Consensus Guided Mutagenesis of *Renilla* Luciferase Yields Enhanced Stability and Light Output

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Abstract

Luciferases, which have seen expansive employment as reporter genes in biological research, could also be used in applications where the protein itself is conjugated to ligands to create probes appropriate for use in small animal imaging. As the bioluminescence activity of commonly used luciferases is too labile in serum to permit this application, specific mutations of *Renilla* luciferase, selected using a consensus sequence driven strategy, were screened for their ability to confer stability of activity in serum as well as their light output. Using this information, a total of 8 favorable mutations were combined to generate a mutant *Renilla* luciferase (RLuc8) that, compared to the parental enzyme, is 200-fold more resistant to inactivation in murine serum and exhibits a 4-fold improvement in light output. Results of the mutational analysis were also used to generate a double mutant optimized for use as a reporter gene. The double mutant had half the resistance to inactivation in serum of the native enzyme while yielding a 5-fold improvement in light output. These variants of *Renilla* luciferase, which exhibit significantly improved properties compared to the native enzyme, will allow enhanced sensitivity in existing luciferase-based assays as well as enabling the development of novel probes labeled with the luciferase protein.

Keywords: *Renilla* Luciferase, Reporter Gene, Enzyme Stability, Mutagenesis

Introduction

Since the cloning of a luciferase from the firefly *Photinus pyralis* in 1985 [1], luciferase genes have become essential components of biological research. They are used ubiquitously as reporter genes in cell culture experiments, and their use as reporters has been extended into the context of small animal imaging [2].

Recently, it has been proposed that the luciferase protein itself could be conjugated to other proteins such as antibodies or growth factors [3], and these bioluminescently labeled ligands could then be used for imaging receptor targets in small animals. The advantage of using a bioluminescent entity to label a protein over similar fluorescent or radioactive approaches, is that in the context of small animal imaging the bioluminescent approach has the potential to be more sensitive [4].

The beetle luciferases (*e.g.* firefly), however, are not optimal for employment as bioluminescent tags. These luciferases are not particularly small ($\sim$62 kDa) and are dependent on ATP, molecular oxygen, and magnesium for activity. The dependence on ATP especially would hinder the application of beetle luciferases as bioluminescent tags *in vivo*, as serum ATP concentrations are generally below 10 nM [5].

Luciferases that use coelenterazine as their substrate are more appropriate for application as bioluminescent tags, as these enzymes are not ATP dependent and in general require only molecular oxygen in addition to coelenterazine for luminescence. From this group of proteins, the luciferase from the sea pansy *Renilla reniformis* (RLuc) [6, 7] is the best characterized as well as being of a size (36 kDa) more appropriate for use as a tag.

The limiting factor for use of RLuc as a bioluminescent tag is the rapid inactivation ($\tau_{1/2}$=0.5-1 hour) of its activity in murine serum at 37°C (see results). A single point mutation of RLuc (C124A) that increases the enzyme's resistance to inactivation approximately 6-fold has been reported [8], however even this level of resistance is insufficient for the tagging of large proteins (*e.g.* antibodies) that require time scales on the order of days to sufficiently distribute. For this reason, we pursued a semi-rational, consensus sequence driven mutagenesis strategy [9] in order to identify mutations that could alter the stability of RLuc's activity in serum. Through the course of this work, we also identified mutations that increased the light output of RLuc, particularly when used with analogs of coelenterazine. By combining these mutations appropriately, we were able to generate a mutant RLuc with enhanced light output and resistance to serum inactivation that is optimized for use as a bioluminescent tag, as well as a mutant RLuc with increased light output and accelerated inactivation that may find use as a reporter gene.

Materials and Methods

Materials

Coelenterazine was from Prolume (Pinetop, AZ). Benzyl-coelenterazine (coelenterazine-*h*) was a generous gift from Dr. Bruce Bryan. Coelenterazine-*n* and coelenterazine-*cp* were from Biotium (Hayward, CA). Bisdeoxycoelenterazine (coelenterazine-*400a*, di-dehydro coelenterazine, DeepBlueC) was from Perkin Elmer (Boston, MA). The chemical structures of these compounds are shown in Figure 1. Coelenterazine and the analogs were dissolved in propylene glycol and stored in small aliquots at -80°C.

Luminometer Calibration

Light measurements were made using a Turner 20/20 and later a Turner 20/20n luminometer (Turner Designs, Sunnyvale, CA). The luminometers were calibrated to absolute units (photons/s) using the luminol light standard performed in dimethyl sulfoxide (DMSO) [10, 11]. No corrections were applied for the spectral sensitivity of the luminometer, as the spectral peak of luminol chemiluminescence in DMSO (486 nm) is close to the spectral peak of *Renilla* luciferase bioluminescence (482 nm).

Computational Prediction

A BLAST search [12] was performed using the "nr" database, and all sequences with an E value of $< 1^{-10}$ were initially retained. Redundant sequences were then removed, as well as sequences that did not contain a conserved catalytic triad known to be required for activity in bacterial haloalkane dehalogenases. An alignment between RLuc and the 14 remaining sequences was then generated using CLUSTAL W [13].

A homology model of RLuc was built with SWISS-MODEL (v3.5) [14] using the default parameters (Figure 4a). In generating this homology model, SWISS-MODEL utilized several crystal structures of the haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* (PDB files 1iz8, 1k63, 1k6e, 1iz7, and 1mj5).

Construction of *Renilla* Luciferase Mutants

The *hrluc* gene from the plasmid phRL-CMV (Promega, Madison, WI) was used as the initial template for cloning. This gene is a human codon usage optimized version of *rluc*, and encodes a protein identical to RLuc with the exception of a T2A substitution. To construct a bacterial expression plasmid, PCR was used to remove the stop codon and to replace the N-terminal methionine codon with a pelB leader sequence. The pelB leader sequence, consisting of the first 22 codons of the pectate lyase B gene from *Erwinia carotovora* [15], directs protein expression into the bacterial periplasm and is cleaved from the final protein product. Using NcoI and HindIII restriction sites, the PCR product was inserted into the pBAD/Myc-His A plasmid (Invitrogen, Carlsbad, CA), which adds a Myc epitope, a 6xHis tag, and a stop codon to the C-terminus of the gene. In some later constructs, the plasmid's SalI site was used for insertion in order to remove the Myc epitope from the construct. Site directed mutagenesis was performed using a QuikChange II XL kit (Stratagene, La Jolla, CA). When needed, cytoplasmic expression plasmids were generated by reverting the pelB leader sequence back to a methionine codon using PCR. All constructs and mutations were confirmed by sequencing.

