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REVIEW

Unveiling the complexity of G protein-coupled receptor heteromers: advances in live cell imaging technologies and biochemical methods

Hyunbin Kim[1,2] · Huimin Lee[2,3] · Jihye Seong[1,2,3,4]

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Abstract

G protein-coupled receptors (GPCRs), crucial for diverse physiological responses, have traditionally been investigated in their monomeric form. However, some GPCRs can form heteromers, revealing complexity in their functional characteristics such as ligand binding properties, downstream signaling pathways, and trafficking. Understanding GPCR heteromers is crucial in both physiological contexts and drug development. Here, we review the methodologies for investigating physical interactions in GPCR heteromers, including co-immunoprecipitation, proximity ligation assays, interfering peptide approaches, and live cell imaging techniques based on resonance energy transfer and bimolecular fluorescence complementation. In addition, we discuss recent advances in live cell imaging techniques for exploring functional features of GPCR heteromers, for example, circularly permuted fluorescent protein-based GPCR biosensors, TRUPATH, and nanobody-based GPCR biosensors. These advanced biosensors and live cell imaging technologies promise a deeper understanding of GPCR heteromers, urging a reassessment of their physiological importance and pharmacological relevance.

✉ Jihye Seong
jihye.seong@snu.ac.kr

1 Neuroscience Research Institute, Seoul National University College of Medicine, Seoul 03080, Republic of Korea

2 Department of Pharmacology, Seoul National University College of Medicine, Seoul 03080, Republic of Korea

3 Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul 03080, Republic of Korea

4 Seoul National University Bundang Hospital, Seongnam 13620, Republic of Korea

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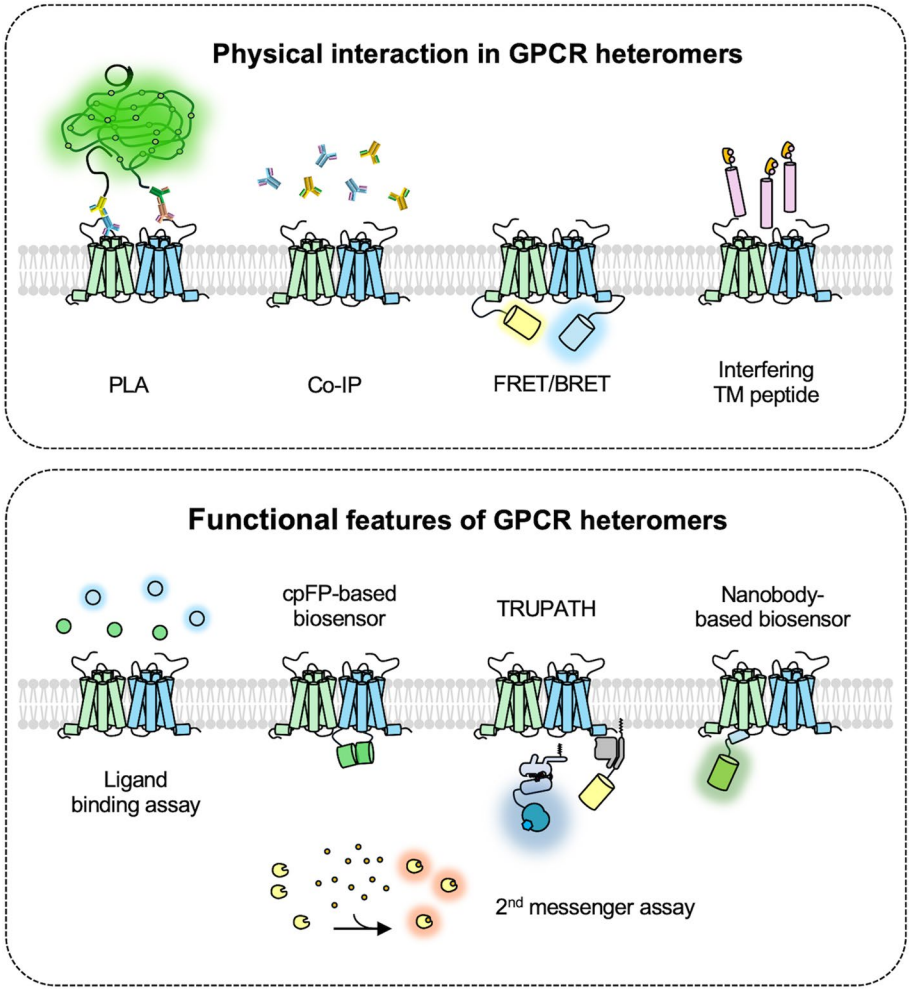
Graphical Abstract



Highlights

- The discovery of G protein-coupled receptor (GPCR) heteromers has revealed a new level of complexity in their functional characteristics.
- This review explores a variety of methodologies and live cell imaging technologies for the investigation of physical interactions in GPCR heteromers and their functional alterations.
- Live cell imaging technologies such as fluorescent protein-based biosensors promise a deeper understanding of GPCR heteromers, urging a reassessment of their physiological importance and pharmacological relevance.

Keywords GPCR · Heteromer · Fluorescent biosensor · Circularly permuted fluorescent protein · Functional crosstalk

Introduction

G protein-coupled receptors (GPCRs) play pivotal roles in diverse physiological responses upon ligand binding [1, 2]. They are classified into four main classes—Class A, B, C, and F—based on sequence homology and structural features [3]. Class A, also known as rhodopsin-like receptors, represents the largest group and includes receptors for many hormones and neurotransmitters. Class B contains secretin-like receptors primarily involved in hormone regulation. Class C GPCRs, distinguished by their large extracellular domains, are well-characterized for forming obligatory

dimers essential for their activation as observed in metabotropic glutamate receptors (mGluRs) and γ-aminobutyric acid receptor B ($GABA_B$) [4]. Finally, Class F, or the Frizzled/Taste2 family, is involved in Wnt signaling pathways crucial for cell differentiation and development. GPCRs in Class A, in contrast to dimeric Class C, have been known to exist as monomers, thus previous research has focused on understanding their functions in the monomeric state [5]. However, it has become clear that Class A GPCRs can also form homodimers or heterodimers [6]. In this review, we aim to examine the methodologies for exploring GPCR interactions, particularly in Class A, which offering new insights into their functional roles and therapeutic potential.

