Published in final edited form as:

Nat Methods. 2010 January ; 7(1): 5–6. doi:10.1038/nmeth0110-05.

A Red-shifted *Renilla* Luciferase Variant Optimized for Transient Reporter Gene Expression

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To the Editor

The emergence of bioluminescence as the primary modality for performing reporter gene imaging has been due to the high sensitivity and relative ease of use that luciferases offer. Two luciferases are principally employed for performing these experiments, firefly luciferase, and the luciferase from *Renilla reniformis* (*Renilla* luciferase, RLuc). The primary limitation when utilizing RLuc for imaging in small animal models has been that, because of its blue-peaked (481 nm) emission spectrum and the preferential absorption of short-wavelength photons by biological tissue, RLuc exhibits poor sensitivity at non-superficial locations relative to firefly luciferase [1]. To overcome this issue, we recently developed the red-shifted *Renilla* luciferase variant RLuc8.6-535 [2] that, in addition to displaying enhanced enzymatic activity compared to the native luciferase, demonstrated an additional 3-fold increase in sensitivity at depths of ~2 mm of tissue because of its green-peaked (535 nm) emission spectrum. Moreover, when combined with the substrate analog coelenterazine-*v* [3], we showed that an additional red-shift could be gained resulting in a yellow-peaked (570 nm) emission spectrum.

RLuc8.6-535 was derived from RLuc8, another RLuc variant developed in our laboratory that demonstrates vastly increased protein stability compared to the native luciferase [4]. RLuc8.6-535 retains this stabilized phenotype. In many experiments, such as when following cell-trafficking in living subjects, this enhanced stability is advantageous. However, in situations where the researcher is aiming to follow transient changes in gene expression (e. g. following the modulation of gene expression by chemotherapeutics), the extended intracellular half-life of RLuc8.6-535 (>50 hrs) may obscure the experimental results. The development of a red-shifted RLuc variant displaying comparable intracellular stability to the native enzyme would benefit researchers aiming to follow short-term fluctuations in promoter activity. Therefore we proceeded on a sequence of mutagenesis screens to develop such a variant.

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Competing Financial Interests

The authors declare competing financial interests.

All mutants were created by site-specific random mutagenesis and selection using previously described methods [2] (see Supplementary Methods). The starting template for mutagenesis was the destabilized variant RLuc/M185V/Q235A [4], as this variant exhibits ~1/2 the stability of RLuc and some inadvertent gain in stability was anticipated during the several rounds of mutagenesis screening planned.

A full description of the mutagenesis screens is given in Supplementary Results. Briefly, ten rounds of mutagenesis were done at the following residue pairs; D162/I163, V185/L186, D154/E155, I159/L163, E160/E161, W156/P157, A164/L165, K136/I137, F286/S287, and P220/R221. D162 and residues in close proximity were mutated as D162 has previously been found to lead to large shifts in the emission spectrum [2], as was the case here. Mutations at D162 that led to blue-shifts in the emission spectrum were also identified (Supplementary Fig. 1), as this was not a focus of this study these variants were not pursued further. Mutations at residue 185 were probed as this residue is known to lie at the top of the active site [5] and substitutions at this residue have been found during previous consensus-sequence guided mutagenesis screens to yield improvements in quantum yield and enzymatic activity [4]. Mutations at the residue pair D154/E155 were tested based on a prior random mutagenesis screen that found alterations at these sites could yield improvements in activity [2]. K136 and S287 were targeted based on data from consensus-sequence guided mutagenesis screens that found increases in stability and enzymatic activity with alterations at these sites [4]. Finally, we performed mutations at P220 based on a recently published report that found increased activity with alterations at this residue [6]; we, however, found no variations at this site that increased activity or red-shift. The end result of this selection process was the variant RLuc/E155G/D162E/A164R/L165I/M185V/Q235A/S287A that we have denoted as RLuc7-521.

RLuc7-521 purified by nickel affinity chromatography was used for assessing the bioluminescence emission spectrum, enzymatic activity, and quantum yield (Supplementary Table 1). RLuc7-521's emission spectra (Fig. 1a) showed a 40 nm red-shift compared to RLuc with both coelenterazine and coelenterazine-v. The variant's signal was increased 1.6-fold on a photons/sec/mole basis compared to similarly purified RLuc. RLuc7-521's quantum yield of $3.9 \pm 0.1\%$, while an improvement over that of RLuc8.6-535 ($3.1 \pm 0.2\%$), was lower than the quantum yield of RLuc ($5.3 \pm 0.1\%$) indicating that further improvements may be achievable by additional mutagenesis.

