Time-resolved Measurements Using the Agilent Cary Eclipse Fluorescence Spectrophotometer
A Versatile Instrument for Accurate Measurements

Technical Overview

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Introduction

Time-resolved fluorescence is a key analytical technology that is commonly used to label and track key biomolecules. Fluorescence labeling offers obvious, significant safety advantages over radioactive labeling and, by examining the time-dependence of fluorescence, we can exploit the same high level of molecular selectiveness and analytical sensitivity that is intrinsic to fluorescence measurements and gain a deeper understanding of sample chemistry and molecular environment.

A considerable advantage of time-resolved fluorescence measurements is that they provide a reliable mechanism with which to eliminate the common problem of background fluorescence (autofluorescence from the cells or the media) that can complicate such analyses and interfere with the steady state emission from common probes (such as fluorescein and rhodamine) and contribute significantly to a high background signal (and so reducing the efficacy of the measurement). Given their significant potential to enhance measurement capabilities, it is easy to understand why lanthanide complexes (used in time-resolved fluorescence) find common usage in life science applications such as immunoassays, receptor-ligand binding assays, protein-protein binding assays, cytokine assays, adherent cell assays, enzyme assays, DNA hybridization assays, and cytotoxicity assays.

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The Concept of Long-lived Emission Measurements

Time-resolved assays measure the temporal response of a labeled molecule to interaction with incident light (this contrasts with steady state fluorescence assays, which only record the prompt emission of a fluorescent probe). In the Agilent Cary Eclipse Fluorescence spectrophotometer, a Xenon flashlamp switches on and off (from full illumination to total darkness) 80 times every second. This means that in every measurement made using the Cary Eclipse, the fluorescent label is excited by a short light pulse from a flashlamp with a peak power in excess of 75 kW which, with its small arc size, provides an extremely bright beam (The brightness of lamp is critical in fluorescence measurements because the signal from a sample is proportional to the amount of light that interacts with it - more light onto the sample equals more light coming off of it). The high pulse frequency, along with the short lamp decay time of the Cary Eclipse Xenon flashlamp, allows time-resolved measurements on timescales ranging from as short as almost 0.1 ms to infinity in duration. When we examine the time-based response of a sample using the Cary Eclipse we use two different collection modes:

- **Fluorescence mode**: The steady state fluorescence emission is recorded at the instant of illumination.

- **Phosphorescence mode**: The software instructs the spectrometer to wait for a user-defined period of time, usually defined by the decay time of the lamp, before the light that has been emitted from the sample is collected. This mode is used for the time-resolved 'delayed' fluorescence measurements commonly exhibited by lanthanide-based probes.

Measuring these different, but near-simultaneous fluorescence responses, using a Cary Eclipse is easy, and can be done without any user interaction. As we demonstrate below, everything is programmed into the software in a few easy steps, then runs automatically.

Figure 1 shows a typical profile of fluorescence excitation-decay with time, and a schematic illustration of the relative differences between fluorescence and phosphorescence measurements.
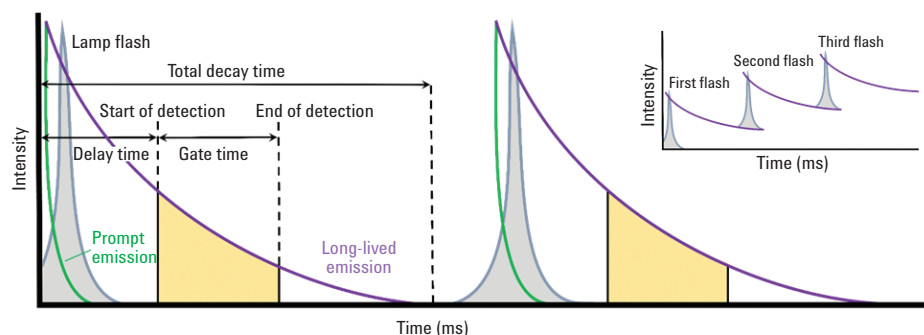

