

**Fluorescence ratiometric detection of ligand-induced receptor
internalization using extracellular coiled-coil tag–probe labeling**

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Abstract

We report a new method for the detection of ligand-induced receptor internalization by fluorescence ratiometric imaging of pH in endosomes in combination with a recently developed posttranslational labeling system based on the formation of a heterodimeric coiled-coil structure. The N-terminus of the β 2-adrenergic receptor expressed on the cell surface was doubly labeled with pH-sensitive fluorescein and pH-insensitive tetramethylrhodamine. A significant increase in the tetramethylrhodamine-to-fluorescein fluorescence intensity ratio was observed after incubation with agonists in a concentration-dependent manner. This simple and accurate method of detecting the agonistic activity of receptors will be useful for high-throughput screening of drug candidates.

Keywords: endocytosis, tag-probe labeling, ratio imaging, membrane receptors, high-throughput screening

Abbreviations: β 2AR, the human β 2-adrenergic receptor; CHO, Chinese hamster ovary; E3, (EIAALKE)₃; E3- β 2AR, the E3-tagged human β 2 adrenergic receptor; FL, fluorescein; K4, (KIAALEK)₄; TMR, tetramethylrhodamine

1. Introduction

Membrane receptors, principal molecular regulators for intracellular signaling cascades, are the most important drug-target proteins [1]. Therefore, there has been considerable interest in developing new chemical tools that detect receptor activities in living cells in order to elucidate the dynamic behavior of receptors and to refine drug-screening systems [2]. A useful indicator of receptor activity is the endocytosis of receptors, often involved in desensitization [3]. Because receptor internalization is a ubiquitous process irrespective of downstream signaling pathways, it is applicable to the monitoring of activities of a wide variety of membrane receptors including orphan receptors. Fluorescence imaging of receptors fused with fluorescent proteins has often been used to visualize the internalization of target receptors [4]. However, fluorescent proteins inevitably label receptors even in intracellular compartments, which can partially obscure observations of internalization. An alternative strategy to image receptor activity is the observation of translocation of arrestins fused with fluorescent proteins from cytosol to endosomes containing activated receptors [5], although the method can detect signals from endogenous receptors other than the target receptors.

Posttranslational labeling of genetically tagged proteins in living cells with exogenous probes that specifically bind to the tag has a number of advantages over fusion with

fluorescent proteins, such as a smaller size, cell surface-specific labeling, the availability of diverse fluorophores, and ease in controlling the labeling ratio in multicolor labeling [6]. Several distinct labeling principles including protein–ligand interaction, metal chelation, and enzymatic reaction have been devised and applied to membrane receptors [7]. We recently developed a coiled-coil tag–probe labeling method based on the peptide pair (E3 tag (EIAALKE)₃ and K4 probe (KIAALEK)₄) that forms a heterodimeric coiled-coil structure (Figure 2A) [8]. The tight interaction (apparent $K_d \sim 6$ nM) is suitable for long-time observation. The tag–probe pair (5–6 kDa) is much smaller than fluorescent proteins (e.g., 27 kDa for GFP). Quick (within 1 min) and cell-surface specific labeling is advantageous when observing the internalization of cell-surface receptors. Here we report a simple and sensitive method for the detection of receptor internalization based on fluorescence ratiometric imaging of pH in endosomes using the coiled-coil labeling method.

2. Materials and methods

2.1. Peptide synthesis

The K4 peptide (KIAALKE)₄ was synthesized by a standard Fmoc-based solid phase method [8]. Fluorophores (tetramethylrhodamine (TMR), Rhodamine 6G, fluorescein (FL), and CypHer 5) were labeled at the N-terminus by treatment with succinimidyl ester derivatives of fluorophores [8]. The purity of the synthesized peptides (higher than 95%) was determined by analytical HPLC and ion spray mass spectrometry.

2.2 Cell culture

Chinese hamster ovary (CHO) cells were maintained in F-12 medium with 10% heat-inactivated fetal bovine serum in 5% CO₂ at 37 °C. Confluent cells were harvested after treatment with 1 mM EDTA and 2.5 g L⁻¹ trypsin for 2 min, resuspended in new medium, and plated to a new dish (3 X 10⁵ cells in a 10-cm dish).

