Supplementary Information

Central C-C Bonding Increases Optical and Chemical Stability of NIR Fluorophores

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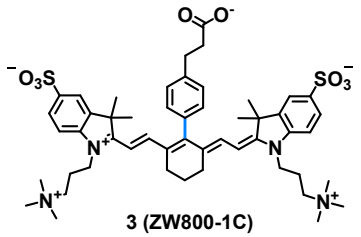
Supplementary Methods

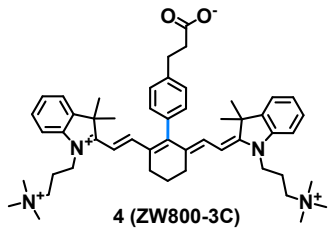
The chemical reagents used in the synthesis of these compounds were obtained from Sigma-Aldrich (Saint Louis, MO) and Alfa Aesar (Ward Hill, MA). A 100 mM phosphate saline buffer solution (PBS) was prepared from monobasic sodium phosphate (Fisher Scientific, Chicago, IL, Certified A.C.S., 99.6%) and dibasic sodium phosphate (JT Baker Phillipsburg, NJ, 98 – 102%) to a final pH of 7.4. Dimethyl sulfoxide (DMSO) and methanol (MeOH) was obtained from Sigma Chemical Co, St. Louis, MO, 99.9%. The reactions were followed with a Varian Cary 50 spectrophotometer with an extended wavelength detector. Open column chromatography was utilized for the purification of all final compounds using 60-200 μm, 60Å reversed phase silica gel (Fluka, Sigma Aldrich). The ^{1}H NMR and ^{13}C NMR spectra were obtained using high quality Kontes NMR tubes (Kimble Chase, Vineland, NJ) rated to 500 MHz and were recorded on a Bruker Avance (400 MHz) spectrometer using DMSO-d_6 or D_2O containing tetramethylsilane (TMS) as an internal calibration standard set to 0.0 ppm. High-resolution accurate mass spectra (HRMS) were obtained using a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer. Liquid chromatography utilized a Waters 2487 single wavelength absorption detector with wavelengths set at 700 nm. The column used in LC was a Waters Delta-Pak 5 μm 100Å 3.9 x 150 mm reversed phase C_{18} column.

Synthesis of C-C coupled ZW800 analogs. Precursor chloro dye **1** or **2** (1.0 mmol) and 3-(4-boronophenyl)propanoic acid (1.8 mmol) in H_2O were heated under reflux in the presence of $Pd(PPh_3)_4$ (0.065 mmol) for 72 h. The reaction progress was monitored by visible/near-infrared spectroscopy with aliquots diluted with methanol until absorption of the starting chlorocyanine disappeared. The reaction mixture was then cooled to room temperature, and H_2O was removed under reduced pressure. The solid was isolated by precipitation with MeOH/acetone, and the precipitate was further washed with acetone. Open-reverse phase column chromatography (eluting with acetonitrile/water) was used to obtain the final fluorophores in analytical purity.



3 (ZW800-1C)

3-((E)-6'-((E)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-2-yl)vinyl)-2'-(2-((E)-3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)-2',3',4',5'-tetrahydro-[1,1'-biphenyl]-4-yl)propanoate (**3**) was obtained in 72% yield; mp 276-278°C; ^{1}H NMR (400 MHz, D_2O): δ 1.31 (s, 12H), 2.40-2.50 (m, 6H), 2.96-3.04 (m, 2H), 3.06-3.16 (m, 4H), 3.45-3.55 (m, 20H), 3.79 (tt, *J* = 8.0 Hz, *J* = 7.2 Hz, 4H), 4.12 (t, *J* = 7.2 Hz, 4H), 6.46 (d, *J* = 13.6 Hz, 2H), 7.16-7.24 (m, 4H), 7.54 (d, *J* = 8.0 Hz, 2H), 7.77 (d, *J* = 8.0 Hz, 2H), 8.01-8.09 (m, 4H). ^{13}C NMR (100 MHz, D_2O): δ 21.02, 21.41, 24.71, 27.55, 32.40, 40.47, 40.97, 48.74, 53.69, 63.67, 100.95, 110.64, 120.19, 127.44, 129.05, 129.56, 134.42, 135.77, 140.56, 141.08, 143.29, 144.15, 150.07, 165.19, 172.69, 181.33. HRMS calculated for $C_{51}H_{66}N_4O_8S_2^{2+}$ $[M]^{2+}$ *m/z* 464.1562, found *m/z* 464.1584



