doi:10.1246/cl.2010.291
Published on the web February 20, 2010

Quantum Yield Measurements of Firefly Bioluminescence Reactions Using a Commercial Luminometer

Kazuki Niwa,[1] Yoshiro Ichino,[1] and Yoshihiro Ohmiya*[2]
[1]*National Metrology Institute of Japan (NMIJ), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba 305-8563*
[2]*Research Institute for Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-higashi, Toyohira-ku, Sapporo 062-8517*

(Received December 28, 2009; CL-091162; E-mail: y-ohmiya@aist.go.jp)

Quantum yields of firefly bioluminescence reactions were determined using a commercially available luminometer whose absolute responsivity was calibrated with respect to the spatially and spectrally integrated photon flux emitted from the reaction solution.

The quantum yield of bioluminescence and chemiluminescence reactions, defined as the probability of a single photon production from a single reactant molecule, is a basic characteristic for interpreting the molecular mechanism of luminescence reactions. Firefly bioluminescence is one of the most well known luminescence reactions with the highest quantum yield. Recently, Ando et al. have reported its value as being 0.41 using *Photinus pyralis* native luciferase,[1] which is markedly different from the value of 0.88 previously reported by Seliger and McElroy.[2] However, despite a number of curious beetle luciferases with various properties,[3] these are the only values reported based on original experimental data.

The total number of photons emitted diffusively from a reaction solution has to be measured absolutely to determine the quantum yield of bioluminescence. Ando et al. used a multichannel spectrometer, whose spectral responsivity was calibrated absolutely, in conjunction with a geometric treatment for determining the light collection efficiency precisely.[1,4] More recently, Daniel et al. reported a method to determine the bioluminescence photon number based on the absolute spectral responsivity of a photodetector using a double-integrating-sphere system.[5] However, these methods require a complicated series of calibrations and measurements, which makes it difficult to accumulate quantum yield data and to validate them for those who do not have expertise in physical measurements and instrumentation. Luminol chemiluminescence reaction is often used as a secondary light standard.[4,6] However, complicated series of measurements are also required for correction due to the difference of spectral profile between the standard luminol solution and the sample solution.

Luminometers are sensitive photon-counting detectors widely employed to observe weak bio/chemiluminescence of reaction solutions. However, they are neither spectrometric nor designed with ideal point-source geometry, with the result that they are not considered to be "absolute" instruments.

In order to determine bio/chemiluminescence quantum yields using a luminometer, there are two requirements as follows. The first is that the detector must collect the entire luminescence signal throughout the reaction. To do this, one must start the data collection before injecting solution to trigger the reaction under dark conditions, which is possible on most commercial luminometers. In addition, the reactant molecules must be totally consumed within a practical period of time for measurement. In the case of firefly bioluminescence, this is achievable when the concentration of D-luciferin is extremely low and that of the enzyme luciferase is extremely high. The second requirement is that the sensitivity of the luminometer must be calibrated absolutely. In this report, we describe a novel method to calibrate the absolute responsivity of the luminometer and the results of quantum yield measurements for firefly bioluminescence reactions. Here we define the "responsivity" of the luminometer as the sensitivity (in counts·photons^{-1}) to the spatially and spectrally integrated photon flux (hereafter referred to as the "total photon flux") emitted from the reaction solution. We should note that the responsivity is not uniquely determined for the luminometer solely but particular to the type of test tube employed, the volume of the solution filling in the tube, and the luminescence spectrum.

To determine the absolute responsivity of a luminometer, a reference light source with a known total photon flux is required, although such a calibrated standard light source is not available commercially.[7] The reference light source to be employed should have power level within the detection range as well as spectral and angular distribution profiles similar to those of the luminescence solution under examination. The spectral matching is essential because a luminometer is not spectrometric and its responsivity depends strongly on the spectrum to be measured.

