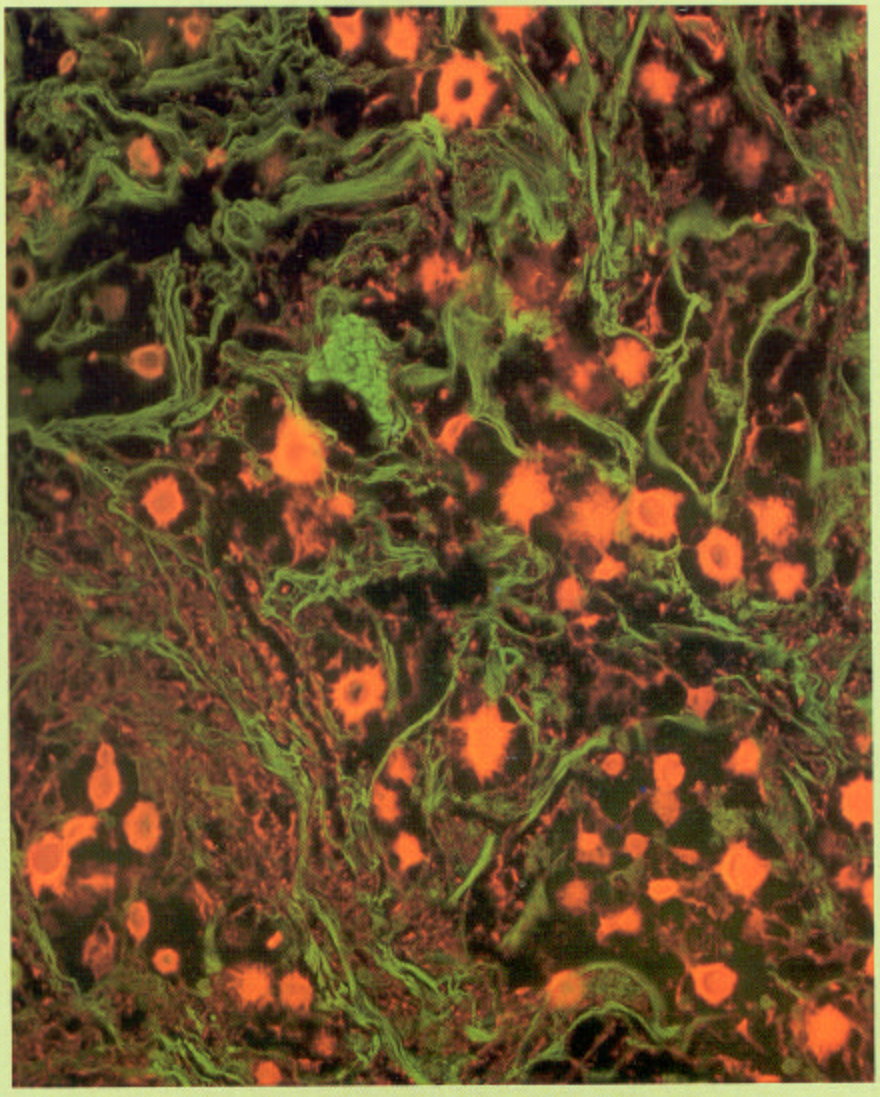
Fluorescence Microscopy

The essentials

by Mortimer Abramowitz
Fellow, New York Microscopical Society

For Olympus America Inc.

Volume 4



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The essentials

By Mortimer Abramowitz
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For Olympus America Inc.
Volume 4, Basics and Beyond Series

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Executive Vice President
of Olympus America Inc.,
whose support
and inspiration
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Introduction

When specimens, living or non-living, organic or inorganic, absorb and subsequently re-radiate light, we describe the process as photoluminescence. If the light emission persists for up to a few seconds after the excitation light is withdrawn, the phenomenon is known as phosphorescence. Fluorescence, on the other hand, describes light emission which continues only during the absorption of the excitation light. The time interval between absorption of excitation light and emission of re-radiated light in fluorescence is of extraordinarily short duration, usually less than a millionth of a second.

The phenomenon of fluorescence was known by the middle of the 19th century. It was Stokes who made the observation that the mineral fluorspar fluoresces when ultraviolet light is directed upon it; he coined the word "fluorescence." Stokes observed that the fluorescing light is in longer wavelengths than those of the excitation light. (Figs. A&B)

Fig. A
Stokes' Observation (Diagrammatic)

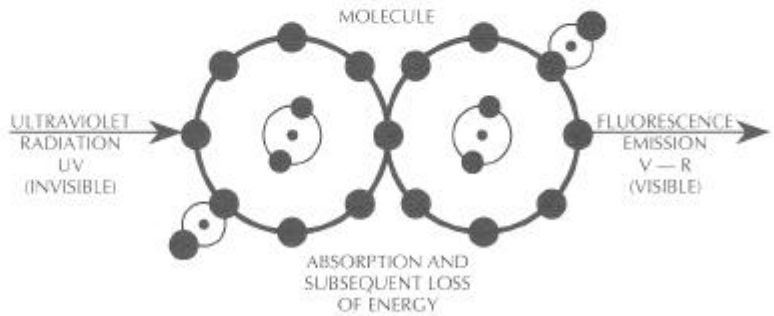
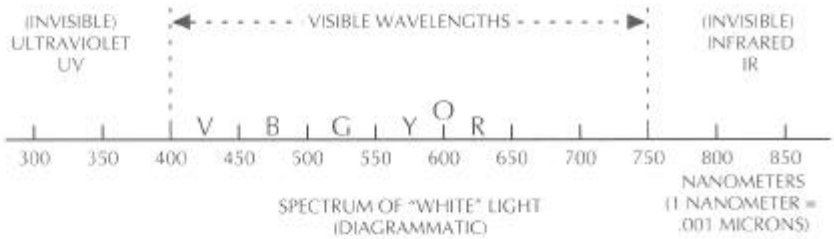


Fig. B
Spectrum of "White" Light (Diagrammatic)

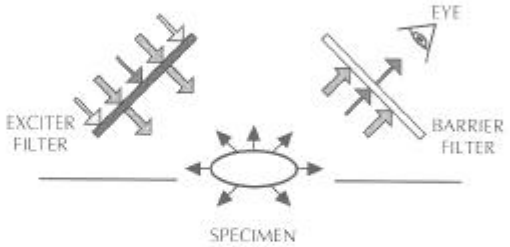


Fluorescence microscopy is basically a method of studying material which can be made to fluoresce, either in its natural form (primary or autofluorescence) or when treated with chemicals capable of fluorescing (secondary fluorescence). The fluorescence microscope was devised in the early part of the 20th century; Koehler, Reichert and Lehman were among the scientists associated with such development. However, the potential of this instrument was not realized for several decades.

Early investigations showed that many specimens (microminerals, crystals, resins, crude drugs, butter, chlorophyll, vitamins, inorganic compounds, etc.) autofluoresce when irradiated with ultraviolet light. However, it was not until the 1930's that Haitinger and others developed the technique of secondary fluorescence—employing fluorochrome stains to stain specific tissue components, bacteria, and other pathogens which do not autofluoresce. These fluorochrome stains, tagged to specific objects, spurred the use of the fluorescence microscope. The instrument's value was significantly enhanced by the 1950's when Coons and Kaplan demonstrated the localization of antigens in tissues that were stained with a fluorescein (the fluorochrome) tagged antibody.

The basic task of the fluorescence microscope is to permit excitation light to irradiate the specimen and then to separate the much weaker re-radiating fluorescent light from the brighter excitation light. Thus, only the emission light reaches the eye or other detector. The resulting fluorescing areas shine against a dark background with sufficient contrast to permit detection. The darker the background of the non-fluorescing material, the more efficient the instrument. (Fig. C)

Fig. C
Principle of Fluorescence Microscope



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 re-radiates spherically in all directions, regardless of the direction of the exciting light.

ADVANTAGES OF FLUORESCENCE MICROSCOPY

Fluorescence microscopy is a rapidly expanding and invaluable tool of investigation. Its advantages are based upon attributes not as readily available in other light microscopy techniques. The use of fluorochromes has made it possible to identify cells and sub-microscopic cellular components and entities with a high degree of specificity amidst non-fluorescing material. What is more, the fluorescence microscope can reveal the presence of fluorescing material with exquisite sensitivity. An extremely small number of fluorescing molecules (as few as 50 molecules per cubic micron) can be detected. In a given sample, through the use of multiple staining, different probes will reveal the presence of different target molecules. Although the fluorescence microscope cannot provide spatial resolution below the diffraction limit of the respective objectives, the presence of fluorescing molecules below such limits is made visible.

Techniques of fluorescence microscopy can be applied to organic material, formerly living material or to living material (with use of in vitro or in vivo fluorochromes) or to inorganic material (lately, especially in the investigation of contaminants on semiconductor wafers). There are also a burgeoning number of studies using fluorescent probes to monitor rapidly changing physiological ion concentrations (calcium, magnesium, etc.) and pH values in living cells.

There are specimens that fluoresce when irradiated with shorter wavelength light (primary or autofluorescence). Autofluorescence has been found useful in plant studies, coal petrography, sedimentary rock petrology, and in the semiconductor industry.

In the study of animal tissues or pathogens, autofluorescence is often either extremely faint or of such non-specificity as to make autofluorescence of minimal use. Of far greater value for such specimens are the fluorochromes (also called fluorophores) which are excited by irradiating light and whose eventual yield of emitted light is of greater intensity. Such fluorescence is called secondary fluorescence.

Fluorochromes are stains, somewhat similar to the better-known tissue stains, which attach themselves to visible or sub-visible organic matter. These fluorochromes, capable of absorbing and then re-radiating light, are often highly specific in their attachment targeting and have significant yield in absorption-emission ratios. This makes them extremely valuable in biological applications. The growth in the use of fluorescence 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.

A chosen fluorochrome should have a high likelihood of absorbing the exciting light and should remain attached to the target molecules; the fluorochrome should also be capable of providing a satisfactory yield of emitted fluorescence light.

DETERMINATION OF ABSORPTION (EXCITATION)-EMISSION CURVES

Because of their different electron configurations, fluorochromes have unique and characteristic spectra for absorption (usually similar to excitation) and emission. These absorption and emission spectra show relative INTENSITY OF FLUORESCENCE, with relative intensity as the vertical axis versus wavelength as the horizontal axis. For a given fluorochrome, the manufacturers indicate the wavelength for the peak of excitation/fluorescence intensity and the wavelength for the peak of emission/fluorescence intensity.

It is important that you understand the origin of the graphs/curves showing the excitation and emission spectra for a given fluorochrome.

To determine the EMISSION spectrum of a given fluorochrome, the dye absorption maximum wavelength (usually the same as the excitation maximum) is found and the fluorochrome is excited at that maximum. A monochromator (a device for allowing narrow wavelengths of light to pass) is then used to scan the fluorescence emission intensity at successive emission wavelengths. The relative intensity of the fluorescence is measured at the various wavelengths to draw the EMISSION spectrum. (Fig. D2)

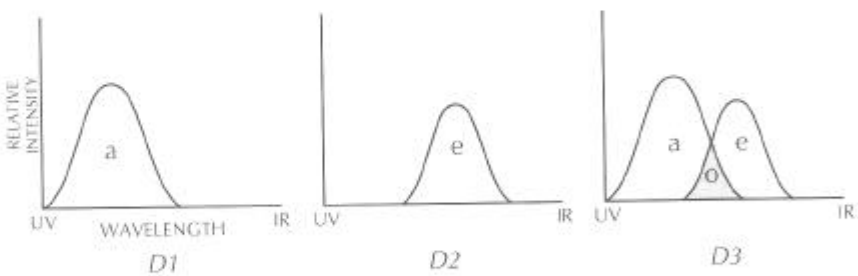


Fig. D 1,2,3 Absorption/ Emission Spectral Diagrams

a= Absorption Spectrum (for molecules usually similar to Excitation Spectrum)

e = Emission Spectrum

o = Overlap

The EXCITATION spectrum of a given fluorochrome is determined in a similar manner. The emission maximum is chosen and only emission light at that emission wavelength is allowed to pass to the detector. Then excitation is induced—by means of a monochromator—at various excitation wavelengths and the intensity of the emitted fluorescence is measured. The result is a graph/curve which depicts the relative FLUORESCENCE intensity caused by excitation at the successive excitation wavelengths. (Fig. D1)

Several observations can be made from a typical excitation/emission set of curves. There is usually an overlap at the higher wavelength end of the excitation spectrum

and the lower wavelength end of the emission spectrum. This overlap of excitation and emission intensities/wavelengths must be eliminated, in fluorescence microscopy, by means of appropriate selection of excitation filter, dichromatic beam splitter (in reflected light fluorescence), and barrier filter. Otherwise, the much brighter excitation light overwhelms the weaker emitted light and significantly diminishes the contrast. (Fig. D3)

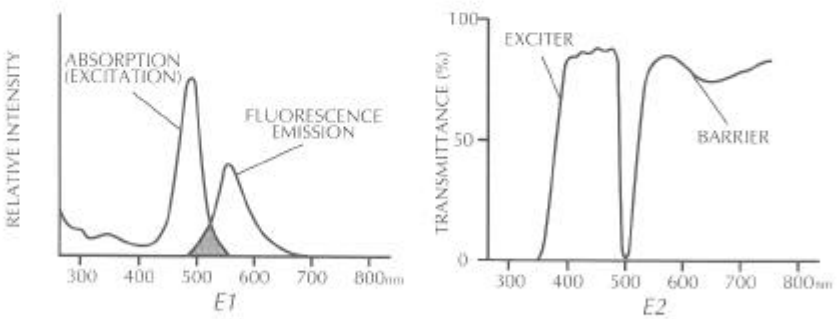
When electrons go from the excited state to the ground state (see later section on molecular explanation), there is a loss of vibrational energy. As a result, the emission spectrum is shifted to longer wavelengths than the excitation spectrum (wavelength varies inversely to radiation energy). This phenomenon 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 the longer wavelengths. To achieve maximum fluorescence intensity, the fluorochrome is usually excited at the wavelength at the peak of the excitation curve, and the emission is selected at the peak wavelength (or other wavelengths chosen by the observer) of the emission curve. The selections of excitation wavelengths and emission wavelengths are controlled by appropriate filters.

