

Optimizing the Sensitivity of Photoluminescent Probes using Time-Resolved Spectroscopy: A Molecular Beacon Case Study

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Steady-state photoluminescence spectra were obtained in a HORIBA Jovin Yvon Fluorolog 3. Time-resolved studies were performed using an Edinburgh Instruments OD470 single-photon counting spectrometer with a high speed red detector, and using a 370 nm picosecond pulse diode laser when exciting MB-Ir-BHQ2 and MB-Ir-Cy5 and a 444 nm picosecond diode pulse laser when exciting MB-Ru-BHQ2 and MB-Ru-Cy5. Steady-state and time-resolved spectra were obtained in 4mm quartz cuvettes, in solutions of 1 μ M MB and 5 μ M target in Tris buffer 100 μ M Tris-HCl buffer (pH 8.0) with 5 mM $MgCl_2$. Detection limit experiments were performed in 0.1 μ M MB solutions with different concentrations of target. Detection limit was extrapolated to the concentration that would produce a S/B ratio of 3. Metal complexes and MBs synthesis is described in Supporting Information.

Synthetic procedure

All reagents and chemicals were obtained from commercial sources and used without further purification. Potassium hexafluorophosphate was purchased from TCI America. 2-phenylpyridine, iridium(III) chloride and N-hydroxysuccinimide were purchased from Alfa Aesar, USA. And all other chemicals were purchased from Sigma-Aldrich. The oligonucleotides were purchased from Integrated DNA Technologies and their sequences are described below:

MB-NH2-BHQ2: 5'-NH₂-CCC CTC ATC ATC ATC TAC ACT TTT CCC AGG GG-BHQ2-3'

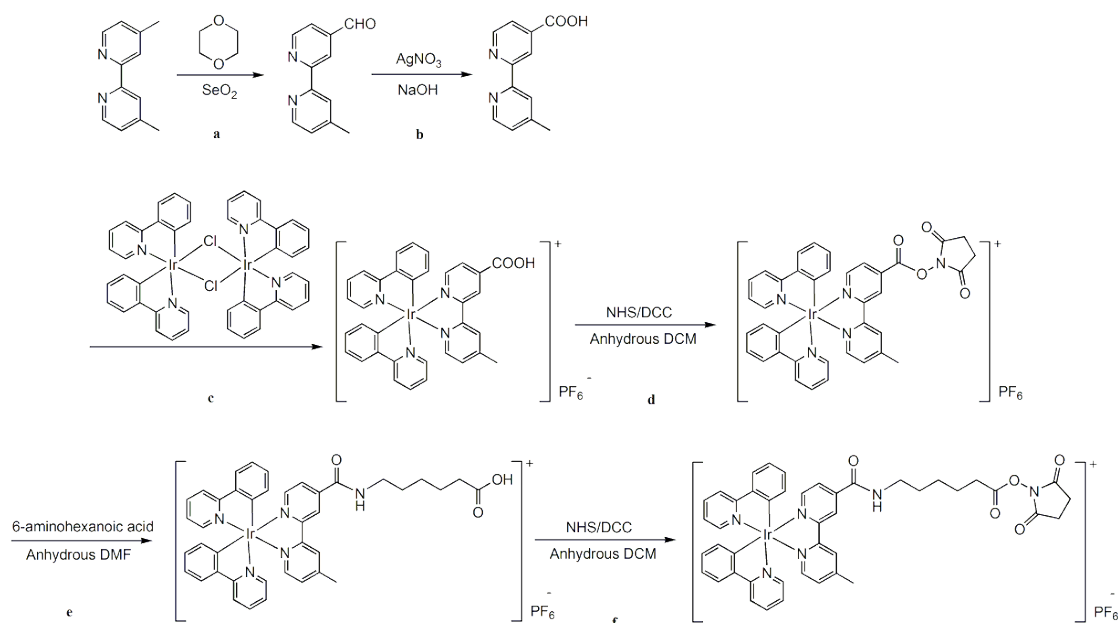
MB-NH2-Cy5: 5'-NH₂-CCC CTC ATC ATC ATC TAC ACT TTT CCC AGG GG-Cy5-3'

Target: 5'-GGGAAAAGTGTAGATGATGAT-3'

a. Synthesis of 4'-Methyl-2,2'-bipyridine-4-carboxaldehyde.

This compound was synthesized based on literature methods.¹ In a typical synthesis 20 mL of dioxane were added to 0.5 g of 4,4'-dimethyl-2,2'-bipyridine in a 50 mL round bottom flask and the solution was purged with argon for 15 min by submerging a piece of Teflon tubing in the flask and bubbling argon into the dioxane with stirring. Selenium dioxide (toxic) (0.34 g) was added to the flask and the argon sparging was continued for 20 minutes. The solution was then heated at

Scheme 1. Synthesis of $[\text{Ir}(\text{ppy})_2(\text{mbpyCONH}(\text{CH}_2)_5\text{CO-NHS})]\text{PF}_6$.



a gentle reflux for 24 hours and as the reaction progressed, solid selenium metal precipitated on the sides of the flask. After the solution cooled down to room temperature, it was filtered by gravity and the solvent was removed under reduced pressure. The remaining solid was dissolved in ethyl acetate, heated at reflux for one hour and gravity filter while hot. Next, the filtrate was washed with 0.1 M sodium carbonate (2×12 mL) to extract any acid bipyridine formed and then extracted into a solution of 0.3 M sodium metabisulfite. After using solid bicarbonate to adjust the pH to about 10, the product was extracted into methylene chloride. Additional sodium carbonate was added to the aqueous solution to maximize partitioning into the organic layer. The solvent layers were separated and the organic solvent was removed under reduced pressure. Yield: 0.26g (48%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) 10.179 (s, 1H), 8.893 (d, $J = 4.8$, 1H), 8.827 (s, 1H), 8.575 (d, $J = 4.8$, 1H), 8.276 (s, 1H), 7.720 (d, $J = 4.8$, 1H), 7.193 (d, $J = 4.8$, 1H), 2.463 (s, 3H). ESI-MS: m/e 199.1 ($\text{M} + \text{H}^+$).

b. Synthesis of 4'-methyl-2,2'-bipyridine-4-carboxylic acid (mbpyc).

