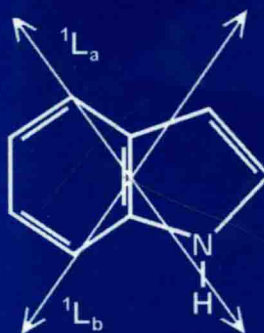
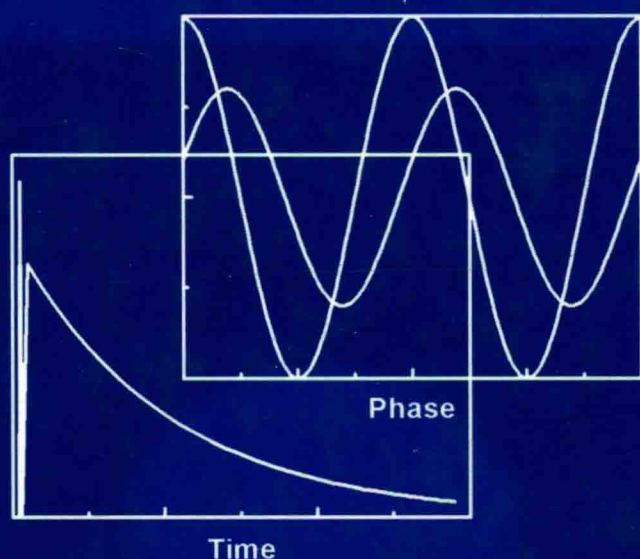


Principles of Fluorescence Spectroscopy

Second Edition



Joseph R. Lakowicz

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Second Edition

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Kluwer Academic/Plenum Publishers
New York, Boston, Dordrecht, London, Moscow

Library of Congress Cataloging-in-Publication Data

Lakowicz, Joseph R.

Principles of fluorescence spectroscopy / Joseph R. Lakowicz. --
2nd ed.

p. cm.

Includes bibliographical references and index.

ISBN 0-306-46093-9

1. Fluorescence spectroscopy. I. Title.

QD96.F56L34 1999

543'.08584--dc21

99-30047

CIP

ISBN 0-306-46093-9

©1999 Kluwer Academic/Plenum Publishers, New York
233 Spring Street, New York, N.Y. 10013

10 9 8 7 6 5 4 3 2

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Printed in the United States of America

*Principles of
Fluorescence
Spectroscopy*

Second Edition

To Professor Aleksander Jabłoński
on the occasion of his 100th birthday

Preface

It has been 15 years since publication of the first edition of *Principles of Fluorescence Spectroscopy*. This first volume grew out of a graduate-level course on fluorescence taught at the University of Maryland. The first edition was written during a transition period in the technology and applications of fluorescence spectroscopy. In 1983, time-resolved measurements were performed using methods which are primitive by today's standards. The dominant light sources for time-resolved fluorescence were the nanosecond flashlamps, which provided relatively wide excitation pulses. Detection was accomplished with relatively slow response photomultiplier tubes. In the case of phase-modulation fluorometry, the available instruments operated at one or two fixed light modulation frequencies and thus provided limited information on complex time-resolved decays. Data analysis was also limited because of the lower information content of the experimental data.

Much has changed since 1983. The dominant light sources are now picosecond dye lasers or femtosecond Titanium:sapphire lasers. In the case of phase-modulation fluorometry, frequency-domain instrumentation now operates over a range of light modulation frequencies, allowing resolution of complex decays. The time resolution in both the frequency and the time domain has been increased by the introduction of high-speed microchannel plate photomultiplier tubes. Data analysis has become increasingly sophisticated, not only because of the availability of more powerful computers, but also because of the availability of additional data and the increased resolution available using global analysis. These advanced experimental and analysis capabilities have been extended to provide resolution of complex anisotropy decays, conformational distributions, and complex quenching phenomena.

Another important change since 1983 has been the extensive development of fluorescent probes. Early fluorescent probes were those derived from histochemical staining of cells, a limited number of lipid and conjugat-

able probes, and, of course, intrinsic fluorescence from proteins. Today the menu of fluorescent probes has expanded manyfold. A wide variety of lipid and protein probes have been developed, and probes have become available with longer excitation and emission wavelengths. There has been extensive development of cation-sensing probes for use in cellular imaging. The nanosecond barrier of dynamic fluorescence information has been broken by the introduction of long-lifetime probes.

Another example of the rapid expansion of fluorescence is DNA sequencing technology. Prior to 1985, most DNA sequencing was performed using radioactive labels. Since that time, sequencing has been accomplished almost exclusively with fluorescent probes. The fluorescence technology for DNA sequencing is advancing rapidly owing to the goal of sequencing the human genome. Finally, who would have expected in 1983 that the gene for the green fluorescent protein could be introduced into cells, with spontaneous folding and formation of the fully fluorescent protein?

