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CHAPTER VI

Directed molecular evolution reveals Gaussia luciferase variants with enhanced light output stability

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ABSTRACT

Gaussia Luciferase (Gluc) has proven to be a powerful mammalian cell reporter for monitoring numerous biological processes in immunology, virology, oncology and neuroscience. Current limitations of Gluc as a reporter include its emission of blue light, which is absorbed by mammalian tissues, limiting its use *in vivo*, and a flash-type bioluminescence reaction, making it unsuited for high-throughput applications. To overcome these limitations, a library of Gluc variants was generated using directed molecular evolution and screened for relative light output, a shift in emission spectrum, and glow-type light emission kinetics. Several variants with a 10-15 nm shift in their light emission peak were found. Further, a Gluc variant that catalyzes a glow-type bioluminescence reaction yielding over 10 minutes of stable light output, suited for high-throughput applications, was also identified. These results indicate that molecular evolution could be used to modulate Gluc bioluminescence reaction characteristics.

INTRODUCTION

Bioluminescence imaging (BLI) is currently one of the most valued and widely used techniques in basic biomedical research.[1-3] Bioluminescence relies on the conversion of chemical energy into visible light in culture or in living animals. This reaction is dependent on a luciferase enzyme which, in the presence of oxygen, causes a biochemical conversion of the luciferin substrate resulting in emission of light.[1] In the past decade, bioluminescence imaging has become indispensable for non-invasive monitoring of biological processes including gene expression,[4, 5] protein-protein interactions,[6-8] T-cell and stem cell trafficking,[9, 10] tumorigenesis and response to therapy,[4, 11, 12] and has further been used as a read-out for high-throughput screening assays in drug discovery.[13, 14] Although many luciferases exist, only a few are currently in use. As a mammalian cell reporter, luciferase needs to meet certain criteria including a distinct emission spectrum ideally with a significant red component (for *in vivo* applications), and a high quantum yield without intracellular accumulation of substrate to allow for real-time monitoring of enzyme expression[1-3]. Examples of frequently used luciferases are from the American firefly

Photinus Pyralis (Fluc), *Renilla reniformis* (Rluc), *Gaussia princeps* (Gluc) and *Vargula hilgendorfi* (Vluc)[1]. Recently, differences between properties of these luciferases including specificity to different substrates, spectral emission, or bioluminescence half-life, were used to develop multiplex reporter systems by which several cellular processes can be monitored simultaneously.[15-17] The next step to further improve the field of bioluminescence is to generate more stable, brighter and variants with different light emission properties suited for different applications.

Gaussia luciferase from the marine copepod *Gaussia princeps* has many advantageous properties over other luciferases including high signal intensity, a favorable enzyme stability, and a secretion signal, making it suitable for real time *ex vivo* monitoring of biological processes in medium of cultured cells and blood or urine in animals.[18-20] Gluc is a monomeric protein composed of 185 aa (19.9 kDa) that uses coelenterazine as a substrate. It is the smallest luciferase known with a peak light emission at around 470 nm and a broad spectrum extending up to 600 nm.[19] Since Gluc does not require ATP for activity, in contrast to the commonly used Fluc, it can be used as a reporter from cells as well as their immediate environment.

Current limitations of Gluc include signal quenching and absorption of its blue light by pigmented molecules when used *in vivo* and its rapid light decay, making the use of a luminometer with a built-in injector essential for immediate reading of signal once substrate is added, one well at a time. Several successful attempts have been made to optimize Gluc as a mammalian cell reporter. To overcome signal quenching when measuring Gluc in the blood *ex vivo*, we have developed an alternative microtiter well-based binding assay in which Gluc is captured from the blood before coelenterazine is added leading to around 10-fold increased sensitivity[18]. We have also characterized a Gluc mutant, GlucM43I, which catalyzes stable light output in the presence of Triton-X 100 detergent, suited for high-throughput applications.[21] Gluc was also shown to potentially contain 2 catalytic domains, the first covering amino acids 27-97 and the second domain covering amino acids 98-168.[22] Recently, Kim and colleagues developed a semi-rational consensus sequence driven mutagenesis strategy to synthesize potent mutant Gluc by comparing sequence similarities between the chromophore region of *Aequorea* green fluorescent protein (GFP) and coelenterazine.[23] Using this rational and hydrophobicity search, they

