Non-Descanned FLIM Detection in Multiphoton Microscopes

Abstract. Multiphoton microscopes use a femtosecond NIR laser to excite fluorescence in the sample. Excitation is performed via a multi-photon absorption process. The advantage of multiphoton excitation is that fluorescence is excited only in the focal plane, and that the laser light penetrates deep into the sample. Multiphoton excitation becomes particularly efficient in combination with non-descanned detection (NDD). This application note describes the principles of non-descanned detection in combination with the bh fluorescence lifetime imaging (FLIM) devices and shows a number of typical NDD FLIM results.

Multiphoton Excitation

A multiphoton microscope excites the fluorescence in the sample via simultaneous absorption of several (usually two) photons of a femtosecond-pulsed near-infrared laser. Absorption and scattering in the NIR is substantially lower than at visible or ultra-violet wavelengths. Therefore multiphoton excitation penetrates substantially deeper into a sample than the visible or UV laser of a confocal microscope, see Fig. 1, left and middle. Moreover, depth resolution is obtained via the nonlinearity of the excitation process, see Fig. 1, middle. In other words, fluorescence is excited only in the focus of the femtosecond laser.

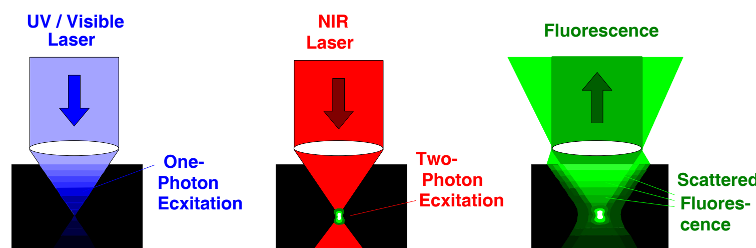

Fig. 1: Left: One-photon excitation. The effective excitation power decreases rapidly with increasing depth. Middle: Two-photon excitation. The NIR laser penetrates deeply into the sample. Right: The fluorescence from a deep focus is scattered on the way out of the sample. It leaves the back aperture of the microscope lens in a wide cone.

Principle of Non-Descanned Detection

When multiphoton excitation is used to reach deep sample layers scattering of the fluorescence photons becomes important, see Fig. 1, right. The scattering coefficient at the fluorescence wavelength is higher than at the excitation wavelength. Thus, a large amount of the fluorescence photons is scattered on the way out of the sample. These photons leave the back aperture in a diffuse cone of light. Because the scattered photons originate from the focus of the femtosecond laser they bear useful information. However, scattered photons cannot be collimated and thus not be fed back through the beam path of the scan head of laser scanning microscope.

The situation is shown in Fig. 2, left. Even if the pinhole is opened wide mainly ballistic photons would reach the confocal detector, scattered photons would be lost. The problem of detecting scattered photons is solved by 'non-descanned' detection (NDD). NDD makes use of the fact that

fluorescence in a multiphoton microscope is only excited in the focus. It is therefore not necessary to use a pinhole to reject out-of-focus fluorescence.

The principle of NDD is shown in Fig. 2, right. A second dichroic mirror diverts the fluorescence light immediately behind the microscope lens, and a transfer lens projects these photons on the detector. With a sufficiently large transfer lens and a reasonably large detector area, all photons emerging from the back aperture of the microscope lens are transferred to the detector.