GPCR heteromers can lead to unique functional features such as altered ligand binding, downstream signaling pathways, and trafficking patterns [7–11]. These changes can play critical roles in both physiological and pathophysiological contexts [12, 13]. First, GPCR heteromers can induce the alteration in their ligand binding. The physical interactions between transmembrane domains within the GPCR heteromer may induce slight but crucial structural changes in their ligand binding site, thereby positively or negatively modulating the binding of ligand [14]. Second, the alteration in ligand binding can subsequently influence downstream signaling pathways. This may be attributed to the structural changes of GPCRs induced by altered ligand binding, which affect their coupling properties with Gα proteins [14]. This modulation can result in either the enhancement or inhibition of downstream signaling, or it may cause a shift to alternative signaling pathways [15, 16]. Consequently, in GPCR heteromers, one GPCR can induce positive or negative effects on the function of another GPCR [17, 18]. Furthermore, heteromerization can influence GPCR internalization and trafficking. For example, alterations in the kinetics of GPCR internalization can affect the duration of GPCR activation and function [19, 20]. Additionally, some GPCR heteromers facilitate their translocation from intracellular part, where they are non-functional, to plasma membrane for enabling their activation and function [21]. These examples illustrate the relevance of GPCR heteromers in both normal physiological functions and disease states. Thus, GPCR heteromer research holds significant relevance for understanding GPCR biology and the development of novel therapeutics targeting GPCR heteromers.

Diverse experimental methods have been applied to elucidate the formation of GPCR heteromers and explore their functional features [22, 23]. Co-immunoprecipitation (co-IP) assay and proximity ligation assay (PLA) have been widely used to confirm the existence of GPCR heteromers. For the real-time detection of GPCR heteromers in live cell conditions, imaging techniques have been developed based on resonance energy transfer (RET) and bimolecular fluorescence complementation (BiFC). In addition, various methods have been employed to explore functional alterations of GPCR heteromers, for example, changes in ligand binding affinity, downstream signaling pathways, and trafficking. This review seeks to explore the strengths and limitations of these methods employed for the investigation of GPCR heteromers. Notably, we will introduce recent advances in fluorescent biosensors capable of capturing real-time GPCR activity, which can be applied to study the functional crosstalk between GPCR heteromers in live cells [24]. Therefore, the objective of this review is to underscore the importance of GPCR heteromer research and the potential applications of cutting-edge live-cell imaging techniques in uncovering their presence and functional implications, thereby advancing our understanding of GPCR biology and its therapeutic relevance in modulating diseases.

Experimental methods for the detection of physical interactions in GPCR heteromers

Co-immunoprecipitation

Co-immunoprecipitation is a classical biochemical technique to elucidate physical interactions in GPCR heteromers [25] (Fig. 1a). The formation of GPCR heteromers can be detected by co-immunoprecipitation using a specific antibody against one GPCR, followed by immunoblotting to identify the presence of another GPCR. This method has played a crucial role in demonstrating the existence of various GPCR heteromers. We have summarized several examples of GPCR heteromers that have been detected using co-IP [26–32] in Table 1.

While co-IP holds significant value in exploring GPCR heteromer interactions, one challenge with this technique is the potential for false-positive signals due to the limited specificity of GPCR-targeting antibodies. Antibodies may cross-react with unintended targets, leading to the co-IP of proteins that are not interaction partners. This limited specificity of GPCR antibodies can complicate the interpretation of experimental results. To enhance the reliability of co-IP experiments in studying GPCR heteromer interactions, researchers need to validate the antibodies and include appropriate negative controls to distinguish specific interactions from nonspecific binding.

Proximity ligation assay

Proximity ligation assay is an important method to confirm the physical interactions in GPCR heteromers [84] (Fig. 1b). In PLA, target GPCRs are recognized by two primary antibodies, and secondary antibodies coupled with oligonucleotides (PLA probes) bind to primary antibodies. When these PLA probes come into proximity, connector

