Fluorescence Microscopy

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INTRODUCTION

When organic or inorganic specimens absorb and subsequently reradiate light, the process is typically a result of fluorescence or phosphorescence. Fluorescence emission is nearly simultaneous with the absorption of the excitation light as the time delay between photon absorption and emission is typically less than a microsecond. When the emission persists long after the excitation light is extinguished, the phenomenon is known as phosphorescence. Stokes coined the term ''fluorescence'' in the middle of the 19th century when he observed that the mineral fluorspar emitted red light when it was illuminated by ultraviolet (UV) excitation. Stokes noted that the fluorescence emission always occurred at a longer wavelength than that of the excitation light. Early investigations showed that many specimens (minerals, crystals, resins, crude drugs, butter, chlorophyll, vitamins, inorganic compounds, etc.) fluoresce when irradiated with UV light. In the 1930s, the use of fluorochromes began in biology to stain tissue components, bacteria, or other pathogens. Some of these stains were highly specific and they stimulated the development of the fluorescence microscope.

Fluorescence microscopy has become an essential tool in biology as well as in materials science as it has attributes that are not readily available in other optical microscopy techniques. The use of an array of fluorochromes has made it possible to identify cells and submicroscopic cellular components and entities with a high degree of specificity amid nonfluorescing material. The fluorescence microscope can reveal the presence of a single fluorescing molecule. In a sample, through the use of multiple staining, different probes can simultaneously identify several target molecules. Although the fluorescence microscope cannot provide spatial resolution below the diffraction limit of the respective objects, the detection of fluorescing molecules below such limits is readily achieved.

There are specimens that autofluoresce when they are irradiated and this phenomenon is exploited in the field of botany, petrology, and in the semiconductor industry. In the study of animal tissues or pathogens, autofluorescence is often either extremely faint or nonspecific. Of far greater value for such specimens are added fluorochromes (also called fluorophores), which are excited by specific wavelength irradiating light and emit light of useful intensity. Fluorochromes are stains that attach themselves to visible or subvisible structures, are often highly specific in their attachment targeting, and have significant quantum yield (the photon emission/absorption ratio). The growth in the use of fluorescent microscopes is closely linked to the development of hundreds of fluorochromes with known intensity curves of excitation and emission and well-understood biological structure targets.

EXCITATION AND EMISSION FUNDAMENTALS

The basic task of the fluorescence microscope is to irradiate the specimen with the desired wavelength and then to separate the much weaker emitted (fluorescent) light from the excitation light. Only the emission light should reach the eye or other detector so that the resulting fluorescing areas are contrasted against a dark background. The detection limit is largely determined by the darkness of the background. The exciting light is typically 10^5 or 10^6 times brighter than the emitted light.

Fig. 1 is a graphical representation of the design of an epi-fluorescence microscope. This is basically a reflected light microscopy mode in which the wavelength of the reflected light is longer than that of the excitation. J.S. Ploem is credited with the development of the vertical illuminator for reflected light fluorescence microscopy. In this device, light of a specific wavelength or set of wavelengths, often in the UV, is produced by passing light from a lamp or other source through a wavelength selective exciter filter. The desired wavelength light reflects off a dichromatic (''dichroic'') mirror, through the microscope objective lens to the specimen. If the specimen fluoresces, the collected emission light then passes through the dichromatic mirror and is subsequently filtered by a barrier filter that blocks the excitation wavelengths. It should be noted that this is the only mode of microscopy in which the specimen, subsequent to excitation, gives off its own light. The emitted light reradiates spherically in all directions, regardless of the direction of the exciting light.

