

Contents

I. Introduction to Fluorescence

1.1. Phenomena of Fluorescence.....	1
1.2. Jablonski Diagram.....	3
1.3. Characteristics of Fluorescence Emission.....	6
1.3.1. The Stokes Shift.....	6
1.3.2. Emission Spectra Are Typically Independent of the Excitation Wavelength.....	7
1.3.3. Exceptions to the Mirror-Image Rule.....	8
1.4. Fluorescence Lifetimes and Quantum Yields.....	9
1.4.1. Fluorescence Quenching.....	11
1.4.2. Timescale of Molecular Processes in Solution.....	12
1.5. Fluorescence Anisotropy.....	12
1.6. Resonance Energy Transfer.....	13
1.7. Steady-State and Time-Resolved Fluorescence.....	14
1.7.1. Why Time-Resolved Measurements?.....	15
1.8. Biochemical Fluorophores.....	15
1.8.1. Fluorescent Indicators.....	16
1.9. Molecular Information from Fluorescence.....	17
1.9.1. Emission Spectra and the Stokes Shift.....	17
1.9.2. Quenching of Fluorescence.....	18
1.9.3. Fluorescence Polarization or Anisotropy.....	19
1.9.4. Resonance Energy Transfer.....	19
1.10. Biochemical Examples of Basic Phenomena.....	20
1.11. New Fluorescence Technologies.....	21
1.11.1. Multiphoton Excitation.....	21
1.11.2. Fluorescence Correlation Spectroscopy.....	22
1.11.3. Single-Molecule Detection.....	23
1.12. Overview of Fluorescence Spectroscopy.....	24
References.....	25
Problems.....	25

2. Instrumentation for Fluorescence Spectroscopy

2.1. Spectrofluorometers.....	27
2.1.1. Spectrofluorometers for Spectroscopy Research.....	27
2.1.2. Spectrofluorometers for High Throughput ...	29
2.1.3. An Ideal Spectrofluorometer.....	30
2.1.4. Distortions in Excitation and Emission Spectra.....	30

2.2. Light Sources.....	31
2.2.1. Arc Lamps and Incandescent Xenon Lamps.....	31
2.2.2. Pulsed Xenon Lamps.....	32
2.2.3. High-Pressure Mercury (Hg) Lamps.....	33
2.2.4. Xe-Hg Arc Lamps.....	33
2.2.5. Quartz-Tungsten Halogen (QTH) Lamps.....	33
2.2.6. Low-Pressure Hg and Hg-Ar Lamps.....	33
2.2.7. LED Light Sources.....	33
2.2.8. Laser Diodes.....	34
2.3. Monochromators.....	34
2.3.1. Wavelength Resolution and Emission Spectra.....	35
2.3.2. Polarization Characteristics of Monochromators.....	36
2.3.3. Stray Light in Monochromators.....	36
2.3.4. Second-Order Transmission in Monochromators.....	37
2.3.5. Calibration of Monochromators.....	38
2.4. Optical Filters.....	38
2.4.1. Colored Filters.....	38
2.4.2. Thin-Film Filters.....	39
2.4.3. Filter Combinations.....	40
2.4.4. Neutral-Density Filters.....	40
2.4.5. Filters for Fluorescence Microscopy.....	41
2.5. Optical Filters and Signal Purity.....	41
2.5.1. Emission Spectra Taken through Filters.....	43
2.6. Photomultiplier Tubes.....	44
2.6.1. Spectral Response of PMTs.....	45
2.6.2. PMT Designs and Dynode Chains.....	46
2.6.3. Time Response of Photomultiplier Tubes.....	47
2.6.4. Photon Counting versus Analog Detection of Fluorescence.....	48
2.6.5. Symptoms of PMT Failure.....	49
2.6.6. CCD Detectors.....	49
2.7. Polarizers.....	49
2.8. Corrected Excitation Spectra.....	51
2.8.1. Corrected Excitation Spectra Using a Quantum Counter.....	51
2.9. Corrected Emission Spectra.....	52
2.9.1. Comparison with Known Emission Spectra.....	52
2.9.2. Corrections Using a Standard Lamp.....	53
2.9.3. Correction Factors Using a Quantum Counter and Scatterer.....	53

