



Manifestation of Peaks in a Live Firefly Flash

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Received: 28 November 2018 / Accepted: 10 March 2019 / Published online: 18 March 2019
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Abstract

Several studies have been carried out on flashing of fireflies till now. From patterns of its flashes in different ambient conditions, different conclusions have been drawn and hypotheses put forward. Here we observe flashes emitted by two Indian species of firefly *Luciola praeusta* and *Asymmetricata circumdata*. For the species *L. praeusta*, as the temperature is lowered below about 21 °C, simple pulses become compound or combination ones. Males begin to emit bi-modal flashes while females emit both bi- and tri-modal flashes, along with the simple ones, in a regular manner. For the species *A. circumdata*, this feature is evident even at their normal flashing temperatures. Though rare, three-peaked flashes do appear from male specimens of both these species, and decay times of all the three peaks in a male- or a female-flash come out to be a few tens of, or a hundred-odd, milliseconds — in contrast to the nanosecond lifetimes determined by different workers for different analogs of the light emitter molecule oxyluciferin. We propose that the tri-modal feature in a flash represents three luminescent forms of the excited state of oxyluciferin, which in the normal flashing state in the living firefly decays via a pathway followed by molecules exhibiting phosphorescence.

Keywords Firefly bioluminescence · Three-peaked flash · Oxyluciferin forms · Lifetime · Phosphorescence

Introduction

The enchanting light of the firefly has been fascinating human beings for millennia. This light is the outcome of a very efficient chemiluminescent reaction. In this reaction, molecular oxygen (biochemical trigger) excites the luciferin molecule (substrate) in presence of the enzyme luciferase (catalyst) and Mg-ATP, generating an unstable dioxetanone intermediate that decomposes to form an electronically excited oxyluciferin (OxyLH₂) species [1]. While decaying to its

ground state, OxyLH₂ emits a photon in the visible part of the electromagnetic spectrum.

It is well known that OxyLH₂ can exist in six different forms: phenol-enol-OxyLH₂, phenolate-enol-OxyLH⁻, phenol-enolate-OxyLH⁻, phenol-keto-OxyLH₂, phenolate-keto-OxyLH⁻ and OxyL²⁻, and out of these the phenolate-keto species was historically considered the most viable form for the emitting state [2–4]. However, a few investigations showed that the enol tautomer should also be included as emitting species produced in the excited state [5–7]. While agreeing with the suggested red emission from the phenolate-enolate or phenolate-keto forms, the phenol-enolate form of oxyluciferin was identified as a yellow-emitting species [8]. The OxyLH₂ molecule, being a chemically unstable product, is difficult to study separately as well as with precision. Hence spectroscopic experiments of more suitable analogues were carried out as its relevant models [9–14]. In those works, fluorescence spectra of oxyluciferin and their pH dependence were investigated. It was reported that emission properties of this molecule were completely immune to external magnetic field of up to 53 T [15].

In recent times, time-resolved measurements were carried out on the light of the Indian species of firefly *Luciola praeusta* at varying temperatures [16–19]. It was observed that the pulse-width increased with decrease in temperature,

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10895-019-02364-6>) contains supplementary material, which is available to authorized users.

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which indicated that the speed of the enzyme-catalysed chemiluminescence reaction decreased in a certain manner with lowering of the temperature. Furthermore, low temperatures were found to enhance the broadening of pulses in an irregular manner, resulting in the ‘burning of a hole’ in the extended temporal profile. For another Indian species of firefly *Asymmetricata circumdata*, male flashes appeared as bimodal while the female ones appeared as tri-modal in the normal range of flashing temperature itself [20, 21].

