Principles of Fluorescence Spectroscopy

Third Edition

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Dedicated to Mary,
for her continuous support and encouragement,
without whom this book would not have been written

Preface

The first edition of *Principles* was published in 1983, and the second edition 16 years later in 1999. At that time I thought the third edition would not be written until 2010 or later. However, the technology of fluorescence has advanced at an accelerating pace. Single-molecule detection and fluorescence-correlation spectroscopy are becoming almost routine. New classes of probes have appeared, such as the semiconductor nanoparticles, or QDots, and genetically engineered green fluorescent probes. Additionally, it is now becoming possible to control the excited states of fluorophores, rather than relying only on spontaneous emission. These developments are changing the paradigm of fluorescence, from a reliance on organic fluorophores, to the use of genetic engineering, nanotechnology, and near-field optics.

I wish to express my appreciation and special thanks to the individuals who have assisted me in the preparation of the book. These include Ignacy Gryczynski for assistance with the figures, Krystyna Gryczynski for drawing the figures, Joanna Malicka for proofreading the chapters, Kazik Nowaczyk for the cover design and color digitizing of all figures, Tim Oliver for typesetting, and the NIH for their support of my laboratory. And finally, Mary, for her endless hours of typing, correspondence and support.

Joseph R. Lakowicz

Glossary of Acronyms

A	acceptor
AA	anthranilic acid
2-AA	2-acetylanthracene
Ac	acetonitrile
Ac	acetone or acridine
ACF	acriflavine
AcH	acridinium cation
ACTH	adrenocorticotropin hormone
Alexa-Bz	Alexa-labeled benzodiazepine
ADC	analog-to-digital converter
Adx	adrenodoxin
I-AEDANS	5-((((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid
AFA	aminofluoranthene
AN	anthracene
2-AN	2-anilinonaphthalene
2,6-ANS	6-(anilino)naphthalene-2-sulfonic acid
AO	acridine orange or acoustooptic
2-AP	2-aminopurine
4-AP	4-aminophthalimide
APC	allophycocyanin
APDs	avalanche photodiodes
9-AS	9-anthroyloxy stearic acid
ASEs	asymptotic standard errors
AT	antithrombin
B	benzene
BABAPH	2-(sulfonatobutyl)-7-(dibutylamino)-2-aza-phenanthrene
BABP	sulfonatobutyl)-4-[4'-(dibutylamino)-phenyl]pyridine
BCECF	7'-bis(2-carboxyethyl)-5(6)-carboxyfluores-cein
BSA	bovine serum albumin
BODIPY	refers to a family of dyes based on 1,3,5,7,8-pentamethyl pyrromethene-BF_2, or 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; BODIPY is a trademark of Molecular Probes Inc.
β-PE	β-phycoerythrin
BPTI	bovine pancreatic trypsin inhibitor
Bromo-PCs	brominated phosphatidylcholines
Bu	butanol
C102	coumarin 102
C152	coumarin 152
C153	coumarin 153
9-CA	9-cyanoanthracene
CaM	calmodulin
cAMP	cyclic AMP
CFD	constant fraction discriminator
CG	calcium green
CHO	Chinese hamster ovary
CC	closed circular
CCDs	charged-coupled devices
CH	cyclohexane
Chol	cholesterol
CLSM	confocal laser scanning microscopy
CNF	carboxynaphthofluorescein
ConA	concanavalin A
CRABPI	cellular retinoic acid binding protein I
CSR	continuous spectral relaxation
CT	charge transfer
CW	continuous wave
D	donor
Dansyl	5-dimethylaminonaphthalene-1-sulfonic acid
DAPI	4',6-diamidino-2-phenylindole
DAS	decay-associated spectra
DBS	4-dimethylamino-4'-bromostilbene
DC	deoxycytosine
DDQ	distance-dependent quenching
DEA	diethylaniline
DEE	diethyl ether
DHE	dihydroequilenin
DHP	dihexadecyl phosphate
DiI or $DiIC_{12}$	1,1'-didodecyl-3,3,3',3'-tetramethy lindo-carbocyanine
DM	dodecylmaltoside
DMA	dimethylaniline
DMAS	N-dimethylaniline sulfonate
DMF	dimethylformamide
DMPC	dimyristoyl-L-α-phosphatidylcholine
DMP	dimethyldiazaperopyrenium
DMSO	dimethyl sulfoxide
DMQ	2,2'-dimethyl-p-quaterphenyl
10-DN	10-doxylnonadecane

