

**IMAGING THE PROTEOME:  
METABOLIC TAGGING OF NEWLY SYNTHESIZED PROTEINS  
WITH REACTIVE METHIONINE ANALOGUES**

Thesis by

**Kimberly E. Beatty**

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2008

(Defended February 8, 2008)

© 2008

Kimberly E. Beatty

All Rights Reserved

**Dedicated to**  
**Jeff Miller and Marissa Mock**  
**for their generous and constant friendship**

## ACKNOWLEDGEMENTS

The best decision I made when I started my research at the California Institute of Technology (Caltech) was choosing to work for David Tirrell. I have really enjoyed learning and working in his group, in large part due to his fairness, integrity, and calm demeanor. Dave provided me with a collegial environment filled with both the people and resources that I needed to complete the research described in this thesis. Moreover, he has been extremely supportive of the additional coursework, teaching, and traveling that I did to augment my studies. I am very grateful to him for his advice and assistance.

Next, I am grateful to have the best thesis committee I can imagine. Robert Grubbs, Dennis Dougherty, and Scott Fraser have all been very generous with their time and wisdom. I also want to thank Henry Lester. Although he was not a member of my committee, he really made me feel like a valued member of the Caltech community.

I had several fantastic mentors before I arrived at Caltech, and I must acknowledge their role in my academic life. My undergraduate research at UC Santa Barbara was with Alison Butler. In addition to supervising my research, she convinced me to major in chemistry and to apply to Caltech. Clearly, she has had a very positive impact on my career. When I worked for Alison, her graduate student, Jayme Carter-Franklin, was a valuable resource and mentor to me. I am really glad to have worked with both Alison and Jayme. I would also like to thank David Kohl (UC Santa Barbara) for encouraging me to apply for my first summer research program. That summer internship lead to many great research opportunities and experiences. I would also like to

thank Paul Carter and Tori Sharma (Genentech) and Naomi Balaban (UC Davis Medical Center) for guiding my research during my time with them.

My research has benefited from many fantastic collaborations. Specifically, I would like to thank Carolyn Bertozzi, Matt Hangauer, Jeremy Baskin (UC Berkeley), Qian Wang, Fang Xie (University of South Carolina), Julie Liu, Ying Lu, Nick Fisk, Erin Schuman (Caltech), and Sanne Schoffelen (Radboud University of Nijmegen). I am looking forward to continuing some of these collaborations during my postdoctoral research at UC Berkeley.

My research has also benefited from other graduate students I have worked with at Caltech. I would like to give special thanks to Marissa Mock and Rebecca Connor for their generous help during my entire time at Caltech. I would also like to thank Sarah Heilshorn, Paul Nowatzki, Dave Flanagan, Tae Hyeon Yoo, Inchan Kwon, Stacey Maskarinec, Pin Wang, Caglar Tanrikulu, and Isaac Carrico. Generally, I would like to thank the Tirrell group, past and present, for being such good colleagues.

My family has been very supportive of my thesis research. I would like to thank my parents, Dan and Shirley Beatty, for supporting my entire education. They taught me the importance of hard work, creativity, and being motivated to succeed. I would also like to thank my grandparents, Hazel and John Czapowski, who have taught me many wise guiding principles. Next, I would like to thank my sisters, Alyson and Jessica, as well as Tom and Pat Miller. Finally, I would like to thank my boyfriend, Jeff Miller, for giving me the support I needed to complete my work.

I am grateful to the Fannie and John Hertz foundation for supporting my graduate research. In addition to funding, the Hertz foundation has done a wonderful job

providing me with access to a network of researchers, through retreats, conferences, and dinners. I am also grateful to the PEO and the AAAS (Alan E. Leviton Student Research Award) for funding my research.

## ABSTRACT

Many strategies have been described for identifying proteins isolated from tissues, cells, or organelles, but the cellular proteome undergoes complex dynamic changes in response to disease or environment. A more complete analysis of the proteome requires complementary, time-resolved images of cellular proteins. In one method for obtaining dynamic proteomic data, cellular proteins are selectively tagged with small, reactive amino acid analogues. Co-translational incorporation of reactive methionine (Met) analogues [e.g., azidohomoalanine (Aha) or homopropargylglycine (Hpg)] is reminiscent of conventional pulse-labeling with radioactive amino acids; the endogenous cellular machinery places reactive Met analogues at sites normally occupied by Met within proteins. Susceptibility to tagging is determined not by the identity of the protein, but rather by the extent to which the protein is translated during exposure to the analogues. The analogue is then labeled using a copper-catalyzed or strain-promoted azide-alkyne ligation; both ligations enable selective, minimally invasive protein labeling in complex biological mixtures. In my dissertation research, I have developed new methods to tag and dye-label proteins in order to visualize one or more subsets of the proteome. In this thesis, I will first describe the tagging of newly synthesized proteins with reactive analogues in bacterial (Chapter 2) and mammalian cells (Chapter 3). In recent work, I have further expanded the method to enable two-fluorophore labeling of two distinct protein populations (Chapter 4). Two-dye labeling of proteins should enable changes in the proteome to be tracked over time. Finally, I will describe how the dye-labeling method has been adapted for live cell compatibility through the use of cyclooctyne-

conjugated fluorophores (Chapters 5 and 6). Labeling in live cells will enable dynamic processes to be monitored in real time. Proteomic visualization is complementary to proteomic identification, and it should be useful for examining many different aspects of biological systems, including translational responses to disease or environment.

