Electronic Supplementary Material (ESI) for RSC Advances.

Non-Covalent Functionalization of Carbon Nano-Onions with Pyrene-BODIPY Dyads for Biological Imaging

Juergen Bartelmess,[a,§] *Marco Frasconi,*[a,§] *Preethi B. Balakrishnan,*[b] *Angelo Signorelli,*[a] *Luis Echegoyen,*[c] *Teresa Pellegrino,*[b] *Silvia Giordani*[*,a]

[a] Istituto Italiano di Tecnologia (IIT), Nano Carbon Materials, Via Morego 30, 16163 Genova, Italy.

[b] Istituto Italiano di Tecnologia (IIT), Drug Discovery and Development, Via Morego 30, 16163 Genova, Italy.

[c] University of Texas at El Paso, Department of Chemistry, 500 W. University Ave., El Paso, TX 79968, USA.

§These authors contributed equally to this work.

Materials, instrumentation and methods.

Absorption spectra were recorded on a Varian Cary 300scan UV-Visible spectrophotometer or on an Agilent Cary 8454 UV-Vis diode array spectrophotometer. Corresponding fluorescence spectra were taken on a Varian Cary Eclipse fluorescence spectrophotometer or on a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer in 1.00 x 1.00 cm quartz glass cells. Fluorescence quantum yields were determined by the comparative method of Williams *et al.*.[1] Toward this, the integrated fluorescence intensities of a known dye and the tested compound were compared and fluorescence quantum yields were calculated using the following equation:

$$\Phi_x = (\Phi_{st}) (Grad_x / Grad_{st}) (\eta^2_x / \eta^2_{st})$$

st and x denotes the standard and test respectively, while Φ is the fluorescence quantum yield. Grad is the gradient obtained from the plot of integrated fluorescence intensity *vs.* absorbance of the dye at the excitation wavelength. η represents the refractive index of the used solvents. The fluorescence quantum yields of **3** was measured relative to *meso*-phenol BODIPY with $\Phi_{St} = 0.64$ in toluene at an excitation wavelength of 485 nm.[2]

For the fluorescence lifetime measurements, a home-built scanning confocal microscope was used.[3] The excitation source was a super-continuum laser producing 50 ps pulses. The 488 nm excitation beam used for the measurements was obtained by spectrally filtering (Bright Line HC 488/6 nm, AHF analysentechnik) the super-continuum beam. Successively, the beam was reflected by a dichroic mirror (zt-488-RDC, AHF analysentechnik) and focused by an oil immersion objective. The fluorescence was collected by the same objective. The histograms of photon arrival times were accomplished by a time-correlated single photon card (TCSPC) (SPC-

830, Becker & Hickl). The fitting to the TCSPC data included a single-exponential decay and a convolution with the instrument response function.

ATR FT-IR spectroscopy was carried out on a Bruker Vertex 70v FT-IR spectrometer equipped with a Platinum ATR accessory.

The Accurate Mass measurements (HRMS) were performed on a Waters SYNAPT G2 High Resolution Mass Spectrometry instrument equipped with an Electrospray Ionization interface and coupled to a Waters ACQUITY UPLC. Electrospray ionization in positive mode was applied in the mass scan range 50-1200 Da. The analysis were performed on a Waters ACQUITY UPLC BEH C18 column 100 x 2.1 mm ID (particle size 1.7 μm) with an in-line filter. The mobile phase was 0.1% formic acid in H_2O and 0.1% formic acid in acetonitrile.

NMR spectroscopy was performed on a Bruker Avance III 400 MHz system (400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C) in $CDCl_3$, purchased from Sigma-Aldrich.

Table S1. Spectroscopic data of **3** in toluene and DMSO.

Solvent	λ_{Abs} max [nm]	ε [M^{-1} cm^{-1}]	λ_{Em} max [nm]	Φ_F*	Stokes Shift [nm]	τ_F [ns]
toluene	503	94.7	515	0.72	12	3.35 +/- 0.02
DMSO	501	87.0	513	0.71	12	3.75 +/- 0.05

* measured relative to 4,4-difluoro-8-(4-(hydroxy)phenyl)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene, which is reported with a fluorescence quantum of 0.64 in toluene.[2] Excitation at 485 nm.

