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Yeast-Based Fluorescence Reporter Assay of G Protein-coupled Receptor Signalling for Flow Cytometric Screening: *FAR1*-Disruption Recovers Loss of Episomal Plasmid Caused by Signalling in Yeast

Jun Ishii[1], Tsutomu Tanaka[2], Shizuka Matsumura[3], Kenji Tatematsu[4], Shun'ichi Kuroda[4], Chiaki Ogino[3], Hideki Fukuda[2] and Akihiko Kondo[3,*]

[1]Department of Molecular Science and Material Engineering, Graduate School of Science and Technology; [2]Organization of Advanced Science and Technology; [3]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University; and [4]Department of Structural Molecular Biology, Institute of Scientific and Industrial Research, Osaka University, Japan

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Here, we describe a yeast-based fluorescence reporter assay for G protein-coupled receptor (GPCR) signalling using a flow cytometer (FCM). The enhanced green fluorescent protein (*EGFP*) gene was integrated into the *FUS1* locus as a reporter gene. The engineered yeast was able to express the EGFP in response to ligand stimulation. Gene-disrupted yeast strains were constructed to evaluate the suitability of the yeast-based fluorescence screening system for heterologous GPCR. When receptor was expressed by episomal plasmid, the proportion of the signalling-activated cells in response to ligand stimulation decreased significantly. The GPCR-signalling-activated and non-activated cell clusters were individually isolated by analysing the fluorescence intensity at the single-cell level with FCM, and it was found that the plasmid retention rate decays markedly in the non-activated cell cluster. We attributed the loss of plasmid to G1 arrest in response to signalling, and successfully improved the plasmid retention rate by disrupting the *FAR1* gene and avoiding cell cycle arrest. Our system will be a powerful tool for the quantitative and high-throughput GPCR screening of yeast-based combinatorial libraries using FCM.

Key words: G protein-coupled receptor, yeast, enhanced green fluorescent protein, signalling, flow cytometer.

Abbreviations: EGFP, enhanced green fluorescent protein; FCM, flow cytometer; GPCR, G protein-coupled receptor; G-proteins, guanine nucleotide-binding proteins; HTS, high-throughput screening; RGS, regulator of G-protein signalling.

Signal transduction regulates various physiological functions in organisms and receptors are widely recognized as the sensors of external information to generate intracellular signalling. G protein-coupled receptors (GPCRs) represent the largest family of integral membrane proteins, and are considered to be attractive pharmaceutical and therapeutic targets (*1*). GPCRs are the starting points of signals transduced from external stimuli into internal responses via heterotrimeric guanine nucleotide-binding proteins (G-proteins).

Ligand binding triggers conformational changes in receptors, and their interactions with intracellular G-proteins are commonly conserved among various eukaryotic species. The eukaryotic yeast cell has a pheromone signalling pathway mediated by G-proteins and the signalling mechanisms are well understood (*2*). Yeasts are used for not only fundamental studies of signalling but also are applied to research into ligand screening and receptor mutagenesis (*3–7*). Yeasts are particularly suited to GPCR studies due to the highly developed range of powerful genetic manipulation techniques, because they exhibit the functional expression of heterologous GPCRs, for example, from mammalian cells that can transduce G-protein signalling (*8–10*).

Figure 1 illustrates the pheromone signalling pathway in the yeast. The haploid **a**-cell of *Saccharomyces cerevisiae* has Ste2p, which is known to be the yeast endogenous GPCR. Ste2p binds α-factor, known to be a mating pheromone, and activates the pheromone signalling pathway mediated by the intracellular heterotrimeric G-protein (*2*). The yeast G-proteins, which consist of Gpa1p, Ste4p and Ste18p subunits, are structurally and functionally similar to the mammalian Gα-, β- and γ-subunits, respectively. The activated signalling triggers cell cycle arrest (G1 arrest) caused by the phosphorylation of Far1p (*11*). Simultaneously, the signalling induces the expression of the *FUS1* gene, which follows the phosphorylation of a transcription factor, Ste12p (*11*). The principal negative regulator of the pathway is the Gpa1-specific GTPase-activating protein Sst2p, a member of the RGS (regulator of G-protein signalling) family (*12*). Pheromone signalling is inactivated through the proteolysis of α-factor to recover from cell cycle arrest (*13*).

*To whom correspondence should be addressed. Tel: +81 78 803 6196, Fax: +81 78 803 6196, E-mail: akondo@kobe-u.ac.jp

