Software-Integrated FLIM for Nikon A1+ Confocals

Abstract: FLIM operation with the bh TCSPC/FLIM modules has been implemented in the new 'NIS-Elements' software of the Nikon A1+ confocal laser confocal laser scanning microscopes. The systems use one or two bh BDS-SM picosecond diode lasers, two bh HPM-100 hybrid detectors, and two fully parallel SPC-150N TCSPC data recording channels. All FLIM components are controlled via Nikon's NIS-Elements software. Intensity images, FLIM images, and decay curves from regions of interest are displayed online. For precision multi-exponential data analysis, the system incorporates bh's SPCImage FLIM data analysis software.

Principle of Signal Recording

The sample is scanned with the focused beam of a high-frequency pulsed laser. The laser excites fluorescence in the sample. The fluorescence signal is collected by the microscope lens, fed back through the beam path of the scanner, separated from the excitation light, sent through a confocal pinhole, and, finally, detected by an optical detector. Compared with wide-field imaging by a camera the scanning process has several advantages. The first one is that the pinhole passes light only from the focal plane of the microscope lens. The images are thus free of the typical out-of focus-haze seen in normal visual microscopes. Moreover, there is no problem with scattering. A wide-field system, in every pixel, records scattered light from all other pixels and focal planes. A scanning system does not have this problem. It excites only one pixel at a time, and detects only from this pixel and within a well-defined sample plane. Thus, confocal scanning delivers clean images from a selected plane in the sample which are free of lateral and longitudinal crosstalk.

The detection system is based on bh's multi-dimensional TCSPC process. The light passing the pinhole is sent to a single-photon sensitive detector. The TCSPC system receives the photon pulses from the detector together with the pixel, line, and frame clock pulses from the scanner. For every photon, it determines the time of the photon after the laser pulse and the position of the laser beam in the sample in the moment when the photon was detected. The result is a photon distribution over the detection time and the scan coordinates. The distribution can be considered an array of pixel, each of which containing a fluorescence decay curve in the form of photon numbers in consecutive time channels [1, 2, 3, 4].

TCSPC FLIM has a number of other intriguing features. The most important ones are the time resolution and the sensitivity. The time resolution of the TCSPC process is only limited by the laser pulse width and the transit-time jitter in the detector. The resulting instrument-response function (IRF) is 10 to 20 times narrower than for a system that uses the same detector in combination with an analog-recording system [1, 5]. At the same time, the system delivers near-ideal sensitivity. Every photon seen by the detector is ending up in the result. For a given number of photons, N, in a particular pixel the signal-to-noise ratio for the obtained fluorescence lifetime is close to $N^{1/2}$, the ideal value for any optical measurement [1, 2].

There is a number of other advantages of TCSPC FLIM, such as the capability to record multi-wavelength FLIM [6], to quasi-simultaneously record a several excitation wavelengths [1, 2], to record extremely fast time-series [1, 9], or simultaneously record FLIM and PLIM [7, 8]. These features of the bh FLIM technique are not (yet) implemented in Nikon's NIS-Elements software.

However, they can be used by running bh SPCM data acquisition software in parallel with Nikon 'Elements' microscope software.

Architecture

The basic system uses a bh BDS-SM picosecond diode laser [10] for excitation, two bh HPM-100-40 hybrid detectors [1] for detecting the photons, and two parallel bh SPC-150N TCSPC / FLIM modules for recording the data [1]. The laser and the detectors are controlled by one DCC-100 Detector/Laser controller module each. The SPC and DCC modules are operated in an extension box connected to the NIKON system computer. The general system architecture is shown in Fig. 1.