In determining the spectral response of an optical system, technical corrections are required to take into account such factors as glass transmission and detector sensitivity variables for different wavelengths.

A TYPICAL ABSORPTION (EXCITATION)-EMISSION SPECTRAL DIAGRAM

A typical fluorochrome absorption-emission spectral diagram is shown. (Fig. E1) Note that the curves of fluorescence intensity for absorption (usually similar to the excitation curve for pure compounds) and emission for this typical fluorochrome are somewhat similar in shape. The wavelength shift between excitation and emission has been known since the middle of the nineteenth century (Stokes' Law). Also note that the excitation and emission curves overlap somewhat at the upper end of excitation and the lower wavelengths end of the emission curve.

Figs. E1 & E2



The separation of excitation and emission wavelengths is achieved by the proper selection of filters to block or pass specific wavelengths of the spectrum. (Fig. E2) The design of fluorescence illuminators is based on 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 ability of the fluorochrome to absorb the excitation light is known as the extinction coefficient. The greater the extinction coefficient, the likelier the absorption of light (a prerequisite to ensuing fluorescence emission).

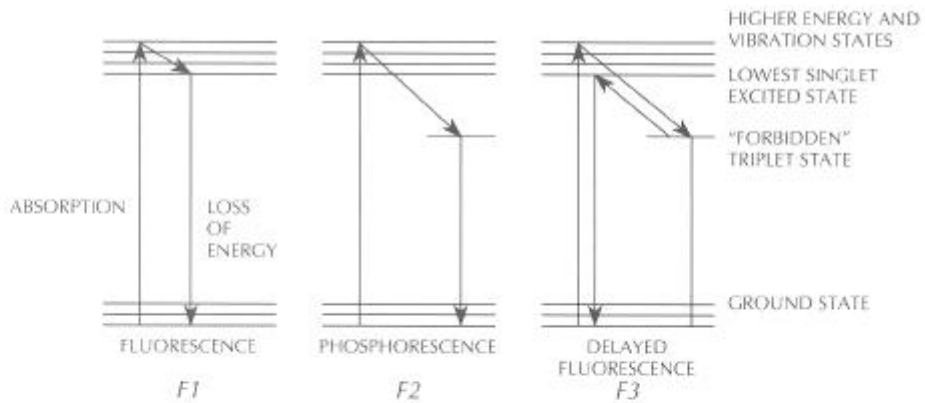
The yield is referred to as the quantum yield, the ratio of the number of quanta ("packets" of energy) emitted compared to the number of quanta absorbed (usually the yield is between 0.1 and 0.7). Quantum yields below 1 are the result of the loss of energy through non-radiative pathways (e.g. heat or photochemical reaction) rather than the re-radiative pathway of fluorescence.

Extinction coefficient, quantum yield, mean luminous intensity of the light source, and fluorescence lifetime are all important factors contributing to the intensity and utility of fluorescence emission.

MOLECULAR EXPLANATION

Fluorescence activity is sometimes depicted diagrammatically as shown in Fig. F (a so-called Jablonski-type diagram). Prior to excitation, the electron configuration of the molecule is described as being in the ground state. Upon absorbing the excitation light, usually of short wavelengths, electrons may be raised to a higher energy and vibrational excited state; this may take a trillionth of a second (10^{-12} sec.). In fluorescence, in an interval of approximately a billionth of a second (10^{-9} sec.), the excited electrons may lose some vibrational energy and return to the so-called lowest excited singlet state. From the lowest excited singlet state, the electrons "drop back" to the ground state with simultaneous emission of fluorescent light. (Fig. F1) The emitted light is always of longer wavelengths than the excitation light (Stokes' Law). If the exciting radiation is halted, the fluorescence ceases.

Fig. F 1,2,3 Molecular Electron Action Jablonski-Type Diagram



If the excited electrons, instead of "dropping back" to the lowest singlet state, "drop" into the so-called "forbidden" triplet state and then to the ground state, the emission of radiation may be considerably delayed—up to several seconds or more. This phenomenon is characteristic of phosphorescence. (Fig. F2) In some instances, the excited electrons may go from the "forbidden" triplet state to the lowest excited singlet state and then return to the ground state, emitting fluorescent light. This action takes a little longer (about 10^{-6} seconds) than the usual fluorescence and is called delayed fluorescence. (Fig. F3)

Under some circumstances, e.g. photobleaching or the presence of salts of heavy metals, etc., emitted light may be significantly reduced or halted altogether.

FADING

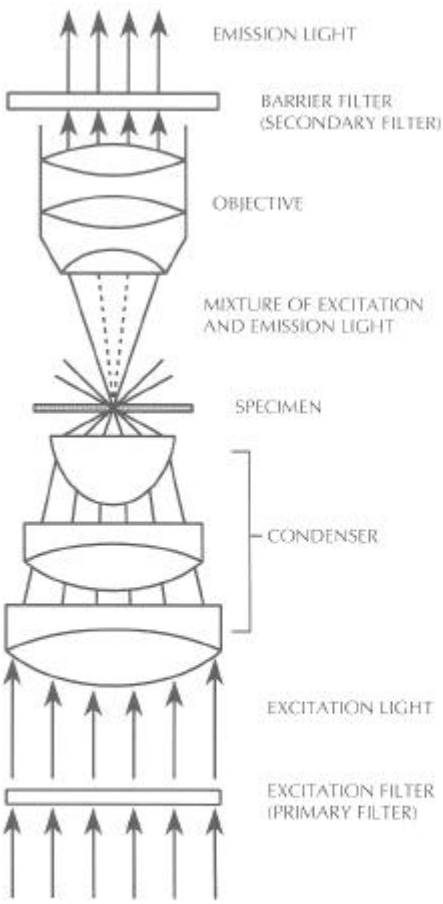
There are conditions which may affect the re-radiation of light and thus reduce the intensity of fluorescence. This reduction of emission intensity is generally called fading. Some authors further subdivide fading into quenching and bleaching. Bleaching is irreversible decomposition of the fluorescent molecules because of light intensity in the presence of molecular oxygen. Quenching also results in reduced fluorescence intensity and frequently comes about as a result of oxidizing agents or the presence of salts of heavy metals or halogen compounds.

Sometimes the 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 for a newer technique of measuring distances far below the lateral resolution of the light microscope.

The occurrence of bleaching has led to a technique known as FRAP, Fluorescence Recovery After Photobleaching. FRAP is based upon bleaching by short laser bursts and subsequent observation of the recovery of fluorescence caused by the diffusion of fluorophores into the bleached area.

To lessen fading in some specimens, it may be advisable to use a neutral density filter in the light path before the light reaches the excitation filter, thus diminishing the excitation light intensity. In other instances, fading effects may be reduced by changing the pH concentration of the mounting medium or by using anti-bleaching agents. For photomicrography or visual observation, rapid changing of the field of view may also avoid fading effects.

Fig. G
Transmitted Light
Brightfield
Fluorescence
(Diagrammatic)



FLUORESCENCE MICROSCOPES

The early fluorescence microscope utilized transmitted light illumination (diascopic fluorescence). A primary filter to select the excitation light wavelengths was placed in the light port of the microscope and a secondary or barrier filter was positioned above the microscope nosepiece to block residual excitation light and to select the emission wavelengths reaching the eye or camera. (Fig. G)

In brightfield transmitted light fluorescence, it was difficult to separate the excitation light from the fluorescing light because both kinds of light directly entered the objective. Transmitted light brightfield condensers were soon replaced by high numerical aperture oil darkfield condensers.

The oil darkfield substage condenser directed excitation light at steep angles toward the specimen. Because of the darkfield design, most of the excitation light never entered the objective. As the specimen absorbed excitation light and emitted only longer wavelength light, it was the longer wavelength light that gained admittance to the objective and thus passed through the barrier filter to the eye or other detector. The resulting image showed as a more or less brightly fluorescing object on an otherwise dark background. Any scattered excitation light was blocked by the barrier filter. (Fig. H)

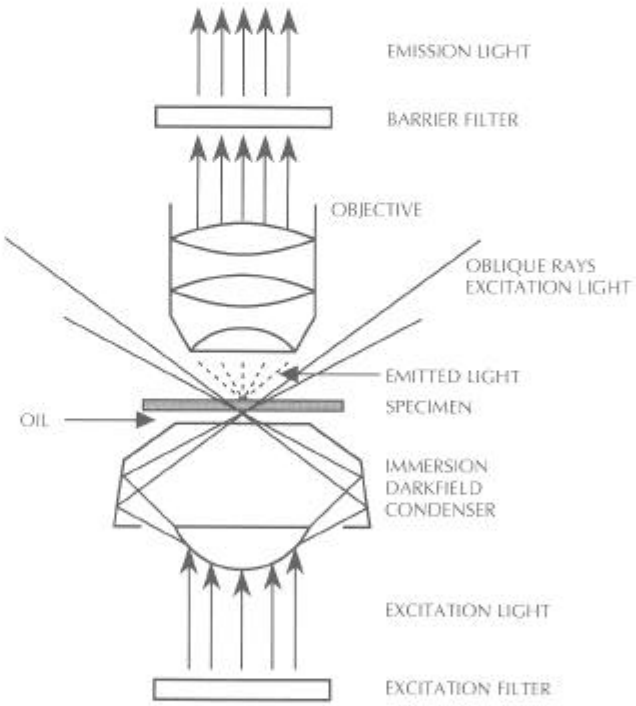


Fig. H
Transmitted Light
Darkfield
Fluorescence
(Diagrammatic)

Although the equipment for transmitted light darkfield fluorescence is relatively simple, the technique has significant disadvantages. Many users find it difficult to properly align the oiled condenser to the optical axis of the microscope. In addition, the numerical aperture of the higher magnification oil or water immersion objectives has to be reduced by a built-in iris diaphragm (with consequent loss of light intensity and resolution) in order to prevent excitation light from entering the objective directly. Transmitted light darkfield technique also precludes the use of simultaneous fluorescence viewing along with phase microscopy or Nomarski differential interference

contrast microscopy. The darkfield method is also very wasteful of light, since the excitation light irradiates much of the specimen outside of the field of view being observed, thus reducing the usability of excitation intensity.

REFLECTED LIGHT FLUORESCENCE ILLUMINATOR

The name of J.S. Ploem is almost synonymous with the use of the vertical illuminator for reflected light fluorescence microscopy. Ploem, Brumberg and others were closely associated with the development of dichromatic beamsplitters (dichroic mirrors) which overcame the light loss problems inherent in the use of ordinary half-mirrors in reflected light microscopy. Reflected light fluorescence microscopy is overwhelmingly the choice of today's fluorescence workers. This mode of fluorescence microscopy is also known as incident light fluorescence, epi-fluorescence, or episcopic fluorescence.

The universal reflected light vertical illuminator is interposed between the observation viewing tubes and the nosepiece carrying the objectives. (Figs. I, K)

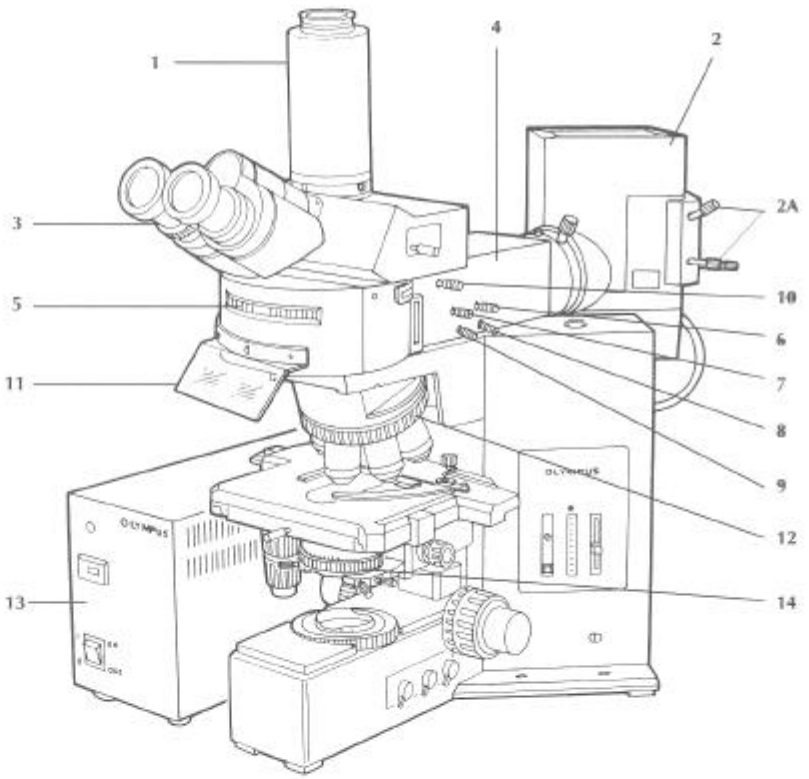
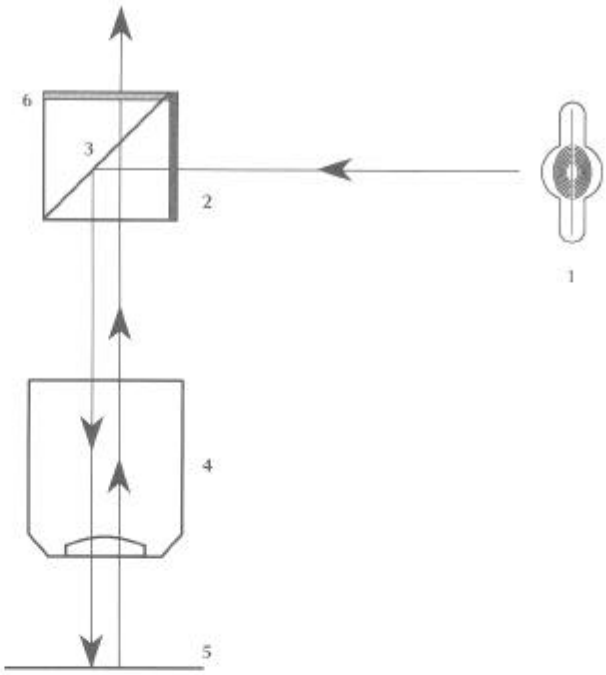


Fig. I
Universal Reflected Light Illuminator mounted on an upright microscope.
1. Trinocular tube
2. Lamphouse
2A. Burner centering knobs
3. Binocular tube
4. Illuminator
5. Cube rotator
6. Aperture diaphragm knob
7. Field diaphragm knob
8. Aperture diaphragm centering knob
9. Field diaphragm centering knob
10. Light shutter
11. UV shield
12. Slot for Nomarski prism (in nosepiece)
13. Power supply
14. Universal condenser

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 light being emitted by the specimen. (Fig. J)

Fig. J
Fluorescence Reflected Light (Diagrammatic)

1. *Mercury Light Source*
2. *Exciter Filter*
3. *Dichroic Mirror*
4. *Objective*
5. *Specimen*
6. *Barrier Filter*



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 relative to each of these functions; most of the unwanted or unused excitation light reaching the specimen travels away from the objective (such "front-face" fluorescence excitation is particularly good with thick specimens); the area being illuminated is restricted to the area being observed; the full numerical aperture of the objective, in Koehler illumination, is utilizable; and, it is possible to combine or alternate reflected light fluorescence with transmitted light phase or Nomarski differential interference or Hoffman Modulation contrast observation. (Figs. K & M)

The universal reflected light illuminator (see Fig. I) has at its far end a universal lamphouse which contains a light source, usually a mercury burner. (Other light sources might be a xenon burner or a halogen bulb.) The light travels along the illuminator parallel to the table top and perpendicular to the optical axis of the microscope. The light passes through collector lenses and a variable, centerable aperture diaphragm and then through a variable, centerable field diaphragm. It is incident upon the excitation filter which selects those excitation wavelengths that are wanted to reach the specimen and blocks the wavelengths not wanted to reach the specimen. The selected wavelengths reach the dichromatic beamsplitting mirror. This mirror is a special type of interference filter which efficiently reflects shorter wavelength light and efficiently passes longer wavelength light. The dichromatic beam splitter (also sometimes called the dichroic mirror) is tilted at a 45 degree angle to the incoming excitation light and reflects the excitation light at a 90 degree angle directly through the objective and onto the specimen. The fluorescent light emitted by the specimen is gathered by the objective, now serving in its usual image forming function. Since the emitted light consists of longer wavelengths, it is able to pass through the dichroic mirror.

