J|A|C|S ARTICLES

Published on Web 03/15/2010

Monitoring a Coordinated Exchange Process in a Four-Component Biological Interaction System: Development of a Time-Resolved Terbium-Based One-Donor/Three-Acceptor Multicolor FRET System

Sung Hoon Kim, Jillian R. Gunther, and John A. Katzenellenbogen*

Department of Chemistry, University of Illinois, 600 South Mathews Avenue, Urbana, Illinois 61801

Received January 11, 2010; E-mail: jkatzene@uiuc.edu

Abstract: Hormonal regulation of cellular function involves the binding of small molecules with receptors that then coordinate subsequent interactions with other signal transduction proteins. These dynamic, multicomponent processes are difficult to track in cells and even in reconstituted in vitro systems, and most methods can monitor only two-component interactions, often with limited capacity to follow dynamic changes. Through a judicious choice of three organic acceptor fluorophores paired with a terbium donor fluorophore, we have developed the first example of a one-donor/three-acceptor multicolor time-resolved fluorescence energy transfer (TR-FRET) system, and we have exemplified its use by monitoring a ligand-regulated protein−protein exchange process in a four-component biological system. By careful quantification of the emission from each of the three acceptors at the four channels for terbium donor emission, we demonstrate that any of these donor channels can be used to estimate the magnitude of the three FRET signals in this terbium-donor triple-acceptor system with minimal bleedthrough. Using this three-channel terbium-based, TR-FRET assay system, we show in one experiment that the addition of a fluorescein-labeled estrogen agonist displaces a SNAPFL-labeled antiestrogen from the ligand binding pocket of a terbium-labeled estrogen receptor, at the same time causing a Cy5-labeled coactivator to be recruited to the estrogen receptor. This experiment demonstrates the power of a four-color TR-FRET experiment, and it shows that the overall process of estrogen receptor ligand exchange and coactivator binding is a dynamic but precisely coordinated process.

1. Introduction

The regulation of cellular function by hormones and drugs typically proceeds through multicomponent interactions involving small molecules that bind to receptors and then effect subsequent interactions of the ligand−receptor complex with other proteins in signal transduction cascades. Because of the dynamic nature of these processes and the number of components involved, it is challenging to track these interactions at the molecular level in cells and even in reconstituted in vitro systems. Most methods enable only two-component interactions to be followed, and they often have limited capacity to follow dynamic changes or to monitor whether they proceed in a coordinated fashion.

A paradigmatic example of a cellular regulatory system involving four components is the estrogen receptor (ER), a regulator of gene transcription in target tissue cells (Figure 1). The activity of the ER is modulated by ligand binding: When *agonists* are bound, the ER ligand-binding domain adopts a conformation in which its C-terminal helix, helix-12, is positioned so as to enable the recruitment of coactivator proteins (*State 1*); recruitment of such a steroid receptor coactivator (e.g., SRC) brings to the ER-agonist complex interactions and cellular activities required for upregulation of gene transcription (*State 2*). By contrast, when *antagonists* are bound, helix-12 is placed in a conformation that blocks coactivator recruitment (*State 3*). Unliganded ER (*State 0*) is also unable to recruit coactivators. The interactions among some of these components have been followed in cells[1−6] and in vitro,[7,8] but typically only by methods capable of monitoring two components at the same time. In particular, it has not been possible to monitor a ligand-exchange process in which an antagonist ligand bound to ER (*State 3*) is replaced by an agonist ligand (*State 1*), following, at the same time, how this ligand exchange is coordinated with the recruitment of the coactivator (*State 2*). In other words, it has not been possible to ask whether the displacement of an antagonist by an agonist is precisely coordinated with the recruitment of a

(1) Wang, Y.; Botvinick, E. L.; Zhao, Y.; Berns, M. W.; Usami, S.; Tsien, R. Y.; Chien, S. *Nature (London, U.K.)* **2005**, *434*, 1040–1045.
(2) Zwart, W.; Griekspoor, A.; Rondaij, M.; Verwoerd, D.; Neefjes, J.; Michalides, R. *Mol. Cancer Ther.* **2007**, *6*, 1526–33.
(3) Hoffmann, C.; Gaietta, G.; Buenemann, M.; Adams, S. R.; Oberdorff-Maass, S.; Behr, B.; Vilardaga, J.-P.; Tsien, R. Y.; Ellisman, M. H.; Lohse, M. J. *Nat. Methods* **2005**, *2*, 171–176.
(4) Paulmurugan, R.; Gambhir, S. S. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 15883–15888.
(5) Kofoed, E. M.; Guerbadot, M.; Schaufele, F. *J. Biomed. Opt.* **2008**, *13*, 031207.
(6) Quaedackers, M. E.; van den Brink, C. E.; van der Saag, P. T.; Tertoolen Leon, G. J. *Mol. Cell. Endocrinol.* **2007**, *273*, 42–50.
(7) Gowda, K.; Marks, B. D.; Zielinski, T. K.; Ozers, M. S. *Anal. Biochem.* **2006**, *357*, 105–115.
(8) Tamrazi, A.; Carlson, K. E.; Rodriguez, A. L.; Katzenellenbogen, J. A. *Mol. Endocrinol.* **2005**, *19*, 1516–1528.

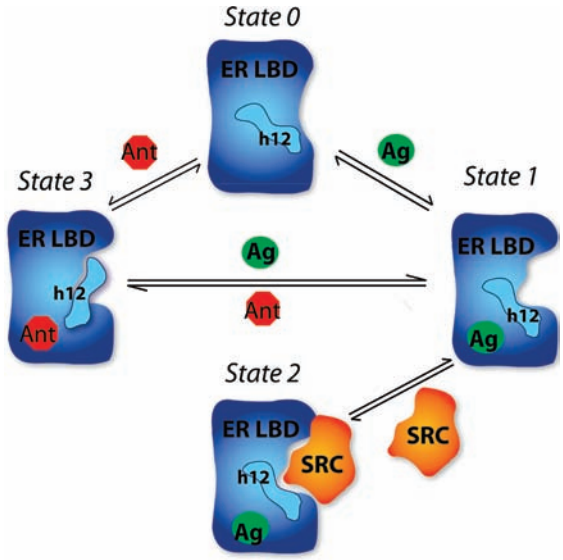


Figure 1. Schematic illustrating the interactions of the estrogen receptor ligand binding domain (ER-LBD, blue) with an antagonist (Ant, red) or an agonist (Ag, green) and a steroid receptor coactivator (SRC, orange). These four components are eventually studied simultaneously with the one-donor/three-acceptor time-resolved FRET system we have developed. In Figures 3, 4, and 6, we have retained the same color scheme, but we also indicate with a designated color code the fluorophores used to label each of the components in each experiment.

