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Red-emitting chimeric firefly luciferase for *in vivo* imaging in low ATP cellular environments

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Abstract

Beetle luciferases have been adapted for live cell imaging where bioluminescence is dependent on the cellular availability of ATP, O₂, and added luciferin. Previous *Photinus pyralis* red-emitting variants with high K_m values for ATP have performed disappointingly in live cells despite having much higher relative specific activities than enzymes like Click Beetle Red (CBR). We engineered a luciferase variant PLR3 having a K_m value similar to CBR and ~2.6-fold higher specific activity. The red-emitting PLR3 was ~2.5-fold brighter than CBR in living HEK293T and HeLa cells, an improvement consistent with the importance of the K_m value in low ATP environments.

Keywords

Bioluminescence; firefly; luciferase; ATP; red-emitting; imaging

Bioluminescent proteins, epitomized by the beetle luciferases (Lucs), are now proven reagents for noninvasive imaging studies. As reporters, bioluminescent enzymes can visualize genetic activity and many other cellular biochemical events; while in living animals, they can be used to track specific types of cells including tumors [1–5]. Major reasons for the current success of bioluminescence imaging (BLI) include: the great detectability (signal to noise) due to essentially nonexistent endogenous background; wide dynamic range; and the availability of reasonably priced commercial CCD-based detection devices [1–3]. Moreover, a particular advantage of the beetle Lucs, which produce light by oxidizing the luciferin substrate (LH₂) in reactions that also require Mg-ATP and molecular

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¹Abbreviations used: BLI, bioluminescence imaging; CBR, Promega's click beetle red, recombinant *Pyrophorus plagiophthalmus* luciferase (GenBank: AY258591); LH₂, D-firefly luciferin; Luc, luciferase; Luc2, Promega's *Photinus pyralis*-based luciferase (GenBank: AY738222); PLG2, recombinant *P. pyralis* luciferase variant (GenBank: KY486507); PLR3, recombinant *P. pyralis* luciferase variant (GenBank: KY486508); PpyRE9, recombinant *P. pyralis* luciferase variant (GenBank: GQ404466); and RLU, relative light units. All luciferases were expressed from the human codon optimized sequences indicated above.

Competing interests statement

The authors declare no competing interests.

O₂, is that there are reliable protocols for introducing LH₂ into cultured living cells and live animals [6].

Mainly for reasons of improved detectability that can be achieved by the more efficient transmission of light through animal tissues, light emission at wavelengths greater than 600 nm is highly advantageous [7, 8]. For this reason, there is great interest in developing Luc variants and LH₂ substrate analogs that efficiently produce red bioluminescence [9–13]. Building on our experience making *Photinus pyralis* Luc variants with altered emission properties, we made a thermostable variant called PpyRE9 that produces red-shifted bioluminescence ($\lambda_{\text{max}} = 617$ nm) with beetle LH₂ at 25 °C and 37 °C [9]. In soluble lysates of HEK293T cells using optimized assay conditions, including substrate concentrations, the intensity of the stable PpyRE9 emission was ~90-fold greater than that of CBR, a commercial click beetle Luc. CBR bioluminescence is a good standard for comparison because the cDNA is commercially available in plasmids containing several promoters, it emits red-shifted bioluminescence ($\lambda_{\text{max}} = 619$ nm) with beetle LH₂, is a popular genetic reporter, and performs well in BLI studies [5, 14, 15].

Methods have been developed by Kelly and Taylor for highly sensitive BLI of *Trypanosoma brucei* [4] with PpyRE9. Subsequent studies have extended the utility of PpyRE9 for BLI-based studies of parasite infections [5, 16–18]. It was very encouraging to learn that in *in vivo* BLI studies of mice infected with *T. brucei*, PpyRE9 provided 10- to 20-fold higher signals than CBR [5]. BLI results of transplanted stem cells [19] and HEK293 cells [20] in mouse brain, however, indicated that while PpyRE9 provided more stable signals over a wide pH range than Luc2, it produced ~4-fold less intense light emission. In our own model BLI experiments using living HEK293T cells, PpyRE9 light emission was barely (1.3-fold) greater than that of CBR (Fig. 1A) despite its ~9-fold higher *in vitro* specific activity at pH 7.4 (Table 1). In these and subsequent experiments, equal numbers of living cells that expressed the human codon optimized *luc* genes in the pF4Ag vector were treated with 5 mM LH₂ [21]. With these standardized conditions, it was possible to make meaningful comparisons of signal intensity and stability.

We noted that *in vitro* at pH 7.4, PpyRE9 had elevated K_m values for LH₂ (~3-fold) and Mg-ATP (~90-fold) compared to CBR (Table 1). Recognizing that in BLI Lucs may have to function in environments with very low ATP concentrations, we considered that the disappointing performance of PpyRE9 in HEK293T cells might be related to the K_m values, and that the ATP value could be especially problematic since ATP cytoplasmic levels are ~5 fmol per viable cell [21, 22]. We therefore focused on producing a Luc variant with K_m values nearer to those of CBR without seriously compromising the favorable specific activity, emission color, and thermostability of PpyRE9. Because PpyRE9 was so highly optimized from *P. pyralis* Luc, we undertook mutagenesis studies on a new template called PLG2 [21], which is a thermostable and specific activity enhanced green ($\lambda_{\text{max}} = 559$ nm) light-emitting Luc that was engineered from a chimeric protein consisting of the large N-terminal domain of *P. pyralis* Luc fused to the small C-terminal domain of *Luciola italica* Luc. After numerous mutagenesis studies, we succeeded in transforming PLG2 into a novel Luc variant called PLR3 with the introduction of 5 amino acid changes (Supplementary Table S1). While PLR3 maintained the excellent thermostability of PpyRE9 that is important

for good expression and stability at 37 °C, the specific activity was ~3.5-fold lower and the emission maxima was slightly blue-shifted (Table 1). Importantly, we succeeded in reducing both K_m values: ~30-fold for Mg-ATP (3-fold greater than that for CBR) and ~7-fold for LH₂ (~2-fold lower than the CBR value). The mutations primarily responsible for the red-shifted emission were Y255F and S284T, while the lowered K_m values resulted from the G246A, F250H, and V351I amino acid changes that also contributed to the relative drop in specific activity.

