receptor subtype, cell type, and other proteins.

Comparing RET methodologies with single-molecule microscopy for studying GPCR oligomerization

For over a decade, RET-based approaches have been used to study the dimerization and oligomerization of GPCRs. The two oldest and most established techniques are fluorescence resonance energy transfer

(FRET) and bioluminescence resonance energy transfer (BRET). These approaches, instead of visualizing the relative positions of individual receptors, are dependent on receptor proximity, whereby, when they are near enough, energy transfer can occur between fluorescent or bioluminescent donors and acceptors labelling the two different receptors (Fig. 1). Both FRET and BRET exploit the strong distance dependence ($1/\text{distance}^6$) of RET between two identical (e.g. homo-FRET) or different (e.g. hetero-FRET) fluorophores to monitor any close interaction occurring between the proteins that they label. Although multiple types have been developed over the years, the basic premise, advantages and disadvantages have not dramatically changed. The advantage of BRET is that it can be performed with live cells over a large range of expression of the receptors. In addition, unlike FRET-based approaches, it does not require exposure to a laser, as the enzymatic catalysis by luciferase of its substrate provides the excitation energy. The disadvantage of BRET is that it requires large fusion proteins and examines the total receptor pool within the cell (e.g. plasma membrane, endoplasmic reticulum, Golgi, and endocytic system). In addition, it also requires a number of controls to validate the results. Despite these drawbacks, the technique has proven to be robust. BRET was one of the initial tools used to identify dimers, and its robust signal over a large range of receptor expression allows the delineation of specific and nonspecific interactions. Importantly, BRET interactions are confirmed via other approaches, such as coimmunoprecipitation and proximity ligation assays [24–26]. The proximity ligation assay has been particularly useful in helping to validate potential complexes in primary cells or in tissue [27]. More recently, BRET has been combined with both fluorescence complementation and FRET to provide the composition of oligomeric structures [28,29]. In addition to providing information about the architecture of GPCR complexes, BRET has hinted at the stoichiometry of receptor–G-protein interactions and G-protein activation. Cristóvão-Ferreira *et al.* found that two different G-proteins could be bound to A_1–A_{2A} heterodimers [30], an architecture surmised to involve four receptors. This model was confirmed by Guitart *et al.* [28] for dopamine D_1 and D_3 receptor heteromers, and was nicely reviewed in Ferre 2015 [31].

Others have used BRET to characterize the mechanisms of G-protein activation [32,33], arrestin recruitment [34], and scaffold association [35]. Indeed, the future of BRET may lie in developing powerful biosensors that will prove invaluable in deciphering the details of the function of GPCR oligomers.

FRET was applied simultaneously with the application and development of BRET to study GPCRs. As with BRET, many reviews have highlighted the findings and importance of FRET. In the context of oligomerization, FRET studies have also shown dimers for many GPCRs. An early application of the method was reported by Patel *et al.*, whereby ligands for somatostatin receptors (SSTRs) were conjugated to fluorophores of two colours (green fluorescent FITC and red fluorescent Texas Red), providing evidence of agonist-induced oligomerization [36]. On the other hand, investigation of neurokinin receptors (neurokinin-1 receptor) at near physiological concentration labelled by use of the acyl carrier protein labelling technique showed a prevalent monomeric arrangement of this receptor, with no dependence of the aggregation state upon agonist stimulation. No emission was observed at native expression levels (25 000 receptors per cell), whereas an increase in FRET was measured at higher expression levels (> 60 000 receptors per cell), indicative of oligomerization [2]. This study was important in pointing out problems associated with the overexpression of fluorescently labelled receptors when GPCR oligomerization is studied. In another study, Herrick-Davies *et al.* used FRET and showed that $5HT_{2C}$ can form dimers [37]. Although it was not related to GPCR association, a recent study investigating membrane protein aggregation found that the results of FRET analysis mimicked those of PALM, at least with regard to the level and number of dimers detected [38]. FRET has also been used in the setting of purified proteins. In an elegant example, using lanthanide as a donor, Rahmeh *et al.* have shown how ligands achieve different efficacies in G-protein activation and arrestin recruitment by stabilizing distinct conformations of V2R [39].

An important and powerful cousin of classic FRET is time-resolved FRET (TR-FRET) [40]. An early application of TR-FRET has demonstrated the advantage of this approach (reduced background), leading to an increased signal-to-noise ratio. More recent applications have used labelled ligands to show that dimers can form in tissue. This is a relevant step to confirm the actual existence of receptor dimers *in vivo* and to discover new molecules with therapeutic applications. In addition, TR-FRET is able to measure ligand affinities in individual protomers, which were previously inferred only through mathematical modelling in radioligand assays [41]. When RET approaches that use fluorescent fusion proteins are compared with super-resolution techniques for application to macromolecular questions, it is clear that the

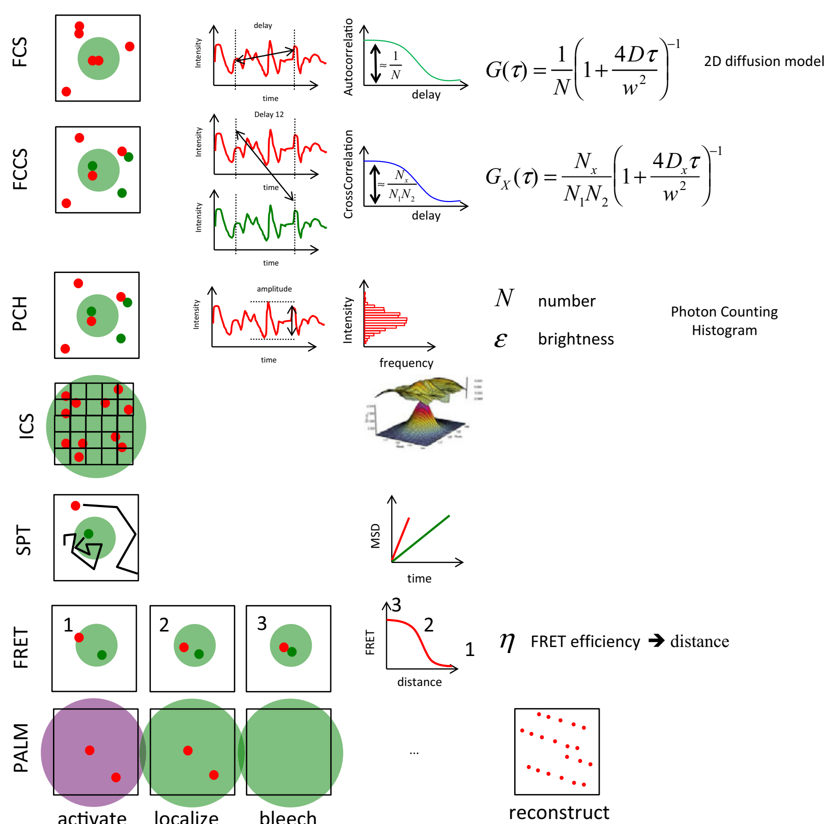


Fig. 1. Schematic overview of fluorescence microscopy methods for the study of receptor oligomerization. In FCS, fluorescence fluctuations arise when a molecule (or an oligomer) crosses through the excitation volume. The average duration of the fluctuations reflects the diffusion properties of the receptors. The experimental autocorrelation function can be fitted to a model to extract the actual diffusion coefficient (*D*) of the diffusing molecule. In FCCS, this concept is extended to two spectral channels. The PCH allows measurement of the amplitude of fluorescence fluctuations by obtaining the average oligomerization state of the aggregates that are diffused within the excitation volume. In ICS and related methods, the spatiotemporal information contained within an image series can be extracted, and the diffusion information can be obtained, and the size of the aggregates can also be measured. In SPT, individual molecules (if isolated) can be followed over time, and their trajectories and diffusion modes can be reconstructed. In FRET methods, the close proximity of two species can be assessed by exploiting the extreme sensitivity of RET to the distance between two dye molecules (nanometre scale). In PALM, the positions of a large number of molecules in a small region of space can be reconstructed by exploiting the sequential activation and bleaching of sparse subsets of photoactivatable fluorescent proteins.

