Fluorescence: Basics, techniques, advantages

Introduction

Most of the newly developed microscopic techniques make use of fluorescence. Microscope and accessories performance is also increasing in accordance with the requirements of these applications and the fast growing number of fluorochromes available. In the next two parts of our “Basics of Light Microscopy and Imaging” series by experts from Olympus and Soft Imaging Systems, we will be concentrating on fluorescence microscopy and the essential role digital imaging plays.

Fundamentals

A Development Both Remarkable and Ongoing

Within the last few decades numerous new techniques such as confocal, deconvolution, ratio-imaging, total internal reflection and applications such as the use of fluorescent proteins (e.g. GFP) have initiated a real renaissance in the microscopy field. All of these techniques make use of fluorescence, a phenomenon first observed by Sir George Gabriel Stokes in 1852 and physically described by Alexander Jablonski in 1935. Compared with nowadays, the number of widely used fluorochromes was restricted to just a few in the 1990's. What is referred to as the fluorochrome FITC filter set for fluorescence microscopy can now be used for a wide range of different fluorochromes with green emission spectra.

Why use Fluorescence?

Using fluorescence is like the situation where a teacher asks whether the students have done their homework. The rapidly changing facial colours of the “guilty” students provides conclusive “results”.

But fluorescence techniques are not really for answering questions like the above. They help address specific questions regarding life science or materials science specimens and to visualise the result in a specific colour. To identify the distribution of a specific protein within a tissue, for example, a fluorochrome can be used to mark the protein via an antibody (immunohistochemistry).

Histological staining procedures for transmission light microscopy do have a long history in microscopy. One essential advantage of fluorescence microscopy, however, is the presence of fluorescent molecules themselves.
Although a structure is too small to be resolved in a light microscope, the emission light remains visible. Fluorescent molecules act like light sources that are located at specific specimen areas, indicating their location via light of a specific colour. These indicators require energy to emit light and this is given to the fluorochrome by the excitation light, provided by the microscope light source. A specific range of wavelengths is needed to excite a specific fluorochrome. A range of blue wavelengths around 480 nm can excite the FITC fluorochrome, for example. This means dealing with two different light beams and having to separate them. On the one hand, we need to direct the light of the microscope light source onto the specimen and on the other hand we have to observe the light that is originating from the fluorochromes. This separation is possible due to the “Stokes shift”, which describes the fact that the wavelength of fluorescent light (emission) is always longer than that of the excitation. Using a blue excitation light will thus result in a green emission for the FITC fluorochrome. Every fluorochrome has its own excitation and emission spectra. The microscope must be perfectly equipped to visualise this fluorescence accordingly.

**Fluorochromes**

There are two options for using fluorescent microscopy: either the specimen itself already contains molecules that show fluorescence; or specific fluorochromes have to be added to the specimen, depending on what is being investigated. Autofluorescence is widely found on materials such as plant sections or electrical circuits, for example. The resin on circuits is fluorescent and can easily be inspected under blue excitation (Fig. 2b). The green emission of the resin allows detection of the tiniest cracks which may influence material quality. Fluorochromes themselves can be divided into at least three groups. The first are fluorochromes that require other molecules such as antibodies or lectines to bind to specific targets. This rapidly growing group of fluorochromes includes longstanding ones such as FITC and TRITC. Most of these fluorochromes are sold together with the specific target-finding molecule (e.g. a goat anti-mouse IgG antibody Cy5 labeled). Quantum dots are also partial members of this group but different in structure and theory. They are nanometer-sized crystals of purified semiconductors and exhibit long-term photo stability as well as bright emission. The main difference featurewise is their capacity for being excited by wavelengths up to the blue range and having different emission colours depending on their size. Due to their flexible capabilities they can also be used for direct staining of cells (e.g. cell viability). This takes us to the second group.
The second group contains fluorochromes that have inherent binding capacities such as the DAPI nucleic acid stain or the DiI anterograde neuron stain. This group also contains fluorochromes that change their fluorescent properties when bound to different amounts of molecules such as calcium (e.g. Fura-2). This means these fluorochromes are used directly and do not necessarily require a transportation system such as an antibody.

