Electronic Supplementary Information

Generalizable synthesis of bioresponsive near-infrared fluorescent probes: sulfonated heptamethine cyanine prototype for imaging cell hypoxia

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1. General
Reagents and solvents were purchased from Sigma-Aldrich, VWR, Oakwood, Alfa Aesar, and Thermo Fisher and used without further purification unless stated otherwise. Column Chromatography was performed using Biotage SNAP Ultra columns. $^1$H NMR spectra were recorded on a Bruker 400 NMR spectrometer. $^{13}$C NMR spectra were recorded on a Bruker 500 NMR spectrometer. Chemical shifts are presented in ppm and referenced by residual solvent peaks. High-resolution mass spectrometry (HRMS) was performed using a time-of-flight (TOF) analyzer with electrospray ionization (ESI). Absorption spectra were recorded on an Evolution 201 UV/vis spectrometer with Thermo Insight software. Fluorescence spectra were collected on a Horiba Fluoromax-4 fluorometer with FluoroEssence software. Analyte solutions were prepared in HPLC grade dimethyl sulfoxide (Alfa Aesar), HPLC grade methanol (Sigma-Aldrich), or phosphate buffered saline (Thermo Fisher). All absorption and fluorescence spectra were collected using quartz cuvettes (1 mL, 1 cm path length; for emission and excitation spectra, slit width = 5 nm, unless stated otherwise).

2. Synthesis and Characterization

Scheme S1. General scheme for the synthesis of heptamethine cyanine dyes 1-6
Compound S1. To a mixture of dry 2,4-dinitrophenol (9.24 g, 50.19 mmol) and tosyl chloride (10.52 g, 51.83 mmol, 1.01 eq), in DCM (100 mL), triethylamine (17.3 mL, 124.12 mmol, 2.5 eq) was added and stirred overnight. The reaction mixture was mixed with 50 mL of water, and the layers separated after shaking. The aqueous layer was washed two times with 60 mL portions of DCM and all organic layers were combined. The organic layer was then washed with 100 mL NaHCO₃, followed by 100 mL of brine. The organic layer was separated and evaporated under reduced pressure. A trituration was performed with hot (40°C) methanol to give the product, S1, as an off yellow white powder (15.42 g, 91%). ¹H NMR (400 MHz, Chloroform-d) δ 8.70 (d, J = 2.8 Hz, 1H), 8.42 (dd, J = 9.1, 2.8 Hz, 1H), 7.73 (d, J = 8.4 Hz, 2H), 7.66 (d, J = 9.0 Hz, 1H), 7.33 (d, J = 8.1 Hz, 2H), 2.43 (s, 3H).

¹H NMR spectrum (400 MHz, CDCl₃, 25°C) of S1
Compound S2. 2,3,3-Trimethyl-3H-indole (1.5 mL, 9.34 mmol, 1 eq) was added to iodomethane (2.9 mL, 46.7 mmol, 5 eq) in acetonitrile (30 mL). The reaction mixture was refluxed overnight. The precipitate was filtered and washed with diethyl ether (20 mL) and hexanes (5 mL) and air-dried to give S3 as a reddish-purple solid (2.76 g, 98%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.94 – 7.88 (m, 1H), 7.85 – 7.80 (m, 1H), 7.67 – 7.58 (m, 2H), 3.97 (d, $J = 1.0$ Hz, 3H), 2.76 (d, $J = 1.0$ Hz, 3H), 1.52 (s, 6H).

$^1$H NMR spectrum (400 MHz, DMSO-$d_6$, 25°C) of S2
Compound S3. A mixture of 1,3-propanesultone (1.0 mL, 7.5 mmol, 1 eq), 2,3,3-Trimethyl-3H-indole (2.4 mL, 15 mmol, 2 eq), and acetonitrile (20 mL) was refluxed for 24 hr. The solution was then cooled to room temperature and stored at 0°C for 30 min. A mixture of methanol (20 mL), hexanes (100 mL), and diethyl ether (200 mL) was added to the cooled reaction mixture and sonicated for 10 min. The mixture was then filtered leaving the pink precipitate as the product, S3 (1.90 g, 90%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.05 (d, \(J = 7.2\) Hz, 1H), 7.82 (d, \(J = 5.9\) Hz, 1H), 7.69 – 7.56 (m, 2H), 4.65 (t, \(J = 8.0\) Hz, 2H), 2.83 (s, 3H), 2.62 (t, \(J = 6.5\) Hz, 2H), 2.15 (t, \(J = 7.8\) Hz, 2H), 1.53 (s, 6H).

