

Functional Analysis of Olfactory Receptors Expressed in a HEK-293 Cell System by Using Cameleons

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Abstract Cameleon is a genetically engineered Ca^{2+} sensing molecule consisting of two variants of the green fluorescent protein (GFP), calmodulin and calmodulin-binding protein, M13. HEK-293 cells stably expressing three types of cameleons, yellow cameleon-2, cameleon-3er, and cameleon-2nu, were constructed, and the expression and localization of these cameleons were confirmed by fluorescent imaging. Among the cameleons, the yellow cameleon-2 was selected for analyzing the change in Ca^{2+} induced by the olfactory receptor-mediated signal transduction, because it is localized in the cytosol and binds to cytosolic Ca^{2+} ions. Cells stably expressing yellow cameleon-2 were transfected with each of the test olfactory receptor genes, *odr-10* and *17*, and the expression of the olfactory receptor genes were examined using immunocytochemical methods and RT-PCR. Stimulating each olfactory receptor with its specific odorant caused an increase in the intracellular Ca^{2+} level, which was measured using yellow cameleon-2. These results demonstrate that yellow cameleon-2 can be conveniently used to examine the function of the olfactory receptors expressed in heterologous cells.

Keywords: Cameleon, olfactory receptor, HEK-293 cells, Ca^{2+}

Ca^{2+} signals in the cytosol and organelles are important for cell signal transduction and are usually measured using synthetic fluorescent chelators or recombinant aequorin [4–6, 10, 20, 24]. Measuring the change in the intracellular Ca^{2+} level is also a useful tool for examining the function of the olfactory receptor proteins expressed in a heterologous cell system. The function of heterologous olfactory cells has been measured by the Ca^{2+} -assay method using the fura method [5]. Functional heterologous olfactory cells would provide an effective olfactory biosensor. Various methods have been used in the development of biosensors [1–3, 7, 9, 12], and apoptosis is also an important issue, particularly for cell-based biosensors [11].

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The fura method for a Ca^{2+} assay is time-consuming, tedious, and expensive. Recent developments in molecular biological synthesis have now provided a more convenient method for measuring the level of intracellular Ca^{2+} ions using “cameleons” [18, 19, 21]. Cameleons are genetically engineered proteins that can sense free Ca^{2+} and are comprised of tandem fusions of a blue or cyan mutant of the green fluorescent protein (GFP), calmodulin, calmodulin-binding M13 fragment of myosin light chain kinase [8], and enhanced green or yellow emitting GFP. A specific wavelength (458 nm) of light excites one of the two GFP variants, which emits a photon with a longer wavelength (480 nm) of light. If Ca^{2+} binds to the calmodulin of these cameleons, the distance between the two GFP variants becomes short. Fluorescence resonance energy transfer (FRET) then occurs. The light emitted from the first GFP variant excites the other GFP variant, and the second variant emits a photon with an even longer wavelength (520 nm). Hence, the FRET between the GFP variants can be used to monitor the localized Ca^{2+} signals in living cells. These cameleons can be readily directed to interesting sites by fusing them to the appropriate organellar targeting signal sequences.

In this study, cameleon was applied to the functional analysis of the olfactory receptor proteins in a living heterologous HEK-293 cell system. When odorants interact with an olfactory receptor expressed on the cell surface, signal transduction occurs through the cAMP or IP_3 pathway. A subsequent change in the intracellular concentration of Ca^{2+} ions occurs within the cell. Cameleon was used to detect these changes in the intracellular Ca^{2+} level for a functional analysis of the olfactory receptor proteins.

MATERIALS AND METHODS

Transfection of HEK-293 Cells with Cameleon Genes

Three types of cameleon genes, yellow cameleon-2, yellow cameleon-3er, and yellow cameleon-2nu, were kindly provided by Roger Y. Tsien (Department of Biochemistry, University

