Renilla Luciferase Variants with Green Emission Peaks for Improved Imaging in Living Subjects

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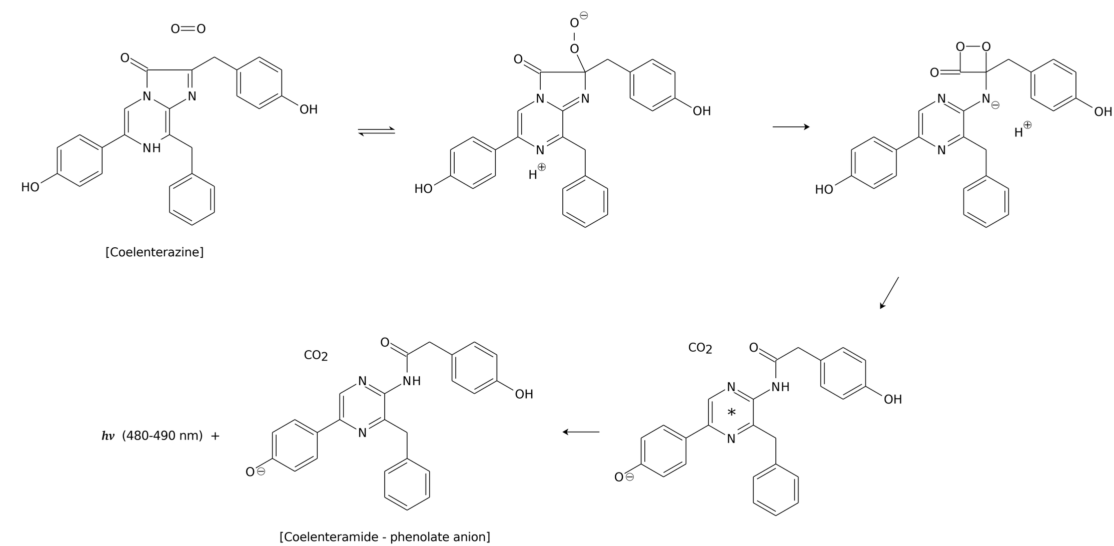
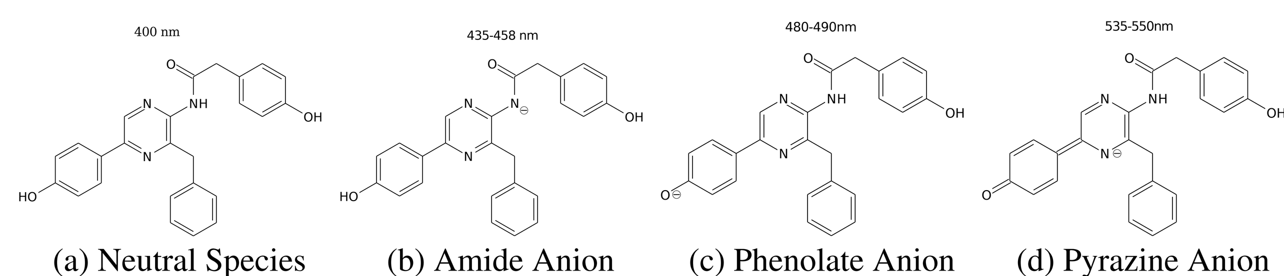
INTRODUCTION

Renilla luciferase (RLuc) is commonly used as a reporter gene either on its own or in conjunction with firefly luciferase. Its use in bioluminescence imaging, however, has been hampered by the blue-peaked emission spectrum generated when the enzyme oxidizes its substrate coelenterazine, as blue wavelength photons are strongly attenuated in biological tissues. To overcome this difficulty, we have explored red-shifting the emission spectrum of RLuc to increase its utility for small animal imaging applications.

As shown below, the reaction catalyzed by RLuc involves a dioxetane intermediate, and the energy released by the break-down of this bond leaves the reaction product coelenteramide in an excited state. The excited electron/coelenteramide system can lose energy and return to the ground electronic state through a number of processes, with the desired transition in bioluminescence resulting in a photon of light. The wavelength of this photon depends on the energy difference between the excited and ground states, which in turn depends on the local chemical environment the coelenteramide finds itself in. The phenolate anion of coelenteramide is shown below as the emitting species, although assignment of the exact state of the emitting species is a matter of contention.



