Design Genetic Fluorescent Probes to Detect Protease Activity and Calcium-Dependent Protein-Protein Interactions in Living Cells

Ning Chen
Georgia State University

Follow this and additional works at: https://scholarworks.gsu.edu/chemistry_diss

Part of the Chemistry Commons

Recommended Citation
Chen, Ning, "Design Genetic Fluorescent Probes to Detect Protease Activity and Calcium-Dependent Protein-Protein Interactions in Living Cells." Dissertation, Georgia State University, 2008. https://scholarworks.gsu.edu/chemistry_diss/43

This Dissertation is brought to you for free and open access by the Department of Chemistry at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Chemistry Dissertations by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact scholarworks@gsu.edu.
DESIGN GENETIC FLUORESCENT PROBES TO DETECT PROTEASE ACTIVITY AND CALCIUM-DEPENDENT PROTEIN-PROTEIN INTERACTIONS IN LIVING CELLS

by

NING CHEN

Under the Direction of Professor Jenny J. Yang

ABSTRACT

Proteases are essential for regulating a wide range of physiological and pathological processes. The imbalance of protease activation and inhibition will result in a number of major diseases including cancers, atherosclerosis, and neurodegenerative diseases. Although fluorescence resonance energy transfer (FRET)-based protease probes, a small molecular dye and other methods are powerful, they still have drawbacks or limitations for providing significant information about the dynamics and pattern of endogenous protease activation and inhibition in a single living cell or in vivo. Currently protease sensors capable of quantitatively measuring specific protease activity in real time and monitoring activation and inhibition of enzymatic activity in various cellular compartments are highly desired. In this dissertation, we report a novel strategy to create protease sensors by grafting an enzymatic cleavage linker into a sensitive location for changing chromophore properties of enhanced green fluorescent protein (EGFP) following protease cleavage, which can be used to determine protease activity and track protease
activation and inhibition with a ratiometric measurement mode in living cells. Our designed protease sensors exhibit large relative ratiometric optical signal change in both absorbance and fluorescence, and fast response to proteases. Meanwhile, these protease sensors exhibiting high enzymatic selectivity and kinetic responses are comparable or better than current small peptide probes and FRET-based protease probes. Additionally, our protease sensors can be utilized for real-time monitoring of cellular enzymogen activation and effects of inhibitors in living cells. This novel strategy opens a new avenue for developing specific protease sensors to investigate enzymatic activity in real time, to probe disease mechanisms corresponding to proteases in vitro and in vivo, and to screen protease inhibitors with therapeutic effects. Strong fluorescence was still retained in the cleaved EGFP-based protease sensors, which stimulated us to identify the EGFP fragment with fluorescence properties for further understanding chromophore formation mechanisms and investigating protein-protein interactions through fluorescence complementation of split EGFP fragments. Through fusing EF-hand motifs from calbindin D9k to split EGFP fragments, a novel molecular probe was developed to simultaneously track the calcium change or calcium signaling pathways and calcium-dependent protein-protein interaction in living cells in real time.

INDEX WORDS: Protease sensor, Ratiometric measurement, EGFP, Calcium-dependent, Protein-protein interactions, Fluorescence complementation
DESIGN GENETIC FLUORESCENT PROBES TO DETECT PROTEASE ACTIVITY AND CALCIUM-DEPENDENT PROTEIN-PROTEIN INTERACTIONS IN LIVING CELLS

by

NING CHEN

A Dissertation Submitted in Partial Fulfillment of the Requirement of the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2008
Copyright by
Ning Chen
2008
DESIGN GENETIC FLUORESCENT PROBES TO DETECT PROTEASE ACTIVITY AND CALCIUM-DEPENDENT PROTEIN-PROTEIN INTERACTIONS IN LIVING CELLS

by

NING CHEN

Committee Chair: Jenny J. Yang
Committee: Giovanni Gadda
           Zhi-ren Liu
           Yujun George Zheng
           Robert Wohlhueter

Electronic Version Approved:

Office of Graduate Studies
Colleges of Arts and Sciences
Georgia State University
December 2008
ACKNOWLEDGEMENTS

All of the work in this dissertation is carried out under the direction of Dr. Jenny J. Yang. I would like to express my high appreciation to Dr. Jenny J. Yang for her kindly providing this research opportunity, demonstrative guidance, valuable suggestion, discussion and support throughout this research.

I would like to thank Dr. Giovanni Gadda for the helpful guidance of catalytic kinetic studies on protease sensors and valuable discussion.

I would like to thank Dr. Zhi-Ren Liu for the helpful guidance of mammalian cell culture and transfection.

I would like to thank Dr. Lily Yang from Emory University for kindly providing MIA PaCa-2 cell lines, valuable guidance and discussion on caspase activation and inhibition.

I would like to thank Dr. Rihe Liu from The University of North Carolina at Chapel Hill for kindly offering active caspase-3 and caspase-8 enzyme.

I would like to thank Dr. Jin Zou, Yun Huang, Shen Tang, and Mike Kirberger from biosensor group for much research technical guidance, and lots of good suggestion and discussion. I would like to specially thank Mike Kirberger for his proofreading and suggestion.

I would like to thank Dr. Shunyi Li for helpful guidance to protein purification, mass spectroscopy, amino acid analysis and discussion.

I would like to thank Dr. Yiming Ye from the Centers for Disease Control and Prevention (CDC) for providing the graft approach on the design of EGFP-based protease sensor variants and protein sequence analysis.
I would like to thank Dr. Robert Wohlhueter from the Centers for Disease Control and Prevention (CDC) for providing a lot of comments on my dissertation and editing.

I would like to thank Dr. Siming Wang and Sarah Cepada at Georgia State University for mass spectrometry analysis and discussion on protease sensors.

I would like to thank Dr. Wei Yang for the help of NMR data analysis, much valuable guidance and discussion.

I would like to thank Dr. Yang’s group members for their helpful discussion.

I would like to thank my dissertation committee members, Drs. Jenny J. Yang, Giovanni Gadda, Zhi-Ren Liu, Yujun George Zheng and Robert Wohlhueter. I would like to thank my qualify examination committee, Drs. Giovanni Gadda, Binghe Wang and Kathryn B. Grant. I would also like to thank the Department of Chemistry at Georgia State University for providing the opportunity to complete my doctorate degree study and research.

I would like to thank the Molecular Basis of Disease program in Georgia State University for supporting my study and research.

I would like to specially thank my wife, Suqin Yao, for her help and support to my study. I would like to specially thank her for taking care of my son although she is busy in her work. The special appreciation also would give my parents for their support.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ............................................................................................................ IV

LIST OF TABLES ...................................................................................................................... XIII

LIST OF FIGURES .................................................................................................................. XIV

LIST OF ABBREVIATIONS ...................................................................................................... XIX

Chapter 1  Background of monitoring intracellular processes related to protease activity and calcium signaling in living cells ................................................................. 1

1. 1  Intracellular processes ................................................................................................... 1

1. 2  Proteases ...................................................................................................................... 2

   1.2.1  Protease functions ............................................................................................... 2

   1.2.2  Significance of protease investigations ................................................................. 4

   1.2.3  Protease classifications ....................................................................................... 6

   1.2.4  Substrate specificity of proteases ...................................................................... 8

   1.2.5  Current methods for protease detection ............................................................. 8

   1.2.6  Criteria for an ideal protease sensors ................................................................. 13

1. 3  Calcium signaling ........................................................................................................ 14

   1.3.1  Regulation of intracellular calcium .................................................................. 16

   1.3.2  Major players to mediate intracellular calcium accumulation ......................... 17

   1.3.3  Calcium signaling and related diseases ............................................................. 19

1. 4  Fluorescent proteins .................................................................................................. 20
1.4.1 Properties of green fluorescent proteins ........................................................................ 20
1.4.2 Chromophore formation of green fluorescent proteins.............................................. 22
1.4.3 Spectral properties of fluorescent proteins ................................................................. 26
1.4.4 Applications of green fluorescent protein .................................................................. 27
1.4.5 Current protease sensors based on GFP ................................................................. 29
1.5 Limitations of FRET-based fluorescent protein biosensors ........................................... 30
1.6 Novel strategy for protease sensor design ................................................................. 32
1.7 Objectives of this dissertation ......................................................................................... 34

Chapter 2 Materials and methods ......................................................................................... 37
2.1 Design and construction of protease sensors ................................................................. 37
2.2 Construction of plasmid DNA EGFP fragments .......................................................... 39
2.3 Competent cell preparation ......................................................................................... 39
2.4 Subcloning for protease sensors ............................................................................... 40
2.5 Expression in E. coli and purification with His-tag column .................................... 41
2.6 Protease sensor purification with Hitrap Q column .................................................. 42
2.7 Protein refolding with urea ....................................................................................... 43
2.8 UV-visible spectroscopy ............................................................................................ 44
2.9 Fluorescence excitation and emission spectroscopy .................................................. 44
2.10 MALDI-TOF mass spectroscopy .............................................................................. 45
2.11 Change in optical properties of sensors following protease cleavage .................... 45
2.12 Cleavage identification with SDS-PAGE analysis .................................................... 46
2.13 Separation of EGFP fragments after protease digestion ......................................... 46
2.14 Identification of cleavage sites of EGFP-T1 ................................................................. 47
2.15 Optical properties of purified EGFP fragments ............................................................. 47
2.16 Determination of steady-state kinetic parameters of protease sensors ....................... 48
2.17 Inhibition of protease cleavage .................................................................................... 48
2.18 Cleavage specificity of protease sensors ...................................................................... 49
2.19 Extinction coefficient calculation of cleaved products ............................................... 50
2.20 Relative signal change calculation ............................................................................... 51
2.21 Mammalian cell culture ............................................................................................... 51
2.22 Preparation of cell plates and transfection of protease sensors ................................... 53
2.23 Transfection of EGFP fragments ................................................................................ 54
2.24 Fluorescence imaging .................................................................................................. 54
2.25 Immunofluorescence ................................................................................................... 56
2.26 Confocal microscope imaging ..................................................................................... 57
2.27 Protease assay in cell lysates ....................................................................................... 57
2.28 Western blot ................................................................................................................ 58
2.29 Detection of half-time for maximum caspase-3 activation in living cells .................... 59
2.30 pH change examination in living cells ......................................................................... 60
2.31 Fluorescence spectral scanning of protease sensors in living cells .............................. 61
2.32 Statistical analysis ....................................................................................................... 63

Chapter 3  Design sensitive EGFP-based trypsin sensors for living cell imaging .......... 64
3.1 Introduction ..................................................................................................................... 64
  3.1.1 Trypsin function ......................................................................................................... 64
3.1.2 Substrate specificity of trypsin................................................................................... 65
3.1.3 Trypsin measurements ............................................................................................... 68
3.1.4 Objectives .................................................................................................................. 70
3.2 Design and construction of trypsin sensors..................................................................... 70
3.3 Optical properties of trypsin sensors............................................................................... 72
3.4 Optical change of trypsin sensors following the action of trypsin.................................. 78
3.5 Verification of trypsin sensor cleavage........................................................................... 80
3.6 Kinetic study of trypsin sensors ...................................................................................... 82
3.7 Inhibition of trypsin sensors ........................................................................................... 85
3.8 Selectivity of trypsin sensors .......................................................................................... 87
3.9 Trypsinogen activation in living cells............................................................................. 88
3.10 Fluorescence imaging of trypsin sensor.......................................................................... 91
3.11 Trypsinogen activation in pancreatic cancer cells .......................................................... 95
3.12 Localization of trypsinogen activation............................................................................ 96
3.13 Plasmid DNA construction of trypsin sensors with optimal cleavage linkers.............. 99
3.14 Optical properties of trypsin sensors with optimal cleavage linkers............................... 99
3.15 Kinetic parameters for trypsin sensors with optimal cleavage linkers......................... 102
3.16 pH effects on trypsin sensors ....................................................................................... 105
3.17 Extinction coefficient constants of products after trypsin sensor cleavage ................. 106
3.18 Relative signal change calculation of trypsin sensors.................................................. 108
3.19 Optical signal change EGFP-T1 upon the action of chymotrypsin................................. 108
3.20 Conclusions and significance......................................................................................... 111
Chapter 4  Developing caspase sensors for imaging apoptosis at early stage in living cells

4. 1  Introduction

4.1.1  Apoptosis, caspase actions and tumors

4.1.2  Apoptosis pathways

4.1.3  Inducers for caspase-3 activation

4.1.4  Structure and substrate specificity of caspases

4.1.5  Current methods for detection of caspase activity and their limitations

4.1.6  Objectives to the development of caspase sensors

4. 2  Design and construction of caspase-3 sensors

4. 3  Optical properties of caspase sensors

4. 4  Optical signal change following caspase digestion

4. 5  Verification of caspase sensors cleavage

4. 6  Kinetic study of caspase sensors

4. 7  Fluorescence imaging of caspase-3 sensors

4. 8  Caspase-3 inhibition tracking in living cells

4. 9  Shorter half-time for maximum caspase-3 activation in MIA PaCa-2 cells

4.10  Fluorescence imaging of caspase-3 sensors with optimal cleavage linkers

4.11  Fluorescence imaging of caspase-8 sensors

4.12  Another new pathway for caspase-8 activation in living cells

4.13  Confirmation of caspase-3 activation

4.14  Analysis of pH change following STS induction in living cells

4.15  Spectral signal change of caspase-3 sensor in living cells
Chapter 5  EGFP-based thrombin sensors ................................................................. 198

5. 1  Introduction ............................................................................................................. 198
   5.1.1 Biological function of thrombin ......................................................................... 198
   5.1.2 Thrombin structure and active sites ................................................................. 199
   5.1.3 Thrombin substrate specificity ......................................................................... 201
   5.1.4 Current methods for detection of thrombin ...................................................... 202
   5.1.5 Significance of this study ................................................................................. 203
5. 2  Construction of thrombin sensors ......................................................................... 203
5. 3  Optical properties of thrombin sensors ............................................................... 204
5. 4  Optical change of thrombin sensors .................................................................... 205
5. 5  Verification of thrombin sensors cleavage .......................................................... 205
5. 6  Kinetic study of thrombin sensors ....................................................................... 209
5. 7  Extinction coefficient constants of products after thrombin sensor cleavage ..... 211
5. 8  Relative signal change calculation of thrombin sensors ..................................... 212
5. 9  Conclusions and significance ............................................................................. 215

Chapter 6  Fluorescence properties of EGFP fragments ............................................. 216

6. 1  Introduction ............................................................................................................. 216
6. 2  Protease sensitivity of EGFP ............................................................................... 217
Chapter 7  Investigation of protein-protein interactions using fluorescence complementation of split EGFP fragments

7.1 Introduction
7.2 Protein-protein interactions
7.3 Current investigations of split GFP
7.4 Strategy for calcium-dependent protein-protein interaction
7.5 Fluorescence imaging of protein-protein interaction
7.6 Calcium effect on protein-protein interaction
7.7 Optical properties of N-EGFP-EF1 and C-EGFP-EF2 expressed in bacteria
7.8 EF-hand dimerization and EGFP fragment complementation
7.9 Conclusions

Chapter 8  Conclusion and significance

References

Publications

Patents
LIST OF TABLES

Table 1.1. Fluorescent protein properties ........................................................................................................28
Table 3.1. Ratio of deprotonated and protonated chromophore of trypsin sensors ................. 74
Table 3.2. Comparison of kinetic parameters between trypsin sensors and commercial kits ... 85
Table 3.3. Substrate specificity of selected proteases ........................................................................... 88
Table 3.4. Designed trypsin sensors with different cleavage linkers ................................................ 100
Table 3.5. Kinetic parameters for first generation of trypsin sensors ........................................ 103
Table 3.6. Optimization of residues in the P and P’ region of cleavage linkers .......................... 104
Table 3.7. Comparison of catalytic parameters between trypsin sensors and commercial kits 105
Table 3.8. Catalytic parameters for trypsin under various pH conditions ................................ 106
Table 3.9. Extinction coefficients of cleavage products from trypsin sensors .......................... 107
Table 3.10. Relative signal changes of trypsin sensor variants ................................................... 109
Table 4.1. The specific recognition sites of various caspases .......................................................... 126
Table 4.2. Designed caspase sensor variants and their cleavage linker sequences .................. 133
Table 4.3. Composition of various reaction buffers for caspase-3 .............................................. 140
Table 4.4. Catalytic kinetic parameters of caspase-3 sensors ...................................................... 145
Table 5.1. Designed thrombin sensors and their cleavage linkers ............................................. 204
Table 5.2. Catalytic parameters for thrombin in thrombin reaction buffer ................................ 211
Table 5.3. Extinction coefficients of cleaved products from thrombin sensors .......................... 212
Table 5.4. The relative signal change of thrombin sensor variants ............................................. 213
Table 6.1. The molecular mass of fragments during EGFP trypsin digestion ............................ 225
Table 6.2. The molecular masses of fragments during EGFP-T1 chymotrypsin digestion ...... 228
LIST OF FIGURES

Figure 1. 1. Specificity diagram between enzyme and substrate...................................................9
Figure 1. 2. The model of FRET-based protease sensor..............................................................12
Figure 1. 3. The ON and OFF mechanisms and modulation of intracellular calcium levels......15
Figure 1. 4. Schematic topology of the secondary structure of GFP..............................................21
Figure 1. 5. The proposed chromophore formation mechanism of EGFP......................................24
Figure 1. 6. Three-step mechanism of GFP chromophore formation...........................................25
Figure 1. 7. Design of ratiometric EGFP-based protease sensors..................................................33
Figure 3. 1. Interaction between substrate and catalytic binding pocket in trypsin.......................66
Figure 3. 2. Grafting location selection and cleavage linker for the design of trypsin sensors........71
Figure 3. 3. Optical properties of designed trypsin sensors..........................................................73
Figure 3. 4. Effects from inserted linkers on spectral change of EGFP-based trypsin sensors........76
Figure 3. 5. Switch between protonated and deprotonated forms of chromophore in EGFP...........77
Figure 3. 6. Optical signal change of trypsin sensors following trypsin digestion........................79
Figure 3. 7. Cleavage verification of trypsin sensors using SDS-PAGE and MALDI-MS..............81
Figure 3. 8. Kinetic studies of EGFP-T1 conducted in trypsin digestion buffer...............................83
Figure 3. 9. Inhibition effect on the cleavage of EGFP-T1............................................................86
Figure 3. 10. The selectivity of EGFP-T1 for proteases...............................................................89
Figure 3. 11. Trypsin activity determination in cell lysates using trypsin peptide substrate.............90
Figure 3. 12. Fluorescence imaging and signal change of EGFP-T1 in living cells.........................92
Figure 3. 13. Subcellular location targeting of EGFP-T1-Mito and EGFP-T1-ER..............................94
Figure 3. 14. Localization of trypsin activation and expression of EGFP-based trypsin sensor......98
Figure 3. 15. UV-visible spectra of EGFP-based trypsin sensor variants. .........................101
Figure 3. 16. Optical signal change of EGFP-T1 following chymotrypsin digestion. ..........110
Figure 4. 1. Diagram of caspase-3 activation and apoptosis pathways. .........................115
Figure 4. 2. The molecular structures of inducers for caspase-3 activation. .....................119
Figure 4. 3. The process from pro-caspase to active caspase tetramer. .........................123
Figure 4. 4. The three-dimensional structure of active caspase-3 (PDB, 2J30)...............124
Figure 4. 5. Interactions between the substrate and residues in the active site of caspase-3. 125
Figure 4. 6. Spectral characteristics of EGFP-based caspase-3 sensors. .........................135
Figure 4. 7. Fluorescence emission spectra of representative caspase-3 sensors. ..........136
Figure 4. 8. Spectral change of EGFP-C3B under the caspase-3 reaction buffer system
containing 10% sucrose following caspase-3 digestion. .............................................139
Figure 4. 9. The cleavage confirmation of EGFP-C3B following caspase-3 digestion. .......141
Figure 4. 10. Cleavage of EGFP-C3B following evaluation by MALDI-MS and SDS-PAGE. 143
Figure 4. 11. Fluorescence imaging and signal change of EGFP-C3B in HeLa cells following
STS induction. .......................................................................................................147
Figure 4. 12. Fluorescence imaging and signal change of EGFP-wt in HeLa cells following STS
induction. ................................................................................................................148
Figure 4. 13. Fluorescence imaging and signal change of EGFP-C3B in MIA PaCa-2 cells
following STS induction. ........................................................................................149
Figure 4. 14. Inhibition of caspase-3 activation detected with EGFP-C3B in living cells.....153
Figure 4. 15. Shorter half-time for maximum caspase-3 activation in MIA PaCa-2 cells. ....154
Figure 4. 16. Fluorescence imaging and signal change of EGFP-C3C in HeLa cells following
STS induction. .......................................................................................................155
Figure 4. 17. Fluorescence imaging and signal change of EGFP-C3C in MIA PaCa-2 cells following STS induction. ......................................................................................156

Figure 4. 18. Comparison of fluorescence ratiometric signal change of EGFP-C3C in different cell lines.................................................................................................................157

Figure 4. 19. Caspase-3 activation in cytosol followed by migration from the cytosol to the nucleus........................................................................................................................................158

Figure 4. 20. Fluorescence imaging and signal change of EGFP-C8A in HeLa cells following STS induction. .......................................................................................................161

Figure 4. 21. Fluorescence imaging and signal change of EGFP-C8A MIA PaCa-2 cells following STS induction. ......................................................................................162

Figure 4. 22. Verification of caspase-3 activation in living cells following STS induction......166

Figure 4. 23. Diagram of DEVD-NucView488 caspase-3 substrate for living cell imaging. ....168

Figure 4. 24. Detection of caspase-3 activation using DEVD-NucView488 caspase-3 substrate in living cells following STS induction..................................................................... 169

Figure 4. 25. Inhibition of caspase-3 was evaluated using DEVD-NucView488 caspase-3 substrate in living cells following STS induction. ................................................171

Figure 4. 26. Comparison of half-time for maximum caspase-3 activation in living cells using EGFP-C3C and DEVD-NucView488 caspase-3 substrate. .........................172

Figure 4. 27. Caspase-3 activation in different cell lines detected using Western blot. .................174

Figure 4. 28. pH change analysis in living cells following STS induction.............................................177

Figure 4. 29. pH change analysis in living cells following NH₄Cl stimulation.................................178

Figure 4. 30. The excitation spectra of EGFP-based caspase-3 sensors in living cells. ..........179
Figure 4. 31. Fluorescence emission spectrum scanning of EGFP-C3C in MIA PaCa-2 cells following 1 μM STS induction.................................................................181

Figure 4. 32. Fluorescence emission spectrum scanning of EGFP-wt in MIA PaCa-2 cells following 1 μM STS induction.................................................................182

Figure 4. 33. Ratiometric fluorescence signal change in maximum fluorescence emission. .....183

Figure 4. 34. Fluorescence signal change of cells transfected with EGFP-C3C following TNFα stimulation........................................................................................186

Figure 4. 35. Caspase-3 activity determination in HeLa and MIA PaCa-2 cells following 10 ng/ml TNFα induction..............................................................................188

Figure 4. 36. Fluorescence signal change of HeLa cells transfected with EGFP-C3C following TBT stimulation. .................................................................190

Figure 4. 37. Fluorescence signal change of DU-145 cells transfected with EGFP-C3C following DHA stimulation. .................................................................191

Figure 4. 38. Spectral signal change of EGFP-C3B under the caspase-3 reaction buffer containing 10% glycerol following caspase-3 digestion..............194

Figure 4. 39. Conformational change analysis in various caspase-3 reaction buffers using CD spectroscopy.................................................................195

Figure 5. 1. The model structure of thrombin (PDB, 2UUF). .........................................................200

Figure 5. 2. Absorption spectra of EGFP-Th2 following thrombin digestion.........................206

Figure 5. 3. Fluorescence spectra of EGFP-Th2 following thrombin digestion.........................207

Figure 5. 4. The cleavage confirmation of EGFP-Th2 thrombin digestion.................................208

Figure 5. 5. Kinetic studies of EGFP-Th2 conducted in thrombin digestion buffer.................210

Figure 6. 1. The cleavage patterns and kinetics of EGFP-T1 by various proteases. ...............219
Figure 6.2. Effects of trypsin concentrations on digestion pattern and kinetics of EGFP-T1...220

Figure 6.3. The verification for the assemble of two major fragments (P20 and P8) through SDS-PAGE under denaturing and non-denaturing conditions.................................223

Figure 6.4. The structural model of EGFP-T1 with a protease cleavable linker......................226

Figure 6.5. The cleavage pattern of EGFP-T1 by chymotrypsin..............................................229

Figure 6.6. The characteristics of UV-visible spectra of the P20 fragment..............................230

Figure 6.7. The fluorescence characteristics of the P20 fragment............................................232

Figure 7.1. The molecular model of calbindin D9k and interaction with calcium ions........237

Figure 7.2. The design for calcium-dependent protein-protein interactions.........................243

Figure 7.3. The fluorescence complementation of split EGFP fragments fused to EF-hand motifs was confirmed in living cells via co-transfection...............................246

Figure 7.4. The effects of calcium on fluorescence complementation of EGFP fragments in living cells............................................................................................................247

Figure 7.5. Fluorescence signal change in extract of HeLa cells with co-transfection of N-EGFP-EF1 and C-EGFP-EF2 through EGTA titration......................................................248

Figure 7.6. Fluorescence spectra of purified N-EGFP-EF1 fragment from E. coli expression.250

Figure 7.7. Fluorescence signal change of purified N-EGFP-EF1 and C-EGFP-EF2 upon calcium action.............................................................................................................252

Figure 7.8. Relative fluorescence emission change of purified N-EGFP-EF1 and C-EGFP-EF2 during calcium titration.................................................................253
LIST OF ABBREVIATIONS

GFP  Green fluorescent protein
EGFP  Enhanced green fluorescent protein
CaM  Calmodulin
TnC  Troponin C
BFP  Blue fluorescent protein
CFP  Cyan fluorescent protein
YFP  Yellow fluorescent protein
FRET  Fluorescence resonance energy transfer
FACS  Fluorescence-activated cell sorting
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
IPTG  Isopropyl-β-D-thiogalactopyranoside
O.D.  Optical density
DTT  Dichlorodiphenyltrichloroethane
UV-vis  Ultraviolet-visible
FPLC  Fast performance liquid chromatography
HPLC  High performance liquid chromatography
EDTA  Ethylenediaminetetraacetic acid
MALDI-MS  Matrix assisted laser desorption ionization-mass spectroscopy
K_d  Dissociation constant
Wt  Wild type
PAR  Protrinase activation receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNA</td>
<td>p-nitroaniline</td>
<td></td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
<td></td>
</tr>
<tr>
<td>AFC</td>
<td>7-amino-4-trifluoromethylcoumarin</td>
<td></td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
<td></td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
<td></td>
</tr>
<tr>
<td>DED</td>
<td>Death effector domains</td>
<td></td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domains</td>
<td></td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate-labeled casein</td>
<td></td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
<td></td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin-streptomycin</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
<td></td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
<td></td>
</tr>
<tr>
<td>BAPNA</td>
<td>Benzoyl-DL-arginine-4-nitroanilide hydrochloride or Bz-DL-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>mRFP</td>
<td>Monomeric red fluorescent protein</td>
<td></td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
<td></td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
<td></td>
</tr>
<tr>
<td>BCECF-AM</td>
<td>2’, 7’-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>VOC</td>
<td>Voltage-operated channel</td>
<td></td>
</tr>
<tr>
<td>ROC</td>
<td>Receptor-operated channel</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>SOC</td>
<td>Store-operated channel</td>
<td></td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol bisphosphate</td>
<td></td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase</td>
<td></td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol 1,4,5-trisphosphate</td>
<td></td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>IP3 receptors</td>
<td></td>
</tr>
<tr>
<td>RyRs</td>
<td>Ryanodine receptors</td>
<td></td>
</tr>
<tr>
<td>CIF</td>
<td>Calcium influx factor</td>
<td></td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
<td></td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
<td></td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor type 1</td>
<td></td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signaling complex</td>
<td></td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting kinase</td>
<td></td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor (TNF)-related apoptosis-inducing ligand</td>
<td></td>
</tr>
<tr>
<td>FCCS</td>
<td>Fluorescence cross-correlation spectroscopy</td>
<td></td>
</tr>
<tr>
<td>PARISS</td>
<td>Prism and reflector imaging spectroscopy system</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1  Background of monitoring intracellular processes related to protease activity and calcium signaling in living cells

1.1  Intracellular processes

Cells are the basic compositions of human body, which is involved in many pivotal biological processes including calcium homeostasis, calcium signaling, protease generation, activation, regulation and inhibition, and complicated cross-talks between proteins and signal pathways (Radosevic, de Grooth et al. 1995; Kruger, Albrecht et al. 2000; Putney and Ribeiro 2000; Chakraborti, Das et al. 2007). All of these intracellular processes have direct or indirect contributions on the different levels of cellular functions, ranging from genome sequence to transcriptional and translational profiles (Chin, Cade et al. 1987; Peiretti, Alessi et al. 1997; Dijkmans, van Hooijdonk et al. 2008). Many diseases, such as acute or chronic pancreatic diseases, cardiovascular diseases, neurodegenerative diseases and cancers, can be attributed to the misfunction of these intracellular processes (Basu, Castle et al. 2006; Leger, Covic et al. 2006; Chakraborti, Das et al. 2007). A large number of components in the cellular processes, their strong regulatory coupling, the intricate stoichiometry, and the nonlinear nature of the underlying mechanisms, are some of the key elements of cellular complexity that limit our understanding of intracellular systems despite the large amount of information about the elements of these systems. Although advances in genomic or proteomic technologies and instrumentation have provided the tools to know more information for understanding of intracellular processes and corresponding cellular functions (Afjehi-Sadat, Gruber-Olipitz et al. 2004; Chen, He et al. 2004; He, Rosen et al. 2007), direct, novel and effective methods to real-time monitor protease activation or inhibition and calcium-dependent protein-protein interactions
in living cells are still highly desired. These methods will be greatly benefited for the understanding of intracellular processes, which can be used to probe mechanisms of diseases related to these intracellular processes and to develop treatment strategies for these diseases.

1.2 Proteases

1.2.1 Protease functions

Proteases, also called proteolytic enzymes or proteinases, are a group of enzymes that conduct catalysis for the breakdown of proteins through hydrolysis of peptide bonds. Proteases can be found in many different cellular locations in all organisms and constitute approximately 2% of the genomes based on the bioinformatic analysis of mouse and human genomes (Puente, Sanchez et al. 2003; Rawlings, Tolle et al. 2004). As enzymatic proteins, proteases are involved in the control of basic biological processes and the regulation of cellular signaling in living cells, tissues or organisms at a post-translational level (Coughlin 2000; Sakakihara, Fujii et al. 2001; Camerer 2007; Traynelis and Trejo 2007). Therefore, proteases play pivotal regulatory roles in both normal physiological development and pathological processes through the tight regulation of protease activation and inhibition (Laxman, Hall et al. 2002; Buhling, Groneberg et al. 2006; Nixon and Wood 2006; Lopez-Otin and Matrisian 2007). Protease signaling in living organisms is accomplished through comprehensive behaviors, such as the generation and release of proteases, availability of cofactors, presence of protease inhibitors, and activation or inactivation of protease-activated receptors (PARs), which are the four-member family of G protein-coupled receptors (Ossovskaya and Bunnett 2004). Under certain circumstances, proteases are not only involved in protein degradation, but may also participate in activation, growth, cell division, differentiation, migration, signaling and other cellular processes. Under normal physiological
conditions, the control of proteolysis in an organism or tissue is achieved through the balance of protease production, degradation and inactivation as a result of interactions with corresponding endogenous inhibitors for maintaining normal tissue homeostasis (Hirota, Ohmuraya et al. 2006; Nixon and Wood 2006). Alteration in protease expression and activity patterns are linked to a plethora of human pathological processes, including arthritis, bacterial and viral infections, osteoporosis, cardiovascular diseases, cancers, Alzheimer’s disease and other neurodegenerative diseases (Puente, Sanchez et al. 2003; Ossovskaya and Bunnett 2004; Qiu, Owen et al. 2007; Turk and Stoka 2007). Therefore, a detailed understanding of the temporal and spatial functions, or activation and inhibition modes or pathways for a particular protease in a complex cellular environment is of fundamental importance to our understanding of disease progression. The most powerful and direct way to address these issues is to develop sensitive and effective methods that allow for tracking the dynamic imaging of protease activity within a living cell or organism, which will improve our ability to understand the role of proteases, physiological control mechanisms of proteases and pathological modulation methods of imbalanced protease activities. From this we anticipate the development of novel and effective treatments or drugs for protease-related diseases including pancreatic diseases, cardiovascular diseases, neurodegenerative diseases and cancers. The efficiency of such new drugs or treatments, however, will depend on our detailed understanding of protease activation or inhibition in living cells, which emphasizes the importance of developing probes or sensors capable of monitoring the cellular protease activity in real time.
1.2.2 Significance of protease investigations

Due to the elegant balance between activation and inhibition of proteases under normal physiological conditions, and the alteration involved in either initiation or progression of the diseases, proteases are becoming important potential targets for medical intervention and for studies aimed at revealing important physiological functions regulated by these important enzymes. Several protease inhibitors are already available as drugs. For example, angiotensin-converting enzyme (ACE) inhibitors capable of successfully lowering blood pressure (Atkinson, Brown et al. 1980; Staffileno 2005) and the recently-introduced human immunodeficiency virus (HIV) protease inhibitors (Havlir and O'Marro 2004; Poveda, Briz et al. 2005; Temesgen and Feinberg 2007) have attracted extensive attention. Although most inhibitors are available to target ACE or the HIV protease, we still face problems to fight the diseases related to these proteases because the complete human genome contains approximately 500 different members belonging to various protease families with multiple activation and inhibition pathways and more than 400 unknown human proteases (Korenberg, Chen et al. 1995; Robinson, Wu et al. 2000; Southan 2000; Southan 2001; Rossi, Deveraux et al. 2004). These large gaps in our knowledge suggest the potential for new discoveries related to protease involvement in disease processes, their evaluation as drug targets, drug discovery, and the development of therapies for diseases related to protease dys-regulation (Southan 2001). On the other hand, it is already known that a single amino acid mutation in human proteases could result in hereditary or genetic diseases, which also provides our new directions to investigate the mechanisms of diseases resulting from the dysfunction of proteases (Dluhy 2002; Molinari, Meskanaite et al. 2003; Howes, Lerch et al. 2004; Kokame and Miyata 2004). Meanwhile, abnormal physiology and disease can also be resulted from the dysfunction of a particular crucial protease or imbalance in the levels of natural
activators and inhibitors of proteases due to either genetic disorder or environmental conditions (Fujishima, Tochikubo et al. 1983; Ahn, Bae et al. 2007). Currently, many other protease inhibitors are being developed to treat parasitic, fungal and viral infections; inflammatory, immunological, respiratory, cardiovascular and neurodegenerative disorders including Alzheimer's disease, and cancers (Elkin, Miao et al. 2000; Fear, Komarnytsky et al. 2007). Human proteases have also been identified as important prognostic indicators of diseases, such as kallikreins (e.g. prostate specific antigen), cathepsin B, and matrix metalloproteinases (MMPs), which are promising diagnostics for prostate and breast cancers, respectively (Lah, Cercek et al. 2000; Ayala, Tuxhorn et al. 2003; Stephan, Yousef et al. 2004; Talvensaari-Mattila and Turpeenniemi-Hujanen 2005). Hence, protease sensors with high sensitivity and high selectivity for specific proteases are highly desired to improve our ability to diagnose illness and develop targeted drugs and/or therapies.

Moreover, some proteases are also responsible for generating and destroying receptor agonists, and activating and inactivating receptors on the surface of cells, both of which are critical to signal transduction (Ossovskaya and Bunnett 2004). Certain proteases generated from the circulation, inflammatory cells, epithelial or neural tissues, or from other unspecified sources can regulate cell behaviors through the cleavage of PARs. The cleavage performed within the extracellular amino terminus of PARs has provided an exposed tethered ligand domain to bind to, and activate PARs for initiating multiple signaling cascades (Bunnett 2006). Although the activation of PARs through a cleavage-induced mode is an irreversible process, the termination of protease activation receptor signaling can be successfully realized through receptor desensitization, as seen with receptor phosphorylation and uncoupling from G proteins, or downregulation such as receptor degradation by cell-surface and lysosomal proteases (Coughlin
1999; Qualmann, Kessels et al. 2000; Trejo 2003; Stalheim, Ding et al. 2005). Meanwhile, since protease signaling in tissues is also closely-related to the generation and release of proteases, availability of cofactors, presence of protease inhibitors, and activation and inactivation of PARs, tissue injury or damage can result in the generation of proteases and activation of protease-activated receptors (Marutsuka, Hatakeyama et al. 2005; Tsuboi, Naito et al. 2007). Therefore, protease-activated receptors make pivotal contributions to physiological and pathological responses due to tissue damage, homeostasis alteration and cell survival. Mimicking or interfering with the protease activation receptor processes or pathways are two strategies that have been attracted attention with respect to developing effective therapies for diseases. The exacerbated cleavage of PARs and imbalance between activation and inactivation of PARs can be successfully halted through selective protease inhibitors and PAR antagonists. Developing selective agonists and antagonists can be used to probe functions of PARs and treat diseases, and investigating proteases and PARs may lead to improve medicine and drug development, such as small-molecule or mimic peptide inhibitors for targeting PARs (Leger, Covic et al. 2006). In addition, understanding the role of protease and protease activation receptors in physiological control mechanisms and pathological processes of human diseases will also be a major challenge in the future. However, all of these investigations on protease activators or inhibitors, protease activation receptor agonists or antagonists, are closely associated with protease activity in organisms.

1.2.3 Protease classifications

Proteases are classified according to their catalytic mechanisms. Four mechanistic classes have been recognized by the International Union of Biochemistry and Molecular Biology: serine
proteases, cysteine proteases, aspartic acid proteases and metalloproteases. Their mechanisms are demonstrated to cleave a peptide bond through creating an amino acid residue. Serine or cysteine (peptidases) or a water molecule (aspartic acid proteases and metalloproteases) is used as a nucleophilic to attack the peptide carbonyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine or threonine as a nucleophile. However, two additional classes of proteases, threonine and glutamic acid proteases, were not described until 1995 and 2004, respectively (Hedstrom 2002; Hooper 2002; Barrett, Rawlings et al. 2003).

Serine proteases play critical roles in many important functions in mammals, especially in digestion and blood clotting system. The digestive enzymes include trypsin, chymotrypsin and elastase, which are responsible for protein digestion. The common activated clotting factors are thrombin, which are in charge of blood clotting. Caspases, cathepsins, calpains and papain are the major members of the cysteine protease family. Aspartic acid proteases have two highly-conserved aspartate residues in the active site, and function with increased activity in acidic pH conditions. The major members of this family are composed of HIV-1 protease, chymosin, renin, cathepsin D, pepsin and plasmesin. Metalloproteases bind a metal ion such as Zn$^{2+}$ in the active site. The most widely-studied metalloproteases are digestive enzymes such as carboxypeptidases A, carboxypeptidases B and thermolysin. Metalloproteases are important in many aspects of biology, ranging from cell proliferation, differentiation and remodeling of the extracellular matrix to vascularization and cell migration. Currently, the matrix metalloproteases (MMPs) have been attracted extensive attentions in the biomedical field due to their involvement of tumor development (Chang and Werb 2001).
1.2.4 Substrate specificity of proteases

The substrate specificity of an enzyme is determined by its capacity to form a stable complex between the substrate and the enzyme with a particular ligand in both the ground state and the transition state. Steric constraints, intermolecular forces, and hydrophobic effects contribute to the stability of a given complex. Therefore, in order to improve the specificity between the substrate and the enzyme, the catalytic site should be able to be flanked on one or both sides by specificity subsites for accommodating the sidechain of a single amino acid residue. Traditionally, substrate specificity mainly based on the primary sequence structure. Secondary and tertiary structural information of the substrate specificity should be involved in. Based on their specific accommodation, these sites are numbered from the catalytic site, S_1...S_n towards the N-terminus of the substrate, and S'_1...S'_n towards the C-terminus. The residues they accommodate are numbered P_1...P_n, and P'_1...P'_n, respectively, as shown in Figure 1.1. However, differences in the catalytic sites of the different proteases exhibit the various preferences of the residue in their substrates.

1.2.5 Current methods for protease detection

Because many diseases, including inflammationary diseases, arteriosclerosis, cancers and neurodegenerative diseases, have long been associated with altered proteolytic activity of proteases or disruption in the balance and control between protease activation and inhibition, it is highly-desirable to track protease signaling, protease activation and inhibition using very sensitive and effective methods. Dynamic visualization of intracellular protease activity can provide valuable information about their physiological roles and pathological processes. Objective and quantitative noninvasive imaging of protease activity would represent a significant
Figure 1.1. Specificity diagram between enzyme and substrate. The amino acid residues (P_n-P_n') around the scissile bond in the substrate should have high complementary with the subsites of the enzyme.
advance towards rapid and dynamic screening as well as monitoring the effects of therapeutic agents. The studies of protease actions, signaling and pathways in living systems are frequently hindered by the lack of tools or probes capable of monitoring dynamic protease processes in various cellular locations.

Currently, protease activity is determined by several methods in cells. Real-time polymerase chain reaction (RT-PCR) (Gelmini, Tricarico et al. 2003; Peluffo, Young et al. 2005; House, Catchpole et al. 2007) and Western blot (Kossakowska, Edwards et al. 1998; Persad, Liu et al. 2004) were used to monitor the elevated gene expression, and protein expression of proteases and protease inhibitors, respectively. Although RT-PCR and Western blot are sensitive to assess the existence of proteases and their substrate products, limited information for understanding the dynamic activity of proteases in cells or in vivo is still provided.

One of the most commonly-used and commercially-available substrates for protease activity measurement is the application of chromogenic or fluorogenic peptide kits in cell lysate assays (Goddard and Reymond 2004). These methods generally measure the actions of most proteases in cell lysates using: chromophores conjugated to short peptides; peptide mimics encompassing enzymatic cleavage sites (Talanian, Quinlan et al. 1997; Thornberry, Rano et al. 1997). The most common chromophores linked to short peptide fragments of 3-6 amino acids that mimic the sequence encompassing the P₁ to Pₙ cleavage sites (usually up to P₃) are: p-nitroaniline (pNA), 7-amino-4-methylcoumarin (AMC), 7-amino-4-trifluromethyl coumarin (AFC), rhodamine or fluorescein isothiocyanate-labeled casein (FITC). Although newly-developed peptide probes are able to penetrate cells, these probes are not ideal for the continuous dynamic imaging of enzyme actions due to limited lifetime, specificity and stability of these protease substrates, resulting from a lack of defined structure in solution because of their short
sequences. However, these short peptides usually degrade rapidly and can not be delivered into specific sub-cellular locations. Moreover, determination of protease activity using the chromophores conjugated with the mimic, small peptide dye is accomplished through the elimination of quenching to emit the absorbance or fluorescence following protease cleavage of the linker. This method is also hampered by poor specificity due to the difficulties in considering the P' region amino acid residues of the cleavage linker. On the other hand, the diagnosis of diseases, such as chronic pancreatitis, are largely restricted to later stages of the illness due to limitations inherent in the currently available peptide kits designed for the detection of trypsin activity in cell lysate (DiMagno 1988; Lemaitre and D'Armiento 2006). Activation of trypsinogen and chymotrypsinogen by caerulein was previously reported in pancreatic cancer cells, MIA PaCa-2 via PAR1 and the PAR pathway using cell lysate assays (Yamaguchi, Kimura et al. 1989; Halangk, Sturzebecher et al. 1997; Kruger, Lerch et al. 1998; Namkung, Han et al. 2004; Yamasaki, Takeyama et al. 2006).

Due to the limitations in real-time determination of protease activation and inhibition, a powerful method using fluorescence resonance energy transfer (FRET) of fluorescent protein pairs has been extensively investigated to track protease activity in vitro and in vivo. FRET-based protease probes are created using a GFP pair connected by a enzymatic cleavage linker (Figure 1.2). The properties of fluorescent proteins that allow for cofactor-independent chromophore formation and expression to provide the capability of monitoring numerous cellular events in living cells or organisms via living cell imaging (Shimomura, Johnson et al. 1962; Chalfie, Tu et al. 1994; Inouye and Tsuji 1994; Wang and Hazelrigg 1994; Chalfie 1995; Tsien 1998; Akemann, Raj et al. 2001; Shimomura 2005). Taking advantage of wild type GFP’s resistance to the cleavage for proteases and denaturation, the cleavage of the peptide bond at the
Figure 1.2. The model of FRET-based protease sensor.
FRET-based protease sensors are mostly constructed by fluorescent protein pair (eg. cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP)) through the connection of a cleavage linker for proteases. Once the cleavage linker is cleaved, the change of fluorescence resonance energy transfer is used to monitor protease activity.
connecting linker between GFP pair results in a change of fluorescence resonance energy transfer. This method has been applied to track the activity of many proteases including trypsin (Pollok and Heim 1999; Eggeling, Jager et al. 2005), caspase-3 (Xu, Gerard et al. 1998; Harpur, Wouters et al. 2001; Luo, Yu et al. 2001), caspase-8 (Luo, Yu et al. 2003) and thrombin (Zhang 2004; Wang, Cao et al. 2005), and offers the advantage of being able to monitor the dynamic processes of protease activation in vitro and targeting of specific locations in vivo. Although FRET-based protease sensors can be used to track the protease activity in living organisms, this strategy has still not been extended to practical applications, and no FRET-based protease sensors are commercially-available. Additionally, the catalytic kinetic parameters ($k_{cat}$, $K_m$ and $k_{cat}/K_m$) of FRET-based protease sensors have not been extensively discussed in the literature.

1.2.6 Criteria for an ideal protease sensors

To design functional protease probes capable of monitoring dynamic protease activation or inhibition in living cells, several factors must be considered. First, the protease sensor must be able to monitor the dynamics of enzymatic activity over the entire natural course of activation or inhibition in intact living cells in real time. Also, the addition of signal peptide sequences can be used to develop probes capable of targeting specific cellular compartments. Some currently-available detection methods simply rely on passive diffusion of the probe through the membrane, and permit only short snapshots of enzymatic actions without the capability of detecting reactions at targeted cellular locations. Second, protease sensors should have high sensitivity and exhibit a large dynamic change following protease actions. Ideally, the fluorescence emission or excitation wavelength of the chromophore will be changed by the cleavage reaction and provide a ratiometric measurement. Such a ratiometric change permits more precise quantitative
measurement of enzymatic activity by normalizing the analytical response to account for variations in sensor concentrations, fluorescence signal changes due to quenching by different cellular factors and conditions, and instability associated with the instrumentation. A single wavelength, by contrast, allows for less precise quantitative measurement. Third, the optical signal change must rapidly respond to catalytic cleavage associated with an optimal kinetic activity. Fourth, the designed protease sensor must have good selectivity for the enzymatic action of interest.

