Chapter 13

Basic Fluorescence Microscopy

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I. Introduction

A. Attributes of Fluorescence Microscopy

There are five major attributes of fluorescence as a tool in microscopy:

1. Specificity. Fluorescent molecules absorb and emit light at characteristic wavelengths. Therefore, fluorescent probes can be selectively excited and detected in a complex mixture of molecular species.

2. Sensitivity. It is possible to detect a small number of fluorescent molecules. Approximately 50 molecules can be detected in a cubic micrometer volume of a cell with the fluorescence microscope. This number should decrease with further advances in probe chemistry, experimental methods, and photodetectors.

3. Spectroscopy. Fluorescent molecules can be designed to be extremely sensitive to the immediate physical-chemical environment. A variety of spectroscopic parameters that are discussed in detail in Volume 30 of this series can be employed to measure chemical and molecular properties such as pH, free calcium ion concentration, membrane potential, hydrophobicity, charge distribution, microviscosity, molecular distances, diffusion coefficients, and molecular orientations.

4. Temporal Resolution. Fluorescence measurements are limited in temporal resolution to those events that occur with a frequency equal to or greater than the inverse of the time between absorption and emission of light. Therefore, those biological processes occurring at a rate on the order of $\sim 10^8$ seconds$^{-1}$ or slower can be detected and measured. This time domain includes many chemical and molecular changes occurring in living cells.

5. Spatial Resolution. Fluorescence signals can be measured from cellular domains as small as single molecules if the molecules contain a sufficient number of fluorophores. The resolution of structures is still limited to the resolving power of the light microscope, which is a function of the numerical aperture of the objective and the wavelength of emission. However, within the limiting resolution, molecular distances can be determined by resonance energy transfer (see Herman, Volume 30, this series).
Immunofluorescence has been the most common application of fluorescence microscopy in cell biology. It combines the specificity, sensitivity, and spatial resolution of fluorescence microscopy with the selective binding of antibodies to restricted regions of antigen molecules termed epitopes. Multiple epitopes can be localized in the same cell, on the same or on different molecules, by choosing fluorophores with different fluorescence colors (see Waggoner et al., Volume 30, this series). The same concept has recently been used to perform fluorescence-based in situ hybridization.

Fluorescence microscopy is also an important biophysical tool for studies in living cells and in reconstituted preparations in vitro. Fluorescent molecules can be incorporated into living cells to measure local physiological changes in cystolic pH (see Tsien and Poenie, as well as Bright et al., Volume 30, this series), Ca\(^{2+}\) (see Tsien and Poenie, as well as Bright et al., Volume 30), membrane potential (see London et al., 1986), cell structure (see Luby-Phelps, Chapter 4, this volume), as well as the dynamics of fluorescent analogs of specific biological macromolecules (see Wang, Maxfield, Angelides, and Pagano, this volume), lateral mobility of lipids and proteins in membranes (Wolf, Volume 30, this series), and rotational diffusion (Axelrod, Volume 30, this series). Dimensional changes in the association of molecules on the order of 5.0 nm can also be detected by resonance energy transfer measurements (Herman, Volume 30, this series).

Recent advances in video image recording methods and digital image processing techniques now provide a means of obtaining multispectral, two- and three-dimensional, time-resolved measurements of the distribution of fluorescent probes in cells (see Waggoner et al., and Agard and Sedat, Volume 30, this series).

This chapter presents the basic principles of fluorescence microscopy and the fundamental practical considerations required for fluorescence microscopy in cell biology. A variety of biological applications of fluorescence microscopy are provided elsewhere in Volumes 29 and 30, this series. Some basic information concerning general fluorescence technology has been summarized recently (Taylor et al., 1986).

**B. Nature of Fluorescence**

Fluorescence is a type of luminescence where light is emitted from molecules for a short period of time following the absorption of light. When the delay between absorption and emission is on the order of \(10^{-8}\) seconds or less, the emitted light is termed fluorescence. If the delay is \(10^{-6}\) seconds it is termed delayed fluorescence, while a delay of greater than \(10^{-6}\) seconds results in phosphorescence.

When light interacts with matter, it may be either scattered (diffracted
light) or absorbed. Light absorption occurs in discrete amounts termed quanta. The energy in a quantum is given by:

\[ E = h\nu = \frac{hc}{\lambda} \]

where \( h \) is Planck's constant, \( c \) is the velocity of light in a vacuum, \( \lambda \) is the wavelength of light, and \( \nu \) is the frequency of the vibration of light.

As an example, the energy in ergs of a photon of wavelength equal to 200 nm is:

\[ \nu = \frac{c}{\lambda} \]

\[ \nu = \frac{3.0 \times 10^{10} \text{ cm/seconds}}{2.0 \times 10^{-5} \text{ cm}} \]

\[ \nu = 1.5 \times 10^{15} \text{ seconds}^{-1} \]

\[ E = h\nu \]

\[ E = (6.6 \times 10^{-27} \text{ erg seconds})(1.5 \times 10^{15} \text{ seconds}^{-1}) \]

\[ E = 9.9 \times 10^{-12} \text{ ergs/photon} \]

This amount of energy is imparted to an atom or molecule when a single photon of this wavelength is absorbed. There is less energy in photons of longer wavelengths.

The power of light in watts is defined as the amount of energy produced per unit of time or the rate of energy production:

\[ 1 \text{ W} = 10^7 \text{ ergs/second} \]

Microscopy is concerned with the power of light per unit of cross-section since the light is spread out over some area of the specimen. The power per unit cross-section is defined as intensity (\( I \)) or irradiance, such that

\[ I = \frac{W}{\text{cm}^2} \]

When a quantum of light is absorbed by a molecule, a valence electron is boosted up into a higher energy orbit forming an excited state. When this electron returns back down to its original lower energy orbit, termed the ground state, a quantum of light may be emitted.

