**Electronic Supplementary Information**

# NIR Fluorescent Biotinylated Cyanine Dye: Optical Properties and Combination with Quantum Dots as Potential Sensing Device

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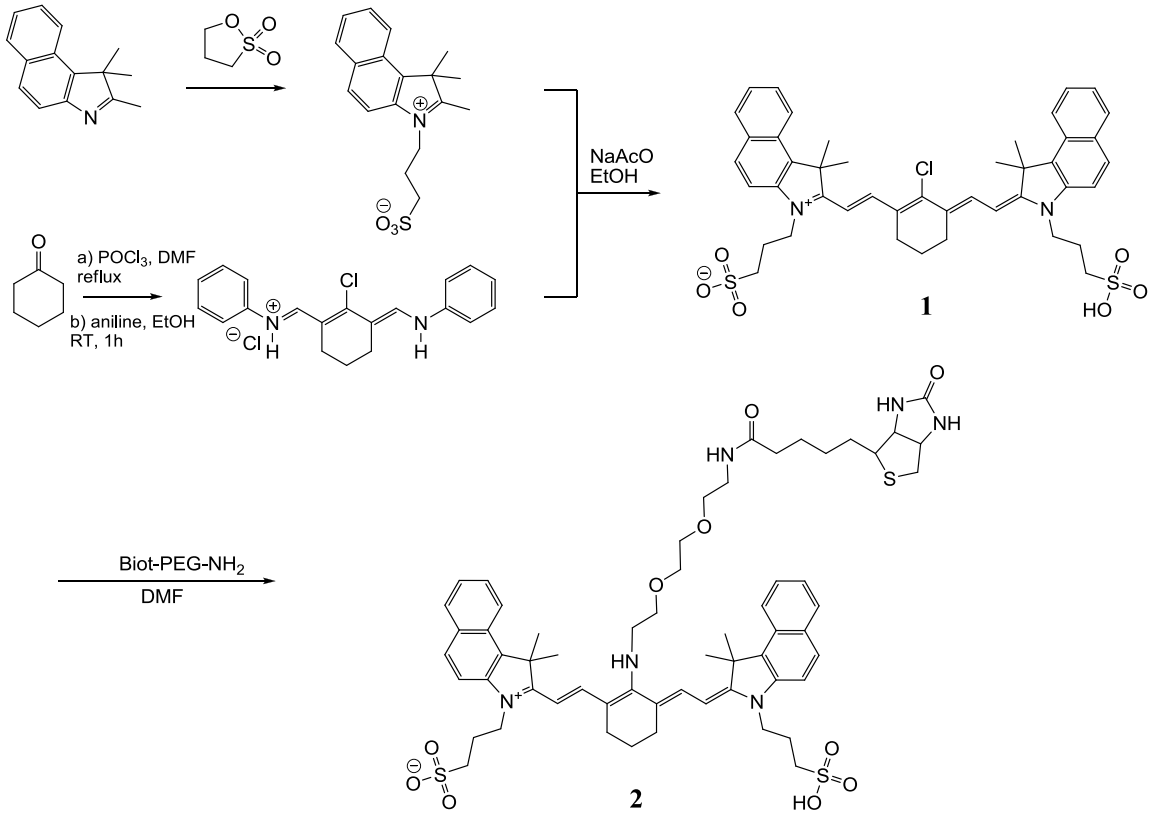
## Materials

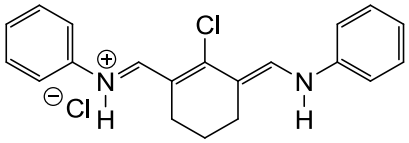
All starting materials for the dye synthesis were purchased from Sigma-Aldrich and were used as received. Quantum dots were purchased from Invitrogen as 1μM and 2μM solutions for PEG and ITK QDs, respectively. Thin layer chromatography (TLC) was performed on aluminum-backed plates with silica gel 60 with F254 indicator. $^{1}$H-NMR spectra were measured with a Varian Bruker AC-200 and Bruker Avance II-500 spectrometers. Mass spectra were measured with a Bruker Daltonik microTOF mass spectrometer (ESI+, ESI-).

**Synthesis and Characterization of CyBiot**

The synthesis of compound **2** (CyBiot) was performed according to Scheme S1.

