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Subsecond Analyses of G Protein Couple Receptor Ternary Complex Dynamics by Rapid Mix Flow Cytometry

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Abstract

The binding of full and partial agonist ligands (L) to G protein coupled receptors (GPCRs) initiates the formation of ternary complexes with G proteins (LRG complexes). We describe the assembly of detergent solubilized LRG complexes on beads. Rapid mix flow cytometry is used to analyze the subsecond dynamics of guanine nucleotide-mediated ternary complex disassembly. Ternary complexes were assembled using three formyl peptide receptor constructs (wild type, FPR-G α_{i2} fusion, and FPR-GFP fusion) and two isoforms of the α subunit (α_{i2} and α_{i3}) and $\beta\gamma$ dimer ($\beta_1\gamma_2$ and $\beta_4\gamma_2$). Experimental evidence suggests that thermodynamic stability of ternary complexes is dependent on subunit isoform. Comparison of assemblies derived from the three constructs of FPR and G protein heterotrimers composed of the available subunit isoforms demonstrate that the fast step is associated with the separation of receptor and G protein and that the dissociation of the ligand or of the α and $\beta\gamma$ subunits was slower. These results are compatible with a cell activation model involving G protein conformational changes rather than disassembly of G $\alpha\beta\gamma$ heterotrimer.

Introduction

GPCR Biology

G Protein Coupled receptors (GPCRs) belong to the largest family of transmembrane signaling molecules. G proteins are comprised of 27 α , 5 β , and 13 γ subunit proteins that combine to form functional heterotrimeric units (Sprang et al., 2007). The functional proclivities of the various combinations of α , β and γ heterotrimers is dictated by subunit isoform (Robishaw and Berlot, 2004). Interaction of ligand-stimulated GPCRs with specific heterotrimeric G proteins triggers the exchange of GDP for GTP at the nucleotide binding pocket of G α subunits resulting in dissociation (Oldham and Hamm, 2007; Oldham and Hamm, 2008) or rearrangement (Lohse et al., 2007a; Lohse et al., 2007b) of the G α subunit from G $\beta\gamma$ enabling the separated/rearranged G proteins to interact with effectors (Oldham and Hamm, 2007; Oldham and Hamm, 2008).

We have used rapid mix flow cytometry measurements on detergent solubilized ternary complexes of {ligand}:{GPCR}:{nucleotide-free G protein}, assembled on beads, to examine the differential kinetics of nucleotide induced dissociation of components (Buranda

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et al., 2007; Wu et al., 2007a). The most significant aspects of this work are: a) the fastest step associated with ternary complex disassembly is the subsecond departure of the receptor from the $G\alpha$ subunit. b) The dissociation of $G\alpha$ from $G\beta\gamma$ occurs on a time scale ($t_{1/2} \approx$ tens of seconds) not relevant to cell signaling for $G\alpha_i$ subunits used in this study. c) In this system GDP was shown to initiate the disassembly of ternary complexes, in a manner nearly analogous to GTP (Wu, et al., 2007a). Because isolated ternary complexes lack the biochemical milieu in which cellular signaling occurs, the results of this study are insensitive to potential collateral effects of downstream contact with effectors (Sprang, et al., 2007), (Sunahara et al., 1997). While it has been generally assumed that neither $G\beta\gamma$ nor $G\alpha$ can interact with effectors, prior to GPCR activation (Sprang, et al., 2007), resting state complexes of $G\alpha_q$ and the effector phospholipase $C\beta$ ($PLC\beta$) (Dowal et al., 2006) have been reported prior to GPCR activation.

It is the intent of this chapter to provide experimental details for the use of modular molecular assemblies of nucleotide-free ternary complexes on beads, and rapid mix flow cytometry to examine the differential kinetics of nucleotide-induced disassembly at specific junction points of the complex. This chapter will focus on the formyl peptide receptor (Buranda, et al., 2007; Wu, et al., 2007a). This receptor system has been well characterized in cells, membranes and in detergent solubilized systems in solution and on beads. Reagents, fluorescently small molecule peptide ligands, (*e.g.* FITC conjugated formyl-Met-Leu-Phe-Lys; fMLFK-FITC), fluorescently labeled antibodies, GFP fusion constructs, and G protein subunits have made this system uniquely suitable as a model system to study on beads.

Analysis of GPCR Function by Flow Cytometry

Introduction

Flow cytometry is a well-established technique for sensitive and quantitative kinetic analysis used in cellular biochemistry involving cell activation, ligand binding, or macromolecular assembly (Seamer et al., 1999; Sklar et al., 2002; Sklar et al., 1998). Because flow cytometers can discriminate between free and cell or particle bound fluorophores, homogenous analysis of real time events and fixed time points can be used to assess ligand/receptor interactions as well as receptor processing and receptor-mediated cell activation without wash steps (Nolan et al., 1999; Sklar, et al., 2002). Generally the behavior of a GPCR that is expressed on the surface of a cell or immobilized on a bead can be probed by: (1) binding of a fluoresceinated ligand; (2) cell expression (or surface coverage on a bead) of a GPCR-GFP fusion, and (3) disassembly of a ternary complex. Because of the ready availability of fluorescent ligands displaying affinities from μM to pM , the FPR is a model system where flow cytometry has been used to study ligand binding on cells, membranes, and detergent-solubilized molecular assemblies (Buranda, et al., 2007)

