# A cyanine based fluorophore emitting both single photon near-infrared fluorescence and two-photon deep red fluorescence in aqueous solution

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## **Materials and General Experimental Methods**

All starting materials for the dye synthesis were obtained from Aladdin Reagent (Shanghai, China) unless otherwise specified. 2,3,3-trimethyl-3H-indolium, 5-Cl-2,3,3-trimethyl-3H-indolium, and 2,3,3,5-tetramethyl-3H-indolium were purchased from Beijing Chengyu Specialty Chemical Co., Ltd (Beijing, China). Column chromatography was performed on silica gel (300-400 mesh). NMR spectra (<sup>1</sup>H-400 MHz and <sup>13</sup>C-100 MHz) were recorded on a Varian Mercury 400 spectrometer instrument using tetramethy silane as the internal reference. The high resolution electron spray ionization (HR-ESI) mass spectra were obtained on a Micromass Q-TOF 2 mass spectrometer with water as the carrier solvent. UV-Vis absorption spectra were collected on a SHIMADZU UV-2550 spectrophotometer. The emission spectra were performed on a SHIMAZDU RF-5301PC spectrofluorophotometer (Kyoto, Japan) equipped with a photomultiplier tube. Sing-photon fluorescence microscopic images were obtained from a Leica DMF4000B inverted laser-scanning microscope (Leica Inc., Wetzlar, Germany) equipped with epifluorescence and phase contrast optics using 40× lenses. Single photon near-infrared fluorescence was captured by using a Cy7 filter cube (excitation:  $710 \pm 37.5$  nm and emission:  $810 \pm$ 45 nm). Two photon fluorescence was acquired from a Leica TCS SP5 II confocal fluorescence microscope. The excitation beam at 900 nm, passing through  $40 \times$  oil immersion objective to excite the fluorophore, was produced from a femto-second Ti: sapphire pulsed laser and the red emissions of fluorophores ranged at 580-660 nm were acquired by a PMT channel.

# Synthesis and Characterization

General synthetic procedure of compounds **4a**–**4c**. 2,3,3-trimethylbenzoindole (200 mg, 1.25 mmol), and 2.5 mmol 5-bromopentanoic acid were mixed with 6.0 mL 1,2-dichlorobenzene solution and were heated at 110 °C in an oil bath with refluxing and stirring for 12 h. After cooling to room temperature, the mixture was dropped into methyl ether in an ice bath. The crude product was precipitated and filtered to get pink color powder, which was further purified by washing repeatly with a mixed solution of CH<sub>3</sub>CN and diethyl ether (V:V = 1:1). The yields of compound **4a**–**4c** were measured in a range of 40–60%.

General synthetic procedure of compound **5a–5c**. 2,3,3-trimethylbenzoindole (200 mg, 1.25 mmol), 1,4-Butane sultone (544 mg, 4.0 mmol) and 6.0 mL 1,2-dichlorobenzene were mixed and heated at 110 °C with stirring for 12 h. At the end of reaction, the solution was cooling down and dropped into

methyl ether in an ice bath. After precipitation, the red product was filtered and re-dissolved in 20 mL saturated sodium chloride and was extracted with chloroform ( $3 \times 60$  mL). After that, the water extract was evaporated and the product was dried under vacuum without further purification. The yields of **1a–1c** after chromotography purification were determined in a range of 75–79%.

Preparation of bisaldehydes **6**. Cyclohexanecarboxaldehyde was synthesized according to previous report<sup>1</sup>. A mixture of 40 mL dimethylformamide (0.52 mol) and 40 mL of methylene chloride was chilled in an ice bath for 30 min. 37 mL phosphorus oxychloride (0.41 mol) and cyclohexanone (10 g, 0.10 mol) was added dropwise to above mixture with stirring. The solution was refluxed for 3 h, cooled, poured onto 200 g of ice, and allowed to stand overnight. The yellow solid was collected with a yield of 8.0 g (46.5%).