Figure 1.   The typical response profile during fluorescence (green line) and phosphorescence (purple line) measurements. ***Prompt emission*** refers to the steady state fluorescence. ***Long-lived emission*** refers to the phosphorescence (or delayed fluorescence) response. ***Delay time*** is the time that elapses between the pulse of the Xenon flashlamp and the start of data collection (this is easily optimized to remove any signal from the short-lived background fluorescence and any scattering effects). ***Gate time*** is the length of that the detector collects light from the emitter (the longer the gate time the more light is collected and, thus, the higher the intensity of the signal). The ***total decay time*** is the duration of the phosphorescence response. In general, the experiment is configured to ensure that the lamp does not flash, and no new measurement will start until the total decay time has elapsed. A key attribute of time-dependent analyses is that they allow the user to increase sensitivity by sequentially co-adding measurements (inset).

Key Attributes of the Agilent Cary Eclipse for Time-resolved Measurements

- The Agilent Cary Eclipse eliminates photodegradation, and is safe for all biological samples.

- The rapid frequency (80 Hz) Xenon flashlamp is ideal for steady state kinetics and time-resolved experiments.

- Both the prompt emission (fluorescence that occurs on the nanosecond timescale) and phosphorescence emission (millisecond timescale) from the same sample can be measured in a single measurement.

- Phosphorescence mode is directly implemented with no need for expensive or additional accessories.

- The flashlamp technology ensures that each intense pulse is followed by complete darkness. This impeccably controlled and absolutely reproducible timing protocol is key to accurate time-resolved measurements.

Long-lived Emissions from Lanthanide Complexes

Many time-resolved fluorescence measurements in the life science sphere take advantage of the unique chemical characteristics of lanthanide complex labels. The particular lanthanide that is of specific interest to life science applications is europium (Eu^{3+}). Eu^{3+} labels have relatively long decay times (on average, the total decay time is about 10^{-5}–10^{-3} seconds compared to 10^{-9} seconds for many conventional fluorescent molecules), and the difference in the wavenumber of the absorbed and emitted light is large. This large separation between excitation and emission spectra allows the instrument to be perfectly configured for extremely low detection limits and, when coupled to the absence of any contribution from background emission (see below), incredible and enhanced sensitivity.

By itself, Eu^{3+} absorbs very weakly, but when it bonds with certain organic ligands it forms a strongly absorbing and highly emissive complex. Of particular interest is the fact that although the absorption characteristics of Eu^{3+} complexes are mainly determined by the attached organic ligands, the emission is uniquely characteristic for Eu^{3+}. This ensures a comparably large difference between the absorption and emission bands (~350 to 600 nm), a feature that removes inferences from excitation light. The process of obtaining a measureable signal is described briefly in the next section.

Obtaining a Measurable Fluorescence Response from Lanthanide Complexes

In most time-resolved emission assays, a solution containing the Eu^{3+}-labeled substrate is combined to the biomolecule of interest (which is normally only very weakly or completely nonfluorescent). After a period of reaction time, the unreacted Eu^{3+} is removed and only Eu^{3+}-labels that have bound with biomolecules remain. At this stage, the Eu^{3+}-bound target molecules may not be much more fluorescent than they were in the original state, and need to be converted into a highly emissive, easy detectable complex using one of a great variety of different ligand solutions that are available. Once this short and simple reaction has taken place, the complexes are fully prepared for the high sensitivity and low background measurements that are characteristic of time-resolved fluorescence assays.

Worked example: using the Agilent Cary Eclipse to collect time-resolved fluorescence spectra

In Figure 1, we graphically illustrated some of the key parameters of a time-resolved experiment, and schematically demonstrated how these can be optimized to maximize the efficacy of a measurement. In the following section, we demonstrate, by working through an example, how optimizing these parameters can extract the maximum amount of relevant data from a sample.