hypothesized that Gluc active site is between amino acids 71-140, the most hydrophilic domain. Site-directed mutagenesis of this active core, similar to those performed for GFP, lead to efficient alteration of Gluc properties including variants with a shift in emission spectrum [Y97W, I90L and Monsta (combination of F89W, I90L, H95E and Y97W mutations)], and enhanced light output (I90L). In this study, we used directed molecular evolution to create a Gluc library based on the sequence of wild type Gluc and screened around 5000 newly generated clones for increased activity, a shift in the emission spectrum, and increased light emission stability. Several variants were selected and sequenced for their mutations. One of these mutants carrying three different mutations (L30S, L40P, M43V; Gluc4) showed a glow-type bioluminescence reaction (as compared to flash reaction for wild-type Gluc) with stable light output up to 10 minutes without the need of a detergent, suited for high-throughput applications.

EXPERIMENTAL SECTION

Construction of Gluc library. A library of Gluc variants was created by shuffling cDNA fragments using error-prone PCR. First, the humanized codon-optimized cDNA sequence encoding *Gaussia* luciferase[19] (Nanolight, Pinetop, AZ) was amplified by PCR (without the Gluc signal sequence) using Taq polymerase (5 PRIME, Fisher Scientific, Pittsburgh, PA) and two flanking primers, which introduced an EcoRI (upstream primer) and XhoI (downstream primer) restriction sites, using the following conditions: 1 cycle of 94 °C-2 min; 35 cycles of: 94 °C-30 s, 58 °C-30 s and 72 °C-30 s; 1 cycle of 72 °C -7 min. The PCR product was then digested using 0.3 Units of DNaseI (New England Biolabs, Ipswich, MA) for 10 min at room temperature followed by heat inactivation at 75°C for 15 min after the addition of EDTA. The digested DNA was separated on a 2% agarose gel by electrophoresis. DNA fragments from ~50–150 base pairs were carefully excised using a sterile scalpel. The gel slice was placed in 3,500 MWCO dialysis tubing (Fisher Scientific, Pittsburgh, PA) and the DNA was eluted into TBE by electrophoresis for 15 min at 120V. The DNA was then ethanol precipitated and re-suspended in nuclease-free water. The PCR fragments were reassembled into the full-sized product using Extensor Hi-Fidelity PCR enzyme mix (Thermo Scientific, Portsmouth, NH) without

primers using the following conditions: 1 cycle, 94 °C-2 min; 40 cycles of: 94 °C-30 s, 45 °C-30 s and 68 °C-30 s. One microliter from this reaction then served as a template for a second PCR using the same primers as for error prone PCR above and the following conditions: 1 cycle, 94 °C-2 min; 25 cycles of: 94 °C-30 s, 58 °C-30 s and 68 ° C-30 s; 1 cycle, 68 °C-7 min. The PCR product was gel extracted after electrophoresis, digested with EcoRI and XhoI and ligated into a similarly digested pHGCx expression vector.[19]

Screening procedure. HMS174 bacterial cells were transformed with the newly created Gluc library by electroporation and spread on ampicillin agar plates. The next day, different colonies were collected by a Hudson RapidPick colony picker (Hudson Robotics, Inc., Springfield, NJ) and grown for 12 hours in 1 ml of LB media at 37 ^{0}C in deep 96 well plates. The bacterial cell culture media was then used directly to screen for emission spectrum shift using the Flexstation III plate reader (Molecular Devices, Sunnyvale, CA). Samples of 50 µl were transferred to regular white 96 well plates and read with 50 µl 40 µM coelenterazine (CTZN). The remaining bacterial media in the deep 96 well plates were mixed with glycerol and frozen at -80 ^{0}C. The clones showing differential spectrum shift as compared to wild-type (wt) Gluc were selected, sequenced for mutations, and cloned into a CSCW lentivirus mammalian expression backbone under the control of CMV promoter[20] using upstream primers which re-introduced the signal sequence to the cDNA. 293T cells (obtained from ATCC) were then transfected with the different Gluc variants using calcium phosphate protocol and two days later, the cell-free conditioned medium was collected and used for the different bioluminescent assays. Lentivirus vectors were packaged as previously described.[18-20]

