An improved instrument for measuring time-resolved lanthanide emission and resonance energy transfer

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Time-resolved fluorescence, particularly of millisecond lanthanide emission, is of considerable interest because of potentially increased sensitivity when short-lived autofluorescence is a problem. Lanthanides have also previously been shown to be excellent donors in resonance energy transfer measurements, where a lanthanide donor transfers energy to a conventional fluorophore. An instrument for the detection of micro- to millisecond lanthanide luminescence and delayed fluorescence, amidst a background of large prompt fluorescence, is described. Time-delayed emission spectra can be measured with 16 μs dead time while suppressing prompt fluorescence by 25 000-fold. This is accomplished by using a mechanical chopper placed very close to the entrance slit of a diffraction spectrometer, and a charge coupled device detector at the exit port. Excited-state lifetimes can also be measured with a 22 μs dead time while reducing detector ringing due to prompt fluorescence to an insignificant level. This is accomplished by high-voltage gating of a photon-counting photomultiplier tube in combination with a fast constant-fraction discriminator and amplifier. These improvements, which utilize all commercially available equipment, reduce the dead time by a factor of 10 for delayed emission measurements and a factor of 5 for lifetime measurements over a previously described instrument. The current instrument is particularly well suited for lanthanide-based resonance energy transfer measurements. © *1999 American Institute of Physics.* [S0034-6748(99)00610-3]

I. INTRODUCTION

Terbium and europium can have millisecond lifetime and sharply spiked emission spectra, even in aqueous solution. Consequently these lanthanides are of considerable interest in time-resolved fluorescence measurements, particularly for discrimination against prompt (nanosecond) background fluorescence.[1,2] They have also been shown to be excellent donors in luminescence resonance energy transfer measurements (LRETs), for measuring distances in the 20–100 Å range between and within biological macromolecules,[3–7] or for measuring diffusion rates.[8,9] In LRET, which is a modified form of the conventional fluorescence resonance energy transfer, energy is transferred via a dipole–dipole coupling from a lanthanide donor to an organic-based fluorescence acceptor. The efficiency of energy transfer E depends on the distance between donor and acceptor. Energy transfer leads to a decrease in the donor's lifetime and intensity, and a simultaneous increase (or ''sensitization'') in the acceptor's emission intensity. This sensitized emission of the acceptor temporally follows the relatively long lifetime of the donor from which it receives energy. The highly unusual characteristics of lanthanide emission enables LRET to overcome many drawbacks of conventional resonance energy transfer, which relies on two organic fluorophores.[5–7]

Whether used as a long-lived emitter in time-resolved fluorescence, or as a donor in energy transfer, detection of delayed luminescence is desired while discriminating against prompt fluorescence. Without proper gating, this prompt fluorescence saturates detectors and results in significant (>100 μs) dead time before the signal can be acquired. We have previously described an instrument which can acquire time-delayed emission spectra using a diffraction grating spectrometer and a charge coupled device (CCD), and which can also measure micro- to millisecond excited-state lifetimes using a photomultiplier tube.[5–7]

We have now improved this instrument to reduce the dead time, and hence are now able to measure emission spectra or excited-state lifetimes with shorter decay times. Excitation is with a pulsed nitrogen laser and two detection methods are employed to discriminate against prompt fluorescence. For emission spectra, a mechanical chopper is placed very close to the entrance slit of an imaging spectrometer and is timed to block the entrance slit during and shortly after the laser pulse. The emission is then acquired on a CCD. Greater than 25 000-fold suppression of prompt fluorescence has been achieved with a dead time of 16 μs. For excited-state lifetime measurements, a photomultiplier tube (PMT) is used with a high-voltage gate, a constant-fraction discriminator, and a fast amplifier, resulting in a dead time of 22 μs. These improvements enable signals with lifetimes of <100 μs to be acquired with complete discrimination against prompt fluorescence. In LRET, this enables signals to be accurately acquired in species with energy transfer over 90% and also significantly improves curve fitting of samples with longer lifetimes as well.

