Using the Synergy™HT to Measure Time-Resolved Fluorescent Compounds

Time-resolved fluorescent reagents have been used in many biological systems as a means to reduce background fluorescence signal and increase the sensitivity of a variety of different assays. Here we describe the use of the Synergy HT Multi-Detection Microplate Reader to measure time-resolved fluorescent compounds.

Introduction

Under many experimental conditions autofluorescence of the sample or the microplate can cause unwanted background fluorescence. High background results in variable inter and intra-experimental signals, as well as a decrease in sensitivity. While several options are available to reduce background fluorescence, including the use of low-background microplates and different excitation and emission wavelengths, these measures often fail to adequately reduce the background signal. Another approach to this problem is to use time-resolved fluorescent reagents.

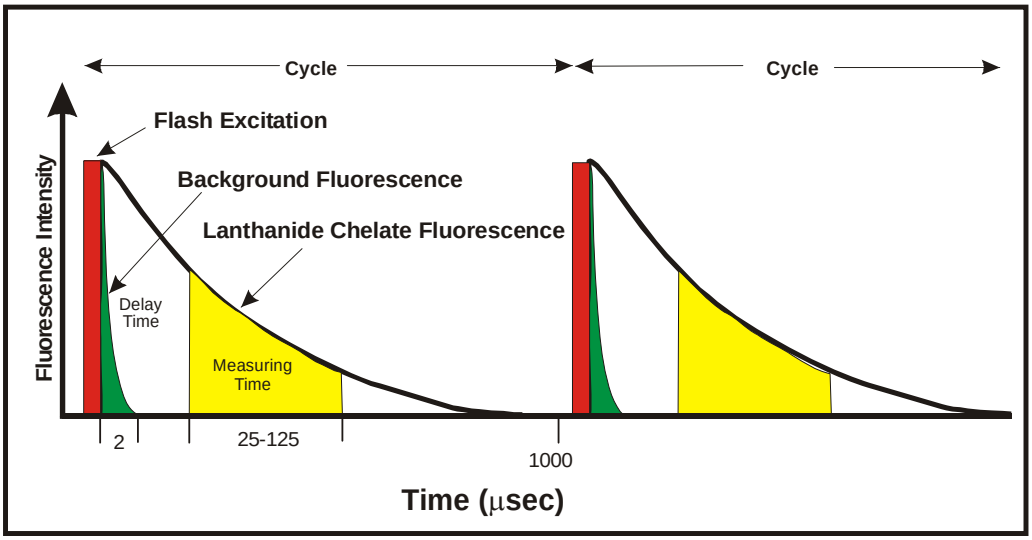

Figure 1. Fluorescence Decay of Lanthanide Chelate. The fluorescence of lanthanide chelate lasts considerably longer than conventional fluorophors. This allows for measurements to begin after the cessation of interfering background fluorescence.
*Model SIAFRT with the Time-Resolved Fluorescence.

Lanthanide ions, particularly Europium (Eu), Samarium (Sm), Terbium (Tb), and Dysprosium (Dy), emit fluorescence of specific wavelength when bound to specific ligands. Compared with traditional fluorescence compounds, the lanthanide compounds have a much longer emission lifetime. These emission lifetimes are often measured in hundreds of microseconds whereas

COPYRIGHT © 2006 TEL: 888-451-5171 FAX: 802-655-7941 Outside the USA: 802-655-4740 E-mail: customercare@biotek.com

traditional organic reagents have lifetimes measured on the scale of several nanoseconds. However, these compounds in and of themselves absorb light very weakly and are usually not directly excited, but rather they are excited through chelated organic compounds, often referred to as “chelates” or “ligands”. Hence the absorption spectrum of the complex generally reflects the absorption spectrum of the ligand rather than the lanthanide itself [1]. In addition, these compounds exhibit large Stokes shift, with excitation occurring by absorbance of UV light and emission wavelengths greater than 500 nm. The emission peaks of Eu, Sm, Tb, and Dy are 615 nm, 643 nm, 545 nm, and 574 nm respectively. The fluorescent emission peak profiles are also quite sharp, with half-widths being 10 nm to 20 nm (Figure 2).

