



TCSPC adds a new dimension to 3D laser scanning microscopy

Confocal 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. Adding ps time resolution by combining the confocal microscope with an advanced Time-Correlated Single Photon Counting (TCSPC) imaging technique yields a new powerful instrument for the investigation of molecular interactions in biological systems.

Introduction

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 fluorescence spectra and fluorescence lifetime of the dyes. Energy transfer between the dye molecules and the proteins changes the fluorescence quantum efficiency and thus the fluorescence lifetime. Due to the variation of the dye concentration these effects cannot be distinguished in simple intensity images. Therefore, recording time-resolved patterns of the full fluorescence decay functions rather than simple intensity imaging is required to investigate molecular interactions in biological systems.

Recording time-resolved fluorescence images can be achieved by combining a confocal laser scanning microscope, a femtosecond Titanium Sapphire (TiSa) Laser and an advanced Time-Correlated Single Photon Counting (TCSPC) imaging technique.

The Confocal Microscope

The optical principle of a confocal microscope is shown in fig. 1.

The laser is fed into the optical path via a dichroic mirror and focused into the sample by the microscope objective lens. The light from the sample goes back through the objective lens, through the dichroic mirror and through a pinhole in the upper image plane of the objective lens. Light from outside the focal plane is not focused into the pinhole plane and therefore substantially suppressed. Imaging is achieved by scanning the laser spot over the sample.

With a fs TiSa laser the sample can be excited by two-photon absorption. Due to the small diameter of the Airy disk the photon density in the focus is very high, so that the two-photon excitation works with a high

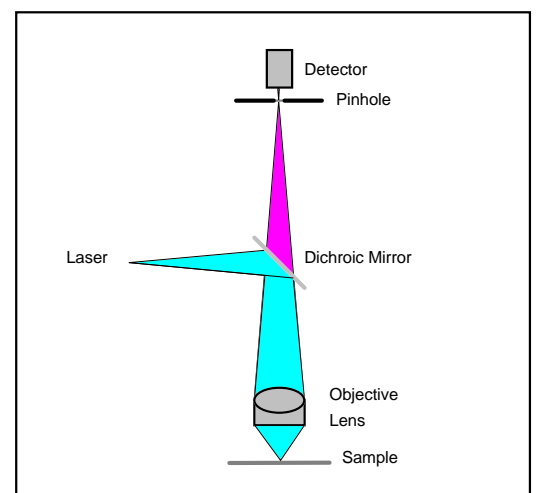


Fig. 1: Principle of a confocal microscope (scanning setup not shown)

efficiency. Furthermore, there is no substantial excitation above and below the focal plane, and scattering effects are small due to the long wavelength. This makes the pinhole in front of the detector unnecessary. The absence of the pinhole allows to record the fluorescence light even if the fluorescent spot in a deep sample layer appears fuzzy due to scattering at the emission wavelength. Therefore, sample layers as deep as 100 μm can be imaged by two-photon excitation. By changing the depth of the focal plane and scanning subsequent slices of the sample a three-dimensional image is recorded.

TCSPC Imaging

The principle of the SPC-730 TCSPC Lifetime Imaging module shown in fig. 2.

