Electronic Supplementary Information

In vivo imaging of tumour bearing near infrared fluorescence- emitting carbon nanodots derived from tire soot

Hae Young Ko, Young Wook Chang, Gokul Paramasivam, Myoung Seok Jeong, Sujeong Cho and Soonhag Kim*

Department of Biomedical Science, CHA University, 605-21 Yoeksam 1-dong, Gangnam-gu, Seoul 135-081, Republic of Korea

*Corresponding author

Laboratory of Molecular Imaging, Department of Biomedical Science, CHA University, 605-21 Yoeksam 1-dong, Gangnam-gu, Seoul, Korea.

Tel.: +82-2-555-5063, fax: +82-2-3468-3373,

E-mail address: kimsoonhag@empal.com

Experimental procedures

Chemicals and Materials

All of the following chemicals were obtained from Sigma Aldrich (Sigma, St. Louise, MO): nitric acid (HNO_3), sodium bicarbonate ($NaHCO_3$), sodium phosphate monobasic and sodium phosphate dibasic. All the chemicals were of analytical reagent grade and were used without further purification.

Preparation of the tC-dots from tire soot

The tire soot was collected by sitting a rectangular glass plate (l =70 cm and b =65 cm; h =15 cm from the burning vessel) on top of flames from burning tire. The tire soot (100 mg) was mixed with 5 M HNO_3 and refluxed for 12 h at 120 °C which turned into a homogeneous black aqueous suspension. Upon initial centrifugation at 3000 rpm for 10 min the suspension separated into a black carbon precipitate and a light-green supernatant. For maximum recovery of this fluorescent material, the precipitate was further mixed with solvent (acetone: water, 3:1) and centrifuged at 14,000 rpm for 15 min. Then the collected supernatant were neutralized with sodium bicarbonate for adjusting pH and extensively dialyzed with 3 kDa membrane against water.

Emission and Absorbance

Emission and absorbance spectra were recorded with 200 µl of the tC-dots (1 mg/ml) obtained from tire soot by a multi-well spectrometer (Synergy MX, Biotek Ltd., Korea). The emission spectra were recorded at different excitation wavelengths ranging from 340 to 660 nm. The absorbance spectra were recorded in the range between 250 to 800 nm. Furthermore, the effect of pH value on the emission properties of the tC-dots were measured in the pH range from 3 to 10 at 420 nm excitation using sodium phosphate monobasic and sodium

phosphate dibasic.

Transmission Electron Microscope (TEM)

TEM measurements were performed on JEM-3010 (JEOL Ltd., Japan) at operating voltage of 300 kV. The size was presented as the mean ± standard deviation. High resolution transmission electron microscope (HR-TEM, JEOL Ltd., Japan) measurements were performed at operating voltage of 300 kV. Drops of a dilute aqueous solution of the tC-dots were deposited on a carbon-coated copper grid and dried in air for overnight.

X-ray Photo electron Spectroscopy (XPS)

XPS measurements were performed with an Al Kα source (1486.6 eV) from Escalab 220i-XL (Thermo VG, U.K.). Spectra were acquired with pass energy- 100 eV, step size: 1 eV for survey scan 50 eV, step size: 0.1 eV for detailed scan and a Shirley type background were subtracted to each region.

Fourier Transform Infra Red Spectrometer

The tC-dots solutions were mounted on a sample holder and the spectra were measured by a Perkin-Elmer Spectrum 2000 FTIR spectrophotometer. A baseline correction was applied after measurement.

Cell culture

C6 cells were purchased from the American Type Culture Collection (ATCC). The culture medium for cells consisted of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY) and 1% Antibiotics (Invitrogen, Grand Island, NY) in a standard incubator (5%

CO_2 atmosphere at 37 °C). The cells were split at regular intervals.

In vitro cell viability assay

C6 cells were cultured using established procedures. In the 96-well plates with 100 µl of culture medium, C6 cells were plated at 1 × 10^4 cells per well overnight. At different concentrations of the tC-dots (20, 40, 100, 200 and 400 µg/ml, respectively) were incubated for 24 h. These cells were exposed to 10 µl of CCK-8 (DOJINDO, Japan) solution and incubated for 4 h at 37 °C. The optical density (OD) was read at 450 nm on a multi well spectrometer. All assay performed in triplicate. The results were presented as reduction of metabolic activity in percentage when compared to control cells cultured in growth medium only (100%).

In vitro fluorescence imaging

The tC-dots (200 µl of 1mg/ml) were taken in a microtube and allowed to fluorescence imaging using the IVIS® spectrum imaging system (Caliper Life Sciences, MA). The excitations wavelengths include 430, 465, 500, 535, 570, 605, 640, 675 and 710 nm with a emission filters from 500, 520, 540, 560, 580, 600, 620, 640, 660, 680, 700, 720, 740, 760, 780 and 800 nm, respectively.

Confocal microscope for C6 cell labeling

C6 cells (0.5×10^5) were seeded into 4-well plates containing 25-mm-diameter cover glass and cells were grown for 24 h at room temperature. The cells were treated with the tC-dots (20 µg/ml) and incubated for 3 h at 37 °C. Then, the treated cells were washed with Dulbecco's phosphate-buffered saline (D-PBS) twice for 15 min. After washing, the cells were fixed by gentle shaking for 20 min with 4% formaldehyde solution (Sigma, Saint Louis, MO). The

cells were then washed three times with PBS for 10 min and cover-slips placed with mounting medium. The confocal laser scanning microscopy (LSM 510, Carl Zeiss Inc., Germany) images were obtained by the excitation wavelength settings at 488, 514, 543 and 633 nm with the emission filter of 508-580, 561-593 and 636-700 nm.

In vivo fluorescence imaging

All animals used in the *in vivo* experiments were housed under specific pathogen-free conditions. The planned experiments were approved by the institution's animal care and use committee of CHA University. For *in vivo* imaging of the tC-dots, 200 μl of the tC-dots (1 mg/ml) were mixed with a matrigel (200 μl) and subcutaneously injected into left thigh of male BALB/c nude mice (7 weeks old, n=3). Matrigel alone was injected into right thigh of mice as background signal.

For *in vivo* imaging of C6 cells bearing the tC-dots, the tC-dots (200 μg/ml) were incubated with 1×10^7 of C6 cells for 3 h at 37 °C. After washing with Dulbecco's phosphate-buffered saline (D-PBS), the cells were harvested and transplanted subcutaneously into the right thigh of a male BALB/c nude mice (7 weeks old, n=3). Only C6 cells were transplanted into the right thighs of mice. To acquire the image of the tC-dots in the mice thighs, we performed *in vivo* fluorescence imaging using the IVIS® spectrum imaging system (Caliper Life Sciences, MA). After the nude mice were anesthetized with 2.5% of isofluorane, the optical imaging was performed using various excitation wavelengths at 430, 465, 500, 535, 570, 605, 640, 675 and 710 nm with a emission filters from 500, 520, 540, 560, 580, 600, 620, 640, 660, 680, 700, 720, 740, 760, 780 and 800 nm, respectively. A camera was used to acquire captured images at constant exposure times (10s).

Ex vivo fluorescence imaging

The mice bearing the tC-dots in the matrigel or C6 cells were sacrificed by cervical dislocation a week after the implantation of the tC-dots. *Ex vivo* tissue imaging with the same excitation and emission for *in vivo* imaging of the tC-dots was acquired for 10 s in the IVIS® spectrum imaging system (Caliper Life Sciences, Waltham, MA).

