

MEASUREMENT OF BIO- AND CHEMILUMINESCENCE - LIQUID  
SCINTILLATION COUNTERS VS. DEDICATED LUMINOMETERS

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INTRODUCTION

Bio- and chemiluminescence has been a topic at previous conferences on liquid scintillation counting<sup>1-5</sup>. Of course, the discussion was centered on the use of LS counters, and therefore, limited to areas of bio- and chemiluminescence where LS counters could be used. Excellent books covering the entire field of bio- and chemiluminescence (from now on "luminescence" only) have recently been published<sup>6-8</sup>. To allow a better understanding of the specifications needed in luminescence instrumentation, an overview of the main areas of luminescence assays will be given first.

ATP-determinations, Microbiology

The determination of ATP (adenosine triphosphate) via the firefly luciferine/luciferase bioluminescence is well-known<sup>6-8</sup>. With presently available high-purity reagents and quality instrumentation, as little as 0.1 pg ATP can be detected. In practice, the sensitivity is limited by ATP-contaminations on dispenser tips, vials, vial phosphorescence and their like.

The main area of practical interest is not the assay of ATP as such, but the determination of microbes via their ATP content. This requires the samples to undergo some procedure which first releases the ATP from the cells. This analysis is complicated by the fact that many samples contain somatic cells in addition to microbial cells, so that the ATP-contribution from somatic cells has to be eliminated. Today, luminescence assays are promoted in clinical and industrial microbiology. An example is the diagnosis of bacteriurea, the bacterial infection of the urinary tract. Bioluminescence allows rapid screening of the vast majority of negative samples to eliminate those with less than about  $5 \times 10^4$  colony-forming units per mL.

Positive samples have to be analyzed further with regard to bacterial identification and antibiotic susceptibility. Two commercial bacteriurea screening tests based on bioluminescence have already obtained approval by the U.S. Federal Drug Administration.

#### Luminescence Immunoassay

Many alternatives to radioimmunoassay (RIA) have been advocated, the main motives being:

1. Regulations concerning handling, storage, and disposal of radionuclides are frustrating to many users
2. The shelf-life of labelled antigens or antibodies is limited (half-life of  $^{125}\text{I}$  is approx. 60 days).

Enzyme immunoassay is already established as one of the so-called "non-isotopic" immunoassays, and fluorescence immunoassays are another alternative. In general, RIA is still the most precise and sensitive immunoassay, so that any non-isotopic immunoassay is usually compared against RIA. Recently, luminescence immunoassay has emerged as one of the most promising non-isotopic immunoassays. Luminescent substances have been used in different ways, either directly replacing the radioactive label, or indirectly by labelling antigens or antibodies with enzymes or cofactors which can take part in a luminescence reaction. Unlike radioactive decay, the emission of light in bio- or chemiluminescence does not occur spontaneously, but is initiated but what may functionally be called an activator. It is interesting to observe the kinetics of chemiluminescence reactions as applied in chemiluminescence immunoassays. Using proper reagent concentrations, the maximum light emission is typically reached in 1 s after activator injection and drops quickly thereafter, so that a total measuring time of 10 s is adequate. In view of the relatively fast kinetics, it is mandatory that the activator is injected while the sample is already in the light-sealed measuring position in front of the photomultiplier, a condition which cannot be met by commercial automatic liquid scintillation counters.

The great promise of luminescence immunoassay is the potential to

surpass even RIA with regard to sensitivity. Using an acridinium ester label, Woodhead and Weeks have recently demonstrated a luminescence immunoassay for TSH (thyroid stimulating hormone) which is 50 times more sensitive than RIA<sup>13</sup>. Some combinations of luminogenic labels and activators are shown in Table 1.

Table 1. Some Lumigenic Labels and Activators used in Luminescence Immunoassay

Label	Activator	Reference
Luminol, Isoluminol	H <sub>2</sub> O <sub>2</sub> /microperoxidase	Schroeder et al. <sup>9</sup>
Firefly Luuciferase	ATP/luciferine	Wannlund et al. <sup>10</sup>
Acridinium Ester	H <sub>2</sub> O <sub>2</sub> /NaOH	Woodhead et al. <sup>11</sup>
Fluorecein- iso-thiocyanate	NaOCl	Berthold et al. <sup>12</sup>

Luminescence associated with cell stimulation:

Certain types of cells (mostly phagocytes) emit light when stimulated, e.g. when their defense mechanism is triggered. This light emission can be substantially "amplified" by adding luminol or lucigenine. One usually follows the kinetics of light emission of each sample over an extended period of time, up to 2 hours. The use of standard or modified liquid scintillation counters for this application has found excellent coverage at previous liquid scintillation conferences<sup>2-5</sup>.

