Supplementary Information

An engineered nanoplatform for bimodal anticancer phototherapy with dual-color fluorescence detection of sensitizers

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S1. Chemicals.
All chemicals were purchased by Sigma-Aldrich and used as received. Compound **2** was purchased from Frontier Scientific and used as received. All solvents used were spectrophotometric grade.

S2. Instrumentation.
NMR spectra (^{1}H NMR recorded at 500 MHz, ^{13}C NMR recorded at 125 MHz) were obtained on Varian Instruments and are referenced in ppm relative to TMS or the solvent signal.

UV/vis absorption spectra were recorded with a Jasco V 560 spectrophotometer. Fluorescence emission spectra were recorded with a Spex Fluorolog-2 (mod. F-111) spectrofluorimeter. Nanoparticle sizes were measured by a dynamic light scattering using a Horiba LS 550 apparatus equipped with a diode laser with a wavelength of 650 nm. Fluorescence quantum yields of **2** and **3** under our experimental conditions were determined by using **2** in DMF and rodamine B in ethanol as standards, respectively. Fluorescence lifetimes were measured in air-equilibrated solutions with a time correlated single photon counting system (IBH Consultants Ltd.). A pulsed laser source at 407 nm (Hamamatsu, 306 mW) operating at 1 MHz frequency was used for excitation and the emission was collected at right angle at 528 or 690 nm. TCSPC was set to 0.0063 ns/channel. The software package for the analysis of the emission decays was provided by IBH Consultants Ltd. Decay profiles were fitted using a multiexponential function and deconvolution of the instrumental response.

$$I(t) = \sum_i a_i \times \exp(-t/\tau_i) \qquad (1)$$

$$f_i = (a_i \times \tau_i)/ \sum_j (a_j \times \tau_j) \qquad (2)$$

Laser flash photolysis.
All of the samples were excited with the second harmonic of a Nd–YAG Continuum Surelite II–10 laser (532 nm, 6 ns FWHM), using quartz cells with a path length of 1.0 cm. The excited solutions were analyzed with a Luzchem Research mLFP–111 apparatus with an orthogonal pump/ probe configuration. The probe source was a ceramic xenon lamp coupled to quartz fiber-optic cables. The laser pulse and the mLFP–111 system were synchronized by a Tektronix TDS 3032 digitizer, operating in pre-trigger mode. The signals from a compact Hamamatsu photomultiplier were initially captured by the digitizer and then transferred to a

personal computer, controlled by Luzchem Research software operating in the National Instruments LabView 5.1 environment. The solutions were deoxygenated by bubbling with a vigorous and constant flux of pure argon (previously saturated with solvent). In all of these experiments, the solutions were renewed after each laser shot (in a flow cell of 1 cm optical path), to prevent probable auto-oxidation processes. The sample temperature was 295 ±2 K. The energy of the laser pulse was measured at each shot with a SPHD25 Scientech pyroelectric meter.

IR luminescence Singlet oxygen detection.

Photogeneration of $^1O_2$ upon laser excitation of the photosensitizer was monitored by luminescence measurements in oxygen-saturated solutions. The near-IR luminescence of singlet oxygen at 1.27 μm resulting from the forbidden transition $^3\Sigma_g^- \leftarrow {}^1\Delta_g$; this was probed orthogonally to the exciting beam with a pre-amplified (low impedance) Ge-photodiode (Hamamatsu EI-P, 300 ns resolution) maintained at –196 °C and coupled to a long-pass silicon filter (>1.1 μm) and an interference filter (1.27 μm). Pure signal of $^1O_2$ were obtained as difference between signals in air- and Ar- saturated solutions. The temporal profile of the luminescence was fitted to a single-exponential decay function with the exclusion of the initial portion of the plot, which was affected by scattered excitation light, fluorescence, and the formation profile of singlet oxygen itself. The luminescence at initial time (LΔ at t = 0) was extrapolated from the curve fitting.

Comparative experiments of $^1O_2$ photogeneration were performed at different intensity of the laser pulse. The values of LΔ at t = 0, corrected for the slight difference of photons absorbed by the different samples at the excitation wavelength, were then plotted against the laser intensity, and the related slopes (χ) were compared. The energy of the laser pulse was measured at each shot with a SPHD25 Scientech pyroelectric meter.