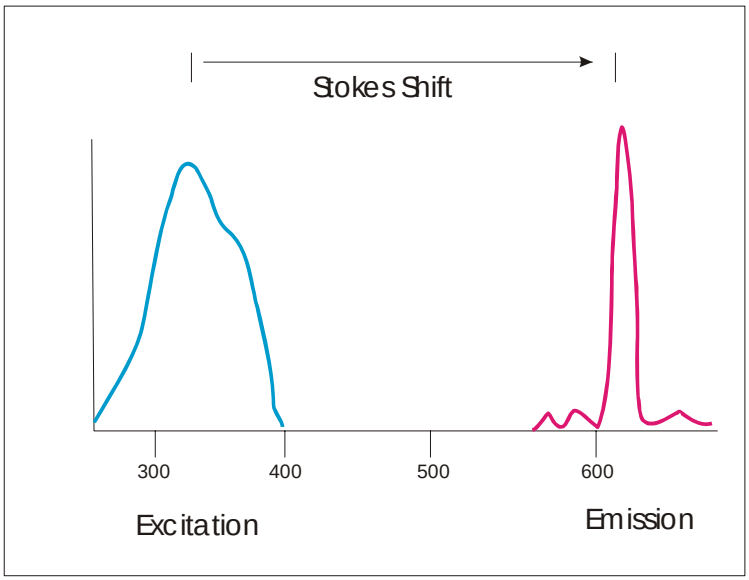

Figure 2. Excitation and Emission profile of Europium. Cartoon depicting the excitation and emission spectrums of Europium. Note the very narrow emission peak, as well as the large Stokes shift of the fluorophor.

The Synergy HT configured for time-resolved fluorescence is a multi-detection reader capable of measuring fluorescence in either the conventional mode, where the fluorescent emission is measured with the excitation source still present, or in time-resolved mode, where the fluorescence is measured at some point following the cessation of excitation. When the reader is in conventional fluorescence mode, it uses a tungsten-halogen lamp as a light source and band-pass filters in a filter wheel cartridge to provide wavelength specificity. When the reader is used in time-resolved mode it automatically switches to a xenon-flash lamp light source with a monochromator to select wavelength. The excitation filter cartridge is replaced with the TR-cassette that, in addition to directing the light from the different light source to the microplate, can also hold a single excitation filter if necessary. When in time-resolved mode, the user has the ability to control the time between the cessation of excitation and the initiation of fluorescence measurement (delay time), as well as the length of time the fluorescent signal is accumulated (collection time).

Materials and Methods

Europium chelate standard, 1 nmole/L (catalog number B119-100), Samarium chelate standard, 10 nmole/L (catalog number B115-100), and Chelate solution (catalog number 1244-105) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Dilutions of the ready to use standards were made using chelate solution as the diluent. Time-resolved fluorescent measurements were made using a Synergy HT* multidetection reader manufactured by BioTek Instruments, (Winooski, VT) configured for time resolved fluorescence. Sodium Fluorescein was

purchased from Molecular Probes (Eugene, OR). In all experiments, opaque white Corning-Costar (Cambridge, MA) microplates (catalog number 3912) were used.

Chelate	Excitation (nm)	Emission (nm)	Fluorescence Lifetime (τ) (µsec)	Suggested Emission Filter
Europium (Eu)	340	615	730	620/40
Samarium (Sm)	340	642	50	645/40
Terbium (Tb)	320	545	1050	545/40
*(Dyprosium (Dy)	320	572	16	575/15
*Ruthenium (Ru)	459	620	0.4	620/40
* Not recommended for time resolved measurements with the Synergy HT				

Table 1. Comparison of fluorescent properties of several lanthanide chelates. Different lanthanide chelates exhibit different properties, including not only excitation and emission wavelengths, but also lifetime constants, which allows for multiplexing of different labels.

Results

In order to carry out time-resolved measurements it is necessary to cease the excitation prior to the measurement of the fluorescent emission. The cessation of excitation can be accomplished either by shuttering a continuous light source or by using a light source that can be extinguished rapidly. The Synergy HT uses a xenon-flash lamp that has a flash-time of 2-5 µsec, after which any light emission very rapidly diminishes. This allows for time-resolved fluorescence measurements for compounds that have a fluorescence lifetime greater than 20 µsec. Traditional organic compounds, such as sodium fluorescein, will have decayed during the delay-time interval prior to the time-resolved fluorescence measurements. This is demonstrated in Figure 3, which depicts the fluorescence of sodium fluorescein with a delay of 0, 20, or 40 µsec between the cessation of excitation and measurement of fluorescence. A delay of 0 is equivalent to a standard fluorescence determination. Note that the sodium fluorescein dilutions provide a linear response when measured without a delay; however, with either a 20- or a 40-µsec delay there is virtually no fluorescent signal (Figure 3).