2.9.4. Conversion between Wavelength and Wavenumber.....	53	4.1.3. Examples of Time-Domain and Frequency-Domain Lifetimes	100
2.10. Quantum Yield Standards	54	4.2. Biopolymers Display Multi-Exponential or Heterogeneous Decays	101
2.11. Effects of Sample Geometry	55	4.2.1. Resolution of Multi-Exponential Decays Is Difficult	103
2.12. Common Errors in Sample Preparation	57	4.3. Time-Correlated Single-Photon Counting	103
2.13. Absorption of Light and Deviation from the Beer-Lambert Law.....	58	4.3.1. Principles of TCSPC	104
2.13.1. Deviations from Beer's Law.....	59	4.3.2. Example of TCSPC Data	105
2.14. Conclusions	59	4.3.3. Convolution Integral.....	106
References	59	4.4. Light Sources for TCSPC	107
Problems	60	4.4.1. Laser Diodes and Light-Emitting Diodes	107
		4.4.2. Femtosecond Titanium Sapphire Lasers	108
		4.4.3. Picosecond Dye Lasers	110
		4.4.4. Flashlamps.....	112
		4.4.5. Synchrotron Radiation	114
		4.5. Electronics for TCSPC.....	114
		4.5.1. Constant Fraction Discriminators	114
		4.5.2. Amplifiers.....	115
		4.5.3. Time-to-Amplitude Converter (TAC) and Analyte-to-Digital Converter (ADC).....	115
		4.5.4. Multichannel Analyzer	116
		4.5.5. Delay Lines	116
		4.5.6. Pulse Pile-Up.....	116
		4.6. Detectors for TCSPC.....	117
		4.6.1. Microchannel Plate PMTs.....	117
		4.6.2. Dynode Chain PMTs.....	118
		4.6.3. Compact PMTs.....	118
		4.6.4. Photodiodes as Detectors	118
		4.6.5. Color Effects in Detectors.....	119
		4.6.6. Timing Effects of Monochromators	121
		4.7. Multi-Detector and Multidimensional TCSPC	121
		4.7.1. Multidimensional TCSPC and DNA Sequencing.....	123
		4.7.2. Dead Times, Repetition Rates, and Photon Counting Rates.....	124
		4.8. Alternative Methods for Time-Resolved Measurements.....	124
		4.8.1. Transient Recording	124
		4.8.2. Streak Cameras.....	125
		4.8.3. Upconversion Methods.....	128
		4.8.4. Microsecond Luminescence Decays	129
		4.9. Data Analysis: Nonlinear Least Squares.....	129
		4.9.1. Assumptions of Nonlinear Least Squares	130
		4.9.2. Overview of Least-Squares Analysis	130
		4.9.3. Meaning of the Goodness-of-Fit	131
		4.9.4. Autocorrelation Function	132
		4.10. Analysis of Multi-Exponential Decays	133
		4.10.1. p-Terphenyl and Indole: Two Widely Spaced Lifetimes	133
		4.10.2. Comparison of χ_R^2 Values: <i>F</i> Statistic	133
		4.10.3. Parameter Uncertainty: Confidence Intervals	134
		4.10.4. Effect of the Number of Photon Counts	135
		4.10.5. Anthranilic Acid and 2-Aminopurine: Two Closely Spaced Lifetimes.....	137
3. Fluorophores			
3.1. Intrinsic or Natural Fluorophores.....	63		
3.1.1. Fluorescence Enzyme Cofactors	63		
3.1.2. Binding of NADH to a Protein	65		
3.2. Extrinsic Fluorophores	67		
3.2.1. Protein-Labeling Reagents	67		
3.2.2. Role of the Stokes Shift in Protein Labeling.....	69		
3.2.3. Photostability of Fluorophores	70		
3.2.4. Non-Covalent Protein-Labeling Probes	71		
3.2.5. Membrane Probes.....	72		
3.2.6. Membrane Potential Probes	72		
3.3. Red and Near-Infrared (NIR) Dyes.....	74		
3.4. DNA Probes	75		
3.4.1. DNA Base Analogues	75		
3.5. Chemical Sensing Probes.....	78		
3.6. Special Probes	79		
3.6.1. Fluorogenic Probes.....	79		
3.6.2. Structural Analogues of Biomolecules.....	80		
3.6.3. Viscosity Probes	80		
3.7. Green Fluorescent Proteins	81		
3.8. Other Fluorescent Proteins	83		
3.8.1. Phytofluors: A New Class of Fluorescent Probes	83		
3.8.2. Phycobiliproteins.....	84		
3.8.3. Specific Labeling of Intracellular Proteins	86		
3.9. Long-Lifetime Probes	86		
3.9.1. Lanthanides	87		
3.9.2. Transition Metal-Ligand Complexes	88		
3.10. Proteins as Sensors	88		
3.11. Conclusion.....	89		
References	90		
Problems	94		
4. Time-Domain Lifetime Measurements			
4.1. Overview of Time-Domain and Frequency-Domain Measurements.....	98		
4.1.1. Meaning of the Lifetime or Decay Time	99		
4.1.2. Phase and Modulation Lifetimes	99		

4.10.6. Global Analysis: Multi-Wavelength Measurements.....	138
4.10.7. Resolution of Three Closely Spaced Lifetimes.....	138
4.11. Intensity Decay Laws.....	141
4.11.1. Multi-Exponential Decays.....	141
4.11.2. Lifetime Distributions.....	143
4.11.3. Stretched Exponentials.....	144
4.11.4. Transient Effects.....	144
4.12. Global Analysis.....	144
4.13. Applications of TCSPC.....	145
4.13.1. Intensity Decay for a Single Tryptophan Protein.....	145
4.13.2. Green Fluorescent Protein: Systematic Errors in the Data.....	145
4.13.3. Picosecond Decay Time.....	146
4.13.4. Chlorophyll Aggregates in Hexane.....	146
4.13.5. Intensity Decay of Flavin Adenine Dinucleotide (FAD).....	147
4.14. Data Analysis: Maximum Entropy Method.....	148
References.....	149
Problems.....	154

5. Frequency-Domain Lifetime Measurements

5.1. Theory of Frequency-Domain Fluorometry.....	158
5.1.1. Least-Squares Analysis of Frequency-Domain Intensity Decays.....	161
5.1.2. Global Analysis of Frequency-Domain Data.....	162
5.2. Frequency-Domain Instrumentation.....	163
5.2.1. History of Phase-Modulation Fluorometers.....	163
5.2.2. An MHz Frequency-Domain Fluorometer....	164
5.2.3. Light Modulators.....	165
5.2.4. Cross-Correlation Detection.....	166
5.2.5. Frequency Synthesizers.....	167
5.2.6. Radio Frequency Amplifiers.....	167
5.2.7. Photomultiplier Tubes.....	167
5.2.8. Frequency-Domain Measurements.....	168
5.3. Color Effects and Background Fluorescence.....	168
5.3.1. Color Effects in Frequency-Domain Measurements.....	168
5.3.2. Background Correction in Frequency-Domain Measurements.....	169
5.4. Representative Frequency-Domain Intensity Decays.....	170
5.4.1. Exponential Decays.....	170
5.4.2. Multi-Exponential Decays of Staphylococcal Nuclease and Melittin.....	171
5.4.3. Green Fluorescent Protein: One- and Two-Photon Excitation.....	171
5.4.4. SPQ: Collisional Quenching of a Chloride Sensor.....	171
5.4.5. Intensity Decay of NADH.....	172
5.4.6. Effect of Scattered Light.....	172

