DCS-120 Confocal FLIM System with Wideband Beamsplitter

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The use of a wide range of excitation wavelengths in a confocal laser scanning system leads to a number of design problems. The most critical one is connected to the main dichroic beamsplitter that separates to fluorescence signals from the excitation beam. For use with several lasers the beamsplitter must either be switchable or tuneable, or a multiband dichroic must be used. The result is either alignment instability, or spectral gaps in the fluorescence detection channels. We developed a version of the DCS-120 confocal FLIM scanner that bypasses most of these problems by using a wideband beamsplitter. The design allows the user to switch lasers without compromising alignment stability. The sensitivity of the system is sufficient to record autofluorescence images of single cells.

Motivation of Using Wideband Systems

There is a wide variety of fluorescence markers used in laser scanning microscopy. The fluorescent proteins alone span over an excitation wavelength range of almost 200 nm [4, 6]. Moreover, there is increasing interest in using near-infrared dyes, with absorption maxima up to 800 nm [5, 9]. Users of laser scanning microscopes therefore want their system to be ready for working with a wide range of different laser wavelengths. Increasing the number of excitation wavelengths is often considered easy: Add more lasers to the system, make lasers interchangeable, or just use a tuneable laser.

Unfortunately the task is not as simple as it may appear. The reason is the main dichroic beamsplitter of the scanning system. The beamsplitter is designed to reflect the laser beams down the beam path of the microscope, and transmit the fluorescence returned from the sample to the detectors, see Fig. 1.

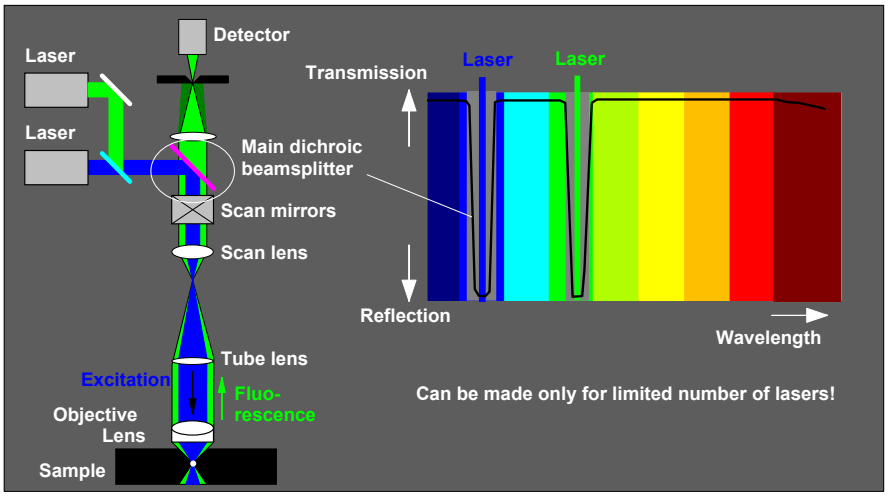

Fig. 1: Basic function of the main dichroic beamsplitter in a confocal scanning system

To work with several lasers of different wavelength the beamsplitter either must be replaced when the laser is changed, or the beamsplitter must be designed to reflect several lasers. Both approaches have advantages and disadvantages.

Switching the beamsplitters (i.e. by placing several dichroics on a wheel) requires extraordinary mechanical precision. If the direction of the beam changes only by a few arc seconds the laser spot in the sample moves, and the fluorescence beam is no longer focused into the pinhole. That means in practice that there must be some kind of automatic re-alignment that corrects for angle variations between the different dichroics. There is also a second disadvantage: Fast multiplexing of lasers of different wavelengths within pixels, lines, or frames is impossible.

Another way of dealing with several wavelengths is to use a main dichroic beamsplitter that has several reflection and transmission bands. The standard versions of the bh DCS-120 confocal scanner use this design [1, 2]. It delivers high efficiency and excellent mechanical stability, and allows lasers to be multiplexed at high rate. The problem of the multi-band dichroic is, however, that it can be made only for a very limited number of laser wavelengths. Fluorescence cannot be transmitted within the laser reflection bands, and the reflection bands cannot be made narrower than about 10 nm. This is especially the case for ps diode lasers diode lasers that can vary in wavelength and have several nm of spectral bandwidth. Moreover, a reasonable manufacturing tolerance for the dichroic must be left. That means in practice that the dichroic can only be made for two or three laser wavelengths which are reasonably spaced from each other.

