Ascertaining Free Histidine from Mixtures with Histidine-Containing Proteins Using Time-Resolved Photoluminescence Spectroscopy

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1. General instruments, spectroscopic and experimental methods

^{1}H NMR spectra were recorded with a Bruker 400 MHz NMR spectrometer. Electrospray ionization mass spectra (ESI-MS) were measured on a Bruker MicroToF system.

UV-Visible spectra were recorded on a Shimadzu UV-2450 UV-Vis spectrophotometer. Steady-state photoluminescence spectra were obtained in a HORIBA JovinYvonFluorolog 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, (IRF FWHM ~ 400 ps).

Preparation and incubation of samples

Stock solutions for complexes **3** and **4** were prepared from the solids obtained from the syntheses detailed in the experimental section. For stock solutions of **1** and **2** an alternative method was devised. 5.36 mg of Iridium chloro-bridged dimer (prepared as detailed in the experimental section) were dissolved in 10 mL of DMSO in a 20 mL vial. The resulting suspension was sonicated at room temperature for 1 hour until all the Iridium chloro-bridged dimer is dissolved in DMSO. After that, the solution was kept in dark at room temperature overnight yielding 1 mM of **1** in DMSO solution. Examination of the product by NMR using d^6-DMSO as solvent showed quantitative conversion of the Iridium chloro-bridged dimer to **1**. A similar procedure is also followed for **2**.

The histidine detection assays were performed by preparing 2.97 mL samples of different concentrations of histidine (from 0 to 10 μM) with BSA (0.25 μM) in 4 mL glass vials in PBS (PBS 6.7 mM, pH 7.2). Then 30 μL of 1mM probe **1** solution in DMSO were added in each vial to give 10 μM of probe **1**. The solutions were incubated at room temperature for 6 hours before measurement. Similar procedures were followed for other reactions in the presence of insulin and lysozyme).

2. Mathematical Fittings

Example of how to calculate the proportionality factor, δ.

$[P] = \delta\tau A$

Therefore, the slope (b) of Figure 1S is $1/\delta\tau$. Since τ is fixed as 487 ns. The δ can be obtained as follows:

$\delta = 1/b\tau = 1/(616.757\ \mu M^{-1})\ (487\ ns) = 1/[(6.168 \times 10^{8}\ M^{-1})\ (4.87 \times 10^{-7}\ s)] = 3.329 \times 10^{-3}\ M\ s^{-1}$

The calibration curve and δ value are specific of the instrumentation and experimental conditions used. Therefore the δ value is calculated with experimental conditions specific for the day of the experiment.

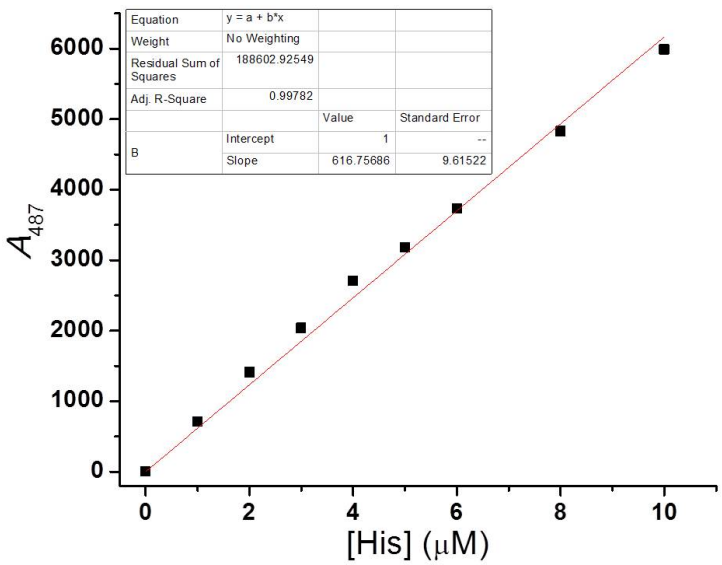

Figure S1. Calculated pre-exponential factors (A_{487}) of a 10 μM solution of probe **1** as a function of histidine concentration (fixed lifetime τ at 487 ns) and fitting to a linear model.

Example of non-linear least square fitting procedure of a decay curve:

As an example of the procedure to separate the pre-exponential factor corresponding to the probe bound to free-histidine (**1-His**) from other components, we show the non-linear least square fit of entry 5 of table S1: 10 μM probe **1** with 0.25 μM BSA and 4 μM histidine. The curve was tail fitted to $I = A_0 + A_1 \cdot e^{(-t/t1)} + A_2 \cdot e^{(-t/t2)} + A_3 \cdot e^{(-t/t3)}$ with a fitting range at least 5 times as long as the longest lifetime component (generally 10,000 ns range). The instrument response function obtained from a scattering solution has a FWHM of ca. 0.4 ns, and therefore doesn't contribute significantly to the decay curve, however to reduce contribution from scattering the fitting is performed a few nanoseconds from the maximum. The black curve is the experimental decay while the red curve is the fit. The number of exponentials used in the fit was decided from the minimization of χ^2 and visual inspection of the decay curve and residuals. The minimum number of exponentials required to fit satisfactorily the data in Figure 3 were three.

