Signaling Assays for Detection of Human G-protein-coupled Receptors in Yeast

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[Abstract] G-protein-coupled receptors (GPCRs) are the largest group of cell-surface proteins and are major molecular targets for drug development. The protocol described herein is for the detection of human GPCR signaling in the yeast *Saccharomyces cerevisiae*. Using *Zoanthus* sp. green fluorescent protein (*ZsGreen*) as the reporter, engineered yeast cells expressing human GPCRs emit strong fluorescence in response to stimuli leading to receptor signal activation. This assay method would allow screening for agonistic ligands and critical mutations required for human GPCR signaling.

Materials and Reagents

1. Engineered *Saccharomyces cerevisiae* strain in which the *ZsGreen* reporter genes were integrated into the genome (IMFD-72ZsD: *MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 sst2*Δ::*AUR1-C ste2*Δ::*LEU2 fig1*Δ::*ZsGreen his3*Δ::P_{FIG1}-*ZsGreen far1*Δ *gpa1*Δ::*Gi3tp*) (Nakamura *et al.*, 2013)
   Note: The expression of ZsGreen is controlled by the signal-responsive FIG1 promoter.
2. Multi-copy expression plasmid (pGK421 containing the *PGK1* promoter, *2 μ* origin and *MET15* marker) (Togawa *et al.*, 2010) encoding the GPCR of interest [*e.g.*, somatostatin receptor subtype-5 (SSTR5); somatostatin receptor subtype-2 (SSTR2); or neurotensin receptor type-1 (NTSR1)] (Ishii *et al.*, 2012; Ishii *et al.*, 2014)
3. GPCR ligands [*e.g.*, somatostatin (SST) (Merck KGaA, Calbiochem®, catalog number: 51110-01-1) and neurotensin (NTS) (Merck KGaA, Calbiochem®, catalog number: 39379-15-2)]
4. 10 mg/ml carrier DNA (Takara Bio Company, Clontech, catalog number: 630440)
5. Tris (hydroxymethyl) aminomethane (Tris·HCl) (Nacalai Tesque, catalog number: 35409-45)
6. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA $2Na{\cdot}2H_2O$) (Nacalai Tesque, catalog number: 15111-45)
7. 1 M Hydrochloric acid (HCl) (Nacalai Tesque, catalog number: 37314-15)

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8. Lithium acetate dihydrate (Sigma-Aldrich, catalog number: L6883)
9. Acetic acid (Nacalai Tesque, catalog number: 00212-85)
10. Polyethylene glycol (PEG) #4000 (Nacalai Tesque, catalog number: 28221-05)
11. DMSO (Nacalai Tesque, catalog number: 13445-74)
12. BD FACSFlow sheath fluid (BD, catalog number: 342003)
13. Immersion oil (Olympus, catalog number: IMMOIL-F30CC)
14. Distilled water (dH_2O)
15. Yeast extract (Nacalai Tesque, catalog number: 15838-45)
16. Peptone (BD, catalog number: 211677)
17. D-Glucose (Nacalai Tesque, catalog number: 16806-25)
18. Yeast nitrogen base without amino acids (YNB) (BD, catalog number: 291940)
19. L-Histidine (Nacalai Tesque, catalog number: 18116-92)
20. L-Leucine (Nacalai Tesque, catalog number: 20327-62)
21. Uracil (Nacalai Tesque, catalog number: 35824-82)
22. 3-(*N*-Morpholino)-2-hydroxypropanesulfonic acid (Mopso) (Nacalai Tesque, catalog number: 23421-64)
23. Sodium hydroxide (NaOH) (Nacalai Tesque, catalog number: 31511-05)
24. Agar (Nacalai Tesque, catalog number: 01028-85)
25. 10 x TE buffer (see Recipes)
26. 10 x LiAc (see Recipes)
27. 50% PEG (see Recipes)
28. TE/LiAc solution (see Recipes)
29. LiAc/PEG solution (see Recipes)
30. YPD medium (see Recipes)
31. Synthetic dextrose (SD) selective medium supplemented with histidine, leucine and uracil (see Recipes)
32. SDM71 selective medium supplemented with histidine, leucine and uracil (see Recipes)