Figure S1. Comparison of the absorption and fluorescence spectra of ***p-CNO/3*** (red) of a mass concentration of 10 µg/mL and pyrene-BODIPY **3** (purple) in DMSO. Both samples have a comparable absorption at the excitation wavelength of 490 nm.
Top: Absorption spectra. Bottom: Fluorescence spectra.

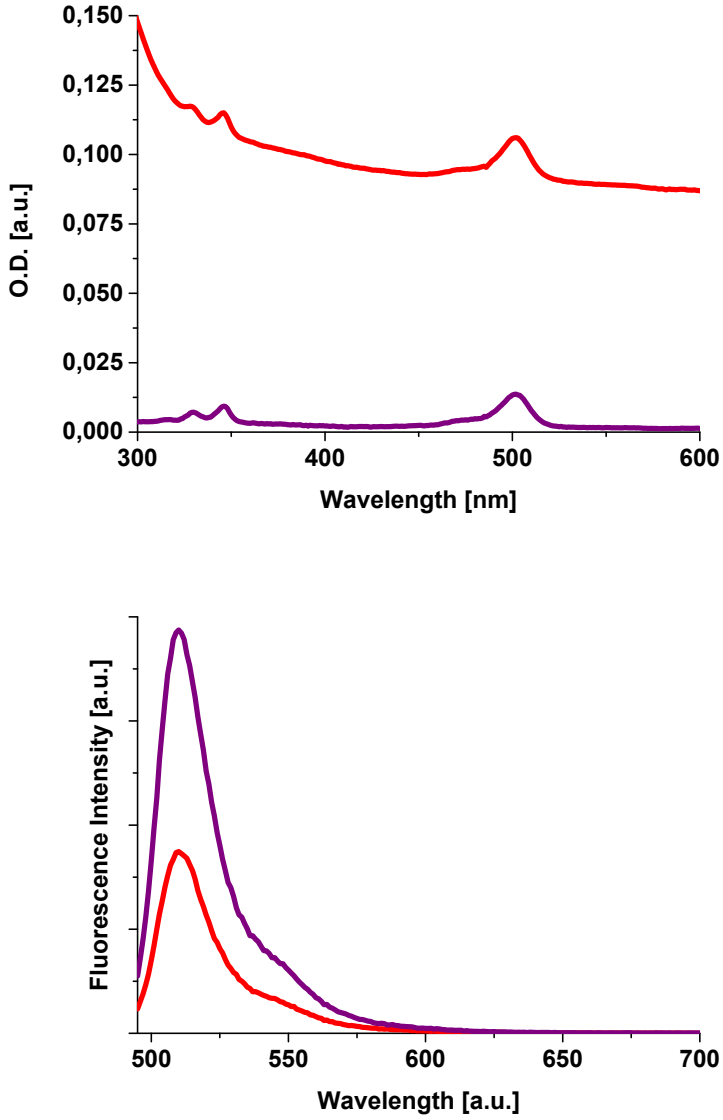
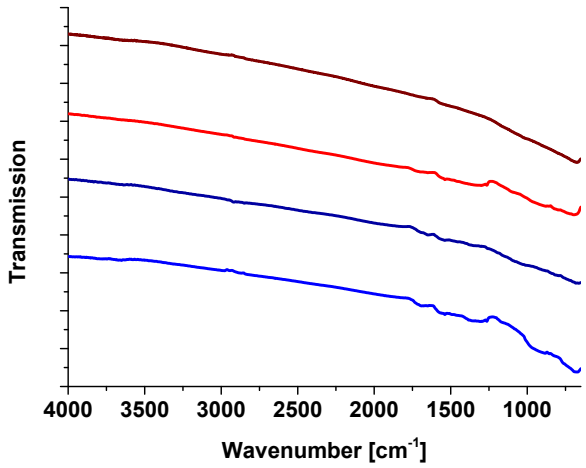
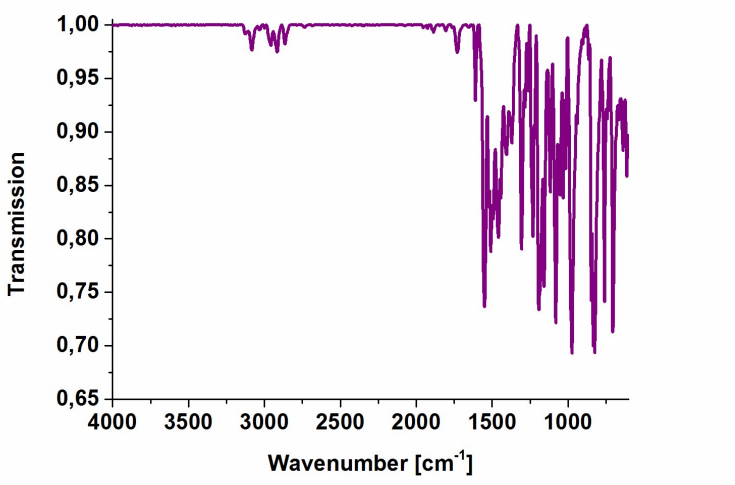


