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## Diheteroarylethenes as Thermally Stable Photoswitchable Acceptors in Photochromic Fluorescence Resonance Energy Transfer (pcFRET)

Luciana Giordano,<sup>†</sup> Thomas M. Jovin,<sup>‡</sup> Masahiro Irie,<sup>§</sup> and Elizabeth A. Jares-Erijman<sup>\*,†</sup>

Contribution from the Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, PROPLAME-CONICET, Ciudad Universitaria-Pabellón II, 1428 Buenos Aires, Argentina, Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany, and Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Higashi-ku, 812, Japan

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**Abstract:** We have employed diheteroarylethenes as acceptors for photochromic FRET (pcFRET), a technique introduced for the quantitative determination of fluorescence resonance energy transfer (FRET). In pcFRET, the fluorescent emission of the donor is modulated by cyclical transformations of a photochromic acceptor. Light induces a reversible change in the structure and, concomitantly, in the absorption properties of the acceptor. Only the closed forms of the selected diheteroarylethenes **2a** and **2b** have an absorption band overlapping the emission band of the donor, **1**. The corresponding variation in the overlap integral (and thus critical transfer distance  $R_0$ ) between the two states provides the means for reversibly switching the process of FRET on and off, allowing direct and repeated evaluation of the relative changes in the donor fluorescence quantum yield. The diheteroarylethenes demonstrate excellent stability in aqueous media, an absence of thermal back reactions, and negligible fatigue. The equilibration of these systems after exposure to near-UV or visible light follows simple monoexponential kinetics. We developed a general conceptual scheme for such coupled photochromic-FRET reactions, allowing quantitative interpretations of the photostationary and kinetic data, from which the quantum yields for the cyclization and cycloreversion reactions of the photochromic acceptor were calculated.

### Introduction

Fluorescence resonance energy transfer (FRET) is a physical process by which energy is transferred nonradiatively from an excited molecular fluorophore (donor) to another chromophore (acceptor) via long-range dipole–dipole coupling.<sup>1a–l</sup> The FRET efficiency varies with the 6th power of the donor–acceptor separation over the range of  $\sim 1.5$ –10 nm, a range of distances corresponding to most biologically significant molecular interactions within cells. Applied in the fluorescence microscope, FRET provides a tool for resolving molecular interactions (proximities)

with a spatial resolution by far exceeding the optical diffraction limit. The photophysical behavior of extrinsic or intrinsic (e.g., genetically expressed) fluorophores also reflects conformational changes and perturbations of the microenvironment.

There are two fundamental problems associated with quantitative FRET determinations in the microscope: (i) The local donor and acceptor concentrations (densities) of donor and acceptor moieties located on separate molecules can vary greatly. Thus, the formalism for estimating the FRET efficiency  $E$  must be appropriate for arbitrary stoichiometries. (ii) For some preparations, for example, living cells undergoing a signaling, metabolic, or other time-dependent process, continuous methods of observation are required. Condition (i) can be met by various combinations of the donor and sensitized emission signals, but at least three separate images are required.<sup>1f,2</sup> Alternatively,  $E$  can be evaluated via the quenching of the donor alone, for example, from determination of the fluorescence lifetime (FLIM<sup>1c,e,h,j,3a–h</sup>) or employing various strategies based on systematic photobleaching of the donor and/or acceptor (pbFRET).<sup>1h,2,4a–h</sup> In the simple acceptor pbFRET procedure, the local (pixel-by-pixel) donor signal is measured prior to ( $I_{DA}$ )

\* To whom correspondence should be addressed. E-mail: eli@qo.fcen.uba.ar.

<sup>†</sup> Universidad de Buenos Aires.

<sup>‡</sup> Max Planck Institute for Biophysical Chemistry.

<sup>§</sup> Kyushu University.

- (1) (a) Förster, T. *Naturwissenschaften* **1946**, *6*, 166–175. (b) Förster, T. *Discuss. Faraday Soc.* **1959**, *27*, 7–17. For recent reviews of relevance to cell biology and imaging, see: (c) Centonze, V.; Takahashi, A.; Casanova, E.; Herman, B. *J. Histotechnol.* **2000**, *23*, 229–234. (d) Chamberlain, C.; Hahn, K. *Traffic* **2000**, *1*, 755–762. (e) Goedhart, J.; Gadella, T. W. J., Jr. In *Root Hairs: Cell And Molecular Biology*; Ridge, R.; Emons, A., Eds.; Springer-Verlag: Tokyo, 2000; pp 65–94. (f) Gordon, G.; Berry, G.; Liang, X.; Levine, B.; Herman, B. *Biophys. J.* **1998**, *74*, 2702–2713. (g) Kenworthy, A.; Edidin, M. *Methods Mol. Biol.* **1999**, *116*, 37–49. (h) Lippincott-Schwartz, J.; Snapp, E.; Kenworthy, A. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 444–456. (i) Miyawaki, A.; Tsien, R. *Methods Enzymol.* **2000**, *327*, 472–500. (j) Selvin, P. *Nat. Struct. Biol.* **2000**, *7*, 730–734. (k) Szöllösi, J.; Damjanovich, S.; Matyus, L. *Cytometry* **1998**, *34*, 159–179. (l) Wouters, F.; Verveer, P.; Bastiaens, P. I. H. *Trends Cell Biol.* **2001**, *11*, 203–211.

- (2) Jovin, T. M.; Arndt-Jovin, D. J. In *Cell Structure and Function by Microspectrofluometry*; Kohen, E.; Hirschberg, J. G.; Ploem, J. S., Eds.; Academic Press: London, 1989; pp 99–117.

and following ( $I_D$ ) photodestruction of the acceptor.<sup>4a–f</sup> For the case of total conversion ( $I_{DA} \rightarrow I_D$ ), the FRET efficiency  $E$  is related to the fractional increase ( $\beta = I_D/I_{DA} - 1$ ;  $0 \leq \beta \leq \infty$ ) or, if the final  $I_D$  state serves as the reference, to the fractional decrease ( $\gamma = 1 - I_{DA}/I_D$ ;  $0 \leq \gamma \leq 1$ ) in the donor signal.

$$E = (1 + \beta^{-1})^{-1} = \gamma \quad (1)$$

However, the use of irreversible photobleaching of the acceptor precludes reexamination of the same region at a later time. Photochromic FRET overcomes this limitation by providing the required reference state (donor alone) in a local (single pixel) and reversible manner. This is achieved by continued, repeated, and light-driven on–off switching of the FRET process. Our strategy is based on the use of photochromic compounds as “programmable” acceptors for FRET.

A photochromic compound is characterized by the ability to undergo a reversible transformation – in response to illumination at appropriate wavelengths – between two different structural forms having different absorption (and, in some cases, fluorescence) spectra. The two FRET probes (donor and acceptor) are selected according to certain spectroscopic criteria, including the key requirement that only one of the photochromic forms of the acceptor has absorption properties rendering it competent for the transfer of excitation energy from the donor. As a consequence, the fluorescence emission (degree of quenching) of the donor can be modulated reversibly by systematic photochemical manipulation of the photochromic acceptor. That is, the donor fluorescence is measured in the “presence” and “absence” of the acceptor, the latter case corresponding to the reference state described above.

We selected thermally stable thiophene-based diheteroarylethenes as acceptors for pcFRET. We employed a perfluorocyclopentene as the central bridging substructure because of its demonstrated resistance to fatigue.<sup>5</sup> The electron-withdrawing property of the perfluoro substituents also promotes the cyclization reaction.<sup>5</sup>

## Results

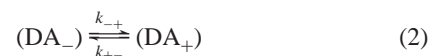
**Molecular Design, Syntheses, and Spectral Properties.** The diheteroarylethenes selected as photoswitchable acceptors for FRET have closed forms with absorption maxima in the range 540–570 nm. The open and closed forms are both thermally stable.

The absorption spectra of both forms of **2a** and **2b** (Figure 1), determined according to the formalism and procedures elaborated in this study, are given in Figure 2b and c, respectively. The spectral bands of the closed forms of **2a** and **2b** in the visible are at 546 nm ( $\epsilon$  12 000 M<sup>−1</sup> cm<sup>−1</sup>) and 568 nm ( $\epsilon$  11 000 M<sup>−1</sup> cm<sup>−1</sup>), respectively. The closed and open forms of **2a** have isosbestic points at 248, 274, and 312 nm and the corresponding forms of **2b** at 233 and 290 nm. The spectral constants are given in Figure 2.

