Supporting information

Ligand-protected Nanoclusters-mediated Photoswitchable Fluorescent Nanoprobes towards Dual-color Cellular Imaging

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Materials and experiments

Materials

All the materials were of analytical grade. Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) and Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, USA). 1-(2-Hydroxyethyl)-3,3-dimethylindolino-6'-nitrobenzopyrylospiran (SP) was purchased from TCI (Tokyo, Japan). 6-Aza-2-thiothymine (ATT) was purchased from Alfa Aesar (Massachusetts, USA). Arginine and DSPE-mPEG (MW: 2000) were purchased from Aladdin (Shanghai, China). Succinic anhydride, N-hydroxy-succinimide (NHS), 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Aladdin (Shanghai, China).

Synthesis of DPF NPs

ATT/Arg AuNCs were synthesized according to the previous literature.[1] Mixture of ATT/Arg-AuNCs in water/ethanol (1: 1) and spiropyran in ethanol was added into an aqueous solution of DSPE-mPEG (MW: 2000) under continuous ultrasonication. In a typical process, 200 μL as-prepared ATT/Arg-AuNCs (5.63 mM of Au) in water/ethanol was mixed with 25 μL spiropyran (20 mM) in ethanol under continuous ultrasonication at room temperature. Then, 45 μL of above mixture solution was slowly added into DSPE-mPEG solution (500 μL, 3.2 mg/mL) under ultrasonication. Next, the solution was purified by passing through a 0.25 μm syringe filter to remove undissolved dyes and other large aggregates. The resultant products were then stored at 4 °C in the dark. The concentration of DPF NPs for the following experiments was estimated based on the concentration of Au. According to characteristic absorption peak of MC state of DPF NPs, the ratio of SP to AuNCs in DPF NPs was calculated to be ca. 20:1. The concentration of spiropyran was chosen mainly based on the solubility of spiropyran in the mixture solution of AuNCs and spiropyran, in order to ensure a high loading amount of spiropyran with a good stability.

Determination of FRET parameters

overlap integral was calculated by equation 1:

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \quad (1),$$

where $F_D(\lambda)$ is the wavelength dependent donor emission spectrum, $\varepsilon_A(\lambda)$ is the extinction coefficient spectrum of the acceptor. The $J(\lambda)$ was calculated to be 6.5×10^{14} $nm^4/(M\cdot cm)$ by FluorTools. Then, R_0 (Förster distance) was calculated to be 6.4 nm according to equation 2:

$$R_0 = 0.2108(n^{-4}\varphi_D k^2 J)^{\frac{1}{6}} \quad (2),$$

where n is the index of refraction of the solution (for water, n=1.33), φ_D is the fluorescence quantum yield of the donor, k^2 is the orientation factor for the transition dipoles (k^2 = 0.67) and J is the overlap integral. The FRET efficiency in DPF NPs was calculated as 90% based on equation 3：

$$E = 1 - \frac{I_{FRET\ ON}}{I_{FRET\ OFF}} \quad (3),$$

where E is FRET efficiency and I is the fluorescence intensity of fluorescent AuNCs. According to the FRET efficiency (90%) of DPF NPs, distance (r) between AuNCs and spiropyran was calculated as 4.4 nm by equation 4:

$$E = \frac{1}{1 + (\frac{r}{R_0})^6} \quad (4)$$

Synthesis of SP-BSA

Carboxylic acid-terminated SP (SP-COOH) was synthesized according to the previous literature.[2] After being activated by EDC and NHS in DMSO for overnight, SP-COOH was covalently conjugated to BSA in PBS solution (10 mM, pH=7.45) at molar ratio 1:1 for 1 h. Further, SP-BSA was obtained after purification by PD MiniTrap G-25 (GE, USA).

Characterization

All UV-Vis absorption spectra were measured by a U-3900H spectrophotometer (Hitachi, Tokyo, Japan). Fluorescence measurements were performed on a FLS980 Fluorometer (Edinburgh, UK) with a 1.0 cm quartz cell. Fluorescence decay curves were recorded on DeltaFlex modular fluorescence lifetime system (Horiba, Kyoto, Japan). Dynamic light scattering (DLS) sizes were measured in water solution by Zetasizer Nano ZS (Malvern, UK). Morphology and elements analysis of samples were obtained by transmission electron microscope (FEI, Talos

F200X) at an operating voltage of 200 kV. The absolute quantum yield of as-prepared AuNCs was determined by the integrating sphere of Quantaurus-QY equipment (Hamamatsu, Japan).

