Organotin(IV) Differential Fluorescent Probe for Controlled-Subcellular Localization and Nuclear Microviscosity Monitoring

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Experimental Procedures

Materials and Methods

Cell Culture
SK-LU-1 and HeLa cells were cultured in Minimum Essential Medium Alpha (MEM alpha, Gibco, Gaithersburg MD) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad CA) at 37°C with 5% CO_2.

Live Cell Measurements
Live SK-LU-1 and HeLa cells were seeded in three different 8-well µ-slides at a density of 10 000 cells per well for 12 hours prior experiments using RPMI medium supplemented with 10 FBS. Then, specific concentrations of **HQN**, nBu$_2$(Sn)Cl$_2$, adenosine monophosphate (AMP), and specific metal ions and polyols in RPMI media were added on each slide 30 minutes before imaging experiments. For nuclear and cytoplasmic imaging, cell cultures were washed two times with RPMI. During confocal imaging the microscope parameters were maintained constant using a 63x oil immersion objective.

Spectrophotometric and Fluorometric Titrations.
All titration experiments were performed at 25 °C and ionic strength 0.05 M created either by bu□er or NaCl. The experiments were performed with and without 5 mM hexadecyltrimethylammonium bromide (HTAB). An aliquot of 1 mM stock solution of **HQN** in acetonitrile was added to a 5 mM MOPS aqueous buffered solution in appropriate pH intervals, allowing to equilibrate for 15 minutes before titrations. The final content of acetonitrile remained less 1%. Fluorescence quantum yields of HQN and the equimolar nBu$_2$Sn(HQN) complex were 0.17 and 0.08, respectively, obtained according to the relative protocol [S. Fery-Forgues, D. Lavabre, *J. Chem. Educ.,* 1999, **76**, 1260.] using coumarin 6 in ethanol as standard fluorophore.

Quantum Chemical Calculations
Quantum Chemical Calculations were obtained by using DFT and TD-DFT with Polarizable Continuum Model[1] as performed in the Gaussian 09 code,[2] using a PBE0/6-31+G(d,p)/IEF-PCM (water) level of theory to determine the optimized molecular geometry of **HQN**. Then, a frequency analysis corroborates that the geometry corresponds to an energy minimum, finding no imaginary frequencies. As a first step in the analysis of the electron charge distribution in the molecules, the electrostatic potentials were computed to compare the local charge distribution between these molecules. Finally, Natural Transition Orbital (NTO)[3] analysis was computed at the same level of theory to further understand the optical properties for probe **HQN**.

*General Probe Synthesis*
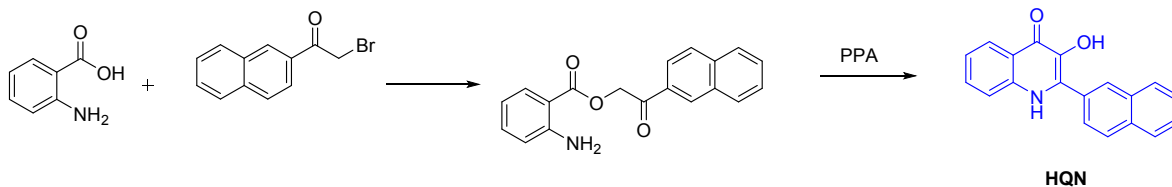
Scheme S1. Synthetic methodology for probe **HQN**.

*Synthetic procedure for 2-naphthyl-3-hydroxy-4(1H)-quinolone (**HQN**).* Compound **HQN** was synthetized as described in literature for other similar structures [P. Hradil, J. Hlaváč, K. Lemr, *J. Heterocyclic Chem.*, 1999, **36**, 141–144.]. A solution of 3-Anthranilic acid (2.00 g, 14.50 mmol) dissolved in DMF (20 mL) was mixed with potassium carbonate anhydrous (2.16 g, 15.6 mmol). Then, the reaction mixture was heated to 90 °C and stirred for 1 h. After that, the solution was cooled to 20 °C and 2-Bromo-2′-acetonaphthone (2.00 g, 8.02 mmol) was added. The generated exothermic reaction was stirred for 30 min, at this temperature. Then, the reaction was heated to 60 °C and stirred

for 30 min more. The product was poured in cooled water and the precipitated product was collected by filtration, washed with water (3 x 30 mL) and dried. After that, the anthranilate (0.5 g, 1.48 mmol) was added to a previously heated polyphosphoric acid (5.0 g) and stirred at 120 °C during 2 h. The reaction mixture was poured into cooled water (10 g), then the pH was adjusted to 7 – 8 with a NaOH 10% solution and the precipitated solid was collected by filtration, washed with water (3 x 30 mL), dried and recrystallized from DMF. ^{1}H NMR (300 MHz; DMSO-d$_6$; Me$_4$Si): δ 9.21 (br. s, 1H), 8.19-7.42 (m, 12H), ^{13}C NMR (300 MHz; DMSO-d6; Me$_4$Si): δ 171.0, 138.5, 138.2, 132.8, 132.2, 132.0, 131.0, 130.4, 129.7, 129.2, 128.3, 128.2, 127.6, 127.5, 124.9, 124.6, 122.4, 122.3, 118.9. IR (vmax/cm-1) 3440 (O–H stretching), 2994, 2913, 2845 (C–H stretching), 1663 (C=O stretching). MS (ESI, m/z) for $C_{19}H_{13}N_1O_2$: [M]$^+$ calculated: 287.03, found [M+H]$^+$: 288.0918, error = 0.012 ppm m.p. 262–264 °C.
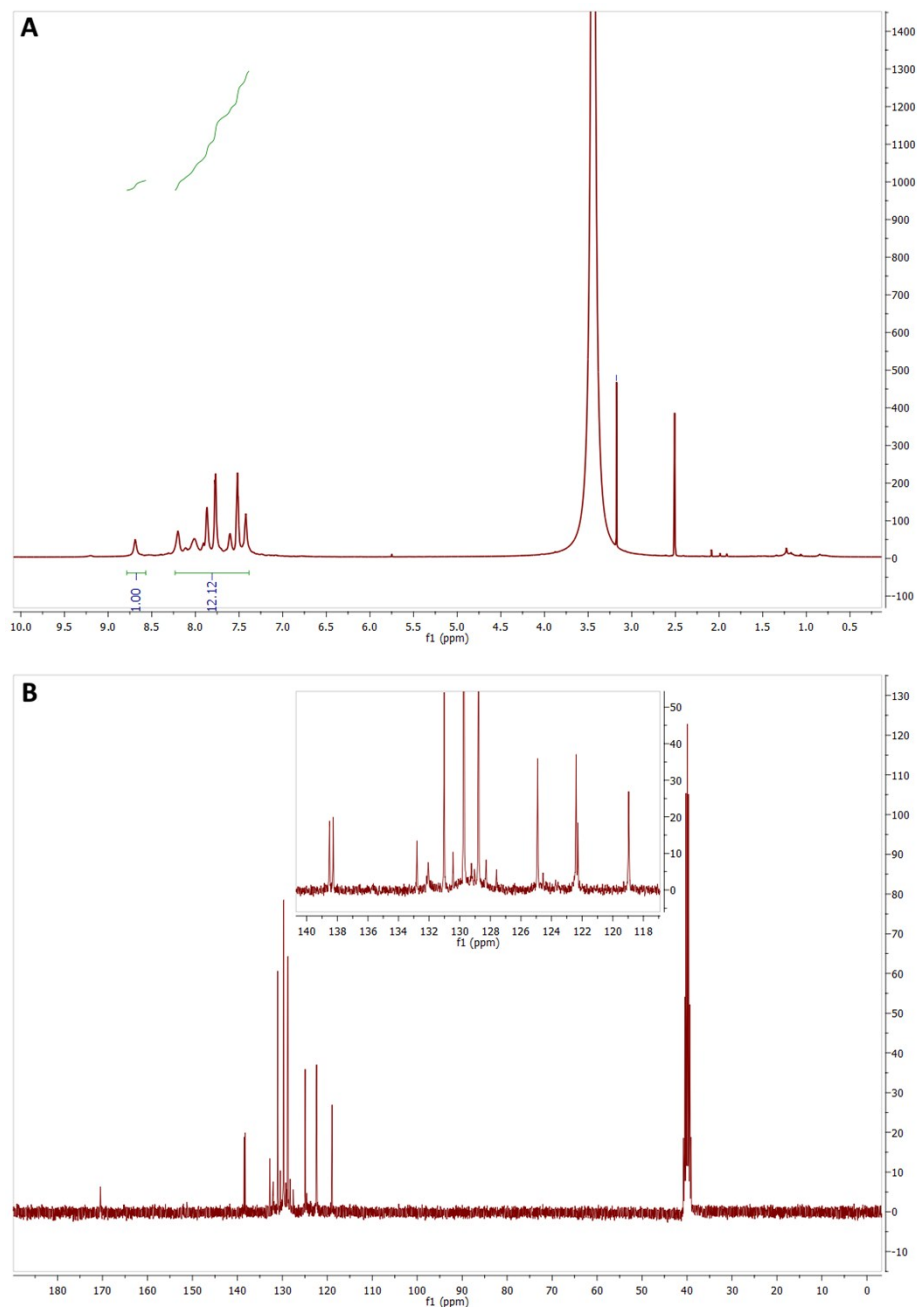
(**A**) ^{1}H and (**B**) ^{13}C NMR spectra of probe **HQN** in DMSO-δ6.

