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Exploring GPCR conformational dynamics using single-molecule fluorescence

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ABSTRACT

G protein-coupled receptors (GPCRs) are membrane proteins that transmit specific external stimuli into cells by changing their conformation. This conformational change allows them to couple and activate G-proteins to initiate signal transduction. A critical challenge in studying and inferring these structural dynamics arises from the complexity of the cellular environment, including the presence of various endogenous factors. Due to the recent advances in cell-expression systems, membrane-protein purification techniques, and labeling approaches, it is now possible to study the structural dynamics of GPCRs at a single-molecule level both *in vitro* and in live cells. In this review, we discuss state-of-the-art techniques and strategies for expressing, purifying, and labeling GPCRs in the context of single-molecule research. We also highlight four recent studies that demonstrate the applications of single-molecule microscopy in revealing the dynamics of GPCRs. These techniques are also useful as complementary methods to verify the results obtained from other structural biology tools like cryo-electron microscopy and x-ray crystallography.

1. Introduction

G protein-coupled receptors (GPCRs) are a large group of membrane proteins in eukaryotic cells that are involved in the sensation of light, taste, and smell [1]. GPCRs mediate physiological and pathological processes like pain transmission and cardiovascular regulation and are implicated in several diseases, including Alzheimer's, schizophrenia, and cancer [2–5]. They have a conserved structure of seven transmembrane (TM) helices linked by three intracellular and three extracellular loops [6]. Human GPCRs are classified into four different groups based on their sequence homology and functional similarity. Class A GPCRs (rhodopsin-like receptors) constitute the largest and most extensively studied group of mammalian GPCRs. The other groups include class B (secretin and adhesion family), class C (glutamate family), and class F (frizzled family) [6,7]. GPCRs mediate cellular responses by interacting with various external stimuli, including photons and neurotransmitters, at their orthosteric binding sites. These binding sites are the main molecular targets that drive the activation and signaling properties of GPCRs [6].

GPCRs initiate cellular signaling cascades upon stimulation with ligands by coupling to cytoplasmic transducer proteins like heterotrimeric G proteins and arrestins (Fig. 1) [8]. G protein-dependent signaling begins with the binding of an agonist to the orthosteric binding site of the GPCR. This binding results in conformational changes within the TM helices, which primes the receptor for G protein binding and activation. The activation of G proteins promotes the exchange of GDP for GTP at the G protein's α subunit, causing it to dissociate from the $G_{\beta\gamma}$ subunits. This process then stimulates the production of second messengers such as cyclic adenosine monophosphate (cAMP), which triggers several physiological changes at the cellular level [1,9]. Alternatively, GPCR signaling through arrestins is triggered by the phosphorylation of the receptor's C terminal tail by G protein-coupled receptor kinases (GRKs). The recruited arrestins block G protein-mediated signaling, leading to receptor desensitization and internalization (Fig. 1) [1].

The development and progress of biophysical methods have provided valuable insights that have transformed our understanding of the dynamic processes in GPCRs. Various biophysical techniques, such as X-ray crystallography [10–13] and cryogenic-electron microscopy (cryo-EM) [14–18] have been employed to capture static snapshots of receptor conformations at high resolution. Additionally, studies using nuclear magnetic resonance (NMR) [19–22], single-molecule fluorescence (SMF) [23–26], and molecular dynamics (MD) simulations [27–30]

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have shown that GPCRs exhibit structural flexibility and undergo transitions between multiple conformational states during receptor activation, providing a clear picture of the complexity of GPCR activity. Of these techniques, SMF has emerged as a powerful complementary tool to other biophysical approaches, as it allows for real-time observation of GPCR structural dynamics and their interactions with other signaling molecules [31]. Moreover, SMF requires minimal structural modifications and can be performed in the context of complex native environments.

In this review, we will discuss the strategies and methods successfully implemented for preparing GPCRs for single-molecule studies. Our focus will be mainly on the expression systems, purification approaches, and labeling strategies, as well as the methods available for the structural and functional characterization of GPCRs at the single-molecule level. Special emphasis will be placed on single-molecule total internal reflection fluorescence (TIRF) microscopy, a high-precision method for determining distance changes between fluorescent probes. We will also highlight its applications and limitations. The techniques discussed in this review will provide researchers with an overview of the pipeline for conducting single-molecule research on GPCRs.

2. Expression strategies for GPCR production

Isolating pure and adequate amounts of GPCRs for analysis is challenging due to their nature as integral membrane proteins. A diverse range of heterologous expression systems have been extensively explored to facilitate GPCR production. The selection of an appropriate expression system depends on several factors, such as the GPCR of interest, expression yield for downstream applications, and the specific requirements for achieving correct folding, post-translational modifications, and integration into the cell membrane [32].

GPCRs like rhodopsin can be obtained in significant quantities from natural sources due to their abundance in the eye's retina [33]. However, most GPCRs are naturally produced in small amounts within cell membranes, requiring heterologous expression systems to generate larger quantities, particularly for structural and functional characterization. Commonly employed expression systems include mammalian, insect, and yeast cells (Table 1) [34]). Beyond these, alternative systems have been successfully used to achieve GPCR expression. For instance, bacterial cells, specifically *Escherichia coli,* have successfully been used to express a water-soluble variant of the mu-opioid receptor [35,36]. Additionally, an *E. coli*-based cell-free system has proven effective for expressing the human neuropeptide Y4 (NPY) receptor and human melatonin 1B (MTN) receptor [37]. Notably, the protozoan eukaryotic system LEXSY (*Leishmania tarentolae*) has been used to achieve remarkably high-yield expression of a stable and functional human A_{2A} adenosine receptor (A_{2A}AR) [38]. These diverse systems contribute significantly to our understanding of GPCR structure and function and facilitate the development of high throughput screening methods for therapeutic improvement and novel drug discovery.

2.1. Mammalian cell expression

Mammalian cells represent one of the most widely used eukaryotic expression systems for GPCR production. They possess the necessary cellular components to correctly translate, fold, modify, and incorporate proteins into the cell membrane. The intrinsic machinery within these cells enables the processing and maturation of proteins, ensuring their functional integrity and appropriate localization within the cellular environment. This capability of mammalian cells to perform these essential tasks makes them a good choice for producing GPCRs. Despite being optimal expression systems, several concerns have been raised

