Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Tracking and recording of intracellular oxygen concentration variations to cell organelles: Preparation and function of azide-modified fluorescent probes

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Additional Experimental Procedures

General Methods. Regents were purchased from Wako pure chemical industries, Tokyo chemical industries, Sigma Aldrich, and eurofins Genomics. Rat liver microsomes were purchased from Veritas. The course of reaction was monitored by Thin-layer chromatography on silica gel plates (Silica gel 60 F254). Wakogel 60 was used for silica gel column Chromatography. NMR spectra were recorded on a JNM-ECX500II (^{1}H: 500 MHz, ^{13}C: 125 MHz) spectrometer (JEOL) and chemical shifts are expressed in ppm downfield from tetramethylsilane, using residual protons in the solvents as internal standards ($CDCl_3$: δ 7.26 in ^{1}H NMR, δ 77.0 in ^{13}C NMR, DMSO-d_6: δ 2.49 in ^{1}H NMR, δ 39.5 in ^{13}C NMR). FAB mass spectrometry was performed with a JMS-700A mass spectrometer (JEOL), using nitrobenzyl alcohol as a matrix. Fluorescence measurement was carried out on a FP-8300 spectrofluorophotometer (JASCO). UV-visible spectra were obtained at 400-1000nm using a JASCO V-630 UV/VIS spectrophotometer. The cells were incubated using CO_2 incubator (MCO-18AIC, SANYO) at 37 ˚C in 5% CO_2. Cell observation were used by C2 confocal laser scanning microscope (Nikon). Hoechst-NH_2 (*J. Am. Chem. Soc.*, **1996**, *118*, 7055.) and Cy-N_3 (*Nanoscale*, **2018**, *10*, 13462.) were prepared according to the reported procedure.

Cell culture. A549, HCT116, and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10 % fetal bovine serum (FBS), and 1 % penicillin-streptomycin. The cells were maintained at 37 ˚C in 5% CO_2 and were kept in a logarithmic growth phase by routine passages every 3−4 days. Prior to the use of cells, the densities of cells were determined using a hemocytometer.

Intracellular localization. A549 was incubated with Cy-N_3 and mitotracker green as a mitochondria staining dye for 30 min. After the incubation, the medium was washed using DPBS two times, and then the cells were observed using a confocal laser scanning microscope. (Excitation wavelengths were 685 nm for Cy-N_3 and 488 nm for mitotracker green respectively). On the other hand, A549 was incubated with Hoechst-N_3 for 30 min and the medium was washed using DPBS. After washing, the A549 cell was further incubated with RedDot in DPBS for 5 min and then the cells were observed using a confocal laser scanning microscope without washing. (Excitation wavelengths were 405 nm for Hoechst-N_3 and 640 nm for RedDot respectively)

Imaging of A549 cells with various oxygen concentrations. A549 cells were Incubated with 5 μM of Hoechst-N_3 or 1 μM of Cy-N_3 under various oxygen concentrations (0.3 and 21 %) for 30 min and 4 h respectively. Then, the medium was washed using DPBS twice and fluorescence images were obtained. For the imaging of hypoxic cells, we made hypoxic cells using Ruskinn Invivo 400. The cells were incubated under hypoxic conditions (0.3% O_2) for 24 h using hypoxic chamber (Ruskinn Invivo 400) to be hypoxic cells. Then, the probes were added to the medium and the cells were further incubated under hypoxic conditions. After the cells were washed, the imaging by microscopy was conducted.

Analysis of the location of enzymatic reduction for Hoechst-N_3 and Cy-N_3. A549 cells were incubated with 5 μM Hoechst-N_3 or 1 μM of Cy-N_3 under Aerobic conditions for 30 min. After washing with DPBS twice, A549 cells were further incubated in N_2 gas parged DMEM for 15 min. Then the medium was replaced by DPBS and fluorescence images were obtained.