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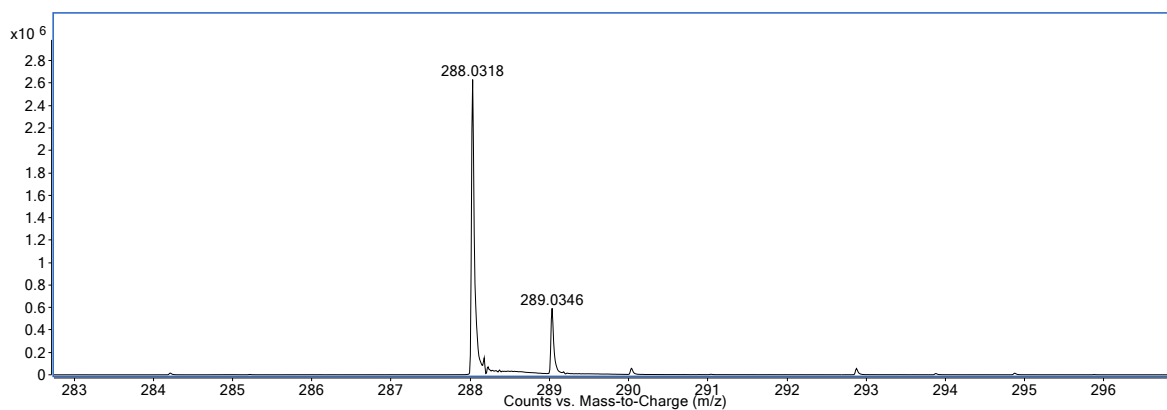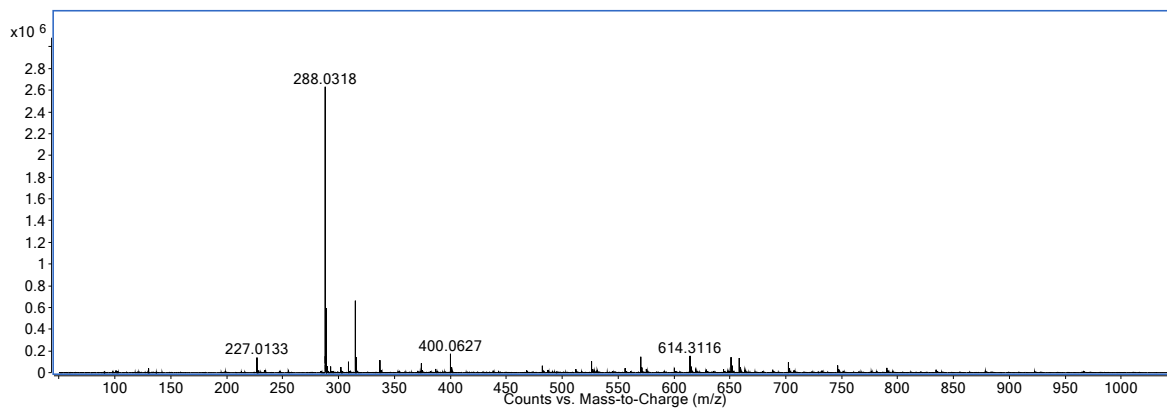
HRMS ESI[+] spectrum of probe **HQN** for $C_{19}H_{13}N_1O_2 = M$; $[M+H]^+$: 288.0318
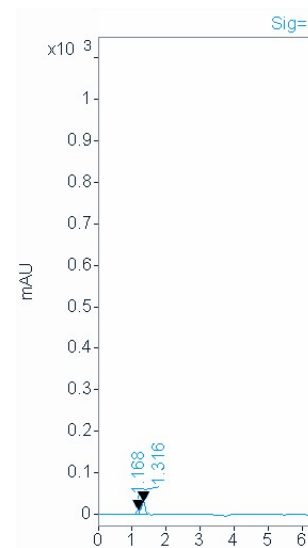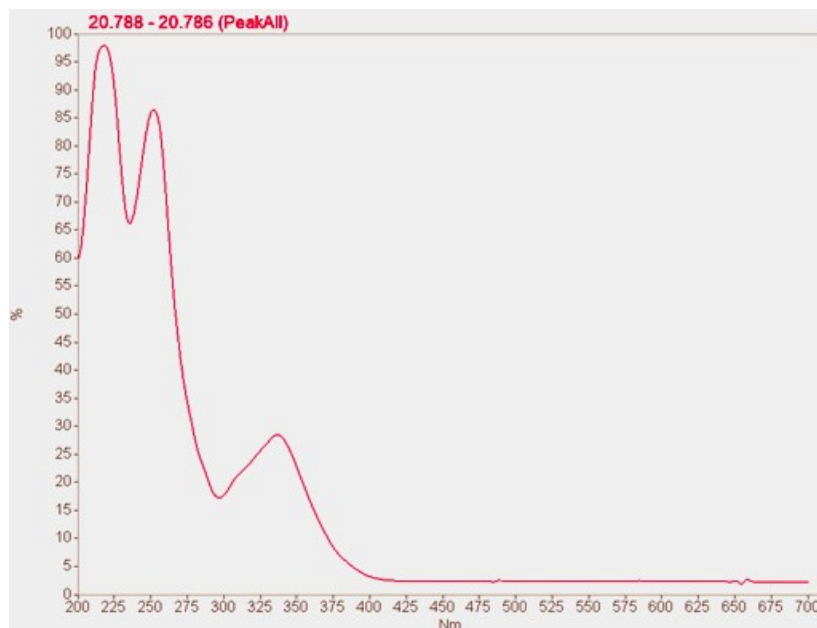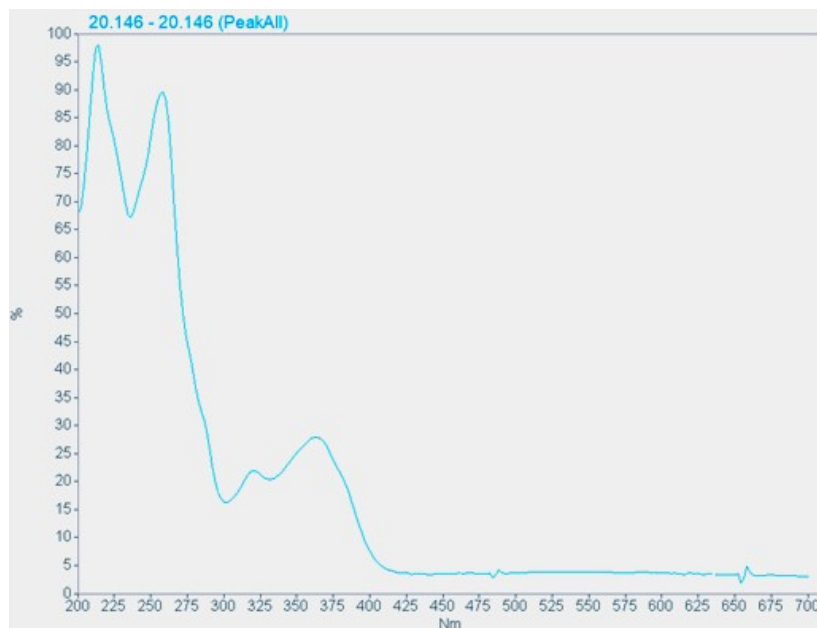
HPLC Purification

