Supporting Information

Novel three-fluorophore system as a ratiometric sensor for multiple protease detection

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Table of contents

Materials and methods	2
Reagents	2
Equipment	2
Proteolytic experiments	3
Determination of FRET efficiency	3
Determination of Michaelis-Menten constants (K _m)	3
Quantitative assay	3
Synthesis of the probe	4
Characterization of the intact probe	12
LC-MS of the intact probe	12
LC/HRMS analysis	13
¹ H NMR spectrum of intact probe	15
IR spectrum of intact probe	16
HPLC analysis of the intact probe stock solution used for proteolytic experiments	17
LC-MS spectra of the probe after cleavage with enzymes.	19
Figure S1	25
Figure S2	26
Figure S3.	27
Figure S4.	28
Procedure for simultaneous proteases detection	29
Table S1	31
Table S2	31
Figure S5	32
List of abbreviations	33
References	

Materials and methods

Reagents

Wang resin and Fmoc-amino acids were purchased from AAPPTec (Louisville, KY). DEAC^[1] and succinimide ester of fluorescein^[2] were synthesized according to literature procedures. α -Chymotrypsin (from bovine pancreas, TLCK treated, type VII, \geq 40 units/mg protein) and trypsin (from bovine pancreas, TPCK treated, \geq 10000 BAEE units/mg protein) were purchased from Sigma Aldrich. All other chemicals and solvents were purchased from Sigma Aldrich (Milwaukee, IL).

Synthesis was carried out on Domino Blocks (Torviq, Niles, MI) in polypropylene disposable syringes equipped with a frit. The volume of the wash solvent was 10 mL per gram of the resin. For washing, the resin slurry was shaken with fresh solvent for at least 1 min before the solvent was replaced.

Equipment

The LC-MS analyses were carried out on UHPLC-MS system consisting of UHPLC chromatograph Accela with photodiode array detector and triple quadrupole mass spectrometer TSQ Quantum Access (both Thermo Scientific, CA, USA), using Nucleodur Gravity C18 column at 30°C and flow rate of 600 μ L/min (Macherey-Nagel, 1.8 μ m, 2.1 x 50 mm, Germany). Mobile phase was (A) 0.1% ammonium acetate in water, and (B) 0.1% ammonium acetate in acetonitrile, linearly programmed from 10% to 80% B over 2.5 min, kept for 1.5 min. The column was re-equilibrated with 10% of solution B for 1 min. The APCI source operated at discharge current of 5 μ A, vaporizer temperature of 400°C and capillary temperature of 200°C. Purity of the compounds was determined as a ratio of the given peak area to the total area of all peaks of the mixture. For the LC-MS analysis a sample of resin (~1 mg) was treated by TFA/DCM/TES (45:45:10, v/v/v) for 30 min, the cleavage cocktail was evaporated by a stream of nitrogen, and cleaved compounds extracted into MeOH/water (1:1, v/v).

Purification was carried out on semipreparative HPLC with a Waters 1500 series HPLC instrument equipped with an Autosampler 2707, a Binary HPLC pump 1525, a Waters Photodiode Array Detector 2998 and a Waters Fraction Collector III with a YMC C18 reverse phase column, 20×100 mm, with 5 μ m particles.

HR-MS analysis was performed using an Orbitrap Elite high-resolution mass spectrometer (Thermo Fischer Scientific, MA, USA) operating at positive full scan mode (120 000 FWMH) in the range of 2000-3000 m/z. The settings for electrospray ionization were as follows: oven temperature of 300 °C, sheath gas of 8 arb. units and source voltage of 1.5 kV. Samples were diluted to a final concentration of 20 μ mol/l with 0.1% formic acid in water and methanol (50:50, v/v). Infrared spectra were measured on FTIR spectrometer Nicolet iZ10 (Thermo Scientific). Samples were scanned 32 times in the range of 400–4000 cm-1 and worked up with OMNIC 8.3 software.

Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a thermostat (FL1009M015). Excitation and emission slits were 5 nm. Absorption spectra were recorded on a Cary 300 UV/VIS spectrophotometer (UV111M031, Agilent).

Proteolytic experiments

Fluorescence and absorption measurements were performed in a 500 μ L quartz cell in 100mM Tris-HCl buffer, 10mM CaCl₂ (pH 8.0) at 37°C. Samples for spectral experiments were prepared by dilution of 1 mM stock solutions of the probe in DMSO prior to measurements. Chymotrypsin and trypsin were dissolved in 1 mM HCl to obtain a final concentration of $1\mu g/\mu L$ and stored at minus 20°C. Proteolysis experiments were performed as follows: an assay mixture was transferred to the cell connected with a thermostat, after 15 min warming an enzyme solution was added to the mixture to obtain a final assay volume of 500 μ L, the final solution was mixed and the recording started immediately.

Determination of FRET efficiency

FRET efficiency (E) was obtained by comparison of the absorption and excitation spectra and calculated from the following equation^[3]:

$$= \frac{A_A(\lambda_A)}{A_D(\lambda_D)} \times \left[\frac{I_A(\lambda_D, \lambda^{em}_A)}{I_A(\lambda_A, \lambda^{em}_A)} - \frac{A_A(\lambda_D)}{A_A(\lambda_A)} \right]_{,}$$

where $A_A(\lambda_A)$ is the acceptor absorbance at the acceptor excitation wavelength, $A_D(\lambda_D)$ is the donor absorbance at the donor excitation wavelength, $A_A(\lambda_D)$ is the acceptor absorbance at the donor excitation wavelength, $I_A(\lambda_D, \lambda_A^{em})$ is the acceptor intensity at the donor excitation wavelength and $I_A(\lambda_A, \lambda_A^{em})$ is the acceptor intensity at the acceptor excitation wavelength.

Determination of Michaelis-Menten constants (K_m)

Chymotrypsin (0.5 µg/mL) or trypsin (4 ng/mL) was incubated with different concentrations the probe (0.5–10 µM) in 100mM Tris-HCl buffer, 10mM CaCl₂ (pH 8.0) at 37°C for 30 min. Fluorescence emission signals at 475 nm and 590 nm were collected every 30 s upon excitation at 435 nm. Measurements were repeated three times for each concentration. Concentrations of the products of proteolysis were determined from the scatterplot comparing F_{590}/F_{475} vs. percent cleavage. Initial velocities were calculated from the linear portion of the curve on a scatterplot comparing concentrations vs. time. K_m values of the probe were then determined by fitting resulting data to the GraphPad Prism 6 software.

Quantitative assay

Probe was incubated with varying concentrations of chymotrypsin or trypsin in 100mM Tris-HCl buffer, 10mM CaCl₂ (pH 8.0) at 37°C for 20 min. Fluorescence emission signals at 475 nm and 520 nm were collected every 30 s upon excitation at 435 nm. Rates of F_{475}/F_{520} change (ΔF) were calculated from the linear portion of the curve on a scatterplot comparing F_{475}/F_{520} vs. time. Measurements were repeated three times for each concentration. Detection limit was determined at lowest concentration, where the slope was significantly non-zero, p < 0.05 (calculated by GraphPad Prism 6 software).

Synthesis of the probe

The probe was synthesized according to the following reaction sequence:





i) Fmoc-Lys(Mtt)-OH, HOBt, DIC, DMAP, DCM/DMF 1:1, rt, 16h; ii) TES/HFIP/TFE/DCE 20:10:5:65, 60°C, µW, 24h; iii) DEAC, HOBt, DIC, DMSO/NMP 1:1, rt, 2h; iv) 50% piperidine/DMF, rt, 20 min; v) aminoacid, DIC, HOBt, DCM/DMF 1:1, rt, time; vi) NsCl, 2,6-lutidine, DCM, rt, 1h; vii) MeOH, PPh₃, DIAD, THF, -20°C to rt, 3h; viii) 2-mercaptoethanol, DBU, DMF, rt, 20 min; ix) rhodamine B, HOBt, DIC, DMAP, DCM/NMP 1:1, rt, 16h; x) MeOH, PPh₃, DIAD, THF, -20°C to rt, 2h; xi) succinimide ester of fluorescein, DMSO/NMP 1:1, rt, 16h; xii) 50%TFA/DCM, rt, 1h;

