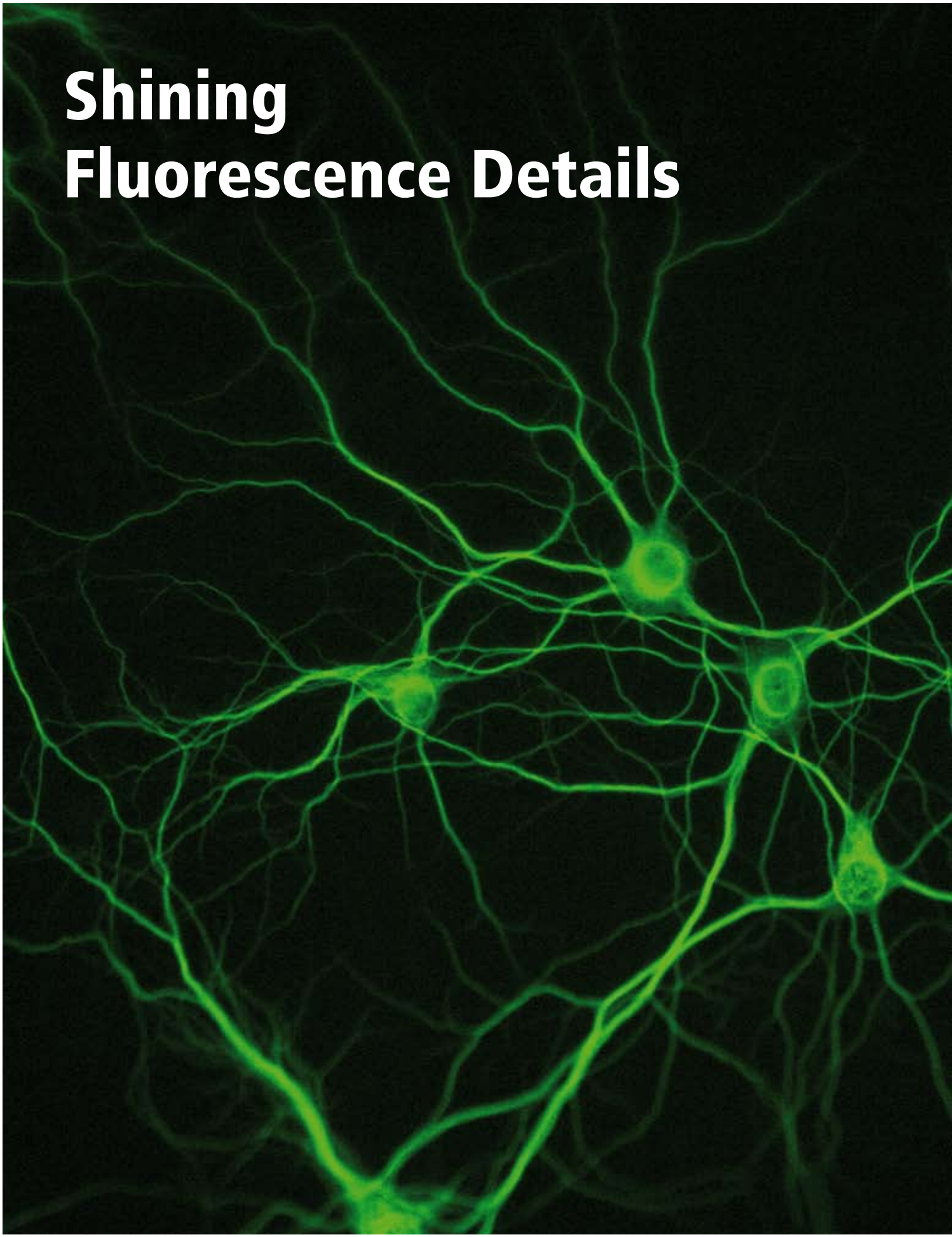
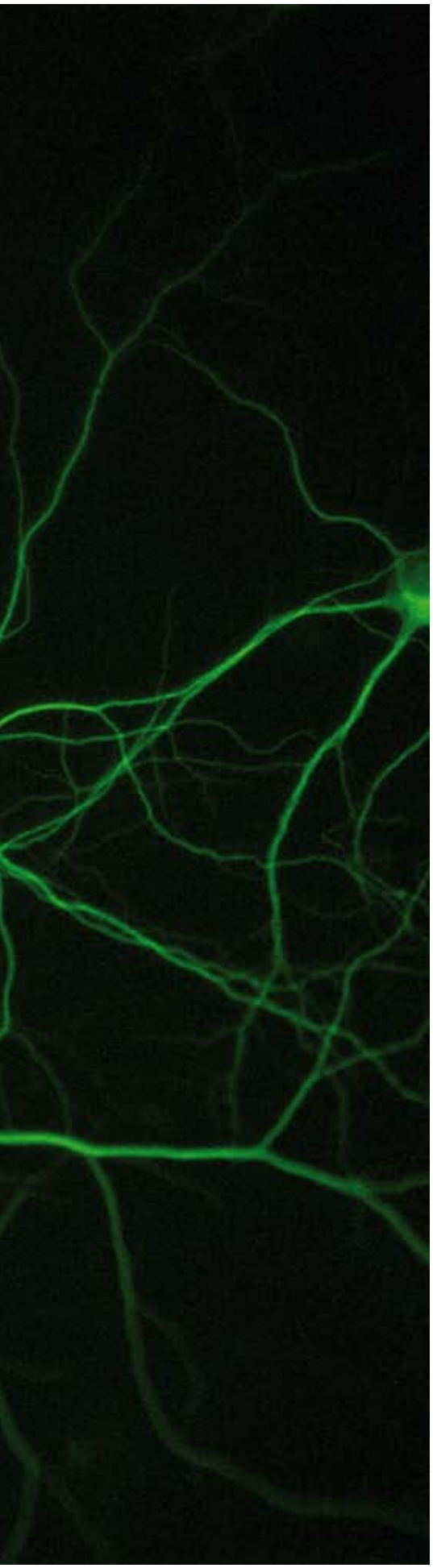
Shining Fluorescence Details