A 50 µM stock solution of Compound **5** in PBS was run through RP-HPLC on a C18 column with a 10-70% MeCN/H_2O 2%/min gradient with 0.1% TFA. Retention time is shown below The peaks were identified through a UV-Vis detector at different wavelengths (below is shown 360 nm before and after purification).

Sample:
HQN

HPLC	**Agilent 1260 Infinity II**
Detector	**UV-Vis**
Wavelength	**360 nm**
Column	**Kinetex XB C18 50 x 2.1 mm 2.6 um**

Eluent		acetonitrile	Water
	Inicial	10	90
	30 min	70	30

Flux	0.2 mL/min

Date 26-01-2019
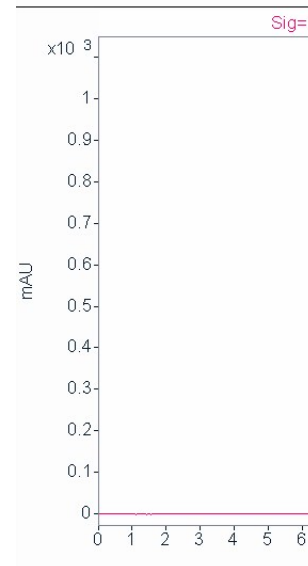
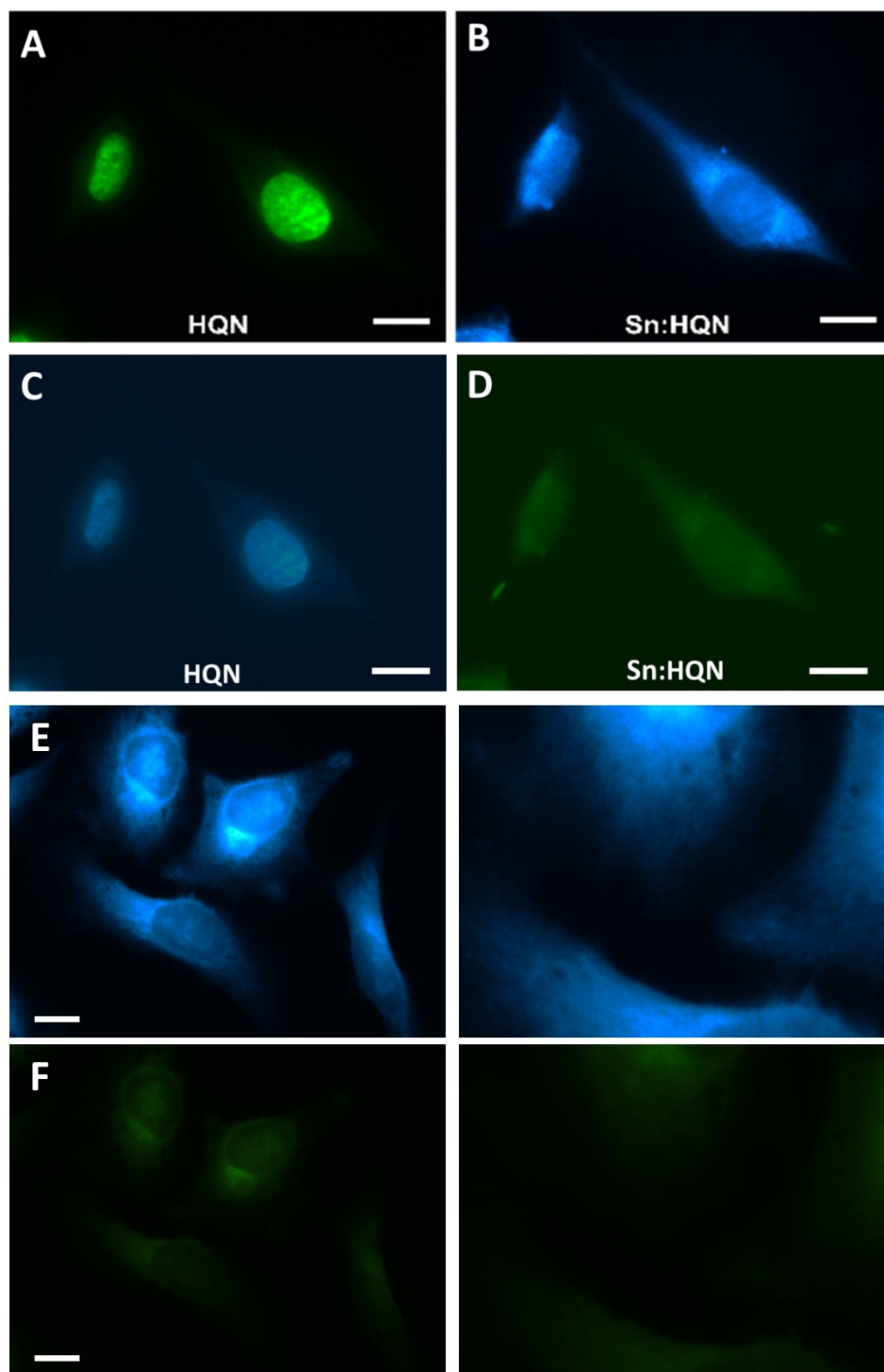UV-Vis traces for the HQN before (blue) and after purification (red).

