Communication

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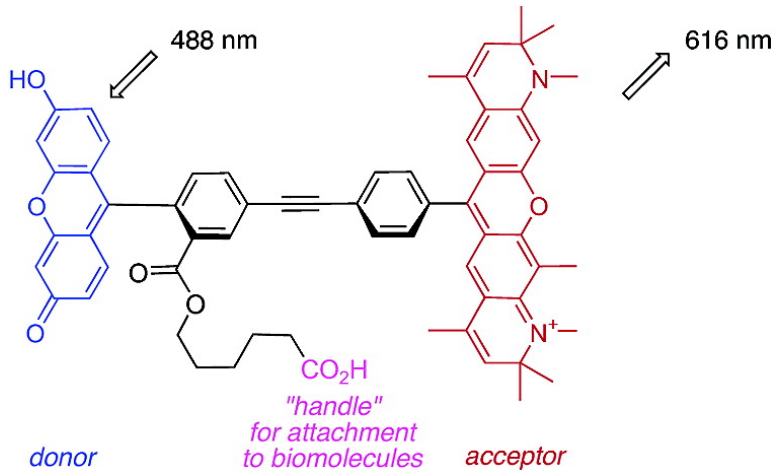

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Fluorescent, Through-Bond Energy Transfer Cassettes for Labeling Multiple Biological Molecules in One Experiment

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Many biochemical experiments require labeling of molecules with distinct fluorescent tags, that is, multiplexing. A convenient approach to multiplexing is to tag each compound with a fluorescent group, and then excite each at one wavelength using, for instance, an argon laser (488/514 nm).[1] However, there is a problem with this strategy: the dyes that emit at longer wavelengths absorb at the excitation wavelength less effectively, so their fluorescence intensities are diminished. This becomes especially important if detection of low levels of fluorescence (sensitivity) is also an issue. A partial solution is to use fluorescence resonance energy transfer (FRET) between two dyes, a donor and an acceptor.[2,3] Unfortunately, the resolution of emissions with multiplexed, FRET-based cassettes is still constrained by the requirement that the fluorescence of the donor must overlap with the absorption of the acceptor (corresponding to the overlap integral in Förster energy transfer).[4]

Our interest in this area is focused on dyes for biotechnology that have donors connected to acceptors via electronically conjugated linkers. Energy transfer (ET) in such systems occurs through-bonds and through-space (Figure 1).[5] If the through-bond ET is fast relative to nonradiative decay pathways, then, unlike standard FRET-based cassettes, through-bond ET is apparently not constrained by these requirements. If so, multiplexing with through-bond energy transfer cassettes would not necessarily involve loss of sensitivity at high resolutions. This Communication reports a simple design concept for through-bond conjugated dye cassettes and structures that could be useful for multiplexing in many areas of molecular biology and biotechnology.

Requirements for good through-bond energy transfer cassettes for labeling biological systems include donor components with strong absorbance at the excitation wavelength, acceptor components that fluoresce strongly, functional groups that allow attachment of the cassettes to biological molecules, and a suitable conjugated linker. The linker must prevent the donor and acceptor fragments from becoming planar because if they did the system would behave as a single conjugated dye. Conversely, the linker must allow through-bond energy transfer from the donor to the acceptor that is rapid relative to nonradiative decay processes. These considerations led us to design systems with fluorescein conjugated to rhodamine-like acceptors, of which **1**−**4** are typical (Figure 2).

Syntheses of cassettes **1**−**4** featured Sonogashira couplings[6] to link the donor and acceptor components. This relied upon prior generation of regioisomerically pure 5-halofluoresceins[7] and similar halogenated compounds such as **5**−**8** (Figure 3). Dye system **8** is new to this work.

