Single-molecule analysis of fluorescently labeled G-protein–coupled receptors reveals complexes with distinct dynamics and organization

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G-protein–coupled receptors (GPCRs) constitute the largest family of receptors and major pharmacological targets. Whereas many GPCRs have been shown to form di-/oligomers, the size and stability of such complexes under physiological conditions are largely unknown. Here, we used direct receptor labeling with SNAP-tags and total internal reflection fluorescence microscopy to dynamically monitor single receptors on intact cells and thus compare the spatial arrangement, mobility, and supramolecular organization of three prototypical GPCRs: the β1-adrenergic receptor (β1AR), the β2-adrenergic receptor (β2AR), and the γ-aminobutyric acid (GABA) receptor. These GPCRs showed very different degrees of di-/oligomerization (β2ARs) and membrane dynamics (β1ARs, GABA receptors). The size of receptor complexes increased with receptor density as a result of transient receptor–receptor interactions. Whereas β1/2 ARs were apparently freely diffusing on the cell surface, GABA receptors were prevalently organized into ordered arrays, via interaction with the actin cytoskeleton. Agonist stimulation did not alter receptor di-/oligomerization, but increased the mobility of GABA receptor complexes. These data provide a spatiotemporal characterization of β1/2 ARs and GABA receptors at single-molecule resolution. The results suggest that GPCRs are present on the cell surface in a dynamic equilibrium, with constant formation and dissociation of new receptor complexes that can be targeted, in a ligand-regulated manner, to different cell-surface microdomains.

live cell imaging | protein–protein interactions

G-protein–coupled receptors (GPCRs) constitute the largest family of cell-surface receptors and important pharmacological targets (1). Whereas research performed over the past 30 y has revealed in great detail the basic mechanisms and kinetics of GPCR signaling (1, 2), fundamental aspects, such as receptor di-/oligomerization or G-protein coupling and dissociation, remain controversial, mostly due to technical limitations to directly observe these phenomena (3–5).

Although GPCRs were initially thought to be monomeric, evidence accumulated over the past two decades suggests that they can form dimers or oligomers in intact cells (6–10). In a few cases, such as the γ-aminobutyric acid type B (GABA_B) receptor and other family-C GPCRs, di-/oligomerization is essential for receptor function (10–12). It is now well established that functional GABA_B receptors consist of a GABA_A subunit (which binds GABA but cannot activate G proteins) and a GABA_B subunit (which is binding deficient but can signal to G proteins) (10–12). Interestingly, heterodimerization of GABA_A with GABA_B masks an endoplasmic reticulum retention signal on the C-terminal tail of GABA_A, and this is required for GABA_A to reach the cell surface (10–12). For family-A GPCRs, the situation is more controversial, even for well-studied members such as the β1-adrenergic receptor (β1AR) and the β2-adrenergic receptor (β2AR) (6–10). For instance, β2 ARs have been suggested to be monomers (13), constitutive dimers (14, 15), or higher-order oligomers (16). Similarly, β1 ARs have been suggested to form either stable (15) or transient interactions (16). Moreover, whereas the di-/oligomerization of family-A GPCRs has been proposed to play roles in receptor trafficking (17) and/or signaling (18), it is apparently not required for receptor function (19–21). The current uncertainty on these topics calls for new methods capable of directly monitoring the size and stability of GPCR supramolecular complexes at physiological expression levels in living cells.

Evidence for GPCR di-/oligomerization has been mostly obtained with biochemical methods or with biophysical techniques, such as resonance energy transfer (RET) (6–10). Compared with biochemical methods, fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) have the advantage of analyzing GPCR di-/oligomerization in intact cells. However, even though these methods have provided important insights on GPCR di-/oligomerization, they are based on average proximity measurements and usually require high receptor expression levels. Moreover, an intense debate arose a few years ago on the possible occurrence of RET due to random collisions (13, 22, 23).

Besides RET and FRET, other optical methods have been proposed to study protein–protein interactions in living cells (4, 16, 24, 25). Of these, single-molecule microscopy has the great potential of directly observing the state and behavior of individual proteins. Interestingly, two recent single-molecule microscopy studies using fluorescent ligands showed dynamic dimerization of M1 muscarinic and N-formyl peptide receptors (26, 27).

Here, we used the SNAP-tag technology (28) to directly label cell-surface GPCRs with small organic fluorophores and visualize individual receptors on the surface of living cells by total internal reflection fluorescence microscopy (TIRF-M). Three prototypical GPCRs, i.e., the β1AR, the β2AR (both family-A GPCRs), and the GABA_B receptor (family C), which are implicated in fundamental physiological processes such as heart contraction and neurotransmission and represent major pharmacological targets, were analyzed. This allowed us to precisely monitor over time and thus accurately compare the spatial arrangement, mobility, and supramolecular organization of these GPCRs, both under basal conditions and after agonist stimulation.

Author contributions: D.C. and M.J.L. designed research; D.C., F.R., J.W., T.S., U.Z., and A.Z. performed research; A.B. and E.C. contributed new reagents/analytic tools; D.C. and F.R. wrote the paper.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.orglookup/suppl/doi:10.1073/pnas.1205798110/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1205798110
Results

Validation of Single-Molecule Analysis. To directly and efficiently label cell-surface GPCRs with a bright fluorophore, we used the SNAP-tag (28). The SNAP-tag is a 20-kDa protein derived from the enzyme O'-alkylguanine-DNA alkyltransferase (AGT), which can be fused to a protein of interest and covalently labeled with fluorescent bensylguanine (BG) derivatives. We initially calibrated the method, using single molecules of the cell-impermeant Alexaflour 647 BG derivative (Alexa647-BG) spotted on glass coverslips that were imaged by TIRF-M (SI Results and Fig. S1).

Then, we evaluated the possibility of using the SNAP method to visualize single cell-surface proteins in living cells. A construct coding for a monomeric cell-surface receptor, CD86 (16), with an N-terminal SNAP-tag (SNAP-CD86) was used. After transfection of CHO cells with this construct, labeling with saturating concentrations of Alexa647-BG produced a highly specific staining (Fig. S2). When low-expressing cells were visualized by TIRF-M, individual fluorescent particles were visible in SNAP-CD86–transfected (Fig. S2 and Movie S1) but not in mock-transfected cells. Particles were automatically detected and tracked with algorithms developed by Jaqaman et al. (29). This analysis was able to correctly recognize (Fig. 1A and B, blue circles) and track (Fig. 1A and B, blue splines) a large majority of particles (typically >90%) present in SNAP-CD86 image sequences (Fig. 1A and B and Movie S2).

