Development of small-molecule reporters for fluorescence imaging of enzymes function and application

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**NANYANG
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SINGAPORE

**DEVELOPMENT OF SMALL-MOLECULE REPORTERS
FOR FLUORESCENCE IMAGING OF ENZYMES
FUNCTION AND APPLICATION**

CHEONG HAOLUN

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2020

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FUNCTION AND APPLICATION**

CHEONG HAOLUN

SCHOOL OF PHYSICAL AND MATHEMATICAL
SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Doctor of Philosophy

2020

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Chapter 2 is published as H. Cheong, J. Kim, J. Mu, W. Zhang, J. Li, H. Yang, B. Xing, Spatiotemporal-Controlled Reporter for Cell-Surface Proteolytic Enzyme Activity Visualization. *ChemBioChem*, 20(4), 561-567 (2019). DOI: 10.1002/cbic.201800445

The contributions of the co-authors are as follows:

- A/Prof Xing Bengang provided the initial project direction and revised the manuscript drafts.
- The manuscript was revised by Dr Zhang Wenmin, A/Prof Li Juan and Prof Yang Huanghao.
- Dr Mu Jing co-designed the initial project design.
- Kim Jisu, a final year fyp student, assisted in the peptide synthesise and the collection of the HPLC data.

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Abstract

Much attention has been placed in understanding the various enzymes activities due to their vital roles in mammalian and bacterial cells, which is closely associated with the progression of many pathological conditions such as tumor proliferation, bacterial infection and neurological disorder. Therefore, developing a selective and sensitive platform to detect and monitor enzyme activities is essential in elucidating the biological functions and crucial for early diagnostic and treatments of diseases.

Among the various techniques developed to monitor enzyme function, optical imaging, which utilize light to obtain the image of the cellular function in the living cells has been widely exploited to detect and visualize enzyme activity in living cells. Its widespread use is mainly due to its ease of operation, high sensitivity, low cost and high-throughput capabilities. Hence, in this thesis, I had utilized the advantages of optical imaging to design various new novel and sensitive fluorescent reporter capable of visualizing selected enzyme, furin and β -lactamase that have similar catalytic activities, at the target cellular compartment.

Furin is located at different organelle and each location contributed to different biological roles in the pathological condition. Therefore, the specific detection of enzyme activity at targeted compartment helps to elucidate furin biological function. Thus in chapter 2, a light-activatable fluorescent reporter was constructed to control the visualization of furin activities on the cellular surface. The peptide-based fluorescent reporter was conjugated with a fatty acid moiety and a photo-removable moiety so that it can efficiently immobilize on the lipid compartment of the cellular membrane. Moreover, the photo-removable moiety enables spatiotemporal control of the fluorescent reporter through external light illumination to control the time and location to conduct imaging of endogenous

proteolytic enzymes in the living cells.

Furthermore, as furin involvement in the pathological pathway differs at different compartment, specific detection of furin at Golgi apparatus is also important for in-depth analysis of furin biological roles. Hence in chapter 3, I had designed a peptide-based fluorescent reporter to localized on the Golgi apparatus to conduct real-time visualization of furin activities located at Golgi. The probe was a conjugation of Golgi targeting sphingosine moiety and a furin-responsive peptide probe to efficiently localize on the Golgi apparatus in order to specifically conduct imaging of proteolytic enzyme in the living cells.

Moreover, furin is closely associated with the progression of various cancers including non-small cell lung carcinomas, head and neck squamous cell carcinomas, and breast cancer. Therefore, in chapter 4, we developed a reporter capable to specifically target tumor cells for visualization and other potential biomedical applications such as disease treatment. We utilized a peptide-caged metabolic precursor that can be activated by furin-like proteolytic activities on selected cells. Upon triggered by furin, the unnatural glycan will be released and will undergo intrinsic metabolism by the cells to exogenously expressed the azide (N_3^-) groups on the cell surfaces, which can be used for visualization or subsequent therapeutic option.

Similarly, in bacterial cells, enzymes also play vital roles in various biological functions. Of interests, β -lactamases activity is a serious medical concern as it promotes the bacteria resistance against the conventional antibiotic drugs. Therefore, in order to better understand the enzyme functions and combat against drug resistance, we demonstrated the use of metabolic-mediated fluorescent reporter to specifically detect and visualize class C β -lactamases enzyme in resistant bacteria for *in vivo* application in chapter 5. A bulky DBCO group was conjugated to the 7' position of the cephalosporin β -lactam ring on the fluorescent

reporter, which can be used for subsequent labeling on the bacterial cell surface and will also acts as a steric hindrance to improve the selectivity toward class C β -lactamases.

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Table of Contents

Abstract	1
Acknowledgements	4
Table of Contents	5
Abbreviation	8
 Chapter 1 Introduction	
1.1 A mammalian cells enzyme, protease	11
1.1.1 Furin, a serine protease	13
1.1.2 Biological roles of furin in diseases	15
1.1.2.1 Furin involvement in infectious diseases	16
1.1.2.2 Furin involvement in cancer	18
1.2 A bacterial cells enzyme, Beta-lactamase (β -lactamase)	19
1.2.1 Biological actions of β -lactamase	20
1.3 Molecular imaging technique to visualize enzyme activity	22
1.3.1 Application of fluorescence imaging to detect furin activity	23
1.3.2 Application of fluorescence imaging to detect β -lactamase	26
1.4 Research objectives	29
1.5 References	31
 Chapter 2 Spatiotemporal-controlled Reporter for Cell Surface Proteolytic Enzyme Activity Visualization	
2.1 Introduction	37
2.2 Spatiotemporal-controllable probe design	39
2.3 Result and discussion	41

2.4	Conclusion	50
2.5	Experimental details	51
2.6	References	58

Chapter 3 Small-Molecule Reporter for Real-Time Visualization of Golgi Apparatus Proteolytic Enzyme Activity

3.1	Introduction	63
3.2	Golgi apparatus probe design	65
3.3	Result and Discussion	67
3.4	Conclusion and future work	71
3.5	Experimental details	72
3.6	References	76

Chapter 4 Proteolytic Enzyme Based Metabolic Labeling for Tumor-Specific Fluorescence Imaging

4.1	Introduction	78
4.2	Metabolic precursor design	79
4.3	Result and discussion	80
4.4	Conclusion and future work	85
4.5	Experimental details	86
4.6	References	89

Chapter 5 Metabolic-Mediated Localization of Fluorescent Reporter on Pathogenic Bacteria

5.1	Introduction	91
5.2	Fluorescent reporter design	93
5.3	Result and Discussion	94
5.4	Conclusion and future work	101

5.5	Experimental details	101
5.6	References	109
Chapter 6	Summary and Perspectives	112
	List of Publications	116

Abbreviation

δ	chemical shift
λ	wavelength
Å	angstrom
β	beta
ACLE	7-Amino-3-chloromethyl-3- cephem-4-carboxylic acid p-methoxybenzyl ester
BHI	brain heart infusion
Boc	<i>t</i> -butoxycarbonyl
Calcd	calculated
Cat.	Catalytic
Cmk	chloromethylketone
d	doublet
Dabcyl	(4-(dimethyl-amino)azobenzene-4-carboxylic acid)
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCM	dichloromethane
dd	doublet of doublet
ddd	doublet of doublet of doublet
Dde	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl
Dec	decanoic acid
DIPEA	<i>N, N</i> -diisopropylethylamine
DMEM	dulbecco's modified eagle medium
DMF	dimethylformamide
DMND	4, 5-dimethoxy-2-nitrobenzyl
EA	ethyl acetate
EDC	<i>N</i> -Ethyl- <i>N'</i> -(3-dimethylaminopropyl)carbodiimide
em	emission

eq	equivalent
ER	endoplasmic reticulum
ESI	electrospray ionization
EA	ethyl acetate
ex	excitation
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Fmoc	fluorenylmethyloxycarbonyl
FRET	förster resonance energy transfer
g	gram
h	hour
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5b]pyridineium 3-oxid hexafluorophosphate
HBTU	<i>O</i> -(benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
Hex	hexane
HOBt	1-hydroxybenzotriazole monohydrate
HPLC	high-performance liquid chromatography
Hz	hertz
<i>J</i>	coupling constant
LSM	laser scanning microscopy
M	molarity (concentration)
m	multiplet
MeOH	methanol
mL	millilitres
min	minutes
mmol	millimoles

MS	mass spectroscopy
Mtt	4-methyltrityl
NHS	<i>N</i> -Hydroxy-succinimide
nm	nanometers
NMM	<i>N</i> -methylmorpholine
NMO	<i>N</i> -methylmorpholine <i>N</i> -oxide
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PC	proprotein convertase
r.t	room temperature
s	singlet
SPPS	solid-phased peptide synthesis
t	triplet
td	triplet of doublet
TEA	triethylamine
TFA	trifluoroacetic acid
TIPS	triisopropylsilane
UV	ultraviolet
UV-Vis	ultraviolet-visible spectroscopy
mW	milliwatts

Chapter 1

Introduction

Enzymes are an important class of macromolecules consisting of a long chain of amino acids residues, which are extensively found in both eukaryotic and prokaryotic cells. They play fundamental and critical roles in numerous cellular and biological processes.^[1] For example, enzymes like proteases or beta-lactamases hydrolysis large proteins or molecules into smaller fragments, which regulate a wide array of functions including protein digestion, activation of protein functions and signaling of cellular processes. However, any abnormalities or malfunction of enzymes activities can cause dysfunction in biological pathways that may lead to the progression of diseases like inflammation, osteoarthritis and cancer, etc. Hence, the elucidation of enzyme function and the development of methodologies that can specifically monitor enzyme activities are crucial in understanding the biological functions, which can provide new insight in developing a novel design for early diagnostic or therapeutic application.^[2, 3] Therefore, in this thesis, I focused my study on furin and beta-lactamases, which has similar enzyme activities and is found in mammalian and bacterial cells respectively.

1.1 A mammalian cells enzyme, proteases

Proteases, also known as proteolytic enzymes, catalyze the hydrolysis of large proteins or peptides substrates thus releasing their bioactive entities, which will influential many physiological and pathological processes including blood coagulation, inflammation, cell proliferation, and cancer progression.^[4-6] In general, these mammalian proteases can be classified into threonine, serine,

cysteine, metalloproteases and aspartic proteases based on their catalytic mechanism in hydrolyzing the substrate. The substrates bind to the proteases active site through hydrogen binding, hydrophobic and electrostatic interaction, which will leads to the hydrolysis of the amide bond. Typically, N-terminal threonine, serine and cysteine proteases will undergo covalent based catalysis by engaging in nucleophilic attack by the active site side chain. In contrast, metalloproteases and aspartic protease proceeded by non-covalent catalysis through the generation of activated water molecules show in Figure 1.1.^[7]

Figure 1.1. Illustration on the catalytic mechanism of proteases. a) Interaction of protease substrates (P and P' residues) with protease catalytic pocket (S and S' pockets). The dashed line denotes a scissile site. (b-f) Catalytic mechanisms of five different types of proteases.^[7]

Among these five classes of proteases, serine proteases has one of the largest

families of proteolytic enzymes, which constituted more than one-third of all known proteases.^[8] Serine proteases hold critical roles in physiological processes within our body that includes digestion, immune response and blood coagulation.^[9] Conversely, any abnormalities of the proteases activities can lead to serious issues such as tumor proliferation, invasion and metastasis.^[6]

Notably, furin, a type serine protease, had gathered much attention in recent years due to its close association with many pathological processes such as viral infection, inflammation and cancer. Therefore, the ability to monitor and study furin can aid in understanding their involvement in disease development better, which can assist in developing new therapeutic targets or design for biomedical application.

1.1.1 Furin, a serine protease

A family of subtilisin-like serine proteases, Proprotein Convertases (PC) is responsible for the activation of pathogenic precursor proteins such as growth factors and their receptors, polypeptide hormones, and various proteins such as viral and bacterial pathogen. The family consisted of nine members namely proprotein convertase 1 (PC1), PC2, furin, PC4, PC5, paired basic amino acid cleaving enzyme 4 (PACE4) PC7, subtilisin kexin isozyme 1 (known as SKI-1 or S1P) and proproteins convertase subtilisin kexin 9 (PCSK9).^[10] Among these nine convertase members, many efforts had been focused on furin due to their pivotal roles in diseases progression.

Furin, a membrane-localized enzyme, is ubiquitously expressed within the endocytic, secretory pathways and at the cellular surface. It functions by site-specific cleavage of proproteins into their bioactive form, which will perform various biological functions. Some of the known substrates that will undergo furin cleavage include precursors of extracellular matrix proteins, receptors, hormones and even bacterial toxin.^[11]

Due to its large substrates scope and involvement in many pathological processes, understanding the cleavage site motif will help better understand the mechanism of furin associated diseases and crucial for the rational design of various reporters to monitor and control the proteolytic activity. Based on the statistical data of furin cleavage sites, a sequence consisted of 20 amino acids for furin cleavage site was revealed. The motif comprises of an 8 amino acids core region at P6 – P2' position and two polar regions at P7 – P14 and P3' – P6' position. A summary of the properties required for each position for furin recognition is shown in Figure 1.2.^[12]

Figure 1.2. Summary of the physical properties of the 20 amino acid residues for furin recognition.^[12]

At P1' position, a small hydrophilic residue such as serine is preferred due to the small hydrophilic pocket found in the furin binding pocket. Any bulky or hydrophobic residues like leucine, isoleucine, valine or tryptophan at P1' position was found to inhibit furin cleavage. Whereas P2' position favors hydrophobic valine residue.

Remarkably, all of the biological active substrates contained positively charged arginine residue at the P1 position. Furthermore, studies had shown that any mutation of arginine (Arg) at P1 led to an undetectable furin cleavage, which

indicates the importance of arginine at the P1 position. For P2 position, a positively charged arginine and lysine (Lys) residue were most commonly found in the active substrates. Besides arginine and lysine, small flexible residue such as glycine, alanine, serine and threonine were also favored.

