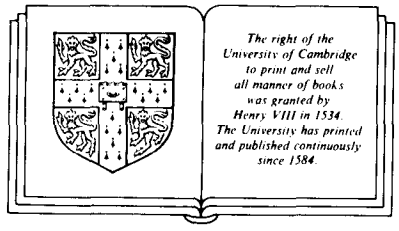
Quantitative fluorescence microscopy

F. W. D. ROST

Quantitative fluorescence microscopy

F.W.D. ROST

Professor of Anatomy, University of New South Wales



CAMBRIDGE UNIVERSITY PRESS

Cambridge,

New York, Port Chester, Melbourne, Sydney

Published by the Press Syndicate of the University of Cambridge
The Pitt Building, Trumpington Street, Cambridge CB2 1RP
40 West 20th Street, New York, NY 10011-4211, USA
10 Stamford Road, Oakleigh, Melbourne, Victoria 3166, Australia

First published 1991
Printed in Great Britain at the University Press, Cambridge

British Library cataloguing in publication data
Rost, F.W.D.
Quantitative fluorescence microscopy.
1. Fluorescence microscopy
I. Title
502.823

Library of Congress cataloguing in publication data
Rost, F.W.D.
Quantitative fluorescence microscopy / F.W.D. Rost.
p. cm.
Includes bibliographical references and index.
ISBN 0 521 39422 8
1. Fluorescence microscopy – Technique. I. Title.
QH212.F55R67 1990
578'.4—dc20 90-15056 CIP

ISBN 0 521 39422 8 hardback

SE

Preface

This book is one of a pair of books dealing with fluorescence microscopy. The companion volumes (*Fluorescence microscopy*; Rost, 1991a,b) deal with fluorescence microscopy in general and include a brief overview of quantitative fluorescence microscopy, with emphasis on those aspects which are important to an understanding of fluorescence microscopy. The present volume deals with quantitative fluorescence microscopy in much greater detail. Scanning microscopy is dealt with in this volume because scanning usually involves measurements of fluorescence intensity.

My goals in preparing this book have been to provide, firstly, a detailed description of the structure and use of microfluorometric apparatus; secondly, a description of the basic physical and chemical principles involved; and thirdly, a broad overview of the various applications of quantitative fluorescence microscopy, to alert users to techniques which might be useful to them, and to give references to facilitate obtaining more information on any topic. As in the companion volume, I have assumed that the reader consulting this book will usually have a practical problem and desire a pragmatic answer. Accordingly, the main part of the text describes current techniques and applications; most of the history of the development of quantitative fluorescence microscopy and of its applications has been placed separately in Chapter 16.

For starting me off on the way to these books, I have to thank Professor A. G. Everson Pearse, of the Royal Postgraduate Medical School, London. I had the great privilege of working under him for almost a decade (1965–1974), and it was in his laboratory that I became involved in microspectrofluorometry.

I am very much indebted to those of my friends and colleagues who kindly read and commented on chapters or sections of the book: (in alphabetical order) Associate Professor C.G. dos Remedios, Dr E. Kohen, Mr W. Loeb, Mr R.J. Oldfield, Dr G.L. Paul, Professor P.J. Stoward, Dr A.A. Thaer, and Dr N.G.M. Wreford. Of course, I alone must accept responsibility for all errors of commission and omission.

I am very grateful to numerous friends and colleagues who have given me the hospitality of their laboratories and provided much valuable information. I particularly wish to thank (in alphabetical order): Professor G. Bottiroli and his colleagues of the University of Pavia; Professor T. Caspersson and Dr Martin Ritzén of the Karolinska Institute, Stockholm; Mr Karl-Heinz Hormel of E. Leitz GmbH, Wetzlar,

formerly of E. Leitz (Instruments) Ltd; Professor M. van der Ploeg and Professor J.S. Ploem of the University of Leiden; Dr Andreas Thaer, of Helmut Hund Kg, Wetzlar, formerly of E. Leitz GmbH and of the Battelle Institut, Frankfurt; Dr F. Walter of E. Leitz GmbH, Wetzlar; and Mr H. Wasmund, formerly of E. Leitz GmbH, Wetzlar.

