Electronic Supplementary Information

Addressing the Autofluorescence Issue in Deep Tissue Imaging by Two-Photon Microscopy: Significance of Far-Red Emitting Dyes

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1. Scheme

Scheme S1. Reagents and conditions: a) Na$_2$S$_2$O$_5$, HNR$_1R_2$, H$_2$O, 150 °C 8 h. b) NaH, DMF, chloromethyl methyl ether, -15 °C, 7 h. c) t-BuLi, Et$_2$O; DMF, -15 °C, 2 h. d) HCl, i-PrOH, 60 °C, 3 h. e) 4-Pyridineacetic acid hydrochloride, EDC, HOBt, Et$_3$N, CH$_2$Cl$_2$, 25 °C, 24 h. f) CF$_3$SO$_3$CH$_3$, CH$_2$Cl$_2$, 25 °C 4 h. g) 4-(2-Ethoxy-2-oxoethyl)pyridine 1-oxide, piperidine, EtOH, 30 °C, 8 h. h) Pd(PPh$_3$)$_4$, NDMBA, 60 °C, 5 h.

Synthesis

6-(Dialkyl- or monoalkylamino)-3-hydroxy-2-naphthaldehydes (1). These compounds were synthesized by following the reported procedure$^1$ using the corresponding amines.

3-(Pyridin-4-yl)-8-(pyrrolidin-1-yl)-2H-benzo[g]chromen-2-one (PyBC590). A solution of 3-hydroxy-6-(pyrrolidin-1-yl)-2-naphthaldehyde (100 mg, 0.41 mmol)$^1$ and 4-pyridineacetic acid hydrochloride (108 mg, 0.62 mmol) in dichloromethane (2 mL) was treated with triethylamine (173 μL, 1.24 mmol). After being stirred for 10 min, the solution was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 119 mg, 0.62 mmol) and 1-hydroxybenzotriazole hydrate (HOBt, 84 mg, 0.62 mmol). The resulting mixture, after being stirred for 24 h at room temperature, was subjected to extraction with dichloromethane. The organic layer was washed with brine, dried over anhydrous Na$_2$SO$_4$, and concentrated. The crude product was purified by silica gel column chromatography (eluent: MeOH/CH$_2$Cl$_2$ = 3/97) to afford PyBC590 as an orange solid (120 mg, 84%). $^1$H NMR (300 MHz, CDCl$_3$, 298 K): $\delta$ 8.68 (d, $J = 4.5$ Hz, 2H), 7.99 (s, 1H), 7.87 (s, 1H), 7.76 (d, $J = 9.2$ Hz, 1H), 7.69 (d, $J = 4.5$ Hz, 2H), 7.44 (s, 1H), 7.01 (dd, $J = 9.2$ 2.4 Hz, 1H), 6.69 (d, $J = 2.4$ Hz, 1H), 3.47 (t, $J = 6.6$ Hz, 4H), 2.14–2.07 (m, 4H). $^{13}$C NMR (600 MHz, CDCl$_3$, 298 K): $\delta$ 159.8, 150.6, 149.5, 147.2, 142.4, 141.5, 137.2, 129.4, 128.5, 122.9, 122.1, 121.6, 115.8, 114.5, 108.7, 102.7, 47.2 (2 carbons), 25.0 (2 carbons). HRMS (ESI) m/z: [M + H]$^+$ calcd for C$_{22}$H$_{18}$N$_2$O$_2$, 342.1368; found, 343.1447.

1-Methyl-4-(2-oxo-8-(pyrrolidin-1-yl)-2H-benzo[g]chromen-3-yl)pyridinium trifluoromethanesulfonate (Py‘BC690). To a solution of PyBC590 (50 mg, 0.15 mmol) in dichloromethane (1 mL) was added methyl trifluoromethanesulfonate (MeOTf,
25 μL, 0.22 mmol) dropwise, and the resulting solution was stirred for 4 h at room temperature. The organic solvent was removed under reduced pressure, and the residue was washed with dichloromethane and hexane (1:9) several times, and then dried in vacuum to give Py\textsuperscript{+}BC690 as a red-violet solid (63 mg, 85%). mp 282 °C. \textsuperscript{1}H NMR (600 MHz, DMSO-d\textsubscript{6}, 298 K): δ 8.95 (d, J = 7.2 Hz, 2H), 8.88 (s, 1H), 8.54 (d, J = 7.2 Hz, 2H), 8.22 (s, 1H), 7.93 (d, J = 9.6 Hz, 1H), 7.52 (s, 1H), 7.12 (ddd, J = 9.6, 2.4 Hz, 1H), 6.82 (d, J = 2.4 Hz, 1H), 4.32 (s, 3H), 3.44 (t, J = 6.6 Hz, 4H), 2.03 (m, 4H). \textsuperscript{13}C NMR (600 MHz, DMSO-d\textsubscript{6}, 298 K): δ 159.5, 151.3, 150.8, 148.7, 147.2, 145.3, 138.8, 132.1, 131.3, 125.4, 123.6, 117.4, 116.9 114.8, 108.7, 103.6, 48.0, 47.6 (2 carbons), 25.4 (2 carbons). IR (KBr): ν = 3129, 3047, 2848, 1701, 1630, 1581, 1551, 1322, 1300, 1262, 1229, 1201, 1185, 1141 cm\textsuperscript{-1}. HRMS (ESI) m/z: [M + H]\textsuperscript{+} calcd for C\textsubscript{23}H\textsubscript{20}N\textsubscript{2}O\textsubscript{2}, 357.1603; found, 357.1603.