Bioluminescence assays. The emission spectrum for the different Gluc variants was confirmed using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc., Palo Alta, CA) that reads the full spectrum within milliseconds (averaging reading time per sample of 5 msec). Emission spectrum analysis was performed using 100 µl of conditioned medium brought up to 500 µl in 1X PBS in a cuvette followed by addition of 500 µl CTZN (8 µg/ml diluted in PBS). The emission slit (nm) was set to 10-round, under a 400-600 nm emission wavelength window, using the bio/chemiluminescence setting. For relative activity comparison, 293T cells were

plated in 6-well plates and co-transfected with 0.5 µg of different Gluc variants and a similar amounts of a plasmid expressing firefly luciferase and mCherry fluorescent protein under control of CMV promoter (CSCW-Fluc-ImCherry)[20] as above. Forty-eight hours later, cells were analyzed by fluorescent microscopy for mCherry expression for transfection efficiency. Ten microliter aliquots (in 5-plicates) of the conditioned medium were assayed for Gluc activity as above. Cells were then lysed in 200 µl 1xRLB lysis buffer (Promega) and 20 µl aliquots of lysates (in triplicates) were assayed for Fluc activity using 80 µl of FLAR reagent (Targeting Systems, El Cajon, CA) and a luminometer (Model MLX, Dynex Technologies) that was set to read for 10 sec and integrate the signal for 2 sec. Fluc reads were used to normalize for transfection efficiency between wells. The enzymatic activities of different Gluc mutants over multiple coelenterazine injections were determined by reading 10 µl samples of conditioned medium in a 96 well microtiter plate using the Dynex luminometer while multiple injections of 20 µl of CTZN (8 µg/ml, in PBS) was added to the same wells and signal was recorded immediately after each injection; we used a 1 sec reading time and 10 different injections of CTZN per well, resulting in a total of 10 reads. The kinetic assay was performed by transferring 10 µl aliquots of conditioned medium (in 5-plicates) to a 96 well white plate, automatically injecting 40 µl 8 µg/ml CTZN in PBS (with or without 0.01% Triton) and recording kinetic intervals of 14 seconds over 10 min using the Flexstation III instrument (Molecular Devices). For validation of Gluc4 for high-throughput applications, 15 µl aliquots of conditioned medium from cells expressing wt Gluc, GlucM43I or Gluc4 were first transferred to a 384 well plate (in triplicates) using an automated dispenser (Multidrop Combi, Thermo Scientific). In a similar fashion, 45 µl 8 µg/ml CTZN diluted in PBS was also dispensed into each well and the plate was then analyzed using the Flexstation III that was set to read for 50 msec/well. In another experiment, U87 human glioma cells (ATCC) expressing either Gluc4 or wt Gluc were plated in a 96-well plate. The next day, several wells/plate were marked and treated with 1 mM temozolomide (Sigma). Seventy-two hours later, 10 µl aliquot of conditioned medium was transferred to a white plate followed by the addition of 45 µl 8 µg/ml CTZN/well using an automated dispenser and acquiring photon counts using the Dynex luminometer which was set to read for 1sec/well column by column.

Cloning multiple mutations. All mutations derived from the screen were located in the first catalytic domain of Gluc as described.[22] Mutations were then transferred to the second catalytic domain by site directed mutagenesis, using PCR to create the desired point mutations for GlucL40P, GlucM43V, and GlucL40S variants, resulting in Gluc1 (L40S, E111S), Gluc2 (L30S, M43V, I114V), Gluc3 (L40P, E111P) and Gluc4 (L40P, M43V, L30S). For each Gluc variant, the desired mutation was incorporated using an overlapping PCR strategy. Using the screen derived Gluc variant cDNA as a template, two flanking Gluc primers which introduce a 5' NheI site and a 3' XhoI site were used for direct cloning of the final PCR product into CSCW plasmid. Two complementary inner primers were designed with a desired sequence mismatch to alter the codon of interest. Three different PCR reactions were performed to incorporate the mutation using specific primer sets (Supplementary Table 2): PCR 1 used Gluc variant cDNA as template and the forward flanking NheI primer combined with the reverse mutagenic primer. PCR 2 using the reverse flanking XhoI primer and the forward mutagenic primer. Specific bands for PCR 1 and 2 were gel extracted and 2 µl of PCR product from PCR 1 and 2 were then used as template for overlapping PCR (PCR 3) using the flanking primers. All PCR reactions were performed using Phusion high fidelity polymerase (New England Biolabs). Correct incorporation of specific mutations was confirmed using DNA sequencing at the Massachusetts General Hospital DNA core facility. The full length PCR product was digested with NheI and XhoI and ligated with similarly digested CSCW lentivirus plasmid. The PCR primer sequences used in this study can be found in Supplementary Tables 1 and 2.

