Supplementary Material (ESI) for Organic & Biomolecular Chemistry

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

Kimberly J. Zanotti[1], Yehuda Creeger[3], Gloria L. Silva[1], Kelly L. Robertson[1], Alan S. Waggoner[2,3], Peter B. Berget[2,3], & Bruce A. Armitage[1,3*]

Departments of Chemistry[1] and Biological Sciences[2] and Molecular Biosensor and Imaging Center[3], Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213

* Tel: 412-268-4196; Fax: 412-268-1061; e-mail: army@cmu.edu

Supplemental Information

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

Synthetic procedures are described below. ^{1}H NMR spectra were recorded on a Bruker Avance DMX-500 NMR spectrometer operating at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C. Standard Bruker software was used. Due to low solubility of Dye 3, data for ^{13}CNMR were obtained indirectly by collecting a HMBC experiment (Figures S2a-S2d). Samples were dissolved in CD_3OD 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). ^{1}H NMR (300 MHz, CD_3OD): 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%); ^{1}H NMR (300 MHz, CD_3OD): 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-SO_3 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). ^{1}H NMR (500 MHz, CD_3OD): 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, brt, *J* = 7.2 Hz), 7.45 (1H, td, *J* = 8.0; 1.0 Hz), 6.76 (1H, s), 4.76 (2H, brt, *J* = 7.8 Hz), 3.91 (3H, s), 2.67 (2H, m), 2.13 (2H, m); ^{13}C NMR δ 163.2, 162.2, 146.7, 140.3, 132.0, 128.5, 126.6, 125.8, 125.2, 125.2, 123.5, 113.8, 111.8, 111.2, 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-SO_3(**3**)
The UV-vis spectra of dye **3** shows λ_{max} 400 nm in 10 mM sodium phosphate buffer with 100 mM NaCl (pH 7); ε_{max} = 92,400 $M^{-1}cm^{-1}$. 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 λ_{max} = 400 nm shows negligible fluorescence emission; Φ_f (CT-DNA) / Φ_f (buffer) = 2.3. A large fluorescence increase is observed when the dye is dissolved in 90% glycerol and excited at 380 nm, λ_{em} = 421 nm; Φ_f (90% glycerol) / Φ_f (buffer) = 56 at *ca*. 20 °C (Figure 2).

Synthesis of 5-*tert*-butyl-1,3-benzoxazole-2(3*H*)-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 Na_2SO_4, filtered and concentrated under vacuum to give an off white powdery solid of 5-*tert*-butyl-1,3-benzoxazole-2(3*H*)-

thione (**4,** 2.3 g, yield 92%), ^{1}H NMR (300 MHz, $CDCl_3$): 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 K_2CO_3 (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 Na_2SO_4, filtered and the solvent evaporated under vacuum to give 5-*tert*-butyl-2-(methylthio)-1,3-benzoxazole (**5**) (0.98 g, 92% yield); ^{1}H NMR (300 MHz, CD_3OD): 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*-toluensulfonate (**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); ^{1}H NMR (300 MHz, CD_3OD): 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).

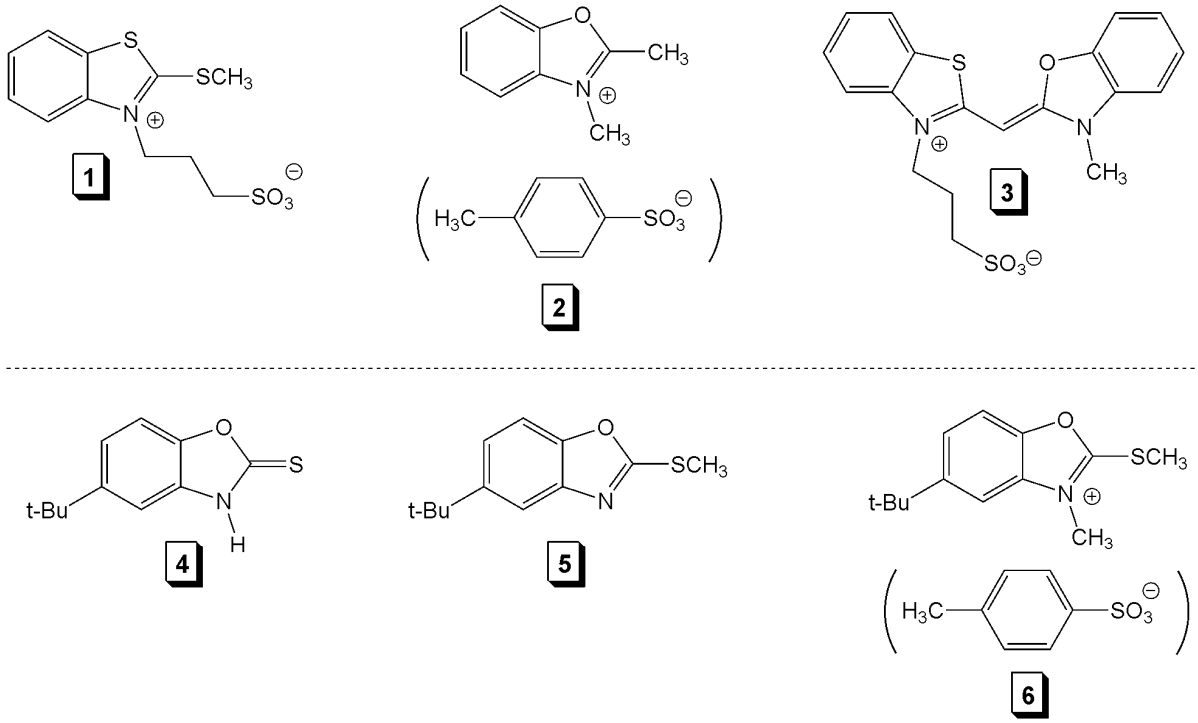
Synthesis of dye *t*-butyl-OTB-CO_2 (**8**)
Salt **6** (30 mg, 0.07 mmol) and 6-(2-methyl-1,3-benzothiazol-3-ium-3-yl)hexanoate (**7**) (26 mg, 0.10 mmol) were dissolved in 2 mL of absolute ethanol and added with triethylamine (20 µL, 0.14 mmol). The mixture was heated with a heat gun for a few seconds 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 dissecator overnight, resulting in t-butyl-OTV-CO_2 dye (**8**) showed to be pure by ^{1}H NMR and HPLC analysis (24 mg, 64% yield). M.p. =264-266 °C (dec.), ^{1}H NMR (500 MHz, CD_3OD): δ 7.94 (1H, d, *J* = 7.7 Hz), 7.74 (1H, d, *J* = 8.4 Hz), 7.67-7.61 (3H, m), 7.54 (1H, dd, *J* = 8.6; 2.0 Hz), 7.47 (1H, ddd, *J* = 8.2, 7.6, 0.8 Hz), 6.10 (1H, s), 4.53 (2H, t, *J* = 7.6 Hz), 3.88 (3H, s), 2.27 (2H, t, *J* = 7.3 Hz), 1.95 (2H, quint, *J* = 7.5 Hz), 1.72 (quint, *J* = 7.5 Hz), 1.57 (2H, m), 1.43 (9H, s); ^{13}C 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 *t*-butyl-OTB-CO_2dye (**8**)
The UV-vis spectra of dye **8** shows a λ_{max} = 404 nm in 10 mM sodium phosphate buffer, 100 mM sodium chloride, pH 7; ε_{max} = 81,000 $M^{-1}cm^{-1}$ in methanol. The dye shows very low fluorescence in buffer or in presence of DNA, Φ_f (CT-DNA) / Φ_f (buffer) = 5. When it is placed in a viscous medium its fluorescence increases considerably; λ_{em} = 429 nm, Φ_f (90% glycerol) / Φ_f (buffer) = 52 at *ca*. 20 °C.

Synthesis of dye *t*-butyl-OTB-SO_3 (**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.), ^{1}H NMR (500 MHz, CD_3OD): 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); ^{13}C NMR δ 165.1, 163.9, 152.1, 146.4, 141.4, 132.7, 129.4, 127.2, 126.3, 123.8, 123.6, 114.2, 111.3, 109.2, 70.93, 48.5 (obscured 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.

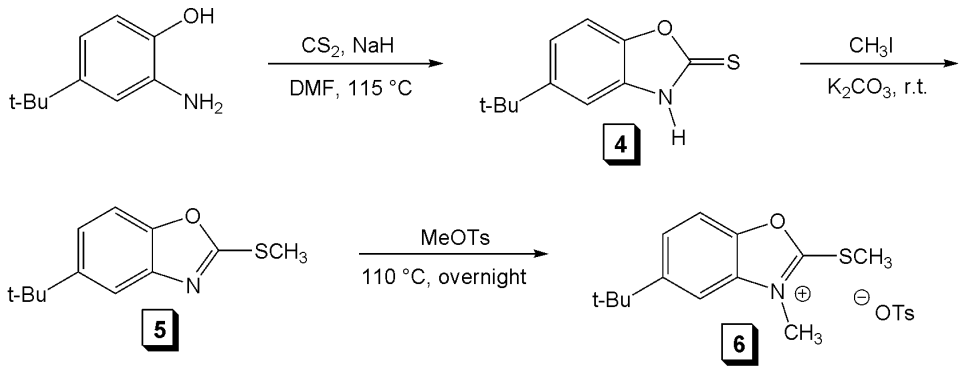
Spectroscopic properties of *t*-butyl-OTB-SO_3 dye (**10**)
The UV-vis spectra of dye **10** shows a λ_{max} = 402 nm in 10 mM sodium phosphate buffer, 100 mM sodium chloride, pH 7; ε_{max} = 78,000 $M^{-1}cm^{-1}$ in methanol. The dye shows very low fluorescence in buffer or in presence of DNA, Φ_f (CT-DNA) / Φ_f (buffer) = 4. In a viscous medium its fluorescence increases considerably; λ_{em} = 425 nm, Φ_f (90% glycerol) / Φ_f (buffer) = 46 at *ca*. 20 °C.

