Supporting Information for

Dual fluorescent labeling of GLP-1R in live cells via enzymatic tagging and bioorthogonal chemistry

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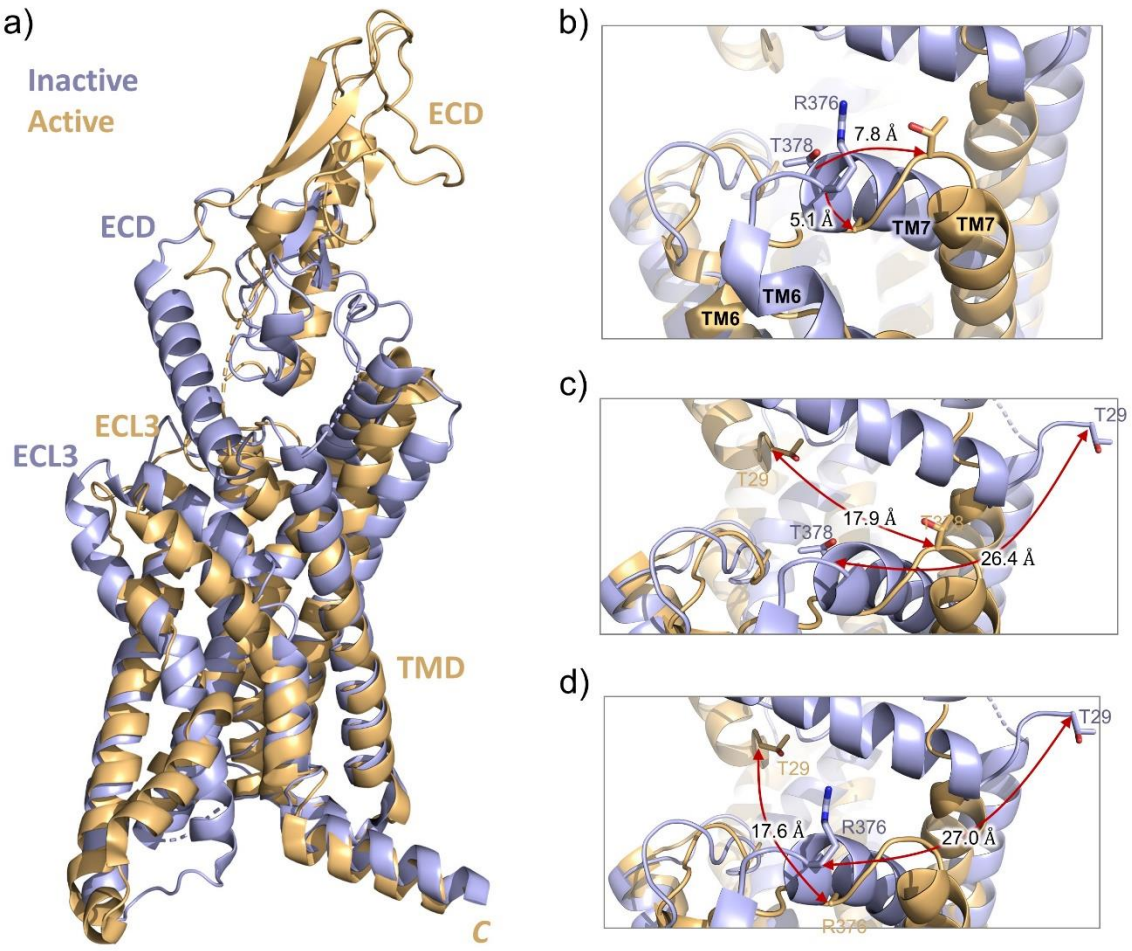


Figure S1. Selection of suitable sites for fluorophore placement. (a) Superimposition of the full-length inactive (PDB code: 6LN2)[1] and active (PDB code: 6VAI)[2] conformations of GLP-1R. (b) A close-up view of the ECL3 regions showing significant outward movement of ECL3 including resides R376 and T378 after receptor activation. (c) A close-up view of the distance change between T29 at the N-terminus and T378 at ECL3 showing a decrease of distance from 26.4 Å to 17.9 Å. (d) A close-up view of the distance change between T29 at the N-terminus and R376 at ECL3 showing a decrease of distance from 27.0 Å to 17.6 Å. All distances were measured between the relevant α-carbons.

