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CHAPTER VII

Codon-optimized Luciola italica luciferase variants for mammalian gene expression in culture and *in vivo*

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ABSTRACT

Luciferases have proven to be useful tools in advancing our understanding of biological processes. Having a multitude of bioluminescent reporters with different properties is highly desirable. Here, we characterized codon-optimized thermostable green- and red-emitting luciferase variants from the Italian firefly *Luciola italica* for *in vivo* imaging. Using lentivirus vectors to deliver and stably express these luciferases in mammalian cells, we showed that both variants displayed similar levels of activity and protein half-lives as well as similar light-emission kinetics. Further, we characterized the red-shifted variant for *in vivo* bioluminescence imaging. Intramuscular injection of tumor cells stably expressing this variant into nude mice yielded a robust luciferase activity. Light emission peaked at 10 minutes post-$_D$-luciferin injection and retained >60% of signal after 1 hr. Similarly, luciferase activity from intracranially-injected glioma cells expressing the red-shifted variant was readily detected and used as a marker to monitor tumor growth over time. Overall, our characterization of these codon-optimized luciferases lays the groundwork for their further utilization as bioluminescent reporters in mammalian cells.

INTRODUCTION

Bioluminescence imaging (BLI) using luciferase reporters has provided crucial information regarding many biological processes including tumorigenesis, bacterial pathogenesis, and transcription factor activation[1-3]. The major advantage of BLI compared to endpoint analysis is that it provides real time, non-invasive analysis of *in situ* biological events, thereby giving a complete "picture" of the kinetics of an entire process. Great strides have been made since the seminal study by Contag et al. published in 1995 which was the first demonstration of *in vivo* BLI[4]. For example, as few as 10 cells expressing an optimized American firefly luciferase can be detected in mice[5]. Additional progress has been made by the discovery and codon optimization of the naturally secreted *Gaussia princeps* luciferase, allowing for sensitive detection of *in vivo* biological processes by simple blood sampling[6]. The discovery of luciferases as well as the genetic engineering of existing luciferases

which emit light at longer wavelengths, thus reducing tissue absorption of light, has enhanced the sensitivity of *in vivo* BLI[7,8,9].

The luciferase with the most biochemical characterization is from the North American firefly *Photinus pyralis (*Fluc*)*. Fluc catalyzes a two-step reaction in which luciferin is first adenylated through an ATP-dependent mechanism. Next, molecular oxygen is used to oxidize the adenylated intermediate yielding oxyluciferin, a molecule in an excited energy state. Upon decay of excited state oxyluciferin, a photon of light is emitted[10]. Due to the high quantum yield of the luminescence reaction, Fluc was pursued as a reporter enzyme. Since its cloning and expression in mammalian cells in 1987[11], Fluc has become the "workhorse" of the molecular biology lab as a reporter for gene expression and ATP levels in cells[12, 13] as well as non-invasive monitoring of *in vivo* processes using bioluminescence imaging technology[14].

Subsequently, other luciferases have been cloned and characterized for bioluminescence imaging in an attempt to increase detection sensitivity as well as dual parameter measurement. Luciferases which catalyze red-shifted light (emission peak >600nm)[15] or mutations of existing luciferases to a red-shifted emission spectra[16] have improved the sensitivity of detection as light absorption by mammalian tissues and hemoglobin decreases greatly above 600 nm[17]. *Renilla reniformis* luciferase (Rluc) and *Gaussia princeps* luciferase (Gluc) utilize coelenterazine as a substrate, in contrast to Fluc, and therefore sequential imaging of Fluc and Gluc or Rluc *in vivo* can be used to monitor two different biological processes[18]. Recently, the cDNA encoding the green-emitting luciferase from the Italian firefly *Luciola italica* (liFluc) was cloned by Branchini et al. which has 64% amino acid identity with the luciferase from *Photinus pyralis*[19]. This luciferase has several advantages over Fluc including thermostability and higher enzyme turnover leading to approximately 2-fold higher light output[19]. Further, using standard mutatagenesis, a red-shifted variant was characterized[20]. liFluc has been expressed and purified from bacteria and was used for cell based assays[20], however, a codon-optimized variant for mammalian gene expression has not been established for *in vivo* imaging. In the current study, we characterize codon-optimized variants of both the green- and red-emitting liFluc for mammalian gene expression and use the ladder for *in vivo* bioluminescence imaging in small animals.

