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Abstract

Firefly luciferase is a prominent reporter on molecular imaging with the advantage of longer wavelength on light emission and the ATP linear correlation, which makes it useful in most of current bioluminescence imaging model. However, the utility of this biomaterial was limited by the signal intensity and stability which are respectively affected by enzyme activity and substrate consumption. This study demonstrated a series of novel synthetic bifunctional enzyme complex of Firefly luciferase (Fluc) and Luciferin-regenerating enzyme (LRE). A peptide linker library was constructed for the fusion strategy on biosynthesis. The findings of both experimental data and structural simulation demonstrated that the intervention of fused LRE remarkably improve the stability of in vitro bioluminescence signal through luciferin recycling; and revealed the competitive relationship of Fluc and LRE on luciferin binding: Fluc performed higher activity with one copy number of rigid linker (EAAAK) at the C terminal while LRE acted more efficiently with two copy numbers of flexible linker (GGGS) at the N terminal. With the advantage of signal intensity and stability, this fused bifunctional enzyme complex may expand the application of firefly luciferase to in vitro bioluminescence imaging.

Keywords	Bioluminescence imaging; Enzyme complex; Structural simulation
Manuscript category	Proteins and Nucleic acids
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Highlights

1. Fusion strategy on biosynthetic bifunctional enzyme complex using peptide linker library.
2. Optimal enzyme structure with high-efficiency for luciferin-recycling to enhance bioluminescence imaging.
3. 3D protein structural simulation to analyze the functional domains and verify the enzyme properties.
4. Organic reagents to strengthen the bioluminescent property of the optimal enzyme complex.

ABSTRACT

Firefly luciferase is a prominent reporter on molecular imaging with the advantage of longer wavelength on light emission and the ATP linear correlation, which makes it useful in most of current bioluminescence imaging model. However, the utility of this biomaterial was limited by the signal intensity and stability which are respectively affected by enzyme activity and substrate consumption.

This study demonstrated a series of novel synthetic bifunctional enzyme complex of Firefly luciferase (Fluc) and Luciferin-regenerating enzyme (LRE). A peptide linker library was constructed for the fusion strategy on biosynthesis. The findings of both experimental data and structural simulation demonstrated that the intervention of fused LRE remarkably improve the stability of *in vitro* bioluminescence signal through luciferin recycling; and revealed the competitive relationship of Fluc and LRE on luciferin binding: Fluc performed higher activity with one copy number of rigid linker (EAAAK) at the C terminal while LRE acted more efficiently with two copy numbers of flexible linker (GGGGS) at the N terminal. With the advantage of signal intensity and stability, this fused bifunctional enzyme complex may expand the application of firefly luciferase to *in vitro* bioluminescence imaging.

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4 **TITLE: Biosynthetic bifunctional enzyme complex with high-efficiency luciferin-**
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6 **recycling to enhance the bioluminescence imaging**
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54 **ABSTRACT**
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63 Firefly luciferase is a prominent reporter on molecular imaging with the
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65 advantage of longer wavelength on light emission and the ATP linear correlation,
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67 which makes it useful in most of current bioluminescence imaging model. However,
68
69 the utility of this biomaterial was limited by the signal intensity and stability which
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71 are respectively affected by enzyme activity and substrate consumption.
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75 This study demonstrated a series of novel synthetic bifunctional enzyme
76
77 complex of Firefly luciferase (Fluc) and Luciferin-regenerating enzyme (LRE). A
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79 peptide linker library was constructed for the fusion strategy on biosynthesis. The
80
81 findings of both experimental data and structural simulation demonstrated that the
82
83 intervention of fused LRE remarkably improve the stability of *in vitro*
84
85 bioluminescence signal through luciferin recycling; and revealed the competitive
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87 relationship of Fluc and LRE on luciferin binding: Fluc performed higher activity with
88
89 one copy number of rigid linker (EAAAK) at the C terminal while LRE acted more
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91 efficiently with two copy numbers of flexible linker (GGGGS) at the N terminal. With
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93 the advantage of signal intensity and stability, this fused bifunctional enzyme
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95 complex may expand the application of firefly luciferase to *in vitro* bioluminescence
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97 imaging.
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106 **Key words:** Bioluminescence imaging, Bifunctional enzyme complex, Firefly
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108 luciferase, Luciferin-recycling, Structural simulation, Peptide linker library.
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111 112 113 **1. INTRODUCTION** 114 115 116 117 118

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122 On molecular imaging, the firefly luciferase (Fluc) was widely studied due to its
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124 ATP linear correlative feature on *in vitro* detection of ATP in live cell [1,2], therefore
125
126 it was used as an indicator for the hygienic index. Meanwhile, the luciferin-
127
128 regenerating enzyme (LRE) [3] that catalyzed converting process from oxyluciferin to
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130 luciferin in the presence of D-cysteine [4] was also reported to improve the
131
132 luminescence signal generated by exogenous Fluc [5]. However, the application of
133
134 Fluc catalyzed bioluminescence imaging was still limited by the bioluminescence
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136 intensity and signal decaying, especially for the *in vitro* detection of ATP at low
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138 concentration.
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144 Fusion strategy has been widely used in a variety of fields to construct artificial
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146 multifunction proteins [6,7]. Fusion protein can be designed and synthesized to
147
148 achieve improved properties or new functionality of multiple proteins by tandem
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150 fusion, domain insertion, or post-translational protein conjugation [8], among
151
152 which, , using connection medium such as peptide linker to produce the combination
153
154 of two or more protein domains in order to enhance bioactivities or generate novel
155
156 functional complex was studied in recent years, with a wide range of
157
158 biotechnological and (bio)pharmaceutical applications [9-11]. The length of linker
159
160 and the residues on the structure play an important role in the stability and
161
162 functionality of a fusion protein by affecting the active domain and the structure of
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164 protein [6,12].
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169
170 In our previous work, we reported that the fusion expressed Firefly luciferase
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172 (Fluc) and luciferin-regenerating enzyme (LRE) could enhance the *in vivo*
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180
181 luminescence imaging [13], mediated by two typical types of peptide linker, a rigid
182
183 linker (hereinafter referred to as R) with alpha helical structure (sequence of EAAAK)
184
185 to maintain distance between domains and a flexible linker (hereinafter referred to
186
187 as S, sequence of GGGGS) that increases spatial separation and allows interaction
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189 between domains [14,15].
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192
193 To study the mediation of peptide linkers on *in vitro* luminescence imaging and
194
195 luciferin-recycling catalyzed by Fluc-LRE fusion complex, and evaluate the efficiency
196
197 of designed linkers to separate domains, a peptide linker library containing rigid
198
199 linker, flexible linker and mixed linker with different length were assessed in this
200
201 study on the model of Fluc and LRE bifunctional enzyme complex, to determine the
202
203 optimal structure of bifunctional fusion protein of Fluc and LRE, thus to optimize the
204
205 catalytic efficiency on *in vitro* luminescence imaging.
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211 **2. MATERIAL AND METHODS**

212 **2.1 Bacteria, plasmids and reagents**

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215 The competent cell of *E. coli* DH5 α and *E. coli* BL21 strain, Isopropyl- β -D-
216
217 thiogalactopyranoside (IPTG) and Kanamycin were purchased from Transgen (Beijing,
218
219 China). The sequence of *Fluc* was cloned from the template of pGL4.17 [*luc2/Neo*]
220
221 Vector purchased from Promega (Cat. E672A). The sequence of *Lre* (GenBank:
222
223 AB062786) was synthesised by Sangon Biotech (Shanghai) Co., Ltd. The primers used
224
225 for PCR (listed in [Table 1the appendix](#)) were synthesised by Sangon Biotech
226
227
228
229 (Shanghai) Co., Ltd. The Takara PrimeSTAR Max DNA Polymerase (Cat. R045A),
230
231 Takara QuickCut restriction enzyme *Nde*I (Cat. 1621) and *Xho*I (Cat. 1635), and
232
233
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237
238
239 Takara ligation kit (Cat. 6022) were used for the construction of expression clones.
240
241 The plasmid pET28a expression vector was from Novagen (Cat. 69864-3). The
242
243 plasmid miniprep kit (Cat. GMK5999) and gel extraction kit (Cat. D2500-02) were
244
245 purchased from Promega.
246
247

248 2.2 Construction of fusion enzyme complex

249
250 The coding sequences A-of a series of fusion proteins with different linker (listed
251 in Table 1) were respectively constructed ~~using the templates and primers listed in~~
252 Table 1 framed with the restriction enzyme cutting site of *NdeI* and *XhoI* by PCR. The
253
254 PCR products were respectively digested by the restriction enzyme *NdeI* and *XhoI*,
255
256 and subsequently ligated between the multiple cloning sites on the pET28a vector by
257
258 Takara ligation kit. The expression clones were respectively driven by T7 promoter,
259
260 and the His-tag coding sequence on the plasmid encoded a histidine to the N-
261
262 terminal of target protein. The engineered plasmids were transferred in *E. coli* BL21
263
264 for protein expression. The engineered plasmids were transferred in *E. coli* BL21
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271 2.3 Bacterial culture

272
273 The bacteria was cultured using LB media (containing 10g/L Tryptone, 5g/L
274
275 Yeast extract and 10g/L NaCl to pH7.0 at 25 °C) with shaking at 180 rpm at 37 °C, 0.2
276
277 mM IPTG was injected to induce the protein expression after 2-hour incubation of
278
279 bacterial culture.
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281
282

283 2.4 Protein expression, purification and qualification

284
285 The bacteria carrying the recombinated enzyme expression clones were
286
287 incubated in triplicate with 0.2 mM IPTG induction at 20 °C with shaking. The
288
289 overnight cultured bacteria were washed and concentrated 5:1 with PBS (pH 7.0),
290
291 performed ultrasonic breaking (3 s × 6 s at 300W for 60 times) on ice and centrifuged
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297
298 at 4°C at 10000 rpm for 15 min to harvest the crude extract from supernatant. The
299
300 His-tagged enzyme were purified by affinity chromatography (GE AKTA prime plus)
301
302 using 5 ml HisTrap™ HP column (GE Healthcare, Sweden). The purified enzyme were
303
304 qualified by SDS-PAGE and the concentration of protein were analyzed using the
305
306 Bradford assay.
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309 2.5 *in vitro* assessment on luminescence imaging

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311 The purified enzyme was added in triplicate to the reaction mixture containing
312
313 0.25 mM D-luciferin, 4 mM ATP, 10 mM MgSO₄ and 2.5 mM D-cysteine in PBS (pH
314
315 7.0). The luminescence signal was measured in triplicate at 37 °C by Tecan Infinite
316
317 M200 Pro. to analyze the specific activity of each luciferase and fusion protein. The
318
319 enzyme activity of Fluc was determined by the relative light unit (RLU) per
320
321 microgram of protein while the enzyme activity of LRE was determined by
322
323 luminescence changing ratio in the presence/absence of D-cysteine.
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328 The equation which can be used for the determination of changing rate of
329
330 luminescence signal to analyze the effect of LRE to the *in vitro* bioluminescence is
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332 given as:
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$$334 \text{ Luminescence changing ratio} = \frac{(\text{RLU}' - \text{RLU})}{\text{RLU}} \times 100\%$$

337
338 Where RLU' is the bioluminescence intensity in the presence of €D-cysteine; RLU
339
340 is the bioluminescence intensity in the absence of D-cysteine.
341

342 2.6 3D Structure simulation

343
344 The 3D structure models of enzyme complexes were simulated and predicted
345
346 by the I-TASSER server [16], the simulated models with a high confidence score were
347
348 analyzed by the software VMD 1.8.3, focusing on the luciferin binding domain I (LBD
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356
357 I) and luciferin binding domain II (LBD II) [5, 17].
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359

360 2.7 Statistic analysis 361

362 One-way ANOVA was applied to compare the effect of different enzyme
363 complex to the *in vivo* and *in vitro* bioluminescence, the data of which were analyzed
364 by the software GraphPad Prism 6 and P value was used to determine the difference
365
366 between each two structures.
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373 3. RESULTS 374

375 3.1 Construction of enzyme complex and protein expression 376 377

378 A panel of Fluc expression clones was constructed on the plasmid of pET28a and
379 induced for expression in *E. coli* BL21, respectively encoding the enzyme of Fluc, dual
380 enzyme of Fluc and LRE, and the fusion proteins through different peptide linker. The
381 serial constructions of Fluc expression clones were listed in Table 21, and were
382 confirmed by sequencing by Sangon Biotech (Shanghai) Co., Ltd.
383
384
385

386 The expressed enzyme complexes were purified through affinity
387 chromatography and the concentration of harvested samples were listed in Table 21.
388
389 The samples of extracted enzyme were analyzed by SDS-PAGE. As was shown in Fig 1,
390 the Fluc at 62 kDa were obtained in lane 2&3, LRE at 38 kDa were obtained in lane 3
391 and the series of bifunctional enzyme complexes at approximately 100 kDa were
392 respectively obtained in lane 4-13.
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405 3.2 Effect of peptide linker on Fluc activity 406

