Multiplexed Analysis of Second Messenger Signaling in Live Cells Using Aequorin and GloSensor™ cAMP on the Hamamatsu μCell™

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1. Abstract

Detection of intracellular second messenger signaling is an established method for measuring G-protein-dependent GPCR activation. Although there are several technologies available for measurement of second messengers via endpoint analysis, technologies for monitoring second messengers in living cells include Promega's GloSensor™ cAMP for quantifying intracellular [cAMP] and technologies such as the photoprotein Aequorin or various fluorescence-based indicators for [Ca2+]. These technologies serve to quantify second messengers in live cells and in real-time following GPCR activation, providing several advantages over lytic endpoint assays. However, it may be challenging when screening for GPCR activity modulators when G-protein-dependent signaling is uncharacterized or when the desired second messenger detection format cannot be predicted (for example, in the case of orphan receptors). Furthermore, for GPCRs capable of modulating both [cAMP] and[Ca2+] pathways concurrently, it would be desirable to measure G protein coupling simultaneously. Few technologies exist that allow for simultaneous measurement of Ca2+ and cAMP in live cells, while maintaining assay robustness and high signal-to-background for use in HTS. To address this limitation, Promega has developed a live cell method for the kinetic measurement of Ca2+ and cAMP by multiplexing of Aequorin and GloSensor™ cAMP bioluminescent sensor technologies. Using the Hamamatsu FDSS/μCell, we report simultaneously analysis of Ca^{2+} and cAMP mobilization following agonism of Parathyroid Hormone Receptor (PTH1R) using a promiscuous compound directing both $G\alpha_q$+$G\alpha_s$ signaling, as well as a biased compound specifically directing $G\alpha_s$ coupling alone. The combination of these bioluminescence-based sensor technologies with the Hamamatsu FDSS/μCell serves as an ideal platform for the analysis of these divergent second messenger signaling events in live cells and in real time.

2. Glosensor™ cAMP Assay

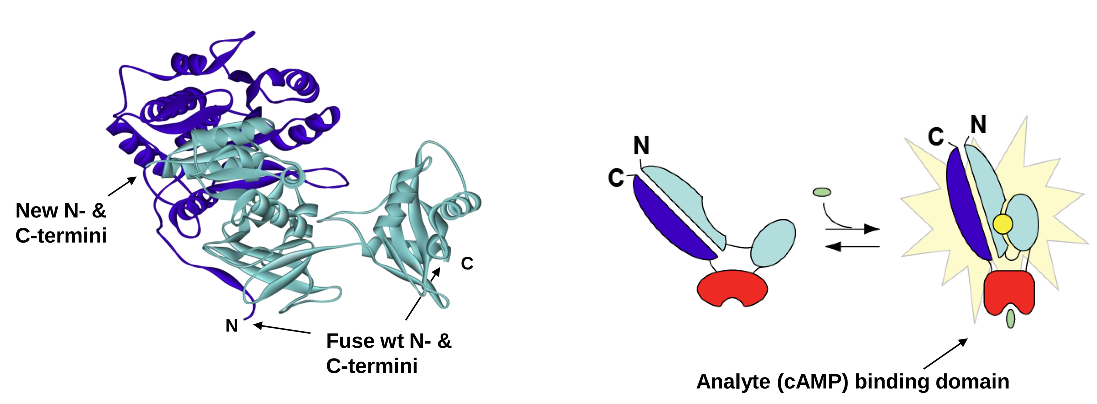
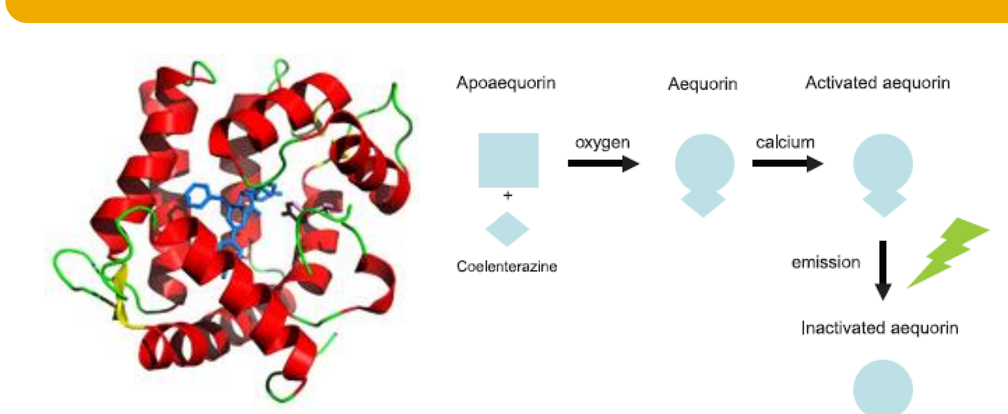


Diagram of Glosensor™ cAMP Activation. The assay is based on the GloSensor™ Technology, a genetically modified form of firefly luciferase which has been modified with a cAMP-binding protein moiety inserted into unique N and C termini. Upon binding of cAMP, conformational change is induced leading to increased luciferase activity.