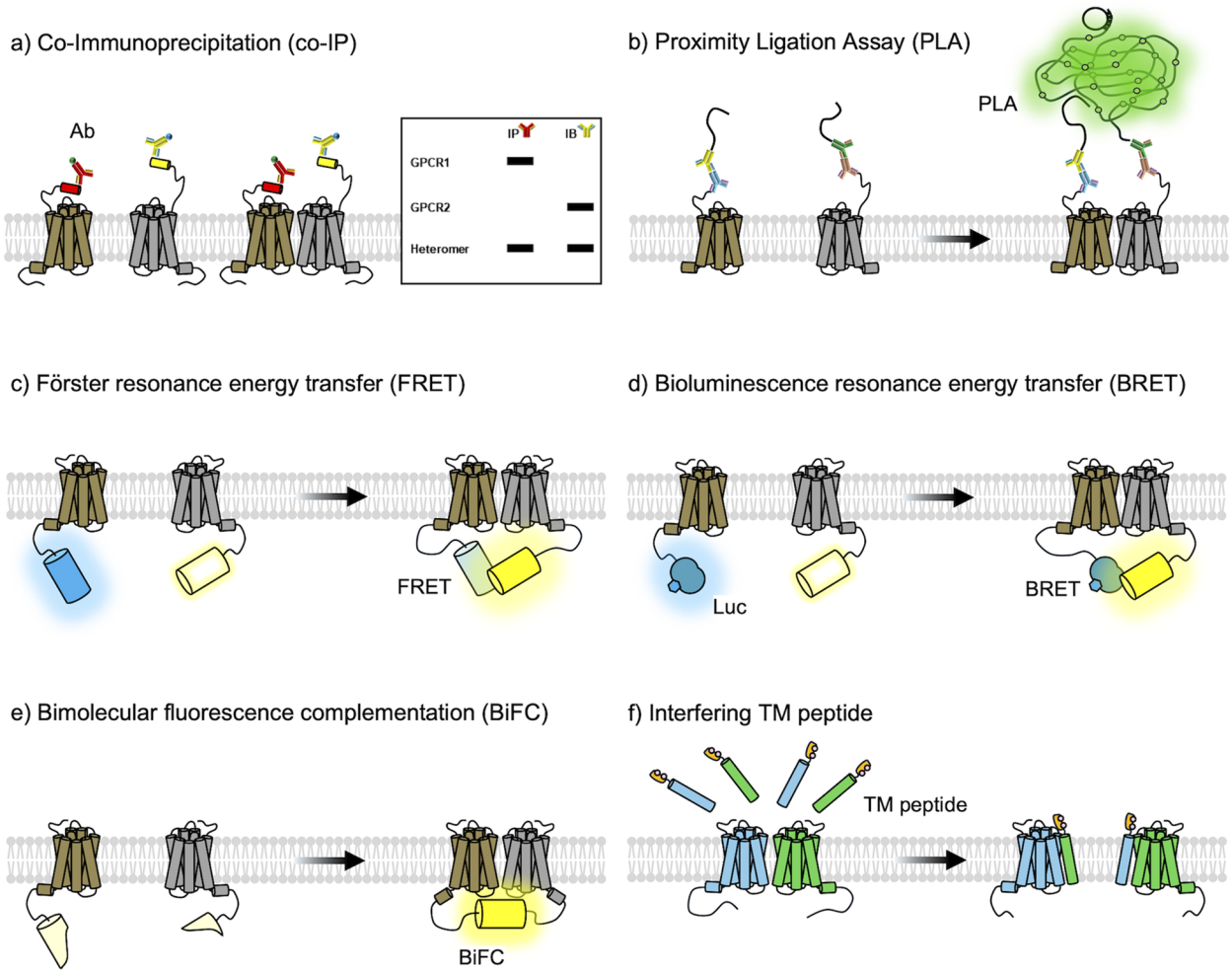


Fig. 1 Experimental methods for detecting physical interactions between GPCR heteromers. Schematic designs of experimental strategies for the detection of the formation of GPCR heteromers, for example, co-immunoprecipitation (co-IP) (**a**), proximity ligation assay (PLA) (**b**), FRET-based detection method (**c**), BRET-based detection method (d), BiFC-based detection method (**e**), and interfering transmembrane (TM) peptide method utilizing the TM sequence of GPCR fused to TAT peptide (**f**)

oligos join them to be ligated resulting closed circular DNA template. After the amplification of the template by DNA polymerase, they hybridize with complementary detection oligos coupled to fluorochromes. Thus, PLA allows for the visualization of the physical interactions between GPCR heteromers, allowing the identification of many GPCR heteromers. For instance, dopamine receptor D1-D2 heteromer was confirmed in rat and macaque brains [40]. The PLA method has been applied to confirm various other GPCR heteromers [37, 43, 44, 46–51] which are summarized in Table 1.

PLA does come with certain limitations that require careful consideration. One notable concern of PLA is the possibility of background signals or non-specific interactions, which may introduce inaccuracies in the obtained results. Thus, it is important to carefully choose specific antibodies for target GPCRs.

Live cell imaging techniques based on resonance energy transfer

Imaging techniques have been used to investigate the real-time formation of GPCR heteromers in live cells, for example, Förster resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) [85, 86] (Fig. 1c, d). The distance between the donor and acceptor is critical for the RET signals, thus these techniques have been applied to visualize the physical interactions in GPCR heteromers. For the FRET measurement, GPCRs are tagged with donor or acceptor fluorescent proteins (FPs), e.g., cyan or yellow FP (CFP and YFP). For the BRET assay, GPCRs are tagged with bioluminescent donor or acceptor FP, e.g., luciferase or YFP. Thus, the existence of GPCR heteromers can be confirmed by the increased RET signals. The GPCR heteromers identified by the FRET-based approach [53, 56,

Table 1 Assessment of GPCR heteromers: techniques for exploring physical interaction and functional characteristics of GPCR heteromers

GPCR heteromer	Physical interaction	Functional characteristics				Reference
		Ligand binding properties	GPCR conformational change	GPCR signaling pathway	GPCR trafficking	
μOR-δOR	co-IP, TM peptide	Radioligand		cAMP assay Western blot Promoter assay	Fluorescence imaging	[26, 33–36]
D2-A2AR	co-IP, PLA, BRET	Radioligand		cAMP assay	Fluorescence imaging	[15, 27, 37–39]
D1-D2	co-IP, PLA, BiFC, FRET, TM peptide		cpFP-based biosensor	cAMP assay Calcium assay Western blot Promoter assay		[24, 28, 40–42]
5HT1A-5HT1B	co-IP					[29]
A1AR-D1	co-IP			cAMP assay	Fluorescence imaging	[30]
AT1-B2	co-IP			Calcium assay		[31]
β2AR-δOR	co-IP	Radioligand		cAMP assay Western blot	ELISA	[32]
D2-D4	co-IP, PLA, BRET			Western blot		[43]
D1-D3	PLA, TM peptide	Radioligand		Western blot		[44, 45]
D2-AT2	PLA, BRET			cAMP assay Calcium assay Western blot	Fluorescence imaging	[46]
CB1-CB2	PLA, BRET			Western blot		[47]
CB1-GPR55	PLA, BRET			Western blot		[48]
5HT1A-5HT2A	PLA, BRET	Radioligand				[49]
5HT1A-D2	PLA, FRET	Radioligand			Fluorescence imaging	[50]
SSTR5-D2	PLA					[51]
OTR-EP2	PLA, BRET			cAMP assay Calcium assay Western blot		[52]
5HT2A-D2	co-IP, FRET, BRET	Radioligand		Calcium assay Western blot Promoter assay		[53–55]
RXFP3-LH	FRET					[56]
A2AR-A2BR	PLA, FRET, BRET, BiFC, TM peptide			cAMP assay		[57, 58]
MT1-MT2	BRET	Radioligand				[59]
CCR5-CXCR4	BRET	Radioligand		Calcium assay		[60]
TAAR1-D2	BRET			cAMP assay Promoter assay	ELISA	[61]
A1R-A2AR	PLA, BRET, BiFC, TM peptide			cAMP assay		[62]
CB1-D2	FRET, BRET, BiFC	Radioligand		cAMP assay Western blot		[63, 64]
CCR2-CXCR4	BRET, BiFC			Calcium assay		[65]
A2AR-CB1	co-IP, BRET, BiFC, TM peptide			cAMP assay Promoter assay		[66, 67]
5HT2A-CB1	PLA, BRET, BiFC, TM peptide			cAMP assay Western blot		[68]
AT1-β2AR	BRET	BRET				[69]
D2-SSTR2	co-IP, FRET	Radioligand		cAMP assay Western blot	Fluorescence imaging	[70]
5HT1A-OX1R	co-IP, PLA, BRET, FRET, TM peptide,			cAMP assay Western blot		[71]
D2-D3	co-IP,	Radioligand		cAMP assay		[72]