The absorption spectrum of a molecule depends on the number of energy levels possible for the electronic state of the molecule. Valence electrons can exist only in discrete energy levels (Fig. 1). Absorption occurs only at wavelengths of light whose quantum energy is equivalent to the difference in energy between the ground electronic state and the excited state. For an atom, there are few possible energy levels, and light absorption occurs only at discrete wavelengths (lines) of equivalent light energy (Fig. 2). The absorption spectrum is much broader for a molecule (Fig. 2), since nuclei
Potential energy of electron

FIG. 1. Diagram of the potential energy of electrons in the ground \((G)\) and the lowest singlet excited state \((S_1)\) of a molecule.

rotate and vibrate relative to the center of molecular mass. Discrete electronic configurations exist, but both the ground state and the excited state are further subdivided into substates of vibrational and rotational energy. This increases the number of possible energy levels and broadens the absorption spectrum in comparison to that exhibited by a single atom.

Fluorescence has been described as a relaxation process starting with the absorption of light and ending with the emission of light. It is the emission of light produced by deexcitation from the lowest singlet excited state to the ground state (path b in Fig. 3). The wavelength of the emitted fluorescence is usually longer than the wavelength of absorbed light (termed Stokes' Law) as shown in Fig. 4. Exceptions occur only when collisions between molecules impart extra energy to the electron in the excited state. Usually such molecular collisions are infrequent and some energy of the excited electron is lost before deexcitation. As a consequence, the quantal energy of emitted light is usually less than the absorbed light. The emission spectrum is shifted to correspondingly longer wavelengths in comparison to the absorption spectrum (Fig. 4). As with the absorption spectra, the emission spectra of molecules exhibits a broad range of wavelengths in comparison to the narrow range of emission spectra exhibited by atoms.

C. Pathways of Deexcitation

Fluorescence is one of several possible pathways of the deexcitation process by which an excited state electron gives up energy and returns to the ground state (Fig. 3). Fluorescence lifetime is the average time that a molecule remains in the excited state. The time required for the process of absorption (about \(10^{-15}\) seconds) is instantaneous compared to the typical lifetime of fluorescence (about \(10^{-8}\) seconds). Fluorescence is delayed (about \(10^{-6}\) seconds) if the excited electron moves to a forbidden triplet state before moving back to the lowest singlet excited state and emitting
light (d in Fig. 3). Phosphorescence (e in Fig. 3) begins with a transition to the forbidden triplet state. In contrast to delayed fluorescence, emission occurs by a transition of the excited electron from this lower energy level to the ground state. Phosphorescence has much longer lifetime of the excited state (up to several seconds) and the emission spectra is red-shifted in comparison to fluorescence.
Excited States

\[ \Delta E \sim h\nu \]

Ground States

Fig. 3. Diagram depicting the possible pathways of deexcitation from an excited state to the ground state of a molecule. (a) Absorption, (b) fluorescence, (c) radiationless loss of energy to the medium, (d) delayed fluorescence, (e) phosphorescence, (f) photobleaching in the presence of \( \text{O}_2 \), and (g) chemical reactions.

There are a variety of nonradiative pathways of deexcitation (c in Fig. 3). Energy of the excited electron can be dissipated by interactions with the solvent or other molecules in the sample. Energy absorbed by one molecule can be passed to another molecule for use in driving chemical reactions such as occurs in the light reactions of photosynthesis in plants. Finally, some fluorescent molecules can be destroyed by the excitation process in the presence of molecular oxygen, a phenomena termed photobleaching. Photobleaching can be a serious technical problem in fluorescence microscopy (see Section III, D), but it can also be used as a powerful tool to measure the mobility of molecules (see Wolf, Volume 30, this series).

D. Fluorescent Probes

Autofluorescence is the fluorescence of naturally occurring molecules in cells. Most of the autofluorescence in mammalian cells excited in the near

\[ \text{Stokes Shift} \]

\[ \lambda \text{ increases} \]

Fig. 4. Generalized excitation and emission spectra of a fluorescent molecule showing the loss of energy between absorption and emission.
UV and blue region of the spectrum is due to NADH, riboflavin, and flavin coenzymes. Changes in autofluorescence have been used to measure molecular reactions of mitochondrial electron transport (see Kohen et al., 1981). However, naturally occurring fluorescence has had limited value in cell biology in comparison to the application of exogenous fluorescent probes. In fact, autofluorescence is a great source of noise in many experiments (see Section III, A, below).

Fluorophores or fluorochromes are fluorescent dyes or probes that are added to cells. Fluorophores are chosen or synthesized for particular applications based on several criteria. These criteria include absorption and emission spectra, extinction coefficient, quantum yield, environmental effects, and chemical reactivity. Some of the most common fluorophores used in cell biology are listed in Table I of Waggoner et al., Volume 30, this series.

E. Parameters of Fluorescent Probes

The extinction coefficient, $\varepsilon$, and quantum yield, $\Phi$, in addition to fluorescence lifetime, $\tau$, are the three fundamental parameters of fluorescence. The extinction coefficient is a measure of the probability of absorption. A large extinction coefficient indicates a high probability of absorption. Large extinction coefficients range from $\sim 40,000$ to $250,000 \text{ M}^{-1} \text{ cm}^{-1}$. If there is a high probability of absorption for fluorescent molecules, then there is also a high probability of emission. Therefore, the intrinsic lifetime of the excited state must be short. In fact, the intrinsic lifetime is inversely proportional to the probability of absorption (see below). In solutions that obey Beer's law in the spectrophotometer, the extinction coefficient is given from:

$$\text{optical density (OD)} = -\log \frac{I}{I_0} = \varepsilon cl$$

and

$$E = \frac{\text{OD}}{cl}$$

where $I$ is the light intensity after passing a distance $l$ through the sample, $I_0$ is the incident intensity, $\varepsilon$ is the extinction coefficient, and $c$ is the concentration of absorber. The common unit of $\varepsilon$ is $\text{M}^{-1} \text{ cm}^{-1}$.

