Electronic Supplementary Material for

Label-free fluorescence imaging of cytochrome *c* in living body and anti-cancer drugs screening with nitrogen doped carbon quantum dots

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Experimental

Materials and chemicals

Ferric nitrate [$Fe(NO_3)_3$], $Cu(NO_3)_2$, $Ni(NO_3)_2$, aconitic acid, L-ascorbic acid (L-AA), and the amino acids including L-Tryptophan (L-Trp), L-Cysteine (L-Cys), L-Arginine (L-Arg), L-Lysine (L-Lys), L-Histidine (L-His), L-Tyrosine (L-Tyr), L-Phenylalanine (L-Phe), L-Methionine (L-Met), and glutathione (GSH) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Etoposide and cytochrome *c* (Cyt *c*) were obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA), bovine immunoglobulin G (bGG), bovine fibrinogen (bFG), lysozyme, trypsin, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were received from Sangon (Shanghai, China). Piceatannol (Pic), myricetin (Myr), dihydromyricetin (Dmy), shikonin (Shi), and gambogic acid (GA) were purchased from Pufei De Biotechnology (Chengdu, China). All reagents were of analytical grade and used as received without further purification. Ultrapure water purified with a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA) was used throughout the experiment.

Apparatus and characterization

The morphologies and sizes of N-doped CDs were characterized by transmission electron microscopy (TEM, Tecnai G2 TF20, FEI, USA). Fourier transform infrared spectroscopy (FT-IR) were collected from a IFS 120HR Fourier transform infrared spectrometer (Bruker, Germany). X-ray photoelectron spectroscopy (XPS) was conducted with an ESCALAB 250Xi spectrometer (ThermoFisher Scientific, USA). Time-resolved fluorescence spectra were carried out in a time-correlated-single-photon-counting (TCSPC) system from FL920P spectrometer (Edinburgh Instruments, U.K.) with λex = 370 nm. All fluorescence spectra were surveyed on an RF-5301PC fluorescence (FL) spectrophotometer using 5/5 nm slit width, and equipped with a 1 cm quartz cell (Shimadzu, Kyoto, Japan). The ultraviolet-visible (UV-vis) absorption spectra were acquired on a Perkin-Elmer Lambda-35 UV-visible double beam

scanning spectrophotometer with a 1 cm quartz cell (Beijing Purkinje General Instrument Co., Ltd., Beijing, China). The cellular fluorescence images were measured on a Perkin-Elmer LS55 scanning spectrofluorometer equipped with a Xenon flash lamp. Fluorescence imaging experiments of zebrafish were performed on an Olympus Fluoview 1000 confocal laser scanning microscope with excitation at 380 nm.

Preparation of fluorescent N-doped CDs

The N-doped CDs were synthesized according to our previous report with some modification. [1] Briefly, aconitic acid and tryptophan with a molar ratio of 2:1 were used as precursors to form a uniform powder. After heated at 220 °C for 6 h in a 50 mL Teflon-lined autoclave, subsequently purified using a 0.22 μm filtration membrane to remove large dots and dialyzed against ultrapure water, N-doped CDs were obtained.

In the control experiments, to investigate the influence of the quantity of nitrogen doping, aconitic acid and tryptophan with various molar ratios (1:0, 5:1, 4:1, 3:1, 1:1) were utilized. Other procedures were the same as those mentioned above.

Fluorescence detection of Cyt *c*

50 μL of the prepared N-doped CDs stock solution (10 μg·mL^{-1}) is mixed with 1.0 mL of 10.0 mM PBS buffer solution (pH 7.0). Then 10.0 μL Cyt *c* with different concentration (0, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, 1×10^4, 2×10^4, 3×10^4 nM) is added. The fluorescence spectrum was recorded with the excitation and emission wavelengths at 370/455 nm. The selectivity of the N-doped CDs probe toward Cyt *c* is evaluated by adding other proteins, amino acids or metal ions solutions instead of Cyt *c* in a similar way. All experiments are performed at room temperature.

Cytotoxicity assays

The cytotoxic effect of N-doped CDs probe was determined by the MTT assay.

