/
Article / Book Information

Title	A Novel Protein-Protein Interaction Assay Based on the Functional Complementation of Mutant Firefly Luciferases: Split Structure Versus Divided Reaction
Authors	Yuki Ohmuro-Matsuyama, Hiroshi Ueda
Citation	Protein-Protein Interaction Assays, pp. 11-27
Pub. date	2018, 7

We are IntechOpen,
the world's leading publisher of
Open Access books
Built by scientists, for scientists

3,500
Open access books available

108,000
International authors and editors

1.7 M
Downloads

Our authors are among the

151
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

A Novel Protein-Protein Interaction Assay Based on the Functional Complementation of Mutant Firefly Luciferases: Split Structure Versus Divided Reaction

Yuki Ohmuro-Matsuyama and Hiroshi Ueda

Additional information is available at the end of the chapter

Abstract

Protein-fragment complementation assays (PCAs) are commonly used to assay protein–protein interaction (PPI). While PCAs based on firefly luciferase (Fluc) in cells or lysates are a user-friendly method giving a high signal/background (S/B) ratio, they are difficult to use in vitro owing to the instability of split Fluc fragments. As a solution to this issue, we developed a novel protein–protein interaction assay named FlimPIA using two mutant Flucs, each of which catalyzes one of the two half-reactions catalyzed by the wild-type enzyme. Upon approximation by the tethered protein pairs, the two mutants yielded higher signal owing to a more efficient transfer of the reaction intermediate luciferyl adenylate. FlimPIA showed many advantages over in vitro split Fluc assays, such as longer detectable distance, more stable probes, and higher signal readout in a shorter time period, and it also worked in cellulo.

Keywords: protein–protein interaction assay, firefly luciferase, protein-fragment complementation assay, FlimPIA, FRET

1. Introduction

When the human genome project was completed in 2003, most researchers expected dramatic developments in various fields such as biology, etiology, and drug discovery. However, progression did not remarkably accelerate. One of the causes is that protein-protein interactions (PPIs) are still not well understood. In the cell, many proteins interact with each other and cooperate to fulfill their roles in biological phenomena. It is reported that there are 150,000–300,000 PPIs in the human interactome [1, 2]. Therefore, PPI assays are very important for biology, diagnosis, and drug discovery.

The conventional PPI assays, which are available both in vitro and in cellulo, are Förster/fluorescence resonance energy transfer (FRET)-based assays, bioluminescent resonance energy transfer (BRET) assays, and protein-fragment complementation assays (PCAs).

For FRET-based assays, two fluorescent proteins or two fluorescent dyes are fused to proteins that interact with each other. When the interaction occurs, the two fluorescent proteins (dyes) are in close proximity, and then the energy transfer is induced, resulting in changes of the fluorescent intensities. In BRET assays, a bioluminescent enzyme and fluorescent protein (dye) are fused to proteins that interact with each other, and the energy is transferred from the bioluminescent enzyme to the fluorescent protein (dye). FRET- and BRET-based assays are the most common and sophisticated methods.

For PCA, the enzyme or fluorescence is divided into two fragments. The split fragments are fused to interacting proteins. Upon interaction, the split fragments come close, and then the full length of the structure is reconstituted, resulting in the recovery of the enzyme activity or fluorescence. PCA in cells and lysates is a user-friendly method that gives a high signal/background (S/B) ratio [3]. Moreover, we reported in vitro PCA using purified firefly luciferase (Fluc) fragments for the first time [4, 5]. The development of PCA is described in Section 2.

Recently, we developed a novel PPI assay, named firefly luminescent intermediate-based protein-protein interaction assay, FlimPIA [6–10]. FlimPIA utilizes the unique reaction of Fluc, which is divided into two half steps. We describe the principle of FlimPIA in Section 3.1 and the several improvements of FlimPIA in Sections 3.2–3.6. Then, the advantages and disadvantages of FlimPIA compared to another PPI assays such as the in vitro PCA are described in the final section.

2. Demonstration of in vitro protein-fragment complementation assay using purified Fluc fragments

Conventional PCA is used in vivo and in cultured cells (in cellulo). Although Porter et al. performed a Fluc-based PCA in vitro, the assay requires cell lysate, and the components in the lysate might affect the PPIs. We succeeded in developing a Fluc-based PCA in vitro using purified probes in a defined solution [4, 5].

For the Fluc-based PCA in vitro, a well-known interacting pair, FKBP12 (a 12 kD domain of FK506-binding protein) and FRB (FKBP-rapamycin-associated protein), was utilized. The association between these proteins depends on the presence of an antibiotic, rapamycin [11, 12]. Two pairs of split *Photinus pyralis* Fluc—the pair of the N-terminal domain (amino acids [aa] 1–437) and the C-terminal domain (aa 394–547) and the pair of the N-terminal domain (aa 1–398) and the same C-terminal domain (aa 394–547)—were selected in several split sites of Fluc [13], which worked well for in cellulo PCA. The gene encoding FKBP12 or FRB was fused to the 5′ end of each domain, and the genes were inserted into the pET32 vector, which originally encodes thioredoxin (Trx), yielding four fusion protein genes, FKBP-N, FKBP-C, FRB-N, and FRB-C. These proteins were expressed in the soluble fraction of *E. coli* BL21(DE3) pLysS and purified by immobilized metal affinity chromatography (**Figure 1A**).

The two interacting pairs, FKBP-N and FRB-C, FKBP-C and FRB-N, were mixed, and rapamycin was added to the pair (**Figure 1B**). The luminescence intensities of the mixture of the interacting pairs and rapamycin were remarkably increased immediately after adding the two
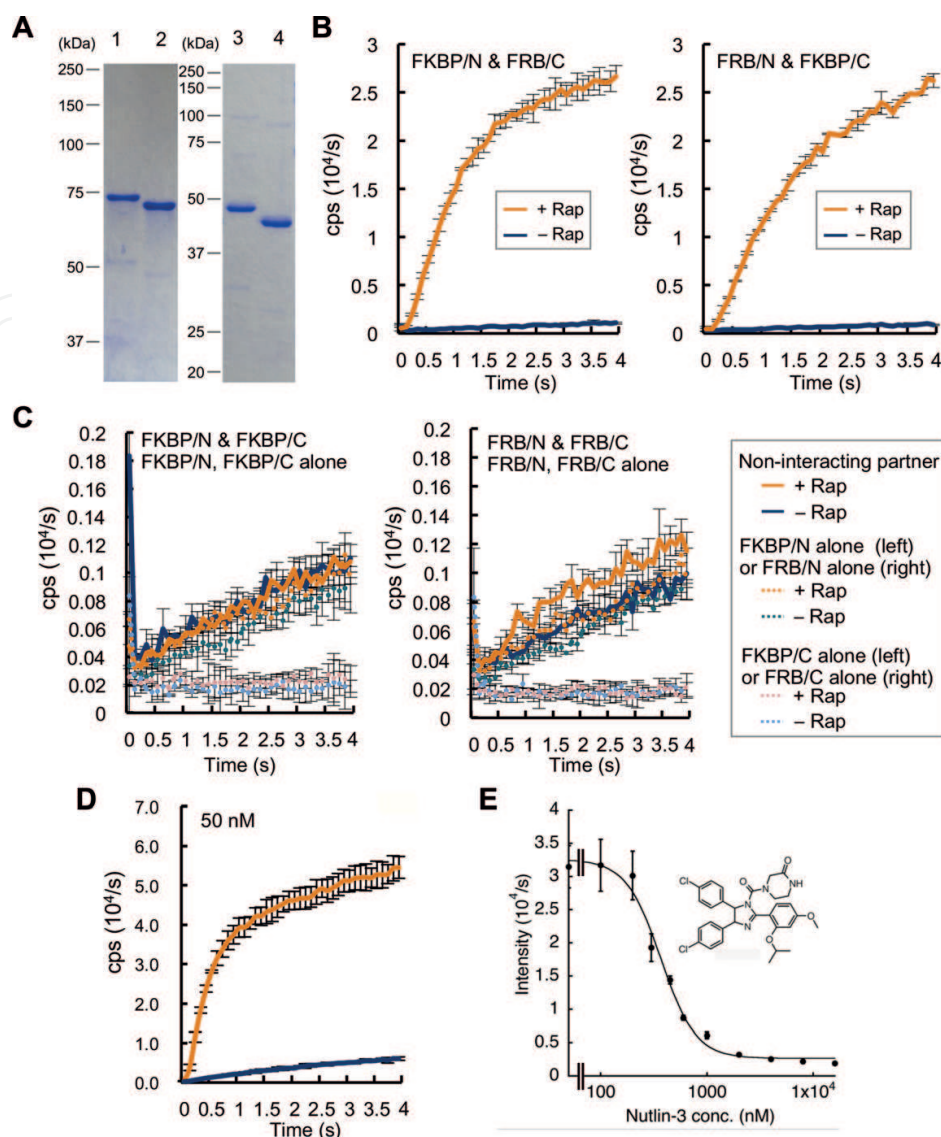
Figure 1. Detection via Fluc-based PCA using purified probes. (A–C) Detection of FKBP-FRB association. (A) Purified probes. Lane 1, FKBP-N; Lane 2, FRB-N; Lane 3, FRB-N; Lane 4, FRB-C. (B) PCA using the purified probes at 50 nM each, with/without equimolar rapamycin (n = 3). (C) Control experiments (n = 3). (D, E) Detection of p53-Mdm2 association. (D) PCA using the purified probes at 50 nM each (n = 3). (E) Inhibition of p53-Mdm2 interaction by Nutlin-3 (n = 3). ©American Chemical Society