#### SPECIFICATIONS FOR LUMINESCENCE INSTRUMENTATION

We are now ready to define specifications for - preferably automatic - luminescence instrumentation (14), and to discuss how these specifications are met with dedicated luminometers as compared with liquid scintillation counters.

## Photomultipliers

Two photomultipliers are required for LSC whereas only one is required for luminescence. If the coincidence gate is switched off, an LS counter is well-suited to count single-photon events. A low noise count-rate is more important for luminescence than for LSC, where coincidence eliminates the noise contribution to background almost completely. A typical background value for a good 1" photomultiplier selected for photon-counting is 180 cps at ambient temperature, and 70 cps when the cathode is cooled to 4°C. With a standard 2" bialkali photomultiplier as used in LSC, on the other hand, 1000 cps at ambient temperature are not uncommon.

**Spectral Sensitivity:** The bialkali cathode used in LSC, with its maximum quantum-efficiency around 400 nm, is well matched to the emission wavelength of liquid scintillators. Table 2 shows certain emission wavelengths occurring in luminescence and typical quantum efficiencies obtained with a standard bialkali and rubidium-bialkali photocathodes. The values shown represent average results and are subject to considerable individual fluctuation.

Table 2. Quantum Efficiency of standard bialkali and rubidium bialkali photomultipliers for different luminescence systems.

Luminescence System	Principal Wavelength	Quantum Efficiency (%)	
		Bialkali	Rubidium
Luminol/H <sub>2</sub> O <sub>2</sub>	425 nm	28	28
Bacterial Luminescence	495 nm	14	16
Firefly Luminescence	562 nm	3	6

**Cathode Size:** A 2" diameter is standard for LSC, and acceptable for

luminometry. However, a 1" cathode area is sufficient in luminometry, and might therefore be preferable since the noise count rate can be reduced by reducing the cathode area.

#### Temperature Control

An LS counter may work at ambient or cooled temperatures although several luminescence assays require sample-heating to 40°C. For research on chemiluminescence associated with cell stimulation, standard LS counters have therefore been modified to allow sample-heating<sup>15</sup>.

#### Automatic Reagent Injection

This feature is not available on LS counters, and not required for studies of chemiluminescence associated with cell stimulation. LS counters have been used to measure ATP<sup>16</sup>, but not in an automatic procedure requiring automatic reagent injection. The following procedure for clinical bacteriurea screening which will make the requirement for automatic reagent injection evident:

1. Aliquot of urine sample is pipetted into vial.
2. Addition of ATP-releasing agent for (only) somatic cells, mixed with an ATPase to destroy ATP from somatic cells.
3. Incubation for 10-45 minutes, according to individual protocol.  
Temperature ambient or above ambient (35°C)
4. Addition of ATP-releasing agent for microbial cells.
5. Addition of Luciferine/Luciferase, within 15-30 s after step 4, to minimize turnover of microbial ATP by ATPase.

For bacteriurea screening, three reagent injectors are needed in clearly definable positions of an automatic instrument, as will be described later. The purpose of injection is not only to add reagents but also to mix them quickly and reproducibly. This can be achieved through high-speed injection, or active mixing devices when long-term homogeneity of the sample is required.

## Quench Correction

The concept of quench correction, so familiar in LSC, can also be used in luminescence assays. Chemical quench would then be related to radiationless de-excitation of molecules caused by contaminants, instead of photon emission. Color quench is related to the absorption of photons within the sample, on their way to the photocathode.

External standardization could be accomplished by measuring the extinction of light through the sample cuvette by means of an external light source which has an emission wavelength as close as possible to the wavelength of luminescence. However, this procedure would only detect color quench. Therefore, the concept of automatic internal standardization has been introduced<sup>17</sup>, involving the following steps with the sample in the measuring position:

1. Addition of activator to the sample
2. Measurement of light output
3. Addition of a known amount of analyte (e.g. ATP)
4. Measurement of the additional light output
5. Calculation of efficiency and quench correction

Lead shielding, coincidence circuitry, external standardization and similar special features are not required in luminometers. Pulse height analyzers, which additionally have the negative effect of limiting the maximum count rate in luminometry to typically  $2 \times 10^6$  counts/min (random pulses), are also not required. Photon counters which do not have pulse height analyzers can measure up to at least  $10^6$  counts/s.