A first introduction

Most of the newly developed microscopic techniques make use of fluorescence. Fluorescence microscopy is more than 'just making colourful images in one, two, three or even more colours', it is an enormously powerful tool for investigations in the biological field. Fluorescence techniques place numerous benefits in the hands of researchers wishing to exploit the upper limits of sensitivity and resolution in microscopy. Beyond the scientific benefits, just studying the fluorescence images can sometimes offer a new insight into a reality which is usually hidden from the view of the world.

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 (see box 13). Compared with today, the number of widely used fluorochromes was restricted to just a few in the 1990's. For example, nowadys the fluorochrome FITC filter set for fluorescence microscopy can also be used for a wide range of different fluorochromes with green emission spectra.

Why use fluorescence?

Using fluorescence can be compared to the situation where a teacher asks if the students have done their homework. The rapidly changing facial colours of the "guilty" students provide conclusive "results". However, fluorescence techniques are not really for answering questions such as the above. They help to address specific questions regarding life science or materials science specimens and to visualise the result in a specific colour. For example, to identify the distribution of a specific protein within a tissue, a fluorochrome can be used to mark the protein via an antibody (immunohistochemistry).

Histological staining procedures for transmission light microscopy have a long history in microscopy. One essential advantage of fluorescence microscopy, however, is the presence of fluorescent molecules themselves. Even if a structure is too small to be resolved by a light microscope, the emission light remains visible.

Fluorescent molecules act like light sources that are located within specific areas of a specimen, indicating their location with 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. For example, a range of blue wavelengths around 480 nm can excite the FITC fluorochrome. This involves using 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.

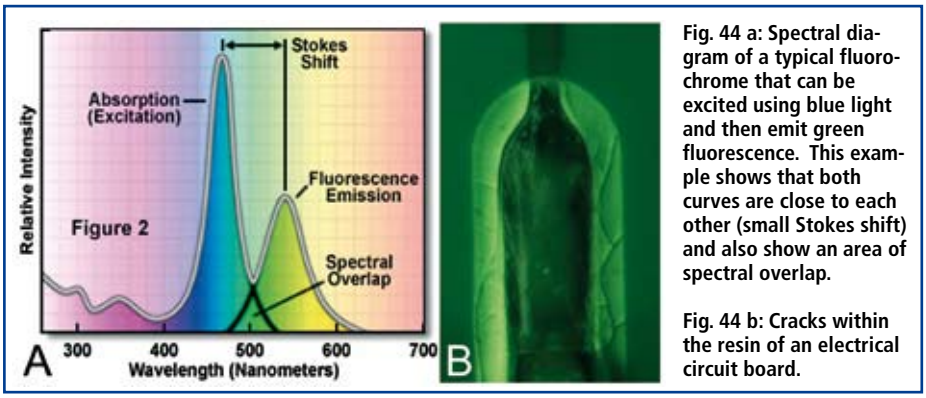


Fig. 44 a: Spectral diagram of a typical fluorochrome that can be excited using blue light and then emit green fluorescence. This example shows that both curves are close to each other (small Stokes shift) and also show an area of spectral overlap.

Fig. 44 b: Cracks within the resin of an electrical circuit board.

Fluorescent molecules

There are two options for using fluorescent microscopy, depending on what is

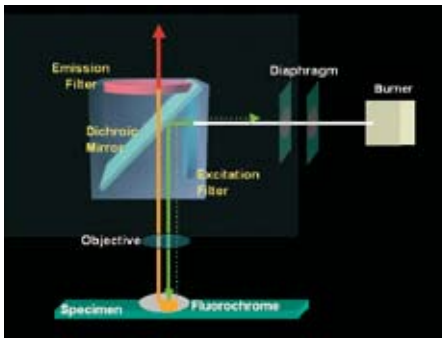


Fig. 45: Light path on a microscope equipped for fluorescence.