Briefly, 5×10^3 HepG-2 cells were incubated with N-doped CDs in triplicate in a 96-well plate for 24 h at 37 °C with a final volume of 100 μL. Cells treated with dimethyl sulfoxide alone were used as controls. At the end of the treatment, 10 μL of MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C. An extraction buffer (100 μL, 10% SDS, 5% isobutanol, 0.1% HCl) was added, and the cells were incubated overnight at 37 °C. The absorbance values of the wells were recorded at 570 nm using a microplate reader (Thermo Scientific Multiskan GO, Finland). HepG-2 cells cultured without N-doped CDs were used as the control sample. The cytotoxic effect (VR) of N-doped CDs was assessed using the following equation: $VR = A/A_0 \times 100\%$, where A and A_0 are the absorbance of the experimental group and control group, respectively.

Cell culture and fluorescence imaging

The HepG-2 cells were seeded in a 12-well plate and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 units/mL), and streptomycin (100 units/mL) at 37 °C in a humidified atmosphere of 5% CO_2 overnight. After washed with 1.5 mL fresh DMEM for 3 times, the cells were incubated with 200 μg/mL N-doped CDs at 37 °C for another 2 h. After washed with 1.5 mL cold phosphate buffer solution (PBS, 10 mM, pH = 7.4) for 3 times, the N-doped CDs stained cells were treated by various concentrations of etoposide (0, 0.5, 1, 2 and 5 μM) in 1.5 mL DMEM at 37 °C for 2 h. Then the cells were rinsed with the medium for three times to remove the remaining N-doped CDs and etoposide. After the cells were washed with fresh PBS (10 mM, pH = 7.4) for three times, the fluorescence images were acquired by a fluorescent microscope (Leica DM 4000B microscope).

Imaging in zebrafish

The wild-type Tuebingen (TU) strain of zebrafish was supported by the Institute of Modern Physics, Chinese Academy of Sciences (Lanzhou, China). Zebrafish embryos were generated by natural pair-wise mating and raised in standard zebrafish embryo

E3 culture medium (5 mM NaCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4 \cdot 7H_2O$, 0.17 mM KCl) medium at 28.5 °C. Three days post-fertilization (3 dpf) embryos were collected and then divided into four groups. The first panel receiving with an E3 culture medium solution was served as a vehicle control. The second panel was incubated with a medium solution containing 50 μg/mL N-doped CDs for 30 min, and then washed with E3 culture medium for three times to remove free probe N-doped CDs. The third panel was pretreated with 50 μg/mL N-doped CDs for 30 min, followed by incubating with Cyt *c* (0.5 μM) for another 30 min. The fourth panel was pretreated with 50 μg/mL N-doped CDs for 30 min, and then etoposide (0.1 μM) for another 30 min. All the zebrafishes were washed with E3 culture medium for three times to remove free probes and immobilized in the center of a microscope slide before imaging operation. Photographs were taken imaged by an Olympus Fluoview 1000 confocal laser scanning microscope using fixed imaging parameters. For probe imaging, the zebrafish was excited by a solid state laser (380 nm, 20 mW) and captured at 390-550 nm.

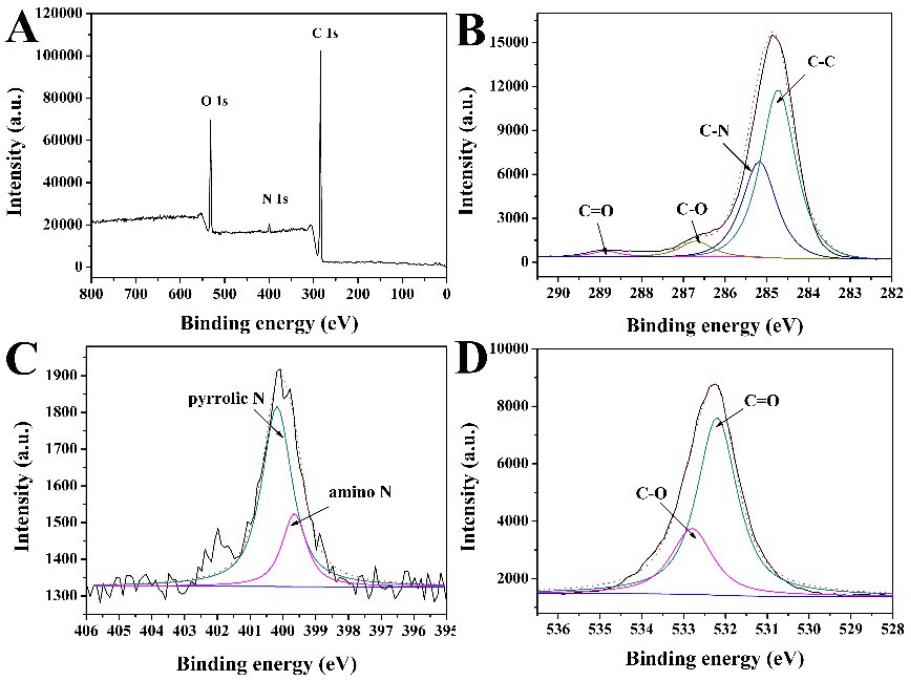

Figure S1. (A) Survey XPS spectra of N-doped C-Dots. High-resolution C 1s (B), N 1 s (C), and O 1s (D) spectra of the N-doped CDs.