We employed a long-lasting luminescence solution as a reference light source optimized for the calibration of the luminometer, which is referred to as the "reference solution." This solution contained the same firefly luciferin–luciferase system as the sample solution whose quantum yield was of interest, resulting in excellent spectral matching. Such system is advantageous in that long-lasting glow is easily obtained with the help of proper additives. Reabsorption correction should be usually taken into consideration for luminescence solution but is negligibly small in the firefly bioluminescence because of the large Stokes-shift, which simplifies data analysis regardless of the concentration of the reagents. In addition, the reference solution was filled into a test tube which is the same type as that used for the sample solution. This can easily reproduce the angular distribution of the sample solution.

Our procedure to determine quantum yield of bioluminescence reaction is as follows: (1) determination of the total spectral photon flux of luminescence from the reference solution, (2) absolute calibration of a commercially available luminometer using the reference solution, and (3) quantum yield measurements using thus calibrated luminometer.

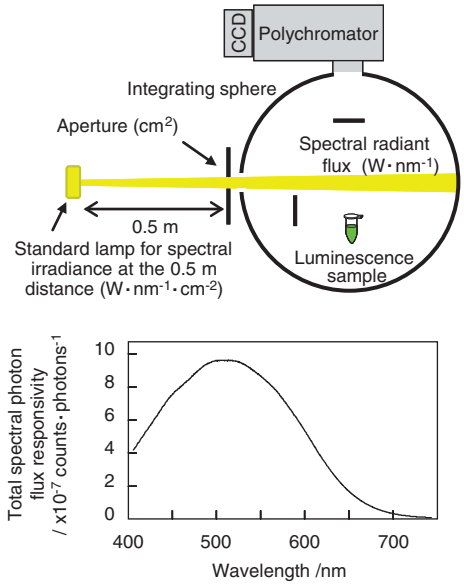

Figure 1. Integrating sphere-based multichannel spectrometer system employed in this study and its spectral total photon flux responsivity.

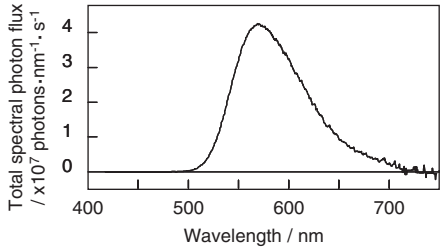

Figure 2. Absolute spectrum of a reference solution of a bioluminescence reaction containing 0.1 ng *L. mingrelica* luciferase in 100 µL of PicaGene reagent, which was used for the calibration of the luminometer.

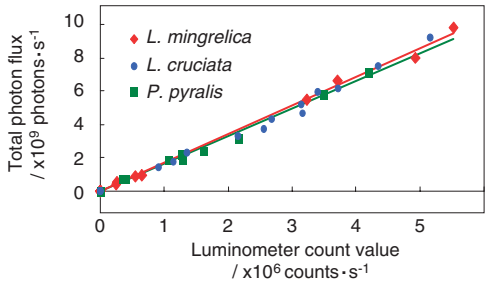

Figure 3. Calibration plot of the total photon flux responsivity of the luminometer with various intensity levels for the three beetle luciferases employed in this study. The red and green lines indicate the least square linear fit of each data for *L. mingrelica* and *P. pyralis*, respectively. The fitting gradient for *L. cruciata* is close to that for *P. pyralis* and is not shown in this figure.

The total photon flux measurement system for the reference solution is illustrated in Figure 1. This system comprises an integrating sphere and a multichannel spectrometer equipped with a cooled CCD detector. The integrating sphere-based absolute total radiant flux calibration is well established in photometry and radiometry.[8] The spatially-integrated spectral photon flux responsivity of this system with respect to an internal light source located inside the integrating sphere is also shown in Figure 1. This spectral responsivity was obtained by using a spectral irradiance standard lamp as the external light source coupled with an aperture with a known area at the entrance of the sphere. The power level of the radiant flux through the aperture was significantly reduced to be comparable to that of the luminescence from the reference solution. The total spectral photon flux responsivity of the system was sufficiently linear in the intensity range of interest, which was confirmed by changing the radiant flux through the aperture. An absolute luminescence spectrum of a reference solution was obtained by employing thus calibrated measurement system, as shown in Figure 2.