## Vials

Sample volumes between 0.2 and 1 mL cover most applications. The standard LSC vial seems to be oversized for luminescence work. Most vials used in luminescence have diameters between 6 and 12 mm. As in LSC, smaller vial diameters lead to smaller path lengths for the emitted photons on their way to the cathode, and therefore to less color-quenching.

## Number of samples

A sample number of 300 has become well-accepted for beta or gamma-counters, and it has also turned out to be reasonable for automatic luminescence analyzers in microbiology or immunoassay work. The situation is different for cell stimulation studies. A six-channel luminometer measuring 6 samples simultaneously has been designed<sup>18</sup>. It is also possible to follow the light output of a limited number of samples which are cycled and measured repeatedly. Anderson and Brendzel<sup>19</sup> have designed a special luminometer for 12 samples contained in a carousel requirement range from 6 to 50 samples in cell stimulation studies.

## SINGLE PHOTON STUDIES

Before describing the design of modern automatic luminometers, I would like to discuss the basic principles of single photon counting<sup>20-23</sup> more accurately and to compare this method with the conventional d.c. measurement techniques used in photometry.

When a photocathode is exposed to single photons (low-level light), single photoelectrons are released from the photocathode. In a photomultiplier, each photoelectron hitting the first dynode produces an average of  $m$  secondary electrons,  $m$  being the secondary-emission ratio, or gain, of the first dynode. This process is repeated throughout the series of dynodes, until a fairly large charge pulse arrives at the anode. For 10 dynodes and  $m = 4$ , the average pulse height at the anode would be  $1.68 \times 10^{-13}$  Coulomb. The ratio of photoelectrons released to the number of photons incident on the cathode area is called quantum efficiency, which has to be distinguished from photon counting efficiency, the ratio of photon originated output pulses to the number of photons incident on the cathode area. The photon counting efficiency is lower than the quantum efficiency because of essentially 3 reasons:

1. Incomplete collection of photoelectrons on first dynode,
2. Small probability of photoelectrons hitting the first dynode to produce zero secondary electrons,
3. Electronic circuitry not optimized, or not operated at the optimum

condition.

With the best modern equipment, the photon counting efficiency approaches the quantum efficiency. The pulse-height distribution for single photoelectron pulses obtained at the anode is typical as in Figure 1. The width of the peak is related to the statistical nature of secondary-electron emission on the dynodes, especially the first dynode. For this reason, photomultipliers with very high first dynode gain have been developed. As an example, the RCA 8850 has a first dynode gain of 35 using a Gallium phosphide dynode, while the gain of standard CsSb or CuBe dynodes is only around 4.

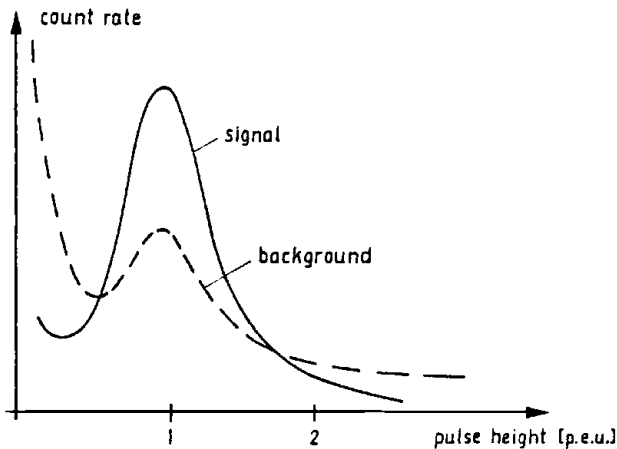


Figure 1. Pulse height distribution for single photons and background, exhibiting maximum at 1 p.e.u. (photoelectron units). Background is shown on a higher sensitivity scale.

As a first approach, Poisson-statistics have been applied<sup>24-26</sup> to calculate the shape of the single photoelectron peak. But mainly due to inhomogeneities of the dynode surface, and to fluctuations in the collection efficiency from dynode to dynode, the peak is generally much wider than attributable to a pure Poisson distribution. According to Morton<sup>27</sup>, the relative FWHM is expected to be:

$$\Delta \approx 2.36 \left( \frac{g}{g_1(g-1)} \right)^{1/2}$$

with  $g_1$  being the first dynode gain and  $g$  the mean dynode gain. Our results generally agree well with this formula. When a photomultiplier is exposed to low-level light, the output pulses observed over a longer term period can be classified in three amplitude-ranges (Figure 2).

