

## PHOTOMETERS FOR MEASURING SINGLE PHOTON EVENTS

### A DISCUSSION OF THEIR PRESENT DESIGN STATUS WITH SOME COMMENTS ON THE NEED FOR A CONVENIENT LIGHT STANDARD

Philip E. Stanley\*, John G. Vossen\*\* and  
Seppo E. Kolehmainen\*\*\*

- \* Consultant Scientist, Cambridge, England
- \*\* Lumac/3M b.v., Schaesberg, The Netherlands
- \*\*\* 3M Company, St. Paul, Minnesota, U.S.A.

#### ABSTRACT

Ten years ago there was only a handful of luminescence photometers on the market and many users had to construct their own instruments. Today, worldwide, around fifteen companies are manufacturing approximately thirty different photometers. Thus the potential user has now a wide choice. An extensive review of these instruments has recently been published (Stanley, P.E., "Clinical and Biochemical Luminescence", Eds. L.J. Kricka and T.J.H. Carter, Marcel Dekker, New York, pp. 219-160, 1982,). However, in this short presentation, we discuss in general terms the salient and important features currently available in this group of instruments.

The following topics will be broached :- cuvettes (size, shape, optical characteristics, mixing of reagents), detector, (photo-multiplier operated in pulse or current mode, types used, background, sensitivity), detector chamber (shape and size), temperature control requirements (detector(s), cuvette(s), reagent(s), type and range of control), injection of reagent(s) (pumps, syringes), sensitivity (definition and present usage of the term), flow monitoring, automation (including preparation of samples, software, hardware) and processing of data.

The topic of and need for a standard light source will be discussed in terms of variation in photomultiplier response, wavelengths of light emitted from sample, the geometry of the typical sample and the geometry of the detector chamber.

## INTRODUCTION

The techniques of analytical luminescence or luminometrics and scintillation counting of radionuclides emerged at the same time in the early-mid 1950's and scintillation analysis developed rapidly into routine use, while luminometrics grew only slowly because of the lack of purpose-built instruments, commercial reagents and routine methods. As a result of the ready availability of liquid scintillation counters in many laboratories, these instruments were often used for research and specialized applications of luminometrics<sup>1</sup>. However, they do not have all the features required for such analyses, e.g. in-chamber injection, and such instruments were generally in heavy demand for radionuclide work. Some workers built their own instruments, most of which were based on measuring the light signal as a current derived from the detector, a photomultiplier. However, from the onset a few workers counted pulses rather than measuring the current from the photomultiplier, and thus quantified the photons emitted from the analytical reactions<sup>2</sup>. A number of workers have concluded that photon counting is the more sensitive technique and it has now transpired that many practical applications require the highest available sensitivity.

## EARLY INSTRUMENTS

The first commercial instrument, built around 1970 specifically for luminometrics, was the Du Pont Biometer. This unit as well as the Aminco Chem-Glow and SAI (previously JRB) instruments which followed shortly afterwards, were all based on current measurement and designed for ATP measurement with firefly luciferase. It was only as recently as 1978 that Lumac B.V. designed and marketed the first photon counter, and this unit was also marketed by Berthold. The lack of space here precludes further discussion but more details may be had in the extensive review published recently<sup>3</sup>.

The sensitivity offered by this instrumentation allows the realization of rapid microbiology based on the intrinsic ATP-content of microorganisms, i.e. urine<sup>4</sup> and milk<sup>5</sup> and for studies of phagocytosis<sup>6</sup>, as well as a multitude of metabolites<sup>7,8</sup>.

## PRESENT INSTRUMENTATION

Today, the increasing number of methods being developed in luminometrics, based on both chemiluminescence and bioluminescence, means that it is necessary to fulfill all the instrumental requirements in terms of flexibility of function and ease of operation.

In the present paper we provide, albeit briefly, the salient features required for today's and tomorrow's applications and a diagrammatic lay-out of an instrument is given in Figure 1.

### Cuvettes

- Constructed from clear, disposable plastic.
- Easy to handle and fill.
- Shape consistent with rapid mixing by injection.
- Optimal light collection and transmission for all wavelengths (400 - 800 nm).
- Free from static electricity, phosphorescence and fluorescence.
- Suitable for reactant volumes between 0.1 to 1.0 ml.

### Detector

- Photomultiplier selected for very low noise and high sensitivity at room temperature.
- Usually a 1 or 2 inch photocathode employed.
- Generally the photocathode is bialkali.
- Spectral response should ideally reflect the emission spectrum of the light reaction. Generally this is not attainable. Figure 2 shows some responses and spectra and is given to illustrate this problem.

### Detector measuring modes

There are three distinct measuring modes which are illustrated in Figure 3.