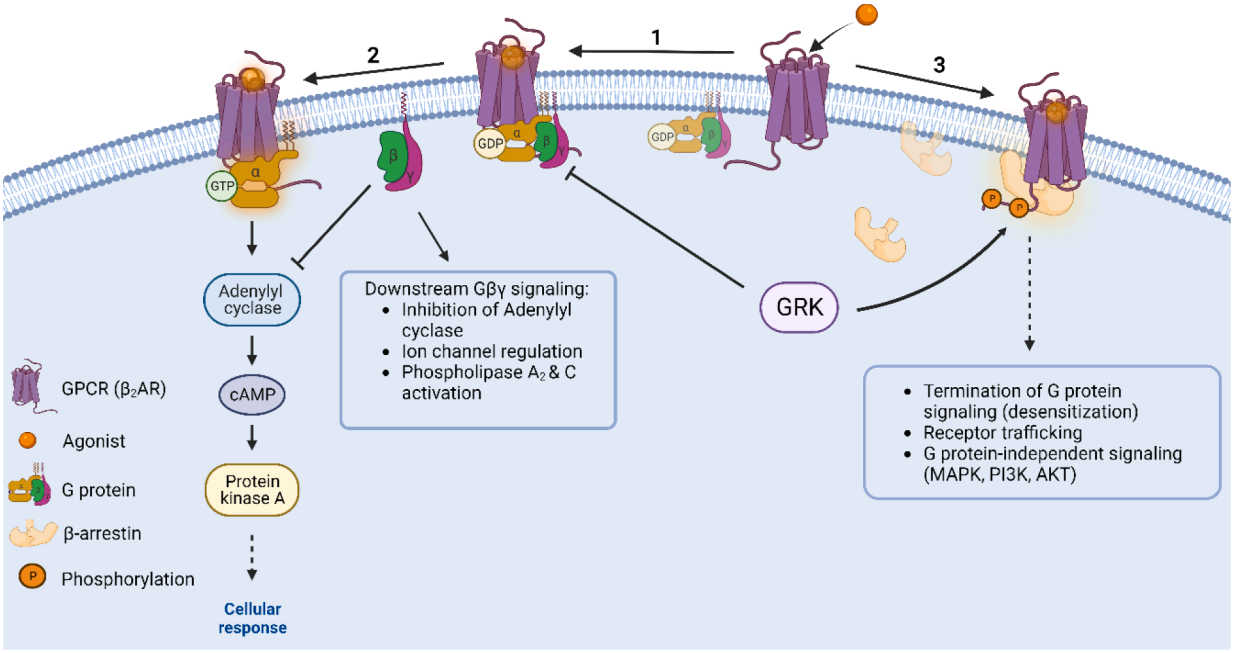

Fig. 1. GPCR signaling at the plasma membrane. (1) Inactive GPCRs form transient pre-coupled complexes with inactive heterotrimeric G proteins through random collisions at the cell surface (Refer to Mafi et al. (2022) [9]). Upon agonist binding, the GPCR undergoes conformational rearrangement of the TM helices, activating the receptor and stabilizing the receptor-G protein complex in a ligand-specific manner. The activated complex then initiates both G protein-dependent and independent signaling pathways. (2) In this example, guanine nucleotide exchange, mediated by the agonist-bound GPCR (β_2AR) initiates G protein-dependent signaling by activating the Gα subunit of the G protein and causing the dissociation of the Gβγ subunit. The activated Gα subunit subsequently transduces downstream signals by activating various effector proteins such as adenylyl cyclase, resulting in diverse cellular responses. (3) Additionally, β-arrestins mediate G protein-independent signaling. During this process, the agonist-activated receptor undergoes phosphorylation of the C-terminal tail by GRKs, inhibiting G protein coupling and facilitating β-arrestin recruitment to the active phosphorylated receptor. β-arrestin coupling then initiates several downstream signaling events, including receptor desensitization and trafficking. Figure created using BioRender.com.

Table 1
Recombinant human GPCRs derived from different expression systems.

GPCR	Class	Expression system	Cell line	Vector	References
β_2-adrenergic receptor (β_2AR)	A	Insect cell	*Sf9*	pFastBac	[24]
Glucagon receptor (GCGR)	B	Insect cell	*Sf9*	pFastBac1	[61]
Metabotropic glutamate receptor 5 ($mGluR_5$)	C	Insect cell	*Sf21*	pFastBac	[62]
Corticotropin-releasing factor receptor (CRF_1)	B	Insect cell	High Five	N/A	[63]
μ-opioid receptor (μOR)	A	Yeast cell	*S. cerevisiae*	pBB161	[64]
A_{2A} adenosine receptor (A_{2A}AR)	A	Yeast cell	*P. pastoris*	pPIC9K	[23]
Histamine H_1 receptor (H_1R)	A	Yeast cell	*P. pastoris*	pPIC9K (SMD1163)	[65]
Metabotropic glutamate receptor 2 ($mGluR_2$)	C	Mammalian cell	CHO (stable)	pcDNA5/FRT/TO-IRES	[66]
Glucagon-like peptide-1 receptor (GLP1R)	B	Mammalian cell	HEK293	pCDNA3	[67]

Abbreviations: CHO – Chinese hamster ovary; HEK293 – Human embryonic kidney 293.

over the production of GPCRs using mammalian cells. The most common issues identified include improper protein folding, incomplete protein insertion into the cell membrane, overloading the post-translational machinery, and cell toxicity, which results in reduced expression and yield [39].

Two general approaches are used when it comes to expressing GPCRs in mammalian cells: transient and stable expression. In transient expression, cells are transfected with the gene encoding the GPCR of interest either with a plasmid DNA complexed with cationic compounds like lipofectamine, enabling membrane insertion, or with a recombinant virus such as the vaccinia virus (for review, see [40,41]) and the Semliki Forest Virus (SFV) (for review see [42]). With this approach, the gene of interest is not integrated into the host cell's genome. Regardless of the transfection efficiency, GPCR expression is short-lived in transiently transfected cells since the cells are only viable for a few days [41]. For instance, transient expression is typically sufficient in the single-molecule studies of GPCRs, where only small amounts of pure, active protein are required. On the other hand, stable expression is recommended if consistently high quantities of functional protein are necessary for high throughput screening or drug discovery assays. Stable expression requires creating stable cell lines and results in the integration of the gene of interest into the genome of the host cell [39]. When conducting long-term experiments, using stable cell lines can be advantageous as they provide consistent expression levels that can be easily replicated. However, it is important to note that generating stable cell lines can be a time-consuming and expensive process.

2.2. Yeast cell expression

Yeast cells like *Pichia pastoris* [43–47], *Schizosaccharomyces pombe* [48–50], and *Saccharomyces cerevisiae* [51,52] are often used to express GPCRs. *P. pastoris* is the most preferred choice due to its ability to produce higher levels of functional GPCRs. It has even been successfully employed to produce different constructs of the human A_{2A} adenosine receptor (A_{2A}AR) for single-molecule studies (Table 1) [23]). On the other hand, *S. cerevisiae* is better suited for cloning and screening protein constructs [52].

Compared to other eukaryotic expression systems, yeast cells are easy to manipulate and can grow to high cell densities in simple and inexpensive media. They can also perform most post-translational modifications, including disulfide bond formation and glycosylation, which are sometimes crucial for the proper folding and activity of GPCRs [53]. It is worth noting that although yeast cells can perform PTMs, their glycosylation patterns differ from those in mammalian and insect cells. Additionally, the lipid composition of yeast membranes differs from that of mammalian cells, with ergosterol being the dominant sterol instead of cholesterol. Since cholesterol is essential for modulating and maintaining the stability of most GPCRs, yeast cells have been engineered to produce cholesterol, increasing the expression of functional human GPCRs [54].

2.3. Insect cell expression

Insect cells are commonly used in structural studies of GPCRs because they provide high yields – milligram amounts of pure protein [39]. They are also ideal expression systems for single-molecule applications that require even smaller amounts of pure protein. GPCRs undergo heavy post-translational modifications, and insect cells can perform essential post-translational modifications, making them an excellent alternative expression system. To ensure efficient expression and membrane localization of GPCRs in insect cells, signal peptide sequences are typically attached to their N-termini [55–57]. However, like yeast cells, insect cells possess distinct membrane lipid compositions and glycosylation patterns that differ from mammalian cells [58].

Insect cells like *Spodoptera frugiperda* (American fall armyworm; *Sf9* and *Sf21* cell lines) and *Trichoplusia ni* (High Five) have been successfully used to express GPCRs (Table 1). Among these cells, *Sf9* and *Sf21* insect cell lines are preferred due to their susceptibility to baculoviral infection and replication [59]. To express GPCRs in Sf9 cells, for instance, a recombinant form of the *Autographa californica* baculovirus is used. Insect cells are typically infected with this virus, which leads to the expression of the viral protein polyhedrin at high levels. However, the gene for polyhedrin can be replaced with a GPCR gene since its expression is not necessary to produce a functional virus [60]. The polyhedrin promoter is a late promoter that only expresses at the end of the infection cycle, specifically 8–24 h post-infection, resulting in the expression of high levels of the target protein [41].

3. Solubilization and purification of structurally intact GPCRs

To study the structural dynamics of GPCRs using single-molecule techniques, they must be purified to maintain their structural and dynamic integrity. As integral membrane proteins, it is beneficial to purify GPCRs in a native-like or native membrane environment for structural studies [68]. Therefore, *in vitro* studies require solubilization methods that utilize either detergent micelles or lipid nanodiscs to shield their hydrophobic regions, preventing precipitation since these proteins are insoluble in aqueous solutions [68]. The most commonly used methods are reviewed below.

3.1. Detergent micelles

The solubilization of membrane proteins requires a disruption of the lipid bilayer for protein removal without immediate reconstitution back into the membrane [69]. Amphiphilic detergent molecules can disrupt the membrane and mimic phospholipid properties to maintain the stability of the membrane protein [69]. A wide variety of detergents commercially available for the extraction of membrane proteins vary by charge and chain length [69]. Some of the most common detergents used for studies of GPCRs are dodecyl maltose (DDM) and lauryl maltose neopentylglycol (LMNG), with cholesteryl hemisuccinate (CHS) to mimic a more membrane-like environment [70]. DDM-CHS is an ideal detergent for solubilization and purification of GPCRs that have been

thermostabilized, while LMNG has been shown to stabilize the less-stable GPCRs [70]. The use of detergents for the solubilization of GPCRs has been incredibly useful in structure determinations.