It has been demonstrated that various chemical environments will lead to different fluorescence emission peaks from coelenteramide; the species thought to be responsible for these distinct emissions are shown below [1].

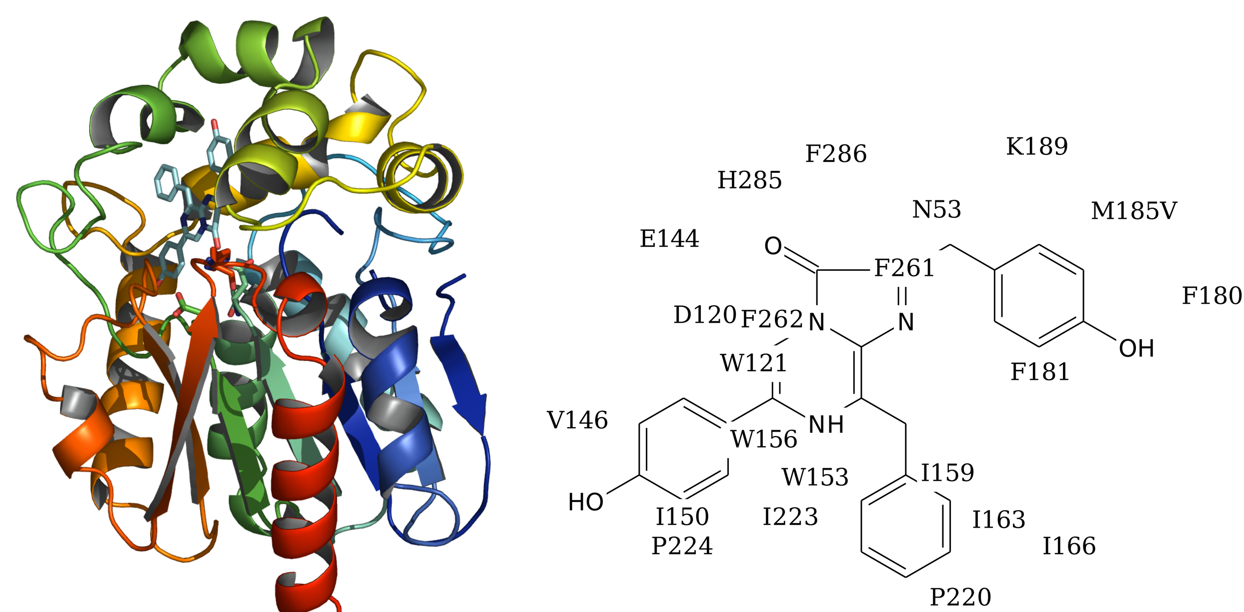


(a) Neutral Species (b) Amide Anion (c) Phenolate Anion (d) Pyrazine Anion

We hypothesized that appropriate alteration of RLuc's enzymatic pocket could alter the chemical environment coelenteramide experiences and in turn favor a product species that would emit a green photon of light. For practical reasons, we focused on modifying a previously described 8-mutation stabilized variant of RLuc called RLuc8 [2]. Initial modifications were done by site-directed mutagenesis in the presumptive active pocket, with later modifications guided by random mutagenesis.

HOMOLOGY MODEL/PUTATIVE ACTIVE SITE

Renilla luciferase shows high homology (34-56% similar) to a number of bacterial haloalkane dehalogenases. This allowed generation of a homology model of RLuc8 using SWISS-MODEL (v3.5) and crystal structures of the haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* (PDB files 1iz8, 1k63, 1k6e, 1iz7, and 1mj5). The resultant model is shown below, with the N-terminus in blue and the C-terminus in red. Based on data from the dehalogenases, the putative active site was identified and the orientation of coelenterazine in the site was estimated. In addition to the estimated substrate location, the catalytic triad of D120, E144, and H285 is shown as well. The neighboring schematic highlights information, derived from the homology model, as to which active site residues likely interact with the substrate and were targets for site-directed mutagenesis.



ENZYMATIC POCKET MUTATIONS

Site-directed mutagenesis of the selected residues was performed on the previously described plasmid pBAD-pelB-RLuc8 [2]. This plasmid allows for periplasmic expression due to the pelB leader sequence, as well as nickel affinity purification using an encoded 6xHis tag. Activity of purified protein was measured with a 10 second integration following addition of 0.5 μg coelenterazine to dilute protein in 100 μl 100 mM Na-phosphate buffer (pH 7), with a correction applied for the spectral sensitivity of the luminometer. Emission spectra at ambient temperature were measured using a Triax 320.

