FLIM in the FIFO Imaging Mode:
Large Images with Small TCSPC Modules

With the introduction of the SPC-150, 152, and 154 modules Becker & Hickl have introduced a new FLIM mode into their TCSPC devices. It differs from the traditional 'Scan Sync In' mode in that it does not build up a photon distribution in the memory of the TCSPC module but transfers the data photon by photon into the computer. The software analyses the incoming data stream and builds up the FLIM data in the memory of the computer. Thus, relatively large FLIM data sets can be acquired with TCSPC modules of moderate memory size. The new 'FIFO Imaging' mode is especially useful for multi-spectral FLIM measurements and for imaging of small particles on a large dark background.

Ways of Recording FLIM Data

Early attempts to record FLIM used a classic TCSPC device in combination with a slow scan procedure. That means, the sample was scanned by a translation stage, and complete decay curves were acquired sequentially for subsequent pixels of the scan [12, 13]. The decay curve was read from the TCSPC device after each pixel and stored on the hard disc of a computer. This procedure was limited to pixel times longer than several 10 ms and thus incompatible with the fast scan rates used in modern laser scanning microscopes.

In 1996 bh introduced a FLIM mode based on their multidimensional TCSPC technique. The mode was named 'Scan Sync In' mode and first implemented in the bh SPC-535 TCSPC modules. The Scan Sync In mode acquires FLIM data by scanning a sample at high pixel rate. The principle is shown in Fig. 1.

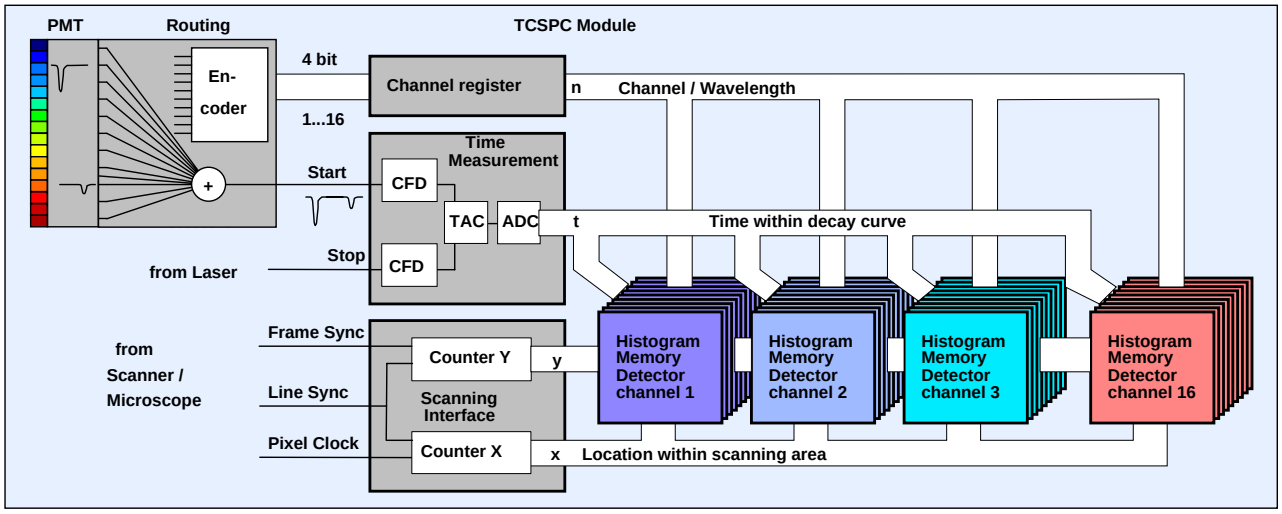

Fig. 1: Structure of the TCSPC module in the Scan Sync In mode. The photon distribution is built up in the memory of the TCSPC module