2.3 Construction of plasmids

The cDNA encoding the E3-tagged human β₂ adrenergic receptor (E3-β₂AR) previously inserted into the pcDNA3 vector was transferred to the Hind III/ApaI site of pcDNA5 (Life

Technologies). Large-scale preparation of the plasmid was performed using a GenElute Endotoxin-Free Plasmid Maxiprep Kit (Sigma, St. Louis, MO).

2.4 Stable expression of receptor

For generating CHO cells that stably express the receptors, the Flp recombinase-mediated integration system (Flp-In System, Life Technologies) was used. A mixture of 0.3 μg of pcDNA5/E3- β 2AR and 2.7 μg of pOG44 encoding Flp recombinase was co-transfected into Flp-In-CHO cells using 5 μL of LipofectAMINE (Life Technologies) for 3 h. Forty-eight hours after transfection, the cells were plated (1×10^4 cells in a 10-cm dish) and cultured in medium containing hygromycin ($500 \mu\text{g mL}^{-1}$). Hygromycin-resistant cell colonies were picked out and expanded.

2.5 Ligand binding

CHO cells expressing E3- β 2ARs were cultured on a 15-cm dish ($\sim 1 \times 10^7$ cells), labeled with TMR-K4/FL-K4 (10 nM each), and incubated with or without 20 μM isoproterenol for 30 min in F-12 media containing 100 μM ascorbate [9]. The cells were washed twice in ice

cold PBS and then incubated in ice cold PBS/EDTA (5 mM) pH 7.2 for 1 h to washout the ligands [9]. The cell suspensions containing 1×10^6 cells in 1 mL PBS/EDTA were prepared in triplicate and incubated with 10 nM [^3H] CGP-12177 [10] (Perkin Elmer, Waltham, MA) for 3 h at 13°C . Nonspecific binding was measured in the presence of 1 μM propranolol. The cells were harvested on Whatman GF/B filters, and the bound radioactivity was quantified by a Perkin Elmer Tri-Carb 3110TR liquid scintillation analyzer.

2.6 Confocal microscopy

CHO cells expressing E3- β 2ARs were plated onto a 35-mm glass bottom dish (1×10^5 cells) and incubated for 36–48h. Before K4 labeling, the cells were washed once with PBS (+) (137 mM NaCl/8.1 mM Na_2HPO_4 /2.68 mM KCl/1.47 mM KH_2PO_4 /0.9 mM CaCl_2 /0.33 mM MgCl_2 , pH 7.4) and then 1 mL of TMR-K4/FL-K4 solution (20 nM each) in PBS (+) was added and incubated for 2 min. After the incubation, 1 mL of ligand solution in PBS (+) was added, gently mixed, and incubated for 30 min at 37°C (final probe concentration = 10 nM). In time-lapse experiments, the cells were immersed in a 1:1 mixture of medium and freshly prepared ascorbate buffer (25 mM HEPES/150 mM ascorbic acid/4.4 mM KCl, adjusted to pH 7.4 by 150 mM NaOH) to suppress photodamage to the receptors that significantly

affected the internalization. The cells were observed under a confocal microscope (Nikon C1, Tokyo, Japan). Green (FL and Rhodamine 6G) and red (TMR) channels were excited by 488-nm and 561-nm lasers, and detected through BP500–550 nm and BP575–615 nm emission filters, respectively.

2.7. Image analysis

Fluorescence intensities obtained for green and red channels (8 bit) were converted to an intensity ratio after the cutoff of background fluorescence (typically <3% of fluorescence intensity observed on membranes). Pseudo-color images were generated using the ‘index color’ command in Adobe Photoshop CS3 (Adobe systems, San Jose, CA). Regions of interest corresponding to intracellular puncta were individually identified by eye using the software provided by manufacturer.

2.8. Analysis of concentration dependence

The degree of receptor internalization in the presence of the agonist was fitted with a sigmoidal curve, $R_{\max} + (R_{\max}-1)/(1+10^{[L]-\log(\text{EC50})})$, where [L] and R_{\max} denote the

concentration of the agonist on a log scale and the maximal value of the ratio in the presence of excess agonist, respectively. Competitive inhibition of receptor internalization with the antagonist was fitted using the equation, $1 + (R_{\max}-1)/(1+10^{[L]-\log(IC_{50})})$.