Model compounds **3a** and **3b** were synthesized to evaluate the performance of diheteroarylethenes as acceptors in FRET (Figure 1). We selected Lucifer Yellow **1** (Figures 1 and 2a) as the donor for the diheteroarylethenes because of the location of its absorption maximum in a spectral region in which the absorbances of both the closed and the open forms of the diheteroarylethenes are minimal. In addition, the donor emission spectrum of LY (Figure 2a) overlaps well the absorption of the closed but not the open forms of **2a** and **2b** (Figure 2). **3a** and **3b** were designed with an aliphatic linker between the fluorescent donor and the photochromic acceptor moiety so as to minimize interactions between the two. The photoconversion difference spectra for **2a** and **3a** and for **2b** and **3b**, generated by irradiation at 366 nm, confirmed that the donor absorption properties did not change during the transition of the acceptor(s) between the open and closed forms. Conversely, the acceptor difference spectra were not perturbed by the presence of the donor.

The computed critical transfer distance  $R_0$  for the closed forms of **3a** and **3b** was 38 and 35 Å, respectively. The corresponding values for the open forms were 9 and 12 Å. According to an energy minimization by Sybyl (AMBER 4.1 package, **3a**: Figure 1 bottom), the distance  $r_{DA}$  between the central atom of the LY and that of the acceptor was ~20 Å in **3a** and 22 Å in **3b**, respectively. Thus, one would predict a FRET efficiency ( $E_+$ , see below for definition) of 0.98 for **3a** and 0.94 for **3b** (closed forms), assuming an invariant  $r_{DA}$  (eq 7).<sup>6</sup> In the case of the open forms, the corresponding computed FRET efficiencies ( $E_-$ ) were very low, 0.008 and 0.02, respectively.

**Quantitative Formalism for the Photostationary State and Photokinetics.** The equilibrium state and kinetic processes characterizing such a system are represented in Figure 3 and by the following set of equations:



$$k_{-+} = (k_{ex}^{DA-} + k_{ex}^D E_-) Q_{-+} \quad k_{+-} = (k_{ex}^{DA+} + k_{ex}^D E_+) Q_{+-} \quad (3)$$

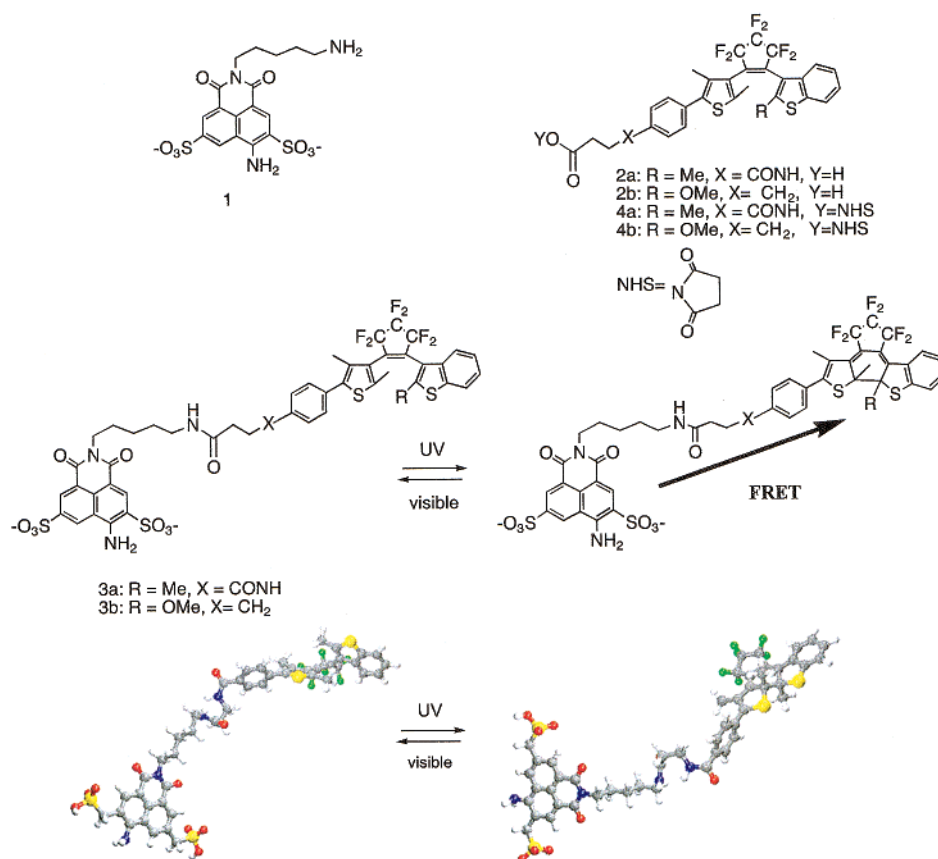
$$Q_{-+} = [1 + k_d^{DA-}/k_{op-cl}]^{-1} \quad Q_{+-} = [1 + k_d^{DA+}/k_{cl-op}]^{-1} \quad (4)$$

$$E_- = [1 + k_d^{D-}/k_{FRET}^{DA-}]^{-1} \quad E_+ = [1 + k_d^{D+}/k_{FRET}^{DA+}]^{-1} \quad (5)$$

in which  $DA_-$  and  $DA_+$  denote the donor paired with the open (−) or closed (+) forms of the acceptor, respectively. The rate constants determining the kinetics of the system are those for excitation, that is, absorption of light ( $k_{ex}^{DA-}$ ,  $k_{ex}^{DA+}$ ,  $k_{ex}^D$ ; see Figure 3 for definitions of other terms in eqs 4 and 5). In this

- (3) (a) Bastiaens, P. I.; Squire, A. *Trends Cell Biol.* **1999**, *9*, 48–52. (b) Gadella, T. W. J., Jr.; Jovin, T. M. *J. Cell Biol.* **1995**, *129*, 1543–1558. (c) Harpur, A. G.; Wouters, F. S.; Bastiaens, P. I. H. *Nat. Biotechnol.* **2001**, *19*, 167–169. (d) Herman, B.; Wang, X. F.; Wodnicki, P.; Gordon, G. W.; Seongwook, K.; Diliberto, P. A.; Periasamy, A. In *CLEO '95. Summaries of Papers Presented at the Conference on Lasers and Electrooptics (IEEE No. 95CH35800)*, Opt. Soc. Am. 1995; pp 274–275. (e) Murata, S.; Herman, P.; Lakowicz, J. R. *Cytometry* **2001**, *43*, 94–100. (f) Schneider, P.; Clegg, R. *Rev. Sci. Instrum.* **1997**, *68*, 4107–4119. (g) Szmajnski, H.; Lakowicz, J. R. *Sens. Actuators, B* **1995**, *29*, 16–24. (h) Verveer, P.; Wouters, F.; Reynolds, A.; Bastiaens, P. I. H. *Science* **2000**, *290*, 1567–1570.
- (4) (a) Kenworthy, A. K.; Edidin, M. *J. Cell Biol.* **1998**, *142*, 69–84. (b) Varma, R.; Mayor, S. *Nature* **1998**, *394*, 798–801. (c) Wouters, F.; Bastiaens, P. I. H.; Wirtz, K. W.; Jovin, T. M. *EMBO J.* **1998**, *17*, 7179–7189. (d) Bastiaens, P. I. H.; Jovin, T. M. In *Cell Biology: A Laboratory Handbook*, 2nd ed.; Celis, J. E., Ed.; Academic Press: New York, 1997; Vol. 3, pp 136–146. (e) Bastiaens, P. I. H.; Wouters, F. S.; Jovin, T. M. *Proc. of the 2nd Hamamatsu Int. Symp. on Biomol. Mechanisms and Photonics*; Research Foundation for Opto-Sci. and Technology: Hamamatsu, Japan, Feb. 20–22, 1997; pp 77–82. (f) Bastiaens, P. I. H.; Majoul, I.; Verveer, P.; Söling, H. D.; Jovin, T. M. *EMBO J.* **1996**, *15*, 4246–4253. (g) Young, R.; Arnette, J.; Roess, D.; Barisas, B. *Biophys. J.* **1994**, *67*, 881–888. (h) Szabo, G., Jr.; Pine, P.; Weaver, J.; Kasari, M.; Aszalos, A. *Biophys. J.* **1992**, *61*, 661–670.
- (5) Irie, M. *Chem. Rev.* **2000**, *100*, 1685–1716.

