Electronic supplementary information

A genetically encoded small-size fluorescence pair reveals allosteric conformational changes of G proteins upon its interaction with GPCR by fluorescence lifetime based FRET

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Experimental

Expression and purification of $G\alpha_{i1}$& $G\alpha_s$ mutations.

The human $G\alpha_{i1}$ and $G\alpha_s$ were cloned into pET28a vector, and N-terminal 6 × His tag were added for purification. The amber codon mutation (TAG) was introduced at specific sites of $G\alpha_{i1}$ and $G\alpha_s$ genes for unnatural amino acid labelling. The pEVOL plasmid, which includes a mutant methanococcus jannaschii tyrosyl amber suppressor tRNA (MjtRNATyr$_{CUA}$) /tyrosyltRNA synthetase (MjTyrRS) pair, could insert fluorescence unnatural amino acid L-(7-hydroxycoumarin-4-yl) ethylglycine (7-HC) into polypeptide chains. At the same time, CCPGCC was introduced after residue E116 of $G\alpha_{i1}$ and the corresponding residue F140 of $G\alpha_s$ for another fluorescence probe fluorescein arsenical hairpin FlAsH labelling. All constructs were confirmed by DNA sequencing.

The TAG-mutant $G\alpha_{i1}$/$G\alpha_s$ plasmid and pEVOL-7HC plasmid were co-transformed into *Ecoli.* BL21 (DE3)-gold. The cells were cultured in Luria-Bertani (LB) medium containing 100 mg/mL kanamycin and 34 mg/mL chloramphenicol at 37°C. When OD_{600} value reach 0.6, 1 mM unnatural amino acid 7-HC (synthesized as described) and 0.02 % arabinose were added into the medium protein expression was induce by addition of 0.2 mM IPTG and 0.02% arabinose when OD_{600} reach 1.0, and then cultured at 20°C overnight.

Cells were collected and resuspended by 20 mM HEPES, pH7.5, 200 mM NaCl, 1 mM PMSF and 5.6 mM β-ME. Cell debris was removed by centrifugating at 14000 rpm for 20 min at 4°C. Proteins were purified using Ni-NTA affinity column (QIAGEN) and eluted by buffer (20 mM HEPES, pH7.5, 200 mM NaCl, 250 mM imidazole, 5.6 mM β-ME). The collected samples were concentrated and further purified by size-exclusion chromatography on Superdex 200 10/300 GL column (GE Healthcare) with buffer 20 mM HEPES, pH7.5, 200 mM NaCl, 10 mM β-Me. The purified proteins were concentrated into 2.0 mg/mL for further experimental analysis. The results of each purification step were monitored by SDS–PAGE.

Expression and purification of β_2AR

The human β_2AR was truncated at C-terminal from residue 348. N-terminal FLAG tag and C-terminal 10 × His tag was added for purification. The mutation E122W was introduced to improve the receptor thermostability. This construct was cloned into pFastBac1 vector and expressed in Sf9 insect cells using the Bac-to-Bac Baculovirus system. The cells were infected with baculovirus at a density of 2.5×10^6 cells per mL at 27 °C for 48 h-60 h.

Cells were resuspended and grinded sufficiently in low salt buffer (10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, protease inhibitors named cocktail (Sigma, S8820)), then ultracentrifuged at 180,000g for 45 min. The membrane precipitate was resuspended and grinded with high salt buffer (10 mM HEPES, pH7.5, 10 mM $MgCl_2$, 20 mM KCl, 1M NaCl, protease inhibitor cocktail) and ultracentrifuged at 180,000g for 45 min to remove solubilized proteins. Finally, purified membranes were resuspended in storing buffer (10 mM HEPES, pH7.5, 10 mM $MgCl_2$, 20 mM KCl, 30% glycerol, protease inhibitor cocktail). Same volume of dissolution buffer (10 mM HEPES, pH 7.5, 1% DDM (n-Dodecyl β-D-maltoside), 0.2% CHS (Cholesteryl Hemisuccinate), 1 M NaCl) were mixed with membrane solution and incubated 2 h at 4°C. After centrifugated at 180,000 g for 45 min, supernatants were pooled and incubated with preprocessed TALON Metal Affinity Resin (Clontech) overnight at 4°C. The resin was collected and washed with washing buffer 1 (50 mM HEPES, PH7.5, 0.05% DDM, 0.01% CHS, 500 mM NaCl, 20 mM IMD, 10 mM $MgCl_2$), washing buffer 2 (25 mM HEPES, PH7.5, 0.05% DDM, 0.01% CHS, 500 mM NaCl, 30 mM IMD), and finally eluted in buffer consisting of 50 mM HEPES, PH 7.5, 0.01% DDM, 0.002% CHS, 150 mM NaCl, 300 mM IMD. The eluted proteins were concentrated and further purified by size-exclusion chromatography on Superdex 200 10/300 GL column (GE Healthcare) in 20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.01% (w/v) DDM and 0.002% (w/v) CHS.