In fluorescence spectra measurement, the fatigue ratio (R_f) was determined by the following equation 5:

$$R_f = \frac{F_n - F_0}{F_0} \quad (5),$$

where F_n is the fluorescence intensity of *n*-th fluorescence-on state and F_0 is the initial fluorescence-on state (n = 9 for 530 nm and n = 10 for 645 nm). Besides, on/off ratio ($R_{on/off}$) was determined by the following equation 6:

$$R_{on/off} = \frac{F_{on} - F_{off}}{F_{on}} \quad (6),$$

where F_{on} and F_{off} are the fluorescence intensity of on-state and off-state in each cycle, respectively.

Photochromic kinetics measurement

For photochromic kinetic measurement, the SP→MC process was triggered by light at 365 nm (1 mW·cm^{-2}) and fluorescence intensity (I) at 645 nm was recorded. The MC→SP process was triggered by light at 520 nm (1 mW·cm^{-2}) and fluorescence intensity at 645 nm was recorded. Photochromic rate constant k was obtained by the equation 7[3]:

$$\ln \frac{I_\infty - I}{I_\infty - I_0} = kt \quad (7),$$

where I_∞ is the final fluoresce intensity, I_0 is the initial fluorescence intensity, and I is the fluorescence intensity at time t.

Cell culture

All operations were performed in a sterile environment. DPF NPs were purified through a 200 nm filter before the cellular experiment. HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM), including 10% fetal bovine serum (FBS), 60 μg/mL of penicillin, and 100 μg/mL streptomycin in a humidified incubator (HF90, Heal Force, China) at 37 °C and 5% CO_2. The cell viability was evaluated by measuring the metabolic activity of HeLa cells using a CCK-8 assay. HeLa cells were seeded in a 96-well plate in cell medium (1×10^4 per well) overnight and then incubated in pure DMEM containing DPF NPs with different concentrations for 3 h at 37 °C and 5% CO_2. After washing thoroughly with phosphate buffer saline (PBS), cells were further incubated in cell medium for 24 h, followed by adding 100 μL fresh medium plus 6 μL CCK-8 solution (Beyotime, China). Upon 3 h incubation, the absorbance of the solution at 450 nm was measured.

Cell imaging

For cell imaging, cells were seeded in eight-well LabTek™ chambers (Nunc, Langenselbold, Germany) and allowed to adhere overnight in a humidified incubator at 37 °C and 5% CO_2 before experiments. Cells were then incubated with 40 μM DPF NPs in pure DMEM without FBS for 3 h at 37 °C and 5% CO_2 before washing thoroughly with PBS. Cell imaging was performed by using a Confocal Laser Scanning Microscope (SP8, Leica, Germany). DPF NPs were excited at 405 nm and the emission was collected in the range of 500-550 nm for the green channel, and 610-700 nm for the red channel.

For colocalization experiments, in order to induce more cellular lipid droplets, HeLa cells were treated with 0.5 mM oleic acid in DMEM for 6 h before incubation with BODIPY 493/503 and DPF NPs. Lipid droplet-specific BODIPY 493/503 in full DMEM medium (2 μM) was incubated with HeLa cells for 15 min before incubation with DPF NPs. Then, HeLa cells were subjected to fluorescence imaging as described above. BODIPY 493/503 was excited at 488 nm and the emission was collected in the range of 500-550 nm.

For distinguishing dynamic and static fluorescence signals, 0.2 μM MitoTracker™ Green (Thermo Fisher, USA) in full DMEM medium was incubated with HeLa cells for 15 min to label mitochondria before incubation with DPF NPs. Then, HeLa cells were subjected to fluorescence imaging as described above. For fluorescence photoswitching, the wavelength of visible light was set as 552 nm.