407 The luminescence intensity of the various of enzyme complex were measured *in*
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415
416
417 *vitro* with the existence of the substrates D-luciferin. The luminescence intensity,
418
419 which indicated the activity of fusion expressed Fluc on catalyzing light-generation,
420
421 were present in Fig 2, compared with the signals produced by the free Fluc and the
422
423 dual expression of Fluc-LRE. The initial luminescence intensity demonstrated the
424
425 activity of Fluc in different structure with the effects of linkers and residues. Among
426
427 the group, the control of Fluc was observed with the strongest initial signal
428
429 (1.21×10¹⁰ RLU) but sharp decaying tendency, whilst Fluc-R-LRE demonstrated a
430
431 signal at 9.66×10⁹ RLU (79.83% of control) with no significant difference (P value was
432
433 0.1739 by T-test), which was 22.59% stronger than the free Fluc at the presence of
434
435 free LRE (7.88×10⁹ RLU, 65.12% of control, P value was 0.9633 to control and 0.1352
436
437 to Fluc-R-LRE). With the effect of peptide linker and the presence of fused LRE, the
438
439 other enzyme complex were observed to have a similar tendency but remarkably
440
441 lower Fluc activity than that of control, as was shown in the magnified view in Fig 2,
442
443 from high to low, respectively at the initial luminescence intensity of 1.15×10⁹ RLU to
444
445 Fluc-RSR-LRE (9.50% of control, P value was 0.0274), 6.96×10⁸ RLU to Fluc-SSS-LRE
446
447 (5.75% of control, P value was 0.0212), 6.36×10⁸ RLU to Fluc-S-LRE (5.26% of control,
448
449 P value was 0.0202), 6.01×10⁸ RLU to Fluc-RR-LRE (4.97% of control, P value was
450
451 0.0173), 4.83×10⁸ RLU to Fluc-RS-LRE (3.99% of control, P value was 0.0183),
452
453 3.27×10⁸ RLU to Fluc-SR-LRE (2.70% of control, P value was 0.0152), 3.01×10⁸ RLU to
454
455 Fluc-RRR-LRE (2.49% of control, P value was 0.0149), 2.40×10⁸ RLU to Fluc-SS-LRE
456
457 (1.98% of control, P value was 0.0139) and 1.32×10⁸ RLU to Fluc-SRS-LRE (1.09% of
458
459 control, P value was 0.0123). The results indicated that all the enzyme complex
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476 showed accordant decaying tendency to luminescence signal under the consumption
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478 of the substrate D-luciferin, and the peptide linker and residues on Fluc affected the
479
480 Fluc activity in most fusion construction, in which the type of one copy rigid linker
481
482 provided the optimal conformation for the activity domain.
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484

485 486 3.3 Effect of D-cysteine on *in vitro* bioluminescence 487

488 The substrate of D-cysteine for luciferin-regenerating was added to the purified
489
490 Fluc solution to assess its effect on the luminescence. As was shown in Fig 3, the
491
492 presence of D-cysteine increased the initial luminescence intensity generated by the
493
494 Fluc with 12.40% enhancement, in which there was no LRE existence, though the
495
496 signals both decayed and showed no difference (P value was 0.9911) in signal
497
498 emission.
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502 503 3.4 Effect of linker on LRE activity and substrate recycling 504

505 The luminescence intensity of the enzyme complexes were measured *in vitro* in
506
507 the presence of both D-luciferin and D-cysteine. The term of Luminescence changing
508
509 ratio was used to indicate the activities of LRE and assess the efficiency of luciferin-
510
511 recycling in different fusion complex.
512
513

514 As was shown in Fig 4, the changing ratio of Fluc (Panel B) verified the effect of
515
516 D-cysteine at the absence of LRE, with this as control, the higher changing ratio
517
518 revealed the higher activity of LRE on luciferin-regeneration that caused extra
519
520 luminescence signal, while the lower ratio demonstrated low activity of LRE and poor
521
522 efficiency in luciferin-recycling. In Panel A, the linear ratio on the complex of Fluc-
523
524 SRS-LRE and Fluc-SS-LRE demonstrated significantly higher effect of LRE than other
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535 complexes, which indicated that 2 copy number of flexible linker enabled the
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537 optimal structure of LRE on luciferin-regenerating. The other complexes in Panel B
538
539 were observed with similar tendency to that of control. Among which, the rigid linker
540
541 enhanced the activities of fused LRE along with the increasing copy number of rigid
542
543 linker, while the flexible linker SSS and S also promoted the LRE activity, which all
544
545 performed better than the free LRE and contributed extract signal during the 6-hour
546
547 test period. The mixed linker of RS demonstrated less but still positive effect on LRE
548
549 activity, while the linker of SR and RSR were observed to inhibit the LRE activity and
550
551 thus decreased the luminescence emission from the bifunctional enzyme.
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556 3.5 Structure feature and bioluminescence kinetics of bifunctional enzyme

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558
559 The structure feature of enzyme complexes were shown in Fig 5, the
560
561 luminescence signals indicated the activities of bifunctional enzyme complex whilst
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563 the difference of signals in the presence/absence of D-cysteine indicated the effect
564
565 of peptide linker to luciferin-recycling on *in vitro* bioluminescence.
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567

568
569 As for the series of rigid linker in Panel A, the intervention of luciferin-
570
571 regeneration did not significantly affect the initial luminescence from each enzyme
572
573 complex, but remarkably slow the decaying in signal recession. The luminescence
574
575 intensity was varied with the copy number of peptide linker in that the signal
576
577 became weaker along with the increasing of rigid linker copy number. In addition,
578
579 the enzyme fused with one copy of rigid linker (Fluc-R-LRE) demonstrated the
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581 optimal light emission, which was observed to have lower initial but higher
582
583 continuous signal than that of control with no linker mediation.
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594 As for the series of flexible linker in Panel B, the luciferin-recycling caused
595
596 extract signal to increase the luminescence emission, and slow the decaying of signal
597
598 recession. The enzyme complex mediated with 3 copies of flexible linker generated
599
600 higher initial luminescence while that with one copy of flexible linker provided more
601
602 continuous signal after 0.5 h. Remarkably, the enzyme fused through 2 copies of
603
604 flexible linker significantly increased the signal emission (P value was 0.0189), though
605
606 its signal was the lowest in the group, which indicated the high efficiency of luciferin-
607
608 recycling in this type of peptide linker.
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613 With regard to the series of mixed linker in Panel C, the fused enzyme complex
614
615 through the peptide linker RSR was observed to decrease of signal generation when
616
617 D-cysteine involving LRE catalyzed luciferin-regeneration (P value was 0.5129). The
618
619 enzyme complex of Fluc-RS-LRE and Fluc-SR-LRE both presented decreasing of
620
621 luminescence at the beginning but the luciferin-regenerating led to an enhancement
622
623 of signal emission, which made no difference in the luminescence generation (P
624
625 value were respectively 0.9527 and 0.5301). However, the complex of Fluc-SRS-LRE
626
627 generated significantly stronger signals when the bifunctional enzyme both worked
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629 (P value <0.0001).
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633

634
635 The bioluminescence kinetics of enzyme complex were analyzed as shown in
636
637 Table [32](#). The decaying of bioluminescent signals were nonlinearly growing with the
638
639 reaction period, the decay kinetics were satisfactorily fitted with a two-exponential
640
641 decay function and consequently described by two rate constants, K1 and K2 as
642
643 shown in Table [32](#). The initial light intensities and half-life of signals also indicated
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652
653 the Fluc-R-LRE as optimal structure on light emission while the Fluc-SRS-LRE was the
654
655 optimal one on luciferin-regeneration.
656

657 658 3.6 3D structural simulation of enzyme complex

659
660 The typical enzyme complexes were structural simulated as shown in Fig 6, and
661
662 the predicted results were consistent with the experimental data. The model of Fluc-
663
664 R-LRE with an average distance of all residue pairs in two structures (RMSD) at 10.3
665
666 $\pm 4.6\text{\AA}$ was observed with open LBD I and LBD II located in both Fluc and LRE. In the
667
668 model of Fluc-RSR-LRE (RMSD at $11.3 \pm 4.5\text{\AA}$), the LBD II in LRE was distant to the
669
670 Fluc domain, which might affect the substrate channeling and thus caused lower LRE
671
672 activity. The model of Fluc-SRS-LRE (RMSD at $11.8 \pm 4.5\text{\AA}$) was observed with a
673
674 covered LBD I in Fluc that might caused lower Fluc activity, but with wilder open LBD
675
676 I and LBD II in LRE for better luciferin-regenerating.
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681 682 3.7 Bioluminescent property of optimal enzyme complex in organic solvents

683
684 The bioluminescent property of Fluc-R-LRE was assessed respectively in the
685
686 solvent of DDT, EDTA, fucose, BSA, Triton X-100 and glycerinum (Fig 7). The results
687
688 demonstrated that the initial signal was enhanced in the presence of DDT (peaked at
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690 0.4 mmol/L), EDTA (peaked at 0.4 mmol/L), fucose (peaked at 0.8 mol/L) and BSA
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692 (peaked at 1.0 mg/L) respectively, whilst decreased along with the increasing
693
694 concentration of Triton X-100 and glycerinum, which indicated the component of
695
696 stabilizing agent for the bifunctional enzyme in further application.
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701 702 703 4. DISCUSSION

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712 LRE was reported to enhance the bioluminescence imaging by regenerating
713
714 luciferin for Fluc catalyzing light emission. Many researches such as codon
715
716 humanization and mutations [18-20], and the replacement of homogenous diverse
717
718 from other firefly species [17, 21-22], had been processed to increase emission
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720 intensity. However, the fast fading of signal catalyzed by free enzyme was still
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722 unsolved (shown as half-life in Table 32).
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725

726 In this study, a serial fused protein of Fluc and LRE mediated by different type of
727
728 peptide linker, were conducted to assess the effect of peptide linker on the enzyme
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730 activities and the bioluminescence imaging. The results of *in vitro* assessment
731
732 demonstrated a relationship of wane and wax between the activity of fused Fluc and
733
734 LRE, which indicated the competitive binding of oxyluciferin between Fluc and LRE.
735
736 Generally, the increasing unit of rigid linker caused decreasing of Fluc activity
737
738 (R>RR>RRR on Fluc activity in Fig 2 and Fig 5) but enhanced the LRE induced luciferin-
739
740 recycling (R<RR<RRR on LRE activity in Fig 4). As for the series of flexible linker, Fluc
741
742 activity peaked at one unit of flexible linker and was lowest at two units of flexible
743
744 linkers (S>SSS>SS in Fig 2 and Fig 5), on the contrary, LRE catalyzed luciferin-
745
746 regeneration peaked in Fluc-SS-LRE and bottomed in Fluc-S-LRE (S<SSS<SS in Fig 4).
747
748 As for the series of mixed linker, the rigid linker on C terminal of Fluc showed more
749
750 effectiveness on Fluc activity than the flexible linker (RSR>RS>SR>SRS in Fig 2), whilst
751
752 the LRE activity showed more effectiveness with flexible linker on the N terminal
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754 (SRS>SR>RS>RSR in Fig 4). Among the group, Fluc showed the highest activity in Fluc-
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756 R-LRE, whilst LRE present the most efficiency in luciferin-recycling in Fluc-SRS-LRE.
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771 The results ~~also~~ revealed that the Fluc activity was related to suitable space between
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773 functional domains by rigid linker, whilst the LRE activity was related to appropriate
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775 folding by the flexible linker. This deduction was consistent with the reported
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777 inhibition of firefly luciferase caused by oxyluciferin and dehydroluciferyl adenylate
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779 [23], that the spacer increase benefits the luciferyl adenylate [24] transferring and
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781 the light emission during coenzyme A (CoA) intermediated dehydrogenation into
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783 oxyluciferin [25].
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788 These findings were also verified on the predicted structural models (Fig 6). The
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790 biological functions of the enzyme complex were reflected by the exposure of
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792 substrate-binding sites and the interactions of luciferin-recycling between domains.
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794 The residues in these domains were also concerned in recent years to study the
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796 biological feature on bioluminescence imaging [5, 20].
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800 Unlike the products, such as oxyluciferin and dehydroluciferyl adenylate [26],
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802 and the substrate pyrophosphate and tripolyphosphate [27], as the inhibitor to the
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804 bioluminescence [28], ~~As~~ the regenerated luciferin supplemented the consumed
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806 substrate for Fluc, therefore the activity of fused LRE and the efficiency of luciferin-
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808 recycling were ~~also~~ important ~~for to~~ the stability of bioluminescence imaging. The
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810 role of LRE in luciferin-regeneration is still unclear [~~23~~29]. As the substrate involved
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812 the LRE catalyzed luciferin-regeneration, D-cysteine was reported to be
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814 characterized as a positive factor to Fluc light-generation in the absence of LRE
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816 [13,~~24~~30]. The results in this article also demonstrated that D-cysteine caused an
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818 extra increase of luminescence intensity to the Fluc structure (Fig 3). Besides, the
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830 optimal enzyme complex, Fluc-R-LRE, ~~performs~~performed better bioluminescence in
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832 the presence of 0.4 mmol/L DDT, 0.4 mmol/L EDTA, 0.8 mol/L fucose and 1.0 mg/L
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834 BSA, and presents negative effect by Triton X-100 and glycerin (Fig 7).
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837 The ATP *in vitro* detection was limited by the bioluminescence intensity and
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839 rapid decaying of signal. The intensity of signal refers to precision while the stability
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841 of signal refers to the accuracy of detection. In this study, the bifunctional enzyme
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843 complex with appropriate structure still generated detectable signal after 6 hours.
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845 Meanwhile, the intervention of fused LRE significantly improved the stability of
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847 luminescence and prolonged the half-life of signal, which might advance and expand
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849 the application of Fluc catalyzed bioluminescence imaging.
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856 5. CONCLUSION

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858 In this study, we demonstrated a novel strategy on fusion expression of Fluc
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860 and LRE to improve the bioluminescence imaging using a series of peptide linker. The
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862 effect of peptide linker on the bioluminescence imaging was analyzed according to
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864 the initial luminescence intensity, decaying kinetic of bifunctional enzyme complex,
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866 and the computer simulation of structural feature. The findings revealed the
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868 relationship of wane and wax between the activity of fused Fluc and LRE, which
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870 indicated the competitive binding of oxyluciferin between Fluc and LRE. The Fluc
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872 catalyzed light emission that determined the sensitivity of detection, and the activity
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874 was the highest in the space made by one copy number of rigid linker; whilst the LRE
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876 catalyzed luciferin regeneration that determined the durability of signal, and the
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889 activity was the most effective with the folding caused by two copy numbers of
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891 flexible linker. The bioluminescent property of the optimal structure of Fluc-R-LRE
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893 was observed with positive effects in DDT, EDTA, fucose and BSA, besides D-cysteine,
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895 and negative effects in Triton X-100 and glycerinum.
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915 **CONFLICT OF INTEREST**

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918 No conflict of interest exists in the submission of this manuscript.
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922 **AUTHOR CONTRIBUTION**

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924
925 XS and CX contributed to the conception of the study; XS performed the experiments,
926
927 analyzed the data and wrote the manuscript; BF helped design the enzyme
928
929 complexes shown in Table 1; PH helped design the experiments shown in Fig 2 & Fig
930
931 4; XT and PH contributed significantly to analysis the results; RH and MR helped
932
933 perform the experiments and analysis with constructive discussions. All authors
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935 reviewed the results and approved the final version of the manuscript.
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through direct effects of D-cysteine on luciferase structure and activity. *Photochem. Photobiol.*, 91(4), (2015), pp. 828–836.