4 (ZW800-3C)

3-((E)-6'-((E)-2-(3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-2-yl)vinyl)-2'-(2-((E)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)ethylidene)-2',3',4',5'-tetrahydro-[1,1'-biphenyl]-4-yl)propanoate (**4**) was obtained in 34% yield; mp 249-253°C; ^{1}H NMR (400 MHz, DMSO-d_6, 50°C): δ 1.14 (s, 12H), 1.91-2.01 (m, 2H), 2.08-2.18 (m, 4H), 2.69 (t, *J* = 7.2 Hz, 2H), 2.74 (t, *J* = 7.8 Hz, 4H), 3.01 (t, *J* = 7.2 Hz, 2H), 3.12 (s, 18H), 3.59 (tt, *J* = 7.4 Hz, *J* = 7.8 Hz, 4H), 4.17 (t, *J* = 7.4 Hz, 4H), 6.23 (d, *J* = 13.6 Hz, 2H), 7.12 (d, *J* = 8.0 Hz, 2H), 7.16-7.26 (m, 4H), 7.34-7.44 (m, 4H), 7.46 (d, *J* = 7.2 Hz, 2H), 7.51 (d, *J* = 8.0 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d_6, 50°C): δ 21.20, 21.45, 27.62, 30.64, 36.15, 48.78, 49.06, 53.13, 63.16, 100.58, 111.36, 122.89, 125.17, 128.92, 129.09, 129.47, 131.86, 136.59, 141.18, 141.87, 142.33, 148.35, 162.73, 171.99, 173.83. HRMS calculated for $C_{51}H_{68}N_4O_2^{2+}$ $[M]^{2+}$ *m/z* 384.7683, found *m/z* 384.7609.

