High-throughput Live Cell Imaging and Analysis for Temporal Reaction of G Protein-coupled Receptor Based on Split Luciferase Fragment Complementation

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For the design of therapeutic drugs, G protein coupled receptors (GPCRs) are notable targets. Many screening methods have been developed to identify effective agents for GPCR signaling. However, analyses of temporal variations of GPCR activity with specific ligands remain insufficient because of monitoring method limitations and difficulties. We previously developed a high-throughput bioluminescence measuring system to detect interactions of GPCR with β -arrestin based on split luciferase fragment complementation. By newly introducing a bioluminescence imaging technique into the system, we demonstrate a method for the temporal monitoring of GPCR- β -arrestin interactions in living cells during stimulation by different ligands.

Keywords GPCR, bioluminescence, luciferase, imaging, protein complementation assay

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Introduction

Discovery of unidentified drugs targeting signal transduction in cells is a principal strategy of pharmacotherapy for several diseases for which no cure exists. The largest transmembrane spanning receptor protein family in humans, G protein-coupled receptors (GPCRs),¹ are related to various physiological functions as a signal converter.^{2,3} Each GPCR protein is activated by extracellular stimuli of several types, thereby eliciting intracellular downstream signals.⁴ Because of their fundamental roles in cells, GPCRs have been centrally positioned as a drug discovery target.⁵ In practice, more than 40% of curative drugs currently under investigation are associated with GPCR signal transduction.⁶

Various strategies to identify GPCR signals are applied to screen effective agents for GPCR from numerous chemical and clinical compound libraries.⁷ Most such strategies specialize in high-throughput measurements that simply judge whether the unknown compound activates or inhibits the target GPCR signal or not. However, the reaction profile of GPCR to the specific ligand differs depending on the type of GPCR and the cellular environment.⁸ Moreover, information related to the temporal variation of the GPCR signal is intrinsically important for the understanding of physiological drug effects. Therefore, time-course analysis of GPCR in living cells is necessary for drug evaluation, although the data remain insufficient because of methodological difficulties.

In several approaches for measuring GPCR signals, split luciferase fragment complementation assays are notable for monitoring GPCR reactions in living cells.^{9–11} The principle is based on genetically encoded luciferase fragment probes fused

with GPCR and β -arrestin, which is a cytoplasmic protein interacting with GPCR for desensitization. Because the interaction of GPCR with β -arrestin induces luciferase fragment complementation on the probes, the timing of interactions can be detected by bioluminescence. Therefore, the method has benefits for monitoring the temporal variation of GPCR activity in living cells. However, in high-throughput analysis, the measuring tool is generally limited by a luminescence plate reader with a photomultiplier, which makes it difficult to measure the multiple samples without a time lag.

This study examined a novel approach for the measurement of GPCR and β -arrestin interactions based on luciferase fragment complementation assays on a 96-well plate. To achieve simultaneous measurement of GPCR reactions in different ligand conditions, a highly sensitive luminescence imaging system was introduced as a detector. We measured the interaction of GPCR with β -arrestin in live cells with temporal-dependent information.

Experimental

Mammalian expression vectors

Constructions of the cDNA encoding β 2-adrenergic receptor (ADRB2) fused with C-terminal of Emerald Luciferase (ELuc) (394 to 542 amino acids) or β -arrestin2 fused with N-terminal of ELuc (1 to 415 amino acids) were established in our earlier report.¹⁰ Mammalian expression vectors, pcDNA4/V5-His B and pcDNA3.1/myc-His B (Invitrogen Corp., Carlsbad, CA) for insertion of these cDNAs were purified using a PureLink HiPurePlasmid Filter Midiprep kit (Invitrogen Corp.).

Cell culture and transfection of DNA

Human embryonic kidney (HEK293) cells were cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal

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bovine serum (FBS), penicillin and streptomycin (P/S) at 37°C with 5% CO₂. Transfection of plasmid DNAs into the cells was done using a reagent with Lipofectamine2000 (Invitrogen Corp.). The cells were screened with 0.8 mg/mL of Geneticin (G418) and 0.5 mg/mL of Zeocin in the medium. The resistant cells were isolated as stable expression cells.

