17. G-Protein Coupled Receptors: Progress in Surface Display and Biosensor Technology

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Signal transduction by G-protein coupled receptors (GPCRs) underpins a multitude of physiological processes. Ligand recognition by these receptors leads to activation of a generic molecular switch involving heterotrimeric G-proteins and guanine nucleotides. With growing interest and commercial investment in GPCRs in areas such as drug targets, orphan receptors, high-throughput screening of drugs, biosensors etc., greater attention will focus on assay development to allow for miniaturization, ultrahigh throughput, and eventually, microarray/biochip assay formats that will require nanotechnology-based approaches. Stable, robust, cell-free signaling assemblies comprising receptor and appropriate molecular switching components will form the basis of future GPCR/G-protein platforms which should be adaptable for such applications as microarrays and biosensors. This chapter focuses on cell-free GPCR assay nanotechnologies and describes some molecular biological approaches for the construction of more sophisticated, surface-immobilized, homogeneous, functional GPCR sensors. The latter points should greatly extend the range of applications to which technologies based on GPCRs could be applied.

The Superfamily of GPCRs

G-protein coupled receptors (GPCRs) represent a superfamily of intramembrane proteins (polypeptides) which initiate many signal transduction pathways in virtually all eukaryotic cells. GPCRs are structurally characterized by their seven transmembrane (*serpentine*) spanning domains (Fig. 17.1). GPCR activation can be initiated by a wide variety of extracellular stimuli such as light, odorants, neurotransmitters, and hormones. In most cases the GPCR uses a transmembrane signaling system which involves three separate components (systems). Firstly, the extracellular ligand is specifically detected by a cell-surface GPCR. Once recognition takes place, the GPCR in turn triggers the activation of a heterotrimeric G-protein complex located on the peripheral intracellular (cytoplasmic) surface of the cell membrane (the term *G-protein* is used since these proteins bind guanine nucleotides such as guanosine di- and triphosphate present in cells, as discussed in detail later). Finally, the *signal transduction* cascade involves the activated G-protein altering the activity of some downstream *effector* protein(s), which can be enzymes or ion channels located in the cell membrane. This then leads to a change in the cellular concentration of cyclic adenosine monophosphate (cAMP), calcium ions or a metabolite such as phospho-

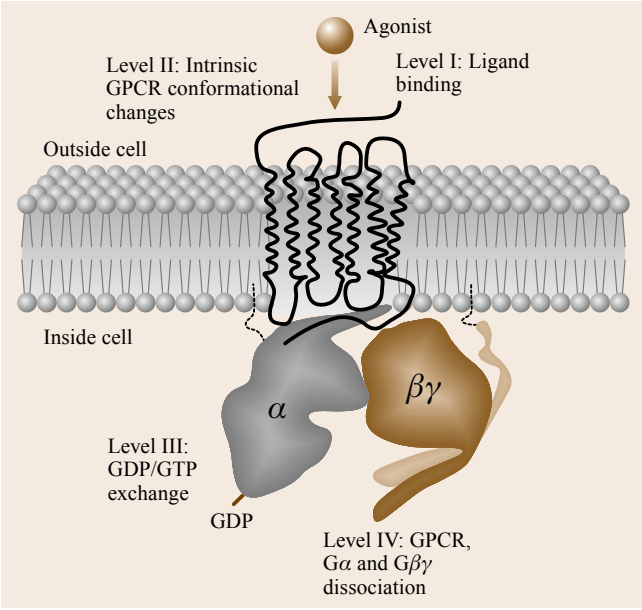

Fig. 17.1 Transmembrane topology of a typical *serpentine* G-protein coupled receptor (GPCR) and representation of the levels of biosensing. The receptor's amino terminal (N-terminal) is extracellular (outside of the cell), and its carboxy (C) terminal is within the cytoplasm (intracellular). The receptor polypeptide chain traverses the plane of the membrane phospholipid bilayer seven times. The hydrophobic transmembrane segments of the GPCR are indicated by spirals. The agonist approaches the receptor from the extracellular surface and binds, depending on the receptor type, to a site near the N-terminal or to a site deep within the receptor, surrounded by the transmembrane regions of the receptor protein. The G-proteins (Gα and G$\beta\gamma$) interact with cytoplasmic regions of the receptor, including the third intracellular loop between transmembrane regions V and VI. The Gα and Gγ subunits contain fatty acid modifications (myristate and palmitate on the Gα and isoprenylate on the Gγ) to help anchor the proteins at the lipid bilayer (shown as *dotted lines*). The *levels* of signaling that may be exploited for detection in a cell-free mode are shown (GDP, guanosine diphosphate) ◀

inositides within the cell, resulting in a physiological response such as stronger and faster contraction of the heart.

Since many disease processes involve aberrant or altered GPCR signaling dynamics, GPCRs represent a significant target for medicinal pharmaceuticals. Furthermore, more than 50% of all drugs currently marketed worldwide are directed against GPCRs [17.1], with this likely to increase with the recent elucidation of high-resolution structural data of the β_2-adrenergic

Table 17.1 Some examples of prescription drugs that target GPCRs for the indicated disease state

Brand name	Generic name	G-protein coupled receptor(s)	Indication
Zyprexa	Olanzapine	Serotonin 5-HT_2 and dopamine	Schizophrenia, antipsychotic
Risperdal	Risperidone	Serotonin 5-HT_2	Schizophrenia
Claritin	Loratidine	Histamine H_1	Rhinitis, allergies
Imigran	Sumatriptan	Serotonin 5-$HT_{1B/1D}$	Migraine
Cardura	Doxazosin	α-Adrenoceptor	Prostate hypertrophy
Tenormin	Atenolol	β_1-Adrenoceptor	Coronary heart disease
Serevent	Salmeterol	β_2-Adrenoceptor	Asthma
Duragesic	Fentanyl	Opioid	Pain
Imodium	Loperamide	Opioid	Diarrhea
Cozaar	Losartan	Angiotensin II	Hypertension
Zantac	Ranitidine	Histamine H_2	Peptic ulcer
Cytotec	Misoprostol	Prostaglandin PGE_1	Ulcer
Zoladex	Goserelin	Gonadotrophin-releasing factor	Prostate cancer
Requip	Ropinirole	Dopamine	Parkinson's disease
Atrovent	Ipratropium	Muscarinic	Chronic obstructive pulmonary disease (COPD)

Table 17.2 A partial list of some of the known endogenous and exogenous GPCR ligands