Time-resolved fluorescence lifetime measurements

The fluorescence lifetime of fluorescent unnatural amino acid (7-HC) was measured using the time correlated single-photon counting fluorimeter (TCSPC) (DeltaFlex, Horiba Scientific). This fluorimeter is equipped with a 374 nm diode-pulsed laser, a picosecond photon detector, and a time-to-amplitude converter which can convert a total of 4096 channels with the range of 27 ps per channel for data acquisition. Fluorescence intensity decay was measured with a vertically orientated polarizer on the excitation and the emission polarizer at the magic angle (54.7° to the vertical) to remove polarization effects, and the emission intensity was detected at 450 nm. For each measurement, a total of 4096 channels with a time-to-amplitude conversion range of 27 ps channel^{-1} were applied for data acquisition. The instrument response time was measured at the excitation wavelength of 374 nm using 0.01% dilution of Ludox AS40 colloidal silica (Sigma-Aldrich) diluted in deionized water. In order to eliminate polarization effects, fluorescence intensity decay was measured with a vertically oriented polarizer on the excitation and a magic angle (54.71º to the vertical) polarizer on the emission. All decays were collected with a 10 000 counts at peak at room temperature (25°C).

The fluorescence intensity decay curve was fitted with a sum of multiple exponential components, and different components of the fluorescence lifetime were derived:

$$F(t) = A + \sum_{i=1}^{n} a_i \, exp\left(-\frac{t}{\tau_i}\right) \quad (1)$$

The overall average lifetime was calculated as the sum of normalized pre-exponential multiplied by the lifetimes:

$$\langle \tau \rangle = \sum_{i=1}^{n} a_i \, \tau_i \quad (2)$$

τ_i is the lifetime, a_i is a pre-exponential factor representing the amplitude of the component at t=0, and n is the number of predicted different lifetimes. The best acceptable lifetime components were evaluated with a data-fitting chi-squared value (χ^2) below 1.2.

Time dependent kinetic analysis of $G\alpha_{i1}$& $G\alpha_s$

Time dependent kinetic lifetime of $G\alpha_{i1}$-K345HC and $G\alpha_s$-R385HC were measured by binding of receptor (β_2AR), agonist Isoprenaline (ISO), and GTPγs sequentially. The lifetime changes with β_2AR binding were acquired at 1 min, 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min, 75 min and 90 min after 1.2-fold excess β_2AR addition. Then, the mixed sample was purified on Superdex 200 10/300 GL column to verify formation of β_2AR-G protein complex. In addition, fluorescence lifetime measurements were acquired with 50 μm agonist isoprenaline (ISO) and 100 μm GTPγS. All measurements were conducted at room temperature 25°C.

Time dependent FRET measurements of $G\alpha_{i1}$& $G\alpha_s$

The purified $G\alpha_{i1}$-E116FlAsH-K345HC and $G\alpha_s$-F140FlAsH-R385HC was labelled with 1.2 fold excess of FlAsH-EDT2 (Invitrogen) and incubated for 1 hour at room temperature in dark. The labelled samples were purified by Superdex 200 10/300 GL column to remove of free FlAsH-EDT2. Then, samples were concentrated for further fluorescence experiments. Time dependent kinetic lifetimes of $G\alpha_{i1}$-E116FlAsH-K345HC and $G\alpha_s$-F140FlAsH-R385HC were measured by adding of receptor (β_2AR), agonist Isoprenaline (ISO), and GTPγs sequentially as described above. The transfer efficiency is typically measured using the fluorescent lifetime of the donor, in the absence (τ_D) and presence (τ_{DA}) of acceptor.