A total of 74 site specific mutations at 22 residues were created and analyzed; the more interesting mutants are presented below.

	Specific Activity	Wavelength (nm) peak	mean	fwhm	%>600 nm
RLuc	1.0±0.1	481	497	93	3
RLuc8	4.3±0.2	486	503	94	4
RLuc8/N53Q	0.10	475	491	92	3
RLuc8/V146M	1.0	481	498	94	3
RLuc8/I150H	0.53	494	514	98	6
RLuc8/I159Y	0.003	513	536	113	13
RLuc8/I163Y	0.13	502	521	103	8
RLuc8/I166Y	0.23	493	508	99	5
RLuc8/F181Y	0.07	497	515	103	6
RLuc8/I223C	3.0	503	524	103	9
RLuc8/I223H	0.07	508	527	105	9
RLuc8/F261W	0.20	504	524	98	8
RLuc8/F262W	0.60	500	521	99	7

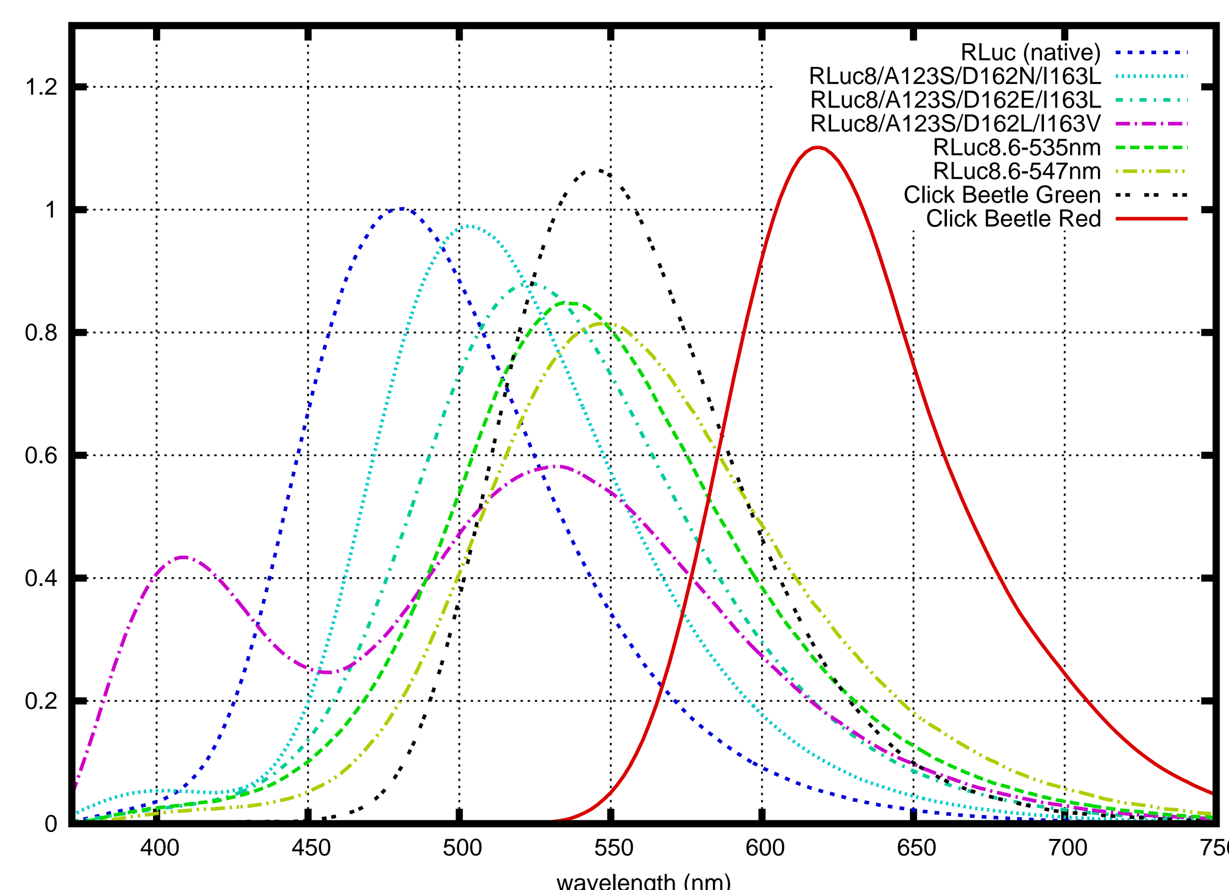
PROTEIN EVOLUTION FOR A GREEN RLUC

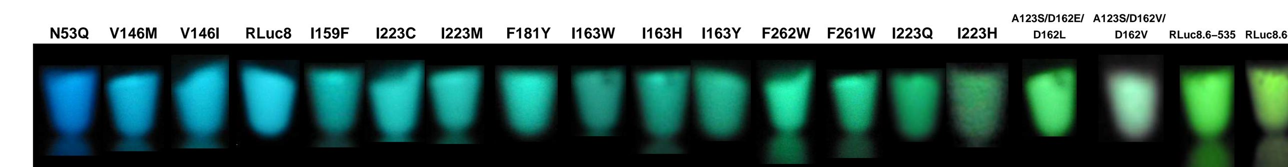
Although a range of emission shifts were created with the single residue mutants, these alterations in the putative active pocket invariably led to significant reductions in enzymatic activity. In the hopes of recovering some of this loss, an initial round of random mutagenesis was done on the F261W and F262W mutants with screening of around 1×10^5 clones. For rapid screening, these clones were initially selected for brightness and color by airbrushing on substrate and imaging using an IVIS-200 (Xenogen). Protein from promising clones was then purified and subjected to further testing.

