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An improved fluorescent protein-based expression reporter system that utilizes bioluminescence resonance energy transfer and peptide-assisted complementation

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In fluorescent protein-based reporter systems used to track gene expression in cells. Here, we propose a modified bioluminescence resonance energy transfer (BRET) reporter as a maturation-less reporter that utilizes a peptide-assisted complementation strategy. Using effective dimerized peptides obtained from library-versus-library screening with more than 4000 candidates, rapid activation of the reporter was achieved.

Genetically encoded fluorescent proteins (FPs) have taken on an important role in the analysis of gene expression or promoter activity in living cells.[1,2] The FPs are either designed to be expressed under the control of promoters of interest or fused with target proteins, which enable quantification of transcription or expression based on the fluorescent intensity of FPs in living cells. The chemical modification of the FP's chromophore, also termed the maturation step, causes FPs to emit fluorescence after translation. This is a rate-limiting step lasting from a few minutes to hours depending on the particular FP, cell type, oxygen concentration, and temperature.[3,4] Therefore, the temporal gap occurs between the timing of actual gene expression and fluorescence emission, and is a significant problem for some applications, such as, early detection of promoter activation or tracking proteins that have transient expression. For example, the gene expression noise caused by the transcription and/or translation burst of *E. coli* fluctuates in a range of minutes and generates heterogeneous subpopulations within a genetically identical population.[5] This contributes to adaptation to fluctuating external environments. Thus, a

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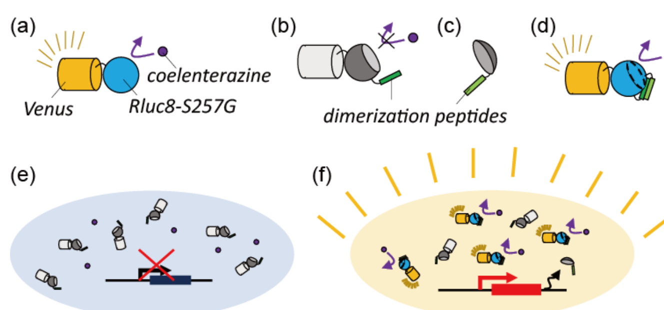

Fig. 1 Schematic illustration of the fast reporter system. (a-f) Schematic illustration of a yellow Nano-lantern (YNL) (a), a partner unit (PU) of split YNL which consisted of Venus and a part of RLuc8-S257G fused with a dimerized peptide at C-terminus (b), a reporter unit (RU) of split YNL which consisted only a fraction of RLuc8-S257G fused with a dimerized peptide at N-terminus (c), a reconstructed YNL *via* dimerized peptides (d), a cell which constitutively expressed PU with inactive RU (e), and a cell after activation of RU (f).

maturation-free reporter is needed to achieve detailed and precise temporal analysis for gene expression dynamics.

So far, most of efforts have been focused on overcoming the maturation problem, which can be categorized into several approaches. The first approach is the directed evolution of FPs with random mutation screening for faster maturation.[6,7] Based on this strategy, a variety of FPs were designed to have shorter maturation times in a particular experimental condition[6], although essential maturation processes persisted. The second approach utilizes the relocation of constitutively expressed and matured FPs into specific locations, such as, the nucleus.[8,9] In this case, while the maturation delay can be avoided, background fluorescence caused by constitutively expressed FPs often degrades the quantification. The third approach is the use of luciferases, which do not need maturation processes, since they catalyze their substrates and produce photons.[10]

Recently, brighter luciferases have been explored and attention is being paid to their applications as bioluminescent reporters.[11] Bioluminescence resonance energy transfer (BRET), where the gene coding for FP as an acceptor for BRET is fused to a *luciferase* gene, improves the brightness of the reporter.[12-14]. Yellow Nano-lantern (YNL),[12,13] one of the BRET probes, is a chimera made up of Venus, a variant of yellow FP with fast maturation,[7] and acts as an acceptor, and RLuc8-S257G, an enhanced mutant based on a stabilized variant of *Renilla*