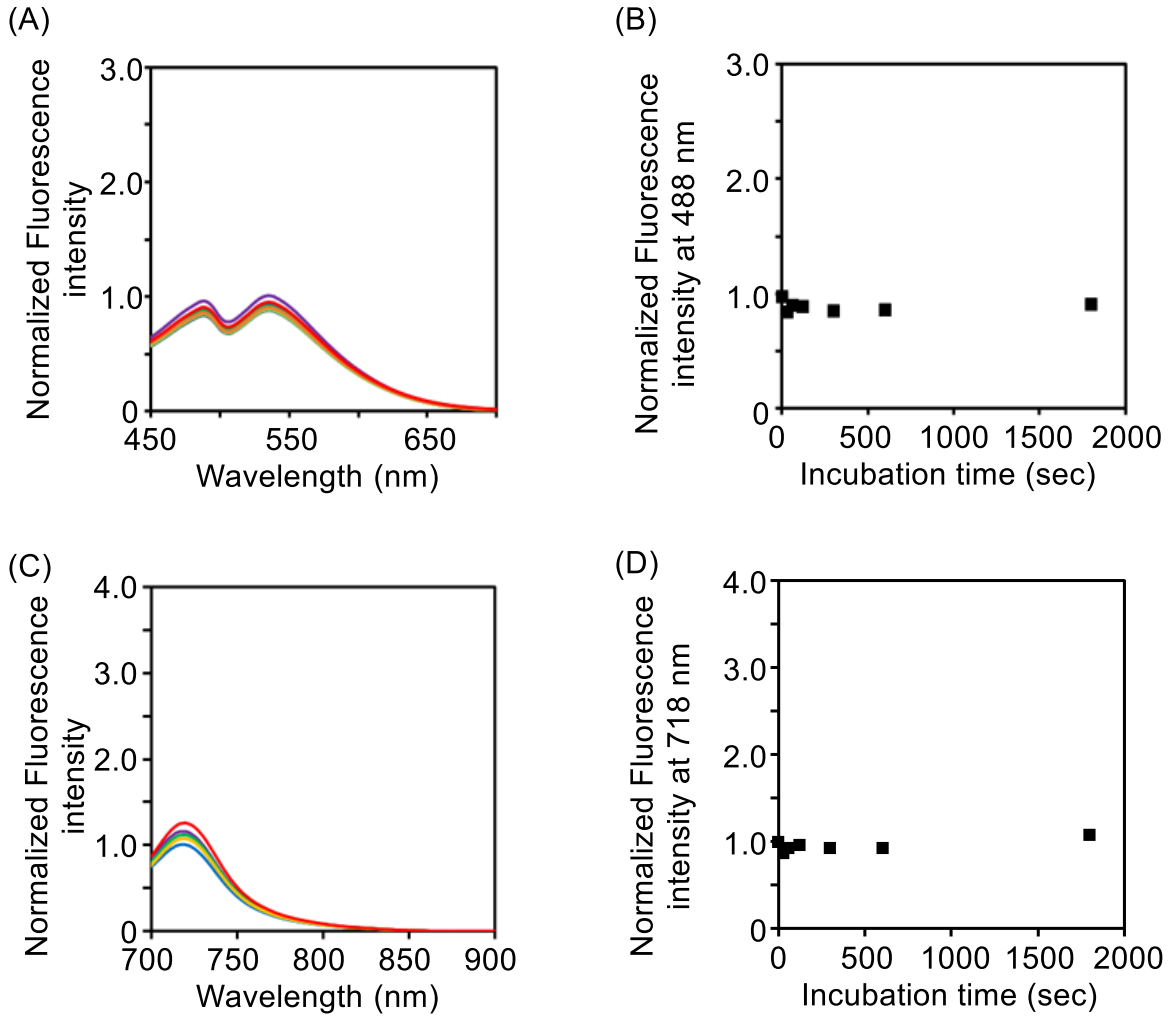

Figure S1. Fluorescence spectra of Hoechst-N_3 (A,B) and Cy-N_3 (C,D) after enzymatic reduction under 10% O_2 conditions: Hoechst-N_3 (500 nM) or Cy-N_3 (500 nM) were incubated with rat liver microsome (1.0 mg/mL) at 37 ˚C in phosphate buffer (pH 7.4) and then the fluorescence spectra were measured. Excitation wavelength was 405 and 685 nm for Hoechst-N_3 and Cy-N_3, respectively. (A) Emission of Hoechst-N_3 upon enzymatic incubation for 0-1800 sec (purple: 0 min, dark blue: 30 sec, blue: 60 sec, green: 120 sec, light green: 300 sec, orange: 600 sec, red: 1800 sec) under 10% O_2 conditions. (B) Plot of fluorescence intensity of Hoechst-N_3 at 488 nm during enzymatic reaction. The data were obtained from Figure S1A. (C) Emission of Cy-N_3 upon enzymatic incubation for 0-30 min (purple: 0 min, dark blue: 30 sec, blue: 60 sec, green: 120 sec, light green: 300 sec, orange: 600 sec, red: 1800 sec) under 10% O_2 conditions. (D) Plot of fluorescence intensity of Cy-N_3 at 718 nm during enzymatic reaction. The data were obtained from Figure S1C.