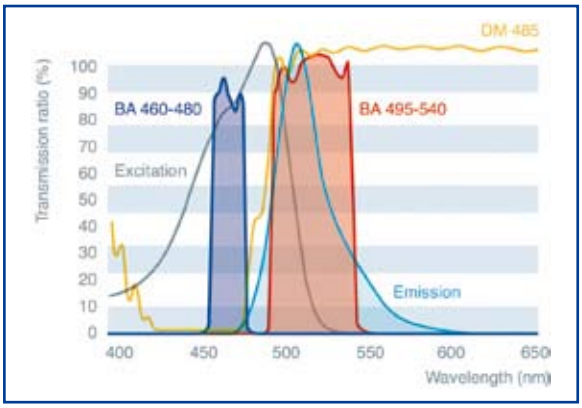


Fig. 46: Characteristics of an Olympus HQ filter set optimised for GFP. By using up to 100 layers of an ion deposition technique with new substrates and coating materials, filters can be created with high transmission and exceptionally sharp cut-off (tolerances < 2 nm). Autofluorescence is significantly reduced and the filters are equipped with a stray light noise destructor to enhance the signal-to-noise ratio.

being investigated: either the specimen itself already contains molecules that show fluorescence; or specific fluorochromes have to be added to the specimen. Autofluorescence is widely found in 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. 44b). The green emission of the resin enables the detection of the tiniest cracks which may influence material quality.

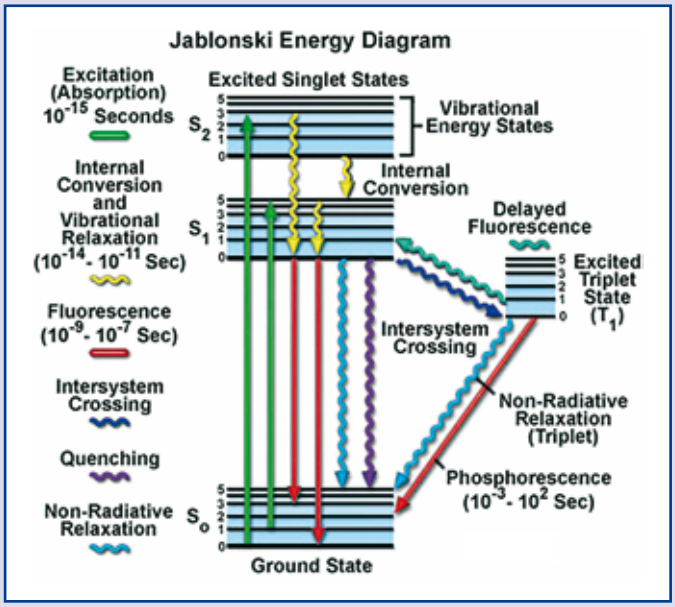
Fluorochromes themselves can be divided into at least three groups. The first are fluorochromes that require other molecules, such as antibodies or lectins, to bind to specific targets. This rapidly growing group of fluorochromes includes longstanding ones such as FITC and TRITC. Most of these fluorochromes are sold together with the specific target-finding molecule (e.g. a goat anti-mouse IgG antibody Cy5 labelled). Quantum dots are also partial members of this group but different in structure and theory. They are nanometre-sized crystals of purified semiconductors and exhibit long-term photo stability, as well as bright emission. The main difference feature-wise is their capacity to be 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).

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 that 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 live cell imaging or developmental studies and molecular biology. All fluorochromes show distinct spectral properties (fig. 44a) and can often be combined for a multicolour specimen analysis.

Box 13: What is fluorescence exactly?

Fluorescence activity can be schematically illustrated using the familiar Jablonski diagram, where absorbed light energy excites electrons to a higher energetic level in the fluorochrome. These lose a proportion of their energy by vibration and rotation and the rest is then given off as fluorescent light as they return to their original state after only about 10 ns.



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 necessary excitation wavelengths to excite the particular fluorochrome. In the first chapter we described the most frequently used light sources for light microscopy and their alignment. A correctly aligned burner plays an essential role in 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, or can show a bad signal to noise ratio.

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 photo-bleaching, which is the 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 duration to the exact amount needed. The amount of light can be efficiently modified with neutral density filters or a motorised attenuator. When the light is not needed for excitation, the shutter is closed. These features

can be predefined in motorised microscopes and help to optimise experiments.

The Filter Sets

In addition to the special light path within a fluorescence microscope (fig. 45), another necessity is having a filter which only permits the required range of excitation wavelengths to pass through. This is achieved using an exciter filter with what are referred to as bandpass filter characteristics (box 14). After restricting the light to the particular colour that is needed to excite the fluorochrome, the light is directed to the specimen via a dichromatic mirror (fig. 45).

As indicated by its 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 undertaken by the emission filter (also called a barrier filter, see fig. 46). This filter restricts the light colour to best fit 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 less optimal due to a reduction in restricted wavelengths). To help find the best filter combination for the fluorochromes in use and the analysis in mind, a variety of web pages is available (e.g. www.olympusmicro.com/primer/java/fluorescence/matchingfilters/index.html). A straightforward example (below) will demonstrate how the combination of filters may differ depending on the context.

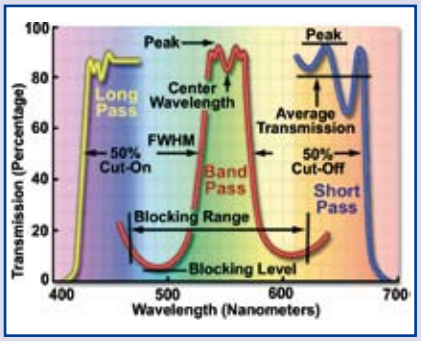
Using the correct filter set – an example

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 long path 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 non-labelled cells are in fact dead, propidumiodide (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

Box 14: How are filters described?

