

# Fluorescence microscopy

The absorption and subsequent re-radiation of light by organic and inorganic specimens is typically the result of well-established physical phenomena described as being either **fluorescence or phosphorescence**. The emission of light through the fluorescence process is nearly simultaneous with the absorption of the excitation light due to a relatively short time delay between photon absorption and emission, ranging usually less than a microsecond in duration. When emission persists longer after the excitation light has been extinguished, the phenomenon is referred to as phosphorescence.

**Fluorescence microscopy** is a rapid expanding technique, both in the medical and biological sciences. The technique has made it possible to identify cells and cellular components with a high degree of specificity. For example, certain antibodies and disease conditions or impurities in inorganic material can be studied with the fluorescence microscopy.

British scientist *Sir George G. Stokes* first described *fluorescence in 1852* and was responsible for coining the term when he observed that the mineral fluorspar emitted red light when it was illuminated by ultraviolet excitation. Stokes noted that fluorescence emission always occurred at a longer wavelength than that of the excitation light. Early investigations in the 19th century showed that many specimens (including minerals, crystals, resins, crude drugs, butter, chlorophyll, vitamins, and inorganic compounds) fluoresce when irradiated with ultraviolet light. However, it was not until the 1930s that the use of **fluorochromes** was initiated in biological investigations to stain tissue components, bacteria, and other pathogens. Several of these stains were highly specific and stimulated the development of the fluorescence microscope.

The *technique of fluorescence microscopy* has become an essential tool in biology and the biomedical sciences, as well as in materials science due to attributes that are not readily available in other contrast modes with traditional optical microscopy. The application of an array of fluorochromes has made it possible to *identify cells and sub-microscopic cellular components* with a high degree of specificity amid non-fluorescing material. In fact, the fluorescence microscope is *capable of revealing the presence of a single molecule*. Through the use of multiple fluorescence labeling, different probes can simultaneously identify several target molecules simultaneously. Although the fluorescence microscope cannot provide spatial resolution below the diffraction limit of specific specimen features, the detection of fluorescing molecules below such limits is readily achieved.

A variety of specimens exhibit **autofluorescence** (without the application of fluorochromes) when they are irradiated, a phenomenon that has been thoroughly exploited in the fields of botany, petrology, and the semiconductor industry. In contrast, the study of animal tissues and pathogens is often complicated with either extremely faint or bright, nonspecific autofluorescence. Of far greater value for the latter studies are added **fluorochromes** (*also termed fluorophores*), which are excited by specific wavelengths of irradiating light and emit light of defined and useful intensity. *Fluorochromes are stains that attach themselves to visible or sub-visible structures, are often highly specific in their attachment targeting, and have a significant quantum yield* (the ratio of photon absorption to emission). The widespread growth in the utilization of fluorescence microscopy is closely linked to the development of new synthetic and naturally occurring fluorophores with known intensity profiles of excitation and emission, along with well-understood biological targets.

**A fluorescence microscope** is a light microscope used to study properties of organic or inorganic substances using the phenomena of fluorescence and phosphorescence instead of, or in addition to, reflection and absorption.

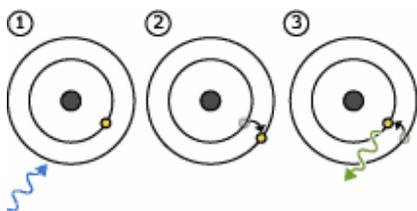


In fluorescence microscopy, the sample used to study is itself the light source. The technique is used to study specimens, which can be made to fluoresce. The fluorescence microscope is based on the phenomenon that certain material emits energy detectable as visible light when irradiated with the light of a specific wavelength. The sample can either be fluorescing in its natural form like chlorophyll and some minerals, or treated with fluorescing chemicals.

## Fluorophores

In analogy to a chromophore, is a *component of a molecule which causes a molecule to be fluorescent*. It is a functional group in a molecule which will absorb energy of a specific wavelength and re-emit energy at a different (but equally specific) wavelength. The amount and wavelength of the emitted energy depend on both the fluorophore and the chemical environment of the fluorophore. This technology has particular importance in the field of biochemistry and protein studies, e.g. in *immunofluorescence and immunohistochemistry*. *Fluorescein isothiocyanate*, a reactive derivative of fluorescein, has been one of the most common fluorophores chemically attached to other, non-fluorescent molecules to create new and fluorescent molecules for a variety of applications. Other historically common fluorophores are derivatives of *Rhodamine*, *coumarin* and *cyanine*.

