Electronic Supplementary Information

of

A dual-FRET based fluorescence probe for sequential MMP-2 and caspase-3 detection

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Experimental section

Materials:

2-chlorotritylchloride resin (100–200 mesh, loading: 0.537 mmol g^{-1}), Rink Amide resin (100–200 mesh, loading: 0.59 mmol/g), *N*-Fluorenyl-9-methoxycarbonyl protected *L*-amino acids (FMOC-Arg(Pbf)-OH, FMOC-Gly-OH，FMOC-Glu(tBu)-OH, FMOC-Leu-OH, FMOC-Pro-OH, FMOC-Val-OH, FMOC-Asp(tBu)-OH, FMOC-Ser(tBu)-OH, FMOC-Lys(Mtt)-OH, o-benzotriazole-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and Diisopropylethylamine (DIEA) were purchased from GL Biochem. Ltd. (Shanghai, China) and used as received. Trifluoroacetic acid (TFA), piperidine, *N,N'*-dimethylformamide (DMF), methanol, dichloromethane (DCM) and anhydrous ether were obtained from Shanghai Chemical Co. (China). TFA, DMF were used after distillation. 1,10-phenanthroline monohydrate, acetic anhydride and 2,6-lutidine were obtained from Aladdin Reagent Co. Ltd. (Shanghai, China). *N*-methylmorpholine (NMM), 4-{[4-(Dimethylamino)-phenyl]-azo}-benzoic acid (Dabcyl) were obtained from TCI corp. (Shanghai, China). 5(6)-Carboxyfluorescein (FAM) were obtained from Aladdin Reagent Co. Ltd. (Shanghai, China). Caspase-3 (human recombinant) was purchased from Biovision corp., the inhibitor Ac-DEVD-CHO and Matrix metalloproteinases (MMP-2) were purchased from R&D Systems. Doxorubicin hydrochloride (Dox) was purchased from Zhejiang Hisun Pharmaceutical Co., Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT), fetal bovine serum (FBS) were purchased from Invitrogen Corp., All other reagents were of analytical grade and used as received.

General characterizations:

HPLC was used for the purity analysis of the probe precursor and the probe. Fluorescence spectra were recorded on a LS55 luminescence spectrometer (Perkin–Elmer). Fluorescence lifetime were measured by Edinbough F900 fluorescence instrument. The matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was used to confirm the probe structure. Fluorescence microscopy images were taken using a confocal laser scanning microscopy (C1-Si, Nikon, Japan). Quantitative flow cytometry were taken by Flow Cytometry (BD FACSAria TM III).

Synthesis of Dabcyl-GPLGVRG-COOH

Dabcyl-GPLGVRG-COOH was synthesized manually using 2-chlorotritylchloride resin and Fmoc-*L*-amino acids by standard solid phase peptide synthesis (SPPS). After the resin was soaked in anhydrous DMF for half an hour, the first peptide residue was coupled to the resin using 4 equiv. (relative to the substitution degree of resin) of Fmoc-protected amino acid (FMOC-Gly-OH) and 10 equiv. of DIEA in a DMF solution for 2 h. Unreacted sites of the resin were then capped by a 30 min incubation by a 30 min incubation with a mixture of methanol, and DIEA in DMF (v/v/v=1:2:7), and then the resin was treated with 20% piperidine in DMF (v/v) for 15 min twice to remove the Fmoc protecting group in attached amino acid, and then the FMOC-Arg(pbf)-OH, FMOC-Val-OH, FMOC-Gly-OH, FMOC-Leu-OH, FMOC-Pro-OH, FMOC-Gly-OH were coupled in turn by reacting with 4 equiv. of Fmoc-protected amino acid, and 4.8 equiv. of HBTU, 4.8 equiv. of HOBt, and 6 equiv. of DIEA for 2 h. Dabcyl (2 equiv.) was conjugated to peptide segments using 2.4 equiv. of HBTU, 2.4 equiv. of HOBt and 6 equiv. of NMM in a DMF solution for 12 h.

After the Dabcyl-GPLGVRG-COOH peptide was synthesized, the resin was washed with DMF, methanol and DCM for 4 times, respectively, and dried under vacuum overnight. Cleavage of the peptides from the resin was performed by stirring the dried resin with a mixture of TFA/DCM (v/v: 0.1%/99.9%) for 5 min for 20 times at room temperature. Collect the filtration and concentrated by rotary evaporation. The residue was obtained by precipitating the viscous solution in cold ether, collected by centrifugation, washed twice with cold ether and then dried under vacuum. The solid obtained was conserved in a -20 ^{o}C freezer in the shielding of light. The purity of the probe was analysized by HPLC.

Synthesis of FAM-K(GPLGVRG-Dabcyl)SDEVDSK(Dabcyl)

FAM-K(GPLGVRG-Dabcyl)SDEVDSK(Dabcyl) was synthesized manually using Rink Amide resin and Fmoc-*L*-amino acids by standard solid phase peptide synthesis (SPPS). After the resin was soaked in anhydrous DMF for half an hour, the resin was treated with 20% piperidine in DMF (v/v) for 15 min twice to remove the FMOC protecting group in the resin, the first peptide residue was coupled to the resin using 4 equiv. (relative to the substitution degree of resin) of Fmoc-protected amino acid (FMOC-Lys(Mtt)-OH) and 4.8 equiv. of HBTU, 4.8 equiv. of HOBt, and 6 equiv. of DIEA for 2 h. Unreacted sites of the resin were then capped by a 30 min incubation with a mixture of 5% acetic anhydride, 6% 2,6-lutidine in DMF, and then the resin was treated with 20% piperidine in DMF (v/v) for 15 min twice to remove the Fmoc protecting group in attached amino acid, and then the FMOC-Ser(tBu)OH, FMOC-Asp(tBu)-OH, FMOC-Glu(tBu)-OH, FMOC-Val-OH, FMOC-Asp(tBu)-OH, FMOC-Ser(tBu)-OH were coupled in turn by reacting with 4 equiv. of FMOC-protected amino acid, and 4.8 equiv. of HBTU, 4.8 equiv. of HOBt, and 6 equiv. of DIEA for 2

