Electronic Supplementary Material (ESI) for Sensors & Diagnostics.

Electronic Supporting Information for

Unveiling Cellular Vitality: Peptide Fluorescent Probes Illuminate Mitochondrial Dynamics and ROS Activity

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Table of Contents:

Acknowledgements

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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. 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. The laser injury was performed through an Erbium:YAG laser (Asclepion) with a frequency of 5 Hz and 7 J/cm2 power density with the corresponding main text reference provided (#22. R. Richardson, K. Slanchev, C. Kraus, P. Knyphausen, S. Eming, M. Hammerschmidt, J. Invest. Dermatol. 2013, 133, 1655.), the Erbium:YAG laser absorption spectrum is shown in Fig. S1.

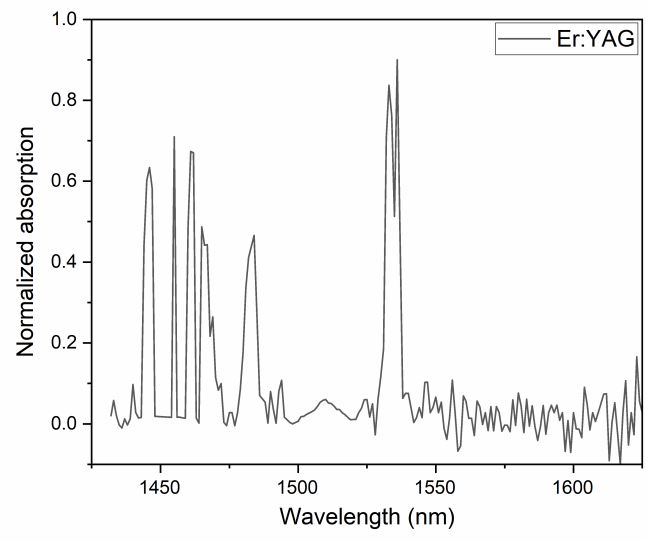


Fig. S1. Normalized absorption spectrum of low dopped Erbium:YAG laser (Asclepion), the specified laser fluorescence is 2950 nm.

Cell Culture, Confocal Microscopy and IC_{50} determination. HeLa cells 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 **DeLiCy5** or **DeLiC-343** concentration of 5 μM was 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 µM **DeLiCy5** or **DeLiC-343** probes for 30 minutes. For experiments with MitoLite™ Blue FX490, 50 nM MitoLite™ Blue (AAT Bioquest) was added 10 minutes before **DeLiCy5** or **DeLiC-343**. 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 **DeLiCy5** or **DeLiC-343** 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 150 nM CCCP (after 5 min. For IC_{50} determination, HeLa cells were treated with **DeLiCy5** or **DeLiC-343**, 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 **DeLiCy5** or **DeLiC-343** 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.

Preparation of ROS-Singlet oxygen. Singlet oxygen ($^1O_2$) was prepared as follows: $^1O_2$ concentration was determined by the reaction: $NaClO + H_2O_2 \rightarrow NaCl + {}^1O_2 + H_2O$. The following concentrations were mixed: NaClO = [14%] (1 mL) with H_2O_2 = [30%] (2 mL) with sodium hypochlorite as the limiting reagent. So, 1 mL NaClO (14%) is equiv. to 0.14 g of pure NaClO, giving 0.6266 M of NaClO. Then, a 1 mL in 40 mL dilution was performed to obtain a 1.6 µM NaClO solution. After that, a linear regression analysis was performed using the commercially available Singlet Oxygen Sensor Green®, as described in literature (Singlet Oxygen Production in Water: Aggregation and Charge-Transfer Effects. The Journal of Physical Chemistry, 1996, 100(16), 6555–6560).

Compound synthesis and chemical characterization

General procedure for the peptide manual synthesis

Resin preparation. To a purification column was added Rink Amid-MBHA resin (125 mg) and DCM (DCM= methylene chloride, 1.0 mL) for resin swelling. For washing the resin, DMF (DMF= N, N-Dimethylformamide, 0.75 mL) was added.

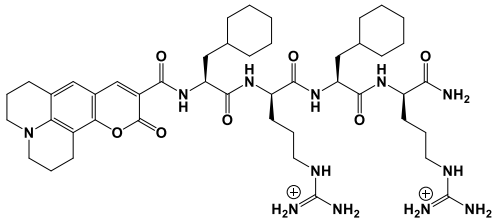
Deprotection/Coupling cycle. For the first and the second Fmoc removal step piperidine/DMF mixture (20 % v/v, 0.75 mL) was added to the resin. The resin was washed five times with DMF (1.0 mL). The amino acids were coupled adding to the resin the solution of the amino acid (0.188 mmol Fmoc-Xxx-OH; 71 mg, 0.188 mmol HBTU= O-(benzotriazol-l-yl)-N,N,N',N'- tetramethyl-uronium hexafluorophosphate); 0.75 mL DMF). DIPEA (66 µL, 0.378 µmol, Sigma-Aldrich, DIPEA = N,Ndiisopropylethylamine) was added as the base during the reaction. The cycle was repeated coupling the amino acids according to the sequence of the peptide.

Removal of the N-terminal Fmoc group and drying of the peptide resin. For the first and the second Fmoc removal step piperidine/DMF mixture (20 % v/v, 0.75 mL) was added to the resin. The resin was washed six times with DMF (1.0 mL) and four times with DCM (1.0 mL). Finally, the resin was air-dried.

*Synthesis of **DeLiCy5** and **DeLiC-343***. These fluorescent peptide conjugates were manually synthesized in solid-phase using Rink Amid-MBHA resin (0.3-1.0 mmol/g, 100-200 mesh, ChemPep) as reported in literature.[i] Purification of the coupled peptide was performed using a Waters e2695 HPLC system equipped with a Waters 2998 PDA UV/Vis detector on a Symmetry C18 Prep column. Electrospray ionization mass spectrometry was obtained by using an Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS equipment. **Coumarin-343** (Cat. 393029, Sigma-Aldrich, St. Louis MO) and **Cyanine5** carboxylic acid (Cat. 63090, Lumiprobe, Maryland, USA) were used as received.

