FLIM on a Spectral Confocal Microscope

FLIM on a Spectral Confocal Microscope. The study of interactions between proteins is becoming increasingly important for live cell research. Since the fluorescent proteins have become available, live cell microscopy has become an important tool. It has been shown that fluorescence resonance energy transfer (FRET) can function as a tool to study protein interactions. In this application note, we focus on different methods to study fluorescence resonance energy transfer between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Fast spectral detection and fluorescence lifetime imaging (FLIM) are competent and complementary methods for the study of interaction between proteins in living cells.

Introduction
In fluorescence microscopy, contrast may be based on different properties of light such as spectral properties, fluorescence lifetime or polarisation. Spectral properties are most commonly used by imaging the fluorescence emission of labelled specimen. Imaging based on contrast by other properties is less frequently applied, mainly because detection techniques are not widely available. For living cell microscopy, the fluorescent proteins (FP’s), such as CFP, GFP, and YFP are increasingly used.

Many new fluorescent protein types are becoming available. Since multiple FP’s may be visualised in living cells, the relation between different (tagged) proteins in (living) cells can now be studied. Imaging of the FP’s, however, may result in problems such as spectral overlap.
In order to visualise the interaction of tagged proteins in living cells, fluorescence resonance energy transfer (FRET) may be studied. Since FRET implies the transfer of energy from a donor to acceptor, this will result in a shift from donor to acceptor emission. Spectral overlap between donor and acceptor emission complicates the measurements of FRET efficiencies.

Fluorescence lifetime imaging (FLIM) has recently gained a lot of interest, mainly because the possibilities of using FLIM for FRET applications.
By looking at the donor lifetimes in the absence and presence of an acceptor, one can (easily) obtain **FRET efficiencies** following the equation:

\[
\text{FRET efficiency} = 1 - \left( \frac{\tau_{DA}}{\tau_D} \right)
\]

where \(\tau\) indicates the fluorescence lifetime.

The Nikon **C1si** confocal microscope may be used to study **FRET** applying different imaging techniques. Since the C1si records full spectra in a fast manner (λ range of 320 nm using 512 x 512 pixels in less than 2 seconds), this confocal may be used to record spectral changes due to **FRET** in living cells. The C1si confocal may also be equipped with a high-speed lifetime detector (Limo) to record donor lifetime changes due to **FRET**. This application note describes the use of lifetime detection by Limo for **FRET** applications using **CFP/YFP** as a **FRET** pair.

**Methods**

Figure 1 shows a schematic overview of the Nikon **confocal microscope system (C1si)** equipped with lifetime detection. Besides the laser setup for continuous (non-pulsed) excitation using a 408 nm diode laser and the 514 nm line of the argon laser, the confocal scanning head is provided with a 440 nm pulsed laser source (70 ps pulse width and 40 MHz repetition rate). The C1si confocal is standardly equipped with a fast spectral detector.

Lifetime detection was performed using the lifetime detection module Limo based on the time gating method for lifetime detection (Gerritsen et al., 2002).

**Fluorescence lifetime measurements** in the time-domain are performed by measuring the decay of the fluorescence intensity following excitation with a short light-pulse. The fluorescence decay curve is recorded after exciting the specimen with a single laser pulse.

The data acquisition time can be reduced when pulsed excitation is employed in combination with time-gated detection techniques.

The Limo system accommodates a time-gated detection technique, using four windows representing gates, in combination with photon counting detection. Each window is delayed by a different time relative to the excitation pulse. The **fluorescence lifetime** is a function of the integrated fluorescence intensities.
Single photon counting is used to acquire the fluorescence intensities for every laser pulse per pixel in the four windows sequentially. Several hundred laser pulses per pixel are used to build up sufficient statistics. One of the attractive features of the time-gated method is that the fluorescence decay is visualised in the four intensity windows (fig. 2B).

Results
Lifetimes of the fluorescence proteins have been reported to vary between approximately 2 and 3.5 ns (Grailhe et al., 2006). To verify the proper setting of the Limo lifetime detection system, we have measured the fluorescence lifetime of two reference probes, eosin and yellow green (YG) respectively. As is shown in figure 2A, the lifetime of eosin (in water) was fitted to be 1.1 ns, which corresponded well with the reported lifetime (Gerritsen et al., 2002). The lifetime histogram revealed a lifetime resolution of approximately 0.15 ns (FWHM). Figure 2B shows the previously discussed fluorescence decay (displayed in 4 windows) and the corresponding lifetime image of YG beads. In addition, the lifetime histogram reveals that the YG lifetime is 4.15 ns, which corresponds to previously reported values (Gerritsen et al., 2002).

For FRET measurements, the combination of cyan fluorescence protein (CFP) and yellow fluorescence protein (YFP) as a FRET pair is often used. To utilise lifetime imaging for the measurement of FRET, we have applied the donor lifetime approach. Measuring the lifetime of the donor only, in the absence and presence of an acceptor, should provide information on the FRET efficiency. Therefore, we have started to measure the lifetime of mock transfected CFP in living cells.

Figure 3 shows the fluorescence decay (as displayed by the four windows) of CFP. The lifetime image shows a homogeneous average lifetime of CFP, which corresponds well with the suggestion that lifetime imaging is independent of the probe concentration. The lifetime histogram reveals that the average lifetime is approximately 2.7 ns, which corresponds to lifetimes reported in literature (Grailhe et al., 2006). It should be noted that the CFP lifetimes were very dependent on both the temperature and pH of the medium.

Large fluctuations in lifetime could be observed when the living cells were imaged in the absence of temperature and CO2 control. Therefore, in order to perform proper FRET measurements, temperature and pH were carefully controlled. To measure FRET between CFP and YFP, a construct was used in which CFP was linked to YFP via a 15 amino acid spacer. This type of construct was reported earlier to function as a positive control for FRET. Cells expressing the donor only or cells expressing the FRET construct were first analysed for the proper emission spectra. The cells were excited with a 408 nm laser and the emission spectra were
recorded using the spectral detector of the C1si confocal system. Figure 4 reveals that the cells expressing the FRET construct produce a different emission spectrum (that corresponds with a transfer of energy from the donor to the acceptor) as compared to the emission of CFP transfectants. After recording the emission spectra of the transfected cells, the identical cells were imaged for fluorescence lifetime using the limo detector on the C1si confocal system. Figure 5 indicates that the cells expressing the FRET pair showed a CFP lifetime of approximately 2.1 ns whereas the CFP expressing cells showed a lifetime of 2.7 ns.

Using the equation to calculate FRET efficiencies “E = 1 - (τDA / τD)”, it could be determined that an efficiency of 1-(2.1/2.7) = 0.23 (23 %) was obtained. This corresponds to efficiencies reported in literature.

Conclusion
Measurements of FRET may be performed using different approaches. Using the confocal setup (Nikon C1si, fig. 1) as described in this application note makes it possible to combine the different FRET methods (based on spectral shift, donor lifetime or acceptor-bleaching).

The different approaches should not be seen as competitive methods, they should be used in a complementary way. However, one remark should be made: the measurement of FRET efficiency based on donor lifetime may be considered as the most practical one.

It has been reported that lifetime imaging (by Limo) was successful in determining FRET for receptor-ligand interactions (Hernanz-Falcon et al., 2004).

References

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