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Cyanine dye conjugates as probes for live cell imaging

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Abstract—A series of fluorescent compounds suitable for live cell imaging is described. Functionalized forms of four different asymmetric cyanine dyes are reported that are amenable to peptide conjugation. The photophysical properties of the modified dyes and conjugates and the use of the compounds as cellular imaging agents are described. The results obtained indicate that these spectrally versatile compounds, which have absorption and emission profiles spanning the visible spectrum, are useful probes for cellular imaging.

Fueled by advancements in imaging technology, examination of live cells by fluorescence techniques is becoming widely used to study cellular processes.[1] Fluorophores that are permeable to intact cell membranes provide an opportunity to perform dynamic analyses in living cells and also avoid limitations associated with fixation. In contrast to fixed cells, which only provide a snapshot of a biological sample, visualization of cellular processes in living cells with techniques such as time-lapse microscopy and flow cytometry allows for real-time analysis. In addition, the process of fixation has been shown to alter the localization of some molecules, which can lead to experimental artifacts.[2,3] While a limited number of dyes for live cell analysis are available commercially, new cell-permeable fluorophores with versatile spectroscopic properties are needed to provide additional options for live cell analysis.

Cyanine dyes, a class of synthetic fluorophores with two heteroaromatic rings joined by a monomethine or polymethine chain, have been used for a variety of biological applications requiring spectroscopic labels.[4–9] These dye molecules have tunable wavelengths across the visible spectrum, which allows for spectral compatibility in multicolor applications. In addition, this family of compounds exhibits high molar extinction coefficients permitting the use of low concentrations. These property, along with high fluorescence quantum yields, make these molecules ideal cellular fluorophores. Although cyanine dyes offer many valuable properties as a cellular imaging tools, those that are commercially available for cell-based studies (e.g., TO-PRO, TO3-PRO) can only be used as an imaging tool with fixed cells as they are unable to penetrate an intact cell membrane.[9] In addition, these commercially available derivatives are not amenable to biomolecule conjugation. Herein, we report the synthesis of four cyanine dye derivatives that can be easily functionalized with peptides or other biomolecules. In addition, we report on the properties of a set of designed cyanine dye–peptide conjugates that serve as live cell mitochondrial markers.

Four cyanine dye derivatives were synthesized. The synthetic approach is based on one described previously for small-scale solid-phase synthesis of modified cyanine dyes.[10] The approach developed here is amenable to the production of much larger quantities of the compounds (the syntheses described here produce 50–90% yields on gram quantities of starting material), and the intermediates can be completely characterized. The dyes made contain either a pyridinium or quinolinium ring linked to a benzothiazolium ring via a monomethine (**4**, **5**) or trimethine linker (**6**, **7**).

Monomethine cyanine dye derivatives were prepared as shown in Scheme 1. The benzothiazole derivative **1a** was

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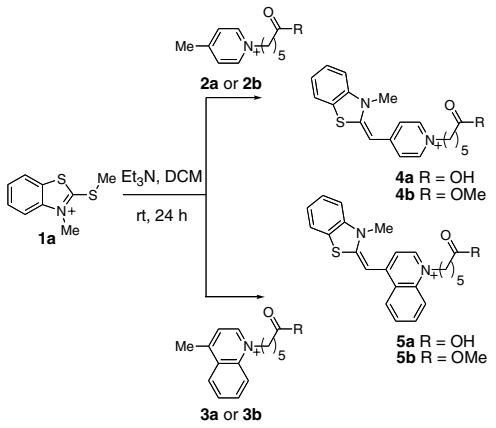
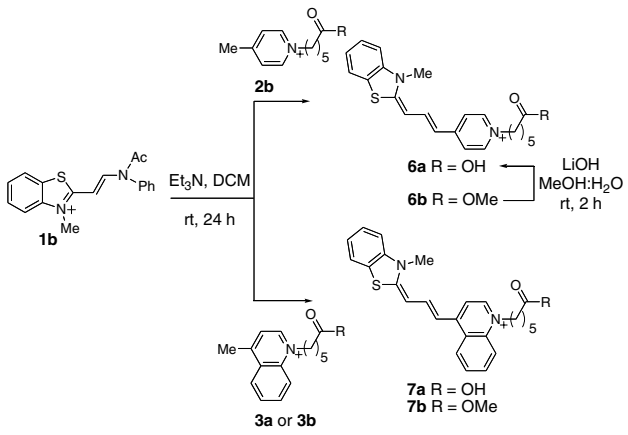