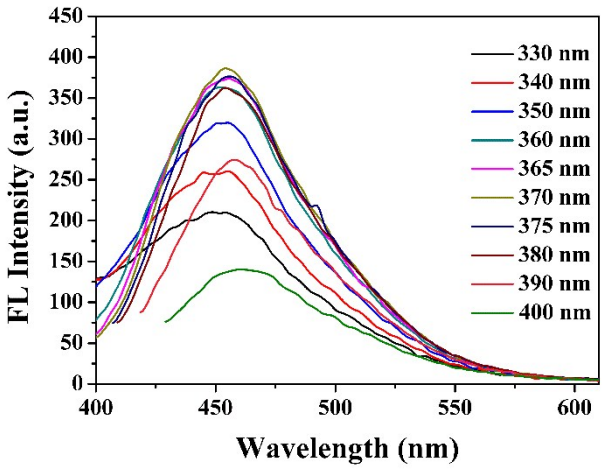

Figure S2. Fluorescent emission spectra of N-doped CDs with progressively longer excitation wavelengths from 330 to 400 nm.

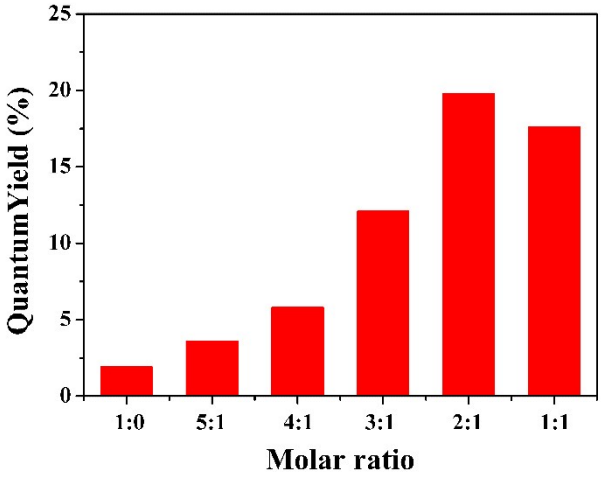

Figure S3. Effect of the mass ratio of aconitic acid and tryptophan in the precursor on the quantum yield of the obtained N-doped CDs.

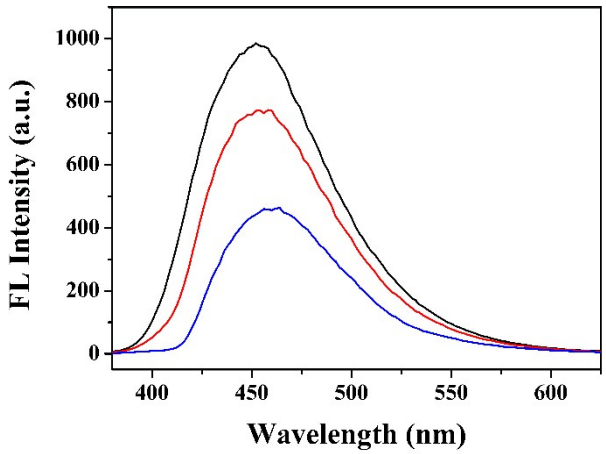

Figure S4. FL spectra of N-doped CDs solution in the absence (black line) and presence of Cyt *c* (red and blue lines).

