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Simultaneous Monitoring of Discrete Binding Events Using Dual-Acceptor Terbium-Based LRET

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The measurement of Förster resonance energy transfer (FRET) between a proximal donor and acceptor fluorophore is an established method for determining distances between fluorophores on the approximately 20−90 Å scale and as such has found application toward measuring geometries in biological systems at the protein or DNA level and of monitoring dynamic processes in living cells.[1] Additionally, because the technique can be used to determine the association or dissociation of species that contain fluorophores that function as a FRET pair, the technology has found widespread use when applied to biochemical assays used in high-throughput screening (HTS) to identify and characterize biologically active small molecules.[2] Lanthanide-based resonance energy transfer (LRET) refers to the method when a lanthanide ion (typically Tb^{3+} or Eu^{3+}) is used as the donor species. When appropriately sensitized and protected from water using a chelate that incorporates a light-harvesting organic moiety, lanthanide ions such as Tb^{3+} or Eu^{3+} have excited-state lifetimes that can range upward to several milliseconds, and by measuring resonance energy transfer to an acceptor in a gated detection mode, nonspecific assay interference from matrix components is reduced.

FRET between a single donor and two different acceptor species ("double FRET") using organic fluorophores has been applied to investigate model systems using hybridized DNA oligonucleotides[3] to study the orientation of transcription factors bound to double-stranded DNA,[4] to monitor conformational changes in DNAzymes or DNA four-way junctions,[5] and to independently monitor both RNA folding and binding to protein.[6] In all cases, spectral bleed-through of donor emission into either of the channels used to monitor acceptor emission, or of the first acceptor into the emission signal of the second acceptor, is substantial and can complicate analysis and require mathematical deconvolution.

In addition to their enhanced excited-state lifetimes, CS124-sensitized Tb^{3+} chelates[7] have unique excitation and emission spectra that make them ideally suited as donors in LRET-based systems that employ multiple distinct acceptor fluorophores. The Tb^{3+} emission spectrum is characterized by four distinct bands, centered at 490, 546, 583, and 620 nm. Because these emissions span a wide range of spectral bandwidth, and because emission is negligible between and beyond these peaks, Tb^{3+} can be paired with a variety of acceptors that have an excitation spectrum that overlaps with the Tb^{3+} emission spectrum and that emit where Tb^{3+} emission is negligible. By choosing acceptors whose emission spectra do not overlap with one another, no deconvolution is required to separate bleed-through emission of one acceptor from the emission of the other. Additionally, by performing measurements in a gated detection mode 100 μs after excitation, interference caused by direct off-peak excitation of the short-lifetime acceptor fluorophores is eliminated.

The excitation and emission spectra of CS124-TTHA*Tb^{3+}, fluorescein, and Alexa Fluor 633 (AF633) are shown in Figure 1.

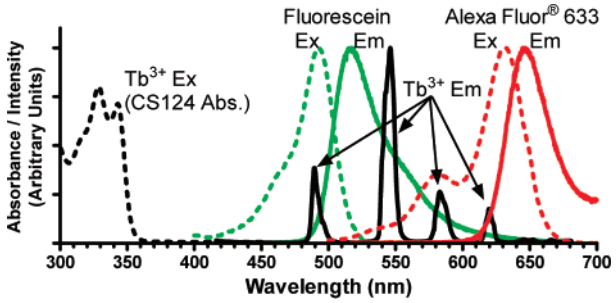

Figure 1. Excitation (Ex) and emission (Em) spectra of CS124-TTHA*Tb^{3+}, fluorescein, and Alexa Fluor 633.

The excitation of Tb^{3+} is sensitized through the CS124 moiety, which absorbs between 300 and 350 nm. Energy transfer from Tb^{3+} to fluorescein can be measured with a filter centered at 520 nm (25 nm bandpass), that captures fluorescein near its emission maximum, and that contains less than 1% of the total Tb^{3+} emission as bleed-through. Energy transfer to AF633 can be measured with a filter centered at 665 nm (10 nm bandpass), that captures <0.2% of the total fluorescein spectra and less than 1% of the total Tb^{3+} emission spectra as bleed through. Overlap of the Tb^{3+} emission spectrum with the excitation spectra of fluorescein or AF633 provides a calculated Förster radius of approximately 49 Å for the Tb^{3+}→fluorescein pair, and 59 Å for the Tb^{3+}→AF633 pair. However, when using long-lifetime donor probes in systems in which the average donor−acceptor distance varies over the time frame of the excited-state lifetime, the Förster radius underestimates the energy transfer efficiency between the fluorophores, as the energy transfer efficiency is determined primarily by the distance of closest approach during the excited-state lifetime and not by the average distance. As a result, RET efficiency can be enhanced in systems in which the donor has a large degree of positional freedom, such as when attached to an antibody.[8]