h. The resin was then treated with 2% TFA in DCM (v/v) for 30 min, and then 1% TFA in DCM (v/v) for 30 min to remove the Mtt protecting group, after washing the resin with DCM and DMF for 4 times, Dabcyl (2 equiv.) was conjugated to peptide segments using 2.4 equiv. of HBTU, 2.4 equiv. of HOBt and 6 equiv. of NMM in a DMF solution for 12 h. Then, remove the FMOC protecting group, FMOC-Lys(mtt)-OH coupled to the resin by 4.8 equiv. of HBTU, 4.8 equiv. of HOBt, and 6 equiv. of DIEA for 2 h. 5(6)-Carboxylfluorescein (2 equiv.) was conjugated to peptide segment in the rest resin using 2.4 equiv. of HBTU, 2.4 equiv. of HOBt and 6 equiv. of NMM in a DMF solution for 12 h. the resin was washed with DMF, methanol and DCM for 4 times, respectively. Treated the resin with 2% TFA in DCM (v/v) for 30 min, and then 1% TFA in DCM (v/v) for 30 min to remove the Mtt protecting group, after washing the resin with DCM and DMF for 4 times, previous synthesized Dabcyl-GPLGVRG peptide segment was coupled to the resin using 2.4 equiv. of HBTU, 2.4 equiv. of HOBt and 6 equiv. of NMM in a DMF solution for 12 h. washed the resin with DMF, methanol and DCM for 4 times, respectively, and dried under vacuum overnight. Cleavage of the peptides from the resin was performed by stirring the dried resin with a mixture of TFA/H_2O (v/v: 95%/5%) for 100 min at room temperature. The filtration was concentrated by rotary evaporation. The residue was obtained by precipitating the viscous solution in cold ether, collected by centrifugation, washed twice with cold ether and then dried under vacuum. The solid obtained was conserved in a -20 ^oC freezer in the shielding of light. The molecular weight of the obtained peptide (FAM-K(GPLGVRG-Dabcyl)SDEVDSK(Dabcyl)) was confirmed by MALDI-TOF-MS, theoretical: 2403.08 and observed at 2403.41. The purity of the probe was analysized by HPLC.

Caspase-3 and MMP-2 induced fluorescence recovery of the probe

The probe was dissolved in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 1 μM. MMP-2 200 ng and caspase-3 100 pM in buffer solutions were added to the 0.5 mL probe solution, Certain volume of PBS buffer was added to adjust the probe solution to 0.5 μM. LS55 luminescence spectrometer was used to record the emission spectra of these solutions at 0 h and 13 h with the excitation wavelength of 480 nm.

Fluorescence lifetime measurement

The probe was dissolved in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 10 μM. And also, 5(6)-Carboxyfluorescein (FAM) was dissolved in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 5 μM. MMP-2 (100 ng/mL) and caspase-3 (50 pM) were added to adjust the probe solutions to 5 μM. After 3 h incubation, the fluorescence decays of the FAM solution and probe solutions with/without caspase-3 and MMP-2 incubation were measured by F900 fluorescence instrument with excitation at 477 nm.

Time dependent fluorescence recovery of the probe

The probe was dissolved in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 1 μM. PBS, MMP-2 (200 ng/mL), caspase-3 (100 pM), MMP-2 (200 ng/mL) and caspase-3 (100 pM) were added respectively to adjust the probe solutions to 0.5 μM, luminescence spectrometer was used to record the emission spectra changes of these solutions by time intervals with the excitation wavelength of 480 nm and emission wavelength of 520 nm.

Time dependent fluorescence change of the probe with MMP-2 and

corresponding inhibitor incubation

The probe was dissolved in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 1 μM. MMP-2 (200 ng/mL) was first incubated with 1,10-phenanthroline monohydrate (MMP-2 inhibitor, 50 μg/mL) containing medium for 2 h. After which, 0.5 mL 1 μM probe solution were added, and certain amount of PBS buffer solution was added to make the final probe concentration at 0.5 μM. Emission spectra of the probe solutions with/without inhibitors were recorded by the time intervals at the excitation wavelength of 480 nm and emission wavelength of 520 nm.

Time dependent fluorescence change of the probe with caspase-3 and corresponding inhibitor incubation

The probe was dissolved in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 1 μM. Caspase-3 (120 pM) was first incubated with Ac-DEVD-CHO (caspase-3 inhibitor, 50 μM) containing medium for 2 h. After which, 0.5 mL 1 μM probe solution were added, and certain amount of PBS buffer solution was added to make the final probe concentration at 0.5 μM. Emission spectra of the probe solutions with/without inhibitors were recorded by the time intervals at the excitation wavelength of 480 nm, the emission wavelength were collected at 520 nm.

Time dependent fluorescence intensity change of the probe with MMP-2 or with simultaneous or sequential addition of MMP-2 and caspase-3

The probe was dissolved in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 1 μM. MMP-2 (200 ng/mL) and caspase-3 (100 pM) were simultaneously added into the probe solution to investigate the combination function of the enzymes, PBS was used to adjust the final probe concentration at 0.5 μM.

MMP-2 (200 ng/mL) was added into the probe solution and certain amount of PBS to adjust the final probe concentration at 0.5 μM. Emission spectra of these solutions were recorded by the time intervals at the excitation wavelength of 480 nm, emission wavelength were collected at 520 nm. After 10.5 h recording, divided the probe solution with MMP-2 enzyme into two groups, one of which added 100 pM caspase-3 and the other one added the same amount of PBS to investigate the sequential fluorescence recovery. By the time, the probe solution with both MMP-2 and caspase-3 enzymes also added the same volume of PBS to make the all the groups with the same original probe concentration. Emission spectra of the all groups were recorded by the time intervals at the excitation wavelength of 480 nm, emission wavelength of 520 nm.

Photo stability of the probe under UV irradiation

The probe was dissolved in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 5 μM. Then, the probe contained solution was irradiated with UV light (the power density of the UV light was 400 μJ/cm^2) in different time periods in cuvette, and the fluorescence of the solution was recorded by as long as the UV light irradiation terminated, LS55 luminescence spectrometer was used to record the emission changes of the solutions with/without irradiation with the excitation wavelength of 480 nm. The emission wavelength was collected at 520 nm.