General preparation of cyanine based fluorophores: *N*-alkylated trimethylbenzoindole (1.0 mmol), sodium acetate (82 mg, 1.0 mmol) and 43 mg (0.25 mmol) of cyclohexanecarboxaldehyde were dissolved in 6 mL acetic anhydride in a flask. The mixture was heated at 70 °C refluxing with constant stirring. After 10 min, another 43 mg (0.25 mmol) of cyclohexanecarboxaldehyde was added into reacting solution. 30 min later, the reaction solution was cooled to room temperature and the mixture was dropped into the methyl ether in an ice bath and rude green powder was filtrated after precipitation. The crude was purified by column chromatography on silica gel with a mixture of  $CH_2Cl_2$  and  $CH_3OH$  as eluent. Removal of solvent under vacuum afforded pure deep green powder.

# Characterization of 1a-1c and 2a-2c.

1b (82.4mg, 21.8%) was obtained from reaction between 5b (309 mg, 1.0 mmol) and



cyclohexanecarboxaldehyde (86 mg, 0.5 mmol). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  = 8.40 (d, *J* = 14.1 Hz, 2H), 7.33 (s, 2H), 7.25 (d, *J* = 8.4 Hz, 4H), 6.29 (d, *J* = 14.1 Hz, 2H), 4.17 (d, *J* = 7.4 Hz, 4H), 2.87 (t, *J* = 7.1 Hz, 4H), 2.74 (s, 4H), 2.41 (s, 6H), 1.93 (s, 10H), 1.71 (s, 12H); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O)  $\delta$  173.78

 $(2 \times C)$ , 150.59 (C, 145.11 (2×C, 142.78 (2×CH) , 141.48 (2×CH) , 136.87 (2×CH) , 130.35 (2×C) , 127.82 (2×C) , 124.11 (2×CH) , 112.08 (2×CH) , 102.20 (2×CH) , 51.78 (2×CH<sub>2</sub>) , 50.54 (2×CH<sub>2</sub>) , 49.84 (2×CH<sub>3</sub>) , 45.04 (2×C) , 28.32 (4×CH<sub>3</sub>) , 27.34 (2×CH<sub>2</sub>) , 23.69

 $(2 \times CH_2)$ , 23.60  $(2 \times CH_2)$ , 21.32  $(CH_2)$ . HRFAB-MS:  $C_{40}H_{51}ClN_2O_6S_2$  [M-H]<sup>-</sup>, found 753.2775 (100.0%), calculated 753.2799.

1c (272 mg, 68.7%) was obtained from reaction between 5c (329 mg, 1.0 mmol) and



cyclohexanecarboxaldehyde (86 mg, 0.5 mmol). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  = 8.43 (d, *J* = 14.1 Hz, 2H), 7.57 (s, 2H), 7.47 – 7.32 (m, 4H), 6.35 (d, *J* = 14.1 Hz, 2H), 4.21 (t, *J* = 7.0 Hz, 4H), 2.89 (t, *J* = 7.0 Hz, 4H), 2.76 (t, *J* = 5.7 Hz, 4H), 2.08–1.85 (m, 10H), 1.74 (s, 12H); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O)  $\delta$ 

173.96(2×C), 151.59 (C) , 145.78 (2×C) , 144.52 (2×CH) , 142.51 (2×CH) , 131.97 (2×CH) , 129.99 (2×C) , 129.06 (2×C) , 124.11 (2×CH) , 113.67 (2×CH) , 102.93 (2×CH) , 51.74 (2×CH<sub>2</sub>) , 50.78 (2×CH<sub>2</sub>) , 45.27 (2×C) , 28.23 (4×CH<sub>3</sub>) , 27.39 (2×CH<sub>2</sub>) , 27.20 (2×CH<sub>2</sub>) , 23.60 (2×CH<sub>2</sub>) , 22.14 (CH<sub>2</sub>) . HRFAB-MS:  $C_{38}H_{45}Cl_{3}N_{2}O_{6}S_{2}$  [M-H]<sup>-</sup>, found 793.1683 (90.0%), calculated 793.1706.

