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Short communication

Label-free functional assays of chemical receptors using a bioengineered cell-based biosensor with localized extracellular acidification measurement

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ABSTRACT

New methods for functional assays of chemical receptors are highly essential for the research of chemical signal transduction mechanisms and for the development of chemical biosensors. This study described a novel bioengineered cell-based biosensor for label-free functional assays of chemical receptors by localized extracellular acidification measurement with a light-addressable potentiometric sensor (LAPS). A human taste receptor, hT2R4, and an olfactory receptor of *Caenorhabditis elegans* (*C. elegans*), ODR-10, were selected as models of chemical receptors, which were expressed on the plasma membrane of human embryonic kidney (HEK)-293 cells. The specific ligand binding function of expressed chemical receptors was monitored by localized extracellular acidification measurement using LAPS chip with a movable focused laser illuminating on the desired single cell. The function of expressed olfactory receptors was further validated using MDL12330A, which can specifically inhibit the activity of adenylyl cyclase. The obtained results indicate that both of chemical receptors were successfully expressed in HEK-293 cells and can be functionally assayed by this bioengineered cell-based biosensor that shows dose-dependent responses to the target ligands of chemical receptors. This bioengineered cell-based biosensor exhibits the sensitivity of 1.0 mV/s for hT2R4 assays, and 9.8 mV/s for ODR-10 assays. The negative control cells without any chemical receptor expression show no response to all the chemical stimuli tested. All the results demonstrate this bioengineered cell-based biosensor can be used to detect the interactions between chemical receptors and their ligands. This provides a valuable and promising approach for label-free functional assays of chemical receptors as well as for the research of other GPCRs.

1. Introduction

Olfactory and taste receptors are typical chemical receptors in biological sensing systems for the detection of environmental chemical signals, which can specifically interact with their target ligands and convert the chemical signals into cellular responses via intracellular signal transduction pathway (Buck and Axel, 1991; Dryer and Berghard, 1999; Matsunami et al., 2000; Chandrashekar et al., 2006; Ishimaru, 2009). Most chemical receptors belong to the large family of G-protein-coupled receptors (GPCRs), which are characterized with seven trans-membrane spanning domains and form one of the largest known families of integral membrane receptors (Hopkins and Groom, 2002; Overington et al., 2006). GPCRs are highly specialized membrane receptors for cellular signal transduction, which mediate a diverse array of cellular functions and have attracted more and more interests due to their significant importance in many fields such as biomedicine, biotechnology, and pharmaceutical industry (Ferguson and Caron, 1998; Rosenbaum et al., 2009; Lappano and Maggiolini, 2011). Specifically, olfactory and taste receptors, which are two main categories of chemical receptors, have been widely applied in the development of novel biomimetic biosensors for the detection of specific chemical substances (Wu, 1999; Wu et al., 2012, 2013; Du et al., 2013). However, there are still many chemical receptors are orphan receptors including most of bitter receptors and olfactory receptors, which means their target ligands or functions are still unknown (Schlyer and Horuk, 2006; Lagerstrom and Schioth, 2008; Ishimaru, 2009). This is mainly due to the lack of efficient and powerful methods for functional assays of GPCRs. The currently available methods usually require careful and special labeling process in order to achieve sufficient responsive signals, which have been proved to be complicated, expensive, and time-consuming (Heilker et al., 2009; Allen and Roth, 2011). Furthermore, most current

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methods are large population cell-based assays, which may submerge some important information from single cell or small population cells to the large population cell data (Elowitz et al., 2002; Fritzsch et al., 2012). Therefore, it is highly essential and favorable to develop novel methods for label-free functional assays of GRCPs, especially for the functional assays of chemical receptors at the single-cell level or small population cell level, which could avoid the loss of information associated with ensemble averaging based on large cell population assays.