of California, San Diego, U.S.A.) and used for transfection. The HEK (human embryonic kidney)-293 cells were grown in DMEM (Dulbecco's modified eagles medium) (Life Technologies, Gaithersburg, MD, U.S.A.), supplemented with 10% FBS (fetal bovine serum; Life Technologies), penicillin (100 U/ml), and streptomycin (100 µg/ml) in 5% CO₂ at 37°C. Prior to transfection, the cells were seeded on a 6-well plate (Nalge Nunc International, Rochester, NY, U.S.A.) and incubated for one day. Transfection was performed using 1 µg of the *cameleon* gene-containing *pcDNA3* vectors with the Lipofectamine plus reagent (Life Technologies). The HEK-293 cell lines stably expressing the cameleons were selected by treating them with G418 (500 µg/ml) for a 2–3-week period. The excitation wavelengths of cameleons are 440±10 nm, 440±10 nm, and 480±15 nm, respectively, and their emission wavelengths are 535±12.5 nm, 535±12.5 nm, and 535±22.5 nm, respectively. Photographs of the fluorescent cells were taken using a Nikon Eclipse TE 300 microscope (Japan) with excitation at 440 nm and emission at 520 nm for yellow cameleon-2 and yellow cameleon-3er, and with excitation at 480 nm and emission at 540 nm for yellow cameleon-2nu.

Molecular Cloning of Olfactory Receptor Genes

A rho-tag sequence, which is the N-terminal 15 residues of rhodopsin, was used at the N-terminus of the receptor protein, to target the olfactory receptor protein to the cell membrane surface [12]. The rho-tag import sequence was obtained by digesting *pBK-CMV* containing *rho-tag* DNA with BamHI-EcoRI. The resulting 60-bp fragment was subcloned into the *pcDNA3* vector (Invitrogen, NV Leek, The Netherlands) and digested with BamHI-EcoRI.

ODR-10 and I7 were used as the model olfactory receptors. The *odr-10* gene was amplified by PCR using *pcDNA3/odr-10* as a template with the following primers: Forward primer, 5'-GTTGC AATTCATGTCGGGAGAATTG-3'; Reverse primer, 5'-TAGAAGGCACA GTCGAGG-3'. PCR amplification was performed using the following conditions: 36 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min. PCR amplification was performed using a mixture of *Taq* polymerase, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 mM of each primer, and 100 ng of the plasmid template DNA. The *pcDNA3/rho-tag/odr-10* construct was sequenced directly.

The rat olfactory receptor I7 gene was obtained by PCR of *pVL-I7* using the following primers: I7-N (EcoRI), 5'-GAATTCATGGACCGAAGGAAC-3'; I7-C (EcoRV), 5'-TCTGATATCGACCTAACC AATT-3'. PCR was performed using the following conditions: 36 cycles of 94°C for 1 min, 49°C for 1 min, 72°C for 1 min. The I7 coding sequence was digested with EcoRI-EcoRV, and the resulting sequence was inserted into the *pcDNA3/rho-tag* construct that had been previously digested with the same restriction enzymes. The *pcDNA3/rho-tag/I7* construct was then sequenced directly.

Transfection of HEK-293 Cell Lines Stably Expressing Cameleons with Olfactory Receptor Genes

The HEK-293 cell lines stably expressing the cameleons were grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in 5% CO₂ at 37°C. Prior to transfection, each cell was seeded on two T-25 culture flasks (Nalge Nunc International, Rochester, NY, U.S.A.) and incubated for one day. Transfection was performed using 2 µg of each *pcDNA3/rho-tag/odr-10* or *pcDNA3/rho-tag/I7* with the Lipofectamine plus reagent.

Detection of Olfactory Receptors Expressed on the Cell Surface

The HEK-293 cells transfected with the olfactory receptor genes were fixed in ice-cold methanol for 5 min and incubated at 4°C for 1 h in a culture soup containing the primary antibody (B6-30) that was produced from a hybridoma cell line. After washing three times with PBS (pH 7.4) for 5 min each, the cells were incubated with a 1:1,000 dilution of the polyclonal anti-mouse Cy2-linked antibody (Amersham Pharmacia Biotech, Uppsala, Sweden). The Rho-ODR-10 and Rho-I7 fusion proteins were visualized by examining pictures of the fluorescent cells taken on a Nikon Eclipse TE 300 microscope (Nikon Corporation, Tokyo, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 506 nm.

