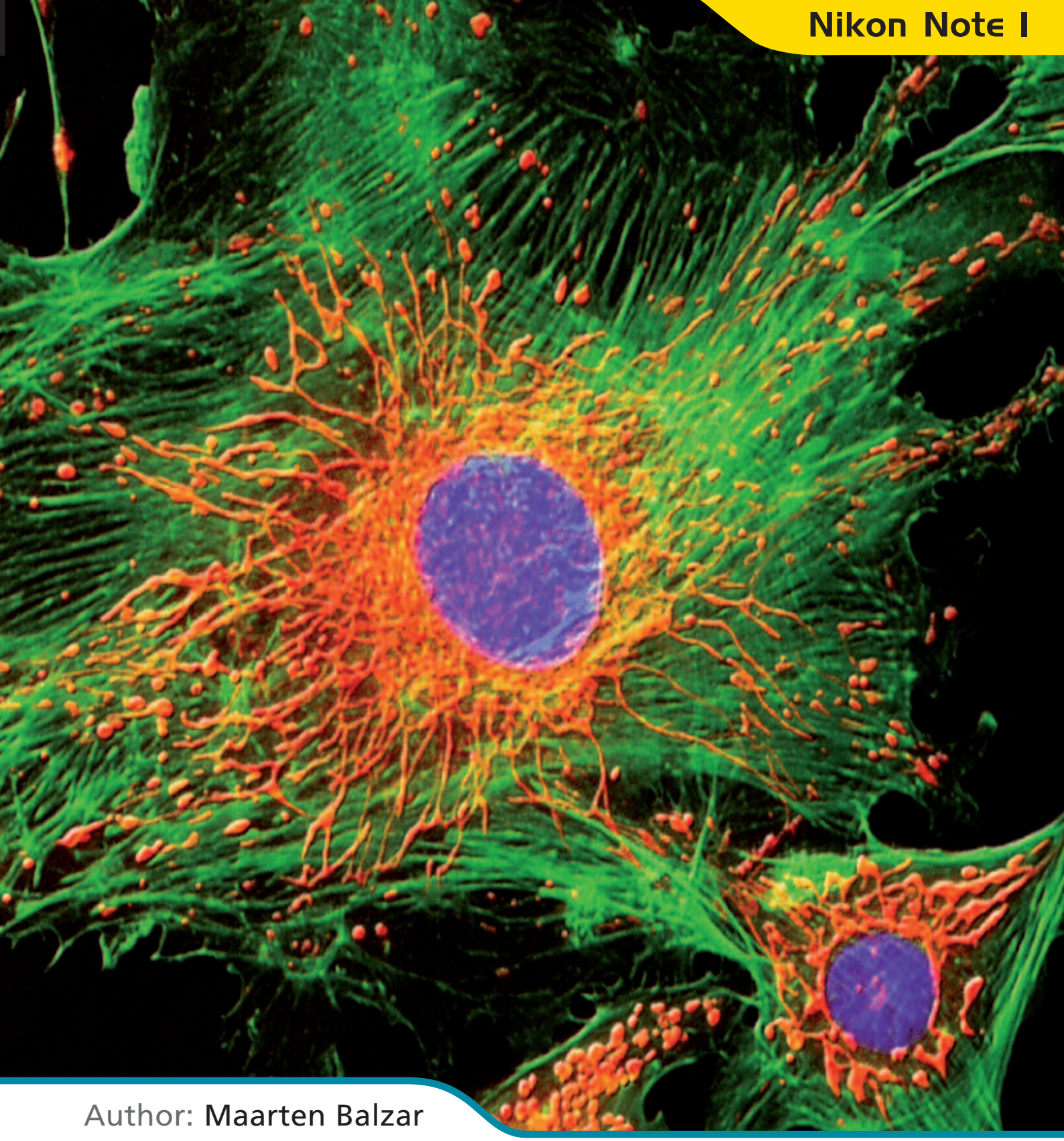

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Fluorescence Lifetime Imaging (FLIM) on a Spectral Confocal Microscope;

the ultimate tool for fluorescence resonance energy transfer (FRET) analysis.