Molecular systems are intrinsically complex, and the measured response from a sample may reflect contributions from different molecules. In a time-resolved experiment that has been prepared following the experimental protocol that was described in the previous section, the Eu^{3+}-bound target molecules are reacted with a ligand solution to ensure that they have properties that allow them to be detected by the instrument. A common side-effect of this is that signals from more than one source may be observed on the resulting spectrum (Figure 2). We can use the time-resolved nature of these to separate each into component parts.

Delay time

Delay time describes the amount of time that elapses between the pulse of the Xenon flashlamp and the start of data collection (Figure 1). In a simple experiment to illustrate the effect of changing the delay time on the spectra that are collected from the Eu^{3+}-ligand solution described earlier, we made a series of measurements with a fixed gate time (Figure 1, this is described in more detail later) and delay times that increased incrementally from 0 µs to 100 µs (Figure 3). With a delay time of 0 µs, the strong peak at ~450 nm caused by the background fluorescence is significant (green spectrum in Figure 3). Because this background emission only occurs over a very short time scale (ns), its contribution to the spectrum decreases as we increase the delay time. The emission from the Eu^{3+}, however, occurs over a much longer period of time (ms), and so is unaffected as the delay time is increased. By modifying the delay time appropriately, the measurement only collects light that is emitted after the short-lived background signal has died down, and the only signal that is measured corresponds to that from particular species of interest (Eu^{3+}).
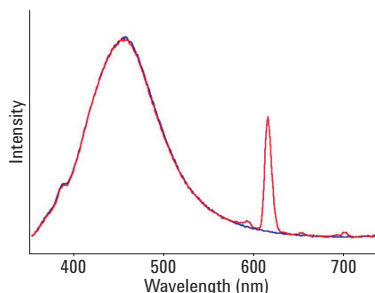

Figure 2. Fluorescence spectra of Eu^{3+} in solution (red) and blank enhancement solution (blue). The broad peak centred at ~450 nm is a strong background emission from the enhancement solution and the sharp, narrow emission peak centred at 615 nm that is attributed to the Eu^{3+} emission.
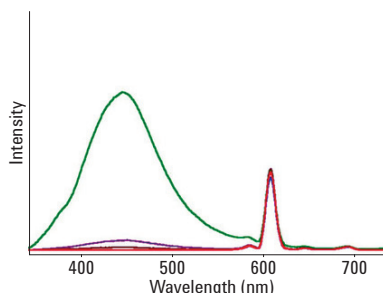

Figure 3. The effect of delay time on the signal that is collected from an Eu^{3+}-ligand solution. The intensity of background fluorescence (the band at ~ 450 nm) band decreases significantly as the gate time is increased from 0 µs (green line) to 100 µs (red line).

Gate time

Gate time describes the length of time that the detector collects light from the particular emitter that is of interest (Figure 1). In essence, the longer the gate time the more light is collected and, thus, the higher the intensity of the signal. In a simple experiment to illustrate the effect of changing the gate time on the resulting spectrum, we collected spectra with a fixed delay time (see above) but different gate times (Figure 4). Changing the gate time has no effect on the intensity of the prompt (background) fluorescence, but can help maximize the intensity of the long-lived (and target) Eu^{3+} emission (Figure 4).