DNS dansyl or 4-dimethylamino-4'-nitrostilbene
DNS-Cl dansyl chloride
DOS trans-4-dimethylamino-4'-(1-oxobutyl) stilbene
DPA 9,10-diphenylanthracene
DPA dipicolinic acid
DPE dansyl-labeled phosphatidylethanolamine
DPH 1,6-diphenyl-1,3,5-hexatriene
DPO 2,5-diphenyloxazole
DPPC dipalmitoyl-L-α-phosphatidylcholine
DPPC dipalmitoylphosphatidylcholine
DP(M,O)PC(E) dipalmitoyl(myrisotyl, oleayl)-L-α-phosphatidylcholine (ethanolamine)
DTAC dodecyltrimethylammonium chloride

EA ethyl acetate
EA ethanol
EAN ethylaniline
EB ethidium bromide
EC ethylcellulose
ECFP enhanced cyan fluorescent protein
EDT 1,2-ethanedithiol
EG ethylene glycol
ELISA enzyme-linked immunoadsorbent assays
eosin-PE eosin-phosphatidylethanolamine
EP 1-ethylpyrene
EPE eosin-labeled phosphatidylethanolamine
ESIPT excited-state intramolecular proton transfer
ESR excited-state reaction
EO electrooptic
EYFP enhanced yellow fluorescent protein

F single-letter code for phenylalanine
Fl fluorescein
Fl-C fluorescein-labeled catalytic subunit
FABPs fatty acid binding proteins
FAD flavin adenine dinucleotide
FC fura-2 with calcium
FCS fluorescence correlation spectroscopy
FD frequency domain
Fn fibronectin
Fs femtosecond
FITC fluorescein-5-isothiocyanate
FLIM fluorescence-lifetime imaging microscopy
FMN flavin mononucleotide
FR folate receptor
FRET fluorescence-resonance energy transfer
FWHM full width of half-maximum intensity
4FW 4-fluorotryptophan

GADPH glyceraldehyde-3-phosphate dehydrogenase
GFP green fluorescent protein
GGBP glucose-galactose binding protein
GM Goppert-Mayer
GOI gated optical image intensifier
GP generalized polarization
GPD glyceraldehyde-3-phosphate dehydrogenase
GPI glycosylphosphatidylinositol
GuHCI guanidine hydrochloride
GUVs giant unilamellar vesicles

H n-hexane
HDL high-density lipoprotein
HeCd helium–cadmium
HG harmonic generator
HITCI hexamethylindotricarbocyanine iodide
HLH human luteinizing hormone
HO highest occupied
HpRz hairpin ribozyme
HPTS 1-hydroxypyrene-3,6,8-trisulfonate
hrIFN-γ human recombinant interferon γ
HSA human serum albumin
17β-HSD 17β-hydroxysteroid dehydrogenase
hw half-width

IAEDANS 5-(((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid
IAF 5-iodoacetamidofluorescein
ICT internal charge transfer
IM insertion mutant
Indo-1-C_{18} indo-1 with a C_{18} chain
IRF instrument response function
IXP isoxanthopterin

KF Klenow fragment
KSI 3-ketosteroid isomerase

LADH liver alcohol dehydrogenase
LCAT lecithin:cholesterol acyltransferase
LDs laser diodes
LE locally excited
LEDs light-emitting diodes
LU lowest unoccupied

M monomer
MAI N-methylquinolinium iodide
MBP maltose-binding protein
MCA multichannel analyzer
MCP microchannel plate
Me methanol
MEM method-of-moments
met RS methionyl-tRNA synthetase
3-MI 3-methyl indole
MLC metal–ligand complex, usually of a transition metal, Ru, Rh or Os
MLCK myosin light chain kinase
MLCT metal–ligand charge transfer (state)
MLE maximum likelihood estimates
MPE multiphoton excitation
MPM multiphoton microscopy
MQAE 6-methoxy-quinolyl acetoethyl ester
MRI magnetic resonance imaging

NADH	reduced nicotinamide adenine dinucleotide
NATA	N-acetyl-L-tryptophanamide
NATyrA	N-acetyl-L-tyrosinamide
NB	Nile blue
NBD	N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)
NBD-DG	1-oleoyl-2-hexanoyl-NBD-glycerol
Nd:YAG	neodymium:YAG
NIR	near infrared
NLLS	nonlinear least squares
NMA	N-methylanthraniloyl amide
NO	nitric oxide
NPN	N-phenyl-1-naphthylamine
NR	neutral red
NRP	neuronal receptor peptide
5-NS	5-doxylstearate
OG	Oregon green
OPO	optical parameter oscillator
ORB	octadecyl rhodamine B
Os	osmium
PBFI	potassium-binding benzofuran isophthalate
PC	phosphatidylcholine
PCSC	photon-counting streak camera
PDA	pyrene dodecanoic acid
PDs	photodiodes
PE	phycoerythrin
PE	phosphatidylethanolamine
1PE	one-photon
2PE	two-photon
3PE	three-photon
PET	photoinduced electron transfer
PeCN	3-cyanoperylene
PG	propylene glycol
PGK	phosphoglycerate kinase
Phe(F)	phenylalanine
PK	protein kinase
PKI	protein kinase inhibitor
PMMA	poly(methylmethacrylate)
PMT	photomultiplier tube
POPC	1-palmitoyl-2-oleoylphosphatidylcholine
POPOP	1,4-bis(5-phenyloxazol-2-yl)benzene
PP	pulse picker
PPD	2,5-diphenyl-1,3,4-oxadizole
PPi	pyrophosphate
PPO	2,5-diphenyloxazole
PRODAN	6-propionyl-2-(dimethylamino)-naphthalene
ps	picosecond
PSDF	phase-sensitive detection of fluorescence
PTP	phosphoryl-transfer protein
Py2	pyridine 2
QDs	quantum dots
QTH	quartz–tungsten halogen
RBC	radiation boundary condition
RBL	rat basophilic leukemia
R-PE	R-phycoerythrin
REES	red-edge excitation shifts
Re I	rhenium
RET	resonance energy transfer
RF	radio frequency
RFP	red fluorescent protein
Rh	rhodamine
RhB	rhodamine B
RhG	rhodamine green
R6G	rhodamine 6G
RNase T_1	ribonuclease T_1
RR	rhodamine red
Ru	ruthenium
SAS	species-associated spectra
SBFI	sodium-binding benzofuran isophthalate
SBP	steroid-binding protein
SBS	substrate-binding strand
SC	subtilisin Carlsberg
SDS	sodium dodecylsulfate
SEDA	dapoxyl sulfonyl ethylenediamine
SMD	single-molecule detection
SNAFLs	seminophthofluoresceins
SNARFs	seminaphthorhodafluors
SP	short-pass
SPQ	6-methoxy-N-[3-sulfopyropyl]quinoline
T	tetramer
TAC	time-to-amplitude converter
TCE	trichloroethanol
t-COPA	16-octadecapentaenoic acid
TCSPC	time-correlated single photon counting
TD	time-domain
TEOS	tetraethylorthosilicate
TFA	trifluoroacetamide
TFE	trifluoroethanol
THF	tetrahydrofuron
TICT	twisted internal charge transfer
TK	thymidine kinase
TL	tear lipocalin
TMA	donor alone
TMR	tetramethylrhodamine
TnC	troponin C
TNS	6-(p-toluidinyl)naphthalene-2-sulfonic acid
TOAH	tetraoctylammonium hydroxide
TOE	tryptophan octyl ester
TPI	triosephosphate isomerase

TRES	time-resolved emission spectra
$TrpNH_2$	tryptophanamide
TRITC	tetramethylrhodamine-5-(and-6)-isothio-cyanate
$tRNA^{fMet}$	methionine tRNA
trp(w)	tryptophan
TTS	transit time spread
TU2D	donor–acceptor pair
tyr(y)	tyrosine
U	uridine
7- UmP	7-umbelliferyl phosphate
w	single-letter code for tryptophan
W	water
WT	wild type
WD	window discriminator
Xe	xenon
y	single-letter code for tryptophan
YFP	yellow fluorescent protein

Glossary of Mathematical Terms

A	acceptor or absorption
B_i	brightness of a fluorophore
c	speed of light
C_0	characteristic acceptor concentration in RET
$C(t)$	correlation function for spectral relaxation
D	donor, or diffusion coefficient, or rotational diffusion coefficient
$D_{\parallel}$ or $D_{\perp}$	rate of rotation diffusion around or (displacing) the symmetry axis of an ellipsoid of revolution
$D(\tau)$	part of the autocorrelation function for diffusion containing the diffusion-dependent terms
E	efficiency of energy transfer
F	steady-state intensity or fluorescence
$F\chi$	ratio of χ_R^2 values, used to calculate parameter confidence intervals
$F(\lambda)$	emission spectrum
f_i	fractional steady-state intensities in a multi-exponential intensity decay
f_Q	efficiency of collisional quenching
G	correction factor for anisotropy measurements
$G(\tau)$	autocorrelation function of fluorescence fluctuations
hw	half-width in a distance or lifetime distribution
$I(t)$	intensity decay, typically the impulse response function
k_{nr}	non-radiative decay rate
k_s	solvent relaxation rate
k_T	transfer rate in resonance energy transfer
k_{st}	rate of singlet to triplet intersystem crossing
k_{ts}	rate of return to the singlet ground state from the triplet state
m_ω	modulation at a light modulation frequency ω
n	refractive index, when used in consideration of solvent effects
N	number of observed molecules in FCS
$N(t_k)$	number of counts per channel, in time-correlation single-photon counting
$P(r)$	probability function for a distance (r) distribution
pK_a	acid dissociation constant, negative logarithm
q	efficiency for detection of emitted photons, typically for FCS
Q	quantum yield
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 multi-exponential anisotropy decay
r_0	fundamental anisotropy in the absence of rotational diffusion
r_{0i}	anisotropy amplitudes in a multi-exponential anisotropy decay
r_∞	long-time anisotropy in an anisotropy decay
r_ω	modulated anisotropy
R_0	Förster distance in resonance energy transfer
T	temperature
T_P	phase transition temperature for a membrane
α_i	pre-exponential factors in a multi-exponential intensity decay
β	angle between absorption and emission transition moments
γ	inverse of the decay time, $\gamma = 1/\tau$
Γ	radiative decay rate
ε	dielectric constant or extinction coefficient
ε_A or ε	molar extinction coefficient for absorption
θ	rotational correlation time
θ_c	critical angle for total internal reflection
κ^2	orientation factor in resonance energy transfer
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 maxima
$\Lambda_?$	ratio of the modulated amplitudes of the polarized components of the emission
η	viscosity
μ_E	excited-state dipole moment
μ_G	ground-state dipole moment
μm	micron
$\bar{\nu}$	specific gravity or wavelength in cm^{-1}
$\bar{\nu}_{cg}$	center of gravity of an emission spectrum in cm^{-1}
σ or σ_A	optical cross-section for absorption
σ_S	optical cross-section for scattering
τ	lifetime or time-delay in FCS
τ_D	diffusion time in FCS
τ_s	solvent or spectral relaxation time

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