latter provide better resolution and faster dynamics. However, many of the fundamental findings obtained with RET have withstood the nanoscopic lens: (a) GPCRs form dimers and, in some cases, higher-order complexes, and these change with receptor density; and (b) heterodimers can also form. This is reinforced by the findings of Renz *et al.* [38]. Although this study did not examine GPCRs, it demonstrated that FRET analysis and PALM provided similar results for the oligomerization of a membrane protein. The areas in which super-resolution microscopy will most likely help will be in determining the half-life of GPCR complexes. Various studies have summarized applications of SMT to GPCR oligomers [23,42,43]. They showed that, over a large population of several thousand receptors at the cell surface, dimers represent ~ 40% of a given receptor. Hence, on the basis of previous RET experiments, it would be reasonable to assume that the RET experiments are monitoring exactly that 40%. In fact, it might be that the dimers, and not the individual monomers, are physiologically important for signalling. Typically, RET approaches provide only an average of a series of populations, whereas single-molecule RET studies can address the real affinities and resonance times of these complexes. SMT showed a strong correlation between receptor density and dimerization. This latter finding is important when cellular compartments (endoplasmic reticulum or endocytic), restricted membranes (synapses) or tissues or cancer cells, in which certain GPCRs can be overexpressed, are considered.

Herrick-Davies *et al*. followed up their 2005 study with FCS and photon-counting histogram (PCH) analysis, which provides single-molecule sensitivity, and obtained similar findings as with FRET [37,44,45]. They then expanded this study to investigate a variety of class A receptors, and found little evidence for monomers; they argued that the basic unit is the homodimer. Importantly, they observed similar results in transfected cells and in native tissues [46]. This is a caveat of many of the existing single-molecule studies, which have not been performed in native conditions. In the future, more studies will be needed to extend these investigations to cell lines other than HeLa or HEK293 cells.

Another important question that RET approaches have yet to clearly answer is how ligand binding alters complex formation. Single-molecule studies suggest that ligands do not alter the dimer distribution. One interesting possibility related to GPCR dimerization is that the ligand determines the differences in temporal signalling or waves of signalling, as measured by Irannejad *et al*. [47]. What if different receptor states contribute to the temporal differences? Future single-molecule studies coupled with signalling assays may be able to address this feature. Alternatively, this may be an area where single-molecule RET studies could contribute. An exciting recent study using a modified PALM technique with photoactivatable dyes demonstrated that asymmetric hetero-oligomeric complexes could be formed by the use of receptor mutants that altered the protomers at the functional level and that this impacted on receptor signalling [48]. These results echo similar findings from other studies [12,49–53]. These important studies highlight the role of dimerization *in vivo* in providing regulation and plasticity for modulating different signalling pathways, and hence provide strong data in support of the importance of dimers, as a response to the questions posed by Lambert and Javitch concerning whether signalling cross-talk is attributable to receptor–receptor interactions [54]. It is clear that dimers can exist *in vivo* [19,25,40,51,55–62]. However, future experiments will need to focus on understanding how these complexes function in intact tissue. The combination of super-resolution techniques with approaches that allow tissue clarification might finally help to address the 'where' of GPCR complexes *in vivo*.

SMT and RET studies together leave a mixed picture, but, when they are coupled with studies in which function was also examined, it becomes more clear that GPCRs *in vivo* may function as dimers or, in some cases, as higher-order entities. As the discussion on dimers has now moved from 'if' to 'how', there still remain a number of questions at the molecular level. Are two and four protomers the only sizes, or do higher-order oligomers also exist? What drives dimer formation/stabilization? What is the function of dimers? There are several examples of the function of heterodimers *in vivo* [25,29,55,58,59,63–65], but very few on the purpose of homodimers. The answer most likely lies with signalling and/or trafficking regulation.

FCS applied to study GPCR mobility and oligomerization dynamics

Fluorescence fluctuation spectroscopy belongs to a set of fluorescence techniques that, although based on a principle that is completely different from that of PALM and single-particle-tracking (SPT) methods, can be effectively used to quantitatively investigate GPCR oligomerization. Fluctuations in the fluorescence signal originating from labelled molecules moving within the excitation volume of a microscope can be used as an indicator of molecular diffusion in a living cell, of binding of a ligand, and even of the molecular aggregation state (Fig. 1), thereby providing an alternative way to establish, or validate, findings related to GPCR oligomerization. The study of fluorescence fluctuations by means of autocorrelation analysis, namely FCS [66], has been extensively used over the last 40 years in a number of research domains, ranging from physics to biochemistry, that also include pharmacological studies of GPCRs [67]. Changes in the diffusion coefficient of a GPCR can have functional significance, as they reflect either a change in oligomerization state or partitioning in compartments of the plasma membrane. For example, A_1-adenosine receptors labelled with a fluorescent antagonist were investigated with FCS [68]. The study identified the existence of two populations of ligand-bound diffusing receptor: the faster population, at 0.9 $\mu m^2 \cdot s^{-1}$, was associated with individual diffusing receptors, whereas the slower population (0.05 $\mu m^2 \cdot s^{-1}$) was associated with either receptor aggregates or receptors partitioning into microdomains. Adenosine receptors were also investigated by Cordeaux *et al*. and Corriden *et al*., who measured the diffusion coefficient of A_3-adenosine receptors in CHO cells when bound, respectively, to a fluorescent agonist and an antagonist [69,70]. In both cases, two receptor species with distinct diffusive behaviour were observed: a fast population diffusing at 2.4 $\mu m^2 \cdot s^{-1}$ to 2.3 $\mu m^2 \cdot s^{-1}$, and a slower population with a diffusion coefficient of 0.13 $\mu m^2 \cdot s^{-1}$ to 0.09 $\mu m^2 \cdot s^{-1}$. The slow population of receptors probably corresponds to oligomeric complexes, and they were also observed when the receptors were fused to a

fluorescent protein in the absence of fluorescent ligands. Additionally, in competitive experiments using unlabelled agonists, antagonists, or allosteric compounds, they provided evidence of allosterism within the A_3-adenosine receptor dimers. It should be noted that, with measurement of only the diffusion coefficients, the changes in oligomerization, such as transitions from monomers to dimers or trimers,are very hard to detect (a dimer diffuses only 0.7 times more slowly than a monomer). Interestingly, Briddon *et al.* demonstrated that homodimers and heterodimers can somehow have different diffusion coefficients [71]. Furthermore, when they are measured with a hybrid technique that combines bimolecular fluorescence complementation and traditional FCS, the diffusion coefficients of heterodimers are higher than those of the monomers. The actual oligomerization state of a GPCR can also be assessed by employing fluorescence cross-correlation spectroscopy (FCCS), namely the extension of FCS to multiple colours. FCCS uses fluorescence fluctuations in two distinct spectral channels to measure codiffusion of two molecular species labelled with fluorophores having distinct emission wavelengths (Fig. 1). An early application of FCCS to study GPCR oligomerization was used by Patel *et al.* in their investigation of homo-oligomerization and hetero-oligomerization of SSTRs [36]. With the use of ligands conjugated to two distinct fluorophores (green fluorescent FITC and red fluorescent Texas Red), FCCS curves revealed that, whereas SSTR1 did not form significant homo-oligomeric complexes when coexpressed in conjunction with SSTR5, it gave rise to a significant degree of hetero-oligomerization on stimulation with somatostatin. Furthermore, SSTR5 was able to form homo-oligomers in the presence of the agonist. They speculated that ligand-induced conformational changes within SSTR1 do not expose a hydrophobic interface that would allow dimer formation. Interestingly, SSTR1 was not internalized after agonist exposure for longer times. This work presented some of the first evidence for ligand-dependent GPCR oligomerization, although this is not the case for many other GPCRs of the same class.

