

Contents

Preface *XI*

List of Contributors *XIII*

1	Fluorescent Sterols for the Study of Cholesterol Trafficking in Living Cells	1
	<i>Avery L. McIntosh, Huan Huang, Barbara P. Atshaves, Stephan M. Storey, Adalberto M. Gallegos, Thomas A. Spencer, Robert Bittman, Yoshiko Ohno-Iwashita, Ann B. Kier, and Friedhelm Schroeder</i>	
1.1	Introduction	1
1.2	Methods for Imaging Fluorescent Sterols in Living Cells: Confocal and Multiphoton Laser Scanning Microscopy	3
1.2.1	Sources of Cholesterol and Fluorescent Sterols	3
1.2.2	Spectral Properties of Fluorescent Sterols	3
1.2.3	Fluorescent Sterol Labeling Methodology	3
1.2.3.1	Method 1: Direct Labeling	3
1.2.3.2	Method 2: Fluorescent Sterol Incorporation by Large Unilamellar Vesicles	5
1.2.3.3	Method 3: Fluorescent Sterol-methyl- β -cyclodextrin (FS-M β CD) Complexes	5
1.2.3.4	Method 4: Fluorescent Labeling of High Density Lipoproteins (HDL)	5
1.2.3.5	Method 5: BC0	6
1.2.4	Confocal Laser Scanning Microscopy (CLSM) and Multiphoton Laser Scanning Microscopy (MPLSM) of Sterol Probes	6
1.3	Cholesterol Structure and Distribution in Membranes	7
1.4	NBD-Cholesterol	8
1.4.1	22-NBD-Cholesterol	9
1.4.2	25-NBD-Cholesterol	11
1.5	Dansyl-Cholesterol	13
1.6	BODIPY-Cholesterol	14

1.7	Dehydroergosterol (DHE)	17
1.8	22-(p-Benzoylphenoxy)-23,24-bisnorcholestan-5-en-3 β -ol (FCBP) Photoactivatable Sterol	20
1.9	BC0	20
1.10	Conclusion	23
	References	25
2	Lipid Binding Proteins to Study Localization of Phosphoinositides	35
	<i>Guillaume Halet and Patricia Viard</i>	
2.1	Introduction: Phosphoinositide Signaling	35
2.2	Monitoring PI Distribution and Dynamics	37
2.2.1	Detection of PI Species Using Antibodies	37
2.2.2	Fluorescent PI Derivatives	38
2.2.3	Fluorescent PI-Binding Domains	39
2.2.3.1	Choosing the Right Domain	39
2.2.3.2	Imaging PI Probes	40
2.3	Detection of PtdIns(3,4,5)P ₃ Synthesis in Transfected Mammalian Cells	41
2.3.1	Transfection with Plasmid DNA Encoding GFP-PH _{GRP1}	41
2.3.2	Detection of PtdIns(3,4,5)P ₃ Synthesis by a Constitutively-active PI3K	42
2.3.3	Detection of PtdIns(3,4,5)P ₃ Synthesis after Stimulation with EGF	43
2.4	Monitoring PtdIns(4,5)P ₂ Dynamics in Mouse Oocytes	43
2.4.1	Making cRNAs	44
2.4.2	PtdIns(4,5)P ₂ Dynamics in Mouse Oocytes at Fertilization and after Treatment with Ionomycin	45
2.5	Limitations of the Technique	45
2.5.1	PI Probes may Detect Only a Subset of the Total PI Pool	46
2.5.2	PI Probes can Interfere with Normal Cellular Function	48
2.5.3	Binding of PI Probes to Inositol Phosphates	48
2.6	Conclusion	48
	References	49
3	The Use of Lipid-Binding Toxins to Study the Distribution and Dynamics of Sphingolipids and Cholesterol	53
	<i>Reiko Ishitsuka and Toshihide Kobayashi</i>	
3.1	Introduction	53
3.2	Cholera Toxin	54
3.2.1	Introduction	54
3.2.2	CTB as a Tool to Study Cell Surface Lipid Rafts	55
3.2.2.1	Use of CTB for Biophysical Characterization of Lipid Rafts	55
3.2.2.2	Electron Microscopic Studies Using CTB	56
3.2.3	Intracellular Trafficking of CT	56
3.2.4	Protocols	57