## TABLE OF CONTENTS

<b>Dedication</b>	<b>iii</b>
<b>Acknowledgements</b>	<b>iv</b>
<b>Abstract</b>	<b>vii</b>
<b>Table of Contents</b>	<b>ix</b>
<b>List of Figures and Schemes</b>	<b>xiii</b>
<b>Chapter 1: Introduction</b>	<b>1</b>
1.1 The Proteome	1
1.2 Identification of Proteins Using Mass Spectrometry	2
1.3 Metabolic Tagging of a Subset of the Proteome	3
1.4 Imaging the Proteome	4
1.5 References	8
<b>Chapter 2: Selective Dye-Labeling of Newly Synthesized Proteins in Bacterial Cells</b>	<b>12</b>
2.1 Abstract	12
2.2 Introduction	13
2.3 Results and Discussion	14
2.4 Conclusion	18
2.5 Materials and Methods	19
2.5.1 Plasmids and Expression Hosts	19
2.5.2 Expression of Barstar	20
2.6 Acknowledgements	20
2.7 References	22

<b>Chapter 3: Fluorescence Visualization of Newly Synthesized Proteins in Mammalian Cells</b>	<b>23</b>
3.1 Abstract	23
3.2 Introduction	24
3.3 Results and Discussion	25
3.4 Conclusion	31
3.5 Materials and Methods	31
3.5.1 Cell Culture	31
3.5.2 Preparation of Cells for Fluorescence Microscopy	32
3.5.3 Preparation of Cells for Flow Cytometry	34
3.5.4 Fluorescence Microscopy	34
3.5.5 Flow Cytometry	35
3.6 Acknowledgements	36
3.7 References	37
<b>Chapter 4: Fluorophore Labeling of Two Distinct Protein Populations in Mammalian Cells</b>	<b>40</b>
4.1 Abstract	40
4.2 Introduction	42
4.3 Results and Discussion	44
4.4 Conclusion	52
4.5 Materials and Methods	53
4.5.1 Cell Maintenance	53
4.5.2 Preparation of Cells for Fluorescence Microscopy	54
4.5.3 Preparation of Cells for Flow Cytometry	56
4.5.4 Fluorescence Microscopy	57
4.5.5 Flow Cytometry	57

4.5.6 Synthesis of Compounds	58
4.6 Acknowledgements	60
4.7 References	61
<b>Chapter 5: Imaging Newly Synthesized Proteins in Living Cells</b>	<b>65</b>
5.1 Abstract	65
5.2 Introduction	66
5.3 Results and Discussion	67
5.4 Conclusion	77
5.5 Materials and Methods	78
5.5.1 Cell Maintenance	78
5.5.2 Preparation of Cells for Fluorescence Microscopy	78
5.5.3 Preparation of Cells for Flow Cytometry	80
5.5.4 Fluorescence Microscopy	80
5.5.5 Image Processing	81
5.5.6 Flow Cytometry	81
5.5.7 Synthesis of Coumarin-Cyclooctyne Dyes	82
5.5.8 <i>In Vitro</i> Reactions and HPLC Analysis	88
5.6 Acknowledgements	89
5.7 References	91
<b>Chapter 6: An Expanded Set of Fluorophores for Labeling Newly Synthesized Proteins in Live Cells</b>	<b>95</b>
6.1 Abstract	95
6.2 Introduction	96
6.3 Results and Discussion	98
6.4 Conclusion	105

6.5 Future Work	106
6.6 Materials and Methods	107
6.6.1 Cell Maintenance	107
6.6.2 Preparation of Cells for Fluorescence Microscopy	108
6.6.3 Preparation of Fixed Cells for Fluorescence Microscopy	109
6.6.4 Preparation of Live Cells for Flow Cytometry	109
6.6.5 Preparation of Fixed Cells for Flow Cytometry	110
6.6.6 Fluorescence Microscopy	110
6.6.7 Image Processing	111
6.6.8 Flow Cytometry	111
6.6.9 Synthesis of BDPY, LR, and FL	112
6.7 Acknowledgements	112
6.8 References	114
<b>Chapter 7: Concluding Remarks</b>	<b>117</b>

## LIST OF FIGURES AND SCHEMES

**Chapter 1: Introduction**

<b>Figure 1.1.</b> Disease and environment can alter the cellular proteome through the translation of new proteins or the degradation of pre-existing proteins.	2
<b>Figure 1.2.</b> Metabolic tagging of proteins for identification by mass spectrometry.	3
<b>Figure 1.3.</b> Selective dye-labeling of newly synthesized proteins in bacterial cells.	6