Abstract:

The interaction between proteins is becoming an increasingly important study in live cell research. The advanced developments of fluorescent proteins have enabled live cell microscopy to become an important tool in these studies. One such technique: fluorescence resonance energy transfer (FRET), provides a vital function as a tool to study these protein interactions. In this application note, we focus on different imaging 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 complementary imaging methods for studying interactions between proteins in living cells.

Introduction:

In fluorescence microscopy, contrast can be achieved by utilising different characteristics of light such as spectral properties, fluorescence lifetime or polarization. Spectral properties are most commonly used by imaging the fluorescence emission of the labelled specimen. Imaging based on contrast by other properties is less frequently applied, mainly because detection techniques are not widely available.

In live cell microscopy, the fluorescent proteins (FP's), such as CyanFP, GreenFP, and YellowFP are the preferred choice for labelling but as new fluorescent proteins are developed this may well change in the future. Since multiple FP's may be visualized simultaneously in living cells, the relation between different (tagged) proteins in these cells can now be studied. Imaging of the FP's however may result in problems such as spectral overlap.
To capture the interaction of tagged proteins in living cells, the 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 attracted a lot of interest, mainly because of the possibilities of using it with FRET applications. By looking at the donor lifetimes in the absence and presence of an acceptor, FRET efficiencies can be easily obtained using the following equation:

$$\text{FRET efficiency} = 1 - (\tau DA / \tau D)$$

: where τ indicates the fluorescence lifetime,
D indicates Donor and A indicates Acceptor.

The Nikon C1si spectral confocal microscope may also be used to study FRET by applying a different imaging technique. Since the C1si records full spectra in a fast manner (Ï range of 320nm using 512x512 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 pairing.

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 408nm diode laser and the 514nm line of the argon laser, the confocal scanning head is provided with a 440nm pulsed laser source (70ps pulse width and 40MHz repetition rate). The C1si confocal is equipped, as standard, 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 and single photon counting is used to acquire the fluorescence intensities for every laser pulse 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 displayed in the four intensity windows (Figure 2B).

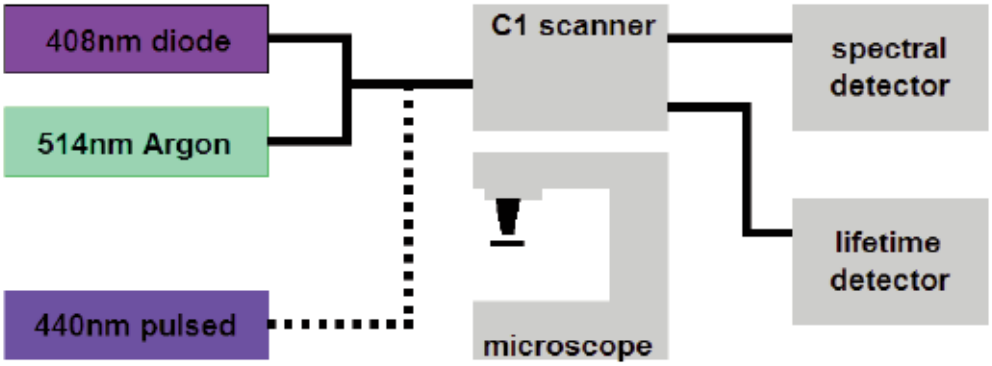


Figure 1. Schematic overview of the C1si system configured with the Limo high-speed lifetime detection module.

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 correct settings 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 reported to be 1.1 ns, which corresponded 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 four 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).

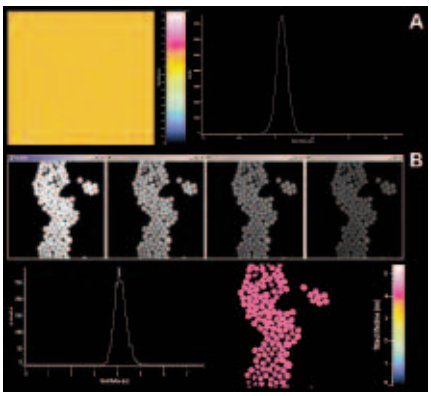


Figure 2: (A) Fluorescence lifetime image and histogram of eosin (dissolved in water). (B) Fluorescence decay, fluorescence lifetime and fluorescence lifetime histogram of Yellow Green beads (in water).