2a (83.8 mg, 25.6%) was obtained from reaction between 4a (260 mg, 1.0 mmol) and



cyclohexanecarboxaldehyde (86 mg, 0.5 mmol). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  = 8.43 (d, *J* = 14.0 Hz, 2H), 7.51 (d, *J* = 7.5 Hz, 2H), 7.45 – 7.33 (m, 4H), 7.27 (t, *J* = 7.3 Hz, 2H), 6.31 (d, *J* = 14.1 Hz, 2H), 4.19 (t, *J* = 7.2 Hz, 4H), 2.74 (d, *J* = 5.9 Hz, 4H), 2.25 (t, *J* = 7.0 Hz, 4H), 2.02 – 1.75 (m, 10H), 1.73 (s, 12H); <sup>13</sup>C

NMR (101 MHz, D<sub>2</sub>O): 174.15 (2×C), 151.04 (C), 145.54 (2×C), 143.66 (2×CH), 142.60 (2×CH), 129.89 (2×CH), 128.06 (2×C), 126.44 (2×C), 123.43 (2×CH), 112.41 (2×CH), 102.38 (2 × CH), 50.63 (2×CH<sub>2</sub>), 45.20 (2×C), 38.17 (2×CH<sub>2</sub>), 28.30 (4×CH<sub>3</sub>), 27.35 (4×CH<sub>2</sub>), 24.82 (2×CH<sub>2</sub>), 22.16 (CH<sub>2</sub>). HRFAB-MS:  $C_{40}H_{47}CIN_2O_4$  [M-H]<sup>-</sup>, found 653.3144 (100%), calculated 653.3146.

2b (62.5 mg, 18.3%) was obtained from reaction between 4b (274 mg, 1.0 mmol) and



cyclohexanecarboxaldehyde (86 mg, 0.5 mmol). <sup>1</sup>H NMR (400 MHz, DMSO)  $\delta = 8.40$  (d, J = 14.2 Hz, 2H), 7.34 (s, 2H), 7.24 (s, 4H), 6.27 (d, J = 14.2 Hz, 2H), 4.16 (t, J = 7.1 Hz, 4H), 2.73 (t, J = 5.9 Hz, 4H), 2.37 (d, J = 36.3 Hz, 6H), 2.25 (t, J = 7.1 Hz, 4H), 2.06–1.75 (m, 10H), 1.72 (d, J = 7.6 Hz, 12H);

<sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ 181.90 (C), 173.74(2×C), 150.50(C), 145.03(2×C), 142.80(2×CH), 141.53(2×CH), 136.84(2×CH), 130.32(2×C), 127.59(2×C), 124.08(2×CH), 112.12(2×CH), 102.11(2×CH), 50.53(2×CH<sub>2</sub>), 49.84(2×CH<sub>3</sub>), 45.24(2×C), 38.51(2×CH<sub>2</sub>), 28.38(4×CH<sub>2</sub>), 28.29(2×CH<sub>2</sub>), 27.35(2×CH<sub>2</sub>), 24.96(2×CH<sub>2</sub>), 21.32(CH<sub>2</sub>). HRFAB-MS:  $C_{42}H_{51}CIN_2O_4$  [M-H]<sup>-</sup>, found 681.3453 (100%), calculated 681.3459.

2c (132 mg, 36.4%) was obtained from reaction between 4c (294 mg, 1.0 mmol) and



from reaction between **4c** (294 mg, 1.0 mmol) and cyclohexanecarboxaldehyde (86 mg, 0.5 mmol). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta = 8.40$  (d, J = 14.3 Hz, 2H), 7.57 (s, 2H), 7.38 (dd, J = 26.8 Hz, 8.5, 4H), 6.31 (d, J = 14.1 Hz, 2H), 4.17 (s, 4H), 2.74 (s, 4H), 2.24 (t, J = 6.8 Hz, 4H), 2.02–1.79 (m, 10H), 1.73 (s, 12H); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O)  $\delta$  173.89(2×C), 151.46(C), 145.65(2×C),

144.48(2×CH), 142.49(2×CH), 131.90(2×CH), 129.91(2×C), 128.79(2×C), 124.02(2×CH), 113.64(2×CH), 102.77(2×CH), 50.73(2×CH<sub>2</sub>), 45.43(2×C), 38.45(2×CH<sub>2</sub>), 28.21(2×CH<sub>2</sub>), 28.16(4×CH<sub>3</sub>), 27.34(2×CH<sub>2</sub>), 24.88(2×CH<sub>2</sub>), 22.11(CH<sub>2</sub>). HRFAB-MS:  $C_{40}H_{45}Cl_3N_2O_4$  [M-H]<sup>-</sup>, found 724.2402 (100%), calculated 721.2367.