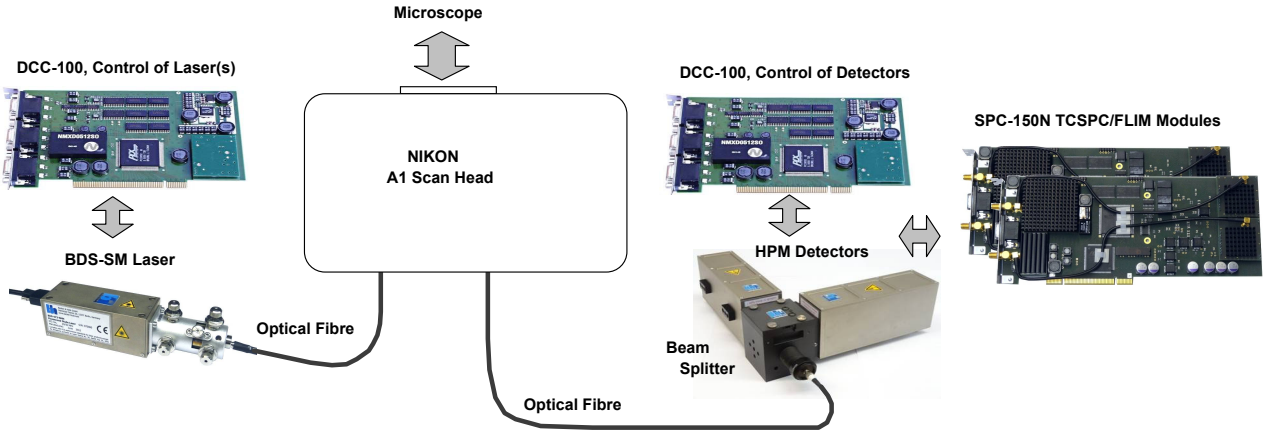

Fig. 1: Architecture of the NINKON integrated FLIM system

Two modifications of the system are possible without changes in the Nikon integrated FLIM software:

- Additional lasers can be added to the system. The output beams of up to four lasers can be combined into a single optical fibre and delivered into the scan head.
- One detector or both detectors can be replaced with ultra-fast HPM-100-06 detectors [5]. With these detectors, the instrument-response function (IRF) is essentially determined by the laser. With 375nm, 405 nm, and 445 nm lasers, the IRF width is about 40 ps. For lasers of 473, 488, and 515 nm the FWHM is between 60 ps and 90 ps.

Other extensions, like a multi-wavelength detector [1], bh FASTAC FLIM [12], or simultaneous FLIM/PLIM [7] can be added and operated via bh SPCM software running in parallel with Nikon's NIS-Elements software.

Software

A typical panel configuration of the FLIM implementation in Nikon's NIS-Elements software is shown in Fig. 2. The FLIM system has two TCSPC channels and two lasers. A lifetime image is shown for one of the FLIM channels is shown on the left. The control panels for the FLIM acquisition and the scan parameters are shown on the right. The panel next right to the FLIM image controls the FLIM acquisition. With the setting shown, 30 Frames are accumulated. One channel records through a 520 nm filter, the other through a 650 nm filter. Lifetime images are calculated online by a first-moment algorithm [11]. The microscope parameters and spatial imaging parameters are defined via the two panels on the right. Image size is 1024 x 1024 pixels, the acquisition is started by clicking on 'Capture' (right panel).

