Supporting Information for

A non-peptide probe for detecting chymotrypsin activity based on protection and deprotection strategy in living systems

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Materials and instruments. Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Ultrapure water was used throughout all experiments. High resolution mass spectrometric (HRMS) analyses were measured on a Finnigan MAT 95 XP spectrometer; NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard; The fluorescence spectra were obtained on a F-4700 spectrofluorometer from Hitachi High-tech Science; The absorption spectra were collected on UV-2700 visible spectrophotometer from Shimadzu Scientific Instruments; The fluorescence imaging of cells was performed with TSC-SP8 (Leica, Wetzler, Germany) confocal laser scanning microscope; The pH measurements were carried out on a PHS-3E PH meter from INESA Instrument; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals. The chymotrypsin used in the experiment was derived from Shanghai Yuanye Biotechnology Co., LTD (S10001-1g USP，1500 U/mg).

Detection limit. The detection limit is based on the reporting method [S1]. According to the results of titration experiments, the fluorescence intensity (I_{587}) measured by CHT with different concentrations of **DT** was normalized between the minimum and maximum intensity. Then, the linear regression curve was fitted to the normalized fluorescence intensity data, and the detection limit was calculated using the formula $3\sigma/k$.

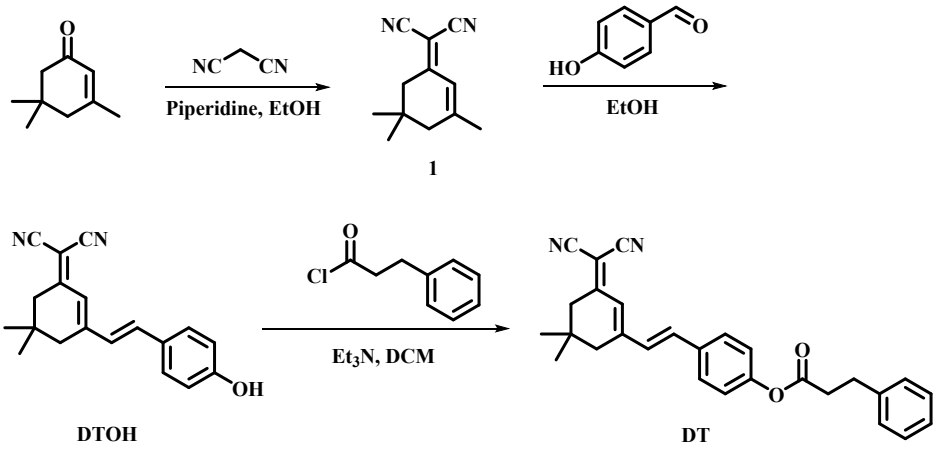
Cell culture and cytotoxicity assays. The living P815、3T3、MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100 U/mL, Hyclone) and streptomycin sulfate (100 U/mL, Hyclone). The cells were cultured at 37 °C and under a 5% CO_2 atmosphere.

The cytotoxicity of **DT** to living cells was performed by standard MTT assays. 2

× 10^4 cells/mL living cells were seeded in 96-well plates and then incubated with different concentrations of **DT** (0-50 μM) for 12 h. Subsequently, P815、3T3、MCF-7 cells were incubated with 5 mg/mL MTT (10 μL per well) and treated for 4 h. After that the supernatants were aspirated and 100 μL DMSO was added to per well. The absorbance of the solution at 490 nm was recorded using microplate reader. The cell viability (%) = (OD_{sample}-OD_{blank}) / ($OD_{control}$-OD_{blank}) × 100 %.

OD_{sample} denotes cells treated with various concentrations of **DT**; OD_{blank} denotes the plates with DMEM; $OD_{control}$ denotes cells without treated with **DT**. Each concentration was conducted with six parallel samples, and the results were expressed as mean ± standard deviation (SD).

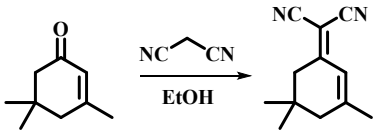
Synthesis



Scheme S1. Synthesis route of the fluorescent probe **DT**.

