

Chapter 12

Fluorescence Microscopy

© C. Robert Bagnell, Jr., Ph.D., 2012

August Köhler investigated fluorescence microscopy in 1904. He is quite likely the first person to have done so.

Fluorescence microscopy has become one of the most powerful techniques in biomedical research and clinical pathology. The technique of attaching a fluorochrome to an antibody was developed by Albert Coons in 1941. Coons and N.H. Kaplan developed the fluorescein isothiocyanate (FITC) immunofluorescence technique in 1950. In this technique the fluorescent molecule is covalently bonded to an immunoglobulin that is made against a specific protein. Today, the localization of molecules by immunofluorescence is one of the most powerful techniques in light microscopy. In 1991, B.J. Trask described a method of fluorescently labeling specific sequences of DNA. The method is known as fluorescence *in situ* hybridization (FISH). Today methods exist for directly labeling the DNA probe with a fluorescent molecule. In 1992, D.C. Parsher et al. cloned the gene that codes for the “green fluorescent protein” (GFP). The gene is from a chemiluminescent jellyfish. Using biotechnology methods the gene is fused with a host gene of interest and this chimera is transfected into the host genome. The resulting protein that the cell produces is fluorescent. This work has been extended by Rodger Tsien into a host of different colors and adapted to a vast array of biological techniques.

Applications of Fluorescence Microscopy

The field of fluorescence microscopy has several major divisions.

Autofluorescence

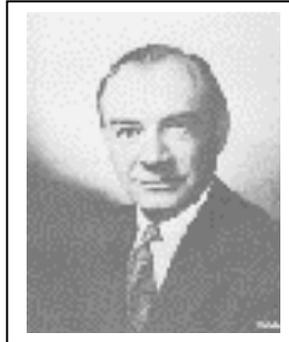
Some specimens naturally fluoresce when illuminated by the proper wavelength of light. This phenomenon is called autofluorescence or primary fluorescence. Table 12.1 is a short list of biological compounds that autofluoresce. Autofluorescence is often a problem in fluorescence microscopy.

Fluorescent Stains

M. Haitinger in 1933 was the first to stain histological specimens with fluorescent dyes. Many common histological stains fluoresce. Table 12.2 is a short list of these. Many fluorescent dyes selectively stain various tissue components. The fluorescence that results from this method of staining is secondary fluorescence.

Immunofluorescence

Albert H. Coons and N. H. Kaplan were the first to attach a fluorescent dye to an antibody, and this antibody subsequently used to localize its respective antigen in a tissue section. The implications of this technique in biology and medicine continue to be astounding. The chromosomal localization of genes with a particular defect is only one example of the power of this technique.



