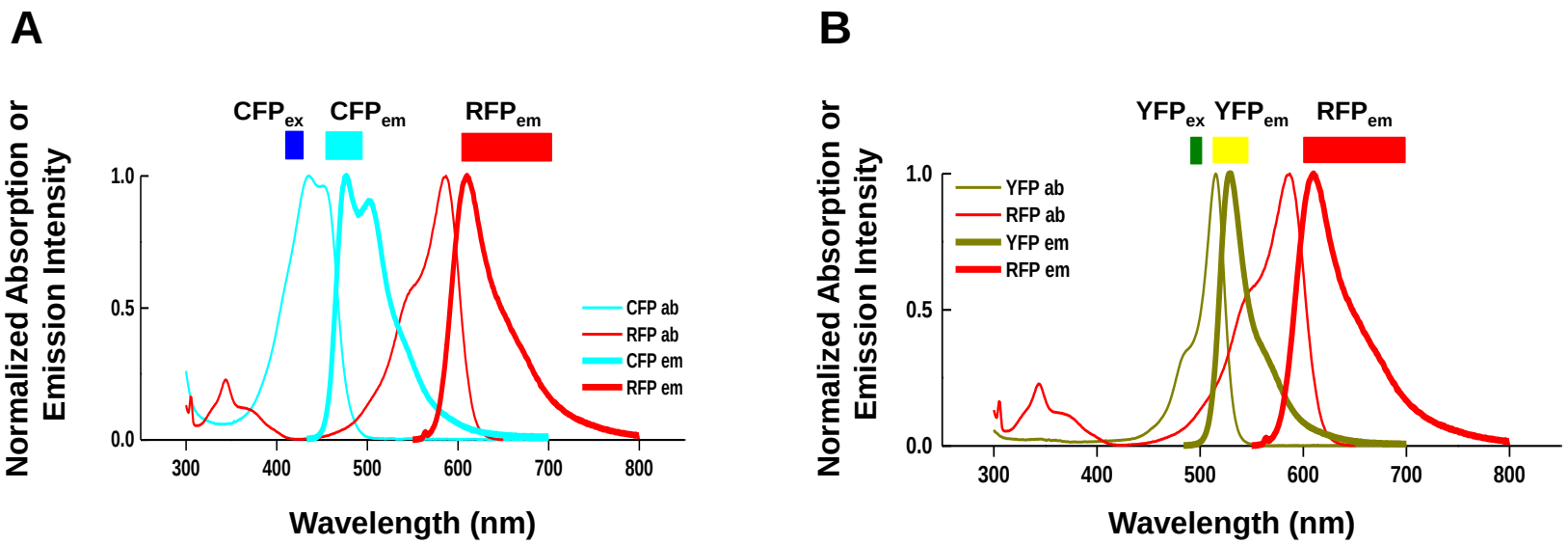
Supplementary Figure 1



Supplementary Figure 1: Absorption and Emission spectra of CFP and RFP (A), and YFP and RFP (B). The solid colored blocks represent the excitation and emission filters used for each FRET pairs. ex: excitation; ab: absorption; em: emission.