Chart S1: Structures described in synthesis

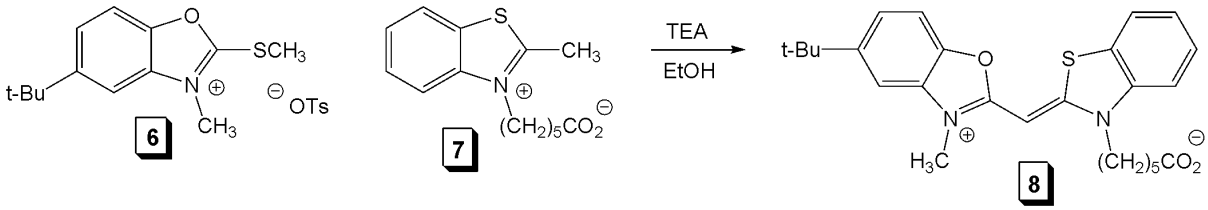


Synthesis schemes:

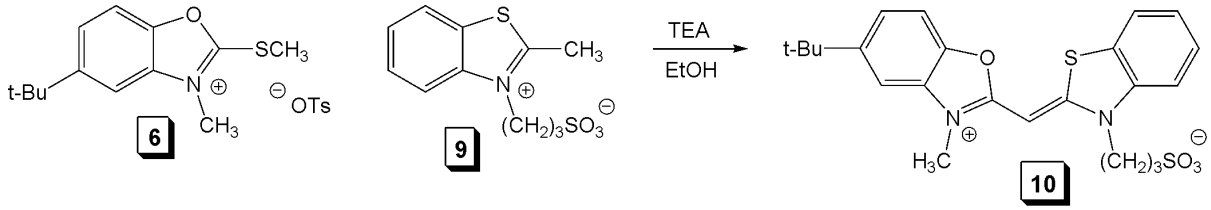
Scheme S1. Synthesis of intermediates **4** to **6**



Scheme S2. Synthesis of *t*-butyl-OTB-CO_2 dye (**8**)



Scheme S3. Synthesis of *t*-butyl-OTB-SO_3 dye (**10**)



NMR Spectra:

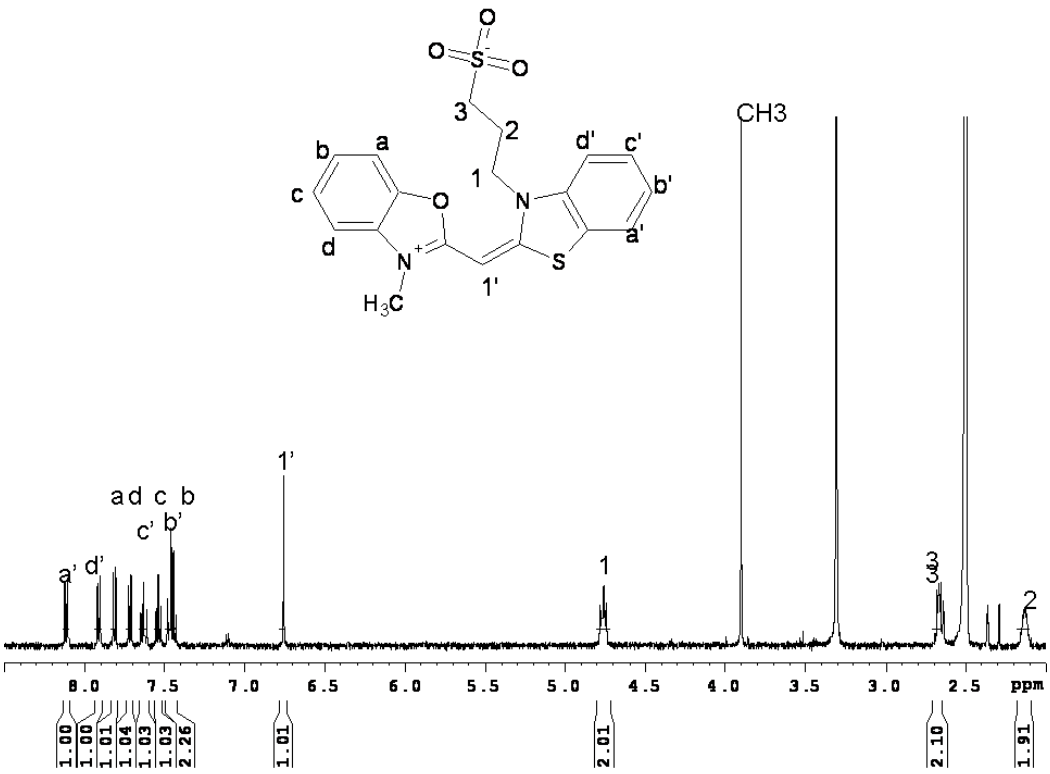


Figure S1. ^{1}H NMR spectrum of Dye **3** OTB-SO_3 (500 MHz, in DMSD-d_6).

carbon#	1H, ppm	13C, ppm
1	4.76	45.5
2	2.13	23.7
3	2.67	47.6
1'	6,76	70.6
N-S		**163.2**
N+-O		**162.2**
a	7.81	111.2
b	7.45	125.2
c	7.54	126.6
d	7.72	111.8
a- -O		**146.7**
d- -N+		**132**
a'	8.11	123.5
b'	7.46	125.2
c'	7.63	128.5
d'	7.91	113.8
a'- -S		**125.8**
d'- -N		**140.3**
N+-CH3	3.91	31.2

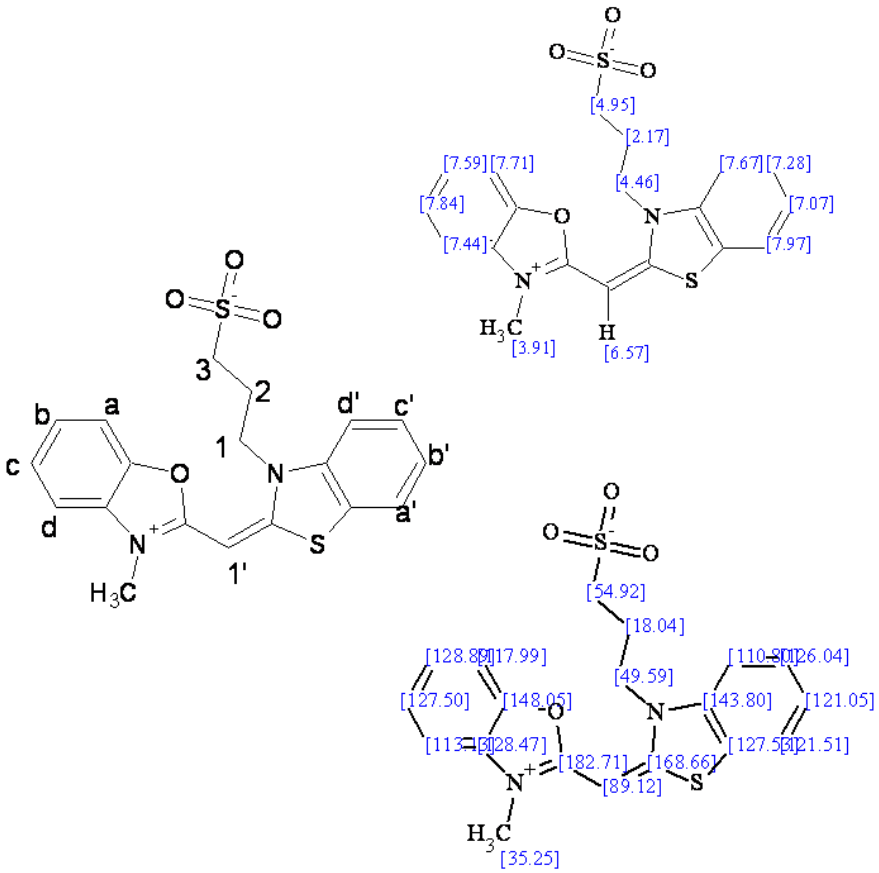


Figure S2a. Proton and carbon NMR assignments for dye **3** OTB-SO_3.

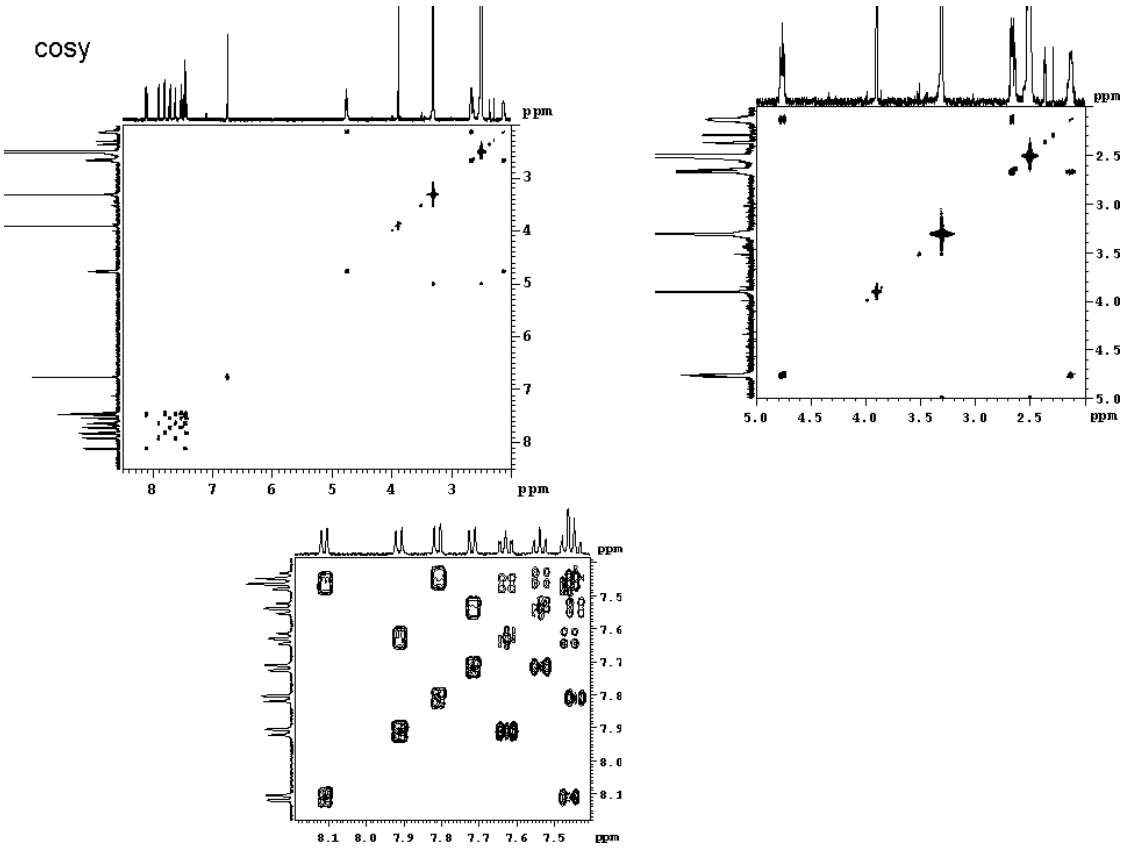


Figure S2b. COSY spectra for dye **3** OTB-SO_3.