In Vitro Cytotoxicity

COS7 cells and SCC-7 cells were seeded into a 96 well plate (6000 cells/well) cultured in DMEM (100 μL) incubated for 24 h (37 ^{o}C, 5% CO_2). After which, DMEM (100 μL) containing a fixed concentration of probe were added in each well.

The cells were co-incubated with probe solutions at 37 ^{o}C for 48 h. Then the probe contained DMEM media were replaced with 200 μL of fresh DMEM. MTT solution (20 μL, 5 mg/mL) was added to each well following and further incubated for another 4 h. Subsequently, the MTT media were removed and DMSO (150 μL) was added to each well. The optical density (OD) was measured at 570 nm with a microplate reader (BIO-RAD 550). The relatively cell viability was calculated as follows: Viability = $(OD_{sample}/OD_{control})\times 100\%$, where OD_{sample} was obtained from the cells treated by probe and $OD_{control}$ was obtained from the cells without any treatments.

Confocal Laser Scanning Microscopy of the Dox induced cell apoptosis imaging

SCC-7 cells were seeded in 35 mm Petri dishes and incubated in DMEM (1 mL) containing 10% FBS for 24 h. Probe dispersed in DMEM medium (1 mL) with 10% FBS at the concentration of 5 μM were added and the cells were further incubated at 37 ^{o}C for 1 h. The probe contained DMEM media were replaced with 1 mL of DMEM containing 10% FBS and 10 μg of Dox, and further incubated for another 24 h, then removing the medium and then washing with PBS (1 mL), Cells were stained with Hoechst 33342 for 15 min in the dark and then removing the medium and washing thrice with DMEM. The cells images were taken by Carl Zeiss fluorescence microscopy, NOL-LSM 710. For Ac-DEVD-CHO inhibited Dox induced apoptosis, Ac-DEVD-CHO (50 μM) and SCC-7 cells were co-incubated for 2 h, Thereafter, probe dispersed in DMEM medium (1 mL) with 10% FBS at the concentration of 5 μM were added and the cells were further incubated at 37 ^{o}C for 1 h. The probe contained DMEM media were replaced with 1 mL of DMEM containing 10% FBS and 10 μg of Dox, and further incubated for another 24 h, then removing the medium and washing with PBS (1 mL). Cells were stained with Hoechst 33342 for 15 min in

the dark and then removing the medium and washing thrice with DMEM, the cells images were taken by Carl Zeiss fluorescence microscopy, NOL-LSM 710.

Quantitative flow cytometry analysis of the Dox induced fluorescence recovery

For the flow cytometry analysis, SCC-7 cells were seeded on 6-well plates, the incubated cells were incubated with probe solution at the concentration of 20 μM for 1 h, then washed thrice by DMEM, one of which was added 10 μg Dox, all the plates were incubated for 24 h, washed thrice by DMEM, and digested by trypsin, collected in centrifuge tubes, washed with DMEM for 3 times and re-suspended in 0.5 mL DMEM, finally analyzed by Flow Cytometry (BD FACS Aria TM III).

Confocal Laser Scanning Microscopy for real time monitoring UV induced cell apoptosis

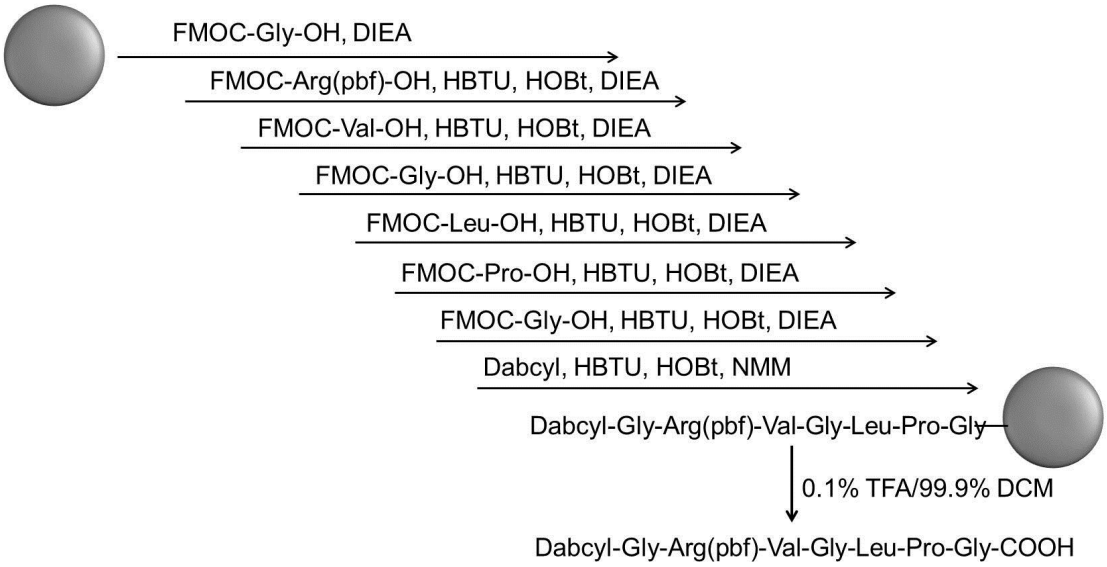
COS7 and SCC-7 cells were seeded in 35 mm Petri dishes and incubated in DMEM (1 mL) containing 10% FBS for 24 h. Probe dispersed in DMEM medium (1 mL) with 10% FBS at the concentration of 5 μM were added and the cells were further incubated at 37 ^{o}C for 1 h. Then removing the medium and washing with PBS (1 mL). Cells were stained with Hoechst 33342 for 15 min in the dark and then removing the medium and washing thrice with DMEM. Then, the dishes were irradiated with UV light (the power density of the UV light was 400 μJ/cm^2) in different time periods, and the fluorescence of the cells was recorded by CLSM as long as the UV light irradiation terminated. The cells images were taken by Carl Zeiss fluorescence microscopy, NOL-LSM 710.

