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Luciferase Calibrants Enable Absolute Quantitation of Bioluminescence Power

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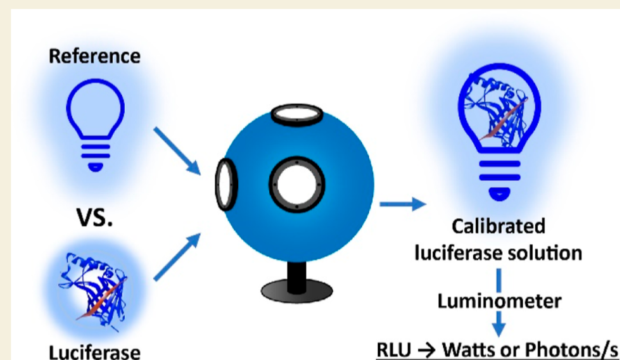
ABSTRACT: Bioluminescence emitted from a luciferase-catalyzed oxidation of luciferin has been broadly utilized to report on biological events, predominantly through relative changes in the light output. Recent advances in protein engineering and synthetic chemistry have yielded bioluminescent systems with markedly improved brightness and bioavailability. These developments have enabled not only the detection of biological events at far lower expression levels but also new opportunities utilizing bioluminescence to power photochemistry in cells. Regardless of the application, bioluminescence analyses have leaned heavily on the use of luminometers to measure the light output of a system. Current luminometers report the light output of a sample in relative units, limiting the ability to compare data between instruments and preventing the absolute power of a bioluminescent system from being quantified. Luminescent solution calibrants comprising luciferases and their cognate luciferins that have been characterized for absolute light output would enable calibration of any given luminometer for absolute photon counting. To this end, we have built a custom light detection apparatus and used it alongside wavelength-matched LED light sources emitting at 450 and 561 nm to characterize the absolute power of a series of NanoLuc and firefly luciferase solutions, respectively. This approach revealed that these two common luciferases produce 3.72×10^{-18} and 7.25×10^{-20} watts/molecule, respectively. Components of these luminescent solution calibrants are commercially available and produce stable bioluminescent signals over 2−5 min, enabling any luminometer to be calibrated for power measurements of bioluminescence emitted by these two luciferases in units of watts or photons per second.



KEYWORDS: *bioluminescence, luminometer, NanoLuc, firefly luciferase, optical power*

INTRODUCTION

Bioluminescent systems have become commonplace in biological research owing to their sensitivity, dynamic range, independence of external light sources, and simplicity of measurement. These systems have been used to track physiological events including gene expression, molecular interactions, and cellular signaling and have been incorporated into numerous biosensors and utilized in cellular and animal imaging.[1−4] Light emitted from bioluminescent systems is the result of the enzymatic turnover of a luciferin substrate by a luciferase enzyme. The brightness and wavelength of the emitted light vary between pairs of luciferase and cognate luciferin, and the choice between them largely depends on the availability and desired downstream application.[5]

Growth in bioluminescence applications has spurred the development of engineered luciferases and synthetic substrates with greatly improved brightness, stability, bioavailability, and emission over a broader range of wavelengths.[6−9] This advance has paved the way for luciferases to claim usefulness beyond traditional cellular sensing. Emerging research has demonstrated that bioluminescence can be harnessed to drive photochemistry, including optogenetic switches, photouncaging of small molecules, and photodynamic therapies.[10−12] In these contexts, photons generated by bioluminescent systems represent an intrinsic energy source for driving chemical reactions inside cells. Although these systems inevitably produce fewer photons than modern light sources (e.g., lasers and LEDs), they have the potential to deliver photons with greater efficiency, primarily due to their capacity as proteins to be expressed or targeted at the site of interest. Despite the widespread use of luciferase-based technologies and their emerging use to drive photochemistry, there is a lack of simple

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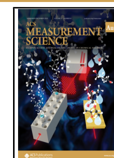

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tools to quantify the absolute power generated by these systems.

Traditional luciferase reporter assays track the extent of a biological event as a relative change compared to a negative control, allowing for robust and sensitive measurements to be made on a luminometer. Although luminometers make consistent measurements across an entire microplate, it is estimated that they detect at most only 2% of the total light generated within a well.[13] This limitation prevents the absolute quantitation of photon generation and requires the brightness to be reported in relative light units (RLU) or counts per second. Furthermore, the light collection efficiency and therefore reported brightness can vary between luminometers (even of the same model), experimental conditions, and the wavelength of the bioluminescent system. Although cumbersome, this variation in light collection efficiency can be mitigated using a single luminometer with consistent internal controls between experimental microplates. As the applications of bioluminescent systems expand beyond tracking relative changes in biology to harnessing photons as an intrinsic energy source for driving downstream reactions, the ability to make quantitative measurements of light energy on a luminometer becomes salient.

