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Cell-based high-throughput odorant screening system through visualization on a microwell array

Eun Hae Oh [a], Seung Hwan Lee [b], Sang Hun Lee [b], Hwi Jin Ko [c], Tai Hyun Park [a,b,c,*]

[a] Interdisciplinary Program of Bioengineering, Seoul, National University, Seoul, Republic of Korea
[b] School of Chemical and Biological Engineering, Seoul National University, Seoul, Republic of Korea
[c] Bio-MAX Institute, Seoul National University, Seoul, Republic of Korea

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ABSTRACT

The development of a cell-based high-throughput screening system has attracted much attention from researchers who study drug screening mechanisms and characterization of G-protein coupled receptors (GPCRs). Although olfactory receptors (ORs) constitute the largest group of GPCRs that play a critical role recognizing and discriminating odorants, only a few ORs have been characterized, and most remain orphan. The conventional cell-based assay system for characterizing GPCRs, including ORs, is very laborious, time consuming, and requires an expensive assay system. In this study, we developed a simple, low-cost miniaturized odorant screening method by combining Micro-Electro-Mechanical system (MEMs) technique and visualization technique for detecting an odorant response. We fabricated PEG microwell from a photocrosslinkable polyethylene glycol diacrylate (PEGDA) solution and applied it to cell culture and a reverse transfection platform for cell-based high-throughput screening. For the first time, the olfactory receptors were expressed on the microwell platform using reverse transfection technique. The various olfactory receptors can be expressed simultaneously using this technique and the microwell spotted with olfactory receptor genes can be used as a high-throughput screening platform. The odorant response was detected via fluorescence analysis on the microwell using a cAMP response element (CRE) reporter assay. We tested this platform using four de-orphaned ORs. This new cell-based screening method not only reduced numerous time-consuming steps but also allowed for simple, efficient, and quantitative screening and patterning of large numbers of GPCRs including ORs, which can help to visualize the OR response to odorants on a microwell.

1. Introduction

A cell-based assay is an effective tool for biochemical studies such as drug screening and characterizing receptors and other cellular responses to physiochemical stimuli (Apostol et al., 2003; Galietta et al., 2001; Sundberg, 2000; Yan et al., 2002). This type of assay not only provides reliable and real cellular signals through ligand stimulation, but it also provides important information about the biochemical activity of cells. There have been many attempts to construct a high-throughput cell-based assay system to monitor cellular activity such as cell adhesion, apoptosis, motility, signaling pathways, and ligand screening (Figueroa et al., 2010; Hertzberg and Pope, 2000). Multi-well culture plates in 96- and 384-well formats have been widely used for high-throughput cell-based screening. This culture plate-based screening technique has been successfully used but is limited as it requires high-cost automatic handling equipment. Therefore, there is demand for the development of simple, miniaturized, economic high-throughput assay system. In 2001, Ziauddin and Sabatini developed a new method to conduct cell transfection at the microarray level, which is called reverse transfection technique (Ziauddin and Sabatini, 2001). By printing various cDNAs onto a slide glass with a transfection reagent followed by seeding of adherent mammalian cells, different proteins can be expressed at each location. It thus became possible to scale down high-throughput gene analysis in a chip-based system (Castel et al., 2006; Mishina et al., 2004; Sturzl et al., 2008). To improve reverse transfection efficiency and to prevent spot-to-spot diffusion, the distance between the different transfection clusters was adjusted and various glass coating materials have been used (Hodges et al., 2005; Peterbauer et al., 2006). However, these methods still have a limitation of a high cost coating material and complex experimental steps. In this study, a polyethylene glycol diacrylate (PEG) microwell was used to generate a spatially separated cell adhesion area (Kwon et al., 2011; Moeller et al., 2008). This method does not require complex chemical treatment or tedious experimental steps. The PEG

* Corresponding author at: School of Chemical and Biological Engineering, Seoul National University, Seoul, Republic of Korea. Tel.: +82 2 880 8020; fax: +82 2 875 9348.
E-mail address: thpark@snu.ac.kr (T.H. Park).

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microwell has been used to culture the stem cells and mammalian cells in several researches (Charnley et al., 2009; Choi et al., 2010; Kobel and Lutolf, 2012). Here, we adopted the PEG microwell as a reverse transfection platform. Due to the cell-repellent property of PEG, cells adhere only to the glass bottomed-wells, where the transfection mixture is spotted, and the microwell system effectively avoids cross contamination of DNA spots. Fabricating a polydimethysioxane (PDMS) stamp-derived PEG microwell is very simple and cheap, and this PEG microwell is convenient for reverse transfection of cells with different kinds of DNA samples. These advantages make the microarray platform of this microwell system suitable for screening various receptors.

Human olfactory receptors (hORs) detect and discriminate thousands of different odorants with high sensitivity and selectivity (Firestein, 2001). Human has about 390 hORs and smells via the combination of hOR-odorant interaction. Many researchers have attempted to develop highly sensitive artificial olfactory screening tools using recombinant olfactory receptors to characterize the ORs (Kim et al., 2009; Ko and Park, 2006; Lee et al., 2006; Lee et al., 2009a; Lee et al., 2009b; Lee et al., 2011; Lee and Park, 2010; Liu et al., 2006; Oh et al., 2011) However, only a few instances of hORs binding to specific ligands have been identified, and most specific hOR ligands are still unknown (Katada et al., 2003; Ko and Park, 2006; Shirokova et al., 2005) and the pattern analysis of OR-odorant interactions was barely conducted. One of the main reasons for the limited ligand information is that conventional ligand screening methods such as the intracellular calcium assay are time-consuming and labor intensive to be used as a high-throughput screening tool. Moreover they include many steps, which make cells unhealthy. It is necessary to assay many ORs simultaneously easily and it is also necessary to visualize the OR-odorant interaction for pattern analysis. We used the reverse transfection technique to express various ORs simultaneously on the PEG microwell. And, to visualize the OR response to odorants, we used the cAMP response element (CRE) reporter assay (Hill et al., 2001), which produced the fluorescence reporter protein upon odorant stimulation.

