Improved mutants of *Renilla* luciferase for imaging applications in living subjects

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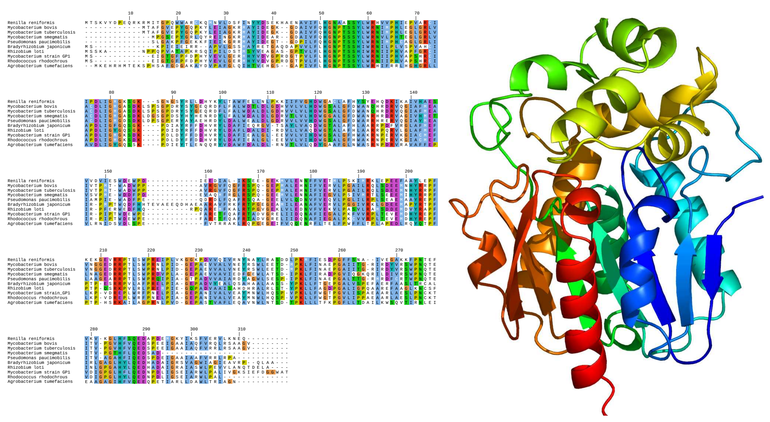
INTRODUCTION

The optical signal generated by *Renilla* Luciferase (RLuc) allows its use as a biomolecular label as well as a reporter gene. Several projects are attempting to make use of RLuc as a label by constructing fusion proteins of RLuc with engineered antibodies [1, 2] or receptor ligands [3, 4]. These RLuc labeled proteins can then be used for studying receptor targets with *in vitro* assays and *in vivo* imaging. Use of RLuc as a label, however, requires high stability and activity in a variety of environments, including mammalian serum. To achieve this, we have characterized mutants of RLuc with respect to serum stability and light output, and have used the results to develop an 8 mutation stabilized variant of RLuc (RLuc8). We have also begun mutation studies of RLuc8's substrate binding site, with the hope of shifting the bioluminescence emission spectrum to longer wavelengths to make this enzyme more favorable for *in vivo* use.

ALIGNMENT/HOMOLOGY MODEL

RLuc shows high homology (34-56% similar) to a number of bacterial haloalkane dehalogenases; this may indicate a horizontal gene transfer event. An alignment generated using CLUSTAL W is shown below.



From the alignment, candidate mutations were chosen at positions where RLuc clearly diverged from the consensus. This semi-rational approach is a fairly efficient mutagenesis strategy [5], as the candidate mutations tend to be compatible with the tertiary structure of the protein. Previous work in the literature [6] has shown that a C124A mutation stabilizes RLuc, and that C73 is required for activity. In light of the alignment data, C73 can be seen to be conserved across the enzymes, while C124 differs from the bacteria consensus.

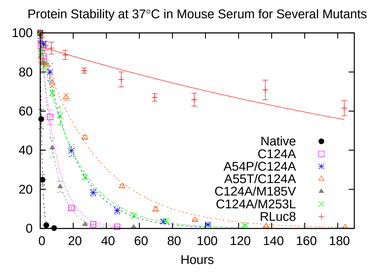
A homology model of RLuc was constructed in order to locate the candidate mutation locations. This model was built using SWISS-MODEL (v3.5) and crystal structures of the haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* (PDB files 1iz8, 1k63, 1k6e, 1iz7, and 1mj5). In the model above, the N-terminus is blue and the C-terminus is red. Based on data from the dehalogenases, the putative active site of RLuc is at the intersection of the red, orange, and yellow helices.