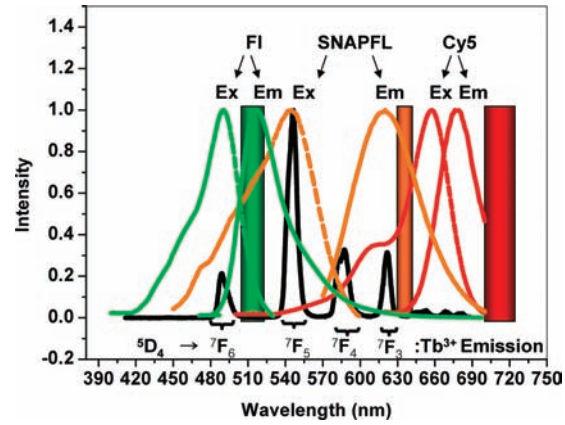


Figure 2. Emission spectrum of streptavidin-Tb^{3+} chelate, sensitized with carbostyril 124 and excited at 340 nm (black solid line). Excitation and emission spectra of three acceptor fluorophores: fluorescein (green), SNAPFL (orange), and Cy5 (red). The solid bars depict the emission filter used to measure the corresponding fluorophore emission signal (green bar for fluorescein, 520/25 nm; orange bar for SNAPFL, 642/20 nm; red bar for Cy5, >700 nm). All emission and absorption spectra are normalized to the same maximum value. For clarity, all figures and cartoons will represent emission signals of terbium, fluorescein, SNAPFL, and Cy5 with lines colored black, green, orange, and red, respectively.

coactivator (*State 3* proceeds directly to *State 2*) or whether the agonist-liganded but coregulator-free state (*State 1*) is present during this ligand-exchange process. We set out to examine the question of the degree to which ligand exchange is coordinated with coactivator recruitment by developing a fluorescence-based system that could be used to monitor simultaneously all four of the components involved in this exchange process.

FRET, or Förster resonance energy transfer technology, has played an important role in the exploration of diverse biological phenomena through its use in proteomic and genomic analyses in both live cells[9,10] and in vitro models.[11] It has also been valuable as a tool for high-throughput screening campaigns for the discovery of new, scientifically relevant compounds.[12] In place of the conventional two-color FRET experiments (one donor/one acceptor), three-color FRET systems (one donor/two acceptors, two donors/one acceptor, sequential energy transfer from high energy level to low energy level) have recently emerged to probe more complicated biological systems and to enable a more complete tracking of the interactions of multi-component biomolecular systems.[13−18]

A serious limitation to the use of multicolor FRET systems based on common organic fluorophores lies in the overlap, or "bleed-through effect", in the excitation or emission spectra of these molecules because of their large bandwidths (>150 nm) and/or small Stokes shifts (<30 nm). This not only interferes with the clear interpretation of biomolecule interactions in two- or three-color systems but also impedes application of FRET technology to systems composed of more than three interacting partners. Although construction of a multicolor FRET system has been deemed theoretically possible,[16] the design and application of a four-color FRET system (one donor/three acceptors) has, to our knowledge, not yet been reported.

Traditional wholly organic dye-based FRET systems have been modified by the adoption of time-gated LRET (luminescence or lanthanide resonance energy transfer) donor systems based on chelates of long-lived (millisecond emission) Tb^{3+} or Eu^{3+} donors.[19−23] The Tb^{3+} chelate preferentially used in lanthanide-based LRET systems is characterized by four discrete emission bands (Figure 2, black solid line: $^5D_4 \rightarrow {}^7F_6$ (489 nm), $^7F_5$ (546 nm), $^7F_4$ (583 nm), $^7F_3$ (620 nm)). These unique features of Tb^{3+}-based LRET offer intriguing possibilities for the design of novel multifluorophore assays.

LRET has three principal advantages: The narrow, multiple emission bands of the lanthanide complexes mean that donor emission has good intensity and can be monitored at more than one wavelength using narrow band-pass filters that minimize overlap with the broader and generally lower intensity acceptor emissions. Similarly, the narrow lanthanide emission bands leave gaps with very low donor emission that are well suited for monitoring emissions from acceptor fluorophores with minimal overlap from donor emissions. In addition, because of the long lifetime of lanthanide emission, time gating can be processed to eliminate direct excitation of the acceptor fluorophores, so that the time-resolved (TR)-FRET signal is more accurate and

(9) Giepmans, B. N. G.; Adams, S. R.; Ellisman, M. H.; Tsien, R. Y. *Science* **2006**, *312*, 217–224.
(10) Vogel, S. S.; Thaler, C.; Koushik, S. V. *Sci. STKE* **2006**, *2006*, re2.
(11) Sacca, B.; Meyer, R.; Feldkamp, U.; Schroeder, H.; Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2008**, *47*, 2135–2137.
(12) Look, G. C.; et al. *Curr. Alzheimer Res.* **2007**, *4*, 562–567.
(13) Ramirez-Carrozzi, V. R.; Kerppola, T. K. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4893–4898.
(14) Horsey, I.; Furey, W. S.; Harrison, J. G.; Osborne, M. A.; Balasubramanian, S. *Chem. Commun.* **2000**, 1043–1044.
(15) Hohng, S.; Joo, C.; Ha, T. *Biophys. J.* **2004**, *87*, 1328–1337.
(16) Liu, J.; Lu, Y. *J. Am. Chem. Soc.* **2002**, *124*, 15208–16.
(17) Watrob, H. M.; Pan, C.-P.; Barkley, M. D. *J. Am. Chem. Soc.* **2003**, *125*, 7336–43.
(18) Klostermeier, D.; Sears, P.; Wong, C.-H.; Millar, D. P.; Williamson, J. R. *Nucleic Acids Res.* **2004**, *32*, 2707–2715.
(19) Robers, M. B.; Machleidt, T.; Carlson, C. B.; Bi, K. *Assay Drug Dev. Technol.* **2008**, *6*, 519–529.
(20) Selvin, P. R. *Annu. Rev. Biophys. Biomol. Struct.* **2002**, *31*, 275–302.
(21) Selvin, P. R. *Nat. Struct. Biol.* **2000**, *7*, 730–734.
(22) Hu, L. A.; Zhou, T.; Hamman, B. D.; Liu, Q. *Assay Drug Dev. Technol.* **2008**, *6*, 543–550.
(23) Chen, J.; Selvin, P. R. *J. Am. Chem. Soc.* **2000**, *122*, 657–660.

