Supporting Information
For
Asymmetric Trimethine 3H-Indocyanine Dyes: Efficient Synthesis and Protein Labeling

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General Experimental Techniques

Mass spectral determinations were made on HP1100 API-ES mass spectrometry. NMR spectra were recorded on a Varian 400 MHz NMR spectrometer (USA). Chemical shifts are expressed in parts per million (ppm) from D2O (δ H = 4.79) or DMSO-d6 (δ H = 2.54) [9]. Fluorescence measurements were performed on a PTI-C-700 Felix and Time-Master system. Visible spectra were measured on a HP-8453 spectrophotometer. The purification of the dyes was performed by conventional column chromatography on C18-RP absorbent (Sinochrom C18, 40-75 mesh, 100 Å, 280 m2 g-1). Protein electrophoresis was tested by the Tanon-2010 Imaging Systems (transmission wavelength: 302 nm, interference filter: 590 nm) and Tanon GIS 1D image analysis software. HPLC experiments were performed on a system composed of an injection valve (Rheodyne, Cotati, CA), a 4-line degasser, an intelligent pump equipped with quaternary gradient unit, an intelligent fluorescence detector (Jasco Co. Ltd., Tokyo, Japan) and a UV detector (Dalian Elite Analytical Instrument Ltd., Dalian, China). A C8 column (5 μ m, 300 Å, 4.6 mm × 250 mm) was ordered from Dalian Elite Analytical Instrument Ltd.

(China). Deionized water was redistilled before using. Acetonitrile were of chromatographic grade. Other chemicals used for the experiments were of analytical grade.

Synthesis

Symmetric dyes 1a-b

3*H*-indolium quaternary salt **2** were synthesized from the indoles and 3-bromopropanoic acid, 6-bromohexanoic acid, p-(chloromethyl) benzoic acid or 1, 4-butane sultone, respectively. The crude salts **2** were used for next step without additional purification. Symmetric Cy3 dyes **1a-b** were synthesized according to the previous procedure. [1]

Dye **1a**: Yield 30 %. 1H NMR (400 MHz, D2O): δ 8.33 (t, 1H, CH=CH, J = 13.6 Hz), 7.74 (s, 2H, Ar-H), 7.69 (d, 2H, Ar-H, J = 8 Hz), 7.19 (d, 2H, Ar-H, J = 8.4 Hz), 6.22 (d, 2H, CH=CH, J = 13.6 Hz), 3.91 (m, 4H, N-CH2), 2.08 (m, 4H, CH2-COOH), 1.64 (m, 4H, N-β-CH2), 1.54 (s, 12H, C(CH3)2), 1.44 (m, 4H, COOH-β-CH2), 1.25 (m, 4H, γ-CH2). API-ES-MS: [M-H]- (m/z = 715.3), [M-2H]2- (m/z = 357.1).