The process of solubilizing GPCRs into detergent micelles is straightforward upon generating membrane fractions from the expression system of choice. The process involves the resuspension and homogenization of the membrane fraction using a solubilization buffer that contains the detergent of interest [71]. After resuspension, the insoluble material is separated from the solubilized portion through ultracentrifugation, followed by affinity purification using affinity tags (e.g., FLAG or poly-histidine tags) (Fig. 2**A**) [71,72].

In single-molecule studies, detergent micelles offer an advantage in that they are quick and effective at solubilizing and maintaining the stability of the receptor after purification. However, they do not accurately represent GPCR dynamic studies due to the lack of native lipids. Lipids are known regulators of many membrane proteins, including GPCRs, and could ultimately play a role in the dynamics and activity of GPCRs.

3.2. Protein nanodiscs

Unlike detergent micelles, the reconstitution of GPCRs into lipid nanodiscs provides insights into how particular lipids affect the structure and dynamics of these receptors. After solubilization of GPCRs using detergent micelles, they can be reconstituted into nanodiscs using desired lipid compositions, membrane scaffold proteins (MSPs), and bio-beads to remove the detergent (Fig. 2**B**) [71,73,74]. The MSPs (human serum apolipoprotein A-I) are amphipathic helical proteins that encircle and stabilize the hydrophobic acyl chains of lipid bilayers upon self-assembly with lipids to form nanodiscs [75,76]. Nanodiscs offer an advantage over detergent micelles by providing a native-like environment for the receptor. This allows for controlled manipulation of the lipid composition to observe how different lipids and lipid compositions affect GPCR activity [47]. Recent NMR studies of the class A human A_{2A}

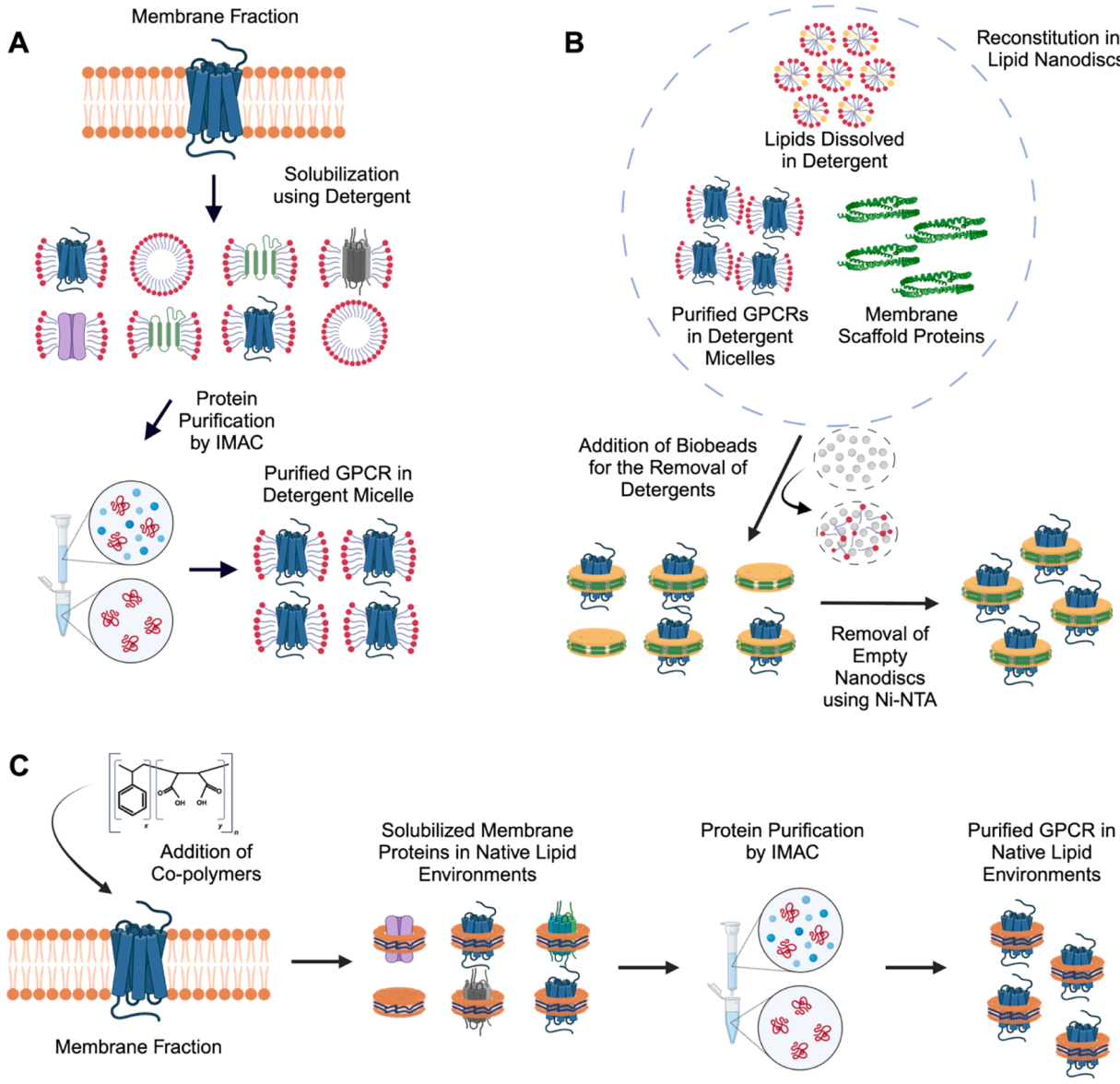

Fig. 2. Schematic showing the solubilization and purification of GPCRs. (A) Stepwise process of the solubilization of GPCRs in detergent micelles (e.g. DDM or LMNG) and the purification of the GPCR through IMAC. **(B)** Reconstitution of GPCRs into lipid nanodiscs purified from detergent micelles, lipids dissolved in detergents, and membrane scaffold proteins. After reconstitution, the detergent is removed using bio-beads and empty nanodiscs are removed with another purification step. **(C)** Stepwise process of the solubilization of membrane proteins using co-polymers (e.g.: SMA, DIBMA, or AASTY) and the purification of GPCRs in native membrane nanoparticles through IMAC. Figure created using BioRender.com.

adenosine receptor in lipid nanodiscs have been used to show how anionic lipids and cholesterol allosterically influence the activity of the receptor, leading to a more active state in which TM6 moves outward to allow for G-protein coupling [47,77]. Nanodiscs also offer the ability to alter lipid ratios to study how the different lipid-GPCR interactions affect receptor dynamics. In addition, controlling the lipid composition within nanodiscs is advantageous for single-molecule experiments. For instance, biotin-conjugated lipids can be incorporated into nanodiscs to aid in the immobilization of individual molecules, as opposed to needing an additional biotin-conjugated antibody [71,73]. Alternatively, biotinylated MSPs can be used for protein nanodisc immobilization. Although the lipid composition within nanodiscs provides a native-like membrane environment, it is essential to note that this environment is not native. Native lipid compositions could affect the dynamics observed during single-molecule experiments.

3.3. Native membrane protein nanodiscs

Co-polymers like styrene-maleic acid (SMA) and diisobutylene maleic acid (DIBMA) are amphipathic compounds that can spontaneously insert themselves into lipid membranes to encapsulate membrane proteins as a means of solubilization into lipid particles (Fig. 2**C**) [78–83]. Once native membrane nanodiscs are formed, they are easier to work with than detergent micelles, as it is not necessary to supplement buffers with additives like detergents at any stage of protein purification and characterization. These co-polymers offer an advantage in maintaining the native lipid environment around the receptor. SMA creates lipid nanoparticles (SMALPs) around 9–12 nm in diameter, while DIBMA can make lipid nanoparticles (DIBMALPs) around 25 nm in diameter [79–84]. Both SMALPs and DIBMALPs effectively solubilize GPCRs that retain their structural integrity and functionality [81]. Depending on the size of the nanodiscs, the choice of co-polymers may vary, as the disc size could potentially alter receptor dynamics [79]. Smaller discs may constrict the dynamics of GPCRs, confounding the interpretation of single-molecule data.