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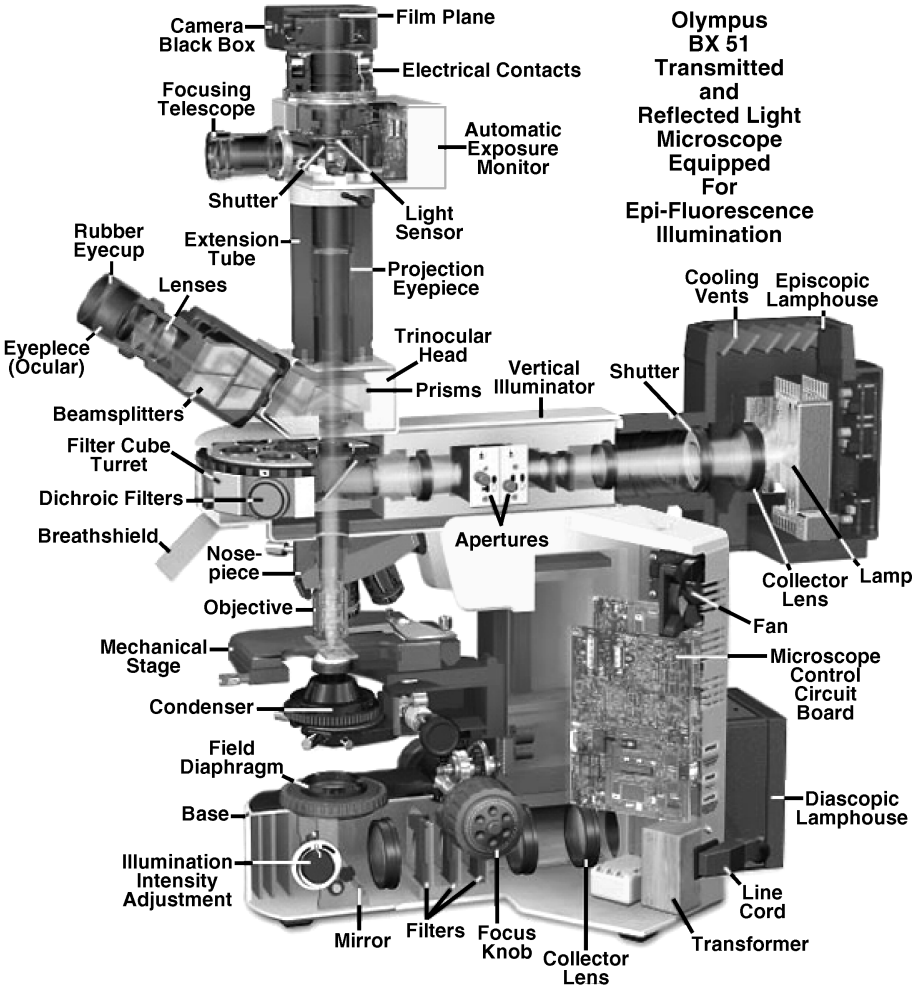

Fig. 1 Cut-away diagram of an upright microscope equipped both for transmitted light and epi-fluorescence microscopy. The vertical illuminator in the center of the diagram has the light source at one end (episcopic lamphouse) and the filter cube at the other.

Epi-fluorescence is the overwhelming choice in modern microscopy and the reflected light vertical illuminator is interposed between the observation viewing tubes and the nosepiece carrying the objectives. The illuminator is designed to direct light onto the specimen by first passing the light through the microscope objective on the way toward the specimen and then using that same objective to capture the emitted light. This type of illuminator has several advantages: the objective—first serving as a well-corrected condenser and then as the image-forming light gatherer—is always in correct alignment; most of the unused excitation light reaching the specimen passes through it and travels away from the objective; the illuminated area is restricted to that which is observed; the full numerical aperture (n.a.) of the objective, in Koehler illumination, is utilizable; it is possible to combine or alternate reflected light fluorescence with transmitted light observation.

The reflected light illuminator has, at its far end, a lamphouse containing a light source, usually a mercury or xenon burner (the episcopic lamphouse in Fig. 1). The light travels along the illuminator perpendicular to the optical axis of the microscope, passes through collector lenses and a variable, centerable aperture diaphragm, and then through a variable, centerable field diaphragm (apertures in Fig. 1). It impinges upon the excitation filter where selection of the excitation wavelength light and blockage of undesired wavelengths occurs. The selected wavelengths reach the dichromatic beamsplitting mirror. This is a special type of interference filter that efficiently reflects shorter wavelength light and efficiently passes longer wavelength light. The dichromatic beam splitter (also sometimes called the dichroic mirror, as shown in Fig. 1) is tilted at 45° to the incoming excitation light and reflects the excitation light at a 90° angle directly through the objective and onto the specimen. The fluorescent light