Scheme 1. Synthesis of monomethine cyanine dye derivatives **4** and **5**.

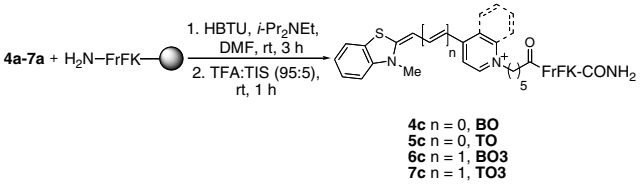
prepared from 3-methylbenzothiazole-2-thione. Condensation of **1a** with pyridinium (**2a**–**2b**) or quinolinium (**3a**–**3b**) derivatives in the presence of triethylamine in DCM afforded the carboxylic acid monomethine dyes **4a** and **5a** or methyl ester dyes **4b** and **5b**.

Trimethine cyanine dye derivatives were synthesized as shown in Scheme 2. The benzothiazole derivative **1b** was prepared in two steps from 2-methylbenzothiazole. Condensation of **1b** with pyridinium (**2b**) or quinolinium (**3a**–**3b**) derivatives in the presence of triethylamine in DCM then yielded the trimethine dyes functionalized as its carboxylic acid (**7a**) or methyl ester (**6b** and **7b**). We were unable to prepare carboxy-functionalized dye **6a** using this method; instead, saponification of the methyl ester **6b** with LiOH in $MeOH/H_2O$ was used to produce **6a**.

Next, cyanine dye–peptide conjugates were prepared using the carboxy-functionalized dyes. These conjugates feature a tetrapeptide sequence with alternating aromatic and basic amino acids, Phe-*d*-Arg-Phe-Lys (FrFK), a sequence known to facilitate mitochondrial localization in cultured human cells.[11] The peptide segments of the dye conjugates **4c**–**7c** were prepared on Rink amide solid support, the N-terminus deprotected and coupled to the carboxy-functionalized dyes **4a**–**7a**



Scheme 2. Synthesis of trimethine cyanine dye derivatives **6** and **7**.



Scheme 3. Solid-phase synthesis of dye–peptide conjugates **4c**–**7c**.

using standard solid-phase Fmoc chemistry, and the resulting peptide conjugate cleaved from the resin (Scheme 3). Purification via HPLC afforded dye–peptide conjugates **4c**–**7c**. The dye conjugates were then characterized using ESI mass spectrometry, UV–vis spectroscopy, and fluorescence spectroscopy. The characterization methods and spectral data are described in the Supplementary information.

These cyanine dyes are frequently utilized as nucleic acid stains, as their fluorescence quantum yield increases upon nucleic acid binding or other environmental changes that rigidify the chromophore.[12,13] In order to determine how the modified dyes and peptide conjugates performed as nucleic acid stains, and to characterize their spectroscopic properties for cellular imaging, we investigated the effect of complexation to double-stranded DNA on the spectroscopic properties of the dye-based conjugates. The modified dyes were examined as methyl esters so that the acidic terminus of the tether would not interfere with DNA binding. Absorption spectra of the dye–peptide conjugates (**4c**–**7c**) are shown in Figure 1. The attachment of the peptide sequence to the dyes causes a slight red-shift of the absorption maxima relative to the dye methyl esters (**4b**–**7b**). All of the dye–peptide conjugates displayed a DNA-dependent enhancement in fluorescence as the methyl esters that is presumably due to rigidification of the heterocyclic ring system upon intercalation (Table 1). Interestingly, BO3 (**6**) exhibits a unique property compared to the other three dyes, having a significant fluorescence quantum yield when free in solution (see Supplementary