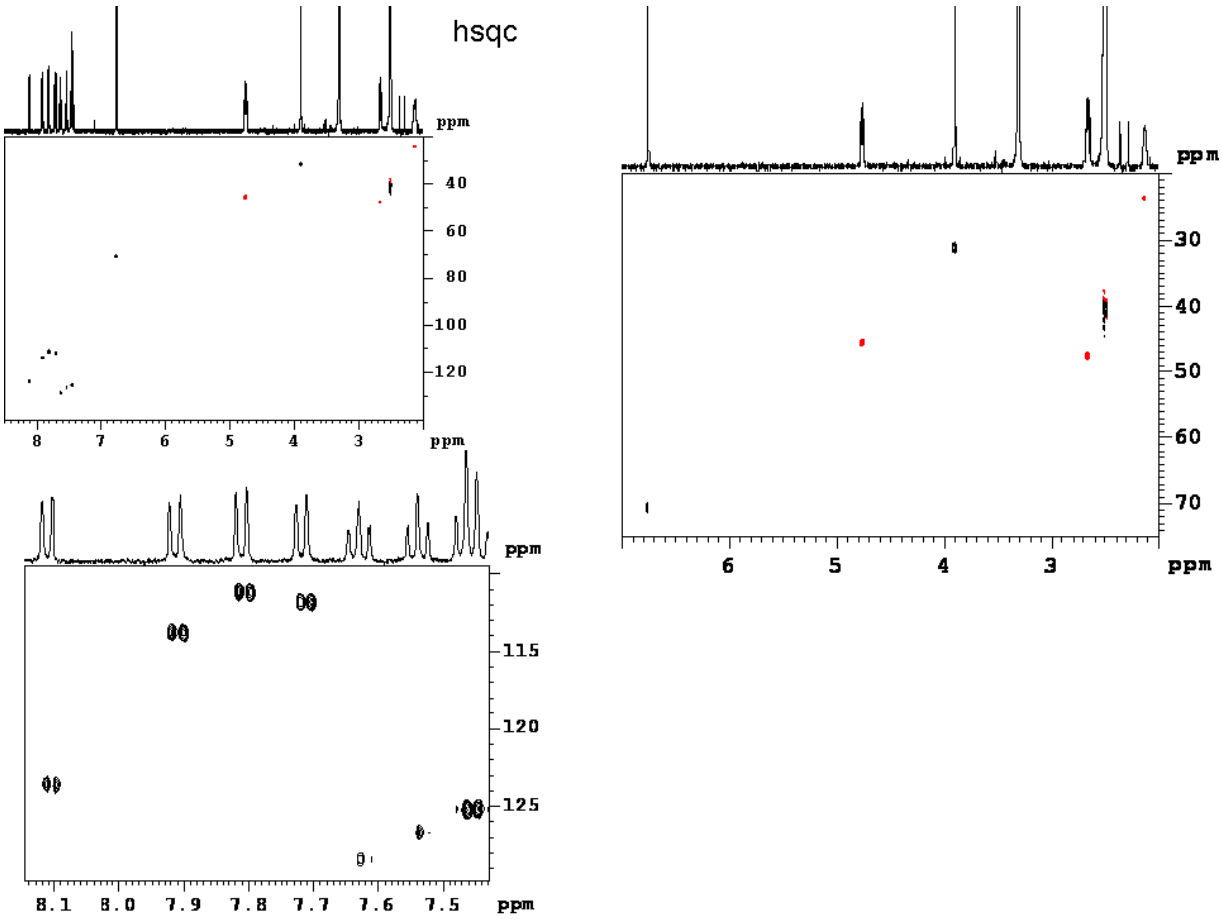


Figure S2c. HSQC spectra for dye **3** OTB-SO_3.

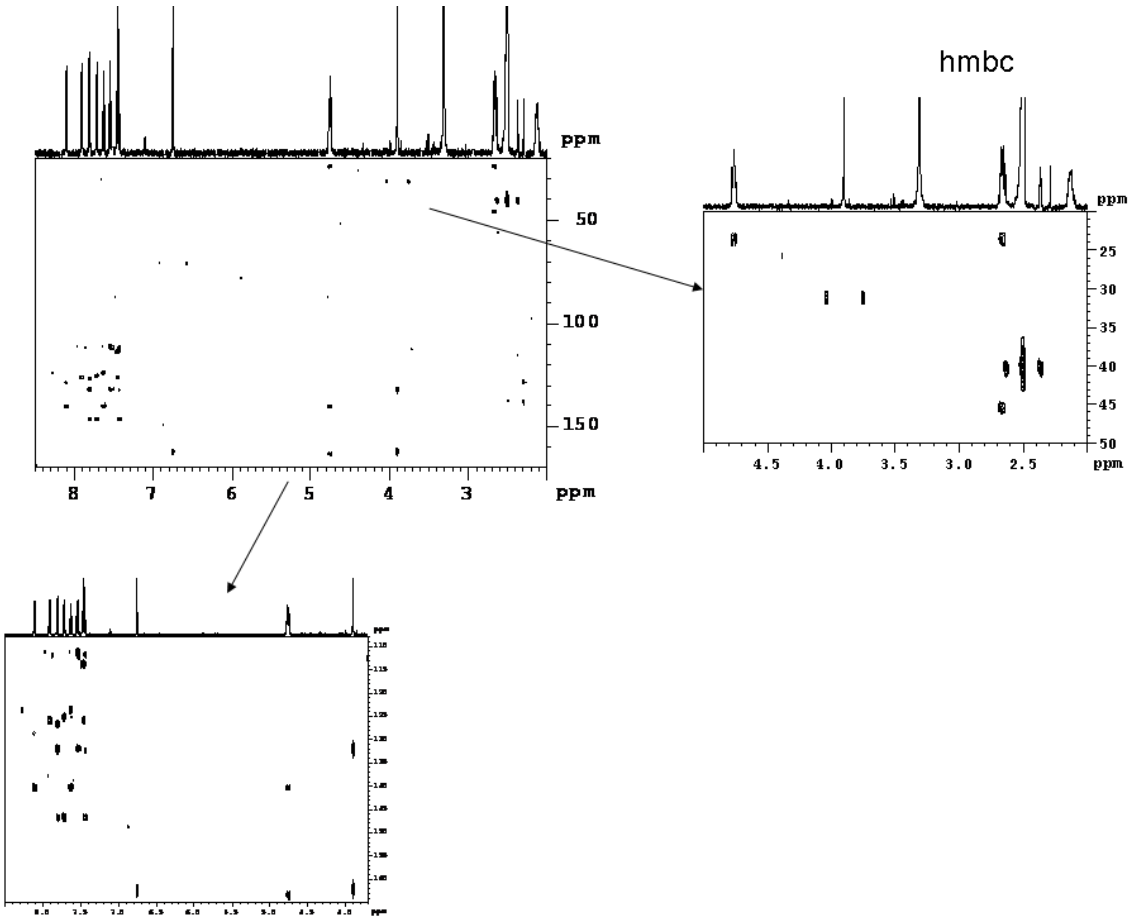


Figure S2d. HMBC spectra for dye **3** OTB-SO_3.

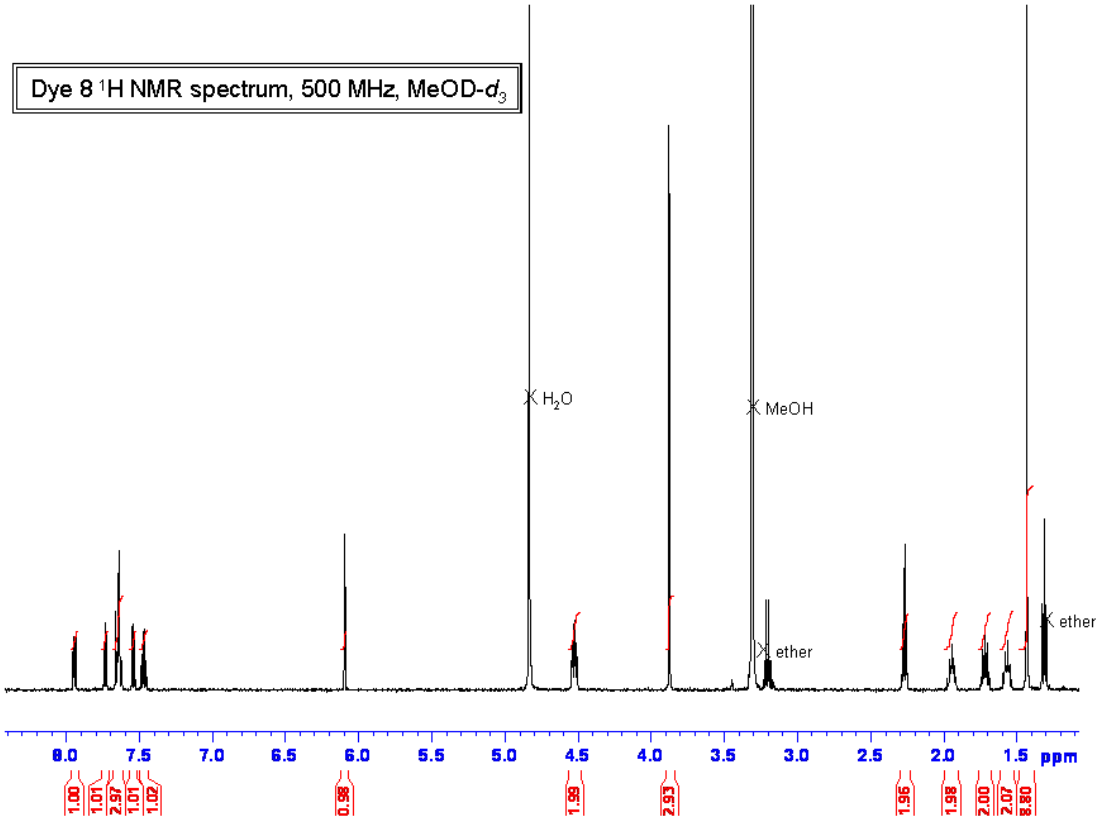


Figure S3. ^{1}H NMR spectrum of dye **8** *t*-butyl-OTB-CO_2 (300 MHz, CD_3OD)

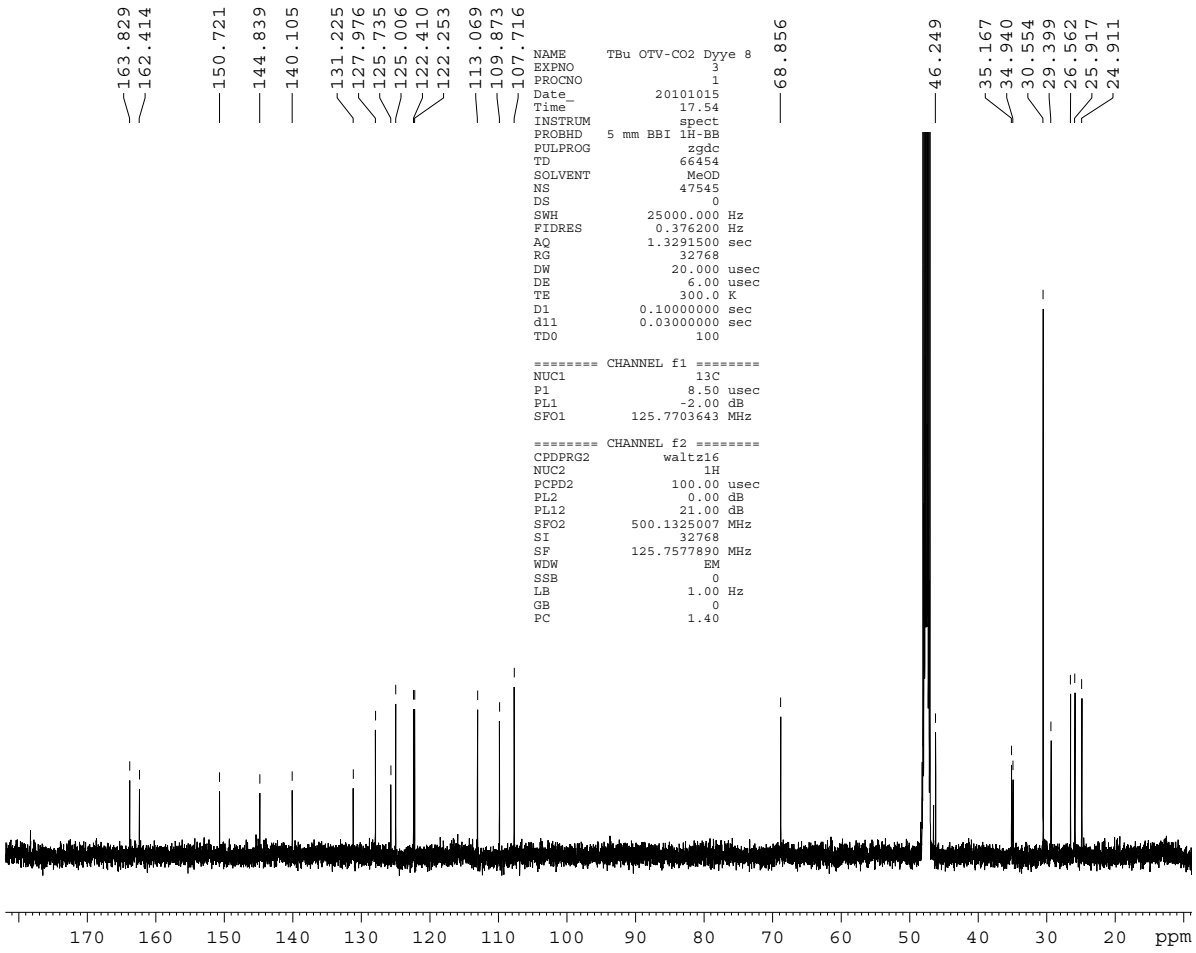


Figure S4. ^{13}C NMR spectrum of dye **8** t-butyl-OTB-CO_2 (125 MHz, CD_3OD)

