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2018

Cheong, H., Kim, J., Mu, J., Zhang, W., Li, J., Yang, H., & Xing, B. (2019).
Spatiotemporal-controlled reporter for cell-surface proteolytic enzyme activity
visualization. *ChemBioChem*, 20(4), 561-567. doi:10.1002/cbic.201800445

<https://hdl.handle.net/10356/143223>

<https://doi.org/10.1002/cbic.201800445>

This is the accepted version of the following article: Cheong, H., Kim, J., Mu, J., Zhang, W., Li, J., Yang, H., & Xing, B. (2019). Spatiotemporal-controlled reporter for cell-surface proteolytic enzyme activity visualization. *ChemBioChem*, 20(4), 561-567. doi:10.1002/cbic.201800445, which has been published in final form at <http://doi.org.remotexs.ntu.edu.sg/10.1002/cbic.201800445>. This article may be used for non-commercial purposes in accordance with the Wiley Self-Archiving Policy [<https://authorservices.wiley.com/authorresources/Journal-Authors/licensing/self-archiving.html>].

Spatiotemporal-controlled Reporter for Cell Surface Proteolytic Enzyme Activity Visualization

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Abstract: Live-cell imaging of cell surface-associated proteolytic enzymes is crucial in understanding of their biological roles and functions in both physiological and pathological processes. However, the complexity of the cell membrane environment increases the difficulties to specifically investigate targeted proteolytic activities within the microenvironment. Towards this end, we designed and synthesized a unique photo-removable, furin-responsive peptide probe (PFP) that can undergo spatio-temporal control via UV light illumination to aid in visualizing of the cell surface-associated protease enzyme, furin, activity in live-cells. Prior to light irradiation, the photo-labile moiety, 4,5-dimethoxy-2-nitrobenzyl (DMND), in the peptide sequence of the reporter will block furin-like enzymatic hydrolysis, thus no fluorescence will be observed. Upon simple light illumination, photolysis will occur, thereby revealing the un-caged peptide probe, which can undergo enzyme hydrolysis and leads to an increase in fluorescence signal, which allows a real-time imaging of endogenous cell surface-associated furin-like enzyme function in living cells through a precise spatial and temporal resolution.

Introduction

Cell membrane is a complex biological entity that consists of various phospholipids, sterols and transmembrane proteins, which separate the organelles from the outside environment. The membrane serves as gatekeepers that protect the integrity of the cells and controls the different molecules from entering and leaving.^[1] This unique regulation of biomolecules plays critical roles in various physiological and pathological pathways.^[2] In addition, the fluidity of the membrane compartment is closely related to a range of biological processes and diseases, which further complicates the environment.^[3] Hence, in order to monitor the dynamic profiles of the membrane structures and further understand their fundamental functions, unique and effective strategies capable of undergoing spatiotemporal visualization of the biological processes embedded in membrane structures, and importantly, precise monitoring of their dynamics occurring in the microenvironment will be highly desirable.

Cell surface proteolytic enzymes, also called proteases, are a special class of enzymes that play critical roles in various

biological functions ranging from cell regulation, signal transduction to protein processing.^[4] These enzymes can catalyze site-specific cleavage of proteins or peptide substrates to either activate or inactivate the protein activities, which will cause significant changes in cellular function. Additionally, the proteolytic events are also involved in many physiological and pathological processes such as extracellular matrix processing, activation of cell receptors or growth factors.^[4b, 5] Furthermore, any abnormal proteolysis will implicate the regulation of bioactive proteins and contribute to the progression of various degenerative diseases including cancer, osteoarthritis and neurological disorders.^[4b, 5-6] Therefore, the capability to study the regulation of proteolysis, especially for proteolytic enzyme localized on the cell surface, will help to better understand the pathological pathways and provide valuable insights for new diagnostic or treatment of cancer and other diseases.^[7] Moreover, some proteolytic enzymes demonstrate very unique biological behavior that is difficult to be monitored through the conventional sensing approaches. For example, intermittent enzymatic activities can be observed due to cellular event such as mitosis for protein kinases.^[8] Hence, having the capability to spatiotemporally control the sensing processes can further help in elucidating the biological functions of the proteolytic enzymes *in vitro* and *in vivo*.^[9]