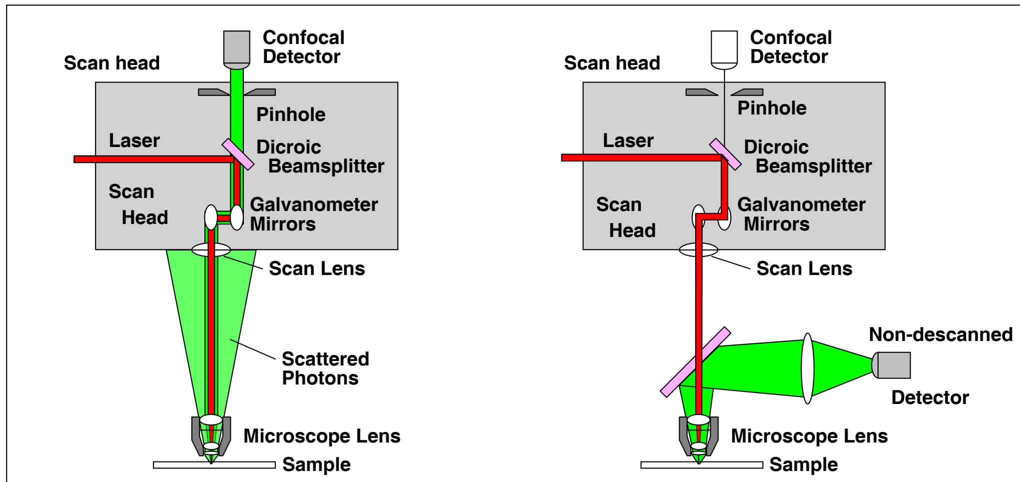

Fig. 2: Left: Confocal detection. Fluorescence scattered in the sample does not reach the detector. Right: Non-descanned detection. Both ballistic and scattered photons are transferred to the detector.

Details of a non-descanned detection beam path are shown in Fig. 3. The fluorescence leaving the back aperture of the microscope lens, L1, is separated from the excitation light by a dichroic mirror. The fluorescence is collected by a transfer lens, L3. This lens projects a de-magnified image of the back aperture of the microscope lens on the active area of the detector. L3 is normally placed directly behind a side port of the microscope. It should be large enough to collect all light transmitted through the internal beam path of the microscope, and have a focal length short enough to obtain a sufficiently small image of L1 on the surface of the detector. With typical values, such as d1 = 180 mm, d4 = 50 mm, and D3 = 30 to 40 mm, a de-magnification of a factor of 3.6 is obtained. That means, a detector diameter of 2.8 mm is sufficient to detect the light emerging from a microscope lens back aperture of 10 mm diameter.

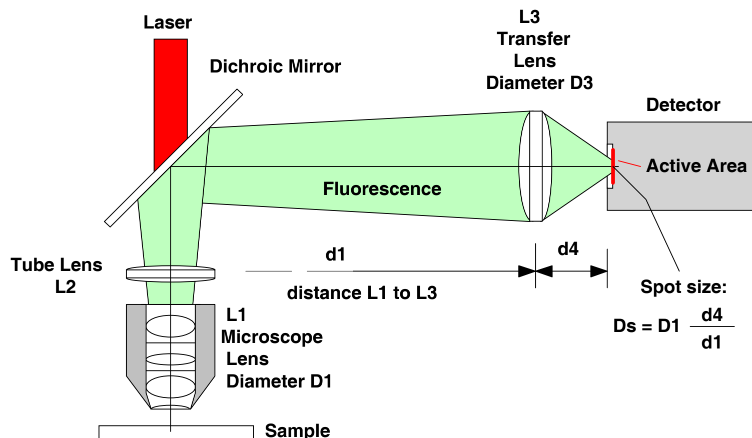

Fig. 3: Details of NDD beam path

It should be noted that the transfer lens, L3, does not need to have perfect optical correction. It is only required that it concentrates the fluorescence light into an area smaller than the active area of the detector. Usually a simple plano-convex or bi-convex lens is sufficient. In extreme cases an aspherical lens can be used.

The location of L1 in the beam path, i.e. the distance d1, has no relation to the location of the upper image plane. L1 can be placed at any distance, either before or behind the upper image plane. However, L1 should not be placed directly in the upper image plane to avoid dust on the lens causing shadows in the images recorded.