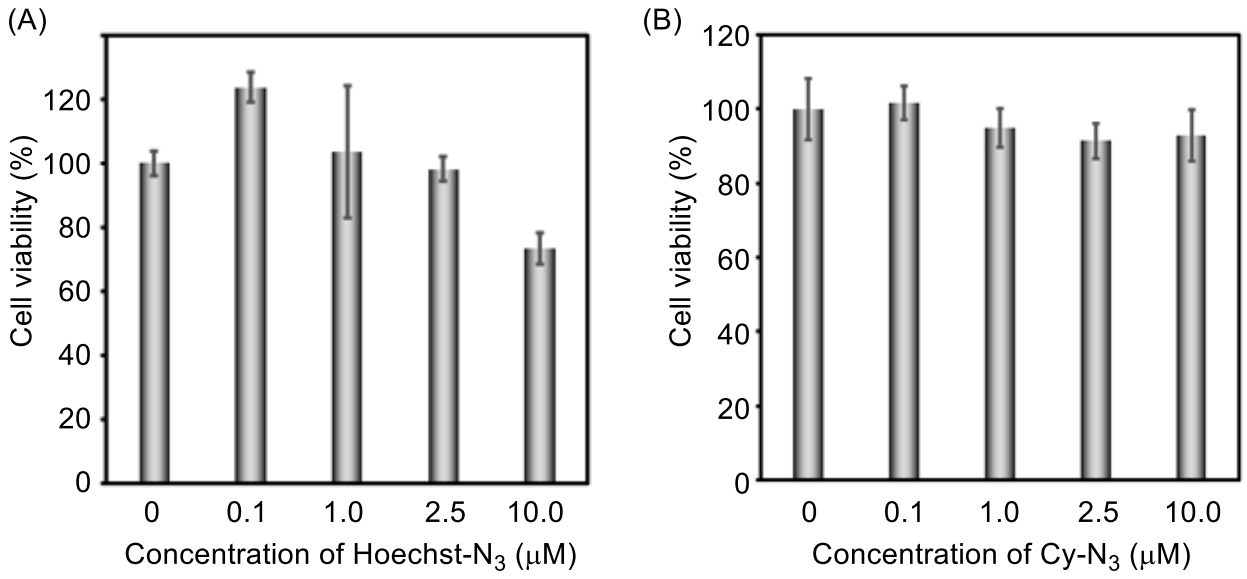

Figure S2. Cytotoxicity of Hoechst-N_3 (A) and Cy-N_3 (B) against A549 cells. The cells were cultured in the presence or absence of the probes (0, 0.1, 1.0, 2.5, and 10.0 μM) at the designated concentration for 24 h at 37 ˚C. Results are shown with the mean ± S.D (n = 3).