Parts of this book were influenced by a course taught at the Center for Fluorescence Spectroscopy, which has been attended by individuals from throughout the world. However, the most important factor stimulating the second edition was the positive comments of individuals who found value in the first edition. Many individuals commented on the value of explaining the basic concepts from their fundamental origins. This has become increasingly important as the number of practitioners of fluorescence spectroscopy has increased, without a significant increase in the number of courses at the undergraduate or graduate level.

In this second edition of *Principles of Fluorescence Spectroscopy*, I have attempted to maintain the emphasis on basics, while updating the examples to include more recent results from the literature. There is a new chapter providing an overview of extrinsic fluorophores. The discussion of time-resolved measurements has been ex-

panded to two chapters. Quenching has also been expanded to two chapters. Energy transfer and anisotropy have each been expanded to three chapters. There is also a new chapter on fluorescence sensing. To enhance the usefulness of this book as a textbook, each chapter is followed by a set of problems. Sections which describe advanced topics are indicated as such, to allow these sections to be skipped in an introductory course. Glossaries of commonly used acronyms and mathematical symbols are provided. For those wanting additional information, Appendix III contains a list of recommended books which expand on various specialized topics.

In closing, I wish to express my appreciation to the many individuals who have assisted me not only in preparation of the book but also in the intellectual developments in my laboratory. My special thanks go to Ms. Mary Rosenfeld for her careful preparation of the text. Mary has cheerfully tolerated the copious typing and numerous revisions of all the chapters. I also thank the many individuals who have proofread various chapters and provided constructive sug-

gestions. These individuals include Felix Castellano, Robert E. Dale, Jonathan Dattelbaum, Maurice Eftink, John Gilchrist, Zygmunt Gryczynski, Petr Herman, Gabor Laczko, Li Li, Harriet Lin, Zakir Murtaza, Leah Tolosa, and Bogumil Zelen. I apologize for any omissions.

I also give my special thanks to Dr. Ignacy Gryczynski and his wife, Krystyna Gryczynska. When I started to write this book, Ignacy said "just go and write, don't worry about the figures." Many of the excellent figures in this book were drawn by Krystyna, with the valuable suggestions of Ignacy. Without their dedicated efforts, the book could not have been completed in any reasonable period of time. I also thank Ms. Suzy Rhinehart for providing a supportive family environment during preparation of this book. Finally, I thank the National Institutes of Health and the National Science Foundation for support of my laboratory.

J. R. Lakowicz

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Glossary of Acronyms

2,6-ANS	6-Anilinonaphthalene-2-sulfonic acid	MLCT	Metal–ligand charge transfer (state)
ASE	Asymptotic standard error	MPE	Multiphoton excitation
BODIPY	Refers to a family of dyes based on 1,3,5,7,8-pentamethylpyrromethene-BF ₂ , or 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene. BODIPY is a trademark of Molecular Probes, Inc.	NADH	Reduced nicotinamide adenine dinucleotide
CFD	Constant fraction discriminator	NATA	<i>N</i> -Acetyl-L-tryptophanamide
Dansyl	5-Dimethylaminonaphthalene-1-sulfonic acid	NATyrA	<i>N</i> -Acetyl-L-tyrosinamide
DAPI	4',6-Diamidino-2-phenylindole	NBD	7-Nitrobenz-2-oxa-1,3-diazol-4-yl
DAS	Decay-associated spectra	NIR	Near-infrared
DNS-Cl	Dansyl chloride	phe	Phenylalanine
DPH	1,6-Diphenyl-1,3,5-hexatriene	PC	Phosphatidylcholine
EB	Ethidium bromide	PMT	Photomultiplier tube
F	Single-letter code for phenylalanine	POPOP	1,4-Bis(5-phenyloxazol-2-yl)benzene
FAD	Flavin adenine dinucleotide	PPD	2,5-Diphenyl-1,3,4-oxadazole
FD	Frequency-domain	PPO	2,5-Diphenyloxazole
FISH	Fluorescence <i>in situ</i> hybridization	Prodan	6-Propionyl-2-(dimethylamino)naphthalene
FITC	Fluorescein-5-isothiocyanate	PSDF	Phase-sensitive detection of fluorescence
FMN	Flavin mononucleotide	RET	Resonance energy transfer
FRET	Fluorescence resonance energy transfer	S ₀	Ground electronic state
GFP	Green fluorescent protein	S ₁	First excited singlet state
HIV	Human immunodeficiency virus	SPQ	6-Methoxy- <i>N</i> -(3-sulfopropyl)quinoline
HSA	Human serum albumin	T ₁	First excited triplet state
IAEDANS	5-(((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid	TAC	Time-to-amplitude converter
IAF	5-(Iodoacetamido)fluorescein	TCSPC	Time-correlated single-photon counting
ICT	Internal charge transfer (state)	TD	Time-domain
IRF	Instrument response function	TICT	Twisted internal charge-transfer state
LE	Locally excited (state)	TNS	6-(<i>p</i> -Toluidinyl)naphthalene-2-sulfonic acid
MCP	Microchannel plate	TRES	Time-resolved emission spectra
MLC	Metal–ligand complex, usually of a transition metal (Ru, Rh, or Os)	TRITC	Tetramethylrhodamine 5- (and 6-)isothiocyanate
		trp	Tryptophan
		tyr	Tyrosine
		W	Single-letter code for tryptophan
		Y	Single-letter code for tyrosine