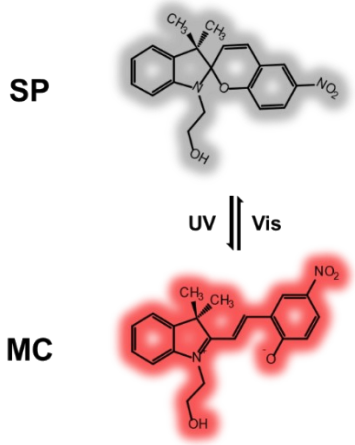

Figure S1. Chemical structures of spiropyran during the photochromism process.

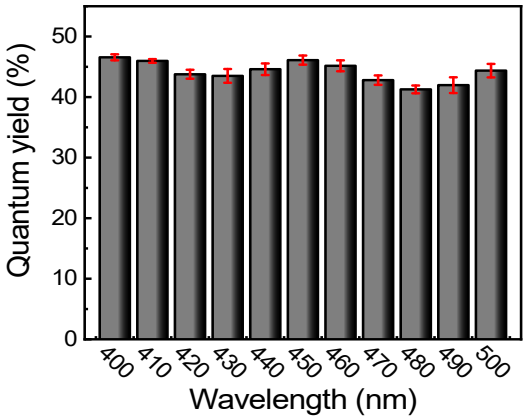

Figure S2. Absolute quantum yield of AuNCs at different excitation wavelengths.

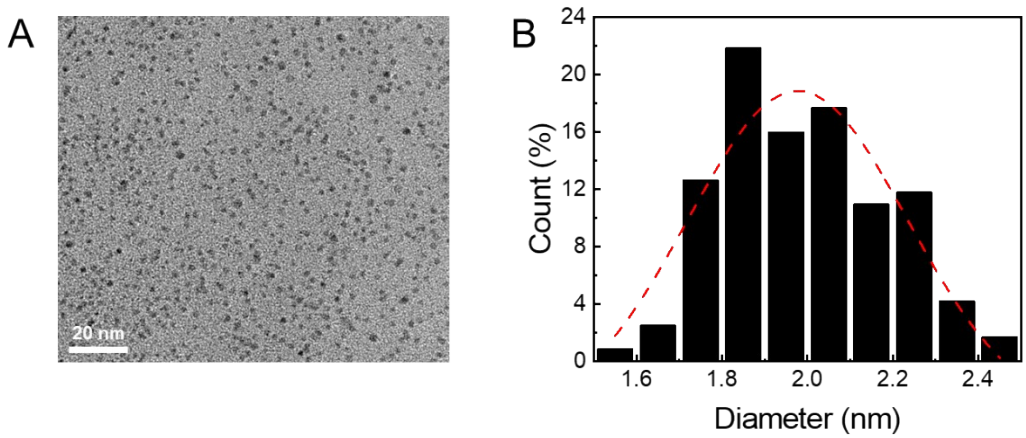

Figure S3. (A)TEM image of ATT/Arg-AuNCs and (B) Size distribution histogram of ATT/Arg-AuNCs.

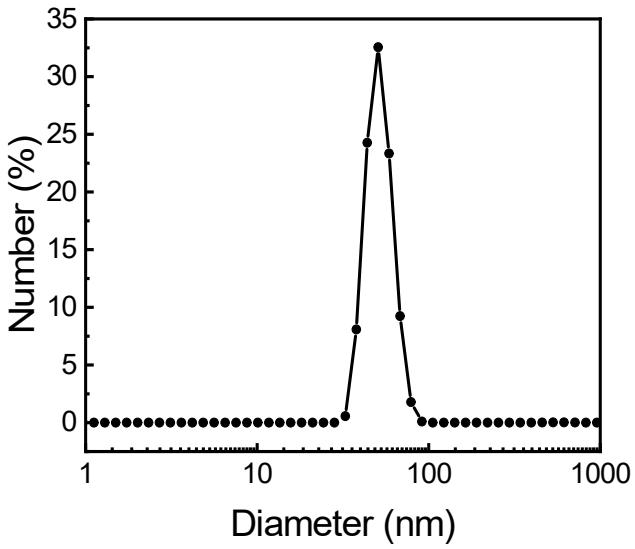

Figure S4. Hydrodynamic size of DPF NPs in aqueous solution measured by DLS.

