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Notes & Tips

A novel label-free live-cell biosensor for G-protein-coupled receptor functional assay with enhanced sensitivity

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ABSTRACT

This study developed a surface plasmon resonance (SPR)-based live-cell biosensor with enhanced sensitivity for label-free ligand binding assay of G-protein-coupled receptors (GPCRs). The β2-adrenoceptor was heterologously expressed in human embryonic kidney-293 cells. The specific ligand binding function of expressed β2-adrenoceptor was monitored by SPR via refractive index measurement. The results indicate the expressed β2-adrenoceptor can respond to isoprenaline with high specificity. The SPR signals can be enhanced more than three times by the use of LY294002. This biosensor can be applied in the functional assay of GPCRs by detecting the specific interactions between GPCRs and their target ligands.

G-protein-coupled receptors (GPCRs) are characterized by seven *trans*-membrane-spanning domains, which form one of the largest known families of membrane receptors. There are more than 700 GPCR sequences that have been identified in the human genome [1,2]. GPCRs are highly specialized integral membrane receptors for cellular signal transduction, which play a key role in many physiological or disease-related processes. GPCRs, whose ligands are various and include hormones, neurotransmitters, peptides, odorants, and light, mediate a diverse array of cellular functions by converting extracellular information into intracellular signals [3]. Because of their decisive importance in cellular responses, GPCRs have become one of the research hot spots in many fields such as cell signaling, drug discovery, and physiology [4–6]. However, many GPCRs are still orphan receptors, whose ligands or functions are still unknown and need to be further investigated. For example, one-third of drug target GPCRs and most olfactory receptors that belong to the large family of GPCRs are still orphan receptors [7–9]. The currently performed methods for ligand binding assays of GPCRs are mostly cell-based assays, which usually require complicated, expensive, and time-consuming labeling to achieve sufficient cellular responsive signals [10,11]. Therefore, it is highly necessary to develop novel tools and approaches for label-free functional ligand binding assay of GPCRs with enhanced sensitivity.

Various techniques and devices have been used in label-free live-cell analysis, such as field-effect transistor, quartz crystal microbalance, and microelectrode array. Among them, surface plasmon resonance (SPR) is one of the most attractive techniques. SPR holds the advantages of simplicity, low cost, high sensitivity, and rapid response, which can realize real-time and label-free dynamic detection. SPR is an optical detection technique based on the evanescent wave phenomenon. SPR measurements are often performed in the Kretschmann configuration, where one face of a prism is coated with a thin metal film [12]. Sensing material is immobilized on the surface of thin metal film. The refractive index at which surface plasmon resonance is achieved is often used as the analytical parameter to investigate changes in the physical properties of the sensing layer. SPR has been widely applied in the research of molecular interactions between GPCRs and their ligands [13–17]. SPR-based biosensors are capable of performing real-time and label-free analysis of molecular binding events occurring on the sensor surface [18–21], although the evanescent field on the SPR sensor surface is limited in the range of several hundreds of nanometers, which means that the SPR is not suitable for sensing signals beyond this range. However, recent reports demonstrated that SPR can be successfully applied in the analysis of live-cell responses derived from the intracellular signaling of the specific binding of a cell membrane receptor with its ligand [22–26]. This makes it possible for SPR to be applied in the live-cell analysis of GPCR function via intracellular signaling.

This study developed a novel SPR-based live-cell biosensor using heterologously expressed GPCRs as sensitive elements for the ligand binding assays. In addition, an intracellular signaling-based method for enhancing SPR signal amplification is proposed for the first time. The specific inhibitor of phosphatidylinositol 3-kinase (PI3K), LY294002, was used as an enhancer for GPCR intracellular signaling [27]. A commonly used GPCR, β2-adrenoceptor, was used as a model for GPCRs and was heterologously expressed on the plasma membrane of human embryonic kidney (HEK)-293 cells [28,29]. Cellular responses were monitored by recording the changes in refractive index of SPR. The sensitivity and specificity of this biosensor were investigated as a GPCR-based biosensor. The enhancing effect of LY294002 on the biosensor sensitivity was studied. This biosensor could be used as a novel tool for label-free functional ligand binding assay of GPCRs.