Next, we exploited the single-molecule data to analyze the size of receptor complexes. In the case of a monomeric protein, particle intensities are expected to be normally distributed with a mean (μ) corresponding to that of single fluorophores. In the case of a mixture of complexes with different size (e.g., monomers, dimers, and oligomers), the distribution of particle intensities is expected to be the sum of n components, each having mean μn. Thus, a mixed Gaussian fitting was performed on the distribution of particle intensities to retrieve the weight of each underlying component. This analysis was validated on monomeric receptors containing either one (SNAP-CD86) or two (SNAP2x-CD86) SNAP-tags fused to their N termini. The latter represents a valuable control, because virtually all of the receptors are expected to be labeled with two fluorophores and should therefore be detected as “dimers”. In cells transfected with the SNAP-CD86 construct (Fig. S2), a predominant peak with an average intensity of 0.0205 ± 0.0057 (mean ± SD) was observed. These values were almost superimposable to those of Alexa647-BG molecules on glass (compare with Fig. S1) and were correctly detected by the mixed Gaussian fitting analysis as monomers (n = 1). In addition, particles bleached in one step, further confirming that they were single molecules (Fig. S3). An analysis of particle intensities at different expression levels (Fig. S2) revealed that, at all particle densities measured (0.15–0.45 particle/μm²), the monomeric fraction (n = 1) was largely predominant (>85%), with a small component of apparently dimeric particles (n = 2; approximately 5–18%). This component was due to random colocalization of two particles below the resolution limit of our method and not to SNAP-CD86 dimerization, as indicated by the fact that the same fractions of apparent dimers were obtained at 10% labeling efficiency (Fig. S2)—these results were also consistent with those of computer simulations (SI Results, Movie S3 and Fig. S4). Moreover, because these simulations indicated that the method was accurate up to 0.45 particle/μm² (Fig. S4), only movies with particle densities below this value were analyzed. The analysis of SNAP2x-CD86 image sequences (Fig. S2) showed a largely predominant peak with μ and σ of intensity values approximately corresponding to two times those measured with SNAP-CD86. The mixed Gaussian fitting analysis correctly identified SNAP2x-CD86 particles as dimers. As expected for particles containing two fluorophores, particles were typically bleaching in two steps (Fig. S3). After partial photobleaching, a peak with the intensity of single fluorophores was observed in SNAP2x-CD86 image sequences (Fig. S2, dashed line). Such data obtained after partial photobleaching were used by the mixed Gaussian fitting algorithm to precisely estimate the intensity of single fluorophores in each image sequence (SI Materials and Methods). These results showed that the vast majority SNAP-tags were functional and were labeled with Alexa647-BG, thus excluding the presence of a relevant fraction of unlabeled, “dark” SNAP-tagged receptors. In addition, they indicated that the method was able to efficiently discriminate between populations of “monomeric” and “dimeric” receptors.

β1 and β2ARs Have Different Di-/Oligomerization States. We then applied our method to two prototypical family-A GPCRs, i.e., β1- and β2ARs. Constructs coding for β1- and β2ARs with the SNAP-tag fused to their N termini were fully functional (Fig. S5). In CHO cells transfected with these constructs (Fig. 2A and F) and labeled with Alexa647-BG, single fluorescent particles were detected by TIRF-M (Fig. S5). Although similar results were also obtained in HEK293 cells, CHO cells were chosen because they have no detectable β1/β2ARs and we observed a dependence of SNAP-tagged β1/-β2ARs. Typical intensity distributions of GPCR particles and mixed Gaussian fits are shown in Fig. 2B and D. Data obtained at different particle densities were used to generate plots of the distribution of particle sizes over density, where the areas filled with different colors indicate the relative abundance of particles containing the indicated number of receptors (Fig. 2C and H). Both receptors were present as mixtures of complexes with different size, which was increasing with particle density. The proportion of di-/oligomeric complexes and the dependency on particle density were much higher than in control SNAP-CD86 and simulated image sequences, as expected for true interactions. The effect of density was more prominent for the β1AR, in which case the fraction of monomers was ∼70% at low densities (0.15–0.3 particle/μm²), whereas dimers predominated over monomers and a small fraction of tri-/tetramers at the highest particle densities measured (Fig. 2C). The β2AR had a higher tendency of forming dimers, which constituted ∼60% at low densities (0.15–0.3 particle/μm²), the rest being represented by monomers (Fig. 2H). At higher densities, β2AR contained a mixture of di-, tri-, and tetrabem, which at 0.4–0.45 particle/μm² accounted for ∼50%, 30%, and 15%, respectively (Fig. 2H). The different abundance of monomers between β1- and β2ARs at low densities was highly statistically significant (Fig. 2G, Inset). These results were confirmed by a separate analysis based on the photobleaching steps (Fig. S3). For this purpose, the intensity profile of each particle was...
fitted with a stepwise model and the number of receptors in each particle was estimated on the basis of the number and size of steps. The results were in very good agreement with those of the mixed Gaussian fitting analyses.

Next we analyzed the movement of receptor particles on the cell surface (Fig. 2 D and J). Plots of their mean square displacement (MSD) against time gave linear relationships, suggesting that the receptors were freely diffusing. MSD data of individual particles indicated no corralling at this temporal resolution. A calculation of their diffusion coefficients from MSD data showed that β1ARs were diffusing quickly on the cell surface (median diffusion coefficient = 0.052 μm²·s⁻¹; peak = 0.032–0.043 μm²·s⁻¹) (Fig. 2D). β2ARs had a slightly slower diffusion speed (median diffusion coefficient = 0.039 μm²·s⁻¹; peak = 0.021–0.032 μm²·s⁻¹) (Fig. 2F). Overall, these values are similar to those reported on β1ARs with other methods (30) as well as on other receptors by TIRF-M (26). The speed of receptor diffusion was negatively correlated with the size of receptor complexes (Fig. 2E and J).

**β1- and β2ARs Undergo Transient Interactions.** Because the observed increase of receptor di-/oligomerization with receptor density was suggesting the presence of transient interactions, we attempted to directly observe and characterize such interactions. An example of a transient colocalization between two β1AR particles is shown in Fig. 3 A–C. For each pair of colocalizing particles, the colocalization time (Δt), i.e., the time between merging and splitting, was automatically calculated and Δt values were used to estimate the apparent lifetime of particle colocalizations (τ*) by fitting the data to an exponential decay function. Colocalization of β1ARs should result from both random colocalizations and true receptor–receptor interactions. To distinguish these true interactions from random colocalizations, we simulated particles with the same characteristics (diffusion coefficients, intensity distribution, and bleaching rate) of β1ARs, but showing no interactions. The estimated τ* value for these random colocalizations (τ*r) was 1.08 s (95% confidence interval: 1.04–1.12) and served as a background value (Fig. 3D). Almost identical τ* values (1.01 s; 95% confidence interval, 0.96–1.05) were obtained with control monomeric (SNAP-CD86) receptors. Then, β1AR data were fitted to the sum of two exponential functions, the first one with a lifetime equal to τ* (Fig. 3E). The apparent lifetime of the second component (τ2), i.e., the one resulting from receptor–receptor interactions, was estimated to be 5.05 s (95% confidence interval: 4.17–5.93). Similar results were obtained with control monomeric (SNAP-CD86) receptors. Then, β1AR data were fitted to the sum of two exponential functions, the first one with a lifetime equal to τ* (Fig. 3E). The apparent lifetime of the second component (τ2), i.e., the one resulting from receptor–receptor interactions, was estimated to be 5.05 s (95% confidence interval: 4.17–5.93). Similar results were obtained with control monomeric (SNAP-CD86) receptors.