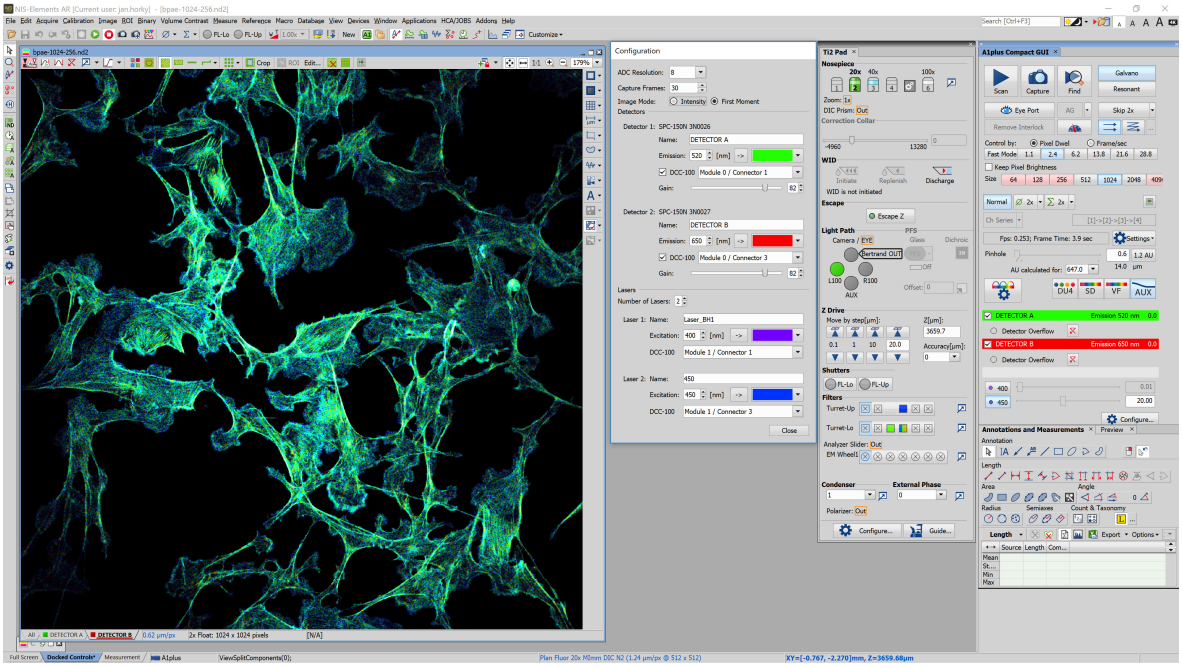

Fig. 2: Implementation of FLIM in Nikon NIS-Elements. Control panels: FLIM configuration, light path configuration, and scan parameter definitions.

Simultaneous display of the lifetime images of both FLIM channels is shown in Fig. 3. The FLIM configuration and the Ti2 Microscope control panel were closed to provide space for the second image.

