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Short Communication

Theoretical considerations on the fluorescence lifetime of labels for time-resolved fluorescence immunoassay

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Introduction

In the past few years there has been a rapid growth of interest in the technique of time resolution as a means of background rejection in fluorescence immunoassay. The concept was described in 1978 by Wieder [1], who reported a fluorescence lifetime for pooled plasma of less than 50 ns, and suggested that pyrene, with a fluorescence lifetime of about 100 ns, or europium tris-thenoyltrifluoroacetone, with a fluorescence lifetime of about 300 μs, would be a suitable label. Subsequently, a number of labelling procedures using long-lived fluorophores have been described [2–6]. The labels have fluorescence lifetimes ranging from about 10 μs to hundreds of milliseconds, and most are chelates of lanthanide elements.

The requirements for a long-lifetime fluorescent label are similar to those for a conventional fluorescent label, i.e. a high absorption coefficient and quantum yield and a large Stokes shift. However, the optimum value for the fluorescence lifetime is less understood. In this study a simplified model and a BASIC computer program are used to simulate the excitation and emission cycle, and calculate fluorescence intensities, for several fluorophores and for different excitation sources.

Several assumptions were necessary to simplify the calculations:

(a) The label is assumed to absorb independently of other solutes and to obey Beer's Law.
(b) Both label fluorescence and background fluorescence are assumed to decay according to a simple exponential law.
(c) The background fluorescence is assumed to have a lifetime of 50 ns.
(d) The absorption of one photon is assumed to lead to the formation of one excited state molecule.
(e) Decay of the excited state population of the long-lived fluorophore during the excitation pulse is assumed to be negligible.

(f) Excitation pulses are assumed to be rectangular for the calculation of excited state populations.
(g) Laser pulses are assumed to have neglible decay time.

The excitation/emission cycle was modelled using these assumptions. The relevant parameters are represented thus:

m	concentration (mol l^{-1}) of the label fluorophore
m_0	concentration (mol l^{-1}) of ground state label molecules
m^*	concentration (mol l^{-1}) of excited state label molecules
ϵ	molar absorptivity ($lmol^{-1}$ cm^{-1}) of the label
q	quantum yield of the label fluorescence
τ	lifetime (s) of the label fluorescence
N	Avogadro number = 6×10^{23}
h, w, b	height, width and breadth (mm) of the fluorimeter cell
p_0	light intensity (photons/s) of the excitation pulse
t_1	duration (s) of the excitation pulse
t_2	delay (s) between the end of the excitation pulse and the start of monitoring
t_3	duration (s) of monitoring.

The cell volume is $(hwb)/10^6$ l and the pulse repetition rate is $1/(t_1 + t_2 + t_3)$ per s. During excitation, the absorption of photons is described by Beer's Law:

$$p = p_0 10^{-\epsilon m_0 b} \tag{1}$$

where p is the flux of photons leaving the cell. The rate of absorption of photons by the label, and hence the rate of production of excited state molecules, is given by

$$\frac{\mathrm{d}}{\mathrm{d}t}\frac{(m^*Nhwb)}{10^6} = \frac{-\mathrm{d}}{\mathrm{d}t}\frac{(m_0Nhwb)}{10^6} = p_0\,(1 - 10^{-\epsilon m_0 b}); \tag{2}$$

expanding the exponential:

$$\frac{\mathrm{d}}{\mathrm{d}t}\frac{(m_0Nhwb)}{10^6} = -p_0\left[1 - \left(1 - 2.3\epsilon m_0\, b + \frac{(2.3\epsilon m_0 b)}{2!} - \frac{(2.3\epsilon m_0 b)}{3!} + \ldots\right)\right] \tag{3}$$

and if $(2.3\epsilon m_0 b)$ is much less than 1

$$\frac{\mathrm{d}}{\mathrm{d}t}\frac{(m_0Nhwb)}{10^6} = -2.3p_0\epsilon m_0 b \tag{4}$$

which can be rearranged and integrated

$$\frac{\mathrm{d}m_0}{m_0} = \frac{-2.3p_0\epsilon.10^6}{Nhw}\,\mathrm{d}t \tag{5}$$

$$\log_e m_0 = \frac{-2.3p_0\epsilon.10^6}{Nhw}\,t + K; \tag{6}$$

when t is zero, $\log_e m_0 = K$, so K, the integration constant, is equal to $\log_e m_{0(t=0)}$ so that

$$m_0 = m_{0(t=0)} e^{-[(2.3p_{0(e.}10^6)/(Nhw)]t} \tag{7}$$

and

$$m^* = m - m_0. \tag{8}$$

Between pulses, the population of excited states decays exponentially, such that

$$m^* = m^*_{(t=t_1)} e^{-t/\tau} \tag{9}$$

and for a fall (Δ) in excited state population, $q \times \Delta$ photons are emitted.