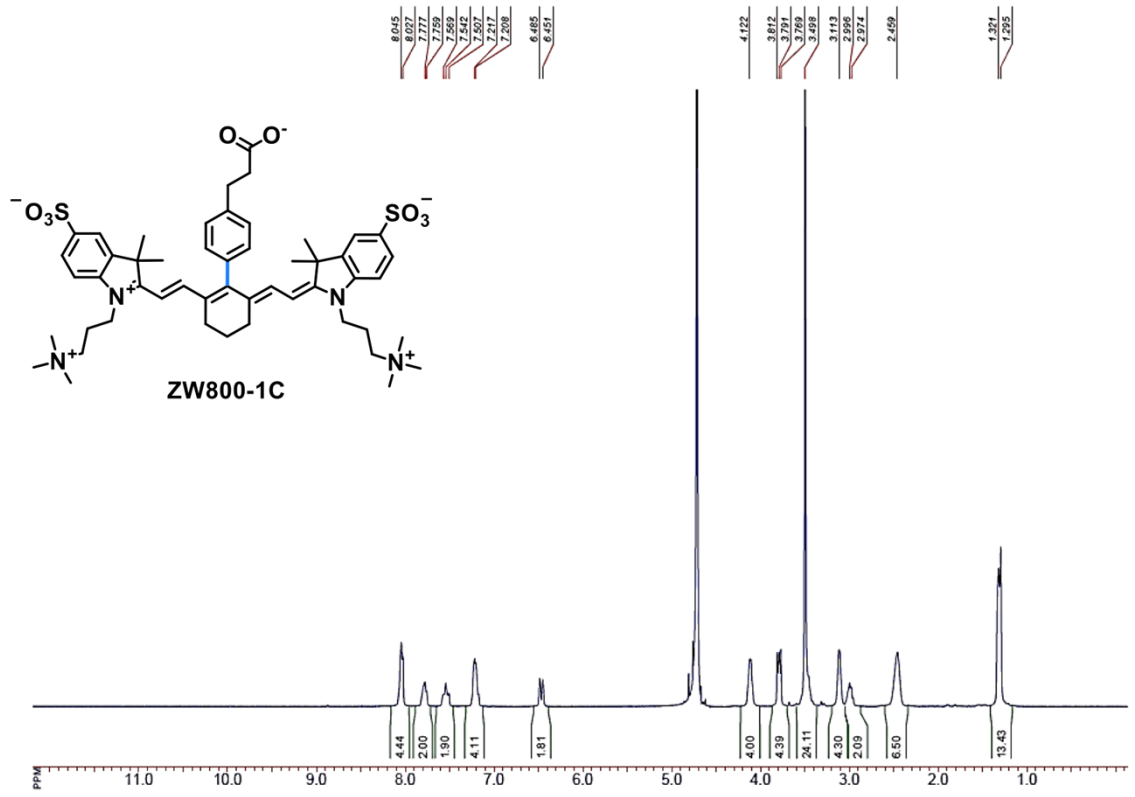


Figure S1. ^{1}H NMR of Compound **3** (**ZW800-1C)** in D_2O.

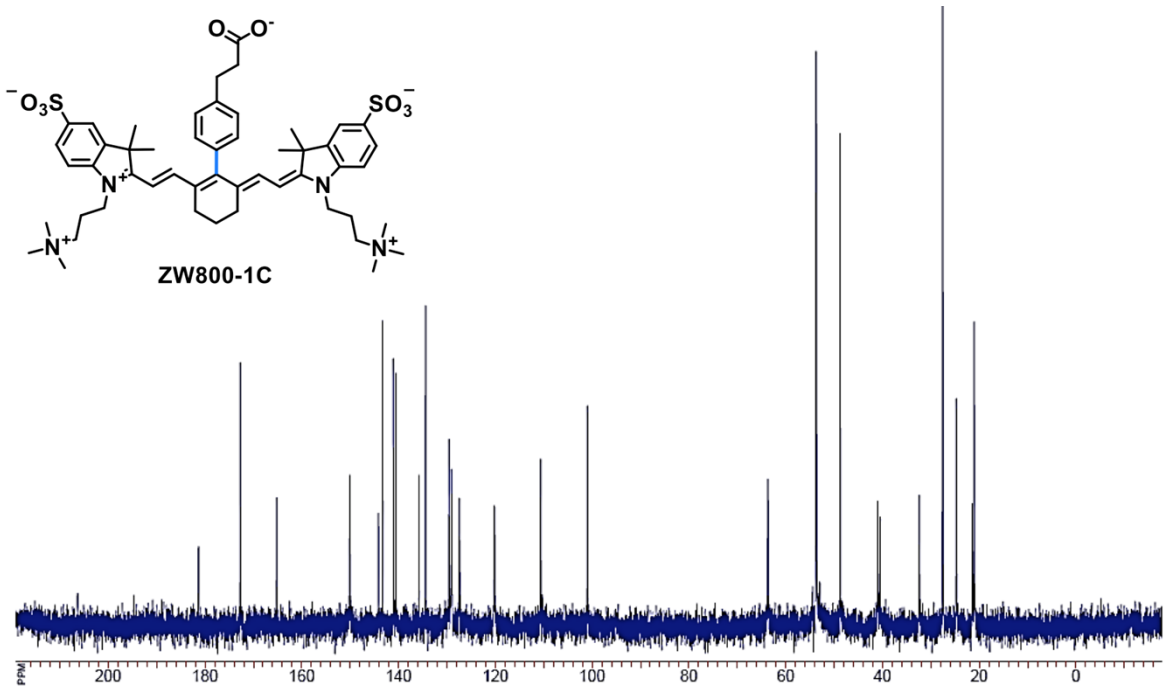


Figure S2. ^{13}C NMR of Compound **3** (**ZW800-1C)** in D_2O.