Statistical analysis

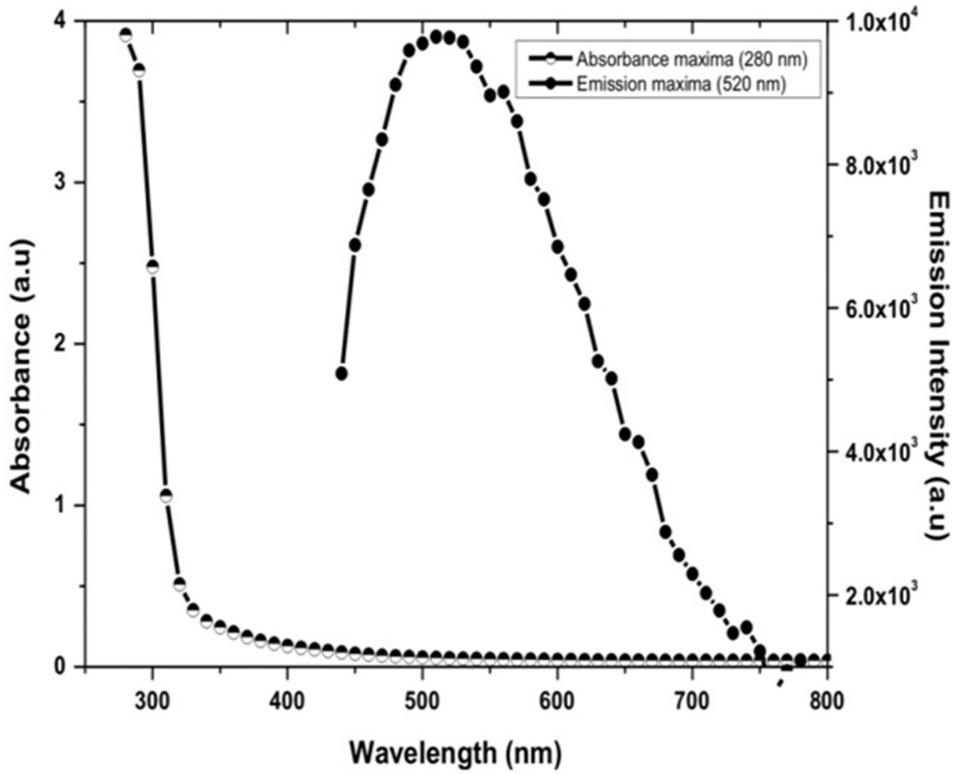
Data are displayed as mean ± standard errors and were calculated with the Student's *t*-test. Statistical significance was accepted at *p*-values above 0.05 *(p >0.05)*.

Supplementary Table 1:

Comparison of the emission peak of the tC-dots for different excitation wavelengths from 340 to 660 nm in 20 nm increments.

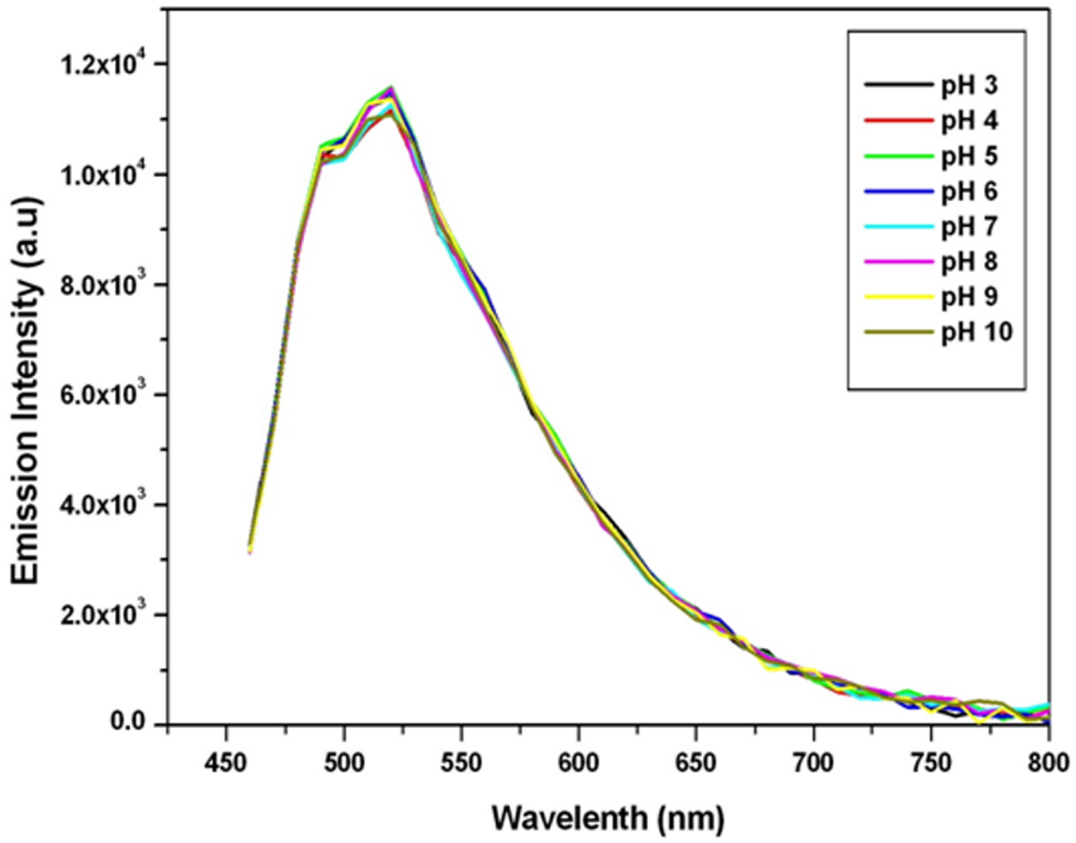
Excitation (λ_{ex}), nm	Emission (λ_{em}), nm
340	420
360	440
380	450
400	490
420	515
440	530
460	550
480	560
500	570
520	590
540	600
560	640
580	640
600	660
620	660
640	670
660	710

Supplementary figure 1



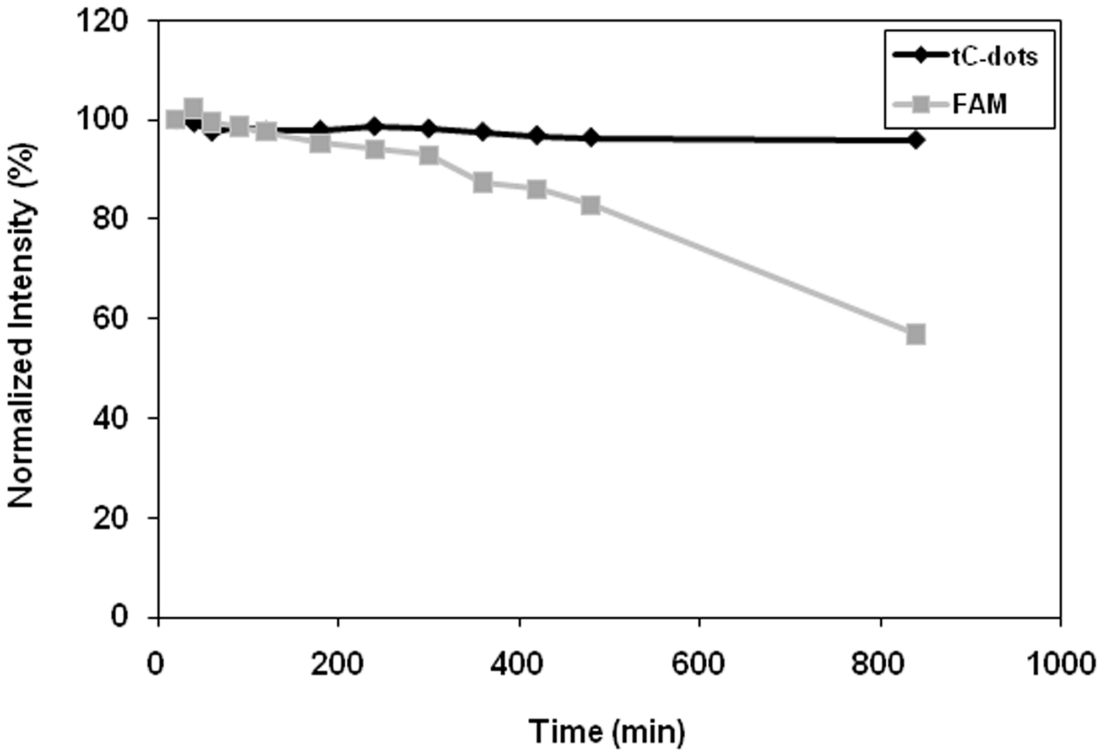
UV/Vis absorbance and emission spectra of the tC-dots. The maximum absorbance of the tC-dots was 280 nm and the maximum emission was 520 nm.