Functional GABAB Heterodimers Form Large Complexes Tethered to the Cortical Actin Cytoskeleton. We then applied our method to the GABAβ receptor, a prototypical family-C GPCR, consisting of heteromers between GABAB1 and GABAB2 subunits (10–12). Initially, we transfected CHO cells with N-terminally SNAP-tagged GABAB1 and untagged GABAB2 subunits (31). As expected, the SNAP-tagged GABABβ subunit alone did not reach the cell surface, whereas the SNAP-tagged GABABβ subunit alone was detectable on the cell surface, where it formed a mixture of mono-/di-/oligomers with high lateral mobility (Fig. S6). Thus, cells cotransfected with both SNAP-tagged subunits (Fig. S7) most likely contained a mixture of labeled homomeric (composed of GABAB1, GABAB2, and GABAB heteromers) and heteromeric complexes. Therefore, to selectively analyze functional GABAB complexes, we took advantage of the fact that the GABABβ subunit requires dimerization with the GABAB2 subunit to reach the cell surface and analyzed cells cotransfected with SNAP-tagged GABABβ and untreated GABABβ subunits (Fig. 4 A and B). Note that in these experiments the labeling stoichiometry is one fluorophore per heterodimer (h.d.). At low densities (0.15–0.2 particle/μm²), GABAB complexes prevalently consisted of heterodimers (n = 1 h.d.) (Fig. 4 C and D). The proportion of higher-order oligomers increased with particle density. At the highest density analyzed (0.4–0.45 particle/μm²), tetramers (dimers of dimers; n = 2 h.d.) and octamers (tetramers of dimers; n = 4 h.d.) constituted a large fraction (30–40% each) of the detected complexes. Very similar results were obtained after correction for random colocalization (SI Results and Fig. S8). These findings were confirmed by an


**Fig. 3.** Dynamic visualization of receptor–receptor interactions. (A) Example of two Alexa647-labeled β2AR particles showing a transient colocalization. A merging event (green) is followed after some frames by a splitting event (red). Images are centered on the particles’ position. (Scale bar, 1 μm.) (B) Same traces as in A on a white background and without centering. (C) Intensity profiles of the traces in A, showing intensity doubling upon merging. (D) Colocalizations between control particles devoid of true interactions. Black, simulated particles with diffusion coefficients, intensity distribution, and bleaching rate analogous to those of β2AR particles. Orange, monomeric SNAP-CD86 receptors. The apparent lifetime of colocalization (τ; 95% confidence intervals in parentheses) was calculated by fitting colocalization time data with an exponential decay function. (E) Lifetime of β2AR colocalizations. Colocalization time data derived from experiments as in A (green) were fitted to the sum (black) of two exponential decays (blue and red, respectively). The obtained apparent lifetimes of particle colocalizations (τ1 and τ2; 95% confidence intervals in parentheses) were then used to estimate the true lifetime of receptor–receptor interactions. (F) Same as E with low-density (<0.35 particles/μm²) β2AR movies. Data in E and F are from 20 and 8 different cells, respectively. Data in E and F were fitted better with two components than with one, as judged by an F-test (P < 1 × 10⁻⁴).

**Discussion**

The extent and functional relevance of GPCR di-/oligomerization is highly debated. Whereas monomeric GPCRs, including rhodopsin (19, 20) and the β2AR (21), efficiently activate G proteins, several studies suggest that GPCRs can form di-/oligomers (6–10). The exact size and stability of such complexes are largely unknown. We used single-molecule TIRF-M combined with direct labeling of the additional analysis, in which the intensity profiles of each receptor particle were fitted with a stepwise model and the number and size of steps were used to estimate the size of the receptor particles (Fig. S3).

Next, we evaluated the diffusion speed of functional GABA<sub>B</sub> complexes. Most particles had limited mobility (median diffusion coefficient = 0.028 μm²·s⁻¹; peak < 0.01 μm²·s⁻¹); particles with diffusion coefficients <0.01 μm²·s⁻¹ = 24.6%; Fig. 4E), which was negatively correlated with particle size (Fig. 4F). Interestingly, GABA<sub>B</sub> receptors showed a tendency to arrange in rows. This was already visible at receptor densities used for the single-molecule analysis and was even better appreciated at higher receptor densities (Fig. 4G). Moreover, GABA<sub>B</sub> receptors colocalized with actin filaments stained with fluorescent phallolidin, suggesting an interaction between these receptors and the cortical actin cytoskeleton. Actin depolymerization with latrunculin A abolished the arrangement of GABA<sub>B</sub> receptors in rows (Fig. 4H), but did not modify the size of GABA<sub>B</sub> complexes (Fig. 4I).

**Agonist Stimulation Increases the Lateral Mobility of GABA<sub>B</sub> Receptors, but not of β<sub>1</sub>-/β<sub>2</sub>ARs.** Labeling the receptors themselves rather than the ligands allowed us to investigate whether the di-/oligomerization or mobility of GPCRs was affected by agonists. CHO cells expressing SNAP-tagged β1-/β2ARs or GABA<sub>B</sub> receptors were stimulated with 10 μM isopropenol or 50 μM GABA, respectively. Agonist stimulation had no effect on the di-/oligomerization state of any receptor (Fig. S9) or on the mobility of β<sub>1</sub>- and β<sub>2</sub>ARs (Fig. 5A and B). However, it did increase the mobility of GABA<sub>B</sub> receptors (median diffusion coefficient from 0.027 to 0.056 μm²·s⁻¹; Fig. 5C).
receptors with small organic fluorophores via SNAP-tags to compare the spatial arrangement, mobility, and di-/oligomerization state of three GPCRs, i.e., the β1AR, β2AR, and GABA_{B} receptors.

The β1AR was found to be prevalently monomeric at low densities and to form an increasing number of dimers at higher densities, due to the occurrence of transient interactions. In contrast, the β2AR had a higher tendency to form dimers or higher-order oligomers already at lower densities. Because the lifetimes of receptor–receptor interactions appear similar for the two receptors, this difference must result from other factors, such as different efficiencies in converting a “collision” into an interaction, different interactions with other proteins capable of interfering with dimerization, or localizations in different subcellular microdomains, as shown for β1- and β2ARs in cardiomyocytes (31), leading to dissimilar effective densities of the two receptors. Moreover, β2AR data were characterized by a relatively higher cell-to-cell biological variability, which might reflect a more dynamic type of di-/oligomerization. These results are consistent with our recent fluorescence recovery after photobleaching (FRAP) study (16) and provide a direct quantification of the size of these complexes and of their dynamics. They are also consistent with the results of the two previous single-molecule studies, providing further evidence that GPCR dimerization is a dynamic process, in which receptors rapidly associate and dissociate.

GABA_{B} receptors are known to be obligate heterodimers (10–12). Recent FRET measurements suggest that they might organize into larger supramolecular complexes (32, 33). Our study provides a direct observation of such complexes and a precise evaluation of their size. On the one hand, we found GABA_{B} receptors to be present in equilibrium between heterodimers and higher-order oligomers, with a relative preference for tetramers (dimers of dimers) and octamers (tetramers of dimers). Whereas GABA_{B} heterodimers are stable due to strong noncovalent interactions, the dependency of the formation of higher-order oligomers on receptor density suggests that these larger complexes are the result of weaker and likely transient interactions among heterodimers. Moreover, the relative predominance of tetramers and octamers might reflect an ordered and asymmetric spatial organization within the oligomers. On the other hand, the analysis of receptor mobility revealed that GABA_{B} receptor heteromers are largely immobile and arranged into rows on the surface of living cells. The latter phenomenon, which is apparently due to an interaction between the GABA_{B1} subunit and the actin cytoskeleton, might play a role in the spatial organization of GABA_{B} receptors at synapses. This interaction was seemingly stronger for oligomers than for heterodimers, as suggested by a pronounced difference in their diffusion coefficients. However, the interaction with actin fibers was not responsible for the formation of the observed large GABA_{B} complexes, as indicated by the results of actin depolymerization with latrunculin A. Thus, these large receptor complexes appear to be due to true oligomerization, either via direct receptor–receptor interactions or with intervention of a scaffold. Finally, our results revealed the presence of cell-surface GABA_{B2} monomers, which is consistent with the lack of an ER-retention signal in the GABA_{B2} subunit and a different type of interaction involved in GABA_{B2} homodimerization (34, 35).