Cross-correlation techniques not only provide a static view of oligomerization state, but can also be used to obtain dynamic information regarding oligomer formation. Recently, FCCS analysis of opsin in live cell membranes showed a dynamic equilibrium between a monomer and a dimer [72]. The dimer population increased linearly with the square of the monomer concentration, similarly to what was demonstrated for FRP with SMT [43]. Notably, the authors used the concentration information contained within the FCCS data to determine the dissociation constant for the monomer–dimer equilibrium, and obtained a value of 1010 molecules per μm^2, which is much higher than those determined for other GPCRs. This difference might be attributable to specific dynamics in opsins. In general, other important information, such as brightness, is also contained within the time series of the fluorescence fluctuations measured in a confocal microscope, illuminating a diffraction-limited spot on the plasma membrane. The brightness of a fluorescently labelled probe is defined as the average number of photons per molecule that is collected. Intuitively, a sample with fewer and brighter particles will show larger fluorescence fluctuations than a sample with many dimmer particles, even if the average fluorescence intensity of the two samples is exactly the same. Therefore, analysis of the fluorescence time trace collected after exciting a confocal volume within the plasma membrane of a living cell expressing fluorescently labelled GPCRs can yield a brightness value that, when calibrated to the reference brightness of the fluorescent label, is able to provide information on the oligomerization state of the receptor (Fig. 1).

Herrick-Davis *et al.* [44] employed a specific type of brightness analysis, termed the PCH [73], to determine the oligomerization state of the 5-HT_{2C} receptor. By examining the distribution of the number of photons collected per unit time in a confocal microscopy setup, they observed that the 5-HT_{2C} receptor forms constitutive dimers in cultured HEK293 cells, and that these dimers were unaffected by drug treatment. This finding was confirmed for endogenous receptors expressed in choroid plexus epithelial cells labelled with anti-5HT_{2C} fragment antigen-binding protein, where the existence of functional homodimers in cells in their native cellular environment was shown [46]. Whereas agonist binding to one protomer resulted in G-protein activation, maximal stimulation required occupancy of both protomers. This evidence regarding the 5HT_{2C} receptor is in contrast to other studies, where it was claimed that a negative allosteric mechanism exists between the two protomers within the dimeric complex for other GPCRs. The approach was extended to other GPCRs, including adrenergic receptors (ARs) (α_{1B}-AR and β_2-AR), muscarinic receptors (M_1 and M_2), and dopamine receptors (D_1). They consistently observed that the existence of stable homodimers was unaffected by agonist stimulation and by their concentration [74]. We will compare these data with similar observations made by the use of SMT [75]. It should be noted that, although the PCH technique can provide information on the fraction of receptors that are, on average, components of

dimeric complexes, it cannot provide direct information on their stability if the lifetime of the complex is greater than the time needed to cross the diffraction-limited excitation spot. Furthermore, these experiments were performed in a concentration range an order of magnitude larger than those used in SMT highlighting the dynamic nature of the dimers, which may explain the increased stability measured for the dimeric complexes. Finally, these are point experiments that do not provide a spatial map of local aggregation within the cell, whereas PALM and SMT do.

In this respect, in recent years, a family of techniques dealing with fluorescence fluctuations within an entire image have been developed, and are closely related to the FCS approach. Image correlation spectroscopy (ICS) [76] methods allow the extraction of dynamic, kinetic and aggregation state information from time series of images, principally removing the need for confocal or two-photon excitation of a femtolitre volume required by FCS studies, and allowing the use of fast cameras to investigate these processes. The idea behind ICS is that the amount and size of the particles present in an otherwise noisy image can be calculated from the autocorrelation function of the image. A member of this family of techniques, temporal ICS (tICS), was employed by Wheeler *et al.* to investigate the impact of the cell cytoskeleton upon the diffusion coefficients of the parathyroid hormone 1 (PTH1) receptor and β_2-AR [77]. In particular, tICS performed both in confocal (60 ms per frame) and total internal fluorescence microscopy (300 ms per frame) setups showed that specific mutations of the PTH1 receptor, or latrunculin treatment, affect its interaction with the cytoskeleton and its diffusion coefficient. In addition, the immobile fraction of β_2-AR increased upon overexpression of the actin-binding protein NHERF1. These data provided by ICS confirmed the relevance of the actin cytoskeleton in receptor partitioning and oligomerization. The framework of tICS can be extended to multiple channels, resulting in image cross-correlation spectroscopy analysis. This approach was employed to study the interaction between the PTH1 receptor and β-arrestin on ligand stimulation, and revealed the formation of a complex between the two.

In conclusion, FCS techniques are very demanding methods with respect to hardware, sample preparation, and interpretation of the data. All sources of fluctuation that do not arise from the actual molecular diffusion of the fluorescently labelled species should be minimized, and, when they are present, the data should be corrected accordingly. In this respect, it is worth noting that even a relatively simple quantity, such as the diffusion coefficient of a GPCR, appears to have significantly different values when it is measured with multiple techniques. On the other hand, FCS approaches are very powerful techniques that allow the capture of GPCR dynamics with very high temporal resolution, down to the millisecond range. In addition, they are free of the isolated-molecule requirement of SMT methods, while still providing information on molecular diffusion.

PALM applied to visualize class A GPCR oligomers on the plasma membrane at the single-molecule level

To characterize GPCR oligomers with PALM, one fundamental requirement is the proper counting of the molecules within the nanoscale structures. The application of PALM is based on the serial and stochastic photoactivation of sparse fluorophores in the sample, temporally separating molecules that would otherwise be spatially indistinguishable. This approach reduces the size of the point spread function spot in a laser scanning microscope image, and allows an improvement of one order of magnitude in the localization of the single molecule (Fig. 1).

Operating PALM in total internal reflection fluorescence (TIRF) microscopy geometry is particularly advantageous for plasma membrane receptors, as it enhances the detection of single fluorescent molecules on the membrane within a thin layer of 100 nm from the coverslip. The precise quantification of the number of molecules in a sample is not simple, so analysis of experimental datasets needs to be performed carefully [8]. Multiple appearances of the same molecule caused by reversible blinking of individual fluorophores complicate quantitative analysis by generating apparent clustering artefacts [78]. As these artificial oligomers are formed within a limited time frame of a few seconds, they can be identified and eliminated just by examining their time domains. Given the stochastic nature of the photoactivation process in PALM, molecules belonging to genuine oligomers will show localization throughout the time span of the experiment, whereas the blinking molecules will show a clustered appearance in time. Sengupta *et al.* developed a pair correlation method (pair correlation PALM) to estimate the size of the aggregates and to determine the reappearance of artificial oligomers [79]. This approach uses image analysis to distinguish between a single protein with multiple appearances and oligomers of actual proteins. The term quantitative PALM was introduced for the quantification of single molecules, and may also represent a unique tool for the characterization of protein stoichiometry in

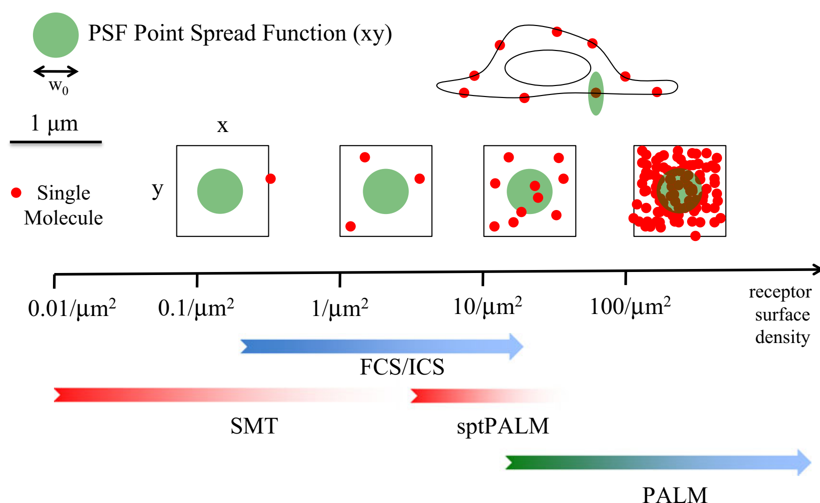


Fig. 2. Receptor surface density (e.g. GPCR) determines the fluorescence microscopy method that can be used to localize single molecules, and to study receptor dimerization/oligomerization. The point spread function has a radius of ~ 250 nm, and corresponds to the uncertainty of localization in a typical diffraction-limited microscope. SMT can be applied to cover a density range from a fraction to a few molecules per μm^2. If receptor density is higher than these values, SMT is not applicable. sptPALM extends this range up to tens of molecules per μm^2. FCS and ICS can be employed in a range of receptor concentrations, from less than one molecule per μm^2 to tens of molecules per μm^2. PALM can provide information on molecular localizations for concentrations up to hundreds of molecules per μm^2.

signalling complexes that are frequent in cellular activities.