Absorption spectra of **1**−**4** show maxima characteristic of the donor and acceptor components. This indicates the compounds are behaving as cassettes and not as planar, totally conjugated dyes. They all have a fluorescein donor component, and hence they absorb strongly at 488 and 514 nm (important wavelengths for excitation via an argon laser). Excitation of the cassettes at 488 or 514 nm

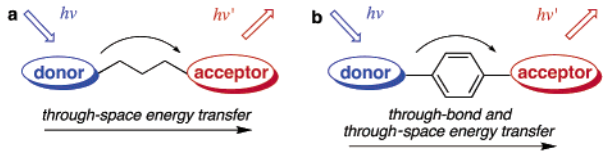

Figure 1. (a) Through-space and (b) through-bond ET cassettes.

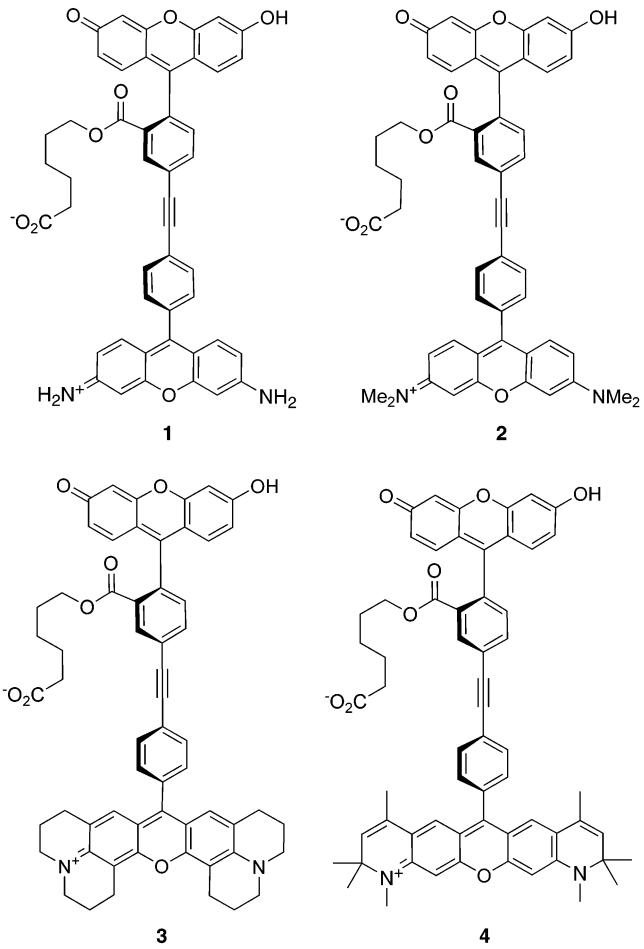

Figure 2. ET cassettes featured in this work.

produces fluorescence characteristic of only the acceptor component, that is, 100% energy transfer efficiency within experimental error.

The value of dye cassettes for multiplexing is mainly determined by resolution and sensitivity. Dispersion of the cassette fluorescence maxima, reflecting the resolution, is easy to evaluate. Cassettes **1**−**4** fluoresce at 538, 582, 603, and 616 nm, respectively; hence their emissions are dispersed over a 78 nm wavelength range (Figure 4), and their fluorescence signals are easily differentiated.

To evaluate the sensitivities that can be obtained for **1**−**4**, their fluorescence emission intensities when excited at the donor

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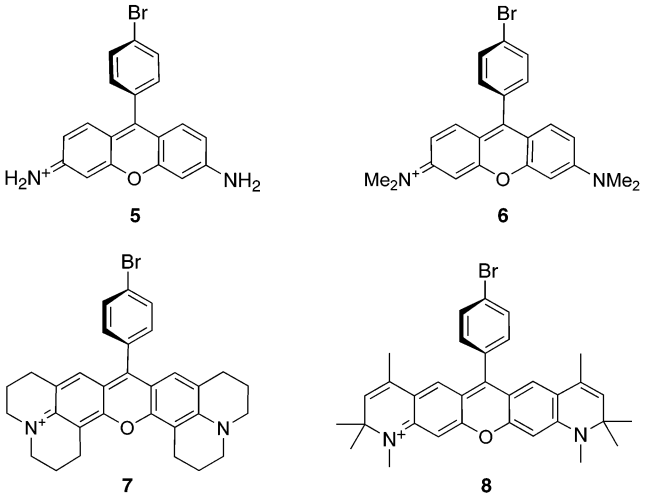

Figure 3. Acceptor synthons for the through-bond ET cassettes.

