

# PROGRESS, PERSPECTIVES AND PROBLEMS IN BASIC ASPECTS OF BIOLUMINESCENCE

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## INTRODUCTION

It is a great pleasure to participate in this 15<sup>th</sup> Symposium on Bioluminescence and Chemiluminescence, thirty years after the first, brilliantly conceived and organized in Brussels by Eric Schram and Philip Stanley, later to be joined by Larry Kricka, and to express my gratitude to the organizing committee for inviting me. It is also an overwhelming experience to see the greatly transformed Shanghai. There has also been a profound transformation in the field of bioluminescence over these thirty years, progressing from the vision in Brussels that luciferase systems could be used for analytical purposes in biochemistry and medicine<sup>1</sup> to the now widespread use of genes of luciferases and GFP as reporters to track expression of other genes in time and location.<sup>2</sup>

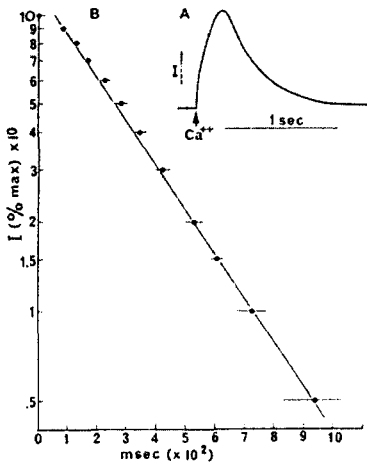
In parallel, there have been many important advances in basic aspects.<sup>3</sup> Color mutants of both luciferases and green fluorescent protein have been put to great advantage in studies where they are used as reporters and, along with other mutants, contribute to our understanding of reaction mechanisms. Crystal structures have been obtained for luciferases from four systems- bacterial, firefly, coelenterate and dinoflagellate, and much has been elucidated concerning the structures of emitters and reaction intermediates. Here I will discuss specific aspects of each of the four systems for which luciferase structures are available, starting with the coelenterate system and the use of the term photoprotein.

**Coelenterates: Aequorin & photoproteins are luciferase intermediates.** For many years the biochemistry of the brilliantly luminescent jellyfish *Aequorea* was a real enigma. Cold-water extracts gave bright and long-lived emission, but the luciferin-luciferase test was frustratingly negative. Shimomura made the seminal discovery that the reaction requires calcium, and found that cold-water extracts made in the presence of EDTA yielded a protein that gave light upon the addition of excess calcium.<sup>4</sup> He named the protein aequorin, and later dubbed it a photoprotein, the precise nature of which was not well appreciated at first. It was later shown to be a luciferase intermediate, effectively the “substrate” in the assay because turnover is slow, and is destroyed in hot water extracts of the luciferin-luciferase test.<sup>5</sup>

Sessions at this symposium are divided into *luciferase-based bioluminescence* and *photoprotein-based bioluminescence*. But both use luciferases; the photoprotein aequorin is simply a stable luciferase-peroxy-luciferin intermediate in which a subsequent reactant has been withheld, as confirmed by its crystal structure.<sup>6,7</sup> Such intermediates in this or other systems, when accumulated, can provide the substrate

for a rapid flash in living cells if the lacking reactant is rapidly added, thus calcium for aequorin.

The flash decay will thus be first order and attributable to the rate constant for the decay of the intermediate formed after calcium addition (Fig. 1), and the total light emitted in the flash will be proportional to the amount of intermediate. Also, it should be noted that for the flash to decay to baseline, the prior enzymatic reaction step(s) must be very slow so that little if any more intermediate will be reformed during the course of the flash, during which time the triggering substance can be withdrawn so that new intermediate can be accumulated.

