Blue Fluorescent Dye-Protein Complexes Based on Fluorogenic Cyanine Dyes and Single Chain Antibody Fragments

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Supplemental Information

1. Dye Synthesis (See Chart S1 for structures and Schemes S1-S3 for reaction sequences.)

Synthetic procedures are described below. ¹H NMR spectra were recorded on a Bruker Avance DMX-500 NMR spectrometer operating at 500.13 MHz for ¹H and 125.76 MHz for ¹³C. Standard Bruker software was used. Due to low solubility of Dye 3, data for ¹³CNMR were obtained indirectly by collecting a HMBC experiment (Figures S2a-S2d). Samples were dissolved in CD₃OD except when stated otherwise. Chemical shifts are given in ppm (δ) downfield from TMS internal standard. Mass spectra were run in a Thermo-Fisher LCQ ESI/APCI Ion Trap working in positive or negative ion mode. Structures and reaction schemes follow the procedures.

Synthesis of 3-[2-(methylthio)-1,3-benzothiazol-3-ium-3-yl]propane-1-sulfonate (1)
A mixture of 2-(methylthio)-1,3-benzothiazole, (546.2 mg, 3.0 mmol) and propanesultone (410.7 mg, 3.4 mmol) in 2 mL of DMF was heated overnight in an oil bath at 115 °C. The reaction mixture was cooled at room temperature, washed several times with ethyl ether and dried to give 1 (742 mg, 81% yield). ¹H NMR (300 MHz, CD3OD): δ 8.23 (1H, brd, J = 8.8 Hz), 8.20 (1H, brd, J = 8.4 Hz), 7.84 (1H, ddd, J = 8.8; 8.7; 1.6 Hz), 7.71 (1H, ddd, J = 8.7; 8.4; 1.0 Hz), 3.12 (3H, s), 2.99 (2H, t, J = 6.8 Hz), 2.37 (2H, quint, J = 7.0 Hz).

Synthesis of 2,3-dimethyl-benzoxazol-3-ium p-toluensulfonate (2)
In a round bottom flask, 2-methylbenzoxazole (1.00 g, 7.5 mmol) was mixed with methyl p-toluenesulfonate (1.11 g, 6.0 mmol) and heated at 110 °C for 3 hours. The solid mixture was triturated and washed five times with ethyl ether to afford solid product 2 (1.29 g, yield 67%); ¹H NMR (300 MHz, CD3OD): δ 7.94 (2H, m), 7.75 (2H, m), 7.61 (2H, d, J = 8.3 Hz), 7.17 (2H, d, J = 8.3 Hz), 4.10 (3H, s), 3.06 (3H, s), 2.33 (3H, s).

Synthesis of OTB-SO₃ cyanine dye (3)
A mixture of salts 1 (30 mg, 0.1 mmol) and 2 (28 mg, 0.09 mmol) in 2 mL of absolute ethanol was added with triethylamine (20 μL, 0.14 mmol) and refluxed for 3 hours. After cooling, the reaction mixture was added with ethyl ether to precipitate dye 3 that was separated by vacuum filtration (4.5 mg, 12% yield). ¹H NMR (500 MHz, CD3OD): δ 8.11 (1H, dd, J = 8.3; 0.9 Hz), 7.91 (1H, d, J = 8.4 Hz), 7.81 (1H, d, J = 8.0 Hz), 7.72 (1H, d, J = 7.7 Hz), 7.63 (1H, ddd, J = 8.4; 7.2; 0.9 Hz), 7.54 (1H, td, J = 7.7; 1.0 Hz), 7.46 (1H, btt, J = 7.2 Hz), 7.45 (1H, td, J = 8.0; 1.0 Hz), 6.76 (1H, s), 4.76 (2H, btt, J = 7.8 Hz), 3.91 (3H, s), 2.67 (2H, m), 2.13 (2H, m); ¹³C NMR δ 163.2, 162.2, 146.7, 140.3, 132.0, 128.5, 126.6, 125.8, 125.2, 123.5, 113.8, 111.8, 111.8, 70.6, 47.6, 45.5, 31.2, 23.7. ESI-MS (positive mode) m/z 403.2 (M+H)⁺, calculated 403.1.

