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Add one more Dimension to 3D Imaging

Fluorescence Lifetime Imaging with the Zeiss LSM 510 Laser Scanning Microscope and the Becker & Hickl SPC-730 TCSPC Module

Introduction

Laser Scanning Microscopes have initiated a breakthrough in biomedical imaging. High contrast due to effective suppression of light scattered from outside the focal plane, simple fluorescence imaging by single photon or two-photon excitation and the 3D imaging capability are features beyond the reach of conventional microscopes.

To investigate molecular interactions in cells and subcellular structures fluorescence markers are used which specifically link to protein structures. Staining the sample with different dyes and recording the fluorescence image reveals the cell structures via the different wavelenth and fluorescence decay time of the dyes. Furthermore, energy transfer between the dye molecules and the proteins changes the fluorescence quantum efficiency and thus the fluorescence decay time. Due to the variation of the dye concentration such effects are not visible in the intensity images. Therefore, parallel imaging of the fluorescence intensity and the fluorescence decay functions significantly enhances the performance of a 3D laser scanning microscope.

The TCSPC Laser Scanning Microscope

Recording time-resolved fluorescence images can be achieved by combining a laser scanning microscope and the Time-Correlated Single Photon Counting (TCSPC) technique. Therefore, Zeiss - the leading manufacturer of laser scanning microscopes - and Becker & Hickl - the leading manufacturer of TCSPC electronics - have cooperated to introduce the first TCSPC Laser Scanning Microscope. The system is based on the Zeiss LSM-510 NLO Confocal Laser Scanning Microscope and the Becker & Hickl SPC-730 TCSPC Imaging Module (fig. 1).

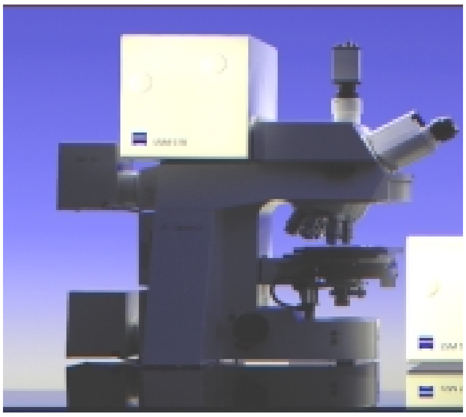
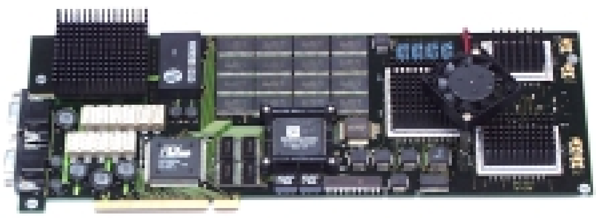

Fig. 1: The LSM-510 Laser Scanning Microscope (left) and the SPC-730 TCSPC Imaging Module (right)

The general setup is shown in figure 2. A Coherent Mira Optima 900F TiSa laser delivers femtosecond pulses over a wavelength range from 700 nm to 1000 nm. The repetition rate is 76 MHz, the typical pulse width is below 200 fs FWHM.

The LSM-510 scans the sample in the x-y plane providing an image of the sample in the focal plane of the objective lens. By changing the depth of the focus in the sample, 3 D imaging of the sample is achieved. Furthermore, the full range of flexible scanning modes of the LSM 510 NLO including time lapse recording capabilities can be used.

The short excitation pulses in conjunction with the high power density in the focus of the microscope lens enable effective two-photon excitation of typical fluorescence marker dyes and of the autofluorescence of biological samples. The near-infrared excitation light easily penetrates deeply into the sample. Fluorescence images can be recorded from sample layers as deep as 100 um.

The LSM-510 has up to four detection channels equipped with individual confocal pinholes, a range of filters, and highly sensitive photomultipliers (PMTs) suitable for photon counting and analog signal recording as well. One of the PMTs can be selected to deliver a single photon signal to the SPC-730 TCSPC module while the other three detectors are used to record steady state images via the standard LSM image recording electronics.

