Instrumentation for Chemiluminescence and Bioluminescence Assays (Part-I)

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Summary: Bioluminescent and chemiluminescent emissions can easily be measured with any instrument sensitive to light. The conventional instrumentation available for measuring chemiluminescence and bioluminescence is described. The advantages and disadvantages, and also a possible development in the foreseeable future for these equipments which are based on batch techniques are also discussed.

Introduction

The term *luminescence* describes the emission of ultraviolet, visible and infrared radiation from an excited electronic state of a molecule. The various types of luminescence can be classified according to the means by which energy is supplied to excite the luminescent molecule [1]. If the excitation energy is obtained from the chemical energy of reaction, the process is termed *chemiluminescence (CL)* [2]. The de-excitation process is accompanied by the emission of a photon:

$$A + B \longrightarrow C^{*} \longrightarrow C + h\nu$$

CL reactions are oxidative, involving a range of organic molecules. The light emitted from these molecules if measured accurately and reproducibly can provide a method of analysis for a variety of samples e.g., clinical, forensic and environmental [3-5]. Similarly, *bioluminescence (BL)* is an enzyme-catalyzed *CL* process found in biological systems, in which a catalytic protein increases the efficiency of the luminescent reaction [6]. The use of *BL* reactions offers a twofold advantages over most *CL* reactions; sensitivity is enhanced by the higher quantum yields and selectivity is increased because of the enzyme involved. The two most important *BL* systems from an analytical stand-point are the enzyme (luciferase)/substrate (luciferin) combinations extracted from fireflies *Photinus pyralis* and a marine bacteria *Vibrio harveyi* which requires ATP (adenosine-5-triphosphate) and NAD(P)H (nicotineamide adenine dinucleotide reduced form) as cofacters [7-8]. Reactions that produce and consume these cofacters can also be coupled to the *BL* reaction to provide assays for a wide range of clinically important species [9-10]. Reaction schemes for the firefly and bacterial systems are given in Figure 1 and 2 respectively.

Analytical method based on *Cl* and *BL* reactions are attractive for possible applications because they are selective, extremely sensitive and rapid as compared with the established methods such as UV-visible and fluorescence spectroscopy. These advantages recommend its use in the analytical laboratory. To fully exploit this analytical potential, however, the ability to quantitate low light levels accurately and efficiently is as important as the effective utilization of these chemical systems. The purpose of writing this article is to introduce *CL* and *BL* techniques, the advantages and dis-ad-

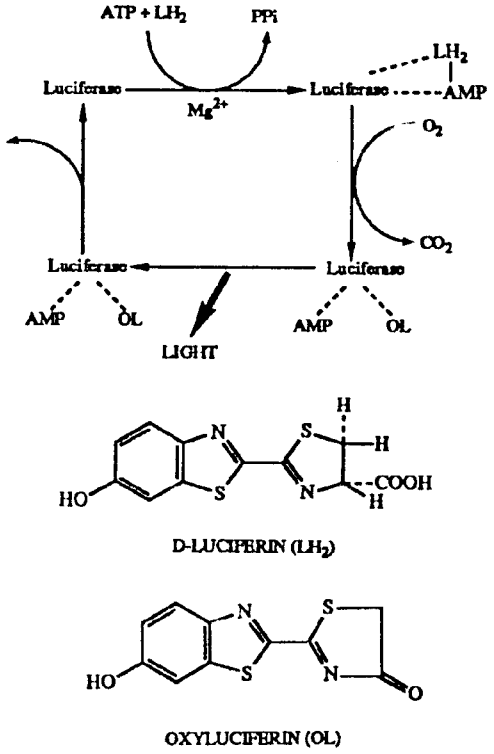

Fig.1: Schematic representation of the firefly luciferase reaction. ATP = adenosine triphosphate, LH2 = Luciferin PPi = Pyrophosphate; AMP = adenosine monophosphate OL = Oxyluciferin.

