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Supporting Information

A peptide-based four-color fluorescent polydopamine nanoprobe for multiplexed sensing and imaging of proteases in living cells

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Experimental

Materials and reagent

Urokinase-type plasminogen activator (uPA), matrix metalloproteinase-2 (MMP-2), cathepsin B (CTB), matrix metalloproteinase-7 (MMP-7), thrombin (Tb), legumain (LGM), cathepsin D (CTD), glutathione (GSH), alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), vascular endothelial growth factor (VEGF), cytochrome C (Cyt-C) and amino acids were obtained from Invitrogen (Carlsbad, CA, USA) and Sigma-Aldrich. (St. Louis, MO, USA). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (MTT) were provided by Sigma-Aldrich. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and Lipofectamine 2000 reagent was purchased from Thermo Fisher Scientific Co. All dye-labeled peptides and unmodified peptides were purchased from Sangon Biotech Co. (Shanghai, China) and Biosynthesis Inc. (Louisville, USA). The peptide 1 (P1: Gly-Gly-Ser-Gly-Arg-Ser-Ala-Asn-Ala-Lys-Phe-Phe-Phe-Phe-Phe) was used to identify uPA, which was labeled with the Alexa Fluor 405 (AF405) dye. The peptide 2 (P2: Gly-Pro-Leu-Gly-Val-Arg-Gly-Phe-Phe-Phe-Phe-Phe) was targeted to MMP-2, which was tagged with the fluorescein isothiocyanate (FITC) dye. The peptide 3 (P3: Gly-Arg-Arg-Gly-Lys-Gly-Gly-Phe-Phe-Phe-Phe-Phe) is was used for the recognition of CTB, which was labeled with (2E)-1-[6-(2,5-dioxopy-rroli-din-1-yl)oxy-6-oxohexyl]-3,3-dimethyl-2-[(E)-3-(1,3,3-tri-methyl-5-sulfona-toindol-1-ium-2-yl)prop-2-enylidene]indole-5-sulfonate (cyanine dye Cy3). The peptide 4 (P4: Val-Pro-Leu-Ser-Leu-Thr-Met-Gly-Phe-Phe-Phe-Phe-Phe) was used for recognition of MMP-7, which was labeled with 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E,5E)-5-(1-ethyl-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)

penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (cyanine dye Cy5). Amiloride, antipain, ilomastat and dopamine hydrochloride (DA) were obtained from J&K (Beijing, China). All other reagents were of analytical grade and used without further purification. Ultrapure water from a Milli-Q plus 185 equip (Milli-pore Co., Bedford, MA, USA) was used throughout the work.

The human hepatocellular liver carcinoma cell line HepG2, human hepatocyte cell line HL-7702, human T24 bladder cancer cell line, human non-malignant urothelial cell line HCV29, human breast adeno-carcinoma cell line MCF-7 and normal human mammary epithelial cell line MCF-10A were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Apparatus

Absorption spectra were measured using a TU-1901 UV-visible spectrophotometer (Beijing Purkinje General Instrument Co, Ltd., China). Scanning electron microscopy (SEM) images was obtained by using a Vario-MicroCube electron microscope (Elementar, Germany). Transmission electron microscope (TEM) images was obtained by using Tecnai G2 SpiritBiotwin 120kV Biology TEM (FEI Company, USA). Raman spectra were recorded with a InVia Raman spectrometer (Reni-shaw, UK). Fourier transform infrared spectroscopy (FT-IR) spectra was recorded on a Spectrum Two spectrometer (Parkin-Elmer, USA). Fluorescence measurements were carried out using a LS-55 spectrofluorometer (Perkin-Elmer, USA) with a 300 μL cuvette. Confocal fluorescence imaging were performed with a LSM710 confocal laser scanning microscopy (Carl Zeiss AG Co., Ltd.

Germany) with an objective lens (63×). Flow cytometry analysis was performed on the LSRFortessa flow cytometer system (BD Biosciences, USA). The absorbance in MTT assays was measured with a EL×800 microplate reader (Bio Tek Instruments). HPLC assays were carried out using an LC-10ATVP system equipped with a RF-10AXL fluo-rescence detector (Shimadzu, Kyoto, Japan) and a C18 column (250×4.6 mm i.d., 5 μm particle sizes, Elite, China).

Synthesis of polydopamine nanoparticles

Polydopamine nanoparticles (PDANPs) were synthesized according to a previously reported method with some modifications.[1] Briefly, 4 mL ethanol and 0.3 mL ammonium hydroxide were added to 9 mL ultrapure water with stirring at 30 ℃. After 30 min at room temperature, 50 mg dopamine were added to the mixture, and the resulting mixture was continuously stirred overnight. Then, the obtained suspension was centrifuged and the precipitate was washed with ultrapure water five times. PDANPs were obtained and the precipitate was dried for the following experiments.

Preparation of the peptide-based four-color polydopamine nanoprobe

To prepare the peptide-based four-color polydopamine nanoprobe, the suspension (100 μL) of PDANPs (400 μg/mL) introduced into the 20 mM PBS solution (100 μL, pH 7.4) containing 200 nM each dye-labeled peptide, and then incubated at room tempera-ture for 20 min to form the four-color nanoprobe. After that, the four-color nanoprobe was stored at 4 °C before further usage.

Kinetics

The nanoprobe (100 nM) was incubated with four protease targets (200 ng/mL uPA, 1 ng/mL MMP-2, 4 ng/mL CTB, and 1 ng/mL MMP-7), then the fluorescence intensity was measured with increasing time (0, 10, 20, 30, 40, 50, 60 min). The fluorescent dyes in the sample solution were excited at 405 nm for AF405, 488 nm for FITC, 543 nm for Cy3 and 633 nm for Cy5, and the fluorescence signals were measured at 422 nm for AF405, at 516 nm for FITC, 563 nm for Cy3 and 663 nm for Cy5, respectively. And slits for both excitation and emission were set at 8 nm. All experiments were repeated three times.

In vitro detection of proteases by the four-color nanoprobe

For uPA assay, different concentrations of uPA (5 μL) were added into 100 nM the four-color nanoprobe in 20 mM PBS solution (95 μL, pH 7.4) and the mixture was incubated at 37 °C for 40 min. After incubation, the fluorescence of the resulting solution was measured by using a spectrofluorometer. The AF405 dye in the sample solution was excited at 405 nm, and the fluorescence signals were measured at 422 nm for uPA quantification. For MMP-2 detection, different concentrations of MMP-2 (5 μL) were added into 100 nM the four-color nanoprobe in 20 mM PBS solution (95 μL, pH 7.4) and the mixture was incubated at 37 °C for 40 min. After that, the resulting solution was used for fluorescence measurements. The FITC dye in the sample solution was excited at 488 nm, and the fluorescence signals were measured at 516 nm for MMP-2 quantification.

For CTB assay, different concentrations of CTB (5 μL) were added into 100 nM the four-color nanoprobe in 20 mM PBS solution (95 μL, pH 5.0) and the mixture was incubated at 37 °C for 40 min. Then, the resulting solution was used for fluorescence measurements at the excitation wavelength of 543 nm. The fluorescence signals of the Cy3 dye were measured at 563 nm for CTB quantification. For MMP-7 detection, different concentrations of MMP-7 (5 μL) were added into 100 nM the four-color nanoprobe in 20 mM PBS solution (95 μL, pH 7.4) and the mixture was incubated at 37 °C for 40 min. The resulting solution was used for fluorescence assays. The Cy5 dye in the sample solution was excited at 633 nm, and the fluorescence signals were measured at 663 nm for the quantification of MMP-7. For specificity study of each target protease assay, the procedures for detection of other non-specific analytes were carried out under other identical conditions (for each target protease assay) but the replacement of protease targets with other non-specific analytes. All experiments were repeated five times.

HPLC assay

HPLC was used to confirm the adsorption of dye-labeled peptides on PDANPs and the target protease-catalyzed cleavage reaction of dye-labeled peptides on the surface of PDANPs. Samples for HPLC assays were prepared as following: (1) the suspension (100 μL) of PDANPs (400 μg/mL) introduced into the 20 mM PBS solution (100 μL, pH 7.4) containing 200 nM each dye-labeled peptide, and then incubated at room temperature for 20 min. After that, the mixture solution was centrifuged at 12,000 rpm for 30 min at 4 ℃ and the supernatant was collected and analyzed by HPLC. (2) 5 μL of target

proteases (10 ng/mL) was introduced into 20 mM PBS solution (100 μL, pH 7.4) containing 100 nM the four-color nanoprobe, and the mixture solution was incubated at 37 ℃ for 40 min. After that, the mixture solution was centrifuged at 12,000 rpm for 30 min at 4 ℃ and the supernatant was collected for HPLC analysis. Blank samples were prepared similarly to the procedure mentioned-above except in the absence of target proteases. HPLC assays were carried out on a C18 column at a rate of 1.3 mL/min, with a 25 min gradient from 10 to 17% acetonitrile in 0.1 M triethylammonium acetate at pH 7.0.

Cell culture

The cells including HepG2 cells, HL-7702 cells, T24 cells, HCV-29 cells, MCF-7 cells and MCF-10A were cultured in DMEM with 10% FBS and 1% antibiotics penicillin/streptomycin solution and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was replenished every other day, and cells were subcultured after reaching confluence.

MTT assay

MTT assays were used to assess the cytotoxicity of the four-color nanoprobe on HepG2 cells, HL-7702 cells, T24 cells, HCV-29 cells, MCF-7 cells and MCF-10A cells. The cells (1×10^6 cells/well) were first seeded in the 96-well microtiter plates (200 μL/well) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After that, the original culture medium was discarded, and the cells were incubated with free PDANPs

(0.2 mg/mL) or 100 nM the four-color nanoprobe in the culture medium for 12 and 24 h. The cells incubated with only culture medium were used as the control groups. Subsequently, the cells were washed three times using sterilized PBS and 100 μL MTT solution (0.5 mg/mL in culture medium) was added to each well. After incubation of 4 h, the MTT solution was discarded. Finally, 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a EL×800 microplate reader.

Live cell protease imaging with the four-color peptide nanoprobe

All cells were planted on glass bottom cell culture dishes and grown at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. Then, the original culture medium was discarded, and 200 μL of fresh culture medium containing 100 nM the four-color nanoprobe was added to the dishes. After incubation at 37 °C in a humidified atmosphere containing 5% CO_2 for different times, PBS (pH 7.4) solution was used to wash the cells three times. The fluorescence imaging of the cells was performed on a confocal scanning laser microscope (CSLM) with an objective lens (63×) under different excitation wavelengths. The fluorescence of the dyes in cells was excited at at 405 nm for AF405, 488 nm for FITC, 543 nm for Cy3 and 633 nm for Cy5, and the fluorescence of AF405, FITC, Cy3 and Cy5 was measured from 412 nm to 495 nm, 500 nm to 540 nm, from 550 nm to 640 nm, and from 645 nm to 710 nm, respectively.

For inhibition assay, T24 cells or HepG-2 cells were seeded into the dishes and incubated in culture medium for 24 h at 37 °C in a humidified atmosphere containing 5%

CO_2. After removing original culture medium, T24 cells or HepG-2 cells were divided into four groups for the following treatments: group 1, incubation with 200 μL of fresh culture medium containing 100 nM the four-color nanoprobe; group 2, adding 10 μM amiloride for 48 h and then incubation with 200 μL of fresh culture medium containing 100 nM the four-color nanoprobe; group 3, adding 10 μM antipain for 48 h and then incubation with 200 μL of fresh culture medium containing 100 nM the four-color nanoprobe; group 4, adding 10 μM ilomastat and incubation with 200 μL of fresh culture medium containing the four-color nanoprobe. After incubation at 37 °C for 12 h, the cells were washed with PBS (pH 7.4) solution three times and then analyzed by CSLM imaging. The conditions for CSLM imaging were shown as those mentioned above.

For imaging of proteases in co-cultured cells, HepG2 cells and HL-7702 cells were co-seeded into the same dish and were co-cultured for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. After removement of original culture medium, the co-cultured HepG2 cells and HL-7702 cells were incubated with 200 μL of fresh culture medium containing 100 nM the four-color nanoprobe for 12 h at 37 °C. Then, the cells were washed with PBS (pH 7.4) solution three times and subsequently analyzed by CSLM imaging. The conditions for CSLM imaging were shown as those mentioned above.

For imaging of extraneous proteases in HL-7702 cells, HL-7702 cells were seeded into the dishes and were incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. The original culture medium was removed, and the cells were incubated in the transfection mixture (containing 10 μL of Lipofectamine 2000 reagent and 5 μL of mixed standard protease target (3.5 ng/mL uPA, 50 pg/mL MMP-2, 200 pg/mL

CTB, 50 pg/mL MMP-7)) at 37 °C in a humidified atmosphere containing 5% CO_2 for 3 h. Then, the transfection mixture was removed, and the cells were incubated with 200 μL of fresh culture medium containing 100 nM the four-color nanoprobe for 12 h at 37 °C. Finally, the cells were washed with PBS (pH 7.4) solution three times and subsequently analyzed by CSLM imaging. The conditions for CSLM imaging were shown as those mentioned above.

Flow cytometric assays

Flow cytometric assays were performed to confirm the detection of four intracellular protease targets with the four-color nanoprobe. In flow cytometry experiments, HepG2 cells, HL-7702 cells, T24 cells, HCV-29 cells, MCF-7 cells and MCF-10A cells were chosen. The cells were planted on a 6-well plate and cultured in DMEM medium at 37 °C in 5% CO_2 for 24 h. After that, the original culture medium was discarded, and 200 μL of fresh culture medium containing 100 nM the four-color peptide nanoprobe was added to the plate. The cells were further incubated for another 12 h and washed three times with sterilized PBS (pH 7.4). The cells obtained were collected and analyzed by a flow cytometer with different laser transmitters. The fluorescence signals of the dyes in cells were excited at 405 nm for AF405, 488 nm for FITC, 543 nm for Cy3, and 633 nm for Cy5.

Results and discussion

Characterization of PDANPs

PDANPs were prepared by using a previously reported method with minor modification.[1] The prepared PDANPs showed spherical-like morphology with the average size of 120 nm in diameter (Fig. S1). This indicates that PDANPs falls within a size range that favors cellular uptake by mammalian cells.[2] PDANPs showed the characteristic bands of C=C (1622 cm-1), C-N (1385 cm-1) and N-H (3426 cm-1) in the FTIR spectrum (Fig. S2), suggesting that the PDANPs are π-rich polymers. Moreover, PDANPs also exhibited broad-band absorbance in the UV-vis spectrum that overlaps with fluorescence spectra of multiple different fluorophores (Fig. S3). This result indicates that PDANPs would be used as the fluorescence resonance energy transfer acceptor for the quenching of the fluorescence of multiple fluorophores.[1,3,4] In addition, the Raman spectrum of PDANPs, which was consistent with that of other reports,[1] provided another evidence of successful synthesis of PDANPs (Fig. S4).

Characterization of the dye-labeled peptide probes and the four-color nanoprobe

The dye-labeled peptide probes were charaterized by UV-Vis spectra. As shown in Fig. S6, each dye-labeled peptide probe exhibited the adsorption peaks of both peptide and the corresonding dye. That is, AF405-labeled P1 had a peak of AF405 dye at ~400 nm and a peak of peptide at ~280 nm, FITC-labeled P2 had a peak of FITC dye at ~490 nm and a peak of peptide at ~280 nm, Cy3-labeled P3 had a peak of Cy3 dye at ~549 nm and a peak of peptide at ~280 nm, and Cy5-labeled P4 had a peak of Cy5 dye at ~648 nm

and a peak of peptide at ~280 nm. In addition, the proposed four-color nanoprobe was also charaterized by UV-Vis spectra and the results are shown in Fig. S7. Comapared with unmodified PADNPs (Fig. S3), the four-color nanoprobe had the adsorption peaks of all dye-labeled peptides. These results demonstrated the successfull formation of the four-color nanoprobe. Furthermore, we carried out th experiments for measurement of the fluorescence spectra of the four dyes on the nanoprobe under individual different excitation wavelengths and the results are shown in Fig S8. Upon excitation of each dye in the nanoprobe, the peak of the corresponding dye in the fluorescence spetra was observed and no peaks of other dyes was obtained. This was attributed to the proximity of the dyes to polydopamine nanoparticles and the significant fluorescence quenching of the dyes by polydopamine nanoparticles. This result indicated that no fluorescence resonance energy transfer (FRET) occured between the four dyes in the proposed four-color nanoprobe.

HPLC characterization

To evaluate the viability of our sensing strategy, the adsorption of dye-labeled peptide substrates on PDANPs and the cleavage of dye-labeled peptide substrates on the PDANPs by protease targets were further confirmed by HPLC fluorescence assays. To test adsorption of dye-labeled peptide substrate on PDANPs, the suspension of PDANPs was added to a mixture of three types of dye-labeled peptide substrates and then incubated the mixture. After centrifugation, the supernatant was collected and analyzed by HPLC at different detection wavelengths. As shown in Fig. S9, a peak from

each dye-labeled peptide substrate appeared in the chromatograms before the addition of PDANPs (traces a-d). However, upon the addition of PDANPs, the peaks from dye-labeled peptide substrates disappeared in the chromatograms (traces e-h). These results indicate that dye-labeled peptide substrates can be adsorbed onto PDANPs. This adsorption was due to the π-π stacking interactions between the aromatic amino acid residues of peptide substrates and PDANPs. For the cleavage study, a solution of the four-color nanoprobe was incu-bated with four protease targets (uPA, MMP-2, CTB, and MMP-7) and then centrifuged to collect the supernatant for HPLC assays. The assay results are shown in Fig. S11. It was clear that a peak appeared in each chromatogram (traces e-h) after incubation of the four-color nanoprobe with the target proteases. However, no peaks in the chromatograms (traces a-d) were observed from blank sample (that is, the four-color nanoprobe solution without target proteases). These results demonstrate that dye-labeled peptide substrates on PDANPs can be cleaved by target proteases, releasing the dye-labeled short peptide fragments. This provides a solid foundation for our sensing strategy.

