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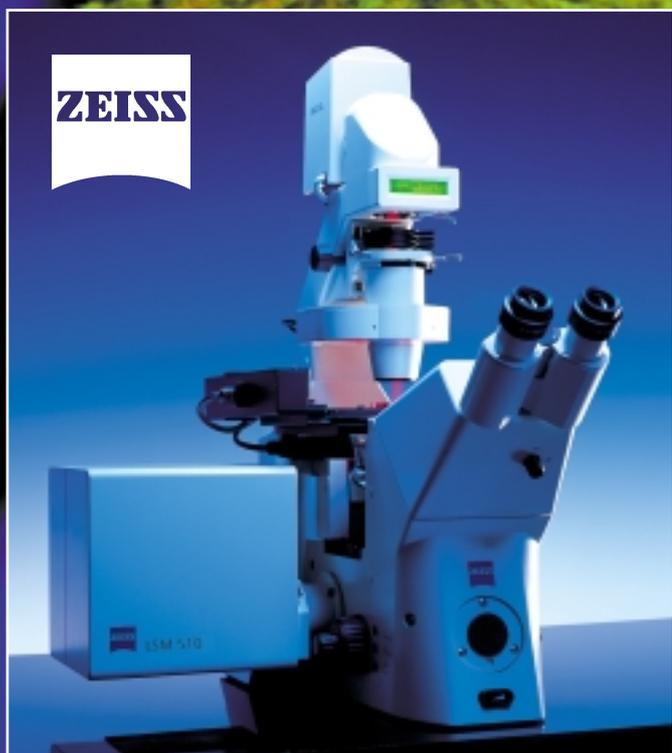
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FRET

Fluorescence Resonance Energy Transfer (FRET) Analysis with the ZEISS LSM 510

Richard Ankerhold

When and where proteins associate with each other in living cells is a key question to learn about their function. To address that question in conventional fluorescence microscopy proteins are labeled with different fluorophores (e.g. with the help of antibodies). However, the optical resolution of light microscopes limits the detection of protein proximities to about 0.2 µm. A higher resolution is necessary to study the physical interaction of protein partners.

FRET using fluorescent proteins

Fluorescence resonance energy transfer (FRET) is a technique to resolve the proximity of proteins beyond the optical limit of light microscopes (in the range of 1–10 nm). It can be used to reveal interactions between proteins or structural changes within a molecule. FRET imaging measures the non-radiative transfer of photon energy from an excited fluorophore (the donor) to another fluorophore (the acceptor). It is very sensitive to the distance between the fluorophores and, thus, can serve as an indicator for close proximity (Fig. 1). There are only certain pairs of fluo-

rophores suitable for FRET experiments since besides other prerequisites the donor emission spectrum has to overlap the excitation spectrum of the acceptor.

Fluorescent proteins (FPs) like the green-fluorescent-protein (GFP) are very attractive for FRET experiments. They can be genetically fused to proteins of interest and expressed in cells making them an excellent reporter system for gene expression and protein localization in living cells. Several enhanced FP variants with different spectral properties are available. The cyan-colored CFP as donor and the yellow YFP as acceptor are best suited for FRET experiments in living cells.

The LSM 510 – an analytical instrument for FRET

Conventional three dimensional FRET imaging can be probably done with dif-

ferent confocal laser-scanning microscopes. The LSM 510 from Carl Zeiss (see journal cover), however, is designed not just as an imaging machine but as a versatile and extendable analytical instrument. It is equipped with several unique software and hardware functions that are advantageous for FRET studies. Examples are the build-in MULTITRACKING technology (patent pending), pixel-precise illumination and bleaching procedures, the REUSE function for reproducing experimental setups, on-line ratiometric analysis during image acquisition, all kinds of mathematical image operation and data export functions. There are LSM 510 configurations available equipped not only with the basic 458 nm laser line for CFP excitation but with an additional 413 nm laser that is ideal for cross-talk free FRET analysis. Finally, the instrument can be extended with new software and hardware options

Keywords

Fluorescent protein, green fluorescent protein, CFP, YFP, protein-protein interaction, spectral analysis, FLIM, FRET

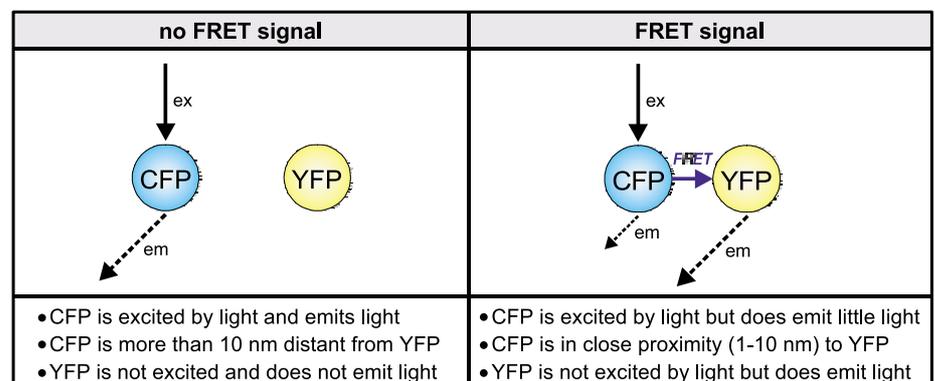


Fig. 1: The principle of FRET

to an analytical system specifically set up for the spectral analysis of FRET signals or for detecting FRET using fluorescence lifetime imaging (FLIM).