The effect of ligands on GPCR di-/oligomerization is controversial (6–8, 10). For instance, previous experiments on the β2AR in intact cells (14) or with purified receptors (36) indicate that agonists have minor effects that could be either due to small changes in steady-state di-/oligomerization or due to conformational changes in individual protomers. No effects of ligands were instead observed in FRAP experiments (16). Our data support the view that agonist binding has no major acute effects on the di-/oligomerization of β1AR, β2AR, and GABA_{B} receptors. In contrast, we observed an increase in the lateral mobility of GABA_{B} receptors after agonist stimulation, which suggests the occurrence of ligand-regulated interactions with the actin cytoskeleton.

The method used in this study is complementary to those based on RET and FRAP. Specifically, it allows to dynamically quantify the size of protein complexes and to identify subpopulations thereof, at receptor densities that do not exceed physiological levels. For instance, considering β2AR complexes, a density of 0.45 particle/μm^{2} corresponds to ~1 receptor/μm^{2}, which is in the same range as found in different cell types (37).

Another characteristic of our approach is the use of the SNAP-tag technology (28) to directly label cell-surface GPCRs, which has several advantages compared with the use of fluorescent ligands (26, 27). First, whereas the latter methods are associated with partial labeling and/or possible influences of negative cooperativity in ligand binding (38, 39), virtually complete receptor labeling can be achieved. Second, our approach allows us to analyze ligand-free receptors and to study the effects of agonists or antagonists. Third, the present method can be easily extended to other GPCRs and cell-surface proteins. Although beyond the scope of the present study, a similar approach combined with dual color labeling, e.g., using SNAP- and CLIP-tags (25, 40), might also be used to study interactions between different proteins such as those occurring between receptors and G proteins or among G-protein subunits. A limitation of our method is that because its spatial resolution is dictated by the diffraction of light, it cannot directly distinguish between complexes due to direct receptor–receptor interactions and complexes due to receptor interactions with a scaffold.

Taken together, our study shows that a method based on labeling with SNAP-tags and single-molecule TIRF-M can be used to dynamically quantify and thus compare the spatial arrangement, mobility, and di-/oligomerization of different GPCRs and possibly other cell-surface proteins. Data obtained on β1AR, β2AR, and GABA_{B} receptors indicate that the cell-surface topology and mobility as well as type and degree of receptor di-/oligomerization vary considerably among different receptors. GPCRs appear to be highly dynamic, constantly associating and dissociating with other receptors to form new supramolecular complexes as well as with other proteins to maintain their specific location on the cell surface.
Materials and Methods

Cell Culture and Transfection. For single-molecule experiments, CHO cells were cultured in DMEM/F12 medium supplemented with 10% (vol/vol) FCS, penicillin, and streptomycin at 37 °C, 5% (vol/vol) CO2. Cells were plated at a density of 10^4 per well onto 24-mm clean glass coverslips and transfected using the Lipofectamine 2000 reagent (Invitrogen), following the manufacturer's protocol. For each well, 2 μg DNA and 6 μL Lipofectamine 2000 were used. Cells were analyzed 8–12 h after transfection to achieve high expression levels.

SNAP Labeling. Cells were labeled with 1 μM Alexa647-BG (Alexafluor 647-SNAP Surface; New England Biolabs) in complete (+FCS) phenol-red-free medium for 30 min at 37 °C. At the end of the incubation, the cells were washed three times with complete phenol-red-free medium and immediately imaged. These conditions resulted in saturating labeling of cell-surface SNAP-tagged receptors (Fig. S2).

Total Internal Reflection Fluorescence Microscopy. A commercial TIRF microscope (Leica TIRF) equipped with an EM-CCD camera (Cascade 512B; Roper Scientific), a 100× oil-immersion objective (HCX PL APO 100×/1.46), and a 635-nm diode laser was used. To avoid photobleaching before image acquisition, cells were searched and focused in bright field and a fine focus adjustment in TIRF mode was performed using only 2% laser power, an intensity insufficient for detecting single molecules. This procedure resulted in negligible photobleaching. Afterward, laser power was set to 83% and image sequences (400–600 frames) were acquired with an exposure time of 50 ms, resulting in the acquisition of an image every 96 ms. The penetration depth of the evanescent field was ~110 nm. Illumination intensity was homogeneous over the image area (maximum difference = 10%). Under these conditions, the photobleaching half-life was 6.82 ± 0.12 s. The microscope was equipped with an incubator and a temperature control unit. Experiments were performed at 20 ± 0.3 °C. Cells were imaged in a buffer containing 13 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM Hepes, pH 7.3. Only cells with less than 0.45 receptor particle/μm² were analyzed.

Additional Methods. Details about plasmids, cover slip cleaning, determination of Alexa647-BG labeling efficiency, radioligand binding, measurement of CAMP concentrations, Latrunculin A treatment/actin staining, and computational analyses are available in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Bianca Klüpfel, Monika Frank, and Christian Dees for excellent technical support. This work was supported by grants from the European Research Council (Advanced Grant Towards the Quantal Nature of Receptor/CAMP Signals) and the Deutsche Forschungsgemeinschaft (SFB487). T.S. was partially supported by the Royal Golden Jubilee Ph.D. Program.
Supporting Information

Calebro et al. 10.1073/pnas.1205798110

SI Results

Calibration with Single Fluorophores. To calibrate the method, the cell-impermeant Alexafluor 647 BG derivative (Alexa647-BG) was spotted on clean glass coverslips that were imaged by total internal reflection fluorescence microscopy (TIRF-M). At low densities, diffraction-limited particles were visible (Fig. S1A). Here and below, particles were automatically detected and tracked using recently described algorithms (1). These particles represented single molecules, as indicated by the fact that they bleached in one step (Fig. S1B). The corresponding particle intensity distribution is shown in Fig. S1C. A single peak of intensity 0.0192 ± 0.0050 (mean ± SD) was observed, further supporting the notion that these were single fluorophore molecules.

Validation with Simulated Particles. Simulated data, consisting of computer-generated image sequences of single particles with Brownian motion, were created (Fig. S4A and Movie S3) and analyzed as described for SNAP-CD86 image sequences. Particles were simulated with an intensity distribution, diffusion speed, and bleaching behavior similar to those of SNAP-CD86 particles. Random noise was added to the image sequences to obtain a signal-to-noise ratio comparable to that of SNAP-CD86 images. The analysis with the Gaussian fitting algorithm revealed the presence of a largely predominant peak corresponding to monomeric particles and a minor peak of approximately double intensity (Fig. S4 B and C), ascribable to the random colocalization of two particles at a distance below the resolution limit of the system. As predicted on the basis of the calculation of the probability that two particles are found in such close proximity, the fraction of particles that for this reason were erroneously identified as apparently dimeric was growing nonlinearly with increasing particle density (Fig. S4 D and E). These data provided an estimate of the accuracy and limits of the analysis. For densities below 0.45 particle/μm², the occurrence of apparent dimers was particularly low, ~5–13%, and most particles were correctly identified as monomers. The small differences observed between simulations and experimental data here as well as elsewhere (2) could be due to a number of reasons. A possible explanation is that the plasma membrane is a highly crowded (about 30% of its surface is covered by proteins) and complex environment. Interestingly, previous studies have found that although cell-surface G-protein-coupled receptors (GPCRs) and other cell-surface molecules apparently diffuse randomly (at least when observed with the temporal resolutions commonly attainable and used in the present study), they undergo “hop diffusion” when observed at very high speed (in the microsecond range) (3). On the basis of this observation, these authors proposed a model in which the diffusion of plasma-membrane molecules occurs as “hop” movements between adjacent microcompartments of the size of ~200–300 nm, delimited by the cortical cytoskeleton. The occurrence of such hop diffusion would not cause appreciable changes in the apparent diffusion of GPCR particles measured at our temporal resolution, but could alter the probability of “random colocalizations” between two particles. To verify this possibility, we have performed an additional series of simulations in which particles macroscopically diffuse with the same apparent diffusion coefficient as in previous simulations, but diffuse 10 times faster within 300-nm compartments in which they reside for 100 ms. Interestingly, these simulations were associated with colocalization rates (Fig S4 D and E, red data) that are almost identical to those obtained with SNAP-CD86. Thus, the apparent small discrepancy between simulated and SNAP-CD86 data can be solved by using a more complex model of particle behavior. Importantly, such deviations from pure random diffusion occur on a temporal (and spatial) scale, which is below that of our measurements and much lower than that involved in random colocalization or true interactions. Thus, these deviations do not significantly affect the lifetimes of colocalizations such as those reported in Fig. 3. These simulations also allowed us to verify the accuracy of our analysis at different receptor densities. Note-worthy, the percentage of apparent dimers as well as the accuracy of particle detection and tracking began to deteriorate above 0.45 particle/μm². For this reason, only movies with densities below this value were analyzed in all subsequent experiments.