The information garnered from this initial random mutagenesis screen was utilized for planning a sequence of saturation mutagenesis studies at particular residue pairs on RLuc8 as well as the F261W and F262W mutants. Following several rounds of evolution, the most promising variants were:

	Specific Activity	Wavelength (nm) peak	mean	fwhm	shoulder	Shoulder/ Peak Ratio	%>600 nm
RLuc	1.0±0.1	481	497	93			3
RLuc8	4.3±0.2	486	503	94			4
RLuc8/A123S	2.8	484	502	92			4
RLuc8/A123S/D162N/I163L	3.1	507	523	93	404	0.07	7
RLuc8/A123S/D162E/I163L	3.6	523	538	102			12
RLuc8/A123S/D162L/I163V	0.35	532	515	124	409	0.75	13
RLuc8/A123S/D162E/I163L/V185L	3.4	532	545	106			15
RLuc8.6-535	6.0	535	550	104			17
RLuc8.6-545	1.9	545	560	106			21
RLuc8.6-547	1.2	547	564	111			23

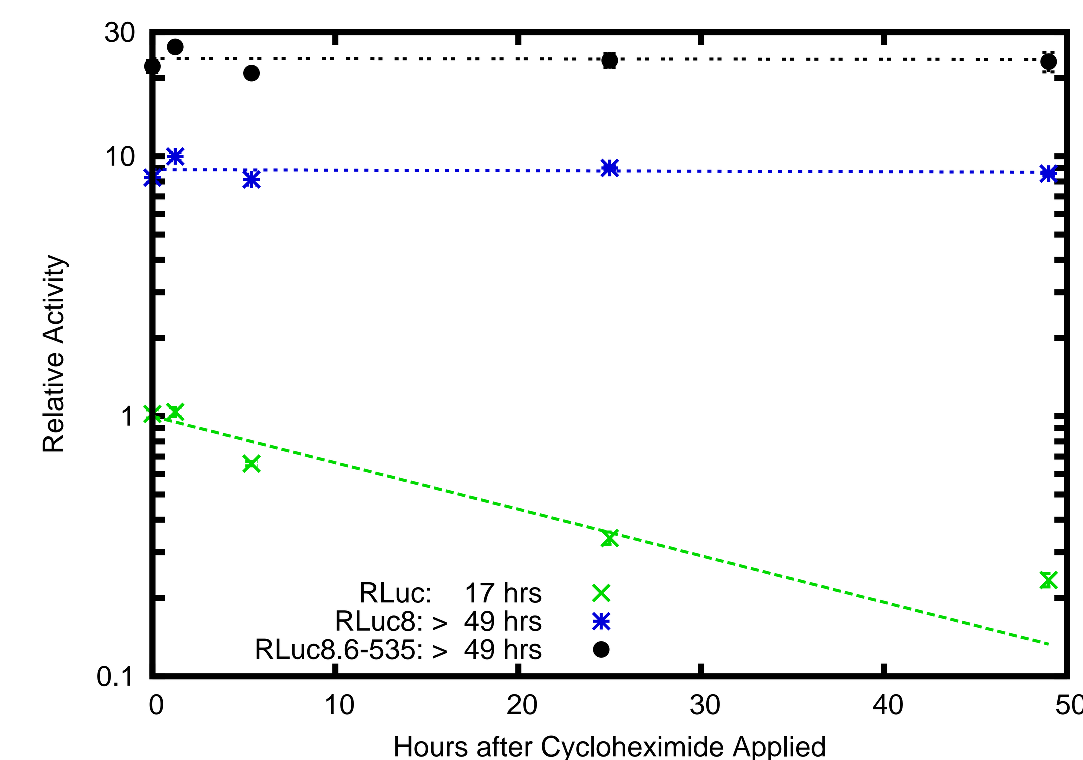
RLuc8.6-535 = RLuc8/A123S/D154M/E155G/D162E/I163L/V185L
RLuc8.6-545 = RLuc8/A123S/D154K/E155N/D162E/I163L/F261W
RLuc8.6-547 = RLuc8/A123S/D154A/E155G/D162E/I163V/F262W