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TABLE LEGENDS

~~Table 1. Sequences of PCR primers in this study.~~

Protein	Template	Primers	Sequences
Fluc	Fluc	Fluc-FusionP1	GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-FusionP2	CCCCTCGAGTTA CACGGCGATCTTGCCGCCCTT
LRE	Lre	LRE-P1	GGAATTCATATGGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCCCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc-LRE	Fluc	Fluc-FusionP1	GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-LRE-Rev-P2	CGATCTTTTCAACTACGGGGCCCATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCG CTCACAATTCCTTATATAGTGAGTCGTATTATTACAGGGCGATCTTGCCGC
	Lre	Fluc-LRE-For-P1	GCCGCAAGATCGCCGTGTAATAATAGGACTCACTATATAAGGGGAATTGTGAGCGGATAACAATTCCCCTCTA GAAATAATTTGTTTAACTTTAAGAAGGAGATGGGGCCCCGTAGTTGAAAAGATCG

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		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc-R-	Fluc	Fluc-FusionP1	GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-R-FusionP2	CGATCTTTTCAACTACGGGGGCCATTTTAGCAGCAGCTTCCACGGCGATCTTGCCGG
LRE	Lre	LRE-R-FusionP1	GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAAATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc-	Fluc	Fluc-FusionP1	GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RR-FusionP2	CGATCTTTTCAACTACGGGGGCCATTTTAGCAGCAGCTTCTTTAGCAGCAGCTTCCACGGCGATCTTGCCGG
RR-LRE	Lre	LRE-RR-FusionP1	GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAGAAGCTGCTGCTAAAATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc-	Fluc	Fluc-FusionP1	GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RRR-FusionP2	CGATCTTTTCAACTACGGGGGCCATTTTAGCAGCAGCTTCTTTAGCAGCAGCTTCTTTAGCAGCAGCTTCCACGG
RRR-		Fluc-RRR-FusionP2	CGATCTTGCCGG
LRE	Lre	LRE-RRR-FusionP1	GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAGAAGCTGCTGCTAAAATGGGCCCCG

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			TAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc-S-	Fluc	Fluc-FusionP1	GGAATTCATATG-GAAGATGCCAAAAACATTAAGAAGG
		Fluc-S-FusionP2	CGATCTTTTCAACTACGGGGCCCC<u>ATAGAACCACCACCACC</u>CACGGCGATCTTGCCGG
	LRE	LRE-S-FusionP1	GCGGCAAGATCGCCGTGGTGGTGGTGGTCTATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc-	Fluc	Fluc-FusionP1	GGAATTCATATG-GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SS-FusionP2	CGATCTTTTCAACTACGGGGCCCC<u>ATAGAACCACCACCACC</u>ATAGAACCACCACCACCACGGCGATCTTGCCGG
	SS-LRE	LRE-SS-FusionP1	GCGGCAAGATCGCCGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTCTATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc-	Fluc	Fluc-FusionP1	GGAATTCATATG-GAAGATGCCAAAAACATTAAGAAGG
			CGATCTTTTCAACTACGGGGCCCC<u>ATAGAACCACCACCACC</u>ATAGAACCACCACCACCATAGAACCACCACCAGG
	LRE	Fluc-SSS-FusionP2	CACGGCGATCTTGCCGG

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	Lre	LRE-SSS-FusionP1	GGGGCAAGATCGCCGTGGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTATGGGCCCCG
		LRE-P2	CGGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
	Fluc	Fluc-FusionP1	GGAATTCATATG-GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RS-FusionP2	CGATCTTTTCAACTACGGGGCCCATTTTAGCAGCAGCTTCAGAACCACCACCACCCACGGGGATCTTGCCGG
RS-LRE	Lre	LRE-RS-FusionP1	GGGGCAAGATCGCCGTGGAAGCTGCTGCTAAAGGTGGTGGTGGTTCTATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CGGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
	Fluc	Fluc-FusionP1	GGAATTCATATG-GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SR-FusionP2	CGATCTTTTCAACTACGGGGCCCATAGAACACCACCACCTTTAGCAGCAGCTTCACGGGGATCTTGCCGG
SR-LRE	Lre	LRE-SR-FusionP1	GGGGCAAGATCGCCGTGGGTGGTGGTGGTTCTGAAGCTGCTGCTAAAATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CGGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc	Fluc	Fluc-FusionP1	GGAATTCATATG-GAAGATGCCAAAAACATTAAGAAGG
RSR		Fluc-RSR-FusionP2	CGATCTTTTCAACTACGGGGCCCATTTTAGCAGCAGCTTCAGAACCACCACCACCTTTAGCAGCAGCTTCACG

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LRE			GCGATCTTGCCGG
	Lre	LRE-RSR-FusionP1	GCGGCAAGATCGCCCGTGGAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAATGGGCCCGG TAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG
Fluc-SRS-LRE	Fluc	Fluc-FusionP1	GGAATTCATATG-GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SRS-FusionP2	CGATCTTTTCAACTACGGGGCCCATAGAACCCACCACCCTTTAGCAGCAGCTTCAGAACCCACCACCACCACC
	Lre	LRE-SRS-FusionP1	GCGGCAAGATCGCCCGTGGAGCTGCTGCTAAAGAAGCTGCTGCTAAAGGTGGTGGTGGTTCTATGGGCCCGG TAGTTGAAAAGATCG
LRE-P2		CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAG	

Table 21. Construction and qualification of enzyme complex.

Fusion enzyme	sketch map	abbreviation	Amino acid sequence	Oligonucleotide sequence	Concentration (µg/ml)
Fluc	Fluc	/ /	/		597.26

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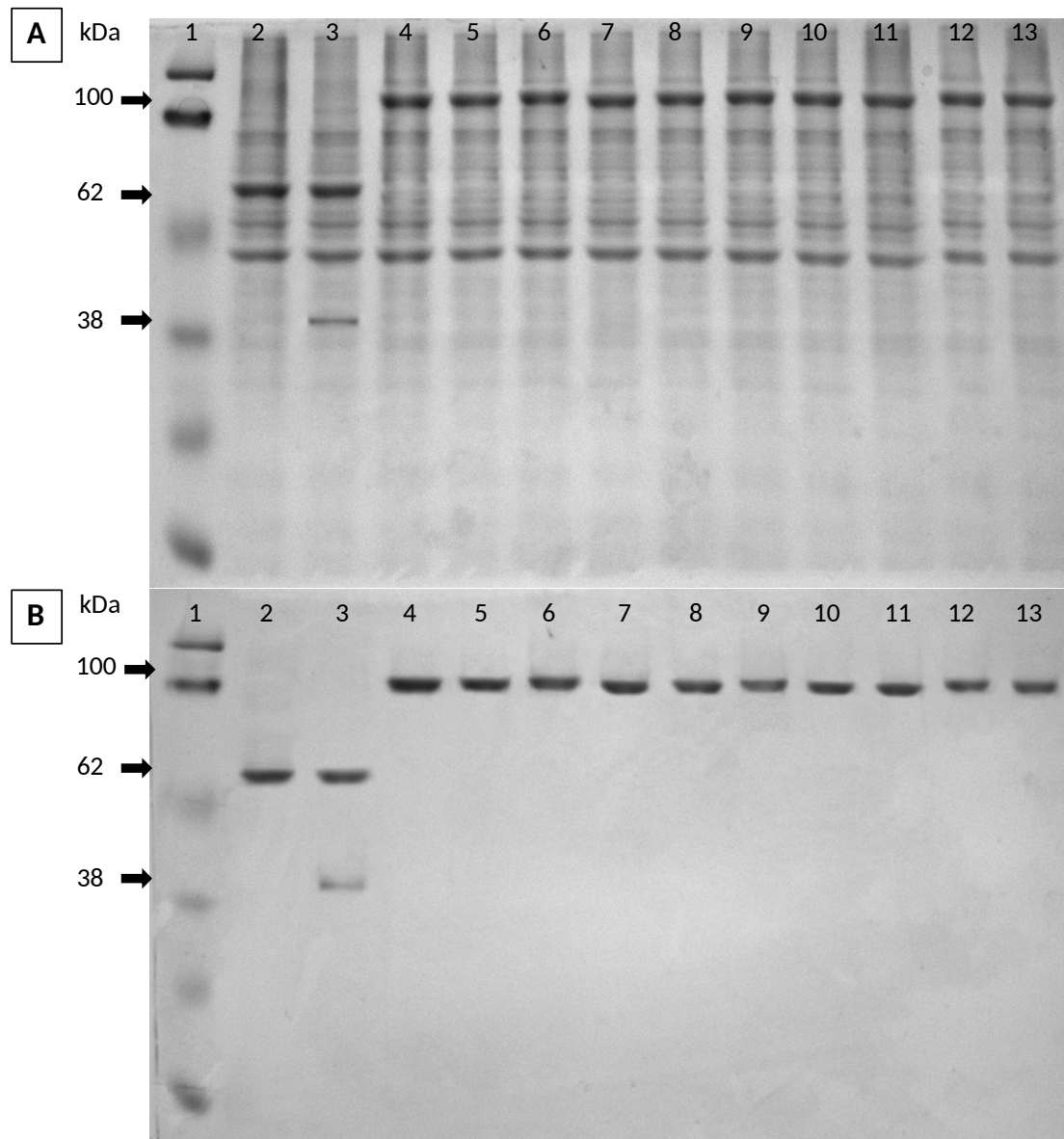
Fluc-LRE		/	/	/	594.04
Fluc-R-LRE		R	(EAAAK)	GAAGCTGCTGCTAAA	885.75
Fluc-RR-LRE		RR	(EAAAKEAAAK)	GAAGCTGCTGCTAAAGAAGCTGCT GCTAAA	580.60
Fluc-RRR-LRE		RRR	(EAAAKEAAAKE AAAK)	GAAGCTGCTGCTAAAGAAGCTGCT GCTAAAGAAGCTGCTGCTAAA	599.31
Fluc-S-LRE		S	(GGGGS)	GGTGGTGGTGGTTCT	610.30
Fluc-SS-LRE		SS	(GGGSGGGGS)	GGTGGTGGTGGTTCTGGTGGTGGT GGTTCT	551.22
Fluc-SSS-LRE		SSS	(GGGSGGGGSG GGGS)	GGTGGTGGTGGTTCTGGTGGTGGT GGTTCTGGTGGTGGTGGTTCT	581.81
Fluc-RS-LRE		RS	(EAAKGGGGS)	GAAGCTGCTGCTAAAGGTGGTGGT GGTTCT	648.61
Fluc-SR-LRE		SR	(GGGGSEAAAK)	GGTGGTGGTGGTTCTGAAGCTGCT GCTAAA	577.09
Fluc-RSR-LRE		RSR	(EAAKGGGGSE AAAK)	GAAGCTGCTGCTAAAGGTGGTGGT GGTTCTGAAGCTGCTGCTAAA	590.98
Fluc-SRS-LRE		SRS	(GGGGSEAAAK GGGGS)	GGTGGTGGTGGTTCTGAAGCTGCT GCTAAAGGTGGTGGTGGTTCT	959.96

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Table 32. Kinetic properties of enzyme complexes depending on enzyme structure.

