Non-Descanned FLIM Systems for Olympus FV-1000 and FV-300 Multiphoton Microscopes

Abstract. Recently multiphoton versions of the Olympus FV 1000 and FV 300 laser scanning microscopes have become available. Based on their TCSPC FLIM devices and detector assemblies, Becker & Hickl have designed non-descanned FLIM systems for these microscopes. The systems are available with a number of different detectors, including ultra-fast MCP PMTs, ultra-high efficiency hybrid PMTs, and the bh multi-spectral FLIM detector assembly.

General Features

Multiphoton microscopes use a femtosecond Titanium-Sapphire lasers for fluorescence excitation. The obvious advantage for FLIM is that the titanium-sapphire laser is an almost ideal pulsed excitation source for fluorescence lifetime detection. Moreover, the titanium-sapphire laser is tuneable and can therefore adjusted to the absorption spectrum of the fluorophores of interest. Combined with non-descanned detection multiphoton excitation offers superior detection efficiency for deep-tissue imaging. Multiphoton microscopes with non-descanned detection are therefore excellently suitable for FLIM. In addition to their existing one-photon and multiphoton FLIM systems [7, 8, 9, 10, 11] bh have therefore designed non-descanned FLIM systems for the Olympus FV 1000 and FV 300 multiphoton microscopes. The FLIM systems can easily be attached to all FV 1000 and FV 300 systems based on inverted IX series microscopes. The systems are available with a number of different detectors, including the bh multi-spectral FLIM detector. A typical system is shown in Fig. 1.

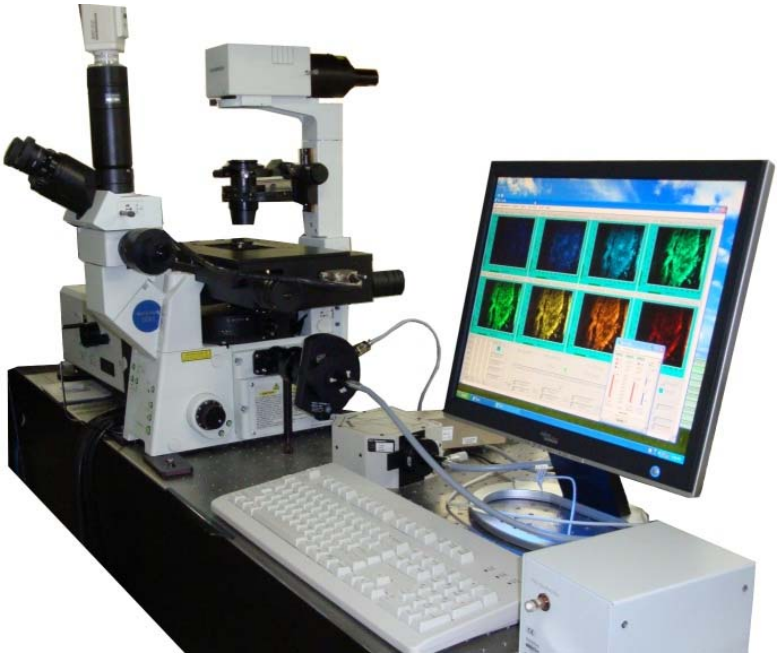

Fig. 1: bh multi-spectral FLIM system at an Olympus FV300 multiphoton microscope

Principle of Non-Descanned Detection

A multiphoton microscope excites the fluorescence in the sample via simultaneous absorption of several (usually two) photons of a femtosecond pulsed near-infrared laser. Multiphoton excitation has the advantage that the near-infrared laser penetrates substantially deeper into a sample than the visible or UV laser of a confocal microscope, see Fig. 2, left and middle. Moreover, depth resolution is obtained via the nonlinearity of the excitation process Fig. 2, middle. It is therefore not necessary to use a pinhole to reject out-of-focus fluorescence.

The fact that no pinhole is required becomes important when deep tissue layers are to be imaged. In these cases, a large fraction of the fluorescence photons is scattered on the way out of the sample, see Fig. 2, right. The scattered photons cannot be collimated and thus not be fed through the beam path of a descanned confocal system, see Fig. 3, left. Only ballistic, i.e. non-scattered photons would reach the confocal detector.