8-(Dimethylamino)-3-(pyridin-4-yl)-2H-benzo[g]chromen-2-one (PyBC580a). Starting from 6-(dimethylamino)-3-hydroxy-2-naphthaldehyde (30 mg, 0.14 mmol), this compound was similarly synthesized as an orange solid (33.4 mg, 74%). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}, 0): 8.69 (d, J = 4.5 Hz, 2H), 8.01 (s, 1H), 7.90 (s, 1H), 7.77 (d, J = 9.3 Hz, 1H), 7.69 (d, J = 4.5 Hz, 2H), 7.48 (s, 1H), 7.16 (dd, J = 9.3, 2.3 Hz, 1H), 6.84 (d, J = 2.3 Hz, 1H), 3.15 (s, 3H). \textsuperscript{13}C NMR (500 MHz, CDCl\textsubscript{3}+MeOD, 0): 160.4, 151.0, 150.3, 149.5, 143.3, 142.4, 137.5, 129.8, 129.0, 123.7, 122.9, 122.1, 116.2, 115.4, 109.7, 104.1, 40.2 (2 carbons). HRMS (ESI) m/z: [M + H]\textsuperscript{+} calcd for C\textsubscript{16}H\textsubscript{17}N\textsubscript{2}O\textsubscript{2}, 316.1212; found, 317.1290.

4-(8-(Dimethylamino)-2-oxo-2H-benzo[g]chromen-3-yl)-1-methylpyridinium trifluoromethanesulfonate (Py\textsuperscript{+}BC680a). Starting from PyBC580a (30 mg, 0.095 mmol), this compound was prepared as a red-violet solid (37 mg, 81%). mp 319°C, \textsuperscript{1}H NMR (300 MHz, DMSO-d\textsubscript{6}, 298 K): δ 8.97 (d, J = 7.2 Hz, 2H), 8.90 (s, 1H), 8.53 (d, J = 7.2 Hz, 2H), 8.25 (s, 1H), 7.96 (d, J = 9.3 Hz, 1H), 7.58 (s, 1H), 7.30 (dd, J = 9.3, 2.4 Hz, 1H), 6.99 (d, J = 2.4 Hz, 1H), 4.33 (s, 3H), 3.13 (s, 3H). \textsuperscript{13}C NMR (300 MHz, DMSO-d\textsubscript{6}, 298 K): δ 159.0, 150.7, 150.2, 146.7, 144.9, 138.1, 131.3, 130.6, 130.1, 125.0, 123.1, 117.0, 116.4, 114.7, 108.7, 103.6, 47.2. IR (KBr): ν = 3127, 3048, 1701, 1644, 1615, 1580, 1554, 1507, 1472, 1440, 1392, 1323, 1299, 1260, 1223, 1198, 1182, 1141, 1060, 1029 cm\textsuperscript{-1}. HRMS (ESI) m/z: [M + H]\textsuperscript{+} calcd for C\textsubscript{19}H\textsubscript{22}N\textsubscript{2}O\textsubscript{2}, 331.1447; found, 331.1447.

8-(Allyl(methyl)amino)-3-(pyridin-4-yl)-2H-benzo[g]chromen-2-one (PyBC580b). Starting from 6-(allyl(methyl)amino)-3-hydroxy-2-naphthaldehyde (130 mg, 0.54 mmol), this compound was synthesized as an orange solid (144 mg, 78%). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}, 298 K): δ 8.69 (d, J = 5.7 Hz, 2H), 8.00 (s, 1H), 7.89 (s, 1H), 7.76 (d, J = 9.3 Hz, 1H), 7.69 (d, J = 5.7 Hz, 2H), 7.47 (s, 1H), 7.12 (dd, J = 9.3, 2.4 Hz, 1H), 6.85 (d, J = 2.4 Hz, 1H), 5.96–5.84 (m, 1H), 5.24–5.16 (m, 2H), 4.10 (d, J = 4.8 Hz, 2H), 3.13 (s, 3H). \textsuperscript{13}C NMR (300 MHz, CDCl\textsubscript{3} + MeOD, 298 K): δ 160.6, 150.9, 149.5, 149.2, 143.6, 142.7, 137.6, 132.7, 130.0, 129.1, 123.7, 123.0, 121.6, 116.5, 116.3, 115.3, 109.6, 103.9, 54.8, 38.2. HRMS (ESI) m/z: [M + H]\textsuperscript{+} calcd for C\textsubscript{23}H\textsubscript{20}N\textsubscript{2}O\textsubscript{2}, 342.1368; found, 343.1447.