25 µmol of NH_2-Fx-r-Fx-r-Fx-r on resin was reacted with HBTU (4 eq), and DIPEA (8 eq) in 1 mL DMF. The reaction was stirred for 30 minutes and reacted with Coumarin-343 (4 eq) or Cyanine5 (4 eq) for 12 hours. The peptide was washed (2 x DMF/MeOH/DCM), cleaved from resin using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5) and precipitated in ether at -20°C for 1 hour. The precipitate was purified by HPLC and lyophilized.

The **DeLiCy5** and **DeLiC-343** peptides were identified by high-resolution ESI mass spectrometry. **DeLiC-343** HRMS ESI+(m/z): calcd. 452.28, found 452.2868 for $[M]^{2+}$. **DeLiCy5** HRMS ESI+(m/z): calcd. 353.25, found 353.2488 for $[M]^{4+}$.



Chemical Formula: $C_{46}H_{72}N_{12}O_7^{2+}$
Exact Mass: 904.56
m/z: 452.28 (100.0%),
452.78 (49.8%), 453.29 (12.1%), 452.78 (4.4%), 453.28 (2.2%), 453.28 (1.4%), 453.79 (1.1%)

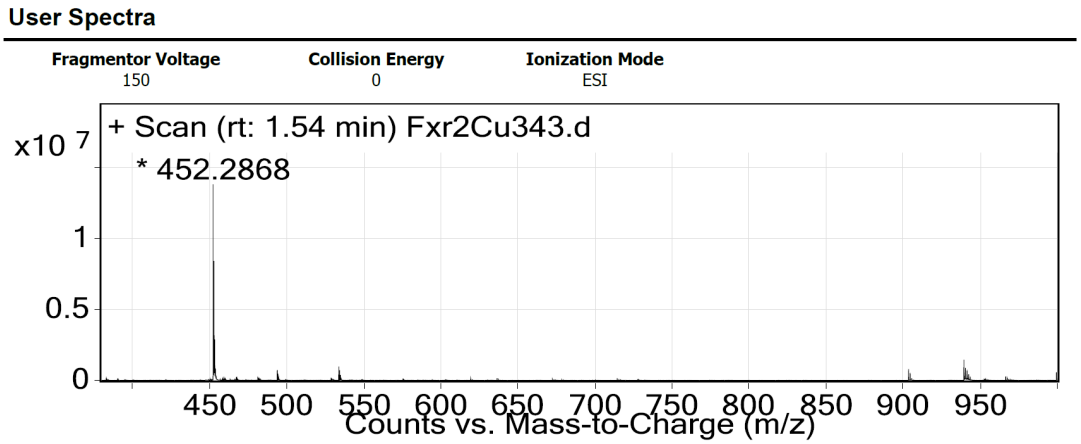


Fig. S1. HRMS for **DeLiC-343**: ESI+(m/z): calcd. 452.28, found 452.2868 for $[M]^{2+}$.

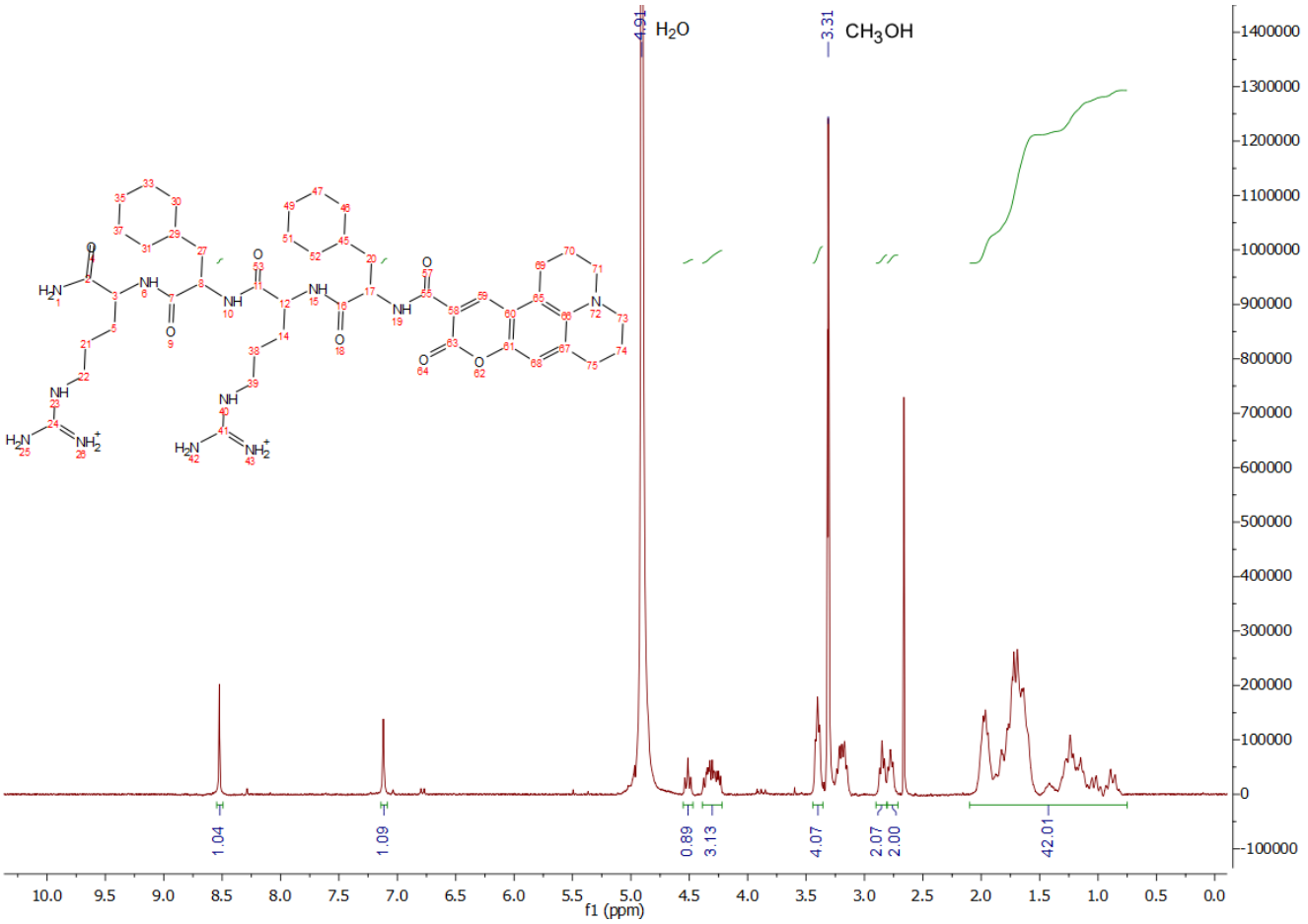
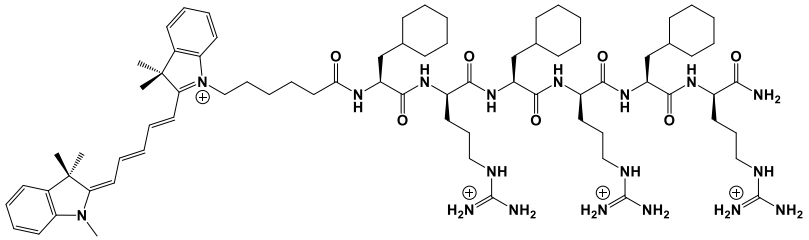


