Fluorescence Microscopy

Secondary article

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Fluorescence microscopy in biomedical research is a light microscope technique designed to view fluorescence emission from a biological specimen. It has become an extremely useful tool to localize genes, messenger RNA and proteins within live and fixed cells and tissues and to visualize spatiotemporal variations in intracellular calcium as well as other ions and metabolites.

Introduction

Fluorescence microscopy for biomedical research is a light microscope technique designed to view fluorescence emission from a cell or tissue specimen. While it can be used to image endogenous fluorescence from a sample, its major use is to image the fluorescence from various exogenous probes or labels. These include dyes staining various parts of the cell or tissue or fluorescently labelled antibodies or nucleotides for immunofluorescence or fluorescence *in situ* hybridization, respectively. Other fluorescent reagents are employed to investigate intracellular dynamics of calcium and other ions and metabolites. Advanced spectroscopic techniques applied through the fluorescence microscope are used to measure translational and rotational diffusion of labelled molecules as well as proximity relationships between cellular macromolecules.

Outline of Methods

Fluorescence and fluorescent probes

Fluorescence (Jameson, 1984) is the property of some atoms and molecules to absorb light of particular wavelength and, after a brief interval (the fluorescence lifetime), to emit light at longer wavelengths. The amount of fluorescence emitted is proportional to the product of the light absorbed by the fluorophore, characterized by the extinction coefficient, and its quantum yield. The quantum yield is a measure of the emission efficiency of the fluorescent molecule and is expressed as the ratio of photons emitted to photons absorbed; it can range from 0 to 1. A 'bright' fluorophore will have both a large extinction coefficient and a good quantum yield.

Compounds that are fluorescent (fluorophores) generally contain multiring systems with conjugated double bonds. Examples of fluorescent compounds used in fluorescent microscopy include fluorescein, rhodamine and their derivatives. The solubility and spectral properties of the fluorophore are determined in part by the substituents on the rings.

The microscope

A fluorescence microscope is basically a filter fluorometer; that is, the desired spectral range (band) of excitation wavelengths, typically produced by a mercury or xenon arc lamp, is isolated using a narrow-band exciter filter, and the emission is detected through an emission or barrier filter that blocks all wavelengths below the emission band. A schematic diagram of the basic fluorescence microscope is depicted in **Figure 1**. The most commonly employed illumination system was invented by Ploem and is called epi-illumination. In this setup, the exciting light is reflected into the back aperture of the objective (which acts as a condenser) by a dichroic beam splitting mirror. The resulting fluorescence is then collected by the objective, and light forming the image passes through the dichroic mirror to either the eyepieces or a camera. The dichroic mirror has the property that it will reflect the shorter-wavelength excitation light and transmit the longer-wavelength emitted light. Thus, the intense excitation light is directed away from the eye or other detector so that it does not have to be filtered out of the fluorescent image reaching the eye or other detector. The most important optical elements of the microscope are the objective lens, as it determines the resolution and brightness of the image, the filters and the dichroic mirror.

Illumination

Light sources that are used for fluorescence microscopy include, most commonly, mercury and xenon arc lamps as well as incandescent halogen lamps and lasers. The optimal light source for a particular application will depend on the spectral output of the source and its brightness or luminance in the desired spectral range. Mercury arc lamps and lasers provide radiation at a few discrete wavelengths or lines; for example, mercury has a strong line at 546 nm that is useful for exciting rhodamine. In contrast to mercury lamps (and lasers), the radiation of xenon arc lamps and halogen lamps is continuous across the visible spectrum, allowing a variety of fluorophores to be excited, but with lower intensity than that provided by the strongest mercury lines.