## Principle of Fluorescence

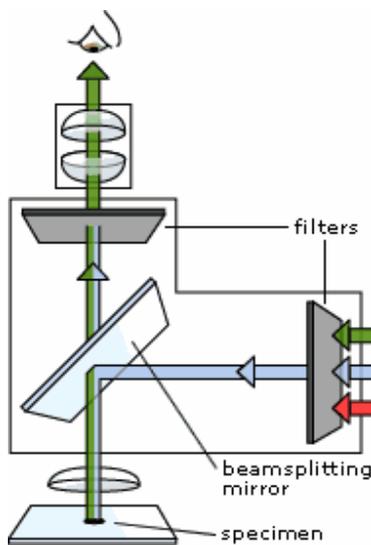


1. Energy is absorbed by the atom which becomes excited.
2. The electron jumps to a higher energy level.
3. Soon, the electron drops back to the ground state, emitting a photon (or a packet of light) - the atom is fluorescing

The basic task of the fluorescence microscope is to let excitation light radiate the specimen and then sort out the much weaker emitted light to make up the image. First, the microscope has a filter that only lets through radiation with the desired wavelength that matches your fluorescing material. The

radiation collides with the atoms in your specimen and electrons are excited to a higher energy level. When they relax to a lower level, they emit light. To become visible, the emitted light is separated from the much brighter excitation light in a second filter. Here, the fact that the emitted light is of lower energy and has a longer wavelength is used. The fluorescing areas can be observed in the microscope and shine out against a dark background with high contrast.

The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light. In a properly configured microscope, only the emission light should reach the eye or detector so that the resulting fluorescent structures are superimposed with high contrast against a very dark (or black) background. The limits of detection are generally governed by the darkness of the background, and the excitation light is typically several hundred thousand to a million times brighter than the emitted fluorescence.



In order to generate sufficient excitation light intensity to produce detectable emission, powerful compact light sources, such as high-energy short arc-discharge lamps, are necessary. The most common lamps are *mercury burners*, ranging in wattage from 50 to 200 Watts, and the *xenon burners* that range from 75 to 150 Watts. These light sources are usually powered by an external direct current supply, furnishing enough start-up power to ignite the burner through ionization of the gaseous vapor and to keep it burning with a minimum of flicker.

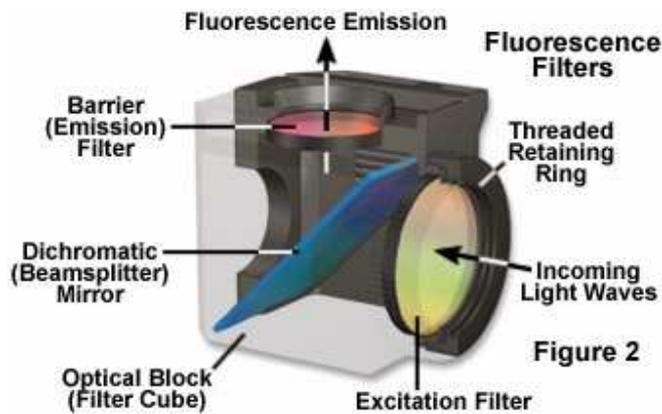


Figure 2

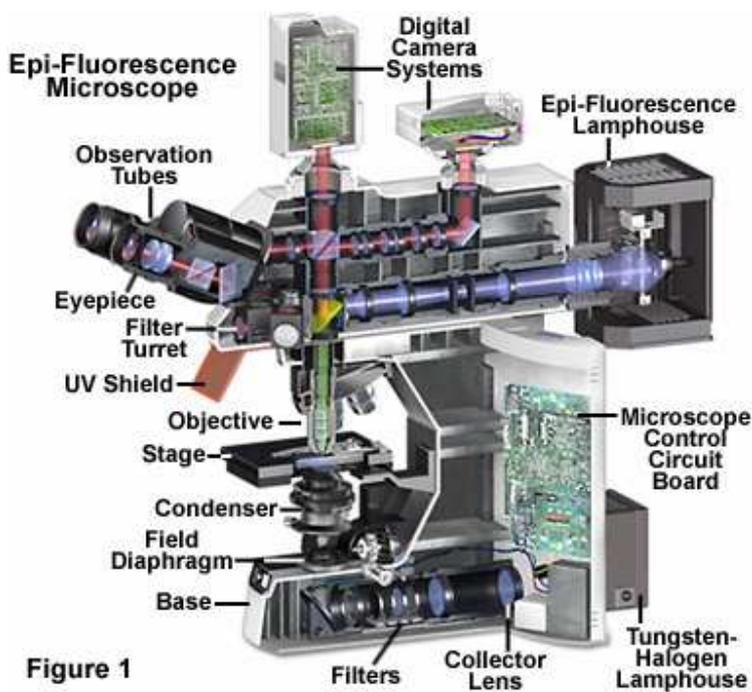


Figure 1

Figure 1 is a cut away diagram of a modern **epi-fluorescence microscope** equipped for both transmitted and reflected fluorescence microscopy. The vertical illuminator in the center of the diagram has the light source positioned at one end (labeled the episcopic lamphouse) and the filter cube turret at the other. The design consists of a basic reflected light microscope in which the wavelength of the reflected light is longer than that of the excitation. In a fluorescence vertical illuminator, light of a specific wavelength (or defined band of wavelengths), often in the ultraviolet, blue or green regions of the visible spectrum, is produced by passing multispectral light from an arc-discharge lamp or other source through a wavelength selective excitation filter. Wavelengths passed by the excitation filter reflect from the surface of a dichromatic (also termed a dichroic) mirror or beam splitter, through the microscope objective to bath the specimen with intense light. If the specimen fluoresces, the emission light gathered by the objective passes back through the dichromatic mirror and is subsequently filtered by a barrier (or emission) filter, which blocks the unwanted excitation wavelengths.