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

(3)

τ_{DA} is the lifetime in the presence of fluorescent acceptor, τ_D is the lifetime in the absence of fluorescent acceptor.

The FRET efficiency curves of $G\alpha_{i1}$-E116FlAsH-K345HC and $G\alpha_s$-F140FlAsH-R385HC were fitted by sigmaplot (Version12.5) with the equation $f = y_0 + a * (1 - exp(-b * x))$.

References

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2. T. Koopmans, M. van Haren, L. Q. van Ufford, J. M. Beekman and N. I. Martin, *Bioorganic & medicinal chemistry*, 2013, **21**, 553-559.
3. M. A. Hanson, V. Cherezov, M. T. Griffith, C. B. Roth, V.-P. Jaakola, E. Y. Chien, J. Velasquez, P. Kuhn and R. C. Stevens, *Structure*, 2008, **16**, 897-905.
4. J. R. Lakowicz, *Principles of fluorescence spectroscopy*, Springer Science & Business Media, 2013.

Supplementary figures

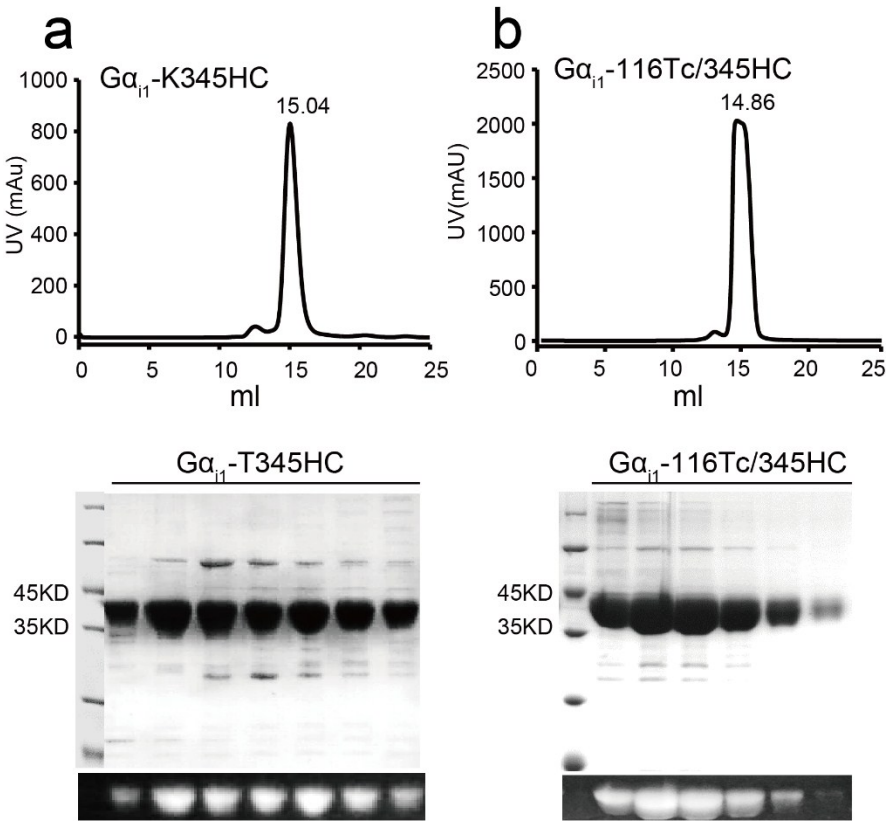

Fig. S1 Purification of $G\alpha_{i1}$ protein. (a) Gel filtration chomatography of $G\alpha_{i1}$K345HC (upper), coomassie-blue stained SDS-PAGE (middle) and the fluorescence image (down) of $G\alpha_{i1}$-K345HC. (b) Gel filtration chromatography of $G\alpha_{i1}$-116Tc/345HC (upper), coomassie-blue stained SDS-PAGE (middle) and the fluorescence image (down) of $G\alpha_{i1}$-116Tc/345HC.