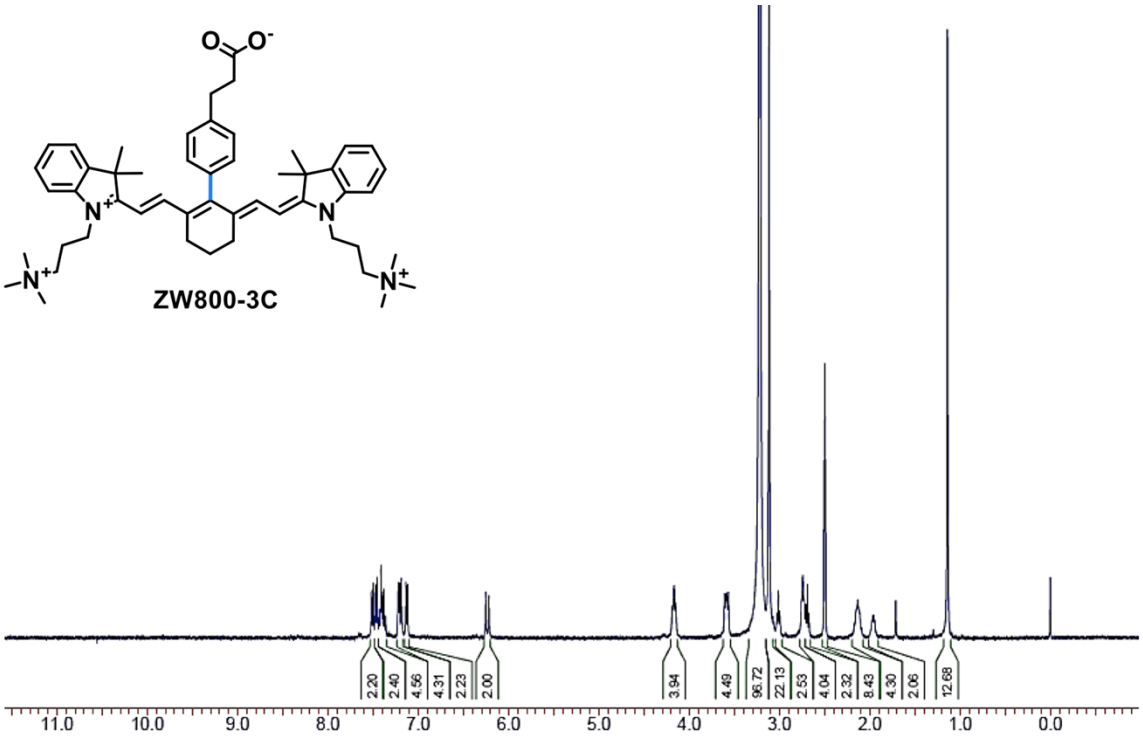


Figure S3. ^{1}H NMR of Compound **4** (**ZW800-3C**) in DMSO-d_6.

