SUPPORTING INFORMATION

Radioactivity Synchronized Fluorescence Enhancement using a Radionuclide Fluorescence-Quenched Dye

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MATERIALS and METHODS

Materials All solvents and reactants, were purchased from commercial sources and used without further purification. Copper (II) chloride dehydrate, nickel (II) chloride, and zinc chloride, were purchased from Sigma-Aldrich, Co., St. Louis**.** Fluorescent NIR dye HITC ((1,1',3,3,3',3'-hexamethylindotricarbocyanine), and rhodamine 101 were obtained from Exciton Inc. Solvents of spectroscopic purity and water (18.2 mΩ water, Millipore Inc.) were used throughout the work. $^{64}CuCl_2$ was produced in a biomedical cyclotron CS-15, as previously reported [1]. All organic precursors and solvents were obtained from commercial sources and used as received. Flash chromatography was done on a Biotage SP1 system. Preparative HPLC was performed on

an Agilent Technologies 1200 Series equipped with a multi-wavelength detector set to 254 nm and 740 nm. ^{1}H NMR spectra were acquired using a Varian spectrometer operating at 400 MHz. Chemical shifts are reported in parts per million (δ) and referenced to tetramethylsilane (TMS). APCI mass spectrometry (APSI-MS) was performed on a LC/MSD TrapXCl Agilent Technologies system. N,N-Bis[(*tert*-butyloxycarbonyl) methyl]-2-bromoethylamine (**1**) [2], 1,2,3,3-tetramethyl-5-nitro-3H-indolium iodide (**2**)[3], 5-amino-1,2,3,3-tetramethyl-3H-indolium (**3**) [3] and 1,3,3-trimethyl-2-(6-(N-phenylacetamido)hexa-1,3,5-trienyl)-3H-indolium (**5**) [4] were obtained according to literature methods. 5-(Bis(2-bis((tert-butyloxycarbonyl)methyl)amino)ethyl)amino)-1,3,3-trimethyl-2-(7-(1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trien-2-yliumyl)-3H-indolium (**6**) [5] was synthesized with modifications to the published procedure.

Steady-state optical measurements. Absorption spectra were recorded on a Beckman Coulter DU 640 UV-visible spectrophotometer and fluorescence spectra were recorded on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Inc., Edison, NJ), excitation 675 nm, emission scan 690-950 nm. All fluorescence measurements were conducted at room temperature. Quantum yield was measured relative to rhodamine 101 in ethanol (0.01% HCl) as recommended by the manufacturer of the fluorimeter [3].

Fluorescence lifetime measurement. Fluorescence lifetimes (FLT) were measured using time-correlated single-photon-counting (Horiba) with a 700 nm excitation source NanoLed® (impulse repetition rate 1 MHz) at 90º to the detector (Hamamatsu Photonics, Japan). For the FLT measurements, the dyes were dissolved in water and the absorbance of the measured solutions was maintained below 0.15 at a 700 nm excitation wavelength. The detector was set to 760 nm with a 26 nm bandpass and data collected until the peak signal reached 10,000 counts. The FLT

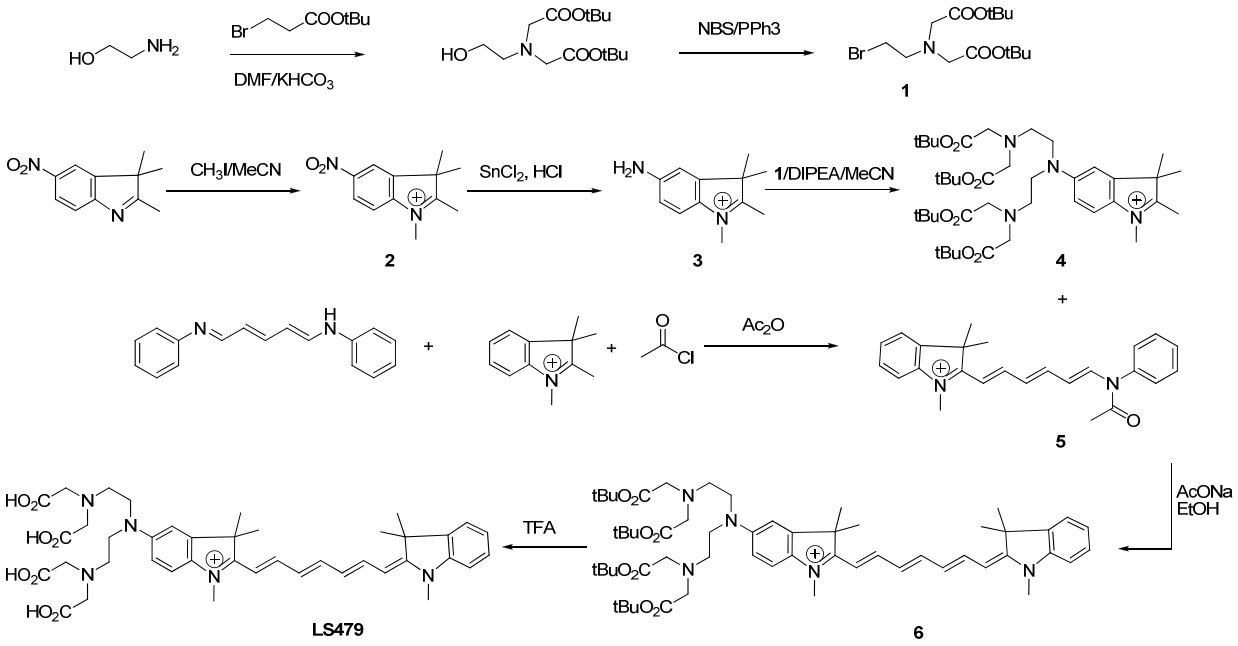
was recorded on a 50 ns scale. The instrument response function was obtained using a Rayleigh scatter of Ludox-40 (0.05% in MQ water; Sigma-Aldrich) in an acrylic transparent cuvette at 700 nm emission. Decay analysis software (DAS6 v6.1; Horiba) was used for lifetime calculations. The goodness of fit was judged by χ^2 values, Durbin–Watson parameters, as well as visual observations of fitted line, residuals, and autocorrelation functions.