Filters for fluorescence microscopy are described using letters and numbers: e.g. BA 460-480. In this case, BA stands for bandpass filter and the numbers indicate the 50 % cut on and the 50 % cut off (fig. 46, box 14 – Figure 1). For a longpass filter, just the number for the 50 % cut on is indicated. Some companies use a different description for the same overall filter characteristics (e.g. 470/20), 470 indicating the central wavelength and 20 indicating the range of the full width at half maximum (FWHK). The correct transmission characteristics of the filter can only be provided using a transmission/wavelength diagram (box 14 – Figure 1).



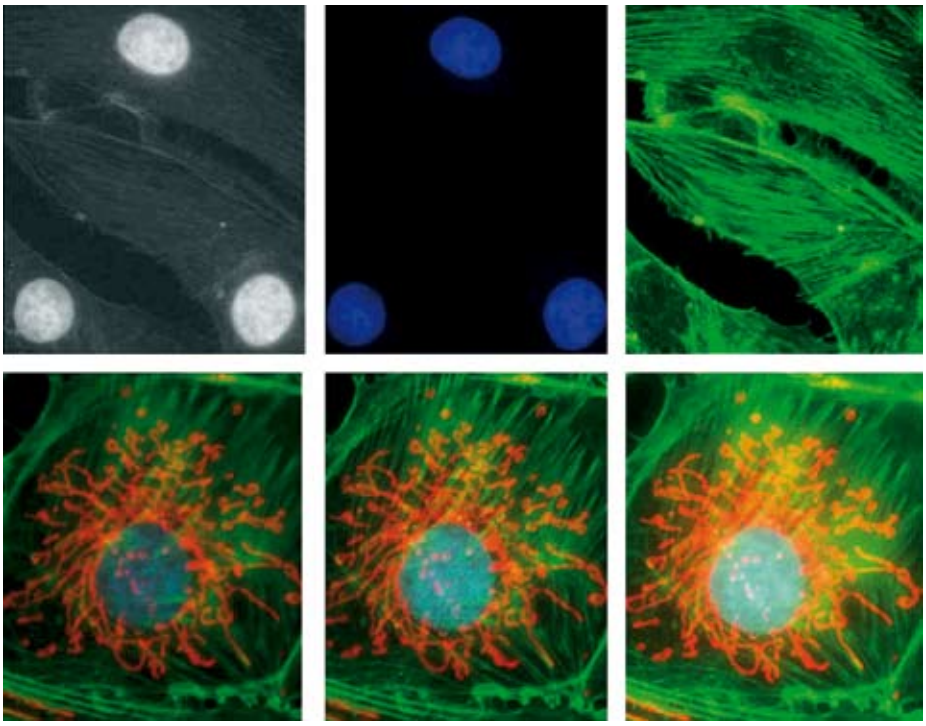
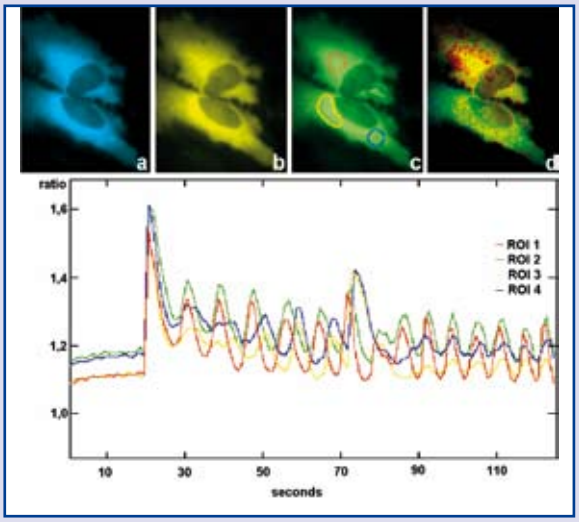


Fig. 47: Upper row: Images of a double-labelled specimen (DAPI for DNA staining and Alexa 488-labelled antibody for F-Actin staining). The first image shows the detection of the fluorescence using a filter set with the appropriate excitation filter for DAPI and with a long pass emission filter. Analysed with a monochrome camera, even the Alexa-488-labelled structures are visible and prevent effective analysis. The blue and the green image show the same specimen with optimised emission filters, allowing the separation of each signal.
The lower row shows an example of an image area analysed with an achromat (left), a fluorite (centre) and an apochromat objective (right) at the same magnification.
Note that the signal intensity and colour transmission increases from achromat to apochromat due to the increasing NA and quality of the objectives, whereas the background intensity in the upper left corner remains unchanged.

Box 15: How to measure FRET.

Ratio measurement

An epifluorescence microscope equipped with highly specific fluorescence filter sets, a digital camera system and image analysis software is required. The ratio between the intensity of the sensitised acceptor emission (= FRET signal) and the intensity of the donor emission is calculated (box 15, fig. 1). The results have to be corrected for spectral cross-talk by spectral unmixing software.



Acceptor photo-bleaching.