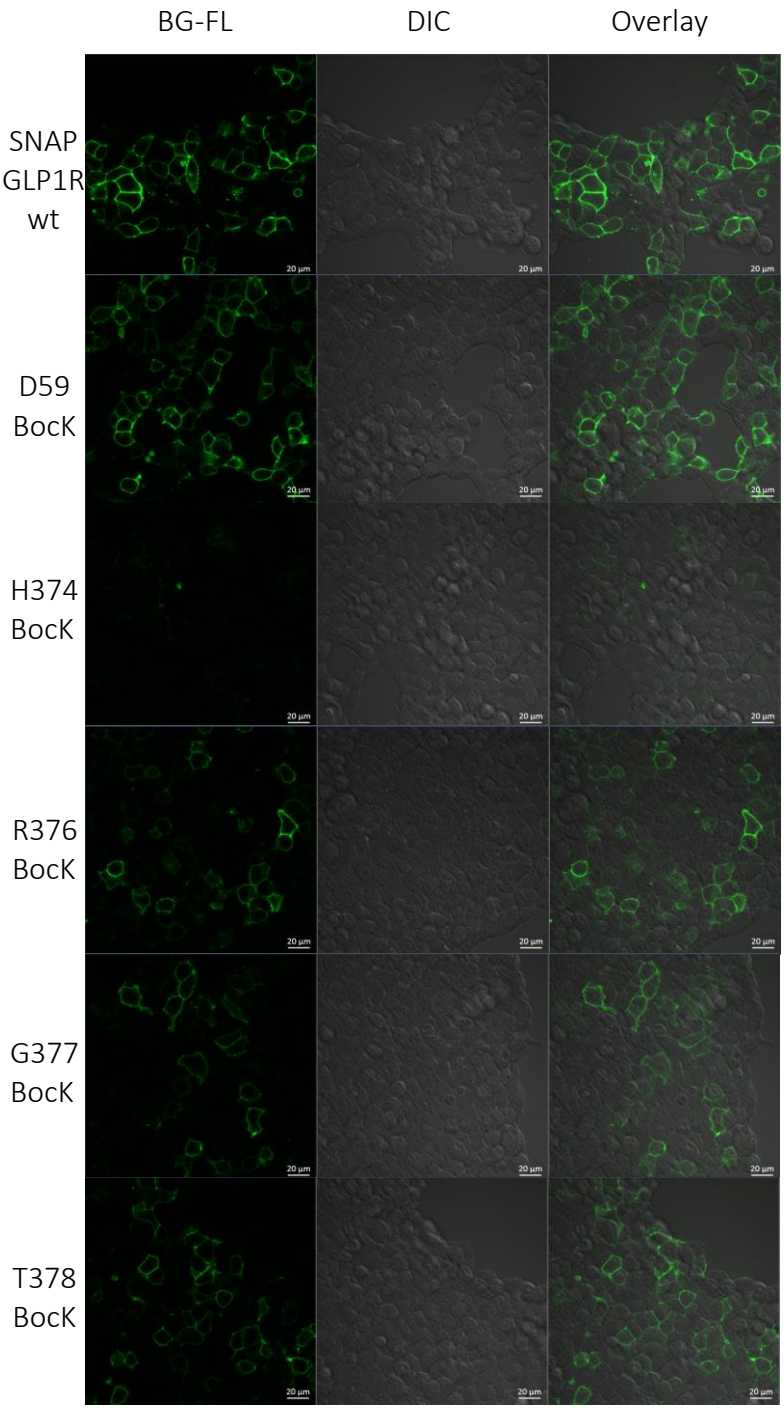


Figure S2. Confocal micrographs of HEK293T cells expressing SNAP-GLP-1R WT or mutants encoding BocK at various positions. Cells were treated with 5 µM BG-FL for 30 min before image acquisition. Scale bar = 20 µm.

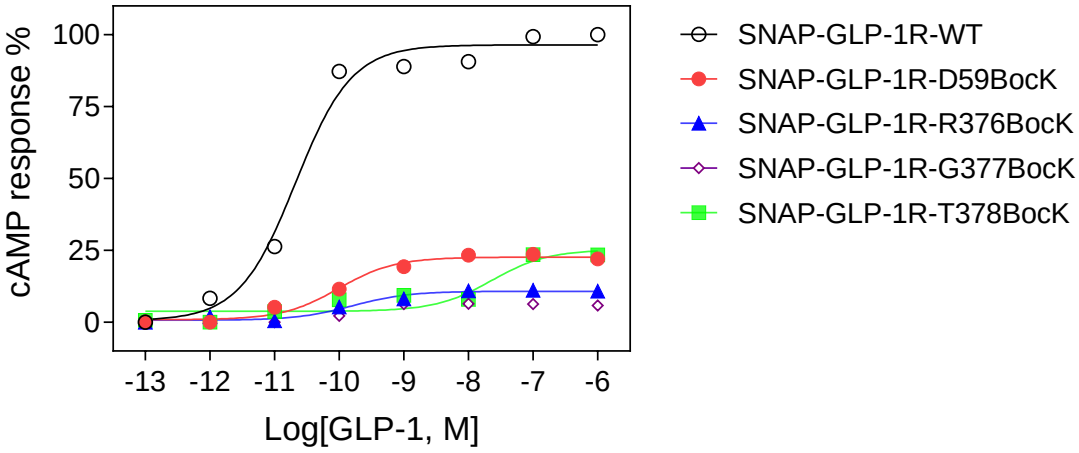
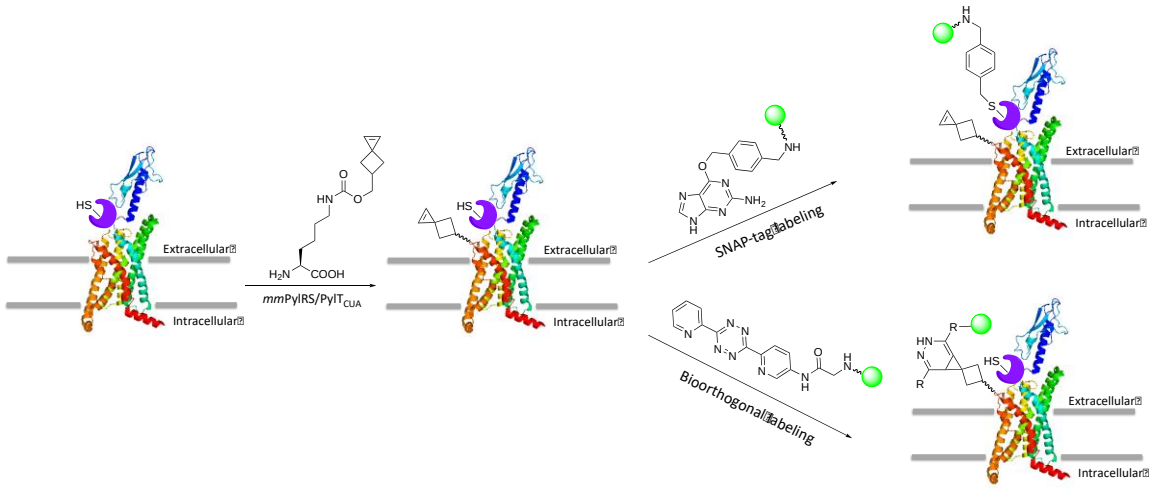