Sample preparation and titration experiments. Stock solutions of the NIR dyes (1 mM) were prepared by dissolving in DMSO and kept as stock solution in the freezer. Dye was added to a final concentration of 1.5 μM in 1 mL of water in a 1.5 mL acrylic cuvette. Titration of the dye with aqueous non radioactive metal solution was conducted directly in a cuvette. For that, a calculated amount of metal salts dissolved in fresh water was added to the cuvette in a stepwise manner, so that each addition would correspond to a known fraction of the amount of dye and the total amount of added solution did not exceed 5% of the initial volume. Titration was stopped when spectral changes ceased.

Monitoring fluorescence sensitivity to $^{64}Cu^{2+}$ decay. All work involving the use of radioactive materials at Washington University is conducted under Radiation Safety Committee approved authorizations in accordance with the University's Nuclear Regulatory Commission license. $^{64}CuCl_2$ was produced in a biomedical cyclotron CS-15, as previously reported [1]. One sample of $^{64}CuCl_2$was obtained completely decayed in aqueous solution 7 months after initial bombardment and treated as a sample of "cold" metals (Fig. 3). To determine whether the fluorescence of LS479 was sensitive to the decay of ^{64}Cu, ^{64}Cu-LS479 was prepared and the fluorescence was monitored (>10-half-lives). $^{64}CuCl_2$ (207 mCi/mg) was diluted with water (~20 mCi/mL, final concentration) to prepare a stock solution. The concentration of $^{64}CuCl_2$ at the earliest time point (2 h, 100 μL, ~2 mCi, ~0.15 μM) was adjusted to substantially quench the

fluorescence of LS479 (~55% quenching). The fluorescence at the time-zero point corresponded to unquenched LS479 (See Supplementary Methods online for details on calculation.) After the addition of $^{64}CuCl_2$, LS479 (0.5 μM final concentration, water) was added directly to the cuvette, followed by ethanol (5%, final concentration) and sodium ascorbate (23 mM, final concentration) to inhibit radiolysis (10% dilution) [6]. Absorbance measurements were performed immediately after fluorescence measurements to verify that there was no observable radiolysis. The experiment was repeated periodically (21, 72, and 144 hours) with a fresh solution of LS479 and a constant amount of $^{64}CuCl_2$ (100 μl) solution with the aforementioned incipients. Relative fluorescence was measured as an area under emission curve after addition of $^{64}CuCl_2$ stock divided by area before addition (ex. 675 nm).

SYNTHESIS



N,N-Bis[(*tert*-butyloxycarbonyl) methyl]-2-bromoethylamine (1). *Tert*-butyl bromoacetate (36.5 mL, 225 mmol) was dissolved in anhydrous dimethylformamide (500 mL), followed by