Statistical Analysis. All experiments were repeated at least 5 times to achieve statistical significance. Data are presented as the mean relative light units (RLU) ± standard deviation (SD) from 5 different replicates in each experiment. P values were calculated using Student's t-test. Relative reaction kinetics, turnover rates, and Drug screening results are shown as representative data of 5 independent experiments.

RESULTS AND DISCUSSION

Recently, through hydrophobicity search and by comparing sequence similarities between the chromophore region of *Aequorea* green fluorescent protein (GFP) and coelenterazine, Kim et al suggested that the Gluc active core ranges between amino acids 71-140.[23] We took an alternative strategy and used directed molecular evolution to shuffle Gluc cDNA to find potent Gluc variants. Table 1 shows potential Gluc variants and their corresponding mutations derived from our screen.

Name	Mutation
GlucL40P	L40P
GlucL40S	L40S
GlucL30S, M43V	L30S, M43V
Gluc1	L40S, E111S
Gluc2	L30S, M43V, I114V
Gluc3	L40P, E111P
Gluc4	L30S, L40P, M43V
Gluc5	S16K, M43V, V159M

Table 1. Overview of the different Gluc variants and corresponding mutations.

Interestingly, all these Gluc variants were not part of the active core discovered by Kim et al., suggesting that Gluc could have another potential putative core region. Importantly, none of these mutations were found in 10 random clones from the original Gluc plasmid library showing that our library is fairly diverse (data not shown). Another study suggested that Gluc contains two catalytic domains and therefore two coelenterazine binding pockets.[22] According to this study, all mutations derived from our screen are found in the first catalytic domain (amino acids 27-97; Fig. 1). We therefore attempted to enhance our mutants by also changing the corresponding amino acids in the second catalytic domain (amino acids 98-168) creating Gluc1, Gluc2 and Gluc3 variants (Fig. 1 and Table 1). Finally, we attempted to amplify our mutants by combining different mutations into a single Gluc cDNA creating Gluc4 (Fig. 1 and Table 1).

Figure 1

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                SS
                MGVKVLFALICIAVAEA

                1)
Gluc wt         KPTENNEDFNIVAVASNFATTDLDAD
GlucL40P        KPTENNEDFNIVAVASNFATTDLDAD
GlucL40S        KPTENNEDFNIVAVASNFATTDLDAD
GlucL30S,M43V   KPTENNEDFNIVAVASNFATTDLDAD
Gluc1           KPTENNEDFNIVAVASNFATTDLDAD
Gluc2           KPTENNEDFNIVAVASNFATTDLDAD
Gluc3           KPTENNEDFNIVAVASNFATTDLDAD
Gluc4           KPTENNEDFNIVAVASNFATTDLDAD
Gluc5           KPTENNEDFNIVAVAKNFATTDLDAD

                27)
Gluc wt         RGKLPGKKLPLEVLKEMEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD
GlucL40P        RGKLPGKKLPLEVPKEMEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD
GlucL40S        RGKLPGKKLPLEVSKEMEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD
GlucL30S,M43V   RGKSPGKKLPLEVLKEVEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD
Gluc1           RGKLPGKKLPLEVSKEMEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD
Gluc2           RGKSPGKKLPLEVLKEVEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD
Gluc3           RGKLPGKKLPLEVPKEMEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD
Gluc4           RGKSPGKKLPLEVPKEVEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD
Gluc5           RGKLPGKKLPLEVLKEVEANARKAGCTRGCLICLSHIKCTPKMKKFIPGRCHTYEGDKESAQGGIGEAIVD