Apoptosis induced fluorescence recovery of the probe for cells apoptosis imaging

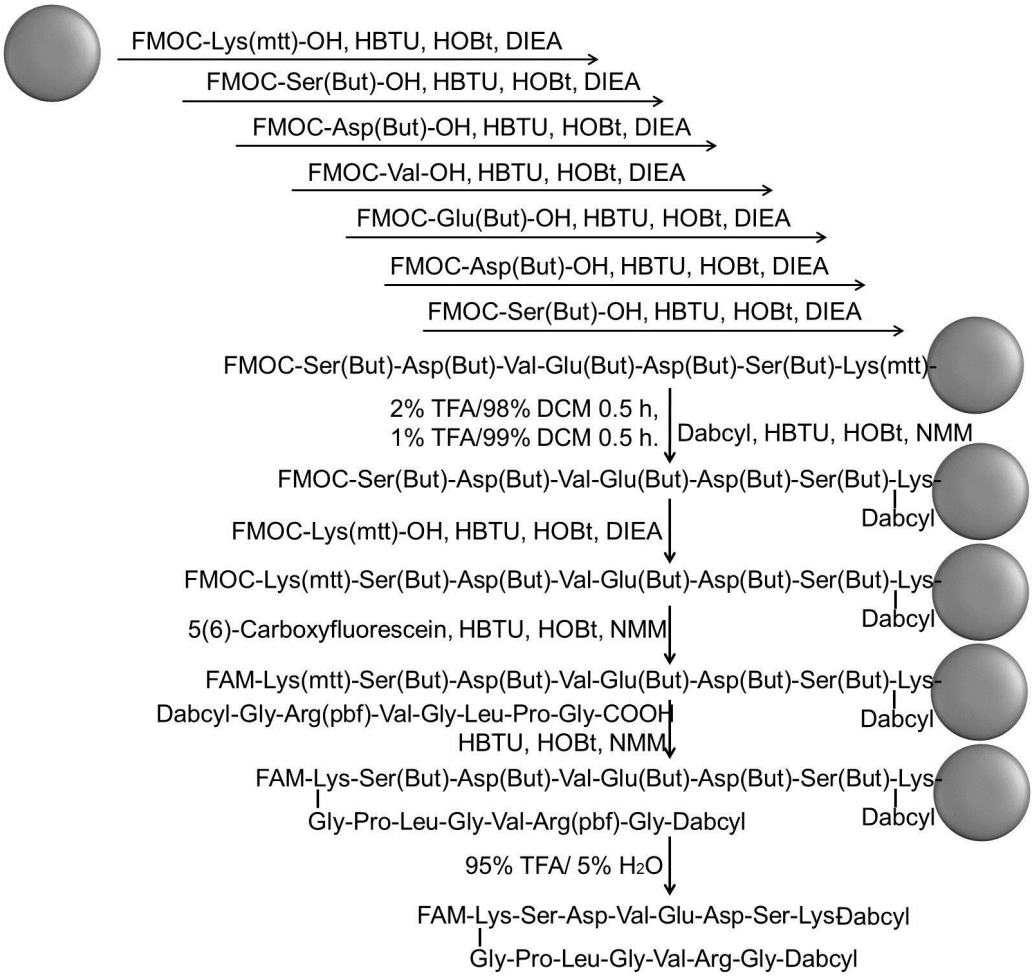
SCC-7 cells were seeded in 35 mm Petri dishes and incubated in DMEM (1 mL) containing 10% FBS for 24 h. Probe dispersed in DMEM medium (1 mL) with 10% FBS at the concentration of 5 μM were added and the cells were further incubated at 37 ^{o}C for 48 h. The cells images were taken by Carl Zeiss fluorescence microscopy, NOL-LSM 710.

Western blotting analysis of the caspase-3 expression in probe and Dox treated SCC-7 cells

SCC-7 cells were seeded in a 6-well plate at a density of 5×10^5 cells per well and incubated for 24 h prior to experiments. Then, probe-containing medium (20 μM) was added and the cells were cultured for 2 h. After washing with PBS for 3 times, Dox-containing medium (10 μg/mL) was added. The probe treated SCC-7 cells without Dox were used for the control. After 16 h incubation, the cells were lysed using 50 μL RIPA buffer (1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate, 2 μg/mL apportioning, 10 μg/mL PMSF, 100 mM Na-orthovanadate, 1×PBS) and resuspended in 50 μL 2 × SDS sample buffer containing 1% β-mercaptoethanol. Subsequently, the samples were boiled for 5 min and separated on a 10% SDS-PAGE (15 μL per lane). After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore) by semi-dry transfer cell (Bio-rad). After blocking in PBS solution (containing 5% skim milk) for 1 h, the caspase-3 was detected by respectively incubating the membranes with mouse monoclonal anti-caspase-3 antibody (1:3000 dilution, Cell Signaling Technology) overnight at 4 ^{o}C and subsequently treated with the secondary antibody HRP-labeled goat anti-rabbit IgG (1:3000 dilution, Santa Cruz Biotechnology) for 1 h. Specific protein was detected by enhanced chem-iluminescence (ECL; Pierce). β-actin was used as protein loading control.



Scheme S1: Synthesis details of Dabcyl-Gly-Pro-Leu-Gly-Val-Arg(pbf)-Gly-COOH.



Scheme S2: Synthesis details of the probe.