Figure S3. cAMP accumulation activity of the WT and BocK-encoded SNAP-GLP-1R mutants. Data were fitted to the following equation: $Y = Y_{min} + \frac{(Y_{max} - Y_{min})}{1+10^{logEC_{50}-log\,[A]}}$, where Y_{min} represents minimal stimulation in the absence of ligand; Y_{max} represents maximal stimulation in the presence of ligand; [*A*] is molar concentration of the ligand, and EC_{50} represents molar concentration of the ligand required to generate a response halfway between Y_{min} and Y_{max}.

a)



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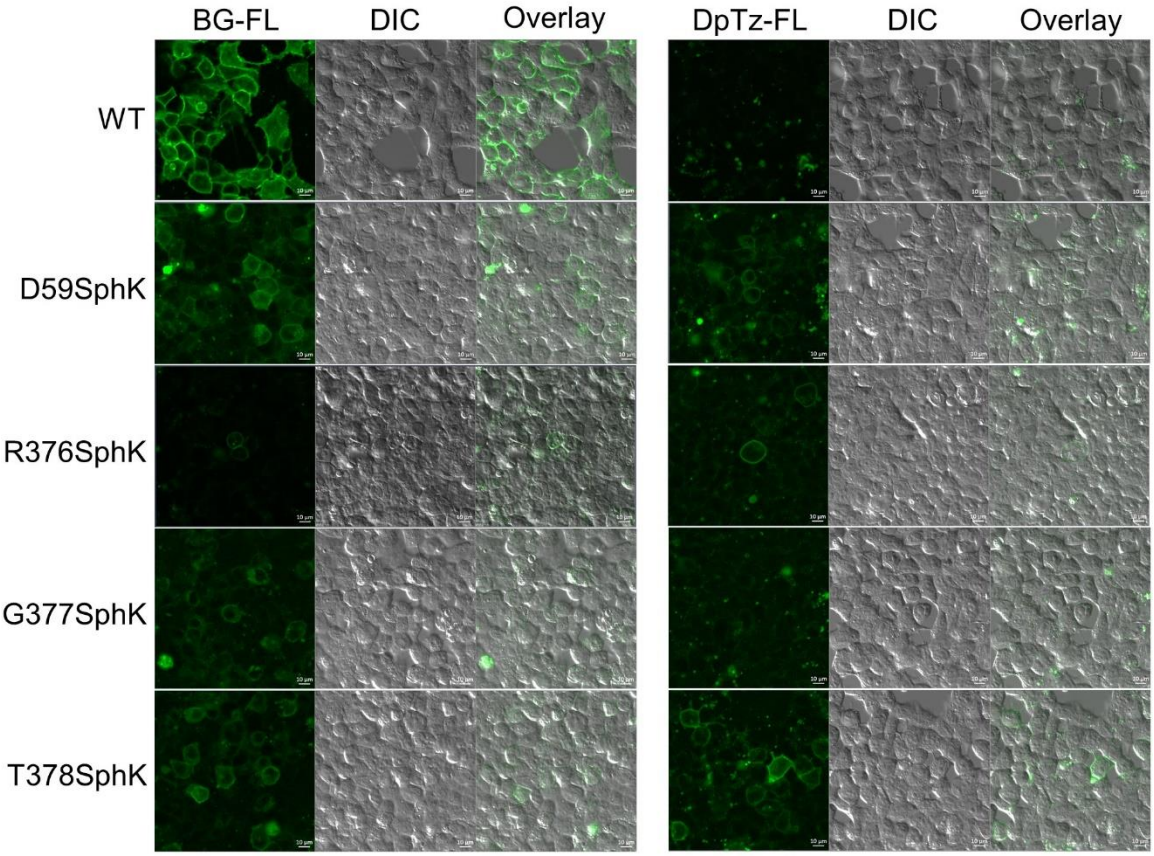
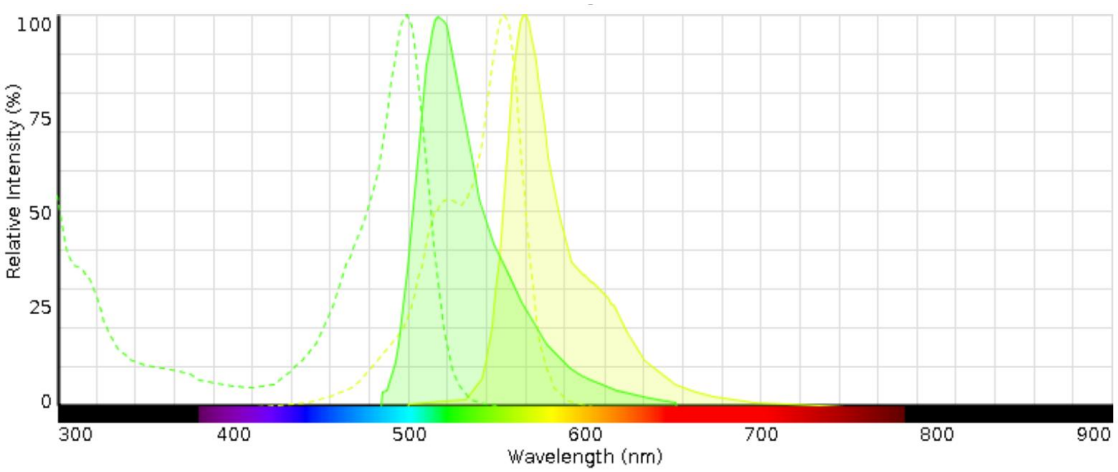


Figure S4. (a) Scheme for SphK incorporation and subsequent labeling by either BG-FL or DpTz-FL. (b) Confocal micrographs of HEK293T cells expressing WT SNAP-GLP-1R or mutants encoding SphK at the indicated positions. Cells were treated with 5 µM BG-FL (left) for 30 min or 5 µM DpTz-FL (right) for 1 h before image acquisition. Scale bar = 10 µm.

a)