Acetylcholine	Ghrelin	Opioids
Adenosine	Glucagon	Orexin
Adrenaline	Glutamate	Oxytocin
Adrenocorticotropic hormone	Gonadotropin-releasing hormone	Parathyroid hormone
Angiotensin II	Growth hormone-releasing factor	Photons (light)
Bradykinin	Growth-hormone secretagogue	Platelet activating factor
Calcitonin	Histamine	Prolactin releasing peptide
Chemokines	Luteinising hormone	Prostaglandins
Cholecystokinin	Lymphotactin	Secretin
Corticotropin releasing factor	Lysophospholipids	Serotonin
Dopamine	Melanocortin	Somatostatin
Endorphins	Melanocyte-stimulating hormone	Substances P, K
Endothelin	Melatonin	Thrombin
Enkephalins	Neuromedin-K	Thromboxanes
Fatty acids	Neuromedin-U	Thyrotropin
Follitropin	Neuropeptide-FF	Thyrotropin releasing hormone
γ-Aminobutyric acid (GABA)	Neuropeptide-Y	Tyramine
Galanin	Neurotensin	Urotensin
Gastric inhibitory peptide	Noradrenaline	Vasoactive intestinal peptide
Gastrin	Odorants	Vasopressin

receptor [17.2]. Table 17.1 lists some commonly prescribed drugs acting on GPCRs. GPCRs are associated with almost every major therapeutic category or disease class, including pain, asthma, inflammation, obesity, cancer, as well as cardiovascular, metabolic, gastrointestinal, and central nervous system diseases [17.3]. It is this vitally important function of these cell-surface receptors, i. e., transduction of exogenous signals into an intracellular response, which makes GPCRs so physiologically significant. Indeed, there are reported to be $\approx$ 747 different human GPCRs as predicted from gene sequencing analyses, 380 of which are thought to be chemosensory receptors, whereas the remaining 367 GPCRs are predicted to bind endogenous ligands such as neurotransmitters, hormones, fatty acids, and peptides [17.4]. With about 230 of these GPCRs having been identified already (i. e., they have known ligands), this currently leaves about 140 *orphan* GPCRs with as yet undiscovered ligands. A summary of some of the known GPCR ligands is presented in Table 17.2.

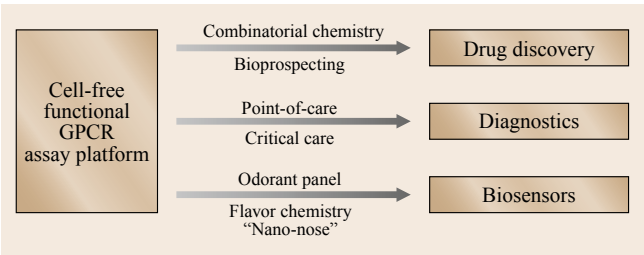

Fig. 17.2 Future applications of GPCR platforms. The development of functional assay platforms for integral membrane proteins, particularly those of the GPCR class, which are compatible with future high-throughput microarray formats will offer significant opportunities in a number of areas. It is expected that such advances in assay technology will likely impact on drug discovery, diagnostics, and biosensors. There is a strong requirement for technologies that enable screening of multiple GPCR targets simultaneously (multiplexing). Therefore, it would be advantageous in the future to design new biosensor platforms using miniaturized nanotechnology approaches. Furthermore, to achieve this aim successfully, it will be an absolute requirement for cross-disciplinary fields of research (including biology, physics, and chemistry, as well as mathematics for molecular modeling and bioinformatics) to be highly integrated ▶

This chapter focuses firstly on possible cell-free approaches which could be used in biosensor applications, diagnostic platforms, and for high-throughput screening (HTS) of GPCR ligands, with particular emphasis on GPCR signaling complexes and associated enabling nanotechnologies (Fig. 17.2). Additionally, we include molecular biology approaches involving G-proteins and GPCRs with reference to biosensor and HTS applications. One of the most important breakthroughs permitting these developments for GPCR and G-protein signaling is the ability to produce these GPCRs and G-proteins in relatively high amounts and in purified form using recombinant DNA techniques. Also, it is becoming increasingly more routine to produce recombinant modifications of such proteins using basic molecular biological approaches. These modifications can include biotin tags, hexahistidine tags or fluorescent protein fusions which can allow site-specific interaction of the recombinant protein(s) with appropriately derivatized biosensor surfaces such as glass or gold or the generation of a biosensor signal.

17.1 The GPCR:G-Protein Activation Cycle

In order to understand how we measure the activation of GPCRs and their associated G-proteins, a first step is to revise the GPCR:G-protein activation cycle in more detail. At the cellular level, GPCRs are integral membrane proteins which reside within the cell membrane lipid bilayer and are closely associated with the peripheral G-protein heterotrimeric complex consisting of the Gα and the G$\beta\gamma$ dimer subunits (Fig. 17.1). Owing to the very high affinity between Gβ and Gγ, these two subunits are almost exclusively considered as the G$\beta\gamma$ dimer. (The Gα subunits are $\approx$ 41 kDa and have a theoretical diameter of $\approx$ 4.7 nm, whilst β subunits are $\approx$ 37 kDa and γ subunits are 8 kDa, giving G$\beta\gamma$ dimers an approximate diameter of 4.6 nm.) Figure 17.3 depicts the cycle of activation/inactivation of the heterotrimeric G-protein complex. In the resting inactive state (i. e., when there is no agonist bound to the receptor), the G-proteins Gα and G$\beta\gamma$ have high affinity for each other and remain tightly bound, forming the heterotrimeric G-protein complex. In this state, guanosine diphosphate (GDP) is tightly bound to the Gα subunit associated with the G$\beta\gamma$ dimer. Both Gα and G$\beta\gamma$ subunits can bind to the GPCR. When the agonist (a GPCR ligand which activates the GPCR signaling pathway) approaches the GPCR from the extracellular fluid and binds to the active site on the GPCR, the GPCR is in turn *activated*, possibly leading to a change in its conformation. The GDP-liganded Gα subunit responds with a conformational change which results in a decreased affinity, so that GDP is no longer bound to the Gα subunit. At this point guanosine triphosphate (GTP), which is in higher concentration in the cell than GDP, can rapidly bind to the Gα subunit, thus replacing the GDP. This replacement of GDP with GTP activates the Gα subunit, causing it to dissociate from the G$\beta\gamma$ subunit as well as from the receptor. This in effect results in exposure of new surfaces on the Gα and G$\beta\gamma$ subunits which can interact with cellular effectors such as the enzyme adenylate cyclase, which converts adenosine triphosphate (ATP) to cAMP. The activated state of the Gα subunit lasts until the GTP is hydrolyzed to GDP by the intrinsic GTPase

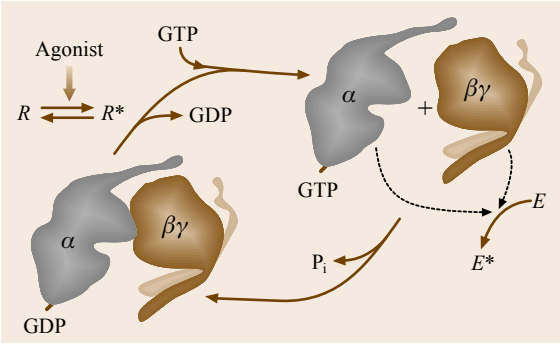

Fig. 17.3 Molecular switching: the regulatory cycle of agonist-induced (receptor-activated) heterotrimeric G-proteins. The binding of the agonist to the unoccupied receptor (R) causes a change in conformation, thus activating the receptor (R^*), which promotes the release of GDP from the heterotrimeric G-protein complex and rapid exchange with GTP into the nucleotide binding site on the Gα subunit. In its GTP-bound state, the G-protein heterotrimer dissociates into the Gα and G$\beta\gamma$ subunits, exposing new surfaces and allowing interaction with specific downstream effectors (E). The signal is terminated by hydrolysis of GTP to GDP (and P_i) by the intrinsic GTPase activity of the Gα subunit followed by return of the system to the basal unstimulated state. Asterisk indicates activated state of receptor (R) or effector (E); P_i, inorganic phosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate ◀

activity of the Gα subunit. The various families of Gα subunits, i. e., Gα_s, G$\alpha_{i/o}$, G$\alpha_{q/11}$, and G$\alpha_{12/13}$, are all GTPases, although the intrinsic rate of GTP hydrolysis varies greatly from one type of Gα subunit to another. Following the hydrolysis of GTP to GDP on the Gα subunit, the Gα and G$\beta\gamma$ subunits re-associate and are able to return to the receptor-associated state.