\(^1\)H NMR spectrum (400 MHz, DMSO-\(d_6\), 25°C) of S3
Compound S4. A mixture of isonicotinic acid (6.43 g, 52.23 mmol, 1 eq) and thionyl chloride (7 mL, 95.97 mmol, 1.9 eq) was refluxed for 3 hours under an inert argon atmosphere. The thionyl chloride was evaporated at a reduced pressure and dry DCM (40 mL) was added. 4-Nitrobenzyl alcohol (8.68 g, 56.68 mmol, 1.1 eq) was added at once and triethylamine (14.3 mL, 102.59 mmol, 2 eq) was then added dropwise under an inert argon atmosphere and stirred overnight. DCM (40 mL) was added and the reaction mixture was washed with 3 x 100 mL of NaHCO₃ and 3 x 100 mL portions of brine. The organic extracts were dried over anhydrous MgSO₄ and filtered. Solvent was removed under reduced pressure to give a pale yellow white solid as the product, S4 (12.04 g, 91%). ¹H NMR (400 MHz, Chloroform-d) δ 8.84 (d, J = 6.0, 2H), 8.30 (d, J = 8.7 Hz, 2H), 7.91 (d, J = 6.0, 2H), 7.64 (d, J = 8.7 Hz, 2H), 5.51 (s, 2H).

¹H NMR spectrum (400 MHz, CDCl₃, 25°C) of S4
Compound S6. A mixture of S5 (464 µL, 3.93 mmol, 1 eq) and S1 (1.40 g, 4.14 mmol, 1.1 eq) in toluene (20 mL) was refluxed for 48 hr. The reaction mixture was then allowed to cool to room temperature then stored in a -20°C freezer for 20 min. The precipitate was vacuum filtered, washed with diethyl ether (10 mL), and the remaining solvent was removed in vacuo to afford the dry pale yellow white solid as product S6 (1.63 g, 25%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.59 (d, $J = 6.9$ Hz, 2H), 9.13 (d, $J = 2.5$ Hz, 1H), 8.99 (dd, $J = 8.7$, 2.5 Hz, 1H), 8.80 (d, $J = 6.9$ Hz, 2H), 8.40 (d, $J = 8.7$ Hz, 1H), 7.46 (d, $J = 8.1$ Hz, 2H), 7.11 (d, $J = 7.9$ Hz, 2H), 4.05 (s, 3H), 2.29 (s, 3H).

$^1$H NMR spectrum (400 MHz, DMSO-$d_6$, 25°C) of S6
Compound S7. The carboxylic acid S7 was synthesized by the hydrolysis of S6 (1.93 g, 4.05 mmol) in aq HCl (6 M, 20 mL) at 50°C for 48 hr. The solvent was removed under reduced pressure and the crude product was recrystallized in methanol (10 mL) to afford the pure pale yellow white solid as the product S7 (1.74 g, 93%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ 9.55 (d, $J = 6.9$, 2H), 9.13 (d, $J = 2.5$ Hz, 1H), 8.99 (dd, $J = 8.7$, 2.6 Hz, 1H), 8.75 (d, $J = 6.9$, 2H), 8.39 (d, $J = 8.7$ Hz, 1H), 7.46 (d, $J = 8.1$ Hz, 2H), 7.11 (d, $J = 7.8$ Hz, 2H), 2.29 (s, 3H).

$^1$H NMR spectrum (400 MHz, DMSO-d$_6$, 25°C) of S7
Compound S8. A mixture of S4 (12.04 g, 46.26 mmol, 1 eq) and S1 (17.28 g, 51.07 mmol, 1.1 eq) in toluene (50 mL) was refluxed for 48 hr. The reaction mixture was then allowed to cool to room temperature then stored in a -20°C freezer for 20 min. The precipitate was vacuum filtered, washed with diethyl ether (10 mL), and the remaining solvent was removed in vacuo to afford the dry white solid as product S8 (11.45 g, 41%). ¹H NMR (400 MHz, DMSO-d₆) δ 9.61 (d, J = 6.9 Hz, 2H), 9.14 (d, J = 2.5 Hz, 1H), 8.99 (dd, J = 8.7, 2.5 Hz, 1H), 8.87 (d, J = 6.9 Hz, 2H), 8.40 (d, J = 8.7 Hz, 1H), 8.32 (d, J = 8.8 Hz, 2H), 7.88 (d, J = 8.8 Hz, 2H), 7.46 (d, J = 8.1 Hz, 2H), 7.11 (d, J = 7.7 Hz, 2H), 5.68 (s, 2H), 2.29 (s, 3H).