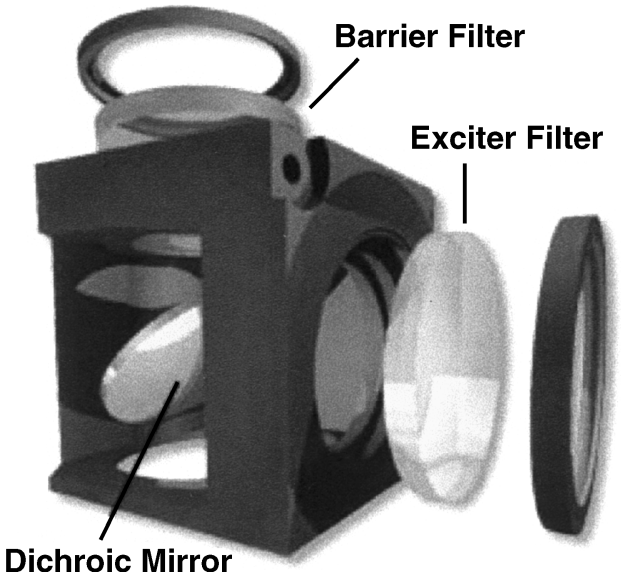

Fig. 2 A microscope filter cube containing the exciter and barrier filters as well as the dichromatic mirror.

emitted by the specimen is gathered by the objective, now serving in its usual image-forming function. Because the emitted light consists of longer wavelengths, it is able to pass through the dichroic mirror.

Any scattered excitation light reaching the dichroic mirror is reflected toward the light source. Before the emitted light can reach the eyepiece or detector, it is incident upon and passes through the barrier or suppression filter. This filter blocks (suppresses) any residual excitation light and passes the desired longer emission wavelengths. In most reflected light fluorescence illuminators, the excitation filter, dichroic mirror, and barrier filter are incorporated in a cube, as illustrated in Fig. 2. Most microscopes accommodate three or four fluorescence cubes (on a revolving turret or on a slider as shown in Fig. 1) and permit the user to attach replacement custom-made exciters, barrier filters, or dichroic mirrors.

The design of the illuminator should permit the user to employ the desirable Koehler Illumination, providing bright and even illumination across the field of view. The corrected condensing lenses of the system ensure that the image of the centerable aperture diaphragm is conjugate with the back aperture of the focused objective. The image of the prefocused, centerable field diaphragm is conjugate with the focused specimen and the plane of the fixed eyepiece diaphragm.

The illuminator lamphouse usually incorporates an infrared suppression filter. The lamphouse itself should not leak harmful UV wavelengths and, preferably, should incorporate a switch to automatically shut down the lamp if the housing is inadvertently opened during operation. The lamphouse should be sturdy enough to withstand a possible burner explosion during operation. The lamp socket should have adjustment screws to permit centering the image of the lamp arc or halogen lamp coil to the back aperture of the objective (in Koehler illumination, these planes are conjugate). In the light path, closer to the lamphouse and before the excitation filter, it is desirable to have a shutter for complete blocking of excitation light as well as neutral density filters on a wheel or slider to permit reduction of light intensity.

STOKES' SHIFT

When electrons go from the excited state to the ground state, there is a loss of vibrational energy. As a result, the emission spectrum is shifted to longer wavelengths than the excitation spectrum (wavelength varies inversely to radiation energy). This phenomenon is known as Stokes' Law or Stokes' shift. The greater the Stokes' shift, the easier it is to separate excitation light from emission light. The emission intensity peak is usually lower than the excitation peak; and the emission curve is often a mirror image of the excitation curve, but shifted to longer wavelengths (Fig. 3). To achieve maximum fluorescence intensity, the dye is usually excited at wavelengths near or at the peak of the excitation curve, and the widest possible range of emission wavelengths that include the emission peak are selected. The selection of excitation wavelengths and emission wavelengths is typically based on interference filters. In addition, the spectral response of an optical system will depend on such factors as glass transmission and detector responsivity.