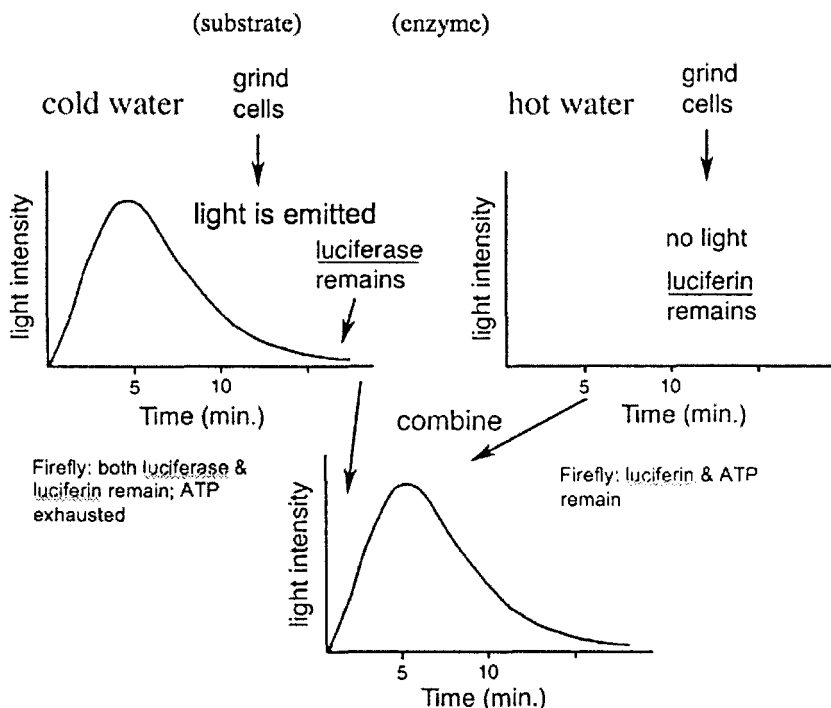


**Fig. 1.** Kinetics of the reaction of aequorin with calcium mixed in a stopped-flow apparatus at 23° C.

**Firefly: the regulation of the flash.** Although the luciferin-luciferase reaction appeared to “work” in firefly extracts, it turned out that the components were not those specified in the long-established protocol. McElroy discovered<sup>8</sup> that ATP is the component exhausted in cold water extracts of fireflies, while both luciferin and luciferase remain (Fig. 2), while the hot-water extract contains ATP.

In McElroy’s lab, we established that the reaction of ATP and luciferin with purified luciferase involves two steps;<sup>9</sup> the first forms an active intermediate, later determined to be the adenylate, and the second is the reaction with oxygen, leading to an excited state and light emission. The prompt decline of luminescence over the first minutes was shown to be due to luciferase inhibition, not substrate exhaustion. All evidence indicates that the flash of the firefly is initiated by the introduction of oxygen into the photocytes, triggered by a nerve impulse, which actually does not end on the photocytes, but on adjacent cells.<sup>10-12</sup> More recently, nitric oxide (NO)

has been proposed to be a humoral agent involved in transmission of the signal from the nerve ending to the photocyte to initiate a flash.<sup>13,14</sup> The evidence for this is not strong, and I believe the proposed mechanism to be incorrect.



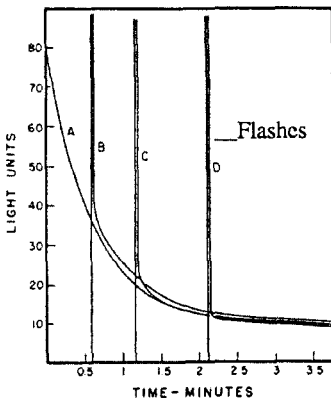
**Fig. 2.** Depiction of the steps and conditions for a luciferin-luciferase reaction in which an exhausted cold-water extract is mixed with a hot-water extract to give light emission. How it differs in firefly extracts is also noted.

Briefly, the NO mechanism postulates that mitochondrial oxygen consumption maintains photocytes anaerobic in spite of a continuous input of oxygen from tracheoles. A flash is initiated through a cascade of transduction steps from the nerve ending that result in NO production in the photocytes, where it inhibits this respiration, allowing oxygen to reach luciferase and initiate the reaction. As NO production ceases, along with some other possible factors, the mitochondrial utilization of oxygen resumes and the luciferase reaction declines.