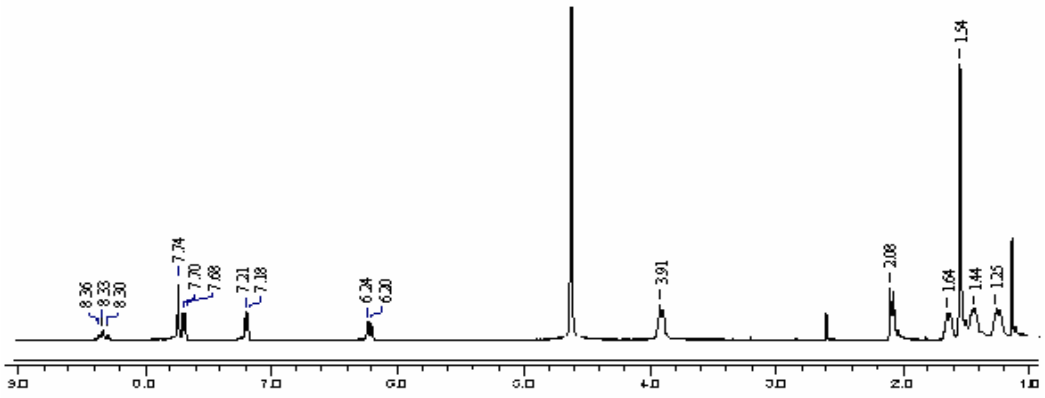


Figure S1. [1]H NMR of **1a**

Dye **1b**: Yield 48 %. 1H NMR (400 MHz, D_2O): δ 8.24 (t, 1H, CH=CH, J = 10 Hz), 7.76 (s, 2H, Ar-H), 7.68 (d, 2H, Ar-H, J = 8 Hz), 7.62 (d, 4H, Ar-H, J = 9.6 Hz), 7.14 (d, 6H, Ar-H, J = 8.2 Hz), 6.09 (d,

2H, CH=CH, J = 13.6 Hz), 5.25 (s, 4H, N-CH2), 1.51 (s, 12H, C(CH3)2). API-ES-MS: [M-H]- (m/z = 755.2), $[M-2H]^{2-}$ (m/z = 377.0).

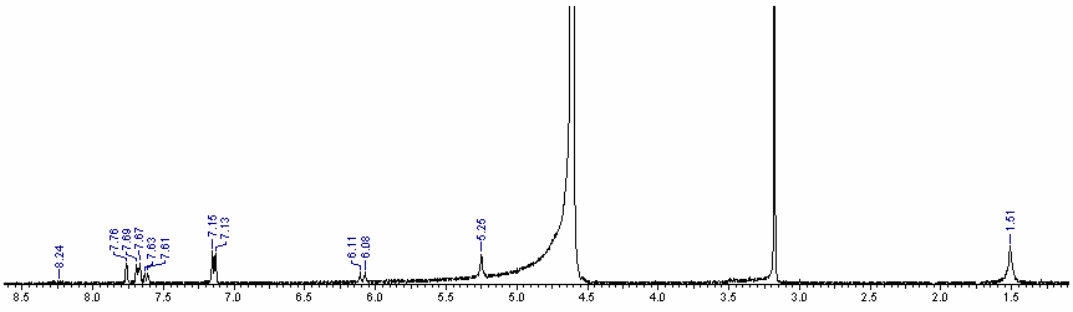


Figure S2. ^{1}H NMR of **1b**

Hemicyanine intermediate 3.

The quaternary salt **2a** (4.1 g, 10 mmol) and N, N'-diphenylformamidine (2.2 g, 11.4 mol) in a mixture of acetic acid (5 mL) and acetic anhydride (5 mL) was heated under reflux. The reaction was checked for completion by TLC. Extended heating produced some symmetrical dye (< 5 %). After 90 min, the mixture was cooled and diluted with several volumes of diethyl ether. The supernatant fluid was removed by decantation, and the product was triturated with a mixture of ethyl acetate and water (50:50). Excess N, N'-diphenylformamidine was dissolved in ethyl acetate while water removed any symmetrical dye and unreacted intermediate **2a**. The crude orange powder thus obtained was simply separated by flash-column.

Asymmetric dyes 1c-f

A solution of crude hemicyanine intermediate **3** (1.44 g, 2.77 mmol) and quaternary salt **2** (2.77 mmol) in acetic anhydride (5 mL) was heated under reflux for 30 min. The reaction completion was checked by TLC. The mixture was cooled and diluted with several volumes of diethyl ether. A product separated from which the supernatant fluid was removed by decantation. Crude dye was dissolved in water and then purified on C18-RP column using methanol–water mixture as eluent.

Dye **1c** yield 6 %. ^{1}H NMR (400 MHz, D_2O): δ 8.42 (t, 1H, CH=CH, J = 12.8 Hz), 7.76 (s, 2H, Ar-H), 7.71 (d, 2H, Ar-H, J = 8 Hz), 7.26 (m, 2H, Ar-H,), 6.29 and 6.25 (2d of 1H each, CH=CH, J = 13.2 Hz), 4.00 (m, 4H, N-CH_2), 2.83 (t, 2H, CH_2SO_3, J = 7.2 Hz), 2.03 (t, 2H, CH_2COOH, J = 7.2 Hz), 1.63 (s, 12H, $C(CH_3)_2$), 1.85-1.10 (m, 10H, $5CH_2$). API-ES-MS: $[M-3]^{3-}$ (m/z = 245.2).