Supplementary Figure 2



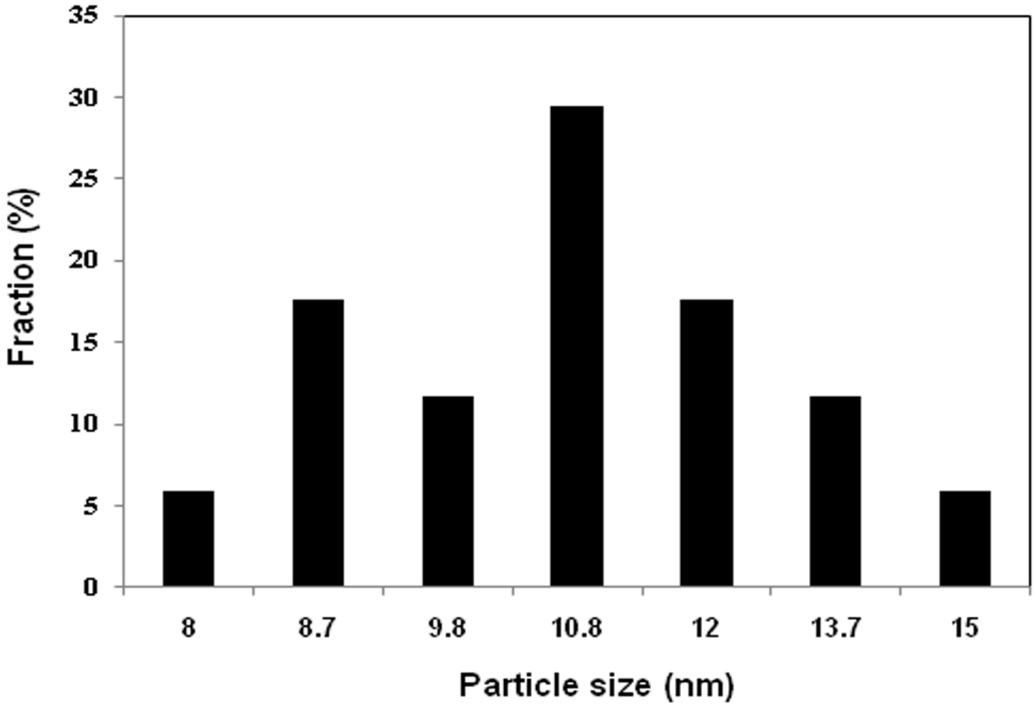
Effect of pH value on the tC-dots fluorescence. Maximum emission was taken from the excitation at 420 nm between pH 3 and pH10.

Supplementary Figure 3



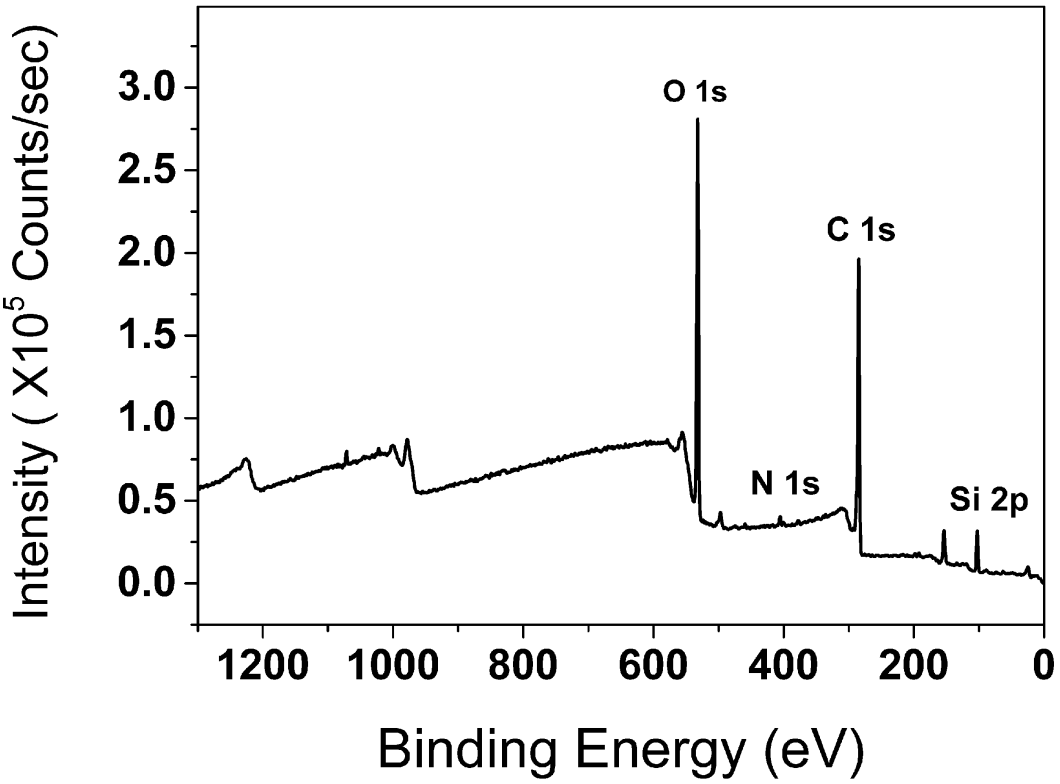
Optical stability of the tC-dots. One of the traditional dye, FAM, was used as a control. The fluorescence intensities of both the tC-dots and the FAM were acquired at the excitation wavelength of 420 nm for 14 hrs.