Figure S2. ATR FT-IR spectum of ***p*-CNO** (dark red), ***p*-CNO/3** (red), ***benz*-CNO** (dark blue) and ***benz*-CNO/3** hybrids (blue).



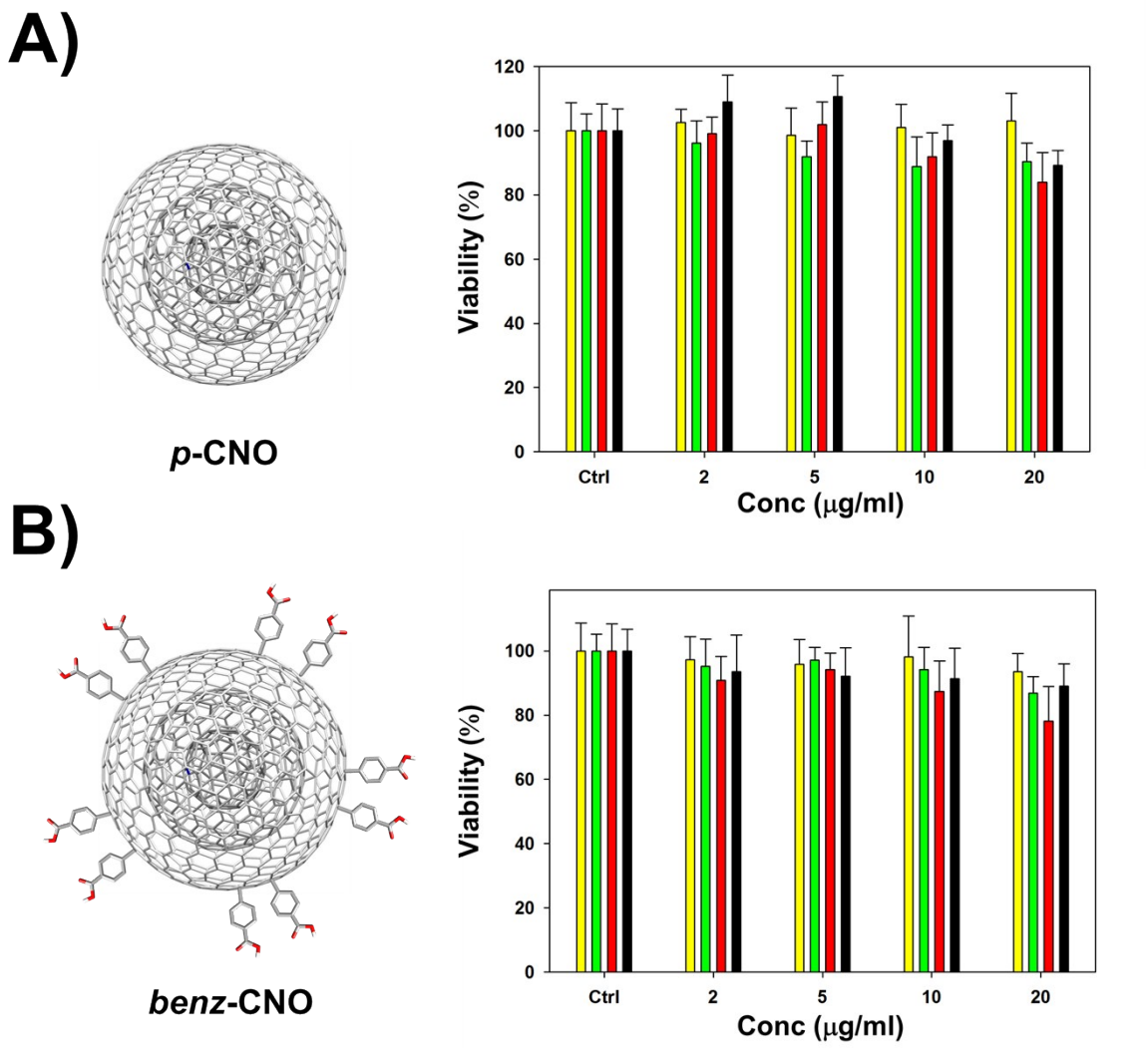
The IR spectrum of ***p*-CNO** (dark red) reveals the typical broad absorption of CNOs over the whole area without significant IR absorption bands. CNO functionalization with benzoic acid moieties (***benz*-CNO** – dark blue) results in some weak bands between 600 and 1750 cm^{-1}. The spectrum of ***p*-CNO/3** (red) and ***benz*-CNO/3** hybrids (blue) instead clearly shows BODIPY centered IR bands in the same area like the ones shown in the IR spectrum of **3** shown below. The resolution, however, is not high, a usual effect observed for all kinds of carbon nanomaterial / chromophore systems, due to the large background absorption of the carbon nanomaterial. No baseline correction was carried out.