We used three beetle luciferases: *P. pyralis* (product No. L9506, Sigma-Aldrich, St. Louis, MO, USA), *Luciola cruciata* (product No. 122-03913, Wako, Osaka, Japan), and *L. mingrelica* (product No. L4899, Sigma-Aldrich) for the firefly bioluminescence reaction solutions whose quantum yields were to be determined in this study. These luciferases exhibited their own luminescence spectral profiles. Therefore, our luminometer needed to be calibrated for each luciferase using its long-lasting reference solution. As the spectral profiles of the firefly bioluminescence reactions using these enzymes are influenced by pH condition of each reaction solution, each test solution for quantum yield measurement contained 0.1 M Tris-HCl (pH 8.0) buffer so that its luminescence spectrum was identical to that of the corresponding reference solution.

PicaGene® reagent (Wako) was employed to obtain the long-lasting luminescence intensity for the calibration of the luminometer (AB-2200, ATTO, Tokyo, Japan). This reagent contains coenzyme-A and dithiothreitol as additives to obtain long-lasting luminescence with a half-decay time of more than 10 min.[9] The luminescence intensity of a bioluminescence reaction, however, tends to be influenced by the quality of the luciferase to be added and hence has poor reproducibility. To avoid this disadvantage, the reference solution was simply duplicated by dividing the homogeneous reaction solution into two test tubes. And then, one of them was put into the integrating sphere system and the other into the luminometer to determine simultaneously the absolute total photon flux of the reference solution and the absolute responsivity of the luminometer, respectively.

To confirm the linearity of the total photon flux responsivity of the luminometer, reference solutions with a wide range of luminescence intensities for each luciferase were prepared by controlling the concentration. In addition, serial measurements of each single divided reference solution with gradual decay of its luminescence intensity enabled coverage of a wider intensity range. As a result, the luminometer employed in our study exhibited a good linear response at the usual detection level for bioluminescence measurements, as indicated in Figure 3. By linear fitting of the plots, the absolute responsivity (in counts·photons^{-1}) of the luminometer was obtained for each luciferase (Table 1).

Using our calibrated luminometer, the time-integrated total number of photons emitted from the luminescence reaction solution of each firefly luciferase was measured, followed by the

Table 1. Quantum yield and related properties of luciferases

Luciferase	λ_{Max}[a] /nm	Photon[b] /$\times 10^{10}$	Quantum yield[c]	Responsivity[d] /$\times 10^{-4}$ counts $\cdot$photons^{-1}
L. mingrelica	571	2.58	0.43	5.82
L. cruciata	565	2.60	0.43	6.02
P. pyralis	566	2.89	0.48	6.05

[a]Luminescence maximum of the bioluminescence reaction of each luciferase. [b]Average of accumulated total number of photons emitted from 6.02×10^{10} reacted D-luciferin molecules. [c]Coefficient of variation and number of samples were 3.4%, $n = 6$; 7.5%, $n = 6$; and 8.1%, $n = 5$, respectively. [d]Responsivity of the luminometer for the bioluminescence reaction of each luciferase.

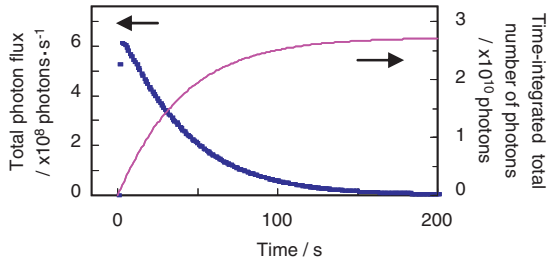

Figure 4. Data for quantum yield measurement collected using the absolute calibrated luminometer. Temporal behavior of total photon flux (dots, left-hand axis) and time-integrated total number of photons (solid line, right-hand axis) for *L. mingrelica* luciferase. The reaction was initiated after initiating data collection and was complete within a period of 4 min in the presence of 6.02×10^{10} molecules of the substrate D-luciferin.

