

## Water-Soluble Through-Bond Energy Transfer Cassettes for Intracellular Imaging

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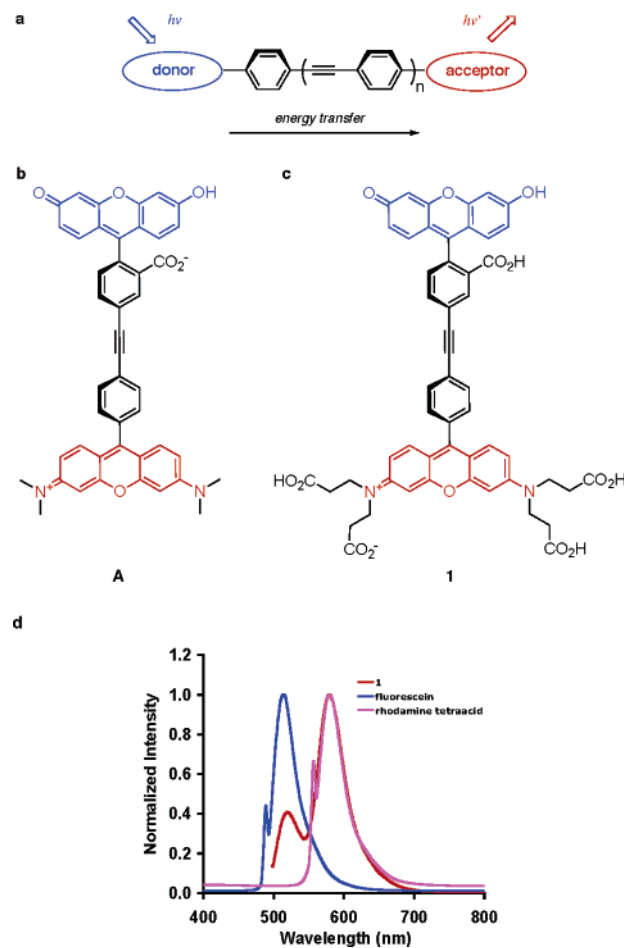
Imaging synthetically labeled proteins in living cells<sup>1</sup> requires ways of labeling the protein such that it functions in much the same way as the unlabeled one. Further, some technique must be mastered to import the tagged protein into cells without significantly perturbing their biology. Finally, labels on the proteins must be readily observable from outside the cell. This paper addresses issues relating to all of these aspects, especially that relating to the design of the labels.

The following characteristics are highly desirable for labels designed for intracellular imaging: (i) water solubility for ease of coupling and to avoid aggregation effects, (ii) absorbance of light with a high extinction coefficient at a wavelength that is conveniently excited within a cell, (iii) emission of fluorescence at a significantly longer wavelength than the excitation source so that observation of the label is not complicated by backscattering effects from the excitation source, and (iv) fluorescence maxima at above 550 nm so that the label can be observed in a region that is not complicated by autofluorescence from within the cell.<sup>2</sup>

Previous studies from one of us introduced a new concept in dyes for biotechnology: “through-bond energy transfer cassettes”.<sup>3,4</sup> These are particularly useful with respect to parameter (iii) listed above. They have donor and acceptor fragments joined via  $\pi$ -bonds but steric effects prevent them from becoming flat and conjugated (Figure 1a). These cassettes absorb at wavelengths characteristic of the donor and then emit it via the acceptor.

Compound **A** is typical of the cassettes that were prepared previously.<sup>4</sup> It has a fluorone (fluorescein-derived) donor fragment connected to a pyronin (rhodamine-derived) acceptor part. Fluorescence energy transfer between the donor and acceptor parts in compound **A** appears to occur with near-complete efficiency in MeOH. It also occurs extremely fast, too fast in fact, to measure by routine methods, and significantly faster than similar cassettes wherein energy can only be transferred “through-space”, i.e. via Förster energy transfer.<sup>5,6</sup> It is likely that the predominant mode of energy transfer in system **A** is a rapid mechanism operating through bonds. This is desirable because fast rates of energy transfer from the donor to the acceptor in through-bond energy transfer cassettes favor fluorescence over undesired nonradiative decay mechanisms. Further, the wavelength difference between the donor absorption and the acceptor emission is greater than Stokes shifts in a single dye molecule, and is not limited by any known constraints corresponding to the overlap integral in Förster energy transfer, i.e. fluorescence resonance energy transfer (FRET).<sup>7,8</sup> Thus, there is no known upper limit to how far to the red the acceptor fluorescence can be shifted relative to the donor fluorescence.

Briefly, cassette **1** was assembled by coupling the fluorescein alkyne **2** to the bromorhodamine derivative **3** (Scheme 1).<sup>9</sup> The most difficult part of this was isolation and purification of the



**Figure 1.** (a) Generalized structure of some through-bond energy transfer cassettes; (b) cassette **A**, prepared previously, has poor water solubility; (c) water-soluble target cassette **1** featured in this work; (d) comparison of the fluorescence of cassette **1** in pH 8 phosphate buffer with fluorescein and a tetraacid from the rhodamine derivative **3** (Scheme 1) under the same conditions.

