Dual Channel Kinetic assays for detecting ligand bias at GPCRs

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- **Genetically-encoded, single-color fluorescent biosensor detects β-arrestin signaling in living cells**
- **β-arrestin sensor multiplexed with Gq sensor simultaneously detect arrestin and G-protein signaling**
- **Kinetic analysis of arrestin and G protein signaling on the CLARIOstar® reliably detects agonist bias**

Introduction

Activated G-protein coupled receptors (GPCRs) can signal through both G-protein and arrestin pathways. Some ligands bias the receptor toward signaling through one or the other pathways which can affect downstream cellular effects[1]. For example, arrestin-biased agonists at the Angiotensin II type 1 (AT1R) receptor reduce arterial pressure and increase cardiac performance while unbiased or G-protein biased ligands fail to improve cardiac performance[2]. The need for better tools to reliably identify and quantify agonist bias inspired us to develop methods that capture signals from β-arrestin and G-protein second messengers in real time. Signals from each pathway can be collected simultaneously and with high fidelity with a BMG LABTECH CLARIOstar plate reader.

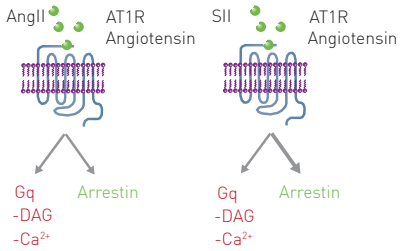

Fig. 1: The AT1R (Angiotensin) receptor is activated by angiotensin II peptide (AngII) or an arrestin biased angiotensin variant (SII). G-protein pathway signalling is detected in red and arrestin pathway in green.

Assay Principle

Temporal patterns of GPCR signaling processes can influence detection and quantification of biased agonism[3] and these patterns vary with different receptors and agonists. However, this "kinetic context" is absent with standard endpoint measurements. To capture second messenger signaling responses over time, we developed red and green fluorescent biosensors[4,5] for β-arrestin, cAMP, DAG, Ca^{2+} and PIP_2. Each biosensor is constructed with a single fluorescent protein that changes in fluorescence intensity when signaling occurs. Red and green biosensors can be combined in a single assay to simultaneously measure response functions of both arrestin and G-protein mediated signals in the same population of cells. We combined a green biosensor for β-arrestin with a red DAG sensor, or R-GECO, a red biosensor for Ca^{2+}, in live cells expressing the Angiotensin receptor AT1R. We activated the receptor with known ligands and monitored fluorescence intensity over time on the BMG LABTECH CLARIOstar microplate reader.

Materials & Methods

- HEK293T cells
- Montana Molecular green β-arrestin sensor, red DAG sensor, and R-GECO calcium sensor.
- Greiner CELLCOAT 96-well microplate, black, µClear bottom (Cat. No. 655946)[1]
- BMG LABTECH CLARIOstar microplate reader
- Angiotensin II and Trevena peptides TRV120026, TRV120045, and TRV120055 were obtained from GenScript. SII was obtained from MyBioSource.

Experimental Procedure

Sensor expression: HEK293T cells were transduced in suspension with BacMam vectors carrying the indicated sensors and plated in 96-well plates. After 24 hrs, cell culture media was exchanged for DPBS and cells were placed at room temperature for 30 minutes prior to drug addition.

Drug addition and dose response: For all experiments, baseline fluorescence measurements were acquired, after which the plate was removed and drug was added using an electronic multichannel pipette. Changes in fluorescence intensity were measured immediately after drug addition and over several minutes to capture response functions for each of five ATR1 ligands.

Instrument settings

Optic settings	Fluorescence intensity with bottom optic		
	Monochro-mator settings	β-Arrestin sensor (Alexa 488 presets)	Ex:488-14 Dichroic:auto Em: 535-50 Gain: appr. 2500
		Red DAG or R-GECO Ca^{2+} sensor	Ex: 566-18 Dichroic:auto Em: 620-40 Gain: appr. 2800
General settings	Number of flashes	40	
	Setting time	0 s	
Kinetic settings	Number of cycles	42-205	
Incubation	25 °C		

Results & Discussion

β-arrestin response was captured over several minutes on a BMG CLARIOstar and is indicated by a robust change in fluorescence intensity and a Z' value of 0.92 (Fig. 2). Dose response measurements were made with two AT1R agonists, generating reliable EC50 values (Fig. 3).