3. Results and discussion

3.1. Fluorescence ratiometric detection of ligand-induced receptor internalization

To detect receptor internalization, we took advantage of the pH decrease in endosomes following internalization [3]. Here we used the human β 2-adrenergic receptor (β 2AR), a G-protein-coupled receptor, and detected the change in endosomal pH by fluorescence ratiometric imaging. The N-terminally tagged receptors (E3- β 2AR) were stably expressed in CHO cells. The expression level was determined to be 1.3×10^5 receptors per cell by the [3 H]-CGP-12,177 whole cell binding assay. The cell-surface receptors were specifically labeled with either the pH-sensitive green dye FL (pKa ~ 6.5 and 4.2) or the pH-insensitive red dye TMR by use of a 1/1 mixture of FL-K4 and TMR-K4 in medium at pH 7.4. FL-K4 and TMR-K4 exhibited similar binding affinities for the E3 tag (Fig. 1). The FL–TMR pair is

conventionally used to monitor pH in living cells [11,12]. Lowering the pH in endosomes attenuates FL fluorescence resulting in an increase in the fluorescence ratio (red/green). Time-lapse fluorescence ratio images following agonist stimulation (Fig. 2D, normalized to 1 before addition of ligands) were obtained based on confocal images for FL (Fig. 2B) and TMR (Fig. 2C). Fifteen to thirty minutes after stimulation with the agonist isoproterenol, the fluorescence ratio in puncta that appeared in the cytosol (arrowheads in Fig. 2D) was significantly enhanced up to three fold of the initial value. The degree of loss of the surface receptors after the agonist stimulation for 30 min as measured by the [³H]-CGP-12,177 binding assay was 40 ± 6 % (n = 3), which was similar to that for wild-type β 2AR (40–50%) [9], confirming that the coiled-coil labeling did not inhibit the receptor internalization. No puncta appeared and no significant change in the ratio was observed for at least 30 min in the absence of the agonist (Fig. 2E). We also examined K4 probes labeled with the fluorophore CypHer 5 that become fluorescent at lower pHs [13]. However, the CypHer 5-K4 probes tended to aggregate in aqueous phase and were significantly endocytosed even in the absence of agonist (Supplementary Fig. S1).

To estimate pH around the N-termini of the internalized receptors, calibration experiments were performed both on the cell surface and in vitro (Supplementary Fig. S2). We estimated

that the threefold increase corresponded to a pH decrease from 7.4 to 5–6, the latter value being consistent with the pH in sorting/late endosomes [3].

The change in the TMR/FL ratio with the agonist could be due to an increase in Förster resonance energy transfer from FL to TMR, and/or originating from self-association of the receptors and/or the formation of domains concentrated in the receptors [14]. In addition to energy transfer, receptor dimerization can also influence the TMR/FL ratio by self-quenching and/or environmental changes, if a fraction of dimers are labeled with two identical fluorophores. Because the labeling degree in the experimental conditions is estimated to be 77% (assuming $K_d = 6$ nM), a significant fraction of dimers (~60 %) should be labeled with two fluorophores. To check these possibilities, the receptors were doubly labeled with a pH-insensitive pair of fluorophores (Rhodamine 6G–TMR) having a similar Förster distance of ~55 Å (calculated according to [15]) to that of the FL-TMR pair (~54 Å [16]) and stimulated with the agonist. No change in the ratio was observed in endosomes (arrowheads in Fig. 2F), indicating that the increase in the ratio observed in Fig. 2D predominantly reflects the drop in pH. Consistent with this, neutralization of endosomes by addition of NH_4Cl after agonist stimulation decreased the TMR/FL ratio in intracellular puncta (Supplementary Fig.S3).

3.2. Ligand- and concentration- dependences of the ratio change

To confirm the usefulness of the ratiometric imaging system for drug screening, similar experiments using the FL–TMR pair were performed for several drugs including agonists for β 2AR. Fig. 3 shows the observed % increase in the ratio in whole images (A) and in endosomes (B). Addition of unrelated drugs (flunarizine, a Ca^{2+} channel blocker; phenytoin, a Na^+ channel blocker) did not affect the ratio, whereas treatment with agonists (isoproterenol, norepinephrine, and epinephrine) significantly increased the ratio. The changes in the ratio measured for whole images were slightly different among agonists (Fig. 3A) although those in endosomes were similar (Fig. 3B), consistent with agonist-specific alterations in the degree of endocytosis [17]. To further examine the reliability of the method, the concentration-dependence of the ratio was measured. The ratio change vs. log concentration of isoproterenol (Fig. 4A) followed a sigmoid curve assuming a Hill constant of 1 to give an EC_{50} value of 176 ± 76 nM, which is slightly smaller than typical binding constants for isoproterenol (377–556 nM) obtained with a radioligand binding to human β 2AR [18]. Competitive inhibition of internalization by adding the antagonist propranolol (Fig. 4B) was also fitted with a sigmoid curve ($\text{IC}_{50} = 33 \pm 17$ nM).