Standardization of flow cytometry data with fluorescence calibration beads

Standard fluorescence calibration beads are used to quantify the number of receptor sites on a cell or bead and to account for day-to-day variation in bead fluorescence, changes in detector settings and use of different flow cytometers (Wu et al., 2007b). The most common standard calibration beads are commercial standards from Bangs Labs

(www.bangslabs.com) QuantumTM FITC MESF beads. These beads are optimized for fluorescein but can be used for other fluorophores being analyzed under the *same excitation and detection optics* as long as a suitable correction factor is applied to account for the spectroscopic differences between the fluorescein and target fluorophore (e.g. eGFP). The calibration scheme has been recently described in detail (Wu, et al., 2007b). The measured fluorescence intensity of any target molecule is proportional to, $I_0 \epsilon \phi \%T$; where I_0 is the intensity of the light source, ϵ_{ex} is the absorption coefficient of the fluorophore at the excitation wavelength and ϕ is the quantum yield of the fluorophore and $\%T$ is the percent fraction of fluorescence light transmitted by the 530 nm, 30 nm wide bandpass filter (up to 80% maximum transmittance) and is used to account for the spectral mismatch between the sample fluorophore and the fluorescein standard. To apply Quantum FITC MESF beads to eGFP labeled assemblies, an appropriate correction factor (cf) must be applied as shown in Equation 1 (Wu, et al., 2007b):

$$cf = \frac{(\epsilon_{488} \phi \%T)_{eGFP}}{(\epsilon_{488} \phi \%T)_{\text{fluorescein}}} = \frac{56,000 \times 0.60 \times 40}{85,000 \times 0.93 \times 28} = 0.60 \quad (1)$$

Equation 1 corrects for the spectroscopic mismatches in ϵ , ϕ and $\%T$ between the calibration standard beads and eGFP. This formalism is accurate when samples are irradiated with a non-saturating light source. It is worth noting that most fluorescein conjugates tend to have lower quantum yields than the parent molecule where simple derivitization of fluorescein typically attenuates the yield by at least 18% e.g. fluorescein biotin (Buranda et al., 1999) or greater e.g. 80% for FITC antibody conjugates (f/p ratio 4:1) (Piyasena et al., 2004). Furthermore the reader should also be aware of the dependence on pH of fluorescein's extinction coefficient and quantum yield. Thus the quantum yield values used in Equation 1 correspond to values measured for monomeric fluorophores under neutral pH conditions. Most spectroscopic parameters are available at the Invitrogen company website (www.invitrogen.probes.com), through the *Fluorescence SpectraViewer* tool.

Small Volume Rapid-Mix Device Flow Cytometry

General considerations

In this section we detail the essential elements of a rapid mix flow cytometer, which is used to delineate the kinetics of disassembly of the ternary complex upon guanine nucleotide activation (Wu et al., 2005b). Typical kinetic flow cytometry measurements generally include a 5–10s deadtime (the time interval between the start of sample mixing and the point of detection), and consume wasteful quantities of precious reagents (Wu, et al., 2005b). To minimize the dead time, we have established a small volume rapid mix flow cytometry device that is capable of: (a) limiting total dead time to 250ms, (b) minimizing total dead volume to 14 μ L and (c) optimization of the efficiency of sample mixing in the laminar flow regime. Dead volume normally includes the unusable volume of the syringes and the volume lost to the connectors and the valves, which is largely determined by the dimensions of the respective components. The dead volume in the rapid mixing device determines the limiting volume of reagent that can be analyzed. The technical details of this device have been described elsewhere (Wu, et al., 2005b). The prototypical device is optimized to handle $\approx$

20,000–27,000 particles in 35–45 μL aliquots, and then to mix and deliver sample to the flow cytometer using an automated rapid mixing sequence. Only 40% of the beads are actually used in the kinetic analysis. 40% of the beads are lost to dead volume in syringes and valves, and 20% are lost during the initial fast boost phase, which enable the device to overcome the effects of laminar flow in order to attain high enough Reynolds numbers necessary to achieve fluid turbulence for efficient mixing (Ottino, 1990; Peltier and Caulfield, 2003) (see Box 1).

Box 1

Optimizing sample mixing and delivery to the flow cytometer under laminar flow conditions

In small volumes, under the conditions of laminar flow, mixing is largely achieved by diffusion alone and is extremely slow and not practical for subsecond kinetics. The Reynolds number (Re) is often used to determine the physical characteristics of the flow (laminar or turbulent regime) (Brody et al., 1996; Purcell, 1977). For continuous flow in circular tubes, the Reynolds number is defined as:

$$Re = \rho d (4v / \pi d^2) / \mu \quad (2)$$

where v is the velocity of the fluid through the tube (mL/s), ρ is the fluid density ($\approx 1 \text{ g/cm}^3$), d is the diameter of the tube; and μ is the viscosity of the fluid ($\approx 10^{-2} \text{ g/cm}^2\text{s}$). The transition from laminar to turbulent flow generally occurs for Re values between 1,800 and 2,300 and is strongly dependent on the geometry of the system (Ottino, 1990). Transition from laminar to turbulence at relatively low flow rates can be mediated by introducing obstacles or irregularities in the system (Ottino, 1990; Peltier and Caulfield, 2003). 3-way connecting valves used in the rapid-mix device provide the necessary irregularities in the system to achieve efficient mixing.