addition of potassium bicarbonate (25.0 g, 250 mmol). The suspension was cooled in an ice-bath and ethanolamine (6.0 mL, 100 mmol) was added via syringe over a five-minute period. The reaction was stirred for 30 minutes on ice and then for 20 hours at room temperature. The reaction was evaporated to one fifth its original volume, saturated sodium bicarbonate solution (300 mL) was added and the mixture was extracted with diethyl ether (3x300 mL). The organic fractions were combined, washed with saturated sodium carbonate (2x250 mL) and dried over anhydrous sodium sulfate. The solvents were removed by rotary evaporation to afford the intermediate di-*tert*-butyl ester (**1**) as an oil. It was used in the next step without further purification. The crude oil was dissolved in dichloromethane (300 mL) at room temperature and triphenylphosphine (28.82 g, 110 mmol) was added with stirring. The reaction was purged with argon and cooled to 0 ^{o}C in an ice-bath. N-bromosuccinimide (19.6 g, 110 mmol) was added portion-wise over 5 minutes. The reaction was stirred for 1.5 hours at 0 ^{o}C and the solvents were removed by rotary evaporation to obtain a purple oil. The oil was triturated with diethyl ether (3x250 mL) with constant manual stirring. The diethyl ether solutions were decanted and the solid was washed with diethyl ether (250 mL). The ethereal fractions were combined, the volume reduced to 80 mL, and allowed to stand overnight at 0 ^{o}C. Diethyl ether (100 mL) was added to the cold mixture, mixed, and the solid residue was filtered and washed with diethyl ether (10x4 mL). The ethereal solution was percolated through a silica gel column (250 g) and eluted with 250 mL portions of ether. The fractions containing the product by TLC (hexane:ether=5:1, R_f=0.29) were combined and concentrated by rotary evaporation to obtain an oil residue which was further purified by flash chromatography (hexane:ether from 10:0 to 9:1). The fractions containing the product were combined and the solvents were removed under reduced pressure. The title compound was obtained as a colorless oil (29 g, yield=82 %). ^{1}HNMR (400 MHz,

$CDCl_3$): δ 3.48 (s, 4H), 3.44 (t, 2H), 3.13 (t, 2H), 1.46 (s, 18H). *m/z* (MS APCI) 352.2 ($[M+H]^+$).

1,2,3,3-Tetramethyl-5-nitro-3H-indolium iodide (2). 5-Nitro-2,3,3-trimethylindolenine (8.16 g, 40 mmol) and methyl iodide (11.44 g, 80 mmol) were dissolved in anhydrous acetonitrile (200 mL). The reaction mixture was refluxed overnight. The color of the reaction mixture turned brown, and it was cooled to room temperature. The final product was collected by filtration as grey-yellowish crystals (8.6 g, yield=60%). ^{1}HNMR (400 MHz, CD_3OD): δ 8.69 (s, 1H), 8.56 (d, *J* = 8 Hz, 1H), 8.10 (d, *J* = 8 Hz, 1H), 4.12 (s, 3H), 1.37 (s, 9H). *m/z* (MS APCI) 220.2 ($[M+H]^+$).

5-Amino-1,2,3,3-tetramethyl-3H-indolium (3). 1,2,3,3-Tetramethyl-5-nitro-3H-indolium iodide **2** (2.2 g, 6.35 mmol) was added to a solution of stannous chloride (8.43 g, 44.45 mmol) in hydrochloric acid (32%, 40 mL) with stirring. The mixture was refluxed for 16 hours and allowed to cool to room temperature. The reaction mixture was poured onto crushed ice and the solution was rendered alkaline with sodium hydroxide (4M). The mixture was filtered and the solid residue dissolved in *tert*-butyl methyl ether and washed with water (3x30 mL). The organic phase was dried over anhydrous sodium sulfate and decolorized with a small amount of active carbon. The solvent was removed by rotary evaporation and the title compound was obtained by crystallization from ethyl acetate as grey crystals (1.03g, yield=79%). ^{1}HNMR (400MHz, $CDCl_3$): δ 8.32-8.26 (m, 2H), 7.62 (d, *J* = 8 Hz, 1H), 4.84 (s, 2H), 3.33 (m, 3H), 2.38 (s, 3H), 1.41 (s, 6H). *m/z* (MS APCI) 190.3 ($[M+H]^+$).

5-(Bis(2-(bis(tert-butyloxycarbonyl) methyl)amino)ethyl)amino)-1,2,3,3-tetramethyl-3H-indolium (4). 5-Amino-1,2,3,3-tetramethyl-3H-indolium **3** (380 mg, 1.7 mmol) was added to a

solution of N,N-Bis[(*tert*-butyloxycarbonyl) methyl]-2-bromoethyl-amine **1** (1.94 mg, 5.5 mmol) and N,N-diisopropylethylamine (6 mL) in anhydrous acetonitrile (100 mL). The reaction mixture was refluxed under argon for 20 hours and the solvent was removed by rotary evaporation. The residue was purified by flash chromatography (dichloromethane:hexane:methanol=5:1:1). The title compound was obtained as a grey solid (1.13g, yield = 91%). ^{1}HNMR (400MHz, CD_3OD): δ7.53 (d, *J* = 8 Hz, 1H), 7.20 (s, 1H), 6.91-6.89 (dd, 1H), 3.76 (s, 3H), 3.64 (t, *J* = 12 Hz, 4H), 3.45 (s, 8H), 2.91 (t, *J* = 8 Hz, 4H), 1.57 (s, 6H), 1.50 (s, 3H), 1.35 (s, 36H). *m/z* (MS APCI) 731.7 ($[M]^+$).