- (6) Realistically, one would envision a dynamic distribution of distances during the excited-state lifetime of the donor because of conformational flexibility of the spacer linking the donor and acceptor; this effect would serve to increase  $E$  even further.



**Figure 1.** Chemical structures of model compounds for pcFRET. Photochromic acceptors: **2a** and **2b**. The donor, Lucifer Yellow (LY) cadaverine (LYC, **1**), is covalently bound to the diheteroarylethene photochromic molecules in **3a** and **3b**. Initially, the diheteroarylethenes are in the open form (middle left). Upon near-UV irradiation, the photochromic compound is converted to the closed form (right), which now acts as an energy acceptor for the donor. Both states are thermally stable. Lower panel: energy minimized structures of the open and closed forms of **3a** (see text for details).

(diheteroarylethene) system, the thermal back reaction (–) → (+) is negligible. As a consequence, both the off and the on states are stable in the absence of light.

**Photochromic Conversion Analyzed by Absorption Kinetics and <sup>1</sup>H NMR.** The photochromic conversion efficiencies of **2a**, **3a**, **2b**, and **3b** were determined in methanol. The kinetics of photoconversion of **2a** upon irradiation at 366 nm are shown in Figure 4a. Two new bands emerged at 412 and 548 nm and attained maximum absorbance at 40 min (photostationary state). Compound **3a** displayed identical behavior (data not shown), confirming that the coupling of **2a** to **1** did not alter the photoconversion process. The photogenerated closed ring form was stable at room temperature in the dark for at least 120 h.

The time-dependent absorption spectra during a cycloreversion of **2a** are shown in Figure 4b. Once in the photostationary state induced by near-UV light (panel a), **2a** was exposed to green light, and the spectra were recorded every 10 s. After 50 s, the depletion of the 548 nm band was complete, and the 266 nm band recovered its original intensity. The identity of the final and original spectra of the open form confirmed the completion of the back conversion and the absence of photo-degradation.

The light-induced conversion between the open and closed forms of **2b** by irradiation at 340 nm is shown in Figure 4c. A photostationary state ( $\alpha_{ps} = 0.99$ ) was achieved after 30 min. Cycloreversion to the initial open form (Figure 4d) with visible light (>520 nm) occurred within 2 min. The back reactions of **2a**, **2b**, **3a**, and **3b** induced by irradiation at >520 nm were

also monitored continuously by absorption at a single wavelength. Monoexponential fitting yielded equilibration times ( $k_{eq}^{-1}$ ) of 5–30 s.

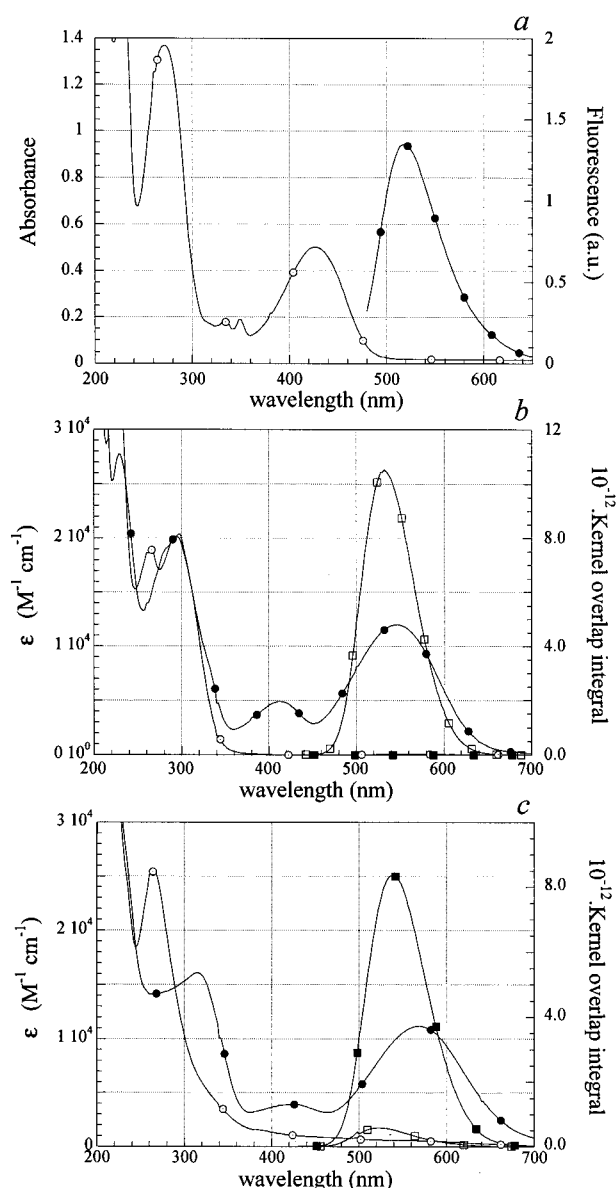
Compound **2a** was also converted by irradiation at 313 nm. Except for scaling factors, the difference spectra for the photostationary states of **2a** and **2b** achieved at 313 and 366 nm were indistinguishable (Figures S1c and S2a,b). However, there was a significant increase in the conversion efficiency ( $\alpha_{ps}$ ) from 0.33 to 0.65 upon passing from 366 to 313 nm irradiation.

The degree of conversion in these reactions was established by <sup>1</sup>H NMR spectroscopy before and after irradiation with UV light in the photostationary state. Integration of the signals of the aliphatic methyl groups and selected aromatic resonances of the spectra of **3a** and **3b** were evaluated before and after photoconversion. On the basis of these measurements,  $\alpha_{ps} = 0.85$  was determined for **3b**.

Six singlet resonances corresponding to methyl groups suggested the presence of two conformations of **2a**, probably parallel and antiparallel, as reported previously for other diheteroarylethenes.<sup>7</sup> The integration of the methyl group resonances indicated an almost equal population of both conformations. Irradiation with UV light to the photostationary state led to a decrease in the integrated signals of all the methyl groups corresponding to the open form. A new group of four singlets between 2.15 and 2.00 ppm appeared and was assigned to methyl resonances corresponding to two configurations of

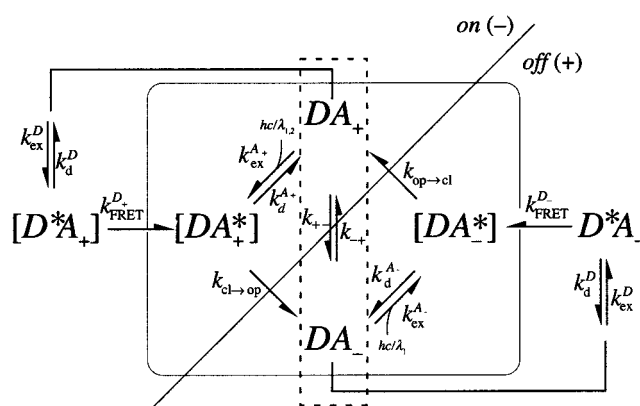
(7) Matsuda, K.; Irie, M. *J. Am. Chem. Soc.* **2000**, *122*, 8309–8310.