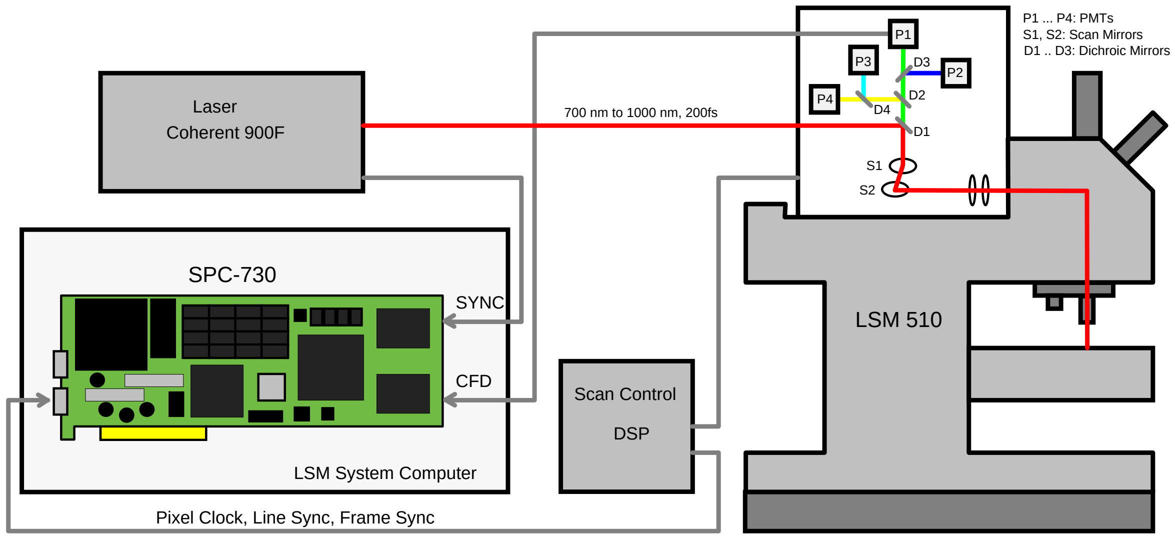

Fig. 2: Basic setup of the TCSPC Laser Scanning Microscope

To record the image data with ps resolution, the SPC-730 TCSPC Imaging Module is used. It is installed in the LSM computer and connected to the microscope via the scan control signals (Pixel Clock, Line Sync, Frame Sync) of the microscope scanning controller and the PMT output signal. The single photon pulses from the PMT are fed to the CFD (Constant Fraction Discriminator) input of the SPC-730 module. Synchronisation with the laser pulse sequence is achieved by the SYNC signal from a fast photodiode in the excitation path or directly from the Coherent laser .

To synchronise the recording in the SPC-730 with the scanning process in the microscope, the SPC-730 module accepts the 'Pixel Clock', 'Line Sync' and 'Frame Sync' signals from the scan control system (DSP: Digital Signal Processor) of the LSM 510. For each pulse at the CFD input (i.e. for each photon), the SPC-730 determines the time of the photon within the laser pulse sequence and the location within the scanning area. These values are used to address a histogram memory in which the events are accumulated. Thus, in the memory the distribution of the photon density over X, Y, and the time within the fluorescence decay function builds up. The result can be interpreted as a two-dimensional (X, Y) array of fluorescence decay curves or as a sequence of fluorescence images for different times after the excitation pulses.

The time resolution depends on the detector and is 25 ps (fwhm) with external Multi Channel Plate (MCP) detectors and 200 ps (fwhm) with the built-in PMTs. For comparison, typical lifetimes of fluorescence markers are in the range of 3...15 nanoseconds.

Interestingly, there is practically no loss of photons in the TCSPC imaging process. As long as the photon detection rate is not too high all detected photons are processed and accumulated in the histogram thus providing maximum sensitivity. This is a key advantage of the combination of TCSPC Lifetime Imaging and Laser Scanning Microscopy and a striking benefit over gated image intensifiers which gate away the majority of the fluorescence photons.

Results

Figure 3 shows the unprocessed TCSPC image of a single cell layer (double staining with Hoechst for DNA and Alexa488 for Tubulin) obtained by simultaneous two-photon excitation at 800 nm. The overall acquisition time was 60 seconds. The image consists of 128 x 128 pixels each containing a complete fluorescence with 256 time channels. The fluorescence decay curves over a selected horizontal stripe of the image are shown in figure 4.

The Becker & Hickl control software offers user-friendly data handling and display as well as system control and subsequent data analysis. It is working in the multitasking mode with the LSM 510 control software on the LSM computer.