This effect occurs when the acceptor is irreversibly destroyed by extensive illumination from excitation light. After bleaching of the acceptor fluorophore, FRET is stopped. Excitation of the donor will result in donor fluorescence emission. After the acceptor photo-bleaching, the donor shows more intensity than before.

Donor-bleaching.

The sample is irradiated with the specific excitation wavelength of the donor. Images of the donor are continuously acquired and the intensity decay is quantified. FRET decreases the mean lifetime of the donor and protects the donor from photo damage. As a result, when using FRET, the rate of photo-bleaching is lower; without FRET, the rate of photo-bleaching is higher.

(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 the emission filter for the detection of the fluorochrome with the shorter wavelength is a band pass filter (fig. 47 upper row).

The objectives

The light gathering capacity of the objective plays an essential role in fluorescence microscopy. For optimal signal strength, a 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 will result in a 35 % increase in light intensity, assuming that all other factors are the same. Furthermore, the type of glass that is used requires good transmission for the wavelengths used. Fluorite or apochromat 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. 47 lower row)). 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. Consequently, the fluorescence microscopist 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 within a specific colour space using the appropriate software. The resulting multicolour images can be displayed, printed out and analysed or further processed. Every channel of colour represents the result of one fluorochrome. This result can be obtained if the fluorescence filters are chosen correctly. Returning 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. 47 (first image) – 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

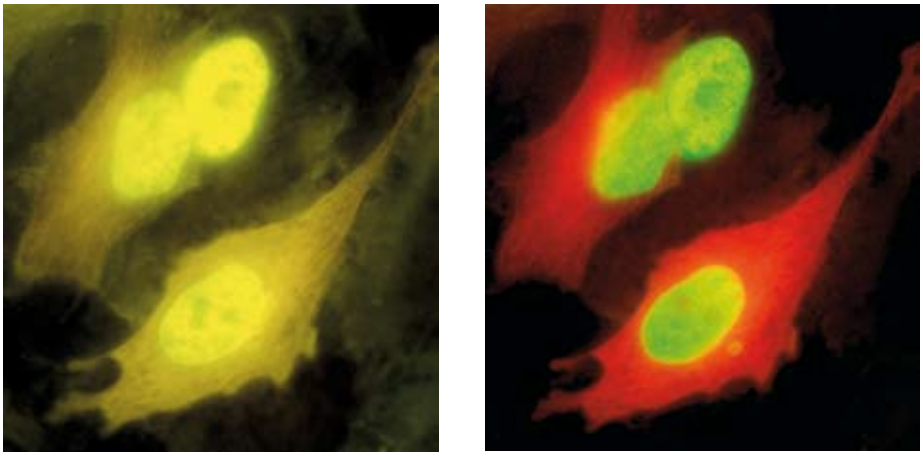


Fig. 48: Multi-colour image of a GFP/YFP double-labelled sample, before (left) and after spectral unmixing (right). In this sample, GFP was fused with the H2B histone protein and YFP with tubulin. The visible result is a pronounced chromatic resolution of both structures now displayed in green and red. The tubulin on top of the bright green nuclei is even detectable.

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. 48 left side). 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 overcome this effect is called spectral imaging and linear unmixing.

Spectral imaging and linear unmixing 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 achieved by redistribution of the intensity (fig. 38 right side). 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 not only remains possible, but also becomes more precise.

How to use light as a tool

After having laid the fundamental principles and the microscopic requirements we can now go deeper into different state-of-the-art qualitative and quantitative imaging methods for fluorescence microscopy. These approaches require advanced microscopic and imaging equipment which varies from case to case. So only general statements can be made here about the hardware and software used, along with a description of the applications and methods.

Life under the microscope

Wherever there is life there are dynamic processes – observation of living cells and organisms is a tremendous challenge in microscopy. Microscopy offers different techniques to reveal the dynamic processes and movements of living cells. The use of fluorescent proteins and live fluorescent stains enable the highly specific labelling of different organelles or molecules within a living cell. The intensity of the emission light of these markers can be used to image the cells. In addition to the application protocol and the fluorescent technique to be used there are further general considerations to be aware of.

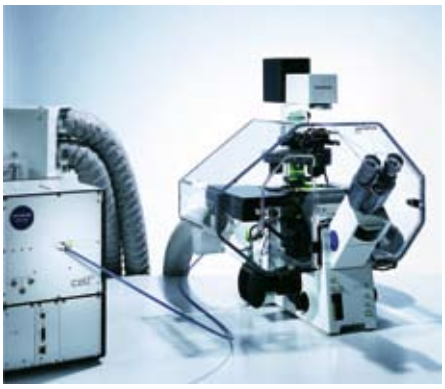


Fig. 49: A climate chamber offers features like control of temperature, humidity and CO_2 content of the atmosphere.

First of all, there is the definition of the needs for the specific environmental conditions and controlling these with regard to specimen handling. Assuming you are using a cell line and would like to analyse processes over time, it may be necessary to provide appropriate environmental conditions for these cells. Dynamic processes in single cells can occur within the millisecond range – such as shifts in ion concentrations. Or they may take minutes – such as the active or passive transport of proteins or vesicles. Microscopes can be equipped with heating stages and/or minute chambers, or with complete cultivation chambers to ensure cultivation of living cells with all the appropriate parameters on the microscope while observation is conducted for hours or days (fig. 49).