The quantum yield, $\Phi$, is a measure of the efficiency of fluorescence relative to all the possible pathways of deexcitation. Quantum yield can be expressed as the ratio of the number of quanta emitted divided by the number of quanta absorbed. Fluorescent molecules usually studied in biomedical samples have quantum yields less than 1, but useful fluorescent
molecules usually have quantum yields greater than 0.1. The fluorescence intensity is also a function of the quantum yield. Therefore, the product of $\Phi$ and $\epsilon$ will determine the fluorescence intensity of a probe:

$$I_{\text{flourescence}} = I_0 \Phi \epsilon \tau$$

Fluorescence lifetime, $\tau$, is the average time that a molecule remains in the excited state. The intrinsic lifetime, $\tau_0$, is the maximum possible average lifetime, $\tau$, and this occurs when the $\Phi$ is a maximum. This requires that all of the pathways of deexcitation other than fluorescence be eliminated. Most useful fluorescent probes have lifetimes on the order of $1-100 \times 10^{-9}$ seconds.

II. Microscope Design

A. Basic Concepts

The fundamental principle in the design of a fluorescence microscope is to maximize the collection of fluorescent light while minimizing the collection of excitation light. This is achieved by optimizing the optical configuration and components of the fluorescence microscope.

A major constraint in the design of a fluorescence microscope is the fact that the intensity of the fluorescence from the specimen is usually several orders of magnitude less intense than the intensity of illumination. Fluorescence images must be recorded using low light level video cameras or cooled CCD imagers and digital image processing to minimize photobleaching damage. Image contrast depends critically on the ability of the microscope to pass fluorescent light to the detector while substantially blocking the excitation light.

The sensitivity and contrast of fluorescence microscopy depends on the optical configuration of the illumination and imaging paths in the microscope and the optical performance of the microscope components. We will first describe the various optical arrangements used in fluorescence microscopy and then discuss the parameters to be considered to obtain optimal performance of the optical components.

A fluorescence microscope is a compound microscope. The optical paths for specimen image formation are similar to that of a standard bright field microscope. There are three basic types of illumination methods used in fluorescence microscopy: (1) full aperture transmitted light illumination (Fig. 5); (2) darkfield (or darkground) transmitted light illumination (Fig. 6); and incident light illumination (Fig. 7).
Fig. 5. Diagram of a fluorescence microscope assembled by the addition of an excitation filter and a barrier filter in a standard bright field microscope. Solid line, excitation light; broken line, emitted light.

Fig. 6. Diagram of darkfield fluorescence microscope. The NA of the objective is designed to miss the direct rays from the darkfield condenser (thin lines). Only emitted fluorescence can enter the objective (thick line).
B. Full Aperture Transmitted Light Illumination

Fluorescence microscopy can be performed with a conventional bright field light microscope by inserting excitation filters between the illuminator and the condenser, as well as the insertion of emission filters (also called barrier or suppression filters) between the objective and the recording device as shown in Fig. 5. Excitation filters are band-pass filters chosen to pass light at the absorption spectra of the fluorophore, while blocking the longer wavelength light of the fluorescence spectrum. In contrast, emission filters are chosen to pass the light in the emission spectrum while blocking the light of the excitation spectrum. As seen in Fig. 8, the absorption and emission spectra of a typical fluorophore usually overlap and their peaks may be separated by only 20 nm as shown by the spectra for fluorescein. Since the excitation light is usually much higher in intensity than the fluorescent light, band-pass filters with very sharp cut-offs must be used to achieve usable image contrast. Practically, this has proven difficult to achieve, and other illumination methods have been developed to prevent light from the illumination beam from entering the objective. The advantages of full aperture transmitted light illumination are the simple modification of a bright field microscope required to perform fluorescence and the
Fig. 8. Excitation and emission spectra of fluorescein with common filter sets employed to separate the excitation and emission light. SP, Short pass filter; LP, long pass filter.

ability to use high numerical aperture (NA) objectives. The disadvantages include the possible presence of inner filter effects in a thick sample (see Section II, C, below) and the difficulty in aligning the condenser and the objective. However, recent improvements in the production of interference filters makes this approach potentially very useful.

C. Darkfield Illumination

Until about 1970, the problem of removing excitation light was solved primarily by using darkfield illumination configurations as shown in Fig. 6. A darkfield condenser produces an annular cone of illumination whose aperture is greater than the aperture of the objective. Unscattered light from the illumination beam does not enter the objective. Image contrast was far superior than with full objective aperture illumination using the excitation and emission filters available in the early 1970s.

The major disadvantage of this design is that the efficiency of collecting the fluorescence emission by the objective is greatly reduced. Weakly fluorescent specimens are difficult to detect, since the ability of the objective to collect emitted light from the specimen depends on the NA of the objective to the second power. In order for the exciting light in a dark-field microscope to miss the objective, the objective aperture must be less than the illumination aperture. Objectives with aperture diaphragms must then be used. The working aperture is typically no greater than NA = 0.7, where good quality objectives can have NA = 1.4. The reduction in potential fluorescent light intensity for equivalent illumination and magnification is
(0.7/1.4)^2 = 0.25. This reduction is a major disadvantage for the detection and measurement of weak fluorescence and the prevention of photobleaching. Darkfield fluorescence is no longer a viable choice in most applications in cell biology.

D. Incident or Epiillumination

The illumination and contrast problems were optimally solved in the late 1960s by modifying conventional vertical illuminators with the addition of newly developed interference filters (Ploem, 1967). As seen in Fig. 7, incident or epiillumination occurs through the objective. The objective is both the condenser lens and the objective lens of the system. The novel component of the system is the chromatic beam splitter (also called a dichroic mirror). The chromatic beam splitter is constructed with an interference coating that has a high reflectance at 45° for the wavelengths transmitted by the excitation filter and not the emission filter. Conversely, it transmits the wavelengths passed by the emission filter, but not the wavelengths passed by the excitation filter.

In combination with excitation and emission interference filters, excellent contrast of weakly fluorescent specimens can be achieved by epiillumination with the full aperture of the objective. Since the objective serves as the condenser, alignment of the instrument is simple. In addition, the fluorescence for thick specimens is brighter than in transmitted light excitation schemes, since the illuminating beam does not have to pass through the specimen.