Confocal FRET imaging

FRET imaging usually starts with the visualization of YFP, CFP and the energy transfer signal. Due to their physical properties, the spectra of the FPs partly overlap. Their simultaneous excitation and detection in a co-expressing cell can lead to significant cross-talk. With MULTITRACKING Carl Zeiss has introduced a technology to effectively tackle the cross-talk problem. In addition, MULTITRACKING is ideal for the easy setup and modification of entire experimental FRET configurations. In a MULTITRACK configuration for FRET first YFP is excited and detected, then CFP and finally a signal is recorded under the excitation conditions for CFP and the detection conditions for YFP – the FRET signal (Fig. 2). If the fluorophores are more than 10 nm distant from each other only YFP and CFP signals should be detectable but the FRET channel should not show any signal. If, however, both fluorophores are located within a few nanometers to each other, the YFP signal should be the same, but the CFP signal should decrease since energy is transferred to YFP leading to a signal in the FRET channel. The latter indicates that the labeled protein partners are potentially interacting.

The acceptor bleach method

An elegant and easy-to-use approach for FRET detection is the acceptor bleach method. Pixel-precise illumination in regions of interest (ROIs) with automatic bleach control (e.g. in complex time-series) is a key feature for this method. In an acceptor bleach experiment, YFP and CFP signals are visualized before and after YFP has been bleached in a region of the cell. If CFP and YFP are in close proximity, the donor (CFP) fluorescence should increase in the ROI where the acceptor (YFP) has been bleached. Prior to the bleaching event energy from the donor is not resulting in fluorescence signal but is transferred to the acceptor via FRET. After depletion of the acceptor, however, the full energy is available for fluorescence of the donor. Consequently, the CFP signal increases in the bleached regions (Fig. 3).

The LSM 510 is equipped with all functions to easily perform acceptor bleach experiments and also with extensive measurement and export tools to quantify the changes in fluorescence intensities and to transfer the results to