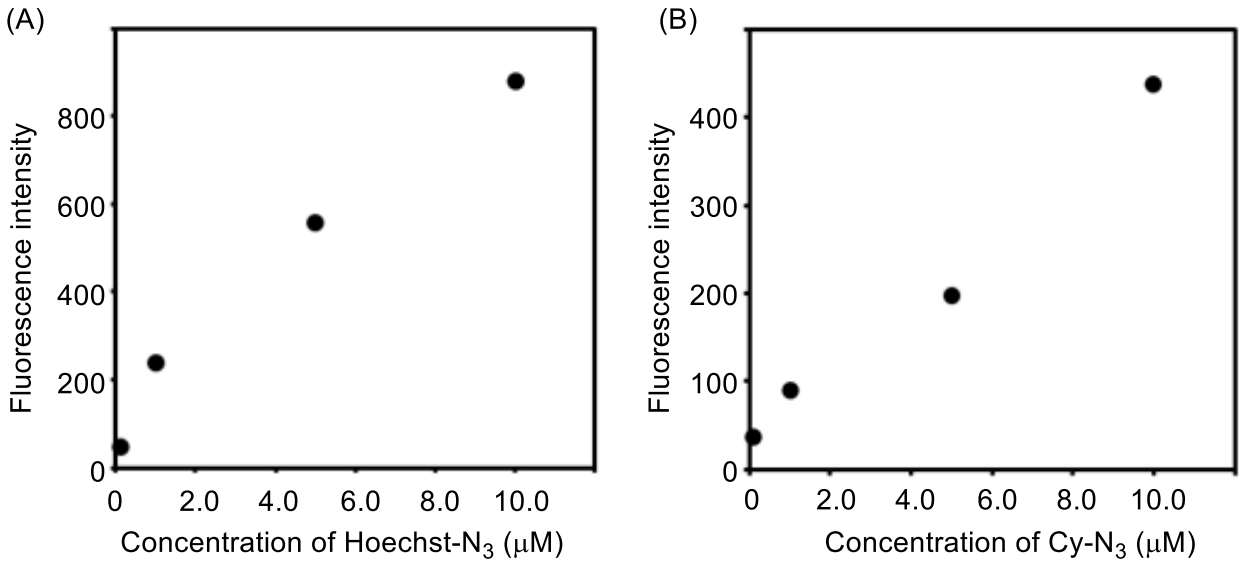

Figure S3. Fluorescence intensity of Hoechst-N_3 (A) and Cy-N_3 (B) in A549 cells under hypoxic conditions. The cells were cultured in the presence of the probes (0, 1.0, 5.0 and 10.0 μM) at the designated concentration for 0.5 h (A) or 4 h (B) at 37 ˚C. After wash, the cells were subjected to microscopy.