#### Peak height

Historically, this method was the most frequently used since the reagents employed were generally crude enzyme preparations such as firefly luciferase extracts. Today, this mode is mainly used when measuring the luminol/hydrogen peroxide system for chemiluminescence immunoassays (see reference (8) for examples).

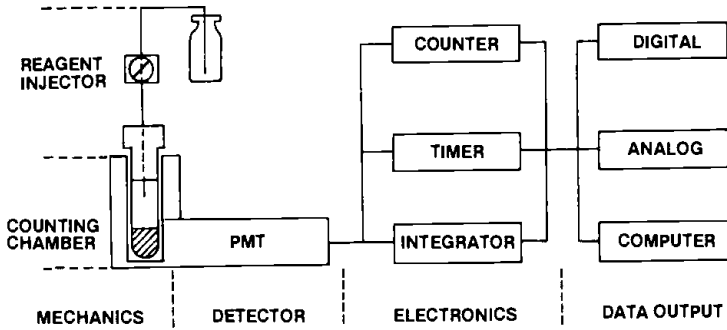


FIGURE 1. Schematic diagram of a luminescence photometer.

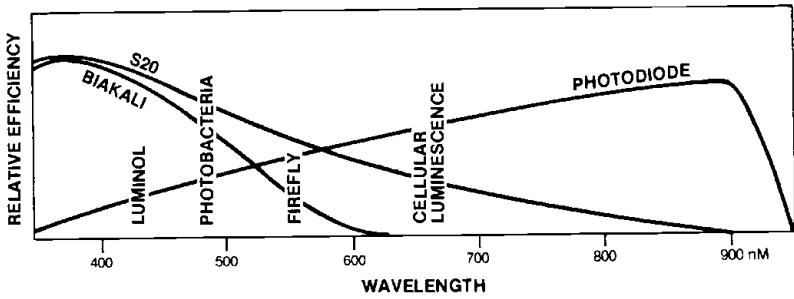


FIGURE 2. Relative sensitivity of two types of PMT's and that of the silicon photodiode at different wavelengths of light. Wavelengths of four bio- and chemi- luminescence systems are also given.

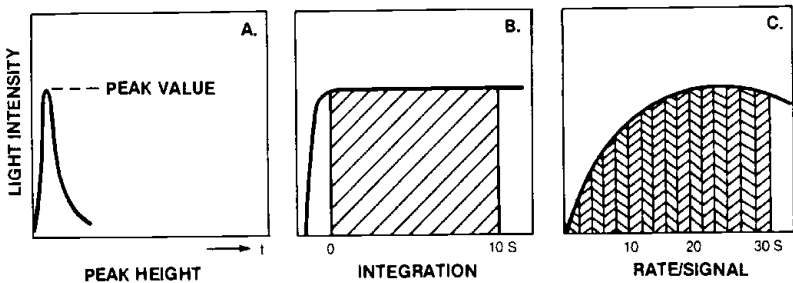


FIGURE 3. Different measuring modes of luminescent light. peak height (A), integration (B), and rate/signal (C). Reaction kinetics represent the luminol, firefly ATP assay and photobacterial NADH assay, respectively.

## Integration

With the availability of purified reagents and the "constant" light signal (ATP) the integration mode is now that most frequently used. The signal is integrated for a fixed time interval, often 10 seconds, after a suitable delay following the reagent mixing. The delay can be just a second or so.

## Rate

This mode is used to follow the kinetics of the light production, for example in phagocytosis, oxidative phosphorylation in mitochondria or the measurement of enzyme kinetics. In instruments having current measurement, this is called the signal mode.

## Counting chamber

This part of the instrument includes the cuvette, the photomultiplier and reflective surfaces together with the injector if present.

- It must be perfectly light-tight.
- It must permit easy transfer of the cuvette in and out of the instrument.
- It must optimize the collection of light from the cuvette and focus it on the photocathode by reflectors if necessary.
- Temperature control may be possible. Usually, this is done by heating alone (operation above ambient temperature). Chamber design should minimize the time taken for the sample to reach this temperature.<sup>6</sup> A fixed temperature is used for enzyme assays and living cells.
- Coefficient of reflectivity may vary with wavelengths of light and may also be affected when the chamber becomes dirty.
- The chamber should be easily cleaned.

## Injection

- The device must deliver reproducible and accurate volumes of reagent and be free from the hazards of contamination.
- The last injection to produce the light reaction is usually in the darkened counting chamber. Injection should be with a force to give sufficiently good mixing but should avoid splashing the walls of the cuvette.
- Injection of several reagents by automation may be possible and has been reported<sup>9</sup>. The benefits accruing from automatic injection include:

1. Saving time.
2. No operator fatigue.
3. Accurate timing becomes possible.
4. Good coefficient of variation in results.

