Supplementary Information for:

Red fluorescent cAMP indicator with increased affinity and expanded dynamic range

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Contents

1. Supplementary Figures

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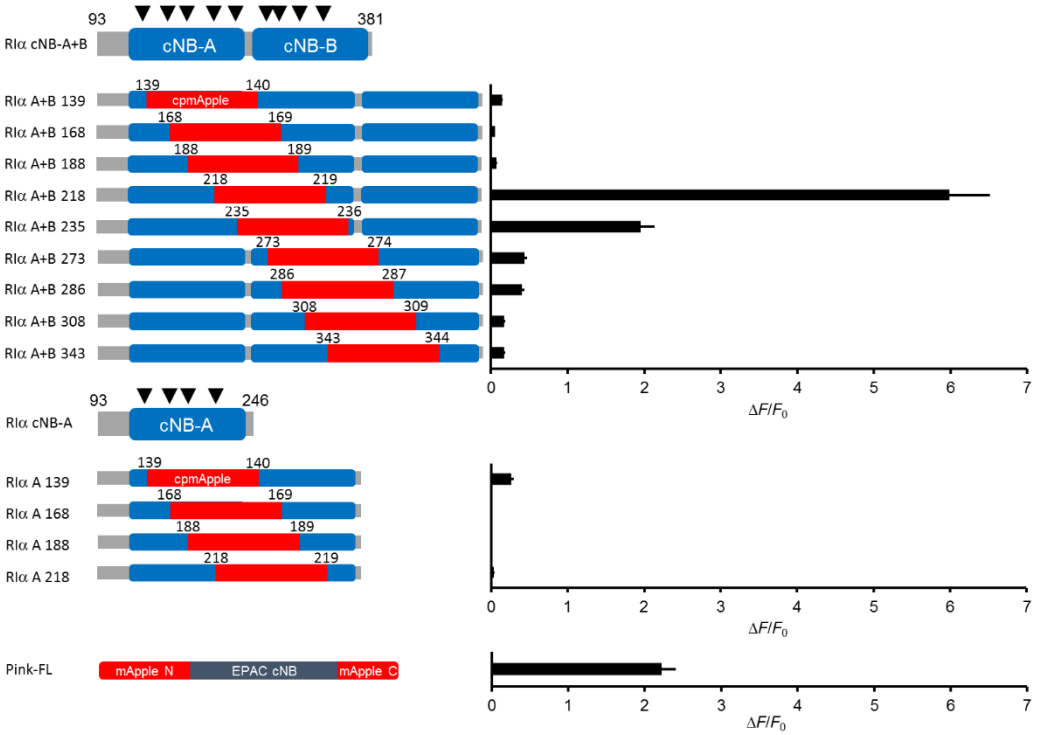
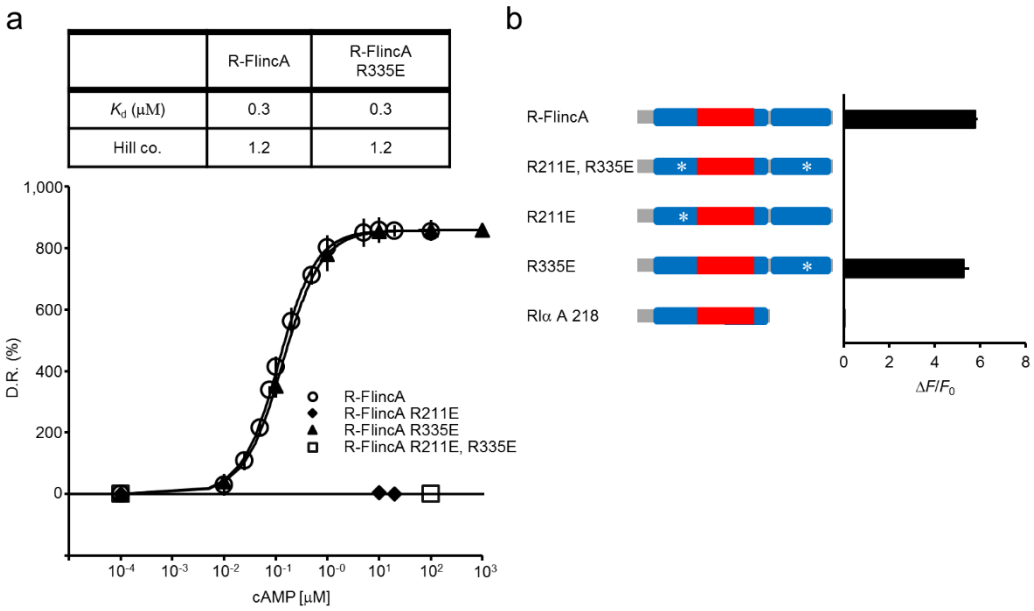


Supplementary Figure S1. Development of an improved cAMP indicator.

