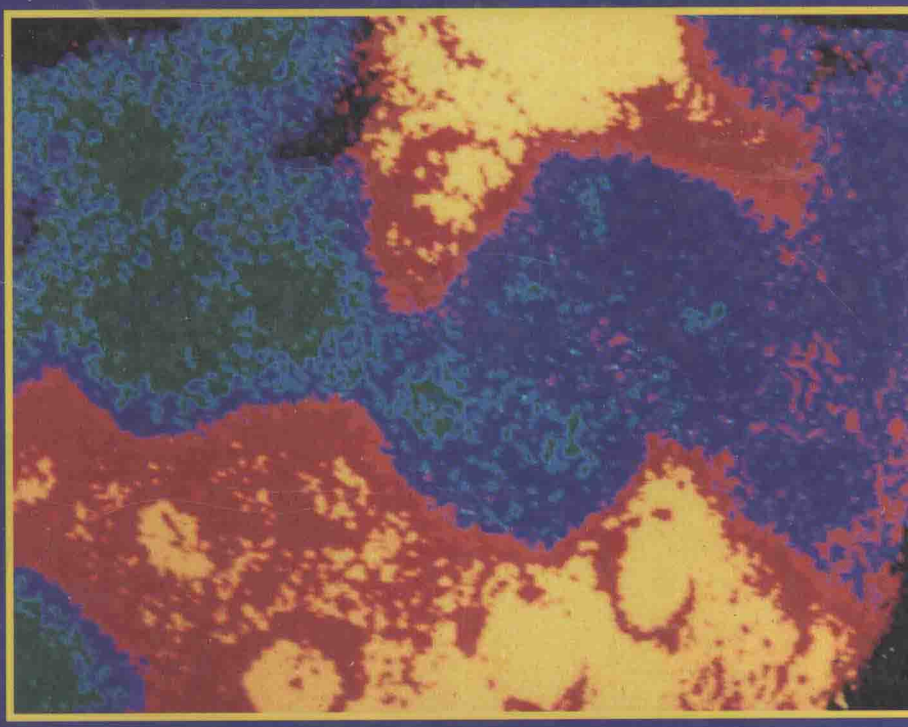
# Fluorescence Microscopy

## Second Edition



B. Herman

世界图书出版公司

# Fluorescence Microscopy

SECOND EDITION

**B. Herman**
CB # 7090, 232 Taylor Hall,
University of North Carolina,
Chapel Hill, NC 27599, USA
e-mail: bhgf@med.unc.edu

BIOS SCIENTIFIC PUBLISHERS

Springer

In association with the Royal Microscopical Society

B. Herman
*University of North Carolina, Chapel Hill, NC 27599, USA*



A CIP catalogue record for this book is available from the British Library.

ISBN 981 3083 83 2 Springer-Verlag Singapore Pte Ltd

Production Editor: John Leonard.
Typeset by Poole Typesetting (Wessex) Ltd, Bournemouth, UK.
Printed by Biddles Ltd, Guildford, UK.

**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.

MICROSCOPY HANDBOOKS 40

# Fluorescence Microscopy

SECOND EDITION

# 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-*N*, *N*, *N*′, *N*′-tetraacetic acid |
| BCECF | 3′-*O*-acetyl-2′,7′-bis(carboxyethyl)-4- or -5-carboxy-fluorescein |
| BCPDA | 4,7-bis(chlorosaltophenol)-1,10-phenanthidine-2,9-dicarboxylic acid |
| BFP | blue fluorescent protein |
| *c* | 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 |
| *D* | diffusion coefficient |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DIC | differential interference contrast |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide trisphosphate |
| DTNB | 5,5′-Dithiobis(2-nitrobenzoic acid) |
| DTT | dithiothreitol |

| | |
|---|---|
| DVM | digitized video microscopy |
| EGF | epidermal growth factor |
| EGFP | enhanced GFP |
| EGTA | *O*,*O*′-Bis(2-aminoethyl)ethyleneglycol-*N*, *N*, *N*′, *N*′-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 *in situ* hybridization |
| FITC | fluorescein isothiocyanate |
| FL | fluorophore |
| FLIM | fluorescence lifetime imaging microscopy |
| FMN | flavin mononucleotide |
| FRAP | fluorescence recovery after photobleaching |
| FRET | fluorescence resonance energy transfer |
| FRETM | fluorescence resonance energy transfer microscopy |
| FWHM | full width half maximum |
| GFP | green fluorescent protein |
| Gly | glycine |
| $G_o$ | 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 |
| $r_{\text{Airy}}$ | 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 |

| | |
|---|---|
| $\varepsilon$ | extinction coefficient |
| $\eta$ | refractive index of medium |
| $\lambda$ | wavelength |
| µlux | microlux |
| $\nu_1$ | vibrational energy level of excited state |
| $\nu_o$ | vibrational energy level of ground state |
| $\phi$ | rotational correlation time |
| $\tau$ | fluorescence lifetime |
| $\tau_D$ | lifetime of donor |
| $\tau_{DA}$ | lifetime of donor in presence of acceptor |
| $\tau_F$ | fluorescence lifetime |
| $\tau_o$ | 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 DiGuiseppi, 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

# Contents

# 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 ($\lambda$). Light normally consists of a mixture of electromagnetic waves of many wavelengths. Planck's law states that $E = h\upsilon$, where $E$ is the energy in ergs, $h$ is Planck's constant ($6.6 \times 10^{-27}$) and $\upsilon$ 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 ($6.02 \times 10^{23}$) at 500 nm is ~ 60 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