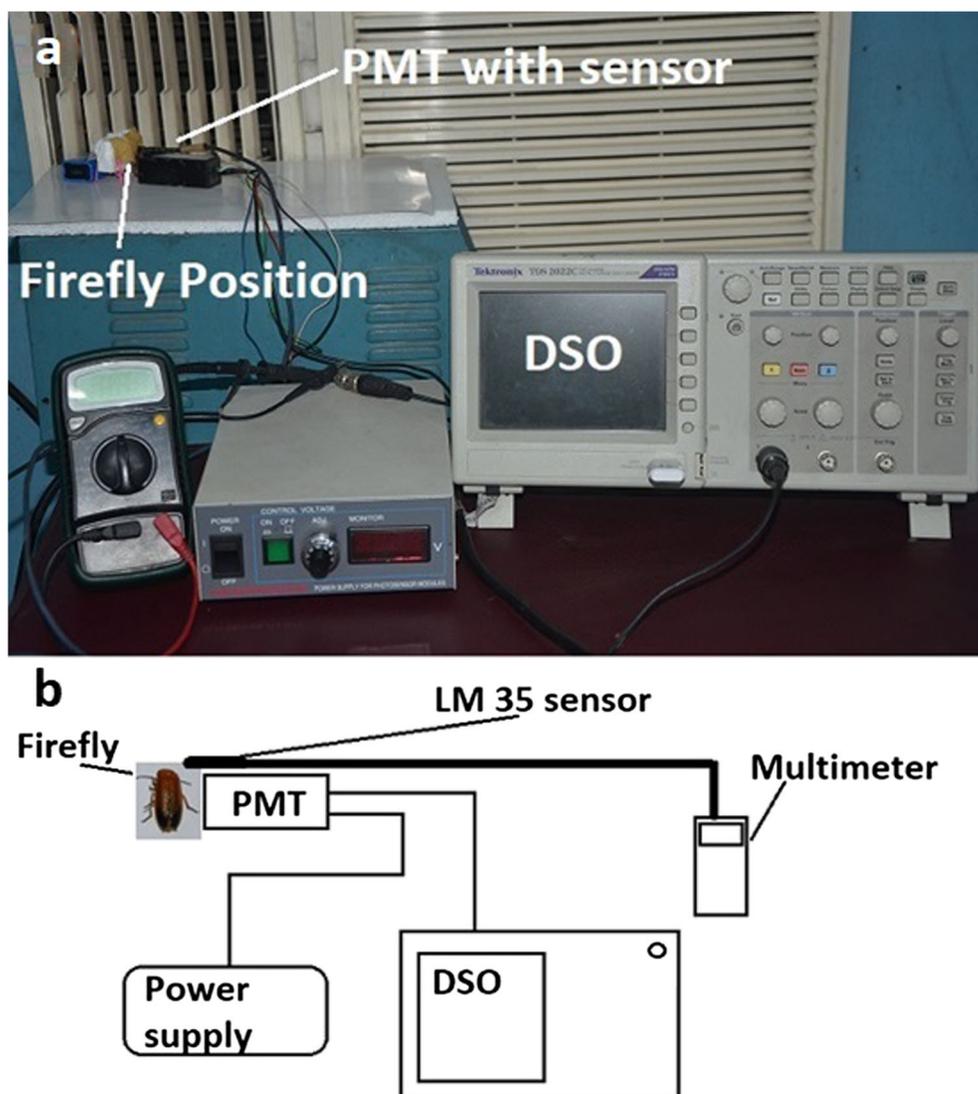
It is well known that the phenomenon of bioluminescence is greatly influenced by the properties and microenvironment of the emitting species in the active site of the enzyme [22–24]. Although a number of studies have been carried out in the recent past on the emission properties of different chemical forms of the oxyluciferin under various laboratory conditions, scant attempt has been made to carry out in vivo studies on firefly bioluminescence. Hence, it is a necessity to carry out such experiments to understand in a greater detail

how the emission characteristics of oxyluciferin are influenced by protein microenvironments of living organisms. In this article, we report our results of in vivo experiments on living fireflies to correlate the more than one-peaked flashes with different chemical forms of the emitter oxyluciferin, which decay in times of the order of milliseconds.

Materials and Methods

The experimental arrangement for recording in vivo flashes of the firefly is shown in Fig. 1. Details of this arrangement were given elsewhere [19]; briefly these were as follows. Just after the sunset, a few male and female specimens of the species *L. praeusta* were caught in the campus of Gauhati University. One of those was fixed in a piece of sponge with sellotape, and its light emitting organ was positioned in front of a photomultiplier tube (Hamamatsu H10722–20 with power

Fig. 1 Experimental arrangement for recording flashes from live fireflies. **a** Photograph of the set up. The photomultiplier tube was placed near the window of the airconditioner. The immobile firefly was positioned in front of it, and the LM35 temperature sensor was kept close to it. The temperature in the locality of the firefly could be read off in the multimeter. Depending on the intensity of flashes, the control voltage of the PMT could be adjusted. Flashes appeared in the digital storage oscilloscope, and the values of the waveforms could be saved in a pen drive. **b** Schematic diagram of the set up



supply C10709). The PMT converted the optical signal to an electrical one, and a digital storage oscilloscope (Tektronix TDS 2022C) was used to record the waveforms. To have good pulse intensity in the DSO (digital storage oscilloscope), the controlling voltage applied in the PMT was varied from 0.25 to 0.35 V. The values of the waveforms were saved in an external drive SONY_4GR in .csv file extensions, and were analysed in Origin 8.0. For recording steady-state spectra, the lantern of the immobile specimen was directed towards the entrance face of the fiber of an HR4000 spectrometer (Ocean Optics). For the experiment in the control state, a piece of cottonwool dipped in ethyl acetate was brought near to the firefly. The flashing was initially rapid; it slowed down gradually and, after 5–6 min, an apparently continuous glow appeared. Emission spectra of this continuous light were recorded in the high-resolution spectrometer by placing the face of the fiber close to the lantern. Because of the low intensity, the integration time in the spectrometer was set at 2 s. Low temperatures in the laboratory were realised with the help of an air conditioner. A curved reflector was used to focus the air from the air conditioner to a small region around the location of the firefly in order to make the temperature very low. For noting down the temperature, a digital thermometer, made by using an IC LM35 sensor of accuracy 0.5 °C and connected to a digital multimeter (MASTECH MAS 830 L), was placed very close to the insect. A photograph of the ongoing experiment, which shows the firefly lantern facing the window of the PMT, is presented as Supplementary Information, Fig. S1. 50 male and 30 female specimens of *L. praeusta* were used for recording steady state and time-resolved spectra. Specimens of *A. circumdata* were caught at Khatkhati, a place about 60 km south-west of Gauhati University. These fireflies remained alive in captivity for about a day only, and the experiments had to be performed in the same night; otherwise light emissions from them would stop. Because of the difficulty and risk involved in catching them in the jungle and bringing to our laboratory, the experiments were carried out in a house near to their habitat at the natural temperatures of 22–28 °C. 12 each of male and female specimens of this species were used in the experiment.