The third group contains fluorescent proteins produced by organisms themselves such as GFP. This makes it possible to set up experiments in an entirely different way. It is most often used for life cell imaging or developmental studies and molecular biology. All fluorochromes show distinct spectral properties (Fig. 2a) and can often be combined for a multicolour specimen analysis.

**Requirements for a Fluorescence Microscopy System**

**The Light Source**
To excite the fluorescence of a fluorochrome, an intense light source is needed that provides the wavelengths to excite the fluorochrome in use. In the first chapter of this series we described the most frequently used light sources for light microscopy and their alignment.

A correctly aligned burner plays an essential role for creating good fluorescent images. If the burner is not correctly aligned, the signal from the fluorochrome may be excited in a very weak way and the field of view will not be homogeneously illuminated.

Due to the very different specimens and applications that can be analysed using fluorescent techniques there is no one-size-fits-all strategy. All fluorochromes are subject to the process of photobleaching, chemical destruction which takes place during excitation. Living cells, too, may be damaged by the intense light. This makes it of supreme importance to restrict the excitation brightness and time length to just the right amount needed. The amount of light can be efficiently modified with neutral density filters or a motorised attenuator. Anytime the light is not needed for excitation the shutter is closed. These features can be predefined in motorised microscopes and help optimise experiments.

**The Filter Sets**
Following the light path within a fluorescence microscope (Fig. 3), the next and critical step is having a filter which only permits the range of excitation wavelengths to pass through. This is done by an exciter filter using what are referred to as bandpass filter characteristics (Fig. 4). After restricting the light to the colour that is needed to excite the fluorochrome, the light is directed to the specimen via a dichromatic mirror. As indicated by the name, the dichromatic mirror treats different colours differently. It reflects light below a given wavelength
and is able to let longer wavelengths pass through. The excitation light travels through the objective to the specimen, acting like a condenser. This is where the fluorescence phenomenon takes place.

Excited by the light, the fluorochromes emit the fluorescence light of longer wavelengths. This is captured by the objective, moving on to the dichromatic mirror, now letting the longer wavelengths pass through. The last step of filtering is done by the emission filter (also called a barrier filter). This filter restricts the light colour to fit best with the fluorochrome emission and the question being investigated. It ensures that no unwanted wavelengths are observed and analysed. The emission filter can be designed as a bandpass filter (precisely restricted to one spectrum) or as a longpass filter (brighter in effect but with less optimised restrictability).

To help find the best filter combination for the fluorochromes in use and the analysis in mind, a variety of web pages is available. A straightforward example (below) will demonstrate how the combination of filters may differ depending on the context.

If you wish to make a vital analysis of a cell culture you may choose a Fluorescein-diacetate (FDA) to stain vital cells. This fluorochrome is excited by blue light and will have a green emission. The vital cells will be the ones appearing green. The filter set could thus be chosen as follows: an exciter with BP460-490 bandpath characteristics, a dichromatic mirror with DM505 characteristics and an emission filter with LP510 longpath characteristics. This will result in a bright green image of all green fluorescent molecules. So far so good. The vital cells are stained. To verify that nonlabelled cells are in fact dead, propidiumiodide (PI) dye may be used. This dye cannot pass through intact cell membranes. The DNA of dead cells only will be labelled and appear red. This means it can be used along with the FDA. When doing so, however, the excitation of the FDA with the filter mentioned will cause some problems. PI will already be excited by the blue light and the red emission is also visible. This is caused by the emission filter because in this set up, all wavelengths above 510 nm are allowed to pass through. Both dyes are thus excited and visible. A definite separation of both signals is not possible and as we will see later on, this can cause problems during imaging.

To separately identify both signals from the cell culture, the emission filter required for FDA is a bandpass filter (e.g. BP510-550). This filter will only allow the green emission of the FDA to pass through and the emission of the PI will be blocked. A second filter set can then be used to analyse the PI signal efficiently (e.g. BP530-550 excitation filter, LP575 emission filter, DM570 dichromatic mirror).