# **Photospectroscopic Studies**

**Absorption spectra.** All absorption spectra were recorded on a SHIMADZU UV-2550 spectrophotometer in a quartz cuvette ( $10 \times 10$  mm) at 25 °C. Stock solutions of compound **1a–1c**, **2a–2c**, and ICG were prepared and equilibrated at 25 °C. Working solutions in a range of 0.25–10 µM were prepared by diluting the stock solution with PBS (pH = 7.4). Maximum absorbance wavelength and corresponding molar extinction coefficient were determined for each compound at the concentration of 1.0 µM. All absorption spectra were recorded from 400 nm to 900 nm.

**SP Emission spectra**. All SP emission spectra were performed on a SHIMAZDU RF-5301PC spectrofluorophotometer equipped with a photomultiplier tube at 25 °C. Fluorescence emission spectra of all samples (1.0  $\mu$ M) were collected at a 90-degree angle relative to the excitation light path. All fluorophores were excited at 765 nm and the emission spectra were recorded from 780 nm to 900 nm.



Figure S1. Absorption (A) and SP fluorescence (B) spectra of 1a, 1b and 1c (1.0  $\mu$ M) with different substituents at 5-position of indole ring in PBS (pH 7.4).

**Quantum yield measurements**: We determined the fluorescence quantum yield according to established techniques<sup>2</sup>, which measure the efficiency of photon energy transfer. The fluorescence quantum yields of **1a–1c**, **2a–2c**, and ICG in PBS pH = 7.4 were referenced to ICG in PBS solution (Q. Y. = 0.041)<sup>3</sup>. The absorbance of five serial dilutions of each compound was measured in the concentration range between  $0.25-1.5 \mu$ M, which were excited at the optimal excitation wavelength. The fluorescence intensity of each fully corrected fluorescence spectrum was integrated. The integrated fluorescence intensity was plotted against the absorbance maximum (Figure S3), and fitted with a straight line. The quantum yield was determined according to Equation 1:

$$\phi_{s} = \phi_{r} \left( \frac{Grad_{s}}{Grad_{r}} \right) \left( \frac{\eta_{s}^{2}}{\eta_{r}^{2}} \right) \left( \frac{q_{s}}{q_{r}} \right) \quad \text{Equation 1}$$

In Equation 1, subscripts s and r refer to sample and reference, respectively.  $\phi$  represents the fluorescence quantum yield. *Grad* represents the gradient from the plot of integrated fluorescence intensity *vs* absorbance.  $\eta$  represents the refractive index of the solvent, and q represents a correction factor accounting for the excitation wavelength used. We assumed  $q_s/q_r$  to be 1 since all spectra were recorded with very similar excitation wavelengths  $\lambda_{exc}$ . This method typically provides an estimate of the fluorescence quantum yield lying within an error of 10%.



Figure S2. Integrated fluorescence vs. absorbance plots were used to determine relative quantum yields of compound 1a (A), 1b (B), 1c (C), 2a (D), 2b (E), 2C (F). ICG in PBS was used as a standard (G).

#### TP induced fluorescence spectra and TP absorption cross section ( $\sigma_{TP}$ )

To obtain TP induced fluorescence spectra of all the fluorophores, a femtosecond mode-locked Ti:Sapphire laser system (output beam~150 fs duration and 1 kHz repetition rate) was used. All the fluorophores ( $10^{-4}$  M) were prepared in PBS, and R6G with the same concentration in methanol was taken as reference. The excitation light (tuned at 900 or 950 nm) was focused to spot size of 50  $\mu$ m onto the fluorophore containing cuvette. The input power of the incident light was measured by a power meter and the emission light were collected with a backscattering configuration and monitored by a PMT.

Xu and Webb<sup>4</sup> reported a standard protocol to determine TP absorption cross section ( $\sigma_{TP}$ ) based on two-photon induced fluorescence (TPIF) method. For details,  $\sigma_{TP}$  of the reference and samples are given by

$$\frac{\sigma_{TP}^{s} \cdot \Phi_{TP}^{s}}{\sigma_{TP}^{R} \cdot \Phi_{TP}^{R}} = \frac{C^{R} \cdot n^{s} \cdot F^{s}(\lambda)}{C^{s} \cdot n^{R} \cdot F^{R}(\lambda)}$$