Supplementary Figure 4



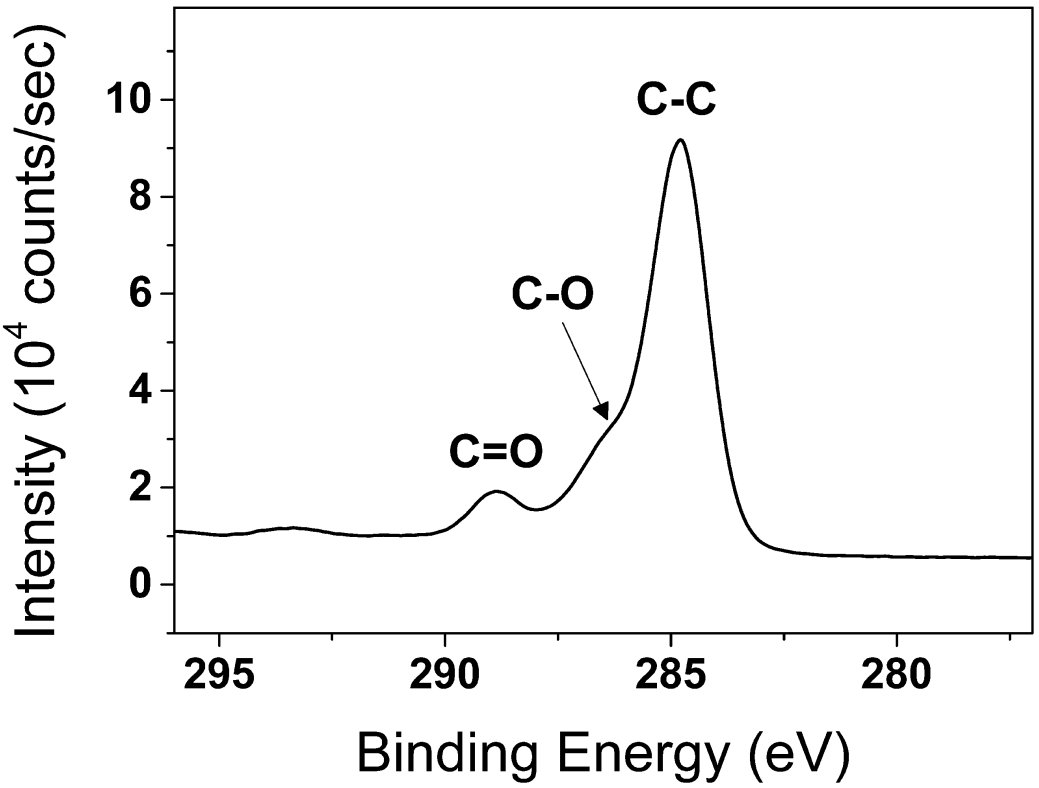
Particle size histogram of the tC-dots. The size was measured from TEM images. The particle size distribution of the tC-dots was analyzed using 95 particles from 10 TEM images.

Supplementary Figure 5



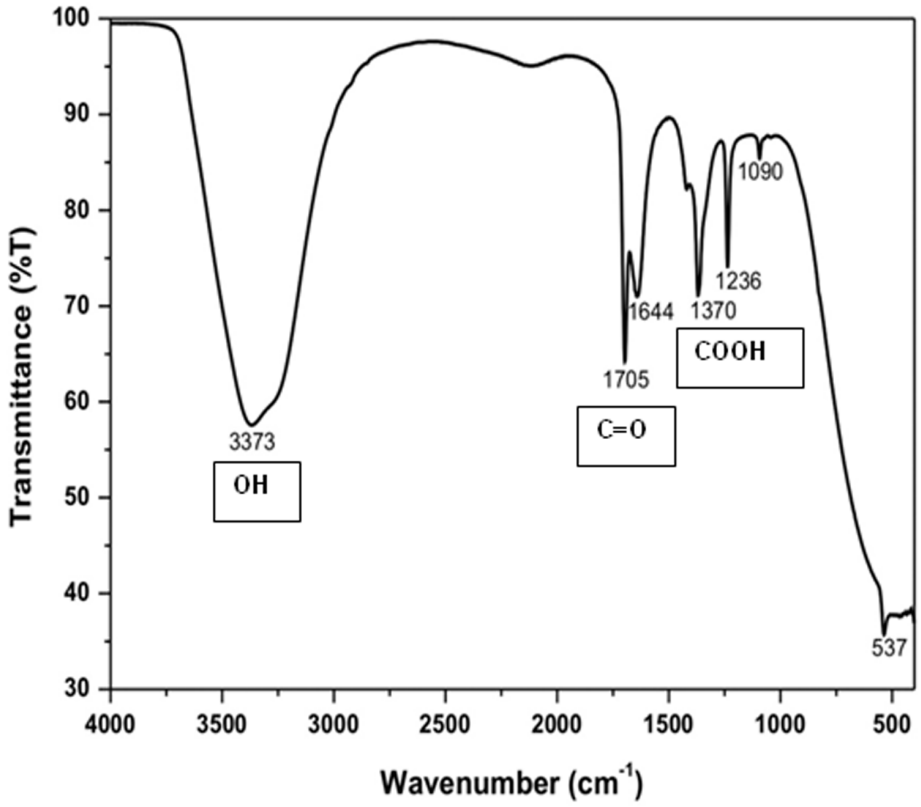
XPS spectra of the tC-dots. The spectra confirmed the presence of elements as Carbon (C), Oxygen (O), Nitrogen (N) and Silicon (Si) with the composition of 31, 58, 2 and 9 atom %, respectively.

Supplementary Figure 6



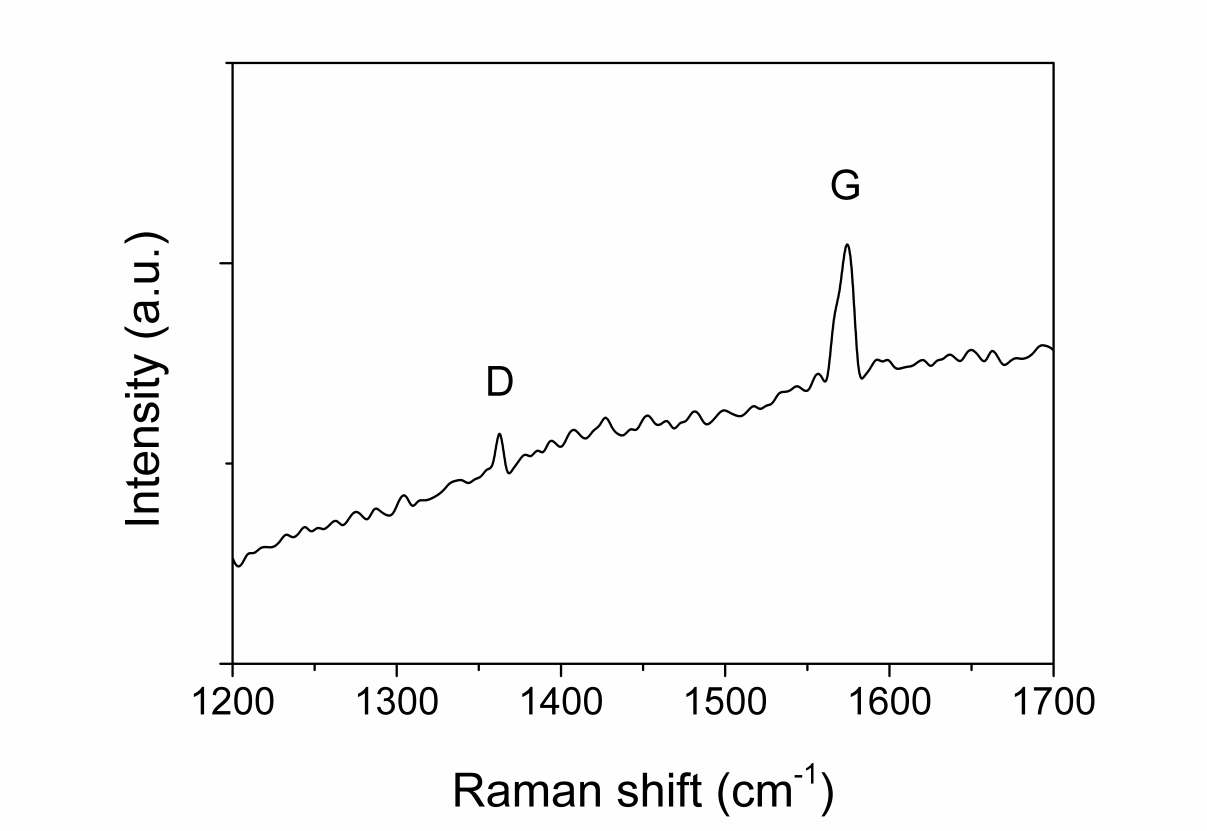
High-resolution XPS C1s spectrum of the tC-dots. The C–C, C–O and C=O carbon peak were clearly absorbed at 284.7, 286.4 and 288.8 eV, respectively.

