Published in final edited form as:

Methods Enzymol. 2020 January 01; 633: 271–280. doi:10.1016/bs.mie.2019.11.009.

Site-directed labeling of β -arrestin with monobromobimane for measuring their interaction with G protein-coupled receptors

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Abstract

β -arrestins (β arrests) are multifunctional proteins that interact with activated and phosphorylated G protein-coupled receptors (GPCRs) to regulate their signaling and trafficking. Understanding the intricate details of GPCR- β arr interaction continues to be a key research area in the field of GPCR biology. Bimane fluorescence spectroscopy has been one of the key approaches among a broad range of methods employed to study GPCR- β arr interaction using purified and reconstituted system. Here, we present a step-by-step protocol for labeling β arrests with monobromobimane (mBB) in a site-directed fashion for measuring their interaction with GPCRs and the resulting conformational changes. This simple protocol can be directly applied to other protein-protein interaction modules as well for measuring interactions and conformational changes in reconstituted systems in vitro.

1 Introduction

Interaction of β arrests with activated and phosphorylated GPCRs is a central mechanism for exerting a precise control on downstream signaling (Kang, Tian, & Benovic, 2014; Lefkowitz & Shenoy, 2005; Luttrell et al., 2018). In the classical paradigm, β arrests sterically hinder the coupling and activation of heterotrimeric G-proteins to the receptors, while also directing internalization of the activated receptors thereby reducing their surface levels (DeWire, Ahn, Lefkowitz, & Shenoy, 2007; Reiter, Ahn, Shukla, & Lefkowitz, 2012). In addition, a critical contribution of β arrests in GPCR mediated signaling has also been established for a large number of GPCRs (DeWire et al., 2007; Reiter et al., 2012; Shukla, Singh, & Ghosh, 2014). More recent paradigm of sustained GPCR signaling, for example, from endosomal compartments also involves an important role of β arrests, potentially via formation of GPCR-G-protein- β arr supercomplexes (Ranjan, Dwivedi, Baidya, Kumar, & Shukla, 2017; Thomsen et al., 2016). Thus, understanding the intricate details and molecular mechanisms of GPCR- β arr interaction is an important area in GPCR biology research.

GPCR- β arr interaction typically depends on two distinct activation-dependent features of GPCRs, first, receptor phosphorylation and second, conformational change in the transmembrane domain (Chen, Iverson, & Gurevich, 2018; Gurevich & Gurevich, 2004, 2018; Ranjan et al., 2017; Shukla et al., 2013, 2014). Receptor attached phosphates

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primarily interact with the positively charged residues on the N-domain of β arrs while the opening on the intracellular side of the receptor transmembrane domain accommodates finger loop of β arrs (Chen et al., 2018; Gurevich & Gurevich, 2018; Shukla et al., 2013, 2014). A number of methods have been employed to measure GPCR- β arr interaction in cellular context and in vitro. For example, confocal microscopy, enzyme complementation, FRET, BRET and Tango assay are widely used to measure the recruitment of β arrs to GPCRs with spatio-temporal resolution in cellular context. Methods like co-immunoprecipitation, ELISA, isothermal calorimetry and fluorescence spectroscopy are most commonly used to measure this interaction in vitro using purified components.

Fluorescence spectroscopy, especially the one using monobromobimane (mBBr) labeled β arrs, has been extensively employed to report the interaction of GPCRs with β arrs as well as to measure the conformational changes in β arrs arising upon their interaction with the receptors (Ghosh et al., 2019; Kumari et al., 2016, 2017; Rahmeh et al., 2012; Sente et al., 2018; Staus et al., 2018). mBBr is an environmentally sensitive fluorophore that exhibits a change in its fluorescence intensity and emission maxima upon alteration of its physico-chemical environment (Chinn, Pigiet, & Fahey, 1986; Mansoor & Farrens, 2004; Mansoor, McHaourab, & Farrens, 2002). Site-directed labeling of β arrs has allowed deciphering the engagement of various loops in β arrs with the receptor, the contribution of receptor phosphorylation and activation in their interaction, and conformational differences among β arr isoforms (Ghosh et al., 2017, 2019; Kumari et al., 2016, 2017; Rahmeh et al., 2012; Staus et al., 2018). These studies involving GPCRs and β arrs have been designed based on extensive studies on rhodopsin-visual-arrestin system described in the literature (Sommer & Farrens, 2006; Sommer, Farrens, McDowell, Weber, & Smith, 2007; Sommer, Smith, & Farrens, 2005; Sommer, Smith, & Farrens, 2006).

Here, we present a step-by-step protocol for labeling β arrs in their finger loop using monobromobimane based on our previous studies (Kumari et al., 2016, 2017) (Fig. 1). The method presented here should allow other researchers to establish this technique in their laboratory for investigating GPCR- β arr interaction, and moreover, it should also be adaptable to other protein-protein interaction systems involved in cellular signaling (Fig. 2).