We examined the potential of PLR3, PpyRE9, and CBR for live cell imaging applications in which bioluminescence is dependent on the cellular availability of ATP, O₂, and exogenously added LH₂. Equal numbers of HEK293T cells were transfected with pF4AG plasmids encoding the three red-emitting Lucs and grown overnight. Cells were counted, diluted, and triplicate wells of 4,000 cells (0.1 ml) were grown for 24 h in 96-well plates. An equal volume of media containing 10 mM LH₂ was then added to each well and the bioluminescence intensity was monitored over 1 h at 37 °C (Fig. 1A). PLR3 catalyzed emission was ~2- and ~2.6-fold brighter than that of PpyRE9 and CBR, respectively, and it was more stable than that of PpyRE9 (Fig. 1A and Table 1). We also confirmed that the *in vitro* bioluminescence emission maxima of the Lucs (Table 1) were maintained in the living cells (Fig. 1B). The BLI potential of PLR3 was further demonstrated by performing experiments with HeLa cells transfected with the same plasmids (Fig. 1C). Briefly (see Supplementary material for additional details), cells (50,000) were grown in 24-well plates for 1 day and 0.2 ml of 1 mM LH₂ solution was added. After 5 min, the intact live cells were imaged for 30 s with a EM-CCD camera equipped with a 10× objective. The data were analyzed with ImageJ software and the calculated relative mean bioluminescence intensities (Table 1) were quite similar to those obtained for HEK293T cells.

It appears that the 2.6-fold greater bioluminescence intensity of PLR3 over CBR does result from the lower (than PpyRE9) engineered K_m values for substrates ATP and LH₂ that are more similar to those of CBR. With K_m values comparable to those of CBR, the 2.6-fold greater specific activity of PLR3 is realized in the live cell BLI assay format. We also considered that differences in the expression and stability of the Lucs could influence the relative intensities of the *in vivo* BLI signals. Unfortunately, we were unable to confirm our expectation that the Lucs were expressed at similar levels because CBR was not stable in the lysates used to quantitate the proteins (Fig. S1). It is likely that the BLI results mainly reflect specific activity and K_m values.

We suggest that PpyRE9 is the red-emitting beetle Luc of choice for reporter gene applications in lysate format and BLI where ATP levels are sufficient and that PLR3 is an excellent choice for the BLI format in low ATP environments where as few as 1,000 cells can be readily detected (Fig. S2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Bioluminescence activity, emission spectra, and BLI of living cells expressing luciferases.

(A) Bioluminescence was initiated by the addition of 0.1 ml of 10 mM LH_2 to wells of assay plates containing equivalent numbers of live HEK293T cells expressing PLR3 (red), PpyRE9 (gray), and CBR (black) in 0.1 ml of media and monitored using a SynergyTM 2 Multi-Mode Microplate Reader operated at 37 °C. (B) Normalized bioluminescence emission spectra of equal numbers of live HEK293T cells expressing PLR3 (red), PpyRE9 (gray), and CBR (black). The spectra were collected 1 min after the addition of 10 mM LH_2 (0.25 ml) to a cuvette containing 0.25 ml of cells (~75-fold more cells than in A) in media at 37 °C. CBR did not appear to be stable under the latter growth and assay conditions as the signal intensity was ~2.5-fold lower than expected. Additional experimental details are included in the Supplementary material. (C) BLI of live HeLa cells transfected with pF4Ag plasmids expressing luciferases. Cells (50,000) were grown in 24-well plates for 1 day. Then, 0.2 ml of 1 mM LH_2 solution in pH 5 citrate buffer was added. After 5 min, the intact live cells were imaged for 30 s with an Imagem X2 EM-CCD camera equipped with a 10× objective and data were analyzed as described in the Supplementary material.

Table 1

Comparison of *in vitro* properties and live cell intensities of CBR, PpyRE9, and PLR3.^a

Enzyme	Relative Specific Activity	Km (μM)		Bioluminescence Emission Maxima	Thermal Inactivation at 37 °C (h)	Mean Bioluminescence Intensity in Living Cells	
		LH ₂	Mg-ATP			HEK293T cells ^b	HeLa cells ^c
CBR	100 ± 9	34 ± 6	4 ± 1	619 (62)	20	100 ± 9	100
PpyRE9	890 ± 80	112 ± 6	364 ± 32	617 (57)	22	130 ± 12	120
PLR3	256 ± 30	17 ± 3	12 ± 2	614 (58)	>24	260 ± 25	240

^aRelative specific activity, Km values, bioluminescence emission maxima, and thermal inactivation were determined from light-based assays at pH 7.4, as described in the Supplementary material.

^bThe mean bioluminescence intensity from 4,000 cells at 60 min relative to CBR.

^cThe mean bioluminescence intensity from 50,000 cells at 5 min relative to CBR.