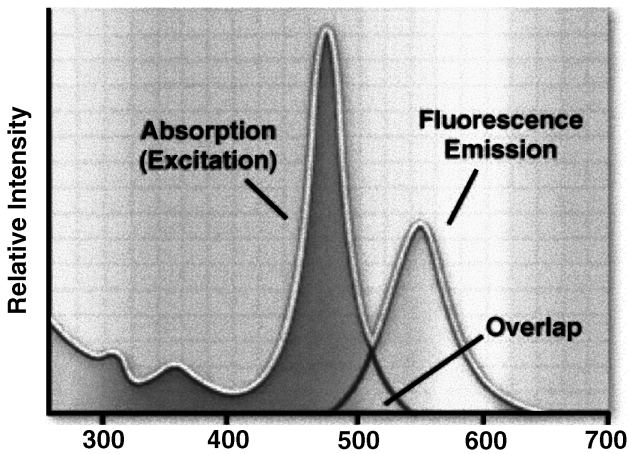

Fig. 3 Absorption and emission spectra are shown for fluorescein.

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The separation of excitation and emission wavelengths is achieved by the proper selection of filters to block or pass specific wavelengths of the spectrum. The design of fluorescence illuminators is based on the control of excitation light and emission light by readily changeable filter insertions in the light path on the way toward the specimen and then emanating from the specimen. It is important, in view of low emission intensities, that the light source chosen for excitation be of sufficient brightness so that the relatively weak emission light can be maximized; and that fluorochromes of satisfactory absorption and yield be chosen.

The efficiency with which the fluorochrome absorbs a photon of the excitation light is a function of molecular cross-section, and the likelihood of absorption is known as the extinction coefficient. Larger extinction coefficients mean that the absorption of light in a given wavelength region is more likely. The quantum yield denotes the ratio of the number of quanta emitted compared to those absorbed (usually the yield is between 0.1 and 1.0). Quantum yields below 1 are the result of the loss of energy through nonradiative pathways (e.g., heat or photochemical reaction) rather than the reradiative pathway of fluorescence. Extinction coefficient, quantum yield, mean luminous intensity of the light source, and fluorescence lifetime are all important factors that contribute to the intensity and utility of fluorescence emission.

FADING

There are conditions that may affect the reradiation of light and thus reduce the intensity of fluorescence. This reduction of emission intensity is generally called fading and is further subdivided into quenching and bleaching. Bleaching is the irreversible decomposition of the fluorescent molecules in the excited state because of their interaction with molecular oxygen. The occurrence of bleaching is exploited in a technique known as fluorescence recovery after photobleaching (FRAP), to study diffusion and motion. It is based upon bleaching a sharply defined region of the specimen by an intense burst of laser light and subsequently observing the rate and pattern of the recovery of fluorescence in the bleached area. Quenching reduces fluorescence intensity by a variety of mechanisms involving nonradiative energy loss and frequently comes about as a result of oxidizing agents or the presence of salts of heavy metals or halogen compounds. Sometimes quenching results from the transfer of energy to other so-called acceptor molecules physically close to the excited fluorophores, a phenomenon known as resonance energy transfer. This particular phenomenon has become the basis of the technique called fluorescence resonance energy transfer (FRET) for studying molecular interactions and associations at distances far below the lateral resolution of the light microscope.

LIGHT SOURCES

In most fluorescence microscopy applications, the number of photons that reach the eye or detector is usually very low. This is because the collection efficiency of microscopes is less than 30% and the concentration of most fluorochromes in the optical path is low (usually micromolar or nanomolar). To generate sufficient excitation light intensity to produce detectable emission, powerful compact light sources are needed, usually short arc lamps. The most common lamps are the mercury burners, ranging in wattage from 50 to 200 W and the xenon burners ranging from 75 to 150 W. These light sources are powered by a direct current (d.c.) supply, furnishing enough start-up power to ignite the burner (by ionization of the gaseous vapor) and to keep it burning with a minimum of flicker. The power supply should have a timer to track the number of hours the burner has been in use. Arc lamps lose efficiency and are more likely to shatter, if used beyond their rated lifetime. The mercury burners do not provide even intensity across the spectrum from UV to infrared and much of the intensity of the mercury burner is expended in the near UV. Prominent peaks of intensity occur at 313, 334, 365, 406, 435, 546, and 578 nm. At other wavelengths of visible light, the intensity is steady although not nearly so bright, but still usable. Mere lamp wattage is not the prime consideration; instead, the critical parameter is the mean luminance that takes into account the source brightness, arc geometry, and the angular spread of emission.