Amperometric NO detection.

NO release was measured with a World Precision Instrument, ISO-NO meter, equipped with a data acquisition system, and based on direct amperometric detection of NO with short response time (< 5 s) and sensitivity range 1 nM – 20 μM. The analog signal was digitalized with a four-channel recording system and transferred to a PC. The sensor was accurately calibrated by mixing standard solutions of $NaNO_2$ with 0.1 M H_2SO_4 and 0.1 M KI according to the reaction:

$$4H^+ + 2I^- + 2NO_2^- \rightarrow 2H_2O + 2NO + I_2$$

Irradiation was performed in a thermostated quartz cell (1 cm pathlength, 3 ml capacity) under gentle stirring by using the monochromatic radiation of 400 nm of the above described fluorimeter (mod. F-111) as light sources. NO measurements were carried out with the electrode positioned outside the light path in order to avoid false NO signal due to photoelectric interference on the ISO-NO electrode.

Confocal fluorescence microscopy

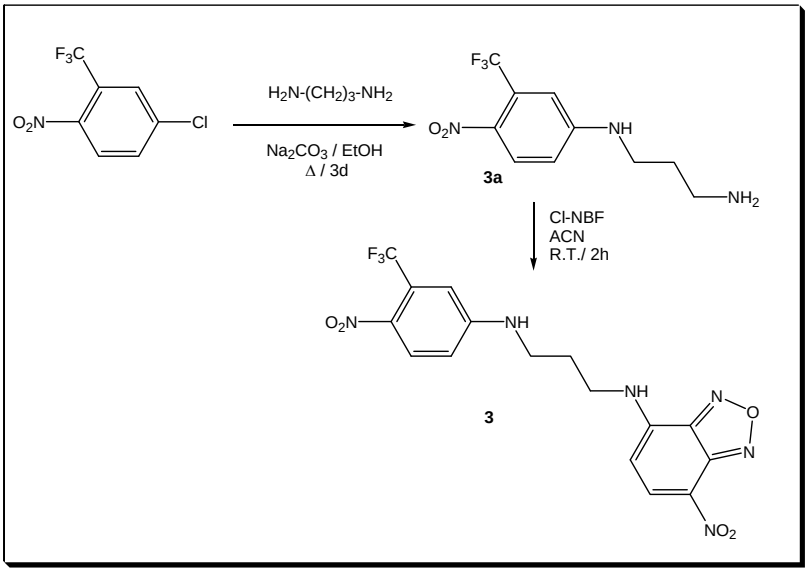
Confocal fluorescence imaging was performed on an inverted Nikon A1 laser scanning confocal microscope equipped with a CW argon ion laser for excitation at 457, 488 and 514 nm (Melles Griot, 40 mW), a CW red diode laser for excitation at 640 nm (Melles Griot, 12 mW) and a diode laser for excitation at 405 nm (LDH-D-C-405 of Picoquant GmbH Berlin, Germany) operating both in continuous mode (50 mW) and pulsed at 40 MHz (1.0 mW average power for pulse FWHM of 70 ps). Confocal fluorescence imaging was carried out on the samples at room temperature. The images were collected using a Nikon PLAN APO VC 60× NA 1.40 oil immersion objective. Images of 512*512 or 1024*1024 pixels have been acquired applying scan speed of 1 frame in 2-8 s and pixel dimension of the xy plane falls in the range 0.1-0.4 μm. Hexagonal pinhole dimension was set to 0.8-1.0 au corresponding to 25-38 mm and optical thickness of 330-440 nm. Two dichroic mirrors reflecting either 405, 488, 541 and 640 or 457 and 514 nm were used. Bandpass filters in front of the PMT selected fluorescence in the ranges 425-475 nm, 500-550 nm and 665-735 nm. Spectral imaging was done with Nikon 32-PMT array detector with resolution varying from 6 to 10 nm per channel. For fluorescence lifetime imaging a time-correlated single photon counting (TCSPC) system of Picoquant GmbH Berlin was used exciting at 405 nm. Photons were detected in TTTR mode with two Single Photon

