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MICROSYSTEMS

Spectral fluorescence lifetime imaging microscopy: new dimensions with Leica TCS SP5

The new Leica TCS SP5 spectral confocal and multiphoton microscope system is based on the widely used SP® spectral detection module. This module allows selection of any spectral emission band in the visible range. Five spectral bands can be selected simultaneously. As an extension, two of these detectors may operate as a sensor for fluorescence lifetime imaging microscopy (FLIM). This SP-FLIM combination opens up a completely new dimension in wavelength-dependent lifetime imaging.

Spectral detection

Leica's system for spectral confocal detection, available since 1997 under the Leica TCS SP brand, has been adopted and modified throughout the confocal marketplace, and various concepts with different benefits and disadvantages have been introduced. The SP system allows the tuning of emission bands to any specification within the range of detection. Because of this and other advantages, it has again been implemented as a basic design element of the Leica's latest spectral confocal and multiphoton imaging system: the Leica TCS SP5, a system with tunable emission bands at maximum transparency for optimum signal efficiency is the primary goal of the invention.

The SP detector follows a series of other inventions for confocal microscopy developed by Leica—Acousto Optical Tuneable Filters (AOTF), for fast selection and stepless attenuation of laser lines, and the Acousto Optical Beam Splitter (AOBS®), for excitation-emission separation[1,2]. The principle of SP detection is shown in **Figure 1**. The emission is spread spectrally by a prism and then guided to a spectrometer slit that allows any emission band to be selected. A prism was chosen rather than a grid, as prisms offer better transmission and therefore produce images with much better signal-to-noise ratios as compared to alternative technologies. To operate with multiple bands simultaneously, the slit barriers consist of high-reflectance mirrors. This opens up the possibility of using the residual fraction of the spectrum to apply to further photometer slit devices. The Leica TCS SP5 can operate up to five such tunable high-efficiency channels simultaneously.

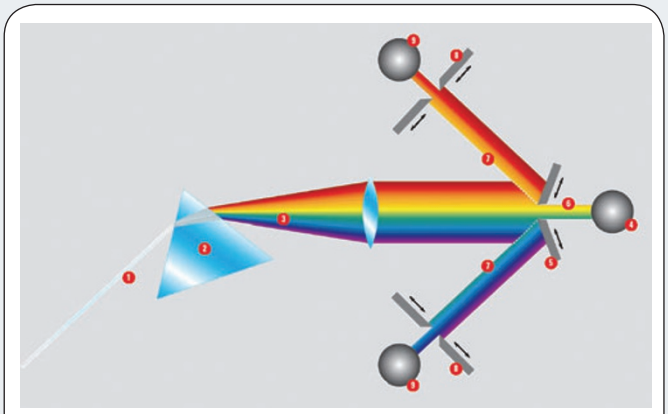

Figure 1 | The SP principle. Emission from fluorochromes in the sample (1) is dispersed by a prism (2) into a spectrum (3). Detectors (4, 9) receive sections of the spectrum (6, 7) defined by movable mirrors (5, 8), which form spectrometer slits.

Fluorescence lifetime

The SP detector allows the detection of emissions resulting from the spectral properties of the fluorochromes in the sample. An additional parameter of this fluorescence is the decay time of fluorescence. During the process of absorption of a photon by a fluorochrome, the electron system of that fluorochrome is excited from a ground state (S_0) to an excited state (S_1). From this excited state, the fluorochrome decays back into the ground state. The lifetime of the excited state depends on the fluorochrome and the environment in which the fluorochrome is embedded.

The mean lifetime can be measured as follows. Upon a brief laser pulse, the time is measured from laser pulse to the first photon recorded by the sensor (a fast photomultiplier tube). To get a significant decay curve, this measurement is repeated sufficiently often. The resulting data can be plotted as a histogram (counts versus delay time), which shows an exponential decay. The decay time is then measured by a least-squares fit to the histogram. Of course, under normal sample conditions, the decay is often not sufficiently described by a single exponential fit. Therefore, two or three decay times are required for a good representation. Typical decay times for pure fluorochromes are

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in the range of 0.5–5 ns. Quenching processes—non-radiative energy transfer from the excited state of the fluorochrome to other molecules in the vicinity—will speed up the depletion of the excited state. This causes the measured lifetime to shrink substantially.

As the fluorescence lifetime is independent of the concentration of the fluorochromes, this parameter is an alternative for measuring metabolites in living samples. The influence of quenching molecules, including fluorescence resonance energy transfer (FRET) to other fluorochromes, allows identification of microenvironmental properties and structural distances in the sample. Scanning imaging systems allow lifetime measurement in all picture elements separately. The fluorescence lifetime can be coded in colors and displayed as a fluorescence-lifetime image, also known as a tau map. The tau map is created by repeatedly scanning the full image and recording histograms at each pixel position. The histograms of all scans are merged for averaging to enhance the significance of the data.