Glossary of Mathematical Terms

A	Acceptor or absorption	r	Anisotropy (sometimes distance in a distance distribution)
c	Speed of light	\bar{r}	Average distance in a distance distribution
C_0	Characteristic acceptor concentration in resonance energy transfer	$r(0)$	Time-zero anisotropy
$C(t)$	Correlation function for spectral relaxation	$r(t)$	Anisotropy decay
D	Donor, diffusion coefficient, or rotational diffusion coefficient	r_c	Distance of closest approach between donors and acceptors in resonance energy transfer, or fluorophores and quenchers
$D_{ }$ or D_{\perp}	Rate of rotational diffusion around (displacing) the symmetry axis of an ellipsoid of revolution	r_{0i} or r_{0gi}	Fractional amplitudes in a multiexponential anisotropy decay
E	Efficiency of energy transfer	r_0	Fundamental anisotropy in the absence of rotational diffusion
F	Steady state intensity or fluorescence	r_{0i}	Anisotropy amplitudes in a multiexponential anisotropy decay
F_x	Ratio of χ_R^2 values, used to calculate parameter confidence intervals	r_{∞}	Long-time anisotropy in an anisotropy decay
$F(\lambda)$	Emission spectrum	r_{ω}	Modulated anisotropy
f_i	Fractional steady-state intensities in a multiexponential intensity decay	R_0	Förster distance in resonance energy transfer
f_Q	Efficiency of collisional quenching	α_i	Preexponential factors in a multiexponential intensity decay
G	Correction factor for anisotropy measurements	β	Angle between absorption and emission transition moments
hw	Half-width in a distance or lifetime distribution	Γ	Radiative decay rate
$I(t)$	Intensity decay, typically the impulse response function	γ	Inverse of the decay time: $\gamma = 1/\tau$
k_{nr}	Nonradiative decay rate	ϵ	Dielectric constant or extinction coefficient
k_S	Solvent relaxation rate	θ	Rotational correlation time
k_T	Transfer rate in resonance energy transfer	κ^2	Orientation factor in resonance energy transfer
m_{ω}	Modulation at a light modulation frequency ω	Λ_{ω}	Ratio of the modulated amplitudes of the polarized components of the emission
n	Refractive index, when used in consideration of solvent effects	λ	Wavelength
$N(t_k)$	Number of counts per channel, in time-correlated single-photon counting	λ_{em}	Emission wavelength
Q	Quantum yield	λ_{em}^{max}	Maximum emission wavelength
$P(r)$	Probability function for a distance (r) distribution	λ_{ex}	Excitation wavelength
pK_a	Acid dissociation constant, negative logarithm	λ_{ex}^{max}	Maximum excitation or absorption wavelength for the lowest $S_0 \rightarrow S_1$ transition
		λ_{max}	Emission maximum
		μ_E	Excited-state dipole moment
		μ_G	Ground-state dipole moment

$\bar{\nu}$	Wavenumber, in cm^{-1}	τ_N	Radiative or natural lifetime
$\bar{\nu}_{\text{cg}}$	Emission center of gravity	τ_S	Solvent relaxation time
$\bar{\nu}_{\text{cg}}(t)$	Time-resolved emission center of gravity, in cm^{-1}	Δ_ω	Differential polarized phase angle, difference in phase between the parallel and perpendicular components of the emission
τ	Decay time	ϕ_ω	Phase angle at a light modulation frequency ω
$\bar{\tau}$	Average lifetime	χ_R^2	Goodness-of-fit parameter, reduced chi-squared
τ_ϕ	Apparent lifetime calculated from the phase angle at a single frequency	χ^2	Sum of the squared weighted deviations
τ_D	Donor decay time or solvent dielectric relaxation time	ω	Light modulation frequency in radians per second; 2π times the frequency in cycles per second
τ_L	Solvent longitudinal relaxation time		
τ_m	Apparent lifetime calculated from the modulation at a single frequency		

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Contents

Most sections of this book describe basic aspects of fluorescence spectroscopy, and some sections describe more advanced topics. These sections are marked “Advanced Topics” and can be omitted in an introductory course on fluorescence. The advanced chapters on quenching (Chapter 9), anisotropy (Chapter 12), and energy transfer (Chapters 14 and 15) can be skipped in a first reading. Depending on the interest of the reader, Chapters 18 to 22 can also be skipped.