Fig. K
Universal Reflected Light Illuminator

1. Illuminator

2. Slot for Polarizer

3. Slot for Analyzer

4. Polarizer

5. Mount for Viewing Tube Head

6. Cube (4 attachable)

7. Cube Attacment Rail

8. ND Filters

9. UV Shade

10. Light Cut Slide

11. Microscope Stand

12. Attachment Site for Lamphouse

13. Cube Rotator Wheel

14. Slot for Upper DIC Prism (in nosepiece)

15. Universal Condenser with DIC prisms

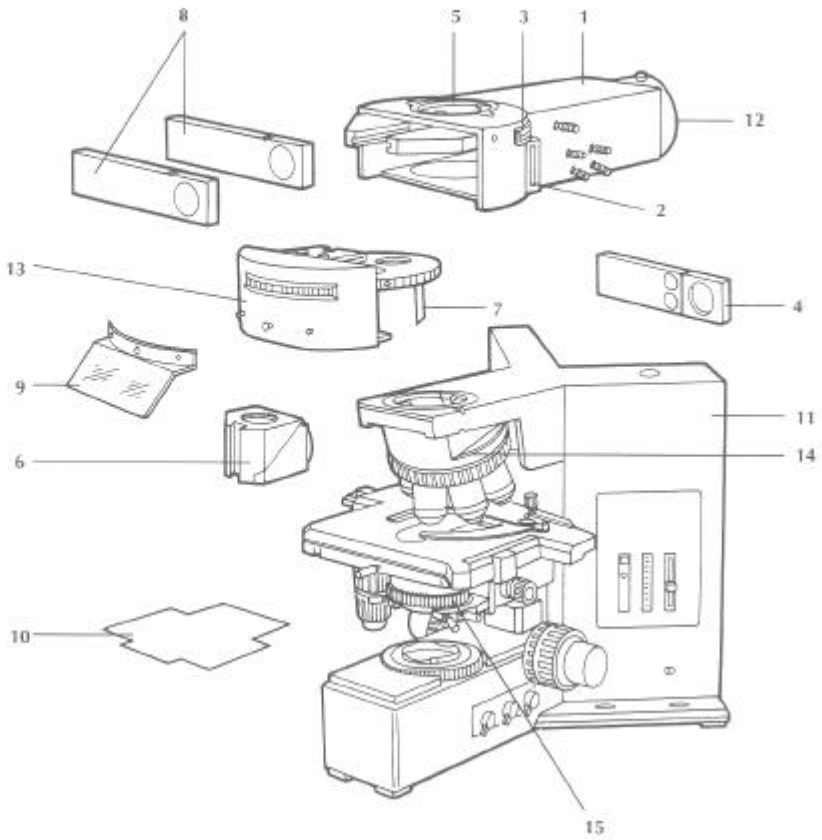
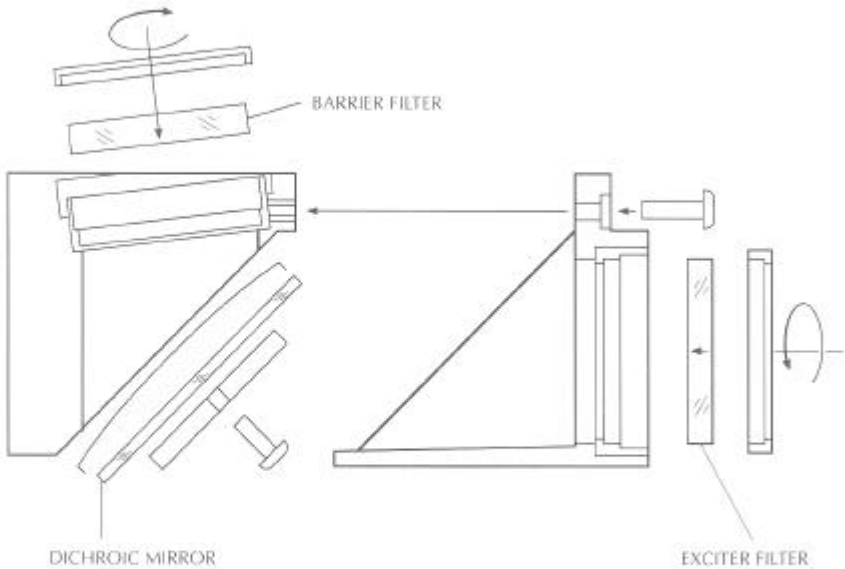


Fig. L
"Exploded" View of Fluorescence Cube (Barrier Filter, Dichroic Mirror, and Exciter Filter are User-Removable)



Any scattered excitation light reaching the dichroic mirror is reflected back toward the light source. Before the emitted light can reach the eyepiece, 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 toward the eyepieces. In most reflected light fluorescence illuminators, the excitation filter, dichroic mirror, and barrier filter are incorporated in a cube. (Figs. L,T)

The more sophisticated systems accommodate three or four fluorescence cubes (on a revolving turret or on a slider) and permit the user to attach replacement custom-made exciters, barrier filters or dichroic mirrors.

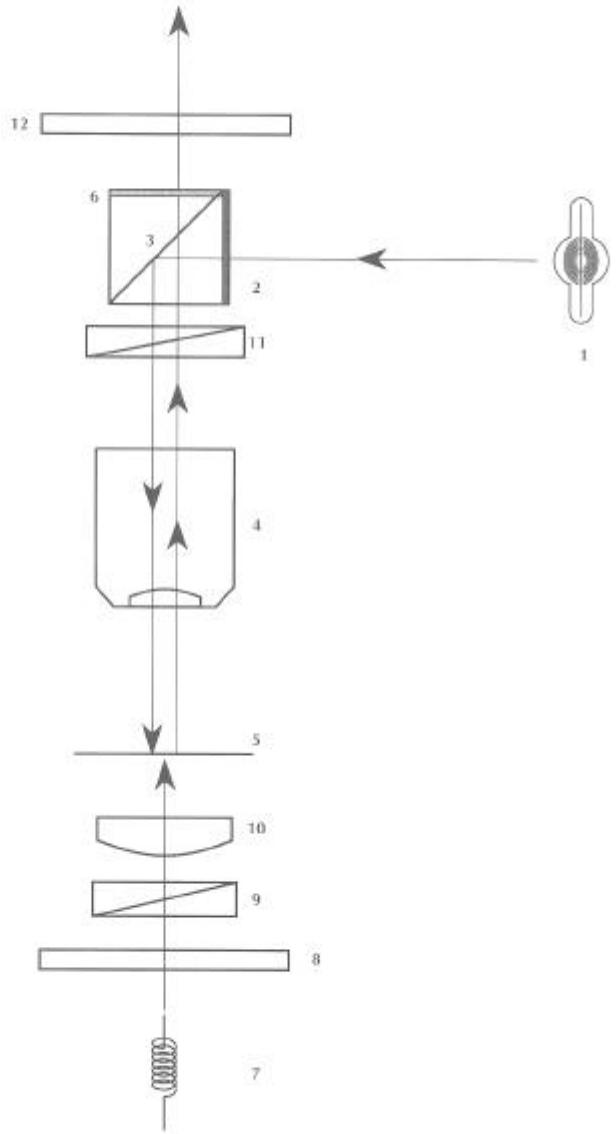


Fig. M
Fluorescence Reflected Light and Nomarski DIC (Diagrammatic)

1. Mercury Light Source
2. Exciter Filter
3. Dichroic Mirror
4. Objective
5. Specimen
6. Barrier Filter
7. Halogen Bulb
8. Polarizer
9. Lower Modified Wollaston Prism
10. Substage Condenser
11. Upper Modified Wollaston Prism (in nosepiece)
12. Analyzer

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 make certain that the image of the centerable aperture diaphragm is conjugate with the back aperture of the focused objective. The image of the pre-focused, centerable field diaphragm is conjugate with the focused specimen and the plane of the fixed eyepiece diaphragm.(Fig. N)

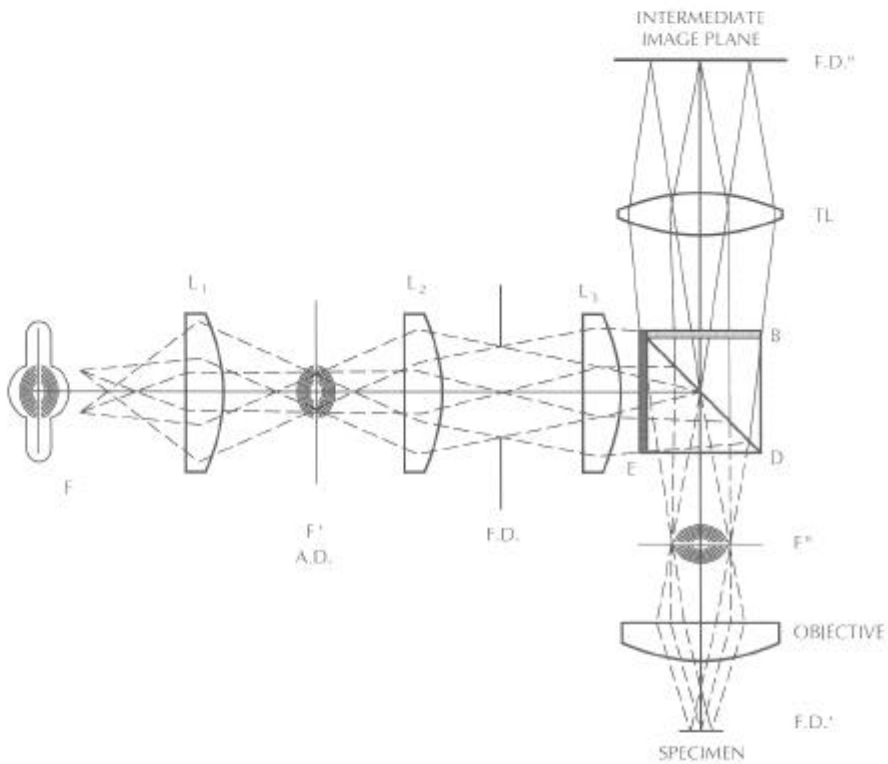


Fig. N
Koehler Illumination for Brightfield Reflected Light Fluorescence (Diagrammatic - for Infinity-Corrected Objective System)

F = Light Source

F' = Image of Light Source at Aperture Diaphragm

F" = Image of Light Source at Back Focal Plane of Objective

A.D. = Aperture Diaphragm

F.D. = Field Diaphragm (Conjugate with Specimen Plane)

L_1,L_2,L_3 = Lenses of Vertical Illuminator

F.D.', F.D." = Conjugate Plane to Field Diaphragm

B = Barrier Filter

D = Dichroic Mirror

E = Exciter Filter

The universal illuminator lamphouse should incorporate an infra-red filter to block the very long, heat generating wavelengths. Some lamphouses have a built-in red suppression filter (e.g. BG38), or a slot for such a filter, to eliminate a reddish background seen in the field of view in some applications. The lamphouse itself should not leak harmful ultra-violet 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 lamp centering 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.) (Fig. P)

An ultra-violet protection shield is fitted into the front of the illuminator to protect the user's eyes from any inadvertent leakage of potentially dangerous short wavelength radiation. In the light path, closer to the lamphouse and before the excitation filter, it is desirable to have a light shutter for complete blocking of excitation light. The light shutter thus permits you to block the burner light without switching the burner off; neutral density filters permit reduced intensity to diminish fading in some specimens.

