# Revealing GPCR 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], Roberto Maggio[4]

[1]Department of Translational Research and New Technology 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 NR4 7TJ

[4] Biotechnological and Applied Clinical Sciences Department, University of L'Aquila, L'Aquila, Italy

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*Corresponding author: Marco Scarselli, Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Via Roma 55, Pisa (56126), Italy

Tel: +39-050-2218716 Fax: +39-050-2218717

marco.scarselli@med.unipi.it

## Abstract

The introduction of super resolution fluorescence microscopy has allowed to visualize 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 coming from Photoactivated Localization Microscopy (PALM) that allows the visualization of single molecules in dense samples, and Single-Molecule Tracking (SMT) that 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, cell-type, actin cytoskeleton and other proteins. Conversely, SMT has revealed the transient dynamics of dimer formation, where receptors display 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 di-/oligomers is also supported by using well-known techniques, such as Resonance Energy Transfer (RET) 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 function and druggability of homo- and hetero-oligomers.


**Keywords:** G protein-coupled receptor; Oligomerization; Single-molecule Microscopy; Photoactivated Localization Microscopy; $\beta_2$ adrenergic receptor; Actin cytoskeleton

## Abbreviations

| | |
|---|---|
| BRET | Bioluminescence Resonance Energy Transfer |
| PALM-TIRF | Photoactivated Localization Microscopy Total Internal Reflection Fluorescence |
| FCCS | Fluorescence Cross-Correlation Spectroscopy |
| FCS | Fluorescence Correlation Spectroscopy |
| FPR | Formyl Peptide Receptor |
| FRET | Fluorescence Resonance Energy Transfer |
| GPCR | G-protein Coupled Receptor |
| GTP | Guanosine Triphosphate |
| HIV-1 | Human Immunodeficiency Virus - Type 1 |
| ICCS | Image Cross Correlation Spectroscopy |
| ICS | Image Correlation Spectroscopy |
| LHR | Luteinizing Hormone Receptor |
| PALM | Photoactivated Localization Microscopy |
| PCH | Photon Counting Histogram |
| PC-PALM | Pair Correlation Photoactivated Localization Microscopy |
| PD-PALM | Photocontrollable Dyes Photoactivated Localization Microscopy |
| qPALM | Quantitative Photoactivated Localization Microscopy |
| RET | Resonance Energy Transfer |
| SMT | Single Molecule Tracking |
| SPT | Single Particle Tracking |
| sptPALM | Single Particle Tracking Photoactivated Localization Microscopy |
| tICS | Temporal Image Correlation Spectroscopy |
| TIRF | Total Internal Reflection Fluorescence |

| | |
|---|---|
| TM | Transmembrane |
| TR-FRET | Time Resolved Fluorescence Resonance Energy Transfer |
| VSVG | Vesicular Stomatitis Virus G-protein |
| PDs | Photocontrollable Dyes |
| wt | Wild Type |
| wt-LHR | Wild Type Luteinizing Hormone Receptor |
| FRAP | Fluorescence Recovery After Photobleaching |

## Introduction

Fluorescence microscopy investigation of G protein-coupled receptor (GPCR) oligomerization should be able to visualize individual molecules and their relative proximity. Although individual fluorescent molecules are easily resolved when isolated, diffraction-limited methods are not able to determine their relative position 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 up to hundreds of receptors/$\mu m^2$ [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 labeling 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 microscopies (i.e. PALM, Photoactivated Localization Microscopy; STORM, Stochastic Optical Reconstruction Microscopy; STED, Stimulated Emission Depletion Microscopy) recognized by the 2014

Nobel Prize in Chemistry, has provided an extraordinary tool to visualize biological structures in the nanometer range, and to characterize protein behavior at the single-molecule level, independent of local density [3–5]. These new techniques permit: (i) counting the number of molecules in a protein cluster,(ii) probing spatial interactions between different protein species, (iii) determining precise protein stoichiometry in signaling complexes, (iv) visualizing interactions between receptors and their ligands, and (v) 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 artifacts, certain issues need to be taken into consideration before applying these methodologies 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 actual molecule size (about 250 nm). In contrast, the new super-resolution microscopy techniques, in particular those exploiting the stochastic activation of photo-controllable fluorophores, allow to extend the resolving power of conventional optics and hence, localization of single-molecules can be determined with a precision up to 5-10 nm, much closer to their molecular size [9,10]. The advantages that can be gained by employing these novel approaches in the field of receptor signaling 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, where the presence of receptor dimers and higher-order oligomers has been demonstrated [11–13]. On one hand, evidence points to the fact that GPCR monomers are functional, but on the other hand, much data supports 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? Which are the factors controlling their formation? Do they exist *in vivo*?

This review addresses most of these questions by examining the findings obtained using PALM and Single-Molecule Fluorescent Tracking (SMT) approaches. PALM uses photocontrollable fluorescent proteins, while SMT allows tracking of isolated single proteins in live cells. PALM is probably the most powerful method to visualize single molecules in dense samples where many receptors are localized within a few microns with a density similar to their physiological concentration [20–23]. In live cells, and at lower molecular concentrations, SMT allows visualization of receptors as single molecules, making it an ultimate tool to understand their behavior and interactions on the plasma membrane in the presence of different ligands [24]. This review also attempts to compare the results of super resolution microscopy with classical approaches such as RET and FCS methodologies. Most of the experiments performed using fluorescence microscopy methods, including those using super resolution imaging, support the existence of receptor oligomers of varying size depending on receptor subtype, cell-type and other proteins.

## Comparing RET methodologies with single-molecule microscopy to study GPCR oligomerization

For over a decade, RET based approaches have been used to study 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 position of individual receptors, are dependent upon receptor proximity, where when near enough energy transfer can occur between fluorescent or bioluminescent donors and acceptors labeling the two different receptors (Fig. 1). Both FRET and BRET exploit the strong distance dependence ($1/\text{distance}^6$) of resonance energy transfer 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 flavours 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 done using 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 of luciferase on 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 (eg. plasma membrane, endoplasmic reticulum, Golgi, endocytic system, etc.). In addition, it also requires a number of controls to validate observed 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 delineation of specific and non-specific interactions. Importantly, BRET interactions are born out via other approaches like co-immunoprecipitation and proximity ligation assay [25–27]. The proximity ligation assay has been particularly useful in helping to validate potential complexes in primary cells or in tissue [28]. More recently, BRET has been

combined with both fluorescence complementation and FRET to provide the composition of oligomeric structures [29,30]. In addition to gaining information about the architecture of GPCR complexes, BRET also has hinted at the stoichiometry of receptor/G protein interactions and on G protein activation. Cristóvão-Ferreira et al. found that two different G proteins could be bound to $A_1$-$A_{2A}$ heterodimers [31], an architecture surmised to involve four receptors. This model was confirmed by Guitart et al. [29] for dopamine $D_1$ and $D_3$ receptors heteromers and nicely reviewed in Ferre 2015. [32].