For each photon the TCSPC module determines the time in the fluorescence decay, the coordinates of the laser beam in the scanning area, and, if bh's routing technique is used, the wavelength of the photon [3, 4, 5]. The recording process builds up a photon distribution over these parameters. The time of the photon is obtained by bh's fast TAC/ADC principle, the position of the laser beam by counting synchronisation pulses (frame clock, line clock and pixel clock) from the scanner. The complete data array is held in the memory of the TCSPC modules. The pixel rate can be (and in practice is) higher than the photon rate. To obtain enough photons per pixel the data are just acquired over as many frames as necessary. The Scan Sync In mode works not only with fast galvanometer scanners, but also with resonance scanners and polygon scanners. The Scan Sync In mode was first used for ophthalmic scanning [19, 20] and later became a standard FLIM technique of laser scanning microscopy [1, 2, 3]. Because the recording process of the Scan Sync In mode is fully hardware-controlled photons can be collected at a sustained rate up to the saturated count rate of the TCSPC module.

The size of a FLIM image recorded in the Scan Sync In mode is limited by the size of the on-board memory of the TCSPC module. For the bh SPC-830 module with its 32 Mb of memory this is not really a problem. However, for modules with smaller memories, as the SPC-140 or the SPC-150 the limitation can be noticeable, especially if multi-wavelength FLIM measurements with the PML-16 detector [5, 6] are performed.

Another way of recording FLIM is by transferring the data of the individual photons into the computer and building up the photon distribution in the memory of the computer. This is possible in the 'FIFO' or 'Time Tag' mode. This mode delivers the time of the photon in the fluorescence decay, the time of the photon form the start of the experiment, and, if routing is used, the detector channel, i.e. the wavelength of the photon [4]. The data can be used to assign the photons to the individual pixels of the scan and thus to build up FLIM images [15]. The FIFO mode has been introduced in 1996 with the bh SPC-431 and SPC-432 TCSPC modules. Because of its ability to provide absolute timing of the photons it has mainly been used for single-molecule experiments [16, 17].

The problem of the FIFO mode is the enormous amount of data that has to be transferred into the computer. For every photon four or six bytes have to be transferred. These data either have to be processed online or be written onto the hard disc. Early TCSPC modules, especially those with ISA interfaces, had relatively slow bus interfaces. The sustained count rate in the FIFO mode was limited by the bus transfer rate of the computer.

With modern computers and fast bus interfaces the data transfer is less a problem than it was in the 90s. bh have recently introduced a 'FIFO Imaging' mode that not only transfers the photon data but also the synchronisation pulses from a fast galvanometer scanner [4, 7]. These data are used to build up a photon distribution in the memory of the computer exactly in the same way as the Scan Sync In mode does in the memory of the TCSPC module. The principle is shown in Fig. 2.

When a photon is detected the TCSPC module tags it with its time in the fluorescence decay, its wavelength channel number, and its time from the start of the experiment. When a scan clock pulse (frame, line, or pixel clock) is detected, the TCSPC module tags it with the time from the start of the experiment and with a marker that identifies it either as a frame clock, line clock or pixel clock.

The memory of the TCSPC module is used as a first-in-first-out (FIFO) buffer. Each photon and each scan clock pulse causes an entry in the FIFO buffer, which contains the parameters of the photon or the pulse in a compressed data format [4].

