Codon-Optimized \textit{Gaussia} Luciferase cDNA for Mammalian Gene Expression in Culture and \textit{in Vivo}

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Photoproteins have played a major role in advancing our understanding of biological processes. A broader array of biocompatible, nontoxic, and novel reporters can serve to expand this potential. Here we describe the properties of a luciferase from the copepod marine organism \textit{Gaussia princeps}. It is a monomeric protein composed of 185 aa (19.9 kDa) with a short coding sequence (555 bp) making it suitable for viral vectors. The humanized form of \textit{Gaussia} luciferase (hGLuc) was efficiently expressed in mammalian cells following delivery by HSV-1 amplicon vectors. It was found to be nontoxic and naturally secreted, with flash bioluminescence characteristics similar to those of other coelenterazine luciferases. hGLuc generated over 1000-fold higher bioluminescent signal intensity from live cells together with their immediate environment and over 100-fold higher intensity from viable cells alone (not including secreted luciferase) or cell lysates, compared to humanized forms of firefly (hFLuc) and \textit{Renilla} (hRLuc) luciferases expressed under similar conditions. Furthermore, hGLuc showed 200-fold higher signal intensity than hRLuc and intensity comparable to that of hFLuc \textit{in vivo} under standard imaging conditions. \textit{Gaussia} luciferase provides a sensitive means of imaging gene delivery and other events in living cells in culture and \textit{in vivo}, with a unique combination of features including high signal intensity, secretion, and ATP independence, thus being able to report from the cells and their environment in real time.

\section*{INTRODUCTION}

Photoproteins have become essential tools in biotechnology, for example, in biochemical assays, cell screening, and \textit{in vivo} imaging. In particular, engineered proteins with either fluorescent (e.g., enhanced green fluorescent protein (eGFP), RFP, and YFP) \cite{1,2} or bioluminescent properties (e.g., luciferases) \cite{3,4} have become indispensable in monitoring biologic phenomena. Here we report on a luciferase with greatly increased bioluminescent signal over firefly and \textit{Renilla} luciferases when expressed in mammalian cells under most conditions, which, like other coelenterazine luciferases, is ATP independent.

Luciferases encompass a wide range of enzymes that catalyze light-producing chemical reactions in living organisms \cite{5,6}. To date, all known luciferases use molecular oxygen to oxidize their substrates while emitting photons. Luciferases are commonly found in lower organisms such as bacteria, fungi, insects, and marine crustaceans. They do not share sequence homology either within or between groups and can use different substrates. Firefly luciferase (FLuc; 550 aa, 62 kDa), found in the light-emitting organ of the firefly \textit{Photinus pyralis}, is one of the best studied luciferases due to its high quantum yield (>88\%) generated by oxidation of its substrate, beetle \textit{d}-luciferin (benzothiazole), with a peak emission at 562 nm \cite{7,8}. FLuc requires ATP and Mg\textsuperscript{2+} as cofactors for its activity \cite{9,10}. Another well-studied luciferase is from the sea pansy, \textit{Renilla reniformis} (RLuc; 311 aa, 34 kDa) \cite{11}. RLuc uses coelenterazine as a substrate and emits light with a peak at 480 nm. As all coelenterazine luciferases, it does not require ATP for activity; however, RLuc has a low enzymatic turnover and quantum yield (6\%) \cite{12}. Since they use different substrates, humanized forms of FLuc and RLuc have been...
combined as “dual reporters” for imaging in cultured cells, in vivo, and in cell lysates using commercially available kits [13,14]. The biological requirements for in vivo imaging of luciferases are particularly stringent and the availability of more efficient luciferases would extend current capabilities.

Here we characterize a luciferase from the marine copepod Gaussia princeps (Figs. 1A and 1B). Gaussia luciferase (GLuc; 185 aa, 19.9 kDa) is the smallest luciferase known and is naturally secreted [15]. This luciferase emits light at a peak of 480 nm with a broad emission spectrum extending to 600 nm (see Fig. 3C). Gaussia luciferase has been cloned, overexpressed in bacteria, and used as a sensitive analytical reporter for hybridization assays [16]. Up to now, this reporter had not been used for monitoring mammalian cell expression in culture or in vivo. In the present study, cDNAs encoding the wild-type [15] and the humanized version of GLuc (hGLuc) were cloned into HSV-1 amplicons under the cytomegalovirus (CMV) immediate early (IE) promoter. DNA-transfected and vector-infected cells were evaluated by bioluminescence imaging in cell lysates, in cultured cells, and in vivo. We show that hGLuc is more highly expressed in mammalian cells compared to its wild-type form and gives a markedly higher bioluminescence signal intensity compared to other well-studied luciferases under most conditions. Since hGLuc is secreted and like other coelenterazine luciferases does not require ATP for activity, it can report from the cells themselves as well as their immediate environment.