Avalanche Diodes manufactured by Micro Photon Devices (MPD), Bolzano, Italy. Fluorescence was filtered with the opportune fluorescence SEMROCK bandpass filters 480/40 nm, 520/40 nm, and 716/40 nm. PicoHarp 300 photon processor completes the TCSPC system. SymPhoTime v. 5.1 analysis software was used for image processing and lifetime fitting. A tail fit with multi-exponential functions was performed to analyze fluorescence decays of selected ROI. The system allowed measurement of fluorescence lifetimes from 300 ps up to several nanoseconds.

S3. Synthetic procedures.

The CD polymer **1** was synthesized by polycondensation of cyclodextrin with epichlorohydrin under basic conditions, using a previously reported method.[S1] The polymer has been characterized by ^{1}H NMR and GPC showing an average molecular mass around 700,000 g/mole and a cyclodextrin content of 70 wt%. As previously reported, the obtained polymer can be readily dispersed in water where it is under the form of spherical nanoparticles (NPs) of around 30 nm.[S1c] Drugs and molecules of interest were successfully loaded in the CD polymer by forming inclusion complexes with the available CDs and/or by insertion in confined microdomains between the CDs and the corsslinks.[S1] This was achieved by a "green" method, without using neither organic solvents nor surface active molecules, simply mixing with aqueous solutions of the polymer.[S1a] In our case, a CD polymer solution has been prepared by stirring overnight 4 mg of **1** in PBS at pH 7.4. Compounds **2** and **3** were dissolved in methanol and slowly evaporated to form a thin film. These films were then hydrated with an aqueous solution of **1** in PBS at a pH of 7.4. The mixtures were stirred for 5 hours at 40 °C and then the final solutions were left to equilibrate at room temperature.

4-(N-(aminopropyl)-3-(trifluoromethyl)-4-nitrobenzenamine)-7-nitrobenzofurazan (**3**) was synthesized in a two steps synthesis as reported in Scheme 1S. Syntheses were carried out under a low intensity level of visible light. The N-(aminopropyl)-3-(trifluoromethyl)-4-nitrobenzenamine **3a** (200 mg, 0.8 mmol) obtained as previously described[2S] was solubilized in 10 mL of acetonitrile and added of in 4 mL of acetonitrile solution of 4-chloro-7-nitrobenzofurazan (Cl-NBF) (184 mg, 0.9 mmol, 1.1 eq.). Although the starting compound was not fully converted, the reaction was stopped after 2h of stirring at R.T. to avoid the formation of undesirable side-products. The organic solution was concentrated under reduced pressure and purified by column chromatography over silica gel (chloroform:methanol, 99:1) to give **3** (yield 15 %, 50 mg) as a orange solid. ^{1}H-NMR CD_3CN: δ 2.07 (2H, qt, $^{3}J_{H\text{-}H}$ = 6.5 Hz, $NHCH_2C\underline{H}_2CH_2NH$), 3.37 (2H, dt, $^{3}J_{H\text{-}H}$ = 6.5 Hz, $C\underline{H}_2NHC_6H_3NO_2CF_3$), 3.64 (2H, m, $C\underline{H}_2NH$-NBF), 5.90 (1H, s broad, $N\underline{H}$ $C_6H_3NO_2CF_3$), 6.31 (1H, d, $^{3}J_{H\text{-}H}$ = 9.0 Hz, $C_{Ar}H$ (NBF)), 6.78 (1H, dd, $^{3}J_{H\text{-}H}$ = 9.0 Hz, $^{4}J_{H\text{-}H}$ = 2.5 Hz, $C_{Ar}H$), 6.99 (1H, d, $^{4}J_{H\text{-}H}$ = 2.5 Hz, $C_{Ar}H$),7.44 ($N\underline{H}$(NBF),1H, s broad), 7.99 (1H, d, $^{3}J_{H\text{-}H}$ = 9.0 Hz, $C_{Ar}H$), 8.48 (1H, d, $^{3}J_{H\text{-}H}$ = 9.0 Hz, $C_{Ar}H$ (NBF)). ^{13}C-NMR CD_3CN: δ 28, 41, 42, 99, 112, 113, 122, 130, 135, 138, 145, 154.