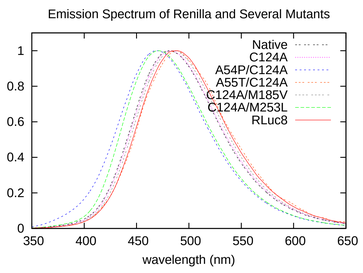
ALTERATION OF RLUC STABILITY

A human codon optimized version of rluc (Promega) was cloned into the pBAD expression vector (Invitrogen), adding a c-terminal Myc epitope and 6xHis tag, and an n-terminal pelB leader sequence for periplasmic expression. Mutations were introduced by site-directed mutagenesis. Proteins were expressed in *E. coli* LMG 194 cells at 32°C, separated from cells by osmotic shock [7], and purified by nickel affinity chromatography. Human serum albumin was added to 1% as a carrier protein and samples were stored at 4°C. Activity was measured with a 10 second integration following addition of 0.5 μg substrate to dilute protein in 100 μl 100 mM Na-phosphate buffer (pH 7). Substrates tested were coelenterazine (native), benzyl-coelenterazine (h), coelenterazine-cp (cp), coelenterazine-n (n), and bisdeoxycoelenterazine/DeepBlueC (bdc). Serum stability was assessed by mixing 0.5 μl dilute luciferase with 20 μl mouse or 50 μl rat serum and placing in a 37°C incubator.

The 7 mutations with the most favorable increases in specific activity and/or stability were combined along with the C124A mutation into a single protein (RLuc8). The 8 mutations present in RLuc8 are A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L. Since the Myc epitope encoded by the expression plasmid was removed during the cloning of RLuc8, an equivalent C124A construct (C124A-ΔMyc) was made in order to facilitate a valid comparison.

In many cases where a reporter gene is employed, the goal is to follow the dynamics of gene activation and repression. In these contexts, it is desirable for the reporter protein to be labile so that levels of the reporter more accurately track the current state of gene activation. In order to generate a less stable luciferase with equal or better light output than the native RLuc, we combined the M185V and Q235A mutations.





	Activity (photons/s/mole enzyme)					Serum $\tau_{1/2}$ (h)		Wavelength (nm)	
	native	*bc*	*cp*	*n*	*bdc*	mouse	rat	peak	mean
Native RLuc	$(3.2\pm0.3)\times10^{22}$	5.4×10^{22}	1.7×10^{22}	8.3×10^{21}	5.8×10^{19}	0.9	0.4	482	497
Initial Mutations	Activity (relative to RLuc)								
C124A	1.2±0.1	0.75	0.79	0.63	0.68	7.1±0.4	6.6±0.5	482	498
C124A-ΔMyc	1.3±0.1	0.91	1.1	0.87	1.0	4.0	4.5	481	499
F33R/I34M/C124A	0.15	0.15	0.16	0.12	0.20	0.3	0.3	481	497
E44G/C124A	0.94	0.78	0.74	0.66	0.98	2.6	3.3	486	502
A54G/A55G/C124A	0.12	0.10	0.06	0.15	0.19	2.4	3.0	476	492
A54P/A55T/C124A	0.21	0.15	0.11	0.38	0.22	119	129	470	483
A54P/C124A	0.05	0.04	0.05	0.08	0.06	14	13	468	482
A55T/C124A	1.7	1.2	0.58	1.4	2.4	30	29	486	504
F116L/C124A	1.3	1.0	1.3	0.88	1.8	11	9.4	486	502
C124A/S130A	1.7	1.4	1.7	1.4	2.6	18	14	482	498
C124A/K136R	2.5±0.3	2.1	1.9	1.9	2.6	12	11	482	498
C124A/A143M	1.7	1.3	0.95	1.5	1.6	30	29	480	497
C124A/F180A	0.02	0.01	0.03	0.01	0.01	1.6	1.6	488	504
C124A/M185V	3.4	3.0	15	7.8	44	5.7	3.7	485	500
C124A/M191L	1.1	0.99	0.97	1.0	1.2	6.5	5.1	480	496
C124A/E195S/P196D	0.12	0.10	0.12	0.10	0.15	1.0	0.7	482	498
C124A/F199M	0.58	0.44	0.53	0.49	0.46	6.7	6.0	480	495
C124A/L203R	0.55	0.55	0.52	0.41	0.43	2.7	2.2	484	501
C124A/G229E	0.02	0.01	0.03	0.03	0.01	1.9	1.8	473	490
C124A/Q235A	1.2	1.1	1.1	1.0	1.2	3.3	3.6	473	489
C124A/M253L	1.9	1.4	1.6	1.6	1.7	15	10	471	488
C124A/S257G	1.1	0.95	1.3	1.1	3.0	1.3	1.4	477	493
C124A/F261L/F262L	0.00	0.00	0.00	0.00	0.00	N/D		N/D	
C124A/F262L	0.03	0.03	0.01	0.06	0.03	5.8	6.4	478	495
C124A/S287L	3.9	2.8	3.4	5.0	9.5	28	20	478	496
C124A/M295I	1.0	0.83	0.57	0.72	0.86	5.0	4.9	480	497
C124A/K300A	1.1	1.0	1.1	1.0	1.3	3.5	3.9	481	497
Stabilized Luciferase									
RLuc8	4.3±0.2	3.0	5.8	8.8	59	253 ±58	88 ±12.4	487	503
Destabilized Luciferases									
M185V	4.4	2.6	12	4.1	20	0.8	0.3	N/D	
M185V/Q235A	4.8	2.7	14	7.1	20	0.5	0.2	N/D	

