Development of an optimized backbone of FRET biosensors for kinases and GTPases

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Abstract: Biosensors based on the principle of Förster (or fluorescence) resonance energy transfer (FRET) have shed new light on the spatiotemporal dynamics of signaling molecules. Among them, intramolecular FRET biosensors have been increasingly used due to their high sensitivity and user-friendliness. Time-consuming optimizations by trial and error, however, obstructed the development of intramolecular FRET biosensors. Here we report an optimized backbone for rapid development of highly sensitive intramolecular FRET biosensors. The key concept is to exclude the “orientation-dependent” FRET and to render the biosensors completely “distance-dependent” with a long, flexible linker. We optimized a pair of fluorescent proteins for distance-dependent biosensors, and then developed a long, flexible linker ranging from 116 to 244 amino acids in length, which reduced the basal FRET signal and thereby increased the gain of the FRET biosensors. Computational simulations provided insight into the mechanisms by which this optimized system was the rational strategy for intramolecular FRET biosensors. With this backbone system, we improved previously reported FRET biosensors of PKA, ERK, JNK, EGFR/Abl, Ras, and Rac1. Furthermore, this backbone enabled us to develop novel FRET biosensors for several kinases of RSK, S6K, Akt, and PKC and to perform quantitative evaluation of kinase inhibitors in living cells.

Introduction: Förster (or fluorescence) resonance energy transfer (FRET) is a process of nonradiative energy transfer between donor and acceptor fluorophores (Jares-Erijman and Jovin, 2003). This process depends on the proper spectral overlap of the donor emission and acceptor excitation, the distance between them, and the relative orientation of the fluorophore’s transition dipole moments (Miyawaki, 2003). With the advent of a myriad of fluorescent proteins (FPs), genetically encoded biosensors based on FRET (hereafter referred to as FRET biosensors) have been increasingly used to visualize the activities of cellular signaling molecules such as Ca^{2+}, phospholipids, small GTPases, protein kinases, and so forth (Miyawaki, 2003; Aoki et al., 2008). These FRET biosensors have contributed to our understanding of the spatiotemporal dynamics of signaling molecules in living cells, which could not be adequately investigated using the techniques of conventional biochemistry.

The genetically encoded FRET biosensors are classified into two types: intramolecular (or unimolecular) FRET biosensors and intermolecular (or bimolecular) FRET biosensors (Miyawaki, 2003). The former contain both donor and acceptor FPs within a single biosensor, whereas the latter consist of a pair of molecules conjugated with a donor FP and an acceptor FP, respectively. To date, intramolecular FRET biosensors have been widely used in cell biology due to the following advantages: high signal-to-noise ratio, easy loading of the biosensor into the cells, and simple ratiometric image analysis (Miyawaki, 2003; Aoki et al., 2008).
A serious disadvantage of the intramolecular FRET biosensors is that it is difficult to render them highly sensitive. Although several research groups have generated a number of FP variants that are optimized for FRET applications (Karasawa et al., 2004; Rizzo et al., 2004; Nguyen and Daugherty, 2005), the development of FP pairs for FRET is still one of the greatest challenges in FRET biosensor development (Li et al., 2006). Even if we could choose optimal FP pairs, there would remain the harder task of designing the composition of domains to be included in the biosensors. The FRET efficiency of intramolecular biosensors is influenced primarily by the distance and the relative orientation of the two fluorophores (Jares-Erijman and Jovin, 2003; Nagai et al., 2004). Because these two parameters are hard to predict, the developers are forced to spend large amounts of time in optimizing the biosensors by trial and error.

To circumvent these problems, we exploited an optimized backbone of intramolecular FRET biosensors. We first optimized the FPs and then developed a long, flexible linker that markedly increased the gain of FRET signals. This new backbone enabled us to improve the previously reported FRET biosensors of PKA, ERK, JNK, EGFR/Abl, Ras, and Rac1 and to develop new FRET biosensors for RSK, S6K, Akt, and PKC Ser/Thr kinases in a short period of time. Furthermore, by using cells stably expressing a FRET biosensor, we quantitatively and rapidly evaluated the effect of several kinase inhibitors on ERK activity.