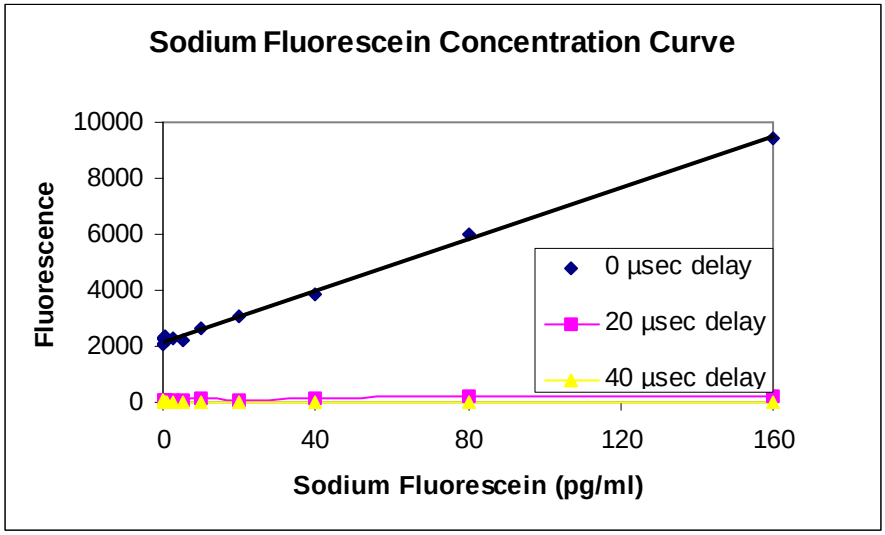

Figure 3. Fluorescence signal of sodium fluorescein with various delay times before fluorescent measurements. A series of dilutions of sodium fluorescein were made with PBS as the diluent. Using a 485/20 filter in the TR-cassette and a 528/20 emission filter, the fluorescence was measured with a Synergy HT with a 20 µsec collection time, a sensitivity setting of 225, and a delay time of either 0, 20- or 40-µsec. The fluorescence was collected using KC4 to control the reader, exported to Microsoft© Excel and plotted.

Because the Synergy HT reader when used for time-resolved fluorescence utilizes optics that has a monochomator and the ability to use a filter to select excitation wavelength, the effect of wavelength specific filter on performance of the Synergy HT to make time-resolved fluorescent measurements on lanthanide chelate was examined. Using the same sensitivity setting and a common emission filter (620/40 nm) the fluorescence of a series of dilutions of Europium chelate was measured using either the monochromator alone set at 360 nm to select excitation wavelength or the monochromator set at 360 nm in conjunction with a 360/40 nm bandpass filter. As demonstrated in Figure 4, both methods provide linear signal through the concentration range (0-1000 fmoles/well) tested. Not surprisingly, the signal with the filter present was diminished relative to that where no filter was present, most likely due to incomplete transmittance of light by the filter. Because there was no loss of linearity without the filter, which implies that there is no overlap between the excitation light and the emission signal and a greater signal, subsequent experiments were performed without a bandpass filter being present.