To express β2-adrenoceptor on the plasma membrane of HEK-293 cells, an expression plasmid was constructed. Briefly, PCR was performed in a 50-μl reaction volume to obtain the full-length sequence of β2-adrenoceptor

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from genomic human DNA using Pyrobest DNA polymerase (Takara, Japan). A rho-tag sequence was inserted into the N-terminus of β2-adrenoceptor. The rho-tag sequence was synthesized by Takara and inserted into the multiple cloning site of pFLAG-CMV-3 (Invitrogen, USA) between *Hin*dIII and *Eco*RI. The full-length sequence of β2-adrenoceptor was then subcloned into pFLAG-CMV-3/rho-tag digested with *Eco*RI and *Bam*HI. Finally, the constructed expression plasmid pFLAG-CMV-3/rho-tag/β2-adrenoceptor was sequenced to confirm the correction of the sequence.

For transfection, HEK-293 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml), in 5% CO_2 incubator. One day before transfection, HEK-293 cells were seeded on six-well plates. Lipofectamine 2000 (GIBCO) was used as the DNA carrier. When 80% confluent, the HEK-293 cells were transfected with 4 μg of constructed plasmid DNA for each well. The cells were incubated with the mixture for 6 h at 37 °C. The transfected cells were used for the selection of stable clones by Geneticin (G418) at a final concentration of 0.5 mg/ml.

GPCRs are integral membrane receptors, which contain seven *trans*-membrane domains [7]. The hydrophobic environment of the cell membrane is important to the maintenance of the function of GPCRs. To assay the function of GPCRs, it is necessary to express GPCRs on the plasma membrane of a heterologous cell system. In this study, a commonly used cell expression system, HEK-293 cells, was employed as a heterologous cell system for the expression of GPCRs. β2-Adrenoceptor was selected as a model of GPCRs and expressed on the plasma membrane of HEK-293 cells. To visualize the subcellular distribution of expressed β2-adrenoceptor, the rho-tag sequence was inserted into the N-terminus of β2-adrenoceptor [30].

The specific anti-rho-tag antibody labeled with fluorescence was used to visualize the cell surface expression of β2-adrenoceptor. Fluorescent staining experiments were performed to determine whether the expressed β2-adrenoceptor was distributed on the cell surface. For fluorescent staining experiments, transfected HEK-293 cells were seeded on six-well plates and fixed with a freshly prepared mixture of methanol:acetone (1:1) for 5 min at room temperature. After being washed with phosphate-buffered saline (PBS; pH 7.4), the cells were incubated with anti-rho-tag M2 monoclonal antibody–FITC conjugate (Sigma–Aldrich, USA) at 10 μg/ml for 1 h at 4 °C. To visualize the surface expression of β2-adrenoceptor, cells were washed twice with PBS and examined under a confocal fluorescence microscope (Leica TCS-SP, Germany) with an excitation wavelength of 492 nm and an emission wavelength of 520 nm. The results indicate the high expression efficiency of β2-adrenoceptor on the plasma membrane of HEK-293 cells.

To test the function of expressed β2-adrenoceptor, intracellular calcium assays were performed using Fluo-3/AM (Invitrogen) as a Ca^{2+} indicator to monitor the changes in intracellular Ca^{2+} concentration under laser confocal fluorescence microscopy (Olympus Fluoview 300, USA). The responses of HEK-293 cells expressing β2-adrenoceptor to isoprenaline, noradrenaline, and phenylephrine were tested at the concentration of 100 nM. HEK-293 cells without any transfection were tested as negative control cells. Upon application of isoprenaline to HEK-293 cells transfected with plasmids encoding β2-adrenoceptor, a transient increase in intracellular Ca^{2+} was observed. We calculated the amplitude of the increase in intracellular Ca^{2+} as a measure of the cell response. The experimental results showed that HEK-293 cells with β2-adrenoceptor have significantly higher responses to isoprenaline, but no similar response could be observed when other stimuli were applied. In addition, negative control HEK-293 cells showed no significant calcium response to any stimuli tested.