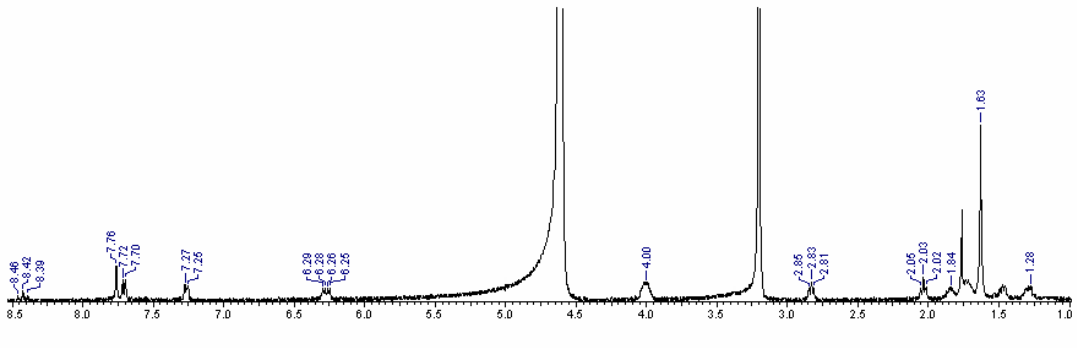


Figure S3. ^{1}H NMR of **1c**

Dye **1d**: Yield 21 %. ^{1}H NMR (400 MHz, D_2O): δ 8.40 (t, 1H, CH=CH, J = 12 Hz), 7.78 (s, 2H, Ar-H), 7.74 (d, 2H, Ar-H, J = 8 Hz), 7.30 (m, 2H, Ar-H,), 6.32 and 6.28 (2d of 1H each, CH=CH, J = 14 Hz), 5.30 (s, 2H, N-CH_2-Ar), 3.93 (m, 2H, N-CH_2), 2.85 (t, 2H, CH_2SO_3, J = 7.4 Hz), 1.68 (s, 12H, $C(CH_3)_2$), 1.90-1.20 (m, 4H, 2CH2). API-ES-MS: $[M–2]^{2-}$ (m/z = 378.1).

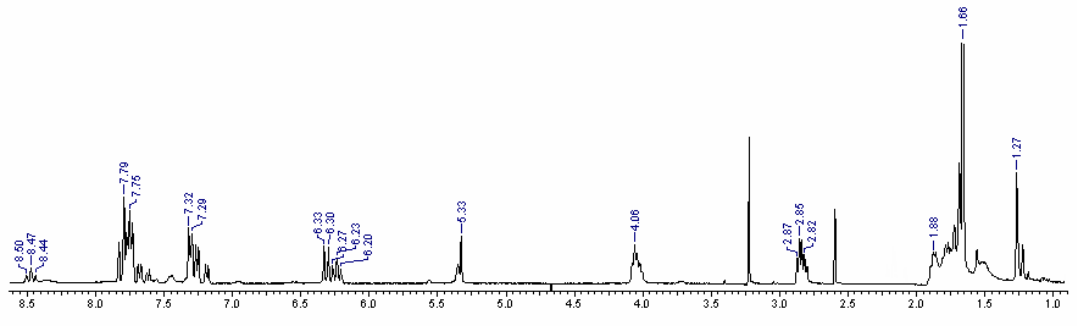


Figure S4. ^{1}H NMR of **1d**

Dye **1e**: Yield 35 %. ^{1}H NMR (400 MHz, D_2O): δ 8.28 (t, 1H, CH=CH, J = 10 Hz), 7.80-7.00 (m, 6H, Ar-H), 6.22 (d, 1H, SO3Ar-CH=CH, J = 13.6 Hz), 6.10 (d, 1H, Ar-CH=CH, J = 12.8 Hz), 3.91 (m, 4H, N-CH_2), 2.79 (t, 2H, CH_2SO_3, J = 7.4 Hz), 1.99 (t, 2H, CH_2COOH, J = 7.4 Hz), 1.51 (s, 12H, $C(CH_3)_2$), 1.80-1.20 (m, 10H, $5CH_2$). API-ES-MS: $[M-1]^-$ (m/z = 657.2).