RESULTS

Strategy for rational design of intramolecular FRET biosensors

To accelerate the development of the intramolecular FRET biosensors, we attempted to provide an optimized backbone structure. The prototype FRET biosensor used in this study comprises 1) a sensor domain and a ligand domain connected by a flexible linker, and 2) cyan and yellow FPs (CFP and YFP) serving as a donor and an acceptor, respectively (Figure 1A). The sensor domain changes its conformation upon perception of the signal. This sensitized sensor domain interacts with the ligand domain, thereby inducing a global change of the biosensor conformation and concomitant increase (or decrease in some cases) of the FRET efficiency from the donor to the acceptor.

The FRET efficiency of the intramolecular FRET biosensor is dependent primarily on the distance and the relative orientation of the donor and the acceptor (Miyawaki, 2003). Although the “orientation-dependent” FRET biosensor may be able to exhibit higher sensitivity than does the “distance-dependent” FRET biosensor (Jares-Erijman and Jovin, 2003), we could hardly predict and control the orientation of the donor and the acceptor for the development of an optimal FRET biosensor because we usually do not know the three-dimensional structures of the biosensor of which the sensor domain is bound (“ON”) and not bound (“OFF”) to the ligand domain. Thus the main job of our intramolecular FRET biosensors is to eliminate the orientation-dependent FRET and to render the biosensors completely distance-dependent with a long, flexible linker.

Before the evaluation of FRET biosensors described in this study, we will define the technical terms related to their performance. In the present FRET biosensors, the CFP and YFP variants are mostly used as the donor and the acceptor, respectively; therefore we will describe CFP and YFP as the default donor and the acceptor, respectively. FRET is detected by ratiometry (Jares-Erijman and Jovin, 2003): Cells are excited at a 440 nm wavelength, and the ratio of fluorescence intensity of the YFP channel (FRET) versus fluorescence intensity of the CFP channel (CFP), FRET/CFP, is used to represent the level of FRET ON state. Here, the “dynamic range” of the FRET biosensor is the theoretical range of FRET/CFP in the ON state biosensor and that in the OFF state (Figure 1C). In practical use, the change of activity or concentration of the molecule is monitored by the change of FRET/CFP after stimulation. This “gain” of the FRET signal is the relative increase or decrease in FRET/CFP after stimulation and is expressed as a percentage of the FRET/CFP value before stimulation. Therefore the gain of a FRET biosensor expressed in a certain cell type depends on both the dynamic range of the FRET biosensor and the increase in the fraction of the ON state after stimulation versus that before stimulation (Figure 1C). Meanwhile, “sensitivity” of FRET biosensors denotes a concentration of stimulants that increases the FRET/CFP value to 50% of the dynamic range (Figure 1C).

Optimization of FRET donor and acceptor pairs

We first optimized the donor and acceptor FPs in the distance-dependent intramolecular FRET biosensor. The prototype biosensor is based on the structure of a PKA activity sensor, AKAR3 (Allen and Zhang, 2006), which contained a consensus peptide of PKA phosphorylation (Zhang et al., 2001) and the phosphate binding domain of FHA1 (Figure 2A). We initially used a 72-amino-acid (a.a.) poly-lysine linker used in an ERK activity sensor, EKAR, as a flexible long linker (Harvey et al., 2008). We tested teal fluorescent protein (TFP)- and CFP-derived FPs, including enhanced CFP (ECFP), Turquoise-GL, and CyPet, as donor FPs, and we examined YFP-derived FPs, including Venus, circularly permuted Venus mutants, mCitrine, and YPet (see Materials and Methods and Supplemental Table S1), as acceptor FPs. The gain of the biosensors was quantified in HeLa
cells stimulated with dibutyryl-cyclic AMP (dbcAMP), a membrane-permeable cAMP analogue. Except for the biosensor containing cp50Venus as the acceptor, the FRET/CFP ratio was increased upon dbcAMP stimulation in all biosensors. Among them, the FRET biosensors containing ECFP/YPet and Turquoise-GL/YPet exhibited the largest gain in FRET/CFP (Figure 2B). A substantial amount of the FRET biosensor with YPet was cleaved at the linker region via a currently unknown mechanism (Supplemental Figure S1). Notably, YPet did not show any superiority to Venus when mTFP, an FP derived from coral (Ai et al., 2006), was used as the donor (Figure 2, A and C), suggesting that a pair of dimerization-prone FPs is suitable for a distance-dependent FRET biosensor (see Discussion). Taken together, these results led us to conclude that the ECFP/YPet or Turquoise-GL/YPet pair is suitable for the donor and acceptor pair of the distance-dependent intramolecular FRET biosensor.