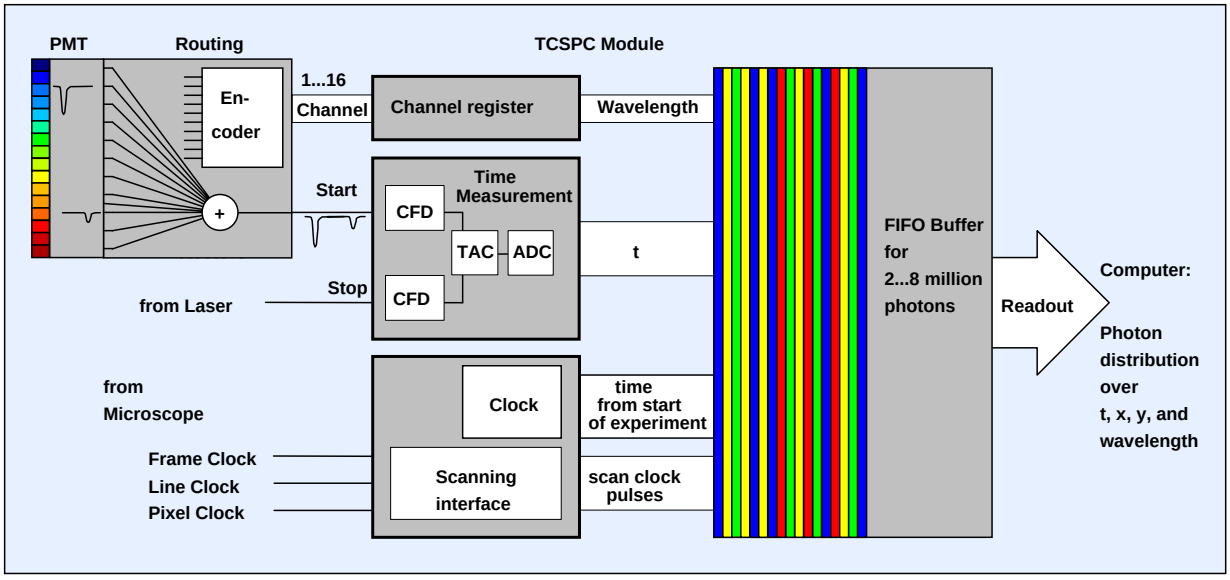

Fig. 2: Structure of the TCSPC module in the FIFO Imaging mode. The photon distribution is built up in the memory of the computer.

At the output the FIFO buffer is continuously read by the computer. By analysing the incoming data stream, the software builds up a photon distribution over the time in the fluorescence decay, the image coordinates, and the wavelength. The result is exactly the same as in the Scan Sync In mode. However, the size of the image is not limited by the size of the TCSPC memory but by the available amount of memory in the computer. Thus, larger FLIM images with more pixels or more time channels can be recorded.

Because the data acquisition is synchronised with the scanner in the same way as for the Scan Sync In mode the FIFO Imaging mode is fully compatible with all previous bh FLIM applications.

The prize to be paid for the large image size is a possible limitation of the sustained count rate by the bus transfer rate and the speed of the online-processing in the computer. For very bright images (average count rates above 2 or 3 MHz) the bus may therefore saturate, and the FIFO can overflow. Fortunately, extremely large pixel numbers are usually required only for imaging small objects on a large, dark background. The bh TCSPC imaging modules have FIFO sizes ranging from 2 million to 8 million photons. As the scanning is performed at high speed the FIFO is able to buffer the photons of a large portion of one frame, if not of a whole frame. Thus, the average count rate usually remains below the critical level, even if the objects are very bright.

Other applications include FRET to investigate protein interactions. FRET experiments require double exponential decay analysis and thus benefit from the larger number of time channels available. Because FRET samples contain fluorescent proteins in highly specific parts of the cells the fluorophore concentrations are low, and so are the count rates. The count rates obtained from FRET samples are usually an order of magnitude below the maximum sustained count rate of the FIFO Imaging mode.

FIFO Imaging in Single-Detector Systems

Typical TCSPC system setup parameters for FIFO Imaging with a single detector are shown in Fig. 3. The image is built up via the ‘Runtime Display’ function of the SPCM software. The incoming

data stream is analysed online, and a photon distribution over the image coordinates and the arrival times of the photons within the laser periods is built up. The image size is 512x512 pixels; each pixel contains 256 time channels. Intermediate results are displayed in intervals of 'Display Time'.