QUANTUM YIELD

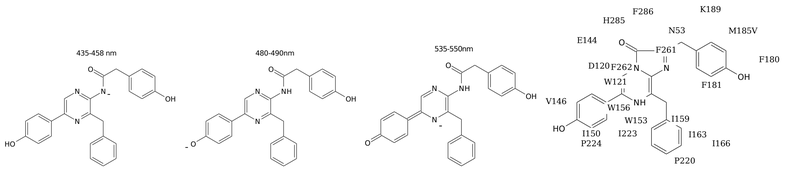
To understand the basis of RLuc8's increased light output, quantum yield measurements were undertaken. The results indicated that some but not all of RLuc8's increased output arises from quantum yield gains. The remainder is assumed to arise from kinetic enhancements.

	Quantum Yield (%)				
	native	*bc*	*cp*	*n*	*bdc*
Native RLuc	5.3±0.1	3.2±0.04	4.7±0.03	6.1±0.2	$(6.1\pm0.9)\times10^{-3}$
C124A	5.4±0.3	3.6±0.1	5.2±0.1	6.4±0.01	$(7.7\pm0.5)\times10^{-3}$
A55T/C124A	5.7±0.2	3.9±0.1	4.5±0.1	5.7±0.1	$(1.0\pm0.9)\times10^{-3}$
C124A/S130A	5.3±0.1	3.4±0.04	5.0±0.1	5.9±0.2	$(6.7\pm0.3)\times10^{-3}$
C124A/K136R	5.4±0.1	3.3±0.1	5.1±0.1	6.0±0.1	$(7.1\pm0.3)\times10^{-3}$
C124A/A143M	5.2±0.3	3.5±0.1	4.8±0.1	5.8±0.2	$(6.3\pm0.7)\times10^{-3}$
C124A/M185V	6.9±0.3	6.3±0.1	10.1±0.2	9.4±0.4	$(174.4\pm6.7)\times10^{-3}$
C124A/M253L	5.5±0.1	3.5±0.1	5.1±0.2	5.8±0.1	$(7.6\pm0.3)\times10^{-3}$
C124A/S287L	6.1±0.2	5.0±0.1	7.2±0.3	7.7±0.2	$(20.9\pm0.6)\times10^{-3}$
RLuc8	6.9±0.1	6.1±0.1	8.9±0.1	9.6±0.4	$(198.2\pm8.5)\times10^{-3}$

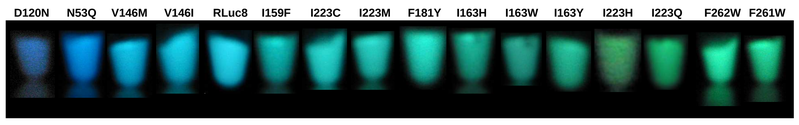
ENZYMATIC POCKET MUTATIONS

The haloalkane dehalogenases contain a conserved aspartate, glutamate, and histidine triad in their enzymatic core. Based on the alignment data, the equivalent triad in *Renilla* (D120, E144, and H285) were mutated, and shown to be critical for luciferase activity.

The bioluminescence emission spectrum of *Renilla* luciferase is believed to be related to the fluorescent spectrum of coelenteramide, the product of coelenterazine degradation. Specifically, the blue light emission (482 nm peak) is thought to arise from the coelenteramide existing primarily in its phenolate anion form within the enzyme [8]. We speculated that alteration of the enzymatic pocket could alter the chemical environment of coelenteramide, favoring different ionic states of coelenteramide with resulting shifts in the bioluminescence emission spectrum. With the aid of the homology model, we estimated the location of coelenterazine in the enzymatic pocket, and proceeded to make a total of 74 site specific mutations at the 22 residues we felt interact with coelenterazine/coelenteramide. From this screen, 21 specific mutations at 10 different amino acid locations resulted in observable shifts in the emission spectrum. Several of these mutants are presented below.