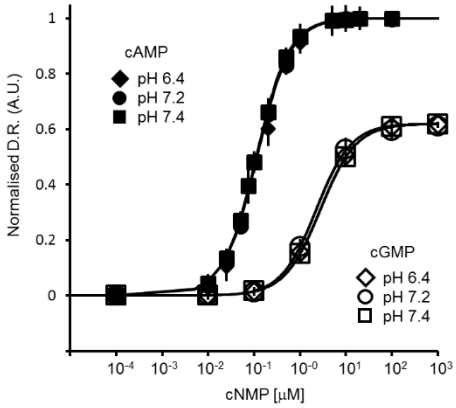
(**a**) cAMP-binding motif of PKA RIα utilized in this study. D/D: dimerization/docking domain, cNB-A/B: cyclic nucleotide binding domain. (**b**) The candidate constructs with the cp146mApple insertion at different positions of RIα (left). The response of these constructs in the cell-based assay using 293T cells. The fluorescence signal change ($\Delta F/F_0$) to the forced elevation of cellular cAMP by FSK treatment (right). Bars represent + SEM (N = 20 cells, respectively). The a.a. numbering represents the position in the full-length RIα. Pink-FL was used as a positive control.



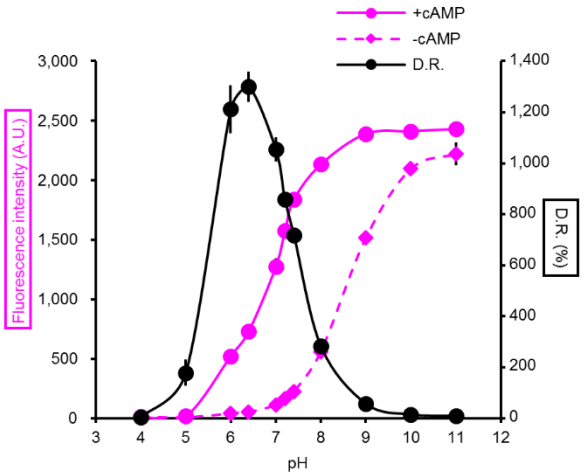
Supplementary Figure S2. The contribution of two-binding sites on the properties of R-FlincA. (**a**) Titration curves for cAMP of R-FlincA variants at pH 7.2. R211E and R335E (in RIα numbering) are the mutations in cNB-A and cNB-B that eliminate cAMP-binding, respectively. For R-FlincA, K_d = 0.30 ± 0.02 (mean ± SEM), Hill coefficient = 1.19 ± 0.06; For R-FlincA R335E, K_d = 0.30 ± 0.04 μM, Hill coefficient = 1.16 ± 0.07. Bars represent ± SEM for triplicate experiments. (**b**) Dynamic range of R-FlincA variants assayed in 293T cells. Bars represent + SEM (N = 20–40 cells) for duplicate experiments. Deletion of cNB-B domain (RIα A 218) abolished the fluorescence increase upon FSK stimulation, indicating that the presence of cNB-B is necessary for increase in the fluorescence intensity of R-FlincA, although a cAMP-binding to cNB-B is not essential. The a.a. numbering represents the position in the full-length RIα.

a

K_d (μM)	cAMP	cGMP
pH 6.4	0.3	6.4
pH 7.2	0.3	6.6
pH 7.4	0.3	6.9

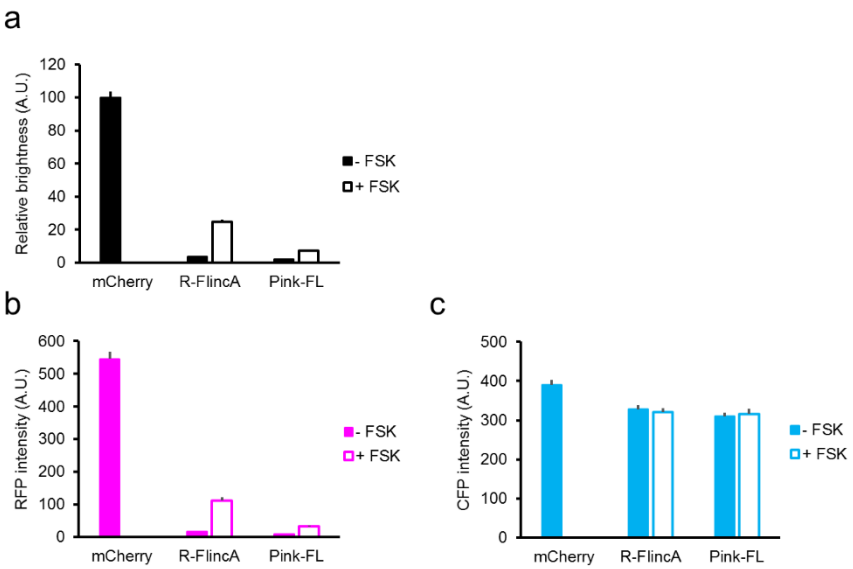


b



Supplementary Figure S3. Additional *in vitro* properties of R-FlincA.