substrates, luciferin and ATP. On the other hand, the luminescence intensities of the mixture of the interacting pair (FKBP12 and FRB) without rapamycin and noninteracting pair were very low (**Figure 1C**). The signal and stability of the pair of the N-terminal domain (aa 1–437) and the C-terminal domain (aa 384–547) were higher than those of the other pair of N-terminal domain (aa 1–398) and the same C-terminal domain. When the first pair was used, the luminescence signal displayed rapamycin dose dependence, and the limit of detection was determined as 250 pM. These results clearly showed that the PPI could be detected with a high S/B ratio and high sensitivity using the purified probes.

Because the rapamycin-dependent FKBP-FRB association is very strong, another interacting pair, p53 and Mdm2, was investigated (**Figure 1D** and **E**) [14, 15]. p53 suppresses cell growth as a tumor suppressor. The oncoprotein Mdm2 binds to p53 and downregulates the function

of p53 in certain cancer cells. In the assay of p53-Mdm2 interaction using p53-C and Mdm2-N, the signal intensity and S/B ratio rose with higher concentrations of the probes of the interacting pair. To investigate the reversibility of the PCA, an inhibitor of the p53-Mdm2 interaction, Nutlin-3, was added to the mixture of p53-C and Mdm2-N. The luminescence intensity decreased depending on the concentration of Nutlin-3.

The in vitro PCA opens the way to study PPIs of cytotoxic proteins, which is impossible to perform in cells. Furthermore, the possibility that the cellular components affect PPIs can be excluded.

3. Development of a novel PPI assay FlimPIA

In this section, we describe a novel PPI assay, FlimPIA, which we recently developed and continue to improve.
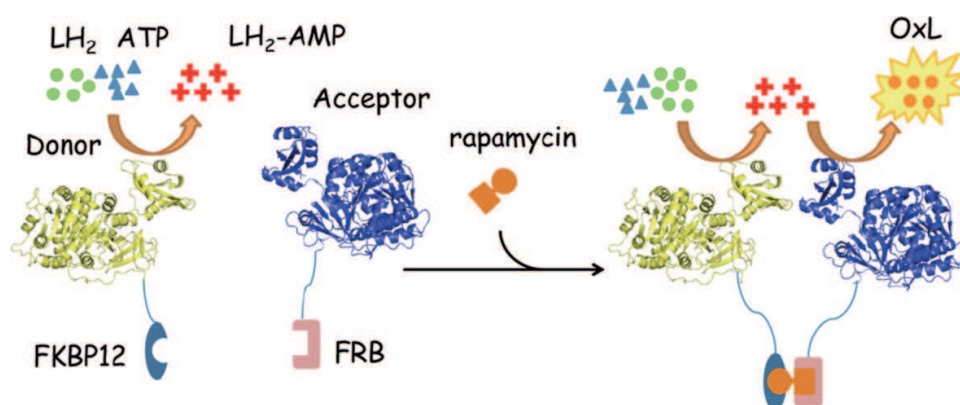
3.1. Principle of FlimPIA

In contrast to PCA, in which the structure of Fluc is divided into two domains as the probes, FlimPIA divides the reaction catalyzed by Fluc into two half-reactions. Fluc catalyzes the conversion of firefly D-luciferin (LH_2) to the excited state oxyluciferin (OxL) by a two-step catalysis, namely, an adenylation step and oxidative luminescence steps. In the adenylation step, LH_2 is converted to D-luciferyl adenylate (LH_2-AMP), and in the oxidative luminescence steps, LH_2-AMP is converted to OxL, and then excited OxL emits light. It was recently supposed that Fluc, which consists of a large N-terminal domain and a small C-terminal domain connected by a flexible hinge region, rotates its C-terminal domain by ~140° to proceed from the adenylation step to the oxidative luminescence steps (**Figure 2**) [16, 17]. One reason for this hypothesis is that the active site of each step in acyl-adenylate-forming enzymes, including Fluc, is different. In the adenylation step, K529 is an important amino acid residue, and on the other hand, K443 and H245 are key residues for the oxidative luminescence steps [18–20].

Two mutant *Photinus pyralis* Flucs were designed for FlimPIA; one is H245D/K443A/L530R, which can produce LH_2-AMP but cannot catalyze LH_2-AMP to form OxL, and the other is K529Q, which very slowly produces LH_2-AMP but maintains the catalytic steps in the oxidative luminescence half-reaction. Each mutant is fused to proteins that interact with each other. The interaction brings the mutants close together, and then LH_2-AMP, which H245D/K443A/L530R produces, is utilized by K529Q, resulting in OxL production (**Figure 3**). The mutant H245D/K443A/L530R acts as the "Donor" providing LH_2-AMP, and the mutant K529Q works as the "Acceptor" of LH_2-AMP [7].

When FKBP12 and FRB are fused to the Donor and Acceptor, respectively, the luminescence intensity increased depending on the concentration of rapamycin (**Figure 4A**, **B**). The EC_{50} values of the cognate pairs were 10.2 ± 0.6 and 16.0 ± 2.1 nM, respectively, which correspond well with the reported K_D value of the association between FKBP12/rapamycin and FRB. FK506 (tacrolimus) is commonly used as an immunosuppressant to prevent the rejection of organ transplants and inhibits the rapamycin-dependent association between FKBP12 and FRB [14]. The luminescence intensity decreased upon FK506 addition (**Figure 4C**). The S/B ratio increased depending on the concentration of PPI when the concentration of probes and
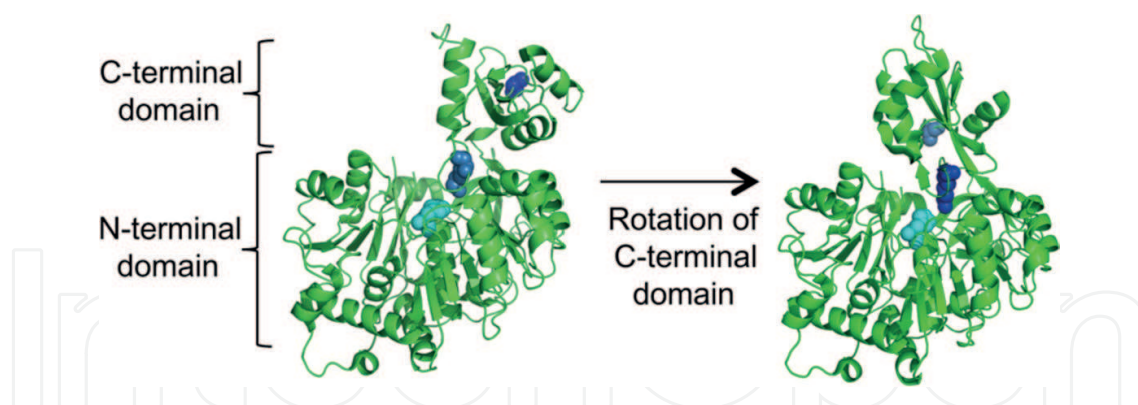
Figure 2. The conformational change of Fluc. Fluc is composed of a large N-terminal domain and a smaller C-terminal domain, which rotates ~140° according to the reactions to proceed from the adenylation reaction to the oxidative luminescent reactions: Key Lys residues (K529 and K443) are shown in light and dark blue, respectively. Another key residue H245 is shown in cyan. ©American Chemical Society.