Enzyme structure	Initial light intensity(RLU)	K1	K2	half-life (h ⁻¹)
Fluc	1.36E+10	1.526±0.056	9.617±0.632	0.14
Fluc-LRE	1.49E+10	3.888±1.106	1.061±0.138	0.37
R	1.06E+10	0.742±0.037	4.357±0.997	0.54
RR	5.99E+08	2.976±0.153	0.661±0.009	0.61
RRR	2.72E+08	0.403±0.016	1.705±0.061	0.64
S	6.66E+08	0.643±0.010	3.057±0.141	0.55
SS	3.21E+08	1.776±0.043	0.300±0.017	0.54
SSS	7.56E+08	2.683±0.114	0.643±0.020	0.44
RS	3.78E+08	0.865±0.015	3.710±0.484	0.61
SR	2.14E+08	4.359±0.532	0.787±0.012	0.64
RSR	9.92E+08	1.096±0.026	6.719±1.173	0.54
SRS	2.04E+08	0.292±0.005	4.803±2.948	2.20

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1548 **FIGURE LEGENDS**
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1585 Fig 1. SDS-PAGE analysis of the enzyme complex expressed with IPTG induction.
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1587 Panel A: crude samples; Panel B: purified samples. Lane 1, Protein Marker; lane 2,
1588 Fluc; lane 3, Fluc & LRE; lane 4, Fluc-R-LRE; lane 5, Fluc-RR-LRE; lane 6, Fluc-RRR-LRE;
1589 lane 7, Fluc-S-LRE; lane 8, Fluc-SS-LRE; lane 9, Fluc-SSS-LRE; lane 10, Fluc-RS-LRE; lane
1590 lane 11, Fluc-SR-LRE; lane 12, Fluc-RSR-LRE; lane 13, Fluc-SRS-LRE.
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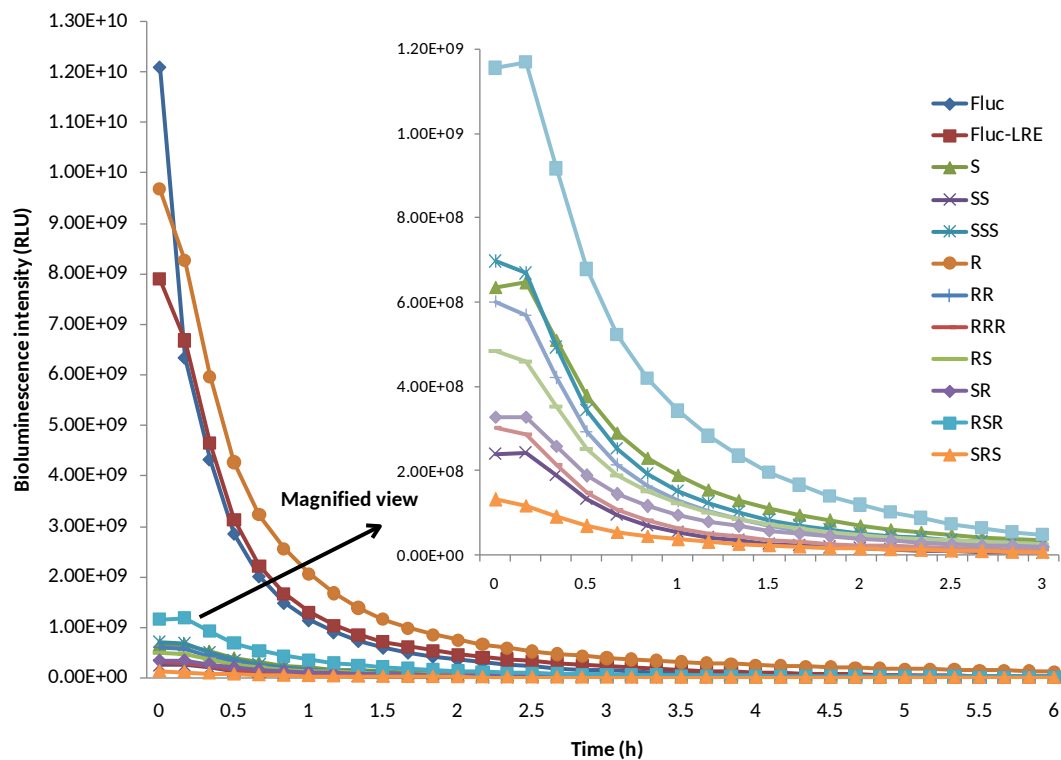


Fig 2. *In vitro* luminescence assessment of signal generated by purified enzyme complex in the absence of D-cysteine.

40 μL of purified enzyme was added to 160 μL reaction mixture containing with 0.25 mM D-luciferin, 2.5mM D-cysteine, 4 mM ATP and 10 mM MgSO_4 in microplate. The luminescence generated by each enzyme complex was measured by Tecan Infinite M200 Pro. at 37 $^\circ\text{C}$.

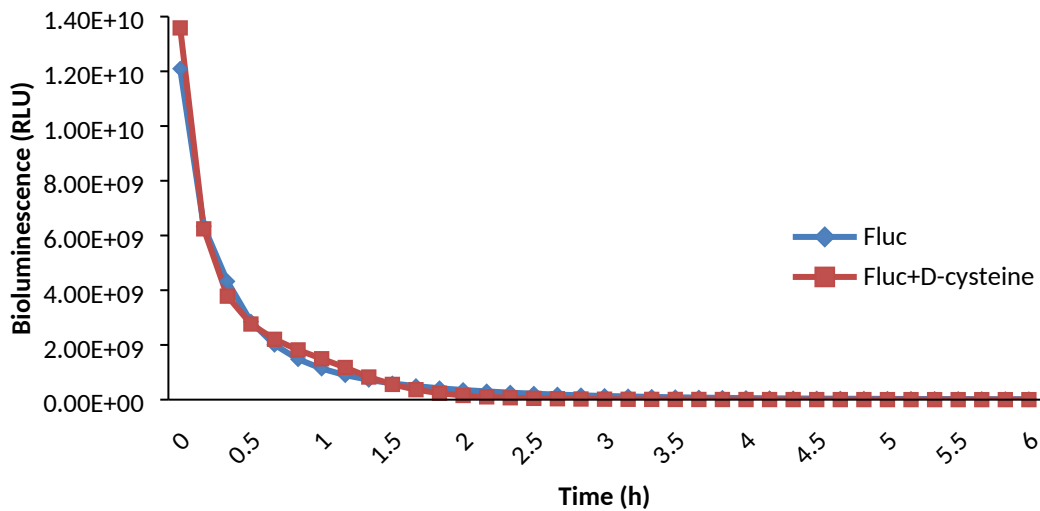
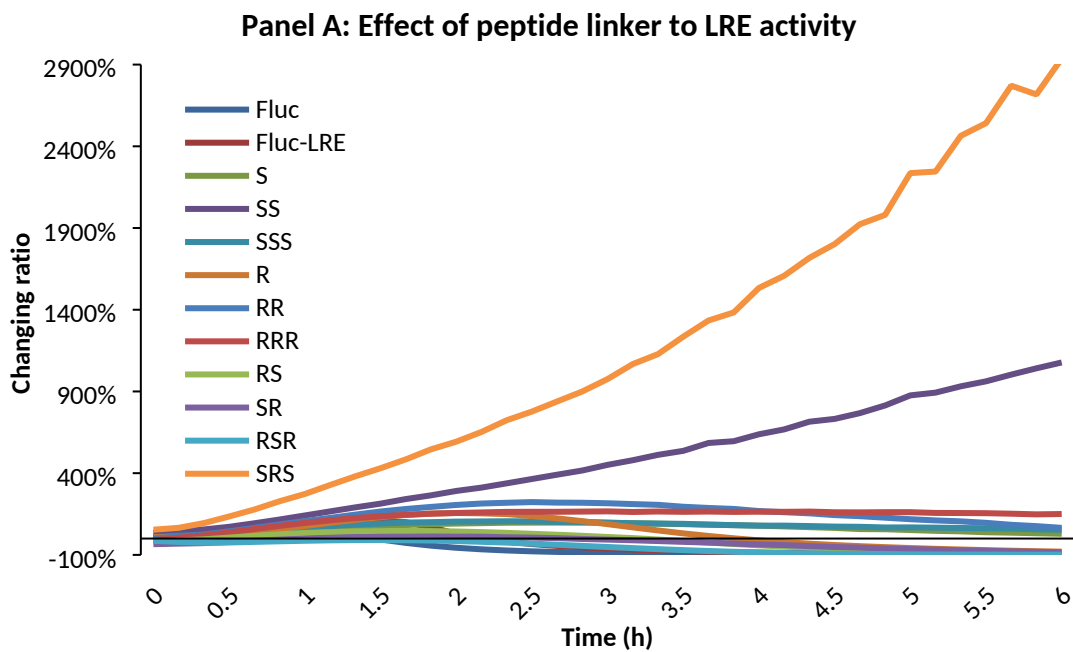


Fig 3. Effect of D-cysteine to the *in vitro* luminescence in the absence of LRE

2.5mM D-cysteine was added to the mixture of purified Fluc containing with 0.25 mM D-luciferin, 4 mM ATP and 10 mM MgSO₄. Luminescence generated by Fluc in the absence of D-cysteine and in the presence of D-cysteine was measured by Tecan Infinite M200 Pro. at 37 °C.



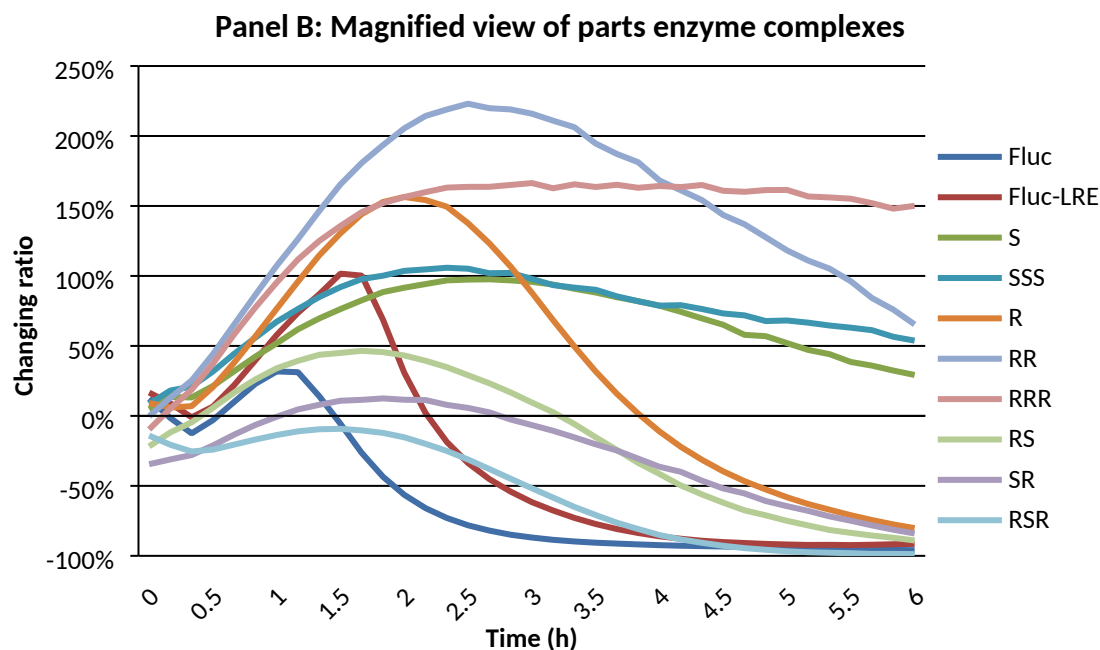
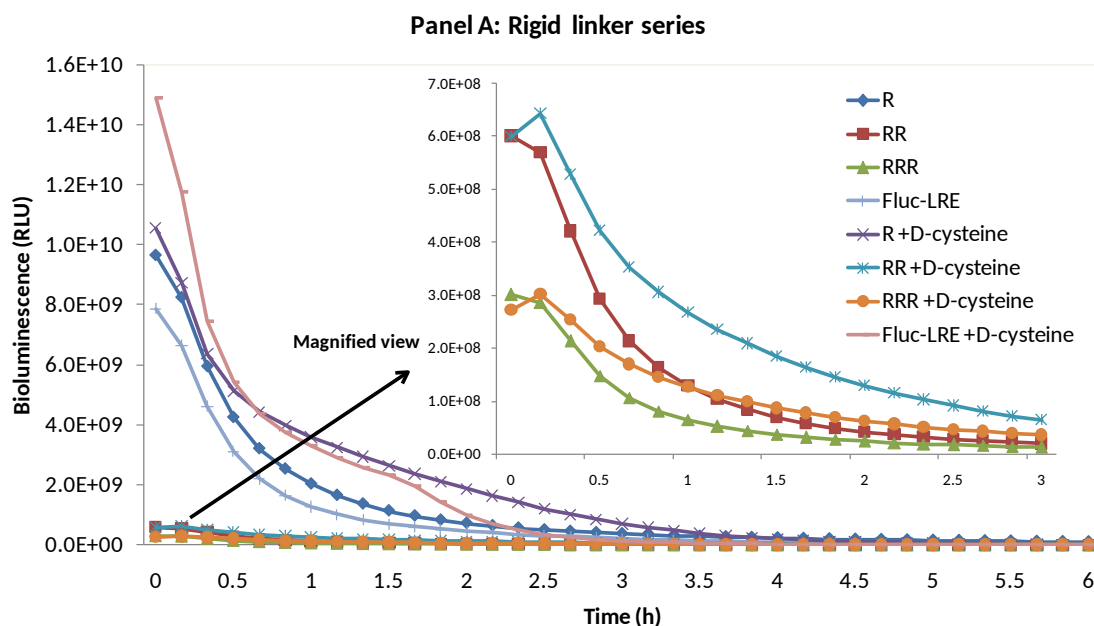


Fig 4. Effect of peptide linker on the *in vitro* activities of fused LRE and the luciferin-regeneration

0.25 mM D-luciferin and 2.5 mM D-cysteine were injected simultaneously to the enzyme mixture and trigger the catalytic reactions by both Fluc and LRE. The luminescence intensity was measured by Tecan Infinite M200 Pro. at 37 °C.