Optimization of the length of flexible linkers

Previously we showed that the basal GTP/GDP ratio of the Ras FRET biosensor was markedly larger than that of the endogenous Ras protein, implicating that the close proximity of the sensor and ligand domains could increase the proportion of FRET biosensors in the basal state. To clarify which mechanism pertained, we separated the phosphorylated FRET biosensor from the nonphosphorylated one in HeLa cell lysates with Phos-tag immunoblotting analysis with an anti-GFP antibody and a fluorescence-tagged secondary antibody. Positions of phosphorylated (p) and nonphosphorylated biosensors (np) are indicated on the right of the representative gel image. Bottom, average values of the fraction of phosphorylated biosensors are shown with SD for three independent experiments. P value was calculated by a one-tailed paired t test.

The observed decrease of FRET/CFP before stimulation could be caused by two mechanisms. First, the long linker might reduce the linker owing to the decrease in FRET/CFP in the absence of dbcAMP (Figure 3C). The 116 aa linker showed a larger gain in FRET/CFP than the 72 aa polyglycine linkers (Levskaya et al., 2009; data not shown). Thus we prepared (SAGG), linkers (n = 13–61) and inserted them into the prototype PKA biosensor. As expected, FRET/CFP in the absence of stimulation correlated inversely with the length of the linker (Figure 3B and Supplemental Figure S2). The gain of the FRET biosensors upon dbcAMP stimulation correlated with the length of the linker owing to the decrease in FRET/CFP in the absence of dbcAMP (Figure 3C).

The observed decrease of FRET/CFP before stimulation could be caused by two mechanisms. First, the long linker might reduce the fraction of ON state FRET biosensors in the basal state. To clarify which mechanism pertained, we separated the phosphorylated FRET biosensor from the nonphosphorylated one in HeLa cell lysates with SDS polyacrylamide gels containing a phosphorylated amino acid and Coomassie blue R-250 as a counterstain. No significant difference in the basal FRET/CFP ratio of the biosensors was observed among the different linkers (Supplemental Figure S2). Second, the long linker might reduce the FRET/CFP ratio after dbcAMP stimulation correlated inversely with the length of the linker (Figure 3B and Supplemental Figure S2). The gain of the FRET biosensors upon dbcAMP stimulation correlated with the length of the linker owing to the decrease in FRET/CFP in the absence of dbcAMP (Figure 3C).
To understand the mode of action of the Eevee backbone, we mathematical validation of the Eevee backbone system. We used this linker in the following study. Because the increase in the gain reached a zenith at 116 of extraFRET (Eevee). We also named the long linkers "EV tem was designated as the extension for enhanced visualization by igning of a long, flexible linker and an optimized pair of FPs. This sys- comparison with the 52 a.a. linker. Because the reduction of the phosphorylated biosensor in the presence of dbcAMP was much lower, the gain of the biosensor containing the 116 a.a. linker was larger than that of the biosensors containing shorter linkers (Figure 3D). The levels of phosphorylated biosensors in the presence of the Ser/Thr phosphatase inhibitor, Calyculin A, were almost equal among the three biosensors. These results indicated that the preferable effect of the long linker was brought about by reducing the fraction of ON state FRET biosensors in the basal state (Figure 3D).

Through these analyses, we established the optimized backbone of the distance-dependent intramolecular FRET biosensors, consisting of a long, flexible linker and an optimized pair of FPs. This system was designated as the extension for enhanced visualization by evading extraFRET (Eevee). We also named the long linkers "EV linkers." Because the increase in the gain reached a zenith at 116 a.a. (Figure 3C), we used this linker in the following study.

Mathematical validation of the Eevee backbone system

To understand the mode of action of the Eevee backbone, we built and simulated a mathematical model of distance-dependent intramolecular FRET biosensors (see Supplemental Information). Computer simulations suggested that linker length and FP dimerization exerted distinct effects of FRET increase: sensitivity and dynamic range (Supplemental Figure S3, A–C). More importantly, these numerical simulations predicted the presence of an optimal length of linker to obtain the maximal gain of FRET, depending on the strength of FP dimerization (Supplemental Figure S3D). In line with this prediction, the dimerization-prone FP pair (i.e., YPet/ECFP) demonstrated a higher gain for longer length of linker in comparison to the Venus/ECFP pair (Supplemental Figure S3E). Convincingly, a gain of AKAR3 with Venus/ECFP was saturated at the linker length of 84 a.a., showing that the gain of the biosensor reached its highest point (Supplemental Figure S3E). Thus this simple simulation provided a plausible model to understand the mechanism by which the combination of EV linker and the dimerization-prone FPs increased the gain of distance-dependent intramolecular FRET biosensor.