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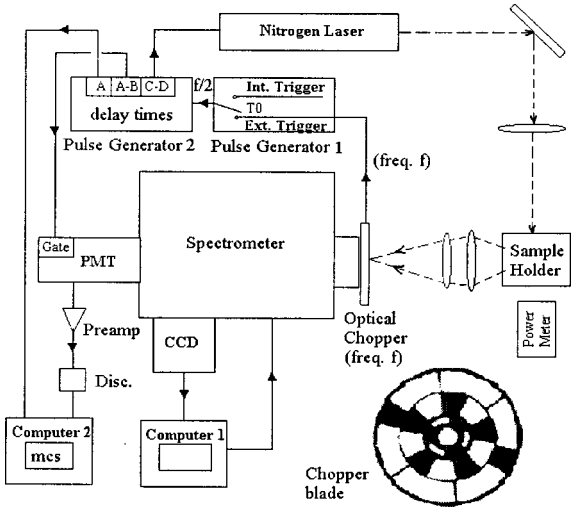

FIG. 1. Instrument setup and signal timing (see text for details). Generator No. 1 is set to external trigger for CCD measurements; internal trigger for PMT measurements. (D) output of generator No. 1=trigger+0.023 95 s. Generator No. 2 is always set to external trigger and triggered by T_o output of generator No. 1. Output settings for generator No. 2: (A)=trigger+1.371 ms (exact time adjusted for optimal suppression of prompt fluorescence and minimum dead time—see text); (B)=(A)+60 μs; (C)=(A)+30 μs; (D) =(C)+10 μs.

II. RESULTS

A. Instrument setup

We have built a spectrometer capable of measuring time-delayed emission spectra at many wavelengths simultaneously, or measuring excited-state lifetimes at a single wavelength (Fig. 1). The excitation source is a nitrogen laser (Laser Photonics, Orlando FL: 337 nm, 40 Hz, 7 mW average power, 5 ns pulse width, or Oriel Model 79111). Two mirrors are used to rotate the rectangular beam profile so that the larger dimension is vertical. After passing through a neutral density filter and slit, the power after the sample cuvette (typically 5–10 μJ per pulse) is monitored via a power meter (Molectron, Portland, OR, Model J4-09 with JD1000 display). 100–200 μl of sample solution is placed in a 2 mm$\times$2 mm or 3 mm$\times$3 mm (i.d.) quartz cuvette. The temperature of the solution is controlled by a laboratory-built water-cooled cuvette holder. The emission light is detected at right angles with a f/1 condenser lens and then f matched with a second lens and focused onto the side entrance slit of the spectrometer (ISA, SPEX Triax 320, f/4.1, with modified side entrance slit—see below). A mechanical chopper (Stanford Research System, Palo Alto, CA, model SR540) with a modified chopper blade is placed as close as possible to the side entrance slit (see Fig. 1, inset). The chopper is timed (see below) such that the entrance slit is blocked during and immediately after the laser pulse to eliminate prompt fluorescence of the LRET acceptor and any other short-lived background such as Raman scattering of water. The sample fluorescence/luminescence is detected either with a water-cooled photon-counting photomultiplier tube (Hamamatsu R943-02; gallium–arsenide photocathode with Products for Research TE104 cooling housing, and Model GSA-02 ''normally on'' PMT gating circuit and resistor chain) for time-resolved measurements at a single wavelength, or a liquid-nitrogen cooled CCD (SPEX Spectrum one: 1024$\times$256, 27 μm/pixel, front illuminated 16-bit digitization; noise: le/hr/pixel; readout noise $<$5 electrons) for time-delayed emission spectrum. A computer-controlled moveable mirror inside the spectrometer directs the light to either (but not both simultaneously) of the detectors. The output of the PMT is amplified by a preamplifier (MITEQ 500 MHz, model Au1054, Hauppauge, NY) and discriminated with a constant-fraction discriminator (EG&G model 548). The resulting TTL pulses are counted in a multichannel scalar with 2 μs resolution (EG&G MCS-plus pc-board OPT2). Because of memory incompatibility between the CCD and the multichannel scalar boards, they were controlled by two different PCs.