Protein Production and Purification

All protein was produced from the periplasmic (pelB containing) expression plasmids unless otherwise noted. To express proteins periplasmically, plasmid containing *E. coli* LMG 194 cells were grown at 32°C in Terrific Broth. Cultures were allowed to reach an OD_{600} of 0.7 and then induced by addition of L-(+)-Arabinose to a final concentration of 0.2%. 12-14 hours later, cells were harvested and the periplasm extracted by osmotic shock [16].

The periplasmic fraction was brought to the same concentration as the wash buffer (WB: 300 mM NaCl, 20 mM HEPES, 20 mM imidazole, pH 8) using a 10x stock, and Phenylmethylsulphonylfluoride (PMSF) was added to 1 mM. The solution was clarified by 0.2 μm filtration and passed over a nickel affinity column (Ni-NTA Superflow, Qiagen, Valencia, CA). The column was washed with WB and eluted with elution buffer (EB: 300 mM NaCl, 20 mM HEPES, 250 mM imidazole, pH 8). Protein concentration measurements were made using the Bradford assay [17] with human serum albumin (HSA: Baxter Healthcare Corporation, Glendale, CA) as the standard. Aliquots were taken at this point for gel electrophoresis (Figure 2). To the remainder of the elution, HSA was added to 1% as a carrier protein. All samples were stored at 4°C.

For those instances in which cytoplasmic expression plasmids were used, the cells were grown as above. After harvesting, the cell pellet was frozen, thawed in WB containing 1 mg/ml lysozyme, 10 μg/ml RNase A, and 5 μg/ml DNase I, sonicated, and centrifuged to remove debris. These lysates were then cleared by filtration and purified by nickel affinity chromatography as above.

Characterization of *Renilla* Luciferase Mutants

Luciferase activity was measured by adding 1 μl of sample (diluted as necessary in EB containing 1% HSA) to 100 μl room temperature 100 mM sodium phosphate buffer (pH 7) [18], adding 1 μl of 0.5 μg/μl coelenterazine or analog, manually mixing, and reading for 10 s in a luminometer. The time between the addition of the luciferin and the start of measurement was approximately 4 s.

Measurements of inactivation in serum were done by mixing 0.5 μl dilute luciferase with either 20 μl mouse serum or 50 μl rat serum (Equitech-Bio, Kerrville, TX), placing the sample in a 37°C incubator, and removing aliquots for activity testing. To calculate the serum inactivation half-life, a mono-exponential decay model was fit to the data using a Nelder/Mead Simplex non-linear least squares minimization algorithm provided by the Octave numerical programming language [19]. Emission spectra at ambient temperature were measured using a Triax 320 (Horiba Jobin Yvon, Edison, NJ), which incorporates an optical grating device with a liquid N_2 cooled CCD detector.

Protein size and monodispersity were confirmed using a Superdex 200 analytical grade gel-filtration column (GE/Amersham Biosciences, Piscataway, NJ) followed by in-line multi-angle light scattering and refractive index detectors (DAWN EOS and Optilab DSP, Wyatt Technologies, Santa Barbara, CA). A dn/dc value of 0.185 mL/g was assumed in all calculations, and all processing was performed using the ASTRA software package (Wyatt Technologies).

For quantum yield measurements, separate 1 μl drops of protein ($\geq$2 pmol) and substrate (0.2 pmol) were placed in a tube, 100 μl of 100 mM sodium phosphate buffer (pH 7) was injected by the luminometer to mix, and the total light output was integrated (generally 5-10 min). For coelenterazine-*n*, the protein amount was increased 10-fold and the acquisition time lengthened to insure the reaction approached completion.

Kinetics

Kinetics were assessed by injecting 100 μl of 100 mM sodium phosphate buffer (pH 7) containing coelenterazine onto 1 μl of protein (diluted appropriately in EB containing 1% HSA), and recording the light output for 20 min. The final coelenterazine concentrations tested were 118, 24, 4.7, 0.94, 0.19, and 0.038 μM. The final luciferase concentrations were in the range of 1-7 pM. Coelenterazine absorbance was corrected for, although this was only significant for the highest concentration (10% attenuation). The values were converted from photons/s to molecules/s using the data from the quantum yield measurements, converted from flux units to mass units via integration, and processed using the kinetic curve fitting program Dynafit [20].

Mammalian Expression

In order to construct mammalian expression vectors, bacterial expression vectors containing the desired mutations were used as templates for PCR, with primers designed such that a methionine codon replaced the N-terminal pelB sequence and a C-terminal stop codon replaced the Myc epitope and 6xHis tag. The primers also contained appropriate NheI and HindIII restriction sites to allow insertion of the product into the pcDNA 3.1 plasmid (Invitrogen). The resultant plasmids were transiently transfected using SuperFect (Qiagen) into 293T cells [21] growing in 24 well plates following the manufacturer's protocol. Transfection media was replaced with fresh media (Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum) after 3 hours. At several time points, cells were lysed using passive lysis buffer (Promega), measured for total protein content using the Bradford assay, and assessed for luciferase activity using coelenterazine in the same manner as described above for bacterially expressed luciferase. Intracellular stability of the luciferases was assessed by adding cycloheximide to 100 μg/ml, and lysing cells at several time points thereafter. Westerns were run on lysates with a monoclonal antibody to RLuc (MAb 4400, Chemicon, Temecula, CA) in order determine the luciferase protein content, with purified bacterially produced RLuc8 used as the standard.

Results

Computational Predictions for *Renilla* Luciferase

Via sequence similarity searches, RLuc was predicted to contain a characteristic α/β-hydrolase fold from around amino acid 71 to 301 [22], and was found to have a high level of similarity to a number of proteins, most of which are known or putative haloalkane dehalogenases. An alignment between these sequences is shown in Figure 3, and a homology model is presented in Figure 4a.