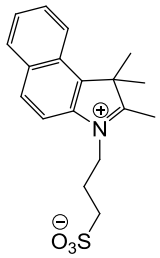



**Scheme S1** Synthesis of compounds **1** and **2**



**N-[5-Anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadiene-1-ylidene]anilinium Chloride.** At 0 °C, phosphorus oxychloride (2 mL, 22 mmol) was added dropwise from a pressure-equalizing addition funnel to anhydrous DMF (2.4 mL, 31 mmol). After 30 min, cyclohexanone (1 mL, 9.6 mmol) was added and the mixture was refluxed for 1 h. Next, an aniline/EtOH [1:1 (v/v), 3.3 mL] mixture was added dropwise with constant cooling at 20 °C. Reaction was continued for an additional 30 min after aniline addition, and then the deep purple mixture was poured into ice cold

$H_2O$/concentrated HCl (10:1, 20 mL). Crystals were allowed to form for 2 h in an ice bath, then filtered, washed with cold $H_2O$ and $Et_2O$, and then dried in vacuo: yield 2.95 g (84%).
$^1$H NMR (200 MHz, MeOD-d4) δ 8.68 (s, 2H), 7.58 – 7.17 (m, 10H), 2.74 (t, J = 6.2 Hz, 4H), 2.11 – 1.75 (m, 2H). HRMS (ESI+): calcd ($M+H^+$) 323.1310; found, 323.1308.



**1,1,2-Trimethyl-3-(3-sulfopropyl)- 1H-benzo[e]indol-3-ium-, Inner Salt**. Toluene (5 mL), 1,1,2-trimethylbenzo[e]indol (1.3 g, 0.062 mol), and 1,3-propane sultone (1.1 mL, 0.093 mol) were heated under reflux for 18 h. The reaction mixture was allowed to cool to room temperature. The resulting bluish crystals were filtered and washed with ether (3 x 10 mL). The filtered product was crystalized from MeOH and ether. The crystals were collected and dried in vacuo to yield 1.68 g (82%). $^1$H NMR (300 MHz, MeOD-d4) δ 8.30 (d, J = 8.4 Hz, 1H), 8.19 (d, J = 9.0 Hz, 1H), 8.11 (d, J = 8.9 Hz, 2H), 7.78 (t, J = 7.1 Hz, 1H), 7.68 (t, J = 7.5 Hz, 1H).
HRMS (ESI+): calcd ($M+H^+$) 332.1315; found, 332.1315.

**Compound 1**: A solution of 1,1,2-trimethyl-3-(3-sulfopropyl)- 1H-benzo[e]indol-3-ium-, inner salt (1.69 g, 6 mmol), N-[5-anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadiene-1-ylidene]anilinium chloride (1.079g, 3 mmol), and anhydrous sodium acetate (600 mg, 7 mmol) in absolute EtOH (60 mL) was heated under reflux for 3.5 h under a $N_2$ atmosphere. The EtOH was removed under reduced pressure, the residue was washed with ether, and the crude was purified by recrystallization from methanol:ether. The crystals obtained were filtered and dried under vacuum, yielding a pure crystalline material (1.35 g, 93%).
$^1$H NMR (500 MHz, DMSO) δ 8.32 (d, J = 14.1 Hz, 2H), 8.25 (d, J = 8.5 Hz, 2H), 8.05 (d, J = 8.9 Hz, 2H), 8.01 (d, J = 8.3 Hz, 2H), 7.82 (d, J = 9.0 Hz, 2H), 7.60 (td, J = 6.8, 1.2 Hz, 2H), 7.47 (td, J = 6.8, 1.2 Hz, 2H), 6.52 (d, J = 14.3 Hz, 2H), 4.47 (t, 4H), 2.74 (t, J = 5.6 Hz, 4H), 2.60 (t, J = 6.7 Hz, 4H), 2.05 (quin, J = 7.2 Hz, 4H), 1.90 (s, 12H), 1.82 (quin, J = 5.8 Hz, 3H).
HRMS (ESI-): calcd 797.2486; found, 797.2493.

**Compound 2**: Compound **1** (27 mg, 0.03 mmol) was dissolved in 1 mL of anhydrous DMF, then triethylamine was added (32 μL, 0.24 mmol) and (+)-N-biotinyl-3,6-dioxaoctanodiamine ($NH_2$-

$PEG_2$-Biotin, Pierce). The resulting solution was stirred for 7 h protected from light. The solvent was removed in vacuo, and then purified on Sephadex LH-20 column using methanol as eluent to afford the desired product (11 mg, 55% Rend).