- **Live Cell Assay**: Excels at kinetic and modulation studies of $G\alpha_s$-coupled receptors signaling through cAMP.
- **Transient or Stable Expression**: GloSensor™ cAMP Assay is utilized by transiently expressing a receptor of interest and the biosensor in the cell line of choice. Alternatively, stably transfected cell lines with both the biosensor and the receptor of interest can be made.
- **Simple Protocol**: Cells are pre-equilibrated with GloSensor™ cAMP Reagent , then cells are treated with specific agonists/antagonists or compounds, and luminescence is measured in real-time (typically 10-30 minutes).

3. Aequorin Assay for $[Ca^{2+}]$

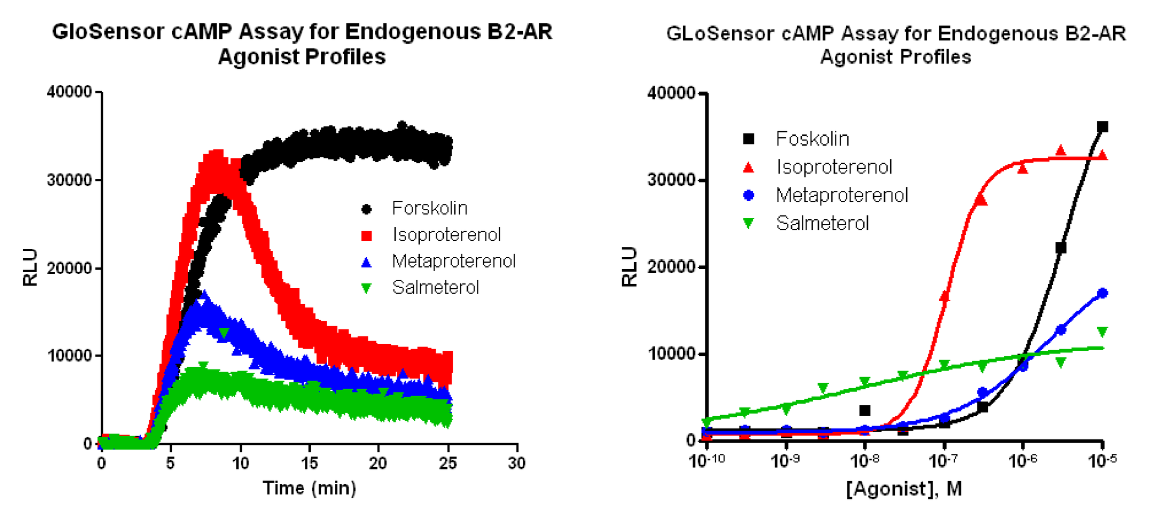


Reference: R.M.Eglen, Assay and Drug Development Technologies , 2008

Diagram of Ca2+-mediated Aequorin Activation. Photoproteins such as Aequorin are widely used for measuring rapid GPCR-induced , transient changes in [Ca2+] from $G\alpha q$-coupled receptors. Aequorin is composed of two distinct units which reconstitute spontaneously, providing a method to quantify changes in [Ca2+] in live cells.