Preparation of the book was commenced while I was employed at the Royal Postgraduate Medical School, London, and completed at the University of New South Wales. I am indebted to Library staff of the University for much assistance, and particularly to Mr Andrew Holmick for searches of the literature. Mr Patrick de Permentier, Ms Jenny Flux and Ms Stacey McClelland provided technical assistance and help with bibliographic work. A substantial amount of the text was typed by Mrs Lorraine Brooks. For conversion of word-processor text from an older system to WORD on a Macintosh, I am very grateful to the late Mr Peter Hughes of Macquarie University and to Mr Paul Halasz. I am also indebted to Mrs Gillian Rankin of Macquarie University and to Ms Alicia Fritchle for redrawing diagrams, to Mr Collin Yeo for assistance in photography, and to Mrs Mary Armstrong for secretarial assistance.

Last but not least I wish to express my appreciation of the collaboration of Dr Alan Crowden and Mrs Sandi Irvine of CUP.

Fred Rost
Sydney, 1990

Abbreviations and symbols

a	area of measured field
A	(1) optical absorption (2) cross-sectional area of light bundle at the prism
AC	alternating current
A-D	analogue-to-digital
ANS	1-anilinonaphthalene-8-sulphonate
AO	Acridine Orange
atm	atmosphere (1 atm $\approx 10^5$ Pa)
B	background photon count
b	constant
BAO	bis-aminophenyl-oxdiazole
BCECF	2'-7'-bis-(2-carboxymethyl)-5- (and -6-) carboxyfluorescein
BMT	bone marrow transplant
BrdU	5-bromodeoxyuridine
c	concentration of fluorophore
CCD	charge-coupled device
CCTV	closed circuit television
CD	compact disc
CPM	3-(4-maleimidylphenyl)-7-diethylamino-4-methylcoumarin
CRO	cathode ray oscilloscope
d	optical path length (depth)
DANS	dansyl chloride
DAPI	4,6'-diamidino-2-phenylindole dihydrochloride
DASPMI	dimethyl-aminostyryl-methylpyridinium iodide
DASS	defined-substrate sphere system
DC	direct current
DIC	differential interference-contrast
Di-I-LDL	3,3-dioctadecylindocarbocyanine-labelled low-density lipoprotein
DIN	deutsches Industrie Norm
DIPI	4,6-bis-(2-imidazolinyl-4,5H)-2-phenylindole

DNA	deoxyribonucleic acid
DPNH	nicotinamide adenine dinucleotide, reduced form
DTE	dithioerythritol
DTT	dithiothreitol
e	base of natural logarithms (2·71828 . . .)
E	(1) optical extinction (optical density) (2) quantum energy of photon (3) radiant energy at entrance slit per wavelength unit
EHT	high-voltage supply for photomultiplier tube (extra-high-voltage)
ELISA	enzyme-linked immunosorbent assay
Em	emission maximum
Ex	excitation maximum
eV	electron volts
F	relative fluorescence intensity
f	focal length (of collimator)
FDA	fluorescein diacetate
F-DIM	fluorescence digital imaging microscopy
FIF	formaldehyde-induced fluorescence
FITC	fluorescein isothiocyanate
FPD	fluorescence photoactivation and dissipation
FPR	fluorescence photobleaching recovery
FRAP	fluorescence redistribution after photobleaching
FRP	final reaction product
GVHD	graft versus host disease
h	Planck constant
5-HT	5-hydroxytryptamine (serotonin)
HPD	haematoporphyrin
Hz	Hertz (cycles per second)
I_0	intensity of incident light
I_a	intensity of absorbed light
I_f	intensity of fluorescence
I_r	intensity of reflected light
I_t	intensity of transmitted light
IR	infrared
J	joules
K	(degrees) Kelvin
k	Boltzmann constant
m	metres
MDy	microdensitometry
MFy	microfluorometry
mm	millimetres
MPV	Mikrophotometer mit variable Messblende
MRC	Medical Research Council