Algert H Coons

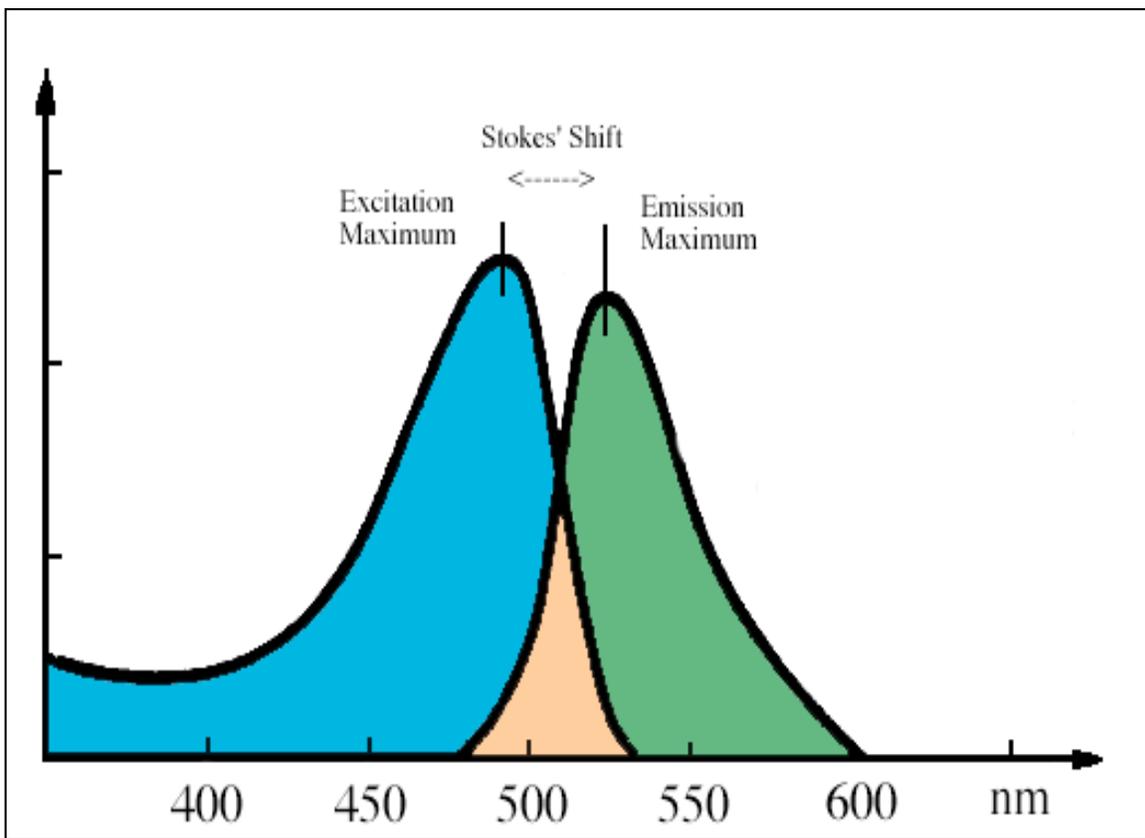


Figure 12.1 Excitation and Emission Spectrum for FITC

Compound	Fluorescing Color	Remarks
<i>Vitamins:</i>		
Ascorbic Acid (C)	Weak yellow	
Carotene (provitamin A)	Intense golden yellow	
Nicotinamide	White	
Nicotinic Acid	Intense bluish white	
Pyridoxine (B4) hydrochloride	Intense violet white	
Riboflavin (B2)	Intense yellow	
Sodium pangamate	Bluish white	
Thiamine (B1) hydrochloride	Light yellow	
Thiamine (B1) mononitrate	White	
<i>Hormones:</i>		
Prolactin	Intense white	
<i>Other Organics:</i>		
Barium platino-cyanide	Intense yellow green	Fluoresces with blue light
Chlorophyll	Intense blood red	Blue light – red
Cinchonine	Cream	
Coumarin	Intense violet	
Diacetylmorphine hydrochloride	Intense bluish white	
Ephedrine	Intense light yellow	
Hippuric Acid	Intense light blue	
Magnesium platinocyanide	Intense orange red with occasional green, yellow and violet colors	Blue light – red.
Morphine	Light buff	
Quinine and salts	Intense blue	
Sodium benzoate	Weak white	
Sodium fluorescein	Intense yellow green	Blue light – green.
Sucrose	Bluish white	
Uranium acetate, nitrate, phosphate	Intense yellow green	Blue light – deep yellow
<i>Inorganic:</i>		
Barrium sulfate	Brownish violet	Barytes
Barrium sulfate		
Zinc sulfide	Lemon yellow	Lithopone
Boric acid	Bluish white	
Cadmium iodide	Light orange	
Lead basic carbonate	Yellow	White lead
Mercerous chloride	Intense oragne	
Mercuric chloride bromide	Intense orange	
Silver nitrate	Intense orange	
Zinc oxide	Intense yellow green	

Table 12.1 - Biological Compounds that Fluoresce - Needham

Dye	Concentration	Fluorescing Color 365 m μ Line
Congo Red 4B	1:1000 aqueous	Orange red
Eosin	1:1000 aqueous	Yellow
*Morin	1:1000 alcohol	Vivid yellow green
*Neutral Red	1:1000 aqueous	Red
*Savarinin	1:1000 aqueous	Red
*Fluid Extract Sanguinaria		Deep yellow
*Differentiate in alcohol		

Table 12.2 - Histological Stains that Fluoresce - Needham

Other Fluorescent Materials

In addition to small organic dyes used to label antibodies and fluorescent proteins such as GFP and its derivatives, quantum dots are now available. Quantum dots have a large absorption spectrum and a very narrow emission spectrum. An excellent review of fluorescent tools for the biologist is: “The Fluorescent Toolbox for Assessing Protein Location and Function” (R Tsien et.al 10.1126/science.1124618).