Among various devices used in label-free analysis of single cell or small population cells such as field-effect transistors (FET) and microelectrode array (MEA), light-addressable potentiometric sensor (LAPS) is one of the most promising and attractive devices due to its decisive advantages such as simple structure, light addressability, and low cost. LAPS is a surface-potential sensitive semiconductor device, which has been widely applied in the development of single cell-based biosensors for extracellular potential measurement (Xu et al., 2005; Wu et al., 2009; Du et al., 2013). LAPS has also been applied in the acidification measurement of large population cells by monitoring the proton generation originated from the metabolism of cells cultured on LAPS surface (Owicki et al., 1990; Fanigliulo et al., 1996; Wu et al., 2001; Yu et al., 2009). Based on acidification measurement of large population cells, LAPS has also been successfully applied in the functional assays of some GPCRs such as M_1-muscarinic acetylcholine receptors, muscarinic receptors, β_2-adrenergic receptors, and prostaglandin (E) receptors (Owicki et al., 1990; Parce et al., 1990; Miller et al., 1991). However, there is no report on the investigation of using LAPS for the functional assays of chemical receptors based on extracellular acidification measurement, especially at the single-cell level or small population cell level.

In this study, a novel bioengineered cell-based biosensor incorporated with LAPS localized extracellular acidification measurement was developed for the label-free functional assays of chemical receptors at the single-cell level or small population cell level. A human bitter receptor, hT2R4 (Ueda et al., 2003; Conte et al., 2006), and an olfactory receptor of *Caenorhabditis elegans* (*C. elegans*), ODR-10 (Sengupta et al., 1996), were expressed on the plasma membrane of human embryonic kidney (HEK)-293 cells. The function of the expressed chemical receptors was demonstrated by intracellular calcium assay using various chemical substances as stimuli. The function of expressed chemical receptors was monitored by localized extracellular acidification recording of cell metabolism using LAPS with a movable focused laser to select the desired target single cell for measurement. The inhibitory effect of MDL12330A on the olfactory receptor signal transduction was employed to further demonstrate the origination of LAPS extracellular recording signals is from the specific interactions between expressed chemical receptors and their target ligands (Guellaen et al., 1977).

2. Materials and methods

2.1. Expression of chemical receptors

A human bitter receptor, hT2R4, and an olfactory receptor of *C. elegans*, ODR-10, were expressed on the plasma membrane of HEK-293 cells according to our previous reports (Wu et al., 2011, 2013). Briefly, the expression plasmids that contain the full-length sequences of hT2R4 or ODR-10 were constructed, which are *pCEP4/rho-hT2R4* and *pFLAG-CMV-3/rho-ODR-10*, respectively. After confirmed by DNA sequencing, the correct expression plasmids were used to transfect HEK-293 cells using Lipofectamine™ 2000 (Invitrogen) as the DNA carrier. The HEK-293 cells were used for further measurement 24 h after transfection.

2.2. LAPS chip fabrication and cell culture

LAPS chip was fabricated by microfabrication process as previously described (Wu et al., 2009). LAPS chip was fabricated on the basis of p-type silicon wafer (⟨1 0 0⟩, 1–10 Ωcm). First, the surface of the silicon wafer was thermal oxidized with a layer of 30 nm SiO_2. Then, the silicon wafer was grinded to a 100-μm thickness from its rear side. A layer of Si_3N_4 was deposited on the upside with a thickness of 60 nm. Finally, the rear side of the silicon wafer was deposited with a thin layer of Al to generate Ohmic contact. After fabrication, the wafer was cut into small square slices and packaged with a cell culture chamber.

Before cell culture, LAPS surface was treated with poly-*l*-ornithine and laminin mixture (100 μg/mL poly-*l*-ornithine and 8 μg/mL laminin mixed by the rate of 1:1) to faciliate the cell seeding and attachment on the sensor surface (Ismail et al., 2003). HEK-293 cells were cultured on the surface of LAPS chip for 24 h at 37 °C in Dulbecco's Modified Eagle's Medium (GIBICO) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL), in 5% CO_2 incubator.