Equipment

1. 16.5 x 105 mm test tubes (AGC Techno Glass, catalog number: 9820TST16.5-105NP)
2. 10-ml conical flasks (AGC Techno Glass, catalog number: 4980FK10)
3. 2-ml microcentrifuge tubes (WATSON, catalog number: 332-720C)
4. 1.5-ml microcentrifuge tubes (WATSON, catalog number: 131-815C)
5. 96-well cell culture plate (Corning, catalog number: 3596)
6. Microplate sealing tape (AS ONE Corporation, catalog number: 1-6774-05)

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7. 12.5 x 75 mm test tubes with 2-position caps (B & M Equipment, catalog number: 222-2036-050)
8. Microscope glass slides (Matsunami Glass, catalog number: S091150)
9. 18 x 18 mm coverslips (Matsunami Glass, catalog number: C218181)
10. Centrifuge (Eppendorf, model: MiniSpin plus)
11. Block incubator (Astec Industries, model: BI-516C)
12. Shaking incubator for test tubes and conical flasks (TAITEC, model: BR-43FL)
13. Shaking incubator for a 96-well cell culture plate (TAITEC, model: M·BR-022UP)
14. Spectrophotometer (Shimadzu, model: UVmini-1240)
15. BD FACSCanto II flow cytometer (BD)
16. Fluorescence microscope (Keyence Corporation, model: BZ-9000)
17. 100x objective lens (Nikon Corporation, model: CFI Plan Apo VC 100x H)
18. BZ filter cube (excitation filter, absorption filter, dichroic mirror) (Keyence Corporation, model: OP-66836 GFP-BP)

Software

1. BD FACSDiva software (v5.0) (BD)

Procedure

A. Yeast transformation

Transformation was performed using the lithium acetate method (Gietz *et al.*, 1992).

1. Inoculate 5 ml YPD medium in a 16.5 x 105 mm test tube with a single colony of *S. cerevisiae* strain IMFD-72ZsD.
2. Incubate the culture overnight at 30 °C with shaking at 150 opm.
   Note: Shaking incubator (TAITEC, model: BR-43FL).
3. Transfer 2 ml of the cell culture to a 2-ml microcentrifuge tube and pellet the cells by centrifugation at 3,000 rpm for 5 min at room temperature.
4. Remove the supernatant and wash the cells with 1 ml sterilized dH_2O.
5. Pellet the cells by centrifugation at 3,000 rpm for 5 min at room temperature.
6. Remove the supernatant and resuspend the cells in 1.5 ml of TE/LiAc solution.
7. Aliquot 100 µl of yeast suspension to a fresh 1.5-ml microcentrifuge tube.
8. Add 500 ng of plasmid DNA (pGK421-SSTR5, -SSTR2 or -NTSR1) and 20 µg of carrier DNA to each tube.
   Note: Before using the carrier DNA, denature it at 95 °C for 5 min, and then chill it quickly on ice.

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9. Add 600 µl of LiAc/PEG solution to each tube and vortex at high speed for 10 sec to mix.
10. Incubate the cell suspension at 30 °C for 30 min.
11. Add 70 µl of DMSO. Mix well by gentle inversion. Do not vortex.
12. Heat shock at 42 °C for 15 min.
13. Centrifuge the cell suspension at 14,000 rpm for 5 sec at room temperature. Remove the supernatant completely.
14. Resuspend the cells in 0.5 ml of 1x TE buffer.
15. Spread 100 µl of cell suspension on each SD selective agar plate.
    Note: Since the yeast strain has his3Δ leu2Δ met15Δ ura3Δ alleles and the plasmid has MET15 as a selection marker, the selective medium requires supplementation with histidine, leucine and uracil.
16. Incubate the plates at 30 °C until colonies appear (generally, 2-4 days).