Fig. S2. ^{1}H NMR spectrum for **DeLiC-343** in deuterated methanol (CD_3OD).



Chemical Formula: $C_{77}H_{124}N_{18}O_7^{4+}$
Exact Mass: 1412.99
m/z: 353.25 (100.0%),
353.50 (83.3%), 353.75 (34.2%), 354.00 (8.4%),
353.50 (6.6%), 353.75 (5.5%), 354.00 (2.3%)

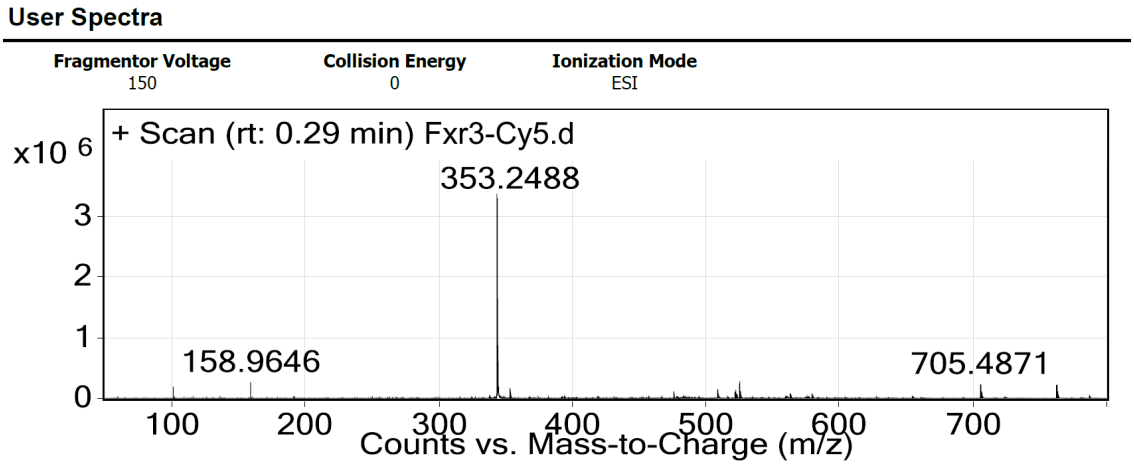


Fig. S3. HRMS for **DeLiCy5**: ESI+(m/z): calcd. 353.25, found 353.2488 for $[M]^{4+}$.

