Supporting Information

Peptide-Fluorophores/AuNP conjugate-based Two-Photon Excited Fluorescent nanosensor for Caspase-3 Activity Imaging Assay in Living Cells and Tissue

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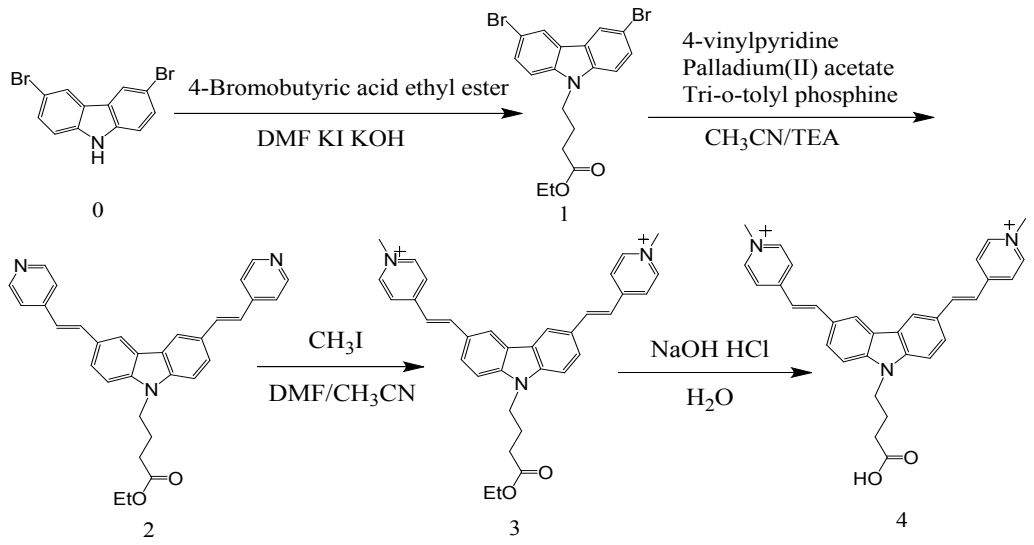
Materials and instruments

Recombinant human caspase-3 (active form, 0.234mg/mL) was purchased from R&D Systems (Minneapolis, USA). The TPdye-labeled caspase-3 specific peptide sequence of CANLNDEVDK-BMVC was purchased from ChinaPeptides (Shanghai) Co., Ltd. 3-[(3-Cholamidopropyl)dimethylammonio] propanesulfonate (CHAPS) was purchased from Sun Chemical Technology Co., Ltd. (Shanghai, China). Chloroauric acid (HAuCl4), sodium citrate, and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich. PEITC (beta-phenethyl isothiocyanate) was purchased from J&K Scientific (Guangdong) Co., Ltd. Other chemicals were of analytical reagent grade and used as received. HeLa cells (cervical cancer) and liver tissue slices resulted from the Rat hepatic ischemia reperfusion model were provided by the Hunan Provincial Tumor Hospital, Central South University (China). The study was approved by the Ethics Committee of Hunan Provincial Tumor Hospital. All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.3 MΩ. All concentrations for caspase-3 used in vitro assays were calculated directly using the amount of protein (active form) indicated by the provider (R & D Systems) with no calibration.