$^1$H NMR (500 MHz, MeOD) δ: 8.15 (d, J = 8.6 Hz, 2H), 7.97 (d, J = 9.7 Hz, 2H), 7.92 (d, J = 9.0 Hz, 6H), 7.92 (d, J = 8.0 Hz, 6H), 7.56 (t, J = 7.2 Hz, 4H), 7.54 (d, J = 8.9 Hz, 3H), 7.38 (t, J = 7.6 Hz, 2H), 6.03 (d, J = 9.8 Hz, 2H), 4.40 (dd, J = 7.8, 4.9 Hz, 1H), 4.30 (t, 4H), 4.18 (dd, J = 7.8, 4.5 Hz, 1H), 3.97 (t, J = 4.6 Hz, 2H), 3.86 (t, J = 4.3 Hz, 2H), 3.78 (t, J = 4.3 Hz, 2H), 3.71 (t, J = 4.7 Hz, 2H), 3.56 (t, J = 5.7 Hz, 2H), 3.38 – 3.33 (m, 3H), 3.04 (dd, J = 9.4, 5.0 Hz, 1H), 3.00 (t, J = 6.9 Hz, 5H), 2.81 (dd, J = 12.7, 5.0 Hz, 1H), 2.62 (t, J = 6.0 Hz, 5H), 2.28 (quin, J = 14.4, 7.0 Hz, 5H), 2.14 (t, J = 7.5 Hz, 2H), 1.98 (s, 13H), 1.90 – 1.79 (m, 3H), 1.68 – 1.61 (m, 1H), 1.61 – 1.54 (m, 2H), 1.53 – 1.43 (m, 2H). HRMS (ESI+) Calcd, 1137.4858; Found, 1137.4837. $\lambda_{max}$ 660 nm (33600 $M^{-1}cm^{-1}$), fluorescence quantum yield (QY) 0.05 (methanol, reference Alexa 647, QY 0.33 in PBS[1,2])

## Photophysical Data and Evaluations

### Steady-state measurements

Fluorescence measurements were performed on a Cary (Varian) Eclipse fluorometer. Absorption spectra were measured on a Cary (Varian) 50 spectrophotometer.

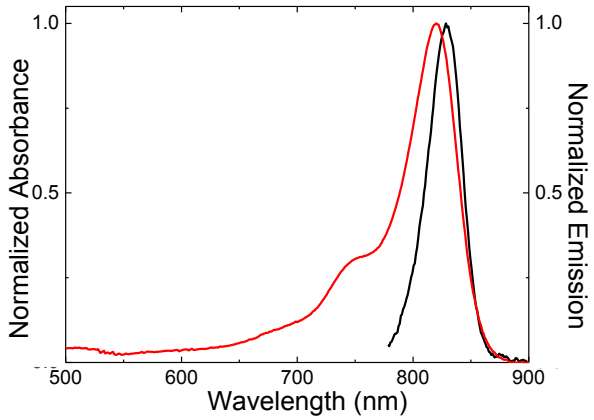



**Figure S2** Normalized absorption(red line) and emission (black line, $\lambda_{Exc}$: 750nm) spectra of compound **1** in methanol.

**Determination of the pKa of compound CyBiot**

To determine the pKa of CyBiot, the fluorescence emission of 20 µM solutions in buffer HEPES pH 2.3-11.2 were measured and the data from $I_{785}$ vs pH were fitted to calculate the value of pKa.

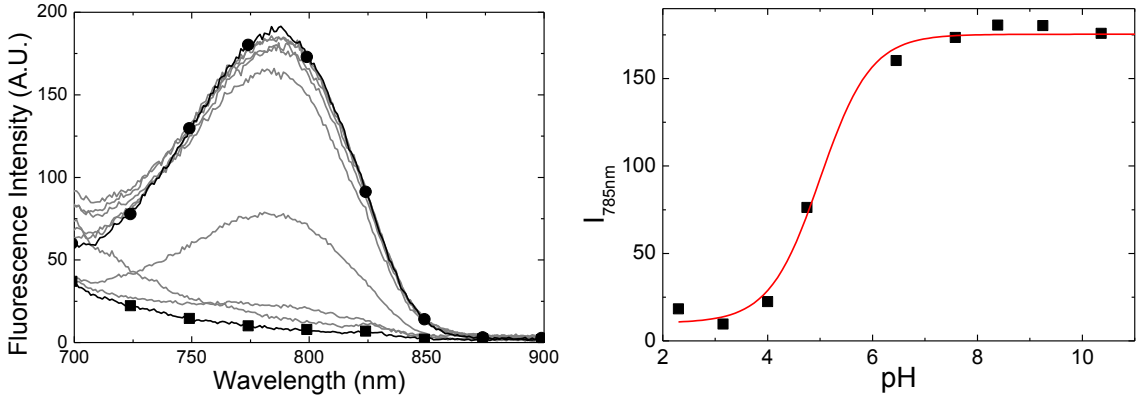



**Figure S3** Left panel. Emission spectra ($\lambda_{Exc}$: 650nm) of CyBiot 20 µM in buffer HEPES at different pH, from 11.2 (●) to 2.3 (■). Right Panel. Changes in the fluorescence intensity at 786 nm as a function of the buffer pH (calculated pKa: 5.0±0.1).