1.3 Calcium signaling

It is well known that Ca$^{2+}$ is the most ubiquitous and pluripotent cellular signaling ion, as the second messenger controlling a wide variety of physiological reactions such as muscle contraction, cellular motility, metabolism, gene transcription, regulation of enzyme activity, ion pump functions, cell proliferation and cell death (Berridge 1993; Clapham 1995; Simpson, Challiss et al. 1995). Ca$^{2+}$ plays the critical roles in pathological processes as the major causes of many human diseases including osteoporosis, cardiovascular diseases, Alzheimer’s disease, neurodegenerative diseases, and cancers (Matsuura 2006; Blair, Schlesinger et al. 2007; Capiod, Shuba et al. 2007; Monteith, McAndrew et al. 2007; Yamamoto, Wajima et al. 2007). The calcium signaling system is operated through highly conserved transporters and channels, or a large number of calcium-sensitive effectors or calcium buffers to execute the movements across biological membranes of calcium ions. The movements across biological membrane of calcium ions are controlled by its ON and OFF mechanisms, which are often organized to produce brief spikes and waves of calcium. The ON and OFF mechanisms are shown in Figure 1.3.
Figure 1.3. The ON and OFF mechanisms and modulation of intracellular calcium levels. Interacellular calcium signaling was regulated by the ON and OFF modulation of calcium ions. The ON mechanisms are dependent on calcium ion entry through channels in the plasma membrane or calcium ion release through receptors. The OFF mechanisms remove calcium from cytoplasm using pumps. The symbols + in red and – in blue demonstrate the activation and inhibition processes, respectively. During calcium signaling, VOC (voltage-operated channel), ROC (receptor-operated channel) and SOC (store-operated channel) are major calcium channels. Phosphatidylinositol bisphosphate (PIP2) is a substrate of phospholipase (PLC) on the receptors (R). Following the cleavage of PIP2, inositol 1,4,5-trisphosphate (IP3) is generated to bind the IP3 receptors (IP3R) or ryanodine receptors (RyRs), which is modulated by their agonists and antagonists. Calmodulin (CaM) and troponin C (TnC) are major Ca2+ sensors. Modified after (Berridge, Lipp et al. 2000; Berridge, Bootman et al. 2003)
1.3.1 Regulation of intracellular calcium

The alteration of intracellular calcium ion concentration ([Ca\(^{2+}\)]\(_i\)) can result in the occurrence of calcium signaling through the activation of ion channels and the release of calcium ions from intracellular stores due to stimulation or indirect signal transduction pathways like G protein-coupled receptors (Brown and MacLeod 2001; Jorgensen, Teilmann et al. 2003). As shown in Figure 1.3, [Ca\(^{2+}\)]\(_i\) increases can be generated from sources both within and outside the cell: Ca\(^{2+}\) release from intracellular calcium stores and Ca\(^{2+}\) influx across the plasma-membrane. The calcium levels outside cells are 10,000 times higher than free intracellular Ca\(^{2+}\) in the cytosol (Artalejo and Garcia-Sancho 1988). However, free [Ca\(^{2+}\)]\(_i\) in the cytosol is the physiologically active form of calcium, which is strictly regulated and maintained low level (~100 nM) through the action of a number of calcium-binding proteins and ion exchange mechanisms (Clapham 1995). Since Ca\(^{2+}\) signal transduction is based on rises in free cytosolic Ca\(^{2+}\) concentration, Ca\(^{2+}\) efflux from the extracellular space or release from intracellular stores are responsible for calcium signal transduction.

The endoplasmic reticulum (ER) is well known to be a major site for sequestered calcium ions (Brostrom and Brostrom 1990). Recent studies indicate that the Golgi apparatus may also be a calcium ion store (Pinton, Pozzan et al. 1998). The accumulation of calcium ions at intracellular stores can be mediated in the following manners: calcium ion pumps, receptors, calcium-binding protein or calcium buffers (Clapham 1995; Clapham 1995; Putney 1999).
1.3.2 Major players to mediate intracellular calcium accumulation

**Extracellular calcium ion channels:** Three major extracellular calcium ion channels are located in the plasma membrane of the cells to mediate the intracellular calcium accumulation from high calcium ion concentration of extracellular matrix.

Voltage-operated channels (VOCs): This is the family of calcium ion channels that are responsible for the channel opening in response to membrane depolarization. This channel family can selectively mediate the entry of calcium ions from extracellular matrix to result in calcium signaling (Catterall 1998). Based on their kinetics and pharmacological properties, this family of channels has several sub-types, L, N, P/Q, R and T types. Channel agonists or channel blockers such as ionomycin for this channel family can modulate calcium signaling.

Receptor-operated channels (ROCs): This is the family of calcium ion channels that are opened by the binding of specific agonists, usually neurotransmitters, such as glutamate (the NMDA receptor) (Sucher, Lei et al. 1991) or ATP (Sela, Ram et al. 1991).

Store-operated channels (SOCs): This family of calcium ion channels exists in the plasma membrane of many cells, which are opened by the emptying of internal stores (Parekh and Putney 2005). However, the mechanism for mediating calcium signaling between the empty stores and the membrane SOCs is still unclear. The calcium influx factor (CIF) or information transfer through a direct protein-protein interaction has been proposed in the previous investigation (Bolotina 2004).

**Intracellular calcium ion channels:** External calcium ions arriving at the cell plasma membrane receptors initiate cell-signaling pathways to achieve the final result, increased intracellular calcium concentration through receptor-mediated calcium ion channels. Three major families of receptor-mediated intracellular channels responsible for releasing calcium ions from
the internal store located in the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR) (Berridge 1993; Pozzan, Rizzuto et al. 1994).

**Inositol 1,4,5-trisphosphate (IP$_3$) receptors:** IP$_3$ receptors (IP$_3$R) belong to the family of receptor-mediated calcium ion channels, which perform energy-requiring transduction to activate phospholipase C (PLC) and hydrolyze the lipid precursor phosphatidylinositol 4,5-biphosphate (PIP$_2$) to generate both diacylglycerol (DAG) and IP$_3$. Following the binding between IP$_3$ and its receptors, stored calcium ions are mobilized to promote an influx of external calcium ions. Meanwhile, IP$_3$ also can be generated through the stimulation of G-protein-linked or tyrosine kinase-linked receptors upon the actions of agonists (Michikawa, Miyawaki et al. 1996).

**Ryanodine receptors (RyRs):** Ryanodine also binds to its receptors and can initiate calcium release by locking the calcium channel in an open configuration. Cyclic ADP ribose is a putative second messenger for regulating the activity of RyRs (Galione and White 1994; Bootman and Berridge 1995). RyR-induced calcium ion release can be activated by caffeine, and RyR channels can be modulated by various associated proteins such as FK506 binding protein 12 (FKBP12). Originally described in muscle cells, these RyRs are also found in neurons and other cell types (MacKrill 1999).

**Calcium-binding proteins:** A variety of calcium-binding proteins function either as sensors to carry out the messenger role of calcium ions or as buffers to shape the cellular response in cells. Both the cytoplasm and the lumen of the ER or sarcoplasmic reticulum (SR) have proteins capable of buffering calcium ions (Baimbridge, Celio et al. 1992; Koster, Hartog et al. 1995; Rakhilin, Olson et al. 2004). For example, the major intracellular mediator for calcium ions is calmodulin (CaM). When calcium ions transfer to the cytosol, they are rapidly buffered by proteins such as calbindin, calretinin, and parvalbumin.
1.3.3 Calcium signaling and related diseases

Abnormalities or mutations in one of above-mentioned calcium-transporting channels, receptors or proteins may lead to diseases associated with calcium. Recent investigations elucidated the important role for calcium dysregulation in the pathogenesis of Alzheimer's disease, because the dysregulation of calcium signaling can result in the disorder of the gating of IP3 receptor and calcium-conducting channels (Missiaen, Robberecht et al. 2000; Green and LaFerla 2008). Abnormal intracellular calcium ion concentration handled by SR is a critical factor in the development of heart failure. Several mutations of RyRs have been reported in patients with lethal cardiac arrhythmia (Yano 2008). In addition, alteration of calcium homeostasis, disorder of calcium ion channels, and changes of calcium signaling pattern resulted from extracellular stimuli, have a direct effect on ER calcium ion level and protein expression of Bcl-2 family, which can remodel the expression of many cancer cells and mediate the cell proliferation and cell death (Rizzuto, Pinton et al. 2003; Roderick and Cook 2008). The large cytoplasmic domain of the RyR serves as a scaffold protein, which binds to calstabin [FK506-binding proteins (FKBPs)], calmodulin, phosphodiesterase, kinases, phosphatases, and their targeting proteins for modulating functions of calcium ion channels. Recent progresses have also described the disease mechanisms associated with the dysregulation of RyR and channels (Zalk, Lehnart et al. 2007).
1.4 Fluorescent proteins

1.4.1 Properties of green fluorescent proteins

GFP was first discovered and isolated from the jellyfish *Aequorea victoria* in 1961 as a companion protein to aequorin (Shimomura, Johnson et al. 1962; Shimomura 2005) and exhibited absorption or excitation peaks at 400 and 480 nm, and an emission peak at 510 nm with a quantum yield of 0.72 when excited at 470 nm, after it was purified and crystallized successfully (Morise, Shimomura et al. 1974; Prendergast and Mann 1978). GFP is a stable, soluble, globular, monomeric protein with 238 amino acid residues with a molecular mass of 27 kDa (Prendergast and Mann 1978). This protein is composed of an 11-stranded anti-parallel β-barrel, a single central α-helix, which contains the chromophore in the β-barrel, and short helices that cap the ends of the barrel (Figure 1.4) (Yang, Moss et al. 1996). Discovery of GFP has opened a new era in biology, medicine, pharmaceuticals, drug discovery and material sciences (Shimomura, Johnson et al. 1962; Akemann, Raj et al. 2001; Shimomura 2005). The chromophore self-formation and GFP expression in the absence of any substrates or cofactors make it possible to examine physiological, pathological change during cellular and organ development (Inouye and Tsuji 1994; Sengupta, Colbert et al. 1994; Wang and Hazelrigg 1994; Chalfie 1995; Tsien 1998; Tucker 2001; Wouters, Verveer et al. 2001; Zhang, Campbell et al. 2002). Therefore, GFP and its variants have been attracted extensive attention on studies such as protein-protein interactions, spatial and temporal change of protein translocation and transport, and proteolysis, as well as protein phosphorylation in intact cells (Tsien 1998; Ni, Heard et al. 1999; Periasamy and Day 1999; Zacharias, Baird et al. 2000; Belmont 2001; Toomre and Manstein 2001; Wouters, Verveer et al. 2001; Periasamy, Elangovan et al. 2002; Zimmer 2002; Lippincott-Schwartz and Patterson 2003; Fehr, Ehrhardt et al. 2004; Shimi, Koujin et al. 2004).
Figure 1.4. Schematic topology of the secondary structure of GFP. GFP is composed of 11 β strands (green ribbon) and 3 helices (red cylinder) connected through loops (purple line), which formed a compacted β-can structure. The chromophore of GFP is located in the middle of β-can. The tripeptide chromophore (Ser65-Tyr66-Gly67) is highlighted in blue cylinder.
1.4.2 Chromophore formation of green fluorescent proteins

Due to the extensive applications of GFP, scientists are making their efforts to probe chromophore formation mechanisms for accelerating chromophore maturation and developing various fluorescent protein variants with characteristic optical properties, such as enhanced fluorescence intensity, improved thermo-stability, or multiple colors. Therefore, a more powerful fluorescent reporter or genetic probe capable of tracking a variety of intracellular dynamic events in a non-invasive mode can be developed and extended through the modification of GFP.

Although GFP has attracted tremendous attention due to its potentially useful characteristics, and the chromophore structure has been confirmed, rather little is known about the detailed mechanisms of folding and chromophore formation of GFP \textit{in vitro} and \textit{in vivo}. The mechanisms of chromophore formation are still not well understood. The chromophore of GFP was first demonstrated to form through the cyclization of the tripeptide Ser65-Tyr66-Gly67 (Shimomura 1979; Perozzo, Ward et al. 1988; Cody, Prasher et al. 1993; Inouye and Tsuji 1994; Inouye and Tsuji 1994) located inside of the β-barrel composed of 11 anti-parallel strands and a single central α-helix. Short helices cap the ends of the barrel (Ormo, Cubitt et al. 1996; Yang, Moss et al. 1996). The active chromophore in GFP is a tripeptide, which requires the presence of oxygen to mature (Inouye and Tsuji 1994). The cylinder of GFP has a diameter of about 30 Å and a length of about 40 Å (Yang, Moss et al. 1996). Physical and chemical studies of purified GFP also indicated that several important characteristics are related to its specific structure. Extensive hydrogen bond interactions within protein frame or between protein frame and water molecules have significant effects on chromophore resulting in various folding status (Wood, Barondeau et al. 2005). An enhanced green fluorescent protein (EGFP) was obtained through a
mutation S65T in GFP. Following this mutation, EGFP has improved fluorescence intensity and thermo-sensitivity. Tsien proposed an autocatalytic mechanism accounting for spontaneous chromophore formation in various GFP-expressing organisms (Heim, Prasher et al. 1994), which suggested chromophore formation through autocatalytic cyclization and oxidation from residues Thr65, Tyr66, Gly67 (Figure 1.5), consisting of an internal tripeptide motif that enables the protein to emit fluorescence in the absence of a cofactor (Dopf and Horiagon 1996). Certain mutation of the chromophore has significant effect on the rate of the chromophore formation in vivo and can alter the spectral characteristics of the matured GFP variants. The chromophore formation of S65T-GFP was monitored in vitro, where an ordered reaction consisting of three distinct steps was observed (Figure 1.6). Protein folding occurs fairly slowly ($k_f = 2.44 \times 10^{-3} \text{ s}^{-1}$) prior to any chromophore modification. Then, an intermediate step occurs including cyclization of the tripeptide chromophore motif ($k_c = 3.8 \times 10^{-3} \text{ s}^{-1}$). The final and slower step ($k_{ox} = 1.51 \times 10^{-4} \text{ s}^{-1}$) involves oxidation of the cyclized chromophore (Reid and Flynn 1997). To gain a comprehensive understanding of the reaction mechanism of GFP chromophore formation, its biosynthesis is of fundamental scientific interest. In addition, knowledge of the reaction pathway and identification of intermediates have significant practical and medical implications. Wachter’s group probed the crystal structure of the Y66L variant of GFP and their results supported the Cyclization-Oxidation-Dehydration mechanism for chromophore maturation (Wachter, King et al. 1997). To understand chromophore formation, Enoki and coworkers used pH 2.0 Tris-HCl to denature GFP and carried out research on kinetic refolding of GFP in vitro. They investigated the kinetic mechanisms of a mutant (F99S/M153T/V163A) of GFP, which is known to mature more efficiently than the wild type protein from the acid-denatured state. The kinetics of the refolding of the mutant suggests at least five kinetic phases (Enoki, Saeki et al. 2004).
Figure 1.5. The proposed chromophore formation mechanism of EGFP. The chromophore of EGFP is formed by a tripeptide (Thr65-Tyr66-Gly67) through folding, cyclization, dehydration and oxidation processes. It exists in both the neutral state (protonated form) and the anionic state (deprotonated form) due to effects from the hydrogen bond network. Modified after (Heim, Prasher et al. 1994).
Figure 1.6. Three-step mechanism of GFP chromophore formation. The three steps of GFP chromophore formation exhibit the formation process of a pre-cyclization structure, an intermediate structure and the mature chromophore. Modified after (Reid and Flynn 1997; Zhang, Patel et al. 2006).
Due to the chromophore being buried in the tightly-constructed β barrel, GFP is highly resistant to denaturation and its fluorescent properties are unaffected in the presence of 6 M guanidine hydrochloride, 8 M urea or 1% SDS at 90 °C; is stable in a broad range of pH from 5 to 12; and can tolerate various proteases such as trypsin, chymotrypsin, papain, subtilisin, thermolysin and pancreatin (Ward and Bokman 1982; Ward, Prentice et al. 1982). The tightly-constructed β barrel protects the chromophore well, which contributes to the overall stability, temperature- and denaturant-resistance, and prohibition of proteases, oxygen or water molecules access. Shimomura also analyzed the acid-denatured GFP by proteolysis and deduced the structure of the chromophore in 1979 and the result indicated that the chromophore is a 4-(p-hydroxybenzylidene) imidazolidin-5-one attached to the peptide backbone through the 1- and 2-positions of the ring (Shimomura 1979). The structure of the chromophore was also confirmed by X-ray and time-resolved fluorescence analysis. The results indicated that the chromophore-containing fragment is a cyclized hexapeptide formed from residues Phe64-Ser-Tyr-Gly-Val-Gln69 of GFP (Perozzo, Ward et al. 1988).

1.4.3 Spectral properties of fluorescent proteins

Although the chromophore is located on the central helix within a couple of angstroms of the cylinder center, both apolar and polar amino acid side chains such as S202, T203, I167 or G148 and immobilized water molecules surround the chromophore. The mutations around the GFP chromophore were shown to alter the optical properties of the protein. Some mutations result in the loss of the excitation at 475 nm and the preservation of the excitation at 395 nm, such as S202F and T203I. Moreover, mutation I167T causes a reversed ratio of the sensitivity at
395 nm to 475 nm. The shift of excitation peaks can provide us multiple color-fluorescent proteins, such as blue fluorescent protein (BFP), cyan fluorescent protein (CFP), green fluorescent protein (GFP), yellow fluorescent protein (YFP) and red fluorescent protein (RFP). The fluorescent protein properties of different GFP variants are shown in Table 1.1 (Piston, Patterson et al. 2004).

In summary, different mutations resulted in various improved GFP variants with the characteristics of high intensity, low photobleaching, and high thermo-stability. These variants also have various excitation or emission wavelengths. Therefore, any proteins tagged with different GFP variants can be visualized in cells with high light intensities over many hours and with little photobleaching. This permits intracellular protein pathways or dynamics to be analyzed in detail. The increased stability and brightness of EGFP also enables the intracellular fluorescent signal from chimeras to be correlated to standard GFP solution, permitting the quantification of molecules visualized in cells. Meanwhile, fluorescent proteins can be used for fluorescence resonance energy transfer (FRET) to monitor intracellular interactions.

1.4.4 Applications of green fluorescent protein

The isolation and cloning of GFP from Aequorea victoria has created a unique new research tool using GFP as an active or a passive reporter of cell function in molecular and cell biology. It has been shown that GFP can be used as a gene marker, which can be easily detected by fluorescence microscopy or flow cytometry. Viable GFP-positive cells have been isolated through fluorescence-activated cell sorting. Therefore, GFP has served as a versatile tool for the study of gene expression, gene transfer, protein folding and trafficking, and protein-protein interactions due to its spectral characteristics, small size, and acquisition of fluorescent activity
Table 1.1. Fluorescent protein properties

<table>
<thead>
<tr>
<th>Protein (Acronym)</th>
<th>Excitation Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
<th>Molar Extinction Coefficient</th>
<th>Quantum Yield</th>
<th>In vivo Structure</th>
<th>Relative Bright (% of EGFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP (wt)</td>
<td>395/475</td>
<td>509</td>
<td>21,000</td>
<td>0.77</td>
<td>Monomer</td>
<td>48</td>
</tr>
<tr>
<td><strong>Green Fluorescent Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP</td>
<td>484</td>
<td>510</td>
<td>56,000</td>
<td>0.60</td>
<td>Monomer</td>
<td>100</td>
</tr>
<tr>
<td>Emerald</td>
<td>487</td>
<td>509</td>
<td>57,500</td>
<td>0.68</td>
<td>Monomer</td>
<td>116</td>
</tr>
<tr>
<td>Azami Green</td>
<td>492</td>
<td>505</td>
<td>55,000</td>
<td>0.74</td>
<td>Monomer</td>
<td>121</td>
</tr>
<tr>
<td>CopGFP</td>
<td>482</td>
<td>502</td>
<td>70,000</td>
<td>0.60</td>
<td>Monomer</td>
<td>125</td>
</tr>
<tr>
<td>AcGFP</td>
<td>480</td>
<td>505</td>
<td>50,000</td>
<td>0.55</td>
<td>Monomer</td>
<td>82</td>
</tr>
<tr>
<td>ZsGreen</td>
<td>493</td>
<td>505</td>
<td>43,000</td>
<td>0.91</td>
<td>Tetramer</td>
<td>117</td>
</tr>
<tr>
<td><strong>Blue Fluorescent Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBFP</td>
<td>383</td>
<td>445</td>
<td>29,000</td>
<td>0.31</td>
<td>Monomer</td>
<td>27</td>
</tr>
<tr>
<td>Sapphire</td>
<td>399</td>
<td>511</td>
<td>29,000</td>
<td>0.64</td>
<td>Monomer</td>
<td>55</td>
</tr>
<tr>
<td>T-Sapphire</td>
<td>399</td>
<td>511</td>
<td>44,000</td>
<td>0.60</td>
<td>Monomer</td>
<td>79</td>
</tr>
<tr>
<td><strong>Cyan Fluorescent Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AmCyan1</td>
<td>458</td>
<td>489</td>
<td>44,000</td>
<td>0.24</td>
<td>Tetramer</td>
<td>31</td>
</tr>
<tr>
<td>ECFP</td>
<td>439</td>
<td>476</td>
<td>32,500</td>
<td>0.40</td>
<td>Monomer</td>
<td>39</td>
</tr>
<tr>
<td>Cerulean</td>
<td>433</td>
<td>475</td>
<td>43,000</td>
<td>0.62</td>
<td>Monomer</td>
<td>79</td>
</tr>
<tr>
<td>CoralHue Cyan</td>
<td>472</td>
<td>495</td>
<td>27,300</td>
<td>0.90</td>
<td>Dimer</td>
<td>73</td>
</tr>
<tr>
<td><strong>Yellow Fluorescent Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EYFP</td>
<td>514</td>
<td>527</td>
<td>83,400</td>
<td>0.61</td>
<td>Monomer</td>
<td>151</td>
</tr>
<tr>
<td>PhiYFP</td>
<td>525</td>
<td>537</td>
<td>130,000</td>
<td>0.40</td>
<td>Monomer</td>
<td>155</td>
</tr>
<tr>
<td>Citrine</td>
<td>516</td>
<td>529</td>
<td>77,000</td>
<td>0.76</td>
<td>Monomer</td>
<td>174</td>
</tr>
<tr>
<td>Venus</td>
<td>515</td>
<td>528</td>
<td>92,200</td>
<td>0.57</td>
<td>Monomer</td>
<td>156</td>
</tr>
<tr>
<td>ZsYellow1</td>
<td>529</td>
<td>539</td>
<td>20,200</td>
<td>0.42</td>
<td>Tetramer</td>
<td>25</td>
</tr>
<tr>
<td><strong>Orange and Red Fluorescent Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoralHue Orange</td>
<td>548</td>
<td>559</td>
<td>51,600</td>
<td>0.60</td>
<td>Monomer</td>
<td>92</td>
</tr>
<tr>
<td>mOrange</td>
<td>548</td>
<td>562</td>
<td>71,000</td>
<td>0.69</td>
<td>Monomer</td>
<td>146</td>
</tr>
<tr>
<td>DsRed</td>
<td>558</td>
<td>583</td>
<td>75,000</td>
<td>0.79</td>
<td>Tetramer</td>
<td>176</td>
</tr>
<tr>
<td>DsRed2</td>
<td>563</td>
<td>582</td>
<td>43,800</td>
<td>0.55</td>
<td>Tetramer</td>
<td>72</td>
</tr>
<tr>
<td>DsRed-Express</td>
<td>555</td>
<td>584</td>
<td>38,000</td>
<td>0.51</td>
<td>Tetramer</td>
<td>58</td>
</tr>
<tr>
<td>mTangerine</td>
<td>568</td>
<td>585</td>
<td>38,000</td>
<td>0.30</td>
<td>Monomer</td>
<td>34</td>
</tr>
<tr>
<td>mStrawberry</td>
<td>574</td>
<td>596</td>
<td>90,000</td>
<td>0.29</td>
<td>Monomer</td>
<td>78</td>
</tr>
<tr>
<td>AsRed2</td>
<td>576</td>
<td>592</td>
<td>56,200</td>
<td>0.05</td>
<td>Tetramer</td>
<td>8</td>
</tr>
<tr>
<td>mRFP1</td>
<td>584</td>
<td>607</td>
<td>50,000</td>
<td>0.25</td>
<td>Monomer</td>
<td>37</td>
</tr>
<tr>
<td>mCherry</td>
<td>587</td>
<td>610</td>
<td>72,000</td>
<td>0.22</td>
<td>Monomer</td>
<td>47</td>
</tr>
<tr>
<td>mRaspberry</td>
<td>598</td>
<td>625</td>
<td>86,000</td>
<td>0.15</td>
<td>Monomer</td>
<td>38</td>
</tr>
<tr>
<td>HeRed-Tandem</td>
<td>590</td>
<td>637</td>
<td>160,000</td>
<td>0.04</td>
<td>Monomer</td>
<td>19</td>
</tr>
</tbody>
</table>

Modified after (Piston, Patterson et al. 2004).
In absence of any other cofactors. *In vivo*, applications of GFP can be divided into two categories based on its use, as either a tag or an indicator. As a tag, GFP fluorescence reflects levels of gene expression or sub-cellular localizations of the host proteins to which GFP is fused. As an indicator, GFP fluorescence is modulated post-translationally by its chemical environment and protein-protein interactions (Tsien 1998). The first proposed application of GFP was the detection of gene expression as a tag *in vivo* by Chalfie (Chalfie, Tu et al. 1994). Currently, GFP variants are used for determining calcium ion level in various compartments of cells, tissues, or organs (Zou, Hofer et al. 2007).

### 1.4.5 Current protease sensors based on GFP

It is well known that green fluorescent protein and its variants play important roles in a variety of biological process, including dynamic detection of protease activation or inhibition *in vitro* or in living cells. The earliest tandem GFP FRET pair, BFP donor and EGFP acceptor, are connected a linker with 25 amino acid sequence, which exhibited a 4.6-fold change in the ratio of fluorescence emission from the respective GFPs upon the complete cleavage by trypsin (Heim and Tsien 1996). Later, a similar GFP-FRET pair was constructed as a tendem BFP-EGFP substrate for Factor Xa to reveal a three-fold fluorescence emission ratio change between 505 and 405 nm due to the disruption of FRET (Mitra, Silva et al. 1996). Due to the pioneer works related to protease activity detection with FRET of GFP pairs, FRET-based protease sensors have been extensively investigated and developed. Currently, many investigators are developing tandem molecules of green fluorescent protein (GFP) as the genetically encoded sensors to detect caspase activity and screen caspase triggers or inhibitors both *in vitro* and in living cells (Mahajan, Harrison-Shostak et al. 1999; Jones, Heim et al. 2000; Tawa, Tam et al. 2001;
Karasawa, Araki et al. 2004; Kawai, Suzuki et al. 2005; Ai, Hazelwood et al. 2008; Ray, De et al. 2008). For example, four-amino-acid recognition sequences, YVAD for caspase-1 and DEVD for caspase-3 were introduced between CFP and YFP was used for high-throughput screening of reagents that modulate caspase activity in live mammalian cells (Mahajan, Harrison-Shostak et al. 1999). Based on various color versions of the FRET-based probes for caspase activity, a simultaneous real-time detection of initiator- and effector-caspase activation by double fluorescence resonance energy transfer analysis was also developed for single living cell (Kawai, Suzuki et al. 2005) and a new violet-excitible yellow-fluorescing variant of GFP was also used to construct a dual FRET-based caspase-3 biosensor for living cells (Ai, Hazelwood et al. 2008). Besides the development of FRET-based biosensors for activity detection trypsin, Factor Xa and caspases, FRET-based GFP pairs have been also used to design thrombin probe for high throughput screening of thrombin inhibitors (Zhang 2004) and genetic MMP substrate to sensitively monitor MMP activation in living cells or high throughput screening of MMP inhibitors for anti-cancer treatments (Yang, Zhang et al. 2007). Furthermore, several elegant studies have only reported the on and off measurements of protease activity by linking GFP with ubiquitin or proteasome cleavage peptide for protein degradation or by using bacterial protease sequences such as PEST and N-terminal rules (Dantuma, Lindsten et al. 2000; Lee, Beem et al. 2002; Spencer, Theodosiou et al. 2004).

1.5 Limitations of FRET-based fluorescent protein biosensors

Due to the unique characteristics, different variants of fluorescent proteins have attracted extensive interest in real-time imaging the spatiotemporal events of living cells. A large number of FRET-based biosensors based on various fluorescent proteins have been designed and
developed to detect calcium signaling or track protease activation or inhibition in living cells in real time. Unfortunately, FRET-based protease sensors are hampered with respect to practical applications due to the following drawbacks or limitations (Felber, Cloutier et al. 2004; Piston and Kremers 2007):

(1) The donor and the acceptor of FRET-based GFP pairs exhibit imbalanced fluorescence intensity due to their 1:1 of stoichiometry. In addition, the quantitative measurement of protease activity is partially limited by problems associated with photobleaching (Jones, Heim et al. 2000; Harpur, Wouters et al. 2001; Kohl, Heinze et al. 2002; He, Wu et al. 2004; Karasawa, Araki et al. 2004; Kawai, Suzuki et al. 2005).

(2) FRET-based fluorophores require spectral overlap between emission wavelength of the donor and excitation wavelength of the acceptor, which results in the issue of cross-talk in FRET signal;

(3) FRET-based methods also require the optimal distance between the donor and the acceptor. Larger fluorescent protein variants with an approximately 40 Å long and 30 Å diameter barrel occupy much of the useful FRET distance and result in the decrease in practical maximal FRET efficiencies.

(4) FRET-based methods also need accurate orientation for their FRET efficiency, both in solution and in a living organism system. In addition, fluorescence energy transfer may be disrupted due to environmental interruptions or complexities in components. Then, fluorescence resonance energy transfer efficiency in certain environments may be changed due to poor orientation. Therefore, empirical optimization on the linker depends on the system of interest.
The application of this class of protease sensors for *in vivo* imaging of protease action may be limited by problems of translocation *in vivo* due to the typical large size of fluorescent protein pair.

### 1.6 Novel strategy for protease sensor design

It is well known that many physiological or pathological processes are protease dependent. A novel and effective probe for investigating protease activation pathways or a diagnostic tool for diseases related to proteases is highly desired to determine protease activity or to track protease activation or inhibition pathways in living cells or *in vivo*. Fluorescence technology is an increasingly appealing strategy for tracking protease activity in living cells. GFP is one of the best candidates due to its intrinsic fluorescence from the chromophore. Currently, many protease sensors or probes are based on FRET of GFP pairs, while the protease sensor based on a single GFP is not reported. In order to develop more effective methods for studying and monitoring intracellular events, a protease sensor with a specific protease cleavable linker in a single GFP with high sensitivity and selectivity are highly desired for the monitoring the real-time dynamic change of protease activity inside living cells. We hypothesize that a protease specific cleavage linker engineered into the chromophore-sensitive locations of a single EGFP could provide a significant ratiometric optical signal change in the absorbance or fluorescence of the chromophore upon the cleavage of this inserted linker, thereby allowing for the determination of protease activity or the monitoring of protease activation and inhibition pathways with high sensitivity, specificity and selectivity inside living cells in real time. A diagram of the grafting approach for developing protease sensors is shown in Figure 1.7.
Figure 1.7. Design of ratiometric EGFP-based protease sensors. A specific cleavage linker for proteases is grafted into a sensitive location for optical signal change of the chromophore in EGFP. Following protease cleavage, a ratiometric optical signal change in absorbance or fluorescence is applied to determine protease activity.
Recently, EGFP with specific cleavage linkers for proteases have been gained prominence, as a sensitive protease sensor to monitor different stages of protease activation.

1.7 Objectives of this dissertation

In this dissertation, several classes of protease sensors, such as trypsin sensors, caspase-3 sensors, caspase-8 sensors and thrombin sensors are systematically designed and studied using a single EGFP scaffold at its sensitive location for ratiometric optical signal change of the chromophore. Meanwhile, based on the association capability of both fragments, another molecular probe based on the split fragments of EGFP was also created for studying calcium-dependent protein-protein interactions in living cells.

Chapter 1 presents the basic background of proteases and current methods of protease activity determination *in vitro* and *in vivo*. Based on the drawbacks and limitations of currently available protease sensors or protease substrate kits, specific criteria for an ideal protease sensor are proposed and a novel design for sensitive protease probes both *in vitro* and in living cells is established. The significance of the sensitive protease sensors built in a single EGFP scaffold is also described.

Chapter 2 discusses the materials and methods utilized in the overall research of this dissertation. The molecular biology to obtain the DNA plasmids encoding specific protease cleavage linkers is detailed. Expression and purification of the designed EGFP-based protease sensor variants, along with differences between fluorescent and non-fluorescent designed proteins, and EGFP fragments are described. The analyses of optical properties for protease sensors before or after protease digestion and fragments of split EGFP are discussed. Mammalian cell culture, transfection and expression of designed protease sensors or EGFP fragments,
protease activation and inhibition measurement in living cells are also illustrated in detail. The comparison of various protease sensor variants and different cell lines was conducted to examine the enzymatic response capability or specificity of protease sensors and basal protease activity in different cell lines.

Chapter 3 discusses the design of sensitive trypsin sensors in detail. Based on the design of various trypsin sensors, the sensitive location for protease sensor development is identified. The trypsinogen activation in pancreatic cancer cells is illustrated and their subcellular compartments are localized. Meanwhile, kinetic studies were used to determine catalytic kinetic parameters and to optimize their cleavage linkers for improving specificity of various trypsin sensors.

Chapter 4 discusses the rational design of various caspase-3 and caspase-8 sensors. The activation or inhibition of caspase-3 and caspase-8 in living cells is probed in detail. The timescale of caspase-3 activation is confirmed using different currently available caspase-3 substrate kits in vitro and in living cells. The basal caspase-3 activity is tested in different cancer cell lines.

Chapter 5 describes the design of thrombin sensors based on our developed protease sensor strategy. The specificity of cleavage linkers in thrombin sensors was investigated through catalytic kinetic studies to optimize the cleavage linker.

Chapter 6 demonstrates the separation of the EGFP fragments of trypsin sensors following trypsin digestion, and the accurate cleavage sites of these EGFP-based trypsin sensors are identified. Optical properties of the large fragment containing the chromophore were studied to reveal the weak fluorescence and the strong potential for reassociation of the split EGFP
fragments. A mini-domain with essential sequence for chromophore formation and fluorescence of EGFP was identified.

Chapter 7 demonstrates a creative strategy for studying calcium-dependent protein-protein interactions in living cells based on the characteristics of association and fluorescence complementation of both split EGFP. The calcium-dependent protein-protein interactions is imaged through the fluorescence complementation of EGFP fragments in living cells and the calcium effect on the interactions of both fragments is confirmed through purified two fragments in vitro and calcium triggers in living cells.

Chapter 8 summarizes the analyses and conclusions in this dissertation. Meanwhile, the major discoveries and future promising are described in this chapter.
Chapter 2  Materials and methods

2.1  Design and construction of protease sensors

In order to design genetic protease sensors, a cleavable linker for specific proteases was genetically generated in pet28a or pcDNA 3.1 (+) plasmid (Invitrogen) encoding EGFP at three locations after residues (Glu172, Gln157 or Asn144) using polymerase chain reaction (PCR) technique. The forward and backward primers of cleavable linkers for various proteases were designed to be length of about 20 base pairs with approximately 70 °C of annulling temperature (T_m). The designed primers of the cleavage linkers for various proteases were purchased from Sigma-Genosys. Before phosphorylation, the primers were dissolved in EB buffer (10 mM Tris, pH 8.5) to final concentration of 100 μM and allowed for incubation in the room temperature for 2 h. After the incubation at room temperature, phosphorylation reaction of the primers was conducted by T4 phosphonucleotide kinase (PNK) (Promega) at 37 °C incubator for 30 min according to the manufacturer’s protocol. Then, the phosphorylation reaction was halted through putting the mixture of reaction in 65°C water bath for 15 min to inactivate T4 phosphonucleotide kinase. KOD hot start polymerase (Invitrogen) was used for the amplification of desired protease sensor DNA plasmids, and the reaction mixture of PCR amplification was prepared following the manufacturer’s protocols. PCR amplification was completed in a MiniCycler thermocycler without heated lid (MJ Research) or TC-3000 thermocycler with heated lid (TECHNE) under a program set up a cycle of activation of polymerase for 2 min at 95 °C; 30-repeated cycles with denatureation at 95 °C for 30 seconds, annulling for 30 seconds at 58-62 °C based on various primers and extension with a rate of 20 sec/kbp at 72 °C; and then a cycle of final extension at 72 °C for 10 min. A drop of mineral oil was added to the top of the mixture of PCR reaction to
prevent evaporation if the reaction was conducted in the MiniCycler thermocycler without heated lid. Following PCR amplification, the synthesized plasmid DNA products were separated on a 0.8% agarose gel and extracted from the gel using a Qiagen gel extraction kit (Qiagen, Valencia, USA). The linear plasmid DNA was then ligated with T4 DNA ligase (Promega) following the manufacturer’s instructions for 22 hours at 4 °C with the addition of approximately 3 mM ATP to provide energy for the reaction. The resulting circular plasmid DNA was transformed into *E. coli* (DH5α) competent cells with a method of heat shock and plated on Luria-bertani (LB) plates with the appropriate antibiotic for corresponding resistance of host vectors, usually kanamycin for pet28a vector or ampicillin for pcDNA vector. Plates with transformed cells were incubated at 37 °C for 16-24 hours to allow for colony growth. A single colony was selected to inoculate in 10 ml LB media with appropriate antibiotic at 37 °C shaker with agitation at 200 rpm and allow for cell growth. The plasmid DNA was purified from DH5α cells using a Qiagen Miniprep kit (Qiagen, Valencia, USA). The constructed plasmid DNA was verified through automated DNA sequencing at Georgia State University (GSU) core facility.

Alternatively, for tracking the protease activation in various subcellular compartments, the ER retention sequence, KDEL, was connected to the C-terminus, and the ER targeting sequence of calreticulin (CRsig), MLLSVPLLLGLLGAAAD, was fused to the N-terminus of EGFP-based protease sensors via PCR technique. The Kozak consensus sequence was placed at the N-terminus of the calreticulin sequence for optimal initiation of protein expression in mammalian cells. Similarly, the sequence of MLSSLRSIRFFKPATRTLCSRYLL for mitochondria targeting was connected to the N-terminus of EGFP-based protease sensors via PCR technique. The commercial marker Mitotracker Red (Invitrogen, Molecular Probe) and DsRed-ER-marker (BD Bioscience, Clontech) were used as positive markers for mitochondria or
ER to confirm the right co-localization of our designed protease sensors with signal peptide sequences.

2.2 Construction of plasmid DNA EGFP fragments

DNA sequences encoding the large (amino acid residues 1-172) and small (amino acid residues 173-238) EGFP fragments (not including the engineered EF-hand motif III of CaM) were fused with the whole EF1 (KSPEELKGIFEEKYAAKEGDPNLQSLKKEELKLLQTEFSLKGP) and EF2 (STLDELFEELKNGDEVSFEFQVLKKISQ) motifs of calbindin D9k, or with partial EF1 (KSPEELKG) and partial EF2 motifs (STLDELFE), respectively, and cloned into pcDNA3.1 (+) vector for mammalian cells. The resulting variants containing whole EF-hand motifs or partial EF-hand motifs were designated N-EGFP-EF1, C-EGFP-EF2, N-EGFP-EF1p and C-EGFP-EF2p. A linker (GGSGSGSS) was interposed to connect the N-EGFP fragment and EF1, or the C-EGFP fragment and EF2 in order to improve the flexibility of EF-hand motifs. The newly-synthesized plasmid DNAs were ligated with T4 DNA ligase, transformed into DH5α competent cells grown in Luria-Bertani (LB) media containing kanamycin or ampicillin, and then purified with a QIAprep Miniprep Kit (Qiagen, USA). The constructed plasmid DNA was verified through automated DNA sequencing.

2.3 Competent cell preparation

A glycerol stock of *E. coli* cell strain is thawed and plated on a fresh LB plate without antibiotic. The plate with *E. coli* cells is incubated overnight at 37 °C for cell growth. A single colony is selected from the plate and inoculated into 10 mL of LB media without antibiotic to allow for cell growth overnight at 37 °C with shaking at 200 rpm. Following the overnight cell
growth, 1 ml of the cell suspension is transferred to 100 ml of fresh LB media without antibiotic and allow to cells grow to an optical density (O.D.) of 0.5 at 600 nm with 200 rpm of shaking at 37 °C. The cell culture is aliquoted to 2 X 50 mL in the prechilled sterile polypropylene tubes and then standed on ice for 5-10 min. The cells are subjected to centrifugation at 3000 rpm for 7 min at 4 °C. The collected cell pellet is resuspended in 10 mL of 100 mM ice-cold calcium chloride solution with a gentle swirling. After another centrifugation at 2500 rpm for 5 min at 4 °C, the cell pellet is resuspended in 10 mL of 100 mM ice-cold calcium chloride solution again and incubated in ice-water bath for 30 min. The cell suspension is subjected to centrifugation at 5500 rpm for 7 min and the cell pellet is then resuspended in 1 mL of 100 mM ice-cold calcium chloride solution containing 15 % glycerol with a gentle swirling. Finally, 100 μL of competent cells is transferred to 1.7 mL prechilled sterile polypropylene centrifuge tubes and immediately freeze at -80°C for later use.

2.4 Subcloning for protease sensors

In order to obtain plasmid DNA of protease sensors with specific cleavage linkers or EGFP fragments for the expression in mammalian cells, the plasmid DNA encoding EGFP-based protease sensors or EGFP fragments in pet28a vector, which contains a kanamycin-resistance gene and a multiple restriction site, was transferred to pcDNA 3.1 (+) vector through subclone technology. The plasmid DNA encoding EGFP-based protease sensors or EGFP fragments and the pcDNA 3.1 (+) vector were subjected to double digestion using BamHI and EcoRI restriction enzymes under their corresponding optimal digestion buffers at 37 °C for 3-4 hours according to the manufacturer’s instructions. Following double digestion, the digested DNA and vector were separated on a 0.8% agarose gel. The bands with desired gene of EGFP-based protease sensor or
EGFP fragments and the vector were sized from the gel and purified with a Qiagen gel extraction kit (Qiagen, USA), respectively. The desired gene was then ligated to the pcDNA 3.1 (+) vector with T4 DNA ligase through the incubation for 22 hours at 4 °C with the addition of approximately 3 mM ATP to provide energy for the reaction. The resulting circular plasmid pcDNA encoding EGFP-based protease sensor or EGFP fragments was then transformed into DH5α competent cells and allowed to grow in Luria-bertani (LB) plates with ampicillin at 37 °C overnight. Individual colony was then grown in 10 mL LB media with ampicillin (100 mM/L) overnight, and the pcDNA encoding EGFP-based protease sensors or EGFP fragments was purified with the Qiagen Miniprep kit (Qiagen, USA). The sequence of desired pcDNA through subcloning was verified by automated sequencing at GSU core facility. On the other hand, in order to obtain plasmid DNA of protease sensors with specific cleavage linkers or EGFP fragments for the expression in *E. coli*, the desired pcDNA encoding EGFP-based protease sensor or EGFP fragments and pet28a vector were subjected to the double digestion, ligation for subcloning into pet28a in the same manner as the subclone in pcDNA vector.

### 2.5 Expression in *E. coli* and purification with His-tag column

EGFP-based protease sensor variants subjected to insertion of a nucleotide sequence encoding a cleavage linker were expressed in *E. coli* BL21 (DE3). A single colony was inoculated into 20 ml of LB media containing kanamycin with concentration of 30 μg/ml at 37 °C with agitation at 200 rpm overnight and then transferred to 1 L of LB media containing kanamycin with a final concentration of 30 μg/ml. The cell culture was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when optical density (O.D.) at 600 nm reached 0.6 and allowed to grow at 30 °C for another 16 to 20 hours. To purify the EGFP-based protease
sensors, cell pellets were resuspended in 10 ml of lysis buffer (20 mM Tris, 10 mM NaCl, 0.1% Triton X-100, pH 8.8) and sonicated to disrupt the cell membrane. The solution was centrifuged at 20000 ×g for 20 min, and the supernatant was filtered and injected into a nickel-chelating column loaded with 0.1 M nickel sulfate solution on fast protein liquid chromatography (FPLC). After washing with buffer A (50 mM phosphate, 250 mM NaCl, pH 7.4), the bound protein was eluted with a gradient of imidazole from 0 to 0.5 M in phosphate buffer. The purity of the fractions was monitored by SDS-PAGE. The protein collected from FPLC was dialyzed in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4) to remove imidazole. The concentration of purified protein was determined by UV-visible absorbance at 280 nm and calculated using Beer’s Law (A = εcl) with an extinction coefficient of 21,890 M⁻¹ cm⁻¹.

2.6 Protease sensor purification with Hitrap Q column

EGFP-based protease sensor variants obtained through the purification from His-tag column were conducted a further purification using an anion exchange column, Hitrap Q column, to improve their purity. Prior to purification, an Hitrap Q column connected to FPLC was subjected to sequentially inject 25 ml (5 column volumes) of start buffer (20 mM Tris, pH 8.0), 25 ml of elution buffer (20 mM Tris, 1 M NaCl, pH 8.0), and 25 ml of start buffer again to equilibrate the column. In order to maintain a similar buffer condition, the EGFP-based protease sensor sample was diluted with start buffer and then loaded on the column. The continual injection was performed until all proteins were bound to the column. Then, the column bound EGFP-based protease sensors was initially washed with start buffer to remove containminating proteins, and then eluted using elution buffer with a gradient of NaCl from 0 to 1 M. Usually, the gradient of approximately 400 mM of NaCl is the optimal condition for the elution of EGFP-
based protease sensors from column. Following the elution of the desired EGFP-based protease sensor, the column was kept to wash using elution buffer with 100% gradient of NaCl to remove any remaining contaminants. The purified EGFP-based protease sensor was concentrated down to desired concentration based on experimental requirements with the Amicon pressure concentrator and dialyzed against Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4) to remove NaCl. The concentration of purified EGFP-based protease sensor was determined by UV-visible absorbance at 280 nm and calculated using Beer’s Law (A = εcl) with an extinction coefficient of 21,890 M⁻¹ cm⁻¹.

2.7 Protein refolding with urea

Some EGFP protease sensor variants grafted into cleavage linkers or EGFP fragments exhibit a tendency to express in inclusion bodies with low solubility, which results in low efficiency of extraction from cells. In order to improve the solubility, the cell pellet of these EGFP protein variants was thawed on ice water and 8 M urea was added to dissolve. Once the cell pellet was dissolved, the mixture was subjected to the refolding through dropwisely adding to appropriate refolding buffer (10 mM Tris, 10 mM DTT, pH 7.4) with a 10X volume of the mixture while stirring. The refolded mixture was centrifuged at 17K rpm for 15-20 min to separate the solubilized protein from the cell pellets or insoluble proteins. The supernatant and pellet were collected to evaluate the extract efficiency using SDS-PAGE. The supernatant containing refolded soluble protein was dialyzed against in a large volume of buffer (10 mM Tris, 1 mM DTT, pH 8.0) overnight at 4 °C to remove the urea. The dialyzed supernatant containing the refolded soluble protein was loaded on His-tag column to conduct the purification. The concentration of purified EGFP-based protease sensor was determined by UV-visible
absorbance at 280 nm and calculated using Beer’s Law \( A = εcl \) with an extinction coefficient of 21,890 M\(^{-1}\) cm\(^{-1}\).

### 2.8 UV-visible spectroscopy

In order to obtain optical properties of EGFP-based protease sensors, UV-visible spectra were monitored using a UV-1700 spectrophotometer scanning from 600 to 200 nm. Samples were buffered in 10 mM Tris, 1 mM DTT, at pH 7.4. The concentrations of the EGFP-based protease sensors were determined by UV-visible absorbance at 280 nm using the molar extinction coefficient of 21,890 M\(^{-1}\)cm\(^{-1}\) calculated from the contribution from aromatic residues (1 Trp and 11 Tyr) (5500 and 1490 M\(^{-1}\)cm\(^{-1}\) for Trp and Tyr, respectively). The extinction coefficients at 398 or 490 nm of the EGFP-based protease sensor variants were obtained with Equation 1, respectively:

\[
ε_p = \frac{ε_{p,280nm}}{A_{p,280nm}} A_p
\]

**Equation 1**

In which, \( ε_p \) is the extinction coefficient at 398 or 490 nm of EGFP-based protease sensor variants, \( ε_{p,280nm} \) is the extinction coefficient at 280 nm of EGFP-based protease sensor variants, \( A_p \) is the absorption of EGFP-based protease sensor variants at 398 or 490 nm, and \( A_{p,280nm} \) is the absorption of EGFP-based protease sensor variants at 280 nm. EGFP was used as a reference in the measurement of the extinction coefficients of the variants.

### 2.9 Fluorescence excitation and emission spectroscopy

In order to obtain optical properties of EGFP-based protease sensors, fluorescence spectra of these EGFP-based protease sensor variants were scanned in the emission range of 410-600 nm.
with an excitation of 398 nm or for emission between 500-600 nm with an excitation of 490 nm. Samples were buffered in 10 mM Tris, 1 mM DTT, at pH 7.4. A fluorescence cuvette with 1 cm pathlength was used.