Although the use of co-polymers for solubilization of membrane proteins has made great strides in purification, there are some other limitations. SMA and DIBMA are sensitive to divalent metal ions (e.g., Mg^{2+} and Ca^{2+}) and pH. Below a pH of 6.5, SMA is insoluble [85]. These properties can complicate experiments as the result would affect the charge of the outward-facing maleic acid group [81,86]. Another limitation of these co-polymers is their light-absorbing properties due to the styrene group absorbing at 260 nm, which overlaps with UV protein absorption at 280 nm. The absorption of the styrene group could interfere with UV absorption and light scattering assays. [81,86] Despite the disadvantages of some co-polymers such as SMA and DIBMA, the field continues developing new co-polymers for membrane protein solubilization. Overall, using co-polymers for GPCR solubilization allows the receptors to retain their native lipid environment, which is a significant advantage when studying the dynamic nature of GPCRs using single-molecule approaches.

4. Site-specific labeling of GPCRs

To study the dynamics of GPCRs using fluorescence microscopy, regions of the receptor that are exposed outside the lipid bilayer are fluorescently labeled with fluorescent probes. If the structure of the extracellular or intracellular loops has been resolved, then these regions can be fluorescently labeled. However, if the structure of these loops is unknown, then the most commonly labeled regions for GPCR research are the ends of the TM helices or the N- or C-terminus regions. The labeling strategy for studying GPCRs varies depending on whether it is an *in vitro* or live cell study [31]. The currently available labeling approaches in live cells allow for the study of receptor dynamics [66], interactions with cytosolic proteins like G proteins [87], oligomerization [88], and hetero-dimerization [89] of GPCRs. On the other hand, single-molecule level dynamics of TM helices [24,25,73], extracellular domains [90], and C-terminal tails [91] are studied with purified GPCRs, as detailed in the previous section.

Alternatively, GPCRs can be directly labeled by fusing the receptor with a fluorescent protein (e.g., Green Fluorescence Protein (GFP)) connected by a linker peptide [92,93]. This approach eliminates the need for additional dyes and allows fluorescence to be detected in live cells after protein expression. However, due to the large size of the fluorescent proteins and their propensity to form homo-oligomers, they may influence the dynamics of the receptor. As a result, this method is usually used for studying protein–protein interactions [93]. Regardless of the labeling strategy used, additional validation methods are needed to ensure that the modifications made to the receptor do not alter its function. These methods include detecting intracellular activation by assaying for cAMP levels [94], G protein activation [95], and ligand binding assays [96,97]. The most used GPCR labeling strategies for *in vitro* and live cell studies are detailed here:

4.1. Maleimide-based click chemistry

For maleimide-based labeling of GPCRs with a fluorescent dye, an amino acid at the region of interest is modified to cysteine. The thiol group of the exposed cysteine is conjugated to a maleimide-containing fluorescent dye via a thiol-maleimide reaction (click chemistry) (Fig. 3**A**). This labeling method does not affect the cysteine residues that form disulfide bonds and those buried within the lipid bilayer, as the thiol moieties are inaccessible. However, other cysteines with the exposed thiol group must be modified to different amino acids to avoid non-specific labeling. This labeling strategy is widely used for labeling GPCRs *in vitro* but requires extensive knowledge of the protein's structural information to ensure that amino acid modifications do not affect its function [23,91].

4.2. Unnatural amino acid-based click chemistry

A novel labeling approach involves using genetic code expansion to incorporate unnatural amino acids such as p-azido-L-phenyl alanine (azF) at the desired site [98]. This is achieved by inserting the low-abundance amber stop codon, TGA, in the gene that encodes for a GPCR. The expressed protein will then contain azF, which can be tagged with a fluorescent dye using the strain-promoted azide-alkyne *cyclo*-addition (SpAAc) method (Fig. 3**B**). This labeling strategy has been successfully implemented for labeling GPCRs in both *in vitro* and in-cell studies and has minimal impact on protein structure [99,100]. However, protein expression may require extensive optimization, and the amount of expressed protein may be lower than other labeling strategies. Moreover, only a limited number of expression systems can be used for this method (e.g., bacteria [101], yeast [102], or mammalian cells [103]).

4.3. Tetra-cysteine peptide motifs

An alternative labeling method uses short tetra-cysteine peptide motifs (CCXXCC, where X denotes any amino acid), which can be conjugated to a small fluorescein derivative called fluorescein arsenical hairpin binder-ethane dithiol (FlAsH-EDT). Rhodamine-based ReAsH can also be used in place of FlAsH. FlAsH-EDT is non-fluorescent by itself and becomes fluorescent on binding to the tetra-cysteine motif [61] (Fig. 3**C**). Flanking amino acid sequences around the tetra-cysteine motif have been shown to improve the binding efficiency of the FlAsH-EDT molecule (e.g., FLNCCPGCCMEP and HRWCCPGCCKTF). This method has been implemented to label GPCRs *in vitro* [91], in live cells [104], and in intracellular proteins like arrestins [105,106]. It is not necessary to know the full structure of the protein to implement this labeling method, however, it must be noted that the exposed cysteine-rich regions in the receptor may interact non-specifically with the FlAsH-EDT

