**Fluorescence microscopy: biological applications and imaging methods**

**Fluorescence microscopy: biological applications and imaging methods - An Introduction**

Fluorescence microscopy is an enormously powerful tool for investigations in the biological field. Fluorescence microscopy is more than “just making colorful images in one, two, three or even more colors”. Fluorescence techniques place numerous benefits in the hands of researchers wishing to exploit the outer limits of sensitivity and resolution in microscopy. In this article, which was put together by experts from Olympus and Soft Imaging System, we focus on different state-of-the-art qualitative and quantitative imaging methods and we give an overview on principles, methods and biological applications. All of these require advanced microscopic and imaging equipment which varies from case to case. This means only general statements can be made here about the hardware and software used along with a description of the applications and methods.

**Life at the Microscope**

Wherever there is life there is dynamics – observation of living cells and organisms is a tremendous challenge in microscopy. Microscopy offers different techniques to reveal dynamic processes and movements of living cells. The use of fluorescent proteins and fluorescent live stainings allows a 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. For more information about imaging of fluorescence microscopy please refer to [Fluorescence - Part 1 (G.I.T Imaging & Microscopy 1/2005)](https://www.imaging-git.com). Besides the application protocol and the fluorescent technique you should keep in mind that there are some general considerations. 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 analyze 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.

**Where Do All the Ions Go?**

Fluorescent dyes such as FURA, INDO or Fluo show a spectral response upon binding Ca2+ ions and are a well established tool to investigate changes in intracellular Ca2+ concentration. The cells can be loaded with a salt or dextran conjugate form of the dye – e.g. by microinjection, electroporation or ATP-induced permeabilization.

Furthermore, acetoxymethylester of the dyes can be added to the medium, are loaded passively into the cells and are cleaved enzymatically to cell-impermeant compounds. For the analysis of a typical two channel FURA experiment it is necessary to switch between the excitation of 340 nm and 380 nm. When observing FURA loaded cells without any stimulus, Ca2+ is bound in cell compartments. The FURA molecules show strong fluorescence at an emission of 510 nm when excited with 380 nm, and weak fluorescence when excited with 340 nm. As the cell releases Ca2+ from storage compartments due to a reaction of a stimulus, FURA molecules form complexes with the released Ca2+ ions.

The fluorescence signal in the emission channel of 510 nm increases when excited with 340 nm, and decreases when excited with 380 nm. 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 then smaller parts of a cell; they simply contain more fluorochromes due to the larger volume, however.
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. After a calibration procedure, even quantitative results are obtainable. There is a range of fluorochromes with different spectral properties and 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, enzymesubstrate reactions, folding/unfolding of molecules. Receptor-ligand interactions, for example, occur in a very close proximity of two proteins in the Angstroem range. Colocalization studies do not reveal interactions of molecules in the Angstroem 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 Angstroem 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 nonradiative 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 (“sensitized emission”). The donor is quenched and does not show any fluorescence. The radiationless energy transfer occurs within the very limited range of 1–10 nm distances between the donor and the acceptor. A positive FRET signal gives information about the range of distance of 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.

**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) the fluorophore remains on the higher energy level before it returns to the ground state. A fluorochrome's lifetime is a highly specific parameter for each 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. Analyzing a fluorochrome’s lifetime is thus critical to distinguish the localization of those fluorochromes in a cell (Fig. 3.).

**FRAP, FLIP and FLAP**
FRAP, FLIP and FLAP are 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.

**From Black to Green, from Green to Red...**
The first time we take a lengthy sunbath the color of our skin changes from white to red and it's referred to as sunburn. If we take a UV laser and irradiate a cell with Kaede protein, the color of the Kaede protein changes from green to red - and we call it photoconversion (see inset on Photoconversion (Fig. 4)). 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 while 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 via 405 nm diode laser (Fig. 5). 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 (see inset on photoactivation).

**Imaging at the Outer Limits of Resolution**
The above-described bleaching and photoactivation techniques can be done easily with 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 tunable filters (AOTF) permit concentration of the laser onto one or more selected regions of the cell without exciting other areas in the 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 that has only image the focal plane (Fluorescence – Part 1 (G.I.T Imaging & Microscopy 1/2005)). 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 (but 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 Fluorescence – Part 1 (G.I.T Imaging & Microscopy 1/2005). The evanescent field used within 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. 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