B. Signal timing

The goal of signal timing (Fig. 1) is to coordinate the timing such that prompt fluorescence does not create a signal. The laser pulse is synchronized with the mechanical chopper and/or detector electronics such that the detectors are turned off or blocked during and shortly after the laser excitation pulse. The timing of the signals is achieved by using the chopper TTL output and two pulse generators (Stanford Research System DG535).

When using the CCD as a detector, the TTL output of the chopper (output setting ''f'') is used as the master clock. (The ''f'' output is actually at six times the rotation frequency of the wheel since the original wheel has six blades.) By triggering the laser with an output derived from the chopper, any jitter in the chopper is inconsequential. The chopper is set to a near maximum rotation rate of 478/6=79.6 Hz to minimize dead time. The TTL output of the chopper triggers pulse generator No. 1. The pulse generator downconverts the pulse rate to 40 Hz, the maximum laser repetition rate, and triggers pulse generator No. 2, which fires the laser (output C–D) with a delay time such that the entrance slit is blocked during and shortly after the laser fires. This delay time is determined by exciting a solution containing a prompt fluorophore (e.g., tetramethylrhodamine, TMR) and eliminating all signal, while minimizing dead time (see also Fig. 2).

When using the PMT as a detector, the chopper is turned off with the slit uncovered and pulse generator No. 1, set to 40 Hz internal trigger, acts as the master clock. Generator No. 1 triggers generator No. 2 and the multi-channel scalar (MCS) and PMT high-voltage gate are simultaneously triggered. 30 μs later the laser is fired, and another 20–30 μs later the PMT gate is turned off and data collection is begun (Fig. 2).

C. Improvement in delayed-emission spectral measurements

The collecting lens focuses a cone of emitted luminescence onto the entrance slit of the spectrometer. If the chopper blade is at the apex of the fluorescence cone, it can block almost all of the fluorescence; otherwise, some of the unwanted prompt fluorescence can leak into the entrance slit. The efficiency of the optical chopper in blocking the prompt fluorescence depends on how close it can be placed to the slit. In our previous setup, the entrance slit of the spectrometer (ISA SPEX 270M) was about 1 cm inside the spectrom-

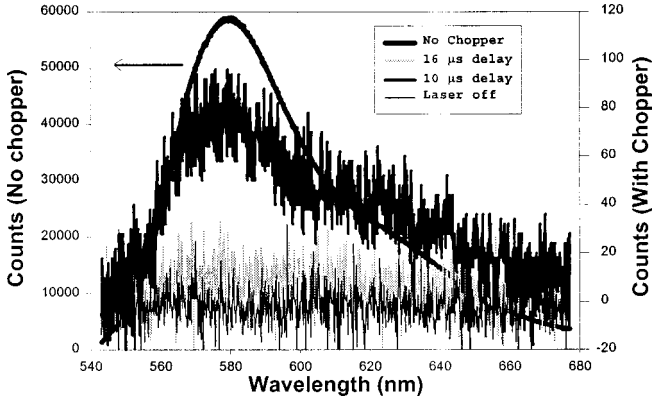

FIG. 2. Suppression of prompt (ns) fluorescence with optical chopper. Free tetramethylrhodamine (lifetime of 4 ns) is excited at 337 nm with a 5 ns pulse from a nitrogen laser. The chopper frequency is 79.6 Hz and the slit is uncovered for a given delay time after the laser is fired. The CCD collects emitted light, either with no chopper or with a chopper blocking light at different delay times after the laser pulse excites the prompt fluorescence. A delay of 10 μs yields a 4000-fold rejection of prompt fluorescence. A delay of 16 μs yields greater than 25 000-fold suppression of prompt fluorescence. The CCD integration time is 5 s except for no chopper, which is 1 s. An entrance slit width of 0.1 mm and a 600 groove/nm diffraction grating is used. TMR concentration is 1.5 μM.

