Electronic Supporting Information for

Fluorescent Probe for Early Mitochondrial Voltage Dynamics

Cinthia Hernández-Juárez, Ricardo Flores-Cruz and Arturo Jiménez-Sánchez*

Instituto de Química - Universidad Nacional Autónoma de México.

Circuito Exterior s/n, Coyoacán 04510. Ciudad Universitaria, Ciudad de México, México. E-mail: arturo.jimenez@iquimica.unam.mx

Table of Contents:

Materials, physical measurements and cell culture methods **S2**

Figure S1 **S3**

Figure S2 **S4**

Table S1 and Compound synthesis and chemical characterization **S5**

Figures S3 **S10**

Table S2 **S11**

Determination of pK_a and partition coefficients (log$P$) **S13**

Full references in the main text and supplementary references **S14**

Materials, physical measurements and cell culture methods.

Commercially available starting materials, components of buffer solutions (CHES, MOPS, MES from Sigma, Mexico) and solvents were used as supplied. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a 700 MHz Bruker unity spectrometer. Chemical shifts (ppm) are relative to $(CH_3)_4Si$. High resolution mass spectrometry (ESI-TOF) was obtained by using an Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS equipment. Fluorescence experiments were measured either on a FluoroMax spectrofluorometer from HORIBA Scientific or in a Cary Eclipse fluorimeter, UV-Vis absorption spectra were taken on a Thermo Scientific Evolution diode array UV-Vis spectrophotometer.

Determination of the fluorescence quantum yield

Fluorescence quantum yield for **RVolt** were determined by using Coumarin 102 ($\phi_F = 0.124$ 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.

Cell Culture, Confocal Microscopy and IC_{50} determination. 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 **RVolt** and **Control** probes 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 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 **RVolt** or **Control** probes for 30 minutes. For experiments with TMRM, 50 nM TMRM (Thermo Fisher Scientific) was added 10 minutes before **RVolt**. 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

[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 spectral detector unit. On treatment day for fluorescence time course experiments, cells were incubated with 7 µM probe **RVolt** 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 using 100nM nigericin, 150 nM CCCP (after 5 min) and 5 µg/mL oligomycin A at 20 min. For IC_{50} determination, HeLa cells were treated with **RVolt**, untreated cells were considered to have 100% survival. Cell viability was determined by a redox indicator (Alamar Blue). For cytotoxicity assays, the cells were plated in 96-well plates at 5000 cells/well in RPMI-1640 medium. About 24 h after plating, varied doses of **RVolt** 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 using Alamar Blue fluorescent assay (Life Technologies, Carlsbad, CA, USA). The obtained IC_{50} was 98.5 ± 4.8.

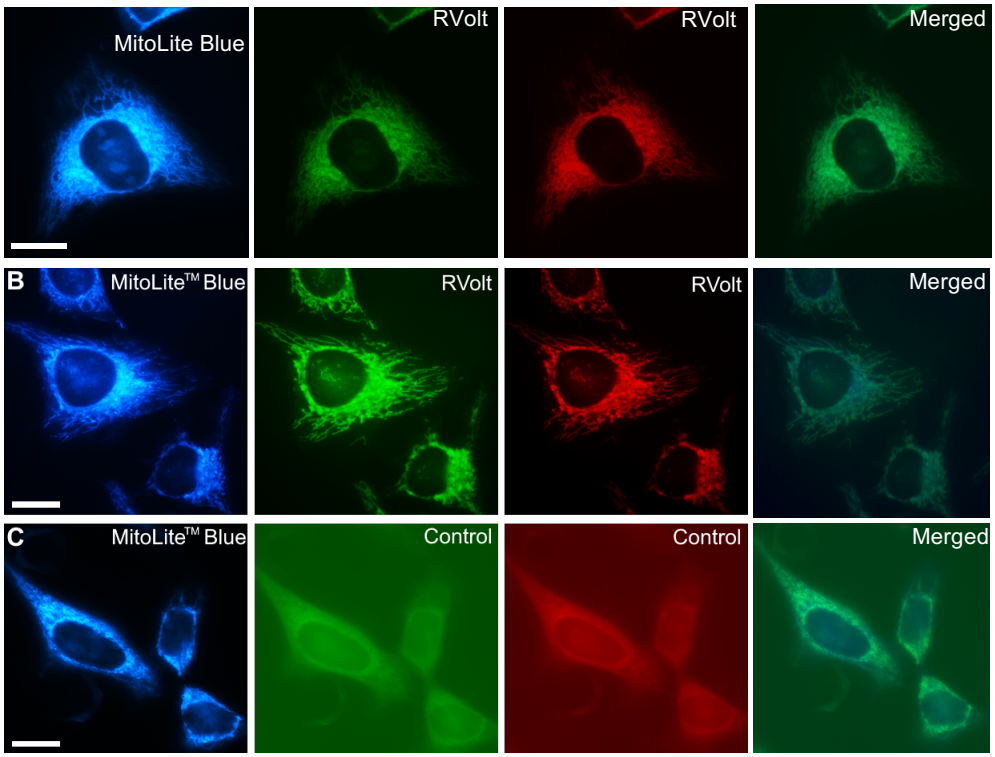


Figure S1. Co-localization imaging of the **RVolt** in live (A) SK-Lu-1 cells and, (B) HeLa cells using MitoLite™ Blue (blue panel, λ_{exc} = 344 nm, λ_{em} = 469 nm) co-localizer indicating that **RVolt** is mitochondria-specific with Pearson's coefficient of 0.96 and 0.94, respectively. (C) HeLa cells stained with MitoLite™ Blue and **Control** probe in the blue, green and red channels, Pearson's coefficient is lower than 0.5. Right panels represent the Blue (λ_{exc} 334 nm) – green (λ_{exc} 488 nm) merged channels. Scale bar represents 20 µm.

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) level of theory to determine the optimized molecular geometry of **RVolt**. 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 **RVolt**.

