Development of green fluorescent protein-based cAMP indicators with expanded dynamic range and various working ranges

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ABSTRACT

There is a wide range in the concentration of intracellular cyclic adenosine 3', 5'-monophosphate (cAMP), which mediates specific effects as a second messenger in pathways affecting many physiological processes. Here, we developed green fluorescent cAMP indicators, named Green Falcan (**Green** **f**luorescent protein-b**a**sed indicator visua**l**izing **cA**MP dy**n**amics) with various EC_{50} (0.3, 1, 3, 10 μM) for covering the wide range of intracellular cAMP concentrations. The fluorescence intensity of Green Falcans increased in a cAMP dose-dependent manner, with the dynamic range of over 3-fold. Green Falcans showed a high specificity for cAMP over its structural analogues. When we expressed Green Falcans to HeLa cells, these indicators visualized dynamics and distinct kinetics of cAMP in various pathways in living cells with high spatiotemporal resolution. Furthermore, we demonstrated that Green Falcans are applicable to dual-color imaging with R-GECO, a red fluorescent Ca^{2+} indicator, in the cytoplasm and the nucleus. This study shows that Green Falcans open up new avenue for understanding hierarchal and cooperative interaction with other molecules in various cAMP signaling pathways by multi-color imaging.

Cyclic adenosine 3', 5'-monophosphate (cAMP) plays an important role as a second messenger in living cells. Many hormones or neurotransmitters stimulate G protein-coupled receptors in the cell membrane, leading to the activation of adenylate cyclase which catalyzes the conversion of ATP to cAMP[1]. In living cells, cAMP binds to protein kinase A (PKA)[2], exchange proteins directly activated by cAMP (Epac)[3], or cyclic nucleotide-gated (CNG) ion channels[4], and causes many cellular functions such as hormone secretion[5], cell motility[6], and memory formation[7], etc.

To date, many single-fluorescent protein (FP) based indicators have been developed as tools for detecting various intracellular signaling molecules (Ca^{2+}, cAMP, cGMP, etc.)[8-14]. Since single-FP based indicators require single wavelength emission for detection, it is suitable for multicolor imaging to simultaneously visualize the hierarchal and cooperative relationship with other molecules in the same or distinct intracellular compartments, with high spatiotemporal resolution. The single-FP based indicators show increased and decreased fluorescence intensity (FI) in the presence of analytes, called turn-on type and turn-off types, respectively. A few turn-off type green fluorescent cAMP indicators have been produced[11,12,15], but distinguishing photobleach or the degradation of the indicator from analyte decrease is problematic. A turn-on type green fluorescent cAMP indicator has been developed so far, but its utility is limited by low dynamic range (~1.5-fold)[16].

Here, we utilized our previously described method of semi-rational molecular design and molecular evolution[17] in developing indicators such as Green cGull[14], MaLionG[18], and Green Glifon[19], to generate powerful turn-on type green fluorescent cAMP indicators with expanded dynamic range and various the half maximal effective concentration (EC_{50}) employing PKA as a binding domain, named them Green Falcan0.3, 1, 3, 10. We demonstrated that this useful method

is not limited to cAMP indicators, but hopefully lead to the development of new indicators for a myriad of molecules.