Review of Fluorescence

Some substances have the property of luminescence. They emit light of one color when exposed to light of a different color. If light emission occurs within one millionth of a second of light exposure, the luminescence is fluorescence. If light emission takes longer than this, the luminescence is phosphorescence. The color of the emitted light has a longer wavelength than the color of the exciting light. For example, fluorescein isothiocyanate (FITC) is excited by blue light and emits green light; rhodamine isothiocyanate is excited by green light and emits red light. This relationship is known as Stokes' law.

Fluorescent substances are excited by a range of wavelengths known as their absorption spectrum. They also emit a range of wavelengths known as their emission spectrum. Figure 12.1 illustrates the absorption and emission spectra for FITC in aqueous solution. For any fluorescent substance the two spectra will show an absorption (excitation) maximum and an emission maximum and some portions of the spectra will usually overlap. The difference between the absorption maximum and emission maximum is the Stokes' shift. For any absorption wavelength there is a certain amount of fluorescence throughout the entire emission spectrum. However, the intensity of fluorescence is at a maximum when the excitation light is at the absorption maximum's wavelength.

Two Types of Fluorescence Microscopes

Diascopic Fluorescence

K. Reichert and O. Heimstadt demonstrated a fluorescence microscope using autofluorescent specimens in 1911. This first type of fluorescence microscopy used transmitted light. This is diascopic fluorescence. Figure 12.2 illustrates the optical set-up. Light from the illumination source first passes through an excitation filter and subsequently to the specimen through a dark field condenser. This eliminates most of the excitation light from the imaging side of the system. Fluorescent light passes through the objective lens and subsequently through a barrier filter (emitter) that further selects only the fluorescent wavelengths. Diascopic fluorescence is rarely used now. It does have some advantages as well as disadvantages.

There are several advantages to diascopic fluorescence. First, the method produces a very good dark field image that improves contrast. Second, any objective lens can be used as long as its NA is less than the condenser's, even for ultra violet (UV)

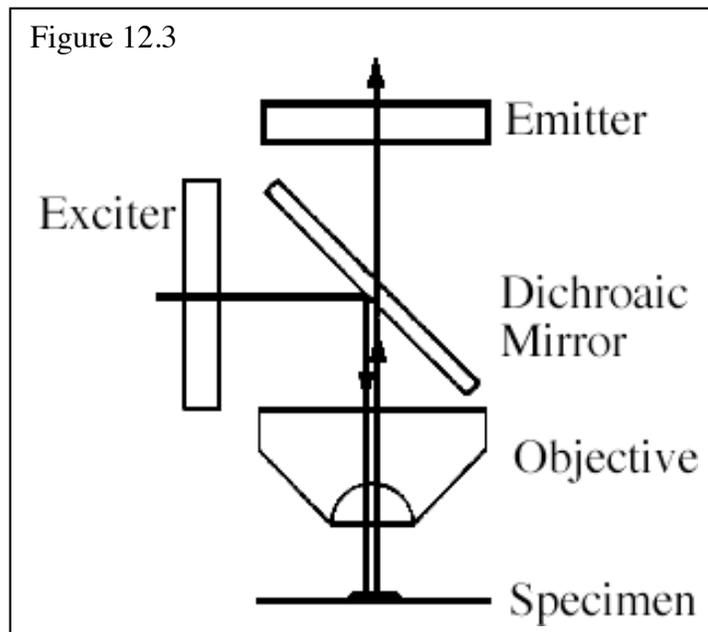
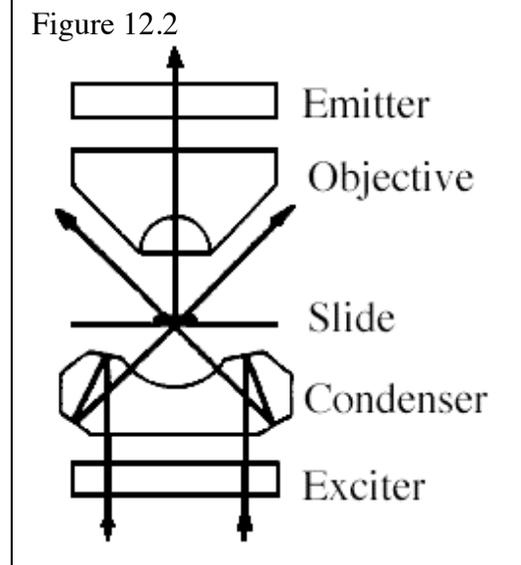
excitation. Of course, in the case of UV, the condenser and specimen slide must be capable of passing UV light. Finally, this method produces very bright images at low magnification.

