Chapter 1
General Introduction
General Introduction

1.1 The firefly

Fireflies, usually called lighting bugs with their amazing capacity of emitting light enthral each and every one of us in our childhood. They were the ‘Fairies’ of our grandmother’s stories. In reality they are neither bugs nor flies but actually beetles of the nocturnal variety and scientifically classified under family *Lampyridae*, a family of insects of the order *Coleoptera* or winged beetle. The classification of lampyridae is based on morphological characteristic\(^1\). *Lampyridae* has three subfamilies; *Luciolinae, lampyridae* and *Photurinae*. The subfamily of Luciolinae is the largest subfamily which are scattered mostly in Eurasia, Europe, East Asia and Australia. In our study we use firefly whose genus is *Luciola*. This is remarkable in the sense that the larvae of several species are aquatic. Luciolin morphology of firefly is related to function; their behaviour and mating process are related to structural adaptation\(^2\). The thesis is about the study of some characteristic of the Indian species, *Luciola praeusta*. Spectra of two Japanese fireflies *Luciola cruciata* and *Luciola lateralis* are also presented in support of these in the third and last chapter.

1.1.1 Firefly lifecycle

There are about 2,000 firefly species. This insect lives in a variety of warm environments, as well as in more temperate regions, and is a familiar sight of summer evening. Fireflies love moisture and often live in humid regions of Asia and America. In drier regions, they are found around wet or damp areas that retain moisture.
Firefly life begins with an Egg. The female deposit her eggs singly or in cluster on moist soil most likely on a leafy litter where the soil is less likely to become dry. Eggs of some beetles are bioluminescence. The eggs hatch within 3-4 weeks.

The larval period after hatching is the longest period. Normally larvae emerge from their eggs in late summer and go through winter to the next phase in late spring. Sometimes it takes one to three years to go to the pupating stage. In this period larvae crawls on ground hunting their prey by injecting an enzyme to their bodies.

After larval period pupating stage comes. Some remarkable transformation takes place in the body in pupating stage. In a process called histolysis, the larva's body is broken down, and special groups of transformative cells are activated. These cell groups, called histoblasts, trigger biochemical processes that transform the insect from a larva into its adult form. When the metamorphosis is complete the pupae converts to an adult firefly. Adult firefly is ready to emerge from pupating stage within 10 days to several weeks.

The Adult firefly emerges only with the purpose to reproduce. The main focus is on searching its partner by signalling with their own coded language. The signalling pattern of male and female firefly is species-specific which establishes that luminescence is neurally controlled. Although they produce light in a very specific manner there are some correlations between firefly signals which develop interest among researchers in this field.

1.1.2 Anatomy of the adult firefly

Despite the great number of different species of fireflies, the anatomy of Lampyridae is relatively conserved. The whole body is about 5-18 mm long and is divided into three main parts; the head, thorax and abdomen which in turn is subdivided into various segments. The species (*Luciola praeusta*) chosen in this study is about 8 mm long. It is
mostly brown.

The head of the adult firefly is concealed beneath the pronotum, a plate like structure that covers the head. The visible elements are the finely faceted eyes (the distinguishing feature of most lampyridae), mouthparts and antenna (Figure 1).

The thorax is the middle part of the body which bears the legs and the wings. Almost all insects have a pronotum covering the top of the first segment of thorax. Near the base of the middle leg there is a small breathing hole called the thoracic spiracle through which they breath and not through their mouth.