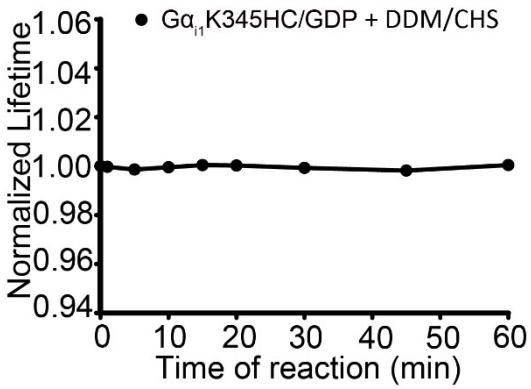

Fig. S2 Time dependent kinetic analysis of $G\alpha_{i1}$-K345HC by adding of 0.1% DDM /0.02% CHS was as experiment control.

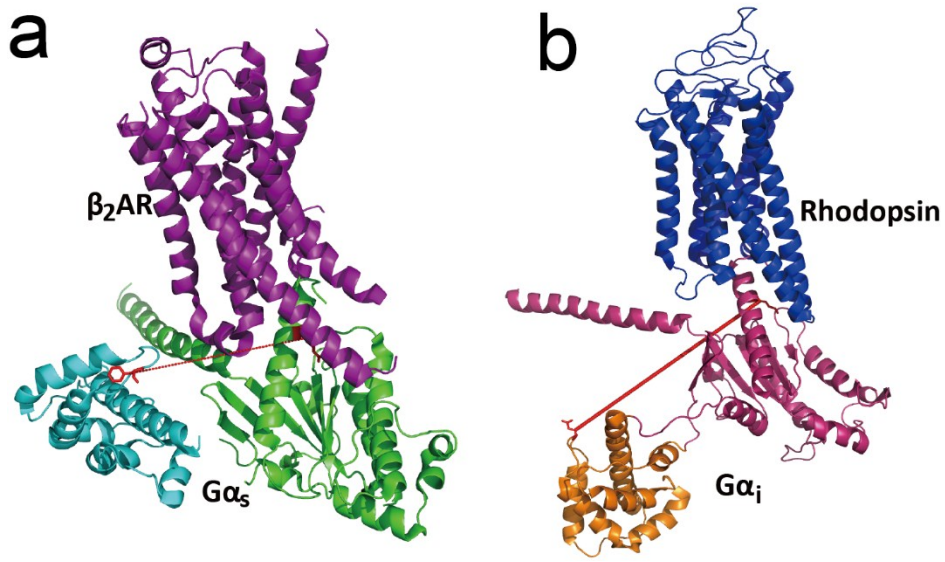

Fig. S3 Comparison of the receptor–G-protein binding interfaces of the β_2AR–Gα_s and Rhodopsin–Gα_i complexes. (a) Crystal structure of the β_2AR–Gα_s complex (PDB number 3SN6, Gβ and Gγ subunits were removed) showed a much deeper insertion of α-helix5 into β_2AR and a larger swing of helix-domain in Gs. **(b)** Cryo-EM structure of the Rhodopsin–Gα_{i1} complex (PDB number 6CMO, Gβ and Gγ subunits were removed) displayed markedly smaller interfaces with GPCR than the structure of GPCR-Gs complexes.