There are also disadvantages that mostly involve problems associated with any type of dark field microscopy. First, high NA dark field condensers are difficult to align and impossible to do so if the glass slide is too thick. Second, the specimen must be transparent. Finally, the method illuminates a very large area of the specimen. This is troublesome if the fluorescence tends to fade quickly.

Episcopic Fluorescence

In episcopic fluorescence microscopy, the excitation light comes from above the specimen through the objective lens. This is the most common form of fluorescence microscopy today. Figure 12.3 illustrates the optical set-up. This type of fluorescence microscopy became feasible with the invention of the dichroic mirror (chromatic beam-splitter) by E.M. Bromberg in 1953.

Besides the lack of a complex condenser set-up, episcopic fluorescence has several important advantages over the diasopic method. First, high NA objectives are used at their full aperture, therefore the expected resolution is much better, and images are brighter at high magnification. Second, Köhler illumination is achieved by use of a field iris in the episcopic optical system. This limits excitation of the specimen to only that area viewed by the objective lens. Third, the invention of the epi-illumination filter cube by J. S. Ploem in 1970 has made it easy to interchange filter combinations using the episcopic apparatus. Finally, it is easy to combine the fluorescent image with a transmitted light image of the specimen.



Since the objective lens acts as both condenser and objective in episcopic fluorescence, quartz objective lenses are required for deep ultraviolet excitation ≤ 320 nm. However, sufficient UV above 320 nm passes through fluorite lenses to provide adequate fluorescence. New types of glass and new lens coatings are leading to better UV transmission in apochromats.

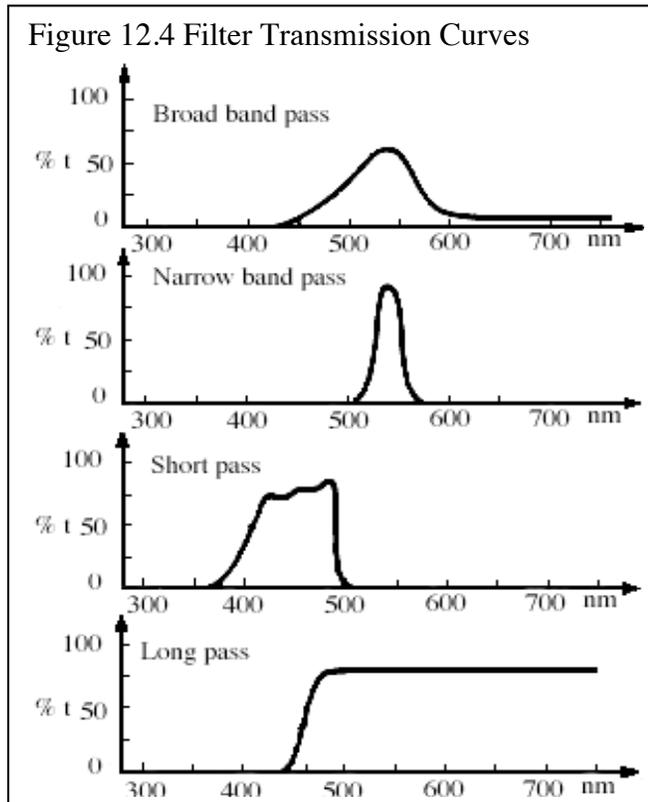
Apparatus of Fluorescence Microscopy

Filters

Optical filters that pass only selected wavelengths of light are necessary in fluorescence microscopy. An excitation filter must select wavelengths of light from a suitable light source that fall in the maximum absorption region of a fluorescent dye. An emission filter must pass the fluorescent wavelengths but not the excitation wavelengths. Several types of filters accomplish this in fluorescence microscopy. Each type is characterized by the wavelengths and intensity of light that it transmits. Figure 12.4 illustrates the basic types of filter transmission curves used in fluorescence microscopy.

Some filters are simply colored glass, but the best type of filter for fluorescence microscopy is the interference filter. These are made by depositing layer upon layer of carefully selected dielectric materials onto a glass surface. These materials have different refractive indices. Constructive and destructive interference of different wavelengths of light caused by the dielectrics result in the filters passing or reflecting a very selective group of wavelengths.