Figure S1. Intracellular localization redistribution of the **HQN** in live SK-LU-1 cells under 30 minutes incubation observed in (A) the confocal green channel (λ_{exc} = 503 nm, λ_{em} = 550 nm) and in (C) the blue channel (λ_{exc} = 404 nm, λ_{em} = 470 nm), indicating a strong nuclear localization. Then, the Sn(nBu)$_2$Cl$_2$ addition (45 minutes incubation) enable a clean cytosol distribution observed in (B) the blue channel (λ_{exc} = 404 nm, λ_{em} = 470 nm) and in (D) the green channel (λ_{exc} = 503 nm, λ_{em} = 550 nm). Control experiments showing the localization using the equimolar performed Sn:**HQN** complex observed under the blue (E) and green (F) channels. Scale bars represent 20 µm.
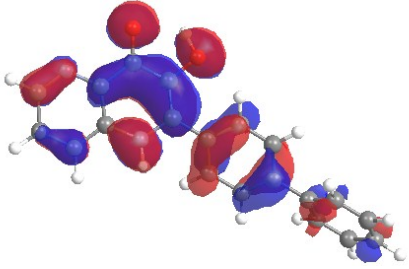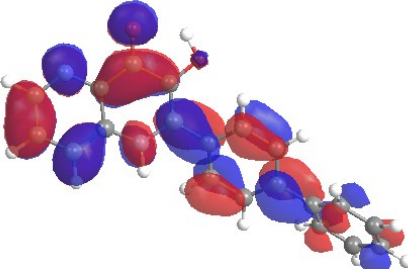
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	NTO – Hole	NTO – Particle
NTO eigenvalue $w = 0.98$ $f = 0.289$ 3.314 eV For the HOMO – LUMO levels		

Figure S2. The dominant natural transition orbital pair for the first excited singlet state of **HQN**. The left panels quote in sequence the NTO eigenvalue (*w*), oscillator strength (*f*), transition energy, and associated MO levels.
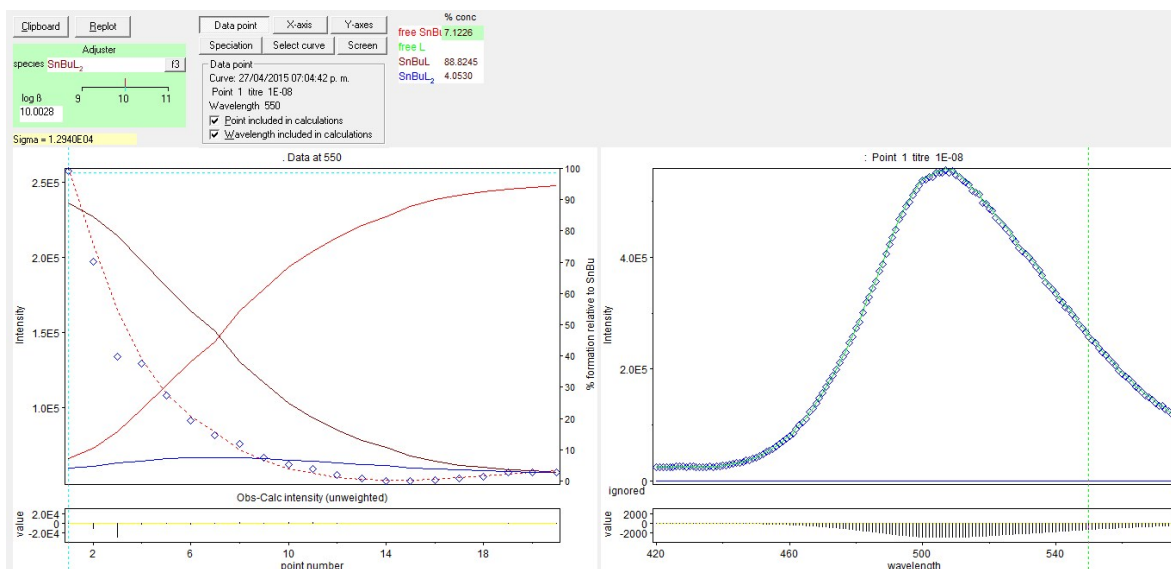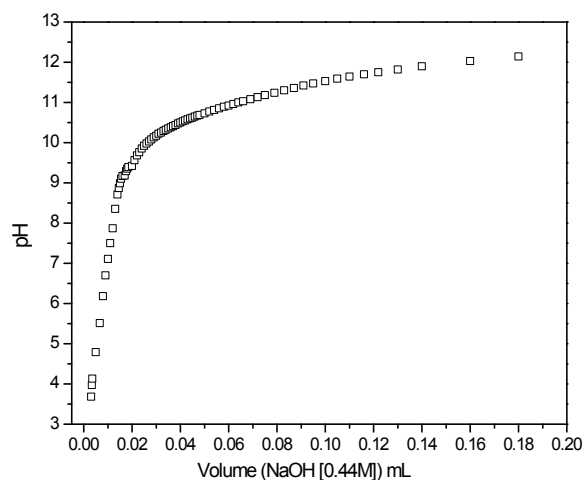
Figure S3. Potentiometric titration in 10 mL **HQN** thermostatted at 25 °C under N_2 using 0.05 M NaCl as background electrolyte to maintain ionic strength, pH was 7 under 5 mM HTAB media. Below: the potentiometric data analyzed with HyperQuad software when detecting at 550 nm. L stands for the **HQN** ligand while SnBu for $Sn(nBu)_2Cl_2$. As can be seen from the iteration data and the Figure below, about 89% of the equimolar [nBu_2Sn]HQN complex is strongly favored over competing [nBu_2Sn]**HQN**$_2$ stoichiometry and the free ligand, indicating that this complex is dominant under such conditions.

Table S1. Protonation constants of ligands and stability constants of metal complex determined potentiometrically in 20% vol. acetonitrile, 0.05 M NaCl at 25°C.

| species	p q r	log β_{pqr}	equilibrium	log K_{obs}
LH	0 1 1	11.08±0.01	L + H = LH	11.90
LH$_2$	0 1 2	20.42±0.01	LH + H = LH$_2$	10.66
nBu$_2$Sn LH	1 1 1	24.17±0.09	nBu$_2$Sn + LH = nBu$_2$Sn(LH)	13.09

The results of potentiometric titrations were analyzed in terms of traditional *pqr* scheme expressed by equations (1) and (2), where L is a completely deprotonated dianionic form of the HQN ligand and M is the metal ion (nBu$_2$Sn^{2+}). The overall binding constants and the pK_a values of free HQN and the complex are collected in Table S2.

$$p\text{M} + q\text{L} + r\text{H} \rightleftharpoons \text{M}_p\text{L}_q\text{H}_r \qquad (1)$$
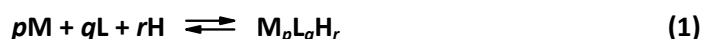
$$\beta_{pqr} = [\text{M}_p\text{L}_q\text{H}_r]/[\text{M}]^p[\text{L}]^q[\text{H}]^r \qquad (2)$$

The simple 1 : 1 nBu$_2$Sn^{2+} complex of the type M(LH)$^+$ is much more stable and is a dominating species in acid and neutral solutions. However, with excess of the ligand and pH above 7 the M(LH)$_2$ complex will be favored.

