GPCRs Interaction Measurement by Fluorescence Resonance Energy Transfer (FRET)

Manuel D. Gahete, Raúl M. Luque* and Justo P. Castaño*

Department of Cell Biology, Physiology and Immunology, University of Córdoba, Reina Sofía University Hospital, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), and CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Córdoba, Spain
*For correspondence: raul.luque@uco.es; justo@uco.es

[Abstract] This is a protocol to determine the physical interaction of a G-protein coupled receptor (GPCR) with itself (homodimerization) or with other GPCR (heterodimerazation) using fluorescence resonance energy transfer (FRET). FRET is a distance-dependent interaction between the electronic excited states of two dye molecules (in this case, CFP and YFP) in which excitation is transferred from a donor molecule (CFP) to an acceptor (YFP) molecule without emission of a photon that can be used to determine interaction among YFP- and CFP-tagged GPCRs. Nowadays, FRET microscopy technique can be used to determine interaction between any proteins that retain biological function when expressed as a fusion to the fluorescent protein.

Materials and Reagents

1. A cell line lacking the expression of the GPCR of interest
2. Expression plasmid containing E-CFP
3. Expression plasmid containing E-YFP
4. Expression plasmid containing E-YFP and E-GFP coupled in frame (CFP-YFP or Positive control)
5. Expression plasmid containing the first GPCR of interest tagged with E-CFP (GPCR1-CFP)
6. Expression plasmid containing the second GPCR of interest tagged with E-YFP (GPCR2-YFP)
7. Lipofectamine 2000
8. 4% paraformaldehyde
9. PBS
10. Fluoromount
11. poly-l-lysine

Equipment

1. Round coverslips coated with poly-l-lysine
2. Nikon Eclipse TE2000 E scope equipped with a 400 DCLP dichroic filter (Chroma)
3. ORCA II BT digital camera

Software

1. MetaMorph software (Imaging Corporation)
2. Image J software

Procedure

1. Plate cells at 100,000 cells/ml onto round coverslips previously coated with poly-l-lysine.
2. Transfect the cells with 1 µg of each plasmid using Lipofectamine 2000, 48 h after plating. The following transfections are needed:
   a. GPCR1-CFP + GPCR2-YFP
   b. CFP-YFP (Positive control)
   c. CFP (Donor)
   d. YFP (Acceptor)
   e. CFP + YFP (Negative control)
3. 24 h after transfection, eliminate the culture medium and fix the cells for 5 min in 4% paraformaldehyde.
4. Rinsed twice in PBS.
5. Mount onto a slide using Fluoromount to reduce photobleaching.
6. Acquire images of cells transfected with each pair of receptors of interest and controls with an inverted Nikon Eclipse TE2000 E scope equipped with a 400 DCLP dichroic filter and recorded with an ORCA II BT digital camera, both controlled with MetaMorph software (Imaging Corporation). Specifically, acquire three sequential images at 1 sec of exposure with the suitable filters sets for the Donor (E-CFP; excitation at 440 and emission at 510 nm), Acceptor (E-YFP/F46L; excitation at 495 and emission at 540 nm) and raw FRET (excitation at 440 and emission at 540 nm) and under a 60x oil immersion objective.
7. Quantify the signal intensity of each cell using the Image J software in order to calculate the net FRET by using the three filters method with the methodology developed previously (Farhan *et al.*, 2004). Specifically, Raw FRET images are corrected with the fully specified bleedthrough method, using the following equation: FRET = Raw FRET - [Acceptor - (DA * Donor)] * [AF] - [Donor - (AD* Acceptor)] * [DF], where DA is the proportion in which the

donor signal contributes to the acceptor, AD is the proportion in which the acceptor signal contributes to the donor, AF is the proportion in which the acceptor contributes to the raw FRET signal, and DF is the proportion in which the donor contributes to the raw FRET signal. These coefficients were calculated from cells expressing Donor (E-CFP) or Acceptor (E-YFP) alone. Specifically, DA is the proportion of Acceptor signal to Donor signal in cells expressing Donor (E-CFP) alone; AD is the proportion of Donor signal to Acceptor signal in cells expressing Acceptor (E-YFP) alone; DF is the proportion of raw FRET to Donor signal in cells expressing Donor (E-CFP) alone; AF is the proportion of raw FRET to Acceptor signal in cells expressing Acceptor (E-YFP) alone.

8. FRET efficiency is calculated in relation to the positive control consisting in a vector with E-CFP and E-YFP coupled in frame (CFP-YFP), which provided the upper FRET efficiency limit.
9. For image analysis and coefficient calculation, background is always subtracted in each picture. A 1:1 E-YFP/E-CFP ratio and equal E-YFP and E-CFP intensities between all samples need to be used for FRET measurements.

Acknowledgments

This protocol is adapted from Duran-Prado *et al*. (2007) and Duran-Prado *et al*. (2012).

References

1. Duran-Prado, M., Bucharles, C., Gonzalez, B. J., Vazquez-Martinez, R., Martinez-Fuentes, A. J., Garcia-Navarro, S., Rhodes, S. J., Vaudry, H., Malagon, M. M. and Castano, J. P. (2007). Porcine somatostatin receptor 2 displays typical pharmacological sst2 features but unique dynamics of homodimerization and internalization. *Endocrinology* 148(1): 411-421.
2. Duran-Prado, M., Gahete, M. D., Hergueta-Redondo, M., Martinez-Fuentes, A. J., Cordoba-Chacon, J., Palacios, J., Gracia-Navarro, F., Moreno-Bueno, G., Malagon, M. M., Luque, R. M. and Castano, J. P. (2012). The new truncated somatostatin receptor variant sst5TMD4 is associated to poor prognosis in breast cancer and increases malignancy in MCF-7 cells. *Oncogene* 31(16): 2049-2061.
3. Farhan, H., Korkhov, V. M., Paulitschke, V., Dorostkar, M. M., Scholze, P., Kudlacek, O., Freissmuth, M. and Sitte, H. H. (2004). Two discontinuous segments in the carboxyl terminus are required for membrane targeting of the rat gamma-aminobutyric acid transporter-1 (GAT1). *J Biol Chem* 279(27): 28553-28563.