Supplementary Figure 7



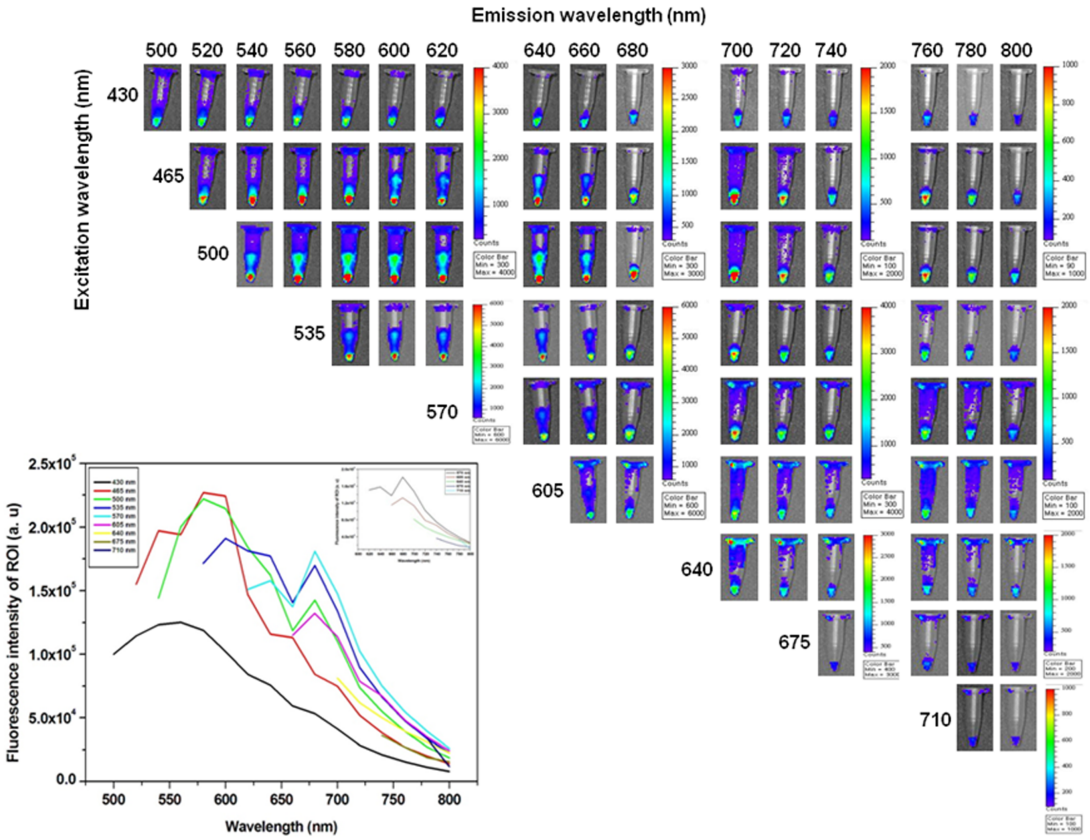
FTIR spectrum of the tC-dots. The peak of the spectrum at 3373, 1705, 1644, 1370, 1236, 1090 and 537 cm^{-1} indicated OH, C=O, H_2O, COOH, aryl-O, C-O and iodine, respectively.

Supplementary Figure 8



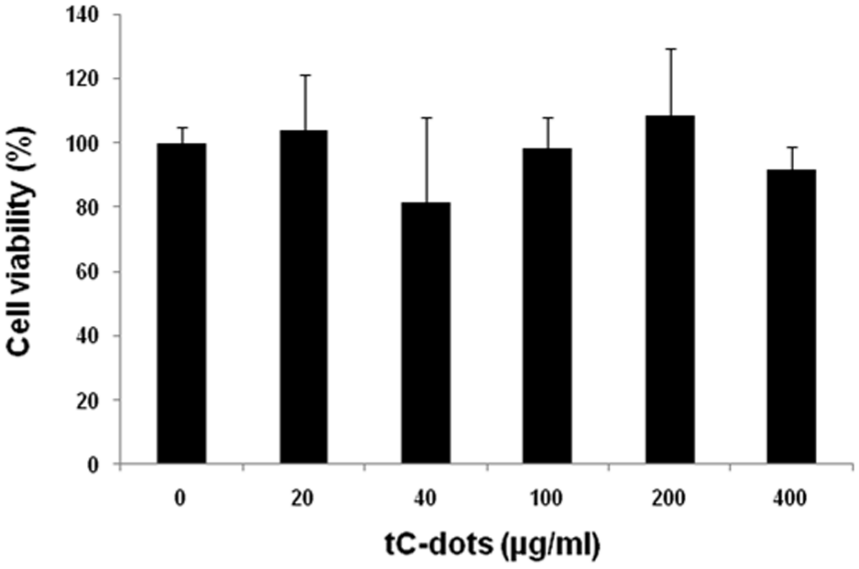
Raman spectra of tC-dots at 532 nm excitation wavelength. Raman spectra of the tC-dots showed both the G band at 1590 cm^{-1} and the D band at 1320 cm^{-1} which indicate sp2 and sp3 carbon, respectively.