Where do all the ions go?

Fluorescent dyes such as FURA, INDO or Fluo show a spectral response upon binding Ca^{2+} ions and are a well established tool to investigate changes in intracellular Ca^{2+} concentration. The cells can be loaded with a salt or dextran conjugate form of the dye – e.g. by microinjection, electroporation or ATP-induced permeabilisation. Furthermore, the acetoxymethylester of the dyes can be added to the medium, loaded passively into the cells and cleaved enzymatically to produce cell-impermeable compounds.

For the analysis of a typical two channel FURA experiment it is necessary to switch between the excitation of 340nm and 380nm. When observing FURA loaded cells without any stimulus, Ca^{2+} is bound in cell compartments. The FURA molecules show strong fluorescence at an emission of 510nm when excited with 380nm, and weak fluorescence when excited with 340nm. As the cell releases Ca^{2+} from storage compartments due to a reaction to a stimulus, FURA molecules form complexes with these released Ca^{2+} ions. The fluorescence signal in the emission channel of 510nm increases when excited with 340nm, and decreases when excited with 380nm. The ratio between the signals of the two excitation channels is used to quantify the change of intensity.

Why use a ratio? Lamp fluctuations or other artificial intensity changes can cause a false signal which can be misinterpreted as a change in ion concentration when intensity is measured in one channel only. The second drawback of a single channel analysis is that it displays the amount of the fluorescence only. Therefore, thicker parts may look brighter than smaller parts of a cell; however, they simply contain more fluorochromes due to the larger volume. Physiologically relevant changes in ion concentration in small areas such as growth cones of a neuron may then not be visible over time because they are too dim in fluorescence compared to the bright centre of the cell. After background subtraction, the calculation of a ratio between two channels corrects the result for overall, artificial fluctuations and specimen thickness. Following a calibration procedure, even quantitative results are ob-

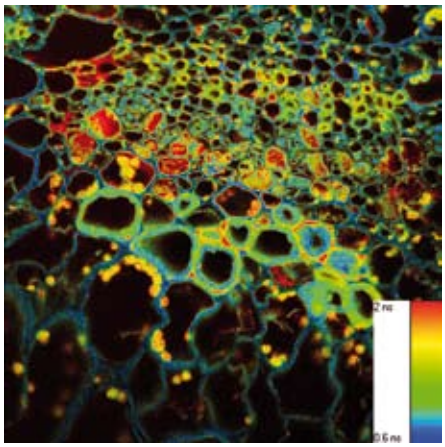


Fig. 50: Autofluorescence of an apple slice (Lieder). The image was taken with an Olympus FluoView FV1000 and the TCSPC by PicoQuant. The colour bar in the lower right corner is a key, showing the distribution of the various lifetimes within the image.

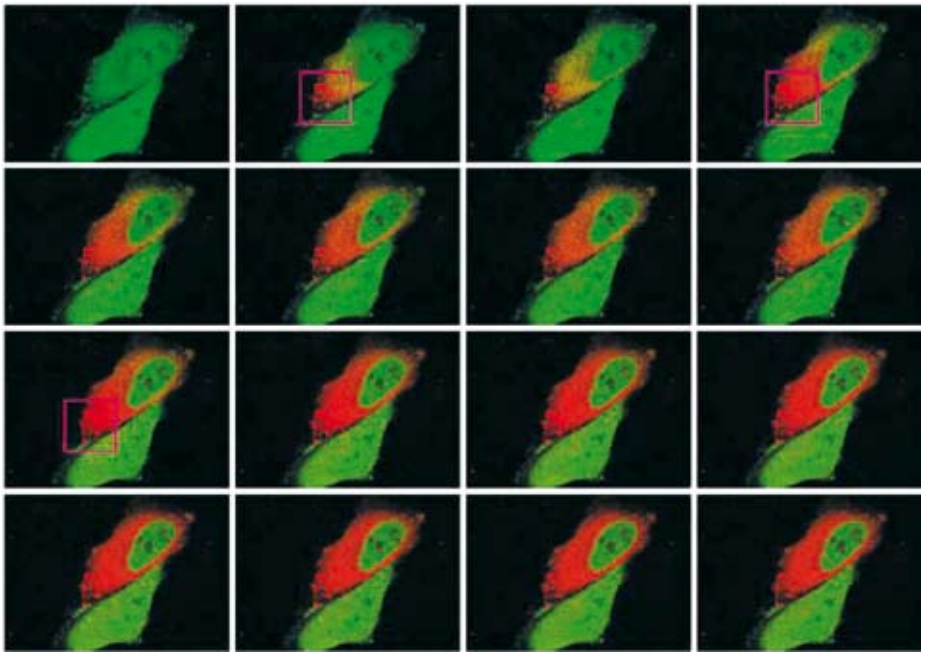


Fig. 51: Photoconversion: In Miyawaki's lab, Ando et al [1] succeeded in cloning the gene that encodes the Kaede fluorescence protein of a coral. By irradiation with UV light, the green fluorescence can be converted to red fluorescence. By using excitation wavelengths in the blue and green spectral range, the movement and distribution of the protein can be observed in a living cell. The figure shows the protein diffusion in Kaede-expressing HeLa cells. Stimulation via 405 nm laser in the region of interest converts the Kaede protein from green to red emission. Confocal images were recorded every three seconds using 488/543 nm laser excitation, showing the activated Kaede protein spreading throughout the HeLa cell. By using two synchronised scanners (one for photo-conversion, one for image acquisition), fluorescence changes that occur during stimulation can be observed.
Data courtesy of: R. Ando, Dr A. Miyawaki, RIKEN Brain Science Institute Laboratory for Cell Function Dynamics.