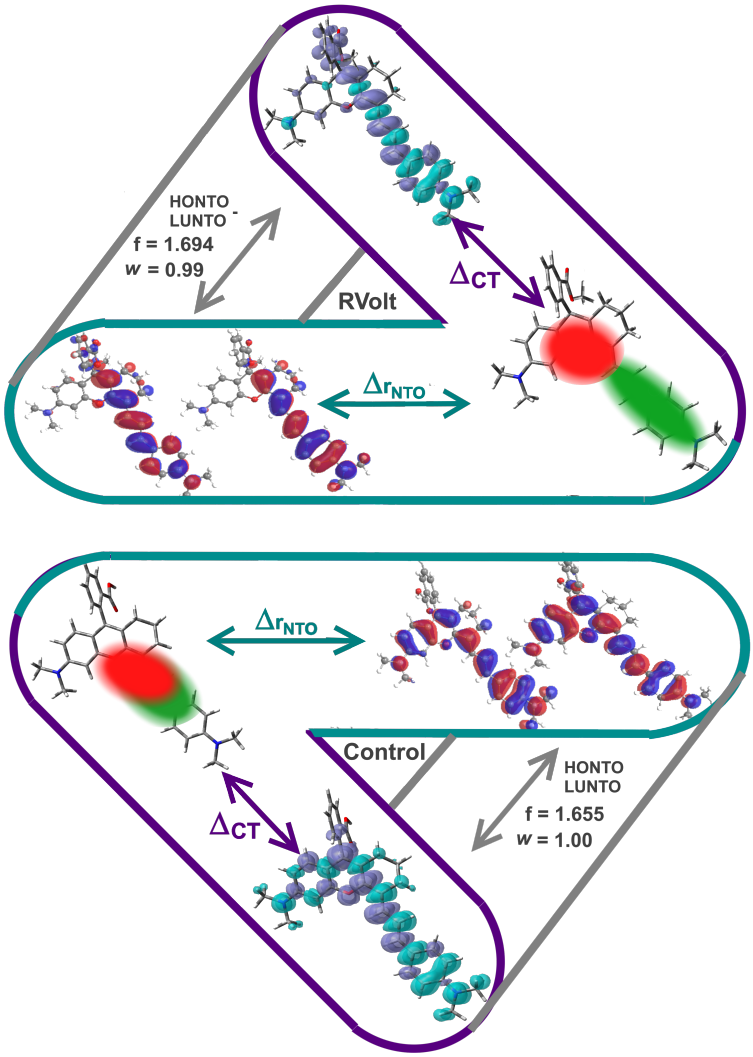


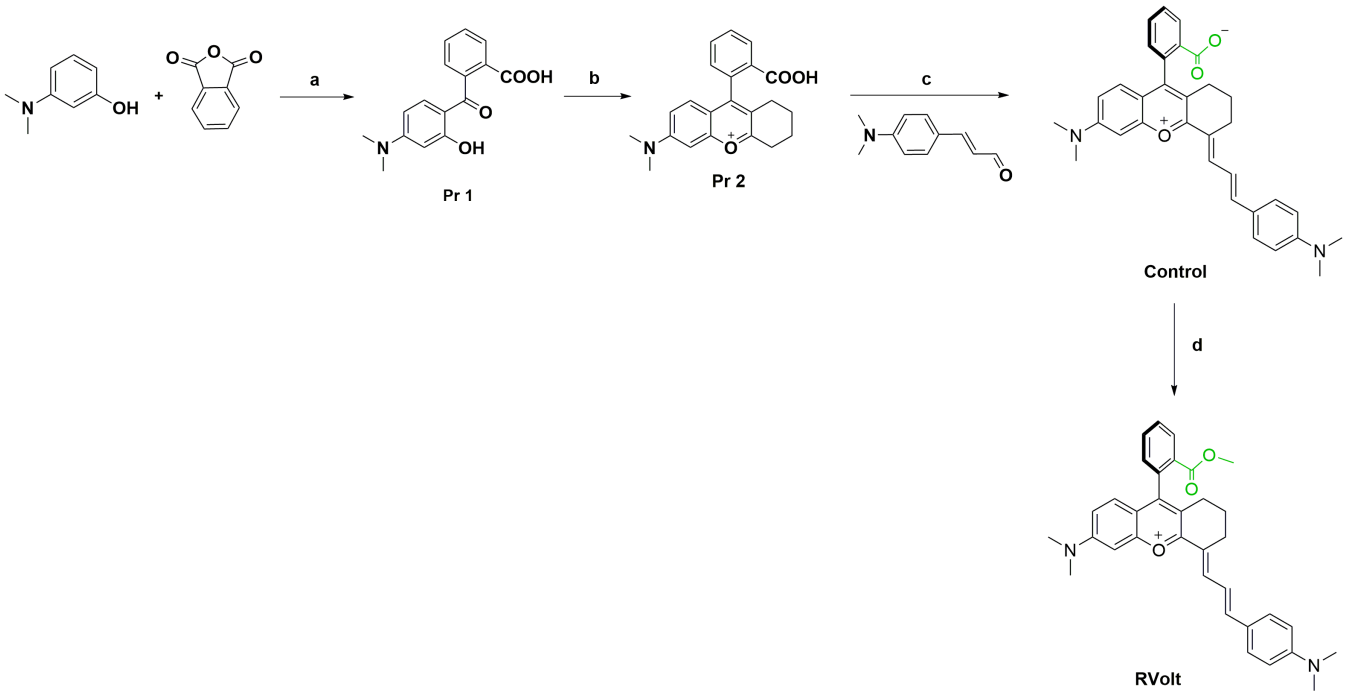
Figure S2. TD-DFT three-model diagram of the synthesized **RVolt** (above) and **Control** (below) probes at PBE0/6-31+G(d,p) level of theory. State energy difference (Δ) are taken for the ground (S_0) and first excited states (S_1). Arrows highlight the complementarity of the electron density difference distribution (green-purple structures), charge transfer index upon photoexcitation (in green and red) and natural transition orbital pairs (in blue and red). Parameters f and *w* are oscillator strength and NTO eigenvalues (the extent to which a given electronic excitation can be seen as a single excitation), respectively.