**Figure 2.** Spectral properties for the completely off (–) and on (+) states (open and closed forms of **2a** and **2b**, respectively) in pcFRET. (a) Absorption (–○–) and emission (–●–) properties of the donor **1**; fluorescence excitation at 430 nm. (b) Absorption spectra of **2a** in the open (–○–) and closed (–●–) forms; kernel of the overlap integral in the open (–□–) and closed (–■–) forms of **2a**. (c) Absorption spectrum of the open (–○–) and closed (–●–) forms of **2b**; kernel of the overlap integral in the open (–□–) and closed (–■–) forms of **2b**. Peaks of **2a** spectra: open form, 226, 266, 298 nm; closed form, 230, 294, 413, 546 nm. Peaks of **2b** spectra: open form, 264 nm; closed form, 314, 424, 568 nm. Spectra calculated from eq S6 based on extinction coefficients for the closed (+) forms: **2a**,  $\epsilon_{\text{cm}}^{546} = 12\,000\, M^{-1}\, cm^{-1}$ ; and **2b**,  $\epsilon_{\text{cm}}^{568} = 11\,000\, M^{-1}\, cm^{-1}$ . In the initial (–) state, there is no substantial overlap between the donor emission spectrum and the absorption spectrum of the open form of the diheteroarylethene. Near-UV irradiation (320–380 nm) generates the closed (+) form, whose absorption spectrum overlaps well with the donor emission, thereby potentiating FRET. Subsequent exposure to green light (500–580 nm) leads to a reversion of the closed to the open form, turning off the FRET process. Applying eq S6 to the spectra of panels a and b, the following relationships for **2a** and **2b** were derived, for which the selected reference isosbestic points were 312 nm (**2a**) and 290 nm (**2b**). **2a**,  $\alpha_{\text{ps}} = -0.0048 + 1.219A_{\text{ps}}^{546}/A_{\text{ps}}^{312}$ ,  $\epsilon_{-}^{546} = 39$ ,  $\epsilon_{+}^{312} = 14\,700$ ,  $\epsilon_{+}^{546} = 12\,000$ ; **2b**,  $\alpha_{\text{ps}} = -0.029 + 1.390A_{\text{ps}}^{568}/A_{\text{ps}}^{290}$ ,  $\epsilon_{-}^{568} = 310$ ,  $\epsilon_{+}^{290} = 14\,900$ ,  $\epsilon_{+}^{568} = 11\,000$  (all  $\epsilon$  in units of  $M^{-1}\, cm^{-1}$ ).

the closed form. The integrated signals indicated a 33% conversion to the closed ring form.



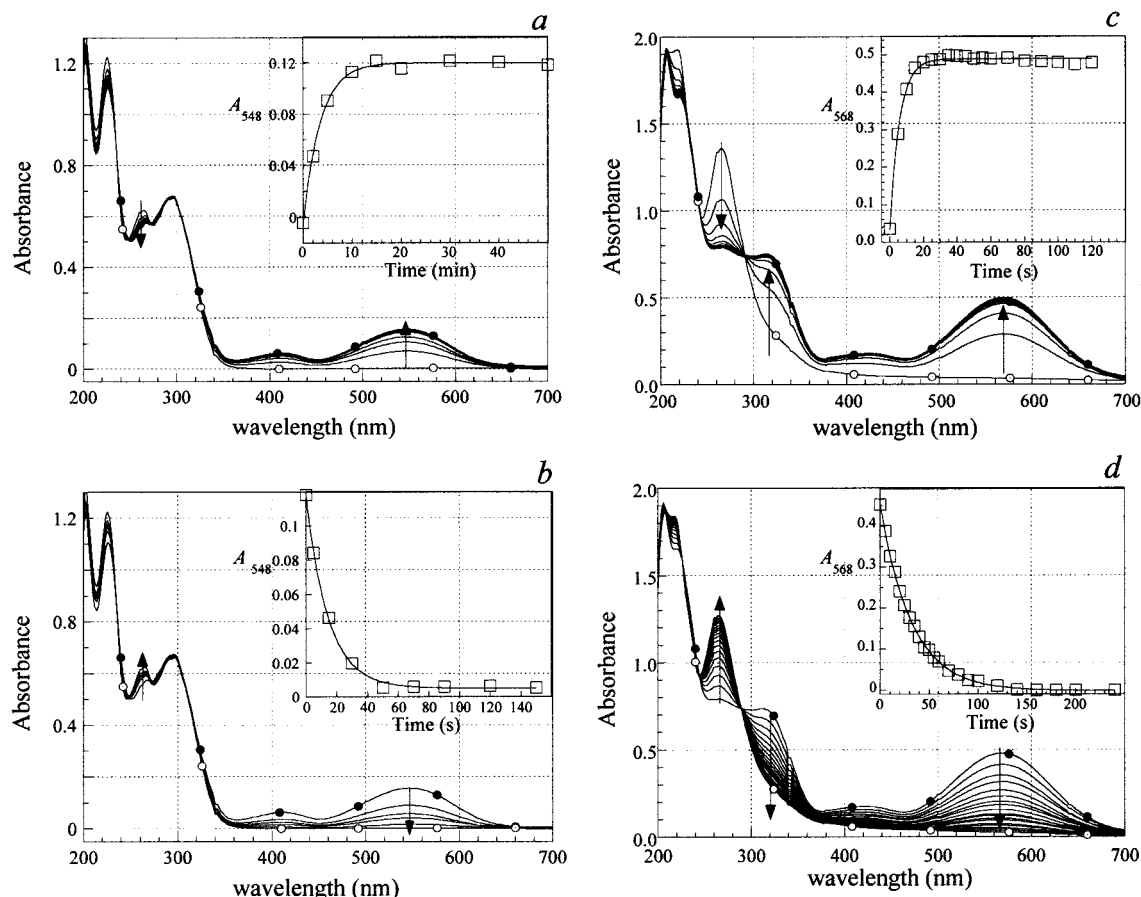
**Figure 3.** Photophysical-photochromic scheme incorporating donor–acceptor FRET (pcFRET). The central region comprises the ring closure and opening reactions of the photochromic acceptor. The external pathways represent the intervention of the donor via excitation and resonance energy transfer reactions. (–) refers to states devoid of significant FRET between donor and acceptor and (+) to states with very high FRET. In the present system, the (–) state is the open molecular form and the (+) the closed form (Figure 1). Because of steady-state conditions, the overall interconversion between the (–) and (+) forms can be represented by an apparent first-order reaction, designated in the scheme by the pair of vertically oriented arrows. The function of near-UV and visible light as reactants is denoted by  $hc/\lambda_i$  ( $i = 1$ , near-UV;  $i = 2$ , visible).

The  $\alpha_{\text{ps}}$  values derived from the NMR measurements permitted computation of the pure open and closed absorption spectra of **2a** and **2b** (Figure 2b,c) according to eq S6a. The results of these calculations, using data for the photostationary states induced by 313 and 366 nm, were the same within experimental error. The photoconversion efficiencies are summarized in Table 1.

**FRET Efficiencies for Open and Photostationary State of 3a and 3b.** The changes in the photostationary state absorption spectra recorded after irradiation at 366 and 313 nm were reflected in the fluorescence signals. Fluorescence emission spectra of **3a** and **3b** were acquired for the initial (open ring form) and the photostationary states achieved after irradiation at 366 nm. **3a** was quenched by 33% after irradiation at 366 nm and 65% after irradiation at 313 nm. The quenching of **3b** after exposure to 320 nm was 84%. From the conversion of **3a**, a value of  $E = 1$  was computed for the diheteroarylethene closed form. Similarly,  $\alpha$  was 0.85 for **3b** with an  $E = 1$  for the closed form. Irradiation for 60 s with visible light led to a complete recovery of the initial fluorescence signal ( $E = 0$ ).

Frequency and time domain fluorescence lifetime analyses of the donor **1** were carried out on the initial state (open form of the diheteroarylethene acceptor) and the photostationary state generated by irradiation at 366 nm. In both cases, a single component sufficed for the analysis of the data ( $\chi^2 < 1.2$  and symmetrical residuals). The fluorescence lifetimes of **3a** and **3b** in the initial (open form) were  $10.2 \pm 0.4$  and  $9.5 \pm 0.6$  ns, respectively, with no significant changes upon conversion to the closed form (at 366 nm for **3a** and 340 nm for **3b**) and after regeneration of the original open form.