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luciferase called RLuc8,[15] acts as a donor of BRET (Fig 1A).[12,13] The chemical energy produced by RLuc8-S257G catalyzing substrate, coelenterazine, is transferred to Venus resulting in a 10-fold brighter emission than RLuc8 itself,[12] while the maturation problem recurs because the chimera contains FP. The complementation of YNL split in the middle of an RLuc8-S257G region into two components avoids this maturation problem. One component is a partner unit (PU) composed of the split RLuc8-S257G and Venus (Fig. 1B), designed to be expressed constantly inside the cell. The other component is a reporter unit (RU), which is the residual fraction of RLuc8-S257G (Fig. 1C), and is designed to be expressed by the promoter of the target protein. If both units are fused with complementation peptides or binding domain pairs, the split YNL can emit fluorescence, but only when both the units interact (Fig. 1D). The split YNL was utilized as a functional indicator of small molecules, such as Ca^{2+}, cyclic adenosine monophosphate (cAMP), and adenosine triphosphate (ATP).[12]

Although self-assembling luciferase platforms, with no interactions with peptides or other binding domains, have been reported,[16] the use of BRET probes is thought to be more suitable for future prospects of multi-color reporting by choosing a desired spectral FP as an acceptor.[13,14] The complementation efficiency and reversibility depends on the presence and the types of peptide pairs or binding domain pairs. The utilization of protein fragment-assisted complementation (PFAC) strategy is necessary to complement our aim of optical monitoring of gene expression with less delay. The PFAC is also a valuable tool for future prospects involving multiple usage of BRET reporters since it provides multiple alternatives of reconstruction with less crosstalk. Hence, in this paper, we propose a strategy to avoid the maturation delay of YNL as a model for BRET reporters by utilizing PFAC technique.

The initial experiment was library-versus-library screening for effective reconstruction of peptide pairs for luminescent competence of split YNL. Since the primary application of this strategy is to report protein expression levels, the aim of the initial experiment was to identify brighter peptide pairs which achieve the highest luminescence regardless of the reversibility, to detect even the initial small increase in a target protein expression. As a candidate library of the peptide pairs, we elected 24 heterodimeric peptides with α-helical coiled-coil structures from the artificial peptide pairs previously reported to form heterodimers, including two types of WinZip series,[17] four types of E/K-peptide series,[18,19] and 14 types of SINZIP series[20,21] (Table S1). According to the literatures, these peptides only achieve stable interaction but also provide a specific interaction profile with less *in vitro* crosstalk.[17-21] Each peptide was fused with the C-terminus of PU or the N-terminus of RU, in forward or reverse direction. Additionally, we investigated the two regions that split YNL, one was between the 91st and 92nd amino acid of RLuc8-S257G and the other was between the 228th and 229th amino acid, both of which are in a loop structure.[22] Thus, in total, we prepared 4608 candidate pairs for library screening.

To evaluate the complement efficiency of these peptide pairs, we simultaneously expressed PU and RU (both fused with peptide candidates) *in vitro* by using a cell-free protein expression system, and measured its luminescent intensity, assuming that brighter samples consist of effective reconstruction peptide pairs. For comparison, all luminescent intensities were normalized with the luminescent intensity of intact RLuc8-S257G measured for each test sample. As few as 92 candidates exhibited intensity values of greater than 0.1, and only 7 candidates had intensities more than 0.5 (Fig. 2, Table S2). Most of these effective pairs appeared in the split YNL between amino acids 228 and 229 of RLuc8-S257G, which is consistent with a previous report showing that this site is a more efficient site for reconstruction than the amino acids 91 and 92 in split YNL.[12] Additionally, most of these pairs were among different peptide families, and 13 of the top 14 efficient peptide pairs were occupied by combinations of E-peptide series for PU and SYNZIP series for RU (Table S2). Some of these multiple efficient peptide pairs possibly applied to multiple reconstructions of split BRET probes in the case of multi-color imaging although we have not investigated that here. Taken together, our library-versus-library screening successfully found a limited number of effective candidates for reconstruction of luminescent competency in split YNL.