Figure 3. The working principle of FlimPIA. ©American Chemical Society.

rapamycin was up to 500 nM (**Figure 4D**). In addition, the association between FKBP12 and FRB could be detected in 40% fetal bovine serum diluted in phosphate buffered saline, suggesting the applicability of the assay to clinical samples.

Next, p53 and Mdm2 were used as interacting proteins. The luminescence intensity of the mixture of the interacting pair (p53-Donor and Mdm2-Acceptor) was higher than the intensities of noninteracting pairs (p53-Donor and p53-Acceptor, Mdm2-Donor and Mdm2-Acceptor) (**Figure 4E**). The inhibition of the p53-Mdm2 interaction by Nutlin-3 was observed (**Figure 4F**). The result clearly shows that FlimPIA is a versatile system and can analyze transient interactions.

3.2. Improved FlimPIA by the entrapment of Fluc conformation

The original FlimPIA had exhibited high background signal, which was mainly caused by the remaining adenylation activity of the Acceptor. As mentioned above, the C-terminal domain rotates according to the reactions proceeding from the adenylation to the oxidative luminescence reactions (**Figure 2**). Therefore, we tried to entrap the Acceptor conformation into the oxidation conformation [10].
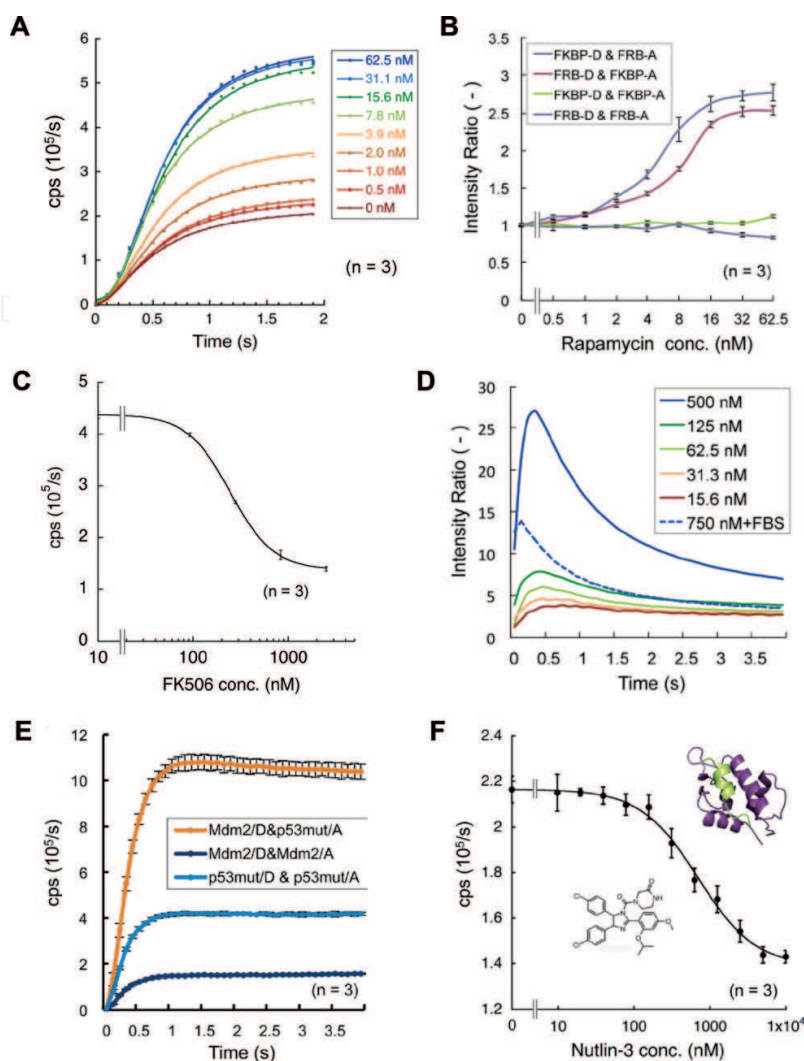
Figure 4. Detection via FlimPIA in vitro. (A-D) Detection of FKBP-FRB association. (A) Luminescence time course at several rapamycin concentrations. A mixture of FKBP/Donor and FRB/Acceptor (50 nM each) was used. (n = 3). (B) Specific detection of FKBP12-FRB interaction. The four possible combinations of four Fluc mutants, namely, FKBP/Donor, FRB/Donor, FKBP/Acceptor and FRB/Acceptor (50 nM each) were tested for their rapamycin dose-dependency. The relative luminescence integrated for 1.5–1.6 s after substrate addition is shown (n = 3). (C) Competition of PPI (protein–protein interaction) by FK506. Rapamycin (80 nM) and FK506 at indicated concentration were added to the mixture of FKBP/Donor and FRB/Acceptor (80 nM each). The luminescence integrated for 0.8–0.9 s after substrate addition is shown (n = 3). (D) Time course of S/B (signal/background) ratio obtained with the mixture of FKBP/Donor and FRB/Acceptor with and without equimolar rapamycin. The ratio of the two light intensities at the indicated time point is shown. Sample with 40% fetal bovine serum and 750 nM proteins is also shown (n = 3). (E–F) Detection of p53-Mdm2 association. (E) Luminescence time course of the cognate (Mdm2/Donor and p53/Acceptor) and control pairs (25 nM each) (n = 3). (F) Competition of PPI by a specific inhibitor. Nutlin-3 (bottom) at indicated concentration was added to the mixture of p53/Donor and Mdm2/Acceptor (25 nM each) (n = 3). The luminescence integrated for 0.8–0.9 s after substrate addition is shown. The ribbon model of Mdm2 (purple)-p53 peptide (light green) complex is also shown. ©American Chemical Society.

According to the report by Branchini et al. that the structure of Fluc could be fixed into the oxidative luminescence conformation by chemical trapping, we first took the same approach to entrap Acceptor mutant [21]. Specifically, all cysteine residues in the Acceptor were substituted with serine or alanine residues. Then, the residues at positions 108 and 447 were substituted with cysteine residues and cross-linked by 1,2-bis-(maleimide)ethane (BMOE) (**Figure 5A**).

The luminescence of the cross-linked Acceptor was almost diminished compared to the non-cross-linked Acceptor (**Figure 5B**). The Acceptor and Donor were fused to FRB and FKBP12, respectively. In a FlimPIA using the cross-linked Acceptor, the background signal was eliminated, and the signal induced by the interaction was significantly higher than the background signal (**Figure 5C**). Taken together, the results clearly showed that the Acceptor can be trapped into the oxidation conformation and the sensitive FlimPIA was successfully developed, giving a high S/B ratio.