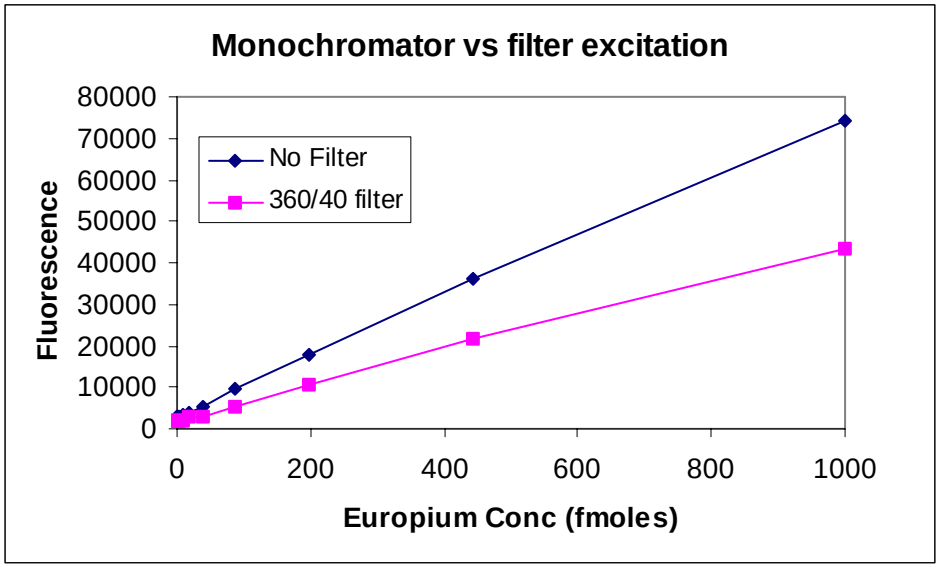

Figure 4. Effect of the presence of a band pass filter for excitation wavelength specificity. A Synergy HT configured for time-resolved fluorescence was used either with or without a 360/40 bandpass excitation filter to measure a microplate containing a series of dilutions of europium chelate. For each determination, the sensitivity gain setting was set at 200, the monochromator excitation setting was 360 nm and a 620/40 emission-filter was used. Data was collected using KC4 data reduction software, exported to Microsoft© Excel and the data plotted.

While the literature indicated that 360 nm was optimal in regards to an excitation wavelength, we wanted to make sure that the lack of a bandpass filter did not have any affect on the excitation maxima. Using an appropriate bandpass filter to select the emission wavelength (620/40 for Europium and 645/40 for Samarium) the fluorescent emission at several different excitation wavelengths was compared. As demonstrated in Figure 5, the returned fluorescent signal increases as the excitation wavelength approaches 360 nm, and then rapidly decreases with wavelengths above 360 nm. Note that there is virtually no fluorescence above background with an excitation wavelength of 400 nm. In addition, with excitation wavelengths in the UV-range (280 nm or below) there is significant background fluorescence as a result of the microplate (data not shown), necessitating the subtraction of a buffer-only blank well at each wavelength tested. For subsequent experiments, an excitation wavelength of 360 nm was used for both Europium and Samarium chelate.

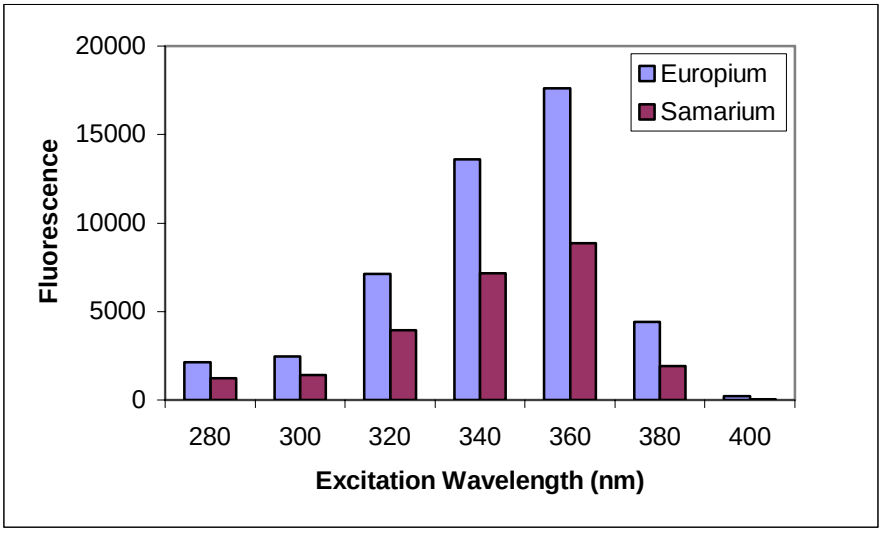

Figure 5. Comparison of Fluorescence Excitation Wavelengths for Europium and Samarium Chelates. Using a Synergy HT, various excitation wavelengths were used to excite Europium chelate (300 fmole/well) or Samarium chelate (3000 fmole/well). Fluorescence measurements were made with the indicated excitation wavelength, and a 620/40 or a 645/40-emission filter for europium and samarium respectively. For either compound, a PMT sensitivity setting of 225, with a delay time of 60 µsec and an integration time of 100 µsec was used. Note that the data at each wavelength presented represents the mean of 8 replicates after subtraction of the mean of control wells, which contain chelate only.

