Reversible Dimerization of *Aequorea victoria* Fluorescent Proteins Increases the Dynamic Range of FRET-Based Indicators

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Genetically encoded Förster resonance energy transfer (FRET)-based indicators have become useful tools for biological research. FRET-based detection is one of the few techniques that enables the microscopic and noninvasive visualization of physiological events such as second messenger dynamics and enzyme activity in real time and space. Since the pioneering work of Tsien's group on the Ca²⁺ indicator cameleon (1), several FRET-based indicators have been developed, and their use has contributed to our understanding of various biological questions. These indicators are generally composed of a sensor domain that is sandwiched between donor and acceptor fluorescent proteins (FPs), such as calmodulin-M13 fusion domain, which is used for Ca²⁺ sensing in cameleon. Conformational changes of the sensor domain alter both the distance and orientation between the donor and acceptor FPs, which are in turn translated into a change in FRET efficiency that is typically detected by a change in the emission intensity ratio. In practice, however, most FRET-based indicators show only a small difference in the emission ratio upon their conformational change. Therefore, improving the dynamic range of the signal change is important for using these indicators to detect biological events with precision and sensitivity.

FRET efficiency is known to decay as a function of the inverse sixth power of the distance between the donor and acceptor, which is generally within 10 nm (2). In addition, the relative orientation of the chromophore’s transition dipole moment affects the FRET efficiency; thus, careful design and construction of the indicator, especially of the FPs, in which the chromophore is fixed in a β-can shell (3), are required. To im-

**ABSTRACT** Fluorescent protein (FP)-based Förster resonance energy transfer (FRET) technology is useful for development of functional indicators to visualize second messenger molecules and activation of signaling components in living cells. However, the design and construction of the functional indicators require careful optimization of their structure at the atomic level. Therefore, routine procedures for constructing FRET-based indicators currently include the adjustment of the linker length between the FPs and the sensor domain and relative dipole orientation of the FP chromophore. Here we report that, in addition to these techniques, optimization of the dimerization interface of *Aequorea* FPs is essential to achieve the highest possible dynamic range of signal change by FRET-based indicators. We performed spectroscopic analyses of various indicators (cameleon, TN-XL, and ATeam) and their variants. We chose variants containing mutant FPs with different dimerization properties, *i.e.*, no, weak, or enhanced dimerization of the donor or acceptor FP. Our findings revealed that the FPs that dimerized weakly yielded high-performance FRET-based indicators with the greatest dynamic range.

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prove the dynamic range of the indicator, adjustment of the linkers between the domains (4) and optimization of the dipole orientation by using circularly permuted FPs (5) are routine. In addition to these techniques, a pair of cyan- and yellow-FP variants, CyPet and YPet (6), respectively, was developed as an optimized FRET pair through a directed-evolution process. A protease indicator containing this FP pair showed a 20-fold change in the emission ratio upon cleavage of the linker peptide between CyPet and YPet, whereas only a 4-fold change was observed when conventional enhanced cyan-FP (ECFP) and enhanced yellow-FP (EYFP) were used. This increase in the dynamic range observed for CyPet and YPet was attributed to the enhanced dimerization of these FPs by substituting serine at 208 and valine at 224 for phenylalanine (S208F) and leucine (V224L), respectively (7), that are present at the dimer interface (8, 9) (Figure 1, panel a). Moreover, it was reported that utilizing ECFP/YPet pair in some FRET-based indicators also increases the dynamic range of the signal change (10). On the other hand, monomeric FPs, which are made by substituting alanine at 206 for lysine (A206K) (11), are also widely accepted to be used as the FRET donor and acceptor (12), because the formation of Aequorea FP dimers at high concentration (dissociation constant \((K_d) = 110 \, \mu \text{M}) (11)\) can induce artifactual FRET due to oligomerization of the FPs in the indicators. One report suggests that A206K mutation leads to a better dynamic range (13), while another report suggests the mutation almost abolishes the FRET signal (14). Thus, it is currently unclear how desirable FP dimerization is for the construction of FRET-based indicators.

To date, there have been no reports systematically examining the effect of the FP dimerization on the performance of FRET-based indicators in terms of the dynamic range of the signal change and the FRET efficiency. Here, we systematically compared the relationship between the dimerization property of the Aequorea FPs and the performance of FRET-based indicators. Our results using FP variants with no, weak, or enhanced dimerization revealed that FPs with a weak dimerization property yielded efficient indicators with the greatest dynamic range of signal change.