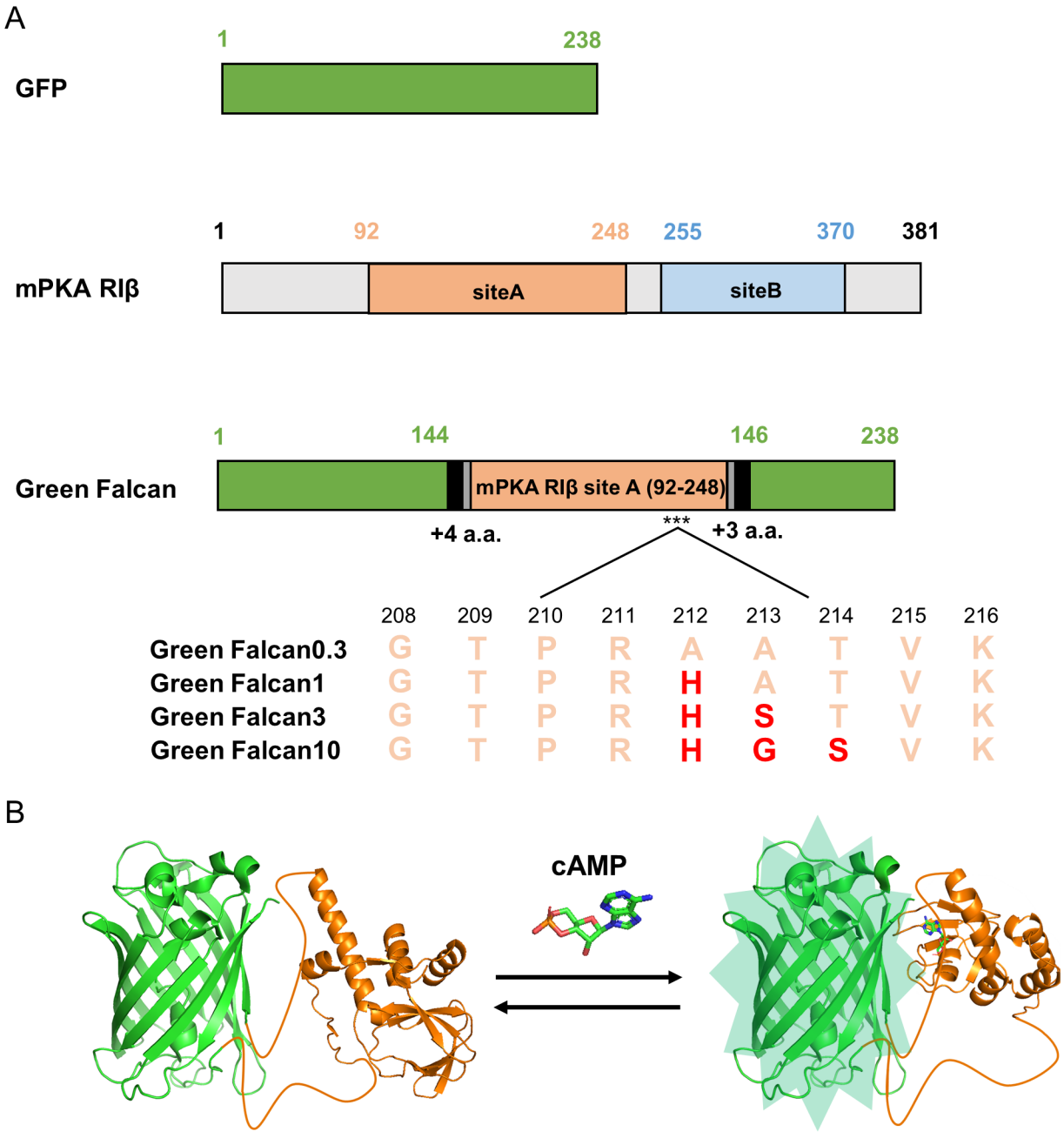

Figure 1. Schematic design of Green Falcan proteins (A) Diagram for GFP, mouse cAMP-dependent protein kinase (PKA) type I-beta regulatory subunit (mPKA RIβ), and Green Falcan proteins. Red letters indicate mutated amino acids. (B) Schematic 3D structures of Green Falcan

unbound (left) and bound (right) to cAMP. The 3D structure diagrams were created using the X-ray structures of cpGFP (PDB 3EVP) and mPKA (cAMP-unbound PDB 6NO7 and bound PDB 1RGS) with manually drawn linkers between cpGFP and mPKA.