2. 10 MALDI-TOF mass spectroscopy

All MALDI-TOF mass spectra were recorded on a Voyager-DE™ Pro biomass spectrometry Workstation (Applied Biosystems, Foster City, CA) in a linear-positive mode with delayed extraction. Sinapinic acid in 50% acetonitrile with 1% trifluoroacetic acid (TFA) was used as the matrix. 1 μL of the sample and matrix mixture with various ratios was spotted and air-dried before the analysis. 25KV was applied as acceleration voltage. Proteins (insulin, thioredoxin and apomyoglobin) were used as external standards. A mass range from 300 to 35000 Da was scanned and recorded. The spectra were processed with Explore software.

2. 11 Change in optical properties of sensors following protease cleavage

The digestion reactions of protease sensors were performed by adding stock proteases in their appropriate reaction buffers. The UV-visible spectra of protease sensors following protease digestion were measured in a 1 cm pathway cuvette and the fluorescence spectra of protease sensors with excitation at 398 and 490 nm were recorded. Comparisons with and without protease digestion were conducted to confirm optical changes in the protease sensors following protease cleavage.
2.12 Cleavage identification with SDS-PAGE analysis

The protease sensitivity and cleavage status of EGFP-T1 were monitored and evaluated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Trypsin, chymotrypsin or thrombin was added into 15 μM EGFP-T1 to a final protein: enzyme (w/w) ratio of 100:1 and the digestion was conducted in each of their respective optimal buffers at room temperature. Different ratios of protein and trypsin (w/w) (2000:1, 1000:1, 200:1 and 100:1) were used to investigate the effects of enzyme concentration. Samples were taken at different time intervals for SDS-PAGE. EGFP-T1, not subjected to protease digestion and stored at -20 °C, was evaluated in parallel as a control. The optical density for bands with molecular mass of 20 kDa in SDS-PAGE was determined using Scion Image software to analyze the cleavage rate of EGFP-T1.

2.13 Separation of EGFP fragments after protease digestion

After natural degradation or protease digestion of EGFP-T1, the mixture of products retained strong green fluorescence. In order to investigate cleavage sites and the source of fluorescence, a method combining fast performance liquid chromatography (FPLC) and high-pressure liquid chromatography (HPLC) was utilized to separate protein fragments under denatured conditions. 6 M Urea was added to the digested products of EGFP-T1 and heated in a 90 °C water bath for 5 min to denature protein fragments. The denatured protein fragments were injected into a prepared FPLC connected to a Hitrap Sephadex 75 size exclusion column and eluted with 10 mM Tris buffer containing 6 M urea at pH 7.4. The major fragments were collected and further purified by reversed-phase HPLC equipped with a Whatman C4 column through gradient elution of mobile phase A (H₂O containing 0.1% TFA) and mobile phase B
(100% acetonitrile containing 0.1% TFA). The fractions with purified protein fragments were lyophilized for the analyses of protein sequence, mass spectroscopy and spectral properties.

2.14 Identification of cleavage sites of EGFP-T1

In order to investigate trypsin cleavage sites of EGFP-T1, the molecular mass of digested products was measured by MALDI-MS at the mass spectroscopy center of Georgia State University (GSU) and N-terminal sequence analysis was conducted using Edman degradation at the Centers for Disease Control and Prevention (CDC). The cleavage sites were identified through the calculation of molecular masses of digested fragments from N-terminal to the cleavage site and comparison with observed results from MALDI-MS.

2.15 Optical properties of purified EGFP fragments

The optical properties of P20 were monitored with a UV-1700 spectrophotometer (Shimadzu Scientific Instruments Inc., Japan) and a fluorescence spectrophotometer (Photon Technology International Inc., Canada). The UV-visible spectra of the fragments were scanned from 600 nm to 200 nm in 10 mM Tris, 1 mM DTT, pH 7.4. The UV-visible spectra at different pH conditions were monitored by gradiently adding 0.5 M NaOH into 1 ml of P20 solution in 10 mM Tris, 1 mM DTT at pH 7.32 to final NaOH concentrations of 1.49, 2.98, 4.46, 5.93, 7.39 and 8.84 μM. The final pH value was 10.82 after addition of NaOH. The UV-visible spectra under various pH conditions were scanned to investigate the optical properties of P20. Fluorescence spectra of P20 were measured in the emission region of 410 to 600 nm with an excitation wavelength of 398 nm, and in the emission region of 500 to 600 nm with an excitation wavelength of 490 nm, respectively.
2. 16 Determination of steady-state kinetic parameters of protease sensors

In order to investigate the catalytic kinetic change and to determine the steady-state kinetic parameters, Michaelis constants ($K_m$), turnover numbers ($k_{cat}$) and specificity constants ($k_{cat}/K_m$) for hydrolysis of EGFP-based protease sensors upon the action of proteases, initial rates of trypsin digestion reaction for EGFP-based protease sensors at various concentrations were measured using a time-course model to monitor the change of absorbance at 490 nm for 10 min on a UV-1700 spectrometer (Shimadzu, Japan), where the initial rate was equivalent to the resulting slope of absorbance increase. These data were then used to calculate the steady-state kinetic parameters, $k_{cat}$, $K_m$ and $k_{cat}/K_m$ for hydrolysis of EGFP-based protease sensors following protease digestion, by fitting data to the Michaelis-Menten equation (Equation 2) by nonlinear regression using KaleidaGraph 3.5 software (Synergy software).

$$\frac{v_0}{e} = \frac{k_{cat} [S]}{K_m + [S]}$$

Equation 2

where $v_0/e$ is per mole enzyme initial reaction rate (s$^{-1}$), $k_{cat}$ turnover number (s$^{-1}$), [S] substrate concentration (M), and $K_m$ the Michaelis constant (M).

2. 17 Inhibition of protease cleavage

In order to examine the inhibition effects of leupeptin (Sigma, St. Louis), a trypsin inhibitor, leupeptin at various concentrations (0, 12.5, 25, 50 and 100 nM) was mixed with EGFP-T1 with various concentrations from 2 to 20 μM buffered in 10 mM Tris, 20 mM CaCl$_2$, pH 7.4. Subsequently, EGFP-T1 with leupeptin was digested by 5 nM trypsin. Kinetic studies of EGFP-T1 at various concentrations were performed at each inhibitor concentration level and the
absorbance changes at 490 nm were monitored using a time course mode. Initial rates and trypsin sensor concentrations were fitted with built in competitive inhibition equation (Equation 3), uncompetitive inhibition equation (Equation 4) and Non-competitive inhibition equation (Equation 5) to obtain a plot to confirm inhibition type and $K_i$ values were calculated with Enzfitter (Bio-soft, Cambridge).

### Competitive inhibition equation

\[
\frac{v}{e} = \frac{k_{cat} [S]}{[S] + K_m (1 + [I]/K_i)}
\]  
Equation 3

### Uncompetitive inhibition equation

\[
\frac{e}{v} = \frac{k_m}{k_{cat}[S]} + \frac{1 + [I]/K_i}{k_{cat}}
\]  
Equation 4

### Non-competitive inhibition equation

\[
\frac{v}{e} = \frac{k_{cat}^{app} [S]}{K_m^{app} + [S]} = \frac{k_{cat} [S]}{1 + [I]/K_i} = \frac{K_m}{1 + [I]/K_i} + [S]
\]  
Equation 5

where \(v_0/e\) is per mole enzyme initial reaction rate (s\(^{-1}\)), \(k_{cat}\) is turnover number (s\(^{-1}\)), [S] is substrate concentration (M), [I] is the inhibitor concentration (M), \(K_m\) is the Michaelis constant (M), \(K_i\) is binding affinity of inhibitor, \(k_{cat}^{app}\) and \(K_m^{app}\) are apparent \(k_{cat}\) and \(K_m\), respectively.

### 2.18 Cleavage specificity of protease sensors

In order to examine cleavage specificity of EGFP-T1 exposed to different proteases, thrombin (GE healthcare, USA), cathepsin B, tryptase, tissue plasminogen activator, kallikerin,
elastase or chymotrypsin (Sigma, St. Louis) was used to digest trypsin sensor, EGFP-T1. Thrombin, cathepsin B, tryptase, tissue plasminogen activator, kallikrein, elastase or chymotrypsin was added to 15 µM EGFP-T1, buffered in their optimal reaction conditions (20 mM Tris, 20 mM CaCl₂, pH 7.5 for trypsin or chymotrypsin; 20 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0 for thrombin; 50 mM Tris, 120 mM NaCl, 20 µg/ml Heparin for tryptase; 10 mM Tris, 75 mM NaCl, 2 mM EDTA and 2 mM Cysteine for cathepsin B; 20 mM Tris, 5 mM NaCl, 0.02% NaN₃, pH 8.0 for tissue plasminogen activator; 20 mM Tris, 100 mM NaCl, 0.1% BSA, pH 7.5 for kallikrein and 20 mM Tris, 400 mM NaCl, pH 7.5 for elastase), to a final concentration of 20 nM, respectively. The cleavage of EGFP-T1 following trypsin, thrombin, cathepsin B, tryptase, tissue plasminogen activator, kallikrein, or chymotrypsin overnight digestion was verified using SDS-PAGE. To confirm optimal reaction buffer conditions for EGFP-T1 cathepsin B digestion, a fluorescent substrate (Z-Phe-Arg-7-amino-4-methyl coumarin or Z-FR-AMC) was used for its cleavage in identical buffer condition by cathepsin B. The fluorescence signal change of Z-FR-AMC at 460 nm was detected by fluorescence spectrophotometer with excitation of 380 nm following cathepsin B digestion.

2.19 Extinction coefficient calculation of cleaved products

In order to determine the concentrations of cleaved products following trypsin digestion, 5 µL of 40 µM trypsin was added to trypsin sensor samples to conduct overnight digestion. Following the complete digestion of trypsin sensor samples, the absorbance at 490 nm of digested trypsin sensors was measured using a UV-1700 spectrometer. Extinction coefficient constants of cleaved products from trypsin sensors were calculated using Beer-Lambert law. Based on the calculated extinction coefficient constants and absorbance change, the cleaved
product concentrations of trypsin sensors following trypsin digestion can be obtained for initial rate calculations.

2.20 Relative signal change calculation

In order to evaluate the optical signal change of our designed trypsin sensors, the relative signal change for absorbance or fluorescence change upon trypsin digestion is calculated by Equation 6:

\[
D = \frac{A_{490a} / A_{398a}}{A_{490b} / A_{398b}}
\]

or

\[
D = \frac{F_{490a} / F_{398a}}{F_{490b} / F_{398b}}
\]

where \(A_{490a}\) and \(A_{398a}\) are the absorbances at 490 and 398 nm following trypsin cleavage; \(A_{490b}\) and \(A_{398b}\) are the absorbances at 490 and 398 nm before trypsin cleavage; \(F_{490a}\) and \(F_{398a}\) are the fluorescence intensities at excitation of 490 and 398 nm following trypsin cleavage; and \(F_{490b}\) and \(F_{398b}\) are the fluorescence intensities at excitation of 490 and 398 nm before trypsin cleavage, respectively.

2.21 Mammalian cell culture

The HeLa cell line was purchased from the American Type Culture Collection (ATCC). The MIA PaCa-2 cell line, a pancreatic cancer cancer line, was provided by Dr. Lily Yang at Emory University. Both cell lines are adherent, and stocks are grown on 100 mm cell culture dishes containing their optimal cell culture media in a 37 °C humidified incubation chamber equipped with 5% CO2. The cell culture media for HeLa cells is Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma Chemical Co., St. Louis, MO) with 1 g/L glucose and L-glutamine, sodium bicarbonate, supplemented with 10% fetal calf serum (FCS), and 100 U/ml penicillin and
0.1 mg/ml streptomycin (Pen-Strep or PS). The cell culture media for MIA PaCa-2 cells has the same components as the media for HeLa cells except the glucose is 4.5 g/L. When the cells are reached the confluence of approximately 95%, the passage is required to reduce the confluence for allowing to the continuing growth of the cells. As for the passage procedure, 10 ml of Hank’s Balanced Salt Solution without calcium chloride and magnesium sulfade (HBSS⁻) (Sigma Chemical Co., St. Louis, MO) was used to replace the cell culture medium in the dish and wash the cells for 2 times. Following the washing of HBSS⁻ buffer, 1 mL of 0.05% trypsin-EDTA solution is added to the cell dish to detach the cells. After the cell detaching, the cells with trypsin solution are diluted with 9 ml of HBSS⁻ buffer and mix the cells well to fully suspend. One miniliter of the diluted cell suspension solution was peptited out and plated plate back to the cell dish with the addition of 9 ml of fresh cell culture media for regular cell growth. The rest of diluted cell suspension solution is discarded. The passaged cells are placed back into a 37 °C humidified incubation chamber equipped with 5% CO₂ until they reach confluence again. In general, the cell passage is required every 2-3 days.

To prepare the cell culture media for HeLa or MIA PaCa-2 cells, 13.4 g DMEM (Sigma Chemical Co., St. Louis, MO) powder with 1 or 4.5 g/L glucose and glutamine was dissolved in 800 mL of ddH₂O. 3.7 g of NaHCO₃ was added to the media to a final concentration of 44 mM. 6 M HCl was applied to adjust pH to 7.2-7.3. The prepared media was brought to 1 L with ddH₂O. The media was filtered in the laminar flow hood with a Sterivex 0.22 μm filter and stored in an autoclaved glass bottle at 4 °C. Before cell culture, 500 mL of the cell culture media were supplemented with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

To prepare the HBSS⁻ buffer, 9.7g Hank’s Balanced Salt Solution without divalent cations (Sigma Chemical Co., St. Louis, MO) was dissolved in 800 mL of ddH₂O. Additionally,
0.0238 g of HEPES, 0.42 g of NaHCO₃, and 0.38 g of EGTA were also dissolved into the buffer solution to final concentrations of 10 mM, 5 mM and 1 mM, respectively. Once all reagents were dissolved, the pH of the buffer was adjusted between 7.2 and 7.3 with 5 M NaOH. Finally, the volume of the buffer was brought to 1 L with ddH₂O and filtered with a Sterivex 0.22 μm filter into an autoclaved glass bottle for storage at 4 °C.

2.2 Preparation of cell plates and transfection of protease sensors

For transfection, the cell plates were prepared one or two days in advance. The cells were grown on glass coverslips (0.5 - 1.0 × 10⁶ cells/dish) in 35 mm culture dishes in DMEM (Sigma, St. Louis, MO) with 44 mM NaHCO₃, pH 7.2, and supplemented with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Pen/Strep) at 37 °C with 5% CO₂ in a humidified incubation chamber. After the cells were seeded for 24-48 hours, the desired DNA plasmids were transfected to the cells. During the transfection, 2 μl of liposome, lipfectamine-2000, (Invitrogen Life Technologies) was mixed with 1 ml of serum-free Opti-MEMI media (Gibco Invitrogen Corporation) and allowed to equilibrate for 5 min at room temperature. 2 μg of desired plasmid DNA was then mixed well with Opti-MEMI media containing liposome. The mixture of DNA and liposome solutions was allowed to equilibrate for 20 min in a light-exclusion environment at room temperature. During the equilibrium, the cell plates were washed twice with 1-2 ml Opti-MEMI. The DNA-liposome mixture was then transferred to the cell plates. The additional Opti-MEMI was supplied to cell plates to 2 ml of the final volume. The cell plates were incubated for 4-5 hours in an incubator supplied with 5% CO₂ at 37 °C. Following 4-5 hours incubation, the Opti-MEMI media was replaced with fresh DMEM cell culture media for protein expression at 30 °C for 24-48 hours.
2. 23 Transfection of EGFP fragments

HeLa cells were grown on glass coverslips (0.5 - 1.0 x 10^6 cells/dish) in 35 mm culture dishes in Dulbecco’s Modified Eagles Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) with 44 mM NaHCO₃, pH 7.2, and supplemented with 10% FCS (v/v), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Pen/Strep) at 37 °C with 5% CO₂ in a humidified incubation chamber. After the cells were seeded and grown overnight, N-EGFP-EF1 and C-EGFP-EF2 were co-transfected into HeLa cells with lipofectamine-2000 (Invitrogen Life Technologies) and serum-free Opti-MEM (Gibco Invitrogen Corporation) according to the manufacturer’s instructions. During the transfection, 2 µl of liposome, lipfectamine-2000, (Invitrogen Life Technologies) was mixed with 1 ml of serum-free Opti-MEM media (Gibco Invitrogen Corporation) and allowed to equilibrate for 5 min at room temperature, then 2 µg of N-EGFP-EF1 and 2 µg of N-EGFP-EF2 plasmid DNA were added to the serum-free Opti-MEM media containing lipofectamine-2000, respectively. Following overnight incubation at 37 °C, the serum-free Opti-MEM media containing DNA and lipofectamine complex was replaced by fresh DMEM cell culture media enriched with FCS and Pen/Strep. The cells were then grown 24 to 48 hours in a humidified chamber with 5% CO₂ at 30 °C before fluorescence microscope imaging. Individual transfection of N-EGFP-EF1 or C-EGFP-EF2 or co-transfection of N-EGFP-EF1p and C-EGFP-EF2p were used as controls.

2. 24 Fluorescence imaging

Cells were transfected with protease sensors or EGFP-wt, and then allowed to grow for 24-48 hours before imaging. EGFP-wt was used as the control. Cells were imaged on a Zeiss
Axiovert 200 Inverted Microscope with a 40x oil objective lens and a CCD camera (AxioCam HRc). Ratiometric emission imaging was acquired through a FITC filter set (excitation wavelength: 480 ± 10 nm; emission wavelength: 510 ± 10 nm) and 395 nm filter set (excitation wavelength: 400 ± 10 nm; emission wavelength: 510 ± 10 nm) from a light source (FluoArc, Zeiss) in a time course mode using Axiovision software. During image acquisition, protease activation triggers or calcium triggers were added to the cell plate to an appropriate final concentration for inducing protease activation or opening calcium channels in the subcellular membrane to adjust calcium concentration in different subcellular compartments. The ratiometric change between fluorescence emission for excitation at 488 nm and 398 nm was calculated at different time intervals to detect protease activation in living cells and average ratio values were calculated from individual cells (Hanson, McAnaney et al. 2002). All fluorescence emissions at different time intervals were normalized by dividing the initial fluorescence intensity to set the basal ratio to 1 (Allen, DiPilato et al. 2006).

The fluorescence ratiometric change (R) is expressed by equation 7:

\[ R_{t-488/398} = \frac{NF_{t-488}}{NF_{t-398}} \]

Equation 7

where NF\(_{t-488}\) is the normalization fluorescence intensity of various time points at 488 nm excitation, and NF\(_{t-398}\) is the normalization fluorescence intensity of various time points at 398 nm excitation.

However, while investigating calcium-dependent protein-protein interactions, calcium ion channel triggers were applied to activate the calcium influx of cellular compartments and examine the calcium effect on dimerization of EF1 and EF2 from calbindin D9k. During imaging acquisition, the signal change in fluorescence intensity under 488 nm excitation was used to an
indicator of calcium dependent protein-protein interaction when ionomycin and calcium were added to stimulate the cells co-transfected N-EGFP-EF1 and C-EGFP-EF2 to final concentrations of 1 μM and 5 mM, respectively.

2.25 Immunofluorescence

In order to identify the same subcellular location of trypsinogen activation and EGFP-based trypsin sensor expression, immunofluorescence was used to confirm the localization of trypsinogen activation following induction with 10 nM caerulein in MIA PaCa-2 cells transfected EGFP-T1. EGFP-T1 was transfected into MIA PaCa-2 according to above statement for its expression 48 hrs. Following protein expression, MIA PaCa-2 cells was conducted to induction using caerulein in a final concentration of 10 nM for 1 hr. The cell culture media was removed and the cells were washed one time using PBS buffer (140 mM NaCl, 2.7 mM KCl, 20 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4). After the washing, 500 μl of 3.7% paraformaldehyde was added in the cell chamber to fix the cells for 30 min at room temperature and then the fixed cells were incubated with 0.1% Triton X-100 for 15 min at room temperature. The fixed cells were washed with TBS buffer three times for 15 min each time. 500 μl of TBS buffer with 5% BSA was added in the cell chamber to block the cells for 3 hrs, and then primary antibodies, rabbit polyclonal to α-antitrypsin and goat polyclonal to GFP conjugated with FITC (Abcam Inc.) was added into cell chamber with TBS buffer containing 2.5% BSA with 1:200 dilutions and then incubated with fixed cells for another 3 hrs at room temperature. Following the incubation with primary antibodies, the cells were washed with TBS buffer three times for 15 min each time. Secondary antibody, rabbit IgG antibody conjugated with Texas Red (Abcam Inc.) was used to against the primary antibody, rabbit polyclonal antitrypsin for incubating 1 hr at room
temperature. After the incubation, the extra secondary antibody was removed through the washing three times with TBS buffer for 15 min each time. One drop of mounting regent was added on the glass coverslip and then covered for cell imaging.

2.26 Confocal microscope imaging

The cells transfected with protease sensors were transferred from DMEM cell culture media to Ringer solution (10 mM HEPES, 0.4 mM KH$_2$PO$_4$, 2.4 mM K$_2$HPO$_4$, 120 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM Glucose, pH 7.4), and the living cell imaging experiments were conducted on an LSM 510 laser confocal microscope (Carl Zeiss Inc., Thornwood, NY) using a 100X oil-immersion objective (Zeiss, Fluar, 1.30 NA). Prior to imaging, the cells and solutions were allowed to equilibrate to room temperature. The localization of EGFP-based protease sensors was visualized by excitation of EGFP with the 488 nm line of an argon laser, and the narrowest bandpass filter (505-530 nm) was employed for emission. Mitotracker Red and DsRed-ER marker were excited with the 543 nm line of a He-Ne laser, and emission was detected through a long-pass filter (emission above 560 nm). Zeiss LSM 510 software (Carl Zeiss, Inc.) was used to control the image acquisition parameters. All images were acquired at high resolution (1024 × 1024).

2.27 Protease assay in cell lysates

To examine the protease and confirm the protease activation pattern in living cells, protease activity was determined using chromogenic or fluorogenic peptide substrates in cell lysates following protease inducer stimulation. HeLa Cells or MIA PaCa-2 cells ($1 \times 10^6$) were cultured in 100-mm cell culture dishes for 24 h and then treated with 1 µM staurosporine (STS)
or 10 nM caerulein to activate caspase-3 or trypsinoenin, respectively. The cell pellets from each cell line at 80% confluence following STS or caerulein induction at various interval times were harvested and lysed with lysis buffer (10 mM Tris, 10 mM NaH₂PO₄/NaHPO₄, 150 mM NaCl, 1% Triton X-100, 10 mM Na₂P₂O₇·10H₂O, pH 7.5) containing phosphatase inhibitors (1 mM sodium orthovanadate, 30 mM NaF, 1 mM phenyl methylsulfonyl fluoride). The supernatant containing protein was obtained through centrifugation at 13000 ×g. The total protein concentration was determined using BSA protein assays (Bio-Rad, USA) according to the protocol of the manufacturer. The cell lysates containing 100-μg protein of each sample at various time intervals were mixed with trypsin substrate (Z-Arg-pNA) or caspase-3 substrate kit (Z-DEVD-AFC) in the indicated substrate reaction buffer (10 mM Tris, 20 mM CaCl₂, pH 7.5) for trypsin activity assays or (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% sucrose, pH 7.4) for caspase-3 activity assays, and then incubated under light-free conditions for 1 hour at 37 °C. The absorbance or fluorescence results of each sample at various induction time intervals were measured using a fluorescence microplate reader. Relative fluorescence units (RFU) were to express the trypsin or caspase-3 activity. To normalize the activated trypsin or caspase-3 amount in cell lysates, active trypsin or caspase-3 was used as a positive control. The amount of trypsin or caspase-3 activation can be estimated at various time points following caerulein or STS induction in living cells.

2.28 Western blot

To confirm the caspase-3 activation pattern in living cells, caspase-3 activation was examined using western blot in cell lysates. HeLa Cells and MIA PaCa-2 cells (1 × 10⁶) were cultured in 100-mm cell culture dishes for 24 h and then treated with 1 μM STS. Cells were
harvested at various time points (0, 30, 60 and 120 min) following STS stimulation and then lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1.0% NP40, 0.5% deoxycholic acid, 0.1% SDS, pH 8.0) containing phosphatase inhibitors (1 mM sodium orthovanadate, 30 mM NaF, 1 mM phenyl methylsulfonyl fluoride). The total amount of protein of each cell lysate supernatant was measured with BSA protein assays. 100 μg of protein from each sample was electrophoresed through a polyacrylamide-SDS gel for 3 h at 60 V and electroblotted onto nitrocellulose membranes in transfer buffer (50 mM Tris, 100 mM glycine, and 20% methanol) for 4 h at 100 mA. The membrane with proteins was blocked overnight at 4 ºC using block buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, 5% BSA, pH 7.5). Then the proteins on the membrane were exposed to a monoclonal primary antibody, anti-mouse caspase-3 (Cell Signaling, USA), for 4 h at room temperature, which can interact with the full length of caspase-3 and the large fragment of cleaved caspase-3. Following the washing of primary antibody, HRP-conjugated secondary antibody was added to interact with primary antibody for 2 h at room temperature. The interaction between primary and secondary antibody was detected with the enhanced chemiluminescence (ECL) system after the addition of anti-mouse or anti-rabbit IgG (Cell Signaling, USA). Meanwhile, the primary antibody, anti-β-actin was used as the loading control.

2.29 Detection of half-time for maximum casapse-3 activation in living cells

Cells were transfected with EGFP-based caspase-3 sensors and then allowed to grow for 24-48 hours before imaging. In order to detect the rate of caspase-3 activation in living cells following 1 μM STS stimulation, the half-time maximum for caspase-3 activation was used to evaluate. Cells were imaged on a Zeiss Axiovert 200 Inverted Microscope with a 40x oil objective lens and a CCD camera (AxioCam HRc). Ratiometric emission imaging was acquired
through a FITC filter set (excitation wavelength: 480 ± 10 nm; emission wavelength: 510 ± 10 nm) and 395 nm filter set (excitation wavelength: 400 ± 10 nm; emission wavelength: 510 ± 10 nm) from a light source (FluoArc, Zeiss) in a time course mode using Axiovision software. During image acquisition, STS was added to the cell plate to a final concentration of 1 μM for inducing caspase-3 activation. The fluorescence ratiometric change between excitation at 398 and 488 nm was calculated at different time intervals to detect protease activation in living cells. The time point of 50% change between minimum and maximum fluorescence ratiometric change is designated as half-time for maximum caspase-3 activation. The average half-time for maximum caspase-3 activation is calculated using individual half-time for maximum caspase-3 activation from various cells (at least 12 cells), which can also used to compare the potential caspase-3 activation capability of different cell lines.

2.30 pH change examination in living cells

In order to determine whether inducers for trypsinogen or caspase-3 activation have an effect on pH change in living cells, one of the pH probes, 2', 7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Invitrogen, Molecular Probe, USA) was used to examine the pH change following protease trigger stimulation. HeLa or MIA PaCa-2 cells were grown on glass coverslips (0.5 - 1.0 × 10^6 cells/dish) in 35 mm culture dishes in Dulbecco’s Modified Eagles Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) with 44 mM NaHCO₃, pH 7.2, and supplemented with 10% FCS (v/v), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Pen/Strep) at 37 °C with 5% CO₂ in a humidified incubation chamber. After the cells were seeded and grown overnight, the cells were incubated with BCECF-AM pH probe to a final concentration of 10 μM in modified Ringer solution at 37 °C for 45 min. Then
the fresh modified Ringer solution was added to the cell dishes and incubated for another 20 min at 37 °C to ensure complete hydrolysis of the probe and release of the fluorescent group in living cells. Cells were imaged on a Leica Inverted Microscope (Leica Microsystems, Germany) with a 40x oil objective lens and an EM CCD camera (Hamamatsu Corporation, Japan). Ratiometric emission imaging was acquired though a monochromater set (excitation wavelength: 490 ± 10 nm and 440 ± 10 nm; emission wavelength: 535 ± 10 nm) from a light source in a time course mode under the control of SimplePCI software (Hamamatsu Corporation, Japan). During image acquisition, protease activation triggers (10 nM caerulein for trypsinogen activation and 1 µM STS for caspase-3 activation) were added to the cell plate to an appropriate final concentration for inducing protease activation. The ratiometric change between fluorescence emission for excitation at 490 and 440 nm was calculated at different time intervals to express pH change due to effects of protease inducers in living cells.

2.31 Fluorescence spectral scanning of protease sensors in living cells

The living cells cultured on glass coverslips with transfected protease sensors were transferred from DMEM cell culture media to Ringer solution (10 mM HEPES, 0.4 mM KH₂PO₄, 2.4 mM K₂HPO₄, 120 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Glucose, pH 7.4). The glass coverslip with living cells containing transfected protease sensors was vertically placed in a cuvette with 1 cm of pathway. Two miniliter of Ringer solution was added into the cuvette to make the solution level over the glass coverslip for maintaining cells under normal culture conditions. Fluorescence excitation spectra and emission spectra of protease sensors in living cells were measured using a fluorescence photospectrometer (Photon Technology International Inc., Canada). The fluorescence excitation spectra ranging from 300 to 500 nm
were scanned with an emission wavelength of 508 nm, while the fluorescence emission spectra ranging from 500 to 600 nm were scanned with excitation wavelengths of 398 and 490 nm. In order to track the fluorescence spectral change of caspase-3 sensors in living cells following stimulation with 1 μM STS, fluorescence emission spectra of caspase-3 sensors were measured at various time intervals of 0, 5, 10, 20, 30, 45, and 60 min following STS induction. The fluorescence spectrum change of EGFP-wt in living cells was also monitored in parallel under identical conditions as a control. The relative change in maximum fluorescence intensity at 508 nm with excitations at 398 and 490 nm was calculated and normalized to reveal relative percentage change following protease activation through equation 8:

$$ NF_t = \frac{F_t}{F_0} $$

Equation 8

where \( NF_t \) is the normalization fluorescence intensity at various time points; \( F_t \) is the fluorescence intensity of various time points and \( F_0 \) is the initial fluorescence intensity.

Meanwhile, the ratiometric change of fluorescence emission between excitations at 398 and 470 nm was calculated at different time intervals to detect protease activity in living cells. The fluorescence ratiometric change (R) is expressed by equation 9:

$$ R_{t-398/490} = \frac{NF_{t-398}}{NF_{t-490}} $$

Equation 9

where \( NF_{t-398} \) is the normalization fluorescence intensity at 508 nm for various time points with 398 nm of excitation; \( NF_{t-490} \) is the normalization fluorescence intensity at 508 nm for various time points with 490 nm of excitation.
2.32 Statistical analysis

The data for normalization of fluorescence intensity and fluorescence ratio change of living cells transfected with EGFP-based protease sensors and EGFP-wt are represented as means ± SD with at least 6 living cells. Student’s t-test analysis was performed to determine the statistical significance between EGFP-based protease sensors and EGFP-wt. Differences were considered statistically significant at $P < 0.05$. 
Chapter 3  Design sensitive EGFP-based trypsin sensors for living cell imaging

3. 1  Introduction

3.1.1  Trypsin function

Digestive proteases are produced as inactive proenzymes inside the membrane-bound zymogen granules (Thrower, Diaz de Villalvilla et al. 2006). The proenzyme becomes active after enzymatic maturation during exocytosis (Raraty, Ward et al. 2000; Sherwood, Prior et al. 2007). Usually protease inhibitors coexist with proteases at concentrations sufficient to prevent autodigestion (Hirota, Ohmuraya et al. 2006). Fatal human diseases such as acute pancreatitis result from autodegradation of trypsin, chymotrypsin and elastase derived from pancreatic tissues. Trypsin is one of the major digestive enzymes that are responsible for regulating protein degradation (Hirota, Ohmuraya et al. 2006). Intracellular misbalanced trypsinogen activation, inhibition, turnover and secretion can result in acute and chronic pancreatic diseases (Goldberg 2000). Approximately 35000 new cases of pancreatic cancer related to serine proteases, including trypsin, are reported annually, which ranks as the fourth leading cause of cancer deaths in the United State (Sarkar, Banerjee et al. 2006). The overall survival rate for pancreatic cancer patients is less than 4%, but diagnosis at an early stage may significantly improve these odds (Shaib, Davila et al. 2006). Clearly, finding an effective approach to investigate pathological mechanisms, improve early diagnosis and develop new treatment strategies for pancreatic cancer would have significant medical value.
3.1.2 Substrate specificity of trypsin

Examination of the three-dimensional structures of the enzyme-inhibitor and enzyme-pseudosubstrate complexes can reveal important information on hydrogen bonds, van der Waals interactions, and electrostatic interactions. Intensive investigations of trypsin have resulted in significant achievements in elucidating the amino acid and nucleotide sequences, three-dimensional structures, and kinetic and thermodynamic constants for trypsin substrates and trypsin inhibitors over the past four decades. Long-term investigation into this enzyme and its family members has thus far produced spectacular achievements. Much attention is also drawn to crystallographic studies or to site-directed mutagenic studies, which can narrow, broaden or change protease specificity in a predictable way.

Based on the interaction between substrate recognition sites and the subsites of trypsin (c.f. Figure 1.1), trypsin is a proteolytic enzyme showing substrate specificity for Arg or Lys with high preference at the P₁ position. A negatively charged amino acid at the base of the catalytic binding pocket of trypsin is important for catalytic activity (Graf, Jancso et al. 1988; Evnin, Vasquez et al. 1990). According to the available crystal structures for trypsin complexes, Asp189 is a crucial amino acid residue in the pocket and always contributes a cyclic network of hydrogen bonds with the guanidinium group of Arg, as also indicated in Figure 3.1 (Perona and Craik 1997). In order to examine the significance of Asp189 in trypsin, extensive investigations were conducted using both site-directed mutagenesis (Graf, Craik et al. 1987; Graf, Jancso et al. 1988; Wilke, Higaki et al. 1991) and genetic selection (Evnin, Vasquez et al. 1990). The mutant of trypsin, D189K, displays no catalytic activity on lysyl or arginyl substrates. Approximately 10⁵-fold decrease in the catalytic efficiency toward peptidyl substrates containing the Arg or Lys residue at the P₁ position can be observed following replacement of Asp189 with different
Figure 3.1. Interaction between substrate and catalytic binding pocket in trypsin. A cyclic network of hydrogen bonds is formed between the guanidinium group of P₁ Arg in substrate and Asp189 at the catalytic binding pocket in trypsin. Modified after (Perona and Craik 1997).
residues, such as Asn, Ala, or Tyr (Graf, Craik et al. 1987; Graf, Jancso et al. 1988). The catalytic activity with respect to arginine and lysine substrates can also be partially restored by either the presence of Asp or Glu at position 189 or 190 due to the electrostatic complementarities. The amino acid residue at position 190 also has a regulatory effect on the catalytic specificity of trypsin (Evnin, Vasquez et al. 1990). Surprisingly, the activity of the D189S mutant can also be partly rescued using a high concentration of acetate (up to 3 M) (Perona, Hedstrom et al. 1994). In this case, the restored activity may result from the negative potential provided by the acetate molecule, which appears to cause up to 300-fold rescue of the catalytic rate.

The contributions from acidic residues in the catalytic binding pocket to the catalytic activity or efficiency were also intensively probed through charge relocation. The side chain of Gly226 in trypsin may potentially protrude into the pocket, based on the analysis of the crystal structure of G226A (Wilke, Higaki et al. 1991). Charge relocation was fulfilled through site-directed mutagenesis, and high-level catalytic efficiency was revealed in a D189G/G226D variant for substrates with P₁ positively-charged side chains. Similar kinetic enhancements were obtained for P₁ Arg and Lys substrates in the case of genetically-selected variants with Asp relocated to position 190 (Evnin, Vasquez et al. 1990). Therefore, electrostatic availability through charge interactions can contribute to more interaction between the enzyme and substrate than the direct hydrogen bond interactions involving Asp226, or can modify the hydrogen bond arrangement between the enzyme and substrate. Such hypotheses can also explain the importance for the conservation of Asp189 in trypsin-like serine proteases.

Taken into consideration the characteristics of the catalytic binding pocket in trypsin, a growing body of methods is used to evaluate substrate specificity for trypsin and to develop
sensitive substrates or probes for determining trypsin activity. Since the P₁ position of the substrates for trypsin selectively prefers Arg or Lys, the P₂ and the P₃ sites were first found to exhibit high preference to Ala and Pro residues at the P₂ site and Gln at the P₃ site based on the comparison of kinetic parameters of Boc-X-X-Arg-Mec substrate (Kawabata, Miura et al. 1988). The trypsin chromogenic substrate selection using combinatorial chemistry approach was used to design an optimal substrate with high specificity (Zablotna, Dysasz et al. 2004) and obtained the most sensitive substrate Phe-Val-Pro-Arg-Anb-NH₂ with 125-fold more activity than BAPNA, (Bz-D, L-Arg-pNA). Peptide microarray and peptide-profiling library were also applied to assay protease substrate specificity (Gosalia, Salisbury et al. 2005; Thomas, Francis et al. 2006). Taking into consideration substrate specificity for trypsin, the kinetic parameter, $k_{cat}/K_m$, of most substrates conjugated to preferable amino acid residues and chromogenic or fluorogenic dyes, ranged from $10^3$ to $10^6$ M⁻¹ s⁻¹.

### 3.1.3 Trypsin measurements

The study of protease actions in living systems requires sensitive probes capable of real-time monitoring the activation or inhibition of proteases in various cellular locations. Although proteases and protease inhibitors can be monitored through real-time PCR and Western blot, quantitative methods to measure protease activity and its inhibition in living cells in real time are highly desired. The actions of most proteases are typically measured using chromogenic or fluorogenic dyes such as p-nitroaniline, AFC, AMC or rhodamine conjugated to short peptides/peptide mimics encompassing the P₁ to Pₙ cleavage sites (usually up to P₄) in cell lysates, or using fluorescence antibody detection of the cleaved product in fixed cells (Abe, Baba et al. 2000; Zablotna, Dysasz et al. 2004). Although some of these peptide probes are able to
diffuse into cells by passive diffusion through the membrane, they cannot provide continuous
dynamic imaging of enzyme actions due to limited cellular lifetime, nor are they capable of
detecting reactions at targeted cellular locations (Miyawaki 2003; Giepmans, Adams et al. 2006).
Fluorescent proteins with self-encoded chromophores are capable of monitoring numerous
cellular events in living cells or organisms via real-time imaging (Chalfie, Tu et al. 1994).
Currently, several protease probes using fluorescent protein pairs have been developed, which
utilize fluorescence resonance energy transfer (FRET) change upon protease actions (Luo, Yu et
al. 2003; Zhang 2004; Allen, DiPilato et al. 2006; Wu, Simone et al. 2006; Vinkenborg, Evers et
al. 2007; Yang, Zhang et al. 2007). Meanwhile, fluorescent complementation techniques based
on ligand-induced chromophore formation by protein fragments have also been applied to
develop sensors to detect calcium, kinase and protein-protein interactions in various cellular
processes (Nagai, Sawano et al. 2001; Kawai, Sato et al. 2004). Calpain proteolytic activity in
living mice muscle can be monitored through FRET-based methods using multiphoton
microscopy (MPM) (Stockholm, Bartoli et al. 2005). However, the practical application of these
FRET-based protease probes is limited by FRET efficiency due to cross-talk in FRET signals,
sensitivity to photobleaching and cellular environmental perturbation (Felber, Cloutier et al.
2004; Piston and Kremers 2007; Vinkenborg, Evers et al. 2007). Therefore, the study of protease
actions in living systems is frequently hindered by the lack of ideal tools or probes capable of
monitoring dynamic processes of proteases in various cellular locations. The capability of
assessing the functional roles of proteases by quantitative, real-time methods to measure protease
activity and its inhibition in living cells is highly desired (Boonacker, Elferink et al. 2003).
Investigations on mechanisms and diagnosis of chronic pancreatic diseases are largely restricted
to later stages of the illness, partly due to inherent limitations in the currently available peptide kits designed for the detection of trypsin activity in cell lysates.

3.1.4 Objectives

In this chapter, we first report a novel strategy to develop trypsin sensors by grafting an enzymatic cleavage site into a sensitive location for chromophore optical property change of EGFP. The ratiometric optical signal change of designed trypsin sensor variants will be examined. Their high enzymatic selectivity and kinetic responses for enzyme activation and inhibition will be investigated. The application of developed trypsin sensors to cellular activation of trypsinogen and the effects of inhibitors in living pancreatic cells will be examined.

3.2 Design and construction of trypsin sensors

To design protease sensors for monitoring cellular processes related to protease activation and inhibition with fluorescence signaling, trypsin sensor variants were engineered by grafting an enzymatic cleavage sequence onto EGFP, as seen in Figure 3.2A. We hypothesized that proteolytic cleavage occurring within designed sequences at sensitive locations near the chromophore could result in a chromophore signal change due to local conformational change. Based on cited criteria for optimal design of a protease sensor, three grafting sites on the wild type EGFP (EGFP-wt) scaffold were selected to confer these essential sensor properties. The three sites are immediately following Glu172 within loop-9, Gln157 within loop-8, and Asn144 within loop-7. Trypsin demonstrates a strong preference to cleave the peptide bond immediately following an Arg or Lys residue in a solvent-accessible region. Consequently, we selected a trypsin cleavage sequence derived from EF-hand motif loop-III of calmodulin as the basic
Figure 3.2. Grafting location selection and cleavage linker for the design of trypsin sensors. (A) The diagram demonstrates three EGFP-based trypsin sensors grafted the cleavable linker for trypsin at positions Asn144, Gln157 and Glu172. Trypsin cleavage occurs following residues R or K underlined in sequence. (B) The model structure shows insertion locations for the grafting of trypsin linker in the EGFP scaffold.
grafted structure because it contains three Arg and one Lys residues in the loop and flanking helices, and has good solvent accessibility based on the model structure shown in Figure 3.2B. Three potential trypsin sensors were obtained through a grafting approach using PCR techniques, which are named after EGFP-T1, EGFP-T2 and EGFP-T3 based on location 172, 157 and 144, respectively.

3.3 Optical properties of trypsin sensors

The genetically engineered trypsin sensors EGFP-T1, EGFP-T2 and EGFP-T3 along with wild type EGFP in the pet28a vector were successfully expressed in *E. coli*. Following grafting of trypsin cleavage sites, EGFP-T1 and EGFP-T2 retained strong fluorescence both in bacteria and as purified proteins, but EGFP-T3 did not. The UV-visible absorption spectra of the three classes of purified trypsin sensors are shown in Figure 3.3A. EGFP-T3 did not exhibit strong absorption peaks in the range 350-500 nm. Compared to the absorption spectra of EGFP-wt, EGFP-T2 exhibited a similar maximum absorption wavelength at 490 nm due to its deprotonated form of the chromophore. In contrast, EGFP-T1 had two strong absorption peaks at 490 and 397 nm, where the peak at 397 nm corresponds to the protonated form of the chromophore (Tsien 1998). The insertion of the EF-loop at position 172 in EGFP increased the population of the protonated form of the chromophore while correspondingly decreasing the deprotonated form of the chromophore observed at 490 nm. The maximum emission wavelengths of EGFP-wt, EGFP-T1 and EGFP-T2 were 508, 508 and 506 nm, respectively, resulting from excitation at 398 nm, as shown in Figure 3.3B. However, it should be noted that part of the fluorescence emission signal at 508 nm of EGFP-T1 also originated from excitation at 490 nm. Furthermore, such alteration of the ionic state of the chromophore is related to the addition of the flanking helices.
Figure 3.3. Optical properties of designed trypsin sensors.
UV-visible absorption (A) and fluorescence (B) spectra of EGFP-wt (○), EGFP-T1 (□), EGFP-T2 (◊) and EGFP-T3 (●) were measured in 10 mM Tris, 1 mM DTT, pH 7.4. EGFP-wt and EGFP-T2 showed the similar absorption peak at the wavelength of 490 nm. On the other hand, EGFP-T1 exhibited two strong absorption peaks at 398 and 490 nm. However, no strong absorption peaks were observed in EGFP-T3. Fluorescence maximum emission peaks of EGFP-wt (○), EGFP-T1 (□), EGFP-T2 (◊) occurred at 508 nm while excited 398 nm.
The EGFP-based trypsin sensor variants with both flanking helices exhibited a greater absorption ratio of $A_{397\text{nm}}/A_{490\text{nm}}$ than trypsin sensor variants having only a single flanking helix, or no flanking helix in the inserted loop (Figure 3.4). The absorption ratio of $A_{397\text{nm}}/A_{490\text{nm}}$ of different trypsin sensor variants was shown in Table 3.1. According to the absorbance and fluorescence properties, EGFP-T2 has optical properties similar to that of EGFP-wt where the dominant species has a protonated chromophore, whereas the insertion of a cleavage linker for trypsin following position 172 results in a population reduction of deprotonated chromophore species and a corresponding increase of the protonated species. This result suggests that position 172 in EGFP is a sensitive location at which the ionic states of the chromophore species can be altered by the addition of the grafting sequence.

Table 3.1. Ratio of deprotonated and protonated chromophore of trypsin sensors

<table>
<thead>
<tr>
<th>Trypsin sensors</th>
<th>E-helix</th>
<th>Loop</th>
<th>F-helix</th>
<th>Ratio ($A_{397\text{nm}}/A_{490\text{nm}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-T1</td>
<td>EEIERAFRVF</td>
<td>$\underline{DKDGNYIYAAE}$</td>
<td>LRHVMTNL</td>
<td>0.75</td>
</tr>
<tr>
<td>EGFP-T1Fa</td>
<td>EEIERAFRVF</td>
<td>$\underline{DKDGNYIYAAE}$</td>
<td>LRHVMTNL</td>
<td>0.64</td>
</tr>
<tr>
<td>EGFP-T1Ea</td>
<td>EEIERAFRVF</td>
<td>$\underline{DKDGNYIYAAE}$</td>
<td></td>
<td>0.40</td>
</tr>
<tr>
<td>EGFP-T1L</td>
<td>EEIERAFRVF</td>
<td>$\underline{DKDGNYIYAAE}$</td>
<td></td>
<td>0.31</td>
</tr>
<tr>
<td>EGFP-wt</td>
<td></td>
<td>$\underline{DKDGNYIYAAE}$</td>
<td></td>
<td>0.15</td>
</tr>
</tbody>
</table>
The dual chromophore forms in GFP has been demonstrated in previous reports (Tsien 1998). A complicated hydrogen bond network allowing for proton transfer between the chromophore and its neighboring side chains provides a great contribution to the equilibrium between protonated and deprotonated chromophore forms (Brejc, Sixma et al. 1997). The predominant protonated form of the chromophore exhibits its maximum absorbance at 397 nm, which is governed by the carboxylation of Glu222 through electrostatic repulsion and hydrogen bonding between a bound water molecule and Ser205. In contrast, the maximum absorbance peak of the deprotonated form of the chromophore is observed at 490 nm. Under this condition, the charge of Glu222 was proposed to donate to the chromophore through proton abstraction and a hydrogen bond network, involving in Ser205 and bound water. In order to stabilize the deprotonated state, a rearrangement of the side chains of Thr203 and His148 may be involved in, as shown in Figure 3.5. Besides the proton relay between Ser205 and Glu222, the side chains of Asp117, Thr118, Glu172 and Asp190 were also proposed to be involved in the alteration of different conformations of the chromophore under the observation of electron density of crystal structure (Brejc, Sixma et al. 1997). Our observation of the switch of dual chromophore forms following the insertion of cleavage linker at position 172 is likely due to a result from the alteration of ionization state of the chromophore. Following the insertion of the cleavage linker at position 172 of EGFP, the population of both states of the chromophore may be significantly altered through the proton relay between Ser205 and Glu222 and arrangement of hydrogen bond networks due to the environmental change of Glu172 (Brejc, Sixma et al. 1997).
Figure 3.4. Effects from inserted linkers on spectral change of EGFP-based trypsin sensors. Compared to EGFP-wt, the ratio ($A_{397\text{nm}}/A_{490\text{nm}}$) of absorbance of deprotonated and protonated chromophore forms was exhibited as an increased order, EGFP-T1 (■) containing loop flanked both E-helix and F-helix, EGFP-T1-Fa (▲) containing loop flanked alone F-helix, EGFP-T1-Ea (♦) containing loop flanked alone E-helix, EGFP-T1L (▼) containing a loop without helices, and EGFP-wt (●).
Figure 3.5. Switch between protonated and deprotonated forms of chromophore in EGFP. Proton relay between Ser205 and Glu222 of EGFP results in two chromophore forms, deprotonated and protonated forms, which provides dual excitation states or maximum absorption peaks of EGFP. The side chains of Asp117, Thr118, Glu172 and Asp190 also involved in the alteration of different conformations of the chromophore through the observation of electron density of crystal structure. Modified after (Brejc, Sixma et al. 1997).
3.4 Optical change of trypsin sensors following the action of trypsin

To evaluate optical signal changes related to the action of trypsin cleavage, the UV-visible absorption spectra of EGFP-T1 were measured before and after trypsin cleavage in trypsin digestion buffer (10 mM Tris, 10 mM CaCl$_2$, pH 7.4). Following trypsin cleavage, the maximum absorption of EGFP-T1 at 490 nm increased by 82% and concurrently decreased by 53% at 398 nm (Figure 3.6A). Similarly, EGFP-T1 exhibited a ratiometric fluorescence change with a 51% decrease in maximum fluorescence emission intensity at 508 nm at an excitation wavelength of 398 nm (Figure 3.6C) and a corresponding 36% increase at an excitation wavelength of 490 nm (Figure 3.6D). In contrast, the maximum absorption of EGFP-T2 showed no obvious change although it was cleaved by trypsin under identical digestion conditions (Figure 3.6B). As expected, EGFP-wt, which was not cleaved, exhibited no optical signal change. This result suggests that position 172 in EGFP is a sensitive location at which the ionic states of the chromophore species can be altered by the addition of the grafting sequence. The observed optical changes in the maximum absorption and fluorescence emission intensities may be due to the recovery of proton relay between Ser205 and Glu222 with the dissection of original interaction of Glu172, thereby recovering relative proportions of the two chromophoric species of EGFP-T1 following cleavage by trypsin, which revealed that the insertion at position Glu172 should be an optically sensitive location with respect to chromophore properties.
Figure 3.6. Optical signal change of trypsin sensors following trypsin digestion.