Others have used BRET to characterize mechanisms of G protein activation [33,34], arrestin recruitment [35], or scaffold association [36]. 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.

Simultaneous to the application and development of BRET to study GPCRs was the application of FRET. 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., where ligands to somatostatin receptors were conjugated to fluorophores of two colors (green fluorescent FITC and red fluorescent Texas Red) providing evidence of agonist-induced oligomerization [37]. On the other hand, investigation of neurokinin (NK1R) receptors near physiological concentration labeled using the AcylCarrier protein labeling 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 (25000 receptors/cell), whereas an increase in FRET was measured at higher expression (>60000 receptors/cell), indicative of oligomerization [2]. This study was important in pointing out problems associated with over-expression of fluorescently labeled receptors when studying GPCRs oligomerization. In another study, Herrick-Davies et al. used FRET and showed that

$5HT_{2C}$ can form dimers [38]. Although not related to GPCR association, a recent study looking at membrane protein aggregation found that FRET analysis mimicked that found by PALM, at least in regards to the level of amount and number of dimers detected [39]. FRET has also been used in the setting of purified proteins. In an elegant example, using lathanide 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 the V2R [40].

An important and powerful cousin of classical FRET is time resolved FRET (TR-FRET) [41]. An early application of TR-FRET has demonstrated the advantage of this approach (reduced background) leading to 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, previously inferred only through mathematical modelling in radioligand assays [42]. When applied to macromolecular questions and comparing RET approaches that use fluorescent fusion proteins with super resolution techniques, it is clear that the latter provide better resolution and faster dynamics. However, many of the fundamental observations discovered by RET have withstood the nanoscopic lens: 1) GPCRs form dimers and in some cases higher order complexes, and these change with receptor density 2) heterodimers can also form. This is reinforced by the findings by Renz et al. [39]. Although this study did not examine GPCRs, it has demonstrated that FRET analysis and PALM provided similar results on the oligomerization of a membrane protein. The areas where 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 [24,43,44]. They showed that over a large population of several thousand receptors, at the cell surface, dimers represent about 40% of a given receptor. Hence, based on 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 are physiologically important for signaling, and not the individual monomers. Typically, RET approaches only provide an average of a series of populations; whereas single molecule RET studies can address the real affinities and resonance times of these complexes. SMT reported a strong correlation to receptor density and dimerization. This latter finding is important when considering cellular compartments (endoplasmic reticulum or endocytic), or restricted membranes (synapses), or in tissues or cancer cells where certain GPCRs can be overexpressed. Herrick-Davies et al. followed up their 2005 study with FCS and photon counting histogram analysis, that provides single molecule sensitivity and observed similar findings as FRET [38,45,46]. They then expanded this study to look at a variety of Class A receptors and found little evidence for monomers and argued that the basic unit is the homodimer. Importantly, they observed similar results in transfected cells and in native tissues [47]. This is a caveat of many of the existing single molecule studies lacking in biologically relevant systems. In the future, more studies will be needed to extend these investigations to cell lines other than HeLa or HEK 293 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 seem to alter dimer distribution. This could be explained by the paper of Kasai et al., where transmembranes (TMs) drive interaction within the dimer, not extra-cellular regions and the short lifetime of dimers and G-protein association may not be dramatically changed by ligand binding [43]. Another interesting possibility related to GPCR dimerization is that the ligand determines the differences in temporal signaling or waves of signaling measured by Irannejad et al. [48]. What if different receptor states contribute to the temporal differences? Future single molecule studies coupled with signaling 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 using receptor mutants that altered the protomers at the functional level and that this impacted receptor signalling [49]. These results echo similar findings from other studies [12,50–54]. These important studies highlight the role of dimerization *in vivo* for providing regulation and plasticity in modulating different signalling pathways, and hence provides strong data in support for the importance of dimers as a response to the questions posed by Lambert and Javitch concerning whether signalling crosstalk is due to receptor-receptor interactions [55]. It is clear that dimers can exist *in vivo* [19,26,41,52,56–63]. However, future experiments will need to focus on understanding how these complexes function in an 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 coupled with studies where function was also examined, it becomes more clear that GPCRs *in vivo* may function as dimers or in some cases higher order entities. As the discussion on dimers has now moved from not "if", but 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? Several examples of the function of heterodimers exist *in vivo* [26,30,56,59,60,64–66], but very few on the purpose of homodimers. The answer most likely lies with signaling 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 completely different from PALM and single particle-tracking methods, it can be effectively used to quantitatively investigate GPCR oligomerization. Fluctuations in the fluorescence signal originating from labeled molecules moving within the excitation volume of a microscope can be used as an indicator of molecular diffusion in a living cell, in binding of a ligand and even in 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 [67] 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 [68]. Changes in the diffusion coefficient of a GPCR can have a 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 labeled with a fluorescent antagonist, were investigated using FCS by [69]. The study identified the existence of two populations of ligand-bound diffusing receptor: the faster one at 0.9 $\mu m^2/s$ was associated with individual diffusing receptors, while the slower population (0.05 $\mu m^2/s$) 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 [70,71]. In both cases, two receptor species with distinct diffusive behavior were observed, a fast population diffusing at 2.4 $\mu m^2/s$ - 2.3 $\mu m^2/s$ and a slower one with a diffusion coefficient of 0.13 $\mu m^2/s$ - 0.09 $\mu m^2/s$. The slow population of receptors likely 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 unlabeled agonists, antagonists or allosteric compounds, they provided evidence of allosterism within the $A_3$-AR dimers. It should be noted that, by measuring 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 slower than a monomer). Interestingly, Briddon et al. demonstrated that homo and heterodimers can somehow have different diffusion coefficients [72]. Furthermore, when measured with a hybrid technique that combines bimolecular fluorescence complementation and traditional fluorescence correlation spectroscopy, 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 colors. FCCS uses fluorescence fluctuations in two distinct spectral channels to measure co-diffusion of two molecular species labeled 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- and heteroligomerization of somatostatin receptors [37]. Using ligands conjugated to two distinct fluorophores (green fluorescent FITC and red fluorescent Texas Red), FCCS curves revealed that while SSTR1 receptor did not form significant homoligomeric complexes, when co-expressed in conjunction with the SSTR5 receptor, it gave rise to a significant degree of heteroligomers upon stimulation with somatostatin. Furthermore, SSTR5 receptors were able to form homoligomers 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 upon agonist exposure for longer times. This work presented some of the first evidence of 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 they can also be used to obtain dynamic information regarding oligomer formation. Recently, FCCS analysis of opsin in live cell membranes determined their dynamic equilibrium between a monomer and a dimer [73]. The dimer population increased linearly with the square of the monomer concentration, similar to what was demonstrated for FRP receptor by using SMT [44]. Notably, the authors used the concentration information contained within the FCCS data for determining the dissociation constant for the monomer-dimer equilibrium, obtaining a value of 1010 molecules/$\mu m^2$, which is much higher than those determined for other GPCRs. This difference might be attributed to specific dynamics in opsins. In general, another 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 labeled probe is defined as the average number of photons/molecule that is collected. Intuitively, a sample with fewer and brighter particles will display 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, the analysis of the fluorescence time trace collected from exciting a confocal volume within the plasma membrane of a living cell expressing fluorescently-labeled 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. [45] employed a specific type of brightness analysis, termed the Photon Counting Histogram (PCH) [74], 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 HEK 293 cells, and that these dimers were unaffected by drug treatment. The