In recent years, there has been increasing use of lasers, particularly the argon-ion and argon–krypton-ion lasers as light sources. They have the virtues of small source size, low divergence, monochromaticity, and high mean luminance. They have become essential in scanning confocal microscopy, a technique that has proved to be a powerful tool in rendering very sharp fluorescence images by rejecting out-of-focus light. This is accomplished through point or line scanning and coincident imaging through a conjugate aperture. Optical sections of the specimen are stored in a computer and reconstituted into the whole image that then can be displayed on a monitor.

FILTER TERMINOLOGY

The terminology applied to fluorescence filters has become a jumble as a result of various initials utilized by

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different manufacturers to identify their filters. Basically, there are three categories of filters: exciter filters, barrier filters, and dichromatic beam splitters (dichroic mirrors). Fluorescence filters were formerly almost exclusively made of colored glass or colored gelatin sandwiched between glass plates. Now, interference filters are used for exciter filters to pass or reject wavelengths of light with great selectivity and high transmission. Dichromatic beam splitters are specialized interference filters. Barrier filters may be either made of colored glass or interference filters.

Exciter Filters

Abbreviations used by manufacturers to denote the properties of their filters include: UG (UV glass) and BG (blue glass), which are glass exciter filters. KP (K is an abbreviation for kurz, short in German) and SP are short pass filters; and EX indicates an exciter filter. Some manufacturers label their interference filters with the designation IF. Narrow band-pass interference filters are especially helpful if the Stokes' shift is small.

Barrier Filters

Acronyms or abbreviations for barrier filters include: LP or L for long pass filters, Y or GG for yellow or gelb (German) glass, R or RG for red glass, OG or O for orange glass, K for kante, a German term for edge (filter), and BA for barrier filter. When the filter type is also associated with a number, e.g. BA475, that designation refers to the wavelength (in nanometers) at 50% of its maximum transmission.

Dichromatic Beam Splitters

Abbreviations used to describe and identify beam splitters include: CBS for a chromatic beam splitter, DM for a dichroic mirror, TK for ''teiler kante,'' German for edge splitter, FT for ''farb teiler,'' German for color splitter, and RKP for reflection short pass. All of these terms should be considered interchangeable. These filters are always the interference type. The coatings are designed to have high reflectivity for shorter wavelengths and high transmission for longer wavelengths. Dichromatic beam splitters are oriented at a 45° angle to the path of the excitation light entering the cube through the reflected light fluorescence illuminator. Their function is to direct the selected excitation (shorter wavelengths) light through the objective and onto the specimen. They also have the additional functions of passing longer wavelength light to the barrier filter, and reflecting any scattered excitation light back in the direction of the lamphouse.

THE LIGHT BUDGET

It is a useful exercise to estimate the light fluxes in a typical fluorescence microscope as one obtains considerable insight into the constraints that any microscopist or optical engineer encounters.

The excitation source is assumed to be a 75-W xenon lamp with a mean luminous density of approximately 400 cd/mm^2. When the lamp output is collected and directed through a 490-nm interference filter (10-nm bandwidth, 75% transmission), about 2 mW of light will pass. After reflection by a 90% efficient dichromatic mirror, a light of 1.8 mW enters the back aperture of the microscope objective lens as the excitation beam. With an objective lens of $100\times/1.4$ n.a., the area of specimen illuminated will be 12×10^{-6} cm^2, assuming a circular field of view of 40 μm in diameter. The light flux on the specimen is then 1.8 mW/12×10^{-6} cm^2 = 150 W/cm^2. This corresponds to a flux of 0.36×10^{21} photons/cm^2 sec, about 1000 times higher than that incident on the Earth's surface on a sunny day.