To generate turn-on type green fluorescent cAMP indicators with expanded dynamic range, we first constructed a prototype indicator by inserting the cAMP binding domain (amino acids 92-248) derived from site A of mouse cAMP-dependent protein kinase (PKA) type I-beta regulatory subunit[20] (NP_001240819.1) into the vicinity of the chromophore of green fluorescent protein (GFP) (Figure 1A). We expected fluorescence intensity (FI) change caused by environment change around the chromophore induced by conformational change of the inserted binding domain responding to ligand binding. When this prototype indicator was expressed in bacteria, the FI measured in the lysates increased slightly in the presence of cAMP. Since this response was insufficient for easy detection of minor changes in cAMP level, we planned to improve the prototype indicator by protein engineering. To begin, we selected the linker length between GFP and PKA (N-terminus: 4 amino acids, C-terminus: 3 amino acids) that displayed the largest FI change (1.3-fold). For further improvement of the dynamic range, we optimized the amino acid sequence around the N- and C-terminus linkers by molecular evolution (Figure S1A, B). After 40 cycles of random mutation, we obtained a green fluorescent cAMP indicator with more than 3-fold FI change in the presence of cAMP (Figure S1C). When the dose-response relationship to cAMP was examined, we found that the half maximal effective concentration (EC_{50}) of this indicator was 0.3 μM (Figure 2I). Generally, cAMP is known to function in a wide range of concentration from sub μM to μM range in living cells[21]. Therefore, to cover this range, we introduced random mutations around R211 of PKA, which is important for cAMP binding[22]. Consequently, by selection based on the response to several concentrations of cAMP, we

succeeded in obtaining additional three green fluorescent cAMP indicators with various EC_{50} (1, 3, and 10 μM) (Figure 1A, 2J-L). The indicators, Green Falcan0.3, 1, 3, and 10 were named after their EC_{50} value and abbreviation of **Green** **f**luorescent protein-b**a**sed indicator visua**l**izing **cA**MP dy**n**amics(Figure 1B). These new indicators have larger dynamic range than an existing turn-on type green fluorescent indicator[16], allowing us to detect small changes in cAMP concentration. Additionally, utilizing the same molecular design we used to develop our previous indicators for cAMP[11-13], cGMP[14], ATP[18], glucose[19], pyruvate and lactate[23] demonstrates its versatility for generating genetically-encoded indicators based on fluorescent protein.