**Cyclic Modulation of Acceptor Absorption and Donor Fluorescence.** Absorption and fluorescence spectra determined for the initial open form after several cycles of irradiation with UV light and after irradiation with green visible light are shown in Figures 5 and 6. The cyclic change in acceptor absorption (Figure 5) and concomitant change in donor fluorescence intensity and thus in FRET efficiency (Figure 6) induced by



**Figure 4.** Time-resolved spectroscopic monitoring of the forward and backward photochromic interconversions between open and closed forms of the diheteroarylethenes. (a) Absorption spectra of **2a** undergoing photoconversion from the initial (—) form upon exposure to 366 nm light (—○—; 0.3 mW, 2 mW cm<sup>−2</sup>); 5 min intervals between spectra. After 50 min, the maximal change was achieved at 548 nm; spectra of the photostationary state (—●—). Inset:  $A_{548}$  as a function of irradiation time (—□—). (b) Back photoconversion from the photostationary state of **2a** produced by irradiation at >520 nm (—●—; 4 mW, 27 mW cm<sup>−2</sup>); interval between curves, 10 s. After 50 s, the spectrum of the original open (—) form was recovered (—○—). Inset:  $A_{548}$  as a function of irradiation time (—□—). (c) Absorption spectra of **2b** undergoing photoconversion from the initial (—) form upon exposure to 340 nm light (—○—; 16 mW, 107 mW cm<sup>−2</sup>); 5 s intervals between spectra. After 40 s, the maximal change was achieved at 568 nm; spectra of the photostationary state (—●—). Inset:  $A_{568}$  as a function of irradiation time (—□—). (d) Back photoconversion from the photostationary state of **2b** produced by irradiation at >520 nm (—●—; 40 mW, 80 mW cm<sup>−2</sup>); interval between curves, 10 s. After 220 s, the spectrum of the original open (—) form was recovered (—○—). Inset:  $A_{568}$  as a function of irradiation time (—□—). The solid curves in the insets represent monoexponential fits according to the photokinetic formalism. The analyses of these and other reactions are given in Table 1.

**Table 1.** Photochromic Conversion Efficiencies (from Absorption and <sup>1</sup>H NMR) and Photokinetic Parameters (from Absorption)<sup>a</sup>

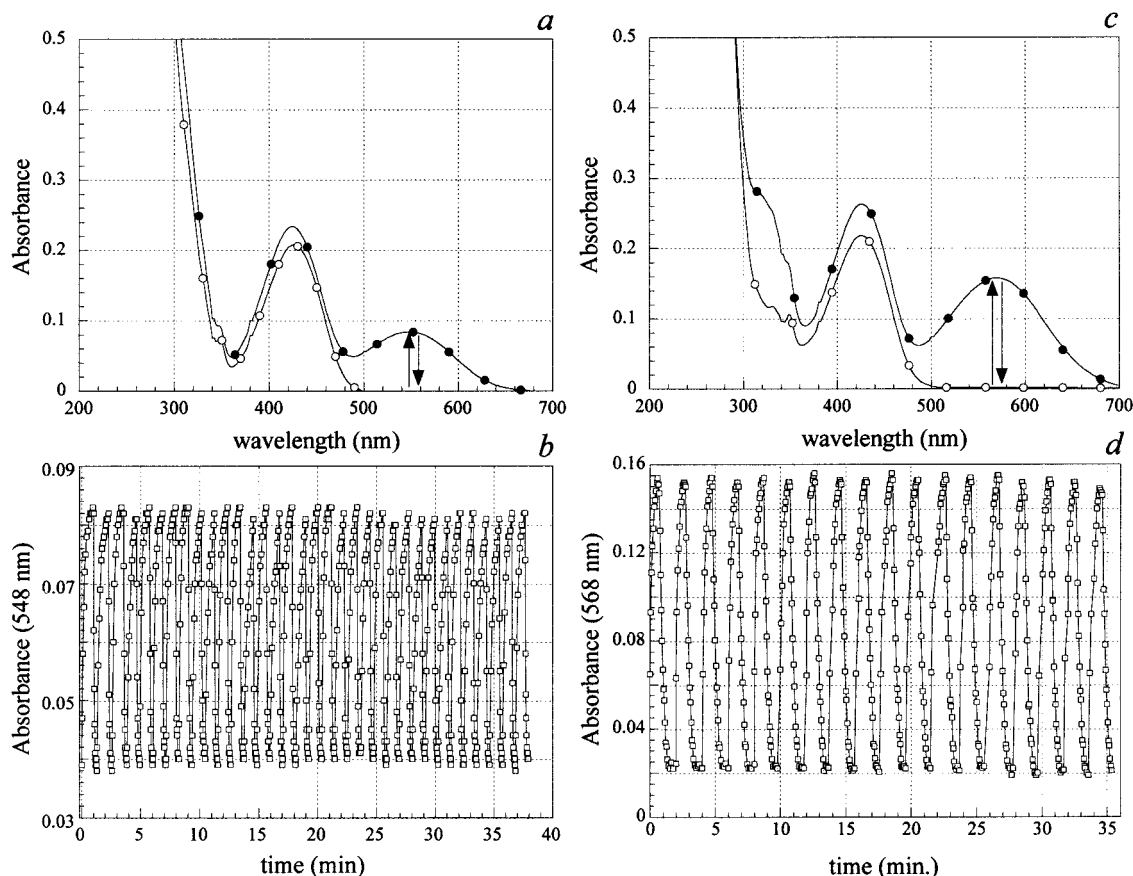
compd	irrad. mW cm <sup>−2</sup>	$\lambda_{\text{irr}}$ nm	$\epsilon_{-}$ M <sup>−1</sup> cm <sup>−1</sup>	$\epsilon_{+}$ M <sup>−1</sup> cm <sup>−1</sup>	$\epsilon_{\text{LY}}$ M <sup>−1</sup> cm <sup>−1</sup>	$k_{\text{ex-}}^1$ s <sup>−1</sup>	$k_{\text{ex+}}^1$ s <sup>−1</sup>	$k_{\text{eq}}$ s <sup>−1</sup>	$\alpha_{\text{ps}}$ abs	$Q_{+}$	$Q_{-}$	$\alpha_{\text{ps}}$ NMR
<b>2a</b>	2	366	410	3200		0.002	0.018	0.002	0.38	0.31	0.065	0.34
	110	340	1800	5000		0.49	1.36	0.16	0.92	0.31	0.025	
	27	548	40	12 100		0.004	1.34	0.069			0.05	
<b>3a</b>	3.7	366	410	3200	2600	0.004	0.033	0.005	0.28	0.31	0.05	0.33
	110	340	1800	5000	2900	0.52	1.45	0.20	0.76	0.29	0.029	
	27	548	90	12 100		0.01	1.35	0.069			0.05	
<b>2b</b>	110	340	4000	10 500		1.17	3.1	0.20	0.97	0.17	0.002	
	80	568	310	11 000		0.11	3.8	0.03			0.008	
<b>3b</b>	110	340	4000	10 500	2900	1.1	2.9	0.21	0.89	0.17	0.006	0.85
	80	568	350	11 000		0.12	3.8	0.036			0.009	

<sup>a</sup> See Figure 3 and text for definitions and formalism. Repeated measurements yielded estimates of  $Q_{+}$  and  $Q_{-}$  varying by ~20% and ~50%, respectively. The greatest uncertainty was related to the irradiance and spectral distribution of the photochromic source(s).

UV–vis irradiation were repeated 40 times for **3a** (Figure 5a,b and Figure 6a,b) and 25 times for **3b** (Figure 5c,d and Figure 6c,d). The absorption and fluorescence signals corresponding to the initial form and the photostationary state remained stable during these sequential reactions.

**Computation of Photokinetic Parameters.** The photokinetic parameters corresponding to ring-closing reactions induced by UV irradiation and to ring-opening back reactions initiated with

visible light were derived from the evolution of absorption signals as a function of irradiation time (Figures 3, 4, and other data). The initial state of the forward reactions and the final state of the back reactions were the open ring configuration of the acceptor [ $\alpha_o = \alpha_{\text{off}} = 0$ ]. Forward reactions led to photostationary states with values of  $\alpha_{\text{ps}}$  invariably <1, because of the finite absorption of the closed state (and of the donor in **3a** and **3b**) in the near-UV. The corresponding fluorescence and



**Figure 5.** Absorption monitoring of cyclical on and off (initial UV-vis) photoconversions of **3a** and **3b**. Initial and photostationary state spectra of **3a** (a) and **3b** (c). Absorbance at 548 nm (**3a**, b) and 568 nm (**3b**, d) as a function of time during multiple forward and back conversion cycles initiated from the diheteroarylethene open form. Photoconversion achieved by irradiation at 320 or 520 nm.

absorbance changes during such reactions are given by eqs S4 and S5; thus, for  $E_- \approx 0$  and  $E_+ \approx 1$  (our system), the fractional quenching during such a back reaction should have been equal to  $\alpha_{on}$ .