5.5. Simple Frequency-Domain Instruments.....	173
5.5.1. Laser Diode Excitation.....	174
5.5.2. LED Excitation.....	174
5.6. Gigahertz Frequency-Domain Fluorometry.....	175
5.6.1. Gigahertz FD Measurements.....	177
5.6.2. Biochemical Examples of Gigahertz FD Data.....	177
5.7. Analysis of Frequency-Domain Data.....	178
5.7.1. Resolution of Two Widely Spaced Lifetimes.....	178
5.7.2. Resolution of Two Closely Spaced Lifetimes.....	180
5.7.3. Global Analysis of a Two-Component Mixture.....	182
5.7.4. Analysis of a Three-Component Mixture: Limits of Resolution.....	183
5.7.5. Resolution of a Three-Component Mixture with a Tenfold Range of Decay Times.....	185
5.7.6. Maximum Entropy Analysis of FD Data.....	185
5.8. Biochemical Examples of Frequency-Domain Intensity Decays.....	186
5.8.1. DNA Labeled with DAPI.....	186
5.8.2. Mag-Quin-2: A Lifetime-Based Sensor for Magnesium.....	187
5.8.3. Recovery of Lifetime Distributions from Frequency-Domain Data.....	188
5.8.4. Cross-Fitting of Models: Lifetime Distributions of Melittin.....	188
5.8.5. Frequency-Domain Fluorescence Microscopy with an LED Light Source.....	189
5.9. Phase-Angle and Modulation Spectra.....	189
5.10. Apparent Phase and Modulation Lifetimes.....	191
5.11. Derivation of the Equations for Phase-Modulation Fluorescence.....	192
5.11.1. Relationship of the Lifetime to the Phase Angle and Modulation.....	192
5.11.2. Cross-Correlation Detection.....	194
5.12. Phase-Sensitive Emission Spectra.....	194
5.12.1. Theory of Phase-Sensitive Detection of Fluorescence.....	195
5.12.2. Examples of PSDF and Phase Suppression.....	196
5.12.3. High-Frequency or Low-Frequency Phase-Sensitive Detection.....	197
5.13. Phase-Modulation Resolution of Emission Spectra.....	197
5.13.1. Resolution Based on Phase or Modulation Lifetimes.....	198
5.13.2. Resolution Based on Phase Angles and Modulations.....	198
5.13.3. Resolution of Emission Spectra from Phase and Modulation Spectra.....	198
References.....	199
Problems.....	203

6. Solvent and Environmental Effects	
6.1. Overview of Solvent Polarity Effects.....	205
6.1.1. Effects of Solvent Polarity	205
6.1.2. Polarity Surrounding a Membrane-Bound Fluorophore	206
6.1.3. Other Mechanisms for Spectral Shifts	207
6.2. General Solvent Effects: The Lippert-Mataga Equation	208
6.2.1. Derivation of the Lippert Equation	210
6.2.2. Application of the Lippert Equation	212
6.3. Specific Solvent Effects	213
6.3.1. Specific Solvent Effects and Lippert Plots ...	215
6.4. Temperature Effects	216
6.5. Phase Transitions in Membranes	217
6.6. Additional Factors that Affect Emission Spectra....	219
6.6.1. Locally Excited and Internal Charge-Transfer States	219
6.6.2. Excited-State Intramolecular Proton Transfer (ESIPT)	221
6.6.3. Changes in the Non-Radiative Decay Rates.....	222
6.6.4. Changes in the Rate of Radiative Decay	223
6.7. Effects of Viscosity	223
6.7.1. Effect of Shear Stress on Membrane Viscosity	225
6.8. Probe-Probe Interactions	225
6.9. Biochemical Applications of Environment- Sensitive Fluorophores	226
6.9.1. Fatty-Acid-Binding Proteins	226
6.9.2. Exposure of a Hydrophobic Surface on Calmodulin	226
6.9.3. Binding to Cyclodextrin Using a Dansyl Probe	227
6.10. Advanced Solvent-Sensitive Probes	228
6.11. Effects of Solvent Mixtures.....	229
6.12. Summary of Solvent Effects.....	231
References	232
Problems	235
7. Dynamics of Solvent and Spectral Relaxation	
7.1. Overview of Excited-State Processes.....	237
7.1.1. Time-Resolved Emission Spectra	239
7.2. Measurement of Time-Resolved Emission Spectra (TRES)	240
7.2.1. Direct Recording of TRES	240
7.2.2. TRES from Wavelength-Dependent Decays	241
7.3. Spectral Relaxation in Proteins.....	242
7.3.1. Spectral Relaxation of Labeled Apomyoglobin.....	243
7.3.2. Protein Spectral Relaxation around a Synthetic Fluorescent Amino Acid	244
7.4. Spectral Relaxation in Membranes	245
7.4.1. Analysis of Time-Resolved Emission Spectra.....	246
7.4.2. Spectral Relaxation of Membrane-Bound Anthroxyloxy Fatty Acids	248
7.5. Picosecond Relaxation in Solvents	249
7.5.1. Theory for Time-Dependent Solvent Relaxation.....	250
7.5.2. Multi-Exponential Relaxation in Water	251
7.6. Measurement of Multi-Exponential Spectral Relaxation.....	252
7.7. Distinction between Solvent Relaxation and Formation of Rotational Isomers	253
7.8. Comparison of TRES and Decay-Associated Spectra	255
7.9. Lifetime-Resolved Emission Spectra.....	255
7.10. Red-Edge Excitation Shifts	257
7.10.1. Membranes and Red-Edge Excitation Shifts	258
7.10.2. Red-Edge Excitation Shifts and Energy Transfer.....	259
7.11. Excited-State Reactions.....	259
7.11.1. Excited-State Ionization of Naphthol.....	260
7.12. Theory for a Reversible Two-State Reaction	262
7.12.1. Steady-State Fluorescence of a Two-State Reaction	262
7.12.2. Time-Resolved Decays for the Two-State Model	263
7.12.3. Differential Wavelength Methods	264
7.13. Time-Domain Studies of Naphthol Dissociation	264
7.14. Analysis of Excited-State Reactions by Phase-Modulation Fluorometry.....	265
7.14.1. Effect of an Excited-State Reaction on the Apparent Phase and Modulation Lifetimes.....	266
7.14.2. Wavelength-Dependent Phase and Modulation Values for an Excited-State Reaction.....	267
7.14.3. Frequency-Domain Measurement of Excimer Formation.....	269
7.15. Biochemical Examples of Excited-State Reactions	270
7.15.1. Exposure of a Membrane-Bound Cholesterol Analogue	270
References	270
Problems	275
8. Quenching of Fluorescence	
8.1. Quenchers of Fluorescence	278
8.2. Theory of Collisional Quenching.....	278
8.2.1. Derivation of the Stern-Volmer Equation	280
8.2.2. Interpretation of the Bimolecular Quenching Constant	281
8.3. Theory of Static Quenching	282
8.4. Combined Dynamic and Static Quenching.....	282
8.5. Examples of Static and Dynamic Quenching	283
8.6. Deviations from the Stern-Volmer Equation: Quenching Sphere of Action	284
8.6.1. Derivation of the Quenching Sphere of Action.....	285