study was confirmed for endogenous receptors expressed in choroid plessus epithelial cells labeled using anti-$5HT_{2C}$ fragment antigen binding protein, showing the existence of functional homodimers in cells in their native cellular environment [47]. While agonist binding to one protomer resulted in G protein activation, maximal stimulation required occupancy of both protomers. This evidence on $5HT_{2C}$ receptor runs in contrast to other works 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 ($\alpha_{1B}$-AR and $\beta_2$-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 [75]. We shall compare these data with similar observations made by SMT [76]. It should be noted that while the PCH technique can provide information on the fraction of receptors that are, on average, part of dimeric complexes, it cannot provide direct information on their stability if the lifetime of the complex lasts more than the time necessary to cross the diffraction limited excitation spot. Furthermore, these experiments were performed in a concentration range in order of magnitude larger than those by SMT highlighting a 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, but PALM and SMT instead do.

In this respect, in recent years, a family of techniques dealing with fluorescence fluctuations within an entire image has been developed, and it is closely related to the FCS approach. Image Correlation Spectroscopy (ICS) [77] methods allow extracting dynamics, kinetic and aggregation state information from time series of images, principally removing the need for confocal or two photon-excitation of a femtoliter 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 by looking at the autocorrelation function of the image. A member of this family of techniques, temporal Image Correlation Spectroscopy (tICS) was employed by Wheeler et al. to investigate the impact of the cell cytoskeleton upon the diffusion coefficient of the $PTH_1$ and $\beta_2$-AR [78]. In particular, tICS performed both in confocal (60 ms/frame) and Total Internal Fluorescence Microscopy setups (300 ms/frames) were used to observe that specific mutations of the $PTH_1$ receptor, or latrunculin treatment, affect its interaction with the cytoskeleton and its diffusion coefficient. In addition, the immobile fraction of $\beta_2$-AR increased upon overexpression of the actin binding protein NHERF1. These data offered by ICS confirmed the relevance of actin cytoskeleton in receptor partitioning and oligomerization. The framework of tICS can be extended to multiple channels, resulting in Image Cross Correlation Spectroscopy (ICCS) analysis. They employed this approach to study the interaction between $PTH_1$ receptor and β-arrestin upon ligand stimulation, revealing 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 fluctuations that do not arise from the actual molecular diffusion of the fluorescently labeled species should be minimized and, when present, the data 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 measured using multiple techniques. On the other hand, FCS approaches are very powerful techniques that allow capture of GPCR dynamics with very high temporal resolution, down to the ms 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 using 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 (PSF) spot in a laser scanning microscope image and allows an improvement of one order of magnitude in the localization of the single molecule (Fig. 2).

Operating PALM in Total Internal Reflection Microscopy (PALM-TIRF) 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 and hence, 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 artifacts [79]. Since 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 *bona fide* oligomers will display localization throughout the time span of the experiment, while the blinking molecules will display clustered appearance in time. Sengupta et al. developed a pair correlation method (PC-PALM) to estimate the size of the aggregates and to determine the reappearance of artificial oligomers [80]. This approach uses image analysis to distinguish between a single protein with multiple appearances and oligomers of actual proteins. The term qPALM (quantitative PALM) was introduced for quantifying single molecules, and may also

represent a unique tool for characterization of protein stoichiometry in signaling complexes that are frequent in cellular activities.

Given the presence of potential photophysical artifacts, it is of paramount importance to perform appropriate controls while quantitatively investigating receptor oligomerization using PALM. For determining GPCR oligomers, a small monomeric peptide on the plasma membrane can be employed as a negative control for calibration [81,82]. This small peptide consists of 15 amino acids on the N-terminus of the Src protein (SrcN15) that 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 $\beta_2$-adrenergic ($\beta_2$-AR) and $M_3$-acetylcholinergic receptors, do not form higher-order oligomers (size of more than five molecules) in a cellular recipient such as HeLa and CHO cells when compared with a negative control, even when expressed at high density. However, PALM can not rule out whether these receptors might be arranged in dimers, trimers or tetramers as shown by SMT microscopy [76]. In this case, PALM on fixed samples and SMT on live cells are complementary approaches that allow investigating an oligomerization range going from dimers to aggregates of tens of molecules. PALM is ideal for samples where receptor density is medium-high with few molecules present in the same diffraction-limited spot of about 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, notably it was demonstrated the existence of higher-order oligomers of $\beta_2$-AR that might correspond to tetramers, octamers and larger size oligomers as a consequence of a specific cellular micro-environment and proteins present in this specific cell-line (Fig. 3). In addition, it was determined the fractions of receptors that were part of higher-order oligomers as well as isolated monomers. This suggests that GPCR oligomerization might be influenced by the