**Chapter 2: Selective Dye-Labeling of Newly Synthesized Proteins in Bacterial Cells**

<b>Figure 2.1.</b> Fluorogenic labeling of barstar in <i>E. coli</i> cells.	16
<b>Figure 2.2.</b> Fluorescence of induced <i>E. coli</i> cells after reaction with 3-azido-7-hydroxycoumarin.	17
<b>Figure 2.3.</b> Polyacrylamide gel electrophoresis of proteins labeled with 3-azido-7-hydroxycoumarin.	18

**Chapter 3: Fluorescence Visualization of Newly Synthesized Proteins in Mammalian Cells**

<b>Scheme 3.1.</b> Bio-orthogonal labeling of newly synthesized proteins for fluorescence visualization in mammalian cells.	25
<b>Figure 3.1.</b> Fluorescence labeling of proteins in MEF-mitoGFP.	26
<b>Figure 3.2.</b> Flow cytometric analysis of pulse-labeling and dye-labeling conditions.	28
<b>Figure 3.3.</b> Coumarin labeling of newly synthesized proteins in a wide variety of cell types.	29
<b>Figure 3.4.</b> Fluorescence micrographs of nucleolar and coumarin	

labeling in HeLa and HEK 293T cells.	30
--------------------------------------	----

## Chapter 4: Fluorophore Labeling of Two Distinct Protein Populations in Mammalian Cells

<b>Scheme 4.1:</b> Structures of Met, Aha, and Hpg.	43
<b>Scheme 4.2.</b> Two-dye labeling of proteins using a simultaneous or sequential pulse of two reactive metabolic analogues.	43
<b>Scheme 4.3.</b> Structures of the reactive fluorophores.	45
<b>Figure 4.1:</b> Selective dye-labeling of newly synthesized proteins using azide or alkyne fluorophores.	46
<b>Figure 4.2.</b> Mean fluorescence of Rat-1 fibroblasts by flow cytometry.	47
<b>Figure 4.3.</b> Fluorescent images of Rat-1 fibroblasts simultaneously pulse-labeled with two reactive amino acids.	49
<b>Figure 4.4.</b> Fluorophore labeling two distinct populations of proteins in Rat-1 fibroblasts.	50
<b>Figure 4.5.</b> Flow cytometry contour plot of two-dye labeled fibroblasts.	51
<b>Figure 4.6.</b> Fluorophore labeling two distinct populations of proteins in Rat-1 fibroblasts.	52
<b>Figure 4.7.</b> Fluorophore labeling two distinct populations of proteins in pancreatic exocrine cells (AR42J).	53

## Chapter 5: Imaging Newly Synthesized Proteins in Living Cells

<b>Scheme 5.1.</b> Structure of Met, Aha, and the coumarin-cyclooctynes.	68
<b>Figure 5.1.</b> Fluorescence labeling of proteins in Rat-1 Fibroblasts.	69
<b>Figure 5.2.</b> Projections of coumarin fluorescence from labeled	

proteins in Rat-1 Fibroblasts.	70
<b>Figure 5.3.</b> Flow cytometric analysis of coumarin fluorescence as a function of dye-concentration for cells pulse-labeled 4 h with Aha or Met.	72
<b>Figure 5.4.</b> Chromatograms of the <i>in vitro</i> reaction of <b>3</b> with Aha or Cys.	74
<b>Figure 5.5.</b> Flow cytometric analysis of pulse-labeling length and dye-labeling length.	75
<b>Figure 5.6.</b> Histograms of coumarin fluorescence from flow cytometry.	76
<b>Figure 5.7.</b> Viability of cells dye-labeled with coumarin-cyclooctynes.	77

## Chapter 6: An Expanded Set of Fluorophores for Labeling

### Newly Synthesized Proteins in Live Cells

<b>Scheme 6.1.</b> Structures of the reactive fluorophore-cyclooctynes: BDPY, LR, and FL.	97
<b>Figure 6.1.</b> Fluorescence micrographs of fixed Rat-1 fibroblasts dye-labeled with BDPY, LR, or FL.	99
<b>Figure 6.2.</b> Flow cytometric analysis of fixed Rat-1 fibroblasts dye-labeled with BDPY, LR, or FL.	100
<b>Figure 6.3.</b> Fluorescence labeling of proteins with FL and LR in Rat-1 fibroblasts.	101
<b>Figure 6.4.</b> Fluorescence labeling of proteins with BDPY in Rat-1 fibroblasts.	102
<b>Figure 6.5.</b> Flow cytometric analysis of BDPY fluorescence as a function of fluorophore concentration for live cells.	103
<b>Figure 6.6.</b> Flow cytometric analysis of the pulse-labeling and dye-labeling conditions for live Rat-1 fibroblasts.	104
<b>Figure 6.7.</b> Viability of cells dye-labeled with BDPY.	106