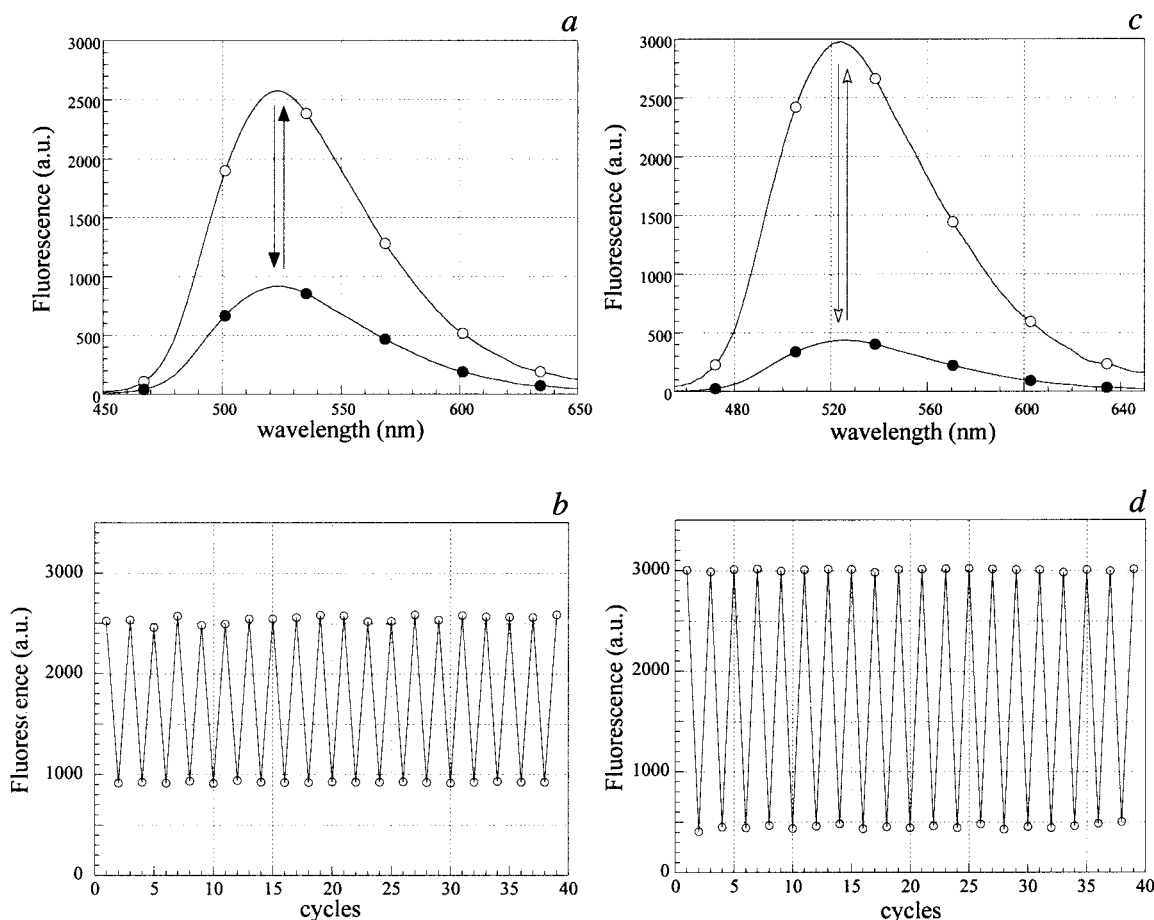
In accordance with the formalism presented in this work (eqs S8–S11), monoexponential processes were observed in all cases (although at some wavelengths, a very slight transient overshoot was observed in forward reactions). The parameters derived from such data using eqs 3–5 and S8–S11,  $k_{+-}$ ,  $k_{+}$ ,  $\alpha_{ps}$ ,  $Q_{+-}$ , and  $Q_{+}$  are given in Table 1. The two isolated diheteroarylethene compounds (**2a**, **3a**) differed in important respects: (i)  $Q_{+-}$  was greater for **2a** (0.31 vs 0.17); both values were  $<0.5$ , in accordance with data reported for most diheteroarylethenes of similar structure.<sup>5</sup> (ii)  $Q_{+}$  was also greater for **2a** (0.01–0.06 vs  $<0.01$ ). (iii) Under identical irradiation conditions for the forward reaction (340 nm), **2b** achieved a higher  $\alpha_{ps}$  and at a somewhat faster rate than **2a**; these differences could be attributed to the higher absorption of **2b** in the near-UV (eq S10). In accordance with the formalism (Figure 3 and eqs 3–5 and S8–S11), conjugation to the donor did not lead to a reduction in  $Q_{+-}$ , but  $\alpha_{ps}$  diminished because of the FRET-mediated donor contribution to the cycloreversion reaction ( $\sim 17$  and  $\sim 8\%$  for **2a**  $\rightarrow$  **3a** and **2b**  $\rightarrow$  **3b**, respectively). There was good agreement between the values of  $\alpha_{ps}$  obtained from the absorption and NMR measurements.

In another series of measurements not represented in Table 1, the forward reactions of **2b** and **3b** induced by identical conditions of irradiation at 340 nm (irradiance  $107 \text{ mW cm}^{-2}$ )

yielded equilibration times of 4.9 and 7.2 s, respectively (average of eight determinations). Photoreversion by irradiation at 568 nm (irradiance  $207 \text{ mW cm}^{-2}$ ) led to equilibration times of  $19.9 \pm 0.8$  and  $13.8 \pm 0.6$  s (**2b** and **3b**, respectively, average of eight determinations). Thus, the cyclization was slightly retarded, and the cycloreversion was slightly accelerated in the donor–acceptor conjugate.

## Discussion

FRET can be activated and deactivated reversibly by switching on and off the closed form of diheteroarylethenes, as demonstrated by the synchronous change in closed form absorbance and donor fluorescence intensity induced by the UV-vis irradiation cycles. Upon photogeneration of the closed form of the diheteroarylethene, a concurrent reduction in the donor fluorescence intensity was observed with  $E$  switching from 0 to 1 for the model compounds **3a** and **3b**, reflecting the changes in the critical transfer distance  $R_0$ . The photogenerated closed form was stable at room temperature in the dark, as evidenced both by absorption and by fluorescence signals, for  $>120$  h. Appropriate measurement conditions and controls excluded inner filter effects arising from absorbance at the excitation and photoconversion wavelengths as well as absorption of donor fluorescence by the diheteroarylethene closed form. Low sample concentrations were used, such that the absorbance at critical wavelengths was  $<0.05$ . Control samples of **1** showed invariant fluorescence emission upon exposure to the same experimental conditions. The positions of the absorption (430



**Figure 6.** Fluorescence monitoring of cyclical on and off (initial UV–vis) photoconversions of **3a** and **3b**. Initial and photostationary state emission spectra of **3a** (a) and **3b** (c); excitation, 430 nm. Emission signals at 520 nm in the initial open form and in the photostationary state recorded for a number of cycles during back and forward conversion initiated from the diheteroarylethene open forms of **3a** (b) and **3b** (d). Photoconversion achieved by irradiation at 320 or 520 nm.

nm) and fluorescence (530 nm) maxima in **1** before and after conjugation were invariant, excluding the presence of ground-state complexes.

We computed the absorption spectra of the closed form using the degrees of conversion determined from NMR.<sup>8a–e</sup> Integration of the resonances corresponding to the closed form were compared with corresponding signals of the open form to determine the contribution of each species. The conversion values agreed well with the extents of fluorescence quenching, indicative of a FRET efficiency close to 100% expected for a system (**3a**) with an  $R_0$  of  $\sim 38$  Å and a maximum donor–acceptor distance of 20 Å (eq S12). The same situation applied to **3b**, with an  $R_0$  of  $\sim 35$  Å and a maximum donor–acceptor distance of 22 Å.