RT-PCR of Olfactory Receptor Genes

The HEK-293 and HEK-293 cell lines stably expressing the cameleons that had been transiently transfected with the olfactory receptor genes were seeded into two T-25 culture flasks and cultured for one day. The cells in each flask were then harvested and lysed. The total RNA was isolated using *PUREscript* (GENTRA Systems, Minneapolis, MN, U.S.A.) and the cDNAs of *rho/odr-10* and *rho/I7* were synthesized from the total RNA and amplified by PCR. The PCR conditions used for amplifying *rho/I7* were 36 cycles of 94°C for 1 min, 49°C for 1 min, and 72°C for 1 min, and the PCR conditions used for amplifying *rho/odr-10* were 36 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min.

Analysis of Ca²⁺ Ions Using Cameleons

After the cells in the T-25 culture flask had been washed three times with a standard solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM glucose, 10 mM HEPES [pH 7.4]), the cell solutions (2.4×10⁶ cells) were transferred into polymethacrylate cuvettes (Sigma-Aldrich). The odorant solutions were prepared as 1 M stock solutions in dimethyl sulfoxide (DMSO) for each experiment. After adding the odorant solutions to the cell solution, the fluorescence in the cells was measured at a wavelength of 520 nm after excitation at a wavelength of 450 nm using a spectrofluorophotometer (RF-5301PC, Shimadzu Corporation, Kyoto, Japan).

RESULTS AND DISCUSSION

Construction of HEK-293 Cell Line Stably Expressing Three Types of Cameleons

The expression and localization of the cameleons in HeLa cells are reported elsewhere [18]. Similar results were obtained using HEK-293 cells, which would be used as a host cell for expressing the olfactory receptor proteins on the cell surface. Yellowameleon-2, yellowameleon-3er, and yellowameleon-2nu are expressed in the cytosolic space, ER (endoplasmic reticulum), and nucleus, respectively, using the signal sequences for their specific localization [18, 21].

The localization of eachameleon in each compartment of the cells was detected by using fluorescent imaging, as shown in Fig. 1. The fluorescent image of yellowameleon-3er appeared to be similar to that of yellowameleon-2. However, the fluorescent image of yellow

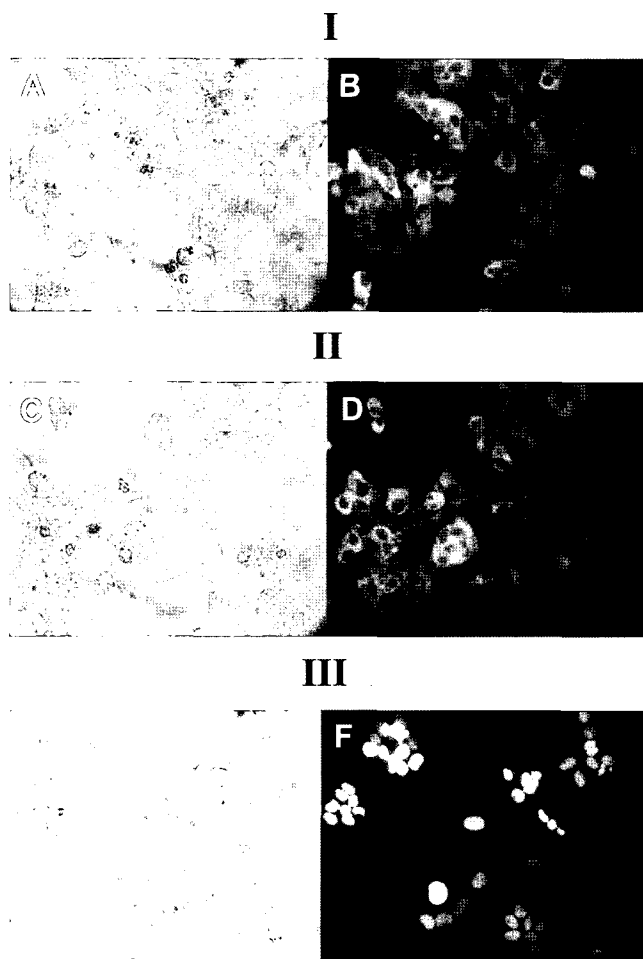


Fig. 1. Fluorescence image of the cameleons expressed in the HEK-293 cells. **I**, yellowameleon-2; **II**, yellowameleon-3er; **III**, yellowameleon-2nu.