Given the presence of potential photophysical artefacts, it is of paramount importance to perform appropriate controls while quantitatively investigating receptor oligomerization by the use of PALM. For determination of GPCR oligomers, a small monomeric peptide on the plasma membrane can be employed as a negative control for calibration [80,81]. This small peptide consists of 15 amino acids on the N terminus of the Src protein (SrcN15), which is myristoylated, and it was shown to be localized to the plasma membrane. The localization accuracy of the single molecules might be 20 nm or better. Under these conditions, PALM experiments have demonstrated that class A GPCRs, such as β_2-ARs and M_3-acetylcholinergic receptors, do not form higher-order oligomers (size of more than five molecules) in cellular recipients such as HeLa and CHO cells as compared with a negative control, even when expressed at high density. However, PALM cannot rule out the possibility of these receptors being arranged in dimers, trimers or tetramers as shown by SMT microscopy [75]. In this case, PALM on fixed samples and SMT on live cells are complementary approaches that allow investigation of an oligomerization range from dimers to aggregates of tens of molecules. PALM is ideal for samples in which the receptor density is medium to high, with few molecules present in the same diffraction-limited spot of ~ 200 nm (Fig. 2). This receptor density is comparable to the physiological conditions of many endogenous GPCRs [1,2]. However, in a cell line similar to cardiomyocytes, namely H9c2, higher-order oligomers of β_2-AR were identified that might correspond to tetramers, octamers and larger-size oligomers as a consequence of a specific cellular microenvironment and proteins present in this specific cell line (Fig. 3). In addition, the fractions of receptors that were components of higher-order oligomers or were isolated monomers were determined. This suggests that GPCR oligomerization might be influenced by the cell type. This was specific for β_2-AR, because another GPCR, the M_3-acetylcholinergic receptor, did not show oligomerization under the same conditions [80]. In these experiments, the receptor density on the plasma membrane ranged from 10 to 60 molecules per μm^2, a concentration similar to that of endogenous β_2-AR in cardiomyocytes and A549 cells [82]. Significantly, the fraction of oligomers was not influenced by receptor density, suggesting that these aggregates tend to be rather stable on the plasma membrane. Although SMT is not applicable in these high-density conditions, when it was applied to β_2-AR expressed at a low level, the degree of oligomerization appeared to increase with receptor density, in a range from 0.1 to 0.5 molecules per μm^2. Taken together, these data indicate that the degree of oligomerization increases with receptor density up to a concentration where it probably reaches a plateau.

At present, the function of higher-order oligomers is still unclear. In fact, although experimental data have

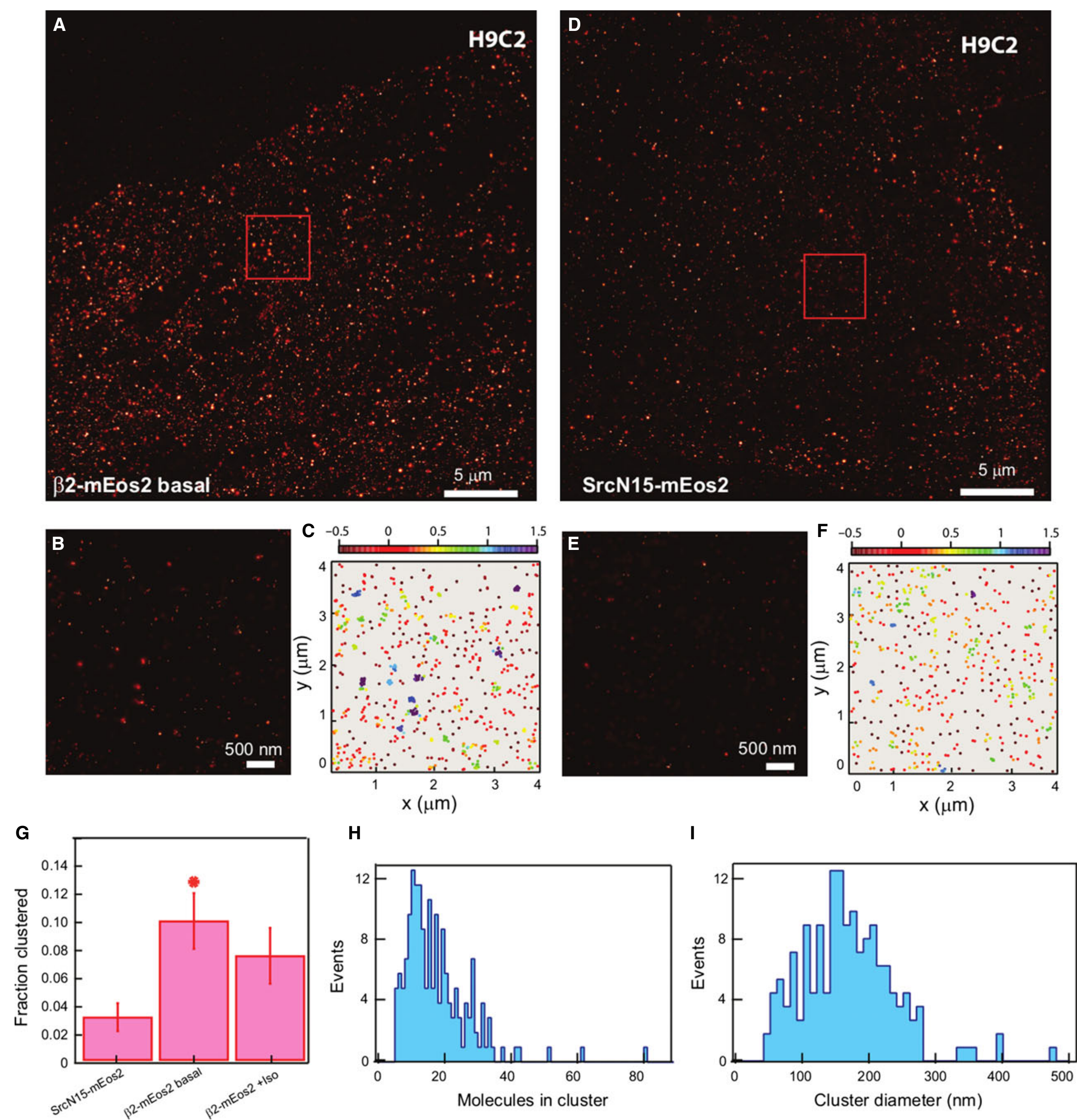


Fig. 3. PALM images in TIRF geometry of the prototypical GPCR β_2-AR labelled with the fluorophore mEos2 on the plasma membrane of the cardiomyocyte-like H9c2 cells, showing receptor oligomers. (A) Image of β_2-AR under basal conditions on the plasma membrane of fixed cells. (B) Magnified view of the boxed region of (A), showing oligomers of different sizes. (C) Schematic representation of the distribution of molecules showing oligomers (different colours represent the degree of oligomerization). (D) Image of the monomeric nonclustering peptide SrcN15 on the plasma membrane of fixed cells. (E) Magnified view of the boxed region of (D). (F) Schematic representation of the distribution of molecules. (G) Quantification of the oligomerization (fraction clustered) of β_2-AR with or without the agonist [isoproterenol (Iso) 10 μM for short time periods] as compared with the negative control SrcN15. Histogram representing the distribution of the number of molecules present in the clusters of β_2-AR for $n > 5$. Most of the oligomers have a size between five and twenty molecules. Histogram representing the distribution of the cluster diameter size of β_2-AR. Reproduced from Scarselli *et al.* [80].