cell-type. This was specific for $\beta_2$-AR because another GPCR, the $M_3$-acetylcholinergic receptor did not show oligomerization under the same conditions [81]. In these experiments, receptor density on the plasma membrane ranged from 10 to 60 molecules/$\mu m^2$, a concentration similar to the expression of endogenous $\beta_2$-AR in cardiomyocytes and A549 cells [83]. 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 low-level expressed $\beta_2$-AR, the degree of oligomerization appeared to increase with receptor density, in a range from 0.1 to 0.5 molecules/$\mu m^2$. 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 the moment, the function of higher-order oligomers is still unclear. In fact, if the functional role of homodimers and heterodimers has found some meaning supported by experimental data, the role of higher-order oligomers is still missing, 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 to concentrate second messengers and potentiate the signaling process in a specific region of the cell membrane, as demonstrated by PALM for T cell antigen receptor clusters in initiating signaling in immune responses [84]. 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 kind of interactions are present within the oligomeric structures and if they are similar or different compared to the ones present in the dimer. If we look at the specific case of class C GPCRs, such as $GABA_B$ receptors, it was proposed that $GABA_B$ heterodimers are stable due to strong noncovalent interactions (same for GluR homodimers), while oligomeric complexes rely on weaker and transient interactions

between heterodimers [76,85]. 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 [86]. 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 where different TM domains are involved [16]. Taken together, these data underline that the interactions within dimers compared to the one present in the oligomer might be different and probably stronger with functional implications. Hence, more studies will be necessary 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 rapid sequestration of receptors from the plasma membrane [87,88]. 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 $\beta_2$-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 of higher-order oligomers is not necessary to activate the downstream cAMP signaling. When receptor oligomerization was inhibited by actin cytoskeleton disruption, cAMP signaling was still intact [81]. In addition, in HeLa cells, where higher-order $\beta_2$-AR oligomers are not present, the GPCR was fully functional. This evidence supports the idea that $\beta_2$-AR oligomerization is not a strict requirement for the activation of the cAMP pathway. However, we still cannot exclude 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 using FRET-based biosensors will enable a more accurate analysis [89].

## Single and dual color PALM to study GPCR oligomers interacting with sub-cellular structures: from actin cytoskeleton interactions to receptor hetero-oligomers

Another subject relevant to GPCR oligomerization is the role that other micro-environmental 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 actin cytoskeleton. In fact, studies examining these factors led to the proposal that cell membrane proteins might be organized into signaling platforms, such as mini-clusters or domains, to maintain the fidelity and efficacy in the transduction of the signal [90,91]. Lipid rafts are an example of highly dynamic cellular nanodomains enriched in cholesterol and sphingolipids that can act as membrane anchors for signaling molecules and induce protein aggregation [92,93]. 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 fluorescent microscopy is evident.

By using PALM, the findings on $\beta_2$-AR oligomerization in H9c2 cardiomyocytes-like cells ruled out this possibility, rather supporting the hypothesis that the GPCR oligomerization was not associated with lipid rafts (Fig. 4C). Similar results were observed for $\beta_2$-AR in HEK 293 cells by Pontier et al. [94] where, even though the receptor itself was found outside lipid-rafts nanodomains, it was still considered part of multimeric complex as it was previously demonstrated by Ianoul et al. [95]. They proposed that maintaining the GPCR outside the cholesterol-enrichment domain, where the G protein was present, it might be necessary to

limit basal receptor activity and to enhance receptor activation in the presence of agonist. These data are in contrast with other papers where an important role for the lipid rafts (and cholesterol) in facilitating GPCR signaling was demonstrated [96,97]. In fact, Nikolaev et al., found that cholesterol removal in cardiomyocytes of healthy rats, caused a redistribution of $\beta_2$-AR [98]. For $\beta_2$-AR expressed in H9c2 cells, although cholesterol removal did not affect receptor oligomerization, the inhibition of actin polymerization decreased the number of receptor oligomers, thereby demonstrating that the 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 and indirect way, the latter being mediated through scaffolding or actin-binding proteins. Notably, bidirectional relationships have been demonstrated between GPCRs and the actin cytoskeleton, where cytoskeleton and associated proteins affect the activities of the receptor and the receptor can reciprocally influence actin cytoskeleton dynamics [99–101]. Interactions between a class C GPCR, named $GABA_B$ receptor, and actin cytoskeleton was also found by Calebiro et al. using SPT [76]. One possible role of 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 receptor encountering its signaling targets (e.g. the G protein). In fact, ours, as well as other groups, found that the $\beta_2$-AR diffusion coefficient measured by SMT in H9c2 cells, where the receptor forms higher-order oligomers, was lower compared to HeLa cells with possible functional implications [76,102]. Notably, while actin disruption with latrunculin A dissociated $\beta_2$-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, while for higher-order oligomers ($n > 10$) subcellular structures, such

as actin filaments, might favor 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) [78,103]. Our group was able to visualize $\beta_2$-AR oligomers in H9c2 cells colocalized with actin filaments labeled with eGFP (Fig. 4B). The same indications were obtained for $GABA_B$ receptors colocalizing with the actin fibers stain phalloidin [76].

Specific scaffolding proteins such as EBP50, AKAP5, AKAP12 and SAP97, might also be involved in GPCR/actin interactions in H9c2 cells [102,103]. Confinement of $\beta_1$- and $\beta_2$-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 a pharmacological target to control $\beta_2$-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 $\beta_2$-AR behave under real physiological conditions. An elegant paper [98] tried to address this difficult question and analyzed cAMP signaling mediated by β-AR in rat cardiomyocytes. The authors found that $\beta_2$-AR activity was localized specifically in the deep transverse tubules, whereas $\beta_1$-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 $\beta_2$-AR dimers. The case of $\beta_2$-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. the 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 [104]. However, the application of dual color PALM is far from trivial and many issues need to be taken into proper consideration to apply 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. Using a totally internally reflected near-infrared laser line, it is possible to build a feedback mechanism keeping the objective stable within 5 nm [105]. Another critical issue is related to the photophysical properties of the two fluorophores chosen [106]. For a proper dual color PALM experiments, the relative photoconversion efficiency of the pair has to be well determined in order to estimate the real degree of colocalization.