Absorption spectra of EGFP-T1 (A) and EGFP-T2 (B) were measured after trypsin digestion at various times from 0 min (solid line) to 120 min (dashed line) in trypsin digestion buffer (10 mM Tris, 20 mM CaCl₂, at pH 7.4). Absorbance of EGFP-T1 exhibited a decrease at 397 nm and an increase at 491 nm following trypsin digestion at different digestion time intervals. In contrast, Absorbance of EGFP-T2 exhibited no change following trypsin digestion at different digestion time intervals. Fluorescence spectra of EGFP-T1 with excitation at 398 nm (C) and excitation at 490 nm (D) were measured following trypsin digestion from 0 min (solid line) to 120 min (dashed line) in trypsin digestion buffer. Fluorescence of EGFP-T1 decreases when excited at 398 nm and increases when excited at 490 nm following trypsin digestion at different digestion time intervals.
3.5 Verification of trypsin sensor cleavage

To verify the cleavage status of EGFP-based trypsin sensors, digestion reactions with 15 μM EGFP-T1 and EGFP-T2 were performed by adding stock trypsin from bovine pancreas (Sigma, St. Louis) up to a final concentration of 20 nM in trypsin reaction buffer (10 mM Tris, 10 mM CaCl₂, pH 7.4). The UV-visible spectra of trypsin sensors following trypsin digestion were measured in a 1 cm pathway cuvette at different time intervals (0, 1, 3, 5, 10, 20, 30, 60, 90 and 120 min) and fluorescence spectra of trypsin sensors with excitation at 398 and 490 nm were recorded at identical time intervals. Similarly, EGFP-wt was subjected to identical digestion as the control. The resulting cleavage products were systematically examined using SDS-PAGE and MALDI-MS. EGFP-T1 and EGFP-T2 were found to specifically cleave into two major fragments by trypsin at the grafted cleavage sites, approximately 20 and 8 kDa from EGFP-T1 (Figure 3.7A), and 18 and 10 kDa from EGFP-T2 (Figure 3.7B) in SDS-PAGE. As a function of increasing digestion time, these two major fragments remain unchanged until digestion up to 24 hours. In contrast, EGFP-wt exhibited only a minor decrease in mass (Figure 3.7C) resulting from the removal of His-tag upon incubation with trypsin based on the SDS-PAGE assays. The cleaved mixtures were subjected to further analysis by MALDI-MS (Dr. Siming Wang provided the spectroscopy data). The results revealed that fragments produced from EGFP-T1 have molecular masses of 20840.79 and 7969.20 Da (Figure 3.7D), and fragments from EGFP-T2 have molecular masses of 18542.65 and 9759.08 Da (Figure 3.7E), which corresponds to the calculated mass values of 20844.68 Da and 18551.00 Da for the large fragments of EGFP-T1 and EGFP-T2, and is consistent with cleavage occurring in the cleavage linker. On the other hand, EGFP-T3, which exhibited no fluorescence, was not suitable as a sensor due to its apparent
Figure 3.7. Cleavage verification of trypsin sensors using SDS-PAGE and MALDI-MS. SDS-PAGE gels show cleavage products of EGFP-T1 (A), EGFP-T2 (B), and EGFP-wt (C) at different digestion time intervals. M is protein marker. Lanes 1, 2, 3, 4, 5, 6, 7, 8, and 9 are samples following trypsin digestion for 0 min, 1 min, 5 min, 10 min, 30 min, 60 min, 2 h, 6 h, and 24 h at room temperature, respectively. The product fragments of EGFP-T1 (D) and EGFP-T2 (E) cleaved by trypsin were identified using MALDI-MS. (F) To block the trypsin cleavage site, all Arg and Lys in the cleavage linker of EGFP-T1 were mutated into Ala to obtain EGFP-T1m. This mutation prevented the cleavage in the inserted linker of EGFP-T1m. M is the protein marker. Lane 1, 2 and 3 are samples of EGFP-T1m without trypsin digestion, EGFP-T1m with trypsin digestion and EGFP-T1 with trypsin digestion, respectively.
unfolded structure revealed by far UV circular dichroism, and its possible digestion into multiple fragments was suggested by the lack of distinct bands in SDS-PAGE. Furthermore, we created a negative control EGFP variant (EGFP-T1m), lacking trypsin cleavage sites, through mutation of Arg and Lys within the designed cleavage linker into Ala. Upon addition of trypsin, EGFP-T1m was not observed to be cleaved into small fragments at the inserted linker region in SDS-PAGE (Figure 3.7F) although a similar minor decrease in mass was observed, as with EGFP-wt, due to removal of the His-tag. The absorption and fluorescence signals of this EGFP variant are also not altered upon addition of trypsin. These results clearly demonstrated that cleavage occurred specifically at the designed cleavage linker in EGFP-T1 and EGFP-T2.

3.6 Kinetic study of trypsin sensors

Kinetic studies of EGFP-T1 were compared with results from three commercially available trypsin substrates, Bz-DL-Arg-pNA (BAPNA), Bz-DL-Arg-AMC and Boc-Gln-Ala-Arg-AMC under identical trypsin digestion buffer conditions. All of these commercially-available substrates exhibit optical signal changes at a single wavelength (i.e. non-ratiometric) upon trypsin cleavage. Kinetic parameters, turnover number ($k_{\text{cat}}$), the Michaelis constant ($K_m$), and the specificity constant ($k_{\text{cat}}/K_m$) for our trypsin sensors (Figure 3.8) and those of commercial trypsin substrates were calculated through fitting with the Michaelis-Menten equation, as listed in Chapter 2.16. Kinetic parameters are shown in Table 3.2. The EGFP-T1 sensor exhibited a 750-fold decrease in $K_m$ and a 150-fold increase in the substrate specificity constant ($k_{\text{cat}}/K_m$) compared to the BAPNA colorimetric kit. Similarly, an approximately 20-fold decrease in $K_m$ and a 20-fold increase in $k_{\text{cat}}/K_m$ were observed in comparison with Bz-DL-Arg-AMC. Boc-Gln-Ala-Arg-AMC, with preferable residues at positions P2 and P3, exhibited 2.3-fold larger $K_m$ and
Figure 3.8. Kinetic studies of EGFP-T1 conducted in trypsin digestion buffer. Kinetic studies of EGFP-T1 were conducted to monitor the absorbance signal change of EGFP-T1 at 490 nm following trypsin digestion in trypsin reaction buffer with 10 mM Tris, 10 mM CaCl$_2$, pH 7.4 at room temperature. The catalytic kinetic parameters, $k_{cat}$, $K_m$, and $k_{cat}/K_m$, are $0.26 \pm 0.01$/s, $4.0 \pm 0.4 \mu$M, and $65000 \pm 5000 M^{-1}s^{-1}$ through Michaelis-Menten equation fitting, respectively. The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.
5.4-fold smaller $k_{cat}$ than EGFP-T1. Since trypsin demonstrates a strong preference to cleave the peptide bond immediately following an Arg or Lys residue in a solvent-accessible region, a trypsin cleavage sequence derived from EF-hand motif loop III of calmodulin was selected as the basic graft structure, which contains highly-accessible Arg and Lys residues in the loop and flanking helices. To eliminate the contribution of calcium binding, Glu at the EF-loop position 12 was replaced by Ala to obtain EGFP-T1nb variant. Consequently, $k_{cat}/K_m$ of EGFP-T1nb for trypsin improved, and was higher than that of Boc-Gln-Ala-Arg-AMC due to the addition of preferable residues for trypsin at P$_2$ and P$_3$ positions before the trypsin cleavage site. On the one hand, based on previous report (Ma, Tang et al. 2005), two loops outside the binding pocket of trypsin plays a critical role in substrate specificity through the cooperative motions between loops and substrate-binding sites for improving the activity and substrate specificity of trypsin. On the other hand, the helix-loop-helix structure of the cleavage linker in EGFP-based trypsin sensors also provides the high flexibility to match the cooperative motion and improve the binding affinity between trypsin and EGFP-based trypsin sensors. Therefore, compared to the commercially available trypsin substrates, a smaller $K_m$ value is contributed by high cooperation and optimal binding between helix-loop-helix linker and trypsin binding pocket, which exhibits a larger $k_{cat}/K_m$ value to reveal a higher substrate specificity of trypsin. Overall, the substrate specificity constants, $k_{cat}/K_m$ of EGFP-based trypsin sensor variants are comparable to that of Boc-Gln-Ala-Arg-AMC.
Table 3.2. Comparison of kinetic parameters between trypsin sensors and commercial kits

<table>
<thead>
<tr>
<th>Trypsin sensors or kits</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-T1</td>
<td>0.26 ± 0.01</td>
<td>3.99 ± 0.38</td>
<td>$(6.52 ± 0.50) \times 10^3$</td>
</tr>
<tr>
<td>EGFP-T1nb</td>
<td>0.85 ± 0.06</td>
<td>4.21 ± 0.71</td>
<td>$(2.01 ± 0.21) \times 10^5$</td>
</tr>
<tr>
<td>Bz-DL-Arg-pNA</td>
<td>1.33 ± 0.17</td>
<td>$(3.00 ± 0.50) \times 10^3$</td>
<td>$(4.44 ± 0.41) \times 10^2$</td>
</tr>
<tr>
<td>Bz-DL-Arg-AMC</td>
<td>0.25 ± 0.01</td>
<td>77.44 ± 8.46</td>
<td>$(3.22 ± 0.21) \times 10^3$</td>
</tr>
<tr>
<td>Boc-Gln-Ala-Arg-AMC</td>
<td>1.41 ± 0.05</td>
<td>9.27 ± 0.95</td>
<td>$(1.52 ± 0.12) \times 10^5$</td>
</tr>
</tbody>
</table>

Conditions: 10 mM Tris, 10 mM CaCl$_2$, pH 7.4, 25°C. The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.

3.7 Inhibition of trypsin sensors

In order to ensure that the developed sensor is able to directly monitor the catalytic step, the inhibition effect of leupeptin on trypsin was examined by monitoring absorbance signal change at 490 nm during the digestion of EGFP-T1 with various leupeptin concentrations (0, 12.5, 25, 50 and 100 nM) in trypsin digestion buffer (10 mM Tris, 10 mM CaCl$_2$, pH 7.4). The rate of the absorbance signal change due to cleavage was found to decrease with increasing leupeptin concentrations (Figure 3.9A). The rates of EGFP-T1 with various leupeptin concentrations were fitted to reveal a competitive inhibition with $K_i$ value of 31 ± 2 nM (Figure 3.9B). This clearly demonstrates that the optical signal change of EGFP-T1 sensor is directly related to the enzymatic cleavage action rather than the binding process, and can be applied to monitor inhibition processes for drug screening.
Figure 3.9. Inhibition effect on the cleavage of EGFP-T1.
Effects of different concentrations of trypsin inhibitor, leupeptin, on the cleavage of EGFP-T1 trypsin digestion were examined in a time course mode (A). EGFP-T1 samples containing leupeptin with 0 nM (○), 12.5 nM (□), 25 nM (Δ), 50 nM (∇), 100 nM (⊠) were digested using 5 nM trypsin in 10 mM Tris, 20 mM CaCl₂, pH 7.4. Absorbance was monitored at 490 nm using a time course model. EGFP-T1 with trypsin inhibitor, leupeptin with 0 nM (○), 12.5 nM (□), 25 nM (Δ), 50 nM (∇), 100 nM (⊠) is evaluated (B) to reveal the competitive inhibition to trypsin, with $K_i$ value of 31 ± 2 nM.
3.8 Selectivity of trypsin sensors

One of the major challenges in developing protease probes is to achieve strong specificity for different proteases (Tung, Mahmood et al. 2000). To examine the enzymatic specificity of EGFP-T1, thrombin, cathepsin B, tryptase, tissue plasminogen activator, kallikrein, elastase and chymotrypsin with different substrate specificities (Table 3.3) were incubated with this sensor at room temperature. Lysosomal cathepsin B was shown to be responsible for the trypsinogen activation (Greenbaum, Hirshkowitz et al. 1959; Hofbauer, Saluja et al. 1998; Criddle, McLaughlin et al. 2007). Neither cathepsin B nor tryptase, as well as thrombin or tissue plasminogen activator was able to cleave EGFP-T1 as monitored by SDS-PAGE (Figure 3.10A). By contrast, cathepsin B substrate, z-FR-AMC, was cleaved in identical buffer condition by cathepsin B monitored by fluorescence signal change (Figure 3.10B). Although EGFP-T1 can be slowly cleaved by kallikerin, elastase and chymotrypsin, which will be examined in more detail in the future. Overall, the designed protease sensor with a specific cleavage linker still exhibited strong enzymatic specificity. Such strong enzymatic selectivity is very exciting since cathepsin B and tryptase are known to cleave the residues after Arg, which are involved in one of the mechanisms of trypsinogen activation. Thus, our developed sensor is ideal for cellular application to understand mechanisms of trypsinogen activation.
Table 3.3. Substrate specificity of selected proteases

<table>
<thead>
<tr>
<th>Proteases</th>
<th>Amino acid sequence of cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pn---P4-P3-P2-P1↓-P1’-P2’-P3’-P4’---Pm’</td>
</tr>
<tr>
<td>Trypsin</td>
<td>-X-X-R↓-X-X- or -X-X-K↓-X-X-</td>
</tr>
<tr>
<td>Thrombin</td>
<td>-X-X-R↓-G-X</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>-X-X-R↓-E-X- or -X-X-V↓-E-X- or -X-X-G↓-K-X-</td>
</tr>
<tr>
<td>Tryptase</td>
<td>-X-X-R↓-X-X- or -X-X-K↓-X-X-</td>
</tr>
<tr>
<td>Tissue plasminogen activator</td>
<td>-X-X-R↓-V-X</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>-X-X-R↓-X-X- or -X-X-M↓-X-X- or -X-X-L↓-X-X-</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>-X-Y↓-X-X- or -X-F↓-X-X- or -X-W↓-X-X-</td>
</tr>
</tbody>
</table>

3.9 Trypsinogen activation in living cells

Caerulein is the most commonly used inducer for trypsinogen activation (Kruger, Albrecht et al. 2000; Thrower, Diaz de Villalvilla et al. 2006). We monitored trypsinogen activation in pancreatic cells using commercially available trypsin MCA peptide substrate Boc-Gln-Ala-Arg-AMC. Trypsin activities in cell lysates of MIA PaCa-2 cells, after induction with 10 nM caerulein at different time points, were examined by monitoring the change in relative fluorescence signal of trypsin MCA substrate at 450 nm under the excitation of 380 nm. The results indicated that the highest level of trypsinogen activation was achieved following caerulein induction for 60 to 120 min based on relative fluorescence unit change of trypsin substrate. Using 4 nM trypsin as a positive reference, trypsinogen was activated to reach a trypsin level of 6 nM following caerulein induction for 1 h (Figure 3.11).
Figure 3. 10. The selectivity of EGFP-T1 for proteases.
SDS-PAGE (A) exhibited no cleavage bands of EGFP-T1 following 20 nM thrombin, cathepsin B, tryptase or tissue plasminogen activator overnight digestion, but EGFP-T1 can be slowly cleaved by kallikerin, elastase and chymotrypsin. M is protein marker. Lane 1 is EGFP-T1 without digestion. Lanes 2, 3, 4, 5, 6, 7, 8 and 9 are EGFP-T1 following overnight digestion with trypsin, thrombin, cathepsin B, tryptase, tissue plasminogen activator, kallikerin, elastase and chymotrypsin, respectively. To confirm optimal reaction buffer for EGFP-T1 cathepsin B digestion, Z-FR-MAC for cathepsin B (B) was cleaved under identical digestion buffer conditions to reveal fluorescence emission signal increase at 460 nm following digestion time increase at 0 (○), 1 (□), 5 (◊), 10 (△), 20 (●), 30 (■), 60 (▲) and 180 min (▼) through detection by fluorescence spectrophotometer at the excitation of 380 nm.
Figure 3.11. Trypsin activity determination in cell lysates using trypsin peptide substrate. The cultured MIA PaCa-2 cells following 10 nM caerulein at various time intervals (0, 10, 20, 30, 60, 120, 240 min and 20 h) were subjected to cell lysis to extract trypsin. Trypsin activity was detected through incubation with trypsin substrate kit, Boc-Gln-Ala-Arg-AMC, at various time points following induction, which revealed that the highest level of trypsinogen activation was reached following caerulein induction for 1 or 2 h based on relative fluorescence emission unit change at 460 nm of trypsin substrate under the excitation of 380 nm. Using 4 nM trypsin as a positive reference, trypsinogen was activated to reach a trypsin level of 6 nM following caerulein induction for 1 h. The change in relative fluorescence units is also observed to be a linear correlation with active trypsin concentrations.
3. 10 Fluorescence imaging of trypsin sensor

In order to track trypsin activity in living cells, our trypsin sensors were transfected into a pancreatic cancer cell line, MIA PaCa-2 cell line using an established method with lipofectamine (Zou, Ye et al. 2005). After transfection, the cells transfected with EGFP-T1 and the controls (EGFP-wt or EGFP-T1m) exhibited strong fluorescence in fluorescence microscopy, following 16 to 48 hours of expression. Upon zymogen activation by 10 nM caerulein, the fluorescence emission signal at 510 nm for EGFP-T1 gradually decreased at 398 nm excitation while concurrently increasing at 488 nm excitation (Figure 3.12A, 3.12B and 3.12C), resulting in a ratiometric fluorescence emission change greater than 25% (Figure 3.12D). In contrast, the fluorescence emission ratio of EGFP-wt or EGFP-T1m remained unchanged. The ratiometric fluorescence change of EGFP-T1 sensor is significantly different from that of EGFP-wt (*\(P < 0.05\)). Trypsinogen activation using 10 nM caerulein induction in living cells (n = 12) exhibited a half-time for maximum caspase-3 activation of 15-20 min, which was in excellent agreement with the results obtained using the peptide substrate, Boc-Gln-Ala-Arg-AMC, to monitor trypsin activity in cell lysates. On the other hand, prior to the imaging, the MIA PaCa-2 cells transfected with EGFP-T1 gene was incubated with cell-permeable trypsin inhibitors such as soybean trypsin inhibitor or leupeptin for 30 mins. During the cell imaging acquisition, 10 nM caerulein was applied to the cell plate for the induction of trypsinogen activation. Fluorescence signal of MIA PaCa-2 cells transfected EGFP-T1 following incubation with trypsin inhibitors and caerulein induction didn’t exhibit any change when excited at both wavelengths (398 and 488 nm). Therefore, no change in fluorescence ratiometric signal was also observed either. These results demonstrated that trypsinogen activation or trypsin activity in the living cells could be
Figure 3.12. Fluorescence imaging and signal change of EGFP-T1 in living cells.
The representative images of MIA PaCa-2 cells transfected with EGFP-T1, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after incubation with 10 nM caerulein. Normalized fluorescence (NF) intensity change (C) at 510 nm increased with excitation at 488 nm and decreased with excitation at 398 nm upon activation by caerulein induction at different times. The arrow indicates the time point of caerulein induction. Relative fluorescence change ratio (D) at 510 nm with excitation of 488/398 nm of MIA PaCa-2 cells transfected with EGFP-T1 following induction with 10 nM caerulein (●) or 5 nM caerulein (★) exhibited significant increase, whereas the controls, EGFP-wt (○), EGFP-T1m (Δ) and EGFP-T1 incubated with 100 µM leupeptin (◊) exhibited no change following 10 nM caerulein stimulation. After induction with 10 nM caerulein for 45 min, the ratiometric change in fluorescence signal of EGFP-T1 was significantly different from that of the control, EGFP-wt (*P < 0.05).
successfully inhibited. By comparing trypsin activity at different time points following caerulein induction, trypsinogen activation in pancreatic cancer cells reached to the highest level (approximately 6 nM) at 1 hr following stimulation with 10 nM caerulein. The trypsinogen activation is strongly dependent on caerulein concentration (Figure 3.12D). Therefore, our trypsin sensor also has the capability of detecting enzymatic activation/inhibition in living cells in real time, which will facilitate further research aimed at understanding the activation or inhibition mechanisms and pathways of protease activation receptors (PAR) (Riewald, Petrovan et al. 2002).

Moreover, our developed trypsin sensor have been specifically targeted to the endoplasmic reticulum (ER) and mitochondria by adding the signal peptide sequence, ER retention sequence, KDEL, and ER targeting sequence of calreticulin, MLLSVPLLGLGGLAAAD (Zou J, Hofer AM et al. 2007), and by fusing to mitochondria signal peptide, PLLRGRCPARRH, from cytochrome C oxidase subunit VIII (COX 8) (Huttemann, Schmidt et al. 2003), respectively. The mitochondrial-targeted sensor (EGFP-T1-Mito) and ER-targeted sensor (EGFP-T1-ER) nicely overlap with commercial marker Mitotracker Red (Invitrogen, Molecular Probe) and DsRed-ER-marker (BD Bioscience, Clontech), respectively (Figure 3.13A and 3.13B). These exciting results indicated that our trypsin sensor provides the potential to map dynamic activation/inhibition processes in specific subcellular environments. Since our trypsin sensor is based on a single EGFP unit, it should not encounter problems with translocation in cellular compartments, as is observed with larger FRET-based sensors. Additionally, our trypsin sensor does not exhibit a tendency towards photobleaching or poor orientation, which is also common with FRET-based sensors.
Figure 3.13. Subcellular location targeting of EGFP-T1-Mito and EGFP-T1-ER. The subcellular location targeting of EGFP-T1-Mito and EGFP-T1-ER was confirmed using mitotracker Red and DsRed-ER. Our trypsin sensors, EGFP-T1-Mito (A) and EGFP-T1-ER (B) exhibited a well overlap with commercial mitotracker Red and DsRed-ER, respectively. The fluorescence emission of EGFP-T1-Mito and EGFP-T1-ER was obtained by using a bandpass emission filter (505-530 nm) under an excitation at 488 nm of an argon laser. The fluorescence emission of mitotracker and DsRed-ER was obtained by using a long-pass emission filter above 560 nm under an excitation at 543 nm of a He-Ne laser.
3.11 Trypsinogen activation in pancreatic cancer cells

Caerulein is the most commonly-used inducer of trypsin activation in living pancreatic acinar cells (Kruger, Albrecht et al. 2000; Thrower, Diaz de Villalvilla et al. 2006). It is widely accepted that zymogen is packed into granules of acinar cells. However, the location of trypsinogen activation has not been conclusively identified despite substantial studies with various methods. Several studies have suggested that zymogen activation might be initiated in zymogen granules (Mithofer, Fernandez-del Castillo et al. 1998; Raraty, Ward et al. 2000). Using antibodies against trypsinogen activation peptide in confocal and immunoelectronic microscopy, initial zymogen activation was observed in a vesicular non-zymogen granule compartment (Hofbauer, Saluja et al. 1998; Otani, Chepilko et al. 1998). The activation of trypsinogen in the large endocytic vacuole was also proposed to be the initiation of acute pancreatitis (Sherwood, Prior et al. 2007). Currently, the pancreatic cancer cell line, MIA PaCa-2, derived from the human pancreatic tumor (Ulrich, Schmied et al. 2002), has been extensively used in developing cancer therapy based on protease-induced apoptosis (Basu, Castle et al. 2006; Rosetti, Tesei et al. 2006). Differing from acinar cells, pancreatic cancer cells such as MIA PaCa-2 do not have a granule structure (Madden, Heaton et al. 1989). However, a high mRNA level of trypsinogen and secretory trypsin inhibitor were reported in this cancer cell line (Ogawa, Matsuura et al. 1987; Swarovsky, Steinhilber et al. 1988). To date, it is still unknown whether pancreatic cancer cells such as MIA PaCa-2 have any elevation of trypsin activity. It is also not clear due to limitations of current commercially available trypsin probes, whether trypsinogen activation of pancreatic cells occurs in the cytosol. The developed trypsin sensors demonstrate strong enzymatic specificity, especially to cathepsin B and tryptase, which are essential for
examining the mechanisms of zymogen activation. Furthermore, the direct observation of trypsinogen activation was first exhibited in MIA PaCa-2 cancer cells by caerulein stimulation even though this cell line lacks zymogen granules or cytosolic vacuole structures (Madden, Heaton et al. 1989). These results correlate well with previous reports related to the high mRNA levels of trypsinogen (Ogawa, Matsuura et al. 1987) and trypsin inhibitors (Swarovsky, Steinhilber et al. 1988). Moreover, the location of trypsinogen activation occurs in the cytosol of this cancer cell, which further adds to new insight regarding zymogen activation and inhibition. Zymogen granules, vacuoles or the combination of cytosol and cellular organelles of pancreatic acinar cells were previously reported to function trypsinogen generation and activation (Hofbauer, Saluja et al. 1998; Mithofer, Fernandez-del Castillo et al. 1998; Otani, Chepilko et al. 1998; Raraty, Ward et al. 2000). Currently, this developed sensor to further investigate the dynamic process and mechanisms for trypsinogen activation was successfully applied at different cellular locations and further development and application of EGFP-based sensors with strong enzymatic specificity will facilitate the understanding of protease actions in living systems. Finally, since MIA PaCa-2 cancer cells have been widely used for understanding cell death, cancer metastasis and drug effects, knowledge gained from zymogen activation and inhibition is expected to impact future development of cancer drugs, especially against pancreatic cancers.

3.12 Localization of trypsinogen activation

The direct observation of trypsinogen activation in MIA PaCa-2 cancer cells by caerulein stimulation was first reported even though this cell line lacks zymogen granules or cytosolic vacuole structures and the activated trypsin can perform the cleavage of EGFP-T1 to result in the ratiometric optical signal change in living cells, which is used for the real-time monitoring of
trypsinogen activation or inhibition. In order to identify whether trypsinogen activation and expression of EGFP-based trypsin sensors in the same subcellular location to accomplish the cleavage of EGFP-based trypsin sensors, immunofluorescence was used to confirm the localization of trypsinogen activation following induction with 10 nM caerulein in MIA PaCa-2 cells transfected with EGFP-T1 according to experimental procedures described in the section of Materials and Methods. On the other hand, the MIA PaCa-2 cells transfected with EGFP-T1 without stimulation using 10 nM caerulein were also conducted immunofluorescence determination as the control. Strong red fluorescence in the cytoplasm of the cells was observed in the confocal fluorescence microscope with an excitation of 560 nm due to the interaction between primary antitrypsin and the secondary antibody (Figure 3.14A), which clearly indicated that a large amount of trypsin was activated in the cytoplasm of the cells following the stimulation with 10 nM caerulein. Similarly, the strong green fluorescence was also observed in the confocal fluorescence microscope with an excitation of 488 nm (Figure 3.14B), which indicated large amount of EGFP-T1 sensor also expressed in the cytoplasm of cells. The well overlap (Figure 3.14C) between the red and the green fluorescence in the cytoplasm of cells demonstrated the same subcellular location between trypsinogen activation and EGFP-T1 expression. All of these results provide us a further confirmation that the real-time optical signal change of EGFP-T1 in living cells following caerulein induction was really resulted in the cleavage of EGFP-T1 sensor by activated trypsin.
Figure 3. Localization of trypsin activation and expression of EGFP-based trypsin sensor. Fluorescence signal of trypsin (A) against anti-trypsin antibody and EGFP-T1 (B) against anti-GFP antibody exhibited a well overlap (C) in the cytoplasm of MIA PaCa-2 cells following 10 nM caerulein stimulation using immunofluorescence, which suggests EGFP-T1 cleavage due to trypsin activation in the same subcellular compartment. The fluorescence emissions of GFP and Texas Red are determined under the excitation of 488 nm and 543 nm, respectively.
3.13 Plasmid DNA construction of trypsin sensors with optimal cleavage linkers

Through the extensive investigation, the position 172 of EGFP has been identified as a sensitive location for chromophore conformation change upon the action of trypsin and a trypsin sensor (EGFP-T1) has been successfully developed for imaging trypsin activation or inhibition in living cells. However, the insertion of optimal cleavage linker at this sensitive location to improve the response rate to trypsin, enlarge the relative signal response and offer a better specificity to trypsin digestion is still an important goal. In order to develop sensitive trypsin sensors with optimal cleavage linkers for imaging trypsinogen activation in living cells, the P region (P1, P2, P3 and P4 positions) and P’ region (P1’, P2’ and P3’ positions) around the cleavage site in trypsin sensors were systematically studied. Based on the bovine trypsin inhibitor or some natural trypsin substrates, the recognition sites of trypsin substrates was designed to be Gly-Pro-Ala-Arg-Leu-Ala-Ile in the specific cleavage linkers for trypsin. In order to evaluate effects from different positions in P region or P’ region, various cleavage linkers for trypsin with Leu-Arg, Gly-Pro-Arg-Leu, Gly-Pro-Ala-Arg-Leu or Gly-Pro-Ala-Arg-Leu-Ala-Ile were designed and grafted at the identified position 172 in EGFP to obtain different trypsin sensor variants, which are shown in Table 3.4.

3.14 Optical properties of trypsin sensors with optimal cleavage linkers

All variants of our designed EGFP-based trypsin sensors have been expressed in E. coli and exhibit strong fluorescence. Through nickel-chelating column purification, all trypsin sensor variants exhibit two strong absorption peaks at 397 and 490 nm, as shown in Figure 3.15. The two strong absorption peaks result from a population distribution between the ionic and neutral
Table 3.4. Designed trypsin sensors with different cleavage linkers

<table>
<thead>
<tr>
<th>Trypsin sensor name</th>
<th>Cleavage linker sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E-helix</td>
</tr>
<tr>
<td>EGFP-T1</td>
<td>EEEIR EAFRVF</td>
</tr>
<tr>
<td>EGFP-T1EFa</td>
<td>EEEIR EAFRVF</td>
</tr>
<tr>
<td>EGFP-T1EFb</td>
<td>EEEIR EAFRVF</td>
</tr>
<tr>
<td>EGFP-T1EFc</td>
<td>EEEIR EAFRVF</td>
</tr>
<tr>
<td>EGFP-T1Ma</td>
<td>EEEIAEFAVF</td>
</tr>
<tr>
<td>EGFP-T1Mb</td>
<td>EEEIAEFAVF</td>
</tr>
<tr>
<td>EGFP-T1Mc</td>
<td>EEEIAEFAVF</td>
</tr>
<tr>
<td>EGFP-T1Fa</td>
<td>DKDNGYISAE</td>
</tr>
<tr>
<td>EGFP-T1Fb</td>
<td>DKDNGYISAA</td>
</tr>
<tr>
<td>EGFP-T1Fc</td>
<td>DKDNGYISAA</td>
</tr>
<tr>
<td>EGFP-T1Fd</td>
<td>DKDNGYISAA</td>
</tr>
<tr>
<td>EGFP-T1Ea</td>
<td>EEEIR EAFRVF</td>
</tr>
<tr>
<td>EGFP-T1Eb</td>
<td>EEEIAEAGPARLF</td>
</tr>
<tr>
<td>EGFP-T1L</td>
<td>DKDNGYISAAE</td>
</tr>
</tbody>
</table>
Figure 3.15. UV-visible spectra of EGFP-based trypsin sensor variants. Through insertion of various cleavage linkers into EGFP, the neutral chromophore form of these trypsin sensor variants with a concentration of 10 μM was partially switched to the ionic chromophore form, which exhibited a decrease of absorption at 490 nm and a concurrent increase of absorption at 397 nm in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4). The population ratio index of both chromophore forms is reduced according to the order: EGFP-wt (○), EGFP-T1Eb (◇), EGFP-T1Fc (▲), EGFP-T1EFb (▷), EGFP-T1Mc (●) and EGFP-T1 (□).
forms of the chromophore. Due to the insertion of cleavage linkers in these trypsin sensor variants, the neutral form of the chromophore is partially converted to the ionic form, resulting in various populations between two chromophore forms. A ratio index between neutral and ionic forms of the chromophore can be expressed by the maximum absorption at 490 nm dividing the maximum absorption at 397 nm to indicate various populations of two states. The linkers containing intact E-III-F hand motif produced the smallest ratio index of chromophore population, where the levels of the chromophore populations were nearly equivalent, such as EGFP-T1. The other trypsin sensor variants, however, with modified or partial E-III-F motifs, exhibited a decrease in neutral chromophore population and an increase in ionic chromophore population due to the length and properties of cleavage linkers.

3.15 Kinetic parameters for trypsin sensors with optimal cleavage linkers

Steady-state kinetic parameters $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$ for trypsin hydrolysis of EGFP-based trypsin sensors can be used for evaluating the optimization of cleavage linkers. When determining catalytic kinetic parameters, $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$, initial rates were measured at various EGFP-based trypsin sensor concentrations in trypsin reaction buffer (10 mM Tris, 20 mM CaCl$_2$, pH 7.4). The initial rate data were fitted through the Michalis-Menten equation to obtain the catalytic kinetic parameters of trypsin sensor variants. Since our first trypsin sensor (EGFP-T1) was constructed on EGFP with a grafted EF-hand motif from calmodulin as the cleavage linker, we also grafted partial or modified EF-hand motifs onto EGFP to obtain different EGFP-based trypsin sensors to test the accessibility for trypsin in the loop or flanking helices regions. The kinetic catalytic parameters of these trypsin sensors are shown in Table 3.5.
EGFP-T1L was not cleaved by trypsin, which is possibly due to less flexibility of the inserted cleavable linker. However, EGFP-T1Fa exhibited the fastest cleavage.

Table 3.5. Kinetic parameters for first generation of trypsin sensors

<table>
<thead>
<tr>
<th>Trypsin sensors</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ ($\times 10^4$ M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-T1</td>
<td>0.26 ± 0.01</td>
<td>3.98 ± 0.74</td>
<td>6.52 ± 0.45</td>
</tr>
<tr>
<td>EGFP-T1Fa</td>
<td>0.82 ± 0.12</td>
<td>11.80 ± 1.91</td>
<td>5.18 ± 0.98</td>
</tr>
<tr>
<td>EGFP-T1Ea</td>
<td>0.70 ± 0.05</td>
<td>5.90 ± 1.31</td>
<td>11.12 ± 2.09</td>
</tr>
<tr>
<td>EGFP-T1Ma</td>
<td>0.09 ± 0.01</td>
<td>2.22 ± 0.06</td>
<td>4.18 ± 0.08</td>
</tr>
<tr>
<td>EGFP-T1L</td>
<td>No cleavage</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conditions: 10 mM Tris, 10 mM CaCl$_2$, pH 7.4, 25°C. The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.

Due to the fast response to trypsin of EGFP-T1Fa, the various cleavage linkers for extending with preferable residues at P and P’ regions were grafted in the identified location of EGFP to obtain different trypsin sensor variants. The results indicated EGFP-T1Fc, with Gly-Pro-Ala-Arg-Leu as a cleavage linker, exhibited the fastest cleavage rate based on the $k_{cat}$ value, whereas, EGFP-T1Fa with Leu-Arg as the linker exhibited the slowest cleavage rate. Similarly, the specificity constant ($k_{cat}/K_m$) of the trypsin sensor with recognition site, Gly-Pro-Ala-Arg-Leu, had the largest value among these trypsin sensor variants. The results clearly demonstrated that P$_2$, P$_3$ and P$_4$ positions with Ala, Pro and Gly and P$_1’$ position with Leu provided strong binding affinity, a fast cleavage rate and better cleavage specificity for trypsin. Although P$_2’$ and P$_3’$ positions were designed to have Ala and Ile, $k_{cat}/K_m$ of these trypsin sensor variants was not
significantly different from those of trypsin sensor variants with cleavage linker, Gly-Pro-Ala-Arg-Leu, which are shown in Table 3.6.

Table 3.6. Optimization of residues in the P and P' region of cleavage linkers

<table>
<thead>
<tr>
<th>Trypsin sensors</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ ($\times 10^4 \text{ M}^{-1} \text{s}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-T1Fd</td>
<td>1.30 ± 0.08</td>
<td>6.16 ± 0.94</td>
<td>21.18 ± 2.07</td>
</tr>
<tr>
<td>EGFP-T1Fc</td>
<td>1.80 ± 0.08</td>
<td>8.16 ± 0.87</td>
<td>22.05 ± 1.42</td>
</tr>
<tr>
<td>EGFP-T1Fb</td>
<td>1.03 ± 0.04</td>
<td>9.17 ± 0.95</td>
<td>11.21 ± 0.76</td>
</tr>
<tr>
<td>EGFP-T1Fa</td>
<td>0.82 ± 0.12</td>
<td>11.80 ± 1.91</td>
<td>5.18 ± 0.98</td>
</tr>
</tbody>
</table>

Conditions: 10 mM Tris, 10 mM CaCl$_2$, pH 7.4, 25°C. The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.

Taking into consideration the preferable residues in P and P’ region for trypsin, EGFP-T1Fc, EGFP-T1Eb, EGFP-T1EFb and EGFP-T1Mc were also designed and constructed. Kinetic parameters, $k_{cat}$, $K_m$ and $k_{cat}/K_m$ of these designed trypsin sensor variants and commercial trypsin kits under identical buffer conditions were determined and calculated through fitting with the Michaelis-Menten equation. The kinetic catalytic parameters are shown in Table 3.7. All of these commercially-available trypsin kits exhibit optical signal changes at a single wavelength (i.e. non-ratiometric) upon trypsin cleavage. Among these trypsin sensor variants or commercial trypsin kits, the EGFP-T1EFb sensor exhibited the smallest value in binding affinity ($K_m$) and the highest substrate specificity constant ($k_{cat}/K_m$), which revealed almost a 750-fold decrease in $K_m$ and a 600-fold increase in $k_{cat}/K_m$ compared to the BAPNA colorimetric kit. Similarly, an approximately 20-fold decrease in $K_m$ and a 75-fold increase in $k_{cat}/K_m$ were observed in
comparison with Bz-DL-Arg-AMC. Boc-Gln-Ala-Arg-AMC with preferable residues at positions P2 and P3 exhibited 2.3-fold larger $K_m$ and 1.6-fold smaller $k_{cat}/K_m$ than EGFP-T1EFb. Consequently, the substrate specificity constant ($k_{cat}/K_m$) of EGFP-T1nb for trypsin improved, and was higher than that of Boc-Gln-Ala-Arg-AMC due to the addition of preferable residues for trypsin at P and P’ region around the trypsin cleavage site. Overall, the substrate specificity constants ($k_{cat}/K_m$) of EGFP-based trypsin sensor variants were comparable to that of Boc-Gln-Ala-Arg-AMC.

Table 3.7. Comparison of catalytic parameters between trypsin sensors and commercial kits

<table>
<thead>
<tr>
<th>Trypsin sensors</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ ($\mu$M)</th>
<th>$k_{cat}/K_m$ ($\times 10^4$ M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-T1Fc</td>
<td>1.80 ± 0.08</td>
<td>8.16 ± 0.87</td>
<td>22.05 ± 1.42</td>
</tr>
<tr>
<td>EGFP-T1Eb</td>
<td>0.87 ± 0.12</td>
<td>6.14 ± 0.98</td>
<td>14.17 ± 1.64</td>
</tr>
<tr>
<td>EGFP-T1EFb</td>
<td>0.98 ± 0.04</td>
<td>4.12 ± 0.45</td>
<td>23.80 ± 1.72</td>
</tr>
<tr>
<td>EGFP-T1Mc</td>
<td>0.85 ± 0.06</td>
<td>4.21 ± 0.71</td>
<td>20.13 ± 2.14</td>
</tr>
<tr>
<td>Bz-DL-Arg-pNA</td>
<td>1.33 ± 0.17</td>
<td>3000 ± 500</td>
<td>0.04 ± 0.004</td>
</tr>
<tr>
<td>Bz-DL-Arg-AMC</td>
<td>0.25 ± 0.01</td>
<td>77.44 ± 8.46</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>Boc-Gln-Ala-Arg-AMC</td>
<td>1.41 ± 0.05</td>
<td>9.27 ± 0.95</td>
<td>15.20 ± 1.20</td>
</tr>
</tbody>
</table>

Conditions: 10 mM Tris, 10 mM CaCl$_2$, pH 7.4, 25°C. The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.

3.16 pH effects on trypsin sensors

In order to examine buffer pH effect on trypsin cleavage, kinetic parameters of our designed trypsin sensors were analyzed following trypsin digestion in PIPES buffer (20 mM PIPES, 20 mM CaCl$_2$, pH 6.7) and Tris buffer (10 mM Tris, 20 mM CaCl$_2$, pH 7.4 and pH 8.0),
respectively. The kinetic parameters of EGFP-based trypsin sensor variants (EGFP-T1 and EGFP-T1Fa) in three pH buffer conditions are shown in Table 3.8. The results indicated that $K_m$ values under different pH conditions did not change, and $k_{cat}$ and $k_{cat}/K_m$ exhibited only a modest increase due to the pH increase. Based on the $k_{cat}$ value of both EGFP-based trypsin sensor variants, the cleavage of EGFP-based trypsin sensors for trypsin was determined to be pH dependent in the range 6.7-8.0.

Table 3.8. Catalytic parameters for trypsin under various pH conditions

<table>
<thead>
<tr>
<th>Trypsin sensors</th>
<th>pH</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ ($\times 10^3$ M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-T1</td>
<td>6.7</td>
<td>0.14 ± 0.01</td>
<td>3.55 ± 0.25</td>
<td>4.16 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>0.26 ± 0.01</td>
<td>3.98 ± 0.74</td>
<td>6.52 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.28 ± 0.01</td>
<td>3.76 ± 0.43</td>
<td>7.45 ± 0.38</td>
</tr>
<tr>
<td>EGFP-T1Fa</td>
<td>6.7</td>
<td>0.50 ± 0.02</td>
<td>6.22 ± 0.69</td>
<td>7.95 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>0.82 ± 0.12</td>
<td>11.80 ± 1.91</td>
<td>5.18 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.87 ± 0.12</td>
<td>6.03 ± 1.98</td>
<td>14.46 ± 1.51</td>
</tr>
</tbody>
</table>

Conditions: 10 mM Tris, 10 mM CaCl$_2$, pH 6.7, 7.4 and 8.0, 25°C respectively.

The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.

3.17 Extinction coefficient constants of products after trypsin sensor cleavage

The quantity of trypsin sensors cleaved or the product can be used to establish the evaluation of the turnover number of trypsin sensors in unit time during trypsin digestion. Due to the monitoring of the change in absorbance of trypsin sensors at 490 nm, the increase in absorbance at this wavelength can be used to calculate the amount of products. Therefore, extinction coefficient constants are necessary. After trypsin sensor samples were cleaved
completely through trypsin overnight digestion, the absorbance of products at 490 nm was measured and then extinction coefficient constants were calculated using Beer-Lambert law. The extinction coefficient constants of the designed trypsin sensor variants differed slightly due to the various cleavage linkers, which are shown in Table 3.9. The extinction coefficients of cleavage products from EGFP-T1Fa, EGFP-T1Fb, EGFP-T1Fc and EGFP-T1Fd after cleavage are similar, revealing that these trypsin sensor variants exhibit similar behavior during trypsin cleavage.

Table 3.9. Extinction coefficients of cleavage products from trypsin sensors

<table>
<thead>
<tr>
<th>Trypsin sensor</th>
<th>Extinction coefficients $(\varepsilon)$ $(\mu M^{-1}cm^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-T1</td>
<td>0.038 ± 0.002</td>
</tr>
<tr>
<td>EGFP-T1Ea</td>
<td>0.033 ± 0.004</td>
</tr>
<tr>
<td>EGFP-T1Fa</td>
<td>0.037 ± 0.003</td>
</tr>
<tr>
<td>EGFP-T1Ma</td>
<td>0.031 ± 0.005</td>
</tr>
<tr>
<td>EGFP-T1Fb</td>
<td>0.037 ± 0.005</td>
</tr>
<tr>
<td>EGFP-T1Fc</td>
<td>0.037 ± 0.003</td>
</tr>
<tr>
<td>EGFP-T1Fd</td>
<td>0.037 ± 0.004</td>
</tr>
<tr>
<td>EGFP-T1EFb</td>
<td>0.038 ± 0.002</td>
</tr>
<tr>
<td>EGFP-T1EFc</td>
<td>0.042 ± 0.002</td>
</tr>
<tr>
<td>EGFP-T1Mc</td>
<td>0.023 ± 0.002</td>
</tr>
</tbody>
</table>

Conditions: 10 mM Tris, 10 mM CaCl$_2$, pH 7.4, 25°C. The data presented in mean ± SD, are extinction coefficients and standard deviations from different samples at various concentrations (2-20 $\mu$M) following trypsin complete digestion, $n = 6$. 
3.18 Relative signal change calculation of trypsin sensors

In order to evaluate the response of trypsin sensors to trypsin, the relative optical signal change in absorbance or fluorescence of different trypsin sensor variants before trypsin cleavage and after trypsin cleavage was measured. Following trypsin digestion, EGFP-T1 has the biggest relative signal change, however, other EGFP-based trypsin sensor variants exhibit the smaller relative signal change. The main reason is possibly due to a similar population of neutral and ionic chromophore forms in EGFP-T1, which provides a larger space for the absorption decrease at 397 nm and increase at 490 nm resulting from the population distribution between the neutral and ionic chromophore forms at both wavelengths. In contrast, the less space for population distribution of the chromophore forms in other trypsin sensor variants resulted in the smaller relative signal change. The relative signal changes of the trypsin sensor variants are shown in Table 3.10. Based on the above analyses, the complete E-III-F motif insertion in EGFP can be attributed to the close level in population between the neutral and ionic chromophore forms of the chromophore and provide the high accessibility for enzymes to enhance the relative signal change.

