

Using Intrinsic Tryptophan Fluorescence to Measure Heterotrimeric G-Protein Activation

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- Heterotrimeric G protein activation is measured via the change in tryptophan fluorescence in the $G\alpha$ subunit
- This method applies to most $G\alpha_i$, $G\alpha_o$ and some small G proteins that have movement in the switch II region
- Alternative, non-radiological method to ^{35}S -GTP γ S and [γ - ^{32}P]GTP assays

Introduction

GTP-binding proteins (G-proteins) are important, well-described cellular signaling molecules.¹ Heterotrimeric G-proteins are composed of three subunits $G\alpha$, $G\beta$ and $G\gamma$ and are typically bound to seven-transmembrane G-protein coupled receptors (GPCRs). The $G\alpha$ subunit binds guanine nucleotides while the $G\beta$ and $G\gamma$ subunits form an obligate heterodimer. In its inactive state, the GDP bound $G\alpha$ subunit is bound to $G\beta\gamma$. Upon agonist activation the receptor acts as a guanine nucleotide exchange factor (GEF), resulting in the release of GDP and subsequent binding of GTP. The binding of GTP causes a dramatic conformational change in three flexible switch regions of $G\alpha$ (Fig. 1 dark red and dark blue) resulting in the dissociation of $G\alpha$ -GTP from $G\beta\gamma$. After their dissociation, $G\alpha$ and $G\beta\gamma$ independently activate downstream effectors such as adenylyl cyclase, phospholipase C, ion channels, RhoGEFs and phosphodiesterases.^{2,3}

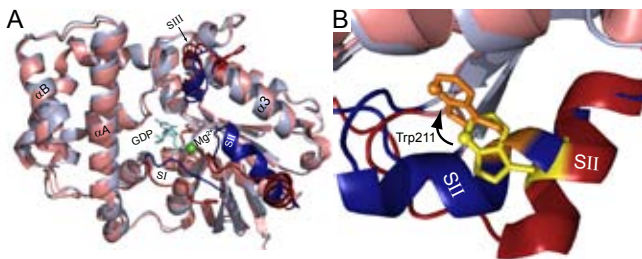


Fig. 1: a) X-ray crystallography structural models of $G\alpha_{i1}$ (an isoform of $G\alpha$) bound to GDP (red) and $GDP \cdot AlF_4^-$ (blue) (PDB ID: 1KJY and 2IK8, respectively). The bound guanine nucleotide and magnesium ion are illustrated in cyan and green, respectively. The switch regions SI, SII, and SIII are in dark red and blue. (b) Close-up view of intrinsically-fluorescent Trp211 located in the switch II region in inactive (yellow) and activated (orange) $G\alpha_{i1}$.

The duration of activation is controlled by the hydrolysis rate of GTP. Two well-described accessory protein families affect the kinetics of $G\alpha$ subunits by either accelerating GTP hydrolysis (the RGS proteins) or retarding GDP release (the GoLoco proteins). Regulators of G-protein signaling rapidly accelerate the GTP hydrolysis of $G\alpha$ subunits by stabilizing the transition state⁴; while GoLoco motifs act as GDIs (guanine nucleotide dissociation inhibitors), preventing GDP dissociation by adding a second arginine side-chain to the contacts made to the bound nucleotide.⁵

The kinetics of nucleotide binding and hydrolysis are traditionally measured using ^{35}S -GTP γ S or [γ - ^{32}P]GTP, and more recently, have been measured using fluorescently-labeled nucleotides.^{6,7} In this

application note, we describe the use of the BMG LABTECH's POLARstar Omega to monitor changes in the intrinsic fluorescence⁸ of a highly-conserved tryptophan located in the switch II region of $G\alpha$ subunits (Fig. 1, "SII"). The conformational change in SII decreases the exposure of the Trp residue to the aqueous environment, resulting in an increase in the quantum yield.⁹ One can quantify this event by measuring the increase in $G\alpha$ protein fluorescence at 350 nm upon excitation at 280 nm. This method is applicable to most $G\alpha_i$ and $G\alpha_o$ proteins as well as some small G-proteins. In this application note, we have optimized the assay by varying concentration of $G\alpha$, changing assay buffers, and shifting excitation and emission wavelengths.