This compound was synthesized based on Peek et al.² In a typical synthesis a solution of AgNO_3 (0.2 g) in water (2 mL) was added to a suspension of 4'-methyl-2,2'-bipyridine-4-carboxaldehyde (0.22 g) in 95% ethanol (9.5 mL). The yellow suspension was stirred rapidly and 5 mL of a 1.0 M NaOH solution were added dropwise over 20 minutes to form Ag_2O . The dark black reaction mixture was stirred rapidly for 15 hours. Ethanol was removed by rotovap and the aqueous residue was filtered through a fine-porosity glass frit to remove Ag_2O and metallic silver. The solid were washed with 1.3 M NaOH (2×2 mL) and water (2 mL). The combined basic filtrates were extracted with CH_2Cl_2 to remove unreacted aldehyde and adjusted to pH 3.5 with 1:1 (v/v) 4 N HCl/acetic acid, which

produced a white precipitate. The mixture was kept at -10 °C overnight, after which the white solid was collected and vacuum dried to afford pure 4'-methyl-2,2'-bipyridine-4-carboxaldehyde (mbpyc). Yield: 0.2 g (84%). ¹H NMR (400 MHz, *d*₆-DMSO) δ (ppm) 8.860 (d, *J* = 5.2, 1H), 8.819 (s, 1H), 8.580 (d, *J* = 5.2, 1H), 8.272 (s, 1H), 7.860 (d, *J* = 4.8, 1H), 7.336 (d, *J* = 4.8, 1H), 2.435 (s, 3H). ESI-MS: *m/e* 215.1 (M + H⁺).

c. Synthesis of [Ir(ppy)₂(mbpyc)]PF₆.

This compound was synthesized based on Zhao et al.³ In a typical synthesis a mixture of 2-ethoxyethanol and water (3:1, v/v) was added to a flask containing IrCl₃ (0.30 g) and ppy (0.34 g). The mixture was refluxed for 24 h. After cooling, the yellow solid precipitate was filtered by vacuum to give crude cyclometalatediridium(III) chloro-bridged dimer. The solution of cyclometalatediridium(III) chloro-bridged dimer (0.1 g) and mbpyc (0.05 g) in CH₂Cl₂/MeOH [15mL, 1:1 (v/v)] was heated to reflux. After 4 h, the orange solution was cooled to room temperature and then potassium hexafluorophosphate (0.08 g) was added. The suspension was stirred for 15 minutes and then filtered to remove insoluble inorganic salts. The filtrate was evaporated to dryness under reduced pressure and the crude product was purified by silica gel chromatography in 70% yield. ¹H NMR (400 MHz, *d*₆-DMSO) δ (ppm) 9.022 (s, 1H), 8.404 (s, 1H), 8.034 (d, *J* = 5.6, 1H), 7.867 (m, 3H), 7.376 (t, *J* = 5.6, 2H), 7.707 (d, *J* = 4.0, 1H), 7.652 (d, *J* = 7.6, 2H), 7.533 (m, 2H), 7.194 (d, *J* = 5.6, 1H), 7.405 (t, *J* = 7.2, 2H), 6.994 (t, *J* = 7.6, 2H), 6.883 (t, *J* = 7.2, 2H), 6.275 (m, 2H), 2.518 (s, 3H). ESI-MS: *m/e* 715.2 (M – PF₆⁻).

d. Synthesis of [Ir(ppy)₂(mbpyCO-NHS)]PF₆.

The mixture of [Ir(ppy)₂(mbpyC)]PF₆ (0.12 g), NHS (0.05 g) and DCC (0.08 g) was dissolved in anhydrous CH₂Cl₂ (10 mL) and stirred at room temperature for 12 hours under N₂ atmosphere. The red solution was filtered to remove the white precipitate and the filtrate was evaporated to dryness under reduced pressure. The crude product was used directly for next step. ESI-MS: *m/e* 812.2 (M – PF₆⁻).

e. Synthesis of [Ir(ppy)₂(mbpyCONH(CH₂)₅COOH)]PF₆.