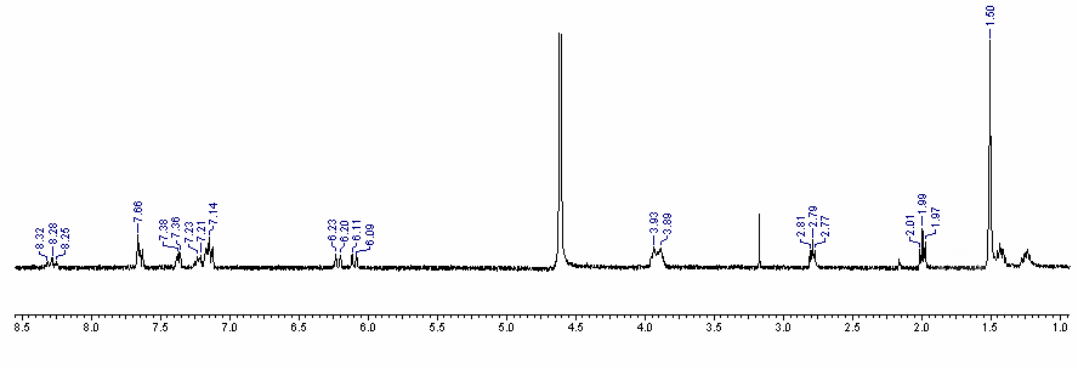


Figure S5. ^{1}H NMR of **1e**

Dye **1f**: Yield 42 %. ^{1}H NMR (400 MHz, D_2O): δ 8.19 (t, 1H, CH=CH, J = 13.8 Hz), 7.80-7.00 (m, 11H, Ar-H), 6.22 (d, 1H, SO_3Ar-CH=CH, J = 13.2 Hz), 6.10 (d, 1H, Ar-CH=CH, J = 13.4 Hz), 5.27 (s, 2H, N-CH_2-Ar), 3.86 (m, 2H, N-CH_2), 2.71(m, 2H, CH_2SO_3), 1.51 (s, 12H, $C(CH_3)_2$), 1.70-1.30 (m, 4H, $2CH_2$). API-ES-MS: $[M-H]^-$ (m/z = 677.2).