As the substitution of the all cysteine residues considerably reduced the luminescence intensity, next, we tried to use the original Acceptor retaining the cysteine residues and put the cysteine residues at positions 108 and 447, which were then cross-linked by BMOE. As a result, one-fifth of the luminescence intensity of the cross-linked Acceptor was retained, probably due to miss- and/or incomplete cross-linking (**Figure 5D**). Although there was some background signal, an apparent improvement in luminescent intensity was observed. When the same concentration (50 nM each) of the probes and rapamycin were used, the maximum S/B ratio was improved from 2.6 to 5.3, compared with the original system (**Figure 5E**).
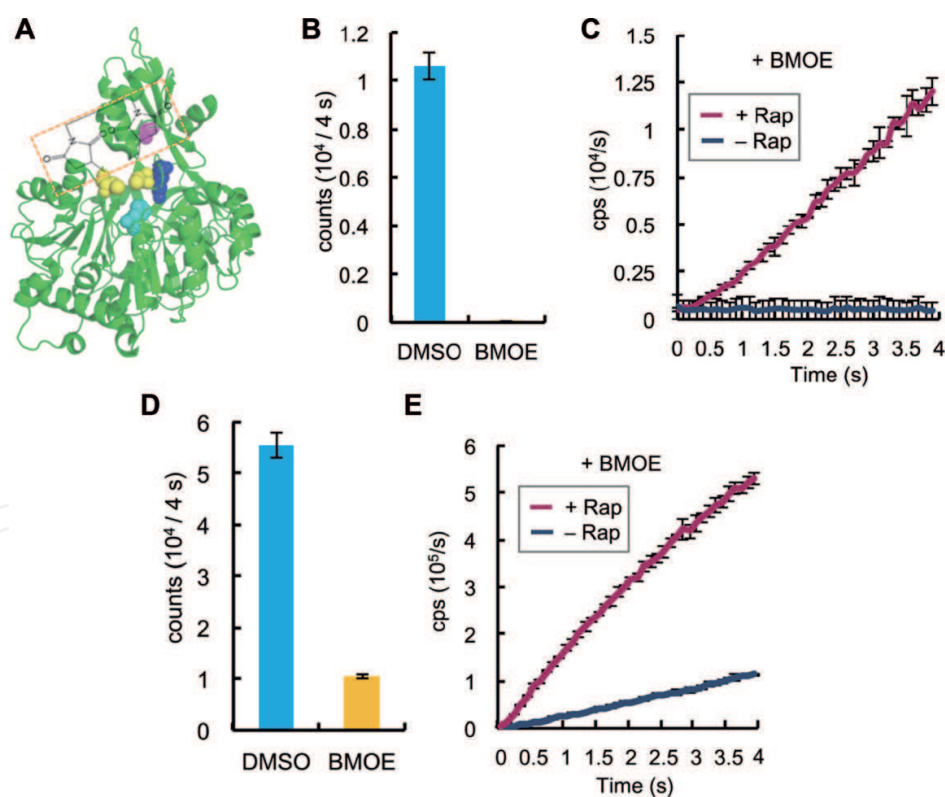
Figure 5. FlimPIA using the trapped Acceptor by bis-maleimide crosslinker (A–C) The trapping by bis-maleimide crosslinker (1). (D–E) The trapping by bis-maleimide crosslinker (2). (A) Scheme of the trapped Acceptors by BMOE. Residues shown in yellow were used for the N−C linkage. (B, D) Suppression of overall luminescent activity by chemical trapping of the Acceptor. The enzyme (10 nM) was reacted with 75 μM LH_2 and 10 mM ATP. The luminescent intensities with and without chemical modification by BMOE were compared (n = 3). (C, E) The mixture of FKBP/Donor and trapped FRB/Acceptor (50 nM each) was added with/without 50 nM rapamycin (n = 3). ©American Chemical Society.

### 3.3. Improved FlimPIA using a mutant acceptor (1)

During another attempt to select paired cysteine residues for possible cross-linking of N-C domains, the introduction of S198C/S440C mutations on the background of original Acceptor was attempted. However, the obtained clone was later found to be contaminated with the S440C mutant retaining only one mutation. The resultant S440C mutant showed higher ability as the Acceptor, whereas the S198C/S440C mutant did not act as the Acceptor. To understand the effect of this mutation, we performed saturation mutagenesis of the S440 residue. The substitution of leucine, phenylalanine, and tryptophan, which have bulky and/ or large side chains, gave a higher maximal S/B ratio in FlimPIA (**Table 1**) [9]. Additionally, not all the mutants with bulky or long side chains showed higher S/B ratios. Although the precise reason is not known, it might be because mutations often affect protein stability and/ or aggregation.

We expected that the bulky and/or large side chains at this position could form steric hindrance with hinge region and the C-terminal domain from the structural modeling based on the adenylation conformation structure of *Luciola cruciata* Fluc with bound substrate analog (**Figure 6A**). On the other hand, there seemed no severe inhibition in the model of the oxidative luminescence conformation.

Then we examined the adenylation and oxidative luminescence activities of the S440L Acceptor. The amounts of LH_2-AMP produced by the new and conventional Acceptors were examined according to the method using the N-terminal domain of Fluc as a selective detector of LH_2-AMP [18]. The LH_2-AMP produced by the new Acceptor was less than one-fifth of the LH_2-AMP produced by the conventional Acceptor (**Figure 6B**). On the other hand, the kinetics against LH_2-AMP are shown in **Table 2**. Because the concentration of the LH_2-AMP that the

S440X	S/B ratio	S440X	S/B ratio
L	7.93 ± 0.60	Q	2.11 ± 0.01
F	5.69 ± 0.12	R	2.08 ± 0.41
W	4.94 ± 0.06	S	1.87 ± 0.24
M	3.65 ± 0.35	N	1.86 ± 0.08
K	3.45 ± 0.20	V	1.85 ± 0.25
A	2.86 ± 0.22	D	1.80 ± 0.13
Y	2.81 ± 0.37	G	1.67 ± 0.26
H	2.57 ± 0.19	I	1.55 ± 0.21
C	2.32 ± 0.13	T	1.52 ± 0.22
E	2.31 ± 0.10	P	1.09 ± 0.11

Table 1. Comparison of maximum S/B ratios obtained by S440 mutants.
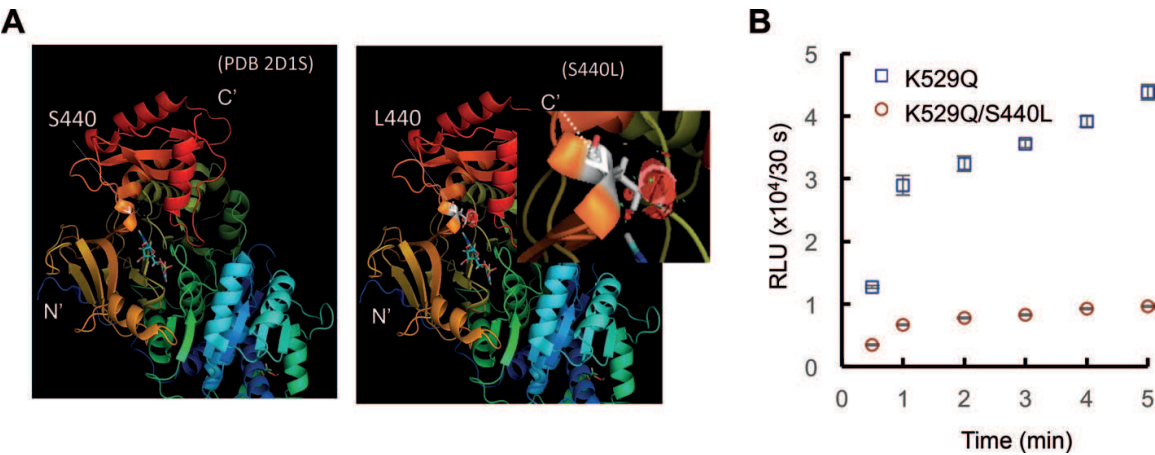
Figure 6. Possible steric hindrance of adenylation conformation with the S440 L mutation. (A) Structure of Fluc (left) and a model of Fluc S440L (right), each at adenylation conformation. The Leu440 residue (shown in white) is enlarged in the inset. Drawn with PyMOL software. (B) Adenylation activity measured by the N-terminal domain method. Error bars mean ±SD (n = 3).