8.7. Effects of Steric Shielding and Charge on Quenching	286
8.7.1. Accessibility of DNA-Bound Probes to Quenchers.....	286
8.7.2. Quenching of Ethenoadenine Derivatives.....	287
8.8. Fractional Accessibility to Quenchers.....	288
8.8.1. Modified Stern-Volmer Plots	288
8.8.2. Experimental Considerations in Quenching	289
8.9. Applications of Quenching to Proteins	290
8.9.1. Fractional Accessibility of Tryptophan Residues in Endonuclease III.....	290
8.9.2. Effect of Conformational Changes on Tryptophan Accessibility.....	291
8.9.3. Quenching of the Multiple Decay Times of Proteins	291
8.9.4. Effects of Quenchers on Proteins.....	292
8.9.5. Correlation of Emission Wavelength and Accessibility: Protein Folding of Colicin E1.....	292
8.10. Application of Quenching to Membranes	293
8.10.1. Oxygen Diffusion in Membranes.....	293
8.10.2. Localization of Membrane-Bound Tryptophan Residues by Quenching	294
8.10.3. Quenching of Membrane Probes Using Localized Quenchers	295
8.10.4. Parallax and Depth-Dependent Quenching in Membranes	296
8.10.5. Boundary Lipid Quenching.....	298
8.10.6. Effect of Lipid-Water Partitioning on Quenching	298
8.10.7. Quenching in Micelles	300
8.11. Lateral Diffusion in Membranes	300
8.12. Quenching-Resolved Emission Spectra	301
8.12.1. Fluorophore Mixtures.....	301
8.12.2. Quenching-Resolved Emission Spectra of the <i>E. Coli</i> Tet Repressor.....	302
8.13. Quenching and Association Reactions	304
8.13.1. Quenching Due to Specific Binding Interactions	304
8.14. Sensing Applications of Quenching	305
8.14.1. Chloride-Sensitive Fluorophores.....	306
8.14.2. Intracellular Chloride Imaging.....	306
8.14.3. Chloride-Sensitive GFP.....	307
8.14.4. Amplified Quenching	309
8.15. Applications of Quenching to Molecular Biology	310
8.15.1. Release of Quenching upon Hybridization.....	310
8.15.2. Molecular Beacons in Quenching by Guanine	311
8.15.3. Binding of Substrates to Ribozymes.....	311
8.15.4. Association Reactions and Accessibility to Quenchers.....	312
8.16. Quenching on Gold Surfaces.....	313
8.16.1. Molecular Beacons Based on Quenching by Gold Colloids	313

8.16.2. Molecular Beacons Based on Quenching by a Gold Surface.....	314
8.17. Intramolecular Quenching	314
8.17.1. DNA Dynamics by Intramolecular Quenching	314
8.17.2. Electron-Transfer Quenching in a Flavoprotein.....	315
8.17.3. Sensors Based on Intramolecular PET Quenching	316
8.18. Quenching of Phosphorescence	317
References	318
Problems	327

9. Mechanisms and Dynamics of Fluorescence Quenching

9.1. Comparison of Quenching and Resonance Energy Transfer	331
9.1.1. Distance Dependence of RET and Quenching	332
9.1.2. Encounter Complexes and Quenching Efficiency	333
9.2. Mechanisms of Quenching.....	334
9.2.1. Intersystem Crossing.....	334
9.2.2. Electron-Exchange Quenching.....	335
9.2.3. Photoinduced Electron Transfer.....	335
9.3. Energetics of Photoinduced Electron Transfer	336
9.3.1. Examples of PET Quenching.....	338
9.3.2. PET in Linked Donor-Acceptor Pairs	340
9.4. PET Quenching in Biomolecules.....	341
9.4.1. Quenching of Indole by Imidazolium	341
9.4.2. Quenching by DNA Bases and Nucleotides	341
9.5. Single-Molecule PET	342
9.6. Transient Effects in Quenching.....	343
9.6.1. Experimental Studies of Transient Effects.....	346
9.6.2. Distance-Dependent Quenching in Proteins.....	348
References	348
Problems	351

10. Fluorescence Anisotropy

10.1. Definition of Fluorescence Anisotropy	353
10.1.1. Origin of the Definitions of Polarization and Anisotropy	355
10.2. Theory for Anisotropy	355
10.2.1. Excitation Photoselection of Fluorophores.....	357
10.3. Excitation Anisotropy Spectra.....	358
10.3.1. Resolution of Electronic States from Polarization Spectra	360
10.4. Measurement of Fluorescence Anisotropies	361
10.4.1. L-Format or Single-Channel Method.....	361
10.4.2. T-Format or Two-Channel Anisotropies.....	363
10.4.3. Comparison of T-Format and L-Format Measurements	363