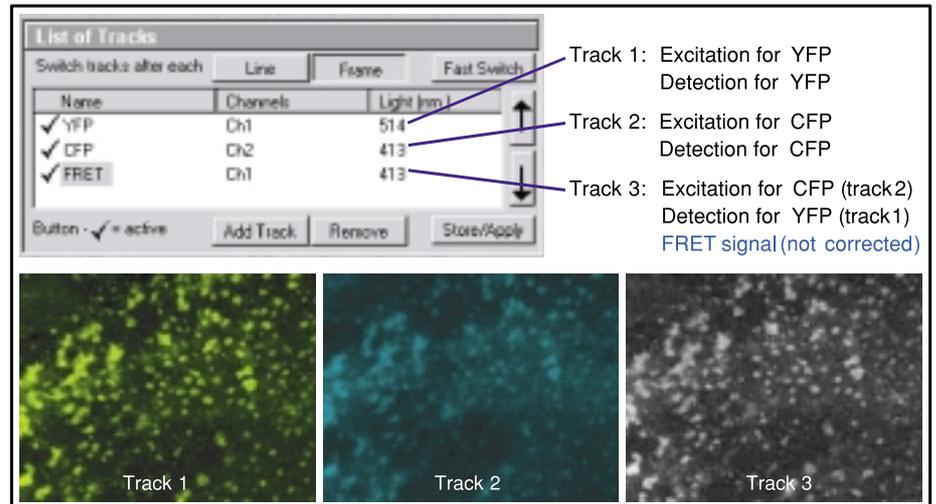


Fig. 2: MULTITRACKING setup for FRET experiments

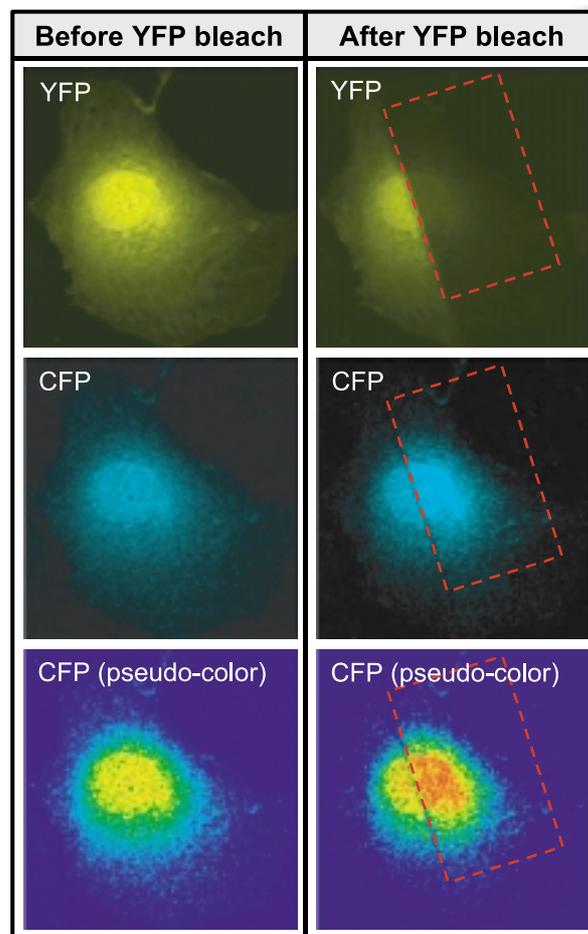


Fig. 3: The acceptor bleach method. CFP/YFP expressing cell before and after YFP (acceptor) bleach. As an indication of FRET the intensity of CFP (donor) signal is increased in the bleached region (red box). (B. Giese, G. Müller-Newen, Institute for Biochemistry, RWTH-Aachen, Germany)

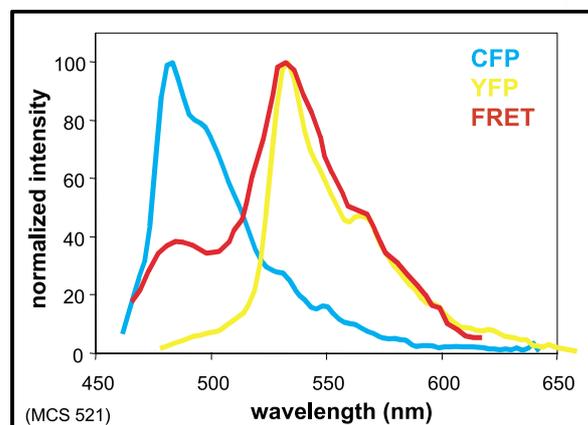


Fig. 4: Spectral emission analysis with the LSM 510 equipped with MCS. Spectra of CFP, YFP and a CFP/YFP co-expressing sample under FRET conditions.

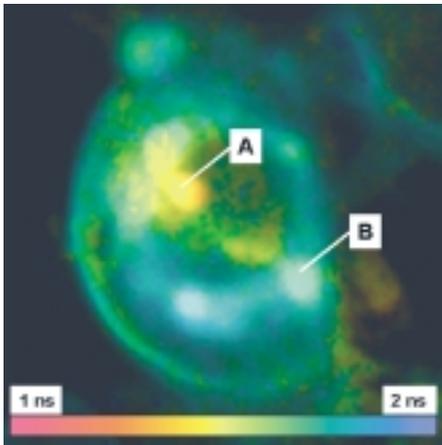


Fig. 5: FRET measurement via FLIM. Color-coded intensity-lifetime image of the donor (CFP) in a CFP/YFP expressing cell. Note that in position A the CFP-lifetime is shorter than in position B indicating FRET in position A.

statistics programs for further processing.

FRET analysis with the multi-channel-spectrometer

The LSM 510 fiber-out-coupling device allows the analysis of the optical signal with any kind of external detector. By connecting a multi-channel-spectrometer (MCS) from Carl Zeiss the emission signal of any user-defined ROI can be spectrally recorded. The z-dimension of this ROI is defined via the opening of an individual motorized confocal pinhole (multiple pinhole concept). Since the MCS employs a simultaneously illuminated diode array for spectral detection, the acquisition is fast and the spectral patterns are not distorted by bleaching artifacts. This is a prerequisite for quantitative analysis. Spectral analysis with automatic peak detection, integration, and normalization can be done with the Aspect Plus software that is part of the MCS package for the LSM 510.

Spectral emission analysis represents an excellent tool not only to characterize the spectral properties of different fluorophores but also for the quantification of the spectral changes that occur in FRET experiments (Fig. 4).

FRET analysis with FLIM

Fluorescence lifetime imaging (FLIM) is a new high-end

application that can be adapted to the multi-photon-system from Carl Zeiss – the LSM 510 NLO equipped with a femtosecond laser and the FLIM add-on from Becker & Hickl GmbH.

FLIM measures the time a fluorophore stays in the excited state. The fluorescence lifetime (τ) is concentration independent and characteristic for different fluorophores. Typical lifetimes range between 1 and 5 ns. In the case of FRET, the excited state energy of the donor (CFP) is transferred to the acceptor (YFP) primarily via dipole-dipole interaction. This process shortens the lifetime of donor (CFP) fluorescence and can be used as an indicator for FRET (Fig. 5). Thus, FLIM represents yet another method to visualize and quantify FRET in living cells with the LSM 510.

Conclusion

The application oriented concept of the LSM 510 allows to carry out FRET experiments at different levels of complexity and to verify the results by different approaches for FRET measurements. Several functions predestine the LSM 510 for FRET analysis with FPs like CFP and YFP. Even with a basic configuration FRET imaging and acceptor bleach experiments can be easily performed. New software and hardware options for spectral emission analysis or FLIM measurements make the LSM 510 from Carl Zeiss to a versatile imaging and fluorescence analysis center.

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