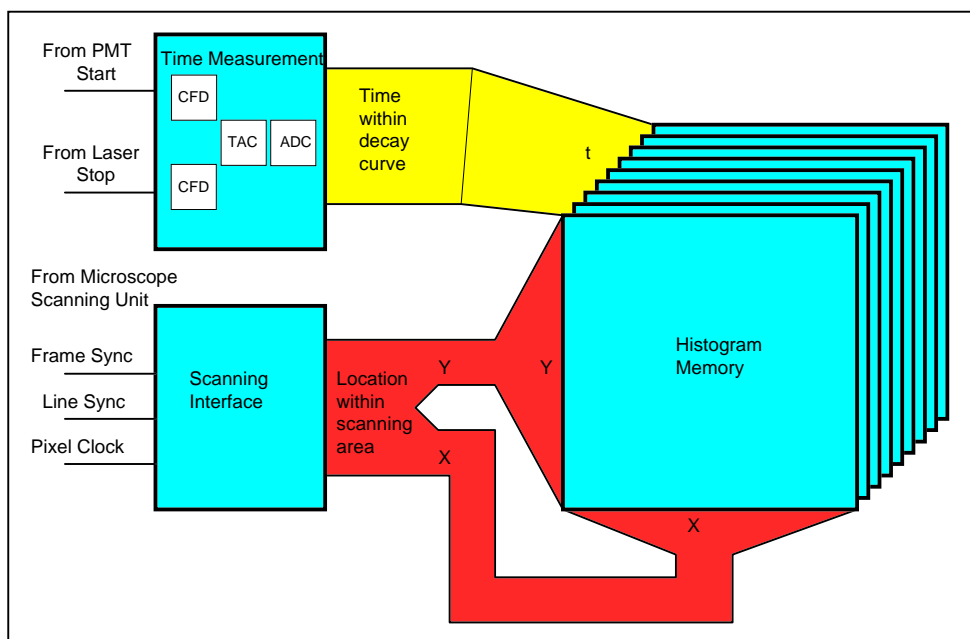


Fig. 2: Principle of TCSPC Lifetime Imaging

The module employs an advanced TCSPC technique featuring both high count rate and low differential nonlinearity. It contains the usual building blocks (CFDs, TAC, ADC) in the ‘reversed start-stop’ configuration together with a scanning interface and a large histogram memory integrated on one board.

The TCSPC module receives the single photon pulses from the photomultiplier (PMT) of the microscope, the reference pulses from the laser and the Frame Sync, Line Sync and Pixel Clock signals from the scanning unit of the microscope. For each PMT pulse (i.e. for each photon), the TCSPC module determines the time of the photon within the laser pulse sequence and the location within the scanning area. These values are used to address the 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 (t) after the excitation pulse.

The TCSPC Microscope

The general setup of the TCSPC Microscope is shown in figure 3. A TiSa laser delivers femtosecond pulses over a wavelength range tuneable from 700 nm to 1000 nm. The repetition rate is 76 MHz, the pulse width is below 200 fs FWHM.

The microscope scans the sample in the x-y plane providing an image of the sample in the focal plane of the objective lens. 3 D imaging is achieved by changing the depth of the focus in the sample.

Most confocal laser scanning microscopes have several detection channels equipped with individual confocal pinholes, filters, and photomultipliers (PMTs). For TCSPC imaging one of the PMTs is used to deliver a single photon signal to the TCSPC module while the other detectors are used to record steady state images via the standard image recording electronics of the microscope.

