Supplementary Information

Nitroxide amide-BODIPY probe behavior in fibroblasts analyzed by advance fluorescence microscopy

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Table of Contents

S1: Chemicals and reagents

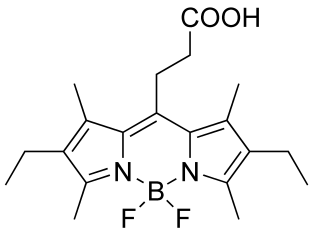
Solvents were dried by standard methods or by elution through a Pure Solv Innovative Technology column drying system. For the synthesis of compound **1** the precursors 3-ethyl-2,4-dimethylpyrrole (97%, Aldrich) and triethylamine (99%, Fluka) were purified by distillation and boron trifluoride diethyl etherate (Fluka) was used as received. Ethyl chloroformate, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, 4-carboxy-2,2,6,6-tetramethylpiperidine 1-oxyl, 4-(dimethylamino)pyperine and dicyclohexylcarbodiimide were purchased from Aldrich and employed without further purification. The dye PM650 (laser grade) was from Exciton.

S2: Characterization methods

TLC was performed on silica gel precoated aluminum foils, Merck 60F 254, 0.25 mm. Flash column chromatography was carried out on silica gel from Merck, 230-400 mesh. Yields are referred to isolated pure compounds. ^{1}H and ^{13}C NMR spectra were registered at room temperature in $CDCl_3$ solution in Varian INOVA-300 and -400 spectrometers. Chemical shifts are reported in parts per million (ppm) using as internal reference the peak of the trace of undeuterated solvent (δ 7.26) or the carbon signal of the deuterated solvent (δ 77.0). The following abbreviations were used to describe: s (singlet), d (doublet), t (triplet), q (quartet), m (complex multiplet). Assignments were based on HSQC and HMBC experiments. IR spectra were recorded in a Perkin Elmer FT 681 spectrophotometer. The fluorescence quantum yield (Φ_f) (excitation at 490 nm) was evaluated relative to that of the PM567 dye in ethanol solution ($\Phi_f = 0.86$).[1] Low-resolution mass spectra were registered in an Agilent HP 1100 LC/MSD spectrometer

using ESI or APCI sources. High resolution mass spectra (HRMS) were recorded in an Agilent 6520 Q-TOF instrument with a ESI source, or in a QTOF QSTAR model (Applied Biosystems) by electro spray ionization in the positive mode (ESI+) using as phase acetonitrile plus 0.1% formic acid, or in an AutoSpecEQ EI apparatus by electron impact (EI, 70 eV).

S3: Synthesis of nitroxide amide-BODIPY probe (BA)



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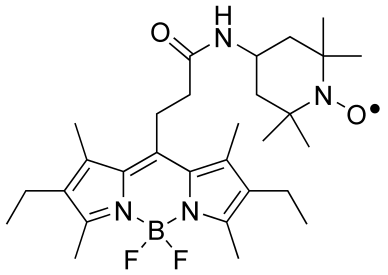
3-(2',6'-diethyl-1',3',5',7'-tetramethyl-4',4'-difluoro-4'-bora-3'a,4'a-diaza-s-indacen-8'-yl)propanoic acid (**1**)[2]: A mixture of 3-ethyl-2,4-dimethylpyrrol (1.7 mL, 13 mmol) and succinyl chloride (0.35 mL, 3.4 mmol) in dichloromethane (50 mL) was refluxed under argon for 30 min. Triethylamine (2 mL, 15.4 mmol) was then added at room temperature and, after 30 min, boron trifluoride diethyl etherate (4.4 mL, 34.7 mmol), and the mixture was refluxed for 2 h. The subsequent workup yielded a red residue that was purified by flash column chromatography (silica gel, hexane-ethyl acetate 8:2 as eluent). Red crystals, mp 183-185 ºC, yield 0.21 g (18%). TLC (hexane-ethyl acetate 1:1): $R_f = 0.19$. ^{1}H NMR (300 MHz): δ = 1.04 (t, 6 H, J = 7.6 Hz, 2×CH_3-CH_2), 2.36 (s, 6 H, CH_3-C1', CH_3-C7'), 2.40 (q, 4 H, J = 7.6 Hz, 2×CH_3-CH_2), 2.50 (s, 6 H, CH_3-C3', CH_3-C5'), 2.66 (m, 2 H, CH_2-CO), 3.37 (m, 2 H, CH_2-C8'), (OH not observed) ppm. ^{13}C NMR (75 MHz): δ = 12.9 (*C*H_3-C3', *C*H_3-C5'), 13.8 (*C*H_3-C1', *C*H_3-C7'), 15.2 (*C*H_3-CH_2), 17.6 (CH_3-*C*H_2), 23.9 (*C*H_2-C8'), 35.7 (*C*H_2-CO), 131.1 (C-7'a, C-8'a),

133.5 (C-2', C-6'), 136.0 (C-1', C-7'), 141.4 (C-8'), 153.6 (C-3', C-5'), 178.3 (C=O) ppm. IR (KBr) ν_{max} = 3408, 2965, 2931, 1712, 1548, 1479, 1197, 980 cm^{-1}. MS (EI), m/z (%) = 376 [nominal mass, M^+] (93), 361 (100).