MATERIALS AND METHODS

Cell culture and reagents. 293T human kidney fibroblasts cells, U87 human glioma cells (both from American Type Culture Collection, Manassas, VA) and Gli36 human glioma cells (kindly provided by Dr. Anthony Capanogni, University of California at Los Angeles, Los Angeles, CA), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma), referred to as complete DMEM. All cells were grown at 37 ^{0}C in a 5% CO_2 humidified atmosphere.

Lentivirus vector construction. Human codon optimized cDNA variants of both green emitting *Luciola italica* luciferase (G-liFluc) and red emitting (R-liFluc) were kindly provided by Dr. Rampyari Walia (Targeting Systems, El-Cajon, CA). These cDNAs were cloned into a lentivirus vector plasmid, CSCW[21]. Transgene expression in this plasmid is driven by a cytomegalovirus (CMV) promoter with the inclusion of an internal ribosomal entry site (IRES) for co-expression of mCherry red fluorescent proteins to allow for titering and transduction confirmation[6]. Both G-liFluc and R-liFluc coding sequences were PCR-amplified using Pfu polymerase (Agilent technologies, Santa Clara, CA) and the following primers: Forward primer, 5' ATAGCTAGCGATCCATGGAAACAGAAAG3'; reverse primer, 5'TACTCGAGACTACCCACCTGCTTGAGGT 3'. The thermalcycler conditions were 1 cycle of 94ºC for 2 min; 30 cycles of 94 ºC for 45s, 60 ºC for 45s, 72 ºC for 90s; 1 cycle of 72 ºC for 10 min. The forward primer was synthesized with an NheI site and the reverse with an XhoI restriction site for ligation with similarly-digested CSCW-ImCherry plasmid. Constructs were named CSCW-GliFluc-ImCherry and CSCW-R-liFluc-ImCherry. A lentivirus vector encoding the Gaussia luciferase (Gluc) and the Cerulean fluorescent protein (CFP) separated by an IRES (CSCW-Gluc-ICFP) was described previously[22]. Lentivirus vectors were produced as described before[21] and vector titers (transducing units/ml) were determined by performing serial dilutions of vector stocks on 293T cells followed by counting the number of mCherry or CFP positive 293T cells 3 days later using fluorescence microscopy.

Lentivirus vector transduction. For side by side comparison of G-liFluc and R-liFluc luciferases, Gli36 and U87 human glioma cells were engineered to stably express both Gluc and either one of these luciferases. Around 350,000 cells were plated in a 6 well plate. The Next day, cells were first transduced with the CSCW-Gluc-ICFP

lentivirus using a multiplicitiy of infection (MOI) of 50 in the presence of 10 µg/mL Polybrene in 3 mL. The plate was centrifuged at 1800 rpm for 1 hr at room temperature. Afterwards, cells were cultured overnight. Seventy-two hrs later, the same protocol was applied to transduce the same cells with either CSCW-RliFluc-ImCherry or CSCW-GliFluc-ImCherry. The CFP (a marker for cell number) and mCherry (marker for transduction efficiency) fluorescence intensities in both cell lines was quantified using a FlexStation® 3 microplate reader (Molecular devices, Sunnyvale, CA).

In vitro Gaussia luciferase assay. 10^4 cells/well were plated in a 96 well plate. After 24 h, cells were washed once with 50 µL PBS and lysed on the plate in 50 µL lysis buffer (Targeting systems, El Cajon, CA). From each well, 20 µL cell lysates were transferred to a standard white opaque 96 wells plate for endpoint luminescence measurement using the FlexStation® 3 microplate reader. 50 µL 10 µM coelenterazine in PBS was injected into each well and photon count was measured immediately for 500 ms.

