Simultaneous detection of GPCR second messengers in living cells

P. Tewson[1], S. Martinka[1], S. Tillo[1], T. Hughes[1], A.M. Quinn[1], C. Peters[2]
[1]Montana Molecular, Bozeman, MT [2]BMG LABTECH, Cary, NC

- **Quantification of Gq signalling in living cells on BMG LABTECH's CLARIOstar® microplate reader**
- **Multiplexing to monitor signal transduction cascades using kinetic readouts**

Introduction

Live cell assays constructed with genetically encoded, fluorescent biosensors can provide significant advantages over end point assays measured in cell lysates because functional information about the timing and location of cellular responses can be monitored in cells that are relevant to disease. Fluorescent biosensors have been tools in basic research and have now been optimized for detection on automated plate readers.[1] We previously demonstrated that Montana Molecular's live cell cADDIS assay for cAMP on the CLARIOstar produces high Z' values characteristic of a robust screening assay.[2] Here we show how second messengers relevant to the Gq signalling pathway can be detected in living cells using assays for DAG, PIP_2 and Ca^{2+}. By combining spectrally distinct sensors in a single assay, we show that two responses can be detected simultaneously. Sensitive detection of these responses is enabled by employing the CLARIOstar microplate reader using filters or the LVF monochromator.

Assay Principle

Montana Molecular's biosensors achieve optimal expression in mammalian cells due to packaging in a modified baculovirus (BacMam). Figure 1 shows the schematic of the mechanism for the DAG sensor, in which a circularly permuted fluorescent protein is inserted near the DAG binding domain of protein kinase C. Similar sensors are used for detecting Ca^{2+} and PIP_2.

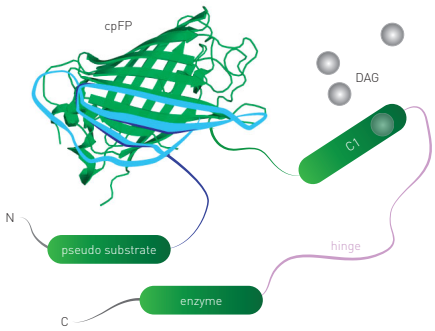

Fig. 1: Schematic of DAG sensor. Upon binding DAG, the sensor undergoes conformational changes that lead to changes in fluorescence intensity of the engineered sensor.

Each sensor comes in either a red or green version which can be spectrally resolved. By combining red and green sensors in a single transduction step, changes in two second messengers can be detected simultaneously. The DAG biosensors are available as decreasing in fluorescence (Downward DAG) or increasing in fluorescence (Upward DAG). Both versions indicate increases in diacylglycerol in living cells.

Gq Detection using DAG, PIP_2, and Ca^{2+} sensors

Montana Molecular has developed a suite of biosensors for the unambiguous detection Gq signalling.[3,4] Figure 2 illustrates hM1R mediated Gq signalling in response to Carbachol. We validated sensors for PIP_2, Diacylglycerol (DAG) and Ca^{2+} on the CLARIOstar microplate reader.

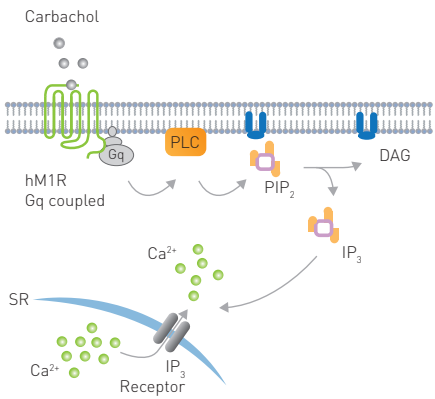

Fig. 2: Signaling from a Gq coupled receptor to PLC after carbachol stimulation leading to production of PIP_2 and subsequently DAG production and Ca^{2+} mobilization

Materials & Methods

- HEK293 cells
- Montana Molecular DAG sensors, PIP_2 sensors, R-GECO Ca^{2+} sensors packaged in BacMam
- Greiner 96-well F bottom plates
- CLARIOstar microplate reader from BMG LABTECH

Transduction of the indicated sensor carried in BacMam was performed on HEK293 cells in suspension. Cells were subsequently plated in 96-well microplates and sensor expression allowed to proceed for 24-36 hours. Thirty minutes prior to the experiment media was exchanged for PBS and cells were allowed to rest at room temperature.

