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Fluorescence polarization assays to measure interactions between $G\alpha$ subunits of heterotrimeric G proteins and regulatory motifs

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Abstract

Fluorescence polarization (FP) is a simple and sensitive method allowing for the quantification of interactions between proteins and fluorescently-tagged small molecules like peptides.

Heterotrimeric G proteins are critical signal transducing molecules and their activity is controlled by a complex network of regulatory proteins. Some of these regulators have defined short motifs (<40 amino acids) that are sufficient to bind G proteins and subsequently modulate their activity. For these cases, FP represents a robust and quantitative method to characterize the G protein-regulator interaction. Here we describe FP assays in a 384-well plate format to quantify interactions between $G\alpha$ subunits of heterotrimeric G proteins and peptides corresponding to the $G\alpha$ Binding and Activating (GBA) or GoLoco motifs, which are present in some proteins with Guanine nucleotide Exchange (GEF) (e.g., GIV/ Girdin) or Guanine nucleotide Dissociation Inhibitor (GDI) (e.g., RGS12) activity, respectively. This assay can be used to determine equilibrium dissociation constants, characterize the impact of single amino acid point mutations on the $G\alpha$ -peptide interaction and is suitable for high-throughput screening.

Keywords

Heterotrimeric G protein; GEF; GDI; fluorescence polarization; protein-protein interaction

1 Introduction

Heterotrimeric G proteins are guanine nucleotide-binding proteins which act as molecular switches and regulate intracellular signaling pathways in response to extracellular stimuli [1,2]. In the inactive “off” state, heterotrimeric G proteins consist of a $G\alpha$ subunit bound to GDP and in complex with $G\beta\gamma$. In the canonical G protein cycle, stimulation of G-protein-coupled receptors (GPCRs), which are guanine nucleotide exchange factors (GEFs), promotes the exchange of GDP for GTP on $G\alpha$. This results in the dissociation of active GTP-bound $G\alpha$ from $G\beta\gamma$, freeing them to interact with downstream effector molecules. The built-in GTPase activity of $G\alpha$ allows it to return to the inactive, GDP-bound state and to reassociate with $G\beta\gamma$, thus completing the activation/deactivation cycle.

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In addition to activation by GPCRs, G protein activity is modulated by “accessory proteins” [3]. The most widely studied of these accessory proteins include GAPs (GTPase Activating Proteins), GDIs (Guanine nucleotide Dissociation Inhibitors) and non-receptor GEFs [3–7]. One of the best characterized group of GAPs is the RGS family (Regulators of G protein Signaling), which is characterized by a conserved domain of ~120 amino acids (“RGS box”) responsible for the acceleration of the intrinsic GTPase activity of Gα [7,8]. Similarly, a well characterized group of GDIs contains a conserved GoLoco (aka GPR) motif (~40 amino acids) which is responsible for binding inactive Gα and locking it in the GDP-bound state [6,9]. On the other hand, the progress of research on non-receptor GEFs has been dampened, at least in part, by the lack of a defined domain or motif responsible for the biochemical activity. This has recently changed by the identification of a subgroup of non-receptor GEFs characterized by a ~30 amino acid motif called the Gα Binding and Activating (GBA) motif [10–12]. To date, 4 mammalian proteins have been shown to contain a GBA motif, i.e., GIV/Girdin [10], DAPLE [11], Calnuc and NUCB2 [12]. In addition, a protein (GBAS-1) exclusively found in some nematodes with no similarity to other non-receptor GEFs has been shown to contain a functional GBA motif, thereby indicating that this motif serves as a GEF module conserved in evolution [13]. Proteins with a GBA motif are cytoplasmic factors that function as activators of G protein signaling by binding to GDP-bound Gα and promoting GDP for GTP exchange. Upon GTP loading, GBA motifs disengage from Gα, which ensures that the signaling pathway proceeds towards activation of effectors.