BA

3-(2',6'-diethyl-1',3',5',7'-tetramethyl-4',4'-difluoro-4'-bora-3'a,4'a-diaza-s-indacen-8'-yl)-N-(2,2,6,6-tetramethyl-1-piperidinyloxy-4-yl) propionamide free radical (BA).

The carboxylic acid **1** (100 mg, 0.26 mmol) was dissolved in anhydrous CH_2Cl_2 (30mL). Ethyl chloroformate (25 μL, 0.26 mmol) was added in presence of triethylamine (36 μL, 0.26 mmol) at 0ºC. Then, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (48 mg, 0.28 mmol) dissolved in the minimum amount of CH_2Cl_2 was added to the reaction mixture drop to drop. When the addition had finished the temperature of the reaction was increased to room temperature. The subsequent workup yielded a red residue that was purified by flash column chromatography (silica gel, hexane-ethyl acetate 1:1 as eluent). A red oil was obtained with a yield 0.1 g (72%). TLC (hexane-ethyl acetate 1:1): R_f = 0.31. IR (KBr) ν_{max} = 3401.5 (NH), 1641.9 (NH), 1547.5 (CO), 1479 (NO) cm^{-1}. ESI-HRMS found M^+ 528.3533 (0.89 ppm from calc. mass of $C_{29}H_{44}BF_2N_4O_2^{\cdot}$), $(M+NH_4)^+$ 547.3872, $(M+Na)^+$ 552.3426.

S4: Cell culture and viability

Human dermal fibroblast cells (PCS-201-012, ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies) containing 10% fetal calf serum (FCS) and 1.0% penicillin/streptomycin. The cells were cultured at 37ºC, 5.0% CO_2, and 100% humidity. CellTiter 96® Aqueous MTS (3-(4,5-dimethyl-2-tiazol)-2,5-diphenil-2H-tetrazolium bromide; tiazol blue) colorimetric viability assay (Promega) was employed to measure cell viability at different times after addition of the BA probe at 10 min, 2 and 24 h. Briefly, from confluent cell cultured skin fibroblasts cells at densities of 16,625 cells/cm^2, 100 µL were seeded in a 96-well plate and incubated overnight, prior experiments. Cell viability was assessed by adding 20 µL of the MTS solution to a 100 µL of fresh non-supplemented DMEM without FCS followed by incubation at 37ºC, 5.0% CO2 and 100% humidity for 2 h. Sample absorbance was read at 490 nm in a microplate reader (SpectroMax 5, Molecular Devices). Hydrogen peroxide (H_2O_2) toxicity was assessed using MTS viability assay in a similar fashion to the described above but DMEM was replaced by Hanks Balanced Buffer Solution (HBBS, Sigma), where a final H_2O_2 concentration of 3.0% v/v was used for this end. Complementary cell toxicity upon addition of H_2O_2 was measured with LIVE/DEAD™ staining kit using Calcein AM and EthD-1 (L-3244, Invitrogen) according to manufacturer's specifications. Imaging was carried out on an inverted fluorescence microscope (Zeiss Observer.A1) and the analysis was done using ImageJ software.

S5: Cells labeling with BA

Live cell labeling was carried out in 90% DEMEN, 10% HBBS 10x buffer with 10 nM solution of the BA probe for 10 minutes at room temperature. After the incubation the samples were washed two times with the same buffer solution.

S5: Spectroscopic measurements.

UV-visible spectra of BA solutions were registered in an Aligent 8453 spectrophotometer (Palo Alto, CA, USA). Fluorescence measurements of BA were carried out in a RF-5310PC spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan).