The universal reflected light illuminator can be attached to the standard modular upright microscope; and a similar version is now used with inverted microscope stands. The inverted stands also permit combining or alternating between reflected light fluorescence and the various contrast techniques of transmitted light microscopy. (Fig. O)

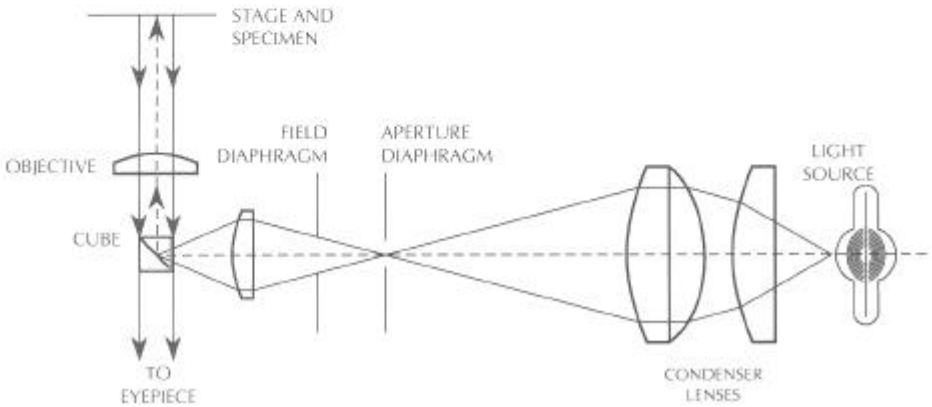


Fig. O
Vertical Illuminator System for Inverted Fluorescence Microscope (Diagrammatic)

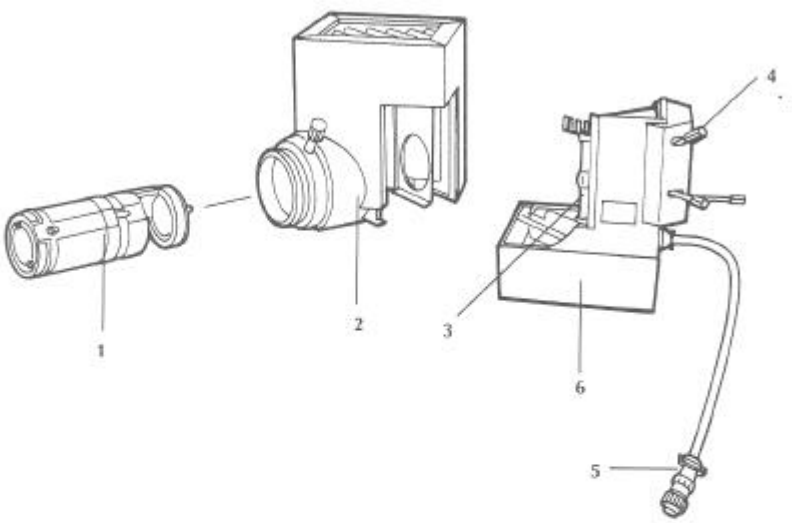


Fig. P
Universal Lamphouse

1. Condenser – Fits into Lamphouse

2. Lamphouse

3. Burner

4. Burner Centering Knob

5. Cord to Power Supply

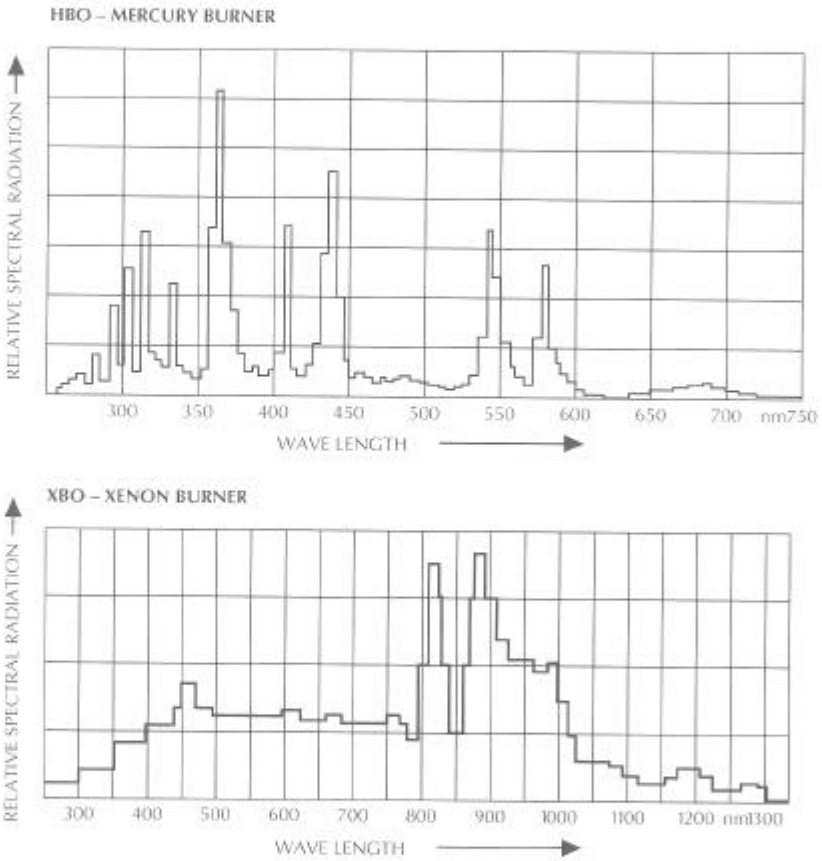
6. Burner Assembly – Fits into Lamphouse

The vertical illuminator, preferably, should have no magnification factor. Some illuminators have a magnification factor of 1.25X and are so inscribed.

FLUORESCENCE- LIGHT SOURCES

In most fluorescence microscopy, the number of photons reaching the eye or other detector, such as a video camera or photomultiplier, is low. The quantum yield of most fluorochromes is low (quantum yield is the ratio of the number of quanta emitted by the specimen as compared to the number of quanta absorbed). To generate enough excitation light intensity to furnish emission capable of detection, powerful light sources are needed, usually arc lamps. The most common lamps are the mercury burners, ranging in wattage from 50 watts to 200 watts and the xenon burners ranging from 75 watts to 150 watts. These light sources are usually powered by a D.C. power supply furnishing enough start-up power to ignite the burner and to keep it burning with a minimum of flicker. The power supply should have a timer to enable you to keep track of 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.

Fig. Q1 Spectral Distribution



The mercury burners do not provide even intensity across the spectrum from ultra-violet to infra-red. Much of the intensity of the mercury burner is expended in the near ultra-violet, with peaks of intensity at 313, 334, 365, 406, 435, 546, and 578 nanometers. At other wavelengths of visible light, the intensity is steady but not nearly so bright, but still usable for blue excitation. (See Fig. Q1 for the emission spectrum of the mercury burner). It also should be understood that mere wattage is not the only consideration for determining brightness. Another important criterion is the size of the arc; the brightness per unit area of the arc encompassed within the back aperture of

the objective is a better measure of the useful brightness of the burner. Using this criterion, you will note that of the three mercury burners listed, the 100 watt burner—other things being equal—is the brightest of the three. (Fig. Q2) Also important in your selection of a burner is whether or not the spectral intensity peaks of the burner's wavelengths match the excitation requirements for the fluorochromes you are using.

Fig. Q2

LAMP TYPE	CURRENT	RATE POWER (watts)	LUMINOUS FLUX (lumens)	LIGHT INTENSITY (Candella)	AVG. BRIGHTNESS (cd/cm_)	ARC SIZE (wxh) mm	LIFE (Hours)
HBO 50W/3	DC	50	1300	150	90000	.20x1.35	200
HBO 100W/2	DC	100	2200	260	170000	.25x .25	200
HBO 200W/2	DC	200	10000	1000	40000	.60x2.20	400
XBO 75W/2	DC	75	950	100	40000	.25x .50	400
XBO 150W/1	DC	150	3000	300	15000	.50x2.20	1200

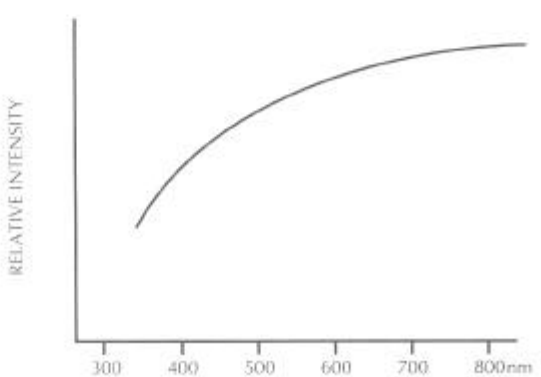
Whenever an illumination system is being evaluated, it is necessary to consider the entire system including collector lenses and the use of aperture and field diaphragms in securing Koehler illumination.

The xenon burners have much more even intensity across the visible spectrum than do the mercury burners; they do not have the very high spectral intensity peaks that are characteristic of mercury burners. (Fig. Q1) Xenon lamps are deficient in the ultraviolet; they expend a large proportion of their intensity in the infra-red, and therefore the use of such lamps requires care in control of heat. Short-gap xenon burners are usually more desirable because the size of the arc is such that its light may be more readily included within the back aperture of the objective, thus avoiding waste of light intensity.

Neither mercury nor xenon lamps should be handled with bare fingers in order to avoid inadvertent etching of the quartz envelope. Mercury and xenon burners require caution during operation because of the danger of explosion. Xenon burners need careful handling, even when cold, because of internal gas pressure.

Sometimes, tungsten-halogen bulbs are used, especially for blue or green excitation with brightly emitting specimens. (Fig.Q3) Their output is relatively even across the visible spectrum; they are deficient in the near-ultraviolet and also have a relatively high proportion of intensity in the infra-red. These lamps do not require expensive power supplies for ignition but are powered by low voltage transformers; the bulbs last for thirty to fifty hours when used at their maximum rated voltage.

Fig. Q3
Spectral Diagram
12 Volt 100 Watt
Halogen Lamp



Mercury burners have a life of 200 hours; xenon burners several hundreds of hours. Frequent on-off switching reduces lamp life. When the burners reach their rated lifetime, the spectral emissions may change and the quartz envelope weakens.

In recent years, there has been increasing use of lasers, particularly the argon-ion laser with powerful emission capability at 488 and 514 nanometers. Laser sources, despite the high cost, have become especially useful in laser scanning confocal microscopy. This technique, with its many variations of equipment, has proved to be a powerful tool in rendering very sharp fluorescence images by ingeniously controlling out-of-focus light. This is accomplished through scanning and imaging extremely small, shallow areas successively. The optical sections of the specimen are stored in a a computer and reconstituted into the whole image which can then be displayed on a video monitor or printed with a video printer.

CENTERING SCREEN FOR LAMP ADJUSTMENT

Microscope companies may offer an optional centering screen to facilitate the centering of the image of the lamp arc to the back aperture of the objective. This accessory has, at its upper end, the standard R.M.S. thread and can be screwed into the nosepiece. It is placed there and rotated into the light path. The lower face of the accessory has a frosted, orange-colored glass with an inscribed crosshair. Light coming down from the dichroic mirror strikes the built-in reflector of the centering screen and is reflected onto the crosshairs. As you manipulate the lamp condenser knob and the centering screws on the lamp socket, you can move that image so that it is centered to the crosshairs. (Figs. R1 & R2) The size of the image of the arc can be made bigger or smaller by manipulating the lamp condenser lever. When you are done with the centering accessory, it can be replaced by a regular objective.

Figs. R1,2,3

If the lamphouse contains a mirror, the mirror position should be adjusted so that the arc image in the mirror is positioned parallel and adjacent to the arc image itself (Fig. R3).

If you do not have a centering screen, the following alternative procedure can be used. Focus the specimen with the 10X objective. Then rotate the nosepiece so that an empty opening on the nosepiece is in the optical path of the microscope. Place a white card on the stage (in place of the specimen) and you will see the image of the arc projected onto the card. By manipulating the lamp condenser knob and the burner centering screws on the lamphouse, you can center the image of the arc to the optical axis of the microscope.

FILTERS

The terminology applied to fluorescence filters has become a jumble as a result of various initials utilized by different manufacturers. Let's try to make some order of this confusing terminology. Basically there are three categories of filters to be sorted out: exciter filters, barrier filters and dichromatic beam splitters. Proper selection of filters is the key to successful fluorescence microscopy.

Exciter filters are filters which permit only selected wavelengths to pass through on the way toward the specimen. Barrier filters are filters which are designed to suppress or block (absorb) the excitation wavelengths and permit only selected emission wavelengths to pass toward the eye or other detector. Dichromatic beam splitters (dichroic mirrors) are specialized filters which are designed to efficiently reflect excitation wavelengths and pass emission wavelengths. Dichromatic beamsplitters are used in reflected light fluorescence illuminators and are positioned in the light path after the exciter filter but before the barrier filter. They are oriented at a 45 degree angle to the light passing through the excitation filter and at a 45 degree angle to the barrier filter. Filter curves show the percentage of transmission (or the log. of percentage) as the vertical axis and the wavelength as the horizontal axis.

Filters formerly were almost exclusively made of colored glass or colored gelatin sandwiched between glass. As a result of more sophisticated filter technology, there have been developed interference filters which consist of dielectric coatings (of varied refractive indices and reflectivity) on glass. These filters are designed to pass or reject wavelengths of light with great selectivity and high transmission. Most of today's exciter filters are the interference type; some barrier filters are also, for special needs, the interference type. Dichromatic beam splitters are specialized interference filters. Sometimes short pass filters (SP) and long pass (LP) filters are combined to narrow the band of wavelengths passing through such a combination.(Fig. S6)

EXCITER FILTERS: (Figs. S1,S4,S5,S6)

UG (ultra-violet glass) and BG (blue glass) are glass exciter filters.

KP (k is abbreviation for kurz, short in German) and SP are short pass filters; or ex for exciter filter.

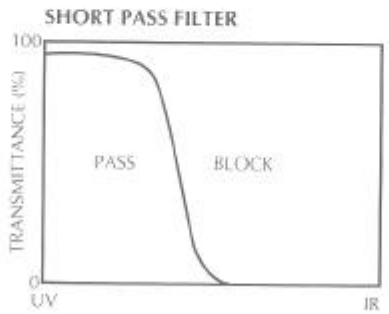
Nowadays, most exciter filters are of the interference type. The transmission curve of a KP or SP filter shows a steep drop at the righthand side of the curve. If the exciter filter is labeled with the letter B or BP, it is a band pass filter. A BP filter is a filter with wavelength cut-off to the left of its curve and to the right of its curve (see diagram). Numbers may refer to the wavelength of maximum transmission for band pass exciter filters. For SP or KP filters, the number may refer to the wavelength at 50% of the maximum transmission. For band pass filters sometimes the bandwidth, in nanometers, at the 50% level of maximum transmission is stated. Band pass filters are designed to pass only a desired band of the spectrum; many interference band pass filters pass a narrow band of the spectrum. Some manufacturers label their interference filters with the designation IF. Narrow interference band pass filters are especially helpful if the Stokes' shift is small.