¹H NMR spectrum (400 MHz, DMSO-d₆, 25°C) of S8
Compound 1. A mixture of S7 (167 mg, 0.35 mmol, 1 eq) and 4-bromoaniline (80 mg, 0.47 mmol, 1.2 eq) in methanol (4 mL) was stirred for 30 min at room temp, followed by the addition of S2 (232 mg, 0.77 mmol, 2.2 eq) and sodium acetate (179 mg, 1.41 mmol, 6 eq) at the same time. The reaction was stirred overnight at room temp in the dark. The reaction vessel was then flushed with diethyl ether (50 mL) and stored in a -20°C freezer for 20 min. The precipitate was collected by vacuum filtration and the filter cake was purified by column chromatography (SiO2, 0-10% MeOH in DCM) to afford 1 as a lustrous purple with a green complexion (104 mg, 66%). \(^1\)H NMR (400 MHz, Methanol-\textit{d}4) δ 8.09 (t, \(J = 13.4\) Hz, 2H), 7.47 (d, \(J = 7.4\) Hz, 2H), 7.40 (t, \(J = 7.7\) Hz, 2H), 7.25 (d, \(J = 7.8\) Hz, 4H), 6.34 (d, \(J = 13.2\) Hz, 2H), 6.24 (d, \(J = 13.6\) Hz, 2H), 3.59 (s, 6H), 1.67 (s, 12H). HRMS (ESI-TOF) m/z: [M + H]\(^+\) calcd for C\(_{30}\)H\(_{33}\)N\(_2\)O\(_2\)\(^+\) 453.2537, found 453.2533.

\(^1\)H NMR spectrum (400 MHz, MeOD-\textit{d}4, 25°C) of 1
 Compound 2. A mixture of S6 (331 mg, 0.70 mmol, 1 eq) and 4-bromoaniline (150 mg, 0.87 mmol, 1.2 eq) in methanol (4 mL) was stirred for 30 min at room temp, followed by the addition of S2 (634 mg, 2.10 mmol, 3 eq) and sodium acetate (350 mg, 4.27 mmol, 6 eq) at the same time. The reaction was stirred overnight at room temp in the dark. The reaction vessel was then flushed with diethyl ether (50 mL) and stored in a -20°C freezer for 20 min. The precipitate was collected by vacuum filtration and the filter cake was purified by column chromatography (SiO₂, 0-10% MeOH in DCM) to afford 5 as a light green powder. (298 mg, 72%). ¹H NMR (400 MHz, Chloroform-d) δ 8.11 (t, J = 13.3 Hz, 2H), 7.36 – 7.26 (m, 4H), 7.16 (t, J = 7.4 Hz, 2H), 7.06 (d, J = 8.0 Hz, 2H), 6.98 (d, J = 13.2 Hz, 2H), 6.69 (d, J = 13.5 Hz, 2H), 3.91 (s, 3H), 3.72 (s, 6H), 1.62 (s, 12H). HRMS (ESI-TOF) m/z: [M + H]⁺ calcd for C₃₁H₃₅N₂O₂⁺ 467.2693, found 467.2663.
$^1$H NMR spectrum (400 MHz, CDCl$_3$, 25°C) of 2

HRMS (ESI-TOF) spectrum of 2
Compound 3. A mixture of S7 (163 mg, 0.35 mmol, 1 eq) and 4-bromoaniline (73 mg, 0.42 mmol, 1.2 eq) in methanol (4 mL) was stirred for 30 min at room temp, followed by the addition of S3 (219 mg, 0.78 mmol, 2.2 eq) and sodium acetate (172 mg, 2.10 mmol, 6 eq) at the same time. The reaction was stirred overnight at room temp in the dark. The reaction vessel was then flushed with diethyl ether (50 mL) and stored in a -20°C freezer for 20 min. The precipitate was collected by vacuum filtration and the filter cake was washed with a 1:1 mixture of dichloromethane (2 mL) and diethyl ether (2 mL). The filter cake was then further purified by reverse phase column chromatography (C18, 0-50% MeOH in H2O) to afford the product 3 as a dark purple solid (124 mg, 51%).

1H NMR (400 MHz, Deuterium Oxide) δ 7.78 (t, J = 13.4 Hz, 2H), 7.41 (d, J = 7.4 Hz, 2H), 7.31 (t, J = 7.8 Hz, 2H), 7.16 (m, J = 12.3, 7.4 Hz, 4H), 6.24 – 6.13 (t, 4H), 4.10 – 4.03 (t, 4H), 2.10 (p, J = 7.1, 6.7 Hz, 4H), 1.52 (s, 12H). HRMS (ESI-TOF) m/z: [M + H]+ calcd for C34H39N2O8S2 - 667.2153, found 667.2180.

1H NMR spectrum (400 MHz, D2O, 25°C) of 3
A mixture of S6 (166 mg, 0.35 mmol, 1 eq) and 4-bromoaniline (75 mg, 0.44 mmol, 1.2 eq) in methanol (4 mL) was stirred for 30 min at room temp, followed by the addition of S3 (173.4 mg, 0.62 mmol, 2 eq) and sodium acetate (173 mg, 2.10 mmol, 6 eq) at the same time. The reaction was stirred overnight at room temp in the dark. The reaction vessel was then flushed with diethyl ether (50 mL) and stored in a -20°C freezer for 20 min. The precipitate was collected by vacuum filtration. The filter cake was then purified by reverse phase column chromatography (C18, 0-50% MeOH in H2O) to afford the product 3 as a dark purple solid with a blue complexation (76 mg, 31%).

