Supporting information

Fluorescent Probe for Transmembrane Dynamics During Osmotic Effects

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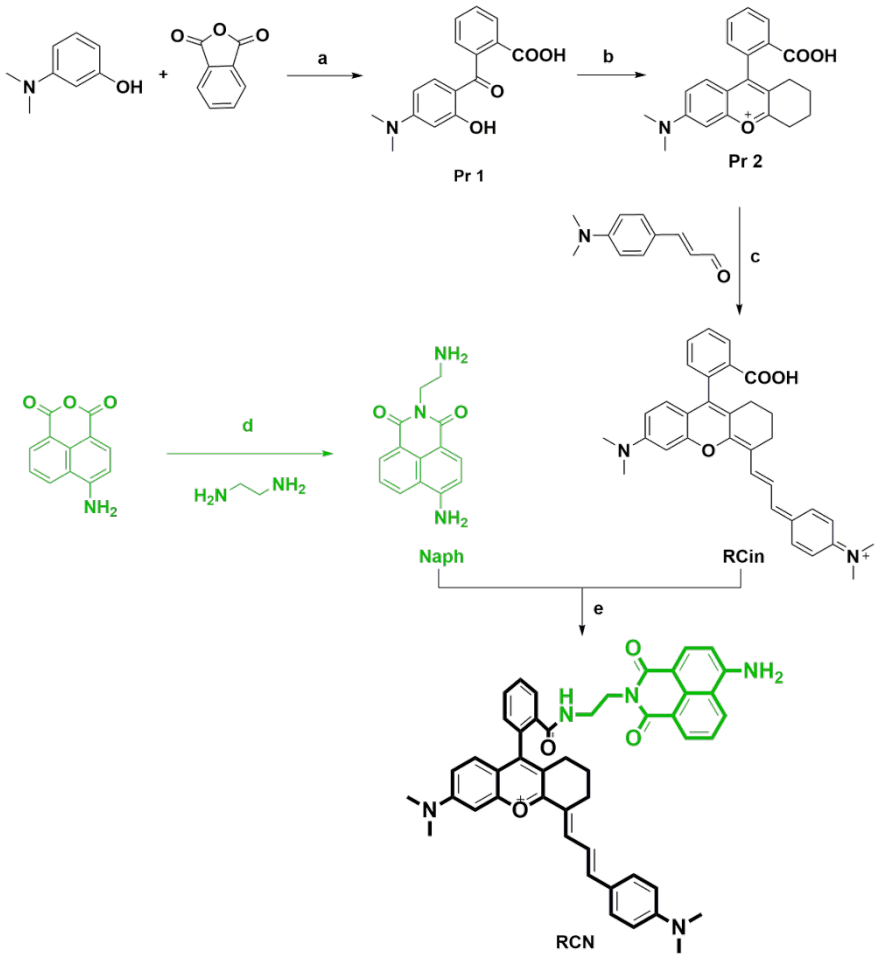
Cell Culture and Confocal Microscopy. HeLa cells as well as live human pulmonary adenocarcinoma epithelial cells (SK-LU-1) were cultured in RPMI-1640 medium (RPMI Medium 1640 (1x), Gibco, Gaithersburg MD) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad CA), L-glutamine (2 μM), penicillin G (100 u/mL), streptomycin sulfate (100 μg/ mL) at 37°C with 5% v/v CO_2. Live SK-LU-1 cells were seeded on 8 Petri dishes of 5 cm diameter with glass bottom for 36 hours before experiments using RPMI-1640 medium supplemented. Then, specific concentrations of **RCN** from 5 to 8 μM were used. Commercial specific organelle localizers were added on each Petri dish 45 minutes before imaging experiments. All dishes were washed two times with RPMI. During confocal imaging, microscope parameters were maintained constant and excitation light was fully-shielded to prevent laser artefacts. Live HeLa cells were seeded in 8 well μ–slides (iBidi, Germany) at a density of 20000 cells per well one day prior to experiments in MEM alpha with 10% FBS. On treatment day, cells were washed once in MEM alpha with no FBS and incubated with 5 to 8 μM probe RCN for 30 minutes. For experiments with TMRM, 10 nM TMRM (Thermo Fisher Scientific) was added 10 minutes before RCN. Cells were then washed twice in MEM alpha with no FBS and imaged maintaining 5% CO_2 and 37°C during the experiments using an inverted Zeiss LSM 880 microscope or a Nikon A1R upgraded with a spectral detector unit. On treatment day for fluorescence time course experiments, cells were incubated with 7 μM probe RCN for 30 minutes in MEM alpha with 5% FBS for the indicated time at 37°C with 5% CO_2, then imaged at the same conditions.



Scheme S1. Structures and synthetic methodology to obtain **RCN** probe: (a) Toluene, 120 °C, 24 h; then NaOH (30% aq), 90 °C, 12 h; (b) H_2SO_4, 60 °C, 2.5h, then $HClO_4$; (c) acetic anhydride, 60 °C, 3 h; (d) 120 °C, 6h; (e) Cs_2CO_3, HBTU, DMF, 90 °C, 24 h.

Compound synthesis and chemical characterization

General Probe Synthesis

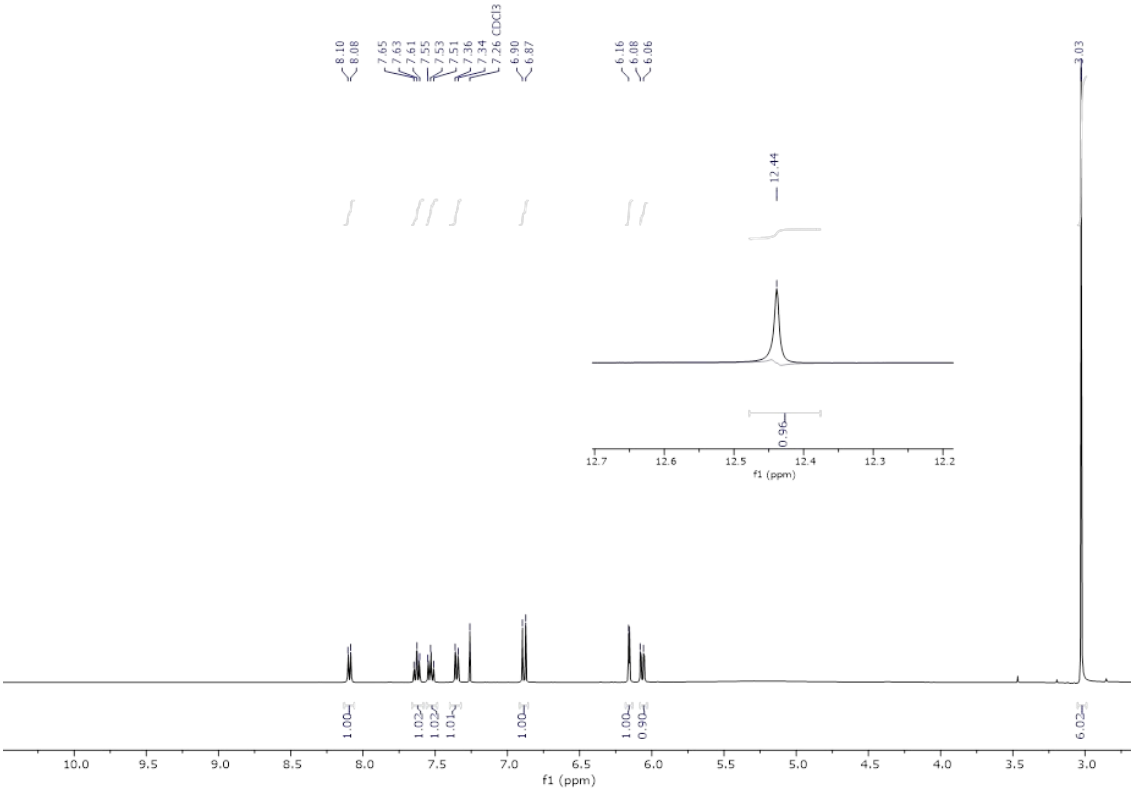
3- (dimethylamino) phenol (1,000 g, 3.5 mmol) and phthalic anhydride (1,079 g, 7 mmol) were mixed in 21 ml of Toluene was stirred at 120 ° C at reflux for 24 h, the toluene was evaporated under reduced pressure, 100 mL of NaOH (35% m / v) was added to the dry product, stirred at 90 ° C for 24 h. The mixture was neutralized with HCl, a violet precipitate was obtained, this was crystallized by a pair of MeOH: H2O solvents. The final product **(Pr1)** had melting point 182-183 ° C and the mass equivalent to 74% yield.

To a solution of **Pr1** (0.5 g, 1.75 mmol) in 3 mL of H2SO4 was added dropwise cyclohexanone (0.36 mL, 3.5 mmol) at 0 ° C, stirred and gradually heated to 90 ° C, kept at that temperature 2.5 h. The mixture was cooled in an ice bath and $HClO_4$ (0.3 mL, 7 mmol) was added dropwise to obtain a red precipitate (**Pr2**), the mixture was filtered under vacuum and the solid was recrystallized from MeOH. The product has 220 ° C of m.p. and a yield of 61%.

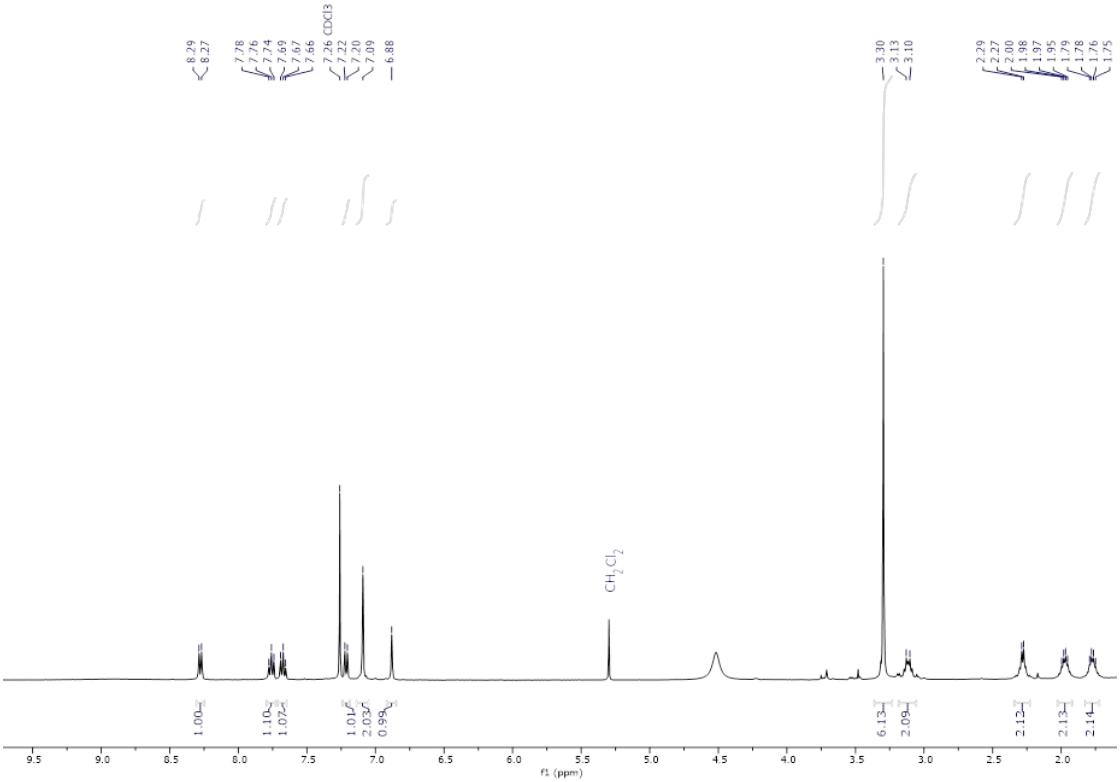
Then, **Pr2** (0.10 g, 0.3 mmol) and cinnamaldehyde (0.05 g, 0.3 mmol) on 10 mL of acetic anhydride were stirred at 0 ° C for 30 minutes in an inert atmosphere. Subsequently, it was heated to 60 ° C and kept stirring for 3 hours. The solvent was evaporated under reduced pressure and the solid was purified by column chromatography (DCM:MeOH = 9: 1). A blue solid (**RCin**) with gold corresponding to 55% and m.p. of 189 ° C was obtained.