10.4.4. Alignment of Polarizers.....	364	11.4.6. Example Anisotropy Decays of Rhodamine Green and Rhodamine Green-Dextran.....	394
10.4.5. Magic-Angle Polarizer Conditions.....	364	11.5. Time-Domain Anisotropy Decays of Proteins.....	394
10.4.6. Why is the Total Intensity Equal to $I_{\parallel} + 2I_{\perp}$	364	11.5.1. Intrinsic Tryptophan Anisotropy Decay of Liver Alcohol Dehydrogenase.....	395
10.4.7. Effect of Resonance Energy Transfer on the Anisotropy.....	364	11.5.2. Phospholipase A ₂	395
10.4.8. Trivial Causes of Depolarization.....	365	11.5.3. Subtilisin Carlsberg.....	395
10.4.9. Factors Affecting the Anisotropy.....	366	11.5.4. Domain Motions of Immunoglobulins.....	396
10.5. Effects of Rotational Diffusion on Fluorescence Anisotropies: The Perrin Equation.....	366	11.5.5. Effects of Free Probe on Anisotropy Decays.....	397
10.5.1. The Perrin Equation: Rotational Motions of Proteins.....	367	11.6. Frequency-Domain Anisotropy Decays of Proteins.....	397
10.5.2. Examples of a Perrin Plot.....	369	11.6.1. Apomyoglobin: A Rigid Rotor.....	397
10.6. Perrin Plots of Proteins.....	370	11.6.2. Melittin Self-Association and Anisotropy Decays.....	398
10.6.1. Binding of tRNA to tRNA Synthetase.....	370	11.6.3. Picosecond Rotational Diffusion of Oxytocin.....	399
10.6.2. Molecular Chaperonin cpn60 (GroEL).....	371	11.7. Hindered Rotational Diffusion in Membranes.....	399
10.6.3. Perrin Plots of an F _{ab} Immunoglobulin Fragment.....	371	11.7.1. Characterization of a New Membrane Probe.....	401
10.7. Biochemical Applications of Steady-State Anisotropies.....	372	11.8. Anisotropy Decays of Nucleic Acids.....	402
10.7.1. Peptide Binding to Calmodulin.....	372	11.8.1. Hydrodynamics of DNA Oligomers.....	403
10.7.2. Binding of the Trp Repressor to DNA.....	373	11.8.2. Dynamics of Intracellular DNA.....	403
10.7.3. Helicase-Catalyzed DNA Unwinding.....	373	11.8.3. DNA Binding to HIV Integrase Using Correlation Time Distributions.....	404
10.7.4. Melittin Association Detected from Homotransfer.....	374	11.9. Correlation Time Imaging.....	406
10.8. Anisotropy of Membranes and Membrane-Bound Proteins.....	374	11.10. Microsecond Anisotropy Decays.....	408
10.8.1. Membrane Microviscosity.....	374	11.10.1. Phosphorescence Anisotropy Decays.....	408
10.8.2. Distribution of Membrane-Bound Proteins.....	375	11.10.2. Long-Lifetime Metal-Ligand Complexes.....	408
10.9. Transition Moments.....	377	References.....	409
References.....	378	Problems.....	412
Additional Reading on the Application of Anisotropy.....	380		
Problems.....	381		
		12. Advanced Anisotropy Concepts	
11. Time-Dependent Anisotropy Decays		12.1. Associated Anisotropy Decay.....	413
11.1. Time-Domain and Frequency-Domain Anisotropy Decays.....	383	12.1.1. Theory for Associated Anisotropy Decay.....	414
11.2. Anisotropy Decay Analysis.....	387	12.1.2. Time-Domain Measurements of Associated Anisotropy Decays.....	415
11.2.1. Early Methods for Analysis of TD Anisotropy Data.....	387	12.2. Biochemical Examples of Associated Anisotropy Decays.....	417
11.2.2. Preferred Analysis of TD Anisotropy Data.....	388	12.2.1. Time-Domain Studies of DNA Binding to the Klenow Fragment of DNA Polymerase.....	417
11.2.3. Value of r_0	389	12.2.2. Frequency-Domain Measurements of Associated Anisotropy Decays.....	417
11.3. Analysis of Frequency-Domain Anisotropy Decays.....	390	12.3. Rotational Diffusion of Non-Spherical Molecules: An Overview.....	418
11.4. Anisotropy Decay Laws.....	390	12.3.1. Anisotropy Decays of Ellipsoids.....	419
11.4.1. Non-Spherical Fluorophores.....	391	12.4. Ellipsoids of Revolution.....	420
11.4.2. Hindered Rotors.....	391	12.4.1. Simplified Ellipsoids of Revolution.....	421
11.4.3. Segmental Mobility of a Biopolymer-Bound Fluorophore.....	392	12.4.2. Intuitive Description of Rotational Diffusion of an Oblate Ellipsoid.....	422
11.4.4. Correlation Time Distributions.....	393		
11.4.5. Associated Anisotropy Decays.....	393		

12.4.3. Rotational Correlation Times for Ellipsoids of Revolution.....	423
12.4.4. Stick-versus-Slip Rotational Diffusion	425
12.5. Complete Theory for Rotational Diffusion of Ellipsoids.....	425
12.6. Anisotropic Rotational Diffusion	426
12.6.1. Time-Domain Studies.....	426
12.6.2. Frequency-Domain Studies of Anisotropic Rotational Diffusion.....	427
12.7. Global Anisotropy Decay Analysis	429
12.7.1. Global Analysis with Multi-Wavelength Excitation	429
12.7.2. Global Anisotropy Decay Analysis with Collisional Quenching.....	430
12.7.3. Application of Quenching to Protein Anisotropy Decays	431
12.8. Intercalated Fluorophores in DNA	432
12.9. Transition Moments.....	433
12.9.1. Anisotropy of Planar Fluorophores with High Symmetry	435
12.10. Lifetime-Resolved Anisotropies	435
12.10.1. Effect of Segmental Motion on the Perrin Plots	436
12.11. Soleillet's Rule: Multiplication of Depolarized Factors	436
12.12. Anisotropies Can Depend on Emission Wavelength	437
References	438
Problems	441
13. Energy Transfer	
13.1. Characteristics of Resonance Energy Transfer	443
13.2. Theory of Energy Transfer for a Donor-Acceptor Pair.....	445
13.2.1. Orientation Factor κ^2	448
13.2.2. Dependence of the Transfer Rate on Distance (r), the Overlap Integral (J), and τ^2	449
13.2.3. Homotransfer and Heterotransfer.....	450
13.3. Distance Measurements Using RET	451
13.3.1. Distance Measurements in α -Helical Melittin	451
13.3.2. Effects of Incomplete Labeling.....	452
13.3.3. Effect of κ^2 on the Possible Range of Distances.....	452
13.4. Biochemical Applications of RET	453
13.4.1. Protein Folding Measured by RET	453
13.4.2. Intracellular Protein Folding	454
13.4.3. RET and Association Reactions.....	455
13.4.4. Orientation of a Protein-Bound Peptide.....	456
13.4.5. Protein Binding to Semiconductor Nanoparticles.....	457
13.5. RET Sensors	458
13.5.1. Intracellular RET Indicator for Estrogens	458

13.5.2. RET Imaging of Intracellular Protein Phosphorylation.....	459
13.5.3. Imaging of Rac Activation in Cells.....	459
13.6. RET and Nucleic Acids.....	459
13.6.1. Imaging of Intracellular RNA	460
13.7. Energy-Transfer Efficiency from Enhanced Acceptor Fluorescence.....	461
13.8. Energy Transfer in Membranes	462
13.8.1. Lipid Distributions around Gramicidin.....	463
13.8.2. Membrane Fusion and Lipid Exchange	465
13.9. Effect of κ^2 on RET.....	465
13.10. Energy Transfer in Solution	466
13.10.1. Diffusion-Enhanced Energy Transfer.....	467
13.11. Representative R_0 Values	467
References	468
Additional References on Resonance Energy Transfer.....	471
Problems	472