Supplementary Figure 9



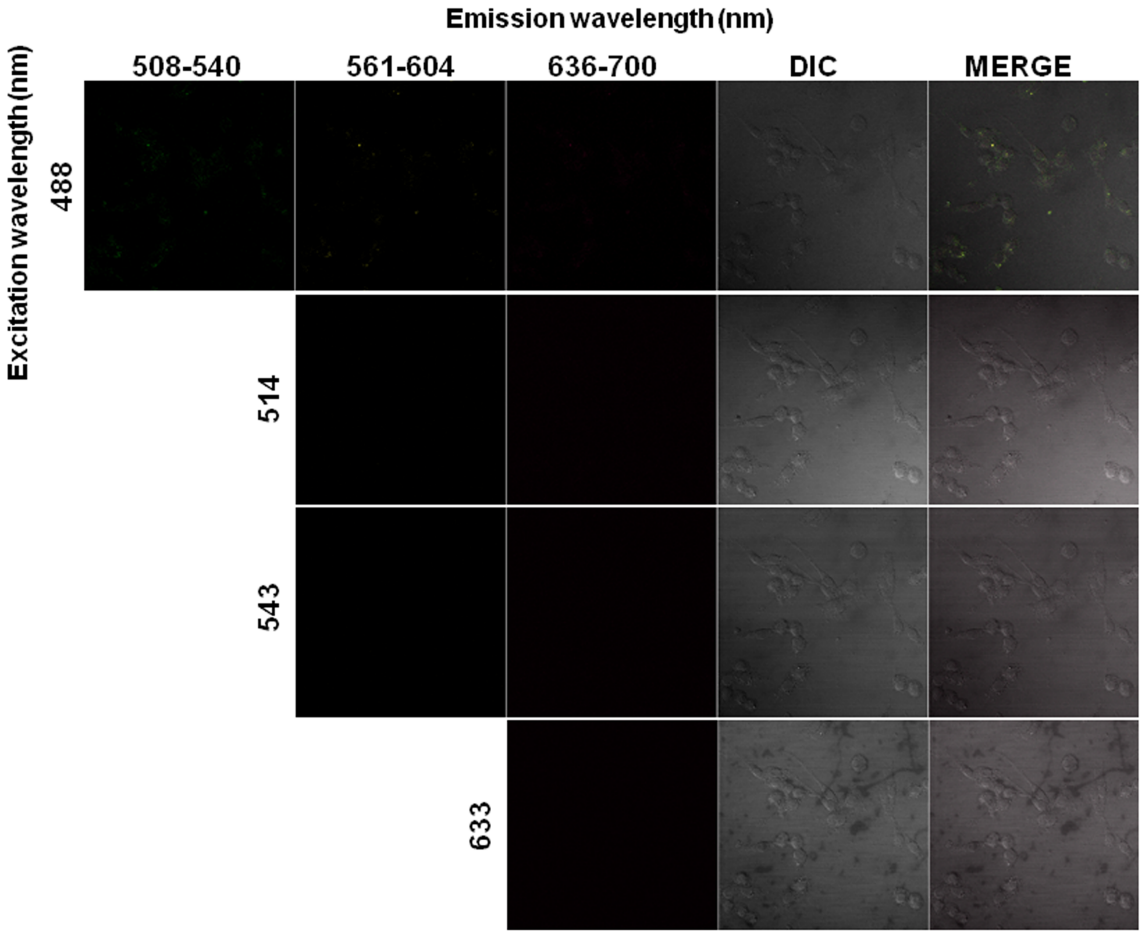
The spectral fluorescence imaging of the tC-dots in a microtube. The tC-dots (200 µl of 1 mg/ml) were taken in a microtube. The images were taken under various excitation wavelengths at 430, 465, 500, 535, 570, 605,640, 675 and 710 nm with emission filters at 500, 520, 540, 560, 580, 600, 620, 640, 660, 680, 700, 720, 740, 760, 780 and 800 nm, respectively. Left panel showed fluorescence intensity of region of interest (ROI) from the spectral tube imaging. The inset is the magnification of the fluorescence intensity with excitation of 570, 605, 640, 675 and 710 nm.

Supplementary Figure 10



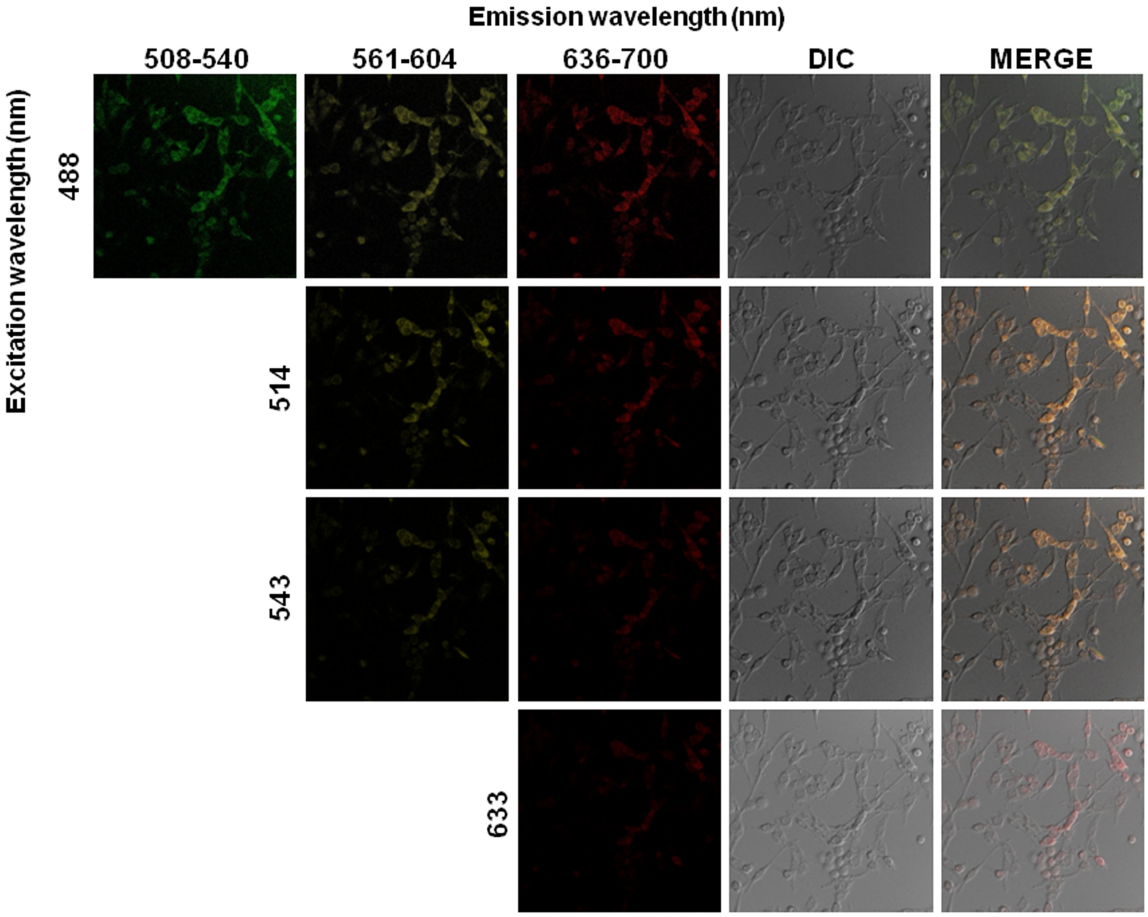
Cytotoxicity of the tC-dots in C6 cells. Various concentrations (0, 20, 40, 100, 200 and 400 μg/ml) of the tC-dots were incubated in C6 cells for 24 h. Data were displayed as means ± standard deviation. No statistically significant difference (*, $p < 0.05$) was observed among groups.

Supplementary Figure 11



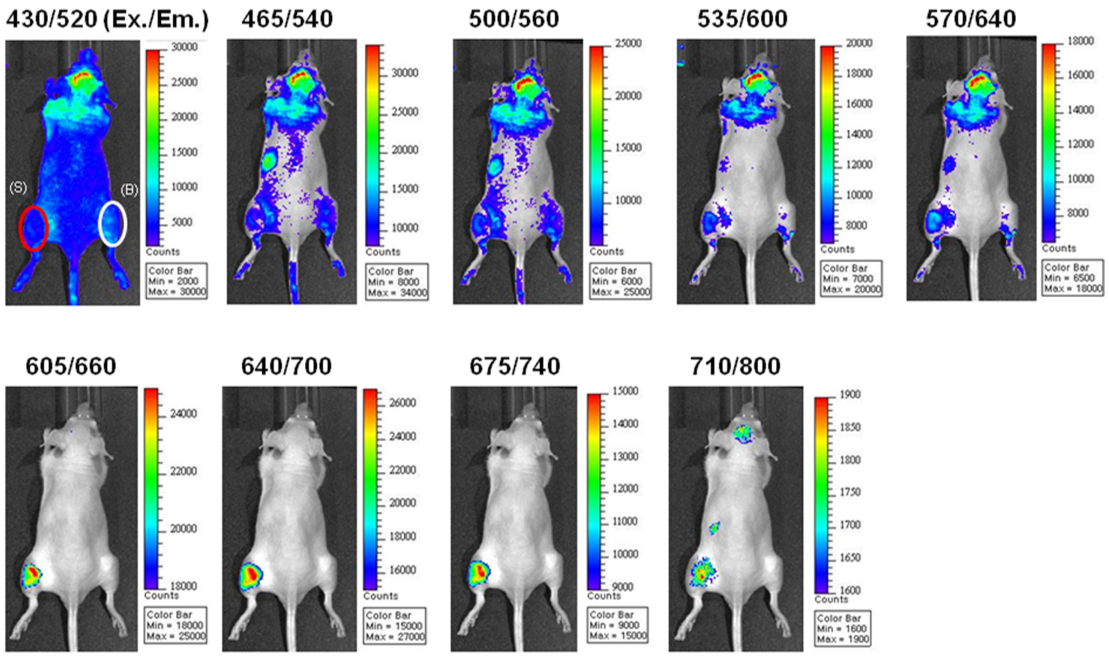
Confocal microscopy image of C6 cells without the incubation of the tC-dots with excitation at 488 (1st row), 514 (2nd row), 543 (3rd row) and 633 nm (4th row) and emission filter of 508-580 (1st column), 561-593 (2nd column) and 636-700 nm (3rd column). DIC shows the bright field image for cellular morphology.