Mutagenesis of *Renilla* Luciferase and Screening

In the hopes of further enhancing the resistance to inactivation of RLuc beyond that achieved with the C124A mutation (denoted C152A in [8]), a number of further mutations were explored. Candidate mutations were chosen from the alignment data at positions where RLuc most clearly diverged from the consensus sequence. For instance, the candidate mutation A55T was chosen because RLuc harbors the aliphatic amino acid alanine at position 55, while nearly all the other proteins harbor a hydroxylic residue of either threonine or serine at this site. Similarly, S287L was chosen as a candidate because RLuc contains a hydroxylic residue at this position, differing from the consensus aliphatic residue. Some of the candidates, such as M253L, are less obvious. This mutation substitutes an aliphatic residue for another aliphatic, but brings the RLuc sequence into consensus with the highly conserved local sequence near this position.

Complete results with respect to activity, inactivation in serum, and emission spectra peaks are summarized in Table 1 for 25 initial mutations, done on a background of RLuc with the C124A mutation. Note that activity was defined as a 10 s integration of the light output curve in order to disfavor mutations that merely increased the burst value at the expense of total light output. Representative data for inactivation in serum and emission spectra are shown in Figures 4b and 4c, respectively.

The assayed values for RLuc reported in Table 1 corresponded well with previous values reported in the literature. In terms of the stability of activity under serum-like conditions, the values reported here for recombinant RLuc ($\tau_{1/2} = 0.4 - 0.9\ h$) are in line with Liu *et al.*, who reported a half-life of 0.6 h for recombinant RLuc in hamster blood at 37°C [23], as well as Lorenz *et al.*, who reported a half-life of 0.5 h for recombinant RLuc in a high ionic strength buffer [24]. The measured emission peak for RLuc with coelenterazine (482 nm) corresponded exactly with the

previously published value of 482 nm for RLuc purified directly from *Renilla reniformis* [25].

Peak light flux from recombinant RLuc was determined to be $(1.2 \pm 0.2) \times 10^{23}$ photons/s/mole enzyme when in the presence of 24 μM coelenterazine. This value corresponds acceptably with the value of 6.5×10^{22} photons/s/mole enzyme reported for RLuc purified directly from *Renilla reniformis* [6], and 9×10^{22} photons/s/mole enzyme reported for recombinant RLuc [24].

Combining Mutations for a Luciferase Resistant to Serum Inactivation

For the purpose of generating a mutant RLuc more appropriate for use as a bioluminescent tag in small animal imaging applications, the initial mutations were judged for their ability to confer resistance to serum inactivation as well as their light output. After excluding A54P due to its strong negative effect on light output and F116L as it yielded only a small increase in resistance to serum inactivation, the 7 remaining mutations that exhibited either increased light output or enhanced resistance to serum inactivation were combined along with the C124A mutation into a single protein designated as "RLuc8". The 8 mutations present in RLuc8 are A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L. Since the Myc epitope was removed during the cloning of RLuc8, a C124A mutant was constructed without the Myc epitope to facilitate a valid comparison (C124A-ΔMyc). The activity, resistance to serum inactivation, and spectra peak values for these two enzymes are shown in Table 1. When compared to the native enzyme, RLuc8 exhibited a greater than 4-fold enhancement in activity, a 200-fold increased resistance to serum inactivation, and a small but measurable 5 nm red shift in the emission spectrum. Compared to the C124A mutant, RLuc8 showed a 3-fold increase in activity and at least a 13-fold improved resistance to inactivation in murine serum.

Light scattering results suggest RLuc8 exists as a monomer in solution, as molar mass moment calculations based on the multi-angle scattering indicate a molecular weight of 33.8 kDa (error: 7%) with a relatively low polydispersity across the gel filtration elution profile ($\sim$11%).

Comparison with Cytoplasmically Expressed Protein

In order to assess what effects the oxidative environment of the bacterial periplasm may be having on the proteins, RLuc and RLuc8 were expressed in the reducing environment of the bacterial cytoplasm using expression plasmids that did not include the pelB leader sequence. Compared to the periplasmically expressed RLuc presented in Table 1, cytoplasmically expressed RLuc had

25% greater activity with similar serum inactivation half-lives (0.8 h and 0.4 h for mouse and rat serum, respectively). Cytoplasmically expressed RLuc8 was also purified and had a similar activity (within 10%) and serum inactivation half-lives (290 h and 65 h in mouse and rat serum, respectively) to the periplasmically expressed RLuc8.

Quantum Yield and Kinetic Parameters of Mutants

To understand the basis for RLuc8's higher activity, both quantum yield and kinetic measurements were undertaken. The results shown in Table 2 indicated that RLuc8 had a 30% improvement in quantum yield for native coelenterazine, and a $\sim$30-fold increase in quantum yield for bisdeoxycoelenterazine. A Michaelis-Menten model was fit to initial reaction velocity data for coelenterazine concentrations in the range of 0.038 to 24 μM. The results for RLuc, the C124A mutant, and RLuc8 were K_m=2.9$\pm$1.0, 2.7$\pm$0.8, 1.6$\pm$0.2 μM, and k_{cat}=3.9$\pm$0.4, 4.7$\pm$0.4, 4.9$\pm$0.1 s^{-1}, respectively, with the errors presented representing the formal standard errors of the fitted parameters. The results for RLuc are roughly consistent with a previously published K_m value of 2 μM for RLuc in the presence of benzyl-coelenterazine [26].

Mutations to Test Proposed Active Site

Based on the catalytic triad of residues known to be critical for activity in the haloalkane dehalogenases, it was predicted that D120, E144, and H285 would be required for activity in *Renilla* luciferase as well. The locations of these residues in a homology model of *Renilla* luciferase are shown in Figure 4a. To test the hypothesis that these residues comprise a portion of the enzyme's active site, further mutations were made at these sites on the RLuc8 construct, with the results shown in Table 1. With respect to maintaining luciferase activity, mutations at these proposed active site residues were deleterious.

Combining Mutants for a Luciferase Prone to Inactivation

In order to construct brighter mutants that were less resistant to serum inactivation, the initial double mutants where compared to the single mutant C124A to identify mutations that led to increased activity without increasing resistance to serum inactivation (*e.g.* M185V) or increased serum inactivation without affecting initial light output (*e.g.* Q235A, S257G). Combining these

mutations in the absence of C124A resulted in the mutants M185V and M185V/Q235A (Table 1) that showed both increased inactivation in serum as well as enhanced activity in comparison to RLuc (Table 1).