1,3,3-Trimethyl-2-(6-(N-phenylacetamido)hexa-1,3,5-trienyl)-3H-indolium (**5**). 1,2,3,3-tetramethyl-indolinium iodide (2.0 g, 6.7 mmol), glutacondialdehyde dianil (1.8 g, 6.34 mmol), and acetyl chloride (2 mL) in acetic anhydride (24 mL) were heated at 110^{o}C for 30 minutes. The reaction mixture was evaporated under reduced pressure. The residue was dissolved in dichloromethane (10 mL) and added to a large volume of rapidly stirred diethyl ether. The precipitate was collected and purified by flash chromatography (dichloromethane:hexane:methanol=5:1:1). The title compound was obtained as a purple solid (2.9 g, yield=87%). ^{1}HNMR (400 MHz, $CDCl_3$): δ 8.20 (d, *J* = 8 Hz, 1H), 7.91-7.79 (m, 1H), 7.60-7.40 (m, 9H), 7.20-7.16 (m, 2H), 6.90-6.82 (m, 1H), 5.43-5.36 (dd, 1H), 4.19 (s, 3H), 1.96 (s, 3H), 1.70 (s, 6H). *m/z* (MS APCI) 372.3 ($[M+H]^+$).

5-(Bis(2-bis((tert-butyloxycarbonyl)methyl)amino)ethyl)amino)-1,3,3-trimethyl-2-(7-(1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trien-2-yliumyl)-3H-indolium (6). 5-(bis(2-(bis(*tert*-butyloxycarbonyl) methyl)amino)ethyl)amino)-1,2,3,3-tetramethyl-3H-indolium **4** (530 mg, 0.724 mmol), anhydrous sodium acetate (125 mg, 1.5 mmol) and 1,3,3-trimethyl-2-(6-(N-phenylacetamido)hexa-1,3,5-trienyl)-3H-indolium **5** (300 mg, 0.725 mmol) were dissolved in

anhydrous ethanol (80 mL) and the mixture was refluxed for 4 hours under argon. The solvent was removed and the residue was purified by flash chromatography (dichloromethane: methanol=19:1). The title compound was obtained as a blue solid (400 mg, yield=74.5%). ^{1}HNMR (400 MHz, CD_3OD): δ 7.92-7.87 (m, 1H), 7.68-7.56 (m, 1H), 7.54-7.50 (m, 2H), 7.33-7.24 (m, 3H), 7.16-6.96 (m, 3H), 6.87-6.80 (m, 1H), 6.58-6.36 (m, 2H), 5.86 (d, *J* = 12 Hz, 1H), 3.72 (s, 3H), 3.62-3.54 (m, 4H), 3.45 (s, 8H), 3.35 (s, 3H), 2.93 (t, *J* = 14 Hz, 4H), 1.73 (s, 6H), 1.62 (s, 6H), 1.48 (s, 36H). *m/z* (MS APCI) 967.3 ($[M+H]^+$).

5-(Bis(2-(bis(carboxymethyl)amino)ethyl)amino)-1,3,3-trimethyl-2-(7-(1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trienyl)-3H-indolium (LS479). 5-(Bis(2-bis((*tert*-butyloxycarbonyl)methyl)amino)ethyl)amino)-1,3,3-trimethyl-2-(7-(1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trien-2-yliumyl)-3H-indolium **6** (120 mg, 0.124 mmol) was dissolved in trifluoroacetic acid (5 mL) and stirred at room temperature for 2 hours. The acid was removed under vacuum. The crude product was dissolved in methanol and purified by preparative HPLC on a Zorbax XDB C-18 column (21.2x50 mm, 5 um) at a 20 mL/min flow rate. The solvent system consisted of solvent A (0.1%TFA in water) and B (0.1% TFA in acetonitrile) with a gradient of solvent B from 25 to 40% over 12 minutes. The title compound was obtained as a blue solid (65 mg, yield=70.6%). ^{1}HNMR (400 MHz, D_2O): δ 7.66-7.52 (m, 1H), 7.38-7.19 (m, 2H), 7.19-6.94 (m, 5H), 6.70-6.56 (bs, 1H), 6.42-6.18 (m, 3H), 6.12 (t, *J* = 12 Hz, 1H), 5.40 (s, 1H), 3.72 (s, 3H), 3.28 (s, 3H), 3.16 (s, 8H), 2.86 (bs, 4H), 2.32 (bs, 4H), 1.44 (s, 6H), 1.10 (s, 6H). *m/z* (MALDI-TOF MS) 742.8($[M]^+$).