4-(8-(Allyl(methyl)amino)-2-oxo-2H-benzo[g]chromen-3-yl)-1-methylpyridinium trifluoromethanesulfonate (Py\textsuperscript{+}BC680b). Starting from PyBC580b (44 mg, 0.13 mmol), this compound was prepared as a red-violet solid (58 mg, 88%). mp 246 °C, \textsuperscript{1}H NMR (600 MHz, DMSO-d\textsubscript{6}, 298 K): δ 8.98 (d, J = 6.6 Hz, 2H), 8.90 (s, 1H), 8.54 (d, J = 6.6 Hz, 2H), 8.25 (s, 1H), 7.94 (d, J = 9.0
Hz, 1H), 7.28 (dd, J = 9.0, 2.4 Hz, 1H), 7.02 (d, J = 2.4 Hz, 1H), 5.94–5.89 (m, 1H), 5.20–5.15 (m, 2H), 4.34 (s, 3H), 4.18 (d, J = 4.8 Hz, 2H), 3.12 (s, 3H). \( ^{13} \)C NMR (600 MHz, DMSO-\( d_6 \), 298 K): \( \delta \) 159.5, 151.2, 150.7, 150.3, 147.2, 145.4, 138.7, 133.8, 131.7, 125.6, 123.7, 117.6, 117.0, 116.7, 115.2, 109.2, 104.1, 54.4, 47.7, 38.6. IR (KBr): \( \nu \) = 3125, 3047, 1701, 1640, 1623, 1579, 1550, 1494, 1436, 1395, 1325, 1305, 1260, 1224, 1185, 1140 cm\(^{-1} \). HRMS (ESI) \( m/z \): [M + H]\(^+ \) calcd for C\(_{22}\)H\(_{20}\)N\(_2\)O\(_2\), 357.1603; found, 357.1603.

8-(Methylamino)-3-(pyridin-4-yl)-2H-benzo[g]chromen-2-one (PyBC560a). A solution of 3-hydroxy-6-(methylamino)-2-naphthaldehyde (50 mg, 0.25 mmol) and ethyl 4-pyridylacetate (45.6 \( \mu \)L, 0.30 mmol) in ethanol (2.5 mL) was treated with 2 drops of piperidine, and the resulting solution was stirred for 8 h at 70 °C. After being cooled to room temperature, the reaction mixture concentrated under reduced pressure. The residue was treated with a mixture of methanol and hexane (1:9) to give PyBC560a as an orange precipitate, which was filtered and dried (57 mg, 76%). \( ^1 \)H NMR (300 MHz, DMSO-\( d_6 \), 298 K): \( \delta \) 8.65 (d, J = 6.2 Hz, 2H), 8.49 (s, 1H), 8.11 (s, 1H), 7.78 (d, J = 6.2 Hz, 2H), 7.51 (s, 1H), 6.99 (dd, J = 9.0 2.0 Hz, 1H), 6.70 (d, J = 2.0 Hz, 1H), 6.66 (dd, J = 9.0, 5.1, 1H), 2.82 (d, J = 5.1 Hz, 3H). \( ^{13} \)C NMR (600 MHz, DMSO-\( d_6 \), 298 K): \( \delta \) 160.0, 151.3, 150.6, 150.1, 150.0, 124.0, 123.1, 119.3, 115.0, 108.8, 100.6. 29.9. HRMS (ESI) \( m/z \): [M + H]\(^+ \) calcd for C\(_{19}\)H\(_{14}\)N\(_2\)O\(_2\), 302.1060; found, 303.1134.

1-Methyl-4-(8-(methylamino)-2-oxo-2H-benzo[g]chromen-3-yl)pyridinium trifluoromethanesulfonate (PyN+BC660a).

Starting from PyBC560a (40 mg, 0.13 mmol), this compound was prepared as a red solid (52 mg, 84%). mp 311 °C, \( ^1 \)H NMR (300 MHz, DMSO-\( d_6 \), 298 K): \( \delta \) 8.94 (d, J = 7.0 Hz, 2H), 8.85 (s, 1H), 8.51 (d, J = 7.0 Hz, 2H), 8.13 (s, 1H), 7.79 (d, J = 9.1 Hz, 1H), 7.45 (s, 1H), 6.99 (dd, J = 9.1 2.0 Hz, 1H), 6.69 (d, J = 2.0 Hz, 1H), 4.32 (s, 3H), 2.83 (s, 3H). \( ^{13} \)C NMR (300 MHz, DMSO-\( d_6 \), 298 K): \( \delta \) 159.1, 150.9, 150.2, 146.8, 144.8, 139.1, 131.3, 130.3, 124.9, 119.1, 116.4, 114.1, 108.3, 100.2, 47.1, 29.3. IR (KBr): \( \nu \) = 3566, 3006, 2990, 1715, 1623, 1586, 1520, 1504, 1475, 1472, 1376, 1260, 1168, 1031 cm\(^{-1} \). HRMS (ESI) \( m/z \): [M + H]\(^+ \) calcd for C\(_{20}\)H\(_{17}\)N\(_2\)O\(_2\), 317.1285; found, 317.1290.