In the rapid mix device (Figure 1) the passage of fluid streams bearing beads and biotin through valve Y3 was characterized by fluid flow of $Re \approx 3800$ ($d = 0.05\text{cm}$; $v = 2\beta 770\mu\text{L/s}$) where the sample streams were expected to be well mixed. During the *sample delivery* and *fast boost* phases, Re ranged from ≈ 1040 to 3140 ($d = 0.025\text{--}0.075 \text{ cm}$; $v = 416.7\mu\text{L/s}$). At the flow cytometer (post mixing) Re was reduced to ≈ 6 ($d = 0.025\text{cm}$; $v = 1.16\mu\text{L/s}$) to match the required sheath flow rate of $\approx 1\mu\text{L/sec}$. The performance characteristics of the rapid mix device are periodically validated by a rapid kinetics fluorescence based biotin assay (Wu, et al., 2005).

General listing of Materials and Abbreviations

Materials

Plasticware was from VWR (West Chester, PA), and all chemicals and reagents were from Sigma (St. Louis, MO) except where otherwise noted. $6.2 \mu\text{m}$ diameter streptavidin coated polystyrene beads (0.5% w/v) were purchased from Spherotech Inc., (Libertyville, IL). α_{i2} and α_{i3} G-protein subunits were purchased from Calbiochem (La Jolla, CA) and were used

without further purification. FLAG-his tagged $\beta\gamma$ subunits ($\beta_1\gamma_2$ or $\beta_4\gamma_2$) were prepared and purified as described previously (McIntire et al., 2001; Simons et al., 2003a). The subunits were stored at -80°C in 2–5 μL volume aliquots depending on stock concentration (3–7 μM). Membrane fractions from U937 cells expressing FPR (R), FPR-GFP (R^{F}), or FPR-G α_{i2} fusion protein (R-G α_{i2}) were prepared as described elsewhere (Simons, et al., 2003a) and used without further purification. The following buffers were used: HPSM (pH7.5, 30 mM HEPES, 100 mM KCl, 20 mM NaCl, 1 mM MgCl_2), DHPSM (HPSM with 0.1% n-dodecyl- β -D-maltoside, DOM), G-buffer (HPSM with 0.1% DOM and 1 mM dithiothreitol, DTT), and AMF solution (20 μM AlCl_3 , 10 mM MgCl_2 and 10 mM NaF).

Abbreviations

GPCR, G protein-coupled receptor; FPR, formyl peptide receptor; α , G α subunit; β , G β subunit; γ , G γ subunit; $\beta\gamma$, G $\beta\gamma$ dimer, $\alpha\beta\gamma$; G $\alpha\beta\gamma$ heterotrimer; GFP, green fluorescent protein; GTP γS , guanosine 5'-O-(3-thio)triphosphate; FITC, fluorescein isothiocyanate; L, ligand; fMLFF, formyl-Met-Leu-Phe-Phe; L^{F} , fluorescent ligand; fMLFK-FITC, FITC conjugated formyl-Met-Leu-Phe-Lys; R, receptor; R^{F} , fluorescent receptor; FPR-GFP, FPR-GFP fusion protein; R-G α_{i2} , FPR-G α_{i2} fusion protein; G, G protein or heterotrimeric regulatory of G protein; LR, ligand-receptor complex; RG, receptor-G protein complex; LRG, ligand-receptor-G protein complex; DOM, n-dodecyl- β -D-maltoside; DTT, dithiothreitol; BSA, bovine serum albumin; FLAG, FLAG peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Lys.

Optimizing for Analysis of Molecular Assemblies by flow cytometry:

Receptor affinity

In order to establish a rational approach to molecular assemblies on cells or beads, it is important to determine the affinity of the fluorophore-derivatized ligand. Affinity measurements are typically measured by generating equilibrium binding curves, which if necessary can be augmented by kinetic measurements of binding and dissociation rate constants (Buranda, et al., 2007). Poor affinities (K_d values greater than approximately 50 nM) and high reagent concentration can lead to excessive background (non-specific) fluorescence signals. The ability of the flow cytometer to discriminate between bound and free ligands may be compromised when the concentration of soluble ligand exceeds $>100\text{nM}$. Nonspecific binding or fluorescent ligand dissociation is determined by incubating the cells or beads with a large excess (i.e. $100\text{--}1000 \times K_d$) of site saturating non-fluorescent ligand. Alternatively, nonspecific binding can be analyzed by incubation of the parental cells, which lack the transfected receptor, or beads, which lack the cognate receptor, in the presence of fluoresceinated peptide alone (Gilbert et al., 1999; Simons et al., 2003c). Affinity measurements can also be quantitatively evaluated by spectrofluorometry, for FPR (Sklar et al., 1981), (Sklar et al., 1985), (Simons, et al., 2003a). The determination of FPR ligand affinities can be accomplished through a soluble ligand competition assay as described in this ref (Simons, et al., 2003a).

Modular Molecular Assemblies of GPCR ternary complexes on beads

Materials

Frozen cell membranes (500 μ L aliquots), HPSM buffer, syringe with 25- gauge needle, and 25% DOM.

The cloning of the FPR, its expression in U937 cells, generation of FPR-G α_{i2} and FPR-GFP fusion constructs and membrane preparations have been described elsewhere (Simons, et al., 2003a). Briefly, wild type FPR receptors and fusion constructs are over expressed (up to 500,000 receptors/cell) in U937 cells. Expression levels are optimized by sorting on the flow cytometer. The cells are lysed by nitrogen cavitation, and crude post-nuclear membrane preparations are stored at -80° C in aliquots of 500 μ L, corresponding to 10^8 cell equivalents.