Kinetics of the four-color nanoprobe

The kinetics of the four-color nanoprobe toward four tumor-related protease targets were investigated. Fig. S14 shows the kinetics of the four-color nanoprobe. It was clear that the four-color nanoprobe responded rapidly to all four protease targets within 40 min. The matched response time for four protease targets makes the four-

color nanoprobe attractive for simultaneous sensing and imaging of four tumor-related protease biomarkers in living cells.

Study of bleed-through of dyes

The four dyes used in this work were similar to those of the previous reported method for multiplexed imaging of mRNAs in living cells.[5] The effect of the fluorescence of the selected dyes on each other for protease detection was studied. First, we measured fluorescence spectra of three dye-labeled peptides (FITC-labeled P2, Cy3-labeled P3, and Cy5-labeled P4, 100 nM each) under different excitation wavelengths. The experimental results are shown in Fig. S17. Upon excitation of FITC dye at 488 nm, FITC-labeled P2 exhibits strong fluorescence. However, no fluorescence of FITC-labeled P2 was observed by using 405 nm as excitation wavelength. Similarly, strong fluorescence of Cy5-labeled P4 was observed by the excitation of Cy5 dye at 633 nm, and no fluorescence of Cy5-labeled P4 was obtianed by using 543 nm as excitation wavelength. These results indicate that the fluorescence of FITC-labeled P2 have no influence on uPA detection and the fluorescence of Cy5-labeled P4 have no influence on uPA detection. For Cy3-labeled P3 assay, strong fluorescence of Cy3-labeled P3 was observed by using 543 nm as excitation wavelength. Only very weak fluorescence of Cy3-labeled P3 was obtianed by using 488 nm as excitation wavelength and the fluorescence intensity was 9.25. The wavelengths of this weak Cy3's fluorescence (from excitation at 488 nm) ranged from 540 nm to 620 nm. This fluorescence wavelength range of Cy3-labeled P3 did not influence the in vitro detection of MMP-2 (using FITC-labeled P2), because the fluorescence wave-

length of FITC dye of 516 nm for quantification of MMP-2 have no overlap with the weak Cy3's fluorescence (from excitation at 488 nm). Then, we carried out fluorescence imaging of the solutions of FITC-labeled P2 (100 nM), Cy3-labeled P3 (100 nM), and Cy5-labeled P4 (100 nM) by using different excitation wavelengths and the same detection wavelenths for the same dye, respectively. The experimental results are shown in Fig. S18. Strong green fluorescence of FITC-labeled P2 was observed by using 488 nm as excitation wavelength and no fluorescence of FITC-labeled P2 was observed by using 405 nm as excitation wavelength. When the Cy3-labeled P3 solution was imaged, strong yellow fluorescence was observed by using 543 nm as excitation wavelength and no fluorescence was observed by using 488 nm as excitation wavelength. For imaging of Cy5-labeled P4, strong red fluorescence was observed by using 633 nm as excitation wavelength and no fluorescence was observed by using 543 nm as excitation wavelength. These results indicate that there is no cross-interference of the fluorescence of the dyes on imaging of the solutions of the dye-labeled peptides. In addition, we also carried out the fluorescence imaging of T24 cells incubated with single-color nanoprobe (FITC-labeled P2/PDANP nanocomplex, Cy3-labeled P3/PDANP nanocomplex, or Cy5-labeled P4/PDANP nanocomplex) by using different excitation wavelengths and the same detection wavelenths for the same dye. The experimental results are shown in Fig. S19. When T24 cells were incubated with FITC-labeled P2/PDANP nanocomplex, high green fluorescence of T24 cells was observed by using 488 nm as excitation wavelength and no fluorescence signal was obtained by using 405 nm as excitation wavelength. Upon incubation of T24 cells with Cy3-labeled P3/PDANP nanocomplexes, high yellow

fluorescence of T24 cells was observed by using 543 nm as excitation wavelength and no fluorescence signal was obtained by using 488 nm as excitation wavelength. Upon incubation of T24 cells with Cy5-labeled P4/PDANP nanocomplexes, high red fluorescence of T24 cells was observed by using 633 nm as excitation wavelength and no fluorescence signal was obtained by using 543 nm as excitation wavelength. These results demonstrate that there is no cross-interference of the fluorescence of the dyes on live cell imaging under the selected assay conditions and the proposed four-color nanoprobe would be used for the simultaneous imaging of multiple tumor-related proteases in living cells.

Imaging of proteases in co-cultured HepG2 and HL7702 cells

The proposed four-color nanoprobe was also applied to image protease targets in co-cultured cells. The cancerous HepG2 cells and normal HL-7702 cells were selected as the model and co-cultured. After incubation with the four-color nanoprobe, the co-cultured HepG2 and HL7702 cells were analyzed by CSLM imaging. As shown in Fig. S20, the blue fluorescence of AF405 for uPA, green fluorescence of FITC for MMP-2, and red fluorescence of Cy5 for MMP-7 were obtained from both HepG2 cells and HL-7702 cells in co-cultured cells. However, yellow fluorescence of Cy3 for CTB was obtained from only HepG2 cells in co-cultured cells. This was attributed to the fact that uPA, MMP-2 and MMP-7 could be secreted from HepG2 cells and entered into HL7702 cells,[6] and then cleaved their dye-labeled peptide substrates to activate the fluorescence of the corresponding dyes. It has been reported that the CTB protease secreted from the cells

needed in an acidic extracellular microenvironment.[7] In our experiment, co-cultured HepG2 and HL7702 cells were incubated in a neutral condition, and the secretion of CTB from HepG2 cells was inhibited. Therefore, no yellow fluorescence of Cy3 for CTB was obtained from HL-7702 cells in co-cultured cells. These results indicate that our propoed four-color nanoprobe could be used for imaging of multiple proteases in the co-cultured HepG2 and HL7702 cells and distinguish HepG2 cells from HL7702 cells.

Specificity study

To investigate the specificity of the proposed four-color nanoprobe, we tested the response of the nanoprobe to protease targets, other several nonspecific proteins, GSH, and 20 amino acids, which often exist in living cells. As shown in Fig. S21, the protease targets led to the significant increase of the fluorescence of the corresponding dyes, while no apparent fluorescence increase was observed in the assay for other nonspecific proteins, GSH and amino acids. These results demonstrate high specificity of our proposed four-color nanoprobe for simultaneous detection of uPA, MMP-2, CTB and MMP-7. To further evaluate the specificity of the four-color nanoprobe, we used T24 cells and HepG2 cells as models to perform the intracellular protease inhibition assays by using specific inhibitors of target proteases and the four-color nanoprobe. The experiment results are shown in Fig. 3 and Fig. S22. Upon the treatment of T24 cells and HepG2 cells with amiloride (uPA inhibitor), the blue fluorescence of AF405 for uPA decreased and the fluorescence of other dyes did not change obviously. When T24 cells and HepG2 cells were treated with antipain (CTB inhibitor), only yellow fluorescence of Cy3 for CTB

weakened. Upon the ilomastat (inhibitor for both MMP-2 and MMP-7) treatment, both green fluorescence of FITC for MMP-2 and red fluorescence of Cy5 for MMP-7 decreased, while fluorescence of other dyes was almost unchanged. These results indicate that the proposed four-color nanoprobe enables the detection of the changes of tumor-related protease expression levels in living cells, and fluorescence signals in cells were generated from intracellular protease targets. In addition, we also used normal HL7702 cells (no endogenous protease targets in HL7702 cellss were detected and imaged by using the proposed four-color nanoprobe) as model cells and treated HL-7702 cells with each standard protease target or the mixture of four protease targets via Lipofectamine transfection. The untreated and treated HL-7702 cells were analyzed by CSLM imaging, and the results are shown in Fig. 23. No fluorescence of all four dyes was detected from untreated HL-7702 cells. Upon transfection of each standard protease target into HL-7702 cells, strong fluorescence of the corresponding dyes for specific protease targets was observed. That is, the transfection of uPA into HL-7702 cells led to high blue fluorescence of AF405, the transfection of MMP-2 into HL-7702 cells led to high green fluorescence of FITC, the transfection of CTB into HL-7702 cells led to high yellow fluorescence of Cy3, and the transfection of MMP-7 into HL-7702 cells led to high red fluorescence of Cy5. When HL-7702 cells were treated with all four protease targets, strong fluorescence of all four dyes was observed. These results further demonstrate that the fluorescence signals of the dyes in cells is specifically generated from protease targets in living cells and the proposed four-color nanoprobe exhibits high specificity for simultaneous imaging of four tumor-related proteases in living cells.

Nanoprobe stability

The stability of the proposed four-color nanoprobe was studied by measurement of the fluorescence of the dyes after storage of the nanoprobe in PBS solution at 4 °C for different time. As shown in Fig. S24, the fluorescence of all four dyes on the four-color nanoprobe kept almost constant when the storage time was up to one weak. This result indicate that the four-color nanoprobe could exist stably in PBS solution at 4 °C within one weak. To investigate stability of the nanoprobe in cells, we applied TEM to image PDANPs in T24 cells after incubation of the cells with the nanoprobe for 24 h, and the results are shown in Fig. S25. Compared with the TEM image of untreated T24 cell, PDANPs were observed in the TEM image of the T24 cell treated with the nanoprobe. PDANPs in cells still had a spherical morphology with a diameter of ~120 nm. This result demonstrate that the PDANPs was stable in cells within 24 h.

Flow cytometric analysis of intracellular proteases

To further verify that the proposed four-color nanoprobe could be used to the simul-taneous detection of multiple intracellular protease biomarkers, flow cytometric assays were performed on T24, HCV29, HepG2, HL-7702 MCE-7 and MCF-10A cells after the incubation with the four-color nanoprobe. Fig. 26 shows the flow cytometric assay results of different cell lines after incubation with the four-color nanoprobe. In all dye channels, strong fluorescence signals were obtained from cancer cell lines, whereas only very weak fluorescence signals in all channels could be detected from all normal cell

lines. This is consistent with the observations from fluorescence images of these cells in the Fig. 2, su-pporting that the proposed four-color nanoprobe could be used for simultaneous sensing of multiple intracellular protease biomarkers, and specifically distinguish between cancer cells and normal cells.

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Supporting figures

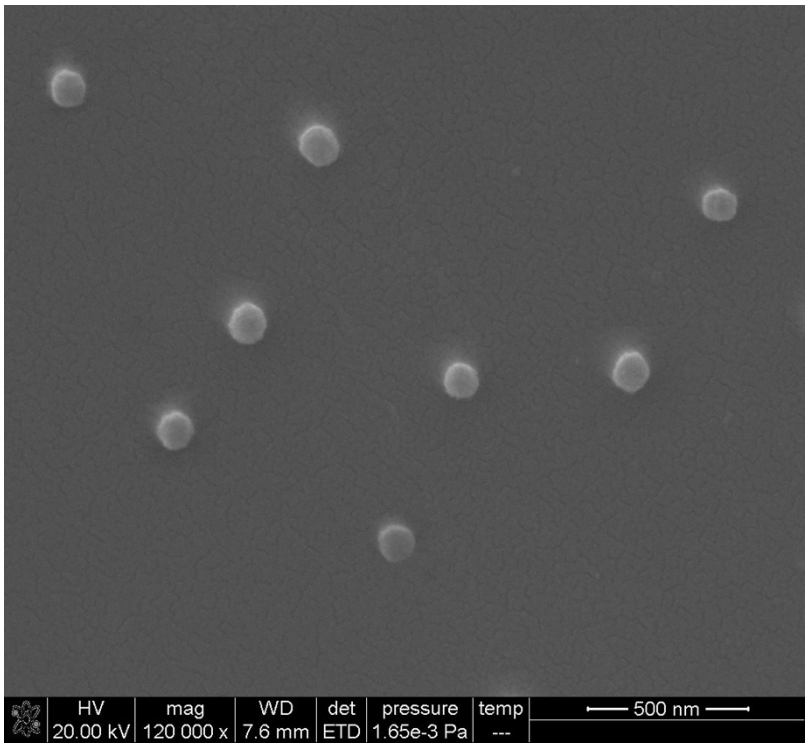


Fig. S1 The TEM image of PDANPs.

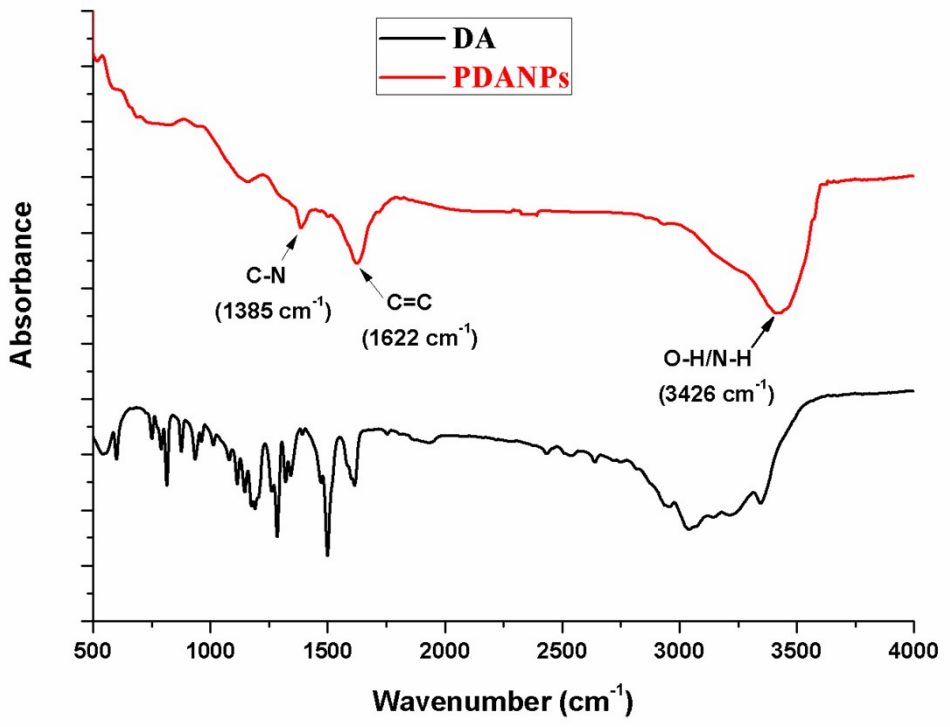


Fig. S2 The FT-IR spectrum of (a) dopamine and (b) PDANPs.

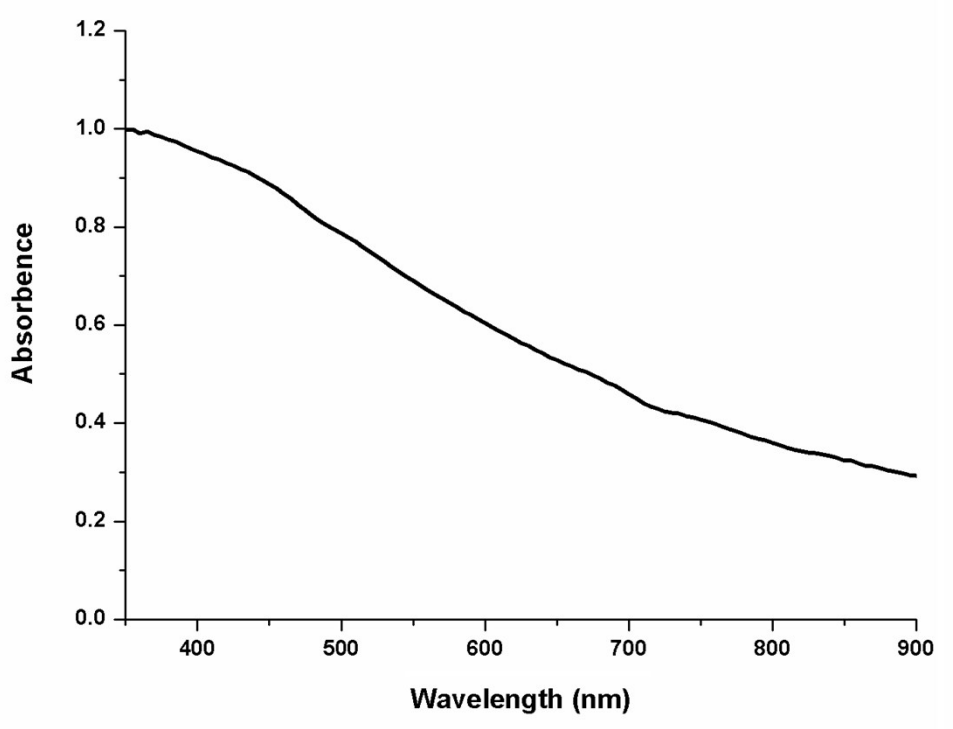


Fig. S3 The UV-Vis spectrum of PDANPs.