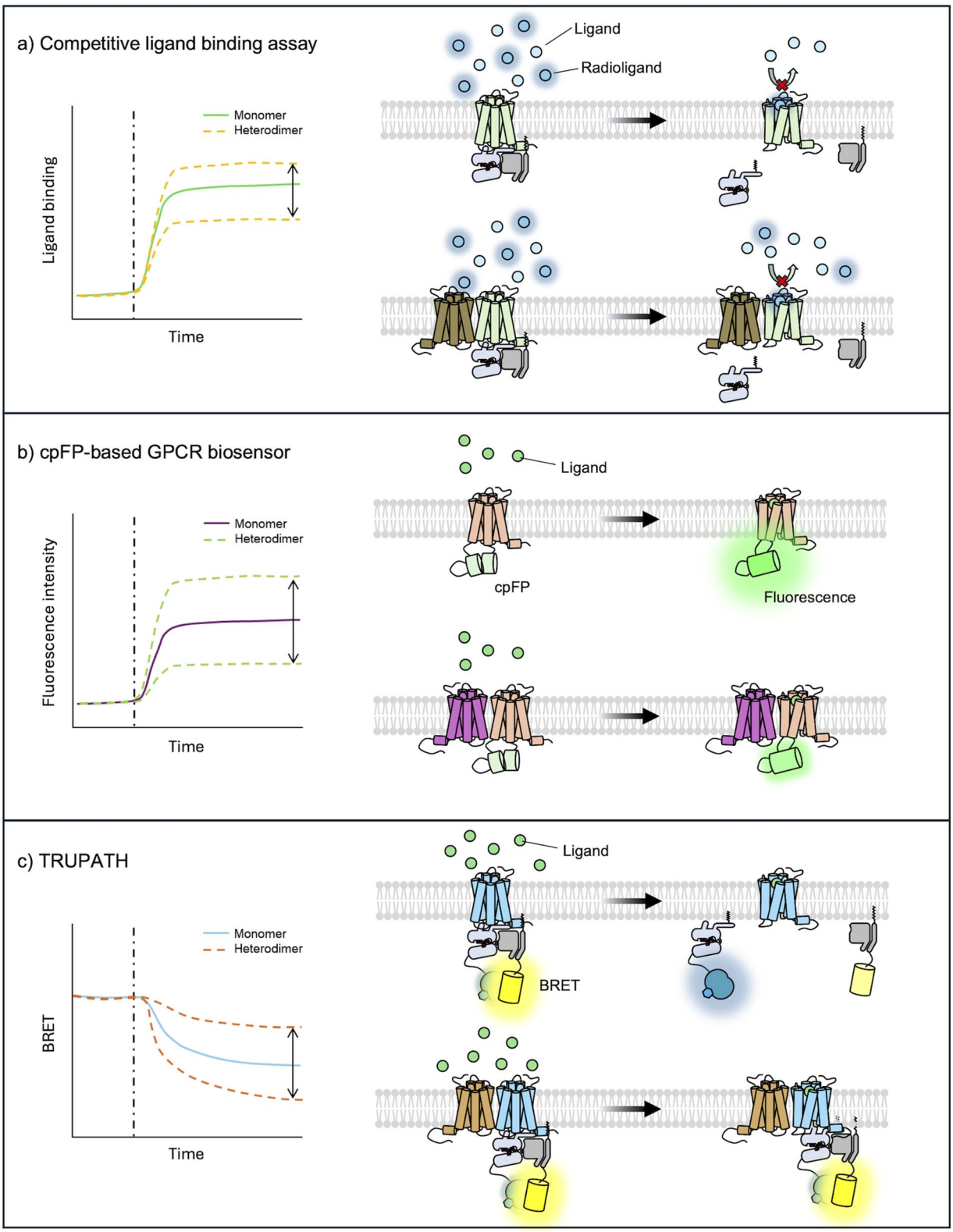


Fig. 2 Experimental methods for detecting functional interactions between GPCR heteromers. **a** Schematic design of competitive radioligand binding assay. The formation of a GPCR heteromer may lead to changes in ligand binding properties. **b** Schematic design of detecting GPCR conformational change using cpFP-based GPCR biosensor. The alteration in GPCR conformational change upon the formation of GPCR heteromer leads to the changes in fluorescence intensity of the biosensor. **c** Schematic design of TRUPATH assay capable of detecting G protein activation based on the BRET change between luciferase and fluorescent protein. The alteration in G protein activation upon the formation of GPCR heteromer leads to the changes in BRET of TRUPATH assay

Table 1 (continued)

GPCR heteromer	Physical interaction	Functional characteristics				Reference
		Ligand binding properties	GPCR conformational change	GPCR signaling pathway	GPCR trafficking	
κOR-NTSR1	co-IP, PLA, FRET, BRET			cAMP assay Western blot		[73]
SSTR2-SSTR5	co-IP, FRET	Radioligand		cAMP assay Western blot	Fluorescence imaging	[74]
5HT2A-mGlu2R	co-IP, FRET, BRET	Radioligand		Calcium assay Promoter assay		[75, 76]
5HT2A-mGlu3R	co-IP, FRET, BRET	Radioligand		Promoter assay		[76]
μOR-GRPR	co-IP, TM-peptide			Calcium assay Western blot	ELISA	[77]
CB2-GPR55	co-IP, BRET			cAMP assay Western blot Promoter assay	Fluorescence imaging	[78]
β1AR-β2AR	BRET			cAMP assay Western blot	BRET	[19]
β2AR β3AR	BRET			cAMP assay Western blot	BRET	[19]
$GABA_{B1}$-$GABA_{B2}$	co-IP, FRET	Radioligand		Western blot	Fluorescence imaging	[79–81]
α1dAR-α1bAR				Calcium assay	Fluorescence imaging	[21]
α1dAR-β2AR					Fluorescence imaging	[82]
δOR-κOR		Radioligand		cAMP assay Western blot	Flow cytometry	[83]
μOR-SSTR2A	co-IP	Radioligand		cAMP assay Western blot	ELISA	[20]

57] are summarized in Table 1. Similarly, the BRET assay has allowed the identification of various GPCR heteromers [52, 54, 57, 59–61], as summarized in Table 1.