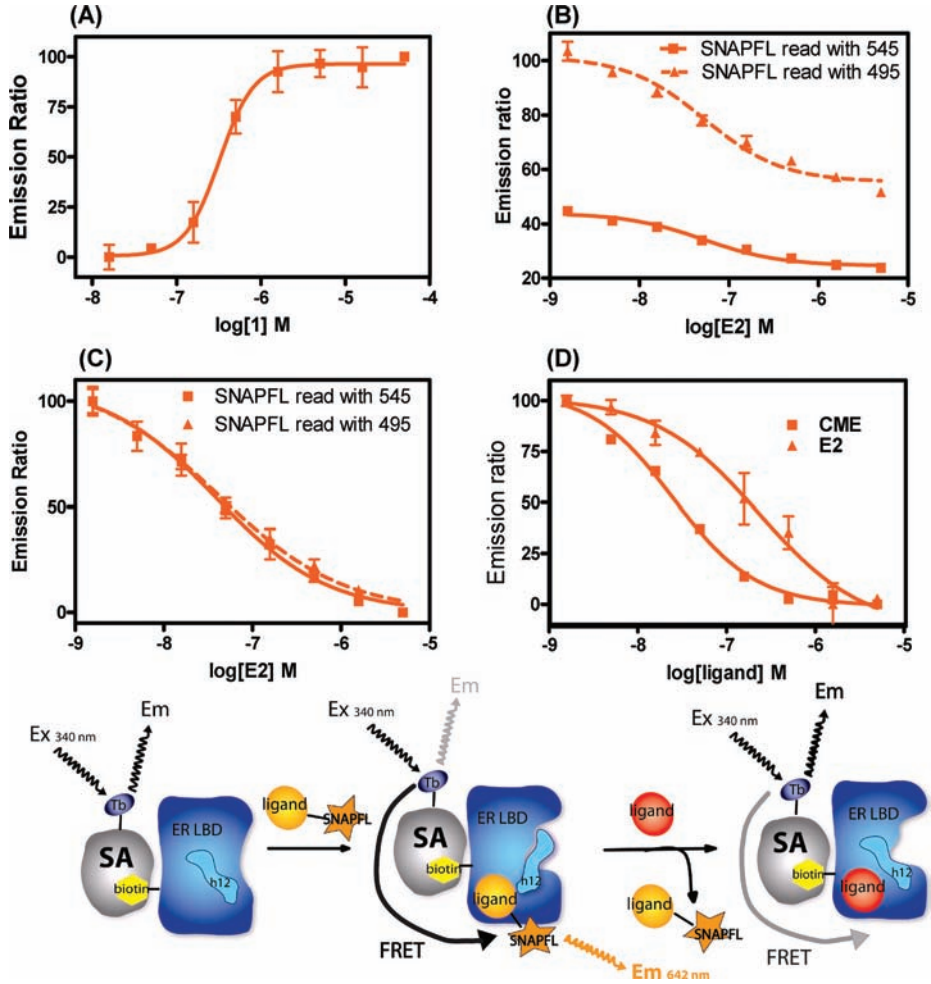


Figure 3. Titration of ERα-SATb with SNAPFL-labeled compound **1** (Panel A). (Panels B−D) FRET curves for the displacement of ligand **1** by E_2 based on the ratio of intensity data measured using a 642/20 nm channel for SNAPFL emission and terbium donor channels 495/20 nm (orange dashed-line curves) and 545/20 nm (orange solid-line curves). Panel B demonstrates the FRET intensity calculated directly from the ratio of SNAPFL raw emission intensity to the raw emission intensity from the two Tb channels. Panel C shows the data, normalized by setting the largest value of each data set as 100% and the lowest value of each set as 0%. (All data were measured as duplicates with SD ± 8% of mean value.) Panel D exhibits the FRET signal obtained from displacement of ERα-SATb saturated with **1** (3 μM) by competing agonist ligands E_2 (estradiol; orange solid line with filled triangle symbols) or 11β-chloromethylestradiol (CME; orange solid line with filled rectangular symbols). (All data measured with SD ± 10% of duplicate mean.) The bottom cartoon illustrates in a pictorial fashion the binding of SNAPFL-labeled antagonist (**1**) (first step; panel A) and then its displacement by an unlabeled ligand (second step, panels B−D). (ER LBD, estrogen receptor ligand binding domain; h12, helix 12. The emissions are shown with the colors designated in the Figure 1 legend.)

less sensitive to incomplete probe labeling than FRET from conventional short-lived organic dyes. These attributes have made LRET or TR-FRET very useful for conformational studies and for high-throughput screening assays using combinations of time-domain and wavelength-domain analyses, and initial efforts have been made for cellular imaging.[24−26] Although the unique quadruple terbium emission can transfer energy to any acceptor that has spectral overlap with any of its four emission peaks, only two-acceptor competition assay systems have, as yet, been reported (using fluorescein and either AlexaFluor 633 or Cy5 dye as the two acceptors).[27,28] In this report, we describe the development of a time-gated one-donor/three-acceptor LRET system using a Tb^{3+} chelate, and we present its application to the analysis of a complex, four-component biological regulatory system involving the estrogen receptor (ER) and the dynamics of its interaction with agonist and antagonist ligands and with a key coactivator protein, SRC. By monitoring three TR-FRET signals at once in real time, we are able to show in a single experiment that the addition of a fluorescein-labeled estrogen agonist displaces a SNAPFL-labeled antiestrogen from the ligand binding pocket of a terbium-labeled estrogen receptor, at the same time causing a Cy5-labeled coactivator to be recruited to the receptor in a precisely coordinated process.

2. Experimental Section

Materials and Methods. LanthaScreen Strepatavidin-Tb^{3+} and iodoacetamide−fluorescein were purchased from Invitrogen Inc. (Carlsbad, CA). Maleimide-Cy5 was purchased from Amersham Biosciences (Piscataway, NJ). Biotin was purchased from Quanta Biodesign (Powell, OH). The 96-well plates were purchased from Molecular Devices, Inc. (Sunnyvale, CA). Time-resolved terbium

(24) Song, B.; Wang, G.; Tan, M.; Yuan, J. *J. Am. Chem. Soc.* **2006**, *128*, 13442–13450.
(25) Poole, R. A.; Bobba, G.; Cann, M. J.; Frias, J. C.; Parker, D.; Peacock, R. D. *Org. Biomol. Chem.* **2005**, *3*, 1013–1024.
(26) Kenjiro, H.; Kazuya, K.; Shigeru, K.; Tetsuo, N. *J. Am. Chem. Soc.* **2007**, *129*, 13502–13509.
(27) Kupcho, K. R.; Stafslien, D. K.; DeRosier, T.; Hallis, T. M.; Szatkowski Ozers, M.; Vogel, K. W. *J. Am. Chem. Soc.* **2007**, *129*, 13372–13373.
(28) Jeyakumar, M.; Katzenellenbogen, J. A. *Anal. Biochem.* **2009**, *386*, 73–78.