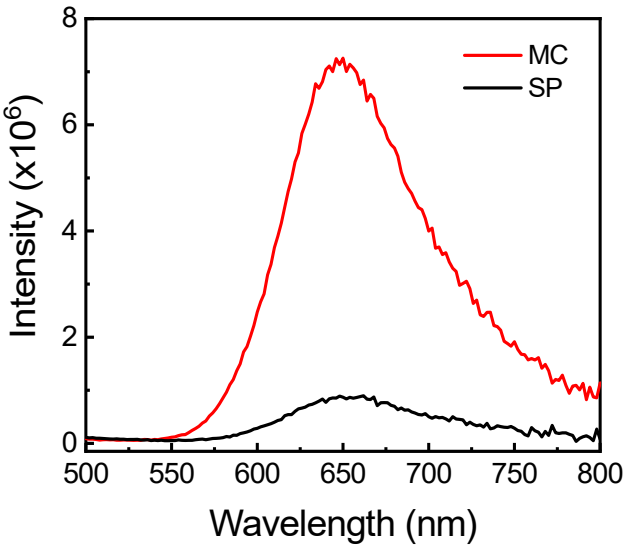

Figure S5. Fluorescence emission spectra of SP/MC in ethanol (Ex: 405 nm). SP→MC process was induced by irradiation of 365 nm light.

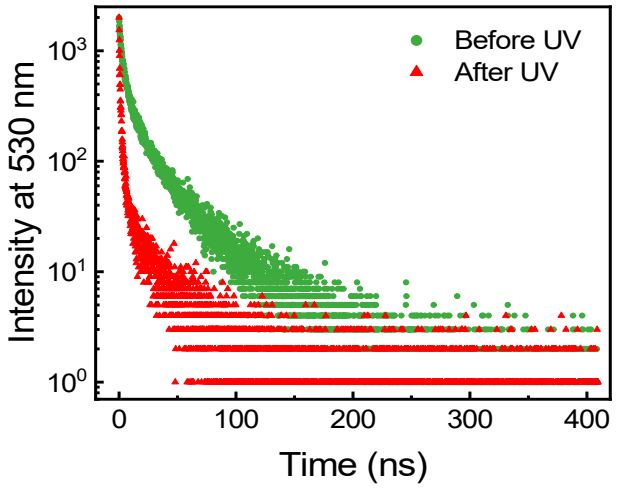

Figure S6. Fluorescence decay curves of DPF NPs before and after UV irradiation. Excitation wavelength: 400 nm.

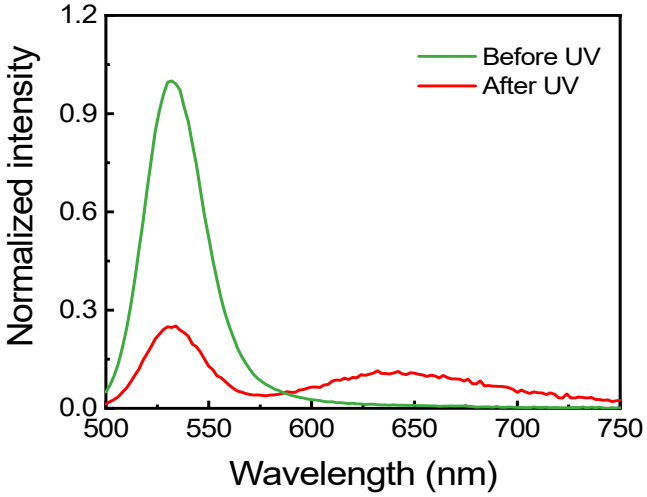

Figure S7. Fluorescence emission spectra of DPF NPs with half amount of spiropyran. Excitation wavelength: 405 nm. The FRET efficiency was calculated as 75%.

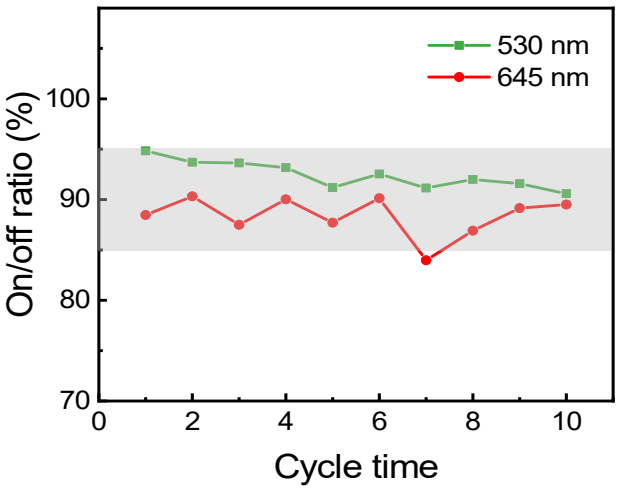

Figure S8. The on/off ratio at 530 nm and 645 nm of DPF NPs during photoswitching cycles.