                98)
Gluc wt         IPEIPGFKDLEPMEQFIAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQVDKIKGAGGD
GlucL40P        IPEIPGFKDLEPMEQFIAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQVDKIKGAGGD
GlucL40S        IPEIPGFKDLEPMEQFIAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQVDKIKGAGGD
GlucL30S,M43V   IPEIPGFKDLEPMEQFIAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQVDKIKGAGGD
Gluc1           IPEIPGFKDLEPMSQFIAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQVDKIKGAGGD
Gluc2           IPEIPGFKDLEPMEQFVAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQVDKIKGAGGD
Gluc3           IPEIPGFKDLEPMPQFIAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQVDKIKGAGGD
Gluc4           IPEIPGFKDLEPMEQFIAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQVDKIKGAGGD
Gluc5           IPEIPGFKDLEPMEQFIAQVDLCVDCTGCLKGLANVQCSDLLKKWLPQRCATFASKIQGQMDKIKGAGGD
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Figure 1. Gluc variants and corresponding mutations in the second active domain characterized in this study. Shown, the Gluc signal peptide covering amino acids 1-27, first active domain covering amino acids 27-97, and second active domain covering amino acids 98-168.

Variants were first screened for a shift in emission spectrum using the Flexstation III device which is a slow reader requiring several minutes to read the full spectrum. Results could therefore be confounded by the kinetic properties of each clone (e.g. flash versus glow emission). All our shifted clones were confirmed using a Cary Eclipse Fluorescence Spectrophotometer, which reads the full spectrum in milliseconds. Upon conformational analysis, we found that GlucL40P, GlucL40S and Gluc4 (L30S, L40P, M43V) showed a 10-15 nm shift in light emission peak as compared to wild-type (wt) Gluc (Supplementary Fig. 1). Mutating the corresponding amino acid in the second active domain (Gluc1, Gluc2 and Gluc3) did not have an additional effect on the emission spectrum (data not shown). These results suggest that this strategy could be used to find Gluc mutants with different emission spectra suited for different applications.

We then compared the relative activities of different Gluc variants to wt Gluc. 293T cells were co-transfected with an expression plasmid for Fluc and each of the Gluc variants, or wt Gluc. Forty-eight hours later, aliquots of conditioned medium were assayed for Gluc activity. Cell lysates were assayed for Fluc activity, which was used to normalize for transfection efficiency. Gluc5 (S16K, M43V, V159M) showed to have around 2-fold enhanced activity as compared to wt Gluc (Fig. 2a). Further, Gluc4 carrying multiple mutations and all variants with corresponding mutations in the second active domain (Gluc1, Gluc2, Gluc3) showed to have 10-100x less activity as compared to wt (Fig. 2a). These results suggest that these amino acids in the second active domain are important for Gluc activity.

To further characterize these new Gluc variants, we compared their light output to wt Gluc after several substrate injection/read intervals to gain insight into enzymatic turnover rate of these variants. GlucL30S/M43V and GlucL40P with similar activity to wt Gluc (Fig. 2a) showed increasing activities after each substrate injection, reaching a plateau after 2-3 rounds of injections (Fig. 2b). Interestingly, the combination of these two variants (Gluc4) showed even higher turnover rate where the signal continued to increase even after 6 rounds of substrate addition, proving that these mutations are important for this characteristic (Fig. 2b). Gluc3 variant containing the corresponding mutation in the second Gluc active domain also showed higher activities over multiple injections as compared to wt Gluc (Fig. 2b). These properties suggest a higher turnover rate of these Gluc variants.

Next, we determined the bioluminescent reaction kinetics of all Gluc variants. Since our laboratory previously reported on a Gluc variant which catalyzes a stable light output in the presence of TritonX-100 (GlucM43I),[21] we decided to include this variant and assay all variants in the presence and absence of this detergent. Remarkably, all Gluc variants, with the exception of Gluc5 (S16K, M43V and V159M) and GlucM43I, showed higher stable light output as compared to wt Gluc, which typically catalyzes a flash-type reaction in the absence of the detergent (Fig. 2c). Gluc variants containing corresponding mutations in the second active domain [Gluc1 (L40S and E111S); Gluc3 (L40P and E111P)], having lower relative activity, showed enhanced light output stability. More importantly, the Gluc4 variant carrying multiple mutations which had lower activity and higher turnover rate (Fig. 2a, b)

displayed a glow-type reaction with ~20% loss in signal over 10 minutes without the need of a detergent (Fig. 2c). All variants including the wt Gluc showed higher stable light output in the presence of TritonX-100, however, L40P, L40S, Gluc1, Gluc2, Gluc3 and Gluc4 exhibited the highest stability (Fig. 2c). As expected, the addition of the detergent decreased the light output of all variants and native Gluc by around one log of magnitude[21] (data not shown).