Spectroscopic properties of OTB-SO₃ (3)
The UV-vis spectra of dye 3 shows λₘₐₓ 400 nm in 10 mM sodium phosphate buffer with 100 mM NaCl (pH 7); εₘₐₓ = 92,400 M⁻¹cm⁻¹. In the presence of 100 μM CT-DNA in buffer the spectral profile does not show significant differences indicating weak or no interaction with nucleic acids. This is further confirmed by fluorescence spectroscopy in the presence of 100 μM CT DNA where excitation at λₘₐₓ = 400 nm shows negligible fluorescence emission; Φᵢ (CT-DNA) / Φᵢ (buffer) = 2.3. A large fluorescence increase is observed when the dye is dissolved in 90% glycerol and excited at 380 nm, λₑₐᵣ = 421 nm; Φᵢ (90% glycerol) / Φᵢ (buffer) = 56 at ca. 20 °C (Figure 2).

Synthesis of 5-tert-butyl-1,3-benzoxazole-2(3H)-thione (4)
In a 250 mL round bottom flask, 2-amino-4-tert-butylphenol (2.0 g, 12.1 mmol) was dissolved in 12 mL of dry DMF. Carbon disulfide (0.73 mL, 12.1 mmol) was added followed by 60% sodium hydride in mineral oil (0.93 g, ~23 mmol) previously washed with hexane; the powder is added stepwise. After the bubbling stopped, the mixture was left to stir at 115°C for 90 min under nitrogen. Color changes were observed during the reaction; the excess NaH was quenched by adding glacial acetic acid. The mixture was diluted with water and extracted with DCM three times; the organic layer was dried over Na₂SO₄, filtered and concentrated under vacuum to give an off white powdery solid of 5-tert-butyl-1,3-benzoxazole-2(3H)-
precipitate started to form immediately; after 2 min, the solid is filtered and washed with cold ethanol and dried under the hood (26.8 mg, 58% yield). M.p. = 368-371 °C (dec.). 1H NMR (500 MHz, CD3OD): δ 10.60 (1H, brs, NH), 7.22 (1H, dd, J = 8.7; 1.8 Hz), 7.18 (1H, dd, J = 8.7; 0.8 Hz), 7.12 (1H, J = 1.8; 0.8 Hz), 1.27 (9H, s).

Synthesis of 5-tert-butyl-2-(methylthio)-1,3-benzoxazole (5)
Compound 4 (1.0 g, 4.8 mmol) was reacted with iodomethane (0.624 mL, 10 mmol) and K2CO3 (140 mg, 1 mmol) at room temperature overnight. The product was extracted by partitioning the reaction mixture between DCM and water. The organic phase was dried with Na2SO4, filtered and the solvent evaporated under vacuum to give 5-tert-butyl-2-(methylthio)-1,3-benzoxazole (5) (0.98 g, 92% yield); 1H NMR (300 MHz, CD2OD): δ 7.74 (1H, d, J = 2.1 Hz), 7.22 (1H, dd, J = 8.5; 2.1 Hz), 7.01 (1H, d, J = 8.5 Hz), 2.26 (3H, s), 1.30 (9H, s).

Synthesis of 5-tert-butyl-3-methyl-2-(methylthio)-1,3-benzoxazol-3-ium p-toluenesulfonate (6)
Compound 5 (1.0 g, 4.5 mmol) was reacted with methyl p-toluenesulfonate (1.0 g, 5.4 mmol) at 110 °C overnight. After cooling, ethyl ether was added and the precipitate was filtered out. Several purification methods led to decomposition of the product. A small portion was purified by RPC18 column eluting with water. The solvent was evaporated under vacuum and product (6) was dried in a dessicator (106 mg collected, 91.4% purity); 1H NMR (300 MHz, CD2OD): δ 7.99 (1H, brs), 7.88 (2H, brs), 7.67 (2H, d, J = 8.2 Hz), 7.22 (2H, d, J = 8.2 Hz), 4.15 (3H, s), 3.08 (3H, s), 2.38 (3H, s), 1.47 (9H, s).