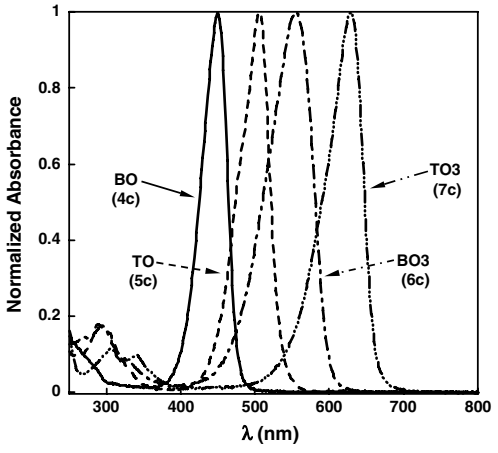


Figure 1. Normalized absorption spectra of the cyanine dye peptide conjugates (**4c**–**7c**) in H_2O; (**4c**) BO (solid line); (**5c**) TO (short dash); (**6c**) BO3 (long dash/short dash); (**7c**) TO3 (long dash/dots).

Table 1. Fluorescence quantum yields (**Φ**) for dye–peptide conjugates[a]

Compound	λ_{max}Abs (nm)	$\lambda_{max}E_m$ (nm)	Φ_{rel} (DNA)	Enh.[d]
BO-CO_2Me (**4b**)	455	480	0.029 (2)[b]	120
BO-FrFK (**4c**)	458	480	0.069 (1)[b]	140
TO-CO_2Me (**5b**)	511	525	0.141 (4)[b]	470
TO-FrFK (**5c**)	512	526	0.206 (2)[b]	430
BO3-CO_2Me (**6b**)	564	593	0.093 (1)[c]	8
BO3-FrFK (**6c**)	567	594	0.116 (2)[c]	4
TO3-CO_2Me (**7b**)	634	574, 649	0.024 (3)[c]	20
TO3-FrFK (**7c**)	638	573, 655	0.008 (2)[c]	6

[a] See Supplementary information for methods used to obtain values tabulated.
[b] Measured in samples containing 1.5 μM dye, 45 μM bp CT DNA and reported relative to a fluorescein standard.
[c] Measured in samples containing 1.5 μM dye, 45 μM bp CT DNA and reported relative to a rose bengal standard.
[d] Fluorescence enhancement observed in presence of DNA relative to buffered solution calculated using values tabulated in Table S1.

information). In general, the emission profiles were not strongly affected by the presence of the appended peptide, and the emission enhancements observed in the presence of DNA were similar for the dyes and the dye–peptide conjugates.

In order to test the utility of this series of cyanine dyes for cellular imaging, the two sets of compounds (i.e., the peptide conjugates and the dye precursors displaying the esterified tether) were incubated with HeLa cells and cellular localization was monitored by confocal laser scanning microscopy (CLSM). As shown in Figures 2 and 3, the four cyanine dyes were each able to cross the plasma membrane when the terminal carboxylic acid moiety was either capped as a methyl ester or conjugated to the N-terminus of a tetrapeptide. All four cyanine methyl esters (**4b**, **5b**, **6b**, **7b**) rapidly entered living cells and **4b**, **6b**, and **7b** primarily accumulated in the mitochondria (Fig. 2). Many lipophilic cations are known to accumulate in the mitochondria, and the cyanine dyes synthesized here appear to adhere to this trend. Compound **4b** also exhibited a limited extent of nucleolar staining. While cells treated with **4b**, **6b**, and **7b** remained viable over the course of imaging experiments, incubation with **5b** resulted in cytotoxicity after a short incubation time (15 min) (see Fig. S10). The onset of cell death and resultant perturbation of the mitochondrial potential may therefore be responsible for the nuclear staining seen for this compound.