P4 position has similar property as P1 position, where most of the residues comprised of positively charged arginine. Aliphatic residues like valine, leucine and isoleucine are also acceptable at the P4 position. In such cases, a positive charge residue is needed at P5 or P6 position to compensate for the lack of positive charge arginine at the P4 position. Lastly, a 3D study on the interaction of furin binding pocket with the residues at P1 – P4 position had shown that the side chain of the residue at P3 position is oriented outward, which does not form any specific interaction with the binding pocket. Based on these principles, a majority of the substrate screening revealed that furin has a preferential cleavage sequence consisting of Arg-X-[Arg/Lys]-Arg↓, where X indicates any amino acids.^[13]

1.1.2 Biological roles of furin in diseases

As furin is able to activate a large number of substrates into their bioactive forms, it is known to play crucial roles in many pathological processes and also associated with the progression of many degenerative diseases like neurological disorder and cancers.^[14, 15] As mention earlier, furin is ubiquitously expressed in different compartment within the cells, which process different substrates based on their location. Like the early endosome, furin activates bacterial toxins including Shiga toxin, diphtheria toxin and *Pseudomonas* exotoxin A. Whereas the cell surface furin cleaves other bacterial toxins like anthrax toxin, aerolysin toxin and *Clostridium septicum* α-toxin as shown in Figure 1.3.^[16]

Figure 1.3. Furin processing at different compartments of the cell includes cell surface, trans-Golgi network and endosomal system.^[11]

1.1.2.1 Furin involvement in infectious diseases

Studies had shown the pivotal roles of furin in activating various bacterial and viral pathogens. Generally, cell-surface furin active the toxin to enable the formation of pores by the toxin in the cell membrane. An example, anthrax toxin consists of three nontoxic proteins: protective antigen (PA), edema factor (EF) and lethal factor (LF). The PA secreted by *Bacillus anthracis* bacterium will bind to the cellular receptor and fragmentized by cell-surface furin proteolytic action. The receptor-bounded fragments were heptameric to form pore precursor (prepore) that can bind up to three factors (LF and/or EF), which the combinations of these factors form the toxic complexes. The complex is subsequently internalized into the host-cell cytoplasm, which results in edema, systemic shock and death (Figure 1.4).^[17-19] Hence, with the absence of furin activity will avert the assembly of the toxin, thus preventing the lethality.^[20]

Figure 1.4. Illustration on the action of anthrax at cellular level.^[19]

In other cases of bacteria toxins, the toxins undergo endocytosis upon receptor binding. Inside the early endosomal compartment, the toxins were processed by furin in the acidic pH environment. The cleavage also enables the translocation of the toxins into the cytosol, which the toxic factors can inhibit the translation process (Figure 1.5).

Figure 1.5. Illustration on the action of SLT toxin at cellular level.^[21]

1.1.2.2 Furin involvement in cancer

Several studies had indicated an increased in the expression level of furin in many different cancers such as head and neck squamous cell carcinomas, breast cancer and non-small cell lung carcinomas.^[22-25] These overexpressed furin will increase the activation of membrane type-1 matrix metalloproteinase (MT1-MMP), which is vital and enhances the tumor metastasis and invasion. Additionally, the matured MT1-MMP further activates the extracellular pro-MMP-2, which can encourage metastasis and tumor growth shown in Figure 1.6. These indicate a close correlation between the expression level of furin and malignancy of tumor. Furthermore, the use of furin inhibitors prevents the activation of MT1-MMP, which will subsequently inhibit MMP-2 activation and leads to a reduction in tumor metastasis.^[26-28] Likewise, Insulin-like growth factor-1 (IGF1), another furin substrate, is also upregulated in cancers such as colon, prostate and lung cancers. IGF1 processing is catalyzed by furin, and inhibition of furin also leads to a reduction in tumor development.

Figure 1.6. Furin processing at different compartments of the cell includes cell surface, trans-Golgi network and endosomal system.^[11]

Given the closely associated involvement of furin in many pathological conditions, it is important to the development of a new and effective strategy to detect, visualize and monitor furin activities in real-time that will allow

elucidation on the functions and dynamics of furin in the living cells. Moreover, the novel reporter design can also provide new insights on diagnostic and effective therapeutic options.

Similar to the hydrolysis activity caused by furin in the mammalian cells, enzyme hydrolysis found in bacterial cells is also a serious medical concern. Notably, β -lactamase is an enzyme secreted by resistant-bacteria, which catalyzed the hydrolysis of β -lactam-based drugs had gathered much attention in the efforts to combat infectious diseases.

1.2 A bacterial cell enzyme, Beta-lactamase (β -lactamase)

Like in the mammalian cells, enzymes are also extensively found within the bacterial cells, which plays critical roles in many biological functions including colonization, proliferation and evasion of host immune defenses, *etc.*^[29] With the vital involvement of enzymes in pathological conditions, it is important to be able to study and monitor enzyme activities in the living system for early detection and treatment of diseases. Especially, there is a growing interest in the detection and monitoring of β -lactamase enzymes due to its active involvement in resisting the conventional antibiotic treatment for infectious diseases.

Since the discovery of the first antibiotic, penicillin, in the early 1900s by Alexander Flemming, antibiotic is widely used in the treatment and prevention of infectious diseases.^[30] Typically, the antibiotic consisted of a β -lactam ring made of a 4-member ring chemical structure capable of mimicking the precursor of D-alanyl-D-alanine, which is essential for cell wall synthesis.

Figure 1.7. Structure of common β -lactam based antibiotics.

During cell wall synthesis, penicillin-binding protein (PBP) catalyzed the cross-linking of two D-alanyl-D-alanine glycosidic units to form the rigid cell wall (Figure 1.8). The formation of this rigid cell wall is important in maintaining the bacterial structure within hypertonic and hostile environment of the human body. The antibiotic works by replacing the D-alanyl-D-alanine substrate during the acylation of PBP, which inhibit the cross-linking reaction. The bacterial cell wall will be compromised with the increased in permeability leading to the cell lysis and antimicrobial effect.^[31, 32]

Figure 1.8. The cross-linking of the tetrapeptide for bacterial cell wall formation.^[33]

With the widespread use of antibiotics for medical treatment, bacteria started to develop resistance against conventional antibiotics with increasing cases each year. One of the primary causes of resistance in bacteria is through the expression of drug-resistant enzymes β -lactamase that will specifically hydrolyze β -lactam based antibiotic rendering it ineffective for antibacterial treatment.^[32, 34-36] Hence, the emergent of antibiotic resistances from pathogenic bacteria is currently a major medical concern and may lead to serious consequences with the reduced treatment efficacy against resistant bacteria.^[36-38]

1.2.1 Biological actions of β -lactamase

β -lactamase enzymes can be classified into four different classes (A, B, C and D) based on its catalytic mechanism and its unique amino acid sequences.^[39, 40] Generally, the catalytic mechanism is classified into two families, serine β -

lactamase consisting class A, C, and D enzyme whereas metallo- β -lactamase comprised of only class B enzyme.

Serine β -lactamase has serine at its active site, which will undergo nucleophilic attack that will open the β -lactam ring and become self-acylated intermediate. These actions prevented the β -lactam based antibiotic from inhibiting the PBP as the drug is hydrolyzed by the β -lactamase, thus rendering it ineffective. Meanwhile, the metallo- β -lactamase has a binuclear zinc cluster at its active site, which hydrolyzes the β -lactam ring through non-covalent reaction. This β -lactamase utilize the zinc at its active site to activate a water molecule, which will indirectly catalyze the ring-opening of the β -lactam drug (Figure 1.9).^[41, 42]

Figure 1.9. Typical mechanism for the hydrolysis of the β -lactam drug by serine and metallo- β -lactamases^[42]

With the rapid spread of β -lactam antibiotic resistant bacteria occurring in the human community may potentially cause an increase in death rate caused by this pathogenic bacteria.^[43] Furthermore, bacteria will continuously be mutating and evolving to fight against β -lactam drugs, which render the current antimicrobial treatment ineffective.^[44-46] Hence, there is a urgency to develop novel strategies to accurately detect and diagnose these bacteria. This development will aid in elucidating the biological process and inspire new concepts for bacterial infection treatments.

Currently, there are various methods developed to measure and monitor enzyme activity like immunoassays, molecular imaging and ion-selective electrodes, *etc.*^[47, 48] Among these methods, molecular imaging has been well established and extensively used to conduct non-invasive imaging of biological functions in living cells.

1.3 Molecular imaging technique to visualize enzyme activity

It is widely known that molecular imaging is a powerful technique, which can be used to monitor important biomolecules such as carbohydrates, proteins, lipids and other metabolites, as well as real-time visualization of biological processes and cellular functions of interest in the complex living systems. Currently, there is a wide selection of imaging modalities and technologies established to elucidate the structure and biological functions, detection and characterization of various diseases, and evaluating the treatment efficacy. Such modalities include x-ray computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), optical imaging and ultrasound (Figure 1.10).^[49-53]

Between the different imaging modalities available, optical imaging has been widely used in real-time visualization of enzyme activities and non-invasive monitoring of cellular events *in vitro* and *in vivo*. Such extensive application of optical imaging is mainly due to the ease of use, high sensitivity, low cost and high-throughput capabilities.^[54-56] Because of these advantages, many kinds of fluorescence imaging probes have been developed to detect furin and β -lactamase activities, which aid in better understanding the biological function of enzymes.

Figure 1.10. Imaging modalities used for oncology^[51]

1.3.1 Application of fluorescence imaging to detect furin activity

One conventional method to detect furin activity is through the use of a bio-activatable fluorescent probe. As highlighted earlier, furin will catalyze the hydrolysis of proteins or peptide substrates. Therefore, using a furin recognizable peptide sequence, a bio-activatable fluorescent reporter can be designed to monitor furin activity. Typically, the reporter will undergo cleavage in the presence of furin activity, resulting in a change in the molecular conformation of the reporter. These changes will lead to a generation or conversion of the fluorescence signal, which can be subsequently used to visualize the furin activity. In 2009, Rao's group had presented a non-invasive bioluminescence probe capable of detecting furin activity *in vivo* (Figure 1.11). In this study, they conjugated a luciferase substrate D-aminoluciferin on a furin cleavable peptide sequence, RVRR. Upon hydrolysis by furin enzyme, D-aminoluciferin will be released, which will produce bioluminescence emission in the presence of firefly luciferase.^[57]

Figure 1.11. a) Schematic of bioluminescence probe for detecting furin activity. b) Bioluminescence imaging of the xenografted tumors in nude mice.^[57]

In 2011, Rao and co-workers further proposed the use of enzyme-controlled cyclization to image furin activity. This concept was derived from the condensation reaction between 2-cyanobenzothiazole (CBT) and free cysteine. As shown in Figure 1.12, upon activation by furin and glutathione (GSH), cysteine will be released for intramolecular cyclization. Thereafter, the cyclized probe will undergo self-assembly in the cells leading to an enhancement in fluorescence emission.^[58]

Figure 1.12. a) Illustration of furin-triggered cyclization. b) Confocal imaging of furin activity and localization in live cells.^[58]

Recently, Liang's group rationally designed a new fluorescent reporter utilizing the furin-triggered CBT-cysteine system. In this study, they include an aggregation-induced emission (AIE) molecule into the probe design. As shown in Figure 1.13a, upon furin and GSH hydrolysis, the cleaved probe will condense to form a dimer where the "dual AIE" enhanced the fluorescence emission and used for visualizing furin activity. This enhancement of fluorescence emission was further validated when in comparison with a single AIE fragmented control probe in Figure 1.13b.^[59]

Figure 1.13. a) Illustration of furin-triggered dual AIE probe. b) Fluorescence imaging of furin activities in live cells.^[59]

Similarly, Xing and co-worker had demonstrated the use of small molecule based Förster Resonance Energy Transfer (FRET) reporter for real-time visualization of furin activities (Figure 1.14). They designed a fluorescent reporter utilizing a furin recognizable sequence, RVRRSVK, conjugated with a pair of FRET dyes. Upon the presence of furin activities, the peptide undergoes hydrolysis, which separates the quencher leading to an increase in fluorescence emission. Furthermore, they were able to efficiently immobilize the reporter on the cellular membrane using a fatty acid moiety to effectively target and monitor furin activities localized on the cell membrane.^[60]

Figure 1.14. a) Illustration of furin-responsive fluorescent reporter detecting furin activity in cellular membrane. b) Fluorescence imaging of furin activities in live cells.^[60]

Furin is ubiquitously expressed within the endocytic, secretory pathways and at the cellular surface, where furin localized at different compartments have unique substrate scope contributing to different pathological conditions. Therefore, there are a large number of fluorescent reporters developed to monitor the activities of furin enzyme. However, monitoring of furin localized at specific cellular compartments remains immature till date, which is important in elucidating the biological functions of furin to inspire novel diagnostic and therapeutic option.

1.3.2 Application of fluorescence imaging to detect β -lactamase

Likewise, the conventional method to detect β -lactamase activity is through the use of a bio-activatable fluorescent reporter. As mentioned earlier, β -lactamase targets antibiotics by hydrolyzing the β -lactam ring. Based on this principle, a small molecule probe can be rationally designed to detect β -lactamase activity by causing a change in fluorescence intensity during ring-opening process. Pioneering in this field, Tsien and co-workers developed a cephalosporin-based fluorogenic substrate (CCF2/AM) to conduct fluorescent imaging of β -lactamase.^[61] The CCF2/AM substrate was first diffused through the membrane and undergo esterases hydrolysis to generate CCF2. Thereafter, in the present of

Blas, CCF2 will be hydrolyzed again to release the blue coumarin fluorescence emission.

Figure 1.15. Schematic illustration of CCF2/AM detecting Blas activity in cell culture.^[61]

Subsequently, several research groups expanded on the idea of using cephalosporin-based reporter in β -lactamase imaging. An example was demonstrated by Xing's group in 2013, where they had designed a novel fluorescent probe that utilizes the hydrolysis process of β -lactamase to covalently label on resistant bacteria for detection (Figure 1.15). In the presence of β -lactamase, the cephalosporin based probe will be hydrolyzed to release fluorescent quinine intermediate, which subsequently labeling on resistant bacteria for fluorescence imaging.^[62]

Figure 1.16. a) Illustration of the enzyme-responsive probe (LRBL1-3) undergoing the labelling process. b)

Fluorescence imaging of resistant bacteria strains *B. cereus* and MRSA as well as non-resistant bacteria *S. aureus*.^[62]

In 2014, Rao and co-workers had presented a β -lactam based probe to selectively monitor class A of β -lactamase BlaC activities (Figure 1.16). In this study, they screen a large number of substrate at 2' and 7' position of the β -lactam ring, and had identified that a cyclopropane ring residue at 2' position together with a methoxy group at 7' position can selectively detect BlaC activity. This probe enables rapid and sensitive detection of BlaC enzyme, which is essential as BlaC is involved in the highly infectious airborne disease called tuberculosis (TB).^[63]

Figure 1.17. The structure of the fluorescence reporter in detecting BlaC activity.^[63]

Recently, outbreaks of new class C β -lactamase resistant bacteria caused by the conjugative transmission of bacterial plasmid had led to an increased risk in antibiotic resistance and efforts in studying and detecting β -lactamase activity.^[64-67] Joining this effort, Li and co-workers had presented a novel probe capable of detecting class C β -lactamase, AmpC enzyme (Figure 1.17). In this study, they demonstrated the conjugation of a bulky group, methoxyimino, on the 7' position of the cephalosporin scaffold, which hinder the activity of class A β -lactamase, TEM-1.^[68]

Figure 1.18. a) Structure of the fluorescent reporter.^[68]

Even with the important roles of β -lactamase, in particular, class C β -lactamase due to the recent outbreaks, investigation on class C β -lactamase was far less extensive as compared to class A β -lactamase, even though both enzymes have similar catalytic active sites.^[69-74] Hence, there is a need to selectively detect and monitor class C β -lactamase enzyme activities, which can help to elucidate the biological process and will assist in combatting microbial resistance.

