G-Protein-Coupled Receptors in Drug Discovery: Nanosizing Using Cell-Free Technologies and Molecular Biology Approaches

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Signal transduction by G-protein-coupled receptors (GPCRs) underpins a multitude of physiological processes. Ligand recognition by the receptor leads to activation of a generic molecular switch involving heterotrimeric G-proteins and guanine nucleotides. Signal transduction has been studied extensively with both cell-based systems and assays comprising isolated signaling components. Interest and commercial investment in GPCRs in areas such as drug targets, orphan receptors, high-throughput screening, biosensors, and so on will focus greater attention on assay development to allow for miniaturization, ultra-high throughput and, eventually, microarray/biochip assay formats. Although cell-based assays are adequate for many GPCRs, it is likely that these formats will limit the development of higher density GPCR assay platforms mandatory for other applications. Stable, robust, cell-free signaling assemblies comprising receptor and appropriate molecular switching components will form the basis of future GPCR assay platforms adaptable for such applications as microarrays. The authors review current cell-free GPCR assay technologies and molecular biological approaches for construction of novel, functional GPCR assays. (*Journal of Biomolecular Screening* 2005:765-779)

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INTRODUCTION

G-PROTEIN-COUPLED RECEPTORS (GPCRs) that are characterized by their 7 transmembrane ("serpentine") spanning domains are a superfamily of membrane proteins involved in signal transduction. GPCRs can be activated by a wide variety of extracellular stimuli such as light, odorants, neurotransmitters, and hormones. The GPCRs closely associate with the G-protein subunits Gα and the Gβγ dimer, and they activate the G-proteins by promoting binding of guanosine-5′-triphosphate (GTP) to the Gα subunit in exchange for guanosine-5′-diphosphate (GDP).[1] This, in turn, leads to the dissociation of the Gα subunit from the Gβγ dimer, with both Gα and Gβγ subunits activating downstream effectors such as adenylyl cyclases, phospholipases, and ion channels. The cycle of formation and reversible interaction of the signal-transducing protein machinery (i.e., the interaction of GPCR with G-proteins) governs the cascade of intracellular responses. As GPCRs represent one of the largest superfamilies in the human genome, they are implicated in a number of physiological disorders and diseases. GPCRs represent a significant target for pharmaceuticals and drug discovery programs, as illustrated by the fact that approximately 60% of all drugs currently in development target GPCRs.[2] Of the 747 predicted human GPCRs, 380 are thought to be chemosensory receptors, with the remaining 367 GPCRs predicted to bind endogenous ligands, with many of these (up to 140 in April 2004) being classified as orphan GPCRs.[3]

Assays for screening the binding of ligands to GPCRs can be broadly classified into whole-cell assays and cell-free assays.[4] In cell-based systems, the (fluorescence) output is usually some downstream signaling event monitored in (multi) well-plate format.[5-10] Assay choice for cell-free GPCR systems is rather limited as downstream targets (e.g., adenylyl cyclase activity or $[Ca^{2+}]_i$) are usually not available in such preparations. New technologies associated with cell-free drug screening may involve increased

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throughput, increased information content, miniaturization, and advances in signal generation and will likely involve fluorescence-based signal technology.

Milligan and Rees[11] listed 6 important attributes for drug screening assays: 1) GPCR pharmacology not affected by molecular modifications, 2) low cost for assay development and reagents, 3) amenability to automation, 4) amenability to miniaturization, 5) appropriate statistical rigor (e.g., signal-to-noise ratio and Z factor), and 6) nonradioactive. It is noteworthy that radioligand assays still appear useful for the drug discovery process[12] as a result of adaptation of new imaging devices and technologies that greatly enable low-volume, high-throughput screening (HTS) such as ImageFlashPlates.[13] In terms of assay miniaturization and cost reduction via microarrays, biochips, or suspension beads, additional characteristics are the design of homogeneous (i.e., "mix and read") and cell-free formats.

This review focuses first on possible cell-free approaches for studying GPCR signaling complexes applicable for biosensor and HTS programs. Molecular biology approaches involving G-proteins and GPCRs are then discussed in the context that this technology has significant impact on the development of HTS via its ability to produce novel proteins in relatively high yield and purity, and this advance complements the development of cell-free GPCR assay formats.

MEASUREMENT OF GPCR SIGNALING

Requirements for GPCR assay measurements

HTS has become an integral part of the drug discovery process,[7] with the driving force toward assay automation, simplification, cost reduction, and miniaturization being the need to screen larger and more diverse compound libraries. HTS assays must be sensitive and robust to accurately identify active compounds without generating false-positive data while maintaining the necessary design features required for high throughput. HTS assays should be developed, optimized, and validated with regard to parameters related to assay robustness, including the signal-to-background ratio, the standard deviation and coefficient of variation, and particularly the Z factor.[14] These statistical factors must also be satisfied when designing novel, cell-free technologies for increasing screening throughput, which may rely on less conventional miniaturized assay formats than well-plate-based technologies.

Levels of GPCR signal measurement in cell-free assays

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 review, we refer to these as "levels" of GPCR activation, as shown in Figure 1.

Level 1 assays—ligand binding

Fluorescence and fluorescence polarization (FP) assays (Fig. 1) offer a nonradioactive alternative to the traditional radioligand binding assays.[15-17] According to the criterion set for intensity-based assays, a signal/noise (S/N) ratio of 10 or greater is required for acceptable performance. FP assays can result in Z-factor values in excess of 0.5, mainly due to the homogeneous "mix-and-read" nature of FP assays,[18-20] allowing such assays to be readily transferable from the assay development stage to HTS formats. Although FP assays of ligand binding to GPCRs offer both a homogeneous format and acceptable Z-factor values,[21] such assay formats lack adaptability to all GPCR ligands, as only a small number of ligands can be chemically tagged with fluors of sufficient intensity and polarizing properties.[20] Furthermore, true K_d estimates can also be compromised when using FP assays due to differences in the fluorescence intensity of the ligand when in the bound and free states.[5] Recently developed alternatives to FP assays use fluorescence intensity distribution analysis (FIDA) and fluorescence correlation spectroscopy (FCS).[22-24] FIDA assays are amenable to HTS and have been used with endothelin, chemokine, and β_2-adrenergic receptors. FIDA assays are sensitive to lower levels of receptor expression and require no separation step of bound from unbound (fluorescent-labeled) ligand,[22,23] but their requirement that the ligand be labeled with an appropriate fluorophore limits their use to only a subset of GPCR ligands that can be chemically tagged.