Synthesis of compound 2: Three millimoles (450 μL) of isophorone and 3 mmol of malononitrile were dissolved in 10 mL of ethanol. The reaction was refluxed for 8 h, and then solvent was removed under vacuum.

Residues were purified by a silica gel column chromatograph using mobile phase (PE/EA = 20/1) to acquire compound 1, white solid, 435.2 mg with a yield: 83%. ^{1}H NMR (600 MHz, DMSO-*d*6) δ 6.56 (s, 1H), 2.54 (s, 2H), 2.24 (s, 2H), 2.05 (s, 3H), 0.96 (s, 6H).



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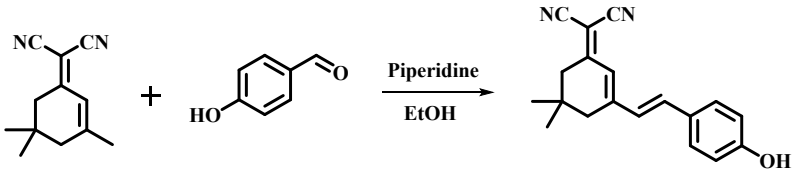
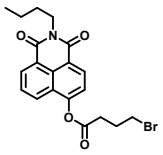
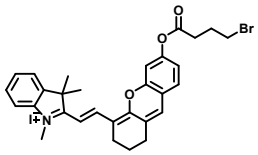
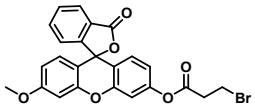
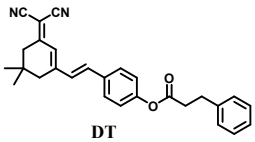
Synthesis of compound DTOH: Dissolve **1** (0.5 mmol, 700 mg) and 4-hydroxybenzaldehyde (0.5 mmol, 62 mg) in 10 mL of ethanol, and then add 2 to 3 drops of piperidine (the mixture solution is red). The mixture was refluxed at 70°C for 6 hours. After the reaction, the mixed solution was cooled to room temperature to produce a red precipitate, which was then washed with absolute ethanol and purified by column chromatography with a mixture of dichloromethane and methanol (DCM/MeOH = 20:1) to obtain **DTOH** as a red solid 78%.^{1}H NMR (600 MHz, DMSO-*d*6) δ 9.98 (s, 1H), 7.56 (d, *J* = 8.7 Hz, 2H), 7.21 (q, *J* = 16.1 Hz, 2H), 6.81 (d, *J* = 3.1 Hz, 2H), 6.79 (s, 1H), 2.60 (s, 2H), 2.53 (s, 2H), 1.02 (s, 6H).



1 DTOH

Table S1. Non-peptide fluorescent probes for the detection of **CHT**.

Probe structure	Synthesis procedures	Wavelength (nm)	Detection limit	Notable experiments	Limitations	References
	Steps; 4, Reactants: 7, Solvents: 3, Time: ≥ 40 hrs.	λ_{ex} = 450 nm, λ_{em} = 500 - 600 nm	8.4 ng/mL	Spectroscopic tests and inhibitors	Not suitable for vivo imaging of chymotrypsin	*Sens. Actuators, B* **2018**, *273*, 204–210
	Steps; 5, Reactants: 10, Solvents: 3, Time: ≥ 40 hrs.	λ_{ex} = 670 nm, λ_{em} = 680 - 720 nm	0.013 U/mL	Detection of endogenous chymotrypsin in mice and cells	Cell experiments has a long response time	*Sens. Actuators, B* **2020**, 306, 127567
	Steps; 3, Reactants: 7, Solvents: 3, Time: 17 hrs.	λ_{ex} = 455 nm, λ_{em} = 500 - 600 nm	50 ng/mL	Spectroscopic tests and inhibitors	Not suitable for vivo imaging of chymotrypsin	*Anal. Chem.* **2017***, 89,* 3687–3693
 **DT**	Steps; 3, Reactants: 5, Solvents: 2, Time: 16 hrs.	λ_{ex} = 410 nm, λ_{em} = 525 - 700 nm	0.071 U/mL	Detection of endogenous chymotrypsin in living cells, mice viscera and zebrafish	-	(This work)