Solubilization of FPR

A typical membrane solubilization preparation involves the following steps:

1. Add 700 μ L of HPSM buffer to an aliquot of thawed membranes.
2. Centrifuge sample in a microcentrifuge tube for 15 min and remove supernatant, and resuspend the pellet in 220 μ L of HPSM using a syringe attached to a 25-G needle.
3. Add 25 μ L of 10% dodecyl maltoside, and 2.5 μ L of 100 $\times$ protease inhibitor cocktail (Calbiochem, San Diego, CA) to the membrane suspension, and gently mix for 2 h at 7° C.
4. Remove the unsolubilized material by centrifugation at 135,000g for 15 min, yielding a supernatant of solubilized FPR at 4×10^8 cell equivalents/ml (~ 5 mg/ml protein), which must be used within 6h of preparation for best results, otherwise samples can be stored at -80° C.

Solubilization may yield up to 100%, of receptors harvested from the membranes. It cannot be assumed that DOM allows binding activity to be retained in other GPCR systems, although it has been confirmed for β_2 -Adrenergic receptor. The typical yield of receptors ranges from 100–300 nM depending on receptor expression levels. The initial number of receptors/cell is usually determined by flow cytometry measurements, which are based on the analysis of bound fluorescent ligands used at saturating concentrations or GFP fluorescence, where applicable (*cf.* section on *Standard calibration beads* above).

Modular assembly of ternary complexes on beads

In cells, the ternary complex of ligand, GPCR, and heterotrimeric G proteins, is expected to have a very short lifetime, which is regulated by the kinetics of nucleotide exchange between the inactive GDP-bound form and the active GTP-bound form (Hamm, 1998; Hamm, 2001). The exchange is initiated by the contact between the heterotrimeric G protein and a ligand activated GPCR. The ternary complex thus survives for the short duration of the dissociative interchange between GDP and GTP under rapid mass transfer considerations

where intracellular $[GTP] \gg [GDP]$ and can thus more readily replace GDP. Under our experimental conditions, *stable* LRG assemblies on beads are created in circumstances where the contact between the GPCR and *G beads* causes the ejection of GDP from the $G\alpha$ nucleotide pocket with little to no chance of rebinding, due to the negligible concentrations of soluble nucleotides (GDP or $GTP\gamma S$), in solution. The nucleotide pocket remains empty until the addition of excess nucleotides, which triggers the disassembly of ternary complexes during the rapid mix experiments.

Preparation of G-protein Coated Beads ($G\alpha\beta\gamma$ beads)

Materials

FLAG tagged subunits ($G\beta_1\gamma_2$ or $G\beta_4\gamma_2$), $G\alpha_{i2}$, $G\alpha_{i3}$, *etc.*, (*n.b.* the variety of subunits isotypes is only limited by availability), G-buffer. We have typically used streptavidin coated beads (Spherotech), where the typical stock concentration is 40,000 beads/ μL (Buranda et al., 2001). The concentration of beads (beads/ μl) is determined by counting on a haemocytometer or a flow cytometer. To use a flow cytometer, the rate at which sample is consumed (i.e. flow rate) must be defined. The typical flow rate on our BD flow cytometers is set at 1 μL /sec on “high flow” setting. Therefore one needs to simply run a sample for a desired time and while the cytometers’ “event count” readout function in CellQuest software is turned on. At present, biotin functionalized anti-FLAG M2 antibodies (*bioM2*) purchased from Sigma need to be purified of up to millimolar levels of biotin impurities, by several washes in YM-30 Microcon centrifugal filter devices (Millipore Corp. Bedford, MA) (Buranda, et al., 2001). Sample purity can be checked by assaying for biotin in successive filtrate solutions using a simple biotin assay (Wu et al., 2005a). The assay is based on the kinetic analysis of the enhancement of fluorescence of streptavidin/fluorescein biotin complexes in the presence of biotin. The kinetic response of fluorescence enhancement is proportional to the concentration of biotin.

Preparation of M2 beads

We have previously used titration binding curves to show that Spherotech beads are surface-saturated with $\approx 4 - 4.5$ million bioM2 molecules (Buranda, et al., 2001)(Wu, et al., 2007b). The number of bioM2 antibodies/bead can be assessed by using a fluorescently labeled FLAG peptide ($K_d \approx 8.0nM$) in a centrifugation assay (Buranda, *et al.*, 2001). In brief, a centrifugation assay involves the determination of absolute numbers of bead-associated ligands from the analysis of fluorescence of residual supernatant solutions after the removal of beads by centrifugation. Paired samples (ligand binding samples and samples blocked with excess biotin) of beads are allowed to equilibrate, before centrifugation. The difference in the fluorescence intensity of residual supernatant solutions of binding samples and blocked samples is used to determine the quantity of bound samples. The solution measurements are in turn correlated to a parallel analysis of bead-associated fluorescence by flow cytometry (Buranda, *et al* 1999).

For routine preparations of M2 beads, a simple 50% stoichiometric excess of bioM2 is sufficient to saturate the bead sites due to the very tight multivalent binding of streptavidin

and the ≈ 3 biotins/bioM2. The M2 beads are washed twice and resuspended in PBS buffer and stored in 25 μ L aliquots (40,000 beads/ μ L) at 4°C until needed.

Preparation of G($\alpha\beta\gamma$) on M2 beads

A typical preparation of G protein coated beads, involves the incubation of an equimolar amount of M2 beads (e.g. a 25 μ L aliquot of M2 beads contains ≈ 13 pmol FLAG binding sites) and FLAG tagged subunits ($\beta_1\gamma_2$ or $\beta_4\gamma_2$) in 25 μ L of G-buffer for an hour at 4°C under mild vortex. The beads can then be stored long term at -80°C . Because these experiments allow for the use of various isoforms of G α_{ij} ($j=1,2,3$) subunits, target α_{ij} subunits can be added as needed.