In vitro Luciola italica luciferase assays. A 20 µL aliquot from cell lysates was transferred to a white opaque 96 well plate. 80 µL FLAR-1 Luciferase Assay Kit reagent (Targeting systems, El Cajon, CA) was added and total luminescence was measured for 500 ms.

Luciferin dosing studies. 80 µL of different $_D$-luciferin concentrations (Gold biotechnology, St Louis, MO; diluted in PBS containing 2 mM ATP) ranging from 0.5 µM to 50 mM were added into wells containing 20 µL of lysates from Gli36 cells expressing either G-liFluc or R-liFluc. Photon count was acquired for 500 ms using the microplate reader.

Stability of luciferases. $5x10^5$ cells plated in a well of a 6 well plate were lysed in 500 µL of cell lysis buffer. A 65 µL aliquot of cell lysates was frozen immediately. Lysates were incubated for 30 min, 1h, 2h, 4h and 6h at 37 °C. In triplicate, 20 µL aliquots from each time point was transferred into an opaque 96 well plate and 80 µL FLAR reagent was injected per well. Luminescence was acquired as above.

Kinetic assays. 20 µL aliquots of lysates from Gli36 cells stably expressing G-liFluc or R-liFluc (10^4 cells lysed in 50 µL) were mixed with 80 µL 830 µM $_D$-Luciferin (in PBS containing 2 mM ATP). Luminescence was acquired for 500 ms every 20 seconds for 4 minutes using the microplate reader.

Spectral analysis of G-liFluc and R-liFluc light emission. 80 µL of FLAR reagent was added to 20 µL of cell lysates and light emission was measured using the microplate reader every 10 nm using the device's emission monochromator.

In vivo experiments. All animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Athymic nude mice were anesthesized with a mixture of Ketamine (100 mg/kg) and Xylazine (5 mg/kg) in 0.9% sterile saline. For intramuscular injection (n=4), $3x10^6$ (in 25 µL) Gli36 cells expressing R-liFluc and Gluc were mixed with equal volume of Matrigel™ (BD Biosciences, San Jose, CA) and injected using an insulin syringe. For the brain tumor model, 10^5 U87 cells expressing R-liFluc and Gluc (in 1 µL PBS) were intracranially injected in the left midstriatum of nude mice (n=4) using the following coordinates from bregma in mm: anterior-posterior +0.5, medio-lateral +2.0, dorso-ventral −2.5. These injections were performed using a Micro 4 Microsyringe Pump Controller (World Precision Instruments, Sarasota, FL) attached to a Hamilton syringe with a 33-gauge needle (Hamilton, Rena, NV) at a rate of 0.2 µL/min.

In vivo bioluminescence imaging. Bioluminescence images were obtained at different time points post-implantation of tumor cells. Mice were anesthesized as above. Initially, 20 µL blood was drawn from the tail vein of each mouse and mixed with 2 µL 20 mM EDTA which was stored on ice until assayed. Mice were then intraperitoneally (i.p.) injected with 150 µL of $_D$-luciferin (150 mg/kg body weight). Ten min later, photon count was acquired using a cryogenically-cooled CCD camera for 1 min as described[23]. For *in vivo* light emission kinetics, imaging was performed every 5 minutes for 1h, starting immediately upon substrate injection. CMIR-Image software, developed by the Center for Molecular Imaging Research at the Massachusetts General Hospital, was used to process the data and quantify signal intensity. Total bioluminescent signals, representing the sum of 1 minute photon counts, are shown in a pseudo-color photon count manner. Images were fused with a gray-scale white light image to allow anatomic localization.

Gluc blood assay. The Gluc-blood assay was performed as described[24]. Briefly, 5 µL blood was transferred into a white opaque 96-well plate and 100 µL 100 µM coelenterazine was injected to each well and the total luminescence was acquired for 10 sec using a luminometer (Dynex, Richfield, MN).