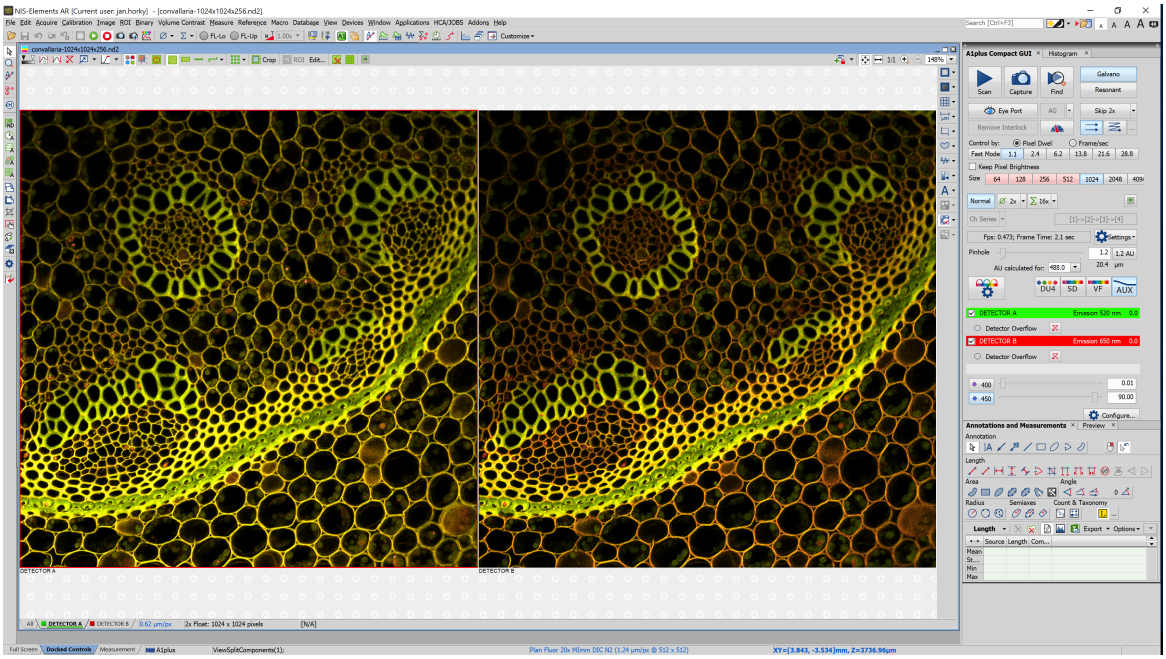

Fig. 3: Convallaria sample, scanned with 1024 x 1024 pixels, Lifetime by First-Moment analysis, images of both FLIM channels displayed.

Online FLIM display can be combined with displaying decay curves from the entire images or from regions of interest. An example is shown in Fig. 4.

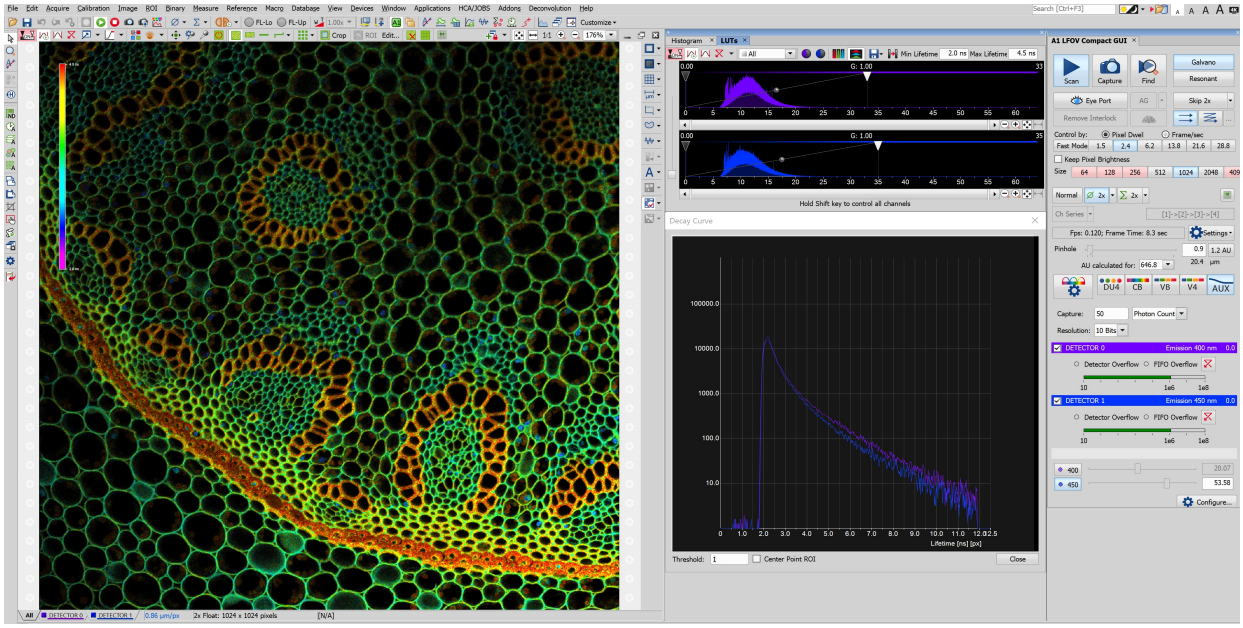

Fig. 4: Display of decay functions from the entire images or from regions of interest.

FLIM with the 'Large-Image' function of the Nikon system is shown in Fig. 5. The principle is equivalent to the ‘Mosaic FLIM’ capability of the bh SPCM software [1]: A part of the sample is scanned, then the sample is offset by the scan-field diameter, and the sample is scanned again. the process is continued until the desired are of the sample has been imaged. The corresponding scans are combined into a single, large image. The advantage is that a large sample are can be covered by a high-NA microscope lens, delivering high lateral and longitudinal resolution, and high light-collection efficiency.