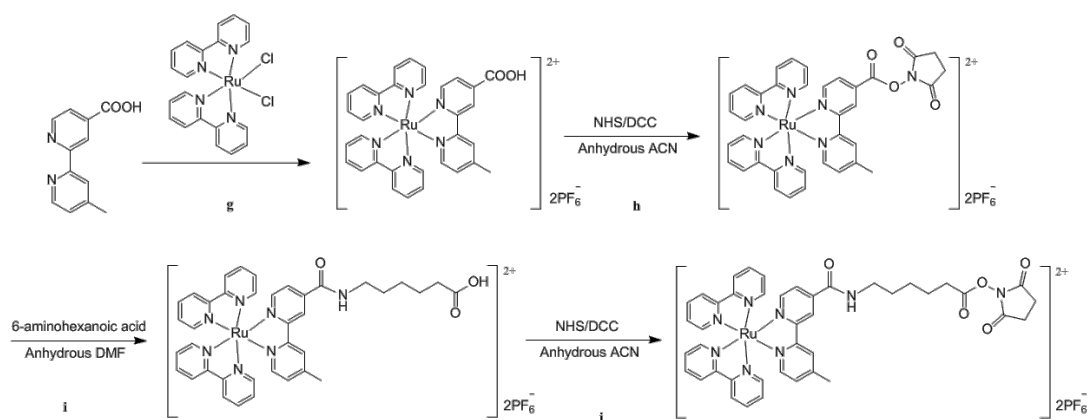
The mixture of [Ir(ppy)₂(mbpyc)]PF₆ (0.05 g) and 6-aminohexanoic acid (0.05 g) was dissolved in anhydrous DMF and stirred at room temperature for 12 hours under N₂ atmosphere. The solution was filtered to remove the unreacted 6-aminohexanoic acid. The solvent was evaporated under reduced pressure and the solid was purified by silica gel chromatography in 70% yield. ¹H NMR (400 MHz, *d*₆-DMSO) δ (ppm) 8.736 (s, 1H), 8.599 (s, 1H), 8.024 (d, *J* = 5.6, 1H), 7.909 (m, 2H), 7.840 (d, *J* = 7.2, 1H), 7.767 (d, *J* = 5.6, 2H), 7.747 (d, *J* = 7.2, 1H), 7.682 (d, *J* = 7.6, 2H), 7.541 (d, *J* = 5.6, 1H), 7.460 (d, *J* = 5.6, 1H), 7.214 (d, *J* = 5.6, 1H), 7.031 (m, 4H), 6.915 (m, 2H), 6.288 (d, *J* = 7.6, 2H), 3.485 (m, 2H), 2.615 (s, 3H), 2.340 (t, *J* = 7.2, 2H), 1.675 (m, 4H), 1.422 (m, 2H). ESI-MS: *m/e* 828.3 (M – PF₆⁻).

f. Synthesis of [Ir(ppy)₂(mbpyCONH(CH₂)₅CO-NHS)]PF₆.

The mixture of [Ir(ppy)₂(mbpyCONH(CH₂)₅COOH)]PF₆ (0.12 g), NHS (0.05

g) and DCC (0.08 g) was dissolved in anhydrous CH_2Cl_2 (10 mL) and stirred at room temperature for 12 hours under N_2 atmosphere. The red solution was filtered to remove the white precipitate and filtrate was evaporated to dryness under reduced pressure. The crude product was purified by silica gel chromatography in 90% yield. ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.717 (s, 1H), 8.571 (s, 1H), 8.027 (d, $J = 5.6$, 5H), 7.914 (dd, $J = 7.2$, 3.6, 2H), 7.844 (dd, $J = 5.6$, 1.6, 1H), 7.766 (m, 3H), 7.688 (d, $J = 5.6$, 2H), 7.546 (d, $J = 5.6$, 1H), 7.470 (d, $J = 5.6$, 1H), 7.225 (d, $J = 5.6$, 1H), 7.031 (m, 4H), 6.914 (m, 2H), 6.290 (dd, $J = 7.6$, 2.8, 1H), 3.489 (q, 2H), 2.777 (s, 4H), 2.610 (s, 3H), 2.605 (t, $J = 7.6$, 2H), 1.708 (m, 2H), 1.713 (m, 2H), 1.493 (m, 2H). ESI-MS: m/e 925.5 ($\text{M} - \text{PF}_6^-$).

Scheme 2. Synthesis of $[\text{Ru}(\text{bpy})_2(\text{mbpyCONH}(\text{CH}_2)_5\text{CO-NHS})](\text{PF}_6)_2$.



g. Synthesis of $[\text{Ru}(\text{bpy})_2(\text{mbpyC})](\text{PF}_6)_2$.

This compound was synthesized based on literature methods.⁴ In a typical synthesis, a solution of cis-dichlorobis(2,2'-bipyridine)ruthenium(II) dihydrate (0.104 g) and mbpyC (0.043 g) in $\text{H}_2\text{O}/\text{MeOH}$ [15mL, 1:1 (v/v)] was heated to reflux. After 4 h, the deep red solution was cooled down to room temperature and then potassium hexafluorophosphate (0.08 g) was added. The suspension was stirred for 15 minutes and then filtered to remove insoluble inorganic salts. The solution was evaporated to dryness under reduced pressure and the crude product was purified by silica gel chromatography in 70% yield. ^1H NMR (400 MHz, CD_3CN) δ (ppm) 8.836 (s, 1H), 8.468 (m, 5H), 8.026 (t, $J = 8.0$, 4H), 7.907 (d, $J = 5.6$, 1H), 7.733 (dd, $J = 5.6$, 1.6, 1H), 7.695 (m, 4H), 7.545 (d, $J = 6.0$, 1H), 7.376 (m, 4H), 7.259 (d, $J = 5.6$, 1H), 2.522 (s, 3H). ESI-MS: m/e 773.1 ($\text{M} - \text{PF}_6^-$), 314.1 ($[\text{M} - 2\text{PF}_6^-]/2$).

h. Synthesis of $[\text{Ru}(\text{bpy})_2(\text{mbpyCO-NHS})](\text{PF}_6)_2$.

The mixture of $[\text{Ru}(\text{bpy})_2(\text{mbpyC})](\text{PF}_6)_2$ (0.108 g), NHS (0.051 g) and DCC (0.083 g) was dissolved in anhydrous acetonitrile (20 mL) and stirred at room temperature for 12 hours under N_2 atmosphere. The red solution was filtered to remove the white precipitate and the filtrate was evaporated to dryness under

reduced pressure. The crude product was used directly for next step. ESI-MS: m/e 870.1 ($M - PF_6^-$), 362.6 ($[M - 2PF_6^-]/2$).

i. Synthesis of $[Ru(bpy)_2(mbpyCONH(CH_2)_5COOH)](PF_6)_2$.