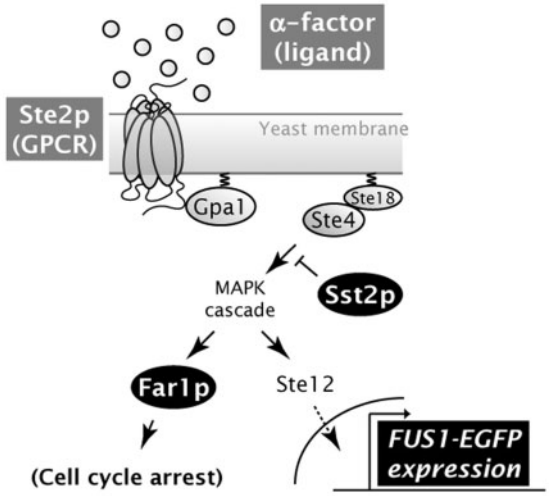


Fig. 1. **Schematic illustration showing the yeast pheromone signalling pathway and the genetic modifications of fluorescence reporter gene in response to α-factor.** This pathway is extensively used for GPCR assay; substitution of the *FUS1* gene by the *FUS1-EGFP* fusion gene enables signalling analyses through Ste12p transcription factor. Ste2p is an endogenous yeast GPCR, which induces signalling by binding of α-factor. Yeast cells lacking Far1p, which is a G1-cyclin-dependent kinase inhibitor mediating cell cycle arrest in response to α-factor, are prevented from inducing G1 arrest in the presence of α-factor. Yeast cells lacking Sst2p, which is a principal negative regulator of the pathway, exhibit hypersensitivity in the presence of α-factor.

In many cases, G-protein signalling in the yeast has been detected and evaluated by growth selections or enzymatic reactions using *lacZ* or *HIS3* controlled by transcriptional activation of the *FUS1* promoter as the reporter gene (*3–10*, *14*, *15*). The enzyme reporters allow highly sensitive and quantitative assays of receptor function (*3*, *8–10*, *14*). However, growth selections using auxotrophic reporters are suitable for screening large libraries rather than quantitative assay (*3–10*, *15*).

The fluorescence protein is a powerful and convenient tool to instantaneously quantify and visualize target proteins *in vivo* without onerous procedures. Fluorescent proteins enable both quantitative assay and high-throughput screening (HTS) using a flow cytometer (FCM) (*16–19*). Especially, the advantage of FCM experiment is not only to measure the fluorescent intensity at the single-cell level but also to obtain cells having the desired fluorescent level by setting the sorting area. However, in terms of GPCR signalling analyses using yeast, there are few reports of quantitative signalling studies using fluorescence reporter genes on FCM (*20–22*), except for investigations into expression and localization on microscopy (*23–25*).

In this study, we demonstrated the advantages of FCM experiment in a fluorescence assay system for exogenous GPCR using yeast, with the aim of establishing quantitative HTS for GPCR or its ligands using a yeast combinatorial library. The *EGFP* gene was integrated as a fluorescence reporter gene into the yeast cell, and the engineered yeast was evaluated for suitability in the fluorescence assay system for heterologous GPCR by disrupting related genes. When the receptor was expressed by episomal plasmid, the proportion of the signalling-activated cells in response to ligand stimulation decreased significantly. We individually isolated the signalling-activated and non-activated cell clusters by FCM sorting and found that the plasmid retention rate drastically decays in the non-activated cluster. The loss of plasmid is a critical problem in combinatorial library screening. We attributed the problem to G1 arrest in response to signalling, and successfully improved the plasmid retention rate by disrupting the *FAR1* gene and avoiding cell cycle arrest. Thus, our system is useful for analysis of yeast GPCR signalling, and our findings can ensure yeast-based quantitative screening for cells displaying the desired GPCR-signalling level.

MATERIALS AND METHODS

Yeast Gene Disrupted Strains—Strains IM-5 and IM-7 constructed by disrupting the *SST2* gene of BY4741 (*26*) and BY4741*far1*Δ have been described previously (*22*). Strains IM-50 and IM-70, which are yeast cells IM-5 and IM-7 with respectively disrupted *STE2* genes, were constructed by the homologous recombination of the *LEU2* marker sequence amplified by PCR from pRS405 (American Type Culture Collection, Manassas, VA, USA) with the following oligonucleotides: 5′AATTGGTTACT TAAAAATGCACCGTTAAGAACCATATCCAAGAATCAA AAtcgactacgtcgtaaggccgtttctgacag and 5′ACCTTATACCG AAGGTCACGAAATTACTTTTTCAAAGCCGTAAATTTT GAtcgacggtcgaggagaacttctagtatatc.

Integration of Fluorescence Reporter Controlled by FUS1 Promoter—The plasmid used for fusing the fluorescence reporter gene to the *FUS1* gene on the yeast chromosome was constructed as follows: a DNA fragment encoding *EGFP* was amplified by PCR from pEGFP (Takara Bio, Shiga, Japan) with oligonucleotides 5′GGTCGTCGACATGGTGAGCAAGGGCGAGGAGCTGT TCACC and 5′CGCGGTCGACTTACTTGTACAGCTCGT CCATGCCGAGAGT. The amplified fragment was digested with *Sal*I and ligated into pUGP3 (*27*), resulting in plasmid pUGP3-EGFP. A fragment encoding the open reading frame (ORF) of *FUS1* was amplified by PCR from BY4741 genomic DNA with oligonucleotides 5′GTGGGAATTCTAATAATCAGAACTCCAACA and 5′GGCGGAGCTCTTTGATTTTCAGAAACTTGA, followed by digestion with *Eco*RI/*Sac*I. A fragment encoding *EGFP* was amplified by PCR from pUGP3-EGFP with oligonucleotides 5′CTCTGAGCTCATGGTGAGCAAGGGC GAGGA and 5′TCTTGTCGACTTAGATATCCTTGTACA GCTCGTCCATGCCGAGAGTGATC, followed by digestion with *Sac*I/*Sal*I. These two digested fragments were simultaneously inserted at the *Eco*RI/*Sal*I sites of pUC119 (Takara Bio, Shiga, Japan), resulting in plasmid pUC119-mFUS1-EGFP. DNA fragments encoding the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) terminator region, the *HIS3* marker and the 1-kb region of downstream of the *FUS1* ORF were respectively amplified by PCR from pUGP3-EGFP, pRS403 (American Type Culture Collection, Manassas, VA, USA) and BY4741 genomic DNA with the following oligonucleotide pairs: 5′ACACGTCGACTTGGTTGAACACGTT