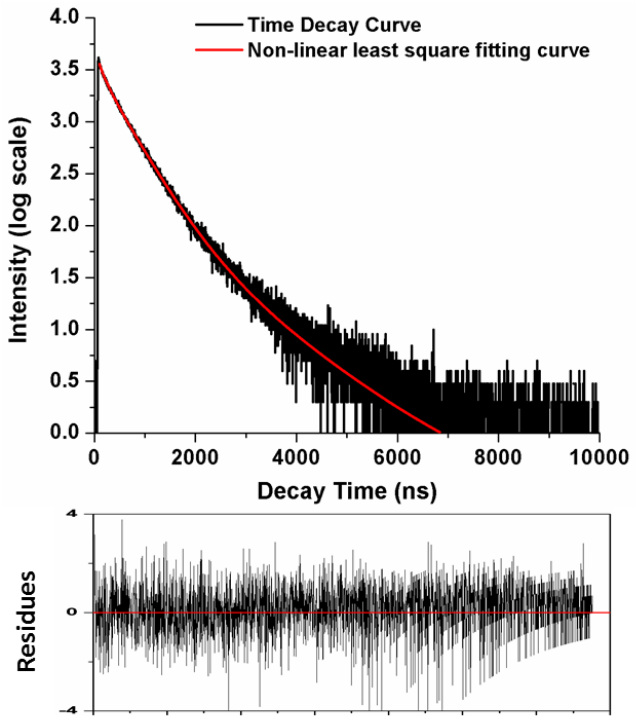

Figure S2. Example of non-linear least square fitting.

Fitting Equation	Fit = $A_0 + A_1 \cdot e^{(-t/t1)} + A_2 \cdot e^{(-t/t2)} + A_3 \cdot e^{(-t/t3)}$				
Fitting Range	From 0 to 10000 ns				
	Value / ns		A_i Value	Std. Dev	Rel %
τ_1	487	A_1	2758	22	81.41
τ_2	1229	A_2	184	9	13.71
τ_3	119	A_3	676	18	4.88
		A_0	0.257		
		χ^2	1.075		

3. Extended Discussion

Table S1. Time-decay titration of histidine (0 – 10 μM, 0.25 μM BSA) to 10 μM solution of probe **1** in PBS (three exponential fit).

[His] (μM)	A_{487} (without BSA, τ_1= 487 ns fixed) (error)[£]	A_{487} (with BSA, τ_1= 487 ns fixed) (error)	% (τ_1) (error)	τ_2, ns (error)	% (τ_2) (error)	τ_3, ns (error)	% (τ_3) (error)	χ^2
0 *	0	0	0	841 (6)	71.8 (0.6)	166 (3)	28 (1)	1.196
1	863 (2)	1060 (20)	65 (2)	1120 (30)	25 (1)	115 (4)	9.6 (0.4)	1.038
2	1538 (3)	1690 (20)	72 (1)	1160 (20)	20.8 (0.9)	108 (4)	6.9 (0.3)	1.030
3	2181 (3)	2240 (20)	78 (1)	1160 (20)	16.4 (0.8)	104 (4)	5.3 (0.3)	1.022
4	2642 (4)	2760 (20)	81 (1)	1230 (20)	13.7 (0.7)	119 (6)	4.9 (0.3)	1.075
5	3190 (4)	3180 (20)	81 (1)	1190 (20)	14.2 (0.7)	128 (6)	4.8 (0.3)	1.102
6	3502 (4)	3580 (20)	83.5 (0.9)	1220 (20)	12.4 (0.6)	128 (7)	4.1 (0.2)	1.046
8	4741 (5)	4190 (40)	83 (1)	1210 (20)	12.2 (0.6)	170 (10)	4.3 (0.3)	1.003
10	5382 (5)	4570 (30)	84 (1)	1260 (20)	12.3 (0.9)	160 (10)	4.0 (0.3)	0.972

[£] χ^2 values for **1** with different concentrations of His are ≤ 1.011.

* Probe **1** in the presence of BSA (no free histidine) presents a biexponential decay. If one of this components is fixed to 487 ns, the non-linear least square routine doesn't provide a satisfactory fit. Therefore we assigned a value of 0 to the A_{487} preexponential factor of **1** with BSA. If a three exponential fit is forced on the time-decay profile of BSA with **1**, and a value of 487 ns if fixed, an amount of about half of the value found for 1 μM His is obtained for A_{487}. This would correspond to a histidine concentration of 0.5 μM, and thus fits that yield A_{487} values around this number < 500) should not be trusted. Therefore for these experimental conditions we estimate a detection limit of 1 μM.

An interesting observation is that the lifetime of the long component corresponding to probe **1** bound to BSA tend to increase with histidine concentration. We believe that as histidine is added, less **1** is available to react with BSA. This suggests that when the amount of **1** reacting with BSA is high, the lifetimes are relatively shorter. Conversely, when the amount of **1** available to react with BSA is low, then the lifetimes are longer. The population of different sites in BSA with different microenvironments (and different lifetimes) when different concentrations

of **1** are available is one possible explanation for this behavior. This observation is also consistent with self-quenching. We confirmed this lifetime dependency leaving BSA constant and changing the concentration of **1** (without free histidine). As can be seen in Table S2 the lifetime of the long component decreases as the concentration of **1** added to BSA increases, which is consistent with the behavior observed in Table S1.

Table S2. 0.25 μM BSA in the presence of different concentration of **1**.

$[Ir(ppy)_2(DMSO)_2^+]$	τ_1 (ns)	%-τ_1	τ_2 (ns)	%-τ_2
1 μM	255.4	15.7	1309.4	84.3
2 μM	262	19.8	1254	80.2
3 μM	224.2	23.2	1118.3	76.8
4 μM	207.6	25.6	1042	74.4
5 μM	193.7	27.4	988.8	72.6
6 μM	168.9	26.4	940.7	73.6
7 μM	157	26.0	880.1	74.0
8 μM	156.9	27.1	876.6	72.9
9 μM	160.7	29.3	866.1	70.7
10 μM	144.1	27.5	815.6	72.5

To further explore the photoluminescence of this system we plotted the steady state photoluminescence intensity as a function of the histidine concentration and in the presence of BSA obtained from a Horiba Fluorolog3 Spectrofluorometer and reproduced from lifetime decays. From equation 2 and 4 we can obtain that the total steady-state intensity can be calculated by:

$$I = \sum_{i=1}^{n} A_i \tau_i \qquad \text{Eq S1}$$

From Figure S3 we can observe that the Fluorolog 3 and lifetime-reproduced steady-state photoluminescence intensity (from Eq S1) match one another. Since we know that the 487 ns component is due to **1** bound to free histidine (**1-His**), then using the other 2 components we can calculate the steady-state intensity behavior of **1** bound to BSA (**1-BSA**). There are two things that are evident from this plot (Figure S4). First, upon addition of 1 μM His, the photoluminescence of **1-BSA** decreases. Second, as more histidine is added to the system the photoluminescence intensity of **1-BSA** increase gradually. Although we don't fully understand this behavior, it is important to recognize that BSA is a complex protein, with cavities capable of binding a variety of molecules.[1] BSA also aggregates in solution.[2] We speculate that the aggregation state of **1-BSA** could be different free in solution than in the presence of histidine or **1-His**. At 0 μM His concentration, many molecules of **1** are bound to BSA (forming **1-BSA**) changing its surface charge and structure, and plausibly inducing the formation of aggregates.