Bioluminescence imaging assay with 96-well plate

HEK293 cells stably expressing the GPCR luminescence probes were cultured overnight on a 96-well plate with 100 µL/well of DMEM supplemented with 10% FBS and P/S at 37°C with 5% CO₂. The medium was exchanged to 100 µL/well of Hank's balanced salt solution (HBSS) with 10% FBS and 10 mM (M = mol/L) of D-luciferin (Wako Pure Chemical Industries Ltd., Osaka, Japan). The cells were incubated on the stage of the imaging system at RT for 10 min. Some wells were treated preliminarily with propranolol (Wako Pure Chemical Industries Ltd.). Subsequently, isoproterenol (Wako Pure Chemical Industries Ltd.) was added directly to each well at different concentrations.

Bioluminescence imaging system

Bioluminescence images were obtained using a multi-functional *in vivo* imaging system (Miis; Molecular Devices Corp., Tokyo, Japan). An electron-multiplying CCD camera (EM-CCD, gain set to 800, iXon Ultra888; Andor Technology) was selected as a bioluminescence detector. Region dependent differences of luminescence count were kept within $\pm 5\%$. Time-lapse bioluminescence images were acquired every 100 s, with 10 s exposure for each. The air temperature of the dark box was maintained at 24°C. All imaging processes and analyses of luminescence counts were conducted using software (Metamorph; Molecular Devices Corp.).

Results and Discussion

We established a bioluminescence system to detect the interaction of GPCR with β -arrestin by split luciferase fragment complementation (Fig. 1A).^{8,10-12} For construction of a pair of probes, the C-terminal of fragment of Luciferase (LucC) is fused with GPCR. The N-terminal fragment of Luciferase (LucN) is then fused with an isoform of β -arrestin, β -arrestin2 (ARRB2). The HEK293 cells, which stably express the probe pair, are stimulated with an agonist in a dose-dependent manner. The luminescence on the 96-well plates is measured. These luminescence data are acquired at fixed time points by lysing cells. A conventional luminescence plate reader with a photomultiplier is used as a detector. Therefore, the standard protocol for the measurement of 96-well plates is performed while reading each well sample in turn. This procedure consequently generates severe time lags among the samples. Particularly, in split luciferase fragment complementation assays, longer exposure time is necessary to detect the signal because of weak luminescence. The time lag presents a risk of overlooking important information from the luminescence profile.

To overcome those shortcomings and difficulties, we introduced a luminescence imaging system that is ordinarily used for *in vivo* imaging to monitor the luminescence from living cells on a 96-well plate (Fig. 1B). As a detector, the EM-CCD camera was set in this system. Such a camera has already been used in microscopic systems for single-cell luminescence imaging of GPCR- β -arrestin2 interactions,^{10,11} but no report in the literature describes its use for bioluminescence

Fig. 1 Establishment of temporal bioluminescence imaging analysis for monitoring of GPCR reactions. (A) Schematic model and construction of probes in split luciferase fragments complementation assay to detect the interaction of GPCR with β -arrestin2. The N-terminal and C-terminal fragments of luciferase are fused respectively with β -arrestin2 (ARRB2) and GPCR. These probes were developed in an earlier study.¹⁰ (B) Technical procedures of temporal bioluminescence imaging. Cells were incubated on a 96-well plate and were stimulated by GPCR ligand. Time-course variation of bioluminescence was detected continuously using an EM-CCD camera.

imaging of a 96-well plate. Using the EM-CCD camera with a wide-field lens, the entire luminescence image of the 96-well plate can be detected. The luminescence count of each well is extracted from the interest region of images. Accordingly, simultaneous imaging of multiple samples resolves the difficulty posed by the detection time lag.