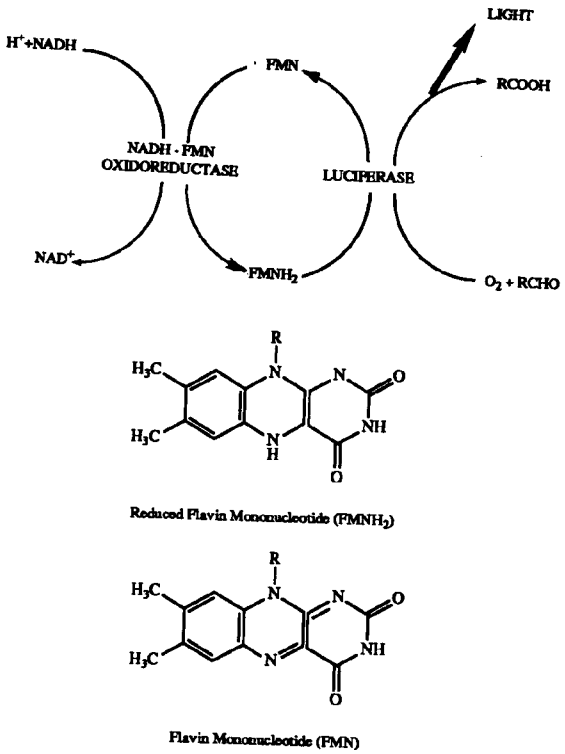

Fig. 2: Schematic representation of the bacterial luciferase reaction. NAD = nicotineamide adenine dinucleoptide; NADH = reduced NAD; FMN = Flavin in Mononucleotide; $FMNH_2$ = reduced FMN; RCHO = decanal.

vantages of conventional instrumentation available for measuring *CL* and *BL* reactions and also to stimulate its interest in teaching and research.

The detection of light

Light emitted as a consequence of chemical reaction, e.g. *CL* and *BL* may be detected and measured by the use of several devices e.g., photographic films, photocells, photomultiplier tubes and multichannel detectors [11-12]. Each of these detection methods depends on the amplification of the energy absorbed when an electron in an atom, molecule or complex is raised to a higher energy state. The primary reaction is always same in principle; that is the movement of electrons within an absorber to a more improbable position, followed by the coupling of the disturbance by induced light absorption in the system to the release of a much larger amount of energy by the amplifier portion of the detector. Among the devices used, the following three are the most suitable for analytical luminescence.

Photographic films

This was one of the original artificial means of detecting luminescent reactions. The amplifier portion of the photographic process depends upon the fact that electrons, which have moved to improbable position as a result of light absorption within a photographic grain, act as catalysts for the reduction of additional Ag ions, existing within that photographic emulsion grain. The energy for this amplification comes from the reaction between the emulsion components (silver salts) and the reducing material present in the developer. With the advent of "instant" emulsions (e.g., the polaroid 'land' system) a potentially useful means for light quantitation became available, in which the depth of the

exposure is proportional to the analyte concentration. Green *et al.*, [13] used polaroid land film type 612 (ASA 2000) as a detector for the bioluminescent determination of ATP using immobilized firefly luciferase and for the determination of NADH and ethanol using co-immobilized bacterial luciferase and oxidoreductase. Photographic method has the advantages of simplicity, providing a permanent record and needing no ancillary equipment. The disadvantage is that it is only semi-quantitative.

Solid-State Devices

The potential of multichannel array detectors, e.g., vidicons and diode array for spectrophotometric measurement has been reviewed and demonstrated elsewhere [14-15]. The disadvantage of these detectors is that the noise levels are quite high. Some of this noise is always generated within the more complex amplification system required for these devices [16]. An intensifier (silicon intensified target vidicon) has been placed in front of the basic detector to improve the detector at low levels and use for the chemiluminescent determination of quinine sulphate [17]. These detectors are relatively cheap, give a linear response over a wide range of robust and require only simple electronics. Ryan *et al.*, [17] compared the intensified diode array with a conventional PMT for the measurement of quinine sulphate and gave reasons for the superior performance of the photomultiplier tube.

Photomultiplier tubes

Photomultiplier tubes (PMT) are the most commonly used detectors based on photoelectronic effect. Stimson [18] discussed the properties and sensitivities of PMT. They are relatively a vacuum-type photocells incorporating several amplification stages (dynods) and an electron collector (anode). In a simple phototube one measures the current associated with electrons ejected from the photocathode, whereas in a photomultiplier the ejected electrons are accelerated over a substantial voltage drop (100-V) towards the first dynode. When this electron hits dynode its accumulated kinetic energy in turn causes the ejection of a number of additional electrons which moves toward the second dynode where the process is repeated. At the terminus of the chain (the anode of the PMT), the photoelectrons are collected and cause a small negative pulse to be generated. The negative current resulting from successive pulses is of sufficient magnitude to be registered in two different ways, either time- averaging of the electron pulses which are converted into a continuous voltage or by using a circuit with high time resolution to collect the pulses, photon counting. The most common equipment based on the latter principle is the liquid scintillation counter.