One way to overcome the relative nature of luminometers is to characterize the photophysical parameters of a luminescent solution using more sophisticated equipment and then use this solution as a calibrant. This strategy was employed by Suzuki et al. using a previously characterized luminol solution to calibrate a plate reader for the absolute counting of photons emitted by various luminescent proteins.[13−15] Due to the mismatch in wavelength between the luminol calibrant and the light emitted by these luminescent proteins, an additional step utilizing a photonic multichannel analyzer was required to account for the varying responsivities of the detector across wavelengths. This approach can be nontrivial for laboratories lacking photonic expertise. Furthermore, luminol calibrant solutions are sensitive to impurities in the luminol reagent and the concentration of hydrogen peroxide.[13] A similar method was used to calibrate the CCD camera of a bioluminescent imager for absolute quantitation of photons generated by single cells expressing luciferases.[16] While this clever approach utilized a stable LED light source as a calibrant rather than a luminescent solution, this strategy cannot be easily extended to luminometers.

Here, we describe the absolute quantitation of energy emitted from two commonly used luciferases: NanoLuc (Nluc) and firefly luciferase (Fluc). For reference light sources, we opted to use calibrated lasers as they offer stable and reproducible light outputs. The lasers were matched to the peak emission wavelength of each luciferase. Although the emission spectrum of a laser is significantly narrower, we assumed that the bell-shaped spectra of luciferases have roughly equal blue-shifted (higher energy) as well as red-shifted (lower energy) emissions. This distribution of photons is expected to have a mean photon energy equivalent to that of the peak wavelength. First, we calibrated the lasers using a custom light detection apparatus consisting of an integrating sphere interfaced with a sensitive photomultiplier tube (PMT) detector, and then, used the calibrated lasers to establish a relationship between light power in the integrating sphere and the PMT signal. Next, the calibrated light detection apparatus was used to quantify the light emitted from the two luciferases. We report the energy emitted from Nluc and Fluc on a per molecule basis and use this photophysical characterization to employ these luciferase solutions as calibrants for the absolute quantitation of samples on a common plate reader. The information provided here will allow any user using Nluc or Fluc systems to easily calibrate their plate reader for absolute quantitation of luminescence using commercially available reagents.

RESULTS AND DISCUSSION

Strategy for Absolute Quantitation of Bioluminescence

A typical luminometer houses an experimental microplate within a dark chamber, where a light detector indexes across the top of the microplate, measuring the relative light output of each well. Luminometers make consistent measurements across a microplate assuming the wavelength of emitted light is uniform. Ando et al. estimated that a maximum of 2% of the light generated in each well is detected.[13] This inefficient light capture can be attributed to multiple factors: (i) photon absorption by the microplate, (ii) photon evasion of the detection pathway by penetrating the well wall or escaping between the top of a well and the detector (crosstalk), (iii) steep incidence angle of photon to the detector, failing to trigger a count, and (iv) detector failure to register photon due to an imperfect quantum yield (Figure S1). In addition, many of these factors depend on the color of the light being emitted since photons of varying wavelengths have individual absorption characteristics, and light detectors have varying responsivity across the spectrum. The overall light collection efficiency will therefore depend on the model of the luminometer, the geometry and color of the microplate containing the light-emitting sample, and the color of light emitted from the sample. These factors together make the absolute quantitation of light using a luminometer alone a challenging task that necessitates the use of well-characterized wavelength-match standards.

We sought to quantify the optical power of Nluc and Fluc luciferases for the subsequent generation of luciferase solution calibrants. Such calibrants would allow a user to convert the typical RLU output of their luminometer to absolute units of photons per second, or watts, for experiments using blue-emitting Nluc (460 nm) or green-emitting Fluc (561 nm). To this end, we constructed a custom light detection apparatus and used it to determine the absolute optical power of the Nluc and Fluc solution calibrants. This apparatus consists of an integrating sphere with individual ports for interfacing a PMT and either a reference light source or a luciferase solution. The integrating sphere uniformly scatters the photons from the reference light source or luciferase solution such that a small but consistent fraction of the total light is detected by a PMT interfaced to the integrating sphere. The absolute light output of a luciferase can then be quantified by direct comparison with the known light output of a calibrated reference light source. The laser reference light sources for the blue-emitting Nluc and green-emitting Fluc were chosen such that their peak emission matched that of the luciferase. Together, the wavelength-matched reference lasers and the integrating sphere form a calibrated light detection system, which accounts for variability in pathloss and PMT responsivity at varying wavelengths.