These methods, based on sensitive and reversible RET signals, are broadly employed to elucidate the formation of GPCR heteromers. However, inserting fluorescent or bioluminescent tags may interfere with the native structure of GPCRs, thus the insertion sites should be carefully chosen. In addition, the RET techniques have limitations of potential artifacts arising from non-specific interactions or background signals.

Live cell imaging technique based on bimolecular fluorescence complementation

Fluorescent protein can be split into two non-fluorescent fragments, which are capable of reacquiring fluorescence when they are reassembled in proximal distance [87]. This is called bimolecular fluorescence complementation, and by fusing the BiFC fragments to proteins of interest, their physical interaction can be measured (Fig. 1e). This technique has illuminated the presence of diverse GPCR heteromers [62, 63, 65] as summarized in Table 1.

While the BiFC technique provides a valuable tool for visualizing GPCR heteromers, the irreversible binding property of the BiFC fragments impedes subsequent experiments to investigate the dynamics of GPCR heteromers. Another limitation of the BiFC assay is that the fused FP fragments may alter the natural configuration of GPCRs, leading to false positive or negative results. Thus, cautious interpretation and supplementary experiments are required for the validation of the BiFC signals between GPCR heteromers.

Interfering peptide with the transmembrane sequence of GPCRs

The existence of GPCR heteromers can be further verified by interfering peptides with the transmembrane (TM) sequences of GPCRs, which can disrupt the BiFC or RET signals between GPCR heteromers [88] (Fig. 1f). Synthetic peptides with amino acid sequences of TM (TM1-7) of GPCRs are prepared and fused to the human immunodeficiency virus (HIV) transactivator of transcription (TAT) peptide for their insertion in the plasma membrane [89]. If the inserted TM sequence is critical