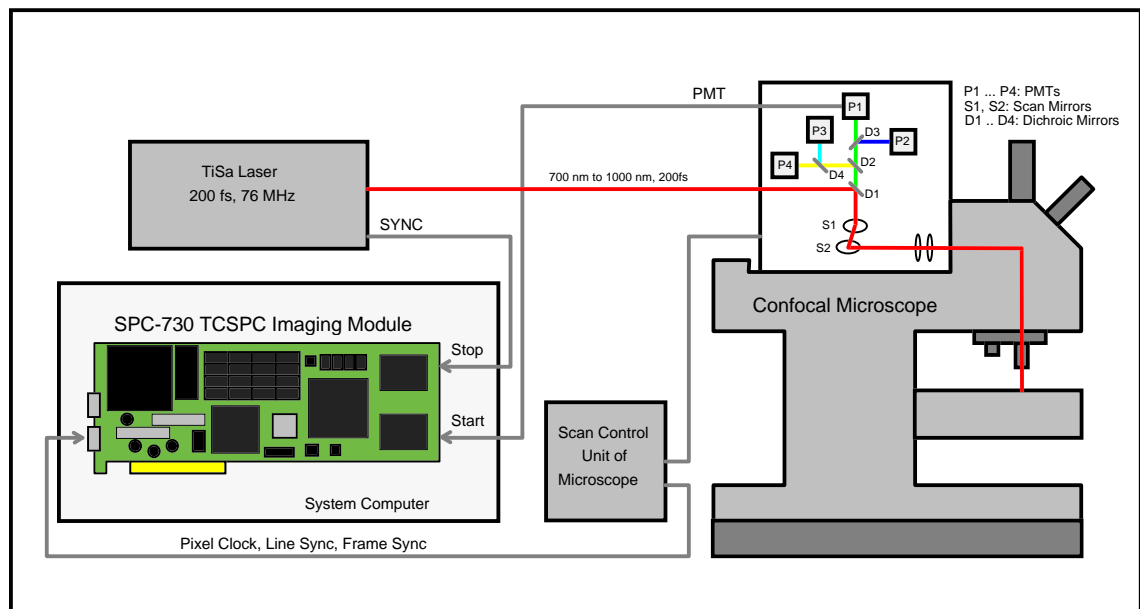


Fig. 3: General setup of the TCSPC Laser Scanning Microscope

The TCSPC imaging module is connected to the microscope via the PMT output signal and the scan control signals (Pixel Clock, Line Sync, Frame Sync) from the microscope scanning controller. Synchronisation with the laser pulse sequence is achieved by the SYNC signal from the monitor diode of the TiSa laser. Connecting the TCSPC module does not require modifications in the microscope hardware and software. The scan parameters are controlled as usual via the microscope software.

The time resolution depends on the detector and is usually 200 ps (fwhm) with the built-in PMTs and 25 ps (fwhm) with external Multi Channel Plate (MCP) detectors. Interestingly, there is practically no loss of photons in the TCSPC imaging process. Due to the short dead time of the TCSPC module (180 ns) nearly all detected photons are processed and accumulated in the histogram. This provides maximum sensitivity and is a benefit compared to gated image intensifiers which gate away the majority of the fluorescence photons.

Results

Fig. 4 shows a TCSPC image of a single cell layer (double staining with Hoechst for DNA and Alexa 488 for Tubulin) obtained by simultaneous two-photon excitation at 800 nm in a Zeiss LSM-510 microscope. The overall acquisition time was 60 seconds. The intensity image (containing the photons of all time channels) is shown left.

Deconvolution analysis delivers the fluorescence lifetime τ in the individual pixels of the image. This allows to generate intensity- τ images that display the fluorescence intensity and the fluorescence time as brightness and colour (fig. 4, right). 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.