Time-resolved spectra of the target molecule

By setting the Cary Eclipse to phosphorescence mode, using a 0 s delay time, and then adjusting the gate time to maximize the collected Eu^{3+} emission, we can accurately capture both the prompt (fluorescence) emission (that is active on the nanosecond timescale) and the target emission from the Eu^{3+} (that is active across on the millisecond timescale). The Cary Eclipse is able to collect fluorescence and phosphorescence spectra from the one sample in one measurement. With absolutely no user interaction, the signal from an appropriate molecule of interest can be discretely and expressly quantified, monitored, and recorded. Figure 5 shows the time resolved spectrum of Eu^{3+} in a ligand solution that was collected with the express intent of removing all background contributions. An added bonus of this technique is that the user can define the length of time the detector measures the desired signal. This ensures that time-resolved analyses can provide essential qualitative and quantitative molecule-specific measurement capabilities, and that the sensitivity of these critical experiments can be optimized to ensure that every piece of information is extracted from the sample of interest.
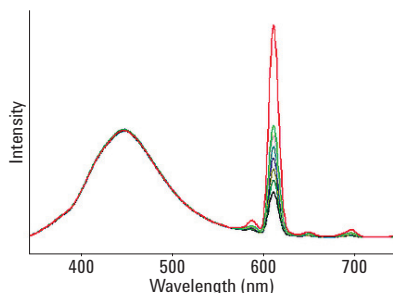

Figure 4.   The effect of gate time on the signal that is collected from an Eu^{3+}-ligand solution. Spectra were collected with a fixed delay time of 0 µs, and with gate times that varied from 40 µs (purple line) to 200 µs (red line). As described in the text, increasing gate time does not change the intensity of the prompt (background) fluorescence, but can be adjusted to maximize the long-lived Eu^{3+} emission.
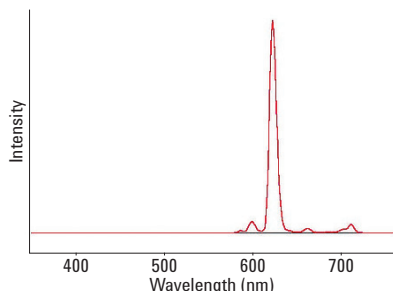

Figure 5.   The time-resolved emission spectrum of Eu^{3+} in a ligand solution. The spectrum was collected after optimizing the time-resolved collection parameters so that all contributions from nontarget sources have been eliminated.

Worked example: using the Agilent Cary Eclipse to measure the decay of europium (Eu^{3+}) in PMMA block

The fluorescence response of europium decays as a function of time. The unique fluorescence properties of europium (Eu^{3+}) and other lanthanide metals have decay times in the order of 0.5–3 ms in aqueous solutions and in solid matrices such as PMMA. The Cary Eclipse Fluorescence spectrophotometer can collect a data point every 40 µs. This means that a decay reaction that lasts in the order of 5 ms can be recorded have 100 decay curves averaged, each containing 100 data points, and all of this displayed on the screen in less than a second. If a gate time of less than 40 µs is used, the decay curve is collected in stages.

Figure 6 shows the results of two experiments that were conducted to measure the fluorescence decay curves of Eu^{3+} in a PMMA block. Data were collected with a 50 µs gate time (left hand panel) and a 2 µs gate time (right hand panel) and illustrate that by collecting 1,000 points over 2 ms, the Cary Eclipse can achieve excellent signal-to-noise ratio (S/N) with very small gate times. The data from the 50 µs gate time experiment were then used for rate and lifetime calculations, the results of which are shown in Table 2.

The data shown in Figure 6 illustrate that the Cary Eclipse effectively has the capacity to collect data faster than the decay time of Eu^{3+} in the PMMA block. The right panel shows that the decay curve has not yet reached a point where it is parallel to the x-axis (and would indicate equilibrium). This highlights the capabilities of the Cary Eclipse to reliably record data from samples with extremely short and infinitely longer lifetimes. It should also be remembered that when these measurements are made using a Cary Eclipse Fluorescence spectrophotometer all of this can be achieved without changing any hardware components.
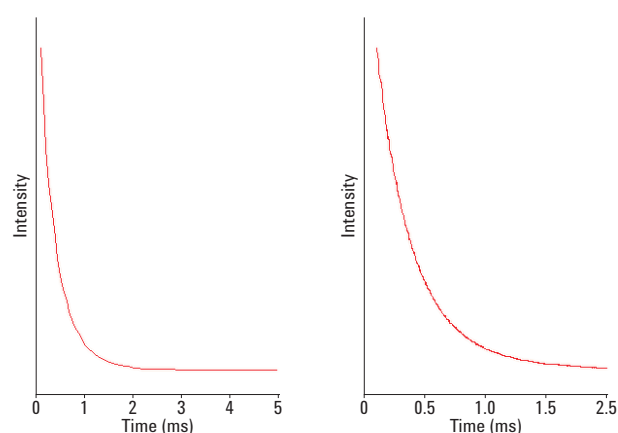