1. Introduction to Fluorescence

1.1. Phenomenon of Fluorescence	1
1.2. Jabłoński Diagram	4
1.3. Characteristics of Fluorescence Emission . .	6
1.3.A. Stokes’ Shift	6
1.3.B. Emission Spectra Are Typically Independent of the Excitation Wavelength	7
1.3.C. Exceptions to the Mirror Image Rule . .	8
1.4. Fluorescence Lifetimes and Quantum Yields .	10
1.4.A. Fluorescence Quenching	11
1.4.B. Time Scale 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.A. Why Time-Resolved Measurements? . .	15
1.8. Biochemical Fluorophores	15
1.8.A. Fluorescent Indicators	16
1.9. Molecular Information from Fluorescence . .	17
1.9.A. Emission Spectra and the Stokes’ Shift .	17
1.9.B. Quenching of Fluorescence	17
1.9.C. Fluorescence Polarization or Anisotropy	18
1.9.D. Resonance Energy Transfer	19
1.10. Fluorescence Sensing	19
1.11. Summary	20
References	20
Problems	21

2. Instrumentation for Fluorescence Spectroscopy

2.1. Excitation and Emission Spectra	25
2.1.A. An Ideal Spectrofluorometer	27
2.1.B. Distortions in Excitation and Emission Spectra	28
2.2. Light Sources	28
2.2.A. Arc and Incandescent Lamps	28
2.2.B. Solid-State Light Sources	31
2.3. Monochromators	32
2.3.A. Wavelength Resolution and Emission Spectra	33
2.3.B. Polarization Characteristics of Monochromators	33
2.3.C. Stray Light in Monochromators	34
2.3.D. Second-Order Transmission in Monochromators	35
2.3.E. Calibration of Monochromators	35
2.4. Optical Filters	36
2.4.A. Bandpass Filters	36
2.4.B. Interference Filters	36
2.4.C. Filter Combinations	38
2.4.D. Neutral Density Filters	38
2.5. Optical Filters and Signal Purity	39
2.5.A. Emission Spectra Taken through Filters .	40
2.6. Photomultiplier Tubes	41
2.6.A. Spectral Response	42
2.6.B. PMT Designs and Dynode Chains . . .	43
2.6.C. Time Response of Photomultiplier Tubes	44

2.6.D. Photon Counting versus Analog Detection of Fluorescence	45	3.6.A. Fluorogenic Probes	78
2.6.E. Symptoms of PMT Failure	46	3.6.B. Structural Analogs of Biomolecules	81
2.6.F. Hybrid Photomultiplier Tubes	47	3.6.C. Viscosity Probes	81
2.6.G. CCD Detectors	47	3.7. Fluorescent Proteins	82
2.7. Polarizers	47	3.7.A. Phycobiliproteins	82
2.8. Corrected Excitation Spectra	49	3.7.B. Green Fluorescent Protein	84
2.8.A. Use of a Quantum Counter to Obtain Corrected Excitation Spectra	50	3.7.C. Phytofluors—A New Class of Fluorescent Probes	85
2.9. Corrected Emission Spectra	51	3.8. Long-Lifetime Probes	86
2.9.A. Comparison with Known Emission Spectra	51	3.8.A. Lanthanides	87
2.9.B. Correction Factors Obtained by Using a Standard Lamp	51	3.8.B. Transition-Metal–Ligand Complexes	88
2.9.C. Correction Factors Obtained by Using a Quantum Counter and Scatterer	51	3.9. Proteins as Sensors	88
2.9.D. Conversion between Wavelength and Wavenumber	52	3.10. Conclusion	89
2.10. Quantum Yield Standards	52	References	89
2.11. Effects of Sample Geometry	53	Problems	92
2.12. Common Errors in Sample Preparation	55		
2.13. Absorption of Light and Deviation from the Beer–Lambert Law	56	4. Time-Domain Lifetime Measurements	
2.13.A. Deviations from Beer's Law	57	4.1. Overview of Time-Domain and Frequency-Domain Measurements	95
2.14. Two-Photon and Multiphoton Excitation	57	4.1.A. Meaning of the Lifetime or Decay Time	96
2.15. Conclusions	59	4.1.B. Phase and Modulation Lifetimes	97
References	59	4.1.C. Examples of Time-Domain and Frequency-Domain Lifetimes	97
Problems	60	4.2. Biopolymers Display Multiexponential or Heterogeneous Decays	98
		4.2.A. Resolution of Multiexponential Decays Is Difficult	100
3. Fluorophores		4.3. Time-Correlated Single-Photon Counting	101
3.1. Intrinsic or Natural Fluorophores	63	4.3.A. Principles of TCSPC	101
3.1.A. Fluorescent Enzyme Cofactors	63	4.3.B. Example of TCSPC Data	101
3.1.B. Binding of NADH to a Protein	65	4.3.C. Convolution Integral	103
3.2. Extrinsic Fluorophores	66	4.4. Light Sources for TCSPC	104
3.2.A. Protein-Labeling Reagents	67	4.4.A. Picosecond Dye Lasers	104
3.2.B. Role of the Stokes' Shift in Protein Labeling	69	4.4.B. Femtosecond Titanium:Sapphire Lasers	106
3.2.C. Solvent-Sensitive Probes	71	4.4.C. Flashlamps	107
3.2.D. Noncovalent Protein-Labeling Probes	71	4.4.D. Solid-State Lasers	109
3.2.E. Membrane Probes	72	4.5. Electronics for TCSPC	109
3.2.F. Membrane Potential Probes	72	4.5.A. Constant Fraction Discriminators	109
3.3. Red and Near-Infrared (NIR) Dyes	74	4.5.B. Amplifiers	110
3.3.A. Measurement of Human Serum Albumin with Laser Diode Excitation	75	4.5.C. Time-to-Amplitude Converter (TAC)—Standard and Reversed Configurations	110
3.4. DNA Probes	76	4.5.D. Multichannel Analyzer (MCA)	110
3.4.A. DNA Base Analogs	77	4.5.E. Delay Lines	110
3.5. Chemical Sensing Probes	78	4.5.F. Pulse Pileup	111
3.6. Special Probes	78	4.6. Detectors for TCSPC	111
		4.6.A. MCP PMTs	111