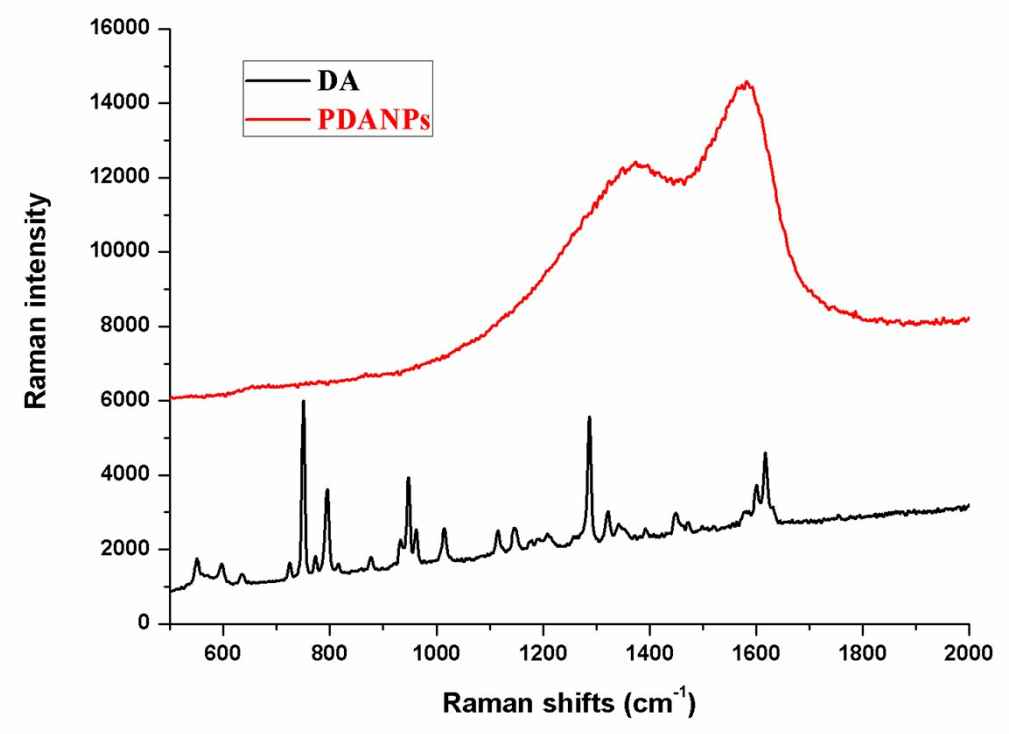
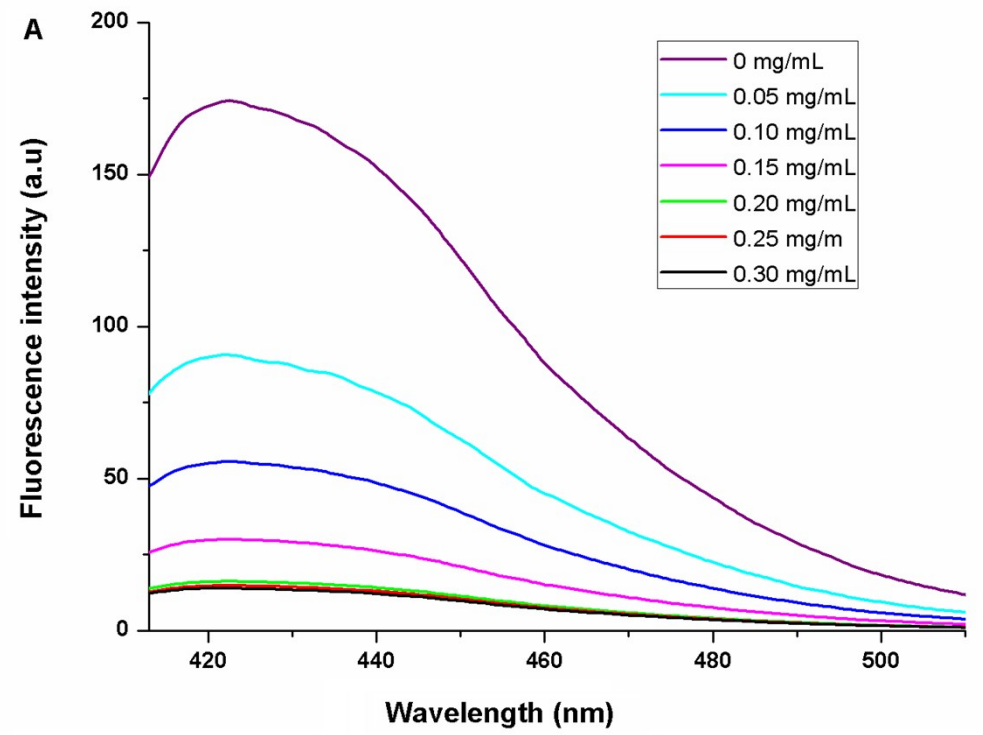
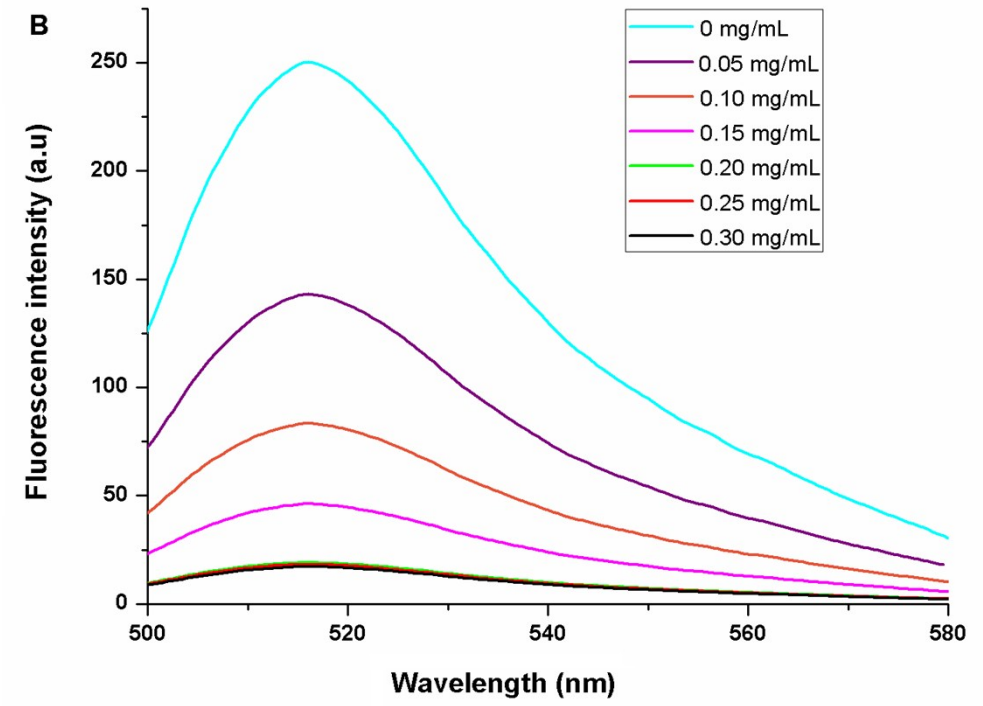
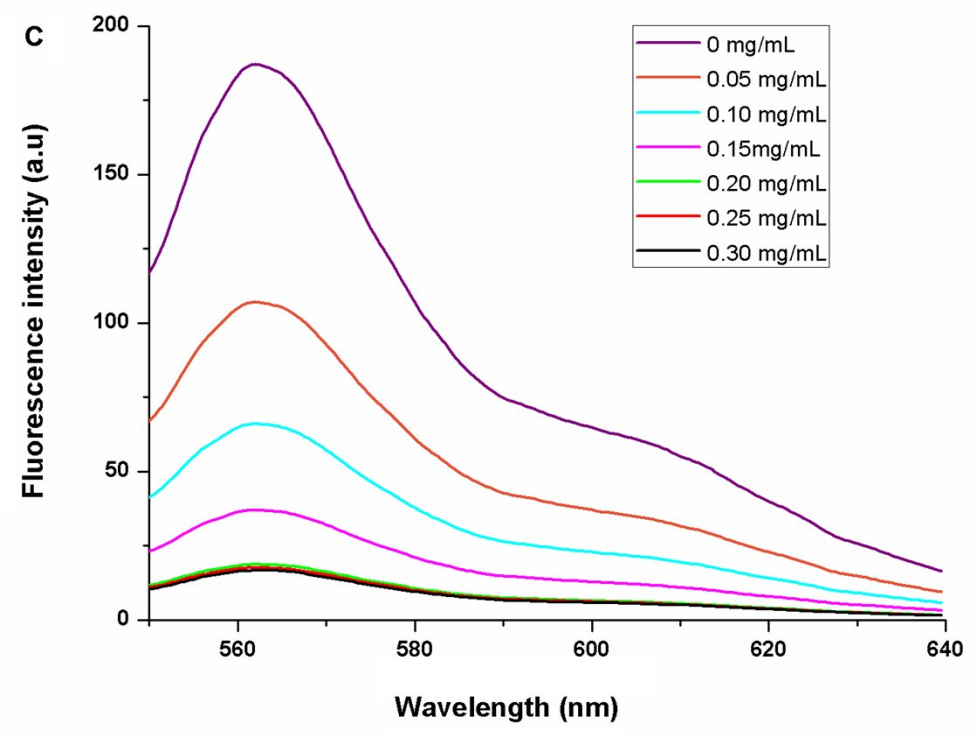
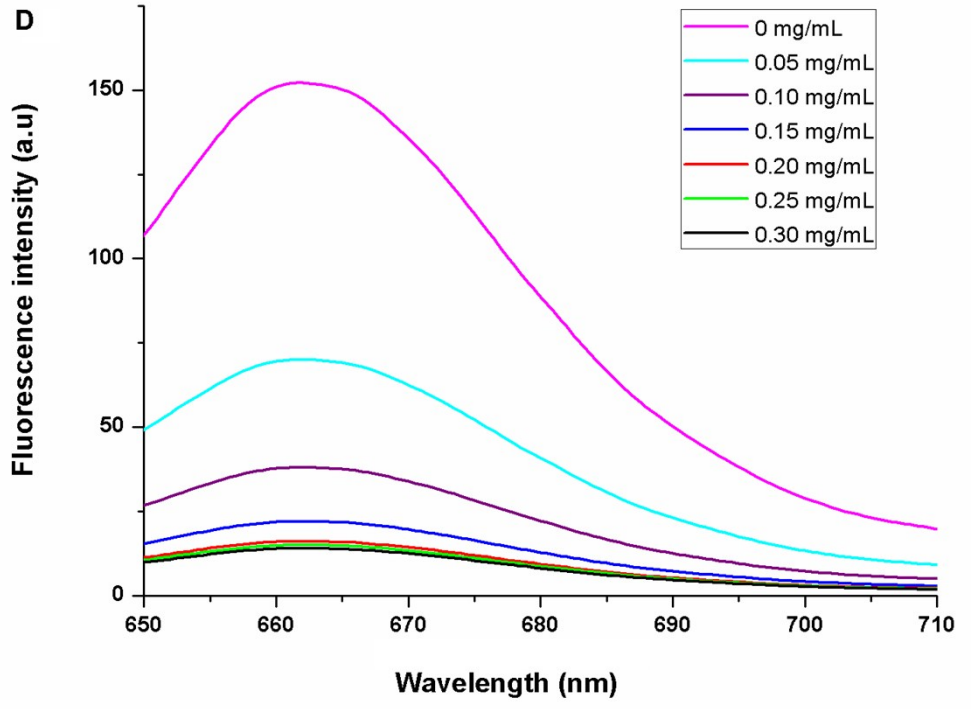


Fig. S4 The Raman spectra of (a) dopamine and (b) PDANPs.


A
Fluorescence intensity (a.u)
Wavelength (nm)
0 mg/mL
0.05 mg/mL
0.10 mg/mL
0.15 mg/mL
0.20 mg/mL
0.25 mg/m
0.30 mg/mL
0
50
100
150
200
420
440
460
480
500


B
Fluorescence intensity (a.u)
Wavelength (nm)
0 mg/mL
0.05 mg/mL
0.10 mg/mL
0.15 mg/mL
0.20 mg/mL
0.25 mg/mL
0.30 mg/mL
0
50
100
150
200
250
500
520
540
560
580


C
Fluorescence intensity (a.u)
Wavelength (nm)
0 mg/mL
0.05 mg/mL
0.10 mg/mL
0.15mg/mL
0.20 mg/mL
0.25 mg/mL
0.30 mg/mL
0
50
100
150
200
560
580
600
620
640


D
Fluorescence intensity (a.u)
Wavelength (nm)
0 mg/mL
0.05 mg/mL
0.10 mg/mL
0.15 mg/mL
0.20 mg/mL
0.25 mg/mL
0.30 mg/mL
0
50
100
150
650
660
670
680
690
700
710

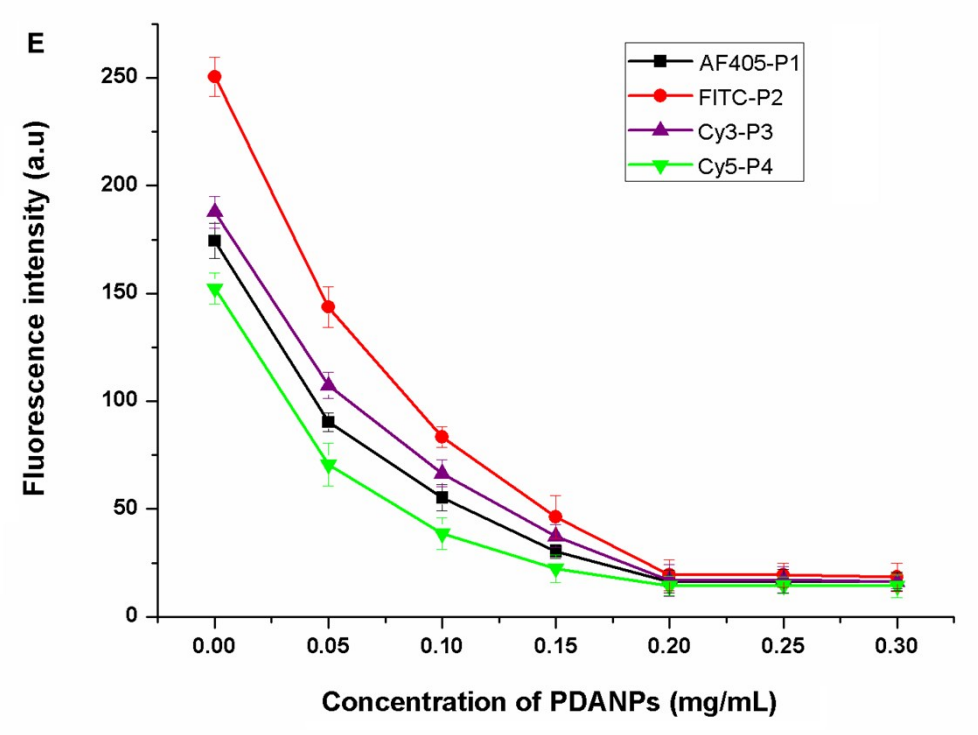
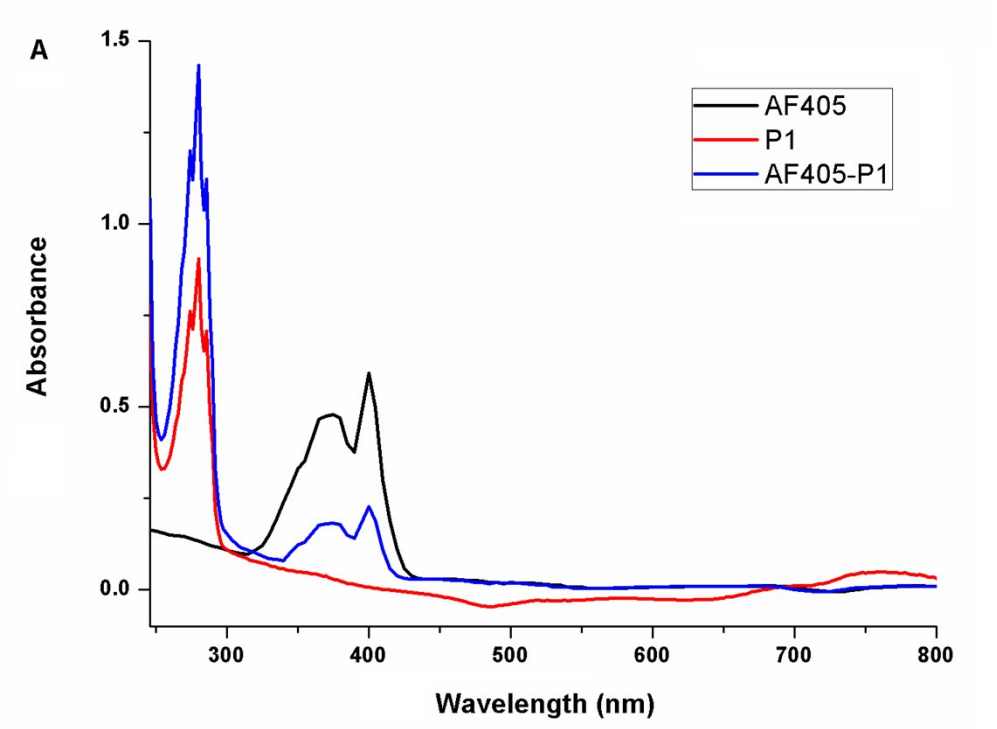
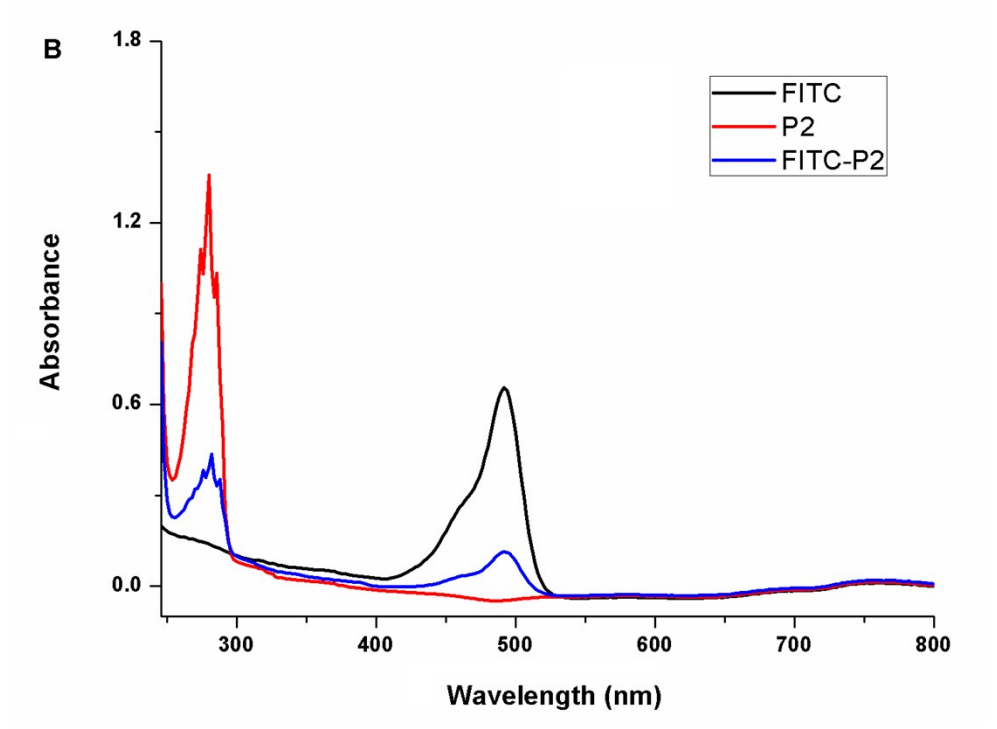