Liquid scintillation counters

A liquid scintillation counter (LSC) is, in essence, a two channel photon counter with a variable discriminator. The sample is placed immediately between two detectors such that optical efficiency is high. As a radiation counter, the LSC is operated in the coincidence mode. A count is only registered when photon simultaneously strike both photocathodes. Thus, radiation decay processes that generate a burst of photons will cause a signal to occur at both detectors essentially simultaneously and will be counted while dark and background pulses will not generate a count except in the relatively improbable case that occur simultaneously.

BL and *CL* is composed of single discrete photons, whereas in liquid scintillation spectrometry the discrete scintillation consist of a burst of photons, some of which goes to one PMT and some to another. When the LSC is used to measure *CL* and *BL*, the coincidence feature is normally disabled and the LSC is operated at a straight photon counting [19-21]. The discriminator level is adjusted to transmit single photon events and reject small dark pulses. In this mode, normally only one PMT is used although some LSC allow the use of both PMT in the non-coincidence mode, thus doubling the effective counting rate. However, automated LSCs e.g., Packard liquid scintillation counters (LSCs) model 2002, 3320 and 3330 are available which allow the measurement of initial burst *CL*. Some of these counters provide not only the advantage of automation, but also may be coupled with graphic recording devices which chart the *CL* curve directly from the duration of the reactions. The use of LSCs for *BL* and *CL* measurement has been considered in more detail in the LSC literature [22-23] and has been used for *CL* and *BL* measurement of enzymes and metabolites [24-26].

The use of LSCs for *CL* and *BL* measurement does have the disadvantages of no provisions for internal mixing of the sample and reagents. The other limitation of LSC is that as a photon counter it is limited to relatively low intensities. If the intensities are too high as in case of *BL*, the phenomena of pulse pile up and dead time loss occurs causing errors in counting rate and a non-linear relationship between intensities and total counts occurs.

Batch luminometers photometers

Numerous luminometers or photometers (light measuring devices) based on PMT have been designed for *CL* and *BL* measurement (Table 1). These devices provides with in *situ* mixing so that the reaction is initiated immediately infront of the detector, and a complete intensity curve can be recorded. Stanely [23] reviewed the instrumentation available for *CL* and *BL* measurement. These devices gives high sensitivity, good reproducibility, convenience and speed as compared to LSCs. The basic design of all luminometers given in Table-1 is the same as shown in Fig. 3, that is the injection of reagents into a reaction vessel or cuvette containing the sample, positioned in front of the PMT. The difference lie in the arrangement of mixing reagents and sample and in the presentation of results. The essential components of luminometer are:

Table 1: Commercially available luminometers

Model	Company
Biolumat LB9500	Berthold Laboratorium.
Biometer 760	Du-Pont.
Chem-Glow Photometer	American Instrument Company.
Photometer Pico-ATP	Jobin Yvon SA
Luminometer 1250	LKB Wallac.
Luminescence Analyzer 302, 340/50	Marwell International AB
Automated Luminescence Analyzer	Alpkem Corp.
Pico-Lite 6100	Packed Instrument Co. Inc.
ATP-Photometer 100, 2000,3000	SAI Technology Inc.
ATP-Photometer 20	Turner Designs.
Automated Luminescence Analyzer	Klpkem Corp.
Automated 101, Moonlight 201, 301, 401	Analytical Luminescence Lab.
Bioluminescence Analyzer XP-200-2	Skan AG
Lumitran 2000	New Brunswick Scientific Co.Inc.
Surface Reaction Photometer.	Vitatect Corp.
Lumacounter Biocounter 1070, 2000, 2080	Lumac.

1. Cuvette

This is the reaction vessel and is usually a disposable cylindrical tube constructed from glass or polystyrene. Although it can contain upto a few ml of reagents, the typical reaction volume is 0.05-0.5