A special type of filter is the dichroic mirror or chromatic beam-splitter. It was first used in fluorescence microscopy by E. M. Bromberg in 1953. This interference filter will reflect light of shorter wavelengths than its cut off frequency while transmitting light of longer wavelengths. Dichroic mirrors have very specific reflection and transmission wavelength characteristics. J. Rygaard and W. Olsen were the first to develop a special interference filter for FITC in 1969. J. S. Ploem used this type of mirror in the construction of his filter cube illustrated in figure 12.5.



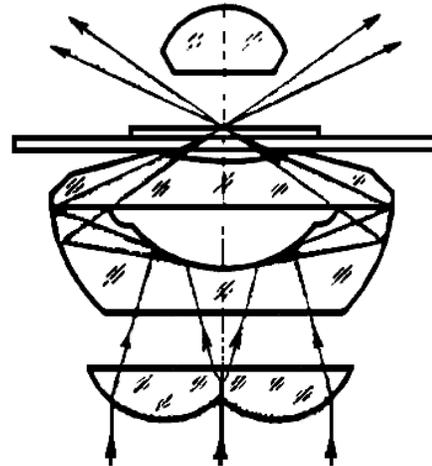
Dark Field Condenser

Diascopic fluorescence microscopy requires the use of a dark field condenser. Dry dark field condensers can have a maximum NA of 0.95 and they must be used with objective lenses of NA less than 0.75. Oil dark field condensers may have an NA as high as 1.4 and can be used with objectives of NA less than 1.1. Standard paraboloid and cardioid dark field condensers work; however, the opaque central disk in them limits the amount of light that reaches the specimen. Special super-wide dark field condensers are available that have no central disk and therefore can deliver more light to the specimen (Fig 12.6).

Illuminators for Fluorescence Microscopy

Light sources for fluorescence microscopy must produce light within the absorption region of the fluorochrome(s) being used and the intensity of the light should be high. Several light sources are available that meet these criteria. The emission spectra for the most common sources are illustrated in figure 12.7. Tungsten halogen lamps can be used for FITC. High-pressure mercury lamps are a common source since they produce radiation in the UV as well as the visible spectrum. Because of the discontinuous nature of the Hg lamp's spectrum, it is not suitable for all fluorochromes. For example, it has no spectral lines between 440 and 540 nm. The high-pressure xenon lamp offers an alternative to the Hg lamp, but it has low emission in the UV. A second alternative to the Hg lamp is the CSI lamp. This is a metal-halide arc lamp.

Figure 12.6 Super-Wide Dark Field Condenser



WARNING: Hg, Xenon, and CSI lamps can explode! They must be allowed to cool off at least 15 minutes before re-igniting them. The power supply must match the type of lamp. A Hg power supply can be destroyed if used with an Xe lamp!

The optical arrangement of the diascopic illuminator is the same as that for any other darkfield illumination. Arrangement for an episcopic system is illustrated in figure 12.8a for an upright stand and in figure 12.8b for an inverted stand.

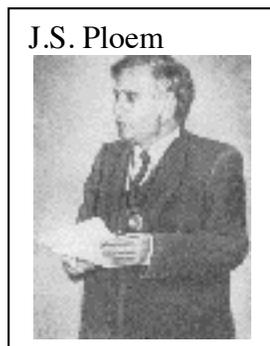
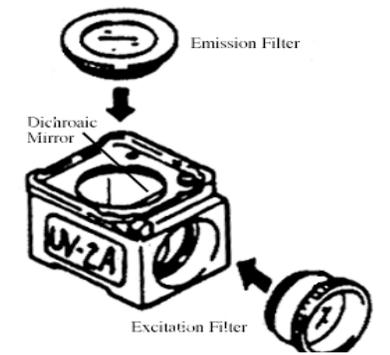