Determination of Luciferase Optical Power

A blue (450 nm) laser diode and a green (561 nm) solid-state laser were selected as reference light sources due to the

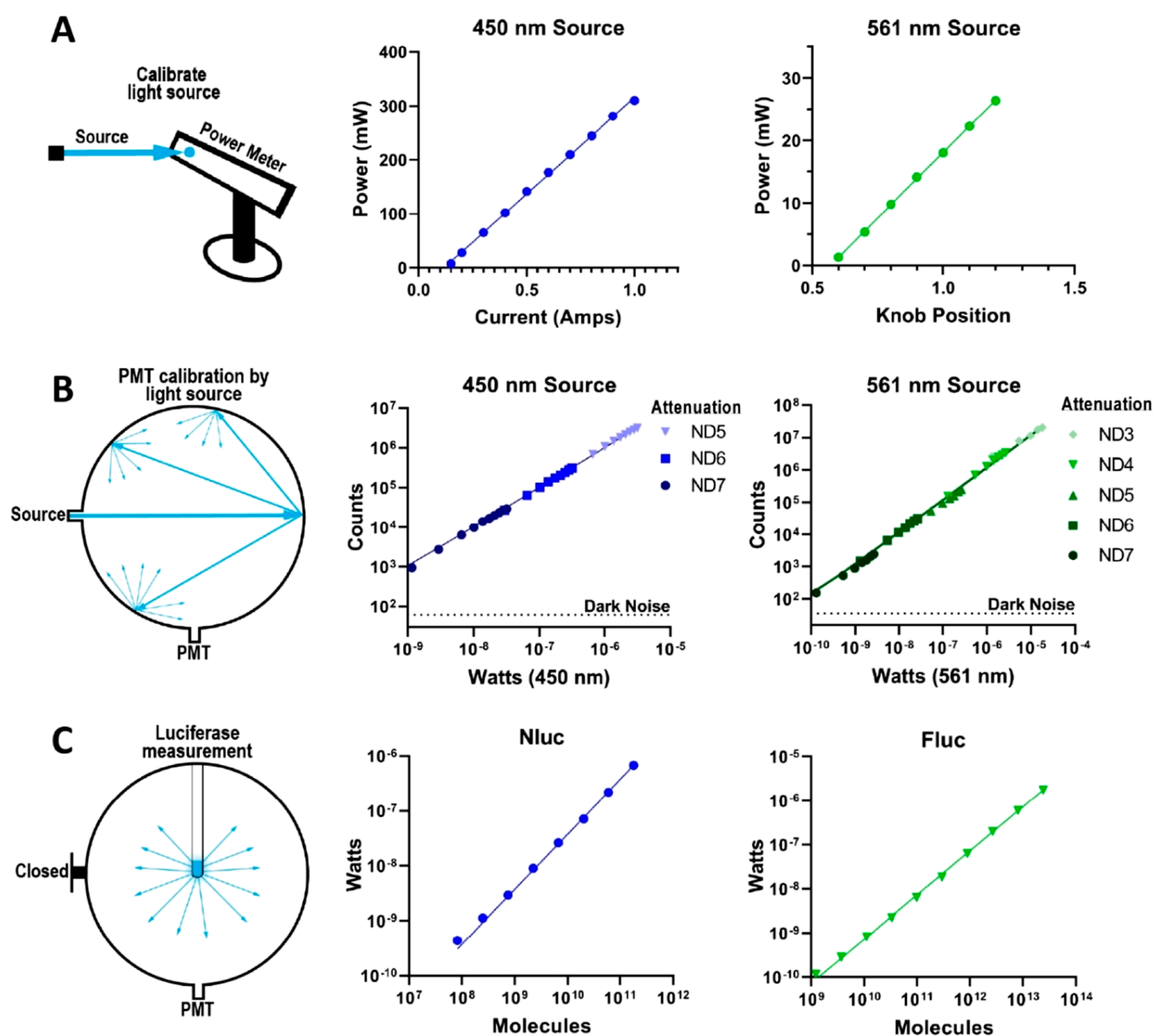
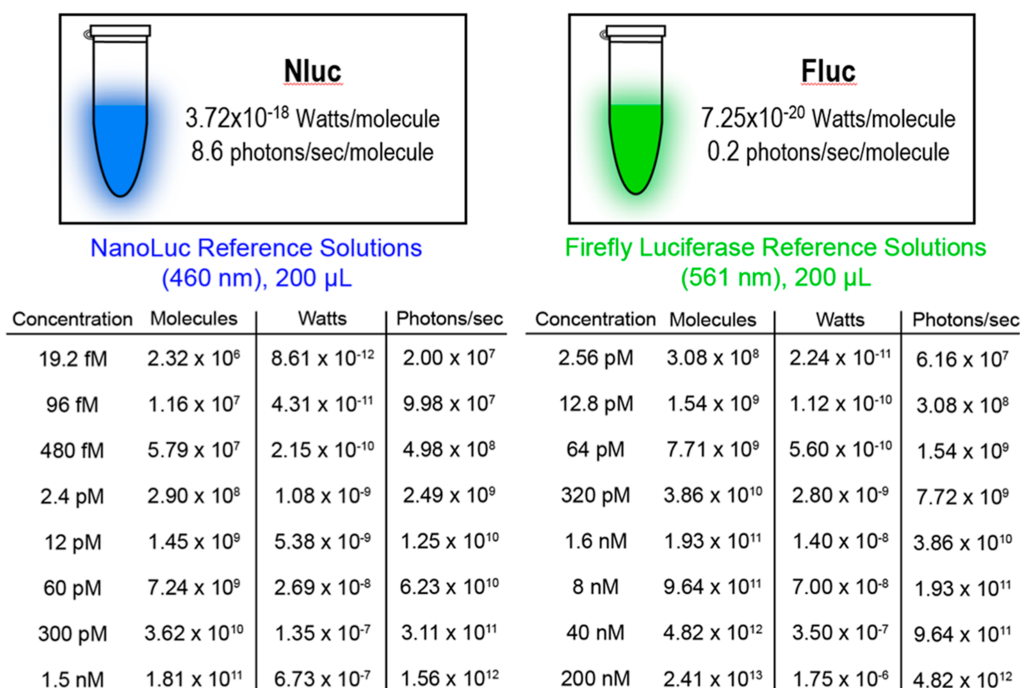