4.6.B. Dynode Chain PMTs	113
4.6.C. Photodiodes as Detectors	114
4.6.D. Color Effects in Detectors	114
4.6.E. Timing Effects of Monochromators	116
4.7. Alternative Methods for Time-Resolved Measurements	116
4.7.A. Pulse Sampling or Gated Detection	116
4.7.B. Streak Cameras	117
4.7.C. Upconversion Methods	118
4.8. Data Analysis	118
4.8.A. Assumptions of Nonlinear Least-Squares Analysis	119
4.8.B. Overview of Least-Squares Analysis	119
4.8.C. Meaning of the Goodness of Fit, χ^2_R	120
4.8.D. Autocorrelation Function	121
4.9. Analysis of Multiexponential Decays	121
4.9.A. <i>p</i> -Terphenyl and Indole—Two Widely Spaced Lifetimes	121
4.9.B. Comparison of χ^2_R Values— <i>F</i> -Statistic	122
4.9.C. Parameter Uncertainty—Confidence Intervals	122
4.9.D. Effect of the Number of Photon Counts	124
4.9.E. Anthranilic Acid and 2-Amino-purine—Two Closely Spaced Lifetimes	124
4.9.F. Global Analysis—Multiwavelength Measurements	126
4.9.G. Resolution of Three Closely Spaced Lifetimes	126
4.10. Intensity Decay Laws	129
4.10.A. Multiexponential Decays	129
4.10.B. Lifetime Distributions	130
4.10.C. Stretched Exponentials	131
4.10.D. Transient Effects	131
4.11. Global Analysis	132
4.12. Representative Intensity Decays	132
4.12.A. Intensity Decay for a Single-Tryptophan Protein	132
4.12.B. Green Fluorescent Protein—Systematic Errors in the Data	133
4.12.C. Erythrosin B—A Picosecond Decay Time	133
4.12.D. Chlorophyll Aggregates in Hexane	134
4.12.E. Intensity Decay of FAD	134
4.12.F. Microsecond Luminescence Decays	135
4.12.G. Subpicosecond Intensity Decays	135
4.13. Closing Comments	136
References	136
Problems	140