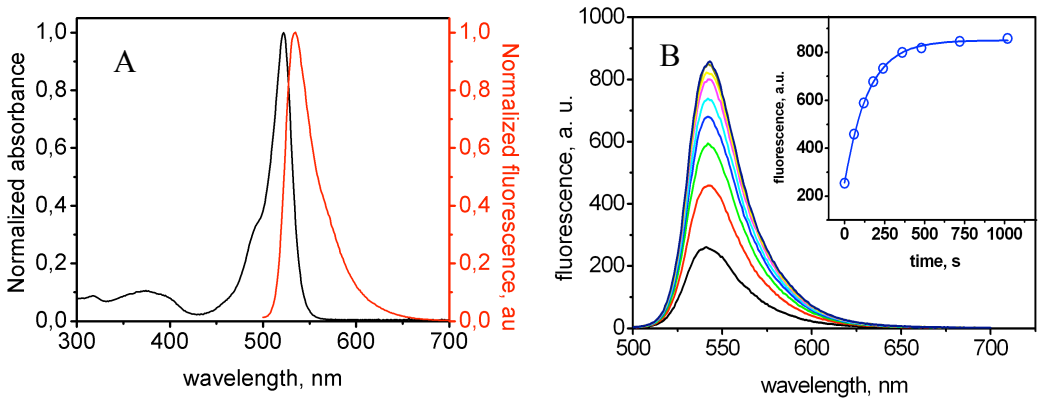


Figure S1. (A) Normalized absorption (black line) and fluorescence spectra (red line) of the BA in methanol. (B) BA fluorescence emission spectrum measured at different incubation times with 500 µM ascorbic acid in 20 mM phosphate buffer, pH 7.0. The inset displays the increase of the fluorescence intensity measured at 543 nm as a function of the incubation time. The blue lines correspond to the monoexponential fit of the experimental data.

S6: Fluorescence Microscopy

Fluorescence lifetime and total internal reflection fluorescence imaging experiments were conducted in borosilicate chambers with 4.2 cm^2 well area (Thermo Scientific) with an Olympus FV1000 TIRF (Olympus). FLIM was performed in a Fluorescent Lifetime Imaging System (PicoQuant). BODIPY-TEMPO probes were excited with a frequency doubled picosecond pulse diode laser (530.6 nm, 100 ps, 20 MHz, LDH-P-FA-530L, PicoQuant) coupled and collimated into the microscope through a fiber optic unit. A beam splitter Z532rdc (Chroma) was used to reflect the excitation light into the oil immersion TIR (total internal reflection) objective (100**X**, NA1.45, Olympus, PLAPO). The fluorescence signal was passed through a 560 nm long pass filter and recorded with a single-photon-counting module (τ-SPAD-100, PicoQuant) and Time Correlated Single Photon Counting Module (TSCPC). Fluorescence decay analysis was performed with SymPhoTime program (PicoQuant). Emission spectra of BODIPY-TEMPO probes incorporated into human skin fibroblast cells were collected by a Shamrock 163 spectrograph (Andor). TIRF images were recorded with a highly sensitive Rolera EM-C^2 camera (Q-Imaging). TIR illumination was achieved with CW laser (488 nm Ar laser). The emission is collected by the camera after passing through a dichroic mirror Chroma ZT488rdc and an emission filter Chroma ET525/50m.

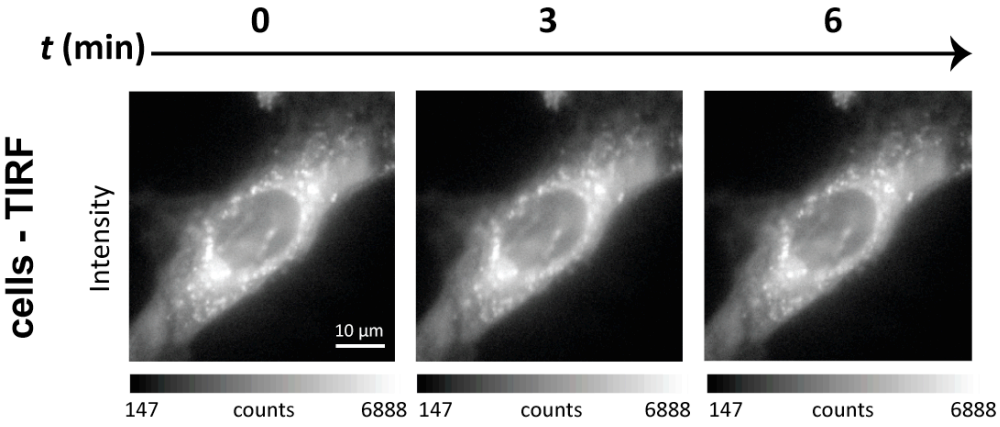


Figure S2. TIRF images of HSF cells incubated for 10 min with [1 nM] of BA as a function of the irradiation time.