Figure 1. Quantitation of the luciferase power. (A) Power output of both blue (450 nm) and green (561 nm) lasers was determined with increasing power input using a benchtop power meter. (B) Integrating sphere/PMT apparatus was calibrated using the previously defined power output of reference light sources. A series of neutral density filters were used to quantitatively attenuate the light output of these light sources to levels similar to those of bioluminescent reactions. (C) Luminescent reactions of Nluc and Fluc were measured by the calibrated integrating sphere/PMT apparatus, and the watt output of each reaction was determined. All measurements were performed in technical triplicate, with data representing the mean value. Error bars represent the standard deviation of measurement and may not be visible if the error is smaller than the dot representing the mean value.

similarity between their peak emission and the emission of Nluc and Fluc, respectively (Figure S2). Additionally, a feedback circuit was built for the blue laser diode to maintain a stable light output throughout fluctuations in operating temperature. We first determined the wattage of each reference light source as a function of input current using a benchtop power meter (Figure 1A). For each reference, a linear relationship was established between the input current and output wattage, allowing its use as a stable downstream calibrated light source.

Following calibration, each reference light source was mounted to our light detection apparatus and used to determine the relationship between the output wattage of the light source and the counts detected by the PMT. To attenuate the intensity of the reference light source to levels comparable to luciferase light output, neutral density filters were affixed to the interface between the light source and the integrating sphere. Neutral density filters, which block a defined amount of visible light generated by the reference light source from passing into the integrating sphere, were assembled in series to achieve levels of light comparable to those of each luciferase. Linear relationships were determined between the output wattage of each reference light source and counts detected by the PMT over 3.5−5 orders of magnitude (Figure 1B). Using this analysis, we calibrated our apparatus to quantify light sources emitting 10^{-10} to 10^{-5} watts. Notably, the degree of light output attenuation could be optimized to fit the brightness of any given luciferase of choice.

Next, we used the calibrated light detection apparatus to quantify the light generated by the two luciferases. Due to their enzymatic nature, luciferases consume substrate to produce photons and, in the process, deplete the substrate, resulting in light-emitting kinetics that can decrease over time. Therefore, reaction conditions for both Nluc and Fluc were optimized to



NanoLuc Reference Solutions (460 nm), 200 µL

Concentration	Molecules	Watts	Photons/sec
19.2 fM	2.32 x 10^6	8.61 x 10^-12	2.00 x 10^7
96 fM	1.16 x 10^7	4.31 x 10^-11	9.98 x 10^7
480 fM	5.79 x 10^7	2.15 x 10^-10	4.98 x 10^8
2.4 pM	2.90 x 10^8	1.08 x 10^-9	2.49 x 10^9
12 pM	1.45 x 10^9	5.38 x 10^-9	1.25 x 10^10
60 pM	7.24 x 10^9	2.69 x 10^-8	6.23 x 10^10
300 pM	3.62 x 10^10	1.35 x 10^-7	3.11 x 10^11
1.5 nM	1.81 x 10^11	6.73 x 10^-7	1.56 x 10^12

Firefly Luciferase Reference Solutions (561 nm), 200 µL

Concentration	Molecules	Watts	Photons/sec
2.56 pM	3.08 x 10^8	2.24 x 10^-11	6.16 x 10^7
12.8 pM	1.54 x 10^9	1.12 x 10^-10	3.08 x 10^8
64 pM	7.71 x 10^9	5.60 x 10^-10	1.54 x 10^9
320 pM	3.86 x 10^10	2.80 x 10^-9	7.72 x 10^9
1.6 nM	1.93 x 10^11	1.40 x 10^-8	3.86 x 10^10
8 nM	9.64 x 10^11	7.00 x 10^-8	1.93 x 10^11
40 nM	4.82 x 10^12	3.50 x 10^-7	9.64 x 10^11
200 nM	2.41 x 10^13	1.75 x 10^-6	4.82 x 10^12