Table S1. Summary of charge-transfer (CT) indexes parameters upon photoexcitation for **Control** and **RVolt**.

Charge transfer results - Control					
			Electron goes from		
			X	Y	Z
CT charge (e)	0.74	Center (Ang)	3.29827	0.08548	-0.09196
CT distance (Ang	3.39	± spread (Ang	4.63889	1.73527	0.26733
CT dipole (Debye	11.97				
			to		
H index (Ang)	3.79		X	Y	Z
		Center (Ang)	-0.08325	-0.05940	-0.18165
t index (Ang)	-0.40	± spread (Ang	2.94190	0.89535	0.27187

Charge transfer results - Rvolt					
			Electron goes from		
			X	Y	Z
CT charge (e)	0.38	Center (Ang)	-7.10309	0.11830	0.09568
CT distance (Ang	6.77	± spread (Ang)	3.15268	1.21544	0.33485
CT dipole (Debye	12.41				
			to		
H index (Ang)	3.47		X	Y	Z
		Center (Ang)	-0.36350	-0.52650	0.05912
t index (Ang)	3.30	± spread (Ang)	3.78388	1.16678	0.77507

Compound synthesis and chemical characterization



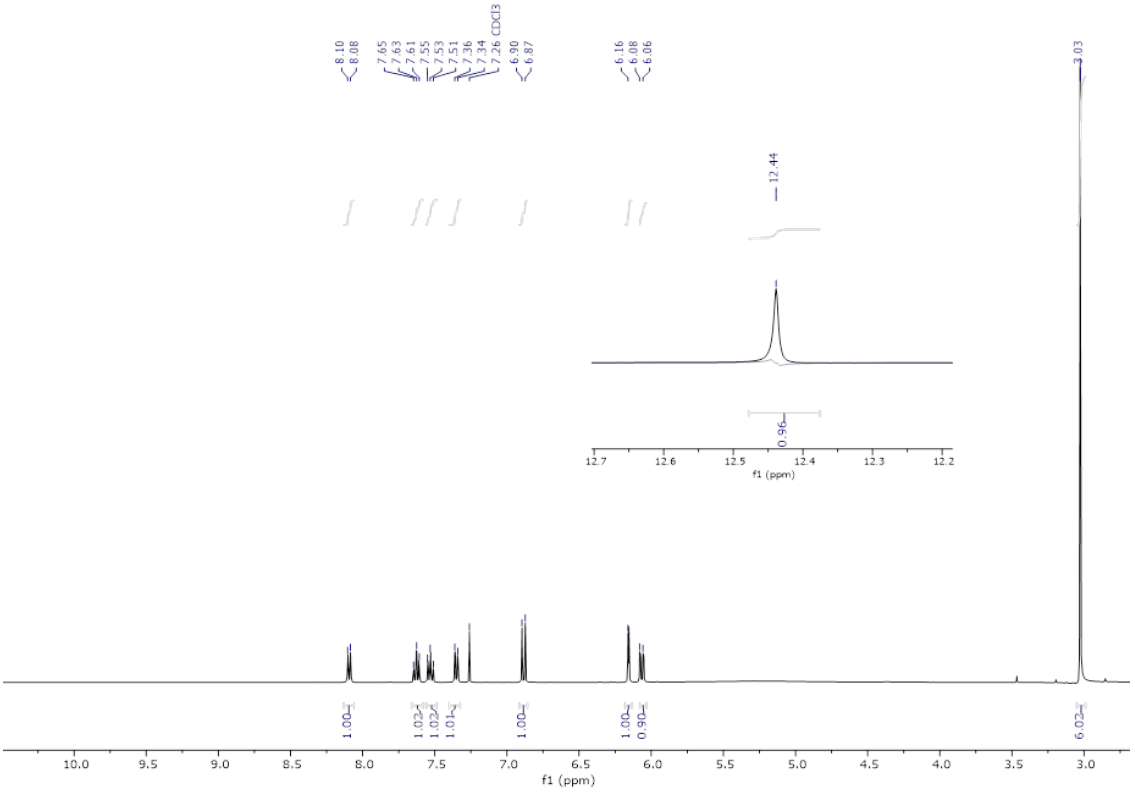
Scheme S1. Structures and synthetic methodology to obtain **RVolt** and **Control** probes: (a) Toluene, 120 °C, 24 h; then NaOH (30% aq), 90 °C, 12 h; (b) H_2SO_4, 60 °C, 2.5h, then $HClO_4$; (c) acetic anhydride, 60 °C, 3 h; pH 7.4 (d) MeOH, HCl, 100 °C, 5h, Ar.