2. Materials and methods

2.1. PDMS stamp fabrication

The master mold for the PDMS stamp was fabricated using standard soft lithography. A photoresistor (SU-8 2075, Microchem, Newton, MA, USA) was spin-coated onto a silicon wafer (500 rpm, 5 sec; 2000 rpm, 30 sec), baked (65 °C, 5 min; 95 °C, 20 min), and was then exposed to UV light using a mask aligner (MA6, Suss MicroTec AG, Garching, Germany). After post-baking (65 °C, 1 min; 95 °C, 10 min), the stamp patterns were formed through a development process (10 min) using SU-8 developer (Microchem). The PDMS prepolymer (Sylgard 184, Dow-Corning, Corning, NY, USA) was mixed with its curing agent in a 10:1 ratio. After removing the bubbles by degassing, the mixture was poured onto the patterned silicon wafer and baked at 80 °C for 2 h. The cured PDMS was peeled off the master mold and used as a stamp.

2.2. PEG microwell fabrication

PEG microwells were fabricated using micromolding on UV-photocrosslinkable polyethylene glycol diacrylate (PEGDA, MW700) (Sigma-Aldrich Co., St. Louis, MO, USA) solution containing 0.5% (w/w) of the photoinitiator 2-hydroxy-2-methyl propiophenone. Glass substrates were treated with 3-trimethoxysilyl propylmethacrylate (TMSPMA) (Sigma-Aldrich) for 5 min and baked at 70 °C for 2 h. The PDMS stamp was placed on glass with 30% PEGDA solution, and the PEGDA solution was photocrosslinked upon exposure to 365 nm wavelength UV at an intensity of 10 mW/cm for 10 min. The PDMS stamp was peeled off after polymerization of the PEGDA solution, and the polymerized microwell was washed using distilled water and dried with air. The diameter of the PEG microwell was 500 μm and the depth was 100 μm. The fabricated microwell on the glass substrate allowed cells to adhere to the bottom of each well.

2.3. Plasmid construction

A Rho-tag was subcloned into the pcDNA3 mammalian expression vector using BamHI and EcoRI restriction enzyme sites. hOR genes were amplified by polymerase chain reaction (PCR) from human genomic DNA using primers containing EcoRI and XhoI or EcoRV and XhoI sites. After digestion with the above restriction enzymes, the receptor genes were inserted into pcDNA3 digested with the same restriction enzymes. RTP1S was amplified from the RTP1L plasmid (Origene, Rockville, MD, USA) and subcloned into pcDNA3. The pcDNA3-CRE reporter vector was generated by PCR-based subcloning of the destabilizing domain (DD)-CRE-ZsGreen gene from pCRE-DD-ZsGreen1 (Clontech, Palo Alto, CA, USA) into pcDNA3. The original pcDNA3 promoter was deleted by mutagenesis technology. Based on reports that the number of CRE sites impacts the reporter gene signal (Dinger and Beck-Sickinger, 2002; Shan and Storm, 2010), we constructed the pcDNA3-CRE vector containing 12 CRE sites to enhance expression of the fluorescent reporter protein ZsGreen.

2.4. Reverse transfection on PEG microwells

The aqueous gelatin solution was prepared as follows. Gelatin (1.2%, w/v) was dissolved in pure water by heating at 55 °C for 20 min. The solution was slowly cooled to room temperature and was filtered with a 0.45 μm cellular acetate membrane. A 4 μl aliquot of OptiMEM (Invitrogen, Carlsbad, CA, USA) containing 0.4 M sucrose, 2 μg plasmid DNA (pEGFP-N1 or pDsRed-N1), and 4 μl Lipofectamine 2000 (Invitrogen) were mixed to test the reverse transfection efficiency on the PEG microwell. After incubating the mixture at room temperature for 20 min, 2 μl of 1.2% gelatin solution was added and gently mixed to make a final gelatin concentration of 0.2%. To assay the signal generated from binding of the hOR and odorants, 2 μg pcDNA3/hOR vector, 0.3 μg pcDNA3.1/$G_{\alpha olf}$, and 0.2 μg pcDNA3/RTP1S were mixed with transfection reagents and gelatin solution. The plasmid DNA/gelatin mixtures were spotted onto the PEG microwells using a Micro-Caster™ 8-pin System (Whatman, Florham Park, NJ, USA). The spotted glass was dried in a vacuum desiccator for at least 12 h. HEK293-12CREs cells were seeded on the spotted PEG microwell glass and cultured for 48 h to express the hORs or fluorescence proteins.

2.5. Immunocytochemistry

The cells were fixed in −20 °C ethanol for 15 min for immunocytochemistry. After fixation, the cells were washed with phosphate buffered saline (PBS) and incubated in PBS containing primary rabbit anti-rho antibody at 1:500 for 2 h. After washing with PBS, the cells were incubated with the secondary anti-rabbit antibody conjugated with Alexa-594 at 1:1000 for 1 h and were imaged by fluorescence microscopy.

2.6. CRE reporter assay

HEK293 cells were maintained in Dulbecoco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected with pcDNA3-CRE. Stable cell lines containing the reporter gene system (HEK293-12CRE cells) were selected using the antibiotic