Technical Note

Compact FLIM NDD Upgrade Kit for Olympus FluoView FV1000MPE

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Introduction

High-resolution deep tissue imaging is very often impossible for classical confocal microscopy due to strong scattering of the visible excitation light. One possibility to overcome this limitation is to use near infra-red (NIR) light along with 2-photon excitation (2PE) schemes. This approach allows deep tissue imaging with penetration depths up to 1 mm. In addition, low-energy NIR excitation light and the highly localised excitation strongly reduces global photo-bleaching of the fluorescent dye as well as tissue damages. 2PE-microscopy also permits to perform Fluorescence Lifetime Imaging (FLIM) in deep tissue using the method of Time-Correlated Single Photon Counting (TCSPC). Due to the nonlinear process of 2PE the generated fluorescence is restricted only to the small focal excitation volume. As a consequence a pinhole in front of the detector to block out-of focus fluorescence is not necessary to achieve confocal detection.

To preserve the full image contrast from considerable depth within thick specimens, 2PE-microscopy is usually performed using non-descanned detection (NDD). In this configuration no pinhole is inserted into the beam path and large active area detectors such as Photomultiplier Tubes (PMTs) are used. These large area detectors lead to an increased fluorescence detection probability, even if the fluorescence light is scattered inside the tissue. However, compared to single molecule sensitive SPAD (Single Photon Avalanche Diode) detectors which are typically used in the FLIM and FCS upgrade kit from PicoQuant, the PMTs have a lower detection efficiency and a lower temporal resolution. PMTs can therefore only be used for FLIM but not FCS. SPAD detectors can, on the other hand, not be used in NDD set-ups, as the active sensor area of these detectors is simply too small.

Technical Realization

LSM system

The FLIM NDD set-up can be mounted both on an inverse or upright Olympus FluoView FV1000MPE system.

As a prerequisite, the FV1000MPE must be equipped with one of the following, newer types of the Olympus NDD units:

1. Two channel reflected NDD units, FV10MP-BXD2CH (upright BX61WI microscope) or FV10MP-IXD2CH (inverse IX81 microscope)
2. Four channel reflected NDD units FV10MP-BXD4CH (upright BX61WI microscope) or FV10MP-IXD4CH (inverse IX81 microscope)

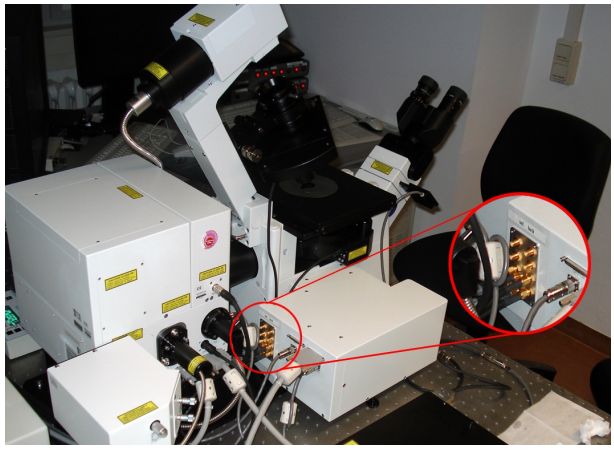

Fig. 1: Olympus FV1000MPE upgraded with NDD FLIM. The modification for the signal output is marked in red.

The 2PE-excitation laser of the FV1000MPE system can be directly used for TCSPC FLIM without any modification. The internal Olympus Photomultiplier Tubes (PMT) in the NDD box can also be used for TCSPC FLIM as they can be operated in a photon counting mode. However, a slight modification of the LSM NDD detector box is still necessary as the output of the internal PMT detectors can not be connected to an external TCSPC electronics (see Fig. 1). The adaptation basically includes a modified top cover of the NDD box which makes the PMT outputs available with an external connector. The PMT output can then either be connected to an external TCSPC electronics or fed back into the NDD box, restoring the original functionality. Switching between TCSPC FLIM mode and conventional NDD imaging is thus easily possible by a simple cable change. Depending on the LSM configuration up to