Synthesis of dye tert-butyl-OTB-CO2 (8)
Salt 6 (30 mg, 0.07 mmol) and 6-(2-methyl-1,3-benzothiazol-3-ium-3-yl)hexanoate (7) (26 mg, 0.10 mmol) were dissolved in 2 mL of absolute ethanol and added with triethylamine (20 µL, 0.14 mmol). The mixture was heated with a heat gun for a few seconds until a yellow color appeared. When the heat was removed a precipitate formed. The mixture was cooled, left standing for 1 h and washed with ethyl ether (3x 2mL). The yellow solid was filtered and dried in a dessicator overnight, resulting in tert-butyl-OTV-CO2 dye (8) showed to be pure by 1H NMR and HPLC analysis (24 mg, 64% yield). M.p. = 264-266 °C (dec.). 1H NMR (500 MHz, CD3OD): δ 8.6; 2.0 Hz), 7.47 (1H, ddd, J = 8.0, 0.8 Hz), 6.52 (1H, s), 4.76 (2H, brt, J = 7.5 Hz), 1.72 (quint, J = 7.5 Hz), 1.57 (2H, m), 1.43 (9H, s); 13C NMR δ 178.4, 163.8, 162.4, 150.7, 144.8, 140.1, 131.2, 128.0, 125.7, 125.0, 122.4, 122.3, 113.1, 109.9, 107.7, 68.9, 46.2, 35.2, 34.9, 30.6, 29.4, 26.6, 25.9, 24.9. ESI-MS (positive mode) m/z 451.3 (M+H)+, calculated 451.2.

Spectroscopic properties of tert-butyl-OTB-CO2 dye (8)
The UV-vis spectra of dye 8 shows a λmax = 404 nm in 10 mM sodium phosphate buffer, 100 mM sodium chloride, pH 7; εmax = 81,000 M-1cm-1 in methanol. The dye shows very low fluorescence in buffer or in presence of DNA, Φf (CT-DNA) / Φf (buffer) = 5. When it is placed in a viscous medium its fluorescence increases considerably; Φf (90% glycerol) / Φf (buffer) = 52 at ca. 20 °C.

Synthesis of dye tert-butyl-OTB-SO3 (10)
To a mixture of 6 (36 mg, 0.10 mmol) and 3-(2-methyl-1,3-benzothiazol-3-ium-3-yl)propane-1-sulfonate (9) (27 mg, 0.10 mmol) in absolute ethanol (4 mL), triethylamine (20 µL, 0.27 mmol) was added. A precipitate started to form immediately; after 2 min, the solid is filtered and washed with cold ethanol and dried under the hood (26.8 mg, 58% yield). M.p. = 368-371 °C (dec.). 1H NMR (500 MHz, CD2OD): δ 7.93 (1H, dd, J = 8.0, 0.7 Hz), 7.79 (1H, d, J = 8.3 Hz), 7.66-7.59 (3H, m), 7.53 (1H, dd, J = 8.6; 2.0 Hz), 7.45 (1H, dt, J = 8.0, 0.8 Hz), 6.52 (1H, s), 4.76 (2H, brt, J = 8.3 Hz), 3.90 (3H, s), 3.04 (2H, m), 2.34 (2H, m), 1.43 (9H, s); 13C NMR δ 165.1, 163.9, 152.1, 146.4, 141.4, 132.7, 129.4, 127.2, 126.3, 123.6, 114.2, 111.3, 109.2, 70.93, 48.5 (observed by solvent peak), 46.2, 36.4, 32.0, 31.2, 23.8. ESI-MS (positive mode) m/z 459.3 (M+H)+, calculated 459.1.

Spectroscopic properties of tert-butyl-OTB-SO3 dye (10)
The UV-vis spectra of dye 10 shows a λmax = 402 nm in 10 mM sodium phosphate buffer, 100 mM sodium chloride, pH 7; εmax = 78,000 M-1cm-1 in methanol. The dye shows very low fluorescence in buffer or in presence of DNA, Φf (CT-DNA) / Φf (buffer) = 4. In a viscous medium its fluorescence increases considerably; Φf (90% glycerol) / Φf (buffer) = 46 at ca. 20 °C.
Chart S1: Structures described in synthesis

1. 