The crude $[Ru(bpy)_2(mbpyc)](PF_6)_2$ (0.101 g) from step h and 6-aminohexanoic acid (0.050 g) were dissolved in anhydrous DMF and stirred at room temperature for 12 hours under N_2 atmosphere. The solution was filtered to remove the unreacted 6-aminohexanoic acid. The solvent was evaporated under reduced pressure and the solid was purified by silica gel chromatography in 70% yield. 1H NMR (400 MHz, CD_3CN) δ (ppm) 8.767 (s, 1H), 8.517 (m, 5H), 8.080 (t, $J = 8.0$, 4H), 7.875 (d, $J = 6.0$, 1H), 7.749 (m, 4H), 7.653 (dd, $J = 6.0$, 2.0, 1H), 7.589 (d, $J = 6.0$, 1H), 7.418 (m, 4H), 7.300 (d, $J = 5.6$, 1H), 3.412 (q, 2H), 2.575 (s, 3H), 2.305 (q, 2H), 1.624 (m, 4H), 1.406 (m, 2H). ESI-MS: m/e 886.2 ($M - PF_6^-$), 370.6 ($[M - 2PF_6^-]/2$).

j. Synthesis of $[Ru(bpy)_2(mbpyCONH(CH_2)_5CO-NHS)](PF_6)_2$.

The mixture of $[Ru(bpy)_2(mbpyCONH(CH_2)_5COOH)](PF_6)_2$ (0.108 g), NHS (0.050 g) and DCC (0.081 g) was dissolved in anhydrous acetonitrile (20 mL) and stirred at room temperature for 12 hours under N_2 atmosphere. The red solution was filtered to remove the white precipitate and the filtrate was evaporated to dryness under reduced pressure. The crude product was purified by silica gel chromatography in 85% yield. 1H NMR (400 MHz, CD_3CN) δ (ppm) 8.750 (s, 1H), 8.525 (m, 5H), 8.073 (t, $J = 7.2$, 4H), 7.874 (d, $J = 5.6$, 1H), 7.749 (m, 4H), 7.660 (dd, $J = 5.6$, 1.6, 1H), 7.591 (d, $J = 5.6$, 1H), 7.427 (m, 4H), 7.300 (d, $J = 6.0$, 1H), 3.419 (q, 2H), 2.736 (s, 4H), 2.635 (t, $J = 7.2$, 2H), 2.566 (s, 3H), 1.744 (m, 2H), 1.651 (m, 2H), 1.475 (m, 2H). ESI-MS: m/e 983.2 ($M - PF_6^-$), 419.1 ($[M - 2PF_6^-]/2$).

Synthesis of MB-Ir-X and MB-Ru-X (X = BHQ2 or Cy5)

MB-Ir-X was prepared by coupling MB-NH₂-X with $[Ir(ppy)_2(mbpyCONH(CH_2)_5CO-NHS)]PF_6$ in mixture solution of DMSO/phosphate buffer (pH 6.0) (2/3, v/v) at room temperature overnight. The crude product was desalted by size exclusion chromatography PD-10 column with water as solvent and then purified by HPLC. An XBridge OST C18 Column (2.5 μm , 4.6 x 50 mm) was used. The elution was performed over 90 min at a flow rate of 0.5 mL min⁻¹ and a fixed temperature of 60 °C, using a linear gradient (12-50%) of methanol in a buffer containing 8.6 mM triethylamine and 100 mM hexafluoroisopropyl alcohol (pH = 8.1).⁵ The pure MB-Ir-X was identified by MALDI-TOF [for MB-Ir-BHQ2 11169.7 (found), 11171.1(Calcd)] and [for MB-Ir-Cy5 11279.3 (found), 11275.3 (Calcd)]. MB-Ru-X was prepared by the same procedure using $[Ru(bpy)_2(mbpyCONH(CH_2)_5CO-NHS)](PF_6)_2$ and identified by MALDI-TOF [for MB-Ru-BHQ2 11083.3 (found), 11084.1(Calcd)] and [for MB-Ir-Cy5 11186.6 (found), 1118823 (Calcd)].

Table S1. Photoluminescence lifetime of different photoluminescent species in the MBs

Name	Lifetime, ns ^d (%) ^e [Ir(ppy) ₂ (dmbp)] ⁺ $\lambda_{em} = 590$ nm	Lifetime, ns ^d (%) ^e [Ru(bpy) ₂ (dmbp)] ²⁺ $\lambda_{em} = 630$ nm	Lifetime, ns ^d (%) ^e Cy5 $\lambda_{em} = 660$ nm
[Ir(ppy) ₂ (dmbp)] ⁺ ^a	340.6	—	—
[Ru(bpy) ₂ (dmbp)] ²⁺ ^b	—	337.1	—
MB-NH ₂ -Cy5 ^c	—	—	0.70 (27.06%) 1.6 (74.94%)
MB-Ir-BHQ2 (no T)	8.1 (53.19%) 54.1 (46.81%)	—	—
MB-Ir-BHQ2 (with T)	7.1 (40.55%) 76.2 (59.45%)	—	—
MB-Ir-Cy5 (no T)	1.8 (25.34%) 15.5 (40.11%) 90.5 (34.55%)	—	1.5 (77.24%) 6.8 (13.73%) 87.4 (9.03%)
MB-Ir-Cy5 (with T)	1.5 (4.80%) 17.4 (21.67%) 86.2 (73.53%)	—	1.2 (74.39%) 3.6 (11.59%) 81.4 (14.02%)
MB-Ru-BHQ2 (no T)	—	263.6	—
MB-Ru-BHQ2 (with T)	—	443.2	—
MB-Ru-Cy5 (no T)	—	5.5 (47.10%) 147.5 (52.90%)	2.8 (41.20%) 17.0 (20.84%) 114.9 (37.97%)
MB-Ru-Cy5 (with T)	—	1.3 (7.63%) 425.6 (92.37%)	2.0 (13.09%) 375.1 (86.91%)

a. In DMSO/H₂O (1/9, v/v)

b. In H₂O

c. All MBs were measured in buffer.