Scheme S 1

S4. Experiments with cells.

A375 human melanoma cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2.0 mM L-glutamine, 100 U/ml penicillin, 100 lg/ml streptomycin, and 25 lg/ml fungizone (Sigma–Aldrich, Italy), and incubated at 37 °C and 5% CO_2/95% air. Cells from confluent cultures were detached using 0.25% trypsin–1 mM EDTA and seeded in complete DMEM medium. For cell staining, the experimental cells were cultured in a 12-well culture dishes for 24 hours. The medium was removed and replaced with medium without phenol red containing the dispersion of NPs loaded with the PS (the concentrations are the same as in Fig 1) for 12 hours. The cells were first washed with PBS, then fixed with 4% paraformaldehyde in PBS for 20 min. After washing with PBS, the cells were incubated with 4-6-diamino-2-phenylindole (DAPI) (1:10,000; Invitrogen) for 10 min.

The mortality experiments were carried out by irradiating the cells incubated with the NPs with a 150 W Xe lamp with a cut-off filter at 390 nm. Under these experimental conditions the NO photodonor chromophore and the $^1O_2$ photosensitizer absorbs comparable light dose. This was calculated on the basis of the intensity of the irradiation source in the range 650-700 nm (absorption range of the photoactive monomer of **2**) and in the range 400-430 nm (absorption range of the component **3**) and by taking into account the related absorbance of the photoactive components in the same range.

Cell proliferation was tested by MTT assay, based on the conversion by mitochondrial dehydrogenases of a substrate containing a tetrazolium ring into blue formazan, detectable spectrophotometrically. Briefly, cells were seeded at an initial density of 8×10^3 cells / microwell in flat-bottomed 200 µl microplates, incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 hours. After this time, some cells, as a control, were incubated in complete DMEM without phenol red. Some cells were incubated with complete DMEM without phenol red containing the polymer nanoparticles. 4 hours before the end of the treatment time, 20 µl of 0.5% 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide in PBS were added to each microwell. After 4 h of incubation at 37 °C, the supernatant was removed and replaced with 100 µl of DMSO. The optical density of each well sample was measured with a microplate spectrophotometer reader (Digital and Analog Systems, Rome, Italy) at 550 nm. The cell viability (%) was calculated according to the following equation:

$$\text{Cell Viability (\%)} = [A_{\text{Before}} - (A_{\text{After}} / A_{\text{Before}})] \times 100$$

where, A_{Before} and A_{After} are the absorbance values of the wells treated with samples before and after irradiation respectively.