RESULTS

Expression of codon-optimized variants of the Luciola italica luciferase in mammalian cells

To ascertain the utility of the codon-optimized green- and red-emitting variants of the *Luciola italica* luciferase as mammalian cell reporters, we cloned the cDNA of either variant into a lentivirus vector plasmid. Cells were first transduced with a lentivirus vector encoding *Gaussia princeps* luciferase (Gluc) and the Cyan fluorescent protein expressed using an internal ribosomal entry site (IRES) element (**Fig. 1A & B**). The expression of Gluc and CFP is used as an internal control to normalize for cell number both *in vitro* and *in vivo* as previously described[6]. These cells were then engineered using lentivirus vectors to stably express either the green-emitting G-liFluc or red-shifted R-liFluc as well as the mCherry Red fluorescent protein separated by an IRES element (**Fig. 1A**). mCherry is used as a marker for transduction efficiency for the liFluc construct (**Fig 1B**). Two weeks post-transduction, cells were plated in a 96-well plate. First, the mCherry fluorescence intensity was measured using a microplate reader which showed that Gli36 cells had 1.5-fold higher transduction efficiency with R-liFluc as compared to G-liFluc (**Fig. 1C**). Then, aliquots of lysates from same cells were assayed for Gluc activity to normalize for cell number (**Fig. 1D**). Finally, the same lysates were analysed for liFluc activity. Luciferase activity was $3.6x10^4$ RLU for G-liFluc and $4.4x10^4$ RLU for R-liFluc (**Fig 1E**). When the liFluc values were normalized to transduction efficiency (mCherry, Fig. 1C) and cell number (Gluc, Fig 1D), we obtained $2.2x10^4$ RLU for G-liFluc and $1.2x10^4$ RLU for R-liFluc (**Fig. 1F**). This 1.83-fold difference was found to be statistically significant (P=0.018).