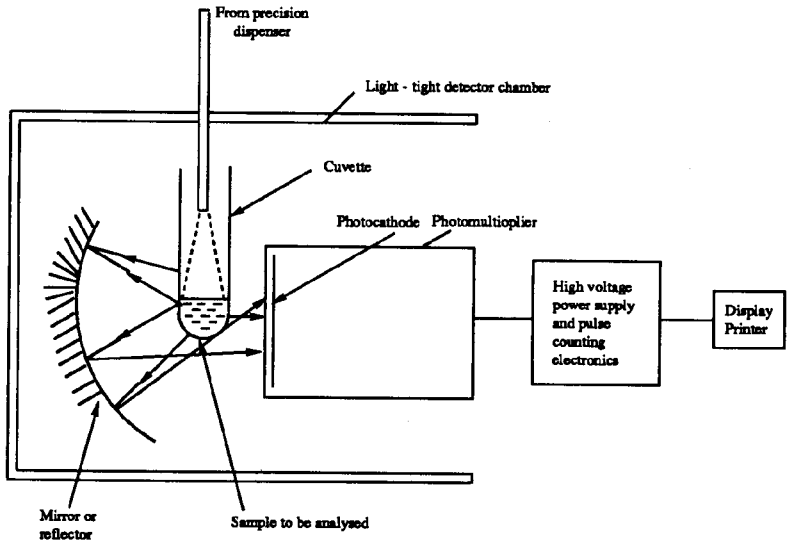

Fig. 3: Basic design of conventional luminometer.

ml. The ratio of cuvette length to diameter is generally around 5:1, to obviate the problem of the injection stream hitting the walls of the cuvette prior to reacting the other reagent which would result in less than adequate mixing.

2. Detector

This is the heart of the photometers. The detector frequently used is the PMT which must be driven by a very stable high voltage power supply. The PMT signal can be obtained in two distinct ways [27]. First there is the photon counting mode where the PMT produces a small pulse of electricity when the photocathode responds to a photon. By counting those pulses or measuring their rate of production, the amount of analyte can be calculated. In the other signal moded, the anode current of PMT is measured. The singal is registered in conjunction with an electric time constant to produce a smooth signal which is particularly necessary at very low signal levels. Microprocessors have been incorporated into some luminometers in order to provide the required flexibility in data handling (e.g., the Lumac 2010 is available linked to a "Pet" micro-computer).

3. Mixing

This is an important aspect of many analytical systems. In *CL* and *BL* reactions it is particularly important because these reactions are transient and the mixing may effect the rate of light output and thus peak light. Most luminometers do not have any mixing devices, apart from the mixing achieved by forceable injection of one reagent into sample exception to this is the LKB luminometer 1250 which has a mixing device as an optional extra.

Detector chamber

This is a light tight chamber protecting the cuvette and PMT from stray light. The cuvette is placed directly infront of the detector. Photons emitted as *CL* and *BL* disperse in all directions and only a fraction will proceed directly towards the detector (PMT). To minimize the loss of other photons the walls of the chamber are usually mirrored in order to minimize the collection of photons at the photocathode. The chamber must be completely light tight during measurement and must be capable of being open easily to insert or remove the cuvette.

Temperature Control

BL and *CL* reactions has a temperature optimum between 25°C to 28°C. Most luminometers do not have this facilities. Some have present and selectable temperature for the reaction chamber, e.g., Lumac Bio-counter 2080 (25,30,37°C). Some allows selection of a temperature within a specific range, e.g., Pico-Lite (5- 44°C).

Automation

This is generally inadequate in most instruments and are designed for a single batch, but some are capable of processing a small batch of samples e.g. The Pico-Lite (Packard Instruments) will take upto 6 samples [28].

Disadvantages of commercial luminometer

Although batch luminometers have widely been used for the determination of enzymes and metabolies [29-31]. There are disadvantages associated with the use of these device for analytical purposes. Firstly, the relative standard deviations obtained in assaying enzymes and metabolites using these devices typically greater than 5%. The reason for poor reproducibility is that most luminometers do not have mixing devices apart from the mixing achieved by forceable injection of one reagent into another. The purpose of injection is not only to add reagents but also to mix them quickly and reproducibly. Thus, one of the main disadvantages of commercial instruments is the lack of precision. Another disadvantage of commercial instruments is the detector chamber in which the light emitting reaction occurs in a curvette placed in front of the detector. The geometry of cuvette is such that only a fraction of the photons emitted as a result of any reaction fall on the photocathode and the rest of the photons emitted are lost in other directions. Thirdly, the assay principle in batch procedures is such that it gives low sample throughout and thus is time consuming. Finally, all these luminometers are designed to be used with soluble bioluminescent and chemiluminescent reagents. This will make the assay very expensive.

Future Trends

The conventional instrumentation for monitoring *CL* and *BL* reactions are the LSC and Luminometers based on batch procedures. Batch

procedures can give poor reproducibility and are less convenient specially using immobilized *CL* and *BL* reagents. A possible development in the foreseeable future is the introduction of commercial luminometers based on continuous flow techniques rather than batch techniques. This would undoubtedly stimulate interest in analytical methodologies using chemiluminescent and bioluminescent reactions.