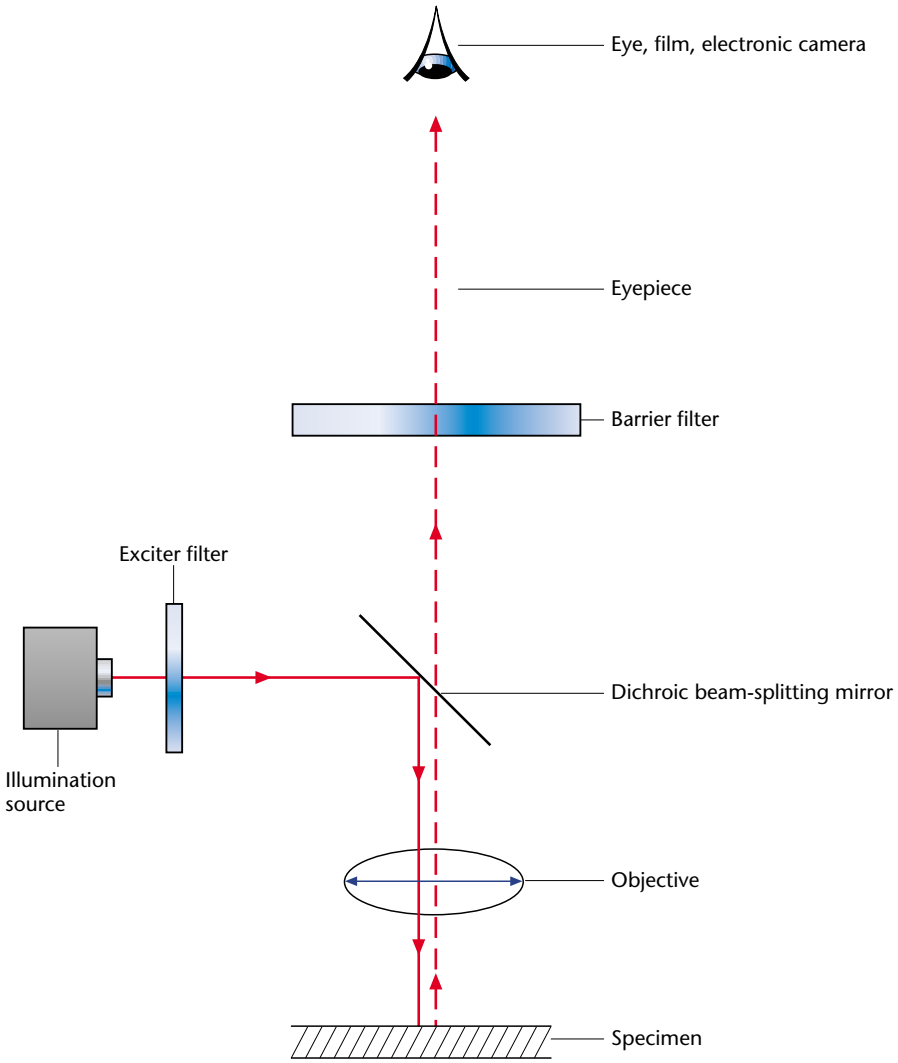

Figure 1 Schematic diagram of the basic fluorescence microscope. —, exciting light; – – –, fluorescent light.

Two other factors must be considered to maximize image brightness in fluorescence microscopy. First, a brighter image will be observed with higher-aperture objectives because the light-concentrating power of the objective (serving as a condenser) is proportional to the square of the numerical aperture, $(NA)^2$, and the light-gathering power of the objective is also proportional to $(NA)^2$. Thus for all but the highest NA objectives, brightness varies as $(NA)^4$. Second, the more the intermediate image produced by the objective lens is magnified by the eyepieces, the dimmer the final image will be. (The brightness of the image will be inversely proportional to the square of the eyepiece magnification.) Therefore, the lowest power of eyepieces consistent with convenient observation should be used.

Filters

Several exciter filter – dichroic mirror – barrier filter combinations that have been optimized for widely used fluorophores are available from each microscope manufacturer. Filters are designed to 'pass' or transmit certain wavelengths of light, while blocking out others. Filter selection is absolutely crucial to good fluorescence microscopy because it contributes to both specific brightness of the image of the fluorophore as well as to contrast between the emission image and the background (Kramer, 1999). The illumination (excitation) is defined by a narrow-bandpass interference filter selected to pass only light in the region of the spectrum where the absorption maximum of the fluorophore is located. Typically, a longpass or barrier filter that passes wavelengths in the emission spectrum of the fluorophore but no shorter wavelengths defines the fluorescence. It is also possible to view multiple, different coloured emissions simultaneously using more complicated multibandpass filters (Kramer, 1999).

Detectors

The fluorescent image can be viewed directly through the eyepieces of the microscope. However, low-light-level images can also be recorded on sensitive film or by recording the image digitally after capturing it with a low-light-level electronic camera in 'black and white' or with a colour camera. Fluorescent intensities can be measured directly using a photometer employing a photomultiplier that is coupled to the microscope.

Some Applications

Immunofluorescence microscopy and green fluorescent protein

Immunofluorescence is probably the most widely used biological application of fluorescence microscopy. In direct immunofluorescence, purified antibodies are directly conjugated to fluorophores and used to localize the antigens in fixed or live biological specimens. A more frequently used technique is indirect immunofluorescence, which offers a considerable amplification of signal compared to direct immunofluorescence. In this approach, the unlabelled primary antibodies are applied to the fixed specimen; labelled secondary polyclonal antibodies are then incubated with the specimen to form a complex at the site of the antigen. An example of the power of immunofluorescence microscopy is given in **Figure 2**.

The green fluorescent protein (GFP) technology is a related methodology of immense power for localizing gene products in live cells or tissues and also for emerging biosensor applications (Tsien and Miyawaki, 1998). GFP is a small, green-emitting protein isolated from chemiluminescent jellyfish. The gene for GFP can be incorporated into an expression vector containing the gene for the protein of interest and the vector transfected into cells. In most cases, the signal from GFP will localize the protein of interest in the fluorescent image of the cell or tissue.