3.19 Optical signal change EGFP-T1 upon the action of chymotrypsin

Since EGFP-T1 can be slowly cleaved by chymotrypsin during the specificity determination for various proteases, this strategy also has the potential to develop the chymotrypsin sensors. In order to evaluate optical signal changes related to the action of chymotrypsin cleavage, the UV-visible absorption spectra of 15 μM EGFP-T1 were measured before and after chymotrypsin cleavage in chymotrypsin digestion buffer (10 mM Tris, 10 mM CaCl₂, pH 7.4). Following chymotrypsin cleavage, the maximum absorption of EGFP-T1 at 490
nm increased by 102% and concurrently decreased by 55% at 398 nm (Figure 3.16A). In contrast, the maximum absorption of EGFP-T2 showed no obvious change although it was cleaved by chymotrypsin under identical digestion conditions (Figure 3.16B). This result further confirms that position 172 in EGFP is a sensitive location at which the ionic states of the chromophore species can be altered by the addition of the grafting sequence and can be used for the development of different biosensors for proteases. In addition, compared to trypsin digestion of EGFP-T1 with an 83% increase in absorbance change at 490 nm and the relative signal change of 3.76 following trypsin digestion, an enhanced absorbance increase at 490 nm of EGFP-T1 and following chymotrypsin digestion was observed so that resulting in the relative signal change of 4.51. The improved relative signal change is possible due to multiple cleavage sites (following residues Met, Leu, Tyr and Thr) on the helix-loop-helix structure, which is different with the cleavage sites with positively-charged residues Arg and Lys.

Table 3.10. Relative signal changes of trypsin sensor variants

<table>
<thead>
<tr>
<th>Trypsin sensor</th>
<th>Relative signal change ($R_D$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-T1</td>
<td>3.76 ± 0.22</td>
</tr>
<tr>
<td>EGFP-T1EFb</td>
<td>3.57 ± 0.36</td>
</tr>
<tr>
<td>EGFP-T1Eb</td>
<td>2.12 ± 0.17</td>
</tr>
<tr>
<td>EGFP-T1Fc</td>
<td>2.06 ± 0.08</td>
</tr>
<tr>
<td>EGFP-T1Mc</td>
<td>1.80 ± 0.29</td>
</tr>
</tbody>
</table>

Conditions: 10 mM Tris, 10 mM CaCl₂, pH 7.4, 25°C. The data presented in mean ± SD, are relative signal changes and standard deviations from different samples at various concentrations (2-20 μM) following trypsin complete digestion, $n = 6$. 
Figure 3.16. Optical signal change of EGFP-T1 following chymotrypsin digestion. Absorption spectra of EGFP-T1 (A) and EGFP-T2 (B) were measured after chymotrypsin digestion at various times from 0 min (●) to 120 min (□) in chymotrypsin digestion buffer (10 mM Tris, 20 mM CaCl₂, at pH 7.4), respectively. Absorbance of EGFP-T1 exhibited a decrease at 397 nm and an increase at 491 nm following chymotrypsin digestion at different digestion time intervals. In contrast, Absorbance of EGFP-T2 exhibited no any change following chymotrypsin digestion at different digestion time intervals.
3. 20 Conclusions and significance

Based on the investigation of trypsin sensor variants, a novel strategy is established to develop protease sensors by grafting an enzymatic cleavage linker on an EGFP scaffold at various locations hypothesized to be sensitive to the EGFP chromophore. The sensitive location, position 172, for the signal change of EGFP chromophore, is confirmed to be suitable location for the development of protease sensors. The designed EGFP-based trypsin sensors are capable of tracking trypsinogen activation and inhibition in various subcellular compartments in living cells in real time. Although several elegant studies reported the on and off measurement of protease activity by linking GFP with ubiquitin or proteasome cleavage peptide for protein degradation or by using bacterial protease sequences such as PEST and N-terminal rules (Dantuma, Lindsten et al. 2000; Spencer, Theodosiou et al. 2004), our developed trypsin sensors exhibit a large ratiometric change which allows for quantitative measurement of enzymatic actions. The substrate specificities our protease sensors are better or comparable than those of currently-available peptide probes. Meanwhile our trypsin sensors exhibit both strong enzymatic selectivity and significant ability to monitor enzymatic inhibition. Moreover, this novel strategy for trypsin sensors is the first example where the dynamic activation or inhibition of trypsin action and function in pancreatic cancer cells via living cell imaging can be determined. This novel approach can also be applied to develop other sensors for real time probing of enzymatic mechanisms, tracking the progress of diseases related to protease activity and monitoring the effects of protease inhibitors in drug development (Roberts, Martin et al. 1990).
Chapter 4 Developing caspase sensors for imaging apoptosis at early stage in living cells

4. 1 Introduction

4.1.1 Apoptosis, caspase actions and tumors

Apoptosis, or programmed cell death, is responsible for many essential physiological processes that play a critical role in excluding damaged or harmful cells for tissue development and maintaining homeostasis in a biological system. Many human diseases can also be attributed directly or indirectly to a derangement of apoptosis, resulting in either cell accumulation, cell eradication, cell turnover impairment, or cell loss (Fadeel and Orrenius 2005). It is increasingly clear that apoptosis plays a central role in the pathogenesis of various human diseases including cancers, autoimmune diseases and neurodegenerative diseases. The progress of apoptosis is regulated by activation of a series of multiple caspases in a stringently temporal and spatial order under certain circumstances. Caspases, closely associated with apoptosis, are aspartate-specific cysteine proteases. The activation and function of caspases, involved in the delicate caspase-cascade system, are regulated by various kinds of molecules, such as inhibitors of apoptosis protein, Bcl-2 family proteins, calpain, and calcium ions (Zhang, Haskins et al. 2004; Fan, Han et al. 2005). Therefore, the caspase-cascade system plays vital roles in the induction, signal transduction and amplification of intracellular apoptotic signals. For example, an increase in apoptosis leads to cell loss accompanied by neurodegenerative diseases (Alenzi 2005). While the relationship between apoptosis and caspases has been demonstrated among some diseases, the apoptosis mechanisms by which caspase activity is mediated are not fully understood. One of the major challenges in monitoring apoptosis pathways is the involvement of series of caspases and
their activation or inhibition mechanisms. Initiator caspases (caspase 8, 9 and 10) and effector caspases (caspase 3 and 7) have different pathways and also share substrate specificity to some extent. On the other hand, progress in this area has been limited due to the lack of a convenient and reliable system to quantify these protease activities and quantitatively detect caspase activity in real time in living cells or \textit{in vivo} (Gurtu, Kain et al. 1997). Therefore, there is a strong need to develop specific caspase sensors for monitoring activation and inhibition in living systems in real-time.

\section*{4.1.2 Apoptosis pathways}

During the last decades, a great deal of attention has been paid to the apoptosis pathways and its regulation in governing survival and death of cells (Cohen 1993; Eischen and Leibson 1997; Burns and El-Deiry 1999; Cummings, Ward et al. 2004; Jegathesan, Liebenthal et al. 2004; Oberst, Bender et al. 2008). Although it is clear that apoptosis is a process with typical morphological characteristics including plasma membrane blebbing, cell shrinkage, chromatin condensation and fragmentation, its pathways and regulation are still being extensively investigated (Sedlakova, Kohut et al. 1999; Kanduc, Mittelman et al. 2002; Elmore 2007). Caspases are key proteins for the signal transduction and the apoptotic signals induced by several stimuli. These proteins are present within the cell as inactive precursors that require proteolytic cleavage for activation. There are two main caspase groups, initiators and executors (Boatright and Salvesen 2003). The former are activated by autoproteolysis when translocated to specific cell compartments or through the coupling of adapters and/or activators. Therefore, although the activation or inhibition of a series of caspases is tightly regulated by other factors, caspases are
still the central component for apoptosis response, protein degradation, chromatin fragmentation and final cell death.

Currently, three pathways are reported for apoptosis. As shown in Figure 4.1, two major apoptosis pathways, the intrinsic pathway (mitochondria-mediated apoptotic pathway) and the extrinsic pathway (receptor-mediated apoptotic pathway) were well demonstrated and established. Both apoptosis pathways had close relationships with the activation or inhibition of caspase-cascade system (Budihardjo, Oliver et al. 1999; Shi 2002; Boatright and Salvesen 2003; Wang, Liu et al. 2005; Logue and Martin 2008). As for the mitochondria-mediated apoptotic pathway, mitochondria participation is the major characteristic of this apoptotic pathway to mediate the interactions among various cytoplasmic organelles, including the endoplasmic reticulum, Golgi apparatus and lysosomes (Ferri and Kroemer 2001). Meanwhile, within this apoptotic pathway, Bcl-2 family proteins have pivotal roles in regulating the behavior of some small molecules, such as cytochrome C, Smac/Diablo, and apoptosis-inducing factor, resulting in apoptosome formation or to activate caspase cascades (Schuler and Green 2001; Harada and Grant 2003; Antonsson 2004). The receptor-mediated apoptotic pathway is initiated to form a death-inducing signal complex through the recruitment and ligation of a death ligand to its transmembrane death receptor (Sartorius, Schmitz et al. 2001; Falschlehner, Emmerich et al. 2007). FasL and other TNF superfamily ligands are instrumental in activating the caspase-cascades by inducing the trimerization of cell-surface death receptors and perturbing the release of cytochrome C from mitochondria or neutralizing the inhibitors of apoptotic protein or activators of caspasess. Because Fas and TNF superfamily ligands have an indirect effect on the release of cytochrome C, cross-talk between the intrinsic and extrinsic pathways has also been extensively investigated (Khosravi-Far and Esposti 2004; Basu, Castle et al. 2006). For example,
Figure 4.1. Diagram of caspase-3 activation and apoptosis pathways.

The activation of caspase-3, one of the major players during apoptosis, can be accomplished through two main pathways, death receptor-mediated pathway and mitochondria-mediated pathways. In the receptor-mediated pathway, procaspase-8 can be activated through the binding between death ligands and death receptors and the formation of death inducing signaling complex (DISC). The activated caspase-8 cleaves procaspase-3 to result in caspase-3 activation and apoptosis. In the mitochondria-mediated pathway, inducers or extracellular stress can promote the release of cytochrome C from mitochondria and then form the apoptosome through cytochrome C, Apaf-1 and caspase-9. The activated caspase-9 performs the cleavage of procaspase-3 to achieve caspase-3 activation and induce apoptosis.
the BH3-domain of protein BID is only cleaved by the activation of pro-caspase-8 through the extrinsic pathway, and then translocates to the mitochondrion to promote cytochrome C release (Li, Zhu et al. 1998; Tan, Tan et al. 1999).

In addition to the above well-characterized apoptotic mechanisms, another mechanism of the apoptotic process was also suggested where the calcium or endoplasmic reticulum (ER)-regulated pathway made a significant contribution to activation or inhibition of caspase-cascade system (Jimbo, Fujita et al. 2003; Tantral, Malathi et al. 2004; Murakami, Aizu-Yokota et al. 2007). The ER provides storage for Ca\(^{2+}\) and is essential for calcium signaling. Ca\(^{2+}\) dynamics can also be modulated through Bcl-2 family proteins, one of the crucial factors in the apoptotic pathway, which results in apoptosis due to the massive accumulation of Ca\(^{2+}\) (Oakes, Opferman et al. 2003; Bassik, Scorrano et al. 2004). Since Ca\(^{2+}\) dynamics can also be mediated by mitochondria to result in apoptosis, some calcium-binding proteins should also have significant effects on the activation or inhibition of the caspase-cascade system and apoptotic pathways (Heath-Engel and Shore 2006). On the other hand, due to the complicated caspase-cascades system and apoptosis networks, a caspase-independent apoptotic process also has been demonstrated (Assefa, Vantieghem et al. 2000; Bidere and Senik 2001; Ajiro, Bortner et al. 2008). Hence, comprehensive investigation and understanding of activation or regulation of apoptotic pathways is essential for developing therapeutic strategies or novel and effective drugs to treat autoimmune diseases, cancers and neurodegenerative diseases.
4.1.3 Inducers for caspase-3 activation

The central contribution of the activation or regulation of caspase-cascade system in the apoptotic process has been well recognized. Since caspase-3 is a pivotal enzyme in the execution stage of the apoptotic pathway, this effector caspase is a good biomarker of apoptosis. In order to understand the contributions and regulations of caspase-3 activation in the apoptotic process, the inducers or triggers for caspase-3 activation are also extensively investigated. The following list is the brief summary of commonly-used inducers for caspase-3 activation through different apoptotic pathways.

Inducers for caspase-3 activation through mitochondria-mediated pathway

1) Staurosporine (STS) (Figure 4.2A) is a small molecule anticancer drug and has been used as a broad-spectrum inhibitor of protein kinases (Feng and Kaplowitz 2002). It has been well-established and widely-used to promote intracellular apoptosis through protein kinase C inhibition to result in caspase-3 activation, the alteration of mitochondrial function to promote the release of cytochrome C (Deshmukh and Johnson 2000), the loss of mitochondrial membrane potential (Matsuyama, Llopis et al. 2000; Rego, Vesce et al. 2001; Smaili, Hsu et al. 2001), the increase of ATP concentration (Shiraishi, Tatsumi et al. 2001), the decrease of extracellular pH (Matsuyama, Llopis et al. 2000) and production of reactive oxygen species (ROS) (Kruman, Guo et al. 1998; Li, Maasch et al. 1999; Gil, Almeida et al. 2003).

2) Etoposide (Figure 4.2B) is a drug for inducing DNA damage to treat cancers, which is mainly focused on the inhibition of topoisomerase activity (Kim, Emi et al. 2005; Karpinich, Tafani et al. 2006).
3) UV irradiation is an approach for inducing the inactivation of mitogen-activated protein kinase or extracellular signal-regulated kinase to result in apoptosis (Franklin, Srikanth et al. 1998; He, Huang et al. 2004; Sethi and Sodhi 2004).

4) Tributyltin (TBT) (Figure 4.2C) can rapidly activate caspase-3 by damaging the functions of mitochondria, increasing free calcium concentration in the cytosol, inducing the production of ROS, improving the release of cytochrome C, and promoting mRNA expression of TNFα and c-jun (Stridh, Kimland et al. 1998; Stridh, Gigliotti et al. 1999; Gennari, Viviani et al. 2000; Tiano, Fedeli et al. 2003).

5) Artemisinin and its derivatives are well-known antimalarial drugs and particularly useful for the treatment of infection of malaria parasites resistant to traditional antimalarials (Klayman 1985; Balint 2001; Liu, Zhao et al. 2006). Currently, dihydroartemisinin (DHA) (Figure 4.2D), a water-soluble active metabolite of artemisinin derivatives, is used to induce apoptosis in many cancer cell lines like leukemia HL60 cells or MD-DBA-451 cells through the expression of caspase-3 (Lu, Meng et al. 2008; Zhou, Wang et al. 2008). The mechanism of apoptosis or caspase-3 activation is reported to downregulate transferin receptor (TfR) expression through mitochondrial pathway (Nakase, Lai et al. 2008). In addition, the report demonstrated that DHA-induced apoptosis was an iron-dependent process and reactive oxygen species (ROS) was contributed little to DHA-induced apoptosis in HL-60 cells (Lu, Meng et al. 2008). Moreover, DHA time-dependently activates mitogen-activated protein kinases (MAPKs) (Lu, Meng et al. 2008; Mu, Zhang et al. 2008).

**Inducers for caspase-3 activation through death receptor-mediated pathway**

1) TNFα (tumor necrosis factor type 1) superfamily is a well-defined caspase trigger through death receptor pathway. Caspase activation is initiated by the binding between death
Figure 4.2. The molecular structures of inducers for caspase-3 activation.
Four different small molecules, staurosporine (A), etoposide (B), tributyltin chloride (C) and dihydroartemisinin (D) were used for triggering apoptosis and caspase-3 activation in living cells.
ligands belonging to the tumor necrosis factor superfamily and its corresponding death receptor, followed by the ligation and the formation of a multiprotein complex, death inducing signaling complex (DISC), which promotes the caspase-8 activation in upstream followed by downstream caspase-3 or caspase-7 activation (Boatright and Salvesen 2003; Philchenkov, Zavelevich et al. 2004). In addition, TNFα also functions to increase the expression level of p53 and p53-associated proapoptotic genes and indirectly stimulates the caspase cascade in cells (Menon, Lombardi et al. 2002). Moreover, since TNFα is an autocrine growth factor for a series of tumors and performs critical functions for tumorigenesis, extensive investigations have also indicated that it exhibits a strong capability to activate receptor-interacting kinase (RIP), resulting in the activation of NF-κB and the MAPK family members such as JNK, p38 and MAPK, which regulate the caspase pathways (Higashimoto, Panopoulos et al. 2006). Furthermore, it has also been reported that chromosomal abnormalities can be induced by TNFα independent of the mediations from ROS, JNK and p38; however, a clear mechanism for this is still being probed.

2) Anti-Fas exhibits similar functions and regulation modes as TNFα superfamily members for caspase-cascade systems. The best-characterized death-receptor signaling pathway is triggered through interaction between Fas and its ligand FasL, followed by the production of DISC to accomplish the caspase-cascade. Currently, typical anti-Fas ligands for caspase activation are anti-CD95 and anti-APO-1 (Boatright and Salvesen 2003; Philchenkov, Zavelevich et al. 2004).

3) TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) is an important member of TNF family of cytokines for promoting caspase activation and apoptosis. TRAIL is specific to a wide variety of tumor cells, but not normal cells for caspase activation and apoptosis induction through the mediation of death receptors such as DR4 and DR5.
(Philchenkov, Zavelevich et al. 2004). The assembly of the signaling complex of DR4/DR5 can be achieved through the binding of TRAIL, followed by initiation of caspase-8 activation. In addition, activation of caspases can be initiated via TRAIL, which also have been reported to have relationships with the mitochondria pathway, such as the loss of mitochondrial transmembrane potential, the cleavage of BID, and the redistribution of mitochondrial cytochrome C (Suliman, Lam et al. 2001; Nencioni, Wille et al. 2005). Therefore, TRAIL-induced caspase activation and apoptosis can be accomplished via both mitochondria-dependent and -independent pathways.

4.1.4 Structure and substrate specificity of caspases

Caspases are synthesized in relatively large amounts as inactive precursors within the cytoplasm. As shown in Figure 4.1 and 4.3, these precursors contain three domains: an extended N-terminal prodomain, a large subunit (~17-20 kDa) and a small subunit (~10 kDa). In an initiator caspase, the prodomain is generally larger, with more than 90 amino acid residues, whereas an effector caspase frequently contains 20-30 residues in its prodomain sequence (Chang and Yang 2000). The long prodomains in initiator caspases are composed of death effector domains (DED), which are found in caspase-8 and caspase-10, or the caspase recruitment domains (CARD), which are found in caspase-9 and caspase-2. These domains can mediate specific protein-protein interactions between procaspases and their adapters to promote the formation of a multimeric complex that is necessary to bring two caspase precursors together for procaspase activation (Stennicke and Salvesen 1998; Donepudi and Grutter 2002; Shi 2002; Shi 2004). On the other hand, the short prodomains in executioner caspases do not mediate protein-protein interactions. Following a proapoptotic stimulus, they are sequentially processed
into a precursor, noncatalytic domain and two catalytic subunits: a large subunit containing the active site Cys, and a smaller 10 kDa subunit.

The three-dimensional structures of active caspases have been determined and shown in Figure 4.4, which are composed of a tetramer formed from two heterodimers of these two subunits with molecular masses of 20 kDa and 10 kDa, respectively (Concha and Abdel-Meguid 2002). Two small subunits are surrounded by two large subunits (Chang and Yang 2000). This catalytic tetramer is arranged in opposite direction and each heterodimer has an active site, which is characterized by a deep pocket for binding the Asp (P₁) at the cleavage site in its specific substrate and a broader, shallower irregular pocket to bind the preceding three amino acid residues (P₂-P₄) (Stennicke and Salvesen 1999). The proteolytic process for the activation of procaspases to produce the active caspase tetramers is shown in Figure 4.3. Each caspase has a conserved active site, pentapeptide QACXG (X is always R, Q, or G) containing the active Cys residue that is necessary for the proteolytic activity of caspases. Therefore, caspases have an essential requirement for cleavage after an aspartate residue (D). Both the large and the small subunits contribute to form the substrate-binding pockets, which recognize at least four amino acids (P₄P₃P₂P₁) as the cleavage site in the substrates. In the S₁ site, the amino acid residues, which form the Asp substrate-binding pocket, are R179, Q283, R341 and S347, with the first two residues from the large subunit and the latter two residues from the small subunit. R179 and R341 can form hydrogen bonds with the Asp residue in the P₁ site of the substrate. The major interactions in the active site are shown in Figure 4.5 (Nicholson 1999). The amino acids from the small subunit forming the S₂ and S₃ sites can be varied considerably (Cohen 1997; Donepudi and Grutter 2002). Therefore, caspases have less strict requirement at the P₂ and P₃ locations in substrates. However, the high specificity of substrates is mainly due to the significant difference
Figure 4.3. The process from procaspase to active caspase tetramer. The pro-caspase composed of prodomain, large subunit and small subunit was proteolytically cleaved at the Asp-X cleavage sites to obtain large and small subunits, respectively. The large subunit (α chain) and small subunit (β chain) are connected to form a heterodimer. Two heterodimers then form a tetramer to produce an active caspase. The active site is composed of four residues, R179, H237, C285 and R341.
Figure 4.4. The three-dimensional structure of active caspase-3 (PDB, 2J30). The active caspase-3 tetramer is composed of two heterodimers, which are formed by the large subunit (highlighted in green and cyan color) and the small subunit (highlighted in red and purple color).
Figure 4.5. Interactions between the substrate and residues in the active site of caspase-3.
The residues at P₁ and P₃ position of caspase-3 substrates provide the critical interactions between substrate and active sites of caspase-3. The Glu residue at the P₃ position of substrate forms the bident hydrogen bonds with Arg341 in the active site of caspase-3. Arg179 and Arg341 are also involved in hydrogen network formation with the Asp residue at the P₁ position of caspase-3 substrates. Modified after (Nicholson 1999).
in the S₄ site. For example, the large and shallow hydrophobic depression contributes to caspase-1 specificity due to correct accommodation with Tyr or Trp in the P₄ site. The narrow and hydrophilic pocket accounts for caspase-3 recognition specificity because of the interaction with the side chain of Asp residue (Earnshaw, Martins et al. 1999). Hence, a unique pocket for each caspase member was recognized, and the specific recognition sites are shown in Table 4.1.

Table 4.1. The specific recognition sites of various caspases

<table>
<thead>
<tr>
<th>Specificity group</th>
<th>Caspase name</th>
<th>Optimal recognition motif (P₄-P₁)</th>
<th>Consensus motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Caspase-1</td>
<td>WEHD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caspase-4</td>
<td>WEHD</td>
<td>WEHD</td>
</tr>
<tr>
<td></td>
<td>Caspase-5</td>
<td>WEHD</td>
<td>WEHD</td>
</tr>
<tr>
<td></td>
<td>Caspase-13</td>
<td>WEHD</td>
<td>WEHD</td>
</tr>
<tr>
<td>Group II</td>
<td>Caspase-2</td>
<td>DEHD</td>
<td>DEXD</td>
</tr>
<tr>
<td></td>
<td>Caspase-3</td>
<td>DEVD</td>
<td>DEVD</td>
</tr>
<tr>
<td></td>
<td>Caspase-7</td>
<td>DEVD</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>Caspase-6</td>
<td>VEHD</td>
<td>(I/V/L)EXD</td>
</tr>
<tr>
<td></td>
<td>Caspase-8</td>
<td>LETD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caspase-9</td>
<td>LETD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caspase-10</td>
<td>LEXD</td>
<td></td>
</tr>
</tbody>
</table>

Modified after (Nicholson 1999).
One of the most attractive features for caspases is that these enzymes have high substrate specificity. Peptide substrates are named by designating the amino acid extending from the cleaved peptide bond to N-terminal side as P₁, P₂, P₃, P₄, etc. and the amino acid residues extending following the cleaved peptide bond to the C-terminal side as P₁’, P₂’, P₃’, P₄’, etc. Initial studies have shown that caspases have very high P₁ specificity. The rate of catalysis decreases more than 100-fold when the P₁ aspartic acid residue is substituted by any other amino acid (Stennicke and Salvesen 1999). To examine the substrate specificity at other positions, caspase-1 substrates were synthesized, and then analyzed to determine their $V_{\text{max}}/K_{m}$ (Howard, Chartrain et al. 1991). Amino acids spanning P₁ to P₄ were proven to be critical for substrate recognition by caspase-1, as deletions of these amino acid residues diminished substrate activity. No significant reduction in catalysis was observed when C-terminal deletions were examined except for the P₂’ position. It is important to note that the P₅ deletion significantly reduced the relative rate of hydrolysis, but placing an acetyl group on the N-terminus of P₄ restored optimal activity. In addition, the activity investigations on caspase-3 and caspase-7 indicated caspase-3 prefers hydrophobic residues, such as Leu or Val, at the P₅ site, however, there is no obvious requirements for the P₅ site of caspase-7 substrates (Fang, Boross et al. 2006). Although extensive investigations indicated that DEVD in substrates is the optimal sequence residues for caspase-3 recognition and cleavage, proteins with less optimal sequences like DKVDV, DGFDS, DMPDG and DNIDA from zebrafish or the nucleobindin-2 protein with sequence of TDGLDP also have high potential to be cleaved by caspase-3 (Valencia, Bailey et al. 2007; Valencia, Cotten et al. 2008). Therefore, investigating the highly-conserved recognition sequence and the specificity for caspase-3 in these natural caspase-3 substrates should provide important
information for highly-specific caspase-3 sensor development, which will enhance our ability to monitor caspase-3 activation or inhibition in real time in living systems.

4.1.5 **Current methods for detection of caspase activity and their limitations**

The majority of caspase actions are mainly monitored in semi-quantified or indirect modes. Antibody against caspases by Western blot is a common method with high sensitivity, which provides a qualitative rather than quantitative assay for caspases of apoptosis pathways in apoptotic cells, tissues or organs. Caspase inhibitors or affinity labels to monitor caspase actions are limited by their relatively low specificity for each individual caspase and toxic effects especially during long-time measurement in living cells or *in vivo* (Lee, Long et al. 2000; Tawa, Tam et al. 2001; Kohler, Orrenius et al. 2002). The current majority of caspase-3 sensors or probes for monitoring caspase actions are also composed of chromogenic or fluorogenic dye conjugated cleavage linkers for caspase-3, which can be summarized under the following major classes. The first class, that is commercially available, is chemical synthetic substrates linked to chromophores, which utilizes a synthetic tetrapeptide, Asp-Glu-Val-Asp (DEVD), labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin (AFC), or a colorimetric molecule, p-nitroanilide (pNA) as substrates. DEVD-dependent protease activity is assessed by the detection of the free AFC or pNA cleaved from substrates for rapid quantification of caspase-3 activity during the onset of apoptosis (Gurtu, Kain et al. 1997). To analyze caspase activity with this method, apoptotic cells are required to be lysed and then subjected to enzymatic action. Although some caspase peptide probes can be diffused into living cells, caspase activity cannot be well-quantified due to difficulties in delivery, especially in the desired sub-cellular compartments such as mitochondria and ER. Quantification of caspase activity in living cells is also affected by
background interference. In addition, specificity of these commercial substrates for caspases is poor due to lack of defined structure and in inability to insert the preferable amino acid residues in both P region and P’ region of the cleavage linkers (Liu, Bhalgat et al. 1999; Kohler, Orrenius et al. 2002; Kawai, Suzuki et al. 2004; Kawai, Suzuki et al. 2005). Another class of fluorescence probes with a fluorophore and a quencher was developed and applied to on-chip detection of caspase-3 activity. This probe is non-fluorescent in the absence of caspase-3. However, when it is treated with active caspase-3, the fluorescence intensity increases dependent on the caspase-3 activity due to the cleavage of the quencher-containing moiety on a glass slide (Han, Sonoda et al. 2006).

To date, real-time determination of caspase-3 pathway in vivo or in living cells has been largely elusive. Due to the limitations of the above methods for caspase-3 activity assay in living cells, and the unique capability of GFP to be expressed in many organisms without the requirement of cofactors, GFP has attracted extensive attention for developing novel and effective methods to determine caspase-3 activity in vitro or in vivo in real time. The amino acid peptide linker containing caspase-3 recognition sequence (DEVD) for linking fluorescence protein pairs, such as GFP and BFP, ECFP and EYFP, has been used for monitoring the apoptosis processes by FRET assay (Luo, Yu et al. 2001; Zhang, Haskins et al. 2004). Dual color fluorescence cross-correlation spectroscopy (FCCS) has also been used to directly detect caspase-3 activation in a single live cell using a fusion protein comprised of enhanced green fluorescent protein (EGFP) and monomeric red fluorescent protein (mRFP). Several reports have also been presented the FRET resulting in the fusion fluorescent protein pairs for monitoring protease activity with flow cytometry, fluorescence microplate reader in apoptotic cells (Jones, Heim et al. 2000; He, Wu et al. 2004; Wu, Simone et al. 2006). Another reported method
allowing for real-time observation of caspase activation *in situ* in living cells was based on FRET measurement using the prism and reflector imaging spectroscopy system (PARISS) (Zhang, Haskins et al. 2004). These FRET-based methods have advantages of monitoring the dynamic processes of caspase activation *in vitro* or targeting the right locations *in vivo*. Unfortunately, as discussed in Chapter 1 (Section 1.5), practical applications for *in vivo* imaging of protease actions with FRET-paired protease probes are limited due to the relatively small change in signal, problems associated with photobleaching, imbalance in fluorescence intensity between the donor and the acceptor, cross-talk in the FRET signal due to spectral overlap between the donor and the acceptor, and the restriction of optimal distance for FRET efficiency due to the large size of the fluorescent proteins (Felber, Cloutier et al. 2004; Piston and Kremers 2007).

### 4.1.6 Objectives to the development of caspase sensors

It is well known that caspase-cascade system has a pivotal function to cell survival and cell death in the cell cycle. The generation, activation, inhibition and regulation of caspases in living systems are complicated, which presents difficulties for real-time monitoring of pathways and mechanisms of apoptosis. On the other hand, due to caspase activation, homeostasis alteration like acidification or alkalization of different subcellular compartments during the late stage of apoptosis, a sensitive genetic caspase probe with a ratiometric measurement mode that circumvents the effect from background and normalizing the alteration of cellular contents is highly desired.

The insertion of EF-hand motifs at position Glu172 of the EGFP is sensitive to proteases, such as trypsin, as is reported in Chapter 3. According to the high sensitivity and large relative signal change of trypsin sensors *in vitro* or in living cell studies, we hypothesized that EGFP can
be applied to design the protease-susceptible molecules for monitoring specific proteolytic activities in vitro, in living cells and in vivo, and to understand mechanisms of protease-related diseases.

In this chapter, we would like to extend the design and application of the strategy for protease sensors. Herein, we have presented our strategy to develop caspase-3 and caspase-8 sensors by grafting a caspase-3-specific or caspase-8-specific cleavage linker at the identified chromophore-sensitive location 172 in a single EGFP. The optical and kinetic properties of these sensors were first examined using purified proteins under different buffer conditions. Because EGFP exhibits high resistance to various proteases and presents the possibility of cleavage with adding a cleavable linker for proteases at the inserted loop region of EGFP, the insertion of caspase-3-specific or caspase-8-specific cleavable linkers in EGFP was conducted to develop sensors for detecting initiator caspase, caspase-8 and effector caspase, caspase-3 activation and inhibition in living cells in real time. In addition, the fluorescence ratiometric changes of caspase-3 at 398 and 490 nm for excitations were also examined using both fluorescence microscopy and fluorimeter assays in several different cell lines. Furthermore, the performance of developed sensors in monitoring caspase-3 activation was compared with peptide kits. Moreover, the data in living cells were compared with the data in vitro using purified proteins.

4.2 Design and construction of caspase-3 sensors

In order to develop EGFP-based caspase sensors, different cleavage linkers containing caspase-3 recognition sequence (DEVD) were grafted into the identified location Glu172, a sensitive position on EGFP for optical signal change of the chromophore, through PCR techniques, to obtain various caspase-3 sensors, as summarized in Table 4.2. Due to the
resistance of EGFP to proteases and the characteristics of better flexibility of EF-hand motifs in the trypsin sensors reported in Chapter 3, the recognition sequence (DEVD) as the cleavage site for caspase-3 was extended with an E-helix and F-helix on both sides to improve the exposure area of the cleavage site and increase accessibility to proteases. The caspase-3 recognition sequence (DEVD) with flanking EF-hand motifs was grafted in EGFP to produce a caspase-3 sensor, designated EGFP-C3A. Meanwhile, in order to improve the possibility of exposure or accessibility for caspase-3 cleavage and to compare the cleavage efficiency of EGFP-based caspase-3 sensors containing one cleavage site and two cleavage sites, another recognition sequence unit of caspase-3 (DEVD) was further grafted into EGFP-C3A to obtain another caspase-3 sensor with double cleavage sites, designated as EGFP-C3B. Both cleavage sites for caspase-3 were linked through a short glycine linker (GG). Based on the investigation of trypsin sensors, the cleavage sites on F-helix can offer fast response to trypsin and produce a large relative signal change. Therefore, the caspase-3 recognition sequence (DEVD) was also located on F-helix to evaluate caspase-3 cleavage efficiency and relative signal change of our designed caspase-3 sensors. Since the preferred residues, Leu or Val on P5 and Gly or Ser on P1’ positions of substrates have an important effect on caspase-3 cleavage, the preferred residues at P5 and P1’ positions, in recognition linkers for caspase-3 have been also considered to provide high specificity to caspase-3 digestion while designing caspase-3 sensors with a cleavage site on F-helix. The designed EGFP-based caspase-3 sensors with preferred residues at P5 and P1’ positions were designated as EGFP-C3C and EGFP-C3D. On the other hand, in order to further confirm the effects of helix-loop-helix motifs on caspase-3 cleavage, caspase-3 sensors without helix-loop-helix motifs were also designed. The alone caspase-3 recognition sequence (DEVD) and the alone caspase-3 recognition sequence (DEVD) extended with the linker (GGSGG) on
both sides were grafted into the identified sensitive location, Glu172 of EGFP to obtain EGFP-based caspase-3 sensors, EGFP-C3E and EGFP-C3F, respectively. A linker without recognition sequence was also designed and inserted onto EGFP to obtain a control, EGFP-C3N.

Table 4.2. Designed caspase sensor variants and their cleavage linker sequences

<table>
<thead>
<tr>
<th>Caspase sensor name</th>
<th>Cleavage linker sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-C3A</td>
<td>EEEIREAFRVFDEVDLRHVMTNL</td>
</tr>
<tr>
<td>EGFP-C3B</td>
<td>EEEIREAFRVFDEVDGGDEVDLRHVMTNL</td>
</tr>
<tr>
<td>EGFP-C3C</td>
<td>EEEIREAFRVFDKDGNGYISAAEVDEVDGVMTNL</td>
</tr>
<tr>
<td>EGFP-C3D</td>
<td>EEEIREAFRVFDKDGNGYISAAELLDEVDSVMTNL</td>
</tr>
<tr>
<td>EGFP-C3E</td>
<td>DEVD</td>
</tr>
<tr>
<td>EGFP-C3F</td>
<td>GGSGGDEVDDGSGG</td>
</tr>
<tr>
<td>EGFP-C3N</td>
<td>EEEIREAFRVFDKDGNGYISAAEVGVMTNL</td>
</tr>
<tr>
<td>EGFP-C8A</td>
<td>EEEIREAFRVFDKDGNGYISAAELLETDGVMTNL</td>
</tr>
<tr>
<td>EGFP-C8B</td>
<td>EEEIREAFRVFDKDGNGYISAAELLETDSVMTNL</td>
</tr>
</tbody>
</table>

Because caspase-3 activation is a landmark event in apoptosis, assaying caspase-3 activity in living cells has been extensively applied as an effective tool or a powerful biomarker for detecting programmed cell death, and understanding comprehensive mechanisms or pathways of apoptosis. However, caspase-3, an effector caspase, is first generated from an inactive pro-caspase-3 which is proteolytically activated by initiator caspase, caspase-8. Therefore, an EGFP-based caspase-8 sensor should also have its significance in visualization of caspase-8 activation and inhibition to study apoptosis mechanisms or pathways. A similar method is exploited to construct caspase-8 sensors. Caspase-3 and caspase-8 sensors were
successfully designed and amplified with PCR in pcDNA 3.1 (+) and pET28a vectors, for mammalian cell expression and bacterial expression systems, respectively. All cleavage linker sequences for our designed caspase-3 and caspase-8 sensors are listed in Table 4.2.

4.3 Optical properties of caspase sensors

The genetically engineered EGFP-based caspase-3 and caspase-8 sensors in the pET28a vector were successfully expressed in *E. coli*. Following the grafting of the cleavage linkers in EGFP, all of these caspase sensors, EGFP-C3A, EGFP-C3B, EGFP-C3C, EGFP-C3D, EGFP-C3E, EGFP-C3F, EGFP-C8A and EGFP-C8B reveal strong fluorescence both in bacteria and as purified proteins. The purified caspase-3 and caspase-8 sensor variants show two chromophore forms, deprotonated and protonated chromophores, resulting in strong absorption peaks at 490 and 398 nm, respectively, as shown in Figure 4.6. Like trypsin sensors, the insertion of the caspase-3 or caspase-8 cleavage linker at position Glu172 onto EGFP increases the proportion of the protonated form of the chromophore and reveals a strong absorption at 397 nm while correspondingly decreasing the deprotonated form of the chromophore with a strong absorption observed at 490 nm. Furthermore, such alteration of the ionic state of the chromophore is also related to the properties of cleavage linkers that are inserted. The EGFP-based caspase-3 or caspase-8 sensor variants with both flanking helices exhibit a greater absorption ratio ($A_{398\text{nm}}/A_{490\text{nm}}$) than caspase sensor variants that did not have a flanking helix in the inserted loop. The maximum fluorescence emission wavelengths of all caspase sensor variants were approximately 508 nm resulting from excitation at 398 or 490 nm, respectively, as shown by the representative variant, EGFP-C3B, in Figure 4.7. Consistent with our results from the developed trypsin sensors, as shown in Chapter 3, the close population level between deprotonated
Figure 4.6. Spectral characteristics of EGFP-based caspase-3 sensors. Compared to EGFP-wt (○), spectra of EGFP-C3A (◇), EGFP-C3B (●), EGFP-C3C (■), EGFP-C3D (□) and EGFP-C3E (△) buffered in 10 mM Tris, 1 mM DTT, pH 7.4, have both protonated and deprotonated chromophore forms. The different lengths and net charge of the cleavage linkers have significant effects on the population of both chromophore forms. All caspase-3 sensors with concentration of 10 μM are buffered in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4).
Figure 4.7. Fluorescence emission spectra of representative caspase-3 sensors. The fluorescence emission spectra of EGFP-C3B were scanned in the range 500 to 600 nm with excitation of 398 (●) and 490 nm (■) in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4). The maximum emission peak is observed at 508 nm when excited at both wavelengths.
chromophore form and protonated chromophore form provides a large relative signal change. Therefore, EGFP-C3B, EGFP-C3C and EGFP-C8A have the larger relative signal change following the digestion of caspases.

4.4 Optical signal change following caspase digestion

In order to examine the spectral characteristics of various caspase-3 sensors, all of the caspase-3 sensors with 15 μM in caspase reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.4) were monitored to observe absorbance or fluorescence spectral changes following caspase-3 digestion at a final concentration of 31.25 nM. Following caspase-3 digestion, the maximum absorption of EGFP-C3B at 490 nm increased and concurrently decreased at 398 nm, as shown in Figure 4.8A. Similarly, EGFP-C3B exhibited a ratiometric fluorescence signal change with a decrease in maximum fluorescence intensity at 508 nm at an excitation wavelength of 398 nm (Figure 4.8B) and a corresponding increase at an excitation wavelength of 490 nm (Figure 4.8C). Meanwhile, EGFP-C3C exhibited similar absorbance and fluorescence signal change trends as EGFP-C3B following caspase-3 digestion under identical reaction buffer conditions. In contrast, other EGFP-based caspase-3 sensors, EGFP-C3A, EGFP-C3E and EGFP-C3F exhibited very little optical signal change in absorbance or fluorescence following caspase-3 digestion, and EGFP-C3E, EGFP-C3F and EGFP-C3N exhibited no optical signal change following the addition of caspase-3, which clearly indicated that EGFP-C3B and EGFP-C3C are suitable for the development of caspase-3 sensors, but not EGFP-C3E and EGFP-C3F in spite of the caspase-3 recognition sites in the linkers. These results demonstrated that the larger exposure or high accessibility of recognition sites for caspase-3 could be improved through the extension of helix-
loop-helix structure in the cleavage linker to result in faster response to caspase-3 action, or provide a larger relative optical signal change following caspase-3 digestion.

In order to form an active caspase-3, a tetramer is generated through interaction between the heterodimers composed of a large subunit and small subunit of pro-caspase-3. Therefore, the optimal compositions of caspase-3 reaction buffers play a pivotal role in caspase-3 activation and its cleavage efficiency for substrates. According to previous investigations for the digestion of caspase substrates (Thornberry, Bull et al. 1992; Stennicke and Salvesen 1997; Okun, Malarchuk et al. 2006), the removal of CHAPS in caspase reaction buffers resulted in the loss of more than 40% caspase activity (Stennicke and Salvesen 1997). In order to improve the interaction between heterodimers, 10% glycerol or 10% sucrose was added in the reaction buffers (Kohler, Orrenius et al. 2002). In addition, because the caspases are proteases of the cysteine family, a relatively high concentration of DDT is required for full activity of caspases to avoid oxidation of catalytic cysteine (Nobel, Kimland et al. 1997; Okun, Malarchuk et al. 2006). Meanwhile, EDTA incorporated into caspase reaction buffers is also reported to avoid to the inactivation of trace metals in the buffer systems and NaCl is used to maintain the consistent ionic strength or pH change (Stennicke and Salvesen 1997; Feeney and Clark 2005). In order to obtain an optimal caspase-3 reaction buffer for our EGFP-based caspase-3 sensors, we have conducted caspase-3 digestion of 15 μM EGFP-C3B through the addition of stock caspase-3 (Sigma, St. Louis) up to a final concentration of 31.25 nM in 7 different reaction buffer conditions with the above-mentioned important compositions and mostly-used caspase-3 reaction buffer conditions in literature (Stennicke and Salvesen 1997; Shin, Okada et al. 2003; Du, Wang et al. 2004; Okun, Malarchuk et al. 2006), which are summarized in Table 4.3. Based on overnight digestion, SDS-PAGE examination indicated EGFP-C3B is specifically cleaved by active caspase-3 into two
Figure 4.8. Spectral change of EGFP-C3B under the caspase-3 reaction buffer system containing 10% sucrose following caspase-3 digestion.

The maximum absorption (A) of EGFP-C3B exhibited a decrease at 398 nm and an increase at 490 nm following caspase-3 digestion in the caspase-3 reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.4). The maximum fluorescence emission at 508 nm exhibited a decrease when excited at 398 nm (B) and an increase when excited at 490 nm (C) after caspase-3 cleavage. The spectra of EGFP-C3B before and after caspase-3 digestion are in solid and dashed lines, respectively.
major fragments approximately 25 and 8 kDa in mass (Figure 4.9) in 7 various caspase-3 buffer conditions. The UV-visible spectra of EGFP-C3B following caspase-3 digestion were measured in a cuvette with 1 cm pathlength and fluorescence spectra of EGFP-C3B with excitation of 398 and 490 nm were recorded. The largest relative signal change for caspase-3 digestion of EGFP-C3B was observed in reaction buffer 4 (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.4), as also summarized in Table 4.3.

Table 4.3. Composition of various reaction buffers for caspase-3

<table>
<thead>
<tr>
<th>Composition</th>
<th>Buffer-1</th>
<th>Buffer-2</th>
<th>Buffer-3</th>
<th>Buffer-4</th>
<th>Buffer-5</th>
<th>Buffer-6</th>
<th>Buffer-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES (mM)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>PIPES (mM)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Tris (mM)</td>
<td></td>
<td>20</td>
<td>20</td>
<td></td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>NaCl (mM)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DTT (mM)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>EDTA (mM)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CHAPS (%)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycerol (%)</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Sucrose (%)</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>6.8</td>
<td>7.5</td>
<td>7.5</td>
<td>6.8</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Relative signal change</td>
<td>1.50</td>
<td>1.85</td>
<td>1.44</td>
<td>2.10</td>
<td>1.84</td>
<td>1.75</td>
<td>1.51</td>
</tr>
</tbody>
</table>
Figure 4.9. The cleavage confirmation of EGFP-C3B following caspase-3 digestion.
In order to investigate optimal buffer conditions for caspase-3 digestion, the digestion of 20 μM EGFP-C3B was conducted by active caspase-3 in 7 buffers with various compositions. Following caspase-3 digestion with the concentration of 20 nM, EGFP-C3B is specifically cleaved in two major fragments with approximately 25 kDa and 8 kDa molecular masses, respectively. M is the protein marker; C3B is the sample of EGFP-C3B sensor; B1, B2, B3, B4, B5, B6 and B7 are the samples of EGFP-C3B following caspase-3 digestion under 7 different reaction buffer conditions listed in Table 4.3.
4.5 Verification of caspase sensors cleavage

Although EGFP-C3B can be cleaved into major fragments in 7 various different reaction buffer conditions, the accurate cleavage locations still need to be verified. The cleaved fragments for EGFP-C3B were subjected to further analysis by MALDI-MS (Dr. Siming Wang provided the mass spectroscopy), which revealed molecular masses for intact EGFP-C3B and the two major fragments of 34440.02 (Figure 4.10A), 25607.73 (Figure 4.10B) and 8226.51 Da (Figure 4.10C), respectively, where the latter two mass values correspond to the calculated mass values of 25494.56 and 8211.15 Da for the major fragments of EGFP-C3B. This result is in good agreement with the analysis of SDS-PAGE. Both mass spectrometry and SDS-PAGE analyses reveal that the molecular masses of the cleavage fragments are consistent with cleavage at the desired caspase-3 cleavage location. When examining the selectivity for different members of caspase family, both purified EGFP-C3B and EGFP-C3C at concentration of 20 μM were specifically cleaved by 20 nM caspase-3 into two major fragments in optimal caspase-3 reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.5) following overnight digestion at room temperature, as revealed by SDS-PAGE (Figure 4.10D). In contrast, wild type EGFP (EGFP-wt) and EGFP-C3N that both lack caspase-3 recognition sites did not exhibit similar cleavage upon overnight incubation with caspase-3. Moreover, our caspase-3 sensors can not be cleaved by 20 nM caspase-6, caspase-7, caspase-8 and caspase-9 (These enzymes are provided by Rihe Liu from The University of North Carolina at Chapel Hill); however, under identical buffer conditions, 20 nM caspase-8 cleaves its substrate EGFP-C8A. Although there is considerable similarity between recognition sites for various caspases, our EGFP-C3B and EGFP-C3C offer a high selectivity and specificity for caspase-3,
Figure 4.10. Cleavage of EGFP-C3B following evaluation by MALDI-MS and SDS-PAGE. 20 μM EGFP-C3B was subjected to 20 nM caspase-3 digestion overnight at room temperature in its optimal reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.5) and the cleavage status was determined using MALDI-MS. The molecular mass of intact EGFP-C3B, cleaved major large fragment and major small fragment are 34440.02 (A), 25607.73 (B) and 8226.51 Da (C), respectively, suggesting a specific cleavage at the recognition site of caspase-3 in the inserted linker. The specificity and selectivity of caspase-3 sensors for different caspases was examined in SDS-PAGE (D). Lanes M, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 are: protein marker, EGFP-C3B, EGFP-C3B with caspase-3 digestion, EGFP-C3B with caspase-8 digestion, EGFP-C3C, EGFP-C3C with caspase-3 digestion, EGFP-C3C with caspase-8 digestion, EGFP-wt, EGFP-wt with caspase-3 digestion, EGFP-C8A with caspase-8 digestion, EGFP-C3B with caspase-6 digestion, EGFP-C3B with caspase-9 digestion, EGFP-C3B with caspase-7 digestion, EGFP-C3C with caspase-6 digestion, EGFP-C3C with caspase-9 digestion and EGFP-C3C with caspase-7 digestion, respectively. All caspase sensors at the concentration of 20 μM are buffered in optimal reaction buffer conditions and subjected to overnight digestion with 20 nM caspases at room temperature.
which may be due to the preferred residue extension outside $P_1$ and $P_4$ position in our EGFP-based sensors. Moreover, the grafting cleavage linker inside the EGFP $\beta$-barrel structural environment possibly provides an improved match to the caspase-3 active pocket. Such enzymatic specificity is very important for further application in monitoring caspase-3 action in live systems.