S5. Photophysical data

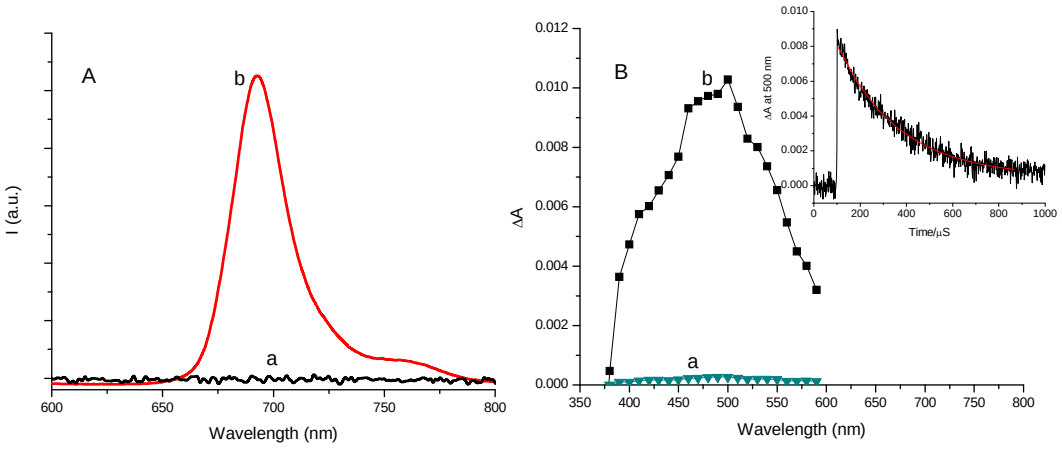


Fig. 1S. (A) Fluorescence spectra (λ_{exc} = 575 nm) and (B) transient absorption spectra (λ_{exc} = 532 nm, E_{532} ~ 12 mJ/pulse, Ar saturated solution) of an aqueous solution of **2** observed in the absence (a) and in the presence (b) of **1** (4 mg mL^{-1}). The inset shows the decay trace monitored at 500 nm and the related first order fitting. The transient spectrum is in according to what reported in the literature.[3S]

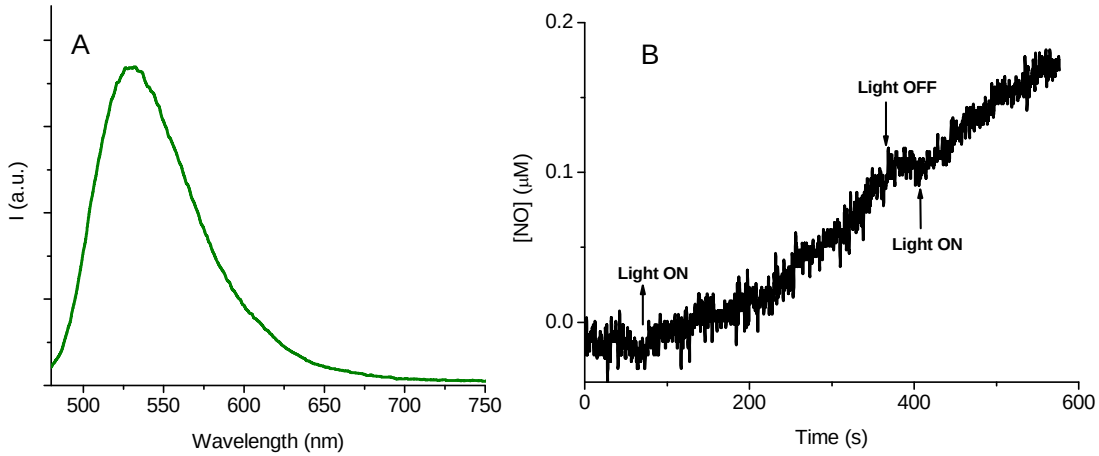


Fig. 2S. (A) Fluorescence spectrum (λ_{exc} = 470 nm) and (B) NO release (λ_{exc} = 400 nm) of an aqueous solution suspension of **1** (4 mg mL^{-1}) in the presence of **3**.

