Time-Resolved Fluorometer for Lanthanide Chelates—A New Generation of Nonisotopic Immunoassays

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Pulsed-light time-resolved fluorometry of lanthanide chelates has proved to be very sensitive for use with nonisotopic immunoassays. We describe a manually operated fluorometer with a conventional xenon flash tube. Sensitivity for 1-s determinations is similar to that of radioisotopic methods.

Chelates of europium and terbium are a potential alternative to radioisotopic compounds as labels in immunoassays. The main advantages of using chelates as fluorescent probes are the high quantum yield, exceptionally large Stoke's shift, narrow emission peaks, and optimal emission and excitation wavelengths for use with biological material. These characteristics make the lanthanide chelates preferable to any conventional fluorescent probe for use with ordinary fluorometers. In this paper we discuss the design and operation of a suitable fluorometer; the preparation and use of the lanthanide chelates will be presented elsewhere.

When used with biological material, conventional fluorescent probes suffer from serious limitations to sensitivity, owing to the natural fluorescence from various compounds in biochemical samples such as blood serum. This interference can, to some extent, be reduced by the careful selection of optical filters in the fluorometer, but ordinarily fluorometric methods are not much more sensitive than radioisotopic methods if conventional fluorescent probes are used. With lanthanide chelates, however, it is possible to reduce the background level significantly by selective detection of long-decay fluorescence. The fluorescence decay time of lanthanide chelates is often in the order of 10–1000 µs, whereas the decay time of natural fluorescence in a typical biological sample is in the order of 1–20 ns. For this reason pulsed-light source, time-resolved fluorometers with lanthanide chelates are potentially several orders of magnitude more sensitive than conventional fluorometers.

The use of time-gating to distinguish between fluorescence and Rayleigh and Raman scattering in a sample of biological fluid has been mentioned in several articles. London (1) suggested the possibility of using time-gating to reduce the background fluorescence in Raman spectroscopy, and Van Duyne et al. (2) designed a practical system involving pulsed laser excitation for temporal resolution between the short-lived Raman signal and the relatively long-lived fluorescence signal. They included an electronic time-gate in the photomultiplier tube circuit and synchronized it with the pulsed laser source in such a way as to ensure that signals caused by Raman photons were preferentially recorded.

Many papers have also been published on pulsed-source time-resolved phosphorimetry. In this technique, xenon-discharge lamps are used as a pulsed-light source and, after a short delay following each pulse, a photomultiplier detector measures the luminescence. This method has been used effectively to eliminate interfering background light scatter-

ing and fluorescence (3–5). Similar systems have also been used to study delayed fluorescence and are useful for removing scattered light and therefore for separating normal and delayed fluorescence (6).

Another application of time-resolving techniques has been the measurement of the rotation of proteins and the degree of flexibility of their active sites. This can be achieved by measuring fluorescence polarization as a function of time after a light pulse of 1 ns. In time-resolved fluorescence spectra all the different components of the emissions that occur during the counting period can be distinguished by their different decay times from the prompt emission component, which occurs during the delay period and which consists mainly of scattered light (7–10). Equipment for time-resolving fluorescence spectroscopy and decay-time determination is available from many manufacturers, one of the earliest suppliers being Ortec (11).

Brown et al. (12) used a variable nanosecond gated photomultiplier technique for separating weak, prompt fluorescence from the total fluorescent and phosphorescent emission. Lytle and Kelsey (13) reported a time-resolved system for measuring the fluorescence spectrum without interference from scattering and Raman lines, and emphasized its usefulness with macromolecular samples where scattered radiation is a severe problem. The concept of using time-resolving fluorometry in the field of fluoroimmunoassay was examined in this laboratory during 1974 and 1975, and some conference reports resulted (14–16). Parallel studies have been carried out by Wieder (17). Time-resolving fluorometry has also been reviewed by Soini and Hemmilä (18).

Principles and Instrumentation

We have developed a simple, manually operated fluorometer for fluoroimmunoassays with lanthanide chelates as fluorescent probes. The particular unit shown in the block diagram in Figure 1 was designed for research purposes only.