Furthermore, the established clinical techniques to detect Blas enzyme, includes the use of susceptibility tests such as double-disk synergy test and modified hodge test,^[75-78] lack the selectivity and were often time-consuming for accurate diagnosis of bacterial infections. Therefore, it is necessary to develop a new efficient and selectively strategy in detecting resistant bacteria for *in vitro* and *in vivo* application.

1.4 Research objectives

Enzymes play essential roles in many physiopathological processes in both mammalian and bacterial cells. Thus, is it important to be capable to detect and visualize the activities of the enzymes for a further in-depth understanding of the biological function, which can inspire new approaches for diagnostic and treatment for diseases. Furthermore, challenges remain in the ability to

specifically target and monitor enzymes of interests within the complex biological environment in the living system. Therefore, our group dedicated our research focus on the detection and visualization of enzymes in both mammalian and bacterial cells. In this dissertation, I will present various novel small-molecule based fluorescent reporters design to specifically target and visualize enzymes of interest in both mammalian and bacterial cells, namely furin and β -lactamase owing to their similar catalytic mechanism in hydrolyzing substrates and its importance in pathological conditions.

Furin is located at different organelle and each location contributed to different biological roles in the pathological condition. Therefore, the specific detection of enzyme activity at targeted compartment helps to elucidate furin biological function. Thus in chapter 2, a light-activatable fluorescent reporter was constructed to control the visualization of furin activities on the cellular surface. The peptide-based fluorescent reporter was conjugated with a fatty acid moiety and a photo-removable moiety so that it can efficiently immobilize on the lipid compartment of the cellular membrane. Moreover, the photo-removable moiety enables spatiotemporal control of the fluorescent reporter through external light illumination to control the time and location to conduct imaging of endogenous proteolytic enzymes in the living cells.

Furthermore, as furin involvement in the pathological pathway differs at different compartment, specific detection of furin at Golgi apparatus is also important for in-depth analysis of furin biological roles. Hence in chapter 3, I had designed a peptide-based fluorescent reporter to localized on the Golgi apparatus to conduct real-time visualization of furin activities located at Golgi. The probe was a conjugation of Golgi targeting sphingosine moiety and a furin-responsive peptide probe to efficiently localize on the Golgi apparatus in order to specifically conduct imaging of proteolytic enzyme in the living cells.

Moreover, furin is closely associated with the progression of various cancers including non-small cell lung carcinomas, head and neck squamous cell carcinomas, and breast cancer. Therefore, in chapter 4, we developed a reporter capable to specifically target tumor cells for visualization and other potential biomedical applications such as disease treatment. We utilized a peptide-caged metabolic precursor that can be activated by furin-like proteolytic activities on selected cells. Upon triggered by furin, the unnatural glycan will be released and will undergo intrinsic metabolism by the cells to exogenously expressed the azide (N_3^-) groups on the cell surfaces, which can be used for visualization or subsequent therapeutic option.

Similarly, in bacterial cells, enzymes also play vital roles in various biological functions. Of interests, β -lactamases activity is a serious medical concern as it promotes the bacteria resistance against the conventional antibiotic drugs. Therefore, in order to better understand the enzyme functions and combat against drug resistance, we demonstrated the use of metabolic-mediated fluorescent reporter to rapidly and specifically detect and visualize class C β -lactamases enzyme in resistant bacteria for *in vivo* application in chapter 5. A bulky DBCO group was conjugated to the 7' position of the cephalosporin β -lactam ring on the fluorescent reporter, which can be used for subsequent labeling on the bacterial cell surface and also argument as a steric hindrance to will improve selectivity toward class C β -lactamases.

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Chapter 2

Spatiotemporal-controlled Reporter for Cell Surface Proteolytic Enzyme Activity

Visualization

2.1 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.^[40] This unique regulation of biomolecules plays critical roles in various physiological and pathological pathways.^[41] In addition, the fluidity of the membrane compartment is closely related to a range of biological processes and diseases, which further complicates the environment.^[42] 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.^[43] 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.^[43b, 44] 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.^[43b, 44-45] 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.^[46] 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.^[47] 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*.^[48]

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.^[7-8] 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.^[8, 12, 13b, 49] Considering the close association of furin in many pathophysiology pathways, development of effective strategies to quantify and visualize furin activities is of great importance.^[31, 34, 50] 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.^[51] 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.^[52]

2.2 Spatiotemporal controllable probe design

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.^[9, 53] 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.^[54] 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.^[55] 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 2.1), 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. The proposed photolysis and enzyme hydrolysis mechanisms for PFP probe are shown in Scheme 2.2 and 2.3.

Scheme 2.1. 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 black line denotes carbon chains; the purple line denotes the lipid anchor; the blue chemical structure denotes the photo-labile moiety.

Scheme 2.2. Proposed photolysis mechanism of PFP probe through UV light irradiation.

Scheme 2.3. Proposed enzyme hydrolysis mechanism of PFP probe after UV light irradiation.

2.3 Results and Discussion

Generally, the photo-labile moiety was functionalized on the amino acid via the synthetic route shown in Scheme 2.4 and 2.5 which was further linked to the peptide sequence, RSRRSVK, of the PFP probe via standard solid phase peptide

synthesis.^[56] 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) and spectroscopic analysis (Figure 2.1).

Scheme 2.4. Synthesis route for photo-labile amino acid, Fmoc-Ser(DMND)-OH.

Scheme 2.5. Synthesis route for photo-caged furin activity probe (PFP). a) DIPEA, 30mins in DCM; b) HBTU/DIPEA in DMF; c) 20% hydrazine in DMF, 20mins then FITC/DIPEA in DMF; d) 2% TFA in DCM; e) Dabcyl SE/DIPEA in DMF, 2h; f) 95% TFA, 2.5% H₂O, 2.5% TIPS, 2h.

Figure 2.1. a) UV absorbance spectra of Fmoc-Ser(DMND)-OH; b) UV absorbance spectra of FITC (in red) and PFP (in black); c) Fluorescence intensity of 2 μ M of FITC (in red) and PFP (in black).

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 uncaging process was monitored through HPLC analysis. As shown in Figure 2.2a, 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 2.2b). 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%.

Figure 2.2. a) HPLC profile of the photo-caged probe based the UV irradiation by UV lamp emitting at 365 nm at 21mW/cm² with different time intervals. b) Conversion of photo-caged probe based on UV irradiation.

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 2.1c and 2.3a, there was no fluorescence observed in the PFP probe, mainly due to the efficient FRET between FITC and its corresponding quencher Dabcyl. 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-RVKR-Cmk (decanoyl-Arg-Val-Lys-Arg-chloromethylketone)^[57], 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.

Figure 2.3. 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.

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 2.3b, there was no observable fluorescence change until the PFP probe was irradiated with light at the stipulated time-point. In addition, we examined the enzyme kinetics of the caged and un-caged PFP probe to furin hydrolysis by presented in Figure 2.4 and Table 2.1. From the Michaelis-Menten plot, the enzyme catalytic efficiency was calculated and it was found that the un-caged probe is 15 times more efficient in undergoing enzyme hydrolysis. 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 2.4. Michaelis-Menten plot of the hydrolysis of 3, 6, 15, 30 and 60 μM of PFP probes in the presence and absence of UV irradiation by 0.759 nM furin at 37°C in 100 mM of HEPES buffer.

	K_M [μM]	K_{cat} [s^{-1}]	K_{cat}/K_M [$M^{-1}s^{-1}$]	K_{cat}/K_M Relative	R^2
Caged Probe	119.9	13.35	1.11×10^5	1	0.9401
Un-caged Probe	18.97	32.46	1.71×10^6	15.37	0.9269

Table 2.1. Kinetic properties of PFP probe by Furin (Data from Figure 2.4).

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,^[16a, 58] while furin-deficient human colorectal adenocarcinoma LoVo cells were used as negative control.^[59] Firstly, we investigated the effect of PFP probe through a live-cell assay. As shown in Figure 2.5, 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.

Figure 2.5. 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°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.

Furthermore, we evaluate the potential cytotoxicity of PFP probe and the external light illumination by using a standard *in vitro* toxicology assay (Figure 2.6). 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 2.6. a) Cytotoxicity for different concentrations of PFP with MDA-MB-468 and Lovo cell lines. b) Cytotoxicity for different UV irradiation timing with MDA-MB-468 and Lovo cell lines.

Another significance 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.^[31, 34, 50b, 50d] 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,^[60] to monitor the localization of PFP probe within the cells. As shown in Figure 2.7, 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 2.7. 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 $\mu\text{g/mL}$) was used to image the membrane. Scale bar in all images = 20 μm .

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 2.8, 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.

Figure 2.8. 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 μm .

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 (Figure 2.9).

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 2.9. Fluorescence imaging of MDA-MB-468 cells segregated by a silicone insert. In the cell culture dish (cells incubated with 600 nM of PFP probe), one side of the cells was isolated in darkness while the other side of the same culture dish simultaneously exposed to light irradiation 10 min. The imaging result was taken by confocal microscopy FITC = green fluorescence signal. Scale bar in all images is 100 μ m.

2.4 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.

2.5 Experimental details

Materials: 2-Chlorotrityl Chloride Resin and all Fmoc amino Acids were purchased from GL Biochem Ltd (Shanghai). All chemical reagents were purchased from Sigma-Aldrich. Commercially available reagents were used without further purification, unless noted otherwise. Anhydrous solvents for organic synthesis were stored over activated molecular sieves (4 Å). Furin was purchased from Biolabs (2000 Unit/mL), The definition is one unit will release 1 pmol of AMC from the fluorogenic peptide Boc-Arg-Val-Arg-Arg-7-Amino-4-methylcoumarin (Boc-RVRR-AMC) (ITS Science & Medical Pte Ltd) per minute at 30°C. The furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-Cmk) was purchased from Bachem.

Instruments: Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DPX 300 and Bruker AVIII 400 spectrophotometer (CDCl₃ as solvent). UV irradiation was done with a portable high intensity UV lamp (Analytikjena, USA). Mass spectra (ESI-MS) were measured with a Thermo LCQ Deca XP Max for ESI. Analytical reverse-phase HPLC analysis was performed on a Shimadzu HPLC system with an Alltima C-18 (250 x 10 mm) column at a flow rate of 3.0 mL/min. UV-Vis absorption spectra were recorded in a 10 mm path quartz cell on Cary 60 UV-Vis Spectrophotometer. Fluorescence emission spectra were performed on a Varian Cary eclipse fluorescence spectrometer. Confocal fluorescence imaging of cells was acquired on Carl Zeiss LSM 800 confocal laser microscope.

Synthesis and characterization of photo-labile amino acid, Fmoc-Ser(DMND)-OH

N-(9-Fluorenylmethyloxycarbonyl)-L-Serine allyl ester (1) N- α -Fmoc-L-Serine (1.64 g, 5.00 mmol) and NaHCO₃ (0.44 g, 5.23 mmol) in water (16 mL) were combined with a solution of tricaprilmethylammonium chloride [aliquot 336] (2.00g) and allyl bromide (3.20 g, 26.5 mmol) in 30 mL of CH₂Cl₂, and the suspension was vigorously stirred at r.t for 24 h. Water (50 mL) was added to the reaction mixture, and the suspension was extracted with CH₂Cl₂ (3 $\times$ 50 mL). The combined organics were dried (Na₂SO₄) and concentrated in *vacuo*. The product is purified by flash column chromatography to afford **1** as a white solid (0.96 g, 52%). ¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, *J* = 7.4 Hz, 2H), 7.59 (dd, *J* = 7.7, 3.4 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.29 (tt, *J* = 7.3, 1.3 Hz, 2H), 5.96 – 5.78 (m, 2H), 5.36 – 5.28 (m, 1H), 5.24 (ddd, *J* = 10.4, 2.6, 1.4 Hz, 1H), 4.67 (d, *J* = 5.7 Hz, 2H), 4.50 – 4.35 (m, 3H), 4.21 (t, *J* = 6.9 Hz, 1H), 3.95 – 3.89 (m, 2H), 2.50 (t, *J* = 6.0 Hz, 1H). ¹³C NMR (100MHz, CDCl₃) δ 47.31, 56.31, 63.41, 66.52, 67.41, 119.15, 120.17, 120.18, 125.27, 127.26, 127.28, 127.93, 131.52, 141.48, 141.51, 143.86, 143.99, 156.48, 170.44. ESI-MS: *m/z* calcd: 368.14[M+H]⁺; found 368.56 [M+H]⁺.

4,5-dimethoxy-2-nitrobenzyl trichloroacetimidate (2) 4,5-dimethoxy-2-nitrobenzyl alcohol (2.13 g, 10 mmol) was dissolved in 40 mL of anhydrous CH₂Cl₂ and stirred at r.t under N₂. Anhydrous K₂CO₃ (3.46 g, 25 mmol), trichloroacetonitrile (3.65 g, 17.4 mmol) and anhydrous triethylamine (TEA) (1.67mL, 12 mmol) were added to the solution and the reaction mixture stirred at r.t for 24 h. CH₂Cl₂ (60 mL) was added to the reaction mixture, and the suspension sequentially washed with 1M HCl (20 mL) and saturated NaCl (20 mL). The organic layer was dried (Na₂SO₄) and concentrated in *vacuo*. The product was purified by flash column chromatography to afford **2** as a yellow solid (1.72 g, 83%). ¹H NMR (400MHz, CDCl₃) δ 3.97 (s, 6H), 5.75 (s, 2H), 7.23 (s, 1H), 7.73

(s, 1H), 8.55 (s, 1H). ^{13}C NMR (100MHz, CDCl_3) δ 56.34, 56.37, 67.48, 91.19, 108.04, 109.16, 127.20, 139.29, 148.03, 153.73, 161.59.