Testing of Mutants in Mammalian Expression

In order to determine whether the *in vitro* data gathered for the RLuc mutants and RLuc8 would translate into the context of a mammalian reporter gene, expression vectors were constructed for RLuc, C124A, C124A/M185V, M185V, M185V/Q235A, and RLuc8 in a pcDNA 3.1 backbone. These mammalian expression plasmids were then transiently transfected into 293T cells. Measurements of light output over time, as shown in Figure 4d with respect to the RLuc plasmid, demonstrated that the mutations conferred increased light output following transfection in mammalian cells. A cycloheximide study was performed to assess the resistance to inactivation for the luciferase variants in the context of the mammalian cytoplasm. As shown in Figure 4e, the relative differences in inactivation resistance, but not the absolute differences, were consistent with the serum inactivation experiments. Through densitometry measurements of western blots performed on the cell lysates, the amount of luciferase was estimated for the different conditions and used to calculate the specific activity values shown in Table 3. These values were roughly consistent with the *in vitro* data for bacterially expressed protein.

Discussion

The amino acid sequence of *Renilla reniformis* luciferase, along with that of the closely related *Renilla mülleri* luciferase, contains a characteristic α/β-hydrolase fold sequence [27]. This fold pattern is found in enzymes that catalyze a diverse range of chemical reactions in all kingdoms of life. Interestingly, within the α/β-hydrolase family RLuc shows a high level of similarity to the bacterial haloalkane dehalogenases, enzymes that catalyze the hydrolytic detoxification of halogenated compounds. This similarity even extends to the conserved haloalkane dehalogenase catalytic triad, present as D120, E144, and H285 in RLuc, being required for appreciable luciferase activity. The level of similarity is unexpected as RLuc is an oxygenase and *Renilla reniformis* is not a bacterium.

The evolution of a coelenterazine using oxygenase from an enzyme that catalyzes an unrelated

reaction would not, in fact, present a considerable challenge for evolution. Coelenterazine chemiluminesces easily in aprotic solutions, and an initial enzyme would have to provide little more than a hydrophobic environment for coelenterazine to achieve some low level of bioluminescence [28]. The high level of primary sequence similarity between RLuc, the bacterial haloalkane dehalogenases, and the protein sequence derived from the *S. purpuratus* genome is harder to explain. As the bacteria, sea pansies (*e.g. Renilla* species), and sea urchins (*e.g. S. purpuratus*) diverged at least 500 million years ago, horizontal gene transfer could be one possible explanation for this similarity. However, this would seem to imply two separate gene transfer events for the ancestral luciferase gene, which seems rather unlikely. On another note, it has not escaped our attention that the gene from *S. purpuratus* could potentially be a luciferase, a finding that might explain the blue bioluminescence emission that has been observed in the fertilized eggs of this species [29].

In a 1999 paper, Liu and Escher reported results from sequentially mutating the three cysteines to alanine in a version of *Renilla* luciferase engineered for mammalian cell secretion [8]. Their hypothesis was that, in a secreted version of *Renilla*, these cysteines would have a propensity to form inactivating disulfide bonds in the oxidizing environment of the protein secretion pathway. They reported a complete loss of activity when mutating out the second cysteine residue (C73). Interestingly, many of the aligned proteins contain a cysteine near this location (Figure 3). They also reported enhanced stability of the enzyme when the third cysteine (C124) was replaced, and suggested that this was due to the blocking of unintended disulfide bond formation in oxidative environments. The homology model, however, indicates that the third cysteine is buried and removal of a potential disulfide bond formation is unlikely to explain the increased stability seen for this mutant after protein folding. More likely, the C124A mutation increases stability by allowing better packing of the hydrophobic core. Interestingly, the alignment data shows that an alanine is favored at this position, coincidentally the amino acid chosen to substitute by Liu and Escher.

Liu and Escher also suggested that a disulfide bond formed under oxidative conditions between C24 and C73 could increase the resistance of the enzyme to inactivation, and showed a number of experiments consistent with this hypothesis. We, however, did not see any significant changes with respect to inactivation between protein produced in the reducing environment of the cytoplasm and the oxidative environment of the periplasm. Cytoplasmically produced RLuc was found to have a 25% higher activity than the same protein produced periplasmically, but this is most likely due to some amount of activity loss during the comparatively longer periplasmic purification process. We have been unable to reconcile the discrepancies between our results and those of Liu and Escher, although they may in part arise due to differences between the mammalian secreted expression

system used by Liu and Escher, and the bacterial expression systems used here.

In any case, the interpretation of the Liu and Escher results for the C124A mutation in light of the protein alignment data led us to examine a mutation strategy in which candidate mutations would be picked at locations where RLuc clearly diverged from the consensus sequence. This strategy was initially predicated upon the hypothesis that mutations would accumulate in the ancestral RLuc gene that are either neutral only in the specific context of the *Renilla* lumisomes, or required for the association with and resonance energy transfer to the green fluorescent protein (GFP) homo-dimer that is the normal light emitter in *Renilla* [30].

Previous studies have utilized similar consensus guided semi-rational mutagenesis strategies [31, 32, 9, 33], albeit with differing experimental methods utilized as measures of stability (*e.g.* thermal denaturation, chemical denaturing, proteolytic cleavage). The common rationale behind all these studies, equally applicable to the study here, is that evolution tends to disfavor amino acids that destabilize a protein [34]. By looking at a family of similar proteins, one can pick candidate mutations that have already been screened by nature and are therefore less likely to be deleterious to the protein than a residue picked at random.

Three of the mutations, K136R, M185V, and S287L, showed sizable increases in the light output of the enzyme. In the case of M185V, a portion of the increase in light output can be explained by enhanced quantum yield, particularly for the coelenterazine analogs, with the difference assumed to arise from enhanced kinetics. The disproportionate increase in light output seen with M185V for several of the coelenterazine analogs leads us to speculate that the light increase and specificity decrease are related, and that M185 may be positively selected for in RLuc and *Renilla mülleri* luciferase to insure specificity of the reaction. This trade off between substrate recognition and light output could arise if the 185 position was important in substrate recognition. The alignment data gives some credence to our hypothesis, as M185 is located in the "cap" of the enzyme, a domain often used for substrate specificity in the haloalkane dehalogenases [27]. The location of M185 in the homology model lends further support, as its placement atop the presumptive catalytic site (formed by D120, E144, and H285) would be an appropriate position for conferring substrate specificity to the luciferase.