A significant advantage of the incident or epiillumination scheme is that it can be combined with conventional transillumination methods such as phase contrast, polarization, and differential interference contrast. It is often valuable to compare the distribution of fluorescence in a specimen with the structure of the specimen which can be seen by the above transmitted light contrast methods.

Sensitivity and compatibility with transmitted light contrast methods usually make incident illumination the preferred optical configuration for fluorescence microscopy. As a consequence, incident illuminators are standard equipment for almost all types of research microscopes manufactured today for the biological sciences.

E. Koehler Illumination

The illumination path for an incident light illuminator is designed according to the principles of Koehler as shown in Fig. 9. In Koehler illuminat-
Fig. 9. Diagram of the illumination pathway and imaging pathway of light in an incident or epiillumination fluorescence microscope. Conjugate planes for the specimen or object plane are labeled \((O, \text{SP}, O', \text{and } O'')\) and for the lamp \((L, L', L'', L''')\). Everywhere in the optical system where the specimen plane is in focus the plane of the lamp is out of focus. The field diaphragm (FD) is conjugate with the specimen, while the image of the lamp filament or arc \((L)\) is conjugate with the back focal plane of the objective \((L'', L''')\). This relationship is called Koehler illumination. Lenses A, B, and C, the excitation filter (EX), the dichroic mirror (DM), and the barrier filter (BF), make up the key optical components of the epiillumination system (see text).

In Koehler illumination, the image of the light source \((L)\) is focused at the iris diaphragm \((L')\) which is also conjugate to the entrance pupil (or back aperture, \(L''L''')\) of the objective lens (Obj), while the image of the field diaphragm (FD) is focused at the specimen plane (SP). When the field diaphragm is in focus at the specimen plane, the image of the light source is out of focus. This arrangement produces even illumination of the specimen field in spite of the uneven illumination intensity typical of most light sources. The field diaphragm controls the size of the illuminated area of the specimen without affecting the intensity of the illuminated area. The iris diaphragm (Iris) controls the size of the light source projected into the objective entrance pupil. Opening and closing the iris diaphragm increases and decreases the intensity of illumination of the specimen without affecting the size of the field illuminated. If the iris diaphragm is not included in the illuminator design, then the intensity of illumination of the specimen is usually adjusted by inserting neutral density filters into the illumination path at a convenient position where the image of the filter surfaces will be out of focus at the specimen plane.
F. Illuminators

Shutters are critical in fluorescence microscopy. Continuous illumination of the specimen can greatly reduce specimen fluorescence due to photobleaching and continuous illumination is not healthy for living cells. Furthermore, fluorescence may not be desirable while the specimen is being viewed or photographed by transillumination methods. Usually a mechanical shutter is placed in the illumination path to provide on–off control of illumination. Electronic shutters (i.e., Vincent Associates, Rochester, New York) are not expensive and they can provide rapid (10 msec), remote control of illumination (see Wampler and Kutz, this volume).

Modern fluorescence microscopy requires a range of light sources to meet the demands of a variety of applications from ultralow light irradiance over a broad range of wavelengths from the UV to the infrared to intense irradiance at specific wavelengths. Several types of white light lamps and lasers have emerged as standard sources of illumination.

Four major characteristics of lamps must be considered when choosing a white light source: (1) the spectral concentration of radiance from the lamp; (2) the size of the filament or arc compared to the area of the back focal plane of the objective (condenser in epillumination); (3) the stability of the light source over time and space within the illuminated field; and (4) the uniformity of illumination of the field.

Lamps can be characterized in radiometric terms by the spectral concentration or spectral density of radiance \( B_\lambda \). This is the proper way to define the light output of a lamp since it is a measure of the radiant intensity per area, a physical measurement independent of the human visual sensitivity. This characteristic is specific for an individual lamp. A portion of the area of the luminous surface of a lamp emits light within a solid angle with a particular spectral range:

\[
B_\lambda = \frac{\phi}{Fw\Delta \lambda}
\]

where \( \phi \) is the radiant flux in watts (W), \( F \) is the area of luminous surface in \( \text{cm}^2 \), \( w \) is the solid angle of emitted cone of light in steradians (sr), and \( \Delta \lambda \) is the spectral bandwidth selected in nanometers (nm).

The light output of the lamp is expressed in the units: \( \text{W cm}^{-2} \text{ sr}^{-1} \text{ nm}^{-1} \). This value represents the radiant flux per unit area, unit solid angle, and unit spectral bandwidth. The absolute value will depend on how the microscope is adjusted. The radiant flux is determined by the size of the luminous surface selected by the iris diaphragm of the epilluminator. The solid angle of the emitted cone of light is determined by the NA of the collecting lens of the illuminator. The spectral bandwidth is determined by the filters and/or monochromometer employed.

The light housing is usually fitted with a well-corrected collector lens that
is positioned so that the filament or arc is near its principal focal point. In Koehler illumination the lamp collector lens serves as an enlarged secondary light source. An image of the filament or the arc must then be projected onto the back focal plane of the objective which serves as the condenser during excitation in epillumination. The filament or arc should fill the back aperture of the objective to both maximize the radiance and to ensure an even illumination of the field by Koehler illumination (see above). A ground glass is sometimes inserted in front of the lamp to maximize the uniform illumination, especially with arc lamps that tend to produce "hot spots" of illumination.