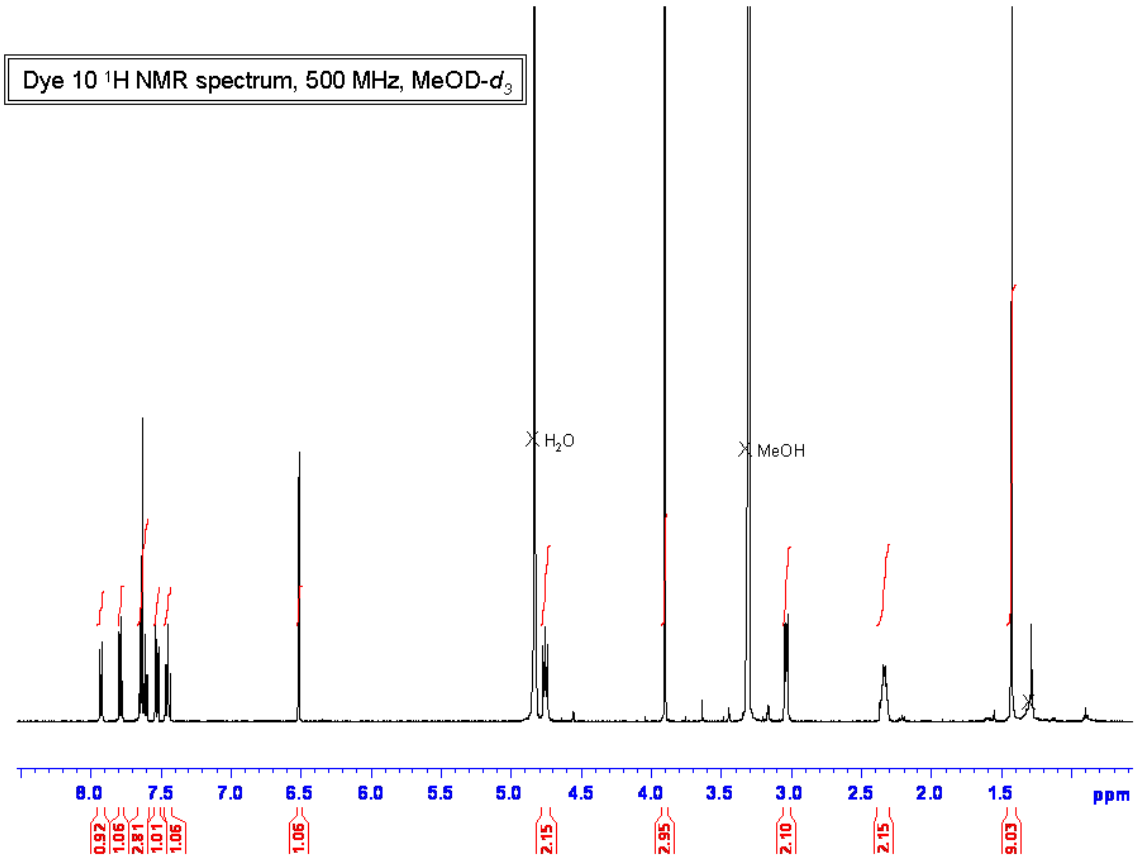


Figure S5. ^{1}H NMR spectrum of dye **10** *t*-butyl-OTB-SO_3 (300 MHz, CD_3OD)

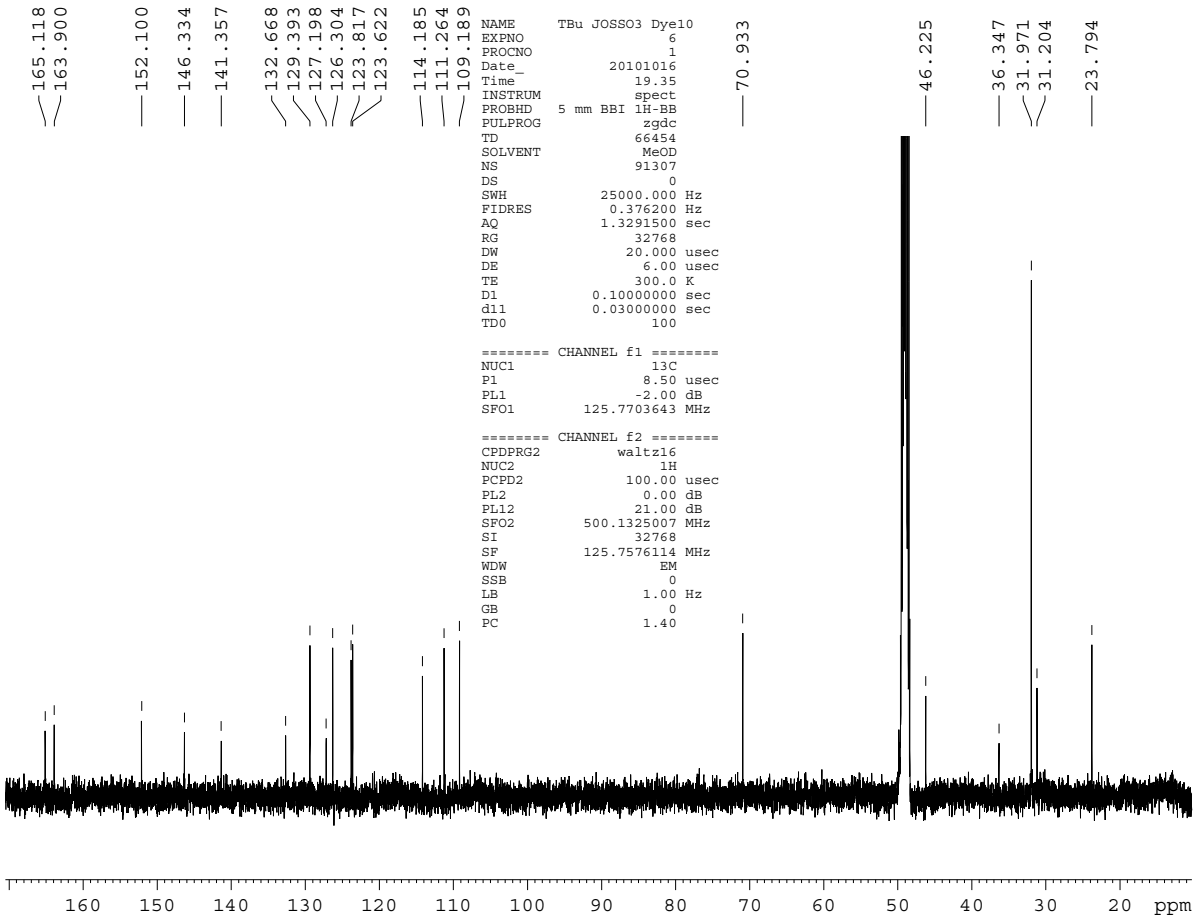


Figure S6. ^{13}C NMR spectrum of dye **10** t-butyl-OTB-SO_3 (300 MHz, CD_3OD)

UV-vis Spectra of OTB Dyes in Methanol

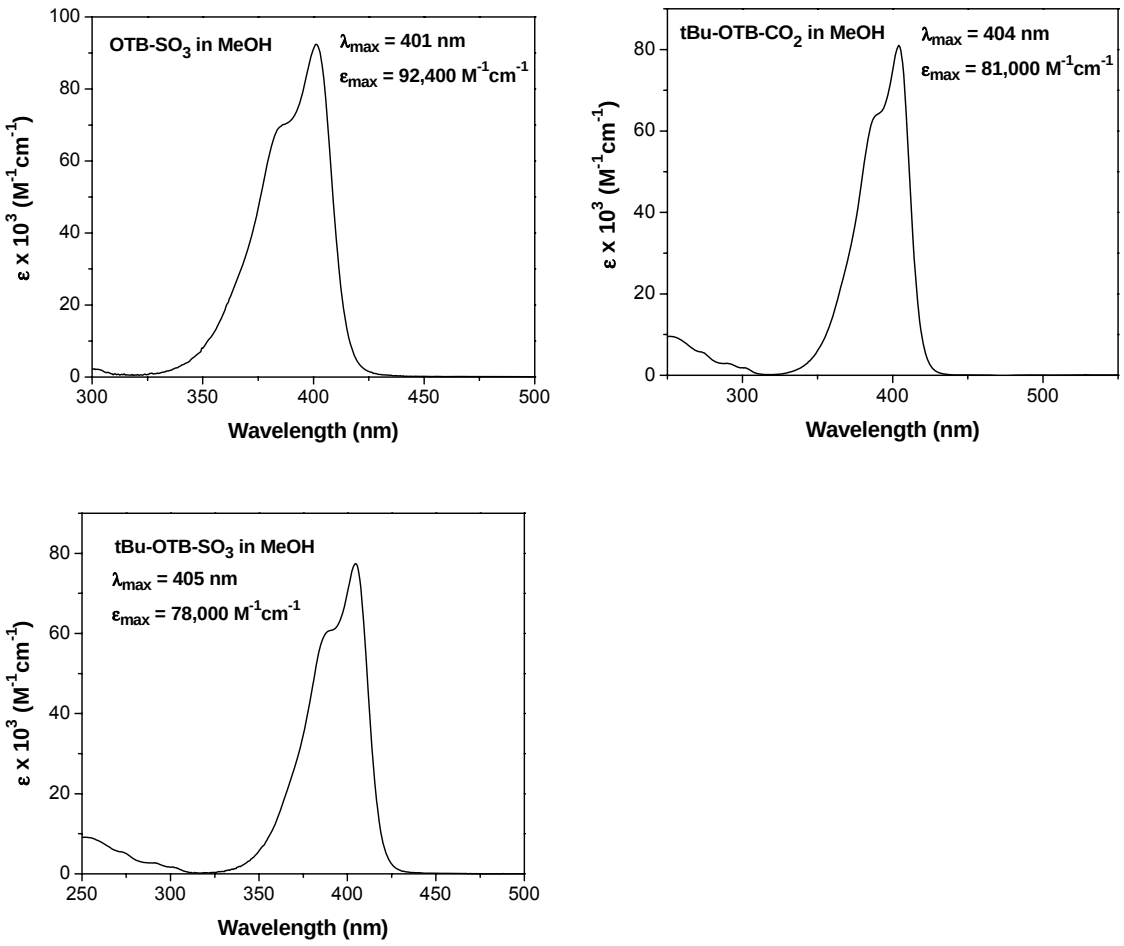


Figure S7. UV-vis spectra recorded of fluorogenic cyanine dyes in methanol.