Naph was obtained mixed 0.1 g (0.391 mmol) of 6-amino-1H, 3Hbenzo [de] isochromoen-1,3-dione with ethylenediamine (3 mL), closed under pressure and stirred at 120 ° C for 6 h. The reaction mixture was cooled and poured into acetone, the pellet was collected by filtration and dried under reduced pressure to provide a yellowish solid, purified by column chromatography (DCM: MeOH = 8: 2) m.p. 190 ° C and 77% yield.

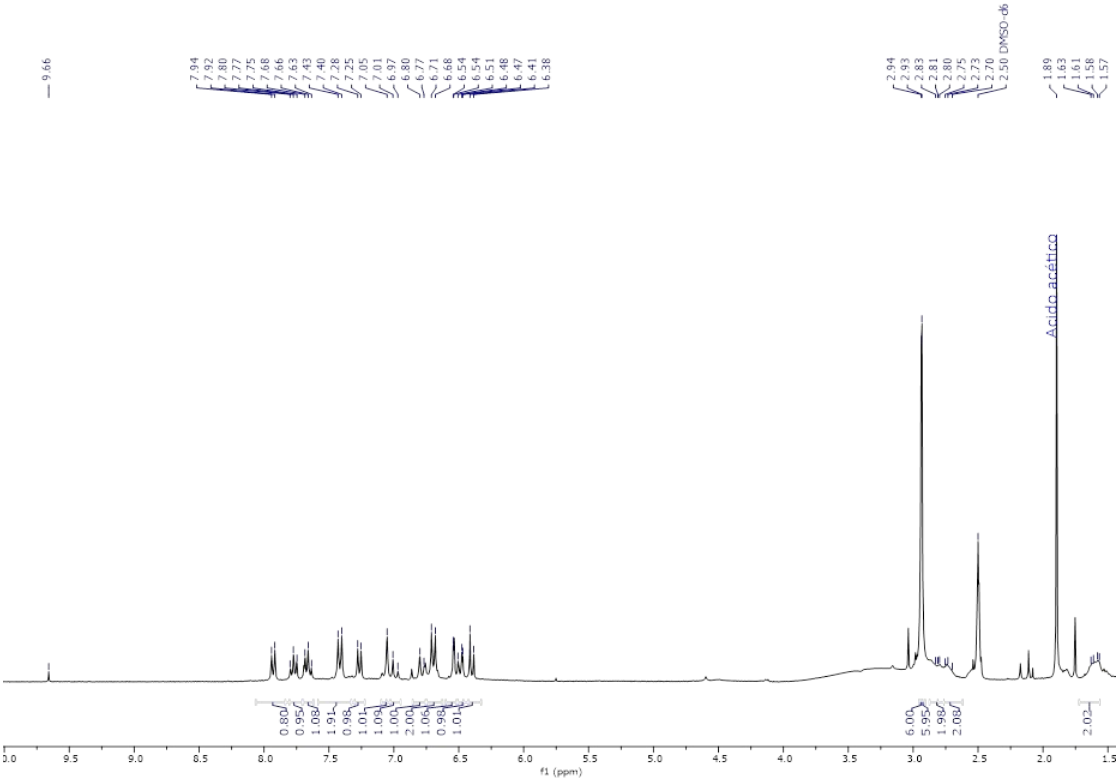
Naph (0.107 g, 0.4 mmol) and **RCin** (0.100 g, 0.2 mmol) were mixed with Ca_2CO_3 (0.128 g, 0.4 mmol) and HBTU(0.379 g, 0.4 mmol) for 1 hour at room temperature and under argon, in 8 mL of dimethylformamide (DMF), subsequently heated to 90 ° C for 24h. A DCM: H2O (2X) extraction was performed, the solvent was evaporated under reduced pressure to obtain a yellowish-brown solid (**RCN**) which was purified by column chromatography (DCM: MeOH = 95: 05) m.p. 152 ° C and 40% yield. ^{1}H NMR (700 MHz, DMSO): δ/ppm 8.57 (d, 1H, J = 8), 8.30 (d, 1H, J = 8), 8.07 (d, 1H, J = 8), 7.76 (d, 1H, J = 8) 7.60 (t, 1H, J = 8), 7.56 (t, 2H, J = 12), 7.51 (d, 1H, J = 8) 7.04 (d, 1H, J = 8), 7.00 (t, 1H, J = 8), 6.97 (s, 1H), 6.80 (d, 2H, J = 8) 6.72 (d, 2H, J = 8), 6.21 (d, 1H, J = 8), 6.11 (d, 1H, J = 8) 5.66 (d, 1H, J = 8), 3.43 (t, 2H, J = 8), 2.98 (t, 2H, J = 8), 2.94 (s, 6H), 2.73 (t, 2H, J = 8), 2.61 (s, 6H), 1.67 (t, 2H, J = 12), 1.57 (t, 1H, J = 12). ^{13}C NMR (400 MHz, DMSO): δ/ppm 207.30, 169.30, 164.90, 163.91, 152.70, 152.87, 150.99, 150.45, 147.84, 134.93, 133.78, 132.40, 131.24, 130.23, 128.96, 128.42, 127.90, 126.56, 124.91, 123.75, 123.31 , 121.08, 120.30, 112.74, 109.53, 108.88, 107.57, 105.99, 98.61, 77.34, 67.37, 51.22, 40.78, 40.23, 39.33, 32.25, 31.26, 29.99, 29.60, 26.52, 23.42, 23.02, 22.58, 14.45. HRMS (ESI +) *m/z* for $C_{47}H_{45}N_5O_4$, 742.34 calculated, 742.3413 found.



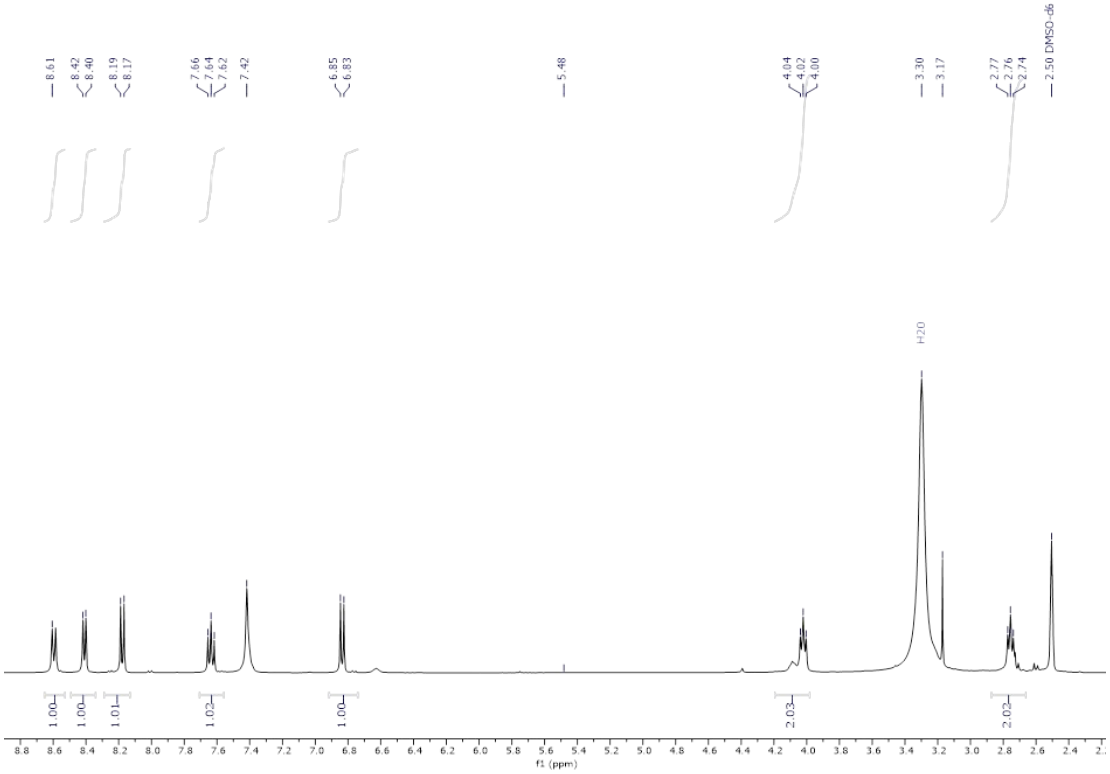
^{1}H NMR spectrum of **Pr1** in CDCl3-δ_6, 400 MHz.



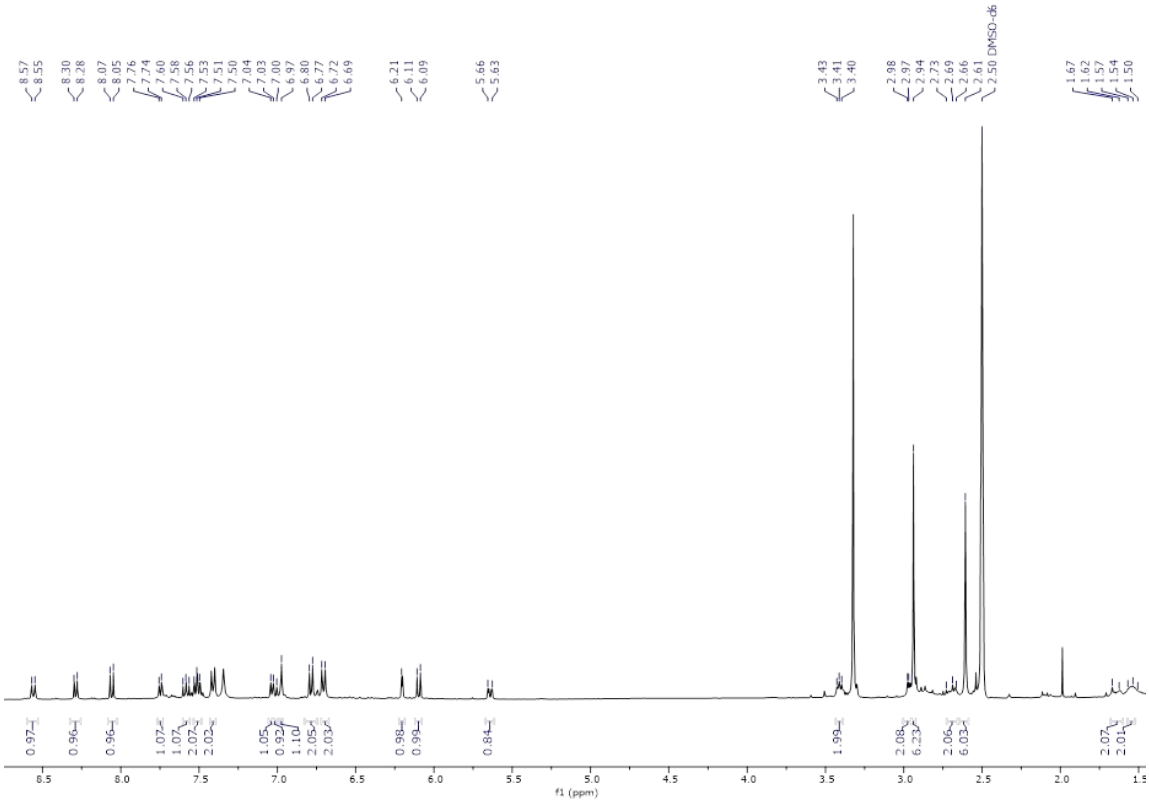
^{1}H NMR spectrum of **Pr2** in DMSO-δ_6, 400 MHz.