provided some information on the functional role of homodimers and heterodimers, the role of higher-order oligomers is still unknown, as it is unclear whether the functional subunits of these aggregates might still be monomers and/or dimers. An intriguing hypothesis is that receptor oligomerization might be relevant for

concentrating second messengers and potentiating the signalling process in a specific region of the cell membrane, as demonstrated by PALM for T-cell antigen receptor clusters in initiating signalling in immune responses [83]. If this is the case, then, in the oligomeric structure, the GPCR might still function as a monomer and/or a dimer. To understand the role of oligomerization in GPCR function, it is important to determine what types of interaction are present within the oligomeric structures, and whether they are similar to or different from those present in the dimer. For the specific case of class C GPCRs, such as $GABA_B$ receptors, it was proposed that $GABA_B$ heterodimers are stable because of strong noncovalent interactions (as for GluR homodimers), whereas oligomeric complexes rely on weaker and transient interactions between heterodimers [75,84]. Similar conclusions were also reached by Patowary *et al.* regarding class A GPCRs such as M_3-acetylcholinergic receptors, which might exist as a stable dimeric unit and form tetramers reversibly [85]. They proposed that the interactions within the dimer are quite strong, as they did not find monomeric or trimeric species in the oligomeric mixture (but only multiples of dimers). In another example, the D_2-dopamine receptor was suggested to be organized in tetramers and probably in higher-order oligomers in which different transmembrane domains are involved [16]. Taken together, these data underline that the interactions within dimers might be different from those in the oligomer, and are probably stronger, with functional implications. Hence, more studies will be needed to understand the relevance of these differences. Another function associated with higher-order GPCR oligomers could be clustering during receptor internalization, as demonstrated by Scarselli and Donaldson, and also by Hanyaloglu and von Zastrow, regarding the rapid sequestration of receptors from the plasma membrane [86,87]. In this case, receptor oligomerization might be the consequence of the clustering of other proteins, such as clathrin during the formation of the coated pit. However, direct interactions between receptor protomers might still have a role in receptor internalization.

For β_2-ARs expressed in cardiomyocyte-like cells, the fraction of oligomers did not appear to be affected by the addition of the agonist for short times, indicating that an increase or a decrease in the number of higher-order oligomers is not necessary to activate downstream cAMP signalling. When receptor oligomerization was inhibited by actin cytoskeleton disruption, cAMP signalling was still intact [80]. In addition, in HeLa cells, where higher-order β_2-AR oligomers are not present, the GPCR was fully functional. This evidence supports the idea that β_2-AR oligomerization is not a strict requirement for activation of the cAMP pathway. However, we still cannot exclude the possibility that receptor clustering on a small scale might be necessary to concentrate second messengers in specific domains of the cell membrane. To demonstrate this hypothesis, the use of novel functional assays to measure the spatiotemporal dynamics of intracellular compartmentalized cAMP with FRET-based biosensors will enable a more accurate analysis [88].

Single-colour and dual-colour PALM to study GPCR oligomers interacting with subcellular structures – from actin cytoskeleton interactions to receptor hetero-oligomers

Another subject relevant to GPCR oligomerization is the role that other microenvironmental factors might play in influencing this phenomenon. Considering that GPCRs are localized on the plasma membrane, it is sensible to investigate the role of factors responsible for cell membrane heterogeneity, such as cholesterol and the actin cytoskeleton. In fact, studies examining these factors led to the proposal that cell membrane proteins might be organized into signalling platforms, such as miniclusters or domains, to maintain the fidelity and efficacy of transduction of the signal [89,90]. Lipid rafts are examples of highly dynamic cellular nanodomains enriched in cholesterol and sphingolipids that can act as membrane anchors for signalling molecules and induce protein aggregation [91,92]. As the size of these nanodomains is expected to be below the diffraction limit of light, the advantage provided by the use of super-resolution fluorescence microscopy is evident.

The findings obtained with PALM on β_2-AR oligomerization in H9c2 cardiomyocyte-like cells ruled out this possibility, supporting, rather, the hypothesis that GPCR oligomerization was not associated with lipid rafts (Fig. 4C). Similar results were observed for β_2-AR in HEK293 cells by Pontier *et al.* [93], where, even though the receptor itself was found outside lipid raft nanodomains, it was still considered to be part of the multimeric complex, as previously demonstrated by Ianoul *et al.* [94]. They proposed that maintaining the GPCR outside the cholesterol-enrichment domain, where the G-protein was present, might be necessary to limit basal receptor activity and to enhance receptor activation in the presence of agonist. These data contrast with those of other studies, in which an important role for the lipid rafts (and cholesterol) in facilitating GPCR signalling was demonstrated [95,96].

In fact, Nikolaev *et al.* found that cholesterol removal in cardiomyocytes of healthy rats caused a redistribution of β_2-AR [97]. For β_2-AR expressed in H9c2 cells, although cholesterol removal did not affect receptor oligomerization, inhibition of actin polymerization decreased the number of receptor oligomers, thereby demonstrating that receptor clustering is influenced by the actin cytoskeleton (Fig. 4C). It is widely accepted that some plasma membrane proteins interact with the actin cytoskeleton in both a direct way and an indirect way, the latter being mediated through scaffolding or actin-binding proteins. Notably, bidirectional relationships have been demonstrated between GPCRs and the actin cytoskeleton, whereby cytoskeleton and associated proteins affect the activities of the receptor, and the receptor can reciprocally influence actin cytoskeleton dynamics [98–100]. Interactions between a class C GPCR, named $GABA_B$ receptor, and the actin cytoskeleton were also found by Calebiro *et al.*, using SPT [75]. One possible role of the actin cytoskeleton is to confine GPCRs to specific cellular domains and/or to influence receptor diffusion in the plasma membrane in order to increase the probability of receptors encountering their signalling targets (e.g. the G-protein). In fact, both our group and other groups found that the β_2-AR diffusion coefficient measured by SMT in H9c2 cells, where the receptor forms higher-order oligomers, was lower than that in HeLa cells, with possible functional implications [75,101]. Notably, whereas actin disruption with latrunculin A dissociated β_2-AR oligomers, $GABA_B$ complexes remained intact after the same treatment. This demonstrates how the interactions within the GPCR oligomers might be diverse and, moreover, have different regulators. We might speculate that, for oligomers of a certain size, such as tetramers and octamers, direct protein–protein interactions are responsible for these functional complexes, whereas for higher-order oligomers ($n > 10$), subcellular structures, such as actin filaments, might favour their formation, leading them to concentrate in specific subcellular domains.