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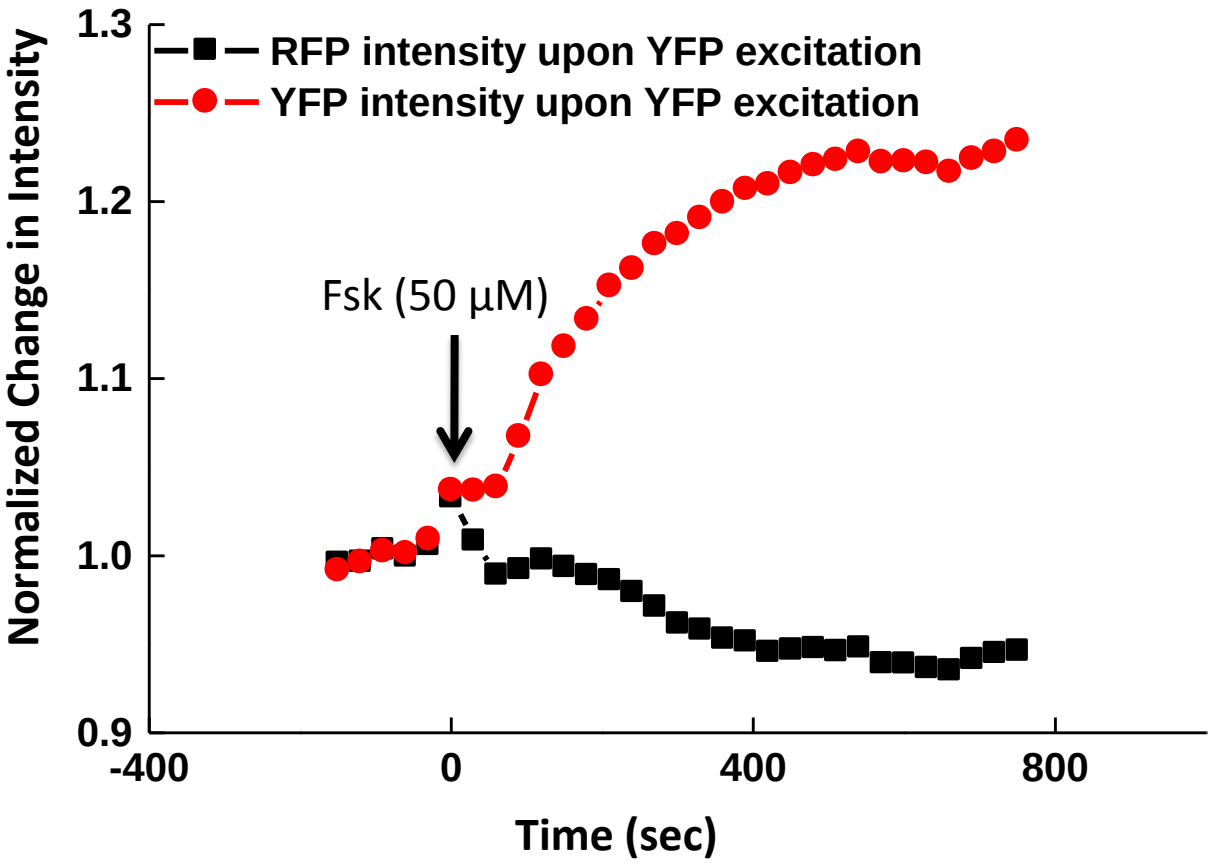
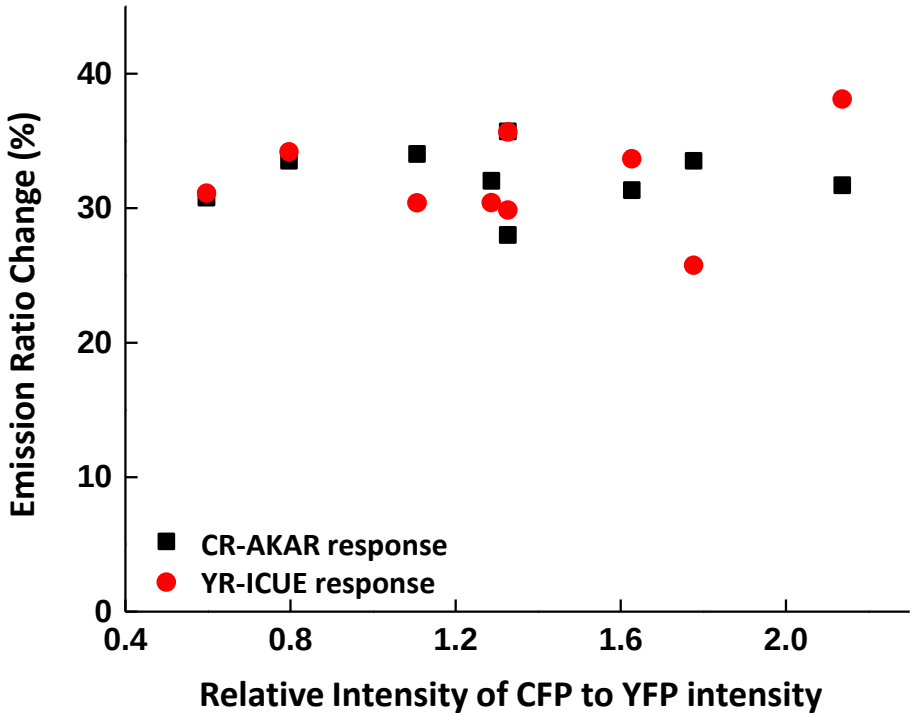
Supplementary Figure 2