1H NMR (400 MHz, Methanol-d4) δ 7.96 (t, J = 13.4 Hz, 2H), 7.40 (d, J = 7.4 Hz, 2H), 7.38 - 7.27 (m, 4H), 7.17 (t, J = 7.9 Hz, 2H), 6.61 (d, J = 13.2 Hz, 2H), 6.48 (d, J = 13.6 Hz, 2H), 4.25 (t, J = 8.0 Hz, 4H), 3.92 (s, 3H), 2.89 (t, J = 6.9 Hz, 4H), 2.15 (p, J = 7.1 Hz, 4H), 1.57 (s, 12H). 13C NMR (126 MHz, Methanol-d4, 25°C) δ (ppm): 172.6, 167.6, 146.3, 142.3, 141.5, 128.8, 125.4, 122.9, 122.3, 111.3, 106.1, 52.0, 49.4, 48.3, 42.9, 27.0, 23.2. HRMS (ESI-TOF) m/z: [M + H]+ calcd for C35H41N5O8S2 681.2310, found 681.2291.

Compound 4. A mixture of S6 (166 mg, 0.35 mmol, 1 eq) and 4-bromoaniline (75 mg, 0.44 mmol, 1.2 eq) in methanol (4 mL) was stirred for 30 min at room temp, followed by the addition of S3 (173.4 mg, 0.62 mmol, 2.2 eq) and sodium acetate (173 mg, 2.10 mmol, 6 eq) at the same time. The reaction was stirred overnight at room temp in the dark. The reaction vessel was then flushed with diethyl ether (50 mL) and stored in a -20°C freezer for 20 min. The precipitate was collected by vacuum filtration. The filter cake was then purified by reverse phase column chromatography (C18, 0-50% MeOH in H2O) to afford the product 3 as a dark purple solid with a blue complexation (76 mg, 31%). 1H NMR (400 MHz, Methanol-d4) δ 7.96 (t, J = 13.4 Hz, 2H), 7.40 (d, J = 7.4 Hz, 2H), 7.38 - 7.27 (m, 4H), 7.17 (t, J = 7.9 Hz, 2H), 6.61 (d, J = 13.2 Hz, 2H), 6.48 (d, J = 13.6 Hz, 2H), 4.25 (t, J = 8.0 Hz, 4H), 3.92 (s, 3H), 2.89 (t, J = 6.9 Hz, 4H), 2.15 (p, J = 7.1 Hz, 4H), 1.57 (s, 12H). 13C NMR (126 MHz, Methanol-d4, 25°C) δ (ppm): 172.6, 167.6, 146.3, 142.3, 141.5, 128.8, 125.4, 122.9, 122.3, 111.3, 106.1, 52.0, 49.4, 48.3, 42.9, 27.0, 23.2. HRMS (ESI-TOF) m/z: [M + H]+ calcd for C35H41N5O8S2 681.2310, found 681.2291.
$^1$H NMR spectrum (400 MHz, MeOD-$d_4$, 25°C) of 4

$^{13}$C NMR spectrum (126 MHz, MeOD-$d_4$, 25°C) of 4
Compound 5. A mixture of S8 (212 mg, 0.35 mmol, 1 eq) and 4-bromoaniline (73 mg, 0.42 mmol, 1.2 eq) in methanol (4 mL) was stirred for 30 min at room temp, followed by the addition of S2 (314 mg, 1.05 mmol, 3 eq) and sodium acetate (173 mg, 2.10 mmol, 6 eq) at the same time. The reaction was stirred overnight at room temp in the dark. The reaction vessel was then flushed with diethyl ether (50 mL) and stored in a -20°C freezer for 20 min. The precipitate was collected by vacuum filtration and the filter cake was then further purified by column chromatography (SiO2, 0-10% MeOH in DCM) to afford the product 5 as a dark purple solid with a green complexion (148 mg, 59%). 1H NMR (400 MHz, Chloroform-d) δ 8.31 (d, J = 8.7 Hz, 2H), 8.23 – 8.13 (t, J = 16.0 Hz, 2H), 7.71 (d, J = 8.7 Hz, 2H), 7.40 (t, J = 8.2 Hz, 2H), 7.35 (d, J = 7.0 Hz, 2H), 7.26 (t, J = 7.4 Hz, 2H), 7.15 (t, J = 9.7 Hz, 2H), 6.85 (d, J = 13.1 Hz, 2H), 5.51 (s, 2H), 3.82 (s, 6H), 1.60 (s, 12H). 13C NMR (126 MHz, CDCl3, 25°C) δ (ppm): 172.6, 166.7, 148.2, 145.4, 143.0, 142.7, 141.3, 129.3, 128.9, 125.6, 124.2, 110.9, 108.1, 105.0, 66.0, 49.4, 33.3, 28.1. HRMS (ESI-TOF) m/z: [M + H]+ calcd for C37H36N3O4+ 588.2857, found 588.2846.
$^1$H NMR spectrum (400 MHz, CDCl$_3$, 25°C) of 5