Fig. 2: BMG LABTECH's POLARstar Omega multidetection microplate reader

Materials and Methods

All experiments were conducted on the POLARstar Omega plate reader (Fig. 2) at ambient temperature using Corning Black Polystyrene 96-well plates (Sigma, Cat#CLS3875). $G\alpha_{i1}$ was purified exactly as previously described¹⁰ and diluted to 1 μM in assay buffer (unless otherwise noted) and plated at an initial volume of 187 μL /well. Experiments were conducted using a 280 ± 5 nm and 350 ± 5 nm filter for excitation and emission, respectively, unless specified otherwise.

To maximize data acquisition during the experiment, typical data collection was divided into three distinct phases – baseline ($-15 - 0$ s), activation ($0 - 132$ s), and plateau phase ($132 - 158$ s). Data was collected at 1, 0.6 and 2 s intervals for baseline, activation and plateau phases, respectively, using the fast kinetics (well mode) function on the Omega. At 0 s, 8 μL of 0.5 M NaF and 5 μL of 1.2 mM $AlCl_3$ were injected sequentially with a 5 s delay. NaF and $AlCl_3$ undergo a chemical reaction to form AlF_4^- , which mimics the leaving phosphate group upon hydrolysis of GTP. This stable complex, $G\alpha_{i1} \cdot GDP \cdot AlF_4^-$, mimics the active, GTP-bound state of $G\alpha_{i1}$. The gain was set to 50% relative to 200 μL of pre-activated $G\alpha_{i1} \cdot GDP \cdot AlF_4^-$ to avoid saturating the signal. The previously described GoLoco motif GDI peptide, AGS3Con, was used and shown to inhibit the formation of $G\alpha_{i1} \cdot GDP \cdot AlF_4^-$.¹¹

Buffers

Phosphate assay buffer (pH 8.0) - 100 mM NaCl, 100 μM EDTA, 2 mM $MgCl_2$, 2 μM GDP, 20 mM K_2HPO_4/KH_2PO_4
HEPES assay buffer (pH 8.0) - 100 mM NaCl, 100 μM EDTA, 2 mM $MgCl_2$, 2 μM GDP, 20 mM HEPES
Tris assay buffer (pH 8.0) - 100 mM NaCl, 100 μM EDTA, 2 mM $MgCl_2$, 2 μM GDP, 20 mM Tris

Instrument Settings

Fluorescence Intensity - Well Mode

Keep default settings except for the following:

No. of kinetic windows - 3

Baseline

No. of intervals - 15, No. of flashes - 10, Interval time - 1 sec

Activation

No. of intervals - 220, No. of flashes - 10, Interval time - 0.6 sec

Plateau

No. of intervals - 13, No. of flashes - 10, Interval time - 2 sec

Injection - use 320 $\mu\text{L/s}$ and keep smart injection unchecked

Pump 1 inject 8 μL at start time 15 s (at $t=0$ in the graphs)

Pump 2 inject 5 μL at start time 20 s (at $t=5$ s in the graphs)

Results and Discussions

In order to measure the effect of sample concentration on maximal response, we made serial dilutions of $G\alpha_{i1}$ from 3 μM to 50 nM in Tris pH 8.0 assay buffer. The most robust response was seen at the highest concentration of $G\alpha_{i1}$ tested (Fig. 3a, red), but a change in fluorescence was detectable at all concentrations. To compare the quality of the signal for each concentration, a Z' -factor was computed for each concentration.

$$Z' = 1 - \frac{3\sigma_{\text{plateau}} + 3\sigma_{\text{baseline}}}{|\mu_{\text{plateau}} - \mu_{\text{baseline}}|}$$

This calculation accounts for the magnitude of the signal change upon excitation ($\mu_{\text{plateau}} - \mu_{\text{baseline}}$) as well as the standard deviation of data collected during the plateau phase (σ_{plateau}) and baseline phase (σ_{baseline}). Using the Z' -factor, 3 μM of $G\alpha_{i1}$ was seen to have no advantage over 1 μM $G\alpha_{i1}$ (i.e., both Z' -factors > 0.9) while the quality of the data decreased at concentrations under 1 μM (Fig. 3b).