Martinez et al[25] used total internal reflection fluorescence (TIRF) to show ligand binding to a biotinylated neurokinin-1 GPCR following immobilization of receptor-containing membrane fragments onto a quartz sensor surface coated with streptavidin. Interestingly, this receptor complex did not require detergent solubilization or reconstitution to bind the fluorescently labeled agonist, substance P. The surface immobilization and uniform orientation of the receptor-containing membranes on the support matrix were template directed via the high-affinity biotin-streptavidin linkage. The highly selective TIRF detection technology was able to resolve the binding of 1 attomol of receptor molecules.[25] An alternative approach involved the ligand-specific capture of solubilized β_2-adrenergic receptors (β-AR) using dihydroalprenolol-conjugated dextran beads.[26] A screening assay for ligands (agonists or antagonists) could then be performed using a flow cytometry-based system by having the captured β-AR expressed as a fusion protein with green fluorescent protein (GFP). Another useful bead-based approach used paramagnetic beads (Dynabeads M-280), which were coated with a surface built up of captured CCR5 receptors.[27] In this system, the CCR5 receptor was tethered via an antibody (directed at the CCR5 receptor) conjugated to the paramagnetic beads and therefore was not free to move laterally in the host bilayer membrane. The resulting "paramagnetic proteoliposome" was quite stable and was used in fluorescence-activated cell sorting (FACS) and competition as-

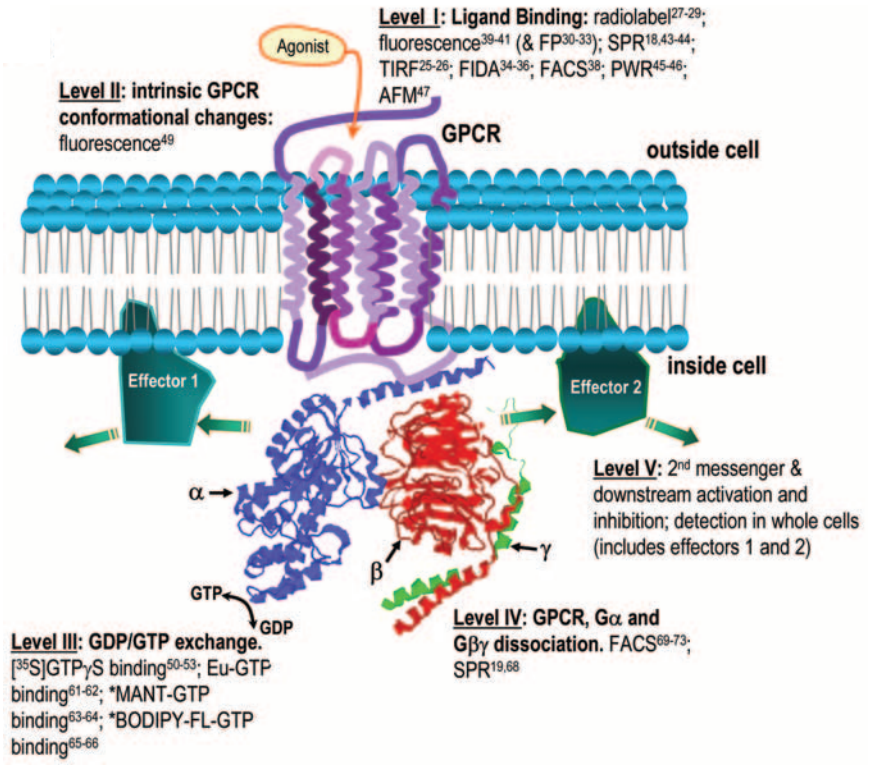

FIG. 1. Schematic representation of the G-protein-coupled receptor (GPCR)/G-protein signaling complex. The "levels" of signaling that may be exploited for detection in a cell-free assay are also shown (only levels 1-4 are discussed in this review). The GPCR is shown as a schematic 7-transmembrane spanning protein, with the associated G-proteins, Gα, Gβ, and Gγ. The Gα and Gβγ subunit ribbon structures were created using "Protein Explorer." The size of the GPCR, G-proteins, effector proteins, and lipid bilayer is not drawn to scale. References to each of the technologies are shown in superscript. AFM, atomic force microscopy; BODIPY-FL-GTP, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene labeled GTP; Eu-GTP, europium-labeled GTP; FACS, fluorescence-activated cell sorting; FIDA, fluorescence intensity distribution analysis; FP, fluorescence polarization; GDP, guanosine diphosphate; GPCR, G-protein-coupled receptor; GTP, guanosine triphosphate; MANT-GTP, *N*-methyl-3′-*O*-anthranoyl-labeled GTP; PWR, plasmon waveguide resonance; SPR, surface plasmon resonance; TIRF, total internal reflection fluorescence. *Indicates that these fluorescent probes have been used for investigating G-protein interactions only.

says, with the paramagnetic properties of the proteoliposomes facilitating rapid buffer/compound exchange as a potential screen for inhibitors generated from a recombinant phage display library.[27]

The intrinsic difficulties in producing, purifying, and manipulating membrane proteins have delayed their introduction into microarray platforms, and hence there are no reports describing purified membrane protein (GPCR) microarrays and their use in functional screening. However, as a 1st step toward such assay analysis, researchers at Corning Incorporated (New York, NY) recently have described the fabrication of GPCR arrays for the screening of GPCR (fluorescent-labeled) ligands.[28-30] Modification of surfaces (mainly glass) with γ-aminopropylsilane allowed the capture and immobilization of a membrane containing a GPCR-G-protein complex, resulting in microspots of approximately 100 μm diameter. The β_1, β_2, and α_{2a} subtypes of the adrenergic receptor as well as the neurotensin-1 receptor and D1-dopamaine receptors were immobilized with this procedure. Atomic force microscopy (AFM) was used to demonstrate that the height of the supported lipid bilayers was approximately 5 nm, corresponding to GPCRs confined in a single, supported lipid layer scaffold (also see Edwardson and Henderson,[31] Santos and Castanho,[32] and Fotiadis et al[33] for other AFM examples). Dose-response curves gave IC_{50} values in the nanomolar range, suggesting that the GPCR-G-protein complex was largely preserved in the microspot.[28,30] Furthermore, there was no change in the performance of the arrays over a 60-day time period, indicating good long-term stability. This procedure does not allow a functional (signaling) assay to be performed, which could distinguish compounds as agonists, antagonists, partial agonists, or inverse agonists. However, the above format could, with appropriate signal detection methods, be a precursor for "functional" cell-free GPCR assay platforms.[34]

In another study, the chemokine receptors (CXCR4 and CCR5) from crude cell preparations were captured onto a biosensor surface using a monoclonal antibody.[35] Conformationally dependent antibodies were then used to determine the conformational states of these receptors using surface plasmon resonance (SPR; see www.biacore.com for a detailed description of SPR). In addition,

SPR has been used to study interactions between GPCR and G-proteins[36] as well as GPCR and ligand.[35] This latter approach is limited to those GPCRs that have cognate ligands of relatively high molecular weight and that are not sterically hindered by streptavidin or antibody anchoring to a surface.