17.2 Preparation of GPCRs and G-Proteins

In cell-free assays, host cells are transfected with DNA, which allows high levels of expression of the GPCR of interest (in a similar manner to that of whole-cell assays). To date GPCRs have proven to be extremely

Table 17.3 Comparison of the main advantages and disadvantages of various commonly used expression systems to obtain GPCRs and/or G-proteins

Expression system	Advantage	Disadvantage
Bacteria, e.g., *Eschericia coli* spp.	• Many host species to chose from • Many DNA expression vectors available • Relatively cheap • Fast process and easy to scale up • Yield can be very high	• Prokaryotic, not eukaryotic • Truncated proteins can be produced • The expressed proteins often do not fold properly and so are biologically inactive • Insufficient posttranslational modifications made, e.g., GPCR glycosylation, G-protein palmitoylation • Overexpression can be toxic to the host cells
Yeast, e.g., *Saccharomyces cerevisiae*	• Eukaryotic • Fast process and relatively easy to scale up • Yield can be very high • Relatively cheap • Performs many of the post-translational modifications made to human proteins	• Cell wall may hinder recovery of expressed proteins • Presence of active proteases that degrade foreign (expressed) proteins, therefore may reduce yield
Insect, e.g., *Spodoptera frugiperda Sf*9, Hi-5	• High levels of expression • Correct folding • Posttranslational modifications similar to those in mammalian cells	• Expensive to scale up • Slow generation time • Difficult to work with
Mammalian, e.g., Chinese hamster ovary (CHO), human embryonic kidney 293 (HEK), CV-1 in origin with SV40 (COS)	• Good levels of expression • Correct folding and post-translational modifications	• Relatively low yields • Very expensive to scale up • Slow generation time • Difficult to work with • Health and safety implications involved

difficult to purify, primarily due to the lipophilic (hydrophobic) nature of these receptors and the fact that they are usually irreversibly denatured (inactivated) when they are removed from their native lipid environment using detergent treatment. However, partial purification of GPCRs is usually carried out in order to obtain a supply of them. This results in small (nanometer-scale) crude membrane fragments being produced. The GPCR membrane fragments will usually contain hydrophobic membrane lipids (which are required for functionality) as well as other native, *contaminating* proteins and can then be manipulated and immobilized by various means (discussed later) onto appropriate surfaces for use as *biosensors*. On the other hand, the G-proteins, which are classified as peripheral as opposed to integral membrane proteins and do not require an absolute lipid environment for activity, can be routinely purified in relatively large amounts (milligram quantities) when expressed using recombinant DNA-based technologies. Therefore, the first step in generating a GPCR biosensor technology is successfully obtaining functional proteins that may also have been engineered to provide new properties that enable surface attachment or a fluorescent signal.

17.2.1 Expression Systems for Recombinant GPCRs/G-Proteins

A prerequisite for molecular approaches to the design of cell-free GPCR assays is an expression system which produces recombinant proteins with the required activity and level of expression. Expression systems utilizing either bacteria, yeast, mammalian or insect cells are detailed in Table 17.3. These systems are generally well characterized and show the greatest promise in terms of their ability to produce large amounts of functional proteins which can be utilized in GPCR biosensing assay formats.

17.3 Protein Engineering in GPCR Signaling

Molecular engineering of proteins is likely to be of great importance for producing receptors and other associated signaling proteins which have a modified structure or function amenable for use in cell-free biosensing applications. Currently, many receptor or G-protein modifications are aimed at enhancing the purification of proteins, facilitating the attachment to a specific surface or to aid in generating the biosensor signal from GPCR activation (e.g., fluorescence). These modifications can range from the attachment of small tags to larger reporter proteins. These fusion proteins can be generated by engineering DNA sequences that encode the receptor and another protein or tag, joining them such that a single protein is expressed (Fig. 17.4).

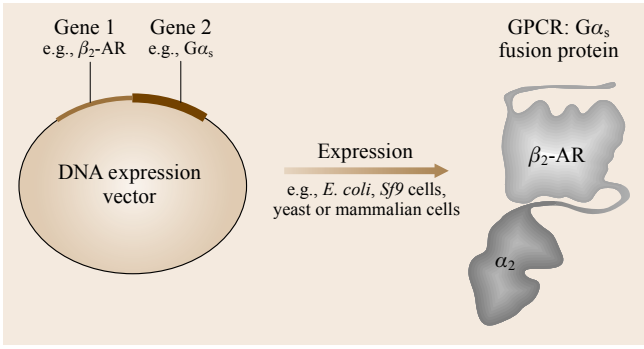

Fig. 17.4 Generation of a fusion protein. Two separate genes of interest are cloned and subsequently ligated into a DNA expression vector *in frame*. In this example, the DNA sequence encoding the GPCR (β_2-adrenergic receptor; β_2-AR) is incorporated into the expression vector within the multiple cloning site. The DNA sequence encoding the Gα_s protein is also cloned into this vector. The resultant recombinant expression vector contains the (carboxy) C-terminus of the β_2-AR fused in frame to the (amino) N-terminus of the Gα_s protein. The recombinant DNA expression vector is then transfected into an appropriate cell line and the fusion protein is expressed

17.3.1 Fluorescent Proteins

Green fluorescent protein (GFP) was first isolated from jellyfish and has been widely exploited in molecular/cell biology research applications due to its efficient fluorescence emission properties. GFPs are particularly useful as they do not require unusual substrates, external catalysis or accessory cofactors for fluorescence as do many other natural pigments [17.5].

Whilst fluorescent proteins provide many advantages, they are limited in their use as protein labels due to their property of being large, multimeric proteins. For this reason alternative methods of site-specific fluorescent labeling are emerging, including the use of

lanthanide binding tags [17.6] and tetracysteine motifs (TCM) [17.7]. Each of these tags are significantly smaller than GFP variants and enable fluorescence through the binding of a lanthanide such as terbium or a fluorescent arsenic derivative such as FlAsH, respectively. In some cases the use of a smaller tag can prevent the loss of function of the proteins of interest. For example, when a TCM was used to label the adenosine A_{2A} receptor, aspects of the receptors function that had been lost when using yellow fluorescent protein (YFP) at the same site were restored [17.8]. In the future it is likely that fluorescence-based assay development involving compounds such as these will increase in efficiency and flexibility, allowing such methods to be at the forefront of technologies for determining molecular interactions using cell-free systems.