There are other solutions, like acousto-optical beamsplitters (AOBS) or variable-wavelength dichroics. However, these have other problems: An AOBS has narrow bandwidth and less-than ideal sideband suppression. Moreover, its common use is prevented by patent issues. A variable-wavelength dichroic has to be moved when the wavelength is changed, which again causes stability problems and makes fast laser multiplexing impossible.

The easiest way to avoid these problems is to go back to Minsky's original design [7, 8] and use a wideband beamsplitter, i.e. a partially reflective mirror, see Fig. 2.

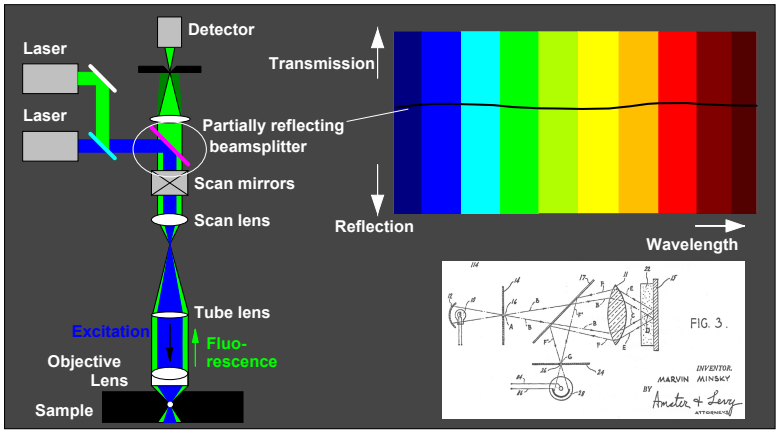

Fig. 2: Minsky's design with partially reflecting mirror as a main beamsplitter

At first glance, a wideband beamsplitter may appear a very poor design: A considerable part of the emission and the excitation light would be lost. A somewhat closer look, however, shows that Minsky's approach is not so poor after all: The vast majority of TCSPC FLIM experiments is performed at less than 10% of the available laser power. Under these conditions, a loss in excitation power at the beamsplitter can easily be compensated for by increasing the laser power at the input of the scanner.

The loss in detection efficiency is more serious: Any loss in efficiency results either in a decrease in fluorescence lifetime accuracy, or in an increase in acquisition time. However, also here practice

teaches different: Consider a 60/40 beamsplitter, with 60% transmission for the fluorescence. The factor of 0.6 in efficiency is the same as the ratio in collection efficiency between an NA=1.0 water immersion lens and an NA=1.3 oil immersion lens. In contrast to beamsplitter efficiency, the dependence of the collection efficiency on the NA is commonly ignored. No one would hesitate to use the water immersion lens to obtain better images of live cells, no matter of what the collection efficiency is. It therefore appears reasonable to sacrifice 40% collection efficiency for obtaining more flexibility in laser wavelengths.

The options for using wideband beamsplitters have also improved by the introduction of new detectors: Hybrid detectors are far more efficient than previously used PMTs. The increase in efficiency is not only due to a better cathode quantum efficiency but also to the absence of afterpulsing background [1, 3]. The result is that a given accuracy in fluorescence lifetime is obtained at a substantially lower sample emission rate. A scanning system with wideband beamsplitter and a hybrid detector therefore delivers at least the efficiency as a system with a dichroic beamsplitter and a conventional PMT.

Technical Issues

Using a wideband beamsplitter in a confocal scanning system solves the problem of alignment stability for different laser wavelengths. However, this does not mean that that there are no pitfalls. The technical issues associated with wideband systems are discussed in the section below.

Wideband Emission of Diode Lasers

All laser diodes are more or less plagued by a spectrally broad background of wideband emission. The background results from luminescence of the semiconductor material. It is excited both by the laser radiation and by late recombination of electron-hole pairs. The background emission is orders of magnitude weaker than the laser emission but shows up clearly when the laser wavelength is blocked by a filter. Typical spectra for the bh BDL-SMC lasers (with Nichia laser diodes) are shown in Fig. 3.