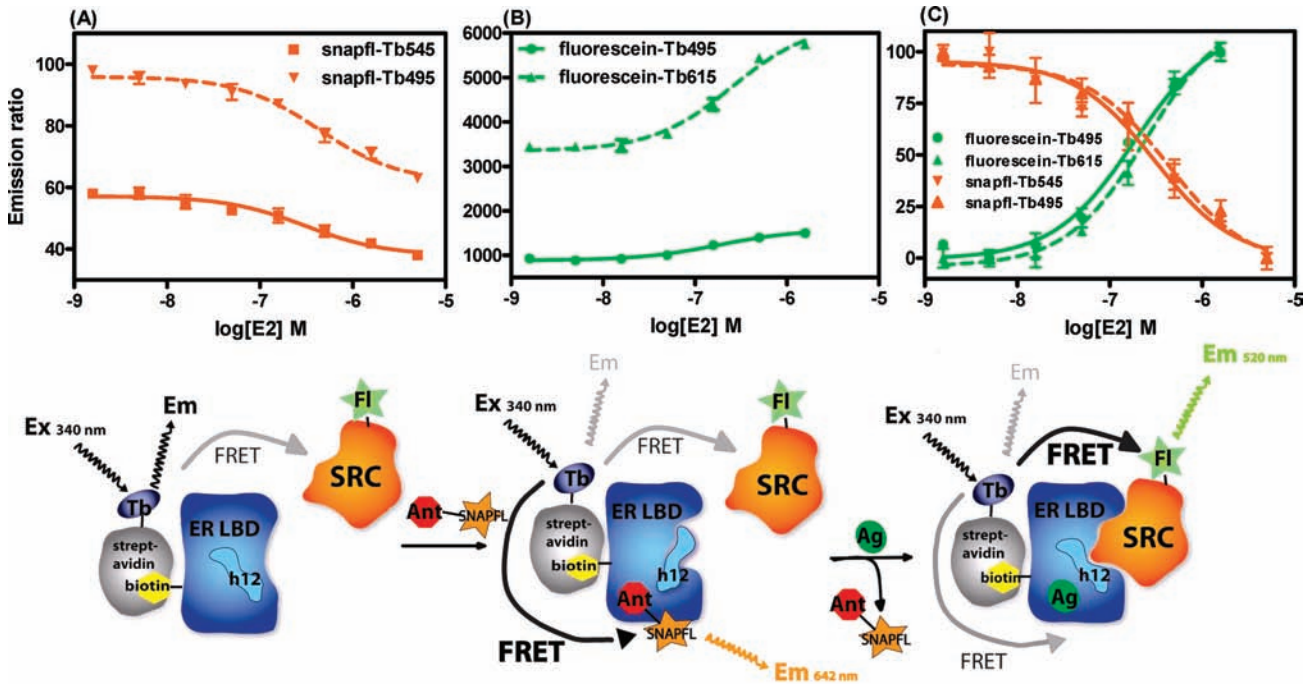


Figure 4. Change in emission ratios as titration by unlabeled agonist estradiol displaces antagonist ligand cyclofenil-SNAPFL **1** from ERα-SATb, producing a decrease in FRET signals (panel A) with simultaneous recruitment of Fl-SRC3, producing an increase in FRET (panel B). In each case, terbium emissions are monitored at two wavelengths (green and orange curves; see legend). Panel A: The ratio of intensity data measured using 642/20 nm channel for SNAPFL emission and terbium donor channels 495/20 nm (orange dashed-line curves) and 545/20 nm (orange solid-line curves). Panel B: The FRET intensity data measured using 525/25 nm channel for FL emission and terbium donor channels 495/20 nm (green solid-line curves) and 615/20 nm (green dashed-line curves). Panel C: All spectra were normalized by making largest number and smallest number among each data set 100% and 0%, respectively. (All data measured showed SD values ± 8% of mean.) The bottom cartoon depicts this one-donor/two-acceptor FRET experiment where in the second step E_2 displaces **1** with concomitant recruitment of SRC3-Fl. (ER LBD, estrogen receptor ligand binding domain; h12, helix 12; Ag, agonist; Ant, antagonist. The emissions are shown with the colors designated in the Figure 1 legend.)

resonance was measured with a Wallac Victor^{2}V, 1420 multilabel HTS counter (Perkin-Elmer, Waltham, MA). All samples were prepared in duplicate to measure the LRET. The filters used for donor channel measurements were provided by Perkin-Elmer except for a >700 nm filter (Omega Optical, Brattleboro, VT). All data were analyzed by GraphPad Prizm V (GraphPad software, Inc., San Diego, CA) and fitted by using nonlinear regression (curve fit) with an equation for a one-site sigmoidal dose−response curve. The binding curves were plotted as the emission ratio (A/D*1000) (where A and D mean the emission intensity of acceptor and donor) against the log value of the ligand concentration. The quantum yield and ε_λ for ligands **1**, **2**, or steroid receptor coactivator 3 (SRC3)-Fl, and SRC3-Cy5 were determined as 0.21 and 58000 M^{-1} cm^{-1} (ε_{542}) in pH 9 Tris buffer, 0.97 and 75000 $M^{-1}cm^{-1}$ (ε_{489}) in 0.1 N NaOH, and 0.28 and 250000 $M^{-1}cm^{-1}$ (ε_{650}) in pH 9 Tris buffer.

Protein Expression and Purification. N-Terminally His-tagged constructs of estrogen receptor α (ERα)-417 and SRC3 NRD were expressed and purified as described previously.[29] Briefly, the ligand binding domain of ERα (residues 304−554; N-terminally His-tagged, with previously described C381,530S mutations that do not affect activity but leave one reactive cysteine) is site-specifically labeled (Cys417)[29] with biotin while bound to a nickel column during protein purification and subsequently tagged with a streptavidin−terbium complex. The SRC-3 nuclear receptor domain (NRD) (residues 627−829, which include all three NR-boxes) is nonspecifically labeled through the four available cysteines using either 5-iodoacetamidofluorescein or Cy^{TM}5-maleimide monoreactive dye.[29]

TR-FRET Assays. A. One-Acceptor FRET. Determination of Ligand 1 Binding Affinity (Figure 3A). A 10-μL volume of a stock solution mix of ERα-417 (4 nM) and LanthaScreen streptavidin−terbium (1 nM) in TR-FRET buffer (20 mM Tris, pH 7.5, 0.01% NP40, 50 mM NaCl) was placed in separate wells of a black 96-well Molecular Devices HE high-efficiency microplate (Molecular Devices, Inc., Sunnyvale, CA). In a second 96-well Nunc polypropylene plate (Nalge Nunc International, Rochester, NY), a 1 mM solution of ligand **1** was serially diluted in a 1:10 manner into DMF. Each concentration of ligand **1** was then diluted 1:10 into TR-FRET buffer, and 10 μL of this solution was added to the stock ERα solution in the 96-well plate. This mixture was allowed to incubate for 1 h at room temperature. TR-FRET was measured using an excitation filter at 340/10 nm and emission filters for terbium at 545/20 and SNAPFL at 642/20 nm, respectively. The final concentrations of the reagents were as follows: ERα-417 (2 nM), streptavidin−terbium (0.5 nM), ligand **1** (15 nM to 50 μM).