The above results demonstrate that the fluorescence ratiometric detection of pH in endocytotic vesicles employing coiled-coil tag–probe labeling is useful for an accurate evaluation of receptor internalization. We emphasize that the simple method is superior to a conventional granularity analysis because our method is not affected by the presence of receptors that are not sorted to cell membranes therefore there is no agonist-independent signal change. Recently, another tag–probe labeling method (SNAP and CLIP protein tagging (~ 20 kDa)) has also been reported to be useful to quantify internalization of orexin OX₁ and cannabinoid CB₁ receptors [19], although the labeling time (30 min) is longer than that of coiled-coil labeling (2 min). We have confirmed that our unique method is applicable to 96-well plate format for high-throughput screening (Takeda, Yano, and Matsuzaki, manuscript in preparation). It will be also useful for both basic research into, and the discovery of drugs for, various membrane receptors.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

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Figure legends

Figure 1. Increase in fluorescence intensity (FI) for (A) TMR-K4 and (B) FL-K4 on CHO cell membranes expressing the human E3-tagged β 2-adrenergic receptors as a function of the probe concentration [P] (n = 10). The dissociation constant (K_d) was determined by fitting the data with the equation $FI = A*[P]/([P] + K_d)$, where A indicates the maximal fluorescence intensity.

Figure 2. (A) Schematic illustration of two-color labeling by the coiled-coil method. (B, C) Confocal images for agonist-induced receptor internalization. The E3-tagged β 2-adrenergic receptors stably expressed in Chinese hamster ovary cells were labeled with the pH-sensitive FL (B) and pH-insensitive TMR (C) by adding the fluorophore-labeled K4 probes (final concentration, 10 nM each). The agonist isoproterenol (10 μ M) was added at time zero. (D–F) Ratiometric detection of agonist-induced receptor internalization. The cells were labeled with the pH-sensitive FL-TMR pair (D and E) or the pH-insensitive Rhodamine 6G–TMR pair (F). The agonist (D and F) or the vehicle (E) was added at time zero. Scale bar = 10 μ m.

Figure 3. Changes in the TMR/FL ratio after incubation with drugs (100 μ M in 0.5 % dimethyl sulfoxide (DMSO)) for 30 min measured in whole images (A) and intracellular puncta (B) (n = 5).

Figure 4. Concentration-dependence of the fluorescence ratio after treatment with the agonist isoproterenol (A) and the antagonist propranolol in the presence of 10 μ M isoproterenol (B) after incubation for 30 min (n = 5).