**Fluorescence quenching in the presence of streptavidin**

All the experiments were done in PBS buffer pH = 7.4, prepared with MilliQ water.

300 µL of streptavidin 1 µM were titrated with stocks solutions of CyBiot (250 µM) by adding aliquots of 1 µL, at time intervals of 2-3 minutes. The fluorescence signal was corrected by the dilution factor at each point. Control experiments were performed in the same conditions, in absence of the protein.

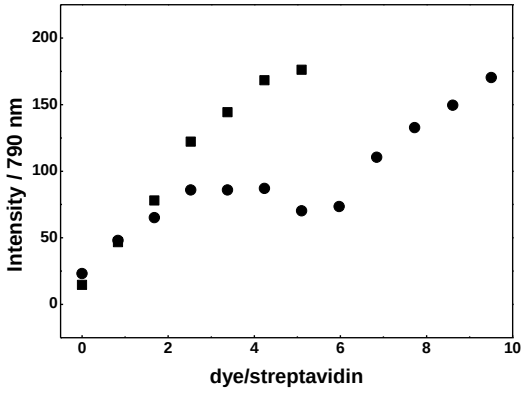



**Figure S4** Quenching of the emission of CyBiot upon binding to SAv. (●) with protein, (■) without the protein.

**QD stability in PBS buffer**

The stability of the fluorescent nanoparticles in PBS buffer were assessed by measuring the fluorescence emission during the titration time. The results are shown in figure S5.

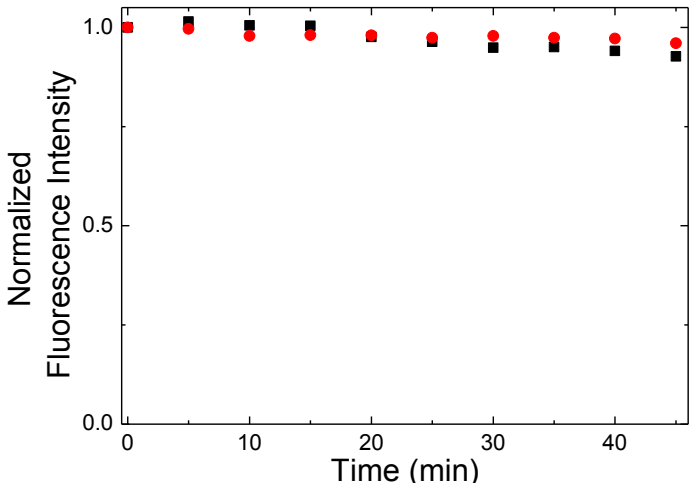



**Figure S5** Fluorescence emission of QDSAv ITK (black) and PEG (red) as a function of time in buffer PBS pH 7.4

**Time-resolved measurements**

Fluorescence lifetimes were determined by TCSPC in a Horiba Jobin Yvon IBH 5000 U apparatus. The excitation source was a NanoLED N-01 Aqua (373 nm) and the detector a TBX-04-A PMT with a Chroma D655/20nm band-pass filter. The decay data were fitted to a three exponential components using the Nonlinear ModelFit routine of Mathematica (Wolfram Research) to adequately represent the time course of excited state deactivation.

All the experiments were done in PBS buffer pH = 7.4, prepared with MilliQ water.

300 µL of QDs 5 nM were titrated with a stock solution of CyBiot (900 nM) by subsequent aliquots, at time intervals of 2-3 minutes.

**FRET calculations**

*J* is the spectral overlap integral between donor and acceptor, given by

$$J = \int F_\lambda^D \, \varepsilon_\lambda^A \, \lambda^4 \, d\lambda$$

where $F^D_\lambda$ is the normalized donor fluorescence spectrum and $\varepsilon^A_\lambda$ is the wavelength-dependent molar extinction coefficient ($M^{-1}$ $cm^{-1}$) of the acceptor.