The illumination of the specimen must be constant over time and space across the field of view. The stability of the light source is also critical for ensuring the precision of measurements. Instability over time reflects the temporal fluctuations of radiance of the lamp and is derived primarily from the variations in the electrical supply to the lamp. Instability of the lamp in space is often detected in arc lamps and is called "flicker." Flicker occurs when different surface elements of the arc lamp exhibit different fluctuations arising from the migration of plasma across the electrodes. These spatial variations can be caused by small variations in the resistance of the electrodes, fluctuations of the power supply, and/or mechanical vibrations. The filament-based light sources are very stable, especially when they are operated under constant current with a power supply. In contrast, the arc lamps are inherently less stable. DC-powered arc lamps have improved the stability considerably, but they must be tested. It has been demonstrated that the smaller the distance between electrodes the more stable the arc lamp (lower wattage lamps). Xenon arcs are more stable than mercury arcs. The addition of a small percentage of xenon to a mercury arc lamp can increase the stability of the arc (Oriel, Stratford, Connecticut). All types of lamps exhibit aging which occurs by the gradual deposition of metal on the surface of the bulb. Aging can be detected by direct observation of the bulb.

Choosing a light source and collector lens that causes the back focal plane of the objective to be filled with an image of the filament or arc is the first step. Taking precautions to stabilize the lamp radiance over time and space is the second step. Finally, the microscope should be adjusted for Koehler illumination. Careful attention to these characteristics of lamps will optimize the use of fluorescence microscopy as a quantitative tool.

1. Specific White Light Sources

Based on the characteristics of lamps described above, several different white light sources have become standards in fluorescence microscopy. The
choice depends on the spectral radiance of excitation over the range of wavelengths required and the stability of radiance required. The major lamps include: tungsten; quartz–halogen; 50 and 100 W mercury arcs; and 75 W xenon arc (Fig. 10).

**Fig. 10.** Diagrams showing the relative spectral output of (a) tungsten, (b) 75 W xenon-arc (XBO-75), and (c) 100 W mercury arc (HBO-100) lamps. Note the large output of near infrared light by the xenon arc.
Lasers provide stable, intense monochromatic light. There are two major intensity peaks in an argon ion laser, one at 488 nm and one at 514 nm. The laser emission can contain one or both laser lines by tuning the resonant frequency of the laser. The 488 line is ideal for exciting fluorescein isothiocyanate (FITC). The 514 line is marginal for exciting rhodamine isothiocyanate (RITC).

In general, a different type of laser is required for different spectral emissions. High power, stable laser light sources are expensive ($5,000–$20,000) and difficult to maintain. Because of their cost and limited spectral output, lasers have thus far been used mainly by biophysicists in applications such as measurement of fluorescence redistribution after photobleaching to analyze the mobility of molecules in living cells and in reconstituted preparations in vitro (see Wolf, Volume 30, this series). In the near future, technological developments in lasers and laser diodes should bring them closer to the ideal light source required for the majority of applications in fluorescence microscopy, particularly in conjunction with laser-based confocal scanning microscopy (see Brakenhoff et al., Volume 30, this series).

G. Filters

There are four different types of filter construction: neutral density, glass filters, colored glass filters, gelatin filters, and interference filters. Neutral density filters attenuate all colors or the spectrum uniformly. They are frequently made by depositing a thin film of metal on a flat glass surface. This surface is sealed by covering the metal surface with another flat glass plate.

Colored glass filters transmit light in limited regions of the spectrum because of light absorption, which is usually due to the metal composition of the glass. Gelatin filters consist of a gelatin layer containing organic dyes. The gelatin layer is usually mounted between two flat glass plates.

Interference filters consist of many layers of films with different refractive indices sequentially deposited upon a flat glass surface and sealed by another flat glass surface. The transmission characteristics of the filter are produced by interference of light reflected at the surfaces of the different film layers. It is important to recognize that light not transmitted by an interference filter is reflected. Interference filters designed for selecting spectral regions in part by reflection are called dichroic beam splitting mirrors. Interference filters can be designed to transmit or reflect light in discrete bandwidths from the UV to the infrared regions of the spectrum.
Filters are characterized by their transmission and reflection characteristics. Band-pass filters are identified by their peak wavelength of transmission, the percent of incident light at that wavelength transmitted, and the half-bandwidth of transmitted wavelengths. High-quality band-pass filters have sharp cutoff transmission characteristics. High-quality short-pass filters and long-pass filters also have sharp cutoffs between the range of wavelengths transmitted and those absorbed or reflected. Colored glass filters and gelatin filters usually do not have sharp cutoffs, but they are inexpensive. Interference filters provide the best performance, but they are expensive (typically $100 or more).

The bandwidths and cutoff wavelengths of filters are chosen to maximize energy transmission without spillover of fluorescence between different filter sets. Figure 11 shows the practical filter sets recommended for Hoechst, FITC, and RITC. They are designed to isolate the excitation and emission spectra for each fluorophore as can be seen by comparing the transmission characteristics of these filter sets with the absorption and emission spectra of the probes. The excitation and emission spectra of Hoechst and FITC are well separated so that broad band filters for excitation and emission of Hoechst can be used to maximize light intensity. The excitation and emission spectra of FITC and RITC overlap to a small degree and narrow band-pass filters must be used. Be careful in using the filter sets supplied with many microscopes. The manufacturers often sacrifice spectral separation for fluorescence throughput. Also, be careful to use blocking filters for the infrared. Many detectors are very sensitive in the infrared and can detect radiation that is invisible to the human eye.

The excitation filter set, dichroic mirror (DM), and emission or blocking filter set (BF) are usually mounted in a mechanical cube positioned above the objective. In most commercially available illuminators, a slider or rotatable filter holder is used to select between two to four different sets. This permits rapid switching of filter sets to provide views of fluorescence from multiple fluorescent probes in the same specimen. Lenses (A, B, and C in Fig. 9) make the light propagate parallel to the optical path, perpendicular to the emission filter surface and at 45° to the dichroic mirror. For all the filter sets in an epiilluminator, the excitation and emission filters should be oriented perpendicular and the dichroic mirror oriented at 45° to the optic axis of the microscope. Otherwise, the image of the specimen will be displaced laterally in the image plane to different degrees for the different filter sets.