$^{13}$C NMR spectrum (126 MHz, CDCl$_3$, 25°C) of 5
Compound 6. A mixture of S8 (216 mg, 0.36 mmol, 1 eq) and 4-bromoaniline (77 mg, 0.45 mmol, 1.2 eq) in methanol (4 mL) was stirred for 30 min at room temp, followed by the addition of S3 (296 mg, 1.05 mmol, 3 eq) and sodium acetate (174 mg, 2.12 mmol, 6 eq) at the same time. The reaction was stirred overnight at room temp in the dark. The reaction vessel was then flushed with diethyl ether (50 mL) and stored in a -20°C freezer for 20 min. The precipitate was collected by vacuum filtration. The filter cake was then purified by column chromatography (SiO2, 0-10% MeOH in DCM) to afford the product 6 as a dark purple solid with a blue tint (113 mg, 38%). 1H NMR (400 MHz, DMSO-d6) δ 8.33 (d, J = 8.7 Hz, 2H), 7.89 (d, J = 14.9 Hz, 2H), 7.54 (t, J = 7.5 Hz, 4H), 7.42 (t, J = 7.6 Hz, 2H), 7.27 (t, J = 7.5 Hz, 2H), 6.75 (d, J = 14.7 Hz, 2H), 6.70 (d, J = 13.5 Hz, 2H), 5.62 (s, 2H), 4.29 (t, J = 8.0 Hz, 4H), 2.56 (t, J = 7.0 Hz, 4H), 2.01 (p, J = 7.3 Hz, 4H), 1.49 (s, 12H). 13C NMR (126 MHz, CDCl3, 25°C) δ (ppm): 172.3, 167.0, 148.1, 145.1, 143.7, 142.8, 141.9, 130.2, 129.3, 125.8, 124.6, 123.0, 112.3, 107.5, 66.6, 49.5, 48.5, 43.7, 30.5, 27.8, 24.4. HRMS (ESI-TOF) m/z: [M + H]+ calcd for C41H38N3O10S8 826.2439, found 826.2444.

HRMS (ESI-TOF) spectrum of 5
$^1$H NMR spectrum (400 MHz, CDCl$_3$, 25°C) of 6

$^{13}$C NMR spectrum (126 MHz, CDCl$_3$, 25°C) of 6
HRMS (ESI-TOF) spectrum of 6
3. COSY NMR of Dyes 5 and 6

Figure S1 $^1$H-$^1$H COSY NMR spectrum (500 MHz, CDCl$_3$, 25°C) of dye 5 (aromatic region).
Figure S2 $^1$H-$^1$H COSY NMR spectrum (500 MHz, DMSO-$d_6$, 25°C) of dye 6.
4. Photophysical Properties

Table S1 Spectral properties of dyes 1-6

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PBS = Phosphate buffered saline (pH 7.4). Dye concentrations were 5 µM. All measurements performed at room temperature. SS = Stokes shift.

Quantum yield measurements used dye 1 (Štacková, L.; Muchová, E.; Russo, M.; Slavíček, P.; Štacko, P.; Klán, P. J. Org. Chem. 2020, 85, 9776–9790) ($\Phi_f = 13.2\%$ in methanol) as a reference standard. The concentrations of 1 and other cyanine dyes were adjusted to the absorption value of 0.075 at 650 nm. The fluorescence spectrum of each solution was obtained with excitation at 650 nm, with a slit width of 5 nm and the integrated area was used in the quantum yield calculation by the following equation:

$$\Phi_{\text{sample}} = \Phi_{\text{ref}} \times \frac{\eta^2_{\text{sample}} I_{\text{sample}} A_{\text{sample}}}{\eta^2_{\text{ref}} I_{\text{ref}} A_{\text{ref}}}$$

where $\eta$ is the refractive index of the solvent, $I$ is the integrated fluorescence intensity, and $A$ is the absorbance at a chosen wavelength. The estimated error for this method is ± 10%.
5. Absorption and Emission Spectra of Dyes 1 – 6

Note that the emission spectra in Figs S3-S8 stop at 850 nm because it is the wavelength detection limit of our fluorescence spectrometer.

**Figure S3** Absorbance (blue) and emission (red) spectra of 1 (5 µM) in methanol (left) and PBS, pH 7.4 (right). \(\lambda_{\text{ex}} = 734\) nm.

**Figure S4** Absorbance (blue) and emission (red) spectra of 2 (5 µM) in methanol (left) and PBS, pH 7.4 (right). \(\lambda_{\text{ex}} = 760\) nm.

**Figure S5** Absorbance (blue) and emission (red) spectra of 3 (5 µM) in methanol (left) and PBS, pH 7.4 (right). \(\lambda_{\text{ex}} = 739\) nm.
Figure S6 Absorbance (blue) and emission (red) spectra of 4 (5 µM) in methanol (left) and PBS, pH 7.4 (right). $\lambda_{ex}$ = 765 nm.