GCCAA and 5′CCACGGATCCAAGCTTTCAATCAATGA ATC; 5′GGCTGGATCCAATTCCCGTTTTAAGAGCTT and 5′AAGACCGCGGTGATCCGTCGAGTTCAAGAG; 5′G CGACCGCGGTGAAAATAATATTGACGTTC and 5′TTAT GCATGCTATTCACCAGACCCGCTCCT. The fragments were digested with *Sal*I/*Bam*HI, *Bam*HI/*Sac*II and *Sac*II/*Sph*I, and were then co-instantaneously inserted into pUC119 at the *Sal*I/*Sph*I sites, resulting in the plasmid pUC119-tGAP-HIS3-tFUS1. pUC119-tGAP-HIS3-tFUS1 was digested with *Sal*I/*Sph*I and the digested fragment was inserted into pUC119-mFUS1-EGFP at the same sites, resulting in the plasmid pUC119-FUS1-EGFP-HIS3. A liner fragment containing the *FUS1* ORF, *EGFP*, *GAPDH* terminator, *HIS3* marker and downstream sequence of *FUS1* was prepared by digestion of pUC119-FUS1-EGFP-HIS3 with *EcoR*I/*Sph*I and the fragment was transformed into BY4741, IM-5, IM-50 and IM-70. Strains substituted with the *FUS1-EGFP* fusion gene on the *FUS1* locus were designated IMG-1, IMG-5, IMG-50 and IMG-70, respectively.

Construction of Plasmid Expressing Ste2p—The plasmids used for Ste2p expression were constructed as follows: two DNA fragments encoding the yeast *PGK1* promoter (*PGK5′*) and *PGK1* terminator (*PGK3′*) were amplified by PCR from BY4741 genomic DNA with the following oligonucleotide pairs: 5′TTTTCTCGAGAAAG ATGCCGATTTGGGCGC and 5′GCCCGCTAGCGTTTTA TATTTGTTGTAAAA; 5′GCCCAGATCTGAAATAAATTG AATTGAATT and 5′TTTTGCGGCCGCAGCTTTAACGA ACGCAGA. A fragment encoding the multiple cloning site (MCS) was prepared by annealing with the following oligonucleotides 5′TTTTGCTAGCGTCGACACTAGTGG ATCCCCCGGGTCTAGAGAATTCAGATCT and 5′TTTT AGATCTGAATTCTCTAGACCCGGGGGATCCACTAGTG TCGACGCTAGC. The *Xho*I-*Nhe*I *PGK5′*, the *Nhe*I-*Bgl*II MCS and the *Bgl*II-*Not*I *PGK3′* fragments were digested with *Nhe*I, *Nhe*I-*Bgl*II and *Bgl*II, respectively, and then ligated and cloned into a pTA2 vector (Toyobo, Osaka, Japan). The resulting plasmid (pTA2-PGK) was digested with *Xho*I/*Not*I, and the fragment was inserted at the same sites on pRS411 (American Type Culture Collection), producing pGK411. A DNA fragment encoding *STE2* was amplified by PCR from BY4741 genomic DNA with oligonucleotides containing 5′TTATGCTA GCATGTCTGATGCGGCTCCTTC and 5′GGGGAGATCT TCATAAATTATTATTATCTT. The amplified fragment was digested with *Nhe*I/*Bgl*II and inserted into pGK411 at the same sites, creating pGK411-STE2.

Signalling Assay and FCM Analysis—Yeast strains IMG-1, IMG-5 and IMG-7 were grown in YPD media (containing 1% yeast extract, 2% peptone and 2% glucose; w/v) at 30°C overnight, and were inoculated into 5 ml of YPD media containing 5 μM or 50 nM α-factor (Zymo Research, Orange, CA, USA) or the same media without α-factor in 10 ml Erlenmeyer flasks to give an initial optical density of 0.03 at 600 nm. Yeast cells were grown at 30°C on a rotatory shaker set at 150 rpm for up to 24 h. Cultured cells were temporally harvested to measure the optical density at 600 nm and to analyse fluorescence on a FCM (FACSCalibur; Becton Dickinson and Co., Franklin Lakes, NJ, USA), as described previously (*22*), with minor optimization of detector voltage. The green fluorescence signal was collected through a 530/30 nm band-pass filter (FL1) and the 'fluorescence intensity' was defined as the FL1-height (FL1-H) geometric mean of 10,000 cells.