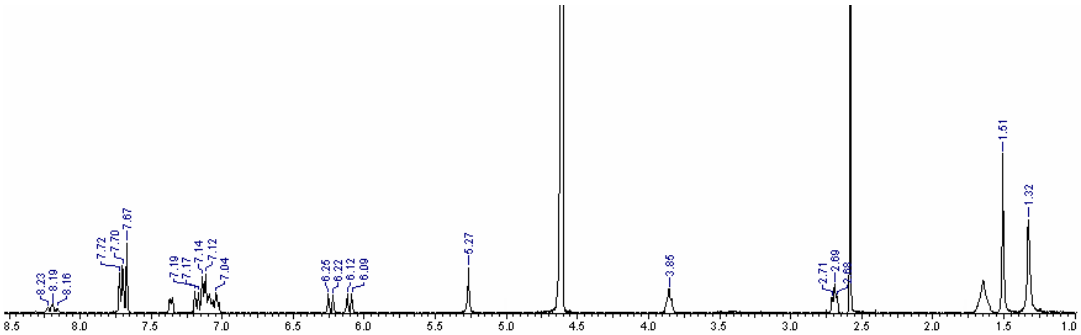


Figure S6. ^{1}H NMR of **1f**

Succinimidyl esters of Cy3 dyes

In separate centrifuge tubes, sulfocyanine with carboxyl group was dissolved in dry DMF (2 mL/100 mg of dye). N, N'-dicyclohexyl carbodiimide (DCC) (5 equiv/carboxyl group) and NHS (N-hydroxysuccinimide, 10 equiv/carboxyl group) was added. The tubes were capped, vortexed and left to stand at room temperature for 10 hours. After diluting the mixture with dry ethyl acetate, the supernatant was centrifuged to collect. The supernatant liquors were decanted; the pellets were washed with fresh ethyl acetate and dried under vacuum. By thin-layer chromatography (TLC), a nearly 100% yield of the active esters of Cy3 dyes, Cy3-NHS esters, was obtained. The products were used to label protein directly without farther purification.

Spectra properties

The Cy3 dyes synthesized were dissolved in methanol to prepare stock solutions (1×10^{-3} M), and kept at 4 °C. The stock solutions were diluted to 1×10^{-6} M in different solvents to obtain samples. The absorption spectra of Cy3 dyes were recorded (220-750 nm) in deionized water, DMF and Methanol at 25 °C. Fluorescent spectra were recorded under the same conditions after excitation at a wavelength 10 nm below the corresponding λ_{max} (excitation and emission slit = 5 nm).

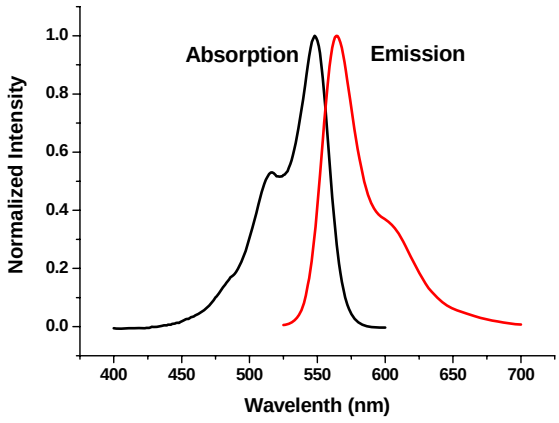


Figure S7. Normalized absorption and fluorescence emission spectra of dye **1f** in water.

Table S1. Spectra properties of water-soluble Cy3 dyes in different solvents

Entry	solvent	$\lambda_{abs}/\lambda_{em}$ (nm)	Stocks shifts (nm)	ε ($\times 10^5$ $mol^{-1}cm^{-1}$ L)	Φ[a]
1a	DMF	565/583	18		0.43
	Methanol	556/571	15		0.58
	water	551/565	14	1.50	0.20
1b	DMF	569/589	20		0.49
	Methanol	562/577	15		0.63
	water	552/566	14	1.64	0.14
1c	DMF	567/584	17		0.29
	Methanol	556/572	16		0.47
	water	549/564	15	1.42	0.18
1d	DMF	560/579	19		0.50
	Methanol	551/569	18		0.22
	water	545/563	18	1.29	0.10
1e	DMF	561/579	18		0.32
	Methanol	551/569	18		0.31
	water	547/564	17	1.75	0.12
1f	DMF	560/579	19		0.40
	Methanol	554/571	17		0.41
	water	549/564	16	1.71	0.11

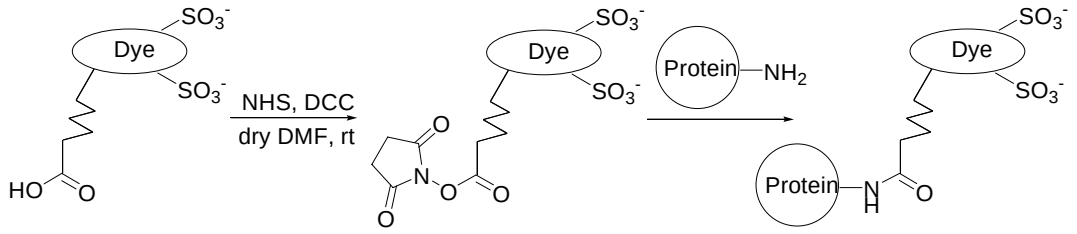
[a]the fluorescence quantum yields were determined in reference to Rhodamine B in ethanol (Φ=0.97). λ_{em}: 540 nm, error ca. ±10 %.