One of the parameters available when using the Synergy HT for time-resolved fluorescence measurements is data collection time. This parameter, which is the time that the reader is collecting fluorescent signal, can be as short as 20 microseconds to as long as 16 milliseconds. Using a series of dilutions of Europium chelate, the effect of increasing the collection time on the fluorescent signal returned was investigated (Figure 6). When measuring the fluorescence of Europium after a 20-µsec delay, an increase in the fluorescence signal is observed as one increases the collection time. Note that the increase in signal is not necessarily linear relative to the collection time, as the compound fluorescence is also decaying over time [1]. However, increasing the collection time is a useful means to increase sensitivity when measuring small amounts of lanthanides, particularly those with longer fluorescent half-lives.

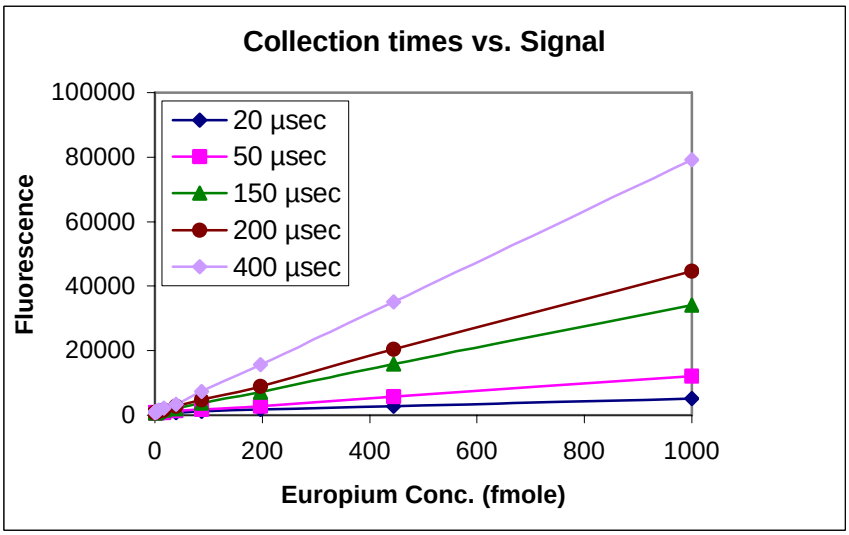

Figure 6. Effect of Collection time on the fluorescence signal of Europium Chelate. Using a Synergy HT the fluorescence of same microplate with a series of Europium dilutions was measured repeatedly with a delay time of 20 µsec and increasing collections times ranging from 20 µsec to 400 µsec. The reader

was controlled and the data collected using KC4 data reduction software. After collection the data was exported to Microsoft© Excel and plotted.

The delay time is the other parameter unique to the time-resolved mode of fluorescent measurement. This parameter is the time interval from the cessation of the excitation light to the initiation of the read measurement. By keeping the collection time and sensitivity settings constant but changing the delay time, one can observe the decay of fluorescence of compounds with long half-lives, such as the lanthanides (Figure 7). As demonstrated in Figure 7, different concentrations of Europium chelate loose their fluorescence as the time from the cessation of excitation to the measurement increases in a first-order kinetic rate. Note that the fluorescence for each concentration is approximately 50% lower every 500 µsec increase in the delay. This agrees reasonably well with the reported fluorescence lifetime (τ) value of 730 µsec. The τ value is a time estimate wherein approximately 67% of the molecules have decayed. When different lanthanide compounds, each with different τ values, are compared remarkably different decay curves are observed (Figure 8). When normalized data for Europium and Samarium are compared to one another the much shorter fluorescence lifetime of Samarium verses Europium becomes apparent (Figure 8).

