Supporting Information

Facile Synthesis of Symmetric, Monofunctional Cyanine Dyes for Imaging Applications

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General Materials and Methods

All chemicals and solvents were purchased from Aldrich, Acros or TCI and were used without further purification. Thin layer chromatography (TLC) was performed on aluminum-backed plates with silica gel 60 with F₂₅₄ indicator. All of the dyes for characterization were purified by high-performance liquid chromatography (HPLC) on a Waters prep LC instrument equipped with a Waters 2998 photodiode array detector. If not otherwise noted, HPLC solvent A is 25 mM triethylammonium acetate (TEAA) in water and solvent B is MeOH. All moisture- or air-sensitive reactions were carried out under a static argon atmosphere. ¹H NMR (400 MHz) spectra were recorded at room temperature on a Bruker ADVANCE-400 spectrometer with residual proton resonances in deuterated solvents (DMSO-d₆) as the internal standard. LC/MS spectra were obtained with a Waters micromass ZQ instrument. Absorption and emission spectra were determined using a PerkinElmer Lambda 35 UV–visible spectrophotometer and a PerkinElmer LS 55 fluorescence spectrophotometer, respectively. The molar extinction coefficient was obtained from HPLC-purified compounds using Beer's law at 0.1-1.0 μM concentration. The relative fluorescence quantum yield was determined using the model:

$$\Phi_{F(X)} = (A_s/A_x) (F_x/F_s) (n_x/n_s)^2 \Phi_{F(s)}$$

where Φ_F is the fluorescence quantum yield, A is the absorbance, F is the area under the emission curve, and n is the refractive index of the solvents used in the measurement.¹ The subscripts s and x represent the standard and the unknown, respectively. Cy5 was used as a reference standard.²

Synthesis of intermediate (3).

2,3,3-Trimethyl-1-(3-sulfonatopropyl)-3*H*-indoleninium-5-sulfonate (**1**) was prepared according to a literature procedures.³ Indoleninium **1** (1.6 g, 4.2 mmol), 2-(4-pyridyl) malondialdehyde **2**

(283 mg, 1.9 mmol), and sodium acetate (1 g, 12 mmol) were dissolved in 15 mL acetic anhydride and 15 mL acetic acid. The reaction solution was heated at 115 °C for 4 h in a sealed pressure tube. The crude product was then precipitated by the addition of ethyl acetate (80 mL) and washed with additional ethyl acetate (50 mL $\times$ 2). Preparative HPLC (Phenomenex Luna C18 250 $\times$ 21.2 mm column, 20–40% buffer B over 40 min at a flow rate of 20 mL/min) was used to purify intermediate **3** (1.47 g, 68% yield) giving the blue dye as the triethylammonium salt. ^1H NMR (400 MHz, D_2O): δ 8.79 (d, 2H, J = 6.4 Hz), 8.19 (d, 2H, J = 14.4 Hz), 7.83 (s, 2H), 7.76 (m, 4H), 7.30 (d, 2H, J = 8.8 Hz), 5.70 (d, 2H, J = 14.0 Hz), 3.97 (t, 4H, J = 8.4 Hz), 3.11 (q, 18H, J = 7.2 Hz), 2.75 (t, 4H, J = 7.2 Hz), 1.98 (m, 4H), 1.69 (s, 12H), 1.19 (t, 27H, J = 7.2 Hz). ESI-MS $[\text{M}+\text{H}]^+$ m/z calcd. for 836.16, found 836.23.

Synthesis of CyP1-5 acid (**4a**).