References

1. C.T. Peng, 1975. In "Chemiluminescence, in Liquid Scintillation" (A.A. Naujaim, C. Ediss, and L.I. Weibleds. eds.), Science and Technology, Academic Press, New York, p. 315.
2. Luminescent Assay, Perspectives, 1982. In "Endochrinology and Clinical Chemistry," (J.S. Woodhead, I. Weeks, A.K. Cambell, M.E. T. Ryall, R Hart, A. Richardson and M. Pazzagli eds.) Raven Press, New York, p. 79.
3. U. Isacsson and G. Wettermark, *Anal.Chim.Acta.*, **68**, 399 (1974).
4. H.R. Schroeder and F.M. Yeager, *Anal.Chem.*, **50**, 1114 (1978).
5. R.W. Abbottt, A. Townshend and R. Gill, *Analyst.*, (London), **111**, 635 (1986).
6. P.T. Herring, 1978. "Bioluminescence in Action" Academic Press, New York.
7. H.H. Seliger, W.D. McElroy, *Proc.Natl. Acad.Sci.*, U.S. **52**, 75 (1964).
8. J.W. Hasting, W.H. Riley, J. Massa, *J. Biol.Chem.*, **240**, 1473 (1965).
9. L.J. Kricka, in ference 2, p. 11.
10. A. Lundin, A. Myhrman, G. Linfers, 1982. In "Bioluminescence and Chemiluminescence, Basic Chemistry and Analytical Applications, (M.A. Deluca, W.D. McElory, eds), Academic Pres, New York, p. 453.
11. W.F. Seitz and M.P. Neary, *Methods, Biochem. Anal.*, **23**, 161 (1976).
12. B.L. Strehler, *Methods. Biochem.Anal.*, **16**, 99 (1968).
13. K. Green and L.J. Kricka, *Talanta.*, **31**, 173 (1984).
14. E.L. Wehry, *Anal.Chem.*, **52** , 266 (1980).
15. E.L. Wehry, *Anal.Chem.*, **54**, 131R (1982).
16. T.J.N. Carter, L.J. Kricka, D.G. Bullock, R.A. Bunce, and T.P. Whitehead, 1979. In "Proc. International Symposium on Analytical Application of Bioluminescence and Chemiluminescence," (E. Schram and P. Stanley, eds) State Printing and Publishing, Westlake Village, California, p. 637.
17. M.A. Ryan, R.J. Miller, J.D. Jr. Ingle, *Anal.Chem.*, **50**,(13) 1772 (1978).
18. A. Stimson, 1974. In "Photometry and Radiometry for Engineers," Inter-Science, New York.
19. E. Schram, 1970 In "The Status of Liquid Scintillation Counting," E.D. Bransome, Grune and Stratton, New York, p. 129.
20. P.E. Stanley and S.G. Williams, *Anal.Biochem.*, **29**, 381 (1969).
21. P.E. Stanley, *Anal.Biochem.*, 441 (1971).
22. P.E. Stanley, 1974 "Liquid Scientillation Counting," (M.A. Crook and P. Johnson eds), Vol. 3, Heyden and Sons Ltd., p. 253.
23. P.E. Stanley, 1982, "Instrumentation, in Clinical and Biochemical Luminescence," (L.J. Kricka and T.J.N. Carter eds) Vol. 3, Marcel Dekker, Inc. New York, p. 219.
24. P.E. Stanley, In reference 22, p.421.
25. E. Schem, and H. Roosens, 1972. "Liquid Scientillation Counting," (M.A. Crook, P. Johnson and B. Scales eds), Heyden and Sons Ltd, London, p. 115.
26. K. Van Dyke, 1974. In "Uses of the Liquid Scientillation Counter for the Measurement of Bioluminescence and Chemiluminescence Reactions," Packard Tech. Bull.
27. G.L. Picciolo, 1979. In "Methods in Enzymology" (D.A. Nibley, E.W. Chappelle and M.A. Deluca, eds.), Vol. 75, Academic Press, New York, p. 550.
28. L.J. Kricka, 1982. "In Luminescent Assays, Perspectives in Endochrinology and Clinical Chemistry," (M. Serio and M. Pazzali eds.) Raven Press, New York, P. 11, (1982).
29. L.J. Kricka, G.K. Wienhausen, J.E. Hinklly and M. Deluca, *Anal.Biochem.*, **129**, 392 (1983).
30. A. Lundin, A. Richardson and A. Thore, *Anal.Biochem.*, **75**, 611 (1976).
31. J. Ford, and M. Deluca, *Anal.Biochem.*, **110** 43 (1981).