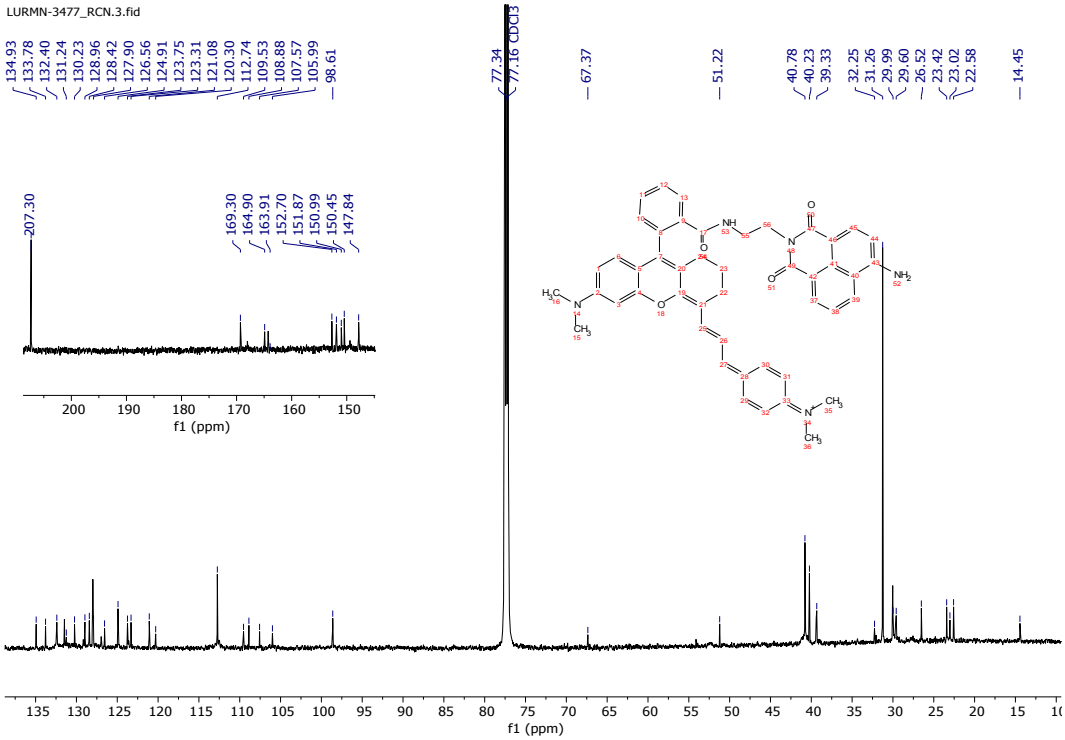
^{1}H NMR spectrum of **RCin** in DMSO-δ_6, 400 MHz.



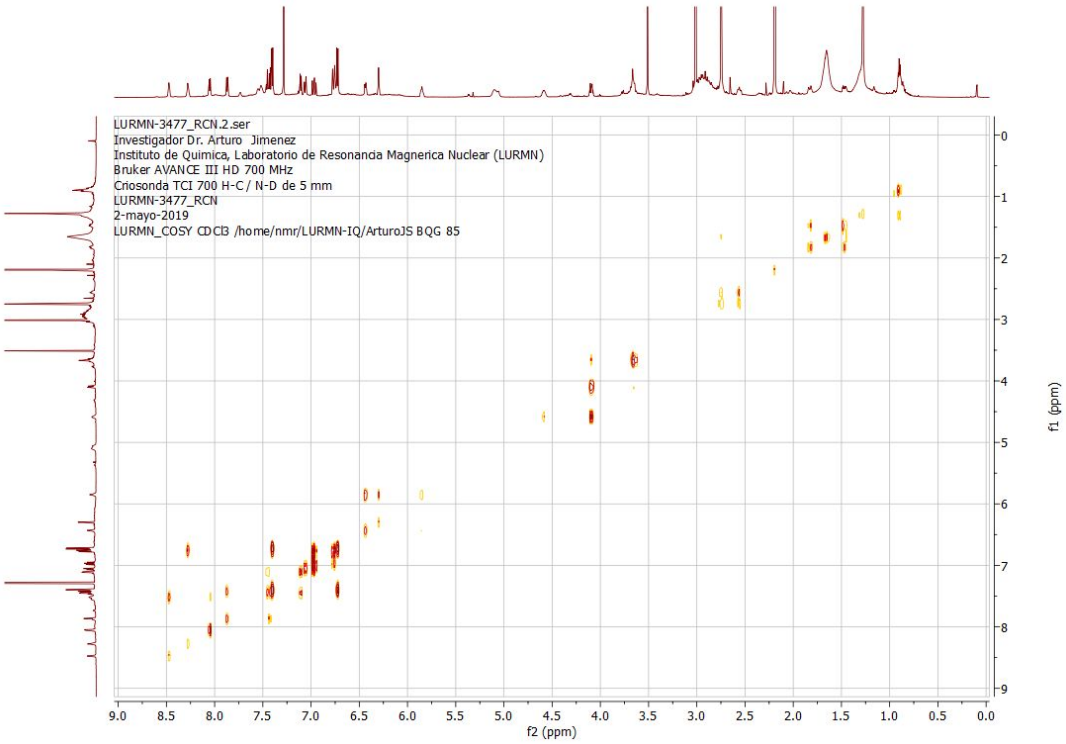
^{1}H NMR spectrum of **Naph** in DMSO-δ_6, 400 MHz.



^{1}H NMR spectrum of **RCN** in DMSO-δ_6, 700 MHz.



^{13}C NMR spectrum of **RCN** in DMSO-δ_6, 700 MHz.



COSY spectrum of **RCN** in DMSO-δ_6, 700 MHz.

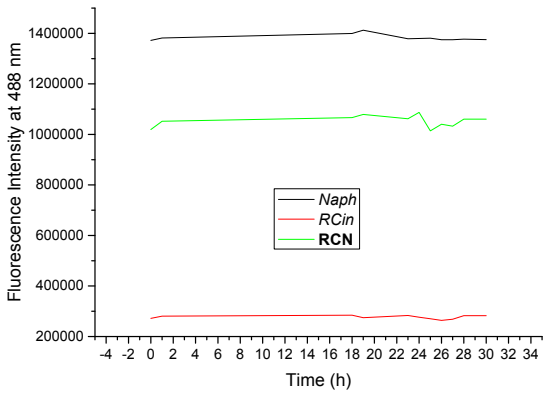
Materials, physical measurements and Quantum Yield Calculation.

Determination of the fluorescence quantum yield

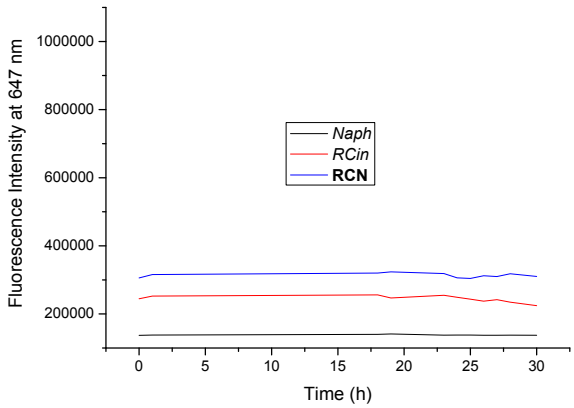
Fluorescence quantum yield for **RCN** were determined by using Coumarin 102 ($\phi_F = 0.764$ in ethanol)[1] as a fluorescence standard. The quantum yield was calculated using the following equation (1):

$$\phi_{F(X)} = \phi_{F(S)}(A_S F_X / A_X F_S)(n_X / n_S)^2 \qquad \text{eq. (1)}$$

where ϕ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used. Subscripts S an X refer to the standard and to the unknown, respectively.



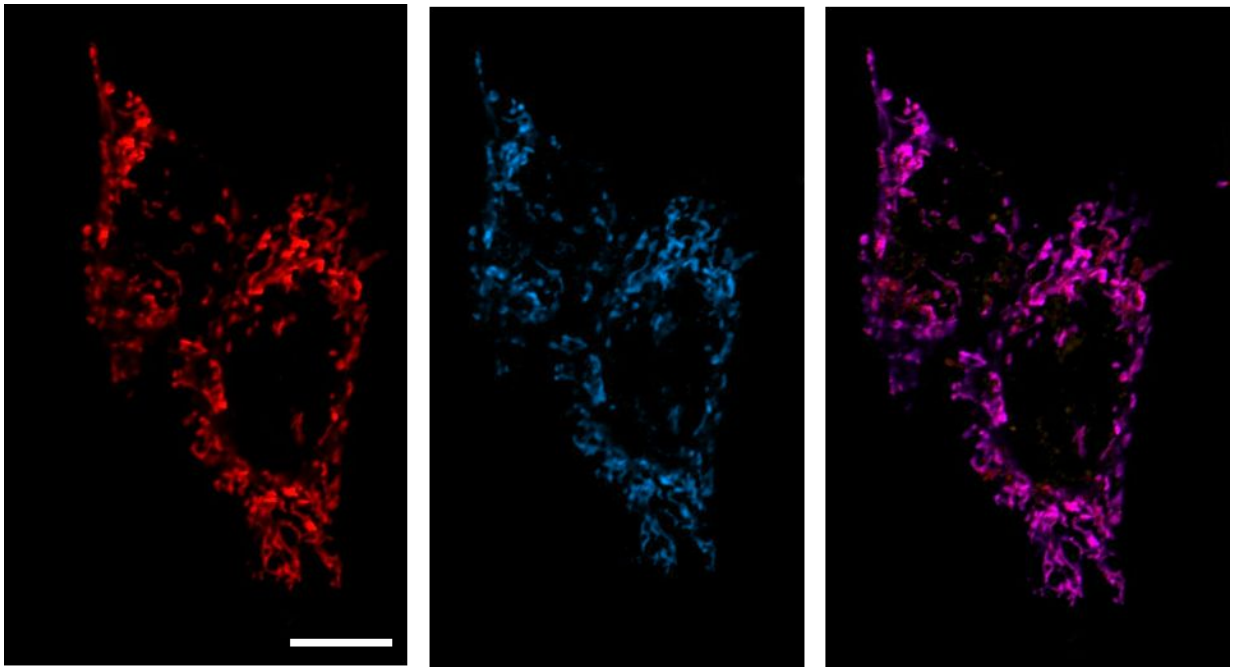
(A)