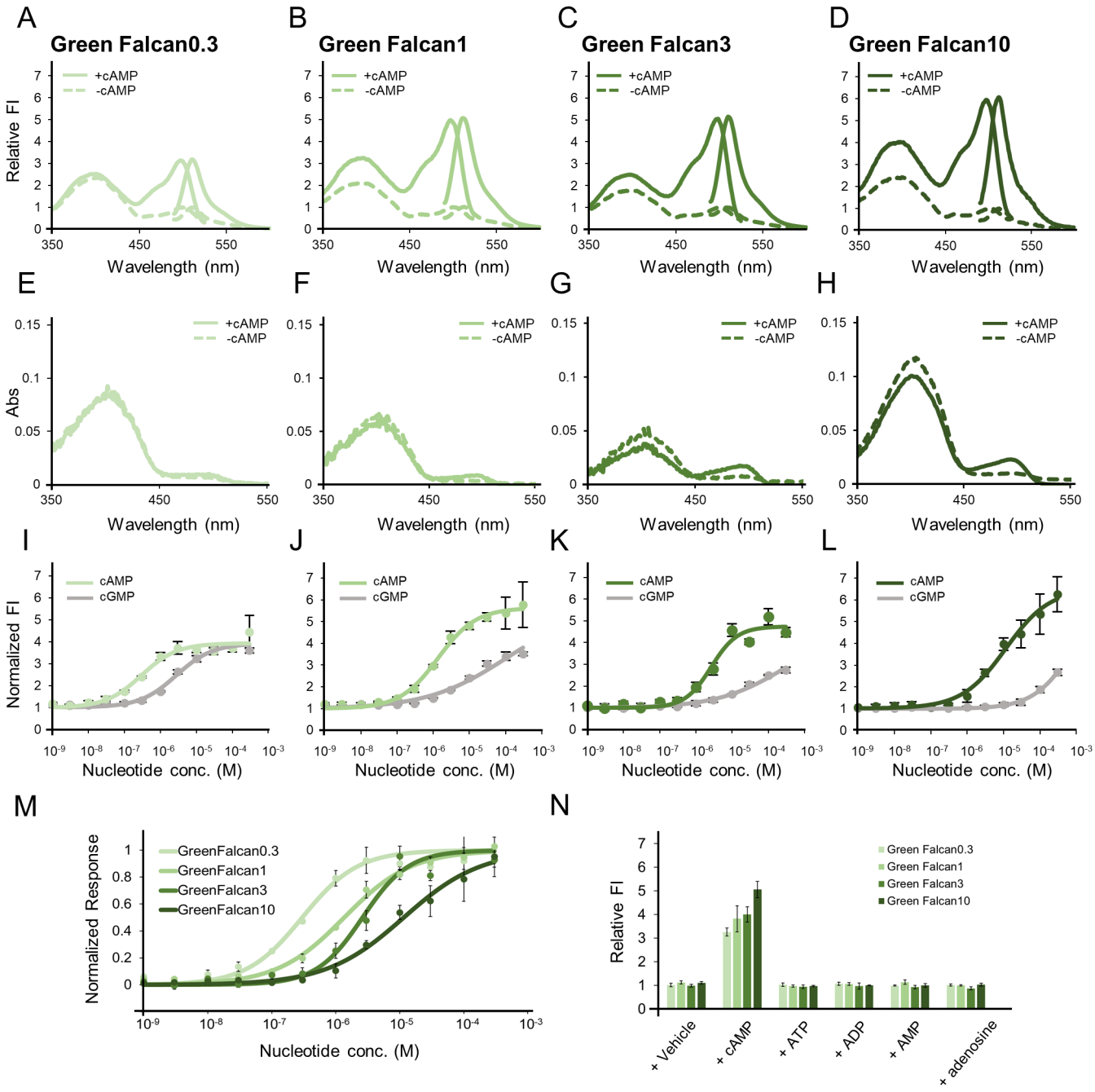

Figure 2. Spectral and functional properties of Green Falcans (A-D) Excitation and emission spectra of purified Green Falcan0.3, Green Falcan1, Green Falcan3, and Green Falcan10 in the presence (solid line) and absence (dashed line) of 100 µM cAMP. The relative fluorescence intensity (FI) was calculated by dividing by the FI of the peak in the absence of cAMP. (E-H) Absorption spectra of purified Green Falcan0.3, Green Falcan1, Green Falcan3, and Green

Falcan10 in the presence (solid line) and absence (dashed line) of 100 μM cAMP. (I-L) Dose-response curves of purified Green Falcan0.3, Green Falcan1, Green Falcan3, and Green Falcan10 for cAMP (green line) and cGMP (gray line). The normalized FI was calculated by dividing by the FI of the peak in the absence of cAMP, and the minimum FI in fitting was normalized to 1. The data represent the means ± standard deviation (n=3). (M) Dose-response curves of all Green Falcans. The normalized response was calculated by dividing by the FI of the peak in the absence of cAMP, and the minimum and maximum FI in fitting was normalized to 0 and 1, respectively. The data represent the means ± standard deviation (n=3). (N) Specificity of purified Green Falcans for cAMP structural analogues. The concentration of these analogues were 10-times higher than the EC_{50} value for each Green Falcan. Each relative FI was calculated by dividing by the FI of the peak in the absence of cAMP analogues. The data represent the means ± standard deviation (n=3).