It is always useful to determine the surface density of the $\beta\gamma$ subunits on each bead, in order to keep track of stability of the beads as well as for the standardization of measurements over a period of several months. Surface coverage of $\beta\gamma$ subunits can be quantitatively determined on the basis of a standard calibration protocol that relies on the fluorescently labeled FLAG peptide described above (Buranda, et al., 2001). Briefly, our standard calibration protocols (Wu, et al., 2007b) for measuring the number of M2 antibody sites on (Spherotech) 6 μ m streptavidin coated beads has typically yielded ≈ 8 –9 million fluorescent FLAG peptides per bead (Buranda, et al., 2001) (Simons, et al., 2003a) covered by 4–4.5 million bivalent antiFLAG antibodies under surface saturating conditions. The site density of subunits is then derived from analyzing the difference in the fluorescence intensity of neat anti-FLAG beads (i.e. beads with known maximal surface density of fluorescent FLAG peptide sites) relative to those beads that are partially covered with FLAG tagged $\beta\gamma$ subunits using the fluorescent FLAG peptide.

In our experience, the functional activity of M2 antibodies drops over time, therefore over the course of more than 6 months, we have noticed a drop in the surface density of fluorescent FLAG peptide and $\beta\gamma$ subunits of 25% (Wu, et al., 2007a). The drop in activity of M2 antibodies does not affect the integrity of the ternary complex assembly because it serves as a simple tether of the $\beta\gamma$ subunits to the beads. The change in surface coverage only affects the initial amplitude of the signal associated with the ternary complexes at disassembly.

Under our normal experimental conditions, the surface coverage of G $\beta\gamma$ varied from 45% to 70% of the total available 9 million anti-FLAG binding sites depending on the concentration of FLAG tagged G $\beta\gamma$ subunits and the age of the M2 antibody stock (Wu, et al., 2007a). The assembly of G α -subunits onto the G $\beta\gamma$ beads can be achieved by mixing G α with an aliquot of beads in 10 μ L G buffer at 4°C for 1 hr. The ratio of beads and G α is governed by the $K_d \approx 32$ nM (Wu, et al., 2007a) of this interaction. The mixture is then centrifuged and resuspended in G buffer at 20,000 beads/ μ L; this is the empirical standard concentration of beads used for rapid mix flow measurements. It is not necessary to saturate sites with G α -subunits as the limiting reagent turns out to be GPCR as detailed below.

Assembly of LRG($\alpha\beta\gamma$) on beads

The assembly of ligand and receptor (LR) complexes on $G\alpha\beta\gamma$ -bearing beads is performed as previously described (Simons, et al., 2003a). Briefly, an aliquot of the detergent-solubilized receptor (1% dodecyl maltoside) is mixed with a saturating concentration of the ligand, ($K_d \approx 4\text{ nM}$) (Bennett et al., 2001; Simons, et al., 2003a; Simons et al., 2004; Sklar et al., 2000) and then mixed with the G protein-complexed beads. For the rapid mix experiments, a typical experimental run uses a volume of $35\mu\text{L}$. The receptor concentration is the limiting reagent, thus one would like to optimize the volume fraction associated with the receptor. The maximum concentration of receptor ever recovered from membrane solubilization preparations was $\approx 300\text{ nM}$. Because the bead component contributes $1\mu\text{L}$ ($20,000\text{ beads}/\mu\text{L}$), and the ligand volume can be similarly minimized by using a sufficiently high concentration, it is possible to minimize the dilution of the receptor during molecular assembly, steps which are typically done in a minimal volume of $10\mu\text{L}$ before increasing the volume to the minimal $35\text{--}40\mu\text{L}$ required for measurement using the rapid mix flow cytometer. As previously noted, the ability of the flow cytometer to discriminate between free and bound fluorophores is limited above 200 nM . It is therefore advisable to maintain the concentration of the fluorophore that is in stoichiometric excess (*i.e.* the fluorescent ligand) below 200 nM . The advantage of using a high affinity ligand (e.g. K_d of fMLFK-FITC is $\approx 4\text{ nM}$) (Bennett, et al., 2001; Simons, et al., 2003a; Simons, et al., 2004; Sklar, et al., 2000) is realized here.

The quantitation of the ternary LRG complexes on beads is carried out using standard calibration beads, based on fluorescence readings of fMLFK-FITC or EGFP (*vide supra*). Although relatively high concentrations of FPR are used, 150 nM , the typical surface coverage (Wu, et al., 2005b) on a bead is notably low and depends on the isotypes of the components subunits of a heterotrimer. For example, LRG complexes derived from fMLFK-FITC, wild type FPR and $G\alpha_{i3}\beta_1\gamma_2$ yielded $20\text{--}30,000$ LRG site occupancies (or 0.7% of 4×10^6 $G\alpha\beta\gamma$ sites) on beads compared $<10,000$ site occupancies when $G\alpha_{i2}$ was exchanged for $G\alpha_{i3}$ in the heterotrimer $G\alpha_{i2}\beta_1\gamma_2$ under similar experimental conditions. The low level of total binding may be related to a potentially *weak* affinity interaction between R and G, with $K_d \approx 1\mu\text{M}$ (Bennett, *et al* 2001). Another contributing factor may be that detergent solubilized receptors originate from cells that are transfected to overexpress the receptor of choice. The unpurified solubilized extracts contain other proteins including endogenous G proteins that can be present in much higher concentrations than even the overexpressed target receptor. In normal cells, the expression of G proteins can be as much as 10 to 100 times higher than receptors (Ransnas and Insel, 1988). Therefore it is possible that the bead-borne $G\alpha_i$ subunits ($\approx 30\text{ nM}$) are in competition for receptor sites with endogenous $G\alpha$ subunits (potentially $\gg 100\text{ nM}$) that are co-solubilized with the receptor. A mitigating factor in this assay is that the local concentration of the bead-borne G proteins is very high relative to the soluble endogenous competitors, thus yielding a practically useful quantity of LRG complexes on beads. Schematics of modularly assembled LRG complexes on beads are summarized in Figure 2 and discussed below.