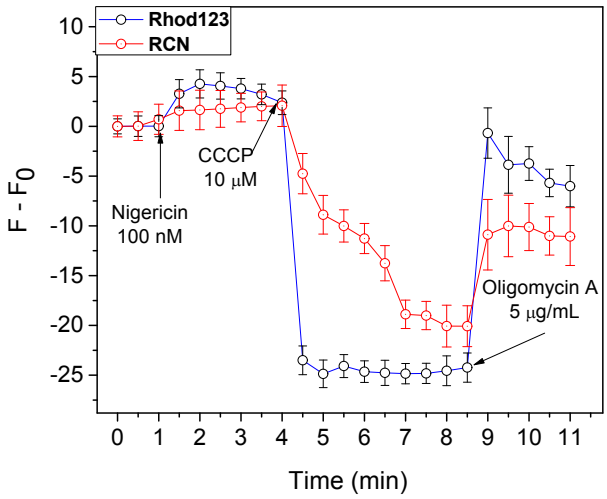
(B)

Figure S1. Fluorescence intensity detected at 550 nm for probe **RCN** and fluorophore fragments Naph and *RCin* at variable time with excitation at (A) (λ_{exc} = 488 nm) and (B) (λ_{exc} = 647 nm)

[1] Rurack, K. and Spieles, M. Fluorescence Quatum Yields of a Series of Red and Near-Infrered Dyes Emittingat 600-1000 nm. *Anal. Chem.* 2011, 83, 4, 1232-1242. https://doi.org/10.1021/ac101329h

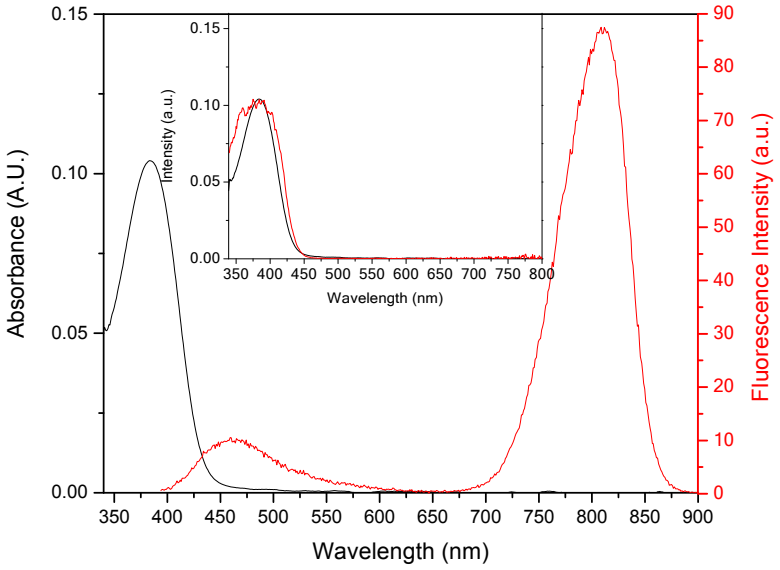


(A)

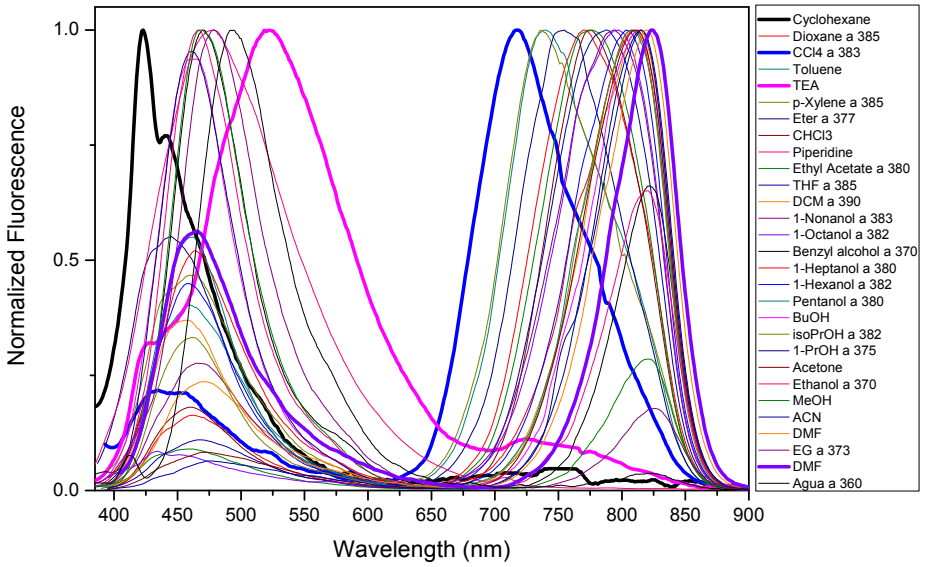


(B)

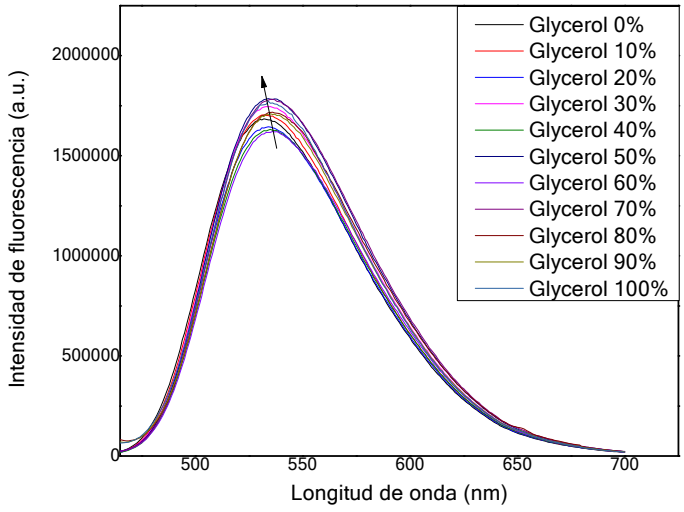
Figure S2. (A) Mitochondrial co-localization differential analysis using MitoLite™ Blue (blue channel, λ_{ex} = 400 nm) and **RCN** (red channel, λ_{ex} = 647 nm). The high degree of overlap indicates that the probe is mitochondria selective. Scale bar represent 20 µm. (B) Effect of nigericin during charge gradient depolarization with CCCP proton uncoupler on the **RCN** and Rhodamine 123 (Rhod123, a slow-equilibrating mitochondrial localizer) fluorescence signals. Then, Oligomycin A was used to hyperpolarize the membrane potential. The $F - F_0$ is the fluorescence intensity difference taking zero as the initial point.


Absorbance (A.U.)
Fluorescence Intensity (a.u.)
Wavelength (nm)
Intensity (a.u.)
Wavelength (nm)

(A)


Normalized Fluorescence
Wavelength (nm)
Cyclohexane
Dioxane a 385
CCl4 a 383
Toluene
TEA
p-Xylene a 385
Eter a 377
CHCl3
Piperidine
Ethyl Acetate a 380
THF a 385
DCM a 390
1-Nonanol a 383
1-Octanol a 382
Benzyl alcohol a 370
1-Heptanol a 380
1-Hexanol a 382
Pentanol a 380
BuOH
isoPrOH a 382
1-PrOH a 375
Acetone
Ethanol a 370
MeOH
ACN
DMF
EG a 373
DMF
Agua a 360

(B)



(C)

Figure S3. (A) Absorption spectrum of 40 µM **RCN** (red line) and Fluorescence spectra in methanol (black line); the inset shows the overlap between absorption and excitation spectra. (B) Fluorescence spectra of 40 µM **RCN** in different solvents (inset). (C) Fluorescence spectra of 40 µM **RCN** for a viscosity variation in a Glycerol : acetone, a solvent mixture having a larger dielectric constant difference. Excitation wavelength was maintained in the absorption maximum between 370 and 385 nm.

Table S1. Catalán solvent parameters {SA, SB, SP, SdP}*

Solvent	SP	SdP	SA	SB	Viscosity (cP)	Em (RCN)	Em (RCin)
Cyclohexane	0.616	0	0	0.056	0.894	25510	22400
Dioxane	0.737	0.312	0	0.444	1.177	19640	18435
Toluene	0.782	0.284	0	0.128	0.56	24510	23900
Diethyl eter	0.617	0.385	0	0.562	0.603	24630	23650
MTBE[a]	0.622	0.422	0	0.567	0.224	24030	24038
Chloroform	0.783	0.614	0.047	0.071	0.36	24330	24331
Buthyl acetate	0.674	0.535	0	0.525	0.537	23250	23550
Ethyl acetate	0.656	0.603	0	0.542	0.685	23640	23640
Tetrahydrofuran	0.714	0.634	0	0.591	0.423	23690	23710
Dichloromethane	0.761	0.769	0.04	0.178	0.793	24150	24250
Octanol	0.713	0.454	0.299	0.923	0.456	23580	23850
i-Propanol	0.633	0.808	0.283	0.83	0.413	23360	23400
Acetone	0.651	0.907	0	0.475	7.288	19720	19840
Ethanol	0.633	0.783	0.4	0.658	5.474	19120	19300
Methanol	0.608	0.904	0.605	0.545	3.619	23040	23100
Acetonitrile	0.645	0.974	0.044	0.286	2.544	22980	22900
DMF[b]	0.759	0.977	0.031	0.613	2.038	23040	23050
Ethyleneglycol	0.777	0.91	0.717	0.534	0.306	23250	23360
DMSO[c]	0.83	1	0.072	0.647	1.074	22720	22730
Water	0.681	0.997	1.062	0.025	0.544	19880	19920

[a] Methyl-tert-butyl ether, [b] N,N-dimethylformamide and [c] Dimethyl sulfoxide

* The mathematical treatment of solvent effects introduced by Catalán is based on four empirical and independent solvent scales:

$$y = y_0 + a_{SA}\,SA + b_{SB}\,SB + c_{SP}\,SP + d_{SdP}\,SdP \qquad \text{Eq. (2)}$$

Here SA, SB, SP and SdP are the solvent acidity, basicity, polarizability and dipolarity properties, respectively. The coefficients a_{SA}, b_{SB}, c_{SP} and d_{SdP} represent the contribution of each type of interactions. Then, a Catalán solvent analysis was carried out in order to understand the solvent parameters that affect the photophysical properties ($\bar{\nu}_{abs}$, $\bar{\nu}_{em}$ and $\Delta\bar{\nu}$,) in probe **RCN** and **RCin**. The {SA, SB, SP, SdP} parameters for each solvent are taken from reference S1. The regression coefficients y_o, a_{SA}, b_{SB}, c_{SP} and d_{SdP}, standard errors and the multilinear correlation coefficient, *r*, are presented in Table S1 (above). In the case of $\bar{\nu}_{abs}$, a good multilinear fit of 0.905 was obtained.

The correlation data analysis *without including* the viscosity parameter in the multilinear regression analysis is:

R (multilinear)	**0.82916022**	**error**
Y0	30564.3622	2708.76289
SP	-8608.99697	3693.84384
SdP	-561.78286	1104.10075
SA	-4481.95321	1054.23579
SB	-1552.93327	1007.55832

The correlation data analysis *including* the viscosity parameter in the multilinear regression analysis is:

R (multilinear)	**0.77873236**	**error**
Y0	28432.1754	4515.23259
SP	-1735.79209	6248.03435
SdP	1541.59337	1645.79514
SA	-6507.98134	1866.33859
SB	105.501192	1638.261

Similar results were found for the free *RCin* fragment (data not presented).