As heterotrimeric G proteins are central hubs of signal transduction, it is not surprising that numerous human diseases are caused by dysregulated G protein signaling [14,15]. In fact, GPCRs are targeted by ~30% of all marketed drugs [16]. G protein regulators, including some accessory proteins described above, have also been implicated in disease. In particular, the role of RGS proteins in health and disease has been extensively studied and they are considered *bona fide* pharmacological targets, as described in recent reviews [17–22]. Proteins with a GoLoco motif have also been linked to autoimmune diseases [23] and in hearing loss [24,25] which supports the increased interest in the development of small molecule inhibitors of the GoLoco-Gαi interface [26]. Among the non-receptor GEFs with a GBA motif, GIV and DAPLE have been the best characterized in terms of biological function and disease linkage. GIV regulates cell migration, autophagy, mitosis, and intracellular trafficking via its GBA motif, and aberrant GIV expression contributes to liver fibrosis and cancer progression towards metastasis through mechanisms that also involve its ability to regulate G proteins (reviewed in [5]). Similarly, the GBA motif of DAPLE also controls tumor cell phenotypes associated with cancer progression [11]. Interestingly, mutations in the GBA motif of GIV or DAPLE that disrupt the GBA-Gα interaction also abolish the associated pro-metastatic phenotypes. Because inhibition of the GBA motif function by mutagenesis blocks the adverse effects of GIV and DAPLE in disease [5,11,27], disruption of the GBA-G protein interaction is emerging as a promising therapeutic avenue [27]. Thus, characterizing the physical interactions between Gα proteins and regulatory motifs derived from accessory proteins may provide leads toward the development of alternative therapies for the treatment of G-protein-related diseases.

Fluorescence polarization (FP) is a method that allows for the characterization and quantification of interactions between proteins and small molecules such as peptides [28,29]. FP is based on the principle that fluorescent molecules, when excited with linearly polarized light, emit polarized light as a function of their molecular weight. While unbound molecules tumble freely in solution and emit depolarized light, their tumbling rate is reduced upon binding to larger proteins, resulting in the emission of polarized light. Thus, the degree of polarization reflects the binding between proteins and peptides, and can be determined by exciting fluorophore-conjugated peptides in solution and measuring light intensity in two different (perpendicular) planes. FP has multiple advantages over other *in vitro* binding assays. Once the reagents are available, the assay is remarkably simple, quick to set up and perform, and inexpensive. The only equipment required is a microplate reader with appropriate polarization filters. The solution-based assay is quick to set up, requiring only the mixing of two components: purified protein and purified fluorescently labeled peptide. After the solutions are prepared, plate reading is also fast and requires only ~ 30 minutes. The microplate-based protocol described here is a simple “mix-and-read” protocol compatible with automation for high-throughput applications. In addition to these general advantages, FP is also well suited to study the interactions between Gα and its regulators. FP is designed to quantify the interactions between small, fluorescent molecules and larger unlabeled proteins, which is the case for peptides derived from GoLoco (aka GPR) or GBA motifs (~3–5 kDa) and Gαi3 (~40 kDa). Importantly, fragments of G protein regulators corresponding to the GBA or GoLoco motifs have been previously shown to recapitulate the binding properties of the native proteins [5,11,12,30–32].

Here we describe the procedures to measure binding of G protein regulator motifs (GBA or GoLoco) to Gα subunits of heterotrimeric G proteins in a 384-well plate and the processing of data to calculate equilibrium dissociation constants for the interactions under investigation. The assay, including data processing, can be completed in approximately 2–2.5 hours.

2 Materials

This protocol assumes that purified His-Gαi3 and the fluorescently labeled peptides are already available. Several papers describe the purification of His-tagged Gαi proteins from bacteria [33,34], which typically yields milligrams of protein per liter with a purity of ~95% after a single step purification. Fluorescently-labeled peptides can be synthesized as described in [35] or custom made from many suppliers (see Note 1).

1. FP binding buffer: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% (v:v) NP-40, 10 mM MgCl₂, 5 mM EDTA. Adjust pH to 7.4. Keep at 4°C for short term storage (<1 month) or freeze aliquots at –20°C for long term storage. Add fresh DTT to 1 mM and GDP to 30 μM before use (see Note 2).

¹The choice of fluorophore depends on the specific application. Here we used peptides labeled with the fluorescein-related molecule carboxyfluorescein (Ex 485nm/Em 528nm) but other fluorophores might be advantageous for other applications. For example, red-shifted fluorophores like TAMRA (Carboxytetramethylrhodamine; Ex 559nm/Em 585nm) are advantageous for HTS because fluorescent small molecules in screening libraries tend to interfere less frequently at red-shifted wavelengths.

