The need to screen large numbers of targets against extensive small molecule libraries has led to the demand for an increase in both the sensitivity and speed of assays. Increasingly, fluorescence-based assays are becoming the method of choice for high-throughput screening. Here, we describe the properties of a number of reagents developed by Amersham Biosciences for use in time-resolved fluorescence assays based on fluorescence resonance energy transfer.

Introduction

Fluorescence resonance energy transfer (FRET) is the radiationless transfer of excitation energy from a donor to an acceptor molecule. This distance-dependent phenomenon occurs over 1–10 nm and is comparable with the dimensions of biological macromolecules, making FRET a valuable tool in studying proximity events in biological systems (1).

The majority of organic dyes used for FRET have a fluorescence lifetime < 10 ns. However, background fluorescence from biological samples, buffer components, and plasticware also have similar lifetimes, and this can limit assay sensitivity (2). To overcome this problem, dyes with considerably longer lifetimes have been used in assays based on time-resolved fluorescence (TRF) whereby the detection and measurement of assay signal is delayed until background fluorescence has dissipated (Fig 1).

Chelate properties

Naked lanthanide ions have a low extinction coefficient and solvent, especially water, quenches their luminescence. Many organic ligands have been synthesized that can chelate a lanthanide ion triplet, resulting in an increase in luminescence by shielding the lanthanide ion from vibronic quenching.

To be effective in biological assays, chelates ideally need to have a high coordination number, efficient light harvesting properties, long triplet excited state lifetime, large Stokes shift, low number of coordinated water molecules, and good solubility in aqueous media. One such chelate that has these desired properties is based upon a complex of Eu³⁺ with terpyridine-his(methyl-enamine)tetraacetic acid (TMT)◊. Furthermore, the 3-amino group of the Eu (TMT) chelate is readily converted to the isothiocyanate (Fig 2), thereby allowing labelling of primary amine groups on a range of biomolecules. The fluorescence lifetime for an aqueous solution of unconjugated Eu (TMT) is 1.4 ms.

Principles of TR-FRET and TRF

Time-resolved fluorescence resonance energy transfer (TR-FRET) combines the benefits of FRET and TRF. The use of europium chelates as donors and Cy5 as a spectrally matched acceptor improves assay sensitivity and, in particular, the ratio of signal-to-background noise (3).

The fluorescence lifetime of most conventional fluorophores can be 10 ns or less; whereas lanthanide chelates exhibit a relatively efficient long-lived fluorescence lifetime of about 200–1500 µs. The advantage of such long-lived emissions is the ability to use time-resolved techniques for measurement (Fig 1).

With TRF, the fluorescent compounds are excited with a short pulse of light from a flash lamp. The rate of fluorescence decay is exponential and proportional to the lifetime of the individual fluor. Therefore, fluor with short lifetimes will completely decay in <100 µs, whereas the longer lifetime lanthanide chelates can take about 1500 µs.

After excitation, measurement of the emission commences 100–400 µs after the flash lamp pulse, which allows all short-lived background fluorescence and light scattering to dissipate. The fluorescence signal from the lanthanide is then counted over a fixed time interval before the sample is re-excited and a new measurement cycle begins (Fig 1). The long-lived lanthanide fluorescence signal can therefore be measured with very high sensitivity.

Fig 1. Excitation measurement of the emission commences following an initial delay of 100–400 µs. The fluorescence signal from the lanthanide is then counted over a fixed time interval before the sample is re-excited and a new measurement cycle begins.

See licensing information.
Properties of Eu (TMT) isothiocyanate

The luminescence intensity of the Eu (TMT) isothiocyanate is not affected by a variety of common buffer components such as Ca²⁺ ions, Mg²⁺ ions, and EGTA. Little effect is seen on the emission spectra of Eu (TMT) in the pH range 5.0–9.6 (Fig 3). In addition, the presence of 10% DMSO has little or no effect on the emission spectrum or luminescence intensity observed.

Properties of Cy5 mono NHS ester

Cy5 monofunctional dye (Fig 4) is a bright, water-soluble dye that is readily conjugated to biomolecules via active ester chemistry. It has a large molar extinction coefficient (250 000) and emits in the far-red region of the spectrum making it ideal as an acceptor in applications based on TR-FRET using Eu chelates as the donor. In buffers of varying pH, little effect is seen on the emission spectra of Cy5 (Fig 5). In addition, the presence of 10% DMSO has little or no effect on the emission spectrum or fluorescence intensity observed.

Preparation of the Eu (TMT) chelate

Terpyridine-bis(methyl-enamine)tetra-acetic acid europium chelate can be prepared as shown in Figure 6 (4, 5).