S287L, while not located in the active site, is close enough to the active site residue H285 that slight alterations in the structure of the enzymatic pocket induced by S287L could explain the modest increase in quantum yield and the larger increase in light output seen with this mutation.

K136, on the other hand, is located on the surface of the protein approximately 20 Å distant

from the presumptive active site. It is still possible that the increased light output seen with the K136R mutation could arise through long range perturbations in the folding of the active site, especially as K136 lies in a loop between the active site residues D120 and E144. Incidentally, *Renilla mülleri* luciferase contains an arginine at this position.

An alternative hypothesis for the increases in light output, especially for M185 and S287, is that these are residues are somehow involved in the transfer of energy to the GFP homo-dimer that interacts with RLuc in nature. As such, these residues have been selected for their role in energy transfer to the GFP fluorophore and not for their ability to optimize the quantum yield of the luciferase on its own.

Using our standard assay, RLuc8 displayed a $\sim$4-fold increase in light output versus RLuc. For RLuc8, combining the increases in quantum efficiency with the enhanced Michaelis-Menten parameters would only predict a $\sim$70% increase in light output over RLuc. Additionally, the Michaelis-Menten model could not satisfactorily fit the reaction progress curves, nor the initial reaction velocity at the highest coelenterazine concentration tested (118 μM), even when product inhibition was incorporated into the model. Previous attempts at elucidating a satisfactory kinetic model for RLuc have failed [35]. We speculate that a complete kinetic model would need to factor in coelenterazine-dependent inactivation of RLuc, a phenomenon known to occur rapidly in anoxic conditions [26].

Transient transfection of plasmids containing RLuc, C124A, C124A/M185V, M185V, M185V/-Q235A, and RLuc8 into mammalian cells demonstrated that the basic trends derived from the *in vitro* mutation analysis are applicable in the context of a mammalian cell as well. The rate of RLuc inactivation in the mammalian cells (Figure 4e) was an order of magnitude slower than that observed in serum (Table 1), which is not surprising given that RLuc is an intracellular eukaryotic enzyme in its native environment. However, the relative inactivation trends between the different luciferase variants were consistent for both sets of experiments. For instance, M185V/Q235A inactivated approximately twice as fast as RLuc whether the experiment was done in serum or in mammalian cells.

The specific activities measured from the mammalian transfection experiment (Table 3) were roughly consistent (within 30-50%) with the activities measured for the bacterially expressed proteins. The differences in the absolute values between the two sets of experiments are most likely due to errors in estimating the amounts of luciferase in the mammalian cellular lysates, and that the mammalian cellular lysates include a mix of active and inactivated protein accumulated during the course of the experiment.

Luciferases are extraordinarily useful in a variety of experiments that require reporter genes. In instances where the reporter gene is constitutively expressed (*e.g.* cell trafficking studies [36]), RLuc8 should be advantageous because of its greatly increased light output compared to RLuc in mammalian cells (Figure 4d). This increased light output stems from both RLuc8's increased specific activity (Table 3) and its decreased rate of inactivation (Figure 4e).

In most reporter gene experiments, however, the investigator wishes to follow the dynamics of gene induction and suppression. In these contexts, a slow rate of inactivation of the reporter would be a detriment to the experiment, as the stability of the signal would obscure transient changes in gene expression. The single mutant M185V and the double mutant M185V/Q235A should be of utility in these cases, as both these mutants show an increase in specific activity as well as an increase in the rate of protein inactivation relative to RLuc.

One issue with the use of coelenterazine catalyzing luciferases for reporter gene assays in mammalian cells is that coelenterazine is a substrate for MDR1 P-glycoprotein (Pgp) [37]. While the resultant transport of coelenterazine out of mammalian cells can be used to measure levels of Pgp, in most studies this phenomenon leads to an inadvertent modulation of signal intensity. For this reason, there has been interest in the coelenterazine analogs coelenterazine-*cp* and coelenterazine-*n* as they are not substrates for Pgp [37]. These analogs, however, suffer from reduced light output when used with RLuc (2-fold for coelenterazine-*cp*, 4-fold for coelenterazine-*n*, see Table 1) as well as $\sim$4-fold higher background rates of auto-chemiluminescence [38]. Combined, these factors lead to a drop in the signal to background ratio of 8 and 16-fold for coelenterazine-*cp* and coelenterazine-*n*, respectively. The M185V mutation greatly reduces the disadvantages of these alternative substrates. In the case of coelenterazine-*cp*, the M185V mutation leads to a signal to noise ratio that it is only a factor of two lower than that of the native substrate. RLuc8 is not as effective at using coelenterazine-*cp* as the M185V mutation alone, most likely because the A55T mutation present in RLuc8 decreases its ability to use this substrate.

Bisdeoxycoelenterazine has been proposed as a better analog to use with bioluminescence resonance energy transfer (BRET) studies because of the increased separation between the bioluminescence and the fluorescence spectra [39]. Bisdeoxycoelenterazine, however, suffers from extraordinary low light output when used with native RLuc (Table 1) because of poor quantum yield (Table 2). Both RLuc8 and the M185V mutation should be of great utility in these BRET assays, as they confer a 20-60 fold increase in light output with bisdeoxycoelenterazine. Interestingly, although C124A alone does not improve utilization of bisdeoxycoelenterazine, it appears to facilitate the M185V mutation, as C124A/M185V has a $\sim$2-fold better light output with this

substrate compared to M185V alone.

In summary, we have characterized mutants of RLuc with respect to resistance to serum inactivation as well as light output, and have used these results to develop luciferases optimized for different purposes. An 8 mutation form of RLuc (RLuc8) was created that has greatly improved characteristics for use as a bioluminescent label. Compared to the native enzyme, RLuc8 exhibited a 200-fold improvement in resistance to murine serum inactivation, a 4-fold improvement in light output, and a 5 nm red shift in the emission spectrum. The enhancement in light output arises from a combination of increases in quantum yield and improved kinetics. A double mutant of RLuc (M185V/Q235A) was created that has improved performance as a reporter gene. Compared to the native enzyme it has twice the rate of inactivation, as measured in murine serum, while incorporating a close to 5-fold improvement in light output. These optimized *Renilla* luciferases represent significant improvements that will increase the sensitivity of luciferase-based assays for both *in vitro* experiments and *in vivo* imaging.