ms	milliseconds (10^{-3} s)
MSA	Microscope Spectrum Analyser
MSF	microspectrofluorometer
MSFy	microspectrofluorometry
N	(1) observed photon count
	(2) total photon count
NA	(1) numerical aperture
	(2) noradrenaline
NADH	nicotinamide adenine dinucleotide, reduced form
n_D	refractive index at the sodium D line
n_E	refractive index at the iron E line
nm	nanometres (10^{-9} m)
ns	nanoseconds (10^{-9} s)
OPT	*o*-phthalaldehyde
p	time
PAS	periodic acid-Schiff
PC	personal computer
P-Con A	pyrene-concavalin A
PMT	photomultiplier tube
PRP	primary reaction product
ps	picoseconds (10^{-12} s)
PVP	polyvinylpyrrolidone
QVIM	quantitative video intensification microscopy
R	percentage polarization
r	a constant
RNA	ribonucleic acid
RNase	ribonuclease
s	second
s	length of capillary tube
S	signal
S_0	singlet ground state of molecule
S_1, S_2	1st, 2nd, excited singlet states of molecule
s.d.	standard deviation
SFM	scanning fluorescence microscopy
SLR	single lens reflex
S_w	slit width
S_h	slit height
T	(1) optical transmission
	(2) absolute temperature
T_0	triplet ground state of molecule
T_1	1st excited triplet state
TMB	tetramethylbenzidine
TPNH	nicotinamide adenine dinucleotide phosphate, reduced form
TRIC	tetramethylrhodamine isothiocyanate

TSRLM	tandem scanning confocal fluorescence microscope
UV	ultraviolet
V	volts
V_{max}	maximum rate of enzyme reaction
VFM	video fluorescence microscopy
VIM	video intensification microscopy
W	watts
W	(1) radiant energy per unit time (2) mass of fluorochrome
WL	wavelength
x	amount of unknown substance

Greek letters

Γ	(gamma)	path difference
Δ_n	(delta n)	difference in refractive index
ϵ	(epsilon)	molar extinction coefficient of fluorophore
λ	(lambda)	wavelength
μ	(mu)	refractive index
μm		micrometres (10^{-6} m)
μs		microseconds (10^{-6} s)
μW		microwatts (10^{-6} W)
π	(pi)	ratio of circumference to diameter of circle (3·14159 . . .)
ν	(nu)	frequency
σ	(sigma)	standard deviation
τ	(tau)	lifetime of fluorescence
τ_E		lifetime of excited state
τ_F		lifetime of fluorescence
ϕ	(phi)	quantum efficiency

Contents

1

Principles of quantitative fluorescence microscopy

Quantitative fluorescence microscopy, as the name implies, is concerned with making measurements from fluorescent specimens in a fluorescence microscope, by measuring fluorescence emission from a defined area or areas of a specimen. The basic measuring technique is called microfluorometry. Quantitative fluorescence microscopy provides part of a range of techniques for microscope photometry; the other microphotometric techniques involve measurement of transmitted or reflected light.

The information to be gained by microfluorometry is of several types. First, microfluorometry is most commonly applied to determining the amount of some specific substance, such as deoxyribonucleic acid (DNA), present in particular regions, such as cell nuclei, by comparison of the intensity of the fluorescence of the specified regions with that of a standard. Secondly, determination of the fluorescence spectra, and possibly of other characteristics described below, may enable the identification of specific fluorescent substances. Thirdly, determination of parameters of fluorescence of a known fluorophore introduced intracellularly as a probe can give information about the micro-environment of the probe, and thereby about the inside of the cell. Fourthly, scanning devices can quantify the distribution of fluorescent components in tissue or other material, and enable digitized images to be built up which can be examined, recorded, corrected, and subjected to image analysis.