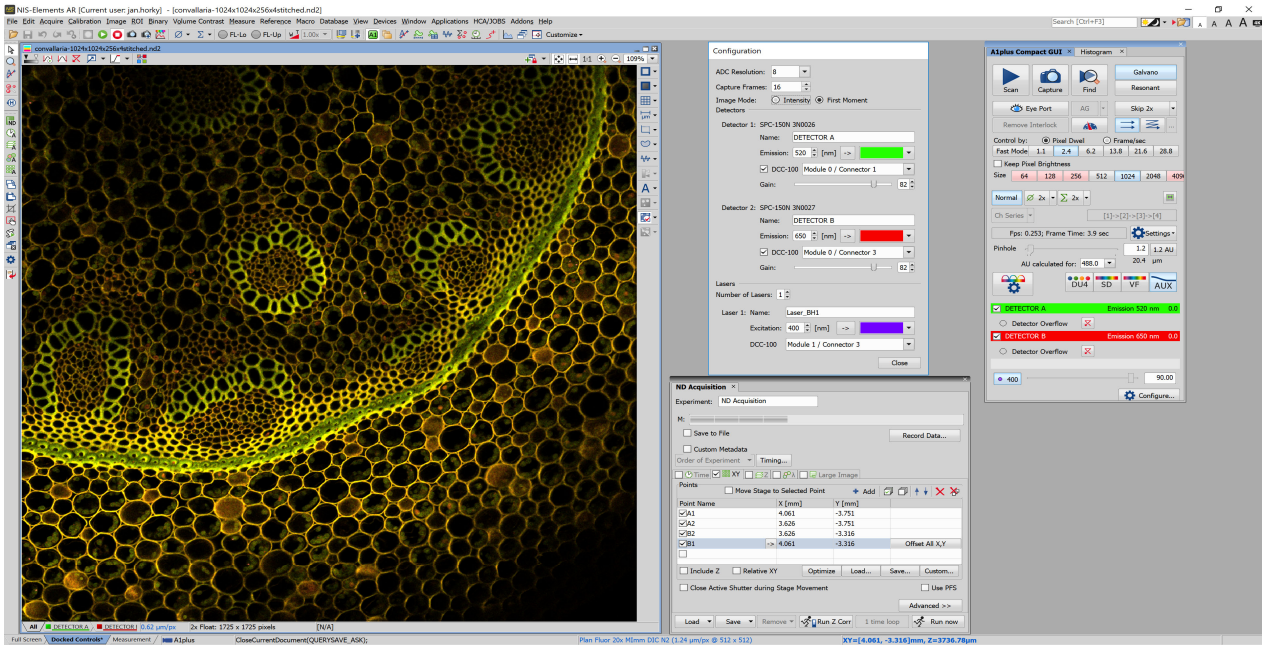

Fig. 5: Mosaic of four 1024x1024-pixel FLIM recordings, stitched together giving a single 2048x2048 pixel image.

Multi-Exponential Data Analysis with bh SPCImage

Online lifetime calculation in NIS-Elements delivers the lifetime for a single-exponential approximation of the decay data in the pixels. For detailed analysis, bh SPCImage [1] has been embedded in the NIKON Elements FLIM software. FLIM data are sent to SPCImage by a right mouse click and selecting 'Send to SPCImage'.

An example is shown in Fig. 6. The data are the same as shown in Fig. 3, left image. The example shows an amplitude-weighted lifetime image obtained from the components of a double-exponential fit.