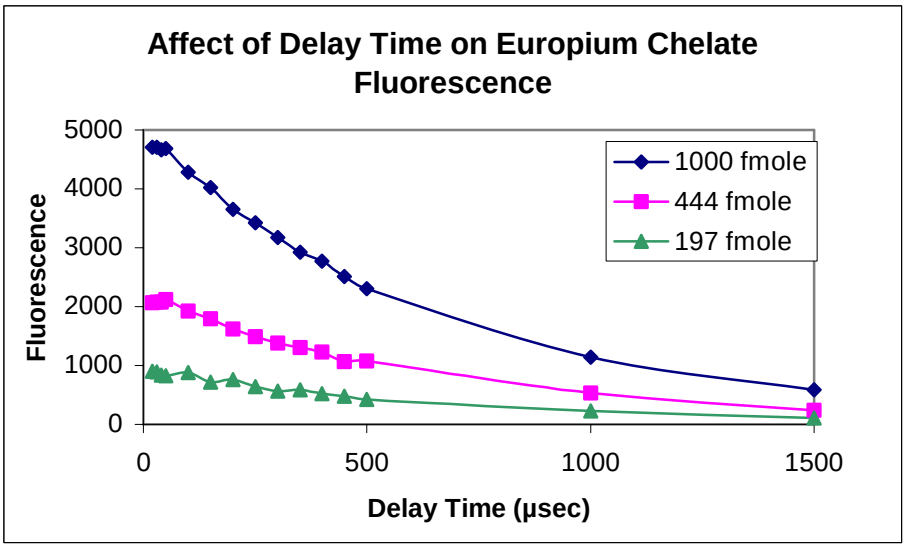

Figure 7. Affect of Delay Time on Europium Chelate Fluorescence signal. The fluorescent signals of three different concentrations of Europium (1000, 444, and 197 fmole/well) were measured using various "delay time" values with a collection time of 40 microseconds. Each data point represents the mean of eight replicates

Discussion

These data demonstrate that the Synergy HT can be utilized to perform time-resolved fluorescence measurements. Technically the process of "time-resolved" measurements is to delay the start of measurements after a flash of excitation and integrate the intensity from the lanthanide. The term "time-resolved" fluorescence is thus a bit of a misnomer, in that there is no measurement of decay times involved. These "time-gated" experiments are essentially steady-state intensity measurements taken after the background fluorescence has diminished. Decay times can be estimated by making measurements with a series increasing delay times, while maintaining the PMT sensitivity and collection time constant. Historically the wavelength selection of fluorescent plate readers has been accomplished using wavelength specific bandpass filters. While there are many reasons for using these filters, the paramount reason has to do with using deep-blocking filters to prevent an overlap between the excitation and emission wavelengths with compounds that have small Stokes shifts. The Synergy HT in time-resolved mode uses an optics path that has a monochromator, which can provide excitation wavelength

specificity. While the large Stokes shift of the lanthanides eliminates the need for deep-blocking filters, the Synergy HT has been engineered to use filters in the TR-cassette if they are required.