Instrument Settings

Detection Mode:	FI (well mode), bottom optic
Number of flashes:	5
Scan mode:	Orbital
Scan diameter (mm):	2
Gain / Focal height:	Adjusted prior to test run
Interval time:	3-11 s

Optical Settings	*Green*	*Red*
Excitation:	F 482-16	560-15
Dichroic:	LP 504	auto580.5
Emission:	F 530-40	618-49

Injection Settings

Volume (µl)	50
Pump speed (µl/s)	85
Injection start time (s)	30

Results & Discussion

The results in figure 3 show that the DAG sensor exhibits excellent assay performance.

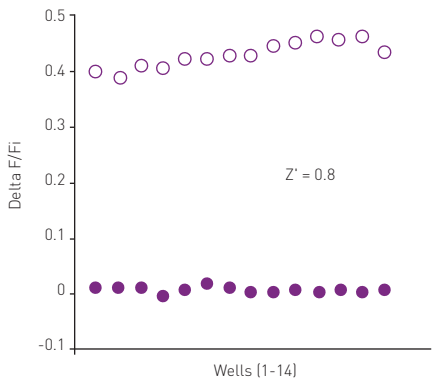

Fig. 3: DAG assay performance. Comparison of 14 replicates treated with Carbachol (○) or vehicle [PBS] (●) indicates robust assay performance. Z' = 0.8.

Figure 4 shows results of a response to carbachol in a multiplexed experiment. The results show the very rapid calcium release as well as rapid production of DAG following compound injection.

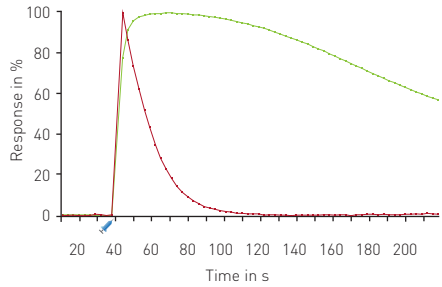

Fig. 4: Multiplexed DAG and Ca^{2+} kinetics. Traces depict the average response to 30 µM Carbachol (n=16) expressed as 100% response. R-GECO (■); green DAG (■). Carbachol was dispensed using on-board reagent injectors at the 30 second time point as indicated.

Figure 5 shows another multiplexed experiment. In this case using the Downward DAG sensor and a sensor for PIP_2.

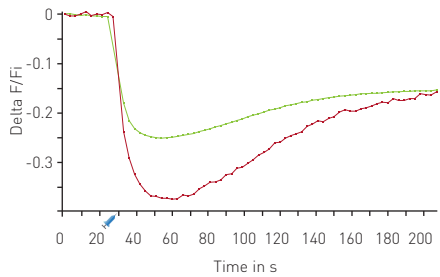

Fig. 5: Multiplexed DAG and PIP_2 kinetics. Traces depict average response to 30 µM Carbachol (n=18). Red DAG (■), green PIP_2 (■). On board reagent injectors dispensed 50 µl of Carbachol after 30 seconds as indicated.

Conclusion

The CLARIOstar supplies high sensitivity detection of the genetically encoded Montana Molecular sensors. Spectrally-resolved variants indicate simultaneous changes in multiple second messengers following Gq activation

References

1. Tewson, P., et al (2016) *J. Biomol. Screen.* **21**:298-305
2. Tewson, P., et al (2016) Real-Time Detection of Gs and Gi Signalling in Living Cells
3. Ding, Y., et al (2015) *Nature Methods* **12**:195-198
4. Tewson, P., et al (2012) Simultaneous Detection of Ca^{2+} and Diacylglycerol Signaling in Living Cells

PHERAstar® *FSX*
PHERAstar® *FS*

CLARIOstar®