Notably, furin, a member of the proproteins convertase (PC) family, which is ubiquitously expressed in cellular membrane with unique functions centralized in the secretory and endocytic pathway compartments, has gathered much interests in recent years.^[10] These furin-like convertases activate an array of proproteins within the intracellular membrane and cell surface into their bioactive forms, which perform a wide variety of biological processes. In addition, furin is also involved in many pathological pathways like processing bacterial and viral pathogens.^[10a, 11] Considering the close association of furin in many pathophysiology pathways, development of effective strategies to quantify and visualize furin activities is of great importance.^[12] Likewise, considering the complexity of biological environment, spatial or temporal control of the proteolysis activities will allow the unique opportunities in visualizing cellular events at defined time point or locations, which will enhance the in-depth elucidation on the functions and facilitate better understanding of furin-dependent dynamics in living cells. Particularly, one most notable and appealing method to achieve such spatiotemporal precision in living system is through the usage of photoactivable or "caged" technique, by which a beam of light irradiation can readily photolyze or modulate the activation of the biological molecules.^[13] So far, the photo-caged strategy has been established for real-time imaging of cellular dynamics, accurately monitoring of complex biological processes and specific labeling protein of interests for better understanding the intrinsic functional mechanism in biological systems.^[14]

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Scheme 1. The structure of photo-caged furin-responsive probe (PFP) and the corresponding cleavage of the probe by furin on the cell membrane after UV light irradiation. F=fluorescein (green structures); Q=Dabcyl quencher molecule (black sphere). The dashed line denotes the cleavage site on the peptide sequence; the purple line denotes the lipid anchor; the blue chemical structure denotes the photo-labile moiety.

In line with these promising advantages, herein, we present a photo-labile peptide-based fluorescent reporter that can effectively undergo spatiotemporal control and specifically visualize furin-like enzyme activities on cell surface. Such well-designed probe includes a lipid anchor and a photo-removable furin-responsive peptide probe (PFP) that can efficiently localize on the lipid compartment of the cell membrane and thus provide great opportunities to conduct imaging of extracellular proteolytic enzyme in living cells. Typically, a unique peptide sequence containing R-(X)-(R/K)-R↓ is preferentially for furin recognition and will undergo specific furin cleavage.^[15] Based on this principle, the fluorescent reporter was designed using furin cleavable peptide sequence, RSRRSVK, conjugated with a pair of dyes, fluorescein (FITC) a fluorophore and Dabcyl (4-(dimethyl-amino)azobenzene-4-carboxylic acid) a quencher, at their respective terminals that will undergo Förster Resonance Energy Transfer (FRET). The combination allows the reporter to image proteolytic activity when the peptide probe undergoes enzyme cleavage and thus separates the FRET pair. Additionally, a fatty acid lipid moiety was added on the PFP to efficiently target and immobilize the fluorescent reporter on the cell membrane.^[16] More importantly, a distinctive class of photo-labile protecting group, 4, 5-dimethoxy-2-nitrobenzyl (DMND), was further functionalized on the peptide probe to prevent the catalytic domain of furin from recognizing the peptide sequence.^[17] When PFP probe was illuminated with UV light, the reporter will undergo photolysis process to remove the photo-labile moiety and thus reveal the furin cleavable peptide sequence (As shown in Scheme 1), which enables enzyme hydrolysis to split the FRET between FITC and Dabcyl, thus allowing the enhancement in green fluorescent signal for real-time imaging of furin-like activity in living cells.

Results and Discussion

Generally, the photo-labile moiety was functionalized on the amino acid via the synthetic route shown in Scheme 2 and S1 which was further linked to the peptide sequence, RSRRSVK, of the PFP probe via standard solid phase peptide synthesis.^[18] The FRET pair was conjugated on the peptide reporter through typical coupling reaction, and further purified by reverse-phase high-performance liquid chromatography (HPLC) and characterized by liquid chromatography-mass spectrometry (LC-MS, shown in Scheme 2).