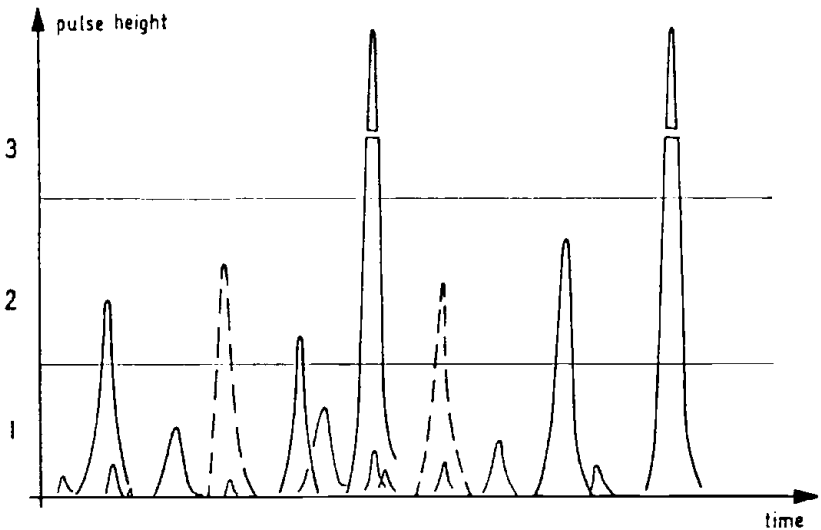


Figure 2. Output pulses from photomultiplier falling into 3 pulse height ranges: (1) dynode noise etc. (2) signal plus cathode noise, (3) high energy events.

- Range 1: Small-amplitude pulses representing dynode noise, leakage currents, photoelectric emission from dynodes (28) etc.
- Range 2: Photoelectrons (signal) and thermal electrons (noise) from photocathode.
- Range 3: High amplitude pulses, multi-photoelectron events from cosmic radiation, radioactivity within the photomultiplier, interacting through Cerenkov radiation.

In a photon counter, a lower discriminator threshold is set to exclude the low-amplitude range 1.

A plateau curve of a good photomultiplier when exposed to low-level light is seen in Figure 3. The background plateau curve is steeper than the photon plateau, because of the added contribution of low and high energy pulses. If background constituted thermal electrons from the cathode alone, both its pulse height spectrum and the plateau curve would be identical in shape with that for photons.

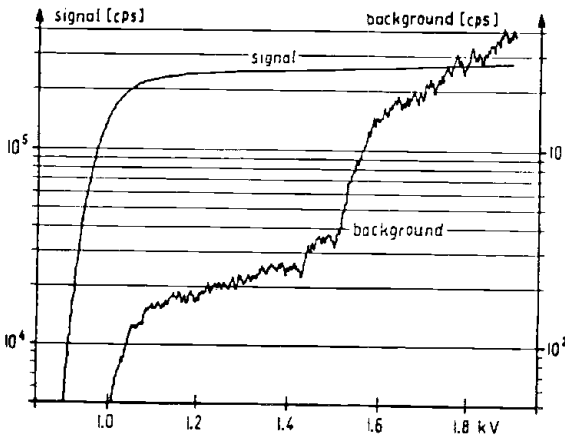


Figure 3. Photon counter plateau curve for signal and background.

#### Single photon counting compared to d.c. measurement

In conventional photometers, the output of the photomultiplier is integrated, leading to a statistically fluctuating d.c. signal when exposed to low-level light. The fluctuation is essentially due to the statistics of photoelectron emission, and is called "shot noise". The signal-to-noise ratio of a photometer in the d.c. mode is inferior to that of a photon counter because:

1. D.C. measurement does not discriminate against low-amplitude pulses
2. In d.c. measurement, the contribution of high amplitude pulses to the output current is proportional to the amplitude, as opposed to single photon counting where each pulse above the discriminator

threshold is standardized. In photon counting, it is furthermore possible to introduce an upper-threshold discriminator, thereby excluding multi-photoelectron events.

The only advantage of d.c. measurement is to be less limited by pile-up effects occurring at the higher end of the measuring range. The use of filters is one way to increase the linear range in photon counting.