The stability constant for nBu$_2$Sn(HL)$^+$ complex is in fact larger than expected for other metal ions present in biological systems (Zn^{2+}, Cu^{2+}, etc).[4] The reason for this effect is the strong hydrolysis of nBu$_2$Sn^{2+} cation in neutral solutions. In accordance with reported hydrolysis constants of nBu$_2$Sn^{2+} in water at pH 7 (logβ_{10-2} = -8.30)[5] the cation is completely transformed into dihydroxo complex and the actual reactions of the formation of M(HL)$^+$ is (3).

$$n\text{Bu}_2\text{Sn(OH)}_2 + \text{LH}_2 + \text{H}^+ = n\text{Bu}_2\text{Sn(LH)}^+ + 2\ \text{H}_2\text{O} \qquad (3)$$
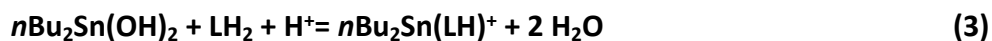
The corresponding values for K$_{obs}$ at pH = 7 are logK$_{obs}$ = logβ_{111} – logβ_{012} – logβ_{10-2} – pH = (24.17) – (20.42) – (-8.30) – 7.0 = 5.05 for nBu$_2$Sn(LH)$^+$.

(A)
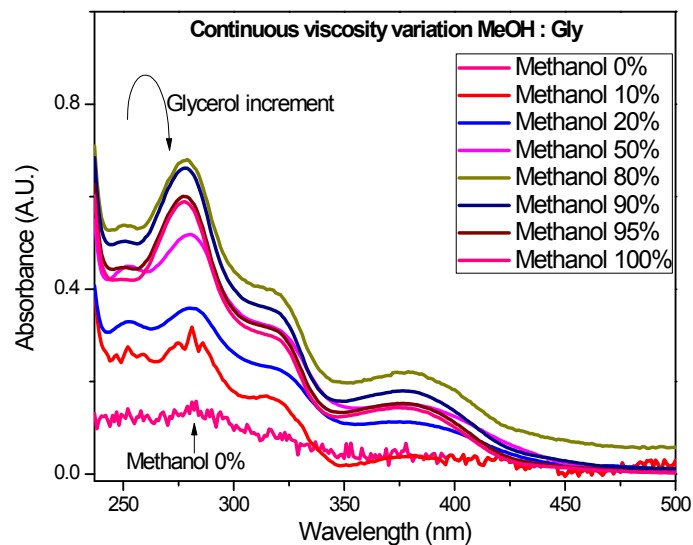
(B)

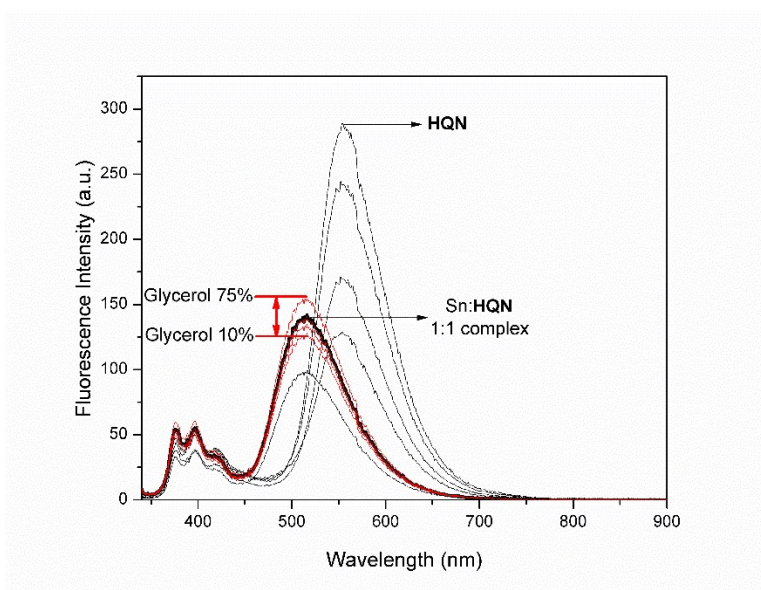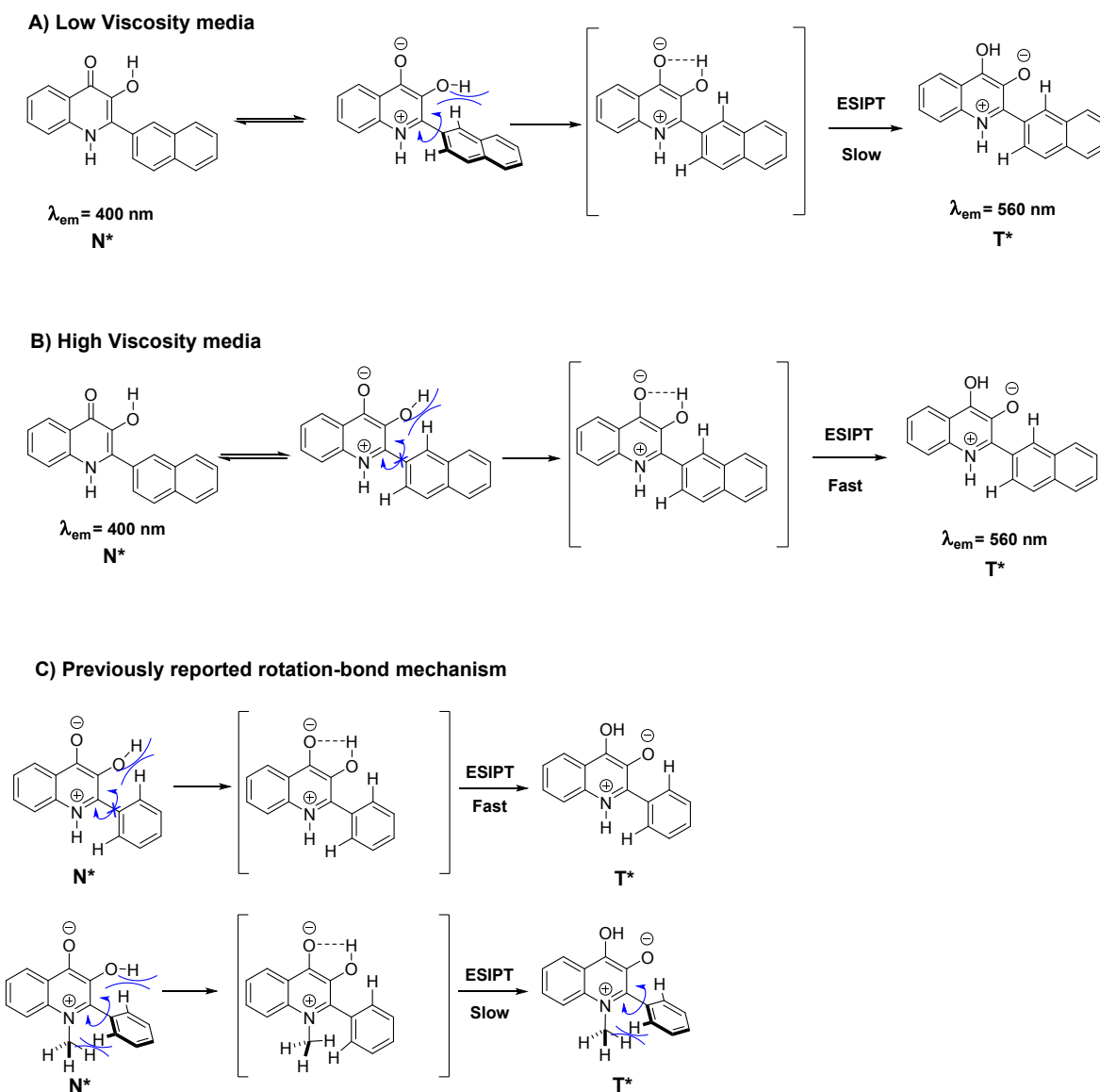**Figure S4**. (A) Absorption spectra of 40 µM **HQN** at variable viscosity increments. (B) Fluorescence spectra of Sn:**HQN** (1:1) complex under a continuous viscosity variation from 10% aqueous glycerol to 75% aqueous glycerol.