Images obtained with PALM-TIRF or SMT clearly showed that some GPCRs can adopt a linear actin-like organization (Fig. 4A) [77,102]. Our group was able to visualize β_2-AR oligomers in H9c2 cells colocalized with actin filaments labelled with EGFP (Fig. 4B). The

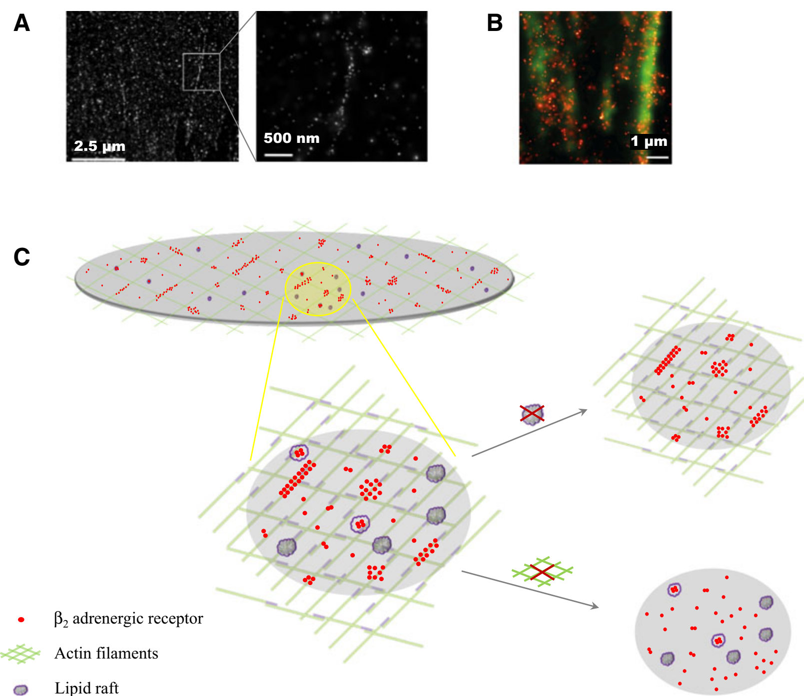


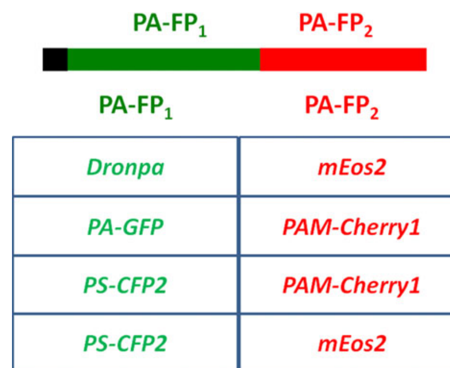
Fig. 4. β_2-AR oligomers formation is influenced by the actin cytoskeleton. (A) β_2-AR labelled with mEos2 when detected with PALM can assume a linear oligomeric actin-like organization in H9c2 cells [102]. This was also found for other GPCRs [75,77]. (B) β_2-AR oligomers detected with PALM (red) are mostly colocalized with actin filaments, labelled with EGFP in the background (green) [102]. (C) The magnified view shows how most of the β_2-AR oligomers interact with actin filaments. Disruption of actin (bottom right) with latrunculin A deletes most of the β_2-AR oligomers. However, only a few oligomers may be present in the lipid rafts (magnified view). The role of lipid rafts in GPCR oligomerization is controversial. Our group showed that removal of cholesterol does not affect β_2-AR oligomerization in H9c2 cells (top right), whereas Nikolaev *et al.* found that the removal of cholesterol led to redistribution of β_2-AR in cardiomyocytes of healthy rats [97].

same findings were made for $GABA_B$ receptors colocalizing with the actin fibre stain phalloidin [75].

Specific scaffolding proteins, such as EBP50, AKAP5, AKAP12, and SAP97, might also be involved in GPCR–actin interactions in H9c2 cells [101,102]. Confinement of β_1-AR and β_2-AR in the plasma membrane of H9c2 cells is mediated by selective interactions with PDZ proteins and A-kinase anchoring proteins, but not caveolae. These scaffolding proteins could become pharmacological targets to control β_2-AR function in specific tissues, with relevant therapeutic applications. Again, PALM data and SMT evidence are complementary, and both methods yielded useful information.

An important outstanding question in this field is how β_2-ARs behave under real physiological conditions. An elegant study [97] tried to address this difficult question, and analysed cAMP signalling mediated by β-ARs in rat cardiomyocytes. The authors found that β_2-AR activity was localized specifically in the deep transverse tubules, whereas β_1-ARs were distributed across the entire cell. They also proposed that cholesterol-rich membrane domains, i.e. lipid rafts, might be responsible for the existence of β_2-AR dimers. The case of β_2-AR in cardiomyocytes suggests that the same receptor can behave differently depending on the cellular environment, with important functional and pharmacological consequences. In fact, if the same receptor has a different quaternary structure depending on the tissue, this may lead to selective pharmacological intervention on specific cellular targets.

The successful application of PALM to study GPCR interactions with other subcellular structures in single-colour microscopy has stimulated our group and others to proceed to the second obvious step, i.e. dual-colour analysis. Soon after Betzig *et al.* proposed dual-colour analysis PALM [20], it was successfully applied by Shroff *et al.* to study adhesion complexes [103]. However, the application of dual-colour PALM is far from trivial, and many issues need to be taken into proper consideration for application of this methodology. Technically, the precision of recording of the two channels can be achieved with a setup that controls the mechanical drift and overall stability over time, particularly in the axial direction. With the use of a totally internally reflected near-infrared laser line, it is possible to build a feedback mechanism that keeps the objective stable within 5 nm [104]. Another critical issue is related to the photophysical properties of the two fluorophores chosen [105]. For a proper dual-colour PALM experiment, the relative photoconversion efficiency of the pair has to be well determined in order to estimate the real degree of colocalization.



PA-FP$_1$	PA-FP$_2$
Dronpa	*mEos2*
PA-GFP	*PAM-Cherry1*
PS-CFP2	*PAM-Cherry1*
PS-CFP2	*mEos2*

Fig. 5. For dual-colour PALM experiments, the relative photoconversion efficiency of the two fluorophores that are used as tags has to be determined in order to estimate the real degree of colocalization. In order to do this, constructs made of two fluorophores (PA-FP$_1$ and PA-FP$_2$) covalently linked for each pair can be used as a positive control for colocalization. A fused pair construct has a constrained 1 : 1 stoichiometry of the two fluorophores, and allows calculation of the relative photoconversion between the two; it also determines the colocalization efficiency of the system. To study these engineered constructs on the plasma membrane, it is possible to insert the small peptide of the Src protein (SrcN15) at the N terminus, which allows localization of the construct on the cell membrane (coloured in black).

In order to do this, a key preliminary experiment is to use genetically engineered constructs composed of two fluorophores covalently linked for each pair that are used as a positive control for colocalization (Fig. 5). A fused pair construct has a constrained 1 : 1 stoichiometry of the two fluorophores, and allows calculation of the relative photoconversion between the two and also determination of the colocalization efficiency of the system.

Our group found, among the different pairs of photoconvertible fluorophores examined, the best one to be PSCFP2–PAMCherry1 [104]. However, the efficiency of the dual-colour system is not yet optimal, and it will be improved by adopting new fluorophores with better photophysical properties, such as being irreversibly activatable. The couple PSCFP2–PAMCherry1 was the only pair tested that did not require sequential imaging, thereby maximizing the speed and, at the same time, increasing the quantitative outcome of the molecular counting process. The best-performing protein pair was applied to investigate the agonist-stimulated GPCR endocytosis known to proceed through clathrin-coated pits. The subdiffraction limit feature size of forming endosomes is an ideal system to be studied with super-resolution techniques. Dual-colour PALM was able to quantify the colocalization of β_2-AR with clathrin during internalization with and without addition of the ligand. It was found that 50% of

the receptor was localized with clathrin in the presence of the agonist, and considerably less without it. These data agree with previous imaging of GPCR endocytosis [86,106]. The same approach was applied by Subach *et al.* to study internalization of transferrin receptors via the clathrin pathway [107]. In addition, they investigated the same process with sptPALM to observe the trajectories of plasma membrane cargos, extracting their diffusion behaviour and their portioning into nanodomains [108].

