

## Contents

### Preface IX

<b>1</b>	<b>Basic Principles of Fluorescence Spectroscopy</b>	<b>1</b>
1.1	Absorption and Emission of Light	1
1.2	Spectroscopic Transition Strengths	5
1.3	Lambert–Beer Law and Absorption Spectroscopy	7
1.4	Fluorophore Dimerization and Isosbestic Points	9
1.5	Franck–Condon Principle	12
1.6	Temperature Effects on Absorption and Emission Spectra	15
1.7	Fluorescence and Competing Processes	17
1.8	Stokes Shift, Solvent Relaxation, and Solvatochroism	20
1.9	Fluorescence Quantum Yield and Lifetime	22
1.10	Fluorescence Anisotropy	27
	References	29
<b>2</b>	<b>Fluorophores and Fluorescent Labels</b>	<b>31</b>
2.1	Natural Fluorophores	31
2.2	Organic Fluorophores	35
2.3	Different Fluorophore Classes	38
2.4	Multichromophoric Labels	49
2.5	Nanocrystals	52
	References	56
<b>3</b>	<b>Fluorophore Labeling for Single-Molecule Fluorescence Spectroscopy (SMFS)</b>	<b>61</b>
3.1	<i>In Vitro</i> Fluorescence Labeling	61
3.2	Fluorescence Labeling in Living Cells	69
	References	80
<b>4</b>	<b>Fluorophore Selection for Single-Molecule Fluorescence Spectroscopy (SMFS) and Photobleaching Pathways</b>	<b>85</b>
	References	91

<b>5</b>	<b>Fluorescence Correlation Spectroscopy</b>	93
5.1	Introduction	93
5.2	Optical Set-Up	98
5.3	Data Acquisition and Evaluation	99
5.4	Milliseconds to Seconds: Diffusion and Concentration	103
5.4.1	Single-Focus FCS	104
5.4.2	Dual-Focus FCS	111
5.5	Nanoseconds to Microseconds: Photophysics, Conformational Fluctuations, Binding Dynamics	120
5.6	Picoseconds to Nanoseconds: Rotational Diffusion and Fluorescence Antibunching	122
5.6.1	Antibunching	122
5.6.2	Rotational Diffusion	125
5.7	Fluorescence Lifetime Correlation Spectroscopy	135
5.8	Conclusion	143
	References	143
<b>6</b>	<b>Excited State Energy Transfer</b>	147
6.1	Introduction	147
6.2	Theory of (Förster) Energy Transfer	148
6.2.1	Mechanism and Mathematical Formalism of FRET	148
6.2.2	Measuring FRET Efficiencies Through Excited-State Lifetimes	153
6.2.3	Spin Rules for FRET	154
6.2.4	Homo-FRET and FRET-Induced Depolarization	154
6.3	Experimental Approach for Single-Pair FRET-Experiments	157
6.3.1	Single-Laser Excitation	157
6.3.2	Alternating-Laser Excitation (ALEX)	160
6.4	Examples and Applications of FRET	161
6.4.1	FRET Processes in Bulk Experiments	162
6.4.1.1	FRET-Based Molecular Biosensors	162
6.4.1.2	Energy Hopping and Trapping in Chromophore-Substituted Polyphenylene Dendrimers	164
6.4.2	Single-Molecule Observation of FRET	168
6.4.2.1	Light-Harvesting Systems: Phycobilisomes and Allophycocyanins	168
6.4.2.2	Hairpin Ribozyme Dynamics and Activity	179
6.4.2.3	Protein (Un)folding and Dynamics	180
	References	183
<b>7</b>	<b>Photoinduced Electron Transfer (PET) Reactions</b>	189
7.1	Fluorescence Quenching by PET	189
7.2	Single-Molecule Fluorescence Spectroscopy to Study PET	192
7.3	Single-Molecule Sensitive Fluorescence Sensors Based on PET	199
7.4	PET Reporter System	202
7.5	Monitoring Conformational Dynamics and Protein Folding by PET	205

7.6	Biological and Diagnostic Applications	209
	References	215
<b>8</b>	<b>Super-Resolution Fluorescence Imaging</b>	219
8.1	Diffraction Barrier of Optical Microscopy	219
8.2	Multi-Photon and Structured Illumination Microscopy	221
8.3	Stimulated Emission Depletion	223
8.4	Single-Molecule Based Photoswitching Microscopy	226
8.5	Background and Principles of Single-Molecule Based Photoswitching Microscopy Methods	229
8.6	Temporal Resolution of Super-Resolution Imaging Methods	236
	References	237
<b>9</b>	<b>Single-Molecule Enzymatics</b>	241
9.1	Introduction: Why Study Enzymes on a Single-Molecule Level?	241
9.2	Biochemical Principles of Enzymatic Activity: the Michaelis–Menten Model	242
9.3	“Looking” at Individual Enzymes	243
9.3.1	Single-Enzyme Studies and Kinetics	244
9.3.1.1	Space-Resolved, but Time-Averaged Single Enzyme Assays	244
9.3.1.2	Single-Turnover Experiments: Space- and Time-Resolved Enzyme Assays	248
9.3.1.3	Results: Revision of the Classical Michaelis–Menten Model	255
9.3.2	Conformational Dynamics	259
9.3.3	Single-Molecule DNA Sequencing	260
9.3.4	Shedding Light on Single-Enzyme Mechanisms	262
9.3.4.1	Movement of Molecular Motor Enzymes on Actin Filaments	263
9.3.4.2	Lipase-Catalyzed Hydrolysis of Phospholipid Bilayers	264
9.4	Data Analysis of Fluorescence Intensity Time Traces of Single-Turnover Experiments	264
9.4.1	Threshold Method	264
9.4.2	Autocorrelation Analysis	267
9.5	Conclusions	267
	References	268
	<b>Index</b>	273