Ligand 1 as a Tracer in Ligand-Binding Experiments (Figure 3B−D). A 5-μL volume of a stock solution mix of ERα-417 (8 nM) and streptavidin−terbium (2 nM) in TR-FRET buffer was placed in separate wells of a black 96-well microplate. A 100 μM solution of each tested ligand (estradiol and 11β-chloromethylestradiol, CME) was serially diluted in a 1:10 fashion into DMF in a second 96-well plate, and each concentration of ligand was then diluted 1:10 into TR-FRET buffer. After 10 μL of this solution was added to the stock ERα solution and incubated for 20 min, 5 μL of 12-μM ligand **1** was added to each well. TR-FRET was measured after a 1-h incubation using an excitation filter at 340/10 nm and emission filters for terbium at 495/10 and 545/20 nm and SNAPFL at 642/20 nm. The final concentrations of the reagents were as follows: ERα-417 (2 nM), streptavidin−terbium (0.5 nM), ligand **1** (3 μM), tested ligands (0−5 μM).

B. Two-Acceptor FRET. Displacement of SNAPFL-Labeled Antagonist Ligand 1 Bound to ERα-SATb with Agonist E_2 along with Concurrent Recruitment of SRC3-Fl (Figure 4). A 10-μL volume of a stock solution mix of ERα-417 (4 nM),

(29) Tamrazi, A.; Carlson, K. E.; Daniels, J. R.; Hurth, K. M.; Katzenellenbogen, J. A. *Mol. Endocrinol.* **2002**, *16*, 2706–19.

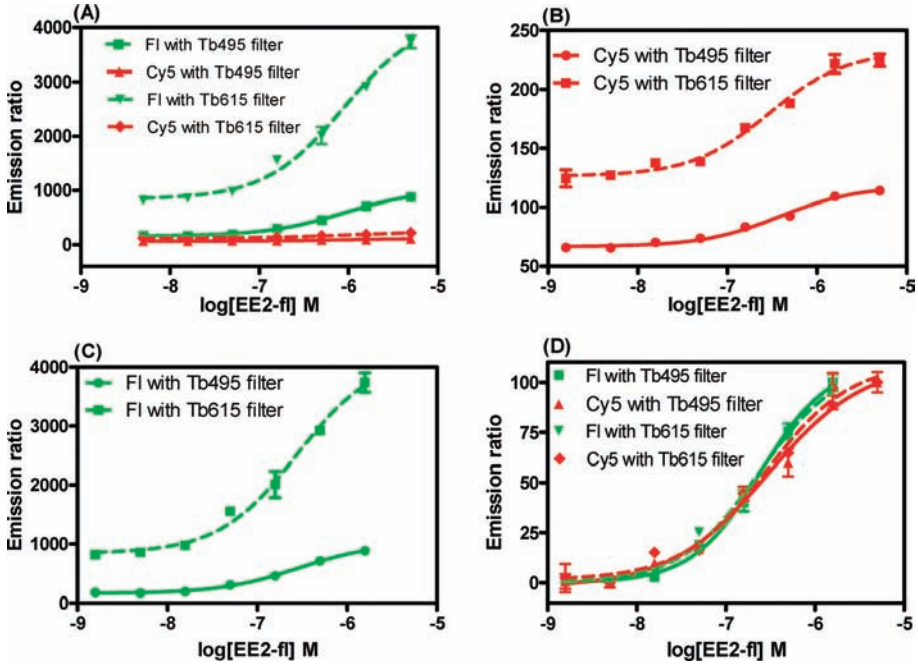


Figure 5. Two-acceptor FRET system with raw and normalized FRET signals. ERα-SATb (2 nM) was saturated with a control unlabeled antagonist ligand TOT (10 nM) to model the three-acceptor FRET system but with an unlabeled antagonist as a control and was then titrated with EE2-Fl in the presence of SRC3-Cy5 (50 nM). Panel A: The FRET signal was determined by measuring the Tb emission intensity using 495/20 nm (red and green solid-line curves) and 615/20 nm filters (red and green dashed-line curves), with the Fl emission intensity using 520/25 nm filter, and with the Cy5 emission intensity measured using 670/10 nm filter. Panels B and C: FRET curves from panel A extracted and plotted on more appropriate scales. Panel D: Plot of normalized FRET curves showing that all EC_{50} values are essentially the same, ranging only from 276 nM to 316 nM. (All data measured had SD ± 10% of duplicate mean.) (The color of the curves follows the colors designated in Figure 1 legend.)

streptavidin−terbium (1 nM), ligand **1** (2 μM), and SRC3-fluorescein (100 nM) in TR-FRET buffer was placed in separate wells of a black 96-well microplate. A 100 μM solution of estradiol (E_2) was serially diluted in a 1:10 fashion into DMF in a second 96-well plate, and each concentration of ligand was then diluted 1:10 into TR-FRET buffer. After 10 μL of this solution was added to the stock solution and incubated for 1 h, TR-FRET was measured by reading the microplate four times (twice for each acceptor using two different donor filter channels). For SNAPFL, the plate was measured using an excitation filter at 340/10 nm and emission filters for terbium at 495/20 and at 545/20 and for SNAPFL at 642/20 nm. It was then measured again using an excitation filter at 340/10 nm and emission filters for terbium at 495/20 and 615/20 and fluorescein at 520/25 nm. The final concentrations of the reagents were as follows: ERα-417 (2 nM), streptavidin−terbium (0.5 nM), ligand **1** (1 μM), SRC3-Fl (50 nM), and estradiol (0−5 μM).

Titration of ERα-SATb with Agonist EE2-Fl (Ligand 2) in the Presence of SRC3-Cy5 (Figure 5). A 10-μL volume of a stock solution mix of ERα-417 (4 nM), streptavidin−terbium (1 nM), TOT (20 nM), and SRC3-Cy5 (100 nM) in TR-FRET buffer was placed in separate wells of a black 96-well microplate. A 100 μM solution ligand **2** (EE2-Fl) was serially diluted in a 1:10 fashion into DMF in a second 96-well plate, and each concentration of ligand was then diluted 1:10 into TR-FRET buffer. After 10 μL of this solution was added to the stock solution and incubated for 1 h, TR-FRET was measured by reading the microplate four times, twice for each acceptor fluorophore at two different donor emission wavelengths. For fluorescein, it was measured using an excitation filter at 340/10 nm and emission filters for terbium at 495/20 and 615/20 and for EE2-Fl at 520/25 nm. For Cy5, it was measured again using an excitation filter at 340/10 nm and emission filters for terbium at 495/20 and 615/20 nm and for Cy5 at >700 nm, respectively. The final concentrations of the reagents were as follows: ERα-417 (2 nM), streptavidin−terbium (0.5 nM), SRC3-Cy5 (50 nM), TOT (10 nM), and EE2-Fl (0−5 μM).