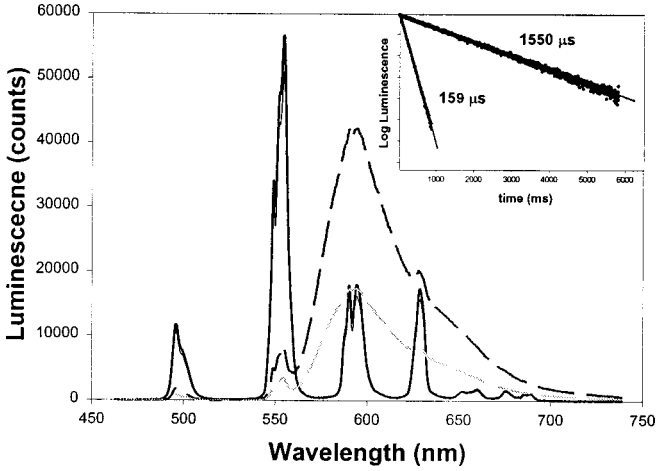

FIG. 3. Detection of delayed emission spectra using CCD and chopper: effect of dead time. Minimizing dead time is important for recovering a relatively short-lived signal (large energy transfer). Solid spectra (thick and thin lines) are terbium emission (1.55 ms lifetime; see inset) collected with 16 and 150 μs dead time, respectively. [Tb bound to chelate (Diethylenetriamine pentaacetate-carbostyril 124) (Refs. 6 and 10)]. The two different dead times show little difference in the signal because in either case the dead time is much shorter than the terbium lifetime. However, for terbium in the presence of an acceptor, tetramethylrhodamine, the donor lifetime is reduced to 159 μs (see inset), and sensitized emission of rhodamine is also present as can be seen in the large emission peak centered around 590 nm (black and gray dashed lines). Collecting with 150 μs dead time (dashed gray) cuts off about 66% of the signal, whereas with 16 μs (dashed black), very little is lost. The CCD spectrum is collected with an entrance slit of 0.1 mm and a 300 groove/mm diffraction grating. Terbium concentration is 1 μM and tetramethylrhodamine, when present, is 3.5 μM. These results show that with a dead time of 16 μs, even a donor–acceptor emission spectrum with a lifetime of $\sim$100 μs(>90% E.T.) can be measured on the CCD.

eter and the chopper construction prevented the chopper blade from being flush with the spectrometer. This meant that the closest distance between the optical chopper and entrance slit was about 1.5 cm, which limited the minimum dead time to about 150 μs with 25 000 times suppression of prompt fluorescence. We replaced the SPEX 270M with a SPEX Triax-320 spectrometer, in which the side entrance slit was custom modified by the manufacturer such that the slit is outside the box and the chopper blade can be placed virtually touching the entrance slit. We have achieved a 16 μs dead time with 25 000 times suppression of prompt fluorescence (Fig. 2). This nearly tenfold improvement (compared to a previous dead time of 150 μs)[7] makes it possible to accurately measure samples with relatively short lifetimes (<100 μs), corresponding in LRET to large (>90%) energy transfer. Figure 3 shows that minimizing dead time is important for recovering relatively short-lived signals (large energy transfer). For Tb chelate emission with a 1.55 ms lifetime (donor only, no acceptor), the difference between 16 and 150 μs dead time is not significant. However, for terbium in the presence of the acceptor such that energy transfer is $\approx$90%, both donor and acceptor sensitized emission decay with a time constant of 159 μs. Consequently, the CCD will record only 33% of the signals if the dead time is 150 μs, missing 66% of the signal in the dead time. In contrast, with 16 μs dead time, very little is lost. The loss with the 150 μs dead time will be particularly significant in LRET when there is a mixture of donor-only and donor–acceptor species. In these samples, the donor emission has a different (longer) temporal decay than the sensitized emission, since the donor emission arises from both donor-only species (Tb lifetime of 1.5 ms) and donor–acceptor species [$\tau = 1.5\,\text{ms} \times (1-E)$], whereas sensitized emission arises only from donor–acceptor species. Consequently, a long dead time will cut off different amounts of the intensity at the donor- and acceptor-emission wavelengths, thereby distorting the shape of emission spectrum and making the calculation of energy transfer based on relative donor and acceptor emission areas inaccurate.[7]