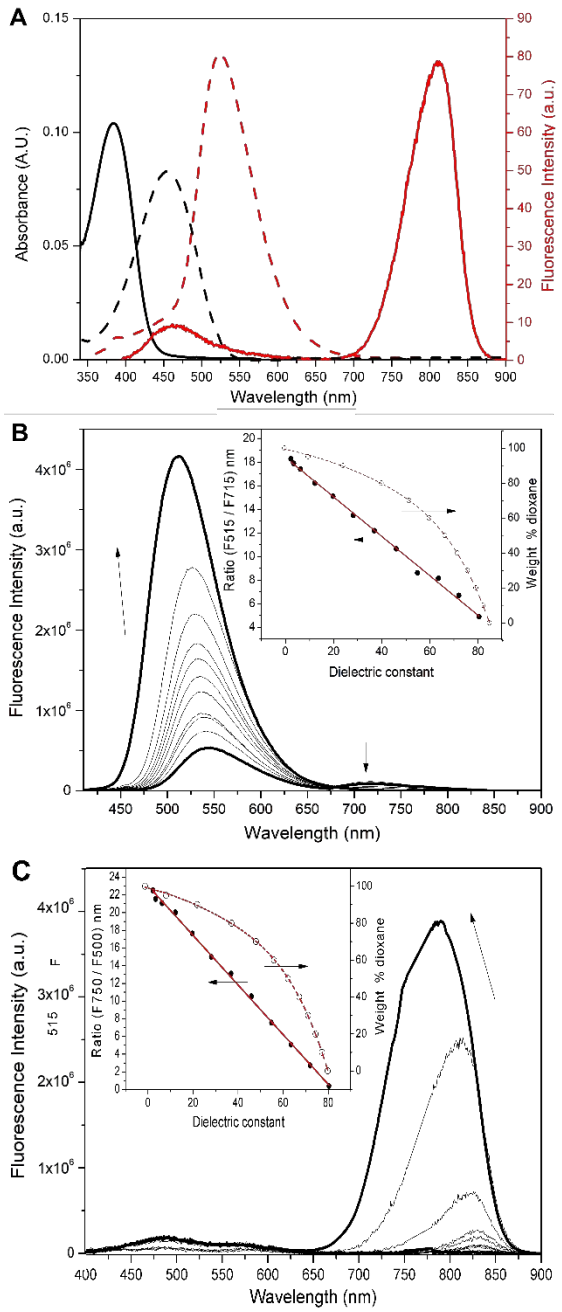


Figure S4. UV-Vis (black lines) and fluorescence (red lines, λ_{ex} = 380 nm) spectra in methanol for the *RCin* (solid lines) and the *Naph* (dashed lines) fluorophore units (A). Calibration of the polarity effect in water : dioxane as a function of dielectric constants for **RCN** (B) and *RCin* (C), where the hollow and filled symbols are weight % and ratio values, respectively.

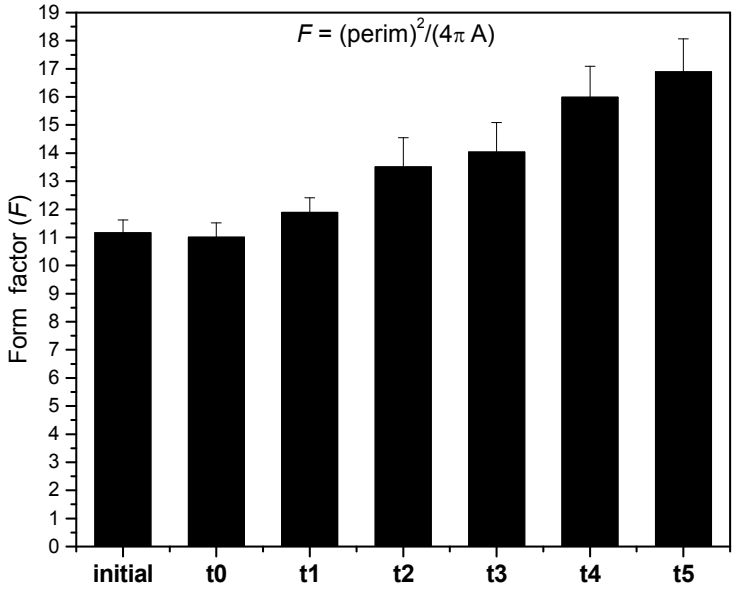
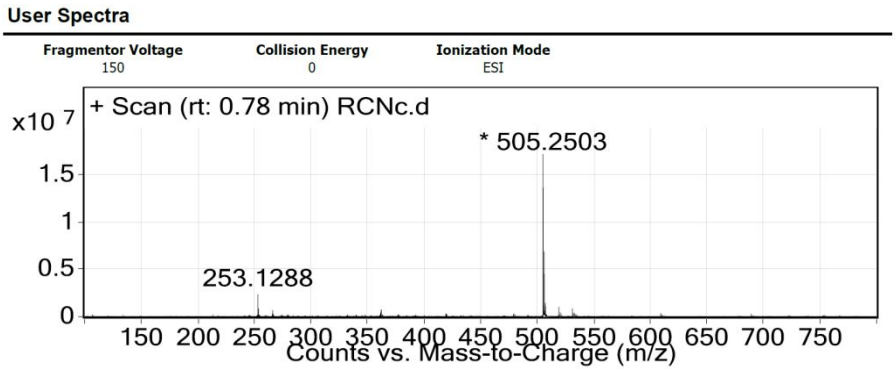
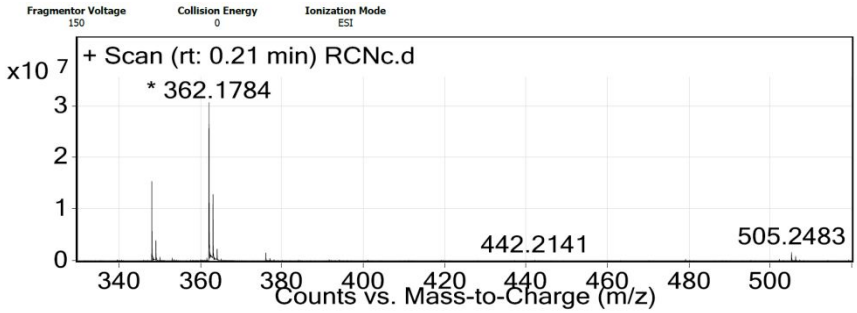


Figure S5. Time dependence of form factor changes of the **RCN** fluorescence profile during the time course monitoring of mitochondrial morphology changes shown in Figure 4 (main text).



Peak List

m/z	z	Abund
253.1288		2356609.03
505.2503	1	17544325.25
505.4285		1158750.93
506.253	1	7017131.01
507.2564	1	1444663.91
519.2661		1028227.79



Peak List

m/z	z	Abund
348.1594	1	15415039.28
348.3112	1	986643.82
349.1623	1	3975837.62
362.1784	1	31208688.71
362.3266	1	2988199.79
363.178	1	13203597.25
364.1787	1	2309123.15
376.1544	1	1582537.95
505.2483	1	1728308.09
506.2518	1	674138.05