5. Frequency-Domain Lifetime Measurements

5.1. Theory of Frequency-Domain Fluorometry	142
5.1.A. Least-Squares Analysis of Frequency-Domain Intensity Decays	144
5.1.B. Global Analysis of Frequency-Domain Data	146
5.1.C. Estimation of Parameter Uncertainties	146
5.2. Frequency-Domain Instrumentation	147
5.2.A. History of Phase-Modulation Fluorometers	147
5.2.B. The 200-MHz Frequency-Domain Fluorometer	147
5.2.C. Light Modulators	149
5.2.D. Cross-Correlation Detection	150
5.2.E. Frequency Synthesizers	150
5.2.F. Radio-Frequency Amplifiers	150
5.2.G. Photomultiplier Tubes	150
5.2.H. Principle of Frequency-Domain Measurements	151
5.3. Color Effects and Background Fluorescence	152
5.3.A. Color Effects in Frequency-Domain Measurements	152
5.3.B. Background Correction in Frequency-Domain Measurements	153
5.4. Representative Frequency-Domain Intensity Decays	154
5.4.A. Exponential Decays	154
5.4.B. Effect of Scattered Light	154
5.5. Analysis of Multiexponential Decays	155
5.5.A. Resolution of Two Widely Spaced Lifetimes	155
5.5.B. Resolution of Two Closely Spaced Lifetimes	157
5.5.C. Global Analysis of a Two-Component Mixture	159
5.5.D. Analysis of a Three-Component Mixture—Limits of Resolution	160
5.5.E. Resolution of a Three-Component Mixture with a 10-Fold Range of Decay Times	162
5.6. Biochemical Examples of Frequency-Domain Intensity Decays	163
5.6.A. Monellin—A Single-Tryptophan Protein with Three Decay Times	163
5.6.B. Multiexponential Decays of Staphylococcal Nuclease and Melittin	163
5.6.C. DNA Labeled with DAPI	164

5.6.D. Quin-2—A Lifetime-Based Sensor for Calcium	165	6.3.A. Specific Solvent Effects and Lippert Plots	196
5.6.E. SPQ—Collisional Quenching of a Chloride Sensor	165	6.4. Temperature Effects	198
5.6.F. Green Fluorescent Protein—One- and Two-Photon Excitation	166	6.4.A. LE and ICT States of Prodan	200
5.6.G. Recovery of Lifetime Distributions from Frequency-Domain Data	166	6.5. Biochemical Examples Using PRODAN	201
5.6.H. Lifetime Distribution of Photosynthetic Components	167	6.5.A. Phase Transition in Membranes	201
5.6.I. Lifetime Distributions of the Ca^{2+} -ATPase	167	6.5.B. Protein Association	202
5.6.J. Cross Fitting of Models—Lifetime Distributions of Melittin	168	6.5.C. Fatty Acid Binding Proteins	202
5.6.K. Intensity Decay of NADH	169	6.6. Biochemical Examples Using Solvent-Sensitive Probes	202
5.7. Gigahertz Frequency-Domain Fluorometry	169	6.6.A. Exposure of a Hydrophobic Surface on Calmodulin	202
5.7.A. Gigahertz FD Measurements	171	6.6.B. Binding to Cyclodextrins Using a Dansyl Probe	203
5.7.B. Biochemical Examples of Gigahertz FD Data	172	6.6.C. Polarity of a Membrane Binding Site	203
5.8. Simple Frequency-Domain Instruments	173	6.7. Development of Advanced Solvent-Sensitive Probes	204
5.8.A. Laser Diode Excitation	173	6.8. Effects of Solvent Mixtures	206
5.8.B. LED Excitation	173	6.9. Summary of Solvent Effects	207
5.9. Phase Angle and Modulation Spectra	175	References	207
5.9.A. Resolution of the Two Emission Spectra of Tryptophan Using Phase-Modulation Spectra	176	Problems	210
5.10. Apparent Phase and Modulation Lifetimes	177		
5.11. Derivation of the Equations for Phase-Modulation Fluorescence	178	7. Dynamics of Solvent and Spectral Relaxation	
5.11.A. Relationship of the Lifetime to the Phase Angle and Modulation	178	7.1. Continuous and Two-State Spectral Relaxation	212
5.11.B. Cross-Correlation Detection	180	7.2. Measurement of TRES	213
5.12. Perspectives on Frequency-Domain Fluorometry	180	7.2.A. Direct Recording of TRES	213
References	180	7.2.B. TRES from Wavelength-Dependent Decays	213
Problems	184	7.3. Biochemical Examples of TRES	215
		7.3.A. Spectral Relaxation in Apomyoglobin	215
		7.3.B. TRES of Labeled Membranes	217
		7.3.C. Analysis of TRES	218
		7.3.D. Spectral Relaxation in Proteins	220
		7.4. Lifetime-Resolved Emission Spectra	222
		7.5. Picosecond Relaxation in Solvents	224
		7.5.A. Theory for Time-Dependent Solvent Relaxation	224
		7.5.B. Multiexponential Relaxation in Water	225
		7.6. Comparison of Continuous and Two-State Relaxation	226
		7.6.A. Experimental Distinction between Continuous and Two-State Relaxation	227
		7.6.B. Phase-Modulation Studies of Solvent Relaxation	227