	V_{max} (× 10⁶ RLU*/sec)	K_m (µM)	V_{max}/K_m (× 10⁶ RLU/s µM⁻¹)
K529Q	1.40 ± 0.16	0.513 ± 0.018	2.11 ± 0.01
K529Q/S440L	0.296 ± 0.031	0.321 ± 0.013	2.08 ± 0.41

*Relative light units

Table 2. Oxidative luminescence activity of K529Q and S440L/K529Q (1 nM each).

Acceptor uses in FlimPIA is low, the V_{max}/K_m is the most important kinetics parameter. The value of the new Acceptor decreased to 33.6% of the value of the conventional Acceptor; therefore, the luminescence intensity in FlimPIA might decrease to some extent. Taken together, the balance of the adenylation and oxidative activities of the new Acceptor gave the highest S/B ratio in the Acceptors, which we have developed.

3.4. Improved FlimPIA using mutated acceptor (2)

When the C-terminal domain of Fluc rotated to proceed from the adenylation step to the oxidative luminescence steps, the flexible hinge region between N- and C-terminal domains is considered highly important (**Figure 2**). Furthermore, the hinge region sits close to the active site in the adenylation conformation. To obtain suitable mutants for the Acceptor, semi-random mutations at the residues 436–439 in the hinge region were introduced [6]. The amino acid residues that enzymes in acyl-adenylate-forming enzyme superfamily contain at the corresponding positions were chosen in the semi-random library. The mutant R437K/L438I was selected from the library, because the mutants showed lower adenylation activity (~15% of the wild-type Fluc) and slightly higher oxidative luminescence activity (116% of the wild-type Fluc).

A single mutation, R437K, or a double mutation, R437K/L438I, was introduced into the conventional Acceptor (K529Q). The overall luminescence activity and the oxidative luminescence activity of the two new Acceptors were compared to that of the conventional Acceptor (**Figure 7A**, **B**). The overall activities of both new Acceptors decreased almost tenfold compared with that of the conventional Acceptor, whereas the oxidative luminescence activities were almost maintained. These results showed that R437K is a key residue for Acceptor activity.

The kinetics properties of the conventional and the new Acceptors fused to FRB are shown in **Table 3**. The lower overall activities and the similar oxidative luminescence activities are probably due to the remarkably lower V_{max} values for LH$_2$ and ATP and similar V_{max} and K_m values for LH$_2$-AMP. Moreover, in the structural model of the adenylation conformation, the mutated residue K437 sits close to the active site residues such as K529, suggesting some inhibition of the adenylation activity (**Figure 7C**).

When the FKBP12-FRB interaction was detected by FlimPIA, the maximum S/B ratio reached approximately 4, whereas it was approximately 2.5 in the conventional assay (**Figure 7D**). Taken together, we succeeded in finding a suitable mutant for the Acceptor in the semi-random library of the hinge region. Furthermore, these results suggest that the hinge region is important for controlling the two half-reactions of Fluc and supports the hypothesis that the C-terminal domain rotates to accomplish the half-reactions.
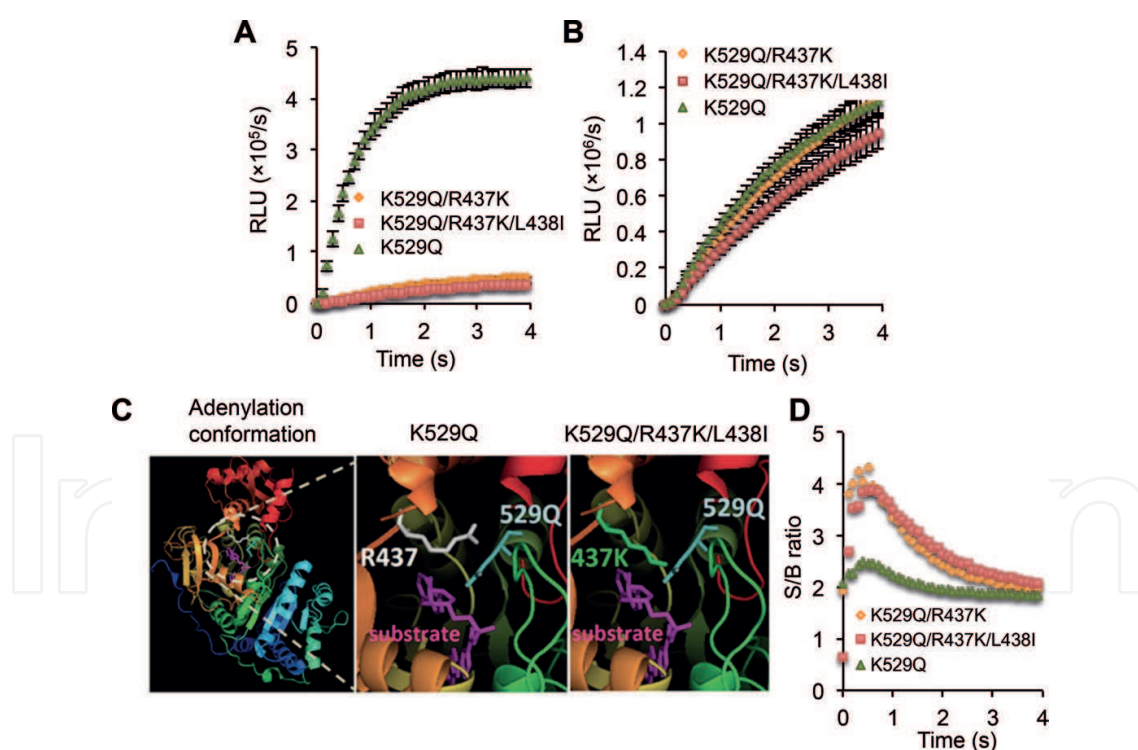
Figure 7. FlimPIA in vitro using the new Acceptor mutated in the hinge region. (A) Overall luminescent activity of the conventional Acceptor and the two new Acceptors. Reactions with LH$_2$ and ATP (n = 3). (B) Luminescent activity of the Acceptors with LH$_2$-AMP as a substrate (n = 3). (C) 3D models of the Acceptors at adenylation conformation. The wild-type Fluc (left), the conventional Acceptor (middle), and the mutant M1 (right) are shown. In the conventional Acceptor, the shortest distance between the active site against LH$_2$ (529Q) and R437 was ~3.8 Å, which was shorter in the mutant (~1.6 Å). (D) FlimPIA with 50 nM each of FKBP/Donor and FRB/the new Acceptor with/ without 50 nM rapamycin (n = 3).

	K_m for LH$_2$	Vmax ($\times 10^4$ RLU/s) for LH$_2$	K_m for ATP	Vmax (10^4 RLU/s) for ATP	K_m for LH$_2$-AMP	Vmax ($\times 10^6$ RLU/s) for LH$_2$-AMP
K529Q	95.0 ± 12.1	3.49 ± 0.20	424 ± 55	2.50 ± 0.11	0.412 ± 0.055	1.04 ± 0.04
K529Q/R437L	115 ± 4.0	5.52 ± 0.08	307 ± 25	3.94 ± 0.11	0.605 ± 0.063	0.737 ± 0.027
K529A/R437K/ L438I	62.7 ± 4.1	35.1 ± 0.7	306 ± 25	39.8 ± 1.1	0.710 ± 0.093	1.28 ± 0.06

Table 3. Kinetics properties of Acceptors fused to FRB.