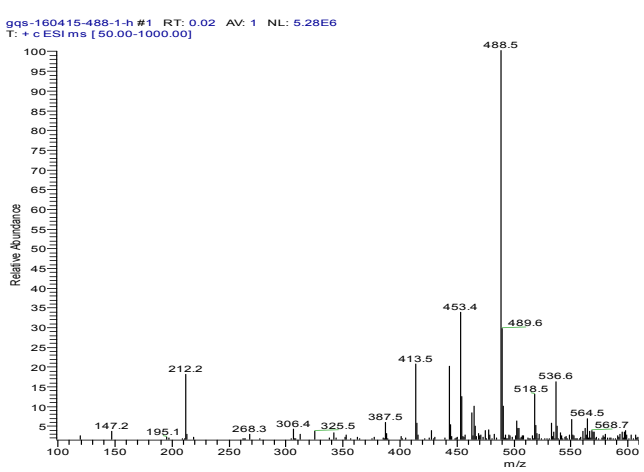
The UV-Vis absorption spectra were measured on a Hitachi U-4100 UV/Vis spectrometer (Kyoto, Japan) using a quartz cuvette having 1 cm path length. The fluorescence spectra were performed on a PTI ASOC-10 Fluorescence System (Photo Technology International, Birmingham, NJ, USA). Transmission electron microscopy (TEM) was performed on a JEOL JEM-3010. The pH values were calibrated with a model 868 pH meter (Orion). For MTT assay, the spectrophotometrical absorbance of each well was measured by using a Tecan microplate reader (Bio-Rad ELISA

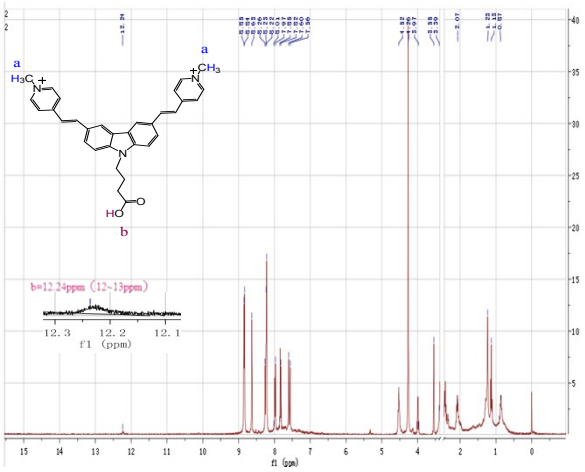
reader, Hercules, CA). Two-photon excitation fluorescence images (TPFI) of cells or tissue slices were obtained using an Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Japan).

Synthesis of the two-photon dye (TPdye: Ethyl-4-[3, 6-Bis (1-methyl-4 -vinylpyridium iodine)-9H-carbazol-9-yl)] butanoic acid, BMVC)