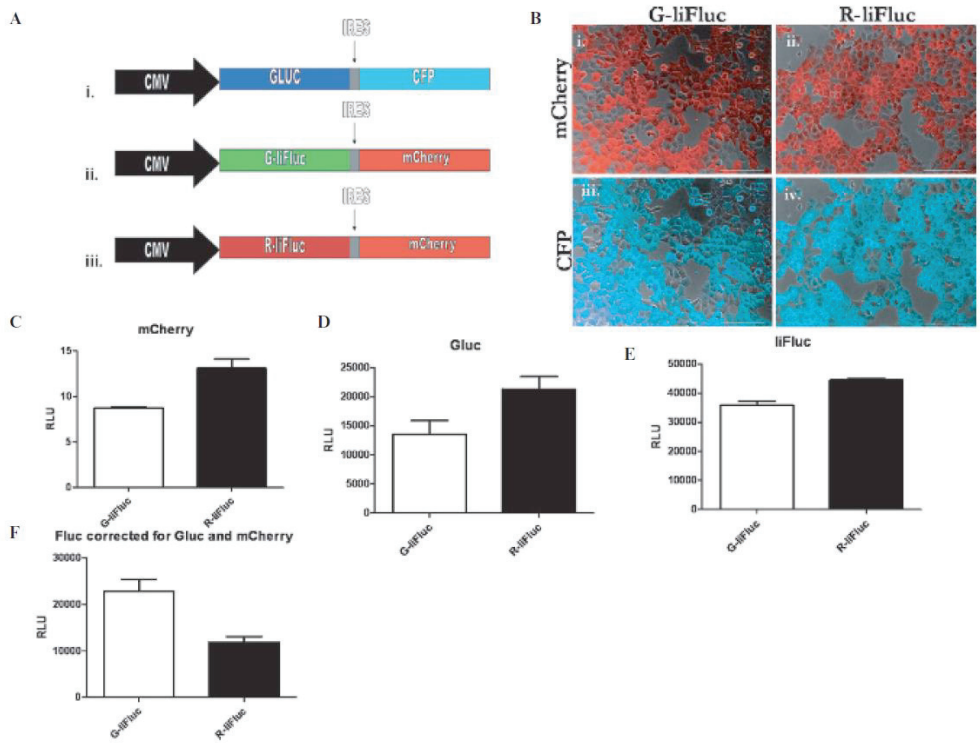

Figure 1. Expression of the codon-optimized *Luciola italica* luciferase green-emitting (G-liFluc) and red-shifted (R-liFluc) variants in mammalian cells. **(A)** Lentivirus expression cassettes used in this study. Gli36 human glioma cells were first transduced with a lentivirus vector encoding *Gaussia* luciferase (Gluc) and CFP **(i.)** followed by either G-liFluc **(ii.)** or R-liFluc **(iii.)** and mCherry encoding vectors. **(B)** Confirmation of successful transduction with these vectors using fluorescence microscopy. Shown are overlays of bright field and mCherry fluorescence (**i., ii**.) and bright field and CFP fluorescence **(iii.-iv.)**. Scale bar, 100 µm. **(C)** Transduction efficiency of Gli36 with vectors encoding G-liFluc or R-liFluc was determined by assaying for mCherry fluorescence using a microplate reader. **(D)** Gluc assay from lysates of same cells using coelenterazine as a substrate. **(E)** *Luciola italica* luciferase assay on separate aliquot of the same cellular lysates in (D) using $_D$-luciferin as a substrate. **(F)** *Luciola italica* luciferase activity normalized for transduction efficiency (mCherry levels from C) and cell number (Gluc levels from D).

Emission and stability of liFluc variants

We first confirmed the spectrum of light output from G-liFluc and R-liFluc in mammalian cells. As expected, we obtained an emission spectra with a peak at 550 nm and 610 nm for G-liFluc and R-liFluc respectively, which are similar to values reported for non-codon optimized variants purified from bacteria[20] (**Fig. 2A**). Stability

assays were then performed for these variants and were compared to the North American firefly luciferase (Fluc). Both G-liFluc and R-liFluc showed an increased thermostability compared to Fluc (5.3 h and 2 h vs 0.5 h; **Fig. 2B**).

Dose response of green and red-emitting liFluc to *D*-luciferin

To gain insight into the performance of each variant under different substrate concentrations, we carried out a dose response analysis of $_D$-luciferin ranging from 1 µM to 50 mM on lysates from Gli36 cells expressing either liFluc. Both G-liFluc and R-liFluc displayed a similar dose/activity profile, although at lower doses the G-liFluc displayed a substantially higher activity than R-liFluc (**Fig. 2C**). For example at a $_D$-luciferin dose of 5 µM, G-liFluc displayed a 5.1-fold higher RLU value than R-liFluc. This difference was reduced to 1.27-fold at the 0.5 mM dose (**Fig. 2C**). At the higher D-luciferin doses of 5 and 50 mM, we observed an apparent inhibition of enzyme activity.