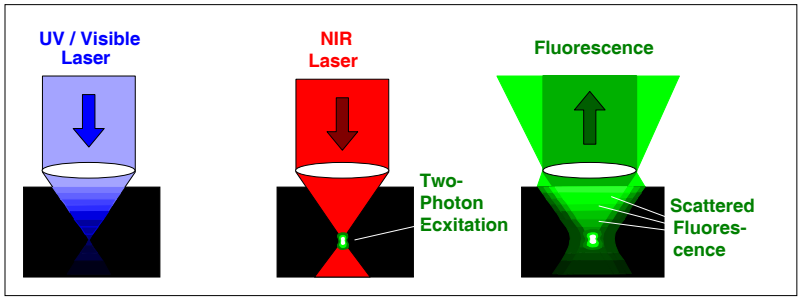

Fig. 2: Left: One-photon excitation. The effective excitation power decreases rapidly with increasing depth. Middle: Two-photon excitation. The NIR laser penetrates deeply into the sample. Right: The fluorescence from a deep focus is scattered on the way out of the sample. It leaves the back aperture of the microscope lens in a wide cone.

The scattering problem is solved by ‘non-descanned’ detection, see Fig. 3, right. A dichroic mirror diverts the fluorescence light directly behind the microscope lens. A lens projects an image of the microscope lens on a large-area detector. Additional advantages of non-descanned over confocal detection is that there are fewer lenses and mirrors in the light path than for confocal detection, and that the light path is far less susceptible to misalignment. Non-descanned detection therefore usually delivers better detection efficiency even for thin samples.

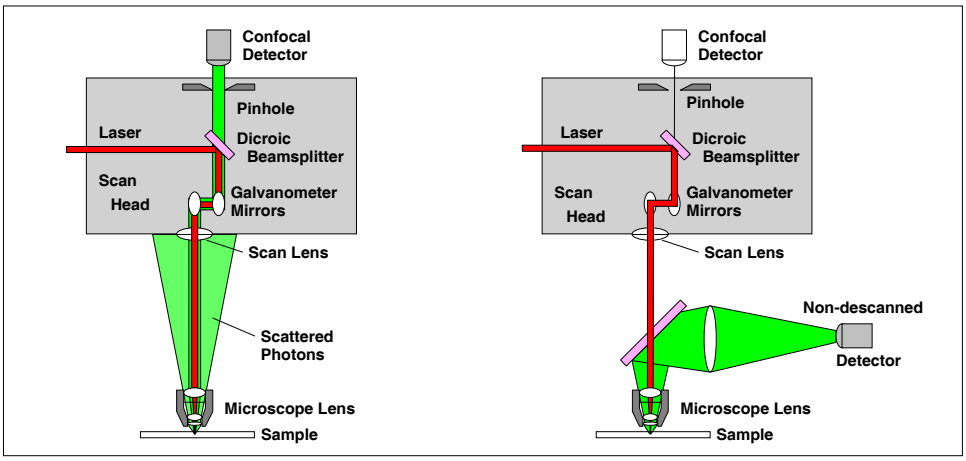

Fig. 3: Left: Confocal detection. Fluorescence scattered in the sample does not reach the detector. Right: Non-descanned detection. Both ballistic and scattered photons are transferred to the detector.

Single-Wavelength FLIM Detectors

As shown in Fig. 3, right, non descanned detection requires a dichroic beamsplitter in the microscope main body, and a suitable port to which the fluorescence light is directed. Suitable beamsplitters positions and side ports are available for the Olympus IX series microscopes. A common problem in conjunction with FLIM is that light from microscope lamps can be transmitted directly to a detector attached to one of these ports. The bh non-descanned FLIM detectors are therefore equipped with overload shutdown and electronically controlled shutters [2, 6] which minimise the risk of detector damage. The shutter assembly also contains the lens that transfers the photons to the detector.

A bh detector/shutter assembly with a PMC-100 cooled PMT module coupled to the side port of the IX81 microscope of an FV 300 multiphoton system is shown in Fig. 4.