Examples of labelling with Eu (TMT) isothiocyanate

The following examples have been generated using reagents from the Amersham Biosciences product.
range. This data is presented as reference information for researchers wishing to establish their own applications. Optimum conditions will need to be determined empirically for each application.

**Streptavidin labelling using Eu (TMT) isothiocyanate**

Labelling of streptavidin is performed using 0.1 M sodium carbonate buffers at pH 9.0. Transfer the streptavidin solution to a vial containing the weighed europium chelate labelling reagent, mix gently, and incubate at 2–8 °C overnight on a roller mixer.

After incubation, transfer the streptavidin europium chelate solution to fresh dialysis tubing that has been rinsed with reagent grade water and fresh phosphate buffered saline (PBS). Dialyze the solution in 10 l PBS for 1.5 h at room temperature. Repeat the dialysis once.

Next, dialyze the streptavidin europium chelate solution against PBS containing 0.01% sodium azide for 1 h at room temperature. Repeat the dialysis twice using fresh buffer.

Recover the dialyzed material and accurately determine the volume. Streptavidin europium chelate conjugate should be stored at 2–8 °C in a sealed container and protected from light.

**Oligonucleotide labelling using Eu (TMT) isothiocyanate**

Amine-modified oligonucleotides should be freeze-dried before labelling. Labelling is carried out in 0.1 M sodium carbonate buffer at pH 9.0 at a concentration of approximately 0.5 mM. Incubate the reaction mixture overnight at 2–8 °C with mixing. Purify the labelled oligonucleotide by polyacrylamide gel electrophoresis.

**Applications**

**Eu (TMT) anti-GST antibody donor and Cy5 streptavidin acceptor**

p65 glutathione S-transferase (GST) fusion protein (500 pM), and Eu (TMT) anti-GST antibody (500 pM), were incubated at room temperature for 60 min. Biotinylated dsDNA NF-κB-specific (HIV-L) consensus sequence (1 nM) was added to the reaction mixture and incubated for a further 60 min at room temperature. Finally, Eu (TMT) Streptavidin (500 pM) was added. A signal:background of 6:1 was observed.

**Eu (TMT) anti-phosphotyrosine antibody donor and Cy5 streptavidin acceptor**

Biotinylated peptide (specific sequence for Lck protein tyrosine kinase) phosphorylated at the tyrosine residue (50 nM), Eu (TMT) anti-PY20 antibody (10 nM), and Cy5 Streptavidin (10 nM) were added to a 384-well microplate and incubated for 60 min at room temperature. A signal:background of 8:1 was observed.

**Eu (TMT) Streptavidin donor and Cy5 anti-phosphotyrosine antibody**

Biotinylated peptide (specific sequence for Lck protein tyrosine kinase) phosphorylated at the tyrosine residue, 50 nM, Cy5 anti-PY20 antibody, 100 nM, and Eu (TMT) Streptavidin, 8 nM, were added to a 384-well microplate and incubated for 60 min at room temperature. A signal:background of 6:1 was observed.

**NF-κB protein: DNA binding assay**

Nuclear factor-κB (NF-κB) is a transcription factor that is considered to be of physiological importance because of its key role as a regulatory molecule in the immune response, inflammation, cancer, and apoptosis (6, 7). A TR-FRET assay to evaluate the binding interaction between the p65 subunit of NF-κB and a dsDNA NF-κB-specific (HIV-L) consensus sequence has been developed◊ (Fig 7).

Assays containing NF-κB p65-GST (University of St Andrews, UK), 10 nM recombinant fusion protein, and 10 nM Eu (TMT)-anti-Glutathione S-transferase antibody were incubated in the dark with agitation for 1 h at 20–25 °C in 10 mM HEPES, 20 mM sodium acetate, 0.2 mM EDTA buffer at pH 7.0 containing 5 mM DTT, 1 mg/ml BSA, and 0.05% NP40.

◊ See licensing information.
Sensitivity of detection was evaluated using a 2ⁿ titration of 40 nM biotinylated dsDNA (containing the NF-κB consensus binding sequence) and the reaction mix was incubated at 20–25 ºC for a further 1 h in the dark with agitation.

For competition and inhibition assays, 20 nM biotinylated NF-κB specific dsDNA was added to the reaction mix and incubated as stated together with competitor dsDNA, IkBα protein (University of St Andrews, UK), or buffer respectively.