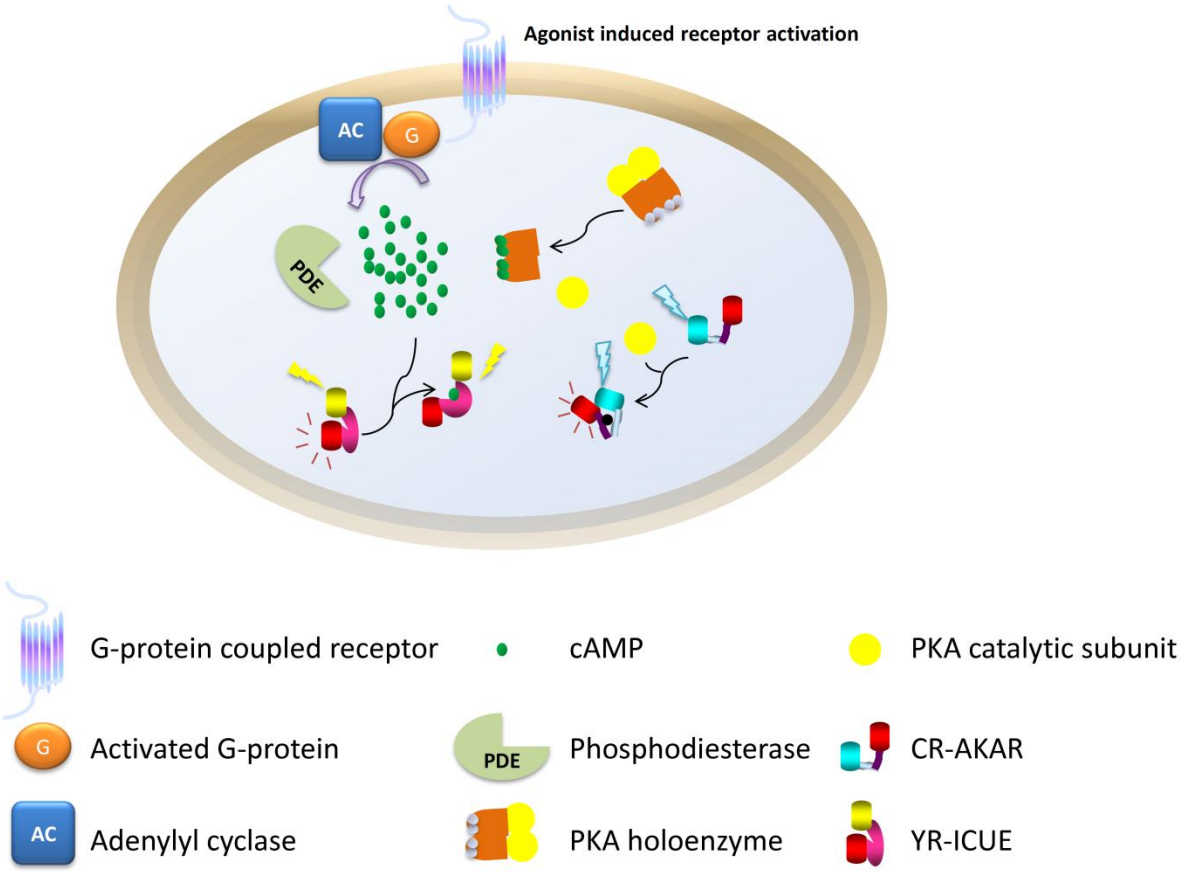
Supplementary Figure 2: YFP emission change appears to be a more important contributor to the YR-emission ratio change.

Supplementary Figure 3



Supplementary Figure 3: During parallel imaging, responses of CR-AKAR and YR-ICUE remain constant over a range of different relative expression levels of the two biosensors. The percent responses of CR-AKAR and YR-ICUE for an individual cell are plotted against relative intensity of CFP to YFP intensity in that particular cell. The relative intensity of CFP to YFP intensity gives indication about relative expression of CR-AKAR with respect to that of YR-ICUE.

Supplementary Figure 4



Supplementary Figure 4: A schematic diagram of the parallel-imaging system using biosensors with orthogonal FRET pairs containing a common acceptor. Using these biosensors, differential dynamics of PKA activity and cAMP can be observed in parallel based on the strength of pathway activation by various receptor agonists.

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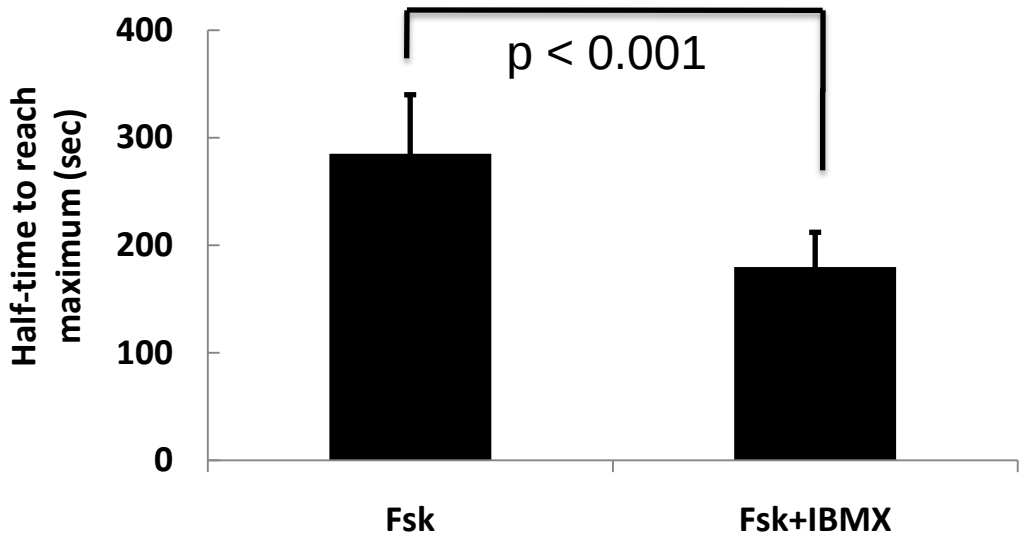
Supplementary Figure 5



Supplementary Figure 5: Fsk-induced cAMP response can be accelerated by addition of a general PDE inhibitor IBMX. In these experiments, HEK293T cells expressing YR-ICUE were treated with either Fsk (50 µM) or Fsk (50 µM)+IBMX (100 µM). The half-times to reach maximum ratio change were 285 ± 55 sec (n = 12) for Fsk alone treatment and 180 ± 32 sec (n = 10) for Fsk+IBMX treatment. P-value was calculated based on Student's t-test.