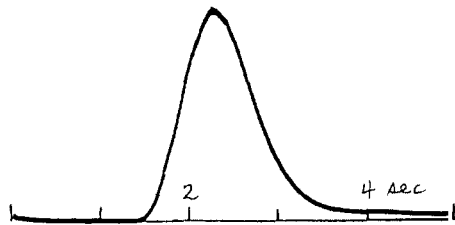
The kinetics of the rise phase of the flash, which in many species is less than 100 msec, seems difficult to attribute to a cascade of signal transduction events. But the extinction of the flash is most certainly not caused by the withdrawal of a reactant. Instead, it has kinetics attributable to the reaction of a luciferase intermediate whose

precursor is accumulated in the absence of oxygen, comparable to the case of the jellyfish flash.

Some years ago I demonstrated that such a "biochemical" flash can be produced in the test tube.<sup>9,15</sup> If oxygen is excluded from a firefly luciferase reaction mixture and then added rapidly back, a bright flash occurs, some 100 to 200 times brighter than the baseline intensity (Fig. 3). This comes from the reaction of the luciferyl adenylate "active" intermediate accumulated in the absence of oxygen. Note that the decay of the flash is not due to the removal of oxygen, but to the utilization of the luciferase-peroxide intermediate, so the baseline returns to a low level (Fig. 4), defined by the slow rate of reaction of ATP with luciferin. It is well known that the kinetics of firefly flashes are species specific and of functional importance in courtship communication, fixed by the rate constant for the first order decay of the peroxide intermediate formed from the adenylate.



**Fig. 3.** Flashes in response to the rapid addition of oxygen to firefly luciferase reactions initiated in the complete absence of oxygen.<sup>9</sup> A: Time course of normal reaction in air. B,C,D: started under strict anaerobic conditions; oxygen added later at times indicated.



**Fig. 4.** Kinetics of a flash obtained by addition of oxygen, as described in Figure 3.<sup>9</sup>

**Bacteria: A peroxide intermediate, quorum sensing and milky seas.** Although the luciferin-luciferase test in bacterial extracts was negative, Strehler<sup>16</sup> discovered that light emission in extracts could be obtained by adding reduced pyridine nucleotide, underlining the fact that bioluminescence is not a phenomenon separate

from all other cell biochemistry, but linked to it in different ways in different systems. Light emission in bacteria is continuous, deriving electrons for the reduction of flavin, the luciferin in this system, from the respiratory pathway, as indicated in Fig 5. Reports that it occurs as pulses have not been confirmed.<sup>17</sup>

This luciferase reaction also forms a semi-stable peroxide intermediate, which we demonstrated some years ago<sup>18</sup> and later isolated.<sup>19</sup> It is reasonably stable in the absence of aldehyde and might, in principle, be accumulated in the cell and triggered to emit a flash by aldehyde addition. Indeed, bioluminescence in tunicates, which utilizes a bacterial luciferase system<sup>20</sup> derived from endosymbionts,<sup>21</sup> emits light as flashes, the biochemical basis for which has not been investigated.

An important phenomenon, now called quorum sensing, was discovered from studies of bacterial bioluminescence, in which it was found that growth and luminescence are controlled separately.<sup>22</sup> After inoculating a culture into fresh medium, growth is exponential with no lag, but the amount of luciferase remains constant for the first three hours, after which its synthesis and light emission increase very, very rapidly (Fig. 6). This was shown to be due to the production and release into the medium of a substance that we named autoinducer; upon reaching a critical concentration, it induces the synthesis of luciferase and other proteins involved in the bioluminescence. Eberhard and colleagues determined the structure to be a homoserine lactone and synthesized it.<sup>23</sup>