Figure 6.   Time-resolved fluorescence decay curves of Eu^{3+} collected using a 50 µs gate time (left hand panel) and a 2 µs gate time (right hand panel). This example shows how the Agilent Cary Eclipse Fluorescence spectrophotometer achieves excellent S/N with very small gate times, collecting 1,000 points over 2 ms.

Table 2.   Rate and Lifetime Data Calculated from the Time-resolved Fluorescence Decay Curves of Eu^{3+} Collected Using a 50 µs Gate Time

Rate calculation

Equation: Intensity = $A1 \times e^{(-k1 \times time)} + C$

Experiment	Start (ms)	Stop (ms)	k1 (ms^{-1})	A1	C	S.D.
	0.00	5.00	2.842 ± 0.0	457.0969 ± 4.6	0.9614 ± 0.4	1.6199

Lifetime calculation

Equation: Intensity = $A1 \times e^{(-time \times TAU1)} + C$

Experiment	Start (ms)	Stop (ms)	TAU1 (ms)	A1	C	S.D.
	0.00	5.00	0.352 ± 0.0	457.0874 ± 4.6	0.9609 ± 0.4	1.6199

Calculations were performed using the Lifetime software application in WinFLR.

Conclusions

Because data collected using phosphorescence lifetime measurements are entirely independent of the absolute concentration of the emitting species and the intensity of the emitted light, the results provide a huge amount of information about the local chemical environment of the emitting species. That makes lifetime measurements an extremely attractive technique with which to:

- Elucidate the number of emitting species in a mixture (for example, to detect the number of receptors a labeled drug binds to)

- Perform Lanthanide Resonance Energy Transfer (LRET) studies

- Analyze the size and shape of the emitting species

- Investigate inter- and intramolecular interactions and binding efficiencies

- Get information about the local environment (viscosity, polarity, and protein conformations)

- Detect the presence of certain analytes (for example, oxygen, carbon dioxide, pesticides, or antibiotics)
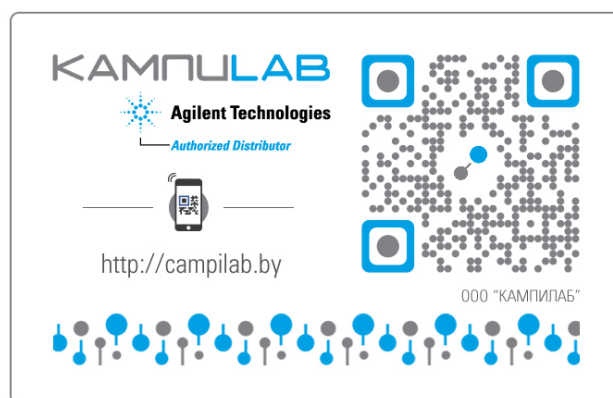
Studying the phosphorescence lifetime of a sample provides an additional layer of information to that one can achieve after examining the time-delayed emission. Indeed, time-resolved delayed emission measurements and phosphorescence lifetimes are ideally complementary, and when these measurements are made using a robust and reliable Agilent Cary Eclipse Fluorescence spectrophotometer (which, because it has been proven not to photodegrade samples, guarantees that you get the correct answer from every sample) allow you to get as much genuine information about your precious sample as possible.

References

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2. Diamandis, E. P. *Clin. Biochem.* **1988**, 21, 139-150

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