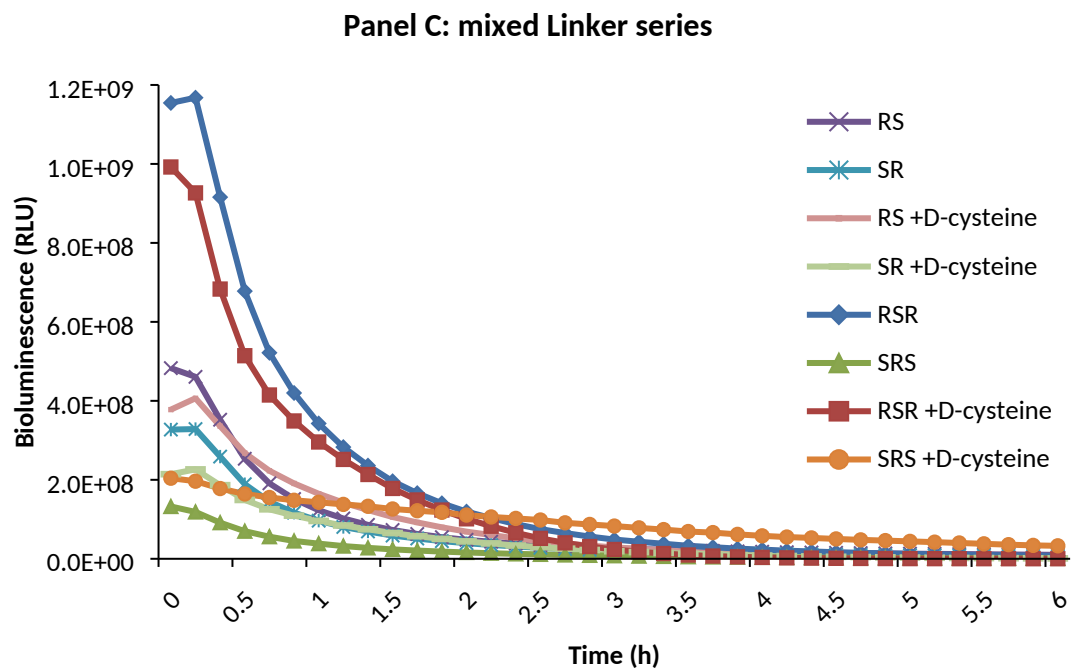
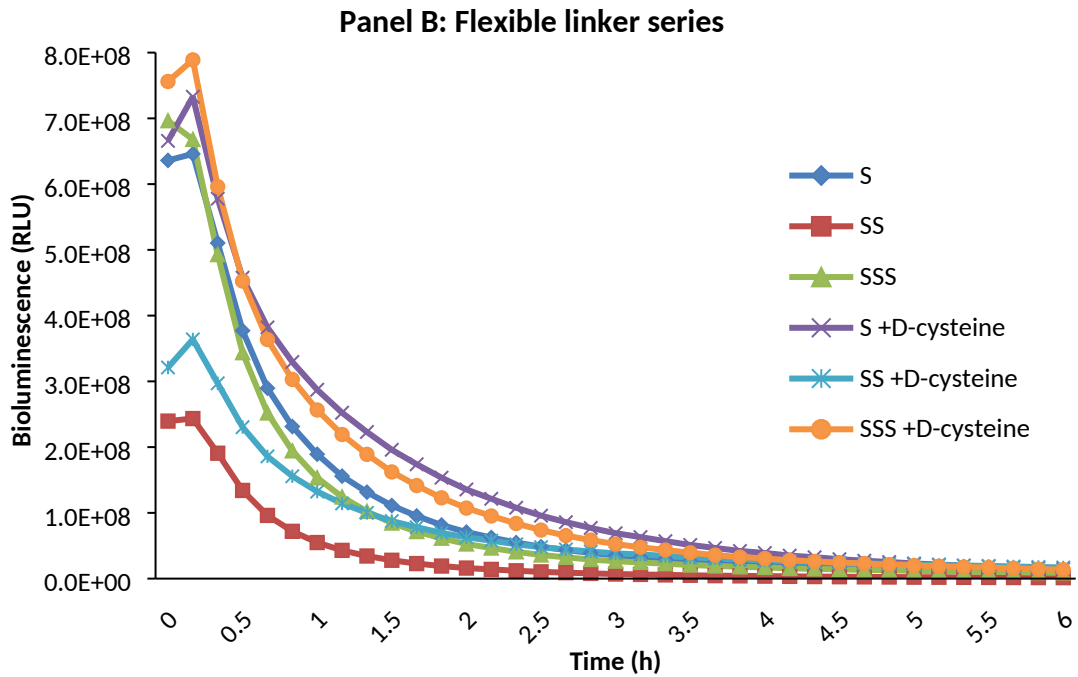


Fig 5. *In vitro* luminescence assessment of signal generated by purified Fluc-LRE enzyme complex in the presence of D-luciferin and D-cysteine.

40 μL of purified enzyme was added to 160 μL reaction mixture containing 0.25 mM D-luciferin, 2.5mM D-cysteine, 4 mM ATP and 10 mM MgSO_4 in microplate. The luminescence intensity was measured by Tecan Infinite M200 Pro. at 37 $^\circ\text{C}$.

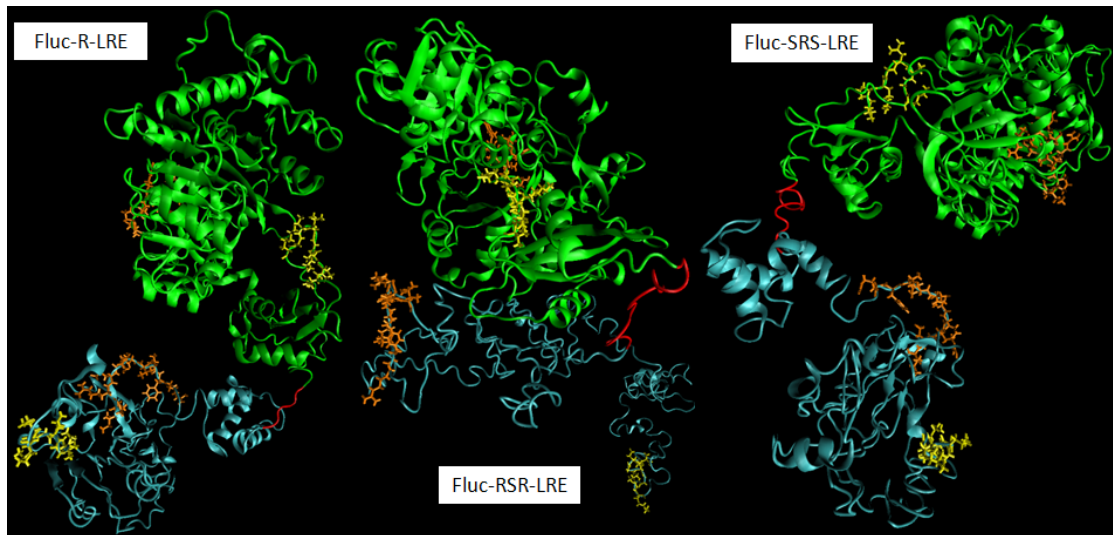
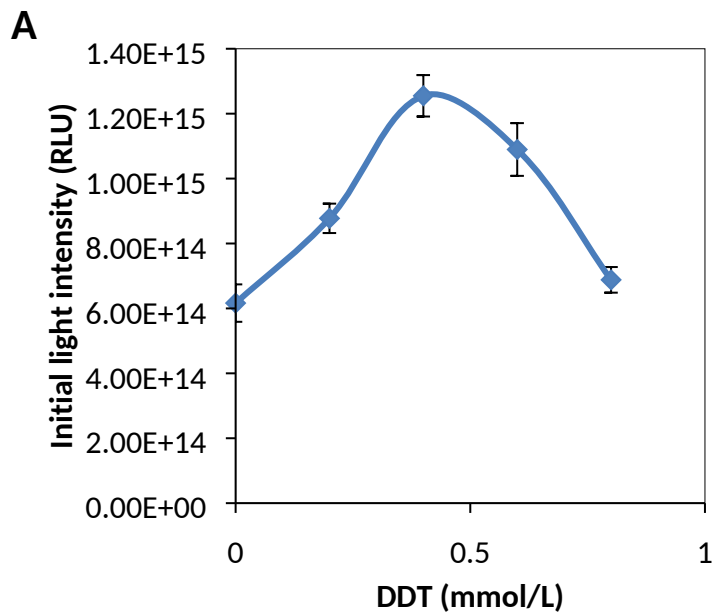
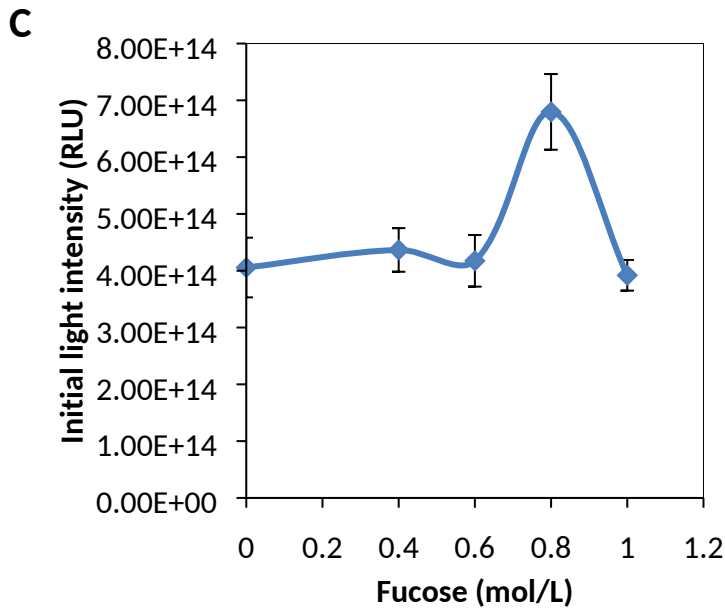
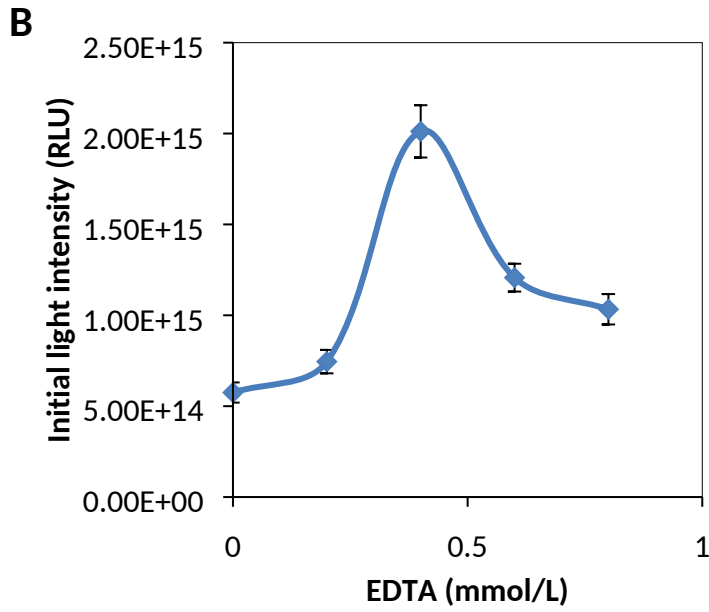


Fig 6. Enzyme complex structural models

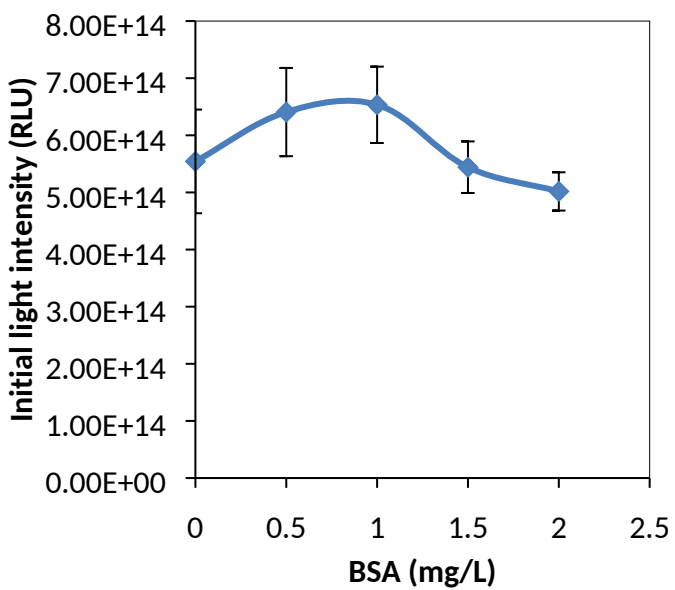
The 3D structure models of enzyme complexes were simulated and predicted by the I-TASSER server and analyzed by the software VMD 1.8.3. Three typical structures with highest confidence were present. The domain of Fluc (in Green), LRE (in Cyan), peptide linker (in Red), LBD I (in Orange) and LBD II (in Yellow) were labeled.



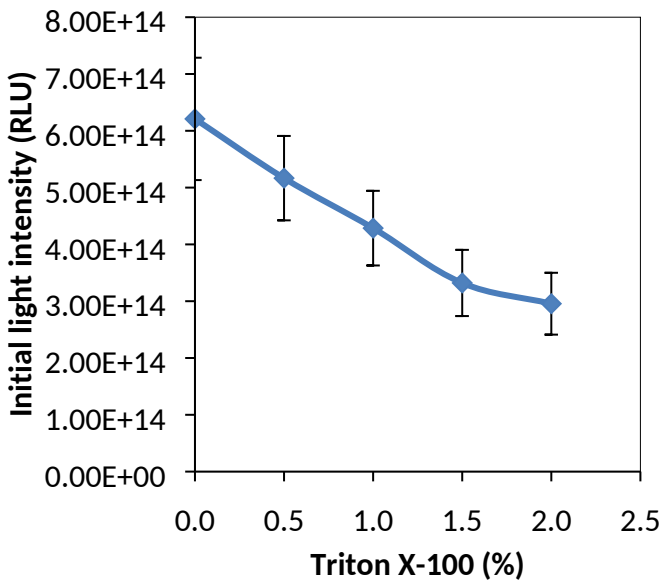


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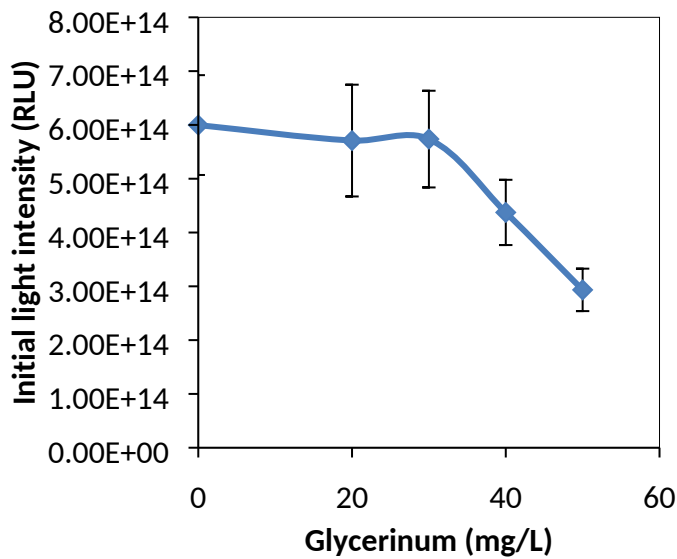


Fig 7. Effect of organic reagents to the bioluminescent property of optimal enzyme complex

Bioluminescent property of the optimal enzyme complex Fluc-R-LRE in organic reagents, different concentration of DDT (A), EDTA (B), fucose (C), BSA (D), Triton X-100 (E) and Glycerinum (F). 180 μ L of purified Fluc-R-LRE was added to 20 μ L reaction mixture of organic reagents containing 0.25 mM D-luciferin, 2.5mM D-cysteine, 4 mM ATP and 10 mM $MgSO_4$ in microplate. The luminescence intensity was measured by Tecan Infinite M200 Pro. at 37 $^{\circ}C$.