### 4.6 Kinetic study of caspase sensors

In order to evaluate the enzymatic response and the specificity of caspase-3 sensors through catalytic kinetic parameters, kinetic studies of EGFP-based caspase-3 sensors at various concentrations from 2 to 30 $\mu$M supplemented the addition of active caspase-3 were conducted in the optimal caspase-3 reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.5) to obtain the turnover number ($k_{\text{cat}}$), the Michaelis constant ($K_m$), and the specificity constant ($k_{\text{cat}}/K_m$). The initial rates of EGFP-based caspase-3 at various concentrations following caspase-3 cleavage were determined using a time course mode to monitor absorbance changes at 490 nm. Kinetic studies of our designed caspase-3 sensors were compared with results from a commercially-available caspase-3 substrate, Ac-DEVD-pNA colorimetric caspase-3 substrate, under identical buffering conditions. Kinetic catalytic parameters, $k_{\text{cat}}$, $K_m$ and $k_{\text{cat}}/K_m$ were determined through fitting with the Michaelis-Menten equation (Chapter 2), as summarized in Table 4.4. Although our designed caspase-3 sensors have smaller $k_{\text{cat}}$ values, EGFP-C3B exhibited a 2.7-fold smaller $K_m$ and a 6.7-fold higher $k_{\text{cat}}/K_m$ than commercial caspase-3 substrates, Bz-DEVD-pNA and Z-DEVD-AMC, respectively, based on previous reports (Fang, Boross et al. 2006). EGFP-C3C exhibited a 3-fold larger $k_{\text{cat}}$ and 0.4-fold smaller $K_m$ with overall substrate specificity 4.8-fold better than that of EGFP-C3B, which is due
either to better solvent accessibility of the cleavage site or a longer linker. Moreover, the substrate specificity $k_{\text{cat}}/K_m$ of EGFP-C3C exhibited 21.8 and 4.5-fold larger than those of commercial caspase-3 substrates, Bz-DEVD-pNA and Z-DEVD-AMC, respectively, due to its smaller $K_m$ value. It is important to note that the same DEVD sequence in two different commercial probes were 2.5 folds difference in $K_m$, suggesting the influence of chromophore on the kinetic property. These results indicate that our designed EGFP-based caspase-3 is comparable or better than currently available commercial caspase-3 substrates without unnecessary influence like the small chromophore. Although fluorescent protein FRET-based caspase-3 probes are extensively investigated for the studies in living cells or in vivo, and this technology has made great achievements in living system detection, no catalytic kinetic parameters corresponding to this method have been reported.

Table 4.4. Catalytic kinetic parameters of caspase-3 sensors

<table>
<thead>
<tr>
<th>Trypsin sensors</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-C3B</td>
<td>0.13 ± 0.01</td>
<td>10.03 ± 1.19</td>
<td>12500 ± 150</td>
</tr>
<tr>
<td>EGFP-C3C</td>
<td>0.39 ± 0.03</td>
<td>6.55 ± 1.53</td>
<td>59600 ± 9300</td>
</tr>
<tr>
<td>Bz-DEVD-pNA</td>
<td>0.73 ± 0.02</td>
<td>26.91 ± 1.20</td>
<td>2730 ± 120</td>
</tr>
<tr>
<td>Z-DEVD-AMC</td>
<td>0.88 ± 0.01</td>
<td>67.1 ± 4.3</td>
<td>13100 ± 790</td>
</tr>
<tr>
<td>FRET-based sensors</td>
<td>No catalytic kinetic parameters reported</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conditions: 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.5, 25 °C

The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.
4.7 Fluorescence imaging of caspase-3 sensors

In order to real-time track caspase-3 activity in living cells, our EGFP-C3B sensor was transfected into HeLa cells. After transient transfection, HeLa cells with EGFP-C3B gene and the control (EGFP-wt) exhibit strong fluorescence via fluorescence microscopy, following 16 to 48 hours of expression. Upon caspase-3 activation by 1 μM staurosporine (STS), the fluorescence emission signal at 510 nm of EGFP-C3B significantly increased by approximately 100% at 398 nm of excitation while concurrently decreasing by 70% at 488 nm of excitation (Figure 4.11A, 4.11B, 4.11C and 4.11D), resulting in a relative signal change of 3.2 expressed in a ratiometric fluorescence emission change, determined by Equation 3, using the fluorescence intensity at 398 nm dividing the fluorescence intensity at 488 nm (Figure 4.11E). During the fluorescence signal change, no obvious change in cell morphology was observed, which suggests that caspase-3 activation can be sensitively detected in the early stage of apoptosis prior to the cell morphology changes and nucleus fragmentation. In contrast, the fluorescence signal change of EGFP-wt at 510 nm with excitation of 398 and 488 nm exhibited no significant change (Figure 4.12A, 4.12B, 4.12C and 4.12D). Moreover, no changes were observed in the ratiometric fluorescence signal of EGFP-wt, as shown in Figure 4.12E. Similarly, the cells transfected with EGFP-C3N that also lacked cleavage sites exhibited no fluorescence signal change at either excitation wavelengths or ratiometric fluorescence signal change.

In order to screen the caspase-3 activity in different cell lines, EGFP-C3B sensor was transfected into a pancreatic cancer cell line, MIA PaCa-2, which is commonly-used for apoptosis research and reported to have high caspase-3 activity based on the analysis of cell lysates. The imaging of the cells transfected with EGFP-C3B was acquired under identical excitation wavelengths and induction via 1 μM STS. The fluorescence emission signal at 510 nm
Figure 4. Fluorescence imaging and signal change of EGFP-C3B in HeLa cells following STS induction.

Representative images of HeLa cells transfected with EGFP-C3B, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after induction with 1 μM STS for 32 min on a Zeiss Axiovert 200 Inverted Microscope. The relative fluorescence signal change at 510 nm exhibited an increase with excitation at 398 nm (C) and a decrease with excitation at 488 nm (D) upon caspase-3 activation by STS induction at different times. The arrows show the time of STS induction. The ratiometric fluorescence signal change ($F_{398\text{nm}}/F_{488\text{nm}}$) (E) at 510 nm of HeLa cells transfected with EGFP-C3B following 1 μM STS induction with excitation of both wavelengths revealed an increase with a relative signal change of 3.2.
Figure 4.12. Fluorescence imaging and signal change of EGFP-wt in HeLa cells following STS induction.

The representative images of HeLa cells transfected with EGFP-wt, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after induction with 1 μM STS for 32 min. The relative fluorescence signal change at 510 nm exhibited a similar pattern, with a slight initial increase followed by a decrease with excitation at 398 nm (C) and 488 nm (D) upon caspase-3 activation by STS induction at different time intervals. The arrows show the time of STS induction. The fluorescence ratiometric change (F_{398nm}/F_{488nm}) (E) at 510 nm of HeLa cells transfected with EGFP-wt following 1 μM STS induction with excitation of both wavelengths revealed no change.
Figure 4. Fluorescence imaging and signal change of EGFP-C3B in MIA PaCa-2 cells following STS induction.

The representative images of MIA PaCa-2 cells transfected with EGFP-C3B, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after induction with 1 μM STS for 32 min. The relative fluorescence signal change at 510 nm exhibited a significant increase with excitation at 398 nm (C) and a slight increase with excitation at 488 nm (D) upon caspase-3 activation by STS induction at different time intervals. The arrows show the time of STS induction. The fluorescence ratiometric change ($F_{398\text{nm}}/F_{488\text{nm}}$) (E) at 510 nm of HeLa cells transfected with EGFP-C3B following 1 μM STS induction with excitation of both wavelengths revealed an increase with a relative signal change of approximately 8.
of EGFP-C3B revealed a significant increase under 398 nm of excitation and a concurrent decrease at 488 nm of excitation. The relative fluorescence signal exhibited a 12-fold increase at 398 nm of excitation while exhibited a slight increase at 488 nm (Figure 4.13A, 4.13B, 4.13C and 4.13D), resulting in a relative signal change of approximately 8 expressed in a ratiometric fluorescence emission change, determined by Equation 3 (Figure 4.13E). Compared to cell imaging results from HeLa cells, the relative signal change of EGFP-C3B in MIA PaCa-2 cells is nearly 3-fold larger than in HeLa cells.

4.8 Caspase-3 inhibition tracking in living cells

In order to understand the activation or inhibition of caspase-3 in living cells, an inhibition study of caspase-3 in living cells was conducted with our designed EGFP-based caspase-3 sensors. After HeLa cells were transfected with EGFP-C3B following 24-48 hours expression, a caspase-3 inhibitor, Ac-DEVD-CHO (Sigma, St. Louis) was added into the cell culture plates to a final concentration of 20 μM and incubated with the transfected HeLa cells for 12 h prior to cell imaging. Fluorescence imaging of HeLa cells treated with caspase-3 inhibitor exhibited a slight increase at excitation with both wavelengths (398 and 488 nm) (Figure 4.14A, 4.14B, 4.14C and 4.14D), which was possibly due to shrinkage and condensation from the cells following 1 μM STS induction, but the ratiometric fluorescence signal \( \frac{F_{398\text{nm}}}{F_{488\text{nm}}} \) did not exhibit obvious change (Figure 4.14E). Since the caspase-3 inhibitor, Ac-DEVD-CHO, is a competitive inhibitor to caspase-3, it can competitively bind caspase-3 and inhibit its activity when caspase-3 is activated following STS induction, which resulted in no cleavage of caspase-3 sensor expressed in cells to maintain intact form. Taken together, caspase-3 activity in living
cells following treatment by STS induction can also be effectively inhibited using caspase-3 inhibitor, AC-DEVD-CHO.

4.9 Shorter half-time for maximum caspase-3 activation in MIA PaCa-2 cells

A comprehensive comparison on ratiometric fluorescence signal change among EGFP-wt, EGFP-C3N, EGFP-C3B and EGFP-C3B incubated with caspase-3 inhibitor, Ac-DEVD-CHO, in both cell lines (HeLa and MIA PaCa-2 cells) was conducted, as shown in Figure 4.15. The ratiometric fluorescence signal change of EGFP-C3B in HeLa cells or MIA PaCa-2 cells following 1 μM STS induction at 45 mins exhibited a significant difference ($P < 0.05$), compared to the controls, EGFP-wt, EGFP-C3N or EGFP-C3B with inhibition. Moreover, caspase-3 activation using 1 μM STS induction in living MIA PaCa-2 cells exhibited a shorter half-life time, compared to living HeLa cells under identical stimulation conditions, which suggested a higher basal caspase-3 activity or higher potential for caspase-3 activation in MIA PaCa-2 cells. Hence, our designed caspase-3 sensor can be used for screening the basal caspase-3 activity or caspase-3 activation rate of different cell lines. Due the different behaviors exhibited by caspase-3 activation or inhibition in different cell lines, our EGFP-based caspase-3 sensors have the capability to detect enzymatic activation in living cells in real time to facilitate an understanding of the activation or inhibition mechanisms and pathways of apoptosis.

4.10 Fluorescence imaging of caspase-3 sensors with optimal cleavage linkers

Based on the analyses of the multiple cleavage sites in our trypsin sensors, the cleavage site on F-helix exhibits a fast enzymatic reaction possibly due to large exposure to the surface and good accessibility for proteases. In order to further confirm the recognition sites on F-helix
with high accessibility for proteases and provide a quick response to caspase-3, EGFP-C3C with a recognition sequence, VDEVDG, at F-helix, was designed and also transfected into HeLa and MIA PaCa-2 cells, respectively. The fluorescence signal of EGFP-C3C following 1 μM STS induction exhibited a significant increase when excited at 398 nm and an obvious decrease while excited at 488 nm, resulting in a larger relative signal change of 14 in HeLa cells (Figure 4.16), which revealed a similar pattern of fluorescence signal change as EGFP-C3B in HeLa cells, although relative signal changes of both EGFP-C3B and EGFP-C3C have nearly 4-fold difference. This result demonstrated that EGFP-C3C with recognition sites at an optimal grafting location, and with good accessibility, could provide fast enzymatic response under the lower basal caspase-3 activity in HeLa cells. In addition, the fluorescence signal of EGFP-C3C following 1 μM STS stimulation also exhibited a significant increase when excited at 398 nm and a significant decrease while excited at 488 nm, resulting in a larger relative signal change of 14 in MIA PaCa-2 cells (Figure 4.17). Compared to the relative fluorescence signal change of EGFP-C3B in MIA PaCa-2 cells, EGFP-C3C showed only 2-fold higher relative signal change and similar fluorescence signal change pattern in this cell line, which provided further confirmation of a fast response to caspase-3 due to optimal cleavage linker in EGFP-C3C and a higher potential caspase-3 activation or a higher basal caspase-3 activity in MIA PaCa-2 cells. Similarly, a slight shorter half-time for maximum caspase-3 activation using EGFP-C3C detection was also observed in MIA PaCa-2 cells (16.3 ± 5.9 min), compared to HeLa cells (18.3 ± 3.4 min), as shown in Figure 4.18. Moreover, based on fluorescence signal diffusion in different subcellular compartments, a strong fluorescence signal increase was first observed in cytoplasm following 1 μM STS stimulation, and then exhibited a dynamic decrease in the cytoplasm and a concurrent increase in the nucleus of the living cells (Figure 4.19). This
Figure 4. Inhibition of caspase-3 activation detected with EGFP-C3B in living cells. Caspase-3 inhibitor, Ac-DEVD-CHO at a final concentration of 20 μM, was incubated with HeLa cells transfected EGFP-C3B sensors for overnight. The representative images of HeLa cells transfected with EGFP-C3B, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after induction with 1 μM STS for 32 min. The relative fluorescence signal change at 510 nm has a similar pattern exhibiting a slight increase with excitation at 398 nm (C) and 488 nm (D) upon caspase-3 activation by STS induction at different time intervals. The arrows show the time of STS induction. The fluorescence ratiometric change (F_{398nm}/F_{488nm}) (E) at 510 nm of HeLa cells transfected with EGFP-C3B with incubation of caspase-3 inhibitor following 1 μM STS induction under excitation of both wavelengths revealed no change.
Figure 4.15. Shorter half-time for maximum caspase-3 activation in MIA PaCa-2 cells. Fluorescence ratiometric signal changes among EGFP-wt (○), EGFP-C3B with DEVD-CHO inhibition (◊), EGFP-C3N without cleavage sites (Δ), EGFP-C3B in HeLa cells (●) and EGFP-C3B in MIA PaCa-2 cells (■) following 1 μM STS induction were compared to reveal a significant shorter half-time for maximum caspase-3 activation in MIA PaCa-2 cells.
Figure 4. Fluorescence imaging and signal change of EGFP-C3C in HeLa cells following STS induction.

The representative images of HeLa cells transfected with EGFP-C3C, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after induction with 1 μM STS for 20 min. The relative fluorescence signal change at 510 nm exhibited a significant increase with excitation at 398 nm (C) and a slight increase with excitation at 488 nm (D) upon caspase-3 activation by STS induction at different time intervals. The arrows show the time of STS induction.
Figure 4. 17. Fluorescence imaging and signal change of EGFP-C3C in MIA PaCa-2 cells following STS induction.
Representative images of MIA PaCa-2 cells transfected with EGFP-C3C, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after induction with 1 μM STS for 20 min. The relative fluorescence signal change at 510 nm exhibited a significant increase with excitation at 398 nm (C) and a slight increase with excitation at 488 nm (D) upon caspase-3 activation by STS induction at different time intervals. The arrows show the time of STS induction.
Figure 4. Comparison of fluorescence ratiometric signal change of EGFP-C3C in different cell lines.

Fluorescence ratiometric signal change of EGFP-C3C was determined in HeLa cells (■) and MIA PaCa-2 cells (●) following 1 μM STS induction. A shorter half-time for maximum caspase-3 activation in MIA PaCa-2 cells (16.3 ± 5.9 min) was observed, compared to the half-time for maximum caspase-3 activation in HeLa cells (18.3 ± 3.4 min). The HeLa cells transfected with EGFP-C3C (♦) without STS induction exhibited no change in fluorescence ratiometric signal, suggesting good photostability of EGFP-C3C under excitation at both wavelengths.
Figure 4. 19. Caspase-3 activation in cytosol followed by migration from the cytosol to the nucleus.
HeLa cells transfected with EGFP-C3C sensor exhibited a strong fluorescence increase in the cytoplasm following 1 μM STS induction in real time and then a fluorescence increase in the nucleus was observed when excited at 398 nm. The subsequent migration demonstrated that caspase-3 activation was initiated in the cytoplasm and transferred to the nucleus of the cells.
fluorescence signal migration in the different subcellular compartments provided a clear demonstration that caspase-3 activation was initiated in the cytosol following STS induction and then the active caspase-3 migrated from cytosol to nucleus to accomplish DNA fragmentation and apoptosis, which is also in agreement with the previous reports of caspase-3 activation patterns in living cells (Kim, Chun et al. 2003; Ramuz, Isnardon et al. 2003). However, the migration mechanisms of activated caspase-3 between different subcellular compartments are still not clear.

Due to the significant fluorescence signal change when excited at both wavelengths, the photostability, as characterized by autofluorescence, photoactivation or photobleaching, is necessary to examine in living cells following 1 μM STS induction. Cell imaging of HeLa cells transfected with EGFP-C3C without STS treatment was acquired. The fluorescence signal resulting from excitation of both wavelengths exhibited similar trends without obvious fluorescence signal change or ratiometric fluorescence signal change, as shown in Figure 4.18. This result clearly indicated that the ratiometric fluorescence signal change resulted from cleavage of the EGFP-based caspase-3 sensors through the activated caspase-3 in living cells, rather than by photoactivation, autofluorescence or photobleaching.

4.11 Fluorescence imaging of caspase-8 sensors

Because caspase-3 is an effector caspase and can be activated by an initiator caspase such as caspase-8, we also designed a caspase-8 sensor, EGFP-C8A, using a similar grafting strategy with PCR amplification. The imaging of HeLa or MIA PaCa-2 cells transfected with EGFP-C8A was acquired following 1 μM STS induction, which showed a similar fluorescence ratiometric signal change pattern as EGFP-C3B and EGFP-C3C although a smaller relative signal change of
EGFP-C8A was observed in both HeLa and MIA PaCa-2 cells (Figure 4.20 and 4.21). During stimulation with 1 μM STS to activate caspase-3 or caspase-8, the half-time for maximum caspase-3 activation ranged between 15-30 min in both cell lines using EGFP-C3B or EGFP-C3C. However, the half-time for maximum caspase-8 activation using EGFP-C8A determination exhibited a little shorter and is approximately 15 min in living cells following stimulation with 1 μM STS. The longer half-time for maximum caspase-3 activation compared with maximum caspase-8 activation is possibly due to the downregulation of procaspase-3 cleavage by activated caspase-8 or caspase-9 in living cells. Therefore, the timescale or half-time for the maximum activation of initiator caspases and effector caspases exhibited minor differences. Although the timescale for caspase-3 or caspase-8 activation in living cells ranged from 1 to 2 hours based on previous reports, the timescale for caspase-3 or caspase-8 activation in both cell lines determined with our designed EGFP-based caspase sensors was less than one hour. From the comprehensive analyses, previously-reported timescales for caspase-3 or caspase-8 activation were determined with caspase recognition peptide substrate kits that detect population-dependent caspase activity in cell lysates, or with GFP-based FRET methods in living cells. Detectable signal change of the population-dependent caspase activity determination in cell lysates can be reduced due to effects from negative cells, weakly responsive cells or the loss of caspase activity during the cell lysis process.

Although EGFP-based FRET caspase probes avoid the limitations of population-dependent caspase activity determination and have been successfully applied to the real-time detection of caspase activation or inhibition in living cells, weak FRET efficiency from imbalanced fluorescence intensity, poor orientation, less optimal distance and wavelength cross-talk between the donor and the acceptor of those FRET-based caspase probes is still an obstacle
Figure 4. 20. Fluorescence imaging and signal change of EGFP-C8A in HeLa cells following STS induction.
Representative images of HeLa cells transfected with EGFP-C8A, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after induction with 1 μM STS for 20 min. The relative fluorescence signal change at 510 nm exhibited an increase with excitation at 398 nm (C) and a decrease with excitation at 488 nm (D) upon caspase-3 activation by STS induction at different time intervals. The arrows show the time of STS induction. The fluorescence ratiometric change (F_{398nm}/F_{488nm}) (E) at 510 nm for HeLa cells transfected with EGFP-C8A following 1 μM STS induction with excitation of both wavelengths revealed a relative signal change of 2.0.
Figure 4.21. Fluorescence imaging and signal change of EGFP-C8A MIA PaCa-2 cells following STS induction.

The representative images of MIA PaCa-2 cells transfected with EGFP-C8A, emitted at 510 nm with excitation of 398 nm (A) and 488 nm (B) before and after induction with 1 μM STS for 20 min. The relative fluorescence signal change at 510 nm showed an increase with excitation at 398 nm (C) and a decrease with excitation at 488 nm (D) upon caspase-3 activation by STS induction at different time intervals. The arrows show the time of STS induction. The fluorescence ratiometric change \( \frac{F_{398\text{nm}}}{F_{488\text{nm}}} \) (E) at 510 nm for HeLa cells transfected with EGFP-C8A following 1 μM STS induction with excitation of both wavelengths revealed a relative signal change of 4.2.
in living systems. Therefore, FRET-based caspase probes can not provide highly-sensitive detection signals or significant FRET signal change at the lower caspase-3 or caspase-8 activity level or lower basal caspase-3 or caspase-8 activity in different cell lines even when caspase-3 or caspase-8 is already activated. Moreover, detection of early stages of caspase-3 activation or apoptosis or the lower caspase-3 activity in living cells can be overlooked due to low FRET efficiency or low sensitivity of FRET-based caspase probes. This may increase the half-time value for maximum caspase activation using those probes. On the other hand, our designed caspase-3 or caspase-8 sensors are built in a single EGFP scaffold and can be cleaved under much lower caspase-3 or caspase-8 activity levels to reveal high sensitivity, which can provide a more accurate activation timescale during determination of caspase-3 or caspase-8 activity in living cells. Therefore, a shorter half-time for maximum caspase activation is exhibited using our designed EGFP-based caspase sensors than that determined with other methods or probes, such as population-dependent caspase determination in cell lysates or FRET-based caspase probes in living systems. Although caspase-3 was activated during the first 1 hour of induction with 1 μM STS, the HeLa or MIA PaCa-2 cells still retain no obvious morphology changes prior to apoptosis, like significant cell shrinkage, which also improved our determination of caspase-3 or caspase-8 activation in real time. Therefore, EGFP-C3B and EGFP-C3C are sensitive caspase-3 sensors, which can be used for real-time detection of caspase-3 activity in living cells and also can be used for detecting much lower caspase-3 activity in the early stages of apoptosis.
4.12 Another new pathway for caspase-8 activation in living cells

According to current investigations, caspase-8 activation is well characterized through the death receptor-mediated pathway during apoptosis. Caspase-8 activation through mitochondria-mediated pathway is not well documented. However, a fluorescence ratiometric signal change was detected in living cells transfected with caspase-8 sensor, EGFP-C8A, following stimulation with staurosporine, a well-studied caspase or apoptosis trigger in the mitochondria-mediated pathway. This important finding that caspase-8 can be activated not only through the death receptor-mediated pathway, but also through the mitochondria-mediated pathway was first demonstrated here. Compared to EGFP-C3B or EGFP-C3C in HeLa or MIA PaCa-2 cells, the relative signal change of EGFP-C8A is much smaller following stimulation with 1 μM STS. One possible reason for the smaller relative signal change of EGFP-C8A is that the mitochondria-mediated pathway for caspase-8 activation can not result in a high caspase-8 activity level in living cells so that the population-dependent caspase-8 determination method or FRET-based caspase-8 probes is not sensitive enough to produce a significant signal change under such lower caspase-8 activity conditions. As for the interactions between both pathways for members of the caspase family, the death receptor-mediated pathway functions to regulate the mitochondria-mediated pathway for the activation of caspases or apoptosis (Figure 4.1). For example, the active caspase-8 from the death receptor-mediated pathway can result in cleavage of regulator protein, Bid, followed by subsequent activation of Bad and Bax to regulate the mitochondria-mediated pathway for accomplishing caspase-9 and caspase-3 activation in the cascade system of caspase regulation and apoptosis. Since this new caspase-8 activation pathway is first described here, verification of this caspase-8 activation pathway and its mechanisms still require to be further investigated.
4.13 Confirmation of caspase-3 activation

In order to confirm caspase-3 activation, a fluorogenic caspase-3 substrate kit, Z-DEVD-AFC, was used to determine caspase-3 activity following 1 μM STS treatment in cell lysates. HeLa or MIA PaCa-2 cells treated with 1 μM STS were harvested at various time points (0, 30, 60, 120 min and 24 h) and lysed to obtain cell lysates. The total protein was extracted from the cell lysates. The mixture of fluorogenic caspase-3 substrate kit, Z-DEVD-AFC and cell lysate supernatant was incubated in a dark box at 37 °C for one hour, and then the fluorescence emission intensity at 508 nm was determined via fluorescence microplate reader at excitation of 380 nm. Because the fluorescent leaving group of Z-DEVD-AFC can be released from the substrate to emit strong fluorescence after cleavage by active caspase-3, the fluorescence intensity at 508 nm of excitation can be used to determine relative units of caspase-3 activity level in the cell lysates, which can be compared to commercially-available active caspase-3 with known concentration. Figure 4.22 showed that the relative fluorescence intensity of HeLa cell lysates following STS induction for 30 min was similar level with that of the standard active caspase-3 solution at 0.03 μg. The fluorescence intensity reached the highest level following STS induction for 120 min. This result indicated caspase-3 was already activated at a picromolar or nanomolar level in intact HeLa or MIA PaCa-2 cells. The caspase-3 in HeLa cells can be fully activated to reach a maximum level following STS induction for 2 hours, which provides strong evidence for confirming that the fluorescence signal change is due to the cleavage of our designed caspase-3 sensors by active caspase-3 in living cells. Moreover, the caspase-3 activation pattern in HeLa cells as determined through the caspase-3 fluorogenic substrate, Z-DEVD-AFC, is further verified by the half-time for maximum caspase-3 activation determined
Figure 4. 22. Verification of caspase-3 activation in living cells following STS induction. The determination of caspase-3 activity using Ac-DEVD-AFC exhibits an initial activation of caspase-3 at 30 min following 1 μM STS induction in HeLa (empty) and MIA PaCa-2 cells (striped). Compared to the positive control, purified caspase-3, a large amount of caspase-3 was activated in HeLa and MIA PaCa-2 cells at 30 min, and MIA PaCa-2 exhibits a high potential for caspase-3 activation following STS stimulation. With increasing induction time, caspase-3 activation increases after 2 h or overnight.
using our caspase-3 sensors in living cells. In addition, the relative fluorescence intensity of MIA PaCa-2 is 3-fold higher than that of HeLa cell lysates following stimulation with STS in the first hour, although similar fluorescence or caspase-3 activation pattern is observed in both cell lines. This result indicated the basal caspase-3 activity in MIA PaCa-2 cells is obviously higher than that of HeLa cells, which should also provide a better explanation for the observed larger relative signal change of our caspase-3 sensors in MIA PaCa-2 cells, which is approximately 3-4 fold higher than the relative signal change of our caspase-3 sensors detected in HeLa cells.

Another confirmation experiment was conducted using a cell permeable caspase-3 probe, DEVD-NucView488 caspase-3 substrate, in living cells. This caspase-3 substrate is composed of a caspase-3 substrate moiety and a dye that fluoresces upon DNA binding through the connection of a caspase-3 cleavable linker. In living cells, the caspase-3 substrate moiety can be cleaved to release the non-fluorescent dye from the conjugation if the caspase-3 is activated. When the non-fluorescent dye migrates to the cell nucleus to bind DNA, the fluorescence is activated as shown in Figure 4.23. Prior to cell imaging, DEVD-NucView488 caspase-3 substrate was incubated with the cultured HeLa cells at 37 °C for 45 min according to the manufacture’s instructions. During the HeLa cell imaging acquisition, 1 μM STS was used to activate caspase-3 in living cells. Fluorescence signal in the stimulated cells with STS induction was observed to reveal a slight gradual increase and become stronger and stronger from no fluorescence conditions (Figure 4.24A, 4.24B and 4.24C). A quick jump of fluorescence signal was observed following STS induction for 30 min (Figure 4.24D). This result also suggests caspase-3 can be activated through STS induction and caspase-3 is activated to the extent that cleavage of the the substrate conjugation occurs, which is also consistent with the timescale or half-time for maximum caspase-3 activation in living cells, determined using both our designed EGFP-based caspase-3
The DEVD-NucView488 caspase-3 substrate is composed of nonfluorescent DNA dye and caspase-3 substrate moiety. In living cell systems, when caspase-3 is activated in the cytoplasm of cells, the nonfluorescent DNA dye is released from the caspase-3 substrate due to cleavage followed by migration to the nucleus of cells. The fluorescence signal is quickly turned on when the DNA dye binds to DNA.
Caspase-3 activation was evaluated and confirmed using DEVD-NucView488 caspase-3 substrate in live HeLa cells following 1 μM STS induction in real time. HeLa cells (A) incubated with DEVD-NucView488 caspase-3 substrate exhibited no fluorescence before induction (B) with 1 μM STS stimulation under excitation of 488 nm in fluorescence microscopy, while the cells showed strong fluorescence following induction (C) with 1 μM STS for approximately 45 min. The relative fluorescence unit (RFU) change (D) was used as the indicator of caspase-3 activation in live HeLa cells in real time. A fluorescent on-off mode was observed following 1 μM STS induction, which mainly resulted from the binding of DNA, not from direct cleavage of the caspase-3 substrate.
sensors and the fluorogenic caspase-3 substrate kit. Similarly, for the co-incubation of DEVD-NucView488 caspase-3 substrate and caspase-3 inhibitor (Ac-DEVD-CHO) in the cultured living HeLa cells, the gradual increase or quick turn-on for fluorescence signal of DEVD-NucView488 caspase-3 substrate is not observed following 1 μM STS induction (Figure 4.25). Therefore, half-life timescale of less than 1 hour for caspase-3 activation is verified by another direct method in living cells, which provides a strong evidence that our designed EGFP-based caspase sensors could be used for real-time detection of caspase-3 activation in living cells at lower activity levels or dynamically imaging the early stages of apoptosis in living cells.

In addition, the half-time for maximum caspase-3 activation is 18.3 ± 3.4 min as detected by EGFP-C3C, which is shorter than 48.5 ± 11.2 min observed with the commercially available DEVD-NucView488 caspase-3 substrate detection under identical inducer concentrations in HeLa cells (Figure 4.26). Similarly, the half-time for maximum caspase-3 activation detected with EGFP-C3C (16.3 ± 5.9 min) in MIA PaCa-2 cells was also shorter, compared to the half-time for maximum caspase-3 activation detected by DEVD-NucView488 caspase-3 substrate (24.8 ± 6.2 min) in MIA PaCa-2 cells. These results were due to the direct monitoring of caspase-3 action in the cytosol by our EGFP-based caspase-3 sensor, rather than real detection of caspase-3 activity after its migration from the cytosol to the nuclei to accomplish DNA dye cleavage and DNA binding.

Moreover, Western blot of cell lysates following 1 μM STS stimulation was also exploited to test caspase-3 activation in HeLa cells and MIA PaCa-2 cells. The results indicated that caspase-3 in both cell lines following STS stimulation at various time intervals revealed a gradient increase in the amount of active caspase-3. Meanwhile, the two cell lines exhibited different capabilities to activate procaspase-3 based on the amount of cleaved caspase-3 band.
Figure 4. 25. Inhibition of caspase-3 was evaluated using DEVD-NucView488 caspase-3 substrate in living cells following STS induction. HeLa cells were incubated with 20 μM caspase-3 inhibitor, DEVD-CHO, overnight. DEVD-NucView488 caspase-3 substrate was added and incubated 45 min prior to cell imaging. The HeLa cells (A) showed no fluorescence signal before induction (B) or after induction (C) with 1 μM STS stimulation. The fluorescence intensity of randomly-selected cells was acquired using fluorescence microscope detection with a time course mode under excitation of 488 nm and exposure of 600 ms following 1 μM STS induction and no fluorescence on-off signal was observed (D).
Figure 4. 26. Comparison of half-time for maximum caspase-3 activation in living cells using EGFP-C3C and DEVD-NucView488 caspase-3 substrate. The half-time for maximum caspase-3 activation in HeLa cells determined using our designed EGFP-C3C sensor and DEVD-NucView488 caspase-3 substrate. EGFP-C3C with an optimal cleavage linker was observed to have a shorter half-time for maximum caspase-3 activation (18.3 ± 3.4 min) than DEVD-NucView488 caspase-3 substrate (48.5 ± 11.2 min) in HeLa cells following 1 μM STS stimulation.
following STS stimulation (Figure 4.27). The caspase-3 activation pattern is highly consistent with the detection of caspase-3 activation using our designed EGFP-based caspase-3 sensors as well as the caspase-3 fluorogenic substrate kit, Z-DEVD-AFC, and DEVD-NucView488 caspase-3 substrate.

Although the caspase-3 fluorogenic substrates, Z-DEVD-AFC, DEVD-NucView488 caspase-3 substrate, and Western blot technique can determine caspase-3 activity for tracking the caspase-3 activation pattern or processes in cells through cell lysates or intact living cells, several drawbacks of these methods were revealed. Z-DEVD-AFC only can be used to cell lysates for determining caspase-3 activity. Caspase-3 activity in cell lysates is not the real activity in living cells because the lysis conditions do not duplicate the environment of intact living cells. Additionally, caspase-3 activity can be damaged during cell lysis. Although cell-permeable caspase-3 substrates are available to determine caspase-3 activity in living cells in real time, problems exist with their cell-permeable efficiency, retention in intact cells and correct translocation. Through the comprehensive comparison between DEVD-NucView488 caspase-3 substrate and designed EGFP-caspase-3 sensors, limitations of the DEVD-NucView488 caspase-3 substrate were also identified. First, the determination of caspase-3 activity using this DEVD-NucView488 caspase-3 substrate does not directly monitor caspase-3 activity in living cells, because the fluorescence signal change is due to the binding between DNA and its dye, not due to the cleavage by caspase-3. Secondly, this substrate is used to detect caspase-3 activity in living cells in a single wavelength mode, which can not eliminate the effects from background or photobleaching. In addition, since no fluorescence in living cells incubated with that substrate was observed in the initial stage before caspase-3 activation, it is more difficult to set up the suitable exposure time for obtaining cell imaging and fluorescence signal change. Moreover, the
Figure 4.27. Caspase-3 activation in different cell lines detected using Western blot. Caspase-3 activation in the lysates of both HeLa and MIA PaCa-2 cells was detected by Western blot using anti-caspase-3 antibody. A faster caspase-3 activation and increased amount of the activated caspase-3 in MIA PaCa-2 cells following 1 μM STS induction were observed.
degradation of this caspase-3 substrate during storage will result in the cleavage of the cell permeable tag or the dye conjugated with it. If the cell permeable tag is removed due to degradation, it will reduce the cell permeability to produce a negative result due to lack of binding between the dye and DNA. On the other hand, if the dye conjugated on the substrate is removed due to degradation, it may move to the nucleus and bind to the DNA to produce fluorescence signal or false positive result before caspase-3 activation with STS stimulation. All of these limitations are successfully avoided by our genetic EGFP-based caspase sensors with the ratiometric measurement mode.

**4.14 Analysis of pH change following STS induction in living cells**

It is well-known that caspase-3 activation in living cells has two pathways: the mitochondria and ligand-binding pathways. Previous investigations have revealed the mitochondria alkalization or cytosolic acidification during apoptosis due to inducers such as UV light, STS and other inducers, through the mitochondria pathway. However, the accurate time points for mitochondria alkalization and cytosolic acidification during apoptosis, and the relationship between mitochondria alkalization and cytosolic acidification compared with the caspase-3 activation time schedule in various cell lines, are still not clear. In order to confirm whether the fluorescence signal change of EGFP-based caspase-3 sensors in HeLa cells or MIA PaCa-2 cells is due to the effect of pH change following 1 μM STS stimulation, the BCECF-AM pH probe was used to test pH change. The BCECF-AM pH probe has the characteristics of a pH-sensitive fluorescence signal change during excitation at 490 nm and pH-insensitive fluorescence signal change during excitation at 440 nm. Therefore, the ratio of fluorescence signal ($F_{490\text{nm}}/F_{440\text{nm}}$) of BCECF-AM pH probe should have a corresponding change following a pH
change due to the effect of inducers in living cells, rather than because of photobleaching or background effects. The results indicated that the ratio of fluorescence signal of BCECF-AM pH probe didn’t show obvious change in HeLa cells or MIA PaCa-2 cells following 1 μM STS stimulation, as shown in Figure 4.28. On the other hand, as a positive control, the ratio of fluorescence signal change in the cells with incubation of BCECF-AM pH probes exhibited a significant increase following the addition of 20 or 50 mM NH₄Cl and obvious decrease due to washing using fresh buffer for removing NH₄Cl, as shown in Figure 4.29. These results clearly demonstrated that the fluorescence signal change of our designed caspase-3 sensors in living cells is due to the cleavage of EGFP-based caspase sensors under the active caspase-3 action following STS stimulation, rather than the pH change effects from STS stimulation. In addition, these results also indicated that the caspase-3 activation in living cells could occur prior to mitochondria alkalization or cytosolic acidification during apoptosis.

4.15 Spectral signal change of caspase-3 sensor in living cells

In order to further confirm the caspase-3 activation and the fluorescence signal change due to cleavage of EGFP-based caspase-3 sensors by activated caspase-3 in living cells, fluorescence spectral signal change of EGFP-C3C expressed in cultured MIA PaCa-2 living cells was measured and evaluated via fluorescence spectrophotometer following the caspase-3 activation using 1 μM STS induction. The fluorescence spectral signal change of the cultured MIA PaCa-2 cells with transfection of EGFP-wt was also evaluated under the identical conditions in parallel as a control. When we conducted the excitation scanning based on the fluorescence emission wavelength of 508 nm and compared to the excitation spectra of EGFP-wt and EGFP-C3C in living cells, the maximum excitation wavelength of EGFP-wt occurs at 470 nm (Figure 4.30A),
pH change analysis was conducted using BCECF-AM pH probe in HeLa or MIA PaCa-2 cells following 1 μM STS induction. No obvious changes in ratiometric fluorescence signal (F_{490nm}/F_{440nm}) of the living cells incubated with BCECF-AM pH probe were observed following STS induction, suggesting that the caspase-3 inducer, 1 μM STS, didn’t result in significant pH change at the early stage of caspase-3 activation or apoptosis.
Figure 4. 29. pH change analysis in living cells following NH₄Cl stimulation. Analysis of pH change was conducted using BCECF-AM pH probe in HeLa or MIA PaCa-2 cells following NH₄Cl stimulation. A ratiometric fluorescence signal change (F₄₉₀nm/F₄₄₀nm) for the living cells incubated with BCECF-AM pH probe exhibited a significant increase following 20 mM or 50 mM NH₄Cl stimulation, and a significant decrease following the buffer washing, which indicated that BCECF-AM pH probe is a sensitive probe for detecting pH change in living cells.
Figure 4. 30. The excitation spectra of EGFP-based caspase-3 sensors in living cells. The EGFP-based caspase-3 sensor, EGFP-C3C, was transfected into MIA PaCa-2 cells. The excitation spectrum of EGFP-C3C cultured on the glass coverslip was scanned using the fluorescence spectrophotometer. EGFP-wt was used as the control under identical conditions. The maximum excitation peak of EGFP-wt (A) occurred at 470 nm and the excitation peaks of EGFP-C3C (B) were observed at 470 nm as a major peak and 398 nm as a minor peak at the emission of 508 nm.
while EGFP-C3C has an observed main peak at 470 nm and a minor peak at 398 nm (Figure 4.30B). The maximum excitation wavelengths of both EGFP-wt and EGFP-C3C are blue-shifted, compared to their maximum excitation peak at 490 nm in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4) or caspase-3 reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 10% Sucrose, pH 7.5). Similarly, the fluorescence emission spectra of EGFP-wt and EGFP-C3C in living cells were also scanned under both 398 and 470 nm excitation, which shows the maximum emission peaks of EGFP-based caspase-3 sensor, EGFP-C3C and EGFP-wt at 508 nm under the both excitation wavelengths.

In order to track the fluorescence signal change and understand the fluorescence signal change pattern of EGFP-based caspase-3 sensors in living cells, fluorescence emission spectra scanning of EGFP-C3C in transfected MIA PaCa-2 cells were acquired using a fluorescence spectrophotometer, with excitations of both 398 and 470 nm. The fluorescence spectral signal changes of EGFP-C3C with both wavelengths of excitation were obtained and analyzed at various time intervals (0, 5, 10, 20, 30, 45, 60 min) following 1 μM STS induction to activate caspase-3 in living cells (Figure 4.31A and 4.31B). The results indicated that maximum fluorescence emission at 508 nm of EGFP-C3C revealed slight increase by 16% with excitation of 398 nm (Figure 4.31C) and simultaneously a slight decrease by 12% with excitation of 470 nm (Figure 4.31D) corresponding to the increasing time of STS induction. In contrast, EGFP-wt, used as the control under identical conditions, revealed no signal change in maximum fluorescence emission under excitation of the two wavelengths following STS stimulation at the various time intervals, as shown in Figure 4.32A and 4.32B. Therefore, the relative fluorescence emission intensity of EGFP-wt with excitation at both 398 and 470 nm was also not observed in intact MIA PaCa-2 cells following STS stimulation, as shown in Figure 4.32C and 4.32D. When
Fluorescence emission spectrum scanning of EGFP-C3C in MIA PaCa-2 cells following 1 μM STS induction. Fluorescence emission spectra of EGFP-C3C at 508 nm in MIA PaCa-2 cells following 1 μM STS induction exhibited a ratiometric change. The fluorescence intensity of EGFP-C3C at 508 nm exhibited a gradual increase with excitation of 398 nm (A) and a corresponding decrease with excitation of 490 nm (B) following 1 μM STS induction at various time intervals, 0 (●), 10 (■), 30 (♦), and 60 min (▲). More than 16% increase of the maximum fluorescence signal change was observed under the excitation of 398 nm (C) and approximately 10% decrease of the maximum fluorescence signal change was observed under the excitation of 490 nm (D).
Figure 4. Fluorescence emission spectrum scanning of EGFP-wt in MIA PaCa-2 cells following 1 μM STS induction.

The fluorescence intensity of EGFP-wt at 508 nm exhibited no change with excitation at either 398 nm (A) or 490 nm (B) following 1 μM STS induction at various time points, 0 (●), 10 (■), 30 (♦), and 60 min (▲). The relative fluorescence signal change showed a flat line in various time at both 398nm (C) and 490 nm (D) of excitation.
Figure 4.33. Ratiometric fluorescence signal change in maximum fluorescence emission. Fluorescence ratiometric change increased by more than 30% in MIA PaCa-2 cells with transfection of EGFP-C3B (■) following 1 μM STS induction at various times; however, no fluorescence ratiometric change was observed in MIA PaCa-2 cells with transfection of the control, EGFP-wt (●).
we evaluated fluorescence signal change in ratiometric measurement expressed in $F_{398nm}/F_{488nm}$, the fluorescence ratiometric signal change reached 30% for EGFP-based caspase-3 sensor, EGFP-C3C, but no change for the control, EGFP-wt (Figure 4.33). These results obtained through fluorescence spectrophotometer acquisition were consistent with those of the fluorescence signal changes of EGFP-based caspase-3 sensors in living cells measured by fluorescence microscopy following STS induction, which showed similar fluorescence signal change patterns and half-life times for caspase-3 activation in living cells. However, the fluorescence signal change, or relative signal change of caspase-3 sensor in living cells acquired with the fluorescence spectrophotometer is much smaller than the relative signal change acquired with the fluorescence microscope. This may be due to that spectrophotometer monitors the behavior of large population of cells including EGFP transfected and no EGFP transfected while fluorescence microscope monitors the performance of the individual cell with EGFP transfected. During the acquisition with fluorescence microscope, the fluorescence signal change following STS induction mainly resulted from the single green cell with EGFP transfected and positive reaction. Conversely, the fluorescence signal acquired using the fluorescence spectrophotometer was produced by the total population of cells with various behavior, which resulted in the smaller fluorescence signal change and smaller relative signal change due to both positive and negative response in living cells. Although the opposite trend in fluorescence signal change between the studies in vitro and in living cells was observed, the fluorescence signal change acquired with both methods in living cells following STS induction exhibited similar trends and half-life timescales, which suggested that the developed EGFP-based caspase-3 sensors are able to monitor caspase-3 activation or inhibition in real time in living cells and can be applied to understand mechanisms or pathways of apoptosis and diseases related to apoptosis.
4.16 Caspase-3 activation using different inducers

Due to the two pathways for caspase-3 activation in living cells, the mitochondrion-mediated pathway and the death-receptor-mediated pathway, different caspase-3 activation triggers can be used through different pathways and detected using our designed EGFP-based caspase-3 sensors. It is well characterized that staurosporine is a commonly-used and fast caspase-3 activation trigger in the mitochondria pathway. The caspase-3 activation and inhibition in living cells by STS is also successfully detected via our developed caspase-3 sensors. On the other hand, we also used the trigger, TNFα, for the death-receptor mediated pathway to stimulate the caspase-3 activation and detected the optical signal change following caspase-3 activation in living cells using our developed caspase-3 sensors. TNFα with a final concentration of 10 ng/ml was used to activate caspase-3 in MIA PaCa-2 and HeLa cells. The MIA PaCa-2 cells transfected with EGFP-C3C sensor were observed to exhibit a slight ratiometric fluorescence signal increase following 10 ng/ml TNFα stimulation for 2 hrs (Figure 4.34A) and a larger ratiometric fluorescence signal increase after TNFα stimulation for 4 hrs (Figure 4.34B) with a relative signal change of 2.5. In contrast, in the HeLa cells transfected with EGFP-C3C sensor, no observed fluorescence signal changes occurred during 2 hrs induction with the same TNFα concentration (Figure 4.34C) and only a slight fluorescence signal increase was observed following TNFα stimulation for 10 hrs (Figure 4.34D) with a relative signal change of 1.2, or a 20% ratiometric fluorescence increase. These results provide further confirmation that the higher potential of caspase-3 activation was exhibited in MIA PaCa-2 cells. However, compared with caspase-3 activation using STS, TNFα has an obvious slow rate and requires longer time for caspase-3 activation in living cells. When comparing caspase-3 activation in both cell lines, the
Figure 4. Fluorescence signal change of cells transfected with EGFP-C3C following TNFα stimulation.

Fluorescence ratiometric signal in MIA PaCa-2 cells transfected with EGFP-C3C following induction with 10 ng/ml TNFα exhibited no obvious change during the first 2 h stimulation (A) and a gradient increase after 4 h stimulation (B) to achieve a relative signal change of 2.5 following TNFα stimulation for 10 h. Similarly, fluorescence ratiometric signal in HeLa cells transfected with EGFP-C3C following induction with 10 ng/ml TNFα exhibited no obvious change during the first 2 h stimulation (C) and a gradient increase after 4 h stimulation (D) to achieve a relative signal change of 1.3 following TNFα stimulation for 10 h.
caspase-3 activity in cell lysates of both cell lines was also determined using the fluorogenic caspase-3 substrate, Ac-DEVD-AFC. No active caspase-3 was detected in the cell lysates of HeLa cells following TNFα induction, and the initiation of caspase-3 activation in MIA PaCa-2 cells was detected with TNF-α stimulation after 2 hrs (Figure 4.35), which is in good agreement with the results obtained using our developed caspase-3 sensor in living cells in real time. On the other hand, the results showing no activated caspase-3 in HeLa cells and a lower level of caspase-3 activation in MIA PaCa-2 cells following TNF-α stimulation may be due to this trigger with a slow response for caspase-3 activation in living cells so that it needs the longer time for caspase-3 activation in many cells lines. According to newly-reported investigation, TNF-α alone exhibited less potential for apoptosis in living cells (Wang, Du et al. 2008). However, TNFα combined with either a protein synthesis inhibitor, cycloheximine, or Smac mimetic, a small molecule mimic of Smac/Diablo protein, can significantly shorten the timescale of apoptosis or increase caspase-3 and caspase-8 activity in a short time period (Wang, Du et al. 2008). Therefore, the cotreatment with both TNFα and protein synthesis inhibitors should accelerate the activation of caspase-3 or caspase-8, and improve the relative signal change of our designed EGFP-based caspase sensors in living cells. All these possibilities will be investigated using fluorescence imaging in parallel with Western blot.