**FRET biosensors of kinases containing the Eevee backbone**

To demonstrate proof of concept of the Eevee backbone, we applied the backbone to the previously reported FRET biosensors of protein kinases, an ERK sensor EKAR (Harvey et al., 2008) and a JNK sensor JNKAR1 (Fosbrink et al., 2010). The new FRET biosensors of PKA, ERK, and JNK were named AKAR3EV, EKAREV, and JNKAR1EV, respectively. HeLa cells expressing EKAREV biosensors were time lapse–imaged and stimulated (Figure 4). The gain of AKAR3EV was approximately sixfold and threefold larger than that of the original AKAR3 and AKAR4, respectively (Allen and Zhang, 2006; Depry et al., 2011; Figure 4A and Supplemental Video S1). Similarly, the gains of EKAREV and JNKAR1EV were larger than those of EKAR and JNKAR1 by four- and threefold, respectively (Figure 4, B and C, and Supplemental Videos S2 and S3). We also found that, in EKAREV, either the FHA1 or WW domain was equally used as the phosphopeptide binding (ligand) domain (data not shown).

The Eevee backbone was also applied to Picchu, an EGFR and Abl tyrosine kinase activity sensor (Kurokawa et al., 2001). In Picchu with an EV linker, the SH2–SH3 region and the substrate peptide (a.a. residues 217–225) of human CrkII were used as the ligand domain and sensor domain, respectively. The resulting PicchuEV exhibited a twofold increase in the gain in EGF-stimulated HeLa cells in comparison to the original Picchu (Figure 4D and Supplemental Video S4). Notably, the time course of FRET/CFP was similar between Picchu and PicchuEV (Figure 4D), suggesting that PicchuEV was also capable of monitoring the rapid kinetics of regulation in the protein kinases and phosphatases like the prototype Picchu.

**FRET biosensors of small GTPases based on the Eevee backbone**

We further applied the Eevee backbone to the intramolecular FRET biosensors of small GTPases. As examples, we adduced the FRET biosensors for Ras and Rac1, Raichu-Ras and Raichu-Rac1, respectively (Mochizuki et al., 2001; Itoh et al., 2002). The new FRET biosensors, RaichuEV-Ras and RaichuEV-Rac1, exhibited two- to threefold larger gains than the original FRET biosensors (Figure 5 and Supplemental Videos S5 and S6). Similarly to AKAR3 with the long linker (Figure 3C), the basal FRET/CFP ratio of Raichu-Ras with the EV linker was considerably reduced by the EV linker (Figure 5C). As expected, the basal FRET/CFP of RaichuEV-Rac1 was also markedly lower than that of the original Raichu-Rac1 (Figure 5F). We further confirmed that the EV linker reduced the ratio of GTP versus GDP bound to biosensors by thin-layer chromatography with the 32P-labeled, unstimulated HeLa cells (Supplemental Figure S4). Thus, in
Because the orientation-dependent FRET could be neglected in the FRET biosensors based on the Eevee backbone, RaichuEV, are shown at the top of panels (A) and (D). RatRBD and PAK CRIB denote the Ras-binding domain of Rafl and the Cdc42/Rac-interactive binding domain, respectively. Cos7 cells expressing RaichuEV or the prototype Raichu were stimulated with 50 ng/ml EGF and time lapse–imaged (Supplemental Videos S5 and S6). Representative FRET/CFP ratio images are shown in the intensity-modulated display mode. Scale bars are 10 μm. (B and E) The FRET/CFP ratio of each cell was normalized by dividing by the averaged FRET/CFP value before stimulation. The mean and SD from at least 10 cells are plotted against time. (C and F) Basal FRET/CFP ratios of RaichuEV and Raichu are plotted. Each dot corresponds to the value from a single cell, and at least six cells were analyzed. The horizontal bar indicates the mean.