General Probe Synthesis

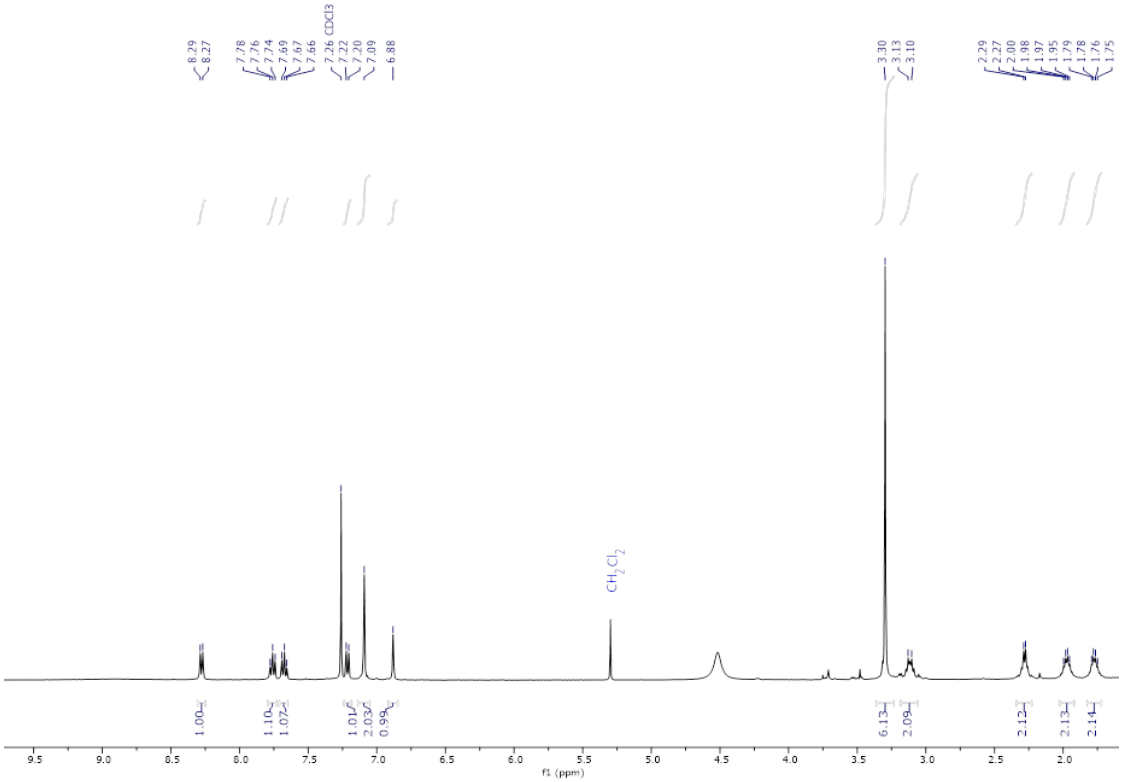
3-(dimethylamino) phenol (1,000 g, 3.5 mmol) and phthalic anhydride (1,079 g, 7 mmol) were mixed in 21 ml of Toluene was stirred at 120 ° C at reflux for 24 h, the toluene was evaporated under reduced pressure, 100 mL of NaOH (35% m / v) was added to the dry product, stirred at 90 ° C for 24 h. The mixture 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 (**Control**) corresponding to 55% and m.p. of 189 ° C was obtained. ^{1}H NMR (DMSO, 400MHz): δ 8.23 (d,1H, *J* = 8), 8.07 (d,1H, *J* = 12), 7.92 (t, 1H, *J* = 8), 7.79 (t, 1H, *J* = 8), 7.66 (d, 2H, *J* = 8), 7.43 (d, 1H, *J* = 8), 7.34 (s, 1H), 7.33 (d, 1H , *J* = 12), 7.19 (t, 1H, *J* = 8), 7.15 (d, 1H, *J* = 8), 6.89 (d, 2H, *J* = 12), 6.78 (d, 2H, *J* = 12), 3.26 (s, 6H), 3.05 (s, 6H), 2.89 (m, 2H), 2.38 (m, 2H),1.81 (m, 2H). ^{13}C NMR (DMSO, 400MHz) 164.5, 163.5, 158.9, 157.9, 153.6, 148.3, 142.9, 142.5, 135.9, 132.7, 132.0, 131.9, 131.5, 130.8, 129.7, 129.4, 127.6, 125.9, 123.7, 120.9, 117.6, 117.0, 113.2, 96.7, 40.8, 40.3, 27.5, 26.2, 22.0. FT-IR (cm^{-1}) COOH (3535.57), methylene groups (2923.57, 2858.12), C=O (1675.82), C-O-$C_{oxonium}$ (1084.64). HRMS [ESI^{+}] *m/z* for $C_{33}H_{33}N_2O_3$, calculated 505.25, found 505.2482.

