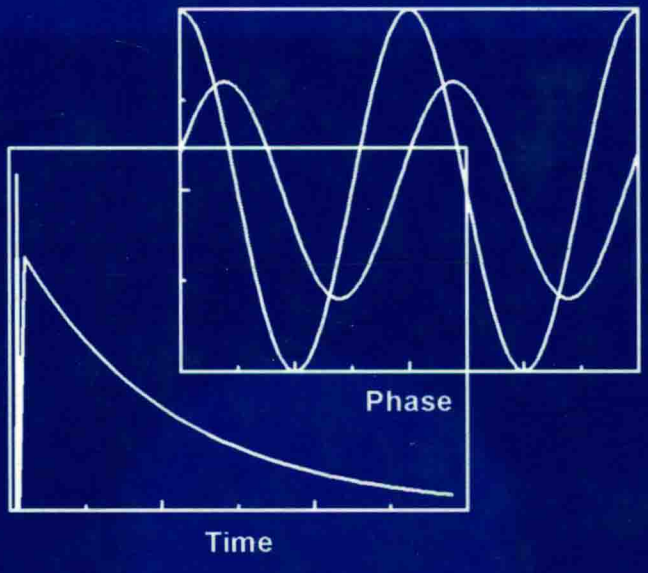
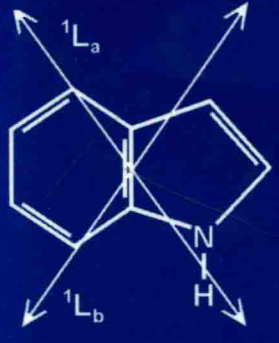
Principles of Fluorescence Spectroscopy

Second Edition





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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 conjugatable 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 suggestions. 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 Zelent. 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.

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Glossary of Acronyms

2,6-ANS	6-Anilinonaphthalene-2-sulfonic acid
ASE	Asymptotic standard error
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.
CFD	Constant fraction discriminator
Dansyl	5-Dimethylaminonaphthalene-1-sulfonic acid
DAPI	4′,6-Diamidino-2-phenylindole
DAS	Decay-associated spectra
DNS-Cl	Dansyl chloride
DPH	1,6-Diphenyl-1,3,5-hexatriene
EB	Ethidium bromide
F	Single-letter code for phenylalanine
FAD	Flavin adenine dinucleotide
FD	Frequency-domain
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein-5-isothiocyanate
FMN	Flavin mononucleotide
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
HSA	Human serum albumin
IAEDANS	5-((((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid
IAF	5-(Iodoacetamido)fluorescein
ICT	Internal charge transfer (state)
IRF	Instrument response function
LE	Locally excited (state)
MCP	Microchannel plate
MLC	Metal–ligand complex, usually of a transition metal (Ru, Rh, or Os)
MLCT	Metal–ligand charge transfer (state)
MPE	Multiphoton excitation
NADH	Reduced nicotinamide adenine dinucleotide
NATA	*N*-Acetyl-L-tryptophanamide
NATyrA	*N*-Acetyl-L-tyrosinamide
NBD	7-Nitrobenz-2-oxa-1,3-diazol-4-yl
NIR	Near-infrared
phe	Phenylalanine
PC	Phosphatidylcholine
PMT	Photomultiplier tube
POPOP	1,4-Bis(5-phenyloxazol-2-yl)benzene
PPD	2,5-Diphenyl-1,3,4-oxadazole
PPO	2,5-Diphenyloxazole
Prodan	6-Propionyl-2-(dimethylamino)naphthalene
PSDF	Phase-sensitive detection of fluorescence
RET	Resonance energy transfer
S_0	Ground electronic state
S_1	First excited singlet state
SPQ	6-Methoxy-*N*-(3-sulfopropyl)quinoline
T_1	First excited triplet state
TAC	Time-to-amplitude converter
TCSPC	Time-correlated single-photon counting
TD	Time-domain
TICT	Twisted internal charge-transfer state
TNS	6-(*p*-Toluidinyl)naphthalene-2-sulfonic acid
TRES	Time-resolved emission spectra
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

c Speed of light

C_0 Characteristic acceptor concentration in resonance energy transfer

$C(t)$ Correlation function for spectral relaxation

D Donor, diffusion coefficient, or rotational diffusion coefficient

$D_{\|}$ or $D_{\perp}$ Rate of rotational diffusion around (displacing) the symmetry axis of an ellipsoid of revolution

E Efficiency of energy transfer

F Steady state intensity or fluorescence

F_χ Ratio of χ^2_R values, used to calculate parameter confidence intervals

$\mathrm{F}(\lambda)$ Emission spectrum

f_i Fractional steady-state intensities in a multiexponential intensity decay

f_Q Efficiency of collisional quenching

G Correction factor for anisotropy measurements

hw Half-width in a distance or lifetime distribution

$I(t)$ Intensity decay, typically the impulse response function

k_{nr} Nonradiative decay rate

k_S Solvent relaxation rate

k_T Transfer rate in resonance energy transfer

m_ω Modulation at a light modulation frequency ω

n Refractive index, when used in consideration of solvent effects

$N(t_k)$ Number of counts per channel, in time-correlated single-photon counting

Q Quantum yield

$P(r)$ Probability function for a distance (r) distribution

pK_a Acid dissociation constant, negative logarithm

r Anisotropy (sometimes distance in a distance distribution)

$\bar{r}$ Average distance in a distance distribution

$r(0)$ Time-zero anisotropy

$r(t)$ Anisotropy decay

r_c Distance of closest approach between donors and acceptors in resonance energy transfer, or fluorophores and quenchers

r_{0i} or $r_0 g_i$ Fractional amplitudes in a multiexponential anisotropy decay

r_0 Fundamental anisotropy in the absence of rotational diffusion

r_{0i} Anisotropy amplitudes in a multiexponential anisotropy decay

r_∞ Long-time anisotropy in an anisotropy decay

r_ω Modulated anisotropy

R_0 Förster distance in resonance energy transfer

α_i Preexponential factors in a multiexponential intensity decay

β Angle between absorption and emission transition moments

Γ Radiative decay rate

γ Inverse of the decay time: $\gamma = 1/\tau$

ε Dielectric constant or extinction coefficient

θ Rotational correlation time

κ^2 Orientation factor in resonance energy transfer

Λ_ω Ratio of the modulated amplitudes of the polarized components of the emission

λ Wavelength

λ_{em} Emission wavelength

λ_{em}^{max} Maximum emission wavelength

λ_{ex} Excitation wavelength

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

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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

2. Instrumentation for Fluorescence Spectroscopy

3. Fluorophores

4. Time-Domain Lifetime Measurements

5. Frequency-Domain Lifetime Measurements

6. Solvent Effects on Emission Spectra

7. Dynamics of Solvent and Spectral Relaxation

8. Quenching of Fluorescence

9. Advanced Topics in Fluorescence Quenching

10. Fluorescence Anisotropy

11. Time-Dependent Anisotropy Decays

12. Advanced Anisotropy Concepts