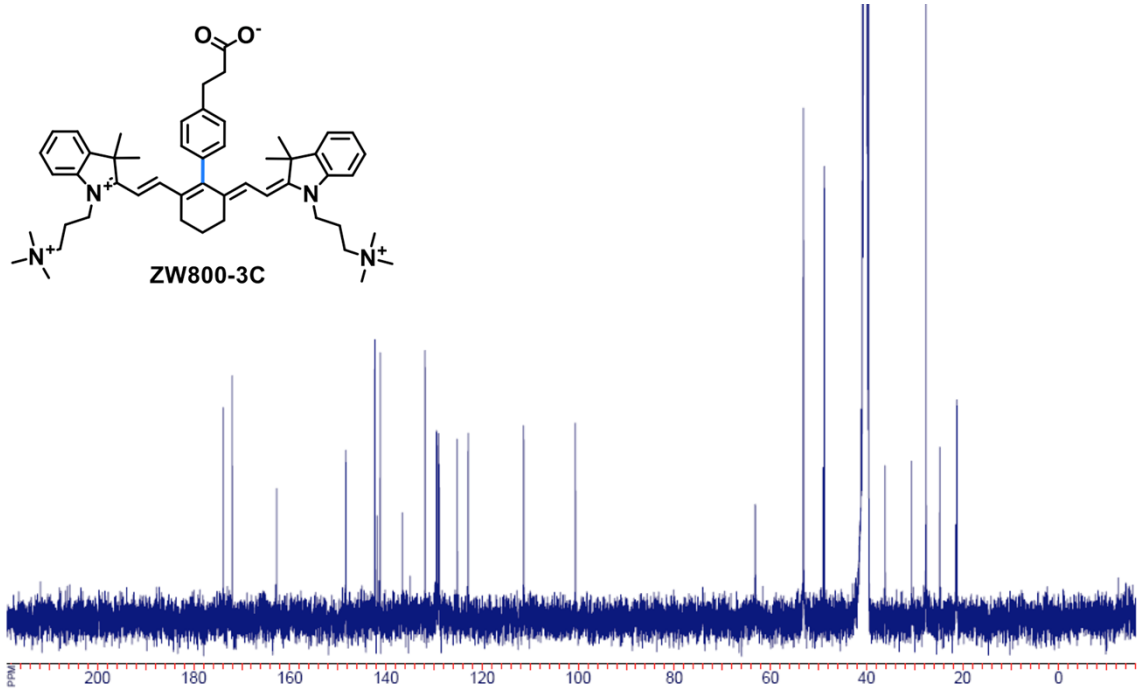


Figure S4. ^{13}C NMR of Compound **4** (**ZW800-3C**) in DMSO-d_6.