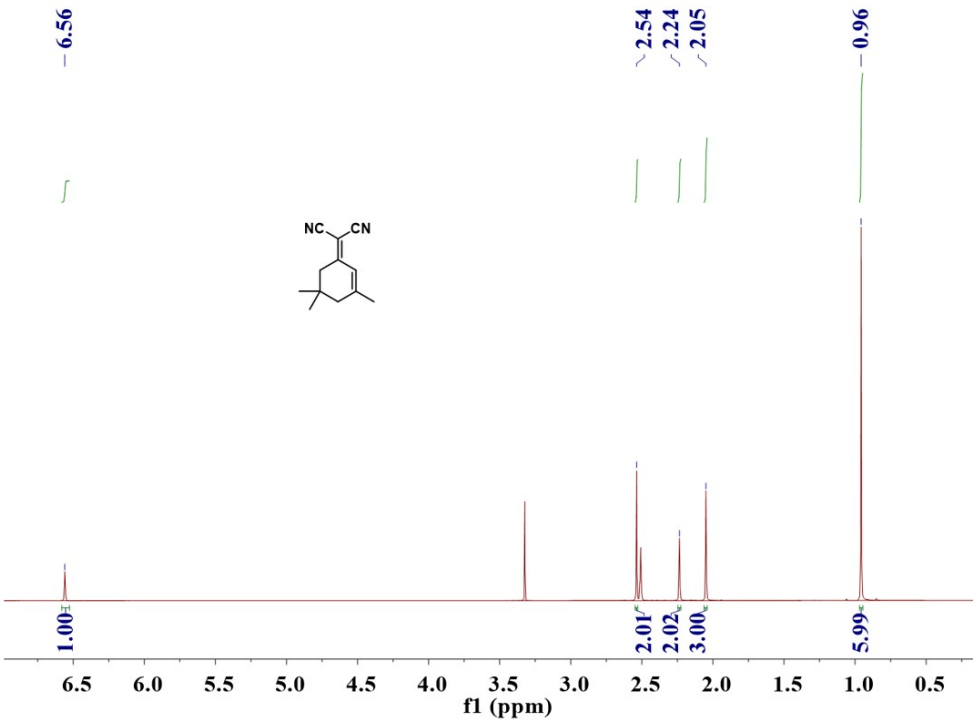


Figure S1. ^{1}H NMR spectrum of **2** in d_6-DMSO.