3.2.4.1	Materials	57
3.2.4.2	Observation of Trafficking of GM1 in Living Cells Using CTB	57
3.3	Lysenin	59
3.3.1	Introduction	59
3.3.2	Lysenin Binds Clustered Sphingomyelin	59
3.3.3	Non-Toxic Lysenin as a Sphingomyelin Probe	60
3.3.4	Protocols	62
3.3.4.1	Materials	62
3.3.4.2	Cellular Staining of Sphingomyelin by Lysenin	62
3.3.4.3	Observation of Sphingomyelin on the Plasma Membrane of Living Cells Using Non-Toxic Lysenin	63
3.4	Perfringolysin O	63
3.4.1	Introduction	63
3.4.2	Non-Toxic Derivatives of PFO Bind Cholesterol-Rich Domains	64
3.4.3	Use of BC0 to Detect Cholesterol-Rich Domains	64
3.4.4	Protocol	65
3.4.4.1	Materials	65
3.4.4.2	Staining of Cholesterol-Rich Domain in the Plasma Membrane Using BC0	65
3.5	Aerolysin	66
3.5.1	Introduction	66
3.5.2	Use of Aerolysin to Detect GPI-Anchored Proteins	66
	References	67
4	"FLAsH" Protein Labeling	73
	<i>Stefan Jakobs, Martin Andresen, and Christian A. Wurm</i>	
4.1	Introduction	73
4.1.1	The Biarsenical-Tetracysteine System	74
4.1.2	Improved TetCys Motifs	75
4.1.3	Fluorescent Biarsenical Ligands	75
4.1.4	Applications of the Biarsenical-Tetracysteine System	76
4.1.5	Staining in Various Model Organisms	78
4.1.6	FLAsH Labeling in <i>S. cerevisiae</i>	79
4.1.7	Outlook	81
4.2	Use of the Biarsenical-Tetracysteine System in <i>S. cerevisiae</i>	82
4.2.1	Materials	82
4.2.1.1	Growth Media	82
4.2.1.2	Buffers (Required Stock Solutions)	82
4.2.2	Labeling Protocols	83
4.2.2.1	Overnight Staining	83
4.2.2.2	Pulse Staining	84
4.2.2.3	Mounting and Microscopy	84
4.3	Short Protocols	85
4.3.1	Overnight Staining	85
4.3.2	Pulse Staining	85