Cell-free signaling by the GPCR, rhodopsin, has been investigated using SPR by coupling rhodopsin to a carboxymethyl-modified, dextran sensor surface via amine linkage.[37] Mixed detergent/lipid micelles facilitated the formation of a lipid bilayer around the immobilized rhodopsin. Light activated the dissociation of the G-protein transducin subunit from the rhodopsin receptor complex, indicating that the detergent solubilization procedure allowed the retention of functional receptor signaling activity. One disadvantage of the amine coupling procedure used in this study was the lack of control of receptor orientation on the biosensor surface. In another study, ligand binding to surface-attached chemokine receptors (CCR5) was studied using SPR.[38] Crude membrane preparations were fused with an alkylthiol monolayer formed on a gold-coated glass surface. The cognate ligand RANTES was shown to bind with higher affinity than the ligands IL-8 and MCP-2, with the rank-order affinity matching that was obtained using a competition radioligand binding assay. To overcome the problem of receptor orientation to the attached surface, a prior selection protocol was carried out using immunoprecipitation to enrich for proteoliposomes having an externally directed CCR5 carboxyl terminus. In this particular example, SPR had a distinct advantage as a screening tool because the technique could detect the ligand binding without requiring fluorescent labeling or radiolabeling. This allows SPR to be used in complex fluids of natural origin, as well as simplifies and potentially speeds the development of assays. However, the sensitivity of SPR in some circumstances may need to be improved to be comparable with the more traditional techniques such as radioligand binding.

Ligand binding to the β_2-adrenergic receptor has been shown using plasmon waveguide resonance spectroscopy (PWR), which detected, in *s*- and *p*-polarized light, changes in refractive index and bilayer thickness upon ligand binding to the surface-immobilized receptor.[39] Ligands with similar molecular weights were studied to determine structural changes in the receptor that differed depending on whether the ligand was an agonist, inverse agonist, or partial agonist. Other studies with PWR detected conformational changes in a proteolipid membrane containing the human δ-opioid receptor following binding of nonpeptide agonists, partial agonists, antagonists, or inverse agonists.[40] Although the ligands had similar molecular masses, there were distinctly different refractive index changes induced by ligand binding, and these were too large to be accounted for by the added ligand mass alone. Therefore, this methodology may have a future use as a drug development tool, particularly in high-information content-screening protocols or to characterize the kinetics and conformational events associated with the binding of ligands to GPCRs.[41]

Other techniques to determine changes at the receptor-ligand interface ("level 1" assay format) have included AFM, in which Lehenkari et al[42] used a specially modified glass ball tip to investigate the interaction between calcitonin and its cognate type B GPCR (secretin-like receptor) in living bone cells. In terms of high-throughput GPCR ligand screening, AFM appears to fall behind other approaches (such as SPR) owing to a number of factors, including difficulty in achieving pure sample preparations and the relative high cost of equipment. Finally, piezoelectric crystal sensing measures a change in mass on molecular binding to the surface due to a change in resonance frequency of the crystal. This technique has been used in an "electronic nose" application with olfactory receptors.[43] Furthermore, an array of 6 sensor elements could be used to characterize each of 6 tested compounds, and such a sensor panel may have potential for GPCR ligand screening. The use of an artificial nose to mimic the human nose may find wide commercial applications in the near future.

Level 2 assays—receptor conformational changes

An example of what we have termed a *level 2* cell-free assay (see Fig. 1) is evident from a recent study in which β_2-adrenergic receptors (β_2-AR) were immobilized onto glass and gold surfaces.[44] Two approaches for β_2-AR immobilization were successful. First, a biotinylated monoclonal antibody directed against the N-terminal FLAG epitope of the β_2-AR was bound to the avidin (surface) layer. The solubilized β_2-AR (in dodecyl-β-D-maltoside) was then immobilized through its amino-terminal FLAG epitope. In the 2nd approach, a β_2-AR (S8C) mutant was generated and biotinylated at the introduced extracellular cysteine (N-terminus, position 8). In both examples above, the receptors were site-specifically labeled with the fluorophore tetramethylrhodamine-maleimide at Cys265 following further purification. By using these 2 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 β_2-AR and β_2-AR (S8C). Moreover, the agonist-induced signal was large enough to detect using a simple ICCD camera. One advantage of this technique was that specific differentiation of agonists and antagonists could be measured (agonists caused the receptor conformational changes, whereas the antagonist reversed this), which could be useful in drug screening with GPCR arrays. Indeed, this method did not require the formation of lipid bilayers or the use of purified G-proteins or fluorescent ligands to detect receptor activation.

Level 3 assays—GTP binding

Guanine nucleotide exchange is a very early event in the signal transduction process and is also a signaling process generic to all GPCRs (depicted as level 3 signaling in Fig. 1). The use of the [^{35}S]GTPγS binding assay typifies this level of assay format. The [^{35}S]GTPγS binding assay measures the level of G-protein activation following agonist activation of a GPCR by determining the binding of the nonhydrolyzable analog [^{35}S]GTPγS to Gα subunits. It is therefore defined as a "functional" assay of GPCR activation by the cognate ligand. Indeed, ligand regulation of the bind-

ing of [^{35}S]GTPγS is one of the most widely used assay methods to measure receptor activation of heterotrimeric G-proteins, as discussed elsewhere in detail.[45,46] It can also provide the basis for measurement of such pharmacological characteristics as potency, efficacy, and antagonist affinity[46] in cell-free assays and artificial expression systems for GPCRs. However, despite the highly desirable attributes of this method and its widespread use to date, ligand regulation of [^{35}S]GTPγS binding has been largely restricted to the analysis of $G\alpha_{i/o}$ proteins (pertussis-toxin sensitive) and their signaling processes and, to a lesser extent, the $G\alpha_s$ and $G\alpha_q$ families of G-proteins. However, these assays can be problematic for HTS as they are usually not of a homogeneous format (i.e., they require a separation step such as centrifugation or filtration to remove bound from free [^{35}S]GTPγS). To overcome this, recent modifications to the [^{35}S]GTPγS binding assay have included scintillation proximity assay (SPA) technology using wheat germ agglutinin-coated beads to capture GPCR-expressing membranes[47] or a modification of this technique using antibody capture SPA.[48]