Supplementary Figure 12



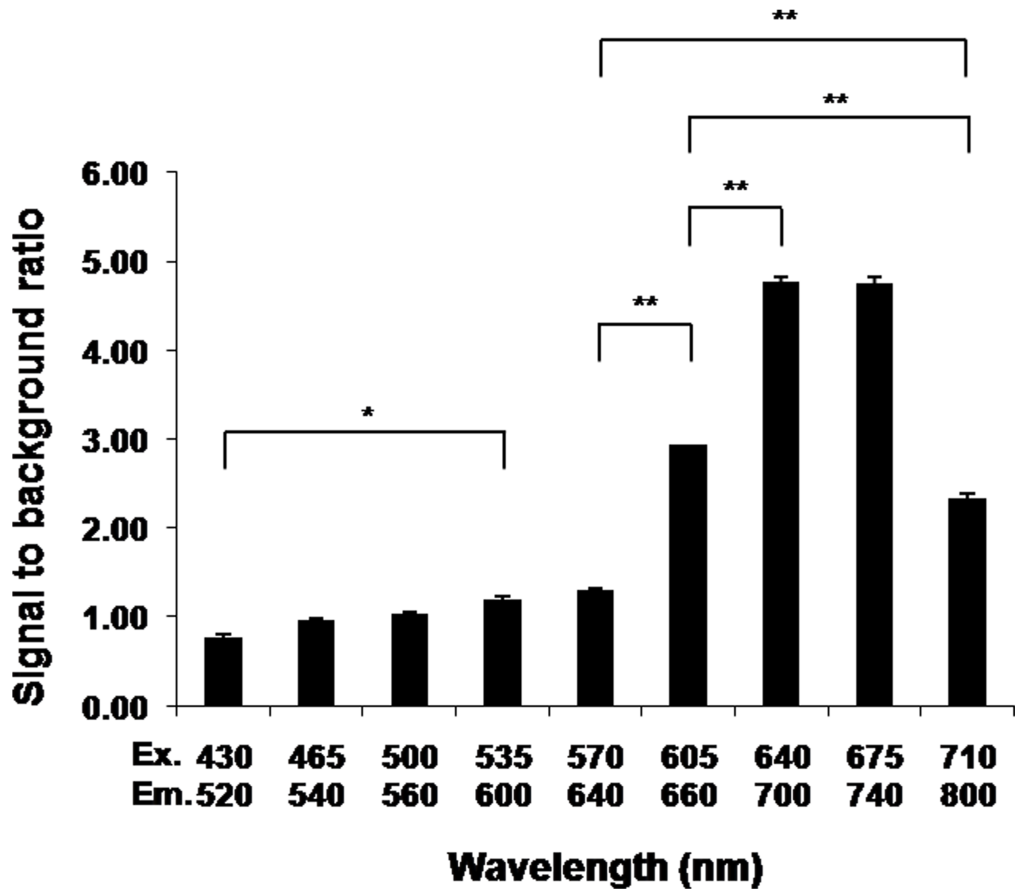
In vitro optical stability of the tC-dots. Confocal microscopy image of the figure 3a was reconducted 6 months later with excitation at 488 (1st row), 514 (2nd row), 543 (3rd row) and 633 nm (4th row) and emission filter of 508-580 (1st column), 561-593 (2nd column) and 636-700 nm (3rd column). DIC shows the bright field image for cellular morphology.

Supplementary Figure 13



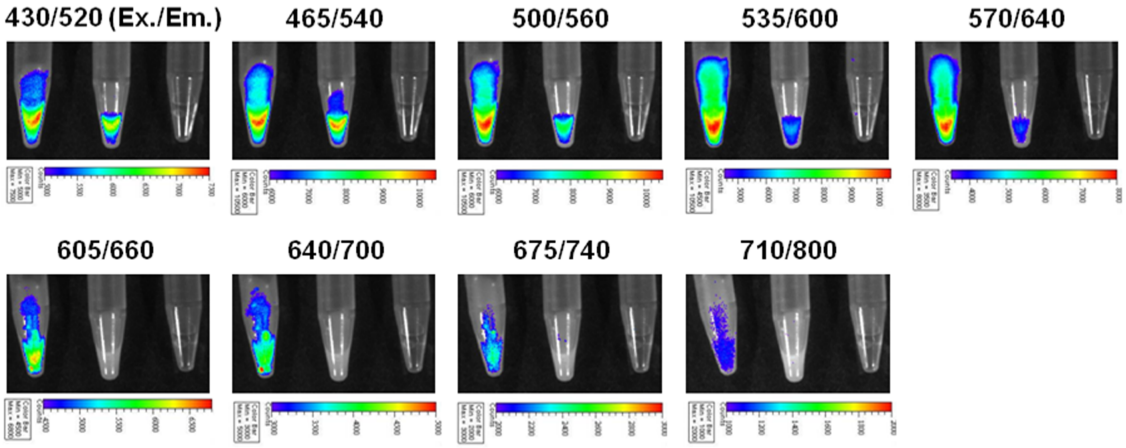
In vivo fluorescence imaging of the tC-dots in the matrigel. The matrigel with and without (used as background signals) incubation of 200 µl of the tC-dots (1 mg/ml) was subcutaneously implanted into a left thigh (indicated by a red circle and an S) and a right thigh (indicated by a white circle and a B) of a nude mice, respectively (n=3).