C. Three-Acceptor FRET. Observation of Ligand Exchange with Concomitant Coactivator Recruitment (Figure 6). This experiment was conducted as described above for the two-acceptor FRET (displacement of SNAPFL-labeled antagonist ligand **1** bound to ERα-SATb with agonist E_2 along with concurrent recruitment of SRC3-Fl (Figure 4)) with the replacement of ligand estradiol with fluorescein-labeled ethynylestradiol (ligand **2**) and use of Cy5-labeled SRC3 NRD instead of SRC3-Fl. After incubation of the microplate, TR-FRET was measured three times, each time using an excitation filter at 340/10 with (1) emission filters for terbium at 495/20 and 545/20 and for SNAPFL at 642/20 nm, respectively, (2) emission filters for terbium at 495/20 and 615/20 and for fluorescein at 520/25 nm, respectively, and (3) emission filters for terbium at 495/20 and 615/20 and for Cy5 at 670/10 nm and >700 nm, respectively. The final concentrations of the reagents were as follows: ERα-417 (2 nM), streptavidin−terbium (0.5 nM), ligand **1** (1 μM), SRC3-Cy5 (50 nM), and ligand 2 (0−5 μM).

3. Results and Discussion

3-1. Construction and Selection of the Proper Dyes and Associated Acceptor Emission Filter Sets To Enable Triple-Acceptor FRET Experiments. To design a triple-acceptor FRET system using wavelength-domain analyses, we planned to employ the terbium/fluorescein FRET pair due to its robust nature and reliable reputation, as well as the terbium/Cy5 FRET pair (as we had before[28,30]), because the longer excitation and emission wavelengths of Cy5 overlap much less with those of other fluorophores. Between these two acceptors, we hoped to include an organic fluorophore with a good excitation spectrum that overlapped with the mainly highest probability terbium emission

(30) Kim, S. H.; Gunther, J. R.; Katzenellenbogen, J. A. *Org. Lett.* **2008**, *10*, 4931–4934.

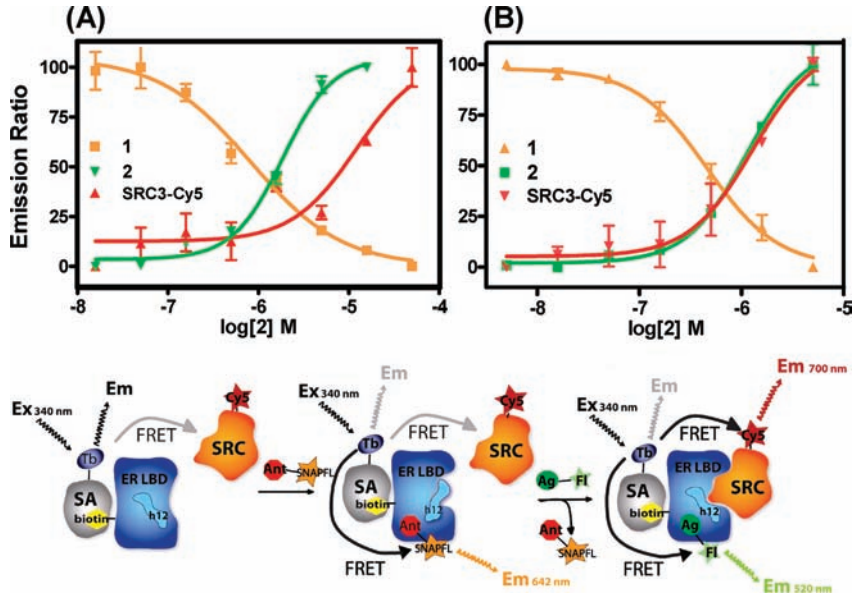


Figure 6. Titration of agonist ligand−fluorophore EE2−fluorescein **2** produces a dose-dependent increase in the fluorescein (green curves) FRET signal as it binds the ER while displacing cyclofenil-SNAPFL **1** (orange curves) and recruiting SRC3-Cy5 (red curves). Panel A: The FRET signal was determined by measuring the Tb emission intensity using a 495/20 nm filter, the SNAPFL emission from **1** with a 642/20 nm filter, the fluorescein emission from Fl-EE2 (**2**) with a 520/25 nm filter, and the emission from Cy5-SRC3 with a 670/10 nm filter; 100-μs delay. Panel B: The FRET signal from Cy5-SRC3 was measured with a >700 nm filter, others were the same; 100-μs delay. (All data were measured within SD ± 10% of raw data error range.) The bottom cartoon shows the one-donor/three-acceptor FRET system used to monitor the binding of agonist ligand **2**, with the concomitant displacement of antagonist ligand **1** and the recruitment of Fl-SRC3. (SA, streptavidin; ER LBD, estrogen receptor ligand binding domain; h12, helix 12; Ag, agonist; Ant, antagonist. The emissions are shown with the colors designated in Figure 1 legend.)

occurring at 546 nm ($^5D_4 \rightarrow {}^7F_5$) and a Stokes shift large enough so that its emission wavelength was longer than any terbium band. After considering a number of organic dye fluorophores, we chose SNAPFL (semi naphthalene fluorescein, ex 545, em 620 nm at pH ~8.5) as the third acceptor (Figure 2).[30]

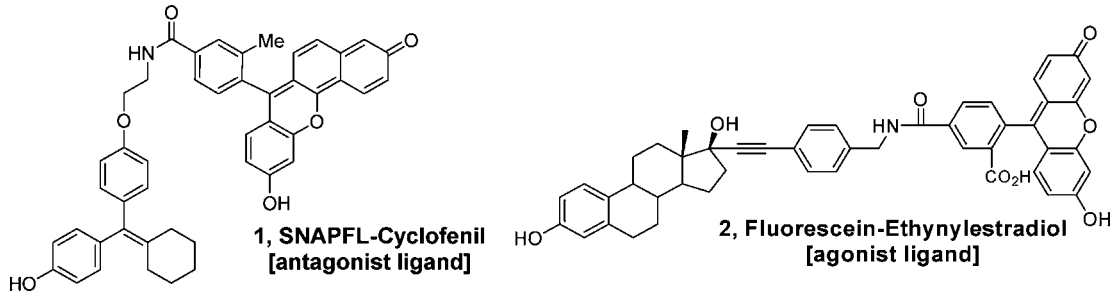
We then carefully chose three filters corresponding to the emission spectra of the three acceptor organic dyes (Figure 2), taking care to minimize spectral overlap with the emissions from Tb and the other acceptors. The emission filter window for fluorescein (520/25 nm) allows less than 2% spectral overlap with SNAPFL emission and none with Cy5 emission; the window for SNAPFL (642/20 nm) allows less than 4% spectral overlap with Cy5 emission and negligible with fluorescein, and the window for Cy5 (>700 nm) allows less than 2% overlap with SNAPFL emission and none with fluorescein. (None of the acceptor emission windows overlap with any of the four terbium donor emission bands.)