RESULTS

Gaussia Luciferase is Naturally Secreted in Active Form

To determine the amount of light production from cells themselves and from cell-free, conditioned medium, we transfected human fibroblasts (293T cells) with amplicon constructs bearing the expression cassette for hGLuc. Forty-eight hours after transfection, we observed over 10 times higher bioluminescence in the cell-free, conditioned medium compared to live cells themselves or lysates prepared from them, confirming secretion of active hGLuc into the medium (Fig. 2). To investigate the relationship between protein location and photon generation, we produced two mutant forms of hGLuc. In one, the signal peptide was deleted (–SP) to confine hGLuc to the cytoplasm. With this form, bioluminescence in the medium decreased to almost background levels, but also decreased in both live cells and cell lysates by 15-fold, resulting in a 1100-fold decrease in combined bioluminescence in the cells and medium compared to the unmodified hGLuc (Fig. 2). When hGLuc was modified for retention in the ER by cloning a KDEL sequence at the C-terminus of the protein (hGLuc–KDEL), the signal from intact viable cells remained identical to that of hGLuc, while that in the cell lysate increased by about 3-fold (Fig. 2). The secretion of hGLuc–KDEL from cells was not completely blocked, however, with one-quarter of the bioluminescence still present in the cell-free, conditioned medium of viable cells compared to hGLuc (Fig. 2A). We concluded from these two experiments that these modifications alter protein conformation or processing in the ER and Golgi and do not enhance the expression level or signal intensity from the cells themselves. We therefore chose the unmodified hGLuc for all subsequent studies.

Gaussia and Renilla Luciferases Induce Flash Kinetics, Which Rapidly Decay with Time, whereas Firefly Luciferase Has Glow Kinetics

We assayed the time kinetics of light production from hGLuc, hRLuc, and hFLuc in cell lysates (Fig. 3A). For the coelenterazine luciferases (hGLuc and hRLuc), the max-

FIG. 1. The marine copepod organism G. princeps. (A) Micrograph of the G. princeps (around 6 mm long from head to telson). Image courtesy of Dr. Steve Haddock, Monterey Bay Aquarium Research Institute (Monterey, CA, USA). (B) Scanning electron microscopic false-colored images of G. princeps. Image courtesy of Tina (Weatherby) Carvalho, University of Hawaii at Manoa (Honolulu, HI, USA).
imum signal was obtained within the first 10 s after the addition of coelenterazine and dropped significantly over the course of 10 min. The signal from hGLuc dropped by nearly 75% within 50 s and reached 10% of starting levels in 90 s. The hRLuc signal decayed slowly (50% within 90 s and 10% of the original signal within 280 s). In contrast, the hFLuc bioluminescence decayed more slowly over time with only 50% loss of the original signal after 10 min.

FIG. 2. Comparison of activity of different versions of humanized Gaussia luciferase. 293T cells (0.4 million) in p60 plates were transfected and viable cells, cell-free conditioned medium, or cell lysates were assayed for luciferase activity with 40 μM coelenterazine using a luminometer. Experiments were repeated three times, and similar results were obtained. (A) Signals obtained from viable cells normalized to cell number. Viable cells versus conditioned medium. (B) Signals obtained from cell lysates normalized to micrograms of protein. RLU, relative light units; -SP, signal peptide at N-terminus removed; +KDEL, with KDEL sequence at C-terminus.

