# Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission

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**Firefly bioluminescence[1–19] is the most well-known ideal photo-emitter system in biophotonics, known in particular for its extremely high quantum yield, 88 ± 25% (refs 2,3) or higher[4–6], and its magnificent pH-dependent emission-colour change[3,7] between yellow-green and red, modelled as the chemical equilibrium between two corresponding states[8–14]. However, the need for re-examination has also been discussed[4–6]. In this letter we quantify quantum yields and colour changes using our new total-photon-flux spectrometer[20,21]. We determine the highest quantum yield to be 41.0 ± 7.4% (1 standard deviation (s.d.) estimate, coverage factor $k = 1$), and find that bioluminescence spectra are systematically decomposed into one pH-sensitive and two pH-insensitive gaussian components. There is no intensity conversion between yellow-green and red emissions through pH equilibrium, but simple intensity variation of the pH-sensitive gaussian peak at 2.2 eV causes the changes in emission colours. This represents a paradigm shift in the concept of colour determination from long-standing interpretation based on pH equilibrium.**

Firefly luciferin is a substrate for bioluminescence reaction[1]. Its oxidation in the presence of luciferase, adenosine triphosphate (ATP) and $Mg^{2+}$ results in the formation of oxyluciferin in the spin-singlet excited state. When it relaxes to the ground state, luminescence occurs. Green and McElroy reported the purification and crystallization of firefly luciferase in 1956 (ref. 16) and Bitler and McElroy purified firefly luciferin in 1957, obtaining 9 mg from 15,000 fireflies[17]. In 1959, Seliger and McElroy[2,3] measured the quantum yield for firefly bioluminescence, which is defined as the probability of photon emission per luciferin molecule reacted, using firefly (*Photinus pyralis*) luciferase. They reported that the quantum yield at pH 7.6 was 88 ± 25%, and as the pH decreased, the luminescence changed from a high-intensity yellow-green emission peaking at 562 nm to a low-intensity red emission peaking at 616 nm (ref. 7).

However, the same group commented in 1961 that the quantum-yield value of 88% should be re-examined[4]. This was because it was later found that luciferin has optical isomers and only the D-form contributes to bioluminescence. Also, the purity of D-luciferin used in the quantum-yield measurement was probably degraded by racemization[4]. Moreover, in 1963, some members of the group characterized luciferin purified in the same way as in the original work[2,3,17] and reported that it contained only 50% D-luciferin owing to complete racemization[5,6]. This suggested that the 88% value should be recalculated to the unrealistic value of 176%, and that the measurement was possibly incorrect. In spite of these problems, the 88% value became so entrenched that no re-examination or confirmation was ever made. A new quantum-yield determination with pure D-luciferin was long overdue.

The recent rise in biotechnology applications[18,19] of various bioluminescence systems indicated the need for quantitative evaluation[22] of luminescence efficiencies. Therefore, we developed a total-photon-flux spectrometer for bio/chemiluminescence[20,21], which directly measures quantitative luminescence spectra scaled by the absolute integrated number of photons in the bio/chemiluminescence total flux, and hence can determine quantum yields. Before its use in bioluminescence experiments, the accuracy of absolute sensitivity was ensured by quantum-yield measurements of an aqueous luminol chemiluminescence standard, for which we obtained 1.23 ± 0.20% (ref. 21), in good agreement with the 1.24% (ref. 23) supported by several independent groups (listed in ref. 24).

Using this spectrometer, we quantitatively measured firefly (*Photinus pyralis*) bioluminescence at various pH values. To ensure that all the luciferin molecules were used in the reaction, all other reagents were present in excess amounts. In fact, we carried out high-performance liquid chromatography (HPLC) measurements on the solutions before and after the reaction, at pH 6, 7 and 8, to confirm that more than 99% of the D-luciferin molecules were consumed by the reaction.