In the case of minimal media, SD media (containing 6.7 g/l yeast nitrogen base without amino acids and 20 g/l glucose) were supplemented with appropriate amino acids and nucleotides depending on the desired selectable marker (20 mg/l histidine, 60 mg/l leucine, 20 mg/l methionine and 20 mg/l uracil). Yeast strains IMG-5 and IMG-50 harbouring pGK411-STE2 and IMG-70 harbouring pGK411-STE2 were grown in appropriate SD media at 30°C overnight and were inoculated into 5 ml of SD media containing 50 nM α-factor or the same media without α-factor in 10 ml Erlenmeyer flasks to give an initial optical density of 0.1 at 600 nm. Yeast cells were grown at 30°C on a rotatory shaker set at 150 rpm for up to 12 h, and cultured cells were analysed by FCM.

Measurement of Plasmid Retention Rate and FCM Sorting—Yeast strains IMG-50 harbouring pGK411-STE2 and IMG-70 harbouring pGK411-STE2 were grown as described earlier. After incubation, cells grown in SD media without or with 50 nM α-factor were harvested and washed with distilled water. Cells were prepared to give an optical density of 0.1 at 600 nm and were then diluted by 10,000-fold. Cell suspensions (800 μl) were then respectively spread on five plates of each YPD medium and methionine-auxotrophic SD medium, and incubated at 30°C for 2–3 days. Colonies were counted and the plasmid retention rates were determined as the colony number on SD plates divided by that on YPD plates. In addition, each value was normalized based on the plasmid retention rate in the absence of ligand and the ratio of the plasmid retention with/without α-factor was calculated. Independent assays were performed in triplicate.

After IMG-50 harbouring pGK411-STE2 was grown in the same medium containing 50 nM α-factor for 12 h, the activated or non-activated signalling cells were separately sorted with the R1 gate or R2 gate on FACSCalibur. Recovered cells were plated on YPD and SD media as described earlier, except for the dilution, and plasmid retention rates were determined.

RESULTS AND DISCUSSION

We have previously reported a detection system for GPCR signalling using the *EGFP–HIS3* fusion gene as a reporter in yeast, and showed that quantitative measurement of the signalling intensity and selection of cell activating signalling were respectively permitted by the *EGFP* and *HIS3* reporters (*22*). The aim of this study was to establish a yeast-based GPCR assay system using *EGFP* as the only reporter gene for application to FCM screening. In yeast cells, we disrupted the *SST2* or *FAR1* gene, the deletion of which is commonly used in yeast-based GPCR assays to increase ligand sensitivity or avoid cell cycle arrest, and examined the effects on fluorescence GPCR assay.

Confirmation of SST2 and/or FAR1 Gene Disruption—The *SST2*-deficient strategy has been widely used in GPCR signalling analysis in the yeast due to

Table 1. **Genotypes of yeast strains used in this study.**

Strain	Genotype
IMG-1	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* ***P$_{fus1}$-FUS1-EGFP***
IMG-5	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* ***sst2Δ***::*AUR1-C* ***P$_{fus1}$-FUS1-EGFP***
IMG-7	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* ***far1Δ***::*kanMX4* ***sst2Δ***::*AUR1-C* ***P$_{fus1}$-FUS1-EGFP***
IMG-50	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* ***sst2Δ***::*AUR1-C* ***ste2Δ***::*LEU2* ***P$_{fus1}$-FUS1-EGFP***
IMG-70	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* ***far1Δ***::*kanMX4* ***sst2Δ***::*AUR1-C* ***ste2Δ***::*LEU2* ***P$_{fus1}$-FUS1-EGFP***

Bold faces highlight the deleted- or integrated-genes.

hypersensitivity for ligand binding (*8*, *9*). First, we confirmed the sensitivity of *SST2*-disrupted cells at the desired ligand concentration by measuring the expression of EGFP fusion protein (Fus1–EGFP) in response to GPCR signalling (Table 1 and Fig. 1). The EGFP fusion protein was expressed in all cells grown in the YPD or SD media with 5 μM α-factor. It was also confirmed that ligand-induced GPCR signalling activation successfully occurs even when IMG-5 is grown in YPD or SD medium with 50 nM α-factor, while EGFP expression in IMG-1 grown in the same medium was never induced (data not shown).