A partially reflecting mirror, which is available from most manufacturers, can be used in place of the dichroic mirror in an incident illuminator. This configuration does not have the selectivity and energy transmission capabilities of a dichroic mirror, but it can be used for fluorescent
FIG. 11. Excitation and emission spectra of Hoechst 33342, fluorescein, and rhodamine (a). The filter sets that we use to image Hoechst 33342, fluorescein, and rhodamine in the same cells are shown in (b).

probes for which no dichroic mirror is commercially available. The excitation and emission wavelengths are isolated by appropriate choice of interference filters for excitation and emission wavelengths.

H. Objective Lenses

The brightness of the fluorescence image varies with the objective employed. The relative brightness of the image can be predicted if we ignore the effects of the transmission and reflectivity of the lens elements. The intensity of light gathered by an objective varies as the square of the
numerical aperture. The NA is a measure of the light gathering power of a
lens and is defined by:

\[ \text{NA} = n \sin \theta \]

where \( n \) is the refractive index between the specimen and the objective and \( \theta \) is the half-angle of the cone of rays entering the objective. In addition, the
brightness of the image also varies as the inverse of the square of the
objective magnification. In transmitted light fluorescence the condenser
usually remains fixed while the objective is changed. When a higher magni-
fication objective is used with a fixed condenser, the illuminating light from
the condenser is spread out over a field larger than that viewed by the
objective. Therefore, some of the illuminating light is lost. In summary, the
brightness of the image in transmitted light fluorescence is related to:

\[ \text{image brightness} \propto \frac{\text{objective NA}^2 \times \text{condenser NA}^2}{\text{magnification}^2} \]

In epillumination the objective also serves as the condenser. When the
objective magnification is increased, the same light intensity is focused on a
smaller field. The result is that the image brightness depends on:

\[ \text{image brightness} \propto \frac{\text{objective NA}^4}{\text{magnification}^2} \]

A practical rule is to maximize the NA of the objective and to minimize
the total magnification for electronic imaging. At very low light levels, when
light is limiting, it is often more important to increase the signal-to-noise
ratio at the expense of magnification. Ploem has described the use of a
demagnifying lens in combination with a high NA objective to maximize
image brightness (Ploem, 1986). The need to minimize the irradiance of
excitation light in order to minimize photobleaching damage must also be
considered when balancing the selection of optical components. It is im-
portant to define the irradiance at the specimen plane and this can be
performed simply using a power meter (see Section IV, B).

Fluorescent specimens act as self-luminous objects. Point sources in the
self-luminous specimen behave as independent sources, like two distant
stars observed with a telescope. Therefore, there is no coherent relationship
between the phases of the emitted light. Light from each point source is
diffracted by the objective aperture, which produces an airy disk image of
the point source. The light from two point sources are incoherent, so that
Raleigh's criterion for resolving power can be applied. The resolution of two
adjacent point sources, \( \gamma \), is given by:

\[ \gamma = \frac{0.61\lambda}{\text{NA}} \]
This consideration of resolution is operable for the transverse \((X \ Y\) axes) resolution only. Axial resolution \((Z\) axis) involves consideration of the depth of field.

The depth of field of an optical system is the physical distance along the \(Z\) axis through the specimen that is in focus at the image plane. Another way of defining depth of field is the distance that a single point object can be moved in the \(Z\) axis before the image changes. This distance is determined primarily by wave optical considerations at high NA, while geometrical optical considerations are significant at low NA. We will consider high NA objectives in our treatment. The depth of field is determined by the refractive index, \(n\), of the medium, the NA of the objective, and the wavelength of the illuminating light. The depth of field can be defined mathematically by:

\[
D = \frac{\lambda}{4n \sin^2(\theta/2)}
\]

Calculating the depth of field for one common objective will illustrate the considerations. We will use a 63 \(X\) objective (water immersion, \(n = 1.33\)) with an \(NA = 1.2\), assuming a wavelength of 500 nm \((0.5 \mu m)\). Since the \(NA = n \sin \theta\), we can solve for \(\sin^2(\theta/2)\) in the equation for depth of field by substitution:

\[
\begin{align*}
NA &= n \sin \theta \\
\sin \theta &= \frac{NA}{n} \\
\sin \theta &= \frac{1.2}{1.33} = 0.9 \\
\theta &= \sin^{-1} 0.9 = 64.5 \\
\sin^2 \frac{\theta}{2} &= 0.28
\end{align*}
\]

therefore,

\[
D = \frac{0.5}{4(1.33)(0.28)} = 0.34 \mu m
\]

This objective will therefore maintain a sharp image of a point source for a distance of 0.34 \(\mu m\) in axial movement of the specimen. It must be emphasized that light emanating from other point sources outside of the distance equal to the depth of field of the objective will be distributed into
I. Detectors

See chapters by Spring and Lowy, Wampler and Kutz, and Aikens et al., in this volume.

III. Practical Issues of Epiillumination Fluorescence Microscopy

A. Autofluorescence

Autofluorescence is the fluorescence of naturally occurring molecules in cells. Most cells will exhibit some degree of autofluorescence when excited with the proper wavelength at high intensity of excitation. Recent investigations indicate that most of the autofluorescence from mammalian cells excited in the near UV and visible region of the spectrum is due to NADH, riboflavin, and flavin coenzymes (Benson et al., 1979; Aubin, 1979). These molecules have a broad excitation spectrum ranging from \( \sim 350 \) to 500 nm. The level of autofluorescence in cells is also a function of the stage of the cell cycle and physiological state. Proteins fluoresce when excited at 250–280 nm due to the presence of tryptophan, tyrosine, and phenylalanine.

All studies with the fluorescence microscope must start with the examination of autofluorescence of the cells under the same conditions that will be
employed with the exogenous fluorescent probes. These conditions include the objective, lamp, irradiance at the specimen plane, and detector. The signal-to-noise ratio will be determined in part by the relative strength of the autofluorescence compared to the fluorescence of the exogenous probe. It may be necessary to correct for the autofluorescence signal or to avoid the autofluorescent region of the spectrum by shifting the exogenous probe to the longer wavelengths (see Waggoner et al., Volume 30, this series). Fluorescence and phosphorescence is often detected in slides, cover slips, immersion oil, and culture media. All components that will be placed in the optical path must be checked separately.