8-Amino-3-(pyridin-4-yl)-2H-benzo[g]chromen-2-one (PyBC560b). Starting from 6-(allylamino)-3-hydroxy-2-naphthaldehyde (100 mg, 0.44 mmol), which was synthesized by following the reported procedure by us, 8-(allylamino)-3-(pyridin-4-yl)-2H-benzo[g]chromen-2-one (\( N \)-allyl-PyBC) was synthesized as an orange solid (120 mg, 84%). \( ^1 \)H NMR (300 MHz, CDCl\(_3 \), 298 K): \( \delta \) 8.55 (d, J = 5.9 Hz, 2H), 8.00 (s, 1H), 7.84 (s, 1H), 7.68 (d, J = 5.9 Hz, 2H), 7.64 (d, J = 9.0 Hz, 1H), 7.38 (s, 1H), 6.88 (dd, J = 9.0 1.8 Hz, 1H), 6.69 (d, J = 1.8 Hz, 1H), 5.98–5.90 (m, 1H), 5.31–5.17 (m, 2H), 3.86 (d, J = 5.0 Hz, 2H). \( ^{13} \)C NMR (500 MHz, CDCl\(_3 \) + MeOD, 298 K): \( \delta \) 161.0, 151.1, 149.3, 148.5, 143.6, 142.8, 138.1, 134.3, 130.1, 129.2, 124.6, 123.1, 122.0, 118.9, 116.8, 115.3, 109.8, 102.6, 45.9. A solution of \( N \)-allyl-PyBC (50 mg, 0.15 mmol) in anhydrous methanol (2 mL) was added to a round bottom flask containing tetrakis(triphenylphosphine)palladium (17.6 mg, 0.015 mmol) and \( N,N' \)-dimethylbarbituric acid (71.2 mg, 0.46 mmol) under argon condition. The resulting solution was stirred for 5 h at 60 °C to afford the crude product as precipitates, which was purified by column chromatography on a short pad of silica gel (eluent: S4
MeOH/CH$_2$Cl$_2$ = 1/9) to give PyBC560b as an orange solid (28 mg, 64%). $^1$H NMR (500 MHz, DMSO-$d_6$, 298 K): δ 8.66 (d, $J$ = 3.6 Hz, 2H), 8.49 (s, 1H), 8.11 (s, 1H), 7.79 (m, 3H), 7.43 (s, 1H), 6.99 (dd, $J$ = 8.7 2.2 Hz, 1H), 6.84 (d, $J$ = 2.2 Hz, 1H), 6.04 (s, 2H). $^{13}$C NMR (500 MHz, DMSO-$d_6$, 298 K): δ 159.5, 150.6, 149.7, 149.6, 143.0, 137.5, 130.1, 129.6, 123.3, 122.5, 118.8, 114.5, 107.8, 104.0. HRMS (ESI) m/z: [M + H]$^+$ calcd for C$_{18}$H$_{12}$N$_2$O$_2$, 288.0899; found, 289.0977.

4-(8-Amino-2-oxo-2H-benzo[g]chromen-3-yl)-1-methylpyridinium trifluoromethanesulfonate (Py$^+$BC660b). Starting from PyBC560b (10 mg, 0.023 mmol), this compound was prepared as a red solid (7 mg, 67%). $^1$H NMR (300 MHz, DMSO-$d_6$, 298 K): δ 8.97 (d, $J$ = 6.7 Hz, 2H), 8.90 (s, 1H), 8.54 (d, $J$ = 6.7 Hz, 2H), 8.18 (s, 1H), 7.84 (d, $J$ = 9.2 Hz, 1H), 7.46 (s, 1H), 7.01 (dd, $J$ = 9.2 2.1 Hz, 1H), 6.84 (d, $J$ = 2.1 Hz, 1H), 6.33 (s, 2H), 4.32 (s, 3H). $^{13}$C NMR (500 MHz, DMSO-$d_6$, 298 K): δ 159.1, 150.8, 150.3, 146.9, 144.9, 138.7, 131.5, 131.0, 125.0, 123.5, 119.1, 116.7, 114.2, 107.9, 104.0, 47.2. IR (KBr): ν = 3406, 3347, 3240, 3127, 1703, 1623, 1586, 1459, 1332, 1317, 1253, 1225, 1153, 1028 cm$^{-1}$. HRMS (ESI) m/z: [M + H]$^+$ calcd for C$_{19}$H$_{15}$N$_2$O$_2$, 303.1133; found, 303.1134.

4-(8-(Allyl(methyl)amino)-2-oxo-2H-benzo[g]chromen-3-yl)pyridine 1-oxide (Py$^+$O$^-$BC600). A solution of ethyl 2-(pyridin-4-yl)acetate (200 mg, 1.21 mmol) and meta-chloroperoxybenzoic acid (mCPBA, 418 mg, 2.42 mmol) in THF (2 mL) was stirred at room temperature for 10 h. The reaction mixture was concentrated under reduced pressure, and the crude product was purified by silica gel column chromatography (eluent: MeOH/CH$_2$Cl$_2$ = 7/93) to afford 4-(2-ethoxy-2-oxoethyl)pyridine N-oxide (146 mg, 67%). A solution of 6-(allyl(methyl)amino)-3-hydroxy-2-naphthaldehyde (50 mg, 0.21 mmol) and 4-(2-ethoxy-2-oxoethyl)pyridine 1-oxide (45 mg, 0.25 mmol) in ethanol (1 mL) was treated with 2 drops of piperidine, and the resulting solution was stirred for 8 h at 30 °C. The reaction mixture was concentrated under reduced pressure, and the crude product was purified by silica gel column chromatography (eluent: MeOH/CH$_2$Cl$_2$ = 3/97) to give Py$^+$O$^-$BC600 as an orange solid (54 mg, 73%). mp 198 °C. $^1$H NMR (300 MHz, CDCl$_3$, 298 K): δ 8.24 (d, $J$ = 7.2 Hz, 2H), 7.99 (s, 1H), 7.88 (s, 1H), 7.80–7.74 (m, 3H), 7.45 (s, 1H), 7.12 (dd, $J$ = 9.3 2.4 Hz, 1H), 6.84 (d, $J$ = 2.4 Hz, 1H), 5.95–5.83 (m, 1H), 5.24–5.15 (m, 2H), 4.10 (d, $J$ = 4.8 Hz, 2H), 3.13 (s, 3H). $^{13}$C NMR (500 MHz, CDCl$_3$ + MeOD, 298 K): δ 160.4, 150.9, 149.7, 142.1, 138.8, 137.9, 135.2, 132.7, 130.2, 129.4, 125.3, 123.9, 119.7, 116.7, 116.5, 115.3, 109.8, 104.1, 54.9, 38.4. IR (KBr): ν = 3420, 3201, 3058, 2925, 2855, 1716, 1630, 1590, 1564, 1489, 1448, 1394, 1374, 1310, 1247 cm$^{-1}$. HRMS (ESI) m/z: [M + H]$^+$ calcd for C$_{22}$H$_{18}$N$_2$O$_3$, 358.1317; found, 359.1396.