Figure 2

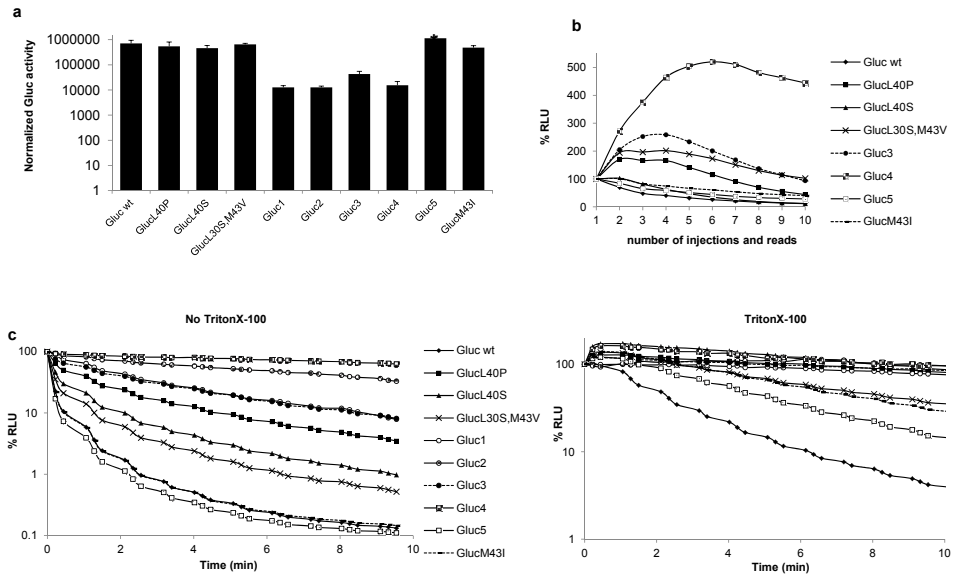


Figure 2. Characterization of different Gluc variants. 293T cells were transfected with different Gluc variants or wt Gluc and 48 hours later, aliquots of the conditioned medium were assayed for different properties. (a) Relative activity of each Gluc variant normalized to Fluc signal, a marker for transfection efficiency. (b) Enzymatic activity over repeated injections of coelenterazine (diluted in PBS) followed by signal acquisition using a plate luminometer with a built-in injector. (c) Bioluminescence light emission kinetics for each Gluc variant was analyzed by adding coelenterazine (diluted in PBS in the presence or absence of TritonX-100) and acquiring photon counts every 14 seconds over a period of 10 minutes using a luminometer.

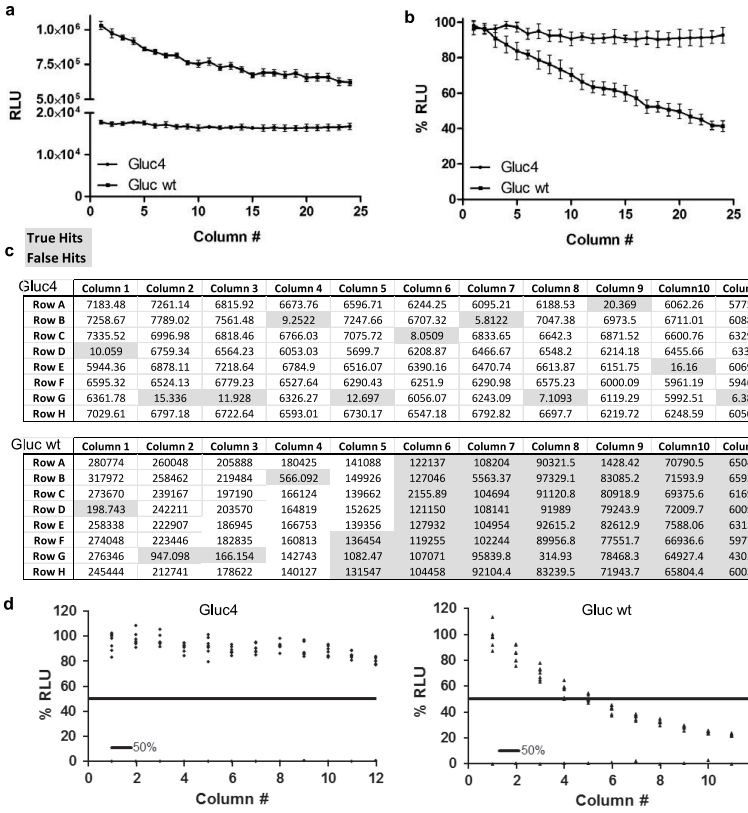
Since Gluc4 showed to have the most stable light output without the need of TritonX-100, and since detergents could interfere with some assays such as live cell analysis, protein assays, or enzymatic activities, we decided to evaluate this variant for high-throughput applications. Our laboratory developed a drug screening assay