A) Low Viscosity media

λ$_{em}$ = 400 nm
N*

ESIPT
Slow

λ$_{em}$ = 560 nm
T*

B) High Viscosity media

λ$_{em}$ = 400 nm
N*

ESIPT
Fast

λ$_{em}$ = 560 nm
T*

C) Previously reported rotation-bond mechanism

N*

ESIPT
Fast

T*

ESIPT
Slow

N*

T*

Scheme S2. Interaction between the 3-hydroxy group and the 2-naphthyl moiety. In (a) a low viscosity media the larger rotational freedom of the naphthyl group decreases the planarity of the dye, enabling a fast ESIPT process, whereas the high viscosity media (b) limits considerably the rotational motion of the naphthyl group giving rise to a slow ESIPT process (T* tautomer is favored). (c) Previously reported rotation-bond mechanism (*J. Phys. Chem. A*, 2007, **111**, 8986.): Above: Interaction between the 3-hydroxy group and the 2-phenyl moiety in the N-H derivative limits considerably the rotational motion of the hydroxyl group. Below: the steric effect of the proximal N-methyl group decreases the planarity of the dye, enabling a larger rotational freedom of the 3-OH group.
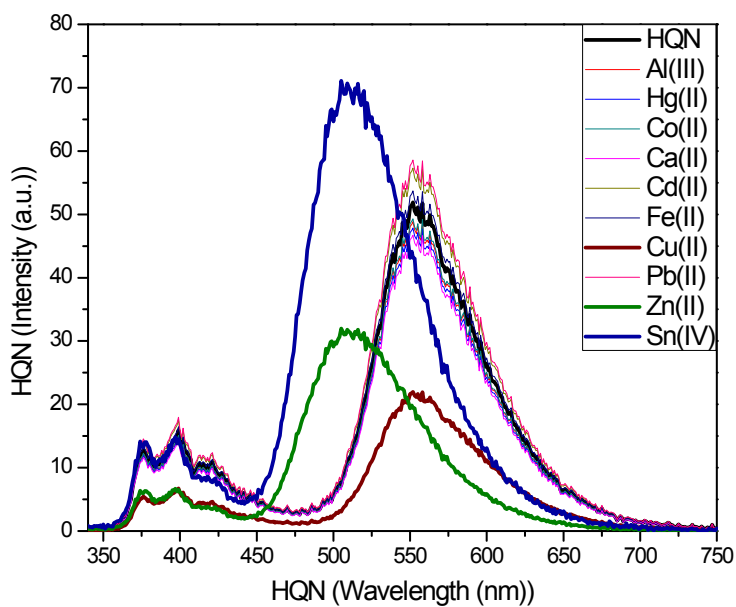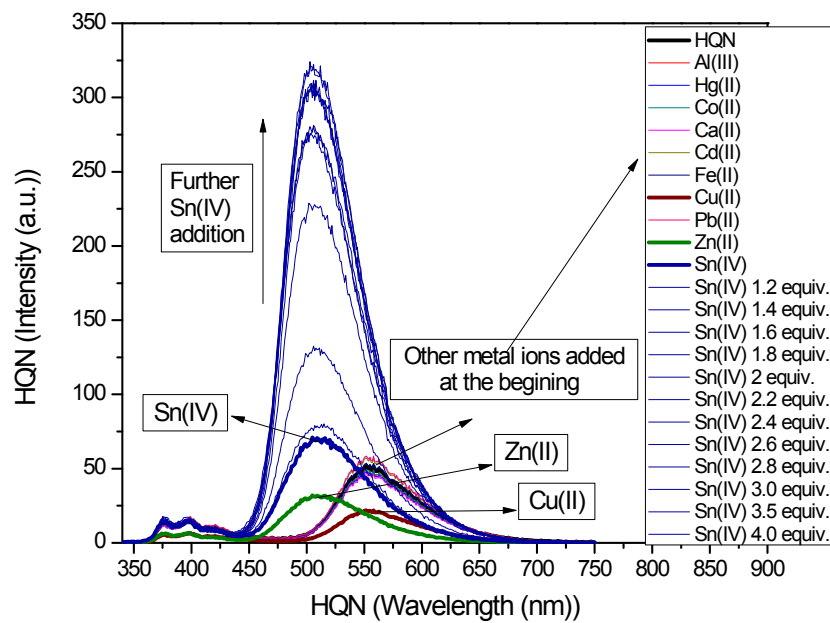
Figure S5. Fluorescence spectra (λ_{exc} = 330 nm) of 40 µM **HQN** in HTAB 5 mM at variable concentration of different metal ions using stock solutions of 10 µM, and 50 mM NaCl at 25 °C.

Table S2. Detailed experimental procedure for *in-vitro* viscosity determinations: The viscosity quantification was done by using the calibration plot obtained in Fig. 1B inset, the Table S1 (below) shows the corresponding viscosity values in cP for each volume fraction of glycerol. Then, the λ-ratiometric method was used by taking the blue signal (at 405 nm) as internal reference since the blue emission channel of the microscope showed no intensity variations during recordings when detecting at the same wavelength λ_{em} = 405 nm.

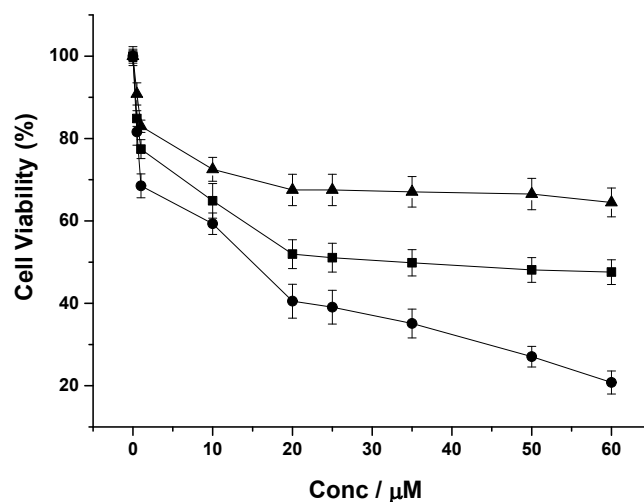| Glycerol volume fraction x_{gly}	Viscosity [cP]
0.0	0.6
0.1	1.8
0.2	4.8
0.3	7.7
0.4	13
0.5	28
0.6	58
0.7	130
0.8	250
0.9	630
1.0	1410