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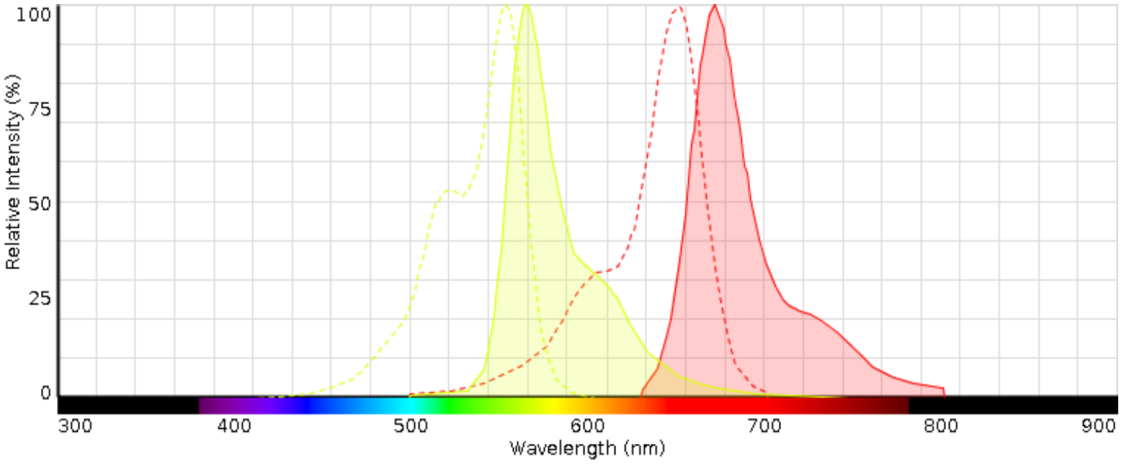
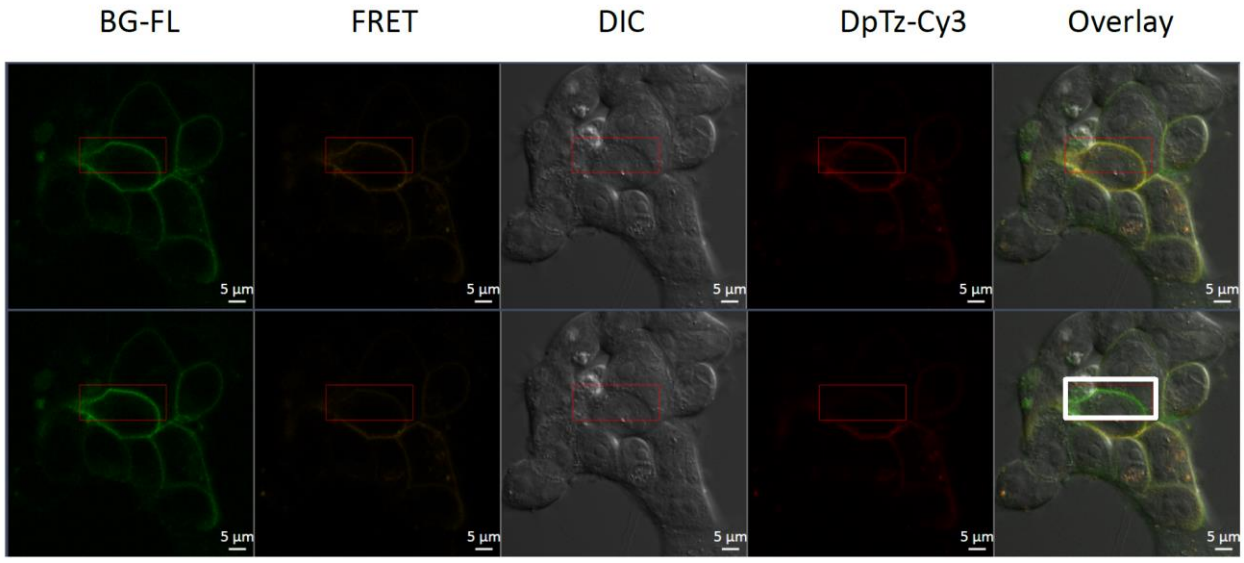


Figure S5. Absorption (dash line) and emission (solid line) spectra of (a) fluorescein (FL)/Cy3 dyes and (b) Cy3/AF647 dyes used in the FRET studies.

a)