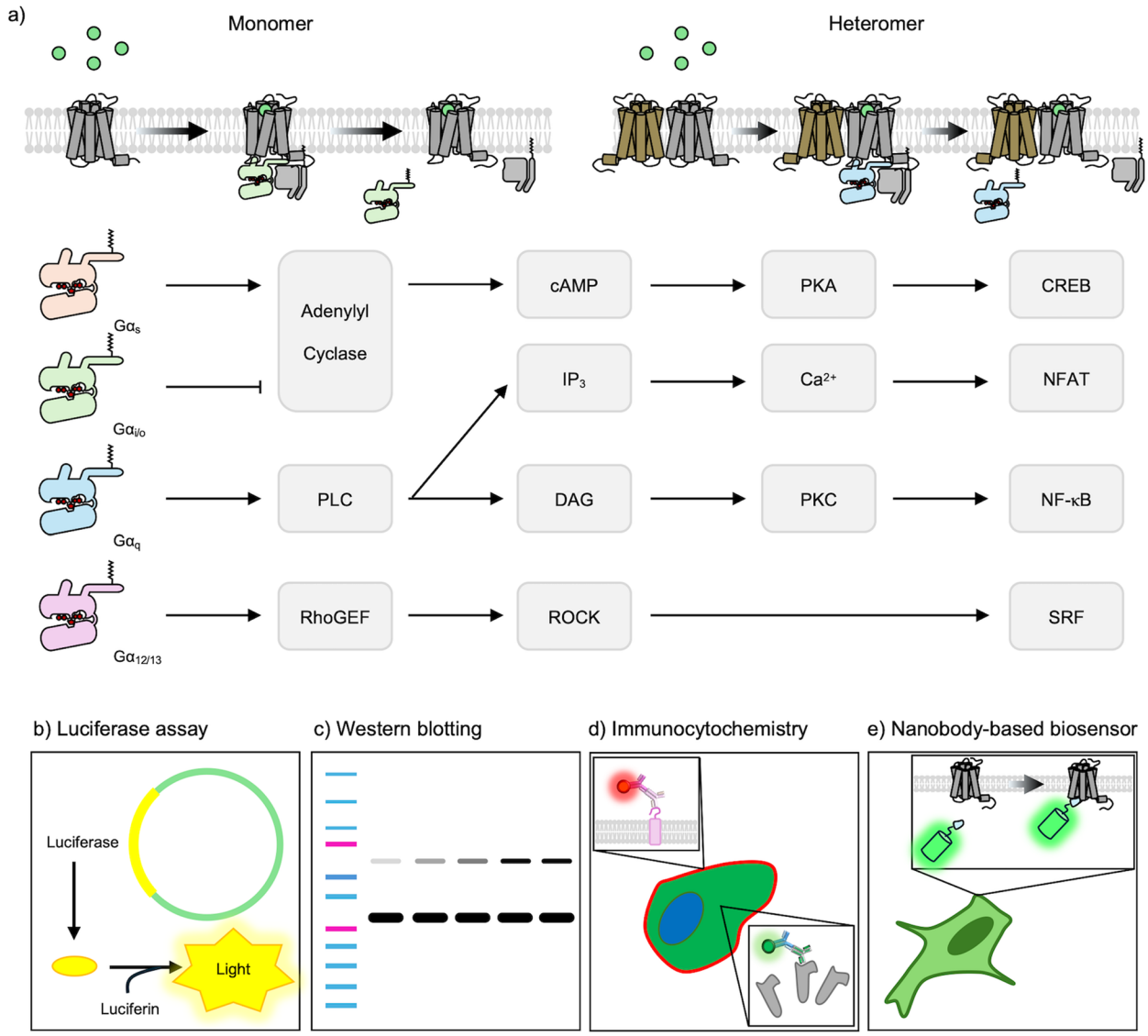


Fig. 3 Experimental methods for detecting the alterations in the GPCR signaling pathways by the formation of GPCR heteromers. **a** An overview of GPCR downstream signaling pathways. Graphical representative of the GPCR downstream signaling pathways. Activated GPCRs interact with heterotrimeric G proteins composed of three subunits (Gα, Gβ, and Gγ). Gα subunits are classified into four subfamilies: Gαs, Gαi, Gαq, and Gα12/13. Gαs and Gαi can regulate the cAMP-PKA-CRE pathway. Gαq regulates intracellular calcium levels and the PLC-Ca^{2+}-NFAT or PLC-IP_3-NF-κB pathway. Gα12/13 initiates the activation of Rho guanine nucleotide exchange factor (RhoGEF) and RhoA-rho-associated protein kinase (ROCK)-serum response factor (SRF) pathway. **b** Schematic design of luciferase-based assays. This principle is used for measuring the levels of cAMP or transcription factors. **c** Western blotting assay for measuring the levels of expression or phosphorylation of GPCR signaling proteins. **d** Immunostaining method for visualizing and quantifying the localization and phosphorylation levels of GPCR signaling proteins. **e** Representative design of nanobody-based GPCR biosensor. The nanobody tagged with fluorescent protein selectively binds to the activated GPCR, allowing the visualization of the location of active GPCRs

for the interaction between GPCR heteromers, it will be selectively interfered with by the TM peptide, thus confirming the existence of GPCR heteromers. This approach also allows the identification of the TM region for the key interaction between GPCRs. For example, TM4 and TM5 were identified as the interface between A2AR and D2 in heteroreceptor complexes [90]. The anti-cocaine effect of an A2AR agonist on the A2AR-D2 heteromers was completely blocked by microinjecting the TM5 peptide into the nucleus accumbens of the rat brain [91]. GPCR heteromers validated by this method are summarized in Table 1 [33, 58, 62, 66, 68].

The interfering TM-peptide method provides valuable insight into the configuration of GPCR heteromers. However, there is a potential risk for off-target effects of the TM peptides. These peptides may interact with unintended molecular targets, leading to false-positive or false-negative outcomes. Additionally, the stability of TM peptides

can be influenced by environmental factors or experimental conditions, necessitating careful development and optimization of the peptide sequences tailored for GPCR heteromer studies.

Exploring functional features of GPCR heteromers by live cell imaging technologies and biochemical methods

Ligand binding properties

The pharmacological properties of GPCRs are crucial for their activation and functions [92]. The formation of GPCR heteromers can alter the ligand binding properties, thus experimental methods for assessing the pharmacological properties of GPCRs have been established for exploring the functional features of GPCR heteromers [93] (Fig. 2a). For example, competitive radioligand binding assay measures the affinities of test compounds for target GPCRs by determining IC_{50} for competitive inhibition of radioligand binding [94]. Similarly, the ligand binding property to the target receptor can be measured using fluorescent dye-conjugated ligands. These methods revealed that the binding affinities of μOR- or δOR-specific ligands decrease when binding to μOR-δOR heteromers [34]. The study in the rat striatal neurons discovered that the affinity of D2 ligand decreases when binding to D2-A2AR heteromers [38]. In the case of the AT1-β2AR heteromer, the binding affinities of receptor-selective ligands decrease compared to their affinities for the individual receptors [69]. In contrast, the treatment with selective ligands induced the heteromerization of D2 with somatostatin receptor 2 (SSTR2), which results in increased affinity for dopamine and enhanced D2 signaling [70].

The altered ligand binding properties provide valuable insight into the functional features of GPCR heteromers. However, it may not directly translate into changes in downstream signaling pathways. Thus, complementary experimental methods will be described in the next sections, with an emphasis on live cell imaging techniques.

GPCR conformational change

Upon ligand binding, GPCR changes its conformation to stabilize the interaction with G proteins, facilitating the G protein activation and related downstream signaling pathways [95]. Therefore, assessing the GPCR conformational change will provide direct insights into its activation status. To monitor the real-time conformational change of GPCR in live cells, genetically encoded fluorescent biosensors for various GPCRs have been developed based on FRET or BRET [96]. According to the structural studies of GPCRs, the most significant conformational change during the GPCR activation occurs between TM5 and TM6 [95, 97]. The FRET or BRET-based GPCR biosensors have been designed to include a donor FP/luciferase and an acceptor FP at the C-terminus and the intracellular loop 3 (ICL3) between TM5 and TM6, respectively. Thus, the conformational change between TM5 and TM6 during GPCR activation results in changes in FRET or BRET levels. These biosensors may affect the native conformations of the heteromers due to their bulky sizes.

The GPCR biosensors based on circularly permuted fluorescent proteins (cpFP) [98] were first introduced in 2018 for the monitoring of the real-time activity of dopamine receptors in live cells, tissues, and animals [99, 100], and since then, a variety of cpFP-based GPCR biosensors have been developed [101]. The cpFP is inserted in the ICL3 region of GPCRs in the biosensor, thus the conformational changes between TM5 and TM6 during GPCR activation can be sensitively detected by the fluorescent intensity of cpFP. The cpFP-based GPCR biosensors allow the real-time monitoring of GPCR activity in live cells. Furthermore, these biosensors incorporate only one cpFP, making them smaller in size compared to FRET or BRET-based biosensors.

We previously developed cpFP-based multicolor biosensors for dopamine receptor D1 and D2 to study functional crosstalk within D1-D2 heteromer [24] (Fig. 2b). These biosensors allowed the precise detection of subtle conformational changes in the D1-D2 heteromer, thus we were able to discover differential functional crosstalk in response to varying dopamine levels: D1 activity in the heteromer is selectively inhibited at micromolar dopamine levels, while D2 activity is inhibited by nanomolar dopamine concentration. This suggests a novel function of D1-D2 heteromer in modulating dopamine signaling pathways across physiological dopamine concentrations ranging from nanomolar to micromolar levels.

Because a variety of cpFP-based GPCR biosensors are available [102], this method will provide important insights into functional crosstalk in various combinations of GPCR heteromers. The modification in the ICL3 region of the biosensor may affect the trafficking of some GPCRs [103], hence additional assays for GPCR signaling pathways will further enhance the comprehensive understanding of functional crosstalk in GPCR heteromers.