Iridium complexes bound to Histidine in these aggregates are likely buried and protected from molecular oxygen (therefore having relatively high photoluminescence intensity). Addition of **1** μM His (which will be converted to **1-His**) could disrupt these aggregates, exposing now the previously protected Iridium complexes to molecular oxygen. These complexes have strong triplet character and therefore will be easily quenched by molecular oxygen. The observation that upon addition of further His, the emission intensity of **1-BSA** increases is rather consistent with self-quenching. At low His concentrations there is plenty of **1** to react with BSA, which might lead to a high concentration of Iridium complexes bound to BSA. This could produce self-quenching and therefore a relatively low fluorescence. As His is added to the system, less **1** is available to react with BSA and thus the concentration of **1** in BSA decreases, which would produce an increase in emission intensity. Although this is just a possibility, we tested whether the fluorescence of **1-BSA** depends on the concentration of **1** (Figure S5). To our surprise the emission intensity of **1-BSA** reaches a maximum with 2 μM **1**, with a decrease in photoluminescence intensity upon further addition of **1** (Figure S5). This behavior is actually consistent with self-quenching and our observations about **1-BSA** in Figure S4. Another possibility that could explain these observations is that the observed changes in the photoluminescence emission of **1-BSA** are caused by the inability of the least-square routine to correctly calculate the parameters for **1-BSA**. Although this possibility cannot be completely discarded, the fair correspondence of the blue and red markers in Figure 3d give us confidence in the methodology.

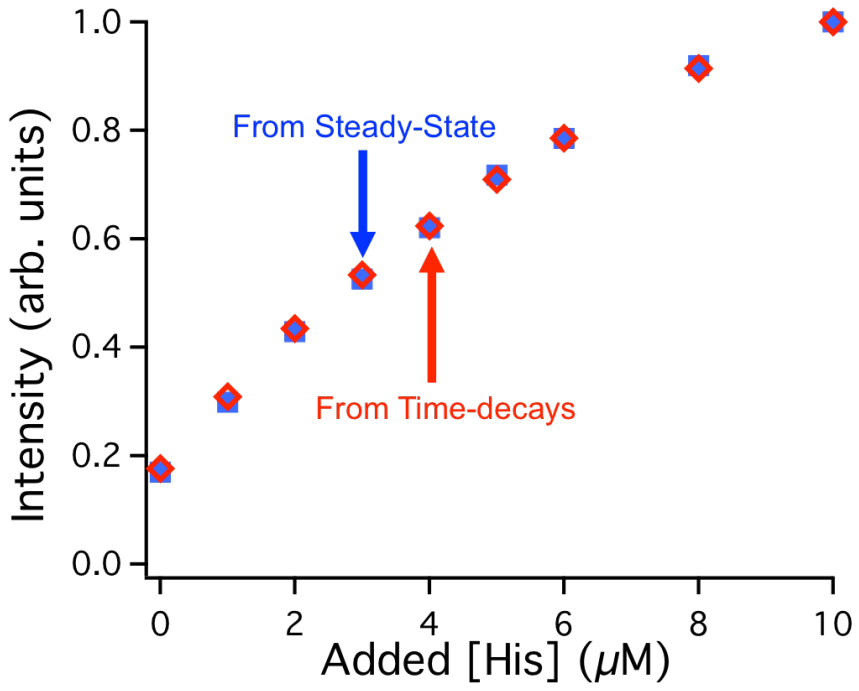

Figure S3. Normalized steady-state photoluminescence intensity at 510 nm (blue square, from Fluorolog3 Spectrometer and data from Figure 3a) and calculated steady-state from time-decays (red open diamonds, data from Figure 3b and Eq S1).