6. Solvent Effects on Emission Spectra

6.1. Overview of Solvent Effects	185
6.1.A. Polarity Surrounding a Membrane-Bound Fluorophore	186
6.1.B. Mechanisms for Spectral Shifts	186
6.2. General Solvent Effects—The Lippert Equation	187
6.2.A. Derivation of the Lippert Equation	189
6.2.B. Application of the Lippert Equation	191
6.2.C. Polarity Scales	193
6.3. Specific Solvent Effects	194

7. Dynamics of Solvent and Spectral Relaxation

7.1. Continuous and Two-State Spectral Relaxation	212
7.2. Measurement of TRES	213
7.2.A. Direct Recording of TRES	213
7.2.B. TRES from Wavelength-Dependent Decays	213
7.3. Biochemical Examples of TRES	215
7.3.A. Spectral Relaxation in Apomyoglobin	215
7.3.B. TRES of Labeled Membranes	217
7.3.C. Analysis of TRES	218
7.3.D. Spectral Relaxation in Proteins	220
7.4. Lifetime-Resolved Emission Spectra	222
7.5. Picosecond Relaxation in Solvents	224
7.5.A. Theory for Time-Dependent Solvent Relaxation	224
7.5.B. Multiexponential Relaxation in Water	225
7.6. Comparison of Continuous and Two-State Relaxation	226
7.6.A. Experimental Distinction between Continuous and Two-State Relaxation	227
7.6.B. Phase-Modulation Studies of Solvent Relaxation	227

7.6.C. Distinction between Solvent Relaxation and Formation of Rotational Isomers	229
7.7. Comparison of TRES and DAS	230
7.8. Red-Edge Excitation Shifts	231
7.9. Perspectives of Solvent Dynamics	233
References	233
Problems	236

8.11.A. Quenching Due to Specific Binding Interactions	255
8.11.B. Binding of Substrates to Ribozymes	256
8.11.C. Association Reactions and Quenching	257
8.12. Intramolecular Quenching	257
8.13. Quenching of Phosphorescence	258
References	259
Problems	264

8. Quenching of Fluorescence

8.1. Quenchers of Fluorescence	238
8.2. Theory of Collisional Quenching	239
8.2.A. Derivation of the Stern–Volmer Equation	240
8.2.B. Interpretation of the Bimolecular Quenching Constant	241
8.3. Theory of Static Quenching	242
8.4. Combined Dynamic and Static Quenching	243
8.5. Examples of Static and Dynamic Quenching	243
8.6. Deviations from the Stern–Volmer Equation; Quenching Sphere of Action	244
8.6.A. Derivation of the Quenching Sphere of Action	245
8.7. Effects of Steric Shielding and Charge on Quenching	245
8.7.A. Accessibility of DNA-Bound Probes to Quenchers	246
8.7.B. Quenching of Ethenoadenine Derivatives	247
8.8. Fractional Accessibility to Quenchers	247
8.8.A. Modified Stern–Volmer Plots	248
8.8.B. Experimental Considerations in Quenching	249
8.9. Applications of Quenching to Proteins	249
8.9.A. Fractional Accessibility of Tryptophan Residues in Endonuclease III	249
8.9.B. Effect of Conformational Changes on Tryptophan Accessibility	250
8.9.C. Quenching of the Multiple Decay Times of Proteins	250
8.9.D. Effects of Quenchers on Proteins	251
8.9.E. Protein Folding of Colicin E1	251
8.10. Quenching-Resolved Emission Spectra	252
8.10.A. Fluorophore Mixtures	252
8.10.B. Quenching-Resolved Emission Spectra of the <i>E. coli</i> Tet Repressor	253
8.11. Quenching and Association Reactions	255

9. Advanced Topics in Fluorescence Quenching

9.1. Quenching in Membranes	267
9.1.A. Accessibility of Membrane Probes to Water- and Lipid-Soluble Quenchers	267
9.1.B. Quenching of Membrane Probes Using Localized Quenchers	270
9.1.C. Parallax Quenching in Membranes	272
9.1.D. Boundary Lipid Quenching	273
9.1.E. Effect of Lipid–Water Partitioning on Quenching	274
9.2. Diffusion in Membranes	276
9.2.A. Quasi-Three-Dimensional Diffusion in Membranes	276
9.2.B. Lateral Diffusion in Membranes	278
9.3. Quenching Efficiency	278
9.3.A. Steric Shielding Effects in Quenching	279
9.4. Transient Effects in Quenching	280
9.4.A. Experimental Studies of Transient Effects	281
9.4.B. Distance-Dependent Quenching in Proteins	285
References	286
Problems	289