2.3. Intracellular calcium assay

The changes in intracellular calcium concentration were monitored by a Ca^{2+} indicator, Fluo-3/AM (Invitrogen, USA). The transfected HEK-293 cells were cultured for 24 h and incubated with 5 μmol/L Fluo-3/AM for 45 min at 37 °C. Then the dye solution was replaced with the imaging buffer (140 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 10 mmol/L HEPES, 10 mmol/L glucose, 1.2 mmol/L KH_2PO_4, pH 7.2), and cells were placed in the dark for 20 min to ensure that all the Fluo-3/AM turned to Fluo-3. During calcium assay, temperature was kept in constant. Various chemical stimuli were all dissolved in water and diluted into the imaging buffer at the desired concentration. Imaging buffer solution without any chemical stimuli was applied to the cells as a control solution. All the measurements were performed under laser scanning confocal fluorescence microscope (IX81-FV1000, Olympus, Center Valley, PA, USA).

2.4. LAPS setup and measurement

In this study, LAPS setup was configurated on the basis of three electrode system (Fig. S1). LAPS chip was used as the working electrode. While an Ag/AgCl electrode and a Pt wire were utilized as the reference electrode and the counter electrode, respectively. A potentiostat (EG & G M273A Princeton Applied Research, USA) was employed to provide the bias voltage for LAPS chip. A He–Ne semiconductor laser (Coherent Co., USA) with a wavelength of 543.5 nm was modulated in 4 kHz and focused to less than 10 μm in diameter. The laser are movable on the LAPS surface and can be used to select the desired single cell for localized extracellular acidification measurement. The ouput of LAPS chip was recorded by peripheral equipments and amplified by lock-in amplifier (model SR830 DSP, Stanford Research Systems). The NI data collection card and the home-made LabVIEW software were used for data collection and analysis (National Instrument Co. Ltd., Austin, TX, USA).

During measurement, LAPS chip packaged with a cell culture chamber was fixed under a microscope. The localized extracellular acidification measurement was carried out by illuminating the desired single cell using the movable focused laser. The responses of HEK-293 cells expressed with chemical receptors to various chemical stimuli were recorded in a real-time manner. Localized cell acidification was monitored by extracellular recording of ptoton generation resulting from the interactions between expressed chemical receptors and their target ligands. HEK-293

cells without any transfection were used as the negative control cells. Various chemical compounds dissolved in cell culture media were applied as the stimuli and introduced into the detection chamber by a syringe pump. After each measurement, the detection chamber was refreshed by the cell culture meida without any chemical stimuli. All the chemical compounds used as the stimuli were purchased from Sigma–Aldrich (USA). All the other chemicals used in this study were of analytical pure grade or better quality. Ultrapure deionized water (18.2 MΩ/cm) was obtained from a Milli-Q system (Bedford, USA). The whole setup was shield with a Faraday box to exclude ambient light and all the measurements were performed at similar environmental conditions with the humidity at 10% and the temperature at 37 °C to minimize the influences of environmental factors to the measurements.

2.5. Statistical analysis

All the data were represented by means ± standard deviation (SD). Student's *t*-test was performed on the data to check the significance of data differences. $p < 0.05$ was considered as statistical significance.

3. Results and discussion

3.1. Functional expression of chemical receptors on cell plasma membrane

The hydrophobic environment of cell membrane is essential to the function of chemical receptors. To maintain the natural structure and function of chemical receptors, it is necessary to express chemical receptors on the plasma membrane of a heterologous cell system. To improve the expression of chemical receptors on cell plasma membrane, *rho-tag* import sequence was inserted into their *N*-terminus. As shown in Fig. S2, immunofluorescent staining results demonstrated the successful expression of hT2R4 and ODR-10 on the plasma membrane of HEK-293 cells, which are in good agreement with previous reports (Wu et al., 2011, 2013). In this study, the expression efficiency of hT2R4 and ODR-10 in HEK-293 cells were estimated to be 36.8% and 45.7%, respectively.