2 Materials and methods

2.1 Chemicals, reagents and equipments

Monobromobimane (Sigma, Cat#B4380)

Fluorometer (e.g., Perkin Elmer, LS-55)

Quartz microcuvette (Perkin Elmer)

E. coli BL21 (DE3) (NEB, Cat#C25271)

LB media

Buffers (Tris-HCL and HEPES buffer)

Isopropyl β -D-1-thiogalactopyranoside (IPTG) (SRL, Cat#67208)

Lysozyme (SRL, Cat#45822)

DMSO (Dimethyl Sulfoxide) Sonicator

Ultracentrifuge

Econo-column (Biorad Cat# 7374251) Glutathione resin (Genescript, Cat#L0026)

Thrombin, Bovine (Merck, Cat#605157)

PD10 desalting column (GE healthcare Cat# 17085101)

2.2 Expression and purification of β arr constructs for bimeane labeling

This protocol is based on our previous studies designed for site-directed labeling of β arrs in their finger loop, i.e., Leu⁶⁸ in β arr1 and Leu⁶⁹ in β arr2 (Kumari et al., 2016, 2017; Sente et al., 2018).

2.2.1 Synthesize or generate by site-directed mutagenesis β arr expression constructs where all the natural cysteines have been mutated to Ala/Val followed by substitution of Leu⁶⁸/Leu⁶⁹ with cysteine. We have used pGEX-4T based vector system for expression of β arrs in *E. coli* (BL21-DE3), however, other expression vectors should work as well.

2.2.2 Express and purify single-Cys variant of β arrs following standard protocol using glutathione sepharose affinity chromatography and on-column cleavage of β arrs with thrombin as described previously (Shukla et al., 2013).

2.2.2a Transform β arr expression plasmid in BL21 (DE3) competent cells, inoculate and grow primary culture overnight at 37 °C. Next day, inoculate secondary culture and grow until OD₆₀₀ reaches 0.6–0.8.

2.2.2b Add 0.1 mM IPTG to the secondary culture and let it grow for 16 h at 18 °C. Afterward, harvest the cell pellet, lyse in binding buffer (25 mM Tris, pH 8.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 1 mM PMSF, 2 mM benzamidine) by sonication and incubate the supernatant with pre-equilibrated glutathione sepharose (GS) resin overnight at 4 °C.

2.2.2c Subsequently, wash the GS-resin extensively with wash buffer (25 mM Tris, pH 8.5, 150 mM NaCl) and incubate it with thrombin for 2 h at room temperature with gentle shaking. Collect the flow through containing β arr and estimate its purity on SDS-PAGE.

2.2.2d β arr can be further purified using anion-exchange chromatography or size-exclusion chromatography as described previously (Shukla et al., 2013).

Caution! Purity of the protein of interest should be better than 80–85% in order to avoid non-specific labeling of impurities which may

otherwise interfere with subsequent fluorescence spectroscopy data collection and interpretation.

- 2.2.3** We typically validate the functionality of bimane-labeled β arrs using co-immunoprecipitation assay involving a phosphopeptide (V2Rpp) and active-conformation of β arr1 specific antibody fragment (Fab30). However, other approaches such as direct interaction with activated and phosphorylated receptors or other interaction partners can also be used to establish the functionality of β arrs after bimane-labeling.

2.3 Site directed labeling of β arrs with monobromobimane (mBBr)

Here, we describe a protocol to label purified β arrs at targeted cysteine residues using thiol-reactive monobromobimane reagent (Fig. 1).

- 2.3.1** Exchange the buffer of purified protein with labeling buffer (20 mM HEPES, pH 7.5, 150 mM NaCl) and concentrate to about 2.0 mg/mL. Alternatively, if the protein is purified in this buffer, it can be directly used for bimane-labeling.

- 2.3.2** Prepare a fresh stock of mBBr at a concentration of 100 mM in DMSO and add it to the purified protein at a molar ratio of 1:10. Incubate the reaction for 1 h in dark at room temperature. Alternatively, bimane labeling can also be performed at 4 °C or on ice depending on the stability of the protein and the labeling pattern.

Caution! Extended labeling time may cause protein aggregation and therefore, it is important to optimize the labeling condition for the protein of interest. A systematic optimization of protein to mBBr molar ratio, incubation temperature and time are recommended. It is also important to protect the reaction from light as it may affect mBBr stability and labeling efficiency.

- 2.3.3** Quench the labeling reaction by adding 100 mM L-cysteine. Subsequently, centrifuge the sample at $14,000 \times g$ for 30 min to remove any aggregated protein.
- 2.3.4** Unreacted mBBr can be removed by desalting on PD-10 columns, standard size exclusion chromatography or dialysis in appropriate buffer.
- 2.3.5** Fluorescence of bimane-labeled β arrs can be measured using a standard Fluorescence spectrophotometer in photon counting mode using excitation and emission band pass of 5 nm. For labeled proteins, it is advisable to read full scan (200–800 nm) of excitation and emission spectrum to precisely measure the excitation and emission maxima, respectively. In our experiments, we have typically used an excitation wavelength of 397 nm and emission wavelength range from 415 to 600 nm with scan speed of 50 nm/min using a Perkin Elmer, LS-55 fluorometer (Fig. 3).