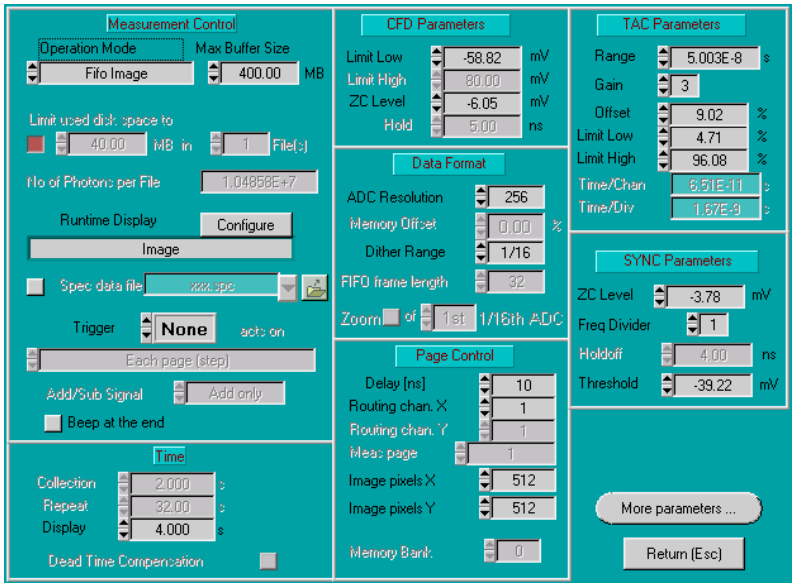

Fig. 3: Typical TCSPC System Parameters for the FIFO Imaging mode

The suggested main panel configuration is shown in Fig. 4. An integral image over all time channels of the pixels is shown on the left. The scanner control panel of a bh DCS-120 scanner [7] is kept open on the right. Please note that the FIFO imaging mode can be used for other scanners [8, 9, 10] as well. In these cases the scanner is controlled via its own software, and you may place the corresponding control panel on the right.

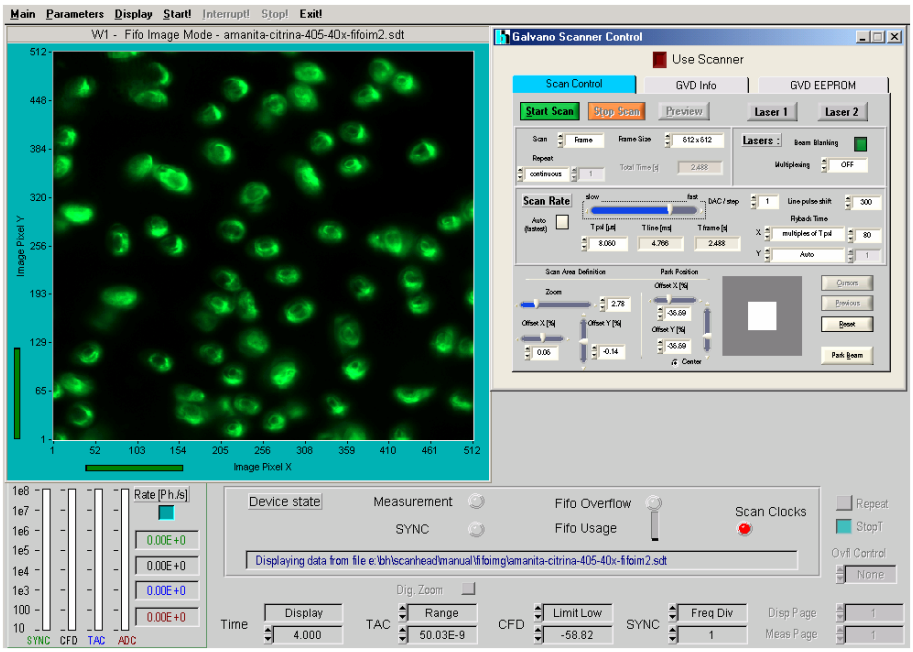

Fig. 4: Main panel configuration

A typical example of a FIFO mode image is shown in and Fig. 5. The figures show autofluorescence images of Amanita Citrina (mushroom) spores. The image was taken by a bh DCS-120 scanning system [7] in combination with an SPC-150 TCSPC module [4].