Resin 1

Wang Resin (100 mg, loading 0.045 mmol/g) was washed with DCM and a solution of Fmoc-Lys(Mtt)-OH (0.2 mmol), HOBt (0.2 mmol), DMAP (0.2 mmol) and DIC (0.2 mmol) in 1 mL DCM/DMF (1:1, v/v) was added. The resin was shaken for 16 h at rt, washed three times with DMF and three times with DCM. After washing, potentially unreacted amino derivative was capped with acetyl group: Ac₂O (0.2 mmol), pyridine (0.4 mmol), DCM (1 mL), 30 min. LC-MS analysis of cleaved product: MS (ESI) exact mass calcd. for C₂₁H₂₄N₂O₄ [M-Mtt+H]⁺ 369.174; found 369.19, $t_R = 1.77$ min, purity: 99%.



Resin 2

Resin 1 (100 mg) was washed with MeOH, dried under stream of nitrogen and was reacted with a solution of TES/HFIP/TFE/DCE (20:10:5:65, v/v, 2 mL) for 24 h at 60°C in microwave (35W). The resin was washed three times with DCM. The analytical amount of resin was then reacted with Fmoc-OSu to control the full conversion. LC-MS analysis of cleaved Fmoc product: MS (ESI) exact mass calcd. for $C_{36}H_{34}N_2O_6$ [M+H]⁺ 590.24; found 591.14, $t_R = 2.94$ min, purity: 92%.



Resin 3

Resin 2 (100 mg) was washed with DMF and a solution of DEAC^[1] (0.2 mmol), HOBt (0.2 mmol), and DIC (0.2 mmol) in 1 mL DMSO/NMP (1:1, v/v) was added. The resin was shaken for 2 h at rt, washed three times with DMF and three times with DCM. LC-MS analysis of cleaved product: MS (ESI) exact mass calcd. for $C_{35}H_{37}N_3O_7$ [M+H]⁺ 611.26; found 612.32, $t_R = 2.70$ min, purity: 95%.



Resin 4

Resin **3** (100 mg) was washed three times with DMF and a solution of 1.5 mL of 50% piperidine in DMF was added to the resin and the slurry was shaken for 20 min. The resin was three times with DMF. The peptide sequence (PEG-Ala-Phe-Ala-PEG-Lys(Mtt)-PEG-Ala-Lys(Boc)-Ala-PEG) was built according to the following procedure: a corresponding aminoacid (0.3 mmol), DIC (0.3 mmol), HOBt (0.3 mmol), DMF/DCM (1:1, v/v, 1.5 mL). Reaction time: 16 h for Fmoc-Lys(Mtt)-OH and 1.5 h for other aminoacids. Fmoc group was removed prior to each coupling by treatment with 50% piperidine in DMF within 15 min and the resin was washed after every coupling step three times with DMF and three times with DCM. LC-MS analysis of cleaved product **4**: MS (ESI) exact mass calcd. for $C_{92}H_{134}N_{16}O_{26}$ [M-Mtt-Boc+H]⁺ 1878,97; found 1880.66, $t_R = 2.54$ min, purity: 85%.







Resin 4 (100 mg) was washed three times with DMF. A solution of 1.5 mL of 50% piperidine in DMF was added to the resin and the slurry was shaken for 20 min. The resin was washed three times with DMF and three times with DCM. A solution of 4-nitrobenzenesulfonyl chloride (0.3 mmol) and 2,6-lutidine (0.3 mmol) in DCM (1.5 mL) was added. The resin was shaken for 1 h at rt and washed three times with DCM. LC-MS analysis of cleaved product: MS (ESI) exact mass calcd. for $C_{83}H_{127}N_{17}O_{28}S$ [M-Mtt-Boc+H]⁺1841.88; found 1843.47, $t_R = 2.27$ min, purity: 80%.