To a mixture of KOH (1.12 g, 20 mmol) and KI (80 mg, 0.48 mmol) dissolved in dry DMF (20mL) was added 3, 6-dibromocarbazole (compound 0) (0.65g, 2mmol), 4-bromobutyric acid ethyl ester (1.15 mL, 8 mmol). The mixture was stirred at 60 °C under argon atmosphere overnight. After the addition of 100 mL H_2O to the final mixture, the mixture was extracted with ethyl acetate and then the organic layer was washed twice with water and once with brine, and dried over anhydrous Na_2SO_4. After filtration, the solution was concentrated under reduced pressure to give crude product The final white powder Ethyl-4-(3,6-dibromo-9H-carbazol-9-yl) butanoate (compound 1) was obtained by chromatography using petroleum/ethyl acetate(5:1, V/V) as an eluent.[1] 4-[3,6-Bis(1-methyl-4-vinylpyridiumiodine)-9H-carbazol-9-yl)] butanoic acid was prepared as indicated in literature.[2] Ethyl-4-(3,6-dibromo-9H-carbazol-9-yl) butanoate (compound 1) (731.7 mg,1.7 mmol) was added into a mixture containing 4- vinylpyridine (667 mg), Palladium(II) acetate (5 mg) and tri-o-tolyl phosphine (50 mg) under the triethylamine (3 mL)/acetonitrile(9 mL) solvent pairs in a high pressure bottle. The mixture was stayed at 105 °C for 48 h. After the reaction, the mixture was transferred to a flask and the solvent was removed under reduced pressure to give a yellow crude product, which was purified by chromatography on silica gel using CH2Cl2/CH3OH (5:1,V/V) as an eluent to give Ethyl-4-[3,6-Bis(4-vinylpyridium iodine)-9H-carbazol-9-yl)] butanoate (compound 2) as earth yellow solid. Excess CH3I and Ethyl-4-[3, 6-Bis (4-vinylpyridium iodine)-9H-carbazol-9-yl)] butanoate (487.0 mg,1 mmol) in acetonitrile /DMF was refluxed for 4h,the orange red powder. Ethyl-4-[3, 6-Bis (1-methyl-4-vinylpyridium iodine)-9H-carbazol-9-yl)] butanoate (compound 3) was obtained with a 90% yield after recrystallization twice using methanol. Then, Ethyl-4-[3,6-Bis(1-methyl-4-vinylpyridium iodine)-9H-carbazol -9- yl)] butanoate (compound 3) (193.0 mg, 0.25 mmol), sodium hydroxide (0.030 g, 0.75 mmol) were put into a 100mL flask containing the mixture solution of THF (4 mL) and water (1 mL) and the mixture was refluxed for 12 h. To get the Ethyl-4-[3, 6-Bis (1-methyl-4 -vinylpyridium iodine)-9H-carbazol-9-yl)] butanoic acid (compound 4), next the diluted hydrochloric acid was added into the mixture to adjust pH=3 giving an orange red solid with a yield of 80%. 1HNMR(d6-MSO,400MHz) δ：12.24(s，1H)，8.84(d，4H)，8.63(s，2H)，8.26(s，2H)，8.22(d，4H)，7.98(d，2H)，7.82(d，2H)，7.58(d，2H)，4.52(t，2H)，4.26(s，6H)，2.50(t，2H)，2.10(m，2H) Anal. calcd. for C32H31I2N3O2: C, 52.19; H, 4.20; N, 5.65. Found: C, 52.19; H, 4.20; N, 5.64.





Preparation of the AuNP/Petide-TPdyes Nanoconjugate

The 15-nm citrate-stabilized gold nanoparticles (AuNPs) was first synthesized according to the classical Turkevich-Frens method reported previously.[3] Briefly, all glassware used in the experiment was first soaked in aquaregia, rinsed thoroughly with water, and oven-dried prior to use. 100 mL of 1 mM HAuCl4•3H2O was added in 250 mL round bottomed flask and was heated to boiling, then refluxed. Next, 10 mL of 38.8 mM sodium citrate was rapidly added to the boiling solution under vigorous stirring and an apparent color changing from light blue to crimson was observed. After the color changed, the solution was refluxed for an addition 15 min, cooled to room temperature naturally, subsequently filtered through 0.45 μm aqueous phase membrane filter. A stable and monodispersed gold nanoparticle colloidal solution was obtained and stored at 4 °C refrigerator.

Then the AuNP/peptide-TPdyes nanoconjugate was prepared via the assembly of BMVC-labeled caspase-3 specific peptide sequences (CANLNDEVDK-BMVC) on the AuNPs surface through the Au–S bond. Briefly, 10 μL of 5 mM peptide solution was added to 1 mL of 13 nM AuNPs, and the mixture was incubation at 700 rpm and 25 °C in shaker overnight. After all, the mixture incubated with Polyvinylpyrrolidone (PVP) for 3 h to keep the peptide-TPdye modified AuNPs stable. For the removal of excess peptides and PVP, peptide-coated particles were subjected to centrifugation at 12 000 rpm for 20 minutes at 4 °C using centrifuge, and the resultant peptide-modified AuNPs (AuNP/CANLNDEVDK-BMVC) were washed with DI water three times and redispersed in phosphate buffer solution (PBS, 20 mM, pH 7.3), and stored in the refrigerator at 4 °C for further use.