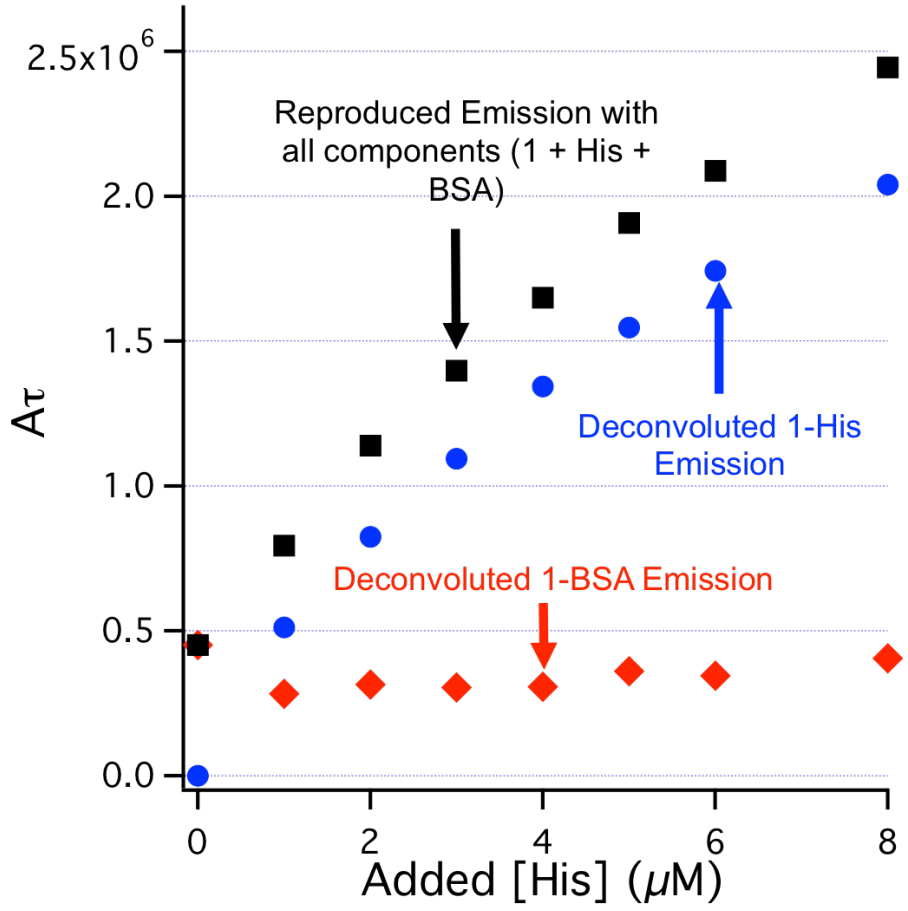

Figure S4. Calculated steady-state photoluminescence intensities for **1-His** (blue circles), **1-BSA** (red diamonds), and steady-state intensity considering all the components (as in Figure S3). Experimental conditions: 0.25 μM BSA and 10 μM **1** in PBS.

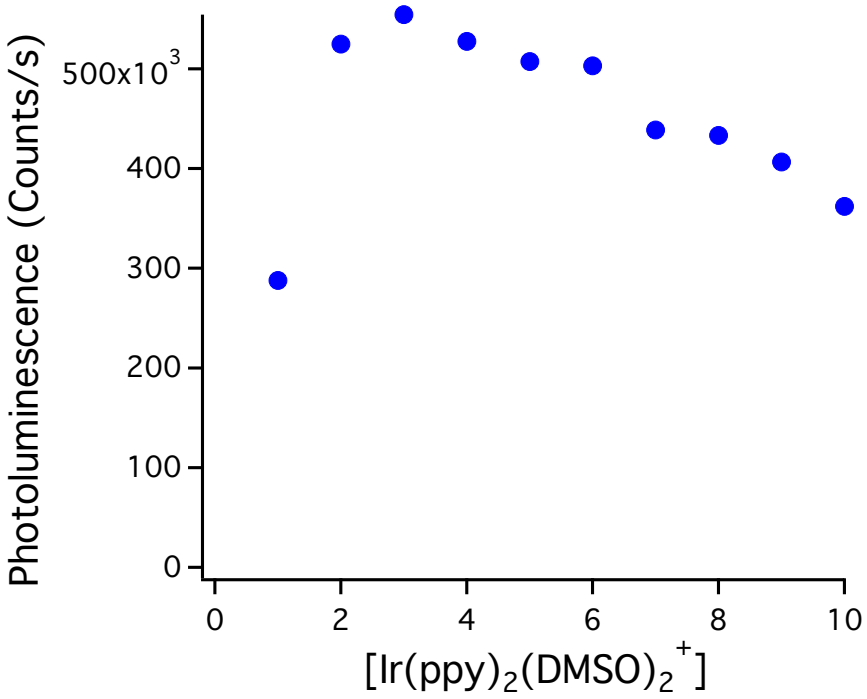

Figure S5. Steady-State photoluminescence intensity of different concentrations of **1** with 0.25 μM BSA.