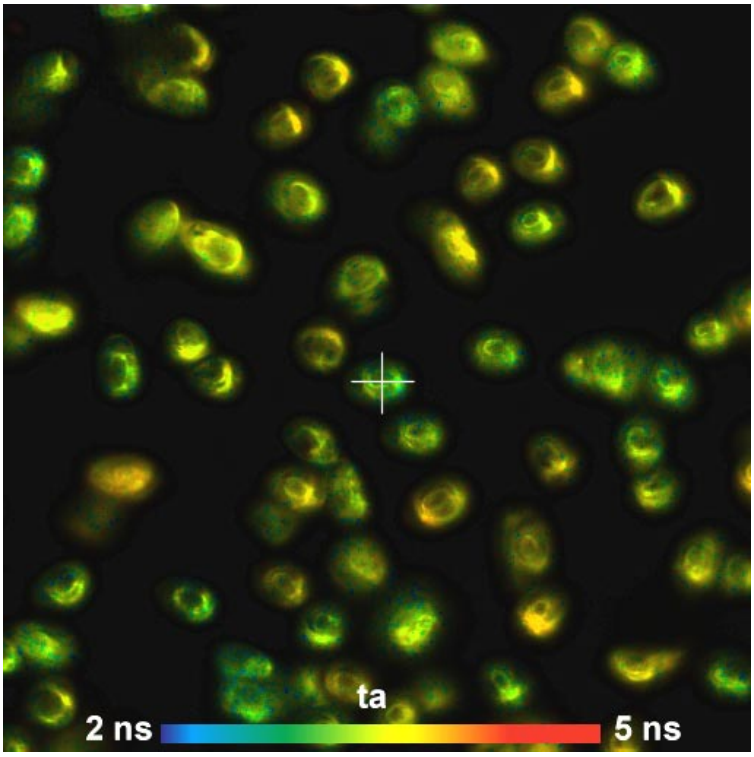

Fig. 5: Lifetime image of spores of Amanita Citrina. SPC-150, Acquisition in the FIFO Imaging mode, analysis by SPCImage

The decay curve in a selected spot and the lifetime components are shown in Fig. 5. It shows the typical multi-exponential decay profile found in autofluorescence data. Triple-exponential decay analysis was used the analyse the data [7]. The lifetime shown in Fig. 5 is an average of the three lifetimes weighted with their intensity coefficients.

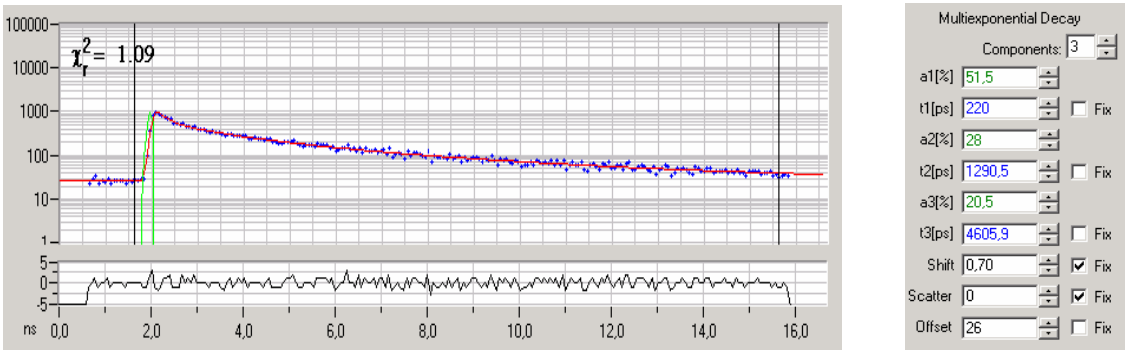

Fig. 6: Fluorescence decay in the spot marked in Fig. 5. Triple-exponential analysis, lifetime components shown on the right.