However, it has been reported that the GPCR signalling in the yeast induces growth arrest by activation of Far1p. Loss of Far1p prevents growth arrest (*8*, *9*; Fig. 1). To confirm disruption of the *FAR1* gene, the growth rates of IMG-1, IMG-5 and IMG-7 (genotypes are listed in Table 1) in the presence or absence of α-factor were examined (Fig. 2). Various types of cell grown in the absence of α-factor smoothly entered the logarithmic growth phase after 6 h of incubation (data not shown). The growth of IMG-1, whose genotype is intact except for the integration of the *EGFP* gene into the *FUS1* locus, was inhibited up to 12 h due to cell cycle arrest caused by signalling in the presence of 5 μM α-factor. The growth of IMG-5, in which the *SST2* gene is deleted, was prevented for up to 18 h due to hypersensitivity to α-factor, even at 50 nM. It was also confirmed that cells lacking Far1p, IMG-7, entered the logarithmic growth phase without growth inhibition by the cell cycle arrest in the presence of α-factor. These data confirm the successful disruption of *SST2* and/or *FAR1* genes.

Average Signalling Intensities of SST2 and/or FAR1 Gene-Disrupted Cells—Figure 3A shows the average intensities of EGFP fluorescence obtained by FCM analyses of 10,000 cells. The average signalling intensity in a cell cluster can be measured using the quantitative enzyme reporter. IMG-1, the intact cells, generated maximum fluorescence at 12 h (average intensity; 87.57), after which fluorescence levels decreased markedly. Similarly, fluorescence levels of IMG-5, the *SST2*-disrupted cells, peaked at 12 h (average intensity; 92.03) before decreasing. In the case of IMG-7, in which both *SST2* and *FAR1* genes were deleted, peak expression levels of EGFP were also seen after 12 h (average intensity; 175.91), and these fluorescence levels were maintained to some extent for up to 24 h, as reported previously (*22*).

Population Analyses of Signalling Cells by Measuring Single-Cell Levels of EGFP Expression—One advantage of the fluorescence GPCR assay using yeast is the elucidation of signalling intensity at the single-cell level,

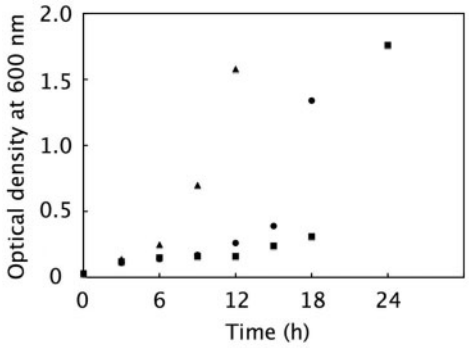


Fig. 2. **Growth curves of IMG-1 (closed circles) grown in YPD media with 5 μM α-factor, and IMG-5 (closed squares; *sst2*Δ) and IMG-7 (closed triangles; *sst2*Δ, *far1*Δ) grown in YPD media with 50 nM α-factor.**

which allows the analysis of activated/non-activated cell populations by FCM. We evaluated the populations of the cells with active signalling using the *EGFP* reporter gene as an indicator of transcriptional activation in *SST2*- and/or *FAR1*-disrupted cells (Fig. 3B).

With IMG-1, IMG-5 and IMG-7 at 12 h, transcriptional activation was induced in almost every cell in response to α-factor. At 18 h, although about 20% of IMG-1 cells were activated, 50% of IMG-5 cells were induced. EGFP expression in both IMG-1 and IMG-5 were largely lost at 24 h, which suggests that signalling was inactivated. In contrast, IMG-7 maintained high fluorescence, with over 85% of cells activated at 24 h, and this suggested that the disruption of the *FAR1* gene is effective in prolonging GPCR signalling and reporter gene expression.

In addition, synchronization of the cell cycle was reflected in histograms displaying the correlation between the signalling level in each cell and the cell population. At 12 h of incubation, the histograms for *FAR1*-intact cells (IMG-1 and IMG-5) were acute, but those for *FAR1*-disrupted cells (IMG-7) were broad. The acutely distributed histograms of IMG-1 and IMG-5 suggest that EGFP expression levels (signalling levels) were uniform in every cell due to arrest in G1 phase and synchronization of the cell cycle. In contrast, the broadly distributed histograms of the *FAR1*-disrupted cells suggest that the cell cycle was not synchronized and the signalling level in each cell was diffuse. The slightly diffuse histogram of IMG-5 suggests that the *SST2* gene disruption prevents the complete synchronization of cell cycle. Although we have no convincing explanation for the variations in synchronization, it is notable that subtle differences in synchronization can be discriminated using our system.