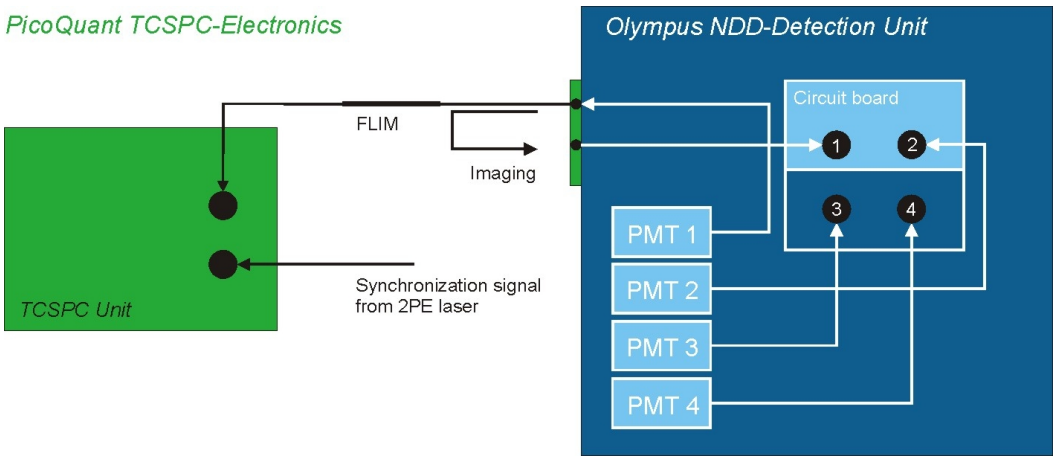

Fig. 2: Schematic overview of a four-channel NDD FLIM set-up. In this scheme, the cabling modification just for PMT 1 is shown.

four PMTs in the NDD box are available for FLIM imaging. With this solution a high system integration of the FLIM upgrade is realized, since the detectors can be addressed by the LSM software in both operation modes (Fig. 3). The modification does not affect other detectors within the LSM. Fig. 2 gives a schematic overview of the NDD FLIM set-up.

Components of the PicoQuant upgrade

Besides the modification of the NDD box, the additional necessary components for performing TCSPC FLIM are the TCSPC unit itself (PicoHarp 300 or HydraHarp 400), signal adapters to pick-up or modify (if necessary) the synchronization signal from the 2PE laser and a suited data acquisition and analysis software (SymPhoTime).

The TCSPC module PicoHarp 300 has one input for a single detection channel. Working with more than one PMT therefore requires the universal signal router PHR 800, which allows connecting and recording the signals of up to four detectors in parallel.

If the multichannel picosecond event timer HydraHarp 400 is used instead of the PicoHarp 300, a router is not needed since the HydraHarp 400 TCSPC device contains up to 8 separate and independent but synchronized detector inputs.

Furthermore, the FLIM NDD upgrade requires the system software SymPhoTime for data acquisition and analysis which is running on a dedicated second computer.

Test measurements

Feed-through test to check NDD imaging

As a first test, the PMT signals from the external connector at the modified NDD cover where fed back into the NDD box (see Fig. 2). This resulted in standard operation of the detectors and allowed for conventional NDD imaging of fluorescence emission. Influence on the imaging performance by the modified NDD box could not be observed.

Detector test to check NDD FLIM performance

As a second test, one PMT signal from the modified NDD cover was connected to the PicoQuant TCSPC electronics. By operating the Olympus NDD PMT in the photon counting mode (Fig. 3), an Instrumental Response Function (IRF) of 500 ps was obtained (see Fig. 4).

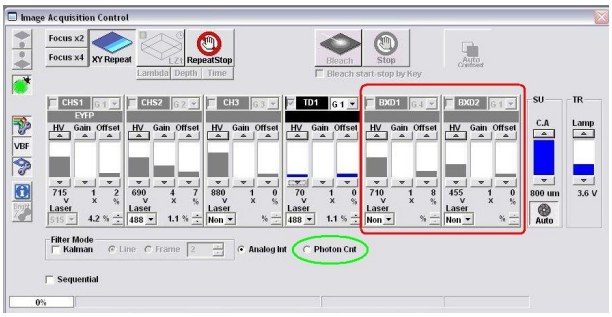

Fig. 3: Screen shot of the LSM software. The two red circled detectors can be used for NDD FLIM if the Photon Counting mode is activated in the Image Acquisition Control window (green circle).