**Figure 5:** FRET biosensors of small GTPases based on the Eevee backbone. Structures of FRET biosensors based on the Eevee backbone, RaichuEV, are shown at the top of panels (A) and (D). RatRBD and PAK CRIB denote the Ras-binding domain of Rafl and the Cdc42/Rac-interactive binding domain, respectively. Cos7 cells expressing RaichuEV or the prototype Raichu were stimulated with 50 ng/ml EGF and time lapse–imaged (Supplemental Videos S5 and S6). Representative FRET/CFP ratio images are shown in the intensity-modulated display mode. Scale bars are 10 μm. (B and E) The FRET/CFP ratio of each cell was normalized by dividing by the averaged FRET/CFP value before stimulation. The mean and SD from at least 10 cells are plotted against time. (C and F) Basal FRET/CFP ratios of RaichuEV and Raichu are plotted. Each dot corresponds to the value from a single cell, and at least six cells were analyzed. The horizontal bar indicates the mean.

**Quantitative evaluation of kinase inhibitors by Eevee biosensor-expressing cell lines**

With FRET biosensors having large gains in hand, we developed a quantitative and rapid assay to evaluate the effects of kinase inhibitors on Ser/Thr kinases in living cells. First, we established HeLa cell lines stably expressing Eevee biosensors. We previously failed to establish such stable cell lines with retrovirus/lentivirus-mediated gene transfer or by the transfection of linearized plasmid DNAs because of frequent recombination between YFP and CFP (data not shown). We recently found that this problem could be readily overcome by the use of a piggyBac transposase system (Yusa et al., 2013).
After single-cell cloning, the cells were seeded onto a 96-well glass base plate and treated with stimulants and kinase inhibitors (Figure 7A). For example, cells expressing EKAREV-nuc, which localized to nucleus, were stimulated with EGF in the presence of decreasing concentrations of various inhibitors and FRET-imaged by an automated epifluorescence microscope (Figure 7B and Supplemental Figure S8). By computer-assisted processing of the FRET/CFP ratio of each cell, we could obtain the IC50 values of kinase inhibitors (Figure 7C). Intriguingly, this single cell–based assay also revealed unexpected differences in terms of the mode of action of the inhibitors. EGF receptor inhibitors such as AG1478 and PD153035 inhibited ERK activity as a bistable (all-or-nothing) response, whereas the MEK inhibitor PD184352 inhibited ERK with a graded response (Figure 7, D and E, and Supplemental Figure S9). Taken together, these results demonstrated that the Eevee system combined with piggyBac transposase enabled rapid and quantitative evaluation of the effect of drugs in living cells.
**DISCUSSION**

We have developed an optimized backbone, Eevee, which allows us to quickly develop FRET biosensors. The flexible long linker and the optimized FP pairs cooperatively served to increase the gain of the FRET biosensors. The Eevee backbone was used to improve FRET biosensors of PKA, ERK, JNK, EGFR/Ab1, Ras, and Rac1 (Figures 4 and 5) and to develop FRET biosensors of RSK, S6K, Akt, and PKC (Figure 6).

The key technology of the Eevee backbone is the flexible long linker EV, which renders FRET biosensors mostly distance-dependent. It has been reported that circularly permuted (cp) FPs improved FRET biosensors of calcium and PKA (Nagai et al., 2004; Allen and Zhang, 2006), which would seem to provide evidence that orientation has a critical impact on the FRET efficiency of the intramolecular FRET biosensors. Consistent with this idea, the FRET efficiency of orientation-dependent biosensors has been reported to be drastically influenced by the addition or deletion of one to several amino acids at the C terminus of FPs (Miyawaki et al., 1997; Horikawa et al., 2010). The developer, however, could not predict whether the FRET of a newly constructed, orientation-dependent biosensor would increase or decrease upon the perception of the signal (Violin et al., 2003; Kunkel et al., 2005). For this reason, we decided to provide an optimized backbone for the distance-dependent type of FRET biosensor. The evidence that FRET of the Eevee backbone is mostly distance-dependent is as follows: 1) All of the FRET biosensors based on the Eevee backbone were associated with an increase in FRET/CFP in the ON state; 2) the basal fraction of OFF-state biosensors was inversely correlated with the length of the linker (Figure 3C); and 3) a series of cpVenus variants did not improve the gain (Figure 2B). Of note, there existed optimal linker lengths, depending on the pair of FPs, which were qualitatively explained by the mathematical model (Supplemental Figure S3). To quantitatively predict an optimal length of linker, we need to measure and/or estimate quantitative parameters and implement a model with those parameters.