7-(Diethylamino)-3-(pyridin-4-yl)-2H-chromen-2-one (PyC). A solution of 4-(diethylamino)salicylaldehyde (100 mg, 0.52 mmol) and ethyl 4-pyridylacetate (118 μL, 0.78 mmol) in ethanol (2 mL) was treated with 2 drops of piperidine, and the resulting mixture was stirred for 24 h at 70 °C. After being cooled to room temperature, the reaction mixture was concentrated under reduced pressure to give the crude product, which was purified by silica gel column chromatography (eluent: EtOAc/Hexane = 2/8) to afford PyC (72 mg, 47%) as an yellow solid. $^1$H NMR (300 MHz, CDCl$_3$, 298 K): δ 8.63 (d, $J$ = 5.6 Hz, 2H), 7.85 (s, 1H), 7.68 (d, $J$ = 5.6 Hz, 2H), 7.35 (d, $J$ = 8.8 Hz, 1H), 6.62 (dd, $J$ = 8.8 2.4 Hz, 1H), 6.52 (d, $J$ = 2.4 Hz, 1H), 5.62 (d, $J$ = 2.4 Hz, 2H), 7.62 (s, 1H), 7.61 (s, 1H), 7.45 (s, 1H), 7.40 (s, 1H), 6.93 (d, $J$ = 8.8 Hz, 1H), 6.84 (d, $J$ = 8.8 Hz, 1H), 6.62 (d, $J$ = 8.8 Hz, 1H), 6.52 (d, $J$ = 2.4 Hz, 1H), 5.62 (d, $J$ = 2.4 Hz, 2H).
3.48–3.41 (q, $J$ = 7.1 Hz, 4H), 1.23 (t, $J$ = 7.1 Hz, 6H). $^{13}$C NMR (500 MHz, CDCl₃, 298 K): $\delta$ 161.0, 156.9, 151.6, 149.9, 143.7, 142.1, 129.8, 122.5, 117.3, 109.5, 108.8, 97.2, 45.2 (2 carbons), 12.6 (2 carbons).
2. Figures

Fig. S1. UV/Vis absorption spectra of (a) Py$^+$BC690, (b) Py$^+$BC680a, (c) Py$^+$BC680b, (d) Py$^+$BC660a, (e) Py$^+$BC660b, and (f) Py$^+$O$^-$BC600, measured in different solvents. All the measurements were conducted at 25 °C for each of the compounds (10 μM) dissolved in the given solvent.
Fig. S2. Fluorescence emission spectra of (a) Py'BC690, (b) Py'BC680a, (c) Py'BC680b, (d) Py'BC660a, (e) Py'BC660b, and (f) Py'O BC600, measured in different solvents. All the measurements were conducted at 25 °C for each of the compounds (10 μM) dissolved in the given solvent. The fluorescence emission spectra were measured under excitation at the maximum absorption wavelength of each dye.
Fig. S3. UV/Vis absorption spectra of (a) Py′BC690, (b) Py′BC680a, (c) Py′BC680b, (d) Py′BC660a, (e) Py′BC660b, and (f) Py′OBC600, at different concentrations (10–100 μM) in HEPES buffer (10 mM, pH 7.4, ≤ 1% DMSO). Note: We were able to measure the solubility of Py′BC690 up to 50 μM where the absorbance was saturated.

Fig. S4. 2PM imaging of HeLa cells incubated with Py′BC dyes. (a, d) Cell images stained with Hoechst 33342, a nucleus staining reference dye. (b, e) Cell images stained with Py′BC690 and Py′BC680b, respectively. Concentration of dyes: 3 μg/mL for the Hoechst dye; 10 μM for Py′BC dyes. Excitation wavelengths: 405 nm for the Hoechst dye and 900 nm for the Py′BC dyes (under TPM). Emission wavelengths collected: 410–450 nm for the Hoechst dye; 565–675 nm for the Py′BC dyes.
Fig. S5. Two-photon action spectra of Py'BC690 and Py'BC680b. The two-photon action cross section (TPACS) values were measured for the Py'BC dyes at 100 μM in DMSO using Rhodamine B (100 μM) in MeOH as a reference dye.

Fig. S6. Autofluorescence analysis for different tissues depending on the emission channels and kinds of tissues, measured under two-photon excitation at 850 nm.
Fig. S7. Autofluorescence in tissue imaging dependent on the emission channels: between the yellow and red channels. The emission from the yellow channel was obtained under excitation at 850 nm and that from the red channel under excitation at 900 nm (at the doubled wavelength of the maximum absorption wavelength of the yellow or the red emitting dye).