Under experimentally optimal conditions, dual-colour PALM is an appealing method with which to study receptor hetero-oligomers, particularly in dense samples. However, this type of investigation, as compared with the study of dense structures such as endosomes, requires careful consideration for the accurate quantification of the number of molecules present in the hetero-oligomeric and/or heteromeric complex. Renz *et al.* used PALM to determine the stoichiometry of the hetero-oligomers of the asiaglycoprotein receptors RHL_1 and RHL_2 [38]. Coexpressed RHL_1 and RHL_2 receptors were fused to PAMCherry1 and PAGFP fluorescent proteins, and the fusion construct PAMCherry1–PAGFP was used to calibrate the relative detection efficiency. They observed a 1 : 1 ratio for homo-oligomer formation and a 2 : 1 RHL_1/RHL_2 ratio for hetero-oligomer formation. The formation of these receptor superstructures was influenced by different ligands modulating selective signalling pathways with relevant pharmacological applications. Notably, these results were confirmed with ensemble FRET imaging, validating the dual-colour PALM analysis.

Very recently, Jonas *et al.* applied dual-colour PALM to study GPCR oligomerization [48]. They adopted a different experimental strategy using CAGE photocontrollable dye (PD) PALM. In particular, PDs have several potential advantages, such as greater brightness and better photostability than fluorescent proteins, therefore enhancing the localization accuracy of the molecules up to 8 nm [48]. PDs also seem to have irreversible activation and bleaching [109], thereby eliminating the problem of multiple counting of the same fluorophores. One drawback, at least for now, is that they are conjugated to a primary antibody, which adds uncertainty to protein localization. With the application of photocontrollable dye PALM, it was found that 80% of wild-type (WT) luteinizing hormone receptor (LHR) homo-oligomers have a size of fewer than six receptors, whereas ~ 15% have a size of more than nine molecules. There were no changes in the relative proportions of dimers and different oligomers after ligand treatment, thereby confirming the general view on GPCR oligomerization following agonist addition. In addition, taking advantage of LHR mutants, one mutated in function and the other mutated in binding, they were able to identify different receptor hetero-oligomeric compositions as determinants for specific receptor functions. The coexpression of the two receptor mutants reconstituted human chorionic gonadotropin-mediated but not luteinizing hormone-mediated $G\alpha q/11$ responses, demonstrating that the organization within the reconstituted hetero-oligomers limits luteinizing hormone activation, probably because of specific geometry within the complex. They proposed that the orientation of the protomers within the receptor complex could influence specific receptor activities. Such a mechanism would provide a fine-tuned system to modulate signalling outputs, which may be adaptable for different cellular responses in physiological or pathological conditions. In addition, the specificity of distinct receptor complexes provides a pharmacological target for new compounds with greater selectivity and/or efficacy.

SMT applied to study GPCR dimerization dynamics in living cells

Unlike other imaging techniques, SMT allows direct inspection of how single proteins move, interact and collide in living cells [42]. There is no doubt that ‘seeing’ receptors as single molecules in live cells is the ultimate tool in cellular fluorescence microscopy for understanding their behaviour and interactions on the plasma membrane. The unprecedented technological advantages providing sensitivity in studying individual protein behaviour with high resolution has, in recent years, generated much new information that has revolutionized cell biology and pharmacology. SMT has been successfully applied to the study of plasma membrane organization, lipid rafts, clathrin-coated pits, focal adhesions, DNA transcription, and cell signalling [23]. In the GPCR field, SMT techniques have been used to help resolve the long-standing debate regarding the dynamics of the receptor quaternary structure, namely dimerization and oligomerization. In fact, by measuring the brightness of the molecules, after proper calibration of the system, it has become possible to determine the monomer, dimer and oligomer fractions for the GPCR examined in living cells, and to understand the dynamics of these complexes, and whether they are transient or stable. An important requirement is that the concentration of the labelled receptors must be low enough (a few receptors per μm^2) (Fig. 2). Under these conditions, it is possible to follow individual molecules and monitor their brightness, and hence observe the frequency and duration of molecular

contacts with an impressive millisecond temporal resolution. Another relevant aspect of SMT methodology is the need for a temporal resolution much higher (~ 20 times) than the duration of the transient dynamics of the biological event at which we are looking. In other words, with a time resolution of 5–10 ms in SMT, it is possible to properly follow transient interactions that are not faster than 50–100 ms. SMT is probably the most suitable method to determine whether GPCRs form dimers, and whether they are transient or stable. The first work on GPCRs was reported by Hern *et al.* on the M_1 muscarinic receptor, expressed in CHO cells, labelled with the fluorescently labelled antagonist telenzepine, by using TIRF microscopy [110]. The choice of this ligand was motivated by its high affinity and slow dissociation kinetics, whereby, after only a few minutes, almost all of the receptors were labelled at equilibrium. The ability of fluorescent ligands to all be fluorescently active is a relevant characteristic, whereas fluorescent proteins fused to the receptor might have folding issues and, in the case of photoactivatable proteins, not show complete photoconversion. The authors of the study observed that receptors exist as transient dimers with an average half-life of 0.5 s at 23 °C, and that they dissociate into monomers rapidly, so that, at equilibrium, 30% of M_1 receptors are dimers. This dynamic interaction was also confirmed by dual-colour imaging with Alexa 488 and Cy3B labels. This evidence was a breakthrough for the GPCR community, and encouraged many groups to use similar approaches to confirm these data.

Following this work, a year later, Kasai *et al.* used a similar approach to study the *N*-formyl peptide receptor (FPR), by labelling the receptor with a fluorescent agonist [43]. They observed that FPRs show a monomer–dimer equilibrium characterized by fast association and dissociation (Fig. 6). At an expression level of two receptors per μm^2, two FPR molecules form a dimer every 150 ms, and the lifetime of the dimer is very short, i.e. ~ 90 ms at 37 °C. This expression level is one to two orders of magnitude lower than under physiological conditions, as found for other GPCRs [1,2]. The authors demonstrated not only that dimer dissociation is rapid, but also that FPR monomers are quickly converted into dimers. At steady state, ~ 40% of the receptors are within dimeric complexes. This confirmed and extended previous data on M_1-acetylcholinergic receptors. Taken together, these two studies showed that GPCR dimerization is a rapid, dynamic process, regardless of the ligand used in the assay.

Surprisingly, the equilibrium between the monomer and the dimer is not changed by the addition of the agonist. The dependence of receptor dimerization on the addition of the agonist is controversial, although these findings are consistent with some previous observations [2]. In reality, to study FPR dimerization dynamics, the authors used an FPR(D71A) mutant that could not activate the G-protein and hence cannot internalize. This was necessary because the WT FPR tends to concentrate in the presence of the agonist, as a possible consequence of the internalization process. This tendency to form clusters with the agonist was also observed with PALM [80]. To confirm the robustness of their data, using the protein fluorescent mGFP, the authors showed that WT FPR formed a similar percentage of dimers as the FPR(D71A) mutant, and that this number did not change in the presence of agonist. Finally, SMT was successfully applied to study monoaminergic GPCRs, such as β-ARs. Calebiro *et al.* performed a comparative study investigating the dimerization and oligomerization state of three GPCRs, the β_1, β_2 and $GABA_B$ receptors, labelled by the use of SNAP-tag technology [75]. All three GPCRs analysed had differing degrees of dimers and higher-order oligomers, underlining how the oligomerization process is receptor-dependent, partly validating the relevance of the application. At equilibrium, although the average lifetimes of these complexes were similar, the percentage of β_2-AR dimers was greater than that of β_1-AR dimers. This might be the consequence of a specific interaction within the β_2-AR complex and/or of other factors. The authors proposed that distinct interactions with other proteins or localizations into different microdomains in the plasma membrane might