d. Lifetime uncertainty < ± 5%

e. % of contribution to the exponential decay law

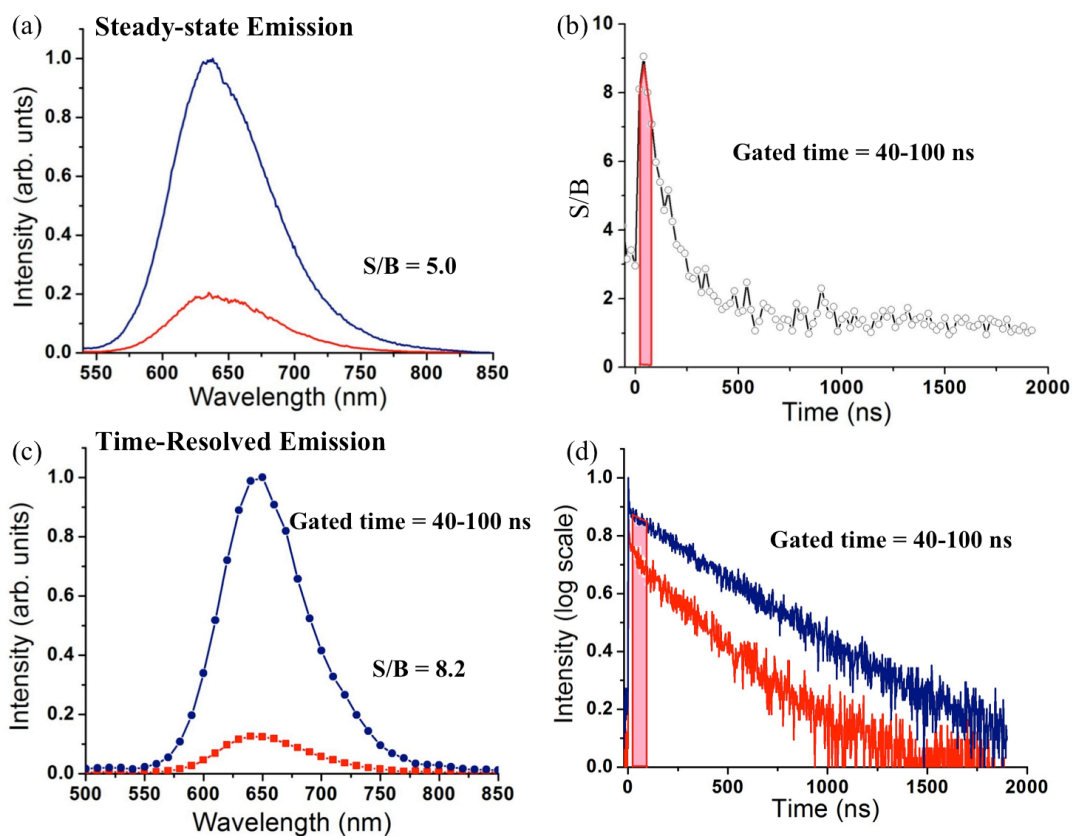


Figure S1. (a) SSPS spectra of MB-Ru-BHQ2 without (—) and with target (—). (b) Time-gating optimization ratio for MB-Ru-BHQ2 portraying a 40-100 ns time window as the optimum time-window for TRPS. (c) TRES obtained with a 40 – 100 ns time-window without (—) and with target (—). (d) Time-resolved photoluminescence decays ($\lambda_{em} = 630$ nm) without (—) and with target (—) portraying the time window used for TRES.