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Legends to Figures

1 Chemical structures of coelenterazine and several analogs. Abbreviations used in the text are given in parenthesis. 21

2 Coomassie stained SDS-PAGE gel of RLuc and RLuc8 at several points during the periplasmic purification process. The lanes are labeled as follows: M - Marker, P - Periplasmic fraction, FT - Flow through from nickel affinity column, W - Wash from column, E - Elution from column. As the elution volume was 5% of the periplasmic fraction, the periplasmic fraction, flow through, and wash were concentrated 20-fold using 3 kDa cut-off centrifugal concentrators (Pall, Ann Arbor, Michigan). The expected sizes for RLuc and RLuc8 are 38.7 kDa and 36.9 kDa, respectively, with the difference in size arising from a Myc epitope added by the expression vector used for RLuc. These protein masses were confirmed by MALDI-TOF. Final recovery of periplasmically produced purified protein was typically 5 mg/L of culture for RLuc, and 50 mg/L of culture for RLuc8. Cytoplasmically expressed RLuc8 generally resulted in a purified protein yield of 500 mg/L of culture. 22

3 Alignment of *Renilla reniformis* luciferase and proteins from 14 other species. When available, the names of the proteins are given in parenthesis. With the exception of RLuc and the protein of unknown function predicated from the *S. purpuratus* (Purple Sea Urchin) genome, all the aligned sequences are from bacterial species. Species 1-5, 12 and 13 are marine organisms. Proteins 6 and 8-11 are known haloalkane dehalogenases. Proteins 3-5, 7, 14 and 15 are putative haloalkane dehalogenases. Amino acids in the sequences corresponding to gaps in the aligned *R. reniformis* sequence have been removed. The bacterial dehalogenases have a conserved catalytic triad [27] corresponding to D120, E144, and H285 in the RLuc sequence. Residues that were selected for mutagenesis in RLuc are shown in boxes. RLuc was 40% identical and 72% similar to the protein from *S. purpuratus*, and 29-43% identical and 49-62% similar to the bacterial proteins. The GenBank Identifiers for sequences 1-15 are 160820, 72160391, 40062609, 83943988, 85826216, 34810153, 16508080, 50399582, 28558081, 14025217, 27349338, 68181465, 50082962, 29608437, and 15163540, respectively. 23

   (a) Homology model of *Renilla* luciferase based on its similarity to the haloalkane dehalogenase LinB. The region of the enzyme from residue 35 to 309 was successfully modeled using Swiss-Model and is shown. The N-terminus is blue and the C-terminus is red. The presumptive catalytic triad of D120, E144, and H285 are marked, along with the mutation site M185. 24

(b) Mouse serum inactivation data for RLuc, RLuc8, and several other mutations. Protein was incubated in mouse serum at 37°C in triplicate, with aliquots removed at various times to determine the remaining luciferase activity. The error bars represent the standard error of the mean, and the lines drawn between points are from the fit to a mono-exponential decay model. . 24

(c) Normalized bioluminescence emission spectra for RLuc, RLuc8, and several other mutations. Most mutations resulted in only small shifts from the emission spectra of the native luciferase. The normalized emission spectrum of RLuc8 when used with bisdeoxycoelenterazine (bdc) is also included for comparison. 24

(d) Mammalian cell expression of native RLuc and several mutants following transient transfection into 293T cells. The luciferases for this study were in pcDNA 3.1 plasmids under the control of the constitutive promoter from cytomegalovirus(CMV). Light output per total cellular protein was recorded for each condition, and is reported as relative to the value of the RLuc condition at the given time point. The absolute values for the RLuc condition at the given time points were (8.6 ± 0.3), (6.7 ± 0.2), and $(1.3\pm0.02)\times10^9$ photons/s/mg of total protein. Samples were done in quadruplicate and error bars represent standard error of the mean. With the exception of the C124A/-M185V and RLuc8 conditions at the 24 hour time point, all differences between groups for a given time point were significant at $p \leq 0.05$ using a two-tailed *t*-test with the incorporation of a Bonferroni-Holms correction for multiple comparisons [40]. 24

(e) Measurements of the intracellular inactivation of luciferase activity. 293T cells, 48 hours after being transiently transfected with the indicated luciferases, were exposed to 100 μg/ml of cycloheximide to inhibit new protein synthesis. Cells were assayed for light output per total cellular protein, with the data fit to a mono-exponential decay model. Samples were done in quadruplicate, and the error bars represent the standard error of the mean. The estimated intracellular activity half-lives are given in the figure key. 24

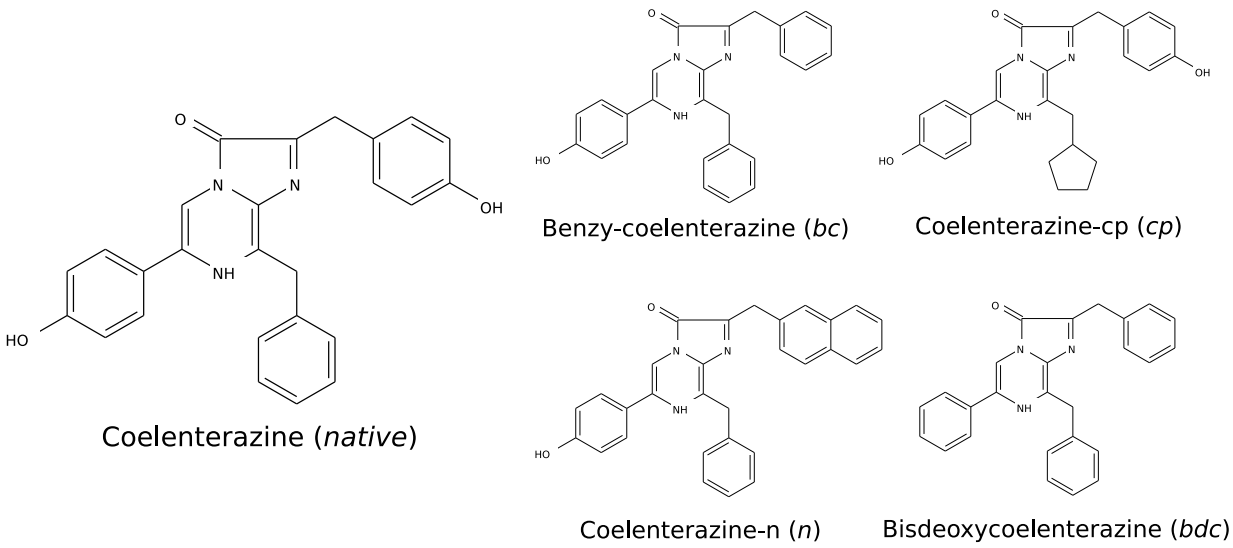

Coelenterazine (*native*)
Benzy-coelenterazine (*bc*)
Coelenterazine-cp (*cp*)
Coelenterazine-n (*n*)
Bisdeoxycoelenterazine (*bdc*)