Correction for Apparent Colocalizations. A mathematical model was developed to correct the results of the mixed Gaussian fitting analyses for the presence of random colocalizations. Results obtained with control monomeric receptors (SNAP-CD86) as well as with simulated monomeric particles showed that the density of apparent dimers was growing with the second power of receptor density (Fig. S8A). On the basis of this information, we developed a system of nonlinear equations that allowed us to estimate the underlying distribution of receptor particles without random colocalizations (details in SI Materials and Methods). This approach was able to efficiently correct the distributions obtained with control SNAP-CD86 and SNAP2×-CD86 receptors (Fig. S8B). Application of the same correction to results obtained with GPCR constructs was associated with limited changes in the corresponding distributions as shown by a representative example in which this correction was applied to data obtained with γ-amino butyric acid (GABAB) receptors (Fig. S8C).

SI Materials and Methods

Plasmids. A plasmid coding for an N-terminally FLAG-tagged human β_2-adrenergic receptor (β_2AR) was previously described (4). A plasmid coding for FLAG-tagged human β_1-adrenergic receptor (β_1AR) was cloned using the same strategy. SNAP-β_1AR and SNAP-β_2AR were generated by inserting the SNAP-tag directly after the FLAG sequence in the previous constructs. These constructs were functional, as shown by normal radioligand binding and cAMP concentration-response dependencies (Fig. S5). Plasmids SNAP-CD86 and SNAP2×-CD86 were generated by replacing YFP with either one or two copies of SNAP in a previously described construct coding for CD86 with YFP fused at its N terminus (5). Plasmids coding for wild-type human GABA_B1A and GABA_B2 receptors as well as human GABA_B1A and GABA_B2 with the SNAP-tag fused at their N termini (SNAP-GABA_B1 and SNAP-GABA_B2) were kindly provided by J. P. Pin (Institut de Génomique Fonctionnelle, Université Montpellier, Montpellier, France) and were shown to be functional in a previous study (6).

Coverslip Cleaning. Twenty-four-millimeter glass coverslips were extensively cleaned to remove any background fluorescence. First, they were sonicated in a solution containing 5 M NaOH for 1 h. After three washes with distilled water, they were dried and further sonicated in chloroform for 1 h. Coverslips were then dried and stored in 100% ethanol until use.

Generation of Stable HEK293 Cell Line. HEK293 cells were cultured in DMEM, supplemented with 10% (vol/vol) FCS, penicillin, and streptomycin at 37 °C and in the presence of 5% (vol/vol) CO₂. For the generation of the stable cell line expressing SNAP-β_1AR,
HEK293 cells were transfected with Effectene (Qiagen), following the manufacturer’s protocol. After selection with 1.3 mg/mL G418, individual clones were obtained by limiting dilution and the clone with best cell-surface SNAPβ2AR expression was used.

**Determination of Alexa647-BG Labeling Efficiency.** HEK293 cells stably expressing β2AR were plated at a density of 100,000 cells per well in 96-well imaging plates (BD Biosciences) precoated with poly-d-lysine. Cells were labeled with different concentrations of Alexa647-BG in complete medium for 30 min at 37 °C and washed twice with complete medium. Thereafter, they were stained with 1 μg/mL Hoechst 33345 for 30 min at 37 °C and washed three times with PBS. Fluorescent images of Alexa647-BG and Hoechst 33345 were acquired with an automated imaging system (Pathway; BD Biosciences).

**Radioligand Binding.** Radioligand binding experiments were performed as previously described (7). Briefly, CHO cells were plated in 20-cm Petri dishes at a density of 1.3 × 10^6 and transfected with 60 μg plasmid DNA + 180 μL lipofectamine 2000. Cells were homogenized 48 h after transfection and membranes of transfected and mock-transfected CHO cells were incubated with 50 pM ^125^Iiodocyanopindolol (I=1-CYP; Amersham Biosciences) and the indicated concentrations of bisprolol or ICI-118551 for 90 min at room temperature, filtered through Whatman GF/F filters, and washed three times with ice-cold assay buffer. Samples were counted in a γ-counter (Wallac 1480 wizard 3 ).

**Measurement of cAMP Concentrations.** cAMP measurements were performed by a RIA, using standard procedures. Briefly, cells were preincubated in Krebs–Ringer–Hepes buffer containing 300 μM 3-isobutyl-1-methylxanthine (IBMX) for 30 min at 37 °C. Thereafter, they were stimulated with different concentrations of isoproterenol in the same buffer for 60 min at 37 °C. At the end of the incubation the medium was discarded and samples were extracted with 0.1 M HCl. The cell extracts were dried in a vacuum concentrator, resuspended in water, and diluted. cAMP concentrations were measured by a RIA according to the method of Brooker et al. (8).

**Latrunculin A Treatment and Actin Staining.** CHO cells were transfected and labeled with Alexa647-BG as described above. Subsequently, cells were incubated with 5 μM latrunculin A (Sigma-Aldrich) for 30 min at 37 °C. For F-actin staining, cells were then fixed with 4% (wt/vol) paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X100 for 3 min, and blocked with 3% (wt/vol) BSA for 20 min, all at room temperature. Cells were then incubated with 16 nM Alexafluor 488-phallolidin (Invitrogen) for 30 min at 37 °C.