### Stability

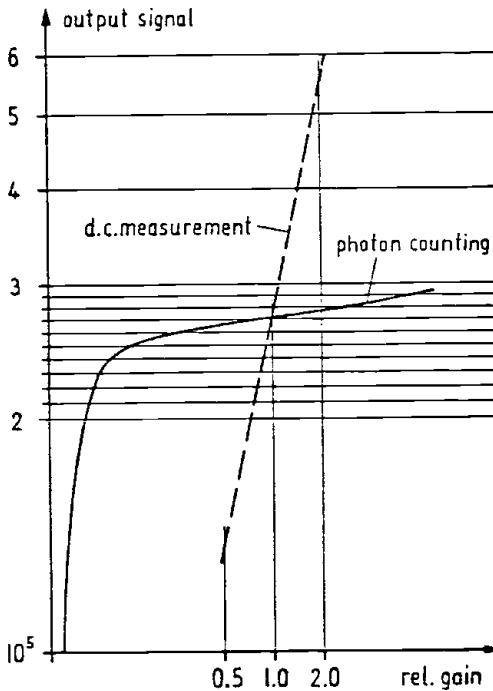


Figure 4. Effect of gain variations on output signal for photon counting vs. d.c. measurement.

The superior stability of single photon counting is explained in Figure 4. In relation to a chosen operating point, a change in high voltage by  $\pm 100$  V changes the output signal:

1. For a photon counter by typically about  $\pm 10\%$ , according to the plateau slope.
2. By about  $\pm 100/-50\%$  for d.c. operation, about proportional to the total gain change.

The same relationships hold true when the stability of both methods is compared with regard to dynode gain variations, these being the main causes for photomultiplier instabilities.

Finally, the statistical variation of the output signal, even considering true photoelectrons alone, is greater in d.c. operation than for photon counting for the following reason. In photon counting, each event producing a pulse height above a discriminator threshold contributes a standard signal, while in d.c. measurement each event contributes in proportion to its pulse height, causing additional variations of the output signal.

#### DESIGN OF A MODERN AUTOMATIC LUMINOMETER

During recent years, several commercial automatic luminescence analyzers have become available<sup>29</sup>, meeting many or most of the requirements set out before. As an example, let me describe the one one which I know best, an automatic luminescence analyzer with three on-line reagent injectors, handling up to 200 samples per batch. The detector is a photomultiplier with a rubidium bialkali cathode, operated in the photon-counting mode. The instrument operates under software control, using an Apple II E computer. All operating parameters are entered in response to questions displayed (in English) on the video monitor. Results are also displayed on the videoscreen and may be either printed out or stored on floppy discs.

#### Automatic sample changer and reagent injection

The sample carrier is a free-standing flexible chain. The vials have a 12 mm diameter and a height of 47-55 mm. Parts of the chain

may be unhooked at any time to be taken out for sample processing, e.g. centrifugation. One section of the sample changer (Figure 5) including the measuring position is precisely temperature-controlled, allowing on-line incubation. When entering this incubation zone, a first reagent may be dispensed automatically. A second dispenser may add another reagent to the sample immediately before the measuring station.

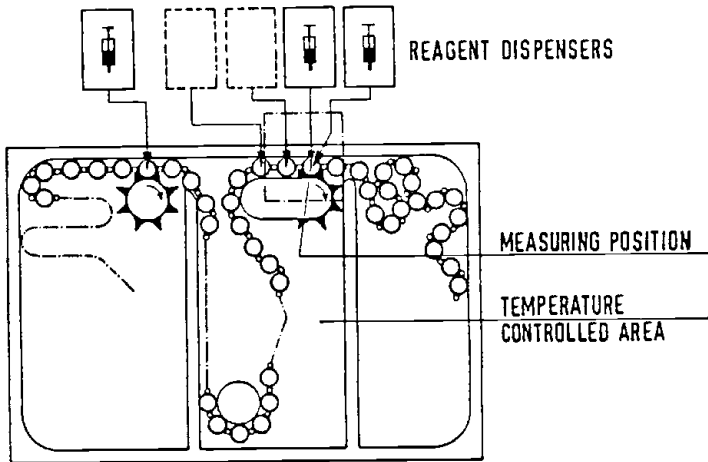


Figure 5. Automatic Luminescence Analyzer for 200 samples, with on-line reagent injection and incubation.

At the measuring position, the sample is lifted upwards into the measuring chamber (Figure 6) where the sample is placed in close proximity with the photocathode. The chamber is automatically sealed against outside light. The light collection is enhanced by a reflector.