The fluorescence emission that results from this light flux depends on the absorption and emission characteristics of the fluorophore, its concentration in the specimen, and the optical path length of the specimen. The fluorescence produced, F, is given by:

$$F = \sigma Q I$$

where σ is the molecular absorption cross-section, Q is the quantum yield, and I is the incident light flux (0.36×10^{21} photons/cm^2 sec, as calculated above). Assuming that fluorescein is the fluorophore, $\sigma = 3\times10^{-16}$ cm^2/molecule, Q = 0.99, and $F = 1\times10^5$ photons/sec molecule. For a preparation with a dye concentration of 1 μM/l uniformly distributed in a 40-μm diameter disk with a thickness of 10 μm (volume of 1.2×10^{-11} l), there are approximately 1.2×10^{-17} moles of dye or 7.2×10^6 molecules in the optical path. If all of the molecules were excited simultaneously, the fluorescence emission rate would be 7.2×10^{11} photons/sec (given by the product of F and the number of dye molecules). How many of these photons would be detected and for how long could this emission rate continue?

The efficiency of detection is a function of the optical collection efficiency and the detector quantum efficiency. A 1.4-n.a. objective lens with 100% transmission has a

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maximum collection efficiency, limited by the acceptance angle, of 30%. The transmission efficiency of the dichromatic mirror is 85% and that of the barrier filter 80%. The overall collection efficiency is then 20% or 14×10^{10} photons/sec. If the detector is a conventional charge-coupled device (CCD), the quantum efficiency is about 50% for the green fluorescein emission, so the detected signal would be 7×10^{10} photons/sec or about 10% of the emitted fluorescence. Even with a perfect detector (100% quantum efficient), only 20% of the emission photons would be detected.

The duration of emission depends on the rate of photodestruction as a result of bleaching. For fluorescein in an oxygenated solution, measurements show that each