Resin 5 (100 mg) was washed with THF and mixed with solution of methanol (0.3 mmol) and PPh₃ (0.3 mmol) in dry THF (1.5 mL) and left at -20°C for 10 min. Then DIAD (0.3 mmol) was added to the mixture and the resin slurry was shaked at rt for 3 h. The resin was then washed three times with THF. LC-MS analysis of cleaved product: MS (ESI) exact mass calcd. for $C_{84}H_{129}N_{17}O_{28}S$ [M-Mtt-Boc+H]⁺ 1855.89; found 1857.89, $t_R = 2.36$ min, purity: 78%.





Resin **6** (100 mg) was washed with DMF and reacted with a solution of 2-mercaptoethanol (0.9 mmol) and DBU (0.3 mmol) in DMF (1.5 mL) for 20 min. After washing three times with DMF, the resin was reacted with rhodamine B (0.3 mmol), HOBt (0.3 mmol), DIC (0.3 mmol) and DMAP (0.15 mmol) in DMF/DCM (1:1, v/v, 1.5 mL) at rt for 16 h. The resin was washed ten times with DMF and three times with DCM. The analytical amount of resin was then cleaved and reacted with Boc₂O. LC-MS analysis Boc product: LC-MS analysis of cleaved product: MS (ESI) exact mass calcd. for C₁₁₁H₁₆₃N₁₈O₂₈ [M-Mtt+H]⁺ 2196.19; found 2297.64, $t_R = 3.15$ min, purity: 60%.



Resin 8

Resin 7 (100 mg) was washed with MeOH, dried under stream of nitrogen and was reacted with a solution of TES/HFIP/TFE/DCE (20:10:5:65, v/v, 3 mL) for 24 h at 60°C in microwave (35W). The resin was washed three times with DMF and three times with DCM. Then the resin was reacted for 1 h with a solution of 4-nitrobenzenesulfonyl chloride (0.3 mmol) and 2,6-lutidine (0.3 mmol) in DCM (1.5 mL). LC-MS analysis of cleaved product: MS (ESI) exact mass calcd. for $C_{112}H_{158}N_{19}O_{30}S$ [M-Boc+H]⁺ 2281.11; found 2282.5, $t_R = 3.03$ min, purity: 75%.





Resin 8 (100 mg) was washed with THF and mixed with solution of methanol (0.3 mmol) and PPh₃ (0.3 mmol) in dry THF (1.5 mL) and left at -20°C for 10 min. Then DIAD (0.3 mmol) was added to the mixture and the resin slurry was shaked at rt for 2 h. The resin was then washed three times with THF. LC-MS analysis of cleaved product: MS (ESI) exact mass calcd. for $C_{113}H_{160}N_{19}O_{30}S$ [M-Boc+H]⁺ 2295.13; found 2297.03, $t_R = 3.14$ min, purity: 66%.







Resin **9** (100 mg) was reacted with a solution of 2-mercaptoethanol (0.9 mmol), DBU (0.3 mmol) in DMF (1.5 mL) for 20 min. After washing, the resin was reacted with succinimide ester of fluorescein^[2] (75 mg) in DMSO/NMP (1:1, v/v, 2 mL) for 16 h. LC-MS analysis of cleaved product: MS (ESI) m/z calcd. for $C_{127}H_{167}N_{18}O_{30}$ [M-Boc+H]²⁺ 1213.11; found 1213.05, $t_{\rm R} = 2.71$ min, purity: 40%.