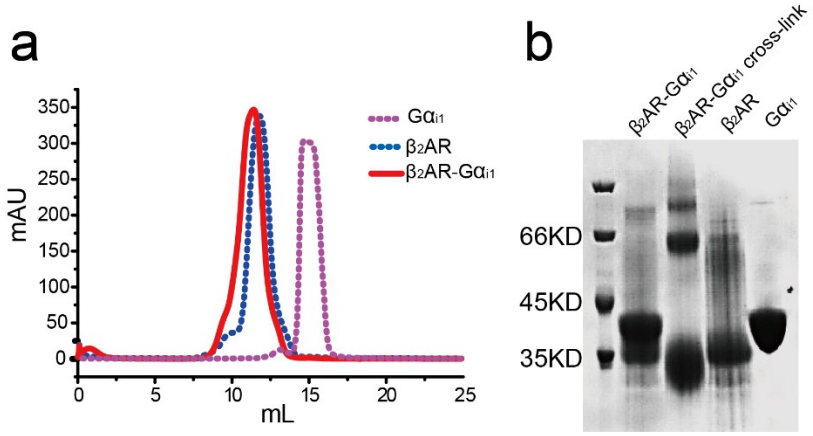

Fig. S4 Identification of β_2AR-Gα_{i1} complex (a) Size exclusive chromatography of Gα_{i1} (dotted magenta), β_2AR (dotted blue), β_2AR-Gα_{i1} complex (red). (b) SDS–PAGE of β_2AR-Gα_{i1} complex (lane 2), β_2AR-Gα_{i1} crosslink by glutaraldehyde (lane 3), β_2AR (lane 4) and Gα_{i1} (lane 5).

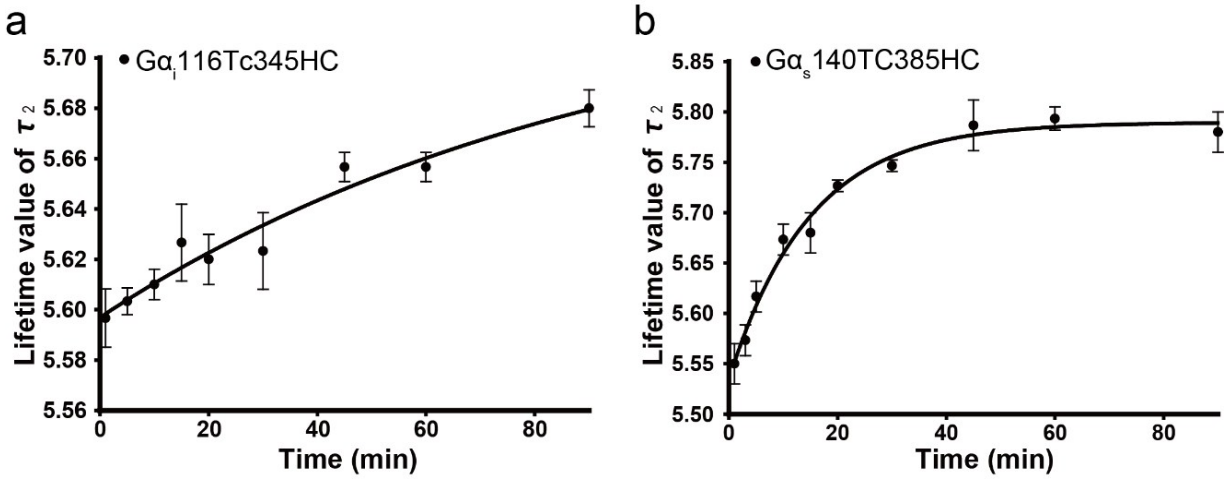

Fig. S5 Time dependent lifetime kinetic analysis of component τ_2. (a) Time dependent changes of component τ_2 of $G\alpha_{i1}$-116TC345HC. **(b)** Time dependent changes of component τ_2 of $G\alpha_s$-140FlAsH385HC.