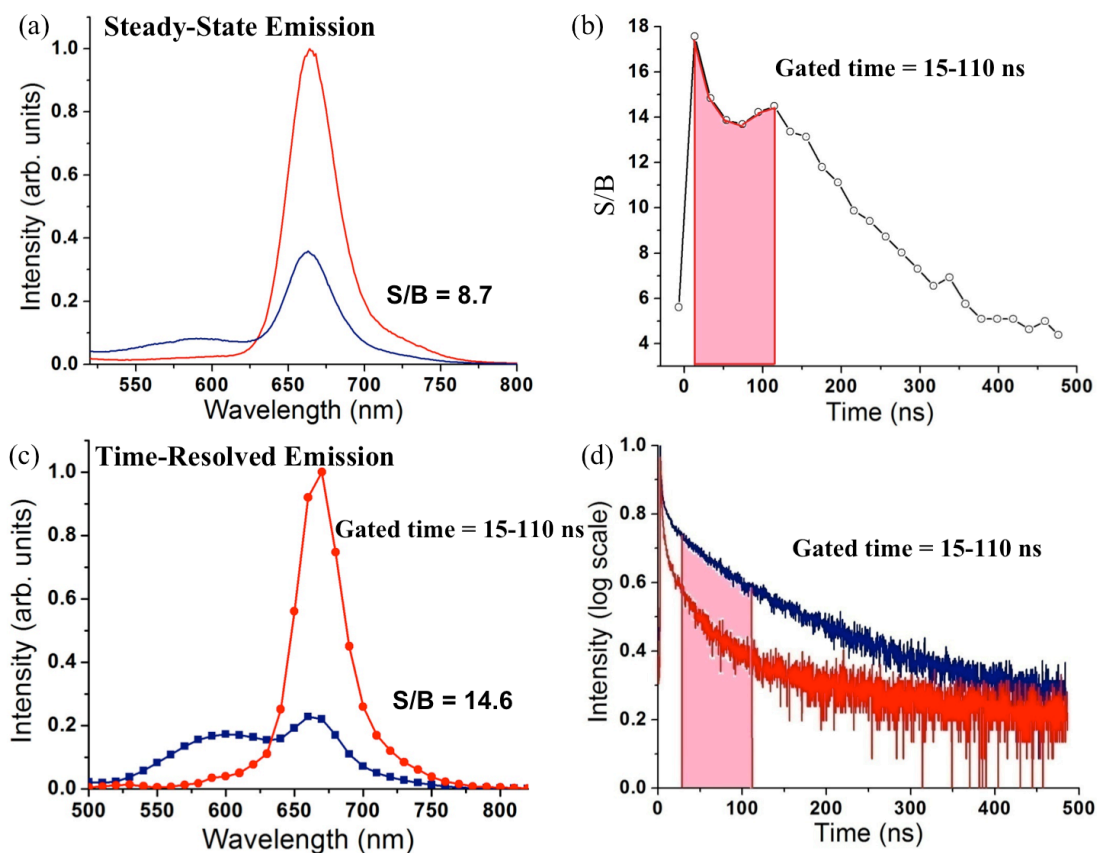


Figure S2. (a) SSPS spectra of MB-Ir-Cy5 without (—) and with target (—). (b) Time-gating optimization ratio for MB-Ir-Cy5 portraying a 15-110 ns time window as the optimum time-window for TRPS. (c) TRES obtained with a 15-110 ns time-window without (—) and with target (—). (d) Time-resolved photoluminescence decays ($\lambda_{em} = 590$ nm) without (—) and with target (—) portraying the time window used for TRES.

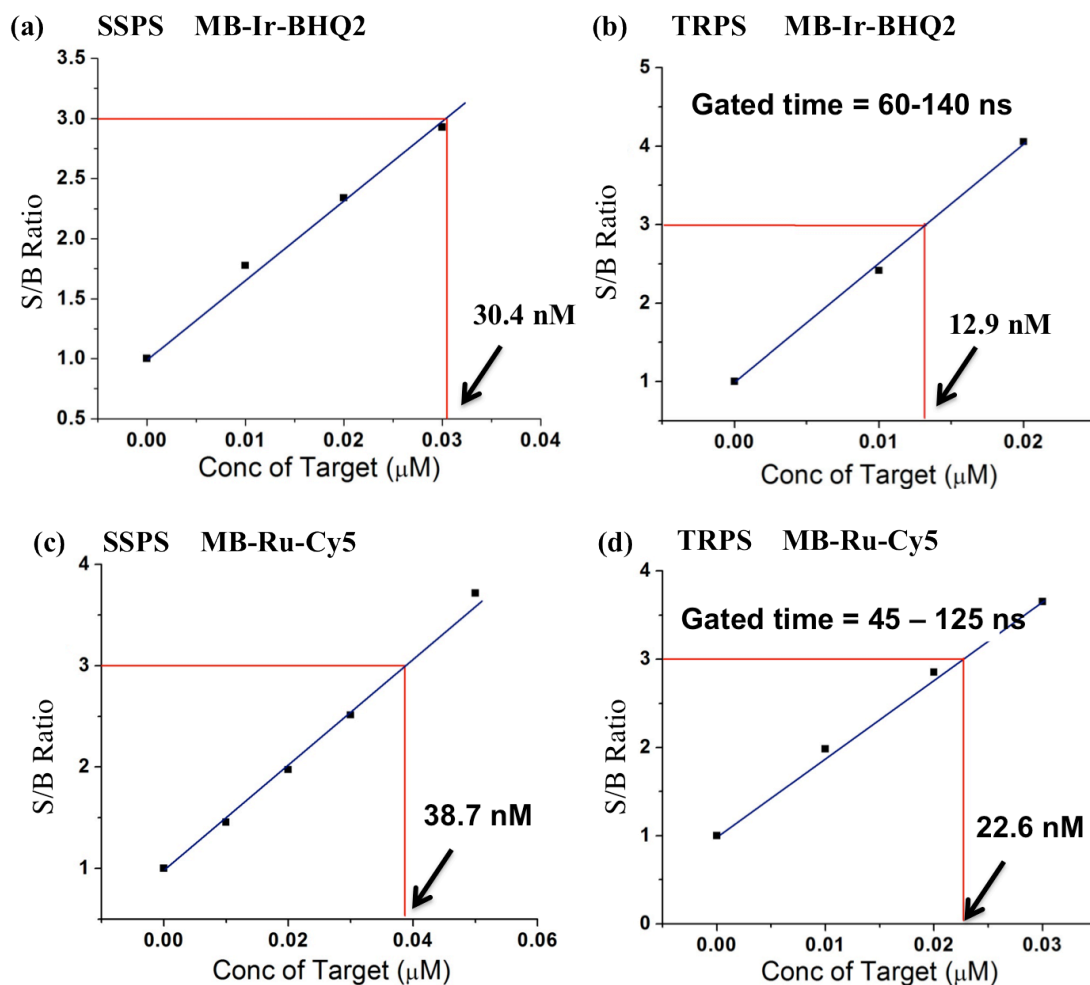


Figure S3. Detection limit determination of MB-Ir-BHQ2 using (a) SSPS and (b) TRPS and for MB-Ru-Cy5 using (c) SSPS and (d) TRPS. The detection limit is defined as the amount of target necessary to raise the S/B ratio to a value of 3. Figure S3a-b and Figure S3c-d represent just one of 3 independent experiments.