N-(9-Fluorenylmethyloxycarbonyl)-O-(4,5-dimethoxy-2-nitrobenzyl)-L-Serine allyl ester (3) To a solution of **1** (0.367g, 1 mmol) in anhydrous CH_2Cl_2 (8 mL) was added **2** (0.715g, 2 mmol) and mixed under N_2 . To the mixture, triflic acid (75 μL , in 1 mL of CH_2Cl_2) was added gradually. The resulting dark green solution was left to stir at room temperature for 30 mins. After the reaction, DIPEA (40 μL) in CH_2Cl_2 (1 mL) and silica gel (100-200mg) was added to the reaction mixture. The dark brown solution was concentrated in *vacuo* and the resulting crude residue purified by flash column chromatography to afford **3** as a pale yellow solid (0.15g, 29%). ^1H NMR (400MHz, CDCl_3) δ 7.79 – 7.72 (m, 2H), 7.70 (s, 1H), 7.60 (t, J = 6.9 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.30 (td, J = 7.5, 1.3 Hz, 2H), 7.16 (s, 1H), 5.89 (ddd, J = 22.7, 10.7, 5.6 Hz, 1H), 5.72 (d, J = 8.4 Hz, 1H), 5.36 – 5.27 (m, 1H), 5.22 (dd, J = 10.5, 1.3 Hz, 1H), 4.93 (q, J = 15.4 Hz, 2H), 4.69 (d, J = 5.6 Hz, 2H), 4.67 – 4.61 (m, 1H), 4.47 (dd, J = 10.6, 7.0 Hz, 1H), 4.38 (dd, J = 10.7, 7.0 Hz, 1H), 4.26 – 4.20 (m, 1H), 4.08 (dd, J = 9.5, 3.4 Hz, 1H), 3.98 – 3.90 (m, 7H). ^{13}C NMR (100 MHz, CDCl_3) δ 47.3, 54.8, 56.6, 56.6, 66.5, 67.4, 70.4, 71.4, 108.1, 109.6, 119.0, 120.2, 125.2, 127.3, 128.0, 130.1, 131.6, 141.5, 143.8, 144.1, 147.9, 148.8, 154.1, 156.2, 170.2. ESI-MS: m/z calcd: 563.20; found: 562.92 $[\text{M}+\text{H}]^+$.

N-(9-Fluorenylmethyloxycarbonyl)-O-(4,5-dimethoxy-2-nitrobenzyl)-L-Serine (4) To a solution of **3** (0.12 g, 0.20 mmol) in CH_2Cl_2 (6 mL) containing acetic acid (54 μL) and N-methylmorpholine (1.44 mL). $\text{Pd}(\text{PPh}_3)_4$ (0.12 g, 0.10 mmol) was then added and the reaction mixture was left to stir for 4 h at r.t. 1M HCl was added to the resulting solution until pH 4 was reached. The solution was then extracted with CH_2Cl_2 (4 $\times$ 30 mL) and the combined organics were dried (Na_2SO_4) and concentrated in *vacuo*. The product was purified by flash column chromatography to afford **4** as a yellow solid (0.47g, 90%). ^1H NMR (300MHz,

CDCl₃) δ 3.78 (m, 1H), 3.84 (s, 3H), 3.88 (s, 3H), 4.08 (d, $J = 7.9$, 1H), 4.19 (t, $J = 6.7$, 1H), 4.38 (dd, $J = 10.4$ and 6.7 , 1H), 4.45 (dd, $J = 10.4$ and 6.7 , 1H), 4.62 (m, 1H), 4.86 (AB quartet, $J_{AB} = 15.2$, 2H), 5.70 (d, $J = 8.3$, 1H), 7.04 (s, 1H), 7.27 (t, $J = 7.3$, 2H), 7.36 (t, $J = 7.3$, 2H), 7.56 (m, 2H), 7.60 (s, 1H), 7.71 (d, $J = 7.3$, 2H), 8.25 (bs, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 47.0, 54.3, 56.2, 67.3, 70.2, 70.8, 107.8, 109.2, 120.0, 125.0, 127.1, 127.8, 129.5, 139.0, 141.3, 143.5, 147.6, 153.7, 156.1, 174.9.^[1] ESI-MS: m/z calcd: 523.16 [M+H]⁺; found: 522.82 [M+H]⁺.

Synthesis and characterization of PFP probe

Synthesis of peptide compound (5) The peptide sequence was synthesized by Fmoc-based solid phase peptide synthesis (SPPS) on a 2-chlorotrityl chloride resin.^[56] 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 O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (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. 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 3.0mL/min. Compound **5** retention time was 17.5 min. ESI-MS: m/z calcd: 1355.67 [M+2H]⁺; found: 1355.13

$[M+2H]^+$.

Synthesis of compound (6) To a solution of **1** in DMF was added Dabcyl 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 (ACN 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 3.0mL/min. The retention time of Compound **6** 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) (7) 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 **7** retention time was 15.5 min. ESI-MS: m/z calcd: 1075.77 $[M+2H]^+$; found: 1074.60 $[M+2H]^+$.

UV-Vis absorbance and fluorescence spectra characterization. The fluorescence spectra of FITC and PFP (6 μ M) probe were recorded in PBS (pH=7.4) buffer. The fluorescence quenching efficiency is about 99.7%, indicating the fluorescence of FITC is almost quenched by Dabcyl moiety.

PFP conversion to un-caged probe. The caged PFP (50 μ L, 60 μ M) was placed in a 100uL cuvette (pathlength = 1cm) 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 21mW/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.

Enzyme Kinetic. The PFP probes undergo photolysis under UV lamp light irradiation at 21mW/cm² for 10 min to obtain the un-caged probes. The caged and un-caged probes of different concentration (3, 6, 15, 30 and 60 µM) was incubated with pure furin enzyme at 37 °C in 100mM 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. The results were processed by Prism 7 to plot the Michaelis-Menten kinetic graph.

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 100mM 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 21mW/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.

***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 x 10⁴ cells per well. After 24 h 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.

Cell viability assay: 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 different concentrations of PFP. After 24-hour incubation, the cell viability was evaluated by *in vitro* toxicology assay kit (TOX8, resazurin based) according to standard manufacturer's protocol^[61].

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 conducted 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).

Spatial controlled imaging of furin-like activity in live-cells. MDA-MB-468 were seeded at 35 mm diameter μ -dish plastic bottom (ibidi GmbH, Germany) with a two-wells silicon insert (ibidi) in complete medium for 24 h incubation at 5% CO₂ and 37°C. The cells were incubated with the probe (600 nM) while one

of the wells was irradiated with UV light at 21mW/cm² for 10 min.

2.6 References

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Chapter 3

Peptide-Based Fluorescent Reporter for Real-Time Visualization of Golgi Apparatus Proteolytic Enzyme Activity

3.1 Introduction

Golgi apparatus is an organelle found in most cells, which functions together with the endoplasmic reticulum (ER) in the processing and transporting of macromolecules such as proteins and lipids.^[1] Golgi will receive various molecules and proteins synthesized in the ER through the cis-Golgi, which is the closest membrane of the Golgi apparatus from ER. These proteins will be transported through the apparatus to the farthest sacs from the ER, the trans-Golgi, where they will undergo various post-translational modifications by different enzymes for examples acylation, glycosylation, and methylation. Lastly, the proteins will leave the trans-Golgi and direct to their intended destination such as lysosomes or plasma membrane as shown in Figure 3.1.^[2] These transported bioactive proteins and molecules are involved in various cellular functions. Hence, any dysfunction in Golgi apparatus such as overexpression of Golgi-associated proteins can cause serious pathological disorders.^[3] Therefore, the capability to specifically study the enzyme activities at the Golgi apparatus will provide new insights in the physiopathological pathways or new diagnostic or treatments for various diseases. Thus, developing unique strategy to conduct real-time visualization enzyme activities and precise monitoring of activity only at Golgi apparatus highly desirable.

Figure 3.1. Illustration of the processing and transportation process by the Golgi apparatus in mammalian cell

Of interests, furin is one of the enzymes that are localized at the trans-Golgi network (TGN), has gathered much attention in recent years.^[4, 5] Furin is a member of the PC family that activate a range of proproteins found in the TGN into their activated protein forms, which will play crucial roles in many biological processes. Furthermore, furin is known to be involved in many pathological functions like processing and activate the bacterial and viral pathogens.^[4, 6] In view of the closely associated involvement of furin in many physiopathological pathways, inventing a new and effective strategy to monitor and visualize the proteolytic activities in real-time will allow elucidation on the functions and dynamic of furin in the living cells.^[7-10]

Thus far, there has been an extensive development in various analytical methods to proficiently monitor cellular event or chemistry through techniques such as CT, MRI, optical imaging, capillary electrophoresis, and mass spectrometry.^[11, 12] Among the various imaging techniques, optical imaging has many distinct advantages like ease of use, capable of conducting non-invasive readout, high sensitivity and high-throughput capability, which makes it an ideal and widely applied method to conduct real-time monitoring of biomolecules and enzymatic activities both *in vitro* and *in vivo*.^[13] One recent example of Golgi apparatus imaging was developed by Schepartz's group using a lipid-based

strategy to visualize Golgi apparatus structure and dynamics in live cells.^[14] Based on the commercial Golgi apparatus fluorescent stain, BODIPY-FL-ceramide, which is widely used for Golgi labeling,^[15, 16] they identified and utilized its Golgi apparatus targeting moiety, sphingosine, in their design. Moreover, they identified a near-infrared (NIR) fluorophore (SiR-Tz) that had good photostability, which is essential for super-resolution imaging. They used two steps labeling to conduct the dynamic imaging of Golgi apparatus: Firstly, they labeled the Golgi apparatus with *trans*-cyclooctene-sphingosine (Cer-TCO) before reacting with SiR-Tz via ‘tetrazine-click’ reaction shown in Figure 3.2.

Figure 3.2. Two steps procedure for Golgi labelling in cell

Currently, the developments in fluorescence imaging for Golgi apparatus mainly focus on organelle dynamic and potential biomarkers for tumor detection.^[17-21] However, specific enzymatic activities detection at the Golgi apparatus remains undeveloped even with the significance roles of proteolytic enzyme activities in Golgi in the progression of diseases.^[22] Therefore, it is important to develop new techniques for real-time monitoring of enzyme activities at the Golgi apparatus to have a better understanding of the enzymatic roles in diseases progression.

3.2 Golgi apparatus probe design

Herein, we intend to develop a peptide-based fluorescent reporter that can visualize furin-like enzyme activities in real time specifically at the Golgi

apparatus. Our probe design includes two main parts; a Golgi targeting ligand and a furin-responsive peptide probe (GAFP) in order to efficiently localized on the Golgi apparatus to conduct imaging of proteolytic enzyme in the living cells. It was known that furin has a preferential recognition of a unique peptide sequence consisting of Arg-(X)-(Arg/Lys)-Arg↓ that will undergo specific furin cleavage.^[23, 24] Hence, using this preferential sequence, a peptide-based reporter was developed, RVRRSVK, conjugated with Cy5.5 a fluorophore and BHQ3 a quencher, at their respective terminals that will undergo Förster Resonance Energy Transfer (FRET). Using this combination, the reporter will be able to monitor the proteolytic activity through the separation of the FRET pair when the peptide probe undergoes enzyme cleavage (As shown in Scheme 3.1). Additionally, Cy5.5, a NIR fluorophore has advantages such as good photostability and reduced interference in absorption and fluorescence in the biological systems, which will further assist in live-cell imaging.^[25, 26] Furthermore, in order for site specific monitoring of enzymatic activities in the Golgi apparatus, we conjugated a lipid moiety, Sphingosine, on the fluorescent reporter to enhance the efficiency and immobilize the reporter on Golgi.^[15, 16]

Scheme 3.1 Illustration of Golgi apparatus furin responsive probe (GAFP) in the presence of furin enzyme. Q = BHQ3 quencher (black sphere); Cy5.5 (red structure); Golgi targeting sphingosine (purple structure). The dashed line dictates the hydrolysis site.

3.3 Results and Discussion

Generally, the peptide sequences, RVRRSVK, of the GAFF probe was synthesized using standard SPPS method shown in Scheme 3.2.^[27] The two dyes were further added on the peptide scaffold through generic amide bond coupling and was purified using reverse-phased HPLC and characterized by LC-MS and spectroscopy analysis.

Scheme 3.2. Synthesis route for golgi apparatus furin activity probe (GAFF). a) DIPEA, 30mins in DCM; b) HBTU/DIPEA, 2 or 4h in DMF then BHQ-3/HATU/DIPEA in DMF; c) 20% hydrazine in DMF, 20 mins then Cy5.5-NHS/DIPEA in DMF, 4h; d) 2% TFA in DCM; e) DCC/NHS, overnight in DMF then Sphingosine/DIPEA, overnight in DMF; f) 95% TFA 2.5% TIPS 2.5% H₂O.

After attaining the GAFF probe, we first examine the FRET properties of the fluorescent reporter. As shown in Figure 3.3, there is minimal impact on the absorption properties of GAFF when compared to free Cy5.5 dye. Furthermore, GAFF has almost no fluorescence as compared to free Cy5.5 due to the efficient FRET quenching by BHQ3 quencher, with a quenching efficiency of 98.3%. These results indicate that GAFF is well designed to monitor the furin's activities through the hydrolysis of the peptide sequence. Moreover, there is an insignificant difference in spectroscopic properties with and without sphingosine conjugation.

Figure 3.3. a) Absorbance spectra of 40nM of GFP (black) and Cy5.5 (red) in PBS buffer; b) Fluorescence intensity of 40nM of GFP (black) and Cy5.5 (red) in PBS buffer

With the effective FRET design of the GFP, we further investigate the efficiency of the enzyme hydrolysis of GFP by measuring the change in fluorescent intensity with the present of furin in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Similar to the PBS buffer, GFP itself is almost non-fluorescence in HEPES buffer. After incubating GFP with furin for 2 h, a significant fluorescence signal increment (approximately 14-fold) was detected with a maximum wavelength at 698nm, corresponding to the emission of Cy5.5. Additionally, when conducting a similar furin hydrolysis experiment with an addition of a standard furin inhibitor (Dec-RVKR-CMK), it was observed that there was limited fluorescence enhancement even after prolong incubation as shown in Figure 3.4a. These results indicated that specific furin hydrolysis had occurred to cut the peptide reporter to release and separate the quencher, thus causing the recovery of the fluorescence emission.

Encouraged by the positive results in enzyme studies, we continued to test the specificity of furin activities on GFP using living cells. For this study, human neuronal glioblastoma U251 cells were used due to their high expression levels of furin.^[28] To examine the specific furin activity effect, we conduct a similar enzyme hydrolysis experiment using U251 cell lysate to observe the effects of live cells on GFP. When GFP was incubated with U251 cell lysate, a

significant fluorescence enhancement was observed (approximately 13-folds). Likewise, when standard furin inhibitor was added to U251 cell lysate, a limited fluorescence enhancement was detected shown in Figure 3.4b. These promising results further indicate that GFP's peptide sequence is specifically cleaved by furin, demonstrating the feasibility of using the fluorescent probe to monitor furin activities in living cells.

Figure 3.4. Fluorescence enhancement of GFP (60 nM, in HEPES buffer, pH7.4): a) when treated with 2 units of Furin with and without Furin inhibitor (Dec-RVKR-CMK) at 37°C; b) when treated with U251 cell lysate with and without Furin inhibitor at 37°C

With the desired enzymatic-responsive probe, we investigate the applicability of GFP in visualizing the proteolytic activities specifically at the Golgi apparatus imaging. To this end, we inspect the ability of sphingosine in assisting the localization of the fluorescent reporter at the Golgi. Generally, GFP probe was incubated with U251 cells for 2 h for the live-cell fluorescence imaging. After 2 h of incubation, a strong red fluorescence signal was observed within U251 cells as shown in Figure 3.5. This imaging result demonstrated that GFP could be employed as a reporter for furin activity in living cells. As contrast, commercially available organelle stain was used to track the localization of the probe within the cells.^[29, 30] Based on the fluorescence, it was observed that the GFP probe is predominantly localized in the lysosome.