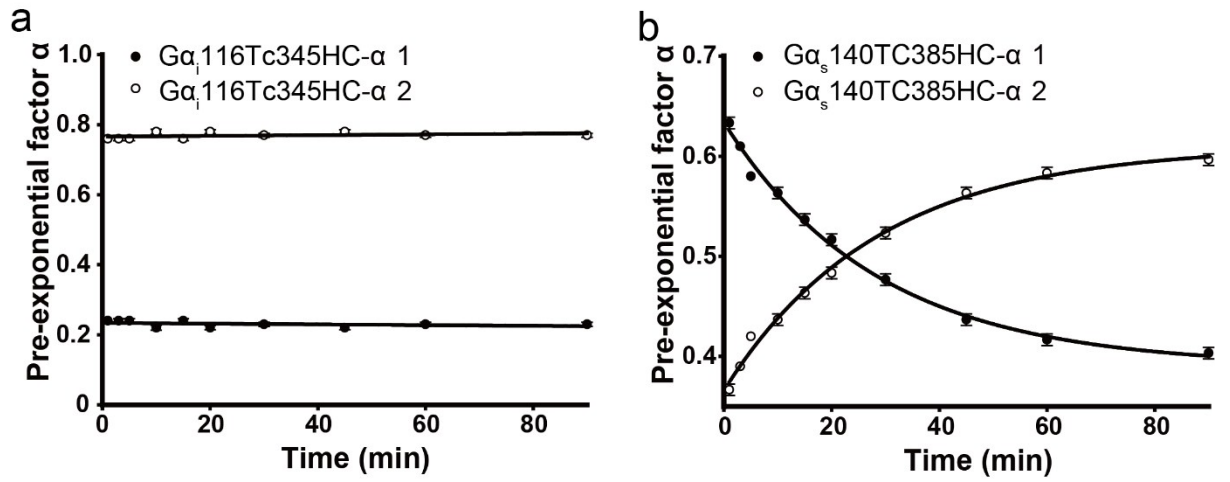

Fig. S6 Time dependent weighting factor analysis of each lifetime component. (a) Weighting factors of $G\alpha_{i1}$-116TC345HC represented the amplitude of each $G\alpha_{i1}$-116TC345HC lifetime component. **(b)** Weighting factors of $G\alpha_s$-140FlAsH385HC represented the amplitude of each $G\alpha_s$-140FlAsH385HC lifetime component.