Experimental

A simple BASIC computer program was used to solve equations (7), (8) and (9) over many excitation/emission cycles. Calculations were performed for a pulsed xenon flash and for a laser source. Both sources were assumed to deliver 10^{14} photons in a pulse lasting either 5 ns (laser) or 5 μs (xenon flash). For the xenon flash source, a literature value of 400 μs [7] was used for the delay time. For the laser source, the fluorescence lifetime of the background was assumed to be the factor determining the required delay. This lifetime was taken to be 50 ns. The ratio, R, of label fluorescence to background fluorescence at time t after the flash is given by

$$R = R_{(t=t_1)} e^{[(\tau - 5\times 10^{-8})/(\tau \times 5 \times 10^{-8})]t}; \tag{10}$$

if τ is much longer than 5×10^{-8} s then this reduces to

$$R = R_{(t=t_1)} e^{[t/(5\times 10^{-8})]}; \tag{11}$$

if $R_{(t=t_1)}$ is as small as 10^{-20}, then the time at which R reaches a value of 100 is given by

$$10^{22} = e^{[(t/(5\times 10^{-8})]} \tag{12}$$

or

$$t/(5 \times 10^{-8}) = 2.3 \times 22 \simeq 50 \tag{13}$$

after about 50 background lifetimes. A value of 100 lifetimes (5 μs) as the delay time was used for calculation.

The sample was taken to be a hypothetical fluorescent label with a molar absorption coefficient of 7500 and a quantum yield of 1 in a fluorimeter cell $5 \times 5 \times 2$ mm ($h \times w \times b$). The rather low absorption coefficient is typical of terbium chelates containing a single aromatic ring; the quantum yield of unity is optimistic, although a value of 0.7 ± 0.1 has been reported for this type of chelate [8]. The sample concentration was taken to be 10^{-15} mol l^{-1}, a value that represents a desirable operating range for a sensitive immunoassay.

Results and Discussion

The results are illustrated in Figs 1–5. Figure 1 shows the approach to equilibrium for a system where the interval between pulses is less than several lifetimes of the label. For long lifetimes (1 s) and slow pulse rates (10 per s) the time taken to reach this equilibrium can be appreciable (2 s or more). However, for most of the cases considered in this study, equilibrium was reached in a very short time (less than 0.05 s).

Figures 2 and 3 show the results for xenon flash illumination, and Figs 4 and 5 for laser illumination. The curves in Figs 2 and 5 are very similar to those presented by O'Haver and Winefordner, who derived expressions for the fraction of total phosphorescence measured in rotating can [9] and flash source [10] phosphorimeters.

Several conclusions follow from these results. Firstly, the potential advantage over chemiluminescent labels is illustrated. The sample described contains about 3×10^4 molecules: consequently, the maximum possible output from a chemiluminescent label (assuming 1 photon per molecule) would be 3×10^4 photons.

The advantage of laser excitation is also apparent. The absolute maximum possible frequency for the xenon flash, at which the fluorescence output falls to zero, is somewhat less than 2500 pulses per s, determined mainly by the delay period. Very little increase in

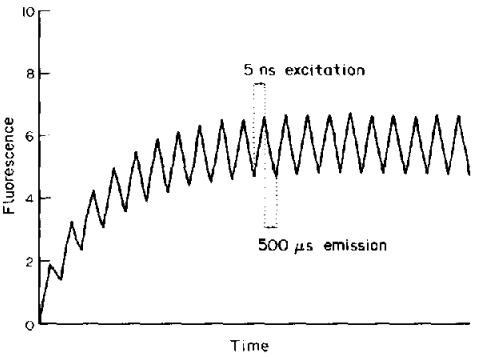

Figure 1
Fluorescence (in arbitrary units) against time for a fluorophore with a lifetime of 1 ms illuminated with pulsed light at approximately 2000 pulses s^{-1}. An equilibrium is eventually established in which the excited state population varies between constant limits. (Note the difference in timescale between excitation and emission).

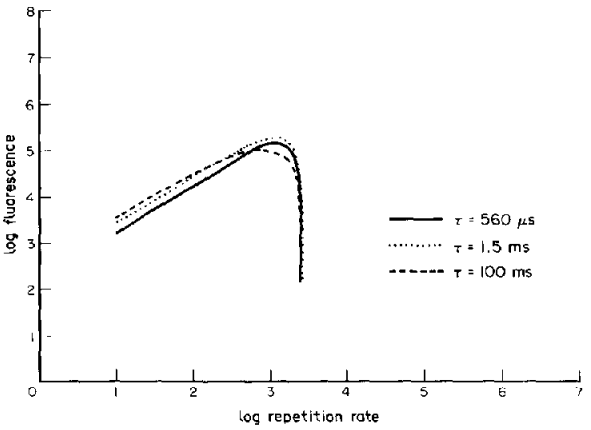

Figure 2
Output of light (photons s^{-1}) against pulse rate for several long-lived fluorescent labels illuminated by a pulsed xenon flash. The delay time (t_2) is 400 μs.