Figure 3.5. Fluorescence imaging of U251 cells with 20nM of GAFP for 2h at 37°C. GAFP = red signal; BODIPY FL-Ceramide (Golgi stain) = green signal; MitoTracker Green FM (Mitochondria stain) = green signal; LysoTracker Green DND-26 (lysosome) = green signal.

Figure 3.6. Pearson's value plotted using Zesis Zen software.

The result was further confirmed after calculating Pearson's value using Zen2.1 shown in Figure 3.6. These outcomes suggest that the design is not effective in targeting and immobilizing the fluorescent reporter at the Golgi

apparatus. Theoretical prediction indicates that small-molecule based probes will be able to retain within the Golgi apparatus if the lipophilicity value stay within a range of 3 – 5.^[31, 32] Hence, it is possible that after the conjugation of sphingosine to the peptide-based fluorescent reporter had resulted in a change in the lipophilicity, which resulted in the GFP unable to localize at the Golgi.

3.4 Conclusion and future work

In this work, we designed a peptide-based fluorescent reporter to visualize furin-like activities at the Golgi apparatus. Based on the preliminary results, we have developed a probe that can effectively visualize furin activities in real-time in living cells. However, the reporter was not able to localize at the Golgi apparatus. The result indicates that the sphingosine moiety in the fluorescent reporter is not efficient in localizing in the Golgi apparatus. However, based on literatures, sphingosine is used to target hCER2, human ceramidase, which is localized in the Golgi apparatus.^[15, 16, 33] Therefore, the lack of selectivity toward the Golgi apparatus may be due to the change in the overall lipophilicity of the fluorescent reporter. Hence, further investigations, such as lipophilicity value testing, are required to be conducted to resolve the localization issue of GFP probe.

In addition, a rational re-design of the reporter may also help to improve the efficiency in localizing the probe at the Golgi apparatus. Several approaches can be used for future works to selectively target the Golgi. One strategy is to design a two-step bioorthogonal imaging probe shown in Figure 3.7. The azide containing sphingosine lipid will be added into cells to localize at the Golgi, before adding the furin-responsive reporter, which will assemble at the Golgi via “click” reaction. This approach utilized a minimalistic targeting moiety, azide modified sphingosine, to avoid any lipophilicity or other potential issues in targeting the Golgi apparatus.

Figure 3.7. Illustration of two steps procedure for “click” chemistry based GFP probe in cell

Additionally, by utilizing the photoactivatable reporter concept in chapter 2, a novel fluorescent reporter can be designed to selectively imaging the Golgi apparatus. Through using precise laser irradiation at the Golgi apparatus, a single cell observation of the enzyme activity could be conducted to study the enzyme dynamic. Lastly, a new targeting ligand can be designed to be directed at a different receptor located on the Golgi apparatus, which may be more selective and efficient then sphingosine.

3.5 Experimental details

Materials: All amino acids and 2-chlorotrityl-chloride resin were purchased from Bio Basic Asia Pacific Pte Ltd (Singapore). All chemical reagents were procured from Sigma-Aldrich. Unless stated otherwise, all commercially purchased chemicals were utilized without any further purification. Furin recombinant enzymes were purchased from Biolabs (2000 Unit/mL), The definition is one unit will release 1 pmol of AMC from the fluorogenic peptide Boc-RVRR-AMC (ITS Science & Medical Pte Ltd) per minute at 30°C. The furin inhibitor (Dec-RVKR-Cmk) was purchased from Bachem.

Instruments: Reverse-phase HPLC purification was done on Shimadzu HPLC system attached with an Alltima C-18 (250 x 10 mm) column. Mass spectroscopy (ESI-MS) was conducted using Thermo LCQ Deca XP Max. UV-Vis absorption spectroscopy was recorded using a 10 mm path quartz cell with Cary 60 UV-Vis Spectrophotometer. Fluorescence emission spectroscopy was conducted on a

Varian Cary eclipse fluorescence spectrometer. Confocal fluorescence imaging of cells was acquired on Carl Zeiss LSM 800 confocal laser microscope.

Synthesis of peptide compound (1) 2-chlorotrityl-chloride resin was used as the solid support. Standard Fmoc-based SPPS protocol was used to synthesis the peptide sequence on the resin. Generally, the amino acids coupling reaction was conduct at r.t for 2 h (4 h for arginine), in the presence of HBTU (1.5 eq) together with DIPEA (1.5 eq). The deprotection of Fmoc-protecting group was achieved by reacting the peptides with 20% piperidine in DMF for 20 min. The coupling reaction for BHQ-3 carboxylic acid (1 eq) was conduct at room temperature for 4 h in the presence of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5b]pyridineium-3-oxid hexafluorophosphate (HATU) (1.5 eq) and DIPEA (1.5 eq). Removal of the Dde protecting group lasted for 20 min in the presence of 20% hydrazine in DMF. Cy 5.5-NHS ester (1 eq) and DIPEA (1 eq) in DMF was then added and reacted for 4 hours. Lastly, 2% TFA in CH₂Cl₂ was added for 10 min to cleave the peptide product from the resin. The product was concentrated in *vacuo* and precipitated with Et₂O. The precipitate was purified by reverse-phased HPLC with the mobile phases comprising of solvent A (H₂O with 0.1% TFA) and B (ACN with 0.1% TFA). A gradient of solvent B mixed with solvent A (30% to 100% for 15 min) with 3.0mL/min flow rate was monitored by UV/Vis absorbance of 650 and 670 nm to attain **1**. Compound **1** retention time was 16.3 min. ESI-MS: m/z calcd: 2920.56 [M+H]⁺; found: 1460.37 [M+2H]⁺, 973.97 [M+3H]⁺.

Synthesis of compound (2) The peptide **1** dissolved in 100 µL of DMF was activated with N,N'-Dicyclohexylcarbodiimide (DCC) (1.5 eq) and N-Hydroxy-succinimide (NHS) (1.5 eq) at r.t for 12 h. After the reaction, the by-product, dicyclohexylurea (DCU), was removed from the mixture by filtration to afford NHS-activated peptide **1**. Sphingosine (1.1 eq) and DIPEA (1.1 eq) was added to the mixture and stirred for additional 12 h at r.t. After the reaction, Et₂O was

added to obtain a blue solid residue **2**. The final product was purified by HPLC with a gradient of solvent B mixed with solvent A (30% to 100% for 15 min) with 3.0mL/min flow rate and monitored by UV/Vis absorbance of 650 and 670 nm to attain **2**. Compound **2** retention time was 18.7 min. ESI-MS: m/z calcd: 3201.83 [M+H]⁺; found: 1600.47 [M+2H]⁺, 810.95 [M+3H]⁺.

Synthesis of Golgi Apparatus Furin Activity Probe (GAFP) (3) The Pbf and Boc protection groups were cleaved by 95% TFA, 2.5% TIPS and 2.5% H₂O for 2 h. After the reaction, Et₂O was added to obtain a blue solid residue **3**. The final product was purified by HPLC with a gradient of solvent B mixed with solvent A (10% to 100% for 20 min) with 3.0mL/min flow rate and monitored by UV/Vis absorbance of 650 and 670 nm to attain **3**. Product **3** retention time was 18.5 min. ESI-MS: m/z calcd: 2389.53 [M+H]⁺; found: 1195.32 [M+2H]⁺, 796.82 [M+3H]⁺.

UV-Vis absorbance and fluorescence spectra characterization. The absorbance and fluorescence spectra of Cy5.5 and GAFP (60 nM) were recorded in PBS (pH=7.4) buffer. The fluorescence quenching efficiency is about 98.3%, indicating the fluorescence of Cy5.5 is almost quenched by BHQ-3.

Fluorescence spectrum for furin activity. The fluorescence spectra were recorded in HEPES buffer (pH 7.4). 2 units of furin enzyme were used with 100 uL of HEPES buffer and incubated with furin activity probe at 37°C for 60 min. The fluorescence emission was recorded at every 5 min interval.

General methods for cell culture. Human Neuronal Glioblastoma U251 Cells was grown in DMEM (Gibico) supplemented with 10% FBS (HyClone) at 5% CO₂ and 37°C.

Incubation of GAFP probe in cell lysates. U251 cells were trypsinized, centrifuged (1200 rpm for 5 min) to remove the medium and subsequently washed with PBS buffer. The commercially bought RIPA buffer (Sigma,

containing 0.1% SDS, 0.5% sodium deoxycholate, 1.0% IGEPAL® CA-630, 150 mM NaCl, 50 mM Tris, and pH 8.0) was added and the mixture was kept on ice for 15 min. U251 cell lysates were collected and centrifuged at 14,000×g for 15 min. Total proteins content in the cell lysates were quantified by the standard procedure of Bio-Rad Protein Assay (Bio-Rad Laboratories, USA). Generally, the dye reagent was diluted 5 times with distilled water before usage. 200 µL of the diluted reagent was mixed with 20 µL of the tested sample in 96-well plates. The mixture was allowed to be incubate at r.t for 5 minutes before recording the absorbance at 595 nm using the microplate reader. The 30 µg total protein was incubated with probe (56 µM) for 3 h. For the control experiment, the U251 supernatant was preincubated with the inhibitor Dec-RVKR-cmk (100 µM) for 30 min before the addition of the probe. Then the fluorescence was measured at 700 nm using the microplate reader.

Fluorescence imaging of live cells. U251 cells were seeded in a 35-mm diameter µ-dish with complete medium and was incubated for 24 h at 5% CO₂ and 37 °C before imaging. The cells were then incubated with the GFP probe (60 nM) for 2 h incubation. Subsequently, the cells incubated with various organelle stains: 1) LysoTracker Green DND-26 (50 nM) was added and incubated for 1 h at 37 °C and washed three times with PBS. 2) BODIPY-FL-Ceramide (5 µM) was added and incubated at 4°C for 30 min before rinsing the cell several times with ice-cold medium. Fresh medium was then added and the cell was further incubated in 37 °C for 30 min before imaging. 3) MitoTracker Green FM (100 nM) was added and incubated for 30 min at 37 °C and washed three times with PBS. (GFP: λ_{ex} = 640 nm, λ_{em} = 650/50 nm; LysoTracker Green DND-26/ BODIPY-FL-Ceramide/ MitoTracker Green FM λ_{ex} = 488 nm, λ_{em} = 525/25 nm).

3.6 References

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Chapter 4

Proteolytic Enzyme Based Metabolic Labeling for Tumor-Specific Fluorescence Imaging

4.1 Introduction

Cancer is one of the leading causes of death worldwide and the number one causes of death in Singapore. Furthermore, the number of new cancer cases increases over the years with very high mortality rate is a serious health threat worldwide.^[74] Therefore, it is crucial to develop efficient treatment modalities to combat against this health concern. Currently, several treatment modalities such as chemotherapy, hormonal therapy, immunotherapy, radiation therapy and surgery are widely used in the hospital. However, although these treatment modalities are effective against cancer, severe side effects were still observed in the patients due to non-specific targeting of the modality.^[75] Hence, it is necessary to develop a precision treatment platform for cancer, which can improve treatment efficacy and reduces side effects.

A promising approach for precision medicine is through the use of enzymatic actions to promote specificity. Of interest, furin has gathered much interest as a potential biomarker in recent years.^[7-8] Furin is ubiquitously expressed within endocytic and secretory pathways as well as the cellular surface, which functions to activate a variety of proproteins into their biologically active forms. These activities contribute to various pathological conditions and progression to degenerative diseases like cancers.^[8, 13b, 49] Furthermore, several reports had

highlighted the overexpression of furin and its involvement in the progression of different cancers including head and neck squamous cell carcinomas, breast cancer and non-small cell lung carcinomas.^[16] With the close association of furin in degenerative diseases, it served as a potential biomarker for diagnostic targets. Hence, designing a platform that utilizes furin activity to trigger the delivery can provide a new approach to precision treatment and early diagnostic.^[34, 50a, 50b, 50d]

4.2 Metabolic precursor design

One notable technique for precision delivery is by using metabolic glycoengineering, which exogenously generate bioorthogonal function groups (such as azide, alkyne and tetrazine) on the cell surface through intrinsic glycan metabolism of unnatural glycan.^[76] Subsequently, biomolecules or nanoparticles can be chemically bound in the living cells through bioorthogonal reaction for visualization or other biomedical applications.^[77]

Based on this principle, we intend to develop a metabolic precursor that can label and functionalize specifically on tumor cells for visualization and other potential biomedical applications such as disease treatment. In our design, we utilized a peptide-caged metabolic precursor (PMAz) that can be activated by furin-like proteolytic activities on selected cells. To achieve this, triacetylated *N*-azidoacetyl-D-mannosamine (Ac₃ManNAz) was conjugated with a furin recognizable peptide sequence, RVRR, through a self-immolative linker.^[9, 53] Upon furin's hydrolysis, the *p*-aminobenzyl alcohol (PABOH) linker will self-eliminate to release Ac₃ManNAz into the targeted cells. As shown in Scheme 4.1, the targeted cells will undergo glycan metabolism of the unnatural glycan and exogenously expressed the azide (N₃-) groups on the cell surfaces, which can be targeted by dibenzocyclooctyne (DBCO)-based dyes (DBCO-Cy3) via "click" chemistry for visualization.^[78] The proposed mechanism for the release of Ac₃ManNAz is also shown in Scheme 4.2.

Scheme 4.1. Illustration of furin-specific hydrolysis of the precursor (PMAz) in tumor cell to trigger the release of Ac₃ManNAz. The released Ac₃ManNAz will undergo intrinsic metabolism, followed by staining of exogenous unnatural glycan with DBCO-Cy3 via bioorthogonal 'click' reaction to label the targeted tumor cell surface.

Scheme 4.2. Proposed enzyme hydrolysis mechanism of PMAz to release Ac₃ManNAz.

4.3 Results and Discussion

Generally, the peptide sequences, RVRR, of the PMAz precursor was synthesized using standard SPPS method.^[56] The PABOH linker conjugated on the peptide typical amide bond coupling reaction and following a combination reaction of the peptide and Ac₃ManNAz is shown in Scheme 4.3. Reverse-phased HPLC was used to purify the final product and subsequently characterized by LC-MS.

After the preparation of PMAz, we first examine the feasibility of un-caging the metabolic precursor by furin through HPLC analysis. The hydrolysis experiment was performed by incubating the caged precursor PMAz with recombinant furin enzyme in HEPES buffer at 37°C. As observed in Figure 4.2, after incubating the precursor with furin for 1 h, a new peak was detected with a retention time of 10.3 min, which can be attributed to the enzyme cleavage of the peptide fragment. However, the majority of the PMAz remains unchanged, indicating that longer incubation period is required to fully un-cage the precursor.