An intramolecular FRET biosensor often suffers from a high basal FRET level, which could be caused by two mechanisms. First, the distance between the donor and acceptor FPs may not be sufficient to eliminate the basal FRET. Second, and more likely, the ligand domain may increase the fraction of ON-state biosensors by masking the sensor domain from negative regulators such as phosphatases and GAPs. Consistent with this idea, the basal GTP/GDP ratio on the Raichu-RhoA biosensor varies significantly according to the affinity of RhoA binding domains for RhoA (Yoshizaki et al., 2003). We presume that a flexible long linker minimizes this masking effect by reducing the effective concentration of the ligand domain around the sensor domain. There have been several reports of a linker peptide improving the FRET biosensor. For example, a “flip-flop” linker composed of a rigid α-helical linker with a flexible diglycine motif (Sato et al., 2003) and an "elastic" linker derived from spider silk protein flagelliform (Grashoff et al., 2010) have been shown to increase the gain of the FRET biosensor. It is unknown, however, whether these linkers also serve to reduce the masking effect of the ligand domain.

We showed that two FP pairs, ECFP/YPet and Turquoise-GL/YPet, were used preferably to the distance-dependent intramolecular FRET biosensors (Ouyang et al., 2008). The wild-type Aequorea FP forms a dimer with congeneric Aequorea FP at high concentration, of which the dissociation constant (K_d) is 110 μM (Zacharias et al., 2002). Recently Kotera et al. (2010) have verified that an Aequorea FP pair possessing reversible dimerization property enhances FRET of intramolecular FRET biosensors. Originally, YPet was reported to enhance FRET without inducing heterodimerization between YPet and CyPet in an intramolecular FRET biosensor (Nguyen and Daugherty, 2005). Recent reports suggested, however, that the increase in FRET gain by using YPet as an acceptor seems to be attributable to an enhanced dimerization with the congeneric FPs (Ohashi et al., 2007; Kotera et al., 2010). In agreement with this report, we have found that YPet did not improve FRET when a TFP, which was derived from Clavulania FP (Ai et al., 2006), was used as a donor (Figure 2C). It should be noted that, beyond a certain threshold, the increasing donor–acceptor FP binding affinity will yield a substantial fraction of FRET biosensors locked to the closed form even in the absence of stimulation.

To develop a novel FRET biosensor of Ser/Thr kinase with the Eevee backbone, we need to find a substrate peptide that is phosphorylated by the said Ser/Thr kinase with high efficiency and specificity. We will adduce S6K as an example. Because the two major substrates of S6K are S6 and Rictor (Ferrari et al., 1991; Dibble et al., 2009), naturally we chose a substrate peptide encompassing Ser-240 of S6 and Thr-1135 of Rictor. Interestingly, the FRET biosensor containing the substrate peptide derived from Rictor exhibited a significant response to EGF and rapamycin (Figure 6), whereas the other FRET biosensor containing a substrate peptide derived from S6 did not respond to EGF due to inefficient phosphorylation (data not shown). Notably, newly developed biosensors, Eevee-RSK, Eevee-S6K, Eevee-Akt, and Eevee-PKC, did not achieve gains as large as did AKAR3EV, EKAREV, and JNKAR1EV. This result is possibly due to the lower phosphorylation efficiency. Hence, screening of an optimal substrate peptide that is efficiently phosphorylated by the kinase of interest remains an important step in the development of FRET biosensors of a kinase. This problem of low phosphorylation efficiency may be overcome by two options. First, by the addition of a subcellular localization domain, the Eevee biosensor could be concentrated at the specific intracellular compartment where the said kinase is activated. Successful examples are Eevee-Akt and Eevee-PKC (Figure 6). Second, we may use a motif that facilitates the association of the substrate with the target kinase as exemplified in EKAREV and JNKAR1EV (Figure 4; Harvey et al., 2008; Fosbrink et al., 2010).

In summary, we described an optimized backbone for intramolecular FRET biosensors. The Eevee biosensor still needs a number of steps and careful characterization to create a sensitive and specific FRET biosensor, such as a choice of efficiently phosphorylated peptide and intracellular targeting. This simple and versatile system should prove useful for developing FRET biosensors with high productivity and for accelerating our understanding of spatiotemporal cellular signaling.