B. GPCR signaling assay

1. Inoculate 5 ml SD selective medium in a 16.5 x 105 mm test tube with a single colony of a positive transformant.
2. Incubate the culture overnight at 30 °C with shaking at 150 opm.
   Note: Shaking incubator (TAITEC, model: BR-43FL).
3. Measure the OD_{600} of the yeast cultures.
   Note: Spectrophotometer.
4. Transfer the cultured cells into 5 ml of fresh SD selective medium in a 10-ml conical flask to give an initial OD_{600} of 0.03.
5. Further incubate the culture at 30 °C for 18 h with rotary shaking at 150 rpm.
   Note: Shaking incubator (TAITEC, model: BR-43FL).
6. Transfer 2 ml of the cell culture to a 2-ml microcentrifuge tube and pellet the cells by centrifugation at 3,000 rpm for 5 min at room temperature.
7. Remove the supernatant and wash the cells with 1 ml sterilized dH_2O.
8. Pellet the cells by centrifugation at 3,000 rpm for 5 min at room temperature.
9. Remove the supernatant and resuspend the cells in ~400 µl sterilized dH_2O.
10. Measure the OD_{600} of the cell suspension, and then dilute it to an OD_{600} of 10.
    Note: Spectrophotometer.
11. Add 10 µl of the resulting cell suspension (to give an OD_{600} of 1) to 80 µl fresh SDM71 selective medium per well of a 96-well cluster dish.
12. Add 10 µl of 10-times concentrated ligands (SST or NTS) or sterilized dH_2O (no ligand control) to each well.
    Note: A final concentration of ~10 µM ligand is recommended (dissolved in and diluted with sterilized dH_2O).

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13. Seal the 96-well cluster dish with sealing tape.
14. Incubate the dish at 30 °C for 4 h with shaking at 150 rpm. After incubation, measure green fluorescence using a flow cytometer or fluorescence microscope (see below).
    Note: Shaking incubator (TAITEC, model: M·BR-022UP).

Flow cytometry analysis

a. Dilute the cell culture in 1 ml sheath fluid in test tubes with 2-position caps.
b. Measure the green fluorescence signal emitted from 10,000 cells using a BD FACSCanto II flow cytometer equipped with a 530/30 nm band-pass filter.
c. Analyze the data and assess the mean ZsGreen fluorescence of 10,000 cells using BD FACSDiva software.

Fluorescence microscopy imaging

a. Transfer the cell culture to a 1.5-ml microcentrifuge tube and pellet the cells by centrifugation at 3,000 rpm for 5 min at room temperature.
b. Remove the supernatant and wash the cells with 100 µl sterilized dH_2O.
c. Pellet the cells by centrifugation at 3,000 rpm for 5 min at room temperature.
d. Remove the supernatant and resuspend the cells in 10 µl sterilized dH_2O.
e. Spot 7 µl of the cell suspension on a glass slide and mount a coverslip over the spot.
f. Observe the cells using a BZ-9000 fluorescence microscope equipped with a 100x objective lens or equivalent and acquire green fluorescence images with a 470/40 band-pass filter for excitation and a 535/50 band-pass filter for emission.
   Note: The 100x objective lens should be immersed in oil.

Representative data

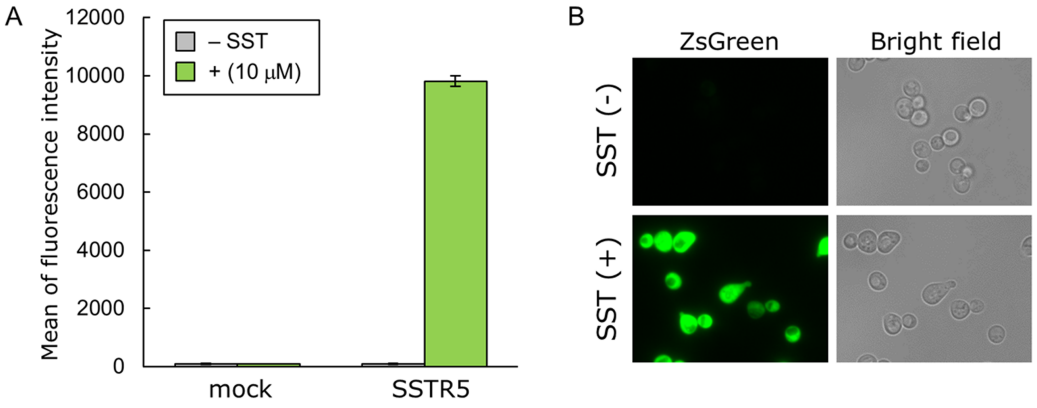

Figure 1. Activation of human SSTR5 produced in yeast following the exogenous addition of SST. Yeast strain IMFD-72ZsD was transformed with pGK421 (mock) or

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pGK421-SSTR5. All transformants were grown in SD medium for 18 h. The cells then were incubated for another 4 h in SDM71 medium with or without 10 μM SST. A. The ZsGreen fluorescence of 10,000 cells was measured by flow cytometry. Data are shown as means ± SDs of triplicate samples. B. Visualization of the ZsGreen fluorescence of IMFD-72ZsD/SSTR5 with or without 10 μM SST. Images were obtained using a 100x objective on a fluorescence microscope. Reprinted with permission from Ishii *et al*. (2012).