GPCR signaling pathways

The active GPCRs interact with heterotrimeric G proteins or β-arrestins, mediating various intracellular signaling pathways [104]. As ligand binding properties and the GPCR conformational change are affected by the formation of GPCR heteromers, we can anticipate the changes in the intracellular signaling pathways. Consequently, assessing the downstream signaling pathways is crucial to understanding functional

alterations induced by GPCR heteromers. In this section, we will discuss various methods including fluorescent biosensors and luciferase-based assays to investigate the GPCR signaling pathways, from G protein-specific second messengers, downstream signaling molecules, and transcription factors.

G protein-induced second messengers

The conformational change of GPCRs induces the recruitment of heterotrimeric G proteins and mediates their activation [105]. When the heterotrimeric G proteins are activated, they dissociate into Gα and Gβγ proteins, and the dissociated Gα proteins mediate differential signaling pathways depending on their subtypes (Fig. 3a). GPCRs coupled to Gαs protein activate adenylyl cyclase (AC), leading to cyclic adenosine monophosphate (cAMP) production. Conversely, GPCRs coupled to Gαi protein inhibit AC activity, consequently reducing cAMP levels. In addition, GPCRs coupled to Gαq protein elevate the intracellular Ca^{2+} concentration via phospholipase C (PLC) signaling pathways. Therefore, second messengers such as cAMP and Ca^{2+} levels serve as important indicators of G protein-mediated signaling pathways.

To measure cAMP levels in live cells, luciferase-based GloSensor assay has been widely employed (Fig. 3b). The GloSensor consists of the cAMP-binding domain (RIIβB) derived from protein kinase A (PKA), which is fused between the N- and C-termini of circularly permuted firefly luciferase [106]. Upon binding to cAMP, the cp-luciferase in the GloSensor undergoes a conformational change, leading to increased luciferase activity. This property has been utilized in the cAMP assays to investigate the functional characteristics of GPCR heteromers. For example, the cAMP assay revealed that functions of D1 and D2 are inhibited in D1-D2 heteromer in response to micromolar and nanomolar concentrations of dopamine, respectively [24]. The cAMP assay has also been widely used to investigate functional alterations of GPCR heteromers [35, 39, 52, 64, 71–74] as summarized in Table 1.

To measure intracellular Ca^{2+} levels, fluorescent dyes such as Fura-2, Indo-1, Fluo-3, and Fluo-4 have been developed. When bound to Ca^{2+}, these dyes change fluorescence intensity or wavelength, thus enabling the detection of Ca^{2+} levels in cells [107]. For example, the Fura-2 assay [108] revealed that the intracellular Ca^{2+} response decreases in the 5HT2A- mGluR2 heteromer, indicating the functional inhibition of 5HT2A in the heteromer [75]. The Ca^{2+} assay has been used to investigate the functional alterations in GPCR heteromers such as μOR-gastrin-releasing peptide receptor (GRPR) [77], D1-D2 [41] and D2-5HT2A [54].

Additionally, dynamic changes in second messenger levels upon the activation of GPCR heteromers can be sensitively monitored by genetically encodable fluorescent protein-based cAMP sensors, such as G-FLamp1, cAMP-inG1, as well as genetically encoded calcium indicators (GECIs) [109–111]. Because these fluorescent sensors are fully genetically encodable, they can allow long-term observation of GPCR heteromer signaling ex vivo or in vivo. These advantages thus enable a more comprehensive understanding of the importance of GPCR heteromers in physiological processes within complex biological systems.

As discussed above, the changes in the G protein activation by GPCR heteromers have been inferred through the alterations in the levels of second messengers. However, the information obtained from these assays can be indirect, because second messengers are influenced by numerous signaling pathways [112, 113]. To measure the G protein activation more directly, the TRUPATH technique has been recently developed, which measures the dissociation of Gα and Gβγ proteins [114]. In TRUPATH, Gα and Gγ proteins are tagged with Renilla luciferase (Rluc) and green fluorescent protein (GFP), thus the BRET signals between Rluc and GFP decrease upon the dissociation of Gα and Gβγ proteins (Fig. 2c). Hence, TRUPATH allows for the direct assessment of both the degree of G protein activation and Gα specificity. This cutting-edge technique will be further useful in evaluating the alterations in the extent and type of G protein activation in GPCR heteromers.

Transcriptional factors activated by G protein signaling

The second messengers induced by G protein activation mediate diverse downstream signaling pathways [104]. For example, the cAMP levels, which are regulated by Gαs and Gαi, mediate PKA activation and cAMP response element-binding protein (CREB)-mediated transcription [115]. Activated Gαq protein interacts with PLC, which produces diacylglycerol (DAG) and inositol triphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). The produced DAG binds to and activates protein kinase C (PKC) [116]. The produced IP_3 binds to the IP_3 receptor in the endoplasmic reticulum (ER), releasing Ca^{2+} from the ER. The elevated Ca^{2+} can be sensed by calmodulin and further activates Ca^{2+}/calmodulin-dependent protein kinase (CaMK), regulating various downstream signaling molecules and transcription factors.

Traditional approaches for investigating GPCR downstream signaling pathways involve using specific antibodies to measure the levels and distribution of downstream signaling proteins via western blot analysis and immunostaining [117] (Fig. 3c, d). Western blotting also allows the measurement of the post-translational modifications such as phosphorylation. Thus, it has played a crucial role in exploring the functional alterations by GPCR heteromers. Examples of GPCR heteromers identified by western blotting are summarized in Table 1 [15, 36, 42, 45, 47, 55, 73, 74].

GPCR heteromers can induce long-term changes in signaling pathways by modulating transcription factors such as signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cAMP response element (CRE), and nuclear factor of activated T-cells (NFAT) [118]. To investigate the alterations in transcription factors, promoter assays have been employed which mediate the expression of a reporter gene upon the increased binding of transcription factors to target promoters [119]. For example, the CRE/CREB assay includes a luciferase gene under the control of a multimerized CRE. The elevated cAMP activates CREB, which binds to CRE and induces luciferase expression, thus the CREB-mediated transcription can be measured by luminescence signals [120]. This method has revealed the transcriptional changes induced by several GPCR heteromers [36, 54, 55, 67, 76, 78] as summarized in Table 1.