Fig. S5 Fluorescence spectra (A-D) and fluorescence intensity (E) of four dye-labeled peptides (100 nM each) upon the introduction of different concentrations of PDANPs. (A) AF405-P1; (B) FITC-P2; (C) Cy3-P3; (D) Cy5-P4. Error bars were derived from N=5 experiments.


A
Absorbance
1.5
1.0
0.5
0.0
300
400
500
600
700
800
Wavelength (nm)
AF405
P1
AF405-P1


B
Absorbance
1.8
1.2
0.6
0.0
300
400
500
600
700
800
Wavelength (nm)
FITC
P2
FITC-P2

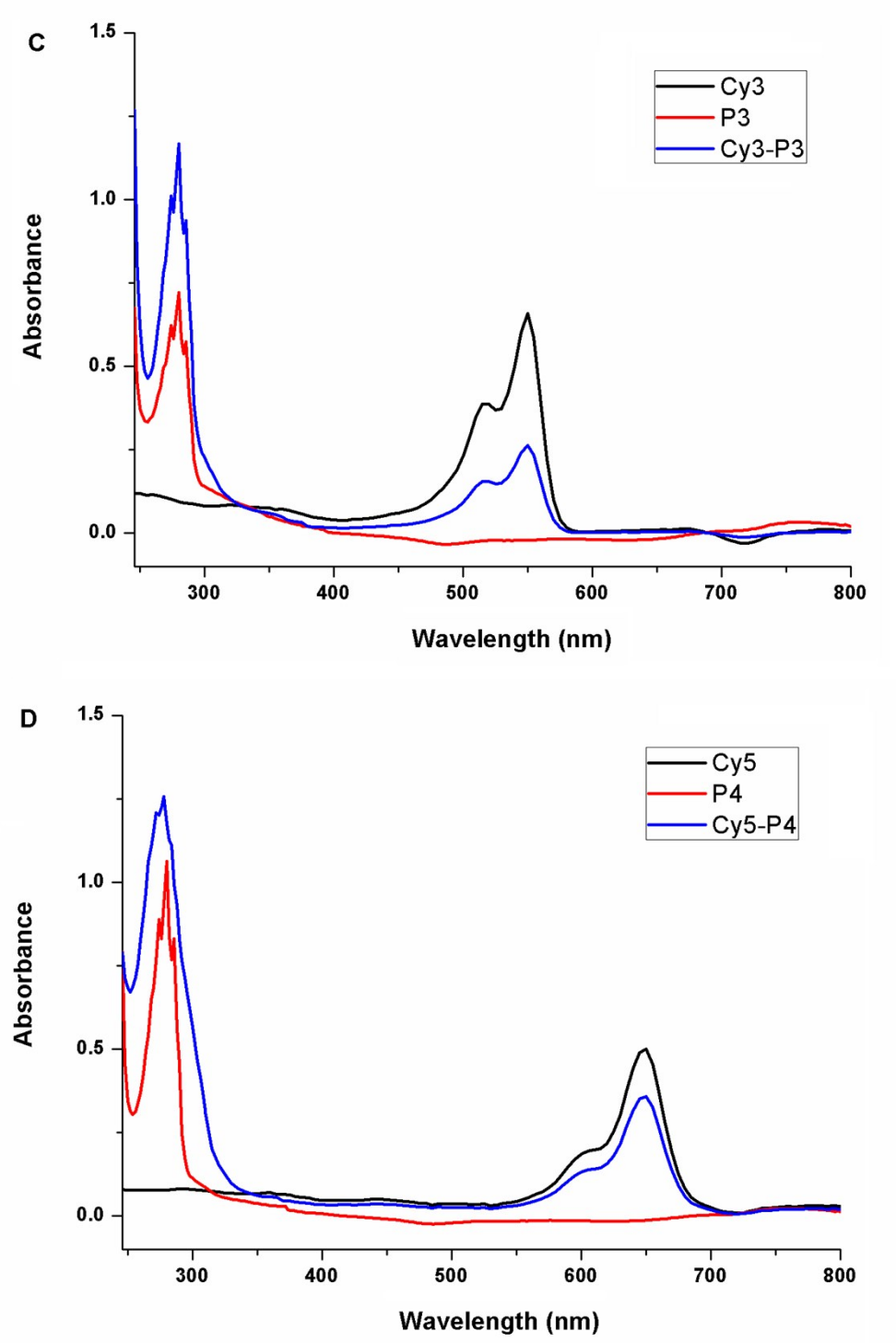


Fig. S6 The UV-Vis spectra of dye-labeled peptides and fluorescent dyes. (A) AF405-labeled P1, P1 and AF405; (B) FICT-labeled P2, P2 and FITC; (C) Cy3-labeled P3, P3 and Cy3;(D) Cy5-labeled P4, P4 and Cy5.

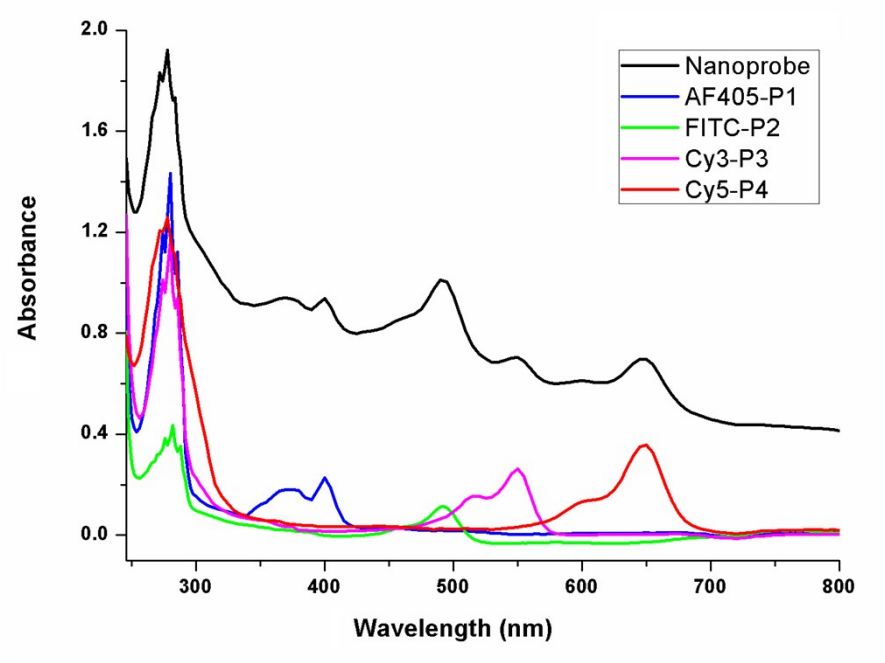


Fig. S7 The UV-Vis spectra of dye-labeled peptides and the nanoprobe.

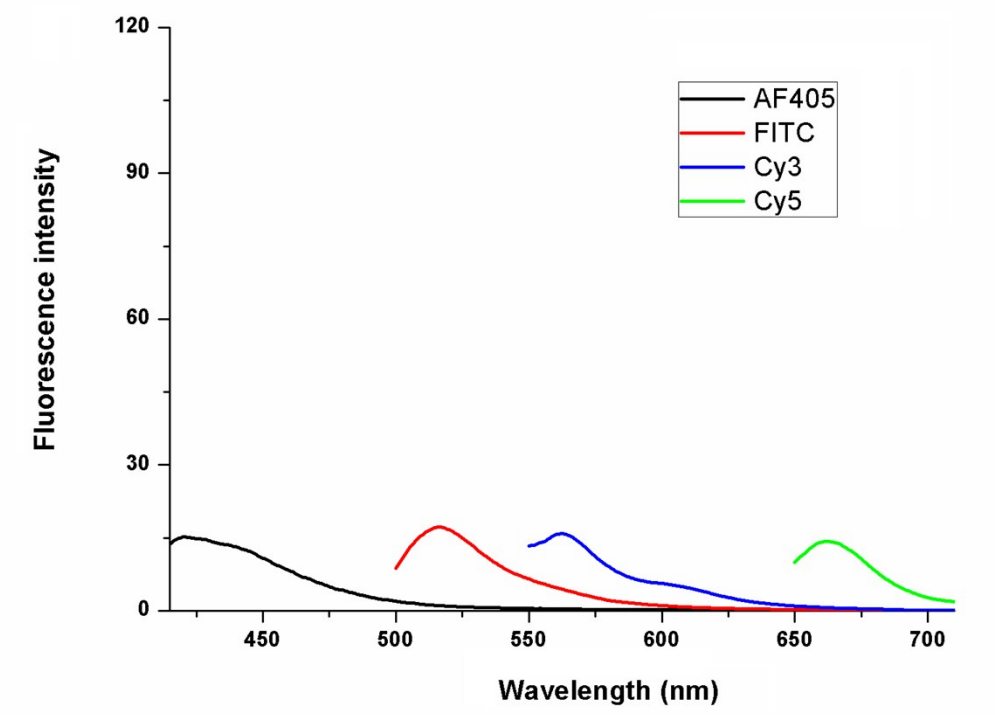


Fig. S8 Fluorescence spectra of the four-color nanoprobe (100 nM). (A) AF405; (B) FITC; (C) Cy3; (D) Cy5.

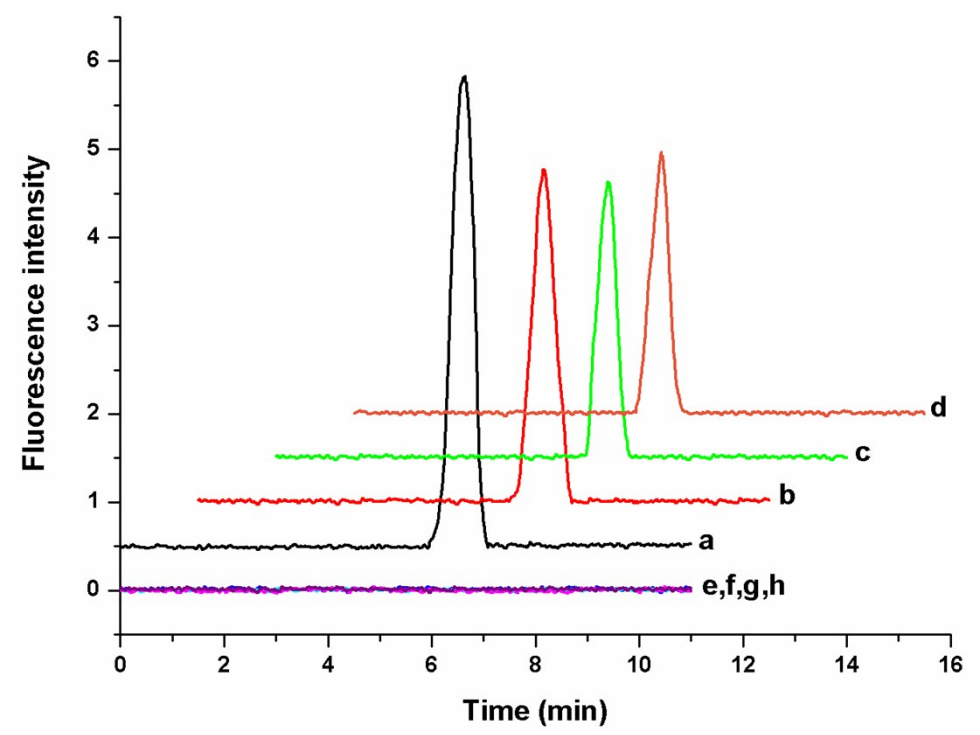


Fig. S9 The chromatograms obtained from the incubation solutions of four dye-labeled peptides in the absence of PDANPs (curves a-d) and in the presence of 0.2 mg/mL PDANPs (curves e-h).

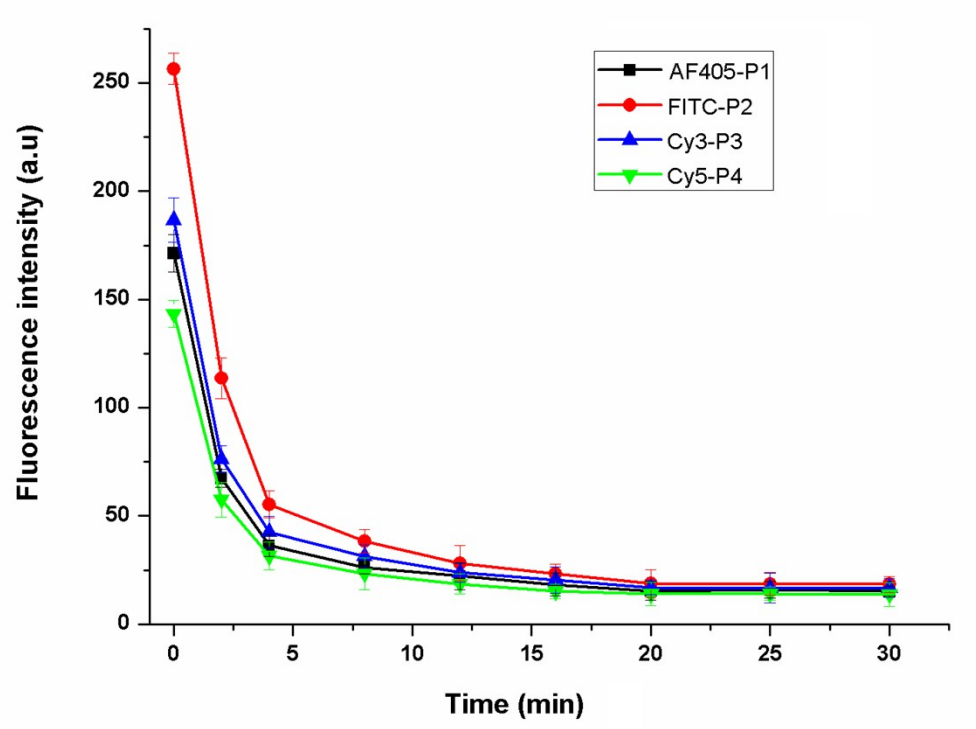


Fig. 10 The fluorescence intensity of the four dye-labeled peptides (100 nM each) upon incubation of 0.2 mg/mL PDANPs for different time. Error bars were derived from N=5 experiments.