FIG. 3. Time kinetics of light production from humanized Gaussia (hGLuc), humanized Renilla (hRLuc), and humanized firefly luciferase (hFLuc). 0.3 million 293T cells were transfected with each of the amplicon plasmids. After 48 h, cells were harvested and lysates assayed for luciferase activity with 40 μM coelenterazine (for hGLuc and hRLuc) or 450 μM 1,β-luciferin (for hFLuc) using a luminometer. Experiments were repeated three times and similar data were obtained. (A) Signals were calculated as % RLU of which 100% corresponds to the signal obtained during the first 10 s. (B) Signals obtained from cell lysates were normalized to microgram of cell protein. (C) Emission spectrum of hGLuc and hRLuc. CPS, counts per second.
Comparing the emission of both coelenterazine luciferases, hGLuc showed 2 orders of magnitude higher bioluminescence in the cell lysates compared to hRLuc (Fig. 3B). Both coelenterazine luciferases showed a broad emission with a peak at around 480 nm with detectable signal out to 600 nm (Fig. 3C).

**Humanized GLuc Gives a 2000-fold Greater Bioluminescent Signal Than Wild-type GLuc When Expressed in Mammalian Cells**

To compare the expression level of hGLuc versus wild-type GLuc in living cells, we infected human glioma cells (line Gli36) with HSV-1 amplicon vectors carrying expression cassettes for either hGLuc or GLuc under the CMV IE promoter at a multiplicity of infection (m.o.i.) of 0.5 transducing units (tu) per cell (yields 80–90% infection of cells in culture). In cultured cells together with their conditioned medium, we observed over 2000-fold higher bioluminescent activity of hGLuc compared to GLuc over a range of substrate concentrations (Fig. 4A), and thus all subsequent studies were carried out with hGLuc.

**Dose Response of Luciferases to Coelenterazine**

To determine the effect of substrate concentration on light production, we tested serial dilutions of coelenterazine (2.5–40 μM) for both hGLuc and hRLuc in live transfected cells in culture (Fig. 4A). Increasing the concentration of coelenterazine raised the signal from hGLuc in a roughly log-linear manner; however, hRLuc reached a plateau at 10 μM. Importantly, the signal obtained from the hGLuc was significantly higher than that obtained from hRLuc at all concentrations tested. At 2 μM coelenterazine the hGLuc signal was 40-fold higher than that for RLuc, while at 40 μM light production by hGLuc was 3 orders of magnitude higher than that for hRLuc. Additional experiments were conducted in cell lysates, thus eliminating the secreted hGLuc component, and still the signal was higher than hRLuc at all coelenterazine concentrations tested, being 100-fold higher in cell lysates at 80 μM substrate (Fig. 4B). At higher concentrations (≥400 μM) of coelenterazine, a decrease in signal intensity was observed for both luciferases, apparently due to nonspecific effects of high substrate concentration in the medium. A dose of 20 μM was chosen for the subsequent cell lysate and culture studies.

Since neither antibody for GLuc or purified enzyme is available, it was not possible to normalize activity to the amount of enzyme. Therefore it was not possible to define the nature of the higher hGLuc signal intensity over hFLuc or hRLuc, when expressed in mammalian cells, which could be due to higher message and protein levels, increased substrate availability, higher substrate turnover, and/or quantum yield of the enzymatic reaction.

**Combined Intracellular and Extracellular Humanized Gaussia Luciferase Produces over 1000-fold Higher Bioluminescent Signal Than Humanized Renilla Or Firefly Luciferases in Mammalian Cells**

We compared the light output from cells expressing hGLuc, hRLuc, and hFLuc, with all codons being optimized for mammalian cell expression, in live 293T cells following transfection with amplicon constructs (Fig. 5A). The mean values combining both intracellular and extracellular activity were over 1000-fold higher for hGLuc compared to either hRLuc or hFLuc using 20 μM coelenterazine for the two former and 450 μM β-luciferin for the latter. This would allow us to detect as few as 3 cells expressing hGLuc, whereas detection would require about 3000 cells expressing hRLuc or hFLuc. In comparing intracellular activity in viable cells (Fig. 5B) or cell lysates (eliminating the contribution of the secreted form, Fig. 4B), hGLuc produced over 100-fold higher bioluminescence than hRLuc or hFLuc.

To corroborate these findings in another cell line and to evaluate another mode of delivery, we infected human glioma cells (Gli36) with HSV-1 amplicon vectors (m.o.i. = 0.5) bearing the same expression constructs. Using a signal-to-background ratio of 5 as the cut-off, we could
detect as few as 20 cells infected with hGLuc at 20 μM coelenterazine when combining both intracellular and extracellular activity. However, with hRLuc, only 40,000 cells were detectable with similar signal-to-background ratio and coelenterazine concentration (Fig. 5C). The generated bioluminescent signal from hGLuc was also shown to be linearly related to the number of infected cells in a range covering over 4 orders of magnitude.