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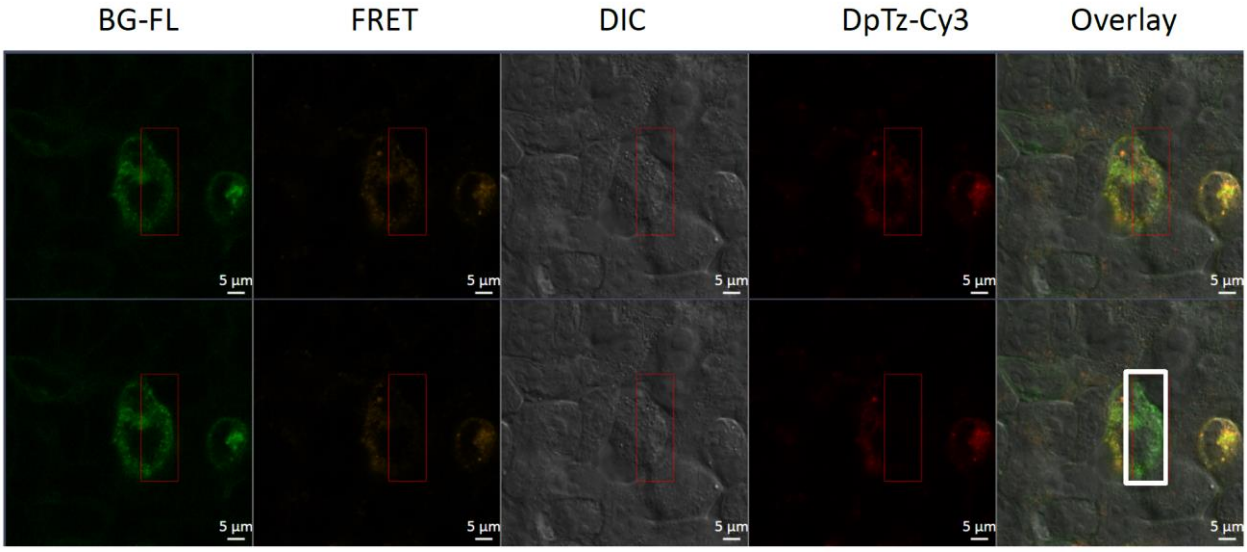
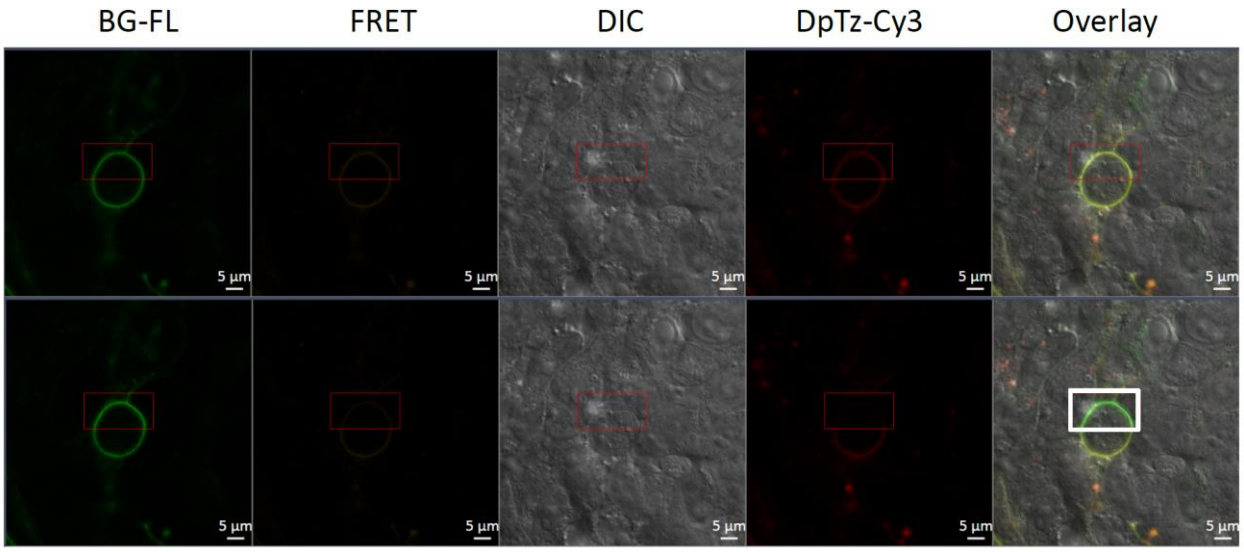


Figure S6. Representative confocal micrographs of the dually FL/Cy3-labeled SNAP-GLP-1R-T378 biosensor's response to GLP-1 stimulation: (a) without treatment; (b) with treatment of 10 µM GLP-1 for 10 min. Top-row micrographs were acquired before acceptor photobleaching while bottom-row micrographs were acquired after acceptor photobleaching. White boxes mark the photobleaching regions. Scale bar = 5 µm.

a)



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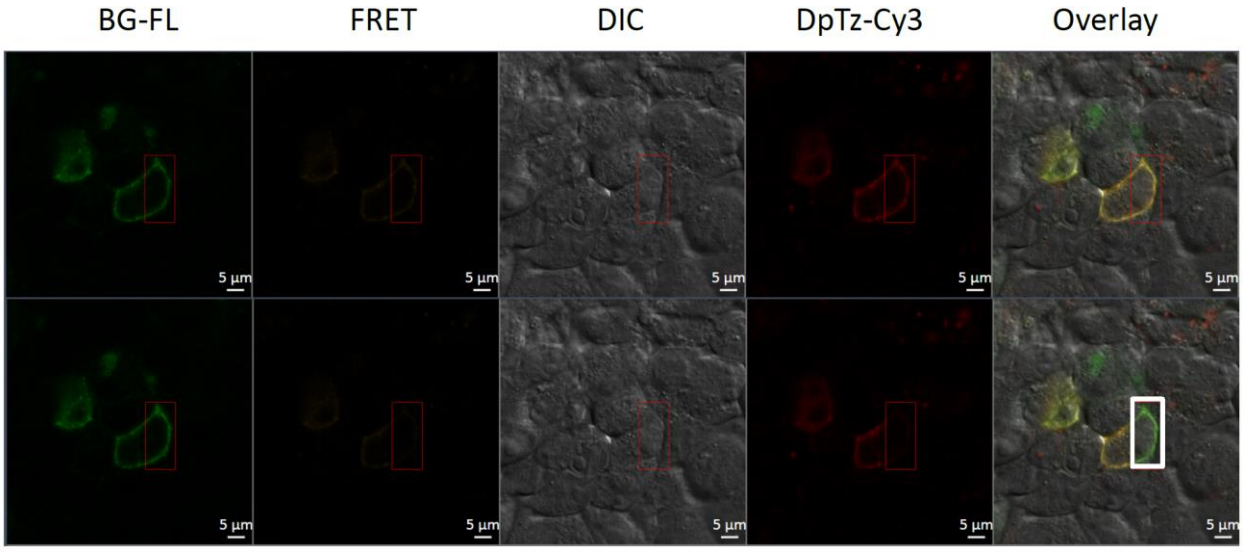
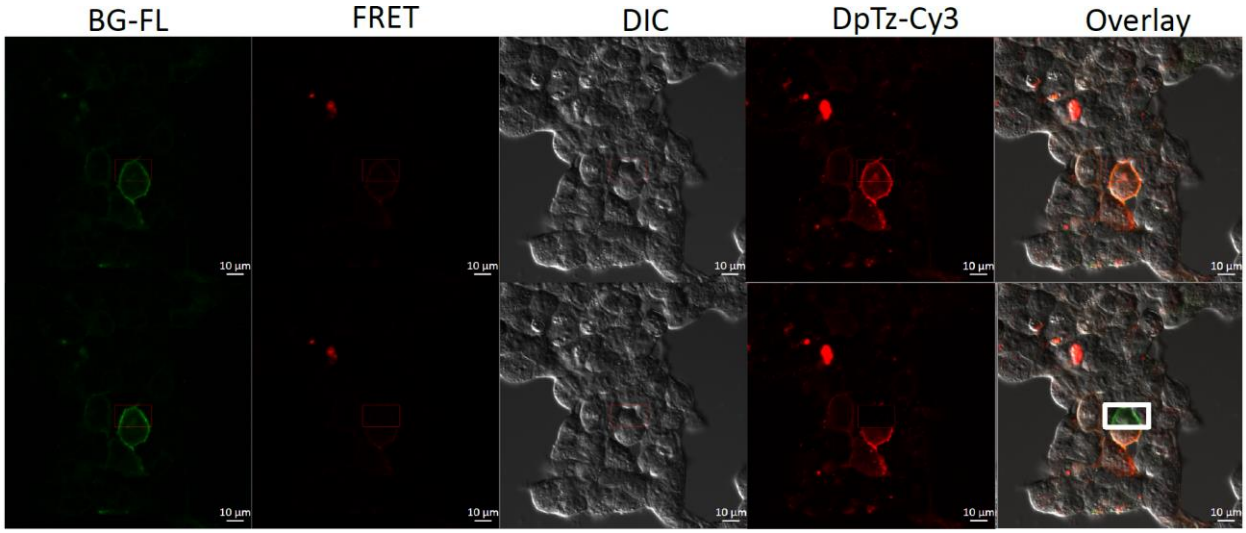