14. Time-Resolved Energy Transfer and Conformational Distributions of Biopolymers

14.1. Distance Distributions	477
14.2. Distance Distributions in Peptides	479
14.2.1. Comparison for a Rigid and Flexible Hexapeptide.....	479
14.2.2. Crossfitting Data to Exclude Alternative Models	481
14.2.3. Donor Decay without Acceptor	482
14.2.4. Effect of Concentration of the D-A Pairs	482
14.3. Distance Distributions in Peptides	482
14.3.1. Distance Distributions in Melittin.....	483
14.4. Distance-Distribution Data Analysis	485
14.4.1. Frequency-Domain Distance-Distribution Analysis	485
14.4.2. Time-Domain Distance-Distribution Analysis.....	487
14.4.3. Distance-Distribution Functions	487
14.4.4. Effects of Incomplete Labeling.....	487
14.4.5. Effect of the Orientation Factor κ^2	489
14.4.6. Acceptor Decays.....	489
14.5. Biochemical Applications of Distance Distributions	490
14.5.1. Calcium-Induced Changes in the Conformation of Troponin C	490
14.5.2. Hairpin Ribozyme	493
14.5.3. Four-Way Holliday Junction in DNA	493
14.5.4. Distance Distributions and Unfolding of Yeast Phosphoglycerate Kinase	494
14.5.5. Distance Distributions in a Glycopeptide ...	495
14.5.6. Single-Protein-Molecule Distance Distribution.....	496
14.6. Time-Resolved RET Imaging.....	497
14.7. Effect of Diffusion for Linked D-A Pairs.....	498

14.7.1. Simulations of FRET for a Flexible D–A Pair.....	499	16.3. Tryptophan Emission in an Apolar Protein Environment.....	538
14.7.2. Experimental Measurement of D–A Diffusion for a Linked D–A Pair.....	500	16.3.1. Site-Directed Mutagenesis of a Single-Tryptophan Azurin.....	538
14.7.3. FRET and Diffusive Motions in Biopolymers.....	501	16.3.2. Emission Spectra of Azurins with One or Two Tryptophan Residues.....	539
14.8. Conclusion.....	501	16.4. Energy Transfer and Intrinsic Protein Fluorescence.....	539
References.....	501	16.4.1. Tyrosine-to-Tryptophan Energy Transfer in Interferon- γ	540
Representative Publications on Measurement of Distance Distributions.....	504	16.4.2. Quantitation of RET Efficiencies in Proteins.....	541
Problems.....	505	16.4.3. Tyrosine-to-Tryptophan RET in a Membrane-Bound Protein.....	543
		16.4.4. Phenylalanine-to-Tyrosine Energy Transfer.....	543
15. Energy Transfer to Multiple Acceptors in One, Two, or Three Dimensions		16.5. Calcium Binding to Calmodulin Using Phenylalanine and Tyrosine Emission.....	545
15.1. RET in Three Dimensions.....	507	16.6. Quenching of Tryptophan Residues in Proteins.....	546
15.1.1. Effect of Diffusion on FRET with Unlinked Donors and Acceptors.....	508	16.6.1. Effect of Emission Maximum on Quenching.....	547
15.1.2. Experimental Studies of RET in Three Dimensions.....	509	16.6.2. Fractional Accessibility to Quenching in Multi-Tryptophan Proteins.....	549
15.2. Effect of Dimensionality on RET.....	511	16.6.3. Resolution of Emission Spectra by Quenching.....	550
15.2.1. Experimental FRET in Two Dimensions....	512	16.7. Association Reaction of Proteins.....	551
15.2.2. Experimental FRET in One Dimension.....	514	16.7.1. Binding of Calmodulin to a Target Protein.....	551
15.3. Biochemical Applications of RET with Multiple Acceptors.....	515	16.7.2. Calmodulin: Resolution of the Four Calcium-Binding Sites Using Tryptophan-Containing Mutants.....	552
15.3.1. Aggregation of β -Amyloid Peptides.....	515	16.7.3. Interactions of DNA with Proteins.....	552
15.3.2. RET Imaging of Fibronectin.....	516	16.8. Spectral Properties of Genetically Engineered Proteins.....	554
15.4. Energy Transfer in Restricted Geometries.....	516	16.8.1. Single-Tryptophan Mutants of Triosephosphate Isomerase.....	555
15.4.1. Effect of Excluded Area on Energy Transfer in Two Dimensions.....	518	16.8.2. Barnase: A Three-Tryptophan Protein.....	556
15.5. RET in the Presence of Diffusion.....	519	16.8.3. Site-Directed Mutagenesis of Tyrosine Proteins.....	557
15.6. RET in the Rapid Diffusion Limit.....	520	16.9. Protein Folding.....	557
15.6.1. Location of an Acceptor in Lipid Vesicles.....	521	16.9.1. Protein Engineering of Mutant Ribonuclease for Folding Experiments.....	558
15.6.2. Location of Retinal in Rhodopsin Disc Membranes.....	522	16.9.2. Folding of Lactate Dehydrogenase.....	559
15.7. Conclusions.....	524	16.9.3. Folding Pathway of CRABPI.....	560
References.....	524	16.10. Protein Structure and Tryptophan Emission.....	560
Additional References on RET between Unlinked Donor and Acceptor.....	526	16.10.1. Tryptophan Spectral Properties and Structural Motifs.....	561
Problems.....	527	16.11. Tryptophan Analogues.....	562
		16.11.1. Tryptophan Analogues.....	564
		16.11.2. Genetically Inserted Amino-Acid Analogues.....	565
16. Protein Fluorescence		16.12. The Challenge of Protein Fluorescence.....	566
16.1. Spectral Properties of the Aromatic Amino Acids... ..	530	References.....	567
16.1.1. Excitation Polarization Spectra of Tyrosine and Tryptophan.....	531	Problems.....	573
16.1.2. Solvent Effects on Tryptophan Emission Spectra.....	533		
16.1.3. Excited-State Ionization of Tyrosine.....	534		
16.1.4. Tyrosinate Emission from Proteins.....	535		
16.2. General Features of Protein Fluorescence.....	535		