17.3.2 Engineering of Promiscuous Gα Proteins

A major impediment to the production of homogeneous, cell-free, GPCR-based screening systems is the coupling between a given GPCR and a subset of Gα subunits. For example, muscarinic receptor subtypes M_1, M_3, and M_5 typically couple to $G\alpha_{q/11}$, whilst M_2 and M_4 subtypes couple to G_i or G_o [17.9]. Biologically, this discrimination is the basis for correct cellular signaling but needs to be modified from the in vivo situation to allow production of a generic GPCR biosensing system. In this regard, recent attempts have been made to produce *promiscuous* Gα subunits capable of transducing signals resulting from extracellular interactions involving any GPCR [17.10, 11]. Many of the promiscuous subunits constructed thus far are based on variants of the human $G\alpha_{16}$ (a member of the $G\alpha_q$ subfamily). This protein was first isolated from hematopoietic cells [17.12] and was shown to couple to a wider range of receptors than other known alpha subunits, and to transduce ligand-mediated signaling through phospholipase C (PLC), resulting in the modification of intracellular calcium concentrations [17.13–17]. Molecular biology approaches have also been utilized to increase the promiscuity of various Gα subunits by altering the sequence of amino acids within the protein [17.11, 18–21]. Although cell-free applications have not been routinely used to date, it is expected that, within the near future, promiscuous G-proteins will be used in a similar manner to in whole-cell applications.

17.3.3 Protein Engineering for Surface Attachment

Ideally, proteins should be uniformly immobilized so that the protein remains functional and is orientated such that the required interaction can occur. To achieve these ends, protein engineering can be a powerful tool. Immobilized metal ion affinity chromatography (IMAC) used for protein purification has been extended to enable functional immobilization of proteins onto a surface. GPCRs and G-proteins are often fused to a 6 histidine tag [17.22–24] that has a high affinity for nitriloacetic acid (NTA) loaded with a divalent cation, often Ni^{2+}, on a surface. The length of the histidine tag can also be adjusted for higher affinity to Ni^{2+}, as has been demonstrated using $G\alpha_{i1}$ [17.25]. Utilizing histidine tags, it has also been possible to functionally reconstitute GPCRs with G-proteins on Ni-NTA beads and observe signaling upon ligand binding [17.24]. Surface immobilization has also been achieved by engineering short peptides such as the C9 peptide or Myc tags onto GPCRs of interest and using surfaces displaying the appropriate antibody to these tags to capture the receptors [17.23, 26].

17.4 GPCR Biosensing

The basic requirement of a biosensor is the use of a biological element, such as an immobilized protein, to act as a sensor for a specific binding analyte. This is coupled with a reporter system which amplifies the initial signal to produce some form of output. Depending on the type of output required for a given screening process, e.g., ligand binding to a GPCR or a functional assay such as G-protein activation, a number of protocols are available to target the site of interest. In this chapter we will refer to these as *levels* of GPCR activation (Fig. 17.1). Examples of each of these levels will be discussed below. Additionally, in this section, the levels of biosensing referred to represent those *cell-free* samples or biological preparations which are derived from cells and are used in the cell-free mode, i. e., the GPCRs and G-proteins have been either partially or fully purified from cells expressing the GPCRs or G-proteins, and then subsequently *reconstituted* at known concentrations, usually within the nanomolar range.

17.4.1 Level 1 Biosensing – Ligand Binding

We have defined level 1 biosensing as ligand binding to the receptor. This includes such techniques as radioligand binding (not discussed here) and fluorescent (and fluorescent polarization) ligand binding assays. Ligand binding can also be detected by techniques such as flow cytometry, two-photon excitation cross-correlation spectroscopy (TPE-FCCS), surface plasmon resonance (SPR), plasmon waveguide spectroscopy, and piezoelectric crystal sensing.

This level of biosensing does not discriminate between compounds which can be pharmacologically defined as agonists, antagonists, partial agonists or inverse agonists. Therefore its use in biosensing of the *activation* of a signaling pathway is somewhat limited. However it is still useful for some specific purposes such as screening for compounds which *interact* with a particular GPCR.

Fluorescence Polarization

Polarization is a general property of most fluorescent molecules. Polarization-based experiments are less dye dependent and less susceptible to environmental interferences (such as pH changes) than assays based on fluorescence intensity measurements. Fluorescence intensity variations due to the presence of samples which may be colored (e.g., in drug screening of compound libraries) tend to produce relatively minor interferences. The degree of polarization (or anisotropy) can be determined from measurements of fluorescence intensities parallel and perpendicular to the plane of linearly polarized excitation light [17.27].

Fluorescence and fluorescence polarization (FP) assays which are based on specific binding of the ligand to a GPCR can offer an alternative to traditional radioligand binding assays which utilize radionuclides (radioisotopes) [17.28]. FP assays usually take the form of a homogeneous or *mix and read* type of assay (and an example of level 1 assays), which indicates that they are readily transferable from assay development to high-throughput screening (HTS). FP allows for the development of protocols which are both real-time measurements (kinetic assays) and insensitive to variations in concentrations. One of the disadvantages of this assay format is the lack of adaptability to all GPCR ligands by virtue of the fact that only a small number of ligands can be chemically tagged with an appropriate fluorophore and still retain their intrinsic binding qualities. Finally, the choice of fluorophore is important, as the intensity of the fluorescent compound must be of sufficient magnitude as well as having good polarizing properties [17.29].

Two-Photon Excitation Fluorescence Cross-Correlation Spectroscopy

Two-photon excitation fluorescence cross-correlation spectroscopy (TPE-FCCS) is used to measure dynamic interactions between molecules, and in the bioscience field has applications in monitoring DNA, protein, and ligand interactions. The technique allows for small measurement volumes and low sample concentrations and has increased detection specificity over classical fluorescence techniques for monitoring molecular dynamics in solution. TPE-FCS is an extension of fluorescence correlation spectroscopy (FCS), which analyzes minute, spontaneous signal fluctuations arising from molecular diffusion. The term "two-photon" refers to the use of different fluorescent molecules with distinct emission properties, each of which can be excited by two photons of half the energy required for a transition to the excited state. Two-photon excitation spectra of many common fluorophores differ considerably from their one-photon spectra without a change in emission. This makes it possible to simultaneously excite two spectrally distinct dyes with a single infrared light source. A cross-correlation of the two fluorophores is only generated when the two detection channels measure synchronous fluorescence fluctuations, which suggests that the different colored species must be spectrally linked.

This technique is useful in the study of association and dissociation reactions such as that of a receptor–ligand pair. To obtain accurate kinetic information regarding the interaction of the human μ-opioid receptor (within nonpurified preparations that were termed *nanopatches*) with its ligand, *Swift* et al. have used TPE-FCCS [17.30]. A pentahistidine-tagged μ-opioid receptor was fluorescently tagged with an Alexa-conjugated antipentahistidine antibody and measured in the presence of fluorescein-labeled antagonists. Similarly to the FP previously mentioned, this fluorescence technique also enables a homogeneous assay platform amenable to HTS.