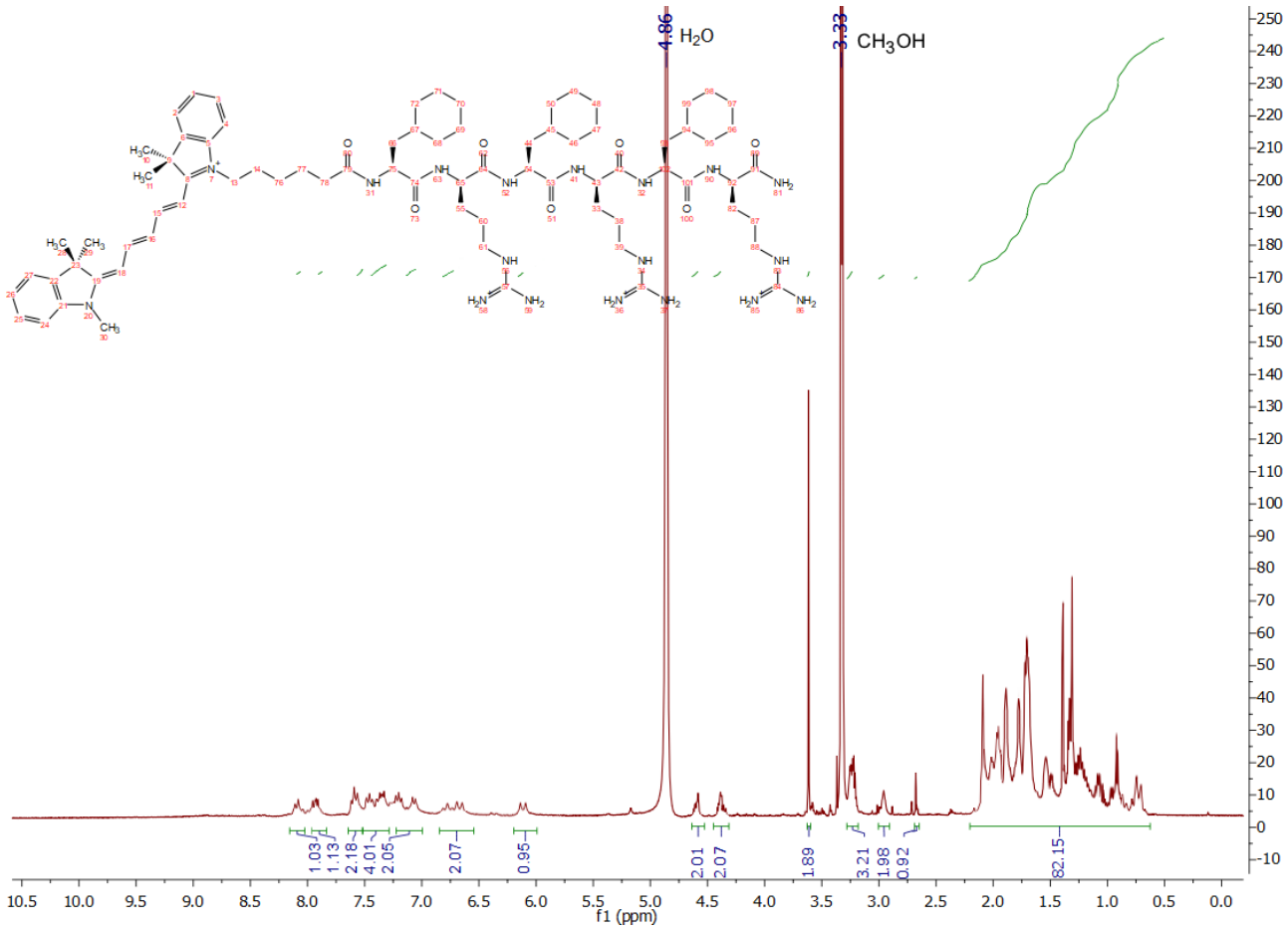
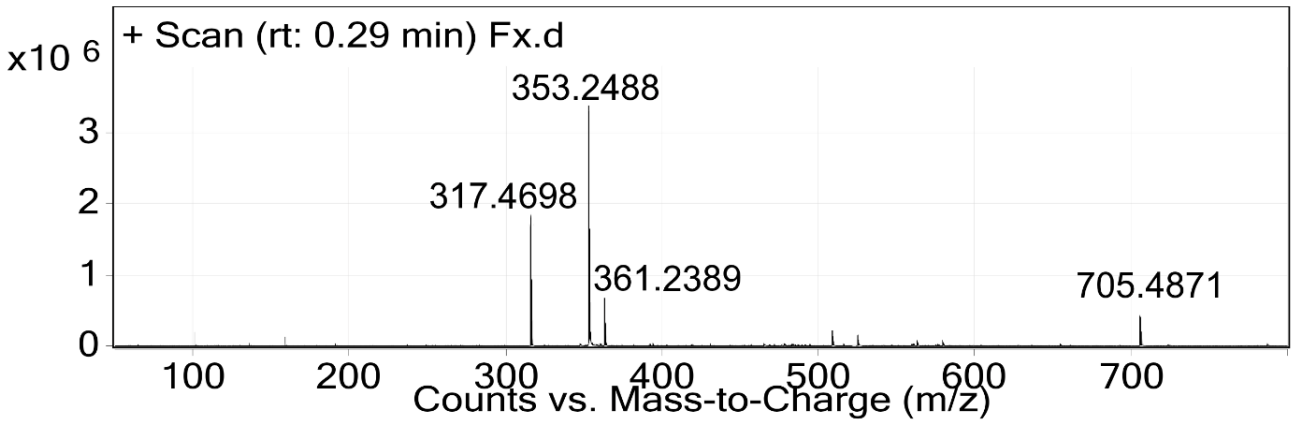
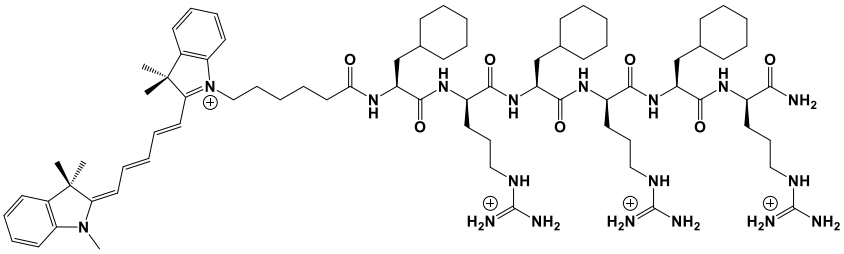
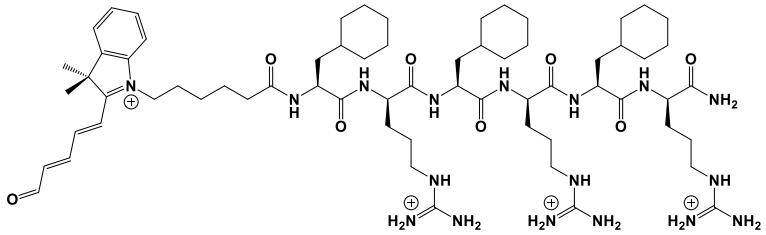


Fig. S4. ^{1}H NMR spectrum for **DeLiCy5** in deuterated methanol (CD_3OD).

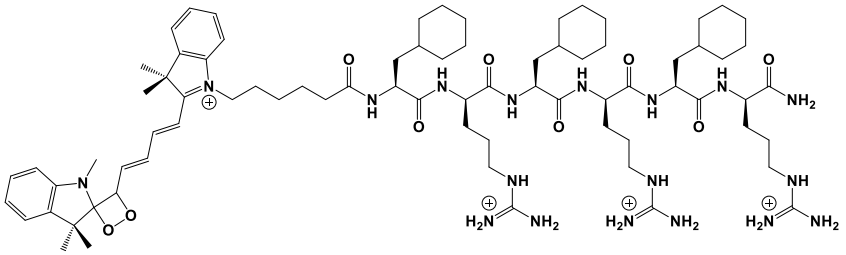




Chemical Formula: $C_{77}H_{124}N_{18}O_7^{4+}$
Exact Mass: 1412.99
m/z: 353.25 (100.0%),
353.50 (83.3%), 353.75 (34.2%), 354.00 (8.4%), 353.50 (6.6%), 353.75 (5.5%), 354.00 (2.3%).



Chemical Formula: $C_{66}H_{111}N_{17}O_8^{4+}$
Exact Mass: 1269.88
m/z: 317.47 (100.0%),
317.72 (71.4%), 317.97 (25.1%), 317.72 (5.9%), 318.22 (5.8%), 317.97 (4.5%), 317.97 (1.6%).



Chemical Formula: $C_{77}H_{124}N_{18}O_9^{4+}$
Exact Mass: 1444.98
m/z: 361.24 (100.0%),
361.50 (83.3%), 361.75 (34.2%), 362.00 (8.4%), 361.49 (6.6%), 361.74 (5.5%), 362.00 (2.3%).

Fig. S5. HRMS ESI[+] spectrum of the **DeLiCy5** after one equivalent $^1O_2$ addition generated by equimolar hypochlorite (HClO) and hydrogen peroxide (H_2O_2) reaction under 300W Xe lamp irradiation for 25 min. Two photooxidized species were mainly observed, the aldehyde fragment [317.4698 m/z] and dioxetane [361.2389 m/z]. For detailed description of $^1O_2$ preparation procedure, please see the '*Preparation of ROS-Singlet oxygen*' section in page S3.