**Computational Analyses.** Particles were detected and tracked using recently described algorithms (1) implemented in Matlab (The MathWorks). Briefly, the subpixel location and intensity above background of diffraction-limited particles are calculated by fitting a 2D Gaussian with the SD of the spread point function of the microscope around local intensity maxima. Tracking is performed on the basis of an approximate of the multiple-hypothesis tracking approach. Specifically, for each step and each particle a cost is assigned to every potential event (e.g., particle blinking, merging, splitting, appearing, or disappearing) and the solution that minimizes the sum of the costs is selected. This allows us also to track a particle beyond a blinking event, which is not possible with other tracking algorithms (details in ref. 1). The blinking frequency measured in our tracks by dividing the number of blinking events by the total duration of the tracks was low—approximately one event every 1,000 frames per fluorophore—and the duration of a blinking event was relatively short, typically 5–10 frames. The outcome of the tracking analysis contains the position and intensity of each particle at each frame as well as information about merging and splitting events. At the signal-to-noise ratio typical of our single-molecule images, two particles were recognized as merged once their distance fell below ~300 nm (details in ref. 1). Particle intensities are expressed as the amplitude values of the Gaussian fit (1) (this Gaussian fitting used for particle detection should not be confused with the subsequent mixed Gaussian fitting analysis that was implemented to assess particle di-oligomerization). This approach allows to get intensities for two or more particles even when they are very close or partially overlapping, due to the fact that if a spot size is statistically bigger than expected on the basis of the measured point spread function (PSF), the detection software attempts to fit more kernels (i.e., partially overlapping Gaussians), the amplitudes of which can be used as estimates of the intensity of the each underlying particle. The cell-surface area was measured by calculating the area of a binary mask that was manually drawn following the contour of the analyzed cell. The particle density was obtained by dividing the number of detected particles located within the binary mask at the beginning of the movie by the cell-surface area. The correctness of this calculation was verified by manual inspection. Most particles were detected until they bleached/disappeared or merged with other particles. The mean track duration was ~4.5 s (min = 0.48 s; max = 57.6 s). The background was estimated on the basis of the values provided by the detection algorithm, which for the detection of local maxima calculates the local background by excluding outliers represented by the fluorescent particles and performing a moving average. Values are expressed as fractions of the maximal intensity value that can be assigned to a pixel. The background values were very similar for both real movies and simulations (about 0.03–0.04 for both). This was also true for the SD of the background (i.e., noise), which was about 0.002–0.004 for both real movies and simulations.

Data were visualized and further analyzed using additional Matlab algorithms that we developed for this purpose and that are described below.

**Mixed Gaussian fitting.** The distributions of particle intensities were analyzed by performing a mixed Gaussian fitting. For each particle, we averaged the intensity from the beginning of the movie to the frame before the first stepwise change in intensity (generally down, due to a photobleaching event) occurred, up to a maximum of 20 frames. Changes in intensity were detected by fitting the intensity data with a step fitting algorithm (below). As there are several sources of variability in the intensity of a single dye, data are dispersed and their distribution can be approximated by a normal distribution. For cells containing a mixture of monomers and oligomers, intensities are expected to have a distribution corresponding to the sum of Gaussians with different mean values. The mixed Gaussian model used for the fitting can be described by the equation

\[
\phi(i) = \sum_{n=1}^{n_{\text{max}}} A_n \frac{1}{\sigma_n \sqrt{2\pi}} e^{-\frac{(i-\mu_n)^2}{2\sigma_n^2}}
\]

where \(\phi(i)\) is the frequency of particles having intensity \(i\), \(n\) is the component number, and \(A_n\) is the area under the curve of component \(n\). \(\mu_n\) and \(\sigma\) are the mean and SD of reference single fluorophores. The maximal number of components \(n_{\text{max}}\) was determined for each image sequence by progressively increasing \(n_{\text{max}}\) until the addition of one component no longer resulted in a statistically better fitting, as judged by an F-test (\(P < 0.05\)). The denominator \(2(\sigma \mu)^2\) was used because, likely as a consequence of the known local inhomogeneities of the cell surface and TIRF illumination, the position of the particles was found to be the major source of variation in particle intensity. Thus, fluorophores located in the same spot were not considered independent (9).
The intensity distribution of monomeric receptors (Alexa647-labeled SNAP-CD86) was used as an initial estimate of $\mu$ and $\sigma$. However, because there can be minor differences in particle intensities among different image sequences, $\mu$- and $\sigma$-values were fine adjusted for each individual image sequence by performing a first mixed Gaussian fitting on the last 60 frames, when a large fraction of fluorophores was photobleached and a predominant peak corresponding to the intensity of single fluorophores should be present. Also in this case, intensity averaging was performed on data only before a change in intensity was detected by the step-fitting algorithm. The obtained $\mu$- and $\sigma$-values were then used in a second mixed Gaussian fitting on the first 20 frames of image sequences to estimate the actual abundance of each particle component. The weight of each individual component was calculated from $A_i$ values. Confidence limits for the intensity of a single fluorophore were on average $\pm 5\%$ (range: 1.6–11.4\%). Concerning the fractions of the components, for fractions of amplitude >5 particles, the confidence limits were on average $\pm 27\%$ (range: 18–37\%). For very small fractions (amplitude <5 particles), they were on average $\pm 59\%$ (range: 26–96\%). $R^2$ values for the mixed Gaussian fitting analyses performed in this study were $0.87 \pm 0.07$ (mean $\pm$ SD).

**Step fitting.** A simple algorithm was developed to perform a step fitting of the intensity profile of each particle. For each iteration $i$, the algorithm assigns an increasing value to the step size $s_i$ within the interval $\mu \pm 3\sigma$, where $\mu$ and $\sigma$ are the mean and standard deviation values of the intensities of a single fluorophore, estimated for each image sequence as described above (Mixed Gaussian fitting). Intensities that fall within $\frac{1}{2}s_i$ and $2s_i$, where $n$ is the number of molecules of intensity $s_i$, are assigned the value $s_i$ in the fitted data. Very fast changes due to noise are excluded from the fitting. For each iteration, the residuals ($r_i$) between the observed and fitted data are calculated. This value is multiplied by two independent penalty factors to give a final score $Z_i$ for each iteration, based on the formula

$$Z_i = r_i \cdot (1 + p_i^{\text{mis}} + p_i^{\text{ss}}),$$

where $p_i^{\text{mis}}$ is a penalty for the presence of missing steps and $p_i^{\text{ss}}$ is a penalty for choosing $s_i$ far from $\mu$. The solution that minimizes $Z_i$ is finally selected.

The penalty $p_i^{\text{mis}}$ was introduced to penalize solutions that contain missing steps and is calculated according to the formula

$$p_i^{\text{mis}} = \alpha^{\text{mis}} \cdot n_i^{\text{mis}},$$

where $\alpha^{\text{mis}}$ is an arbitrary constant and $n_i^{\text{mis}}$ is the number of missing steps.

The penalty $p_i^{\text{ss}}$ was introduced to penalize solutions that use $s_i$ far from $\mu$. Such a penalty is calculated on the basis of the intensity distribution of single fluorophores, according to the formula

$$p_i^{\text{ss}} = \alpha^{\text{ss}} \cdot \left(1 - e^{-\frac{(s_i-\mu)^2}{\sigma^2}}\right),$$

where $\alpha^{\text{ss}}$ is an arbitrary constant.