Time-integrated quantitative bioluminescence spectra of all emitted photons from $2.98 \times 10^{11}$ molecules of luciferin at various pH values are shown in Fig. 1a. Note that the vertical axis is scaled by the absolute number of photons in units of $eV^{-1}$. The series of quantitative spectra reveals that luminescence intensity in the green region changes significantly with pH but

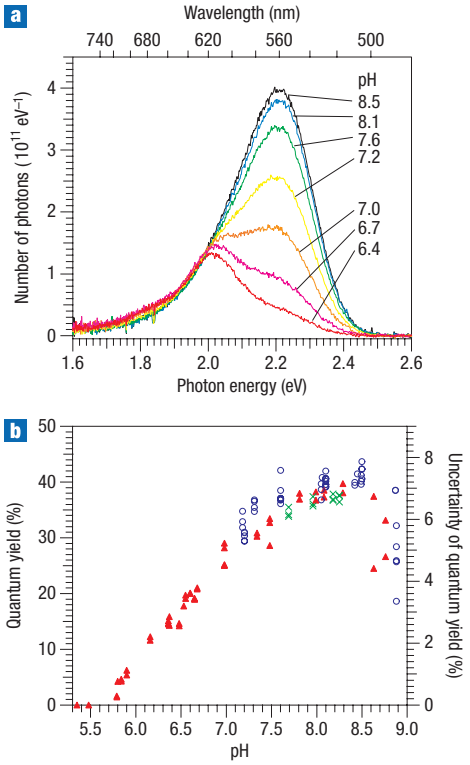


**Figure 1 Quantitative measurements of firefly bioluminescence.** **a**, Quantitative luminescence spectra for $2.98 \times 10^{11}$ luciferin molecules at various pH values: black, pH 8.5; blue, pH 8.1; green, pH 7.6; yellow, pH 7.2; orange, pH 7.0; pink, pH 6.7; red, pH 6.4. The vertical axis is scaled by the absolute number of emitted photons in units of eV$^{-1}$. **b**, Quantum yields at various pH values with Tris (blue open circles), GTA (red filled triangles, 3,3-dimethylglutaric acid, Tris, 2-amino-2-methyl-1,3-propanediol) and glycylglycine (green crosses) buffer solutions at 23.5–26.5 °C. The right vertical axis shows uncertainty in the quantum yields resulting from ±18% uncertainty ($k = 1$) in the calibration.

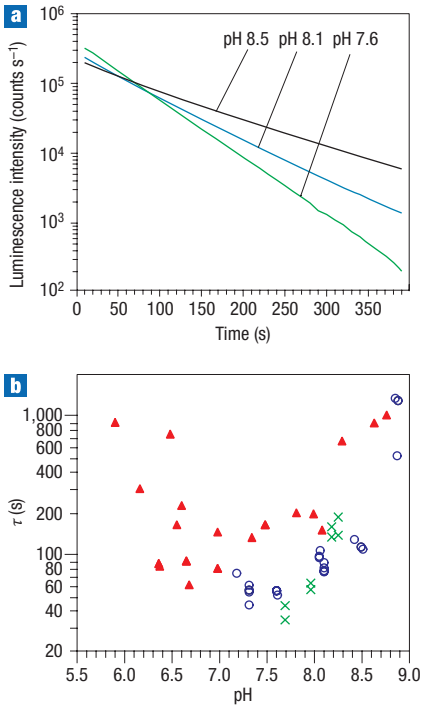


**Figure 2 Luminescence decay of firefly bioluminescence.** **a**, Luminescence decay plots at pH 7.6 (green line), pH 8.1 (blue line) and pH 8.5 (black line) with Tris buffers. **b**, Luminescence decay times at various pH values with Tris (blue open circles), GTA (red filled triangles) and glycylglycine (green crosses) buffer solutions.

that it does not in the red region. A quantitative analysis of this change in spectral shape is given later in this paper.