**MATERIALS AND METHODS**

**FRET biosensor construction**

The cDNAs of YFPs and CFPs were amplified by PCR. Fluorescence proteins used as acceptor or donor were Venus (Nagai et al., 2002), cpVenus (cp50Venus, cp157Venus, cp173Venus, cp195Venus, cp229Venus; Nagai et al., 2004), mCitrine (Griesbeck et al., 2001), mTFP1, YPet, CyPet (Nguyen and Daugherty, 2005), mTFL1 (Ai et al., 2006), mTurquoise, mTurquoise-GL (Goedhart et al., 2010), ECFP, and SECFP (a brighter version of ECFP developed by A. Miyawaki [RIKEN, Saitama, Japan]). The cDNA of SECFP contains additional mutations of K26R, D130G, N165H, and S176G based on the cDNA of ECFP. mTurquoise and mTurquoise-GL were the gifts of T. W. J. Gadella, Jr. (Swammerdam Institute for Life Sciences, Amsterdam,
The mutation of Ala at the 206 position in mTurquoise and mTurquoise-GL was restored to Lys with a two-step overlap PCR, generating Turquoise and Turquoise-GL (Goedhart et al., 2010). Amino acid substitutions in YFP and CFP variants used in this study are summarized in Supplemental Table S1. The cDNAs of fluorophores were inserted into the eukaryotic expression vectors pCAGGS (Niwa et al., 1991) and/or pPBbrs, which harbors basicidcin S-resistant gene (Yusa et al., 2009). The unique restriction enzyme sites are shown in Figure 1B. EKAR was obtained from Addgene (Cambridge, MA; http://www.addgene.org/), AKAR3, AKAR4, and JNKAR1 were gifts from J. Zhang (Johns Hopkins University, Baltimore, MD).

The flexible linkers collectively designated EV consisted of various numbers of the 20 a.a. peptide, SAGGSAGGSAGGSAGGSAGG (Levkaya et al., 2009). The cDNAs of the EV were introduced into an expression plasmid by a combination of standard subcloning and annealed DNA duplex ligation. The DNA of the 20 a.a. EV linker was generated by annealing the sense-oligonucleotide (5’-GTACACGTGCAGATGCACTCACTACCCACTACACACACACACACACACACACCACTTGGTGGTGGTT-3’) and antisense-oligonucleotide (5’-GCAGTGCTGGTGGTAGTGCTGGTGGTA-3’) and represented by the intensity-modulated display mode. In the background subtraction, FRET/CFP ratio images were created by using an Odyssey Infrared Imaging System (LI-COR).

FRET images were obtained and processed using essentially the same conditions and procedures as previously reported (Aoki and Matsuda, 2009). Briefly, HeLa cells or Cos7 cells expressing FRET biosensors were starved for 6–12 h with phenol red–free DMEM/F12 medium or Medium 199 (Invitrogen) containing 0.1% bovine serum albumin (BSA) or phenol red–free M199 (Invitrogen) with 0.1% FBS. The cells were plated on 35-mm glass base dishes or 96-well glass base plates (Asahi Techno Glass, Tokyo, Japan), which were coated with collagen type I (Nitta Gelatin, Osaka, Japan). Plasmids encoding FRET biosensors were transfected into HeLa cells and Cos7 cells by 293fectin or Lipofectamine 2000, according to the manufacturer’s instructions (Invitrogen, San Diego, CA), respectively. EGF was purchased from Sigma-Aldrich. dabcAMP, TPA, Calphostin C, and Calpeptin were purchased from Calbiochem (La Jolla, CA). PLX-4720 was obtained from Toronto Research Chemicals (Ontario, Canada). BI-D1870 was purchased from Sano (Shanghai, China). Rapamycin was obtained from LC Laboratories (Woburn, MA). PHO-24 was purchased from Selleck Chemicals (Houston, TX). The expression vector of piggyBac transposase was provided by A. Bradley (Wellcome Trust Sanger Institute, Cambridge, UK; Yusa et al., 2009). Phos-tag was obtained from the Phos-tag Consortium (Hiroshima, Japan; www.phos-tag.com). Anti-green fluorescence protein (GFP) sera were prepared in our laboratory. LI-COR (Lincoln, NE) blocking buffer and the IRDye680- and IRDye800-conjugated anti–rabbit and anti–mouse immunoglobulin G secondary antibodies were obtained from LI-COR.

Phospho-affinity PAGE

Phospho-affinity gel electrophoresis was performed essentially as described previously (Kinoshita et al., 2006). Conventional SDS-polyacrylamide separation gels were supplemented with 50 μM Phos-tag and 100 μM MnCl2, according to the manufacturer’s protocol. Proteins were detected and quantified by using an Odyssey Infrared Imaging System (LI-COR).