**Diffusion speed.** The diffusion speed of receptor particles was calculated on the basis of their mean square displacement (MSD). For each particle and every time interval $t$, the MSD was calculated according to the formula

$$\text{MSD}(t) = \text{MSD}(n \cdot \Delta t) = \frac{1}{2} \sum_{i=1}^{N} \left[(x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2\right],$$

where $N$ is the number of steps analyzed, $n$ is the step size in frames ranging from 1 to 1/10 of the available frames, $\Delta t$ is the time between two consecutive frames, and $x$ and $y$ describe the particle position at the frame indicated by the indexes. Diffusion coefficients ($D$) were then calculated by fitting data with the following equation:

$$\text{MSD} = 4Dt,$$

**Lifetime of interactions.** The duration of each colocalization between two particles ($\Delta t$) was calculated on the basis of the merging and splitting information derived from the tracking analysis. Particles showing bleaching during the analysis time window were excluded. Using these data, the apparent lifetime of particle colocalizations ($\tau$) was calculated by fitting data with the equation

$$I = I_0 \cdot e^{-\frac{t}{\tau}},$$

where $I$ is the number of colocalizing particles at time $t$ and $I'$ is the initial number of colocalizing particles. Apparent lifetime values obtained with simulated particles ($\tau_i$) provided an estimate of the duration of random colocalizations. Because the colocalizations observed in $\beta_1$-$\beta_2$AR image sequences were expected to result either from random colocalization or from receptor interactions, data from $\beta_1$-$\beta_2$AR image sequences were then fitted to the sum of two-exponential functions, using the equation

$$I = I_1 \cdot e^{-\frac{t}{\tau_1}} + I_2 \cdot e^{-\frac{t}{\tau_2}},$$

where $I_1$, $I_2$, and $\tau_1$ are unknowns. Once both $\tau_1$ and $\tau_2$ were estimated, the true lifetime of receptor interactions ($\tau_{\text{int}}$) was calculated on the basis of the following equation (2):

$$\tau_{\text{int}} = \tau_2 - \tau_1.$$
size \(i + j\) was then modeled by a bilinear colocalization function as follows:

\[
\rho_{i+j} = a_j \rho_i\rho_j .
\]

This function was used to determine the underlying particle densities \(\rho_j\) based on the apparent values \(\rho_{i+j}\). Specifically, we assumed that \(\rho_j\) results from \(\rho_i\) plus all possible colocalizations of particles of different size resulting in the formation of particles of size \(j\) and minus all possible colocalizations of particles of size \(j\) with themselves or with particles of another size. In the case of colocalization of two particles of the same size, we introduced a multiplying factor of \(2\) to take into account the disappearance of two particles of equal size to form one of double size. Thus, we obtained the following nonlinear algebraic equations:

\[
\begin{align*}
\rho_1 - a_1 (2\rho_1 + \rho_2 + \rho_3 + \rho_4 + \rho_5) - \rho_7 &= 0 \\
\rho_2 + a_1^2 - a_2 (\rho_1 + \rho_2 + \rho_3 + \rho_4 + \rho_5) - \rho_7 &= 0 \\
\rho_3 + a_1 \rho_1 - a_3 (\rho_1 + \rho_2 + 2\rho_3 + \rho_4) - \rho_7 &= 0 \\
\rho_4 + a_1 \rho_1 + a_2 \rho_2 - a_4 (\rho_1 + \rho_2 + \rho_3) - \rho_7 &= 0 \\
\rho_5 + a_1 \rho_1 + a_2 \rho_2 + a_3 \rho_3 - a_5 (\rho_1 + \rho_2 + \rho_3 + \rho_4) - \rho_7 &= 0 \\
\rho_6 + a_1 \rho_1 + a_2 \rho_2 + a_3 \rho_3 + a_4 \rho_4 - a_6 (\rho_1 + \rho_2 + \rho_3 + \rho_4) - \rho_7 &= 0 \\
\rho_7 &= 0 .
\end{align*}
\]

In addition, we considered a linear combination of these equations to impose consistency of the formulation of the nonlinear system and conservation of the total number of receptors:

\[
\begin{align*}
\rho_1 + 2\rho_2 + 3\rho_3 + 4\rho_4 + 5\rho_5 + 6\rho_6 + 7\rho_7 &= \rho_1 + 2\rho_2 + 3\rho_3 + 4\rho_4 \\
&\quad + 5\rho_5 + 6\rho_6 + 7\rho_7 .
\end{align*}
\]

For ease of discussion, we denote Eqs. S1 and S2 as

\[
J_f(\rho) = 0, \quad j = 1, \ldots, n + 1 ,
\]

where \(\rho = (\rho_1, \rho_2, \rho_3, \rho_4, \rho_5, \rho_6, \rho_7)\). Further, we denote with \(F(\rho) = (f_j(\rho))_{j=1}^{n+1}\) a column vector of functions. With this setting, the system containing Eqs. S1 and S2 can be formulated as \(F(\rho) = 0\).

\[\text{We remark that the problem } F(\rho) = 0 \text{ may have no solutions because the measured data are affected by observation errors and the bilinear colocalization function represents an approximation to the microscopic colocalization function. For this reason, it is appropriate to solve } F(\rho) = 0 \text{ with a least-squares method; i.e., we consider the following minimization problem. Find } \rho^* \text{ such that }\]

\[\| F(\rho^*) \| = \min_{\rho} \| F(\rho) \| .\]

where \(\| \cdot \| \) denotes the Euclidean norm.

To solve this problem, a Gauss–Newton (GN) iterative scheme was used. Using Taylor expansion \(F(\rho) = F(\rho^k) + F'(\rho^k)(\rho - \rho^k) + O(\rho - \rho^k)^2\), we obtained a sequence of linear least-squares problems:

\[\text{i) Find } s^k \text{ with minimal Euclidean norm, such that }\]

\[\| F'(\rho^k)s^k + F(\rho^k) \|_2 = \min_s \| F'(\rho^k)s + F(\rho^k) \| .\]

\[\text{ii) Set } \rho^{k+1} = \rho^k + s^k \text{ and repeat until convergence.}\]

If the linearized equations have full rank, i.e., \(\text{Rank}(F'(\rho^k)) = n\), a minimal norm solution is obtained by the GN iteration. Otherwise, a Levenberg–Marquardt (LM) scheme is used to obtain the least-squares solution with minimal norm. In fact, the LM solver represents a regularized version of the GN scheme. It solves the following problem. Find \(s^k\), such that

\[\| F'(\rho^k)s^k + F(\rho^k) \|_2^2 + \mu^2 \| s^k \|_2^2 = \min_{s^k} \| F'(\rho^k)s + F(\rho^k) \|_2^2 + \mu^2 \| s \|_2^2 ,\]

where \(\mu > 0\) is a regularization parameter.

This solution process is implemented by the Matlab function lsqnonlin that we used in our code. Note that the additional Eq. S2 improved the stability of the solution process.
Fig. S1. Detection of single Alexa647-BG fluorophores by TIRF-M. (A) TIRF-M image of single Alexa647-BG molecules spotted on a clean glass coverslip. (Scale bar, 5 μm.) The position and intensity of each particle were automatically detected. (B) Representative intensity profiles (blue) of Alexa647-BG particles. Intensity profiles were fitted with a step-fitting algorithm (red). Single-step bleaching is observed, as expected for single molecules. (C) Intensity distribution of Alexa647-BG particles. Besides a largely predominant peak (red curve), an additional small peak of approximately double intensity was present, likely due to the random colocalization of two fluorophores.