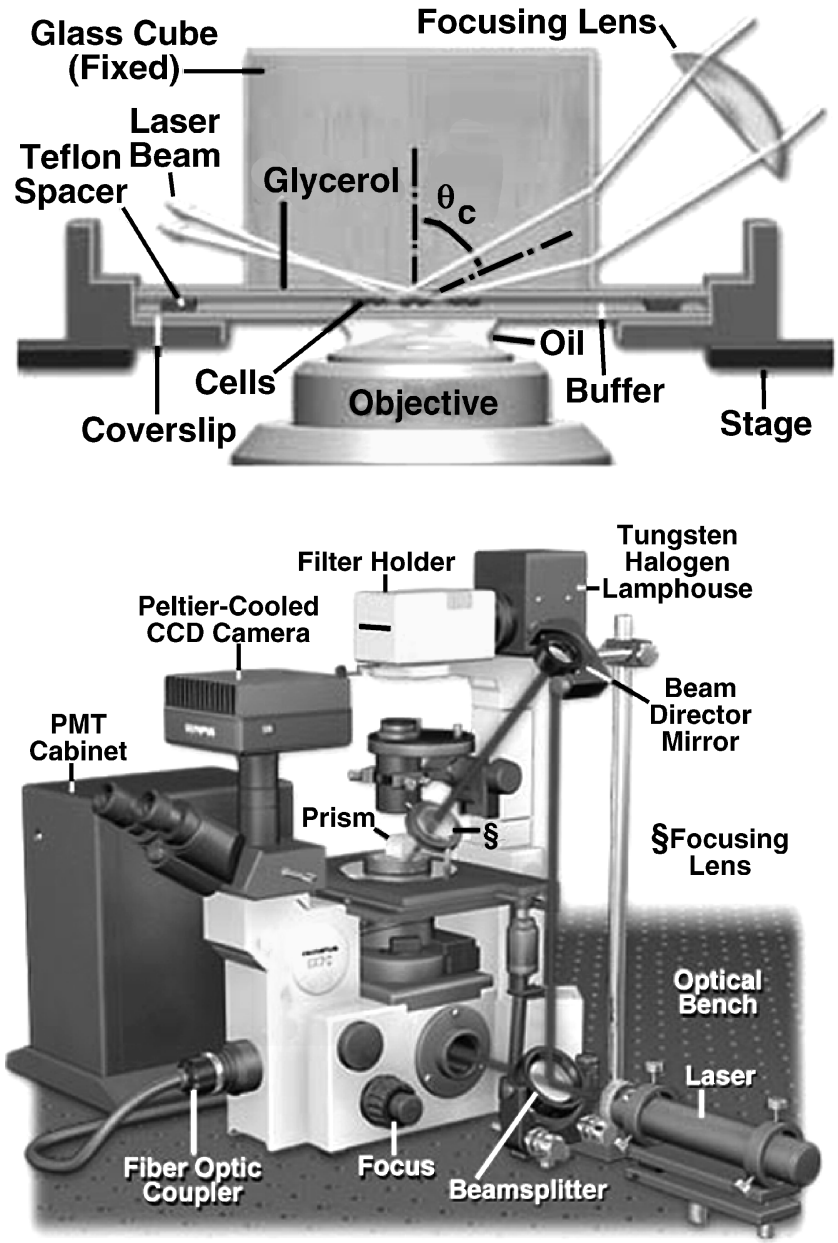

Fig. 4 The principle and design of a total internal reflection fluorescence (TIRF) microscope using an external laser light source are shown. The top portion shows a close-up of the specimen to which a glass cube is approximated with an intervening layer of glycerol. The illuminating laser beam is totally reflected at the boundary between the coverslip and the aqueous buffer solution provided that it enters the glass cube at an angle greater than the critical angle (θ_c).

molecule can only emit about 3.6×10^4 photons before being destroyed. In a deoxygenated environment, the rate of photodestruction diminishes about tenfold, so 3.6×10^5 photons are produced per molecule. The entire dye pool of 7.2×10^6 molecules could then produce a minimum of 2.6×10^{11} photons or a maximum of 2.6×10^{12} photons. Assuming the emission rate of 1×10^5 photons/sec molecule calculated above, fluorescence could continue for only 0.3–3 sec before photodestruction. If 10% of the photon flux were detected, a signal of 7.2×10^{10} electrons/sec would be obtained. If the detector is a 1×1 K CCD camera, this signal would be distributed over 1×10^6 sensors, with 7.2×10^4 electrons/sensor. For a scientific-grade CCD with 9×9 μm square sensors, the full well storage capacity is about 8×10^4 electrons and the read-out noise is less than 10 electrons. The signal/noise ratio would then be largely determined by photon statistical noise equal to the square root of the signal, i.e. 268. Unfortunately, this high signal level could only continue for a very brief time before photodestruction occurs. The compromise utilized by most microscopists to prolong the observation period is a reduction in the incident light flux intensity so that only a fraction of the fluorophore molecules in the dye pool are excited and subjected to photodestruction. Thus, the signal/noise ratio rarely equals the theoretical maximum and typically ranges between 10 and 20 in fluorescence microscopy.

DETECTING SINGLE MOLECULES

It is possible to detect the emissions from a single fluorophore provided that the optical background and detector noise are sufficiently low. As discussed above, a single fluorescein molecule could emit as many as 3×10^5 photons before it is destroyed. Assuming a 20% collection/detection efficiency, about 6×10^4 photons would be detected. Investigators, using an avalanche photodiode detector for these experiments, have been able to monitor the behavior of single molecules for many seconds or minutes. The biggest problems arise from the need to suppress the optical background sufficiently. Because most of the materials used in microscope lenses and filters show some autofluorescence, efforts were initially directed toward the manufacture of very low fluorescence components. However, it soon became evident that fluorescence microscopic methods utilizing total internal reflection provided the desired combination of low background and high exiting light flux.