The abdomen is the most interesting and important body part of a firefly. The firefly lantern (Figure 2) is a particularly favourable material to study excitation-effectors coupling because it excels all other photophores in versatility and precision of control. Also, there exists a large body of detailed information about the biochemistry, physiology and morphology of the light-emitting system. Apart from the diversity of the firefly light organ it can be generalised that they are close to the body parts behind a window of translucent cuticle. They vary from minute pinhead to masses occupying the entire ventral surface in size, from circular to entirely irregular in outline. The firefly lantern consists of a dorsal and a ventral layer. A rough measurement by SEM showed for a Japanese species *Luciola cerata* that the light organ consists of a 200 μm thick reflector layer, and a 40 μm thick photogenic layer (Figure 3). The ventral layer is called the photogenic layer while the dorsal layer is called reflector layer. The ventral layer houses the light reaction while reflector layer is thought to be a specialized tissue for increasing bioluminescence intensity via reflection; this layer is formed by a group of cells filled with opaque white granules called uric acid granules. Photogenic layer and reflector layer can be distinguished by their distinct morphology.
The ventral layer consists of photocytes which are the light producing cells and the cylinders composed of tracheal branch buried deep into the cells. Each cylinder is surrounded by photocytes, ensuring adequate access to gas exchange. The photocyte contains high concentration of mitochondria and peroxisome. Luciferin and luciferase are housed within the peroxisome. A morphological survey shows that photocyte granules are concentrated in the cell interior and are separated from the tracheal end organs by differentiated zone of small mitochondria and small differentiated zone granules\textsuperscript{9}.

The dorsal or reflector layer consists of uric acid granules which are capable of reflecting light emitting from ventral photogenic layer. The sizes of the uric acid granule in the inner region are found to be very regular. For a particular species \textit{Luciola cerata}, it was found to be of the order of 700 nm in diameter\textsuperscript{10} but at the edge it is found to be very large, about 4 \textmu m. TEM showed that the reflector layer consists of cells with an average size of about 40 \textmu m in length and nearly 15 \textmu m in thickness. All these intracellular granules show an empty internal space and are surrounded by a layer of membrane-like structure. Thus firefly lantern consists of all possible organs which can initiate the light producing mechanism as well as increasing the intensity of produced light.
Figure 1. Photograph of firefly *Luciola praeusta*.

Figure 2. Photograph of the lantern of firefly *Luciola praeusta*.
1.2 Luminescence—Bioluminescence

The bioluminescence is the term which is the combination of two terms: Greek word bios meaning life and a Latin word lumen meaning light. The basic mechanism behind the firefly light is bioluminescence or luminescence generated by living organism. The light produced by firefly is referred as “cold” light as there is no heat produced by this mechanism.

Luminescence is a science closely related to spectroscopy, which is the study of general laws of absorption and emission of radiation by matter. It is the phenomena in which light emits from certain substance without emission of any heat. Luminescence requires variety of excitation source. The various luminescence phenomena are given names based on the type of radiation used to excite the emission. The characteristic of luminescent light is its wavelength produced not the radiation of the excited source\textsuperscript{12}. The luminescence is divided into two categories\textsuperscript{13}: fluorescence and phosphorescence.
depending on the nature of the excited state. In fluorescence, the electron in the excited state is paired to the electron to the ground state as they are of opposite spin. Consequently they are spin allowed and hence occur rapidly by emitting a photon. The emission rate of fluorescence is typically $10^8$ sec$^{-1}$. Thus fluorescence life time is about 10 ns. In phosphorescence the transition is from triplet excited state where electron from the excited state is in the same spin orientation as that of the ground state. Thus transition to the ground state is forbidden and therefore it is slow ($10^3$ to $10^0$ sec$^{-1}$). An important feature of fluorescence is high sensitivity detection.

The processes that occur between the absorption and emission of light are usually illustrated by the Jablonski diagram.

![Jablonski Diagram](https://example.com/jablonski.png)

**Figure 4.** Jablonski Diagram.
(Courtesy: J. R. Lakowicz, Principle of fluorescence spectroscopy)

A typical Jablonski diagram is shown in Figure 4. The singlet ground, first and second electronic states are depicted by $S_0$, $S_1$, and $S_2$, respectively. At each of these electronic energy levels the fluorophores can exist in a number of vibrational energy levels, depicted by 0, 1, 2 etc. The transitions between states are depicted as vertical lines to
illustrate the instantaneous nature of light absorption. Transitions occur in about 10–15 sec, a time too short for significant displacement of nuclei.