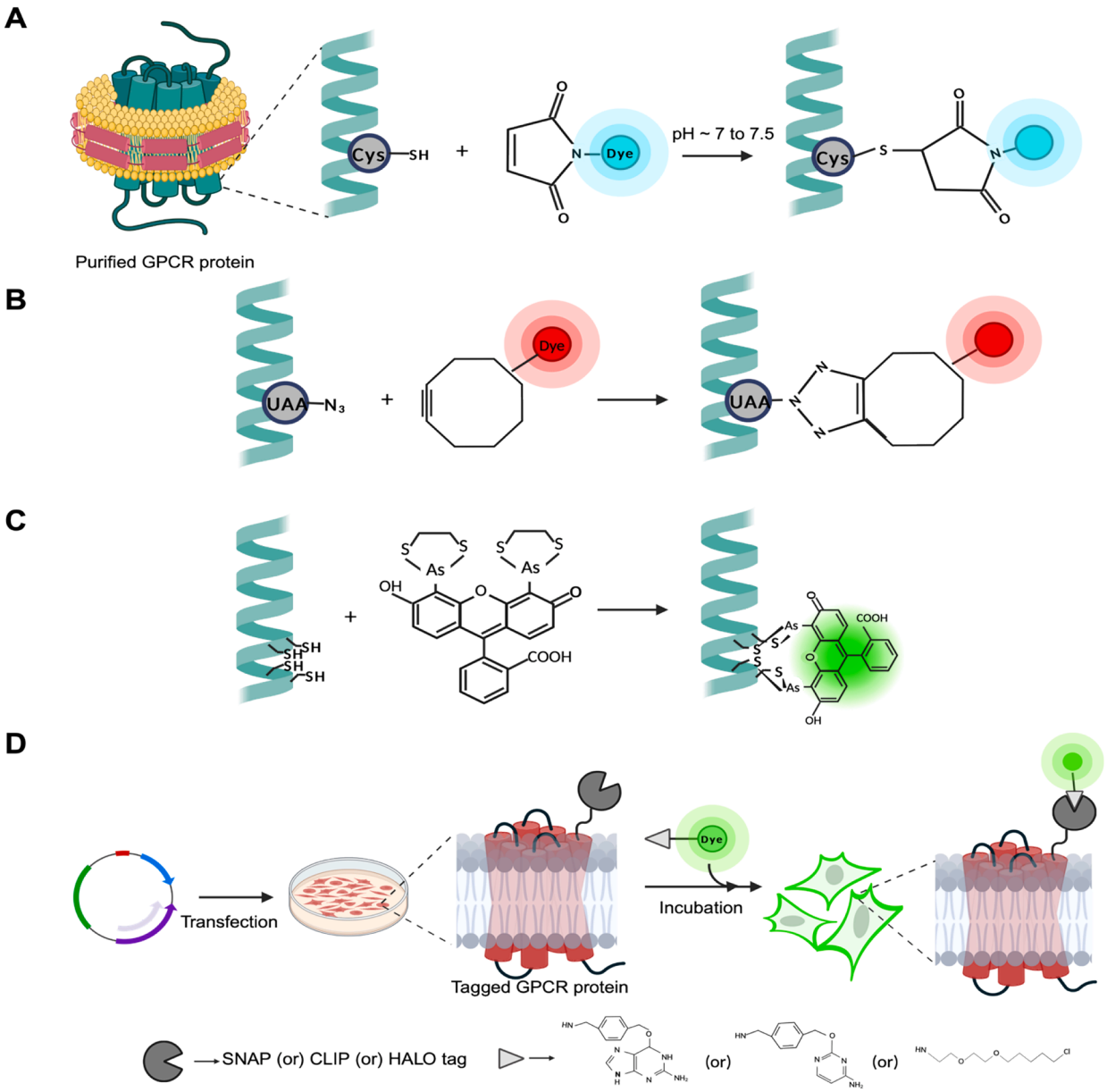

Fig. 3. Labeling strategies for GPCRs. (A) A purified GPCR in an MSP nanodisc is shown with the intracellular region of the transmembrane helix to be labeled highlighted. The exposed cysteine is labeled with a fluorescent dye via a thiol-maleimide reaction. **(B)** The incorporation of an unnatural amino acid (UAA) with an azide moiety in the purified GPCR can be used for labeling with strain-promoted azide-alkyne *cyclo*-addition (SpAAC) reaction. **(C)** The tetra-cysteine (TC) moiety in the exposed transmembrane helix can be labeled with a non-fluorescent fluorescein arsenical hairpin binder-ethane dithiol (FlAsH-EdT$_2$) molecule, which becomes fluorescent after coordinating with the sulfide groups of the TC-motif. **(D)** Illustration of the steps involved in the fluorescent labeling of GPCRs expressed on live cell membranes. Different protein tags and their corresponding fluorescent ligands that can be used for labeling are shown. Figure created using BioRender.com.

[107].

4.4. Snap-tag and CLIP-tag

SNAP-tag is a 182-amino acid long protein tag (Mw. ~ 19.4 kDa) that can be fused to a GPCR using recombinant DNA technology with a flexible linker of 6 to 12 amino acids. The linker peptide sequence is crucial to the dynamics of the tagged GPCR. The SNAP-tag is a genetically modified version of the human alkyl-guanine transferase (AGT) enzyme. It forms a covalent bond with O^6-benzylguanine derivatives, which can then be attached to a fluorescent dye (Fig. 3**D**) [108]. This allows a wide variety of dyes with unique photophysical characteristics to be used for live-cell microscopy. This method is the most common way of labeling GPCRs in live cells [109–111], compared to the fusion of fluorescent tags to proteins. Another modified AGT enzyme, CLIP-tag, binds to fluorescent O^6-benzycytosine derivatives [112]. Typically, the N-terminus of GPCRs, facing the extracellular side of the cell, is used as the fusion site for these protein tags [111,113].

4.5. Halo-tag

The HALO-tag is a type of self-labeling protein tag composed of 293 amino acids (Mw. ~ 33 kDa) and can be used to label GPCRs. It is a modified haloalkane dehalogenase enzyme derived from bacteria. It forms a strong covalent interaction with chloroalkanes, which can be attached to a fluorescent dye (Fig. 3**D**) [114]. The binding reaction is

stable, rapid, and irreversible under physiological conditions. Due to its bacterial origin, it does not undergo any non-specific interactions inside the cell [115]. However, because it is large, it requires a more extended, flexible linker peptide to fuse with GPCRs and reduce any interference it may cause with the functional dynamics of the receptor [115]. Chloroalkanes tagged with cell membrane-permeable dyes are often used to study the intracellular interactions of GPCRs within the cell, as chloroalkanes can easily cross the cell membrane [111,116].

5. Total internal reflection fluorescence (TIRF) microscopy

Single-molecule TIRF microscopy uses the evanescent field generated from a laser excitation to excite immobilized samples. TIRF has several advantages: (i) it excites the fluorophores only at a penetration depth of ~ 100–150 nm, thereby reducing background noise, and (ii) it minimizes photobleaching of fluorophores in solution or photo-toxicity in cells by minimal excitation [117]. Two types of TIRF microscopes are typically used for single-molecule studies: Prism-based TIRF (pTIRF) (Fig. 4**C**) and Objective-based TIRF (oTIRF) (Fig. 4**D**). Both microscope setups provide a high signal-to-noise ratio (SNR), with the prism-based TIRF giving a better SNR due to the possibility of using higher laser powers. Since the pTIRF microscopes use open lasers, they are not commercially available; instead, they are usually custom-built by research labs. For a step-by-step guide on building a pTIRF setup for single-molecule microscopy, refer to [118–120] for more information. Also, it must be noted that pTIRF setups use expensive quartz slides instead of regular glass slides, which are used for oTIRF. The smTIRF movies are recorded by either a scientific Complementary Metal-Oxide-Semiconductor (sCMOS) camera [121] or an Electron-Magnified Charge-Coupled Device (EMCCD) camera [90]. These detectors are sensitive enough to detect a single fluorophore molecule at ~ 200 nm spatial resolution and ~ 20–30 ms temporal resolution, depending on the imaging conditions [122]. The TIRF movies are usually recorded until the fluorophores are bleached. In the case of live-cell microscopy, the imaging is performed until the cell viability is affected.

5.1. Sample preparation and protein immobilization

One significant advantage to a single-molecule TIRF study is that it allows the monitoring of individual GPCR molecules and their dynamic behavior over time [23]. However, to visualize individual molecules, the receptors must be immobilized on microscope slides [123]. There are variations in immobilization techniques, but most involve the passivation of quartz slides with a mixture of biotin-PEG and unmodified PEG to prevent non-specific interactions with the slide while allowing biotin to serve as an anchor [119,124]. The biotin on the PEG can then interact with neutravidin or streptavidin, acting as a bridge between biotin-conjugated antibodies specific for a tag on the receptor, or biotin-conjugated lipids in nanodiscs [23,71,119].

To prepare samples for single-molecule TIRF experiments, flow channels must be created on quartz or glass slides that have been drilled with two holes [71,119]. The channel is prepared using double-sided tape, grease, and a glass coverslip. The samples are then injected through the hole on one end of the slide and allowed to flow out through the other end of the channel [71,119] (Fig. 4**A and 4E**).

5.2. Sample preparation for live cell TIRF microscopy

The development of novel DNA transfection methods in recent years has made it possible to study the dynamics of labeled GPCRs in live cell membranes. This is achieved by transfecting adherent cells with plasmids expressing GPCRs fused with fluorescent tags [66] or tetra-cysteine motifs [104]. The cells are then labeled with fluorescent dyes and imaged under a microscope. Since the over-expression of GPCRs on the