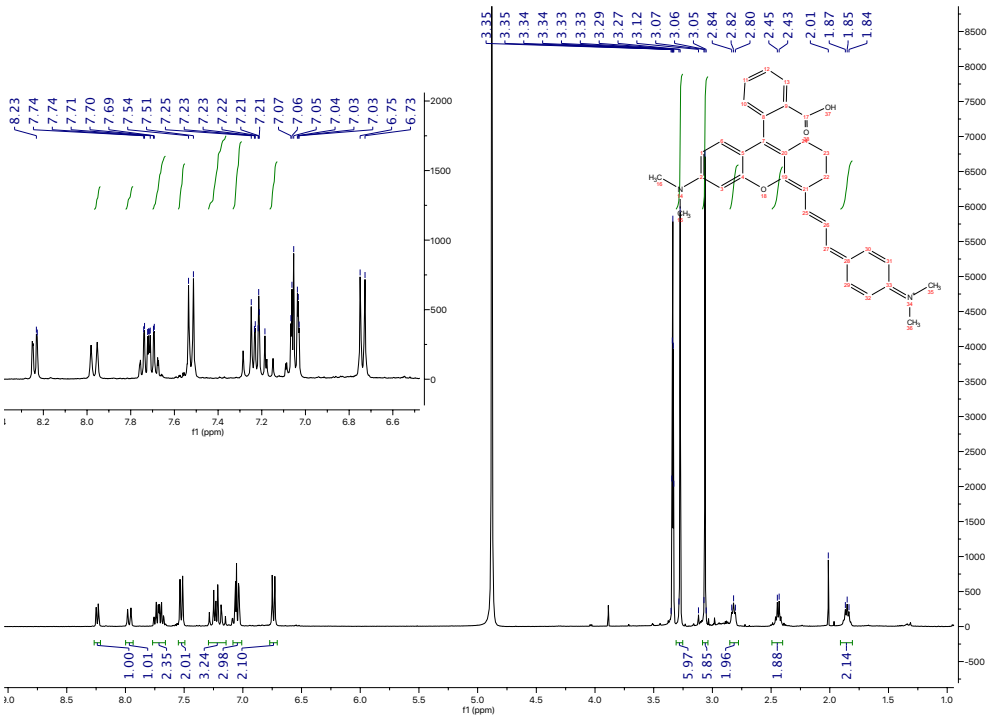
RVolt was obtained by mixing 0.1 g (0.4 mmol) of **Control** with 10 mL methanol, subsequently 3 mL of concentrated HCl were added. The reaction mixture was refluxed for five hours under Argon atmosphere. The solvent was evaporated and then the product was purified by silica gel preparative chromatography using Hexane/AcOEt = 90:10 as eluent to afford 0.530 g of **RVolt** as a blue solid (84%, 1.770 mmol). m.p. = 192 ° C and 90% yield. ^{1}H NMR (DMSO, 400MHz): δ 8.15 (d,1H, *J* = 9.08), 7.99 (d,1H, *J* = 10.4), 7.83 (t, 1H, *J* = 8), 7.72 (t, 1H, *J* = 8), 7.56 (d, 2H, *J* = 9.2), 7.36-7.20 (m, 3H), 7.13-7.07 (m, 2H), 6.80 (d, 1H , *J* = 9.2), 6.71 (d, 1H, *J* = 9.2), 3.60 (s, 3H), 3.19 (s, 6H), 2.98 (s, 6H), 2.82-2.73 (m, 2H), 2.30-2.12 (m, 2H), 1.73-1.63 (m, 2H). ^{13}C NMR (DMSO, 400MHz) 165.7, 162.9, 159.7, 157.3, 156.3, 152.4, 147.7, 141.7, 135.0, 134.0, 131.2, 130.6, 129.7, 129.0, 128.8, 124.4, 124.0, 122.3, 120.6, 117.1, 116.1, 112.6, 96.3, 53.0, 40.8, 26.3, 24.9, 20.8. FT-IR (cm^{-1}) COOH (3400.75), methylene groups (2856.65, 2249.65), C=O (1655.91), C-O-$C_{oxonium}$ (1149.38). HRMS [ESI^{+}] *m/z* for $C_{34}H_{35}N_2O_3$, calculated 519.26, found 519.2911.



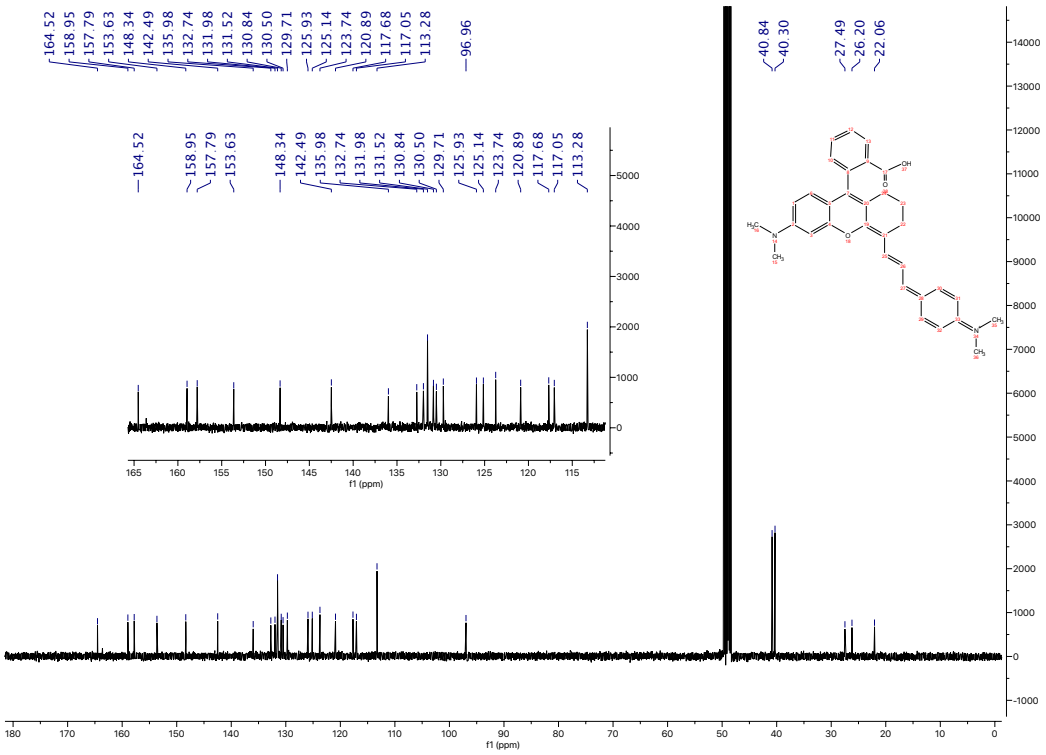
^{1}H NMR spectrum of **Pr1** in CDCl3-δ_6, 400 MHz.