The increasing number of assays being used to screen increasingly large compound libraries has led to safety, handling, waste disposal, and cost concerns when using radioactivity-based assays. To partly overcome some of the above limitations, PerkinElmer (www.perkinelmer.com) recently developed a highly sensitive, nonradioactive functional assay to monitor certain GPCR (e.g., motilin, neurotensin, muscarinic-M1, and α_{2A}-adrenergic receptor) activity using time-resolved fluorometry (TRF). This involved the development of a europium (chelate) conjugated with nonhydrolyzable GTP (Eu-GTP). GTP binding could then be measured after including steps to separate bound from free Eu-GTP (chelate) and subsequent detection by time-resolved fluorescence resonance energy transfer (FRET). With this system, it was necessary to use substantially higher GTP concentrations of between 10 and 30 nM to obtain adequate detectable GPCR-induced GTP binding to Gα subunits compared with [^{35}S]GTPγS binding protocols, which routinely use a final concentration of 0.2 nM. The TRF protocol exploits some of the unique properties of the lanthanide chelates (particularly europium and terbium) and has been well developed and characterized. One of the main advantages of using TRF assays is their low background and high signal-to-noise ratios, 2 important factors that must be considered with HTS scenarios. The high signal-to-noise (low background) ratio is achieved by long fluorescence decay of the lanthanide fluors after excitation and allows for time-delayed signal detection to eliminate much of the natural background fluorescence caused by cell debris, plates, reagents, and screening compounds. The TRF procedures are valid for a broad spectrum of biochemical associations, including signaling, protein-protein interactions, protein-DNA interactions, DNA-DNA interactions, and so on.[49-55] Although the TRF assay using Eu-GTP has somewhat similar basic principles to the [^{35}S]GTPγS assay (but without the use of radionuclides), it is a good alternative to the [^{35}S]GTPγS assay.[56,57] However, both assay systems still require separation of the free GTP from the bound GTP (to the Gα subunit), and therefore these assay systems are not homogeneous.

Other fluorescent nucleotide analogs have also been developed as probes of Gα activation. The N-methylanthaniloyl (MANT) conjugates of GTP and GTPγS have been used to study the concentration and kinetics of $G\alpha_{i/o}$ subunits.[58,59] Although the conjugates are commercially available, there are no studies to our knowledge that have used these analogs to directly study GPCR signaling in cell-free formats. In relation to G-protein subunit interactions, it was reported that although the increase in fluorescence on the binding of MANT-GTP to Gα subunits was only about 20% when excited at 340 nm, fluorescence was substantially increased to approximately 370% if tryptophan residues within the Gα protein were excited at 280 nm, allowing for FRET from tryptophan donor residues in the Gα protein to the MANT-GTP acceptor.[58] One of the disadvantages of using the MANT-GTP probes is their inability to allow detection of signaling activity with all Gα families. In addition, this FRET-based assay requires excitation in the UV region, necessitating the use of specialized equipment. More recently, Molecular Probes (www.probes.com) has developed the BODIPY-FL-GTP analog in which the BODIPY fluorophore is attached via the sulfur atom of GTPγS. BODIPY-FL-GTPγS shows varying affinities for some of the G-protein families.[60,61] The visible excitation and emission spectra and high fluorescence levels of these probes permit robust, real-time detection of nucleotide binding to Gα subunits. Thus, these GTP analogs appear to be ideal candidates for HTS, although very high concentrations of BODIPY-FL-GTPγS, as well as Gα proteins, were required to obtain adequate signals.[61] It is quite possible that the ability to conduct such multiwell assays of guanine nucleotide exchange will facilitate the discovery of modulators of heterotrimeric G-protein signaling such as the family of the regulator of G-protein signaling (RGS) proteins.[62] This has been shown recently for RGS14[61] as well as our unpublished observations using RGS4 and BODIPY-FL-GTPγS (with $G\alpha_{i1}$). Considerable further development will be needed to overcome the present requirements of high-probe concentration and high purity of assay components if screening assays based on GTP binding (level 3) are to prove useful in screening and other applications.

Level 4 assays—GPCR:G-protein dissociation

Functional GPCR assays that directly and specifically report G-protein activation by ligand-bound receptors could offer substantial advantages over the existing simple ligand-binding assays,[34] such as investigating the interaction between receptors and their associated G-protein both as a phenomenon per se and as a new pharmacological target. Further advantages in terms of assay throughput and future adaptability to microarray formats could be gained by the attachment of GPCR:G-protein complexes to various surfaces. However, immobilization of proteins via covalent anchoring by C- or N-terminal-reactive groups may result in random

orientation or altered structure of the protein, which may, in turn, prevent optimal interaction with sample molecules due to steric hindrance. To achieve stable receptor immobilization as well as the necessary accessibility of substrates to all the reaction components, Bieri et al[63] used carbohydrate-specific biotinylation chemistry for the appropriate orientation and functional immobilization of the solubilized bovine rhodopsin receptor. This reconstituted GPCR:G-protein (transducin) system provided relatively stable results (over hours), with the added advantage of obtaining repeated activation/deactivation cycles of the GPCR:G-protein system under study. Measurements were taken using a self-assembled monolayer, which included streptavidin directed to the biotinylated rhodopsin receptor with SPR being used to directly detect dynamic changes in the association/dissociation of the G-proteins as a functional output of receptor activation. Although SPR is useful to study G-protein interactions, it may not be well suited to directly detect the binding of small-ligand molecules, as it is only sensitive to changes in mass concentration. However, it does offer the advantage of being able to report on repeated activation/deactivation cycles of GPCRs so as to facilitate the serial testing of different compounds with the same receptor preparation. In that regard, the approach offers promise for future applications of chip-based technologies in the area of GPCR assay and screening development.

Another example of a GPCR assay format that relied on GPCR:G-protein association/dissociation was the study involving the β_2-adrenergic receptor captured via surface-attached G-proteins. 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.[64] Tethered $\beta_1\gamma_2$ subunits were then used to capture $G\alpha_s$ subunits that, in turn, were capable of binding membrane preparations with an expressed β_2-adrenergic receptor containing a GFP fusion protein; the whole complex being measured using flow cytometry (HyperCyt™). Specificity of β_2-adrenergic receptor binding was confirmed by inhibition of receptor binding by the ligand alprenolol. In addition to the β_2-adrenergic receptor, a similar protocol has been used to quantitatively solubilize and reassemble the (hexahistidine-tagged) N-formyl peptide receptor (FPR) on Ni^{2+}-silica particles using the HyperCyt™ flow cytometry system with dodecyl maltoside as the detergent.[65] The HyperCyt™ flow cytometry system had a sampling rate of approximately 50 to 100 samples per minute, but flow cytometry's greatest advantage in HTS is by virtue of the ability to multiplex in which different molecular assemblies can be made with 1 sample and yet be discriminated by their unique characteristics.[26,64,66] 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 activation kinetics of the ternary complex.[64,67] The study by Simons et al[64] extended the knowledge of ligand-GPCR interactions to involve the G-protein/GPCR/ligand interactions, resulting in a small-volume, homogeneous assay with a bead-based approach to high-throughput flow cytometry. Using such approaches, it may be possible to screen ligands for a known solubilized GPCR or, alternatively, to test which G-proteins preferentially couple with a particular solubilized, reconstituted GPCR. In a similar manner, flow cytometry has been used to assess real-time α_{2A}-AR/G-protein interactions in a complex membrane environment using the α_{2A}-AR expressed in Chinese hamster ovary (CHO) cell membranes.[68] Indeed, HTS and proteomic applications could easily be based on such bead arrays with potential for color-coded particles and multiplexing (e.g., quantum-dot technology). Particle-based screening would be compatible for the identification of agonists promoting assembly of G-protein/GPCR interactions on particles, as well as potential antagonists inhibiting such interactions. Furthermore, this technology would also have potential for use in screening G-protein/RGS protein interactions and inhibitors of those binding events.