To test the function of expressed chemical receptors, intracellular calcium assays were carried out using different chemical molecules as stimuli. The responses of HEK-293 cells expressed with chemical receptors to denatonium (500 nmol/L), quinine (500 nmol/L), diacetyl (100 nmol/L), and butanone (100 nmol/L) were measured. Different concentrations of chemicals were used as stimuli to avoid the toxic effects of chemicals on cells. The concentrations of chemical stimuli used in this study are sufficient to elicit typical cellular responses but much lower to generate cellular toxic effects that are usually at the level of tens of mM. HEK-293 cells without any transfection were also measured using the same protocol to serve as the negative control cells. Upon perfusion of denatonium, which is the natural ligand of hT2R4, HEK-293 cells expressed with hT2R4 show a transient increase in intracellular Ca^{2+} winthin 20 s as indicated by the fluorescence intensity increase. Similarily, HEK-293 cells expressed with ODR-10 show specific responses to diacetyl, which is the natural target ligand of ODR-10. However, around 60% transfected HEK-293 cells show no response to any chemical stimulus tested, which is probably because the expression efficiency of chemical receptors in HEK-293 cells is not as high as 100%. We calculated the amplitude of increasing in intracellular Ca^{2+} by the fluorescence intensity increase and considered the maximum amplitude during the application of chemical stimuli as the cellular responses. As summarized in Fig. 1, the resluts show that HEK-293 cells expressed with functional chemical receptors have significant higher responses to their target ligands and show no responses to other chemical stimuli tested. On the contrary, negative control HEK-293 cells show no significant calcium response to any chemical stimulus tested. All these results demonstrated that chemical receptors have been functional expressed on the plasma membrane of HEK-293 cells, which suggest these cells are suitable to be used as sensitive elements in the development of bioengineered cell-based biosensors for functional assays of chemcial receptors.

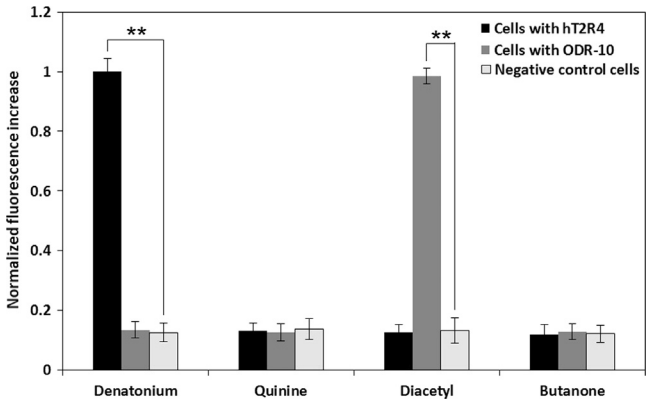

Fig. 1. Intracellular calcium assays of HEK-293 cells with and without expression of functional chemical receptors in response to various chemical stimuli. The responses of various chemical stimuli were normalized to that of HEK-293 cells with hT2R4 upon denatonium stimulation. $^{**}p < 0.05$, Student's *t*-test. The mean and SD of 16 experiments are shown.

3.2. Localized cell metabolism measurement

Chemical receptors can respond to extracellular stimuli and convert the extracellular signals into cellular responses via intracellular GPCRs signal transduction pathway. The specific binding of chemical receptors with their target ligands can initiate a cascade of intracellular biochemical reactions, which will lead to the release of Ca^{2+} from intracellular Ca^{2+} stores and result in the increasing concentration of intracellular Ca^{2+}. This will change the status of cells and finally generate various cellular responses such as cell membrane potential changes, energy consumption changes, and cell metabolism changes. In order to explore the feasibility of monitoring cellular responses by cell metabolism measurement, LAPS chip was employed to monitor the localized cell metabolism by the detection of extracellular proton concentation changes originated from bioengineered cells expressed with functional chemical receptors in response to the specific chemical stimuli.