Scheme 4.3. Synthetic route for PMAz. a) DIPEA, 30mins in DCM; b) HBTU/DIPEA, 2 or 4h in DMF; c) 2% TFA in DCM; d) PABOH/EDC/HOBt, 4h in DMF; e) 95% TFA/2.5% TIPS/2.5% H₂O; f) *p*-Nitrophenyl chloroformate/TEA, overnight in DCM; g) compound 3, overnight in DMF.

Figure 4.2. HPLC analysis of PMAz in the presence of recombinant furin enzyme.

With the promising results of the PMAz in the presence of furin enzyme, we investigate the applicability of the metabolic precursor in living cells. For this study, high furin expression MDA-MB-468 cells were used as the targeted tumor cells, whereas furin-deficient LoVo cells were used as a control. Additionally, NIH/3T3 mouse embryo cells were used as non-tumor cell control.^[59] Firstly, we investigate the concentration of PMAz is required for labeling the cell membrane via native metabolic biosynthesis. Generally, the PMAz precursor of different concentration was supplemented into the medium and incubated at 37°C for 1 day. Next, DBCO-Cy3 was added to perform a copper-free “click” reaction with PMAz-treated MDA-MB-468 cells, which confocal microscopy was then used to monitor the labeling of the N₃-functionalized cell surfaces. As shown in Figure 4.3, different concentration of PMAz may generate different amount of N₃ on the cell surface based on the fluorescent intensity observed and 360 nM of PMAz is the ideal concentration to fully functionalized the cell surface.

Figure 4.3. Confocal imaging of N₃-tagged MDA-MB-468 cells after treatment with different concentration of PMAz. After incubation with PMAz, the cell membrane was stained with DBCO-Cy3 (10 μ M). Scale bar for all images = 20 μ m.

After optimizing the concentration of metabolic precursor required for functionalizing the cell surface, we investigate the ability of PMAz to specifically label on target tumor cells. To this end, PMAz was feed to the medium of different cell lines and fluorescence imaging was conduct to observe the labeling effect. As presented in Figure 4.4, a strong red fluorescent signal was observed when PMAz precursor was pre-incubated with MDA-MB-468 cells. However, only a minimum fluorescence emission was detected in furin-deficient LoVo cells under the similar condition. In addition, a control experiment shown that there was no significant difference with the fluorescent signal when both the cells were incubated with Ac₃ManNAz. The results indicated that furin-like cleavage activities are required to un-cage the metabolic precursor before intrinsic glycan metabolism of unnatural glycan can occur to generate N₃ group on the cell surface. Additionally, no signal was observed in the cells that were not pre-incubated with Ac₃ManNAz, suggesting the fluorescence signals monitored is caused by the covalent linkage of the fluorophore on the cell membrane after native metabolic labeling.

Figure 4.4. Fluorescence imaging of N₃ groups on the membrane of LoVo and MDA-MB-468 cells stained with DBCO-Cy3 (10 μ M) in the presence and absence Ac₃ManNAz (360 nM) or with PMAz (360 nM). Scale bar for all images = 20 μ m.

Encouraged by the favorable results, we further study the effect of the furin expression in different cell lines by quantifying the fluorescent intensity of Cy3 on the cell membrane via ImageJ. Based on the plot, a significantly higher fluorescence signal (approximately 5-folds) in MDA-MB-468 cells was measured in contrast with the control cell lines as shown in Figure 4.5. This observable difference in fluorescence signal strongly suggests that furin can be used as a potential biomarker, which can be utilized to trigger and label specifically on tumor cells for subsequent visualization or treatment.

Figure 4.5. Fluorescence imaging of NIH/3T3, LoVo and MDA-MB-468 cells. PMAz precursor (360 nM) was supplemented to the different cells and incubated for 1 day, before staining with DBCO-Cy3 (10 μ M). Scale bar for all images = 20 μ m. Fluorescence intensity along the arrows was measured and plotted as a function of distance using ImageJ.

4.4 Conclusion and future work

As a proof-of-concept, we designed and developed a precision drug delivery platform through utilizing furin activity in tumor cells. Our design comprised of a furin cleavable peptide-caged metabolic precursor (PMAz) to exogenously generate N_3 groups on the cellular membranes of the tumor cells for subsequent labeling. Based on the preliminary results, we have shown that the PMAz precursor can effectively functionalize N_3 group on furin-expressed tumor cells surface, which can be targeted by DBCO-Cy3 through bioorthogonal “click” reaction. The promising results is an indication that furin can be used as a potential biomarker to trigger tumor-specific imaging or drug delivery.

However, more investigations such as incubating the precursor together with furin inhibitors to test the specificity of furin-triggered PMAz un-caging, feeding PMAz to a wider variety of tumor and normal cells, and quantitative FCM analysis of the treated cell lines are still required to strengthen the hypothesis of

using furin as a biomarker. In addition, *in vivo* testing will be conducted to test the applicability of using PMAz to label targeted cells for disease treatment and other biomedical applications.

4.5 Experimental details

Materials: All amino acids and 2-chlorotriethyl-chloride resin were purchased from Bio Basic Asia Pacific Pte Ltd. Triacetylated N-azidoacetyl-D-mannosamine (Ac₃ManNAz) was purchased from FutureChem Co., Ltd. All chemical reagents were procured from Sigma-Aldrich. Unless stated otherwise, all commercially purchased chemicals were utilized without any further purification. Furin recombinant enzymes were purchased from Biolabs (2000 Unit/mL), The definition is one unit will release 1 pmol of AMC from the fluorogenic peptide Boc-RVRR-AMC (ITS Science & Medical Pte Ltd) per minute at 30°C. The furin inhibitor (Dec-RVKR-Cmk) was purchased from Bachem.

Instruments: Nuclear magnetic resonance (NMR) spectroscopy were conducted on Bruker DPX 300 spectrophotometer (CDCl₃ was used as the solvent). Reverse-phase HPLC purification was conducted using Shimadzu HPLC system attached with an Alltima C-18 column (10 × 250 mm). Mass spectroscopy (ESI-MS) was conducted using Thermo LCQ Deca XP Max.. Fluorescence emission spectroscopy was conducted on a Varian Cary eclipse fluorescence spectrometer. UV-Vis absorption spectroscopy was recorded using a 10 mm path quartz cell with Cary 60 UV-Vis Spectrophotometer. Confocal fluorescence imaging of cells was acquired on Carl Zeiss LSM 800 confocal laser microscope.

Synthesis of Dec-RVRR-COOH (1) 2-chlorotriethyl-chloride resin was used as the solid support. Standard Fmoc-based SPPS protocol was used to synthesis the peptide sequence on the resin. Generally, the amino acids coupling reaction was

conduct at r.t for 2 h (4 h for arginine), in the presence of HBTU (1.5 eq) together with DIPEA (1.5 eq). The deprotection of Fmoc-protecting group was achieved by reacting the peptides with 20% piperidine in DMF for 20 min. The product was cleaved from the resin using 2% TFA in CH₂Cl₂ for 10 min. After concentrating the product in *vacuo*, product was purified by flash column chromatography (DCM: MeOH = 4:1) to afford **1** as a white solid (0.524 g, 70%). ESI-MS: m/z calcd: 1496.95 [M+H]⁺; found: 1496.74 [M+H]⁺.

Synthesis of protected Dec-RVRR-PABOH (2) To a solution of **1** (0.524 g, 0.35 mmol) in DMF (500 μ L) was added *p*-aminobenzyl alcohol (PABOH) (0.065 g, 0.525 mmol), 1-hydroxybenzotriazole hydrate (HOBt) (0.095 g, 0.7 mmol) and *N*-Ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl) (0.134 g, 0.7 mmol) and was stirred at r.t for 2 h. The product was purified by reverse-phased HPLC with the mobile phases comprising of solvent A (H₂O with 0.1% TFA) and B (ACN with 0.1% TFA). A gradient of solvent B mixed with solvent A (30% to 100% for 15 min) with 3.0mL/min flow rate was monitored by UV/Vis absorbance of 220 nm to attain **2**. Compound **2** retention time was 20.5 min. ESI-MS: m/z calcd: 1602.09 [M+H]⁺; found: 1601.84 [M+H]⁺.

Synthesis of deprotected Dec-RVRR-PABOH (3) The Pbf protection was removed by using 95% TFA, 2.5% TIPS and 2.5% H₂O for 2 h. After the reaction, the product was purified by HPLC with a gradient of solvent B mixed with solvent A (30% to 100% for 20 min) with 3.0mL/min flow rate and monitored by UV/Vis absorbance of 220 nm to attain **3**. Compound **3** retention time was 7.1 min. ESI-MS: m/z calcd: 845.11 [M+H]⁺; found: 845.68 [M+H]⁺.

Synthesis of Ac₃ManNAz-PNP (4) To a solution of Triacetylated N-azidoacetyl-D-mannosamine (Ac₃ManNAz) (0.02 g, 0.05 mmol) in DCM (1 mL) was added *p*-nitrophenyl chloroformate (PNP) (0.021 g, 0.1 mmol), TEA (42 μ L, 0.3 mmol) and stirred overnight at r.t. After the reaction, product was purified by

flash column chromatography (Hex: EA = 1:1) to afford **3** as a white solid (0.012 g, 43.4%). ¹H NMR (300 MHz, CDCl₃) δ 8.29 (d, *J* = 9.1, 2H), 7.41 (d, *J* = 9.1, 2H), 6.59 (d, *J* = 9.5, 1H), 6.09 (d, *J* = 1.7, 1H), 5.40 (dd, *J* = 10.1, 4.2, 1H), 5.30 (t, *J* = 9.9, 1H), 4.65 (ddd, *J* = 9.3, 4.1, 2.0, 1H), 4.39 (ddd, *J* = 14.4, 12.1, 3.2, 2H), 4.07 (d, *J* = 5.2, 1H), 2.20 (s, 3H), 2.10 (s, 3H), 2.02 (s, 3H), 1.59 (s, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 20.9, 20.9, 21.0, 49.5, 52.6, 65.3, 66.5, 68.9, 70.3, 91.5, 122.1, 125.6, 145.9, 152.6, 155.6, 167.1, 168.3, 170.0, 170.2.

Synthesis of protected PMAz (5**)** To a solution of **3** in DMF (500 μL) was added Ac₃ManNAz-PNP **4** and stirred overnight at r.t. After the reaction, the product was purified by HPLC with a gradient of solvent B mixed with solvent A (30% to 90% for 20 min) with 3.0mL/min flow rate and monitored by UV/Vis absorbance of 220 nm to attain **5**. Compound **5** retention time was 16.5 min. ESI-MS: *m/z* calcd: 1259.44 [M+H]⁺; found: 1259.35 [M+H]⁺.

General methods for cell culture. Human mammary gland/breast MDA-MB-468 cells (from ATCC) were cultured in DMEM supplemented with 10 % FBS. Mouse embryo NIH/3T3 cells (from ATCC) cultured in DMEM supplemented with 10 % FBS. Human colorectal adenocarcinoma LoVo cells (from ATCC) were cultured in F-12K medium supplemented with 10% FBS. All the cells were incubated with 5% CO₂ at 37°C.

Metabolic glycan labeling on cell membrane and imaging in live-cells. The cells were seeded at a density of 1 × 10⁵ in 35 mm diameter μ-dish plastic bottom (ibidi GmbH) in complete medium and with different concentration of Ac₃ManNAz for 24 h at 5% CO₂ and 37°C. The medium was subsequently replaced with fresh medium with DBCO-Cy3 (10 μM) for 30 min and washed with medium before conducting imaging. For co-localization imaging, CellMask Deep Red (2.5 μg/mL) was added into the cell and was incubated at 37°C for 5 min, washed thrice with PBS before imaging. Fluorescence imaging was conduct

using confocal laser scanning microscope (LSM800). (Cy3: λ_{ex} = 561 nm, λ_{em} = 585/25 nm, CellMask Deep Red: λ_{ex} = 640 nm, λ_{em} = 678/22 nm).

4.6 References

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Chapter 5

Metabolic-Mediated Localization of Fluorescent Reporter on Pathogenic Bacteria

5.1 Introduction

Enzyme plays many important roles in various biological functions in bacteria, notably causing a resistant of bacteria against antibiotics. Antibiotic resistances emerging from pathogenic bacteria had become a major medical concern due to the overuse of antibiotics over the years.^[1-3] One of the primary causes of resistance in bacteria is through the production of drug-resistant enzymes β -lactamase (Blas), which specifically hydrolyze β -lactam based antibiotic rendering it ineffective for antibacterial treatment.^[4-6] Hence, developing new strategies to visualize enzyme activities can help to profile bacterial resistance and provide insight into the biochemical processes and drug resistance mechanism for new antimicrobial treatments.

Figure 5.1. Illustration of different antibiotic resistance mechanism in bacteria^[7]

Blas enzymes can be categorized into four different classes (A, B, C and D) based on its unique amino acid sequences and its catalytic mechanism.^[8, 9] Among these classes of Blas, class A and C Blas are known to be highly encountered in the resistant bacteria found transmitting within the humans.^[10, 11] Even with its importance, investigation on class C Blas was far less extensive as compared to class A Blas, even though both enzymes have similar catalytic active sites.^[12-17] In addition, recent outbreaks of new class C Blas resistant bacteria due to the conjugative transmission of bacterial plasmid led to an increased risk in antibiotic resistance.^[18-21] Therefore, the development of novel approaches to selectively monitor and visualize class C Blas enzyme can help to elucidate the biological process and will assist in combatting microbial resistance.

Currently, established clinical techniques to detect Blas enzyme includes the use of susceptibility tests such as double-disk synergy test and modified hodge test.^[22-25] Such methods lack the selectivity and were often time-consuming for accurate diagnosis of bacterial infections. Hence, it is necessary to develop a new efficient and selectively strategy in detecting resistant bacteria for *in vitro* and *in vivo* application. Furthermore, the ability to localize and immobilize the detection platform on the resistant bacteria can allow in-depth elucidation and profiling of resistant bacteria within the living system, and have a better understanding on the pathological pathway is highly desirable.