3-2. Analysis of FRET Intensity and Efficiency Obtained from Different Donor Channels. The terbium donor, excited using a 340/10 nm filter, produces four emission bands, any one of which could be used to monitor donor emission intensity. Although narrow, these bands show considerable overlap with acceptor emissions (Figure 2), suggesting that, in principle, acceptor emission bleed-through into the donor emission channel would seriously interfere with the determination of FRET magnitude. A number of photophysical factors, the conditions of our experiments, and careful filter selection, however, can make this interference minimal and make it possible to obtain accurate FRET measurements with all three acceptors.

Using components of our assay system at the concentration ranges typically used in our experiments (see the Experimental Section and below), we investigated the extent of bleed-through at three terbium donor emission channels, 495/20, 545/20, and 615/20 nm, that might arise from the emission of all three of our acceptor fluorophores, fluorescein, SNAPFL, and Cy5. Because its bands are narrow, the emission intensity of the terbium donor, present in our assays at 1−2 nM, is in the range 2000−10000 cps in these three channels, depending on the level of FRET (see the Supporting Information for further details; SI Tables 1, 2, and 3, top). By contrast, because their emissions are broad and their FRET efficiency and quantum yields are limited, the emission intensity from acceptors SNAPFL and Cy5 at the concentrations used in our assays is much less, being only ca. 200 cps in the 615/20 nm channel and even less at 545/20 nm. (There is no evidence of diffusion-enhanced FRET at any of the acceptor concentrations we use.) Thus, despite the nominal spectral overlap (Figure 2; note, curves are normalized), acceptor bleed-through from these two fluorophores is, in practice, minimal.

Fluorescein emission intensity, however, is ca. 10-fold brighter, and it gives stronger bleed-through at 495/20 and 545/20 nm but less at 615/20 nm. Nevertheless, because the 495/20 nm filter is recommended for Tb-fluorescein FRET experiments,[23] it is the one we have used in most cases. We did, however, examine other donor/acceptor filter pairs with the set of fluorophores in our assay system, and we found that all produce identical EC_{50} values once the raw acceptor/donor intensity ratios are normalized (see below). Thus, even though acceptor and donor emission band overlap appears to be serious from the normalized curves in Figure 2, because of band shape, quantum yield differences, and FRET intensities, terbium donor emission can be monitored in our experiments with no more than 5−10% bleed-through from emssions from SNAPFL and Cy5. This is actually much less than that typically encountered for fluorescein bleed-through, which is considered acceptable in well established Tb-fluorescein FRET technology (see the Supporting Information for further details).

Scheme 1. Structure of Ligand **1** (SNAPFL-Cyclofenil, an Antagonist) and Ligand **2** (Fluorescein Ethynylestradiol, an Agonist)



3-3. Model System for Multicomponent FRET Measurements. To test our proposed one-donor/three-acceptor FRET system, we used as a model system the estrogen receptor (ER), a ligand-modulated transcription factor that regulates gene expression in response to changing levels of circulating cognate hormones or hormone analogues. As illustrated in Figure 1, when ER binds a ligand, it adopts distinct conformations in which helix-12 either enables or disables the binding of coactivator proteins such as steroid receptor coactivator (SRC) that are required for transcription; these conformations reflect, respectively, the agonist vs antagonist character of the bound ligand. As an antagonist ligand for ER, we prepared a SNAPFL-conjugated cyclofenil (**1**),[30] and as an agonist ligand, we prepared fluorescein-conjugated 17α-ethynylestradiol (**2**) (Scheme 1).[31]

As previously reported by us, we also prepared the site-specific biotin-labeled ligand binding domain of ERα and a fragment of the SRC3 coactivator protein labeled nonspecifically with Cy5 or fluorescein.[29] To conduct the assay, the ER at 1 nM was incubated with 0.25 nM of a streptavidin-Tb^{3+}/Cs124 chelate (Invitrogen, Inc.) that acted as the FRET donor. As described below, these components enabled us to study agonist and antagonist ligand binding to the ER and how these binding events regulated coactivator interaction with ERα. In particular, we could investigate whether the process of exchange of an antagonist ligand for an agonist ligand and the recruitment of the coactivator occurs in precisely a coordinated manner.

3-4. Two- and Three-Color FRET Measurements of Estrogen Receptor Interaction with Agonist and Antagonist Ligands and a Coactivator Peptide. Prior to performing our four-color (one-donor/three-acceptor) FRET experiment, we conducted simple one- and two-acceptor FRET experiments to measure relative FRET intensity obtained from different donor channels and to confirm our estimates of the limited bleed-through of acceptor emission at three different donor emission channels (495/20, 545/20, and 615/20 nm). The results of these preliminary experiments are shown in Figures 3−5 (further details and raw data are given in the Supporting Information).

We first confirmed that we were able to follow the binding of SNAPFL-labeled ligand **1** to the terbium-labeled estrogen receptor (ERα-SATb). We used this assay in both a direct binding and in a competitive binding mode by displacing ligand **1** with other unlabeled ER ligands in one-donor/one-acceptor and one-donor/two-acceptor FRET experiments (Figures 3 and 4, respectively). When SNAPFL-labeled ligand **1** is titrated in the presence of ERα-SATb, FRET signals increase, giving maximum values above ca. 1 μM (Figure 3, panel A). A saturating concentration of ligand **1** (3 μM) bound to the ERα-SATb produced a high FRET signal, and this signal decreased dose-dependently when unlabeled ER ligands were added (Figure 3, panels B−D). Notably, although the actual value for the FRET expressed as raw emission ratios depends on whether the 495/20 or the 545/20 nm donor emission channel was used (panel 3B), the normalized FRET curves are identical (panel 3C).