using Gluc as a cell viability marker.[13] Since Gluc is secreted, aliquots of the conditioned medium can be assayed over time, allowing functional analysis of drug kinetics. In this study, we have used the native Gluc as a viability marker and therefore measurements of one well at a time using a luminometer with a built-in injector was essential due to the flash-type reaction kinetics of this reporter, limiting the numbers of drugs that can be screened.[13] A Gluc variant that catalyzes a glow-type reaction would be extremely helpful in the field of drug discovery allowing for high-throughput approach without the risk of confounding results due to kinetic instability (wt Gluc) or to interactions with detergents such as TritonX-100 (GucM43I). 293T Cells were infected with a lentivirus vector carrying the expression cassette for wt Gluc, GlucM43I or Gluc4 under the control of CMV promoter. Aliquots of conditioned medium from these cells were transferred to a 384 well plate and read under high throughput conditions by adding coelenterazine (diluted in PBS) to all wells simultaneously and acquiring photon counts for 50 msec/well, resulting in a read-time of <1 minute per plate. As expected, the signal from wt Gluc and GlucM43I variant decreased over the course of measurements of different columns in the 384-well plate, with around 50% signal loss between the first and last columns (Fig. 3a,b and Supplementary Fig. 2). On the other hand, there was no significant signal loss of Gluc4 activity among all columns showing the usefulness of this variant for high-throughput applications (Fig. 3a,b). During this experiment, we noticed that the emission of blue light and the stable light emission of Gluc4 is so sensitive that we can simply visualize it by naked eye in the dark. Further, despite the fact that Gluc4 is 100x less sensitive than wt Gluc, this mutant yields 2 logs of magnitude more light output when expressed in mammalian cells under our assay conditions, as compared to firefly luciferase, the most commonly used mammalian cell reporter (Supplementary Fig. 3a). To confirm that this change in relative activity is not due to simply different amounts of protein production/expression or secretion levels, conditioned medium as well as lysates from cells expressing Gluc4 or wt Gluc were analyzed for Gluc protein level by western blotting using anti-Gluc antibody which showed nearly equal Gluc protein levels being produced and secreted (Supplementary Fig. 3b)

To further evaluate Gluc4 mutant for high-throughput screening, we plated U87 glioma cells stably expressing Gluc4 or wt Gluc in 96-well plates in triplicate. We

deliberately added temozolomide, a known chemotherapeutic agent against glioma, to 12 selected wells that were marked. Seventy-two hours later, 10 µl aliquots of conditioned medium were transferred to a new 96-well plate, to which coelenterazine was added to all wells simultaneously using an automated injector, followed by Gluc signal acquisition (column by column) using a plate luminometer. The Gluc4 mutant showed signal stability across all columns in the plate. Upon data analysis for positive hits defined as >50% decrease in Gluc activity and therefore cell viability, Gluc4 revealed 100% of the "true" hits (selected wells to which the drug was added) without any false-positives (Fig. 3c,d). On the other hand, the wt Gluc signal decreased across all columns; the last column of the plate had >80% lower signal compared to the first column. In this case, wt Gluc revealed 51 false positive hits, which is more than half of the screened plate (Fig. 3c,d). These data suggests that Gluc4 is a strong useful reporter for high-throughput applications.