For the time-resolved photoluminescence study, the lantern of a dead firefly was separated from the rest of the body with the help of a sharp blade. The removed lantern was fixed on the sample holder using a both side adhesive carbon tape, keeping the ventral side of the lanterns upward. The spectra were recorded in a fully integrated fluorescence lifetime spectrometer LifeSpec II (Edinburgh). Edinburgh Photonics EPL-405 pulsed diode laser (repetition rates: 10 MHz) having pulse duration of 90 ps was used to excite fluorescence from the lantern of a dead specimen. Emission slit width was 20 nm. The detector was a Micro Channel Plate-Photomultiplier Tube of response width < 25 ps and the instrument response function was < 130 ps. After re-convolution, the shortest

recoverable lifetime was 1/10 of the instrument response function. The goodness of fit quality parameter (χ^2) for all the recorded spectra was approximately 1. The experiments were carried out at the temperature of 26 °C.

Results and Discussion

Typical simple flashes from a male firefly of the species *L. praeusta* were triangular in shape with duration of approximately 97 ms at 30 °C [17], and those became unusually large and nonlinear as the temperature went down below about 21 °C [18]. This implied slowing down of the reaction in an irregular manner and appearance of two-peaked flashes. For the specimens used in the present experiment, two of which are shown in Fig. 2a, this kind of ‘splitting’ of a male-flash was found to be most pronounced between 16 and 17 °C. However, this was heavily dependent on the specimen, and as such one could give an approximate value only. Three-flash combinations were also observed below 15 °C, as shown in Fig. 2b. In this figure, the first flash is bi-modal with peak-intensities of 1.016 and 0.183 V, while the second one is tri-modal with intensities of 1.011, 0.676 and 0.171 V. Such type of flashes for two more male specimens are presented as Supplementary Information, Fig. S2 (a) and (b). Out of the 50 specimens used in the experiment, flashes from 5 strongly flashing ones remained simple up to 10 °C, and for the other ones 1 to 5 three-peaked flashes only could be observed. This indicated that the rate of the reaction had to be very slow for appearance of three peaks in a flash. Compared to males, flashes emitted by females — two of the specimens being shown in Fig. 2c — were distinctly wider: more than twice as big at the usual temperature of flashing [19]. This meant that the speed of the light-producing reaction was considerably slower in females, and as such manifestation of three flash-combinations, along with single- and two-flash ones, became quite regular below about 24 °C for a freshly caught specimen. Typical three-peaked flashes from an *L. praeusta* female firefly are shown in Fig. 2d. The peak-intensities are 0.156, 0.383 and 0.310 V in the first flash and 0.132, 0.390 and 0.812 V in the second one. In the flashes from both male and female specimens, the end-points are never clear or sharp, and as such determination of area under a curve would require too much of approximation. Two more such type of flashes are given as Supplementary Information, Fig. S2 (c) and (d). Here again, the temperature from which the combination flashes began to appear was difficult to pinpoint, it depended very much on the specimen and the condition or state of the specimen. For specimens caught in the previous night and kept in captivity, three-flash combinations were observed even in the normal flashing temperature range ~ 27–32 °C. It could be said that three-peaked female-flashes became pre-dominant when the temperature went down below 18 °C. It was already