GPCR internalization and trafficking

The distribution of GPCRs at the plasma membrane is required for the efficient binding with their ligands in the extracellular space, while the internalization of GPCRs has been recognized as a primary mechanism for desensitization [121]. Consequently, the duration and strength of GPCR functions can be influenced by their trafficking process. The formation of GPCR heteromers can affect their trafficking from the ER to the plasma membrane, as well as the internalization of GPCRs from the plasma membrane to intracellular regions [122]. For the investigation of the GPCR trafficking process, fluorescence imaging-based methods have been employed to directly visualize spatial information of GPCRs tagged with fluorescent proteins or labeled with specific antibodies [123]. Additionally, FRET and BRET techniques can be employed for the investigation of the trafficking of GPCR heteromers as well as to confirm the formation of GPCR heteromers [19, 79]

Utilizing these methods, it has been shown that GPCR heteromers can alter the surface expression of GPCRs. For example, the $GABA_B$ receptor subtypes $GABA_{B1}$-$GABA_{B2}$ reside in the ER, but upon the formation of $GABA_{B1}$-$GABA_{B2}$ heteromers, they translocate to the plasma membrane [79–81]. In addition, alpha1-adrenergic receptor subtype d (α1dAR) requires heteromerization with α1bAR for its translocation to the plasma membrane [21]. Similarly, the surface expression of α1dAR is also enhanced by heteromerization with β2AR [82].

The formation of GPCR heteromers can also alter the GPCR trafficking process from the plasma membrane into the intracellular regions [83]. In some GPCR heteromers, it was observed that the agonist-induced internalization can be diminished. For example, the isoproterenol-mediated internalization of β2AR was decreased when it formed heteromers with other β-adrenergic subtypes such as β1 adrenergic receptor (β1AR) or β3 adrenergic receptor (β3AR) [19]. Similarly, etorphine-induced δOR internalization is diminished when it is co-expressed with β2AR or κOR [32, 83]. In other GPCR heteromers, an agonist can promote the internalization of GPCR. For example, in cells co-expressing μOR with somatostatin receptor 2A (SSTR2A), stimulation with selective agonists for μOR or SSTR2A results in the internalization of both GPCRs [20]. Similarly, in heteromers between the μOR subtype and GRPR, the treatment of morphine led to the internalization of both μOR and GRPR, whereas conversely, gastrin-releasing peptide (GRP) specifically triggers the internalization of GRPR but not μOR [77].

In addition to direct observation through fluorescence imaging techniques, alterations in the trafficking of GPCR heteromers have been also accessed using enzyme-linked immunosorbent assay (ELISA). This method quantitatively measures non-internalized GPCR heteromers using antibodies and conjugated enzymes that change the color of substrates. The signals inversely correlate with the internalization levels of GPCRs, thus the trafficking changes in GPCR heteromers can be evaluated by ELISA [20, 32, 77]. Similarly, flow cytometry can be employed to measure the GPCR heteromer internalization, quantifying the signals from fluorescent antibodies bound to GPCRs at the plasma membrane [83].

Live cell imaging studies have demonstrated that GPCR heteromers can lead to changes in their trafficking. Furthermore, the activation states of GPCRs, which may be also influenced by their altered trafficking, can be assessed by live cell imaging with biosensors, for example, nanobody-based GPCR biosensors [124] (Fig. 3e). FP-tagged nanobodies [125] which specifically bind to the active form of GPCRs can visualize active GPCRs during altered trafficking pathways.

Conclusion

G protein-coupled receptor (GPCR) heteromers have brought a paradigm shift in GPCR research, offering insights into the complexity of GPCR signaling pathways. The formation of GPCR heteromers has been investigated through various experimental techniques such as co-IP, PLA, imaging techniques based on RET, BiFC, and interfering TM expression. These methods, in particular live cell imaging techniques, have provided crucial information on the physical interactions in GPCR heteromers. In addition, the functional consequences of GPCR heteromers, such as ligand binding properties, GPCR conformational changes, downstream signaling pathways, and trafficking processes, have been explored. A variety of experimental methods have been used to investigate these functional aspects, in particular, live cell imaging techniques and luciferase-based assays (Table 1).

Notably, new technologies have significantly advanced our understanding of GPCR heteromers. For example, the interfering transmembrane (TM) peptide method enables the investigation of crucial TM regions that mediate the physical interaction within GPCR heteromers [126]. Additionally, cpFP-based GPCR biosensors allow the real-time monitoring of GPCR conformational changes in the heteromers [24]. It was challenging to detect the GPCR conformational changes in the heteromers until our recent study employing cpFP-based biosensors for the D1 and D2 receptors unveiled the functional crosstalk occurring within D1-D2 heteromers. As many cpFP-based GPCR biosensors are currently available [102], this approach could be extended to investigate conformational dynamics and potential crosstalk in many other GPCR heteromers as well. Furthermore, the distinctive advantage of TRUPATH lies in its ability to measure specific G protein activation [114], while conventional methods rely on inferring G protein activation through second messenger levels or downstream signaling pathways. Thus, TRUPATH will provide a more direct and accurate assessment of G protein activation in GPCR heteromers. In line with these advancements, we can apply these advanced techniques to investigate the physical interactions in many other GPCR heteromers and explore their functional alterations.

Despite these advances, current research methods still face limitations, particularly in distinguishing between dimeric and oligomeric interaction within GPCR heteromers. Understanding whether they are dimers or more complex oligomers remains a significant challenge. While studies on homomers, such as proteinase-activated receptors (PARs), have utilized photobleaching to identify the number of GPCR units forming oligomers by observing the incremental reduction in fluorescence [127], similar approaches need to be further developed for studying heteromers. Continued development in experimental techniques will be crucial to elucidate the complexities of GPCR heteromer formation and functions.

Growing evidence suggests a strong correlation between GPCR heteromer formation and the progression of various neurodegenerative diseases. The potential role of these heteromers in the pathophysiology of conditions such as Alzheimer's disease, Parkinson's disease, and schizophrenia, etc. underlies the importance of further research to explore their biological functions and therapeutic potential in these contexts [12, 13]. The advances in live cell imaging techniques and GPCR technologies will offer a more comprehensive understanding of GPCR heteromers, thereby advancing our knowledge of GPCR biology and its therapeutic implications in disease modulation.

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Declarations

Competing interest The authors declare no conflict of interest.

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