Flow Cytometry

Flow cytometry is a technique used to analyze the fluorescence of individual cells or particles (such as the dextran beads in the example below). Fluorescence can arise from intrinsic properties of the cell, but generally molecules/particles of interest are fluorescently labeled. Hydrodynamic focusing is used to force the cells or particles into a single file, after which they are passed

through a laser beam where both scattered and emitted light are measured. A benefit of this method is that simultaneous measurements can be performed on individual particles.

Waller et al. [17.31] have conjugated dextran beads with the cognate ligand dihydroalprenolol, which allowed for capture of solubilized β_2-adrenergic receptors (β-AR) onto this immobilized surface ligand. To measure the specific binding of the receptor to the bead in this flow-cytometry-based assay system, the receptor was expressed as a fusion protein with GFP. It was then possible to screen for ligands (either agonists or antagonists) to β-AR using a competition assay. Another successful bead-based approach used paramagnetic beads [17.32]. In that study the authors built up a surface containing the captured CCR5 receptor from a cell lysate held within a lipid bilayer. In this instance the CCR5 receptor was not able to freely move laterally in the bilayer as it was tethered via an antibody (directed at the CCR5 receptor) conjugated to the paramagnetic beads (paramagnetic proteoliposome).

Total Internal Reflection Fluorescence

Total internal reflection fluorescence (TIRF) takes advantage of refractive index differences at a solid–liquid interface, with the solid surface being either glass or plastic, e.g., cell culture containers. At a critical angle, when total internal reflection occurs, an evanescent wave is produced in the liquid medium. This electromagnetic field decays exponentially with increasing distance from the surface. The range of this field limits its background fluorescence, as only fluorophores in close proximity to the surface are excited. As such, the technique is used to examine interactions between the molecule of interest and the surface, for example, receptors binding to a surface.

Martinez et al. [17.33] used TIRF to demonstrate ligand binding to the neurokinin-1 GPCR by surface immobilization of membrane fragments containing this receptor protein. In this study, the GPCR expressed as a biotinylated protein using mammalian cells was selectively immobilized on a quartz sensor surface coated with streptavidin (streptavidin binds biotin with extremely high affinity). TIRF measurements were made using a fluorescence-labeled agonist (i. e., the cognate agonist substance-P labeled with fluorescein). Using this approach, it was not necessary to detergent-solubilize and reconstitute the neurokinin-1 receptors, thus avoiding the deleterious effect(s) associated with such processes. This receptor, in the form of a mammalian cell membrane homogenate, was then surface-immobilized without further purification. The selective, high-affinity interaction between biotin and streptavidin allowed template-directed and uniform orientation of the neurokinin-1 receptor on the support matrix. Additionally, the highly selective TIRF fluorescence detection methodology was able to resolve the binding of fluorescently tagged agonist to as little as 1 aM of receptor molecules.

Microspotting of GPCRs on Glass

The intrinsic difficulties in producing, purifying, and manipulating membrane proteins have delayed their introduction into microarray platforms. Hence there are no reports to date describing purified membrane protein (GPCR) microarrays and their use in functional screening or biosensor applications. However, as a first step towards such display technologies, researchers at Corning Inc. (Rochester, USA) have recently described the fabrication of GPCR membrane arrays for the screening of GPCR ligands [17.34–37]. The arraying of membrane GPCRs required appropriate surface chemistry for the immobilization of the lipid phase containing the GPCR of interest (Fig. 17.5). They reported that surface modification with γ-aminopropylsilane (an amine-presenting surface) provided the best combination of properties to allow surface capture of the GPCR:G-protein complex from crude membrane preparations, resulting in microspots of $\approx 100\,\mu$m diameter. Atomic force microscopy (AFM) demonstrated that the height of the supported lipid bilayer was ≈ 5 nm, corresponding to GPCRs confined in a single, supported lipid layer scaffold. Using these chemically derivatized surfaces, it was possible to demonstrate capture of the β_1, β_2, and α_{2A} subtypes of the adrenergic receptor, as well as neurotensin-1 receptors and D1-dopamine receptors. This was achieved by using ligands with fluorescent labels covalently attached and detecting fluorescence binding to the GPCRs with a fluorescence-based microarray scanner. Dose–response curves using the fluorescently labeled ligands gave 50% inhibition concentration (IC_{50}) values in the nanomolar range, suggesting that the GPCR:G-protein complex was largely preserved and biologically intact in the microspot. There was no change in the performance of the arrays over a 60 day period, indicating good long-term stability. Although the use of glass slides for the printing of the GPCR arrays was promoted by this research group, in some instances gold surfaces were required due to nonspecific binding of fluorescent ligands. A current limitation of this technology is the inability to carry out a functional(i. e., signaling) assay, which

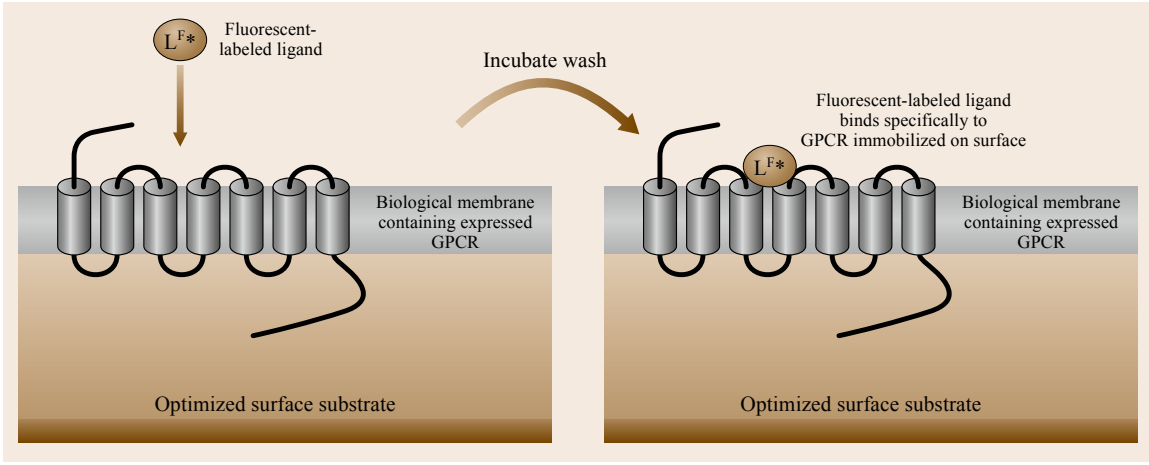

Fig. 17.5 Idealized schematic of an immobilized GPCR with associated G-proteins. The fabricated surface array is printed on a γ-aminopropylsilane (GAPS)-presenting surface. The height of the supported lipid bilayer is ≈ 5 nm. Fluorescently labeled (L^{F*}) ligands (such as 4,4,-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY)-tetramethylrhodamine (TMR)) will bind specifically to the GPCR (for example, a neurotensin receptor) at nanomolar concentrations. The fluorescence is measured following an incubation/washing step to remove unbound fluorescent ligands. When compounds of unknown activity are added to the incubation step, as in drug screening programs, fluorescent-labeled ligand binding is blocked by agents that bind to the GPCR (for example, GPCR antagonists) (adapted from [17.13])

would allow test compounds to be classified as agonists or antagonists. Furthermore, although there are increasing numbers of commercially available fluorescently labeled ligands, the need to always structurally modify the ligand to accommodate some reporter moiety may limit the implementation of the technology. Nevertheless, the above-mentioned GPCR microarrays may have potential as *functional* GPCR assays when complexed with G-proteins and integrated with appropriate signal generation and detection methods.