To investigate the properties of the Green Falcans, we examined excitation and emission spectra for purified protein preparations of these indicators. All Green Falcans had two excitation peaks at around 400 nm and 498 nm with an emission peak at around 511 nm. The peaks in excitation at around 498 nm, and emission at around 511 nm, which are similar to the spectral properties of GFP[24], were increased more than 3-fold in the presence of cAMP (Figure 2A-D). In the presence of cAMP, the absorption spectra of all Green Falcans showed a little decreased and increased peaks at around 400 nm and 500 nm, respectively (Figure 2E-H). It has been demonstrated that the GFP absorption peak observed at around 400 nm corresponds to the protonated state of the chromophore and that observed at 500 nm, to the deprotonated state[24]. Thus, we think that a change in population of the protonated/deprotonated state of chromophore contributes to the fluorescence intensity (FI) change. Interestingly, unlike other GFP-based indicators, Green Falcans have an excitation peak at around 400 nm, which increased in the presence of cAMP. Since the excitation peak derived from the protonated form of chromophore

creates the fluorescence at around 511 nm by excited-state proton transfer[25,26], it seems that the proton transfer was also accelerated by the application of cAMP to induce FI change. When the dose-response relationship of all Green Falcans was examined, we found that the EC_{50} value of each Green Falcan for cAMP was 0.30, 1.4, 2.6 and 11 μM, while those for cGMP were much higher using a four-parameter logistic curve fitting (Figure 2I-M, Table 1). We also found that the dynamic range of Green Falcans in the presence of cAMP was 3.9, 5.6, 4.7, 6.5-fold for Green Falcan0.3, Green Falcan1, Green Falcan3, and Green Falcan10, respectively. To examine the specificity of all Green Falcans for cAMP, we applied cAMP structural analogues (ATP, ADP, AMP, adenosine) at 10 times concentrations of the EC_{50} for each indicator. This demonstrated that all Green Falcans display a high specificity for cAMP (Figure 2N). These results suggest that by choosing the appropriate indicator, according to its EC_{50}, for the expected intracellular cAMP range, these indicators can be used to specifically visualize a wide range of cAMP levels in living cells, during a variety of physiological events. Although we have employed cAMP binding domain from mouse Epac1 for developing cAMP indicator such as Flamindo[11], Flaminodo2[12] and Pink Flamindo[13], that from PKA was done in this study. Since introducing a mutation in the binding domain usually decreases the affinity for ligand, we recommend exploiting a binding domain with a higher affinity to develop indicators covering a wide range of ligand concentrations.

Table 1. EC_{50} of each Green Falcan

Indicator name	EC_{50} (cAMP) (μM)	EC_{50} (cGMP) (μM)
Green Falcan0.3	0.30	2.8
Green Falcan1	1.4	1.0×10^2
Green Falcan3	2.6	3.1×10^2
Green Falcan10	11	8.7×10^2