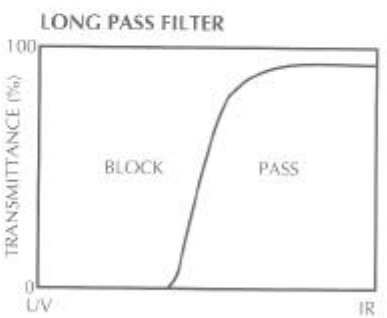
BARRIER FILTERS: (Figs. S2,S4,S5,S6)

LP or L (long pass), GG (yellow or gelb glass) or G (green glass), R or RG (red glass), OG or O (orange glass), Y (yellow glass), K (kante, German for edge), BA (barrier).

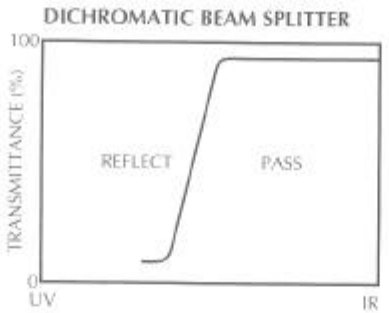
Barrier filters block (suppress) shorter wavelengths and have high transmission for longer wavelengths. When the type also has a number, e.g. BA475, that designation refers to the wavelength (in nanometers) at 50% of its maximum transmission. Curves for barrier filters usually show a sharp edge at the left side, indicating the blocking of wavelengths to the left of that edge. More recently, there have been produced barrier filters of the interference type; also some barriers which are band pass with sharp cut-offs at both the left and right sides of the transmission curve.

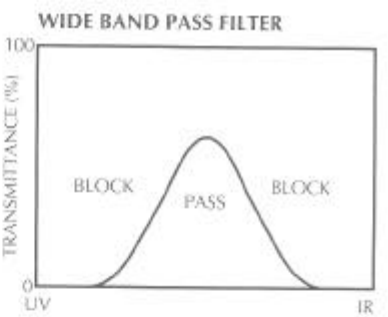
Figs. S1 & S2



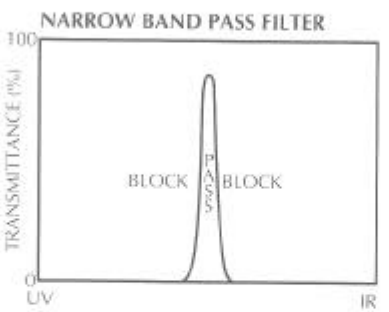


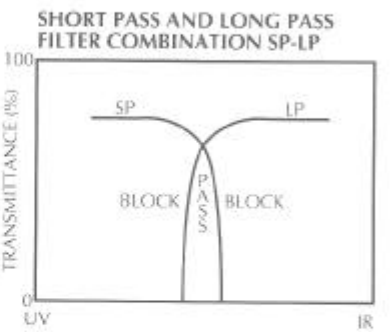
Figs. S3 & S4





Figs. S5 & S6





DICHROMATIC BEAM SPLITTERS: (Fig. S3)

CBS (chromatic beam splitter), DM (dichroic mirror), TK (teiler kante, German for edge splitter), FT (farb teiler, German for color splitter), RKP (reflection short pass)—all of these terms are 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. They are placed at a 45 degree angle to the path of the excitation light coming through the reflected light fluorescence illuminator. Their function is to direct the selected excitation (shorter wavelengths) light through the objective and onto the specimen. Their additional functions are to pass longer wavelength light to the barrier filter, and to reflect any scattered excitation light back in the direction of the lamp-house.

In many of the current reflected light fluorescence illuminators, the exciter filter, the dichroic mirror and the barrier filter are all incorporated into a single cube. (Fig. T) The illuminator, by means of a slider or rotation device may incorporate as many as three or four cubes, thus giving the user the option to conveniently work with fluorochromes of various specifications. Alternative exciters and barriers are easily attachable for optimizing the excitation or emission wavelengths for certain fluorochromes. The standard exciter filters and barrier filters are user-detachable so that custom-made filters can be fitted.

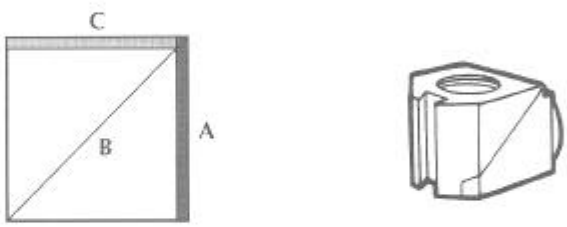


Fig. T Cube Diagrammatic

A. Exciter Filter (Replaceable)

B. Dichroic Mirror (Replaceable)

C. Barrier Filter (Replaceable)

Usually, the lamp housing contains an infra-red or heat filter to protect the fluorescence filters from heat deterioration. Some illuminators may incorporate or accept a red suppression filter (BG38) to eliminate reddening of the field of view background associated with some filter combinations.

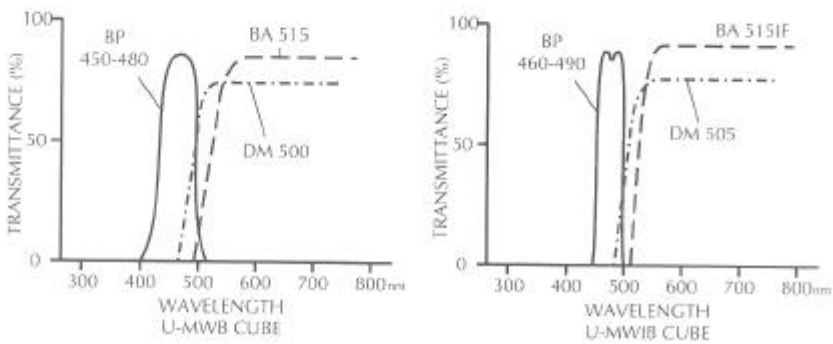
Also, the illuminator may accept a neutral density filter and have an opaque light shutter to reduce or temporarily block the light from reaching the specimen.

It is advisable that you inquire of the manufacturer as to what procedures they use in naming and identifying their particular filters. Samples of such nomenclature use for Olympus are shown in the appendix but you should bear in mind that manufacturers differ in their naming rules. Microscope companies can supply the transmission curves for their exciter and barrier filters and for their dichroic mirrors.

CUBES FOR BLUE EXCITATION

To understand how a cube functions, let's take, as an example, the commonly-used cube for blue excitation. This cube (Olympus designation for the U-URA illuminator) is the U-MWB cube. The U-MWB cube has a band pass 450-480 exciter filter. This designation means that a high percentage of the excitation light falls between 450 and 480 nanometers in wavelength. The dichroic mirror in this cube is the DM500, so named because 500 nanometers is the wavelength at 50% of the maximimum transmission for this mirror. The transmission curve for this mirror shows high transmission above 500 nm., a steep drop in transmission to the left of 500 nanometers, and maximum reflectivity to the left of 500 nanometers but still may have some transmission below 500 nm. The barrier filter in this cube is a BA515. This barrier filter has a steep slope below 515 nanometers and thus passes little light below 515. The BA515 is a long pass filter which transmits a high percentage of wavelengths above 515 all the way up from green into the far red. (Figs. U1,U2)

Figs. U1 & U2



If you wish to narrow the excitation band for blue excitation, you might choose the U-MWIB cube. This cube has an interference excitation filter (very sharp slopes on either side of the excitation band) BP460-490, a dichroic mirror DM505 and a barrier long pass 515IF (interference barrier filter). The sharp slopes of the exciter and barrier filters do a better job in separation of excitation and emission wavelengths with minimum overlap.

If you wished to do blue excitation but wanted to restrict the emitted wavelengths, traversing the barrier filter, to green emission only, you could choose the U-MWIBBP cube. This cube has an identical exciter and dichroic mirror to the U-MWIB cube but, as its barrier filter, it has a band pass BP515-550 (NOT a long pass filter). This barrier filter passes only light in the green wavelengths 515-550 nm. and blocks longer wavelengths above 550 and blocks wavelengths shorter than 515 nm.

There are also other cubes for blue excitation, e.g. U-MNIB, U-MNIBBP (see U-URA cubes listed in the appendix).

If none of the microscope manufacturer's cubes fits your needs, you will have to go to an outside commercial manufacturer (see appendix) for custom-made filters and dichroic mirrors. Most microscope manufacturers now produce cubes which have removable exciter and barrier filters and a removable dichroic mirror.

The function of the cube is to employ the excitation filter to tailor-make the excitation light reaching the fluorochrome; to ensure maximum reflection of the desired excitation light by the dichroic mirror; and finally to employ the barrier filter to pass the desired emission wavelengths yet block unwanted excitation light or specific unwanted emission wavelengths.

IGS CUBE

In addition to the standard fluorescence cubes, manufacturers may offer a cube for immuno-gold staining. This cube, in place of a dichroic mirror, has a standard half-mirror similar to the kind used in metallurgical brightfield reflected light microscopy. In place of an exciter filter, there is positioned a long pass 420 nanometer barrier filter (to block light below 420) and a polarizing filter oriented to pass light vibrating only east-west perpendicularly to the light entering the cube. In place of a barrier filter on the cube, there is another polarizing filter (serving as an analyzer) which allows only light vibrating north-south to the light path to pass to the eye or detector. The analyzer may be placed in not quite crossed position to the polarizer. The immuno-gold (or silver) stain shows up quite clearly as it adheres to specific targets being studied.

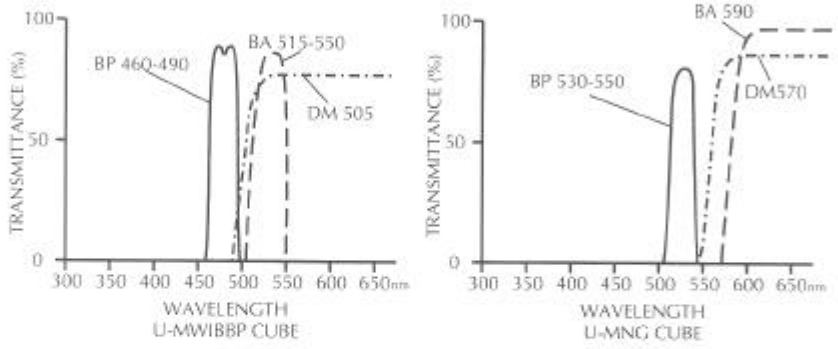
MULTIPLE STAINING

Researchers often run into crossover problems when doing multiple fluorochrome staining. For example, in the common double staining using Fluorescein isothiocyanate (FITC) and a Rhodamine conjugate, it may be that the blue excitation light exciting FITC (green emission) will also cause excitation of the Rhodamine conjugate (red emission). For this combination of stains, you might try the U-MWIBBP cube (see appendix for filter specifications for Olympus U-URA cubes). This cube has a band-pass excitation filter, 460-490, which will excite FITC. The barrier filter for this particular cube is NOT a long pass filter but a band pass 515-550 which will restrict the emission, reaching the eye or other detector, to the green wavelengths and will block red emission from the Rhodamine.

A second cube, the U-MNG, has a band pass excitation filter 530-550 for green excitation of the Rhodamine conjugate. The barrier filter for the U-MNG cube is a long pass BA590 which will permit the red emission of the Rhodamine to reach the eye or other detector (e.g. film or video) and will block any green emission.

By alternately rotating the U-MWIBBP cube and the U-MNG cube into the light path, you should be able to separate the green emission of FITC and the red emission of Rhodamine in a double stained sample. (Figs. V1,V2)

Figs. V1 & V2
Double Staining
FITC/Rhodamine



Similarly, for other combinations of multiple fluorochrome staining, the user must know the excitation-emission spectra for the fluorochromes and the transmission curves for the cubes supplied by the microscope manufacturer.

In some instances, it may be necessary to seek custom-made filters (see appendix for sources) to secure needed excitation wave lengths and/or separation of fluorescence emission wavelengths. Several of the commercial sources now also provide custom-made filters and a dichroic mirror, installed in a SINGLE manufacturer-supplied cube, which are capable of handling double or triple fluorochrome stained specimens without crossover problems (e.g,, DAPI&FITC, DAPI&FITC&TEXAS RED, etc.).

THE MICROSCOPE OBJECTIVE

In view of the low emitted light levels, the role of the objective in fluorescence microscopy is crucial because it is the objective's function to gather light from the specimen. The angle of the cone of light accepted by the objective is determined by the numerical aperture (N.A.) of the objective. (Fig. W) In transmitted light fluorescence microscopy, the intensity of the light reaching the eye or other detector varies directly as the square of the numerical aperture of the objective and the condenser and inversely as the square of the total magnification. On the other hand, in reflected light fluorescence, the intensity of the image varies directly as the fourth power of the numerical aperture of the objective in use, as well as inversely as the square of the total magnification ($I=N.A.^4/Mag.^2$) This difference comes as a result of the objective's initially functioning as a condenser for concentrating light on the specimen, and then as the usual light gatherer for image formation. The implications are clear: high numerical aperture objectives will yield images of much higher intensity than will identical magnification objectives with lower numerical aperture. For example, other things being equal, a 40X objective with an N.A.of 1.0 will yield images more than five times brighter than a 40X objective with a numerical aperture of 0.65. A further implication is that, if possible, the employment of lower magnification visual eyepieces (e.g. 8X magnification) will also increase the brightness of the image as compared to the more commonly used 10X observation eyepieces. Additionally, in reflected light fluorescence, the excitation light is concentrated by the objective (in its function as a condenser) only on the area being observed. As a result the intensity of the excitation light is much higher than in transmitted light darkfield fluorescence where the area being excited does not change as objectives are changed. Also in darkfield, the numerical aperture of the objective must be reduced to below that of the condenser to preserve the darkness of the field of view.

Fig. W
Numerical Aperture and Light Gathering Ability
N.A. = Numerical Aperture
n = Refractive Index of Medium between Front Lens and Specimen
μ = 1/2 the Angle of Cone of Light "Captured" by the Objective

In addition to numerical aperture being an important consideration, it is advisable to have objectives of highest quality chromatic correction since the focusing of the various colors in the same plane will yield sharper images. Further, the better the spherical correction, the sharper the image. Thus, the objectives of choice are plan apochromats or plan-fluorites. These should be designed so that the lens elements and their cements will not themselves autofluoresce when irradiated with light below 400 nanometers in wave length, that is light in the near ultra-violet.