A, C, and E are optical micrographs, and B, D, and F are fluorescent images.

ameleon-3er had many dark and bright green specks (Fig. 1II), whereas that of yellowameleon-2 showed a uniform fluorescent image throughout the cells (Fig. 1I). The bright green specks shown in Fig. 1II indicate the yellowameleon-3ers bound to the Ca^{2+} ions in the ERs. The uniform fluorescent image in the cytosol shown in Fig. 1I indicates that the yellowameleon-2 was expressed uniformly throughout the cytosolic space. Compared with the fluorescent images of yellowameleon-2 and -3er, the fluorescence of yellowameleon-2nu was concentrated on the specific location inside the cells (Fig. 1III), indicating that the yellowameleon-2nu was expressed only in the nucleus.

Expression of Olfactory Receptor Proteins

Each stable cell line expressing the respective type ofameleon was transfected with the olfactory receptor genes for the functional analysis of the olfactory receptors using theameleon system. ODR-10 and I7 were used as the model olfactory receptor proteins for the IP_3 pathway and cAMP pathway, respectively. Diacetyl and octanal were odorant molecules specific to the ODR-10 and I7, respectively [15, 23, 26]. The receptor protein was targeted to the cell membrane surface using a rho-tag import sequence at the N-terminus of the receptor protein [13, 15, 17, 25].

The cell surface expression of the olfactory receptor proteins, ODR-10 and I7, was detected by their immunofluorescence

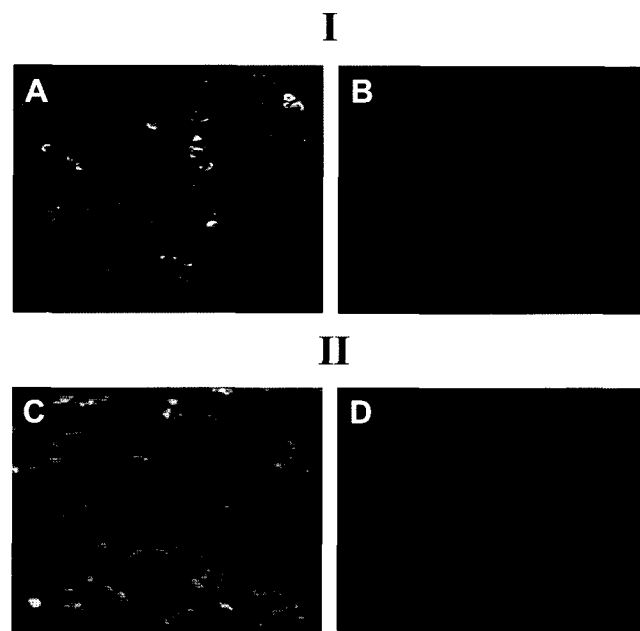


Fig. 2. Expression of the olfactory receptors in HEK-293 cells. **I**, ODR-10; **II**, I7. The cells were treated with a B6-30 primary antibody bound against a rho-tag import sequence, followed by a treatment with a Cy2-labeled goat anti-mouse IgG. **IA** and **IC** are fluorescent images of the cells transiently transfected with olfactory receptor genes. **IB** and **ID** are fluorescent images of the nontransfected control cells. The excitation and emission wavelengths were 488 nm and 506 nm, respectively.