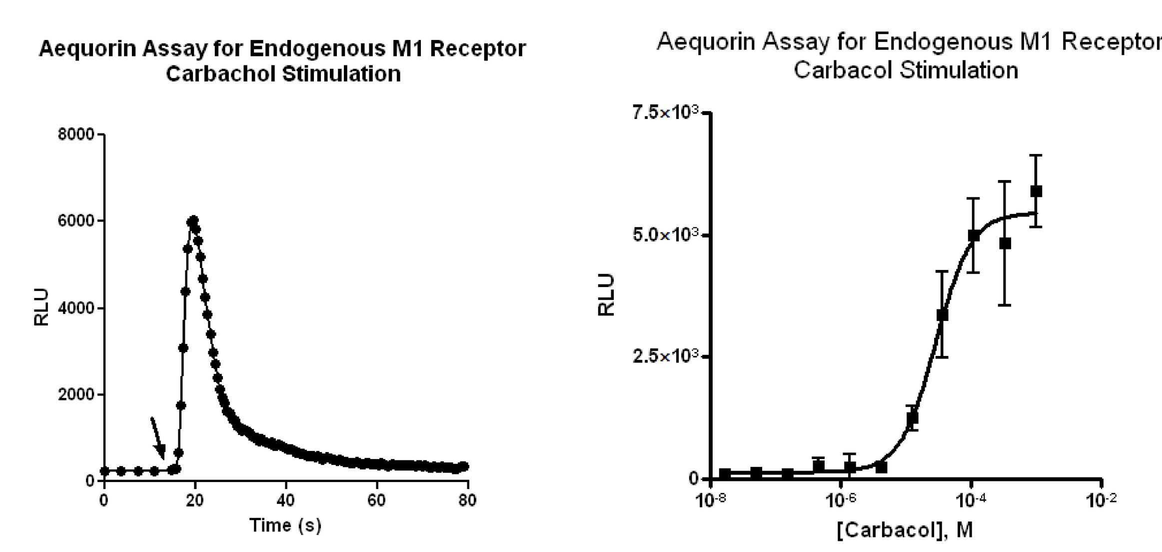
- **Live Cell Assay**: Apoaequorin binds Coelentrazine to produce Aequorin in live cells. When Ca2+ binds Aequorin, the protein undergoes conformational change, resulting in oxidation of coelentazine to the exited form coelentramide.
- **Fast Kinetics**: As an exited coelentramide relaxes to the ground state blue light at wavelength 470 nm is emitted. The intensity of light emission can vary but typically occurs within seconds, enabling a live cell endpoint that can be resolved over time with GloSensor™ cAMP
- **Simple Protocol**: Cells are pre-equilibrated with coelenterazine, then cells are stimulated and luminescence is measured within seconds.

4. Glosensor™ cAMP Assay: Representative Data for $G\alpha_s$ Signaling



Materials and Methods. Left: Kinetic measurement of agonist-induced cAMP mobilization in HEK293 cells stably expressing GloSensor™ cAMP. (L9) In 384 format, cells were preincubated with CloSensor™ cAMP substrate for 1.5h prior to stimulation. Luminescence was measured on the Hamamatsu μCell with 3s of integration time. **Right:** Dose-response measurement of agonist-induced cAMP mobilization under similar conditions (7.5 minute timepoint).

5. Aequorin Assay: Representative Data for $G\alpha_q$ Signaling

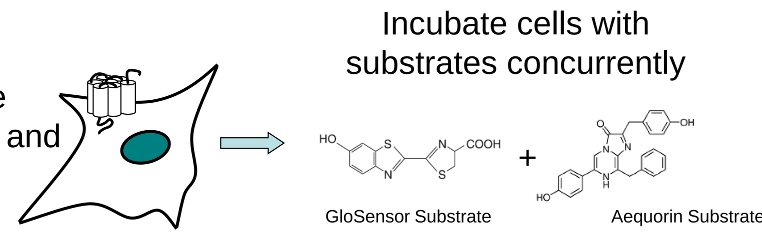


Materials and Methods. Left: Kinetic measurement of Carbacol-induced Ca2+ mobilization in HEK293 cells. Cells were transfected with plasmid DNA encoding Aequorin and seeded in 96-well plates. Following >24h of transfection, cells were preincubated with Aequorin substrate for 3h prior to Carbacol stimulation (0.5s of luminescence integration time). **Right:** Dose-response measurement of Carbacol-induced Ca2+ mobilization as described but at a 3s timepoint.