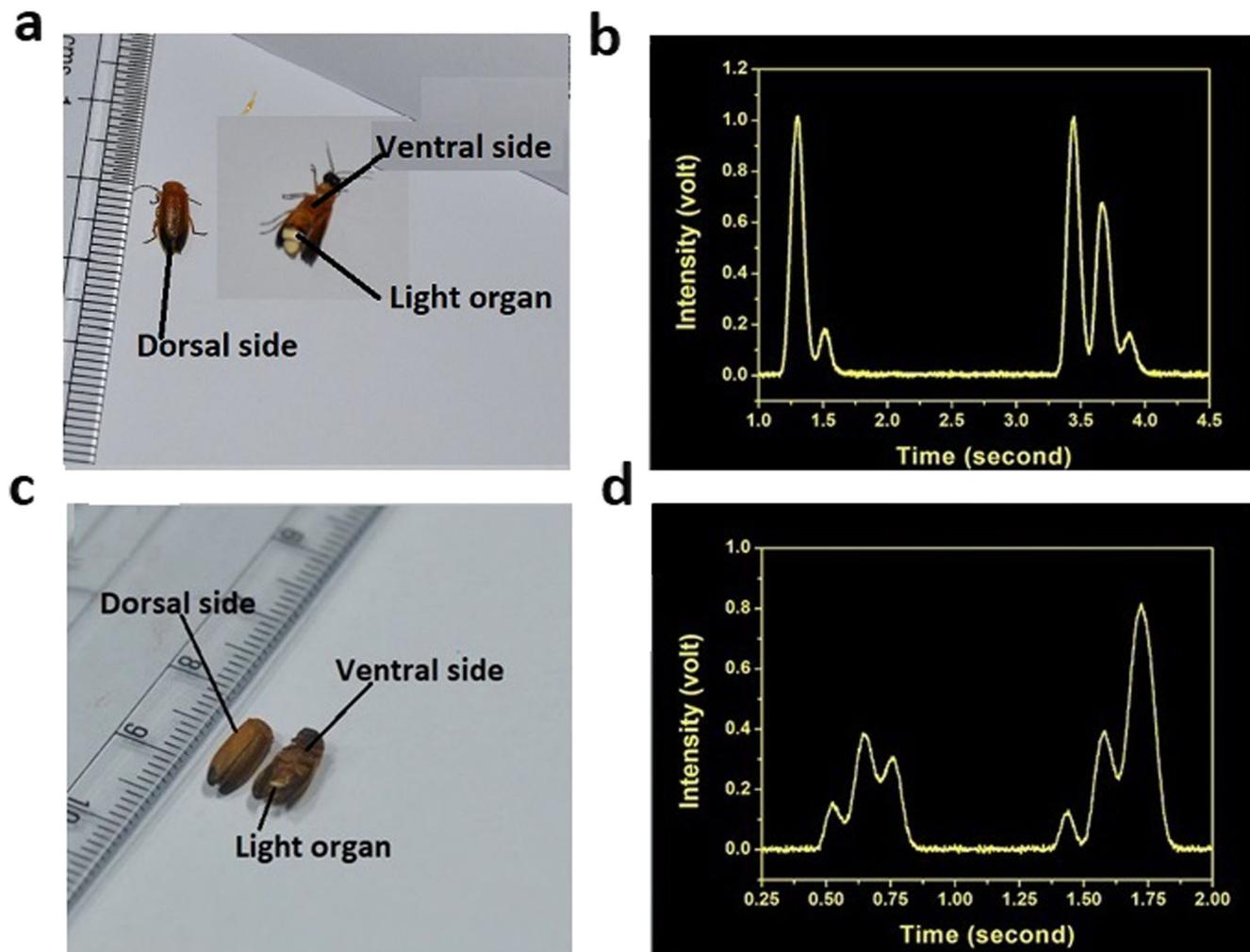


Fig. 2 Firefly species *L. praeusta*, and flashes recorded at temperatures considerably below its normal flashing ones. **a** Photograph of dorsal and ventral sides of two male specimens. The lantern in such a specimen at the lower abdomen consists of two segments. **b** Male-flashes at 13 °C, which

are both bi- and tri-modals. **c** Snap shot of female specimens. The female-lantern consists of only one light-emitting segment. **d** Tri-modal flashes emitted by a female at 17 °C

reported that there was no shift in the peak position for males at low temperatures up to 12 °C [18]. Just as for males [16], for females also the peak position remained at 562 nm up to the temperature of 16 °C (Supplementary Information, Fig. S3) and there was no deviation in this value in the specimens studied. It could be mentioned here that when the temperature became very low for this species, below about 13 °C, widths of the three peaks increased to such an extent that a flash gave the appearance of an envelope, that is, the distinctness of the peaks was lost, resulting in the production of a broad single flash (Supplementary Information, Fig. S4).

For the species *A. circumdata* (Fig. 3a and c), though the male flashes were mostly bi-modal [20], these could, on rare occasions, be tri-modal ones, especially at the present lowest recording temperature of 22 °C. Tri-modal flashes emitted by male specimens of this species of firefly are shown in Fig. 3b. In this figure, the most intense central peaks have the values of 0.678 V in the DSO, while feeble side peaks are of intensities 0.028 V in

the first flash and 0.017 and 0.028 V in the third one. Female flashes, on the other hand, were tri-modal ones at temperatures they usually flash; these are presented in Fig. 3d. In this figure, the most intense flashes have the values 0.5, 0.5 and 0.7 V, and other intensities in the three flashes are of the values 0.2 and 0.4 V (first flash), 0.30 and 0.10 V (second one), and 0.32 and 0.09 V (third one). As in the case of flashes from *L. praeusta*, the end-points of the peak-containing flashes from *A. circumdata* are not clear and hence it is impossible to determine the areas under these curves without making too much of approximation. Three-peaked flashes for two more male and female specimens each are given as Supplementary Information, Fig. S5 (a) and (c), respectively. A close look at the pulse duration brought out the fact that the duration had to be at least 200 ms for the three peaks to manifest. It could be mentioned here that the wavelength peak for a male specimen was 570 nm while that for a female was 565 nm, and there were no variations in these values as found earlier [20, 21].