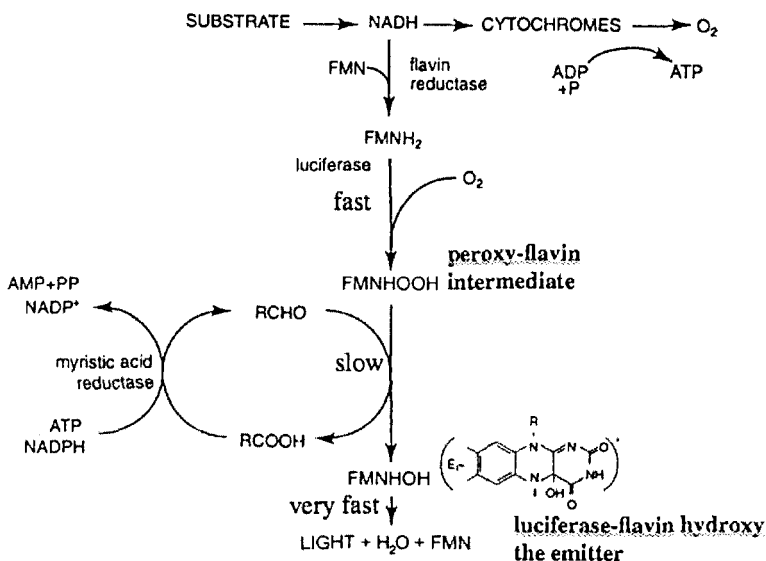
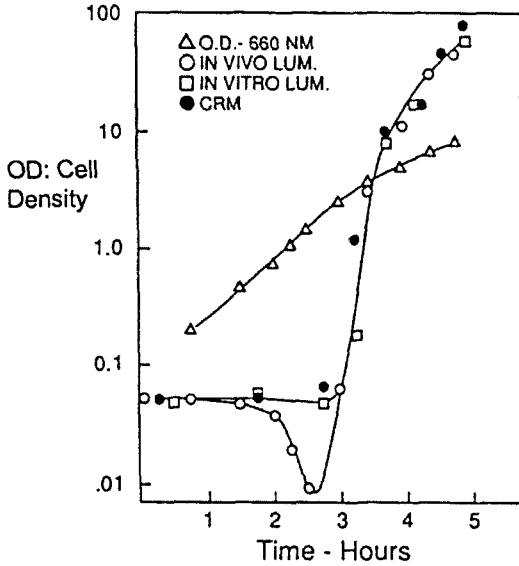


Fig. 5. Pathways and intermediates in the bacterial luciferase reaction.



**Fig. 6.** Time courses showing that the development of luminescence and luciferase (both in vitro activity and by antiluciferase, CRM) lag cell growth.<sup>22</sup>

For many years this phenomenon was believed to be simply a special curious feature of luminous bacteria, but when DNA sequences became available, genes homologous to those responsible for autoinducer production were found to occur widely in the bacterial world. Up to then it had been generally believed that bacterial cells are mostly loners, essentially autonomous in their activities. But this discovery demonstrated that bacteria produce substances that control expression of different genes in many other bacteria, both in the same and different species, thus constituting chemical communication.<sup>24,25</sup>

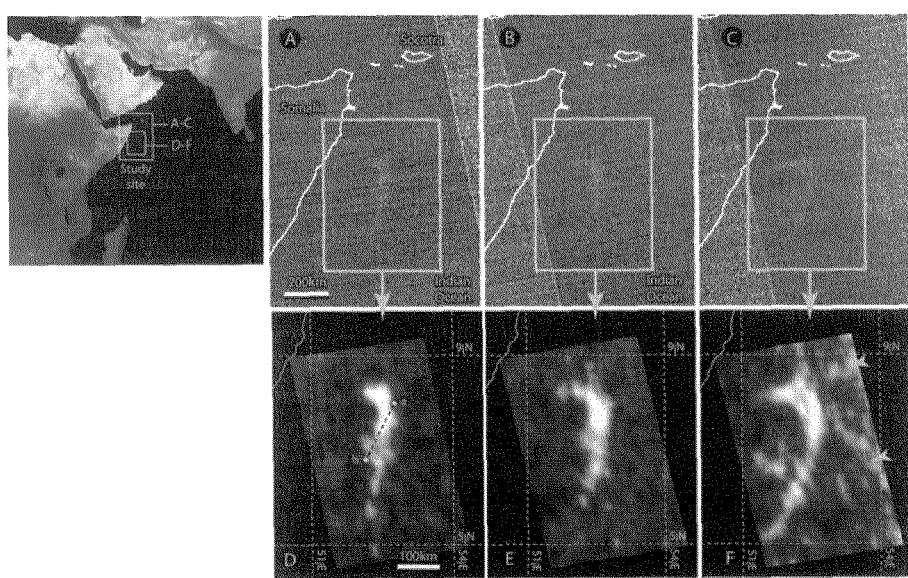
A major function of luminescence in bacteria is to provide light when cultured in specialized light organs of a higher organism. There, the production of luciferase and light are delayed until cell numbers are high enough for the light to be visible to other organisms. In some pathogenic bacteria toxin production may be delayed until the invading population is high: a surprise attack can produce massive amounts of toxin and overwhelm before resistance can be mounted.