Figure S7. Representative confocal micrographs of the dually FL/Cy3-labeled SNAP-GLP-1R-T378 biosensor's response to OXM-7 stimulation: (a) without treatment; (b) with treatment of 10 µM OXM-7 for 10 min. Top-row micrographs were acquired before acceptor photobleaching while bottom-row micrographs were acquired after acceptor photobleaching. White boxes mark the photobleaching regions. Scale bar = 5 µm.

a)



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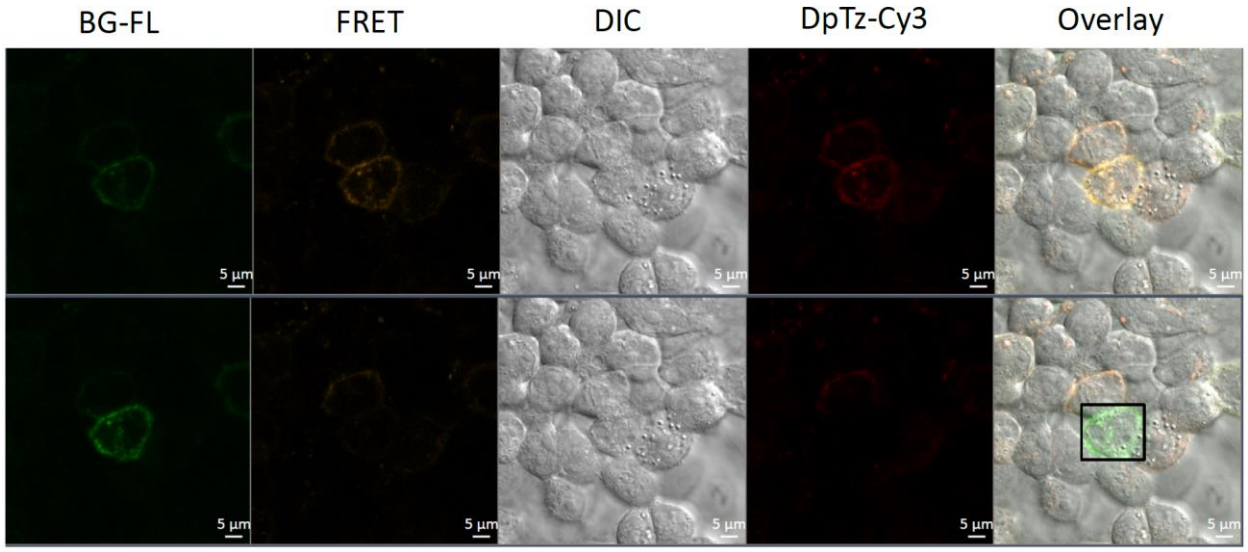
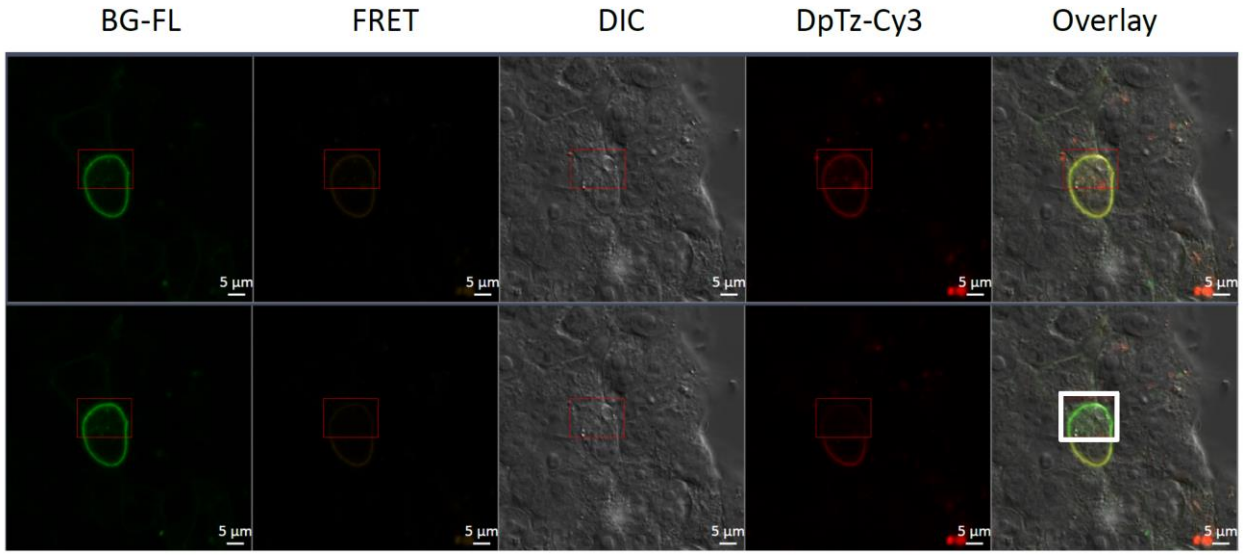