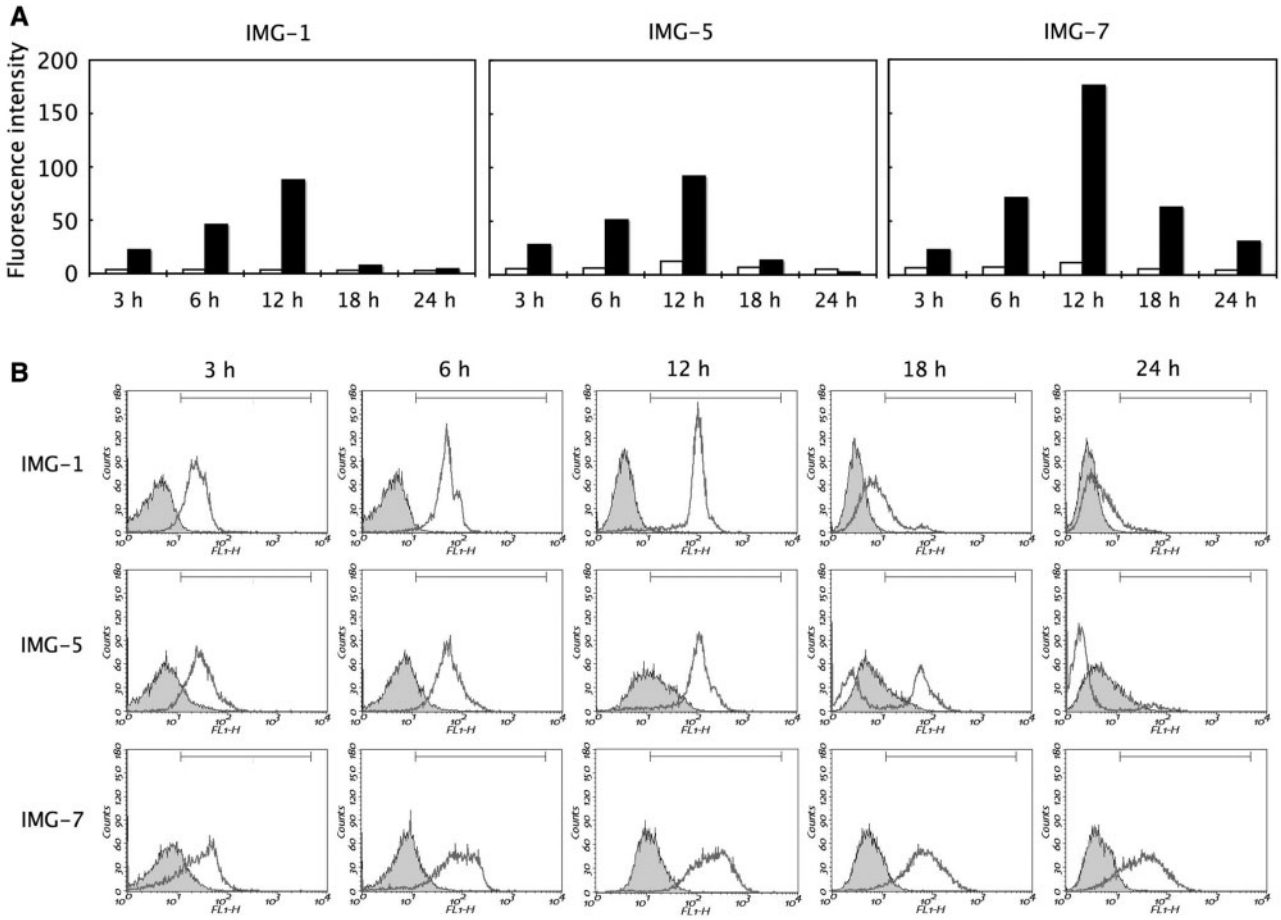


Fig. 3. **(A) Average intensity of EGFP fluorescence by FCM analyses.** White bars indicate the cells in YPD media without α-factor and black bars indicate the cells in YPD media with α-factor. Fluorescence intensity indicates the geometric mean of the green fluorescence signal from 10,000 cells. (B) Histogram plots by FCM analyses of 10,000 cells. Vertical axis indicates number of cells, and horizontal axis indicates green fluorescence signal. Closed histograms (light grey area inside of plots) indicate cells in YPD media without α-factor and opened histograms (grey plots) indicate cells in YPD media with α-factor. α-factor was added at a final concentration of 5 μM for IMG-1 (intact) or at 50 nM for IMG-5 (*sst2*Δ) and IMG-7 (*sst2*Δ, *far1*Δ). Cells were incubated for 3, 6, 12, 18 and 24 h, respectively.

SST2-Disrupted Cells Grown in Rich Media Display Higher Background Signalling in the Absence of Ligand—FCM analysis data for IMG-5, which are *SST2*-disrupted yeast cells, incubated in YPD medium without α-factor and for IMG-5 incubated in YPD medium with 50 nM α-factor are shown in Fig. 3B. Interestingly, after 12 h of incubation, the histogram for IMG-5 without α-factor shows increasing fluorescence, while that of IMG-1 without α-factor shows lower background levels (Fig. 3B; 12 h, closed histogram). Although IMG-1 cells were almost never induced, about 50% of IMG-5 cells were activated. This suggests that disruption of the *SST2* gene conferred background signalling in the absence of ligand, because Sst2p negatively regulates G-protein signalling (*12*). This corresponds to a previous report (*21*). Similarly, in *SST2*- and *FAR1*-disrupted cells (IMG-7), higher background levels were also confirmed (Fig. 3B). Although this phenotype appears in the case of enriched medium, it has little influence in minimal medium (Fig. 4A; IMG-5 grown in SD medium without α-factor after 12 h of incubation).