RESULTS AND DISCUSSION

Comparison of the Dynamic Range Using YC3.60 Dimer Mutants in Vitro. To investigate the effect of the FP dimerization ability on the properties of FRET-based indicators, we first analyzed the effect of the A206K mutation in the Aequorea FP of the Ca\(^{2+}\)/H11001 indicator, yellow cameleon variant YC3.60 (5). We mutated the original YC3.60 (Figure 1, panel b) with a native dimerization interface (YC3.60-nD/nA) to place the monomer-associated A206K on the donor FP (YC3.60-mD/nA), the acceptor FP (YC3.60-nD/mA), or both the donor and acceptor (YC3.60-mD/mA) FPs. In the absence of Ca\(^{2+}\), no obvious spectroscopic difference was observed among the recombinant YC3.60 variants (dotted curve, Figure 2, panels a–d). When Ca\(^{2+}\) was added, however, the YFP/CFP ratio was decreased among the A206K mutants (solid curves, Figure 2, panel b–d). The greatest reduction in the YFP/CFP ratio was observed using the recombinant YC3.60 variants (dotted curve, Figure 2, panels a–d). When Ca\(^{2+}\) was added, however, the YFP/CFP ratio was decreased among the A206K mutants (solid curves, Figure 2, panel b–d). The dynamic range of the emission intensity change in the indicator was calculated for each variant (Table 1). In accordance with previous findings (5), the dynamic range of YC3.60-nD/mA reached 557%, whereas the ranges observed with the YC3.60-nD/mA and YC3.60-mD/mA

![Figure 1](image-url)
mD/nA mutants were less than half that level. The YC3.60-mD/mA mutant further reduced the dynamic range of the indicator to 159%. These results suggest that introducing the monomer-associated mutation into

![Fluorescence spectra of cameleon YC3.60 variants.](Image)

**Figure 2.** Fluorescence spectra of cameleon YC3.60 variants. a) The original cameleon YC3.60-nD/nA. Variants with monomer-associated mutants b) YC3.60-mD/nA, c) YC3.60-nD/mA, and d) YC3.60-mD/mA and e) with the dimer-enhanced mutant YC3.60-dD/dA in Ca²⁺-saturated (solid curves) and Ca²⁺-free (dotted curves) buffer. The spectra are normalized to the CFP peak in Ca²⁺-saturated buffer. F.I. = fluorescence intensity.

**TABLE 1.** Dynamic range (DR) and YFP/CFP ratio of the FRET signal change in FRET-based indicators

<table>
<thead>
<tr>
<th></th>
<th>nD/nA</th>
<th>mD/nA</th>
<th>nD/mA</th>
<th>mD/mA</th>
<th>dD/dA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YF/CFP ratio</td>
<td>YF/CFP ratio</td>
<td>YF/CFP ratio</td>
<td>YF/CFP ratio</td>
<td>YF/CFP ratio</td>
</tr>
<tr>
<td>DR, %</td>
<td>Ca²⁺-saturated</td>
<td>Ca²⁺-free</td>
<td>Ca²⁺-saturated</td>
<td>Ca²⁺-free</td>
<td>Ca²⁺-saturated</td>
</tr>
<tr>
<td>YC3.60</td>
<td>554</td>
<td>8.6</td>
<td>1.3</td>
<td>208</td>
<td>3.8</td>
</tr>
<tr>
<td>TN-XL</td>
<td>460</td>
<td>3.8</td>
<td>0.7</td>
<td>278</td>
<td>2.6</td>
</tr>
<tr>
<td>ATeam</td>
<td>128</td>
<td>3.7</td>
<td>1.6</td>
<td>100</td>
<td>2.9</td>
</tr>
</tbody>
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the FPs reduces the FRET efficiency and also the dynamic range of the indicator.