GPCRs can respond to extracellular stimuli and convert the extracellular signals into cellular responses via an intracellular signal transduction pathway. Fig. S1a shows the schematics of a typical intracellular transduction pathway of GPCRs. The specific binding of GPCRs with their ligands will initiate a cascade of intracellular biochemical reactions. The initial step is the activation of specific G proteins, which can then activate phospholipase C. This will lead to an increase in intracellular concentration of inositol 1,4,5-triphosphate (IP_3). The increased IP_3 will lead to the release of Ca^{2+} from intracellular Ca^{2+} stores. This cascade of enzymatic reactions will ultimately result in an increase in the concentration of intracellular Ca^{2+}. It has been demonstrated that PI3K can inhibit this intracellular transduction pathway. In contrast, LY294002, which is the specific inhibitor of PI3K, can enhance the cellular responses via signal transduction of GPCRs [27].

SPR can detect changes in the intracellular components of cells cultured on the surface of the SPR chip. Fig. S1a also shows the basic detection mechanism of GPCR-based live-cell biosensors on the basis of SPR. In principle, SPR can respond only to the changes within a few hundred nanometers of the sensor surface. In the case of cell measurement, the sizes of cells are usually several tens of micrometers. In this regard, the whole cellular response is not able to be detected by SPR. However, because the cell membranes are usually within a thickness of around tens of nanometers, it is possible to detect the intracellular component changes that are very near the cell membranes attached to the sensor surface. This has been well documented by previous reports [22–26]. In this study, when the intracellular components change because of the GPCRs responding to their ligands via intracellular signal transduction pathways (for example, the increase in intracellular Ca^{2+}), the refractive index of SPR will change accordingly. The changes in refractive index can be read out by the SPR setup. Therefore, the changes in the intracellular components can be monitored by the measurement of refractive index changes.

In this study, a Spreeta SPR device with a bare gold surface (Texas Instruments, USA) was fixed on the bottom of a cell culture chamber with a hole in the center to allow the only exposure of sensor surface to the solution. Fig. S1b shows the full schematic diagram of the SPR-based biosensor measurement setup. On the top of the cell culture chamber, there is a lid with an input pipe and an output pipe to form a sealed detection chamber during measurement. The transfected HEK-293 cells were cultured on the gold surface of SPR sensor with Dulbecco's modified Eagle's medium supplemented with 10% FBS for 24 h to allow the cells to form a good attachment on the sensor surface. HEK-293 cells without any transfection were used as negative controls. The sensor surface was pretreated with ultraviolet irradiation for 24 h for sterilization. The changes in refractive index of SPR were recorded to monitor cellular responses to stimuli. Various stimuli (Sigma–Aldrich) dissolved in PBS buffer (pH 7.4) at the desired concentrations were applied to the cells using a syringe pump. All the measurements were carried out under the same environmental conditions with the humidity at 10% and temperature at 37 °C.

To investigate the performance of this GPCR-based live-cell biosensor, the specificity and sensitivity were tested using various stimulation molecules. To enhance the sensitivity of this biosensor, the specific inhibitor of PI3K, LY294002, was used as an enhancer for GPCR intracellular signaling.

The specificity of this biosensor was tested using various stimuli at the concentration of 100 nM, which included isoprenaline, noradrenaline, phenylephrine, methoxamine, metaraminol, and phentolamine. These stimuli were applied to HEK-293 cells with and without expression of β2-adrenoceptor. The responses of the cells to the stimuli were monitored by recording the refractive index changes in SPR when the stimuli were applied. The maximum amplitude of the refractive index change was used as the measure of the cellular response. As indicated by the typical SPR signals shown in Fig. 1a, under the stimulation of isoprenaline, HEK-293 cells expressing β2-adrenoceptor had significantly higher response signals than under noradrenaline stimulation. As shown in Fig. 1b, the results indicate that HEK-293 cells with β2-adrenoceptor have significantly higher responses to isoprenaline than to the other stimuli tested. HEK-293 cells without expression of β2-adrenoceptor show no significant response to any stimulation molecule tested. These results indicate this biosensor can specifically respond to isoprenaline, which is the specific target ligand of β2-adrenoceptor. This demonstrates the high specificity of the GPCR-based live-cell biosensor. It is also demonstrates that the SPR signals of this biosensor originated from the specific binding of β2-adrenoceptor with its target ligand.