In any case, it must be avoided that the detector is placed in an image plane conjugate with the sample. This would result in scanning the luminescent spot over the detector and thus imprint internal structures of the detector on the images [1, 2]. An NDD setup can easily be checked by placing a sample in the microscope and turning on the lamp in the transmission path. The spot in the detector plane is then examined on a sheet of paper. In a correctly designed beam path the spot should be an image of the microscope lens, not an image of the sample.

Practical Examples of NDD FLIM Detectors

As shown in Fig. 3, 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 most research-grade microscopes. A common problem of NDD FLIM is that light from microscope lamps can be transmitted directly to a detector attached to one of these ports. The light can be so strong that it can damage the detector. The non-descanned FLIM detectors of bh are therefore equipped with overload shutdown, and electronically controlled shutters [2, 7, 8] are available. 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. Please see [11] for details.

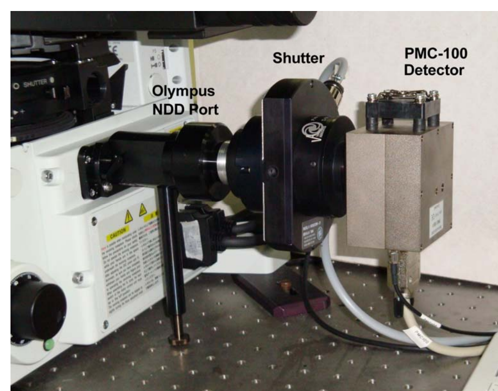

Fig. 4: PMC-100 detector with shutter coupled to the right side port of the IX81 microscope of an FV 300 multiphoton scanning system

bh NDD FLIM systems are available for different microscopes and with a number of different detectors [8, 10, 11]. Examples are shown in Fig. 5. The bh PMC-100-01 module with a Leica RLD adapter is shown in Fig. 5, left. The PMC-100 is a compact PMT module containing the preamplifier, the PMT power supply, overload shutdown circuitry, and thermoelectric cooling [2]. For ultra-high time resolution the Hamamatsu R3809U MCP PMT is available. To provide

maximum safety against overload the R3809U is usually operated via a shutter, see Fig. 5, second left and middle.

Fig. 5: Left to right: bh PMC-100 detector module with Leica RLD adapter, R3809U MCP PMT with shutter and Leica RLD adapter, R3809U MCP PMT with shutter and Zeiss LSM 510 adapter, HPM-100-40 hybrid detector module with Zeiss LSM 710 adapter, PMZ-100 PMT module with Zeiss LSM 710 adapter.

Since 2009 bh have added ultra-sensitive GaAsP hybrid detectors to their TCSPC systems. These detectors combine the sensitivity of a single-photon avalanche photodiode with the large active area of a PMT. They are free of afterpulsing and thus deliver an extremely high dynamic range of fluorescence decay detection [5, 8]. A HPM-100-40 with a Zeiss LSM 710 NDD adapter is shown second right. A PMZ-100 PMT module with an LSM 710 NDD adapter is shown right.

Multi-spectral multiphoton NDD FLIM [6] is available by using the bh MW FLIM multi-wavelength detector assembly [2, 4]. In combination with the bh TCSPC technique, the MW FLIM detects simultaneously in 16 wavelength channels, without any wavelength scanning or time gating [1, 2]. Multi-wavelength detection requires the fluorescence light to be transferred into the entrance slit of a polychromator. The bh multi-spectral FLIM assembly therefore uses a fibre bundle to achieve a transformation of the cross section of the light bundle [1, 2, 4, 6]. The MW FLIM assembly attached to the side port of an IX81 FV300 system [11] is shown in Fig. 6 .