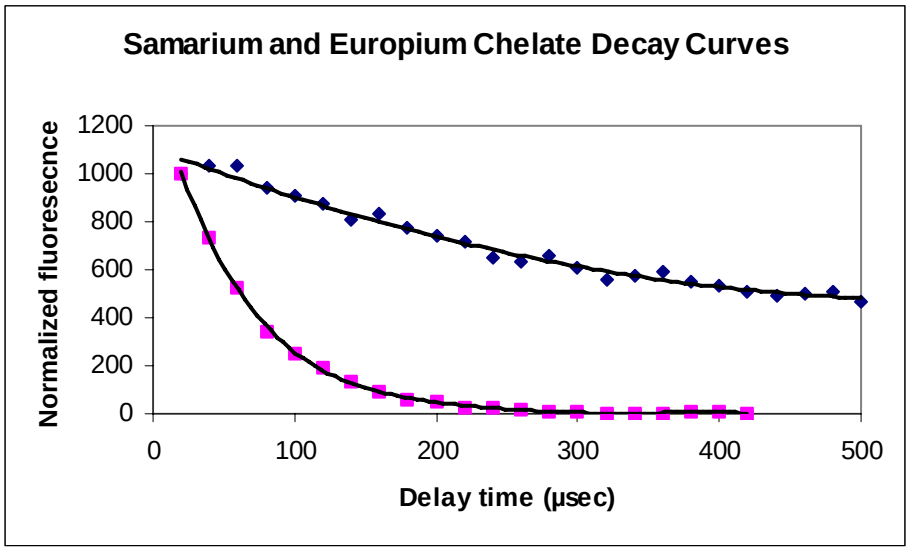

Figure 8. Comparison of Samarium and Europium chelate fluorescence over time. The fluorescence of 3000 fmole/well of Samarium chelate (squares) and 300 fmole/well of Europium chelate (diamonds) were measured using a Synergy HT with an excitation wavelength of 360 nm and either a 620/40 or a 645/40-bandpass-emission filter for Europium or Samarium respectively and a PMT gain setting of 225. Using increasing delay times, as indicated by the x-axis, measurements were taken using a 20-µsec collection-time. For each data point, the mean of chelate-only control wells was subtracted. The resultant fluorescent data was normalized to 1000 by dividing each point by the initial reading and multiplying the ratio value by 1000. The normalized data was plotted using Microsoft© Excel with a polynomial trend line generated. Note that each data point represent, the mean of eight determinations.

The primary advantage of the lanthanide compounds is their long-lived fluorescence emission. This allows for determinations to be made with low background fluorescence. This is particularly useful when experimental conditions require that measurements be made in the presence of interfering agents. Cell based assays require the use of tissue-culture plates which often have high background. There are also many cellular components (e.g. NADH, FADH) that are inherently fluorescent, which can add to unwanted background interference. However, there also are several disadvantages to the use of lanthanides. By themselves, they are only weakly fluorescent due to their poor absorption properties. This necessitates the use of enhancer molecules to improve energy absorption that are chelated to the lanthanide. This requirement of a chelating enhancer limits their utility as substrates for many enzymes. The commonly used chelates also require that the pH be maintained near 7 for maximal fluorescence. These compounds are particularly unstable in acid environments. Heavy metal ions such as manganese (Mn^{+2}), copper (Cu^{+2}), chromium (Cr^{+2}), and iron ($Fe^{+2/+3}$) quench the fluorescent signal and often require EDTA to recover the signal. Fortunately, most physiologic ions such as K^{+}, Na^{+}, Mg^{+2}, and Ca^{+2} do not seem to affect lanthanide signals [2]

Several different methodologies have been developed to utilize the advantages of lanthanides while minimizing their deficiencies. Perkin Elmer Life Sciences, formerly Wallac, has developed a series of assays collectively referred to as DELFIA®, which uses an enhancer agent to exchange and chelate lanthanides from specifically bound ligands to produce a time-resolved signal. Prior to the addition of enhancer, the fluorescent signal of the chelated lanthanide is non-fluorescent. Because these assays generally involve some sort of solid substrate and a means to wash away unbound lanthanide they are referred to as heterogeneous assays. LANCE™ assays, also from Perkin Elmer Life Sciences, are homogeneous assays that utilize a lanthanide

compound as part of a fluorescence resonance energy transfer (FRET) assay. In these assays the signal is transferred from one compound to another only when they are in close proximity, as would be the case during a protein-binding event for example. When a binding event does not take place the signal is not transferred and there is little fluorescent signal. Because these types of assays do not require removal of unbound reagents, they are often referred to as homogeneous assays.

Besides having the capability to perform time-resolve fluorescence measurements, the Synergy HT can perform UV-Vis absorbance measurements, measure glow luminescence, as well as perform conventional fluorescence measurements. With two complete sets of optics, there are no compromises in terms of performance when switching from absorbance to fluorescence. The reader can read any plate format up to 384-wells, automatically adjusting the top probe height setting to accommodate different plates when reading from the top. Temperature control up to 50°C and shaking are also standard features. The Synergy HT is robotics compatible with a fully extending plate carrier and interfacing is via the OLE capabilities of the KC4 software used to control the reader.

References

1) Principles of Fluorescence Spectroscopy 2nd Edition (1999) Lakowicz, J.R. Editor,Kluwer Academic/Plenum Publishers, New York, New York.
2) Application Note: Stability of the Wallac LANCE™ Eu-chelates, Perkin-Elmer Life Sciences, http://www.lifesciences.perkinelmer.com/library/appnotes.asp

Rev. 10/29/02