The detector has approximately 1000 dark counts per second and the time-resolved data acquisition reveals reflections which are generated somewhere inside the LSM. The influence of these reflections can, however, be removed during data analysis. It is crucial to shield the sample sufficiently, as NDD is very sensitive to picking up ambient light.

All in all, the performance (IRF, reflections, dark counts) of the Olympus NDD detectors is sufficient for FLIM measurements of standard dye labels with lifetimes > 0.3 ns.

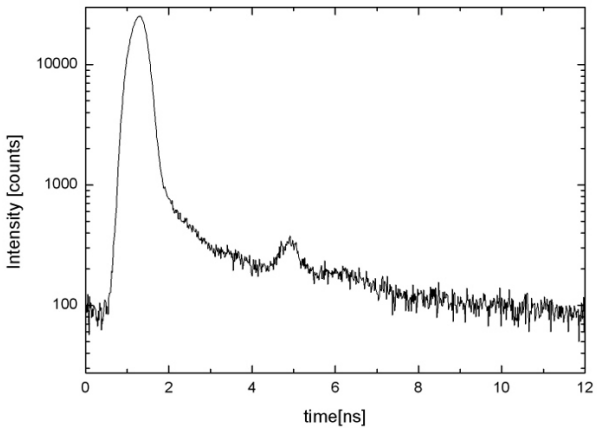

Fig. 4: Measurement of the instrument response function (IRF) via SHG in collagen samples using the internal NDD PMTs operated in photon counting mode. An IRF of 500 ps was obtained with a clearly visible reflection approx. 3 ns after the main peak. The decaying tail of the IRF is due to residual fluorescence from the collagen sample.

FLIM measurement

For FLIM measurements, two PMT signals from the modified NDD cover were connected to the PicoQuant TCSPC electronics. Fixed cells transfected with an EGFP-mRFP fusion construct were excited using a 2PE-laser (Spectra Physics MaiTai, emission wavelength 900 nm, repetition rate 80 MHz) and the donor and acceptor emission was recorded in channel 1 and 2, respectively. This sample acts as a FRET positive control (sample courtesy of Ahmed Sohail, IMB, Singapore) showing an amplitude-weighted average lifetime of the donor EGFP of 1.2 ns in the unbleached region (TCSPC histogram and Lifetime histogram in Fig. 5). Photo-bleaching of the acceptor mRFP in the indicated cellular region resulted in an 23% increase of the average donor lifetime towards 1.6 ns. This result was in good agreement with the intensity based FRET approach: Here an average donor fluorescence intensity increase of 23% could be observed after destroying the acceptor by photo-bleaching.