3.5. Optimization of assay conditions

The overall activities of the improved Acceptor (R4437K/K529Q) mentioned in Section 3.4 showed a tenfold decrease, and the oxidative luminescence activities were almost maintained. However, the S/B ratio increased only 1.6-fold. To investigate this discrepancy, the Acceptor was reacted with (1) LH$_2$ + ATP, (2) LH$_2$-AMP, and (3) LH$_2$ + ATP + LH$_2$-AMP (**Figure 8A**). The luminescence intensity in the case of (3) should be equal to the sum of the intensities of (1) and (2). However, the intensity in (3) was remarkably lower than the sum. Therefore, we thought that some competition may exist in the oxidative luminescence steps. It was reported that dehydroluciferyl-AMP (L-AMP), which is converted from LH$_2$-AMP, competes with LH$_2$-AMP in the oxidative luminescence steps, and coenzyme A (CoA) converts L-AMP to dehydroluciferyl-coenzyme A, which is a less potent competitor of LH$_2$-AMP. First, we added
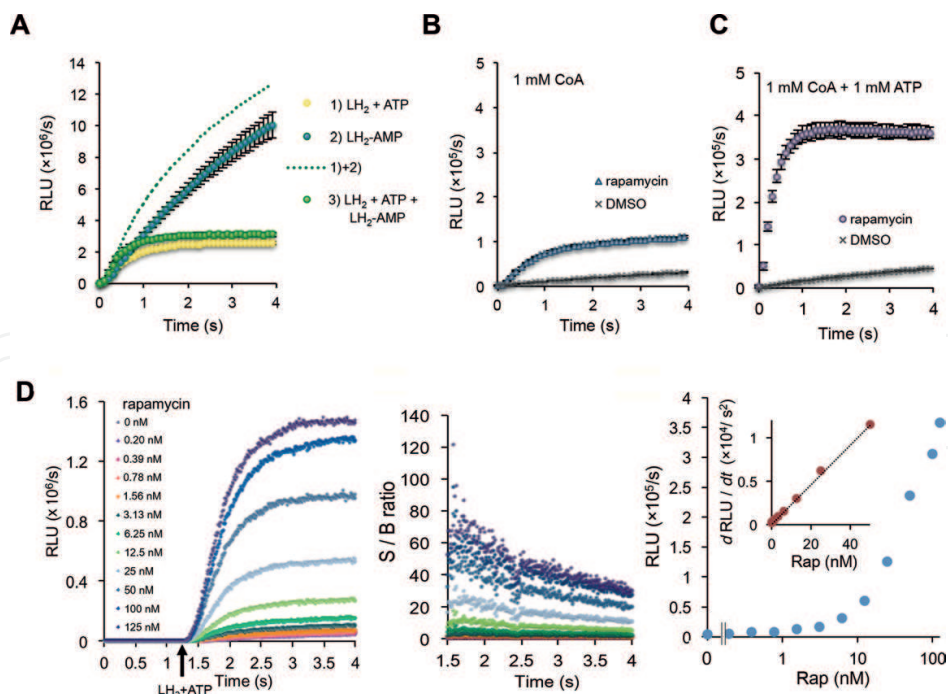

Figure 8. Optimization of assay condition in vitro. (A) An experimental simulation of FlimPIA using the conventional Acceptor. (B) The responses with and without 50 nM rapamycin in the presence of 1 mM CoA and 20 mM ATP. (C) The responses in the presence of 1 mM CoA and 1 mM ATP. (D) The results of tube-based luminometer with rapid mixing of the probes and substrates.

CoA to the mixture of FlimPIA (**Figure 8B**). In the presence of CoA, the maximum S/B ratio reached 8, representing a twofold improvement, when 50 nM of each probe was used.

Next, we optimized the concentration of ATP, as it was designed so that the K_m value of the Acceptor for ATP was lower than that of the wild type to suppress the adenylation activity, but the K_m value of the Donor for ATP was maintained to provide LH_2-AMP. The optimal concentration of ATP was 1 mM, and the maximal S/B ratio reached approximately 40, representing a fivefold improvement, when 50 nM of each probe was used (**Figure 8C**).

Finally, we had optimized the reaction conditions. As the increase of luminescence occurred as soon as substrates were added, a luminometer equipped with a stirrer was used to mix and react the substrates quickly (**Figure 8D**). The luminescence intensity increased quasi-linearly from 0.2 to 0.6 s after the reaction start and then reached a plateau. The maximal S/B ratio reached more than 60 when 100 nM of each probe was used.

Taken together, these improvements achieved a remarkably higher S/B ratio and sensitivity [6].

4. Advantages and disadvantages of FlimPIA

In this section, we describe the advantages and disadvantages of FlimPIA compared to the conventional PPI assay, FRET, and PCA, which are available in cellulo and in vitro.

4.1. FlimPIA in cells

To determine if FlimPIA is applicable in cellulo or in vivo, the FKBP-Donor and FRB-Acceptor were transiently expressed in cultured cells (**Figure 9**) [7]. The response was clearly observed in cells when rapamycin was added, and the luminescence intensity increased depending on the concentration of rapamycin.

However, the maximal S/B ratio was less than 2.5, and the detectable range of the concentration of rapamycin was rather narrow. Although the S/B ratio of FRET is often as low as that of FlimPIA in cells, PCA gives a high S/B ratio both in vitro and in cellulo.

4.2. Stability of probes in vitro

The same Fluc derived from *P. pyralis* was applied to both Fluc-based PCA in vitro and FlimPIA. Then, the thermostability of probes was compared [10]. The probes of Fluc-based PCA (FKBP-C and FRB-N) and the probes of FlimPIA (FKBP-Donor and FRB-Acceptor) were preincubated with or without rapamycin at 37°C (**Figure 10A**). After 30 minutes, half of the luminescence signal was retained in FlimPIA, and on the other hand, the luminescence signal was almost completely diminished in PCA. The rate of the luminescence decay in FlimPIA was approximately one-fourth of the rate of the decay in PCA (**Figure 10B**).

4.3. S/B ratio in vitro

The S/B ratio of FRET is rather low, but on the other hand, PCA shows a high S/B ratio and high sensitivity. The conventional FlimPIA described in Section 3.1 showed that the maximal S/B ratio was 2.5, which is generally lower than the S/B ratio of PCA [7]. However, the S/B ratio dramatically increased by the improvements described in Section 3.2-3.5 and was equal to or higher than the S/B ratio of PCA [6, 9, 10].
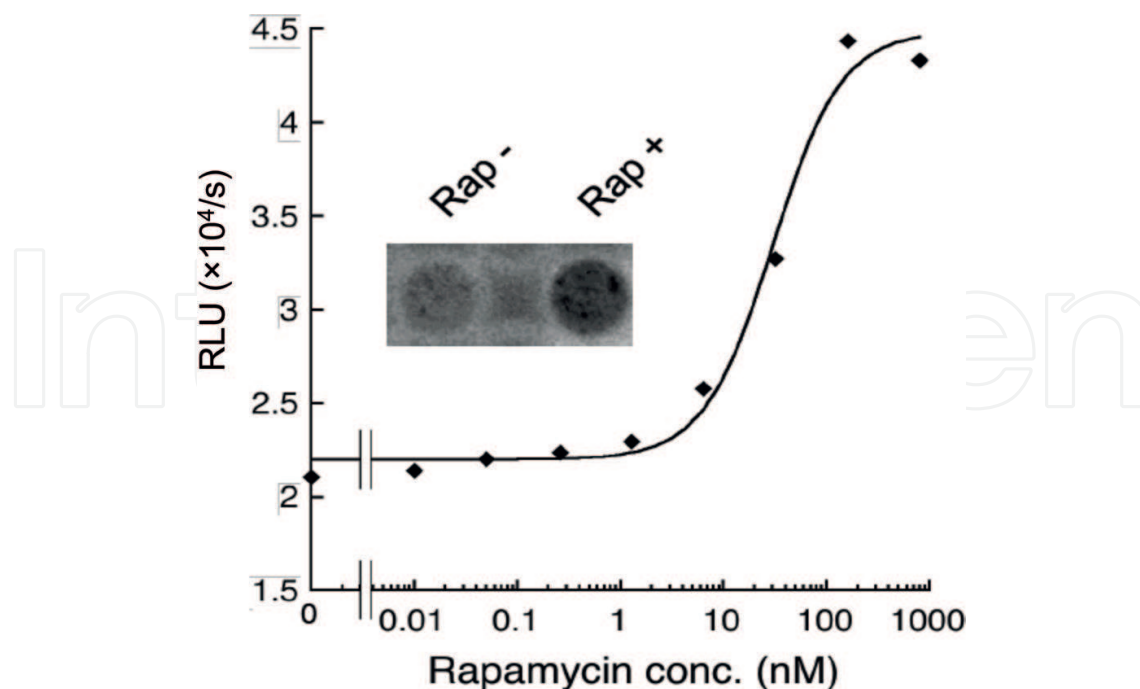
Figure 9. FKBP12-FRB association detected by FlimPIA in cultured cells. ©American Chemical Society.
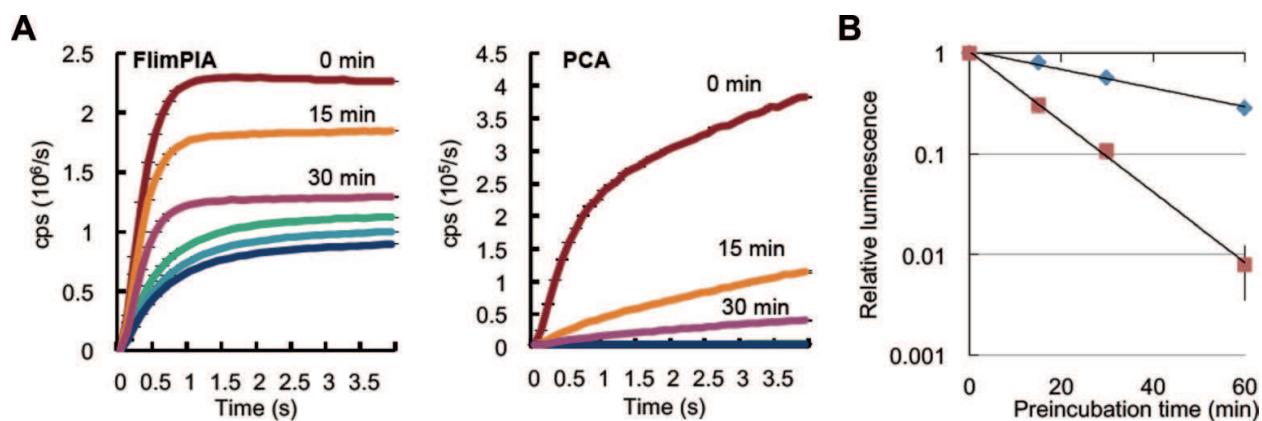
Figure 10. Comparison of thermostability in vitro. (A) Probes (50 nM each) were preincubated at 37°C with or without equimolar rapamycin. The luminescent intensity was measured for 4 s after adding substrates (LH$_2$ and ATP). Left: FlimPIA, Right: Fluc-based PCA. Red: incubation for 0 min with rapamycin, Orange: 15 min with rapamycin, pink: 30 min with rapamycin, green: 0 min without rapamycin, light blue: 15 min without rapamycin, dark blue: 30 min without rapamycin. (n = 3) (B) Inactivation time course. Relative luminescent intensities at 4 s after reaction start were normalized at the value obtained with 0 min pre-incubation (n = 3). ©American Chemical Society.