Figure 1:

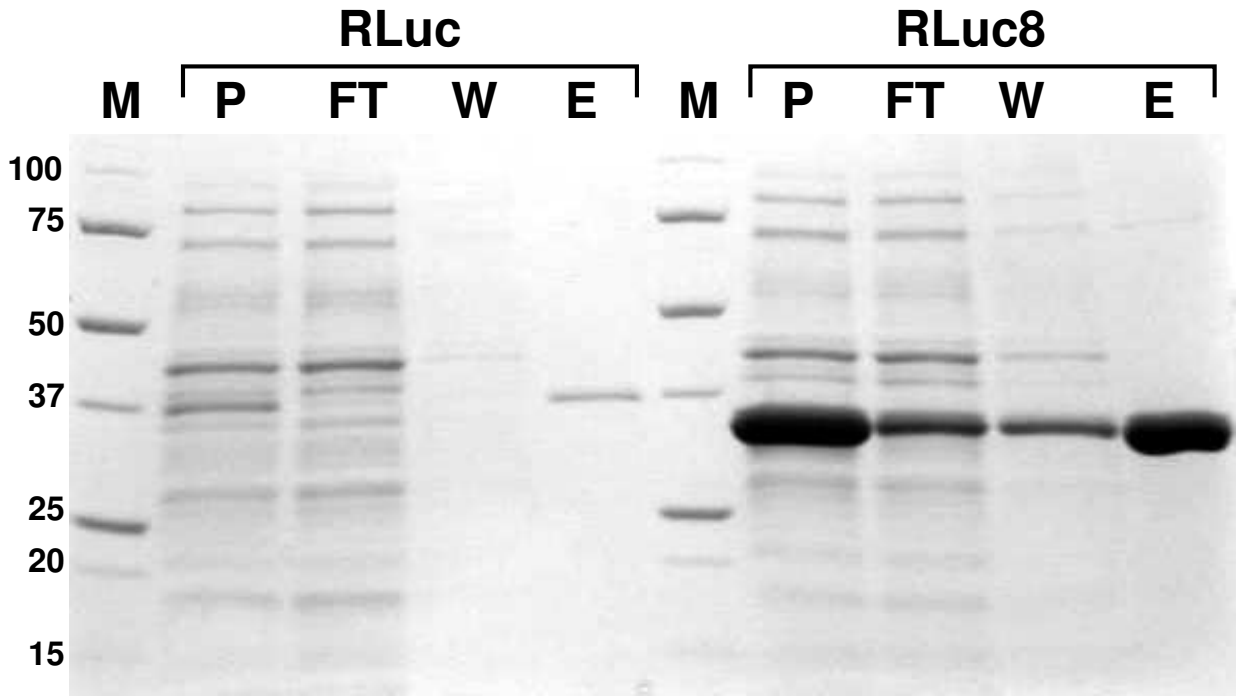


Figure 2:

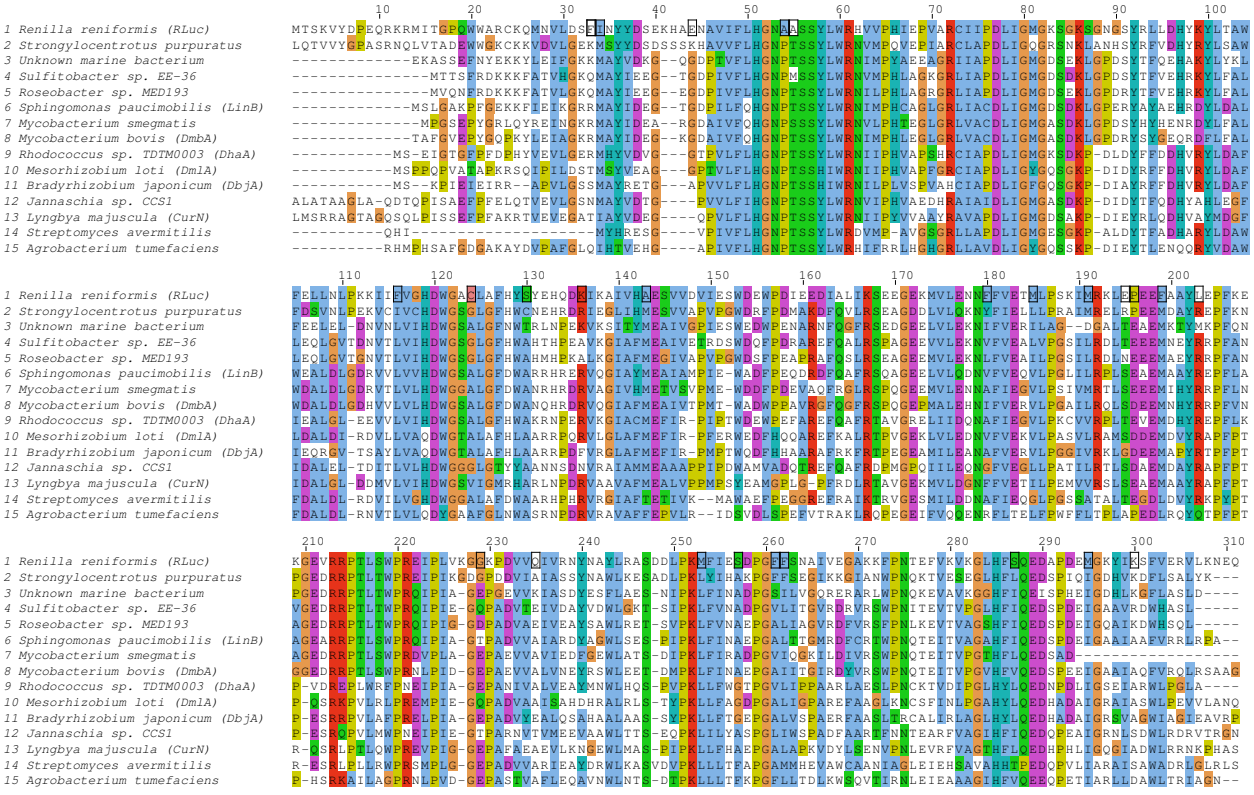
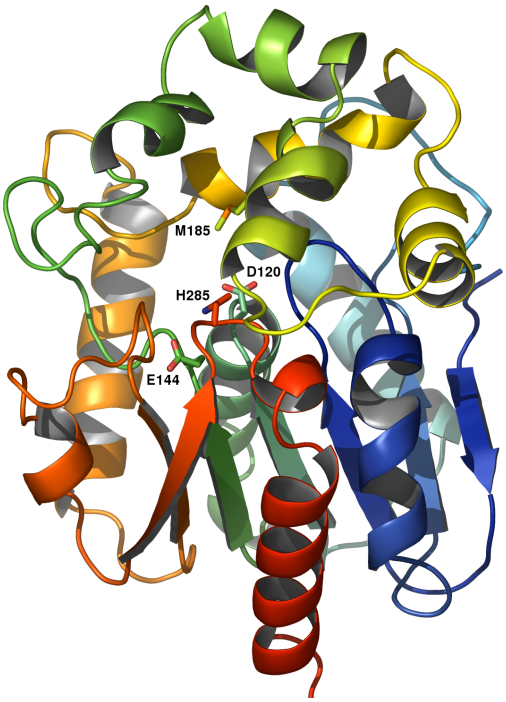
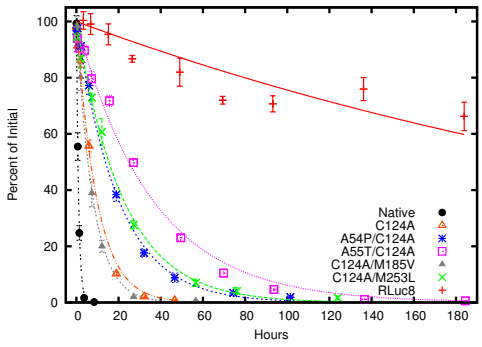