2. Purified His-tagged Gαi3 protein. Stored in aliquots at -80°C , typically at high concentrations ($>4\text{ mg/ml}$ or $\sim 100\mu\text{M}$). Minimize the number of freeze-thaw cycles.
3. Purified fluorescently-labelled peptides. 5,6-carboxyfluorescein-labeled human GIV (residues 1671–1701): KTGSPGSEVVTLLQQFLEESNKLTSVQIKSSS and 5,6-carboxyfluorescein-labeled human RGS12 (residues 1185–1221): DEAEFFELISKAQSNRADDQRGLLRKEDLVLPFLR. Stored in aliquots at -80°C , typically at concentrations $\sim 5\text{--}20\mu\text{M}$ ($\sim 250\text{--}1,000$ times of the final concentration in the assay). Minimize the number of freeze-thaw cycles and protect from light.
4. Black 384-well plates (PerkinElmer OptiPlate 384, Part # 6007270)
5. Plate reader (BioTek Synergy H1 microplate reader) equipped with excitation ($485 \pm 20\text{ nm}$) and emission ($528 \pm 20\text{ nm}$) polarization filters.
6. Microcentrifuge tubes

3 Methods

The following procedure describes how to determine the equilibrium dissociation constant (K_d) for the interaction between a peptide derived from GIV's GBA motif and His-Gαi3 as a typical example and we include some notes on variables to take into account for other cases. It consists of 3 parts: (1) preparation of His-Gαi3 serial dilutions, (2) mixing of fluorescently-labeled GIV GBA peptide and G protein, and (3) FP measurement and data analysis.

3.1 Preparation of His-Gαi3 serial dilutions

Prepare and maintain all working protein solutions on ice.

1. Prepare and label seven microcentrifuge tubes (#1–7) for each interaction pair to be assayed (*see Note 3*).
2. Add $15\mu\text{L}$ of FP buffer (supplemented with DTT and GDP) to tubes #1–6.
3. In tube #7, prepare $30\mu\text{L}$ of His-Gαi3 protein in FP buffer (supplemented with DTT and GDP) at a concentration which is two-fold higher than the maximum Gα protein concentration to be tested (*see Note 4*). For this example, the highest final concentration of His-Gαi3 in the FP measurement described here is $8\mu\text{M}$, so the $30\mu\text{L}$ aliquot must be prepared at a $16\mu\text{M}$ concentration.

²GBA and GoLoco motifs bind to inactive, GDP-bound Gαi subunits. If the goal is to measure binding to active G proteins, GDP is replaced with GTPγS ($30\mu\text{M}$) or GDP plus AlF_4^- ($30\mu\text{M}$ AlCl_3 and 10mM NaF). GTPγS loading requires long incubations (2–3 h at room temperature or 30°C) whereas AlF_4^- is readily loaded (a few minutes at room temperature).

³This protocol is designed to generate an eight-point binding curve spanning a 64-fold coverage of Gα protein concentrations, which is generally sufficient to determine K_d values accurately.

⁴If the Gα protein storage buffer were to contribute more than 10% to the final sample volume in any well during the FP measurement, the FP binding buffer used for the serial dilutions would be mixed with G protein storage buffer so that the composition is kept constant throughout the dilution series.

4. Perform two-fold serial dilutions by first transferring 15 μL from tube #7 to tube #6, mixing and transferring 15 μL from tube #6 to tube #5. Repeat this 15 μL transfer through the tube series. All tubes should now contain 15 μL , with the exception of tube #1 (30 μL). Because the initial tube #7 is at 16 μM , the resulting series will be: 16, 8, 4, 2, 1, 0.5 and 0.25 μM (which will translate into 8, 4, 2, 1, 0.5, 0.25 and 0.125 μM in the final FP measurement) (*see* Note 5).

3.2 Preparation of peptide solution and incubation with G protein in 384-well plates

1. For each tested peptide, prepare a minimum of 90 μL of a 50 nM peptide solution in FP binding buffer (*see* Note 6). Prepare and maintain this solution in ice. Avoid prolonged exposure of the fluorescent peptide to light.
2. In a black 384-well plate, transfer 10 μL per well for each one of the His-G*α*i3 concentrations prepared as described in 3.1 (tubes #1–7). Include an additional well containing only FP binding buffer (supplemented with DTT and GDP), which will correspond to the 0 μM concentration. This step can be carried out at room temperature.
3. Transfer 10 μL of the solution containing the fluorescently labeled peptide to each of the eight wells, including the peptide-only control well (*see* Note 7). This will result in a final concentration of peptide of 25 nM. This step can be carried out at room temperature.
4. Quickly mix the wells by gently tapping the sides of the plate, or by spinning down the plate at 1000 rcf for 1 min.
5. Loosely cover the plate with aluminum foil to protect the samples from light, and incubate the plate at room temperature for 10 min.

3.3 Data collection and analysis

1. Measure the FP of the samples in the plate every 3 minutes for 30 minutes using a Biotek Synergy H1 plate reader (or an equivalent machine with the appropriate set of filters) (*see* Note 8).