CALCULATIONS

Concentration of added decaying copper was calculated according to a known decay equation:

$$[\mathbf{Cu}] = [\mathbf{Cu_0}]e^{-(0.693t/T_{1/2})}, \%$$

where $[Cu_0]$ is the concentration of copper at the moment of production equal to 100 %, t – the time point of the experiment, h., and $T_{1/2}$ - a half-life of ^{64}Cu equal to 12.701 ± 0.002 hours [7].

Relative fluorescence (***F***) was measured by dividing the area under the emission curve after addition of radioactive solution by the area before the addition. (ex. 675 nm, em. 690-900 nm) according to the equation

$$\boldsymbol{F = \left(F_t^*/F_t^0\right) \times 100}, \%$$

where $\boldsymbol{F_t^0}$ is an area under emission curve at the time point t prior to $^{64}Cu^{2+}$ addition, $\boldsymbol{F_t^*}$ is an area under emission curve at the time point t after $^{64}Cu^{2+}$ addition.

FIGURES

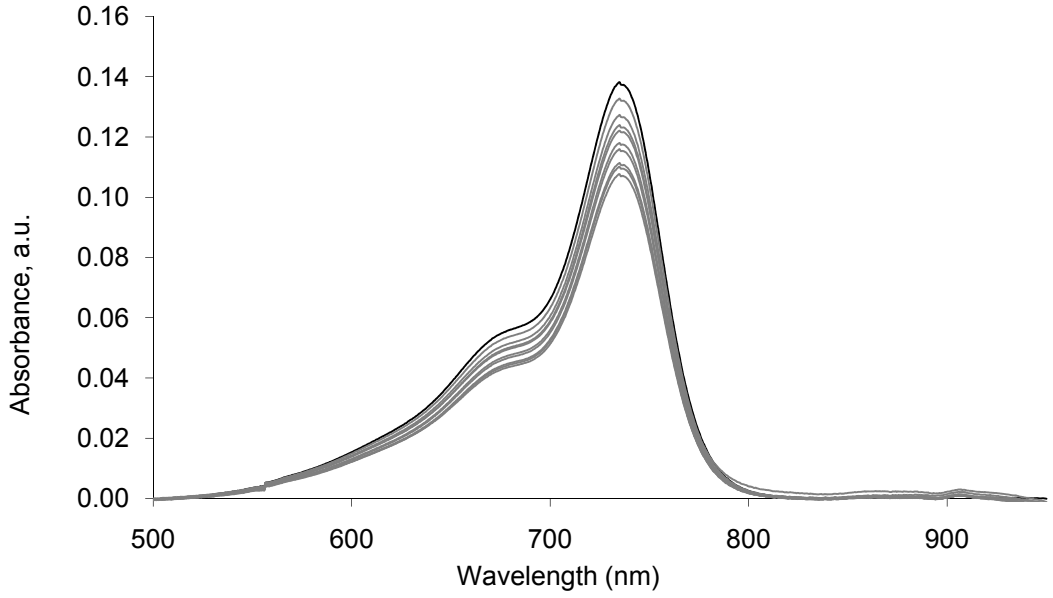


Figure S1: Absorption spectra after addition of Ni^{2+} to HITC. No shift in absorption was observed. The changes in intensity in absorption spectra of HITC upon metal addition was apparently due to a counterion effect, where initial iodides were replaced with chlorides from the added salts. The absence of bathochromic or hypsochromic shifts indicated the lack of complexation. Arrow indicates the change in spectra upon increase of metal concentration from 0 to 1.8 mol/mol metal to dye ratio.