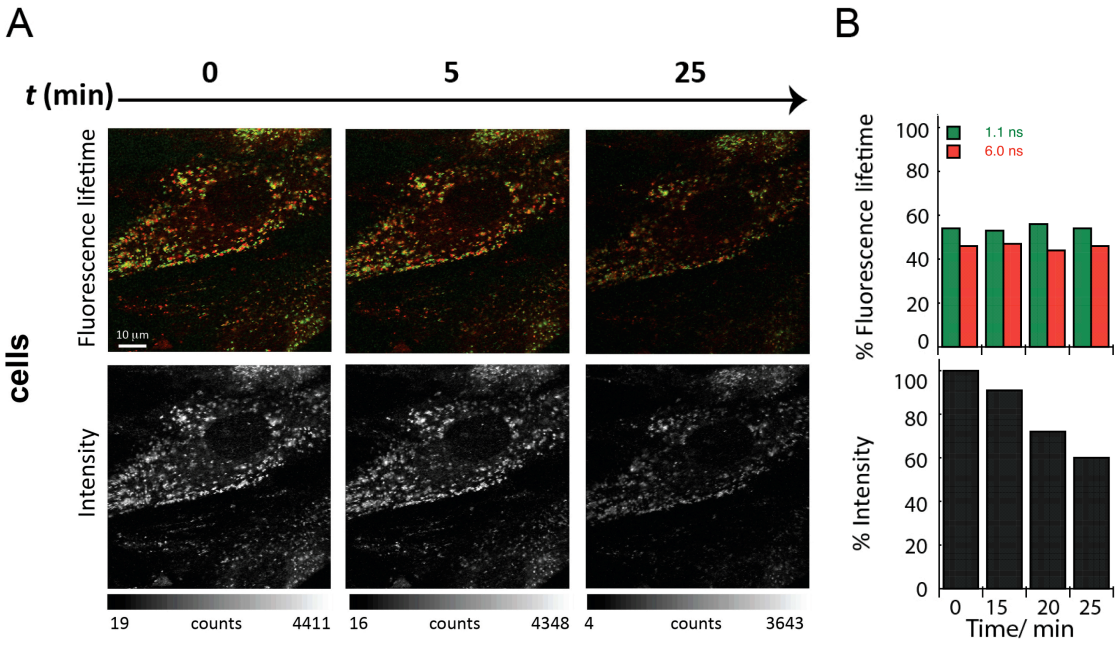


Figure S3. (A) Fluorescence lifetime (top panel) and intensity (bottom panel) images of HSF cells incubated with BA for 25 min and washed to remove excess of dye. The lifetime image uses an RGB false color scheme for the amplitudes of the short (green, t_1 = 1.1 ns) and long (red, t_2 = 6.0 ns) lifetime component. Numbers indicate timing in minutes after 25 min of BA incubation in the cells. (B) Bar plots of the percentage that each lifetime occurs in the image and the percentage of total number of detected photons as a function of the time.