The corresponding fluorescence quantum yields were calculated relative to a standard solution of Rhodamine B in ethanol (Φ = 0.97)[2] and was determined using the formula:[3]

$$QY_u = \frac{(QY_s)(FA_u)(A_s)(\lambda_{exs})(\eta_u{}^2)}{(FA_s)(A_u)(\lambda_{exu})(\eta_s{}^2)}$$

where QY = quantum yield; FA = integrated area under the corrected emission spectrum (in Ep units); A = absorbance at the excitation wavelength; λ_{ex} = the excitation wavelength; η = the refractive index of the solution used in measurement;[21] and the subscripts u and s refer to the unknown and the standard, respectively.

General protein labeling procedures



Scheme S1. Protein labeling with the Cy3 dyes

Stock solutions of 1 mg of Cy3-NHS esters in 100 μL of anhydrous DMF were prepared. A Stock solution of the standard proteins BSA, Myoglobin or Cytochrome C was made in deionized water (5 mg/mL) and stored at 4 °C. The loading buffer (2×) consistes of 50 mM borate buffer (pH 8.4), 2.5 % (w/v) SDS and 1 % (w/v) sucrose.

The 2 μl proteins (2.5 mg/mL) were denatured in equivalent volume of loading buffer (2 μl) at 100 °C for 10 min. Then 1 μl Cy3-NHS esters (12 mM carboxyl NHS ester group) in dry DMF was added during vigorous vortex mixing and reacted with proteins for 30 min at 30 °C in dark.

Gel electrophoresis (SDS-PAGE)

The fluorescent labeling of protein was investigated by SDS-PAGE. The PAGE with 10 % gel concentration and 1 % SDS was performed in 0.38 M Tris-HCl separating buffer (pH 8.8), 0.12 M Tris–HCl stacking buffer (pH 6.8) and in 25 mM Tris-glycin (pH 8.3) electrode buffer.

Optimization on labeling conditions

In our experiments, relative fluorescence response, defined as the product of intensity and pixel (I × P) of fluorescence band measured by SDS-PAGE with Tanon GIS 1D image analysis software, was used to evaluate the labeling reactions.

To obtain the optimal conditions of labeling reactions, BSA as a model protein at final concentrations 1 μg/μL was derivatized with Cy3 dye **1b** respectively under the conditions of pH 8.0-9.0 (buffer), 25-45 °C (reaction temperature), 5-45 min (reaction time) and reagent concentration 0.1 to 5 mM.