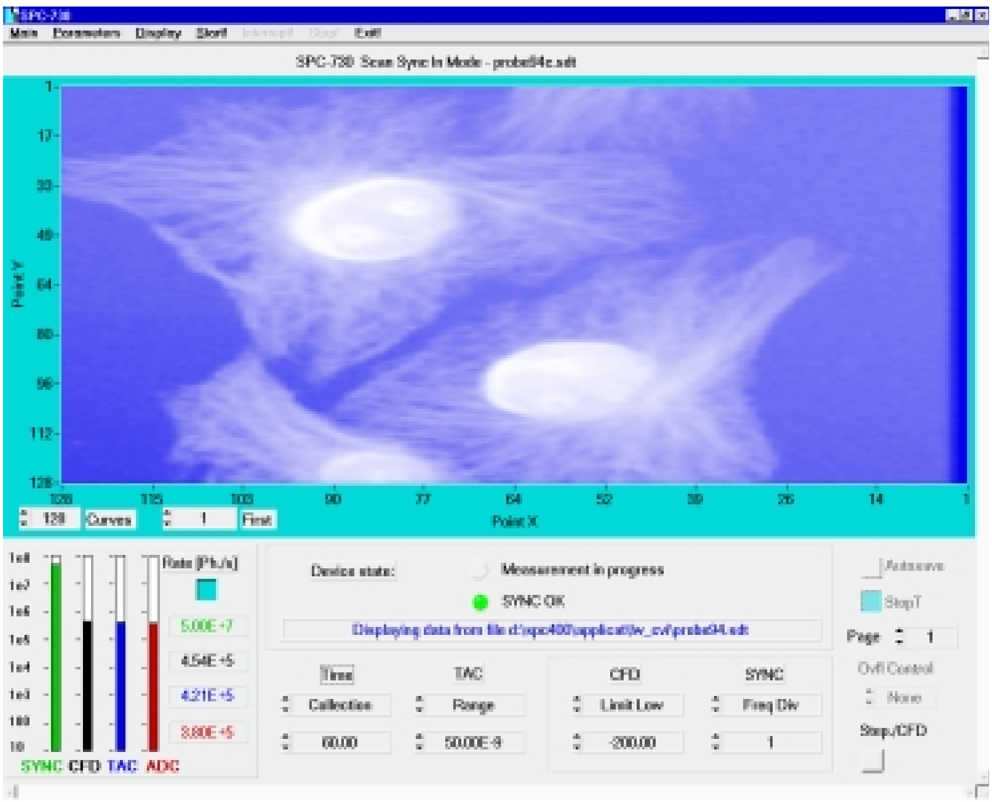

Fig. 3: SPC-730 control panel with the unprocessed TCSPC image

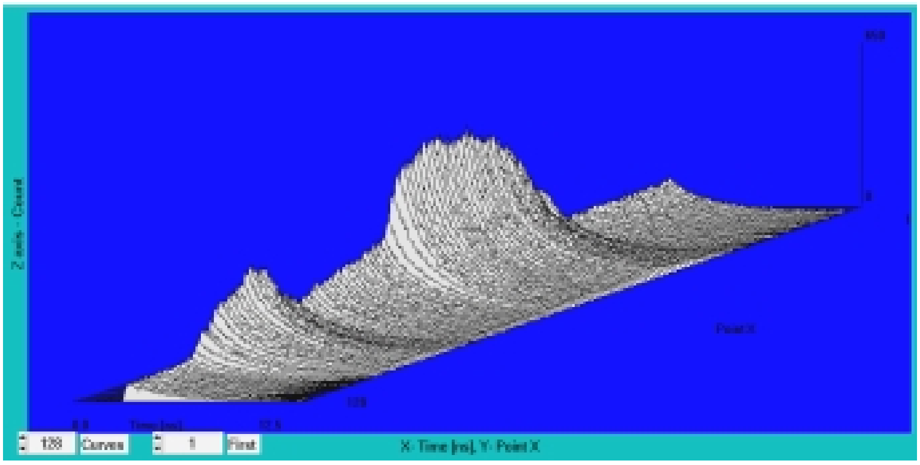

Fig. 4: Fluorescence decay curves over a 32 pixel wide horizontal stripe of the image

Data analysis by the SPCImage software (Becker & Hickl) delivers the fluorescence decay times of the individual pixels of the image. A typical result is shown in fig. 5. The intensity image (containing the photons of all time channels) is shown left. For the image right, both the intensity and the decay time of the pixels was used. The intensity is displayed as brightness, the decay time as colour of the resulting image. The quality of the fit is shown for two selected pixels (fig.4, bottom). The decay times of 2.0 ns and 2.8 ns are clearly distinguished.