⁵The number of concentration points and the range covered depends on the particular protein-peptide to be investigated and its K_d . As a general rule, it is desirable to cover concentrations from ~4–8 times below the K_d to ~8–10 times above the K_d . Similarly, the volumes to be prepared of each concentration depend on the number of replicates and/or different peptides to be tested. The protocol presented here assumes singlet measurements for a single peptide because the high reproducibility of this assay makes technical replicates unnecessary. For calculation purposes, one should prepare a volume of $[(10 \times n) + 5]$ μL of tubes #1–6 and $2 \times [(10 \times n) + 5]$ μL of tubes #7, where n is the final number of wells for each condition. The volume to be transferred from tube to tube should be $[(10 \times n) + 5]$. For example, to make measurements in triplicate we would prepare $(10 \times 3) + 5 = 35$ μL for tubes #1–6 and 70 μL for tube #7, with a transfer volume of 35 μL for the serial dilution.

⁶The concentration of peptide to prepare must be twice the final concentration in the well during the FP measurements. The concentration of peptide to be used must be determined empirically for each specific protein-peptide interaction. To determine the K_d accurately, the concentration of peptide must be low (less than the K_d of the interaction) but sufficiently high to give a robust and reproducible signal.

⁷A final volume of 20 μL provides reproducible results but it can be scaled up if the quantity of reagents is not a limitation.

⁸The purpose of measuring FP as a 30 min kinetic is double. One, it allows to visually inspect if the interaction is stable during a reasonable time frame (if not, the procedure might need to be optimized in terms of buffer composition and/or time of measurement). Two, it permits the calculation of an averaged value, thereby minimizing the effect of noise fluctuations for the final calculations.

2. Export the raw fluorescence polarization values to a spreadsheet program such as Microsoft Excel and calculate the average of the values across the different points for each well.
3. These raw polarization values can be exported into a program like GraphPad Prism to make a non-linear regression curve fit and calculate the K_d (see Note 9). An example of the result is shown in Fig. 1A.
4. Raw FP data can also be normalized to be expressed as a function of maximal binding (% of maximum binding) to facilitate the comparison across different conditions. For this, the FP value of the free peptide (0 μ M His-G α i3) is used as 0% binding and the maximum binding (B_{max}) from the curve fit determined in step 3 is used as 100%.
5. Normalized values can be used again in GraphPad Prism to create a curve fit (see Note 10). An example of this normalization is shown in Figure 1B. Figure 2 shows an additional example of normalized binding for different peptides (GBA motif of GIV and GoLoco motif of RGS12) to G α i3 wild-type (WT) or mutants (see Note 11).

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⁹In GraphPad Prism 6, the equation used for this fit is under “Analysis” → “Non-linear (curve fit)” → “Binding-Saturation” → “One site”. $Y = B_{max} * X / (K_d + X) + \text{Background}$, where B_{max} is the FP signal corresponding to maximal binding, Background is the FP signal in the absence of G protein (peptide alone) and X the concentration of His-G α i3. This equation assumes one binding site for the peptide in G α .

¹⁰The curve fit is done with the same settings as in Note 9 but constraining (“Constrains” tab) B_{max} to 100 and Background to 0.

¹¹In the case of mutants for which binding is significantly decreased and maximal binding is not reached with the concentrations tested, it is acceptable to use the FP values corresponding to maximal binding of WT for the calculation of normalized binding.

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Figure 1. Raw and normalized curve fits for the interaction between Gαi3 and a peptide corresponding to the GBA motif of GIV (residues 1671–1701)

(A) The interaction was assayed using a peptide concentration of 25 nM and Gαi3 concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0 μM (free peptide). Raw FP values were exported into GraphPad Prism 6, and the values were fit to a curve using a “one-site” binding equation (see Note 9). (B) The FP values in panel A were normalized in terms of binding percentage by assigning the FP value of the free peptide (0 μM Gαi3) as 0% binding and the B_{max} value from the curve fit in panel A as 100% (maximum binding).

Figure 2. Normalized curve fits for the binding of wild type or mutant Gαi3 to two different regulatory motifs regulators

(A) Curve fits for the Gαi3-GIV GBA motif interaction using wild type and mutant forms of Gαi3. Normalized Gαi3-GIV binding was determined as in Figure 1, but testing Gαi3 S252D and W211A mutants in addition to Gαi3 WT. (B) Curve fits for the Gαi3-RGS12 GoLoco motif interaction using wild type and mutant forms of Gαi3. Normalized binding of RGS12 GoLoco motif to Gαi3 WT, S252D and W211A was determined as in panel “A”.