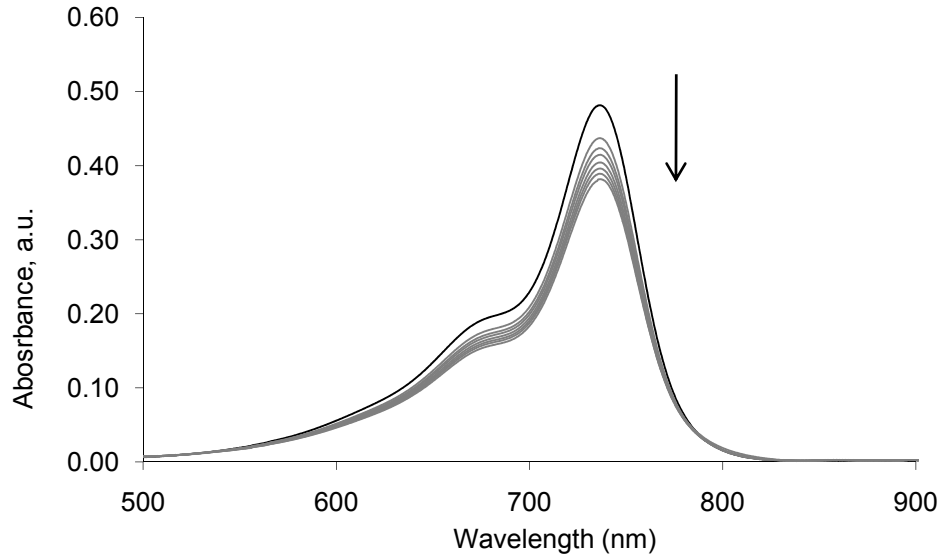


Figure S2: Absorption spectra after addition of Cu^{2+} to HITC. No shift in absorption was observed. The changes in intensity in absorption spectra of HITC upon metal addition was apparently due to a counterion effect, where initial iodides were replaced with chlorides from the added salts. The absence of bathochromic or hypsochromic shifts indicated the lack of complexation. Arrow indicates the change in spectra upon increase of metal concentration from 0 to 2.4 mol/mol metal to dye ratio.

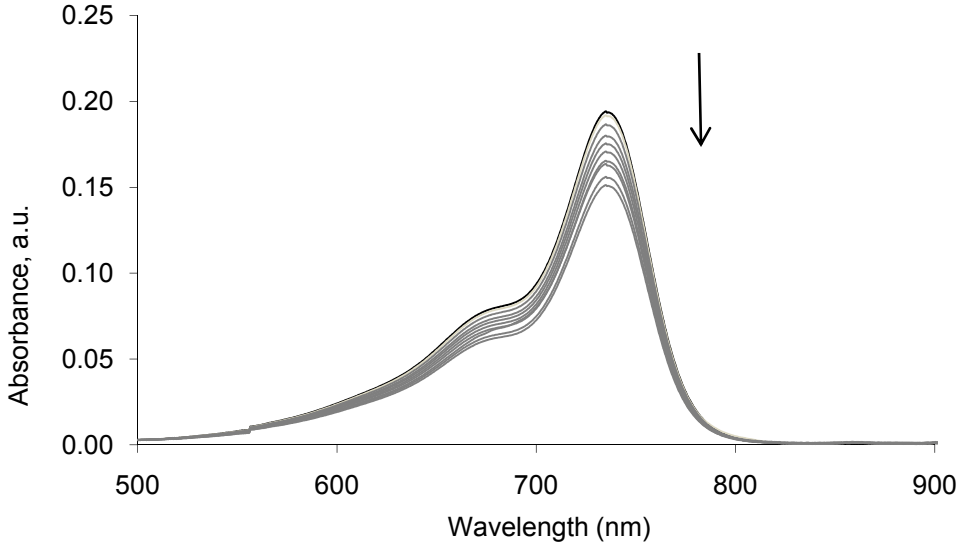


Figure S3: Absorption spectra after addition of Zn^{2+} to HITC. No shift in absorption was observed. The changes in intensity in absorption spectra of HITC upon metal addition was apparently due to a counterion effect, where initial iodides were replaced with chlorides from the added salts. The absence of bathochromic or hypsochromic shifts indicated the lack of complexation. Arrow indicates the change in spectra upon increase of metal concentration from 0 to 1.5 mol/mol metal to dye ratio.

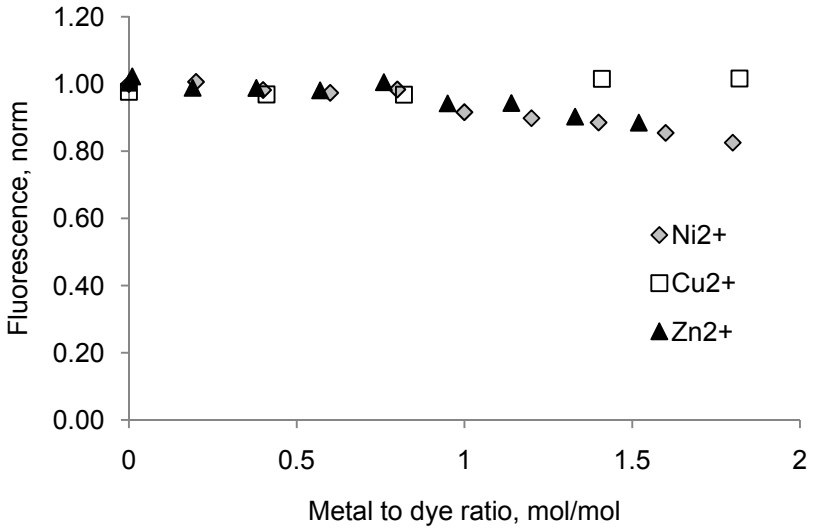


Figure S4 Normalized fluorescence intensity of HITC in the presence of metal salts at different concentrations (ex. 675, em.760 nm) demonstrates no significant change upon metal salts addition.

