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Easy-to-Use Aids to a New Unifying Approach to Quantitative Luminescence Microscopy

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Work has already been reported indicating the attractions of certain easy-to-use, essentially stable, light standards, in forms suited to testing and calibrating the quantitative photometry of a wide variety of (chemi-)luminometers. Appropriate log/log plots of such calibrations not only offer great insight into the photometric performance of such instruments, but also often permit direct comparisons of the analytical results from the various instruments concerned. More recently, this approach has been extended to an analogous evaluation of simple fluorimeters, and this paper reports further application of the approach to fluoresence microscopy.

KEY WORDS: Light standards for microfluorimeters; photometric performance of microfluorimeters; evaluation of the practical fluorimetric equation.

INTRODUCTION

Modern microscopy is increasingly using highly sensitive electronic detector systems for recording and quantitatively analyzing luminogenically labeled items within particular fields of view. Luminogenic analytical methods offer particular advantages over the corresponding absorptiometric procedures for such purposes [1]. However, luminometric methods generally suffer from practical difficulties in calibration, using often unstable standards and instruments with arbitrary readouts [1]. These difficulties have been overcome in a variety of (chemi-)luminometers using a series of essentially stable light standards in different forms [2,3].

Recently, means and methods have been described extending this approach to measurements utilizing a full, practical relationship for fluorescence measurements using a very simple fluorimeter [4]. The present paper shows how such methods can be extended, with analogous advantages, to microfluorimeters.

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MATERIALS AND METHODS

Standard solutions of the 4-methylumbelliferone (MU) fluorogen were prepared as described elsewhere [5]. Biolink light standards have been described previously [2–4]. The cooled, slow-scan, charge-couple device system (CCD) was as reported earlier [6,7], as was the imaging photon detector (IPD) system [8]. The Hamamatsu 50-mm Argus camera system was available by courtesy of Hamamatsu Ltd. of Enfield, Middlesex, U.K. The Photonic Sciences Isis Detector/Nikon Microphot system was available through PTI Ltd. of Sheen, London.

Appropriate parts of focused images were selected, and the detected light is reported as photons per second.

RESULTS AND DISCUSSION

Equation (1) was derived [2,3] and proved useful in practical analyses employing, (chemi-)luminometers. In that equation, the actual light detected (L_D) is related to a solution of the luminogen of concentration c, depth

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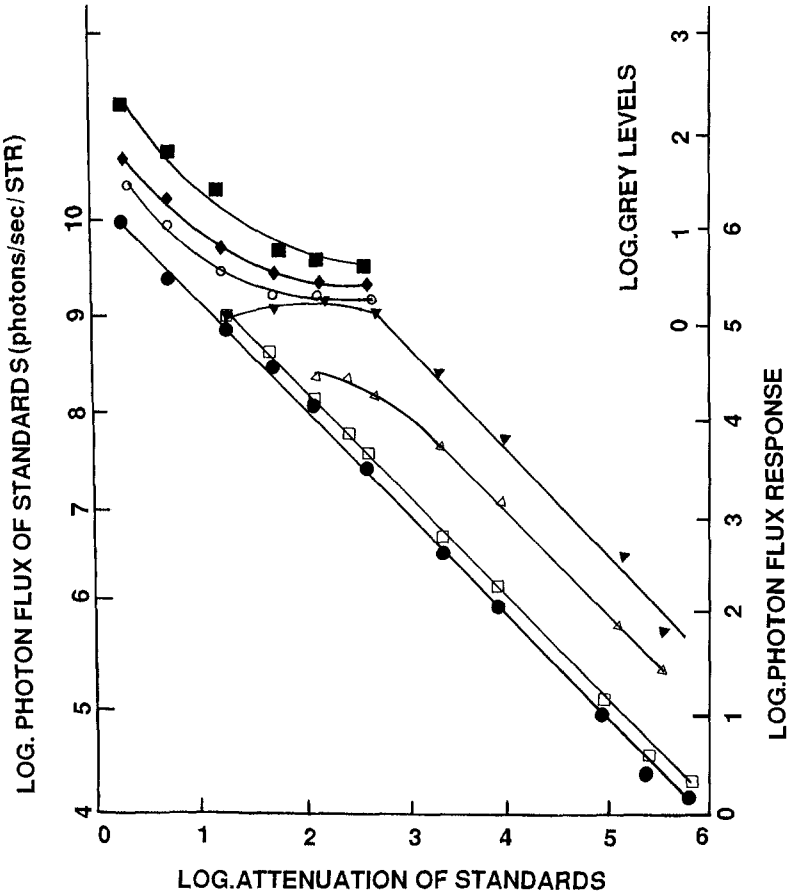