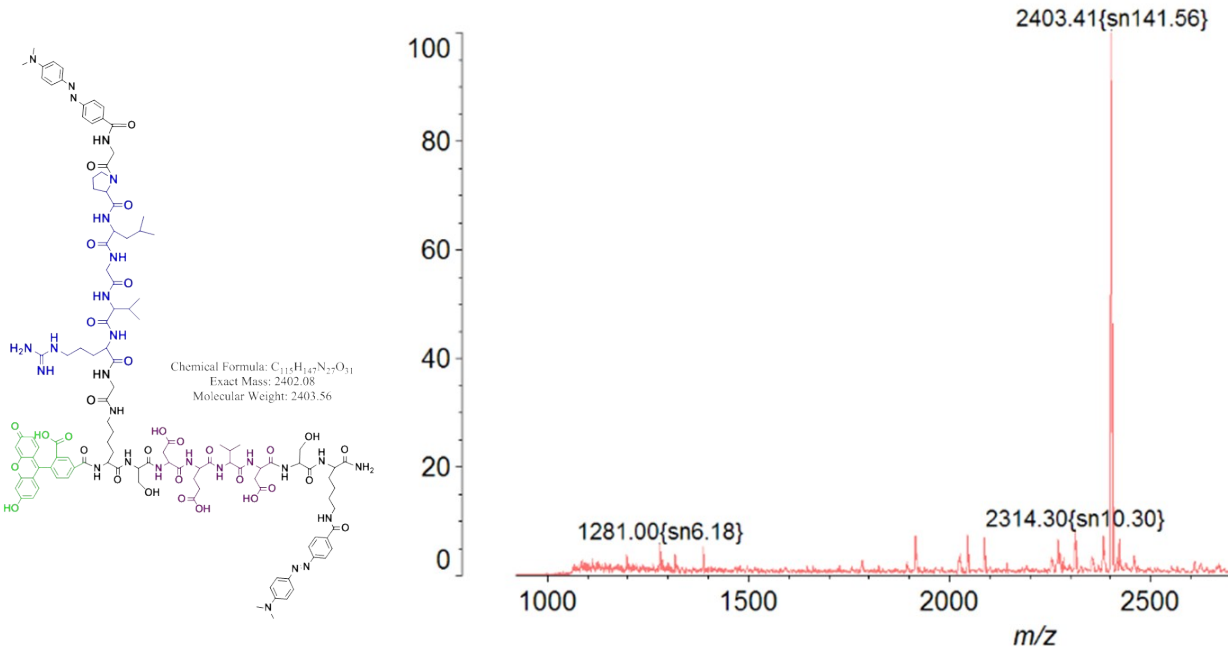


Figure S1. Chemical structure, and MALDI-TOF-MS of the probe.

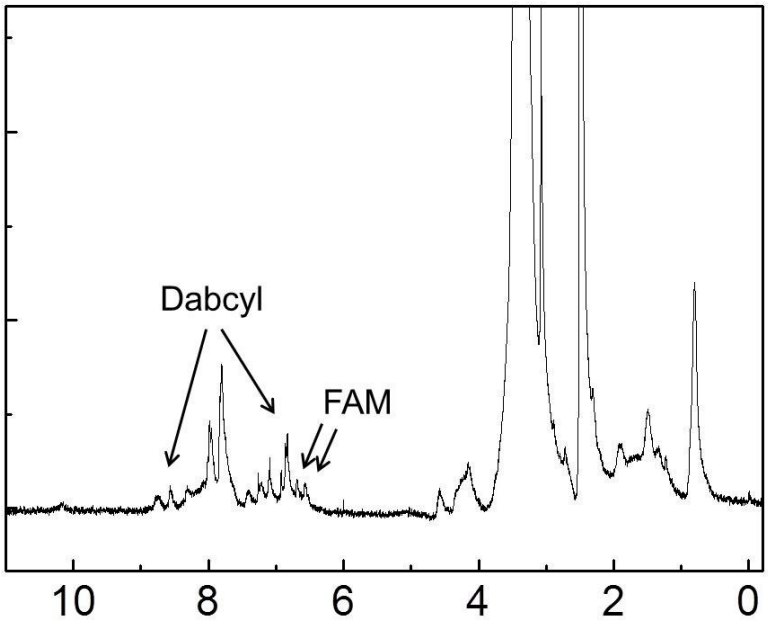


Figure S2. ^{1}H-NMR of the probe.

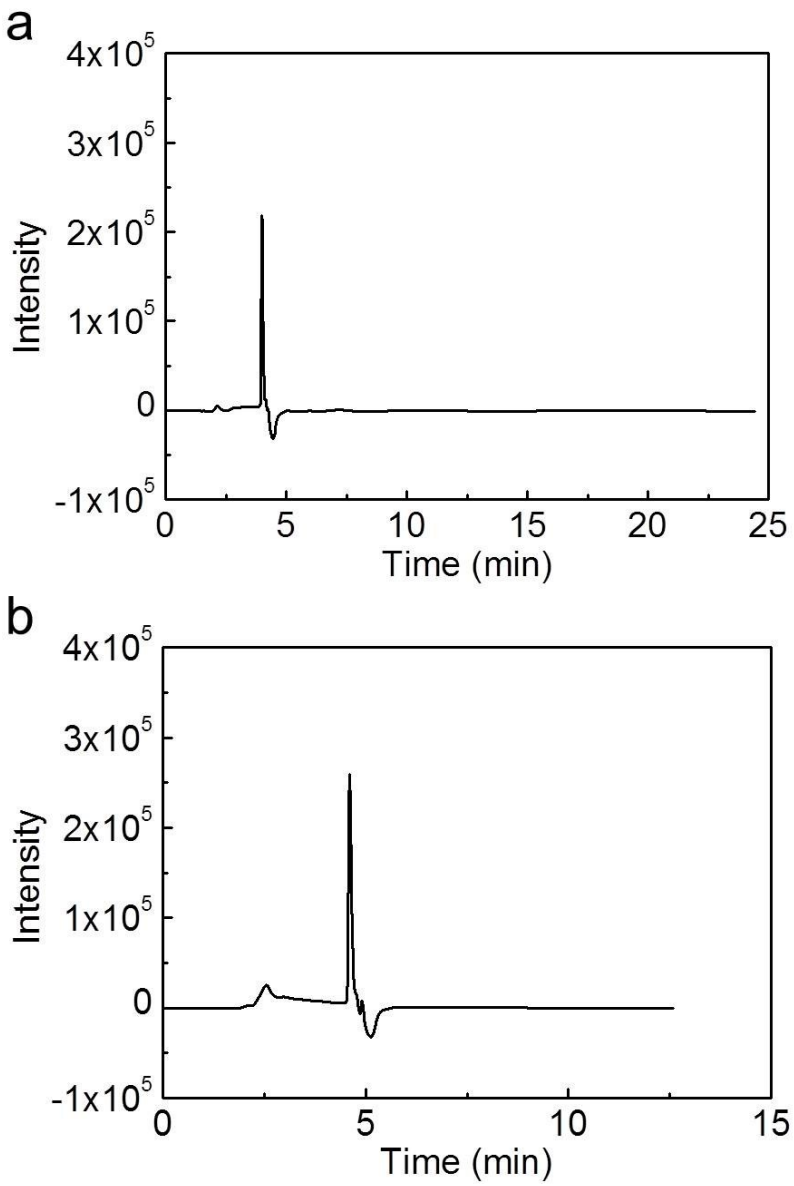


Figure S3. HPLC analysis of a) the Dabcyl-GPLGVRG-COOH moiety and b) the probe FAM-K(GPLGVRG-Dabcyl)SDEVDSK-(Dabcyl).