Surface Plasmon Resonance

One of the most versatile techniques for measuring biospecific interactions in real time are biosensors based on the optical phenomenon of surface plasmon resonance (SPR, Fig. 17.6). Surface plasmon resonance occurs when light interacts with a conducting surface (plasmon interaction) which is positioned between two materials of different refractive index. At a specific angle the intensity of the reflected light decreases, with this angle being dependent on (among other things) the refractive index of the material on the opposite side to which the light is applied. Molecules associating with or disassociating from this material (e.g., receptor to surface or ligand to receptor) will change the refractive index of the material and can be detected by measuring the reflected light. The instrument detects the change in angle of the reflected light minimum. The technique can be used to study interactions between ligands, GPCRs, and G-proteins. SPR experiments do not require a large amount of sample, and detection does not require fluorescent or radioisotopic labeling. A variety of available surface chemistries allows for immobilization of many types of proteins using a range of strategies.

Ligand binding to a GPCR attached to a surface has been reported for the chemokine CCR5 receptor using SPR methodology [17.38]. For such display, purification of the GPCR has not always been necessary, and crude membrane preparations have either been fused with an alkylthiol monolayer (≈ 3 nm thickness) formed on a gold-coated glass surface or onto a carboxymethyl-modified dextran sensor surface [17.39]. One problem of surface-based assays is orientation of the receptor once attached to the surface. One means of overcoming this problem was to specifically select only those proteoliposomes (≈ 300 nm-diameter vesicles) in which the carboxy terminus of the receptor was orientated to the outside of the vesicle. This was performed using conformationally dependent antibodies [17.26]. In this biosensor application, SPR has a distinct advantage as a screening tool since it can detect the cognate ligand without requiring fluorescent or radio labeling. This al-

lows SPR to be used in complex fluids of natural origin and simplifies, and potentially speeds up, the development of assay technologies.

Plasmon-Waveguide Resonance Spectroscopy

Plasmon-waveguide resonance (PWR) spectroscopy measures real-time binding of free molecules to immobilized molecules such as GPCRs without the application of specific labels (reviewed elsewhere [17.40]). PWR has several significant advantages compared with conventional surface plasmon resonance, including enhanced sensitivity and spectral resolution, as well as the ability to distinguish between mass and conformational changes. This latter property is a consequence of the use of both p- and s-polarized excitation to produce resonances. This allows for measurement of refractive index anisotropy, which reflects changes in mass distribution and, therefore, changes in molecular orientation and conformation.

In a recent study, ligand binding to the β_2-adrenergic receptor has been demonstrated using PWR [17.41]. Using this technique, changes in the refractive index upon ligand binding to surface-immobilized receptor results in a shift in the PWR spectra. The authors used ligands with similar molecular weight in order to study structural changes in the receptor caused by agonist, inverse agonist, and partial agonist binding. The technique was used to produce binding curves for five ligands using shifts in the PWR spectra (with both s- and p-polarized light) with increasing ligand concentration, with the results from PWR being compared with those obtained by traditional radioligand binding assays. Differences in s- and p-polarized light measurements demonstrated changes in receptor structure which varied depending on whether the ligand was a full, partial or inverse agonist. Previous work using PWR technology has been reported for the detection of conformational changes in a proteolipid membrane containing the human δ-opioid receptor following binding of nonpeptide agonists, partial agonists, antagonists, and inverse agonists [17.42]. Although the ligands in the above study were of similar molecular weight, there were distinctly different refractive index changes induced by ligand binding and these were too large to be accounted for by differences in mass alone. The infer-

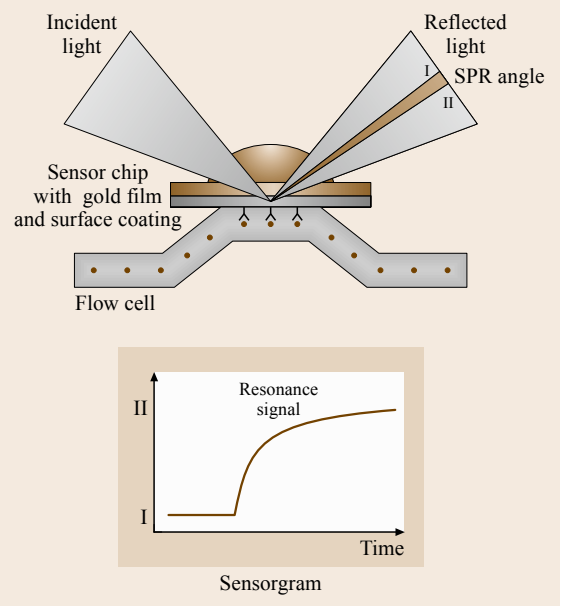

Fig. 17.6 Surface plasmon resonance (SPR) provides mass detection. Most importantly, this technique does not require labeling of the interacting components. Since it is the evanescent field wave and not the incident light which penetrates the sample, measurements can be made on turbid or even opaque samples. The detection principle of SPR relies on electron charge density wave phenomena arising at the surface of a metallic film when light is reflected at the film under specific conditions (surface plasmon resonance). The resonance is a result of energy and momentum being transformed from incident photons into surface plasmons, and is sensitive to the refractive index of the medium on the opposite side of the film from the reflected light. Quantitative measurements of the binding interaction between one or more molecules are dependent on the immobilization of a target molecule onto the sensor chip surface. Binding partners to the target can be captured from a complex mixture as they pass over the chip. Interactions between proteins, nucleic acids, lipids, carbohydrates, and even whole cells can be studied. The sensor chip consists of a glass surface coated with a thin layer of gold. This forms the basis for a range of specialized surfaces designed to optimize the binding of a variety of molecules. The gold layer in the sensor chip creates the physical conditions required for SPR. The *upper figure* shows a detector with sensor chip. When molecules in the test solution bind to a target molecule the mass increases; when they dissociate the mass falls. This simple principle forms the basis of the sensorgram for continuous, real-time monitoring of the association and dissociation of the interacting molecules (*lower figure*). The sensorgram provides quantitative information in real time on the specificity of binding, active concentration of molecules in a sample, kinetics, and affinity. Molecules as small as 100 Da can be studied ◀

ence from this finding was that a ligand-specific conformation change in the receptor protein may have been detected. Therefore this methodology may have use as a future biosensor, particularly with regard to GPCRs.

Piezoelectric Crystal Sensing

Piezoelectric crystal sensing measures a change in mass on molecule binding to the surface due to a change in resonance frequency of the crystal. The technique has been used in an *electronic nose* with olfactory receptors which are typically GPCRs [17.43], where an array of six sensor elements could be used to characterize each of six test compounds, emphasizing the potential for GPCR ligand screening in the sensory area. The use of an artificial nose (*bionose*) to mimic the properties of the human nose may find wide applications in the near future.