1 Renilla reniformis (RLuc)
2 Strongylocentrotus purpuratus
3 Unknown marine bacterium
4 Sulfitobacter sp. EE-36
5 Roseobacter sp. MED193
6 Sphingomonas paucimobilis (LinB)
7 Mycobacterium smegmatis
8 Mycobacterium bovis (DmbA)
9 Rhodococcus sp. TDTM0003 (DhaA)
10 Mesorhizobium loti (DmlA)
11 Bradyrhizobium japonicum (DbjA)
12 Jannaschia sp. CCS1
13 Lyngbya majuscula (CurN)
14 Streptomyces avermitilis
15 Agrobacterium tumefaciens

Figure 3:



(a)



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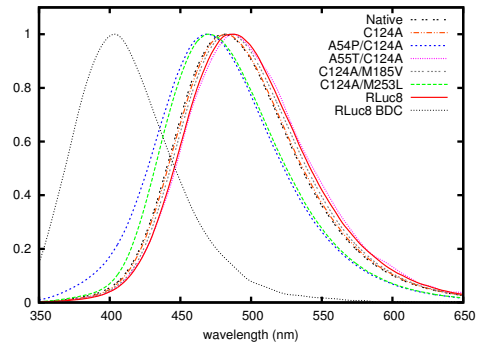

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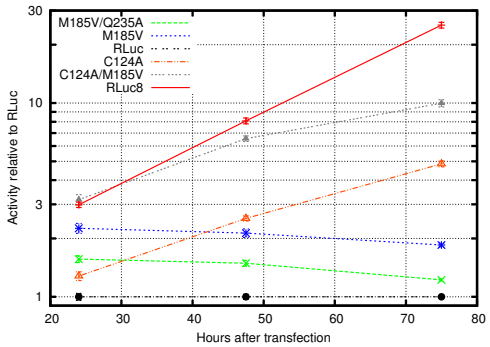

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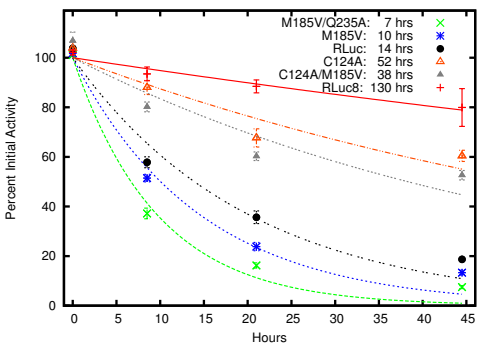

(e)

Figure 4:

Legends to Tables

	Activity (photons/s/mole enzyme)					Serum Inactivation $\tau_{1/2}$ (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±0.1	0.4±0.1	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
Inactivation Resistant									
RLuc8	4.3±0.2	3.0	5.8	8.8	59	281 ±49	86 ±9	487	503
Active Site Mutations									
RLuc8/D120A	0.000	0.001	0.001	0.003	0.21	> 100	> 100	N/D	
RLuc8/D120N	0.023	0.016	0.050	0.34	5.1	> 100	> 100	N/D	
RLuc8/E144A	0.000	0.000	0.000	0.000	0.000	57	13	N/D	
RLuc8/E144Q	0.000	0.000	0.000	0.000	0.002	> 100	> 100	N/D	
RLuc8/H285A	0.023	0.020	0.046	0.028	0.20	> 100	21	N/D	
Inactivation Prone									
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	

Table 1:

	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	$(198.2\pm8.5)\times10^{-3}$

Table 2:

	photons/s/mole enzyme
RLuc	$(4.2\pm0.2)\times10^{22}$
Mutant	Activity (relative to RLuc)
C124A	1.6±0.1
C124A/M185V	4.2±0.6
M185V	2.5±0.2
M185V/Q235A	2.6±0.2
RLuc8	4.4±0.2

Table 3: