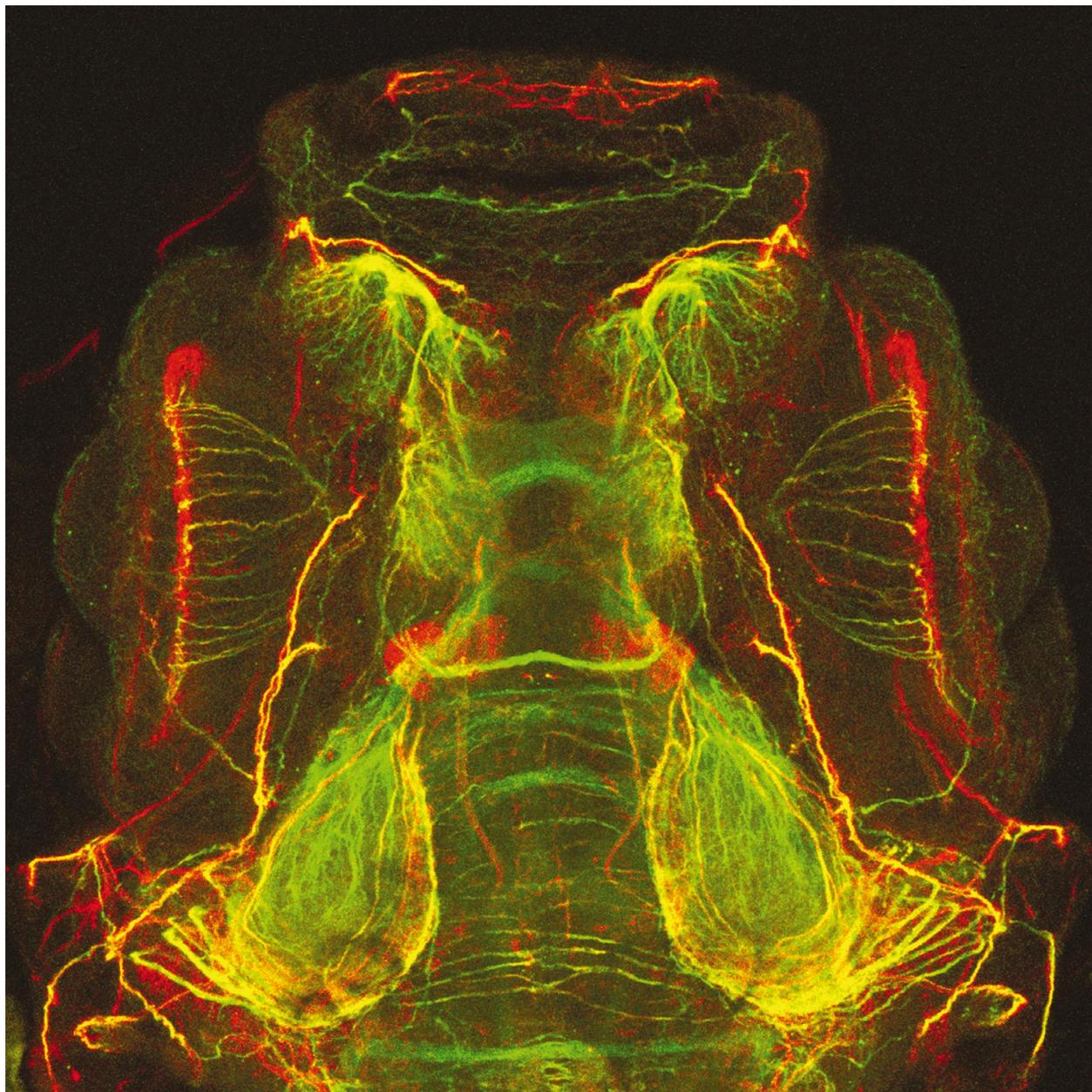


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**Confocal Microscopy Analyzes Cells**



# Confocal Microscopy Reveals Molecular Interactions

by Dr. Richard Ankerhold and Dr. Bernhard Zimmermann

## SUMMARY

When and where specific proteins interact with each other is the central question that needs to be resolved to gain a better understanding of their function. Confocal laser scanning microscopes can support key approaches to the study of functional interactions of bio-molecules.

In living cells, protein activity often depends on their association in a complex of several molecules. For example, the replication of chromosomes prior to cell division requires the cooperation of proteins such as DNA polymerases, helicases and ligases to build the replication machinery. Furthermore, most enzymes need to interact with co-factors to become active. For instance, protein kinase C is a crucial enzyme that influences a great number of cellular processes, including secretion, cell growth and differentiation. The interaction of the kinase with specific lipid co-factors is essential for the regulation of its activity.

To study when and where proteins associate with each other, scientists commonly label them with different fluorophores and subject them to fluorescence microscopy. Labeling can be either indirect, by using fluorescently labeled antibodies that are directed against the protein of interest, or direct via conjugation between protein and fluorophore. A popular method for the latter approach is the

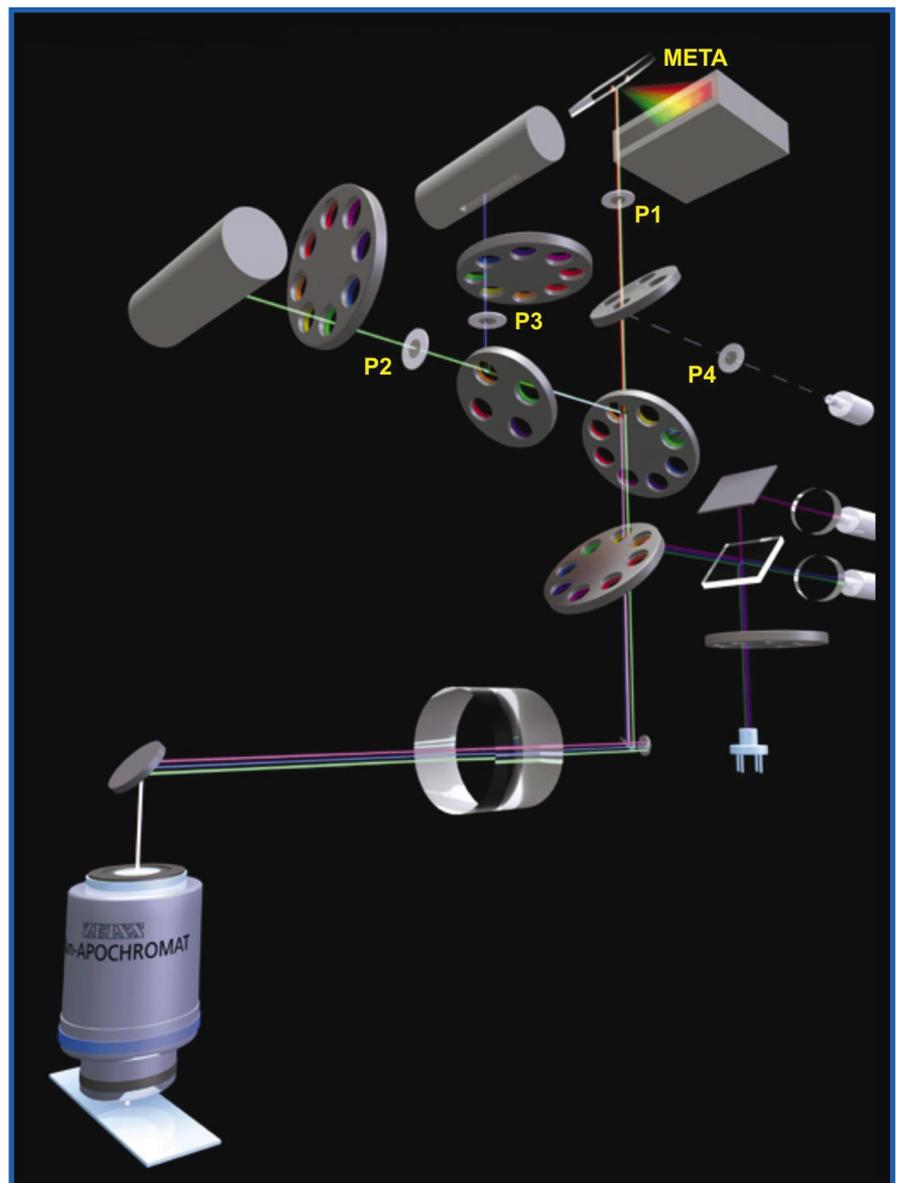


Figure 1. Individual pinholes in front of every detector (P1-P4), including the Meta detector, allow the user control of confocal slice thickness for each channel.

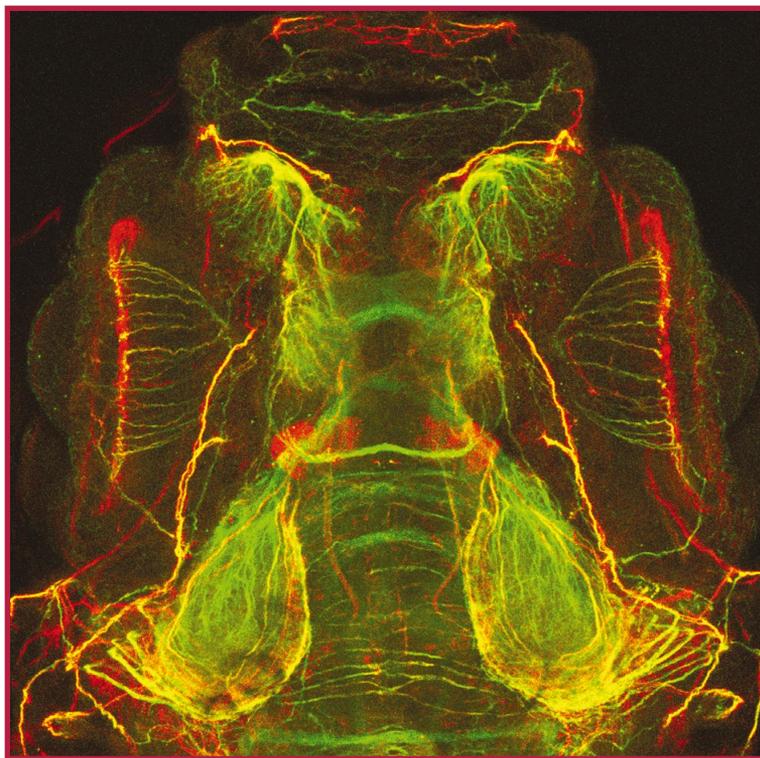
genetic engineering of fusion proteins composed of the protein of interest and green fluorescent protein (GFP) or its variants. The fusion construct can then be expressed in cells, tissues or even whole animals.

A major advantage of confocal methods over conventional fluorescence microscopy is the ability to optically section cells and tissue in three dimensions without mechanically disrupting the structure of interest. In addition, a laser scanning microscope allows the user to fully control the depth of focus and eliminate unwanted light from out-of-focus areas. Quantitative colocalization assays, fluorescence resonance energy transfer (FRET) microscopy and fluorescence correlation spectroscopy are all techniques that can be used where confocal methods are beneficial or essential to investigate the functional interaction of molecules.

### Identifying colocalization

In colocalization experiments, the coincidence of two fluorescently labeled proteins in the same spot is indicative of their potential interaction. Hereby, confocal technology virtually excludes the possibility of false-positive observations that can result from the low depth of focus of conventional fluorescence microscopy.

Colocalization studies require identical dimensions for the detection volumes from which the two signals are collected. Most importantly, the confocal sections must exhibit identical thicknesses; otherwise, the reconstructed 3-D image will show overlapping parts that do not overlap in reality. Also, the two fluorescent signals indicating the position of the proteins must be completely and unambiguously separated, each in its own im-



**Figure 2. A confocal image of a 6-day-old zebra fish (dorsal view) embryo that has been double-labeled with antibodies against cell adhesion molecules shows staining of different subpopulations of axons in the nervous system. Yellow indicates areas of colocalization, while other axons show either green or red fluorescence. Courtesy of M. Marx, University of Konstanz, Germany, and M. Bastmeyer, University of Jena, Germany.**

ing channel. Finally, the method requires advanced software functions specialized for quantitative analysis of the colocalization data.

This may sound simple, but it is not. The thickness of confocal image slices depends upon the excitation and emission of the dyes used, as well as on the size of the confocal pinhole; the pinhole can help compensate for differences in slice thickness that result from different-colored dyes. However, this requires a multipinhole system, so that the user can adjust the optical slice thickness for each color/detector individually. The LSM 510 series from Carl Zeiss provides the required architecture with individual motorized pinholes in front of every detector and automatic adjustment routines (Figure 1). The software allows the researcher to adjust the pinholes of each confocal channel to the same optical

thickness, independent of the color.

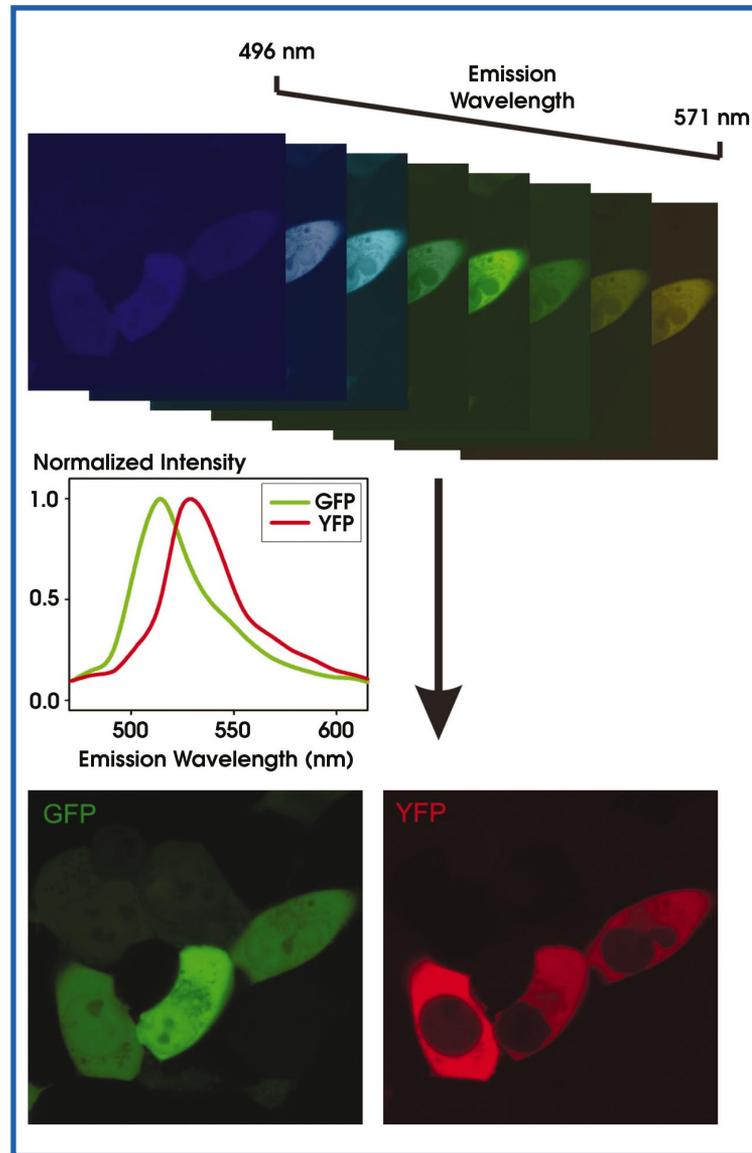
Complete separation of the fluorescent signals requires careful selection of the fluorescent dyes for a colocalization experiment. Because of the spectral properties of dyes, the emission of one dye typically overlaps the emission of the second. This effect is present in most combinations of fluorescent dyes, even for the classical combination of FITC and rhodamine. Simultaneous excitation and detection of these dyes causes the green fluorescence emission to contribute to the detection of the red, a phenomenon known as crosstalk. One solution implemented in the microscope, multitracking, allows the user to alternate between excitation lines in an extremely fast manner that can be applied to live-cell measurements. This procedure can minimize crosstalk and may even eliminate it if the excitation spectra of the dyes do not overlap.

However, this doesn't eliminate crosstalk for all experiments because some studies require fluorophores with overlapping emission and excitation spectra, such as combinations of GFP and its variants.

To address this issue, emission fingerprinting, a detection technology incorporated into the LSM 510 Meta, can separate the spectra even if the overlap is extreme. The system allows the user to completely separate fluorescence emissions even if much of the emission profiles overlap, such as green and yellow fluorescent protein or GFP and Sytox Green.

Emission fingerprinting requires the acquisition of the spectral composition of the fluorophores' emission signatures in the sample and mathematical separation into images representing the distribution of the individual fluorophores (Figure 3). An algorithm allows the user to au-

**Figure 3.** For emission fingerprinting of GFP and yellow fluorescent protein in cultured cells, the user acquires the spectral emission signature in a series of spectrally resolved images (lambda stack) followed by linear unmixing on the basis of reference spectra for the proteins. The result is a pair of images representing the GFP and yellow fluorescent protein distribution in the sample, respectively. Sample courtesy of Frank D. Böhmer, Friedrich Schiller University.



tomatically extract the spectral components. A highly parallel acquisition scheme makes emission fingerprinting ideal for live-cell imaging, particularly in the online mode where the separation occurs instantaneously during data acquisition.

"We are anxious to move into multicolors," said William C. Hyun, director of the Laboratory of Cell Analysis at the University of California, San Francisco. "The ability to tune emissions and pick out the specific pattern of these fluorescent proteins allows us to go in and tease apart multiple probe sets for multiple GFPs. Being able to clear out the complicated spectral overlaps is a tremendous benefit."

### Key to success

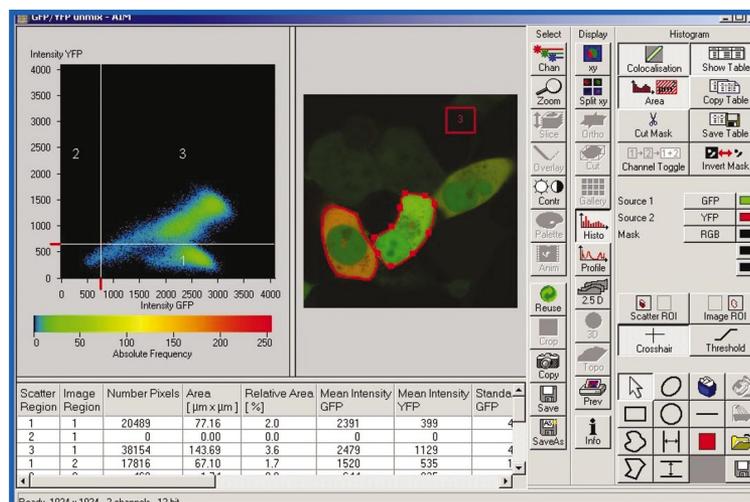
Accurate three-dimensional detection with a multiple pin-hole system and unambiguous separation of spectrally overlapping dyes are the first prerequisites for a successful colocalization experiment. Such studies also require software functions for

analysis of the resulting image. The latest version of the LSM 5 software (Rel. 3.2) introduces a colocalization analysis package (Figure 4).

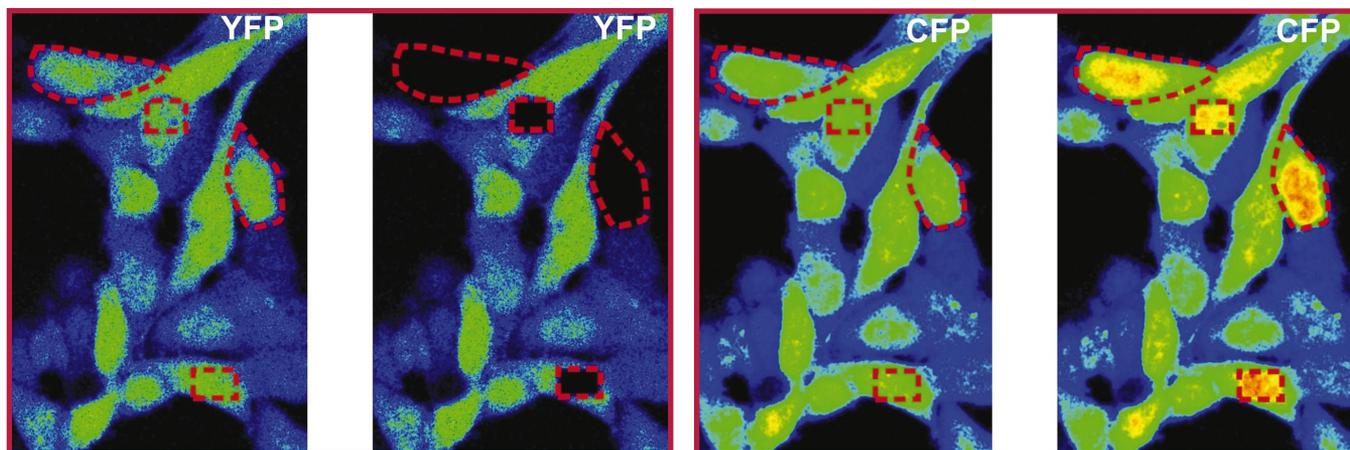
Key is the interactive link of the image display with a two-dimensional scatter plot representing the distribution of individual voxels of the image according to the signal intensities of the image channels. Either in the image display or in the scatter plot, regions can be selected interactively for analysis. A comprehensive set of quantitative data, including various colocalization coefficients and correlation parameters, can be directly extracted from the selected regions. With this, the user can adjust colocalization thresholds according to background intensities within the images or can perform, visualize and quantitatively assay for colocalization in the image set and color-overlay image voxels that are positively tested for colocalization.