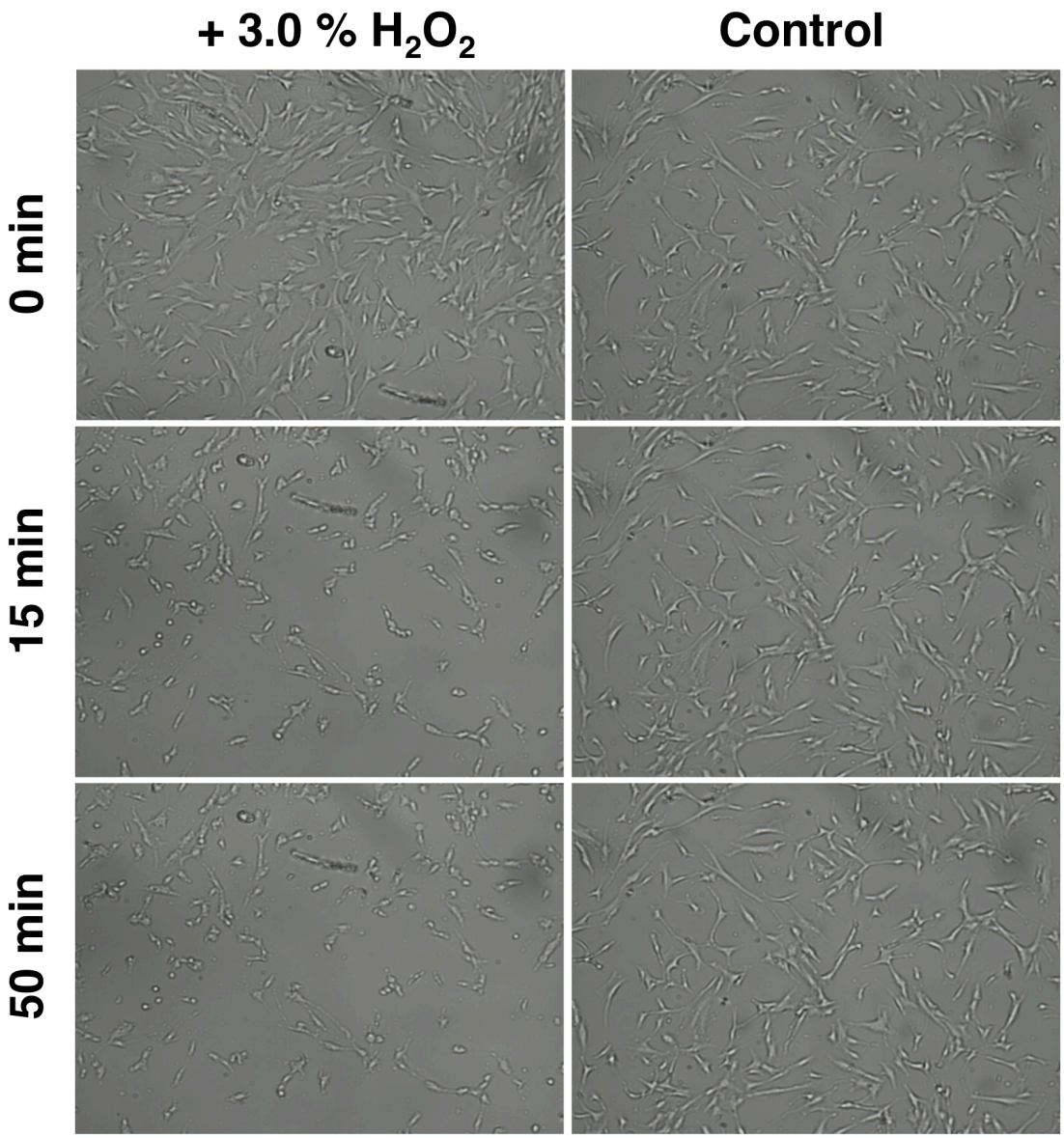


Figure S4. Effect of 3.0 % hydrogen peroxide in the cell morphology of human skin fibroblasts (PCS-201-012, ATCC, USA) at different time points as indicated in the panel. Cells were cultured at densities 16,625 cells/cm2 at 37ºC, 5.0% CO_2 and 95% humidity.

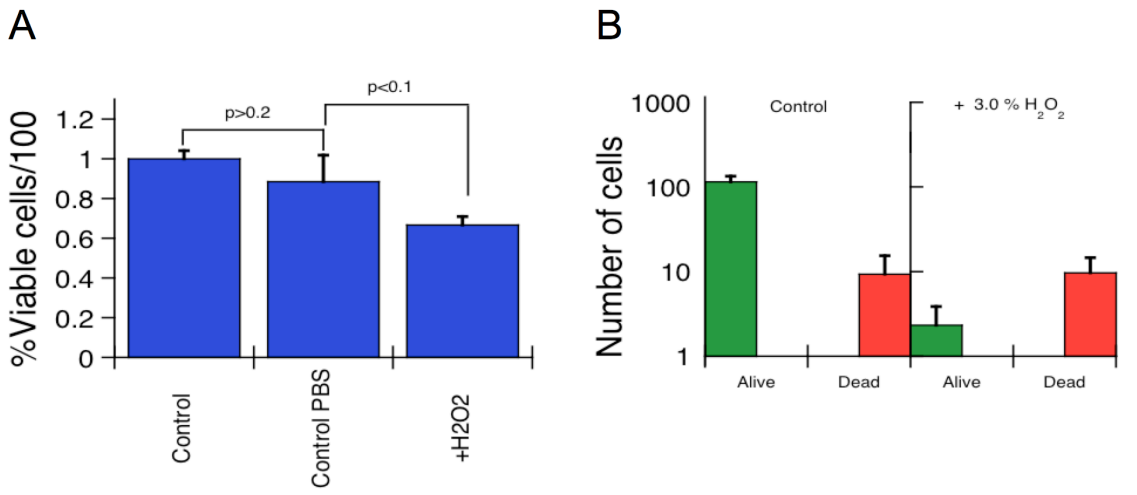


Figure S5. (A) Cell viability evaluated using MTS colorimetric assay and (B) Live-Dead cell assay for cells cultured with and without 3.0 % H_2O_2 for 60 min. All experiments were carried out at cell densities of 16,625 cells/cm^2 in 90 % DMEM and 1X PBS.

References

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