The sensitivity of this biosensor was tested using isoprenaline at a series of concentrations, which ranged from 10 to 100 nM. The responsive SPR signals were recorded by monitoring the refractive index changes during the application of isoprenaline at a certain concentration. The sensitivity was calculated as follows:

$$S = \Delta R/\Delta C,$$

where S denotes the sensitivity of this biosensor, ΔR denotes the refractive index changes of two different isoprenaline concentrations, and ΔC denotes the concentration differences of isoprenaline. We carried out the measurement on the GPCR-based live-cell biosensors with and without enhancement of LY294002. Fig. 1c shows the statistical results of the sensitivity measurement, which indicates that a linear response can be obtained in a dose-dependent manner in a certain concentration range of isoprenaline. Calculated using the above-mentioned equation, the sensitivity of this biosensor with LY294002 enhancement is 13.3 U/nM, whereas the sensitivity reduced to 4.2 U/nM without LY294002 enhancement. The results indicate that the sensitivity of this biosensor can be enhanced more than three times by using LY294002 as an enhancer of intracellular transduction of GPCR responses. These results demonstrate that using LY294002 is an efficient method to

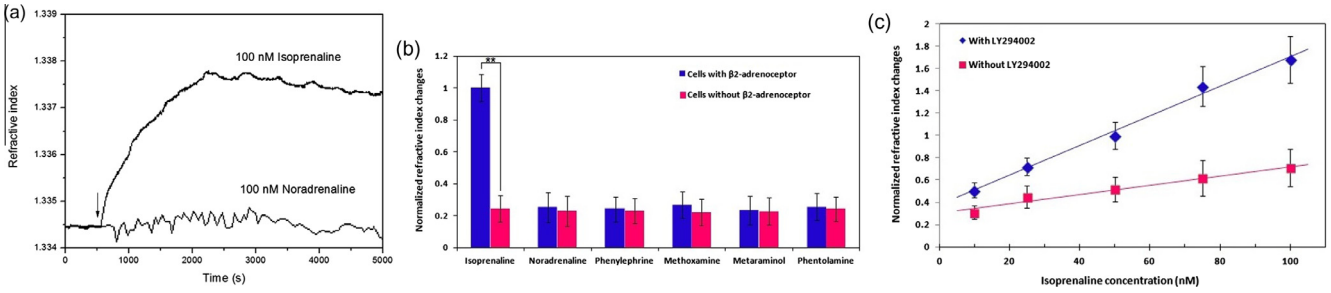

Fig.1. (a) Typical SPR signals of HEK-293 cells expressing β2-adrenoceptor in response to isoprenaline and noradrenaline at the concentration of 100 nM. (b) Responses of SPR biosensors incorporated with HEK-293 cells with and without β2-adrenoceptor to various stimuli. The refractive index changes in SPR were considered the cellular responses. The responses to various stimuli were normalized to that of isoprenaline. All the data are represented as means ± SD. $^{**}p < 0.05$, Student's t test. The means and SD of eight experiments are shown. (c) Dose-dependent responses of GPCR-based live-cell biosensor in response to serial concentrations of isoprenaline. The enhancing effect of LY294002 on the sensitivity of this biosensor was investigated. The responses to isoprenaline at various concentrations were normalized to that of isoprenaline at a concentration of 50 nM when the LY294002 was applied. All the data are represented as means ± SD. The means and SD of six experiments are shown.

enhance the sensitivity of GPCR-based live-cell biosensors. Based on this method, other kinds of GPCRs can also be assayed in this GPCR-based live-cell biosensor. Because of the importance of GPCRs in basic research and drug discovery, this kind of biosensor has great potential to be used as a novel and valuable tool for the functional assay of GPCRs and even for drug discovery.

In this study, we developed a GPCR-based live-cell biosensor on the basis of SPR measurement. A model of GPCRs, β2-adrenoceptor, was functionally expressed on the plasma membrane of HEK-293 cells. It can respond to isoprenaline with high specificity and sensitivity. A novel method for enhancing the sensitivity of this biosensor was also demonstrated, based on the enhancement of intracellular signal transduction by LY294002. This method can enhance the sensitivity of this biosensor more than three times. All the results suggest that this GPCR-based live-cell biosensor can be used to detect the interactions between GPCRs and their ligands and suggest it could be a valuable tool for the functional assay of GPCRs and drug discovery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.12.036.

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