In Vitro Assay of Caspase-3 Using AuNP/Petide-TPdyes Nanoprobe

In a typical caspase-3 activity assay using the AuNPs/Petide-TPdye nanoconjugate, an aliquot of 10 μL of the prepared AuNPs/Petide-TPdye nanoconjugate suspension was added to 80 μL of reaction buffer (pH 7.3, 50 mM HEPES, 10 mM DTT, 100 mM NaCl, 1 mM EDTA, 0.1% w/v CHAPS, 10% sucrose) or cell media (DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin), then 10 μL of caspase-3 (final concentrations ranging from 0 to 2.64 nM) or other species was added and the solution was incubated at 37 °C for 60 min. After reaction, the resulting solution was subjected to fluorescence measurements. The fluorescence spectra were recorded in a quartz cuvette on PTI QM4 fluorescence system with the excitation wavelength of 450 nm and the emission wavelengths in the range from 470 to 700 nm with both excitation and emission slits of 2.0 nm. The time-dependent fluorescence responses were recorded immediately after the addition of capspase-3 at an excitation wavelength of 450 nm (slit 2.0 nm) and an emission wavelength of 560 nm (slit 2.0 nm) with a certain time interval.

Cell Culture and Cytotoxicity Assay

For cytotoxicity assay, HeLa cells were cultured in DMEM supplemented with 10% FBS (Thermo Scientific HyClone) and 1% penicillin-streptomycin. When in the proliferative period, HeLa cells were dispersed within replicate 96-well microliter plates to a total volume of 100 μL/each well and maintained at 37 °C in a 5% CO2/95% air incubator for 24 h. Then, the culture media was removed and the cells were incubated in culture medium containing the as-prepared AuNP/Peptide-TPdyes nanoconjugates, and AuNPs with different concentrations for 48 h and then washed with the

culture medium. An amount of 100 μL of the fresh culture medium containing MTT (0.5 mg/mL) was then added, followed by incubating for 4 h to allow the formation of formazan dye. After the medium was removed, 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a Bio-Rad ELISA microplate reader. Relative cell viability was expressed as $([OD]_{test}/[OD]_{control}) \times 100\%$. Each experiment was repeated at least three times.

Preparation of cell lysates

Tumor cells were cultured in DMEM medium and cell-free extracts were prepared as follows: 1×10^6 cells were harvested with trypsin treatment and centrifuged at 1500g for 2 min. Then, cells were washed 3 times with 10 mL of cold PBS, centrifuged and resuspended in 0.5 mL of ice-cold cell lysis buffer (cell signaling) on ice for 5 min. Cells were pulse-sonicated on ice 5 times for 5 s. Then, centrifuged extracts at 15,000g for 20 min at 4 °C and supernatants were collected. Concentrations of cell-free extracts were quantitated by measuring the absorbance at 595 nm using Coomassie blue protein reagent (Pierce, Rockford, USA).

Live Cell Imaging Assay of Caspase-3 with AuNP/Petide-TPdye Nanoprobe

HeLa cells were seeded on glass-bottom dishes (Mattek) and were grown until 70−80% confluent. Then the growth medium was removed and washed three times with cold PBS. Then, 500 μL of fresh cell growth medium supplemented with AuNP/CANLNDEVDK-BMVC conjugates (~0.5 nM, concentration of the nanoconjugate refers to the concentration of AuNPs) was added in each well. After incubation for 2 h at 37 °C, the medium was replaced with fresh medium containing PEITC of a final concentration of 15 μg/mL, and the cells were incubated for an additional 1 h at 37 °C. The control group without PEITC followed the above operation. Finally, the treated cells were washed three times with cold PBS and two-photon confocal fluorescence imaging of the cells was observed under an Olympus FV1000-MPE multiphoton laser scanning confocal microscope, with a mode-locked titanium-sapphire laser source set at wavelength 800 nm.