4. Supplementary spectra data

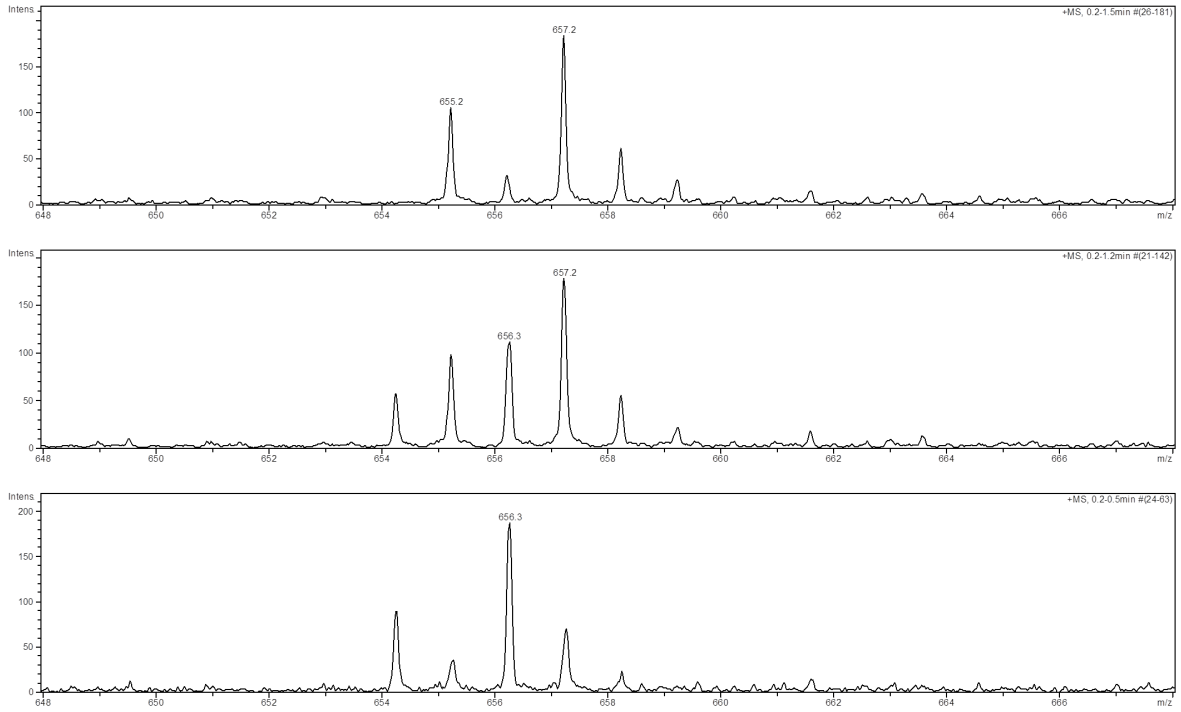

Figure S6. ESI-Mass Spectra of probe **1** in aqueous solution in the absence of histidine (top), in the presence of 0.5 equivalent of histidine (middle) and 1.2 equivalent of histidine (bottom). Notice that the expected mass of **1** ($C_{26}H_{28}IrN_2O_2S_2^+$) is 657.12 and **1-His** ($C_{28}H_{27}IrN_5O_2^+$) is 656.16.

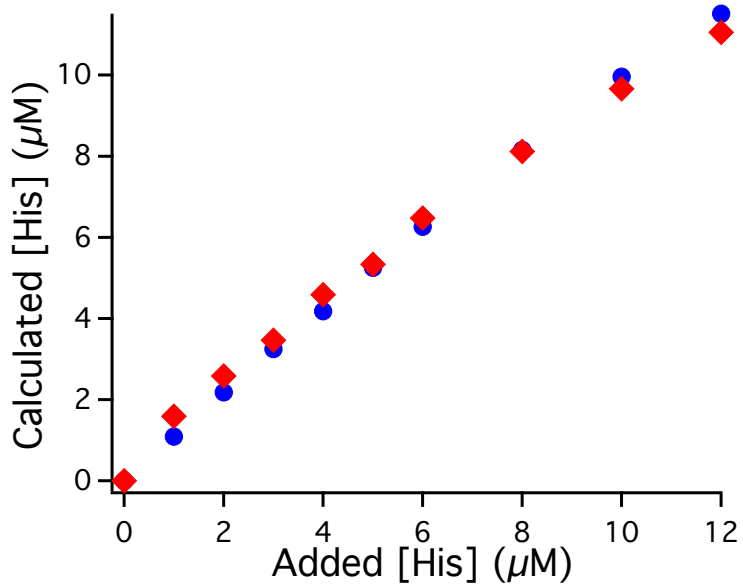

Figure S7. Determination of the free histidine concentration from the A_{487} pre-exponential factors obtained from a 20 μM solution of probe **1** in the presence (red diamonds) and absence (blue circles) of 0.25 μM of BSA.

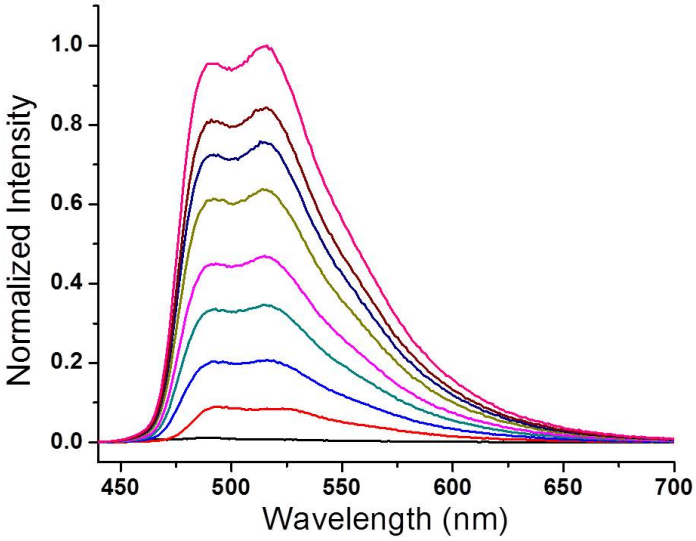

Figure S8. Steady-state photoluminescence of **1** (10 μM) in the presence of different concentrations of histidine (0 – 8 μM) in PBS and with 1.5 μM of insulin.

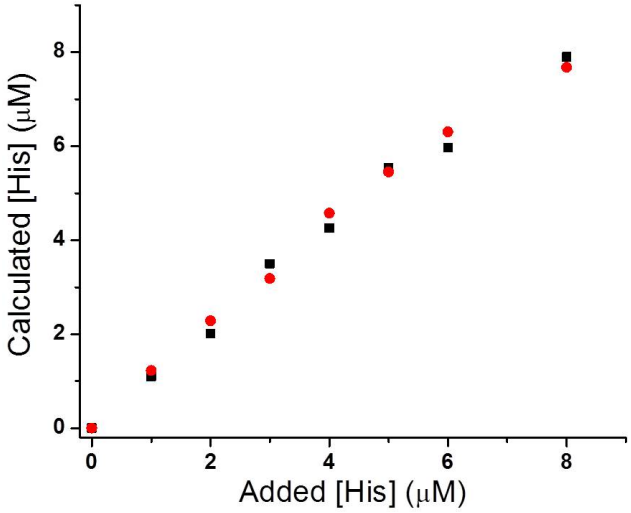

Figure S9. Determination of the free histidine concentration from the A_{487} pre-exponential factors obtained from a 10 μM solution of probe **1** in the presence (red points) and absence (black points) of 1.5 μM of insulin.