The optical resolution of light microscopes, including confocal microscopes, limits the detection of protein proximities to about 0.2  $\mu\text{m}$  in conventional colocalization experiments. However, studying the physical interactions of protein partners requires higher resolution.

FRET can resolve the proximity of proteins beyond the optical limit of light microscopes (in



**Figure 4.** The software allows the user to perform quantitative colocalization analysis.

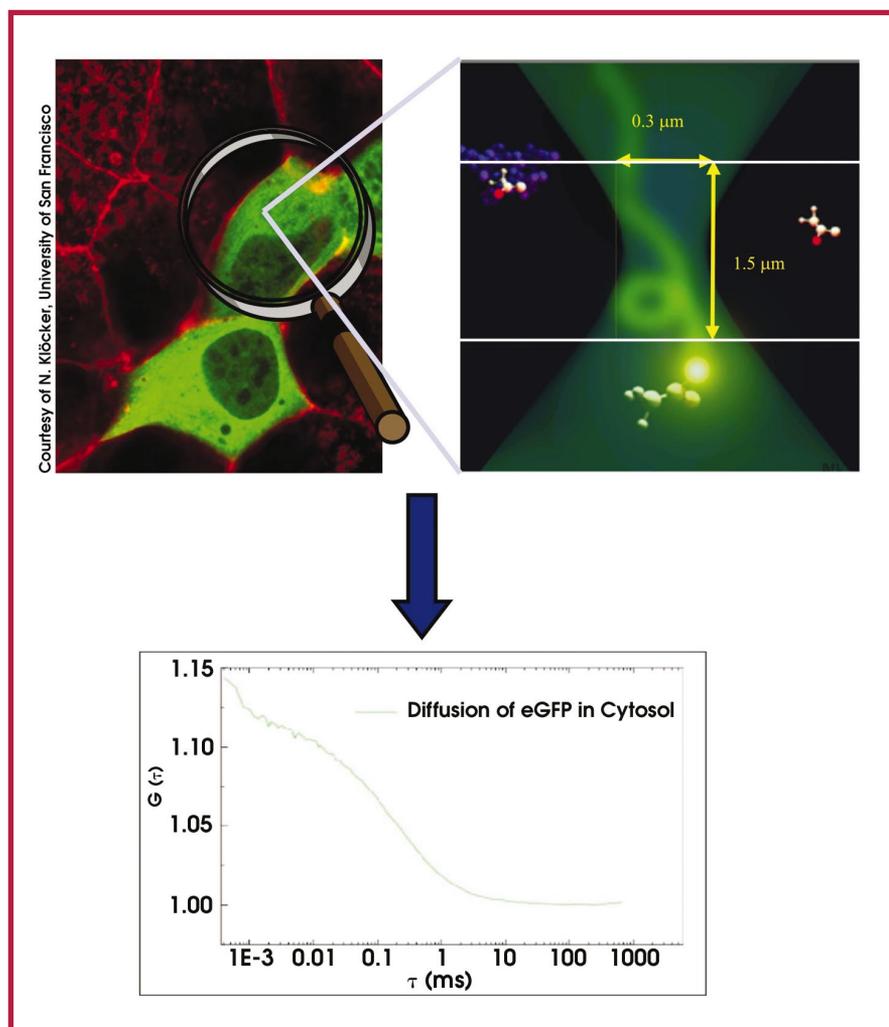


**Figure 5. Emission fingerprinting reveals complete bleaching of yellow fluorescent protein and concomitant dequenching of cyan fluorescent protein, indicating fluorescence resonance energy transfer in the bleached areas (marked by dashes).**