Figure 2. Luciferase calibrant solutions. This chart details the measurements of a dilution series of luminescent calibrant solutions with quantified energy output. Nluc reactions contain 20 μM furimazine, and Fluc reactions contain 1.05 mM Bright-Glo, both in TBS pH 7.4 supplemented with 0.01% acetylated BSA. Energy outputs for 200 μL solutions describe their light levels between 1 and 2 min after initiating the bioluminescent reaction. Light emission data represent the mean value with percent standard deviations of <4 and ≤3% for Nluc and Fluc, respectively. Supporting Information includes a more detailed table with the mean values and their associated standard deviations (Figure S4).

ensure steady light emission over the ∼2 min required to take measurements in the light detection apparatus (Figure S3A,B). Furthermore, each luciferase solution was titrated to determine a linear range in which a series of measurements could be made over a range of brightness (Figure S3C). Notably, Fluc is dimmer than Nluc, and therefore, ∼50-fold higher enzyme concentrations were needed to generate solutions with similar brightness. Once these optimizations were completed, the reactions of each luciferase were performed in thin-walled glass NMR tubes and suspended in the center of the light collection apparatus. Following a 1 min preincubation, light emissions were measured by the PMT and converted to watts using the already established correlation between the PMT counts and the wattage output of the reference light sources (Figure 1C). From this, we determined the watts generated per molecule of Nluc (3.72×10^{-18} W/molecule) and Fluc (7.25×10^{-20} W/molecule) under saturating substrate conditions. These values were easily converted to photons emitted per second per molecule of Nluc (8.6 photons $\times$ sec^{-1}/molecule) and Fluc (0.2 photons $\times$ sec^{-1}/molecule) (Figure 2). This analysis circumvents the need to correct for any absorbance, pathloss, or differences in PMT responsivity that could arise when using mismatched wavelengths between the reference light source and luciferase. Furthermore, this photophysical characterization of luciferases empowers any user with the ability to calibrate their own luminometer for absolute quantitation of Nluc or Fluc energy using a simple and reproducible solution made from commercially available reagents.

Luminometer Calibration with Luciferase Standards

Our characterization of Nluc and Fluc optical power enables the simple assembly of luciferase solutions that produce a defined light output from commercially available reagents (Figure 2). These solutions can be used as standards to calibrate common luminometers to report the bioluminescence of blue or green luciferases in absolute units of wattage or photons/sec. Here, we describe two applications utilizing a calibrated luminometer to quantify the total joules of energy produced by luciferases over a kinetic read and to estimate the number of luciferase molecules expressed in mammalian cells. We also demonstrate how these reference solutions can be used to normalize data between various luminometers.

To determine the total joules of energy produced by equivalent amounts of Nluc and Fluc, we first calibrated our luminometer, converting its RLU output to absolute units of watts (joules/sec). Reference solutions of Nluc and Fluc with defined wattage outputs were assembled in microplates and measured in triplicate (Figure S5A,B). These data generated a linear relationship between luciferase wattage and RLU for both Nluc ($RLU_{Nluc} = 3.06 \times 10^{-15} \times$ watts) and Fluc ($RLU_{Fluc} = 5.16 \times 10^{-15} \times$ watts). The variance in slope relating watts to RLU between luciferases is attributed to differences in pathloss and PMT responsivity at distinct wavelengths and highlights the need for wavelength-matched reference solutions. Bioluminescent reactions containing equivalent amounts of Nluc and Fluc were then assembled and read for 1 h in the same luminometer which had been calibrated (Figure 3A). Using the previously generated standard curve, the RLU output data of the luminometer was converted to units of watts, and the area under the curve was calculated to determine the total joules of energy emitted from Nluc (1132 μJ) and Fluc (26 μJ) over 1 h (Figure 3A). We found that an increased volume of luminescent solution containing the same total moles of luciferase showed a slightly decreased signal in a microplate (Figure S6A). Similarly, doubling the volume and total moles of luciferase resulted in less than twice as much light being detected, together