Figure 1. a) HPLC profile of the photo-caged probe based on the UV irradiation by UV lamp emitting at 365 nm at 65 mW with different time intervals. b) Conversion of photo-caged probe based on UV irradiation.

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After the preparation of the PFP probe, we first examined the conversion efficiency of the caged peptide probe triggered by light irradiation through HPLC analysis. The photolysis of the caged reporter was conducted in phosphate-buffered saline (PBS) buffer, treated with UV light illumination and the un-caging process was monitored through HPLC analysis. As shown in Figure 1a, the fluorescent reporter can be effectively un-caged to the active peptide probe upon exposure to illumination at different time durations. The un-caged probe was further quantified by comparing the normalized HPLC peak areas with their pure reference compounds (Figure 1b). The results demonstrated the effective time-dependent light cleavage and the conversion to the un-caged probe is directly proportionate to the illumination time with a maximum conversion of approximately 80%.

Encouraged by the promising photo-controllable properties, we investigated the effectiveness of the PFP probe to undergo furin hydrolysis. The enzyme hydrolysis of the probe was performed in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer while the fluorescence change was monitored upon treatment with furin and light irradiation at 365 nm. As shown in Figure 2a and S1c, there was no fluorescence observed in the PFP probe, mainly due to the efficient FRET between FITC and its corresponding quencher Dabcyl. In addition, the DMND moiety does not quench the fluorescence of FITC (Figure S2b). After the peptide reporter was incubated with furin enzyme for 1 hour, the limited fluorescence signal was observed. However, when the probe sequence was exposed to light illumination and followed by 1 hour incubation with the furin enzyme, a significant fluorescence enhancement (approximately 37-folds) was detected at a maximum wavelength of $\lambda_{em} = 520$ nm. These results suggested that the photo-caged PFP probe could efficiently block the furin hydrolysis before external light illumination. In addition, the enzyme hydrolysis with standard furin inhibitor (Dec-RVRR-Cmk)^[19] and light irradiation demonstrated a minimal fluorescence change even after prolong incubation, clearly indicating that furin-specific hydrolysis occurred to cleave the FRET probe and release the quencher, therefore caused the recovery of the fluorescence.

To further investigate the photo-controlled properties of PFP probe, we conducted time-dependent *in vitro* fluorescence measurement on the enzyme activities. As shown in Figure 2b, there was no observable fluorescence change found until the PFP probe was irradiated with light at the stipulated time-point. These results corresponded to the ability of the peptide reporter to undergo spatiotemporal control in detecting the enzymatic activities, which prove the possibility of using the PFP probe to conduct furin-specific enzyme imaging through external light stimulation.

Figure 2. a) Fluorescence enhancement of PFP (6 μ M; in 100mM HEPES buffer, pH 7.4) incubated with furin in the presence of UV irradiation at different time intervals (0 – 60 min, $\lambda_{ex} = 470$ nm). PFP incubated with inhibitor or in the absence of UV irradiation were measured after incubated with furin for 60 min. b) Time-dependent fluorescence change before and after light illumination. The photo-caged peptide probe was incubated with furin at 37°C and irradiated with UV light at different time duration, 10 (A), 20 (B), or 30 (C) min indicated by black arrows.