**Humanized Gaussia Luciferase Produces 200-fold Higher Bioluminescent Signal Intensity Than Humanized Renilla Luciferase and Intensity Comparable to That of Humanized Firefly Luciferase in Vivo**

Using a nude mouse model of subcutaneous glioma tumors, we compared the signal intensity produced by Gli36 cells expressing each of the three luciferases, hGLuc, hRLuc, and hFLuc. Thirty-six hours or 5 days after implantation of these tumor cells, we injected 25–200 μg (0.96–7.7 mg/kg body wt) coelenterazine or 4 mg (150 mg/kg body wt) d-luciferin per mouse. At a substrate dose of 200 μg, hGLuc produced 200-fold higher bioluminescent signal intensity than hRLuc (Figs. 6A and 6B) and intensity comparable to that of hFLuc with 4 mg substrate dose (Figs. 6C and 6D). The 200-μg dose had to be given intracardially in 450 μl phosphate-buffered saline (PBS) due to the precipitation of coelenterazine at higher concentration, with no more than 200 μl being able to be injected via the tail vein. For comparison purposes, we also gave d-luciferin to the animal intracardially. Even though different routes of administration can result in different substrate biodistribution patterns, the signal intensity obtained from hGLuc-expressing tumors was higher than that of hRLuc-expressing tumors at all coelenterazine doses and comparable to that of hFLuc tumors under the same conditions. No signal was observed for cells infected with HGCX (control) amplicon vectors at any coelenterazine or d-luciferin dosage.

Furthermore, to assess whether hGLuc could be used as a reporter gene to image deeper tissues, we implanted 1 million Gli36 cells expressing hGLuc into the right basal ganglia of nude mouse brains. Seven days postimplantation, and immediately after iv injection of 100 μg coelenterazine, we observed a high bioluminescent signal from all the brains implanted with these tumor cells (Fig. 6E). We did not observe any toxicity to mice upon repetitive dosage of coelenterazine in vivo as assessed by apparent signs of respiratory distress, disorientation, abnormal movements, or ruffled fur.

**DISCUSSION**

Bioluminescence is the process whereby light is produced by enzyme-mediated chemical conversion of a substrate,
usually through an intermediate highly energized state. Luciferases/luciferins fall into many unrelated classes with different evolutionary origins. The most notable example is firefly luciferase, with other examples including luciferases from bacteria and a variety of marine organisms, such as sponges, corals, jellyfishes, clams, and a few types of fish [17–19]. For luciferases to be useful as mammalian reporters they have to meet certain criteria, including broad emission spectrum, ideally with a significant red component, and high quantum yield without the intracellular accumulation of their substrates to allow real-time monitoring of enzyme expression. Here we describe the properties of the humanized form of Gaussia luciferase, which demonstrates high level expression in mammalian cells.

Gaussia luciferase cDNA was isolated from a cDNA library using expression cloning and the protein was expressed and purified in quantity from bacterial ferments [15]. This enzyme is 188 aa in length and encoded in a cDNA of 555 bp and has been humanized (Nano-light, Pinetop, AZ, USA) by codon optimization for mammalian cell expression. Gaussia luciferase has a broad pH optimum with peak activity at 7.7 [15]. Analysis of the gene sequence indicates a secretory signal which is functional in eukaryotes. Our results demonstrate that the hGLuc provides a new tool for bioluminescence imaging in cultured cells and in vivo with a number of useful properties. First, it generates much stronger signal intensity from cells in culture (1000-fold) compared with hRLuc and hFLuc. Second, despite the
fact that a substantial portion of hGLuc is secreted, its intracellular bioluminescence from viable cells or cell lysate is still over 100-fold higher compared to hRLuc or hFLuc when expressed under similar conditions with saturating substrate concentration. Third, hGLuc shows 200-fold higher bioluminescent signal intensity than hRLuc and intensity comparable to that of hFLuc in vivo under standard conditions with limiting coelenterazine concentration. Fourth, it is unique among luciferases currently in use as it is secreted. This, in combination with the fact that, like other coelenterazine luciferases, it does not require ATP for activity, allows it to report from cell surroundings as well as cells themselves. Fifth, similar to hRLuc, it can be used in dual bioluminescence imaging modalities with hFLuc, as they use different substrates. Sixth, its short coding sequence makes it suitable for small viral vectors, such as AAV. Further, it should be feasible to generate unique multireporter strategies based on separation of spectral emissions and/or luminescence lifetime of different luciferases. Finally, apart from serving as a good reporter in mammalian cells, hGLuc is very heat stable and strongly resistant to acidic and basic conditions [15], which should enhance its use in a number of biological applications. Therefore, the introduction of hGLuc as a bioluminescent reporter gene is an important step toward developing a broader array of real-time reporter tools.