Probably the most exciting recent developments in fluorescence microscopy have been the commercial availability of confocal fluorescence microscopy (described in Chapter 15), which enables three-dimensional analysis by optical sectioning, and the development of video-intensified fluorescence microscopy, which allows living cells to be studied with minimal radiation.

Fluorescence microscopy

Fluorescence microscopy is described in the companion volumes (Rost, 1991a,b). A brief summary of the nature of fluorescence microscopy, to remind the reader (if necessary) of the major features, is given in this section.

In a fluorescence microscope, the specimen is illuminated with light of a short wavelength, e.g. ultraviolet (UV) or blue. Part of this light is absorbed by the specimen, and re-emitted as fluorescence. The re-emitted light has a longer wavelength than that

of the incident light. To enable the comparatively weak fluorescence to be seen, despite the strong illumination, the light used for excitation is filtered out by a secondary (barrier) filter placed between the specimen and the eye. This filter, in principle, should be fully opaque at the wavelength used for excitation, and fully transparent at longer wavelengths so as to transmit the fluorescence. The fluorescent object is therefore seen as a bright image against a dark background.

It follows that a fluorescence microscope differs from a microscope used for

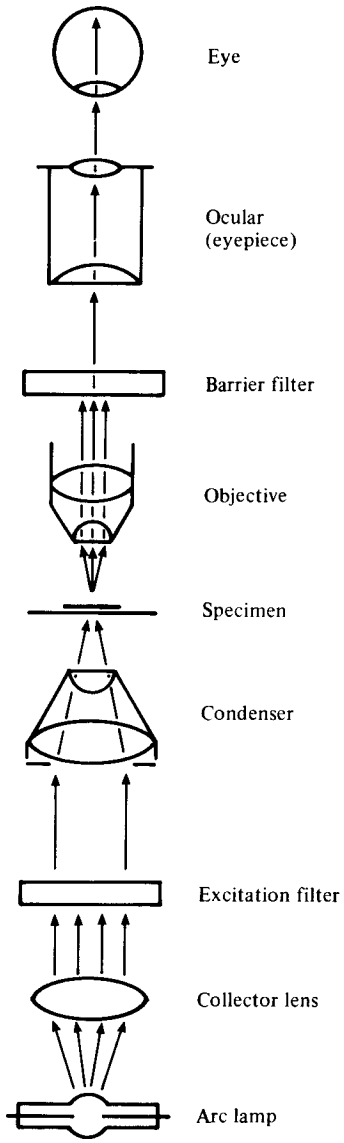


Fig. 1.1. Optical diagram of a fluorescence microscope using dia-illumination (illumination through the specimen) with brightfield substage condenser.

conventional absorption microscopy mainly in that it has a special light source and a pair of complementary filters. The basic arrangement is shown in Fig. 1.1. The lamp should be a powerful light source, rich in short wavelengths: high-pressure mercury arc lamps are the most common. A primary (excitation) filter is placed somewhere between the lamp and the specimen. This filter, in combination with the lamp, should provide light over a comparatively narrow band of wavelengths corresponding to the absorption maximum of the fluorescent substance (fluorophore). The secondary (barrier or suppression) filter prevents the excitation light from reaching the observer's eye (or a photometric device in place of the eye), and is placed anywhere between the specimen and the eye, preferably in the body tube. Its transmission should be as low as possible in the spectral range of the light used for excitation, and as high as possible within the spectral range of the emission from the specimen.

The functions of the excitation and barrier filters may be more clearly demonstrated with the aid of Fig. 1.2. The figure relates to a hypothetical fluorophore (fluorescent object) which absorbs in the blue and fluoresces in the green. The absorption spectrum of the fluorophore is shown on the left; this indicates that the substance absorbs predominantly in the blue, hardly at all in the violet, and the spectrum has a long tail with a secondary peak in the UV. The emission curve is an approximate mirror image of the longest-wavelength peak of the excitation curve.