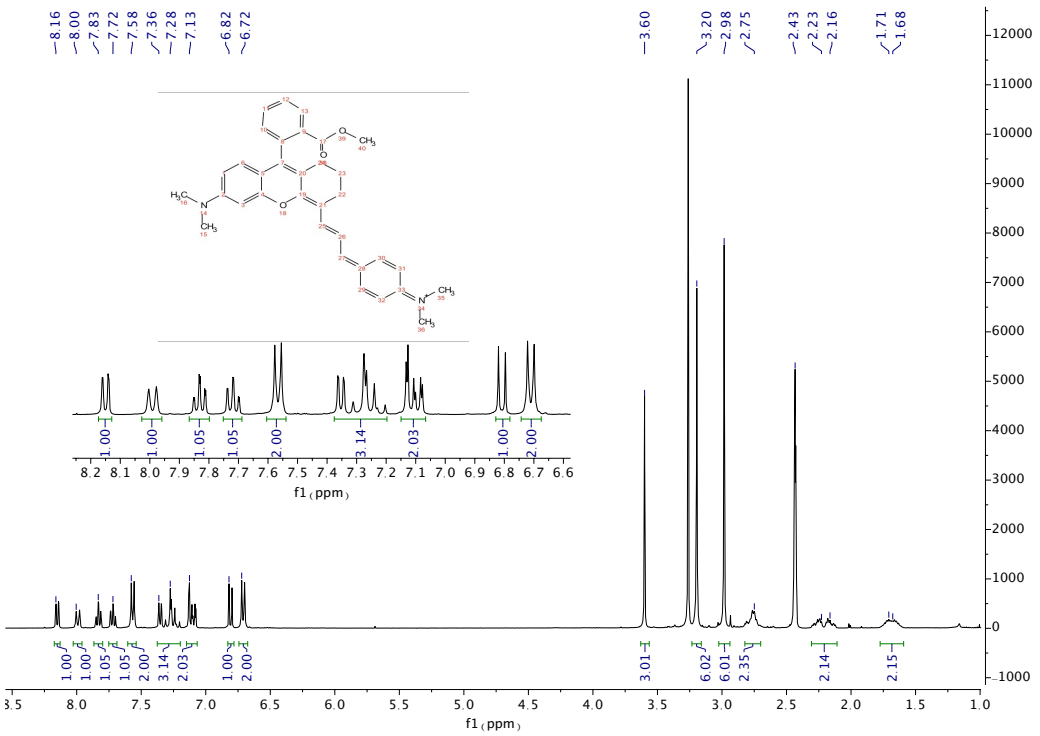
^{1}H NMR spectrum of **Pr2** in DMSO-δ_6, 400 MHz.



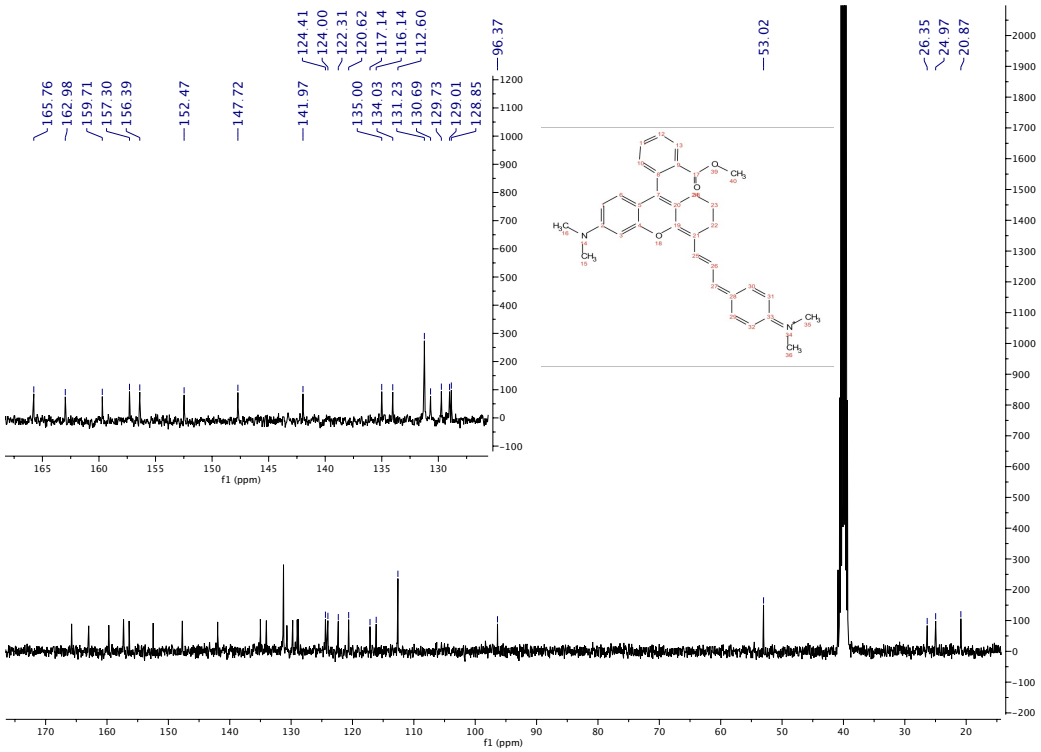
^{1}H NMR spectrum of **Control** in DMSO-δ_6, 400 MHz.



^{13}C NMR spectrum of **Control** in DMSO-δ_6, 400 MHz.

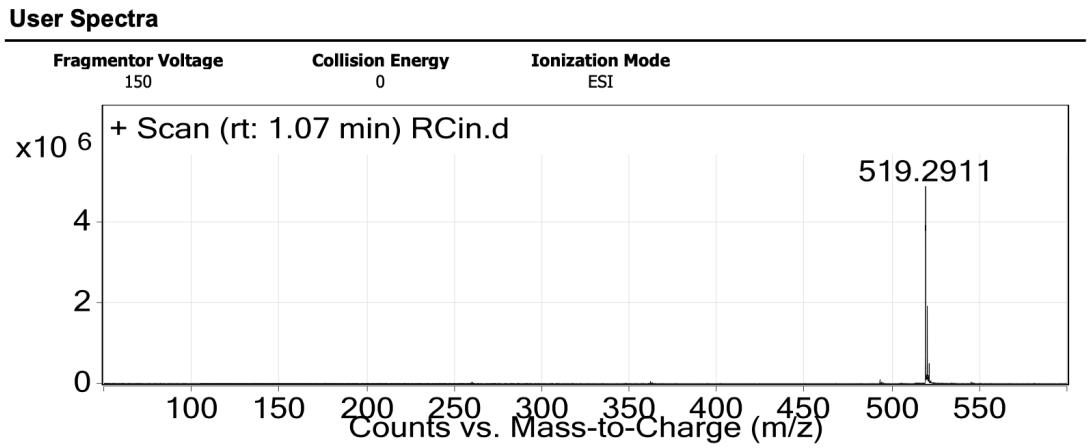


^{1}H NMR spectrum of **RVolt** in DMSO-δ_6, 400 MHz.