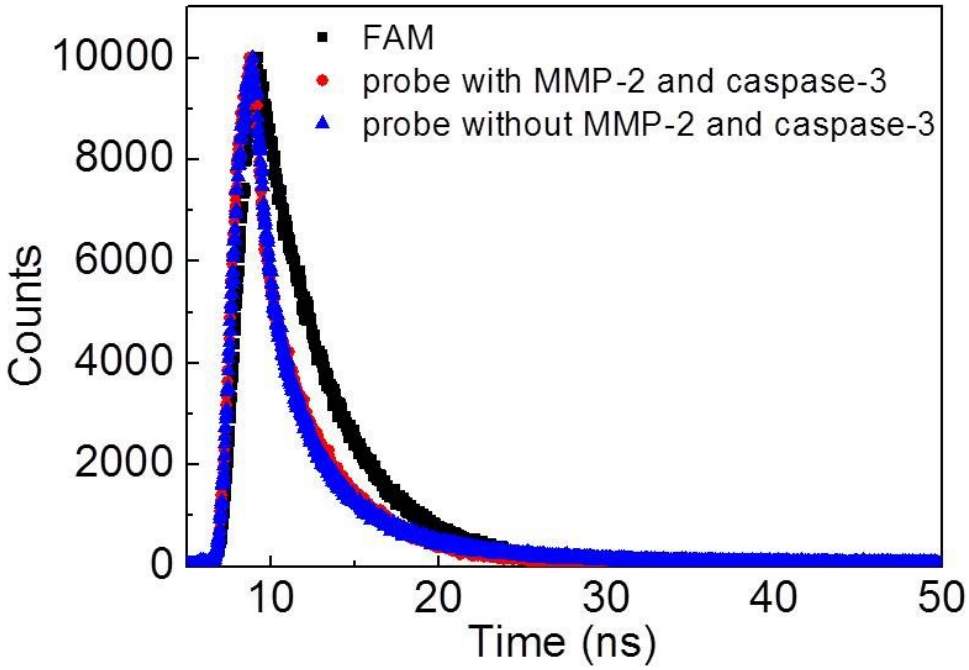


Figure S4. Fluorescence decays of FAM (5 μM) and probe (5 μM) with/without MMP-2 (100 ng/mL) and caspase-3 (50 pM) incubation for 3 h. Excitation: 477 nm.

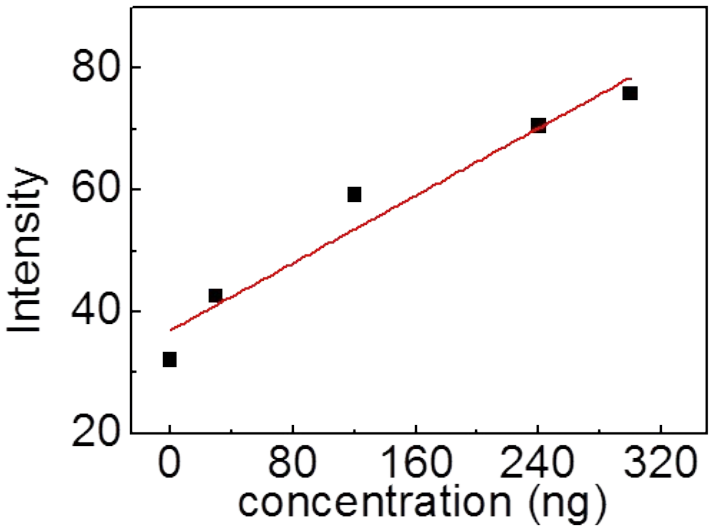


Figure S5. MMP-2 concentration related fluorescence recovery at the probe concentration of 0.5 μM in 1 mL PBS buffer solution for 2 h incubation. Excitation wavelength: 480 nm. Emission wavelength: 520 nm.

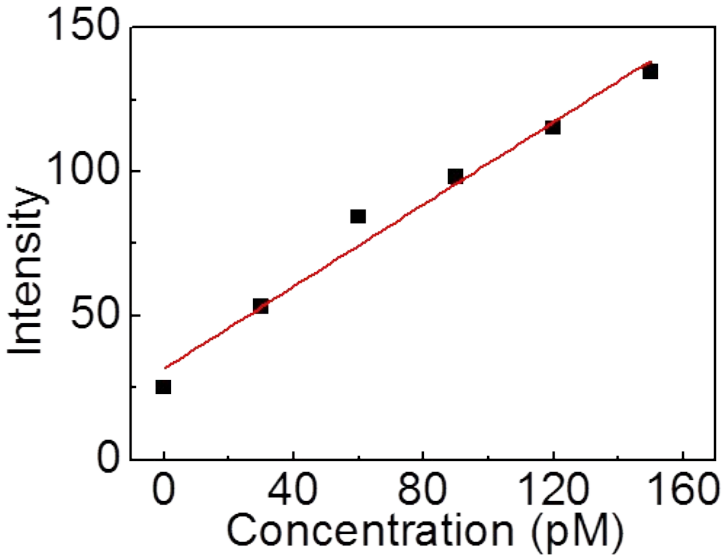


Figure S6. Caspase-3 concentration related fluorescence recovery at the probe concentration of 0.5 μM for 2 h incubation. Excitation wavelength: 480 nm. Emission wavelength: 520 nm.