Model of Receptor Expression by Episomal Plasmid in STE2-Deleted Strain—Most heterologous GPCR analyses in yeast are carried out using the episomal plasmid for receptor expression. The episomal plasmid system is favourable for the construction of larger libraries when compared with the chromosomal integration system (*9*). To demonstrate the applicability of our fluorescence assay to the episomal plasmid expression system, we selected endogenous Ste2p as a model receptor and evaluated signalling levels in the episomal expression and chromosomal expression systems.

IMG-5 expresses Ste2 receptor under the regulation of a genomic sequence. However, IMG-50, whose *STE2* gene is deleted, is designed to avoid the competitive expression of yeast endogenous Ste2 receptor (Table 1). IMG-50 harbouring mock vector did not exhibit EGFP expression in response to α-factor (data not shown). We constructed an episomal plasmid for the expression of the

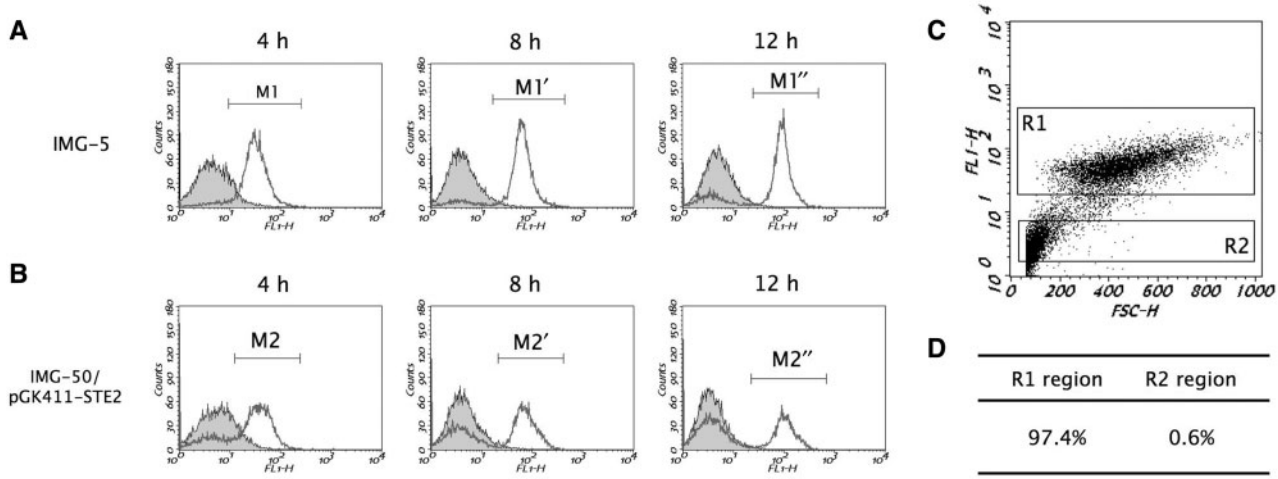


Fig. 4. **(A) Histogram plots of FCM analyses with yeast strain IMG-5 (chromosomally integrated *STE2* gene) grown in SD minimal media for 4, 8 and 12 h, respectively.** Vertical axis indicates number of cells, and horizontal axis indicates green fluorescence signal. (B) Histogram plots of yeast IMG-50 (*sst2*Δ, *ste2*Δ) harbouring pGK411-STE2 (episomal *STE2* gene) grown in SD minimal media for 4, 8 and 12 h, respectively. Closed histograms (light grey area inside plots) indicate cells without α-factor and open histograms (grey plots) indicate cells with 50 nM α-factor. M1, M1′, M1″, M2, M2′ or M2″ indicate regions including activated cells. (C) Dot plot of IMG-50 harbouring pGK411-STE2 grown in SD medium containing 50 nM α-factor for 12 h. Vertical axis indicates green fluorescence signal, and horizontal axis indicates forward-scattered light signal. The R1 gate was used to sort the activated cells and R2 gate was used to sort the non-activated cells. (D) Plasmid retention rates of cells sorted as described for Figure 4C.

STE2 gene as a model for GPCR analysis. IMG-50 harbouring pGK411-STE2 expresses Ste2p by retaining the episomal plasmid bearing the *PGK1* promoter and a single copy of *CEN*/*ARS* origin.

Figure 4A and B show the histograms for population analyses of reporter expressions in cells grown in SD medium with or without α-factor. In the case of IMG-5 grown in the SD medium containing α-factor, the population of the activated cells was 94% after 4 h of incubation (cell population included in M1), 90% after 8 h of incubation (cell population included in M1′) and 80% after 12 h of incubation (cell population included in M1″). However, signalling in IMG-50 harbouring pGK411-STE2 grown in the SD medium containing α-factor was induced in 74% of cells at 4 h (cell population included in M2) and 58% at 8 h (cell population included in M2′), before decreasing substantially to 42% at 12 h (cell population included in M2″). IMG-5, which has chromosomally integrated *STE2*, achieved stable activation of GPCR signalling. In contrast, IMG-50 harbouring episomal pGK411-STE2 exhibited activated signalling at 4 h, but the proportion of activated cells subsequently decreased rapidly.