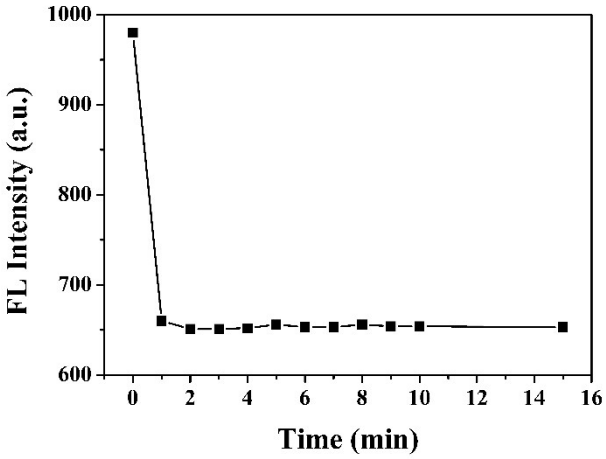

Figure S5. Time-dependent fluorescence intensity of N-doped CDs with the addition of Cyt *c* at room temperature.

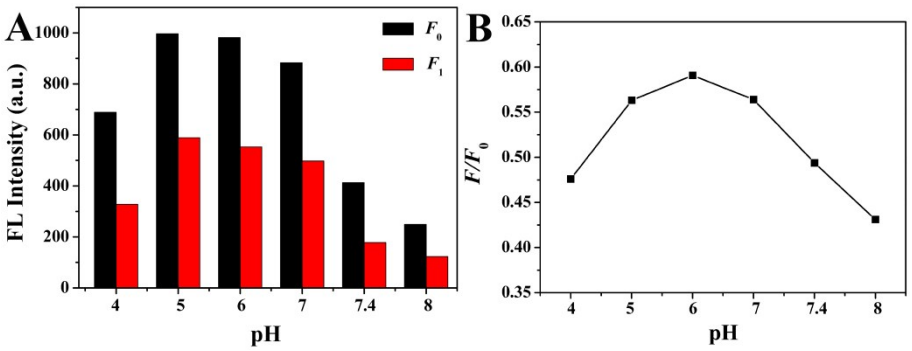

Figure S6. Fluorescence responses of N-doped CDs in the absence and presence of Cyt *c* at different pH values.

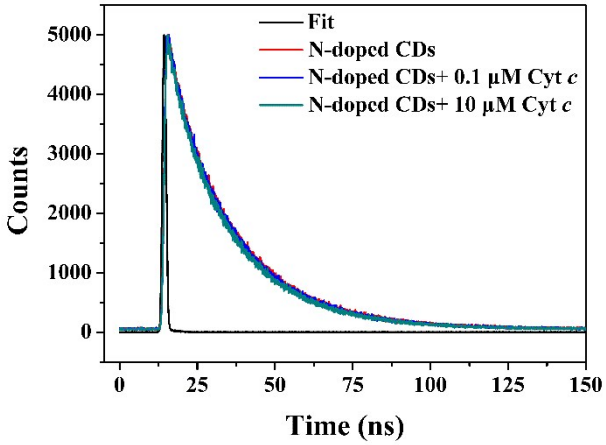

Figure S7. Time-resolved decay curves of N-doped CDs in the absence and presence of varied concentration of Cyt *c* in 10 mM PBS buffer solution (pH 7.0).

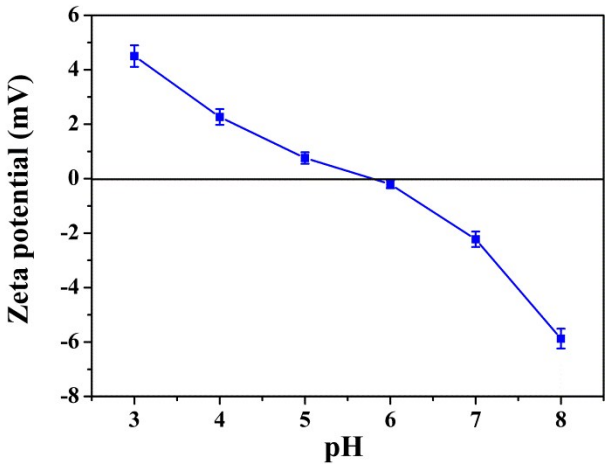

Figure S8. Zeta potential of N-doped CDs at different pH values. The error bars represent the standard deviation of three measurements.

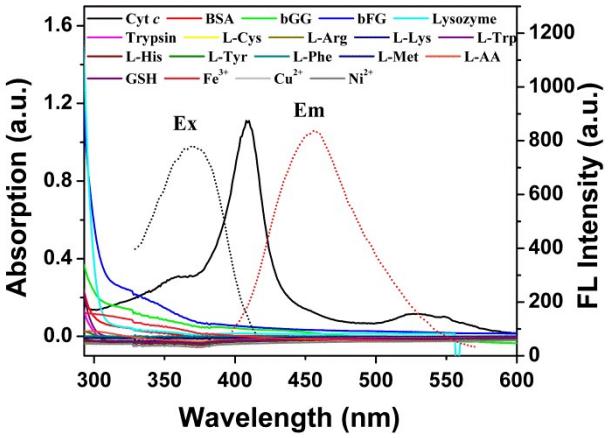

Figure S9. The UV-vis absorption spectra of Cyt *c* (10 μM) and potential interferences (100 μM) and the fluorescence excitation and emission spectrum of the N-doped CDs.