In summary, measurement of GPCR signaling has been represented here as 4 different levels in which many different protocols/assays can be used to target the site of interest. Level 1 assays focus on the binding of ligands to the GPCR, but distinguishing between antagonistic and agonistic ligands at this level is problematic, as these assays are limited in their functionality with respect to GPCR activation. Nonetheless, these assays can be relatively high throughput, fluorescent in nature, and quite adaptable for each receptor, making them the perfect 1st port of call for discovery of novel ligands. Conformational changes in the GPCR that can be measured upon agonist induction form the basis of the level 2 cell-free assays. Very few reports of assays of this type have appeared in the literature. We have classified the functional assays directed at the early signal transduction event of GTP binding as level 3 assays. These assays rely on the principle of monitoring GTP exchange with the use of GTP analogs (radioactive and nonradioactive in nature) and have been used quite routinely and for many years. More recently, the use of nonradioactive assays has been advocated because of the associated problems and limitations associated with radioactivity. However, measuring GPCR activation at this level (GDP/GTP exchange) has become quite stagnant in its potential. The next 2 levels downstream further enhance the amount and type of information generated from functional GPCR assays; therefore, they may set up more possibilities for immobilization and subsequent measurement in the development of HTS cell-free assays.

Nanotechnology approaches

Nanotechnology, particularly nanobiotechnologies employing biomimetic principles, would be expected to facilitate order of magnitude changes in the rate of output of information originating from receptor/signaling assay platforms, particularly for GPCRs.[69] Biochip, microchip, microarray, and suspension microbeads all describe structures that can host greatly increased numbers of reac-

tion centers for binding and signaling events due to their potential for increasing the surface density, surface area, and degree of miniaturization of any platform.

PROTEIN ENGINEERING IN GPCR SIGNALING

Receptor engineering

Molecular engineering of GPCRs, producing receptors that have modified properties, is crucial for the production of cell-free assays. Modifications include the tagging of receptors with peptides designed to allow targeted fluorophore binding, to enhance purification, or to facilitate attachment to a specific surface. Ligation of DNA sequences for GPCRs and interacting proteins, creating fusion protein constructs, has also proved to be useful. An early example was a β_2-adrengeric receptor-Gα_s fusion used to stimulate adenylate cyclase in a mutant cell line lacking expression of Gα_s.[70] Various GPCR-Gα fusion proteins have since been used to elucidate aspects of GPCR pharmacology (see reviews[71,72]). The main advantage of GPCR-Gα fusions is the resultant 1:1 stoichiometry, allowing comparisons between expression systems, although this ratio may not correctly represent the situation in vivo.[71] Other examples of fusion proteins (recently reviewed[73]) include fusion of RGS proteins to GPCRs to examine RGS function.[74] In cell-free screening, regulatory proteins (such as RGS and AGS) may be useful for optimizing receptor signaling response. PCR-based mutation, altering specific amino acids in receptor coding sequences, has also been widely applied to determining aspects of GPCRs signaling,[75] with comparative molecular modeling used to identify important amino acids as mutable targets.[76]

Another approach (used widely in cell-based screens and real-time measurement of cellular protein distribution[77]) is to fuse fluorescent proteins to GPCRs (and interacting proteins) that are able to support FRET/bioluminescence resonance energy transfer (BRET) interactions. For example, Vilardaga et al[78] reported that α_{2A}-adrenergic receptor or parathyroid hormone receptors fused to a cyan fluorescent protein (CFP) donor at the 3rd intracellular loop and a yellow fluorescent protein (YFP) acceptor at the C-terminus were amenable for detection of intramolecular FRET upon receptor-agonist binding. Data suggested a similar conformational change in GPCR structure for each receptor, perhaps indicating that GPCRs undergo generic conformational changes upon activation. At present, only 2 acceptor/donor BRET pairs are widely used to monitor the effect of ligand binding on GPCR activity.[8] The need for downstream cellular processing currently limits the use of BRET in cell-free signaling, but it is likely BRET will become increasingly important in cell-free systems as new donor and acceptor molecules are discovered or engineered.

Engineering of guanine nucleotide-binding subunits

A major impediment to the production of generic, cell-free GPCR screening systems is the tight control of coupling between a given receptor and a subset of Gα subunits. This specificity, crucial for correct cellular signaling, is maintained even between receptor subtypes.[79] As "promiscuous" Gα subunits (coupling to a wide range of receptors) would be an important tool for HTS,[80,81] molecular approaches have been used to increase the promiscuity of various Gα subunits, often by altering amino acids at either terminus.[82-87] Conklin et al[82] showed that replacing the 5 C-terminal amino acids of Gα_q with the corresponding Gα_i residues resulted in a chimeric subunit, allowing some Gα_i-linked receptors to signal through PLC-β. Similarly, replacement of the 5 C-terminal amino acids of Gα_q with those from Gα_s conferred an ability to signal through PLC-β on several GPCRs normally coupling to Gα_s.[83] The same study also showed that the Gα_q-coupled receptors, bombesin and V1a vasopressin, could stimulate adenylate cyclase by coupling to a modified Gα_q subunit containing the 5 N-terminal amino acids from Gα_z in place of wild-type residues. The N-terminus of Gα_q has also been modified to enhance promiscuity. Deletion of the 6 N-terminal amino acids produced a subunit allowing transduction of signaling through PLC-β and inositol (1,4,5)-trisphosphate by G_i- and G_s-linked receptors (sst_1 and β_2-adrenergic, respectively[84]).