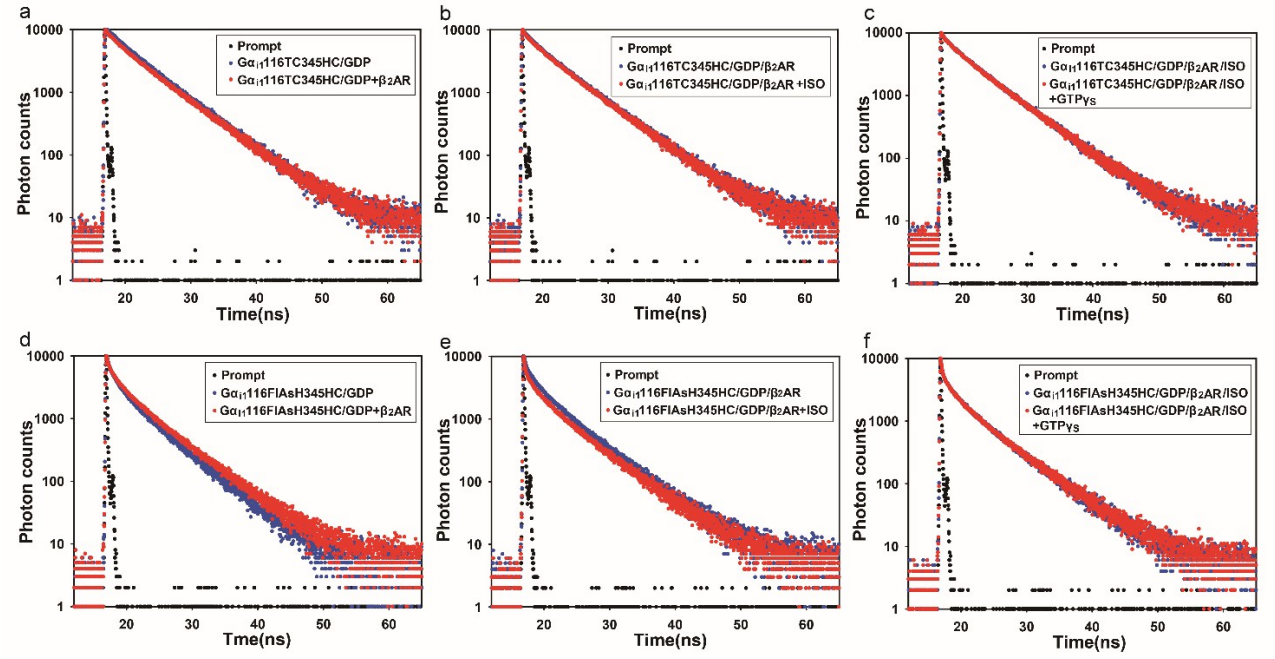

Fig. S7 Fluorescence decay curves of $G\alpha_{i1}$ upon the β_2AR binding, the ligand activation and the GTPγS exchange. (a)(d) Comparing of fluorescence lifetime curves of $G\alpha_{i1}$116TC345HC (a) or $G\alpha_{i1}$116FlAsH345HC (d) before and after β_2AR binding. (b)(e) Comparing of fluorescence lifetime curves of $G\alpha_{i1}$116TC345HC (b) or $G\alpha_{i1}$116FlAsH345HC (e) before and after ligand (ISO) activation. (c)(f) Comparing of fluorescence lifetime curves of $G\alpha_{i1}$116TC345HC (c) or $G\alpha_{i1}$116FlAsH345HC (f) before and after GTPγs binding.

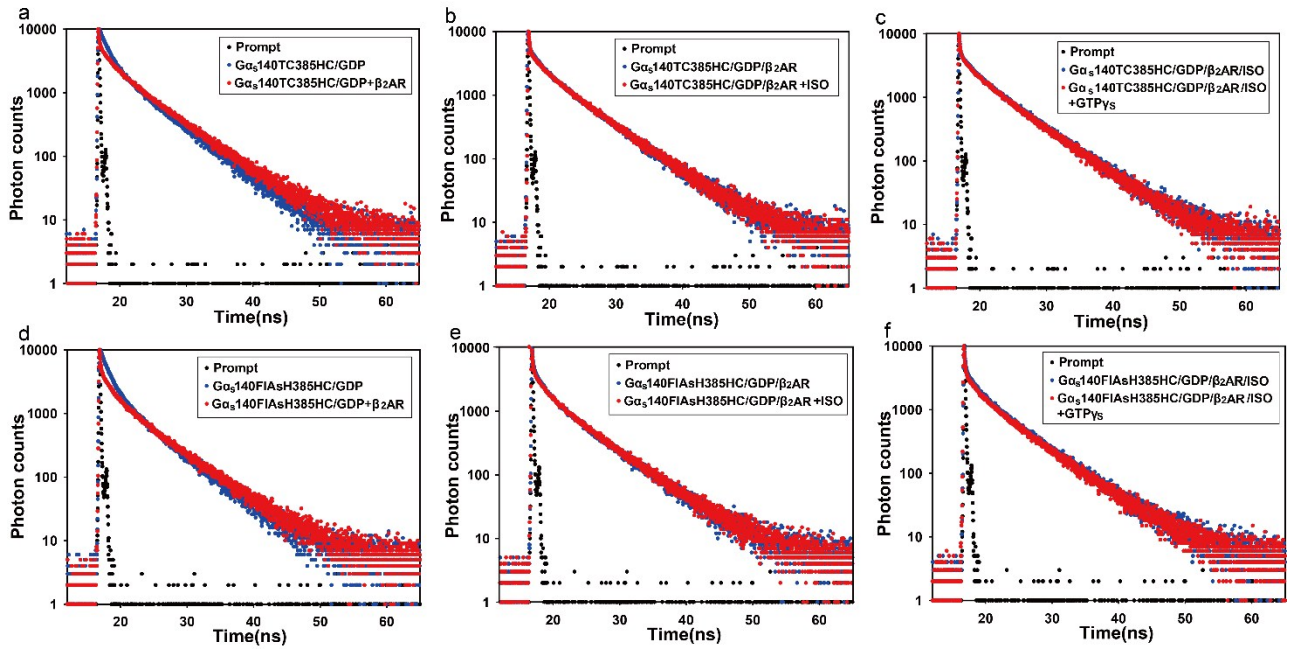

Fig. S8 Fluorescence decay curves of $G\alpha_s$ upon the β_2AR binding, the ligand activation and the GTPγS exchange. (a)(d) Comparing of fluorescence lifetime curves of $G\alpha_s$140TC385HC (a) or $G\alpha_s$140FlAsH385HC (d) before and after β_2AR binding. (b)(e) Comparing of fluorescence lifetime curves of $G\alpha_s$140TC385HC (b) or $G\alpha_s$140FlAsH385HC (e) before and after ligand (ISO) activation. (c)(f) Comparing of fluorescence lifetime curves of $G\alpha_s$140TC385HC (c) or $G\alpha_s$140FlAsH385HC (f) before and after GTPγS exchange.