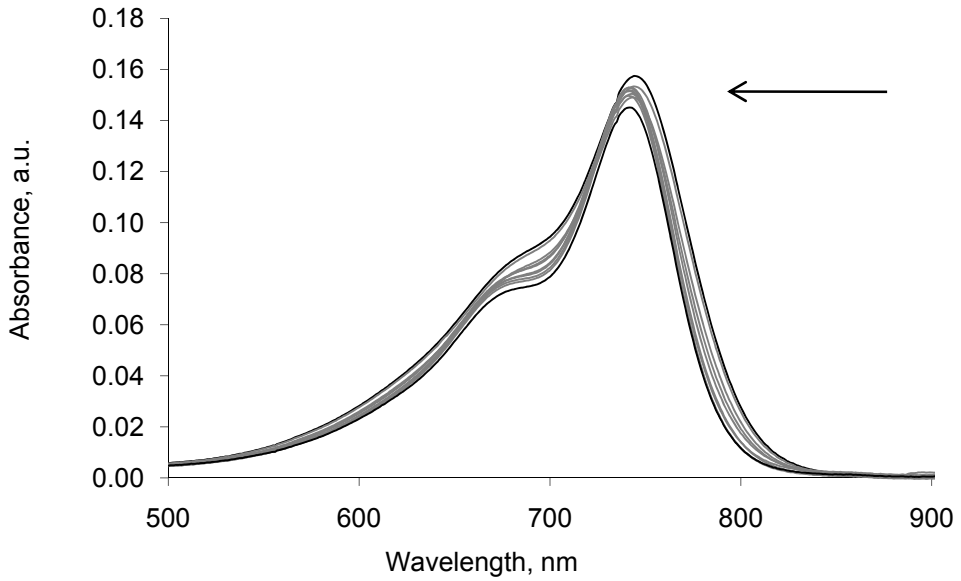


Figure S5 Absorption spectra of Cu^{2+}-LS479 in water. Arrow indicates the change in spectra upon increase of metal concentration from 0 to 2.0 mol/mol metal to dye ratio.

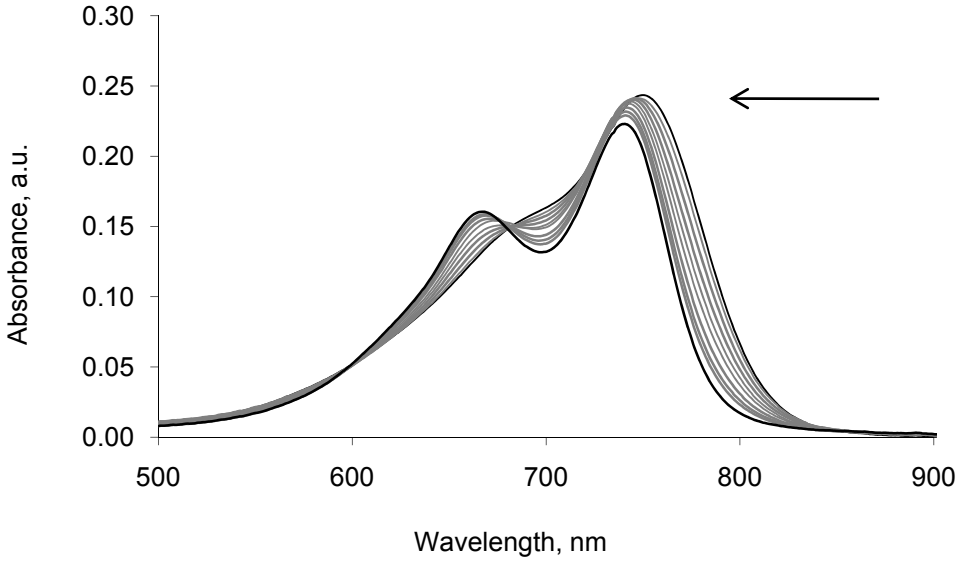


Figure S6 Absorption spectra of Ni^{2+}-LS479 in water. Arrow indicates the change in spectra upon increase of metal concentration from 0 to 1.1 mol/mol metal to dye ratio.