Analytical HPLC of Dye 3 (OTB-SO_3)

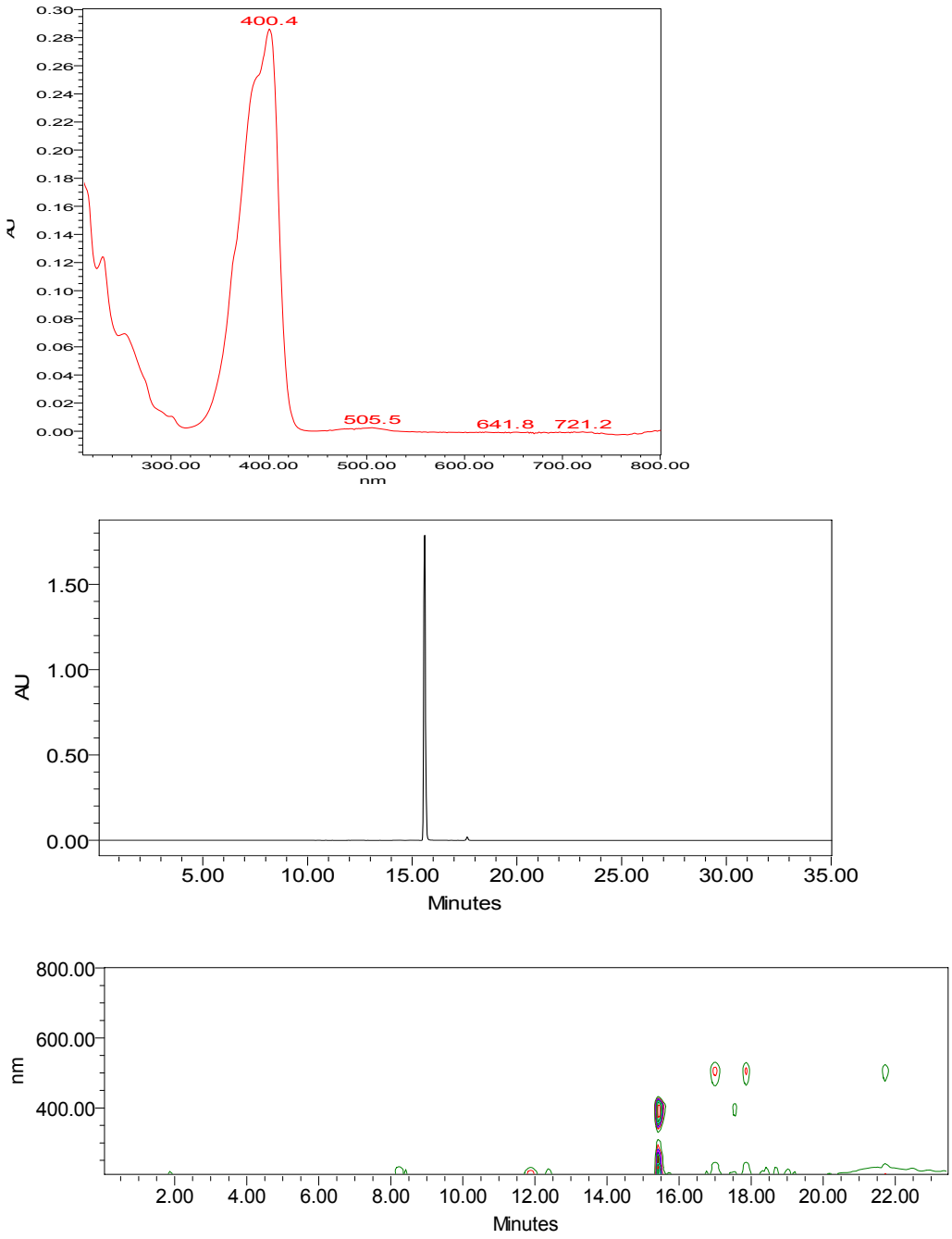


Figure S8. HPLC data for dye **3** OTB-SO_3.

Analytical HPLC of Dye 8 (t-Bu-OTB-CO_2)

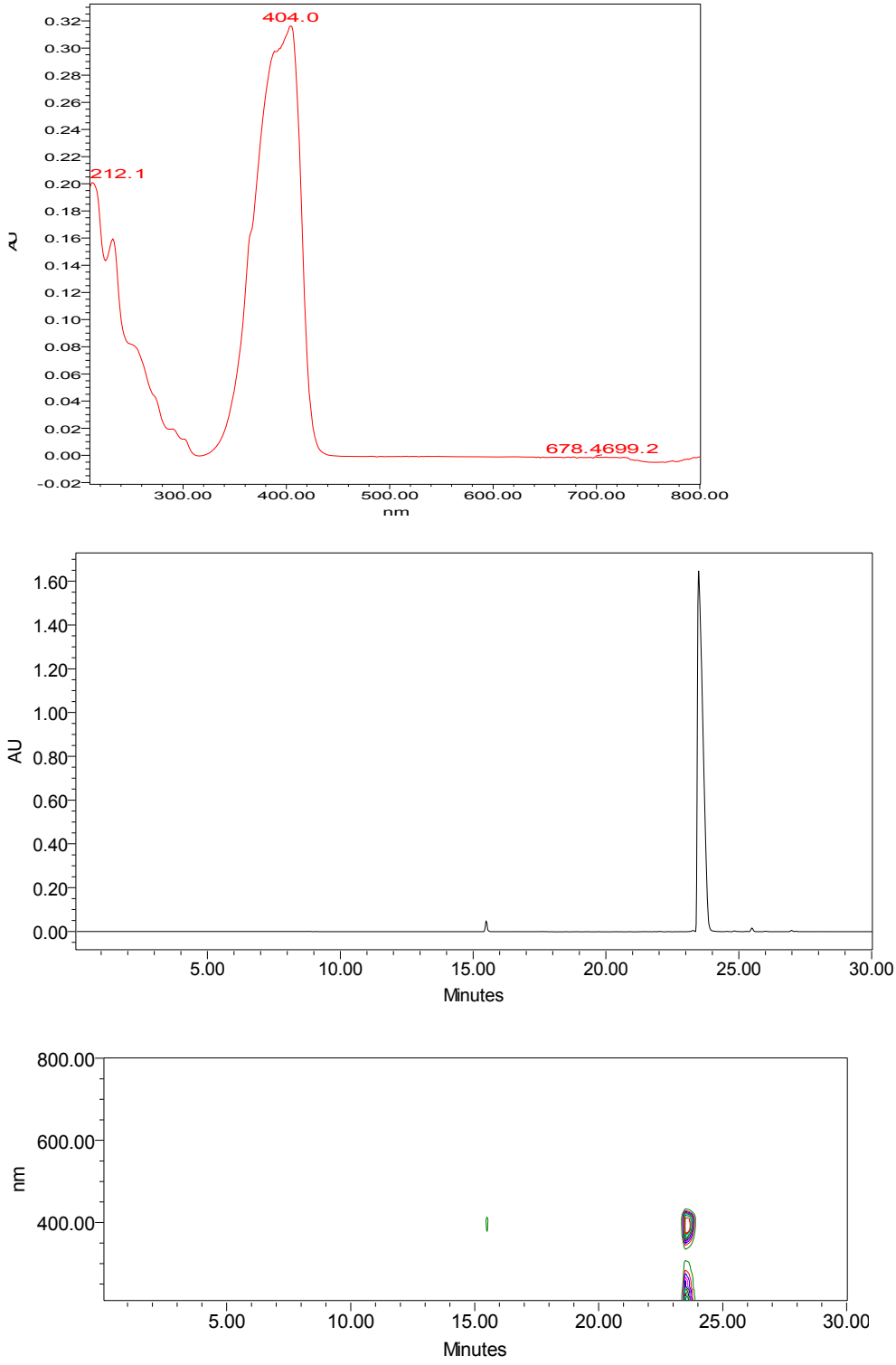


Figure S9. HPLC data for dye **8** t-Bu-OTB-CO_2.

Analytical HPLC of Dye 10 (t-Bu-OTB-SO_3)

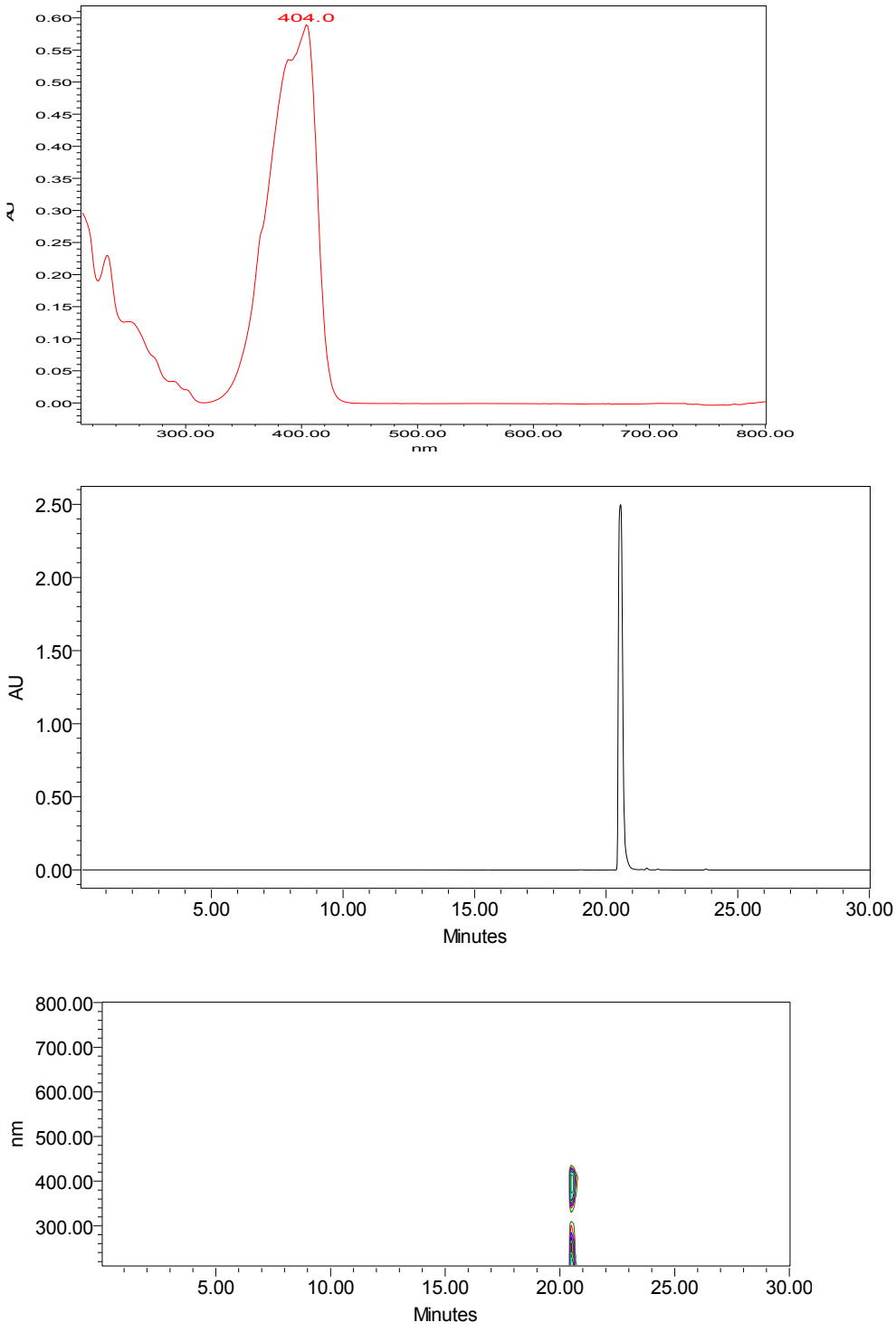


Figure S10. HPLC data for dye **10** t-Bu-OTB-SO_3.