The natural route of synthesis of hGLuc through the secretory pathway has advantages and disadvantages. Advantages include a means to report from the cell environment, which could prove useful in assessing diffusion parameters in a tumor/tissue, fluid volume spaces, such as cysts or ventricles, and the integrity of the secretory pathway in cells. Disadvantages include a lack of direct correlation between number of transduced cells and signal intensity and loss of signal through diffusion away from transduced cells. It may be possible to engineer hGLuc such that it is retained in cells with full activity by other modifications beyond elimination of the signal sequence or addition of an ER retention signal, which proved unsuccessful in this study.

Apart from serving as a reporter gene in its own right, it may also be possible to modify hGLuc in a number of ways to impart molecular specificity and/or influence its physicochemical properties. Use of coelenterazine analogues [20] or mutations in the active site of hGLuc, in a manner similar to that done for hFLuc [21,22] or click beetle luciferase [23], could result in an ultrasensitive, red-shifted luciferase that would be ideal for in vivo imaging. Furthermore, hGLuc could also be used in a bioluminescence resonance energy transfer (BRET) system [24] to study protein–protein interactions in living mammalian cells. In this case, one of the proteins of interest could be fused to hGLuc and the other to an intracellular fluorescent protein, such as GFP, or a secretable form of the same. When the two proteins are in close proximity to each other (100 Å), the energy resulting from the chemically excited state of coelenterazine–luciferase complexes would be transferred to the GFP, which, in turn, would reemit the light in a narrow-green band (510 nm) [25]. This secondary energy transfer, in turn, can further increase hRLuc quantum yield by 36-fold [15].

The pharmacokinetics of coelenterazine is somewhat limiting in vivo, compared to D-luciferin, the FLuc substrate, because coelenterazine is prone to quick inactivation, such as degradation through autoxidation [26]. This substrate is also costly with low solubility and has never been administered in excess of the reporter luciferase in any published reports [20]. Further, coelenterazine binds to serum proteins [27] and is cleared very rapidly from the bloodstream [20], and coelenterazine luciferases tend to have flash kinetics (versus glow kinetics for firefly), which rapidly decay with time [14]. Therefore, when imaging in vivo with coelenterazine luciferases the signal needs to be acquired immediately after substrate administration [20]. On the other hand, this short half-life can be advantageous in cases in which sequential imaging of two luciferases, such as hGLuc and hFLuc, is required to monitor two biological processes in tandem in the same animal [13,14].

Gaussia and Renilla luciferases have limited use for in vivo imaging compared to Fluc due to their blue bioluminescence (480 nm peak), which has high absorbance by pigmented molecules, such as hemoglobin and melanin, and scattering by tissues. In contrast the emission spectra of FLuc extends beyond 700 nm into the red range, which penetrates mammalian tissues efficiently [28]. However, despite these limitations, low levels of light generated by hGLuc and hRLuc do escape the absorbing and scattering environment of tissues in living animals and can be detected externally by the use of the charge-coupled device (CCD) camera [13,14,20,29]. Here we show that hGLuc produces 200-fold higher bioluminescence than hRLuc when they are expressed in mammalian cells under similar conditions in vivo. Furthermore, despite the fact that hFLuc is more red-shifted than hGLuc, and higher levels of the substrate (4 mg of D-luciferin compared to maximum 200 µg for coelenterazine) can be given per animal, the bioluminescent signal of FLuc is in the same range as hGLuc under standard imaging conditions in subcutaneous tumors in vivo. Apparently the secreted portion of hGLuc is largely retained within the tumor, probably due to diffusion constraints [30].