Many engineered promiscuous α subunits are based on human Gα_{16} (a member of the Gα_q subfamily), first isolated from hematopoietic cells[88] and shown to inherently couple to a wider range of receptors than other α subunits, mediating signaling via PLC.[80,81,89-91] Promiscuity of human Gα_{16} was further increased by creating chimeric α subunits with wild-type C-terminal amino acids replaced by corresponding residues from rat Gα_s or Gα_z.[85,87] The fact that Gα_{16} can inhibit signaling of some Gα_q-coupled receptors (e.g., thyrotropin-releasing hormone receptor[81]) means that several promiscuous Gα subunits will probably be required for coupling with all GPCRs. Another complicating factor is that some receptors couple to a wide range of Gα subtypes, eliciting different cellular responses. Thus, cell-free assays may require flexibility, allowing some elements to be varied to detect the full array of responses of receptors to a given ligand. However, it is conceivable that 1 or several modified Gα subunits may be developed to couple to most GPCRs, allowing for development of generic cell-free signaling assays. In cell-free systems, labeled promiscuous G-proteins (possibly with additional mutations to alter function) may be important as generic components that are monitored to maintain assay integrity.

Expression systems for recombinant GPCRs/G-proteins and their regulators

A prerequisite for the molecular engineering of proteins for cell-free GPCR assays is an expression system producing high levels of functional recombinant proteins. Various expression systems are used, although eukaryotic cell types (such as insect or mammalian) provide the most promising results in terms of functional GPCR production.[92-94] Expression systems using bacterial, yeast, mammalian, or insect cells are discussed below, as these systems are generally well characterized and show the greatest prom-

ise for producing large amounts of functional proteins that can be purified for use in HTS cell-free GPCR assay formats.

Bacteria

Bacterial expression is widely used as it is cheap and easy to scale up, and large quantities of protein are produced quickly. However, disadvantages (due to the prokaryotic nature of bacteria) may include low expression levels, truncated protein forms, and insufficient posttranslational modifications such as glycosylation or acylation (both important for GPCR and G-protein function).[92] A further problem is that membrane receptors are often toxic to bacteria, leading to reduced yield.[94] The human adenosine A_{2a} and various human opioid (γ, κ, and μ) and rat neurotensin receptors have been successfully overexpressed in *Escherichia coli*,[95-97] although purified receptors were useful for structural or ligand-binding studies but not for signaling assays. A new approach to producing correctly folded bacterial GPCRs, mainly for determination of 3D crystal structure, has been developed (www.m-phasys.com). This technology (M-FOLD™) relies on a novel in vitro refolding method for detergent-solubilized GPCRs, produced as occlusion bodies in recombinant bacteria, and may become important for the preparation of purified functional GPCRs for use in cell-free HTS assays.

Yeast

Several species of yeast have been used to successfully produce GPCRs such as the serotonin 5-HT_{5A}, α_2-adrenergic, β_2-adrenergic, and dopamine D_2 receptors, each expressed at levels exceeding 10 pmol/mg membrane protein.[92] Advantages of yeast expression include ease of producing large-scale cultures and posttranslational modifications that are closer to those produced by mammalian cells. However, the presence of a cell wall may hinder recovery of cellular proteins.

Mammalian cell culture

Mammalian cell culture is the most widely used expression system and has dual advantages of providing an environment close to that found in vivo and complete posttranslational processing. Thus, receptors may be expressed that not only bind ligands but also will elicit cellular signaling following ligand activation. Many mammalian cell lines are available, including COS (cells from a monkey CV1 cell line), CHO, and human embryonic kidney (HEK), with the latter being amenable to suspension culture.[98,99] Disadvantages of mammalian cell lines in terms of protein production for cell-free assays are the cost and labor involved in their culture and low yields from stably transformed lines.[94]

Various systems have been developed, allowing increased levels of stable or transient expression (inducible and noninducible) in mammalian cells, most often with vectors containing virus-derived genetic elements. A range of adenovirus- and lentivirus-based vectors are available, containing different promoters (or lacking promoters) and allowing flexible mammalian expression of recombinant GPCRs/G-proteins (e.g., ViraPower™ systems by Invitrogen®). Adenovirus infection was used to successfully express κ and μ human opioid receptors, both in their native form and as GFP fusions.[100] Adenovirus infection was also used to reinstate functionality of mutant V2 vasopressin receptors produced in stably transformed CHO cells by expressing a recombinant peptide required for correct function of the mutant V2.[101] A GPCR expressed by Kaposi sarcoma–associated herpesvirus as part of normal infection processes was also expressed in mammalian cells using an adenovirus-based vector.[102] A further example is the use of a replication-deficient lentivirus vector for stable expression of human retinal opsin in COS-7 cells and cell lines derived from human or mouse epithelium.[103]

Several other novel virus-mediated expression systems have been recently developed. An episomal vector based on Epstein-Barr virus was used to overexpress the h5-HT_{1B} neurotransmitter receptor (and a chloramphenicol acetyltransferase reporter) in various mammalian cell lines under control of an interferon-inducible Mx promoter.[104] Furthermore, Semliki forest virus (SFV)–derived vectors have been used to functionally overexpress more than 100 GPCRs, including several human α_{2B}-adrenergic receptor-based constructs (including a GFP fusion) in CHO cells.[99,105] SFV is also being heavily used by the recently established Membrane Protein Network (MePNet) structural genomics initiative.[99,105] Baculovirus vectors have also been developed for gene delivery into mammalian cells, whereby the baculovirus polyhedrin promoter (specific for expression in insect cells—see below) has been replaced by a mammalian cell promoter (e.g., BacMam™ by Novagen). Although not widely used to date, the application of such a vector for the production of GPCRs for use in HTS assays appears promising.[106] BacMam™ vectors were used to functionally express the 5 muscarinic acetylcholine receptor subtypes (M1-M5), P2Y receptors (P2Y1 and P2Y2), and several LPA receptors (EDG-2 and EDG-7) in human U-2 OS osteosarcoma cells.[107]

Baculovirus expression in insect cells

Baculovirus/insect cell expression (usually based on the *Autographa californica* multiple nucleopolyhedrosis virus [AcMNPV]) is routinely used for GPCR production (see review[108]) as it is a highly characterized system that overcomes many disadvantages of mammalian and prokaryotic systems. Two basic cell lines (*Sf* 21 and *Sf* 9) are most commonly used,[108] although various cell lines are available, optimized for either increased production of secreted proteins (High Five™) or improved glycosylation (Mimic™ *Sf* 9). The high level of characterization of the AcMNPV system has allowed the production of kits providing efficient production of recombinant baculovirus DNA (e.g., Bac-to-Bac® and BaculoDirect™ from Invitrogen). A range of baculovirus construction vectors is available, allowing for addition of purification tags (e.g., polyhistidine, FLAG) or expression of multiple genes. The main advantages of baculovirus expression are the following: insect cells grow easily in suspension and are readily adaptable to serum-free conditions, posttranslational modifications are close to wild type, no special biosafety measures are required, and very

high expression levels are generated by the viral polyhedrin promoter.[109] *Sf* 9 cells can also be used for stable expression of recombinant proteins, usually under control of baculovirus immediate-early promoters (IE1, IE2), which drive expression much earlier after infection compared to the polyhedrin promoter.[108,110] This technique was used to stably express human β_2-adrenergic and μ-opioid receptors.[108] Although expression levels are significantly lower under IE1 control (compared to the polyhedrin promoter), this approach has advantages such as continuous expression and no requirements for viral inoculum production.