Leica SP-FLIM

The SP detector is also a very elegant device for performing wavelength-dependent FLIM. The new TCS SP5 confocal and multiphoton microscope system allows two FLIM detectors to be inserted into the SP module. This makes it possible to measure FLIM at any wavelength band in the visible range, independent of the excitation wavelength. Recording FLIM at each wavelength, one can measure a spectrum of lifetime with two channels simultaneously.

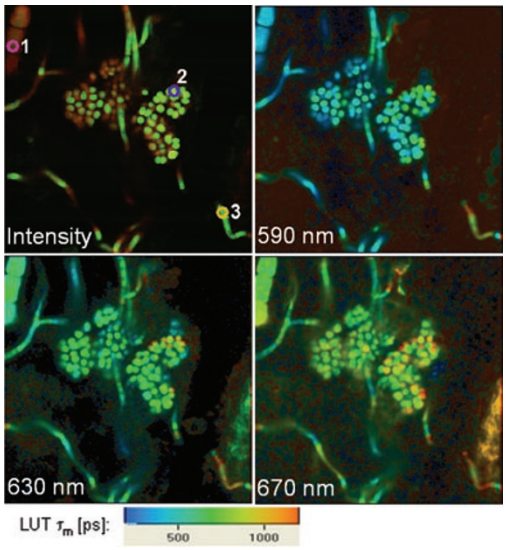

Figure 2 | SP FLIM gallery of cyanobacteria. Fluorescence intensity image with three regions of interest highlighting large cells in short chains (1), spherical types (2) and thin chains of elongated cells (3; top left). The other images in the gallery show tau maps for various emission wavelengths, as indicated. Different cell types exhibit diferential decay, but cells of the same type also have varying decay times. This is most probably due to energy transfer effects occurring in the light-absorbing structures of these complex compounds of different fluorescent molecules.

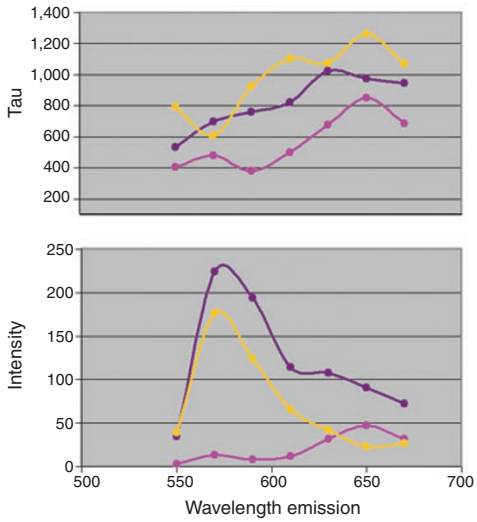

Figure 3 | Dependence of lifetime and intensity in various cell types as a function of emission wavelength in three different cell types. Colors refer to regions of interest indicated in **Figure 2**.

This opens up a new dimension in modern fluorescence imaging technology: SP-FLIM. As an example, wavelength-dependent lifetime was measured in mats of cyanobacteria from Roman catacombs. These samples had already been used for excitation spectroscopy with white-emitting laser sources[3]. The intensity image (**Fig. 2**) shows morphologically distinct types of fluorescent microorganisms: large cells in short chains, spherical types and thin chains of elongated cells.

Lifetime images were recorded at varying emission bands in the range of 550 nm to 690 nm to see possible effects and correlations of emission wavelength and lifetime. **Figure 2** also shows a selection of tau maps at wavelengths of 590 nm, 630 nm and 670 nm. From this tau-map sequence, one can read the mean lifetime for selected regions of interest, as described above. The graph in **Figure 3** shows the intensity and the lifetime as a function of emission wavelength. Obviously, the spectral emission intensity does not correlate with fluorescence lifetime. This implies new insights in structural as well as in kinetic properties of biological samples.

Applications could include separation of different fluorescence species with identical or similar spectral emission properties, quenching effects and interactions of fluorochromes. The novelty of this method creates the potential for a broad range of new experiments and insights.

1. Birk, H. *et al.* Programmable beam-splitter for confocal laser scanning microscopy. *Proceedings of SPIE* **4621**, 16–27 (2002).
2. Borlinghaus, R. Benefits and applications of programmable spectral devices in confocal microscopes. *Proceedings of SPIE* **4964**, 7–13 (2003).
3. Borlinghaus, R. *et al.* Closing the spectral gap – the transition from fixed parameter fluorescence to tunable devices in confocal microscopy. *Proceedings of SPIE*; published online 23 February 2006.