the range of 1 to 10 nm). FRET imaging measures the nonradiative transfer of photon energy from an excited fluorophore (the donor) to another fluorophore (the acceptor). This interaction results in quenching of donor fluorescence and simultaneous sensitized photon emission by the acceptor molecule. Its efficiency depends on various parameters, including a sufficient overlap of donor emission and acceptor excitation spectra (as in cyan and yellow fluorescent proteins, today the most widely used FRET pair).

The amount of energy transferred depends on the distance between the donor and acceptor. Therefore, the technique can be used as a molecular ruler and as an indicator of physical interactions between fluorescently labeled molecules. The use of pairs of fluorescent proteins, such as cyan fluorescent protein and yellow fluorescent protein as donor and acceptor molecules, respectively, has greatly facilitated in vivo FRET studies because they allow labeling of the molecules of interest with excellent specificity while retaining the integrity of the cell and its physiological functions.

Several methods have been developed for imaging FRET in cells. One approach involves either bleaching the acceptor molecule and subsequent monitoring of concomitant dequenching of donor fluorescence or examining relative temporal changes of donor emission and acceptor/FRET signals under donor excitation in dynamic situations (Figure 5). An al-



**Figure 6. By combining a confocal laser scanning microscope and a fluorescence correlation spectrometer, structures can be made visible and, at the same time, molecular interactions can be investigated.**

ternative approach uses various excitation-emission configurations (such as donor excitation-donor emission, acceptor excitation-acceptor emission, donor excitation-acceptor emission) and subsequent evaluation of sensitized acceptor emission via mathematical correction. The LSM 510's abilities, such as multitracking, pixel-precise bleaching and online ratiometric analysis, allow the user to perform the described types of FRET experiments with high three-dimensional resolution.

Until now, however, acceptor and donor with overlapping excitation and emission spectra required that the user select narrow detection bands to differentiate between emissions. Even then, quantitative approaches had to include complex mathematical corrections to account for inevitable crosstalk between channels and for local concentration differences.

Emission fingerprinting overcomes several of these drawbacks. Parallel acquisition of the emissions from donor and acceptor provide the high photon efficiency and the image acquisition speed required in live-cell FRET microscopy. Sorting donor

and acceptor emissions into separate output channels can produce significantly bigger signal changes in dynamic FRET situations. Finally, this method eliminates the need to correct for emission crosstalk in quantitative studies.

While colocalization and FRET experiments still measure the distance between the protein partners of interest as an indicator of their possible interaction, fluorescence correlation spectroscopy directly measures the associated changes of diffusion times. We have combined our system with this technique in one instrument dedicated to fluorescence correlation spectroscopy analysis in living cells.

#### Single-molecule sensitivity

First, the researcher acquires a 3-D image stack of the cell. Then several fluorescence correlation spectroscopy analysis spots can be placed anywhere in or outside the cell at X, Y and Z positions. Each spot represents a measurement volume of less than  $10^{-15}$  l. When starting the measurement, the system excites the spot and measures the fluorescently la-

beled biomolecules passing through the defined volume as a photon shower of a defined time (Figure 6). From this, the system can calculate the characteristic diffusion times of the proteins of interest. If the instrument detects a prolonged diffusion time, the molecule of interest is directly interacting with another.

A software correlator analyzes the photons registered by the detector for time interrelations. Thereby, the instrument can distinguish between populations of biomolecules with different diffusion times; e.g., populations of single molecules and a population of molecules associated in a complex. High-sensitivity avalanche photodiodes allow it to detect single molecules, providing a tool for direct measurement of dynamic molecular interactions within living cells. □

#### Meet the authors

Richard Ankerhold is head of the Training, Application and Support Center, and Bernhard Zimmermann is product manager for confocal laser scanning and advanced imaging microscopy at Carl Zeiss GmbH in Jena, Germany.