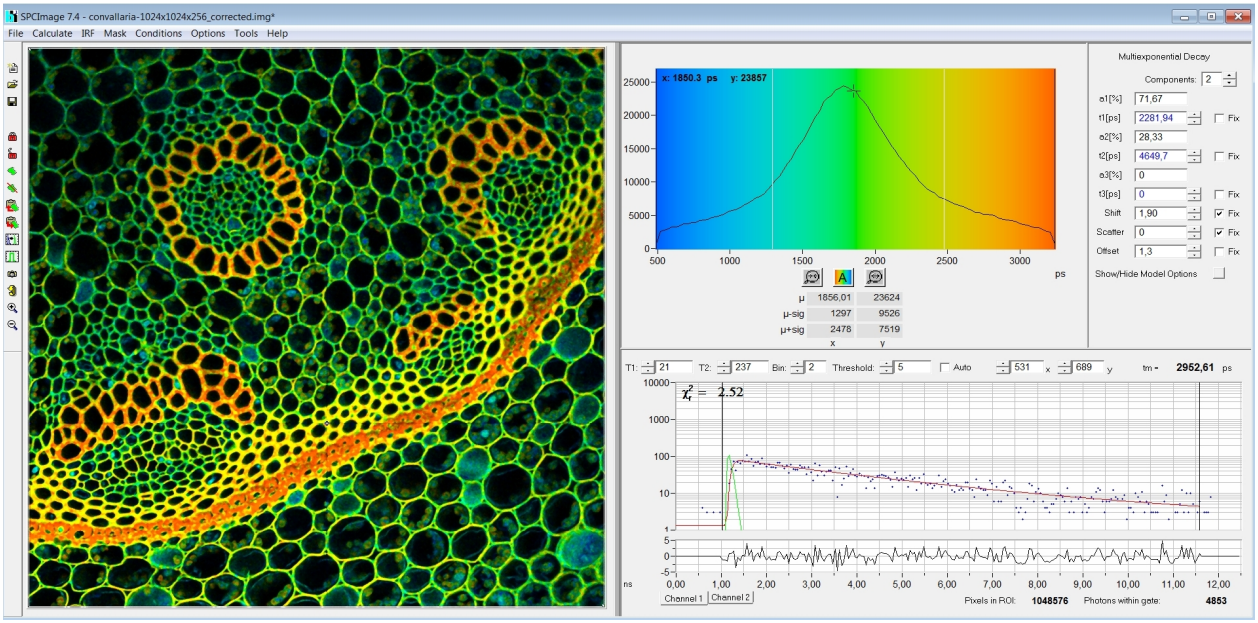

Fig. 6: SPCImage Data Analysis. Main panel, with lifetime image, lifetime histogram, and decay curve at cursor position.

SPCImage is able to show also images of the component lifetimes, t1 and t2, and of the amplitude ratio, a1/a2. Images for these parameters are shown in Fig. 7. Biologically, these parameters are often more meaningful than the amplitude-weighted or intensity-weighted lifetime. Depending on the systems investigated, ratio images of bound and unbound NADH, FRET efficiencies, and the ratio of interacting and non-interacting FRET Donor can be derived. A phasor plot is available as well (Fig. 7 lower right), providing extended options of image segmentation and decay analysis of combined pixels. Please see [1] or [13] for details.