^{13}C NMR spectrum of **RVolt** in DMSO-δ_6, 400 MHz.

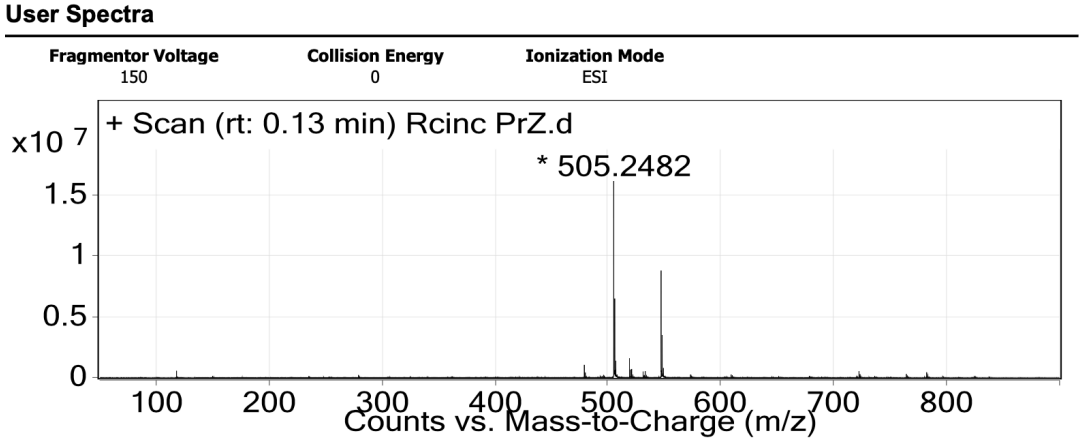
A)



Peak List

m/z	z	Abund
519.2911	1	4909842.63
519.4852		413323.83
520.2058		656300.76
520.3003	1	1920005.78
521.3109	1	495525.8

B)



Peak List

m/z	z	Abund
479.2324		1052164.84
505.2482	1	16474094.11
505.428		1078957.71
506.2509	1	6516568.04
507.2543	1	1364121.01
519.2635		1594840.28
547.2583	1	8820207.24
548.2614	1	3553879.94

Figure S3. High-Resolution Mass Spectrometry (ESI-TOF technique through direct injection) for (A) **RVolt** and (B) **RVolt** in esterase enriched buffer showing the main peak at 505.2482 corresponding to hydrolized-**RVolt** (**Control**).

Table S2. Catalán solvent parameters {SA, SB, SP, SdP}*,[4] for the **RVolt** probe.

Solvent	SP	SdP	SA	SB	Viscosity (cP)	Em (RVolt)
Cyclohexane	0.616	0	0	0.056	0.894	22400
Dioxane	0.737	0.312	0	0.444	1.177	18435
Toluene	0.782	0.284	0	0.128	0.56	23900
Diethyl eter	0.617	0.385	0	0.562	0.603	23650
MTBE[a]	0.622	0.422	0	0.567	0.224	24038
Chloroform	0.783	0.614	0.047	0.071	0.36	24331
Buthyl acetate	0.674	0.535	0	0.525	0.537	23550
Ethyl acetate	0.656	0.603	0	0.542	0.685	23640
Tetrahydrofuran	0.714	0.634	0	0.591	0.423	23710
Dichloromethane	0.761	0.769	0.04	0.178	0.793	24250
Octanol	0.713	0.454	0.299	0.923	0.456	23850
i-Propanol	0.633	0.808	0.283	0.83	0.413	23400
Acetone	0.651	0.907	0	0.475	7.288	19840
Ethanol	0.633	0.783	0.4	0.658	5.474	19300
Methanol	0.608	0.904	0.605	0.545	3.619	23100
Acetonitrile	0.645	0.974	0.044	0.286	2.544	22900
DMF[b]	0.759	0.977	0.031	0.613	2.038	23050
Ethyleneglycol	0.777	0.91	0.717	0.534	0.306	23360
DMSO[c]	0.83	1	0.072	0.647	1.074	22730
Water	0.681	0.997	1.062	0.025	0.544	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 **RVolt**. The {SA, SB, SP, SdP} parameters for each solvent are taken from reference 4. 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 S2 (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)	**0.8317**	**error**
Y0	30610.3321	2645.4688
SP	-8789.5643	3054.5693
SdP	-545.0894	1012.2585
SA	-4459.3479	1002.9566
SB	-1487.5398	1010.3685