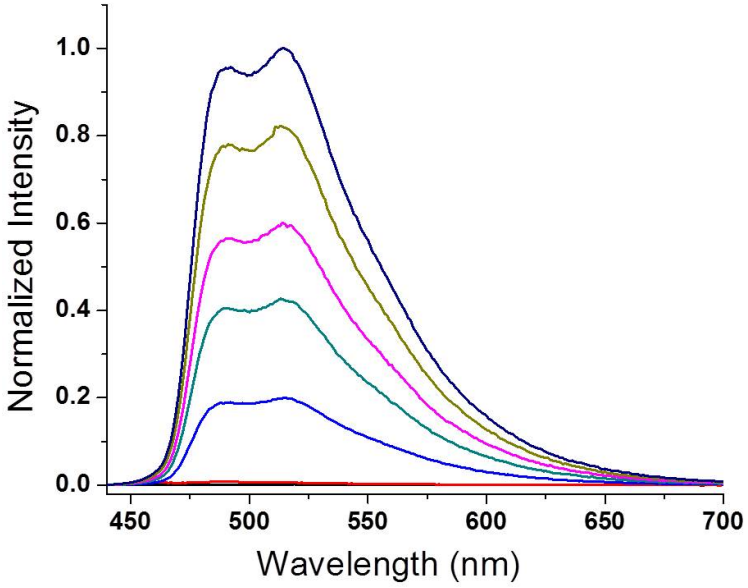

Figure S10. Steady-state photoluminescence of **1** (10 μM) in the presence of different concentrations of histidine (0 – 10 μM) in PBS and with 5 μM of lysozyme.

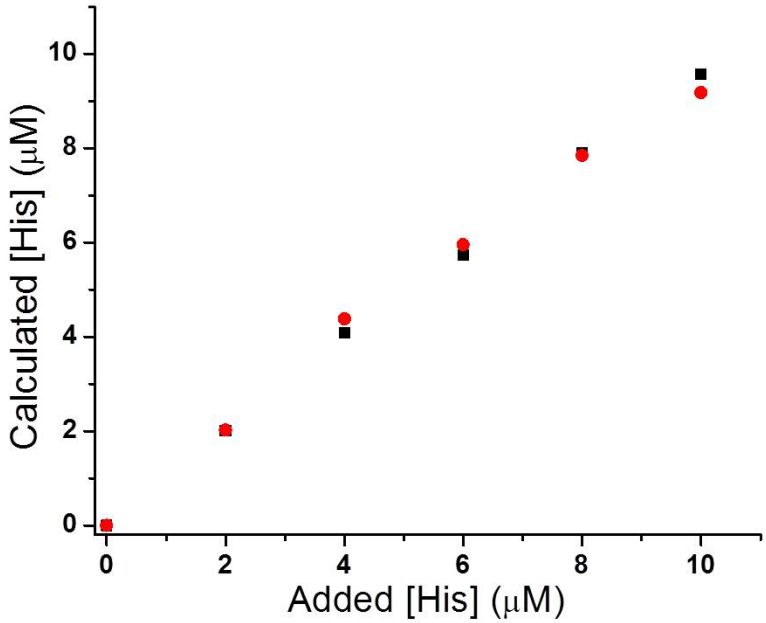

Figure S11. Determination of the free histidine concentration from the A_{487} pre-exponential factors obtained from a 10 μM solution of probe **1** in the presence (red points) and absence (black points) of 5 μM of lysozyme.

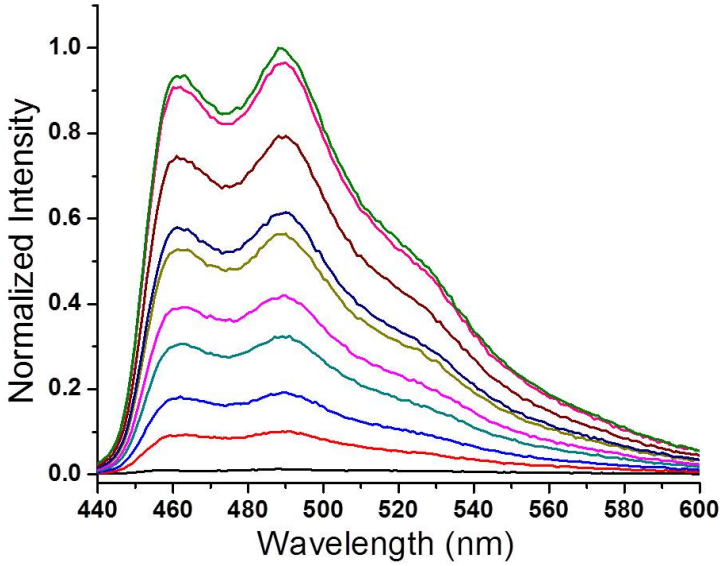

Figure S12. Steady-state spectra of 10 μM probe **2** with different concentrations of histidine (from 0 to 15 μM).

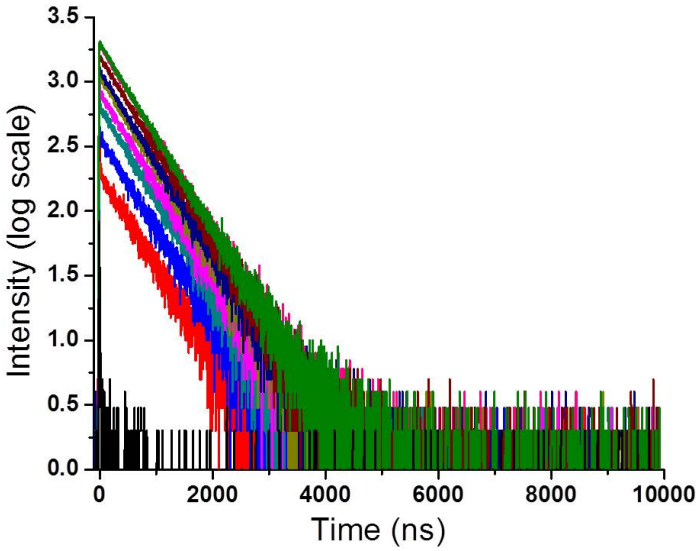

Figure S13. Time-decay profiles of 10 μM probe **2** with different amounts of histidine (from 0 to 15 μM).