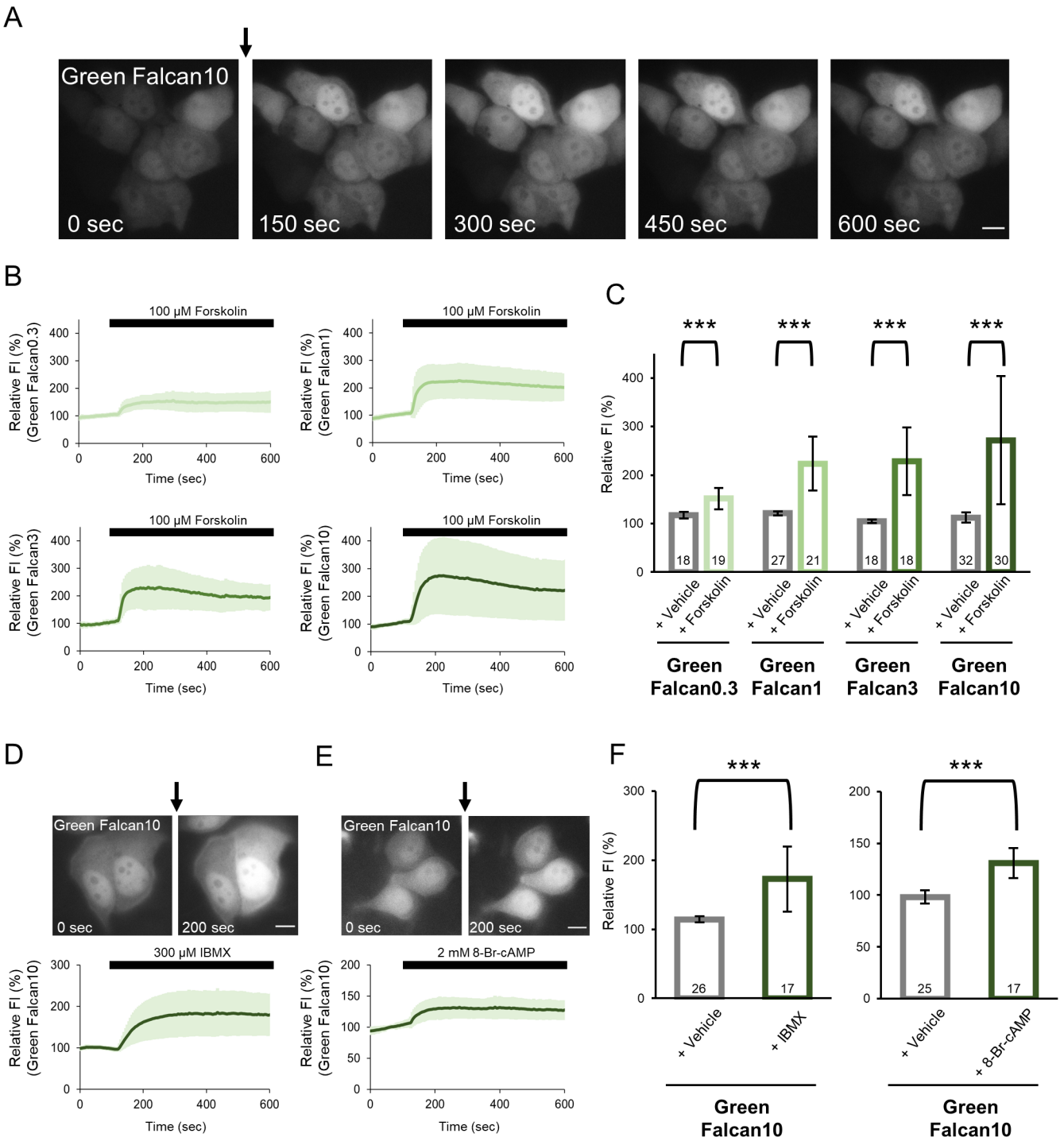

Figure 3. Live-cell imaging using Green Falcans (A) Sequential images of Green Falcan10 expressing HeLa cells. Scale bar represents 10 μm. (B) Time courses of fluorescence intensity (FI) in HeLa cells expressing Green Falcans in response to 100 μM forskolin. Relative FI was calculated by dividing by the mean of the FI during the 2 min prior to administration. The data

represent the means ± standard deviation (Green Falcan0.3; n=19, Green Falcan1; n=21, Green Falcan3; n=18, Green Falcan10; n=30 from three independent experiments). (C) Comparison of relative FI at 2 min after the administration of DMSO (vehicle) or 100 μM forskolin to HeLa cells expressing Green Falcans. The data represent the means ± standard deviation. The numbers in bar graphs represent the number of cells analyzed from three independent experiments. ***p<0.001. (D-E) Images and time course of FI in HeLa cells expressing Green Falcan10 in response to 300 μM IBMX (D) or 2 mM 8-Br-cAMP (E). The data represent the means ± standard deviation (IBMX; n=17, 8-Br-cAMP; n=17 from three independent experiments). Scale bar represents 10 μm. (F) Comparison of relative FI at 2 min after the administration of DMSO (vehicle) or 300 μM IBMX, and after that of dH_2O (vehicle) or 2 mM 8-Br-cAMP to HeLa cells expressing Green Falcan10. The data represent the means ± standard deviation. The numbers in bar graphs represent the number of cells analyzed from three independent experiments. ***p<0.001.