2. Flow Cytometry Data

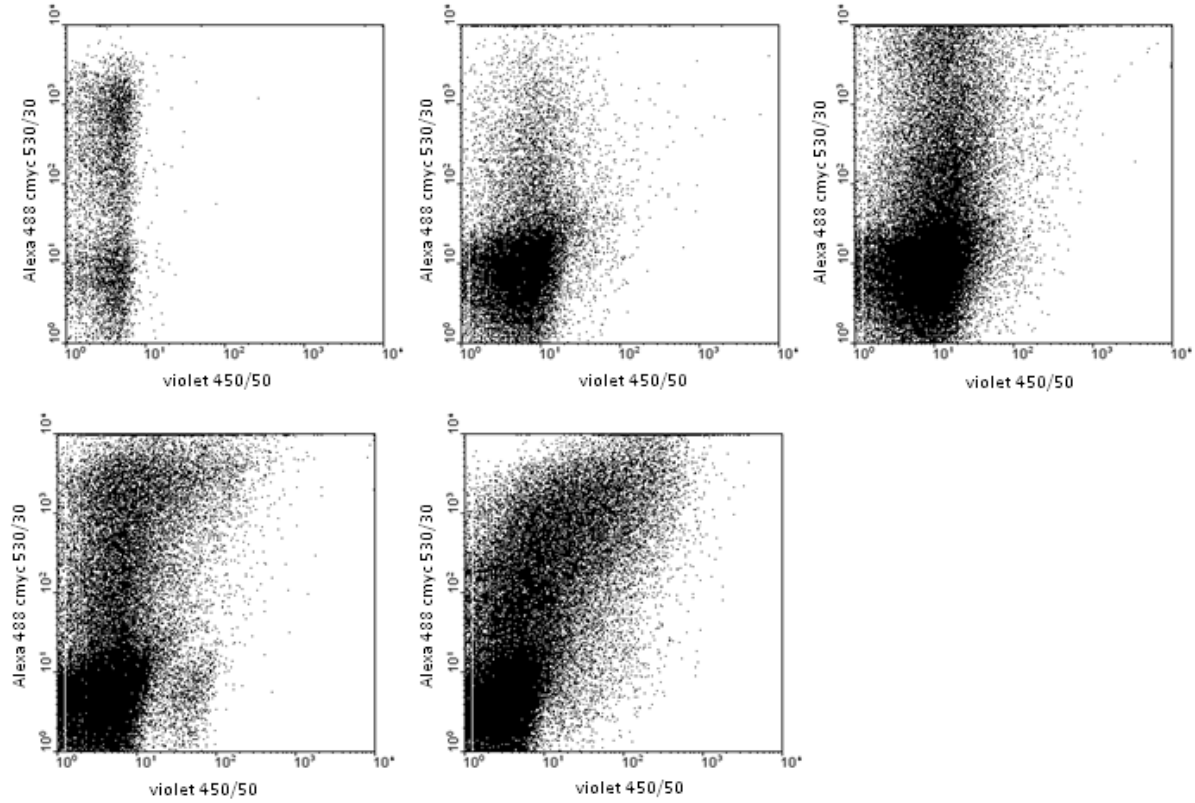


Figure S11. Data from five sequential rounds of flow cytometry of yeast-displayed library sorted for activation of OTB-SO_3 fluorescence. Violet signal is on the x-axis; induction measured by labeling with c-myc epitope tag and Alexa 488 antibody is on the y-axis.

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 bitotinylated 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 ε_{280} values)

A5 (25,737 kDa, ε_{280} = 43,010 $M^{-1}cm^{-1}$)
Q V Q L V E S E G G L V Q P G E S L R L S C A A S G F T F S G S W M A W V R Q P P G K G L E W V
A E L Q P D G S G K Y Y V D S V K G R F T I S R D N A K N S L Y L Q M N N L K A D D T A I Y Y C A
R D P S F G A F D Y W G Q G T L V T V S S G I L G S G G G G S G G G G S G G G G S Q S A L T Q P
A S V S G S P G Q S I T I S C T G T S S D V G G Y N Y V S W Y Q Q H P G K A P K L M I S D V T K R
P S G V P D R F S G S K S G N T A S L T I S G L Q T E D E A D Y Y C S S F T S T S S V I F G G G T K
V T V L S

A6 (26,823 kDa, ε_{280} = 51,260 $M^{-1}cm^{-1}$)
Q V Q L Q Q S G P G L V K P S Q T L S L T C A I S G D S V S S N S A V W N W I R Q S P S R G L E
W L G R T Y Y R S K W N N H Y A E S V K S R I T I N P D T S K N Q V V L T M S N M D P L D T A T Y
Y C A L S Y S S S P N D Y W G Q G I L V T V S S G S A S A P T G I L G S G G G G S G G G G S G G
G G S E I V M T Q S P A T L S L S P G E R A T L S C R A S Q S V S S Y L A W Y Q Q K P G Q A P R L
L I Y D A S N R A T G I P A R F S G S G S G T D F T L T I S S L E P E D F A V Y Y C Q Q Y G S L G T
F G Q G T K V D I K S

B11 (26,400 kDa, ε_{280} = 52,540 $M^{-1}cm^{-1}$)
Q V Q L Q Q S G P G L V K P S Q T L S L T C A I S G D S V S S N S A A W N W I R Q S P S R G F E
W L G R T Y Y R S K W F Y D Y A V S V K S R I T I N P D T S T N Q I S L Q L N S V T P E D T A V Y Y
C S R G R G V Y Y F D Y W D Q G T L V T V S S G I L G S G G G G S G G G G S G G G G S E I V M T
Q S P G T L S L S P G E S A T L S C R A S R S V S G N L A W Y Q Q K P G Q P P R L L I Y G A S T R
A T G I P A R F S G S G S G T E F T L T I S S L Q S E D F A V Y Y C Q Q Y N N L G T F G Q G T K L E
I K S

D10 (26,320 kDa, ε_{280} = 51,260 $M^{-1}cm^{-1}$)
Q V Q L Q Q S G P G L V K P S Q T L S L T C A I S G D S V S S N S V A W N W M R Q S P S R G L E
W L G R T Y Y R S K W F Y D Y A V S V K G R I S I N P D T S K N Q F S L Q L N S I N P D D T A V Y Y
C A R G A A V D G F D Y W G Q G T L V T V S S G I L G S G G G G S G G G G S G G G G S E I V L T
Q S P A T L S V S P G E R A T L S C R A S Q S V S S Y L A W Y Q Q K P G Q A P R L L I Y D A S S R
A A G L S D R F S G S G S G T D F T L T I S R L E P E D V A V Y Y C Q Q Y F R S G T F G Q G T K V
E I K S

H10 (26,277 kDa, ε_{280} = 47,420 $M^{-1}cm^{-1}$)
Q V Q L Q Q S G P G L V K P S Q T L S V T C V I S G D S V S S N S A V W N W I R Q S P S G G L E
W L G R I Y Y R S R W F F D Y A E S V K G R I T I N P D T S K N Q F S L Q L N S V T P E D T A M Y Y
C T R D G D L G L D T L D V W G Q G T M V T V S S G I L G S G G G G S G G G G S G G G G S E I V
L T Q S P A T L S L S P G E R A T L S C R A S Q F V S N S L A W Y Q Q K P G Q A P R L L I Y D A S
T R A T G I P A R F S G S G S A T D F T L T I S R L E P E D F A V Y Y C Q Q Y G G T F G G G T K L E
I K S

J10 (26,273 kDa, ε_{280} = 41,730 $M^{-1}cm^{-1}$)
Q V Q L V Q S G A E V K R P G S S V K V S C K A S G G A F S S S A N S W V R Q A P G Q G L E W
M G G I I P V F G T P N Y A Q K F Q G R V T I T A D E S T R T T Y M E L S S L R S E D T A V Y Y C A
R V L G S G I D L T G Y I D L W G R G T L V T V S S G I L G S G G G G S G G G G S G G G G D S E I V
L T Q S P A T L S V S P G E R A T L S C R A S Q S V D N K L A W Y Q Q K P G Q A P R L L I Y G A S
T R A T G I P A R F S G G G S G T E F T L T I S G L Q S E D F A V Y Y C Q Q Y T D R P S W T F G Q
G T K V E I K S

4. Fluorescence Spectroscopy of DNA-Binding Fluorogens

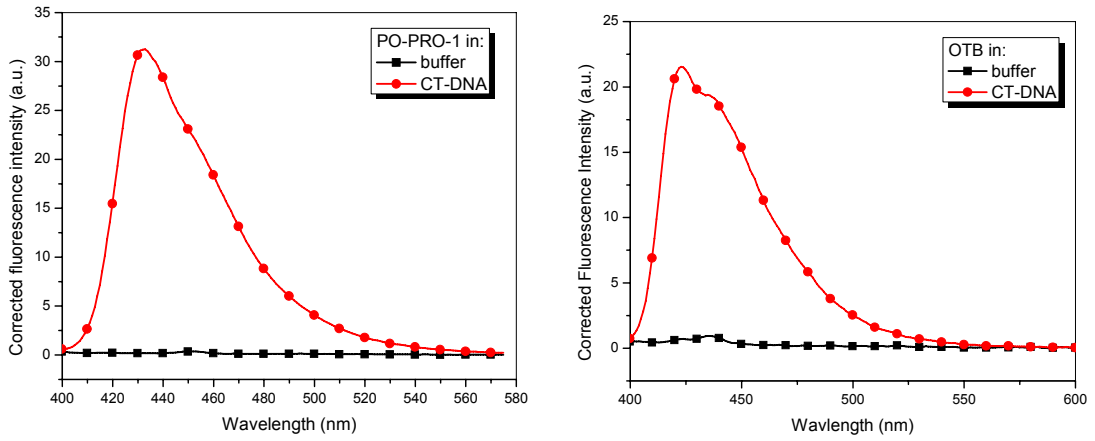


Figure S12. Emission spectra of 200 nM PO-PRO-1 (left) or OTB (right) in buffer or in presence of 100 μM base pairs calf thymus DNA

5. Absorbance Spectra of FAP-Bound Fluorogens

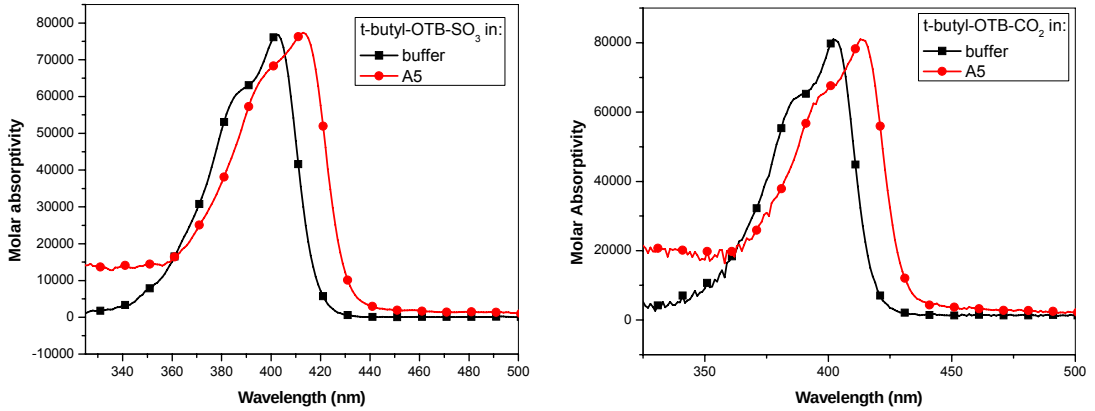


Figure S13. Absorption spectra of 1.2 μM *t*-butyl-OTB-SO_3 (left) or *t*-butyl-OTB-CO_2 (right) in the presence and absence of A5 soluble protein.