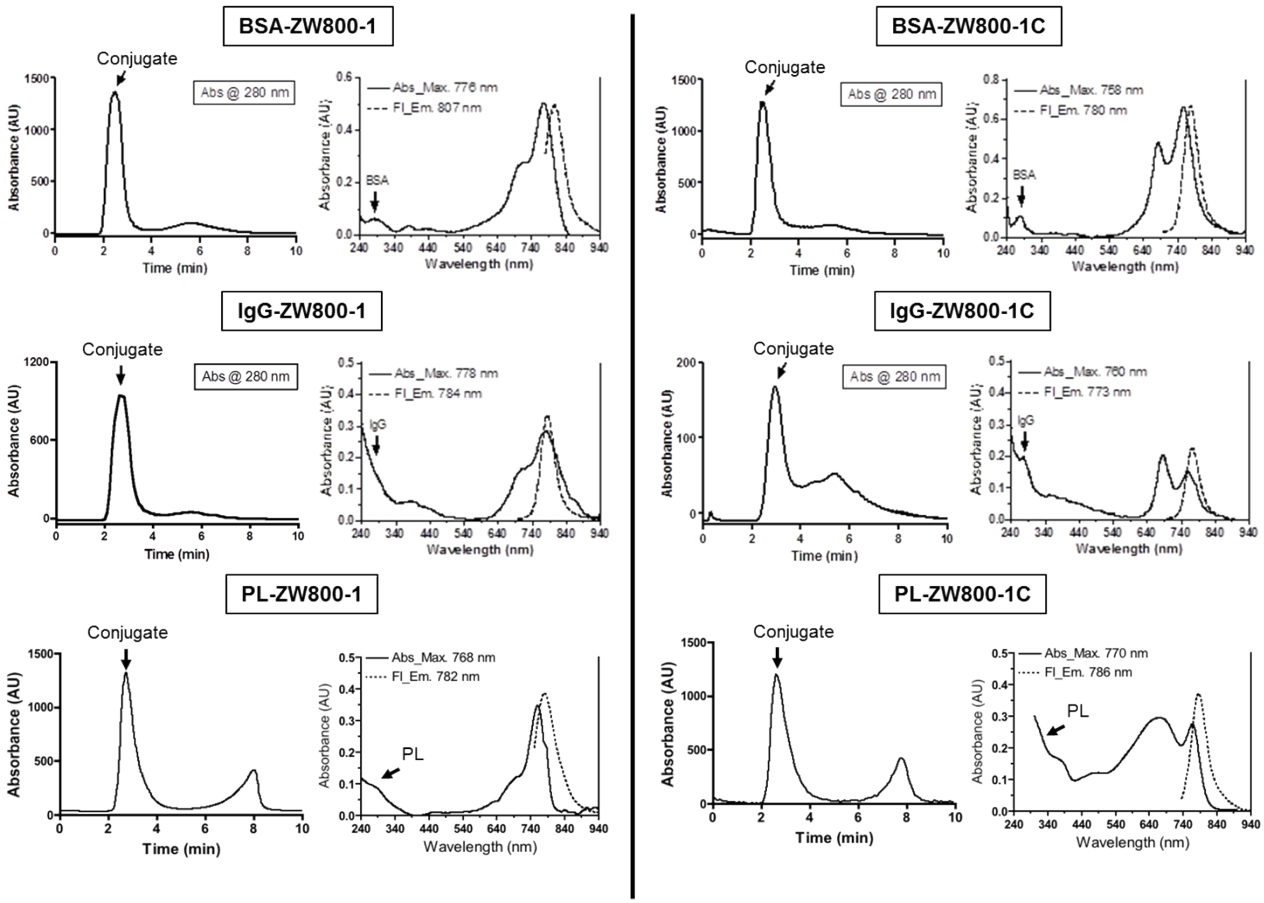


Figure S5. Purification and optical analyses of BSA-, IgG-, and poly-ε-lysine-ZW800 conjugates. Gel filtration chromatography (GFC) and optical analysis of BSA-, IgG-, and poly-ε-lysine-ZW800 conjugates. The labeling ratios of BSA-, IgG-ZW800 conjugates were 2.5 and 3.8 (for BSA-ZW800-1 and BSA-ZW800-1C), 1.7 and 1.5 (for IgG-ZW800-1 and IgG-ZW800-1C), respectively. (BSA: Extinction Coefficient at 280 nm = 43,824 $M^{-1}cm^{-1}$; IgG : Extinction Coefficient at 280 nm = 210,000 $M^{-1}cm^{-1}$)

Table S1. Maximum solubility (mg/mL) of NIR fluorophores in various solvents.

Fluorophore	Water	PBS	D5W	FBS	DMSO	DMSO + 0.1% FA	MeOH	ACN
3: ZW800-1C	3.8	6.8	5.6	7.0	0.5	4.9	0.1	< 0.1
ZW800-1	79.2	84.1	78.7	86.0	32.5	56.4	0.5	< 0.1
4: ZW800-3C	18.1	4.3	3.8	4.0	141.8	217.4	11.5	1.4
ZW800-3	28.7	4.0	6.3	3.8	349.7	302.4	181.4	1.0

PBS = phosphate buffered saline, pH 7.4; D5W = 5% dextrose in water, FBS = fetal bovine serum supplemented with 50 mM HEPES, pH 7.4; DMSO = dimethyl sulfoxide; FA = formic acid; MeOH = methanol; ACN = acetonitrile.

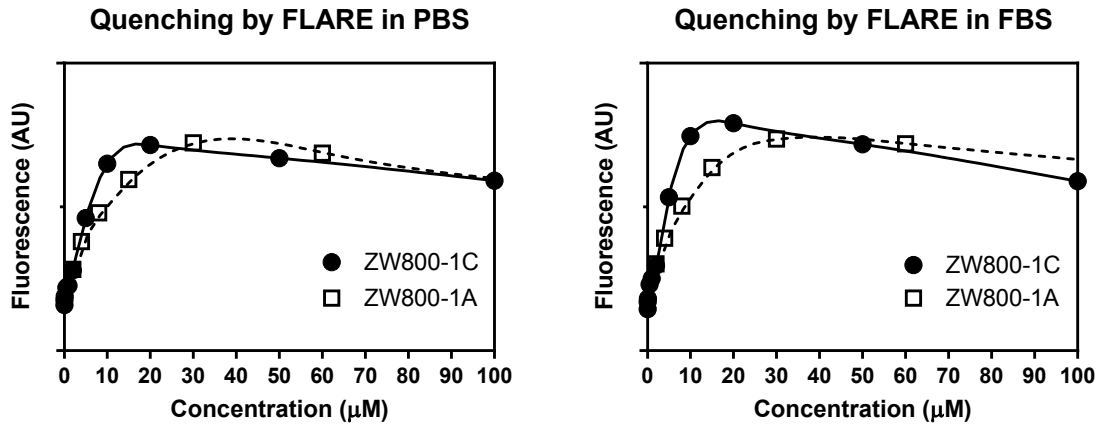


Figure S6. Effect of concentration on total fluorescence yield in PBS and FBS measured by FLARE system

As shown above, ZW800-1C shows higher fluorescence intensity than ZW800-1 at low concentrations in both PBS and FBS, and they have similar fluorescence intensities at high concentrations—despite ZW800 having higher quantum yield. The only difference is the decomposition of the ether linkage and the stability of the C-C coupled fluorophores. This result proves that ZW800-1C has robust chemical structure compared to ZW800-1 resulting in the higher photostability at low concentrations.