Supplementary Figure 14



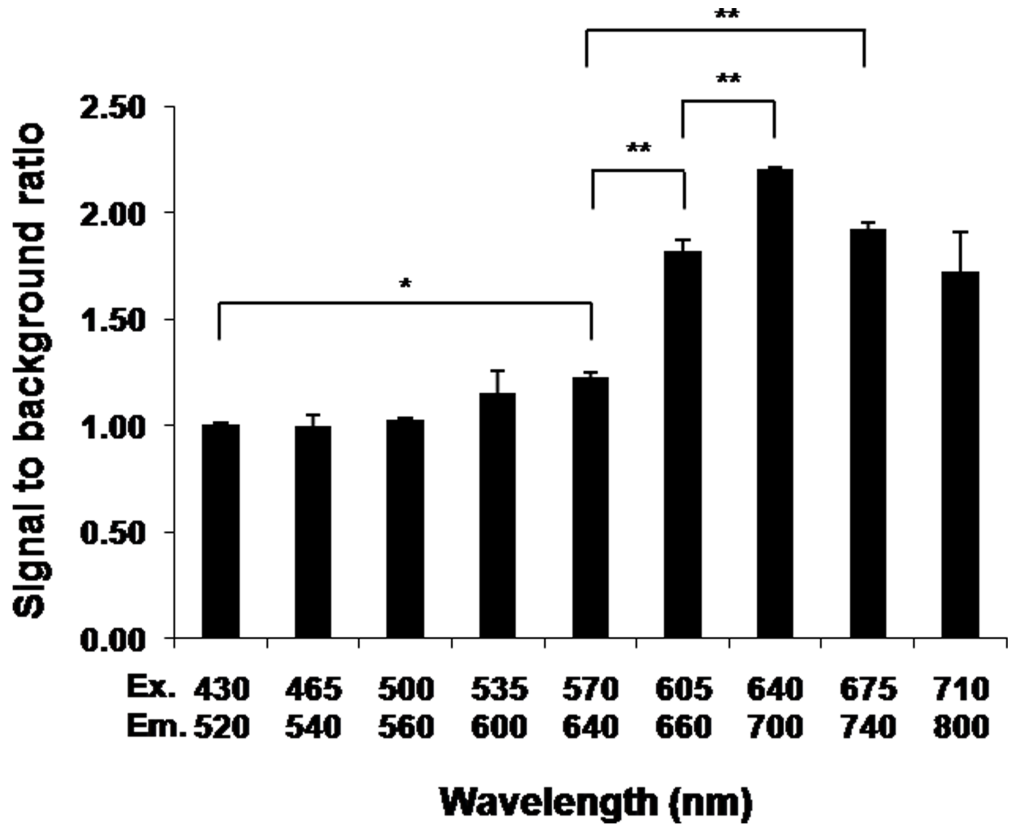
Signal to background ratio of the figure S13. Fluorescence intensity within the red circle from the left thigh containing the tC-dot-incorporated matrigel and within the white circle from the right thigh having only a matrigel was measured for signals of the tC-dots and signals of the background, respectively. Data was expressed as means ± standard deviation (*, p <0.05; **, p <0.005).

Supplementary Figure 15



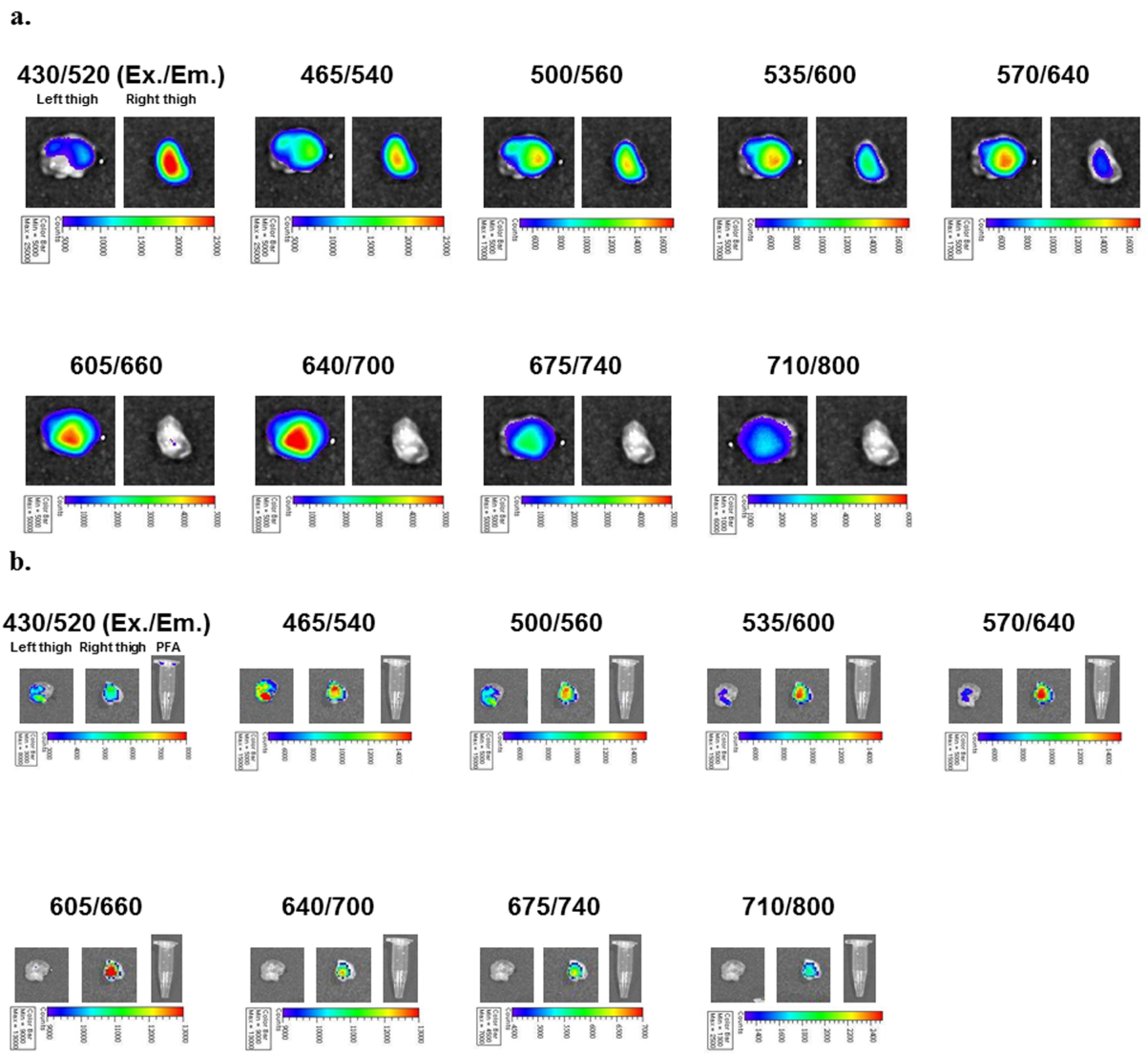
In vitro fluorescence imaging of C6 cells bearing the tC-dots in a microtube. C6 cells were incubated with (1st tube) or without (2nd tube) 200 ug/ml of the tC-dots in a microtube. PBS buffer (3rd tube) was used as a control.

Supplementary Figure 16



Signal to background ratio of the figure 3b. Fluorescence intensity within the red circle from the right thigh containing C6 cells bearing the tC-dots and within the white circle from the left thigh having only C6 cells was measured for signals of tracking C6 cells labeled by the tC-dots and signals of the background, respectively. Data was expressed as means ± standard deviation (*, p <0.05; **, p <0.005).

Supplementary Figure 17



Ex vivo tissue imaging of figure S13 and 3b. The fluorescence imaging from the mice bearing the tC-dots in the matrigel (a) or C6 cells (b) was acquired with a pair of excitation and emission including 430/520, 465/540, 500/560, 535/600, 570/640, 605/660, 640/700, 675/740 and 710 nm/800 nm. Tissues from both thighs were obtained 7 days after the implantation of the tC-dot-uptaken C6 cells or the tC-dot-incorporated matrigel. a) The left thigh (1st lane) contained the tC-dot-incorporated matrigel and the right thigh (2nd lane) had only matrigel. b) The left thigh (1st lane) contained only C6 cells and the right thigh (2nd lane) had C6 cells bearing the tC-dots . 4 % paraformaldehyde buffer (3rd lane) was used as a control.