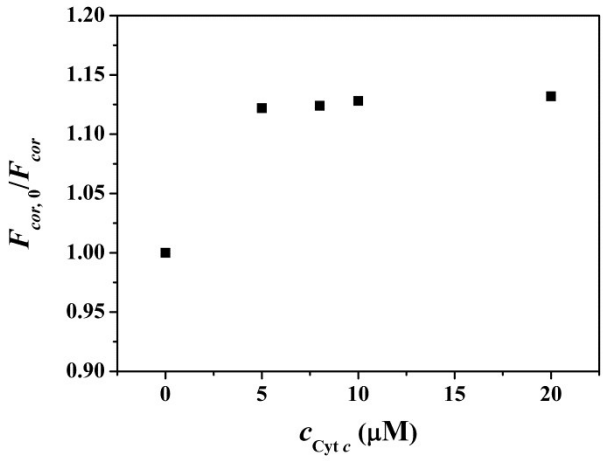

Figure S10. Influence of Cyt *c* concentrations on the corrected fluorescence intensity ratio ($F_{cor,0}$ /F_{cor}, Table S2) of N-doped CDs.

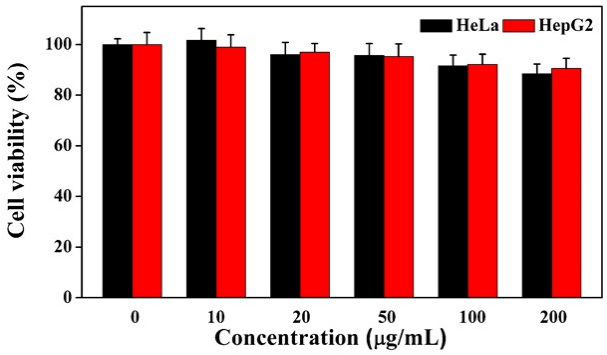

Figure S11. Cell viability of HeLa and HepG-2 cells in the presence of different concentrations of N-doped CDs. The error bars mean standard deviations (n=3).

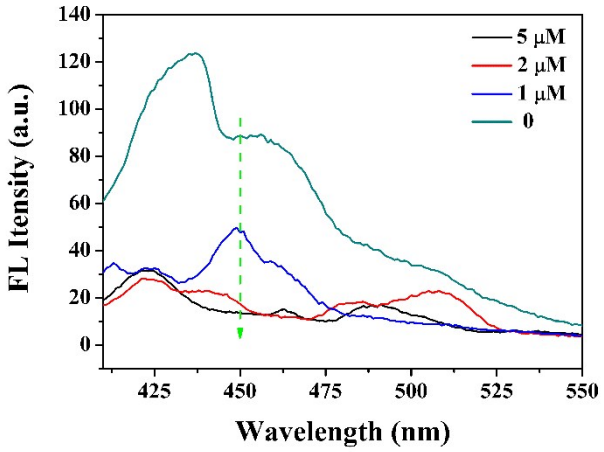

Figure S12. Fluorescence emission spectra of N-doped CDs stained HepG-2 cells treated with various concentrations (0, 1, 2, 5 μM, respectively) of etoposide for 2 h.

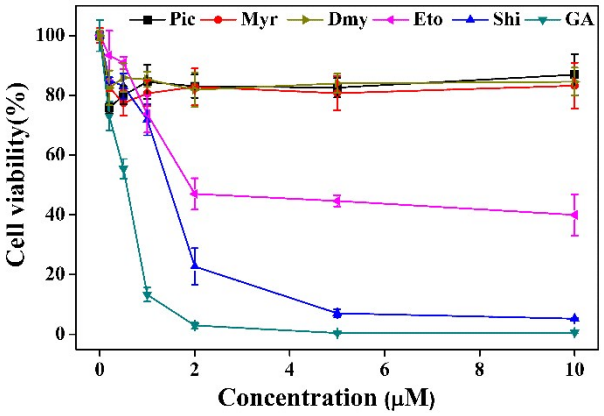

Figure S13. Cell viability of HepG-2 cells in the presence of different concentrations of various candidate drugs. The error bars mean standard deviations (n=3).