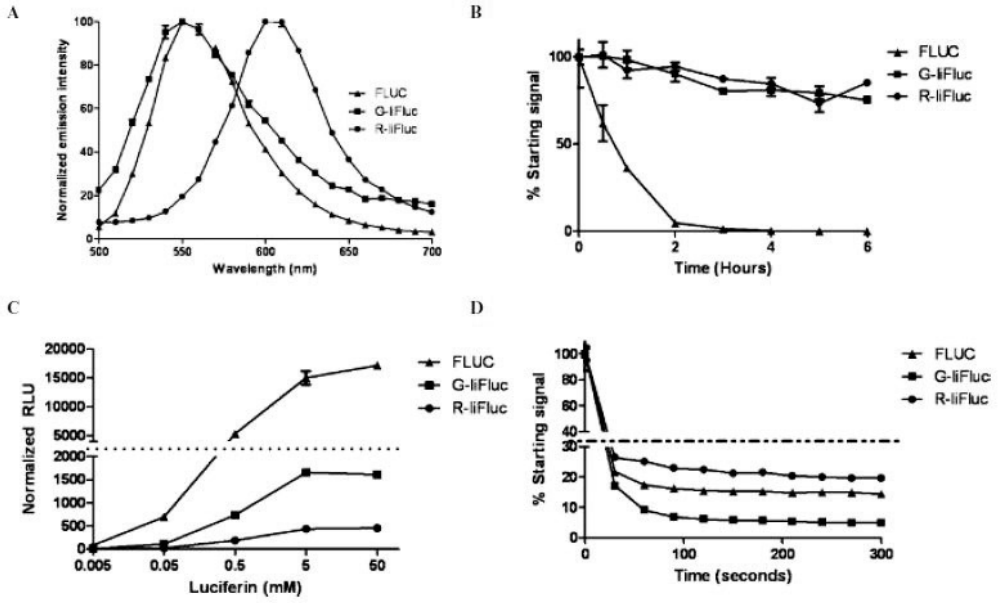

Figure 2. Characterization of *Luciola italica* luciferase activity in mammalian cells. **(A)** Emission spectra of G-liFluc and R-liFluc and the North American firefly luciferase Fluc. **(B)** *Luciola italica* luciferase stability at 37°C. Lysates from cells expressing either G-liFluc, R-

liFluc or Fluc, were incubated at 37 °C and assayed for luciferase activity at different time points. **(C)** $_D$-luciferin dose response of G-liFluc, R-liFluc, and Fluc was assayed on lysates from B.. **(D)** light emission kinetics of liFluc variants. $_D$-luciferin was added to a well containing lysates from cells expressing either G-liFluc, R-liFluc or Fluc. Light emission was measured every 30 seconds for 5 minutes.

Light emission kinetics for liFluc variants

Light emission kinetics after substrate $_D$-luciferin addition was determined for both G-liFluc and R-liFLuc in lysates from cells expressing either luciferases. The relative light units declined to 50% of starting values at 1 minute for G-liFluc and 46 sec for R-liFluc variant (**Fig. 2D**). Interestingly, luminescence signal stabilized for R-liFluc at 2 minutes to 10 minutes post-substrate addition (16% of starting signal) while the luminescence continued to decay for G-liFluc (from 20% to 6%).

***In vivo* imaging of R-liFluc**

Since light of longer wavelengths is known to have much lower absorption by pigmented molecules such as hemoglobin and melanin as well as scattering by mammalian tissues, we characterized the red-shifted *Luciola italica* luciferase R-liFluc for *in vivo* deep tissue bioluminescence imaging. Nude mice were injected into the hamstring with $3x10^6$ Gli36 cells expressing R-liFluc. Forteen-days post-injection, mice were injected intraperitoneally (i.p.) with 150 mg/kg $_D$-luciferin and imaged using a cooled CCD camera. Light emission was readily detected from these muscle tissues ($4.03x10^5$ photons/min; **Fig. 3A**). To determine the optimum acquisition time for imaging R-liFluc in deep tissues, we imaged mice immediately and at 5 min interval for 1 hr after $_D$-luciferin injection. Bioluminescence signal was detected immediately after injection and peaked at 10 minutes post-substrate injection (**Fig. 3B**). Signal began to slowly decline at 30 minutes, although never dropped below 48% for the 1 hour duration (**Fig. 3B**).

Having confirmed its utility for imaging in muscle tissues, we next tested R-liFluc as a reporter to image brain tumors, which requires adequate light to pass through 2.5 mm of brain tissue as well as ~1mm of skull. 10^5 U87 glioma cells stably expressing R-liFluc-ImCherry and Gluc-ICFP were injected into the striatum of nude mice. At two and three weeks post injection, mice were anesthetized and blood was collected and assayed for Gluc activity[6], and then mice were i.p. injected with 150 mg/kg $_D$-luciferin and imaged for R-liFluc 10 min later. At two weeks post tumor cells

implantation, luminescence was detected at an average of $1.23x10^3$ photons/min (**Fig. 4A**). This signal increase to $8.58x10^3$ photons/min at week 3 post tumor injection (**Fig. 4A**) resulting in a 7-fold increase in signal between the two time points proving that R-liFluc can be used as a reporter to monitor biological processes including tumor growth over time. A similar fold increase was observed for Gluc in the tumor as assessed by Gluc-blood assay at the same time points (**Fig. 4B**).