4.4	Troubleshooting	86
	References	88
5	ACT/SNAP-Tag: A Versatile Tag for Covalent Protein Labeling	89
	<i>Arnaud Cautier, Kai Johnsson, and Helen O'Hare</i>	
5.1	Introduction	89
5.2	Labeling SNAP-Tag Fusion Proteins with BG Derivatives	90
5.2.1	Human <i>O</i> ⁶ -Alkylguanine-DNA Alkyltransferase	90
5.2.2	The Principle of SNAP-Tag Labeling	91
5.3	SNAP-Tag for Cell Imaging	95
5.4	Procedures for SNAP-Tag Labeling	97
5.4.1	Standard Protocol for Fluorescent Imaging of SNAP-Tagged Proteins in Transiently Transfected Adherent Mammalian Cell Culture	97
5.4.2	Technical Notes	99
5.4.2.1	Counterstaining or Fixing	99
5.4.2.2	Photobleaching	99
5.4.2.3	Checking Expression of the Fusion Protein	99
5.4.2.4	Labeling AGT <i>in vitro</i>	100
5.4.2.5	Pulse-Chase Labeling	100
5.4.2.6	Labeling on the Cell Surface Using Non-Permeable Dyes (BG-FL, BG-Cy3, BG-Cy5)	100
5.4.2.7	Labeling Intracellular Proteins Using Non-Permeable Dyes	101
5.4.2.8	Multicolor Labeling of More Than One Protein	101
5.4.2.9	Measuring Protein-Protein Interactions or Conformational Changes by FRET	101
5.4.2.10	Sensors	102
5.5	Broader Applications of SNAP-Tag to Study Protein Function	102
5.5.1	Induction of Protein Dimerization by Covalent Labeling in Living Cells	102
5.5.2	SNAP-tag-Mediated Covalent Immobilization of Fusion Proteins on BG-Functionalized Surfaces	104
5.6	Conclusion	105
	References	106
6	Trimethoprim Derivatives for Labeling Dihydrofolate Reductase Fusion Proteins in Living Mammalian Cells	109
	<i>Lawrence W. Miller and Virginia W. Cornish</i>	
6.1	Introduction	109
6.2	Preparation of <i>E. coli</i> Expression Vectors	112
6.2.1	Materials	112
6.2.2	Plasmids for Over-Expression of <i>E. coli</i> DHFR Fusion Proteins in Mammalian Cells	112
6.2.2.1	Nucleus-Localized eDHFR Expression Plasmid (pLM1302). Construction of Nucleus-Localized eDHFR Vector (Plasmid pLM1302)	112

6.2.2.2	Myosin Light Chain Kinase eDHFR Plasmid	112
6.3	Synthesis of Fluorescent Trimethoprim Derivatives	113
6.4	Cell Growth and Transfection	114
6.5	Protein Labeling and Microscopy	114
6.6	Results and Discussion	115
6.7	Conclusion	117
	References	118
7	Phosphopantetheinyl Transferase Catalyzed Protein Labeling and Molecular Imaging	121
	<i>Norman J. Marshall and Jun Yin</i>	
7.1	Introduction	121
7.2	Protein Posttranslational Modification by Phosphopantetheinyl Transferases	123
7.3	Protein Labeling Via Carrier Protein Fusions	127
7.4	Orthogonal Protein Labeling by Short Peptide Tags	131
7.5	Summary and Perspectives	133
	References	134
8	Bioorthogonal Chemical Transformations in Proteins by an Expanded Genetic Code	139
	<i>Birgit Wiltschi and Nediljko Budisa</i>	
8.1	Introduction	139
8.2	Chemical Transformations at the Protein N-terminus Classical Approaches	140
8.2.1	Biomimetic Transamination	140
8.2.2	Enzymatic Modification of the N-terminus	143
8.3	Chemical Transformations Using an Expanded Genetic Code	145
8.3.1	The Genetic Code and Its Expansion	145
8.3.2	Cell-free N-terminal Labeling with Modified Initiator tRNA	147
8.3.3	Cell-free N-terminal Labeling with Suppressor Initiator tRNA	149
8.4	Modifications Internal to Proteins	149
8.5	Chemical Transformations at the Protein C-terminus	155
8.6	Summary and Outlook	158
	References	159
9	Using the Bacteriophage MS2 Coat Protein-RNA Binding Interaction to Visualize RNA in Living Cells	163
	<i>Jeffrey A. Chao, Kevin Czaplinski, and Robert H. Singer</i>	
9.1	Introduction	163
9.2	Construction of an MBS-Containing Reporter RNA	165
9.3	Construction of an MS2 CP-FP Chimera	166
9.4	Co-introduction of MS2 Reporter RNA and MS2 CP-FP	168
9.5	Microscopy Platform for Single Molecule Detection of RNAs in Living Cells	168

9.5.1	Components of the Imaging System	169
9.6	Protocols for Co-expressing MS2 CP-FP- and MBS-Containing Plasmids for Live Cell Imaging	170
9.7	Image Analysis and Quantification of mRNA Molecules	171
	References	172
	Index	175