Figure S8. Representative confocal micrographs of the dually FL/Cy3-labeled SNAP-GLP-1R-R376 biosensor's response to GLP-1 stimulation: (a) without treatment; (b) with treatment of 10 µM GLP-1 for 10 min. Top-row micrographs were acquired before acceptor photobleaching while bottom-row micrographs were acquired after acceptor photobleaching. White boxes mark the photobleaching regions. Scale bar = 5 µm.

a)



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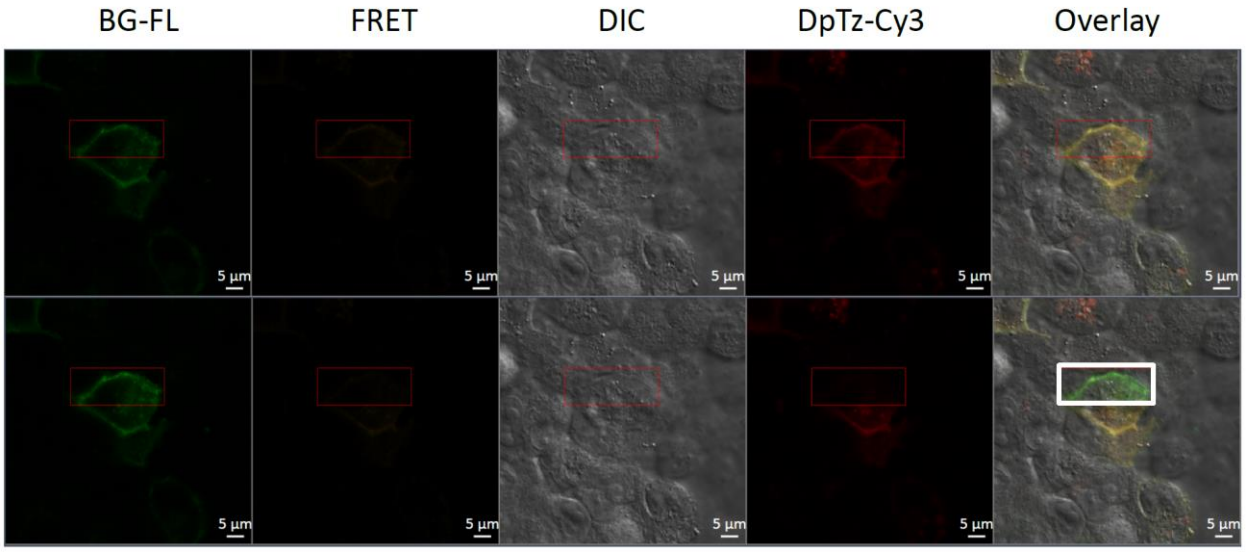

Figure S9. Representative confocal micrographs of the dually FL/Cy3-labeled SNAP-GLP-1R-R376 biosensor's response to OXM-7 stimulation: (a) without treatment; (b) with treatment of 10 µM OXM-7 for 10 min. Top-row micrographs were acquired before acceptor photobleaching while bottom-row micrographs were acquired after acceptor photobleaching. White boxes mark the photobleaching regions. Scale bar = 5 µm.

Table S1. Oligonucleotides used for amber mutations of SNAP-GLP-1R

Amino acid position	Primer sequence
D59	F: 5'- GAGGATCCACCTCCTCCTGCCACATAGTTGTTCTGCAACCGGACCTTC G -3'
	R: 5'- CGAAGGTCCGGTTGCAGAACAACTATGTGGCAGGAGGAGGTGGATCCTC -3'
E373	F: 5’- GTGATGGACTAGCACGCCCGGGGGACCCTGCGCTTC -3’
	R: 5’- CCGGGCGTGCTAGTCCATCACAAAGGCAAAGATGAC -3’
H374	F: 5’- TTTGCCTTTGTGATGGACGAGTAGGCCCGGGGGACCCTGCGCTTC -3’
	R: 5’- GAAGCGCAGGGTCCCCCGGGCCTACTCGTCCATCACAAAGGCAAA -3’
R376	F: 5’- GAGCACGCCTAGGGGACCCTGCGCTTCATCAAGCTG -3’
	R: 5’- CAGGGTCCCCTAGGCGTGCTCGTCCATCACAAAGGC -3’
G377	F: 5’- CACGCCCGGTAGACCCTGCGCTTCATCAAGCTGTTTAC -3’
	R: 5’- GCGCAGGGTCTACCGGGCGTGCTCGTCCATCACAAAG -3’
T378	F: 5’- GCCCGGGGGTAGCTGCGCTTCATCAAGCTGTTTACAG -3’
	R: 5’- GAAGCGCAGCTACCCCCGGGCGTGCTCGTCCATCAC -3’