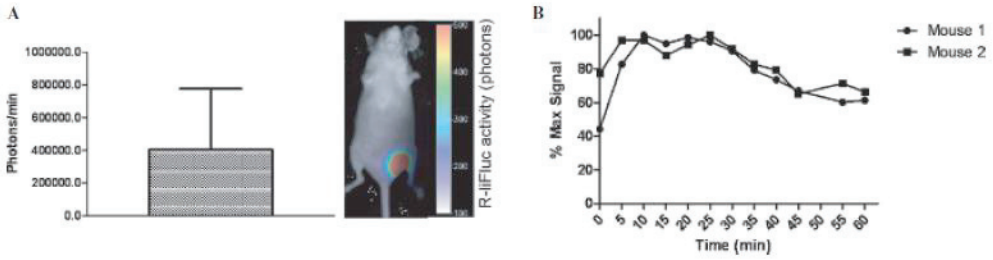

Figure 3. Red-emitting *Luciola italica* luciferase variant for *in vivo imaging*. $3x10^6$ Gli36 cells expressing R-liFluc were injected into the hamstring of nude mice. (**A**) Two weeks later, mice were injected i.p with $_D$-luciferin (150 mg/kg body weight) and imaged 10 min later using a cooled CCD camera. Signal from tumor was quantified using CMIRimage program. Data shown are mean ± SD (n=4). **(B)** Light emission kinetics after injection of $_D$-luciferin. Same experiment in (A) was repeated but the luciferase activity was acquired immediately and every 5 min for 1 hr post-substrate injection. Data shown from 2 representative animals.