quantum yield calculation from the number of reacted D-luciferin molecules (6.02×10^{10}) (Table 1). To complete the reaction within a period of a few minutes (Figure 4), the concentration of the luminescence substrate D-luciferin was extremely low (10 µL of a 10^{-8} mol L^{-1} solution) and that of the enzyme luciferase was extremely high (10 µL of a 1.5×10^{-5} mol L^{-1} solution).[1] The reaction was initiated by adding 80 µL of an ATP–Mg solution (containing 3 mM of ATP disodium salt, 8 mM of magnesium sulfate, and 0.1 M tris-HCl [pH 8.0]). The consumption of all reactant D-luciferin in this reaction condition was confirmed using HPLC, which was described previously.[1] The relative standard uncertainty of the measured quantum yield values was approximately 15%, where the uncertainty of the spectral irradiance of a standard lamp and the nonequivalence of the internal/external spectral flux in the integrating system, as well as the reproducibility of the quantum yield measurements, are the major sources of uncertainty. Although it is well known that the intensity of firefly bioluminescence depends significantly on the enzyme and/or reaction conditions, our results suggest that the quantum yield is not the main determining factor. Detailed biochemical analysis of a wider variety of beetle luciferases based on their quantum yields will be reported elsewhere. In addition, the value of the quantum yield of 0.48 for *P. pyralis* luciferase is consistent with the previously reported value 0.41 ± 0.074,[1] even though their measurement procedure was completely different from ours.

In conclusion, we have successfully measured quantum yields of firefly bioluminescence reactions based on a novel method using a commercially available luminometer. Because of the high sensitivity of luminometers, the proposed method allowed us to reduce the amount of substrate D-luciferin molecules used for the data collection than the previously reported method,[1] which resulted in reducing data collection period. High sensitivity of luminometers also enables the proposed method to be applied for other dim glow luminescence reactions. This method will contribute to the scientific understanding of the mechanism of bio/chemiluminescence, and can be applied to the development of novel luminescent reagents.

This work was partially supported by a Grant-in-Aid for Scientific Research (C) (No. 21510238), Japan Society for the Promotion of Science.

References and Notes

1 Y. Ando, K. Niwa, N. Yamada, T. Enomoto, T. Irie, H. Kubota, Y. Ohmiya, H. Akiyama, *Nat. Photonics* **2008**, *2*, 44.
2 H. H. Seliger, W. D. McElroy, *Biochem. Biophys. Res. Commun.* **1959**, *1*, 21.
3 The luminescence color of firefly bioluminescence reactions depends on the enzymes and/or reaction conditions, and has received much attention from many researchers, e.g.: a) K. V. Wood, Y. A. Lam, H. H. Seliger, W. D. McElroy, *Science* **1989**, *244*, 700. b) B. R. Branchini, D. M. Ablamsky, J. M. Rosenman, L. Uzasci, T. L. Southworth, M. Zimmer, *Biochemistry* **2007**, *46*, 13847. c) K. V. Wood, Y. A. Lam, W. D. McElroy, *J. Biolumin. Chemilumin.* **1989**, *4*, 289. d) V. R. Viviani, *Cell. Mol. Life Sci.* **2002**, *59*, 1833.
4 Y. Ando, K. Niwa, N. Yamada, T. Irie, T. Enomoto, H. Kubota, Y. Ohmiya, H. Akiyama, *Photochem. Photobiol.* **2007**, *83*, 1205.
5 R. Daniel, R. Almog, Y. Sverdlov, S. Yagurkroll, S. Belkin, Y. Shacham-Diamand, *Appl. Opt.* **2009**, *48*, 3216.
6 D. J. O'Kane, J. Lee, *Methods Enzymol.* **2000**, *305*, 87.
7 Although a scintillation light source Glowell™ standards (LUX Biotechnology, Edinburgh, U.K.) is commercially available, it is only calibrated for directional radiant flux (pW), and is not suitable for our purpose.
8 a) Y. Ohno, *Metrologia* **1998**, *35*, 473. b) Y. Zong, C. C. Miller, K. R. Lykke, Y. Ohno, Proceedings of the CIE Symposium '04 on LED Light Sources, **2004**, p. 107.
9 K. V. Wood, *Photochem. Photobiol.* **1995**, *62*, 662.