Where  $\sigma_{TP}$  is the TP absorption cross section at wavelength of  $\lambda$ ,  $\Phi_{TP}$  is the quantum yield of TP induced fluorescence (identical to the SP quantum yield measured at  $\lambda/2$  excitation), *C* is the concentration, *n* is the refractive index of the solution, *F*( $\lambda$ ) is the integration area of TP induced fluorescence spectra. R6G was used as a reference with known QY and TP absorption cross section values at 900 and 950 nm.<sup>4b</sup>



**Figure S3**. The correlation curve of two photon induced fluorescence intensity of **2a** (10<sup>-4</sup> M,  $\lambda_{ex}$ = 950 nm) *vs*. input excitation power.

#### In Vitro Studies

**Cell culture.** Human epithelial cervical cancer HeLa cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown as mono-layers in 75-cm<sup>2</sup> flasks containing Minimum Essential Medium, Alpha 1X (MEM, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA) in a fully-humidified incubator containing 5% CO<sub>2</sub> at 37 °C. Cells were harvested when they reached 80% confluence to maintain exponential growth.

**Cytotoxicity studies.** The MTT cell proliferation assay was applied to determine the viabilities of the cells treated with fluorophore **1a** and **2a**. A cell monolayer in exponential growth was harvested using 0.25% trypsin, and a single-cell suspension was obtained. Cell suspensions containing cell density of  $2 \times 10^3$  cells/well in 100 µL cell culture medium were added to 96-well plates by serial dilutions. Eight replicates were prepared under the same condition. 24 h after the cell attachment, the cells were treated with selected fluorophore that was sterile filtered through MILLEX<sup>®</sup>-HV 0.22 µm syringe filter with final concentrations in a range of 0.05–10 µM. After incubation for 4 days at 37 °C in 5% CO<sub>2</sub>, cells were washed with PBS, and the cell viabilities were measured by MTT assay as we reported previously<sup>5</sup>. The cell viabilities after the treatment of fluorophores were normalized to the value without any treatments (Figure S4).



Figure S4. Concentration dependent cell viabibilities of in HeLa cells after incubation with 1a or 2a for 4 days.

#### SP fluorescence microscopic imaging

To avoid the artifacts that occur during fixation procedures, all the experiments were conducted in live HeLa cells. Cells  $(2 \times 10^4)$  cultured on 35 mm glass bottom culture dishes (14 mm microwell, MatTek, Ashland, MA) to approximately 50% confluence were added with 1.0 µM nanoprobe in 2 mL media supplemented with 10% FBS for seletcted period at 4 or 37 °C. At the end of incubation, the cells were washed with Hanks Balance Salt Solution (HBSS) 3× prior to addition of 1 mL phenol red free media, and the cells were immediately imaged by fluorescence microscopy.

# Determining the subcellular location of fluorophore in cell culture

Human epithelial cervical cancer HeLa cells were seeded on 35-mm glassbottom culture dishes (14mm microwell; MatTek, Ashland, MA). After reaching 70% to 80% confluence, culture media containing 0.1 mg/mL FITC labeled dextran with a molecular weight of 10 kDa (Molecular Probes, Eugene, OR) was added and incubated for 4 hours. Cells were washed three times with HBSS and continually incubated in normal culture media for 16 hours to achieve lysosome labeling from dextran through the endocytic pathway. After washing three times with HBSS, media with 1.0 µM fluorophore was added, and incubated for another 8 to 24 hours. After washing twice with HBSS, phenol red–free culture media was added to the cells before microscopy studies.

#### Live cell TP confocal microscopic imaging studies

HeLa cells were seeded onto sterile glass coverslips in 35-mm tissue culture dish (20,000 cells per dish) and incubated in 2 mL MEM at 37 °C for 24 h. The culture medium was then replaced by 2 mL fluorophore (100  $\mu$ M) containing MEM for another 24 h incubation. Cells in culture medium with no samples were used as controls. After washing with PBS for three times, cells-adhered coverslips were mounted onto slides and immersed in CO<sub>2</sub>-independent medium for imaging experiments. The excitation beam at 900 nm, passing through 40× oil immersion objective to excite the cells, was produced from a femto-second Ti: sapphire pulsed laser and the red emissions of fluorophores ranged at 580–660 nm were acquired by a PMT channel.

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# **Supplemental Spectra**













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