Intermediate **3** (200 mg, 0.17 mmol), 6-bromohexanoic acid (663 mg, 3.4 mmol), and potassium iodide (56 mg, 0.34 mmol) were dissolved in 10 mL NMP. The reaction solution was heated at 115 °C for 5 days in a sealed pressure tube. The crude product was then precipitated by the addition of ethyl acetate (40 mL) and washed with additional ethyl acetate (20 mL $\times$ 2). Preparative HPLC (Phenomenex Luna C18 250 $\times$ 21.2 mm column, 10–40% buffer B over 40 min at a flow rate of 20 mL/min) was used to purify CyP1-5 acid **4a** (182 mg, 90% yield) giving the blue dye as the triethylammonium salt. ^1H NMR (400 MHz, D_2O): δ 8.85 (d, 2H, J = 6.8 Hz), 8.15 (d, 2H, J = 14.4 Hz), 7.93 (d, 2H, J = 6.8 Hz), 7.80 (s, 2H), 7.72 (d, 2H, J = 8.4 Hz), 7.24 (d, 2H, J = 8.4 Hz), 5.72 (d, 2H, J = 14.4 Hz), 4.59 (t, 2H, J = 7.2 Hz), 3.97 (t, 4H, J = 7.6 Hz), 3.09 (q, 24H, J = 7.2 Hz), 2.75 (t, 4H, J = 6.4 Hz), 2.15 (m, 4H), 1.92 (m, 4H), 1.66 (s, 12H), 1.63 (m, 2H), 1.44 (m, 2H), 1.16 (t, 36H, J = 7.2 Hz). ESI-MS $[\text{M}+\text{H}]^+$ m/z calcd. for 950.23, found 950.31.

Synthesis of CyP2-5 acid (**4b**).

Intermediate **3** (200 mg, 0.17 mmol), 4-(bromomethyl)benzoic acid (731 mg, 3.4 mmol), and potassium iodide (56 mg, 0.34 mmol) were dissolved in 10 mL NMP. The reaction solution was heated at 115 °C for 24 h in a sealed pressure tube. The crude product was then precipitated by the addition of ethyl acetate (40 mL) and washed with additional ethyl acetate (20 mL $\times$ 2). Preparative HPLC (Phenomenex Luna C18 250 $\times$ 21.2 mm column, 10–40% buffer B over 40 min at a flow rate of 20 mL/min) was used to purify CyP2-5 acid **4b** (187 mg, 91% yield) giving the blue dye as the triethylammonium salt. ^1H NMR (400 MHz, D_2O): δ 8.84 (m, 2H), 8.14 (dd, 2H, J = 4.4 Hz, 14.4 Hz), 7.95 (m, 4H), 7.81 (s, 2H), 7.74 (d, 2H, J = 8.8 Hz), 7.66 (d, 2H, J = 8.0 Hz), 7.27 (m, 2H), 5.86 (s, 2H), 5.73 (d, 2H, J = 14.4 Hz), 3.98 (t, 4H, J = 7.6 Hz), 3.10 (q, 24H, J = 7.2 Hz), 2.76 (t, 4H, J = 6.4 Hz), 1.94 (m, 4H), 1.66 (s, 12H), 1.16 (t, 36H, J = 7.2 Hz). ESI-MS $[\text{M}+\text{H}]^+ m/z$ calcd. for 970.20, found 970.29.

Synthesis of CyP1-5 succinimidyl ester (**5a**).

CyP1-5 acid **4a** (25 mg, 0.02 mmol), O-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU, 10 mg, 0.03 mmol), and Et_3N (10 μL , 0.07 mmol) were dissolved in 3 mL dry DMF. The reaction mixture was stirred at room temperature for 1 h under argon. The mixture was then precipitated by the addition of ethyl acetate (20 mL) and washed with ethyl

acetate (20 mL), acetone (20 mL), ether (20 mL), and dried under vacuum to afford CyP1-5 succinimidyl ester **5a** as a blue solid (24 mg, 90% yield). ^1H NMR (400 MHz, DMSO- d_6): δ 8.99 (d, 2H, $J = 6.8$ Hz), 8.50 (d, 2H, $J = 14.4$ Hz), 7.90 (d, 2H, $J = 6.8$ Hz), 7.85 (s, 2H), 7.62 (d, 2H, $J = 8.4$ Hz), 7.39 (d, 2H, $J = 8.4$ Hz), 5.89 (d, 2H, $J = 14.4$ Hz), 4.58 (t, 2H, $J = 7.2$ Hz), 4.13 (t, 4H, $J = 7.6$ Hz), 3.09 (q, 6H, $J = 7.2$ Hz), 2.89 (s, 4H), 2.81 (t, 4H, $J = 6.4$ Hz), 2.47 (m, 4H), 2.22 (m, 2H), 1.80 (m, 4H), 1.76 (s, 12H), 1.55 (m, 2H), 1.18 (t, 9H, $J = 7.2$ Hz). ESI-MS $[\text{M}+\text{H}]^+$ m/z calcd. for 1047.25, found 1047.44.