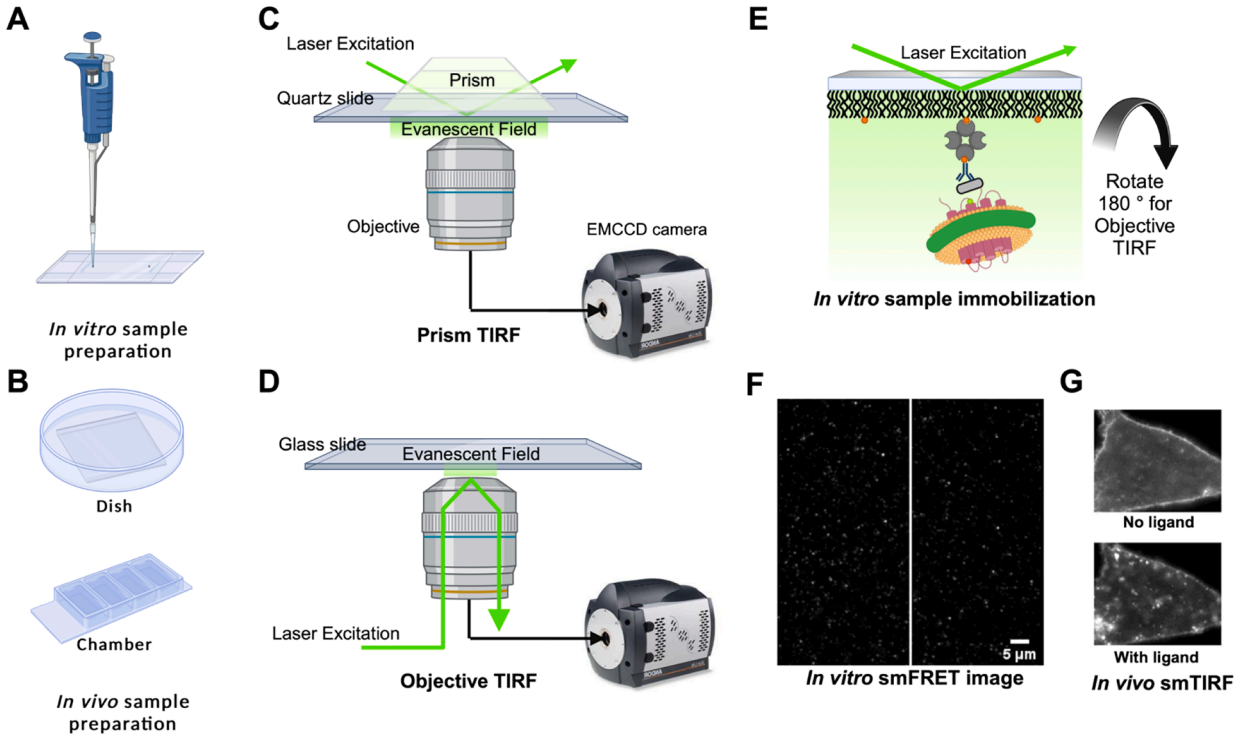

Fig. 4. Illustration of TIRF microscopy. (A) Illustration of the sample preparation method for *in vitro* TIRF microscopy. **(B)** Sample preparation methods in a dish or microscope chamber for in vivo TIRF microscopy. **(C)** Schematic of a prism-based TIRF microscopy setup highlighting different components as well as the excitation path and an evanescent field. **(D)** Schematic of objective-based TIRF microscopy components and the excitation path with an evanescent field. **(E)** In vitro sample immobilization strategy using streptavidin and biotinylated antibodies. **(F)** An example smFRET image of GPCR molecules with no ligand added *in vitro* with donor fluorescence spots (left) and acceptor fluorescence spots (right). **(G)** An example of an in vivo smTIRF image of a live cell with GPCR occurring as bright spots when activated. This figure was reproduced from the article Grimes et al. [111] published in Cell journal with permission. Figure created using BioRender.com.

membrane can give high background fluorescence, the receptors are either moderately expressed or minimally labeled to study their dynamics at the single-molecule level. The cells can then be grown directly in a dish with a coverslip for short-time imaging [125] or special incubation chambers for long-time imaging with a TIRF microscope [109,111] (Fig. 4**B and 4G**).

5.3. *smTIRF techniques*

The SMF raw data obtained from smTIRF microscopy can be very informative. The oligomerization of GPCRs can be studied from the step-photobleaching patterns observed in fluorescence intensity traces [66,88,89]. The number of bleaching steps corresponds to the number of oligomers of the GPCRs localized in a region [88]. Similarly, based on the localization and tracking of the labeled GPCR spots typically on live cell membranes, the receptor dynamics during the activation process can be determined [87,111]. Also, labeling GPCRs and their interacting proteins like G proteins [87] and β-arrestins [111] with different fluorophores enables studies of the colocalization and dynamics between the proteins *in vitro* and live cells.

Further biophysical techniques have been developed by harnessing the photo-physics of novel fluorophores. For instance, the quantum yield of cyanine dyes can vary depending on the environment [126]. With this discovery, researchers have utilized this photophysical property to study the structural dynamics of biomolecules [127] through a technique called single-molecule protein-induced fluorescence enhancement (smPIFE). Recent studies have employed this technique to reveal previously unknown states and molecular kinetics during the activation process of GPCRs [23,24,43,73]. Since only a single dye is required for this technique, it is easy to implement and is versatile for studying GPCRs.

Another important fluorescence microscopy technique that is widely used for studying the structural dynamics of GPCRs both *in vitro* and in live cells is single-molecule fluorescence resonance energy transfer (smFRET) [119]. This technique requires labeling GPCR samples with a unique pair of fluorescent dyes commonly known as a 'FRET-pair'. In the 'FRET-pair,' the 'donor-dye' is excited by the laser beam, and the emitted fluorescence from the donor is absorbed by the 'acceptor-dye' depending on its proximity to the donor. This technique acts as a 'molecular ruler' [119] that allows variations in the emission intensities from both the donor and acceptor dyes to be detected, revealing the proximity dynamics of the labeled regions (Fig. 4**F**).

smFRET can be harnessed to reveal the dynamics of different regions of GPCRs, such as the TM-helices [128,129], extracellular domains (ECDs) [130], and C-terminal domains [131] during the activation process. This technique has also been used to investigate the kinetics of GPCR dimerization [66,109] and ligand binding [110]. This method can determine previously unknown hidden states in GPCR activation, which can aid in developing novel biosensors for drug screening.

6. Applications

Single-molecule techniques have been applied to study class A, B, and C GPCRs in micelles, native-like, and native membrane environments [23,90,110]. Here, we will review some of the studies used to showcase the wide range of experimental techniques that can be conducted to study these receptors at the single molecule level, both in live cells and *in vitro*.

6.1. *Sequential A_{2A}AR TM dynamics in a native-like milieu*

A recent application of single-molecule fluorescence experiments on a class A GPCR involved investigations on the conformational dynamics of the human A_{2A} adenosine receptor (A_{2A}AR) in native-like lipid environments. A_{2A}AR has been the focus of many NMR spectroscopic studies and has become of great interest for single molecule studies. A receptor variant was created with a single cysteine (A289C) positioned on the intracellular surface of helix 7 [23]. (Fig. 5). This variant was created for chemical conjugation with the environment-sensitive fluorescent dye Cy3. The receptor was expressed in *Pichia pastoris* and later solubilized and reconstituted into lipid nanodiscs with a molar ratio of 65:30:5 POPC, POPS, and biotinylated POPE, respectively [23,71].

The nanodiscs containing the receptor labeled with Cy3 were immobilized on a microscope slide and observed using a TIRF microscope [23]. In these experiments, the conformational dynamics of A_{2A}AR were observed in three different conditions – an unliganded (apo) condition, an antagonist-bound (ZM241385) condition, and an agonist-bound (NECA) condition. For the apo- and antagonist-bound A_{2A}AR, there were predominately two intensity states observed – ~59 % for state 1 and ~ 41 % for state 2, with a small population transitioning to a higher intensity state, state 3. However, the agonist-bound A_{2A}AR showed significant changes from the apo- and antagonist-bound receptors. The population of state 1 decreased (49 %), state 2 increased (39 %), and a third state emerged (12 %) [23]. Each condition's fluorescence intensity time trace showed a reversible temporal ordering of the fluorescence emission states. It was observed that the receptor does not transition directly from its inactive state to its active state, i.e., state 1 to state 3, but transitions through an intermediate state, state 2. Here, the single-molecule study has revealed new information upon receptor activation that had not been shown in previous crystal and cryo-EM structures [23].

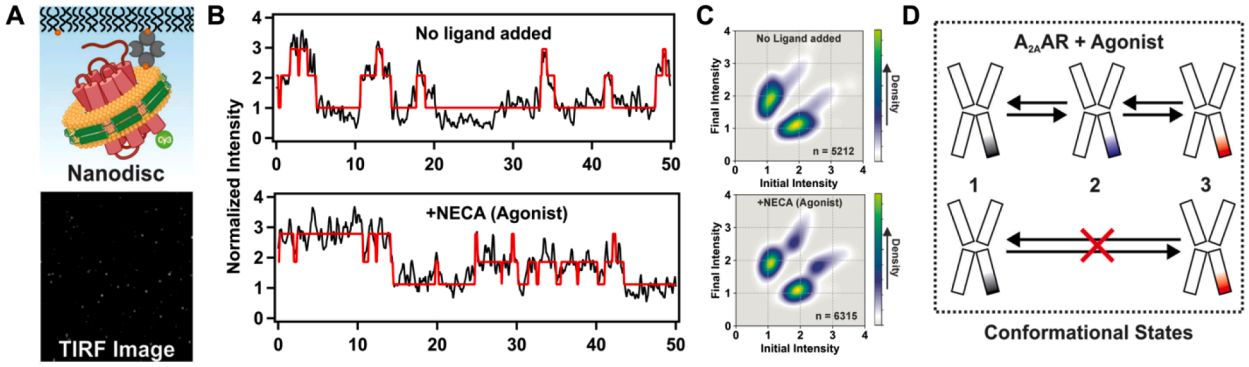

Fig. 5. Sequential A_{2A}AR TM dynamics in a native-like milieu. (**A**) Schematic representation of A_{2A}AR-nanodisc immobilized to a PEGylated slide via biotin-streptavidin interactions. (**B**) Single-molecule time trajectories of apo- and agonist-bound A_{2A}AR. Red lines indicate hidden Markov fitting of the raw traces (Black). (**C**) Representative transition density plots (TDPs) obtained from single–molecule fluorescence intensity trajectories in apo- and agonist-bound A_{2A}AR. (**D**) Model of A_{2A}AR TM helix VII dynamics during receptor activation. The figure is prepared from Wei *et al.* [23] with permission from Structure journal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6.2. Single-molecule visualization of glucagon receptor extracellular domain dynamics