Inspired by the result of PFP probe responding to furin enzyme recognition in the buffer solution, we examined the applicability of the fluorescent reporter towards furin-dependent cell surface imaging. In this study, human mammary gland/breast MDA-MB-468 cells were used as targeted cell lines mainly due to their high level of furin expression,^[20] while furin-deficient human colorectal adenocarcinoma LoVo cells were used as negative control.^[21] Firstly, we investigated the effect of PFP probe through a live-cell assay. As shown in Figure 3, a considerable fluorescence enhancement was measured for MDA-MB-468 cells when exposed to light illumination, whereas a substantially low fluorescence signal was observed under the same condition in LoVo cells as well as when MDA-MB-468 cells was not illuminated. Moreover, the proteolysis activities were considerably suppressed with the addition of furin inhibitor. The promising results indicate that PFP probe can serve as a reliable fluorescence reporter, which can undergo spatio-temporal control by light illumination to monitor furin-like activities in the complex biological environments. Furthermore, we evaluate the potential cytotoxicity of PFP probe and the external light illumination by using a stand MTT assay (Figure S3). There was no obvious cytotoxicity caused by the PFP probe and the external light illumination to both MDA-MB-468 and LoVo cells, indicating that the PFP probe enables spatiotemporally

controlled and real-time visualization of furin activity without affecting the enzyme physiological processes.

Figure 3. Illustration of the fluorescence intensity change of PFP (2 μ M) with time to quantify the furin activity in MDA-MB-468 and LoVo cells at 37 $^{\circ}$ C. The inhibitor Dec-RVKR-cmk (50 μ M) was added to MDA-MB-468 cell for 30 minutes prior to addition of the probe. The specified probes were irradiation with UV light for 10 min.

Another significant feature for the PFP probe is the ability to visualize the proteolysis activities specifically on the cell surface. To this end, investigation was conducted to confirm the ability of the hydrophobic lipid chain to assist in localizing the fluorescent reporter within the cells membrane.^[12b-12d, 12f] Generally, MDA-MB-468 and LoVo cells were incubated with PFP probe for 1 hour during the process of live-cell fluorescence imaging. In this experiment, the cells were incubated with PFP probe for 1 hour after light illumination. As contrast, the cell membranes were also tracked with the commercially available stain, CellMask Deep Red dye,^[22] to monitor the localization of PFP probe within the cells. As shown in Figure 4, after 1 hour incubation, a strong green fluorescence signal was detected on the surface of MDA-MB-468 cells, whereas no fluorescence signal was observed for LoVo cells. The results demonstrated that PFP probe is mainly localized on the cell membrane, thus exhibiting the capability of PFP probe in imaging of cell surface-associated proteolytic activities.

Figure 4. Fluorescence imaging of MDA-MB-468 cells and LoVo cells using PFP probe (200 nM) irradiated with light irradiation for 10 min. Standard CellMask Deep Red (2.5 μ g/mL) was used to image the membrane. Scale bar in all images = 20 μ m.

To demonstrate the ability of the peptide probe to image the cellular dynamics of furin enzyme, the time-lapse fluorescence imaging was conducted to visualize cell surface proteolytic activity. As shown in Figure 5, a green fluorescence signal was observed when PFP probe was illuminated and incubated with MDA-MB-468 cells. Additionally, a significant increase in the fluorescence intensity was monitored with longer incubation time. As a control, the minimum fluorescence signal was observed for furin-deficient LoVo cells under similar conditions and when MDA-MB-468 cells was not exposed to light illumination. The promising result displayed the ability of PFP probe to visualize furin-like enzymatic activity through observing the changes in fluorescence signal, which can also be used for real-time monitoring of furin activity. In addition, the precise control of the light irradiation at different area was also performed in live cells incubated with PFP probe. The visualization of furin-like activity could be achieved at the area where it exposed to light irradiation, whereas, no signal was observed for the area without light exposure (as shown in Figure S10). These indicates that PFP probe is an efficient fluorescent reporter that can undergo spatial and temporal control in conducting real-time visualization of cell surface-associated furin activities in living cells, which can be used to monitor the proteolytic enzyme dynamics, and helped to understand the roles of furin enzyme in various pathophysiology pathways.

Figure 5. Fluorescence imaging of MDA-MB-468 and LoVo cells using the PFP probes. The cells were incubated with PFP (200 nM) in the presence or absence of UV light illumination for 10 min. Fluorescence intensity was plotted as a function of distance (d) along the arrows using ImageJ. Scale bar of all images = 50 μ m.