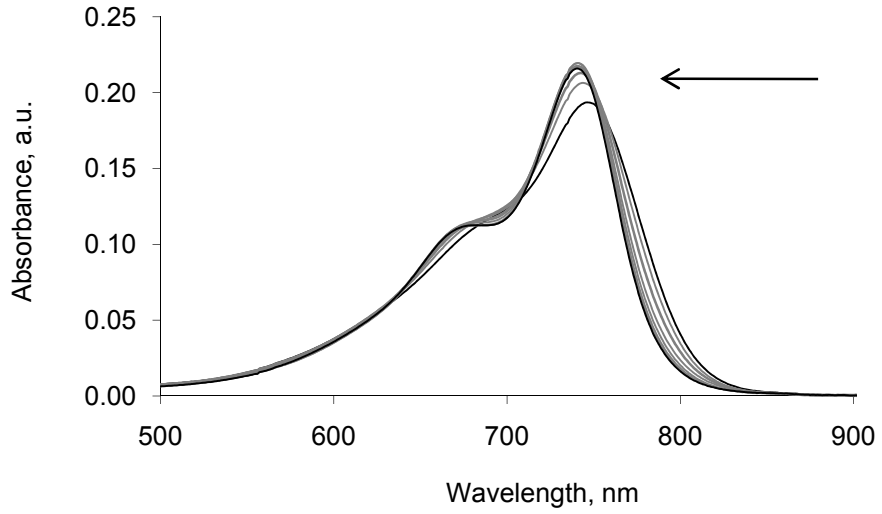


Figure S7 Absorption spectra of Zn^{2+}-LS479 in water. Arrow indicates the change in spectra upon increase of metal concentration from 0 to 1.5 mol/mol metal to dye ratio.

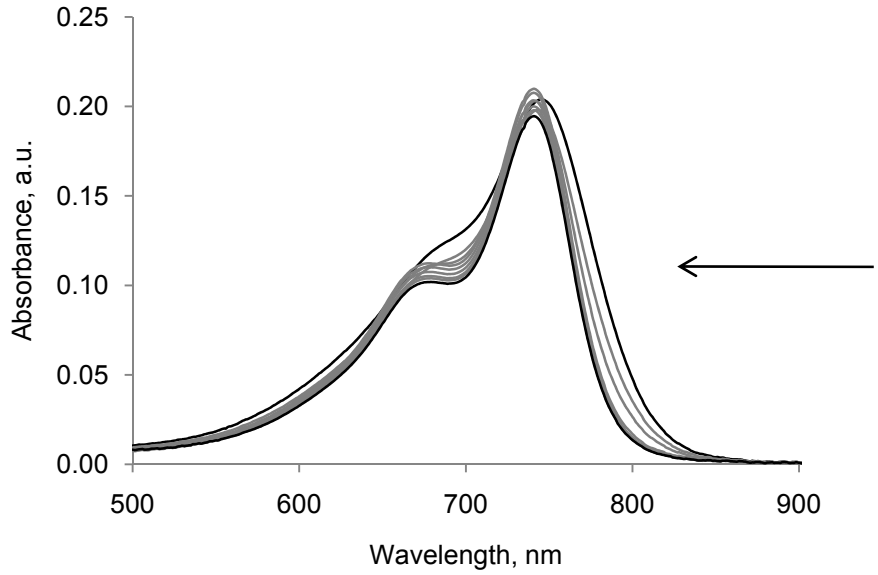


Figure S8 Absorption spectra of [61% $^{nat}Ni^{2+}$/39% $^{nat}Zn^{2+}$] -LS479 in water. Arrow indicates the change in spectra upon increase of metal concentration.

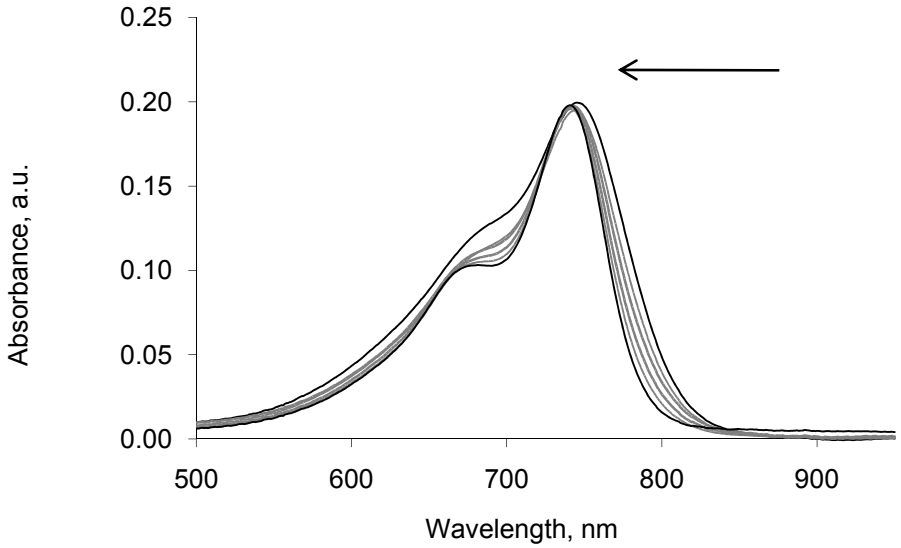


Figure S9. Absorption spectra of decayed $^{64}Cu^{2+}$-LS479 in water. Arrow indicates the change in spectra upon increase of metal concentration.

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