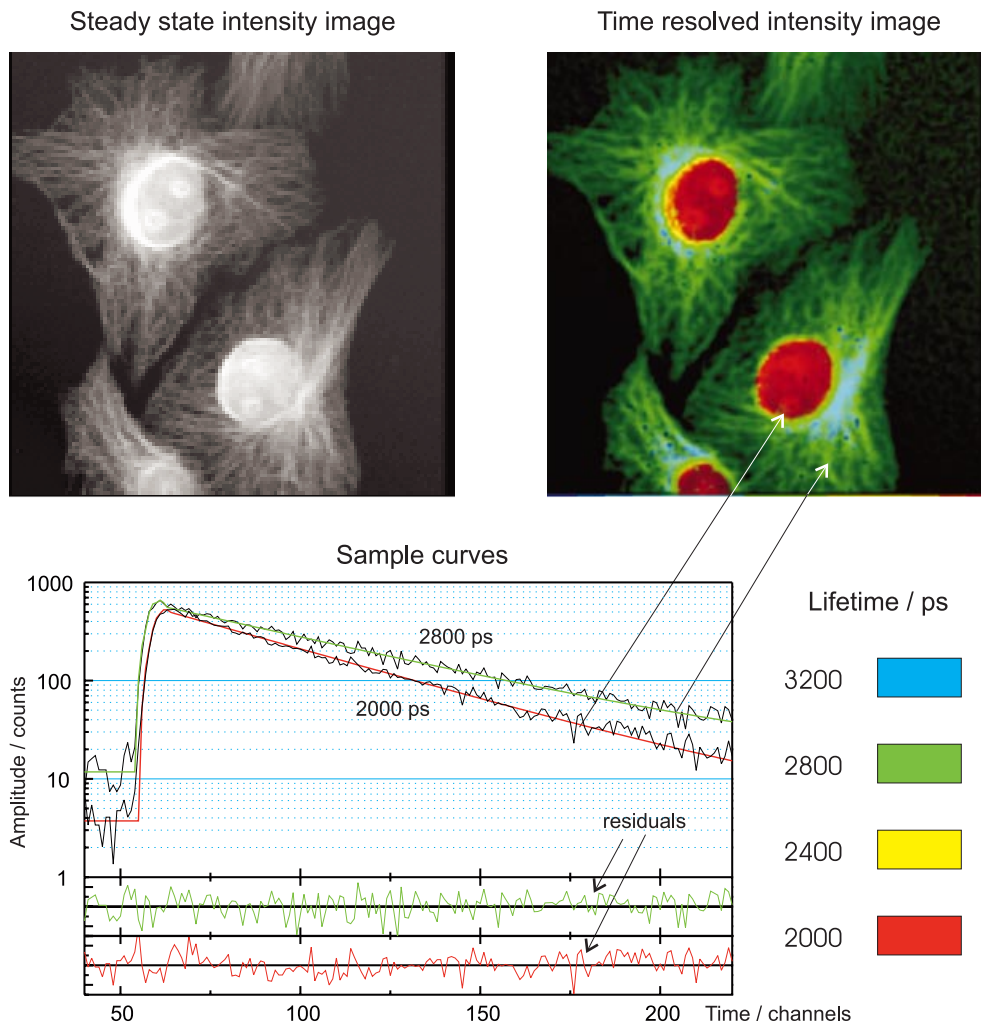


Fig. 4: Intensity image (top left), intensity- τ image (top right) and fitted curves for two selected pixels. Zeiss LSM-510 Scanning Microscope and SPC-730 TCSPC Imaging Module with SPCImage Software of Becker & Hickl, Berlin. Data by courtesy of Zeiss Jena, Germany

Fig. 5 demonstrates the 4 D imaging capability of a TCSPC laser scanning microscope. The images represent subsequent sample layers in a depth from 0 to 50 μm . Again, the intensity is displayed as brightness, the lifetime as colour.

Basically, a sequence of 4 D images could be recorded at different wavelengths thus generating a 5 D fluorescence image of the sample.

The results show the potential of TCSPC Confocal Laser Scanning Microscopy as a new method of fluorescence lifetime imaging. The field of application covers energy transfer measurements (Flourescence Resonance Energy Transfer, FRET), separation of multiple fluorescent labels, imaging of the autofluorescence of cells, single molecule detection, and other fluorescence imaging applications of microscopic samples.

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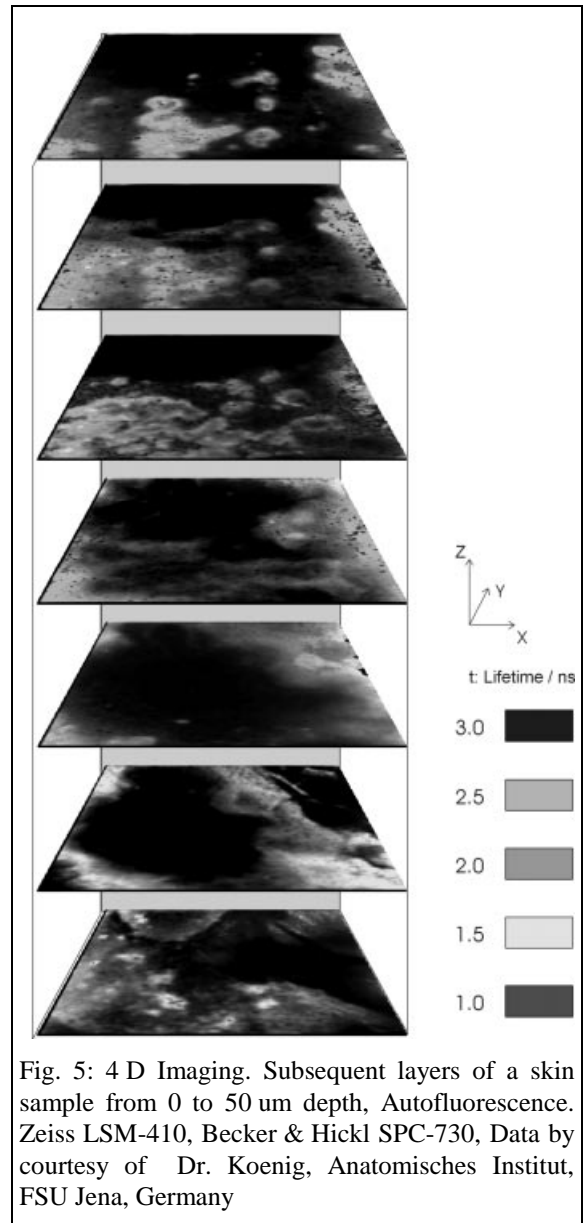


Fig. 5: 4 D Imaging. Subsequent layers of a skin sample from 0 to 50 μm depth, Autofluorescence. Zeiss LSM-410, Becker & Hickl SPC-730, Data by courtesy of Dr. Koenig, Anatomisches Institut, FSU Jena, Germany