tainable. There is a range of fluorochromes, with different spectral properties for numerous different ions, available on the market. Microscopical systems with fast switching filter wheels and real time control permit investigations even in the millisecond range.

Light as a ruler

Many processes in a cell are controlled by inter- and intra-actions of molecules: e.g. receptor-ligand interactions, enzyme-substrate reactions, folding/unfolding of molecules. Receptor-ligand interactions, for example, occur in the very close proximity of two proteins in the Angstrøm range. Colocalisation studies do not reveal interactions of molecules in the Angstrøm range because the spatial resolution of a light microscope is limited to 200 nm. When using a light microscope, how can the proximity of two molecules in the Angstrøm range be proven beyond the physical limits of light microscopy? Fluorescence Resonance Energy Transfer (FRET) helps to find an answer to this question. FRET is a non-radiative energy transfer between two different fluorophores. The first fluorophore (the donor) is excited by light. The donor transfers its energy to the second fluorophore (the acceptor) without radiation, meaning without any emission of photons. As a result, the acceptor is excited by the donor and shows fluorescence ("sensitised emission"). The donor is quenched and does not show any fluorescence. This radiation-free energy transfer occurs within the very limited range of 1–10 nm distances between the donor and the acceptor. A positive FRET signal provides information about the distance between the FRET partners and can be quantified as FRET efficiency. When no FRET signal is achieved, there may be many reasons for that: e.g. too much distance between the FRET partners, insufficient steric orientation, insufficient dipole orientation, insufficient spectral overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor. See box 15.

How long does a fluorochrome live? – count the photons!

When a fluorochrome is excited, it is shifted to a higher energy level. The lifetime of a fluorophore is the average amount of time (in the nanosecond/picosecond range) that it remains at the higher energy level before it returns to the ground state. A fluorochrome's lifetime is a highly specific parameter for that particular fluorochrome. It can be influenced easily by changes of environmental parameters (e.g. pH, ion concentration, etc.), by the rate of energy transfer (FRET) or by interaction of the fluorochrome with quenching agents. Fluorochromes often have similar or identical spectral properties, therefore, analysing a fluorochrome's lifetime is critical to distinguishing the localisation of those fluorochromes in a cell (fig. 50). Here, the different experimental setups are referred to as FLIM – Fluorescence Lifetime Imaging Microscopy.

Fluorescence Lifetime Imaging Microscopy – FLIM.

There are different techniques for fluorescence lifetime imaging microscopy available on the market, for both wide-field and confocal microscopy. Here we focus on Time-Correlated Single Photon Counting (TCSPC). The fluorochrome is excited by a laser pulse emitted by a pulsed laser diode or femto-second pulsed Ti:Sa laser. A photon-counting photo- multiplier or single photon avalanche diode detects the photons emitted from the fluorophore. The time between the laser pulse and the detection of a photon is measured. A histogram accumulates the photons corresponding to the relative time between laser pulse and detection signal. Every pixel of the FLIM image contains the information of a complete fluorescence decay curve. If an image is composed of three fluorochromes with different lifetimes, distribution of all

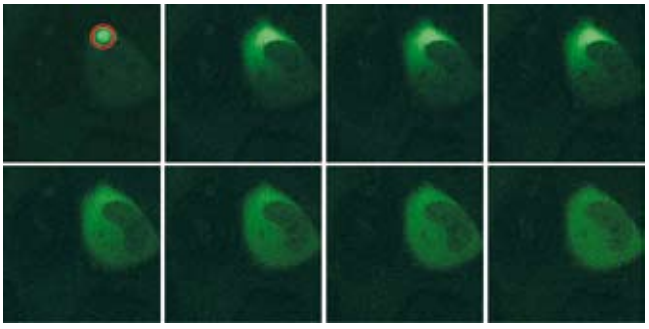


Fig. 52: Photoactivation of PA-GFP expressing HeLa cells. The PA-GFP was activated by 405 diode laser (ROI) and images of the PA-GFP distribution within the cell were acquired at 1 second intervals using the 488nm laser.
Data courtesy: A. Miyawaki, T. Nagai, T. Miyauchi, RIKEN Brain Science Institute Laboratory for Cell Function Dynamics.

dyes can be shown as three different colours (fig. 50).

Time-resolved FRET microscopy

With FRET, the lifetime of the donor depends on the presence or absence of radiation-free energy transfer (see above). Therefore, time-resolved FRET microscopy is a technical approach for quantitative measurement of the FRET efficiency.