Analysis of modular disassembly of LRG modules

Because LRG complexes can be assembled in modular fashion it is possible to assess the kinetic and thermodynamic parameters of their interaction at each junction point (Buranda, et al., 2007). Disassembly of LRG complexes can be analyzed under circumstances driven by guanine nucleotide activation. Junction points are identified in the schematics displayed in Figure 2. The ternary complex of wild type FPR is shown in Figure 2A. Guanine nucleotide mediated disassembly of the ternary complex occurs at three junction points as shown by arrows: 1) $\beta\bullet\alpha$, 2) $\alpha\bullet R$ and 3) $R\bullet L$. The $G\beta\gamma$ heterodimeric subunit, is virtually nondissociable under physiological conditions (Oldham and Hamm, 2007). The junction points are distinguishable by kinetics. To identify the kinetics of each break point we examined the reactivity of isolated component modules of the ternary complex as well as fusion constructs of FPR- $G\alpha_{i2}$ (Figure 2C) and FPR-GFP (Figure 2D) as described below.

Spectrofluorometric and rapid mix flow cytometry can be used to analyze the kinetics of component L, R, and G dissociation from the ternary complexes in solution and beads. To isolate the specific joint associated with a particular kinetic measurement, it is useful to first consider components of the *ligand-receptor* module separately from the components of the *heterotrimeric G protein* unit. With the exception of constitutively active systems, R and G generally remain as separate entities in the absence of ligand. Equations 3–7 represent the dissociating modules under consideration. Final results are summarized in Table 1.

Real time spectrofluorometric measurement of the dissociation of L from R

The interactions between GPCRs and ligands (agonists or partial agonists) have been quantitatively evaluated (Simons, et al., 2003a), (Simons, et al., 2004) and some K_d values have been tabulated in a review chapter (Buranda, et al., 2007). We briefly focus on the measurement of ligand dissociation using a fluorometric anti FITC antibody assay (Sklar, et al., 1981), which has been described in more recent publications (Bennett et al., 2000; Buranda, et al., 1999; Key et al., 2001; Simons, et al., 2003a). The polyclonal antibody to fluorescein that is used in our laboratory is not commercially available. However, alternative antifluorescein antibodies such as clone4-4-20, A-6421 (Invitrogen.probes.com), or clone FIT-22 (Biolegend.com) can be purchased. We are not aware of previous characterizations of these antibodies, thus the reader would need to perform dilution series experiments to optimize.

To measure k_{diss} in Equation 3, it is necessary to remove endogenous G-protein from solubilized receptors (Bennett et al 2001). G-Proteins can be removed from solubilized receptors by incubating anti- $G_{i1,2,3}$ antibody (Calbiochem or Sigma) for 45 min on ice. The immunocomplex of antibody-substrate is removed by incubating with a slurry of Protein A-agarose for 30-min. The sample is centrifuged at 14,000 x g for 30 s and the supernatant was removed. The stoichiometric ratios of anti- $G_{i1,2,3}$ antibody, or Protein A beads to solubilized

receptors is determined by sample size, and typically follows recommended guidelines on product data sheets of the commercial reagents.

In a cylindrical cuvette, a fluorescein labeled ligand (e.g. 10nM) to the formyl peptide receptor, fMLFK-FITC (L^F) is mixed with a 10-fold stoichiometric excess of detergent solubilized receptor cleared of endogenous G-proteins (*vide supra*). At the spectrofluorometer, a baseline reading of the intensity of receptor bound ligand is taken before a 2μL aliquot of the anti fluorescein antibody is added *in situ*, via a Hamilton syringe through a sample port, in thousand fold stoichiometric excess to $L^F R$ complexes and free L^F in the cuvette. Diffusive encounter between anti fluorescein antibodies and $L^F R$ or L^F selectively quenches 95% of the emission intensity of free L^F but not $L^F R$ complexes where the receptor's steric bulk effectively blocks contact between the fluorescein tag on the short peptide and the antibody (Sklar, *et al* 1990). Because the antibody can quench dissociating L^F , the rate at which L^F is quenched, represents a direct measure of its dissociation from R: $t_{1/2} \approx 20\text{sec}$ for fMLFK-FITC.

Real-time spectrofluorometric measurement of the dissociation of L from RG

To measure the dissociation of L^F from FPR in the presence of G proteins, exogenous G proteins e.g. purified $G_{\gamma 13}$ subunits are combined with $\beta\gamma$ in an equimolar ratio (10 μM) and mixed with solubilized FPR protein (Bennett et al 2001) and with 10 nM fMLFK-FITC in a volume of 12 μL and incubated for 2 h at 4 °C. Prior to analysis, samples are diluted to 200 μL and equilibrated to room temperature for 2 min. At the spectrofluorometer, anti-FITC-Ab is added to quench the L^F as it dissociates from LRG. The dissociation rate of L^F from G protein coupled R is typically slower than dissociation from uncoupled R: $t_{1/2} \approx 100$ seconds. The decoupling of R and G by *in situ* addition of 0.1mM GTPγS yields a rate similar to LR complex in Equation 3 (Bennett et al 2001).