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Appendix

Table 1. Sequences of PCR primers in this study.

<u>Protein</u>	<u>Templa</u> <u>te</u>	<u>Primers</u>	<u>Sequences</u>
<u>Fluc</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
		<u>Fluc-FusionP2</u>	<u>CCGCTCGAGTTA CACGGCGATCTTGCCGCCCTT</u>
<u>LRE</u>	<u>Lre</u>	<u>LRE-P1</u>	<u>GGAATTCCATATGGGCCCGTAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-</u> <u>LRE</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
		<u>Fluc-LRE-Rev-P2</u>	<u>CGATCTTTTCAACTACGGGGCCCATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCG</u>
	<u>Lre</u>	<u>Fluc-LRE-For-P1</u>	<u>GCGGCAAGATCGCCGTGTAATAATACGACTCACTATATAAGGGGAATTGTGAGCGGATAACAATCCCCTCTA</u> <u>GAAATAATTTTGTTTAACTTTAAGAAGGAGATGGGCCCGTAGTTGAAAAGATCG</u>

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		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-R-</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
		<u>Fluc-R-FusionP2</u>	<u>CGATCTTTTCAACTACGGGGCCCATTTTAGCAGCAGCTTCCACGGCGATCTTGCCGC</u>
<u>LRE</u>	<u>Lre</u>	<u>LRE-R-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAAATGGGCCCGTAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-RR-LRE</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
		<u>Fluc-RR-FusionP2</u>	<u>CGATCTTTTCAACTACGGGGCCCATTTTAGCAGCAGCTTCTTTAGCAGCAGCTTCCACGGCGATCTTGCCGC</u>
	<u>Lre</u>	<u>LRE-RR-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAAGAAGCTGCTGCTAAAATGGGCCCGTAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-RRR-LRE</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
		<u>Fluc-RRR-FusionP2</u>	<u>CGATCTTTTCAACTACGGGGCCCATTTTAGCAGCAGCTTCTTTAGCAGCAGCTTCTTTAGCAGCAGCTTCCACGG</u>
	<u>Lre</u>	<u>LRE-RRR-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAAATGGGCCCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>

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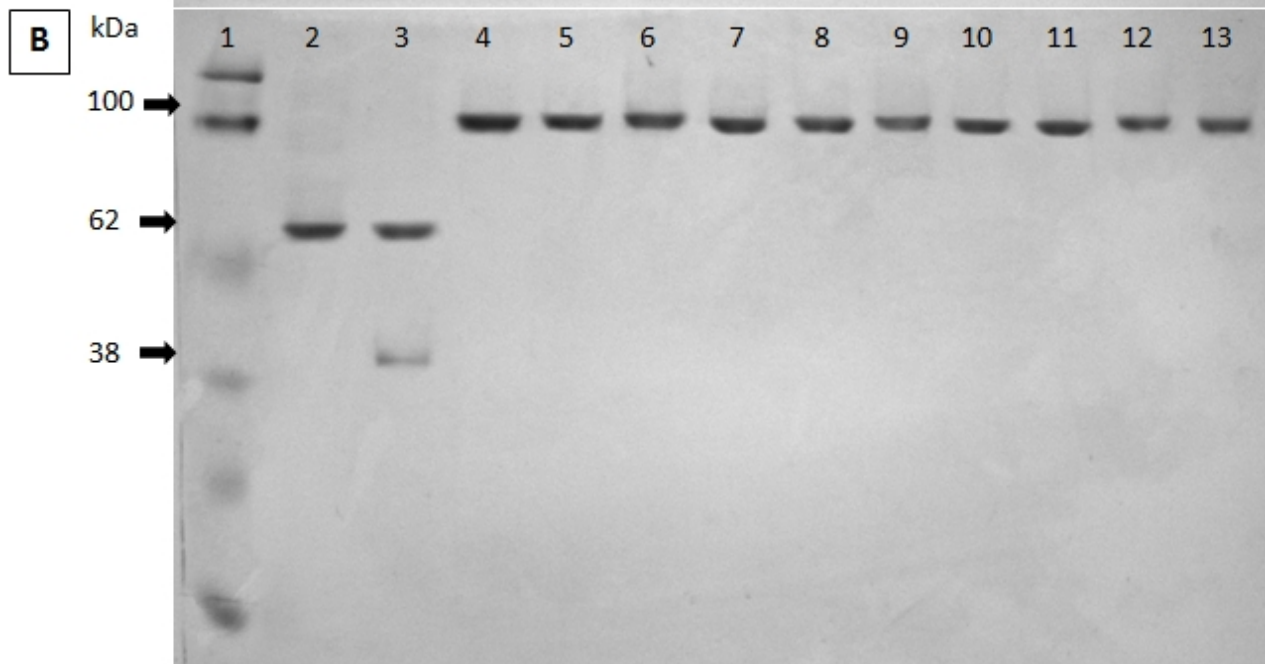
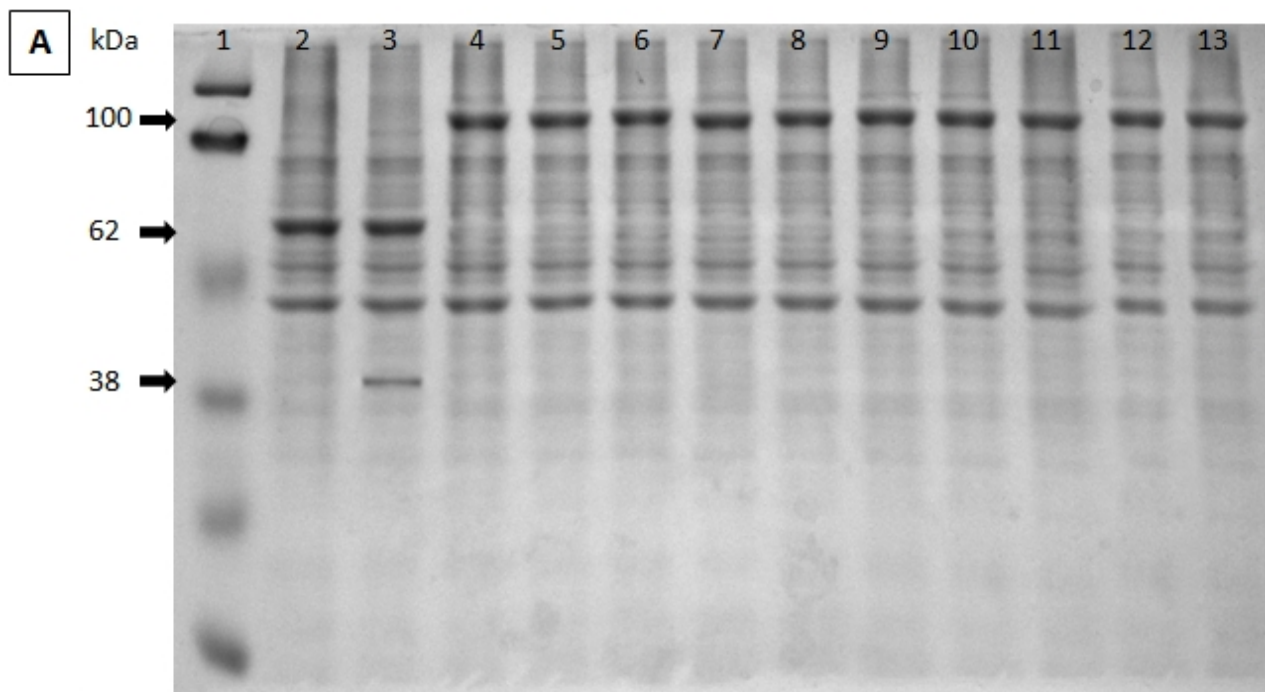
			<u>TAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-S-</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
		<u>Fluc-S-FusionP2</u>	<u>CGATCTTTTCAACTACGGGGCCCATAGAACCACCACCACCACGGCGATCTTGCCGC</u>
<u>LRE</u>	<u>Lre</u>	<u>LRE-S-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGTGGTGGTGGTCTATGGGCCCCGTAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
		<u>Fluc-SS-FusionP2</u>	<u>CGATCTTTTCAACTACGGGGCCCATAGAACCACCACCACCATAGAACCACCACCACCACGGCGATCTTGCCGC</u>
<u>SS-LRE</u>	<u>Lre</u>	<u>LRE-SS-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGTGGTGGTGGTCTGGTGGTGGTGGTCTATGGGCCCCGTAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
<u>SSS-</u>			<u>CGATCTTTTCAACTACGGGGCCCATAGAACCACCACCACCATAGAACCACCACCACCATAGAACCACCACCACC</u>
<u>LRE</u>		<u>Fluc-SSS-FusionP2</u>	<u>CACGGCGATCTTGCCGC</u>