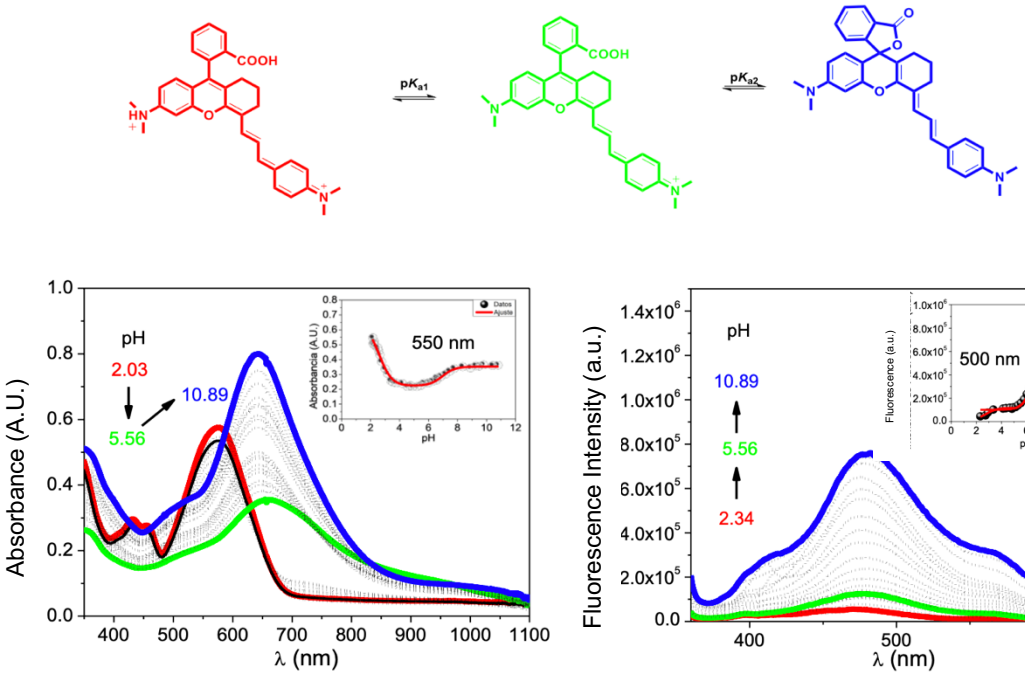
The correlation data analysis *including* the viscosity parameter in the multilinear regression analysis is:

R (multilinear)	**0.7893**	**error**
Y0	28544.8321	4431.3245
SP	-1834.8329	6276.0445
SdP	1532.7329	1523.7832
SA	-6506.4314	1732.8322
SB	101.4292	1374.7532

Similar results were found for the free **Control** probe (data not presented).

Determination of pK_a and partition coefficients (log*P*):

The different **Control** probe species formed during the acid-base titration using spectrophotometric methods and their corresponding pK_a vales is presented as follows:



Then, pK_a values were obtained by the following equations:

$$\mathbf{Abs} = \left(A_{in} + A_0 * 10^{(pK_a - pH)}\right)/\left(1 + 10^{(pK_a - pH)}\right)$$ for UV-Vis absorption spectra

$$\mathbf{FI} = \left(Fl_{in} + Fl_0 * 10^{(pK_a - pH)}\right)/\left(1 + 10^{(pK_a - pH)}\right)$$ for Fluorescence spectra

Then, the obtained pK_{a1} and value pK_{a2} are **2.78 ± 0.18** and **6.78 ± 0.05**.

Finally, Log *P* values were measured via octanol partitioning by a modification of the shake-flask method and as previously described.[5] An aliquot of 100 ml of 300 mM of the probe in Tris buffer (10 mM, pH 7.4) and 100 ml 1-octanol (Aldrich) were added to a 0.5 ml microtube. Buffer was employed in order to measure log *P* of the probes at physiogloical pH where **Control** can be in the *spiro*lactone form. The tubes were vortexed for 1 min and centrifuged; 25 ml of each layer was removed and diluted in 100 ml 3:1 methanol:Tris or methanol:octanol for a final composition of 3:1:1 methanol:octanol:Tris. The aqueous layer was diluted an additional 4-fold. Three dilutions were prepared per layer, 100 ml of each dilution was pipetted into a 96 well plate, and the absorbance read at 488 nm and 625 nm wavelengths. The mean A_{500} of three dilutions was calculated for each layer. The log (A_{500} of the organic layer/A_{500} of the aqueous layer) yielded log *P*. All absorbance measurements used were within the linear range of the instrument.

Acknowledgements

Financial support by PAPIIT-UNAM IA200320 is gratefully acknowledged. C.H-J. acknowledges Conacyt PhD. grant 771165. We also acknowledge the assistance of Ruth Rincón Heredia (PhD. Unidad de Imagenología del IFC-UNAM) in imaging microscopy and Salvador Ramírez Jiménez (Biol. IIBO, UNAM) in tissue culture and Everardo Tapia Mendoza (MS), Elizabeth Huerta Salazar, M.Sc. and Beatriz Quiróz García, PhD. (NMR) Instituto de Química, UNAM.

Full references in the main text

Full reference number 4:

Milica Momcilovic, Anthony Jones, Sean T. Bailey, Christopher M. Waldmann, Rui Li, Jason T. Lee, Gihad Abdelhady, Adrian Gomez, Travis Holloway, Ernst Schmid, David Stout, Michael C. Fishbein, Linsey Stiles, Deepa V. Dabir, Steven M. Dubinett, Heather Christofk, Orian Shirihai, Carla M. Koehler, Saman Sadeghi & David B. Shackelford, In vivo imaging of mitochondrial membrane potential in non-small-cell lung cancer. *Nature,* 2019, **575**, 380–384.

Full reference number 15:

J. P. Norman, S. W. Perry, H. M. Reynolds, M. Kiebala, K. L. De Mesy Bentley, M. Trejo, D. J. Volsky, S. B. Maggirwar, S. Dewhurst, E. Masliah, H. A. Gelbard, *PLoS One*, 2008 **3(11)**: e3731.

Full reference number 16:

D. Zorova, V. A. Popkov, E. Y. Plotnikov, D. N. Silachev, I. B. Pevzner, S. S. Jankauskas, V. A. Babenko,S. D. Zorov, A. V. Balakireva, M. Juhaszova, S. J. Sollott, D. B. Zorov, *Anal. Biochem.* 2018, **552**, 50.

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**, 227-61; b) J. Tomasi, B. Mennucci, R. Cammi, *Chem. Rev.* 2005, **105**, 2999-3094.

[2] M. J. Frisch, et.al. 2. Gaussian 09, Revision **D.01**, Gaussian, Inc., Wallingford CT, 2009.

[3] R. L. Martin, J. *Chem. Phys.* 2003, **118**, 4775-81.

[4] 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.

[5]. Rothbard, J.B., Jessop, T.C., Lewis, R.S., Murray, B.A., and Wender, P.A. *J. Am. Chem. Soc.* 2004, **126**, 9506–9507.