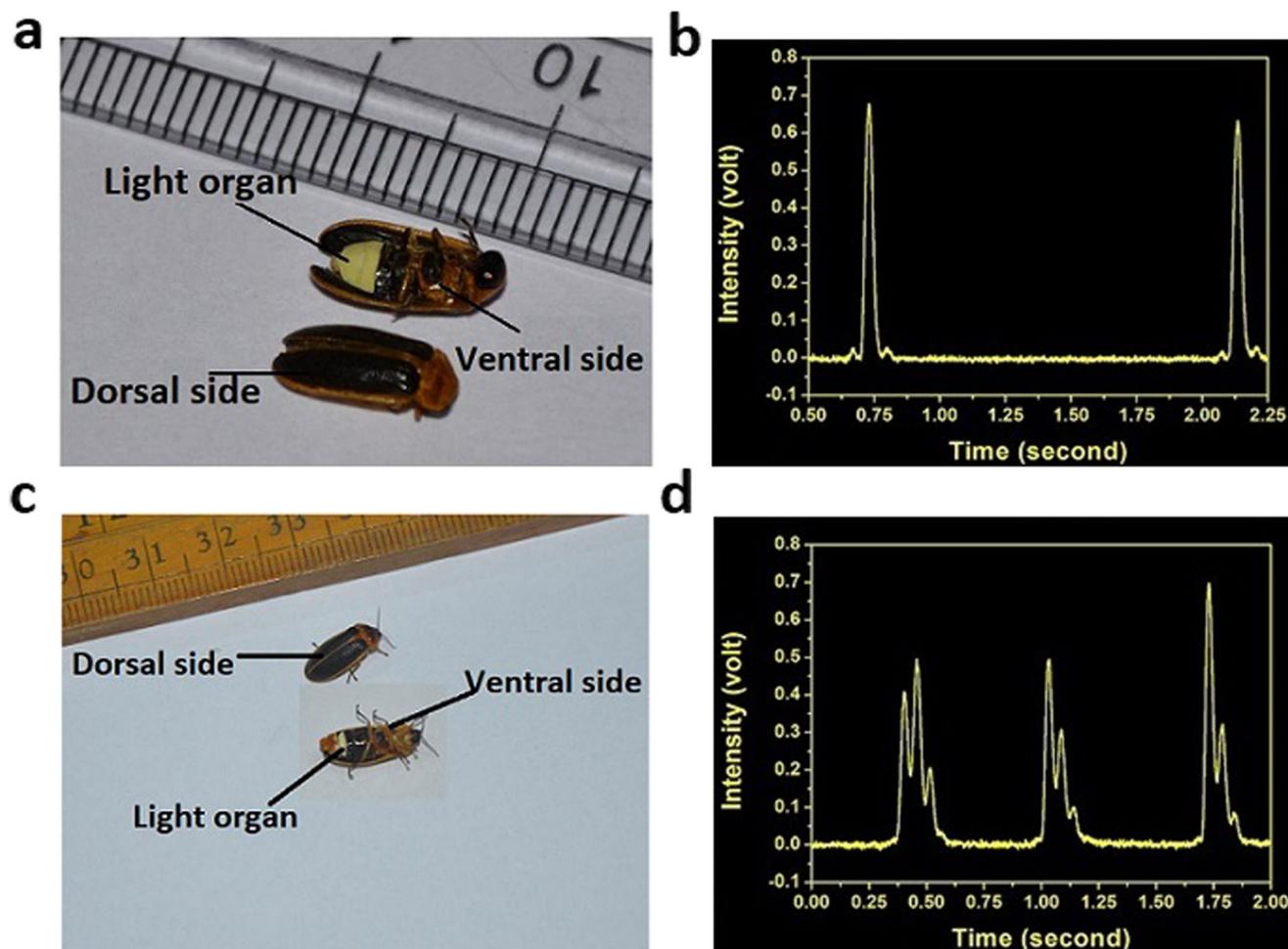


Fig. 3 Firefly species *A. circumdata*, and flashes recorded from it. **a** Photograph of dorsal and ventral sides of male specimens. **b** Male-flashes at the temperature of 22 °C exhibiting a three-peaked shape, which occur rarely. Intensities of the side peaks are observed to be much lower compared to the central one. **c** Image of female specimens.

Just as in the case of the species *L. praeusta*, the female lantern of *A. circumdata* consists of one light emitting segment while the male one contains two segments. **d** Female-flashes at 28 °C: three peaks are quite distinct in a flash even at this temperature around which this firefly-species normally emits

Unlike in the case of a typical luminescence, there is no excitation wavelength in firefly bioluminescence — the light is the production of a chemical reaction.