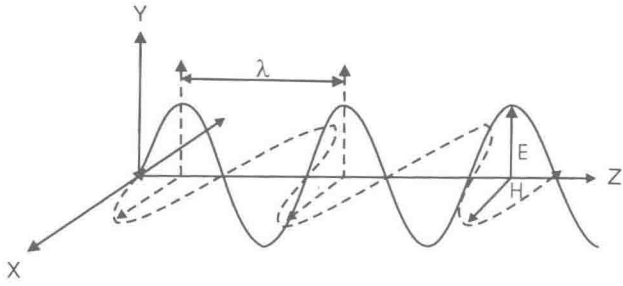



**Figure 1.1.** An electromagnetic light wave with electric field vector **E** in the *yz* plane and magnetic field vector **H** in the *xz* plane. The distance between consecutive wavecrests is defined as the wavelength, λ. Reproduced by permission of Marcel Dekker, New York, from Pesce, A.J., Rosen, 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*

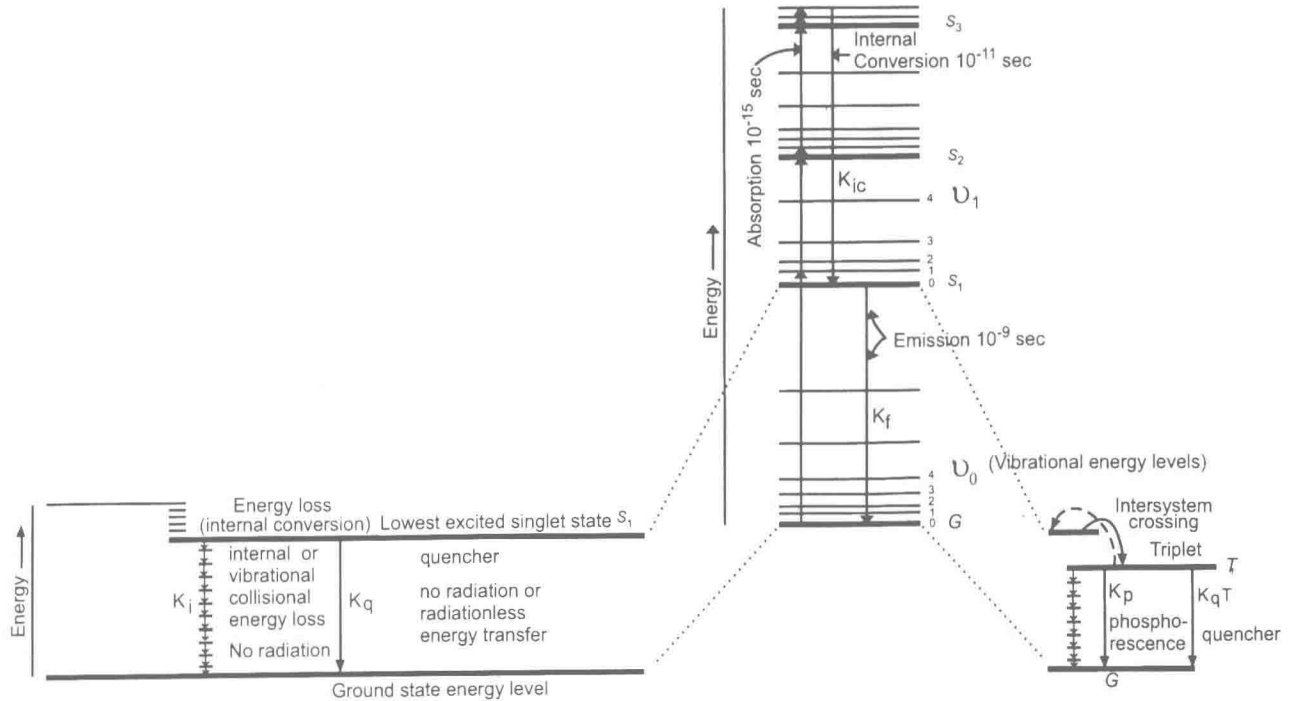



**Figure 1.2.** Modified Jablonski diagram illustrating the lifetimes of electronic transitions and the various processes which compete with fluorescence ($k_f$) ($k_i$ = internal conversion, $k_q$ = quenching, $k_{pT}$ = intersystem crossing into triplet state, $k_p$ = phosphorescence) for deactivation of excited energy state. $\upsilon_0$ and $\upsilon_1$ represent vibrational energy states (*see Figure 1.3*).

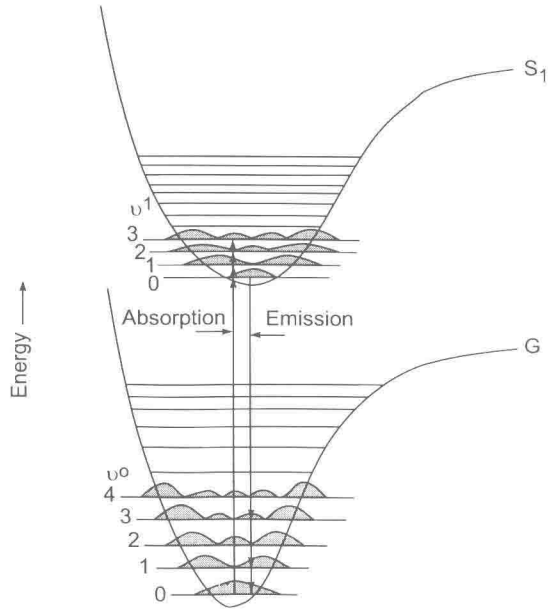



**Figure 1.3.** Energy diagram. Shaded areas represent the probability of the electron position in the ground ($\upsilon_0$) and excited ($\upsilon_1$) vibrational energy states. Transitions from the ground to the excited state occur in such a short time ($10^{-15}$ sec) that the molecule cannot move (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 ($\upsilon_0$ = 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 ($\upsilon_1$ = 0; *Figure 1.3*). The energy