Synthesis of CyP2-5 succinimidyl ester (**5b**).

CyP2-5 acid **4b** (20 mg, 0.017 mmol), O-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU, 10 mg, 0.03 mmol), and Et₃N (10 μL , 0.07 mmol) were dissolved in 3 mL dry DMF. The reaction mixture was stirred at room temperature for 1 h under argon. The mixture was then precipitated by the addition of ethyl acetate (20 mL) and washed with ethyl acetate (20 mL), acetone (20 mL), ether (20 mL), and dried under vacuum to afford CyP2-5 succinimidyl ester **5b** as a blue solid (18 mg, 86% yield). ^1H NMR (400 MHz, DMSO- d_6): δ 8.94 (d, 2H, $J = 6.8$ Hz), 8.50 (d, 2H, $J = 14.4$ Hz), 8.21 (d, 2H, $J = 8.0$ Hz), 8.13 (d, 2H, $J = 8.0$ Hz), 7.95 (s, 2H), 7.85 (s, 2H), 7.63 (dd, 2H, $J = 1.6$ Hz, 8.4 Hz), 7.39 (d, 2H, $J = 8.4$ Hz), 6.06 (s, 2H), 5.93 (d, 2H, $J = 14.4$ Hz), 4.14 (t, 4H, $J = 7.6$ Hz), 3.10 (q, 12H, $J = 7.2$ Hz), 2.89 (s, 4H), 2.73 (t, 4H, $J = 6.4$ Hz), 1.82 (m, 4H), 1.76 (s, 12H), 1.17 (t, 18H, $J = 7.2$ Hz). ESI-MS $[\text{M}+\text{H}]^+$ m/z calcd. for 1067.22, found 1067.41.

Optical Properties

Absorption spectra and extinction coefficients of the fluorophores were obtained on a PerkinElmer Lambda 35 UV–visible spectrophotometer. Extinction coefficients were measured in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4), and were averaged from three sets of parallel experiments. For each trial, 2~3 mg of the HPLC-purified dyes were weighed on a Mettler Toledo analytical balance with an error of ± 0.01 mg and were dissolved in deionized water using a 10 mL volumetric flask to prepare the stock solutions. Emission spectra were collected on a PerkinElmer LS 55 fluorescence spectrophotometer. Quantum yields were measured for each dye derivative with the maximum absorption for each sample less than 0.1, using Cy5 as a standard ($\Phi = 0.27$).² Photostability data were collected on a Nikon Eclipse E400 spectrofluorometer equipped with a Nikon mercury lamp. Dye photo-bleaching was observed by monitoring the fluorescence emission of absorbance-matched dye samples (absorbance ~ 0.03) at an excitation wavelength 630 nm for 3 min.

General Antibody Labeling Procedures

Stock solutions of the sulfoindocyanine succinimidyl esters were made in dry DMSO at 10 mg/mL concentration. Antibody was dissolved in 0.1 M NaHCO₃ buffer at 2 mg/mL concentration. The desired amount of dye was calculated as follows:

$$\text{mL of 10 mg/mL dye} = \frac{\text{mg protein}}{\text{Mw of protein}} \times \text{MR} \times \frac{\text{Mw of dye}}{10}$$

where MR is the molar ratio of dye to protein. For example, using 2 mg of Goat anti-mouse IgG (Mw 145,000) and a 10:1 molar ratio of dye **5a** (Mw 1,249) to protein gives:

$$\frac{2}{145,000} \times 10 \times \frac{1249}{10} = 0.017 \text{ mL of 10 mg/mL dye } \mathbf{5a}$$

The desired amount of dye was added to the above antibody solution and incubated at room temperature for 1 h with gentle stirring. Free dye was separated from the labeled antibody by gel permeation chromatography (Bio-Rad BioGel P-30 resin) using PBS buffer as eluent.