To achieve the localized cell metabolism measurement, the focused laser was illuminated on the desired single cell cultured on LAPS surface for measurement. Cells was cultured on the LAPS surface with a distribution density as low as 10^5 cells/mm^2 to minimize the influeneces of neighbour cells on the localized extracellular metabolism measurement of the single cell. The area of LAPS surface exposed to cell culture medium was around 50 mm^2. Therefore, the approximate number of HEK-293 cells for each LAPS chip prepared was estimated to be 5×10^6 cells. For each LAPS chip, less than 10 measurements were performed due to the limited lifetime of cells outside a cell incubator. Fig. 2a shows typical recordings of LAPS localized extracellular metabolism measurement from HEK-293 cells expressed with functional hT2R4 in response to different concentrations of denatonium. LAPS recordings show obvious differences when various concentrations of denatonium were applied to HEK-293 cells expressed with functional hT2R4. In addition, HEK-293 cells expressed with functional hT2R4 show no singnificant response to other bitter substances including quinine and D-(-)-salicin (Fig. S3). These results indicate that LAPS metabolism measurement can efficiently

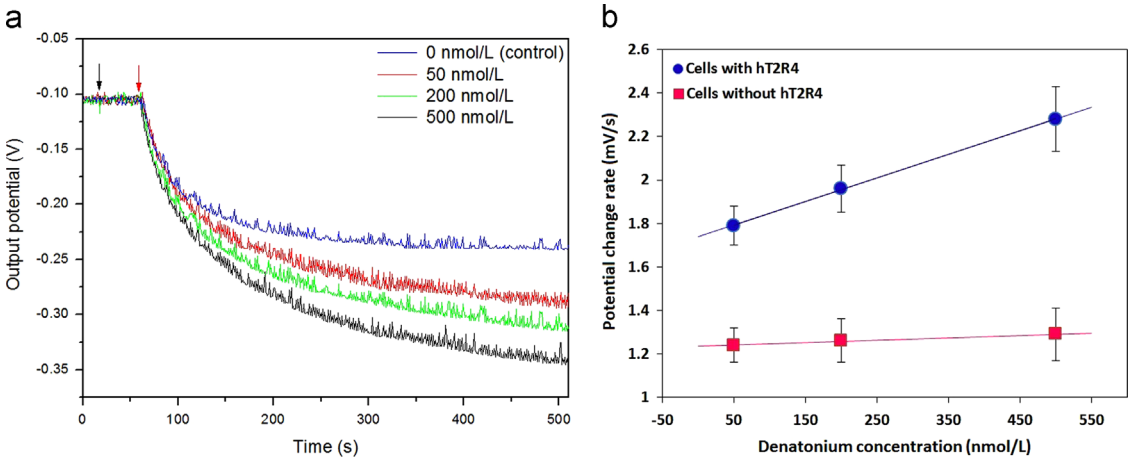

Fig. 2. (a) Typical LAPS recordings of localized cell metabolism from HEK-293 cells expressed with functional hT2R4 in response to different concentrations of denatonium. Cell culture media without denatonium was used as a control solution. The black arrow indicates the time point of the introduction of denatonium stimulation. The red arrow indicate the time point of the stop of pumping solution to the detection chamber. (b) Dose-dependent responses of HEK-293 cells expressed with functional hT2R4 to serial concentrations of denatonium. HEK-293 cells without hT2R4 expression were used as negative control cells. The mean and SD of 16 experiments are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

monitor and discriminate the different cellular responses with high specificity.

We calculate the LAPS ouput potential change rates within 100 s after the introduction of chemical stimuli and choose the maximum potential chage rate as the cellur response to the chemical stimuli. The time period of 100 s was choosed because the LAPS potential chang rates are usually faster at the beginning of cellular responses and then the potential change rates gradually become slower. The maximum potential change rate usually appears within the first 50 s after the introduction of chemical stimuli. As summarized in Fig. 2b, HEK-293 cells expressed with functional hT2R4 show dose-dependent resposes to different concentrations of denatonium. The sensitivity is 1.0 mV/s. On the contrary, no similar responses can be observed in the negative control cells when the same chemical stimuli were applied. All the results demonstrate that this bioengineered cell-based biosensor can successfully monitor the responses of bioengineered cells expressed with functional chemical receptors to the specific chemcial stimuli.