2. 

3. 

4. 

5. 

6. 

7. 

8. 

9. 

10. 

\[ R = (\text{CH}_2)_6\text{CO}_2^- \] 

\[ R = (\text{CH}_2)_3\text{SO}_3^- \]
Synthesis schemes:

Scheme S1. Synthesis of intermediates 4 to 6

\[
\text{Scheme S2. Synthesis of } t\text{-butyl-OTB-CO}_2 \text{ dye (8)}
\]

\[
\text{Scheme S3. Synthesis of } t\text{-butyl-OTB-SO}_3 \text{ dye (10)}
\]
NMR Spectra:

Figure S1. $^1$H NMR spectrum of Dye 3 OTB-SO$_3$ (500 MHz, in DMSD-$d_6$).
Figure S2a. Proton and carbon NMR assignments for dye 3 OTB-SO₃.
**Figure S2b.** COSY spectra for dye 3 OTB-SO$_3$. 
Figure S2c. HSQC spectra for dye 3 OTB-SO$_3$. 
Figure S2d. HMBC spectra for dye 3 OTB-SO$_3$. 
**Figure S3.** $^1$H NMR spectrum of dye 8 $t$-butyl-OTB-CO$_2$ (300 MHz, CD$_3$OD)
Figure S4. $^{13}$C NMR spectrum of dye 8 t-butyl-OTB-CO$_2$ (125 MHz, CD$_3$OD)
Figure S5. $^1$H NMR spectrum of dye 10 $t$-butyl-OTB-SO$_3$ (300 MHz, CD$_3$OD)
Figure S6. $^{13}$C NMR spectrum of dye 10 t-butyl-OTB-SO$_3$ (300 MHz, CD$_3$OD)
UV-vis Spectra of OTB Dyes in Methanol

Figure S7. UV-vis spectra recorded of fluorogenic cyanine dyes in methanol.
Analytical HPLC of Dye 3 (OTB-SO₃)

Figure S8. HPLC data for dye 3 OTB-SO₃.
Analytical HPLC of Dye 8 (t-Bu-OTB-CO₂)

Figure S9. HPLC data for dye 8 t-Bu-OTB-CO₂.
Analytical HPLC of Dye 10 (t-Bu-OTB-SO₃)

Figure S10. HPLC data for dye 10 t-Bu-OTB-SO₃.
2. Flow Cytometry Data

Figure S11. Data from five sequential rounds of flow cytometry of yeast-displayed library sorted for activation of OTB-SO$_3$ fluorescence. Violet signal is on the x-axis; induction measured by labeling with c-myc epitope tag and Alexa 488 antibody is on the y-axis.

Flow Cytometry Methodology
The naïve yeast library was sorted directly on the flow cytometer, in a marked departure from previous methods. In the past, our Center had selected scFvs capable of activating fluorogenic dyes by first conducting two rounds of Magnetic-Activated Cell Sorting (MACS), followed by several rounds of flow cytometry. In the magnetic sorts, a biotinylated version of the dye is attached to magnetic beads coated with either streptavidin or anti-biotin antibody. Yeast cells with scFvs expressed on their surface are then passed through a column containing the beads; only cells expressing scFvs that bind the target dye remain in the column. Although this method worked well, it required additional time and expense for the magnetic sorts. We also encountered problems with contamination by other yeast strains during MACS. By skipping the magnetic sorting step, we were able to avoid these difficulties and did not need to synthesize biotinylated OTB-SO$_3$. 

3. Protein Sequences (Molecular Weights and $\varepsilon_{280}$ values)

A5 (25,737 kDa, $\varepsilon_{280} = 43,010$ M$^{-1}$cm$^{-1}$)
QVQLVESQGGLQVPGESLRLCASAGFTFSGSWMAWVRQPPGKGLEWV
AELOPDGSGKYYVDSVKGRTISRDNKSLYNLQMNKLADDTAIYYCA
RDPSFGAFDYWGQGTIVTSSGILGSGGSGGSGGSGGSALTQPA
SVMGSPQGQSITISOCTGTSDDVGGYNYVSWYQQHPGKAPKLMISDVTKR
PSGVPRDFSAGSKSGNTASLTISGLQTEDAAYCSSFSTSTSSVIFGGGTK
VTVL