In order to do so, a key preliminary experiment is to use genetically engineered constructs made of two fluorophores covalently linked for each pair that are used as a positive control of co-localization (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 to determine the co-localization efficiency of the system.

Our group, among the different pairs of photo convertible fluorophores examined, found the best one to be PSCFP2-PAMCherry1 [105]. However, the efficiency of the dual color system was not yet optimal and 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 sub-diffraction limit feature size of forming endosomes is an ideal system to be studied using super-resolution techniques. Dual color PALM was able to quantify the colocalization of $\beta_2$-AR with clathrin during internalization with and without the 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 [87,107]. The same approach was applied by Subach et al. to study internalization of transferrin receptors via the clathrin pathway [108]. In addition, they investigated the same process with single particle tracking PALM (sptPALM) to observe the trajectories of plasma membrane cargos, extracting their diffusion behavior and their portioning into nanodomains [109].

Under experimentally optimal conditions, dual colour PALM is an appealing method to study receptor hetero-oligomers, particularly in dense samples. However, this kind of investigation, compared to the study of dense structures 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 heterooligomers of the asiaglycoprotein receptor $RHL_1$ and $RHL_2$ [39]. Co-expressed $RHL_1$ 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 homoligomer formation and a 2:1 $RHL_1$:$RHL_2$ ratio for heteroligomer formation. The formation of these receptor super-structures was influenced by different ligands modulating selective signaling pathways with relevant pharmacological applications. Notably, these results were confirmed with ensemble FRET imaging validating the dual color PALM analysis.

Very recently, Jonas et al. applied dual color PALM to study GPCR oligomerization [49]. They adopted a different experimental strategy using CAGE photocontrollable dyes (PDs) PALM (PD-PALM). In particular, photocontrollable dyes 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 [49]. PDs also seem to have irreversible activation and bleaching [110], therefore 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 that adds uncertainty to protein localization. Applying PD-PALM, they found that 80% of wild type (wt) Luteinizing Hormone Receptor (LHR) (wt-LHR) homo-oligomers have a size of less than six receptors while about 15% have a size of more than nine molecules. There was no change in the relative proportions of dimers and different oligomers after ligand treatment, thereby confirming the general view about GPCR oligomerization following agonist addition. In addition, taking advantage from LHR mutants, one in the function and the other in the binding, they were able to identify different receptor hetero-oligomeric compositions as determinant for specific receptor functions. The coexpression of the two receptor mutants reconstituted only hCG- but not LH-mediated Gαq/11 responses, demonstrating that the organization within the reconstituted heterooligomers limits LH activation probably due to 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 signaling 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

Compared to other imaging techniques, SMT allows direct inspection of how single proteins move, interact and collide in living cells [43]. There is no doubt that the possibility to "see" receptors as single molecules in live cells is the ultimate tool in cellular fluorescent microscopy to understand their behavior and interactions on the plasma membrane. The unprecedented technological advantages yielding the sensitivity to study individual protein behavior with high resolution has generated much new data in recent years 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 signaling [24]. In the GPCR field, SMT techniques have been used to probe the long-standing debate regarding the dynamics of the receptor quaternary structure, namely di- and oligomerization. In fact, measuring the brightness of the molecules, after a proper calibration of the system, it has become possible to determine the monomer/dimer/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 labeled receptors must be low enough (a few receptors/um$^2$) (Fig. 2). Under these conditions, it is then possible to follow individual molecules and monitor their brightness, hence observing the frequency and duration of molecular contacts with an impressive ms temporal resolution. Another relevant aspect in SMT methodology is the need of a temporal resolution much higher (about 20 times) than the duration of the transient dynamics of the biological event which we are looking at. In other words, with a time resolution of 5-10 ms in SMT, it is possible to follow properly transient interactions which are not faster than 50-100 ms. SMT is probably the most suitable method to determine whether GPCRs form dimers, and if they are transient of stable. The first work on GPCRs was reported by Hern et al. on $M_1$ muscarinic receptor, expressed in CHO cells,

labeled with the fluorescently-labeled antagonist telenzepine using TIRF microscopy [111]. The choice of this ligand was motivated by its high affinity and slow dissociation kinetics, where, just after a few minutes, almost all the receptors were labeled at the equilibrium. The capability of fluorescent ligands to all be fluorescently active is a relevant characteristic, while fluorescent proteins fused to the receptor might have folding issues and, in the case of photoactivatable proteins, not a complete photoconversion. The authors of the study observed that receptors exist as transient dimers with an average halftime of 0.5 s at 23°C and they dissociate into monomers rapidly where at the equilibrium 30% of $M_1$ receptors are dimers. This dynamic interaction was also confirmed in a dual-color imaging using 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 N-formyl peptide receptor (FPR) labeling the receptor with a fluorescent agonist [44]. They observed that FPR receptors display a monomer-dimer equilibrium characterized by fast association and dissociation (Fig. 6). At an expression level of 2 receptors/$\mu m^2$, two FPR receptor molecules form a dimer every 150 ms, and the lifetime of the dimer is very short, about 90 ms at 37°C. This expression level is about 1-2 order of magnitude less than physiological conditions as found for other GPCRs [1,2]. The authors demonstrated that not only dimer dissociation is rapid but also FPR monomers convert quickly into dimers. At steady state about 40% of the receptors are part of dimeric complexes. This confirmed and extended previous data on $M_1$-acetylcholinergic receptor. 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 a 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 using PALM [81]. To confirm the robustness of their data, using the protein fluorescent mGFP, the authors showed that wt-FPR forms a similar percentage of dimers compared to FPR(D71A) mutant, and 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 di- and oligomerization state of three GPCRs, the $\beta_1$, $\beta_2$ and $GABA_B$ receptors, labeled using SNAP-tag technology [76]. All three GPCRs analyzed had differing degree of dimers and higher-order oligomers underlining how the oligomerization process is receptor-dependent, in a way validating the relevance of the application. At equilibrium, though the average lifetime of these complexes was similar, the percentage of $\beta_2$-AR dimers was greater than $\beta_1$-AR dimers. This might be the consequence of a specific interaction within the $\beta_2$-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 be involved for such differences. Notably, for β-ARs, the authors estimated a dimer lifetime of approximately 5 s at 20.5°C, which is about 40 times longer than FPR dimer (37°C) and about 6 times longer than $M_1$-acetylcholinergic receptor dimer (23°C). This might be due to: the lower temperature employed in this study compared to FPR experiments, the different methodologies used and/or the different molecular interactions within the dimer of the receptor examined. To understand if receptor dimer lifetime is actually different among GPCR family members and what the functional consequences are is a priority of SMT research and in the near future this topic 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 determinant for distinct functions and for targetability with new drugs. As mentioned, no effects of ligands on $\beta_2$-AR dimeric/oligomeric fraction were observed also in Fluorescence Recovery After Photobleaching (FRAP) and in PALM experiments [112]. To interpret this evidence, we might assume that the conformational changes of individual protomers during activation within the dimer neither decreases nor strengthens 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 [50]. 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 [76]. However, with higher receptor density, the proportion of the higher-order oligomers increased. $GABA_B$ receptors oligomers were prevalently 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 we found for $\beta_2$-AR with actin in cardiomyocytes. Notably, while for $\beta_2$-AR oligomers the interaction with actin determined their existence, for $GABA_B$ complexes it did not. In fact, the elimination of actin fibers 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 whereas their functional consequences still need to be clarified.