B. Detectability

A fluorescence microscope is able to collect a higher percentage of emitted fluorescence from a sample than a standard fluorometer due to the high NA of the microscope objective. Several studies have demonstrated the extreme sensitivity of fluorescence microscopy. Barak and Webb were able to detect single low-density lipoproteins (LDL) particles labeled with $\sim 50$ fluorophores (Barak and Webb, 1981). The lower limit of detectability has not been reached and even single fluorophore detection is feasible under the optimal conditions (Mathies and Stryer, 1986). Hirschfeld originally demonstrated the ability to detect single biological molecules by labeling polyethyleneamine with multiple fluorophores and then coupling the labeled “kite tail” to single IgG molecules (Hirschfeld, 1976a,b). This is a technically challenging area in the field of fluorescence microscopy.

C. Quantitation of Fluorescence Intensity

The fluorescence intensity of a solution is directly proportional to its concentration only in highly dilute solutions that follow Beer’s law (Fig. 12); see Section I,E. At higher concentrations of fluorescent probes the fluorescence intensity can actually decrease. There are three major reasons that can account for this phenomenon. The inner filter effect occurs when the excitation light does not penetrate through the sample due to the light absorbance of the sample. Therefore, all of the fluorophores do not absorb to the same extent. This can occur in very thick and/or concentrated microscope samples. Epiillumination is particularly valuable under these conditions since this configuration is similar to “front surface” fluorescence performed in fluorometers. This is the only way to study the fluorescence of this type of sample. The fluorescence can also decrease at higher fluorophore concentrations due to the formation of probe dimers produced in the ground state or dimers formed only in the excited state, which are called
excimers. These complexes usually cause quenching of the fluorescence. In addition, trivial reabsorption of the emitted fluorescence can occur since the emission spectrum of many probes overlaps the excitation spectrum.

The presence of a concentration effect can be tested by increasing and decreasing the concentration of the fluorescent probe used in an experiment. This is a dose response fluorescence control. A sure sign of a concentration artifact is a measured increase in fluorescence intensity when the fluorophore concentration is decreased. These concentration problems are usually not a problem with thin mammalian cells labeled with standard probes. However, each new probe and experimental situation must be evaluated.

D. Bleaching

Bleaching is defined as the permanent destruction of fluorescence by a light-induced conversion of the fluorophore to a chemically nonfluorescent compound. This process requires light and molecular oxygen for most of the commonly used fluorophores. However, the photochemistry is very complex and no absolute mechanism has been defined. Removing oxygen is not a viable option when studying living cells, so the total dose of light must be regulated. This is the main reason for using a shutter on the light source. The sample should be illuminated only during the time of recording an image or making a measurement. Furthermore, the total dose of light must be kept to a minimum, where dose is defined as the intensity of excitation multiplied by the time of illumination (see Bright et al., 1987).
Excitations at low irradiances over a relatively long time interval are required with image intensifiers, since they typically have very low intrascene dynamic ranges. In contrast, high irradiances for very short times can be obtained with cooled CCD cameras, due to the very large intrascene dynamic range (see Bright et al., 1987). The same total dose can be delivered by both of these approaches, yet the temporal resolution is optimized in the latter.

The generation of toxic compounds including free radicals during photobleaching is an additional problem. The loss of fluorescence signal, as well as the loss of physiological significance of the experiment, are two important reasons to control the bleaching of probes introduced into living cells. Solutions to the problem usually involve a combination of choosing a more photostable probe such as rhodamine instead of fluorescein; decreasing the total dose of illumination by decreasing the number and duration of excitation periods; and increasing the sensitivity of the detector. Better probes and approaches to imaging should improve this most serious limitation to fluorescence microscopy.

E. Light Scattering

The scattered light in an epiillumination fluorescence microscope is primarily forward light scattering which passes through the specimen and does not enter the objective. The presence of an optimal dichroic mirror and barrier filter removes any back-scattered light that does enter the objective. However, excessive light scattering due to the content of the specimen could affect spectral measurements and must be corrected. Microspectrofluorometric analyses of the excitation and emission spectra should be performed. It is encouraging that the total light scattering of mammalian cells in culture is very small due to the small specimen path-length.

F. Environmental Effects

The major power of fluorescence spectroscopy is the environmental sensitivity of many fluorescent probes. However, this same sensitivity must be well understood and controlled in order to make the proper interpretations of the fluorescence signals. It is easy to obtain a fluorescence signal, but it is very difficult to interpret it properly. In practice, the behavior of fluorescent probes under different environmental conditions in vitro must be well understood before attempting interpretations in living cells (see Waggoner, 1986). A good example of the environmental sensitivity is the effect of solvent polarity on the quantum yield and excitation, as well as
emission spectrum of some probes. Some of the membrane potential sensitive probes (see Waggoner, 1986) exhibit both an increase in quantum yield and a red shift in the excitation and emission spectra when the probe enters the more hydrophobic environment of the membrane from the cytoplasm. A distribution of this class of probe into different environments would yield a complex fluorescence signal. Microspectrofluorometric measurements of the probe \textit{in vitro} and in different regions of cells can identify environmental changes in probes.

Fluorescence quenching is the loss of fluorescence due to the interaction of the fluorophores with other molecules in the environment. Static quenching is the decrease in fluorescence due to the interactions of fluorophores in the ground state with other molecules. This can include self-association called ground state dimerization. Static quenching causes a decrease in the fluorescence intensity since the quenched fluorescent molecules do not fluoresce with the same quantum yield and/or with the same spectral properties. Fluorescent probes may be more quenched in cells compared to buffer solutions because of the presence of quenching activity in the local cell environment.