We propose that the three peaks in an in vivo flash represent the three forms of the excited state OxyLH_2 , as luminescence decays revealing more than one lifetime indicate more than one structural form [25]. We believe that out of the six interconvertible tautomeric forms of OxyLH_2 , three forms exist in dynamic equilibrium within the protein binding pockets in the presently studied species of fireflies. The interconversion from one particular form to the other is very fast at normal or higher flashing temperatures for which a single peak appears. However, the rate of interconversion can be slowed down significantly by lowering the temperature. When the spectral measurements are carried out at low temperatures, all the existing forms get frozen and hence three distinct peaks appear. Intensity of a peak is dependent on the relative stability of that particular structural form. Recently, broadband emission

spectrum from a live firefly was recorded in the form of kinetic and spectral components [13]. Firefly oxyluciferin is a labile substance; the reason for the lability was clarified as due to the presence of both keto and enol forms in protic solution that permitted a Mannich-type dimerization [26].

As the speed of the reaction was much faster in males compared to females, few three peaked-flashes could be obtained from the male specimens, and those too at noticeably lower temperatures. At normal or higher temperatures, the reaction-rate increased and one could observe a single ‘clean’ flash from both male and female specimens of *L. praeusta*. Manifestations of two- and three-peaked flashes in the cases of male and female specimens, respectively, of *A. circumdata* at the usual flashing temperature implied that transitions from one form to another were slow enough in this species; appearance of three peaks in males at temperatures considerably lower than this range indicated the extent to which the reaction needed to slow down.

Photoluminescence decays from dead male specimens of *L. praeusta* and *A. circumdata* at the emission peak of 524 nm (Supplementary information, Fig. S6) are presented in Fig. 4a and b, respectively, for the 405 nm-excitation. The decay times come out as 0.509, 2.22 and 5.994 ns in the first case, and 0.505, 2.302 and 5.621 ns in the second case. Females of these two species also have the same order of three decay times: 0.431, 2.865 and 8.129 ns for *L. praeusta*, and 0.792, 2.675 and 6.226 ns for *A. circumdata*. Photoluminescence decays from the excited-state OxyLH₂ revealed its following three species: phenolate-OxyLH⁻, phenolate-keto-OxyLH⁻, and phenolate-enolate-OxyL²⁻; with a 510 nm excitation at pH 6 and 7, their decay times were 7.68, 0.53 and 3.95 ns, respectively [12]. Luminescence life times in nanoseconds imply fluorescence while those in milliseconds to seconds indicate phosphorescence decays [25], and hence we could easily conclude that externally excited oxyluciferin decays via a fluorescence pathway. In the case of externally excited luminescence decays from the dead specimens also, decay times vary from a fraction

of an ns to a few ns — approximately in the manner of the above set of values in ref. [12] — which substantiate the above statement. Excitations of firefly luciferin at different pH by 100 fs pulses of wavelengths 365 and 400 nm also showed that decay times at the green wavelength of 540 nm and the red wavelength of 650 nm were from a fraction of a ns to a few ns [11]. In living organisms, the mechanism of interactions between oxyluciferin and protein binding sites might be different from the one existing in the case of a dead specimen or in the pure form. Hence, emission properties of the active species will be greatly influenced by the physiological environment and its interaction with the protein [27, 28].

It is worth mentioning here that in vivo time-decay measurements could be carried out for only those few cases where falling parts of all the three peaks in a flash are complete or are down to at least 1/e of their maximum values. Average decay times of the three peaks with their standard deviations in the 1 °C range of temperature are presented in Table 1. We have taken the 1 °C interval because of the difficulty in getting