Zebrafish lines and maintenance.

The experiments and handling of zebrafish were approved by the Committee for Laboratory Animal of the Universidad Nacional Autónoma de México (UNAM), under the CICUAL-Protocol number: FLC40- 14. (CICUAL: "Comité Institucional para el Cuidado y Uso de los Animales de Laboratorio del Instituto de Fisiología Celular, Universidad Nacional Autonoma de Mexico").

Zebrafish from AB strain were maintained in aquatic habitats recirculation system at 28°C with a dark/light period 14-10hr respectively. Embryos were obtained from natural mating, all the embryonic stages were determined according to (Kimmel et al. 1995).[S1] All embryos were treated with 0.2 mM 1-phenyl-2-thio-urea (Sigma Aldrich) to prevent pigmentation and permit posterior imaging.

Zebrafish imaging.

Embryos were stained with **DeLiCy5** or **DeLiC-343** for 30minutes, 4 hours or overnight with the indicated concentrations of the compound, embryos were washed in zebrafish water 3 times for 5-10minutes. All embryos were anesthetized with tricaine 4.2% (Sigma), some embryos were stimulated with $^1O_2$ for 1 or 2 minutes at the indicated concentrations. Immediately the embryos mounted in 1% low melting point agarose (National Diagnostics), images were acquired from living zebrafish embryos with stereomicroscope Nikon SMZ1500 or confocal microscope Zeiss LSM800.

Photoinduced singlet oxygen determination. The presence of singlet oxygen in the solutions was determined by its emission band at 1270 nm with natural bandwidth of 18 nm. A control air saturated solution (with 0.5 optical density) of Erythrosin B in anhydrous ethanol was used. Then, the single emission peaks at 1270 nm were recorded with a 450 W Xe lamp using a NIR photomultiplier. The emission spectra were measured in a FLS980 Fluorescence Spectrometer and measured in quartz cuvettes of 10 mm path-length. Higher diffraction orders were filtered by long wave-pass filters in the FLS980. Erythrosine B at 530 nm with excitation and emission bandwidths of 15 nm. The $^1O_2$ emission spectra is shown below, Fig. S4.

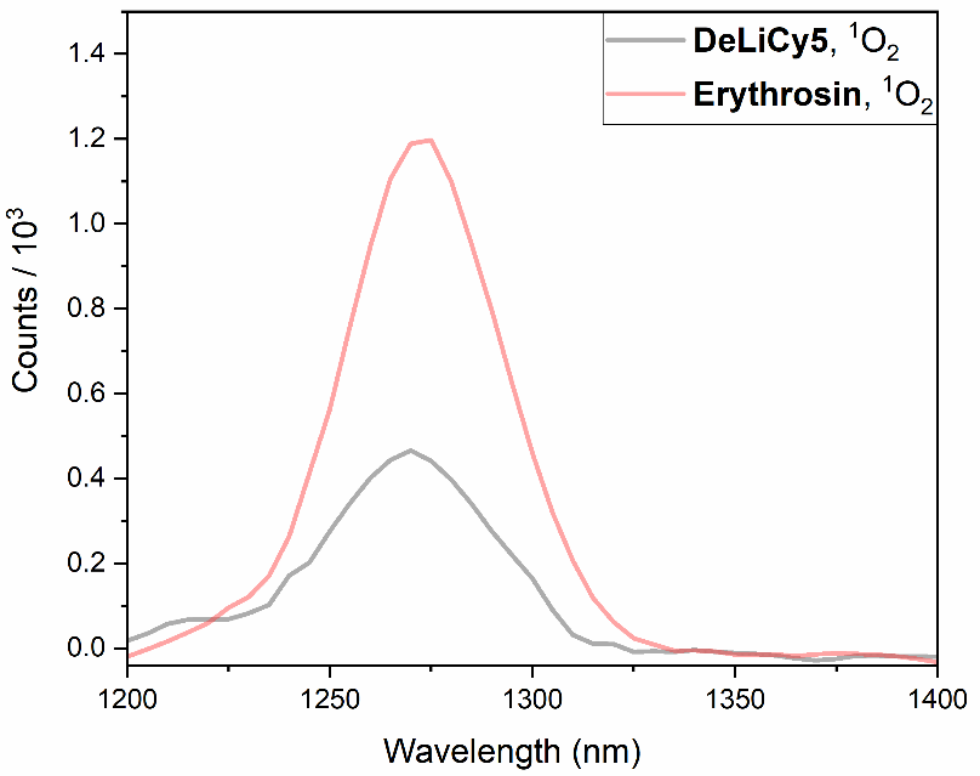


Fig. S6. Emission spectrum of $^1O_2$ in Erythrosin B (a standard photosensitizer) under $\lambda_{exc} = 525$ nm and with **DeLiCy5** (grey spectrum) to estimate the $^1O_2$ consumption. The integration time was 5 s/step and step of 5 nm. The estimated quantum yield of singlet oxygen formation (Φ_Δ) of erythrosine was calculated with the 1270 nm intensity as 0.58, compared to previously reported Φ_Δ: [DeRosa, M. C.; Crutchley, R. J. Photosensitized singlet oxygen and its applications. *Coord. Chem. Rev.* **2002**, *233*, 351-371.]