Figure 12.5 Nikon version of Ploem filter cube



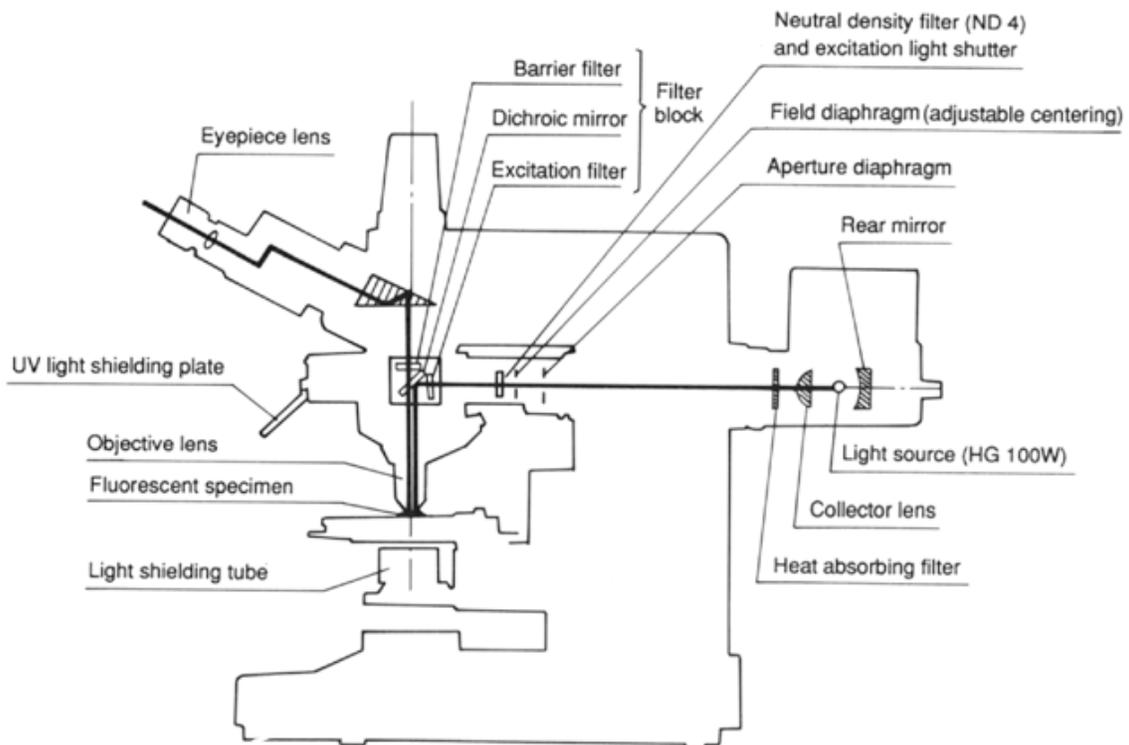
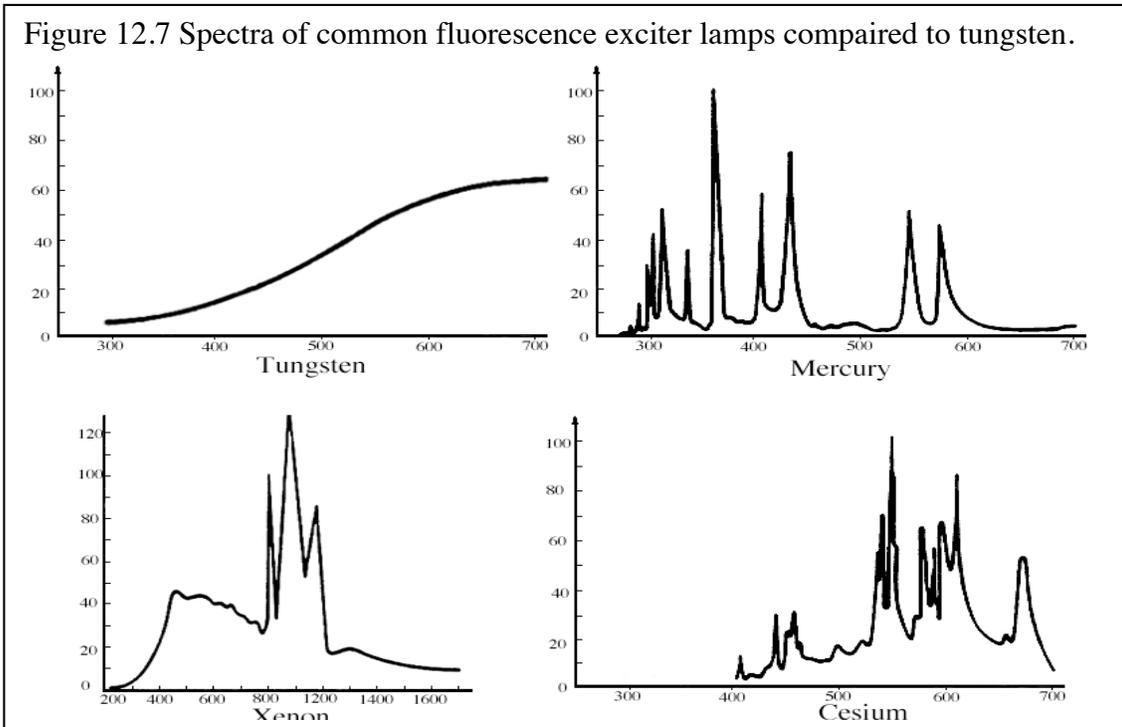


Figure 12.8a. Light path of an upright epifluorescence system.