The next step was to evaluate the improvement in the maturation delay in the screened candidates by analyzing the luminescence response to the initiation of reporter expression in a cell-free expression system. We examined three efficiently

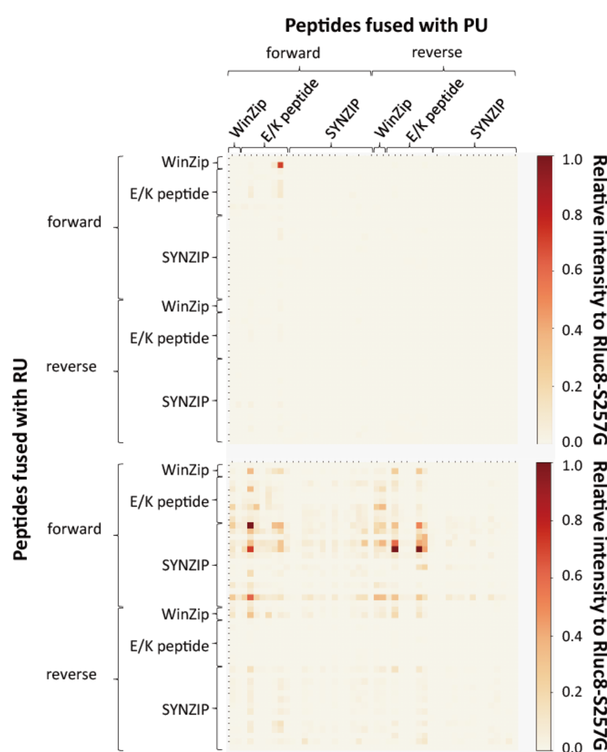

Fig. 2 Library screening for reconstructed peptide pairs to efficiently recover luminescence competence of split YNL. Relative intensities of reconstructed YNL is presented as heat maps. The top panel represents the results with split YNL between amino acids 91 and 92 of the donor (RLuc8-S257G) unit. The bottom represents the results of split YNL between amino acids 228 and 229 of the donor unit. The types of synthesized peptides and the directions to fuse to PU or RU are described.

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reconstructed peptide pairs in the screening experiment, E-peptide/SYNZIP1 (E/SZ1), E-peptide/SYNZIP5 (E/SZ5), and E-peptide/SYNZIP22 (E/SZ22) (stated as “peptide fused with PU” / “peptide fused with RU”) (Table S2). PU was expressed 1 h prior to the induction of RU expression so that the matured PU accumulated in advance and luminescence was measured at various time pointes after induction of RU expression (Fig. 1E, 1F). The controls were intact YNL, which needed the maturation of acceptor (Venus), and RLuc8-S257G, which did not need chromophore maturation. In all the reporters, a clear increase of luminescence was observed without any saturation within 30 min (Fig. S1A, S1B). To visually compare the timing of initial luminescence increment, the measured intensities were normalized by using respective intensities at 30 min after the initiation of RU expression (t = 30). The luminescent increment of E/SZ1, E/SZ5, and RLuc8-S257G exceeded that of YNL (Fig. 3A–D). Focusing on the earliest time point (t = 5), those intensities were significantly higher than that of YNL (Fig. S2). This result indicated that the present reporter systems succeeded in reducing maturation delay and were activated quickly after expression, faster than YNL.

To evaluate the properties of the present reporters in comparison with conventional fluorescent reporters quantitatively, we constructed a simple mathematical model that is adaptable to both the conventional FP reporters and the present developed reporters. Postulating constant expression and negligible degradation of reporter proteins, we considered a two-step model of reporter activation (details are provided in Supplemental text (I)), which resulted in a mathematical description of luminescence intensity (I_L) evolution as a function of t as follows:

$$I_L(t) = k_{LP}\left\{t + \frac{1}{k_M}\left(e^{-k_M t} - 1\right)\right\} \quad \text{(Eq. 1)}$$