The phenomenon of fluorescence displays a number of general characteristics. Study of the Jablonski diagram (Figure 4) shows that the energy of the emitted light is typically less than that of absorbed light. Fluorescence is the phenomena that occur at lower energies or longer wavelengths. This phenomenon was first observed by Sir. G. G. Stokes in 1852 at the University of Cambridge\textsuperscript{14}. Another important feature is that Emission Spectra are typically independent of the excitation wavelength.

Oldest known written observation on bioluminescence started from 1500 to 1000 BCE in China regarding fireflies and glow-worms. Cladistic analysis indicates that the bioluminescence first appears in larvae and then it is carried over to adult\textsuperscript{15}. One of the basic expectations about nascent bioluminescence in any organism is that the organism must be nocturnal\textsuperscript{16}. A comprehensive study of past two decades’ work on firefly light, mate choice and predation is provided in a review\textsuperscript{17}. Firefly bioluminescence is mostly popular because of its high quantum yield value which determines how efficient the reaction is.

1.2.1 Quantum Yield

Quantum yield is perhaps the most important characteristics of a photophore. In bioluminescence the energy required for the production of light is obtained by the enzymatic oxidation of a specific substrate, luciferin. Considering the various mechanisms which are related to the bioluminescence reaction, it is essential to know the number of oxygen and luciferin molecule used to produce each light quantum. The absolute number of light quanta emitted per luciferin molecule oxidized is defined as quantum yield\textsuperscript{18}. It is given as\textsuperscript{19}
\[ \Phi_f = \frac{K_r^s}{(K_r^s + K_{nr}^s)} \]

\[ \Phi_f = K_t^s \tau_s \]

where

\[ K_r^s \] - rate constant for radiative deactivation \( S_1 - S_0 \) with emission of fluorescence

\[ K_{nr}^s \] - rate constant for non-radiative deactivation

\[ \tau_s \] - life time of excited state \( S_1 \) equals to \( 1/(K_r^s + K_{nr}^s) \)

I.e. Quantum Yield is the ratio of number of emitted photons to the number of absorbed photon given by

\[ \frac{i_F(t)}{[\text{I}A^*]_0} = K_t^s \exp \left( -\frac{t}{\tau_s} \right) \]

where

\[ i_F(t) \] is the fluorescence intensity at time \( t \) after excitation by a very short pulse of light at time 0

\[ [\text{I}A^*]_0 \] is the concentration of excited molecules at time 0.

Thus integration of the above relation over the whole duration of the decay yields \( \Phi_f \)

\[ \frac{1}{[\text{I}A^*]_0} \int_0^\infty i_F(t) \, dt = K_t^s \tau_s = \Phi_f \]

An \textit{in vitro} experiment of oxidative reaction and subsequent light emission for quantum yield measurement shows that the average value for frequency distribution for 39 independent measurements is 0.88 ± 20.25. This study demonstrates that one light quantum is emitted for every luciferin molecule oxidized. The possible reason may be that the energy released in the oxidative process is retained more readily in the enzyme-
substrate complex from which it can be trapped at an emitting site. Measuring at different
P_H shows that below 7.0 the quantum yield decreases markedly which shows the
importance of protein in the transfer of energy required for light emission^{20}. In another
report quantum yield values found to be different for different luciferase measured by
absolutely calibrated luminometer ranging from 0.15 to 0.61 for wavelength ranging from
625 nm to 539 nm respectively. These results suggest that the luminescence colour of the
luciferase is associated with the quantum yield of the firefly bioluminescence reaction^{21}.

1.2.2 Chemistry of firefly light

The molecule behind the firefly light is luciferin. Beetle luciferases (including those of
the firefly) use the same luciferin substrate but different enzymes to naturally display
light ranging in colour from green (firefly) to red (click beetle). The basis of the
mechanism is that catalytic enzyme modulates emission colour by controlling the
resonance-based charge delocalization of the anionic keto form of the oxyluciferin
excited state^{22}. A series of chemical reactions takes place in producing this cold light.