Caution! Slit width throughout the path of the light can greatly affect the sensitivity of the experiment. It is advisable to optimize this setting for

individual labeled protein and must be kept constant throughout for a set of experiment.

2.3.6 Bimane fluorescence intensity should be corrected for background fluorescence by measuring the fluorescence intensity of just buffer (blank) and subtracting it from the measured values.

2.3.7 Bimane-labeled protein can be flash frozen with 10% glycerol and stored in small aliquots at -80°C for several weeks.

2.4 Calculation of bimane-labeling efficiency

Bimane-labeling efficiency can be measured in terms of moles of bimane fluorophore per mole of protein of interest using the following formula:

$$\frac{A_x}{\epsilon} \times \frac{\text{MW of protein}}{\text{Mg protein/mL}} = \frac{\text{moles of fluorophore}}{\text{moles of protein}}$$

A_x = fluorescence value at emission maximum wavelength. ϵ = molar extinction coefficient of dye fluorophore.

In our experiments, the labeling efficiency of β arrs was typically in the range of 80–85%.

3 Potential problems, troubleshooting and additional considerations

3.1 If the labeling efficiency is sub-optimal, a number of troubleshooting steps can be implemented.

3.1.1 Carefully assess the buffer conditions of the protein and carry out additional dialysis steps or size exclusion chromatography.

3.1.2 It is advisable to use freshly prepared mBBr solution as stored stocks may not be very stable over time. Additionally, multiple freeze-thaws of mBBr stocks should be avoided.

3.1.3 If the protein of interest exhibits aggregation or precipitation during labeling reaction, the molar ratio of mBBr:protein should be titrated and optimized accordingly.

3.2

It is advisable to avoid excess labeling of protein with mBBr as it may alter the structural and functional properties of the protein. It is also advisable to validate the functionality of mBBr-labeled protein before using it in subsequent experiments.

Acknowledgments

The research program in Dr. Shukla's laboratory is supported by an Intermediate Fellowship of the Wellcome Trust/DBT India Alliance Fellowship (grant number IA/I/14/1/501285) awarded to A.K.S.; the Science and Engineering Research Board (SERB) (EMR/2017/003804); Innovative Young Biotechnologist Award from the Department of Biotechnology (DBT) (BT/08/TYBA/2014-3); and the Indian Institute of Technology, Kanpur. A.K.S. is an Intermediate Fellow of Wellcome Trust/DBT India Alliance, EMBO Young Investigator, and Joy Gill Chair Professor. H.D.-A. and A.S. are supported by DBT-BioCaRE (BT/PR31791/BIC/101/1228/2019) and Early

Career Fellowship of the Wellcome Trust/DBT India Alliance Fellowship (grant number IA/E/17/1/503687) respectively.

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Fig. 1. Site-specific labeling of proteins with monobromobimane (mBBR) for biophysical studies. (A) Chemical structure of mBBR drawn using Marvin JS online tool. (B) A schematic representation of typical bimane-labeling using free thiol group on the protein of interest. (C) Schematic representation of bimane fluorescence assay as a readout of the core interaction between a GPCR and β arr1 labeled in its finger loop as a case example. Here, mBBR is conjugated to Cys⁶⁸ position in the finger loop of β arr1. Upon interaction of β arr1 with activated GPCR, the finger loop interacts with the receptor-core leading to a change in mBBR fluorescence due to a change in its chemical environment. *This figure is adapted from Kumari, P., Srivastava, A., Banerjee, R., Ghosh, E., Gupta, P., Ranjan, R., Chen, X., Gupta, B., Gupta, C., Jaiman, D., Shukla, A.K. (2016). Functional competence of a partially engaged GPCR-beta-arrestin complex. Nature Communications, 7, 13416.*

Fig. 2.

A schematic flow diagram for step-by-step labeling of proteins with mBBR. Purified protein of interest is incubated with mBBR solution under light-protected conditions for specified period of time followed by quenching the reaction with L-cysteine. Subsequently, unreacted mBBR can be separated either by size-exclusion chromatography or dialysis. The labeling efficiency of the mBBR-labeled protein can be measured with a standard fluorometer using experimental settings described in the text.

Fig. 3.

A typical fluorescence scan profile of bimane-labeled β arr1. Purified β arr1 was labeled with mBBr under indicated experimental conditions followed by fluorescence scan using a standard LS-55 fluorometer in photon counting mode. Fluorescence scan profile of free mBBr and that of non-labeled β arr1 is presented as control. RT= room temperature. *This figure is adapted and modified from a previous study (Kumari, P., Srivastava, A., Banerjee, R., Ghosh, E., Gupta, P., Ranjan, R., Chen, X., Gupta, B., Gupta, C., Jaiman, D., Shukla, A.K. (2016). Functional competence of a partially engaged GPCR-beta-arrestin complex. Nature Communications, 7, 13416.*