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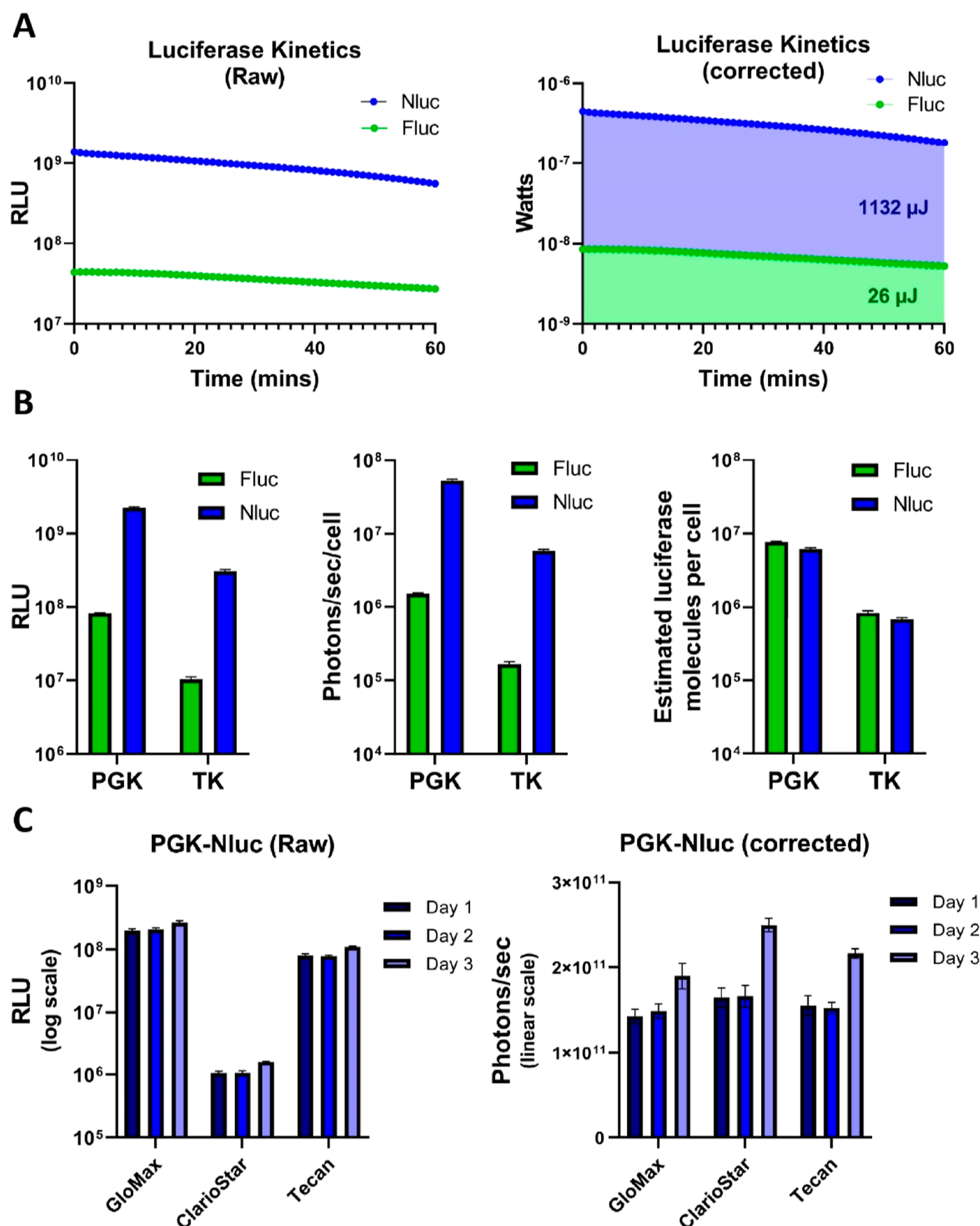

Figure 3. Plate reader calibration enables quantitative measurements (A) 60 min time course for bioluminescent reactions containing 1 nM of either Nluc or Fluc with 20 μM furimazine or 1.05 mM Bright-Glo, respectively. Triplicate RLU measurements were converted to watts using plate reader calibration, and the area under the curves was determined as a measurement of total joules of bioluminescent energy produced over 60 min. (B) Luminescence reads for HeLa cells transfected with luciferase expression vectors. Data were converted to photons per second via the calibration curve and normalized to cell count. (C) Luminescence intensity reads on three plate readers for HeLa cells transfected with PGK promoter-driven Nluc expression vector on three consecutive days. Luciferase standards were used to calibrate each individual plate reader and to convert all data to units of photons per second. Data represents the mean values of three technical replicates, with error bars signifying standard deviations. Error bars which are smaller than the dot representing the mean value may not be visible.

suggesting that larger volumes likely result in photon reabsorption (Figure S6B). As such, these measurements require that the experimental volume and microplate selection match those of the reference solutions.

This characterization of luciferase bioluminescence can enable the estimation of the total luciferase molecules within a biological sample under controlled conditions. To this end, mammalian cells were transfected with either PGK or TK promoter-driven Nluc or Fluc expression vectors prior to luminescence intensity measurements on our previously calibrated plate reader (Figure 3B). The Fluc reference curve was established in Bright-Glo lytic assay buffer, allowing for the same buffer to be used for cellular measurement. However, to facilitate cellular Nluc measurements, digitonin was supplemented to TBS to permeabilize cells after demonstrating that the addition of digitonin did not impair the brightness of Nluc (Figure S5C). As expected, PGK promoter-driven luciferase expression exceeded that of the TK promoter, and in both cases, the Nluc signal surpassed that of Fluc. Using the calibrated luminometer, luminescent intensities were first

converted to photons per second per cell and then to molecules of luciferase per cell. Despite the Nluc signal far exceeding that of Fluc, the total estimated molecules of Nluc and Fluc per cell were similar when either driven by the PGK (6.2×10^6 and 7.7×10^6) or TK (6.7×10^5 and 8.3×10^5) promoter. It is important to note that these estimations assume enzyme behavior is similar between lysed or permeabilized cells and a biochemical environment, and any effects of buffers or media additives should be carefully considered.