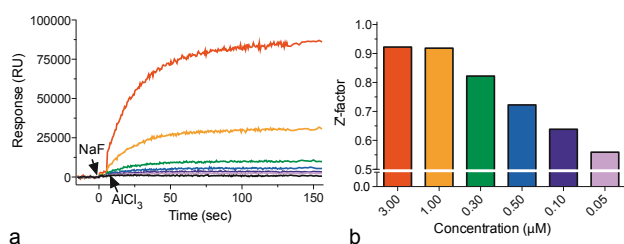


Fig. 3: (a) Signal intensities at varying concentrations of $G\alpha_{i1}$ as activated by aluminum tetrafluoride addition (sequential application of NaF and AlCl_3). (b) Z' -factors of the assay at varying $G\alpha_{i1}$ concentrations.

To assess the effect of assay buffer composition on signal intensity, we measured the activation of 1 μM $G\alpha_{i1}$ in assay buffer prepared with various common buffer salts (Fig. 4a). The quality of the measurements, as determined by the Z' -factor, was similar for all of the buffers (Fig. 4b) although the maximum signal was observed with Tris assay buffer and the lowest magnitude was observed using HEPES assay buffer.

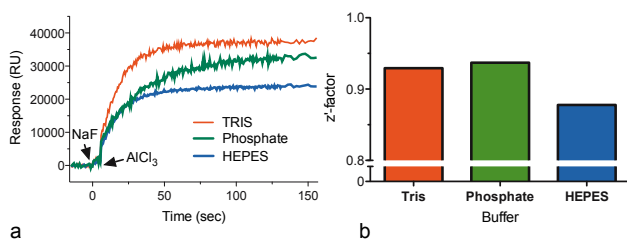


Fig. 4: (a) Signal intensities for 1 μM $G\alpha_{i1}$ in various assay buffers with common salts. (b) Z' -factors of the assay performed in various buffers.

Optimization of the filter set was performed by measuring the activation of 1 μM of $G\alpha_{i1}$ with varying combinations of excitation (280 or 290 nm) and emission filters (340, 350 and 360 nm) (data not shown). The best filter pair found for this assay was 280/350 nm.

To verify that the assay is detecting the rate of $G\alpha$ activation and is sensitive to changes in this rate, we incubated 500 nM of $G\alpha_{i1}$ with 5 μM AGS3Con peptide, a previously described GDI.¹¹ As expected, the addition of AGS3Con (Fig. 5) dramatically dampened the maximal response of $G\alpha_{i1}$, as compared with 500 nM $G\alpha_{i1}$ alone.

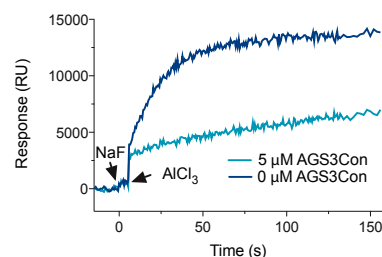


Fig. 5: Effect of 5 μM of the GDI peptide AGS3Con on intrinsic tryptophan signal intensity upon aluminum tetrafluoride activation of 500 nM $G\alpha_{i1}$.

Conclusion

In this application note, we described a robust automated assay system for measuring G-protein α subunit activity. This is a fairly universal assay to measure $G\alpha_{i/o}$ activation as modulated by a variety of inputs (eg. peptides, proteins, small molecules), because the assay relies on the flexible switch region SII to change conformation depending on nucleotide state. The assay is a sensitive and high-quality means to measure G-protein activation without the use of radiolabeled nucleotides.

While this assay has been previously described for cuvette-based instruments, performing the assays with the POLARstar Omega 96-well plate reader with on-board injectors offers the advantage of automating the assays in triplicate on multiple $G\alpha$ mutants or multiple modulators of spontaneous GDP release.

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