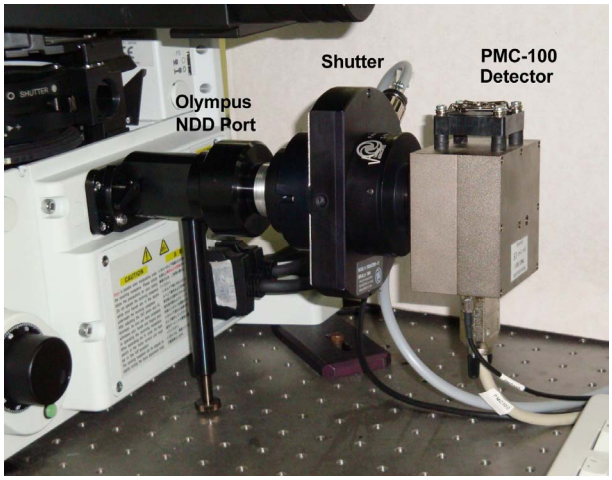

Fig. 4: PMC-100 detector coupled to the side port of the IX81 microscope of an FV 300 multiphoton scanning system

Other detectors, such as the ultra-fast R3809U MCP PMT or the bh HPM-100 hybrid detector module can be used the same way. The output pulses of the detectors are connected directly to the input of a bh TCSPC module. The module uses bh's multi-dimensional TCSPC process to record a photon distribution over the time in the fluorescence decay and the scan coordinates. Please see [1, 2, 3] for details.

Multi-Spectral FLIM Detection

The bh multi-spectral FLIM detector uses a bh PML-16 C multi-anode PMT module [4] in combination with a grating polychromator. The PML-16-C detector is controlled by a DCC-100 detector controller which provides overload shutdown and supplies the operating voltages to the detector. The DCC-100 modules also controls the shutter. The single photon pulses and the routing signals of the PML-16-C detector are directly connected to a bh TCSPC module. bh's multi-dimensional TCSPC process [1] is used to detect FLIM images simultaneously in all 16 wavelength channels of the detector [2, 4, 5].

Connecting a polychromator to a non-descanned port is faced with the problem that the image of the microscope lens delivered by the transfer lens neither in shape nor in size fits to the input slit of the polychromator. The bh multi-spectral FLIM assembly therefore uses a fibre bundle to achieve a transformation of the cross section of the light bundle [2, 4, 5]. The assembly attached to the side port if an IX81 FV300 system is shown in Fig. 5.

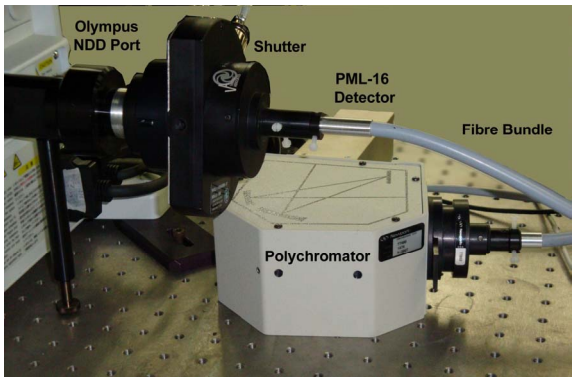

Fig. 5: Multi-spectral FLIM assembly attached to the side port of an IX81 FV300 scanning system

FLIM Electronics

The standard FLIM systems for the FV 300 and FV 1000 multiphoton microscopes use the bh 'Simple-Tau 150' TCSPC systems. The systems contain a bh SPC-150 TCSPC FLIM module and a bh DCC-100 detector controller. The TCSPC electronics is contained in an extension box that is connected to a lap-top computer via a bus extension interface. The Simple Tau 150 is shown in Fig. 6.

Fig. 6: Simple-Tau 150 system. The SPC-150 TCSPC-FLIM module and the DCC-100 detector controller are contained in a bus extension box of a lap-top computer

Because the bh systems are highly modular there are a number of possible modifications of these systems. An SPC-830 module may be used instead of the SPC-150, or two detectors may be used and the signals be processed in two parallel SPC-150 modules. Other modifications are the use of a standard Pentium PC instead of a Simple Tau system. (If you prefer a standard PC please make sure that the PC has dual-core architecture.) Please see [2] for details .

The FLIM systems come with the TCSPC hardware and the software readily installed. The software allows you to select between a large number of image formats with different numbers of pixels and time channels. Single FLIM images and time-series of FLIM images can be recorded. For focusing and sample alignment, a fast live mode allows you to display fluorescence images of the sample as fast as 2 frames per second. You can easily change between these setups by clicking on a button of the 'Predefined Setup' panel.