Notes

1. Work on a clean bench.

Recipes

1. 10x TE buffer (200 ml)
   2.4 g Tris-HCl (0.1 M)
   0.74 g EDTA $2Na \cdot 2H_2O$ (10 mM)
   Dissolved in 175 ml dH_2O
   Adjust pH to 7.5 with 1 M HCl
   Adjust volume to 200 ml with dH_2O
   Sterilized by autoclaving
2. 10x LiAc (200 ml)
   20.4 g lithium acetate dihydrate (1 M)
   Dissolved in 175 ml dH_2O
   Adjust pH to 7.5 with dilute acetic acid
   Adjust volume to 200 ml with dH_2O
   Sterilized by autoclaving
3. 50% PEG (200 ml)
   100 g PEG #4000
   Add dH_2O to 200 ml
   Sterilized by autoclaving
4. TE/LiAc solution (1.5 ml)
   150 μl 10 x TE buffer
   150 μl 10 x LiAc
   1.2 ml sterilized dH_2O
   Note: Prepare fresh just prior to use.
5. LiAc/PEG solution (1 ml)
   800 μl 50% PEG

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100 µl 10 x TE buffer

100 µl 10 x LiAc

Note: Prepare fresh just prior to use.

6. YPD medium (1 L)

   10 g yeast extract

   20 g peptone

   20 g D-Glucose

   Add dH_2O to 1 L

   Sterilized by autoclaving

7. SD selective medium supplemented with histidine, leucine and uracil (1 L)

   20 g D-Glucose

   6.7 g yeast nitrogen base without amino acids (YNB)

   20 mg L-Histidine

   60 mg L-Leucine

   20 mg Uracil

   Add dH_2O to 1 L

   Sterilized by autoclaving

   Note: For solid plates, add 2% agar to the media.

8. SDM71 selective medium supplemented with histidine, leucine and uracil (1 L)

   20 g D-Glucose

   1.7 g yeast nitrogen base without amino acids (YNB)

   45 g 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (Mopso)

   20 mg L-Histidine

   60 mg L-Leucine

   20 mg uracil

   Dissolved in 800 ml dH_2O

   Adjust pH to 7.1 with 10 N NaOH

   Adjust volume to 1 L with dH_2O

   Filter sterilized

Acknowledgments

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References

1. Gietz, D., St Jean, A., Woods, R. A. and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* 20(6): 1425.
2. Ishii, J., Moriguchi, M., Hara, K. Y., Shibasaki, S., Fukuda, H. and Kondo, A. (2012). Improved identification of agonist-mediated $G\alpha_i$-specific human G-protein-coupled receptor signaling in yeast cells by flow cytometry. *Anal Biochem* 426(2): 129-133.
3. Ishii, J., Oda, A., Togawa, S., Fukao, A., Fujiwara, T., Ogino, C. and Kondo, A. (2014). Microbial fluorescence sensing for human neurotensin receptor type 1 using Gα-engineered yeast cells. *Anal Biochem* 446: 37-43.
4. Nakamura, Y., Ishii, J. and Kondo, A. (2013). Bright fluorescence monitoring system utilizing *Zoanthus* sp. green fluorescent protein (*ZsGreen*) for human G-protein-coupled receptor signaling in microbial yeast cells. *PLos One* 8(12): e82237.
5. Togawa, S., Ishii, J., Ishikura, A., Tanaka, T., Ogino, C. and Kondo, A. (2010). Importance of asparagine residues at positions 13 and 26 on the amino-terminal domain of human somatostatin receptor subtype-5 in signalling. *J Biochem* 147(6): 867-873.

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