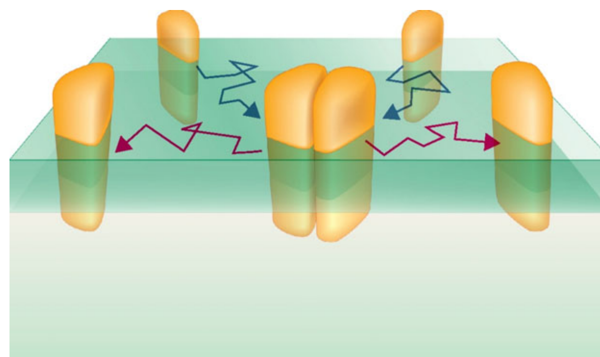


Fig. 6. Schematic picture representing the dynamics of the dimerization process characterized by fast association of two monomers and fast dissociation of the dimeric complex. Class A GPCRs continually form dimers and dissociate into monomers with different kinetic parameters, depending on the receptor subtype and the temperature of the assay. For FPR labelled with a fluorescent agonist, at steady state ~ 40% of the receptors are components of dimeric complexes. Reproduced from Kasai *et al.* [42] .

contribute to such differences. Notably, for β-ARs, the authors estimated a dimer lifetime of ~ 5 s at 20.5 °C, which is ~ 40 times longer than that of the FPR dimer (37 °C) and approximately six times longer than that of the M_1-acetylcholinergic receptor dimer (23 °C). This might be attributable to: the lower temperature employed in this study than in FPR experiments; the different methodologies used; and/or the different molecular interactions within the dimers of the receptors examined. Determining whether receptor dimer lifetime is actually different among GPCR family members and what are the functional consequences is a priority of SMT research, and in the near future these issues will hopefully be clarified. This is particularly relevant for understanding the dynamics of receptor heterodimerization between different receptors, where the lifetime of the heterodimeric complex might be a determinant for distinct functions and for targetability with new drugs. As mentioned, no effects of ligands on β_2-AR dimeric/oligomeric fractions were observed in fluorescence recovery after photobleaching and PALM experiments [111]. To interpret this evidence, we might assume that the conformational changes of individual protomers during activation within the dimer neither decrease nor strengthen the interactions between protomers. In addition, we can assume that changes in the number of dimers are not necessary for G-protein activation. Recently, Xue *et al.* proposed a mechanism of receptor activation through a change in the dimer interface for the class C GPCR $mGluR_2$, which is an obligatory dimer [49]. It remains to be verified whether class A GPCRs have a similar mechanism of activation. Calebiro *et al.* also found that $GABA_B$ receptors exist mostly as dimers and tetramers [75]. However, with higher receptor density, the proportion of the higher-order oligomers increased. $GABA_B$ receptor oligomers were mainly organized into ordered rows, through interactions with the actin cytoskeleton. Under real conditions, actin might be important for the spatial organization of receptors at synapses in the central nervous system, and this situation has some biological similarities with the interactions that we found for β_2-AR with actin in cardiomyocytes. Notably, for β_2-AR oligomers the interaction with actin determined their existence, but for $GABA_B$ complexes it did not. In fact, the elimination of actin fibres with latrunculin A abolished the $GABA_B$ organization in rows, but did not change the degree of oligomerization. This evidence suggests that GPCR oligomerization can be differently regulated among different receptors, but their functional consequences still need to be clarified.

In the near future, research will be carried out with dual-colour SMT to study interactions between different GPCRs in the heterodimerization process, between GPCRs and G-proteins, β-arrestins, or other effectors, and finally between GPCRs and their ligands. This approach is feasible, as was demonstrated with different SNAP and CLIP tags [112], or ligands with different bound fluorophores [110]. However, when the number of labelled molecules exceeds the limits posed by the diffraction limit with a density of 10 molecules per μm^2 or more, then SMT techniques are no longer appropriate (Fig. 2). Here, super-resolution methods such as sptPALM may provide a possible solution to this problem. Manley *et al.* employed sptPALM to investigate, in living cells, the distribution of a membrane protein such as tsO45 vesicular stomatitis virus G-protein and the HIV-1 structural protein Gag [113]. The method demonstrated the ability to observe the trajectories of these receptors within dense aggregates, extracting both their diffusion behaviour and their partitioning into microdomains. A two-colour application of this method was also successfully employed to study the colocalization between clathrin-coated pits and transferrin receptors during endocytosis [108]. For GPCR dual-colour sptPALM, at least for now, only a proof of principle has been demonstrated regarding β_2-AR together with the TfR receptor [114].

Conclusions and future directions

In the past, diffraction-limited microscopy techniques were unable to visualize individual receptors in experimental settings, where many of them are expressed in a few μm^2 on the plasma membrane.

Today, super-resolution microscopy techniques have been successfully applied to study class A GPCR homo-oligomers and hetero-oligomers at single-molecule level with unprecedented resolution. PALM has allowed the localization of receptors in dense samples where the concentration of GPCRs is similar to that under physiological conditions. SMT has shown its efficacy in determining receptor interactions in living cells, with extraordinary new evidence concerning GPCR dimerization. These methods have revealed how class A GPCR dimerization/oligomerization is a dynamic phenomenon depending on the receptor subtype, the cell type involved, and other factors, such as actin filaments. Notably, the presence of many GPCR dimers and oligomers has also been confirmed with other well-established fluorescence microscopy techniques, such as FCS and FRET/BRET, thus extensively validating this concept. The interactions within receptor dimers (and probably tetramers) seem to be

direct and different from those present in higher-order oligomers, with potential functional consequences. In the latter, subcellular compartments, such as the actin cytoskeleton, might favour their formation to concentrate them in certain domains. In fact, although we have found possible biological functions for receptor dimers (and tetramers), such as negative or positive allosterism, we are still searching for a role of GPCR higher-order oligomers; the concentration of the signal in specific cellular domains is, at least for now, only an attractive speculation. However, even though the existence of reversible class A GPCR dimers in living cells has been finally proven, a conclusive demonstration of their biological function is, in many cases, still lacking. On this topic, future research taking advantage of single-molecule microscopy and other biophysical or biochemical methods, particularly using receptor mutants, may be able to provide some answers. A preliminary application of this approach was demonstrated by combining PALM and FRET to study LHR and its mutants, where specific spatial interactions within the hetero-oligomeric complexes were determined to have specific cellular functions. Importantly, receptor dimer lifetimes seem to be different among the class A GPCRs that have been studied so far. However, this needs to be confirmed and extended to other members of the family, and, most importantly, the functional consequences need to be clarified. This is particularly relevant for understanding the dynamics of receptor heterodimerization between different receptors, where the lifetime of the heterodimeric complex could be critical for novel functions and for its targetability with novel drugs. The case of β_2-AR oligomers that are present only in cardiomyocytes and not in other cells offers the possibility of finding compounds with selective action on specific tissues. The application of single-molecule microscopy will make a tremendous contribution to finding new drugs active on GPCR homomers and/or heteromers, and specifically to confirm the interactions between ligands and specific protomers within the oligomeric complex. In the same direction, some groups have started to generate new evidence by using fluorescence ligands, an avenue that seems very promising. These studies can be extended to determine the interactions between the GPCR and the G-protein or β-arrestin, with tremendous impacts on understanding receptor signalling. One example is the recent study of Damian *et al.* investigating the precoupling of G-proteins to ghrelin receptors [115]. Finally, the potential role of the newly discovered GPCR dimer/oligomer needs to be confirmed *in vivo* in animals, and this is a topic that, although beyond the scope of this review, is already being investigated for some GPCR heteromers [25].

Author contributions

M. Scarselli conceived the general plan of the review and wrote most of the manuscript. P. Annibale wrote the section regarding FCS and contributed to the part related to PALM and SMT. P. J. McCormick wrote the section regarding FRET/BRET methodologies. P. Annibale, S. Kolachalam and S. Aringhieri produced the figures. M. Scarselli, P. Annibale, S. Kolachalam, A. Radenovic, G. U. Corsini and R. Maggio reviewed all of the manuscript. All authors contributed to writing the manuscript.

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