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Keywords: **GPCR; Arrestin; Multiplexing; Calcium; DAG; Biosensor; Second messenger**

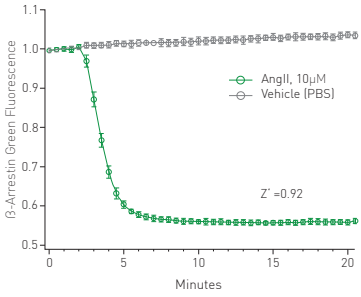

Fig. 2: β-arrestin response in HEK293T cells reports activation of the AT1R receptor following addition of Angiotensin II at t=3 minutes.

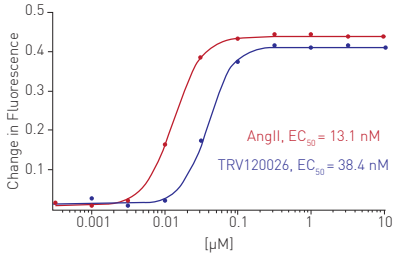

Fig. 3: Dose-responses of AT1R agonosts Angiotensin II and TRV120026, obtained in HEK293T cells with the green fluorescent β-arrestin assay.

Five AT1R receptor agonists (30μM) produce different β-arrestin, DAG, and Ca2+ response functions over time (Fig 4). Kinetic analysis of the response function from each ligand can be used to reliably assess agonist bias[6].

Conclusion

Fluorescent biosensor assays for β-arrestin and G-protein signaling can be combined in living cells and monitored on a BMG LABTECH CLARIOstar with unprecedented precision. The optical path featuring Linear Variable Filter (LVF) monochromators provide both sensitivity and bandwith flexibility that are ideal for detecting genetically-encoded fluorescent biosensors.

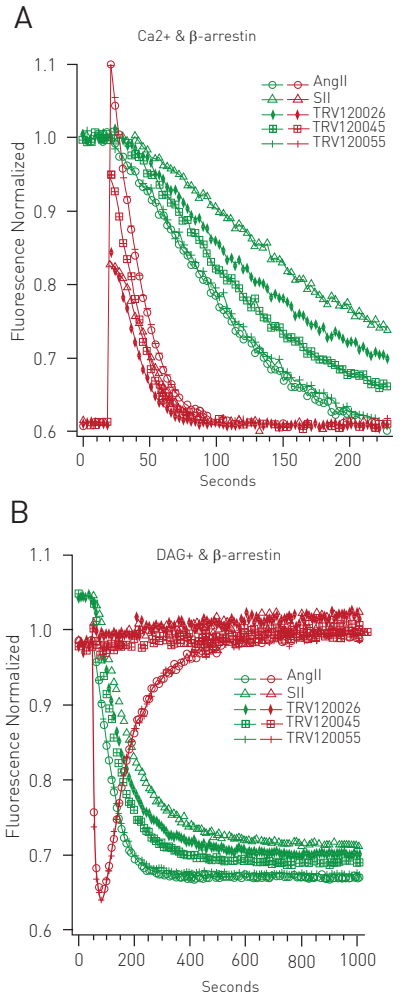

Fig. 4: Differences in signaling activity from known AT1R ligands were captured over time on the BMG CLARIOstar, providing insight into pharmacological differences that are not apparent at assay endpoints. Green β-arrestin and red Ca^{2+} (A) or red DAG (B) responses for each ligand were captured simultaneously in live HEK293 cells.

References

1. Smith, S *et al.* 2018. Nat. Rev. Drug Disc.17: 243-260.
2. Violin, J *et al.* 2010. JPET 335 (3): 572-579.
3. Herenbrink, K.C. *et al.*, 2016. 7, p.10842.
4. Tewson, P. *et al.* 2012. PloS one, 7(8), p.e42791.
5. Tewson, P. *et al.*, 2018. SLAS discovery, 23(9), pp.898–906.
6. Hoare, S.R.J. *et al.*, 2019. https://doi.org/10.1101/772293.