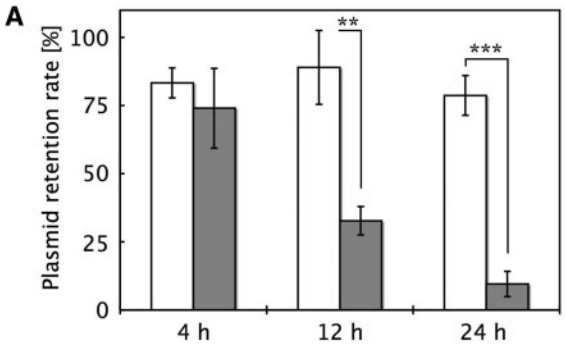
FCM Sorting of GPCR-Signalling-Activated or Non-Activated Cells—We assumed that the significant decrease in GPCR signalling in IMG-50 harbouring episomal pGK411-STE2 was caused by a defect in Ste2p expression rather than the signalling inactivation. Therefore, we examined the plasmid retention rates in cells grown in medium with or without α-factor. The plasmid retention rate of IMG-50 harbouring pGK411-STE2 grown in SD medium without α-factor after 12 h was 89.0%; however, that of the same strain grown in the SD medium with 50 nM α-factor after 12 h was only 32.7% (Fig. 5A; 12 h).

To investigate further, we sorted the activated or non-activated cell populations by FCM. Activated cells were sorted with the region gated by R1, and the non-activated cells were sorted with the region gated by R2 (Fig. 4C). The plasmid retention rate of each sorted cell population was measured. While the plasmid retention rate of the activated cells (R1 region) was 97.4%, that of the non-activated cells (R2 region) was only 0.6% (Fig. 4D). This clearly demonstrates that the loss of plasmids caused significant decreases in GPCR signalling. Furthermore, it successfully demonstrates the usefulness of the fluorescence reporters to sort the specified cell clusters using FCM when compared to other reporter genes, such as *HIS3* or *lacZ*.

Improvement of Plasmid Retention Rate by Avoiding Cell Cycle Arrest—The advantage of the yeast-based GPCR assay is that it can be easily applied to the combinatorial library screening, such as ligand screening or receptor mutagenesis. In combinatorial library screening, the loss of plasmids caused by signalling is a critical problem because the heterologous GPCR is commonly expressed in yeast by using episomal plasmids.

We first confirmed the time course of plasmid retention in IMG-50 harbouring pGK411-STE2 (Fig. 5A). The plasmid retention rate was decreased markedly to <10% at 24 h. We attributed the lack of plasmids in the cells grown with α-factor to G1 arrest in response to signalling. To validate this assumption, we constructed strain IMG-70 by deleting the *FAR1* gene in IMG-50 (Table 1) and examined the plasmid retention rates of IMG-70 harbouring pGK411-STE2 with or without α-factor (Fig. 5B).

When cells were grown without α-factor, the plasmid retention rates of *FAR1*-disrupted cells (IMG-70) were almost the same as those of *FAR1*-intact cells (IMG-50).



C

Time (h)	IMG-50	IMG-70
4	88.3%	96.1%
12	37.2%	81.0%
24	11.6%	72.0%

Fig. 5. **Plasmid retention rates of (A) IMG-50 (*FAR1* intact) harbouring pGK411-STE2, (B) IMG-70 (*FAR1* disrupted) harbouring pGK411-STE2.** White bars indicate cells grown in SD media without α-factor and grey bars indicate cells grown in the same media with 50 nM α-factor for 4, 12 and 24 h, respectively. Plasmid retention rates are given as the means of three different transformants with standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (C) Effects of *FAR1* gene disruption on plasmid maintenance in signalling. Each value is normalized based on plasmid retention rate in the absence of ligand and shows the ratio of the plasmid retention rate with/without α-factor.

However, the plasmid retention rate of IMG-70 was obviously higher than IMG-50 after 12 or 24 h of incubation in the presence of α-factor. This observation was clearly discernible by normalizing the value on the basis of plasmid retention rate in the absence of α-factor (Fig. 5C). It was also confirmed that *FAR1*-disrupted cells (IMG-70) harbouring the *STE2* expression plasmid generated EGFP fluorescence in response to α-factor after 12 h of incubation (data not shown). These results suggest that the cell cycle arrest attributed to Far1p phosphorylated by signalling is closely related to the lack of plasmids. Although it is unclear whether the lack of plasmids results from plasmid dropout or degradation, deletion of Far1p significantly improves plasmid retention rate after ligand stimulation. In addition, to our knowledge, this is the first report directly showing a relationship between G1 arrest by GPCR signalling and plasmid retention rate.

In conclusion, we have established a platform for screening by yeast GPCR assay using a fluorescence reporter. Our fluorescence GPCR assay can distinguish and isolate signalling activated/non-activated cell clusters at the single-cell level. We also improved the plasmid retention rate by avoiding cell cycle arrest. Our system will be a powerful tool for quantitative and high-throughput GPCR screening of yeast-based combinatorial libraries using FCM.

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