(**a**) A set of titration curves for cAMP and cGMP at a given pH, the both were normalised by the D.R. of cAMP-bound form (100 μM). Three different pH conditions were tested: pH 7.2 and 7.4 are the physiologically relevant intra-cellular pH. pH 6.4 is the condition yielding the largest D.R. (1,300%, see also Figure S3b). K_ds for cAMP are 0.29 ± 0.02 (mean ± SEM), 0.30 ± 0.02 and 0.30 ± 0.03, at pH 6.4, 7.2 and 7.4, respectively. K_ds for cGMP are 6.36 ± 0.14, 6.61 ± 0.21 and 6.87 ± 0.25 μM, at pH 6.4, 7.2 and 7.4, respectively. Bars represent ± SEM for three independent experiments. (**b**) The fluorescence intensity (left axis) in the presence (solid magenta) or absence (dashed magenta) of 1 mM cAMP, plotted against pH. The dynamic range (right axis) was calculated by dividing the difference (ΔF) between the intensities of the cAMP-bound form and cAMP-free form by that of the cAMP-free form (F_0). Bars represent ± SEM for triplicate experiments.



Supplementary Figure S4. In-cell brightness of red cAMP indicators.

(**a**) The intensities of RFPs before and after FSK stimulation were divided by those of bi-cistronically expressed ECFP, then were normalised to the brightness of mCherry (100%). The relative brightness of R-FlincA before and after FSK stimulation were 3.60 ± 0.24 (mean ± SEM) and 24.9 ± 1.03, respectively. Those of Pink-FL before and after FSK stimulation were 2.06 ± 0.22 and 7.21 ± 0.40, respectively. Bars represent + SEM (N = 30, 30, and 40 cells for mCherry, R-FlincA, and Pink-FL) for duplicate experiments. (**b, c**) The raw intensities of RFPs and ECFP were shown in (b) and (c), respectively.

2. Legend for Supplementary Video

Supplementary Video S1 (.mp4)

Extra-cellular and intra-cellular dual imaging by PKA RIα #7 and R-FlincA in the population of *D. discoideum* cells. The left and right panels show FRET ratio images of the culture medium containing PKA RIα #7 and R-FlincA/iRFP ratio images of *D. discoideum* cells, respectively. Bar, 100 μm.

3. Supplementary Tables

Supplementary Table S1. Properties of 1-FP cAMP indicators, sorted by the affinity. Modified from Ref. (13).

cAMP indicator	Dynamic range[a] (%)	K_d (μM)	FP	References
R-FlincA	860[b]	0.3[b]	cp146mApple	This study
Flamindo2	300[c]	3.2[c]	Citrine	Odaka *et al*.[26]
Pink Flamindo	320[c]	7.2[c]	mApple	Harada *et al*.[27]

[a]$(F_{max}-F_{min})/F_{min}$, where F_{max} and F_{min} is the maximum and minimum fluorescence intensity, respectively.

[b]The value obtained by *in vitro* spectroscopy at pH 7.2.

[c]The value obtained by *in vitro* spectroscopy at pH 7.4.

Supplementary Table S2. Properties of selected FRET cAMP indicators, sorted by the affinity. Modified from Ref. (13).

cAMP indicator	Dynamic range[a] (%)	K_d (μM)	Donor & Acceptor combination[f]	References
PKA RIα #7	40[b]	0.04[b]	cp173Venus/ECFP	Ohta *et al*.[23]
mlCNBD-FRET	50[c]	0.07[b]	Citrine/Cerulean	Mukherjee *et al*.[28]
R-CFP, C-YFP	~20[d]	0.3[b]	YFP, CFP	Zaccolo *et al*.[17], and Smith *et al*.[25] Mongillo *et al*.[19]
Epac2-camps	~20[d]	0.9[b]	EYFP/ECFP	Nikolaev *et al*.[21]
Epac-S^{H187}	160[c]	~4.0[f]	mTurquoise2/Td-cp173Venus	Klarenbeek *et al*.[22]
CUTie	~20[e]	7.4[c]	EYFP/ECFP	Surdo *et al*.[18]
RII-EBFP, C-S65T	N/A	N/A	EBFP, EGFP	Zaccolo *et al*.[16]
ICUE3	100[c]	N/A	ECFP/cp194Venus	Dipilato *et al*.[24] and Smith *et al*.[25]

[a](R_{max}-R_{min})/R_{min}, where R_{max} and R_{min} is the maximum and minimum emission ratio, respectively.

[b]The value determined by *in vitro* spectroscopy in the referenced study.

[c]The value determined by in-cell observations in the referenced study.

[d]The estimated value (~) from the spectroscopic data in the cited paper.

[e]The estimated value (~) from the in-cell data in the cited paper.

[f]The value (~) of the related construct (Epac-S^{H134}) sharing Q270E mutation on EPAC.

[g]The combination of the donor and acceptor with its order.