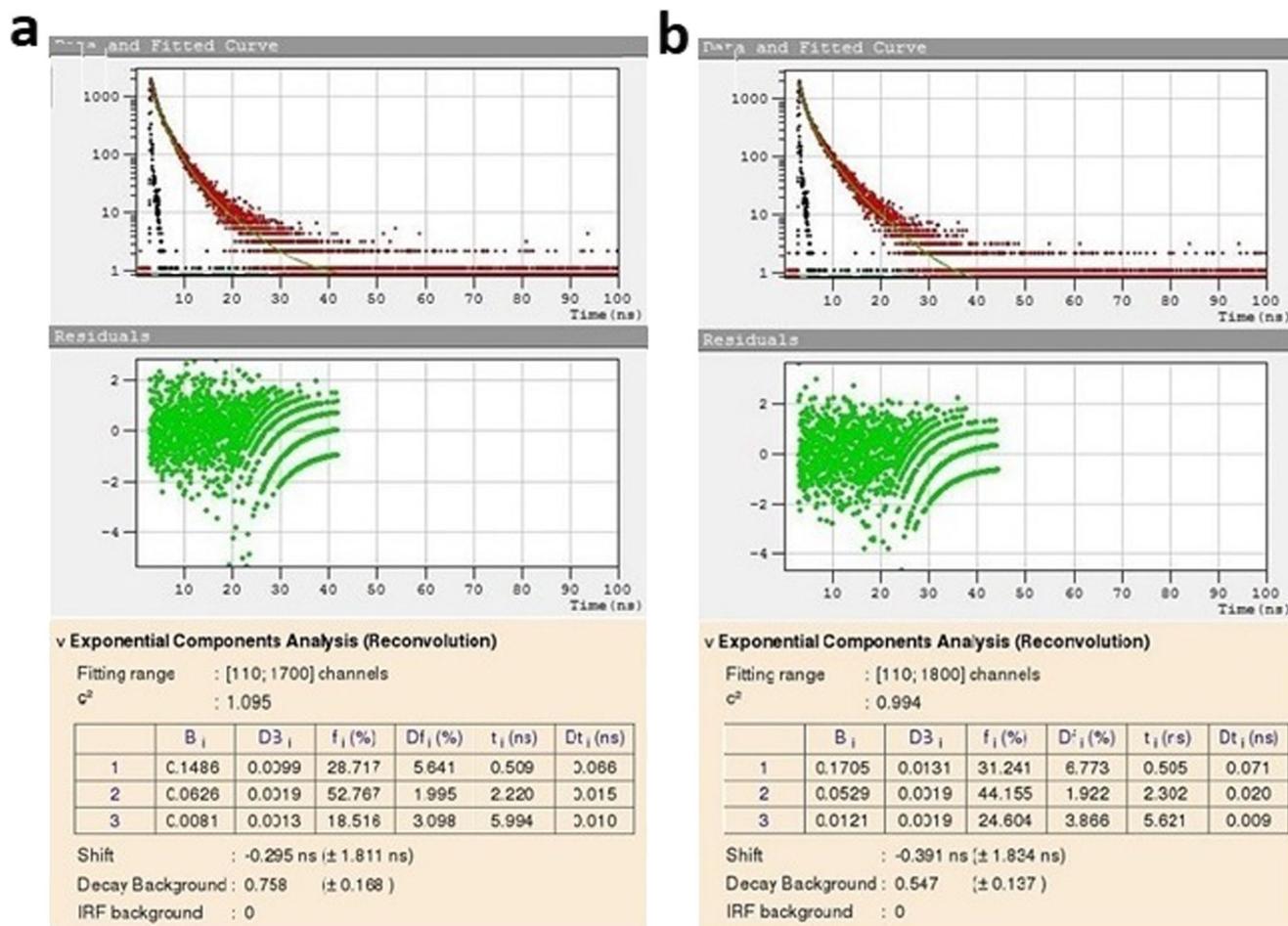


Fig. 4 Excitation of fluorescence from lanterns of dead male fireflies by picosecond-duration pulses from a 405 nm laser source. **a** Decays at the peak wavelength of 524 nm from a specimen of *L. praeusta*. **b** Decays at the same peak wavelength of 524 nm upon excitation of fluorescence

from an *A. circumdata* firefly. It could be noticed that lifetimes for the specimens of both the species are nearly equal: from a fraction of a nanosecond to a few nanoseconds — the necessary requirement for a process of luminescence to be called fluorescence

Table 1 Lifetimes of the peaks in three-peaked flashes of the firefly *L. praeusta* and *A. circumdata*

Species and specimen type	Temperature	Peak 1		Peak 2		Peak 3	
		Mean lifetime (ms)	S.D.	Mean lifetime (ms)	S.D.	Mean lifetime (ms)	S.D.
<i>Luciola praeusta</i> male $N=8, n=14$	14–15 °C	64.4	15.48	79	22.5	74.4	22.76
<i>Luciola praeusta</i> female $N=7, n=15$	16–17 °C	75.2	14.66	105.73	18.24	122.4	25.96
<i>Asymmetricata circumdata</i> male $N=4, n=20$	22–23 °C	13	2.5	22	1.414	15	1.22
<i>Asymmetricata circumdata</i> female $N=5, n=25$	27–28 °C	25	1.92	23	2.25	20	1.37

N = number of specimens, n = number of pulses, S.D. = standard deviation

sufficient number of suitable flashes at a particular temperature. For the female specimens of *A. circumdata*, the lifetimes do not fluctuate much; the stability is probably due to the fact that the three peaks manifest at their normal flashing temperatures. For the males of this species, the standard deviation for the strong central peak is even smaller than that of the females, while the same for the other two are a bit larger probably due to their very low intensities. In the cases of the specimens of *L. praeusta*, standard deviations are quite high and as such their determinations could even be considered as pointless. It is evident that in vivo decay times of all the three peaked-flashes are a few tens of ms, with two being just over a hundred ms. The biochemiluminescence reaction was described by many as the biochemically (O_2) triggered oxyluciferin decaying via a fluorescence pathway, which was consolidated by pulsed excitations and consequent emissions with nanosecond decay times from oxyluciferin analogues. Based on the present evidence, however, one cannot say straightway that the excited state oxyluciferin is a singlet in firefly bioluminescence in vivo. It has been concluded that phosphorescence can be induced by a chemical reaction (chemiluminescence); though chemiluminescence can often be released in the form of spin-allowed