The sample compartment is covered by a light-tight lid and the sample is changed manually. The samples are held in small disposable tubes or cuvettes made of polystyrene, which has a reasonably low long-decay background fluorescence. Because the intensity of the single flashes from the xenon flash tube was not very reproducible, we had to ensure stabilization of the excitation system. An integrator (P1) for a semiconductor photodiode serves as the stabilizer of the flash lamp. The flash lamp is activated about 10³ times at a frequency of 1 kHz. The exact number of flashes (N) is controlled by the integrator P1 so that the integrated intensity of the photon emission is thus fixed. For the stabilization detector we used a photodiode (Model UV-215B; EG & G Inc., Electro-optics Div., 35 Congress St., Salem, MA 01970), operated in the photovoltaic mode and connected to the optical system by a fiber light guide. The integrator is made of an operational amplifier, which provides a control signal for the flashtube circuit. The integrated photon emission from the flashtube is stabilized by this method with a precision of ±(1/N)·100% assuming that the

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deviation of the intensity of single flashes is not greater than ±50%.

This stabilization method has many advantages. First of all, the system is simple; the flashtube and its power supply can be made without any stabilization circuit and less-expensive flash tubes with lower stability can be used. The temperature dependence of the system can be minimized by a single compensator element. The flashtube is operated only during a measurement, thus ensuring a long practical life. The eventual fatigue of the flashtube will be automatically compensated by the integrator.

The pulsed-light source used in this fluorometer was an FX-198 bulb-type xenon flash tube with a 1.5 mm arc cap (EG & G Inc.). An EG & G Lite-Pac Trigger Module produced the high-voltage trigger pulses required to operate the flashtube. We operated the flashtube system at +600 V and a flash duration of 0.5 μs.

To provide optimal excitation and emission bands, we used interference band-pass filters (Ferroperm AS, Copenhagen, Denmark) mounted inside the sample compartment for easy and quick replacement.

The detector is a side-window photomultiplier tube (Model R928; Hamamatsu TV Co. Ltd., 1126 Ichino-cho, Hamamatsu, Japan) operated with negative-bias voltage, thus obtaining a direct analog signal between the anode and ground. We found this to be a practical arrangement for monitoring the total amount of fluorescence and obtaining an indication of counter saturation.

The photomultiplier tube, operated in the single-photon mode, is connected to a fast preamplifier and discriminator and to a fast scaler having a digital display of seven decades. The counting speed of random events is limited to 40 MHz by the preamplifier and single-photon discriminator.

**Results**

This time-resolved fluorometer was designed primarily for use with fluorescent chelates of europium. Typical excitation and emission spectra of europium chelate is shown in Figure 2. The photomultiplier sensitivity for the emission peaks of this chelate depends on the cathode material used. For the tube used in this device the photocathode quantum efficiency q for europium was 8% at 613 nm. The emission filters used were chosen for the longer-wavelength peak.

The time-resolving operation was controlled by pulses from the clock-pulse generator. The delay time from the flash trigger pulse to the start of scaling was typically t₁ = 400 μs and the fast scaler gate open-cycle was 500 μs. In this arrangement, single-photon counting occurred for one-half of the total measuring time and the fraction (g₂) of the photon emission decay spectrum registered for each flash was

\[ g_2 = \sum_{i=0.1, 1} \left( e^{-\frac{0.693}{T_{1/2}}} \right) dt \]

where decay time \( T_{1/2} = 560 \) μs for europium and excitation cycle time \( t_0 = 1 \) ms. The value of \( g_2 = 0.40 \) includes second- and third-order decay fractions. As a consequence the maximum number of pulses registered in the scaler was \( 5 \times 10^6 \) counts during a 1-s measurement.

**Sensitivity Evaluations**

The rate of emitted photons (specific activity) obtained from a fluorescent sample of Eu(β-NTA)$_3$TOPO$_{0.3}$ is

\[ n_e = 2.3 \cdot \varepsilon \cdot d \cdot n_0 \cdot f_1 \cdot g_1 \cdot Q \cdot k \text{ photons/s} \]

where

- \( \varepsilon = \text{molar absorptivity} = 150 \text{ 000 L mol}^{-1} \text{ cm}^{-1} \)
- \( d = \text{path length of cuvette} = 1 \text{ cm} \)
- \( n_0 = \text{photon intensity of the flash in the position of the sample cuvette} = 7.12 \times 10^{11} \text{ photons/flash} \)
- \( f_1 = \text{repetition rate of the flash} = 1000 \text{ flashes/s} \)
- \( g_1 = \text{factor dependent on the transmission and reflections of the cuvette} = 0.74 \)
- \( Q = \text{quantum yield of the fluorescent probe} = 0.06 \) (at the 613 nm emission peak of europium)
- \( k = \text{concentration of the sample (mol/L)} \)

The numerical value of \( n_e \) is \( 1.1 \times 10^{19} \cdot \text{ k photons} \cdot \text{s}^{-1} \)