Figure S6. Percentage of SK-LU-1 cell viability remaining after treatment. Cells were grown under standard conditions and treated with: triangles (Sn:**HQN**), squares (free **HQN**) and circles (*n*Bu$_2$SnCl$_2$). Untreated cells were considered to have 100% survival. Cell viability was determined by a redox indicator (Alamar Blue). For cytotoxicity assays, human lung adenocarcinoma (SK-LU-1) cell lines were plated in 96-well plates at 5000 cells/well in RPMI-1640 medium. About 24 h after plating, varied doses of compound Sn:**HQN**, **HQN** and *n*Bu$_2$SnCl$_2$ at 0.5, 1, 10, 20, 25, 35, 50 and 60 μM concentration were added in triplicate. Cell viability was evaluated after 72-h incubation with the molecules using Alamar Blue fluorescent assay (Life Technologies, Carlsbad, CA, USA).
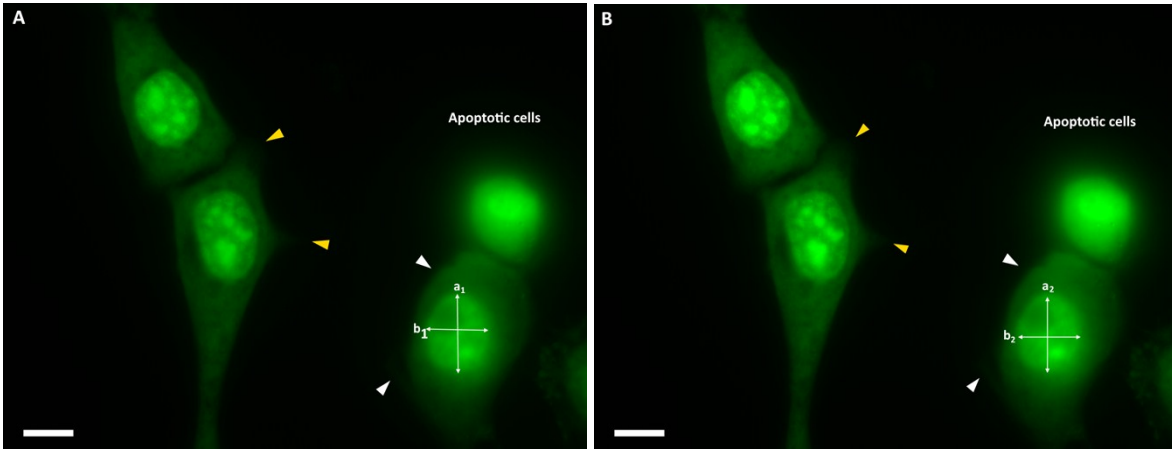
Figure S7. Confocal imaging of the **HQN** in apoptotic HeLa cells treated with high-dose H_2O_2 for 45 min under (A) non-swelling and (B) swelling conditions. Panel (B) shows a 10 minutes time lapse observed in the green channel (λ_{exc} = 503 nm, λ_{em} = 550 nm). Scale bars represent 10 µm. Distances are a_1 = 17 µm and b_1 = 13 µm, while a_2= 21 µ and b_2 = 16 µm. Arrows indicate zones of plasma membrane adhesion (focal points) having almost no variations. However, white arrows (apoptotic cells) show a significant decrease in adhesion. Laser light was fully shielded between recordings to prevent artifacts and photobleaching.
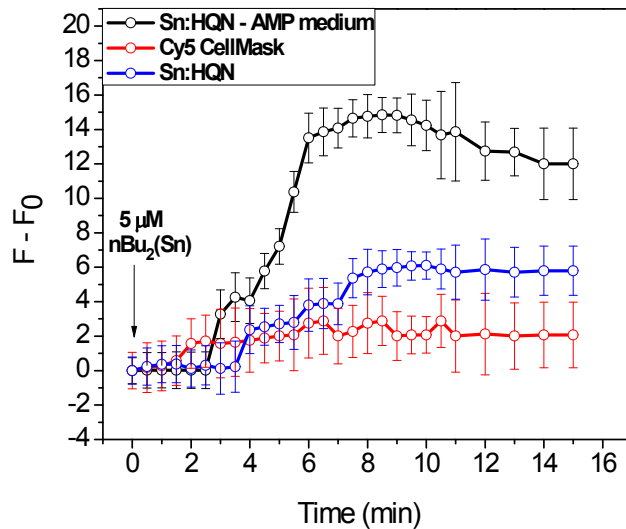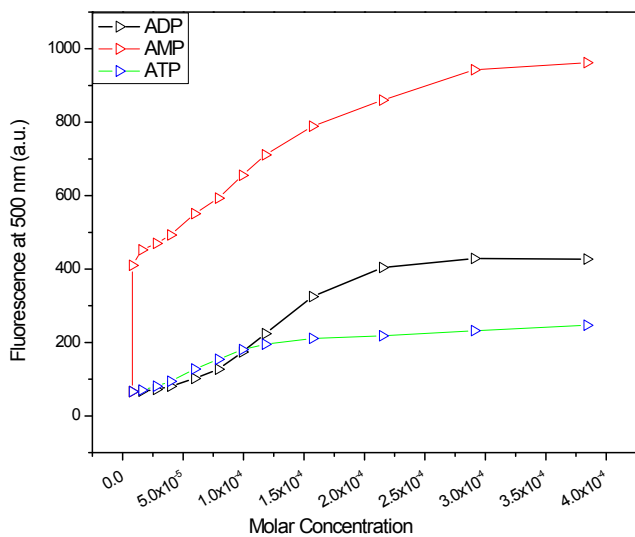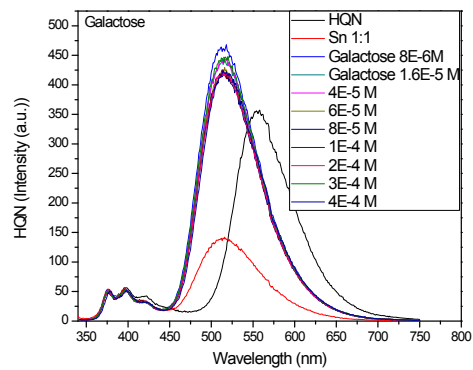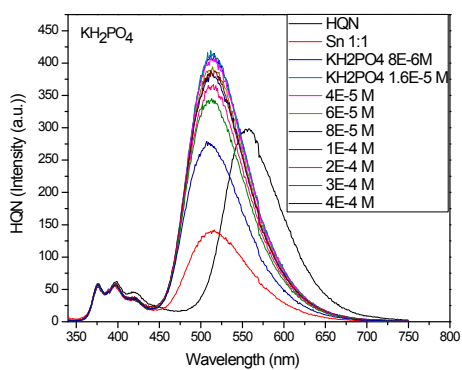

Figure S8. Time-dependent effect of 5 µM nBu$_2$(Sn) ion on the **HQN** fluorescence signal, shown as fluorescence difference (F – F_0). The experiment was performed in cell culture AMP-enriched MEM medium (dark line) and no AMP-enriched, 2 mg/mL, medium (blue line). Data in red correspond to the fluorescence intensity control Cy5 CellMask Deep Red ($\lambda_{ex}/\lambda_{em}$ = 649/666 nm).