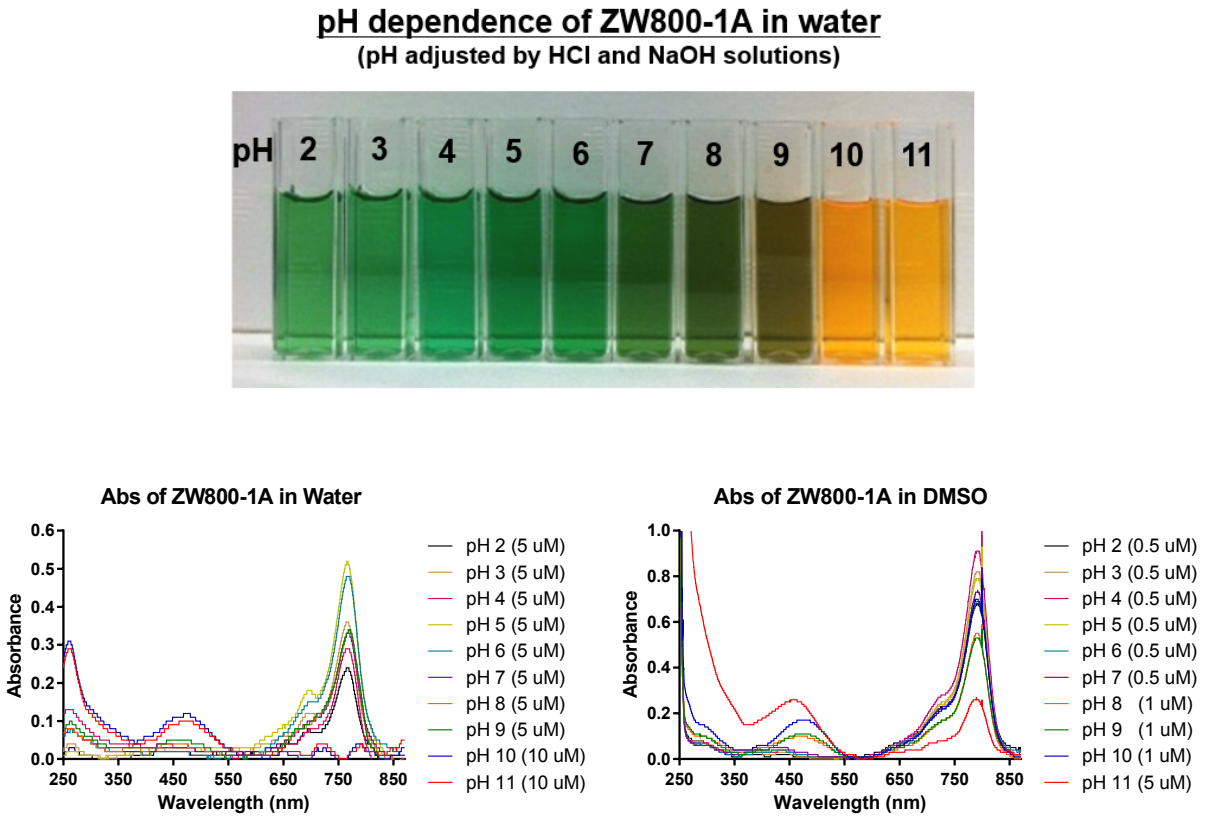


Figure S7. Acid/Base pH profile of ZW800 ether linkage but we expect that the same pH profile would be obtained for the new C-C coupled fluorophores.

Furthermore, under physiological and amide-chemistry conditions this extreme basic solution is not necessary and the compounds are not required to survive under such harsh conditions which does not change the impact of the C-C coupled compounds in image-guided surgery.