The broken lines show ideal curves for the transmission of the two filters. The excitation filter should ideally have complete (100%) transmission in the region of the absorption peak of the fluorophore, while having zero (0%) transmission (complete opacity) at the wavelengths of fluorescence. Conversely, the barrier filter should have 100% transmission at the wavelengths of fluorescence, with 0% transmission in the region used for excitation. The two filters are therefore complementary; they allow the

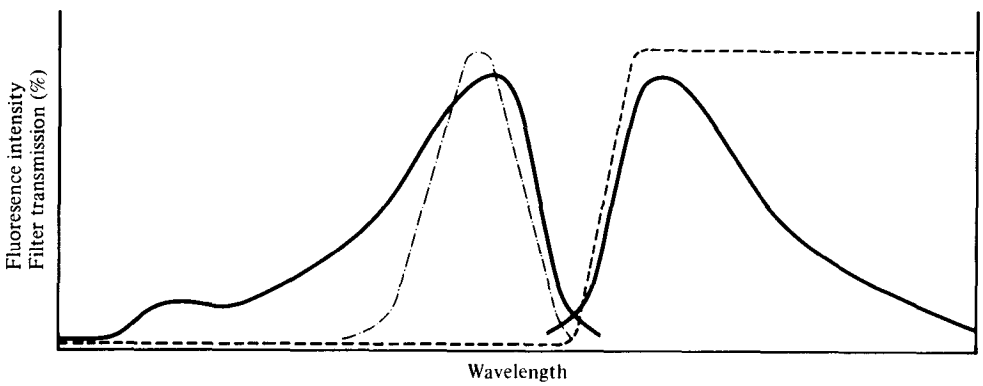


Fig. 1.2. Excitation and barrier filters in relation to the spectra of a fluorophore. Solid lines (–), the excitation (left) and emission (right) spectra of a hypothetical fluorophore. This fluorophore has maximal absorption in the blue, with a smaller absorption peak in the UV; the emission spectrum has a peak in the green, with a long tail extending into the red. The transmission of an excitation filter (·····) with a narrow bandpass in the blue provides optimal excitation. A barrier filter (-----) is opaque at the excitation wavelengths, while transmitting the major part of the emission.

passage of short-wavelength light as far as the specimen, blocking its passage to the eye, and transmitting the fluorescence. For quantitative studies, one or both of the filters is commonly replaced by a monochromator, a device which transmits light in a selected narrow band of wavelengths only.

Opaque or very thick objects can be examined using epi-illumination, a technique whereby the light for excitation is reflected downwards through or around the objective onto the specimen. Although this technique is in principle only essential for examination of opaque objects, such as the surface of intact organs, it has some advantages also for the more conventional transparent specimens, and particularly for quantification, as will be explained below.

Because the image seen in the microscope may consist of only a few small fluorescent areas in an otherwise black field, fluorescence microscopy is sometimes supplemented with other forms of microscopy, e.g. phase-contrast, to enable the specimen as a whole to be visualized and to show the position of fluorescent areas in relation to the rest of the specimen.

Fluorescence microscopy has two particular problems, apart from the purely technical one of having the necessary equipment. The fluorescence image as seen in the microscope is weak compared to that obtained by almost all other kinds of microscopy. This makes particular demands on the efficiency of the system, to avoid loss of light. The dimness of the image may lead to difficulties in interpretation, due to the eye's poor discrimination of colour at low light levels. To make matters worse, the specimen usually fades more or less rapidly under irradiation, producing errors during photometry, and may fade too quickly to be photographed.