Fig. S8. 3D 2PM images of mouse brain tissue stained with Py**BC690 after the BABB clearance procedure. Corner cup images from the (a) top, (b) side, and (c) bottom, which were collected through the red channel (625–675 nm) at every 2 μm of depth while excited at 900 nm. Laser power was gradually increased from 5 mW to 50 mW (compensation excitation).
Fig. S9. Photostability spectra in time under UV irradiation (at 365 nm) with 10 μM of dyes in EtOH. The fluorescence emission was measured under excitation at the maximum absorption wavelength of each dye.

Fig. S10. Photostability spectra under two-photon excitation (900 nm) in HeLa cells incubated with 10 μM of dyes. The fluorescence emission was collected in the range from 410 to 675 nm.
**Fig. S11.** Variation of fluorescent intensity depending on pH. Line and symbol indicate normalized fluorescent intensity (based on pH 7) on each pH and bar graph indicate the standard deviation of normalized fluorescent intensity. Measured in 1X universal buffer with 10 \( \mu \text{M} \) of Py\(^+\)BC dyes.

**Fig. S12.** Cell viability evaluation by CCK-8 assay with HeLa cells treated with Py\(^+\)BC dyes at various concentrations (5, 10, and 30 \( \mu \text{M} \)) within 24 h.
3. Tables

Table S1. Maximum absorbance wavelengths ($\lambda_{\text{abs}}$(nm)) of Py$^\text{BC}$ derivatives in different solvents$^a$

<table>
<thead>
<tr>
<th></th>
<th>Py$^\text{BC690}$</th>
<th>Py$^\text{BC680a}$</th>
<th>Py$^\text{BC680b}$</th>
<th>Py$^\text{BC660a}$</th>
<th>Py$^\text{BC660b}$</th>
<th>Py$^\text{O BC680}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES buffer</td>
<td>510</td>
<td>489</td>
<td>487</td>
<td>454</td>
<td>445</td>
<td>447</td>
</tr>
<tr>
<td>Dioxane</td>
<td>507</td>
<td>492</td>
<td>493</td>
<td>475</td>
<td>485</td>
<td>440</td>
</tr>
<tr>
<td>EtOH</td>
<td>527</td>
<td>513</td>
<td>515</td>
<td>506</td>
<td>499</td>
<td>456</td>
</tr>
<tr>
<td>MeCN</td>
<td>517</td>
<td>500</td>
<td>499</td>
<td>483</td>
<td>484</td>
<td>445</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>562</td>
<td>546</td>
<td>542</td>
<td>517</td>
<td>492</td>
<td>456</td>
</tr>
<tr>
<td>DMSO</td>
<td>513</td>
<td>498</td>
<td>499</td>
<td>492</td>
<td>490</td>
<td>454</td>
</tr>
<tr>
<td>Toluene</td>
<td>525</td>
<td>506</td>
<td>506</td>
<td>435</td>
<td>425</td>
<td>447</td>
</tr>
</tbody>
</table>

$^a$All the measurements were conducted at 25 °C for each of the compounds (10 μM) dissolved in the given solvent.

Table S2. Maximum emission wavelengths ($\lambda_{\text{em}}$(nm)) of Py$^\text{BC}$ derivatives in different solvents$^a$

<table>
<thead>
<tr>
<th></th>
<th>Py$^\text{BC690}$</th>
<th>Py$^\text{BC680a}$</th>
<th>Py$^\text{BC680b}$</th>
<th>Py$^\text{BC660a}$</th>
<th>Py$^\text{BC660b}$</th>
<th>Py$^\text{O BC680}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES buffer</td>
<td>689</td>
<td>678</td>
<td>675</td>
<td>657</td>
<td>645</td>
<td>630</td>
</tr>
<tr>
<td>Dioxane</td>
<td>647</td>
<td>643</td>
<td>637</td>
<td>626</td>
<td>619</td>
<td>543</td>
</tr>
<tr>
<td>EtOH</td>
<td>691</td>
<td>681</td>
<td>680</td>
<td>663</td>
<td>680</td>
<td>606</td>
</tr>
<tr>
<td>MeCN</td>
<td>694</td>
<td>685</td>
<td>680</td>
<td>663</td>
<td>651</td>
<td>591</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>694</td>
<td>685</td>
<td>678</td>
<td>666</td>
<td>656</td>
<td>585</td>
</tr>
<tr>
<td>DMSO</td>
<td>700</td>
<td>664</td>
<td>668</td>
<td>673</td>
<td>664</td>
<td>605</td>
</tr>
<tr>
<td>Toluene</td>
<td>652</td>
<td>645</td>
<td>640</td>
<td>528</td>
<td>530</td>
<td>538</td>
</tr>
</tbody>
</table>

$^a$All the measurements were conducted at 25 °C for each of the compounds (10 μM) dissolved in the given solvent. The fluorescence emission spectra were measured under the excitation at the maximum absorption wavelength of each dye.