Luminous bacteria can be isolated from sea water almost anywhere in the world, but the number is typically very few, so the autoinducer in the water should and does not reach the concentration needed to induce luciferase in isolated cells.<sup>26</sup> Yet ever since records of ship voyages have been kept, there have been repeated reports of continuous luminous light emission in the ocean, all around the ship as far as the eye

can see.<sup>27</sup> This has been called “Milky Sea”, for it does indeed look like the ship is sailing on a sea of milk!

Although no explanation of the phenomenon had been reported in the literature, a group of scientists wondered if earth-imaging satellite cameras might be able to detect the light emission. Checking the archives, they found a ship log reporting the phenomenon in 1995 when a camera had been overhead. They retrieved the satellite images and detected a weak signal on three consecutive nights; with background subtracted it revealed a luminous area of about 14,000 km<sup>2</sup>, its exact structure changing from night to night (Fig. 7).<sup>28,29</sup> The reported positions of the ship when it entered and exited the area corresponded exactly to the coordinates obtained from the satellite data. The location off the Horn of Africa is where reports in logs of Milky Seas have been most frequent.<sup>27</sup>

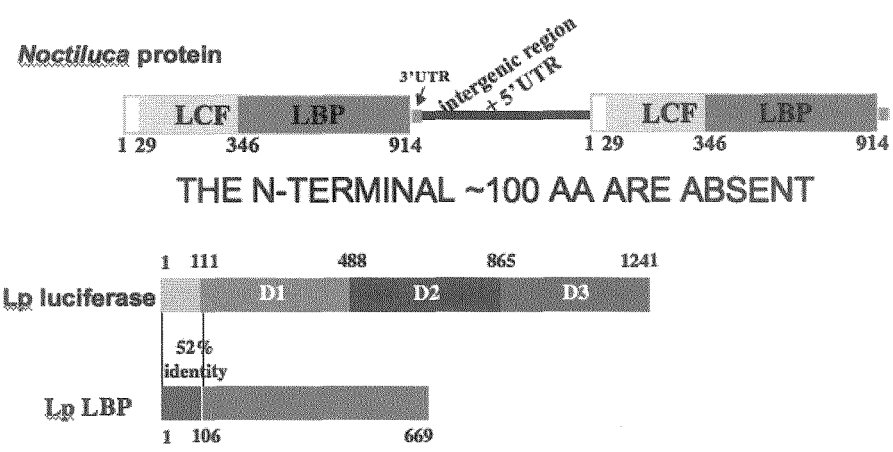
Because the emission is continuous it had been speculated, and many scientists believed, that luminous bacteria might be responsible. But, if so, how might the autoinducer concentrations needed be achieved? The answer to this is not yet known, nor is it certain that the light is actually due to luminous bacteria of the kind already cultured. But a clue comes from reports of merchant sailors, who from time to time reported what they saw in a bucket of water from the milky sea. A typical description was that the water “... contains thousands of very thin lines of light....”