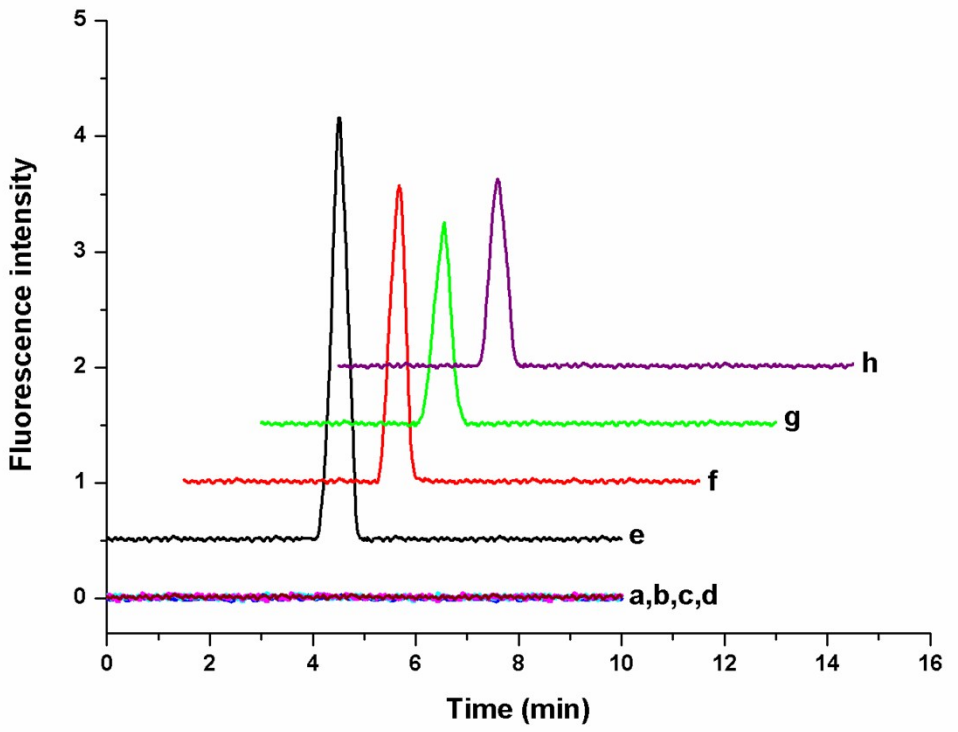
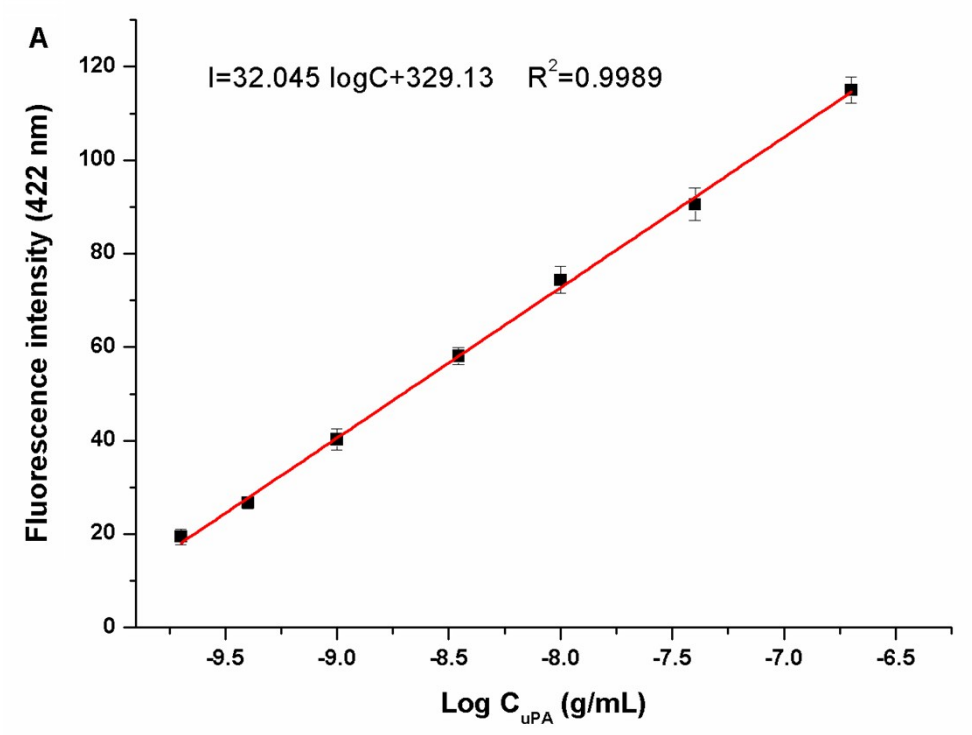
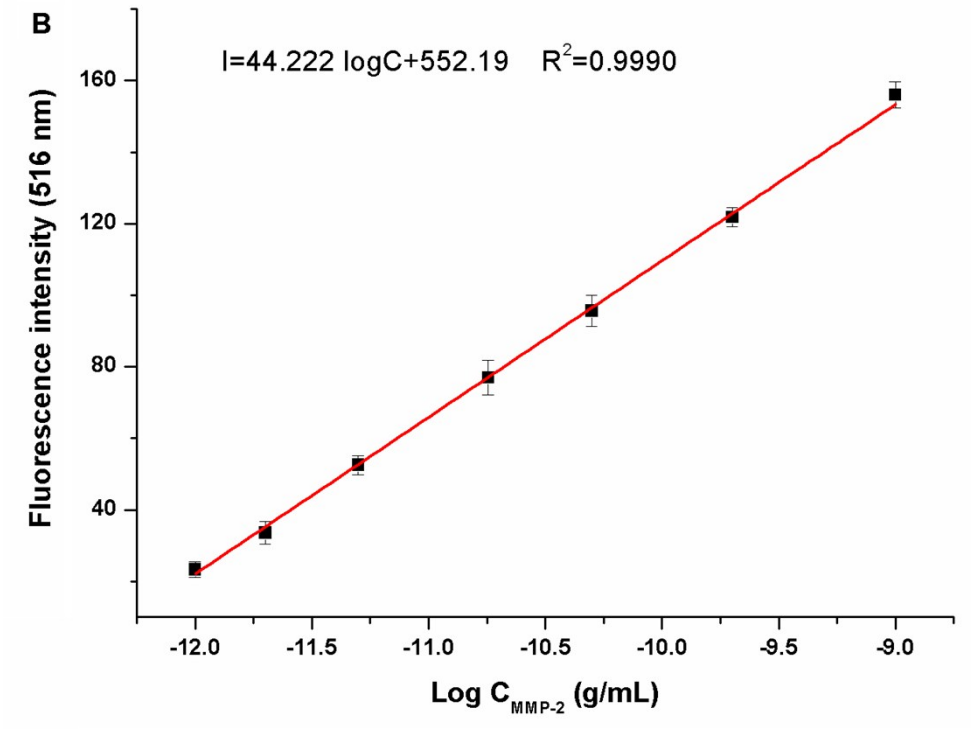


Fig. S11 The chromatograms obtained from an incubation solution of the proposed four-color nanoprobe in the presence of four tumor-associated protease targets (curves a-d) or a blank sample solution (that is, the four-color nanoprobe incubated with the assay buffer only; (curves e-h)).


A
I=32.045 logC+329.13 R2=0.9989
Fluorescence intensity (422 nm)
120
100
80
60
40
20
0
-9.5
-9.0
-8.5
-8.0
-7.5
-7.0
-6.5
Log CuPA (g/mL)


B
I=44.222 logC+552.19 R2=0.9990
Fluorescence intensity (516 nm)
160
120
80
40
-12.0
-11.5
-11.0
-10.5
-10.0
-9.5
-9.0
Log CMMP-2 (g/mL)

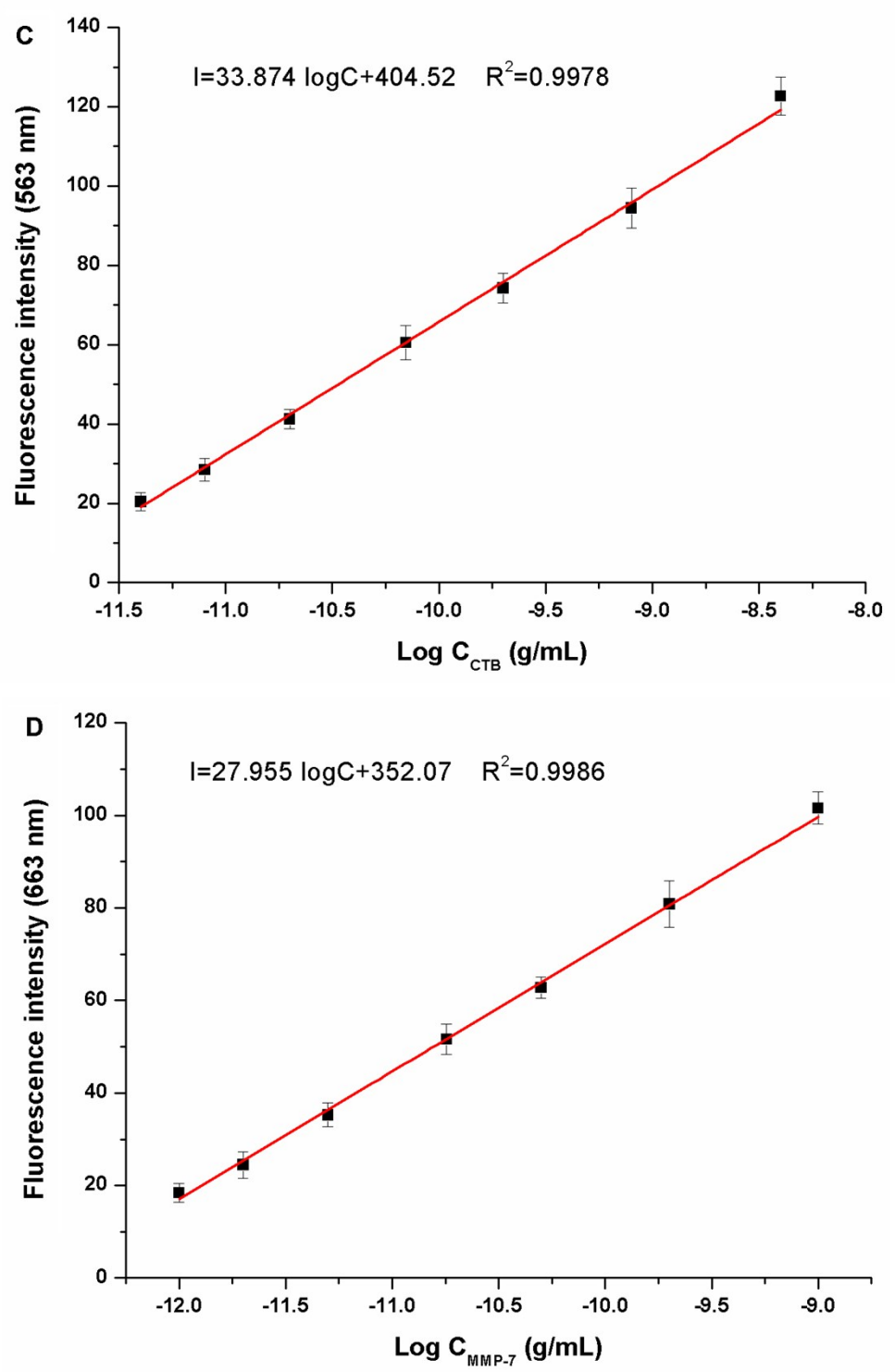
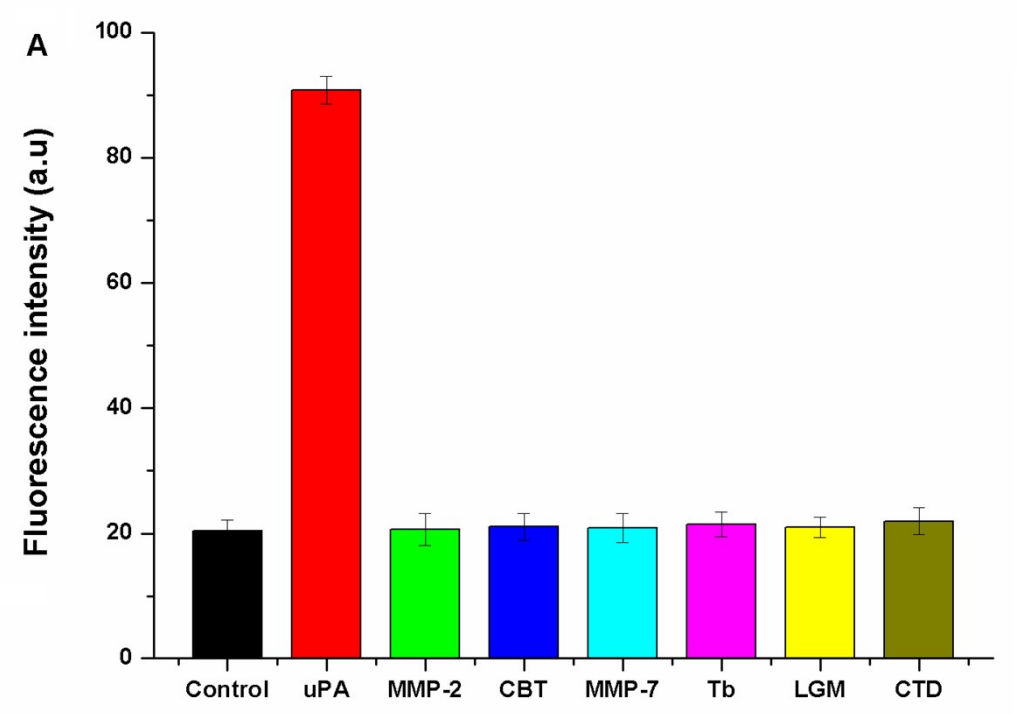
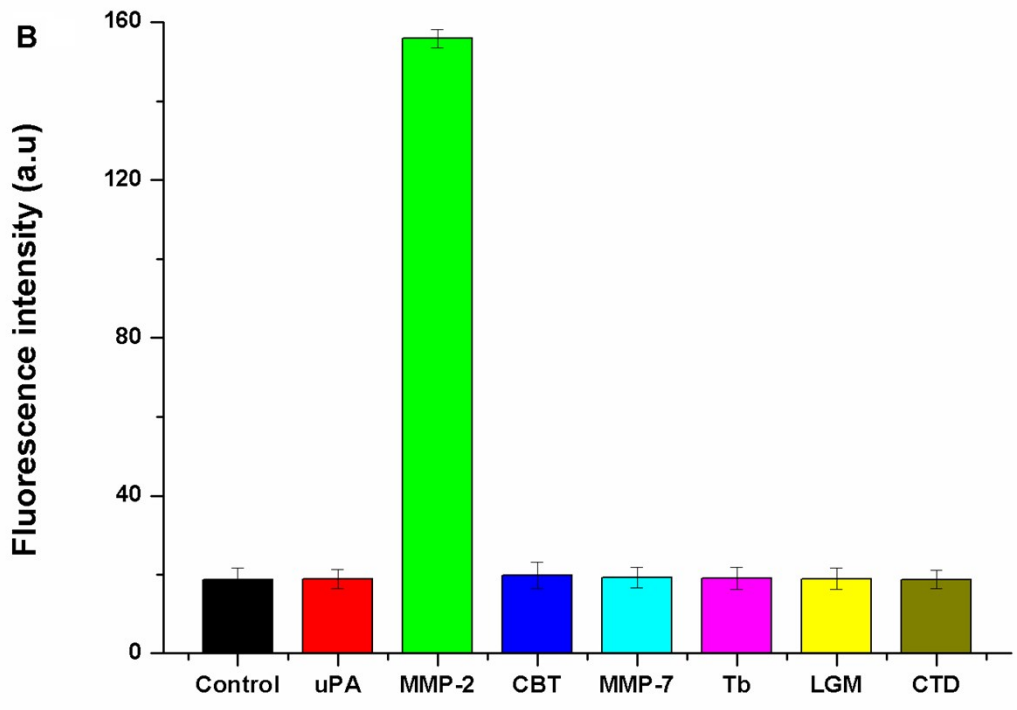


Fig. S12 Calibration curves corresponding to the proposed four-color nanoprobe sensing system. (A) uPA; (B) MMP-2; (C) CTB; and (D) MMP-7. I is the fluorescence intensity of the corresponding dyes, and C is the concentration of target proteases. Error bars were derived from N=5 experiments.