To investigate the applicability of Green Falcans to live-cell imaging, we expressed these indicators in HeLa cells, and monitored with several reagents increasing intracellular cAMP level under a fluorescence microscope. After administration of 100 μM forskolin, an adenylate cyclase activator, the fluorescence intensity (FI) of these cells was significantly increased, suggesting that all Green Falcans could be used in visualizing cAMP dynamics in living cells. We also found that Green Falcan10 showed a greater response compared to the other Green Falcans despite having a similar dynamic range (Figure 3A-C), reflecting that the detection range of Green Falcan10 is suitable for monitoring cAMP dynamics in HeLa cells. After administration of 300 μM 3-isobutyl 1-methylxanthine (IBMX, phosphodiesterase inhibitor) or 2 mM 8-Br-cAMP (membrane-permeable cAMP analogue) to Green Falcan10-expressing HeLa cells, the FI of these cells was significantly increased (Figure 3D-F). Interestingly, kinetics and amplitude of FI change after administration of forskolin, IBMX and 8-Br-cAMP differed from each other,

reflecting the distinct kinetics of various mechanisms for achieving cAMP increase such as production, inhibition of degradation, and membrane permeation. Therefore, these results suggest that the developed indicators are adaptable for detecting a wide range of cAMP dynamics in various physiological phenomena by selecting an indicator with optimal EC_{50}.