A final shortcoming in luminescence detection that we sought to address is the inability to compare relative luciferase measurements between multiple plate readers. This inherent limitation can confuse collaborative work, where multiple plate readers are used, or place a stranglehold on equipment resources during larger experimental campaigns. Here, we calibrated 3 different plate readers with the same series of Nluc luciferase standards (Figure S5D). Mammalian cells transfected with a Nluc expression vector were then used for the luminescence measurement on each of the three calibrated plate readers. Data were subsequently converted to units of photons per second using a Nluc standard curve specific to each plate reader (Figure 3C). Identical analyses were performed independently on three subsequent days. We found that the day-to-day variation in photons per second for Nluc-transfected cells was greater than the variation between plate readers. This analysis demonstrates the simplicity and effectiveness of using luciferase standards to both normalize and apply absolute units to the data from various plate readers.

DISCUSSION

Bioluminescent-based assays have been widely employed in basic research, drug discovery, and healthcare to assess and report biological events with high sensitivity and a large signal over background. Recent advances have demonstrated the use of luciferases as an intrinsic source of energy that can drive photochemical reactions in a range of applications, including the uncaging of small molecules and photodynamic therapies. These developments expand luciferase applications beyond those of biological reporters and necessitate the ability to quantify the available power of a bioluminescent system. Nonetheless, evaluating bioluminescent intensities has been largely constrained to relative units due to the inherent limitations and variations between commonly used luminometers. As applications that harness bioluminescence as an energy source continue to emerge, there is a growing need for simple, sensitive, and quantitative bioluminescent measurements. Our work here, characterizing a series of luciferase calibrants, enables end users to calibrate their existing luminometers to report absolute units of brightness, granting a more thorough comprehension of the energy produced by bioluminescent systems.

METHODS

Evaluation of Optical Power of Light Sources

The optical powers of reference light sources were measured by using a benchtop power meter (Thorlabs S130C). The optical power of a blue laser diode (OSRAM PL-TB450B) was measured as input current from a power supply (RIGOL DP832), which was adjusted from 100 to 1000 mA. Laser power was stabilized by a closed automatic feedback loop where approximately 4% of laser light was directed by a beam splitter to a feedback photodiode with an electronic amplifier producing a signal for a laser power control unit. The optical power of a green solid-state laser (OptoEngine MGL-FN-561) was measured as its control knob was adjusted from 0.6 to 1.2. This solid-state laser did not require any additional modifications to operate at stable power. Each laser was coupled to the optical fiber via an SMA connector before being directed at the benchtop power meter.

Assembly and Calibration of the Light Detection Apparatus

We assembled a light detection apparatus consisting of a central three-port integrating sphere (Labsphere 3P-GPS-033-SL) coupled to a PMT via an optical fiber with SMA connectors (Thorlabs M29L01). The second port of this integrating sphere was used as an input for reference light sources using a custom printed light-tight adapter, which couples to the optical fiber lead of each laser. This adapter was made to accommodate the insertion of neutral density filters. The third and uppermost port was used to mount bioluminescent samples inside the integrating sphere. A light-tight adapter was constructed which secured thin-walled NMR glass tubes using a set screw. The NMR tubes were cut such that the bioluminescent sample was suspended centrally in the integrating sphere.

The PMT mounted to the integrating sphere was calibrated using reference light sources with known optical powers mounted, as described above. The reference light source was attenuated using neutral density filters (Thorlabs NE10A-A through NE50A-A). Attenuation above ND5 utilized two neutral density filters in series. Calibration was performed using both blue and green lasers at a range of intensities achieved both by varying the input current to the laser and by using multiple arrangements of neutral density filters. This analysis calibrated the PMT to report light intensities in units of watts.