Conclusions

In conclusion, a membrane-localizing photo-labile furin activity probe, PFP, was developed to effectively conduct spatiotemporal control in visualizing furin-like activity on the cell surface. In this study, the probe was carefully designed with photo-removable moiety to efficiently control the timing to

release the active reporter to monitor furin activity through external light stimulation. Upon the release of the active fluorescent reporter, a significant fluorescent enhancement can be observed due to the furin-like enzyme hydrolysis, which further demonstrates the potential of using PFP probe for conducting spatiotemporal controlled live-cell imaging of surface localized furin activities. Furthermore, the developed peptide probe can assist in monitoring the enzyme dynamics located at the cellular membrane, which can thus provide valuable new insights to the various pathological pathways and facilitate the discovery of new theranostic strategies for furin-related diseases. In addition, the rational design of a photo-labile reporter can also be used as a general method to develop new approaches for spatiotemporal controlled visualization of other proteolytic enzymes, which is useful in elucidating their functions and biological roles.

Experimental Section

Scheme 2. Synthesis route for photo-caged furin activity probe (PFP) after SPPS. a) Dabcy SE/DIPEA in DMF, 2h; b) 95% TFA, 2.5% H₂O, 2.5% TIPS, 2h.

Synthesis of compound (1) The peptide sequence was synthesized by Fmoc-based solid phase peptide synthesis (SPPS) on a 2-chlorotrityl chloride resin.^[18] The coupling reaction for each amino acid lasted 2–4 hours (4 hours for Arg(Pbf), Ser(DMND) and 2 hours for other amino acids) at room temperature in the presence of HBTU (1.5 eq) as a

coupling reagent and N, N-Diisopropylethylamine (DIPEA) (1.5 eq) as a basic catalyst. The deprotection of Fmoc group was performed with 20 % piperidine in Dimethylformamide (DMF) for 20 min. The Dde protecting group was removed in 20% hydrazine in DMF for 20 min. Fluorescein isothiocyanate (FITC) (~1.5 eq) and DIPEA (1.5 eq) in DMF was added and reacted for 2 h to afford **1**. The product was cleaved from the resin using 2% trifluoroacetic acid (TFA) in CH₂Cl₂ for 10 min. After concentrating the product in *vacuo*, Et₂O was added to obtain the precipitate. The precipitate was purified by HPLC with the eluting system consists of A (water with 0.1% TFA) and B (acetonitrile with 0.1% TFA) under a linear gradient monitored by UV-Vis absorbance at 450 nm. The linear gradient stretched over 25 min from t=0 min at 30% of solution B to t=15 min at 100% solution B with flow rate of 3.0 mL/min. Compound **1** retention time was 17.5 min. ESI-MS: m/z calcd: 1355.67 [M+2H]⁺; found: 1355.13 [M+2H]⁺.

Synthesis of compound (2) To a solution of **1** in DMF was added Dabcy SE (1 eq) and DIPEA (1 eq) and stirred for 4 h at r.t. The mixture was purified by HPLC with the eluting system consists of A (water with 0.1% TFA) and B (acetonitrile with 0.1% TFA) under a linear gradient monitored by UV-Vis absorbance at 500 nm. The linear gradient stretched over 25 min from t=0 min at 30% of solution B to t=15 min at 100% solution B with flow rate of 3.0 mL/min. The retention time of Compound **2** was 20.5 min. ESI-MS: m/z calcd: 1481.31 [M+2H]⁺; found: 1482.69 [M+2H]⁺.

Synthesis of Photo-caged Furin Activity Probe (PFP) (3) The Pbf and Boc protection groups were cleaved by 95% TFA, 2.5% H₂O and 2.5% triisopropylsilane (TIPS) for 2 h. The final product was purified by HPLC monitored by UV absorbance of 500 nm. The linear gradient stretches over 32 min from t=0 min at 30% of solution B to t=20 min at 90% solution B with a flow rate of 3.0 mL/min. Compound **3** retention time was 15.5 min. ESI-MS: m/z calcd: 1075.77 [M+2H]⁺; found: 1074.60 [M+2H]⁺.