For FRET measurements, the combination of cyan fluorescence protein (CFP) and yellow fluorescence protein (YFP) as a FRET pair are often used. To utilize 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 in 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.

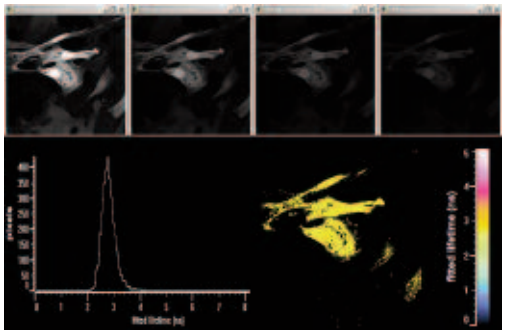


Figure 3: Fluorescence decay (four windows), Fluorescence lifetime, and Fluorescence lifetime histogram of CFP in transfected cells.

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 analyzed for the proper emission spectra. The cells were excited with a 408nm 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 form the donor to the acceptor) when compared to the emission of CFP transfectants.

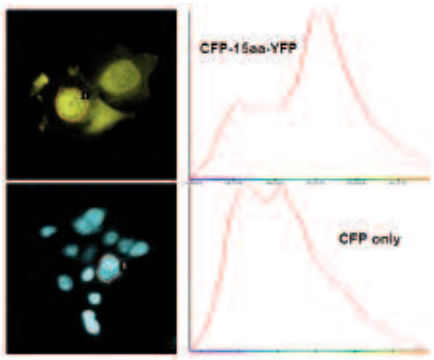


Fig 4: Cells expressing CFP or the FRET pair show different emission spectra. Please note that the cells expressing the FRET construct (top panel) mainly show YFP emission under 408nm laser light excitation only. Control cells (with expression of CFP and YFP without a spacer) showed mainly CFP emission.

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 indicated 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.

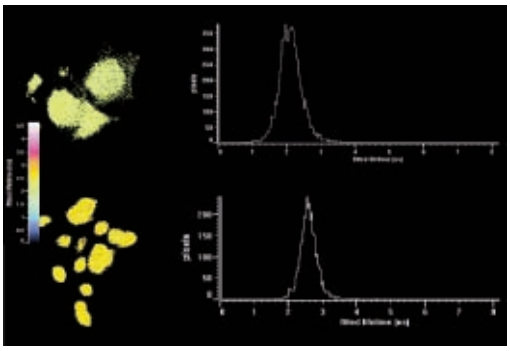
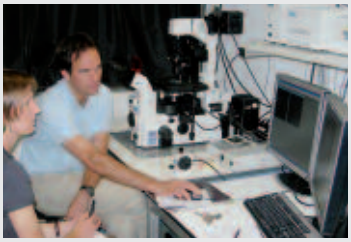


Figure 5: Fluorescence lifetime of FRET positive cells (top panel) and donor-only control cells (bottom panel). Please note that the lifetime histogram reveals a shorter lifetime for the FRET positive cells.

Using the equation to calculate FRET efficiencies
"$E = 1 - (\tau DA / \tau D)$", 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, **figure 1**) as described in this application note, it is 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).



Maarten Balzar at one of the imaging wokstations with Ulrike Engel, Scientific Director of the Nikon Heidelberg Imaging centre.

Authors Background

Maarten Balzar is an application manager for Nikon Instruments and is responsible for (biological) research applications such as TIRF, confocal, and advanced fluorescence microscopy. After finishing his PhD at the department of Pathology of the Leiden University Medical Centre (LUMC) in the Netherlands, Maarten joined Nikon in 1999. His current activities are focussed on new product developments for the (confocal) microscopy product line.

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- C1si records fast spectral images
- C1si configured with Limo combines fast spectral with fast lifetime detection
- C1si allows three different methods for FRET measurements

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