The entire data set, i.e. the .sdt data file of the measurement, has a size of 130 Megabytes. Larger pixel or time channel numbers may be desirable for special applications. They do, however, require a computer with more than 2 Gb main memory and a data storage medium of extremely large size.

Multi-Spectral FIFO FLIM

Multi-spectral FLIM (or spectrally resolved lifetime imaging, SLIM) [4, 5, 11, 18] records in 16 wavelength intervals simultaneously and therefore requires 16 times the memory size of a single-wavelength FLIM image. The FIFO imaging mode is therefore recommended, especially if the SPC-150 is used for data recording. The system parameter setup for FLIM acquisition with 256 x 256 pixels, 64 time channels, and 16 wavelength channels is shown in Fig. 7.

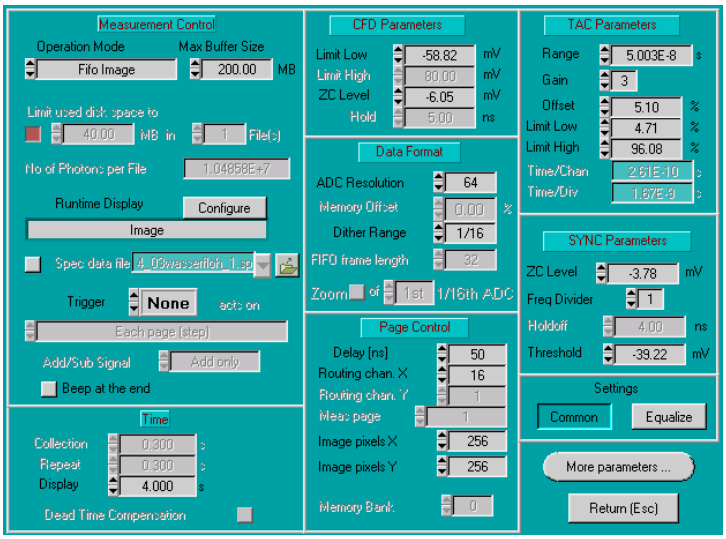

Fig. 7: TCSPC System Parameters for MW FLIM in the FIFO Imaging mode. Example for 256x256 pixels, 64 time channels, and 16 wavelength intervals.

The recommended main panel configuration is shown in Fig. 8. Eight wavelength intervals were defined. The images in the wavelength intervals are displayed in eight display windows. The colours of the images were defined according to the selected wavelength ranges. During the measurement, the images are updated in intervals of ‘Display Time’, i.e. every four seconds for the settings shown in Fig. 7.