Firstly, Luciferase in presence of ATP, firefly D-luciferin gets converted into the
enzyme-bound luciferyl adenylate, giving inorganic pyrophosphate PPi as a byproduct in
presence of ATP and magnesium.

\[
\text{Luc} + \text{LH}_2 + \text{ATP} \rightarrow \text{Luc}.\text{LH}_2\text{-AMP} + \text{PPi}
\]

(Firefly luciferin)

Secondly, Luciferyl adenylate in presence of oxygen gets converted to electronic
excited-state oxyluciferin molecule and carbon dioxide.

\[
\text{Luc}.\text{LH}_2\text{-AMP} + \text{O}_2 \rightarrow \text{Luc.oxyluciferin}^* + \text{AMP} + \text{CO}_2
\]

The excited oxyluciferin losses its energy through fluorescent path which results in the
emission of visible light.

\[
\text{Luc.oxyluciferin}^* \rightarrow \text{Luc.oxyluciferin} + \text{Light}
\]
1.3 Earlier studies on firefly light

Quite a few *in vivo* studies on the firefly light were done till date. Although much progress was done during the past decades on its *in vitro* study, many mysteries have yet remained unsolved. Questions as “which amino acid residues take part in each catalytic step of the reaction?” or “what intermediate enzyme-substrate states are formed during reaction?” are not yet clear\(^{22}\). Such type of interdisciplinary study involves people from various fields like biology, chemistry, physics, electronics etc. In a study on peroxisomes of the firefly *Luciola cerata* showed the role of reflector layer which is done by isolating the luminescent light organ from dying fireflies. The functional study indicates that the dorsal light organ is relatively weaker than the ventral light organ\(^{10}\). In another study, it was reported that the transportation from protein to peroxisome has been highly conserved through evolution\(^{23}\). These energetic results support the generally accepted theory of chemically initiated electron exchange luminescence (CIEEL)\(^{24}\).

Regarding the spectral distribution an extensive study was done for sixteen species of Jamaican fireflies and four species of American fireflies with photoelectric recording spectrometer, which gives a wide range of emission peaks from 5520 Å (*Photuris pennsylvanica*) to 5820 Å (*Pyrophorus plagiophthalamus*). On studying the dorsal light organ and ventral light organ separately for the *P. plagiophthalamus* species, it was observed that dorsal light organ showed three distinct colours ranging from 550.1 nm to 562.4 nm where ventral light organ showed peak ranging from 547 nm to 594 nm\(^{26-27}\). Study on a glass spectrograph of three Indian species gives bioluminescence peak in the range 500 nm to 675 nm\(^{28}\). The species *Luciola praeusta* chosen in this study gives emission peak at 562 nm and FWHM of 55 nm which was reported to be the smallest *in vivo* value among all firefly species till date\(^{29}\). It is found that female flash pattern is different from male\(^{30}\). It
has also been reported that firefly flash is regulated by Nitric Oxide\textsuperscript{31} and Calcium\textsuperscript{32}. Ethyl acetate affects the neural activity of firefly which results a continuous light coming out of the lantern of the firefly\textsuperscript{33}. The affect of magnetic field on the firefly flash shows that magnetic field affects the nerve connected to its lantern\textsuperscript{34}. It is possibly due to the Lorentz force, induced by 10 Tesla magnetic field, the velocity of nerve action potential decelerate when the direction of nerve conduction and magnetic were crossed.

1.4 About the Thesis

The thesis is about some of the important aspects of firefly light which comprises of five chapters. The present chapter is about overall information of firefly, its anatomy including the lifecycle, about bioluminescence and also about the basic chemical reaction involved in this process.