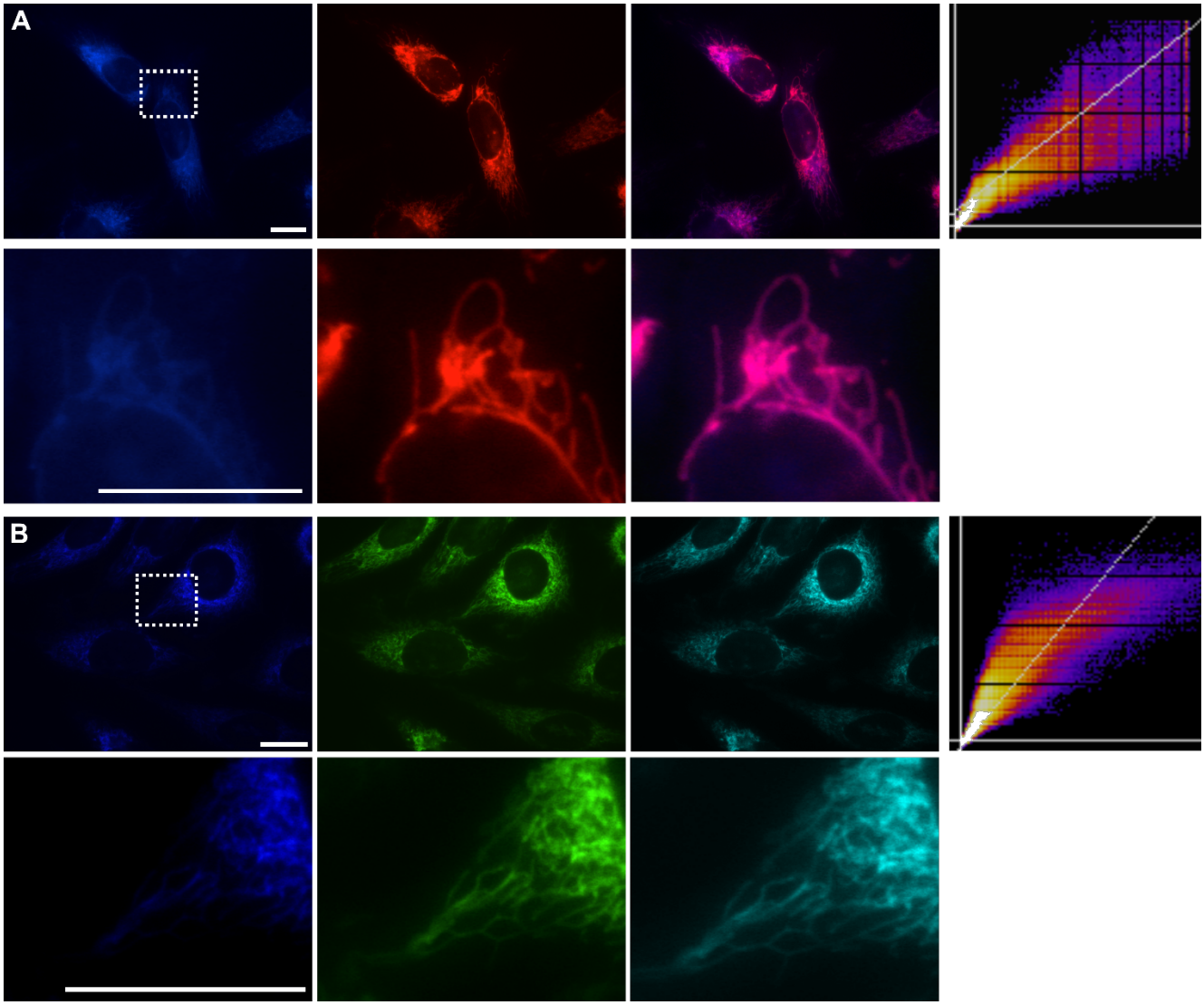


Fig. S7. Colocalization images of (A) **DeLiCy5** (Red-channel, λ_{exc} = 647 nm, λ_{em} = 700 nm) and (B) **DeLiC-343** (Green channel, λ_{exc} = 488 nm, λ_{em} = 500 nm) of live HeLa cells using MitoLite™ Blue (blue channel, λ_{exc} = 410 nm, λ_{em} = 440 nm) mitochondrial localizer. Estimated Pearson's coefficient from Scatter Plot analysis is 0.95 for **DeLiCy5** and 0.93 for **DeLiC-343**. To avoid cell autofluorescence, laser powers were maintained at 0.05 mW (0.2% from a 25 mW laser) and untreated cells were first recorded in order to subtract any native emission signal.

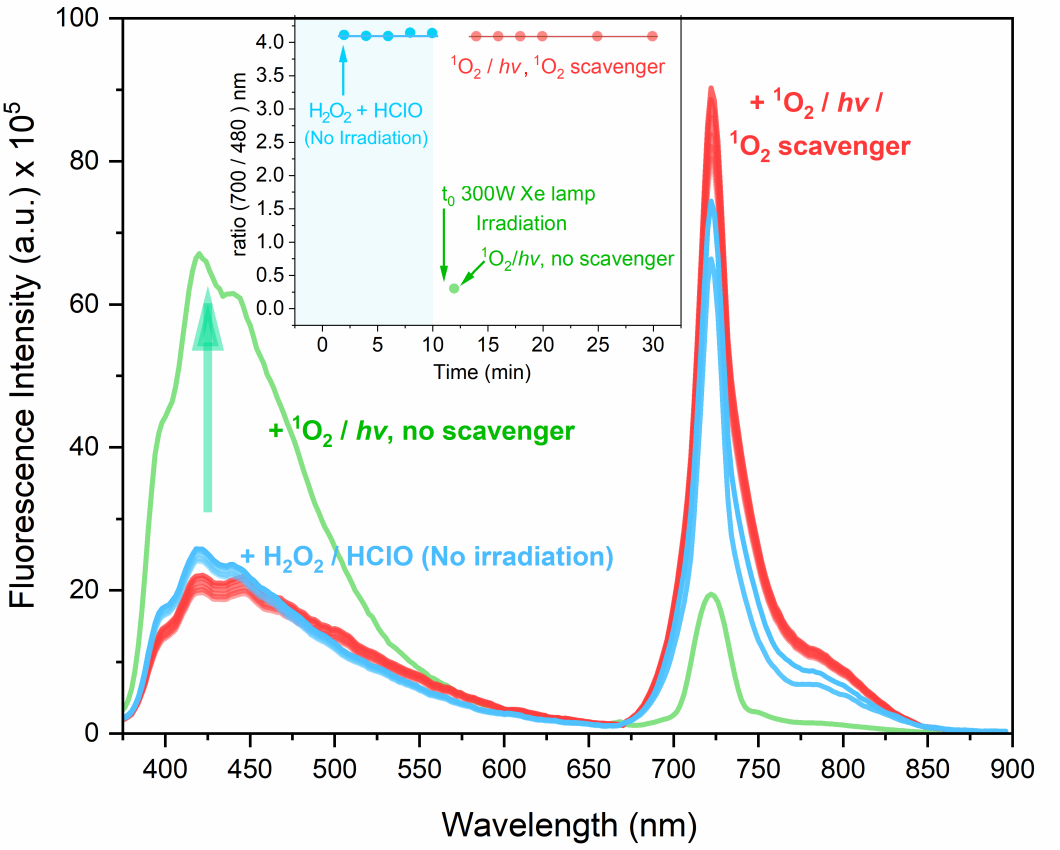


Fig. S8. Time-course fluorescence spectra of 16 mM **DeLiCy5** upon *in situ* addition of 1.6 mM singlet oxygen (H_2O_2 30%, and HClO 14%) during 10 minutes before (blue spectra) and after (green spectrum) starting the 300W Xe lamp light exposure in the absence of NaN_3, a selective $^1O_2$ scavenger. The fluorescence spectra in red colour represent 16 mM **DeLiCy5** with 1 equiv. NaN_3 and under light exposure. For detailed description of $^1O_2$ preparation procedure, please see the '*Preparation of ROS-Singlet oxygen*' section in page S3.