A
Fluorescence intensity (a.u)
100
80
60
40
20
0
Control
uPA
MMP-2
CBT
MMP-7
Tb
LGM
CTD


B
Fluorescence intensity (a.u)
160
120
80
40
0
Control
uPA
MMP-2
CBT
MMP-7
Tb
LGM
CTD

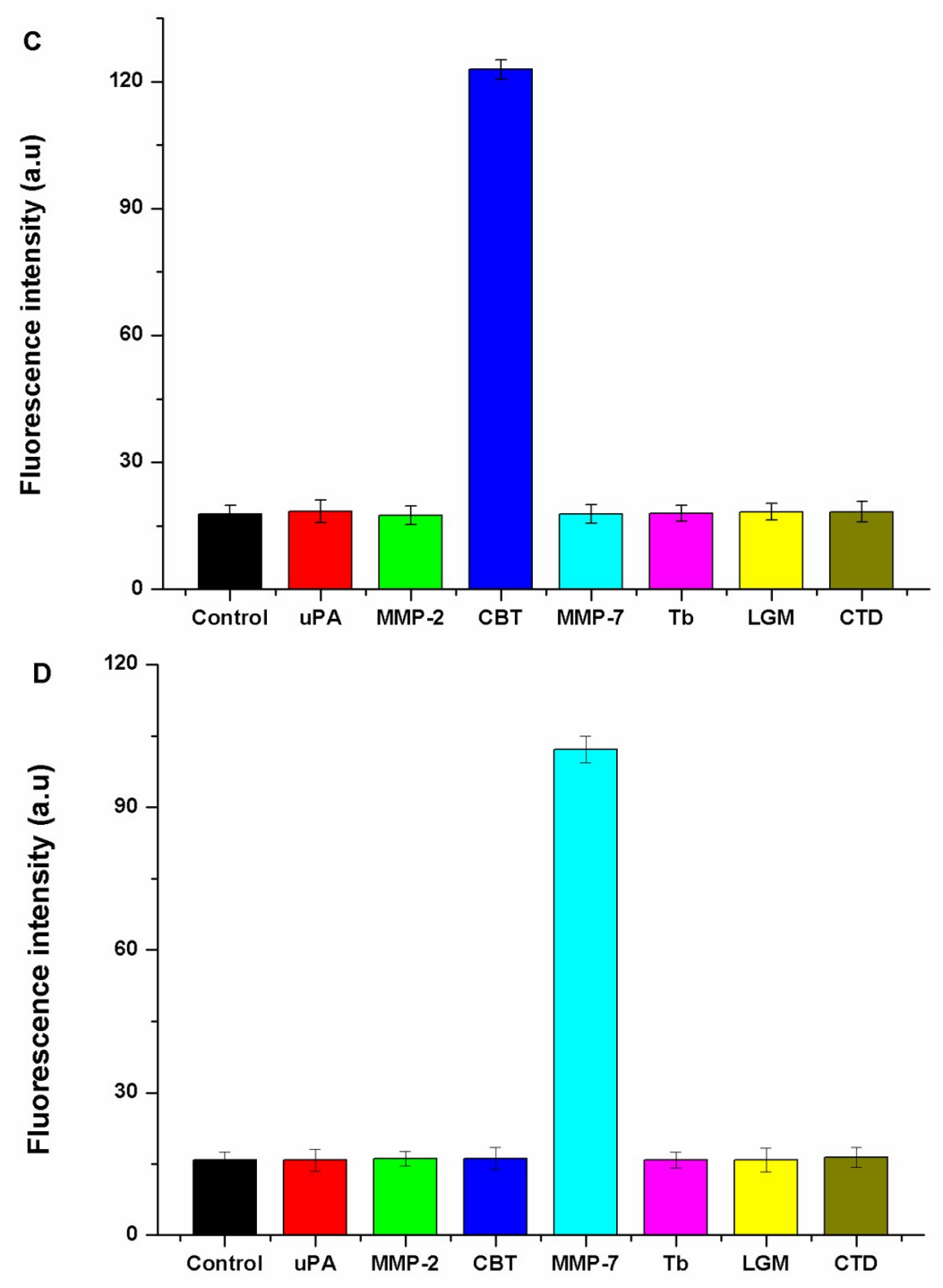


Fig. S13 Specificity of the proposed four-color nanoprobe. The concentration of uPA, MMP-2, CTB and MMP-7 was 40 ng/mL, 1 ng/mL, 4 ng/mL, and 1 ng/mL, respectively. Other nonspecific proteases were 400 ng/mL each. Error bars were derived from N=5 experiments.

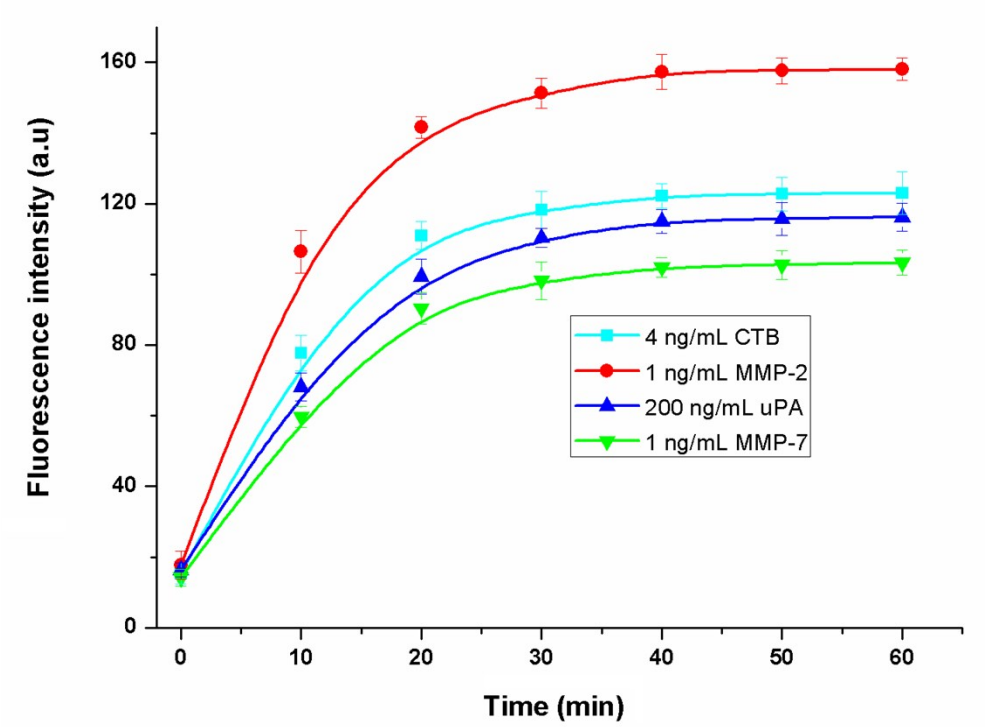


Fig. S14 The kinetics of the proposed four-color nanoprobe toward four tumor-related protease targets. The nanoprobe (100 nM) was incubated with four tumor-related protease targets for different time. Error bars were derived from N=5 experiments.

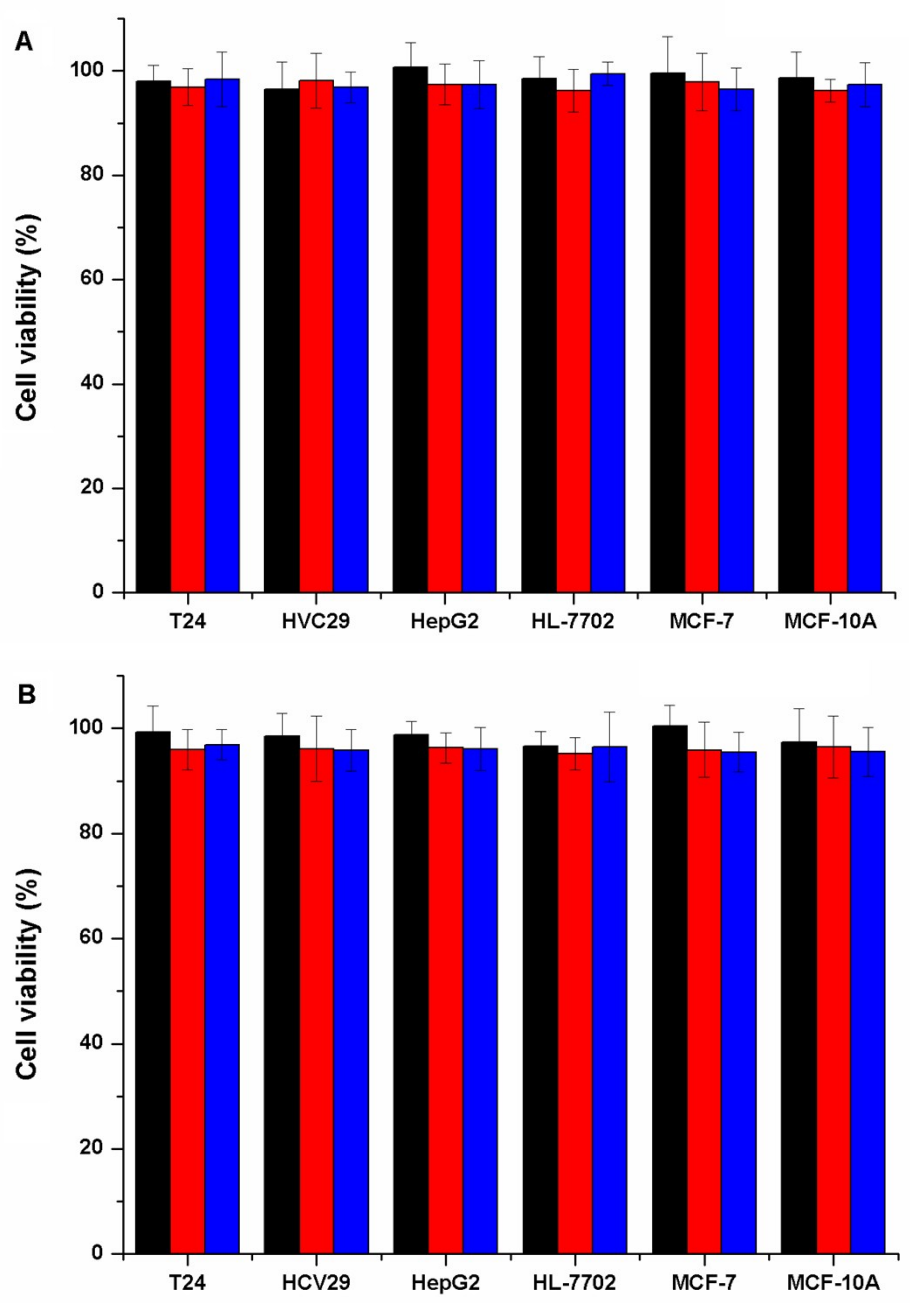


Fig. S15 (A) Viability of different cells incubated with the four-color nanoprobe (100 nM) or free PDANPs (0.2 mg/mL) for 12 h. (B) Viability of different cells incubated with the four-color nanoprobe (100 nM) or free PDANPs (0.2 mg/mL) for 24 h. The cells incubated with culture medium only were used as control groups. Black, red and blue columns represent control groups, the treated group with free PDANPs, and the treated group with the four-color nanoprobe, respectively. Error bars were derived from N=5 experiments.

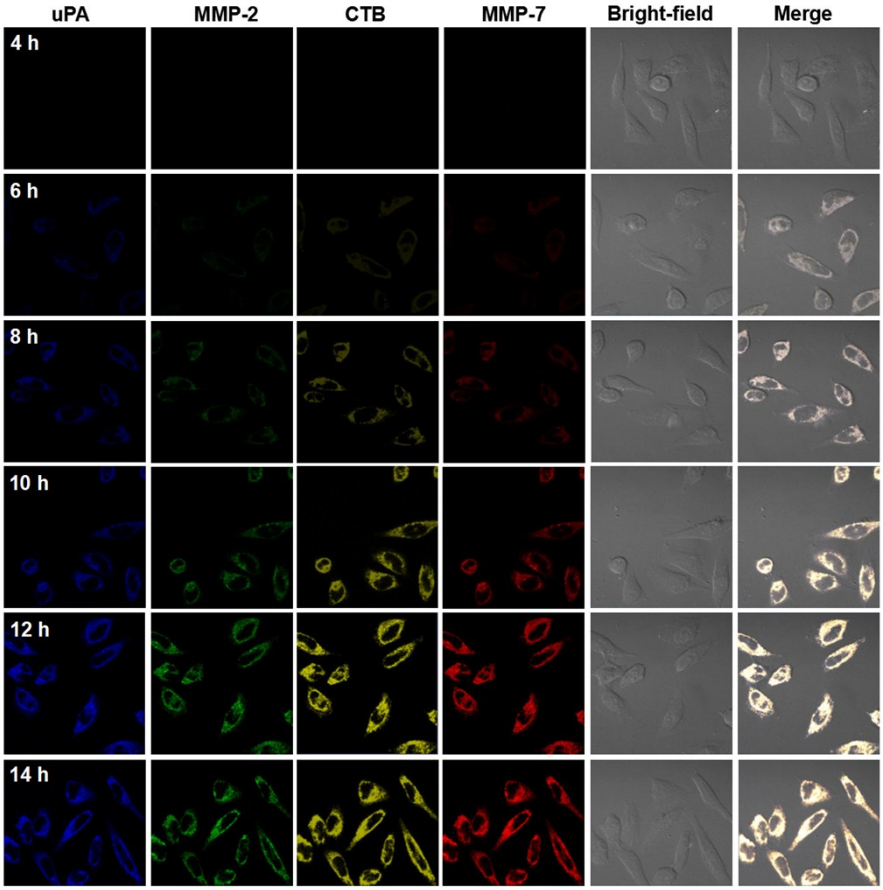
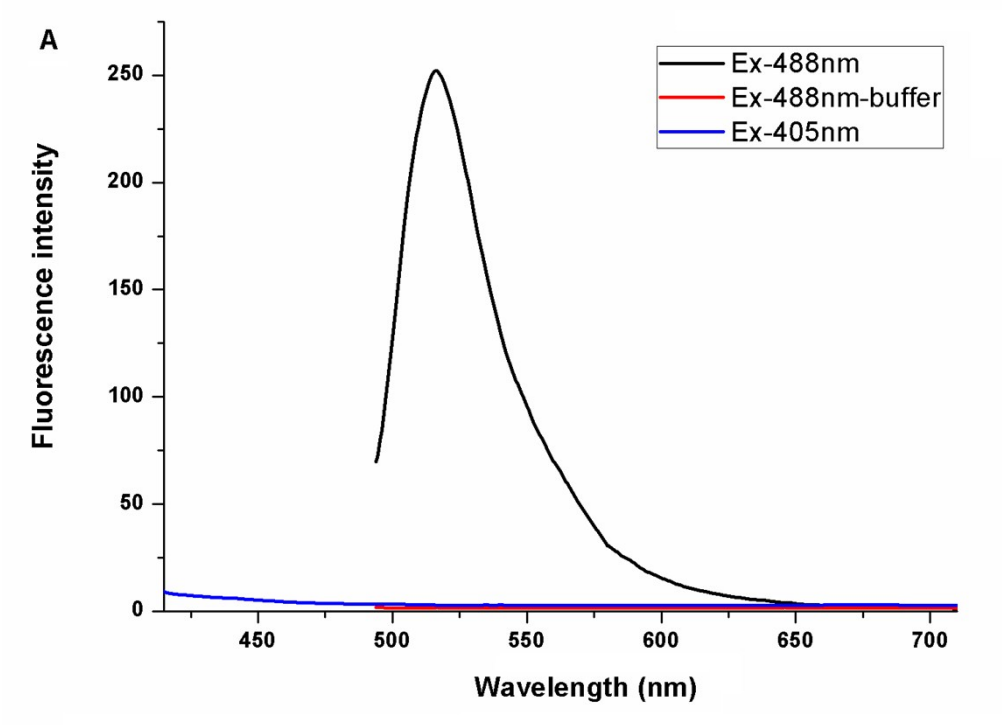
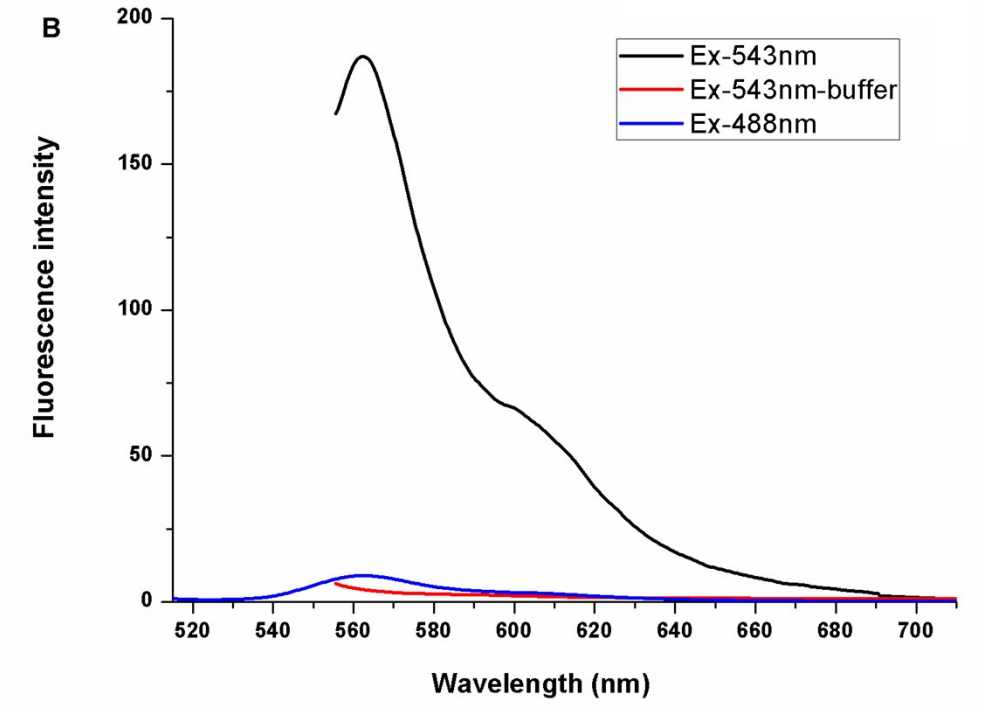


Fig. S16 Fluorescence images of uPA, MMP-2, CTB, and MMP-7 in T24 cells after incuba-tion with the four-color nanoprobe for different time at 37 ℃. These four protease targets were recorded by excitation of AF405 at 405 nm, FITC at 488 nm, Cy3 at 543 nm, and Cy5 at 633 nm, respectively.