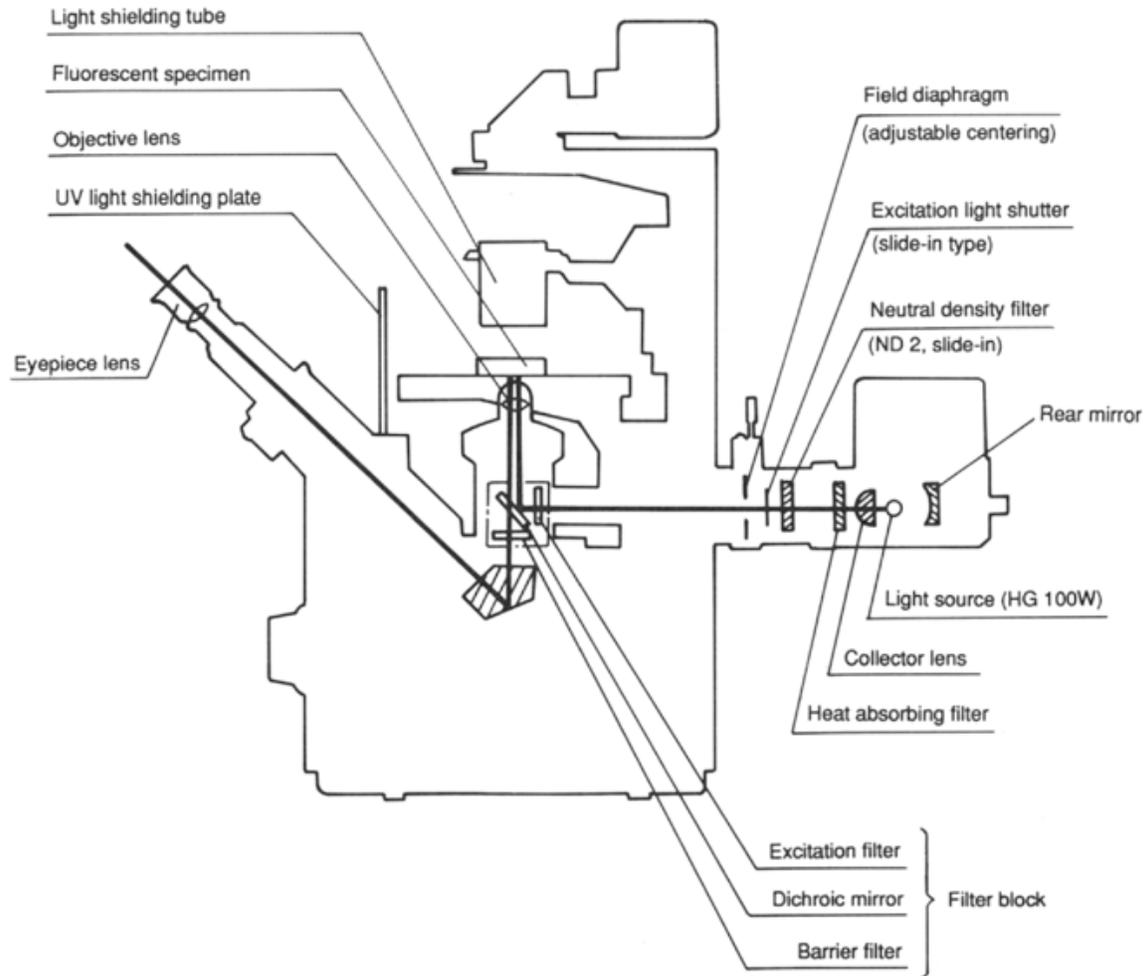


Figure 12.8b. Light path of an inverted epifluorescence system.