Determination of the Degree of Labeling

A simple method for estimating the degree of labeling (DOL) is determined by direct measuring the protein absorbance at 280 nm and dye absorbance at the absorption maximum. The protein concentration is calculated as follows:

$$\text{Protein concentration (M)} = \frac{A_{280} - XA_{\text{dye}}}{\epsilon_{\text{protein}}}$$

where the molar extinction coefficient (ϵ) of a typical IgG at 280 nm is 203,000 $\text{cm}^{-1}\text{M}^{-1}$. The absorbance of dyes **5a** and **5b** is measured at 638 nm, and the correction factor (X) for the dyes **5a** and **5b** is about 0.04.

The degree of labeling (DOL) is calculated as follows:

$$\text{DOL} = \frac{A_{\text{dye}} \epsilon_{\text{protein}}}{(A_{280} - XA_{\text{dye}}) \epsilon_{\text{dye}}}$$

Fluorescence Labeling and Cell Imaging

Bovine Pulmonary Artery Endothelial (BPAEC) cells in Dulbecco's modified eagle medium (DMEM) supplemented with 20% fetal bovine serum (FBS) were cultured on glass coverslips for 24 h to reach 50%-70% confluency. The cells were fixed with 4% formaldehyde in PBS for 15 min at 37 °C, then washed with PBS (2X). The cells were permeabilized with 0.2% Triton-X in PBS for 5 min at room temperature, then washed with PBS (3X). The cells were blocked with blocking solution (5% normal goat serum (NGS)/0.2% BSA in PBS) for 30 min at room temperature. Then, the cells were incubated with mouse anti-tubulin primary antibody (2 $\mu\text{g}/\text{mL}$ in blocking solution) at room temperature for 1 h. The cells were washed with PBS (3X), followed by incubating with dye-labeled goat anti-mouse secondary IgG antibody (10 $\mu\text{g}/\text{mL}$ in blocking solution) at room temperature for 30 min. The cells were washed again with PBS (3X), and incubated with the nuclear stain 4',6-diamidino-2-phenylindole (0.2 $\mu\text{g}/\text{mL}$ DAPI) in PBS for 2 min. The cells were washed with PBS (3X), eH_2O (1X), and 0.1% Tween-20 solution (1X). After the coverslips were air-dried, the coverslips were mounted in Cytoseal onto microscopy slides. Fluorescent images were captured on a Zeiss Axioskop 2 fluorescence microscope with 40X objective equipped with a Hamamatsu ORCA-ER CCD camera using excitation and

emission filters from Omega Optical. The nuclear stain DAPI was imaged using a 365 ± 5 nm band-pass filter for excitation and a 400 ± 5 nm cutoff filter for emission. The NIR fluorophore was imaged using a 610 ± 20 nm band-pass filter for excitation and a 650 ± 20 nm band-pass filter for emission. Exposure times of 100 and 200 ms, for the nuclear stain and carbocyanine dye channels, respectively, were used for image collection. All images from the carbocyanine dye channel were processed with identical leveling.

HPLC profiles and ^1H NMR Spectra

Cell Imaging and Fluorescence Intensity

Fluorescence microscopy of BPAEC cells incubated fluorophore-labeled antibody. Cells incubated with **5a**-labeled antibody (A), **5b**-labeled antibody (B), and Alexa Fluor 647-labeled antibody (C). Imaging in the bottom is a close up view of representative cells. Fluorescence signal from the nuclear stain, DAPI, colored in blue, NIR fluorophores colored in red.

Fluorescence relative intensity of BPAEC cells labeled with **5a**-GAM IgG, **5b**-GAM IgG, and AF647-GAM IgG, respectively. The mean intensity was averaged based on five different images.

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