In contrast, the dimerization-inducing S208F/V224L mutations have been reported to enhance both the FRET efficiency and the dynamic range of a protease indicator (7). Therefore, we next examined the effect of introducing the S208F/V224L dimer-enhancing mutations into the FPs of cameleon (YC3.60-dD/dA). In contrast to the results with the A206K mutants, the S208F/V224L mutants of YC3.60-dD/dA showed a YFP/CFP ratio similar to that of the original YC3.60-nD/nA when Ca²⁺ was added to the solution (solid curve, Figure 2, panel e). However, when Ca²⁺ was excluded from the indicator solution, little reduction in the YFP/CFP ratio was observed (dotted curve, Figure 2, panel e), indicating that some portion of the donor and acceptor were still in contact owing to the enhanced dimerization. As a result, the overall dynamic range of YC3.60-dD/dA was reduced more than 5 times, to 100% (Table 1).

These results suggested that dimerization of the FPs does not always improve the performance of FRET-based indicators. We speculated that the discrepancy with the previous results using a protease indicator (6, 7) were due to the difference in the mechanism of the indicators. Unlike YC3.60, the substrate domain of the protease indicator is irreversibly cleaved between the donor and acceptor, permanently separating them. Once the donor and acceptor are physically disconnected, the dimerization characteristics of the FPs minimally affect the FRET signal if the concentration of the cleaved FPs is lower than the dissociation constant. Thus, a strong tendency for the FPs to undergo dimerization is desirable in the case of protease indicators, because it enhances the FRET signal before the cleavage, while it minimally affects the cleaved FPs. In contrast, change in the FRET signal of YC3.60 is caused by a reversible reaction, i.e., the intramolecular distance between the donor and acceptor reversibly changes upon Ca²⁺ binding and release. In constructing such indicators, the design plan must maintain the delicate balance of the conformational equilibrium within the indicator. Either overdimerization or overmonomerization of the FPs would restrict the dynamic range of the indicators.

To investigate the effects of dimerization interface mutations on the Ca²⁺-sensing domains of YC3.60, we performed Ca²⁺ titration assays and analyzed the affinity of the YC3.60 variants for Ca²⁺ (Figure 3, Supplementary Table 1). The $K_d$ value of the original YC3.60
(YC3.60-nD/nA) was 249 nM, which is comparable to its previously reported value (5), whereas that of the YC3.60 variants with monomer-associated mutations was about 2-fold higher (approximately 450 nM). Among them, YC3.60-mD/mA showed the lowest affinity for Ca\(^{2+}\) (\(K_d = 465\) nM). In contrast, the \(K_d\) value of the YC3.60 with dimeric FPs (YC3.60-dD/dA) was 298 nM, which was similar to the value for the original YC3.60 (Figure 3, Supplementary Table 1). These results suggest that the monomer-associated mutation in the FPs causes the donor and acceptor to repel, which in turn affects the interaction between Ca\(^{2+}\)-calmodulin and the calmodulin-binding M13 peptide in the indicator, lowering the Ca\(^{2+}\) affinity of the sensor domain.

It was reported previously that GFPs dimerize in antiparallel configuration, at least in the crystal (9). In YC3.60, the Venus moiety has been circularly permuted so that Venus has been turned upside down relative to the ECFP moiety. We hypothesized that the high FRET efficiency of YC3.60 was caused by the circular permutation not because of the relative orientation of chromophore dipoles but because of the dimerization of the FPs in antiparallel configuration. To obtain deeper insight, we analyzed the effect of the mutations in indicators whose donor and acceptor cannot assume antiparallel configuration. As expected, the monomeric mutations had minimum effect on YC3.12 and YC3.30 (Supplementary Figure 1), both of which have ECFP and Venus moieties connected on the same side of the barrel with short linkers (5). Nevertheless, the dimeric mutations did increase the FRET efficiency of the indicators both in Ca\(^{2+}\)-free and Ca\(^{2+}\)-saturated condition. Considering the molecular structure of these indicators and the fact that the dynamic range is actually reduced, intermolecular dimerization may have caused such increase in FRET efficiency.

Comparison of the Dynamic Range of YC3.60 Containing FP Dimer Mutants in Live Cells. We next measured the indicator performance in live cells expressing the YC3.60 variants. HeLa cells were treated with 20 \(\mu\)M histamine, a condition that induces a large Ca\(^{2+}\) response in live mammalian cells, enabling the indicators to be adequately saturated with Ca\(^{2+}\) (Figure 4, panel a). By measuring the fluorescence intensity ratios of the donor and acceptor FPs, we could roughly estimate the dynamic range of the indicators in living cells. YC3.60-nD/mA displayed a reduced fluorescence intensity ratio at the peak (Figure 4, panel b). Furthermore, the baseline ratio of YC3.60-dD/dA was clearly higher than that of the other YC3.60 variants, although the peak ratio after histamine stimulation was comparable to that of YC3.60-nD/nA. These results in live cells were in good agreement with the results of our in vitro spectroscopic assays described above and further confirmed the importance of dimer interface optimization in FRET-based reversible indicators.

