Confocal laser scanning microscopes (CLSM) are an essential tool in biological and biomedical research. Their functionality can be further enhanced by adding sensitive time-resolved data acquisition capabilities, enabling Fluorescence Lifetime Imaging (FLIM), lifetime based Förster Resonance Energy Transfer (FRET) and Fluorescence (Lifetime) Correlation Spectroscopy (FL(C)S) down to the single molecule level. Complete and turn-key upgrade packages allow to apply these powerful techniques easily for all modern CLSMs including non-descanned detection using two photon excitation.

However, the measurements were up to now relatively tedious since the user had to operate two systems at the same time. Based on a recently added network interface, the FLIM and FCS data acquisition can now be directly accessed from the CLSM computer. This unique integration enables a seamless workflow recording z-FLIM stacks. Furthermore, the dependence of FLIM images onto the detection wavelengths range (lambda – FLIM stacks) can directly be investigated.

Förster Resonance Energy Transfer (FRET) studies provide a very powerful tool for a broad range of biological applications since this technique enables to measure intra- and intermolecular distances down to several nanometres. In this way, molecular interactions can be determined in vitro as well as in living cells. In addition, so-called FRET sensors allow the monitoring of environmental conditions such as pH and ion concentration. FRET results in donor quenching and leads to changes in its fluorescence intensity and fluorescence lifetimes.

Such distance measurements on a nanometer scale can be improved by applying FLIM-FRET. Here, changes in fluorescence lifetime of the donor are monitored which is in a broad range, concentration independent. This is advantageous since in biological systems like cells, the fluorophore concentration often cannot be accurately determined and compared amongst different cells. Other than intensity-based FRET measurements, FLIM can further reveal sub-populations; thus, allowing to determine the fraction of free donors compared to associated donor molecules within a complex. The result of such an analysis yields not only the lifetime distribution of FLIM-FRET images, but also the fraction and distribution of complete to incomplete FRET complexes. The underlying analysis will be demonstrated using an easy-to-use scripting language of the data analysis software “SymPhoTime”.

KEY WORDS: Fluorescence Correlation Spectroscopy, Fluorescence Lifetime Imaging, Single molecule spectroscopy, Förster Resonance Energy Transfer, Confocal Laser Scanning Microscopy