Figure S7 Absorbance (blue) and emission (red) spectra of 5 (5 µM) in methanol (left) and PBS, pH 7.4 (right). $\lambda_{ex}$ = 700 nm.

Figure S8 Absorbance (blue) and emission (red) spectra of 6 (5 µM) in methanol (left) and PBS, pH 7.4 (right). $\lambda_{ex}$ = 700 nm.
Figure S9 Absorbance and emission spectra for dyes 1, 3, 5, and 6 in methanol, (5 µM). \( \lambda_{\text{ex}} = 700 \) nm

6. Molar Absorptivity Calculations

Figure S10 Dependence of absorption at \( \lambda_{\text{max}} \) on the concentration of 3 in methanol.
**Figure S11** Dependence of absorption at $\lambda_{\text{max}}$ on the concentration of 4 in methanol.

**Figure S12** Dependence of absorption at $\lambda_{\text{max}}$ on the concentration of 5 in methanol.

**Figure S13 a)** Dependence of absorption at $\lambda_{\text{max}}$ on the concentration of 6 in methanol.
7. Dynamic Light Scattering Studies

Figure S14 a) DLS of dyes in PBS shows that aggregation increases with concentration and that 5 is more aggregated than 6.

8. Synthesis of Control Dye 7, A Literature Probe for Nitroreductase Expression in Hypoxic Cells

Compound S9. A mixture of 4-aminoo-1,8-naphthalic anhydride (400 mg, 1.88 mmol, 1 eq) and 2-(2-amino ethoxy) ethanol (378 µL, 3.76 mmol, 2 eq) was suspended in ethanol/ethyl acetate (1:1, 100 mL) in a 250 mL round bottom flask. The mixture was refluxed for 7 hr. After cooling to room temperature, the crude was vacuum filtered on a Buchner funnel and the filtrate was collected. The solvent in the filtrate was evaporated under reduced pressure to yield S9 a greasy orange solid (675.4 mg) which was used for the next step without purification. A mixture of S9 (675.4 mg, 2.25 mmol, 1 eq) and acetic anhydride (723 µL, 7.65 mmol, 3.4 eq) was stirred in anhydrous pyridine (10 mL) at room temperature for 24 hr. The solvent was evaporated under reduced pressure and the crude was purified through flash column chromatography (SiO₂, 0-2% MeOH in DCM) to afford RHF as a yellowish-orange compound (200 mg, 26%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.61 (d, 1H, J = 7.52 Hz), 8.43 (d, 1H, J = 6.58 Hz), 8.19 (d, 1H, J = 8.37 Hz), 7.65 (t, 1H, J = 5.26 Hz), 7.47 (s, 2H), 6.84 (d, 1H, J = 8.50 Hz), 4.20 (t, 2H, J = 6.41 Hz), 4.06 (s, 2H, J = 4.66 Hz), 3.61-3.65 (m, 4H), 1.91 (s, 3H).

¹H NMR spectrum (400 MHz, DMSO-d₆, 25°C) of RHF
Compound 7 (RHP). To compound RHF (60 mg, 0.175 mmol, 1 eq) in toluene (10 mL), DIPEA (122 µL, 0.70 mmol, 4 eq) was added, the mixture was stirred and cooled to 0 °C in an ice-bath. A solution of triphosgene (104 mg, 0.35 mmol, 2 eq) in toluene (4 mL) was added dropwise over 30 minutes at 0 °C. After the addition, the reaction mixture was refluxed for 6 hr. The reaction was cooled to room temperature, diluted with DCM (10 mL) and filtered using a Hirsch funnel. The filtrate was collected and 4-nitrobenzyl alcohol (54 mg, 0.35 mmol, 2 eq) was added to it. This solution was stirred at room temperature for 24 hr. The solvent was evaporated under reduced pressure and the crude was purified through flash column chromatography (SiO₂, 0-50% EtOAc in Hexane) to get an efficient separation and afford 7 (RHP) as a yellowish-white compound (57 mg, 62%). ¹H NMR (400 MHz, Chloroform-d) δ 8.52-8.57 (m, 2H), 8.29 (d, 1H, J = 8.6 Hz), 8.20 (d, 2H, J = 8.8 Hz), 8.13 (d, 1H, J = 8.2 Hz), 7.71 (t, 1H, J = 8.0 Hz), 7.55 (d, 2H, J = 8.9 Hz), 7.49 (br s, 1H), 5.33 (s, 2H), 4.36 (t, 2H, J = 6.3 Hz), 4.11 (t, 2H, J = 4.8 Hz), 3.77 (t, 2H, J = 5.8 Hz), 3.68 (t, 2H, J = 4.6 Hz), 1.91 (s, 3H).

Figure S15 Photographs of 1 mM stock solution of 7 (RHP) and RHF in DMSO under (a) lab light, (b) blue light.
**Figure S16** Absorbance (left) and emission (right) spectra of 7 (RHP) (blue) and RHF (red) in PBS, pH 7.4 (30 µM, \( \lambda_{ex} = 340 \text{ nm}, \text{slit width} = 2.5 \text{ nm} \)).