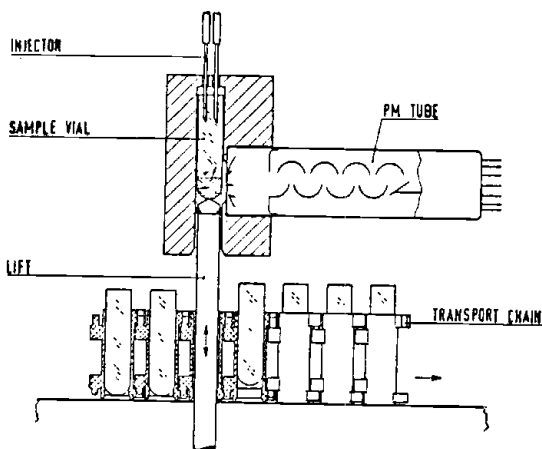


Figure 6. Measuring Station of Automatic Luminescence Analyzer.

When the sample is in the measuring position, a third injector may dispense the activator reagent, starting the luminescence emission. The light output is then measured over a preset time, typically 10 s, using a software multiscaler. This allows representation of the time-dependent light emission both in analogue and digital form, and offers the choice to integrate counts over the entire measuring time or over selected regions of interest only. A fourth injection port, also at the measuring position, allows the injection of an additional reagent. This would be needed for automatic internal standardization.

#### Reagent Injectors

All injectors have been designed to work with corrosive liquids like  $\text{H}_2\text{O}_2$ ,  $\text{NaOCl}$ , or  $\text{NaOH}$ , as used in chemiluminescence immunoassays. Reagent volumes, from 10 - 360  $\mu\text{L}$ , are freely selected via the computer. Different injection speeds may be selected, controlling the degree of reagent mixing in the vial.

#### Temperature Control

The temperature in the on-line incubation area can be selected to

be anywhere from 20 - 43°C, with an accuracy of 0.1°C. The incubation time is determined by the number of samples in the incubation zone, multiplied by the sample sequence time. Both factors can be varied, allowing effective incubation times up to and beyond 1 hour. Long-term incubations are performed outside the instrument. The temperature at the measuring station can be set from 20 to 43°C, independently of the incubation zone. The cathode of the photomultiplier is constantly cooled to 4°C.

#### Closed-loop Operation

For studies on cell stimulation, the instrument allows closed-loop operation (Figure 7). A selected number of chain-elements is coupled together forming a closed loop for up to about 60 samples. This closed loop is contained in the temperature-controlled zone. The computer program sorts the raw data into histograms showing the time-dependent light emission for each individual sample, in analogue and digital fashion, allowing integration over variable time windows and analyses of some curve parameters, like maximum: value, time of maximum, etc.

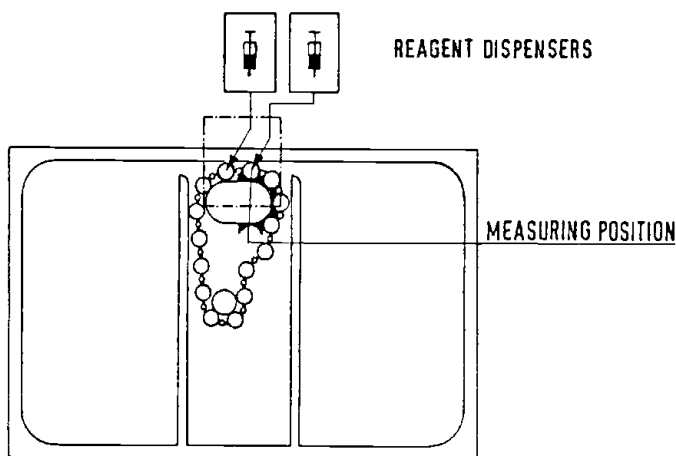


Figure 7. Closed Loop Operation of Automatic Luminescence Analyzer.

Light standards

This subject has been addressed by several authors<sup>1,30,31</sup>. We use green light-emitting diodes operated at very low voltages as relative standards, to determine photomultiplier and instrument performance, and as service tools.

#### CONCLUSION AND OUTLOOK

From what we know now, we can only agree with P. Stanley's statement<sup>29</sup>: "... with the availability of flexible luminometers at moderate cost, it seems likely that LSC use for this purpose will wane". For the future, one might envisage instruments designed to perform both LSC and luminescence under optimum conditions. This might be the subject of a contribution to the next conference on scintillation counting.

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