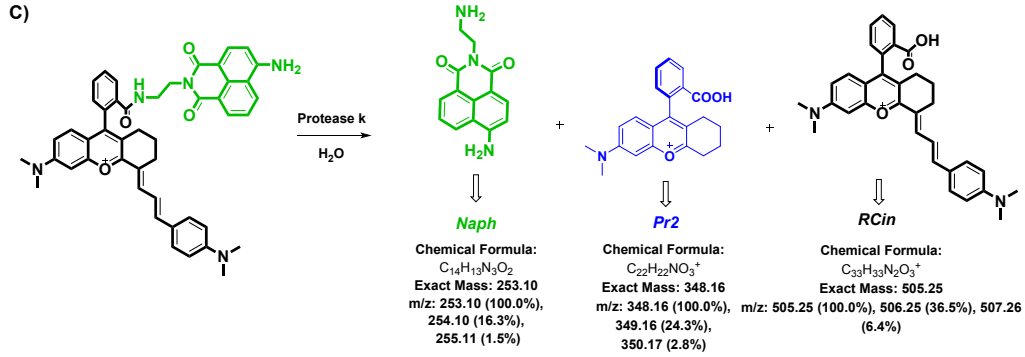
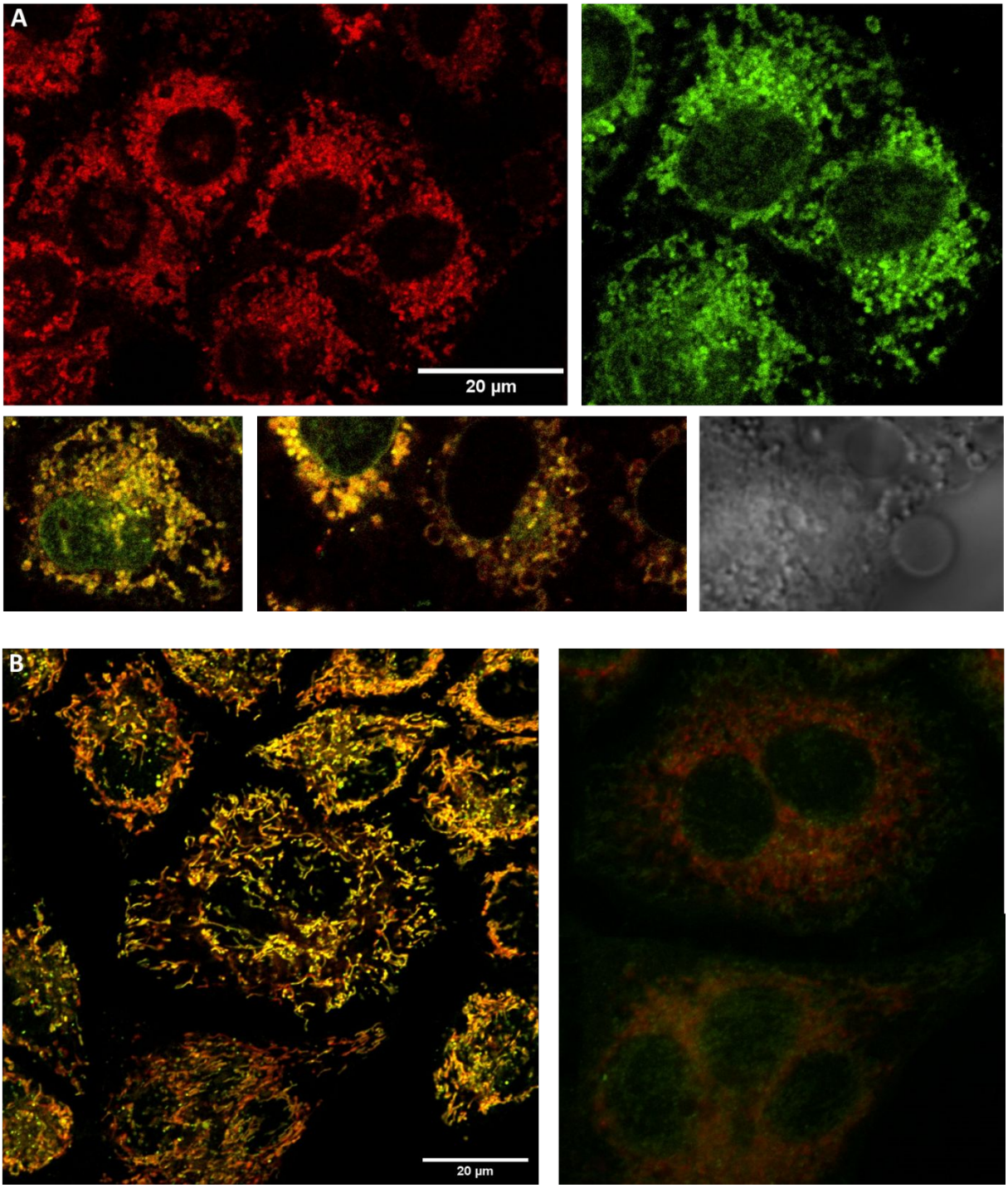
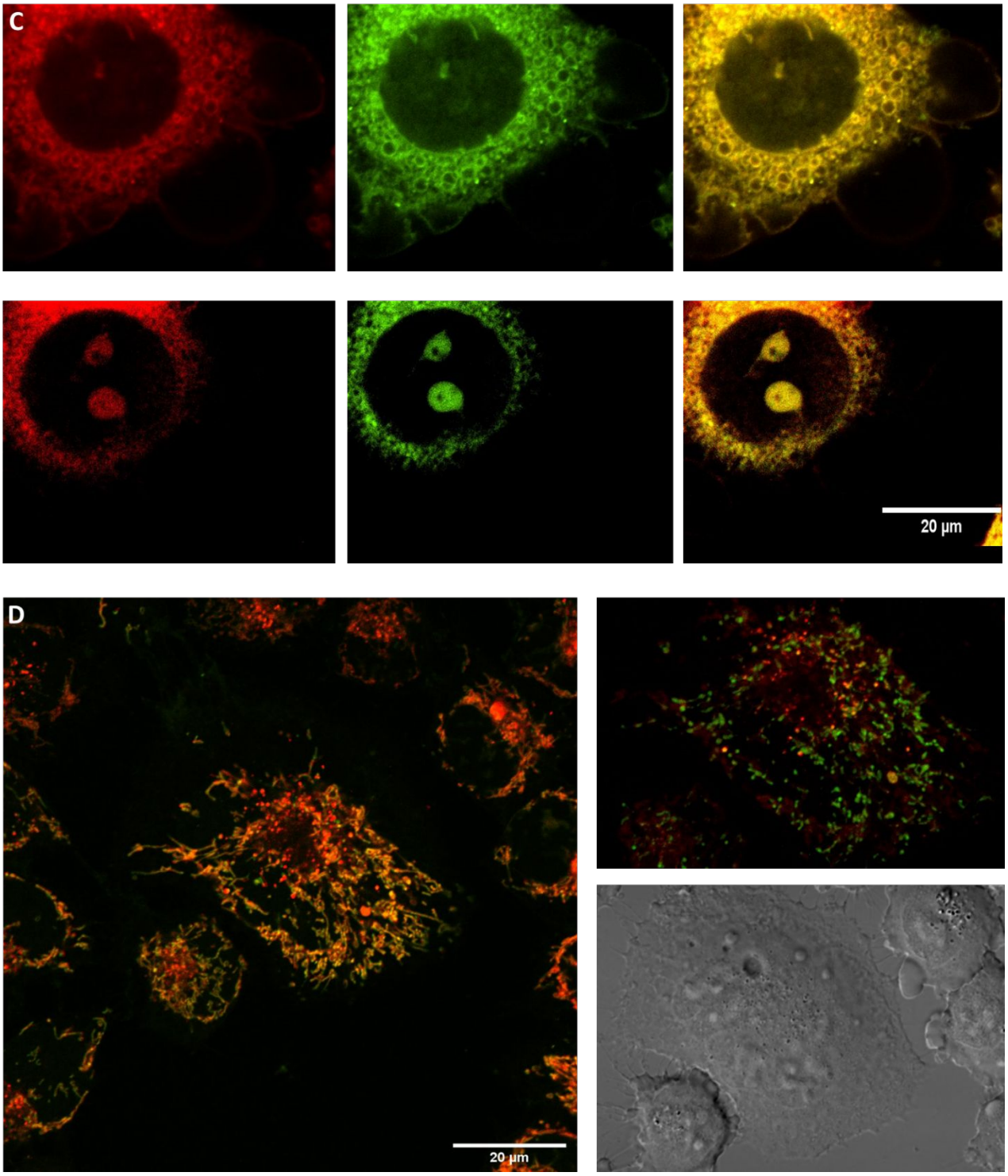
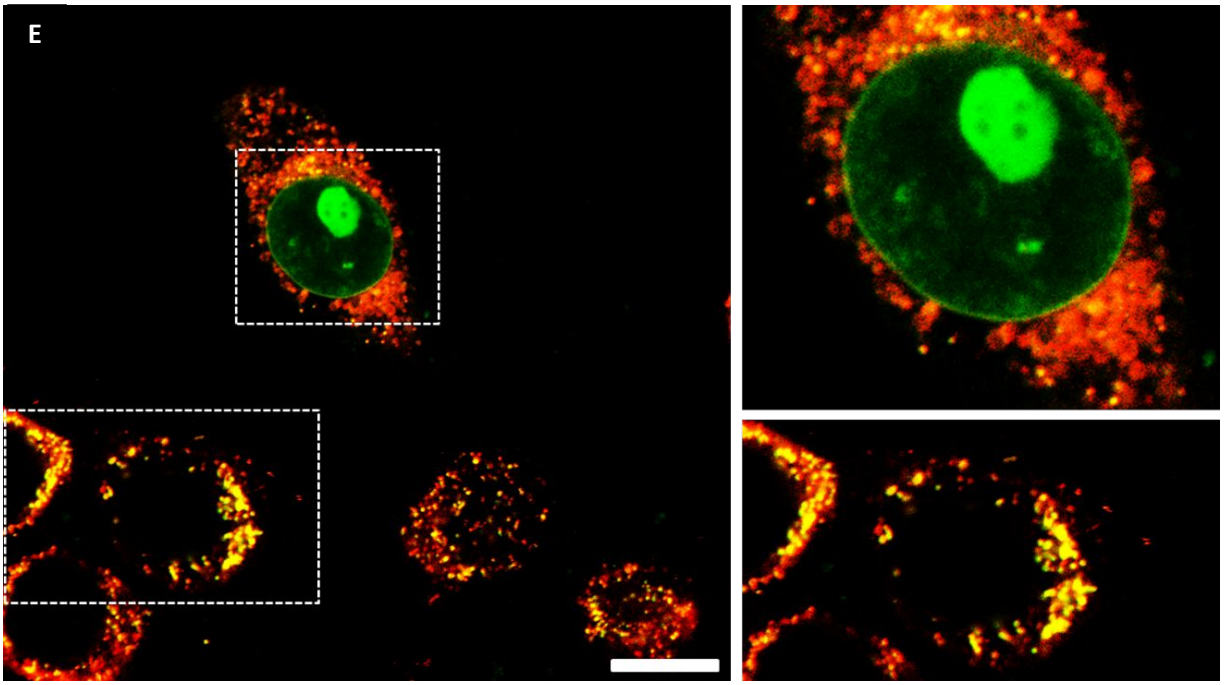
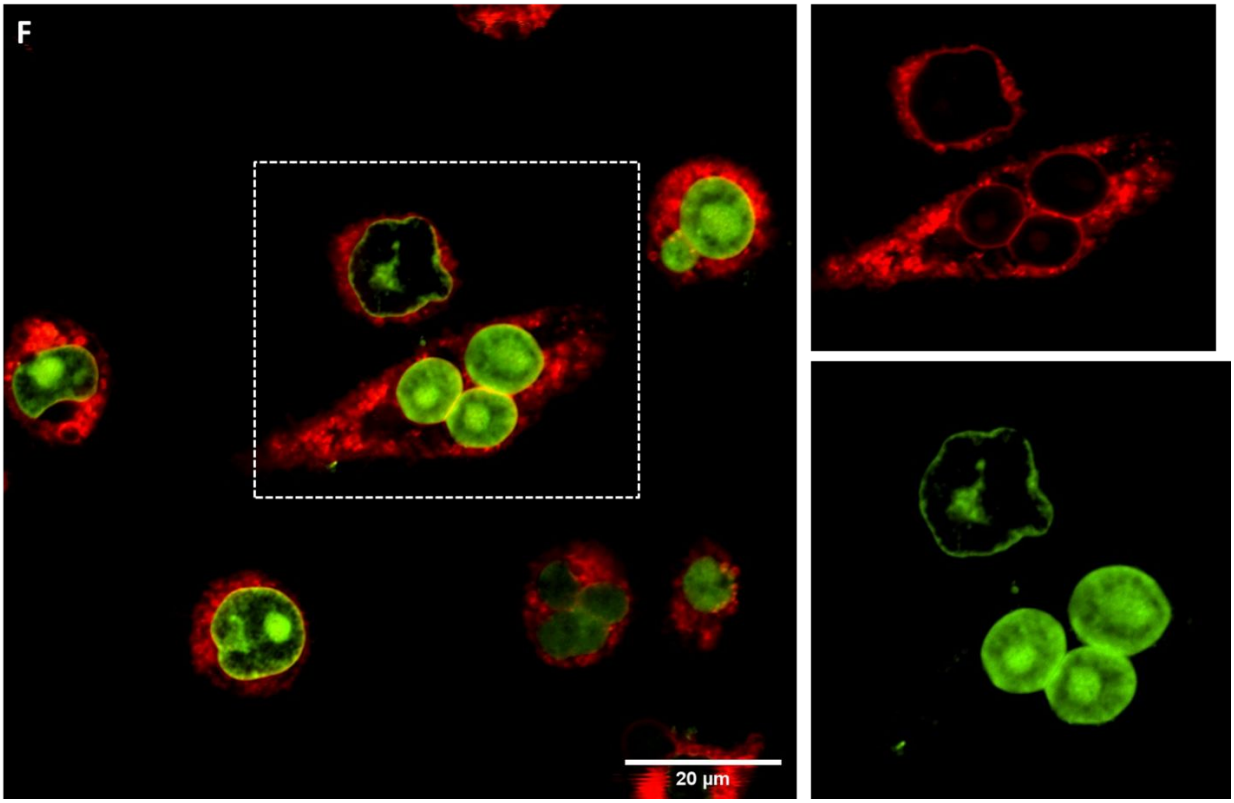