FTIR-spectrum of BODIPY-pyrene dyad **3**

Viability Assay

Figure S3. Cellular viability of HeLa wt cells after exposure to different concentrations (2, 5, 10, 20 μg/ml) of ***p*-CNO** (A) and ***benz*-CNO** (B) after 12 (yellow), 24 (green), 48 (red) and 72 (black) hours of incubation at 37 ºC.



Mechanism of Cellular Uptake

HeLa cells, grown in the 24 well chamber plates, were treated with 500 μL of medium with ***p*-CNO/3** at concentration of 2 and 10 μg/ml. The cells were incubated for 30 min at 4 ^{o}C or 37 ^{o}C. The cells were then washed thoroughly with PBS, tagged with DAPI dye for nuclear staining and fixed using 4% paraformaldehyde. The coverslips containing the fixed cells were than retrieved and glued over a glass-slide for confocal microscopy imaging.

Cellular Localization

The cells were incubated for 30 min with 2, 5 and 10 μg/ml of ***p*-CNO/3** in medium. After the treatment, all the wells containing cells were washed carefully with PBS to remove the adsorbed CNO samples. One set of cells were incubated for an extra 2 hours with fresh cell media. Then all of them were stained with LysoTracker Red DND-99, a dye that tags endosomes and lysosomes and Hoechst 33342 for live nuclear staining. The samples were then fixed following the same procedure described in previous section.

Figure S4. Confocal images of fixed HeLa cells incubated for 30 min with 2 μg/ml (A,B), 5 μg/ml (C,D), 10 μg/ml (E,F) of ***p*-CNO/3** at 37 ºC. After washing the cells from the excess of ***p*-CNO/3,** the cells were stained and fixed.. Blue: Hoechst 33342; Red: LysoTracker Red DND-99 and Green: ***p*-CNO/3**. Merged image with the colocalization (yellow) of ***p*-CNO/3** within the lysosomes. Scale bar 25μm.