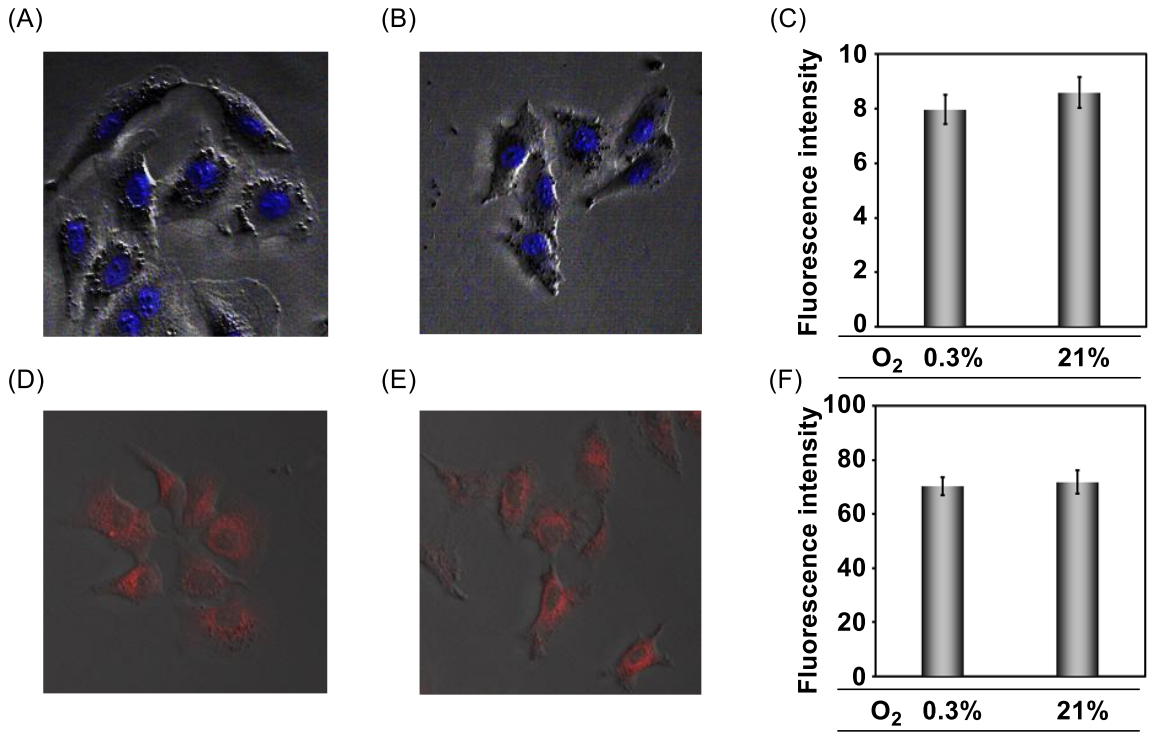

Figure S4. Fluorescence imaging of oxygenation of A549 cells, which was induced by change of medium. The cells were loaded with reduction products of Hoechst-N_3 (5 μM, A, B and C) and Cy-N_3 (1 μM, D, E and F), and then incubated (for A and B: 30 min, for D and E: 4 h) under hypoxic conditions (0.3% O_2). Then, the medium was replaced by flesh medium which were kept under the 21% oxygen concentrations and the cells were further incubated under aerobic conditions for 10 min. Before (A and D) and after (B and E) the replacement, the images were taken by means of microscopy. (C) Fluorescence intensity before and after stimulation. Fluorescence intensities (417-477 nm) were obtained from obtained from Figure S4A and S4B. (F) Fluorescence intensity before and after stimulation. Fluorescence intensities (640-1000 nm) were obtained from obtained from Figure S4D and S4E.

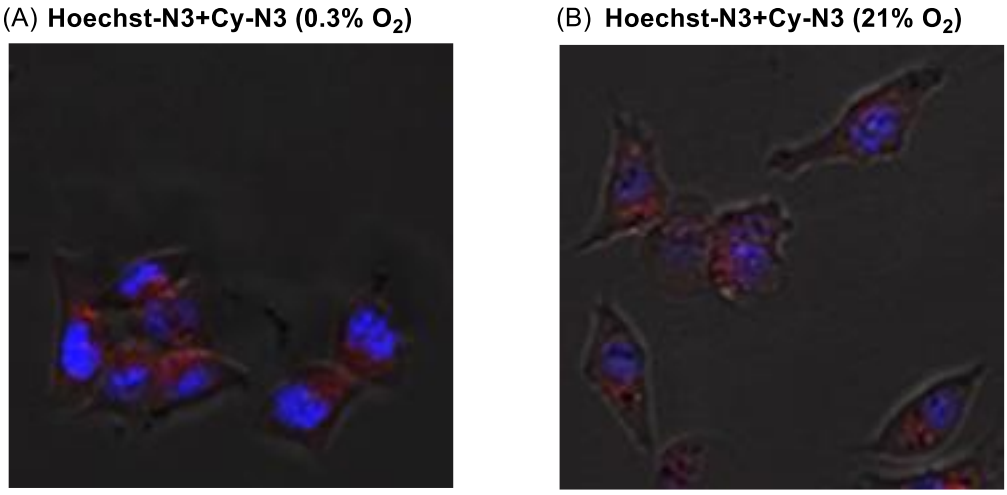

Figure S5. Emission images of HCT116 cells as incubated with Hoecsht-N_3 and Cy-N_3. The cells were incubated with Hoechst-N_3 (5 μM) and Cy-N_3 (1 μM) under hypoxic (A: 0.3% O_2) or aerobic conditions (B: 21% O_2) and then, the images were immediately taken by means of confocal microscopy. Excitation wavelengths were 405 and 640 nm for Hoechst-N_3 (blue) and Cy-N_3 (red), respectively.