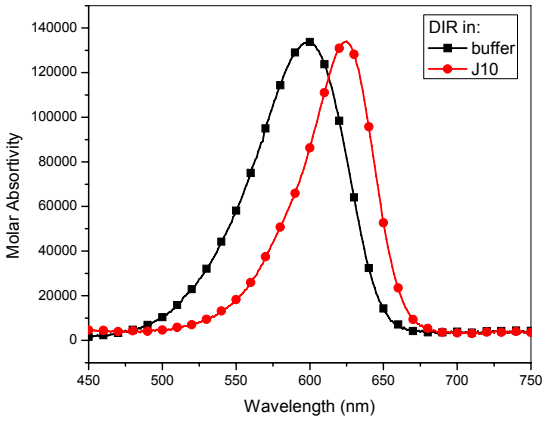


Figure S14. Absorption spectra of 750 nM DIR in the presence of buffer or 1.5 μM soluble **J10** protein.

6. Temperature Dependence of H10-OTB-SO_3 Emission Spectrum

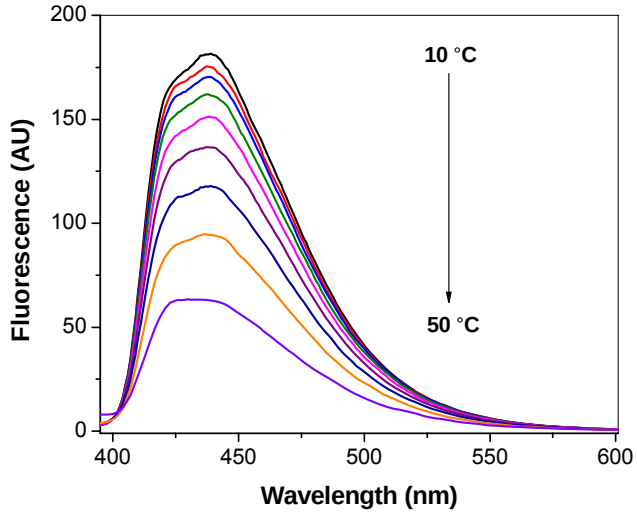


Figure S15. The fluorescence of 200 nM OTB-SO_3/H10 was measured in 5 degree increments from 10°C to 50°C. Only slight changes are observed in the profile at the highest temperature, where the protein begins to undergo

7. pH-Dependence of H10-OTB-SO_3 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 $Na_2B_4O_7$/ 1 M NaCl pH= 9).

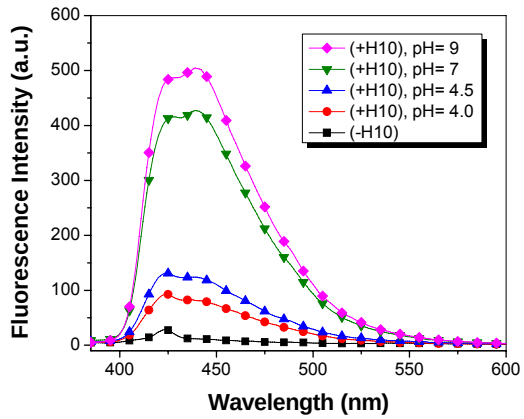


Figure S16. The fluorescence of 200 nM OTB-SO_3 /H10 in various pH buffers. Samples were excited at 370 nm.

8. K_d 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.

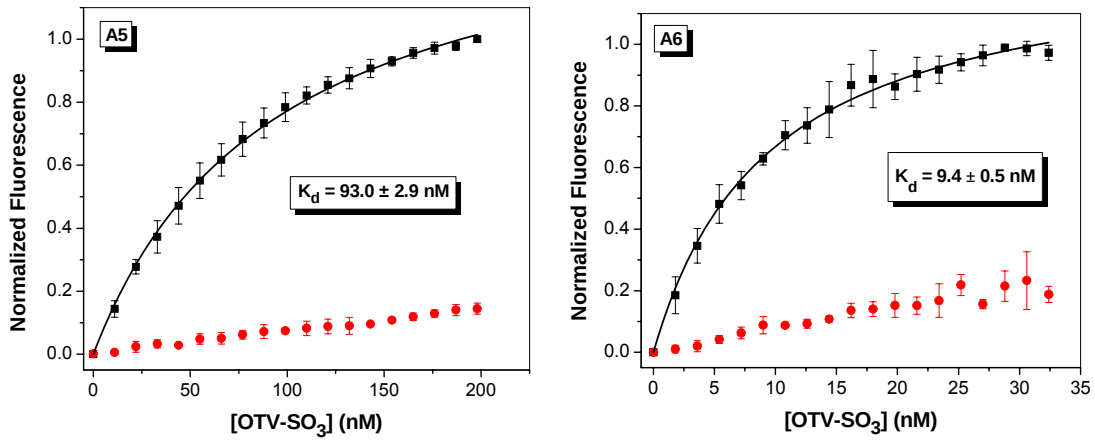


Figure S17. Fluorescence titrations of OTB-SO_3 into 50 nM soluble **A5** (left) or **A6** (right).

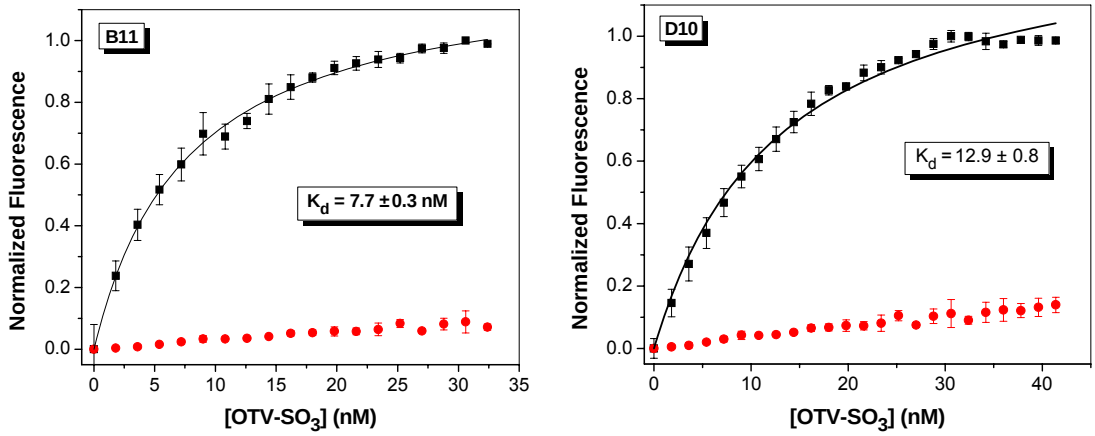


Figure S18. Fluorescence titrations of OTB-SO_3 into 50 nM soluble **B11** (left) or **D10** (right).

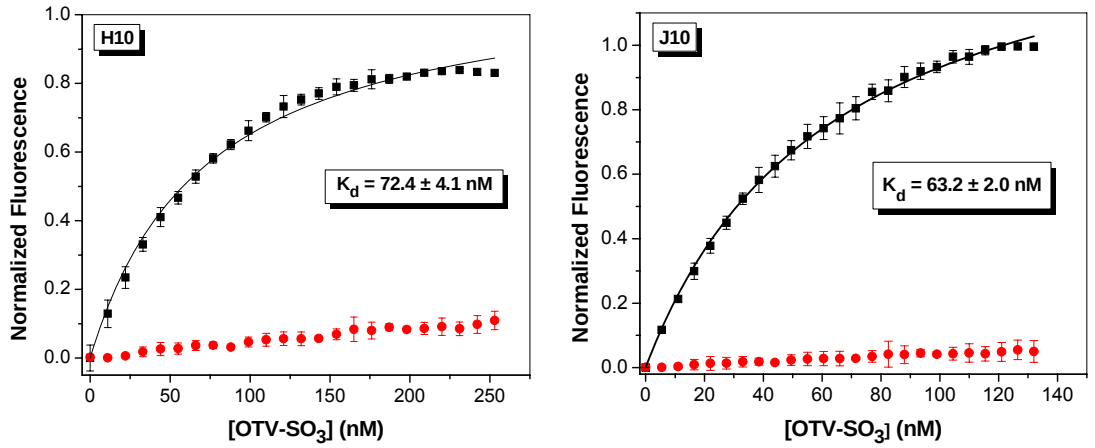


Figure S19. Fluorescence titrations of OTB-SO_3 into 50 nM soluble **H10** (left) or **J10** (right).

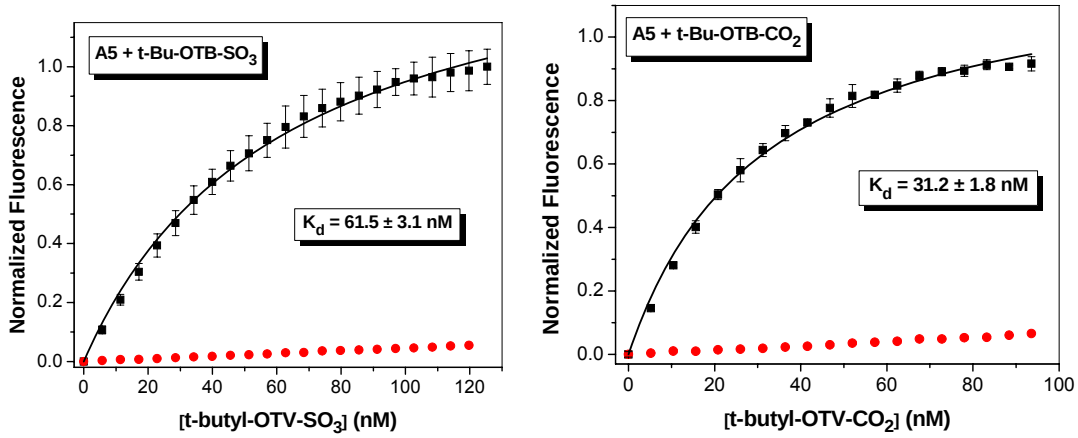


Figure S20. Fluorescence titrations of *t*-Bu-OTB-SO_3 (left) or *t*-Bu-OTB-CO_2 (right) into 50 nM soluble **A5**.

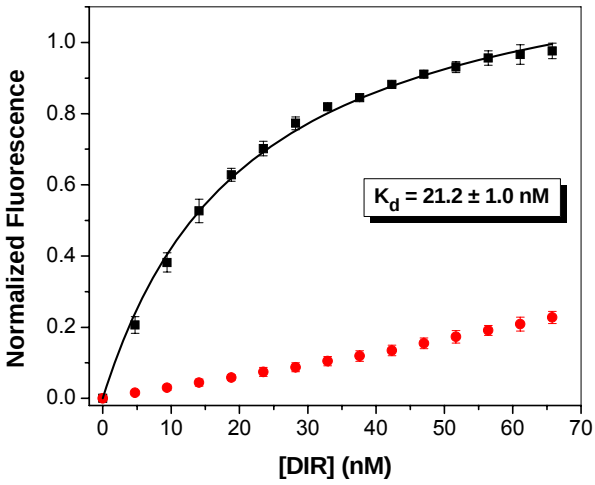


Figure S21. Fluorescence titrations of DIR into 50 nM **J10**.

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.