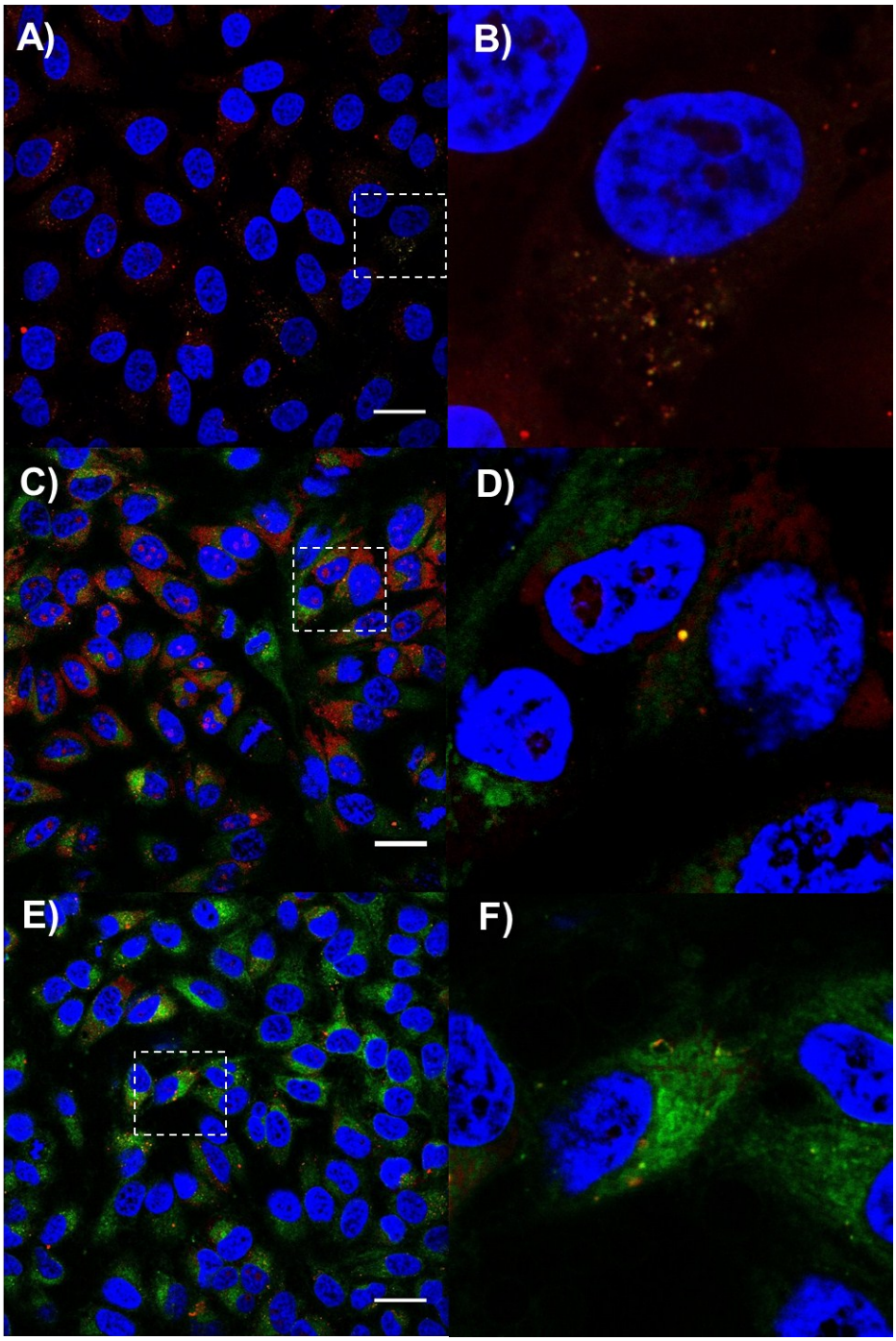


Figure S5. Confocal images of fixed HeLa cells incubated for 30 min with 2 μg/ml (A,B), 5 μg/ml (C,D), 10 μg/ml (E,F) of ***p*-CNO/3** at 37 ºC. After washing the cells from the excess of ***p*-CNO/3**, allowed to stand in DMEM media for an additional 2h, washed, stained and fixed. Blue: Hoechst 33342; Red: LysoTracker Red DND-99 and Green: p-CNO/3. Merged image with the colocalization (yellow) of ***p*-CNO/3** within the lysosomes. Scale bar 25μm.