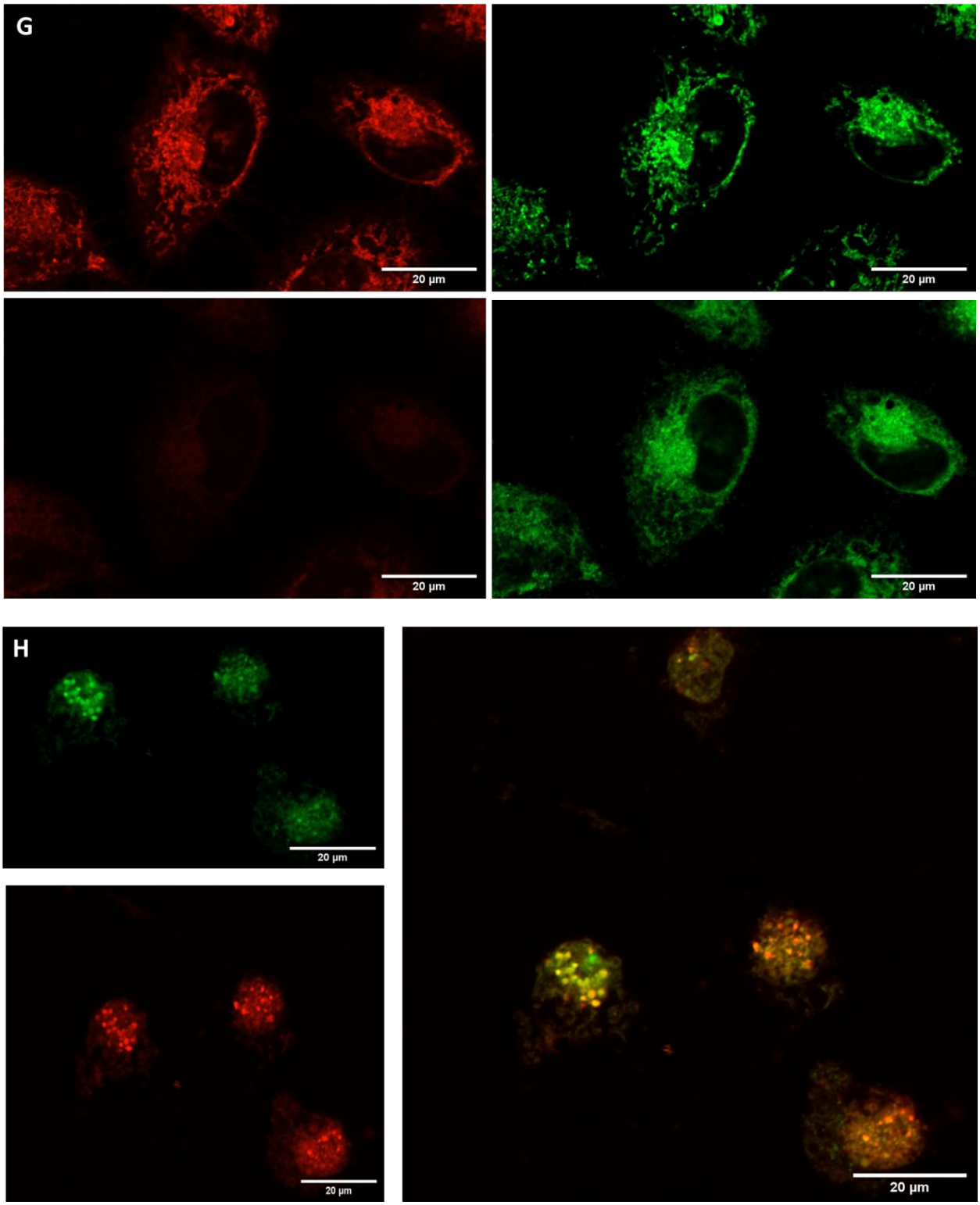
Figure S6. High-Resolution Mass Spectrometry (ESI-TOF technique through direct injection) for **RCN** with Protease K at 37 °C for 30 minutes, collecting at (A) 0.78 min observing the *Naph* and *RCin* fragments and (B) 0.21 min observing *Pr2* fragment in 348.1594 as well as *RCin*. C) Schematic representation for the hydrolysis-derived products.


A
20 µm
B
20 µm


C
20 µm
D
20 µm


E


F
20 µm


G
20 µm
20 µm
20 µm
20 µm
H
20 µm
20 µm
20 µm

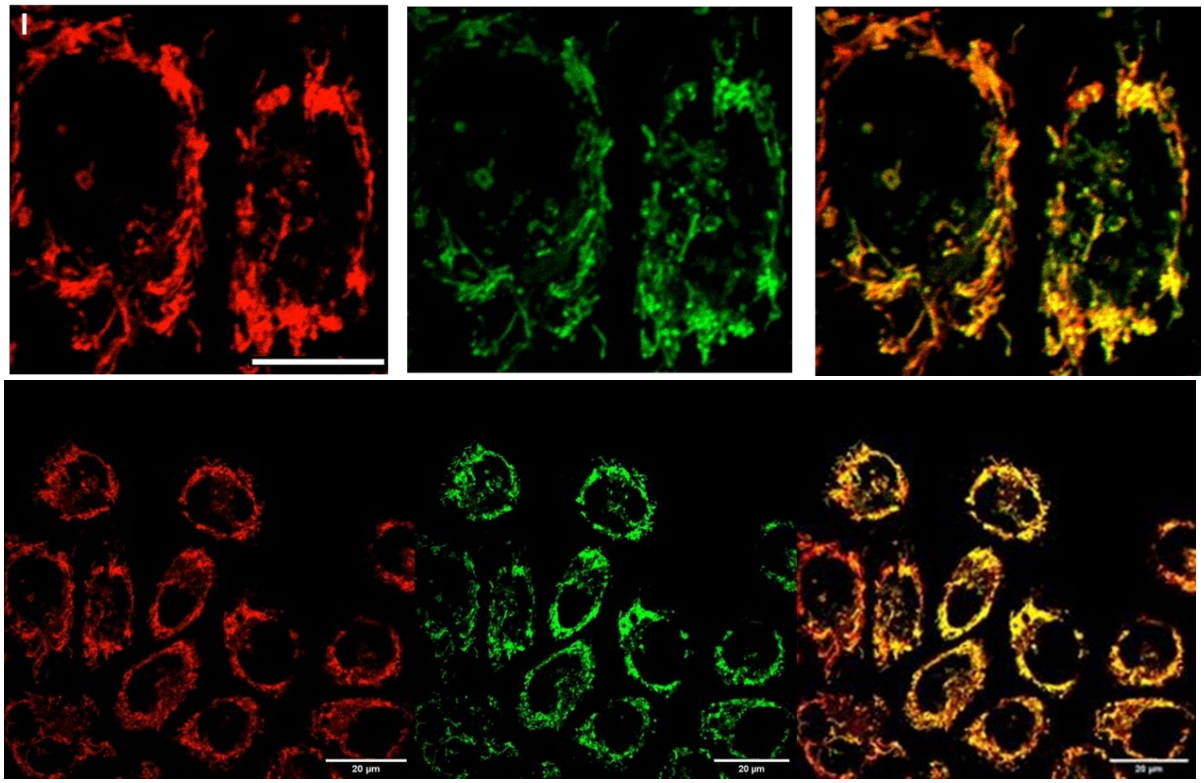
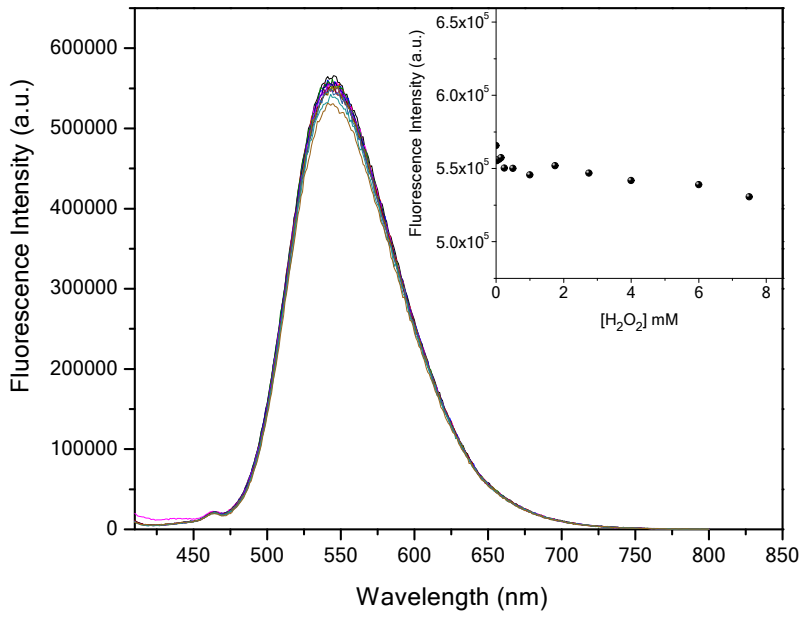
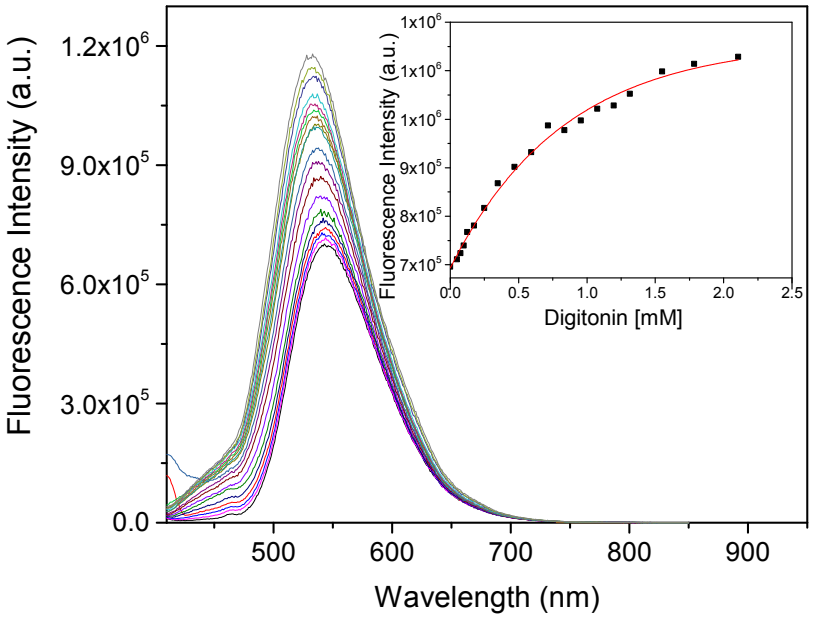


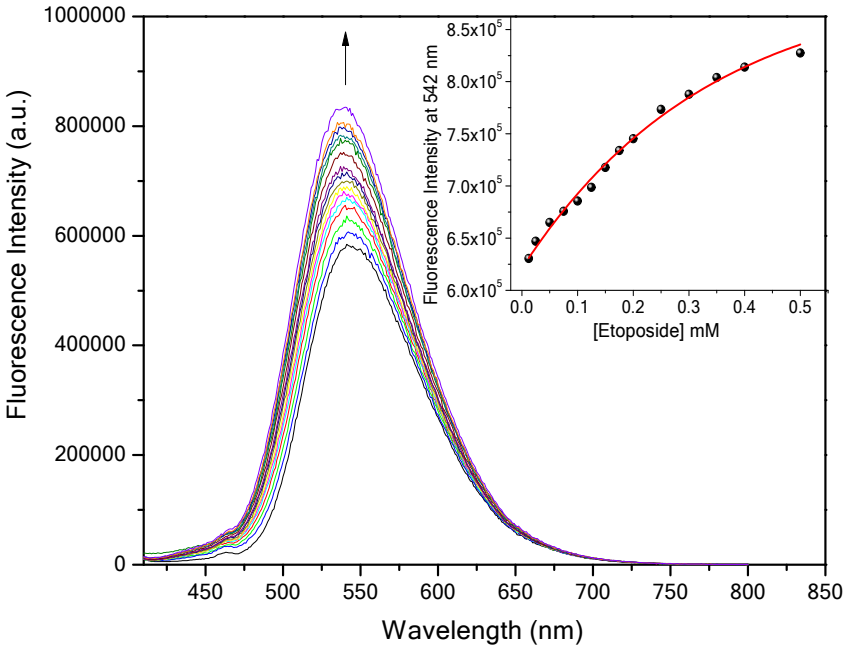
Figure S7. Confocal imaging of **RCN** in the presence of (A) 50 µM H_2O_2 for 40 minutes; (B) 5 µM Digitonin; (C) 5 µM Ca(II) ion and 5 µM Digitonin; (D) 2 µM Etoposide; (E and F) 50 µM Nystatin before **RCN** incubation; (G) **RCN** incubation before 50 µM Amphotericin B; (H) 50 µM Amphotericin B and then **RCN** and, (I) 10 µM CCCP treatment for 30 minutes after **RCN** incubation. Experiments were performed at different concentrations finding no significant differences above the ones presented here. Scale bars represent 20 µm.