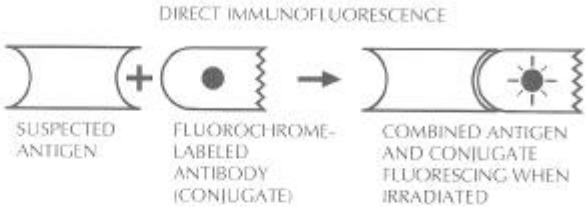
Of course, it is also necessary to employ immersion oil (for oil immersion objectives), slides, and cover slips which do not autofluoresce.

IMMUNOFLUORESCENCE

One of the most important applications of fluorescence microscopy is in the field of immunofluorescence. The living animal manufactures innumerable antibodies which are used, in conjunction with white blood cells, to neutralize any entering foreign bodies (e.g. viruses, bacteria, foreign proteins) which contain or produce antigens. The antigen-antibody reaction is highly specific, often likened diagrammatically to a lock-key relationship. Immunofluorescence owes its success to the "marriage" of the sensitivity of fluorescence microscopy to the specificity of immunology.

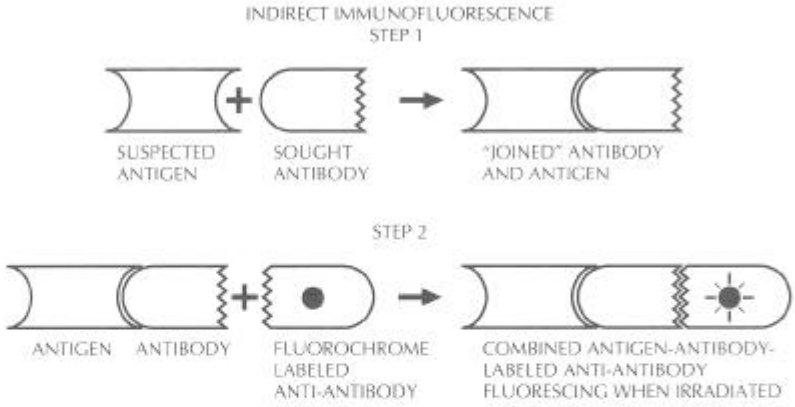
In direct immunofluorescence, a specific antibody is labeled by chemically attaching a fluorochrome. This combination is known as a conjugate. The conjugate is spread on a slide containing the suspected presence of the particular antigen known to stimulate the production of that antibody. If the antigen is present, the labeled antibody conjugate "joins" the antigen and remains on the slide after washing. The presence of the chemically attached fluorescent conjugate and antigen is demonstrated when the fluorochrome is excited at its known excitation peak (Fig. X); and the emission intensities at various wavelengths can then be observed visually or "captured" by still camera or video.

Fig. X
Direct Immuno-fluorescence



Another commonly used immunofluorescence technique is known as indirect immunofluorescence. In this technique, serum possibly containing unlabeled antibody and its related known antigen are incubated together. A fluorochrome conjugated to an anti-human antibody (if the subject being tested is human) is then placed on the slide containing the the unlabeled antibody-antigen. If indeed, there has been an antibody-antigen "joining," the fluorochrome-labeled anti-human antibody attaches itself to this antibody-antigen. Then, the labeled grouping of antigen-antibody-fluorochromed anti-human antibody is excited at the peak wavelength intensity for that fluorochrome and any emission is observed.(Fig. Y)

Fig. Y
Indirect Immuno-
fluoescence



The indirect technique reduces the need to stock large numbers of labeled antibodies; this technique often results in greater fluorescence intensity.

HISTOCHEMICALS

A significant area of fluorescence investigation deals with cytochemical and histochemical staining. Fluorochromes have been used to identify chromosomes, DNA content, proteins, cell structures, hormones, vitamins,etc. The fluorescence microscope is a powerful tool in such studies because of the exquisite sensitivity of selected fluorochromes and their specificity for extremely minute quantities in the sample. Indeed, although the fluorescence microscope is limited in its spatial resolution to the usual rules governed by numerical aperture and diffraction limits, fluorescence probes can, by emitted light, reveal the presence of fluorescing material by making such material visible even in sub-resolution amounts.

IN VIVO, IN VITRO

A burgeoning group of applications for fluorescence involve the use of fluorescent probes (fluorochromes) with living materials, in vivo or in vitro. The difficulties multiply for such probes because of constraints imposed by possible toxicity; and the need for attention to time intervals because of the ever-changing nature of the life processes and movement of intracellular structures. Fluorescence investigations have been applied to changes in ion concentration, bound and unbound, for such chemical components as calcium or magnesium.

Among the best known are studies of intracellular calcium using the probe Fura-2. In this instance the dual excitation dye is excited at approximately 340 nanometers and also at 380 (using a light chopper and dual excitation filters or monochromators) and the single emission is measured for each of these excitations. Attached computers calculate the ratio (the process is called ratio imaging fluorescence) of bound to free intracellular calcium as revealed in the changes in the fluorescence emission intensity. The advantage of this ratio method is that essentially all factors are kept constant except the dual near-ultraviolet excitation wave lengths; each of the two excitation wave lengths causes emission in the green part of the spectrum.

A similar type of ratio imaging is done with Indo-1. For this fluorochrome, also used for determination of intracellular unbound and bound calcium, a single excitation wave length is used, but emission is measured at each of two emission wavelengths to distinguish bound from unbound calcium.

FLUORESCENCE PHOTOMICROGRAPHY

Although fluorescence images may appear to be bright to your eyes because of the eye's exquisite sensitivity to light, you quickly realize that long exposures are often necessary to register an image upon film. Such exposures complicate the photomicrography by increasing the likelihood of vibration and film reciprocity failure effects causing unwanted color shifts in the image.

While the basic problem is often the paucity of light reaching the film, there are steps that can be taken. The use of high numerical aperture objectives, with high transmission for the wavelengths being used, can significantly reduce exposure time because, in reflected light mode, the intensity varies as the 4th power of the numerical aperture. Since intensity also varies inversely as the square of the magnification, reduction of the total magnification on film also results in brighter images. Thus, low magnification photoeyepieces are helpful in photomicrography. When using a trinocular head, it may be desirable to divert all light to the camera rather than have the light split between viewing eyepieces and camera. Since the intensity varies inversely as the square of the distance to the film plane, it is best to keep that distance short; this calls for 35 mm. film format in preference to large film size format.

Another variable you can control is the speed (ASA or ISO) of the film used in the camera. The higher the ASA rating, other things being equal, the less the amount of light needed to register an image on the film. Kodak daylight Ektachrome color, ASA 400 or 200, or Kodachrome 200, 35 mm. size, are positive transparency films often suggested because of speed, good color rendition and good resolution. Other manufacturers make comparable type film. For black/white 35 mm. film, you might try Kodak T-Max 400 or Ilford XP2 in 35 mm. film size. Kodak Ektar 1000 ASA color negative film has also been used successfully, if the local 60-minute processor is willing to cancel the usual automatic color filters of the printing equipment.

Several valuable aids for fluorescence photomicrography are available from some manufacturers. One is the focusing telescope with illuminated framing reticle (attaches as the eyepiece camera's focusing telescope) which allows you to easily see the film frame dimensions in a very dark field. For more common fluorochrome emission intensity, a camera with a silicon blue detector cell (for determining exposure duration) will suffice; for extremely faint specimens a photomultiplier metering detector may be necessary. Since fluorescing specimens usually show as bright objects on an otherwise dark background, a camera with spot metering capability is desirable. If you do not have a spot meter, you may have to cut exposure down to a half or a fourth of the metered reading (for scattered fluorescing objects on a dark background) as you would do for darkfield illumination.

It is advisable to do the setting up and initial observation with one field within the specimen and then to quickly move to a "fresh" field just prior to taking the exposure. This practice may circumvent bleaching or fading effects. It is also helpful to do both observation and photomicrography in a partially darkened room environment.

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Appendix A

Troubleshooting Guide

TROUBLE	CAUSE	REMEDY
OPTICAL SYSTEM		
The bulb is on, but image cannot be seen or is dark.	The shutter knob is closed or an ND filter is in use.	Move the shutter to open aperture. Remove ND filter.
	The cube is not rotated into light path correctly.	Rotate the cube into the light path correctly.
	The exciter filter and barrier filter are incorrectly combined.	Follow the filter combinations for the fluorochrome.
	The aperture iris diaphragm, field iris diaphragm or objective's iris diaphragm opening is not completely opened	Completely open the aperture iris diaphragm and objective's iris diaphragm openings, and open the field iris diaphragm opening until the image circumscribes the field of view.
	A cube unsuitable for the specimen is used.	Change to a suitable cube.
Image is unclear, blurred or has insufficient contrast.	Objectives or filters are dirty.	Wipe them clean.
	The exciter filter and barrier filter are incorrectly combined.	Follow the filter combinations for the fluorochrome.
	The aperture iris diaphragm or field iris diaphragm opening is not opened correctly.	Open the aperture iris diaphragm opening completely, and open the field iris diaphragm until its image circumscribes the field of view.
	A cube unsuitable for the specimen is used.	Change to a suitable cube.
Image is partially obscured or unevenly illuminated.	The objectives are not inserted into the light path correctly.	Rotate the revolving nosepiece until it clicks.
	The cube is not rotated into light path correctly.	Rotate the cube into light path correctly.
	The field iris diaphragm opening is closed excessively.	Open the field iris diaphragm opening as required.
	The shutter slider is not pushed in far enough. The mercury burner is not centered correctly, or focus adjustment has not been completed.	Push the shutter slider all the way in. Center the mercury burner or adjust the focus.
Excessive glaring.	Either exciter filter or barrier filter has not been inserted.	Insert required filter.
ELECTRICAL SYSTEM		
Power switch indicator does not light up.	The power cord is connected incorrectly	Connect correctly.
Power switch indicator lights, but mercury burner does not.	Connectors are connected incorrectly.The burner has not been installed.	Connect correctly. Install the burner.
	The lamp housing interlock is operating.	Tighten the bulb socket locking screw securely.
	Auto ignition is not operating as required.	Turn off the power of the power supply unit. Switch on again. (Repeat as necessary.)
The bulb flickers or is dark.	Insufficient time has elapsed since the burner was turned on.	Wait for 10 minutes after turning on the burner.
	The bulb life has expired.	Replace the mercury burner if the life meter reads over 200 hours.

Appendix B

Examples of Cubes Offered for U-URA

(Olympus America Inc.)

EXCITATION*	CUBE	EXCITATION FILTER**	DICHROIC MIRROR**	BARRIER FILTER**
U	U-MWU	330-385	400	420—
U	U-MNU	360-370	400	420—
U	U-MNUBP	360-370	400	420-460
V	U-MNV	400-410	455	455—
BV	U-MWBV	400-440	455	475—
BV	U-MNBV	420-440	455	475—
B	U-MWB	450-480	500	515—
IB	U-MWIB	460-490	505	515IF—
IB	U-MNIB	470-490	505	515IF—
IB	U-MWIBBP	460-490	505	515-550
IB	U-MNIBBP	470-490	505	515-550
G	U-MSWG	480-550	570	590—
G	U-MWG	510-550	570	590—
G	U-MNG	530-550	570	590—
IG	U-MNIG	520-550	565	580IF—
IY	U-MWIY	545-580	600	610IF—

* Excitation: U=ultra-violet; V=violet; BV=blue violet; B=blue; IB=interference blue; G=green; IG=interference green; IY=interference yellow. SW=ultra-wide; W=wide; N=narrow

** Expressed in nanometers (see section on filters). Where a pair of numbers appears, the band width is indicated. Where a — appears, the filter is a long pass filter.

Appendix B

Dichroic Mirror/Filter Combinations (Olympus for BH-2 and Vanox-3)

CODE	CUBE	DICHROIC MIRROR	EXCITER FILTER	BARRIER FILTER	SUPPLEMENTARY EXCITER FILTER	ADDITIONAL BARRIER FILTER
U	-DMU	DM400	UG1	L420		L435 Y455
V	-DMV	DM455	BP405	Y455		Y475 Y495 515
BV	-DMBV	DM455	BP440	AFC +Y475	EL420	Y495 515
B	-DMB	DM500	BP490	AFC +O515	EY455	B460 530 570 590 G520
IB	-DMIB	DM505	BP495	0515IF	EY475	B460 530 570 590
G	-DMG	DM570	BP545	590	E0515 E0530	R610
IGS	-HMIGS	(Half-mirror)	L420 + PO (Polarizer)	AN + DP		
BF	-BF	(Optical axis adjuster)	(Light beam shutter)	(Image position adjuster)	—	—

For "U" (Ultra Violet)
For "V" (Violet)
For "BV" (Blue-Violet)
For "B" (Blue)
For "IB" (Interference Blue Filter)
For "G" (Green)
For "IGS" (Immuno Gold Staining Method)
For brightfield or phase contrast, and for Nomarski DIC only -BF

Supplementary filters do not come with cubes.

Appendix B

Cross Reference Olympus U-URA Cubes to Olympus BH-2, Vanox-3, IMT-2 Cubes

EXCITATION	CUBE	EXCITATION FILTER	DICHROIC MIRROR	BARRIER FILTER
U	U-MU	BP330-385 (UG-1)	DM400	BA420 (L420)
V	U-MNV	BP400-410 (BP405)	DM455	BA455 (Y455)
BV	U-MWBV	BP400-440 (BP440)	DM455	BA475 (Y475&AFC)
BV	U-MNBV	BP420-440 (BP440&EL420)	DM455	BA475 (Y475&AFC)
B	U-MWB	BP450-480 (BP490&EY455)	DM500	BA515 (O515&AFC)
IB	U-MWIB	BP460-490 (BP495)	DM505	BA515IF (O515IF)
IB	U-MNIB	BP470-490 (BP495&EY455)	DM505	BA515IF (O515IF)
G	U-MSWG	BP480-550 (BP545)	DM570	BA590 (O590)
G	U-MWG	BP510-550 (BP545&EO515)	DM570	BA590 (O590)
G	U-MNG	BP530-550 (BP545&EO530)	DM570	BA590 (O590)

It is advisable to consult the manufacturer's wavelength/transmission curve diagrams for exciter filters, dichroic mirrors and barrier filters to determine how they match the fluorochrome being used.