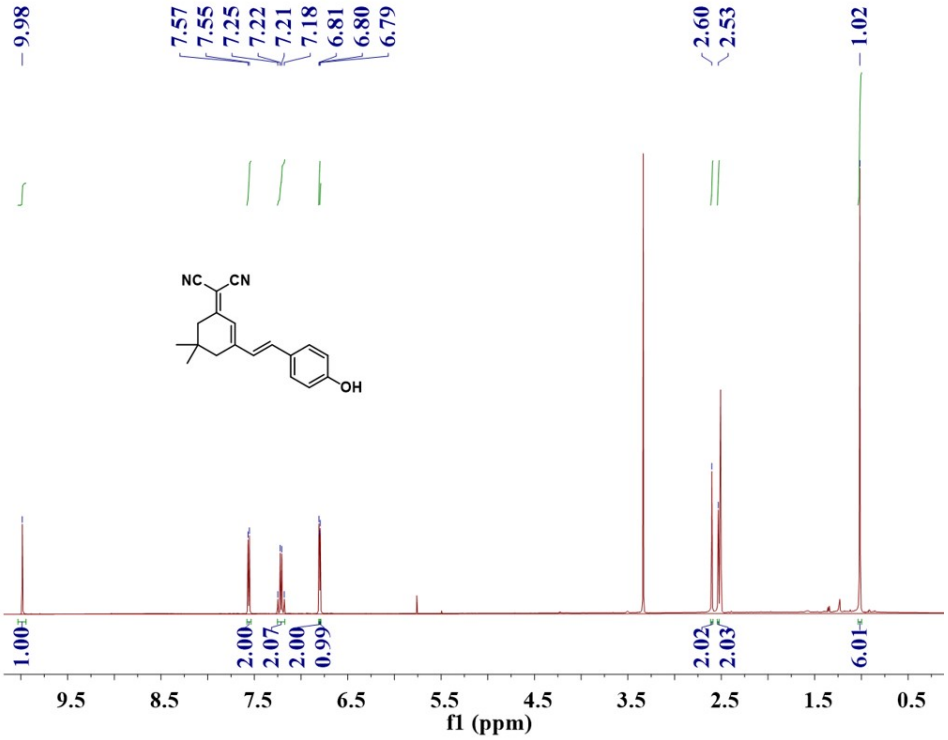


Figure S2. ^{1}H NMR spectrum of **DTOH** in d_6-DMSO.

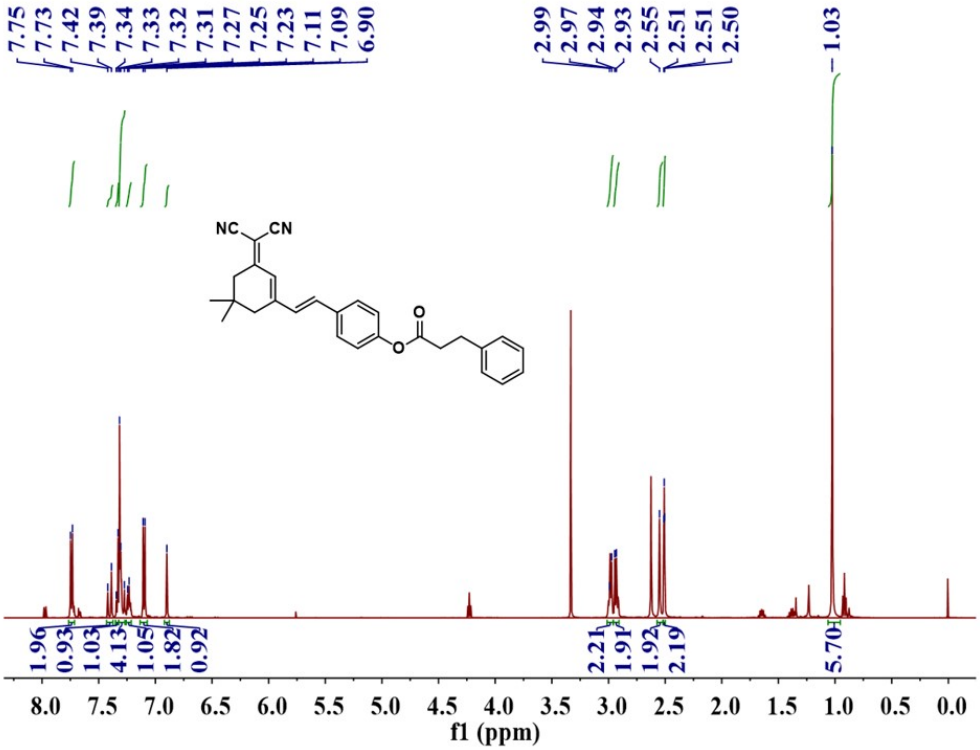


Figure S3. ^{1}H NMR spectrum of **DT** in d_6-DMSO.