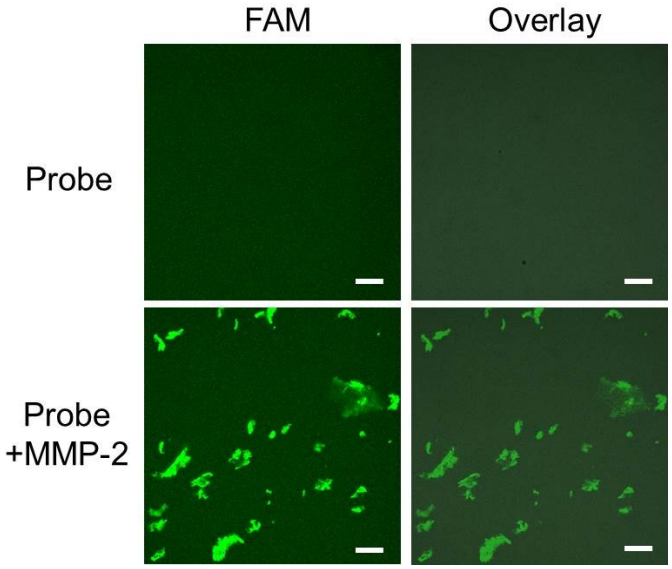


Figure S7. CLSM images of the probe (5 μM) incubated without/with MMP-2 (200 ng/mL) for 24 h. Excitation wavelength: 480 nm. Scale bar: 50 μm.

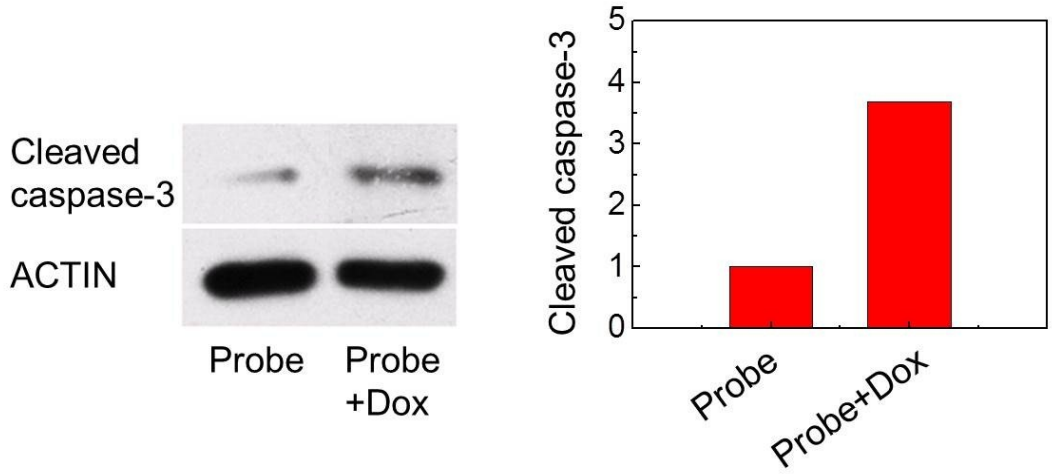


Fig. S8. Western blot analysis of cleaved caspase-3 in SCC-7 cells pre-treated with probe (5 μM) for 1 h and incubated without/with Dox (10 μg/mL) for 24 h.

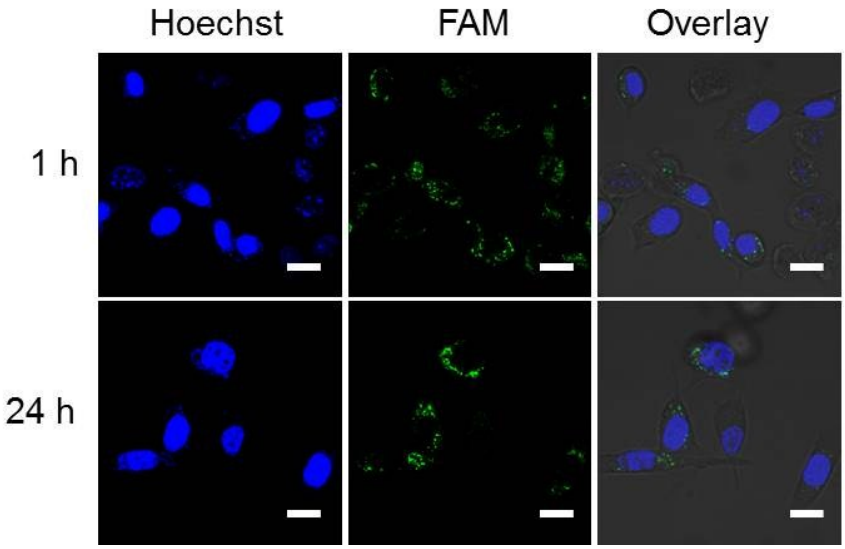


Figure S9. CLSM images of SCC-7 cells pre-incubated with probe (5 μM) for 1 h, caspase-3 inhibitor (Ac-DEVD-CHO, 50 μM) for 2 h and treated with Dox (10 mg/L) for 1 h and 24 h. Excitation wavelength: 480 nm. Scale bar: 40 μm.