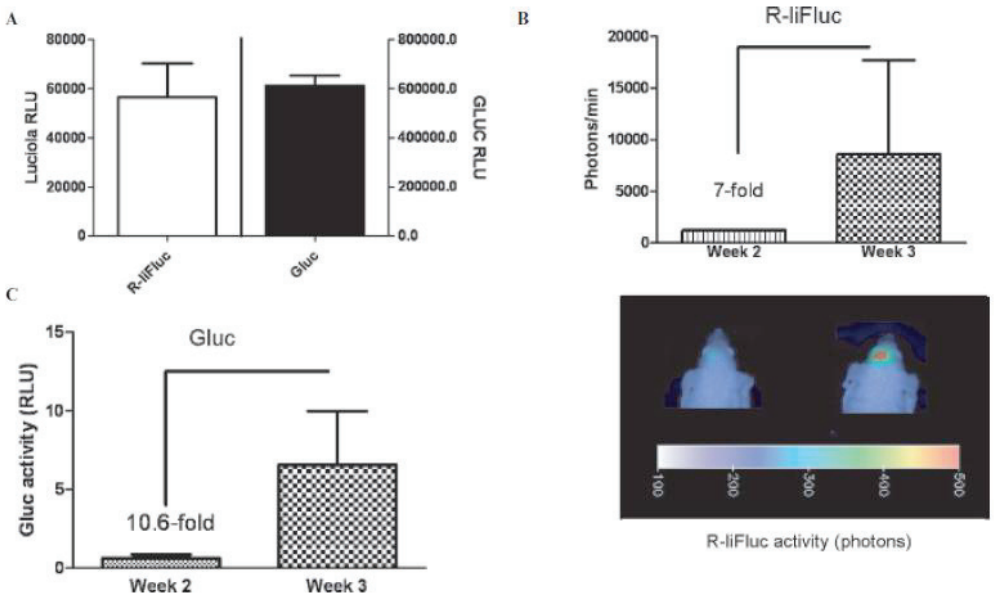

Figure 4. Bioluminescence imaging of brain tumors expressing Red-emitting *Luciola italica* luciferase. A.Luciferase activity from U87 cells stably expressing R-liFluc and Gluc. B. The striatum of nude mice were stereotactically injected with 10^5 U87 human glioma cells expressing both R-liFluc and Gluc. At 2 and 3 weeks post-tumor implantation, mice were intraperitoneally injected with D-luciferin (150mg/kg body weight) and imaged for Luciola Italica luciferase activity 10 minutes later using a cooled CCD camera. Intracranial brain tumor associated R-liFluc signal (bottom) as quantified using the CMIR-image program (top). C. Prior to D-luciferin injection, 5uL blood was collected and assayed for Gluc activity after addition of 100uL of 100uM coelenterazine and acquiring photon counts using a luminometer. Data shown are mean +/- SD (n=4).

DISCUSSION

Luciferases catalyze light producing chemical reaction by oxidizing their substrates, luciferin, while emitting photons. This process, known as bioluminescence, is a natural phenomenon found in many lower forms of life including fungi, bacteria, insects and marine cutaneous. The North American firefly luciferase from *Photinus pyralis* (Fluc) is the best characterized luciferase for luminescence applications and has proven invaluable as a biological reporter protein[25, 26]. Oxidation of $_D$-luciferin by Fluc results in a high quantum yield of light emission leading to a sensitive detection. Less than 10^4 molecules of Fluc protein can be detected using a standard luciferase

assay[27]. However, the yellow-green color of light emission is suboptimal for *in vivo* imaging. Although there is single report of a red-shifted variant of Fluc with enhanced *in vivo* luminescence properties[16], the availability of other luciferases expands the potential of advancing our understanding of biological processes. Our results broaden the characterization of the *Luciola italica* luciferase as a promising biological reporter in mammalian cells. Using green and red-emitting codon-optimized, liFluc variants, we observed a robust luciferase activity in cultured mammalian cells. Additionally, we found that the red-emitting variant was a valuable tool for *in vivo* bioluminescence imaging of tumors in deep tissues.

One important characteristic of luciferases for molecular biological applications is thermostability. For measurement of luciferase activity in cultured cells or tissue homogenates, cells are lysed and the luciferase protein is subject to degradation/inactivation by proteases, pH changes, and temperature fluctuations[28, 29]. This has implications for storage conditions of samples post cell lysis. We found that both the G-liFluc and R-liFluc variants had a 10 and 4-fold increase in half-life activity compared to Fluc. This may lower the amount of signal decay between sample harvest and freezing/assaying, allowing for higher number of samples to be processed simultaneously. Interestingly, at a lower substrate dose, the green-emitting liFluc had a higher activity than R-liFluc, most likely due to a lower K_M for the green-peaked variant. This characteristic may be desirable for *in vitro* assays in the case that lower substrate concentrations are used.

For a luciferase/luciferin combination to be suited for *in vivo* imaging, they need to overcome certain barriers such as light absorption by pigmented molecules (e.g. hemoglobin and melanin) and scattering by deep tissues. Since most light below 600 nm cannot penetrate mammalian tissues, red-emitting luciferases would be highly beneficial for *in vivo* imaging. The codon-optimized red-emitting liFluc characterized here with a peak emission at 610 nm showed to be a robust tool for imaging tumor cells injected intramuscularly or in the brain of nude mice.

Aside from *in vivo* imaging, having multiple luciferases utilizing the same substrate but yielding different emission properties could be highly useful for multicolor applications using spectral unmixing[9, 30]. Since the codon-optimized green and red-emitting liFluc variants have a distinct emission maximum, they can be used together to monitor dual biological processes simultaneously. Further, they can be

combined with other luciferases utilizing different substrates, such as *Gaussia* or *Renilla* luciferase, for triple reporter systems[20].

In conclusion, we have characterized two human codon-optimized *Luciola italica* thermostable variants for bioluminescence applications in mammalian cells and showed that the red-emitting variant is useful for deep tissue imaging. These variants may be useful alternative luciferases where increased thermostability is desired.

Acknowledgments

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