**Table S3.** Photophysical properties of RHF and 7 (RHP) in PBS.

<table>
<thead>
<tr>
<th>Dye</th>
<th>( \lambda_{\text{abs}}^{\text{max}} ) (nm)</th>
<th>( \lambda_{\text{em}}^{\text{max}} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 (RHP)</td>
<td>380</td>
<td>475</td>
</tr>
<tr>
<td>RHP</td>
<td>435</td>
<td>545</td>
</tr>
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</table>

**9. Enzyme Cuvette Studies**

**Figure S17** (next page) HPLC traces showing that probe 6 is cleaved by nitroreductase to cleanly produce 3. (I) HPLC chromatograms of authentic 6 (top, A), authentic 3 (bottom, D), and authentic 6 after the treatment with nitroreductase (NTR) in the presence of NADH at room temperature in 1X PBS buffer (middle, B+C). The samples were analyzed by LC-MS with a linear gradient 0-75% acetonitrile in water with 1mM ammonium bicarbonate. \([6] = 20 \text{ mM}, [\text{NTR}] = 662 \text{ nM}, [\text{NADH}] = 0.4 \text{ mM} \). ESI-MS negative ion mode spectra of authentic 3 (II. Bottom, D) and authentic 6 (III. Bottom, A) and reaction sample with NTR (II., B, & III., C, Top). The molecular weight (MW) of the retention time at 4.6 min is 667.2174, which corresponds to [M-H]⁻ for probe 3. The MW of the retention time at 9.0 min is 802.2536, which corresponds to [M-H]⁻ for probe 6.
Figure S18 NIR fluorescence images of syringes containing solutions of molecular probes 5 plus NADH cofactor or 6 plus NADH cofactor, in the absence or presence of NTR enzyme. The images were acquired using an in vivo imaging station (Ami HT Spectral Imaging) with ex: 745 nm, em: 850 nm, exposure: 3 sec, percent power: 50%, F-stop: 2, binning: small, FOV: 20). The bar graphs show fluorescence mean pixel intensity (MPI) for each syringe image. Not only do these results show that NTR is necessary for fluorescence “turn on” but the increase in NIR fluorescence produces a large change in image signal intensity that is easily detected by a common animal imaging station.

Figure S19 Control cuvette experiments showing that NTR conversion of (a, c) 5 into 1 and (b, d) 6 into 3, requires the presence of NADH cofactor and is inhibited by the NTR inhibitor dicoumarol (DC) (0.25 mM).
**Figure S20** Cuvette experiments showing that NTR (1 μg/mL) and NADH (500 μM) does not result in cleavage and fluorescence “turn on” of 2 (5 μM) or 4 (5 μM). $\lambda_{ex} = 733$ for 2 and $\lambda_{ex} = 750$ nm for 4.

**Figure S21** Determination of Michaelis-Menten parameters. (left) Cleavage of 6 (different concentrations) by NTR (0.5 μg/ml) in the presence of NADH (500 μM) in 1X PBS Buffer + 1% DMSO, pH 7.4. The appearance of product 3 was monitored 782 nm ($\lambda_{ex} = 750$ nm). (right) Lineweaver-Burk plot.
Table S2. Comparison of NTR cleavage times and Michaelis-Menten parameters for probe 6 with related literature probes.

<table>
<thead>
<tr>
<th>Substrate name</th>
<th>Reaction Completion Time/ [NTR] and [NADH]</th>
<th>Km, µM</th>
<th>Vmax, µM s⁻¹</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>10 minutes, 1 µg/mL NTR, 0.5 mM NADH</td>
<td>17.02</td>
<td>0.18</td>
<td>This Study</td>
</tr>
<tr>
<td>RHP</td>
<td>10 minutes, 10 µg/mL NTR, 0.5 mM NADH</td>
<td>Not Reported</td>
<td>Not Reported</td>
<td>Org. Lett. 2011, 13,928-931</td>
</tr>
<tr>
<td>Probe 1</td>
<td>20 minutes, 10 µg/mL NTR, 0.5 mM NADH</td>
<td>18.28</td>
<td>0.03</td>
<td>Anal. Methods, 2015, 7, 10125–10128</td>
</tr>
<tr>
<td>Cy7-NO₂</td>
<td>~30 minutes, 0.25 µg/mL NTR, 0.5 mM NADH</td>
<td>32.58</td>
<td>0.34</td>
<td>Sens. Actuators B Chem., 2019, 286, 337-345</td>
</tr>
<tr>
<td>TP-NO₂</td>
<td>Not stated, 20 µg/mL NTR, 0.5 mM NADH</td>
<td>46.82</td>
<td>0.13</td>
<td>Sens. Actuators B Chem., 2020, 310, 12775.</td>
</tr>
<tr>
<td>Probe 1</td>
<td>~30 minutes, 1 µg/mL NTR, 0.5 mM NADH</td>
<td>7.21</td>
<td>0.020</td>
<td>Chem. Commun., 2017, 53, 11177</td>
</tr>
</tbody>
</table>
Figure S22. Fluorescence spectra of 6 (5 μM, observed at 815 nm, with λ_ex= 750 nm) before and several time points after addition of 1 μg/mL porcine liver esterase in PBS buffer, pH 7.4. After 24 hrs there is essentially no appearance of highly fluorescent 3 which is easily detected at 782 nm with λ_em= 750 nm (the green line shows the spectrum obtained after addition of authentic 3, 5 μM, to the sample after the 24 hr incubation). Thus, 6 is not a substrate for porcine liver esterase.