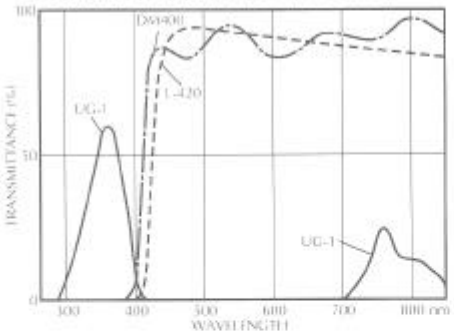
The cube names and nanometer numbers refer to the Olympus U-URA cubes; the figures in parentheses refer to filters still current for BH-2, Vanox 3 and IMT-2 fluorescence cubes.

Use this chart as an aid to guide you in selection of some of the Olympus U-URA cubes if you wish to cross-reference to the extensive listing (appearing in the appendix) of fluorochromes-suggested cubes that was prepared by Olympus Europe for the BH-2 and Vanox 3 fluorescence illuminators.

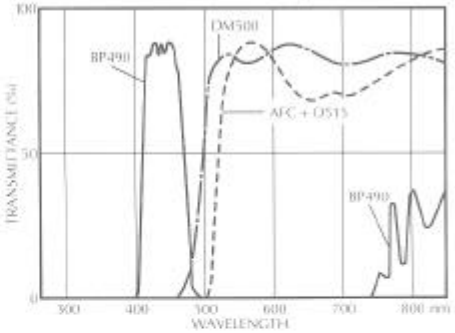
Appendix C

Characteristic Curves of Filters (Olympus BH-2 and Vanox-3)

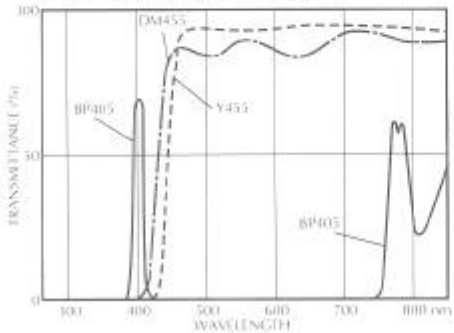
ULTRA-VIOLET EXCITATION (wide band)



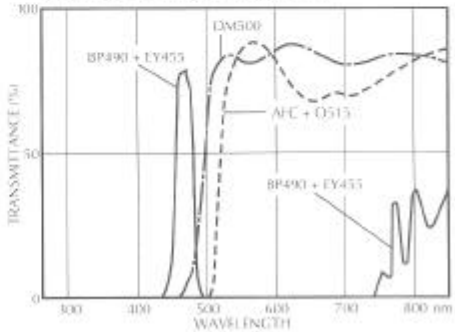
BLUE EXCITATION (wide band)



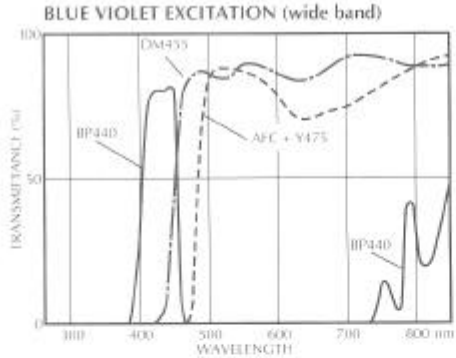
VIOLET EXCITATION (narrow band)



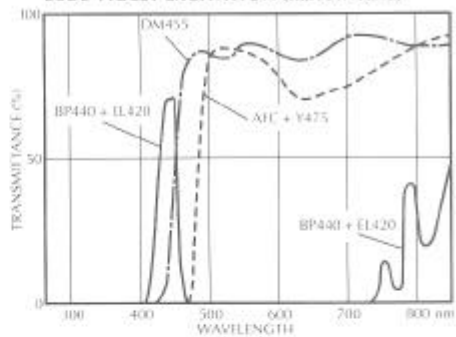
BLUE EXCITATION (narrow band)



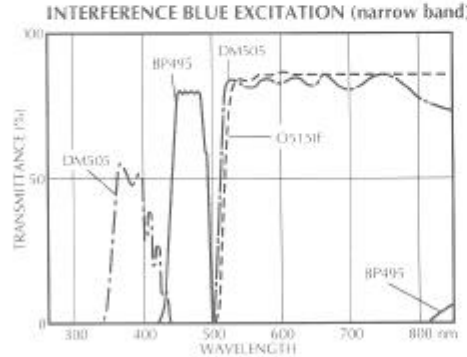
BLUE VIOLET EXCITATION (wide band)



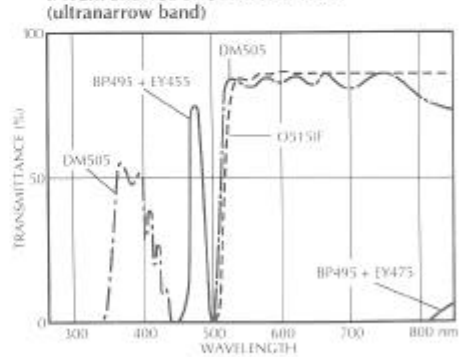
BLUE VIOLET EXCITATION (narrow band)



INTERFERENCE BLUE EXCITATION (narrow band)



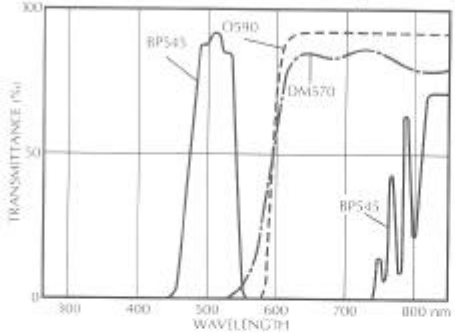
INTERFERENCE BLUE EXCITATION (ultranarrow band)



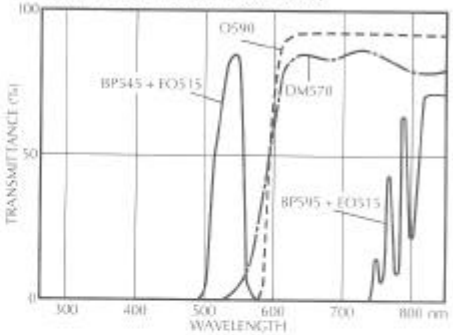
Appendix C

Characteristic Curves of Filters (Olympus BH-2 and Vanox-3)

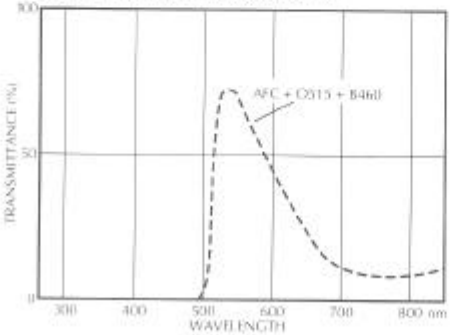
GREEN EXCITATION (ultrawide band)



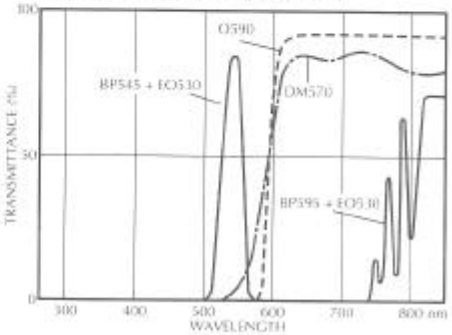
GREEN EXCITATION (wide band)



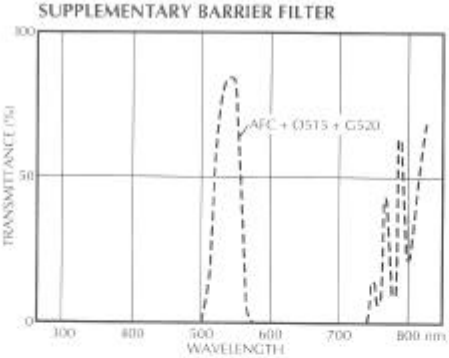
SUPPLEMENTARY BARRIER FILTER



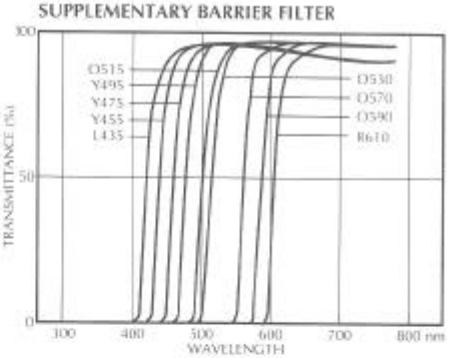
GREEN EXCITATION (narrow band)



SUPPLEMENTARY BARRIER FILTER



SUPPLEMENTARY BARRIER FILTER



EXCITER FILTER

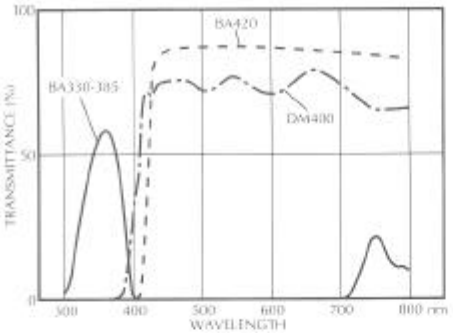
DICHROIC FILTER

BARRIER FILTER

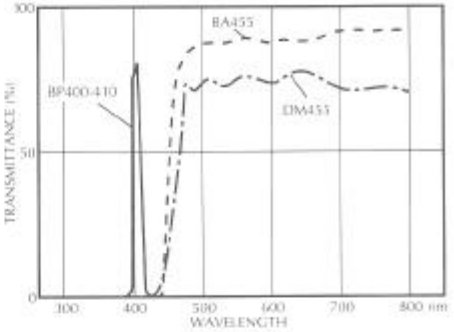
Appendix C

Some Sample Curves for U-URA Cubes (Olympus)

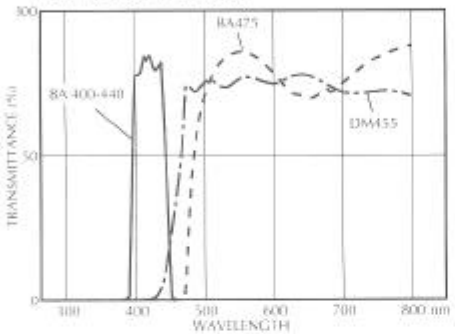
ULTRA-VIOLET U-MWU



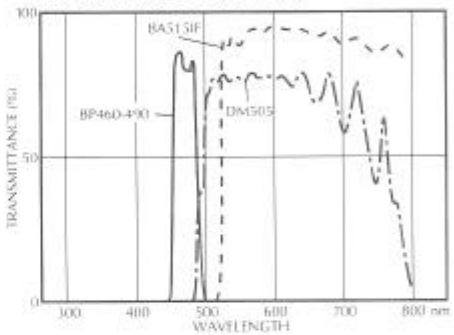
VIOLET U-MNV



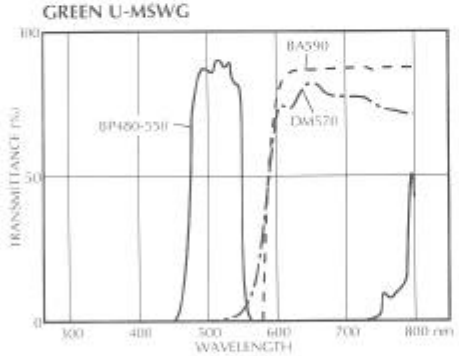
BLUE-VIOLET U-MWBV



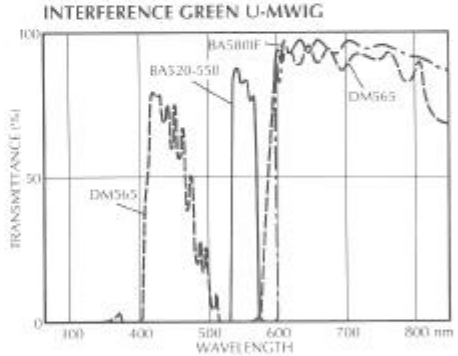
INTERFERENCE BLUE U-MWIB



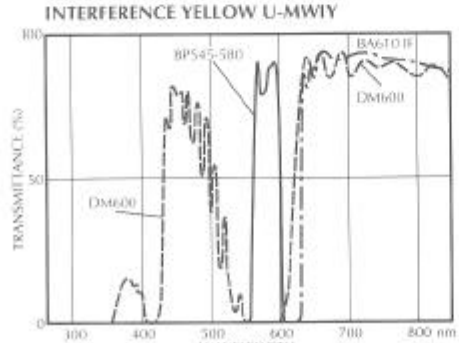
GREEN U-MSWG



INTERFERENCE GREEN U-MWIG



INTERFERENCE YELLOW U-MWIY



Appendix D "Outside Sources" For Filters

Chroma Technology Corp.
RR6, Box 9E
Brattleboro, Vermont 05301
802-257-1800

Omega Optical, Inc.
P.O. Box 573
Brattleboro, Vermont 05301
802-254-2690

Schott Glass Technologies, Inc.
York Avenue
Duryea, Pennsylvania 18642
717-457-7485

Appendix E

Tables of Fluorochromes

You will find in the following listing many of the commonly-used fluorochromes, with their respective peak excitation and emission wavelengths. The author wishes to express particular appreciation to Molecular Probes, Inc. for supplying some of this information; several other sources in the literature were also helpful.

While the author assumes responsibility for the accurate reporting of the data as published in various reliable sources, several caveats must be given. "Fluorochrome dyes are environmentally sensitive and different results will be obtained with different solvents and applications." In reviewing the literature, you will frequently find somewhat different data supplied for the identical fluorochrome. There are also, in many instances, several sub-varieties of a fluorochrome.

The "Handbook of Fluorescent Probes and Research Chemicals," published by Molecular Probes, Inc. in Eugene, Oregon is a rich source of information about fluorochrome dyes. Much information about fluorochromes will also be found in sources cited in the bibliography which you will find in the final pages of this booklet, just preceding the appendices. See particularly the books edited by Taylor et al. and the literature published by microscope companies. Also, consult the publications of Omega Optical and Chroma Technology (listed in the appendix under "outside sources" for filters.)

Included in this section is a copy of a table of fluorochromes compiled by Olympus Europe—with accompanying suggestions for standard Olympus (BH-2 and Vanox-3) cubes and suggested supplementary exciter and/or barrier filters found suitable for each respective fluorochrome. By examining the table of cubes and filters (for Olympus equipment) included in the appendices section, the fluorescence worker should find these data of wide applicability for the fluorescence equipment of other manufacturers.