Rapid Mix Measurement of Guanine Nucleotide-Induced Disassembly of Ternary Complexes

The basic operation of a rapid mix flow cytometer has been outlined above, instrumental details, interface with a standard Beckton Dickinson flow cytometer, setup of automation routine and calibration beads for mixing, have been described elsewhere (Wu, et al., 2005b).

A typical run consumes a minimum volume of 35 μL. The 1000 μL volume double barrel syringes of the rapid mix device can therefore be loaded with enough sample volume for several sequential runs. For example, for 3 consecutive runs, 105 μL of LRG beads in DHPSM buffer (600 beads/μL) are loaded into one of the sample syringe of a small volume rapid mix device (Syringe1 in Figure 1). An equal volume of DHPSM buffer or 0.2 mM guanine nucleotide is loaded into the other sample syringe (Syringe2 in Figure 1). A buffer-only control experiment is used to establish a baseline measurement of the effects of a two-

fold dilution of the bead suspension. Other controls involve the use of LRG assemblies made in the presence of GTP γ S to inhibit the formation of LRG. The experimental run proceeds according to an automated mixing sequence as described elsewhere (Wu, et al., 2005b).

The time course data are then converted to ASCII format using the FCSQuery program (Bruce Edwards, UNM HSC). Data are analyzed and graphed using commercial software such as Prism software (GraphPad Software, San Diego, CA). To determine the dissociation characteristics, the fluorescence in blocked control samples is subtracted point by point from the time resolved fluorescence of ligand binding samples (Wu, et al., 2005b), (Wu, et al., 2007a).

Real-time rapid mix measurement of the dissociation of G α from G $\beta\gamma$: Disassembly of Module in Figure 2B

In cells, heterotrimeric G proteins are composed of GDP bound to G α subunit, and G $\beta\gamma$ heterodimeric subunit, which is virtually nondissociable under physiological conditions (Oldham and Hamm, 2007). On beads, G $\beta\gamma$ and fluorescently tagged G α •GDP subunits form a stable heterotrimer, $K_d \approx 32$ nM (Wu, et al., 2007a). Constitutive dissociation of G α •GDP from G $\beta\gamma$ heterodimers is too slow to be considered for rapid mix ($t_{1/2} > 100$ min) cytometry measurements (Wu, et al., 2007a). Tetrafluoroaluminate, AlF_4^- , which emulates the γ -phosphate of GTP in the nucleotide-binding pocket can be used activate the G-protein, to yield the dissociation of G α •GDP• AlF_4^- from the beads ($20 \text{ sec} < t_{1/2} < 30 \text{ seconds}$) (Wu, et al., 2007a). This time frame of subunit dissociation is one which is not characteristic of early signaling events.

Rapid mix measurement of the dissociation of R from G $\alpha\beta\gamma$: Disassembly of Modules in Figure 2D and 2A

The molecular assembly of the ternary complex shown in Equation(s) 6 has two putative junction points, one involving $\beta\bullet\alpha$ junction and another, which involves $\alpha\bullet R^F$ junction. Either junction could contribute to the loss of the GFP tagged receptor (R^F) from beads. The disassembly rate of $\alpha\bullet R^F$ junction can be distinguished from the already known rate of $\beta\bullet\alpha$ junction if the former process (Eq. 6a) is significantly faster than the latter (Eq. 5). Using rapid mix flow cytometry measurements, the loss of GFP was shown to occur on the subsecond timeframe depending on the isotypes of G α and G $\beta\gamma$ subunits used in the

molecular assembly (*cf.* Table 1)(Wu, et al., 2007a). The magnitude and range of these dissociation processes are much faster than that measured for the dissociation of α^F in Equation 5, which by elimination implicates the disruption of the $\alpha \bullet R^F$ junction (Equation 6b) as the correct target of the measurement. Similar dissociation kinetics were also measured using the wild type complexes (Figure 2A)

Rapid mix measurement of the dissociation of L^F from $R\alpha\beta\gamma$: Disassembly of Module in Figure 2C

To corroborate the conclusions from the analysis of Equation 6 we used a Gai2- fused FPR in the molecular assembly of the heterotrimer. Guanine nucleotide activation of the ternary complex yielded a rate constant that was consistent with the dissociation rate of L^F measured from an isolated $L^F R$ complex (*cf.* Eq. 4). Furthermore, the addition of GTP γ S in the presence of excess L^F appeared to inhibit the loss of fluorescence as one would expect facile rebinding of excess ligand L^F to mitigate the decrease in affinity of the $R L^F$ interaction due to GTP γ S mediated uncoupling of the receptor from the G-protein.

Since the measured separation of R- α and $\beta\gamma$ can be no faster than the dissociation of L^F from $L^F R$, the subsecond dissociation of the ternary complex of Equation 6b must reflect the dissociation of FPR-GFP from G α . (Wu, et al., 2007a). Table 1 shows a summary of the kinetics of disassembly from a variety of junction points from data taken from (Wu, et al., 2007a). The data also show that addition of a large excess (0.1 mM) of either GTP γ S or GDP to the ternary complexes on beads produces essentially the same rapid dissociation of LR from the heterotrimer with notable differences between subunit isotypes that were used.

