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 Gα 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-GTPγS and[γ-^{32}P]GTP assays**

Introduction

GTP-binding proteins (G-proteins) are important, well-described cellular signaling molecules. Heterotrimeric G-proteins are composed of three subunits Gα, Gβ and Gγ and are typically bound to seven trans-membrane G-protein coupled receptors (GPCRs). The Gα-subunit binds guanine nucleotides while the Gβ and Gγ subunits form an obligate heterodimer. In its inactive state, the GDP bound Gα subunit is bound to Gβγ. 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 Gα (Fig. 1 dark red and dark blue) resulting in the dissociation of Gα-GTP from Gβγ.

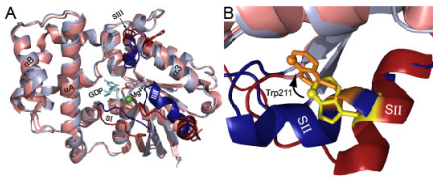

Fig. 1: A) Structural models of $G\alpha_{i1}$ (an isoform of Gα) bound to GDP (red) and GDP · AlF_4^- (blue) (PDB ID: 1KJY and 2IK8, respectively). The switch regions SI, SII, and SIII are in dark red and blue. (B) Close-up view of intrinsically-fluorescent Trp211 located in the switch II region in 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 Gα 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 Gα subunits by stabilizing the transition state; 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.

In this application note, we describe the use of the BMG LABTECH's POLARstar® Omega to monitor changes in the intrinsic fluorescence of a highly-conserved tryptophan located in the switch II region of Gα 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. One can quantify this event by measuring the increase in Gα protein fluorescence at 350 nm upon excitation at 280 nm. In this application note, we have optimized the assay by varying concentration of Gα, changing assay buffers, and shifting excitation and emission wavelengths.

Materials & Methods

All experiments were conducted on the POLARstar Omega plate reader at ambient temperature using Corning Black Polystyrene 96-well plates. $G\alpha_{i1}$ was purified exactly as previously described 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 were 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 AlCl3 undergo a chemical reaction to form AlF_4^-, which mimics the leaving phosphate group upon hydrolysis of GTP. This stable complex, $G\alpha_{i1}$ · GDP · 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 $G\alpha_{i1}$ · GDP · AlF_4^- to avoid saturating the signal. The previously described GoLoco motif GDI peptide, AGS3Con, was used and shown to inhibit the formation of $G\alpha_{i1}$ · GDP · AlF_4^-.

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 pH 8.0

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

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Injection - use 320 µL/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 =5s in the graphs)

Results & Discussion

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. 2, 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

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

This calculation accounts for the magnitude of the signal change upon excitation ($\mu_{plateau}$ - $\mu_{baseline}$) as well as the standard deviation of data collected during the plateau phase ($\sigma_{plateau}$) and baseline phase ($\sigma_{baseline}$). 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 (not shown).

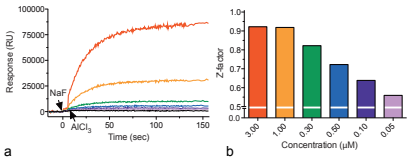

Fig. 2: (a) Signal intensities at varying concentrations of $G\alpha_{i1}$ as activated by aluminum tetrafluoride addition (sequential application of NaF and $AlCl_3$.) (b) Z'-factors of the assay at varying $G\alpha_{i1}$ concentrations.

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. 3a). The quality of the measurements, as determined by the Z'-factor, was similar for all of the buffers (Fig. 3b) although the maximum signal was observed with Tris assay buffer and the lowest magnitude was observed using HEPES assay buffer.

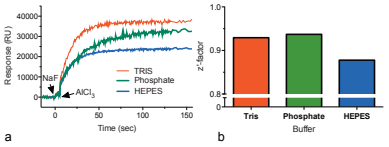

Fig. 3: (a) Signal intensities for 1 µM $G\alpha_{i1}$ in various assay buffers with common salts. (b) Z'-factors of the assay performed in various buffers.

To verify that the assay is detecting the rate of Gα 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. As expected, the addition of AGS3Con (Fig. 4) dramatically dampened the maximal response of $G\alpha_{i1}$, as compared with 500 nM $G\alpha_{i1}$ alone.

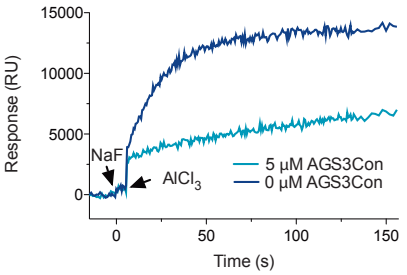

Fig. 4: Effect of 5 µM of the GDI peptide AGS3Con on intrinsic tryptophan signal intensity upon aluminum tetrafluoride activation of 500 nM $G\alpha_{i1}$.

Conclusion

In this application note, we described a robust automated assay system for measuring G-protein α subunit activity. The assay is a sensitive and high-quality means to measure G-protein activation without the use of radiolabeled nucleotides.

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 Gα mutants or multiple modulators of spontaneous GDP release.