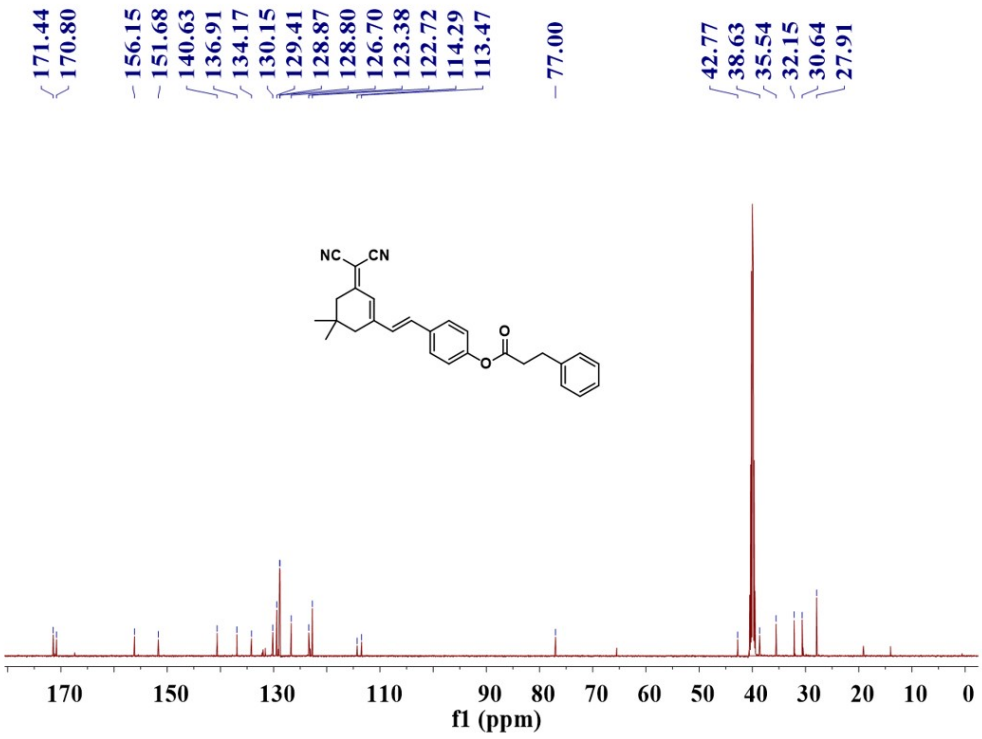


Figure S4. ^{13}C NMR spectrum of **DT** in d_6-DMSO.

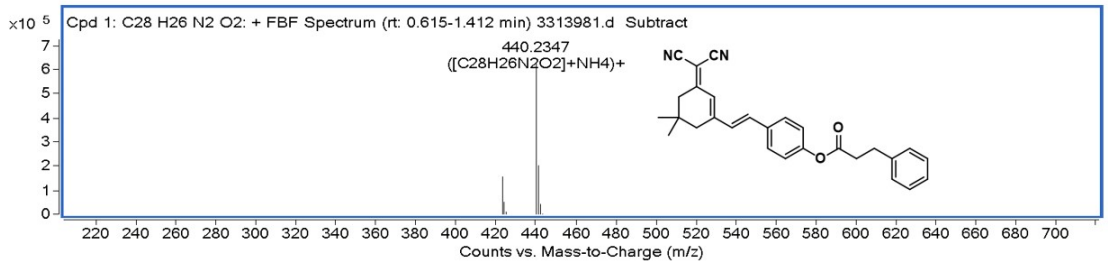


Figure S5. The ESI-MS spectrum of **DT** in DMSO.