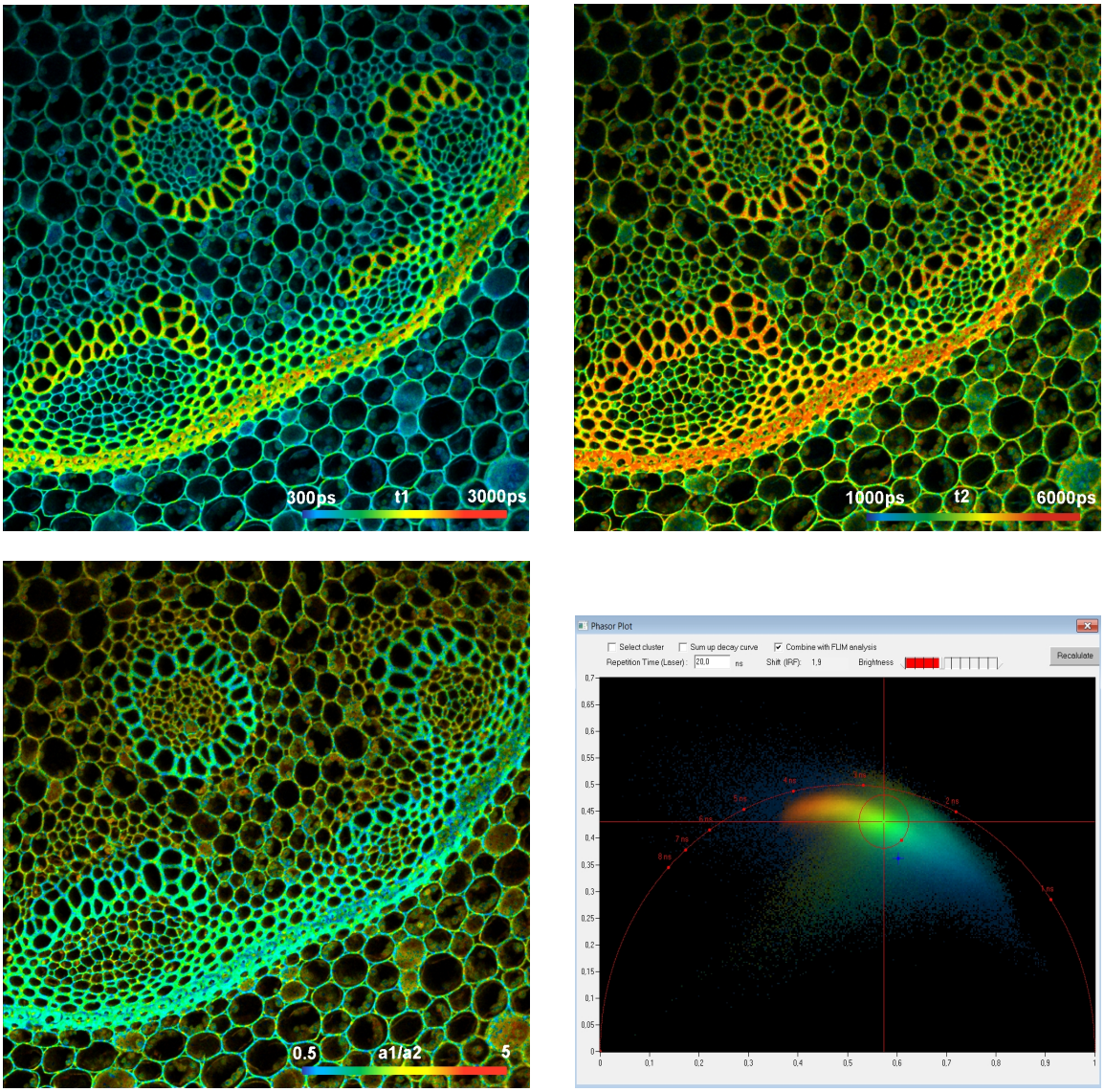

Fig. 7, upper left to lower right: Lifetime image of fast decay component, t1, lifetime image of slow decay component, t2, colour-coded image of amplitude ratio of the components, a1/a2, and phasor plot. All images 1024 x 1024 pixels.

Summary

The bh / Nikon A1 integrated FLIM system provides an easy way to record FLIM data by a laser scanning microscope. Nikon NIS-Elements mainly provides entry-level FLIM functions, and thus does not require special knowledge about TCSPC and data recording procedures. Nevertheless, the system records data with high photon efficiency, high signal-to-noise ratio, and high time resolution. High-level multi-dimensional recording functions are available by simply running bh SPCM data acquisition software in parallel with Nikon Elements.

References

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Contact:

Becker & Hickl GmbH
Nunsdorfer Ring 7-9
12277 Berlin, Germany
Tel. +49 30 212 800 20, Fax. +49 30 212 800 213
email: info@becker-hickl.com, https://www.becker-hickl.com