The signal strength of the fluorometer is given by the formula

\[ n = g_3 \cdot g_4 \cdot g_5 \cdot g_6 \cdot g_7 \cdot n_e \]

where

- \( g_3 = \text{fraction of the photon emission decay spectrum registered for each flash} = 0.40 \)
- \( g_5 = \text{fraction of pulses passed through the single-photon pulse discriminator} = 0.20 \)
- \( g_4 = \text{transmission of the sample solution, cuvette, and optical parts at 613 nm, including reflections} = 0.67 \)
\[ g_s = \text{geometrical photon-collection efficiency} = 0.28 \]
\[ g_o = \text{transmission of the secondary filter} = 0.25 \]
\[ q = \text{quantum efficiency of the photomultiplier tube} = 0.08 \text{ at } 613 \text{ nm} \]

This equation gives a numerical value for \( n = 3.36 \cdot 10^{18} \cdot k \text{ counts} \cdot s^{-1} \), which is in good agreement with the test results with the fluorometer.

A simplified theoretical comparison between \(^{125}\text{I}\) and lanthanide chelates may illustrate the potential of the latter as a very sensitive labeling material. Given insulin (\( M_r = 5800 \)) as a model compound, let us suppose that the insulin is labeled with europium chelate in the molar ratio of 1:1, which has been proved possible without any analytical side effects. The typical sample volume would be 1 mL; an insulin sample of 1 pg/mL corresponds to 1.72 \( \cdot 10^{-13} \) mol/L concentration of both insulin and fluorescent probe. The number of emitted photons (specific activity) is then \( n_E = 1.9 \cdot 10^9 \) photons/s per picogram. The counting rate obtained with the fluorometer would be \( n = 580 \) cps for the 1 pg/mL insulin sample. The background rate of the fluorometer, mainly caused by the afterglow of the xenon flash tube, is 100 cps for distilled water and is about the same for a serum blank. The count-to-background ratio in this model example would thus be 5.8. (For the precision achieved at various counting times, see Table 1.)

A typical maximum specific activity of \(^{125}\text{I}\) label is in the order of 50 Ci/g (1.85 MBq/\( \mu \)g) in the case of insulin. This specific activity corresponds to 0.1–0.2 active labels per molecule and gives a count rate of 80 cpm/\( \mu \)g (1 pg \( = 10^6 \) molecules) at a gamma counting efficiency of 70%. A typical background rate in gamma counting is 40 cpm. Thus the count-to-background ratio is 1.94, which is of the same order as when using the fluorometric method but to attain the same precision as the latter, the counting time would be 100 times longer. Table 1 details differences in the performance of fluorometry and gamma counting.

**Discussion**

The test results and the theoretical example above show clearly the great potential of fluorometry for shortening counting time and for increasing sensitivity. The sensitivity of the fluorometer and fluorescent probe in this work is similar to that obtained by using \(^{125}\text{I}\) as a label, which means that the sensitivity of the present device is sufficient for ordinary immunoassays. However, it is not the specific activity of the label alone that determines the lowest level of detection of an immunoassay; the immunological system is also an important factor. The main advantages of the flurometric assay are, of course, the short counting time and the use of nonradioactive material. The statistical precision can easily be improved by increasing the excitation intensity 10-fold, which increases both the signal and background rate by the same factor, but the statistical effect is a minor one. The excitation level can be increased significantly without any risk of saturating the probe, because in the present system only about 0.01 of the fluorescent probe is excited. The active volume of the sample is about 200 \( \mu \)L, which represents only 20% of the sample volume. By decreasing the sample volume from 1 mL to 0.2 mL and optimizing the optical conditions for smaller samples, the sensitivity of the fluorometer, in terms of picomoles per milliliter, could thus be improved fivefold.

Lanthanide chelates and time-resolved fluorescence detection provide potentially very high detection sensitivity for the following reasons:

- Signal/photon emission ("specific activity") can be increased by a stronger excitation
- Background fluorescence from sample "blank" can be discriminated by using time-resolved detection
- Lanthanide concentrations in biological samples are normally negligible
- Lanthanide labels are biochemically inert (no interaction with the sample)

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**References**

13. Lytle FE, Kelsey MS. Cavity-dumped argon-ion laser as an

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<th>Table 1. Performance of Time-Resolved Fluorometry and Gamma Counting for 1 mL of Labeled Insulin</th>
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<tr>
<td>Typical specific activity</td>
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\[ s = \sqrt{\frac{n+B}{n}} \cdot 100 \]