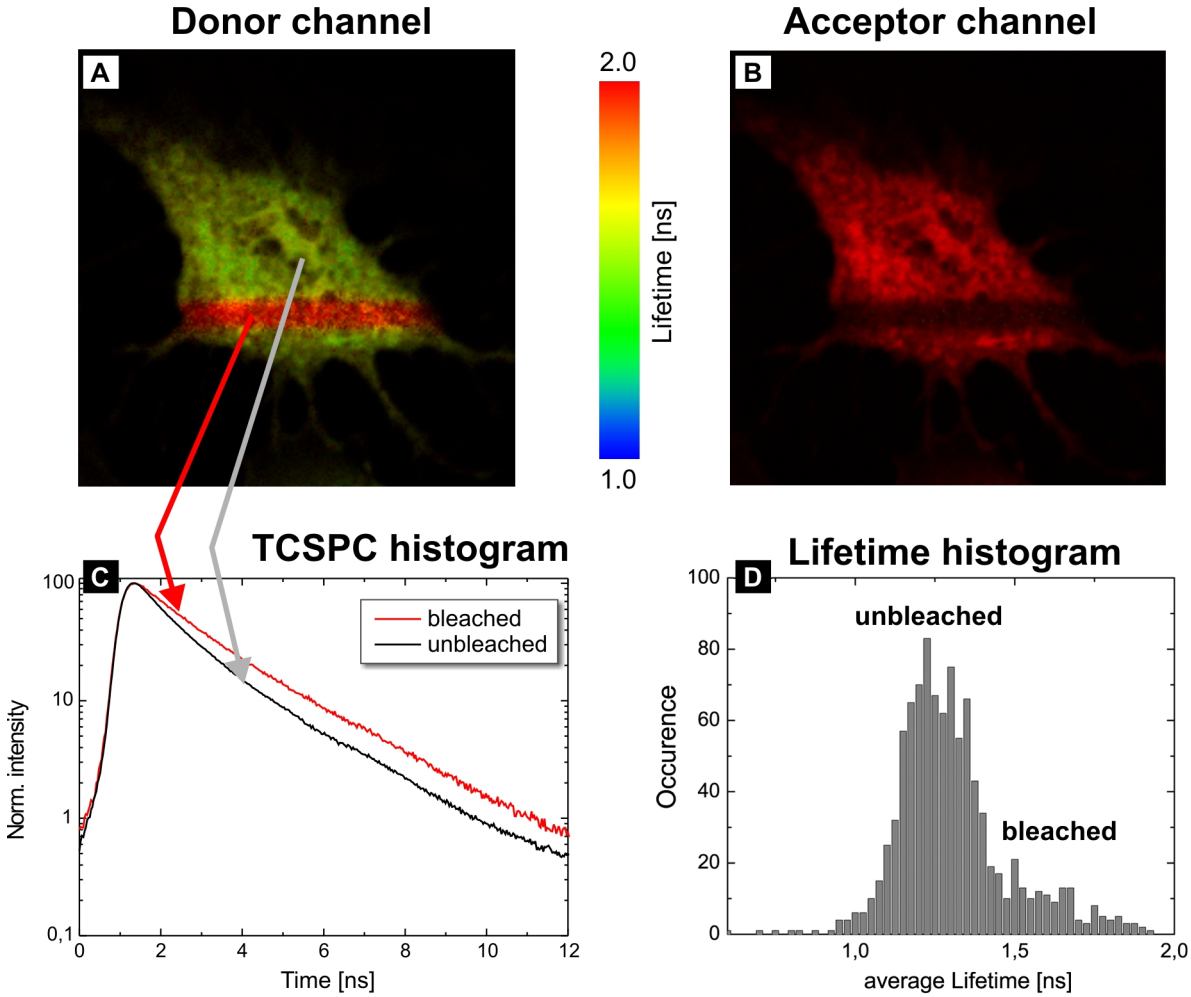

Fig. 5: FLIM NDD measurements of cells containing a EGFP-mRFP tandem construct. The fluorescence lifetime in the EGFP channel (A) and mRFP channel (B) is indicated by a false color representation according to the rainbow marker. The EGFP/RFP filter set in the Olympus FV1000MPE restricted the fluorescence in the corresponding channel to EGFP or mRFP only. The stripe indicates the photo-bleached area where the acceptor was destroyed. The TCSPC histogram (C) displays the fluorescence decay of the donor channel (EFGP) in the unbleached (black curve) and acceptor-bleached (red curve) cellular region. In the lifetime histogram (D) the average intensity-weighted lifetimes of the bleached and non-bleached cellular region were plotted. Recording time 2.9 min, image size 100x100 µm, 512x512 pixel.

Conclusion

The compact FLIM NDD Upgrade Kit for Olympus FluoView FV1000MPE allows deep tissue FLIM imaging at depths beyond 100 µm. Using up to four Olympus internal NDD PMTs, the upgrade provides a cost-effective, highly integrated and compact solution for FLIM imaging. The necessary modification of the NDD box is easily reversible and guarantees normal operation of the detection unit. The performance of the Olympus NDD detectors enables to measure fluorescence lifetimes down to ~0.3 ns but is not sufficient for FCS measurements.

In general, SPAD detection is still advantageous in 2PE systems compared to NDD PMTs for most applications except deep tissue imaging, because SPAD detectors have a significant higher detection efficiency (up to 45%) and a significantly shorter instrument response time (down to 50 ps).

Further reading

[1] Bibliography listing all publications with measurements based on PicoQuant instruments:
http://www.picoquant.com/_biblio.htm

[2] general download link of technical and application notes:
http://www.picoquant.com/appnotes.htm

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