emission, it is in some cases also triggered by the triplet-singlet transition in the reaction products [29, 30]. As no chemical reaction takes place in typical luminescence — excitation is done externally — we could infer that the chemiluminescence reaction taking place in the lantern of the firefly have possibly made the situation favorable for intersystem crossing in the excited state. However, it should be mentioned here that LH_2 in ethanol was found to show phosphorescence at 77 K, and the triplet state, with a lifetime of 28 ms, was assigned as $^3(\pi, \pi^*)$ [9]; the extremely low temperature probably facilitated this singlet-triplet transition. Flash durations for a few other species of firefly were reported to vary from about 70 ms [31] to a few hundred ms [32–34] up to a couple of seconds [35]. Hence should peaks be distinguished in a flash from these or other species of fireflies by lowering the temperature considerably below normal, their decay times will have come out to be of this order. Emission spectra of the two presently studied firefly-species recorded on color films revealed three colored sectors: broad green and red bands with a narrow yellow one in between [20, 36]; we hypothesize that the three peaks in a flash represent the three luminescent forms of the emitter $OxyLH_2$, which are responsible for giving rise to these three color-sectors.

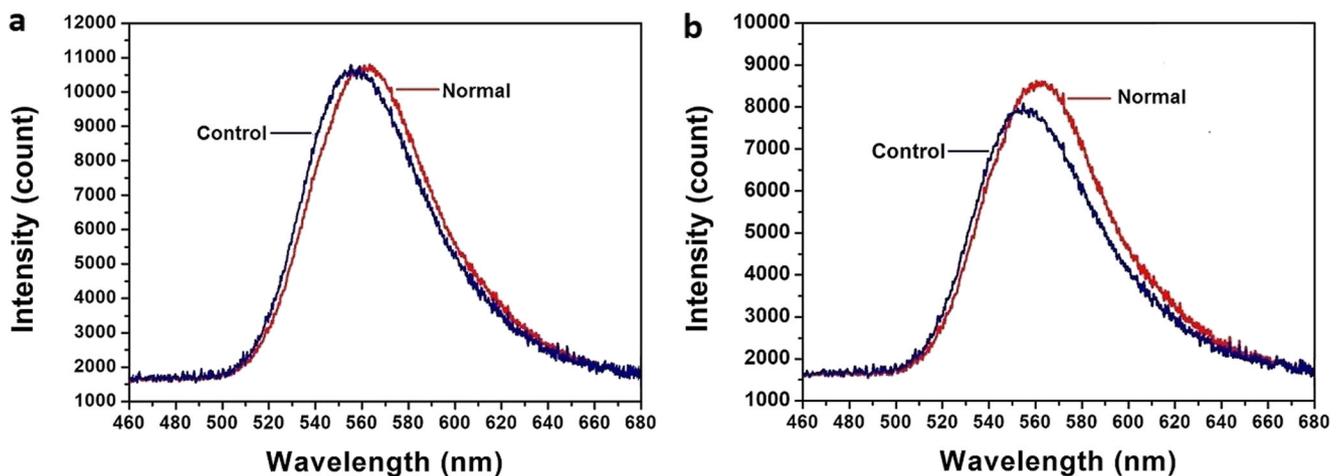


Fig. 5 Emission spectra of *Luciola praeusta* male fireflies in normal (flashing) and control (continuous glow) states. **a** For a specimen, the wavelength peak of 562 nm in the normal state becomes 555 nm in the control state. Thus, there is a shift of 7 nm towards blue when the firefly

was anaesthetized by making it inhale vapors of ethyl acetate. **b** For another specimen, the values of the wavelength-peak and wavelength-shift are the same

In the time-resolved photoluminescence, duration of the excitation-pulse is typically smaller than the decay time. In the case of the firefly bioluminescence reaction, gating time of oxygen, that is, the time for which triggering by O₂ happens, has not yet been determined. However, in a control experiment — a firefly was made to inhale vapors of ethyl acetate whereby it became anaesthetized and a constant glow appeared in its lantern — it was shown that the ‘dc’ light consisted of a continuous train of tiny microsecond-duration pulses [37]. One of those sequences in the DSO is presented as Supplementary Information, Fig. S7. Even though oxygen supply to the luciferin-containing organelles (peroxisomes) was continuous in that case, we still had lifetimes of a few tenths of a microsecond. These lifetimes definitely point towards the process of fluorescence; that is, the situation which was favorable for S → T intersystem crossing in the normal state no longer remained as such in the control state. Steady-state spectra, given in Fig. 5, reveal that the peak appearing at 562 nm in the normal flashing state becomes 555 nm in the ethyl acetate-affected control state, which means that there is a shift of 7 nm in the emission peak towards blue. As the phosphorescence emission-peak is usually red-shifted compared to the one in fluorescence, we could conclude that the process of the bioluminescence reaction is analogous to phosphorescence in the conscious flashing state while the same is analogous to fluorescence in the unconscious continuous-glow state.

Conclusion

In conclusion, low temperatures force the light producing reaction in the firefly to slow down which results in the appearance of three peaks in a flash. These three peaks most probably represent the three luminescent forms of OxyLH₂, which decay in times of the order of milliseconds. Further studies are needed to confirm whether or how a particular wavelength sector is related to a particular peak in a flash in the time-resolved spectrum.

Funding This study was funded by Department of Biotechnology, Government of India (grant number BT/PR4855/BRB/10/1030/2012).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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