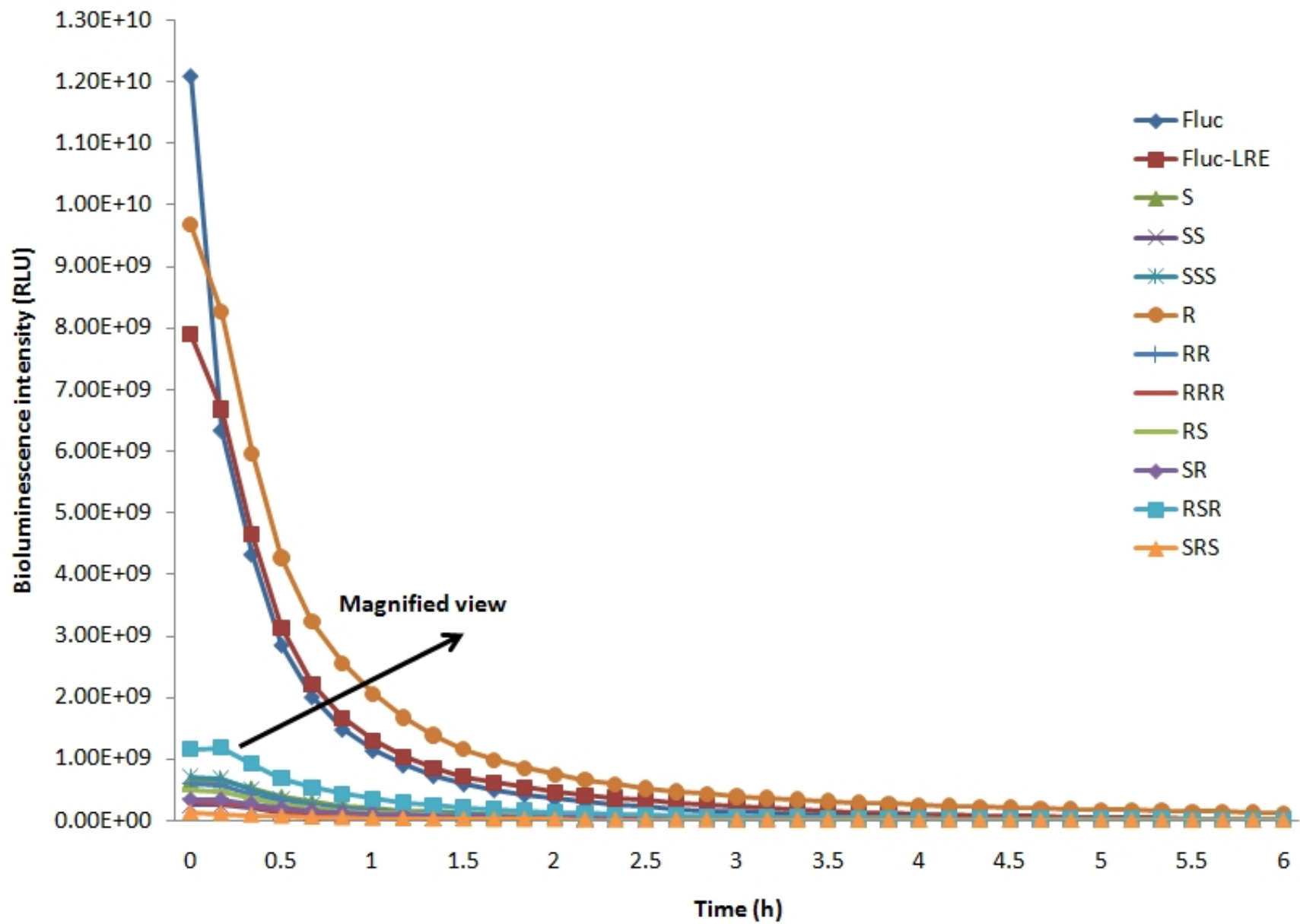
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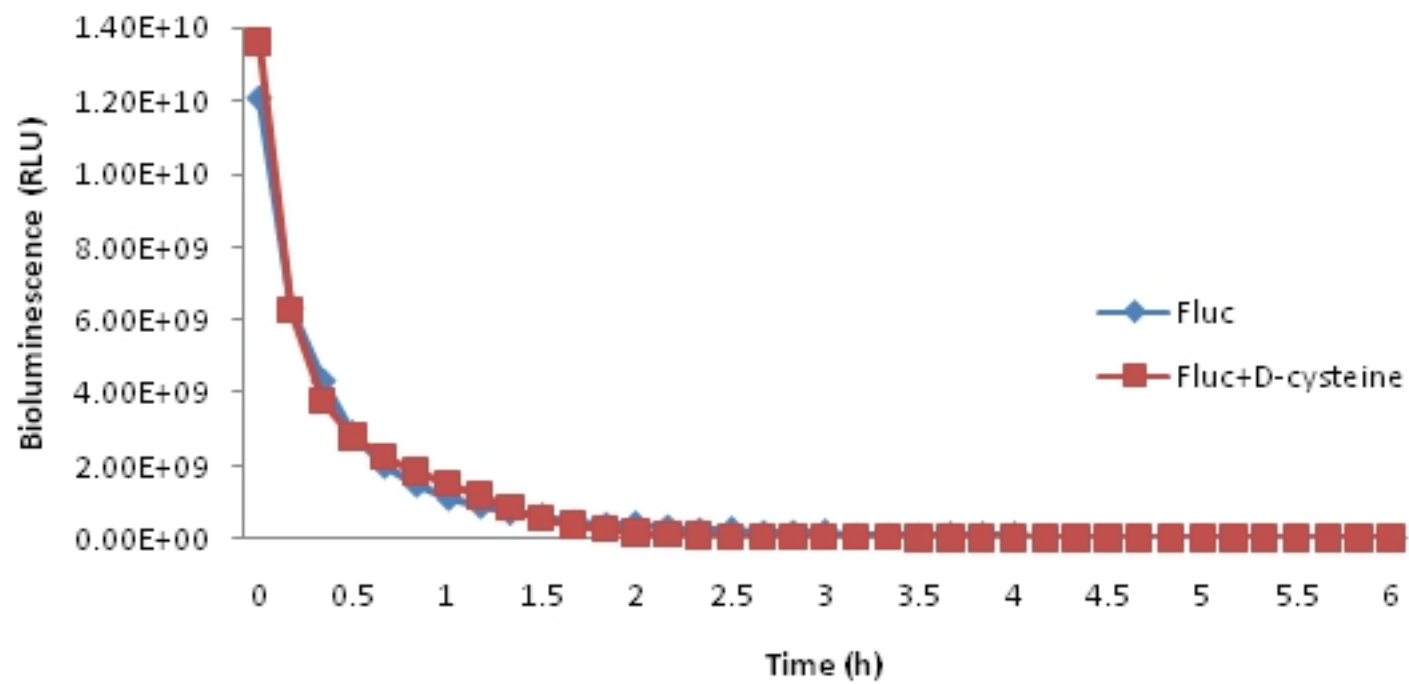
	<u>Lre</u>	<u>LRE-SSS-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTATGGGCCCCG</u>
		<u>LRE-P2</u>	<u>TAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
		<u>Fluc-RS-FusionP2</u>	<u>CGATCTTTTCAACTACGGGGCCCATTTTAGCAGCAGCTTCAGAACCACCACCACCGGCGATCTTGCCGC</u>
<u>RS-LRE</u>	<u>Lre</u>	<u>LRE-RS-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAAGGTGGTGGTGGTTCTATGGGCCCCGTAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
<u>Fluc-</u>		<u>Fluc-SR-FusionP2</u>	<u>CGATCTTTTCAACTACGGGGCCCATAGAACCACCACCACCTTTAGCAGCAGCTTCCACGGCGATCTTGCCGC</u>
<u>SR-LRE</u>	<u>Lre</u>	<u>LRE-SR-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGTGGTGGTGGTTCTGAAGCTGCTGCTAAAATGGGCCCCGTAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
<u>Fluc-</u>	<u>Fluc</u>	<u>Fluc-FusionP1</u>	<u>GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
<u>RSR-</u>		<u>Fluc-RSR-FusionP2</u>	<u>CGATCTTTTCAACTACGGGGCCCATTTTAGCAGCAGCTTCAGAACCACCACCACCTTTAGCAGCAGCTTCCACG</u>

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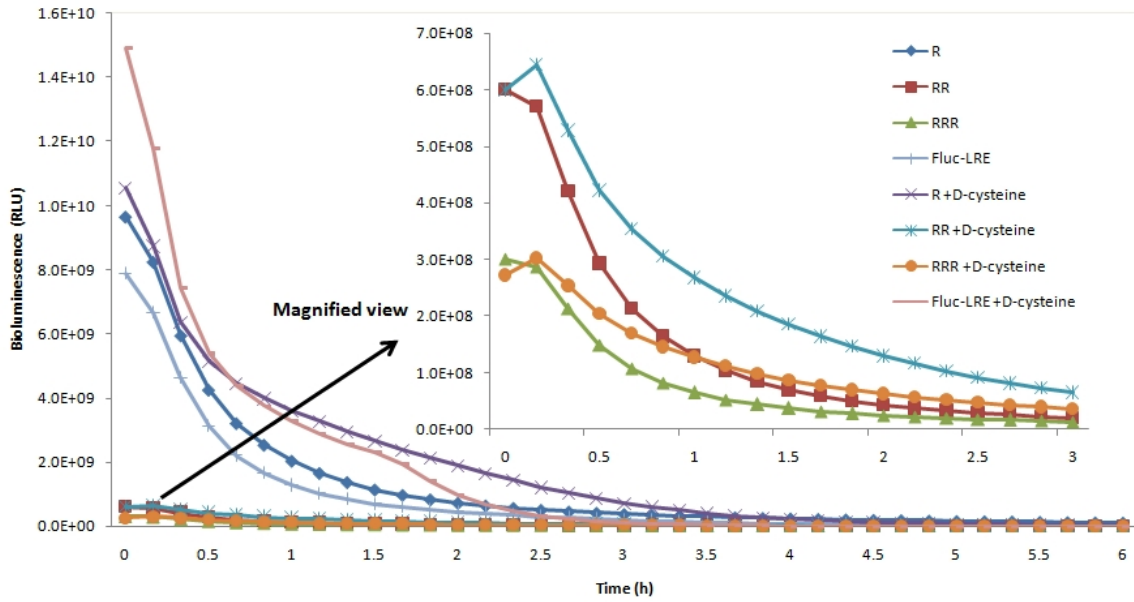
<u>LRE</u>			<u>GCGATCTTGCCGC</u>
	<u>Lre</u>	<u>LRE-RSR-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAAATGGGCCCG</u>
			<u>TAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>
		<u>Fluc-FusionP1</u>	<u>GGAATTCATATG GAAGATGCCAAAAACATTAAGAAGG</u>
<u>Fluc-</u>	<u>Fluc</u>		<u>CGATCTTTTCAACTACGGGGCCCATAGAACCACCACCACCTTTAGCAGCAGCTTCAGAACCACCACCACCCACG</u>
<u>SRS-</u>		<u>Fluc-SRS-FusionP2</u>	<u>GCGATCTTGCCGC</u>
<u>LRE</u>	<u>Lre</u>	<u>LRE-SRS-FusionP1</u>	<u>GCGGCAAGATCGCCGTGGAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGGTGGTGGTGGTTCTATGGGCCCG</u>
			<u>TAGTTGAAAAGATCG</u>
		<u>LRE-P2</u>	<u>CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC</u>