A)

B)
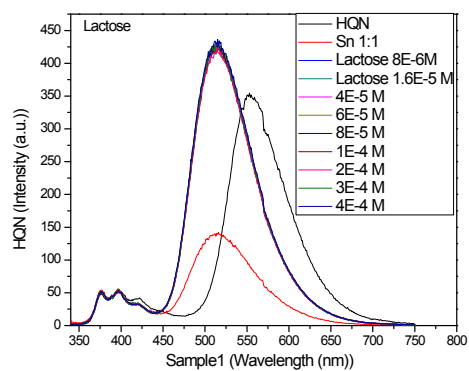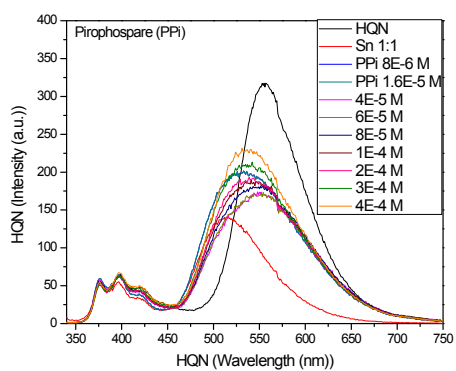
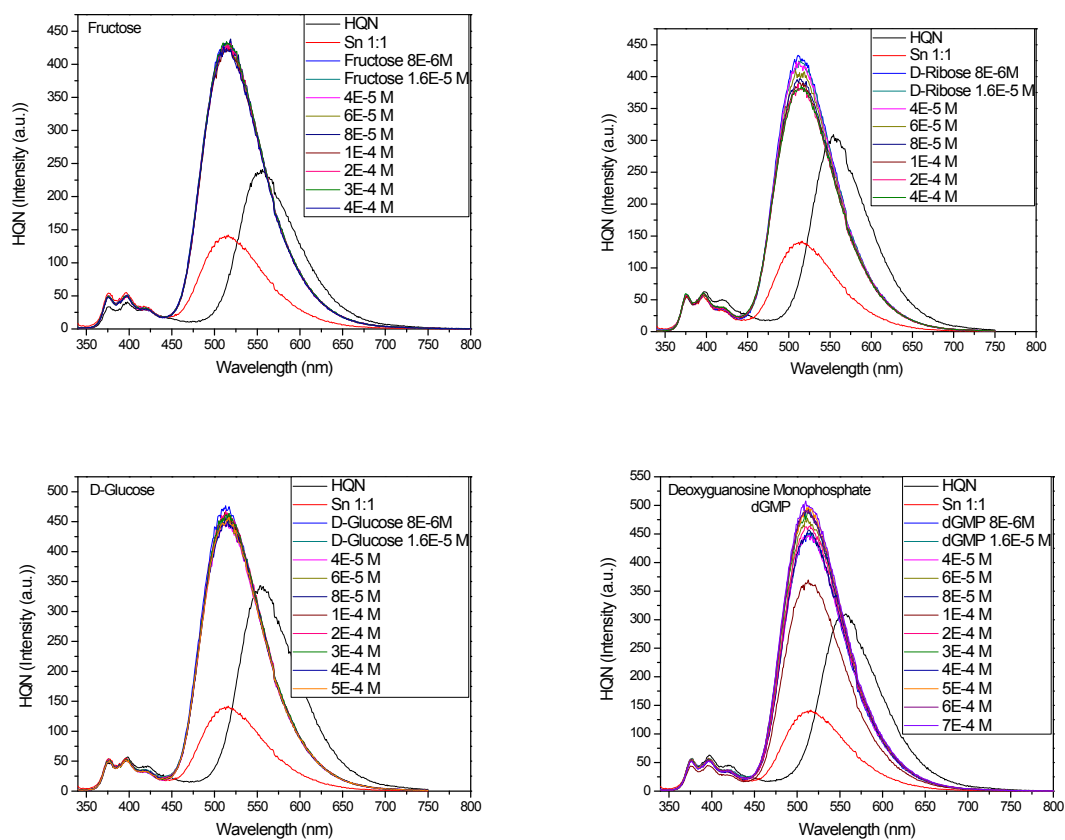**Figure S9**. Fluorescent emission titrations (λ_{exc} = 330 nm) of 10 mM Sn:**HQN**, under variable concentrations of A) AMP, ADP and ATP anions and; B) a series of polyols. The titrations were conducted at pH = 7.0 in HTAB (5mM) and 10 mM NaCl at 25 °C. Analyte concentrations are shown in the insets.

Table S3. Comparison of different adenosine monophosphate (AMP) detection methods previously reported.

Reference	Method	Conditions	log K / limit of detenction, LoD
Yakun Lian, et.al. *Talanta,* 2016, **150**, 485.	^{1}H and ^{31}P NMR	D_2O, peak at 0.9 ppm as quantitative signal of AMP	LoD = 0.40 mM
Israel Carreira-Barral, et.al. *Molecules,* 2018, **23**, 479; doi:10.3390/molecules23020479	Spectrophotometric	pH = 7, MOPS buffer	log K = 4.35
Li Qin, et.al. *Anal. Chem.,* 2018, **90**, 9983.	Colorimetric	pH = 7, pretreated sample (digestion)	LoD = 1 µM
Sujoy Das, et.al. *Chem. Commun.*, 2017, **53**, 7600.	Spectrophotometric	pH 7.0, 10 mM HEPES buffer, CH_3CN/H_2O, 1:6 (v/v)	LoD = 2.8 µM
Eisuke Furuya, et.al. *Anal. Biochem.,* 1985, **145**, 144.	HPLC	Pretreated: glucagon-treated rat liver	LoD = 2 pM
Laxman Gangwani, et.al. *Biochem. Biophys. Res. Commun.,* 1991, **178**, 1113.	HPLC	Pretreated: acidic pH and TLC-purified	LoD = 70-80 pM
Mikio Bakke, et.al. *J. Food Protect.,* 2018, **81**, 729.	Bioluminescence: Total XXXdenylate (ATP+ADP+AMP)	Pretreated: acidic conditions	Not reported Signal rec. 1735 RLU (relative light units)
Shan Sun, et.al. *Anal. Chem.,* 2017, **89**, 5542.	Spectrophotometric	pH = 7.0, Tris buffer,	LoD ~ 1 µM
Dhaval P. Bhatt, *J. Chromatogr. B,* 2012, doi:10.1016/j.jchromb.2012.02.005.	HPLC	Pretreated: pH 4.5	LoD = 0.16–20.6 pM
Subhanjan Mondal, et.al. *Assay Drug. Dev. Techn.*, 2017, **15**, 330.	Bioluminescence: enzyme-coupled assay	pH 7.5 HEPES buffer	LoD = 100 nM
E. Harmsen, et.al. *J. Chromatogr.,* 1982, **230**, 131.	Ion-exchange chromatography	pH 7.5, UV detector 210 nm	LoD = 20 pM
Q. Y. Bai, *J. Microbiol. Meth.,* 1989, **9**, 345.	Spectrophotometric	pH 7.5	LoD = 0.05 µM
This work	Spectrophotometric	pH ~ 7.4 (cyosol)	log K_{obs} = 4.51±0.01 LoD = 0.038 mM

References

[1] a) C. Amovilli, V. Barone, R. Cammi, E. Cancès, M. Cossi, B. Mennucci, C. S. Pomelli, J. Tomasi, *Adv. Quant. Chem.* 1998, **32**, 227–261; b) Tomasi, J.; Mennucci, B.; Cammi, R. *Chem. Rev.* 2005, **105**, 2999–3094.

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[3] R. L. Martin, *J. Chem. Phys.* 2003, **118**, 4775–4781.

[4] R. Villamil-Ramos, V. Barba and A. K. Yatsimirsky. Selective fluorometric detection of pyrophosphate by 3-hydroxyflavonediphenyltin(IV) complex in aqueous micellar medium. *Analyst*, 2012, **137**, 5229–5236.

[5] M. A. El-Gahami, H. M. Albishri. Equilibrium Studies of Dibutyltin(IV)–Zwitterionic Buffer Complexation. *J. Solution. Chem.* 2013, **42**, 2012–2024.