In the near future, research will be carried out using the dual color 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 it was demonstrated using different SNAP- and CLIP tags [113], or ligands with different bound fluorophores [111]. However, when the number of labeled molecules exceeds the limits posed by the diffraction limit with a density of 10 molecules/$\mu m^2$ 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 (VSVG) and the human immunodeficiency virus type 1 (HIV-1) structural protein Gag [114]. The method demonstrated the ability to observe the trajectories of these receptors within dense aggregates, extracting their diffusion behavior as well as their partitioning into micro-domains. A two-color application of this method was also successfully employed to study the colocalization between clathrin coated pits and transferrin receptors during endocytosis [109]. For GPCR dual colour sptPALM, at least for now, only a proof of principle has been demonstrated regarding $\beta_2$-AR together with TfR receptor [115].

## Conclusions and future directions

In the past, diffraction-limited microscopies were unable to visualize individual receptors in experimental settings where many of them are expressed in a few $\mu m^2$ on the plasma membrane.

Today, super resolution microscopy techniques have been successfully applied to study class A GPCR homo- and hetero-oligomers at single molecule level with an unprecedented resolution. PALM has allowed localizing receptors in dense samples where the concentration of GPCRs is similar to their physiological condition. SMT has shown its efficacy in determining receptor interactions in living cells with extraordinary new evidence about GPCR dimerization. These methods have revealed how class A GPCR di-/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 validated using also other well established fluorescence microscopy techniques, such as FCS, FRET/BRET, thus extensively validating this concept. The interactions within receptor dimers (and probably tetramers) seem direct and different from the ones present in higher-order oligomers having potentially functional consequences. In the latter, subcellular compartments, such as actin cytoskeleton, might favor their formation to concentrate them in certain domains. As a matter of fact, if on one hand receptor dimers (and tetramers) have found a possible biological meaning, such as negative or positive allosterism, then on the other hand, GPCR higher-order oligomers are still searching for a role, where 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, the concluding answer for their biological function in many cases is 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 combining PALM and FRET to study LHR and its mutants, where specific spatial interactions within the hetero-oligomeric complexes were determined for specific cellular functions. Importantly, receptor dimer lifetimes seems 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, it has to be clarified which are the functional consequences. 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 $\beta_2$-AR oligomers present only in cardiomyocytes and not in other cells offers the possibility to find compounds with a selective action on specific tissues. To

find new drugs active on GPCR homomers and/or heteromers, the application of single molecule microscopy will make a tremendous contribution 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 using fluorescence ligands, an avenue that seems very promising. These studies can be extended to understand the interactions between the GPCR and the G protein or β-arrestin with tremendous impact in receptor signaling. One example is the recent study of Damian et al. looking at pre-coupling of G proteins to ghrelin receptors [116]. Finally, the potential role of the new GPCR di-/oligomer discovered needs to be confirmed *in vivo* in animals and this is a topic that, though beyond the scope of this review, is already being investigated for some GPCR heteromers [26].

## Author contributions

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

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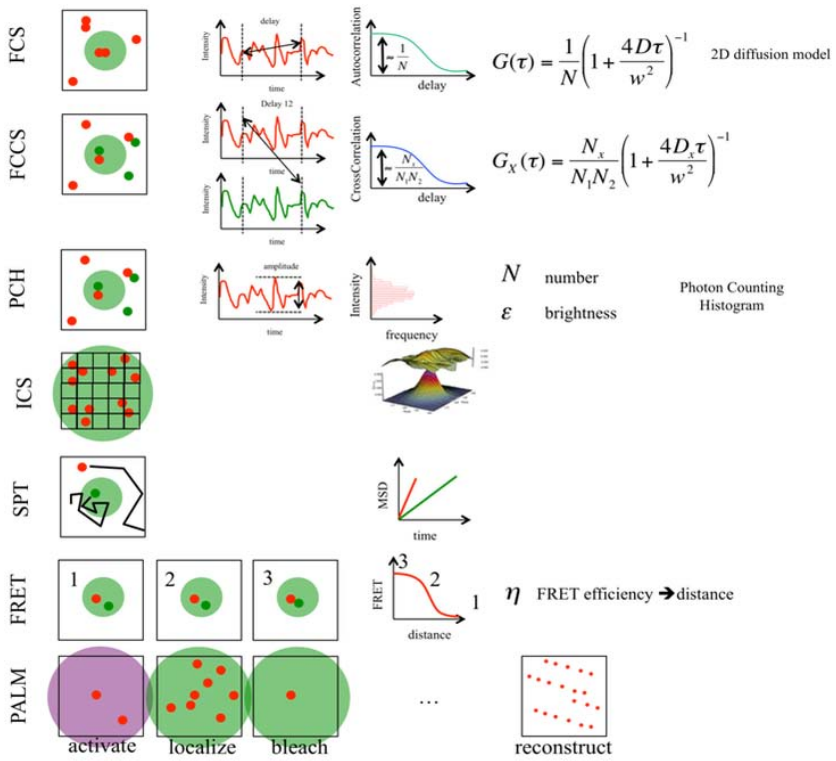