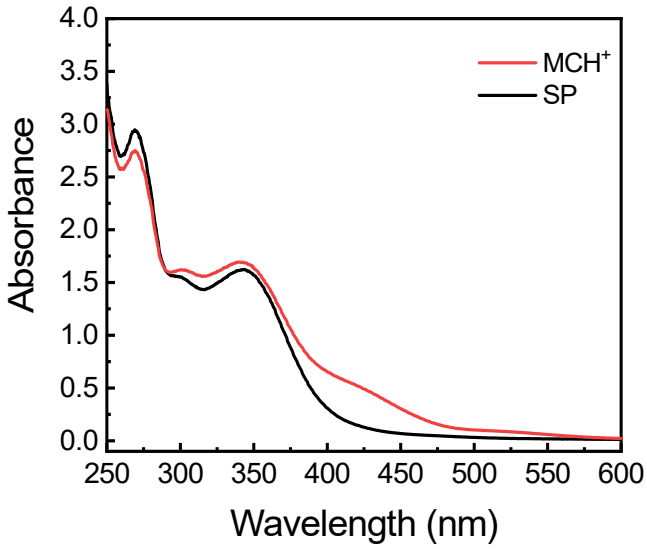

Figure S9. Absorption spectra of SP/ATT-AuNCs@DSPE-mPEG in aqueous solution.

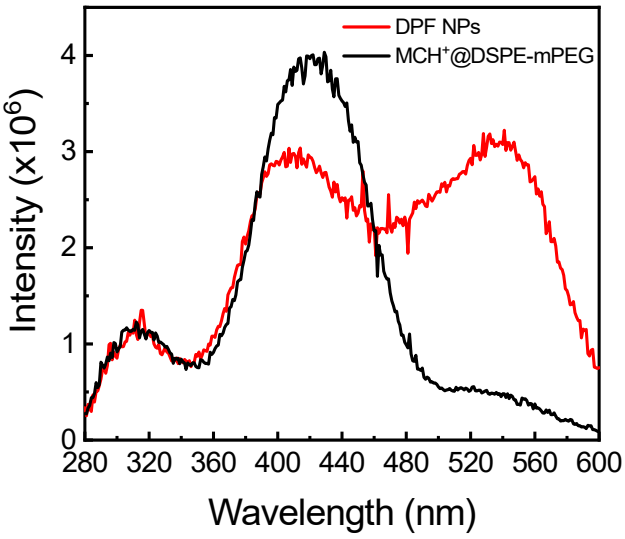

Figure S10. Fluorescence excitation spectra of MCH^+@DSPE-mPEG and DPF NPs in aqueous solution. Emission wavelength: 645 nm.

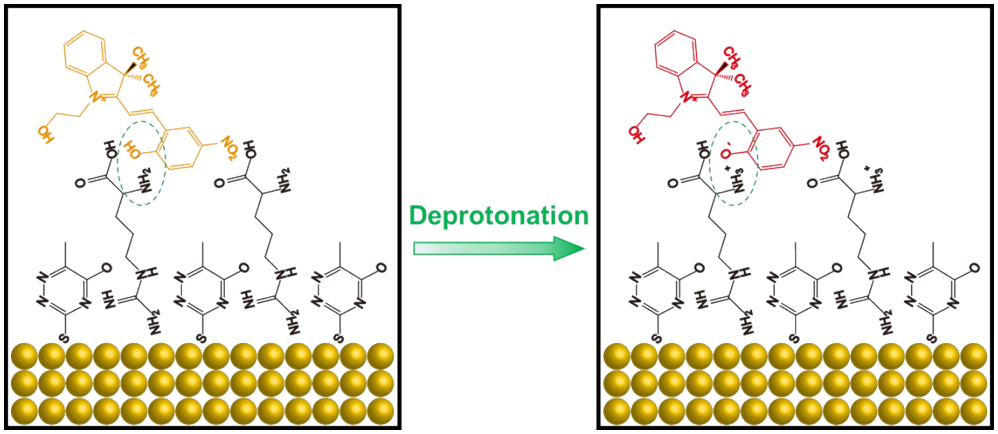

Figure S11. Scheme of the deprotonation of MCH^+.