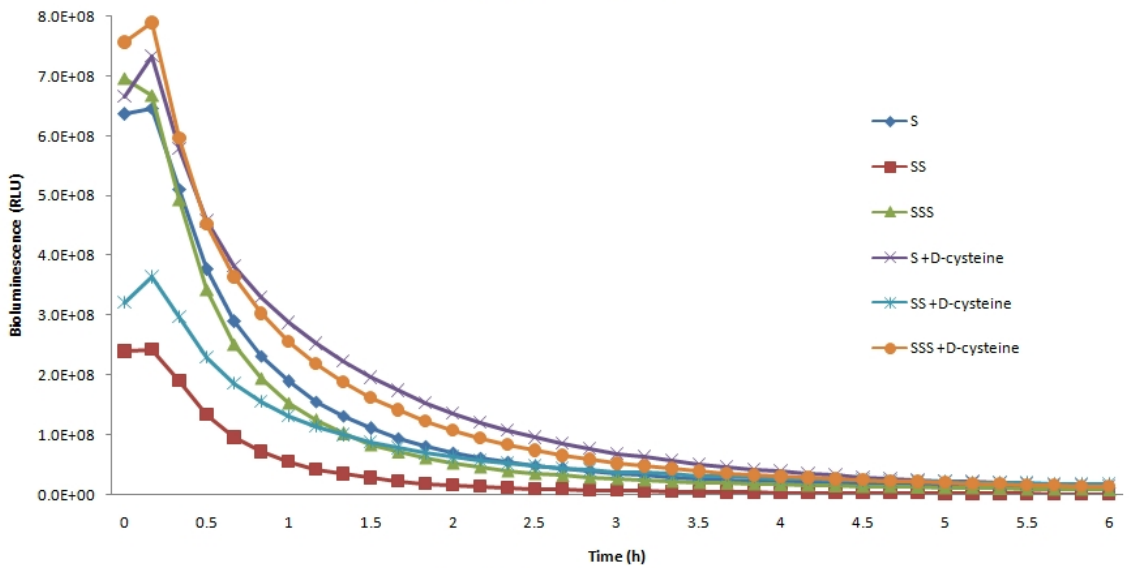




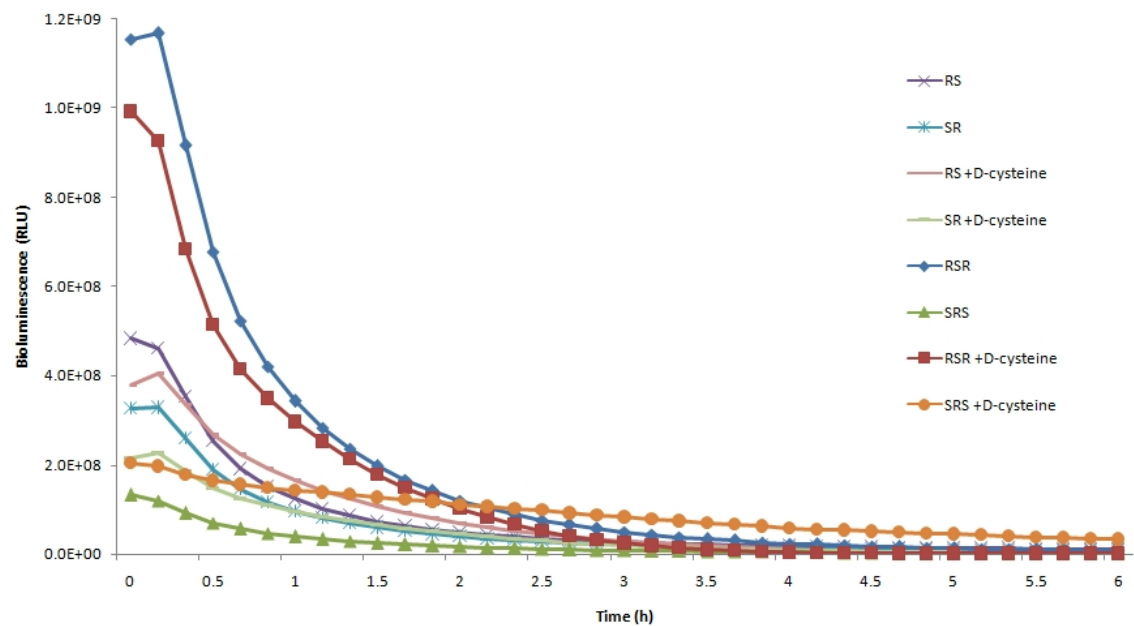
Panel A: Rigid linker series



Panel B: Flexible linker series



Panel C: mixed Linker series



Fluc-R-LRE



Fluc-SRS-LRE



Fluc-RSR-LRE



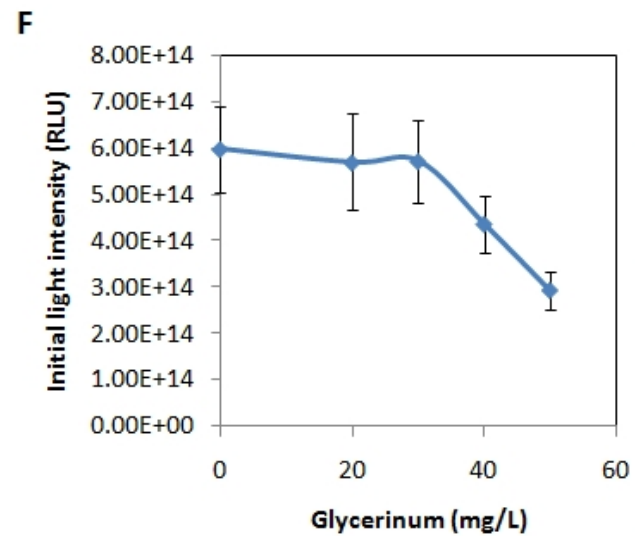
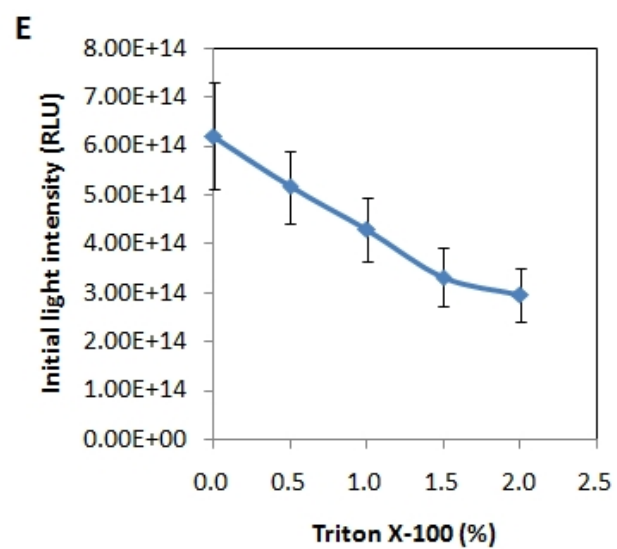
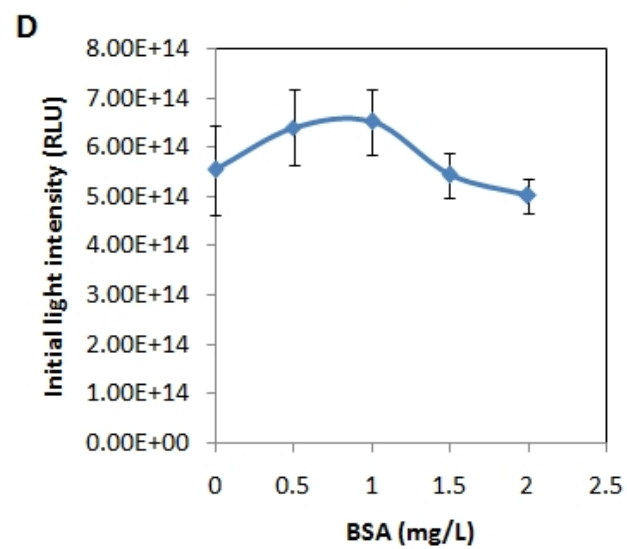
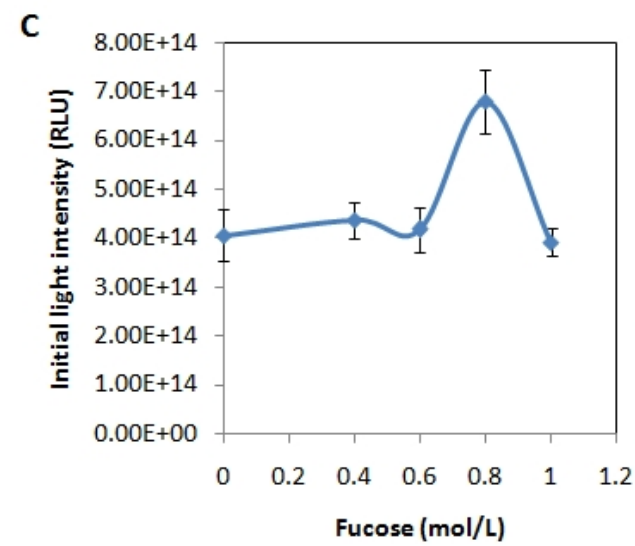
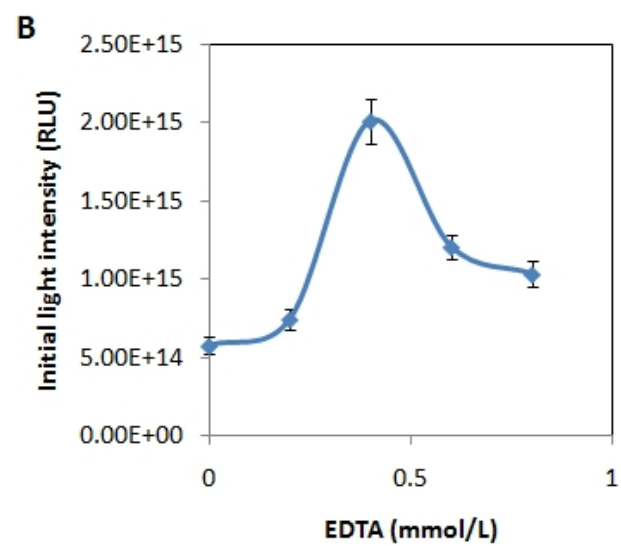
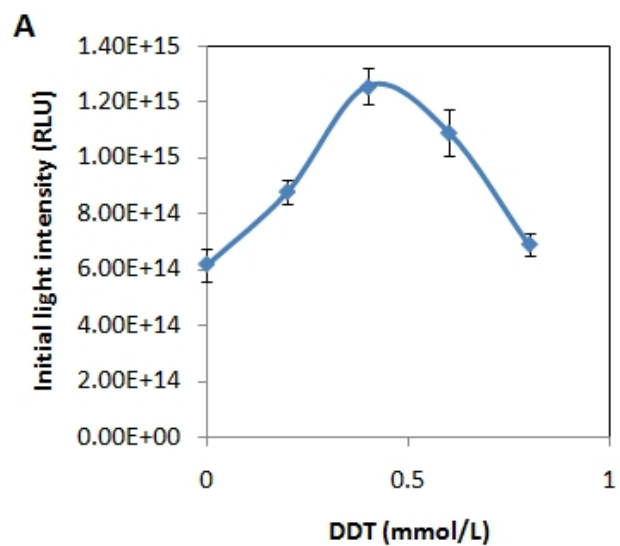



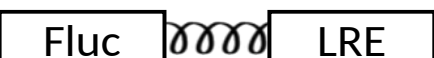
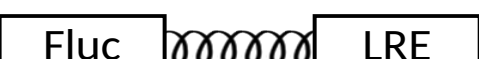
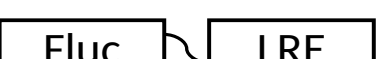




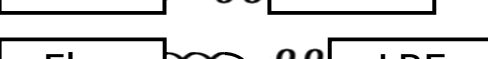
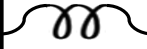


Table 1. Construction and qualification of enzyme complex.

Fusion enzyme	sketch map	abbreviation	Amino acid sequence	Oligonucleotide sequence	Concentration (µg/ml)
Fluc		/	/	/	597.26
Fluc-LRE		/	/	/	594.04
Fluc-R-LRE		R	(EAAAK)	GAAGCTGCTGCTAAA	885.75
Fluc-RR-LRE		RR	(EAAAKEAAAK)	GAAGCTGCTGCTAAAGAAGCTGCTGCTAAA	580.60
Fluc-RRR-LRE		RRR	(EAAAKEAAAKEAAAK)	GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAA	599.31
Fluc-S-LRE		S	(GGGGS)	GGTGGTGGTGGTTCT	610.30
Fluc-SS-LRE		SS	(GGGSGGGGS)	GGTGGTGGTGGTTCTGGTGGTGGTGGTTCT	551.22
Fluc-SSS-LRE		SSS	(GGGSGGGGSGGGGS)	GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTTCTGGTGGTGGTGGTTCT	581.81
Fluc-RS-LRE		RS	(EAAAKGGGGS)	GAAGCTGCTGCTAAAGGTGGTGGTGGTTCT	648.61
Fluc-SR-LRE		SR	(GGGGSEAAAK)	GGTGGTGGTGGTTCTGAAGCTGCTGCTAAA	577.09
Fluc-RSR-LRE		RSR	(EAAAKGGGGSEAAAK)	GAAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAA	590.98

Fluc-SRS-
LRE

Fluc



LRE

SRS

(GGGGSEAAK
GGGS)

GGTGGTGGTGGTTCTGAAGCTGCT
GCTAAAGGTGGTGGTGGTTCT

959.96

Table 2. Kinetic properties of enzyme complexes depending on enzyme structure.

Enzyme structure	Initial light intensity(RLU)	K1	K2	half-life (h ⁻¹)
Fluc	1.36E+10	1.526±0.056	9.617±0.632	0.14
Fluc-LRE	1.49E+10	3.888±1.106	1.061±0.138	0.37
R	1.06E+10	0.742±0.037	4.357±0.997	0.54
RR	5.99E+08	2.976±0.153	0.661±0.009	0.61
RRR	2.72E+08	0.403±0.016	1.705±0.061	0.64
S	6.66E+08	0.643±0.010	3.057±0.141	0.55
SS	3.21E+08	1.776±0.043	0.300±0.017	0.54
SSS	7.56E+08	2.683±0.114	0.643±0.020	0.44
RS	3.78E+08	0.865±0.015	3.710±0.484	0.61
SR	2.14E+08	4.359±0.532	0.787±0.012	0.64
RSR	9.92E+08	1.096±0.026	6.719±1.173	0.54
SRS	2.04E+08	0.292±0.005	4.803±2.948	2.20

Table 1. Sequences of PCR primers in this study.

Protein	Template	Primers	Sequences
Fluc	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-FusionP2	CCGCTCGAGTTA CACGGCGATCTTGCCGCCCTT
LRE	<i>Lre</i>	LRE-P1	GGAATTCCATATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc- LRE	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-LRE-Rev-P2	CGATCTTTTCAACTACGGGGCCCATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCG CTCACAATCCCCTTATATAGTGAGTCGTATTATTACACGGCGATCTTGCCGC
LRE	<i>Lre</i>	Fluc-LRE-For-P1	GCGGCAAGATCGCCGTGTAATAATACGACTCACTATATAAGGGGAATTGTGAGCGGATAACAATCCCCTCTA GAAATAATTTGTTAACTTTAAGAAGGAGATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC

Fluc-R-	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-R-FusionP2	CGATCTTTTCAACTACGGGGCCCAT <u>TTTAGCAGCAGCTTCC</u> CACGGCGATCTTGCCGC
LRE	<i>Lre</i>	LRE-R-FusionP1	GCGGCAAGATCGCCGTG <u>GAAGCTGCTGCTAAA</u> ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT <u>TTTAGCAGCAGCTTCTTTAGCAGCAGCTTCC</u> CACGGCGATCTTGCCGC
RR-LRE	<i>Lre</i>	LRE-RR-FusionP1	GCGGCAAGATCGCCGTG <u>GAAGCTGCTGCTAAAGAAGCTGCTGCTAAA</u> ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc- RRR-	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RRR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT <u>TTTAGCAGCAGCTTCTTTAGCAGCAGCTTCTTTAGCAGCAGCTTCC</u> CACGG
LRE	<i>Lre</i>	LRE-RRR-FusionP1	GCGGCAAGATCGCCGTG <u>GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAA</u> ATGGGCCCCG TAGTTGAAAAGATCG

		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-S-	Fluc	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-S-FusionP2	CGATCTTTTCAACTACGGGGCCC <u>ATAGAACCACCACCACC</u> CACGGCGATCTTGCCGC
LRE	Lre	LRE-S-FusionP1	GCGGCAAGATCGCCGTG <u>GGTGGTGGTGGTTCT</u> ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-	Fluc	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SS-FusionP2	CGATCTTTTCAACTACGGGGCCC <u>ATAGAACCACCACCACCATAGAACCACCACCACC</u> CACGGCGATCTTGCCGC
SS-LRE	Lre	LRE-SS-FusionP1	GCGGCAAGATCGCCGTG <u>GGTGGTGGTGGTTCTGGTGGTGGTGGTTCT</u> ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SSS-LRE	Fluc	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SSS-FusionP2	CGATCTTTTCAACTACGGGGCCC <u>ATAGAACCACCACCACCATAGAACCACCACCACCATAGAACCACCACCACC</u> CACGGCGATCTTGCCGC
	Lre	LRE-SSS-FusionP1	GCGGCAAGATCGCCGTG <u>GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT</u> ATGGGCCCCG

			TAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RS-FusionP2	CGATCTTTTCAACTACGGGGCCCAT <u>TTTAGCAGCAGCTTCAGAACCACCACCACC</u> CACGGCGATCTTGCCGC
RS-LRE	<i>Lre</i>	LRE-RS-FusionP1	GCGGCAAGATCGCCGTG <u>GAAGCTGCTGCTAAAGGTGGTGGTGGTCT</u> ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT <u>AGAACCACCACCACCTTTAGCAGCAGCTTCC</u> CACGGCGATCTTGCCGC
SR-LRE	<i>Lre</i>	LRE-SR-FusionP1	GCGGCAAGATCGCCGTG <u>GGTGGTGGTGGTCTGAAGCTGCTGCTAAA</u> ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
RSR-			CGATCTTTTCAACTACGGGGCCCAT <u>TTTAGCAGCAGCTTCAGAACCACCACCACCTTTAGCAGCAGCTTCC</u> CACG
LRE		Fluc-RSR-FusionP2	GCGATCTTGCCGC

	<i>Lre</i>	LRE-RSR-FusionP1	GCGGCAAGATCGCCGTG <u>GAAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAA</u> ATGGGCCCCG TAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc- SRS- LRE	<i>Fluc</i>	Fluc-FusionP1	GGAATTCCATATG GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SRS-FusionP2	CGATCTTTTCAACTACGGGGCCCAT <u>AGAACCACCACCACCTTTAGCAGCAGCTTCAGAACCACCACCACCACG</u> GCGATCTTGCCGC
	<i>Lre</i>	LRE-SRS-FusionP1	GCGGCAAGATCGCCGTG <u>GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGGTGGTGGTGGTTCT</u> ATGGGCCCCG TAGTTGAAAAGATCG
		LRE-P2	CCGCTCGAGTTACAATTTAACTTTAACACCAGCAAAACCTTTCAC