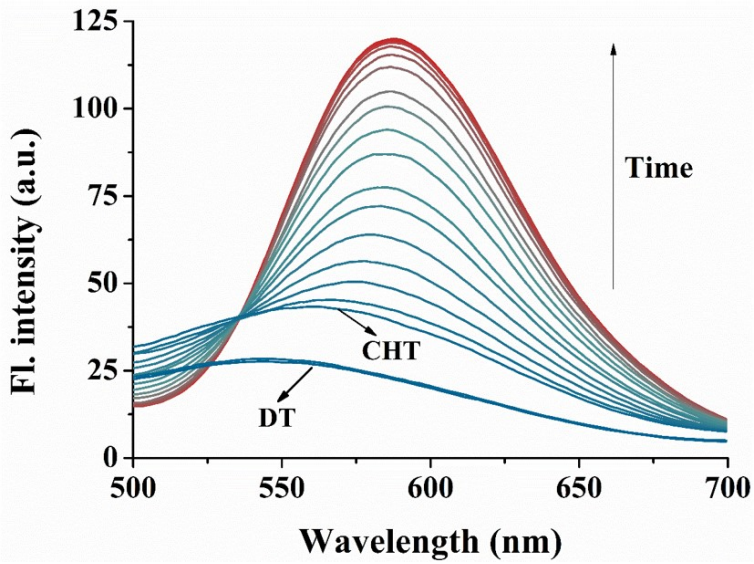


Figure S6. The fluorescence spectral peak changes of the probe **DT** with 45 U/mL CHT during 42 mins. λ_{ex} = 410 nm, slit: 5 nm/5 nm, U = 600 V.

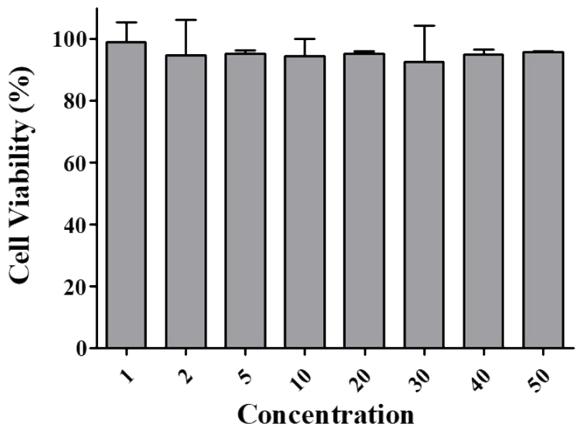


Figure S7. The viability of **P815** cells was treated with different concentrations (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM,50 μM) of **DT** for 12 hours.

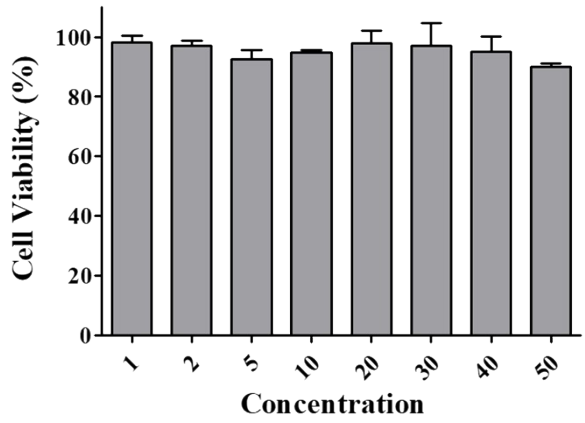


Figure S8. The viability of **3T3** cells was treated with different concentrations (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM,50 μM) of **DT** for 12 hours.

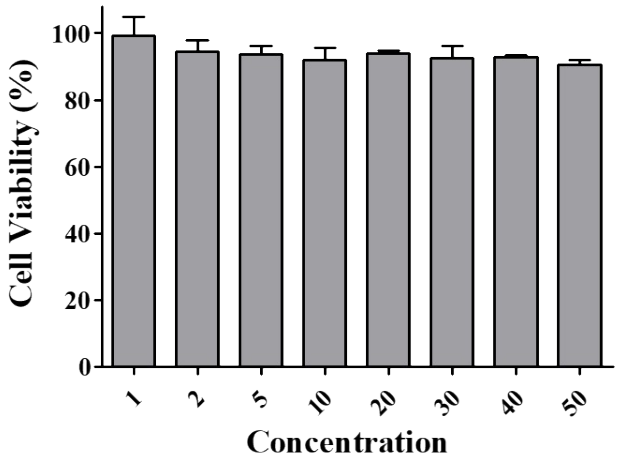


Figure S9. The viability of **MCF-7** cells was treated with different concentrations (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM,50 μM) of **DT** for 12 hours.