This principle also applies to other multicolour staining procedures. Best results are achieved when at least the emission filter for the detection of the fluorochrome with
the shorter wavelength is a band pass filter (Fig. 6)

**The Objectives**
The light gathering capacity of the objective plays an essential role in fluorescence microscopy. For optimal signal strength, high-numerical aperture (high NA) and the lowest useable magnification should be employed. For example, using a 1.4 NA aperture lens instead of a 1.3 NA lens of the same magnification and assuming that also all other factors are the same, results in about a 35% increase of intensity. On the other hand, the type of glass that is used requires good transmission for the wavelengths used. Fluorite or apochromate objectives are used for that reason. Further enhancement can be achieved by selecting objectives with extremely low autofluorescence of the glass material used (Olympus UIS2 Objectives). When all of these factors are provided for – high NA, good transmission and low autofluorescence – this ensures a perfect signal-to-noise ratio (i.e., a strong signal with low background intensity (Fig. 6)). Background noise can also be introduced by the specimen itself due to fixation, autofluorescence of the specimen or non-optimised staining procedures.

**Type of Camera**
The imaging device is one of the most critical components in fluorescence microscopy analysis. This is because the imaging device used determines at what level specimen fluorescence may be detected, the relevant structures resolved and/or the dynamics of a process visualised and recorded. Numerous properties are required to use fluorescence microscopy effectively. These include: high resolution, extreme sensitivity, cooling, variable exposure times and an external trigger function. Generally no single detector will meet all these requirements in fluorescence microscopy. The fluorescence microscopist thus frequently has to compromise. However, the cameras used in fluorescence microscopy should at the very least offer high signal sensitivity, low noise and the ability to quantify intensity of intensity distribution. Colour cameras are less sensitive than their monochrome counterparts because of the additional beam-splitting and wavelength selection components. Therefore monochrome cameras are preferable. They image the fluorescence intensity of each fluorochrome separately and can handle the respective images later on as images within a specific colour space using the appropriate software. The resulting multicolour images can be displayed, printed out and analysed or further processed. And every channel of colour represents the result of one fluorochrome. This result can be obtained if the fluorescence filters are chosen correctly. Coming back to our example of the FDA and PI double labelling: when using the longpass emission filter to detect the FDA signal, the red emission of the PI will also contribute to the resulting digital image.
A monochrome camera would not differentiate between red and green or between blue and green as shown in Fig. 6 - it will only show intensity in grey values. Therefore the image will represent the resulting distribution of both fluorochromes within the same colour. The use of the bandpass emission filter as described above will help in this respect.

**Software-Tools: Spectral Unmixing**

A further problem can occur when using different fluorochromes with overlapping spectra within one sample. The considerable overlap of excitation and emission spectra of different fluorochromes is exemplified by the spectra of enhanced green fluorescence protein (eGFP) and enhanced yellow fluorescent protein (eYFP), two commonly used variants of the green fluorescent protein (Fig. 7). Even with the use of high quality optical filters it is not possible to separate the spectral information satisfactorily. This means that YFP-labelled structures are visible with a GFP filter set and vice versa, affecting the resulting images significantly and detrimentally. This phenomenon, known as “bleed-through”, strongly reduces colour resolution and makes it difficult to draw accurate conclusions.

The solution to overcoming this effect is called spectral imaging and linear unmixing. This is a technique adapted from satellite imaging to wide-field fluorescence microscopy. Using this highly effective method, it becomes possible to ascertain the specific emission of the different fluorochromes to the total signal and to restore a clear signal for each colour channel, unaffected by emission from the other fluorochrome. This is done by redistribution of the intensity. It is important to note that original data is not lost during linear unmixing nor is any additional data added to the image. The original image information is all that is used in this procedure. The overall intensity of pixels is maintained. Thus, the technique does not result in artificially embellished images. After unmixing, quantification analysis does not only remain possible, but also becomes more precise.

*Image source for images 1, 2A, and 4 courtesy of Michael Davidson, PhD., Florida State University, Florida, USA*