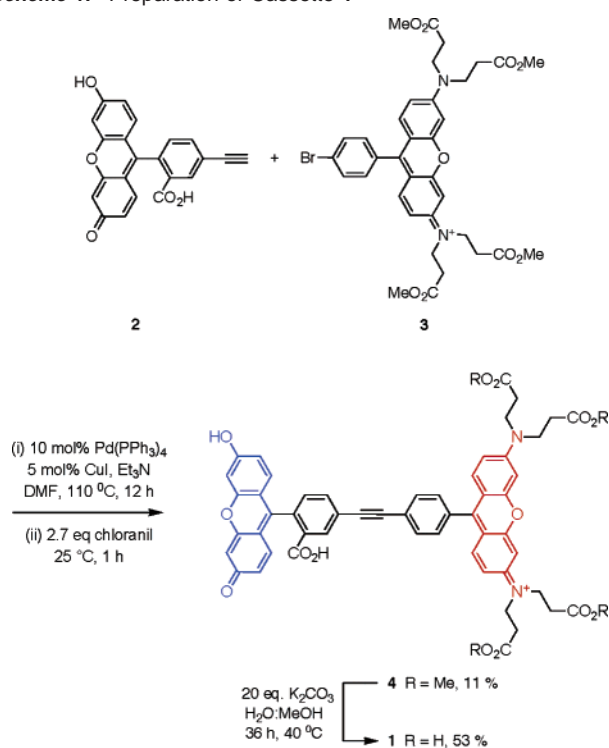
product. Ultimately, we found that the tetraester **4** could be obtained in 80% purity via column chromatography and then purified via preparative reverse phase HPLC. The final product **1** was obtained via an acid/base extraction procedure.

Electronic spectra of **1** showed several desirable characteristics. The dye absorbs strongly at 556 nm and emits at 579 nm in aqueous buffer. However, the energy transfer efficiency was not 100% because some of the fluorescence leaks from the fluorescein donor rather than being transferred to the acceptor. Under the pH 8 buffer conditions shown in Figure 1d, there is about 80% energy transfer efficiency.<sup>10</sup>

Monoactivation of the cassette **1** (DIC/N-hydroxysuccinamide) allowed it to be coupled with an illustrative recombinant protein,

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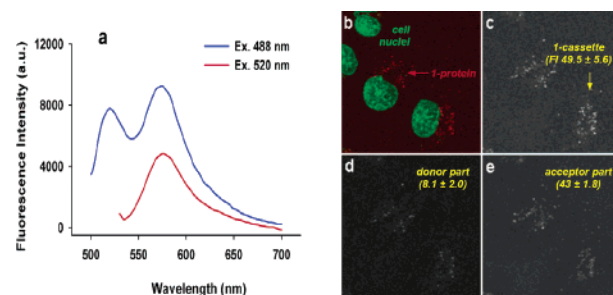
## Scheme 1. Preparation of Cassette 1



mouse acyl-coenzyme A binding protein (ACBP).<sup>11</sup> Gel electrophoresis experiments showed this could be achieved without significant cross-linking (data not shown). In vitro ACBP binds medium- and long-chain fatty acyl-CoA thioesters with a low nanomolar  $K_d$ .<sup>12,13</sup> Titration with a fluorescent ligand, *cis*-parinaroyl-CoA, up to saturation of binding was performed to check if this function was maintained after conjugation of cassette 1. Unlabeled ACBP had a  $K_d$  of 26.6 nM, whereas the cassette-labeled protein bound this ligand with a  $K_d$  of 20.5 nM. Circular dichroism (CD) spectra for the unlabeled- and labeled-ACBP were very similar. Both spectra were analyzed using CDPPO software<sup>14</sup> to detect percentages of different elements of secondary structure, and the program predicted only small differences. In summary, the binding and CD data indicate that labeling ACBP with cassette 1 did not impede its natural binding function or cause it to misfold in vitro.

Figure 2 presents fluorescence spectra and real-time confocal images accumulated for ACBP-1 in living cells (PBS buffer, pH 7.4) excited at 488 nm. It shows emissions characteristic of the fluorescein donor (ca. 520 nm) and rhodamine acceptor cassette constituents, the latter being stronger. These spectra are slightly different from the ones of the free cassette in Figure 1d, indicating its emission properties are affected by its microenvironment.

The next challenge was to load the fluorescently labeled protein into living cells without perturbing them. For this, the cell-penetrating peptide Chariot was used. Chariot is unique insofar as it binds nonselectively and noncovalently to extracellular proteins and mediates their transfer into cells.<sup>16</sup> It is believed that Chariot dissociates from its protein cargo inside the cell and is degraded without causing cytotoxic effects. After some experimentation, Chariot-mediated import of ACBP-1 into COS-7 cells was demonstrated. Figure 2b shows ACBP-1 (pseudo-red color) colocalizes around the nuclei (false-stained green using Hoechst 33342). When



**Figure 2.** (a) Fluorescence emission spectrum of ACBP-1 in PBS buffer (pH 7.4); (b) ACBP-1 localizes near the cell nuclei, as expected;<sup>15</sup> (c) corresponding image of ACBP-1 when the cassette is irradiated at the donor part and observed near the acceptor emission maximum (throughout, values in parentheses are relative fluorescence intensities); d as in c except observed near the donor emission maximum; and, e as in c but excited near the acceptor absorption maximum.

the cells were excited at 488 nm (to excite the donor) and a filter at 598 nm (acceptor fluorescence) was applied, then the image shown in Figure 2c was obtained. Application of a filter corresponding to the donor fluorescence gave a much weaker image (Figure 2d). Figure 2e shows the result of exciting the cells near the acceptor absorption maxima, at 568 nm. Comparison of c and e of Figure 2 shows that ACBP-1 in the cell is more visible when the cassette is irradiated at the donor part; hence, the function of the energy-transfer cassette is demonstrated *in vivo*.

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**Supporting Information Available:** Preparation and characterization of 1, procedure for coupling 1 to ACBP, protocol for determining binding constants, CD spectra and protocol for their analyses, method for import of ACBP-1 into COS-7 cells, and details of the laser confocal microscopy experiments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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