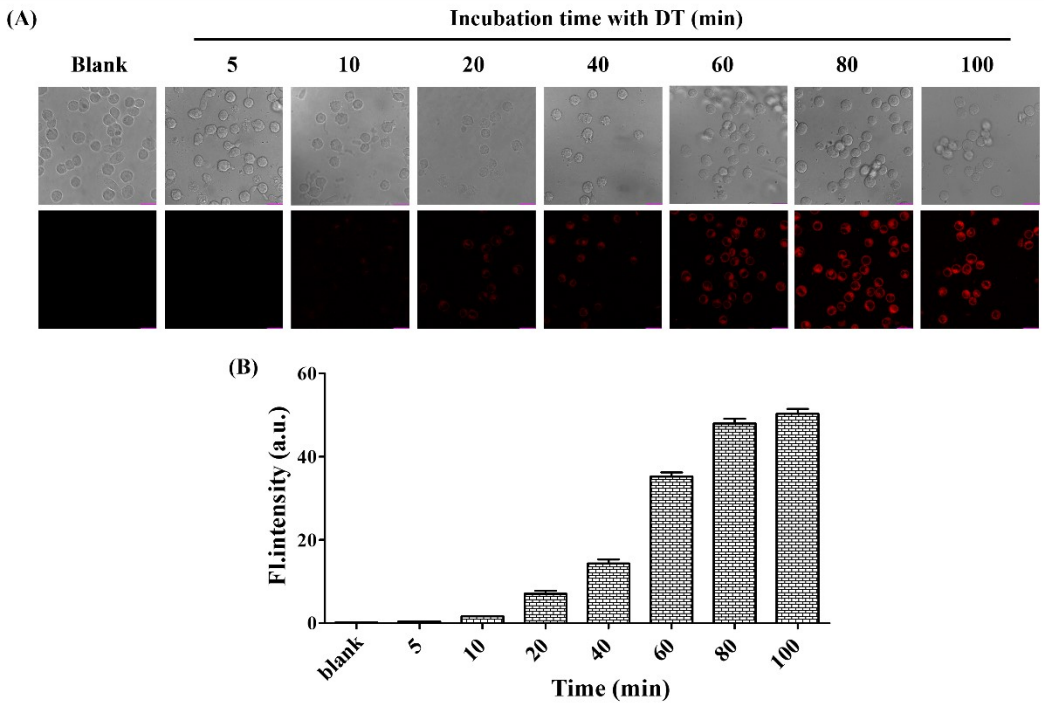
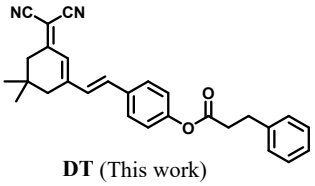


Figure S10. (A) The images of P815 cells incubated with **DT** in different time (5, 10, 20, 40, 60, 80, and 100 mins) were imaged under 100 × oil mirror; (B) Histogram of fluorescence intensity of (A) at different time. Bar = 20 um; λ_{ex} = 405 nm.

Table S2. Properties of peptide fluorescent probes and non-peptide fluorescent probe **DT**.

Item	The article DOI no.	Wavelength (nm)		Biological experiment	Living sample types	Incubation time (mins)
		λ_{ex}	λ_{em}			
1	 **DT** (This work)	410	525 - 700	Detection of chymotrypsin	3T3, MCF-7, P815	60
2	org/10.1016/0003-2697(85)90216-7	325	465/510	Detection of chymotrypsin	-	-
3	org/10.1016/j.bios.2016.08.029	570	600 - 680	Detection of chymotrypsin	-	-
4	10.1039/c8cc01731j	435	450 - 600	Protease detection	-	-
5	10.1038/cr.2013.11	400	450 - 600	Autophagosome-lysosome fusion	E. coli DH5α	180
6	10.1039/c7cc06056d	497	525	Collagen detection	Connective tissues	120
7	10.1016/j.talanta.2018.11.011	260	510 - 570	Carcinoembryonic antigen detection	Human serum	90
8	10.1039/c4cc00670d	410	550 - 700	Lysosomal tracking	A549	60

References

[1] Shortreed M.; Kopelman R.; Kuhn M.; Hoyland B. Fluorescent Fiber-Optic Calcium Sensor for Physiological Measurements. *Anal. Chem.*, **1996**, *68*, 1414-1418.