Effect of Dimer Interface Mutations in Another FRET-Based Indicator, TN-XL. To fortify our hypothesis, we analyzed the effects of dimerization interface mutations on the properties of another FRET-based Ca\(^{2+}\) indicator, TN-XL (15) (Figure 1, panel c), which uses the same...
Aequorea FPs as YC3.60 but differs in its Ca\textsuperscript{2+}/H\textsubscript{11001}-sensing domain, which is troponin instead of calmodulin. The TN-XL was affected by the interface mutations in a manner essentially identical to YC3.60: the YFP/CFP ratio was decreased when Ca\textsuperscript{2+}/H\textsubscript{11001} was added to TN-XL with the A206K mutation on the donor (TN-XL-mD/nA), acceptor (TN-XL-nD/mA), and both the donor and acceptor (TN-XL-mD/mA) FPs, and the YFP/CFP ratio was increased in the TN-XL with the S208F/V224L mutations (TN-XL-dD/dA) when Ca\textsuperscript{2+} was omitted (Figure 5, Table 1).

Improvement of the Performance of the ATP Indicator ATeam by Dimer Interface Optimization. To generalize our hypothesis, we next sought to improve the performance of a FRET-based indicator that already has a wide dynamic range, by optimizing the dimerization interface. ATeam, a recently developed ATP indicator (16) (Figure 1, panel d), has a molecular configuration similar to that of YC3.60, in which the e subunit of the bacterial F\textsubscript{1},F\textsubscript{1}-ATP synthase, used as an ATP-sensing domain, is sandwiched between Aequorea FPs. Upon binding ATP, ATeam undergoes conformational changes that generate a higher FRET signal by decreasing the distance between the donor and acceptor.

Like many other genetically encoded FRET-based indicators, the original ATeam has the monomer-associated A206K mutation in both its acceptor and donor FPs (i.e., ATeam-mD/mA). However, an ATeam containing the native K206A FP variant (ATeam-nD/nA) showed a YFP/CFP ratio higher than that of the original ATeam (ATeam-mD/mA) when ATP was added, whereas the YFP/CFP emission ratios of both ATeam-nD/nA and ATeam-mD/mA in the absence of ATP were almost identical. Dimeric ATeam (ATeam-dD/dA), as in the case of Ca\textsuperscript{2+} indicators mentioned above, showed greatly reduced dynamic range due to high baseline level in the ATP-unbound form (Figure 6, Table 1). Thus, we increased the YFP/CFP ratio by changing the lysine at 206 back to alanine, achieving a 40% gain in ATeam’s dynamic range.

We then compared the dynamic range of the ATeam variants in living HeLa cells. To deplete ATP, the cells were cultured in galactose medium and treated with oligomycin A, an inhibitor of FoF\textsubscript{1}-ATP synthase. Before oligomycin A addition, the ATeam-nD/nA-expressing cells showed an intensity ratio larger than that of cells expressing the original ATeam (ATeam-mD/mA). However, upon ATP depletion, the intensity ratio of both indicators decreased to a comparable value, indicating that ATeam-nD/nA had a higher dynamic range in living cells (Figure 7).

Conclusions and Outlook. Here we demonstrated that the monomer-inducing mutation in Aequorea FPs, which disrupts the dimer interface through introduced positive charges, reduces the FRET efficiency of reversible FRET-based indicators. We also found that the dimer-enhancing mutations S208F and V224L also reduced the dynamic range of the indicators by increasing the basal FRET efficiency, possibly due to the formation of intermolecular dimerization. These observations were in contrast to results obtained using a protease indicator, whose overall performance was improved by enhancing the dimerization property of the FPs (6, 7). The difference seems to depend on whether the signal-inducing reaction of the indicator is reversible or irreversible. For reversible FRET-based indicators, the balance between the enhancement of dimerization and the maintenance of free dissociation is critical. Taking advantage of these observations, we improved the dynamic range of a FRET-based ATP indicator.