Figure S23 (left) Fluorescence spectra of Calcein AM (0.025 μg/mL) and 6 (5 μM) before and after addition of 0.05 μg/mL porcine liver esterase in PBS pH 7.4. (right) Change in fluorescence intensity with time. Esterase cleavage of Calcein AM to create calcein is indicated by the increase in fluorescence at λ_em = 529 nm (λ_ex = 488 nm). But there is no change in fluorescence for 6 at λ_em = 815 nm (λ_ex = 792 nm) indicating no esterase cleavage to create highly fluorescent 3 (appears at 782 nm, λ_ex= 750 nm). Thus, Calcein AM is a fluorogenic substrate of porcine liver esterase. but 6 is not a substrate.
Figure S24 Change in fluorescence intensity for conversion 6 (5 μM) into highly fluorescent 3 (observed at 782 nm, λex = 750 nm) after addition porcine liver esterase (0.05 μg/mL) at time = zero, and then addition of nitroreductase (NTR, 0.05 μg/mL) and NADH (500 μM) at time = 12 minutes. In PBS, pH 7.4. Data shows that 6 is not a substrate for porcine liver esterase but it an efficient substrate for nitroreductase.

Figure S25 Intracellular nitroreductase levels (picograms NTR per milligram of total intracellular protein) under normoxic (20% O₂) and hypoxic (1% O₂) conditions. N = 3. Error bars are too small to
10. Cell Microscopy Studies

**Figure S26** Representative micrographs of hypoxic A549 cell pre-treated with NTR inhibitor dicoumarol (DC) for 30 minutes and then treated with 5 or 6 (5 µM) for 12 hours under hypoxic atmosphere (1% O₂). Red fluorescence is NIR probe and blue fluorescence is cell nucleus stained with Hoechst dye.

**Figure S27** Colocalization of 5 with mitotracker green in A549 cells under normoxic (top) and hypoxic (bottom) conditions. Pearson’s correlation coefficients in the range 0.5 - 0.6 indicate a moderate level of probe accumulation in the cell mitochondria.

**Figure S28** Colocalization of 6 with mitotracker green in A549 cells under normoxic (top) and hypoxic (bottom) conditions. Pearson’s correlation coefficients in the range 0.1 - 0.2 indicate a low level of probe accumulation in the cell mitochondria.
Studies with Literature Control Probe 7

Figure S29 (a) Fluorescence spectra and (b) change in ratio of fluorescence intensity at 550 and 475 nm of 7 (RHP) (5 μM) + NADH (500 μM) + NTR (5 μg/mL) in 1X PBS Buffer pH 7.4 over a period of 10 minutes. λ<sub>ex</sub> = 410 nm.

Figure S30 Representative epifluorescence images of A549 cells that were treated for 12 hours with 7 (RHP) (5.0 μM) under normoxic (20 % O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions. (a) and (e) Images taken with the DAPI filter (blue) indicating fluorescence of 7 under both conditions. (b) and (f) Images taken with the TxRD filter (green) indicating cleavage of 7 (RHP) by intracellular NTR to produce RHF. (c) and (g) Merge of fluorescent images showing difference in overlap of green-blue intensity under normoxic and hypoxic conditions. (d) and (h) Brightfield images merged with overlap of green-blue intensity under normoxic and hypoxic conditions. Length scale bar = 20 μm. (i) and (j) Bar graphs showing quantification of probe fluorescence intensities under normoxic and hypoxic conditions as mean pixel intensity (MPI) under both conditions. Blue fluorescence (Bl) is the amount of 7 (RHP) and Green fluorescence (Gr) is the amount of RHF that is the product of 7 (RHP) cleavage catalyzed by intracellular NTR enzyme.
Figure S31 Cell viability of (a) 1 and (b) 3 under normoxic (20% O₂) and hypoxic (1% O₂) conditions. N = 3.

Figure S32 Photostability comparison. Absorption spectra for separate samples containing 1, 3 or ICG (1 μM in 1X PBS Buffer pH 7.4) and the change in absorbance over time. Each sample was irradiated by a Xenon lamp with a 620 nm long-pass filter. λ_{abs-max} = 778 nm for ICG, 750 nm for 1 and 755 nm for 3.