A6 (26,823 kDa, $\varepsilon_{280} = 51,260$ M$^{-1}$cm$^{-1}$)
QVQLQSGPGLVPQSTLSLTCAISGDSVSSNSAVWNWIRQSPSRGLEY
WLGRTYRSQWNNHYAESVKSRTINPDTSTKNQVLTMNSMDPLDTATY
YCAWSYSSPNDYWQGILVTSSGASAPFTGLGSGGSGGSAGGGS
GGGEIVMTQSPATLSLSPPGERATLSRASQSVSVSYLAWYQQPQGAPRL
LIYDASNRATGIPARFSGSGSGTDFTLTISSELEDFAVYYCQYSGL
TFGGGTKVDIKS

B11 (26,400 kDa, $\varepsilon_{280} = 52,540$ M$^{-1}$cm$^{-1}$)
QVQLQSGPGLVPQSTLSLTCAISGDSVSSNSAAWNWIRQPSRGFY
WLGRTYRSQWNYFYAVSVKSRTINPDTSTNQISLQLNSVTPEDDAVYY
CSRGVRGVYFYDYWDQGLTVSTSSGILSGGGSGGGSGGSGGGSEIVMT
QSPGTLSSPGESATLSRASRVSNGNLAWYQQPGQPRLIIYGAISTR
ATGIPARFSGSGSGTEFTLTISLLQEDFAVYYCQYNNLGTFTFGGQ
GTKLEIKS

D10 (26,320 kDa, $\varepsilon_{280} = 51,260$ M$^{-1}$cm$^{-1}$)
QVQLQSGPGLVPQSTLSLTCAISGDSVSSNSAVNWIRQSPSRGLEY
WLGRTYRSQWNYFYAVSVKSRTINPDTSTKQFQLNSINPDFDTAVYY
CARAEADAIFDYWGQGTSSTSSGGGSAGGGSAGGGSAGGGSAGGGS
GGGEIVLTQSPATLSLSPPGERATLSRASQSVSVSYLAWYQQPQGAPRLIIYDASSR
AAAGLSDRFSGSGSGTDFTLTISRLEPDEDFAVYYCQQYFSGTGGK
GTKLEIKS

H10 (26,277 kDa, $\varepsilon_{280} = 47,420$ M$^{-1}$cm$^{-1}$)
QVQLQSGPGLVPQSTLSVTCISGDSVSSNSAVWNWIRQPSGGLLEY
WLGRIYRSROWFDDAYAESVKSRTINPDTKQFSQSLNSVPEDTAMYY
CTRDGDGDLDLDVDWQQGTMVTVSSTSGGSGGSGGSGGSGGSGGSGG
LTQSPATLSLSPPGERATLSQSFVSVSYLAWYQQPQGAPRLIIYDAS
TRATGIPARFSGSGSATDFTLTISRLEPDEDFAVYYCQYGGTGGK
GTKLEIKS

J10 (26,273 kDa, $\varepsilon_{280} = 41,730$ M$^{-1}$cm$^{-1}$)
QVQLQSGSGAEVKPGSVSVKVSCKASGGAFSSANWVRQAPQGQLEW
MGIIIIPVTPNYAQKFGQRVTADRESTTRFYYELMSSRSEDVAYYCA
RVLSGISDLGYIDLWGRGTVLTVSTSSGILSGGSGGSGGSGGSGGSGG
LTQSPATLSVSPGERATLSQSFVSNLKAWYQQPQGAPRLIIYGA
STRAATGIPARFSGSGGSGTEFTLTISSQLQEDFAVYYCQYTDRTPSWTFGG
GTKVEIKS
4. **Fluorescence Spectroscopy of DNA-Binding Fluorogens**

![Emission spectra of 200 nM PO-PRO-1 (left) or OTB (right) in buffer or in presence of 100 μM base pairs calf thymus DNA](image)

**Figure S12.** Emission spectra of 200 nM PO-PRO-1 (left) or OTB (right) in buffer or in presence of 100 μM base pairs calf thymus DNA

5. **Absorbance Spectra of FAP-Bound Fluorogens**

![Absorption spectra of 1.2 μM t-butyl-OTB-SO₃ (left) or t-butyl-OTB-CO₂ (right) in the presence and absence of A5 soluble protein](image)

**Figure S13.** Absorption spectra of 1.2 μM t-butyl-OTB-SO₃ (left) or t-butyl-OTB-CO₂ (right) in the presence and absence of A5 soluble protein.