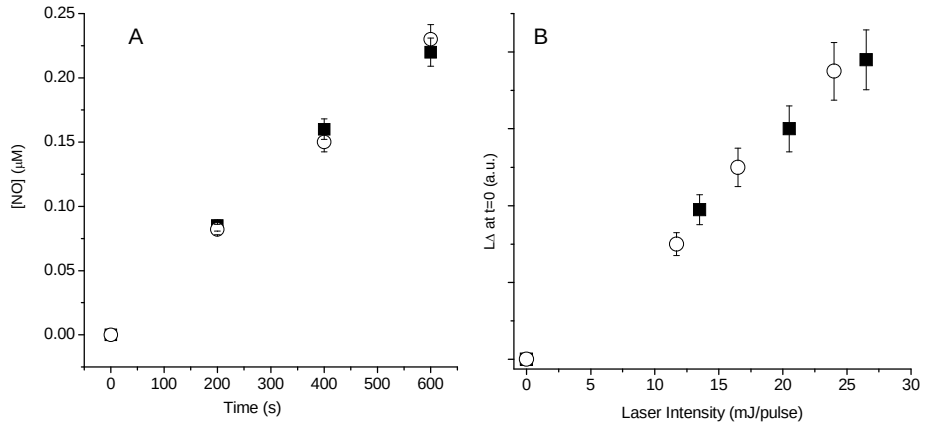
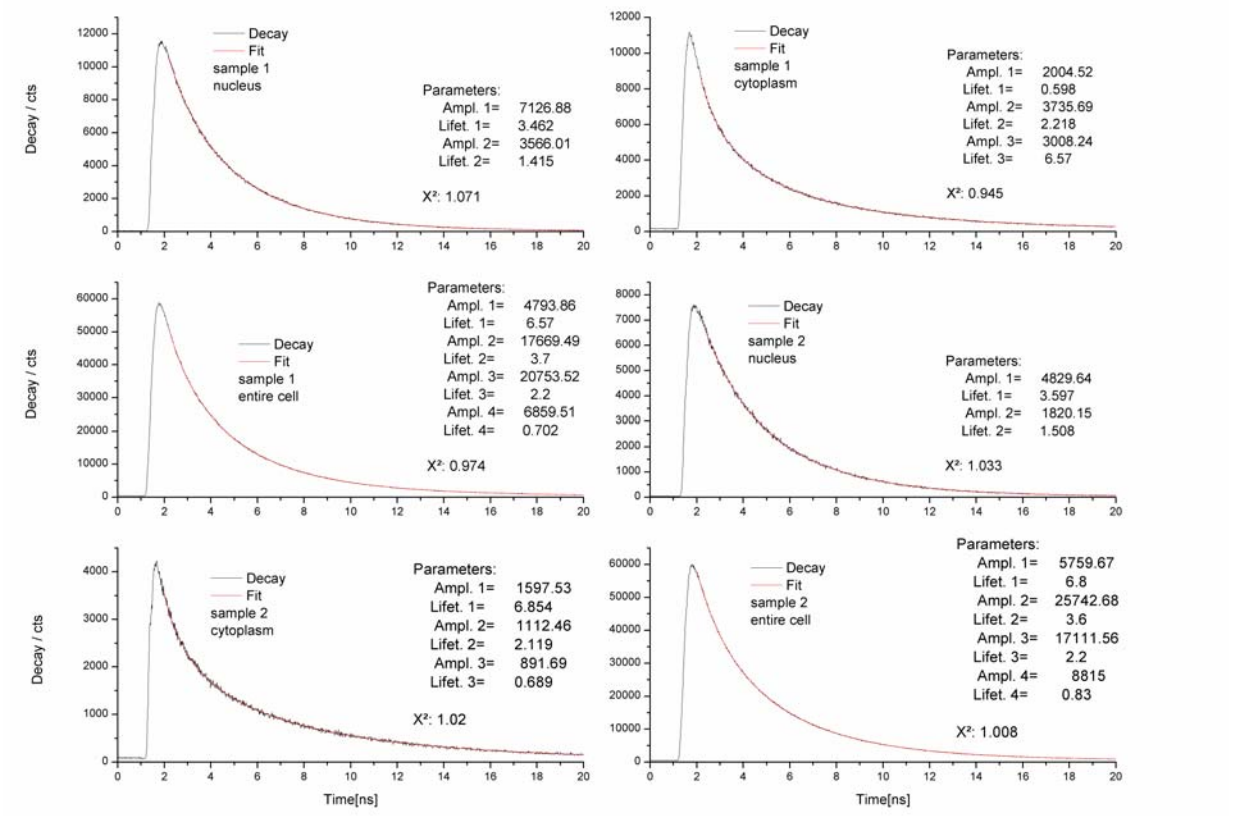


Fig. 3S. (A) NO photoreleased by aqueous dispersions of **1** (3 mg mL^{-1}) in the presence of **3** (○) and **2**+**3** (■). (B) Luminescence intensity of $^1O_2$ at initial time as a function of the laser intensity in the case of aqueous solution dispersions of **1** (4 mg mL^{-1}) in the presence of **2** (○) and **2**+**3** (■).The data are corrected for different absorbance of the two samples at the excitation wavelengths.