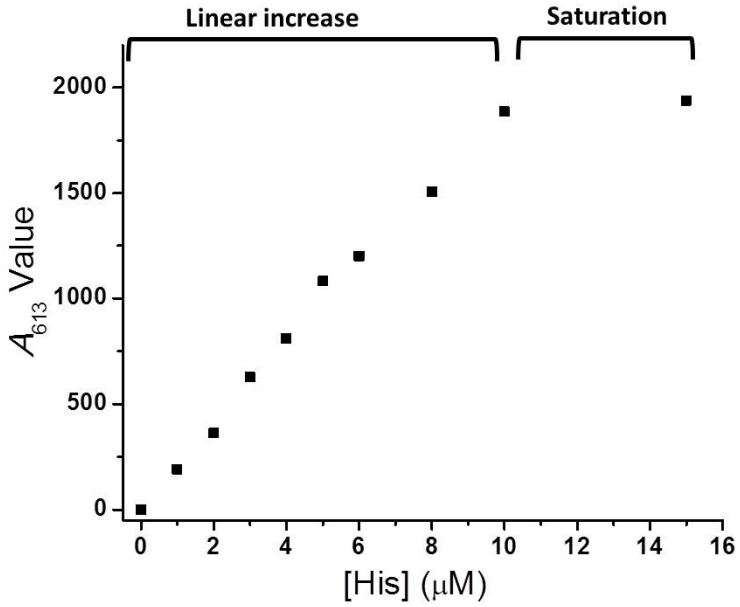

Figure S14. Calculated A_{613} values for 10 μM probe **2** with different amounts of histidine (from 0 to 15 μM).

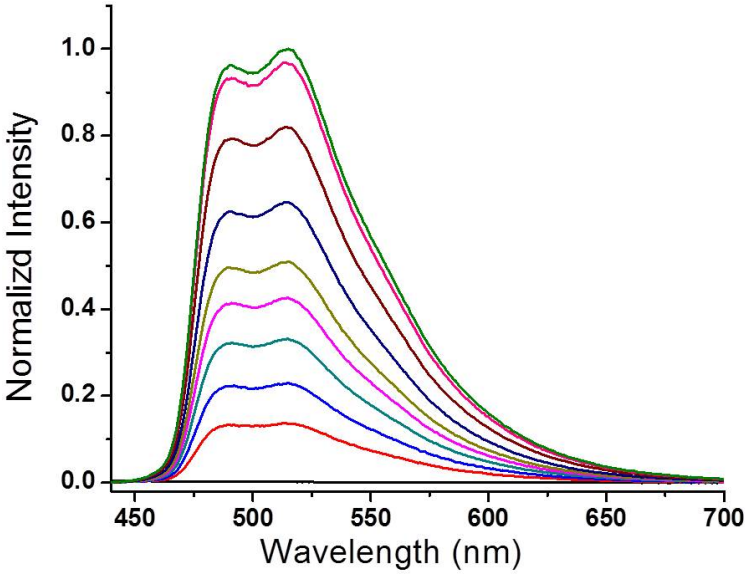

Figure S15. Steady-state spectra of 10 μM probe **3** with different amounts of histidine (from 0 to 15 μM).

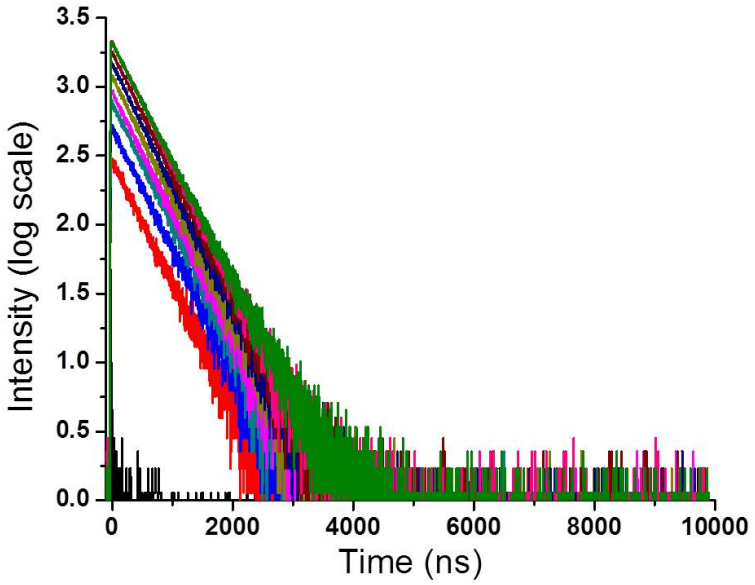

Figure S16. Time-decay profiles of 10 μM probe **3** with different amounts of histidine (from 0 to 15 μM).

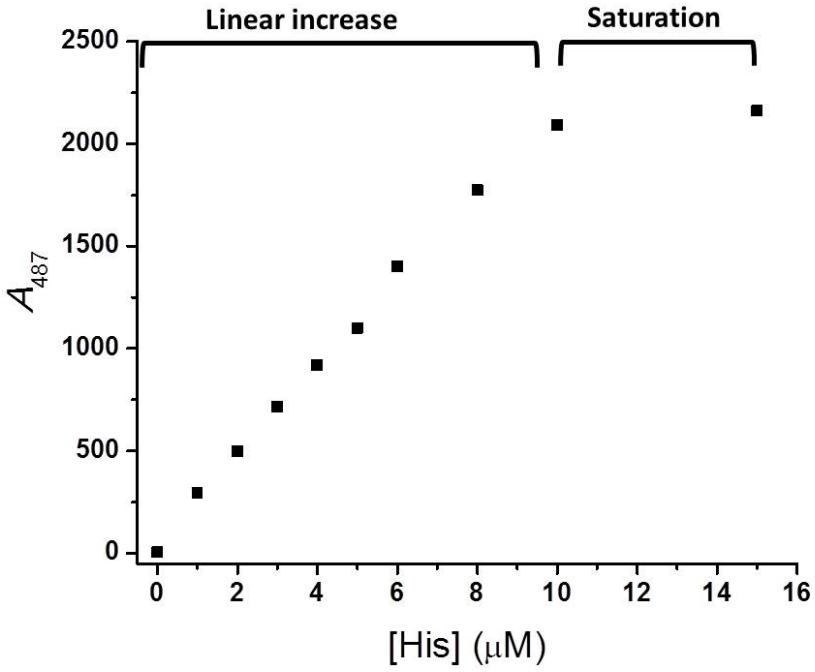

Figure S17. Calculated A_{487} values for 10 μM of probe **3** with different amounts of histidine (from 0 to 15 μM).