Tissue imaging of caspase-3 activity with AuNPs/Petide-TP dye nanoconjugate

1.0 mm-thick liver tissue slices were obtained from the rats with/without hepatic ischemia-reperfusion surgery. Then the slices were incubated with 0.5 nM AuNP/CANLNDEVDK-BMVC nanoconjugates (concentration of the nanoconjugate refers to the concentration of AuNPs) in PBS for 2 h at 37 °C. After the samples were washed with PBS to remove the remaining nanoconjugates, two-photon confocal fluorescence imaging, Z-scan imaging and the 3D two-photon confocal fluorescence images collected along the Z-direction at depth of 0−300 μm (60× magnification) of this treated liver tissue slices were observed under an Olympus FV1000-MPE multiphoton laser scanning confocal microscope, with a mode-locked titanium-sapphire laser source set at wavelength 800 nm.

References

1. Yan, X.; Cheng, S.; Sun, L.; Chen, X.; Qin, J., π-conjugated polymers containing pendant Mn12 cluster: Synthesis and physical properties. *Polymer* **2012,** *53* (1), 241-247.
2. Feng, X. J.; Wu, P. L.; Bolze, F.; Leung, H. W. C.; Li, K. F.; Mak, N. K.; Kwong, D. W. J.; Nicoud, J.-F.; Cheah, K. W.; Wong, M. S., Cyanines as New Fluorescent Probes for DNA Detection and Two-Photon Excited Bioimaging. *Organic Letters* **2010,** *12* (10), 2194-2197.
3. FRENS, G., Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. *Nature Physical Science* **1973,** *241*, 20-22.

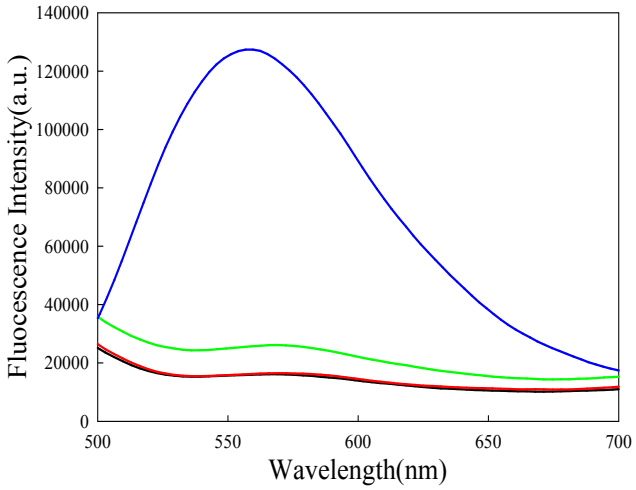


Fig. S1. The fluorescence spectra of BMVC (black curve), "BMVC + random single-stranded DNA" (red curve), "BMVC-labeled peptide" (green curve, CANLNDEVDK-BMVC) and "BMVC + double-stranded DNA" (blue curve, HPDNA: 5'-AATTCAAGCTTCCCCAAGCTTGAATTC-3') in buffer solution.

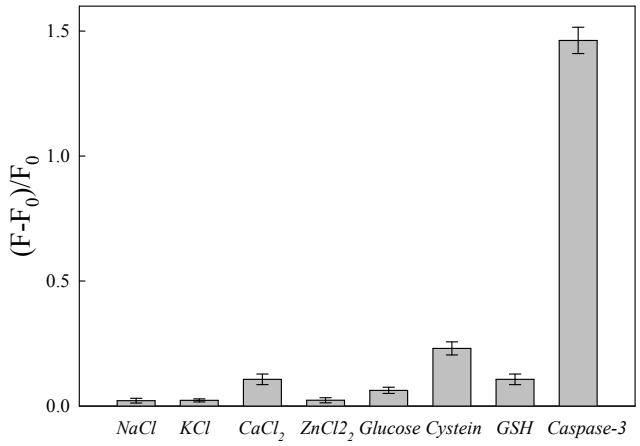


Fig. S2. Fluorescence emission intensity change of the AuNPs/Petide-TPdye nanoconjugate in the presence of various species. Where F_0 represents the fluorescence intensity at 560 nm of the nanoconjugate and F is the fluorescence intensity at 560 nm of the nanoconjugate with inspected species. The concentration of NaCl, KCl, $CaCl_2$, $ZnCl_2$, Glucose, Cystein, GSH and caspase-3 was 80 mM, 80 mM, 2 mM, 2 mM, 20 mM, 1 mM, 1mM, and 4 μg/mL, respectively.