Fluorescence Intensity (a.u.)
600000
500000
400000
300000
200000
100000
0
450
500
550
600
650
700
750
800
850
Wavelength (nm)
Fluorescence Intensity (a.u.)
6.5x10^5
6.0x10^5
5.5x10^5
5.0x10^5
0
2
4
6
8
[H2O2] mM

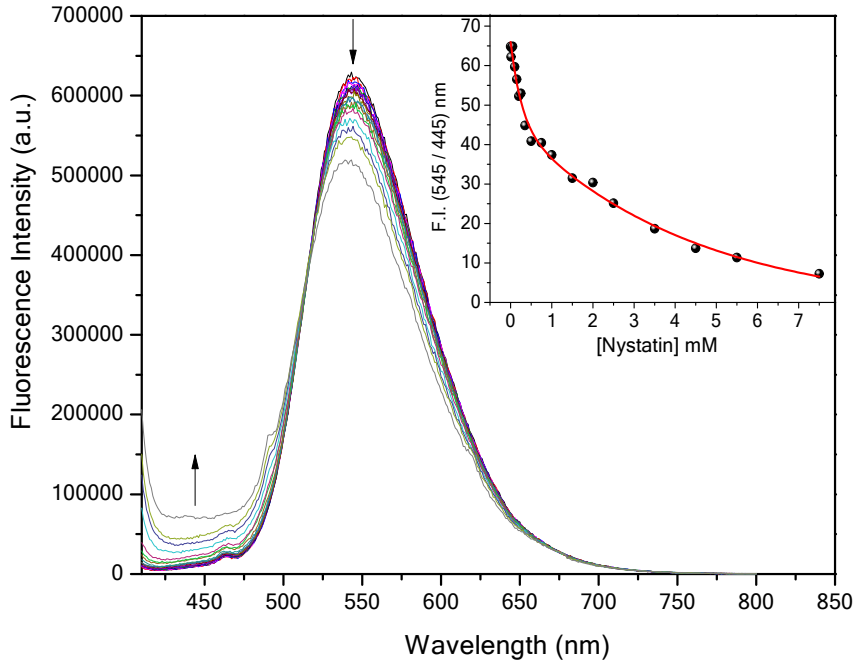
(A)


Fluorescence Intensity (a.u.)
1.2x10^6
9.0x10^5
6.0x10^5
3.0x10^5
0.0
500
600
700
800
900
Wavelength (nm)
Fluorescence Intensity (a.u.)
1x10^6
1x10^6
1x10^6
9x10^5
8x10^5
7x10^5
0.0
0.5
1.0
1.5
2.0
2.5
Digitonin [mM]

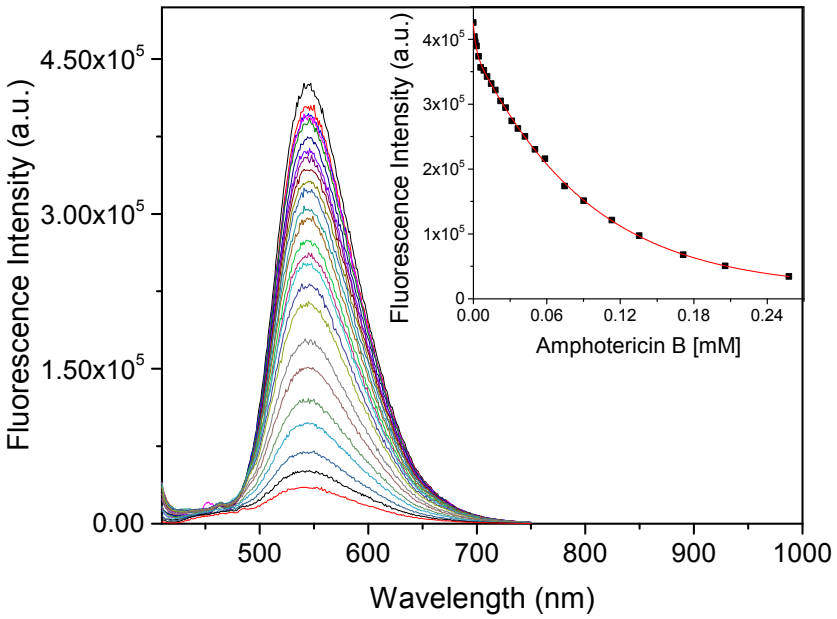
(B)


Fluorescence Intensity (a.u.)
Wavelength (nm)
Fluorescence Intensity at 542 nm
[Etoposide] mM

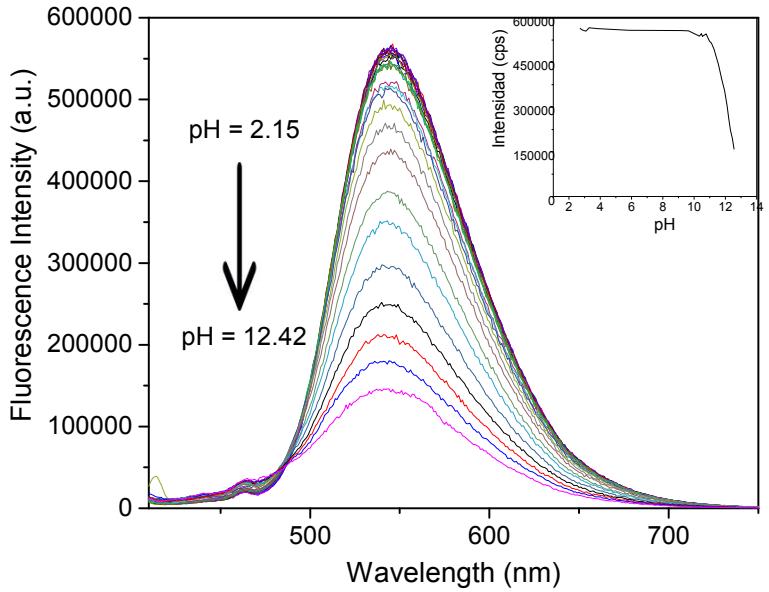
(C)


Fluorescence Intensity (a.u.)
Wavelength (nm)
F.I. (545 / 445) nm
[Nystatin] mM

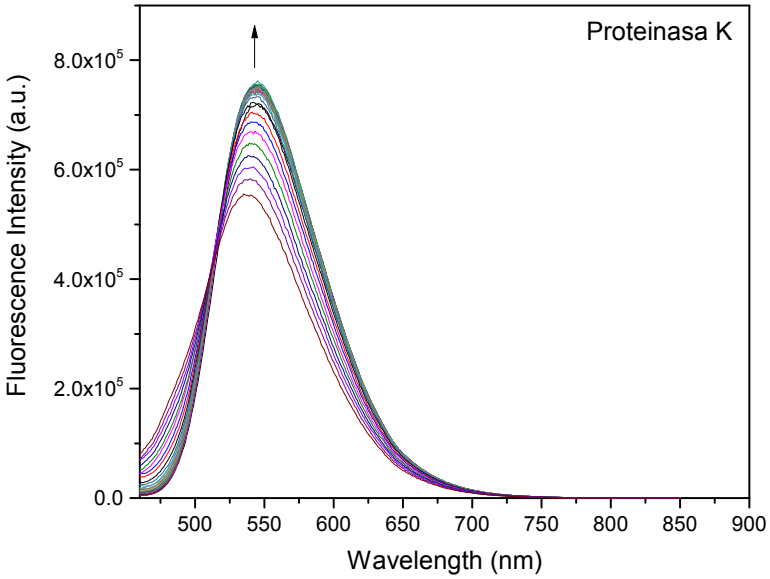
(D)


Fluorescence Intensity (a.u.)
4.50x10^5
3.00x10^5
1.50x10^5
0.00
500
600
700
800
900
1000
Wavelength (nm)
Fluorescence Intensity (a.u.)
4x10^5
3x10^5
2x10^5
1x10^5
0
0.00
0.06
0.12
0.18
0.24
Amphotericin B [mM]

(E)

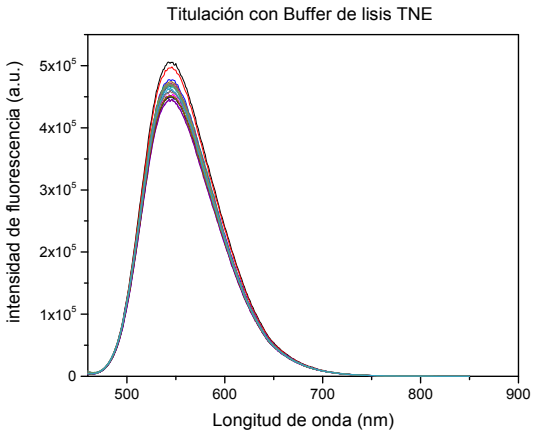

Fluorescence Intensity (a.u.)
600000
500000
400000
300000
200000
100000
0
pH = 2.15
pH = 12.42
500
600
700
Wavelength (nm)
Intensidad (cps)
600000
450000
300000
150000
0
2
4
6
8
10
12
14
pH

(F)

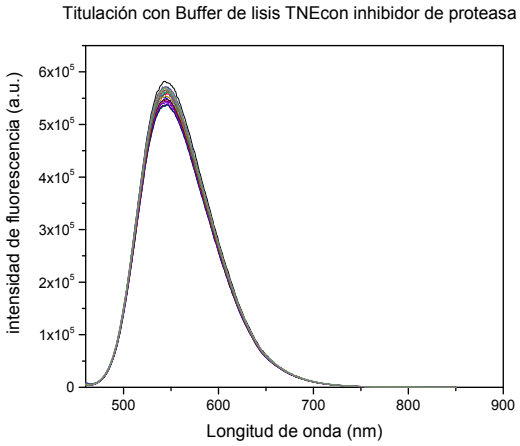


(G)

Figure S8. Fluorescence spectra of the **RCN** probe upon variable concentration of (A) H_2O_2; (B) Digitonin; (C) Etoposide; (D) Nystatin; (E) Amphotericin B; (F) variable pH and; (G) Proteinase K.



A)



B)

Figure S9.- Fluorescence spectra of approx. 50 uM **RCN** with varying concentrations of lysis buffer. A) Without protease inhibitor. B) With protease inhibitor.

References

[S1]. Catalán, J. Toward a Generalized Treatment of the Solvent Effect Based on Four Empirical Scales: Dipolarity (SdP, a New Scale), Polarizability (SP), Acidity (SA), and Basicity (SB) of the Medium. *J. Phys. Chem. B* **2009**, *113*, 5951–5960.