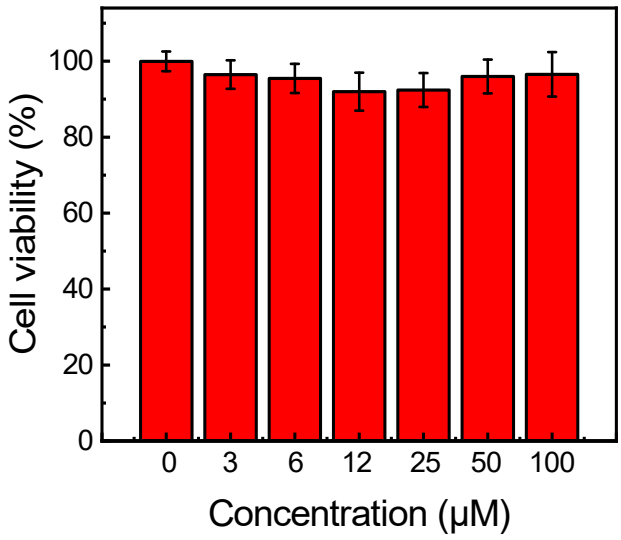

Figure S12. Cell viability of L929 cells after incubation with different concentrations of DPF NPs for 3 h.

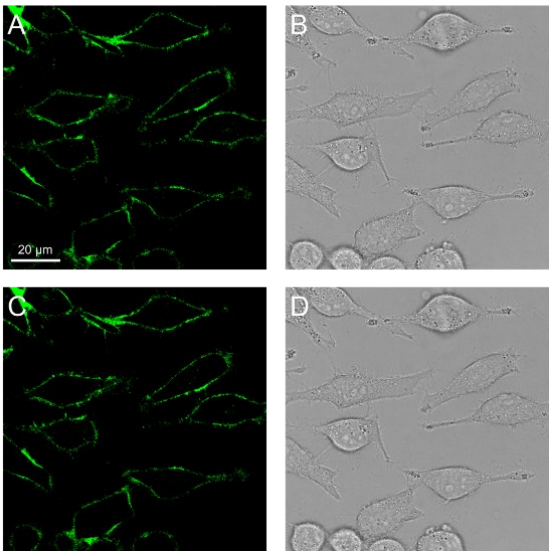

Figure S13. Fluorescence images of (A, C) green channel for CellMask™ Green, (B, D) bright field channel of HeLa cells (A, B) before and (C, D) after continuous irradiation of UV light (405 nm) for 90 seconds.

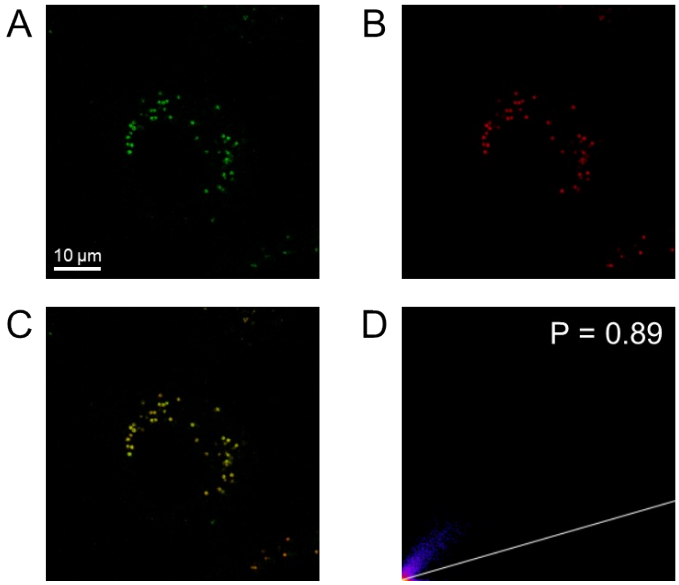

Figure S14. Fluorescence images of HeLa cells after the incubation with DPF NPs: (A) green channel; (B) red channel; (C) overlay channel. (D) Colocalization result of green and red channel signal, which was obtained by the Coloc 2 plugin in Image J.