The integrated areas of the spectra in Fig. 1a divided by the number of luciferin molecules give the quantum yields. Measured quantum yields at various pH values with Tris, GTA (3,3-dimethylglutaric acid, Tris, 2-amino-2-methyl-1,3-propanediol) and glycylglycine buffer solutions are shown in Fig. 1b. They showed maximum values at pH 8.0–8.5, and the average value and its statistical fluctuation at pH 8.5 with the Tris buffer was 41.0 ± 1.1%. The quantum yield decreased with decreasing pH, and luminescence was quenched at around pH 5.6. Although we measured quantum yields with different buffers—Tris, GTA and glycylglycine—the differences were small. Each set of quantum-yield data contained ±18% uncertainty (1 s.d. estimate, or coverage factor $k = 1$) in the calibrations of the spectrometer system[22], which is indicated on the right axis in Fig. 1b. The ±18% calibration uncertainty in the 41% value is ±7.4%. This uncertainty is seven times larger than the statistical data fluctuations in Fig. 1b under pH 8.5. Therefore, the quantum yield at pH 8.5 with the Tris buffer was concluded to be 41.0 ± 7.4% taking the $k = 1$ calibration uncertainty into account. At pH 8.6–8.9, the data had large fluctuations, probably because luciferase was not functional in this pH range, but the spectral shape was the same as that in the pH range 8.0–8.5. Below pH 8.5, the quantum yield was not sensitive to reaction conditions except for pH. In fact, we increased and decreased the concentrations of luciferase, ATP and $MgSO_4$ by one order of magnitude and tried various combinations, but quantum yields for the same pH were similar.

In contrast, the reaction time, or luminescence decay time, changed sensitively with concentrations of buffers and pH. The luminescence decay for Tris buffers at pH values of 7.6, 8.1 and 8.5 is plotted in Fig. 2a. The plots show exponential decay. As the concentration of luciferase was much higher than that of luciferin, usual bioluminescence decay, so called flash-glow, was not observed. Luminescence decay times $\tau$ were derived from the slopes of the decay plots and are shown in Fig. 2b as well as those under other conditions. Note that the decay time $\tau$ varied with pH 7.6–8.5 and was different for Tris, GTA and glycylglycine buffers, in stark contrast to the almost constant quantum yield for pH 7.6–8.5 shown in Fig. 1b. The difference in the sensitivity to concentration, buffers and pH proves that the mechanisms determining the reaction time and quantum yield are independent.

We now focus on the change in spectral shape dependent on pH. To quantitatively analyse the spectra, we tried gaussian curve fitting and found that three components explained the whole spectral change very well. We assumed three gaussian components peaking at around 2.2 eV (560 nm), 2.0 eV (620 nm) and 1.85 eV (670 nm) to reproduce two different peaks at high and low pH values as well as the tail observed in the low-energy region in Fig. 1a. The gaussian fitting spectra are plotted in Fig. 3a, which indicates good agreement with the spectra in Fig. 1a. The three gaussian peak components are shown separately in Fig. 3b. All three gaussian components similarly showed small shifts