Time-lapse FRET imaging

FRET images were obtained and processed using essentially the same conditions and procedures as previously reported (Aoki and Matsuda, 2009). Briefly, HeLa cells or Cos7 cells expressing FRET biosensors were starved for 6–12 h with phenol red–free DMEM/F12 medium or Medium 199 (Invitrogen) containing 0.1% bovine serum albumin (BSA) or phenol red–free M199 (Invitrogen) with 20 mM HEPES and 0.1% BSA. Starved cells were treated with stimulants, followed by the addition of inhibitors if necessary. Cells were imaged with an inverted microscope (IX71 or IX81; Olympus, Tokyo, Japan) equipped with a 60× objective lens (Olympus), a cooled CCD camera (CoolSNAP HQ or CoolSNAP K4; Roper Scientific, Tucson, AZ), an LED illumination system (CoolLED, Exi680-; Molecular Devices, Sunnyvale, CA), an IXZ-ZDC laser-based autofocus system (Olympus), and an MD-XY10010T-Meta automatically programmable XY stage (SIGMA KOKI, Tokyo, Japan). The following filters used for the dual-emission imaging studies were obtained from Omega Optical (Brattleboro, VT): an XF2034 (455DRLP) dichroic mirror, and two emission filters used for the dual-emission imaging studies were obtained from Addgene (Cambridge, MA; http://www.addgene.org/).
intensity-modulated display mode, eight colors from red to blue are used to represent the FRET/CFP ratio, with the intensity of each color indicating the mean intensity of FRET and CFP. For the quantification, the FRET and CFP intensities were averaged over the whole cell area, and the results were exported to Excel software (Microsoft Corporation, Redmond, WA). In some experiments, the FRET/CFP value from before 10 min to the time of stimulation was averaged and used as the reference. The ratio of raw FRET/CFP value versus the reference value was defined as the normalized FRET/CFP value.

**Multiwell FRET imaging**
A HeLa cell line stably expressing EKAREV-nls, which contained the nuclear localization signal, was established according to Yusa et al. (2009). The cells were seeded on a 96-well glass base plate at a cell density of 1.5 × 10^4 cells/well. One day after seeding, the cells were serum starved for 6 h, followed by treatment with stimulant and kinase inhibitors for 15 min. Then, the 96-well plate was imaged by an inverted microscope as described earlier in text, except that an 20x objective lens was used. FRET and CFP images were obtained in one position for every well of the 96-well plate.

**Spectroscopy by confocal microscopy**
Twenty-four hours after transfection, HeLa cells expressing FRET probes were starved for 3–6 h. Fluorescence spectra were acquired by using a FV-1000 confocal imaging system (Olympus) in the lambda scanning mode upon excitation of CFP at a wavelength of 405 nm.

**Quantification of guanine nucleotide bound to GTPases**
Guanine nucleotides bound to Raichu biosensors were analyzed essentially as described previously (Gotoh et al., 1997). Briefly, HeLa cells were transfected with expression vectors for Raichu-Ras, RaichuEV-Ras, Raichu-Rac1, and RaichuEV-Rac1. After 36 h, the cells were metabolically labeled with [32P] orthophosphate for 2 h and then lysed. The cell lysates were clarified by centrifugation, and Raichu biosensors were immunoprecipitated by using anti-GFP antiserum. Guanine nucleotides bound to Raichu biosensors were separated by thin-layer chromatography and quantitated with a BAS-1000 image analyzer (Fujifilm, Tokyo, Japan).

**Mathematical and statistical analysis**
Simulation was implemented by Mathematica software (Wolfram Research, Champaign, IL). Details are described in the Supplemental Information.

Phosphorylation levels (Figure 3) or GTP/GTP+GDP levels (Supplemental Figure S4) at the basal state varied from day to day; the reason for this variation is not clear. Thus we could not combine all data obtained in 3–4 d and we had to handle the data obtained in one experiment as one data set. In this case, a paired t test needed to be applied to examine a statistical significance. The paired t test demonstrated whether a long linker decreased the basal phosphorylation and GTP/GTP+GDP levels. In addition, a long linker was involved in a decrease of basal FRET level, suggesting that the long linker decreased basal phosphorylation level (Figure 3) and basal GTP/GTP+GDP level (Supplemental Figure S4). Therefore a one-tailed t test was applied to the analysis.

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**REFERENCES**
Dibble CC, Asara JM, Manning BD (2009). Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by mS6K.