Supporting Information

A semisynthetic fluorescent protein assembly-based FRET probe for real-time profiling of cell membrane protease functions in situ †

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Experimental Procedures

1. Reagents and instruments

The peptide P (RDHMVLHESVNAAGIT- (GGSGGS)-RVRRSVK-TAMRA) used in present study was synthesized and purified by KareBayBiochem (Zhejiang, China). Furin and its inhibitor decanoyl-Arg-Val -Lys-Arg-chloromethylketone (Dec-RVKR-cmk) were purchased from New England Biolabs (Beijing, China) and Cayman (Michigan, USA), respectively. Furin (B-6), which is a mouse monoclonal antibody raised against amino acids 575-794 of furin of human origin, was purchased from Santa Cruz (California, USA). Phospholipids with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissaminerhodamine B sulfonyl) (PE-RhB) was purchased from Avanti Polar Lipids (Alabama, USA). 3-(4,5-dimethylthiazol-2-yl)-2-diphenyltetrazolium bromide (MTT) and phosphate buffer saline were purchased from KeyGen Biotech (Nanjing, China). Ultrapure water with an electric resistance of 18.2 MΩ was obtained from a Millipore system(Milli-Q).

The UV-vis absorption spectra were recorded on a UV-vis spectrophotometer (Agilent). Fluorescence measurements were performed on Photon Technology International (PTI) QM4 fluorescence spectrophotometer (Hitachi). Flow cytometries were determined using a flow cytometer (Becton Dickinson) and imaging was performed under a confocal laser scanning microscope (CLSM) (Nikon).

2. Plasmid construction, protein expression and purification

The gene MIP-GFP1-10 and MIP-GFP were amplified by PCR using Q5 High-
Fidelity DNA Polymerase and customized specific primers, and inserted into pET28a via Nde I/Hind III restriction sites. Then the constructs pET28a-MIP-GFP1-10 and pET28a-MIP-GFP were transformed into *E. coli* BL21 (DE3) by heat shock, respectively. Cells were incubated in LB medium at 37 °C for 3 h until an OD₆₀₀ of 0.5-0.7 was reached, and then were induced with 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 17 °C for 24 h. Cells were harvested by centrifugation and re-suspended in buffer containing 50 mM Tris (pH 7.4), 2 M NaCl, and 40 mM imidazole, and lysed by sonication on ice. Protein was purified by Ni-NTA agarose chromatography (ÄKTA, GE) and then the buffer was exchanged into 10 mM Tris (pH 7.4), 100 mM NaCl, and 5 % glycerol by desalination chromatography (ÄKTA, GE). Expression level and purity of these proteins were investigated using SDS-PAGE. The purified MIP-GFP was quantified by absorbance at 488 nm with an extinction coefficient of 8.33 × 10⁴ M⁻¹ cm⁻¹, and MIP-GFP1-10 was qualified by BCA Protein Assay Kit, and the purified proteins were stored at -80 °C.

3. Assembly between the GFP fragments

We evaluated the assembly between the purified MIP-GFP1-10 and GFP11 in solution. Different concentrations of GFP11 fragment (0 to 12 μM) was added to 2 μM of MIP-GFP1-10 in PBS buffer (pH 7.4), and incubated at room temperature overnight. The fluorescence spectrum was measured using the fluorescence spectrophotometer.

For preparation of the sFPAP probes, MIP-GFP1-10 and P were assembled in
PBS buffer under the optimized conditions (MIP-GFP1-10: P =1:5) and incubated for overnight at room temperature. After that, the excess P was washed away through centrifuging the complex of MIP-GFP1-10/P at 10000 rpm for 15 min three times with an ultrafiltration tube (10K). Then the sFPAP probe was obtained.

4. Detection of furin activity in buffer

The sFPAP probe (10 μM) was co-incubated with furin (0-0.02 U/μL) at 37 °C for 2 h in 10 μL of the assay buffer (100 mM HEPES, pH 7.4, 0.5 % Triton X-100, 1 mM CaCl₂, and 1 mM 2-mercaptoethanol). The emission spectrum was measured by the fluorescence spectrophotometer with the excitation wavelength of 470 nm.

5. Cell Culture

Human neuronal glioblastoma U251 cells and human colon adenocarcinoma Lovo cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and F-12K Medium respectively. Both media contained 10 % fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured in an incubator (Thermo Scientific) at 37 °C under an atmosphere of 5 % CO₂ and 90 % relative humidity. The cells were regularly sub-cultured approximately every 3 days using Trypsin-ethylenediaminetetraacetic acid (EDTA) at a split ratio of 1:4.