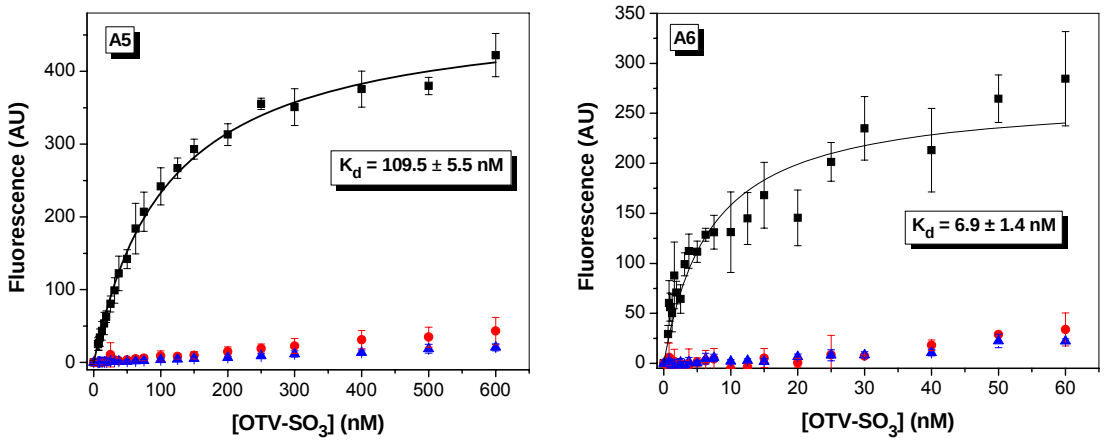


Figure S22. Fluorescence titrations of *t*-Bu-OTB-SO_3 into yeast-displayed **A5** (left) or **A6** (right).

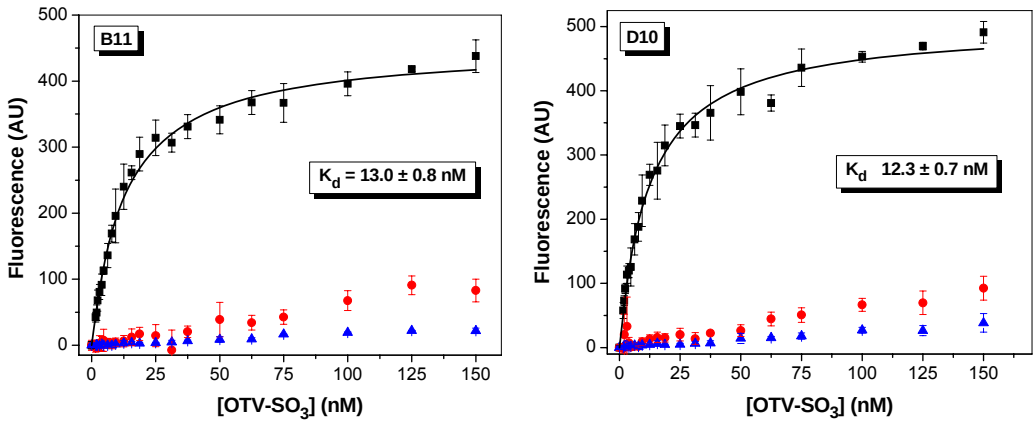


Figure S23. Fluorescence titrations of *t*-Bu-OTB-SO_3 into yeast-displayed **B11** (left) or **D10** (right).

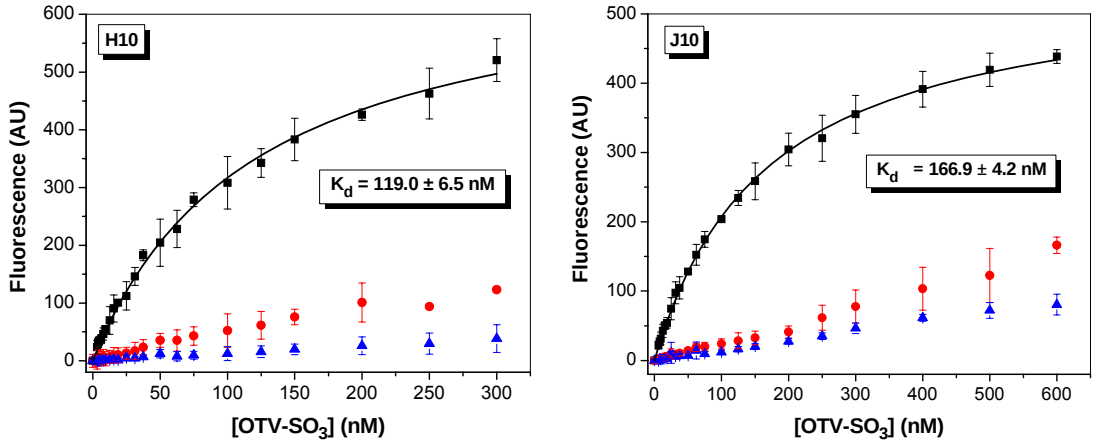


Figure S24. Fluorescence titrations of *t*-Bu-OTB-SO_3 into yeast-displayed **H10** (left) or **J10** (right).

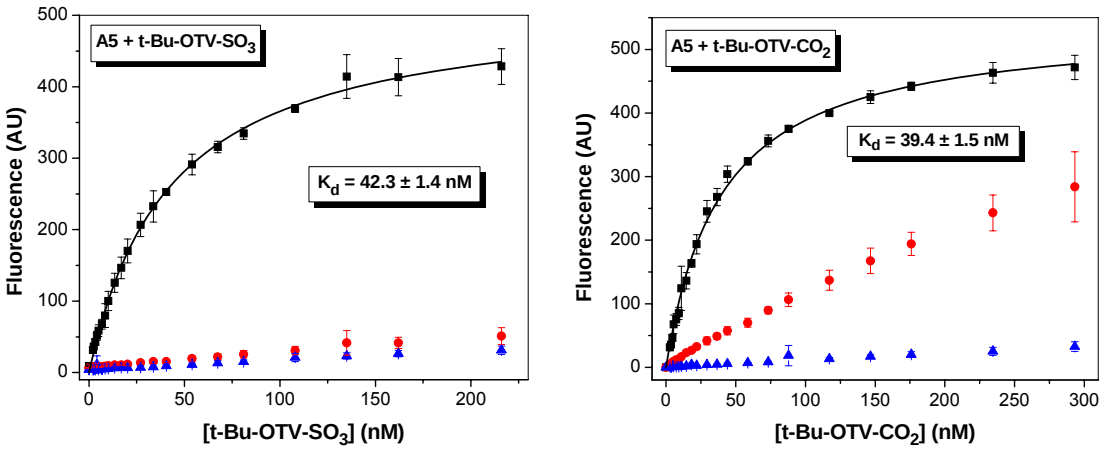


Figure S25. Fluorescence titrations of *t*-Bu-OTB-SO_3 (left) or *t*-Bu-OTB-CO_2 (right) into yeast-displayed **A5**.

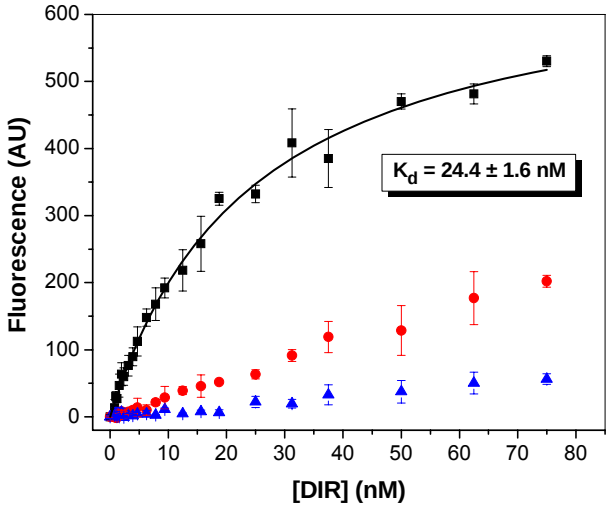


Figure S26. Fluorescence titrations of DIR into yeast-displayed **J10**.

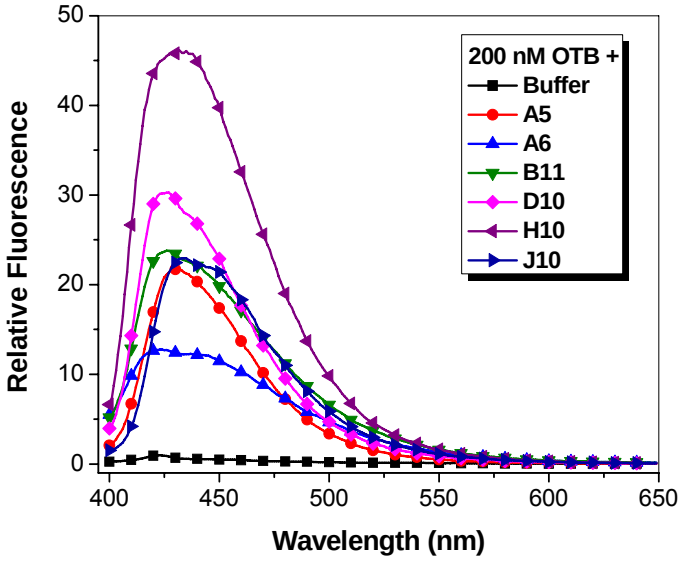


Figure S27. Relative fluorescence spectra were measured for 200 nM OTB and 1 μM soluble FAP. Samples were excited at 370 nm and spectra were divided by the value of the buffer spectrum at 423 nm to give the relative fluorescence values.

9. Microscopy Images

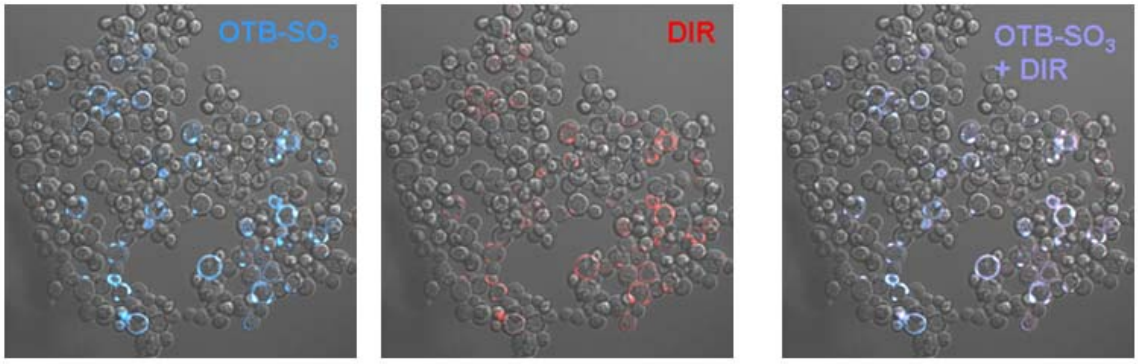


Figure S28. Overlay of fluorescence and differential contrast images of J10 cells labeled with 200 nM DIR (red) and OTB-SO_3 (blue), along with the merged fluorescence signal from both dyes (purple).

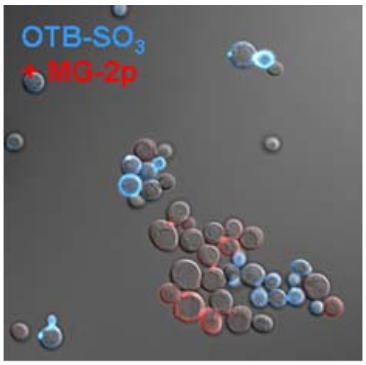


Figure S29. Overlay of fluorescence and differential contrast images of H10 cells labeled with OTB-SO_3 (blue) and H6-MG cells labeled with MG-2p (red).