Quantification of Bioluminescent Energy Using Light Detection Apparatus

The calibrated light detection apparatus was used to quantify the light generated at room temperature by bioluminescent solutions containing various amounts of either Nluc or Fluc (Figure 2; protein sequences are included in Figure S7). Nluc (Promega E499A) was diluted in TBS (50 mM Tris pH 7.4 + 150 mM NaCl) + 0.1 mg/mL acetylated BSA (Promega R3961) to generate a 2× of a 3-fold serial dilution (3000−1.372 pM). Furimazine (Promega N113A) was diluted 125-fold in TBS +0.1 mg/mL acetylated BSA to generate a 40 μM solution (2× final). Fluc (Promega E170B) was diluted in TBS +0.1 mg/mL acetylated BSA to generate a 2× of a 3-fold serial dilution (400−0.18 nM). Luciferin solution was made by resuspending the Bright-Glo substrate (Promega E263A) with 5 mL of Bright-Glo buffer (Promega E264A) to achieve a 2× stock (2.1 mM). Luciferase reactions were initiated by mixing 100 μL of either 2× Nluc solution with 100 μL 2× furimazine solution or 100 μL 2× Fluc solution with 100 μL 2× luciferin solution in a thin-walled NMR tube. This light-emitting solution was quickly hung in the center of the light detection apparatus and sealed from external light below. The wattage of the solution was recorded 1 min after reaction initiation. All measurements were made in triplicate, with the data representing the mean value. Error bars represent the standard deviation of measurement and may not be visible if the error bar is smaller than the dot representing the mean value. Watts were further converted to photons/s using the equation watts = (photon/sec) × $(h \times c)/\lambda$, wherein λ is the wavelength (m), Planck's constant (h) is 6.6261×10^{-34} (J × s.), and the speed of light c is 299,792,458 m/s.

Calibration of a Luminometer to Report Absolute Units

To calibrate a plate reader (Figure S5), 2× serial dilutions of Nluc and Fluc as well as 2× stock solutions of furimazine and luciferin were prepared, as described above. Luciferase reactions were initiated by mixing 100 μL of either 2× Nluc solution with 100 μL of 2× furimazine solution or 100 μL of 2× Fluc solution with 100 μL of 2× luciferin solution in a white, 96-well, flat bottom, nonbinding plate (Corning 3600). Both the Nluc and Fluc series included a control for autoluminescence which did not contain luciferase. Luminescent intensities were measured 1 min after thorough mixing on either GloMax Discover (Promega), Spark (Tecan), or CLARIOstar (BMG

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Labtech) plate readers at 21 °C. Autoluminescence was subtracted from raw intensities, and adjusted data of RLU vs power (watts or photons/s) were fit by linear regression on GraphPad Prism. These relationships were recorded for each instrument and used to convert subsequent RLU measurements to absolute units of power.

Transient Overexpression of the Luciferase Construct in Mammalian Cells

HeLa cells were transfected in bulk using ViaFect (Promega E498) and a DNA construct encoding either Nluc or Fluc that was diluted 10-fold in promoterless carrier DNA (Promega E488). DNA constructs included PGK promoter-driven expression vectors of Nluc (Promega N144) or Fluc (Promega E501) or TK promoter-driven expression vectors of Nluc (Promega N150) or Fluc (Promega E506). After 6 h, cells were harvested, seeded in a 96-well plate (Corning 3917) at 20,000 cells per well, and incubated for 16−18 h at 37 °C and 5% CO_2. Cells were plated in duplicate for experiments in which data were normalized to the total cell count so that cell growth could be measured and accounted for. Prior to bioluminescence reading, media were replaced with 100 μL OptiMEM (Gibco 31985) and plates were equilibrated to room temperature. 2× solutions of luciferase detection reagents were made by either resuspending a vial of the Bright-Glo substrate (Promega E263A) in 5 mL of Bright-Glo buffer (Promega E264A) or by adding 100 μL furimazine (Promega N113) to 5 mL of Nano-Glo buffer (Promega N112). To initiate bioluminescence, cells expressing Fluc or Nluc were treated with 100 μL of either 2× Bright-Glo or 2× NanoGlo detection reagents, respectively. Measurements made to estimate molecules of Nluc per cell used a 2× substrate mix in which 100 μL furimazine (Promega N113) was added to 5 mL TBS with 60 μg/mL digitonin (Promega G9441). Following brief mixing and a 2 min incubation, luminescence was measured on GloMax Discover (Promega), Spark (Tecan), or CLARIOstar (BMG Labtech) plate readers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmeasuresciau.3c00036.

Reference source spectra, luciferase kinetics, and standard curves (PDF)

■ AUTHOR INFORMATION

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All authors have given their approval to the final version of the manuscript. CRediT: **Mark A Klein** conceptualization, data curation, formal analysis, investigation, methodology, writing-original draft, writing-review & editing; **Sergey Lazarev** conceptualization, data curation, formal analysis, investigation, methodology, writing-review & editing; **Charles Gervasi** conceptualization, data curation, formal analysis, investigation, methodology, writing-review & editing; **Cristopher Cowan** project administration, writing-review & editing; **Thomas Machleidt** project administration, writing-review & editing; **Rachel Friedman Ohana** conceptualization, data curation, formal analysis, investigation, methodology, project administration, writing-original draft, writing-review & editing.

Notes

The authors declare the following competing financial interest(s): All authors are employees of Promega.

■ ABBREVIATIONS

Nluc, NanoLuc; Fluc, firefly luciferase; RLU, relative light units; PMT, photomultiplier tube

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