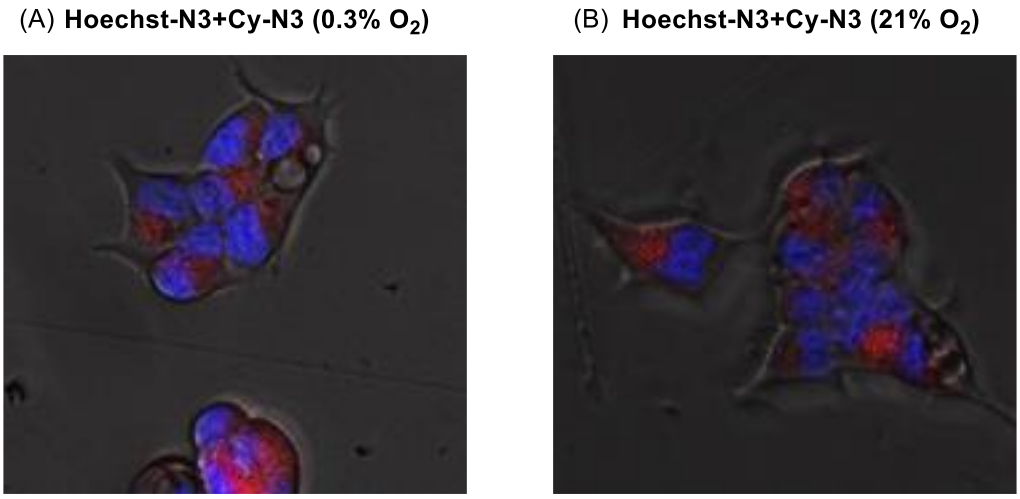

Figure S6. Emission images of HEK293 cells as incubated with Hoecsht-N_3 and Cy-N_3. The cells were incubated with Hoechst-N_3 (5 μM) and Cy-N_3 (1 μM) under hypoxic (A: 0.3% O_2) or aerobic conditions (B: 21% O_2) and then, the images were immediately taken by means of confocal microscopy. Excitation wavelengths were 405 and 640 nm for Hoechst-N_3 (blue) and Cy-N_3 (red), respectively.

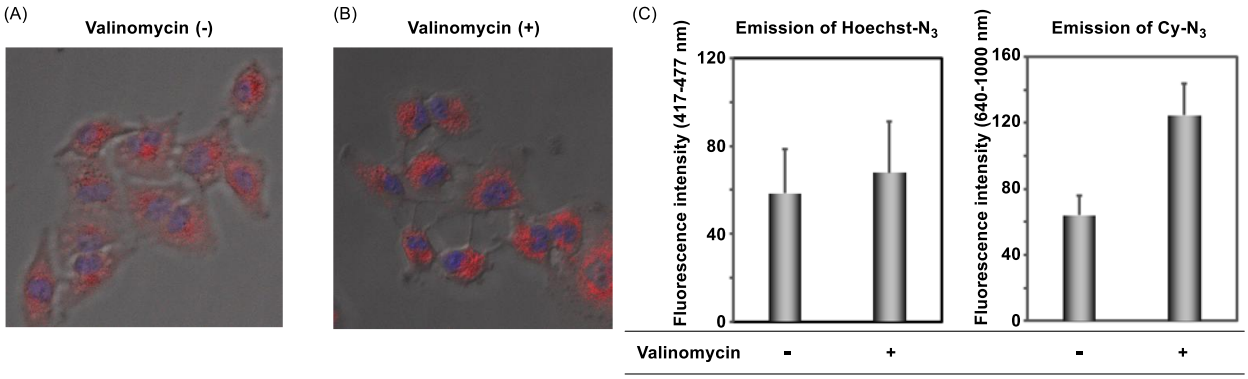

Figure S7. Fluorescence imaging of deoxygenation of A549 cells, which was induced by mitochondrial uncoupler (valinomycin, 1 μM) stimulation. The cells were loaded with Hoechst-N_3 (5 μM) and Cy-N_3 (1 μM), and then incubated for 30 min. After incubation, the cells were stimulated by valinomycine. (A,B) Fluorescence image of the cells before stimulation (A) and after stimulation (B) by valinomycin. Excitation wavelength was 405 and 640 nm for Hoechst-N_3 (blue) and Cy-N_3 (red), respectively. (C) Fluorescence intensity before and after stimulation. Fluorescence intensity of Hoecsht-N_3 (left, 417-477 nm) and that of Cy-N_3 (right, 640-1000 nm) were obtained from Figure S3A and S3B.