6. Simple Workflow for Multiplexing of Aequorin / GloSensor™ cAMP Using The Hamamatsu μCell

Transient or stable expression of Sensors and GPCR of interest

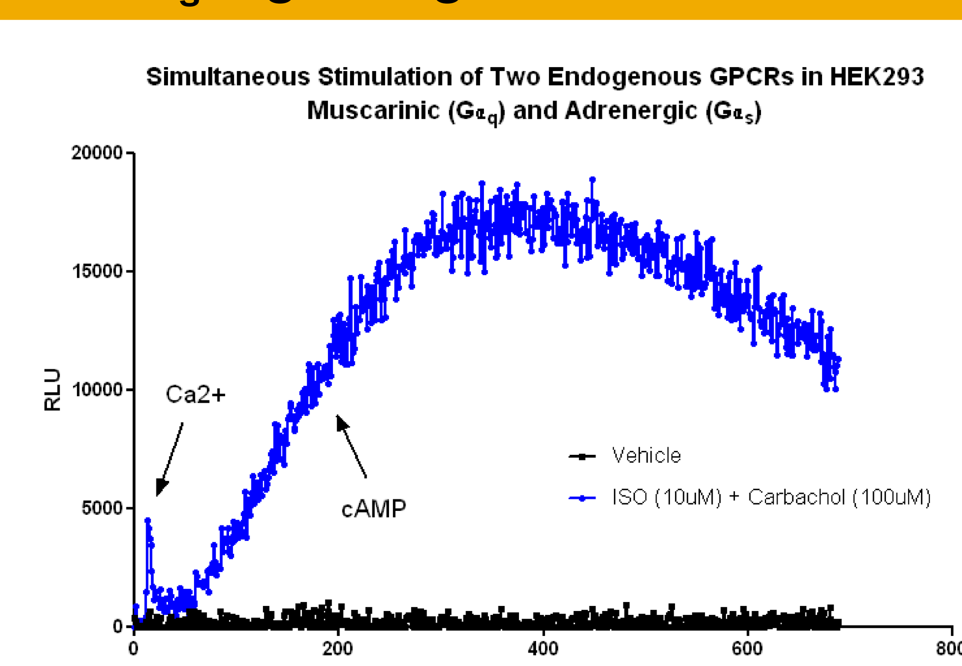
Incubate cells with substrates concurrently



Stimulate cells in 384 format using 384-channel dispenser

Simultaneously gather "flash" and "Glo" Luminescence in kinetic mode using ccd camera

7. Simultaneous measurement of $G\alpha_q$ and $G\alpha_s$ signaling from two GPCRs



Materials and Methods: HEK293 cells were transiently-transfected with plasmid DNA encoding using Aequorin and GloSensor™ cAMP 22Fand seeded in 384-well, clear-bottom plates. 24 h post-transfection, cells were preincubated with GloSensor™ cAMP substrate and coelenterazine for approximately 3 hours. Cells were then stimulated with Isoproterenol + carbacol or vehicle. Luminescence was then measured on a Hamamatsu μCell using 1s of luminescence integration.

7. Biased Agonism at PTH1R

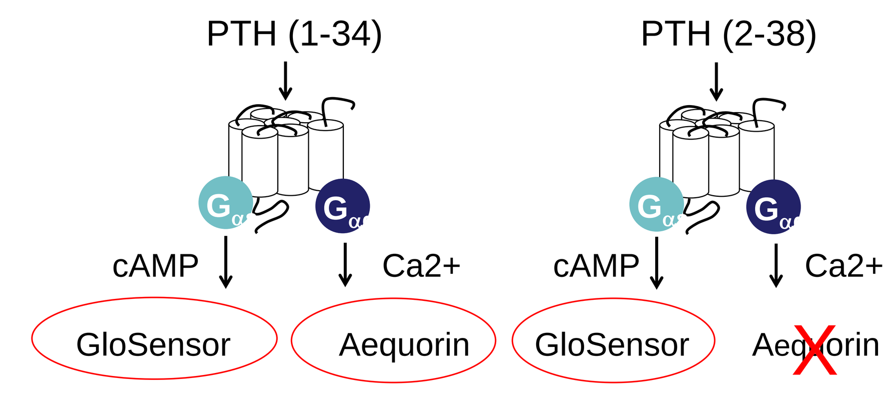
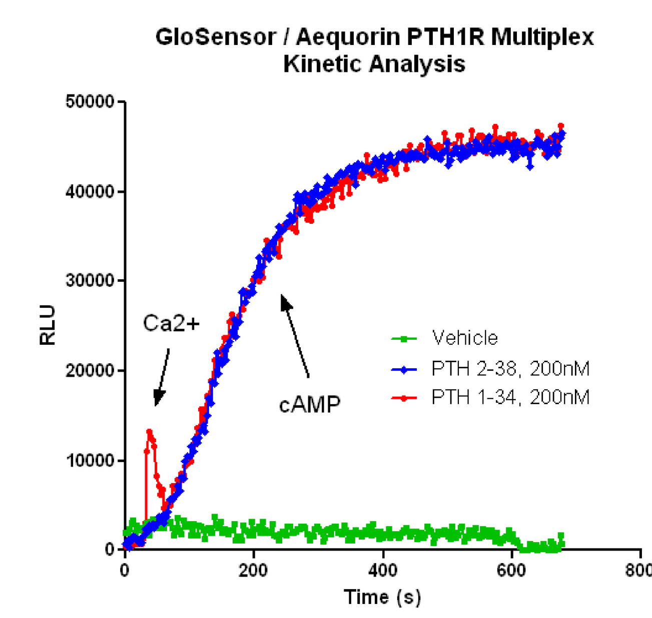


Diagram of biased agonism at Parathyroid Hormone Receptor (PTH1R) using a non-selective and $G\alpha_s$-selective peptide agonist. Peptide agonist PTH(1-34) is expected to activate both $G\alpha s$ and $G\alpha q$ pathways, leading to concurrent activation of both cAMP and Ca2+. Amino-terminal truncation of PTH peptide (PTH-2-38) is expected to induce cAMP pathway selectively. GloSensor™ cAMP and Aequorin can therefore be used to query these distinct signaling mechanisms.