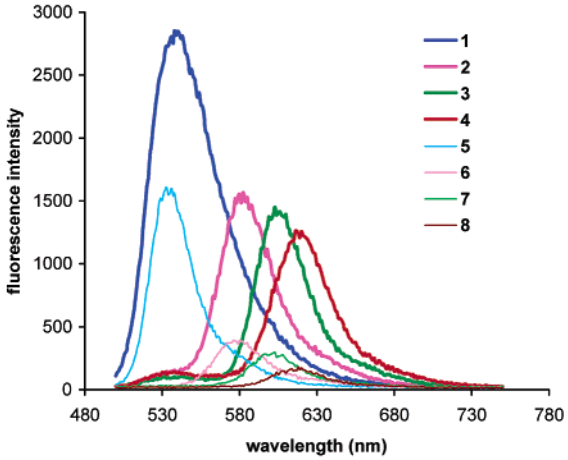

Figure 4. Fluorescence of equimolar EtOH solutions of **1**−**8** excited at 488 nm.

component were compared with the emission of molecules that resemble the acceptors alone irradiated under the same conditions, that is, the "fluorescence enhancement" which is the ratio of the fluorescence intensity of cassettes to that of corresponding acceptors with excitation at 488 nm. Fluorescence spectra of cassettes **1**−**4** irradiated at 488 nm compared with equimolar solutions of **5**−**8** irradiated under the same conditions are shown in Figure 4. The cassettes fluoresce more brightly than the corresponding acceptor components **5**−**8** irradiated at 488 nm. The fluorescence enhancements measured were **1**, 1.8; **2**, 4.0; **3**, 5.0; and **4**, 7.0.

The prevalent application of multiplexed dyes is in DNA sequencing at the genomic level where small improvements in sensitivity can markedly increase throughput. Fluorescence enhancements observed here for cassettes **1**−**4** compare favorably with those of the widely used, through-space (FRET) "Big Dye" cassettes (fluorescence enhancements 2.0−2.5)[8] and similar FRET-based systems with different linkers (up to 4.0).[9] Both sets of dye cassettes have narrower dispersions than those reported here; they have acceptor units similar to **5**, **6**, and **7**, and another which fluoresces at a wavelength between **5** and **6**. Moreover, cassettes **1**−**4** are not complicated by formation of atropisomers.[10]

As single molecule detection methods emerge, multiplexing at this level also becomes important. Photobleaching of dyes is a significant obstacle in such methods.[11] Fluoreceins, for instance, are notoriously poor because their fluorescence fades rapidly as the dyes decompose on irradiation. Cassettes **1**−**4** are considerably more stable to photobleaching than fluorescein itself, even though the cassettes contain a fluorescein donor fragment. In a typical experiment, the fluorescence of fluorescein had diminished by 55% while the fluorescence of cassette **3** in the same solution was at least 95% of its original value. Photobleaching of fluorescein is thought to occur via triplet states.[11] It may be that donor-to-acceptor energy transfer rates in **1**−**4** are so fast that intersystems crossing cannot compete, and hence the triplet states are not significantly populated; this would account for the greater photostability of the cassettes. Experiments are in progress to measure the ET rates, but that requires special equipment and expertise because they are so fast.[12]

Through-bond donor−acceptor systems are well known in materials research,[13,14] but those featuring fluorescein donors and rhodamine-like acceptors are new. We believe that compounds **1**−**4** and similar cassettes have the potential to replace many dyes currently used for DNA sequencing and other applications in biotechnology. Our current efforts are focused on making more through-bond energy transfer cassettes for applications in biotechnology.

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Supporting Information Available: Syntheses of compounds **1**−**8**, absorption spectra of **1**−**8**, tabulated spectroscopic properties of **1**−**8**, and demonstration of the photostability of the cassettes (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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