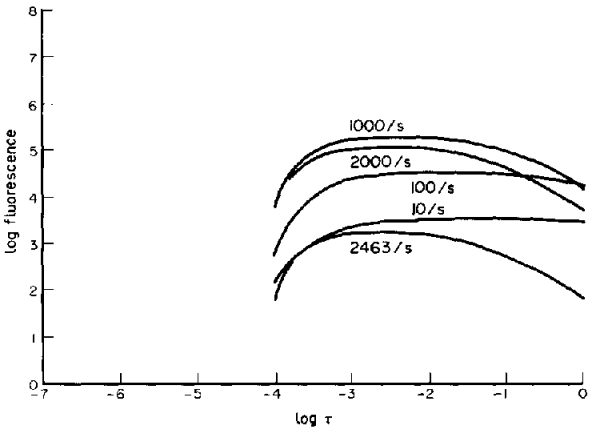

Figure 3
Output of light (photons s^{-1}) against fluorescence lifetime for several pulse rates, using a pulsed xenon flash. The delay time is 400 μs.

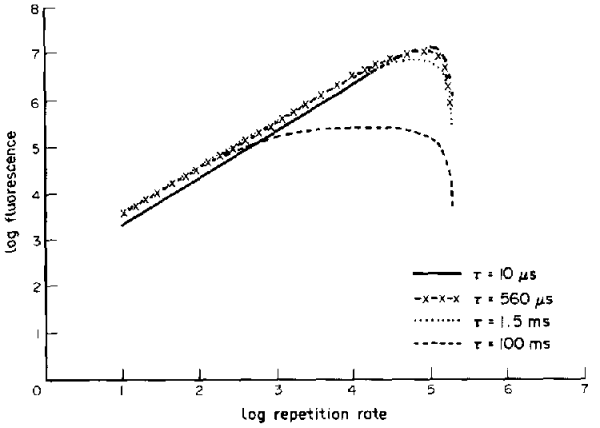

Figure 4
Output of light (photons s^{-1}) against pulse rate for several long-lived fluorescent labels illuminated by a pulsed laser source. The delay time is 5 μs.

output is achieved, for any label lifetime, at pulse rates greater than 1000 per s. The long delay required also means that labels with lifetimes of less than about 100 μs are unlikely to be of use with a pulsed xenon source. Labels with longer lifetimes show small differences in output, but in practice other factors (absorption coefficient and quantum yield) would have greater influence on the output of fluorescence from different labels.

With a laser excitation source, much higher pulse rates are possible. In addition, labels with much shorter lifetimes (e.g. dysprosium chelates) should be usable. The combination of high pulse frequency and relatively short lifetime results in particularly high fluorescence output. The figure of 50 ns used for the background lifetime is a "worst-case" assumption and it is possible that labels with lifetimes shorter than 1 μs could be of use.

For laser sources in particular, there is little advantage in using labels with lifetimes longer than about 100 ms, unless slow repetition rates are required: there is a substantial

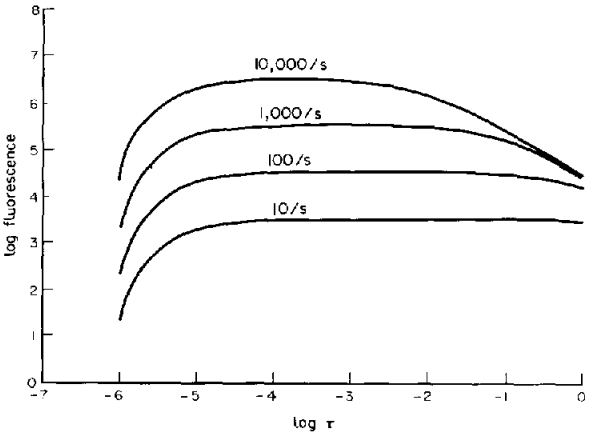

Figure 5
Output of light (photons s^{-1}) against fluorescent lifetime for several pulse rates, using a pulsed laser source. The delay time is 5 μs.

fall in ground state population (>30%) as pulse rate increases with such labels, and the result is a plateau in the pulse rate/output profile which is particularly apparent in Fig. 4. Since labels with such long lifetimes are likely to be phosphorescent organic compounds which have poor quantum yields, their use is likely to be restricted to applications where high sensitivity is not required. For both xenon flash and laser sources, the optimum range of label lifetimes corresponds to that attainable by the use of lanthanide chelates: europium and terbium chelates are suitable for use with a xenon flash, whilst dysprosium chelates, with lifetimes of about 10 μs, may prove useful with laser excitation.

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