17.4.2 Level 2 Biosensing – Conformational Changes in the GPCR

Level 2 involves the detection of intrinsic conformational changes in the GPCR protein following agonist activation and may involve the use of fluorescence-based techniques. Cell-free measurements of conformational changes in the GPCR following ligand (usually agonist or partial agonist) binding have been limited to date.

A good example of a level 2 cell-free assay used β_2-adrenergic receptors immobilized onto glass and gold surfaces. In this study, the receptors were site-specifically labeled with the fluorophore tetramethyl-rhodamine-maleimide at cysteine 265 (Cys265) using a series of molecular biology approaches. It was then possible to show agonist (isoproterenol)-induced conformational changes within the vicinity of the fluorescent moiety (tetramethyl-rhodamine) at position Cys265 of the recombinant β_2-adrenergic receptors. Moreover, the agonist-induced signal was large enough to detect using a simple intensified charge-coupled device (ICCD) camera image. Thus, it was suggested that the technique may be useful for drug screening with GPCR arrays. Indeed this method did not require the formation of lipid bilayers and did not require the use of purified G-proteins or fluorescent ligands to detect receptor activation.

17.4.3 Level 3 Biosensing – GTP Binding

Measurements of GPCR activation further downstream from level 2 are considered for the purposes of this chapter to be truly functional assays since the transducer G-proteins are the first differentiated site of signalling initiated from the GPCR. This therefore means that the GPCR must be in a *functional* form, enabling it to interact and activate a G-protein signaling pathway. Level 3 biosensing involves the use of nonhydrolyzable GTP analogs such as radiolabeled $^{35}S\gamma GTP$ or fluorescent-tagged europium-GTP which bind to the receptor-activated form of the Gα subunit targeting the site of guanine nucleotide exchange (GDP for GTP on the Gα subunit of the G$\alpha\beta\gamma$ heterotrimer). The guanine nucleotide exchange process is generally considered the first major point of G-protein activation following GPCR stimulation (Figs. 17.1 and 17.3).

Guanine nucleotide exchange is a very early, generic event in the signal transduction process of GPCR activation and is therefore an attractive event to monitor as it is less subject to regulation by cellular processes further downstream (we denote this as level 3 biosensing, see Fig. 17.1). The radiolabeled $^{35}S\gamma GTP$ or fluorescent europium-GTP binding assays measure the level of G-protein activation following agonist activation of a GPCR by determining the binding of these nonhydrolyzable analogs of GTP to the Gα subunit. Therefore, they are defined as *functional* assays of GPCR activation. Ligand regulation of the binding of $^{35}S\gamma GTP$ is one of the most widely used assay methods to measure receptor activation of heterotrimeric G-proteins, as discussed elsewhere in detail [17.44, 45]. This methodology also provides the basis for measurement of pharmacological characteristics such as potency, efficacy, and the antagonist affinity of compounds [17.45] in cell-free assays and artificial expression systems for GPCRs (an example of typical data is shown in Fig. 17.7). However, despite the highly desirable attributes of this methodology and its widespread use to date, ligand regulation of $^{35}S\gamma GTP$ binding has been largely restricted to those receptors which signal through the $G\alpha_{i/o}$ proteins (pertussis-toxin sensitive) and, to a lesser extent, the $G\alpha_s$ and $G\alpha_q$ families of G-proteins. As such, the use of these assay platforms can be problematic for high-throughput screening as they are not homogeneous (i. e., they require a separation step to remove bound from free $^{35}S\gamma GTP$). Additionally, the use of radioactive-based assays (including ligand binding assays) has led to safety, handling, waste disposal, and cost concerns. The newly developed, fluorescence-based europium-GTP assay partly overcomes some of the above limitations and has already been successfully used with the following GPCRs: motilin, neurotensin, muscarinic-M_1, and α_{2A}-adrenergic receptors.

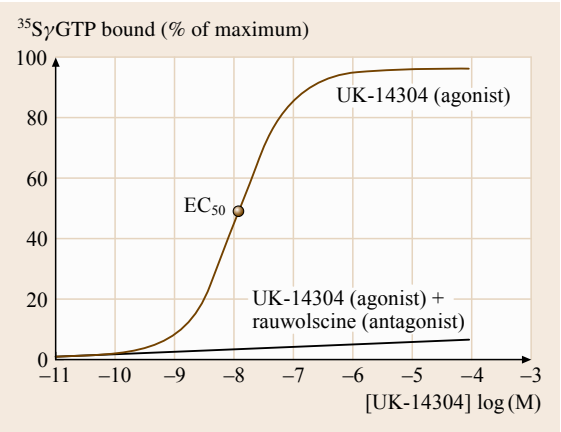

Fig. 17.7 Activation of GPCR-induced GTP binding. The data show results from an experiment which was conducted by incubating 20 nM purified G-proteins ($G\alpha_{i1}$ and $G\beta_1\gamma_2$) reconstituted with 0.4 nM recombinant α_{2A}-adrenergic receptor-expressing membranes (these receptors normally bind adrenaline with high affinity). The assay also contained 0.2 nM $^{35}S\gamma$GTP (a radioactive nonhydrolyzable analog of GTP). An adrenaline analog (UK-14304) was then added to the reconstituted α_{2A}-adrenergic receptor membrane, at the concentrations indicated on the x-axis (0.01 nM to 100 µM) in the presence or absence of the α_{2A}-adrenergic receptor antagonist, rauwolscine (10 µM). Following a filtration step to remove the bound $^{35}S\gamma$GTP:$G\alpha_{i1}$ complex from unbound $^{35}S\gamma$GTP, the filters were subsequently counted in a scintillation counter to measure the level of radioactivity. As the concentration of the agonist (UK-14304) was increased above 1 nM (10^{-9} M), the characteristic sigmoidal dose–response effect was seen. This result shows an increase in receptor-activated binding of $^{35}S\gamma$GTP to the $G\alpha_{i1}$ subunits as the UK-14304 is increased in concentration, indicating functional signaling of the receptor through the G-protein complex. The concentration at which 50% (also the point of inflexion) of the signaling response (effective concentration) was observed (EC_{50}) was ≈ 12 nM. In the presence of excess α_{2A}-adrenergic receptor antagonist (rauwolscine), the signal was completely blocked at the receptor. Therefore, this type of biosensing application demonstrates sensitivity as well as specificity

17.4.4 Level 4 Biosensing – GPCR:G-Protein Dissociation

Procedures that utilize only ligand binding (level 1) do not distinguish between agonist (activates receptor), antagonist (blocks the action of the agonist at the receptor binding site) or inverse agonist [inhibits the intrinsic (nonagonist-stimulated) activity of the receptor signaling, often observed in overexpressed receptors]; however, if a functional GPCR assay is constructed in which G-protein *activation* is an endpoint, i. e., level 4 biosensing, then it is possible to distinguish between these functionally distinct ligands. For cell-free assays, both methodologies (levels 1 and 4) are important in HTS programs, for example, and may have differing extents of applicability. Indeed, novel nanotechnology approaches will be required to achieve level 4 biosensing, including suitable surface derivatization for immobilization of GPCR and G-protein complexes.