Objective Lenses for Fluorescence Microscopy

There are several important considerations in selecting objective lenses for fluorescence microscopy:

First, consider the wavelength of excitation and emission. Use lenses made of quartz for deep UV light at 290 nm or shorter. Glass will not pass light of these wavelengths. Also, use a quartz cover glass over the specimen. Lenses with some fluorite components (semi-apochromats or neofluors) are good for wavelengths in the UV longer than 320 nm (340 - 360 nm). Fluorite passes these wavelengths completely. This minimizes the number of lens components that absorb excitation light compared to apochromats. For visible light excitation, any type of lens will do even achromats since the emission light is usually of only one color.

Second, consider the intensity of the emitted light. Use a high NA objective if the fluorescence is very weak or is coming from very small regions of the specimen. Otherwise, any objective will do.

Finally, consider whether any components of the objective or immersion oil fluoresce at any given excitation wavelength. Background fluorescence will be greatly exaggerated if this is the case.

Microscope Set-Up for Episcopic Fluorescence

Episcopic illuminators are designed for Köhler illumination. The epi-illuminator contains a field iris. An aperture iris may also exist in the system since the objective lens is also acting as the condenser. The aperture iris often helps in reducing background fluorescence. Here are the steps for Köhler alignment of the epi-illuminator using a mercury arc lamp.

1) Centering the arc lamp:

Use **CAUTION** doing this since these lamps produce UV radiation. Never look directly at the light source.

Focus on a specimen with a low power objective lens. Remove the objective lens and replace the specimen with a white paper. Fully open the epi-illuminator's field iris and aperture iris. Place a filter pack in the optical path and open the lamp shutter. Use the collector lens focus knob to focus an image of the lamp's electrodes onto the white paper. If the lamp image is too bright, try closing the field iris, and the aperture iris, or use a different filter pack. One electrode will appear pointed, the other blunt. Use the controls on the side of the lamp housing to place the electrode's image in the center of the circle of illumination. The side controls will move only the real image of the electrode. A second, fainter image of the electrodes should also be visible. This is the reflection from the lamp's concave mirror. It is moved using the mirror controls on the rear of the lamp housing. These controls move both images of the electrodes. It may be necessary to de-center the real image in order to see the reflected image. Use the lamp housing's mirror controls to superimpose the reflected image of the electrodes onto the centered primary image. Some manufacturers recommend that the two images be placed a specified distance from one another. Follow the recommended method for your instrument. Close the lamp shutter.

2) Focusing the collector lens:

Replace the objective lens. Place a fluorescent specimen on the microscope. An ordinary H&E stained specimen will do. Focus the specimen using bright field then switch to fluorescence. Open the epi-illuminator's field and aperture irises. Focus the lamp housing's collector lens to achieve a uniformly illuminated field of view. If the illumination remains uneven, adjust the lamp centering screws while observing the specimen until a uniform illumination is achieved. (On some systems, this requires either very long arms or two people.)

3) Adjusting the epi-field iris:

Focus on the fluorescent specimen. Close the field iris. A sharp image of its edge should enter your field of view. Center the field iris with the centering controls. Open the field iris just out of the field of view.

4) Adjusting the epi-aperture iris:

Stopping down the aperture iris is often helpful in reducing non-specific background fluorescence. This will also reduce the intensity of fluorescence from specifically labeled structures thus the adjustment should be made while observing the fluorescent specimen. It is good practice to check the aperture iris when starting to work to insure that it is fully open.

Tip: On upright stands, lower the transmitted light condenser, or remove it from the stand if it will not be needed. This will prevent light from being reflected off the condenser's surface back into the fluorescent image thus producing a darker background.

Exercises

- 1) Set up your microscope for fluorescence. What type of lamp do you have? Make a list of your filter sets. Does your epi-illuminator have a field iris and an aperture iris? How do you block transmitted light during fluorescence work?
- 2) Check the centering of your arc lamp. Describe the controls you have for doing this on your illuminator.
- 3) Observe a fluorescent specimen on your fluorescence system. Make a list of the colors you see with various excitation / emission filter combinations? Check the color of light coming from the lens as well as what you see in the oculars.
- 4) Examine your photomicrography system for features useful in fluorescence photography. For example, on a digital camera, can you change the gain of the amplifier as well as the exposure time? If you still use film, see if your camera has a way to underexpose the film, see if it has a spot meter, and reciprocity failure compensation.
- 5) Check out the current resources for filters and fluorescent probes. A short list of companies is included in the Resources section at the end of these notes.