10. Fluorescence Anisotropy

10.1. Definition of Fluorescence Anisotropy	291
10.1.A. Origin of the Definitions of Polarization and Anisotropy	292
10.2. Theory for Anisotropy	293
10.2.A. Excitation Photoselection of Fluorophores	294
10.3. Excitation Anisotropy Spectra	295
10.3.A. Resolution of Electronic States from Polarization Spectra	297
10.4. Measurement of Fluorescence Anisotropies	298
10.4.A. L-Format or Single-Channel Method	298

10.4.B. T-Format or Two-Channel Anisotropies	299	11.2.A. Time-Domain Anisotropy Data	325
10.4.C. Comparison of T-Format and L-Format Measurements	300	11.2.B. Value of r_0	327
10.4.D. Alignment of Polarizers	300	11.3. Analysis of Frequency-Domain Anisotropy Decays	327
10.4.E. Magic-Angle Polarizer Conditions	301	11.4. Anisotropy Decay Laws	328
10.4.F. Why Is the Total Intensity Equal to $I_0 + 2I_{\perp}$?	301	11.4.A. Nonspherical Fluorophores	328
10.4.G. Effect of Radiationless Energy Transfer on the Anisotropy	302	11.4.B. Hindered Rotors	329
10.4.H. Trivial Causes of Depolarization	302	11.4.C. Segmental Mobility of a Biopolymer-Bound Fluorophore	329
10.4.I. Factors Affecting the Anisotropy	303	11.4.D. Correlation Time Distributions	330
10.5. Effects of Rotational Diffusion on Fluorescence Anisotropies: The Perrin Equation	303	11.4.E. Associated Anisotropy Decays	331
10.5.A. The Perrin Equation—Rotational Motions of Proteins	304	11.5. Hindered Rotational Diffusion in Membranes	331
10.5.B. Examples of Perrin Plots	306	11.6. Time-Domain Anisotropy Decays of Proteins	333
10.6. Perrin Plots of Proteins	307	11.6.A. Intrinsic Tryptophan Anisotropy Decay of Liver Alcohol Dehydrogenase	333
10.6.A. Binding of tRNA to tRNA Synthetase	307	11.6.B. Phospholipase A ₂	334
10.6.B. Molecular Chaperonin cpm 60 (GroEL)	307	11.6.C. Domain Motions of Immunoglobulins	334
10.6.C. Perrin Plots of an F _{ab} Immunoglobulin Fragment	308	11.6.D. Effects of Free Probe on Anisotropy Decays	335
10.7. Protein Association Reactions	308	11.7. Frequency-Domain Anisotropy Decays of Proteins	335
10.7.A. Peptide Binding to Calmodulin	308	11.7.A. Apomyoglobin—A Rigid Rotor	335
10.7.B. Binding of the Trp Repressor to DNA	309	11.7.B. Melittin Self-Association and Anisotropy Decays	336
10.7.C. Melittin Association Detected from Homotransfer	309	11.7.C. Picosecond Rotational Diffusion of Oxytocin	336
10.8. Anisotropy of Membrane-Bound Probes	310	11.8. Microsecond Anisotropy Decays	337
10.9. Lifetime-Resolved Anisotropies	310	11.8.A. Phosphorescence Anisotropy Decays	337
10.9.A. Effect of Segmental Motion on the Perrin Plots	311	11.8.B. Long-Lifetime Metal-Ligand Complexes	337
10.10. Soleillet's Rule—Multiplication of Depolarization Factors	312	11.9. Anisotropy Decays of Nucleic Acids	338
10.11. Anisotropies Can Depend on Emission Wavelength	312	11.9.A. Hydrodynamics of DNA Oligomers	339
10.12. Transition Moments	313	11.9.B. Segmental Mobility of DNA	340
10.12.A. Anisotropy of Planar Fluorophores with High Symmetry	314	11.10. Characterization of a New Membrane Probe	341
10.13. Anisotropies with Multiphoton Excitation	315	References	342
10.13.A. Excitation Photoselection for Two-Photon Excitation	315	Problems	345
10.13.B. Two-Photon Anisotropy of DPH	315		
References	316		
Problems	318		
		12. Advanced Anisotropy Concepts	
11. Time-Dependent Anisotropy Decays		12.1. Rotational Diffusion of Nonspherical Molecules—An Overview	347
11.1. Analysis of Time-Domain Anisotropy Decays	321	12.1.A. Anisotropy Decays of Ellipsoids	348
11.2. Anisotropy Decay Analysis	325	12.2. Ellipsoids of Revolution	349
		12.2.A. Simplified Ellipsoids of Revolution	349
		12.2.B. Intuitive Description of an Oblate Ellipsoid	351
		12.2.C. Rotational Correlation Times for Ellipsoids of Revolution	351
		12.2.D. Stick versus Slip Rotational Diffusion	353