A
Ex-488nm
Ex-488nm-buffer
Ex-405nm
Fluorescence intensity
0
50
100
150
200
250
450
500
550
600
650
700
Wavelength (nm)


B
Ex-543nm
Ex-543nm-buffer
Ex-488nm
Fluorescence intensity
0
50
100
150
200
520
540
560
580
600
620
640
660
680
700
Wavelength (nm)

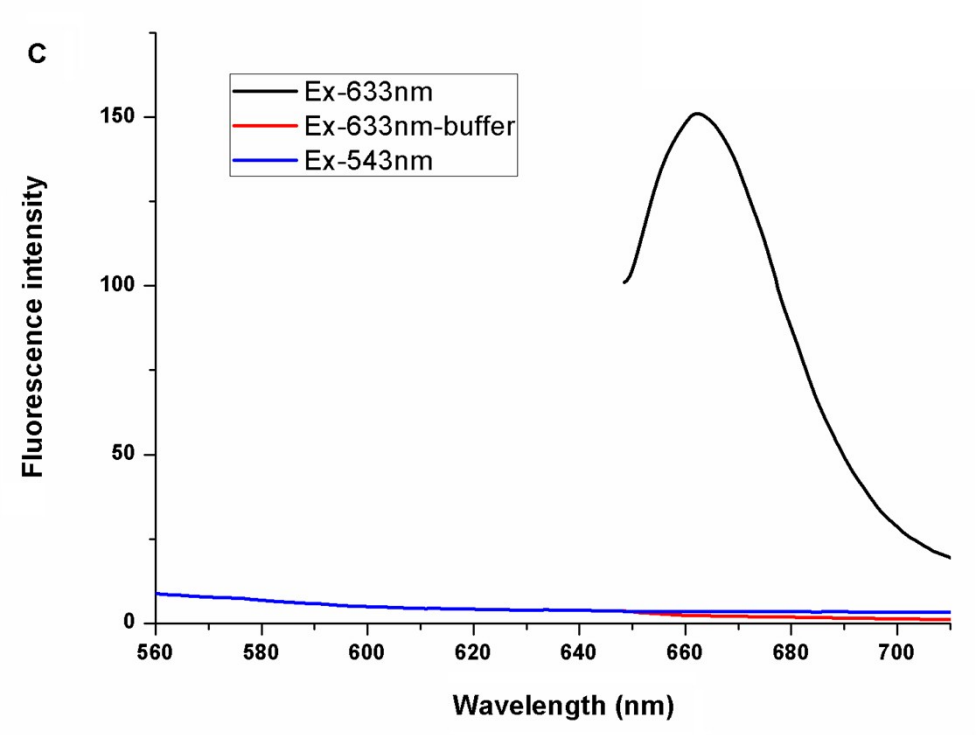


Fig. S17 Fluorescence spectra of three dye-labeled peptides (FITC-labeled P2, Cy3-labeled P3, and Cy5-labeled P4, 100 nM each) under different excitation wavelengths. The three dye-labeled peptides were recorded by excitation of FITC at 405 nm and 488 nm, Cy3 at 488 nm and 543 nm, Cy5 at 543 nm and 633 nm, respectively.

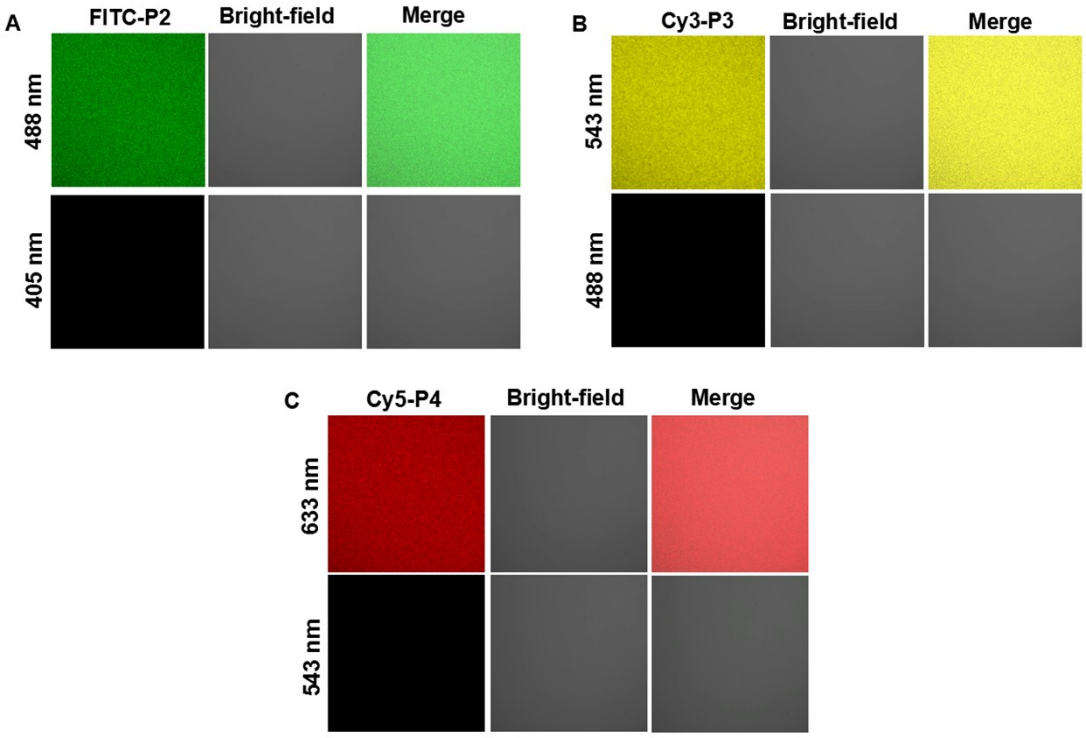


Fig. S18 Fluorescence images of the solution of three dye-labeled peptides (FITC-labeled P2, Cy3-labeled P3, or Cy5-labeled P4) in dishes. (A) FITC-labeled P2; (B) Cy3-labeled P3; (C) Cy5-labeled P4. The three dye-labeled peptides were recorded by excitation of FITC at 405 nm and 488 nm, Cy3 at 488 nm and 543 nm, Cy5 at 543 nm and 633 nm, respectively.

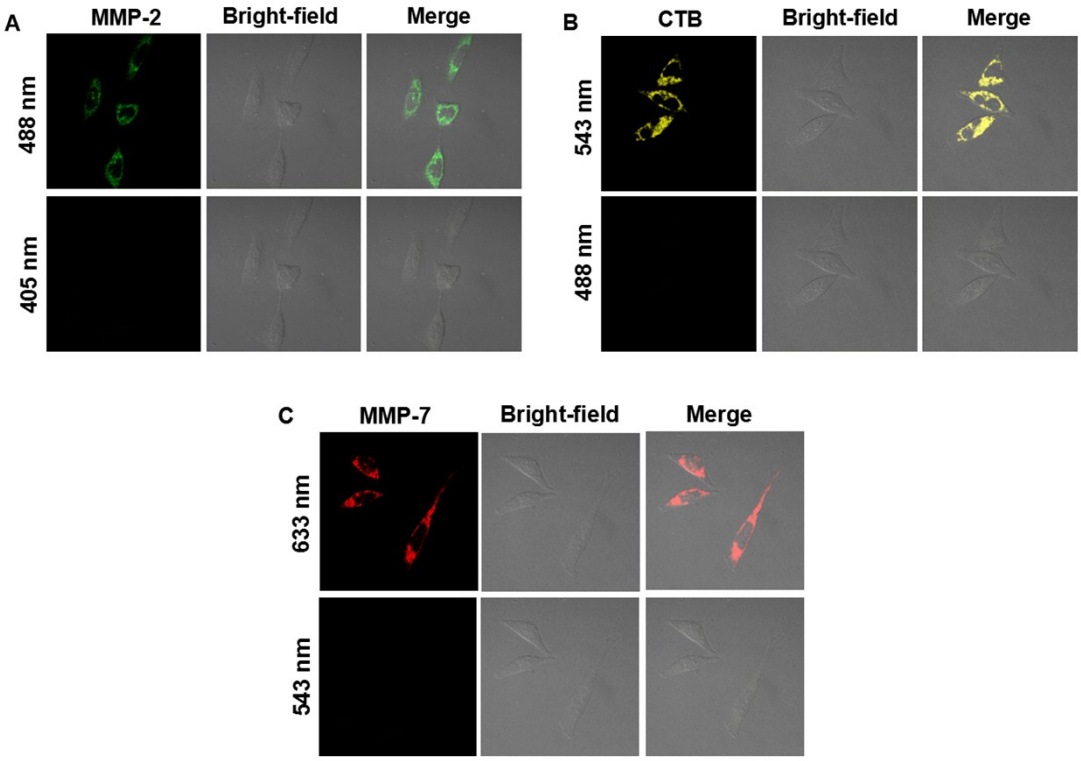


Fig. S19 Fluorescence images of T24 cells incubated with single-color nanoprobe (FITC-labeled P2/PDANP nanocomplex for MMP-2, Cy3-labeled P3/PDANP nanocomplex for CTB, or Cy5-labeled P4/PDANP nanocomplex for MMP-) for 12 h at 37 ℃. The three protease targets (MMP-2, CTB and MMP-7) were recorded by excitation of FITC at 405 nm and 488 nm, Cy3 at 488 nm and 543 nm, Cy5 at 543 nm and 633 nm, respectively.

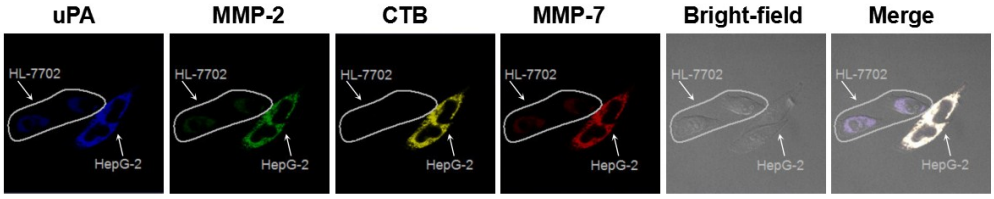
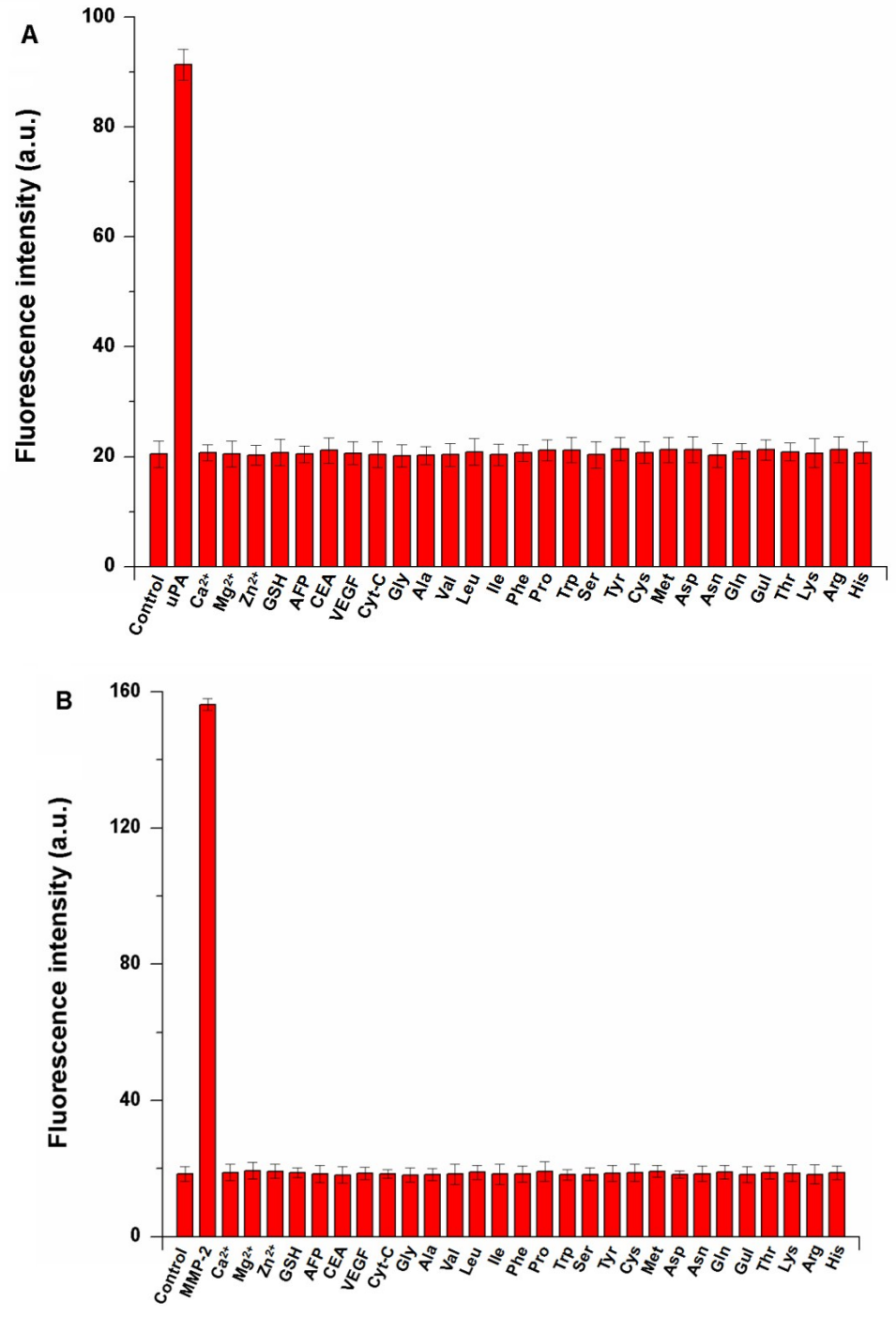


Fig. S20 Fluorescence images of tumor-related proteases in co-cultured HepG2 and HL7702 cells incubated with the four-color nanoprobe for 12 h at 37 ℃. The four protease targets were recorded by excitation of AF405 at 405 nm, FITC at 488 nm, Cy3 at 543 nm, and Cy5 at 633 nm, respectively.


A
Fluorescence intensity (a.u.)
100
80
60
40
20
0
Control
uPA
Ca2+
Mg2+
Zn2+
GSH
AFP
CEA
VEGF
Cyt-C
Gly
Ala
Val
Leu
Ile
Phe
Pro
Trp
Ser
Tyr
Cys
Met
Asp
Asn
Gln
Gul
Thr
Lys
Arg
His
B
Fluorescence intensity (a.u.)
160
120
80
40
0
Control
MMP-2
Ca2+
Mg2+
Zn2+
GSH
AFP
CEA
VEGF
Cyt-C
Gly
Ala
Val
Leu
Ile
Phe
Pro
Trp
Ser
Tyr
Cys
Met
Asp
Asn
Gln
Gul
Thr
Lys
Arg
His

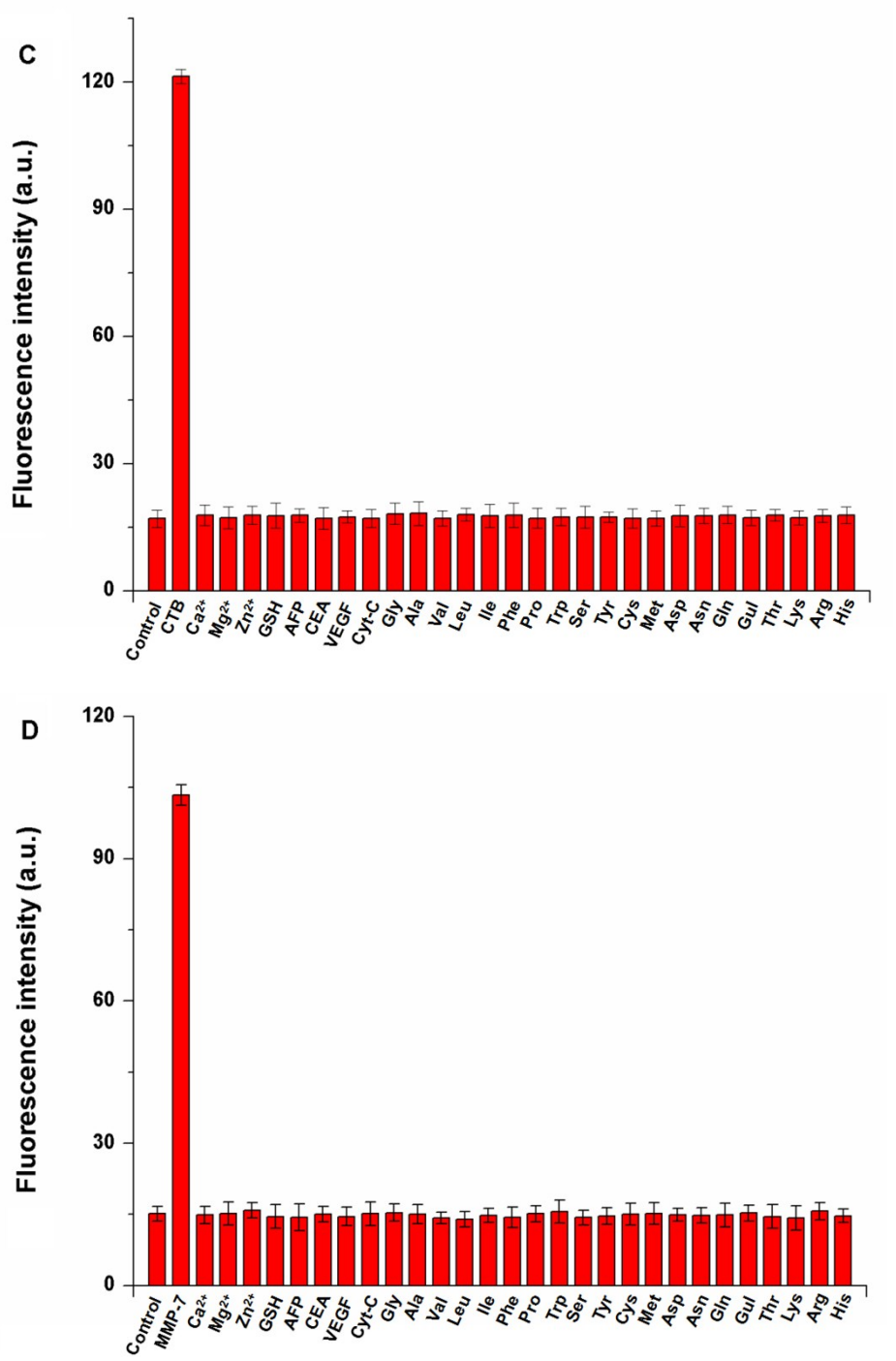


Fig. S21 Specificity of the proposed four-color nanoprobe. The concentration of uPA, MMP-2, CTB and MMP-7 was 40 ng/mL, 1 ng/mL, 4 ng/mL, and 1 ng/mL, respectively. Other nonspecific analytes were 400 ng/mL each. Error bars were derived from N=5 experiments.