D. Improvement in sensitized emission lifetime measurements

In LRET, generally both the donor- and sensitized-emission lifetimes are measured. A significant advantage of LRET, which simplifies data interpretation, is that the sensitized emission can be measured with no interfering background from either donor emission, or from prompt fluorescence of the acceptor. (Prompt acceptor fluorescence, which decays with nanosecond lifetime, is due to direct absorption of the excitation pulse by the acceptor.) For example, with tetramethylrhodamine as acceptor, we can monitor the sensitized emission at 570 nm where the donor emission is near zero, and we can eliminate prompt fluorescence by temporal discrimination. One complication is that the prompt fluorescence is often very large, and hence will cause ringing from the photomultiplier and associated preamplifier and discriminator. This ringing creates dead time. We have previously found, using an integrated Hamamatsu preamplifier and leading-edge discriminator (Model C3866), that recovery from this ringing took 100–150 μs[7] (see also Fig. 4).

Here we have used the combination of a fast bipolar preamplifier (MITEQ 500 MHz) and a constant discriminator (EG&G model 548). This dramatically decreases the ringing while retaining 80% of the gain of the Hamamatsu unit (Fig. 4). In order to completely eliminate the ringing, we employ a

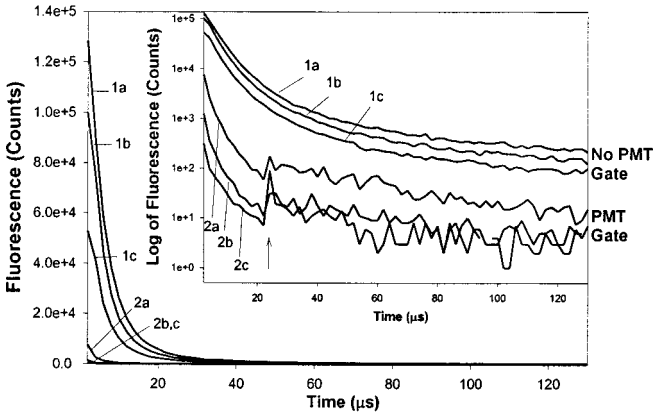

FIG. 4. Effects of preamplifier, discriminator, and PMT gating on the response to prompt fluorescence, plotted on a linear and log (inset) scale. In all cases, data are acquired using 1.5 μM free tetramethylrhodamine in 5 mM Mops, *p*H 7.0, excited with a 5 ns pulse at 337 nm. Since the tetramethylrhodamine has $\approx$4 ns lifetime, any signal on the microsecond time scale is due to electronic ringing. Emission is collected with different combinations of preamplifier and discriminator, with or without PMT high-voltage gating. The top three curves (curves 1a,b,c) were acquired without a PMT gate; the bottom three curves (curves 2a,b,c) used a PMT gate. Curves a correspond to Hamamatsu leading-edge preamp/discriminator; curves b to EG&G leading-edge discriminator and MITEQ preamplifier; curves c to EG&G constant-fraction discriminator and MITEQ preamplifier. The arrow indicates when the PMT gain is reestablished. The data were collected at 570 nm$\pm$1.3 nm, (1 nm slits, 1200 groove/mm diffraction grating). The MCS-plus board was set at 2 μs per point, 8912 points/sweep, 3200 sweeps. The combination of constant-fraction discriminator and high-voltage gating of the PMT can completely suppress PMT ringing due to prompt fluorescence, thereby reducing the dead time from 150 to 22 μs.