It is strongly urged that the data for a particular fluorochrome be confirmed by consulting the dye manufacturer to secure full specifications of curves for excitation and emission.

Appendix E

Fluorochromes — Excitation/ Emission

FLUOROCHROME	EXCITATION	EMISSION
Cascade Blue	400	425
Catecholamine	410	470
Chinacrine	450-490	515
Coriphosphine O	460	575
Coumarin-Phalloidin	387	470
CY3.18	554	568
CY5.18	649	666
CY7	710	805
Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid)	340	525
Dansa (Diamino Naphtyl Sulphonic Acid)	340-380	430
Dansyl NH-CH3 in water	340	578
DAPI	350	470
Diamino Phenyl Oxydiazole (DAO)	280	460
Dimethylamino-5-Sulphonic acid	310-370	520
Diphenyl Brilliant Flavine 7GFF	430	520
Dopamine	340	490-520
Eosin	525	545
Erythrosin ITC	530	558
Ethidium Bromide	510	595
Euchrysin	430	540
FIF (Formaldehyde Induced Fluorescence)	405	435
Flazo Orange	375-530	612
Fluorescein Isothiocyanate (FITC)	490	525
Fluo 3	485	503
Fura-2	340-380	512
Genacryl Brilliant Red B	520	590
Genacryl Brilliant Yellow 10GF	430	485
Genacryl Pink 3G	470	583
Genacryl Yellow 5GF	430	475
Gloxalic Acid	405	460
Granular Blue	355	425
Haematoporphyrin	530-560	580
Hoechst 33258 (bound to DNA)	346	460
Indo-1	350	405-482
Intrawhite Cf Liquid	360	430
Leucophor PAF	370	430
Leucophor SF	380	465
Leucophor WS	395	465
Lissamine Rhodamine B200 (RD200)	575	595
Lucifer Yellow CH	425	528

Appendix E

Fluorochromes — Excitation/ Emission

FLUOROCHROME	EXCITATION	EMISSION
Lucifer Yellow VS	430	535
Magdala Red	524	600
Maxilon Brilliant Flavin 10 GFF	450	495
Maxilon Brilliant Flavin 8 GFF	460	495
MPS (Methyl Green Pyronine Stilbene)	364	395
Mithramycin	450	570
NBD Amine	450	530
Nile Red	515-530	525-605
Nitrobenzoxadidole	460-470	510-650
Noradrenaline	340	490-520
Nuclear Fast Red	289-530	580
Nuclear Yellow	365	495
Nylosan Brilliant Flavin E8G	460	510
Pararosaniline (Feulgen)	570	625
Phorwite AR Solution	360	430
Phorwite BKL	370	430
Phorwite Rev	380	430
Phorwite RPA	375	430
Phosphine 3R	465	565
Phycoerythrin R	480-565	578
Pontochrome Blue Black	535-553	605
Primuline	410	550
Procion Yellow	470	600
Propidium Iodide	536	617
Pyronine	410	540
Pyronine B	540-590	560-650
Pyrozal Brilliant Flavin 7GF	365	495
Quinacrine Mustard	423	503
Rhodamine 123	511	534
Rhodamine 5 GLD	470	565
Rhodamine 6G	526	555
Rhodamine B	540	625
Rhodamine B 200	523-557	595
Rhodamine B Extra	550	605
Rhodamine BB	540	580
Rhodamine BG	540	572
Rhodamine WT	530	555
Rose Bengal	540	550-600
Serotonin	365	520-540
Sevron Brilliant Red 2B	520	595

Appendix E

Fluorochromes — Excitation/ Emission

FLUOROCHROME	EXCITATION	EMISSION
Sevron Brilliant Red 4G	500	583
Sevron Brilliant Red B	530	590
Sevron Orange	440	530
Sevron Yellow L	430	490
SITS (Primuline)	395-425	450
SITS (Stilbene Isothiosulphonic acid)	365	460
Stilbene	335	440
Snarf 1	563	639
Sulpho Rhodamine B Can C	520	595
Sulpho Rhodamine G Extra	470	570
Tetracycline	390	560
TRITC (Tetramethyl Rhodamine Isothiocyanate)	557	576
Texas Red	596	615
Thiazine Red R	510	580
Thioflavin S	430	550
Thioflavin TCN	350	460
Thioflavin 5	430	550
Thiolyte	370-385	477-484
Thiozol Orange	453	480
Tinopol CBS	390	430
TOTO 1	514	533
TOTO 3	642	661
True Blue	365	420-430
Ultralite	656	678
Uranine B	420	520
Uvitex SFC	365	435
Xylene Orange	546	580
XRITC	582	601
YO PRO 1	491	509

Appendix E

Fluorochromes — Related Cubes and Filters (Courtesy, Olympus Europe BH-2 and Vanox-3)

FLUOROCHROME	EXCITATION	CUBE	SUGGESTED SUPP. EXC.	SUGGESTED SUPP. BARR.
7-Aminoactinomycin D	G	DMG	EO515 EO530	R610
9-Aminoacridyl Propanol	U	DMU	— Y455	L435
Acid Fuchsin	G	DMG	EO515 EO530	R610
Acridine Orange	B	DMB	EY455	O530 O570 O590
	IB	DMIB	—	O530 O570 O590
Acridine Orange pH 9.0	B	DMB	EY455	O530 O570 O590
Acridine Yellow	B	DMB	EY455	B460 G520
	IB	DMIB		B460 G520
Acriflavine Feulgen	B	DMB	EY455	O530 O570 O590
	IB	DMIB	EY475	 O570 O590
Acriflavine SO2 (Pseudo-Schiff)	IB	DMIB	—	O530 O570 O590
ANS (Mg salt)	B	DMB	EY455	B460 G520
	IB	DMIB	—	B460 G520
Analine Blue, pH 7.2	B	DMB	EY455	O530 O570 O590
	IB	DIMB	—	O530 O570 O590
Auramine	B	DMB	EY455	O530
BAO (Bisamino-phenoloxadiazole)	U	DMU	—	L435 L455
Berberine Sulphate	B	DMB	EY455	O530 O570
	IB	DMIB	—	O530 O570
B-Phycoerythrin	G	DMG	EO515	— EO530
Brillian Sulfaflavine, pH 2.8	U	DMU	—	—
Brillian Sulfaflavine, pH 8.0	U	DMU	—	—
Carboxy Fluorescein Diacetate	B	DMB	EY455	B460 G520
	IB	DMIB	—	B460 G520
Catecholamine	BV	DMBV	—	—

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FLUOROCHROME	EXCITATION	CUBE	SUGGESTED SUPP. EXC.	SUGGESTED SUPP. BARR.
Chlorotetracycline	BV	DMBV	—	O515 O530
	B	DMB	EY455	O530 O570
Chromycin A	BV	DMBV	EL420	—
Cori Phosphine O	B	DMB	EY455	—
	IB	DMIB	—	—
CPM	U	DMU	—	—
	BV	DMBV	—	—
DACM	BV	DMBV	—	—
DANS (Dinaphthyl-amino)	U	DMU	—	—
Dansyl Cadaverine	U	DMU	—	—
Dansyl Chloride	U	DMU	—	—
Dansyl Hydrazine	U	DMU	—	—
DAPI	U	DMU	— Y455	L435
Dibutyrate	B	DMB	EY455	—
	IB	DMIB	—	—
Dicarbocyanine	U	DMU	—	—
Dilute Basic	G	DMG	EO515	—
DIPI	U	DMU	—	—
DPH	U	DMU	—	—
EB (Ethidium Bromide)	G	DMG	EO515	— EO530
Eosin B	G	DMG	EO515	— EO530
Eosin Y	G	DMG	EO515	— EO530
Eriochrome Black	G	DMG	EO515	— EO530
Euchrysine	BV	DMBV	—	O515
	B	DMB	EY455	—
	IB	DMIB	—	—
Evans Blue	G	DMG	EO515	— EO530
EXC	B	DMB	EY455	—
	IB	DMIB	—	—
Fast Blue	G	DMG	EO515	— EO530
Feulgen	B	DMB	EY455 IB DMIB	— —
Filipin	BV	DMBV	EL420	—
FITC (Fluorescein isothiocyanate)	U	DMU	—	—
	B	DMB	EY455	B460 G420
	IB	DMIB	—	B460 G520
Fluora Bora I	B	DMB	EY455	—
Fluora Bora II	U	DMU	—	—
Fluora Bora T	BV	DMBV	—	Y495 O515
	B	DMB	EY455	—
	IB	DMIB	EY475	—

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FLUOROCHROME	EXCITATION	CUBE	SUGGESTED SUPP. EXC.	SUGGESTED SUPP. BARR.
Fluora Bora T Plus	BV	DMBV	—	Y495 O515
	B	DMB	EY455	—
	IB	DMIB	EY475	—
Fluora Bora P	U	DMU	—	—
Fluorescein Diacetate	B	DMB	EY455	—
	IB	DMIB	—	—
Fluorescent Feulgen	IB	DMIB	—	—
Fuchsin Schiff	G	DMG	EO515	— EO530
Geramine G pH 2.8	G	DMG	EO515 EO530	— —
Haemotoxylin (Ridened)	BV	DMBV	EL420	—
Hoechst 33258	U	DMU	—	—
Hoechst 33342, Neut. pH	U	DMU	—	—
Lissamine Rhodamine B	G	DMG	EO515 EO530	—
Lucifer Yellow	BV	DMBV	—	O515
	B	DMB	EY455	
MBD Chloride	B	DMB	EY455	O530 O570
	IB	DMIB	—	O530 O570
Merocyanine 540	G	DMG	EO515 EO530	—
Methyl Green	G	DMG	EO530	—
Mithramycin	BV	DMBV	EL420	—
Monobrombiamane (Thiolyte)	BV	DMBV	—	—
Neutral Red	G	DMG	EO515 EO530	—
Nuclei	U	DMU	—	—
Olivomycin	BV	DMBV	EL420	—
O-Phaldohyde	U	DMU	—	—
Pararosaniline Feulgen	G	DMG	EO515 EO530	R610
Periodic Acid Schiff (P.A.S.)	IB	DMIB	—	—
Phelanthrene Quinone	B	DMB	EY455	—
Phosphine 3R	B	DMB	EY455	—
Phycoerythrin	G	DMG	EO515 EO530	—
Procion Yellow M4RS	B	DMB	EY455	—
	IB	DMIB	—	—
Proflavine-2 HCL	B	DMB	EY455	—
	IB	DMIB	—	—
Primuline	V	DMV	—	Y475 Y495 O515
	BV	DMBV	—	Y495 O515
Propidium Iodide	G	DMG	EO515 EO530	R610
Proteins	G	DMG	EO515	—
Pyronin Y	G	DMG	EO515 EO530	—

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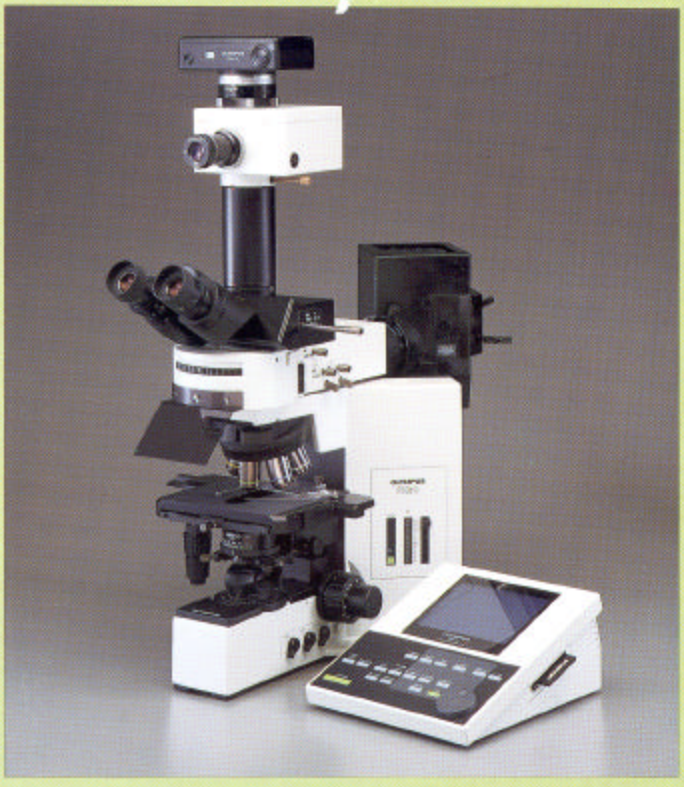
FLUOROCHROME	EXCITATION	CUBE	SUGGESTED SUPP. EXC.	SUGGESTED SUPP. BARR.
Pyronin Y Sitibene	U	DMU	—	—
Quinacrine Mustard	BV	DMBV	EL420	Y495 O515 O530
	B	DMB	—	O530
Quinacrine -2HCL (Atabrin)	BV	DMBV	EL420	Y495 O515 O530
	B	DMB	—	O530
RH-414 Fluorescent Style Dye	G	DMG	EO515 EO530	R610
Rhodamine	G	DMG	EO515 EO530	R610
Rhodamine 123	G	DMG	EO515 EO530	R610
Rhodamine 640	G	DMG	EO515 EO530	R610
Rhodamine B200	G	DMG	EO515 EO530	R610
Rhodanile Blue Sulphate	G	DMG	EO515 EO530	R610
Rivanol pH 9.0	B	DMB	EY455	O530
R-Phycoerythrin	G	DMG	EO515 EO530	—
Serotonin	U	DMU	—	—
SITS	U	DMU	—	—
Sulfaflavine	U	DMU	—	—
TRITC (Tetramethyl Rhodamine Isothiocyanate)	G	DMG	EO515 EO530	—
Tetracycline	BV V	DMBV DMV	—	— Y475
Texas Red Isothiocyanate	G	DMG	EO530	—
Tetramethyl Hexatriene	U	DMU	—	—
Thiazine Red R	G	DMG	EY455	—
Thioflavine S (Alkaline)	B IB	DMB DMIB	EY455 —	— —
True Blue	G	DMG	EO515 EO530	—
Xritc-Labeled Dextrans	G	DMG	EO515 EO530	R610

Price $8.00



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