Reagents to be injected may be held at room temperature or may be cooled when they are labile. Usually, it is necessary to hold them in a dark bottle to obviate unwanted actinic effects.

#### Sensitivity

Most instruments are not calibrated against an absolute light standard but against an ATP standard and the firefly reaction or a secondary light source, such as liquid scintillation solution<sup>10</sup>. Detection limits for ATP, which have been commonly published, are around  $10^{-13}$  grams of ATP, but in most real life situations only  $10^{-12}$  grams is obtainable. Sensitivity is often defined as the amount of analyte required to give a signal equal to twice the standard deviation of the series of blank determinations. Clearly the blank is the paramount influence in this and is made up of the instrument blank as well as the reagent blank and the analyte blank as well.

#### Automation, sample and data processing

Several automated systems are now becoming available for discrete sample analysis<sup>3</sup>, which involves sample transport in cuvettes via a serpentine chain or turn table, reagent addition and incubation at specific times. Operator time and error is reduced substantially. A number of specially made flow systems have also been described<sup>3</sup>. Interaction with the systems is generally via a keyboard, sometimes alphanumeric, which also permits the selection of data processing via a menu selection of inbuilt programs. Printing and videoviewing of results is available as well as plotting of data, such as might be obtained in studies of phagocytosis.

#### THE NEED FOR A STANDARD LIGHT SOURCE

Such a source is required to calibrate, check, service and compare instrumentation. Such a system is not available presently and we wish to indicate the desirable characters that it should possess:

1. Its geometry should be similar to a typical sample and be adjustable to reflect that typical sample.
2. The spectrum of photons emitted from it should be similar to the typical sample. This means there may have to be more than one standard to cover the emitting species involved (firefly 560 nm, bacterial luciferase 490 nm, luminol 425 nm).
3. Light output should be constant.
4. The source should require little or no preparation prior to use.

How close can we come presently to these ideals? If the standard is a liquid, then sample geometry can be readily mimicked. This is not possible with a solid source such as a photodiode. The liquid scintillation solution has been used, but as its scintillations are of a multi-photon nature, they should ideally be quenched to single photon events<sup>10</sup>. However, the wavelength of the light produced (380 nm peak), is substantially removed from that of the firefly luciferase system, the most commonly used assay (560 nm). Unfortunately, the firefly emission spectra is at part of the quantum efficiency curve of the alkali photomultiplier, where that curve is falling away very rapidly, and so it is particularly sensitive to this parameter (see Figure 2).

#### CONCLUSION

Instruments for luminometric analyses have been developed by a number of manufacturers in the past decade and some are rather sophisticated. They have not yet reached the degree of development seen in liquid scintillation instruments, whose physical make-up varies from model to model. The matter is not helped when one considers that the characteristics of the reagents used to specify the instruments are often manufacturer dependent.

#### REFERENCES

1. P.E. Stanley, "Analytical bioluminescence assays using the liquid scintillation spectrometer. A review" in "Liquid Scintillation Counting", Vol. 3, M.A. Crook and P. Johnson (eds.), Heyden and Son, London, 253-272, 1974.
2. B.L. Strehler and J.R. Totter, "Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determinations", Arch. Biochem. Biophys. 40, 28-41, 1952.

3. P.E. Stanley, "Instrumentation" in "Clinical and Biochemical Luminescence", L.J. Kricka and T.J.H. Carter (eds.), Marcel Dekker, New York, 219-260, 1982.
4. P.W. McWalter and C.A. Sharp, "Evaluation of a commercially available semi-automated bioluminescence system for bacteriuria screening", *Eur. J. Clin. Microbiol. I*, 223-227, 1982.
5. R. Bossyut, "A 5-minute ATP platform test for judging the bacteriological quality of raw milk", *Neth. Milk Dairy J.* 36, 355-364, 1982.
6. R.C. Allen, "Chemiluminescence and the study of phagocyte redox metabolism", *Adv. Exptl. Med.* 141, 411-421, 1982.
7. L.J. Kricka and T.J.N. Carter, "Clinical and Biochemical Luminescence", Marcel Dekker, New York, 1982.
8. M. Serio and M. Pazzagli, "Luminescent Assays. Perspectives in Endocrinology and Clinical Chemistry", Raven, New York, 1982.
9. S.E. Kolehmainen, H. Vanstaen and J. Vossen, "Utilization of multi-injection for sample processing and automatic internal standardization (AIS) in luminometry" in "Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications", M.A. DeLuca and W.D. McElroy (eds.), Academic Press, New York, 705-708, 1981.
10. E. Schram, H. Van Esbroeck and H. Roosens, "Single photon standard for luminescence measurements" in "International Symposium on Analytical Applications of Bioluminescence and Chemiluminescence, Brussels, 1978", E. Schram and P. Stanley (eds.), State Printing and Publishing, California, 687-688, 1979.