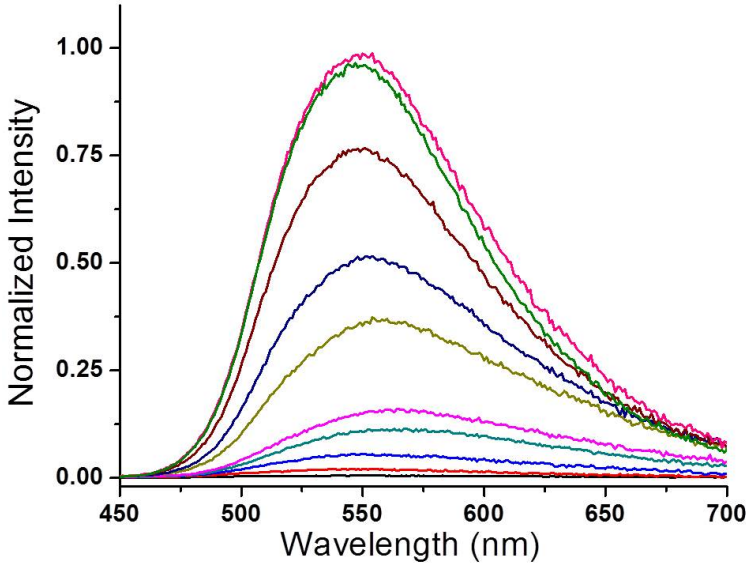

Figure S18. Steady-state spectra of 10 μM probe **4** with different amounts of histidine (from 0 to 30 μM).

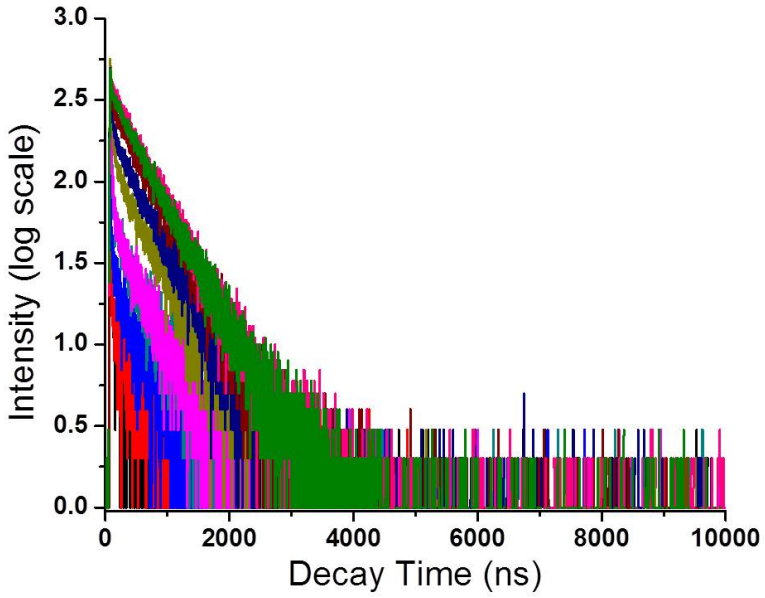

Figure S19. Time-decay spectra of 10 μM probe **4** upon addition of different concentrations of histidine (from 0 to 30 μM).

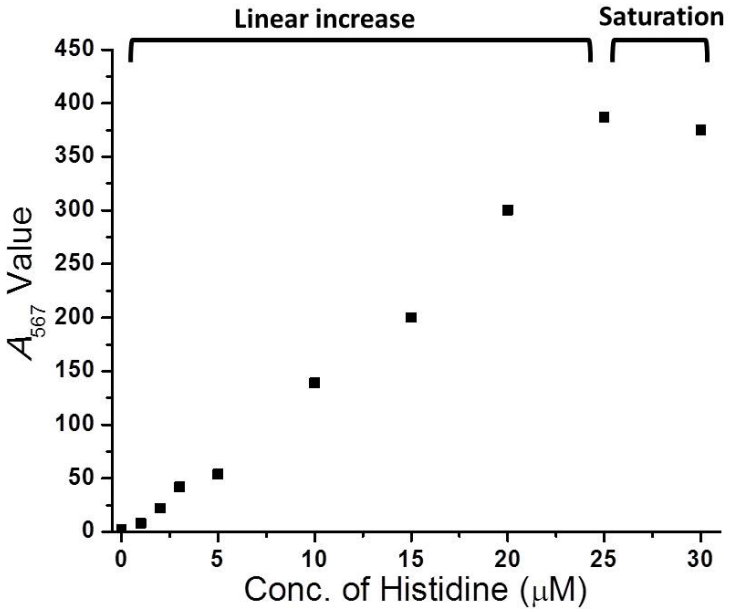

Figure S20. Calculated A_{567} values for 10 μM of probe **4** with different amounts of histidine (from 0 to 30 μM). Note that differently than probes **1**-**3**, the photoluminescence response of **4** with concentration is not stoichiometric.

1. Bujacz, A.; Zielinski, K.; Sekula, B. Structural Studies of Bovine, Equine, and Leporine Serum Albumin Complexes with Naproxen *Proteins* **2014**, *82*, 2199-2208.
2. Levi, V.; González Flecha, F. L. Reversible Fast-Dimerization of Bovine Serum Albumin Detected by Fluorescence Resonance Energy Transfer *Biochim. Biophys. Acta* **2002,** *1599*, 141-148.