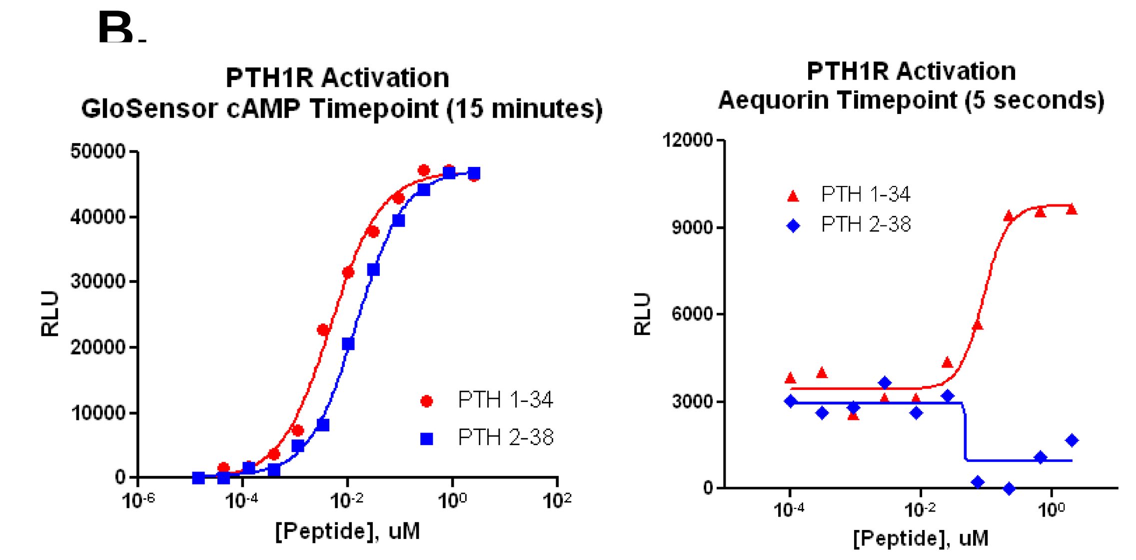
Reference: Takasu H et al. Biochemistry. 1999 Oct 12;38(41):13453-60.Amino-terminal modifications of human parathyroid hormone (PTH) selectively alter phospholipase C signaling via the type 1 PTH receptor: implications for design of signal-specific PTH ligands.

8. Multiplexing of $[Ca^{2+}]$ and [cAMP] Signaling from PTH1R

A.



B.



Simultaneous multiplexing of Ca2+ and cAMP mobilization using GloSensor™ cAMP and Aequorin. Materials and Methods: HEK293 cells were triple-transfected with plasmid DNAs encoding PTH1R, Aequorin, and Glosensor™ cAMP 22F using Fugene HD ,and seeded in clear-bottom 384-well plates. 24h posttransfection, cells were preincubated with Glosensor™ substrate and coelenterazine for 3h. **A.** Kinetic Analysis of PTH1R activation using a peptide agonist activating $G\alpha s$+$G\alpha q$ or an agonist selectively activating $G\alpha s$ only. Cells were then stimulated with 200 nM PTH(1-34) peptide, PTH(2-38) peptide, or vehicle. Kinetic analysis of luminescence was performed on the Hamamatsu μCell using 3s of luminescence integration time. **B.** Simultaneous dose-response profiles for PTH1R activation of cAMP after 15 min (left) or Ca2+ after 5s (right).

9. Conclusions

Glosensor ™ cAMP and Aequorin are complementary bioluminescent technologies for multiplexing of second messenger signaling.

- Kinetic measurements of Ca2+ and cAMP can easily be multiplexed on the Hamamatsu FDSS/ μCell in an HTS-compatible format
- The method described is compatible via transient transfection of DNA encoding the biosensors and the GPCR of interest (no stable cell lines required)
- Two endogenous GPCRs can be measured in a single well, offering a unique solution for analysis of GPCR signaling when G-protein couplings are not fully characterized
- Multiplexing of Aequorin/GloSensor™ cAMP enables a novel approach to GPCR functional selectivity studies

Questions or comments?
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