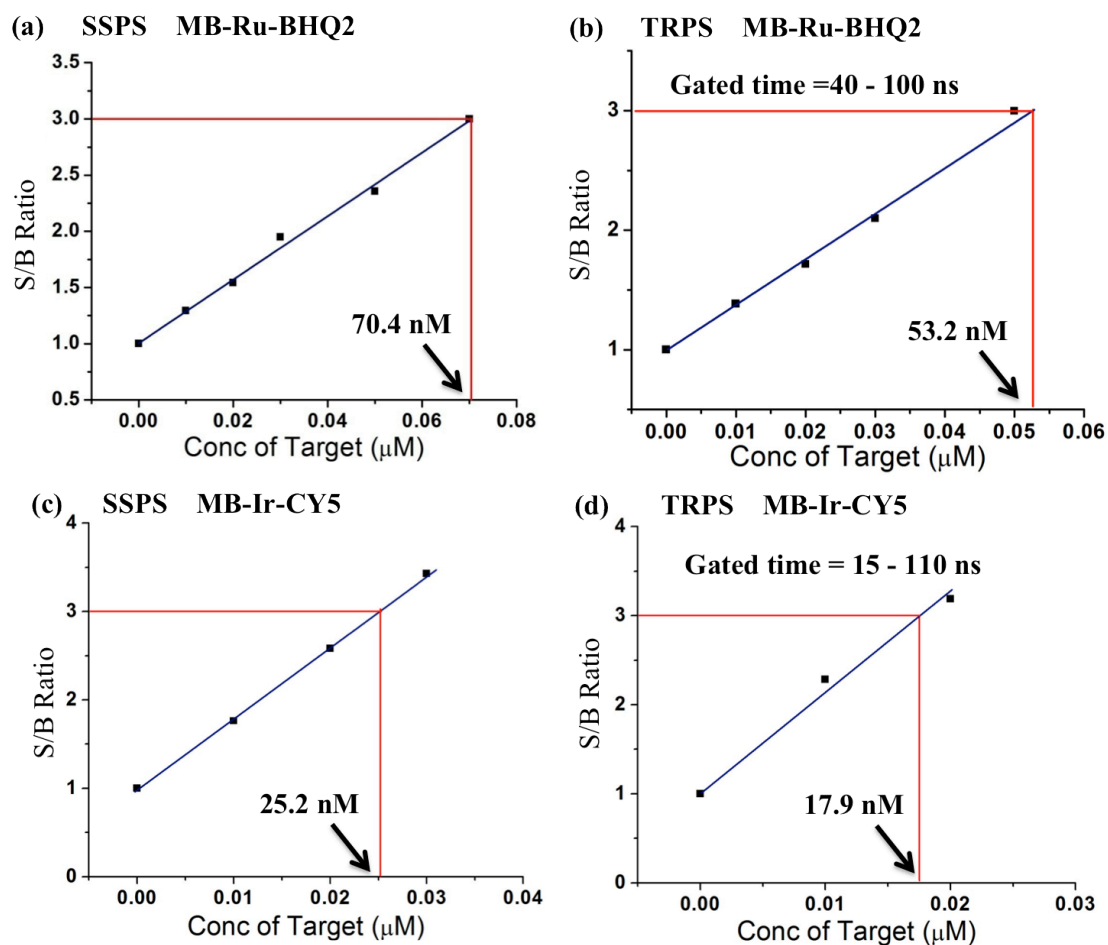


Figure S4. Detection limit determination of MB-Ru-BHQ2 using (a) SSPS and (b) TRPS and for MB-Ir-Cy5 using (c) SSPS and (d) TRPS. The detection limit is defined as the amount of target necessary to raise the S/B ratio to a value of 3. Figure S4a-b and Figure S4c-d represent just one of 3 independent experiments.

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Appendix 1

In this section we will examine in closer detail how the time-dependent S/B change for **type 2** and **type 3** probes. As discussed in the manuscript, in **type 2** probes the lifetime of **P+T** is shorter than the lifetime of **P**. To examine the effect of non-specific background (axis offset) and difference in lifetimes, we have plotted **Figure 2a and b**. Similar to **type 1** probes in the manuscript, the time-resolved S/B in the absence of non-specific background and integrated over the whole time is set to 20. As explained before, this will set the total intrinsic photoluminescence intensity of **P+T** to be 20-fold larger than **P**, regardless of what parameters are changed. **Figure A1a** shows the effect of the axis offset (non-specific background) in the S/B(t). Contrary to **Figure A1a**, where the S/B(t) was very dependent on the offset, here the effect is minimum, which is directly related to **P** having a longer lifetime than **P+T**. On the other hand, it can be observed that the time-dependant S/B increases as the time decreases without the appearance of a maximum in the curve. **Figure A1b** shows the effect of leaving **P+T** fixed at 5 ns and changing the lifetime of **P** with a constant offset 0.02. As the lifetime of **P** increases the S/B(t) also increases which is a consequence of fixing the time-resolved S/B in the absence of non-specific background and integrated over the