Dynamic quenching is the decrease in fluorescence intensity at defined wavelengths due to the interaction of the fluorophore in the excited state with quenching molecules in the environment. Therefore, the excitation spectrum is not altered, but the emission spectrum is changed. Dynamic quenching forms a new molecular species with distinct fluorescence properties. This type of quenching is dependent on the rate of diffusion in solution, so that it is affected by temperature and viscosity. Determination of the quantum yield and spectral properties of the probe in the living cell compared with solution measurements \textit{in vitro} is the only way to characterize this problem (see Wampler, 1986; Kohen \textit{et al.}, 1981).

G. Standards

Standards for quantitative fluorescence microscopy are important to ensure the reproducible performance of the system from day to day and to compare results between laboratories (see Sisken, Volume 30, this series). A variety of standards for fluorescence intensity, quantum yield, detector sensitivity, and detector corrections must be developed. This is an important, yet underdeveloped area in fluorescence microscopy.

H. Spectral Corrections

Excitation and emission spectra must be corrected for instrumental variables including optical components, quality of monochromators or
filters, sensitivity of the detector, spectral output of the lamp, etc. Uncorrected spectra cannot be reproduced in other laboratories with different equipment. There are a variety of methods for correcting both excitation and emission spectra but the simplest are standard solutions that have been calibrated under control conditions. Standardized, "corrected" spectra are published in a number of books (see Argauer and White, 1964; Berlman, 1971). The essence of the correction scheme is to determine the correction factor at each wavelength which converts the experimental spectrum of a standard solution, normalized to a peak height of 1.0, to the published corrected spectrum of the same solution also normalized to a peak height of 1.0. This conversion factor at each wavelength can then be used to correct a spectrum of any other solution measured under the same conditions. This issue only becomes a problem when accurate spectral measurements are attempted (see Herman, Volume 30, this series).

IV. Basic Setup of an Epiillumination Fluorescence Microscope

A. Alignment of Lamp and Microscope

1. A fluorescent bead slide can be prepared by mounting commercially available beads on a standard microscope slide. Beads with a diameter of \( \sim 5 - 10 \mu m \) and labeled with a variety of fluorophores are diluted and dried on a microscope slide (sources for the beads include Polysciences, Inc., Paul Valley Industrial Park, Warrington, Pennsylvania). A drop of UV curing cement is added, a cover slip is applied, and the slide is irradiated for \( \sim 1 \) hour. This standard slide can then be used to set up the microscope from day to day.

2. Select an objective that will be used in the experiment. Align the microscope for Koehler illumination for transmitted light microscopy before setting up epiillumination. This is performed as follows: (a) bring the specimen into focus; (b) close the field diaphragm (FD) and focus the condenser until the edge of the FD is in focus with the specimen; (c) center the condenser with the adjustment screws until the image of the FD is in the center of the field; (d) use a focusing telescope to view and to center the iris diaphragm; and (e) focus an image of the lamp on the plane of the iris diaphragm. This can be accomplished by closing down the FD and adjusting the screws on the lamp housing.
3. Now place a thin piece of white paper on the microscope stage in place of a specimen. Select the lamp for epiillumination. Rotate the chosen objective out of place so that a blank objective slot allows the image of the filament or arc to be visible on the paper by epiillumination. The image of the arc is then centered and focused using the adjustment screws on the lamp. Close down the iris diaphragm if your microscope has one in the incident light path. The image of the filament or arc should be coincident with the image of the iris diaphragm to attain Koehler illumination. The focused and centered filament or arc should now illuminate the microscope field uniformly when the objective is swung back into place. The optimal situation is when the filament or arc just fills the back focal plane of the objective (circular aperture observed on the white paper in the absence of the objective).

4. Place the bead slide on the microscope stage and bring one bead into focus. Check the uniformity of illumination by moving the bead into different quadrants of the field. A properly aligned microscope will produce an even illumination of the field so that the test bead will exhibit uniform fluorescence. Repeat the steps above if you do not achieve uniform illumination.

5. This procedure can be quantified by using a photomultiplier (PMT). The output of the PMT should be constant if the illumination system is optimal. The camera will add the problem of shading due to the characteristics of the detector (see Spring and Lowy, Chapter 15, and Aikens et al., Young, and Jericevic et al., Volumes 29 and 30, this series).

6. The field diaphragm in the epiillumination pathway can now be adjusted to limit the area of the field that is illuminated.

7. The iris diaphragm can be adjusted over a small range to control the irradiance at the specimen plane (see Section IV, B, below). The irradiance can be controlled in microscopes not possessing an iris in the epiillumination pathway by inserting neutral density filters between the light source and the objective. The voltage on filament lamps should never be used to control irradiance, since the color temperature of these lamps changes significantly with operating voltage.

8. At this point you should use some type of low light level camera to view the experimental samples. Remember, you want to minimize the total dose of illumination (irradiance) to the specimen.

9. Check to make sure that the camera is viewing the center of the field.

10. If your microscope has two lamps, one for epiillumination and the other for transmitted light illumination, you can observe the specimen either sequentially or simultaneously by both transmitted and fluorescence microscopy.
B. Measurement of Irradiance at the Specimen Plane

Characterization of the irradiance at the specimen plane is an important parameter in fluorescence microscopy. Publication of this value along with the time of illumination should optimize the ability of other investigators to reproduce experiments. We use an NRC (Newport Research Corporation, Fountain Valley, California) laser power meter, model 820 with a detector area of 1.0 cm², to measure the irradiance.

1. Select the objective and fluorescence filter set to be used and set the irradiance at the specimen plane that will be used in the experiment by using neutral density filters and/or the incident light iris diaphragm.

2. Adjust the field diaphragm to a specific diameter. This can be accomplished with an eyepiece reticle and a stage micrometer. Determine the area illuminated by this setting of the field diaphragm.

3. Place the surface of the detector photodiode on the stage in place of the specimen. Measure the uncorrected power output at the selected excitation wavelength with the laser power meter.

4. Correct power reading for spectral sensitivity using the table supplied with the power meter. Divide the uncorrected value in microwatts by the correction factor.

5. Divide the corrected power by the area of illumination measured above. This will yield the intensity or irradiance in microwatts per squared centimeter.

REFERENCES