Finally, Cy5 Streptavidin (10 nM) was added to each reaction well and incubated for a further 15 min. Reactions were performed in a total volume of 100 µl using Corning black 384-well non-binding surface plates. TR-FRET was measured on FARCyte™ Fluorescence Plate Reader using the standard 340-nm excitation filter and an optimized 670 nm filter (Thermo Corion). Lag time was set at 50 µs and integration time was 400 µs (Fig 8).

The lowest limit of detection of NF-κB specific dsDNA in the assay was 5.2 fmol/well (52 pM). At maximal acceptor concentration (40 nM), signal:background for the assay was 9.6:1. We have also demonstrated the use of this assay on LEADseeker™ Multimodality Imaging System (8).

Competition for binding of specific and non-specific dsDNA to the p65-GST protein indicated that signal is significantly reduced with the addition of the specific dsDNA competitor containing the NF-κB p65 consensus binding sequence (Fig 9). The non-specific dsDNA reduced signal only marginally when added at considerable excess, demonstrating binding specificity.

Inhibition of protein:dsDNA binding was evaluated using IkBα protein. IkBα is a NF-κB regulatory protein found in the cell cytoplasm which inhibits DNA binding activity (9).

Effect of inhibition by IkBα is shown in Figure 10 and demonstrates that increasing concentrations of the inhibitory protein reduced the signal in the assay. The IC₅₀ value for IkBα was 15 nM.

**Detection of phosphorylated peptides**

TR-FRET assays have become a popular method for high-throughput screening (HTS) of kinase enzyme targets (10). Here we demonstrate the use of Eu (TMT) anti-Phosphotyrosine antibody and Cy5 Strep-tavidin for quantitating phosphorylated peptide. This assay is shown diagrammatically in Figure 11.

Assays were set up in buffer (50 mM HEPES [pH 7.0], 10 mM MgCl₂, 1 mM DTT, and 0.1% (w/v) BSA) containing 30 µl of biotinylated peptide at a final concentration of 2 µM. This ‘total peptide’
consisted of two separate peptides, both biotinylated 14-mers. One contained a single phosphotyrosine residue. The other peptide was identical in sequence, except it contained a tyrosine residue in place of phosphotyrosine. The phosphotyrosine-containing peptide was added at 0, 0.31, 0.62, 1.25, 2.5, 5, 10, and 20% of the total peptide concentration.

For detection, 10 µl (20 nM final concentration) Eu (TMT) anti-phosphotyrosine antibody and 10 µl (100 nM final concentration) Cy5 Streptavidin were added. Final reaction volume was 50 µl. Assays were performed in Corning black 384-well non-binding surface plates.

Following the addition of the detection reagents, reactions were incubated for 60 min at ambient temperature (22–25 °C) with agitation, before TR-FRET signals were measured. TR-FRET was measured on FARCyte Fluorescence Plate Reader using 340-nm excitation and 670-nm emission filter sets. Lag time was set at 50 µs and integration time was 400 µs. The resulting signals observed are shown in Figure 12.

A Z’ value of 0.71 (11) was obtained when the phosphorylated peptide was added at 10% of the total peptide concentration.

**Hybridization assays**

Eu (TMT) chelate can also be used in DNA hybridization assays (Fig 13). To demonstrate this application, biotinylated capture oligonucleotides labelled with Eu (TMT) chelate were immobilized on 96-well streptavidin-coated plates. Subsequently, unlabelled or Cy5 labelled target oligonucleotides were added and allowed to hybridize. Following a series of stringency washes, luminescence signals were measured and results are shown in Figure 14.

When luminescence of the Eu (TMT) was measured, wells containing chelate/unlabelled oligonucleotide duplexes gave high luminescence signals compared with those from wells containing chelate/Cy5 oligonucleotide duplexes (Fig 14A). Generally signals were reduced by > 95% suggesting efficient energy transfer between chelate donor and Cy5 acceptor. This observation was confirmed when luminescence was measured at donor wavelengths (panel A, excitation 340 nm; emission 615 nm) and TR-FRET wavelengths (panel B, excitation 340 nm; emission 670 nm).
measured at TR-FRET wavelengths. In this case, wells containing chelate/Cy5 duplexes gave a significant signal compared with those obtained in control wells (Fig 14B).

Conclusion
The Eu (TMT) isothiocyanate (Fig 2) has been demonstrated to be a very sensitive reagent that can be utilized in a wide variety of biological applications as both a stand alone reagent for applications based on TRF and also as a temporally discriminated donor in a FRET pair. Amersham Biosciences range of donor and acceptor generic reagents allows researchers to perform sensitive, non-radiometric screening assays based on TR-FRET.

References