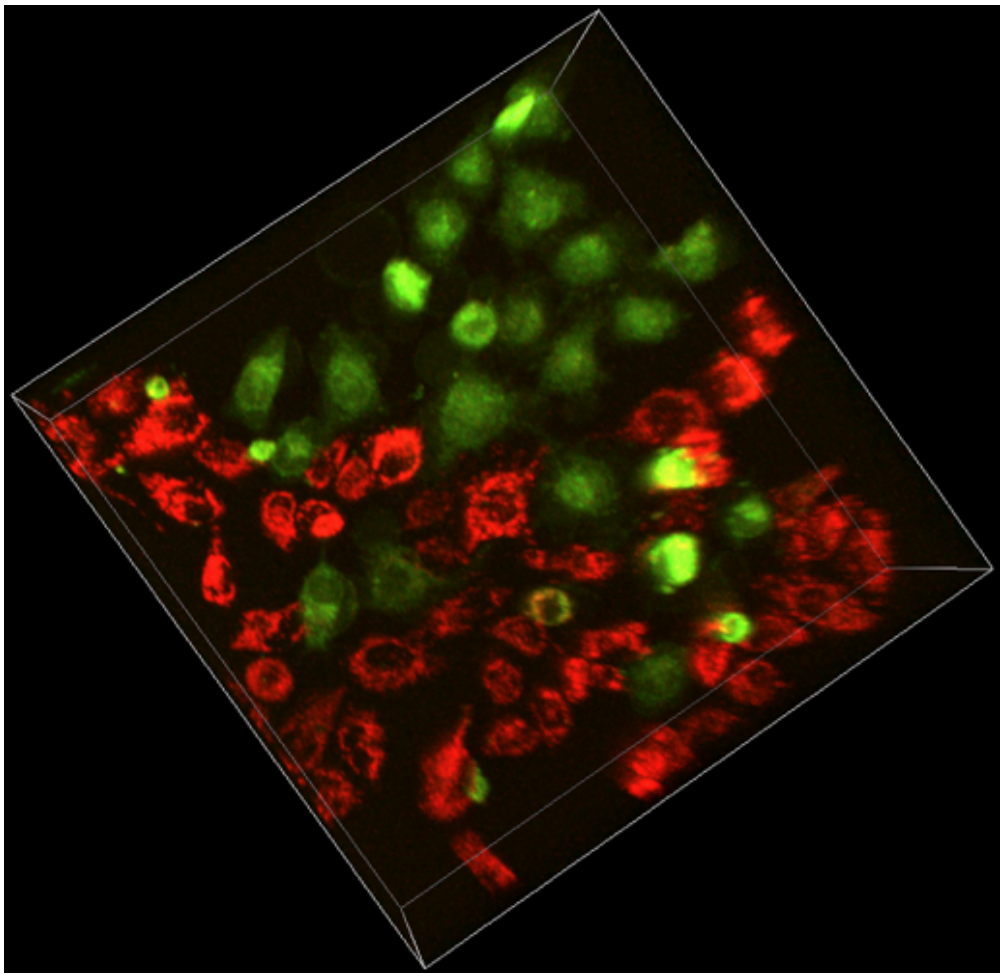


Fig. S9. Dual-channel confocal z-scan imaging of **DeLiCy5** in HeLa cells after 36 hours ALA-photosensitization, showing live (red cells) *vs.* dead (green) cells.