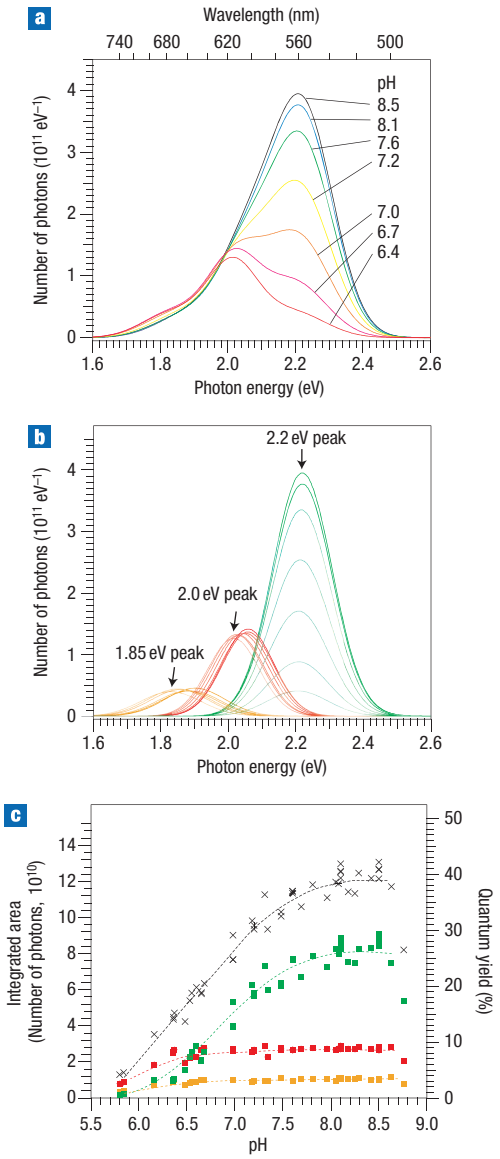


**Figure 3 Gaussian fits of firefly bioluminescence. a**, Gaussian fitting spectra that reproduce the spectra shown in Fig. 1a: black, pH 8.5; blue, pH 8.1; green, pH 7.6; yellow, pH 7.2; orange, pH 7.0; pink, pH 6.7; red, pH 6.4. **b**, Three gaussian components peaking around 2.2 eV (green), 2.0 eV (red) and 1.85 eV (orange). **c**, Absolute integrated areas of the gaussian components shown in **b** at various pH values: black crosses, total integrated area; green filled squares, 2.2-eV peak; red filled squares, 2.0-eV peak; orange filled squares,1.85-eV peak. The right vertical axis indicates quantum yields, which are the integrated areas divided by the total number of luciferin molecules.

in peak energies and very small changes in peak widths with pH. As for peak intensities, however, that of the 2.2-eV peak component was strongly pH dependent, but those of the 2.0-eV and 1.85-eV peak components were not. This feature is shown more quantitatively in Fig. 3c, where absolute integrated areas of the respective components and their total in units of the number of photons are plotted as functions of pH values. The plots show that the integrated area of the 2.2-eV peak component was strongly pH dependent below pH 8.0, but those of the 2.0-eV and 1.85-eV peak components were flat against pH variations for pH 6.5–8.5. The right vertical axis of Fig. 3c indicates quantum yields, which are the integrated areas divided by the total number of luciferin molecules. The changes in the total quantum yield and spectral shape with pH were mostly determined by the pH-dependent intensity change of the single 2.2-eV peak gaussian component.

Most colour determination models for firefly bioluminescence have assumed that the yellow-green emission at high pH and the red emission at low pH correspond to two alternative states of an excited oxyluciferin molecule in luciferase and that pH equilibriums between the two states determine bioluminescence colours. Proposed microscopic models for the two states are the keto and enol forms of oxyluciferin[8], the rotation of the thiazolinone fragment about the C2–C2′ single bond of oxyluciferin[9], states of oxyluciferin in the microenvironment of the luciferase–oxyluciferin complex[10–13] and the structural basis of luciferase[14]. We emphasize, however, that the observed intensities of the 2.0-eV and 1.85-eV peak components were rather insensitive to pH. The red emission components were present even in alkaline conditions and their intensities are almost the same as in neutral or acidic conditions. These results hardly seem to be accounted for by chemical equilibrium with pH between the yellow-green and red emission states, as one must decrease when the other increases according to the law of mass action. (See Supplementary Information for more details on the gaussian fits and colour change[8–15].)

In conclusion, the highest quantum yield of firefly (*Photinus pyralis*) bioluminescence is $41.0 \pm 7.4\%$ ($k = 1$) at pH 8.5. Although this value is still higher than those of other bioluminescence, such as 28% for cypridina[25], 17% for aequorin[26] and 30% for bacteria[27], it is not an extraordinary exception having an ideal quantum yield close to unity. To further confirm this conclusion, an additional experiment comparing differently prepared luciferase was performed (see Supplementary Information). The intensity change of the pH-sensitive single 2.2-eV-peak gaussian component mostly determines the pH dependence of quantum yield and colour change. It will be important to carry out similar quantitative measurements and analyses for other luciferin–luciferase reaction systems and investigate quantitative differences with a view to establishing a universal understanding of bioluminescence.