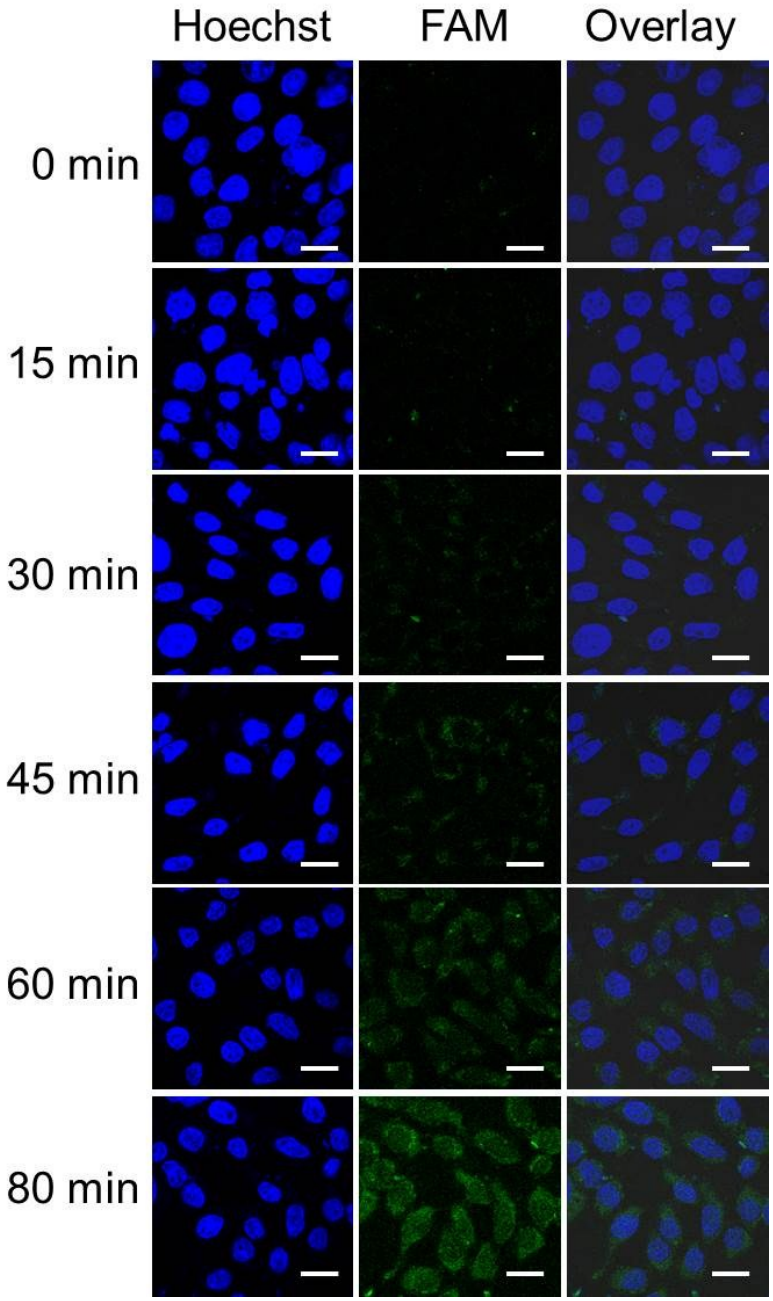


Figure S10. CLSM images of COS7 cells pre-incubated with probe (5 μM) for real time monitoring UV induced apoptosis signal by time intervals. Excitation wavelength: 480 nm. Scale bar: 40 μm.

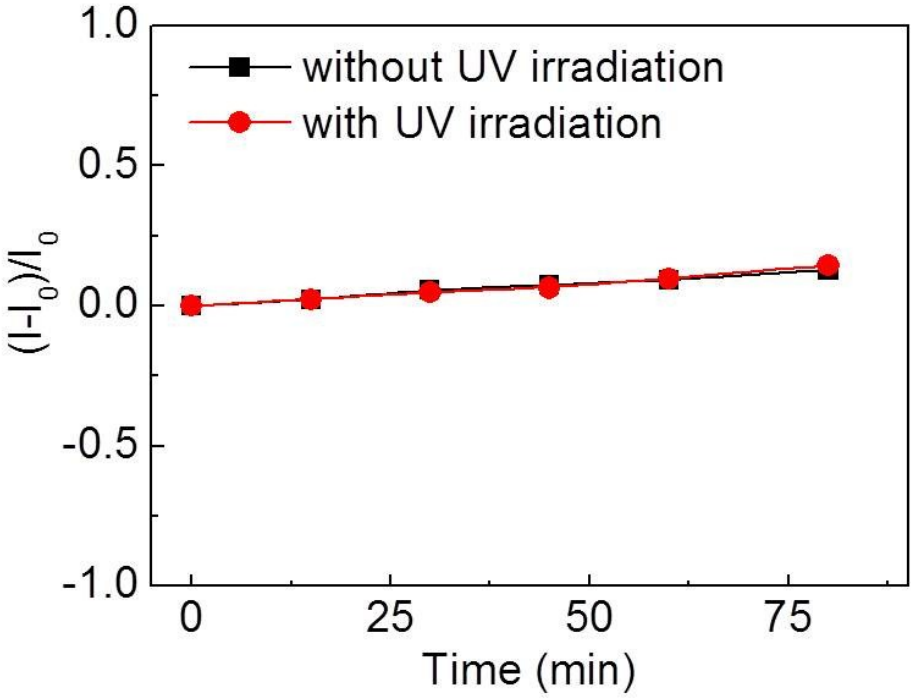


Figure S11. Fluorescence change of the probe in cuvette with (red solid circle)/without (black solid square) UV irradiation by various time. Excitation wavelength: 480 nm. Emission wavelength: 520 nm.

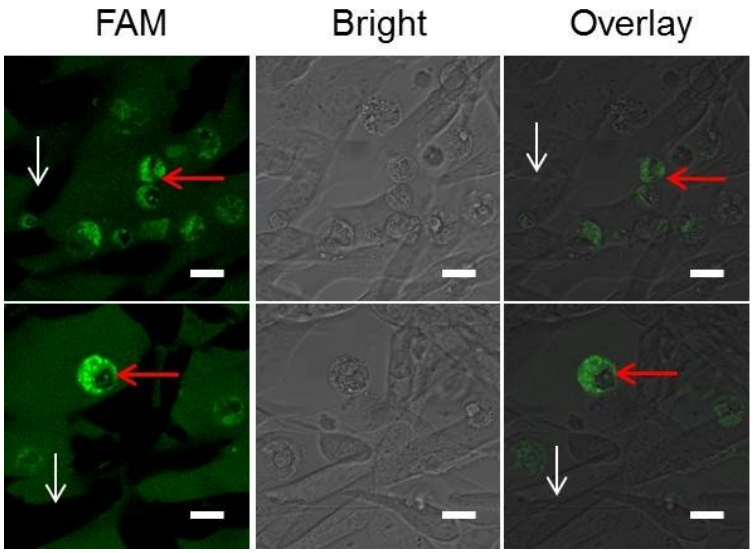


Figure S12. No-washing CLSM images of SCC-7 cells incubated with probe (5 μM) for apoptosis cell imaging. Excitation wavelength: 480 nm. Apoptosis cells: red arrows. Live cells: white arrows. Scale bar: 40 μm.