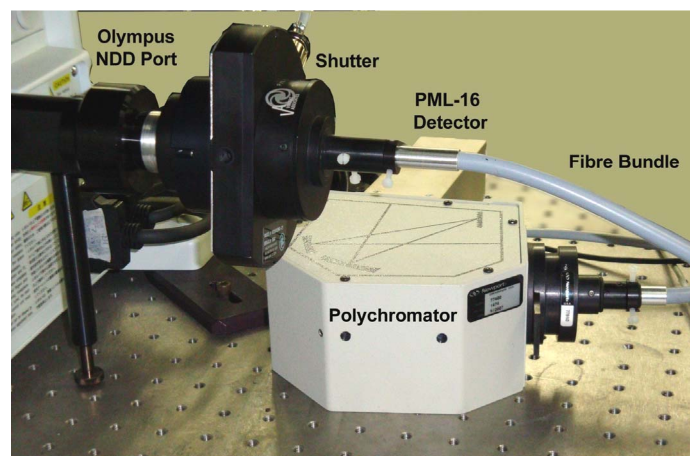

Fig. 6: Multi-spectral FLIM assembly attached to the side port of an IX81 FV300 scanning system

FLIM Electronics

bh NDD FLIM systems for multiphoton microscopes use the bh Simple-Tau 150 and Simple-Tau 152 TCSPC systems. The systems contain one or two bh SPC-150 TCSPC FLIM modules 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. Please see [1, 2] for details of the bh TCSPC technique. The Simple Tau 150 and 152 Systems are shown in Fig. 7.

Fig. 7: Simple-Tau 150 and Simple-Tau 152 systems. One or two SPC-150 TCSPC-FLIM modules and a DCC-100 detector controller are contained in a bus extension box of a lap-top computer

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 positioning, a fast preview mode allows you to display fluorescence images of the sample as fast as 2 frames per second [8, 10, 9]. 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 [8] or [9] for details.

Typical Results

A typical FLIM result obtained with a single-detector NDD FLIM system connected to an Olympus FV 300 multiphoton microscope is shown in Fig. 8. The samples delivers both fluorescence from endogenous fluorophores and an SHG signal from the collagen in the tissue. SHG is an ultra-fast process; the collagen thus shows up via an infinitely fast signal component.

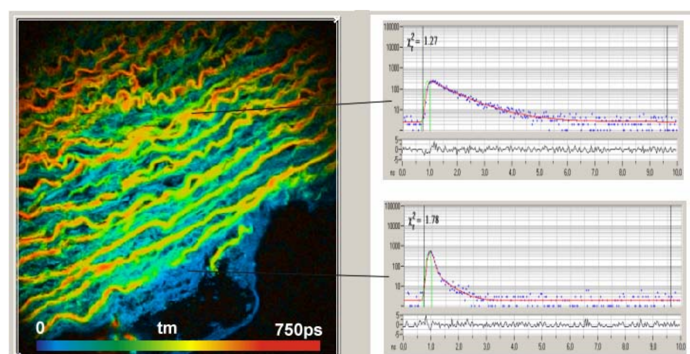

Fig. 8: Heart tissue sample. The sample shows fluorescence from endogenous fluorophores and SHG from the collagen in the tissue. Mean lifetime of double-exponential decay, colour scale from 0 to 750 ps.

Fig. 9 shows NDD FLIM images recorded by a dual-channel Simple-Tau 152 FLIM system attached to a Zeiss LSM 710 NLO. Two images were recorded simultaneously in two wavelength intervals.

The left two images show a pig skin sample excited at 800 nm, and recorded at 400 to 480 nm (left) and 480 to 650 nm (right). The left image shows the fraction of SHG, the right image the fluorescence lifetime. The right two images show a plant sample. The images were recorded at 450 to 550 nm and 550 to 700 nm. The left image shows the flavines in the cell membranes, the right image the chlorophyll in the chloroplasts of a plant sample.