## METHODS

### MATERIALS

A $1 \times 10^{-2}$ M aqueous D-luciferin stock solution diluted into MilliQ water (Milli-Q SP, Millipore) was prepared from D-luciferin sodium salt (HPLC assay 99% purity, 128-03954, Wako, Osaka). Accurate concentration was characterized by absorbance with $\log \varepsilon_{327\mathrm{nm}} = 4.27$ (ref. 4). Using HPLC with a chiral column (Chiralcel OD-RH, Daicel Chemistry, Tokyo), we confirmed that the D-form purity of luciferin in the stock solution was 99%, and that racemization in this solution when kept for about 10 days at $-20\,^{\circ}$C, during measurements, was negligible. We also confirmed that the racemization rates of D-luciferin in diluted solutions of $1 \times 10^{-5}$ M, $1 \times 10^{-6}$ M and $1 \times 10^{-7}$ M with GTA buffers were 0.03% at pH 6 and 0.2% at pH 8 per hour at room temperature. Therefore, the luciferin solution was diluted to $1 \times 10^{-7}$ M with Tris, GTA and glycylglycine buffers every day during the experiments. The uncertainty in determining the number of luciferin molecules was estimated to be $\pm 2\%$.

Natural *Photinus pyralis* luciferase (L9506, crystallized chromatographic-grade, Sigma) was purchased and used. Luciferase solution (1 mg ml$^{-1}$) in a buffer containing 10% glycerol was prepared, and was consumed within 2 weeks without refreezing after the initial thawing. The concentration was estimated to be $1.7 \times 10^{-5}$ M based on a luciferase molecular weight of 60 kDa. The luciferase solution was diluted with a 0.1 M Tris buffer (pH 8.0) for all measurements.

An aqueous solution of ATP (adenosin-5′-triphosphoric acid disodium salt, Oriental) was prepared and neutralized with NaOH or buffers with a high pH to adjust the pH and then diluted to $1 \times 10^{-3}$ M by buffers with adjusted pH.

$MgSO_4$ (Wako) solution was diluted to 0.1 M with the buffers. A Tris–HCl buffer (0.1 M, 2-amino-2-hydroxymethyl-1,3-propanediol, Wako), and GTA–HCl and –NaOH buffers[28] (0.1 M, 3,3-dimethylglutaric acid, Wako; 0.1 M, Tris; 0.1 M, 2-amino-2-methyl-1,3-propanediol, Sigma) were used. To reassess the data reported by Seliger and McElroy[2,3], glycylglycine–NaOH ($2.5 \times 10^{-2}$ M, Wako) buffer was also used.

### MEASUREMENTS

We first premixed 5 μl of $1.0 \times 10^{-7}$ M luciferin, 5 μl of $1.7 \times 10^{-5}$ M (1 mg $ml^{-1}$) luciferase, 5 μl of 0.1 M $MgSO_4$ solutions, and 35 μl of Tris, GTA or glycylglycine buffer, and then added 50 μl of $1.0 \times 10^{-3}$ M ATP solution to initiate the reaction. Luminescence from the solutions was measured with our total-photon-flux spectrometer[20,21]. Luminescence measurement was continued by repeating data acquisition for 10–30 s until the reaction had completed. All the curves in Fig. 1a showed time-integrated spectra. All measurements were performed at room temperature, 23.5–26.5 °C. Details of the optical configurations and absolute intensity calibration of the total-photon-flux spectrometer are given in refs 20 and 21. The total uncertainty in the calibration accuracy was estimated to be ±18% (ref. 20; $k = 1$). The validity of our calibration was confirmed by measuring the aqueous luminol chemiluminescence standard, which showed a quantum yield of 1.23 ± 0.20% (ref. 21), in good agreement with the 1.24% of Lee and Seliger[23], which is supported by several independent groups (listed in ref. 24).

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## Author contributions

Y.A., Y.O. and H.A. conceived and designed the experiments. Y.A. and K.N. performed the experiments. Y.A., K.N., Y.O. and H.A. analysed the data. Y.A., K.N., N.Y., T.E., and H.K. contributed materials and analysis tools. Y.A. and H.A. produced the figures and wrote the paper.