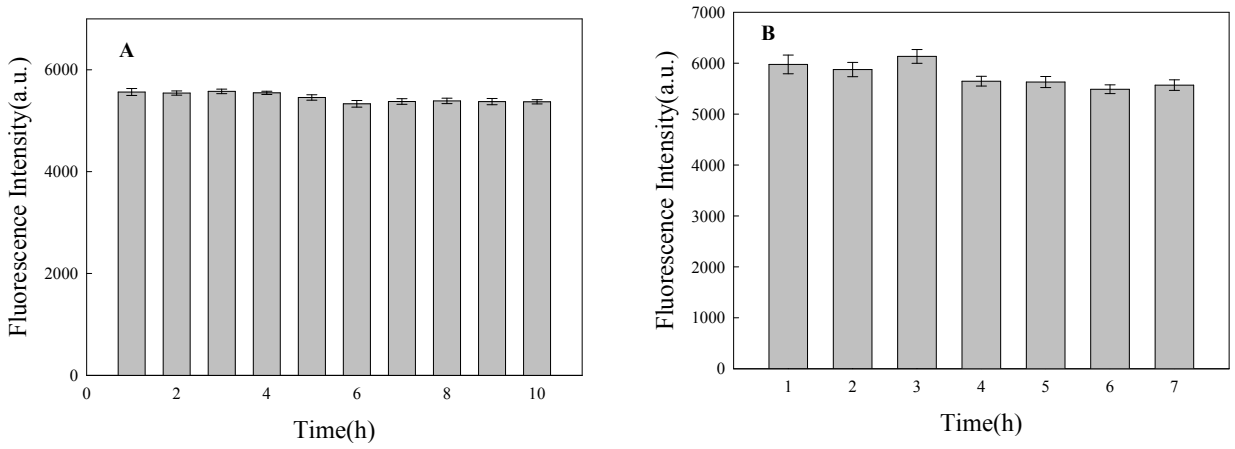


Fig. S3. Fluorescence emission intensity at 560 nm of the AuNPs/Petide-TPdye nanoconjugate in DMEM (A) and cell lysates (B).

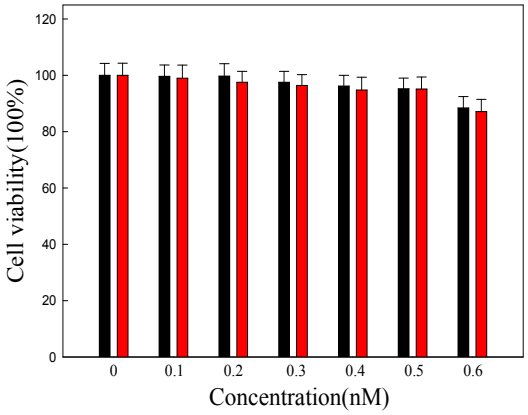


Fig. S4. Cell viability of HeLa treated with different concentrations of AuNP/Peptide-TPdye nanoconjugate (red) and AuNPs (black) for 24 h in fresh medium. Note: The concentration of the nanoconjugate refers to the concentration of AuNPs.