**Fig. 7.** Bioluminescence of milky seas recorded by satellite imaging for 3 consecutive nights. Raw data, A,B,C; with background subtracted, D,E,F. Left, locations of images.<sup>28</sup>

approximately 13 mm long ...”<sup>27</sup> If bacteria are responsible, they might be concentrated on a substrate, perhaps a filamentous alga of some sort, where autoinducer could accumulate. Future studies should give the answer.

**Dinoflagellates: pH triggers the flash; two functions in one protein.** These unicellular marine plankton, which my laboratory has studied for many years, are responsible for the sparkling oceanic luminescence, earlier called phosphorescence. Most of our work has with the photosynthetic species, *Lingulodinium polyedrum*, (formerly *Gonyaulax polyedra*), which emits brief (0.1s) flashes from small (0.4µm) cell organelles named scintillons.<sup>3,30</sup> They contain two major proteins, a luciferase (LCF) and a luciferin binding protein (LBP); the activities of both are pH-dependent. The luciferin is a tetrapyrrole, probably derived from chlorophyll. The sequences of the N-terminal ~100 residues of the two proteins are about 50% identical but the remaining regions have no similarities (Fig.8).<sup>31</sup> In LCF, the rest of the molecule (~137kDa) is comprised of three repeat homologous domains, each with a centrally located independent catalytic site, where the sequences are about 95% identical. Each individual domain has luciferase activity, and each has four conserved histidines, which have been shown to be involved in the regulation of activity by pH.<sup>32</sup>



**Fig. 8.** Structure for *Noctiluca* luciferase (top) showing that it occurs as tandem copies of a gene possessing a sequence homologous to a partial single domain of (*Lp*) luciferase (bottom) together with a sequence homologous to a full length luciferin binding protein. The *Noctiluca* protein lacks the first N-terminal ~100 amino acids found in both *Lp* proteins.<sup>34</sup>

A crystal structure of one of the domains reveals a catalytic pocket and residues responsible for regulation by pH.<sup>33</sup> The LBP has four homologous domains, but their sequence similarities are not great.<sup>34</sup>

The luciferase genes and proteins are very similar in seven different luminous photosynthetic species. They are about the same length and all have three domains, and occur as tandem repeats but with very different intergenic sequences.<sup>35,36</sup> The individual domains of different species are more similar to each other than to either of the other two domains of the same species.

But in the heterotroph *Noctiluca scintillans* the catalytic and luciferin binding sequences are both found in a single gene, and are expressed as a single protein (Fig. 8). The N-terminal ~100 sequences found in *L. polyedrum*, which might be functional for protein-protein association, are completely absent. There is only a single luciferase domain, and it is truncated on the N-terminal side, with three of the four histidines found the three-domain luciferases absent. Aside from the N-terminal ~100 sequences, the luciferin binding sequence is similar in size and homologous to the LBP in *L. polyedrum*, including the four domain structure.

### **Bioluminescence originated independently many different times in evolution**

From a biological point of view bioluminescence is truly unusual by virtue of its evolutionary origins. As well illustrated by the four systems described, the genes, proteins and substrates involved are altogether different, as are the regulatory and functional aspects of the systems. This is most readily explained by assuming that the different systems arose independently,<sup>37</sup> some being related to genes coding for proteins with completely different functions (coelenterates, fireflies), others with no known affinities (bacteria, dinoflagellates).

How could this have been? Why is luminescence different in this respect from many, perhaps most, other genes, which have relationships to genes with similar functions in phylogenetically distant organisms? I propose that this is because the different bioluminescence systems actually have different functions, thus not subject to being carried out by the same proteins. For the systems reviewed, coelenterate flashes may startle predators and deter predation; fireflies communicate in courtship by flash patterns; bacteria provide light for various uses for hosts that culture them in different specialized organs, and dinoflagellates flash in response to mechanical stimulation by their predators, thus revealing their presence to their own predators (the burglar alarm theory).

Some years ago I estimated that there may be up to 30 different bioluminescent systems.<sup>37</sup> Researchers interested in luciferases, as well as mechanisms and functions of light emitting organisms, will thus still find a diversity of new systems for exploration with the prospect of many new and different applications. I hope that researchers will pursue such studies with vigor in the years to come.

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