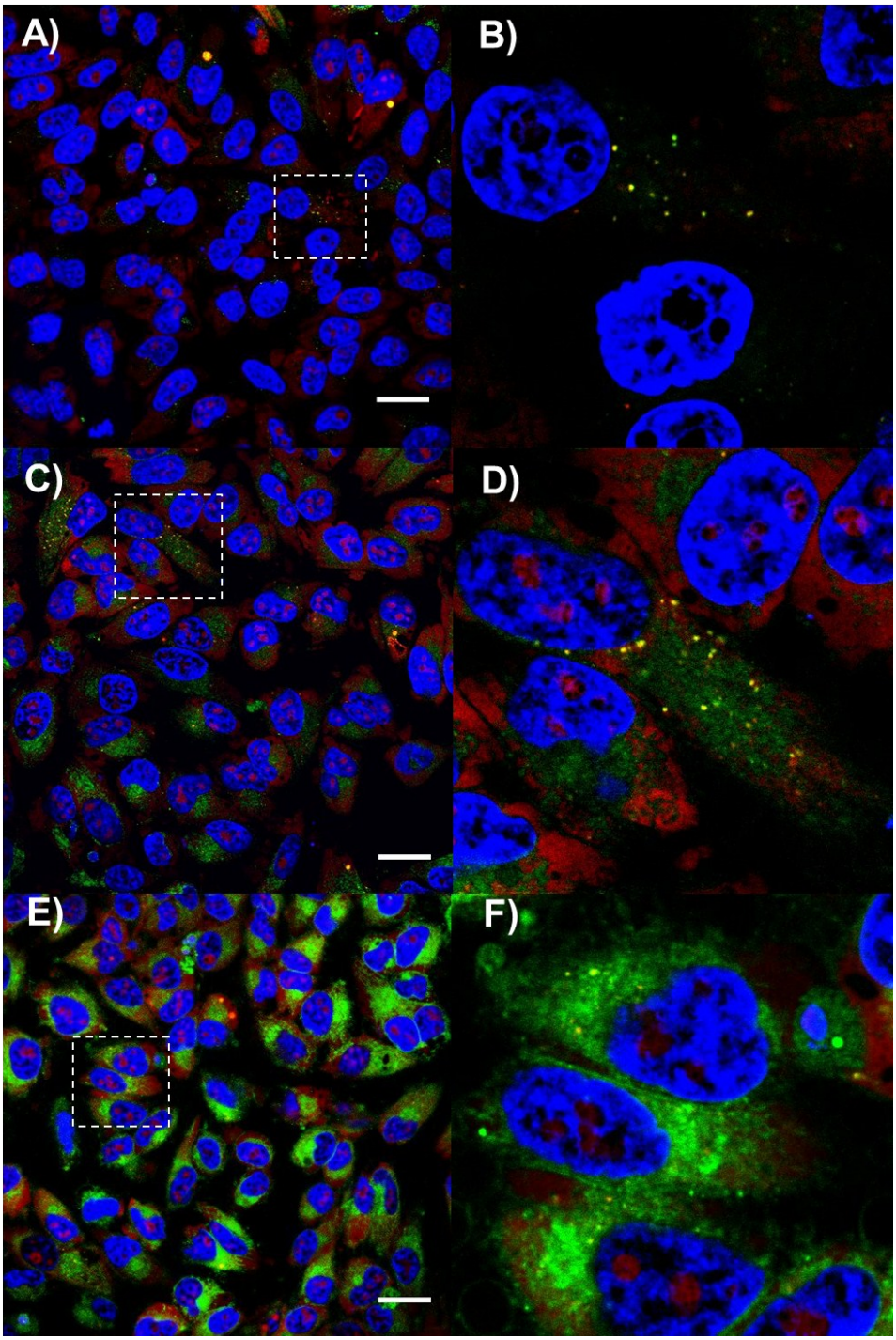


Figure S6. Confocal images of fixed HeLa cells incubated for 30 min with 2 μg/ml of ***p*-CNO/3** at 4 ^{o}C (A,B) and 37 ^{o}C (C,D). Cells were stained with Hoechst (blue). Scale bar 25 μm.