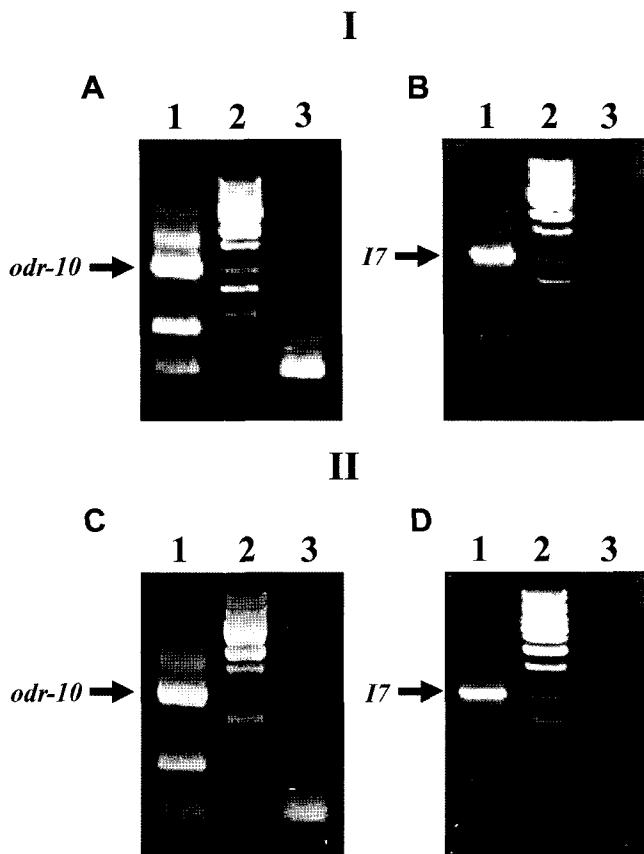


Fig. 3. RT-PCR of the olfactory receptor genes. **I**, HEK-293 cells without cameleon, transfected with an olfactory receptor gene: (A) ODR-10, (B) I7; **II**, HEK-293 cells stably expressing yellow cameleon-2, transfected with an olfactory receptor gene: (C) ODR-10, (D) I7. Lanes 1 and 3 show the RT-PCR results of the transfected and nontransfected HEK-293 cells, respectively. Lane 2 is a 1-kb ladder size marker.

signals (Fig. 2) and RT-PCR (Fig. 3). The immunofluorescence signals for the olfactory receptor expression were measured using HEK-293 cells without cameleon, because it is difficult to distinguish between the fluorescence signals for olfactory receptor expression from those emitted from cameleon. The fluorescence shown in Fig. 2 indicates that the ODR-10 and I7 receptor proteins are expressed on the plasma membrane of the HEK-293 cells, because these antibodies cannot pass through the plasma membrane of these cells *via* the cell fixation used in these experiments. No fluorescence was detected in the control cells, which had not been transfected with the olfactory receptor genes. The mRNAs of the *rho/odr-10* and *rho/i7* fusion genes were detected in the HEK-293 cells without cameleon (Fig. 3I) and those cells stably expressing cameleon (Fig. 3II). The RT-PCR results show that the olfactory expression in the HEK-293 cells without cameleon was similar to that in the HEK-293 cells stably expressing cameleon. These results support the expression of the olfactory receptor in both cell types.

Functional Analysis of Olfactory Receptors Using Yellow Cameleon-2

The stimulation of the olfactory receptor proteins by odorants triggers the signaling pathways within the cells and an inflow of Ca^{2+} ions to the cytosol from either outside of the cells (cAMP pathway) or from internal Ca^{2+} stores (IP₃ pathway). It was reported that the intracellular signaling induced by the binding of odorants to the olfactory receptor proteins can be detected by surface plasmon resonance [16]. The I7 receptor transduces signals *via* a cAMP pathway [15], indicating that the inflow of Ca^{2+} ions from outside of the cell causes an increase in the cytosolic Ca^{2+} level. Recent study showed that I7 receptor expressed in heterologous cells transduced signals *via* an IP₃ pathway as well as a cAMP pathway at high odorant concentrations [14]. The receptor ODR-10 is known to transduce signals *via* the IP₃ pathway [22], suggesting that Ca^{2+} ions are released from the internal Ca^{2+} stores (e.g., endoplasmic reticulum) to the cytosol. Regardless of the cAMP or IP₃ pathway, the function of olfactory receptors including I7 and ODR-10 protein in the heterologous HEK-293 cell system can be analyzed by measuring the change of cytosolic Ca^{2+} ions. Cameleon-2nu, which is expressed in the nucleus, cannot be used for measuring the function of olfactory receptors, but it might be used for any other receptors or proteins inducing the change of Ca^{2+} within the nucleus. Cameleon-3er, which is expressed in the ER, can be used for measuring the function of olfactory receptor only eliciting a signal *via* an IP₃ pathway, in which Ca^{2+} ions are released from ER to cytosol. Among the three types of cameleons, the yellow cameleon-2 is localized in the cytosol and binds to cytosolic Ca^{2+} ions. It can be used for any olfactory receptors triggering either the cAMP or IP₃ pathways. Therefore, the yellow cameleon-2 is the most appropriate for analyzing the changes in the Ca^{2+} level induced by the olfactory receptor-mediated signal transduction.