17. Time-Resolved Protein Fluorescence

17.1. Intensity Decays of Tryptophan:
The Rotamer Model 578

17.2. Time-Resolved Intensity Decays of
Tryptophan and Tyrosine 580

17.2.1. Decay-Associated Emission Spectra
of Tryptophan 581

17.2.2. Intensity Decays of Neutral Tryptophan
Derivatives 581

17.2.3. Intensity Decays of Tyrosine and
Its Neutral Derivatives 582

17.3. Intensity and Anisotropy Decays of Proteins 583

17.3.1. Single-Exponential Intensity and
Anisotropy Decay of Ribonuclease T₁ 584

17.3.2. Annexin V: A Calcium-Sensitive
Single-Tryptophan Protein 585

17.3.3. Anisotropy Decay of a Protein with
Two Tryptophans 587

17.4. Protein Unfolding Exposes the Tryptophan
Residue to Water 588

17.4.1. Conformational Heterogeneity Can
Result in Complex Intensity and
Anisotropy Decays 588

17.5. Anisotropy Decays of Proteins 589

17.5.1. Effects of Association Reactions on
Anisotropy Decays: Melittin 590

17.6. Biochemical Examples Using Time-Resolved
Protein Fluorescence 591

17.6.1. Decay-Associated Spectra of Barnase 591

17.6.2. Disulfide Oxidoreductase DsbA 591

17.6.3. Immunophilin FKBP59-I: Quenching
of Tryptophan Fluorescence by
Phenylalanine 592

17.6.4. Trp Repressor: Resolution of the Two
Interacting Tryptophans 593

17.6.5. Thermophilic β -Glycosidase:
A Multi-Tryptophan Protein 594

17.6.6. Heme Proteins Display Useful
Intrinsic Fluorescence 594

17.7. Time-Dependent Spectral Relaxation of
Tryptophan 596

17.8. Phosphorescence of Proteins 598

17.9. Perspectives on Protein Fluorescence 600

References 600

Problems 605

18. Multiphoton Excitation and Microscopy

18.1. Introduction to Multiphoton Excitation 607

18.2. Cross-Sections for Multiphoton Absorption 609

18.3. Two-Photon Absorption Spectra 609

18.4. Two-Photon Excitation of a DNA-Bound
Fluorophore 610

18.5. Anisotropies with Multiphoton Excitation 612

18.5.1. Excitation Photoselection for
Two-Photon Excitation 612

18.5.2. Two-Photon Anisotropy of DPH 612

18.6. MPE for a Membrane-Bound Fluorophore 613

18.7. MPE of Intrinsic Protein Fluorescence 613

18.8. Multiphoton Microscopy 616

18.8.1. Calcium Imaging 616

18.8.2. Imaging of NAD(P)H and FAD 617

18.8.3. Excitation of Multiple Fluorophores 618

18.8.4. Three-Dimensional Imaging of Cells 618

References 619

Problems 621

19. Fluorescence Sensing

19.1. Optical Clinical Chemistry and Spectral
Observables 623

19.2. Spectral Observables for Fluorescence Sensing 624

19.2.1. Optical Properties of Tissues 625

19.2.2. Lifetime-Based Sensing 626

19.3. Mechanisms of Sensing 626

19.4. Sensing by Collisional Quenching 627

19.4.1. Oxygen Sensing 627

19.4.2. Lifetime-Based Sensing of Oxygen 628

19.4.3. Mechanism of Oxygen Selectivity 629

19.4.4. Other Oxygen Sensors 629

19.4.5. Lifetime Imaging of Oxygen 630

19.4.6. Chloride Sensors 631

19.4.7. Lifetime Imaging of Chloride
Concentrations 632

19.4.8. Other Collisional Quenchers 632

19.5. Energy-Transfer Sensing 633

19.5.1. pH and pCO₂ Sensing by
Energy Transfer 633

19.5.2. Glucose Sensing by Energy Transfer 634

19.5.3. Ion Sensing by Energy Transfer 635

19.5.4. Theory for Energy-Transfer Sensing 636

19.6. Two-State pH Sensors 637

19.6.1. Optical Detection of Blood Gases 637

19.6.2. pH Sensors 637

19.7. Photoinduced Electron Transfer (PET) Probes
for Metal Ions and Anion Sensors 641

19.8. Probes of Analyte Recognition 643

19.8.1. Specificity of Cation Probes 644

19.8.2. Theory of Analyte Recognition Sensing 644

19.8.3. Sodium and Potassium Probes 645

19.8.4. Calcium and Magnesium Probes 647

19.8.5. Probes for Intracellular Zinc 650

19.9. Glucose-Sensitive Fluorophores 650

19.10. Protein Sensors 651

19.10.1. Protein Sensors Based on RET 652

19.11. GFP Sensors 654

19.11.1. GFP Sensors Using RET 654

19.11.2. Intrinsic GFP Sensors 655

22.6. Frequency-Domain Laser Scanning Microscopy 750
 22.7. Conclusions 752
 References 752
 Additional Reading on Fluorescence-Lifetime
 Imaging Microscopy 753
 Problem..... 755

23. Single-Molecule Detection

23.1. Detectability of Single Molecules 759
 23.2. Total Internal Reflection and Confocal Optics..... 760
 23.2.1. Total Internal Reflection..... 760
 23.2.2. Confocal Detection Optics 761
 23.3. Optical Configurations for SMD..... 762
 23.4. Instrumentation for SMD 764
 23.4.1. Detectors for Single-Molecule Detection ... 765
 23.4.2. Optical Filters for SMD 766
 23.5. Single-Molecule Photophysics 768
 23.6. Biochemical Applications of SMD 770
 23.6.1. Single-Molecule Enzyme Kinetics..... 770
 23.6.2. Single-Molecule ATPase Activity 770
 23.6.3. Single-Molecule Studies of a
 Chaperonin Protein..... 771
 23.7. Single-Molecule Resonance Energy Transfer 773
 23.8. Single-Molecule Orientation and Rotational
 Motions..... 775
 23.8.1. Orientation Imaging of R6G and GFP..... 777
 23.8.2. Imaging of Dipole Radiation Patterns..... 778
 23.9. Time-Resolved Studies of Single Molecules 779
 23.10. Biochemical Applications..... 780
 23.10.1. Turnover of Single Enzyme Molecules... 780
 23.10.2. Single-Molecule Molecular Beacons 782
 23.10.3. Conformational Dynamics of a
 Holliday Junction 782
 23.10.4. Single-Molecule Calcium Sensor..... 784
 23.10.5. Motions of Molecular Motors 784
 23.11. Advanced Topics in SMD..... 784
 23.11.1. Signal-to-Noise Ratio in
 Single-Molecule Detection..... 784
 23.11.2. Polarization of Single Immobilized
 Fluorophores..... 786
 23.11.3. Polarization Measurements
 and Mobility of Surface-Bound
 Fluorophores..... 786
 23.11.4. Single-Molecule Lifetime Estimation 787
 23.12. Additional Literature on SMD 788
 References 788
 Additional References on Single-Molecule
 Detection 791
 Problem..... 795