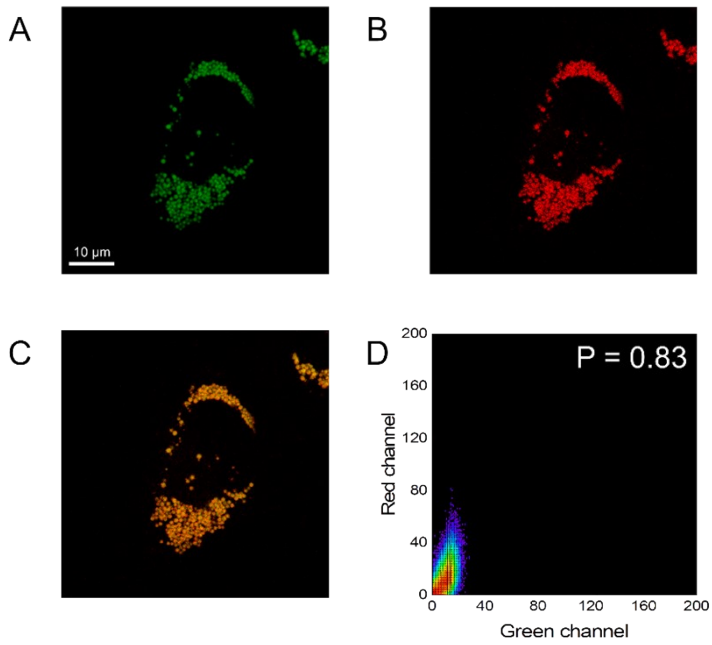

Figure S15. Fluorescence images of (A) green channel for BODIPY 493/503, (B) red channel for DPF NPs, and (C) overlay channel. (D) Colocalization results of green and red channels in HeLa cells, which was obtained by the Coloc 2 plugin in Image J.

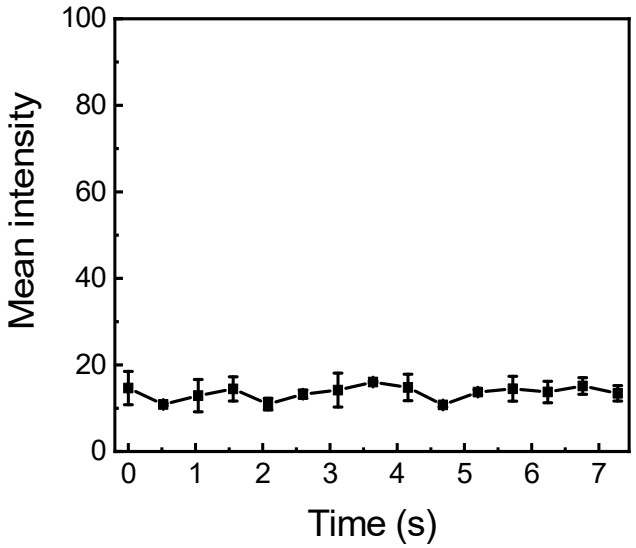

Figure S16. Fluorescence signal change in mitochondria region of Hela cells in the green channel after the incubation with MitoTracker™ Green.

Table S1. Fitting results of fluorescence lifetime.

	τ_1 (ns)	B_1	τ_2 (ns)	B_2	Average time (ns)
FRET ON	5.13±0.02	915.95±61.3	31.75±1.27	246.08±6.96	10.77±0.14
FRET OFF	1.38±0.02	610.69±4.87	20.65±0.16	45.73±2.70	2.72±0.04

The fluorescent lifetime data was fitting based on the exponential function 8:

$$I = A + B_1 exp(-t/\tau_1) + B_2 exp(-t/\tau_2) \quad (8)$$

The average time was calculated with the equation 9:

$$\tau_{avg} = \frac{B_1}{B_1 + B_2}\tau_1 + \frac{B_2}{B_1 + B_2}\tau_2 \quad (9)$$

References

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