Single-molecule studies of the class B glucagon receptor (GCGR) have recently been published, showing the dynamics of the extracellular domain (ECD) at the molecular level [90]. For class B GPCRs, two models have been proposed on the activation mechanism of peptide ligands that target and bind to the ECD and transmembrane domain binding pocket [132,133]. Here, the authors showed the dynamics of the ECD of GCGR in the presence of the peptide ligand glucagon. For these experiments, GCGR with a C-terminal *hexa*-histidine tag (6xHis) and FLAG tag were expressed in HEK293T cells and solubilized into DDM-CHS detergent micelles. The receptor was then purified through Ni^{2+}-NTA affinity chromatography and site-specifically labeled through chemical conjugation at C287 and C28 with a FRET donor (Alexa Fluor 555) and acceptor (Alexa Fluor 647) pair [90].

In the single-molecule FRET experiment, fluorophore-labeled GCGR in detergent micelles were immobilized on a microscope slide via biotin-streptavidin interactions using biotinylated anti-FLAG antibodies (Fig. 6). The donor fluorophore was excited with a 532 nm laser, and emissions from both the donor and acceptor fluorophores were recorded in the presence and absence of glucagon (agonist) and MK0893 (antagonist) [90]. The FRET states of individual molecules were estimated from the donor and acceptor emissions and used to generate population FRET distributions of the molecules. In the absence of glucagon, two distinct states were observed. Based on the distance between the fluorophores, these states corresponded to the open and closed conformations of the ECD of GCGR. Upon the addition of glucagon, additional populations appeared on the FRET distributions of the population between the open and closed state FRET efficiencies, with lower FRET values. This data suggested that the ECD remains dynamic in the presence of glucagon, with multiple open states [90,134]. Single-molecule experiments have proven helpful in understanding ECD dynamics and ligand recognition for class B GPCR activation and signaling.

6.3. Dimerization dynamics of the glutamate receptor

In ensemble FRET experiments, mechanistic information like receptor conformations and transition states cannot be delineated for GPCRs. However, for class C GPCRs, smFRET has recently been applied to investigate the activation of metabotropic glutamate receptor 2 (mGluR2) receptors upon homodimerization and to study the effects of the cysteine-rich domain (CRD) that bridges the transmembrane domain of the GPCR with the extracellular Venus flytrap domain (VFT) [110]. This work used HEK293T cells to express GPCR with azi-CRD, an allosteric modulator of mGluR2 containing a C-terminal FLAG-tag. The cells were then labeled via azide-alkyne click chemistry with the FRET donor (Cy3 alkyne) and FRET acceptor (Cy5 alkyne). After cell lysis, the lysate was applied to the biotin-PEG surface of the slide and pulled down using a biotinylated anti-FLAG antibody, a method called single-molecule pull-down (SiMPull) [109,110,135].

In this study, they observed how negative allosteric modulators that bind to the transmembrane domain act to prevent glutamate-dependent activation of the receptor using MNI-137 [110,136]. Through smFRET experiments, they found that intermediate and saturating concentrations of glutamate and MNI-137 had little effect on the active conformation of mGluR2 but increased the intermediate states 1 and 2 [110]. Transition density plots derived from Hidden Markov Model analysis of fluorescence time traces showed that saturating glutamate conditions resulted in transitions between state 2 and the active conformations of the CRD. However, in the presence of saturating glutamate conditions and MNI-137, the transitions occurred primarily between intermediate states 1 and 2, with very few transitioning to the active conformation of the CRD [110]. Thus, this study provides insights into how negative allosteric modulators influence the activation of class C GPCR homodimers at the single-molecule level (Fig. 7).

Additionally, it is worth noting that as a future direction, the application of TIRF-based single-molecule photobleaching techniques such as Native-nanoBleach [137] could prove to be very useful for studying how GPCRs are organized at nanoscale resolution. By directly visualizing and quantifying GPCR oligomerization in its native environment, this technique could potentially provide valuable insights into the oligomeric states of GPCRs under different ligand-binding conditions, mutations, or interactions with other proteins. Ultimately, this could lead to a deeper understanding of GPCR function and help identify new therapeutic approaches.

6.4. Single-molecule tracking of GPCRs

In addition to using single-molecule imaging to study receptor conformations and dynamics, SMF also serves as a powerful tool for studying receptor interactions at the cellular level [111,121]. It has been found that the fluidity of the cell membrane can affect the dynamic behavior of GPCRs. Recently, single-molecule tracking techniques have been applied to observe how β_2 adrenergic receptor (β_2AR) – β-arrestin2 (βArr2) interactions occur in the plasma membrane of live cells at a temporal resolution of ~ 30 ms and a spatial resolution of ~ 20 nm [111]. For these experiments, Chinese hamster ovary (CHO) cells, which have no detectable β_2AR expression, were transiently transfected with SNAP-β_2AR and Halo-βArr2 constructs and were labeled with saturating concentrations of the organic fluorophores – Alexa Fluor 647 and Janelia Fluor 549. Additionally, the clathrin-coated pits (CCPs) of the cell were also visualized through the transfection of GFP-tagged clathrin

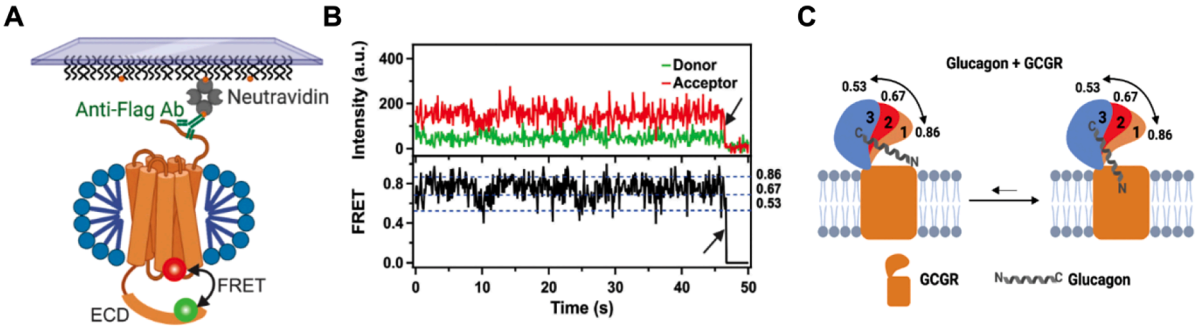

Fig. 6. Single-molecule visualization of glucagon receptor extracellular domain dynamics. The receptor (GCGR) was labeled with two different fluorophores – one on the extracellular face of the TM domain and the other on the ECD – and imaged using TIRF microscopy to monitor the dynamics of the ECD upon ligand binding. **(A)** Schematic of the prism TIRF-based smFRET setup used for imaging individual glucagon receptors. **(B)** Representative single-molecule fluorescence trajectory for a glucagon-bound receptor. **(C)** Proposed model of ligand-induced GCGR ECD dynamics based on single-molecule fluorescence data. The figure is assembled from Liu et al. [90] with permission from JBC journal.