Correct posttranslational modifications are important for correct folding, solubility, stability, and expression of GPCRs. Major posttranslational modifications to GPCRs are phosphorylation of the carboxyl tail and 3rd intracellular loop, covalent attachment of palmitate to cysteine residues (palmitoylation), and N-glycosylation of asparagine residues.[111] Although not as efficient as mammalian cells for N-glycosylation, insect cells efficiently perform phosphorylation and palmitoylation, known to play an important role in the correct association of GPCRs with cell membranes.[108,111] The main disadvantage of baculovirus expression is that viral infection causes cell lysis after 4 to 5 days of culture, leading to degradation of expressed membrane proteins.[108]

Several unique features of baculovirus infection can be exploited to display functional GPCRs on the viral surface or to increase the level of GPCRs inserted into the cell membrane. During baculovirus infection, a viral protein (gp64) is expressed and targeted to the cell membrane (via a signal sequence).[110,112] Fusion of the gp64 signal sequence to the N-terminus of GPCRs was used to increase expression levels of several human receptors, including μ-opioid, 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{5D}.[113-115] Furthermore, several receptors (including β_2-adrenergic, muscarinic M_1, and dopamine D_1) have been recovered from the budded virus surface, with all recovered receptors being fully processed and functional.[116,117] Interestingly, addition of cleavable N-terminus signal sequences (derived from influenza hemagglutinin and *Apis mellifera* mellitin) proved successful in increasing baculovirus expression of human β_2-adrenergic[118] and dopamine D_{2S}[119] receptors, respectively.

Engineering of protein tags and fluorophores

Traditional labeling methods use compounds that covalently link to amino acid residues, producing labeling with limited site specificity. However, improvements to labeling specificity can be achieved by creating specific fusions (with protein fluorophores) or by the addition of a defined peptide acting as a site for labeling (protein and other label classes) by specific enzymes or spontaneous affinity binding.[120,121] Discussed below are various site-specific labeling strategies that may be useful in cell-free HTS assays.

Polyhistidine

Hexahistidine sequences have been commonly used for purification of tagged proteins due to the high affinity of the aromatic ring in histidine residues for Ni^{2+} (and Co^{2+}), which are usually chelated by nitrilotriacetate (NTA) to provide a binding matrix. Such binding can be reversed by free histidine or imidazole. Many GPCRs have been purified using this technology, a recent example being the overexpression and purification of large amounts of pharmacologically viable histamine H1 receptor, containing 10 histidine residues fused to its C-terminus.[122] Purification techniques, allowing enrichment of overexpressed proteins, are crucial in obtaining sufficient recombinant protein for cell-free HTS screening systems. Ni^{2+}-histidine affinity is also a means by which tagged proteins may be tethered to surfaces for production of microarrays or bead-based assays, using readily available technology.[123]

Oligohistidine sequences are also useful, reversible site-specific tags for labeling by NTA-fluorophore conjugates. This approach was first used in vitro to modify GFP such that its fluorescence was quenched (via FRET) upon fluorophore binding.[121] A similar approach was used to label the $5HT_3$ serotonin receptor (not a GPCR) at several sites, allowing study of FRET interactions between the receptor and a fluorescine-labeled antagonist.[121] Thus, existing expression vectors may be used to produce recombinant proteins that can be purified, fluorescently labeled at a known site, attached to surfaces, and used in cell-free systems to infer aspects of GPCR activity, such as conformational changes within or between GPCRs and interacting proteins.

Biotin

Biotin is often used for biotechnology applications due to its extremely high affinity for the specific binding proteins, avidin and streptavidin,[124] which are often used to coat surfaces for capture of biotin-labeled proteins.[25,44,63] Recently, site-specific monobiotinylation has been accomplished by "AviTag" peptide technology (Roche Diagnostics). The tag is inserted into the protein at a site where labeling is desired, and in the presence of biotin and a bacterial biotin protein ligase, biotin is covalently attached to a lysine residue within the AviTag sequence, in an adenosine triphosphate (ATP)–dependent manner.

Green fluorescent protein

GFP is often used in molecular/cell biology applications due to its inherent ability to perform as an internal fluorophore with efficient emission properties (Table 1). GFP can be used passively as a reporter of gene expression, protein production, and/or protein localization or actively as an indicator of metabolic changes.[125] GFPs are particularly useful as they do not require substrates, external catalysis, or accessory cofactors for fluorescence to occur, as do many other natural pigments.[126] GFP fusions have been used for studies of protein dynamics and localization within living cells and to study GPCR/heterotrimer binding in a cell-free context.[26] GFPs have traditionally been limited by an emission maxima extending to only 529 nm and by their large size, leading to problems with correct folding of fused proteins and possible steric hindrance of interactions being analyzed.[127] However, increased knowledge of GFP crystal structures has allowed production of GFP mutants

Table 1. Molecular Engineering of Fluorescent Tags

Fluorescent Tag	*Examples*	*Characteristics*	*Uses*
GFP[26,95,125-128]	GFP, PA-GFP	Relatively large fusion proteins, green fluorescent proteins	Internal fluorophore, GPCR dimerization, good FRET/BRET partners, measurement of intramolecular changes
Anthozoan fluorescent proteins[126,129-134]	AGFP, AYFP, ARFP, CP, asCP, KFP1, DsRed	Fusion protein, multiple colors available, nonfluorescent forms, kindling proteins, often large proteins	Fluorescence quencher, FRET partner, can be irreversibly converted to bright-red form
Biarsenical ligands[127]	FlAsH, ReAsH-EDT_2	Small peptide sequence (tetracysteine) can be expressed anywhere in protein	Site-specific capture point for biarsenical fluorescent ligands, fluorescence quenching of GFP and YFP
Lanthanide probes[51,135,136,139]	Eu^{3+} and Tb^{3+} binding motifs, LBTs	LBTs bind Eu^{3+} and Tb^{3+}, long period of luminescence, small peptide sequence	Time-resolved fluorometry, LRET, intramolecular distance determination

AGFP, anthozoan green fluorescent protein; ARFP, anthozoan red fluorescent protein; AYFP, anthozoan yellow fluorescent protein; PasCP, a mutant CP; BRET, bioluminescence resonance energy transfer; CP, chromoproteins; DsRed, a mutant CP; FlAsH, fluorescein-containing As(III) substituents; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; KFP1, a mutant CP; LBT, lanthanide binding protein; LRET; luminescence resonance energy transfer; PA-GFP, a GFP mutant; ReAsH-EDT2, red analog of FlAsH; YFP, yellow fluorescent protein.