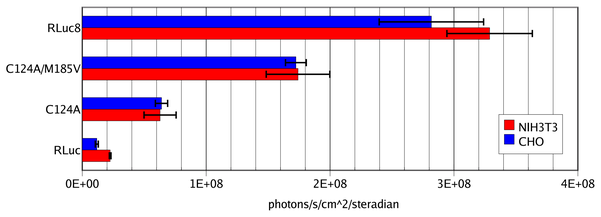


	Specific Activity (relative to RLuc)					Serum $\tau_{1/2}$ (h)	
	native	h	cp	n	bdc	mouse	rat
RLuc8	4.3±0.2	3.0	5.8	8.8	59	197	94
Catalytic Triad							
RLuc8-D120A	0.000	0.001	0.001	0.003	0.21	> 100	> 100
RLuc8-D120N	0.023	0.016	0.050	0.34	5.1	> 100	> 100
RLuc8-E144A	0.000	0.000	0.000	0.000	0.000	57	13
RLuc8-E144Q	0.000	0.000	0.000	0.000	0.002	> 100	> 100
RLuc8-H285A	0.023	0.020	0.046	0.028	0.20	> 100	21
Color Shift Mutants							
RLuc8-N53Q	0.10	0.16	0.52	0.04	0.31		
RLuc8-V146M	1.0	0.66	0.51	0.47	0.43		
RLuc8-I159F	0.60	0.43	0.56	1.7	1.4		
RLuc8-I163Y	0.13	0.16	0.10	0.14	1.2		
RLuc8-F181Y	0.07	0.11	0.03	0.06	1.2		
RLuc8-I223C	3.0	1.7	6.2	3.7	12		
RLuc8-I223H	0.07	0.05	0.09	0.43	1.9		
RLuc8-I223M	0.19	0.37	0.25	0.64	14		
RLuc8-I223Q	0.06	0.06	0.06	0.31	2.3		
RLuc8-F261W	0.08	0.38	0.38	0.02	0.76		
RLuc8-F262W	0.60	0.20	0.23	0.07	0.02		



MAMMALIAN REPORTER GENE

To demonstrate the applicability of these results in the context of a mammalian reporter gene, expression vectors were constructed for RLuc, C124A, C124A/M185V, and RLuc8 in a pcDNA 3.1 backbone, with the gene under the control of a constitutive promotor (CMV). These mammalian expression plasmids were then transiently transfected into 293T or CHO cells. The results 24 hours post transfection are shown below with the bars representing the standard error of the mean. For a given cell line, all differences between mutants were significant at $p \leq 0.06$ using a two-tailed *t*-test. The increased light output for the mutants was consistent with the *in vitro* data.



CONCLUSION

The combination of 8 favorable mutations generated a mutant *Renilla* luciferase (RLuc8) that exhibited a greater than 150-fold stability improvement in murine serum when compared to RLuc (<1 hr versus >100 hr) along with a 4-fold improvement in light output. This optimized *Renilla* luciferase represents a significant improvement that will allow its use for the labeling of biomolecules and following these constructs *in vivo*. This stabilized variant also shows increased light output compared to the native enzyme when used as a reporter gene in mammalian cells.

Mutations conducted in the enzymatic pocket of RLuc8 were able to shift the emission spectrum, albeit with significant reductions in the light output of the enzyme. Work is ongoing in an attempt to restore the light output of these color shift variants by additional rounds of random mutagenesis.

References

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Acknowledgments: This work was supported in part by a Department of Defense NDSEG Fellowship (AML), a Stanford Bio-X Graduate Fellowship (AML), and NIH 5 R01 CA082214-06 (SSG). The authors would like to thank Brad Rice and Olivier Coquoz of Xenogen Corporation for help with the spectra measurements, along with Mark Breidenbach, Tim Fenn, and the Brunger lab for help with the sequence alignment and homology modeling. Benzyl-coelenterazine was a generous gift from Dr. Bruce Bryan.