, where k_{LP} and k_M correspond to light (luminescence or fluorescence) production rates and pseudo- maturation rates, respectively (Fig. S3). Fitting the experimental data with Eq. 1, k_M of YNL was estimated to be 0.125, corresponding to a maturation time of t_{50} = 5.2 min, which was consistent with a previously reported maturation time of Venus in bacteria (t_{50} = 4.1 min).[4] Additionally, an estimation of k_M and k_{LP} of RLuc8-S257G resulted in higher and lower values than those of YNL respectively, which is consistent with the fact that RLuc8-S257G can be more rapidly activated and is darker than YNL. These results suggested that the quantification was reasonable. Maturation rates of E/SZ1 and E/SZ5 were 1.9-fold (k_M = 0.238) and 3.7-fold (k_M = 0.457) higher than that of YNL, while that of E/SZ22 was much lower (k_M = 0.042) (Fig. 3E). This demonstrated that E/SZ1 and E/SZ5 achieved faster responses than Venus, which is one of the fastest maturating FP. The calculated k_{LP} of E/SZ1 (368) and E/SZ5 (194) were significantly lower than that of YNL (1300), and the values were closer to those observed for RLuc8-S257G (285) (Fig. 3F). Meanwhile, E/SY22 exhibited a higher k_{LP} value compared to E/SZ1 and E/SZ5, although the value was still lower than YNL (Fig. 3F). The difference in k_{LP} between YNL and RLuc8-S257G could be due to a difference in the number of photons per molecule (Eq. S14).

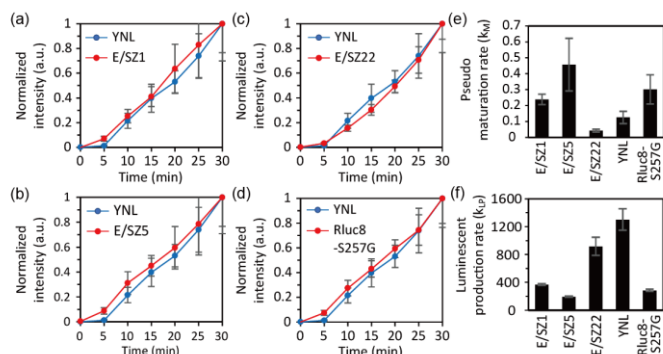

Fig. 3 Evaluation of the response speed of our reporters using screened peptide pairs in a cell free expression system. (a-d) Time course of normalized luminescence intensities of the present reporter systems in a cell free expression system using three peptide pairs of E/SZ1 (a), E/SZ5 (b), E/SZ22 (c), and RLuc8-S257G (d). Each luminescence intensity was normalized by the respective intensities at t = 30. The expression of reporters was initiated at t = 0. (e) The maturation rates, k_M, of the mathematical model (Eq. 1) were extracted by fitting to the experimental results. (f) The light production rates, k_{LP}, of the mathematical model (Eq.1) were extracted by fitting to the experimental data. Error bars in (e, f) are standard errors of the fittings.

Based on the mathematical model, both k_{LP} and k_M are dependent not only on the dissociation and association affinities of the dimerized peptides, but also on the concentration of PU (Eq. S15 and S16). Next, we investigated the dependency of the present reporter system on the concentration of PU using the peptide pair consisting of E/SZ1 by increasing the concentration of template DNA for PU expression from 2 ng/μL to 16 ng/μL (Fig. S1C). Our results showed that the k_M increased with the concentration of PU as the model predicted. At the same time, k_M was saturated around 0.3 (Fig. 4A). The value (0.3) is consistent with k_M of RLuc8-S257G (0.30), indicating that our reporters achieved the same speed of activation as the no maturation reporter. The reason for this saturation might be restricted by other factors such as transcription, translation, and/or folding rates. Meanwhile, in case of the lowest concentration of PU, k_M was much lower (k_M = 0.168) and closer to that of YNL (Fig. 4A). Further, k_{LP} also exhibited a positive correlation with the concentrations of PU, and was ~2-fold and ~8.5-fold higher, at the highest PU concentration tested, than those of YNL and RLuc8-S257G, respectively (Fig. 4B). This result was consistent with the fact that the BRET system could be brighter than the no BRET system (RLuc8-S257G).[12] We concluded that the speed of response of the present system reached as fast as that of a no maturation reporter, such as, RLuc8-S257G, although optimization of PU concentration is needed in case of low expression of PU.