3.3. *Inhibition of cellular responses*

In order to further validate the performances of this bioengineered cell-based biosensor, similar protocol to that of used in hT2R4 was applied to the HEK-293 cells expressed with functional ODR-10. Different concentrations of diacetyl were used as the chemical stimuli. To further demonstrate the LAPS recording responses are originated from the specific interactions between ODR-10 and its target ligand, MDL12330A, which is the specific inhibitor of adenylyl cyclase (an essential component of GPCR intracellular signal transduction pathway) (Guellaen et al., 1977), was used to inhibit the cellular responses. MDL12330A was applied to the target cells 10 s prior to and during the application of chemical stimuli. As summarized in Fig. 3, HEK-293 cells expressed with functional ODR-10 can respond to diacetyl in a dose-dependant fashion. The sensitivity is 9.8 mV/s, which is much higher than that of hT2R4. The reason may be due to the higher expression efficiency of ODR-10 in HEK-293 cells. Furthermoe, this bioengineered cell-based biosensor show no response to other odorant molecules including butanone, hexanal, and pentanedione (Fig. S4). As expected, the negative control cells show no singnificant response to diacetyl at different concentrations as well as to other odorant molecules tested.

In addition, the results show that MDL12330A can efficiently inhibit the LAPS recording cellular responses, which makes the

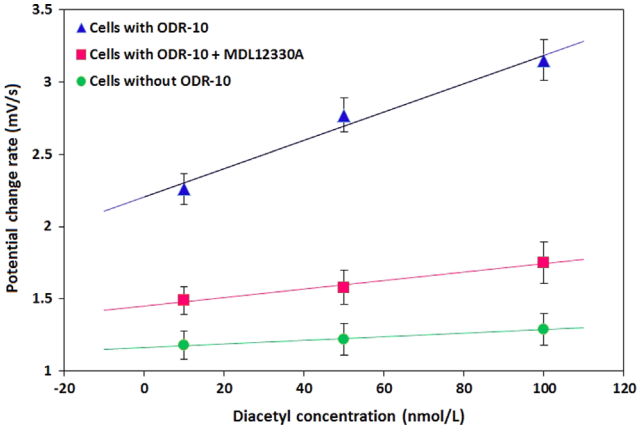

Fig. 3. HEK-293 cells expressed with ODR-10 respond to various concentrations of diacetyl in a dose-dependent fashion. The inhibitory effect of MDL12330A on the cellular responses to chemical stimuli was investigated. HEK-293 cells without ODR-10 expression were used as negative control cells. The mean and SD of 16 experiments are shown.

sensitivity of cellular response reduce to 2.9 mV/s. This means that the olfactory signal transduction pathway was inhibited by MDL12330A but not completely blocked, which will consequently result in the inhibitory effects on the cellular responses to chemical stimuli. It is further demonstrated that the LAPS recordings were originated from the specific interactions between ODR-10 and its natural ligand. All these results suggest that this bioengineered cell-based biosensor could also be applied in functional assays of other chemical receptors as well as other GRCRs underlying simiar signal transduction mechanisms.

4. Conclusion

This study provides a proof-of-concept study on bioengineered cell-based biosensors for label-free functional assays of chemical receptors on the basis of LAPS localized extracellular acidification measurement. Chemical receptors including a human bitter receptor, hT2R4, and an olfacotry receptor of *C. elegans*, ODR-10, were functionally expressed on the plasma membrane of HEK-293 cells. Both of them can respond to their target ligands in a dose-dependent fashion as indicated by the results from LAPS localized

extracellular acidification measurement. All the results demonstrate that this bioengineered cell-based biosensor can be used to detect the interactions between chemical receptors and their ligands, and suggest it could be a valuable and promising tool for the functional assays of chemical receptors as well as other GPCRs. To achieve high consistence and practical applications, it is essential to improve the coupling stability between bioengineered cells and transducers as well as the expression efficiency of chemical receptors. This is the main challenge enconuntered in the development of bioengineered cell-based biosensors and will be the focus of our near future reserch work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.11.049.

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