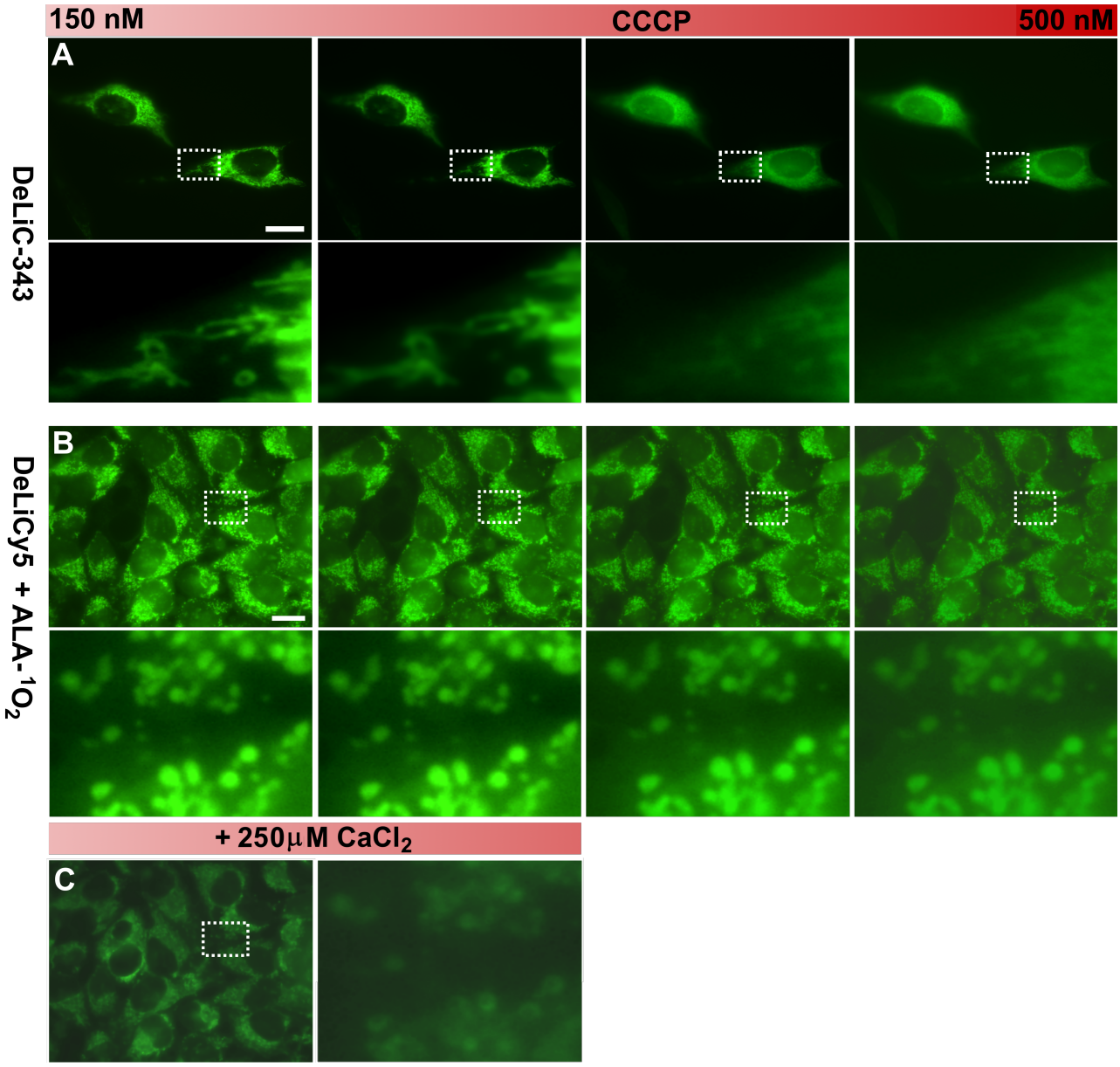


Fig. S10. Time course monitoring of CCCP depolarization changes in HeLa cells detected in the green confocal channel (λ_{exc} = 488 nm) stained with (A) **DeLiC-343** showing a Nernstian behavior or dye release and, (B) **DeLiCy5** in HeLa cells previously subjected to ALA-induced $^1O_2$ stimuli. Then, images were recorded after each 5 min with corresponding CCCP additions from 150 nM to 500 nM. (C) Effect of mPTP opening for HeLa cells before (left-panel) and after (right-panel) 5 min treatment with 250 mM $CaCl_2$. Scale bars represent 20 μm.

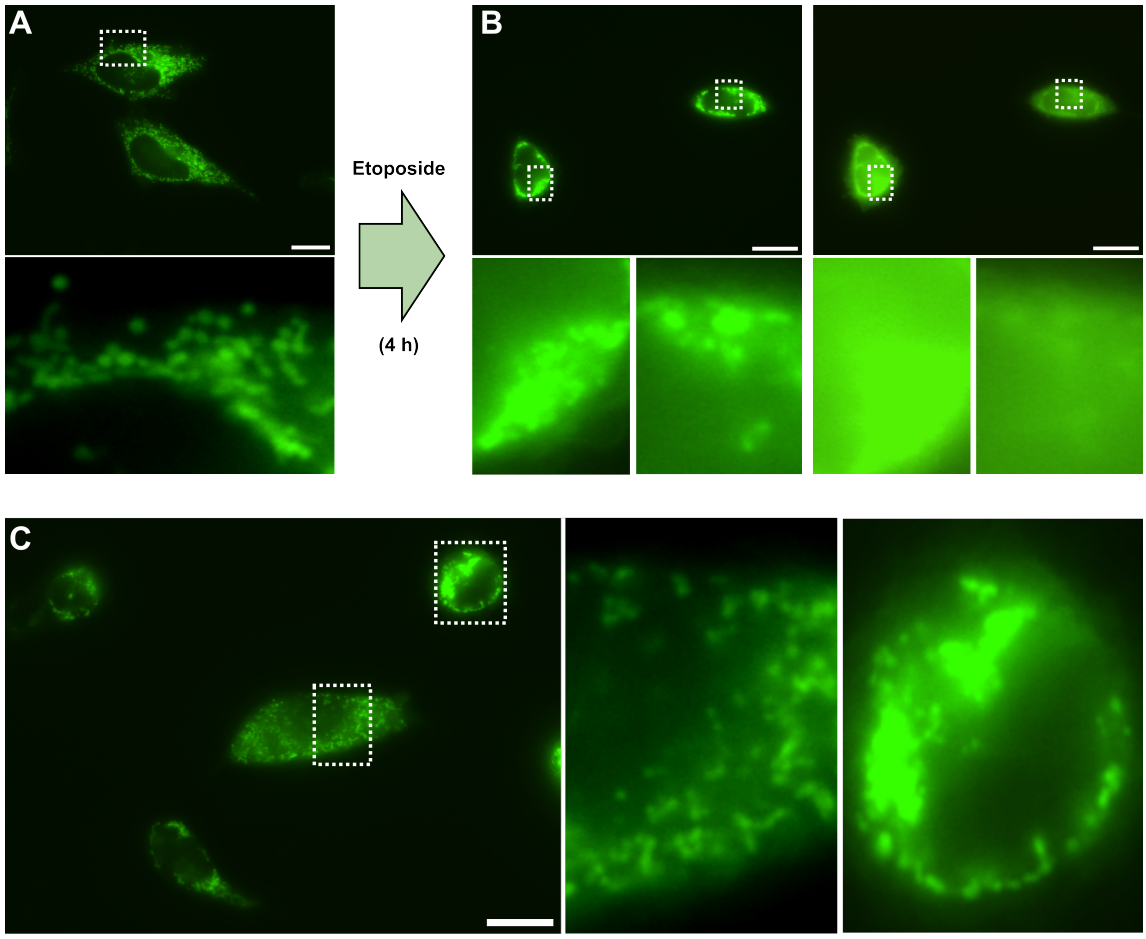


Fig. S11. Imaging microscopy depicting the subcellular distribution of the **DeLiCy5** probe following a 4-hour exposure to 10 μM etoposide in HeLa cells previously subjected to ALA-induced singlet oxygen stimuli. This exposure results in the release of the **DeLiCy5** probe from mitochondria, a phenomenon not observed when the probe undergoes photodegradation alone (panel A *vs.* B). Additionally, panel C demonstrates a subtle loss of cell vitality, evident in the comparison between stressed, rounded mitochondria in the cell at the center and apoptotic cells at the corners, further highlighting the involvement of probe release in this process.