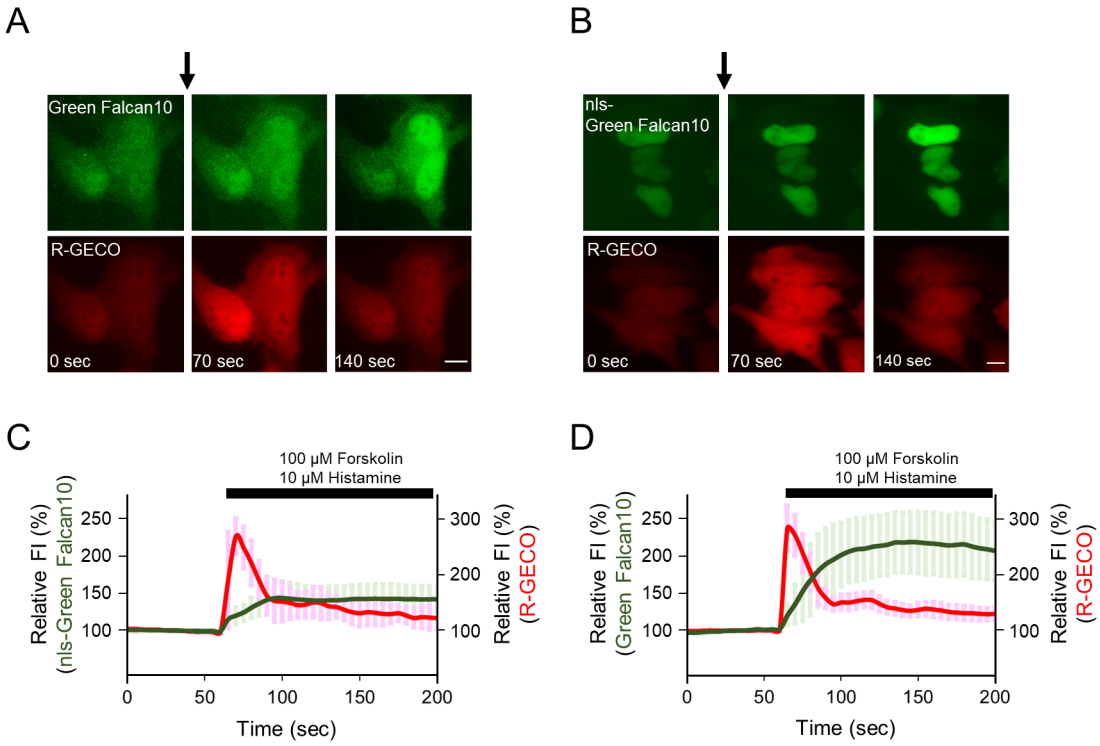

Figure 4. Dual-color imaging of Green Falcan and R-GECO. (A, B) Sequential pseudo-color images of Green Falcan10 and R-GECO (A), nls-Green Falcan10 and R-GECO (B) expressing HeLa cells, Scale bar represents 10 μm. (C-D) Time courses of fluorescence intensity (FI) in Green Falcan10 (C) / nls-Green Falcan10 (D) and R-GECO expressing HeLa cells in response to 100 μM forskolin and 10 μM histamine. Relative FI was calculated by dividing by the mean of the FI during 1 min prior to administration. The data represent the means ± standard deviation (Green Falcan10; n=14, nls-Green Falcan10; n=14 from three independent experiments)

Many single-fluorescent protein (FP)-based indicators have been developed for visualizing with other signaling molecules. To demonstrate the advantage of single-FP indicators, dual-color imaging, we expressed both Green Falcan10 and R-GECO[10], a red fluorescent Ca^{2+} indicator, in HeLa cells. After administration of 100 μM forskolin and 10 μM histamine which causes Ca^{2+} elevation by stimulation of the histamine H1 receptor, the fluorescence intensity (FI) of both Green Falcan10 and R-GECO increased. We next expressed nuclear localization signal (nls)-Green Falcan10 and R-GECO in HeLa cells to examine the applicability for visualization of dynamics of different molecules in distinct intracellular compartments. After administration of 100 μM forskolin and 10 μM histamine, the FI of both nls-Green Falcan10 and R-GECO increased. These results suggest that Green Falcans are applicable to dual-color imaging with other indicators, and to further hierarchical and cooperative analysis by multi-color imaging. Interestingly, the FI increase in the cytoplasm by administration of forskolin and histamine was a little smaller than in the nucleus and was also smaller than administration of forskolin alone. These results could be ascribed to the accelerated degradation of cAMP by phosphodiesterase[27] in the cytoplasm due to calcium elevation.

In this study, we developed green fluorescent cAMP indicators with EC_{50} by protein engineering, named Green Falcan0.3, 1, 3 and 10. These indicators were able to detect cAMP dynamics in the cytoplasm and nucleus of living cells after administration of several reagents and allowed dual-color imaging with R-GECO. Since these indicators are turn-on type and have a larger dynamic range than a previous one[16], we expect that the Green Falcans will become user-friendly tools around the world to unravel new physiological phenomena involved in cAMP signaling.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and supplementary figure.

Accession Code

Mouse cAMP-dependent protein kinase type I-beta regulatory subunit (mPKA RIβ, NP_001240819.1)

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Author Contributions

S.H., T.F. and S.A. performed the experiments and analyzed the data. T.K. and T.T. designed the concept and experiments. S.H., H.U., T.T. and T.K. discuss the results. S.H. and T.K. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

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ABBREVIATIONS

cAMP, cyclic adenosine 3', 5'-monophosphate; EC_{50}, the half maximal effective concentration; PKA, cAMP binds to protein kinase A; Epac, exchange proteins directly activated by cAMP; CNG ion channels, cyclic nucleotide-gated ion channels; FP, fluorescent protein; cGMP, cyclic guanosine 3', 5'-monophosphate; FI, fluorescence intensity; GFP, green fluorescent protein; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IBMX, 3-isobutyl 1-methylxanthine

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