Summary and Outlook

The advantages of modular assemblies on beads to study GPCR ternary complex dynamics in modular systems have been recently elucidated in a review chapter (Buranda et al 2007). The assemblies of specific isotypes of promiscuous G protein subunits can be clearly defined and has the potential of elucidating the differences in dynamic and equilibrium roles played by different isotypes of α and $\beta\gamma$ subunits (Kukkonen et al., 2001; Lindorfer et al., 1998; Mayeenuddin et al., 2006; McIntire, et al., 2001; McIntire et al., 2002; Simons et al., 2003b). As shown in Table 1, the substitution of β_1 for β_4 in the G $\beta\gamma$ dimer has a small but measurable effect ($(\tau_{1/2} = 18.3 \text{ sec vs. } 12.4 \text{ sec})$ on the dissociation rate of L^F , after the receptor uncouples from the G protein ($(\tau_{1/2} < 1 \text{ sec})$). This might arise as a consequence of specific subunit isotypes inducing the receptor to achieve conformational states which display a higher affinity for the ligand (Vauquelin and Liefde, 2005). For example in the molecular assemblies described here, the stability of the LRG complexes improved according to the rank $\alpha_{i1} < \alpha_{i2} < \alpha_{i3}$ (Bennett et al 2001). In these systems, soluble ternary complex formation of the receptors with G proteins allows direct quantitative measurements, which can be analyzed in terms of three dimensional concentrations (molarity). In contrast to the difficulty of analyzing comparable measurements in two dimensional membrane

systems, the output of these flow cytometric experiments can be analyzed via ternary complex simulations in which appropriate parameters can be estimated.

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Figure 1.

Schematic of small volume rapid mix device. Mixing and delivery of fluid samples to the flow cytometer are initiated with the three syringes labeled, “Buffer” and “Double Barrel” (Syringes 1 and 2 define cell/bead and ligand/reagent loading respectively). The volumes shown refer to the maximum capacity of the syringes. Samples are loaded into Syringes 1 and 2 through the sample-loading paths of the loading “Y” connectors. The Buffer Syringe 3 is used to push samples from the delay line into the flow cytometer. A computer program is used to control the sequence in which syringe pumps and valves are activated. Syringe 4 is used to clean the delay line without affecting the loaded samples in the double barrel syringes after each run. Two three-way Teflon solenoid, mixing valves (1 and 2) are used to isolate the delay line from the rest of the sample lines. Mixing Valve 1 is used to switch flow between the Double Barrel syringes and the Buffer syringe. A 13.2 cm (60 µL) delay line is used to carry the mixed samples. Mixing Valve 2 is positioned at the end of the delay line and is used to direct sample flow either into the flow cytometer or to waste.

Figure 2.

Schematics of modular molecular assemblies of ternary complexes on beads. Numbered arrows refer to junction points that are likely to break in the when complexes are activated by guanine nucleotides. A socket and plug connector is utilized to depict the very high affinity interaction of the epitope tag and $\beta\gamma$ -subunits of the G protein (circles labeled with β and γ) that are fused with a FLAG epitope tag, which recognizes the biotinylated M2 antiFLAG antibodies on streptavidin-coated beads. The modular setup of G protein heterotrimers allows for α_i -subunits for capturing receptors (R). Fluorescent components such as GFP or ligand are indicated in green. See text for details.

Summary of experimental results of LRG dissociation kinetics measured by small volume rapid mix flow cytometry. Reproduced from (Wu, et al., 2007a) with permission.

Table 1

	Ligand	Receptor	Gα	βγ	τ _{fast} (sec)	Junction ^c
1 ^a	L ^F	R-Gα ₁₂		β ₁ γ ₂	18.3 (GTP _γ S)	L•RG
					33.8 (GDP)	
2 ^a	L ^F	R-Gα ₁₂		β ₄ γ ₂	12.4 (GTP _γ S)	L•RG
3 ^b	L ^F	R	Gα ₁₂	β ₁ γ ₂	0.8 ± 0.2 (GTP _γ S)	LR•G
					1.5 ± 0.4 (GDP)	
4 ^b	L ^F	R	Gα ₁₂	β ₄ γ ₂	0.7 ± 0.1 (GTP _γ S)	LR•G
5 ^b	L	R ^F	Gα ₁₂	β ₁ γ ₂	0.8±0.1 (GTP _γ S)	LR•G
					1.1 (GDP)	
6 ^b	L	R ^F	Gα ₁₂	β ₄ γ ₂	0.6±0.05 (GTP _γ S)	LR•G
7 ^b	L ^F	R	Gα ₁₃	β ₁ γ ₂	3.1±0.2 (GTP _γ S)	LR•G
					5.8 (GDP)	
8 ^b	L	R ^F	Gα ₁₃	β ₁ γ ₂	2.4±0.2 (GTP _γ S)	LR•G
					5.2±0.6 (GDP)	

^a Kinetic data analyzed using a single-phase exponential model.

^b Kinetic data analyzed using a two-phase exponential model. The data only shows the results of the analysis of the fast component. The slower component was typically two orders of magnitude or more than the fast component (cf. Figure 3 in (Wu, et al., 2007a)). We have attributed the slow component to receptor misfolding (see text for details). Dissociation produced by GTP_γS was always faster and to a greater extent than GDP.

^c Experimentally measured point of dissociation in the ternary complex.