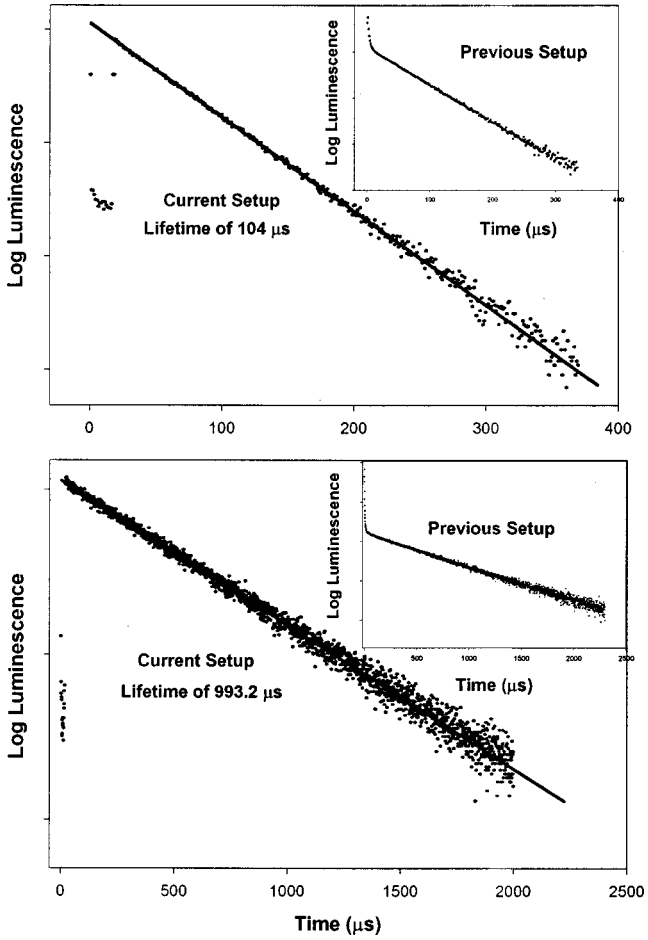

FIG. 5. Effects of preamplifier, discriminator, and PMT gating on lifetime measurements. Single exponential lifetimes were obtained using diffusion-enhanced energy transfer, in which 5 μM free TMR and 1 μM DTPA-cs124 were mixed in different percentages of a glycerol solution. The top graph shows a 104 μs lifetime (0% glycerol) measured using either the previous configuration (Hamamatsu C3866 leading-edge discriminator and integrated preamplifier, no PMT gating) or the current setup (MITEQ 500 MHZ preamplifier, EG&G 584 constant fraction discriminator and PMT gating) (see inset). The bottom graph shows a lifetime of 993 μs (90% glycerol), which was measured in two different instrumental setups (inset is measured using current setup). The acquisition was done under similar conditions as Fig. 4. The combination of constant-fraction discrimination and high-voltage gating of the PMT enables measurements of lifetimes with $\leqslant$100 μs and also improves the quality of data and curve fitting for samples with both short and long lifetimes.

PMT high-voltage gating circuit from Products for Research (GSA-02). A 20–30 μs gating pulse turns the gain of the PMT to near zero during and shortly after the prompt fluorescence. At the end of this pulse, when the PMT gain is reestablished, the effect of the prompt fluorescence is insignificant. Comparing the combination of PMT gating circuit with either the Hamamatsu unit, the MITEQ preamplifier with Ortec leading-edge discriminator, or the MITEQ preamplifier with the Ortec Constant Fraction discriminator, the best combination is the PMT with high-voltage gate in combination with the MITEQ preamplifier and the constant fraction discriminator (Fig. 4).

This system was tested with diffusion-enhanced energy transfer[8,9] by mixing different concentrations of the acceptor tetramethylrhodamine with a terbium donor (terbium bound to chelate DTPA-CS124).[6,10] The amount of energy transfer depends on the acceptor concentration, resulting in various single exponential lifetimes. Figure 5(a) shows that we are able to measure lifetimes as short as 100 μs (>90% E.T.) with no residuals, in contrast to previous instrumentation (inset), which displayed significant initial pulsing. Figure 5(b) shows the improvement for longer lifetimes (993 μs) as well. The elimination of the instrument artifacts leads to significantly simplified and improved curve fitting on multiexponential decays (data not shown). We have also tested the linearity of this new setup by measuring the single exponential lifetime decay of pure Tb-DTPA-cs124. Nonlinearities due to saturating effects of high-count rate lead to distortions from single-exponential decays. We find the lifetime remains perfectly single-exponential decay ($r^2>0.999$; no residual structure) up to 2 million counts/s in the first channel.

ACKNOWLEDGMENT

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