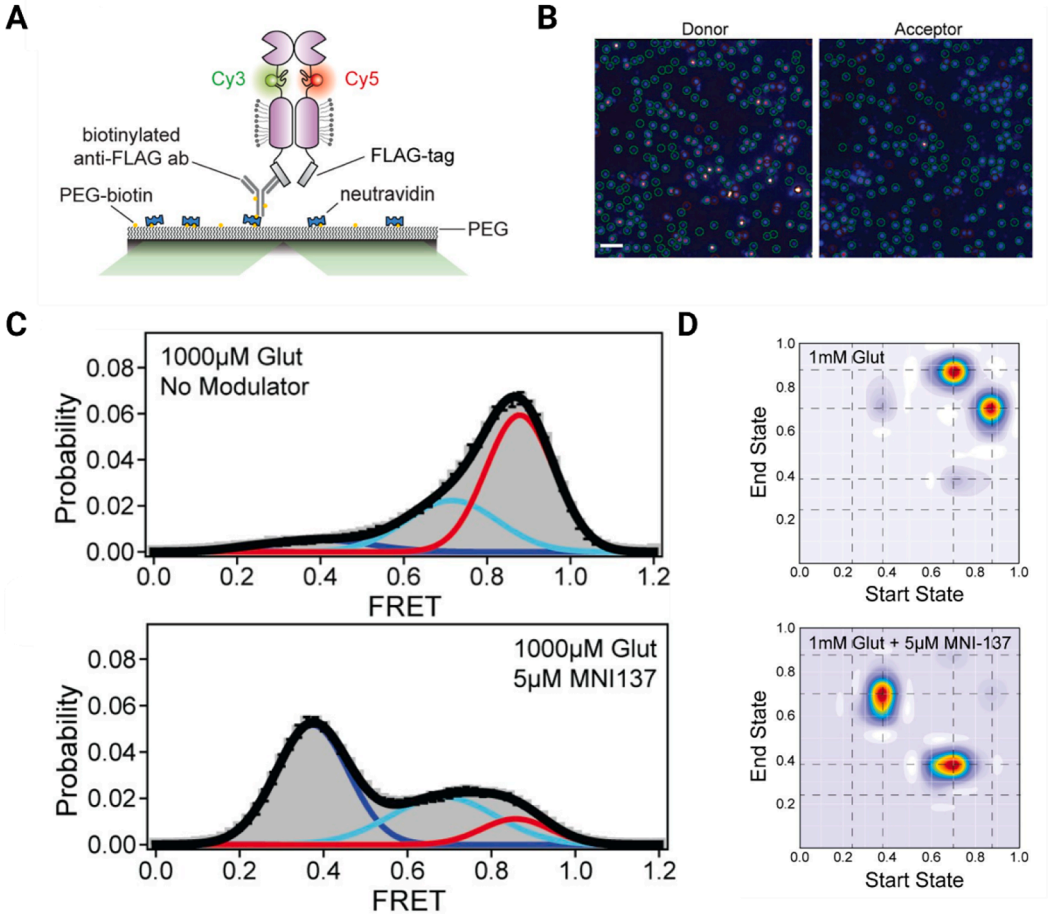

Fig. 7. Dimerization dynamics of the glutamate receptor. (A) Click chemistry labeling with an unnatural amino acid and immobilization of the glutamate receptor. **(B)** smTIRF image of the donor (left) and acceptor (right) molecules. Blue circles indicate selected single molecules of labeled glutamate receptors. **(C)** Population FRET histograms showing the effect of the modulator MNI137 (top: no modulator and bottom: 5 μM MNI137 added) on the dimerization dynamics of the glutamate receptor. **(D)** Transition density plots of the no modulator (top) and the 5 μM MNI137 (bottom) conditions. The figure is reproduced from the article Liauw et al. [110] with permission from eLife journal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

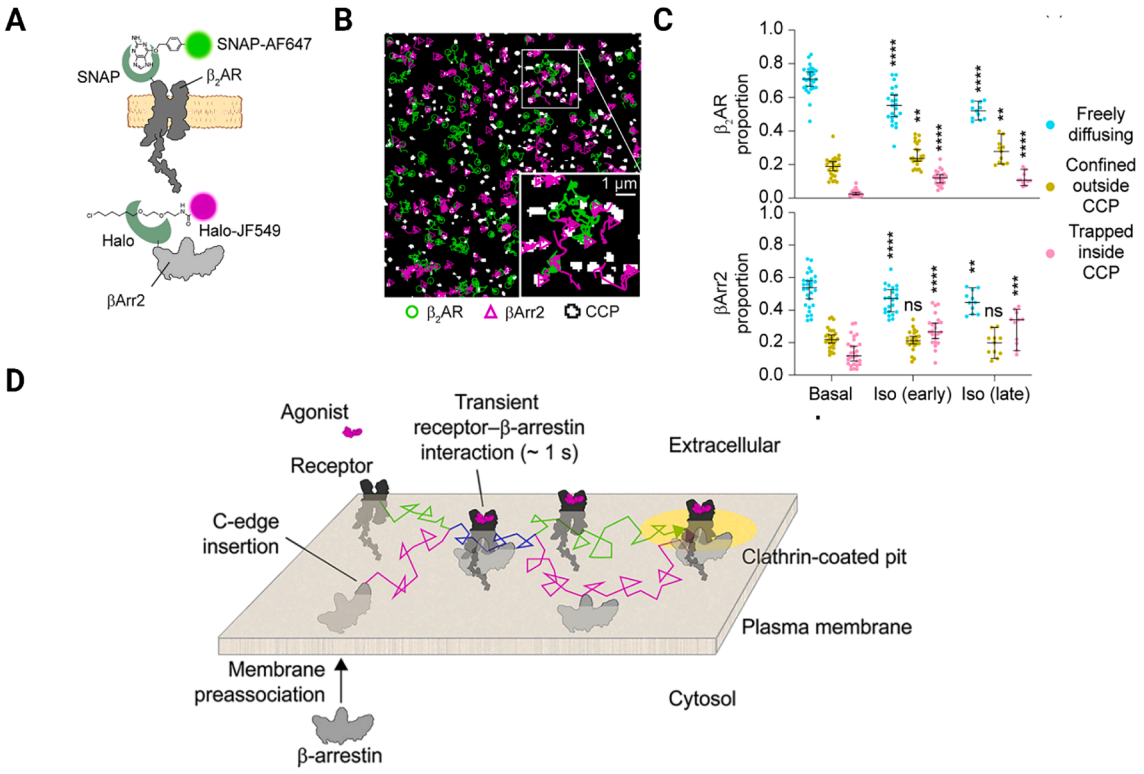

Fig. 8. Single-molecule dynamics of β_2AR and β-arrestins (βArr2) in live cells. (A) Illustration depicting the labeling strategy used to image β_2AR and βArr2. **(B)** Objective-TIRF image of β_2AR (green) and βArr2 (pink) tracks in live cells colocalized with tracks of clathrin-coated pits (CCPs). **(C)** Population proportions of β_2AR (top) and βArr2 (bottom) exhibiting 'freely diffusing', 'confined outside CCP' and 'trapped inside CCP' dynamics. **(D)** Model showing the sequence of dynamic events observed during the arrestin-based desensitization of β_2AR. The figure is adapted from the article Grimes et al. [111] with permission from Cell journal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

light chains. The cells were imaged using fast multicolor TIRF microscopy with single-particle tracking [111].

The results of this single-molecule particle tracking study showed that βArr2 translocated to the plasma membrane before interacting with activated GPCR molecules. The diffusion of β_2AR and βArr2 was heterogeneous. Upon analysis, they were observed to be freely diffusing, confined outside CCPs, and trapped inside CCPs [111]. The addition of isoproterenol, a β_2AR full agonist, increased β_2AR and βArr2 confinement to CCPs. The study showed that βArr2 spontaneously associates with the membrane, and the accumulation of β_2AR and βArr2 to the CCPs is agonist-dependent [111]. The findings from the study demonstrate the usefulness of single-molecule particle tracking in understanding GPCR desensitization in live cells (Fig. 8).

7. Concluding remarks

Single-molecule research on GPCRs will continue to provide valuable information on the real-time dynamics of these receptors and their response to different types of external signals, including ligands. Such studies can improve our understanding of the dynamics of GPCRs in their native environment, enabling the development of assays for high-throughput drug screening and drug development. The single-molecule microscopy techniques discussed in this context can also complement structural studies of the inactive and active states of GPCRs. Furthermore, SMF experiments can help identify key intermediate states that cannot be resolved using X-ray crystallography and cryo-EM methods.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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