In summary, this study characterizes a humanized form of Gaussia luciferase that generates higher light output over the humanized firefly and Renilla luciferases when expressed in mammalian cells in culture, under similar conditions. Also, it shows higher signal intensity than hRLuc and similar intensity compared to hFLuc in
Luciferase activity assays. 293T cells were seeded in either six-well (0.3 million cells/well) or 96 plates (0.6 million cells/plate) and transfected with pHGC-GLuc, pHGC-hGLuc, pHGC-hRLuc, or pHGC-hFLuc plasmids, as well as pHGCX as a control, using Lipofectamine reagent (Invitrogen). Transfection efficiency, as monitored by GFP fluorescence, was consistently in the range of 18-24%. Forty-eight hours after transfection, the cells were trypsinized and an aliquot of intact viable cells, the conditioned cell-free medium, or both was assayed for luciferase activity using a luminometer by introducing coelenterazine to a final concentration of 40 μM and integrating the signal over the first 10 s.

To assay cell lysates, cells were harvested, centrifuged for 5 min at 1500g, washed 3× with PBS, and resuspended in 50 μl 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl, pH 8, and 0.5 μl protease inhibitor (Roche, Indianapolis, IN, USA). After 30 min incubation on ice, the cell homogenate was centrifuged for 30 min at maximum speed using a tabletop microcentrifuge at 4°C. The supernatant (cell lysate) was removed and protein concentration was measured using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Luciferase activity in cell lysates was assayed as above.

Cells transfected in 96-well plates were also assessed for luciferase activity after the addition of 20 μM coelenterazine by using a CCD camera (Roper Scientific, Trenton, NJ, USA) cooled to −120°C with liquid nitrogen and acquiring the signal for 10 s. The mean photon count was calculated in a manner similar to that used for the in vivo bioluminescence imaging (see below).

Dosing studies. One million glioma (Gli36) cells were seeded per p60 plate and infected with amplicon vectors at a m.o.i. of 0.5 pfu per cell to achieve 80-90% infection of cells as observed by GFP fluorescence. Forty-eight hours postinfection, cells were harvested in the conditioned medium and aliquots (50,000 cells in 100 μl) were added to each well of 96-well plates. Subsequently, 100 μl of coelenterazine at different concentrations was added to each well and luciferase activity was assayed as above using the CCD camera.

In vivo studies. Two million Gli36 cells were either stably transfected or infected in suspension with each of the amplicon vectors at an m.o.i. of 0.5 for 1 h. Subsequently, cells were washed, resuspended in 100 μl PBS, and implanted subcutaneously into the flanks of nude mice (6 weeks of age, weighing 25–27 g), anesthetized by ip injection of a mixture of ketamine (25 g/L) and xylazine (5 g/L). In one set of four mice, hRLuc-expressing cells were implanted in the left side and hGLuc cells in the right side of the same mouse. In another set of four mice, hGLuc cells were implanted in the left side and hGLuc in the right side of the same mouse. In another four mice, HGCX control vector-infected cells were implanted in the right side. In another experiment, while the animal was immobilized in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), cells expressing hGLuc (1.0 × 106 in 4 μl of PBS) were stereotactically injected into the right basal ganglia of five nude mice over 15 min at the coordinates 0 mm anterior, 2 mm lateral, and 2 mm deep from the bregma. Before imaging, 25 or 100 μg (0.96 or 3.9 mg/kg body wt) of coelenterazine (in 150 μl PBS) was injected into the tail vein, or 200 μg (7.7 mg/kg body wt, in 450 μl PBS) intracardially, and 2 min later, photon counts were acquired for 1 min and then for an additional 5 min using the CCD camera system. When we imaged hGLuc and hFLuc in the same mouse, 200 μl of coelenterazine and 4 mg (150 μg/kg body wt) of n-luciferin were mixed together and injected intracardially and animals were imaged as above. Conventional white-light surface images were obtained immediately before each photon counting session to provide an anatomical outline of the animal. Following data acquisition, postprocessing and visualization were performed using CMIR-Image, a custom-written program with image display and analysis suite developed in IDL (Research Systems, Inc., Boulder, CO, USA). Images were displayed as a pseudo-color photon count image, superimposed on a grayscale anatomic white-light image, allowing assessment of both bioluminescence intensity and its anatomical source. Regions of interest were defined using an automatic intensity contour procedure to identify
bioluminescent signals with intensities significantly greater than background. The sum of the photon counts in these regions was then calculated.

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