4.4. Sensitivity in vitro

The detectable limits of the concentration of rapamycin in Fluc-based PCA, the conventional FlimPIA, and the improved FlimPIA were compared, when 50 nM of each probe (FKBP-C and FRB-N, or FKBP-Donor and FRB-Acceptor) and rapamycin were used. The limits were 250 pM in Fluc-based PCA and 10 pM in FlimPIA using the K529Q/S440L mutant as the Acceptor [4, 9, 10]. The sensitivity of the improved FlimPIA was higher than the sensitivity of Fluc-based PCA.
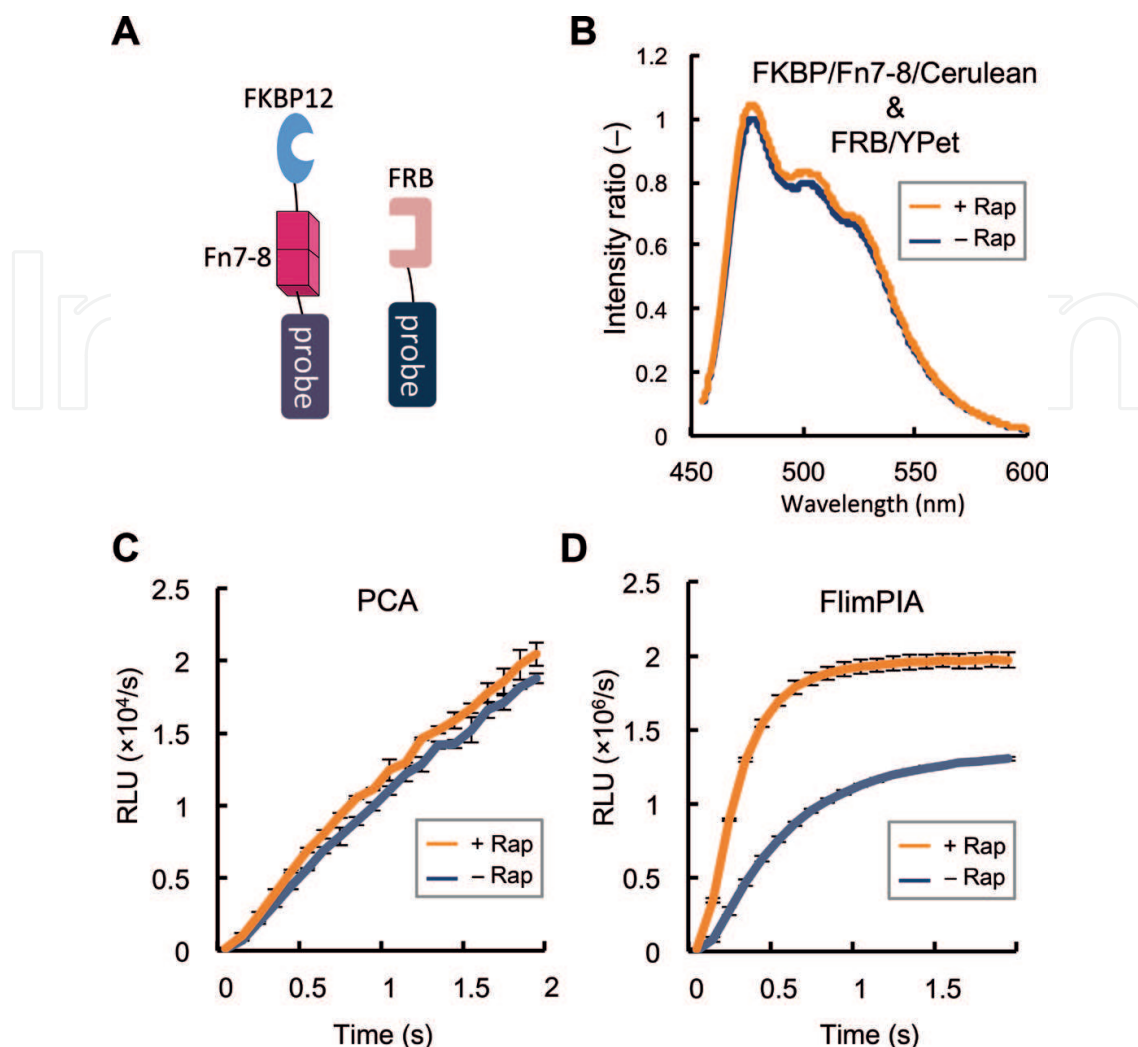
Figure 11. Detectable distance between the probes in vitro. (A) Scheme of the assays. A long (7 nm) Fn7-8 domain is inserted between a binding domain (FKBP12) and a probe. Signals with and without equimolar rapamycin were compared. (B) FRET using 40 nM each of FKBP-Fn7-8-Cerulean and the FRB-YPet as a probe pair. (C) Fluc-PCA using 100 nM each of FKBP-Fn7-8-C and FRB-N (n = 3). (D) FlimPIA using 100 nM each of FKBP-Fn7-8-Donor and FRB-Acceptor (left) (n = 3). ©American Chemical Society.