In situ hybridization

In situ hybridization is a way of looking for defined sequences of nucleotides in RNA or DNA within the nucleus and cytoplasm of fixed cells. It takes advantage of the specific base pairing that can occur between the target sequence and the hybridization probe into which a fluorescent label has been incorporated. To prepare the cells for *in situ* hybridization, the RNA or DNA is first fixed as in immunofluorescence and formamide is added to melt complementary strands of the nucleic acid. The probe nucleic acid is then allowed to hybridize with the target sequence of nucleotides. In this way, specific genes can be located within the nucleus or messenger RNA localized in the nucleus and cytoplasm.

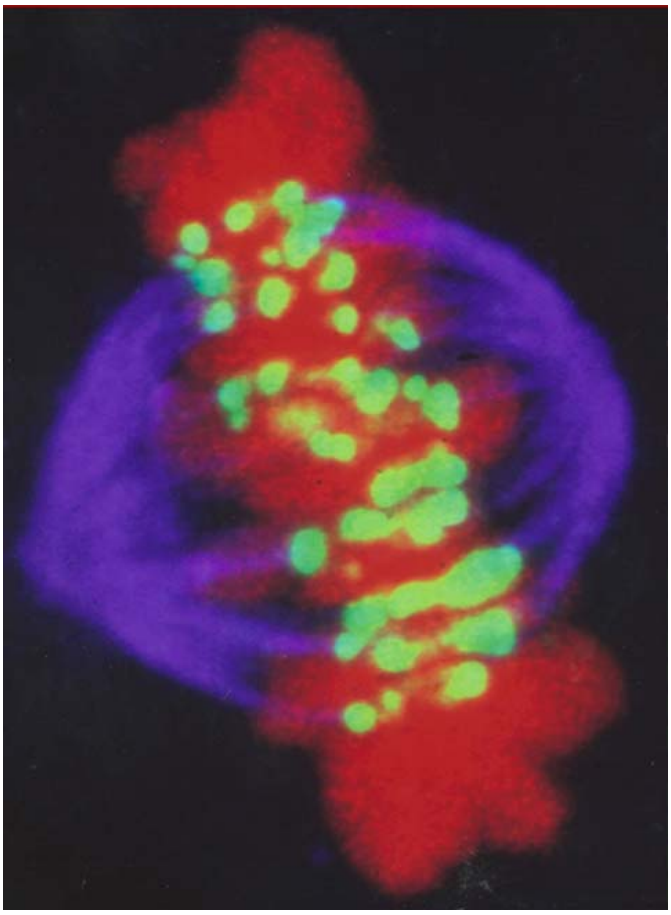

Figure 2 An example of an immunofluorescence micrograph. Optical section of a metaphase cell showing chromosomes stained with an anti-DNA antibody (red), centromeres labelled with an anticentromere antibody (green) and the spindle apparatus stained with an antitubulin antibody (purple). The image was taken using a confocal microscope and pseudocoloured. The micrograph was provided by Dr Vasily Ogryzko, National Institutes of Health, and was taken at the Cold Spring Harbor course on 'Advanced In Situ Hybridization and Immunocytochemistry', 1995.

Ion and metabolite imaging

A number of fluorescent indicators for various ions and metabolites are available; this list will continue to expand in the future. Fluorescence imaging of these indicators allows dynamic cellular processes to be followed spatially and temporally with high resolution. Examples include intracellular calcium and pH imaging, imaging of cyclic AMP concentrations, and membrane potential sensing (Tsien and Waggoner, 1995).

Optical sectioning fluorescence microscopy

Two optical sectioning techniques have had considerable impact on fluorescence imaging from thicker specimens such as tissues or embryos. Confocal microscopy permits clean optical sectioning to be performed without contamination from the portions of the specimen not in focus. Such contamination by out-of-focus fluorescence typically obscures important details in conventional widefield fluorescence microscopy of thicker specimens. In confocal microscopy, the illumination and the detection are limited to a small three-dimensional region. 'Confocal' refers to the fact that the condenser focusing the illumination and the objective collecting light from the illuminated region are both focused on the same spot. Because of this, there is a great gain in vertical resolution (along the optical axis) but the field of view, as normally imaged in a conventional full field microscope, is sacrificed. To obtain spatial information, either the confocal region must be scanned over the specimen or the specimen must be moved across this region. Most commonly, a focused laser is used as the excitation source.

A related, laser scanning technique is termed two-photon microscopy (TPM). This is a field where further advances in availability and convenience can be expected in the near future. In this form of fluorescence microscopy, optical sectioning is achieved by exciting fluorophores with two identical, infrared photons of twice the wavelength (half the energy) required to excite the fluorophore with conventional single-photon absorption. Because the two photons must be absorbed simultaneously, extremely high excitation light fluxes must be employed. This occurs only where the laser beam is most tightly focused on the specimen plane. Thus, emission occurs only from the in-focus plane resulting in excellent optical sectioning. Furthermore, photodamage is limited compared with normal confocal microscopy because light is only absorbed in the actual in focus plane – not in the planes below and above the specimen plane of focus. Hence, TPM is extremely useful for long-term observations, for example in studies on embryo development (Willis, 1999).

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Further Reading

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