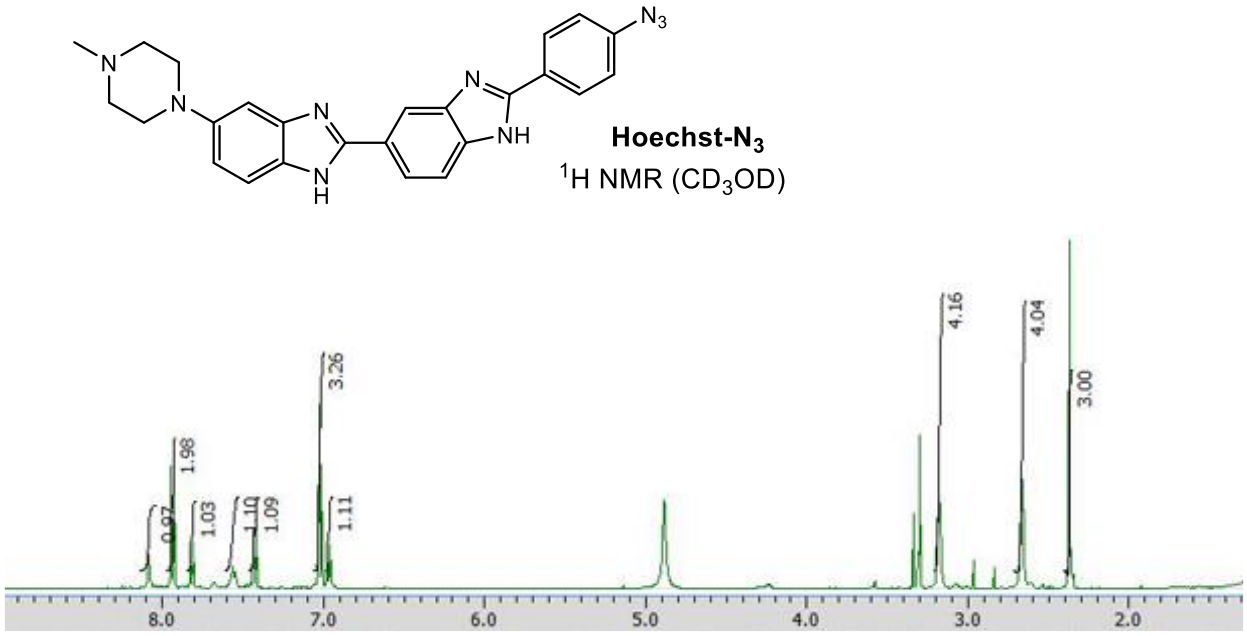

Figure S8. ^{1}H NMR spectrum of Hoechst-N_3.

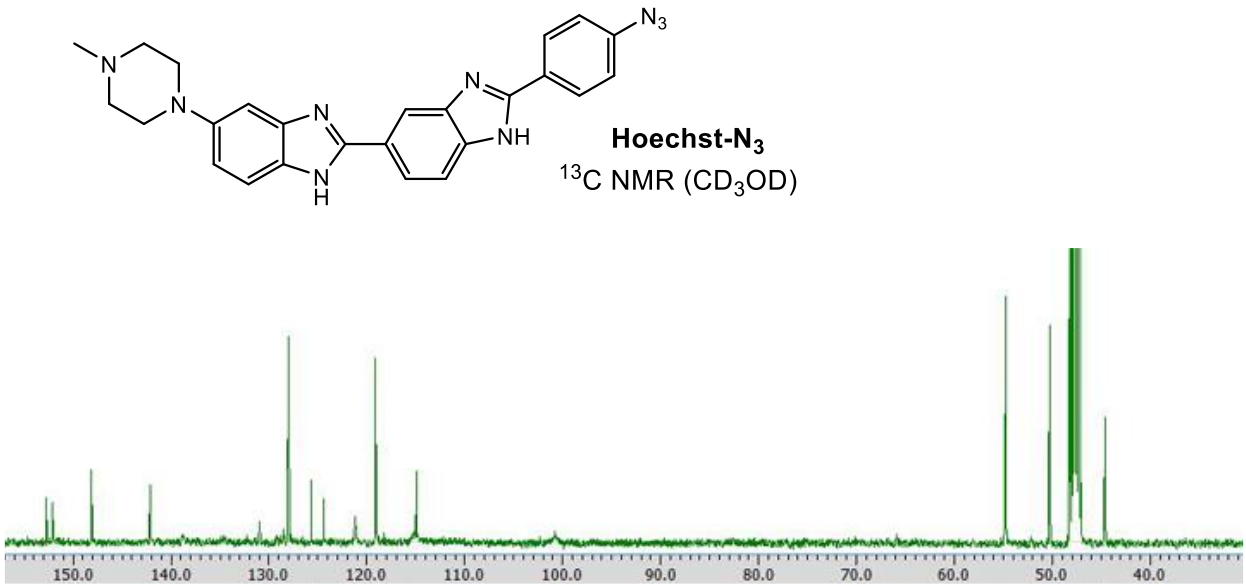

Figure S9. ^{13}C NMR spectrum of Hoechst-N_3.