PFP conversion to un-caged probe: The caged PFP (50 μL, 60 μM) was placed in a 100 μL cuvette (pathlength = 1 cm) before the sample was irradiated with a portable UV lamp for 0, 5, 10, 15 or 20 min. The light source was measure with a real light power output (emitting 365 nm at 21 mW/cm²) by an optical power meter to adjust the distance of light source to sample. The aliquot was removed and analyzed by reverse-phase HPLC (The linear gradient stretches over 20 min from t=0 min at 30% of solution B to t=20 min at 90% solution B with a flow rate of 1.0 mL/min.). PFP and its un-caged analogue was quantified by their absorbance at 450 nm and normalized to the HPLC peak areas of the pure compounds. Each experiment was repeated three times to obtain an average value.

General methods for cell culture, cell assays and live-cell imaging. Human mammary gland/breast MDA-MB-468 cells (from ATCC) grown in Dulbecco's modified eagle medium (DMEM, from Gibco) supplemented with 10 % fetal bovine serum (FBS, from HyClone) at 5% CO₂ and 37°C. Human colorectal adenocarcinoma LoVo cells (from ATCC) were grown in Kaighn's Modification of Ham's F-12 medium (F-12K, from Gibco) with 10% FBS.

Time-dependent change in fluorescence before and after irradiation of PFP. The PFP probe was incubated with pure furin enzyme at 37 °C in 100 mM HEPES, pH 7.4, in 96-well plates. Tecan's Infinite M200 microplate reader was used to measure the changes in fluorescence at 520 nm for 10, 20, or 30 min. The plate was then removed and irradiated with a portable UV lamp emitting 365 nm at 21 mW/cm² for 5 min. The samples were then re-analyzed for the changes in fluorescence intensity as a function of time. Each experiment was repeated three times and the average values were used for analysis.

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In vitro furin activity analysis in cells. MDA-MB-468 Cells and LoVo cells were seeded in 96-wells plates with a density of 5×10^4 cells per well. After 24 hour incubation at 5 % CO₂ and 37°C, the medium was replaced with fresh medium containing 2 μ M PFP or probe together with the inhibitor Dec-RVKR-cmk (5 μ M). The indicated sample's wells were exposed to UV light irradiation for 5 min before measurement. The changes in fluorescence intensity were measured as a function of time, each experiment was repeated three times and the average values were used for analysis.

Imaging and quantification of furin-like activity in live-cells. MDA-MB-468 and LoVo cells were seeded at 35 mm diameter μ -dish plastic bottom (ibidi GmbH, Germany) in complete medium for 24 h incubation at 5% CO₂ and 37°C. The medium was subsequently replaced with fresh medium with PFP probe (200 nM) and the indicated samples were irradiated with UV light (365 nm) at 21mW/cm² for 10 min. Thereafter, the cells were incubated at 5% CO₂ and 37°C, live-cell imaging was then conduct at defined time-point. For co-localisation imaging, cells were incubated with CellMask Deep Red (2.5 μ g/mL) for 5 min and washed three times with PBS before conducting imaging. Live-cell imaging was performed under confocal laser scanning microscope (Carl Zeiss LSM800). (PFP: λ_{ex} = 488 nm, λ_{em} = 525/25 nm; CellMask Deep Red: λ_{ex} = 640 nm, λ_{em} = 678/22 nm).

Acknowledgements

This work was partially supported by NTU-AIT-MUV NAM/16001, RG110/16 (S), (RG11/13) and (RG35/15), NTU-JSPS JRP grant (M4082175.110), Merlion program (M4082162.110) awarded in the Nanyang Technological University, Singapore and the National Natural Science Foundation of China (NSFC) (No. 51628201)

Keywords: photo-caged • proteolytic enzyme • biosensor • FRET • peptides

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Schematic showing the release of the furin cleavable fluorescence reporter upon light illumination

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Page No. – Page No.

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