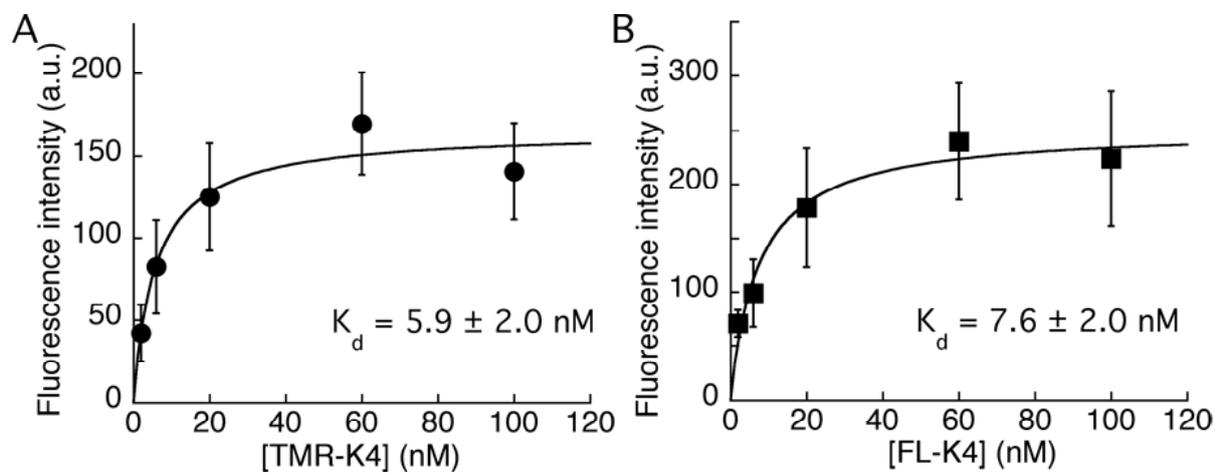


Figure 1

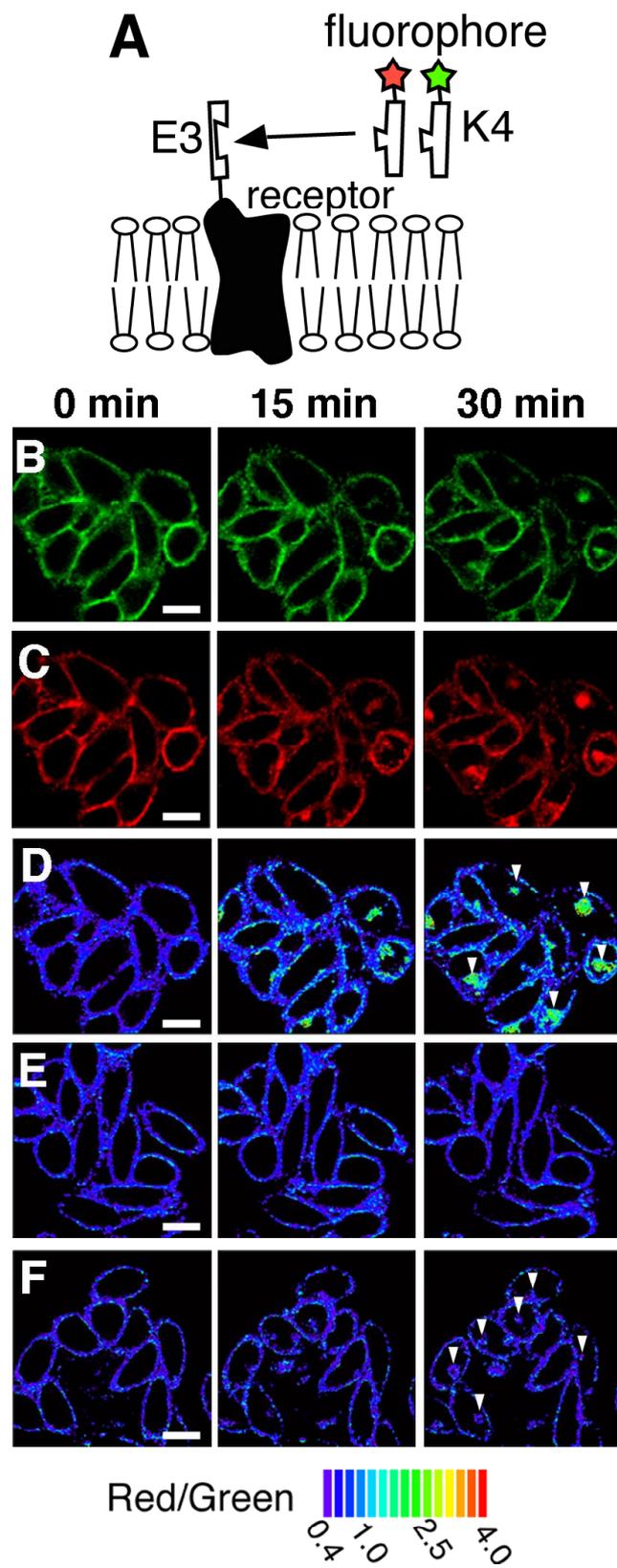


Figure 2

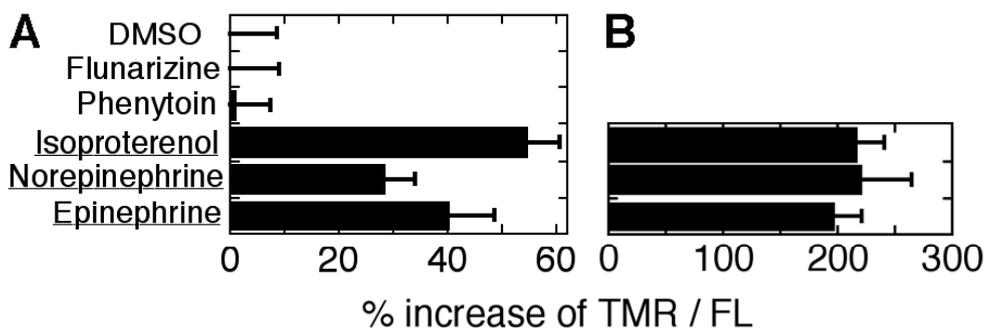


Figure 3

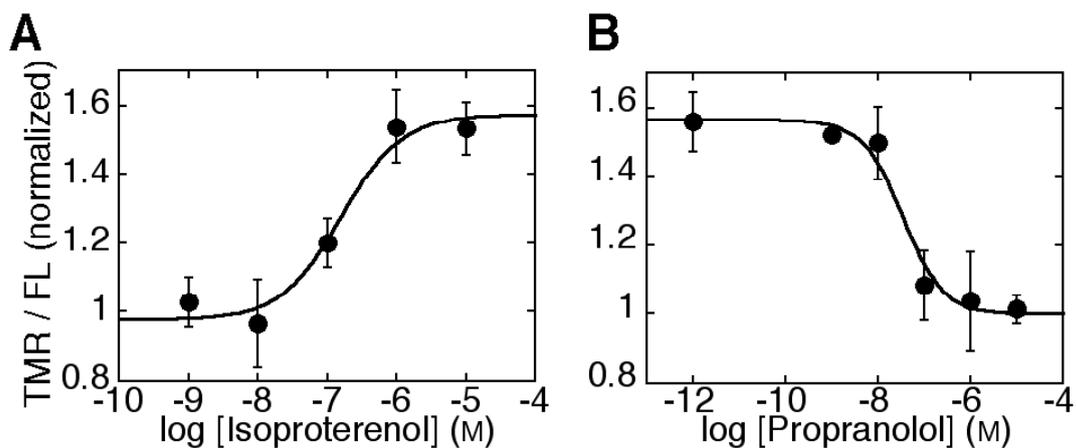


Figure 4

Supplementary data

Fluorescence Ratiometric Detection of Ligand-Induced Receptor Internalization Using Extracellular Coiled-Coil Tag-Probe Labeling

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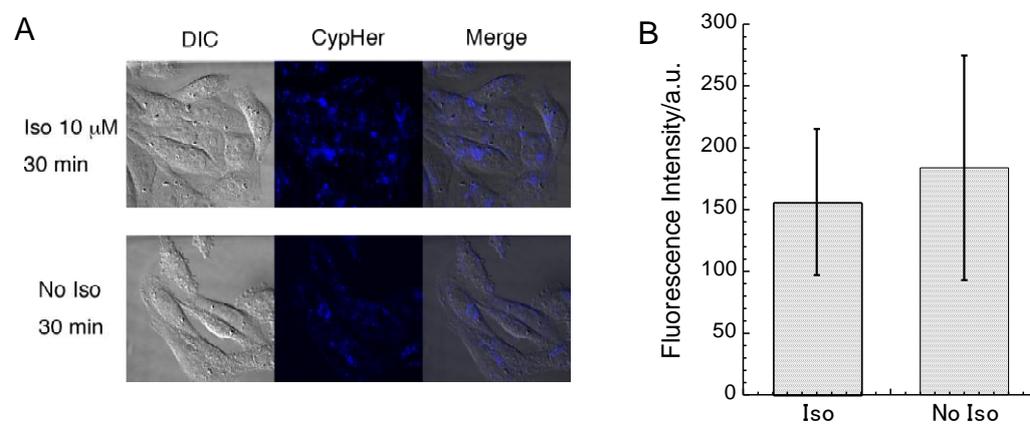


Figure S1. Agonist-induced endocytosis of E3 tagged β 2 adrenergic receptors probed by CypHer 5-K4. (A) Confocal images in the presence and absence of agonist (10 μ M Isoproterenol). A stock solution of CypHer 5-K4 in DMSO (20 μ M) was diluted into PBS (+) to obtain 20 nM labeling solution. The probe was not soluble in H₂O. (B) Fluorescence intensities in cells ($n = 50$).