Previous investigations reported that tributyltin (TBT) is a faster caspase-3 activation trigger and can result in caspase-3 activation at approximately 30 min (Stridh, Kimland et al. 1998; Nopp, Lundahl et al. 2002; Jurkiewicz, Averill-Bates et al. 2004; Nakatsu, Kotake et al. 2007) without pH change prior to caspase-3 activation following induction. The HeLa cells transfected with EGFP-C3C sensor were induced with TBT, and the ratiometric fluorescence signal change exhibited a concentration-dependent mode following TBT stimulation. The
Figure 4. 35. Caspase-3 activity determination in HeLa and MIA PaCa-2 cells following 10 ng/ml TNFα induction.

The caspase-3 activity determination in cell lysates using Ac-DEVD-AFC exhibits an initial activation of caspase-3 at 120 min following 10 ng/ml TNFα induction in MIA PaCa-2 (purple). Compared to the positive control, active caspase-3, 15 nM caspase-3 was activated in MIA PaCa-2 cells at 240 min. However, no caspase-3 activation was determined in lysates of HeLa cells (cyan) following 10 ng/ml TNFα induction at various time intervals. Therefore, further confirmation was obtained that MIA PaCa-2 exhibits a high potential for caspase-3 activation.
ratiometric fluorescence signal exhibited a gradient increase and reached the highest-level as a relative signal change of approximately 1.3 following induction of 2 μM TBT for 60 min in HeLa cells (Figure 4.36A). However, the ratiometric fluorescence signal exhibited a rapid increase to reach the peak within 30 min following 2 mM TBT stimulation in an identical cell line with a relative signal change of 1.8 (Figure 4.36B). These results demonstrate that TBT can be used to activate caspase-3 in living cells during a short time period in a concentration-dependent mode, which is consistent with previous reports.

Although artemisinin was first used as a drug for malaria, currently it is also reported to have the potential to induce apoptosis in different cell lines and is used as an anticancer drug. Since designed EGFP-based caspase-3 sensors have the capability to detect caspase-3 activation or inhibition in living cells, caspase-3 activation status was evaluated using the designed caspase-3 sensor, EGFP-C3C, in DU-145 cells following induction of dihydroartemisinin (DHA). The ratiometric fluorescence signal change of DU-145 cells transfected with EGFP-C3C exhibited a slight increase with a relative signal change of less than 1.1 during real-time fluorescence acquisition following stimulation with 100 μM DHA (Figure 4.37A). However, compared to the control, a 5% ratiometric fluorescence signal change in DU-145 cells transfected with EGFP-wt following identical stimulation was observed (Figure 4.37B). The ratiometric fluorescence signal change due to stimulation of 100 μM DHA was still in the range of less 15%. The small signal change is possibly due to less optimal concentrations of this inducer or trace amount of caspase-3 activation in the early stage of apoptosis at this concentration condition, which still needs to be further evaluated by Western blot or other methods. In addition, since this inducer functions to down regulate transferrin receptor (TfR) expression through the mitochondria pathway, another possible reason is that ferrous is necessary for accomplishing down regulation and apoptosis.
Figure 4. Fluorescence signal change of HeLa cells transfected with EGFP-C3C following TBT stimulation.

Fluorescence ratiometric signal HeLa cells transfected with EGFP-C3C following 2 μM TBT induction exhibited a slight increase with a relative signal change of 1.3 during 2 h stimulation (A) and a fast and enhanced increase following 2 mM TBT induction during the first 1 h (B) to achieve a relative signal change of 1.7, which indicates that caspase-3 activation in living cells is a concentration-dependent inducer. The higher concentration of TBT resulted in shorter half-time for maximum caspase-3 activation (15 min).
Figure 4. Fluorescence signal change of DU-145 cells transfected with EGFP-C3C following DHA stimulation.

Fluorescence ratiometric signal in DU-145 cells transfected EGFP-C3C following 100 μM DHA induction exhibited a slight increase with a relative signal change of 1.1 during 90 min stimulation (A). However, fluorescence ratiometric signal in DU-145 cells transfected EGFP-wt following identical stimulation exhibited no obvious change (B).
4.17 Inconsistent spectral signal change between living cells and in vitro following caspase-3 action

It is so exciting that the developed EGFP-based caspase-3 sensors such as EGFP-C3B and EGFP-C3C exhibited high sensitivity and excellent specificity to monitor caspase-3 activation or inhibition under the induction with STS, TBT, TNFα and DHA in living cell systems. The ratiometric fluorescence signal changes of EGFP-based caspase-3 sensors at excitation of 398 nm and 488 nm provide the enhancement of relative signal response to track the low concentration level of caspase-3 at the early stage of apoptosis in different cell lines, and avoided the problems associated with poor fluorescence intensity, photobleaching and background interference using current commercially-available caspase-3 substrates conjugated with chromogenic or fluorogenic dyes with single wavelength detection mode. Moreover, the developed EGFP-based caspase-3 sensors revealed comparable or better kinetic parameters (smaller \( K_m \) and larger \( k_{cat}/K_m \) values) than those of other caspase-3 substrates.

Unfortunately, although all of above characterizations of the developed EGFP-based caspase-3 sensors will offer tremendous potential to the sensitive detection of caspase-3 activity in living systems in real time, contradict results obtaining in various buffer conditions in vitro, which exhibited fluorescence signal increase at 398 nm excitation and concurrent decrease at 488 nm excitation upon the caspase-3 digestion of the developed EGFP-based caspase-3 sensors in buffer conditions in vitro.

As shown in Figure 4.8, 15 μM EGFP-C3B buffered in caspase-3 reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.4) exhibited a decrease in absorbance at 398 nm and an increase in absorbance at 490 nm following
caspase-3 digestion at the final concentration of 31.25 nM. Similarly, the fluorescence emission at 508 nm exhibited a decrease when excited at 398 nm and an increase when excited at 490 nm. Based on the previous reports, neither 10% sucrose nor 10% glycerol appear to produce any difference in caspase-3 activity in their buffer systems. In order to understand the effect on cleavage of EGFP-C3B from 10% glycerol in the buffer system, another caspase-3 reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Glycerol, pH 7.4) was applied to the caspase-3 digestion of EGFP-C3B. These results exhibited a signal change pattern identical to caspase-3 digestion in the buffer system containing 10% sucrose (Figure 4.38). On the other hand, various compositions in 7 different buffer conditions were investigated to confirm the effects on the absorbance or fluorescence signal change pattern of EGFP-based caspase-3 sensors upon the caspase-3 digestion and still exhibited the similar signal change pattern.

As already discussed in the Section 4.4 of this chapter, the compositions of CHAPS, glycerol or sucrose, EDTA and NaCl play the critical role for caspase-3 activation in buffer system in vitro. A hypothesis is that these inconsistent results may be due to the different environments for caspases activation or conformation changes of the developed EGFP-based caspase-3 sensors between buffer conditions in vitro compared to the cellular environment in living cells. In order to evaluate the conformation change of caspase-3 sensors, EGFP-C3B and EGFP-C3C in the optimal caspase-3 reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, pH 7.5), CD spectroscopy scanning was performed to observe any conformation changes compared to caspase-3 sensors in 10 mM Tris buffer condition (Figure 4.39). Based on the analysis of CD spectroscopy, the necessary components for caspase-3 activation in the reaction buffer system, CHAPS, glycerol and sucrose
Figure 4.38. Spectral signal change of EGFP-C3B under the caspase-3 reaction buffer containing 10% glycerol following caspase-3 digestion. The maximum absorption (A) of EGFP-C3B exhibited a decrease at 398 nm and an increase at 490 nm following caspase-3 digestion in the caspase-3 reaction buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.1% CHAPS, 10% Glycerol, pH 7.4). The maximum fluorescence emission at 508 nm exhibited a decrease when excited at 398 nm (B) and an increase when excited at 490 nm (C) after caspase-3 cleavage. The spectra of EGFP-C3B before and after caspase-3 digestion are in solid and dashed lines, respectively.
Figure 4. Conformational change analysis in various caspase-3 reaction buffers using CD spectroscopy.

The secondary structures of caspase-3 sensor, EGFP-C3B, buffered in B0 (10 mM Tris, 1 mM DTT, pH 7.4) (○), B1 (□), B2 (◊), B3 (●), B4 (■), B5 (♦), B6 (▲), and B7 (▼) were monitored using CD spectroscopy. The conformation exhibited significant changes in buffers B1, B2, B3, B4, B5, B6, with 0.1% CHAPS or 10% glycerol.
possibly result in the change of the secondary structure of our EGFP-based caspase-3 sensors, which is the most probable reason for the observed opposite spectral behavior in living cell or in vitro studies. In addition, it is also possible that caspases have oligomeric states in vitro or in vivo, which requires a detailed investigation if large amount of enzymes are available.

Since the inconsistent spectral signal response directions of EGFP-based caspase-3 sensors were exhibited between the buffer system in vitro and in living cells following caspase-3 digestion and caspase-3 activation via inducters, and the reasons resulting in the inconsistent spectral change in both systems are still not clear, other factors for caspase-3 activation or avoiding the conformational change of the developed EGFP-based caspase-3 sensors will need to be further systematically investigated. This is still a big hamper to use the developed EGFP-based caspase-3 sensor for the simultaneous detection or monitoring of caspase-3 activity or activation both in vitro and in living cells. According to current status of progress, the developed EGFP-based caspase-3 sensors could be separately used to detect caspase-3 activity in vitro and caspase-3 activation or inhibition in living cells under the corresponding optimal conditions.

4.18 Conclusions and significance

Based on the comprehensive analyses of caspase-3 and caspase-8 sensor variants, the novel strategy for protease sensor development by grafting an enzymatic cleavage linker on an EGFP scaffold at the sensitive location 172 for chromophore spectral signal change is further confirmed and extended to the widely applications. Compared to current mimic peptide-based chromogenic or fluorescent caspase probes, DEVD-NucView488 caspase-3 substrate, and GFP-based FRET caspase sensors, our developed caspase-3 and caspase-8 sensors with ratiometric measurement manner exhibited strong advantages in sensitivity, relative signal change range and
specificity. The significant advance for dynamically rapid response to caspase activation or inhibition in living cells is also good at quantitatively determining the lower casapase activity in living cells due to the high sensitivity of our designed caspase-3 sensors. Moreover, because of the high sensitivity of our designed caspase sensors, the half-life times for caspase-3 or caspase-8 activation in individual living cells determined with our noninvasive caspase-3 or caspase-8 sensors are significantly shorter than those determined with current population-dependent cell lysate-based detections, FRET-based caspase probes and DEVD-NucView488 caspase-3 substrate, which will greatly enhance dynamic imaging for early stages of caspase activation or apoptosis, and improve our understanding of the mechanisms of caspase activation and the apoptosis regulation pathways. Furthermore, our sensitive caspase sensors will provide a good opportunity for high-throughput screening caspase activators and caspase inhibitors, and for validating the target or new drugs related to caspase activity or apoptosis in various cell lines and transgenic animals.
Chapter 5  EGFP-based thrombin sensors

5.1  Introduction

5.1.1  Biological function of thrombin

Thrombin, one of the most important members in serine protease family, plays a crucial role in a wide range of physiological and pathological biological processes, which is involved in homeostasis, thrombosis, tumor invasion, cell differentiation, angiogenesis, tissue injury, platelet aggregation and blood coagulation. Its proteolysis activity is highly related to these physiological and pathological processes, and responsible for the conversion of fibrinogen to fibrin and the activation of blood coagulation and plasma factors such as factor V, factor VIII, factor XIII, and protein C. Thrombin has been widely investigated due to its multiple functions since it was discovered, named and sequenced (Goldsack, Chambers et al. 1998). Thrombin is generated from its precursor prothrombin after tissue injury and involved in converting fibrinogen to fibrin during clotting cascade and mediating cellular functions through proteolytically activating protease activated receptors (PAR1, PAR3 and PAR4) on the cell surface (Coughlin 1999; Anderluh and Dolenc 2002). Following the activation of these receptors, numerous cellular effects such as chemotaxis, proliferation, extracellular matrix turnover and release of cytokines are initiated. Therefore, a series of tissue repair processes, the inflammatory pathogenesis and fibroproliferative disorder including pulmonary fibrosis and atherosclerosis are related to these receptor activations. On the other hand, many research reports have revealed that some neurodegenerative diseases such as Alzheimer’s disease, and a number of tumors due to fibrin formation and deposition, could result from the disorder of thrombin activation. Due to these multiple functions of thrombin, physiological and pathological mechanisms of some diseases
related to thrombin, factors or pathways of thrombin activation and its receptors on the cell surface, determination of thrombin activity in living cells or in vivo, investigation of thrombin triggers or inhibitors, development of agonists and antagonists for thrombin receptors are involved in extensive investigation and become a current heated medical field. In order to probe these physiological or pathological processes, thrombin activity determination is highly necessary.

5.1.2 Thrombin structure and active sites

Thrombin, a trypsin-like member of the chymotrypsin family of serine proteases, reveals a similar structure and catalytic mechanism with trypsin. Two polypeptide chains, 36-residue A-chain and 259-residue B-chain, form the basic frame of thrombin through a covalent linkage of a disulfide bridge, as shown in Figure 5.1. This basic structure of thrombin was first demonstrated by the solution of crystallographic structure of thrombin (Bode, Mayr et al. 1989; Bode, Turk et al. 1992). Both chains are organized in two adjacent β-barrels and the functional moiety, catalytic domains of thrombin are located in B-chain of thrombin (Lesk and Fordham 1996). The active site cleft is provided by Ser195, His57 and Asp102 between the junction of both chains. In addition, the large insertion loops shape a deep and narrow, canyon-like active site cleft, which offers the narrow specificity of thrombin (Huntington 2005; Bode 2006). Another particular characteristic of thrombin molecule is its uneven charge distribution on the thrombin surface, which results in high positive and negative electrostatic field strength. Hence, two positively-charged sites, Exosite I and Exosite II, located to the right and left of the thrombin active site cleft, respectively, are responsible for functions of a direct and specific substrate or inhibitor
Figure 5.1. The model structure of thrombin (PDB, 2UUF). The residues of catalytic triad, His^57^, Asp^102^ and Ser^195^, are shown in red, blue and purple color at the cleft region between the two β-barrel domains. The Exosite I and Exosite II are located to both sides of active site of thrombin. The Na^+_\textsuperscript{+} is shown in cyan color and bound to the Na^+_\textsuperscript{-}-binding site.
interaction. Meanwhile, a negative pocket, Na\textsuperscript{+}-binding site has been found to be an important allosteric modulator of thrombin (Di Cera, Dang et al. 1997).

### 5.1.3 Thrombin substrate specificity

A wealth of structural information now is available for thrombin, which is greatly benefited for the rational design of its substrates and inhibitors. Although an unusually deep and narrow active site cleft provides one of the primary causes for the narrow specificity of thrombin, the observed modularity of thrombin allows diversity in this specificity; its "mix-and-match" nature is exemplified by its interactions with macromolecules. Therefore, extensive investigations have been devoted to probe the specificity of thrombin for a sensitive and specific thrombin detection, or a novel and effective thrombin inhibitor development.

First, thrombin specificity was often investigated using chromogenic or fluorogenic peptide substrates. Based on previous literature reports, the substrate P\textsubscript{1} residue should be in agreement with the known primary specificity of thrombin for basic residues, and the P\textsubscript{1} position always prefer to positively-charged residue Arg. Analysis of a synthetic substrate library has indicated that thrombin has a strong preference for aliphatic amino acids at the P\textsubscript{4} position, little preference at P\textsubscript{3}, and strict preference for proline at the P\textsubscript{2} position. The kinetic parameters, $k_{cat}/K_m$, of these substrates conjugated to preferable amino acid residues and dyes, are ranged from $10^3$ to $10^7$ M\textsuperscript{-1}S\textsuperscript{-1} (Sadasivan and Yee 2000). Second, analysis of natural protein substrates such as PAR\textsubscript{1}, PAR\textsubscript{4}, Factor XIII, Factor VIII, and prothrombin, demonstrate highly-conserved ligand preference for proline, arginine and glycine or serine at the P\textsubscript{2}, P\textsubscript{1} and P\textsubscript{1}’ position (Ayala, Cantwell et al. 2001; Bode 2006). Therefore, the substrates with arginine in the P\textsubscript{1} position, proline or a proline homolog in the P\textsubscript{2} position, and an apolar amino acid in the P\textsubscript{3} position were
identified to have better specificity for thrombin (Harris, Backes et al. 2000). However, limited reports are available for protein-based substrate specificity, especially for their kinetic properties.

### 5.1.4 Current methods for detection of thrombin

The most common thrombin determination is still focused on blood or cell lysate assays using chromogenic or fluorogenic small molecule dyes conjugated to a short peptide to monitor fixed cell imaging (Tung, Gerszten et al. 2002). The most common chromophores linked to short peptide fragments of 3-6 amino acids mimic the sequence encompassing the P$_1$ to P$_n$ cleavage sites (usually up to P$_3$) are p-nitroaniline, 7-amino-4-methylcoumarin and fluorescein isothiocyanate-labeled casein. These probes are not ideal for the continuous dynamic imaging of enzyme actions due to limited lifetime, specificity and stability resulting from a lack of defined structure in solution due to their short sequences. Moreover, P$_1$’ to P$_3$’ sequence is still difficult to be optimized in these peptide probes and fast degradation of these short peptides also hinder the delivery to specific sub-cellular locations. Fluorescent proteins with self-encoded chromophores are capable of monitoring numerous cellular events in living cells or organisms via live cell imaging. A GFP-pair probe has been developed to monitor thrombin activity through the change of fluorescence resonance energy transfer to facilitate screening of inhibitors (Zhang 2004). However, no detailed reports about substrate specificity beyond P$_4$ site. DNA aptamer or nano-particle probes for thrombin activity determination are also reported with high sensitivity (Xiao, Lubin et al. 2005). However, their determination mechanism is indirect, which depends on the binding ability from negative charged DNA and positive charged probes with complication of non-specific electrostatic interaction. Therefore, there is a strong need to have thrombin sensors that allow us to better probe substrate specificity.
5.1.5 Significance of this study

In this chapter, we have successfully designed a series of thrombin sensors for the determination of thrombin activity in vitro using our grafting approach. Their optical change upon the action of thrombin was systematically investigated and steady state kinetic studies were conducted to obtain their kinetic parameters, Michaelis constants ($K_m$), turnover numbers ($k_{cat}$) and specificity constants ($k_{cat}/K_m$) for optimizing the cleavage linker in our designed thrombin sensors. The thrombin sensor development can further confirm our hypothesis of novel strategy of protease sensor and extend its application to various proteases. Moreover, the substrate specificity of thrombin can be investigated using our developed thrombin sensors.

5.2 Construction of thrombin sensors

In order to develop sensitive thrombin sensors with optimal cleavage linkers for thrombin digestion for in vitro studies and in living cell imaging, a series of specific cleavage linkers for thrombin were designed and inserted at the position 172 in EGFP to obtain different variants of thrombin sensors. A ratiometric signal change in absorbance or fluorescence was exhibited upon the cleavage of the thrombin linker. Based on previous design of sensitive EGFP-based trypsin sensors, caspase-3 and caspase-8 sensors, novel thrombin sensors are constructed through the insertion of a thrombin cleavage site based on calmodulin EF-hand motif at the position Glu172 of EGFP due to the high flexibility of the EF-hand, which can improve the accessibility of cleavage site for thrombin. On the other hand, amino acid residues from P$_1$ to P$_4$ and P$_1'$ to P$_2'$ were designed to examine their specificity to thrombin and compare the exposure degree of cleavage site for testing thrombin accessibility. In order to design the amino acid residues from
P₁ to P₄ and P₁’ to P₂ for improving substrate specificity, five cleavage linkers with residues, Gly-Arg-Gly, Phe-Asn-Pro-Arg-Gly-Phe, Phe-Thr-Pro-Arg-Gly-Phe, Phe-Asn-Pro-Arg-Ser-Phe and Phe-Thr-Pro-Arg-Gly-Phe, are based on the sequences of thrombin activation receptors (PAR1, PAR3 and PAR4) and some natural thrombin cleavable protein substrates. Different variants of thrombin sensor and their cleavage linkers for thrombin were shown in Table 5.1. The EGFP plasmid encoding the sequence of thrombin cleavage linkers was amplified through PCR for obtaining corresponding EGFP-based thrombin sensors.

Table 5.1. Designed thrombin sensors and their cleavage linkers

<table>
<thead>
<tr>
<th>Thrombin sensor name</th>
<th>The cleavage linker sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-Th1</td>
<td>EEEIREAFRVFDKDGRYISAELRHVMTNL</td>
</tr>
<tr>
<td>EGFP-Th2</td>
<td>EEEIREAFRVFDKDGNYISAFNPRGFMTNL</td>
</tr>
<tr>
<td>EGFP-Th3</td>
<td>EEEIREAFRVFDKDGNYISAFNPRSFMTNL</td>
</tr>
<tr>
<td>EGFP-Th4</td>
<td>EEEIREAFRVFDKDGNYISAFTPRGFMNTL</td>
</tr>
<tr>
<td>EGFP-Th5</td>
<td>EEEIREAFRVFDKDGNYISAFTPRSFMNTL</td>
</tr>
</tbody>
</table>

5.3 Optical properties of thrombin sensors

All variants of our designed EGFP-based thrombin sensors are successfully expressed in *E. coli* and exhibit strong fluorescence. Through nickel affinity column purification, purified EGFP-based thrombin sensors exhibit two strong absorption peaks, which are at 397 and 490 nm. Two absorption peaks are resulted from both states of chromophore, ionic form and neutral form. However, different variants of these EGFP-based thrombin sensors have various proportions of ionic form and neutral form chromophore due to insertion of various cleavage
linkers for thrombin. Due the cleavage sites in different locations between EGFP-Th1, EGFP-Th2 and EGFP-Th3, EGFP-Th1 has the slight more proportion of ionic chromophore form. EGFP-Th2 and EGFP-Th3 has no obvious difference due to cleavage site in the same location although the residue in the P₂’ position.

5. 4 Optical change of thrombin sensors

To detect optical signal change upon the action of thrombin cleavage, the UV-visible absorption spectra of EGFP-Th2 in thrombin reaction buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0) before and after thrombin cleavage. Following thrombin cleavage, the maximum absorption of EGFP-Th2 at 490 nm increased by 30.9% and concurrently decreased by 21.3% at 398 nm (Figure 5.2). Similarly, EGFP-Th2 exhibited a ratiometric fluorescence change by a 25.74% decrease in maximum fluorescence emission intensity at 508 nm at an excitation wavelength of 398 nm (Figure 5.3A) and a corresponding 23.63% increase at an excitation wavelength of 490 nm (Figure 5.3B).

5. 5 Verification of thrombin sensors cleavage

To verify the cleavage site of EGFP-based thrombin sensors, digestion reaction of 10 μM EGFP-Th1 and EGFP-Th2 was performed by adding stock solution of thrombin (GE Healthcare) up to a final concentration of 30 nM in thrombin reaction buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0). The resulting cleavage products were examined using SDS-PAGE and MALDI mass spectrometry. EGFP-based thrombin sensors are specifically cleaved into two major fragments by thrombin, approximately 20 and 8 kDa from EGFP-Th2 (Figure 5.4). The cleaved mixtures subjected to further analysis by MALDI mass spectrometry. MALDI mass
Figure 5.2. Absorption spectra of EGFP-Th2 following thrombin digestion. The 15 μM intact EGFP-Th2 (solid line) was cleaved by 20 nM thrombin to result in a decrease in absorbance at 398 nm and an increase at 490 nm (dash line) in thrombin reaction buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0) at room temperature for overnight digestion.
Figure 5.3. Fluorescence spectra of EGFP-Th2 following thrombin digestion. The 15 μM intact EGFP-Th2 (solid line) was cleaved by 20 nM thrombin in thrombin reaction buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl$_2$, pH 8.0) at room temperature for overnight digestion to result in a decrease (dash line) in fluorescence emission at 508 nm while excited at 398 nm (A) and an increase (dash line) in fluorescence emission at 508 nm while excited at 490 nm (B).
Figure 5.4. The cleavage confirmation of EGFP-Th2 thrombin digestion.
The verification of cleavage of EGFP-Th2 by thrombin in thrombin reaction buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0) at room temperature for overnight digestion was confirmed using SDS-PAGE (A) and MALDI mass spectrometry (B). M is the protein marker. Lanes 1 and 2 are EGFP-Th2 without and with thrombin digestion. Following thrombin digestion, EGFP-Th2 was specifically cleaved into two major fragments with molecular mass of 20 and 8 kDa. The MALDI mass spectrometry analysis shows two major fragments with molecular masses of 21309.4 and 7931.1 Da.
spectrometry analysis reveals that the fragments produced from EGFP-Th2 have molecular masses of 21309.4 and 7931.1 Da, which corresponds to the calculated mass values of 22646.6 and 7914.8 Da for the large and small fragments of EGFP-Th2 and indicates that the cleavage occurs in the cleavage linkers, respectively.

5.6 Kinetic study of thrombin sensors

In order to investigate the optimal cleavage linkers for thrombin and to determine the steady-state kinetic parameters, $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$ for hydrolysis of EGFP-based thrombin sensors upon the action of thrombin, initial rates were measured at various EGFP-based thrombin sensor concentrations in thrombin digestion buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0) at room temperature following digestion with a final concentration of 125 nM thrombin. The kinetic parameters of EGFP-Th2 were fitted using Michaelis-Menten equation, as shown in Figure 5.5. The kinetic parameters, $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$, are $0.29 \pm 0.02 /s$, $2.81 \pm 0.69 \mu M$, and $105000 \pm 14600 M^{-1}S^{-1}$.

In order to design an optimal cleavage linker for thrombin and improve thrombin specificity of EGFP-based thrombin sensors, the $P_1$, $P_2$, $P_3$ and $P_4$ position in $P$ region and $P_1'$ and $P_2'$ position in $P'$ region in thrombin sensor were systematically studied. Another four specific cleavage linkers (Phe-Asn-Pro-Arg-Gly-Phe, Phe-Asn-Pro-Arg-Ser-Phe, Phe-Thr-Pro-Arg-Gly-Phe and Phe-Thr-Pro-Arg-Ser-Phe) for thrombin were designed to obtain different thrombin sensor variants. The studies on kinetic parameters of these thrombin sensors were performed in thrombin digestion buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0). Compared to the thrombin sensor with cleavage linker (Gly-Arg-Gly), the turnover number, $k_{\text{cat}}$, has significant increase of 5 fold and $K_m$ has a significant decrease of 10 fold. Therefore,
Figure 5.5. Kinetic studies of EGFP-Th2 conducted in thrombin digestion buffer. EGFP-Th2 at concentration from 2-32 μM in thrombin reaction buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl$_2$, pH 8.0) at room temperature was conducted thrombin digestion. The absorbance signal change was monitored at 490 nm in time course mode. The catalytic kinetic parameters, $k_{cat}$, $K_m$, and $k_{cat}/K_m$, are $0.29 \pm 0.02$ /s, $2.81 \pm 0.69$ μM, and $105000 \pm 14600$ M$^{-1}$S$^{-1}$ through Michaelis-Menten equation fitting, respectively. The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.
substrate specificity \( (k_{\text{cat}}/K_m) \) of these four thrombin sensor variants is resulted in 50-fold increase. The kinetic parameters for all of these thrombin sensor variants are shown in Table 5.2.

### Table 5.2. Catalytic parameters for thrombin in thrombin reaction buffer

<table>
<thead>
<tr>
<th>Thrombin sensors</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( K_m ) (μM)</th>
<th>( k_{\text{cat}}/K_m ) (( \times 10^4 ) M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-Th1</td>
<td>0.06 ± 0.001</td>
<td>20.92 ± 0.96</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td>EGFP-Th2</td>
<td>0.29 ± 0.02</td>
<td>2.81 ± 0.69</td>
<td>10.49 ± 1.46</td>
</tr>
<tr>
<td>EGFP-Th3</td>
<td>0.23 ± 0.01</td>
<td>2.86 ± 0.28</td>
<td>8.02 ± 0.62</td>
</tr>
<tr>
<td>EGFP-Th4</td>
<td>0.67 ± 0.06</td>
<td>11.37 ± 1.83</td>
<td>5.94 ± 0.05</td>
</tr>
<tr>
<td>EGFP-Th5</td>
<td>0.93 ± 0.14</td>
<td>12.78 ± 3.48</td>
<td>7.32 ± 0.92</td>
</tr>
</tbody>
</table>

Conditions: 50 mM Tris, 150 mM NaCl, 2.5 mM CaCl\(_2\), pH 8.0, 25 °C.
The data presented in mean ± SD, are fitting kinetic parameters and fitting errors.

### 5.7 Extinction coefficient constants of products after thrombin sensor cleavage

After thrombin sensor samples were cleaved completely through thrombin digestion overnight, the absorbance of products at 490 nm was measured and then extinction coefficients were calculated through Beer-Lambert law. The extinction coefficients of the variants of these thrombin sensors have no obvious difference due to similar helix-loop-helix cleavage linkers although the recognition residues are different. Their extinction coefficient constants of these cleavage products were shown in Table 5.3.
Table 5.3. Extinction coefficients of cleaved products from thrombin sensors

<table>
<thead>
<tr>
<th>Thrombin sensor</th>
<th>Extinction coefficients (ε) (μM⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-Th1</td>
<td>0.037 ± 0.005</td>
</tr>
<tr>
<td>EGFP-Th2</td>
<td>0.031 ± 0.004</td>
</tr>
<tr>
<td>EGFP-Th3</td>
<td>0.030 ± 0.003</td>
</tr>
<tr>
<td>EGFP-Th4</td>
<td>0.035 ± 0.007</td>
</tr>
<tr>
<td>EGFP-Th5</td>
<td>0.033 ± 0.004</td>
</tr>
</tbody>
</table>

Conditions: 50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0, 25 °C.
The data presented in mean ± SD, are extinction coefficients and standard deviations from different samples at various concentrations (2-20 μM) following trypsin complete digestion, n = 6.

5.8 Relative signal change calculation of thrombin sensors

In order to evaluate the response of thrombin sensors to thrombin, the relative absorbance change between before thrombin cleavage and after thrombin cleavage was measured and calculated by the Equation 2 in the section of materials and methods. The relative signal change of thrombin sensor variants is shown in Table 5.4. EGFP-Th1, a cleavage site in loop-III region, revealed a large relative signal change. However, EGFP-Th2 and EGFP-Th3, cleavage sites in F-helix, showed no significant difference in relative signal change. The difference in relative signal change of these thrombin sensors are possibly due to slight difference in conformation of cleaved products, which is still not clear. Compared to our trypsin sensors, EGFP-based trypsin sensors have similar relative signal change for these EGFP-based thrombin sensors where cleavage sites are located on the F-helix.
<table>
<thead>
<tr>
<th>Thrombin sensor</th>
<th>Relative signal change ($D_R$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-Th1</td>
<td>2.64 ± 0.10</td>
</tr>
<tr>
<td>EGFP-Th2</td>
<td>1.94 ± 0.23</td>
</tr>
<tr>
<td>EGFP-Th3</td>
<td>1.76 ± 0.18</td>
</tr>
<tr>
<td>EGFP-Th4</td>
<td>1.88 ± 0.25</td>
</tr>
<tr>
<td>EGFP-Th5</td>
<td>1.79 ± 0.14</td>
</tr>
</tbody>
</table>

Conditions: 50 mM Tris, 150 mM NaCl, 2.5 mM CaCl$_2$, pH 8.0, 25 °C
The data presented in mean ± SD, are relative signal changes and standard deviations from different samples at various concentrations (2-20 μM) following trypsin complete digestion, n = 6.

In our lab, EGFP-based calcium sensors were successfully designed and developed through the insertion of calcium binding motif, EF-hand, from calmodulin, which can be expressed in mammalian cells and can be used for tracking calcium signaling *in vitro* and in living cells (Zou, Ye et al. 2005). According to this grafting strategy, the specific cleavable linker for thrombin can be inserted in EGFP to develop sensitive thrombin sensors. On the one hand, EGFP can be easily expressed in bacteria and mammalian cells, which offers EGFP-based thrombin sensors for exploiting thrombin activity *in vitro* and investigating real-time thrombin activation or inhibition in living cells. On the other hand, EGFP-based thrombin sensors have characteristics of double wavelengths change in opposite directions for absorbance and fluorescence, which also provides the ratiometric measurement to determine thrombin activity.

As well known, loop-III has strong binding affinity for calcium and helices connected to loop-III can improve the flexibility of EF-hand (Ye, Shealy et al. 2003; Ye, Lee et al. 2005). The cleavage sites for thrombin were located in the modified EF-hand motif, which was grafted into
EGFP to develop the sensitive thrombin sensors. According to our kinetic studies of thrombin sensors, the cleavage site in loop-III revealed the slow enzymatic reaction and lower substrate specificity to thrombin possibly due to the less accessibility to thrombin at this cleavage site, which is consistent with our previous result that the trypsin sensor with a cleavage site in loop-III can not be cleaved by trypsin. With the addition of flexible helices on both sides of the loop-III, the accessibility to thrombin and the cleavage rate on F-helix are significantly increased. A larger relative signal change of these thrombin sensors with specific cleavage site at F-helix is provided.

Due to the designed thrombin sensor variants based on EGFP, these sensors have great potential to determine thrombin activity, thrombin activation or pathway in living system such as targeted to extracellular space in real time. Meanwhile, compared to catalytic kinetic parameters of commercially available thrombin kits, the binding affinity and specificity of our EGFP-based thrombin sensor variants to thrombin are significantly increased due to the lack of P’ region residue design in thrombin detection kits containing small molecular dye (Rijkers, Wielders et al. 1995). The kinetic parameters of our designed thrombin sensors obviously reach or overpass these substrates. Similarly, our trypsin sensor variants, caspase sensor variants and thrombin sensor variants have obvious larger substrate specificity ($k_{cat}/K_m$) for proteases. The major reason for enhanced specificity is still attributed to the compact β-barrel structure of EGFP. Following the grafting of enzymatic cleavage linker on the chromophore-sensitive location, the cleavage linkers can provide the high accessibility to enzymes. Therefore, the class of protease sensors exhibited a strong binding capability or smaller $K_m$ value to result in larger $k_{cat}/K_m$ value.
5.9 Conclusions and significance

We have successfully developed EGFP-based thrombin sensors with optimal cleavage linkers on F-helix, which exhibit fast response, high sensitivity and specificity, large relative signal change with thrombin cleavage. The catalytic kinetic parameters of $k_{cat}$, $K_m$, and $k_{cat}/K_m$ of EGFP-Th2 are $0.29 \pm 0.02$/s, $2.81 \pm 0.69 \mu$M and $104900 \pm 14600 \text{ M}^{-1}\text{S}^{-1}$, respectively. Since EGFP have the capability to express in both bacteria and mammalian cells without requirements of cofactors and the successful monitoring trysinogen, caspase-3 or caspase-8 activation and inhibition in living cells using our developed EGFP-based trypsin sensors, caspase-3 sensors and caspase-8 sensors, the significant advantages of our engineered thrombin sensors will have the potential to real-time track thrombin activity or activation processes in living system such as extracellular environment with a ratiometric measurement mode, which can avoid the influence from environment, such as expression level, background. Signal peptides for extracellular environment can assist our thrombin sensors to track thrombin activity or investigate thrombin activation pathway in the specific cellular locations for understanding the mechanisms of thrombin regulation pathways. These thrombin sensors will be greatly benefited for probing physiological process and pathological mechanisms of diseases corresponding to the imbalance of thrombin activation and inhibition. Moreover, this method for protease sensor development will be promising to investigate other diseases related to protease activity.
Chapter 6  Fluorescence properties of EGFP fragments

6.1 Introduction

A novel strategy for protease sensor development has been successfully established through a grafting approach to obtain various protease sensors including trypsin sensors, chymotrypsin sensors, caspase sensors and thrombin sensors. Although all of these protease sensors can be cleaved by their specific proteases, they still retained their strong fluorescence in native conditions. Which fragment of EGFP variants can emit the strong fluorescence after digestion? What is the minimum or essential sequence for chromophore formation or fluorescence of EGFP?

Extensive research has been conducted to understand mechanisms of chromophore formation in GFP (Heim, Prasher et al. 1994; Dopf and Horiagon 1996; Reid and Flynn 1997; Wachter, King et al. 1997; Enoki, Saeki et al. 2004), which can be applied to develop various new sensors for monitoring cellular processes. One of the earliest studies demonstrated the cleavage of GFP through proteases, and obtained protease-derived chromopeptides from GFP under denatured conditions and pH-dependent spectral characteristics (Shimomura 1979). The chromophore was determined to be a cyclization of the residues Ser65-dehydroTyr66-Gly67 within the polypeptide through papain digestion of GFP and HPLC isolation (Cody, Prasher et al. 1993). The protease-sensitive positions of GFP were also identified to be the loop areas at positions 157, 172, or 189 through the digestion of modified GFP with inserted protease cleavage linker (Chiang, Okou et al. 2001). A series of deletion studies of GFP determined the minimum domain for fluorescence to be the amino acid residues from 2 to 232 in GFP, which was the earliest investigation of the minimal region of GFP required for its fluorescence and provided the
key amino acids at the termini for its structure and function of GFP (Dopf and Horiagon 1996). A more defined map of the minimal functional domain of GFP based on single amino acid truncation studies from both ends of an enhanced version of GFP indicated that the minimal fluorescent domain spans the region from amino acids 7 to 229 in GFP (Li, Zhang et al. 1997). Truncation of GFP mutants up to nine amino acids at the C terminus had no deleterious effect on the fluorescence intensity of GFP, but a longer deletion up to 11 amino acids caused a decrease in maximal fluorescence as analyzed by spectrofluorimetry and fluorescence-activated cell sorting (FACS) (Kim and Kaang 1998).

In this chapter, we would like to further understand the chromophore formation and mini-domain for chromophore formation or essential sequences for fluorescence of EGFP. We successfully separated the two major fragments following protease digestion and identified accurate cleavage sites of EGFP-T1 containing trypsin cleavage linker. The optical properties of both fragments were examined through spectroscopic methods to reveal the weak fluorescence from large fragment due to the maintenance of the chromophore in this large fragment. It is also the first time report that the large fragment containing 172 amino acid residues of EGFP is the critical sequence for chromophore formation and fluorescence.

6.2 Protease sensitivity of EGFP

Commonly used proteases such as trypsin, chymotrypsin and thrombin have different specificities. The protease sensitivity of EGFP-T1 to trypsin, chymotrypsin and thrombin was monitored and evaluated through SDS-PAGE. Figure 6.1A shows that the intact protein (EGFP-T1, 34 kDa) was first cleaved by trypsin into three fragments with molecular masses of 23, 20 and 8 kDa following digestion for 1 to 6 h. The fragment of 23 kDa was further digested and
converted to 20 kDa, and the fragment of 8 kDa remained unchanged following digestion up to 24 h. Similarly, chymotrypsin first cleaved EGFP-T1 into three fragments of 21, 20 and 10 kDa, and then the 21 kDa band was reduced to 20 kDa when subjected to digestion for 6 h or longer. Finally, EGFP-T1 was cleaved by chymotrypsin and trypsin into two stable fragments. The larger fragments had similar molecular mass (approximately 20 kDa, P20) while the smaller fragments have molecular mass of 8 kDa (P8) or 10 kDa (P10). The P8 and P10 fragments remained unchanged following digestion up to 24 h.

Figure 6.1B shows semi-quantitative measurement of cleavage rates of this protein by trypsin obtained by monitoring the P20 fragment formation based on its optical density in SDS-PAGE. Compared to the final P20 optical density, P20 fragment formation reached 20% and 40% at 1 h, and reached 50% and 80% at 6 h following trypsin or chymotrypsin digestion, respectively; EGFP-T1 was fully converted to P20 fragment after digestion for 24 h.

In order to examine the effects of trypsin concentration on hydrolysis of this protein, as well as the cleavage order, trypsin digestion of EGFP-T1 was performed using different ratios of protein and trypsin (w/w) (2000:1, 1000:1, 200:1, 100:1). Increasing trypsin concentrations had no effect on cleavage locations or patterns of EGFP-T1 although the cleavage rate, or P20 formation rate, was greatly accelerated (Figure 6.2A). An intermediate product (31 kDa) was visualized at low trypsin concentration and with digestion time less than 12 h. The appearance of the 8 and 23 kDa bands occurred simultaneously with the disappearance of the 31 kDa band. An increase of the relative trypsin concentration was found to accelerate the cleavage of EGFP-T1 to produce two final major fragments based on the semi-quantitative optical density of P20 band on SDS-PAGE (Figure 6.2B). Therefore, the cleavage of EGFP-T1 to produce the fragments follows the order from 34 to 31 kDa, then from 31 to 23 and 8 kDa, and finally to 20 and 8 kDa.
Figure 6.1. The cleavage patterns and kinetics of EGFP-T1 by various proteases. The cleavage pattern of EGFP-T1 by various proteases was identified via SDS-PAGE (A). Trypsin, chymotrypsin and thrombin cleavage of EGFP-T1 following digestion for 0, 1, 6, 24 h were shown in SDS-PAGE. M is protein marker. NP is the sample of EGFP-T1 not subjected to protease cleavage as the control. Lane 1, 2, 3 and 4 are the samples of EGFP-T1 subjected to no protease, subjected to trypsin, chymotrypsin, and thrombin digestion at room temperature, respectively. The ratio of protein to enzyme is 100:1 (w/w). The rate of cleavage of EGFP-T1 is semi-quantitatively measured based on the formation of P20 (B) fragment using the optical density of its band in SDS-PAGE following EGFP-T1 trypsin (▨) and chymotrypsin (▧) digestion.
Figure 6.2. Effects of trypsin concentrations on digestion pattern and kinetics of EGFP-T1. The trypsin digestion pattern of EGFP-T1 was analyzed through SDS-PAGE under the various trypsin concentrations (A). EGFP-T1 samples digested with various trypsin concentrations for 0, 1, 6, 12, 24, and 48 h at room temperature were shown in SDS-PAGE. M is protein marker. NP is the sample of EGFP-T1 not subjected to trypsin digestion as the control. Lane 1, 2, 3 and 4 are the samples with trypsin digestion at EGFP-T1/trypsin ratios of 2000:1, 1000:1, 200:1 and 100:1 at room temperature. The rate of cleavage (B) of EGFP-T1 is semi-quantitatively measured based on the formation of the P20 fragment using the optical density of its band in SDS-PAGE with EGFP-T1/trypsin ratios of 2000:1 (▃), 1000:1 (▇), 200:1 (▎) and 100:1 (▨), respectively.
6.3 Separation of EGFP fragments

In order to identify the cleavage sites through protein sequence analysis and mass spectrometry, and to investigate the optical properties of two major fragments, different chromatographic methods were used to separate the fragments of EGFP-T1 following trypsin digestion. P20 and P8 could not be separated under the non-denaturing conditions. Therefore, the separation was performed under denaturing conditions with 6 M urea and heating in 90°C water bath for 5 min. Denatured products were successfully separated in a Hitrap Sephadex 75 size exclusion column. To improve the purity of both fragments, P20 and P8 collected from FPLC were further purified by reversed phase HPLC.

Although our mini-GFP was successfully separated in denaturing conditions, many difficulties in separation were encountered in non-denaturing conditions. The digested protein fragments of EGFP-T1 cannot bind to the Hitrap Nickel chelating column due to the removal of the N-terminal His-tag. In non-denaturing condition, the two digested major fragments are co-eluted while using Sephadex 75 size exclusion column or Hitrap Q column with salt gradient elution so that two bands in SDS-PAGE exhibited molecular masses of 20 and 8 kDa, respectively. Due to the difficulties in separation under the non-denaturing conditions, the pure P20 fragment was obtained using SDS-PAGE first through excising the corresponding band and eluting the protein in a Tubeluta tube with dialysis membrane. Because the amount of P20 is limited from SDS-PAGE electroelution and P8 is still difficult to purify, the purification of P20 and P8 using a combinative FPLC and HPLC under the condition of 6 M urea for denaturation is conducted. Similar results from N-terminal sequence analysis and molecular weight analysis of P20 and P8 fragments were exhibited through the separation using SDS-PAGE electroelution.
and combinative FPLC or HPLC methods. As has been discussed above, two bands after trypsin
digestion of EGFP-T1 were always observed in SDS-PAGE although mixture of both fragments
was conducted separation under non-denaturing conditions. This result suggested that the
cleaved mixtures were not separated in non-denaturing conditions, but separated in denaturing
conditions.

6.4 Cleaved EGFP fragments remain association at the physiological conditions
Proteolytically cleaved EGFP fragments exhibited a strong tendency to interaction with each
other under the physiological conditions, based on several observations. First, the digested
sample mixtures without heating to maintain the native status and with heating in water bath to
convert them into denatured status were loaded on the same SDS-PAGE for verifying the
association capability of two fragments. The samples in the native status have only a single band
with green fluorescence while denatured samples have two bands with molecular masses of 20
kDa and 8 kDa on SDS-PAGE, as shown in Figure 6.3. The strong potential association of P20
and P8 fragments in non-denaturing conditions was confirmed. In addition, when purified under
native conditions, using either Sephadex 75 size exclusion column or Hitrap Q column with salt
gradient elution, the two major digested fragments with molecular masses of 20 and 8 kDa are
co-eluted and appear in SDS-PAGE. Therefore, it is more difficult to separate both fragments in
non-denaturing conditions although EGFP-T1 is cleaved at the insertion linker. Although they
have the strong potential to associate to the full EGFP, this is not a bad thing because we can
exploit this association capability to reconstitute EGFP for tracking protein-protein interactions
in living cells or determining protease activity through a specific signal peptide or linkers to
target the host proteins in specific compartments.
Figure 6. 3. The verification for the assemble of two major fragments (P20 and P8) through SDS-PAGE under denaturing and non-denaturing conditions. M is the protein marker; Lane 1, 2, 3 and 4 are the samples of: uncleaved EGFP-T1 denatured by adding SDS and heat boiling; mixture of cleaved products with denatured treatment from EGFP-T1; mixture of cleaved products without denatured treatment from EGFP-T1, P20 fragment separated through FPLC with denatured treatment. The mixture of cleaved products of EGFP-T1 without denatured treatment is shown in the associated band with green color in SDS-PAGE and has similar molecular mass with uncleaved EGFP-T1. Bands with rectangular boxes are yellow before Coomassie blue staining, and the band with the circular box is green before Coomassie blue staining.
6.5 Cleavage site identification of EGFP-T1 trypsin digestion

In order to identify the cleavage sites through protein sequence analysis and mass spectrometry, and to investigate the optical properties of two major fragments from EGFP-T1 digestion, high purity P20 and P8 solutions were obtained following separation by FPLC and HPLC. These samples were used for amino acid sequence analysis. The molecular masses of the two major fragments were 20842.06 Da for P20 and 7968.58 Da for P8, determined using MALDI-MS. This result indicates that cleavage sites of EGFP-T1 by trypsin are specific, and the two fragments generated by trypsin are stable under non-denaturing conditions although there are 33 potential trypsin cleavage sites in this protein. The N-terminal sequence of P20 is MVSKGEELF based on the Edman degradation analysis. This result indicates that the His-tag was removed by trypsin digestion, which is consistent with the result that P20 can not bind to the Hitrap Nickel chelating column. Moreover, the intermediate product (31 kDa) during trypsin digestion coincides with the removal of His-tag. This result is also consistent with previous literature report that His-tag can be removed by proteases during storage (Battistutta, Negro et al. 2000). Amino acid sequence analysis of P8 reveals that the N-terminal is HVMTNL, which is located at the inserted F-helix of EF-hand motif. The calculated molecular mass for the fragment from HVMTNL in F-helix to the C-terminal is 7946.90 Da, which differs from the observed molecular mass of 7968.58 Da by 21.68 Da. A possible reason for this difference is that P8 binds an ion of Na⁺. The molecular mass from MVSK to the Arg at position –3 in the E-helix is 20844.68, which matches the observed molecular mass (20842.06 Da). However, the calculated molecular mass from MVSK to Arg at position 2 of the F-helix is 22581.58 Da, which is also consistent with the 23 kDa band on SDS-PAGE. All of these results are also consistent with
three cleavage steps shown in SDS-PAGE (Figure 6.1A and 6.2A): a fragment of 31 kDa in the first step, a fragment of 23 kDa in the second step and a fragment of 20 kDa in the third step are listed by cleavage order in Figure 6.4. Taken together, EGFP-T1 was specifically cleaved by trypsin and chymotrypsin at preferable sites and the cleavage process stopped after the preferable sites were completely cleaved. Moreover, the cleaved protein solution still retained its original green color, although EGFP-T1 was completely cleaved into two major fragments by trypsin and chymotrypsin. The chromophore, TYG, is located in the P20 fragment. The identified cleavage sites based on analysis of MALDI-MS, SDS-PAGE, and the N-terminal sequence of P20 and P8 are clearly shown in Figure 6.4 and Table 6.1.