FRAP, FLIP and FLAP

FRAP, FLIP and FLAP are all photobleaching techniques. By using the laser scanner of a confocal microscope, fluorochromes (which are bound to a specific protein, for example) in a selected region of a stained cell can be bleached (destroyed). As a result, the fluorochrome does not show any appropriate fluorescence. Other proteins that are also labelled, but where the fluorescence was not bleached, can now be observed during movement into the previously bleached area. Dynamic processes, such as active transport or passive diffusion in a living cell cause this movement. Therefore the intensity of the fluorochrome recovers in the bleached area of the cell.

FRAP = Fluorescence Recovery After Photobleaching

After bleaching of the fluorochrome in a selected region of a cell, a series of images of this cell is acquired over time. New unbleached fluorochromes diffuse or are transported to the selected area. As a result, a recovery of the fluorescence signal can be observed and measured. After correction for the overall bleaching by image acquisition, a curve of the fluorescence recovery is obtained.

FLIP = Fluorescence Loss In Photobleaching

The fluorophore in a small region of a cell is continuously bleached. By movement of unbleached fluorophores from outside of the selected region into the bleaching area, the concentration of the fluorophore decreases in other parts of the cell. Measuring the intensity loss, outside the bleached area results in a decay curve. A confocal laser scanning microscope which incorporates one scanner for observation and one scanner for light stimulation is especially appropriate for this technique. A simultaneous scanner system allows image acquisition during continuous bleaching.

FLAP = Fluorescence Localization After Photobleaching

The protein of interest is labelled with two different fluorochromes. One is bleached, the other remains undamaged and acts as a reference label. The population of bleached fluorochromes can be identified by subtraction of the image of the bleached dye from the image of the unbleached dye.

FAUD = Fluorescence Application Under Development!

We are looking for inventors of new fluorescence techniques – please keep in touch!

From black to green, from green to red …

If we sunbathe for too long, the colour of our skin changes from white to red and is referred to as sunburn. If we take a UV laser and irradiate a cell with Kaede protein, the colour of the Kaede protein changes from green to red – and we call this photo-conversion (fig. 51). A confocal microscope can be used to stimulate fluorochromes in selected parts of the cell. The Olympus FluoView FV1000 even offers stimulation of one area with one laser whilst observing the result in a different channel with a second laser simultaneously. A recently developed photoactivatable GFP mutant, PA-GFP, can be activated by irradiation using a 405 nm diode laser (fig. 52). The intense irradiation enhances the intensity of the fluorescence signal of PA-GFP by 100 times. PA-GFP is a powerful tool for tracking protein dynamics within a living cell (fig. 52).

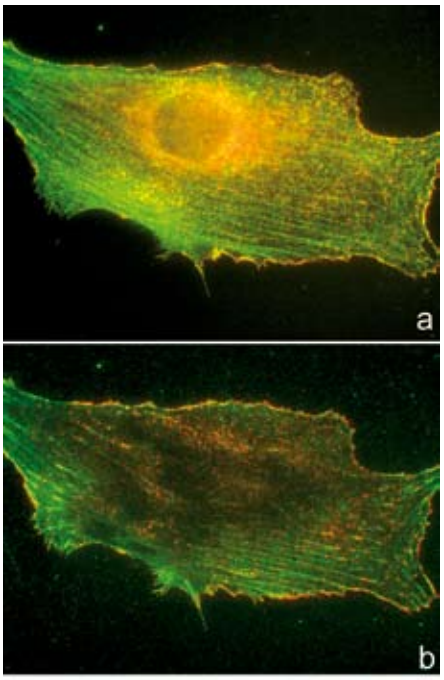


Fig. 53: Imaging at the Outer Limits of Resolution.
(a) Dual emission widefield
(b) TIRF images acquired with a 488nm laser
green Fitc-Actin
red DyeMer 605-EPS8.

Courtesy of M. Faretta, Eup. Inst. Oncology, Dept. of Experimental Oncology, Flow Cytometry and Imaging Core, Milan, Italy.

Imaging at the upper limits of resolution

The bleaching and photoactivation techniques described above can be undertaken easily using a suitable confocal laser scanning microscope. The laser is a much more powerful light source than a fluorescence burner based on mercury, xenon or metal halide. The galvano mirror scanners, in combination with acousto-optically tuneable filters (AOTF), permit concentration of the laser onto one or more selected regions of the cell without exciting any other areas in that cell. This makes the laser scanning microscope the preferred tool for techniques which use light as a tool. Additionally, the confocal principle removes out-of-focus blur from the image, which results in an optical section specifically for that focal plane. This means that high resolution along the x, y and z axes can be obtained. A confocal microscope is an ideal system solution for researchers who want to use light not only for imaging, but also as a tool for manipulating fluorochromes.

To achieve an even thinner optical section (for a single thin section only and not a 3-D stack), Total Internal Reflection Microscopy (TIRFM) may be the technique of choice (for more information on TIRFM, see box 4 or at www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfhome.html). The evanescent field used for this technique excites only fluorochromes which are located very closely to the coverslip (approx. 100–200 nm). Fluorochromes located more deeply within the cell are not excited (fig. 53). This means that images of labelled structures of membranes, fusion of labelled vesicles with the plasma-membrane or single molecule interactions can be achieved with high z resolution (200 nm or better, depending on the evanescent field).

References

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