The photochromic difference spectra for **2a** and **3a** and for **2b** and **3b** were virtually indistinguishable, attesting to the lack of significant ground-state interactions between the LY donor and the diheteroarylethene acceptors.

Irradiation of **2a** at 313 nm gave rise to a greater (albeit slower) conversion to the closed form of the diheteroarylethene

than did exposure to 366 nm. The increase in the photogeneration of the closed form resulted in a concurrent increase of the fluorescence quenching from 33 to 65%. Diheteroarylethenes do not display a thermal back reaction at temperatures below 80 °C, thereby enabling stable FRET determinations over long measurement times. Moreover, the photocyclization reaction takes place without a change in net charge, solvent-driven dark processes are absent, and fatigue is negligible.

We have provided in this work a general, systematic formalism accounting for the coupling between the photochromic interconversion mechanisms and the photophysical states of both donor and acceptor moieties. The photokinetic scheme exhibits in theory and practice a simple monoexponential behavior, from which rate constants and quantum yields for the forward and cycloreversion photochromic reactions can be derived. The formalism allowed the dissection of the photoconversion reaction from the FRET process. The quantum yields for the cyclization and cycloreversion reactions were equal within experimental error for **2a** and **3a** as well as for **2b** and **3b** (Table 1). We conclude that the donor moiety in the model compounds did not affect the inherent properties of the photoconversion reactions and that the observed slight changes in the equilibration rates in the donor conjugate resulted from the differences in the combined absorption cross-sections. However, the conversion to the closed forms was somewhat

(8) (a) Norsten, T. B.; Branda, N. R. *J. Am. Chem. Soc.* **2001**, *123*, 1784–1785. (b) Osuka, A.; Fujikane, D.; Shinmori, H.; Kobatake, S.; Irie, M. *J. Org. Chem.* **2001**, *66*, 3919–3923. (c) Gauglitz, G.; Scheerer, E. *J. J. Photochem. Photobiol., A* **1993**, *71*, 205–212. (d) Rau, H.; Greiner, G.; Gauglitz, G.; Meier, H. *J. Phys. Chem.* **1990**, *94*, 6523–6524. (e) Wilkinson, F.; Hobley, J.; Naftaly, M. *J. Chem. Soc., Faraday Trans.* **1992**, *88*, 1511–1517.



more efficient for **2a** and **2b** than for **3a** and **3b**. FRET-augmented cycloreversion accounted for a <20% decrease in the yield of the closed form upon irradiation with near-UV light. According to the scheme of Figure 3, the FRET process contributes to the back reaction via a second parallel pathway leading to the excited state of the closed form of the diheteroarylethene. The values of  $Q_{-+}$  computed from data obtained with irradiation at 366 and 340 nm were the same, although at wavelengths <340 nm, a distinct trend to lower conversion efficiencies was observed. Under all conditions, the computed values of  $Q_{-+}$  were <0.5, implying the existence of the two states postulated for the open form, only one of which can undergo cyclization. The presumption is that the equilibration between the open-form conformers is very rapid, leading to a preequilibrium that is implicit in the scheme of Figure 3.

The quenching of a donor engaged in a FRET process is generally reflected in a corresponding fractional change (reduction) of its fluorescence lifetime. We observed two donor species in the photostationary state after irradiation in the near-UV, for example, at 366 nm. One of these corresponded to the population of donor **1** attached to the diheteroarylethene in the closed form, the latter acting as an efficient energy acceptor and thus quenching the donor virtually completely. The other fraction represented **1** bound to the diheteroarylethene in the open form, characterized by an  $E_{-} \approx 0$  and thus incapable of generating a significant FRET effect. This case can be visualized in the time domain with an expression for the decay of two fluorescence species excited by a pulse of light:

$$F(t) = F_0[(1 - \alpha_{ps})e^{-t/\tau_{D-}} + \alpha_{ps}e^{-t/\tau_{D+}}] \quad (6)$$

where  $F_0$  is the initial donor fluorescence signal, and  $\tau_{D+} = \tau_{D-}(1 - E_{+})/(1 - E_{-})$ . For the limit  $E_{+} \approx 1$ , computed for both **3a** and **3b** with the diheteroarylethenes in the closed form,  $\tau_{D+}$  would be too short to be detected in the frequency-domain system, regardless of the value of  $\alpha_{ps}$ . That is, the fluorescence decay would be dominated by  $\tau_{D-}$ . The quenching of steady-state donor fluorescence  $F_D$  after photogeneration of the closed form would have been  $\alpha_{ps}E_{+}$  (eq S4). From NMR measurements, we determined  $\alpha_{ps}$  (366 nm) to be 0.33 in **2a** and 0.85 in **2b**, values which were reflected accurately in the extents of fluorescence quenching (33 and 85%, respectively), allowing the determination of the FRET efficiency (100%) for the diheteroarylethene closed form.

Experiments involving cyclical ring closures and openings demonstrated that the FRET process can be switched on and off reversibly. We performed 40 cycles for **3a** and 25 cycles for **3b** with no apparent fatigue (Figures 5 and 6). The number of cycles of FRET activation–deactivation that can be achieved by cyclic irradiation with UV and visible light is of central importance, in that this parameter determines the feasibility of carrying out continuous determinations in dynamic systems, for example, with living cells.

Recently, we succeeded in conjugating diheteroarylethene-based photochromic moieties to biomolecules and creating donor–acceptor pairs via biotin–streptavidin. The donor fluorescence was readily modulated in aqueous solution by cyclic irradiation.<sup>9</sup> The FRET  $E$  was 0 for the diheteroarylethene in the open form and rose to 0.5 with the compound in the closed form. The formalism for pcFRET presented here provides the means for developing experimental strategies and analyzing the

results of such experiments. Other applications of pcFRET include the potential for enhancing signal-to-background relationships in analytical procedures using flow cytometric or imaging technology, which are characterized by very low levels of specific complexes.

## Experimental Section

**Materials.** (3-(4-{4-[3,3,4,4,5,5-Hexafluoro-2-(2-methoxy-benzo[*b*]thiophen-3-yl)-cyclopent-1-enyl]-3,5-dimethyl-thiophen-2-yl}-benzoylamino)-propionic acid (**2a**) was prepared by treatment of acyl chloride of the diheteroarylethene derived from the formyl derivative<sup>10</sup> with  $\beta$ -alanine in ether–water under alkaline condition. 4-(4-{4-[3,3,4,4,5,5-Hexafluoro-2-(2-methoxy-benzo[*b*]thiophen-3-yl)-cyclopent-1-enyl]-3,5-dimethyl-thiophen-2-yl}-phenyl)-butyric acid (**2b**) was prepared from the butanol derivative.<sup>11a,b,12–14</sup> The details of the synthetic procedure will be published elsewhere. Lucifer Yellow cadaverine **1**, *N*-(5-aminopentyl)-4-amino-3,6-disulfo-1,8-naphthalimide, was from Molecular Probes (Eugene, Oregon). *N,N'*-dicyclohexyl-carbodiimide and *N*-hydroxysuccinimide were purchased from Fluka Chemie AG, Buchs, Switzerland. We also refer to substructure **1** as LYC and substructure **2a** or **2b** as DAE.

(3-(4-{4-[3,3,4,4,5,5-Hexafluoro-2-(2-methoxy-benzo[*b*]thiophen-3-yl)-cyclopent-1-enyl]-3,5-dimethyl-thiophen-2-yl}-benzoylamino)-propionic acid-2,5-dioxo-pyrrolidin-1-yl Ester (**4a**). Twenty milligrams (0.032 mmol) of **2a** reacted with 8 mg (0.039 mmol) of DCC and 4 mg (0.035 mmol) of *N*-hydroxysuccinimide in 5 mL of dry acetonitrile at room temperature for 12 h. The dicyclohexylurea was filtered, and the *N*-succinimide active ester **4a** (Figure 1) was evaporated in vacuo.