Fig. 1. Photometric performance of secondary sides of some microfluorimeters. Left ordinate: Logarithmic plots of photon fluxes of Biolink light standards (●) attenuated as indicated on the abscissa. Photon flux responses to the standards, by the secondary sides of the Argus (▲), IPD (△), and CCD (□) systems are indicated by the lower right ordinate scale. Corresponding log gray-level readouts from the ISIS/optics system [with magnifications of ×10 (■), ×20 (◆), and ×40 (○)] are indicated by the upper right ordinate scale. All the curves depicted were limited by electronic overload and statistical variations at their upper and lower ends, respectively.

d, and quantum yield Q, but also to the proportions of emitted light transmitted and detected (p_O and p_D, respectively):

$$L_0 = c \cdot d \cdot Q \cdot P_0 \cdot P_D \qquad (1)$$

where the product $P_D \cdot P_0$ is known as the Photon Detection Efficiency (PDE), and represents the proportion of emitted light detected by the instrument. Calibration of various luminometers with the light standard [2,3] led not only to evaluation of PDE values, but also to insight into the photometric stability of fluorimeters, for particular analytical problems. Accordingly, the full fluorimetric equation was derived [4]:

$$F_0 = 2 \cdot 3 \cdot e \cdot c \cdot d \cdot Q \cdot I_0 \cdot P_0 \cdot P_D \qquad (2)$$

where e is the extinction coefficient of the fluorogen, I_0 is the intensity of activating light, and the other parameters are analogous. The light standards can similarly be used to gain insight into the photometric parameters of the secondary sides of fluorimeters [4]. Moreover, measurements with a fluorogen of known Q can lead to a complete evaluation of Eq. (2).

Figure 1 shows log/log plots of calibrations of the secondary sides of some microfluorimeters. The different positions and extents of the ideal linear, unit slope portions of the curves are significant for assessments of the photometry of the systems; e.g., they yielded PDE values of about 0.7, 0.1, and 0.02% for the respective Argus, IPD, and CCD systems used. Note that curves toward the left of the space indicate less efficient pho-

tometries, and while the gray-level readouts of the ISIS system prevent other comparisons, the positions of the various ISIS curves reflect the relative efficiencies of their optics. As an illustration of the general approach, it is instructive to consider the assay of MU using the IPD imaging fluorimeter [8]. The lower limit of detection was 10 μM. This level showed no signs of photodegradation and gave a detected response of about 2.5E4 photons/s. Feeding also the figures of 1.8E4, 0.2, and 0.8 for e, d, and Q, respectively, into Eq. (2), these give an estimate of about 3E7 photons/s. for I_o. This is in accord with the observation that MU photodegradation typically becomes significant at activating intensities of about 1E13 photons/s [3]. In this case, the sensitivity of the assay is probably limited by stray light—commonly experienced in methods employing epiillumination [9].

A consideration of Eq. (2) shows that as the level of fluorogen in the sample is decreased, for a given requirement of detected fluorescence (F_D), provided the maximal PDE is employed, the only option is to raise I_o. Modern microscopes often have the potential to bring very high intensities of actinic light to bear upon specimens. The approach described here makes it possible to estimate and control activating light intensities. This is important since any fluorogen will probably photodecompose ("bleach") at some intensity of radiation. It has been shown [4] that steady-state fluorogenic assays of MU become impossible at irradiation levels above about 1E14 photons/s. Interestingly, however, Hirschfeld [10] used very high levels of activating radiation (about 1E22 photons/s) in a microfluorimeter to detect single molecules of a fluorogen (fluorescein) undergoing rapid photobleaching.

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