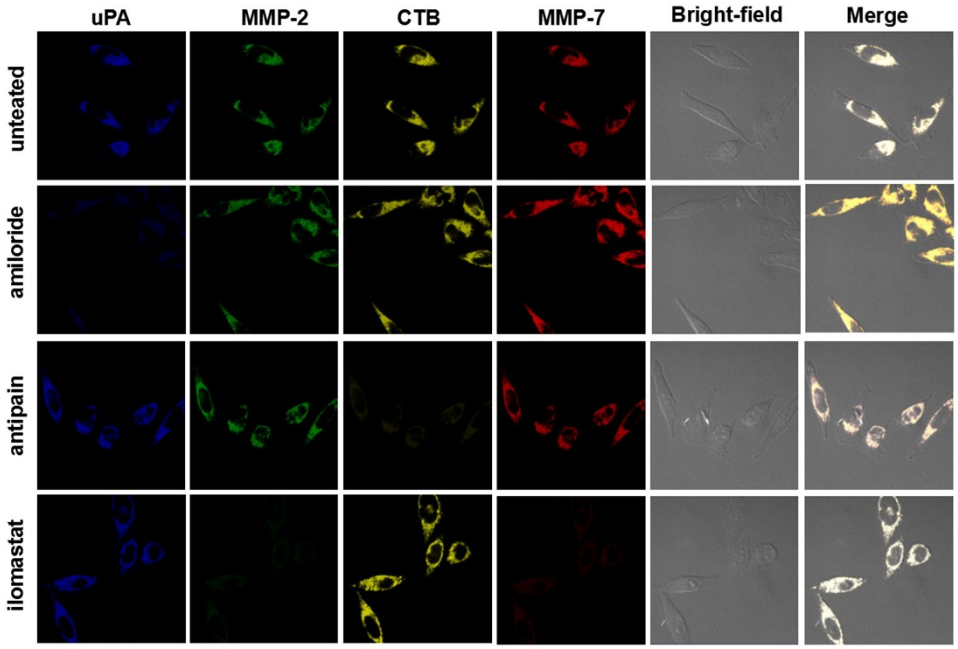


Fig. S22 Fluorescence images of different levels of tumor-related proteases in HepG2 cells. The treated and untreated HepG2 cells were incubated with the four-color nano-probe for 12 h at 37 °C. HepG2 cells were treated with amiloride to decrease the expression of uPA, treated with antipain to decrease the CTB expression and treated with ilomastat to inhibit the expression of both MMP-2 and MMP-7. The four protease targets were recorded by excitation of AF405 at 405 nm, FITC at 488 nm, Cy3 at 543 nm, and Cy5 at 633 nm, respectively.

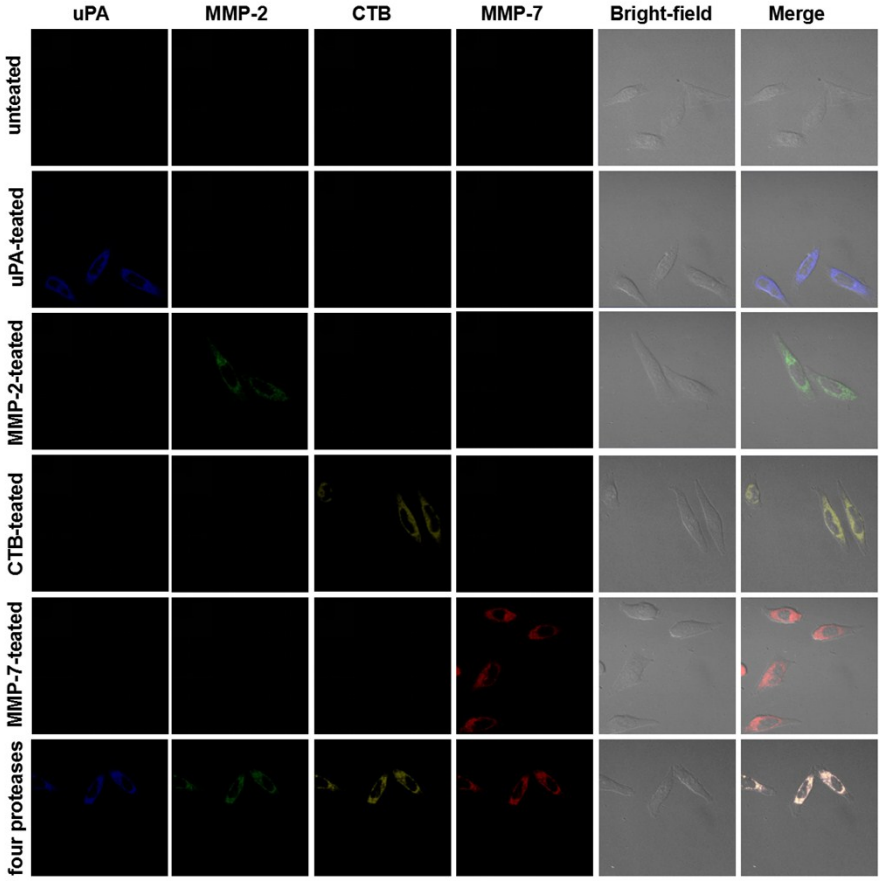


Fig. S23 Fluorescence images of HL-7702 cells before and after transfection with standard target proteases. The treated and untreated HepG2 cells were incubated with the four-color nano-probe for 12 h at 37 ℃. The four protease targets were recorded by excitation of AF405 at 405 nm, FITC at 488 nm, Cy3 at 543 nm, and Cy5 at 633 nm, respectively.

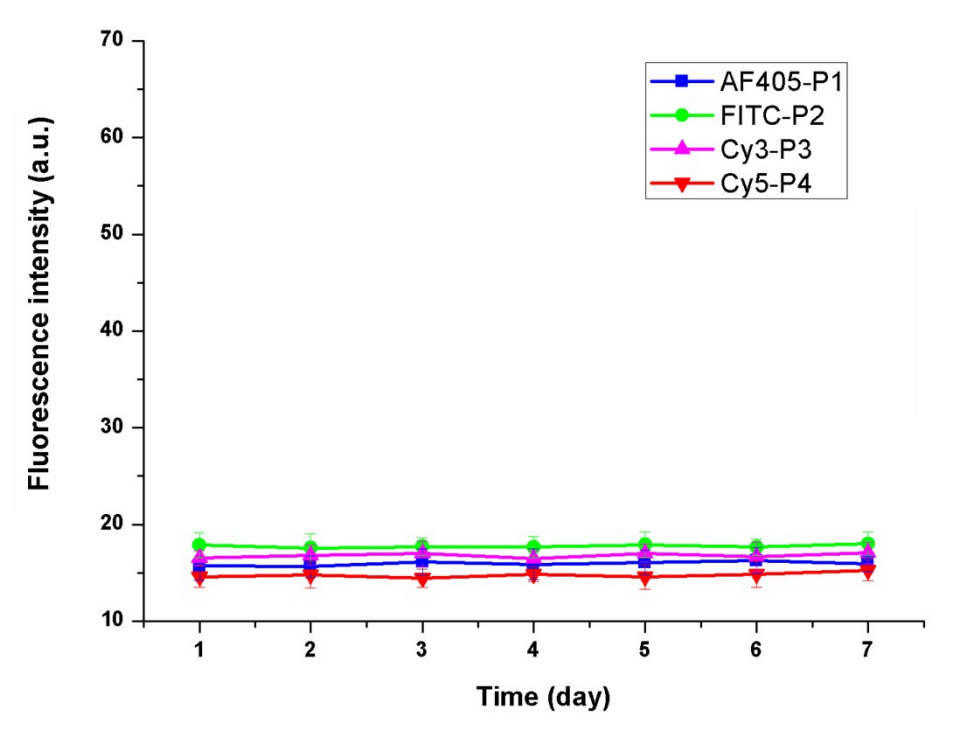


Fig. S24 The fluorescence intensity of all four dyes on the four-color nanoprobe (100 nM) after storage of the nanoprobe in PBS solution at 4 ℃ for different time. (A) AF405; (B) FITC; (C) Cy3; (D) Cy5.

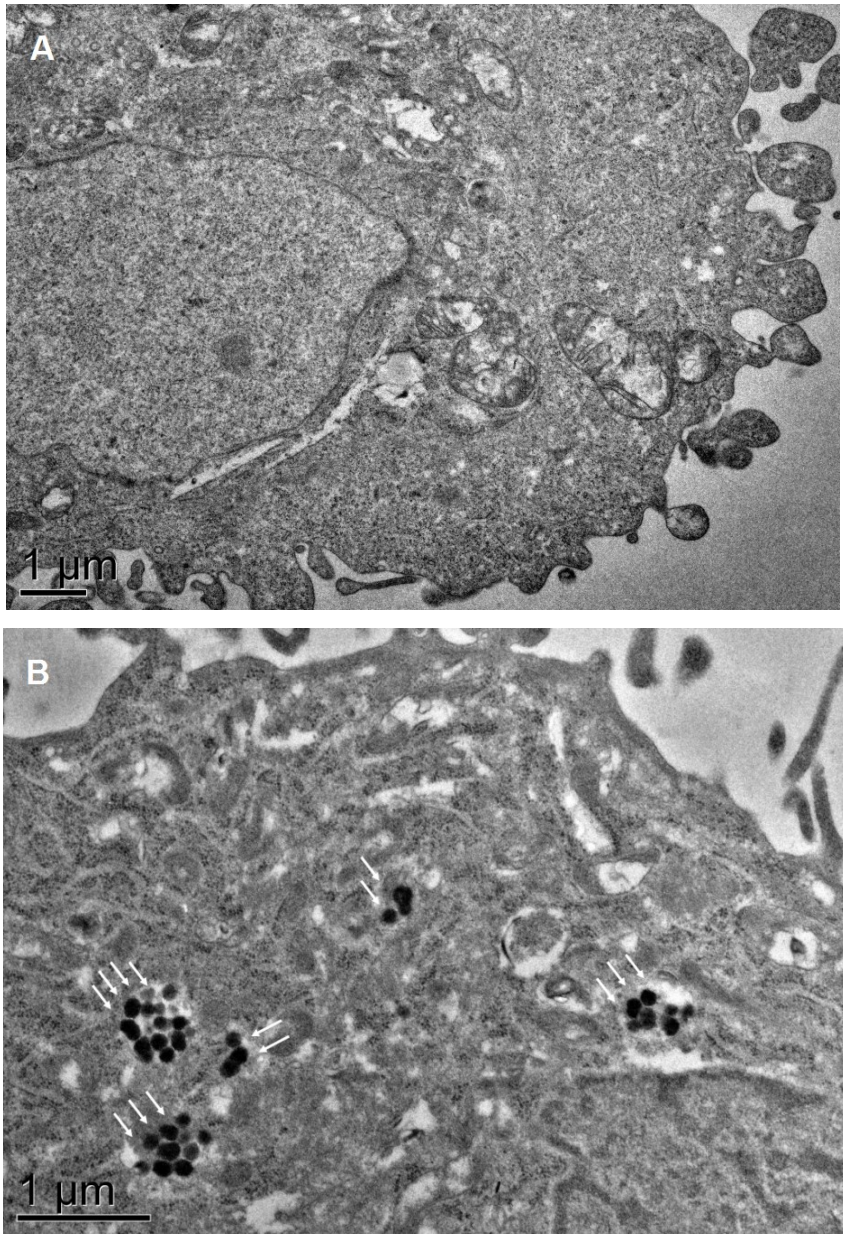


Fig. S25 The Bio-TEM image of T24 cells before (A) and after (B) incubation with the four-color nanoprobe for 24 h. The white arrow pointed to the intraceullar PDANPs.

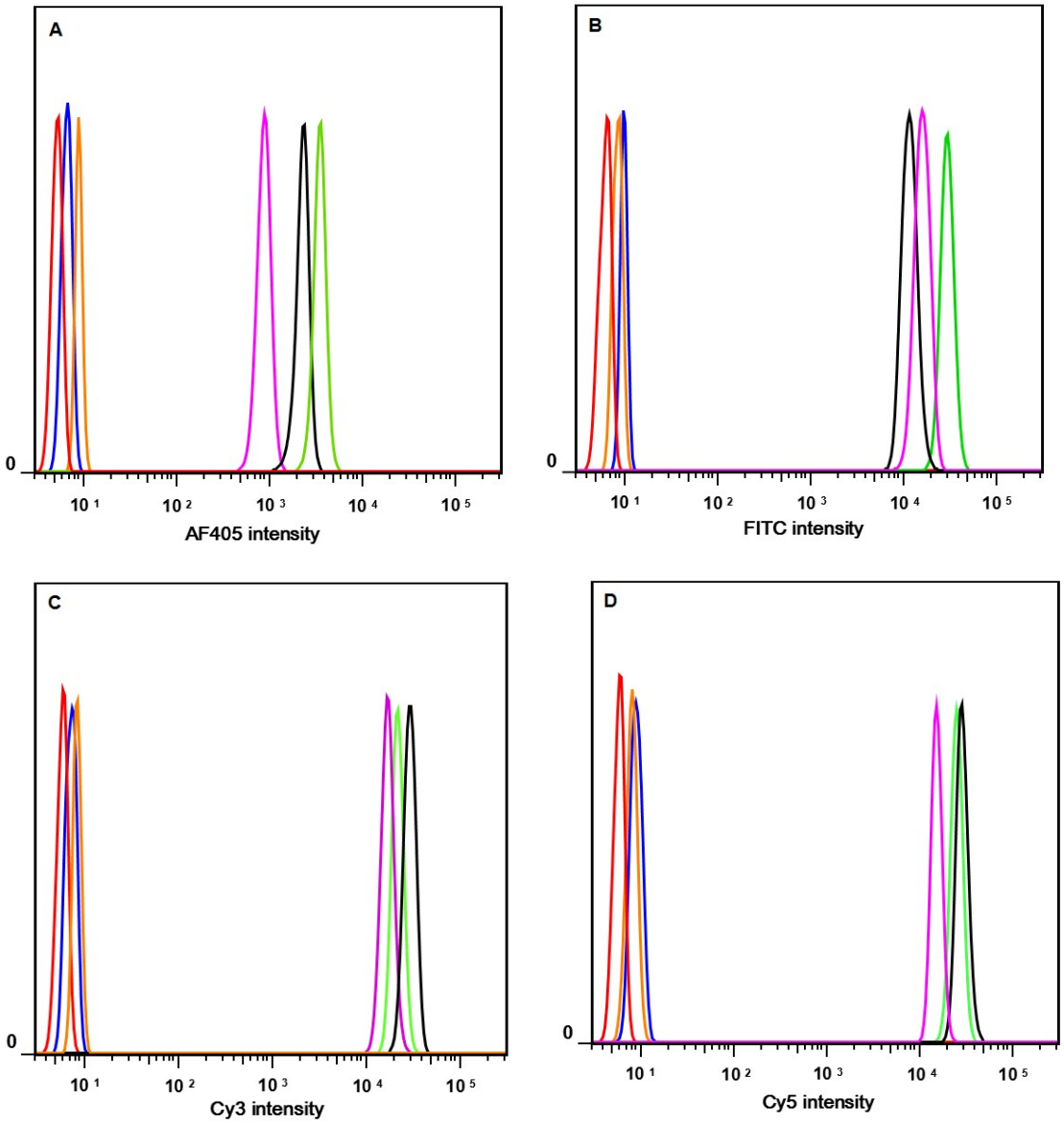


Fig. S26 Flow cytometric assays of uPA, MMP-2, CTB, and MMP-7 in HepG2 cells, HL-7702 cells, T24 cells, HCV-29 cells, MCF-7 cells and MCF-10A cells. (A) uPA; (B) MMP-2; (C) CTB; (D) MMP-7. Red, blue and orange curves were obtained from MCF-10A cells, HCV-29 cells and HL-7702 cells, respectively. Black, green and pink curves were obtained from MCF-7 cells, HepG2 cells and T24 cells, respectively. The cells were incubated with the proposed four-color nanoprobe for 12 h at 37 ℃.