Table S3. Molar extinction coefficients [$\varepsilon$ (Lmol$^{-1}$cm$^{-1}$)] of Py$^\text{BC}$ derivatives in different solvents

<table>
<thead>
<tr>
<th></th>
<th>Py$^\text{BC690}$</th>
<th>Py$^\text{BC680a}$</th>
<th>Py$^\text{BC680b}$</th>
<th>Py$^\text{BC660a}$</th>
<th>Py$^\text{BC660b}$</th>
<th>Py$^\text{O BC680}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES buffer</td>
<td>15991</td>
<td>5307</td>
<td>14761</td>
<td>3755</td>
<td>10477</td>
<td>14085</td>
</tr>
<tr>
<td>Dioxane</td>
<td>26033</td>
<td>35259</td>
<td>17094</td>
<td>11566</td>
<td>7070</td>
<td>25332</td>
</tr>
<tr>
<td>EtOH</td>
<td>27577</td>
<td>34818</td>
<td>17258</td>
<td>12915</td>
<td>11593</td>
<td>35464</td>
</tr>
<tr>
<td>MeCN</td>
<td>24187</td>
<td>34174</td>
<td>15618</td>
<td>10380</td>
<td>9773</td>
<td>21073</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>22311</td>
<td>42870</td>
<td>17441</td>
<td>7803</td>
<td>6594</td>
<td>31209</td>
</tr>
<tr>
<td>DMSO</td>
<td>23402</td>
<td>32800</td>
<td>15234</td>
<td>8972</td>
<td>4038</td>
<td>33535</td>
</tr>
<tr>
<td>Toluene</td>
<td>26554</td>
<td>34615</td>
<td>16571</td>
<td>1531</td>
<td>1696</td>
<td>41244</td>
</tr>
</tbody>
</table>
Table S4. Quantum yields ($\Phi_F$) of Py$^*BC$ derivatives in different solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Py$^*BC690$</th>
<th>Py$^*BC680a$</th>
<th>Py$^*BC680b$</th>
<th>Py$^*BC660a$</th>
<th>Py$^*BC660b$</th>
<th>Py$^*O BC600$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.007</td>
<td>0.009</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
<td>0.085</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.029</td>
<td>0.060</td>
<td>0.068</td>
<td>0.058</td>
<td>0.044</td>
<td>0.097</td>
</tr>
<tr>
<td>MeCN</td>
<td>0.048</td>
<td>0.070</td>
<td>0.063</td>
<td>0.070</td>
<td>0.063</td>
<td>0.398</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>0.037</td>
<td>0.082</td>
<td>0.072</td>
<td>0.073</td>
<td>0.054</td>
<td>0.489</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.022</td>
<td>0.034</td>
<td>0.047</td>
<td>0.041</td>
<td>0.035</td>
<td>0.243</td>
</tr>
</tbody>
</table>

Table S5. Photophysical properties of two-photon absorbing red and far-red emitting dyes.

<table>
<thead>
<tr>
<th>Name</th>
<th>$\lambda_{em}$ (nm)</th>
<th>$\delta\Phi$ (GM)</th>
<th>M.W.</th>
<th>Photo-stability (t$_{1/2}$)</th>
<th>Water Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMT-red</td>
<td>626$^a$</td>
<td>50$^h$</td>
<td>650.1</td>
<td>1200 sec$^d$</td>
<td>6 $\mu$M</td>
</tr>
<tr>
<td>1-NH$_2$</td>
<td>670$^d$</td>
<td>50$^h$</td>
<td>311.1</td>
<td>Stable$^l$</td>
<td>n.d.</td>
</tr>
<tr>
<td>1</td>
<td>725$^a$</td>
<td>180$^h$</td>
<td>359.2</td>
<td>Stable$^l$</td>
<td>n.d.</td>
</tr>
<tr>
<td>PEB</td>
<td>617$^a$</td>
<td>290$^d$</td>
<td>492.3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CP</td>
<td>645$^a$</td>
<td>n.d.</td>
<td>393.2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>NRNO</td>
<td>650$^a$</td>
<td>38$^h$</td>
<td>497.2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>TSP</td>
<td>610$^a$</td>
<td>0.03$^e$</td>
<td>789.6</td>
<td>900 sec$^d$</td>
<td>n.d.</td>
</tr>
<tr>
<td>Py$^*BC690$</td>
<td>700$^a$</td>
<td>150$^e$</td>
<td>357.2</td>
<td>Stable$^a$</td>
<td>&gt;100 $\mu$M</td>
</tr>
</tbody>
</table>

Measured in $^a$aqueous buffer, $^b$Dioxane, $^c$MeOH, $^d$toluene, $^e$DMSO, and $^f$aqueous/organic mixture (50:50). Irradiated under $^g$two-photon excitation, $^h$UV (365 nm), $^i$mercury lamp (100 W), and $^j$tungsten lamp (500 W). n.d.; not determined.
4. Notes

Fluorescence quantum yield measurement.