Increased Ca^{2+} ions bind to the yellow cameleon-2 proteins. After the *odr-10* and *i7* genes had been transfected, the responses of the expressed olfactory receptors to their specific odorants were measured by Ca^{2+} assay using yellow cameleon-2 (Fig. 4). The olfactory receptors expressed on the surface of cells were stimulated by the odorants diacetyl and octanal, which are specific to the olfactory receptors ODR-10 and I7, respectively. Fig. 4 shows the stimulation of each olfactory receptor by its specific odorant, which caused an increase in the intracellular Ca^{2+} level. This indicates that the olfactory receptors expressed on the surface of the HEK-293 cells are functional. The function of the olfactory receptors has usually been examined by measuring the concentration of Ca^{2+} ions using fura, which is a time-consuming, tedious, and expensive technique. This study demonstrated that yellow cameleon-2 could be conveniently used for a functional assay of the olfactory receptors expressed in heterologous cells. Cameleons can

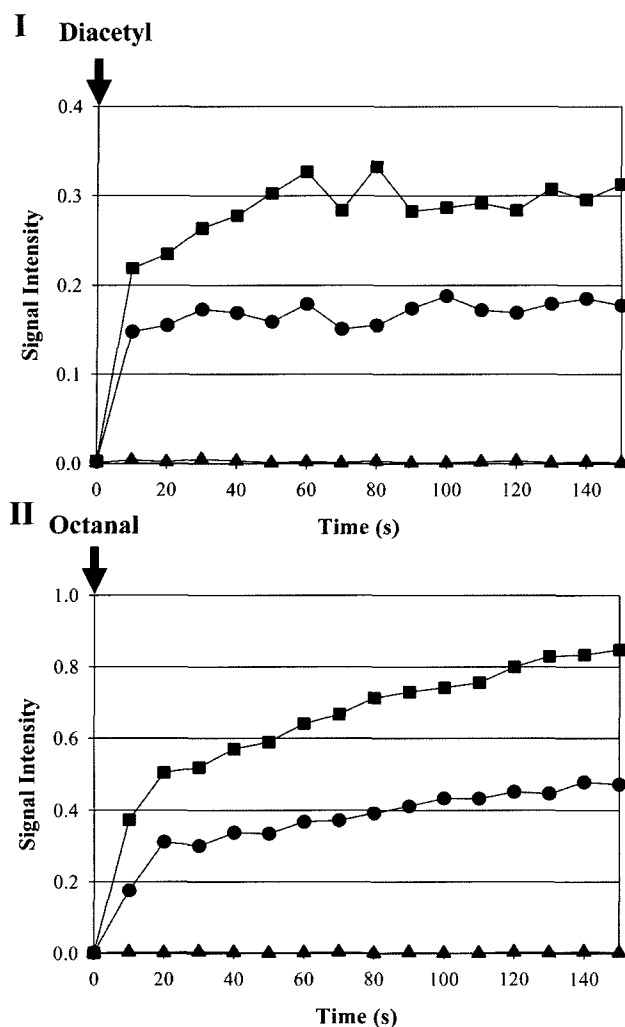


Fig. 4. Functional analysis of the olfactory receptors using yellow cameleon-2.

I, ODR-10; II, I7. *pcDNA3/rho-tag/odr-10* and *pcDNA3/rho-tag/I7* genes were transiently transfected into the HEK-293 cells stably expressing the yellow cameleon-2. Each olfactory receptor expressed on the surface of the cells was stimulated by diacetyl or octanal. The intracellular changes in Ca^{2+} ions were measured by a spectrofluorophotometer. The Y axis represents the fluorescence intensity at 520 nm. The signal intensity was obtained by subtracting the fluorescent intensity before stimulation from that after stimulation. The closed squares (■) and circles (●) represent the changes in the Ca^{2+} level in the cells expressing an olfactory receptor stimulated by 1 mM and 0.1 mM odorant, respectively. The closed triangles (▲) show the changes in the Ca^{2+} level in the control cells without an olfactory receptor with the stimulation by 1 mM odorant.

also be used to monitor the change in Ca^{2+} , which occurs in a specific location inside cells.

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