FRET and FRAP using Emission Fingerprinting with the LSM 510 META

– A Superior Solution for Functional Cell Imaging
Genome sequencing provided fundamental insights into the repertoire of proteins supporting the life of organisms ranging from bacteria to humans. As a major outcome, these investigations boosted activities focusing on the question how cells use their inventory of genes and proteins for the control of development, for sustaining their structural integrity and for fulfilling their functions in a particular tissue environment. In the last years, the immense progress in understanding these topics has been substantially driven by advances in imaging techniques and the availability of genetically encoded, non-toxic fluorescent markers (e.g. GFP and its variants). Combining modern live cell labeling technology with the optical sectioning capabilities and flexibility of modern confocal laser scanning microscopes such as the LSM 510 META from Carl Zeiss enables to visualize and quantify distributions, mobilities and interactions of proteins as well as their spatio-temporal dynamics with excellent sensitivity, speed and 3D resolution.

Emission Fingerprinting: high-efficiency acquisition and quantitative separation

Recent advances in confocal imaging techniques culminated in the introduction of Emission Fingerprinting, an innovative detection technology supported by the new LSM 510 META from Carl Zeiss. Equipped with a detection unit capable of pixel-by-pixel recording of emission spectra, this microscope was specifically developed for studies involving multiple fluorochrome-tagged molecules. The system allows to exactly separate fluo-

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**Fig. 1:** Emission Fingerprinting of GFP and YFP in cultured cells with the LSM 510 META. Acquisition of the whole emission signals a series of spectrally resolved images (Lambda Stack) is followed by Linear Unmixing on the basis of reference spectra for GFP and YFP and results in a pair of images representing the GFP – and YFP distribution in the sample, respectively (Sample: Dr Frank-D. Boehmer, Friedrich-Schiller-University, Jena, Germany).
rescence emissions with extremely overlapping emission profiles such as GFP and YFP or even GFP and Sytox Green to substitute DAPI (Fig. 1). To this end, Emission Fingerprinting relies on the acquisition of the whole spectral emission signatures present in the sample and mathematical separation into images representing the distribution of the individual fluorochrome components [1]. Using the LSM 510 META, the recording of spectrally resolved images, called Lambda Stacks, takes place in a parallel manner. Thus, Emission Fingerprinting ideally fulfills the requirements critical for live cell imaging. This offers new opportunities in functional multifluorescence applications including FRET and FRAP:

**LSM 510 META – The FRET microscopy system**

Fluorescence resonance energy transfer (FRET) microscopy is a technique used to resolve and quantify distances between two molecule species or molecular domains. FRET may be defined as the non-radiative transfer of photon energy from an excited donor fluorophore to an acceptor molecule. 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 and acceptor excitation spectra (as in CFP and YFP). FRET also exhibits a steep dependence on the distance between donor and acceptor. Therefore, it may be used as an indicator of physical interactions between fluorescently labeled molecules. Moreover, intelligent molecular engineering of FRET constructs based on fluorescent proteins has recently produced sensor systems for monitoring enzyme activities, ion concentrations and activities of intracellular messenger molecules.

Several methods have been developed for imaging FRET signals in cells [2]. Unique software and hardware functions of the Carl Zeiss LSM 510 such as linewise switching between laser lines (Multitracking), pixel-precise bleaching and on-line ratiometric analysis provide the features necessary to perform the various types of FRET experiments with high 3D resolution.

So far, however, overlaps of excitation and emission spectra of acceptor and donor required to select narrow detection bands in order to differentiate between emissions. Even then, quantitative approaches had to include complex mathematical corrections accounting for inevitable crosstalk between channels and for local concentration differences.

Emission Fingerprinting with the LSM 510 META overcomes several of these drawbacks: Because of its potential to separate fluorescence signals despite spectral overlap it eliminates the need to correct for emission crosstalk. Sorting donor and acceptor emissions into separate output channels furthermore produces significantly bigger signal changes in dynamic FRET situations (Fig. 2, 3).
FRAP – Fluorescence recovery after photobleaching

FRAP and the related FLIP technique (Fluorescence Loss In Photobleaching) are popular methods that utilize changes and recovery of fluorescence after local bleaching events to measure the dynamics of 2D or 3D molecular mobility e.g. diffusion or transport of fluorescently labeled molecules in membranes or inside living cells. (Fig. 4). Either technique benefits from the LSM 510 META technology as Emission Fingerprinting allows to monitor the dynamics not only of one fluorescent tag, but also of two or even more spectrally overlapping labels. For these types of experiments, speed and highly efficient scanning strategies are of critical importance as any time delay after the bleaching event will shift the starting point of the recovery function. Furthermore, time delays between consecutive image frames should be minimal as they allow changes to take place without being monitored by the system. To minimize the time delay after bleaching, Carl Zeiss introduced excitation/acquisition regions of interest defined to a single pixel (real ROIs) and independent bleach ROIs of any shape and sizes as small as 1 pixel as early as 1997. In addition, the scanner control of the LSM 510 guarantees dead times between the image frames of less than 10 %. This is unique among currently available laser scanning microscopes.

Very recently, a third FRAP-related method was described by Dunn and coworkers [3] and named FLAP (Fluorescence Localization After Photobleaching). In this type of experiment, the molecule of interest is labeled with two fluorophores e.g. CFP and YFP, one to be bleached and the other to act as a reference label. This combination permits to monitor the localization of the bleached molecules themselves as the FLAP signal is represented by a simple difference image (unbleached fluorophore – bleached fluorophore; Fig. 4). Therefore, FLAP is comparable with methods of photoactivation or uncaging fluorescent probes, but avoids exposing the living cells to harmful UV irradiation and is taking advantage of the fluorescent proteins to be expressed by living cells. Even though a standard LSM 510 was used in the study by Dunn and colleagues, the relevance of Emission Fingerprinting for FLAP is obvious as yet again it allows to quantitatively separate the dyes of interest even when their emissions are heavily overlapping.

References