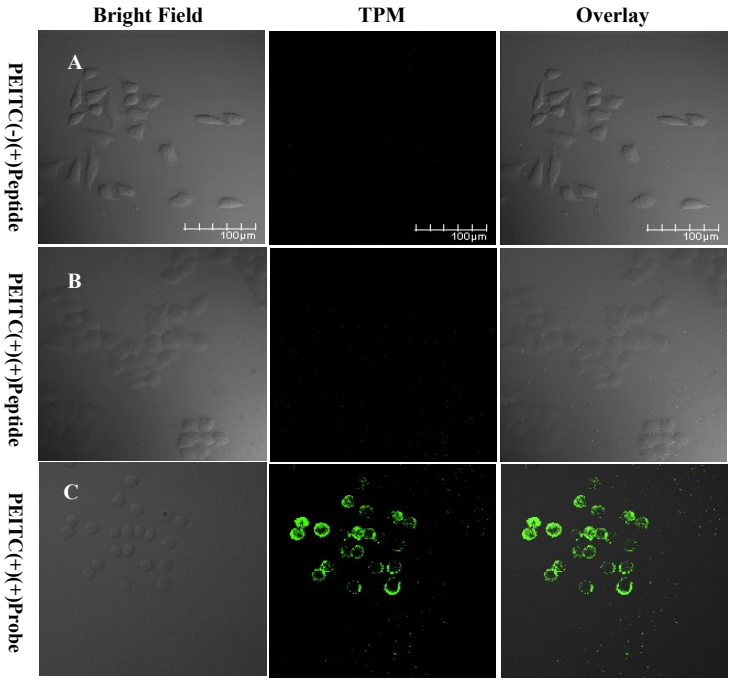


Fig. S5. TPM images of HeLa cells treated without (A)/with (B) PEITC and then incubated with TPdye-labeled peptide of "CANLNDEVDK-BMVC". (C) TPM image of HeLa cells treated with PEITC and then incubated with the AuNP/Peptide-TPdye nanoconjugates.

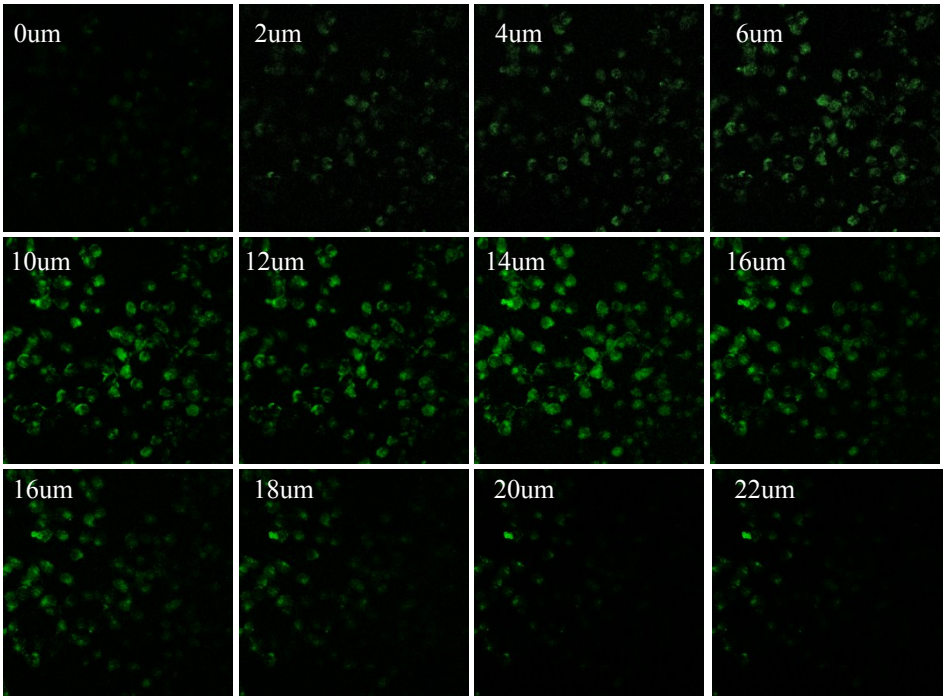


Fig. S6. Z-scanning confocal fluorescence microscopy images of HeLa cells incubated with AuNPs/Peptide-TPdye nanoconjugate and then treated with PEITC.