6. Western blotting

U251 and Lovo Cells were lysed with the lysis buffer (RIPA buffer with 1×Complete protease inhibitor cocktail (Roche)). Lysates were boiled at 98 °C for 5 min and analyzed by SDS–PAGE and standard immunoblotting. The primary antibody (anti-furin ab3467, Abcam, Cambridge, UK,) and the secondary antibody of
Goat anti-Mouse IgG (H&L)-HRP (Bioworld Technology Inc. Shanghai, China) were diluted 1:500 and 1:2000 in PBS with 5 % BSA, respectively. Immunoreactive bands were detected by chemiluminescence using a gel imaging system (MicroChemi 4.2).

7. Cytotoxicity assay

In a 96-well plate, 100 μL of U251 or Lovo cells-containing solution with the cell density of \( \approx 2 \times 10^4 \) cells mL\(^{-1} \) was added to each well and incubated at 37 °C for 24 h. Then, different concentrations of the sFPAP probe were added into the cell-containing plate and incubated for another 12 h. After being washed with PBS, the cells were incubated with 20 μL MTT aqueous solution (0.5 mg mL\(^{-1} \)) for 4 h. Then, 150 μL of dimethyl sulphoxide (DMSO) was added to lyse cell membrane followed by stirring for 20 min. The optical absorbance at 490 nm of the final solution in each well was measured using a microplate reader (Biotek). Cell viability was defined as the percentage of survival cells in the probe treated wells to that in untreated control wells.

8. Detection of furin activity in cells lysates

U251 and Lovo cells were lysed with RIPA buffer. Total protein amounts of the cell lysates were quantified by BCA Protein Assay Kit according to its standard procedure. Then the cell lysate containing 1.0 mg mL\(^{-1} \) total protein was incubated with sFPAP (10 μM). For the enzyme inhibition experiment, the U251 cells lysate was preincubated with the inhibitor Dec-RVKR-cmk (50 μM) for 30 min before the addition of sFPAP. Then the fluorescence intensities at 507 nm and 580 nm were measured immediately every 2 min.
9. Anchoring the sFPAP probe on cell membrane, and imaging and Flow cytometric analysis

Fixed U251 cells (1×10⁴) were incubated with different concentrations of MIP-GFP for different times in DMEM (free FBS) at 37 °C. Then cells were washed with PBS (containing 0.1 mg mL⁻¹ heparin) to remove the free MIP-GFP and analyzed through CLSM. Flow cytometry was performed on a FACScan cytometer by counting 1×10⁴ events. To illustrate the performance of the membrane insertion peptide MIP, 10 µM MIP-GFP or GFP were incubated with U251 cells at 37 °C for 90 min. Cells were washed with PBS (containing 0.1 mg mL⁻¹ heparin) three times and digested with trypsin. Then, the cells were subjected to flow cytometry and the data were processed with FlowJo software.

10. Imaging of cell-surface furin activity in living cells

U251 and Lovo cells (1×10⁴ cells/well) seeded in confocal dishes (Greiner Bio-One) were incubated with the sFPAP probe (30 µM) or the probe together with the inhibitor Dec-RVKR-cmk (50 µM) at 37 °C for 90 min. Next, cells were washed three times with PBS (containing 0.1 mg mL⁻¹ heparin) and imaging was performed under CLSM.

For real-time detection of furin activity on living cell surface, the sFPAP probe (30 µM) was incubated with U251 cells at 37 °C about 7 min, and subsequently the unbound probe was washed away by PBS (containing 0.1 mg mL⁻¹ heparin). Then living cell imaging was performed under CLSM.
11. Calculation of FRET efficiency

The 3D structure of the sFPAP probe was predicted by the software of Rosetta Floppy Tail based on the 3D structure of GFP (crystal structure has been resolved). We utilized the following equation\(^1,2\) to calculate the experimental fluorescence energy transfer efficiency (E):

\[
R = 36.3\text{Å}
\]

\[
J(\lambda) = \int_{0}^{\infty} F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda
\]

\[
R_0 = 9.78 \times 10^3 [k^2 \eta^4 \Phi_{GFP} J(\lambda)]^{1/6}
\]

\[
E = \frac{1}{1 + (R/R_0)^6}
\]

where \(R\) is the distance between donor and acceptor molecules. In this study, it was simulated using the software of PyMOL. \(J(\lambda)\) (in unit of \(\text{M}^{-1}\text{cm}^{1}\text{nm}^{4}\)) is the degree of spectral overlap between donor fluorescence spectrum and acceptor absorption spectrum, \(F_D\) is the emission spectrum of donor, \(\epsilon_A\) is the molar absorption coefficient of acceptor, \(R_0\) is the Förster distance, \(k^2\) is the dipole orientation factor (assumed to be 2/3 based on random orientation distribution of donor and acceptor molecules), \(\Phi_{GFP}\) is the quantum yield of donor fluorescence (without acceptor), and \(\eta\) is the refractive index of the intervening medium.