We applied a Tb-based, dual acceptor strategy to the study of ligand-specific interactions of peptides with nuclear receptor ligand binding domains (NR-LBDs, Scheme 1). The 48 members of the (human) nuclear receptor family function as transcription factors, and their biological activity is due in part to ligand dependent (or independent) interactions with specific coregulatory proteins that enhance (coactivators) or repress (corepressors) transcriptional activity, either directly or through the recruitment of other accessory proteins. These interactions can be studied in vitro using the NR-LBD and synthetic peptides that contain the interacting motif of coactivator ("NR box" sequence) or corepressor ("CoRNR box" sequence) proteins, and over 50 such peptides have been described in the literature.[9] Binding of ligand to the receptor causes a conformational change in the receptor that leads to an increase or decrease in the affinity of the receptor for these motifs. By labeling the receptor with Tb^{3+} through a Tb-labeled anti-GST antibody

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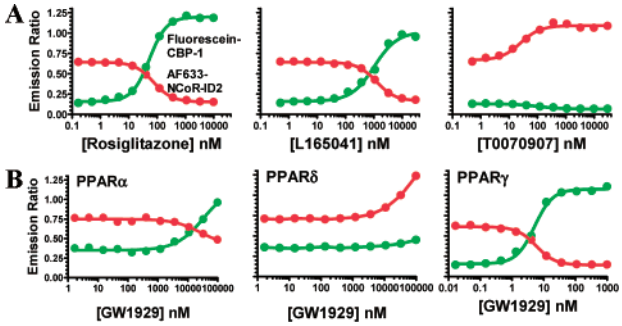
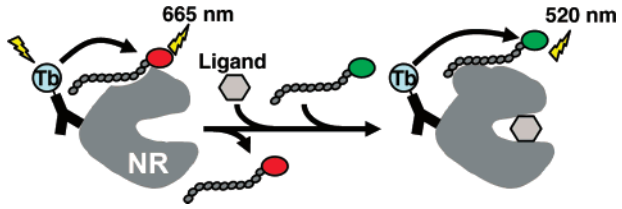


Figure 2. (A) Simultaneous monitoring of ligand-dependent and ligand-independent association of fluorescein-labeled coactivator (CBP-1, green) and AF633-labeled corepressor (NCoR-ID2, red) derived peptides with PPARγ. (B) Differential response of PPARα, δ, and γ to GW1929. The emission ratio for the fluorescein-labeled peptide is taken as the fluorescein-dependent signal at 520 nm divided by the Tb^{3+}-dependent signal at 495 nm. The emission ratio for the AF633-labeled peptide is taken as the AF633-dependent signal at 665 nm divided by the Tb^{3+}-dependent signal at 495 nm. Each datapoint is the average of 4 replicate wells. Error bars marking standard deviations are smaller than the symbols used to mark each datapoint.

Scheme 1. Schematic Illustration of Ligand-Independent and Ligand-Dependent Peptide−Protein Interactions Monitored Simultaneously by Tb-Based LRET



that targets the GST tag of the recombinant receptor and using fluorescein or AF633 labeled peptides, preferential binding of the NR to coactivator or corepressor derived peptides can be monitored simultaneously.

Ligand-independent and ligand-dependent interactions of PPAR (peroxisome proliferator-activated receptor) α, δ, and γ with coactivator and corepressor-derived peptides were studied using a fluorescein-labeled peptide derived from residues 62−80 of the coactivator CBP (CBP-1)[10] and an AF633 labeled peptide derived from residues 2270−2292 of the corepressor NCoR (NCoR-ID2).[11] When a solution containing 5 nM GST-PPARγ LBD, 5 nM Tb-labeled anti-GST antibody, and 125 nM each of the labeled coactivator and corepressor peptides was titrated with either of the known PPARγ agonists rosiglitazone[12] or L165041,[13] there was concomitant disruption of ligand-independent association with the corepressor peptide and a corresponding increase in association with coactivator peptide (Figure 2). In contrast, titration with the PPARγ antagonist T0070907[14] caused an increase in association with corepressor peptide and a decrease in a low basal level of ligand-independent association with coactivator peptide.

We then profiled the response of PPARα, δ, and γ to GW1929, a small molecule PPARγ agonist that has previously been shown to bind to the α and δ isoforms with substantially reduced affinity.[15] When GW1929 was titrated against the γ isoform, signal from fluorescein-labeled CBP-1 increased in concert with a decrease in AF633 labeled NCoR-ID2, with an EC_{50} value of approximately 5 nM. Similar observations were made with PPARα, but only at substantially higher concentrations of GW1929. Interestingly, at high concentrations, GW1929 caused a preferential increase in association with the corepressor peptide, suggesting that GW1929 could have an antagonistic effect on PPARδ activity at higher concentrations. This observation was confirmed in a cell-based assay (see Supporting Information).

To our knowledge, these studies represent the first reported use of Tb-based LRET for the simultaneous monitoring of orthogonal binding events in a biochemical system. The unique properties of the Tb^{3+} chelate emission spectrum provide for a simple way to monitor such events with no mathematical deconvolution of acceptor emission signals. This work sets the stage for the application of this strategy to the study of more complex biochemical processes or for use in applications such as screening libraries of antibodies (or other affinity reagents) for specificity against closely related epitopes. Additionally, the strategy we have developed can be extended to systems in which the terbium donor is present on different receptors, thereby allowing the simultaneous monitoring of independent binding events on different receptors.

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Supporting Information Available: Complete refs 13 and 15; complete experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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