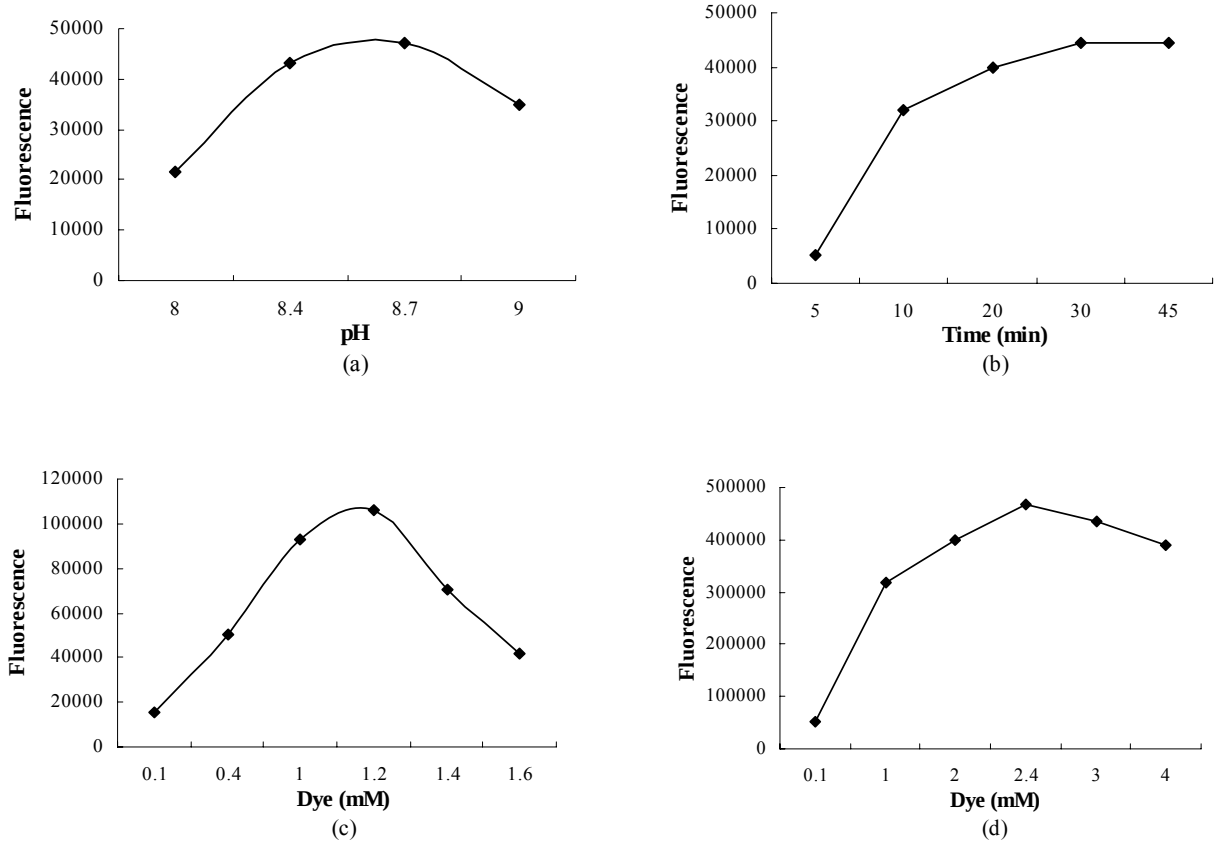


Figure S8. Effects of (a) pH of buffer, (b) reaction time, (c) the concentration of dye **1b** and (d) the concentration of dye **1f** on labeling reaction by SDS-PAGE.

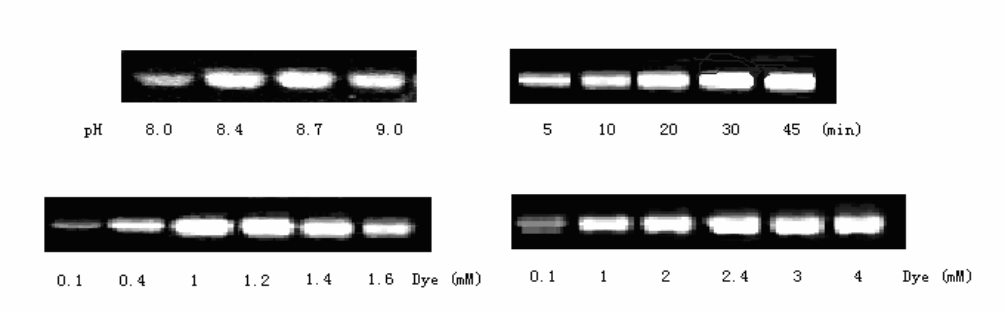


Figure S9. Optimization on labeling conditions by SDS-PAGE: (a) pH of buffer, (b) reaction time, (c) the concentration of dye **1b** and (d) the concentration of dye **1f**

The optimal pH for the formation of the Cy3 derivatives is related to the pKa of the amino residue and the reactivity of NHS groups. Generally the reaction of the amino group is favored under the alkaline condition, but hydrolysis of the Cy3-NHS ester is a competing reaction which speeds up with the increases of pH. From Figure S8, it could be seen that the maximum fluorescence intensity is obtained at pH 8.7 (Figure S8a and Figure S9a) with a reaction time of 30 min (Figure S8b and Figure S9b). The reaction temperature, however, has little effect on the reaction in the range of 25-45 °C. Considering the thermo-sensitivity of proteins, we take 30 °C as the optimal temperature.