Table S1. Fluorescence lifetime measurements of individual Ga_{i1}-7HC mutations using multi-exponential analysis.

		τ_1[a]	$_1$[b]	τ_2[a]	$_2$[b]	<τ>[c]	χ^2	SD
	apo	**2.79**	20	**5.81**	80	**5.22**	1.12	0.004
I19	apo+GDP	**3.02**	11	**5.94**	89	**5.62**	1.16	0.003
	apo+GDP+GTP	**3.15**	10	**5.71**	90	**5.53**	1.11	0.043
	apo	**2.94**	19	**5.77**	81	**5.23**	1.08	0.009
L23	apo+GDP	**3.45**	10	**5.92**	90	**5.66**	1.18	0.008
	apo+GDP+GTP	**4.99**	16	**5.88**	84	**5.73**	1.21	0.012
	apo	**2.00**	73	**5.18**	31	**2.99**	1.13	0.007
Y61	apo+GDP	**2.10**	66	**5.06**	34	**3.12**	1.16	0.010
	apo+GDP+GTP	**2.45**	64	**5.16**	36	**3.42**	1.16	0.010
	apo	**3.43**	7	**5.98**	93	**5.80**	1.17	0.006
F108	apo+GDP	**3.39**	7	**5.98**	93	**5.81**	1.17	0.014
	apo+GDP+GTP	**3.66**	8	**5.99**	92	**5.81**	1.16	0.011
	apo	**3.07**	13	**6.30**	87	**5.87**	1.13	0.014
D150	apo+GDP	**2.51**	15	**6.14**	85	**5.60**	1.09	0.014
	apo+GDP+GTP	**3.30**	11	**6.26**	89	**5.93**	1.14	0.009
	apo	**2.25**	22	**5.80**	78	**5.02**	1.13	0.020
F199	apo+GDP	**2.19**	18	**5.83**	82	**5.18**	1.10	0.015
	apo+GDP+GTP	**3.03**	14	**5.96**	86	**5.54**	1.15	0.010
	apo	**2.12**	21	**5.68**	79	**4.93**	1.09	0.019
K210	apo+GDP	**1.87**	29	**5.68**	71	**4.59**	1.10	0.002
	apo+GDP+GTP	**1.92**	26	**5.77**	74	**4.79**	1.13	0.014
	apo	**1.56**	34	**4.87**	66	**3.74**	1.15	0.007
F215	apo+GDP	**1.57**	31	**5.07**	69	**3.98**	1.19	0.033
	apo+GDP+GTP	**1.47**	54	**4.90**	46	**3.06**	1.22	0.015
	apo	**1.88**	26	**5.80**	74	**4.78**	1.08	0.020
F323	apo+GDP	**1.82**	32	**5.87**	68	**4.55**	1.09	0.013
	apo+GDP+GTP	**1.71**	23	**6.05**	77	**5.05**	1.12	0.010
	apo	**2.15**	29	**5.46**	71	**4.50**	1.15	0.009
F336	apo+GDP	**1.91**	30	**5.50**	70	**4.41**	1.10	0.024
	apo+GDP+GTP	**2.16**	21	**5.60**	80	**4.89**	1.09	0.042
	apo	**2.30**	26	**5.75**	74	**4.86**	1.10	0.005
T340	apo+GDP	**2.48**	27	**5.67**	73	**4.79**	1.06	0.031
	apo+GDP+GTP	**2.65**	20	**5.62**	80	**5.03**	1.18	0.009
	apo	**2.37**	20	**5.62**	80	**4.97**	1.05	0.004
K345	apo+GDP	**2.44**	20	**5.61**	80	**4.97**	1.09	0.015
	apo+GDP+GTP	**2.48**	21	**5.63**	79	**4.96**	1.14	0.004

Fluorescence decay curves were obtained at the steady-state peak emission wavelength (450 nm) for all samples. All curves were fitted to a two exponential function using Eq.1. [a] Lifetimes for τ_1 and τ_2. [b] The weighting factors for τ_1 and τ_2. [c] Average lifetimes calculated using Eq.2. χ^2 was the data-fitting chi-squared value. SD was the standard deviation of average lifetime in three independent detections.