Probes 1 was cleaved from resin 10 by treatment with TFA/DCM/TES (45:45:10, v/v, 5 mL) for 1 h. Cleavage cocktail was collected and resin was washed three times with 50% TFA in DCM. The combined extracts were evaporated by a stream of nitrogen and the crude products were purified by reverse phase HPLC. Purity of crude product: 40%. The mobile phase consisted of (A) 0.01 mol dm⁻³ 0.01% formic acid in water and (B) methanol, with B linearly programmed to shift from 30% to 60% over the course of 6 min. Violet solid, 5 mg (5% yield, 100% purity). HR-MS (ESI) m/z calculated for $C_{127}H_{167}N_{18}O_{30}$ [M]⁺ 2425.2124, found 2425.2158.

Characterization of the intact probe

LC-MS of the intact probe.



LC-MS (gradient 20-80%, 5 min): absorbance at 0-499 nm.



MS (ESI) exact mass calcd. for $C_{127}H_{167}N_{18}O_{30}$ [M+H]⁺ 2425.22; found 2425.60:



LC/HRMS analysis

a) Column: XBridge 3.5 μm, 4.6x50 mm Gradient (flow: 0,6 mL/min):
0. min 80% pufr, 20% ACN,
1,5.min 70% pufr, 30% ACN,
6. min - 20% pufr, 80% ACN,
12. – 15. min 80% pufr, 20% ACN

HR-MS (ESI) m/z calcd. for $C_{127}H_{166}N_{18}O_{30}\ \mbox{[M]}^{\bullet-}$ 2423.2017; found 2423.2651

HR-MS (ESI) m/z calcd. for $C_{127}H_{168}N_{18}O_{30}$ [M] + 2425.2129; found 2425.2178



b) Column: Kinetex 2.6 μm 100 Å, 2.1x50 mm Gradient (flow: 0,6 mL/min):
0. min 80% pufr, 20% ACN,
1,5.min 70% pufr, 30% ACN,
6. min - 20% pufr, 80% ACN,
12. – 15. min 80% pufr, 20% ACN

HR-MS (ESI) m/z calcd. for $C_{127}H_{166}N_{18}O_{30}$ [M] \cdot 2423.2017; found 2423.2695

HR-MS (ESI) m/z calcd. for $C_{127}H_{168}N_{18}O_{30}$ [M] $^{\bullet+}$ 2425.2129; found 2425.2192



¹H NMR spectrum of intact probe

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NMR spectrum was measured in methanol- d_4 using a Jeol ECX-500 (500 MHz) spectrometer

IR spectrum of intact probe

IR spectrum was measured with use of NICOLET iZ10 (Thermo Fisher) via ATR technique.



HPLC analysis of the intact probe stock solution used for proteolytic experiments

a) Precolumn C18 + column: Waters XSelect HSS T3 2.5 μm, 3.0x50 mm Gradient (flow: 0,6 mL/min):
0. min 80% pufr, 20% ACN,
1,5.min 70% pufr, 30% ACN,
6. min - 20% pufr, 80% ACN,
12. – 15. min 80% pufr, 20% ACN



b) Precolumn C18 + column: XBridge 3.5 μm, 4.6x50 mm Gradient (flow: 0,6 mL/min):
0. min 80% pufr, 20% ACN,
1,5.min 70% pufr, 30% ACN,
6. min - 20% pufr, 80% ACN,
12. – 15. min 80% pufr, 20% ACN



LC-MS spectra of the probe after cleavage with enzymes.

After chymotrypsin cleavage:

LC-MS (gradient 20-80%, 5 min): absorbance at 0-499 nm.



Exact Mass: 1621,7916

11

UV-Vis spectrum of compound 11:



MS(ESI) spectrum of compound 11:



12

UV-Vis spectrum of compound 12:



MS (ESI) spectrum of compound 12:



After trypsin cleavage:

LC-MS (gradient 20-80%, 5 min): absorbance at 0-499 nm.



UV-Vis spectrum of compound 13:







Exact Mass: 1836,9135



UV-Vis spectrum of compound 14:





After simultaneous cleavage with chymotrypsin and trypsin:

LC-MS (gradient 5-60%, 5 min): absorbance at 0-499 nm.