In addition, the reagent concentration is another important reaction parameter.13 When the concentration of dye **1b** (containing two NHS groups) reached 1.2 mM to 5 μg BSA, the highest relative fluorescence response was obtained (Figure S8c and Figure S9c). However, with further increased the concentration, the signal intensity began to decrease due to fluorescence self quenching. For asymmetrical dyes (with only one NHS ester group), the highest signal happened to appear at 2.4 mM (Figure S8d and Figure S9d). Accordingly, 2.4 mM was chosen as the optimal concentration for NHS active ester group labeling reactions.

Therefore, the optimal conditions for the labeling reaction are: (i) pH of the buffer: 8.7; (ii) reaction time: 30 min; (iii) reaction temperature: 30 °C; and (iv) the concentration of NHS active ester group: 2.4 mM. The results also show good agreement with those of HPLC method.

Determination of Dye-to-Protein ratios

Absorption spectra of each dye-BSA conjugate was measured by a HP-8453 spectrophotometer in water. Subsequently the absorbances at the absorption maxima of the dye-BSA conjugate ($A_{conj(\lambda max)}$) and at 278 nm ($A_{conj(278)}$) were read. Next the absorbances of the free dye at 278 nm ($A_{dye(278)}$) and at the absorption maximum ($A_{conj(\lambda max)}$) were taken. The dye-to-protein ratios (D/P) were calculated using the following formula (with the assumption that the extinction coefficients for the free and conjugated dyes are about the same):[4]

$$\frac{D}{P} = \frac{A_{conj(\lambda_{max})}\varepsilon_{BSA}}{(A_{conj(278)} - xA_{conj(\lambda_{max})})\varepsilon_{dye}}$$

where ε_{dye} is the extinction coefficient of the dye at λ_{max}, ε_{BSA} = 45,540 M^{-1} cm^{-1} is the extinction coefficient of BSA at 278 nm, $\chi = A_{dye(278)}/A_{dye(\lambda max)}$, and absorbance ($A_{conj(\lambda max)}$) of 0.15-0.20.

HPLC

Column 250×4.6 mm (C8, 5 μm and 300 Å); eluent A, 100 % water with 0.1 % TFA; eluent B, 95 % acetonitrile with 0.1 % TFA; gradient conditions: 0-27 min, 25-80 % B, 27-35 min: 80-80 % B; flow rate: 1.0 mL/min; the column temperature: ambient temperature. Detector: Fluorescence detection: λ_{ex} 554 nm, λ_{em} 570 nm; UV detection: λ_{abs} 214 nm.

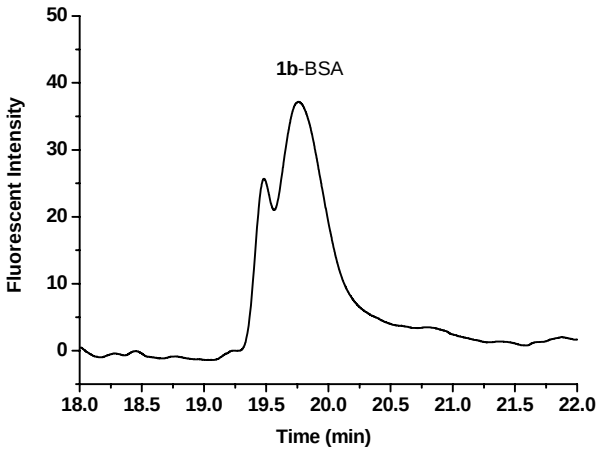


Figure S10. Analysis of NHS ester of dye **1b** labeled BSA with low concentration (2×10^{-8} M) by HPLC

References

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