Figure 6. Fluorescence spectra of ATeam variants. a) The improved ATeam-nD/nA. Variants with monomer-associated mutants b) ATeam-mD/nA, c) ATeam-nD/mA, and d) the original ATeam-mD/mA and e) with the dimer-enhanced mutant ATeam-dD/dA in 10 mM ATP (solid curves) and ATP-free (dotted curves) buffer. The spectra are normalized to the CFP peak measured in 10 mM ATP buffer. F.I. = fluorescence intensity.
by changing the lysine at 206 in the FPs back to alanine.

To construct high-performance FRET-based indicators that reversibly show a large change in the FRET signal, the FPs should have a moderate dimeric property, to enable conformational change in the indicator’s sensor domain and yet prevent undesired interactions between the FPs, for efficient FRET. Among the Aequorea FP variants we examined, those with alanine at 206 most closely matched these requirements, although a better interface might be obtainable, which should be examined in future studies. Our present recommendation is to examine possible combinations of mutations at the interface empirically and to screen for the highest dynamic range in each case. Until more data are available for predicting the results of such mutations, such screening for the optimal interface is essential.

One thing to note for the design of efficient FRET-based indicators is the relative orientation of the FPs. In order for the FPs to dimerize, they have to be bound in antiparallel configuration. Because wildtype GFP has both N- and C-terminals in close proximity, simple fusion of FPs with a short linker will not result in antiparallel dimerization of FP moieties. This fact might explain why circularly permuted FPs are so effective in increasing the FRET efficiency in many indicators: it turns the FP upside down for antiparallel configuration. Although we have shown some evidence to support such notion, the details need to be clarified in future studies.

Our hypothesis might also explain the fact that, despite theoretical competence and many experimental trials, no non-Aequorea FRET pairs with high FRET efficiency have been found to date (17–19), whereas the FRET efficiency of some Aequorea FRET pairs approaches unity (20). It would be interesting to examine whether interface engineering can be applied to improve the performance of FRET pairs that use non-Aequorea FPs with longer absorption and emission wavelengths, for which a larger Förster distance is expected by theory, thereby expanding the applicability of this technique.

**METHODS**

**Generation of Mutant Indicators.** Mutations were introduced by a modified version of QuikChange (Stratagene), to generate multiple mutations with a single cloning step (21). Intervector subclonings were performed using FASTR, an automatic cloning technology, as described previously (22). Briefly, the insert and vector fragments were amplified by PCR (KOD-plus, Toyobo) according to the manufacturer’s protocol. The PCR conditions were as follows: 94 °C/2 min, (94 °C/15 s, 60 °C/30 s, 68 °C/90 s) 35 cycles, 68 °C /5 min. One microliter of the PCR solution from each sample was transferred to a mixture containing the following reagents in a total volume of 20 μL: 17 mM Tris-acetate, 25 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 5 mM magnesium acetate, 33 mM potassium acetate, 5 mM ATP, 5 mM DTT, 63 μg/mL-1 BSA, 5 U of Lgul (Fermentas), 400 U of T4 DNA ligase (New England Biolabs), 20 U of DpnI (NEB), and 5 mM aphidicolin (Calbiochem). The mixture was incubated without agitation at RT for 1 h. After incubation, 5 μL of the mixture was used to transform E. coli (XL10Gold, Stratagene).

**Protein Expression, in Vitro Spectroscopy, and Ca²⁺ Titrations.** Recombinant YC3.60, TN-XL, and ATeam protein variants with N-terminal polyhistidine tags were expressed in Escherichia coli [JM109(DE3)] at RT and purified using a Ni-NTA column (Qia-gen) followed by buffer exchange to PBS(−) with an Amicon YM10 centrifugal filter (Millipore). Fluorescent spectra were measured with an F-2500 fluorescence spectrophotometer (Hitachi). Upon excitation with 430-nm light, the emission spectra of 0.3 μM recombinant cameleon were obtained using the
value of EGTA for Ca\(^{2+}\) fluorescence spectrophotometer (Hitachi). Ca\(^{2+}\) measurements was fitted to the Hill equation in a two-site model using GraphPad Prism (GraphPad Software).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES