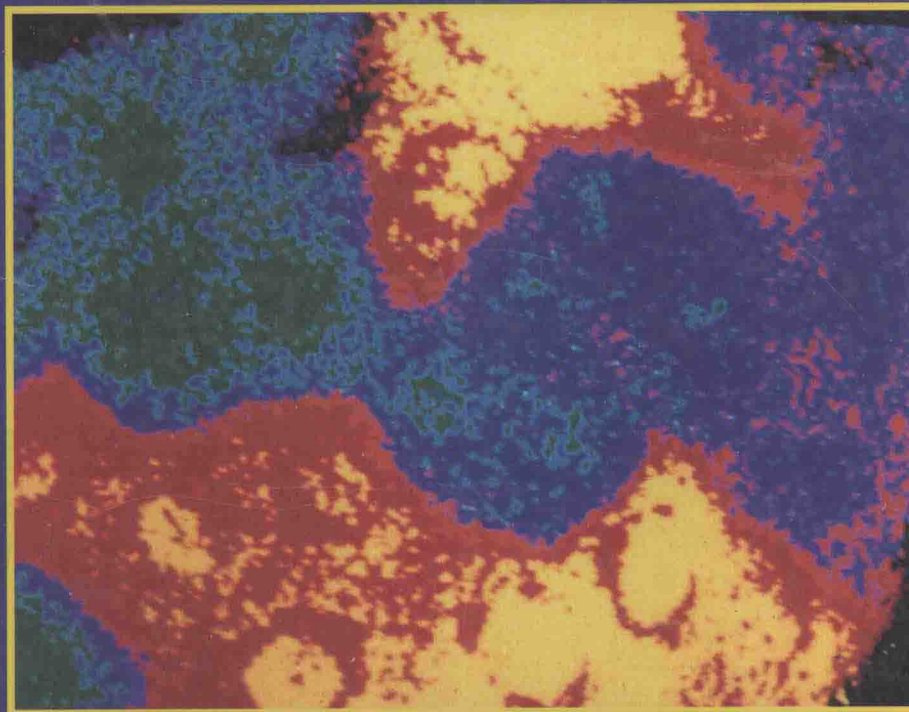




Fluorescence Microscopy

Second Edition



B. Herman



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Fluorescence Microscopy

SECOND EDITION

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Front cover: Alterations in cytosolic calcium in human epidermal keratinocytes following exposure to epidermal growth factor (EGF). Keratinocytes were loaded with Fura-2 and exposed to 10 ng ml⁻¹ EGF. Warm colours (yellow, red) represent areas of high calcium, while cool colours (violet, blue) represent areas of low calcium.

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Abbreviations

A	absorption
AFIC	automated fluorescence image cytometry
A/D	analogue-to-digital converter
Ala	alanine
ALU	arithmetic logic unit
AM	acetoxymethyl
AMCA	amino-methyl-coumarin-acetic acid
AOD	acoustic-optical device
AOTF	acousto-optical tunable filter
ASA	film speed
BAPTA	O,O'-bis(2-aminophenyl)ethyleneglycol- <i>N, N, N', N'</i> -tetraacetic acid
BCECF	3'- <i>O</i> -acetyl-2',7'-bis(carboxyethyl)-4- or -5-carboxy-fluorescein
BCPDA	4,7-bis(chlorosaltophenol)-1,10-phenanthridine-2,9-dicarboxylic acid
BFP	blue fluorescent protein
<i>c</i>	concentration
CAT	computerized axial tomography
CBS or DBS	dichromatic beam splitter
CCD	charge coupled device
CD ROM	compact disk read only material
cDNA	complementary DNA
CID	change injector device
CMYK	cyan, magenta, yellow, black
CPU	central processing unit
CRT	cathode ray tube
CW	continuous wave
CY3	cyanine 3
<i>D</i>	diffusion coefficient
DAPI	4',6-diamidino-2-phenylindole
DIC	differential interference contrast
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol

DVM	digitized video microscopy
EGF	epidermal growth factor
EGFP	enhanced GFP
EGTA	<i>O,O'</i> -Bis(2-aminoethyl)ethyleneglycol- <i>N, N, N', N'</i> -tetraacetic acid
EM	electron microscopy
EPR	exhaustive photon reassignment
E_T	efficiency of FRET
F/P	fluorophore/protein molar ratio
FAD	flavin adenine dinucleotide
fc	foot candles
FEP	fluorescence emission before photodestruction
FER	fluorescence emission rate
FISH	fluorescence <i>in situ</i> hybridization
FITC	fluorescein isothiocyanate
FL	fluorophore
FLIM	fluorescence lifetime imaging microscopy
FMN	flavin mononucleotide
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
FRET _M	fluorescence resonance energy transfer microscopy
FWHM	full width half maximum
GFP	green fluorescent protein
Gly	glycine
G ₀	ground state
HPV	human papillomavirus
HSI	hue, saturation, intensity
I_A	acceptor emission
I_D	donor emission
I_O	incident intensity
I	observed intensity
ISIT	intensified silicon intensified target
ISO	Industrial Standards Organization
$J(\lambda)$	overlap integral
K^2	orientation factor
k_f	rate constant for fluorescence emission
k_i	rate constant for internal conversion
K_M	Michaelis–Menton constant
k_{nr}	rate constant for non-radiative processes
k_p	rate constant for phosphorescence
K_T	rate constant for RET
LCTF	liquid crystal tunable filter
LM	light microscopy
LP	long pass
LSCM	laser scanning confocal microscope
LSM	laser scanning microscope
Mag	magnification

MCP	multichannel plate
MDVM	multiparameter digitized video microscopy
Met	methionine
mf	mobile fraction
MOS	metal oxide semiconductor
mt	mutant
NA	numerical aperture
ND	neutral density
NIH	National Institute of Health
NITR-5	caged calcium
NITR-7	caged calcium
OMDR	optical memory disk recorder
OPD	optical path difference
p	measured polarization
PBS	phosphate-buffered saline
PCB	printed circuit board
PCR	polymerase chain reaction
Phe	phenylalanine
PMT	photomultiplier tube
p_0	limiting polarization
Q	quantum yield
r	measured anisotropy
r_{Airy}	radius of Airy disc
r_0	limiting anisotropy
RET	resonance energy transfer
RGB	red, green, blue
S/N	signal-to-noise ratio
S_1	singlet excited state
SDS	sodium dodecyl sulphate
Ser	serine
SIT	silicon intensified target
SLR	single lens reflex
S_0	ground state
SP	short pass
SSCM	stage scanning confocal microscope
TE	transverse electronic
TeO_2	tellurium oxide
Thr	threonine
TIFF	tagged image file format
TIRF	total internal reflectance fluorescence microscopy
T_m	melting temperature
TM	transverse magnetic
TPEM	two-photon excitation microscopy
Tyr	tyrosine
Val	valine
wt	wild-type
x	pathlength

ε	extinction coefficient
η	refractive index of medium
λ	wavelength
μlux	microlux
ν_1	vibrational energy level of excited state
ν_0	vibrational energy level of ground state
ϕ	rotational correlation time
τ	fluorescence lifetime
τ_D	lifetime of donor
τ_{DA}	lifetime of donor in presence of acceptor
τ_F	fluorescence lifetime
τ_0	intrinsic fluorescent lifetime

Preface

Fluorescence microscopy is a very powerful tool in that it allows quantitative spatial and temporal visualization of fluorescent material in microscope specimens. Because of this capability, numerous investigators have turned to this technology to address questions of fundamental biological importance. Having used fluorescence microscopy in my own research, I am often asked an array of questions about this technology, and especially, where individuals can turn to learn more about this subject. While many excellent texts and monographs exist which cover various aspects of fluorescence and microscopy, the lack of a concise comprehensive source for this information was a major motivation for writing this book. I hope to have relayed my understanding of the important principles and applications of fluorescence microscopy, and that this book will serve as a broad introduction for new users to the variety of information that can be gathered with fluorescence microscopy.

Much of the information presented in this book is the result of the substantial contributions of many of my colleagues. While the list is long, particularly important contributions were made by David Albertini, Richard Berlin, John Bogan, Ludwig Brand, Robert Clegg, Jim DiGuseppi, Pamela Diliberto, Fred Fay, Salvatore Fernandez, Kathryn Florine-Casteel, Hans Gerritsen, Gerry Gordon, Enrico Gratton, Charles R. Hackenbrock, Richard Haugland, Richard Inman, Shinya Inoué, Colin Izzard, Ken Jacobson, Tom and Donna Jovin, Joe Kao, Hans Kapitza, Ernst Keller, Dennis Koppel, Joseph Lakowicz, John Lederer, John J. Lemasters, Leslie Loew, Steve Lockett, Fred Maxfield, Butch Moomaw, Stephen Morris, John Murray, Ammasi Periasamy, David Piston, Ted Salmon, Jan Slavik, Deborah K. Smith, Ken Spring, Hans Tanke, D. Lansing Taylor, Roger Tsien, Jim Turner, Xue Feng Wang, Yu-Li Wang, Pawel Wodnicki and Barney Wray. The support of numerous funding agencies and corporations is also appreciated, including the NIH, NSF, ACS, AHA, The North Carolina Biotechnology Center, and the Gustavus and Louise Pfeiffer Foundation. Dage-MTI Inc., Datacube, Inc., Hamamatsu, Inc., Olympus, Inc. and Carl Zeiss, Inc., are also gratefully acknowledged. This book would not be a reality without the work of an exceptionally talented graphic artist, Tracey Curran. Lastly, I wish to dedicate this book to my wife, Deborah K. Smith, and our beautiful daughter, Lindsey, without whose

understanding, love and patience I would not have been able to undertake this endeavour.

Brian Herman

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1 Fundamentals of fluorescence

1.1 What is fluorescence?

Fluorescence is the property of some atoms and molecules to absorb light of a particular wavelength and after a brief interval, termed the fluorescence lifetime, to re-emit light at longer wavelengths. Fluorescence requires an outside source of energy, is the result of the absorption of light, and involves the emission of electromagnetic radiation (light). This process is different from chemiluminescence, where the excited state is created via a chemical reaction.

1.2 Wavelength, excitation and emission spectra

Light can be described as having characteristics of both particle and wave phenomena (*Figure 1.1*).

As a wave, light has two components, electric (E) and magnetic (H), which travel in space perpendicular to one another. The distance between consecutive wavecrests is defined as the wavelength (λ). Light normally consists of a mixture of electromagnetic waves of many wavelengths. Planck's law states that $E = h\nu$, where E is the energy in ergs, h is Planck's constant (6.6×10^{-27}) and ν is the frequency of light (sec^{-1} ; i.e. the number of waves passing a point in 1 sec). As a reference, the energy of 1 mole of photons (6.02×10^{23}) at 500 nm is $\sim 60 \text{ kcal mol}^{-1}$. Shorter wavelengths (i.e. shorter distances between consecutive wavecrests) have higher amounts of energy versus longer wavelengths.

The absorption of a photon of energy by a fluorescent molecule is an all or none phenomenon and each fluorescent molecule can only absorb incident light of certain specific wavelengths known as absorption

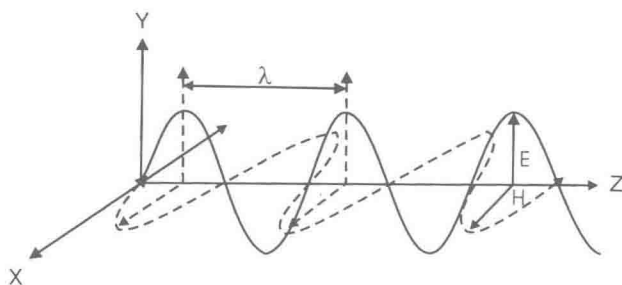


Figure 1.1. An electromagnetic light wave with electric field vector E in the yz plane and magnetic field vector H in the xz plane. The distance between consecutive wavecrests is defined as the wavelength, λ . Reproduced by permission of Marcel Dekker, New York, from Pesce, A.J., Rosen, C.-G. and Pashby, T. (1971) *Fluorescence Spectroscopy: An Introduction for Biology and Medicine*, p. 3.

bands. Emission of fluorescence also occurs at certain specific wavelengths, but these wavelengths are at lower energies (i.e. longer λ values) than absorption band maxima due to loss of energy by the molecule during interactions with its environment before it emits its fluorescence (internal conversion).

Absorption of energy by fluorescent molecules occurs between a number of closely spaced vibrational and rotational excited states in different orbitals. The Jablonski diagram (named after A. Jablonski and described in 1953) demonstrates the different energy levels involved in the absorption and emission of light (*Figure 1.2*). Physically, absorption of light occurs very quickly (approx. 10^{-15} sec) and corresponds to the excitation of the fluorophore from the ground state to an excited state. Relaxation to the lowest excited singlet state, known as internal conversion, occurs within approximately 10^{-11} sec as energy is thermally transferred to the environment. Internal conversion (loss of energy in the absence of emission of light) is due to collision of the excited state probe with solvent molecules. The molecule 'lives' in the lowest excited singlet state for periods of the order of nanoseconds (approx. 10^{-9} sec). Relaxation from this state (the lowest excited singlet, S_1 ; see *Figure 1.2*) to the ground state with emission of a photon is, physically, what is referred to as fluorescence. Each fluorescent molecule (fluorophore) can repeat the excitation/emission process many times, for example for fluorescein isothiocyanate (FITC) approximately 30 000 times, before excited state processes bleach the FITC molecule. In addition to fluorescence, molecules which reside in the lowest excited singlet state can undergo intersystem crossing to the triplet state from which a longed lived emission, phosphorescence, occurs. Fortunately, this is a relatively rare event. Delayed fluorescence can also occur due to transitions from T_1 back to S_1 and then to G_0 .

The probability of movement of an electron from the ground state (G_0) to the excited state (S_1) depends on the degree of similarity of the vibrational and rotational energy states where the electron resides in the ground state versus where it would reside in the excited state (*Figure*

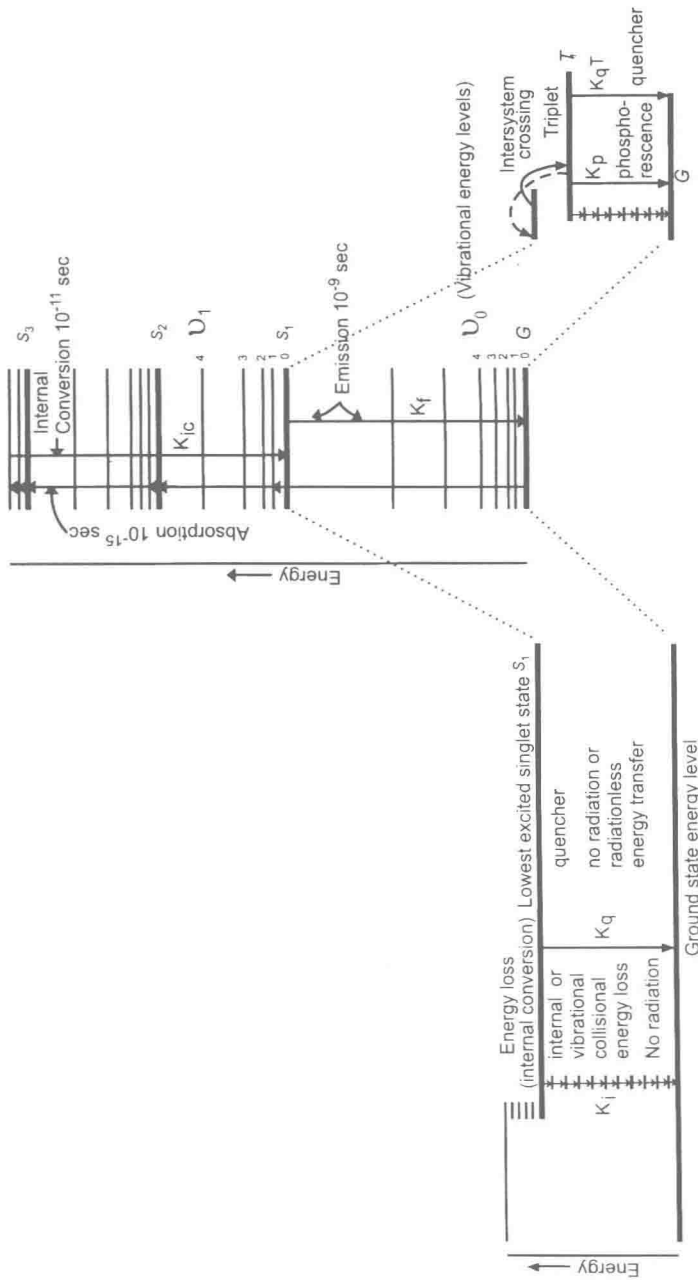


Figure 1.2. Modified Jablonski diagram illustrating the lifetimes of electronic transitions and the various processes which compete with fluorescence (k_i) (k_i = internal conversion, k_{qT} = quenching, k_p = phosphorescence) for deactivation of excited energy state. v_0 and v_1 represent vibrational energy states (see Figure 1.3).

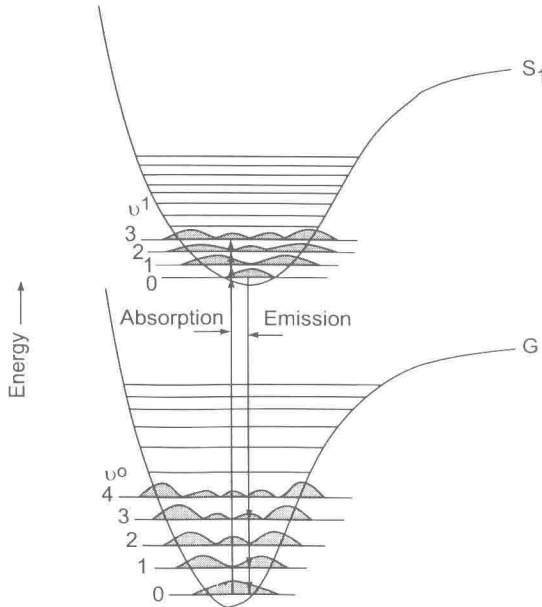


Figure 1.3. Energy diagram. Shaded areas represent the probability of the electron position in the ground (v_0) and excited (v_1) vibrational energy states. Transitions from the ground to the excited state occur in such a short time (10^{-15} sec) that the molecule cannot move (Frank Condon principle). Therefore, the only transitions from the ground state to the excited state which can occur are those where the probability of the electron position in the ground and excited states maximally overlap. Reproduced by permission of Marcel Dekker, New York, from Pesce, A.J., Rosen, C.-J. and Pashby, T. (1971) *Fluorescence Spectroscopy: An Introduction for Biology and Medicine*, p. 42.

1.3). The most favoured electron transitions will be those where the probability of the position of the electron in the ground and excited states (rotational and vibrational) maximally overlap. This position of maximal overlap can be thought of as indicating the excitation energy level that is most likely to be absorbed. The most likely state for an electron at room temperature is the ground state (G_0 or S_0). Within the ground state there exist a number of distinct vibrational energy states ($v_0 = 0, 1, 2$), and thus the amount of energy of absorption for each of these vibrational levels in the ground state will differ. This gives rise to the absorption spectrum containing multiple peaks (Figure 1.4). If we scan through the absorption spectrum while looking at the emission at one wavelength, we generate the excitation spectrum (Figure 1.5). Like the absorption spectrum, the excitation spectrum is broadened. If we hold the excitation constant and scan through the emission wavelengths, we generate the emission spectrum (Figure 1.5).

Following the absorption of energy and movement of the electron to the excited state, internal conversion causes the excited state electron to lose energy, such that the electron comes to rest in the lowest vibrational energy level of the excited singlet state ($v_1 = 0$; Figure 1.3). The energy