24. Fluorescence Correlation Spectroscopy

24.1. Principles of Fluorescence Correlation
 Spectroscopy..... 798

24.2. Theory of FCS 800
 24.2.1. Translational Diffusion and FCS..... 802
 24.2.2. Occupation Numbers and Volumes
 in FCS..... 804
 24.2.3. FCS for Multiple Diffusing Species 804
 24.3. Examples of FCS Experiments 805
 24.3.1. Effect of Fluorophore Concentration 805
 24.3.2. Effect of Molecular Weight on
 Diffusion Coefficients 806
 24.4. Applications of FCS to Bioaffinity Reactions..... 807
 24.4.1. Protein Binding to the
 Chaperonin GroEL 807
 24.4.2. Association of Tubulin Subunits 807
 24.4.3. DNA Applications of FCS 808
 24.5. FCS in Two Dimensions: Membranes 810
 24.5.1. Biophysical Studies of Lateral
 Diffusion in Membranes 812
 24.5.2. Binding to Membrane-Bound
 Receptors 813
 24.6. Effects of Intersystem Crossing 815
 24.6.1. Theory for FCS and Intersystem
 Crossing..... 816
 24.7. Effects of Chemical Reactions 816
 24.8. Fluorescence Intensity Distribution Analysis..... 817
 24.9. Time-Resolved FCS 819
 24.10. Detection of Conformational Dynamics
 in Macromolecules 820
 24.11. FCS with Total Internal Reflection 821
 24.12. FCS with Two-Photon Excitation..... 822
 24.12.1. Diffusion of an Intracellular
 Kinase Using FCS with
 Two-Photon Excitation..... 823
 24.13. Dual-Color Fluorescence Cross-Correlation
 Spectroscopy..... 823
 24.13.1. Instrumentation for Dual-Color
 FCCS 824
 24.13.2. Theory of Dual-Color FCCS..... 824
 24.13.3. DNA Cleavage by a
 Restriction Enzyme 826
 24.13.4. Applications of Dual-Color FCCS 826
 24.14. Rotational Diffusion and Photo Antibunching 828
 24.15. Flow Measurements Using FCS..... 830
 24.16. Additional References on FCS 832
 References 832
 Additional References to FCS and
 Its Applications 837
 Problems 840

**25. Radiative Decay Engineering:
 Metal-Enhanced Fluorescence**

25.1. Radiative Decay Engineering 841
 25.1.1. Introduction to RDE..... 841
 25.1.2. Jablonski Diagram for Metal-
 Enhanced Fluorescence 842
 25.2. Review of Metal Effects on Fluorescence..... 843

25.3. Optical Properties of Metal Colloids 845

25.4. Theory for Fluorophore–Colloid Interactions 846

25.5. Experimental Results on Metal-Enhanced Fluorescence 848

 25.5.1. Application of MEF to DNA Analysis..... 848

25.6. Distance-Dependence of Metal-Enhanced Fluorescence 851

25.7. Applications of Metal-Enhanced Fluorescence..... 851

 25.7.1. DNA Hybridization Using MEF 853

 25.7.2. Release of Self-Quenching..... 853

 25.7.3. Effect of Silver Particles on RET..... 854

25.8. Mechanism of MEF..... 855

25.9. Perspective on RET 856

 References 856

 Problem..... 859

26. Radiative Decay Engineering: Surface Plasmon-Coupled Emission

26.1. Phenomenon of SPCE 861

26.2. Surface-Plasmon Resonance 861

 26.2.1. Theory for Surface-Plasmon Resonance..... 863

26.3. Expected Properties of SPCE..... 865

26.4. Experimental Demonstration of SPCE..... 865

26.5. Applications of SPCE..... 867

26.6. Future Developments in SPCE..... 868

 References 870

Appendix I. Corrected Emission Spectra

1. Emission Spectra Standards from 300 to 800 nm..... 873

2. β -Carboline Derivatives as Fluorescence Standards 873

3. Corrected Emission Spectra of 9,10-Diphenylanthracene, Quinine, and Fluorescein 877

4. Long-Wavelength Standards..... 877

5. Ultraviolet Standards 878

6. Additional Corrected Emission Spectra 881

 References 881

Appendix II. Fluorescent Lifetime Standards

1. Nanosecond Lifetime Standards..... 883

2. Picosecond Lifetime Standards 884

3. Representative Frequency-Domain Intensity Decays 885

4. Time-Domain Lifetime Standards..... 886

Appendix III. Additional Reading

1. Time-Resolved Measurements 889

2. Spectra Properties of Fluorophores..... 889

3. Theory of Fluorescence and Photophysics..... 889

4. Reviews of Fluorescence Spectroscopy 889

5. Biochemical Fluorescence 890

6. Protein Fluorescence 890

7. Data Analysis and Nonlinear Least Squares 890

8. Photochemistry 890

9. Flow Cytometry..... 890

10. Phosphorescence..... 890

11. Fluorescence Sensing 890

12. Immunoassays 891

13. Applications of Fluorescence 891

14. Multiphoton Excitation..... 891

15. Infrared and NIR Fluorescence 891

16. Lasers..... 891

17. Fluorescence Microscopy 891

18. Metal–Ligand Complexes and Unusual Lumophores 891

19. Single-Molecule Detection..... 891

20. Fluorescence Correlation Spectroscopy 892

21. Biophotonics..... 892

22. Nanoparticles..... 892

23. Metallic Particles..... 892

24. Books on Fluorescence..... 892

Answers to Problems 893

Index 923