The fluorescence quantum yields were measured by using rhodamine 6G in ethanol as a reference. The sample solutions were excited by a laser light at the wavelengths tuned to 500, 510, and 520 nm, and fluorescence was detected by a spectrograph equipped with a CCD detector. The full emission spectra were measured and integrated to give the quantum yields. After confirming no uncertainty in the calculated quantum yield of rhodamine 6G compared with the literature values (literature quantum yield is 0.91\textsuperscript{2}), we measured the quantum yields of all the Py\textsuperscript{+}BC derivatives using rhodamine 6G as a reference. The quantum yields of the compounds in various solvents were calculated according to Equation S1 as below. Where \( \Phi \) is the quantum yield, \( r \) represents references, \( I \) is the measured integrated emission intensity, \( n \) is the refractive index, and \( A \) is the optical density

\[
\Phi = \Phi_r \times \frac{A_r}{I_r} \times \frac{1}{A} \times \frac{n^2}{n_r^2}
\]

\[(S1)\]

Determination of two-photon action cross-section value. TPACS values were measured following the known method.\textsuperscript{3,4} Two equations are referred from the references as below.

\[
\frac{<F(t)>_{\text{cal}}}{<F(t)>_{\text{new}}} = \frac{\Phi_{\text{cal}} \eta_{\text{2cal}} \sigma_{\text{2cal}} C_{\text{cal}} <P_{\text{cal}}(t)>^2 n_{\text{cal}}}{\Phi_{\text{new}} \eta_{\text{2new}} \sigma_{\text{2new}} C_{\text{new}} <P_{\text{new}}(t)>^2 n_{\text{new}}}
\]

\[(S2)\]

The Equation S2 is the main equation that calculates TPACS using a reference dye and Equation S3 could be extracted from Equation S2.

\[
\sigma_{\text{2new}}(\lambda) \eta_{\text{2new}} = \frac{\Phi_{\text{cal}} \eta_{\text{2cal}} \sigma_{\text{2cal}}(\lambda) C_{\text{cal}} <P_{\text{cal}}(t)>^2 <F(t)>_{\text{new}} n_{\text{cal}}}{\Phi_{\text{new}} C_{\text{new}} <P_{\text{new}}(t)>^2 <F(t)>_{\text{cal}} n_{\text{new}}}
\]

\[(S3)\]

\( \sigma_2 \) = two-photon absorption cross section; \( \eta \) = quantum efficiency; \( \sigma_{\text{TP}} \) (two photon action cross section = \( \sigma \eta \)); \( <F(t)> \) = time averaged fluorescence emission; \( C \) =fluorophore concentration; \( <P(t)> \) = time averaged laser power; \( n \) =refractive index of sample; \( \Phi \) = fluorescence collection efficiency

\( \Phi_{\text{cal}} \) and \( \Phi_{\text{new}} \) are identical in the same experimental setup, and \( <P_{\text{cal}}(t)> \), \( <P_{\text{new}}(t)> \) are also identical when same laser is applied. TPACS values of samples could be calculated by putting values of known TPACS (two-photon action cross section) \( \eta \), concentration \( C \), detected emission \( <F(t)> \), and known refractive index \( n \) into either of the two equations.

Rhodamine B in methanol (100 \( \mu \)M) was used as a reference, and 100 \( \mu \)M of Py\textsuperscript{+}BC690 or Py\textsuperscript{+}BC680b in DMSO was used for the measurements. Each refractive index of a given solvent was applied (assuming that the refractive index of sample is almost the same as that of pure solvent). 100 \( \mu \)L of a sample was loaded in the well slide and covered with cover glass. The edge of cover glass was coated with transparent manicure to prevent the evaporation of solvent and then mounted on a vibration isolation table. Two-photon excitation was performed with a Ti-sapphire laser (Chameleon Vision II, Coherent) at 140 fs pulse width and 80 MHz pulse repetition rate. The emission intensity was collected through an HCX APO 10\times objective lens (Leica,
Germany) of a two-photon microscopy (TCS SP5 II, Leica, Germany) equipped with HyD detector (Leica, Germany).

**Cell viability evaluation.** Cell viability was assessed by measuring their ability to metabolize CCK-8 (Cell Counting Kit-8, Dojindo molecular technologies, Inc.) in HeLa cell line. Cells were seeded into 96-well plates at a density of $5 \times 10^3$ cells per well in Dulbecco’s Modified Eagle’s Medium (DMEM) and incubated at 37 °C for 24 h in a humidified atmosphere of 5% CO$_2$ in the air. The Py$^+$BC dyes at various concentrations (5, 10 and 30 $\mu$M) were added into the culture media in the plate and control group was treated with PBS buffer (10 mM, pH 7.4). The plates were incubated for 1, 6, 12 or 24 h and 10 $\mu$L of CCK-8 solution was added to each well of the plate. After incubation for 1 h, absorbance at 450 nm was measured using a microplate reader (Multiskan EX, Thermo Elelectron). Results were expressed as a percentage in comparison to the absorbance of non-treated cells (Fig S12).

5. Reference

6. $^1$H and $^{13}$C NMR Spectra

Py*BC690
7. HRMS Spectra

Py'BC690

[Mass Spectrum]
Data: J-HRFAB  Date: 18-Feb-2017 10:10
Instrument: QStarStation
Sample: -
Note: -
Inlet: Direct  Ion Mode: FAB+
Spectrum Type: Normal ion (SF-Lined)
RT: 0.60 min  Scan: 9  Temp: 3276.7 degC
GF: m/z 207.1464  Int: 376.16 (392.222)
Output m/z range: 310 to 420  Out Level: 0.50 %

Py'BC680a

[Mass Spectrum]
Data: J-HRFAB  Date: 18-Feb-2017 09:51
Instrument: QStarStation
Sample: -
Note: -
Inlet: Direct  Ion Mode: FAB+
Spectrum Type: Normal ion (SF-Lined)
RT: 0.15 min  Scan: 4  Temp: 3276.7 degC
GF: m/z 301.1444  Int: 22.68 (251.68)
Output m/z range: 273 to 381  Out Level: 0.00 %