As in other forms of microscopy, there are three basic kinds of fluorescence microscopy: qualitative, quantitative and analytical. The first is concerned with morphology, or with whether or not something (e.g. an immunological reaction) is present. Quantitative fluorescence microscopy is concerned with finding out how much of a specific substance is present in a specified region of the specimen. Analytical fluorescence microscopy is the characterization of a fluorophore by measurement of excitation and emission spectra or other characteristics such as polarization or decay time.

Quantification

Microfluorometry basically involves measuring the brightness of fluorescence emission from a defined area of the specimen under standardized conditions. This process can be extended to the measurement of other characteristics of the fluorescence: the actual experimental variables which can be measured or determined are the fluorescence intensity, excitation and emission wavelengths, the polarization of fluorescence, the time, and the area or volume from which measurement is made. It may be possible to measure more than one variable simultaneously (e.g. see Araki & Yamada, 1986). In principle, microdensitometric measurements can be made from the same specimen (given suitable apparatus, see Rost & Pearse, 1971) and the results combined with microfluorometric measurements.

The measuring field is a region of the field delineated by a field stop (diaphragm) in the magnifying system, within which measurement is made. The diaphragm itself may be either a variable circular iris diaphragm, variable rectangular diaphragm, or one of a set of interchangeable fixed diaphragms (e.g. holes in a metal plate).

The parameters or characteristics of the fluorescence which can be measured from a given region of the specimen are as follows.

1. The intensity of fluorescence (i.e. the emittance, I_f).
2. The emission spectrum.
3. The excitation spectrum.
4. The quantum efficiency (ϕ) of the fluorophore.
5. The polarization of fluorescence.
6. The fluorescent lifetime (υ) of the fluorophore.
7. Structure-correlated information, e.g. the relative areas of fluorescence and non-fluorescence.

Changes in all of the above can be followed over a period of time, either short (comparable to the fluorescent lifetime) or long (e.g. rate of fading). In addition, information may be obtainable concerning the transfer of energy between different probes.

Of the above possible types of measurement: (1) is microfluorometry proper, and is the main subject of the present chapter and Chapters 2–4; (2) and (3) are microspectrofluorometry, and are dealt with in Chapters 6–10; (5) and (6) have only recently been applied to fluorescence microscopy, see Chapter 12; (7) requires scanning, either mechanically or with a video camera, and is dealt with in Chapter 15; (8), kinetic measurement, is dealt with in Chapters 11–15. The measurement of cells *en passant* in a flow system (flow cytofluorometry) is dealt with in Chapter 14.

The standard text on microscope photometry in general is that of Piller (1977). Many topics related to quantitative cytochemistry are discussed in detail in the books edited by Kohen & Hirschberg (1989), Thaer & Sernetz (1973), Wied & Bahr (1970), and Wied (1966), and in the journal *Cytometry*. Brief reviews of microfluorometry were given by Ploem & Tanke (1987), Rost (1980, 1974), Fukada, Böhm & Fujita (1978), Ploem (1977), and Ruch & Leeman (1973). Useful information is also found in the chemical and biochemical literature on fluorometry: see, for example, Miller (1984), Wehry (1982), O'Donnell & Solie (1976), Wotherspoon, Oster & Oster (1972), and Parker (1969a).

Microfluorometry

For the remainder of this chapter, and Chapters 2–5, microfluorometry may be said to be a technique whereby the intensity of fluorescence is measured from a given area of a specimen, usually with a view to measuring the amount of fluorophore present and

thereby estimating the amount of some substance present in that region of the specimen. For example, nuclear DNA may be measured by microfluorometry of nuclei subjected to Feulgen hydrolysis and stained with a fluorescent Schiff-type reagent. Further examples are described in Chapter 5.

A microfluorometer, the instrument required for microfluorometry, is essentially a fluorescence microscope with a measuring device which measures the intensity of fluorescence from a specified area of the specimen (see Fig. 1.3). This measured intensity is compared to the intensity to be measured from a standard containing a known amount of the fluorophore, and the amount of fluorophore present in the specimen is determined by proportion.