To demonstrate the potential of this new strategy, dose-response analysis for GPCR activity based on split luciferase fragment complementation was performed using the imaging system. Because several types of luciferases are available, each having different luminescence intensity and wavelength, we needed to select the best one for real-time imaging that reflected the temporal information. The absolute luminescence intensity of luciferase is correlated directly with the detection time of the image. In addition, luciferase types using substrate D-luciferin show stable luminescence photon counts in sustained measurement.¹³ Therefore, ELuc, which is an extremely bright luciferase using D-luciferin, was chosen for the imaging assay.¹⁴ In an earlier study using ELuc, we demonstrated the potential of a split luciferase fragment complementation assay with GPCR protein, β 2-adrenergic receptor (ADRB2), and β -arrestin2.¹⁰ The luminescence counts increased concomitantly with the dose of the agonist, isoproterenol (Iso). From the report, HEK293

Fig. 2 Bioluminescence images of living cells on a 96-well plate. HEK293 cells stably expressing ELucN-ARRB2 and ADRB2-ELucC probe pair were cultured on a 96-well plate. The luminescence from cells was detected temporarily by an EM-CCD camera after isoproterenol (Iso) and propranolol (Prop) dosing. These luminescence images from three time points (0, 15, and 30 min) are displayed as pseudo-colors. The D-luciferin (D-luc), Iso, and Prop concentrations in each well are shown at the sides of the images.

cells stably expressing the probe pair (ELucN-ARRB2 and ADRB2-ELucC) were cultured on a 96-well plate and were incubated with D-luciferin (Fig. 2). Some wells were treated preliminarily with competitive inhibitor of Iso, propranolol (Prop), of which the concentration was increased stepwisely among rows of the well. Continuous luminescence images of the plate were taken using the EM-CCD camera after the stimulation of Iso (Fig. S1, Supporting Information). The results demonstrated that 50 wells of samples in all were observed simultaneously for the variation of luminescence along the time, enabling acquisition of the image from each time point within 10 s. Therefore, in contrast to a conventional plate reader, this new imaging strategy shortens the total measuring time remarkably.

The simultaneous measurement of wells enables analyses of different samples in the same time course. To compare the temporal changing of ADRB2- β -arrestin2 interaction in different concentrations of Iso, the luminescence photon counts were calculated from each well region (Figs. 3A and S2A (Supporting Information)). Cells without stimulation or with 1×10^{-8} M of Iso showed no increase in the counts during the observation. Over 1×10^{-7} M of Iso induced a time-dependent increase of luminescence, which reached the peak within 50 min. The photon counts in the peak were found to be six-fold higher than

Fig. 3 Temporal variation of bioluminescence that responds to the interaction frequency of ADRB2 and β -arrestin2. All data were measured from results portrayed in Fig. 2. (A) Time-course analysis of ADRB2- β -arrestin2 interaction in different concentrations of Iso, blue filled circle, no addition of Iso; red open circle, 1×10^{-8} M of Iso; green filled square, 1×10^{-7} M of Iso; purple open square, 1×10^{-6} M of Iso; blue filled triangle, 1×10^{-5} M of Iso. Data were measured from five regions of wells that were reacted under 2 mM of D-luc without Prop. (B) Time-course analyses of inhibition for Iso in the presence of several concentrations of Prop, blue filled circle, no addition of Prop; red open circle, 1×10^{-9} M of Prop; green filled square, 1×10^{-8} M of Prop; purple open square, 1×10^{-7} M of Prop; blue filled triangle, 1×10^{-6} M of Prop. Data from wells treated with 2 mM of D-luc and 1×10^{-5} M of Iso were selected for measurements.

those in the initial time points, which showed agreement with the result obtained using a conventional plate reader.¹⁰ Additionally, the temporal patterns of luminescence appeared to change for each concentration of Iso. Inhibition for Iso in the presence of Prop was also analyzed from the results of temporal imaging (Figs. 3C and S2B (Supporting Information)). Over 1×10^{-7} M of Prop inhibited the reaction of Iso remarkably. The inhibitory effects were not displayed at 1×10^{-8} M and less. Unexpectedly, these concentration conditions caused higher luminescence intensities than those without Prop. Moreover, some time points showed acceleration of the luminescence. These results revealed that the effects of inhibitor for GPCR agonist varied dynamically according to the temporary concentration.

Conclusions

We demonstrated real-time bioluminescence imaging for the monitoring of GPCR activation in living cells. Using an

EM-CCD camera, numerous cell samples were visualized simultaneously to assess the time-dependent variation of luminescence. These data suggest that the temporal patterns of GPCR activities are sensitive to the concentrations of GPCR agonist and the inhibitor. Time-course information from the imaging strategy is expected to be useful to evaluate the drug performance of GPCRs in living cells.

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Supporting Information

Figures S1, S2 and brief description of this material. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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