Level 4 biosensing encompasses those assays which measure the final stage of activation of the G-protein heterotrimeric complex, i.e., the putative dissociation or rearrangement of the subunits following GPCR-induced G-protein activation [17.46]. This level of GPCR activation has currently not been investigated in great detail but may prove to be extremely valuable in future functional biosensor applications. Assay methodologies which are examples of level 4 biosensing have been reported using surface plasmon resonance and flow cytometry technologies to demonstrate receptor dissociation from the G-protein complex.

SPR G-Protein Dissociation

Bieri et al. [17.47] used carbohydrate-specific biotinylation chemistry to achieve appropriate orientation and functional immobilization of the solubilized bovine rhodopsin receptor, with high-contrast micropatterns of the receptor being used to spatially separate protein regions. This reconstituted GPCR:G-protein system provided relatively stable results (over hours) with the added advantage of obtaining repeated activation/deactivation cycles of the GPCR:G-protein system. Measurements were made using SPR detection of G-protein dissociation from the receptor surface following the positioning of the biotinylated form of the rhodopsin receptor onto a self-assembled monolayer containing streptavidin. Using this approach, G-protein activation could be directly monitored, giving a functional output, as opposed to ligand–receptor binding interactions, which yield little information on the receptor-activated pathway when screening agonists and antagonists. Although SPR is useful for the study of G-protein interactions, it may not be well suited to detect binding of small ligand molecules directly due to its reliance on changes in mass concentration. An advantage of repeated activation/deactivation cycles of GPCRs is that different compounds can be tested serially with the

same receptor preparation. The above approach appears promising for future applications of chip-based technologies in the area of GPCR biosensor applications.

Flow Cytometry – GPCR:G-Protein Interactions

Modifying the surface of epoxy-activated dextran beads by forming a Ni^{2+}-NTA conjugate was shown to produce beads with a surface capable of binding hexahistidine (his)-tagged $\beta_1\gamma_2$ subunits (Fig. 17.8). Tethered $\beta_1\gamma_2$ subunits were then used to capture $G\alpha_s$ subunits, which in turn were capable of binding membrane preparations with expressed β_2-adrenergic receptor containing a GFP fusion protein (see Sect. 17.3 for a detailed description of fusion proteins); alternatively, a fluorescence-labeled ligand could be detected binding to the tethered β_2-adrenergic receptor, the whole complex being measured using flow cytometry. Additionally, quantitative solubilization and re-assembly of the (hexahistidine-tagged) *N*-formyl peptide receptor (FPR) has been demonstrated on Ni^{2+}-silica particles using flow cytometry with dodecyl maltoside as the detergent [17.48]. Using such approaches, it may be possible to screen ligands for a known solubilized GPCR, or alternatively to test which G-proteins preferentially couple to a particular solubilized, reconstituted GPCR. The flow cytometry system used above had a sampling rate of ≈ 50–100 samples per minute; however, flow cytometry's greatest advantage is its ability to be multiplexed, where different molecular assemblies can be made with one sample and yet be discriminated by their unique spectral characteristics [17.31, 49, 50]. In more detailed studies, the assembly and disassembly of the FPR and his-tagged G-proteins complexed on Ni^{2+}-silica particles provided insight into the activation kinetics of the ternary complex (i. e., receptors and heterotrimeric G-proteins) [17.49,51]. The study by *Simons* et al. [17.49] extended the knowledge of ligand–GPCR interactions to involve G-protein–GPCR–ligand interactions assayed in a homogeneous format with a bead-based approach amenable to high-throughput flow cytometry. Indeed, HTS and proteomic applications could easily be based on such bead arrays with potential for color-coded particles and multiplexing (e.g., by using quantum-dot technology [17.52]).

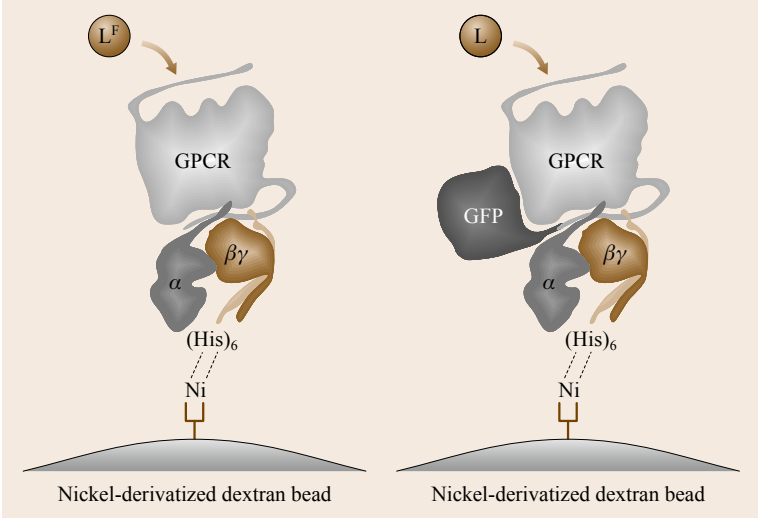

Fig. 17.8 Schematic diagram of two flow cytometry modes for detection of the ligand:receptor:G-protein assembly on nickel-coated beads. The G-proteins are immobilized on the bead surface containing a nickel (Ni) chelate. The exposed Ni binds to an engineered hexahistidine $(His)_6$ sequence on the N-terminal of the $G\gamma$ subunit and is able to capture the heterotrimeric G-protein complex (*left figure*). The fluorescent ligand (L^F) binds to the GPCR following capture of the GPCR with appropriate G-proteins complexed on the surface of dextran beads. This technique is useful for biosensing of the interaction of specific GPCR ligands (agonists and antagonists) and may be useful for demonstrating receptor:G-protein specificity and screening of ligands. In the *right figure*, the assembly uses a GPCR fusion protein containing enhanced green fluorescent protein (GFP). This technique demonstrates the requirement of the heterotrimeric complex for ligand activation and allows quantification of the receptor without the use of fluorescent ligands (adapted from [17.28])

Particle-based screening constitutes an enabling technology for the identification of agonists promoting assembly of G-protein–GPCR interactions as well as antagonists which inhibit such assembly.

17.5 The Future of GPCRs

Although this chapter has focused on the GPCR signaling system for biosensing applications, many other potential biological systems could equally be exploited for biosensing applications, including those involving antibodies, ion channels, and enzymes. We have emphasized that molecular biology, combined with nanobiotechnologies, provides important tools by which every facet of designing and investigating cell-free biosensing approaches can be improved. GPCR and G-protein engineering is a technique which has been employed not only to study GPCR interactions but to enhance the measurement of GPCR activation, which will interface with future biosensing applications. Fusion proteins, promiscuous and chimeric Gα proteins, and molecular tagging are some of the molecular attributes which have been described herein. Structural enhancements to GPCRs and G-protein subunits or effectors are only limited by the creativity of the researcher, and these enhancements will be imperative in the design of novel, cell-free assay technologies. Further research into microarray and chip-based technologies, recombinant protein design and production, assay automation, and new assay methodologies for studying GPCR signaling is rapidly developing. The involvement of GPCR signaling in such a multitude of cellular processes indicates that it is unlikely that the current interest in GPCRs will diminish in the foreseeable future.

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