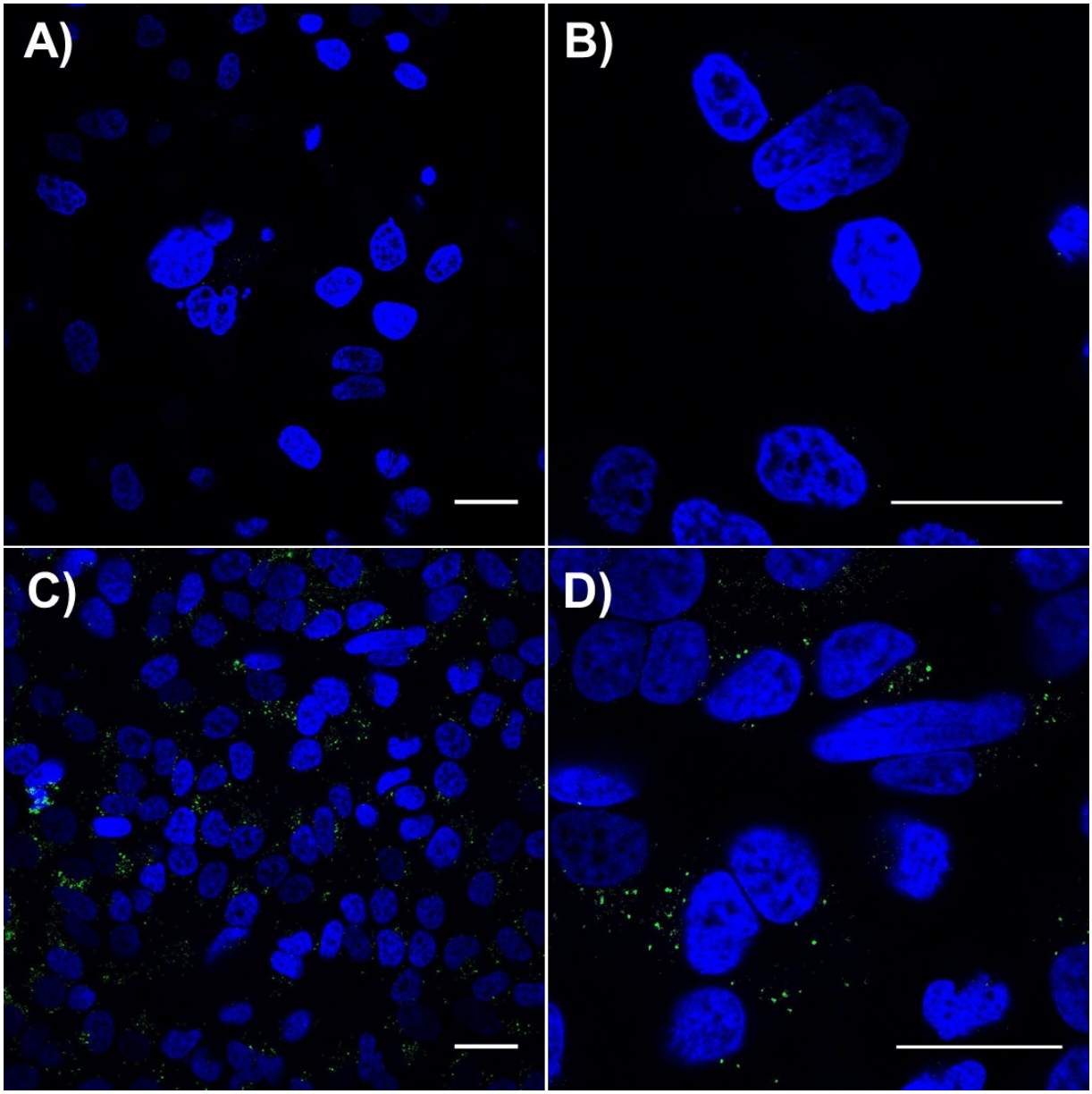
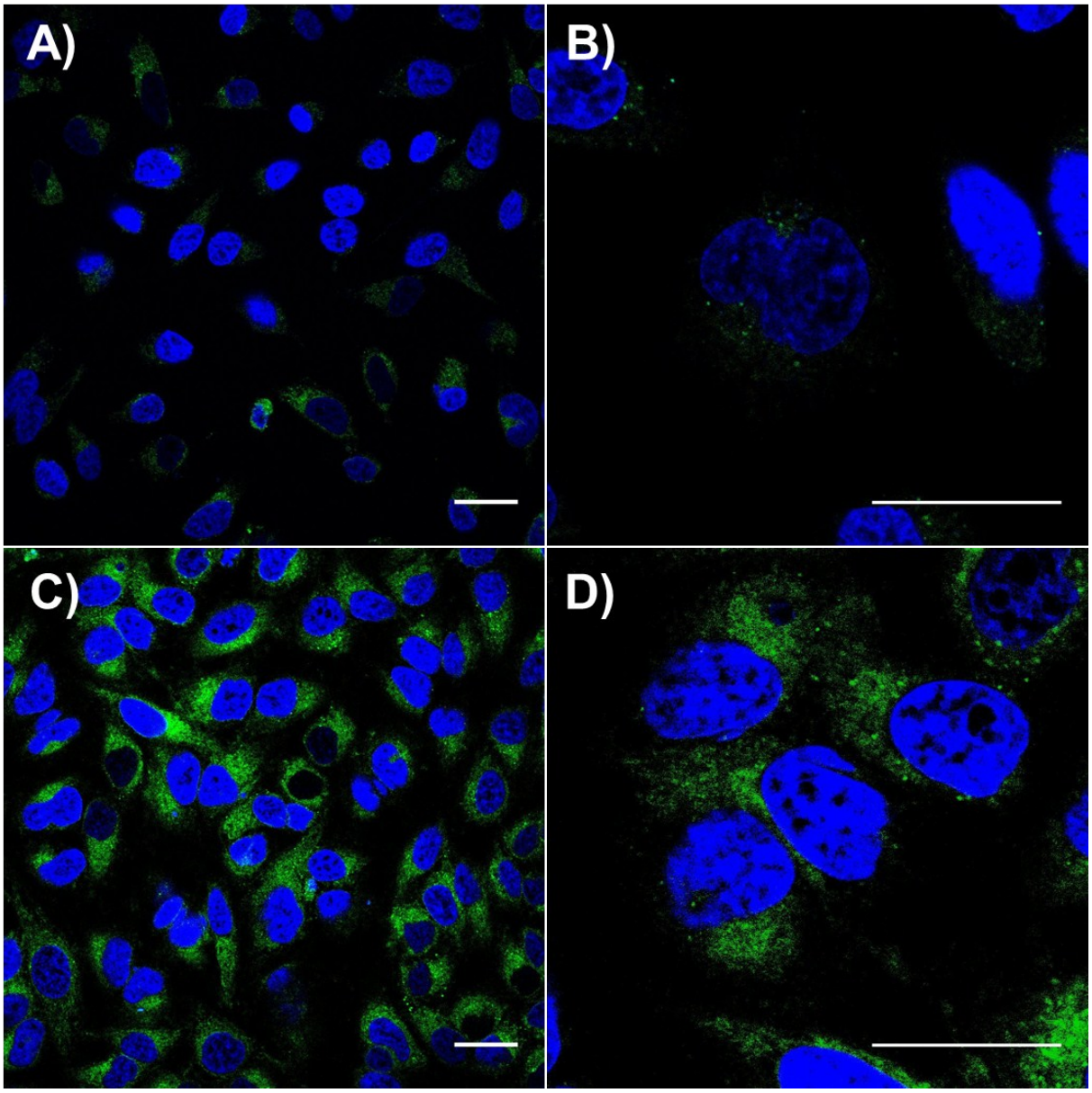


Figure S7. Confocal images of fixed HeLa cells incubated for 30 min with 10 μg/ml of ***p*-CNO/3** at 4 ºC (A,B) and 37 ºC (C,D). Cells were stained with Hoechst (blue). Scale bar 25 μm.



References

1 A. T. R. Williams, S. A. Winfield, J. N. Miller, *Analyst* **1983**, *108*, 1067.

2 Lazarides, T.; Kuhri, S.; Charalambidis, G.; Panda, M. K.; Guldi, D. M.; Coutsolelos, A. G. *Inorg. Chem.* **2012**, *51*, 4193.

3 Vicidomini, G.; Coto Hernandez, I.; d'Amora, M.; Cella Zanacchi, F.; Bianchini, P.; Diaspro, A. *Methods* **2014**, *66*, 124.