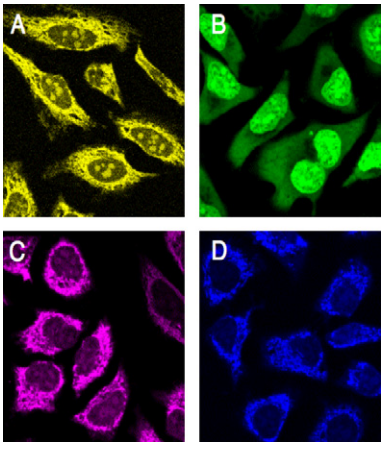


Figure 2. Fluorescence images of (A) BO (**4b**); (B) TO (**5b**); (C) BO3 (**6b**); (D) TO3 (**7b**); HeLa cells were incubated with 1 μM conjugate at 37 °C/5% CO_2/humidity for 15 min and washed with PBS (1x). The cells were then visualized by CLSM using an excitation wavelength of 488 nm for A, B; 543 nm for C; 633 nm for D.

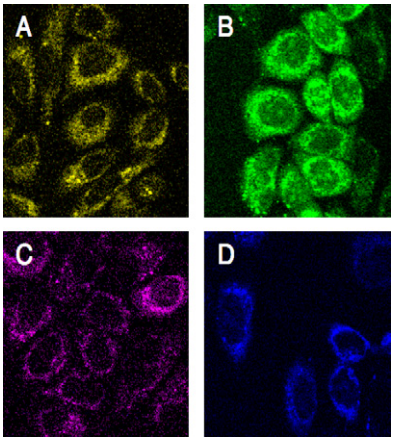


Figure 3. Fluorescence images of (A) BO-FrFK (**4c**); (B) TO-FrFK (**5c**); (C) BO3-FrFK (**6c**); (D) TO3-FrFK (**7c**); HeLa cells were incubated with 5 μM conjugate at 37 °C/5% CO_2/humidity for 90 min and washed with PBS. The cells were then visualized by CLSM using an excitation wavelength of 488 nm for A, B; 543 nm for C; 633 nm for D.

The peptide conjugates of the modified cyanine dyes are also useful mitochondrial dyes and exhibit more specific localization than the parent dyes. As shown in Figure 3, mitochondrial localization was exclusively observed when the peptide sequence Phe-*d*-Arg-Phe-Lys-NH2 was appended. Uptake was reduced, which necessitated longer incubations and higher concentrations of dye, but no toxicity was observed (Fig S11). These conjugates are therefore more effective mitochondrial stains than the parent dyes and are more appropriate for live cell imaging. Moreover, the spectroscopic characteristics of the parent dye are retained upon conjugation, thereby allowing for the visualization of unfixed mammalian cells using these peptide derivatives using common excitation and emission ranges.

We have synthesized a series of novel dye–peptide conjugates that bind nucleic acids and label live human cells. These dyes provide a practical method to visualize

the mitochondria of living cells using a variety of observation wavelengths with excitation in the visible range. The straightforward syntheses of these dye molecules feature a carboxylic acid tether that can easily be conjugated to a peptide sequence, as demonstrated here, or to other biomolecules of interest. Importantly, the cytotoxicity of this type of compound can be reduced by utilizing standard solid-phase peptide synthesis to attach a tetrapeptide sequence. Our results indicate that these untethered dyes and their peptide conjugates can be used to probe living cells, broadening the application for this class of fluorophores.

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Supplementary data

Experimental procedures and full characterization of compounds **1a**–**7c**. as well as DIC images of HeLa cells treated with **4b,c**–**7b,c**. This material is available free of charge via the Internet at http://www.elsevier.com. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.06.097.

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