Finally, we evaluated the rising speed of the present reporter system as a direct result of the improvements in maturation delay in living cells using *E. coli* strain BL21-AI. We designed a transformation plasmid which could induce expression of PU by L-arabinose addition and also of RU in response to isopropyl β-D-1-thiogalactopyranoside (IPTG) addition. The reconstructed peptide pair consisting of E/SZ1, which displayed the second-best k_M, was selected here. PU was expressed for 8 h in a fast growth medium [23] at 37 °C before induction of RU (Fig. S4, *red* and *yellow*). Subsequently, *E. coli* cells were transferred into a slow growth medium[23] to reduce the effect of dilution of accumulated matured PU caused by cell division (Fig. S4, *cyan and blue*). Meanwhile, RU expression was induced after 1 h

incubation in the slow growth medium. As a negative control, PU was not induced in advance in *E. coli* but the induction was done along with the induction of RU (t = 0) to have a slower response. Also, we investigated Venus as a comparison of the present system. All samples after the induction exhibited a clear luminescence or an increase in the fluorescence in comparison to the non-induced samples (Fig. 4C and Fig. S5). The E/SZ1 reporter with pre-incubation of PU exhibited a significantly faster initial increment of luminescence intensity, which started just after RU induction (Fig. 4C, *red*). Meanwhile, the intensity of Venus or E/SZ1 reporter without pre-incubation of PU started to increase around 30 min after induction (Fig. 4C). The similarity in the timing of initiation between the two controls indicates that both controls need maturation of Venus. The saturation in samples with and without pre-maturation was most likely caused by degradation, because split luciferase might reduce structural stability as seen in other species of luciferase.[24] Thus, the present strategy for the development of a maturation-less fast reporter was successful in living *E. coli* cells.

Significantly, the present strategy could direct further improvements in overcoming maturation delay, thereby providing a faster expression reporter than Venus, which is the fastest reporter of FP. This study chose *E. coli* experiment systems for simple comparisons to the previous results of Venus optimized in *E. coli*.[7] Although the peptide pairs screened in this study did not work in mammalian cells (Fig. S6a), we also found the best candidate peptides for mammalian cells (Fig. S6-8). Although the present strategy should be applicable to systems other than *E. coli*, appropriate dimerized peptides are varied depending on cell types and we recommend the well-known working peptides should be tested first. Subsequently, by screening the peptide candidates using an *in vitro* system simulating intracellular environment of the target cells, better variants might be achieved. The detailed discussion is described in Supplemental Text (II).

In summary, we have demonstrated a novel strategy for eliminating maturation of a BRET reporter for expression

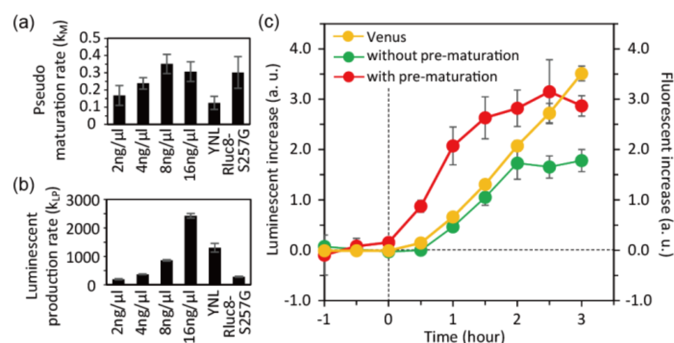

Fig. 4 Evaluation of the present reporter system *in vitro* and in *E. coli* cells. (a, b) Dependence of the maturation rate k_M (a) and light production rate k_{LP} (b) of E/SZ1 reporter on PU concentrations were evaluated using a cell-free expression system. Four concentrations, 2, 4, 8 and 16 ng/µL of template DNA for PU expression, were tested with two controls. Both the parameters were extracted by the model fitting to experimental results. Error bars are standard errors of the fittings. (c) Time course of luminescence or fluorescence increase of reporters. Each plot indicates the intensity differences between induced and non-induced samples at each time point of data as shown in Fig. S5. All reporters were induced at t=0. *Red*, E/SZ1 with pre-maturation; *Green*, E/SZ1 without pre-maturation; *Yellow*, Venus. N=3.

monitoring by utilizing PFAC with screened peptides from more than 4000 candidates. Here, we focused on initial activation responses and have successfully demonstrated that PFAC significantly reduced the maturation delay of YNL both *in vitro* and in cells. The present strategy is applicable to other BRET reporters as well[13,14] and will contribute to detailed and precise temporal analysis for gene expression dynamics.

Conflicts of interest

The authors have no conflicts to declare.

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