**Figure S14.** Absorption spectra of 750 nM DIR in the presence of buffer or 1.5 μM soluble J10 protein.

**Figure S15.** The fluorescence of 200 nM OTB-SO$_3$/H10 was measured in 5 degree increments from 10°C to 50°C. Only slight changes are observed in the profile at the highest temperature, where the protein begins to undergo
7. pH-Dependence of H10-OTB-SO₃ Emission Spectrum

The pH sensitivity of the H10 fluoromodule was examined in a variety of buffers. In order to encompass the pH range where the protein is expected to maintain a stable structure, pH values ranging from 4 to 9 were used. It was necessary to vary the type and concentrations of salts present in the buffers to ensure that they were effective in the desired pH range (10 mM NaAc pH = 4.0, 10 mM NaAc pH = 4.5, PBS pH = 7.4, or 10 mM Na₂B₄O₇/1 M NaCl pH = 9).

![Figure S16. The fluorescence of 200 nM OTB-SO₃/H10 in various pH buffers. Samples were excited at 370 nm.]

8. Kᵯ Determinations

**Soluble Protein.** Figures S9-S13 show data from fluorescence titrations of dye into solutions containing either soluble scFv (black) or buffer (red). Samples were excited at 380 nm, and emission was monitored at 435 nm except for DIR (Figure S14), which was excited at 580 nm and emission monitored at 651 nm.

![Figure S17. Fluorescence titrations of OTB-SO₃ into 50 nM soluble A5 (left) or A6 (right).]
Figure S18. Fluorescence titrations of OTB-SO$_3$ into 50 nM soluble B11 (left) or D10 (right).

Figure S19. Fluorescence titrations of OTB-SO$_3$ into 50 nM soluble H10 (left) or J10 (right).
Figure S20. Fluorescence titrations of t-Bu-OTB-SO$_3$ (left) or t-Bu-OTB-CO$_2$ (right) into 50 nM soluble A5.

Figure S21. Fluorescence titrations of DIR into 50 nM J10.
Yeast Displayed Protein. Figures S15-S19 show data from fluorescence titrations of dye into solutions containing induced yeast (black), uninduced yeast (red), and buffer (blue). Samples were excited at 401 nm and emission was monitored at 435 nm except for DIR (Figure S19), which was excited at 602 nm and emission monitored at 644 nm.

Figure S22. Fluorescence titrations of t-Bu-OTB-SO$_3^-$ into yeast-displayed A5 (left) or A6 (right).

Figure S23. Fluorescence titrations of t-Bu-OTB-SO$_3^-$ into yeast-displayed B11 (left) or D10 (right).
Figure S24. Fluorescence titrations of t-Bu-OTB-SO₃ into yeast-displayed H10 (left) or J10 (right).

Figure S25. Fluorescence titrations of t-Bu-OTB-SO₃ (left) or t-Bu-OTB-CO₂ (right) into yeast-displayed A5.
**Figure S26.** Fluorescence titrations of DIR into yeast-displayed J10.

**Figure S27.** Relative fluorescence spectra were measured for 200 nM OTB and 1 μM soluble FAP. Samples were excited at 370 nm and spectra were divided by the value of the buffer spectrum at 423 nm to give the relative fluorescence values.
9. Microscopy Images

**Figure S28.** Overlay of fluorescence and differential contrast images of J10 cells labeled with 200 nM DIR (red) and OTB-SO$_3$ (blue), along with the merged fluorescence signal from both dyes (purple).

**Figure S29.** Overlay of fluorescence and differential contrast images of H10 cells labeled with OTB-SO$_3$ (blue) and H6-MG cells labeled with MG-2p (red).