Till date, several methods have been developed to aid in the localization such as through genetic modification, protein labeling and usage of targeting ligands.^[26, 27] Among these techniques, metabolic glycoengineering had gathered much interest due to its ease of operation and allow covalent conjugation on bacterial cells for prolong dynamic studies and application.^[28, 29] Generally, the bacteria cell wall is made up of peptidoglycan, which is a mesh-like of polymer consisting of sugars and amino acids. During the cell-wall synthesis, the disaccharide monomers are linked together to form a sugar polymer, which is crosslinked by

transpeptidation through the incorporation of D-glutamic acid and D-alanine. Hence, the introduction of unnatural glycan or D-alanine (D-ala) functionalized with bioorthogonal groups (such as azide, alkyne and tetrazine) will undergo intrinsic metabolism by replacing the D-amino acid required during the crosslinking of the peptidoglycan during cell wall synthesis, which will exogenously generate the chemical receptors on the bacterial cell surface for future covalent labeling. This strategy will allow specific localization on resistant bacteria surface in the complex microenvironment, which can visualize and monitor Blas enzyme activity to aid in understanding the biological functions.^[30-32]

5.2 Fluorescent reporter design

In line with these challenges, herein, we present a metabolic-mediated fluorescent reporter (DBLR) that can specifically visualize class C Blas enzyme in resistant bacteria for *in vivo* application. Typically, Blas enzyme will recognize β -lactam ring and will undergo Blas cleavage. Based on this principle, we designed the fluorescent reporter using cephalosporin as a scaffold and conjugated a pair of dyes, Cyanine 5.5 (Cy5.5) a fluorophore and black hole quencher (BHQ3), which will undergo FRET effect. Thus, in the presence of Blas enzyme, fluorescence will be detected for visualization as the reporter undergoes cleavage thus separating the FRET pair. Furthermore, to selectively monitor class C Blas enzyme, a bulky DBCO group was conjugated to the 7' position of the cephalosporin β -lactam ring for subsequent labeling on the bacterial cell surface and also argument as a steric hindrance, which will improve selectivity toward class C Blas.

Hence, the bacteria will undergo intrinsic metabolism of unnatural D-ala and exogenously expressed the N_3 groups on the cell surfaces. Then, DBLR will be added and covalently labeled on the bacteria surface via “click” chemistry, which

will fluoresce upon detecting class C Blas resistant bacteria as shown in Scheme 5.1. The proposed mechanism of DBLR in the presence of class C Blas is shown in Scheme 5.2.

Scheme 5.1. Illustration of a metabolic-mediated localization of fluorescent reporter on peptidoglycan in bacterial cell. Upon intrinsic metabolism of Azide -D-Ala in the bacteria, DBLR was conjugated on the peptidoglycan through copper-free “click” reaction and will undergo fluorescence enhancement upon activation by AmpC enzyme (class C Blas).

Scheme 5.2. Proposed mechanism of DBLR in the presence of class C Blas.

5.3 Results and Discussion

The synthetic route of the fluorescent reporter DBLR **8** and the control probe

BLR **7** is depicted in Scheme 5.3. Generally, the cephalosporin β -lactam was linked with Mtt-Lys **1** at the 7' position of cephalosporin to yield Mtt-Lys-Lac **2**. Next, the 3' position of cephalosporin was conjugated with BHQ3 to afford **4**. Lastly, Cy5.5 was added to the 7' position to afford BLR **7** followed by the conjugation of DBCO to give the desired product DBLR **8**. The final product was purified using reverse-phased HPLC and characterized by LC-MS.

Scheme 5.3. Synthetic route for BLR (**7**) and DBLR (**8**). a) MttCl/TEA, 4 h in DCM/DMF; b) ACLE/HOBt/TEA/EDC, 6 h in DMF at 0°C; c) 4-aminothiophenol/NMM, 2 h in DMF; d) BHQ 3/HATU/NMO,

24 h in DMF; e) 2% TFA, 2 h in DCM; f) Cy 5.5-NHS/DIPEA, 4 h in DMF; g) 20% TFA and 5% anisole, 1 h in DCM; h) DBCO-NHS/DIPEA, 12 h in DMF.

After attaining the product DBLR and control probe BLR, we first examined the sensitivity of the probes towards class A and C Blas. In this study, the TEM-1 enzyme was used to represent class A Blas and AmpC enzyme was used to represent class C Blas. The enzyme hydrolysis of the probes was performed in PBS buffer while the changes in the fluorescence were monitored upon treatment with the Blas. As shown in Figure 5.2, a limited fluorescence signal was observed in the absence of Blas enzymes indicating an efficient FRET effect occurred between Cy5.5 and its quencher BHQ3. When DBLR was incubated with AmpC enzyme for 1 h, a strong fluorescence enhancement was observed (~150-folds), whereas a minimal fluorescence change was detected (~45-folds) upon incubation with TEM-1 enzyme. In comparison, a significant fluorescence enhancement was measured (~150-folds) for BLR probe upon incubation with either TEM-1 or AmpC enzyme. The results suggest that both DBLR and BLR are sensitive toward Blas hydrolysis, which can be monitored by the recovery of fluorescence emission. Additionally, the presence of a bulky group (DBCO) on the 7' position of the β -lactam drastically improves the selectivity of the reporter towards class C Blas detection.

Figure 5.2. Fluorescence spectrum of (a) DBLR and (b) BLR (10 μ M) before and after incubating with 50 nM of TEM-1 and AmpC enzyme in PBS buffer for 1 h.

Furthermore, to prove that hydrolysis of DBLR caused by Blas, DBLR was incubated with Blas together with their respective inhibitor, Clavulanic acid (CA) for class A Blas and Aztreonam (AZT) for class C Blas. As shown in Figure 5.3, a strong fluorescence signal (approximately 8-folds) was observed for DBLR incubated with AmpC as compared with the presence of its inhibitor (AZT). Additionally, only a slight fluorescence signal enhancement (approximately 1-fold) was detected for DBLR incubated with TEM-1 as compared with the presence of CA. These results indicate that Blas enzymes are necessary for the hydrolysis of the reporter and the fluorescent reporter is more selective toward class C Blas.

Figure 5.3. Fluorescence intensity of DBLR in the presence and absence of Blas inhibitors.

Encourage by the promising results, we investigate the applicability of the fluorescent reporter DBLR in detecting the pathogenic bacteria. In this study, *Enterobacter cloacae* (*E. cloacae*) was used owing to its high level of AmpC enzyme expression, *Escherichia coli* (*E. coli*) BL21 was used due to a high expression level of TEM-1 and *E. coli* DH5 α were chosen as negative control bacteria without Blas expression.^[20, 33, 34] The toxicity of 3-azido-D-ala towards the bacteria was validated through a bacterial cells viability test. Based on the results shown in Figure 5.4, low concentrations of 3-azido-D-ala (0.1mM – 1mM) have relatively low toxicity toward the pathogens, which indicates that 3-azido-D-ala is suitable to use for intrinsic metabolic labeling onto the bacteria surface.

Figure 5.4. Bacteria viability with different concentration of 3-azido-D-ala (0.1 mM – 2 mM).

Next, we investigate the ability of the bacteria to undergo intrinsic metabolism of the unnatural amino acid, 3-azido-D-ala. To this end, the bacteria were incubated with 3-azido-D-ala (1 mM) for 1 h and the functionalized surface were stained with DBCO-Cy3 and observed using fluorescence imaging. As shown in Figure 5.5, a strong red fluorescence signal was detected on the surface of all the bacteria strains treated with 3-azido-D-ala, while no obvious fluorescence was observed for bacteria without pretreatment of 3-azido-D-ala. These results demonstrated the feasibility of the bacteria to uptake 3-azido-D-ala through native metabolism pathway to exogenously generate the chemical receptors on the bacteria surface for subsequent covalent labeling.

Figure 5.5. Fluorescent imaging of 3-azide-D-ala treated and non-treated bacteria strains stained with DBCO-Cy3.

Inspired by the possibility of the fluorescent reporter to be covalently blinded on the resistant bacteria, we studied the selectivity of DBLR towards class C type resistant bacteria strain. Typically, two sets of experiments were conducted with one set of bacteria strains pretreated with 3-azido-D-ala while the other set of bacteria, as a negative control, were not treated with 3-azido-D-ala. These bacteria were incubated with 10 μ M of DBLR separately at 37 °C for 30 minutes and were subjected to confocal imaging. As shown in Figure 5.6, intense red fluorescence emission was observed when DBLR was incubated with *E. cloacae* as compared with other bacteria strains. These results successfully demonstrated the selectivity of DBLR reporter toward class C Blas resistant bacteria. Additionally, minimum fluorescence signal was observed when *E. cloacae* was treated with AmpC inhibitor AZT, which further indicates that the hydrolysis of DBLR reporter is mainly due to class C Blas showed in Figure 5.7.

Figure 5.6. Fluorescent imaging of DBLR (10 μ M) in metabolized and non-metabolized bacteria strains.

Figure 5.7. Fluorescent imaging of DBLR (10 μ M) in metabolized bacteria strains with and without AZT inhibitor.

Encouraged by the promising results in bacteria imaging, we investigate the feasibility of the reporter for *in vivo* application in zebrafish. Generally, we injected the different bacterial cells separately into the yolk sac of the zebrafish at the 72 h post-fertilization. After infection, the zebrafish was bathed with 3-azido-D-ala for 1 h to allow native metabolism and exogenously generate N_3 group on the surface of the bacteria. Thereafter, the DBLR was injected into the zebrafish and incubated for 1 h to allow bioorthogonal conjugation to the bacteria before confocal imaging. As shown in Figure 5.8, a significant fluorescent signal was observed throughout the zebrafish infected by *E. cloacae* pre-treated with 3-azido-D-ala. Whereas, zebrafish infected by BL-21 and DH5 α bacteria shown a relatively weaker fluorescent emission. As a contrast, a weak fluorescence signal was observed in the zebrafishes not treated with 3-azido-D-ala. These results demonstrated the ability to use DBLR reporter for *in vivo* detection of class C Blas.

Figure 5.8. Fluorescent imaging of DBLR in the presence and absence of 3-azido-D-ala in zebra fish.

5.4 Conclusion and future work

In conclusion, we had designed and developed a metabolic-mediated fluorescent reporter (DBLR) that can specifically detect and visualize class C Blas enzyme in resistant bacteria. Based on the preliminary results, we had shown that 3-azido-D-ala can undergo native metabolic pathway to exogenously generate N_3 group on the bacteria surface. Thereafter, the DBLR reporter can target this chemical receptor via “click” chemistry to covalently label on the bacteria to visualize and monitor class C Blas enzyme. Furthermore, the bulky DBCO group on the 7' position of the β -lactam had enhanced the selectivity of DBLR toward class C Blas. These promising results signified the feasibility of using DBLR fluorescent reporter to specifically detect and visualize class C Blas in both *in vitro* and *in vivo* applications. For future works, further *in vivo* testing will be conducted in more animal models to examine the robustness of the reporter for clinical application.

5.5 Experimental details

Materials: Black Hole Quencher®-3 (BHQ®-3) carboxylic acid was purchased from LGC Biosearch Technologies. Cy 5.5 NHS ester and DBCO NHS ester were purchased from Lumiprobe. 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid 4-methoxybenzyl ester hydrochloride (ACLE) was purchased

from Otsuka Chemical Co., Ltd. All other chemical reagents were purchased from Sigma-Aldrich. All commercially purchased chemicals and reagents were used without any further purification, unless noted otherwise. Anhydrous solvents used for organic synthesis were stored over activated molecular sieves (4 Å).

Instruments: Nuclear magnetic resonance (NMR) spectroscopy were conducted on Bruker DPX 300 and Bruker AVIII 400 spectrophotometer (CDCl_3 was used as the solvent). Reverse-phase HPLC purification was conducted using Shimadzu HPLC system attached with an Alltima C-18 column (10×250 mm). Mass spectroscopy (ESI-MS) was conducted using Thermo LCQ Deca XP Max. Fluorescence emission spectroscopy was conducted on a Varian Cary eclipse fluorescence spectrometer. UV-Vis absorption spectroscopy was recorded using a 10 mm path quartz cell with Cary 60 UV-Vis Spectrophotometer. Confocal fluorescence imaging of cells was acquired on Carl Zeiss LSM 800 confocal laser microscope.

Synthesis of compound 1:

Boc-lysine-OH (1.23 g, 5 mmol) and TEA (2.8 mL, 20 mmol) were added to a solution of 4-methyltrityl chloride (MttCl) (2.93 g, 10 mmol) in 2:1 DCM/DMF (10 mL) and stirred at r.t for 4 h. After the reaction is completed, 5mL of methanol was then added to quench the reaction. 50 mL of EA was added to dilute the mixture, washed with water then the organic layers were dried with brine and anhydrous Na_2SO_4 before concentrated in *vacuo*. The product was purified by flash chromatography (EA: Hex = 1:1) to yield **1** as a white crystalline solid. (1.48 g, 59%). ^1H NMR (400 MHz, CDCl_3) δ = 7.42 (d, J =7.1, 5H), 7.28 (dd, J =15.8,

7.8, 6H), 7.20 (d, $J = 6.8$, 2H), 7.09 (d, $J = 7.4$, 2H), 5.21 (d, $J = 5.9$, 1H), 4.02 (s, 1H), 2.34 (s, 2H), 2.29 (s, 3H), 1.65 (s, 1H), 1.52 (s, 3H), 1.39 (s, 9H), 1.21 (s, 2H). ^{13}C NMR (101 MHz, CDCl_3) $\delta = 21.1, 23.1, 28.6, 28.9, 33.2, 44.7, 54.4, 72.9, 79.3, 127.3, 128.3, 128.8, 128.9, 129.0, 137.0, 140.3, 143.6, 155.6, 176.8$. ESI-MS (m/z) calcd: 503.66 $[\text{M}+\text{H}]^+$; found: 503.38 $[\text{M}+\text{H}]^+$.

Synthesis of compound 2:

Compound **1** (1.10 g, 2.2 mmol), 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid 4-methoxybenzyl ester hydrochloride (ACLE•HCl) (0.82 g, 2 mmol) and hydroxybenzotriazole monohydrate (HOBt) (0.54 g, 4 mmol) was dissolved in DMF (5 mL). Then TEA (0.28 mL, 2 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC•HCl) (0.46 g, 2.4 mmol) were added at 0°C. The mixture was stirred for at 0°C for 6 h under N_2 . The reaction mixture was diluted with EA (50 mL), and washed with sat NaHCO_3 and water, then dried with brine and anhydrous Na_2SO_4 before concentrated in *vacuo*. The product was purified by flash chromatography (EA: Hex = 1:1) to afford **2** as a pale-yellow solid. (0.806 g, 47.2%) ^1H NMR (300 MHz, CDCl_3) δ 7.45 (d, $J = 7.4$, 4H), 7.33 (d, $J = 8.5$, 4H), 7.24 (t, $J = 7.5$, 4H), 7.15 (t, $J = 7.2$, 2H), 7.06 (d, $J = 8.1$, 2H), 6.88 (d, $J = 8.7$, 2H), 5.75 (dd, $J = 8.8, 4.9$, 1H), 5.21 (q, $J = 11.8$, 2H), 4.92 (d, $J = 5.0$, 2H), 4.45 (dd, $J = 36.7, 11.8$, 2H), 4.15 – 4.08 (m, 1H), 3.79 (s, 3H), 3.46 (dd, $J = 55.0, 18.3$, 2H), 2.29 (s, 3H), 2.11 (t, $J = 6.6$, 2H), 1.76 (td, $J = 15.1, 6.5$, 1H), 1.66 – 1.18 (m, 15H). ^{13}C NMR (75 MHz, CDCl_3) δ 21.1, 23.5, 27.3, 28.5, 30.5, 32.3, 43.4, 43.6, 55.4, 57.7, 59.5, 68.3, 71.0, 80.7,

114.2, 125.8, 126.3, 126.3, 126.8, 127.9, 128.7, 128.7, 128.7, 130.8, 135.9, 143.2, 146.3, 155.8, 160.1, 161.3, 164.6, 173.1. ESI-MS (m/z) calcd: 853.47 $[M+H]^+$; found: 853.19 $[M+H]^+$.