UV-Vis spectrum of compound **15**:



MS (ESI) spectrum of compound 15:





Figure S1. a) UV-Vis spectrum of the probe; b) Fluorescence excitation spectrum of the probe, $\lambda em = 590$ nm. All data were obtained in Tris-HCl buffer solution (pH 8) at 37 °C at a probe concentration of 2 μ M.



Figure S2. Substrate-dependent rate change fitted to Michaelis-Menten curve for a) chymotrypsin ($0.5 \ \mu g/mL$) and b) trypsin ($4 \ ng/mL$).



Figure S3. Photostability study of the probe. All data were obtained from continuous excitation at 435 nm in Tris-HCl buffer solution (pH 8) at 37 °C.



Figure S4. Dependence of fluorescence response of the probe (1 μ M) on different concentrations of a) chymotrypsin and b) trypsin. Excitation wavelength: 435 nm. dF indicates the rate of F_{475}/F_{520} change, where F_{475} and F_{520} are the emission intensities at 475 nm and 520 nm, respectively. c) and d) present calibration curves zoomed in at low concentrations for chymotrypsin and trypsin respectively.

Procedure for simultaneous proteases detection

a) The probe is incubated with an enzyme in various concentrations for reasonable time (parameters are determined from k_{cat}/κ_{M} value). The values for X and Y (X=X₀/X_t; Y = Y₀/Y_t) are calculated and plotted to the graph (Figure 4). The limit points A and B are defined.

For our system the following characterization is valid: Point A achieved via cleavage by trypsin in concentration 80 ng/ml within 20 minutes: X = 0.3; Y = 10 (Table S1) Point B achieved via cleavage by chymotrypsin in concentration of 4 µg/ml within 20 minutes: X = 3.5; Y = 1.4 (Table S1)

b) The mixture of both enzymes (in concentrations corresponding to limit points A and B) is applied for incubation. The values X and Y are monitored within the time till they get constant. These values define the point C (Figure 4), which corresponds to the full cleavage of both linkers.

Our system was incubated with the mixture of chymotrypsin (4 μ g/mL) and trypsin (80 ng/mL). The cleavage of both linkers was completed in 30 min, affording final constant indexes $X = X_0/X_t = 1.7$ and $Y = Y_0/Y_t = 3.0$ characteristic for point C (Table S2, item 1).

c) The limits of X and Y for unambiguous detection of both enzymes are set up

For our system the following characterization is valid When X > 1, chymotrypsin is present in the mixture (see Figure 4) When Y > 1.4, trypsin is present in the mixture (see Figure 4) When X > 1 and Y > 1.4, both enzymes are surely present in the mixture (see Figure 4)

d) The concentration detection limits are defined by following procedure: Both enzymes in low concentrations are incubated with the probe. The fluorescence is monitored within the time till the X and Y limits (see paragraph c) are reached. The concentrations can be enhanced to get shorter time or the time can be prolonged to get lower detection limits.

For our system the following characterization is valid: Cleavage with enzyme mixtures at concentrations of 0.05 μ g/ml (chymotrypsin) and 0.5 ng/ml (trypsin) was studied first. After 110 min of incubation, it fitted the assay condition X > 1 and Y > 1.4, corresponding to the mixture of enzymes (Table S2, item 2). When a combination of two times higher concentrations was used (Table S2, item 3), the assay condition was fulfilled in 30 min. We preferred shorter time for assays, so the latter combination was chosen as the detection limits.

e) The assay time limit is set up by following procedure: the combination of the lowest concentration of one enzyme and the highest concentration of the other enzyme is used for incubation with the probe. The fluorescence is monitored within the time till the X and Y limits (see paragraph c) are achieved.

For our system the following characterization is valid: In the case of excess chymotrypsin (Table S2, item 4) the assay condition X > 1and Y > 1.4 was fulfilled after 60 min of incubation, and in the case of excess trypsin after 120 min of incubation (Table S2, item 5).

The longer time obtained from paragraphs d and e is chosen as the relevant.