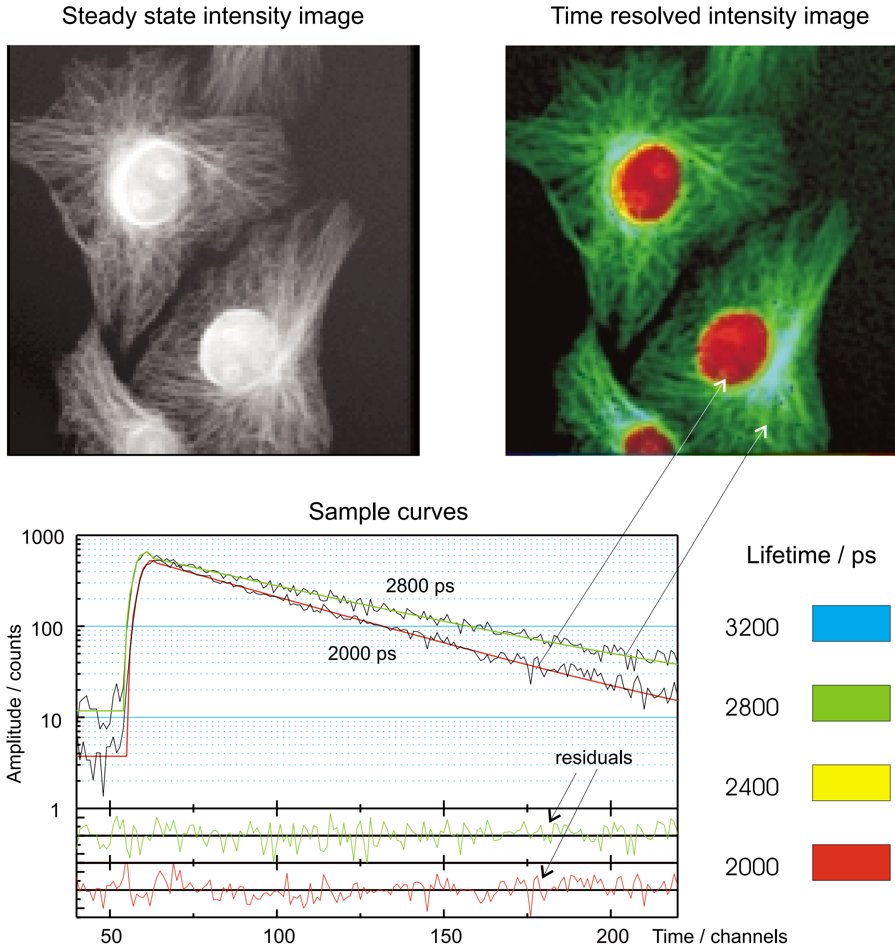

Fig. 5: Intensity image (top left), τ image (top right) and fitted curves for two selected pixels

The table below gives an overview about the accuracy of the decay time that can be expected for various recording times and the numbers of pixels. The values are related to a typical photon count rate of 10^5 to 10^6 cps and a fluorescence decay time from 0.3 ns to 5 ns.

Recording Time	**Image Size (Pixels)**	**Accuracy of τ**
10 min	128 x 128	< 1 %
1 min	128 x 128	2 %
10 s	128 x 128	10 %
10 s	32 x 32	2 %
1 s	32 x 32	15 %
1 s	8 x 8	< 2 %

Table 1: Accuracy of Decay Time τ depending on Recording Time and Image Size

As can be seen from the table above, a high accuracy of τ can be achieved either by long recording times or by imaging the region of interest with a reduced number of pixels. For low pixel numbers the recording time can be less than 1 s, allowing even the investigation of short-term phenomena in living cells.

Summary

The combination of Time Correlated Single Photon Counting (TCSPC) and Confocal Laser Scanning Microscopy offers new possibilities for the detection of weak signals, quantitative measurements and fluorescence lifetime imaging as a novel contrasting method in microscopy. Lifetime imaging is an indicator for the fluorescent dye localisation and the micro environmental condition.

The symbiosis of the Becker & Hickl TCSPC imaging module and the Zeiss LSM 510 NLO Laser Scanning Microscope combines highly sophisticated photon counting technology and state of the art multiphoton excitation microscopy. The field of application covers separation of multiple fluorescent labels (i.e. GFP mutants), energy transfer measurements (FRET) for protein-protein interaction, imaging of autofluorescence of cells, single molecule detection, and other fluorescence imaging applications of microscopic samples.