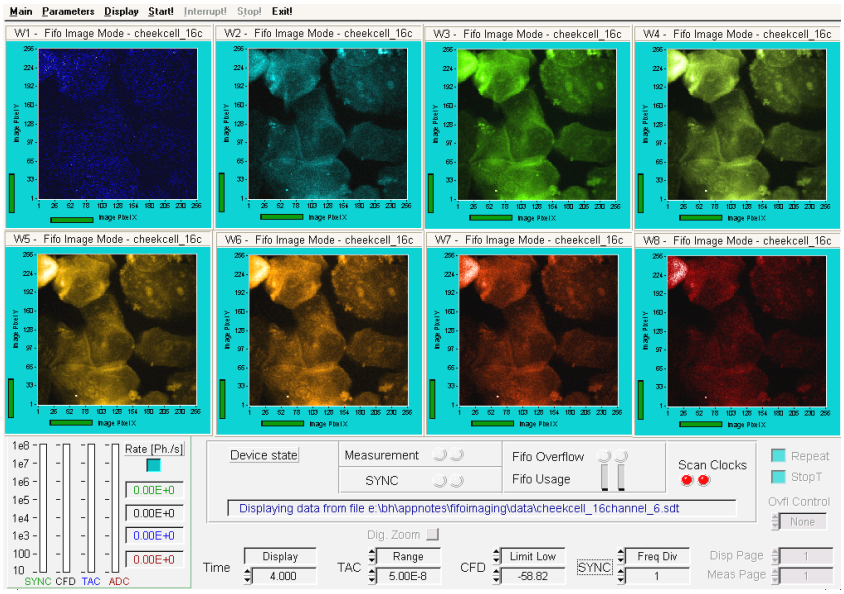

Fig. 8: Recommended main panel configuration for multi-spectral FLIM.

An example of a spectrally resolved FLIM result is shown in Fig. 9. It shows lifetime images of the autofluorescence of human epithelial tissue cells in 16 wavelength intervals. The resolution of the images is 256 x 256 pixels; each pixel contains 64 time channels. The sample was scanned by a bh DCS-120 scanner [7] attached to a Nikon TE2000 U microscope. An x60 water immersion lens was used; the pinhole size was 2 Airy units. The lifetimes shown in Fig. 9 are the intensity-weighted average of the decay components of a double-exponential fit.

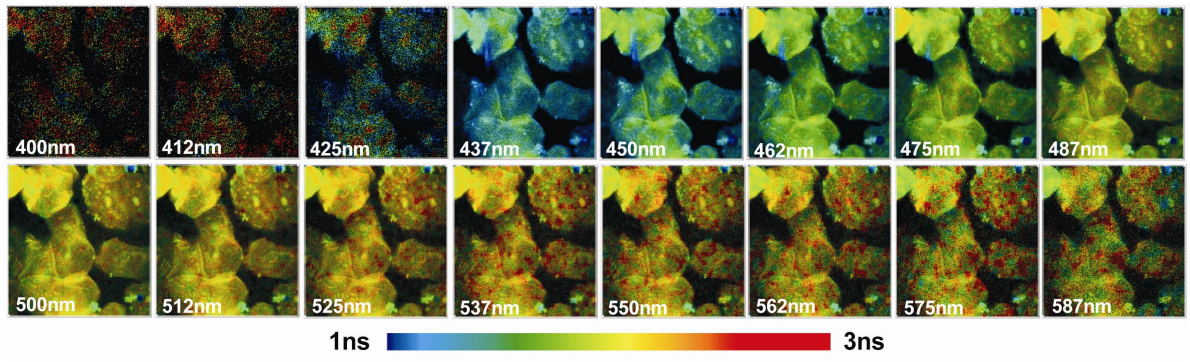

Fig. 9: Multi-wavelength lifetime images of the autofluorescence of human epithelium cells. Lifetime is represented by colour. bh DCS-120 scanner with bh MW-FLIM detector, SPC-150 TCSPC module, excitation wavelength 405 nm, detection wavelength 400 nm to 587 nm.

The fluorescence decay profiles of autofluorescence deviate strongly from a single-exponential decay. Fig. 10 shows a decay curve from a 20 x 20 pixel area in the centre of the 450 nm window of Fig. 9.

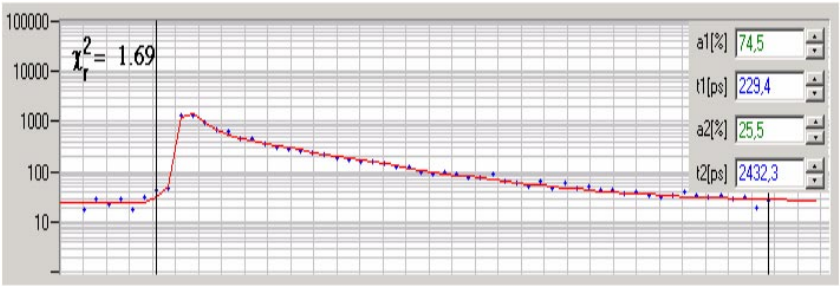

Fig. 10: Fluorescence decay curve of a 20 x 20 pixel area in the centre of the 475 nm window of Fig. 9.

The decay function can be approximated at satisfactory quality by a double-exponential decay model. The fit delivers a fast component of 229 ps and a slow component of 2.43 ns, with amplitudes of 74.5 % and 25.2 %, respectively. The composition of the decay shown in Fig. 10 is typical of autofluorescence measurements. It is likely that the fast component originates from unbound NADH and bound FAD while the slow component comes from bound NADH and unbound FAD. The large amplitude and the wide spacing of the decay times makes double-exponential analysis feasible. In fact, a double-exponential model often delivers a more stable fit of autofluorescence decays than a single-exponential one.

The double-exponential data can be used to obtain images of the relative amplitudes or relative intensities of the lifetime components. An example is given in Fig. 11. The figure shows the relative intensities, i.e. the integrals of the photon numbers, contained in the fast lifetime component.

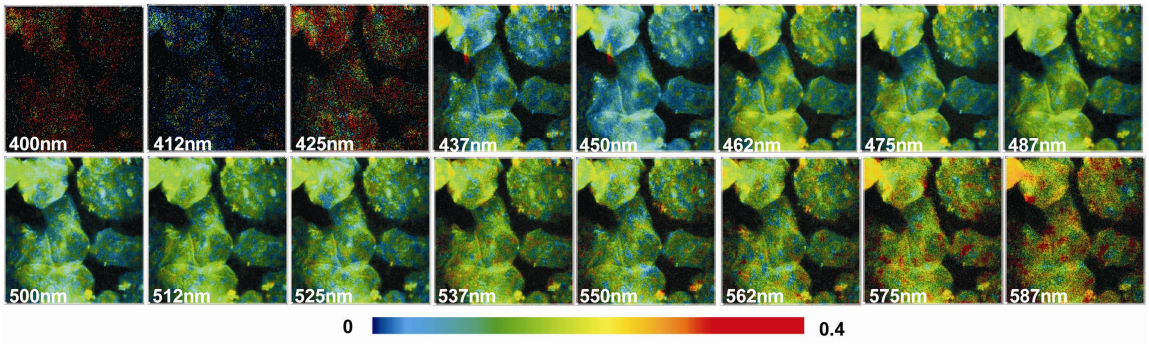

Fig. 11: Relative intensity contained in the fast decay component

Saving Time-Tag Data

For standard FLIM applications the time-tag data of the photons are discarded after being used in the image acquisition process. In other applications it may be desirable not only to save the FLIM data but also the time-tag data of the individual photons. The data may then be used for multi-parameter fluorescence analysis [14, 17], for correlation measurements, or to derive a sequence of FLIM images from a single recording taken over a long acquisition time. The measurement control section of the system parameters for saving the time-tag data is shown in Fig. 12.

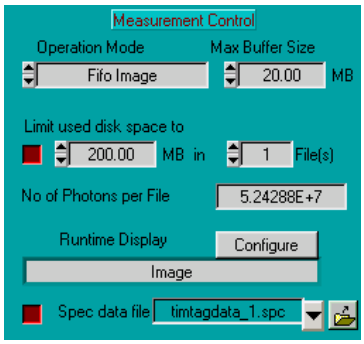

Fig. 12: Setup for saving the time-tag data. Measurement control section of the system parameter panel.

With the setting shown, the data are saved into a file named ‘timetagdata_1.spc’. The maximum file size can be limited by the user, in this case to 200 Mb. The setup shown stops the measurement when the specified amount of data has been recorded.. For details please see [4].

Availability of the FIFO Imaging Mode

The FIFO imaging mode is available in the SPC-150, SPC-152, and SPC-154 modules, and in SPC-830 modules with serial numbers starting from 3D0178 (May 2007). Earlier SPC-830 modules starting from serial number 3D0098 (May 2005) can be upgraded for FIFO imaging. Please contact bh for details.

References

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