**Disodium-6-amino-2-{5-[3-(4-{4-[3,3,4,4,5,5-hexafluoro-2-(2-methoxy-benzo[*b*]thiophen-3-yl)-cyclopent-1-enyl]-3,5-dimethyl-thiophen-2-yl}-benzoylamino)-propionylamino]-pentyl]-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinoline-5,8-disulfonate (**3a**).** Sixteen milligrams of Lucifer Yellow cadaverine (**1**, 32  $\mu$ mol) was dissolved in 30  $\mu$ L of 0.5 M borate buffer (pH 9.2) and reacted with 32  $\mu$ mol of **4a** dissolved in 200  $\mu$ L of dry acetonitrile, for 2 h at room temperature. The product was purified by HPLC using a RP-C18 stationary phase and an elution solvent CH<sub>3</sub>CN:triethylammonium acetate (TEAA, 1 M, pH 7.0) 35:65.

<sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  9.05 (1H, H-7 LYC), 8.94 (1H, d, 1.5 Hz, H-5 LYC), 8.91 (1H, s, H-2 LYC), 8.49 (1H, s, NH, LYC), 7.20–8.00 (8H, Ar in DAE), 4.05 (2H, t,  $J = 8.5$  Hz, NCCCCCH<sub>2</sub>NHCO LYC), 3.72 (2H, m, –CONHCH<sub>2</sub>CH<sub>2</sub>CO– DAE), 3.20 (2H, m, NCH<sub>2</sub>CCCCNHCO LYC), 2.62 (2H, m, CONHCH<sub>2</sub>CH<sub>2</sub>CO DAE), 2.47 (1.5H, s, CH<sub>3</sub> DAE), 2.45 (1.5H, s, CH<sub>3</sub> DAE), 2.42 (1.5H, s, CH<sub>3</sub> DAE), 2.21 (1.5H, s, CH<sub>3</sub> DAE), 2.19 (1.5H, s, CH<sub>3</sub> DAE), 1.93 (1.5H, s, CH<sub>3</sub>– DAE), 1.6–1.8 (6H, CH<sub>2</sub> LYC). EM, FABMS, M–H: 1061.3. Anal. Calcd for C<sub>47</sub>H<sub>38</sub>F<sub>6</sub>N<sub>4</sub>Na<sub>2</sub>O<sub>10</sub>S<sub>4</sub>: C, 52.03; H, 3.73; N, 3.79. Found: C, 52.36; H, 3.74; N, 3.81.

<sup>1</sup>H NMR at the photostationary state by irradiation at 366 nm (500 MHz, CD<sub>3</sub>OD):  $\delta$  9.05 (1H, H-7 LYC), 8.94 (1H, d, 1.5 Hz, H-5 LYC), 8.91 (1H, s, H-2 LYC), 8.49 (1H, s, NH, LYC), 7.17–8.00 (Ar in DAE), 4.05 (2H, t,  $J = 8.5$  Hz, NCCCCCH<sub>2</sub>NHCO LYC), 3.72–3.74 (CONHCH<sub>2</sub>), 3.20 (NCH<sub>2</sub>CCCCNHCO LYC), 2.62–2.63 (CH<sub>2</sub>CH<sub>2</sub>CO DAE), 2.47 (s, CH<sub>3</sub> DAE), 2.45 (s, CH<sub>3</sub> DAE), 2.42 (s, CH<sub>3</sub> DAE), 2.35 (s, CH<sub>3</sub>-DAECF), 2.21 (s, CH<sub>3</sub> DAE), 2.19 (s, CH<sub>3</sub> DAE), 2.11

(9) Giordano, L.; Macareno, J.; Song, L.; Jovin, T. M.; Irie, M.; Jares-Erijman, E. *Molecules* **2000**, *5*, 591–593.

(10) Takeshita, M.; Irie, M. *Chem. Lett.* **1998**, 1123–1124.

(11) (a) Nakashima, N.; Irie, M. *Polym. J.* **1998**, *30*, 985–989. (b) Corey, E. J.; Schmidt, G. *Tetrahedron Lett.* **1979**, 399–403.

(12) Gadella, T. W. J., Jr.; Jovin, T. M.; Clegg, R. M. *Biophys. Chem.* **1993**, *48*, 221–239.

(13) Morgan, C.; Hua, Y.; Mitchell, A.; Murray, J. G.; Boardman, A. *Rev. Sci. Instrum.* **1996**, *67*, 41–47.

(14) Baumann, J.; Calzaferri, G.; Forss, L.; Hugentobler, T. J. *J. Photochem.* **1985**, *28*, 457–473.

(s,  $\text{CH}_3$ -DAECF), 2.05 (s,  $\text{CH}_3$ -DAECF), 2.04 (s,  $\text{CH}_3$ -DAECF), 1.93 (s,  $\text{CH}_3$ -DAE), 1.6–1.8 (6H).

**4-(4-{4-[3,3,4,4,5,5-Hexafluoro-2-(2-methoxy-benzo[*b*]thiophen-3-yl)-cyclopent-1-enyl]-3,5-dimethyl-thiophen-2-yl}-phenyl)-butyric acid 2,5-dioxo-pyrrolidin-1-yl Ester (4b).** Ten milligrams (0.0164 mmol) was reacted in a similar manner as described for **4a**, yielding **4b** (Figure 1).

**Disodium-6-amino-2-{5-[5-(4-{4-[3,3,4,4,5,5-hexafluoro-2-(2-methoxy-benzo[*b*]thiophen-3-yl)-cyclopent-1-enyl]-3,5-dimethyl-thiophen-2-yl}-phenyl)-butyrylamino]-pentyl]-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinoline-5,8-disulfonate (3b).** As described for **3a**, 0.0164 mmol of **4b** was reacted with equimolar amounts of **1**. The product **3b** was purified by HPLC.

$^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  9.06 (1H, d, 1.5 Hz, H-7), 8.90 (1H, d, 1.5 Hz, H-5), 8.87 (1H, s, H-2), 8.52 (0.5H, broad s), 7.70–7.15 ppm (8H, Ar in DAE), 4.10 (2H, t,  $\text{NCH}_2\text{CCCCNHCO}$ ), 3.85 (3H, s,  $\text{OCH}_3$ ), 3.43 (2H, m), 2.6 (2H, t,  $J = 8$  Hz,  $\text{CH}_2$ -Ph), 2.20 (2H, t,  $\text{CH}_2\text{CO DAE}$ ), 2.15 (3H, s,  $\text{CH}_3$  DAE), 1.95 (3H, s,  $\text{CH}_3$ -DAE), 1.5–1.80 (8H). EM, FABMS,  $\text{M}^+$ : 1048.3. Anal. Calcd for  $\text{C}_{47}\text{H}_{39}\text{F}_6\text{N}_3\text{Na}_2\text{O}_{10}\text{S}_4$ : C, 51.60; H, 3.59; N, 3.84. Found: C, 52.05; H, 3.61; N, 3.87.

$^1\text{H}$  NMR of photostationary state by irradiation at 340 nm (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  9.06 (1H, d, 1.5 Hz, H-7), 8.90 (1H, d, 1.5 Hz, H-5), 8.87 (1H, s, H-2), 8.00–7.15 (DAE), 4.10 (2H, t,  $\text{NCH}_2\text{CCCCNHCO}$ ), 3.85 (s,  $\text{OCH}_3\text{DAE}$ ), 3.43 (2H, m), 3.36 (s,  $\text{OCH}_3$  DAECF), 2.6 (2H, t,  $J =$

8 Hz,  $\text{CH}_2$ -Ph), 2.20 (2H, t,  $\text{CH}_2\text{CO}$ ), 2.15 (3H, s,  $\text{CH}_3$  DAE), 1.95 (3H, s,  $\text{CH}_3$ -DAE), 1.5–1.8 (8H).

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**Supporting Information Available:** Quantitative formalism for the photostationary state and photokinetics containing eqs S1–S11. Steady-state spectroscopy methods: photochromic conversion light source, photochromic conversion, and photokinetics. Methods: fluorescence lifetime and FRET methods. Table S1: Integrated values for  $^1\text{H}$  NMR signals of **2a** and **2b**. Figure S1: Spectral properties for the completely off (–) and on (+) states (open and closed). Figure S2: Effects of photochromic conversion wavelength on the degree of photoconversion and donor fluorescence quenching. Figure S3:  $^1\text{H}$  NMR for **2a** and **3b** in the open form and in the photostationary state. Figure S4: Photophysical-photochromic scheme incorporating donor–acceptor FRET (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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