The second chapter includes the study of different colours emitted by firefly. Firefly light basically consists of three colours. These colours have been seen in the coloured spectrum recorded on the Hilger &Watts glass spectrograph as well as in the profile of the Ocean optic HR 2000 Series high resolution spectrometer. The colours observed are Green, Yellow and Red. The Yellow colour was highly intense while green was less intense. The red boundary was surprisingly found to be sharp. While analysing with the software Image J, a highly intense band in the yellow sector has been observed. The spectrometer reading also showed the same line in the yellow sector before. This was probably overlooked or considered to be as a noise thus was not detected. Comparing with the iron spectrum the wavelength of this sharp line was found to be 591 nm, the FWHM of which was measured to be 0.25 nm. This sharp line was hypothesised as the laser light and the system as a random laser. The intensity ratio of the 591 nm peak (observed in this study) and the 562 nm (recorded earlier by clipping off the higher value)
shows that although the ratio is not always constant, 591 nm peak is always more intense than the 562 nm peak. The inner mechanism and possible reason of production of laser light is discussed in this Chapter 2.

As the yellow light was hypothesised as the laser light, the study of its diffracted pattern was necessary to investigate its coherence nature and hence studied in chapter 3. Here we use Hilger analytical grating of 15000 lines per inch to see the diffracted pattern of one Indian species (*Luciola praeusta*). In comparison to this we have studied the diffracted pattern of two Japanese fireflies for better result. The two Japanese species were *Luciola laterelis* and *Luciola cruciata*. The central fringe was predominantly yellow and could be seen up to the first order only. On the other hand the green and the red colours became visible only from the first order. The diffracted patterns of other two species were found to be similar with the result. A significant feature is that in the first order on the right hand side of the principal diffraction maximum for each of the three species the yellow boundary is quite marked. Hence it can be suggested that the yellow photon shows the temporal coherence property. It is proposed that the firefly has a tendency for spectral narrowing around the peak wavelength region. The work presented here seems to substantiate that proposition.

The second and third chapter are about the steady state of firefly light, while the fourth and the fifth chapter include its time resolved study. The third chapter is about the seasonal dependence of firefly light. The whole study was done for the entire season effectively from early March to late October. The room temperature was varied from 21 $^0$C to 34 $^0$C. The six room temperature recorded while doing the experiment were 21 $^0$C, 24 $^0$C, 26 $^0$C, 28 $^0$C, 32 $^0$C and 34 $^0$C. Flash duration measured at these temperatures were 128 ms, 117.7 ms, 107.9 ms, 104.1 ms, 93.1 ms and 86 ms respectively. At these
temperatures the flashes of firefly were recorded. The data were analysed with the software Origin 6.1 which give surprising result. A constant relationship between the temperature and the firefly flash duration was found. The flash duration were almost linearly related with the temperature. The correlation coefficient was 0.983. The decrease in flash duration with the increase in temperature could be due the change in speed of chemilluminescence reaction with the temperature. We propose that a change in temperature affects the functioning of the catalytic activity of enzyme luciferase, which results in a change in the reaction rate - variation in the flash duration being a manifestation of this change.

The fifth chapter is about the effect of magnetic field on the firefly flash. The Fourier spectra of the three firefly species were analysed. The actual experiment was done in the Chiba University, Japan and the flashes recorded there were analysed in Gauhati University. The Fourier transform of the recorded flashes of the three species were analysed with the help of software Origin 6.1. The Fourier spectra of the three species show that under the static magnetic field a few harmonics, which were present in the control state, disappear. Disappearances of the harmonics are found to occur in regular intervals. These regular intervals can be assigned as particular bands. The result shows analogy with multiple notch filters which is required to get rid of more than one frequency. A notch filter highly attenuates/eliminates a particular frequency component from the input signal spectrum while leaving the amplitude of the other frequencies relatively unchanged. Bursts of neural activity releasing octopamine was supposed to produce blocking of certain frequencies and allowing of other frequencies.
References