Unnatural amino acids, fluorescent labeling reagents, and peptide ligands

SphK was synthesized by following the published procedure.[3] BocK was purchased from Chem-Impex and used directly in the study. BG-FL, BG-C3, DpTz-FL, DpTz-Cy3, and DpTz-AF647[4] were prepared by following the published procedure.[4] OXM-7 was prepared by following the published procedure.[5]

SNAP-GLP-1R expression construct

The cloning sites *HindIII* and *NheI* were introduced by PCR into *pCMV6-GLP-1R-myc-DDK* (OriGene) immediately after the signal sequence to make *pCMV6-SP-HN-GLP-1R-myc-DDK* using the following primers:

F: 5'- tcaactaggaccggctagcCGCCCCCAGGGTGCCACTG -3'
R: 5'- gatctggcgcgccgaagcttGGGGCCGGCCCTGCCCAC -3'

Mammalian codon-optimized SNAP-tag gene was custom synthesized (GenScript) and cloned into *pCMV6-SP-HN-GLP-1R-myc-DDK* at the N-terminus to make *pCMV6-SNAP-GLP-1R*.

Amber mutation

Amber codon was introduced into selected positions in GLP-1R by site-directed mutagenesis using Platinum Pfx DNA polymerase (Thermo Fisher Scientific) or Phusion high-fidelity DNA polymerase (New England Biolabs) following the manufacturer' instructions. The primers were listed in Table S1, and the template *pCMV6-SNAP-GLP-1R-myc-DDK* was used in the PCR.

Determination of FRET efficiency

Using the FRETcalc plug-in in ImageJ, %FRET was calculated using the following equation. The calculation is based on the sum of the fluorescence intensities of all the pixels in the selected region-of-interest (ROI). Essentially, in the FRET pair the acceptor is destroyed which then causes the "de-quenching" of the donor. An increase in the donor fluorescence can be observed.

$$\%\,FRET = \left(\frac{I_{Donor\ Postbleaching} - I_{Donor\ Prebleaching}}{I_{Donor\ Postbleaching}}\right) \times 100\%$$

HEK293T Cell Culture and Transfection

HEK293T cells were maintained in a growth medium containing Dulbecco's modified eagle medium (DMEM, Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies) and 10 μg/mL Gentamicin (Life Technologies). Transfection was performed at 70−80% confluency using 3:1 reagent/DNA ratio of Lipofectamine 2000 (Life Technologies) with 2.5 μg of total DNA or polyethylenimine (PEI, Polysciences, Inc.) with 3 μg of total DNA per 35 mm dish. For imaging experiments, cells were kept in an imaging medium (FluoroBrite DMEM supplemented with 10% FBS, 4 mM L-glutamine, and 25 mM HEPES).

UAA incorporation in HEK293T cells

HEK293T cells were co-transfected with a 1:4 ratio of DNA containing the plasmid of interest: synthetase in 200 µL of OPTI-MEM (Life Technologies). The plasmid used contained an amber codon mutation on the protein of interest and the synthetase used was pCMV-mmPlyRS-U6-tRNA that is able to charge BocK and SphK. A 100 mM stock solution of the UAA was first made in DMSO and then diluted to 1 mM using DMEM containing FBS. This solution was filtered through a 0.2 µm PES filter. The medium was removed from the cells and the UAA solution was added followed by the addition of the transfection mixture. Cells were incubated for 24 h before imaging.

Labeling of SNAP-GLP-1R on live cell surface

A fresh solution of BG-FL or BG-Cy3 was prepared by diluting 1 mM stock solution in DMSO to a final concentration of 5 µM in 1 mL growth medium and filtered through 0.2 µM PES. The solution was added to HEK293T cells expressing SNAP-GLP-1R and then incubated at 37 °C and 5% CO_2 for 30 min. The cells were washed once and incubated in 1 mL growth medium for 2 h before changing to the imaging medium.

Labeling of SphK-encoded SNAP-GLP-1R on live cell surface

A fresh solution of DpTz-Cy3 or DpTz- AF647 was prepared by diluting 1 mM stock in DMSO to a final concentration of 5 µM in 1 mL growth medium and filtered through 0.2 µM PES. The solution was added to HEK293T cells expressing SphK-encoded SNAP-GLP-1R and incubated at 37 °C and 5% CO_2 for 1 h. The cells were washed twice and incubated in 1 mL growth medium for 2 h before changing to imaging medium. Confocal imaging was performed using a Zeiss LSM 710 equipped with Plan-Apochromat 20×/0.8 M27 or 40×/1.3 Oil DIC M27 objective with Ex 490/Em 495-635 for the FL channel, Ex 550/Em 555-680 for the Cy3 channel; Ex 635/Em 640-759 for the AlexaFluor 647 channel.

Reference:

1. F. Wu, L. Yang, K. Hang, M. Laursen, L. Wu, G. W. Han, Q. Ren, N. K. Roed, G. Lin, M. A. Hanson, H. Jiang, M. W. Wang, S. Reedtz-Runge, G. Song and R. C. Stevens, *Nat Commun*, 2020, **11**, 1272.
2. Y. Zhang, B. Sun, D. Feng, H. Hu, M. Chu, Q. Qu, J. T. Tarrasch, S. Li, T. S. Kobilka, B. K. Kobilka and G. Skiniotis, *Nature*, 2017, **546**, 248-253.
3. Z. Yu and Q. Lin, *J Am Chem Soc*, 2014, **136**, 4153-4156.
4. C. P. Ramil, M. Dong, P. An, T. M. Lewandowski, Z. Yu, L. J. Miller and Q. Lin, *J Am Chem Soc*, 2017, **139**, 13376-13386.
5. A. Muppidi, H. Zou, P. Y. Yang, E. Chao, L. Sherwood, V. Nunez, A. K. Woods, P. G. Schultz, Q. Lin and W. Shen, *ACS Chem Biol*, 2016, **11**, 324-328.