Table S1. Comparison of the present approach with other reported methods for the detection of Cyt *c*.

Applied material	Method	Linear range	LOD	Ref.
UCNP@PDA@AP	Turn-off fluorescence	0.05-10 μM	20 nM	[2]
Aptamer-GO Nanoassembly	Turn-on fluorescence	0.03-10 μM	10 nM	[3]
Hb/Au NCs	Turn-off fluorescence	0-10 μM	14.3 nM	[4]
DNA/Ag NCs		0-1.0 μM	15.7 nM	
N-GQDs/SiO_2/MIP	Turn-off fluorescence	0.2-60 μM	0.11 μM	[5]
TPP-UC(PS)	Turn-off luminescence	0-10 mM	/	[6]
TGA/CdTe QDs	Turn-off fluorescence	0.5-2.5 μM	0.5 μM	[7]
MIP-coated QDs	Turn-off fluorescence	0.97-24 μM	0.41 μM	[8]
N-doped CDs	Turn-off fluorescence	0.001-30 μM	0.3 nM	Present work

Table S2. Influence of different Cyt *c* concentrations on fluorescence lifetime of N-doped CDs.

Cyt *c* (μM)	Fluorescence lifetime (ns)
0	20.7
0.1	20.6
10	20.2

Table S3. Parameters used to calculate IFE of Cyt *c* on the fluorescence of the N-doped CDs.

Cyt *c* (μM)	A_{ex}[a]	A_{em}[b]	CF[c]	F_{obsd}[d]	F_{cor}[e]	E_{obsd}[f]	E_{cor}[g]	$F_{cor}/F_{cor,0}$
0	0.219	0.016	1.295	846	1096	0.000	0.000	1.000
5	0.351	0.068	1.861	525	977	0.379	0.108	0.892
8	0.437	0.106	2.078	469	975	0.446	0.110	0.890
10	0.455	0.116	2.286	425	972	0.498	0.113	0.887
20	0.681	0.219	2.894	334	968	0.645	0.117	0.883

[a]A_{ex} and [b]A_{em} are the absorbance of the N-doped CDs upon addition of Cyt *c* at 370 and 455 nm, respectively.

[c]Corrected factor (CF) is calculated as F_{cor}/F_{obsd}.

[d]F_{obsd} is the measured FL intensity of the N-doped CDs upon addition of Cyt *c* at 455 nm.

[e]F_{cor} is the corrected FL intensity with Eq. (1) by removing IFE from the measured FL intensity (F_{obsd}).

[f]$E_{obsd} = 1-F_{obsd}/F_{obsd,0}$, in which $F_{obsd,0}$ is the observed FL intensities of the N-doped CDs in the absence of Cyt *c*.

[g]E_{cor} =1-$F_{cor}/F_{cor,0}$, in which $F_{cor,0}$ is the corrected FL intensities of the N-doped CDs in the absence of Cyt *c*.

Calculation of the Förster Radius [2,3,9]

$$E=1-\frac{\tau_{DA}}{\tau_{D}} \quad (1)$$

$$R_0^6=\frac{9000(\ln 10)k^2Q_D}{128\pi^5Nn^4}J(\lambda) \quad (2)$$

$$J(\lambda)=\int_0^\infty F_D(\lambda)\varepsilon A(\lambda)\lambda^4 d\lambda \quad (3)$$

where τ_{DA} and τ_D are the exciton lifetime of the donor in the presence or absence of the acceptor, respectively. R_0 and Q_D are the Förster distance and fluorescence quantum yield in the absence of acceptor, respectively. k^2 is a factor describing the dipole-dipole orientation factor between donor and acceptor (k^2 = 2/3). n is the refractive index of the medium (n = 1.33). N is Avogadro's number. The overlap integral J (λ) expresses the degree of spectral overlap between the emission of donor and the absorption of acceptor. F_D (λ) is the fluorescence intensity of the donor at wavelength λ, and ε_A (λ) is the molar absorption coefficient of the acceptor at the wavelength of λ.

Table S4. Parameters used to calculate FRET between Cyt *c* and N-doped CDs.

E	Q_D	$J(\lambda)$	R_0 (nm)
2.88%	0.20	6.11×10^{-13}	5.3

References

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