This assay can be used to estimate the relative binding affinity of the SNAPFL-labeled ligand **1**, as the concentration of estradiol needed to displace half of the tracer ligand was ca. 230 nM, which corresponds to a binding potential of approximately 7.7% that of estradiol (100%/[3,000 nM ligand **1**/230 nM estradiol]). Because estradiol has a K_D of 0.2 nM for ERα, this corresponds to a K_I value of 2.6 nM for ligand **1**. Similarly, in this assay, 11β-chloromethylestradiol, which has nearly 10-fold higher binding affinity than estradiol, had an IC_{50} of ca. 24 nM (Figure 3, panel D). This corresponds to a K_I value of ca. 0.2 nM, which is consistent with values previously determined by competitive radiometric binding assays.[32]

Ligand **1** was also used as the tracer in a one-donor/two-acceptor FRET assay to monitor ligand binding with simultaneous tracking of ligand-mediated coactivator recruitment. This assay used the same components as in the previous experiment, with the addition of a fluorescein-labeled SRC3 coactivator protein fragment. As the concentration of unlabeled agonist ligand estradiol was increased, competition for the ligand binding site of the ER occurred, and one FRET signal progressively decreased as cyclofenil−SNAPFL (**1**) was displaced (Figure 4A). Concomitantly, as agonist ligand estradiol concentrations increased, there was an increase in the second FRET signal (fluorescein) with the recruitment of SRC3-Fl (Figure 4B). This assay demonstrates that fluorescein and the SNAPFL fluorophore can be used in one-donor/two-acceptor FRET experiments, and it again demonstrates that although the raw emission ratio values depend on which donor emission channel is used to determine FRET (Figure 4AB), the normalized FRET curves are superimposible (Figure 4C).

A related experiment in which an unlabeled ER antagonist, *trans*-hydroxytamoxifen (TOT), is first bound to ERα-SATb and then displaced with increasing concentration of the fluorescein-labeled agonist EE2-Fl (ligand **2**) in the presence of SRC3-Cy5 is shown in Figure 5. The development of FRET signals to fluorescein is evident as the EE2-Fl agonist displaces TOT and to Cy5 is evident as the ERα-agonist complex recruits SRC3-Cy5. The magnitude of the FRET signal as raw emission

(31) Nettles, K. W.; Bruning, J. B.; Gil, G.; Nowak, J.; Sharma, S. K.; Hahm, J. B.; Kulp, K.; Hochberg, R. B.; Zhou, H.; Katzenellenbogen, J. A.; Katzenellenbogen, B. S.; Kim, Y.; Joachmiak, A.; Greene, G. L. *Nat. Chem. Biol.* **2008**, *4*, 241–247.

(32) Bindal, R. D.; Carlson, K. E.; Reiner, G. C.; Katzenellenbogen, J. A. *J. Steroid Biochem.* **1987**, *28*, 361–70.

ratios, however, is quantitatively very different (panel A), with the signal to fluorescein being ca. 20-fold greater than to Cy5. Again, signal strength is dependent on the donor emission channel used as reference for calculating the emission ratios (panels B and C); the normalized FRET curves, as before, are superimposable (panel D), consistent with agonist binding and coactivator recruitment being coordinate events.

Based on the success of these one-donor/one-acceptor or one-donor/two-acceptor experiments, we constructed a one-donor/three-acceptor system in which three FRET signals would be independently and simultaneously measured. In the two-acceptor experiment in Figure 4, one must assume that the decrease in FRET that occurs as the concentration of unlabeled estradiol increases results from the displacement of antagonist ligand **1** from the ligand binding pocket in ER by the agonist estradiol. We can, however, follow the binding of the agonist directly using a third FRET signal, obtained by using the fluorophore-labeled agonist ligand, 17α-ethynylestradiolfluorescein (**2**). Thus, in the three-acceptor FRET experiment (Figure 6), a saturating concentration of SNAPFL-labeled antagonist ligand **1** (1 μM) was added to 2 nM ERα-SATb and incubated with increasing concentrations of fluorescein-labeled agonist ligand **2** in the presence of 50 nM SRC3-Cy5 (Note: Here, SRC3 is labeled with Cy5, not fluorescein, as it was in Figure 4). Three FRET signals were measured independently (Figure 6): As the concentration of agonist ligand (ligand **2**; green line) increases and it binds to ERα, the antagonist ligand (ligand **1**) is displaced (orange line) and the SRC3 coactivator is recruited (red line).

Interestingly, when the most commonly used Cy5 emission filter (670/10 nm) was initially employed, the recruitment curve of the SRC3-Cy5 protein was right shifted with respect to the binding curve of the agonist ligand (green curve; $EC_{50} = \sim 2$ μM) to a lower potency curve (red curve; $EC_{50} = \sim 12$ μM) (Figure 6A). While we considered that this might indicate that coactivator recruitment was not coordinate with agonist ligand (**2**) exchange with antagonist ligand (**1**), we suspected that bleed-through energy from the decreasing SNAPFL signal was interfering with measurement of the emission signal of the Cy5; thus, we substituted a filter with a longer wavelength cutoff (>700 nm). As expected, the longer wavelength filter allowed measurement of only the Cy5 signal, and gratifyingly the signal from the binding of agonist ligand **2** then very nicely overlapped with the recruitment of SRC3 (Figure 6B), both having EC_{50} values of ~ 1 μM. In such a multicomponent system, not only the wavelength of the filter but also its band-pass must be carefully considered to ensure that each signal is well separated so the biological interactions of each component system can be accurately monitored. With these refinements, we were able to demonstrate that the replacement of an antagonist ligand bound to ERα with an agonist ligand occurs in a precisely reciprocal fashion such that ERα occupancy by the agonist is precisely coordinated with the recruitment of the coactivator protein. Referring back to Figure 1, we can conclude that when an agonist displaces an antagonist, coactivator recruitment occurs in a precisely coordinated fashion with ligand exchange, without detectable accumulation of ER-agonist complex without coactivator, or that *State 3* proceeds to *State 2* without detectable *State 1*.

4. Conclusion

In summary, the propitious wavelength of excitation and large Stokes shift of SNAPFL enable the Tb/SNAPFL FRET pair to be included in an assay system with two other known FRET partners for terbium, namely fluorescein and Cy5. Three independent FRET signals can then be followed simultaneously, provided that filters are carefully chosen to monitor the energy transfer from terbium to each of the three acceptor fluorophores, with negligible interference from each other and from the donor, and that acceptor emission intensities in the donor emission channel are minimal. To the best of our knowledge, a terbium-based time-resolved one-donor/three-acceptor system has up to now not been reported. Thus, our novel three-acceptor FRET system, which we use here to observe that the exchange of ligands bound by ERα is precisely reciprocal and coordinated with recruitment of a coactivator, could be a valuable tool for the design of assays intended to reveal the simultaneous interactions of multicomponent biological systems.

Acknowledgment. We are grateful for support of this research by a grant from the National Institutes of Health (PHS 5R37 DK015556). J.R.G. received support from a David Robertson Fellowship and National Institutes of Health Fellowships (NRSA 1 F30 ES016484-01 and NRSA 5 T32 GM070421).

Supporting Information Available: The raw intensities using different donor channels for Fl, SNAPFL, and Cy5 and single FRET for SNAPFL obtained from the displacement experiment with E2 and 11β-chloromethylestradiol. This material is available free of charge via the Internet at http://pubs.acs.org.

JA100248Q