Table 6.1. The molecular mass of fragments during EGFP trypsin digestion

<table>
<thead>
<tr>
<th>Sequence start point</th>
<th>Sequence end point</th>
<th>Observed Mass (Da)</th>
<th>Calculated Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Protein</td>
<td></td>
<td>34451.80</td>
<td></td>
</tr>
<tr>
<td>MVSKGE</td>
<td>C-terminal</td>
<td>31 k (SDS-PAGE)</td>
<td>30520.46</td>
</tr>
<tr>
<td>MVSKGE</td>
<td>SAAELR</td>
<td>23 k (SDS-PAGE)</td>
<td>22581.58</td>
</tr>
<tr>
<td>HVMTNL</td>
<td>C-terminal</td>
<td>7968.58 (MALDI)</td>
<td>7946.90</td>
</tr>
<tr>
<td>MVSKGE</td>
<td>EEEIREAFR</td>
<td>20842.06 (MALDI)</td>
<td>20844.68</td>
</tr>
</tbody>
</table>
Figure 6.4. The structural model of EGFP-T1 with a protease cleavable linker.
The structural model of EGFP-T1 containing a protease-cleavable helix-loop-helix motif from calmodulin, generated via Modeller and PyMol software, depicts the cleavable linker, in red, grafted at position 172 in the loop region of EGFP. His-tag is shown in cyan. The ordinal designations (1st, 2nd, 3rd) are based on trypsin cleavage kinetics experiments, described in the text. Following exhaustive trypsin digestion, two major fragments (P20 and P8) result, shown in green and yellow, respectively.
6.6 Cleavage site identification of EGFP-T1 chymotrypsin digestion

As shown in Figure 6.1, EGFP-T1 exhibited two major cleaved bands (called P20c and P10c fragments) in SDS-PAGE following chymotrypsin digestion. In order to identify the cleavage sites of EGFP-T1 by chymotrypsin, high purity solutions of P20c and P10c fragments were successfully separated through combinative FPLC and HPLC. The samples of P20c and P10c fragments were conducted the amino acid sequence analysis using Edman degradation and molecular mass analysis using MALDI-MS. The molecular mass of large fragment was 20728.52 Da; the small fragment exhibited multiple peaks with molecular masses of 7604.96, 7480.63 and 6771.25 Da. In addition, the N-terminal sequence of P20c is MVSKGEELF based on the Edman degradation analysis. Amino acid sequence analysis of P10c reveals that the N-terminal is TNLDGS, which is also located at the inserted F-helix of EF-hand motif.

Based on the comprehensive analysis between mass spectroscopy and amino acid sequence, the C-terminal of large fragment (P20c) should be EEEIREAF, which indicates the chymotrypsin cleavage site is on the inserted E-helix of EGFP-T1 to obtain the large fragment containing the chromophore of EGFP. The fragment of P10c shown in SDS-PAGE is consistent with molecular mass of the fragment from RVFDKD to the C-terminal of EGFP-T1, which exhibited a calculated molecular mass of 9839.99 Da. The N-terminal sequence analysis of the small fragment purified by FPLC and HPLC is TNLDGS and the multiple peaks with molecular masses of 7604.96, 7480.63, and 6771.25 Da were shown in the mass spectroscopy. Due to the multiple cleavage sites in the inserted linker, the calculated molecular masses of the fragments from TNLDGS to the C-terminal of EGFP-T1, GMDELY, and AAGITL are 7579.94, 7451.26, and 6742.47 Da, which match to 7604.96, 7480.63 and 6771.25 Da in the results of MALDI-MS,
respectively. The identified cleavage sites based on analysis of MALDI-MS, SDS-PAGE, and the N-terminal sequence of P20c and P10c are clearly shown in Figure 6.5 and Table 6.2.

Table 6.2. The molecular masses of fragments during EGFP-T1 chymotrypsin digestion

<table>
<thead>
<tr>
<th>Sequence start point</th>
<th>Sequence end point</th>
<th>Observed Mass (Da)</th>
<th>Calculated Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Protein</td>
<td></td>
<td>34451.80</td>
<td></td>
</tr>
<tr>
<td>MVSKGE</td>
<td>EIREAF</td>
<td>20 k (SDS-PAGE)</td>
<td>20708.49</td>
</tr>
<tr>
<td>RVFDKD</td>
<td>C-terminal</td>
<td>10 k (SDS-PAGE)</td>
<td>9839.99</td>
</tr>
<tr>
<td>MVSKGE</td>
<td>EIREAF</td>
<td>20728.52 (MALDI)</td>
<td>20708.49</td>
</tr>
<tr>
<td>TNLDGS</td>
<td>C-terminal</td>
<td>7604.96 (MALDI)</td>
<td>7579.43</td>
</tr>
<tr>
<td>TNLDGS</td>
<td>GMDELY</td>
<td>7480.63 (MALDI)</td>
<td>7451.26</td>
</tr>
<tr>
<td>TNLDGS</td>
<td>AAGITL</td>
<td>6771.25 (MALDI)</td>
<td>6742.47</td>
</tr>
</tbody>
</table>

6.7 Optical properties of EGFP fragments

Figure 6.6A shows the absorption spectra of purified P20 fragment compared with uncleaved EGFP-T1 and EGFP-wt. A predominant maximum absorbance for EGFP-wt at 488 nm was observed in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4), while the intact EGFP-T1 exhibited two absorbance maxima at 398 and 488 nm. Interestingly, purified P20 has a strong blue shifted absorbance peak at 383 nm under identical Tris buffer conditions at pH 7.4, which is similar to the maximum absorption of EGFP-wt at pH 3.36 (Figure 6.6A). The maximum absorbance peak gradually shifts to 354 nm at a final pH of 10.8 with the addition of NaOH (Figure 6.6B), suggesting that at physiological condition, the chromophore of P20 exists largely in the neutral state (Brejc, Sixma et al. 1997; Tsien 1998).
Figure 6.5. The cleavage pattern of EGFP-T1 by chymotrypsin.
Based on the analysis of N-terminal amino acid sequence of P20c fragment and SDS-PAGE of EGFP-T1 chymotrypsin digestion, the cleavage site was followed by the sequence of EIREAF to obtain two major fragments with molecular masses of 20 and 10 kDa. According to the analysis of N-terminal amino acid sequence of P10c fragment and MALDI-MS of EGFP-T1 chymotrypsin digestion, the possible cleavage sites are followed by sequence LHVM, or GITL or DELY to obtain the observation of multiple peaks in MALDI-MS with molecular masses of 7604.96, 7480.63 and 6771.25 Da, respectively.
Figure 6.6. The characteristics of UV-visible spectra of the P20 fragment.

(A) The characteristics of UV-visible spectra of the P20 fragment (◆) was compared with those of EGFP-wt (●), EGFP-T1 (■) and acid-denatured EGFP-wt (◇) at pH 3.36. The P20 fragment has a maximum absorption peak similar to acid-denatured EGFP-wt at 383 nm, which is different from the maximum absorption peaks of native EGFP-wt and EGFP-T1. (B) The characteristics of UV-visible spectra of the P20 fragment at various pH conditions was also measured. The maximum absorption of the P20 fragment at pH 7.32 (●) gradually shifted from 383 to 454 nm while the P20 fragment in the final pH 10.82 (△) due to the addition of NaOH.
The purified and lyophilized P20 exhibits yellowish green color while P8 is colorless. Under UV light, lyophilized P20 or P20 dissolved in water or 10 mM Tris buffer emitted a weak green fluorescence (Figure 6.7A). The P20 fragment has maximum fluorescence excitation at 490 nm and a shoulder peak at 470 nm, and a fluorescence emission at 505 nm (Figure 6.7B and 6.7C). Both excitation and emission maxima are blue shifted compared with the uncleaved EGFP-wt and EGFP-T1 with an emission maximum at 510 nm from a single excitation at 398 nm. On the other hand, uncleaved EGFP-T1 has an emission at 510 nm that originates from the excitation at 398 and 488 nm. Hence, these results further reveal that P20 fragment contains a mature chromophore in water or 10 mM Tris buffer in the absence of P8.

Several groups have attempted to identify short fragments that are required for chromophore formation in fluorescent proteins (Heim, Prasher et al. 1994; Dopf and Horiagon 1996; Reid and Flynn 1997; Wachter, King et al. 1997; Enoki, Saeki et al. 2004). Amino acid residues 7-229 of GFP were shown to be essential for the fluorescence of the protein (Li, Zhang et al. 1997; Kim and Kaang 1998). Recently, random deletion and substitution in the longest loop of GFP demonstrated that only residues I129 and D130 in the loop can be deleted without altering fluorescence efficiency, and that all other deletions or substitutions irreversibly reduce or lose the fluorescence of GFP, which damaged the characteristics of the chromophore and eliminated the fluorescence of GFP (Flores-Ramirez, Rivera et al. 2007).

Departing from previous reported studies on the determination of the minimum domain for fluorescence by gradually deleting the residues at both termini and loop regions of GFP, we have engineered protease cleavage sites at different loop locations (157 and 172) of the protein frame to investigate the kinetic protease cleavage process and proteolytic effects on the optical properties of chromophore formation. Although GFP was previously reported to be cleaved into
Figure 6.7. The fluorescence characteristics of the P20 fragment.

(A) Fluorescence of P20 and P8 fragments was first examined under the UV lamp. Tube 1, 2, 3 and 4 are the samples of ddH₂O, lyophilized P20, lyophilized P20 dissolved in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4), and lyophilized P8, respectively. The P20 fragment emitted weak green fluorescence after lyophilization or when dissolved in Tris buffer. The excitation and emission spectra of P20 fragment were compared with those of EGFP-wt and EGFP-T1 in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4). Excitation spectra (B) and emission spectra (C) of EGFP-wt (○), EGFP-T1 (□) and P20 (◇) were obtained, revealing that the P20 fragment has a similar maximum excitation peak at 490 nm and a shoulder peak 470 nm. However, EGFP-T1 has maximum excitation peaks at 490 and 398 nm. The fluorescence maximum wavelength of EGFP-wt and EGFP-T1 occurred at 508 nm, however, the maximum emission of the P20 fragment was slightly blue shifted to 505 nm. The fluorescence of P20 fragment was 100-fold weaker than EGFP-T1.
fragments upon adding several cleavage sequences (IEGRS) for pronase, factor Xa and trypsin (Chiang, Okou et al. 2001), the effect on optical properties of GFP by proteolytic degradation was not reported. Our strategy has shown that both trypsin and chymotrypsin are able to specifically cleave EGFP variants into two major fragments (Figure 6.1A and 6.2A). The MALDI-MS and amino acid sequencing results revealed that the grafting region selected is sensitive to protease action, and the chromophore was found to be located in P20 fragment. Another fragment, P18, was also obtained through the digestion of EGFP-T2 with cleavage linker at position 157 of EGFP. Purified P18 and P20 fragments encompass amino acid sequences 1-157 or 1-172, respectively. Despite the availability of more than 21 trypsin or 27 chymotrypsin cleavage sites in our EGFP variants, the two fragments produced in our cleavage process remained stable for more than 90 days without the continuing degradation that was observed in proteolytic cleavage of other globular proteins (Lee, Chen et al. 2002). The proteolytically-cleaved EGFP fragments maintained strong fluorescence, which is likely a result of their strong tendency to remain intact under physiological conditions. Such strong protease stability and specific cleavage likely originated from the unique β-can structure. GFP was reported to remain folded in 8 M Urea, in pH from 5 to 12, and to have strong tolerance for several proteases (Ward and Bokman 1982). Therefore, the specific cleavage at the engineered protease site in the loop of EGFP results in two strongly-interacting fragments, which maintain protein tertiary structure and protect the chromophore environment.

Consistent with previous reports of short protease-derived peptide fragments containing a chromophore (chromopeptides) (Shimomura 1979; Cody, Prasher et al. 1993), the optical properties of P20 fragment were blue shifted from 397 to 383 nm and exhibited only weak absorbance at 490 nm compared with EGFP-wt and EGFP-T1. The absorption wavelength of the
P20 fragment is identical to that of EGFP-wt in acid denatured conditions (pH 3.36). In addition, the absorbance intensity at 383 nm decreases with a simultaneous increase at 454 nm following a gradual increase of pH (Figure 6.6B). Such pH-dependent optical properties indicate that the chromophore in the P20 fragment exists mainly in the neutral state. Moreover, the absorption spectrum of P20 fragment under neutral conditions is very similar to Niwa’s report that the absorption peak of the fragment containing the chromophore is approximately 386 nm (Niwa, Inouye et al. 1996).

The P20 fragment also has excitation peaks at 490 nm and a shoulder peak at 470 nm and an emission peak at 505 nm. This excitation wavelength is slightly blue shifted when comparing with EGFP-wt. Meanwhile, the fluorescence intensity is approximately 100-fold lower than that of the intact EGFP-T1. These results indicate that the P20 fragment is able to emit green fluorescence with weak intensity or low quantum yield, near 0.04. In contrast, Niwa also reported that short peptides or fragments of GFP were not able to emit fluorescence under physiological conditions in solution. The lysyl endopeptidase fragments have no fluorescence in organic solvent or aqueous mixtures, and this fragment only produces fluorescence with low intensity in ethanol glass at lower temperature, 77 K (Niwa, Inouye et al. 1996). Interestingly, the absorbance and fluorescence properties of the isolated P20 and P18 fragments are very similar to that of bacterially-expressed and refolded EGFP fragment (residue 1-158) with peak excitation at 488 nm and peak emission at 507 nm (Demidov, Dokholyan et al. 2006).

6.8 Conclusions

The engineered EGFP-T1 can be specifically cleaved by trypsin and chymotrypsin at the engineered region into two major fragments and can not be cleaved by thrombin. According to
N-terminal sequence and MALDI-MS analysis of P20 and P8, three cleavage locations are at the end of the His-tag, the Arg at position –3 in the E-helix and the Arg at position 3 in the F-helix. The P20 fragment, also named mini-GFP, contains 181 amino acids with a chromophore inside and has a strong absorption spectrum of protonated form mimicking to that of EGFP-wt at pH 3.36. Although the P20 and P8 peptide segments remain the noncovalent association and still maintain strong fluorescence after digestion, P20 can emit weak green fluorescence at 503 nm in neutral Tris buffer in the absence of P8. Compared to previous research achievement that the minimal domain of GFP for fluorescence is amino acid 7-229, our mini-GFP containing 181 amino acid residues was much shorter to exhibit fluorescence. This research provides a new direction for tracking the protein-protein interactions by targeting to host protein with this small domain and for the development of biosensors. However, whether the refolded structure in this EGFP fragment is same as that of EGFP variants is still unknown and need to be further probed.
Chapter 7 Investigation of protein-protein interactions using fluorescence complementation of split EGFP fragments

7.1 Introduction

During the separation of P20 and P8 fragments after EGFP-T1 digestion, both fragments have the strong tendency to associate full EGFP in native conditions, which is confirmed in the Chapter 6. Based on above results, we have the strong desire to confirm that this high potential association capability is due to two alone fragments or highly requires inductions from other functional proteins. We hypothesize this high potential association capability can be exploited to reconstitute EGFP for tracking protein-protein interactions for tracking cellular events or determining protease activity through a specific signal peptide or linkers to target the host proteins in specific compartments.

Calcium ion is an important messenger in a variety of signaling pathways in biological systems with association of specific calcium binding proteins. The common calcium binding proteins have a calcium-binding motif termed EF-hand, possessing a characteristic helix-loop-helix fold (Linse, Brodin et al. 1987; Heizmann and Hunziker 1991; Yang, Jones et al. 2003; Yang and Yang 2005; Yang, Wilkins et al. 2005). Calbindin D9k is a common cytosolic calcium-binding protein, which is important to regulate the availability of calcium ions within cells (Luu, Nie et al. 2004). This protein exhibits the calcium buffering properties and is involved in modulating calcium absorption and regulating the transient cytosolic calcium ion concentration in a number of cells. Calbindin D9k consists of four helices and two EF-hands joined together by a flexible linker region with high calcium binding affinity (Julenius, Thulin et al. 1998; Julenius, Robblee et al. 2002). Figure 7.1 exhibits the model of calbindin D9k structure
Figure 7.1. The molecular model of calbindin D9k and interaction with calcium ions. The molecular model of calbindin D9k exhibited its consists of four helices to form two EF-hand structures connected by flexible loops. Two calcium-binding pockets located inside offered the high binding affinity to calcium ions (in blue color).
and its binding to calcium ions. The binding of calcium ions by EF-hand proteins depends strongly on the electrostatic interactions between calcium ions and negatively charged residues of these proteins. The cooperative binding of calcium ions is an essential functional property of the EF-hand family of calcium-binding proteins. Previous investigations indicated that isolated calcium binding EF-hand peptides have a potential dimerization and the separated fragments containing N-terminal EF-hand of calbindin D9k show the strong tendency to dimerize in the presence of calcium (Linse and Chazin 1995; Kragelund, Jonsson et al. 1998; Julenius, Robblee et al. 2002).

In this chapter, to further identify minimal fragment for fluorescence of fluorescent proteins, we constructed a protease cleavage site in EGFP and identified two major EGFP fragments upon trypsin cleavage first time. The fragment containing the chromophore, called mini-EGFP or P20, still retains weak fluorescence, which is much shorter than previous report that minimal domain for fluorescence of GFP is required amino acid residue from 7 to 229. Our goal is to develop calcium-dependent protein sensor by fusing mini-EGFP fragment with EF-hand motifs from calbindin D9k for real-time monitoring calcium dependent protein-protein interactions in living cells. Our strategy is to fuse EF1 and EF2 of calbindin D9k to N- and C-terminal split EGFP fragments. The chromophore maturation and fluorescence complementation of split EGFP fragments can be accomplished through the dimerization between EF1 and EF2 of calbindin D9k with the aid of the trigger, calcium. The HeLa cells co-transfected with EGFP fragments containing EF-hand motifs exhibited strong fluorescence emission during expression. In addition, the fluorescence intensity is significantly increased after stimulation of ATP, ionomycin and calcium. Therefore, this technology have the potential to use as a sensitive
calcium sensor for tracking calcium signal pathways in living cells in real time and for tracking
the calcium signal pathways in subcellular compartments with the aid of signal peptides.

7.2 Protein-protein interactions

It is well known that protein-protein interactions play crucial roles in the structural and
functional organization of living cells due to proteins associated with many important
physiological and pathological processes. Investigating these complex physiological and
pathological processes from protein-protein communication is very useful for understanding
some physiological processes, identifying the biochemical abnormalities in unhealthy states,
probing the pathological mechanisms and tracking the drug effect in diseased situations or in
treatment processes (Ozawa, Nogami et al. 2000). A large amount of modern biological research
is focused on how, when and where these protein-interaction associations preform regulations of
physiological and pathological mechanisms and processes. Currently, most of these research
tools are two-hybrid system (Fields and Song 1989; Chien, Bartel et al. 1991), split-ubiquitin
system (Johnsson and Varshavsky 1994; Stagljar, Korostensky et al. 1998; Dunnwald,
Varshavsky et al. 1999), staining with exogenous fluorophores or dyes (Sloan and Hellinga
1998; Doi, Takashima et al. 2002; Lata, Gavutis et al. 2006), fluorescence resonance energy
transfer (FRET) (Li, Ng et al. 2001; He, Olson et al. 2003), and so on. The two-hybrid method
facilitates the identification of potential protein-protein interactions and has been proposed as a
method for the generation of protein interaction maps. However, the two-hybrid technique is
limited to detect protein-protein interactions in the nucleus. The FRET assay, another common
method for investigating protein-protein interactions in living cells, is based on two fluorophores
(donor and acceptor), either chemically linked or genetically fused to two proteins whose
interaction is to be examined. A high protein expression level for reliable determination of energy transfer is required in the FRET analysis. In addition, the partial overlap between the excitation wavelength of the donor and the emission wavelength of the acceptor is also required. Moreover, the donor and acceptor should be placed within 100 Å in distance with right orientation. Due to these limitations for detecting protein-protein interaction in living cells, a novel, convenient and powerful method, fluorescence complementation of split green fluorescent proteins is developed for detecting specific protein-protein interactions in living cells. This strategy is based on the reassembly of two fragments with weak fluorescence or without fluorescence to result in fluorescence restoration driven by biomolecular interactions.

7.3 Current investigations of split GFP

The fluorescence complementation between fragments of a fluorescent protein was first discovered and detected in *Escherichia coli* through the fusion to the interacting peptides, antiparallel leucine zippers (Ghosh I., Hamilton A.D. et al. 2000; Magliery, Wilson et al. 2005). The fragments of yellow fluorescent protein (YFP) fused to calmodulin and the M13 calmodulin-binding peptide can produce fluorescent complexes in mammalian cells (Nagai, Sawano et al. 2001). A biomolecular fluorescence complementation assay was exploited and applied to monitor the interactions between bZIP and Rel family transcription factors based on the assembly of split YFP fragments. Later, a multicolor fluorescence complementation assay between fragments of different fluorescent proteins was also created through the dimerization of the bZIP domains of Fos and Jun to track the gene transcription location in living cells (Hu, Chinenov et al. 2002; Hu and Kerppola 2003). The split GFP fragments genetically fused to the N- and C-terminal of maltose binding protein (MBP) were employed to monitor the
conformation change of MBP through the generation of fluorescence upon the maltose binding (Jeong, Kim et al. 2006). The chromophore maturation of the split EGFP fragments and fluorescence emitting can result from the N- and C-terminal halves of interin (VDE) without the endonuclease domain for monitoring the protein-protein interactions and splicing activity (Ozawa, Nogami et al. 2000; Ozawa, Takeuchi et al. 2001). The method for visualizing beta and gamma dimers of G protein was also developed using the fluorescence complementation of split GFP fragment to investigate the role of subcellular localization in regulating the specificity of G protein beta-gamma signaling in vivo (Hynes, Tang et al. 2004). Although the history of studies on fluorescence reconstitution from the split green fluorescent protein fragments was just several years, to date, the fluorescence complementation of split green fluorescence protein fragments can investigate protein-protein interactions in living cells for studying the protein folding and conformation change, understanding the cellular processes and pathways, detecting protease activity on cellular signaling, determining the gene expression levels and tracking the subcellular transcription location. Therefore, this promising fluorescence technique will provide great contributions to physiological and pathological research in the biomedical field.

7.4 Strategy for calcium-dependent protein-protein interaction

EGFP-T1 can be cleaved by trypsin to obtain two major fragments at the insertion linker. Both fragments are difficult to separate at the non-denaturing condition and have the potential to assemble together although EGFP is cleaved by trypsin at the insertion linker. On the other hand, the large fragment also revealed the optical properties, such as strong absorbance at 383 nm and weak fluorescence with maximum emission at 505 nm after being separated. Therefore, we hypothesize that the large fragment and the small fragment can be reconstituted under the
dimerization of EF-hand motifs from calbindin D9k because the EF1 and EF2 of calbindin D9k have strong capacity to dimerization due to swapping of EF-hands from the induction of an extended hydrophobic core (Hakansson, Svensson et al. 2001). Therefore, the experiment was conducted to fuse EF1 to the carboxyl end of the N-terminal fragment of EGFP and EF2 to the amino end of the C-terminal fragment of EGFP to N-EGFP-EF1 and C-EGFP-EF2, respectively. In order to improve the flexibility of EF-hands in both fragment, a linker (GGSGSGSS) was used to connect the N-EGFP fragment and EF1, or C-EGFP fragment and EF2 (Figure 7.2).

7.5 Fluorescence imaging of protein-protein interaction

It is interesting to note that the isolated EGFP fragment P20 has fluorescence in 100 fold weaker than the both fragments obtained from EGFP-T1 following trypsin digestion under associated status. This observation leads us to hypothesize that the newly generated EGFP fragments could be applied to fragment complementation to develop sensors capable of significantly enhanced fluorescence upon association. Since EF-hand motif 1 (EF1) and motif 2 (EF2) from calbindin D9k were reported to exhibit a strong tendency to dimerize (Linse, Brodin et al. 1987; Linse and Chazin 1995), the capacity for reassembly of the EGFP fragments during the dimerization of EF-hand motifs from calbindin D9k could be examined. To test this reassembly capacity, we fused EF1 and EF2 from calbindin D9k to the carboxyl end of the N-terminal fragment of EGFP and the amino end of the C-terminal fragment of EGFP, denoted as N-EGFP-EF1 and C-EGFP-EF2, respectively. In order to improve the flexibility of EF-hands in both fragments, a linker (GGSGSGSS) was used to connect the N-EGFP fragment and EF1, or C-EGFP fragment and EF2, respectively. Meanwhile, in order to further verify the origin of GFP
Figure 7.2. The design for calcium-dependent protein-protein interactions. The design for calcium-dependent protein-protein interaction is diagrammed, based on EGFP fragments fused to EF-hand motifs of calbindin D9k. C-terminus of N-EGFP fragment is connected to EF-hand motif 1 (EF1) following Glu172 and EF-hand motif 2 (EF2) is connected to N-terminus of C-EGFP fragment before Asp173 with an extended GGSGSGGS linker. N-EGFP-EF1p and N-EGFP-EF2p, with truncated flanking helices, were also constructed similarly to explore the minimal requisites for the expected association. Helix-loop-helix structures (EF1 and EF2 from calbindin D9k) are highlighted in blue, red and blue, respectively. The partial EF-hand motifs are highlighted in purple. GGSGSGGS linker is shown in black. The connection sites of Glu172 in the C-terminus of N-EGFP fragment and Asp173 in the N-terminus of C-EGFP fragments are highlighted in green and cyan color, respectively. Fluorescence complementation is postulated to result from the reconstitution of both EGFP fragments guided by the calcium-dependent dimerization of EF1 and EF2.
assembly due to dimerization in living cells, N-EGFP-EF1p and N-EGFP-EF2p with incomplete flanking helices were also designed and created.

In order to track fluorescence complementation of EGFP fragments, N-EGFP-EF1 and C-EGFP-EF2 were co-transfected into HeLa cells according to above methods (Materials and Methods) to allow protein expression for 24-48 h. Fluorescence imaging was acquired using an epifluorescence microscope with excitation at 398 and 488 nm for 50 ms exposure time. HeLa cells with co-transfection of both EGFP fragments revealed strong fluorescence (Figure 7.3A). In contrast, no significant fluorescence was acquired through 488 nm excitation even under 1000 ms exposure time with co-transfection of N-EGFP-EF1p and C-EGFP-EF2p (Figure 7.3B) or individual transfection of N-EGFP-EF1p in HeLa cells (Figure 7.3C). These results clearly indicated that although N-EGFP-EF1 encompasses the entire chromophore sequence, it can not fully fold to form the mature chromophore necessary for emitting strong fluorescence. Moreover, co-transfection of two fragments, N-EGFP-EF1p and C-EGFP-EF2p with an incomplete EF-hand motif, also fails to produce fluorescence complementation due to inability of dimerization from incomplete EF-hand motifs. Taken together, fluorescence complementation or chromophore maturation was achieved by fusion of two identified EGFP fragments to intact EF-hand motifs (EF1 and EF2) from calbindin D9k with strong dimerization capacity.

7. 6 Calcium effect on protein-protein interaction

In order to test the effects of calcium on fluorescence complementation of EGFP fragments in living cells, fluorescence of HeLa cells with co-transfection of EGFP fragments was acquired using epifluorescence microscope in a course model. The fluorescence signal change was analyzed following the addition of calcium triggers and calcium in living cells. As shown in
Figure 7.4, the fluorescence signal shows a 20% increase in response to stimulation with 1 μM ionomycin, a calcium channel trigger whose function stimulates opening of the calcium channel on the cell membrane, which increases the intracellular calcium concentration due to flux of extracellular calcium. Following the stimulation with ionomycin, fluorescence signal exhibits another 10% increase with the addition of 5 mM calcium. Therefore, the fluorescence complementation of EGFP fragments fused to EF-hand motifs from calbindin D9k can be moderated by the change in Ca$^{2+}$ concentration, which is possibly due to an indirect adjustment of reassembly of split EGFP fragments through the calcium-mediated dimerization of EF-hand motifs from calbindin D9k (Linse, Brodin et al. 1987).

In order to further confirm calcium effect on fluorescence complementation of EGFP fragments originated from the dimerization of EF-hand motifs, the HeLa cells with co-transfection of N-EGFP-EF1 and C-EGFP-EF2 was harvested after expression for 72 hours. The proteins were extracted from cell lysates through sonication. The supernatant containing proteins was collected after centrifuging at 14000 rpm for 10 minutes. 200 μl of supernatant was diluted to 1 ml in 1.7 ml fluorescence cuvette with a pathway of 1 cm. Fluorescence spectra were scanned from 600 to 500 nm with excitation at 490 nm. The maximum emission at 508 nm has slight decrease with the various concentrations of EGTA. The decrease in fluorescence intensity at 508 nm is maximal at the 0.5 mM of final concentration of EGTA, which is approximately 5% decrease total, as shown in Figure 7.5. This result suggests that fluorescence reconstitution of EGFP fragments is induced by the dimerization of EF-hands of calbindin D9k upon calcium condition. However, the fluorescence complementation is irreversible due to the chromophore formation although the fluorescence intensity has the minor decrease.
Figure 7.3. The fluorescence complementation of split EGFP fragments fused to EF-hand motifs was confirmed in living cells via co-transfection.

The strong fluorescence complementation in HeLa cells through (A) the co-transfection of N-EGFP-EF1 and C-EGFP-EF2 under excitation of 488 nm proceeded via the dimerization of EF-hand motifs and was monitored by fluorescence microscopy with 50 ms exposure time. The weak fluorescence in HeLa cells with (B) individual transfection of N-EGFP-EF1, and (C) co-transfection of N-EGFP-EF1p and C-EGFP-EF2p at excitation of 488 nm with 1000 ms exposure time.
Figure 7.4. The effects of calcium on fluorescence complementation of EGFP fragments in living cells.

The change in fluorescence signal from HeLa cells upon co-transfection with the genes for N-EGFP-EF1 and C-EGFP-EF2 followed by promotion of calcium influx by ionomycin, and with extracellular addition of calcium. The fluorescence signal is increased by about 20% with addition of 1 μM ionomycin and another 10% with the addition of 5 mM calcium.
Figure 7.5. Fluorescence signal change in extract of HeLa cells with co-transfection of N-EGFP-EF1 and C-EGFP-EF2 through EGTA titration. The fluorescence emission signal at 508 nm of the extract of HeLa cells with co-transfection of both fragments exhibited a decrease by 5% with the addition of EGTA up to 0.5 mM while excited at 490 nm. The smaller fluorescence signal change is possibly due to the irreversible dimerization of EF-hand motifs from calbindin D9k.
7. 7 Optical properties of N-EGFP-EF1 and C-EGFP-EF2 expressed in bacteria

In order to examine the optical properties of two EGFP fragments fused to EF-hand motifs and calcium-dependent protein-protein interactions in buffer conditions, both EGFP fragments were expressed in *E. coli* and purified to evaluate the optical properties *in vitro*. Due to both EGFP fragments expressed in inclusion body, the purification of both fragments was conducted under the urea-denatured conditions to unfold the proteins and then refold back. The refolded protein fragments was separated using Nickel chelating affinity column according to above-stated methods. The purified proteins are evaluated and confirmed through MALDI-MS examination. The fragment of N-EGFP-EF1 exhibited fluorescent spectra with maximum excitation peak at 403 nm (Figure 7.6A) and maximum excitation peak at 468 nm (Figure 7.6B), which further verified EGFP fragment containing chromophore could emit fluorescence, although it only retained 172 amino acid residues (1-172) of original EGFP. This maximum excitation and emission wavelengths is close to previous reports that large split intein-EGFP fragment expressed in *E. coli* (DE3) pLys and purified with intein column exhibited maximum excitation spectrum at 360 nm and emission spectrum at 460 nm (Demidov, Dokholyan et al. 2006). However, the maximum excitation and emission peaks of N-EGFP-EF1 from bacterial expression are blue shifted when compared to the spectra of P20 fragments from separation of EGFP-T1 digestion. These results clearly demonstrated that the chromophore could fold and mature in smaller EGFP fragment with amino acid residues (1-172). Additionally, the chromophore folding, formation and maturation is highly affected by its environment because the matured chromophore of P20 fragment is directly come from fully folded EGFP-T1, while the chromophore folding in N-EGFP-EF1 fragment is weak possibly due to shorter sequence.
Figure 7.6. Fluorescence spectra of purified N-EGFP-EF1 fragment from *E. coli* expression. The maximum excitation wavelength (A) and emission wavelength (B) of 10 μM N-EGFP-EF1 was observed at 403 and 468 nm in Tris-HCl buffer (10 mM Tris, 1 mM DTT, pH 7.4), respectively.
In order to test the calcium effect on the fluorescence signal enhancement due to calcium-dependent heterodimerization of EF-hand motifs from calbindin D9k, we have conducted the calcium titration in the mixture of purified N-EGFP-EF1 and C-EGFP-EF2 fragments. The purified N-EGFP-EF1 and C-EGFP-EF2 fragments buffered in Tris-HCl buffer (10 mM Tris, 100 mM NaCl, 1 mM DTT, pH 7.4) were mixed according to the ratio of 1:1. When the fluorescence signal at 468 nm of mixture of purified N-EGFP-EF1 and C-EGFP-EF2 fragments was compared to the individual N-EGFP-EF1 fragment, the fluorescence signal doesn’t have any difference under the both conditions, which confirms the fluorescence signal of N-EGFP-EF1 fragment containing chromophore doesn’t affected by the small fragment, C-EGFP-EF2 under the calcium-free buffer condition. However, a stepwise increase of the fluorescence signal at 468 nm was observed when calcium titration in the mixture of both fragments was conducted up to calcium concentration of 4 mM (Figure 7.7) and exhibited 20% enhancement through the calcium titration (Figure 7.8). This result gave a clear explanation that fluorescence signal change is calcium-dependent and the enhancement of fluorescence complementation from EGFP fragments can be resulted from heterodimerization of EF1 and EF2 motifs under the calcium induction.

7.8 EF-hand dimerization and EGFP fragment complementation

Fluorescence complementation of split green fluorescent proteins was developed for detecting specific protein-protein interactions in living cells. This strategy is based on the reassembly of two fragments with weak fluorescence or without fluorescence, to result in fluorescence restoration driven by biomolecular interactions. This method is promising since protein-protein interaction in living cells can be directly visualized. This method is promising
Figure 7. Fluorescence signal change of purified N-EGFP-EF1 and C-EGFP-EF2 upon calcium action.
Both EGFP fragments with concentration of 15 μM were incubated in protein refold buffer (20 mM Tris, 100 mM NaCl, 1 mM DTT, pH 7.4) and then calcium titration was conducted in the mixture of both EGFP fragments. Fluorescence emission signal of the incubation mixture with both EGFP fragments at 468 nm gradually increases with the addition of calcium ions when excited at 403 nm. Precipitation was observed when calcium concentration reached to 4 mM.
During calcium titration, fluorescence emission signal of the incubation mixture with both EGFP fragments at 468 nm gradually increases when excited at 403 nm. The fluorescence complementation enhancement of N-EGFP-EF1 and EGFP-EF2 fragments was observed and exhibited nearly 20% increase up to 4 mM calcium.
since protein-protein interactions in living cells can be directly visualized. Currently, fluorescent complementation of split GFP fragments was achieved via various types of molecular recognitions. Antiparallel leucine zippers were added to NGFP and CGFP fragments containing 1-157 and 158-238 amino acid residues without green fluorescence to rebuild a new fluorescent protein with a matured chromophore to understand mechanism of protein assembly (Ghosh, Hamilton et al. 2000; Wilson, Magliery et al. 2004; Magliery, Wilson et al. 2005). NGFP and CGFP fragments connected to maltose binding protein (MBP) were used to monitor the conformation change through fluorescence complementation assay upon maltose binding (Jeong, Kim et al. 2006). Moreover, protein splicing-based reconstitution of split GFP fragments has been applied to monitor protein-protein interactions in bacteria and in vivo (Ozawa, Nogami et al. 2000; Ozawa, Takeuchi et al. 2001; Cabantous, Terwilliger et al. 2005), screen proteins expressed at different cellular environment, improve protein folding and solubility (Wilson, Magliery et al. 2004; Cabantous, Terwilliger et al. 2005; Cabantous and Waldo 2006), and simultaneously visualize interactive biochemical processes (Prasher, Eckenrode et al. 1992; Chalfie, Tu et al. 1994; Heim, Prasher et al. 1994; Marshall, Molloy et al. 1995; Tsien 1998; Pollok and Heim 1999; Hu, Chinenov et al. 2002; Hu and Kerppola 2003). Furthermore, DNA hybrization was also used to facilitate fluorescent complementation (Demidov, Dokholyan et al. 2006) and coiled-coiled interaction was recently used to monitor metal-dependent GFP emission (Mizuno, Murao et al. 2007).

Our strategy has shown that the fluorescence complementation of split EGFP fragment can be accomplished by fragment complementation based on the calcium-dependent heterodimerization of EF-hand motifs from calbindin D9k and the identified split EGFP fragments. EF-hand calcium binding proteins with a helix-loop-helix calcium-binding motif
represent one of the major types of calcium binding proteins that have been found in all organisms, ranking as the 5th most common binding motif found in eukaryotic genomes. Based on sequence prediction, there are more than 3000 calcium-binding proteins in eukaryote and 500 in prokaryocyte (Zhou, Yang et al. 2006). Upon stimulation by first massager, agonist, such as ATP, intercellular calcium concentration increases significantly. Trigger proteins with paired EF-hand motifs, such as calmodulin and troponin C, regulate more than 100 biological processes in response to calcium binding. Other binding proteins with EF-hand motifs, such as calbindin D9k and pavalalbumin, function as buffers to maintain calcium ion concentration. EF-hand motifs in proteins are normally paired and coupled to form a compact domain. For example, the smaller unit with paired two EF-hand motifs exists in calbindin D9k. Such coupled EF-hand motif interactions contribute greatly to the cooperativity of calcium binding which is essential for calcium-controlled activities to switch on/off over a narrow range of free calcium concentration. Isolated EF-hand motifs have a strong tendency of dimerization. EF-hand motif III from skeletal troponin C has been shown to dimerized in the presence of calcium (Delbaere, Vandonselaar et al. 1989; Shaw, Hodges et al. 1990; Shaw, Hodges et al. 1991). The other EF-motifs such as rabbit skeletal troponin C, the C-terminal EF-hand from E6-binding protein (E6bp4), third EF-hand motifs from silver hake parvalbumin and N-terminal EF-hand from human S100B protein were shown to form dimers (Kay, Forman-Kay et al. 1991; Revett, King et al. 1997; Chen, Hong et al. 1998). Using fragment complementation, Linse’s group also showed that the EF-hand motif 1 (residues 1-43) from calbindin D9k has strong dimerization affinity with EF-hand motif 2 (residues 44-75) \((3.6 \times 10^{11} \text{ M}^{-1})\) (Berggard, Julenius et al. 2001) in the presence of calcium. Such heterodimerization constant in the presence of calcium is more than six orders of magnitude greater than the association constant for formation of homodimers (Julenius, Robblee
et al. 2002). About 11 hydrophobic core residues are responsible for their dimerization and the stability of the intact domain, which is the calcium-dependent protein-protein interaction. Figure 7.3 clearly shows that HeLa cells with the co-transfection of the split EGFP fragments fused with EF-hand motifs of calbindin D9k (N-EGFP-EF1 and C-EGFP-EF2) exhibit strong fluorescence signal, suggesting the chromophore maturation or florescence complementation is achieved. In contrast, no fluorescence was observed in HeLa cells transfected with a single fragment of N-EGFP-EF1, or co-transfection with N-EGFP-EF1p and C-EGFP-EF2p with incomplete EF-hand motifs. We further demonstrated that an increase of intracellular calcium concentration after stimulation with ionomycin results in a significant increase of fluorescence. Giving the diverse role of EF-hand proteins in signal transduction, calcium homeostasis and the strong dimerization of isolated EF-hand motifs, this reported technology can be further developed to simultaneously sense calcium concentration change and calcium-dependent protein-protein interactions in living cells in real time.

7.9 Conclusions

Through connecting the EF hand motifs of calbindin D9k on split enhanced green fluorescent protein fragments, a novel fluorescence complementation method was developed to monitor the calcium ion contractions in living cells in real time due to the dimerization of EF hand motifs of calbindin D9k upon effect from calcium ions. Due to the significant increase in fluorescence intensity after the stimulation with ionomycin and calcium, this technology can be used for a sensitive calcium sensor to track calcium signal pathways in living cells in real time. On the other hand, this technology can be used for tracking the calcium signal pathways in subcellular compartments with the aid of signal peptides.
Chapter 8  Conclusion and significance

Based on our previous work on the development of calcium sensor (Zou et al, 2007) and the change in optical properties of protease sensor variants with a grafting cleavage linker at different loop regions in EGFP following protease digestion, the sensitive position immediately following Glu172 of EGFP is identified to be a suitable grafting site for the design of protease sensor and the optical signal of designed protease sensors exhibits the ratiometric change upon protease action. A variety of specific cleavage linkers for trypsin, caspase-3, caspase-8 and thrombin were inserted into the identified chromophore-sensitive location to obtain various trypsin, caspase-3, caspase-8 and thrombin sensors. The cleavage linkers flanking sequences that have highly helical property result in a large optical signal change in chromophore environment, which in turn exhibits the significant ratiometric optical signal change upon protease cleavage. From the screening and analyses of optimal cleavage linker for specific proteases, the cleavage linker flanked both E-helix and F-helix significantly improve the flexibility of cleavage linker and accessibility for proteases, which provides the largest ratiometric optical signal change for developed protease sensors. This ratiometric measurement strategy has been successfully exploited to real-time track trypsin, caspase-3 and caspase-8 activation and inhibition in living cells, which eliminates drawbacks or limitations of the currently-available methods to determine protease activity. The substrate kinetic properties, especially $K_m$, of these developed protein-based protease sensors, are comparable or better than the current commercially-available protease substrates conjugated to chromogenic or fluorogenic dyes. Then, enzymatic specificity of these developed protein-based sensors is usually better than those commercially-available substrates. We have successfully applied the developed EGFP-based protease sensors to monitor
activation of trypsin, caspase-3 and caspase-8 by different inducers at least two cell lines (HeLa and MIA PaCa-2). These developed protease sensors also have potential to target different subcellular compartments such as ER and mitochondria for tracking protease activation or inhibition in the different subcellular environments with the aid of signal peptides. Moreover, trypsinogen activation in pancreatic cancer cells, MIA PaCa-2 cells is the first time observed using our trypsin sensor although this cell line lack of granule structure. The activation of caspase-3 thorough STS induction can be observed a clear translocation from cytosol to nuclei using our caspase-3 sensors and our developed caspase-3 sensors can provide a sensitive detection of caspase-3 activation at low concentration level during the early stage of apoptosis, which is difficult to realize using commercially-available caspase-3 probes such as NucView488 caspase-3 substrate, due to its low sensitivity or indirect detection of caspase-3 activity. In addition, our protease sensors can real-time monitor trypsin or caspase-3 inhibition in living cells. Therefore, A grafting approach to engineer a specific cleavage linker for proteases into a stable scaffold host protein, EGFP, to determine the protease activity, and to investigate protease activation or inhibition pathways in living cells has been successfully established, which opens an avenue for tracking protease-dependent physiological and pathological mechanisms or processes, and provides a novel and effective method for the investigation on mechanisms, new drug development or screening for diseases related to protease activity.

The digested mixture of protease sensors still retains strong fluorescence although they are fully cleaved the designed cleavage site to two major fragments. The separation of both major fragments and identification of cleavage sites or pattern indicate the chromophore is maintained in the large fragment (amino acid residue, 1-172) for its weak fluorescence, which exhibits that the mini-domain for fluorescence of EGFP is much less shorter than that of
currently reported investigations. Meanwhile, both cleaved EGFP fragments have high tendency to associate for emitting strong fluorescence under the physiological conditions. Due to the strong heterodimerization capability of EF-hand motif (EF1 and EF2) from calbindin D9k under the induction of calcium, EF1 and EF2 are fused to the C-terminal of N-EGFP fragment (residue 1-172) and the N-terminal C-EGFP fragment (residue 173-238), respectively. The constructed N-EGFP-EF1 and C-EGFP-EF1 could be used for monitoring calcium-dependent protein-protein interaction in living cells. The fluorescence enhancement through the co-transfection of both N-EGFP-EF1 and C-EGFP-EF1 can be applied to track the change in calcium concentration or calcium signaling pathways, and to simultaneously monitor protein-protein interactions in living cells in real time.

The significance of these works is multi-fold. First, the creative thoughts of designing protease sensors in a single EGFP will provide extensive investigation and knowledge on the key factors of protease activation and inhibition in living organisms for understanding the mechanisms and pathways of many diseases related to protease activity. Additionally, this strategy may be further extended to the application of other important proteases. Second, application of our design approach to the development of protease sensors, without the use of FRET pairs, results in correlative ratiometric measurements, which eliminates many drawbacks and limitations of the currently-available FRET-based protease sensors or protease substrates conjugated with small peptide dyes. In addition, the design of sensitive protease sensors in a single EGFP offers a quick enzymatic response at lower protease concentration with picomolarity level in living cells, especially for caspases, to determine the early stage of caspase activation or apoptosis, which is more difficult to accomplish using FRET-based GFP pairs protease sensors. Also, the designed protease sensors can be targeted to different subcellular
compartments in living cells such as mitochondria or ER, which are the essential subcellular locations for probing enzymogen activation pathways with the aid of signal peptides.

Although our developed protease sensors exhibit multiple advantages in the detection of protease activation or inhibition in living system, there are also several limitations that need to be further addressed for the application in living cells or in vivo. First, trypsin sensors need to be conducted studies in more cell lines, such as Panc-1, Capan-1, Capan-2, CFPAC-1 and BxPC-3 cell lines. Second, as for caspase-3 and caspase-8 sensors, the reverse optical signal change between in living cells and in vitro needs to be further investigated. More control or confirmation experiments such as Western blot need to be further conducted. Third, protease concentration needs to be further quantitatively normalized in single individual cell to understand protease activation or inhibition mechanisms, rather than the normalization of population-dependent protease concentration in a large amount of cells. Fourth, further structural analysis for chromophore change of our developed protease sensor upon cleavage needs to be identified using NMR or X-ray.
References


concentration and pH of target cells during the cytotoxic process: a quantitative study at

of control mediated by regulator of calcium/calmodulin-dependent signaling. *Science*

Constitutive nuclear localization and initial cytoplasmic apoptotic activation of

dependent enzyme activation and vacuole formation in the apical granular region of


potential retention following release of cytochrome c in apoptotic GT1-7 neural cells.


Publications


Patents