12. Steady-state kinetic study

Steady-state kinetic assay was carried out at 37 °C in a 96-well plate. The sFPAP probe (10 \(\mu\text{M}\)) as a special substrate for furin was added into 60 \(\mu\text{L}\) of furin buffer (100 mM HEPES, pH 7.4, 0.5 % Triton X-100, 1 mM \(\text{CaCl}_2\), and 1 mM 2-mercaptoethanol.), and then 0.006 U/\(\mu\text{L}\) furin was added. Kinetic measurement was
conducted immediately by monitoring the fluorescence intensity change both at 507 nm and 580 nm on a microplate reader (Cytation5, BioTek, USA).

13. Supplementary table and figures

Table S1. Theoretical parameters of the sFPAP probe.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>J (M⁻¹cm⁻¹nm⁴)</th>
<th>R₀ (Å)</th>
<th>E_max (%)</th>
<th>R (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semisynthetic GFP</td>
<td>TAMRA</td>
<td>1.27×10¹⁵</td>
<td>44.5</td>
<td>77.5</td>
<td>36.3</td>
</tr>
</tbody>
</table>

Fig. S1. 3D structure of the reassembled GFP.
Fig. S2. Excitation and emission spectra of TAMRA (in red) and GFP (in green), respectively.

Fig. S3. Complementation of MIP-GFP1-10 and GFP11 detected by the fluorescence recovery, where the concentration of MIP-GFP1-10 was fixed to 2 μM and GFP11 concentration changed from 0 to 12 μM (left). The bar graph is the fluorescent changes at 507 nm with the different ratio of MIP-GFP 1-10: GFP11 (right).
Fig. S4. Fluorescence kinetics of the sFPAP probe (10 μM) after addition of furin (final concentration is 0.006 U/μL).

Fig. S5. The changes of $F_{\text{GFP}}/F_{\text{TAMRA}}$ with the different concentrations of furin, corresponding to Fig. 1C.
**Fig. S6.** The bar graph of fluorescent changes of the sFPAP probe to various kinds of proteins or enzymes, including 0.7 nM sortase A (SrtA), bovine albumin (BSA), alkaline phosphatase (ALP), thrombin, matrix metalloproteinase-2 (MMP-2), and glucose oxidase (GOX), and 0.07 nM (0.001 U/μL) furin.

**Fig. S7.** Colocalization coefficient between GFP (with/without MIP) and PE-RhB on U251 cell membranes.
**Fig. S8.** CLSM images of U251 cells incubated with 20 μM MIP-GFP at 37 °C for different time spans (left) and the ratio of corresponding fluorescence signal between the cell membranes (F_{membrane}) and the whole cells (F_{total}) (right). scale bar: 20 μm.

**Fig. S9.** Images of U251 cells incubated with MIP-GFP1-10 (25 μM) at 37 °C for 1 h, washed with PBS, and then incubated with GFP11 for another 2 h at room temperature. U251 cells incubated with the MIP-GFP1-10 alone as the control.
**Fig. S10.** CLSM images of U251 cells incubated with different concentrations of MIP-GFP for 30 min at 37 °C (left). The corresponding fluorescence signal ratio between the cell membranes ($F_{\text{membrane}}$) and the whole cells ($F_{\text{total}}$) (right). scale bar: 20 μm.

**Fig. S11.** Cytotoxicity of different concentrations of the sFPAP probe on U251 and Lovo cells.

**Fig. S12.** Time-lapse imaging of U251 cells after 7 min incubation with the sFPAP probe and subsequent washing procedure. The cell labeled in the yellow box are used in Fig 4A. scale bar: 50 μm.
Fig. S13. Time-lapse imaging of different cells after 7 min incubation with the sFPAP probe and subsequent washing procedure. (A) Lovo cells incubated with 30 μM sFPAP. (B) U251 cells were incubated with 30 μM sFPAP and 50 μM furin inhibitor. Scale bar: 50 μm.
14. References