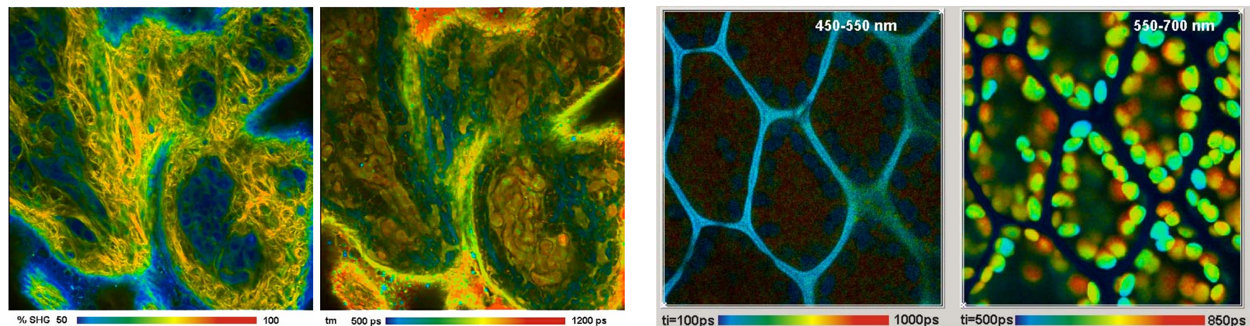

Fig. 9: Multiphoton images, LSM 710 NLO, dual-channel FLIM, HPM-100-40 GaAsP hybrid detectors. Left: Pig skin, 40 um deep, excitation 800 nm. Left image shows percentage of SHG at emission wavelength <480 nm, right image shows fluorescence lifetime at >480 nm. Right: Plant sample, excitation at 860 nm. Left image emission at 450 to 550 nm, right image emission at 550 to 700 nm.

A multi-wavelength FLIM result is shown in Fig. 10. The images show a plant sample in 16 wavelength intervals. The data were recorded by a bh Simple-Tau 510 with bh multi-wavelength FLIM assembly connected to the non-descanned output of a Zeiss LSM 710 NLO.

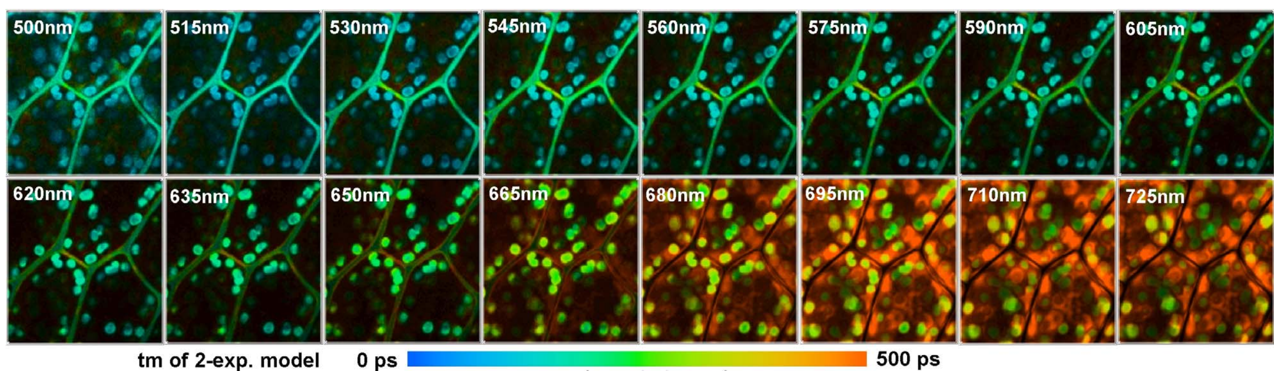

Fig. 10: Multiphoton Multispectral NDD FLIM. LSM 710 NLO, bh MW FLIM detector

Summary

Multiphoton excitation penetrates substantially deeper into a sample than one-photon excitation. The depth penetration capability of multiphoton excitation can only be exploited by non-descanned detection. Non-descanned detection systems project an image of the back aperture of the microscope lens on the detector. With appropriate lens diameters, NDD systems are not only highly efficient for ballistic photons, they also detect photons scattered on the way out of the sample. Non-descanned detectors for the bh FLIM systems are available for all commercial laser scanning microscopes, and can easily be adapted to home-built systems.

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