Fig. S5 represents a graphical visualization of the obtained results. The curves 1-3 represent the already discussed situations of trypsin, chymotrypsin, and the

enzyme mixture. The combination of low enzyme concentrations (curve 4) interferes with the chymotrypsin area at the beginning of incubation and reaches the red area after 30 min of incubation. When the highest concentration of trypsin (80 ng/mL) was used with the lowest concentration of chymotrypsin (DL = 0.1 $\mu g/mL$), the curve significantly interferes with the trypsin curves at the beginning of incubation. However, it turns to the red area in 120 min (Fig. S5, curve 5). We presume that any other combination of protease concentrations (when trypsin is fixed at 80 ng/mL and chymotrypsin > DL) should take less than 120 min, since the cleavage of both linkers would be faster. In the case of a mixture of excess chymotrypsin (4 μ g/mL) over trypsin (DL = 1 ng/mL), the curve also follows the trend of the pure enzyme at the beginning of incubation, but reaches the red area after approximately 60 min of incubation (Fig. S5, curve 6). Again, in the case of the other combination of concentrations (when chymotrypsin fixed at $4 \mu g/mL$ and trypsin > DL), the cleavage should proceed faster and take less than 60 min. The time limit for the simultaneous assay was thus found to be 120 min and the detection limits are 0.1 µg/mL and 1 ng/mL for chymotrypsin and trypsin, respectively.

	Chymotrypsin			Trypsin		
Item	concentration, µg/mL	X	Y	concentration, ng/mL	X	Y
1	0.05	1.4	1.0	0.5	0.9	1.0
2	0.1	1.5	1.1	1	0.7	1.5
3	0.2	1.9	1.1	2	0.6	2.2
4	0.5	2.7	1.2	4	0.5	3.0
5	1	3.3	1.2	10 0.		4.9
6	2	3.5	1.3	20 0.		6.7
7	4	3.5	1.4	40	0.3	9.6
8	8	3.5	1.4	80	0.3	10
9	-	-	-	160	0.3	10

Table S1 Calculated X and Y indexes for various concentrations of chymotrypsin and trypsin used for "one enzyme" cleavage.

 $X = X_0/X_t$, where X_0 is the fluorescence intensity ratio 475/520 before incubation and X_t – after 20 min of incubation. $Y = Y_0/Y_t$, where Y_0 is the fluorescence intensity ratio 590/475 before incubation and Y_t – after 20 min of incubation.

Table S2 Calculated X and Y indexes for mixture of chymotrypsin and trypsin.

Item	Chymotrypsin + trypsin ($\mu g/mL + ng/mL$)	X	Y	Incubation time (t),
1	4 + 80	17	3.0	30
1	4 - 50	1.7	1.7	110
2	0.05 + 0.5	1.3	1.5	110
3	0.1 + 1	1.5	1.7	30
4	4 + 1	2.7	1.7	60
5	0.1 + 80	1.2	4.0	120

 $X = X_0/X_t$, where X_0 is the fluorescence intensity ratio 475/520 before incubation and X_t – after corresponding incubation time (t). $Y = Y_0/Y_t$, where Y_0 is the fluorescence intensity ratio 590/475 before incubation and Y_t – after corresponding incubation time.



Figure S5. Dependence of X and Y indexes on the presence of proteases.

List of abbreviations

- DBU 1,8-Diazabicyclo[5.4.0]undec-7-ene
- DCE Dichloroethane
- DEAC 7-diethylaminocoumarin-3-carboxylic acid
- DIC N,N'-Diisopropylcarbodiimide
- DIAD Diisopropyl azodicarboxylate
- DMAP 4-Dimethylaminopyridine
- FRET Förster resonance energy transfer
- HFIP Hexafluoroisopropanol
- HOBt Hydroxybenzotriazole
- Mtt 4-Methyltrityl
- NMP N-Methyl-2-pyrrolidone
- NsCl 4-Nitrobenzenesulfonyl chloride



- TES triethylsilane
- TFA trifluoroacetic acid
- TFE trifluoroethanol

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