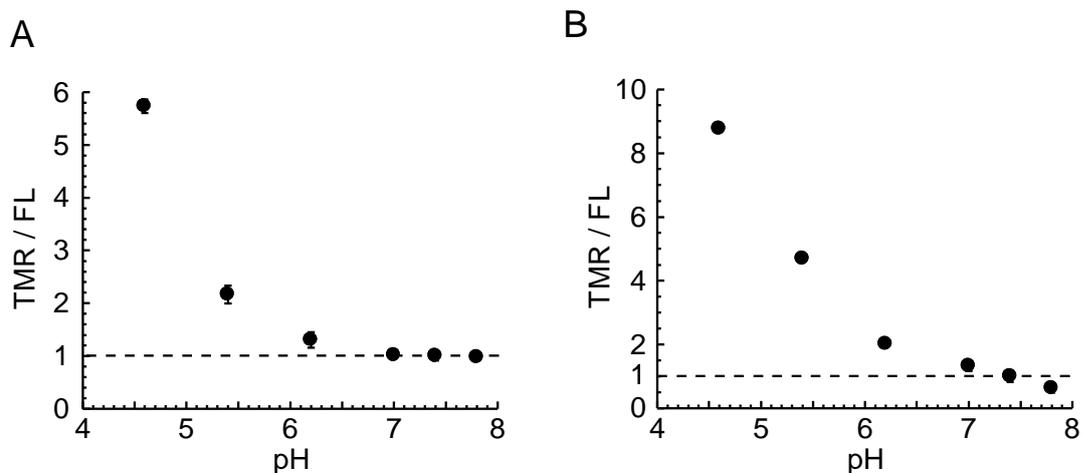


Figure S2. Relationship between pH and the TMR/FL ratio measured on (A) cell surface and (B) in vitro. (A) The TMR/FL intensity ratios were measured for CHO cells stably expressing E3-tagged β 2AR labeled with TMR-K4 and FL-K4 (10 nM each) in buffers having different pH values (pH 4.6–7.8). The buffers were prepared by titrating a 1/1 mixture of 9.57 mM Na_2HPO_4 /137 mM NaCl/2.68 mM KCl/0.9 mM CaCl_2 /0.33 mM MgCl_2 and 4.785 mM citric acid/141.8 mM NaCl/2.68 mM KCl/0.9 mM CaCl_2 /0.33 mM MgCl_2 with 0.15 M NaOH. (B) The ratio determined in a quartz cuvette using a spectrofluorometer at a total probe concentration of 50 nM. Photo-physical properties of a fluorophore often change after being tagged to proteins. Furthermore, negatively charged cell membranes may decrease the surface pH. On the other hand, the calibration performed on cell surface may involve signals from internalized vesicles underneath the cell membrane although the measurements were finished prior to the appearance of intracellular grains and cell surface regions were selected for the intensity analysis. Although a slight difference was observed in the two calibrations, we estimated that the threefold increase in the ratio corresponded to a pH decrease from 7.4 to 5–6.

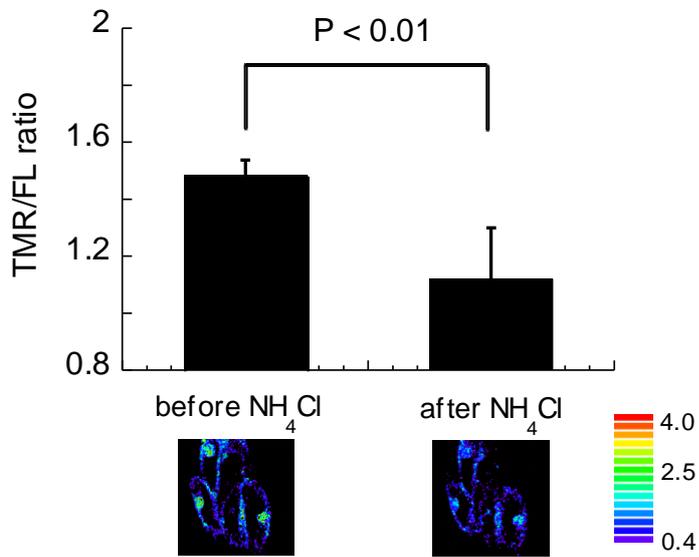


Figure S3. Neutralization of endosomes by addition of NH₄Cl (10 mM). After labeling of E3-tagged β 2AR expressed in CHO cells with TMR-K4 and FL-K4 (10 nM each), the cells were stimulated with 10 μ M isoproterenol for 30 min, the ratio image was obtained (before NH₄Cl). The same area was imaged 1 min after addition of NH₄Cl (after NH₄Cl, $n = 5$).