(usually by random or directed mutagenesis) with useful characteristics, including increased folding under adverse assay conditions and/or shifted wavelength maxima (producing different-colored GFPs).[125] For example, a recently produced GFP mutant (PA-GFP) can be activated by blue and UV light.[128] Development of efficient, flexible cell-free assays would be aided by the availability of a diversity of fluorescent proteins, covering a range of absorption/ emission wavelengths.

Anthozoan GFP-like proteins and nonfluorescent chromoproteins

Useful fluorescent proteins have been isolated from anthozoan corals, with Verkhusha and Lukyanov[126] placing them into 4 broad spectral groups: anthozoan green fluorescent proteins (AGFPs), yellow fluorescent proteins (AYFPs), red fluorescent proteins (ARFPs), and nonfluorescent chromoproteins (CPs) (see Table 1). ARFPs are useful as they are good FRET acceptors when combined with AGFP.[126] ARFPs have also been paired with organic dyes (suitable for FRET) and insect luciferases (suitable for BRET).[126] CPs may act as quenchers in FRET interactions as they have characteristic single-absorption maxima ranging from 560 to 590 nm but emit low levels of light.[126,129] Quenchers are useful for fluorescence-based assays in which separation of fluorophore- and CP-labeled peptides results in production of measurable FRET (a "lights-on" assay), which is desirable in most situations, rather than decreasing FRET (a "lights-off" assay). CPs may also become a source of novel fluorescent proteins in that mutation of CP amino acids may produce fluorescence attributes not seen in wild-type molecules. An example is the creation of far-red fluorescent proteins by mutagenesis of CPs from several anthozoans.[129]

Molecular engineering has facilitated production of several fluorescent proteins that are activated by light, providing direct induction of emission by UV irradiation (e.g., Kaede,[130] mcavRFP and rfloRFP[131]) or altered emission by bleaching (e.g., DsRed[132]). A CP known as "asCP" was mutated to produce KFP1, which can be irreversibly photoconverted from a nonfluorescent form to a stable bright-red fluorescent form by green light excitation.[133] This characteristic is described as "kindling"; thus, KFP1 and related mutants are termed *kindling proteins*. Such proteins are likely to be useful labels for the production of lights-on assays. Site-directed mutagenesis was used to determine that amino acids spatially surrounding the Tyr66 residue of asCP were crucial for kindling.[134] Using this information, kindling ability was transferred to other anthozoan CPs, producing variants capable of both reversible and irreversible kindling and that could be kindled by blue light irradiation.[134]

Although anthozoan fluorescent proteins provide many advantages, they are limited in their use as protein labels due to often being large multimeric molecules (e.g., DsRed and kindling proteins are tetrameric)[126,130] and being limited for FRET analyses by generally having broad absorption spectra leading to cross-excitation. These problems are overcome by the production of smaller oligomers or monomers (e.g., dimeric DsRed) and by the choice of FRET pairs with widely differing excitation and emission maxima.[126] Given the potentially huge array of unknown, naturally occurring fluorescent proteins and CPs, combined with protein engineering techniques used to produce novel fluorescent proteins or quenchers, it is conceivable that FRET/BRET assays involving these compounds will increase in efficiency and flexibility, becoming the method of choice for measurement of interactions with cell-free systems.

Biarsenical fluorescent ligands

Tetracysteine motifs, consisting of any 2 noncysteine residues flanked on each side by 2 cysteine residues, act as specific sites of attachment for biarsenical ligands that fluoresce only after their arsenic groups are bound to cysteine thiols.[127] Therefore, the recognition motif can be expressed internally in the protein or at either terminus, allowing for subsequent labeling of recombinant proteins in cell-free assays (Table 1). FlAsH, consisting of

fluorescein-containing As(III) substituents bound to the 4′- and 5′-positions, was first identified as having high affinity for the attachment sequence.[127] By incorporating FlAsH in a defined series of chemical reactions, both red and blue analogs were produced.[127] The red analog (ReAsH-EDT_2) could quench emission from recombinant GFP or YFP, which had each been engineered to contain a C-terminal tetracysteine sequence. Furthermore, ReAsH-EDT2 underwent sensitized emission at 635 nm, indicating successful FRET.[127]

Directed lanthanide probes

Lanthanide-based probes have been recognized as useful multipurpose protein labels for more than 20 years.[135] Eu^{3+} and Tb^{3+}, when combined with a sensitizing chromophore and placed into an appropriate coordination environment, undergo a relatively long period of luminescence, the energy of which can be transferred to an organic-based acceptor dye.[51,136] This process is termed *lanthanide-based resonance energy transfer* (LRET).[51] Selvin[51] lists several technical advantages of LRET when compared with standard FRET, including larger measurable distances of interaction (> 100 Å), insensitivity to incomplete labeling, greater accuracy, and greater signal-to-background ratio.

Several techniques are available for labeling proteins with lanthanides. The simplest of these is to chemically label single amino acid residues with lanthanide chelates.[137,138] A 2nd method used for directed lanthanide labeling uses motifs based on calcium-binding proteins, which also bind lanthanide ions due to their having ionic radii and coordination preferences comparable to Ca^{2+}.[136] The discovery that mutating the 7th amino acid within a 14-residue calmodulin loop to tryptophan resulted in enhanced terbium luminescence,[139] leading to a series of studies aiming to produce lanthanide binding tags (LBTs) with enhanced Tb^{3+} affinity and luminescence by varying amino acids flanking the calmodulin-based peptide.[135,136] By producing libraries of candidate LBTs combined with iterative screening, LBTs were produced with doubled luminescence,[136] or up to 140-fold increased Tb^{3+} affinity.[135] For cell-free HTS GPCR assay systems, lanthanide-based probes show enormous potential due to the ability to label defined regions within GPCRs (or interacting proteins) and the availability of appropriate receptor molecules providing efficient LRET, allowing elucidation of protein-protein interactions (Table 1). Further advantages of LBTs include their small size (reducing chances of altered functionality of the tagged molecule) and compatibility with polyhistidine tags.[136]

In summary, molecular biology is a means by which many facets of design and implementation of cell-free assays can be improved. GPCR and G-protein engineering have been employed not only to study GPCR interactions but also to enhance the measurement of GPCR activation for improved functional cell-free assays. Fusion proteins, promiscuous and chimeric Gα subunits, and molecular tagging are important molecular approaches that have been described here, each of which has been (and will continue to be) useful for designing novel, cell-free assay technologies.

CONCLUSION

Further research into microarray and chip-based technologies, recombinant protein design and production, assay automation, and new cell-free assay methodologies for studying GPCR signaling are 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 as drug targets will diminish in the foreseeable future.

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