Figure 3



Gluc4

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column10	Column11	Column12
Row A	7183.48	7261.14	6815.92	6673.76	6596.71	6244.25	6095.21	6188.53	20.369	6062.26	5775.35	5526.83
Row B	7258.67	7789.02	7561.48	9.2522	7247.66	6707.32	5.8122	7047.38	6973.5	6711.01	6088.61	26.793
Row C	7335.52	6996.98	6818.46	6766.03	7075.72	8.0509	6833.65	6642.3	6871.52	6600.76	6329.63	5517.78
Row D	10.059	6759.34	6564.23	6053.03	5699.7	6208.87	6466.67	6548.2	6214.18	6455.66	6338.9	5727
Row E	5944.36	6878.11	7218.64	6784.9	6516.07	6390.16	6470.74	6613.87	6151.75	16.16	6069.24	5932.68
Row F	6595.32	6524.13	6779.23	6527.64	6290.43	6251.9	6290.98	6575.23	6000.09	5961.19	5946.49	5726.04
Row G	6361.78	15.336	11.928	6326.27	12.697	6056.07	6243.09	7.1093	6119.29	5992.51	6.3887	5578.05
Row H	7029.61	6797.18	6722.64	6593.01	6730.17	6547.18	6792.82	6697.7	6219.72	6248.59	6050.05	6016.93

Gluc wt

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column10	Column11	Column12
Row A	280774	260048	205888	180425	141088	122137	108204	90321.5	1428.42	70790.5	65043.2	55031.7
Row B	317972	258462	219484	566.092	149926	127046	5563.37	97329.1	83085.2	71593.9	65936.3	613.007
Row C	273670	239167	197190	166124	139662	2155.89	104694	91120.8	80918.9	69375.6	61694.1	54506
Row D	198.743	242211	203570	164819	152625	121150	108141	91989	79243.9	72009.7	60096.4	55473.3
Row E	258338	222907	186945	166753	139356	127932	104954	92615.2	82612.9	7588.06	63150.6	55349.7
Row F	274048	223446	182835	160813	136454	119255	102244	89956.8	77551.7	66936.6	59779.6	51881.1
Row G	276346	947.098	166.154	142743	1082.47	107071	95839.8	314.93	78468.3	64927.4	430.397	53853.4
Row H	245444	212741	178622	140127	131547	104458	92104.4	83239.5	71943.7	65804.4	60031.8	51214.5

Figure 3. Gluc4 variant displays a glow-type reaction suited for high-throughput applications. (a-b) Aliquots from conditioned medium of cells expressing wt Gluc, or Gluc4 were dispensed in 3 different 384-well plates in a high-throughput fashion followed by addition of coelenterazine (diluted in PBS) to all wells and signal acquisition (50 msec/well) using the Flexstation III device. Data presented in (a) as average raw RLUs/column ± SD and in (b) as average %RLU/column ± SD in which the first well was set at 100%. (c-d) U87 human glioma cells expressing Gluc4 or wt Gluc were plated in a 96-well plate and 12 wells/plate were marked and treated with 1 mM temozolomide. Seventy-two hours later, 10 µl aliquots of conditioned medium were transferred to a new 96-well plate to which 45 µl of coelenterazine was added to all wells simultaneously and signal was acquired using the Dynex luminometer column by column. Raw data is showing in (c) highlighting the "true" hits and false-positives using the Gluc4 or wt Gluc. (d) Scatter plot of each plate in which data are presented as %RLU where the first well of the first column was set at 100%.

In conclusion, using directed molecular evolution, we identified several Gluc variants with a shift in emission spectrum (up to 15 nm) and enhanced light-output stability. We showed that mutations in the second active domain of Gluc could have dramatic effect on light emission intensity, but have advantages with respect to turnover rate

and bioluminescence reaction kinetics. We also discovered a Gluc variant (Gluc4) that catalyzes a glow-type reaction with <20% decrease in signal over 10 min, suited for high-throughput applications. Interesting, all these mutations were not part of the Gluc active core recently discovered by Kim et al.,[23] suggesting another potential Gluc putative core domain. Aside from the given useful improvements of the current repertoire of available Gluc variants, these clones and their mutations shed new light on the structure and biophysical characteristics of Gluc giving insight towards the development of potent Gluc variants for different applications.

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