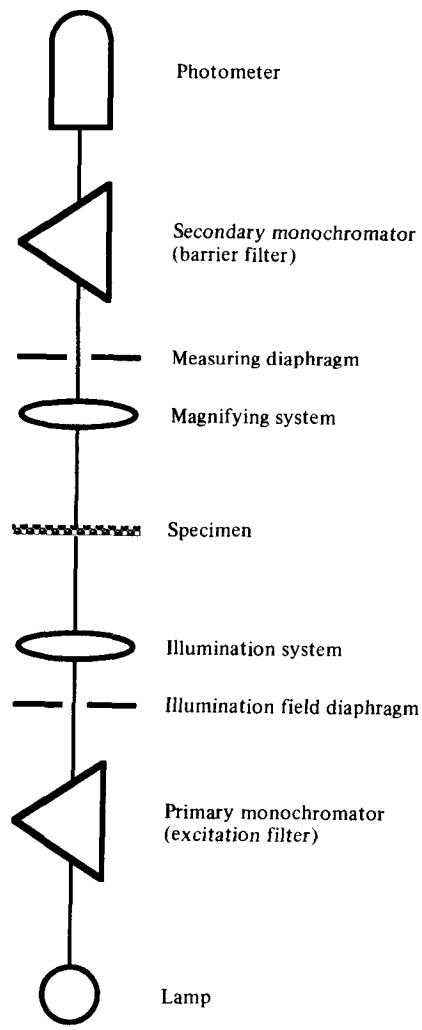


Fig. 1.3. Block diagram of a microfluorometer. See also figures in Chapter 2.

Although simple in principle, microfluorometry presents a number of traps for the unwary, against which due precautions must be taken; these are discussed in Chapter 4.

Sources of error

All microfluorometry is based on the concept of irradiating a specimen with nominally monochromatic light of known intensity, and measuring the intensity of light emitted from a given area of the specimen. It is generally assumed that the intensity of fluorescence is proportional to the amount of fluorophore present. Unfortunately this is only true at low concentrations of fluorophore, as is shown in Chapter 4. If the excitation (absorption) and emission curves of the fluorophore overlap, as they commonly do, some of the emitted light may be reabsorbed (see Chapter 4). For this reason, it is important that the fluorescence emission be measured at a wavelength long enough to be beyond the effective limits of the absorption spectrum of the fluorescent substance. A further difficulty is fading of the specimen as a result of irradiation during measurement, and/or during initial examination to find the area to be measured. Causes of errors are discussed in Chapter 4.

Standardization

In microfluorometry, the observable data are the fluorescence intensity of the specimen and of a standard containing a known amount of the fluorophore, and the ratio of these measurements is calculated. The incident light and the emission are at different wavelengths and radiate in different directions (emission radiates from the specimen in all directions), so that they cannot be compared directly. This contrasts with the situation in densitometry, where incident and transmitted light are measured with the same apparatus, respectively in an empty field and with the specimen present. Accordingly, quantification requires comparison with a standard, measured under the same conditions as those of the specimen. Standards are discussed in Chapter 4.

Instrumentation

Practical instrumentation is the subject of the next chapter. However, the basic principles involved need to be set forth here. A microfluorometer is essentially a fluorescence microscope with a stable, uniform light source and a photometric device for measuring the intensity of fluorescence from a defined area of the specimen (see Fig. 1.3). Usually, a separate illumination system is required for phase-contrast or other examination with light of a longer wavelength, so that the specimen can be set in the measuring field before the excitation light is allowed to irradiate the specimen; this procedure reduces fading.

The direction of illumination

It was Rigler (1966) who first pointed out the significance of the relative directions of the illumination and of the measurement of fluorescence emission in relation to the accuracy of the measurement. He concluded that epi-illumination was to be preferred; see also Benson & Knopp (1984). The reasons are as follows.