Fig. S2. Visualization of SNAP-tagged proteins on the surface of living cells (A) and validation of the single-molecule approach using control proteins with a single or with two labels (B–L). (A) Efficiency of SNAP-tag labeling. HEK293 cells stably expressing SNAP-β1AR were stained with different concentrations of Alexa647-BG and the nuclear dye Hoechst 33345, used for normalization. Shown are data (means ± SEM) from a representative experiment performed in quadruplicate. (Scale bar, 100 μm.) (B) Schematic representation of a CD86 construct carrying one SNAP-tag at its N terminus (SNAP-CD86). (C) Epifluorescence images of cells transfected with SNAP-CD86 and control mock-transfected cells. (D) Schematic representation of a CD86 construct carrying two SNAP-tags at its N terminus (SNAP2x-CD86). Reported are the results obtained in cells transfected with either SNAP-CD86 (E–H) or SNAP2x-CD86 (I–L). (E and I) Representative images of single cells transfected with either construct, labeled and visualized by TIRF-M. (Scale bars, 5 μm.) Insets correspond to higher-magnification images of the areas in the white boxes; here detected particles are indicated by blue circles. Particle densities were 0.23 (E) and 0.31 (I) particle/μm². (F and J) Intensity distribution of Alexa647-labeled CD86 particles in E and I. Data were fitted with a mixed Gaussian model. (G and K) Abundance of individual components of the mixed Gaussian fits in F and J, n = 1, monomers. n = 2, dimers. n = 3, trimers. n = 4, tetramers. (H and L) Dependency of the distribution of particle components on particle density. Shown are the cumulative distributions of mono-, di-, and tetramers of Alexa647-labeled CD86, based on mixed Gaussian fitting analyses like those shown in F and J, as a function of particle density. Data were fitted using third-order polynomial functions. Each data point represents one cell [n = 1,965 particles from 11 different cells (H) and 3,127 particles from 17 different cells (L)]. (H, Inset) Same analysis in cells labeled with a low concentration (75 nM) of Alexa647-BG, resulting in ~10% labeling efficiency.
Fig. S3. Estimation of the size of receptor particles based on bleaching steps. (A–E) (Left) Representative intensity profiles (blue) and the results of the step-fitting algorithm (red) obtained with SNAP-CD86 (A), SNAP2×-CD86 (B), SNAP-β1AR (C), SNAP-β2AR (D), and SNAP-GABAB1 plus wild-type GABAB2 (E). (Right) Cumulative distributions of the abundance of receptor n-mers based on the results of the step-fitting analyses as a function of particle density. Data were fitted using third-order polynomial functions. Each data point represents one cell [n = 1,965 particles from 11 different cells (A), 3,127 particles from 17 different cells (B), 6,181 particles from 27 different cells (C), 7,419 particles from 30 different cells (D), and 4,472 particles from 17 different cells (E)].
Fig. S4. Validation of the single-molecule approach, using simulated particles. Computer-generated image sequences containing particles moving with Brownian motion and characteristics (diffusion coefficients, intensity distribution, bleaching rate) similar to those of Alexa647-labeled SNAP-CD86 particles were analyzed as described in Fig. S2. (A) First image of a typical simulated movie. Particle density = 0.35 particle/μm². (Scale bar, 5 μm.) (B) Intensity distribution of simulated particles in A. Data were fitted with a mixed Gaussian model. (C) Abundance of individual components of the mixed Gaussian fitting in B. n = 1, monomers. n = 2, dimers. n = 3, trimers. n = 4, tetramers. (D) Dependency of the fraction of particles detected as apparently dimeric on particle density. The results obtained with SNAP-CD86 and simulated particles are compared. Red data refer to simulations of particles with “hop” diffusion [n = 12,445 ± 8,615 simulated particles from 39 ± 27 simulations and 1,965 SNAP-CD86 particles from 11 different cells]. (E) Detailed view of the data in D over the particle densities used in the present study.

Fig. S5. Functional characterization and single-molecule visualization of SNAP-tagged β₁- and β₂AR constructs. (A) Radioligand binding. CHO cells were transfected with SNAP-tagged β₁AR, β₂AR, or wild-type receptors. Membranes were incubated with 50 pM [125I]iodocyanopindolol and the indicated concentrations of bisprolol or ICI-118551. Data are means ± SEM of four replicates from two independent experiments. (B) cAMP assay. CHO cells were transfected with SNAP-tagged β₁AR, β₂AR, or wild-type receptors and stimulated with the indicated concentrations of isoproterenol. Data are means ± SEM of three independent experiments. (C) Visualization of individual receptors on the surface of living cells. Shown are representative images of cells transfected with SNAP-β₁AR or SNAP-β₂AR, labeled with Alexa647-BG and visualized by TIRF-M. (Scale bars, 5 μm.)
Fig. S6. Analysis of individually expressed SNAP-GABA<sub>B1</sub> and SNAP-GABA<sub>B2</sub> subunits. (A and B) Cells were transfected with either construct alone, labeled with Alexa647-BG, and visualized by TIRF-M. (C) Representative image (Left, brightfield; Right, TIRF) of a cell transfected with SNAP-GABAB<sub>1</sub>. No cell-surface staining is present. (D) Representative image of a cell transfected with SNAP-GABAB<sub>2</sub>. Single receptor particles are visible. Particle density = 0.42 particle/μm<sup>2</sup>. (E) Dependency of the distribution of particle components in cells transfected with SNAP-GABAB<sub>2</sub> on particle density. Particle composition is based on mixed Gaussian fitting analyses and is represented as in Fig. S2 H and L (n = 3,580 particles from 12 different cells). (F) Distribution of diffusion coefficients of SNAP-GABAB<sub>2</sub> particles. (Scale bars, 5 μm.)

Fig. S7. Single-molecule analysis of coexpressed SNAP-GABA<sub>B1</sub> and SNAP-GABA<sub>B2</sub>. (A) Schematic representation of the used SNAP-tagged constructs. (B) Representative image of cells transfected with both constructs, labeled with Alexa647-BG, and visualized by TIRF-M. (Scale bar, 5 μm.) Particle density was 0.36 particle/μm<sup>2</sup>. (C) Intensity distribution of Alexa647-labeled particles in B. Data were fitted with a mixed Gaussian model. (D) Dependency of the distribution of particle components on particle density. Particle composition is based on mixed Gaussian fitting analyses as in C and is represented as in Fig. S2 H and L (n = 7,399 particles from 25 different cells).
Fig. S8. Correction of particle distributions for random colocalizations. (A) Dependency of the density of apparent dimeric receptors on the density of monomeric control receptors (SNAP-CD86) or simulated particles. Raw data are the same as those used in Fig. S4. (B) Correction of SNAP-CD86 and SNAP2×CD86 particle distributions for the presence of random colocalizations. Left, starting data; Right, data after correction. (C) Same correction applied to the GABA<sub>e</sub> data shown in Fig. 4D.
Supplementary Figure S9. Effect of agonists on receptor di-/oligomerization. (A–C) Cells were transfected with SNAP-β₁AR (A), SNAP-β₂AR (B), or SNAP-GABAB₂ plus wild-type GABAB₂ (C) constructs; labeled with Alexa647-BG; and visualized by TIRF-M. To limit particle photobleaching, images were acquired every 10 s for 300 s, which resulted in less than 15% photobleaching at the end of the acquisition. The particles in the resulting image sequences were detected as in Fig. S1, without tracking. For each time point, particle intensities in three consecutive frames were used to perform a mixed Gaussian fitting analysis. (Left) Results of three representative experiments. The graphs report the composition of receptor particles, derived from the mixed Gaussian fitting analyses, as a function of time. For β₁AR and β₂AR: n = 1, monomers; n = 2, dimers; n = 3, trimers; n = 4, tetramers. For GABAB₂: n = 1, one heterodimer; n = 2, two heterodimers, n = 3, three heterodimers, etc. (Right) Summary of the results obtained from six experiments per each condition. The mean (±SD) abundance of each component before and 300 s after agonist stimulation is reported. Differences between stimulated and basal are not statistically significant by paired two-way ANOVA.
Movie S1. TIRF-M image sequence of Alexa647-labeled SNAP-CD86 receptors on the surface of a living cell.
Movie S2. Result of particle tracking analysis of the image sequence in Movie S1.

Movie S3. Computer-generated image sequence containing simulated particles with Brownian motion and characteristics (diffusion coefficients, intensity distribution, bleaching rate) similar to those of Alexa647-labeled SNAP-CD86 particles.