Data analysis includes fitting with single, double and triple-exponential models. For samples with SHG components a 'Scatter' component can be included in the fit. The results are displayed by false-colour images. Any parameter of the fit model, ratios of parameters, mean lifetimes, average lifetimes, SHG components, and the FRET efficiency of the interacting donor component of FRET experiments can be displayed. Please see [10] for details.

Results

A typical FLIM result obtained with an FV 300 multiphoton NDD FLIM system is shown in Fig. 7. The samples delivers both fluorescence from endogenous fluorophores and an SHG signal from the collagen in the tissue. The collagen thus show up via an infinitely fast signal component.

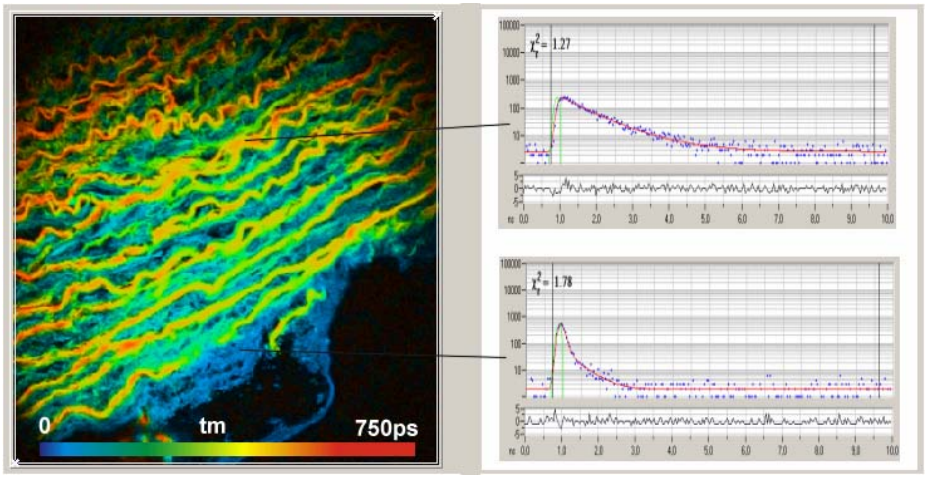

Fig. 7: Heart tissue sample. The sample shows fluorescence from endogenous fluorophores and SHG from the collagen in the tissue. Mean lifetime of double-exponential decay, colour scale from 0 to 750 ps.

References

1. W. Becker, Advanced time-correlated single-photon counting techniques. Springer, Berlin, Heidelberg, New York, 2005
2. W. Becker, The bh TCSPC handbook. 3rd edition, Becker & Hickl GmbH (2008), available on www.becker-hickl.com
3. W. Becker, A. Bergmann, M.A. Hink, K. König, K. Benndorf, C. Biskup, Fluorescence lifetime imaging by time-correlated single photon counting, Micr. Res. Techn. **63**, 58-66 (2004)
4. Becker & Hickl GmbH, PML-16-C 16 channel detector head for time-correlated single photon counting. User handbook. Available on www.becker-hickl.com
5. W. Becker, A. Bergmann, C. Biskup, Multi-Spectral Fluorescence Lifetime Imaging by TCSPC. Micr. Res. Tech. 70, 403-409 (2007)
6. Becker & Hickl GmbH, DCC-100 detector control module, manual, www.becker-hickl.com
7. Becker & Hickl GmbH, Modular FLIM systems for Zeiss LSM 510 and LSM 710 laser scanning microscopes. User handbook. Available on www.becker-hickl.com
8. Becker & Hickl GmbH and Leica Microsystems, Leica MP-FLIM and D-FLIM Fluorescence Lifetime Microscopy Systems. User handbook. Available on www.becker-hickl.com
9. bh NDD FLIM systems for Leica SP2 MP and SP5 MP Multiphoton Microscopes. Application note, Becker & Hickl GmbH (2007), www.becker-hickl.com
10. Becker & Hickl GmbH, Modular FLIM systems for Olympus laser scanning microscope FV1000. User handbook. Available on www.becker-hickl.com
11. Becker & Hickl GmbH, DCS-120 Confocal Scanning FLIM Systems, user handbook. www.becker-hickl.com