Synthesis of compound 3:

Compound **2** (0.85 g, 1 mmol) and 4-aminothiophenol (0.19 g, 1.5 mmol) was dissolved in dry DMF (1.0 mL) to obtain a pale-yellow solution. *N*-methylmorpholine (NMM) (220 μ L, 2 mmol) was added and the mixture was stirred at r.t for 2 h under N_2 . The reaction mixture was diluted with EA (30mL), washed with water, dried with brine and anhydrous Na_2SO_4 before concentrated in *vacuo*. The product was purified with flash chromatography (DCM: MeOH = 50:1) to attain the **3** as pale-yellow residue (0.327 g, 34.7%). 1H NMR (300 MHz, $CDCl_3$) δ 7.44 (d, J = 7.4, 4H), 7.35 – 7.20 (m, 8H), 7.18 – 7.11 (m, 4H), 7.06 (d, J = 8.1, 2H), 6.87 (d, J = 8.7, 2H), 6.50 (d, J = 8.5, 2H), 5.62 (dd, J = 8.6, 4.7, 1H), 5.00 (dd, J = 24.7, 11.9, 2H), 4.83 (d, J = 4.8, 1H), 4.13 (t, J = 14.2, 2H), 3.82 – 3.68 (m, 4H), 3.62 – 3.44 (m, 2H), 3.25 (d, J = 18.0, 1H), 2.29 (s, 3H), 2.09 (t, J = 6.6, 2H), 1.86 – 1.26 (m, 16H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.0, 23.5, 28.4, 28.7, 30.7, 32.5, 39.3, 43.4, 55.3, 57.8, 59.3, 67.6, 70.7, 80.3, 114.0, 115.5, 120.6, 124.1, 126.2, 127.2, 127.8, 128.5, 128.6, 128.7, 130.6, 135.7, 135.9, 143.4, 146.5, 147.3, 155.7, 159.8, 161.5, 164.3, 173.0. ESI-MS (m/z) calcd: 942.39 $[M+H]^+$; found: 942.20 $[M+H]^+$.

Synthesis of compound 4:

Compound **3** (66 mg, 0.07 mmol) was dissolved in 2 mL of DMF. BHQ-3 carboxylic acid (Black Hole Quencher-3) (50 mg, 0.07 mmol), *N*-Methylmorpholine *N*-oxide (NMO) (8 mg, 0.07 mmol) and HATU (27 mg, 0.07 mmol) were added and the mixture and stirred at r.t for 24 h. The reaction mixture was diluted with EA, washed with water and dried with brine and anhydrous Na₂SO₄. The product was concentrated in *vacuo* and purified with flash chromatography (DCM: MeOH = 50:1) to yield **4** as a dark blue residue (52 mg, 50.3%). ESI-MS (*m/z*) calcd: 1471.87 [M+H]⁺; found: 1470.55 [M+H]⁺.

Synthesis of compound 5:

A solution of 2% TFA in DCM (2mL) was added to compound **4** (52 mg, 0.035 mmol) to obtain a deep blue solution and stirred at 0°C for 2 h. After the reaction, the product was concentrated in *vacuo* and dried under high vacuum to afford **5** as a crude product. The crude product was used without any further purification. ESI-MS (*m/z*) calcd: 1215.52 [M+H]⁺; found: 1214.55 [M+H]⁺.

Synthesis of compound 6:

Cy5.5 NHS ester (Cy5.5-*N*-hydroxysuccinimide ester) (10 mg, 0.013 mmol) and DIPEA (2.26 μ L, 0.013 mmol) was added to a solution of compound **5** (42.5 mg, 0.035 mmol) dissolved in DMF (1 mL) to obtain a dark blue solution. The reaction mixture was stirred at r.t for 4 h. After the reaction, the product was purified by HPLC with a gradient of solvent B mixed with solvent A (50% to 100% for 10 min) with 6.0 mL/min flow rate, monitored by UV/Vis absorbance of 670 nm to attain **6**. Compound **6** retention time was 9.6 min. ESI-MS: *m/z* calcd: 1779.85 $[M+H]^+$; found: 1779.72 $[M+H]^+$, 890.29 $[M+2H]^+$.

Synthesis of compound 7 (BLR):

Compound **6** (5 mg, 0.0028 mmol) was dissolved in a mixture of 20% TFA and 5% anisole in DCM (500 μ L) and stirred at r.t for 1 h. The product was purified by HPLC after the reaction, with a gradient of solvent B mixed with

solvent A (50% to 100% for 10 min) with 6.0 mL/min flow rate, monitored by UV/Vis absorbance of 670 nm to attain **7**. Compound **7 (BLR)** retention time was 6.2 min. ESI-MS: m/z calcd: 1559.74 $[M+H]^+$; found: 1559.50 $[M+H]^+$, 780.96 $[M+2H]^+$.

Synthesis of compound 8 (DBLR):

DBCO-NHS ester (2.00mg, 0.00497 mmol) was added to compound **7** (2.00 mg, 0.00108 mmol) dissolved in DMF (1 mL), followed by adding DIPEA (0.38 μ L, 0.00216 mmol). The reaction mixture was allowed to stirred at r.t for 12 h. After the reaction, the final product was purified by HPLC with a gradient of solvent B mixed with solvent A (50% to 100% for 10 min) with 6.0mL/min flow rate, monitored by UV/Vis absorbance of 670 nm to attain **8**. Compound **8 (DBLR)** retention time was 12.3 min. ESI-MS: m/z calcd: 1876.41 $[M+H]^+$; found: 938.42 $[M+2H]^+$.

Fluorescence detection of enzyme activity. The stock solution of BLR and DBLR in dimethyl sulfoxide was diluted with PBS buffer (0.1M, pH = 7.4) to attain 10 μ M of the respective solution. Thereafter, the fluorescence emission was detected using λ_{ex} = 670nm in a 1 cm quartz cuvette after adding TEM-1 Blas and AmpC Blas (10 nM) to the probe solution in PBS solution and incubated at 37°C after 1 h. For negative control experiment, the fluorescence emission was measured after incubating the diluted probe in PBS solution with TEM-1 Blas

together with class A Blas inhibitor Clavulanic Acid (100 μ M), or AmpC Blas in combination with class C Blas inhibitor Aztreonam (100 μ M) at 37°C after 1 h. The excitation and emission slit widths of the spectroscopy were kept at 10.0 nm for both experiments.

General method for intrinsic metabolic labeling of the bacterial and fluorescence imaging. Bacterial cells were seeded and cultured using Brain Heart Infusion (BHI) broth in 5 mL falcon tubes under 37°C and was shaken overnight. Afterward, OD₆₀₀ = 0.5 of bacterial cells were resuspended in BHI broth in a microcentrifuge tube. Thereafter, 3-azido-D-alan hydrochloride (1mM) was added to the bacteria sample and incubated at 37°C for 1 hour allowing native metabolism of 3-azido-D-alan onto bacterial peptidoglycan. Subsequently, the medium was removed and the bacterial cells were washed thrice with PBS buffer. After washing, the bacterial cells were resuspended in HEPES Buffer and incubated for 1 h with DBCO-Cy3, followed by washing thrice with PBS buffer. Finally, the bacteria sample was resuspended in HEPES buffer and imaged using a confocal microscope after it was plated on the poly L-lysine coated glass slides. (Cy5.5: λ_{ex} = 640 nm, λ_{em} = 675/25 nm.)

Metabolic labeling of the bacteria surface with DBLR and fluorescence imaging. Bacterial cells were seeded and cultured using Brain Heart Infusion (BHI) broth in 5 mL falcon tubes under 37°C and was shaken overnight. Afterward, OD₆₀₀ = 0.5 of bacterial cells were resuspended in BHI broth in a microcentrifuge tube. Thereafter, 3-azido-D-ala hydrochloride (1mM) was added to the bacteria sample and incubated at 37°C for 1 hour allowing native metabolism of 3-azido-D-alan onto bacterial peptidoglycan. Subsequently, the medium was removed and the bacterial cells were washed thrice with PBS buffer. After washing, the bacterial cells were resuspended in HEPES Buffer and incubated with BLR or DBLR (10 μ M) for 1 h, followed by washing thrice with PBS buffer. The bacteria sample was resuspended in HEPES buffer and imaged

using a confocal microscope after it was plated on the poly L-lysine coated glass slides. (Cy5.5: $\lambda_{\text{ex}} = 640 \text{ nm}$, $\lambda_{\text{em}} = 675/25 \text{ nm}$.)

Bacterial cell viability test. The bacterial cells with an OD_{600} of 0.7 were diluted with PBS buffer to attain a final concentration of $2 \times 10^8 \text{ CFU/mL}$. 60 μl aliquots of the bacteria solution were mixed together with different concentration of 3-azido-D-alanine (0.1mM~2mM) in the 96-well plate. After 1 h incubation, 25 μl of each suspension mixture was pipetted and plated onto the Lysogeny broth (LB) agar plates and incubated overnight at 37°C . After incubation, the number of colony-forming units was counted.

***In vivo* imaging using zebrafish.** All animal experimental protocols were in compliance with Nanyang Technological University, School of Chemical and Biochemical Engineering, Animal Care Committee. Wild type zebrafish (*Danio rerio*) were grown and maintained following the standards of zebrafish care.

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Chapter 6

Summary and Perspectives

Enzymes are extensively found in both mammalian and bacterial cells, which serve pivotal roles in many pathological conditions. Therefore, developing a new strategy to study the enzyme activities will allow elucidation of the biological processes and provide new insights for diagnostic and diseases treatments. Hence, the research works mentioned in this dissertation had focused on the development of different novel fluorescent reporters to monitor the biological function of enzyme found in both mammalian and bacterial cells, namely furin and class C β -lactamases. Furin is ubiquitously expressed and cyclized from TGN to the endosomal compartment and cell surface of mammalian cells, where furin at each compartment catalyze different unique substrates. Furthermore, furin play vital roles in many pathological conditions including cancer progression and infectious disease, thus it is essential to monitor furin activities at specific organelle to unravel it function in different diseases.

Hence, by taking the advantages of optical imaging, we developed small-molecule peptide-based FRET probe capable to monitor furin activities at the cellular surface upon external light stimulation. The reporter was conjugated with a hydrophobic fatty acid, which helped to localize and immobilized the fluorescent probe on the cell membranes. Furthermore, the photo-removable functionalized peptide sequence of the reporter had allowed for a light-controlled visualization of furin activities. This unique design of a spatiotemporally controlled reporter will enable us to control and monitor furin activities at different stages of disease progression. Furthermore, precisely controlling the laser irradiation within the cells will allow the study of furin dynamic at target areas or organelle. However, there is a limitation in the current probe design as it

utilized UV light irradiation to trigger the release of the photoremovable moiety. The short-wavelength UV irradiation undergoes scattering and absorption in the biological tissue, leading to a low signal to noise ratio and low tissue penetration, which limits the application of the fluorescent reporter for *in vivo* imaging. Therefore, it is important to develop a new strategy to use long-wavelength light irradiation to trigger the photolysis process in order to expand the practical application for *in vivo* or clinical studies.

Subsequently, we wanted to design a fluorescent probe that can visualize furin activities selectively at the Golgi apparatus. Using the principle similar to the spatiotemporal controllable reporter, we developed a furin-responsive fluorescent reporter conjugated with a Golgi-targeting ligand, sphingosine. Although this ligand was commercially used to localize the Golgi apparatus in living cells, our fluorescent reporter was localized at lysosome instead. We hypothesized the changed in the localization area of the reporter could be caused by the change in the overall lipophilicity of the reporter after conjugating the ligand to the furin-responsive peptide. Therefore, rational re-design of the reporter and testing may be required to improve the efficiency in localizing the probe at the Golgi. There are several potential approaches to improve the efficiency in localizing a fluorescent reporter at the Golgi apparatus. One strategy is to use a photoactivatable reporter concept in chapter two to selectively imaging the Golgi apparatus through precise laser irradiation. Another potential approach is the development of new ligand capable to target different receptor located on the Golgi apparatus to improve the selectivity of the fluorescent reporter.

Additionally, furin is closely associated with diseases progression like cancers. Therefore, we explored the viability of using furin as the biomarker to trigger the delivery platform to specifically target tumor cells for early diagnostic and treatment. Hence, we developed a furin-triggered metabolic precursor to exogenously generate azide group on the target cell surfaces for subsequent

labeling. Preliminary results had shown the ability to use furin-triggered metabolic precursor to selectively label on furin expressing tumor cells, which is a positive indication of using furin as a biomarker for drug delivery. The promising result also indicates the potential application of using the precursor for precision therapeutic application or as a visualizing aid to assist doctors in identifying tumor cells during surgery.

In bacterial cells, important enzymes like β -lactamases play pivotal roles in antibiotic drug-resistant, which is a major health concern. Therefore, we developed a fluorescent probe to selectively detect and monitor class C β -lactamases type resistant bacteria. The reporter was conjugated with a bulky DBCO group at the 7' position of the cephalosporin, which shown remarkable improvement in the selectivity of the probe towards class C β -lactamases. The novel fluorescent probe had displayed promising results to selectively visualize class C β -lactamases and is feasibility for *in vivo* application. The successful development of this fluorescent reporter will provide a new rapid test method for hospital to accurately detect class C β -lactamases for better decision in treatment strategy.

In conclusion, although we have successfully developed several unique reporters in monitoring enzymatic activities, more efforts are still required to improve the applicability for *in vivo* and clinical trials. Currently, for the spatiotemporal controllable fluorescent reporter, UV light illumination was used to trigger the probe. The penetration depth of UV light is a potential issue to apply the reporter in living animal studies. As such, NIR photo-removable moiety should be explore as a possible solution to future application.

Lastly, antibiotic resistant remains as a major threat on infectious diseases. Furthermore, new resistant bacteria are emerging every day, which is worsening the issue. Therefore, there is a vital need to development new strategies to combat

against the infectious pathogens. More works can be conduct on less investigate resistant enzymes, like carbapenemase, to understand the biological functions and aid in developing new therapeutic drugs.

List of Publications

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