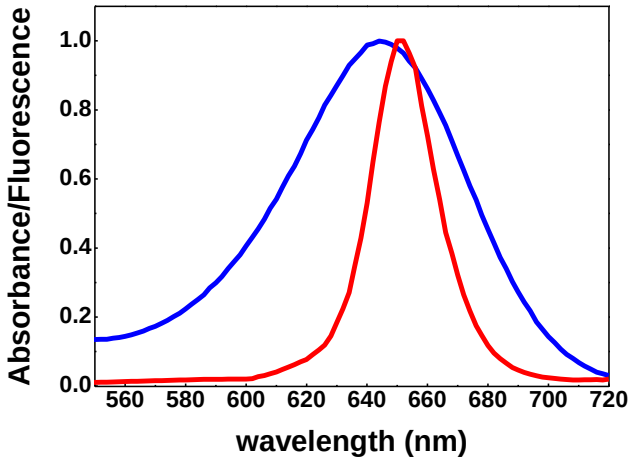



**Figure S6** Spectral overlap between emission of the QD655-streptavidin (red) and the absorbance of CyBiot (blue)

$R_0$ is the critical distance or Förster radius, i.e. the distance at which transfer and spontaneous decay of the excited donor are equally probable. $R_0$ can be determined from spectroscopic data, is given by

$$R_0^6 = 9000(\ln 10)\,\kappa^2\,\varphi_D\,J / 128\,\pi 5\,N_A\,n^4$$

where $\varphi_D = 0.5^3$, $\kappa^2 = 2/3$ and n = 1.33 (water). For an isolated donor-acceptor pair separated by $r_{DA}$, the FRET efficiency $E$ varies according to

$$E = [1 + (r_{DA}/Ro)^6]^{-1}$$

In the steady-state $E$ was calculated as

$$E = 1-(I_{DA}/I_D)$$

Where $I_{DA}$ and $I_D$ are the intensity of the donor in the presence and absence of the acceptor, respectively.

While for time-resolved measurements the following expression was applied

$$E = 1-(\tau_{DA}/\tau_D)$$

Where $\tau_{DA}$ and $\tau_D$ are the fluorescence lifetime of the donor in the presence and absence of the acceptor, respectively.

The use of intensity-averaged or amplitude-averaged lifetimes depends on the case under study. For instance, the intensity-averaged lifetime must be used for the calculation of an average collisional quenching constant, whereas in resonance energy transfer experiments, the amplitude-averaged decay time or lifetime must be used for the calculation of energy transfer efficiency. For

further reading on this calculations, refer to *Molecular Fluorescence: Principles and Applications. Bernard Valeur*.

**Fluorescence Microscopy**

Streptavidin conjugated magnetic nanoparticles (DynaBeads M-280, Invitrogen) were incubated with a solution of CyBiot 10 nM in PBS for 10 min. Then, a magnetic field was applied to separate and wash the free dye. Then, the particles were placed in microwell slides (A10657, Hamamatsu) and observed with a wide field fluorescence microscopy (Olympus IX-71) equipped with a 40x objective Olympus UPlanSAPO 0.95NA. As a negative control, in order to evaluate non-specific binding, the magnetic particles were pre-incubated with biotin 10 μM for 10 min, washed and the incubated with the solution of CyBiot 10 nM in PBS.

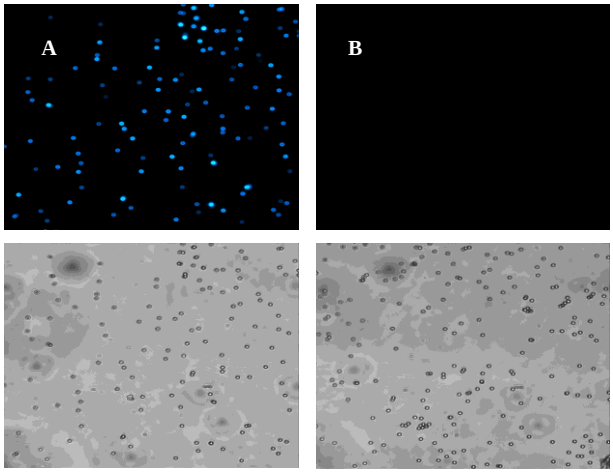



**Figure S7** A. Specific binding of CyBiot to magnetic beads coated with streptavidin. B. negative control. Upper panel: fluorescence image, lower panel: transmission image

**References**

1. The Molecular Probes® Handbook—A Guide to Fluorescent Probes and Labeling Technologies. 11th Edition
2. Fery-Forgues, S; Lavabre, D. *J. Chem Educ*. 1999, **76** (9) 1260-1264.
3. Ballou, B., Lagerholm, B.C., Ernst, L.A., Bruchez, M.P. and Waggoner, A.S. *Bioconjugate Chem.* 2004, **15**, 79-86.