IN VITRO EVALUATION

To demonstrate the applicability of these generated RLuc variants as mammalian reporter genes, expression vectors were constructed for RLuc, RLuc8, and RLuc8.6-535 in a pcDNA 3.1 backbone under the control of a constitutive promoter (CMV). 48 hrs following transfection of these plasmids into 293T cells, cycloheximide was added to allow assessment of the intracellular stability of the proteins. The results, shown below, indicate that RLuc8.6-535 is significantly brighter than RLuc and RLuc8, and has a similar intracellular half-life to RLuc8.



IN VIVO EXPECTATIONS

Theoretical estimates were made as to the benefit these red-shifted RLuc variants could be expected to show *in vivo*, by calculating the expected percentage of photons that would be transmitted through 0.1 cm or 0.5 cm of rat liver tissue [3]. An additional parameter was calculated that took into account the variant's specific activity as well as its emission spectrum. This parameter, "effective output", shows the relative benefit of each variant versus native *Renilla* luciferase.

	Specific Activity	Wavelength mean (nm)	% Transmitted 0.1 cm	0.5 cm	Effective Output 0.1 cm	0.5 cm
RLuc	1.0	497	2.8	0.025	1.0	1.0
RLuc8	4.3	503	3.1	0.029	4.7	5.0
RLuc8/A123S/D162N/I163L	3.1	523	4.2	0.065	4.7	8.1
RLuc8/A123S/D162E/I163L	3.6	538	5.8	0.13	7.4	19
RLuc8/A123S/D162E/I163L/V185L	3.4	545	6.9	0.18	8.4	25
RLuc8.6-535	6.0	550	7.4	0.20	16	48
RLuc8.6-545	1.9	560	8.7	0.26	5.9	20
RLuc8.6-547	1.2	564	9.7	0.31	4.2	15

CONCLUSION

- Mutagenesis of RLuc8 led to emission-shifted variants of the enzyme, with spectra shifts up to ~60 nm. The green-emitting luciferases presumably favor the pyrazine anion resonance form of coelenteramide in their enzymatic pocket.
- Mutations at D162 were critical for generating large red-shifts. This residue is immediately adjacent to the putative active site residue I163.
- Of the developed variants, RLuc8.6-535 is expected to be the best for *in vivo*, as it is 6-fold bright than native *Renilla* luciferase and emits light with a mean wavelength of 550 nm. The green-peaked emission spectra should allow significantly more photons to escape through biological tissue.

References

[1] Shimomura O, et al. *Luminescence*, 15:51–58, 2000

[2] Loening AM, et al. *Protein Eng Des Sel*, 19:391–400, 2006

[3] Cheong WF, et al. Optical properties of biological tissues. on the World Wide Web at http://omlc.ogi.edu/pubs/abs/cheong90a.html, 1993

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