Total internal reflection fluorescence (TIRF) utilizes the evanescent wave that is developed at the interface at which light is totally internally reflected. The principle employing an external light source is illustrated in Fig. 4. In this method, a beam of light (usually an expanded beam of laser light) is directed through a prism of high refractive index, e.g. glass or sapphire, which abuts a lower refractive index medium, e.g. glass or aqueous solution. If the light is directed at higher than the critical angle, the beam will be totally internally reflected at the interface. However, an evanescent wave develops at the interface by the generation of an electromagnetic field that permeates about 200 nm or less into the lower refractive index space. The light intensity in the evanescent wave is sufficiently high to excite the fluorophores within it, but because of its shallow depth, the volume excited is very small. The result is an extremely low-level background because so little of the sample is exposed to the excitation light.

TIRF can also be accomplished by a modification of the epi-illumination approach used in wide field fluorescence microscopy (Fig. 5). This method necessitates a very high numerical aperture objective lens (at least 1.4, but preferably 1.6) and partial illumination of the microscope field from one side by a small spot or more uniform illumination by a thin annulus. High refractive index lens immersion medium and microscope cover

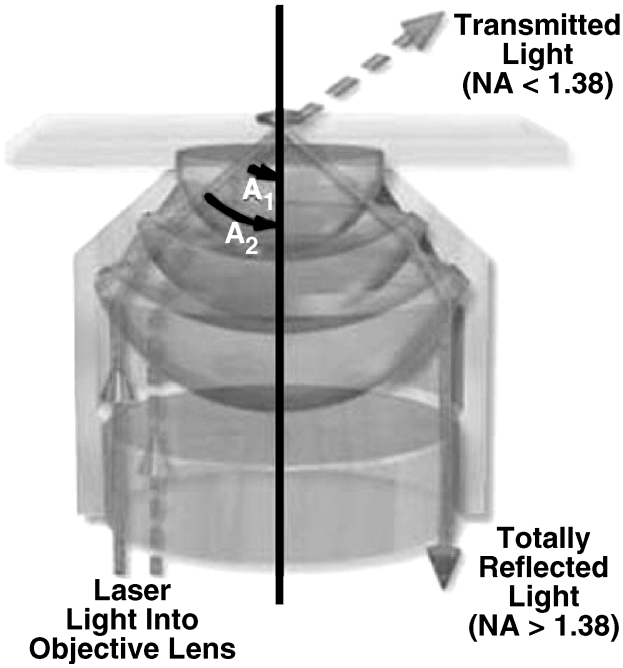

Fig. 5 The basic principle of TIRF through a microscope objective lens is shown. Excitation laser light exiting the objective lens at angle A_1, less than the critical angle, will be transmitted because the effective numerical aperture is < 1.38. Light that exits the lens at angle A_2, equal to or greater than the critical angle, will be totally reflected (labeled NA > 1.38).

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glass are required for achievement of the illumination angle resulting in total internal reflection. As shown in Fig. 5, light rays exiting the objective lens at an angle less than the critical angle (denoted as angle A_1 in Fig. 5) are transmitted. When the angle is increased to or beyond the critical angle (denoted as angle A_2 in Fig. 5), total internal reflection results. TIRF may be combined with other optical methods such as FRAP and FRET as well as spectroscopy. The result is a very powerful tool for the study of individual fluorophores and fluorescently labeled molecules. The advantages of the study of the properties of single molecules are only beginning to be appreciated. The range of optical microscopy now extends from the single molecule to the entire animal.

CONCLUSION

The modern light microscope combines the power of high performance optical components with computerized control of the instrument and digital image acquisition to achieve a level of sophistication that far exceeds that of simple observation by the human eye. The fluorescence microscope depends heavily on electronic imaging to rapidly acquire information at low light levels or at visually undetectable wavelengths. These technical improvements are not mere window dressing, but are essential components of the light microscope as a sytem. The days of the light microscope as a purely descriptive instrument or plaything of the intellectual are past. At present, optical image formation is only the first step toward data analysis. The microscope accomplishes this first step in conjunction with electronic detectors, image processors and display devices that can be viewed as extensions of the imaging system. Computerized control of focus, stage position, optical components, shutters, filters, and detectors is in widespread use and enables experimental manipulations that are not humanly possible. The increasing use of electro-optics in fluorescence microscopy has lead to the development of optical tweezers capable of manipulating sub-cellular structures or particles, the imaging of single molecules, and a wide range of sophisticated spectroscopic applications.

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