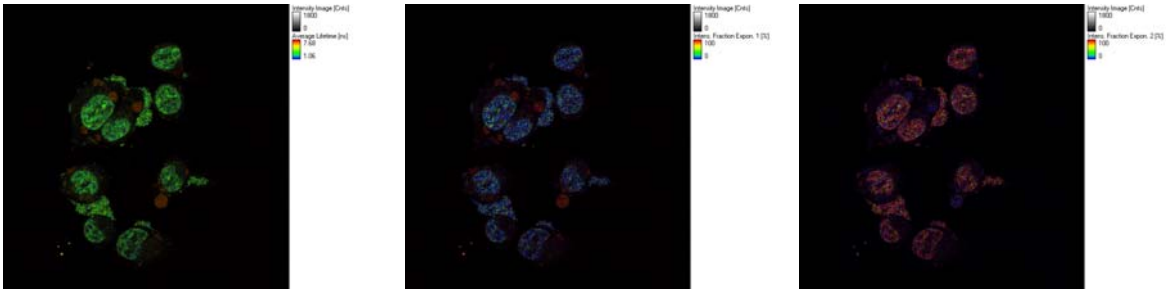
Table 1S: Fluorescence lifetimes obtained from multiexponential fitting of fluorescence decays measured with TCSPC technique exciting at 407 nm

Sample	Lifetime (fractional intensity)	Chi-square	λ_{em} (nm)
2 in PBS	2.44 ns	0.87	690
2 in the presence of **1**	2.9 ns	1.5	690
3 in ACN	8 ps (18%) 556 ps (82%)	1.0	528
3 in the presence of **1**	170 ps (19%) 940 ps (44%) 3.6 ns (37%)	1.0	528
2+3 in the presence of **1**	130 ps (17%) 830 ps (45%) 3.46 (38%)	1.0	528
2+3 in the presence of **1**	110 ps (2%) 3.46 (98%)	1.22	690

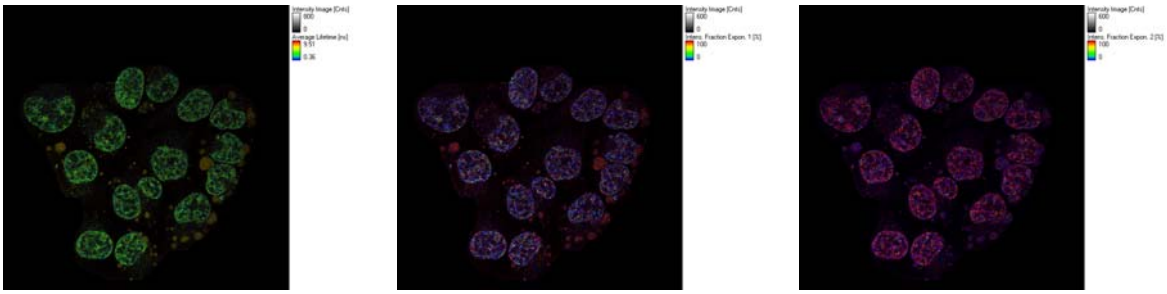
S6. Fluorescence Lifetime Imaging (FLIM)



The graph above shows the multiexponential fitting of fluorescence decays for different regions of interest (ROI) of cell samples 1 and 2 incubated with DAPI and NPs **1** loaded either with compound **3** alone or with both compounds **3** and **2**, respectively. Excitation was performed at 405 nm. Emission was collected with two SPADs in the ranges 460-500 nm and 500-550 nm. A very low number of photons was collected in the range 695-735 nm due to very low absorbance of compound **2** at 405 nm and fitting was not reliable. For fitting of fluorescence originating in the nucleus and cytoplasm we analyzed decays of photons collected in the 460-500 nm channel and 500-550 nm channel, respectively. The images below were then calculated after a 4-exponential fit of decay of all emitted photons collected in both channels fixing the three longest lifetimes to the values obtained from ROI fitting.



Sample 1, DAPI, NPs **1** with **3**; left: average lifetime image; middle: 6.6 ns fractional intensity image; right: 3.7 ns fractional intensity image



Sample 2, DAPI, NPs **1** with **3** and **2;** left: average lifetime image; middle: 6.8 ns fractional intensity image; right: 3.6 ns fractional intensity image

Supplementary bibliography

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