**Fig 1.** Schematic overview of fluorescence microscopy methods for the study of receptor oligomerization. In FCS, fluorescence fluctuations arise when a molecule (or an oligomer) crosses through the excitation volume. The average duration of the fluctuations reflects the diffusion properties of the receptors. The experimental autocorrelation function can be fit to a model to extract the actual diffusion coefficient (D) of the diffusing molecule. In Fluorescence Cross Correlation Spectroscopy (FCCS), this concept is extended to two spectral channels. The Photon Counting Histogram (PCH) allows measurement of the amplitude of fluorescence fluctuations by obtaining the average oligomerization state of the aggregates getting diffused within the excitation volume. In Image Correlation Spectroscopy (ICS) and related methods, the spatiotemporal information contained within an image series

can be extracted and the diffusion information as well as the size of the aggregates can also be measured. In Single Particle Tracking (SPT), individual molecules (if isolated) can be followed over time, and their trajectory and diffusion modes can be reconstructed. In Fluorescence Resonance Energy Transfer Methods (FRET), close proximity of two species can be assessed by exploiting the extreme sensitivity of resonance energy transfer to the distance between two dye molecules (nm-scale). In Photoactivated Localization Microscopy (PALM), the position 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.

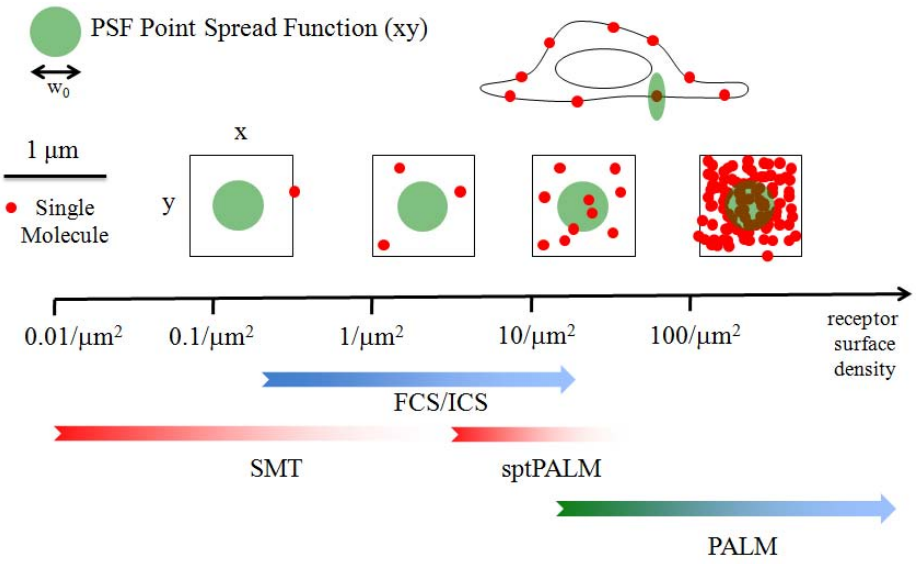


**Fig 2.** Receptor surface density (e.g. GPCR) determines the fluorescence microscopy method that can be used to localize single molecules, and to study receptor di-/oligomerization. The Point Spread Function (PSF) has a radius of approximately 250 nm, and corresponds to the uncertainty of localization in a typical diffraction-limited microscope. Single Molecule Tracking (SMT) can be applied to cover a density range from a fraction to a few molecules/µm$^2$. If receptor density is higher than these values, SMT is not applicable. Single

Particle Tracking Photoactivated Localization Microscopy (sptPALM) extends this range up to tens of molecules/$\mu m^2$. Fluorescence and Image Correlation Spectroscopy methods (FCS and ICS) can be employed in a range of receptor concentrations going from less than 1 molecule/$\mu m^2$ to tens of molecules/$\mu m^2$. Photoactivated Localization Microscopy (PALM) can provide information on molecular localizations for concentrations up to hundreds of molecules/$\mu m^2$.

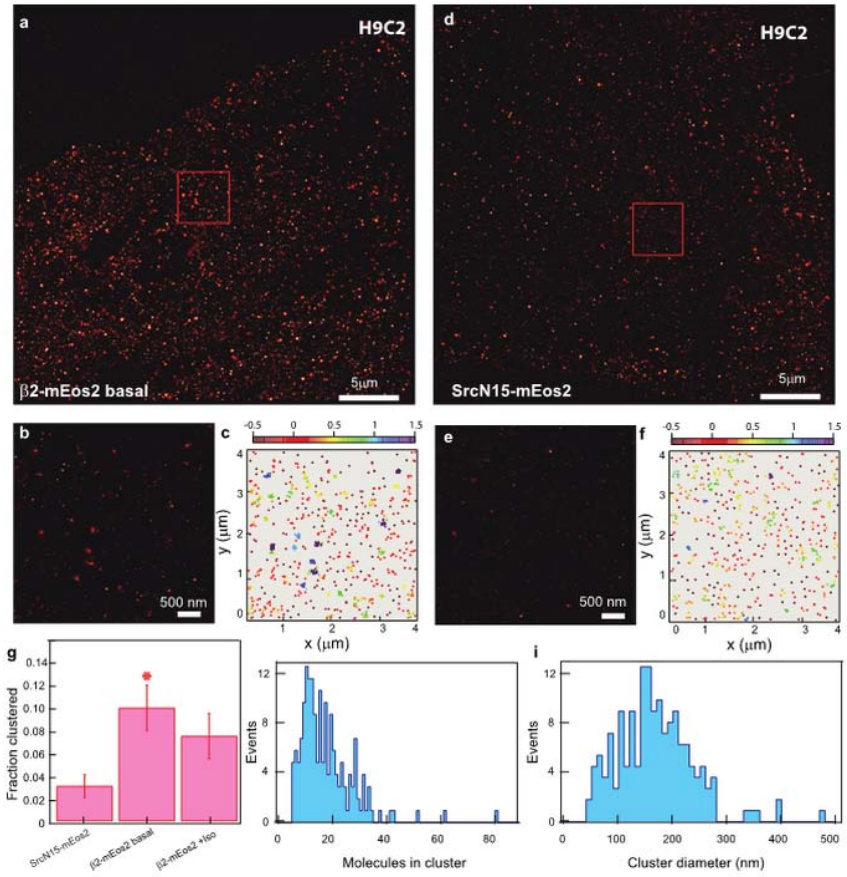


**Fig 3.** PALM images in TIRF geometry of the prototypical GPCR $\beta_2$-adrenergic receptor labeled with the fluorophore mEos2 on the plasma membrane of the cardiomyocytes-like H9c2 cells showing receptor oligomers. a) Image of $\beta_2$-AR under basal conditions on the plasma membrane of fixed cells. b) Magnified view of boxed region of "*a*" showing oligomers of different sizes. c) Schematic representation of the distribution of molecules