To demonstrate the performance of RLuc7-521 as a mammalian reporter gene, the gene was inserted into the pcDNA 3.1 mammalian expression plasmid. The resultant plasmid was then transiently transfected into 293T cells (Fig. 1b). RLuc7-521 exhibited a nearly 2-fold improvement in light output compared to RLuc under these experimental conditions, while retaining an almost identical intracellular stability.

To demonstrate the utility of RLuc7-521 in small-animal imaging, 293T cells were transiently transfected with either the RLuc or RLuc7-521 containing pcDNA plasmids. Western blotting demonstrated equivalent levels of luciferase protein expression (Supplementary Fig. 2). These cells were subsequently injected via tail-vein into nude mice. Previous experiments have shown that a majority of these cells will initially accumulate in

the lungs. Subsequent imaging showed a significant 3.3-fold increase in signal output from the lungs for RLuc7-521 (Fig. 1c), a reflection of both the ~2-fold improved light output of RLuc7-521 combined with reductions in signal tissue attenuation for this red-shifted luciferase.

As the lungs are predominantly air and are less attenuating to optical-wavelength photons than most other tissues, even greater gains in signal output will be obtained for RLuc7-521 relative to RLuc for other body locations. For example, based on previous experiments [2] we expect that for an equal number of emitted photons the 40 nm red-shifted emission spectra of RLuc7-521 will result in a ~3-fold gain in photon transmission at 1–2 mm of tissue depth - with yet greater relative gains at deeper tissue depths. Even further improvements in sensitivity could be achieved via further red-shifting of the emission spectrum by combining RLuc7-521 with novel analogs of coelenterazine currently being developed based on the substrate analog coelenterazine-*v*.

In summary, RLuc7-521 is a 7-mutation variant of RLuc that exhibits a green-peaked (521-nm) emission spectrum, an identical level of intracellular stability, and at least a 2-fold increase in signal with even greater gains to be realized in small-animal imaging due to decreased attenuation of the red-shifted photons. This new variant represents a direct replacement of *Renilla* luciferase for reporter gene applications as it retains the temporal relationship between gene activation and signal generation while increasing the sensitivity of these assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the Stanford Medical Scientist Training Program (AML), NCI CA114747 ICMIC P50 (SSG), NCI CA119367 CCNE U54 (SSG), NCI R01 CA082214 (SSG), and the NCI SAIRP. The authors would like to thank Dr. Brad Rice and Dr. Tamara Troy of Caliper Life Sciences for help with the spectra measurements, and Dr. Zachary Walls and Dr. Jinha Park for assistance in carrying out these experiments.

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Figure 1.

Validation of RLuc7-521

(a) Normalized bioluminescence emission spectra for RLuc and RLuc7-521 in the presence of coelenterazine (c) or coelenterazine- ν (v). The peaks for RLuc were 481 nm (c) and 516 nm (v), with mean emission wavelengths of 500 nm (c) and 535 nm (v). The peaks for RLuc7-521 were 521 nm (c) and 556 nm (v), with mean emission wavelengths of 534 nm (c) and 569 nm (v). Normalization in the figure is to the total area under the curve, not the emission peak.

(b) Mammalian cell expression of RLuc and RLuc7-521 following transient transfection into 293T cells. The luciferases were in pcDNA 3.1 plasmids under the control of the

constitutive promoter from cytomegalovirus (CMV). Cells were exposed to 100 $\mu\text{g/ml}$ of cycloheximide to inhibit new protein synthesis 24 hours following transfection. Cells were assayed for light out-put per total cellular protein. Data was fit to a mono-exponential degradation model and estimated intracellular activity half-lives are given in the figure key. Values are reported as relative to the RLuc condition immediately prior to cycloheximide application ($T=0$). The two conditions were significantly different ($p < 0.05$, two-tailed Student t -test) at all time points except the last. Samples were in quadruplicate, error bars represent standard error of the mean.

(c) Bioluminescence from 293T cells containing either RLuc or RLuc7-521 within the lungs of nude mice. Cells were transiently transfected with pcDNA 3.1 plasmids containing either RLuc or RLuc7-521, 24 h later these cells were injected via the tail-vein with resultant accumulation in the lungs. Mice were injected with coelenterazine 2 h later and imaged. Representative mice from a substrate control group ($n=5$), the RLuc condition ($n=7$), and the RLuc7-521 condition ($n=7$) are shown. Total flux from the thorax was recorded, with substrate-only injected mice used for background subtraction. As shown in the bar graph, the two conditions were significantly different ($p < 0.001$, two-tailed Student t -test).