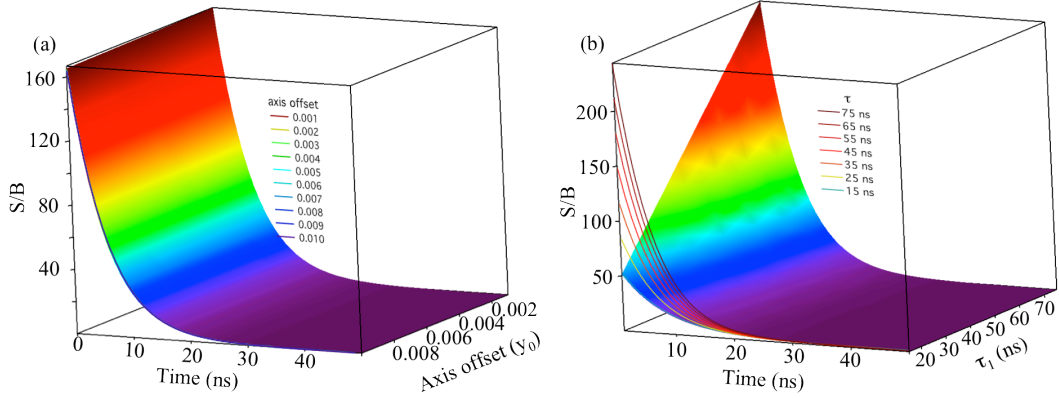


Figure A1. Three-dimensional plot of the S/B (equation 2) of **type 3** probes by varying the time after excitation and (a) the axis offset or (b) the lifetime of **P**. For (a) the lifetimes of **P** is set to 50 ns, **P+T** is 5 ns, and the preexponential factor of **P+T** is set to 200, while for (b) the lifetime of **P+T** is 5 ns, and the offset is 0.01.

$$(a) \quad \frac{S}{B} = \frac{200e^{-\frac{t}{5ns}} + y_0}{e^{-\frac{t}{50ns}} + y_0} \quad (b) \quad \frac{S}{B} = \frac{Ae^{-\frac{t}{5ns}} + 0.02}{e^{-\frac{t}{\tau_1}} + 0.02}$$

Where A (the amplitude of the **P+T** exponential function) is changed such as the S/B in the absence of background and integrated over the whole time is equal to 20:

$$\frac{\int_0^\infty Ae^{-\frac{t}{5ns}} dt}{\int_0^\infty e^{-\frac{t}{\tau_1}} dt} = 20 \rightarrow A = 4\tau_1$$

whole time to 20. Although not apparent from **Figure A1b**, the $S/B(t)$ tends to decrease faster with time as the lifetime of **P** increases. Although **type 2** probes don't show a maximum in their $S/B(t)$ as **type 1** probes do, equation 2 still allows to select the more appropriate time window that would yield the largest possible time-dependant S/B , showing the usefulness of this approach.

Type 3 probes differ from **type 1** and **type 2** in that their photoluminescence lifetimes are the same for **P** and **P+T**. In an ideal **type 3** probe (without non-specific background), the time-dependent S/B should be constant over time and equal to the SSPS time-independent S/B ratio. Even so, in the presence of non-specific background (offset), the $S/B(t)$ will decrease with time, with the decrease being more prominent as the offset increases (**Figure A2a**). Interestingly, **Figure A2b** shows that as the lifetime of **P** and **P+T** increase, the effect of time on the $S/B(t)$ is smaller. Nonetheless, the analysis described in the paper allows to unambiguously identify the time window that will show the best $S/B(t)$, although it should be noted that for **type 2** probes $S/B(t) \leq S/B$, which implies that the sensitivity of these probes cannot be improved using TRPS as efficiently as for **type 1** and **type 2** probes.

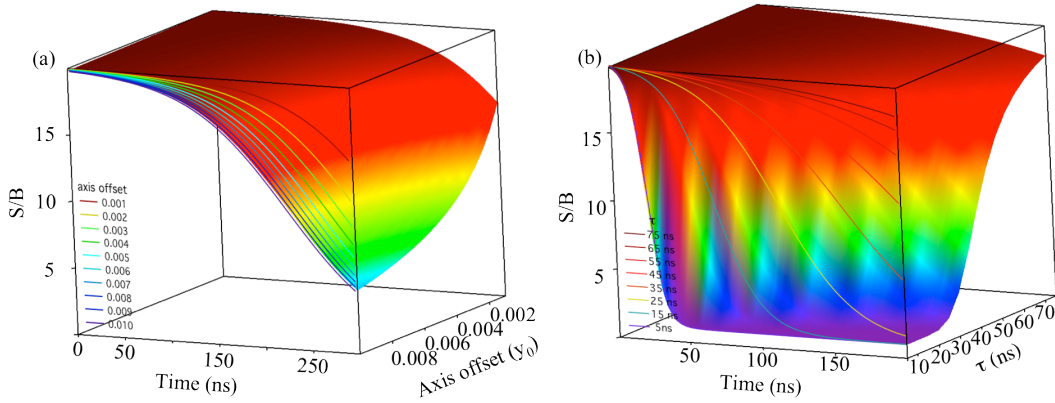


Figure A1. Three-dimensional plot of the S/B of **Type 2** probes by varying the time after excitation and (a) the axis offset or (b) the photoluminescence lifetime of the probe. For (a) the lifetimes of **P** and **P+T** are set both to 50 ns and the amplitude of **P+T** exponential function is set to 20, while for (b) the lifetime of **P** and **P+T** are varied by the same amount, leaving the offset constant at 0.01 and the amplitude of **P+T** exponential function set to 20. The equations that describe these surfaces are:

$$(a) \quad \frac{S}{B} = \frac{20e^{-\frac{t}{50ns}} + y_0}{e^{-\frac{t}{50ns}} + y_0} \quad (b) \quad \frac{S}{B} = \frac{20e^{-\frac{t}{\tau}} + 0.01}{e^{-\frac{t}{\tau}} + 0.01}$$