4.5. Detection limit of dimension of interacting protein in vitro

A fundamental limitation of FRET is that the detectable distance between the two probes is less than several nanometers, because the fluorescent signal is inversely proportional to the sixth power of the distance. A part of fibronectin type III, the seventh and eighth domains (Fn7-8), has a rigid structure with a 7 nm N-C terminal distance [10]. Ohashi et al. reported that a FRET signal using YPet and CyPet could not be observed by inserting Fn7-8 between the two fluorescent proteins [22]. The limit of the detectable distance between the two probes determines the detectable dimensions of the interacting protein.

Therefore, we compared the limit of the detectable distance between the probes in our assay. To examine this, Fn7-8 was inserted between FKBP12 and one of the probes (C-terminal domain for PCA, cerulean for FRET, and Donor for FlimPIA) (**Figure 11**). The large probes

were mixed with FRB-N, FRB-YPet, and FRB-Acceptors, respectively. As expected, the FRET signal was very weak when rapamycin was added to the mixture of FKBP12-Fn7-8-Cerulean and FRB-YPet, whereas the signal derived from the mixture of FKBP12-Cerulean and FRB-YPet was clearly observed (not shown). In the case of PCA using FKBP12-Fn7-8-C and FRB-N, the luminescence intensity was not significantly increased by the addition of rapamycin when the concentrations of the probes were moderate (100 nM each), while some response was observed with higher concentrations (750 nM) of each probe (not shown). However, the response of FlimPIA was clearly observed, even when 100 nM each of FKBP-Fn7-8-Donor and FRB-Acceptor was used.

5. Conclusions

We reported the development of Fluc-based PCA using purified probes for the first time. However, the stabilities of the probes were low due to the split forms. The problem might be overcome by using another enzyme with a highly stable structure.

Furthermore, we developed a unique PPI assay, called FlimPIA, wherein the catalytic reaction of Fluc is divided into two half-reactions. FlimPIA has several advantages, especially in vitro. Our next challenge is to improve the response in cellulo.

Acknowledgements

Conflict of interest

The authors declare that there are no conflicts of interest.

Author details

Yuki Ohmuro-Matsuyama and Hiroshi Ueda*

*Address all correspondence to: ueda@res.titech.ac.jp

Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology, Yokohama, Japan

References

[1]   Venkatesan K, Rual JF, Vazquez A, Stelzl U, Lemmens I, Hirozane-Kishikawa T, Hao T, Zenkner M, Xin X, Goh KI, et al. An empirical framework for binary interactome mapping. Nature Methods. 2009;**6**:83-90. DOI: 10.1038/nmeth.1280

[2]   Zhang QC, Petrey D, Deng L, Qiang L, Shi Y, Thu CA, Bisikirska B, Lefebvre C, Accili D, Hunter T, et al. Structure-based prediction of protein-protein interactions on a genome-wide scale. Nature. 2012;**490**:556-560. DOI: 10.1038/nature11503

[3]   Porter JR, Stains CI, Jester BW, Ghosh I. A general and rapid cell-free approach for the interrogation of protein-protein, protein-DNA, and protein-RNA interactions and their antagonists utilizing split-protein reporters. Journal of the American Chemical Society. 2008;**130**:6488-6497. DOI: 10.1021/ja7114579

[4]   Ohmuro-Matsuyama Y, Chung CI, Ueda H. Demonstration of protein-fragment complementation assay using purified firefly luciferase fragments. BMC Biotechnology. 2013;**13**:31. DOI: 10.1186/1472-6750-13-31

[5]   Dale R, Ohmuro-Matsuyama Y, Ueda H, Kato N. Mathematical model of the firefly luciferase complementation assay reveals a non-linear relationship between the detected luminescence and the affinity of the protein pair being analyzed. PLoS One. 2016;**11**:e0148256. DOI: 10.1371/journal.pone.0148256

[6]   Kurihara M, Ohmuro-Matsuyama Y, Ayabe K, Yamashita T, Yamaji H, Ueda H. Ultra sensitive firefly luciferase-based protein-protein interaction assay (FlimPIA) attained by hinge region engineering and optimized reaction conditions. Biotechnology Journal. 2016;**11**:91-99. DOI: 10.1002/biot.201500189

[7]   Ohmuro-Matsuyama Y, Nakano K, Kimura A, Ayabe K, Ihara M, Wada T, Ueda H. A protein-protein interaction assay based on the functional complementation of mutant firefly luciferases. Analytical Chemistry. 2013;**85**:7935-7940. DOI: 10.1021/ac4016825

[8]   Ohmuro-Matsuyama Y, Ueda H. A protein-protein interaction assay FlimPIA based on the functional complementation of mutant firefly luciferases. Methods in Molecular Biology. 2016;**1461**:131-142. DOI: 10.1007/978-1-4939-3813-1_10

[9]   Ohmuro-Matsuyama Y, Ueda H. Ultrasensitive firefly luminescent intermediate-based protein-protein interaction assay (FlimPIA) based on the functional complementation of mutant firefly luciferases. Methods in Molecular Biology. 2017;**1596**:119-130. DOI: 10.1007/978-1-4939-6940-1_8

[10] Ohmuro-Matsuyama Y, Hara Y, Ueda H. Improved protein-protein interaction assay FlimPIA by the entrapment of luciferase conformation. Analytical Chemistry. 2014;**86**:2013-2018. DOI: 10.1021/ac403065v

[11] Chen J, Zheng XF, Brown EJ, Schreiber SL. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proceedings of the National Academy of Sciences of the United States of America. 1995;**92**:4947-4951

[12] Chiu MI, Katz H, Berlin V. RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. Proceedings of the National Academy of Sciences of the United States of America. 1994;**91**:12574-12578

[13] Paulmurugan R, Gambhir SS. Combinatorial library screening for developing an improved split-firefly luciferase fragment-assisted complementation system for studying protein-protein interactions. Analytical Chemistry. 2007;**79**:2346-2353. DOI: 10.1021/ac062053q

[14] Banaszynski LA, Liu CW, Wandless TJ. Characterization of the FKBP.Rapamycin.FRB ternary complex. Journal of the American Chemical Society. 2005;**127**:4715-4721. DOI: 10.1021/ja043277y

[15] Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ, Pavletich NP. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science. 1996;**274**:948-953

[16] Branchini BR, Rosenberg JC, Ablamsky DM, Taylor KP, Southworth TL, Linder SJ. Sequential bioluminescence resonance energy transfer-fluorescence resonance energy transfer-based ratiometric protease assays with fusion proteins of firefly luciferase and red fluorescent protein. Analytical Biochemistry. 2011;**414**:239-245. DOI: 10.1016/j.ab.2011.03.031

[17] Sundlov JA, Fontaine DM, Southworth TL, Branchini BR, Gulick AM. Crystal structure of firefly luciferase in a second catalytic conformation supports a domain alternation mechanism. Biochemistry. 2012;**51**:6493-6495. DOI: 10.1021/bi300934s

[18] Ayabe K, Zako T, Ueda H. The role of firefly luciferase C-terminal domain in efficient coupling of adenylation and oxidative steps. FEBS Letters. 2005;**579**:4389-4394. DOI: 10.1016/j.febslet.2005.07.004

[19] Branchini BR, Murtiashaw MH, Magyar RA, Anderson SM. The role of lysine 529, a conserved residue of the acyl-adenylate-forming enzyme superfamily, in firefly luciferase. Biochemistry. 2000;**39**:5433-5440

[20] Branchini BR, Magyar RA, Murtiashaw MH, Anderson SM, Zimmer M. Site-directed mutagenesis of histidine 245 in firefly luciferase: A proposed model of the active site. Biochemistry. 1998;**37**:15311-15319. DOI: 10.1021/bi981150d

[21] Branchini BR, Rosenberg JC, Fontaine DM, Southworth TL, Behney CE, Uzasci L. Bioluminescence is produced from a trapped firefly luciferase conformation predicted by the domain alternation mechanism. Journal of the American Chemical Society. 2011;**133**:11088-11091. DOI: 10.1021/ja2041496

[22] Ohashi T, Galiacy SD, Briscoe G, Erickson HP. An experimental study of GFP-based FRET, with application to intrinsically unstructured proteins. Protein Science. 2007;**16**:1429-1438. DOI: 10.1110/ps.072845607