showing oligomers (different colors represent the degree of oligomerization). d) Image of the monomeric non-clustering peptide SrcN15 on the plasma membrane of fixed cells. e) Magnified view of boxed region of "*d*". f) Schematic representation of the distribution of molecules. g) *Left,* Quantification of the oligomerization (fraction clustered) of $\beta_2$ adrenergic receptor with or without the agonist (Isoproterenol 10μM for short times) compared to the negative control SrcN15. *Center,* histogram representing the distribution of the number of molecules present in the clusters of $\beta_2$-AR for n > 5. Most of the oligomers have a size between 5 and 20 molecules. *Right,* histogram representing the distribution of the cluster diameter size of $\beta_2$-AR. Reproduced from Scarselli et al. [81].

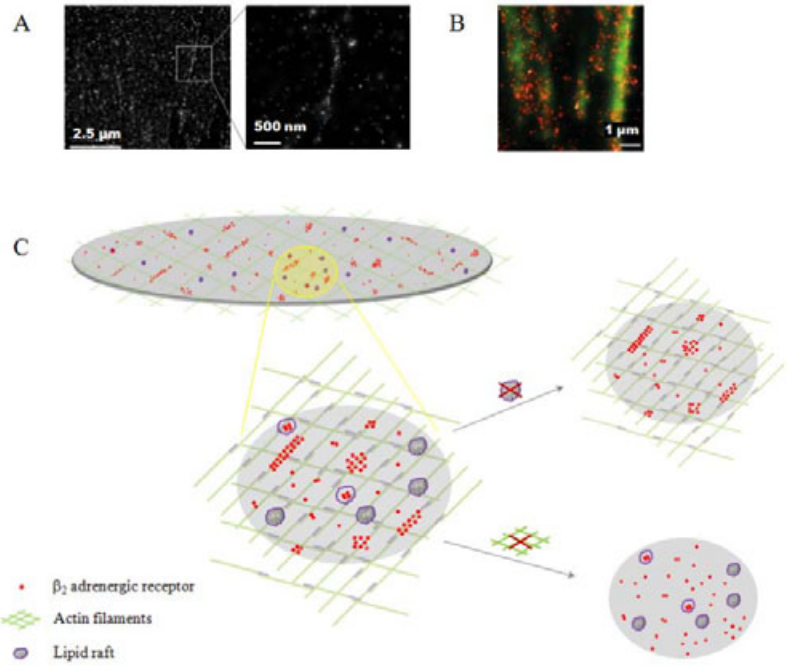


**Fig 4.** $\beta_2$-AR oligomer formation is influenced by actin cytoskeleton. a) $\beta_2$-AR labeled with mEos2 when detected with PALM can assume a linear oligomeric actin-like organization in H9c2 cells [103]. This was also found for other GPCRs [76,78]. b) $\beta_2$-AR oligomers detected with PALM (red) are mostly colocalized with actin filaments, labeled with eGFP in the background (green) [103]. c) The magnified view shows how most of the $\beta_2$-AR oligomers

interact with actin filaments. Disruption of actin (bottom right) with latrunculin A deletes most of the $\beta_2$-AR oligomers. However, only few oligomers may be present in the lipid rafts (magnified view). The role of lipid rafts in GPCR oligomerization is controversial. Our data show that removal of cholesterol does not affect $\beta_2$-AR oligomerization in H9c2 cells (top right) while Nikolaev et al. found that the removal of cholesterol led to redistribution of $\beta_2$-AR in cardiomyocytes of healthy rats [98].


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

**Fig 5.** For dual color 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 so, constructs made of two fluorophores (PA-FP$_1$ and PA-FP$_2$) covalently linked for each pair can be used as a positive control of co-localization. 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 co-localization 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 that allows to localize the construct on the cell membrane (colored in black in the figure).

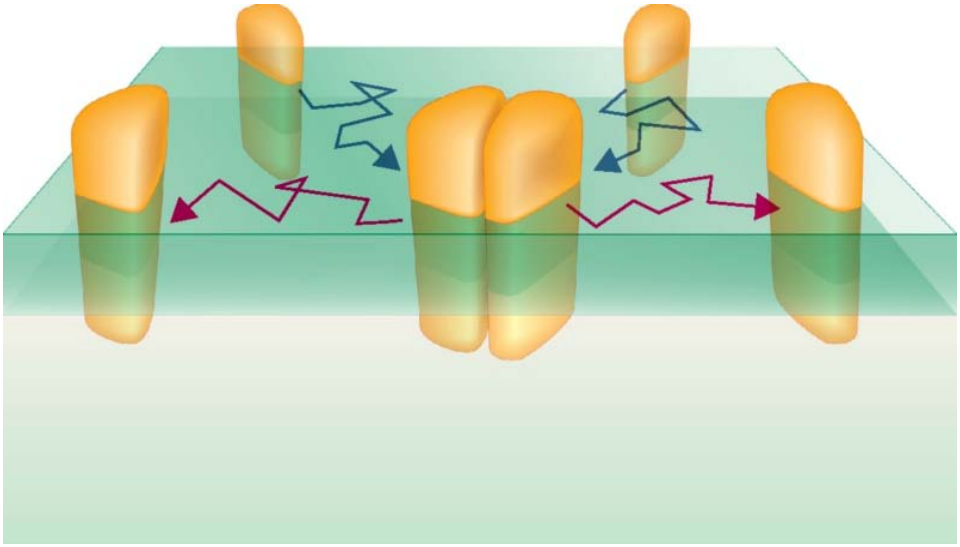

**Fig 6.** Schematic picture representing the dynamics of the dimerization process characterized by fast association of two monomers and fast dissociation of the dimeric complex. Class A GPCRs continually form dimers and dissociate into monomers with different kinetic parameters depending from receptor subtype and the temperature of the assay. For N-formyl peptide receptor (FPR), labeled with a fluorescent agonist, at steady state about 40% of the receptors are part of dimeric complexes. Reproduced from Kasai et al. [43].