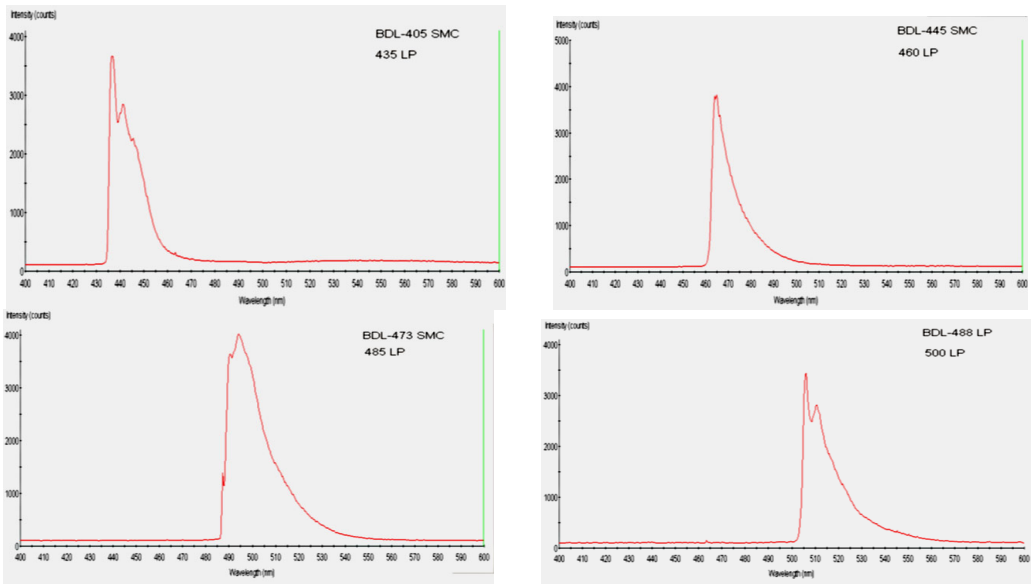

Fig. 3: Spectra of wideband emission from picosecond diode lasers. The laser wavelength was blocked by long-pass filters as indicated in the diagrams. Wavelength scale from 400 to 600 nm.

The spectra were recorded in the picosecond mode with the laser wavelength blocked by long-pass filters. Filter wavelengths are indicated in the diagrams. Please note that some ripple may be induced in the spectra by the filter characteristics. Typical waveforms of the background are shown in Fig. 4. The waveforms contain a background from long-lifetime luminescence, and short-decay components. The ripple on the curves is real and probably comes from ringing of the diode reverse voltage after the driving pulse.

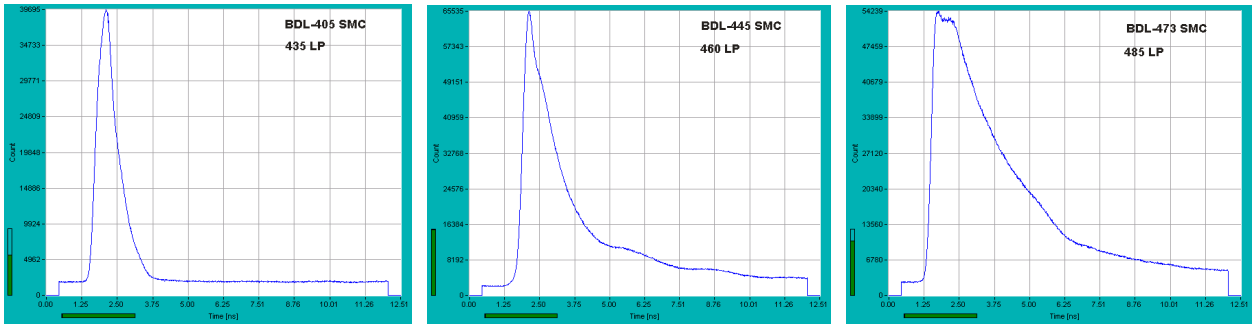

Fig. 4: Waveforms of wideband emission from 405nm, 445nm, and 473nm ps diode lasers. Laser wavelength blocked by filters, as indicated in the diagrams

For a confocal scanner that uses a dichroic beamsplitter spectral background from the laser is not normally a problem. The main dichroic beamsplitter acts as a bandpass or low-pass filter for the laser and thus suppresses the background. However, there is no such background suppression by a wideband beamsplitter. Systems with wideband beamsplitters must therefore be operated with filters in the laser beam path.

There are versions of the BDL-SMC lasers that have a cleaning filter integrated. For lasers without integrated filters a cleaning filter can be inserted in the collimator barrels of the fibre couplers. Suitable filters and filter holders are available from bh.

Variation of Laser Wavelength, Spectral Width

The emission wavelength of different laser diodes of the same wavelength type can vary considerably. Variations up to 10 nm are not unusual. Moreover, the spectral width is larger in the picosecond mode and can reach 10 nm in some cases, see Fig. 5, right.

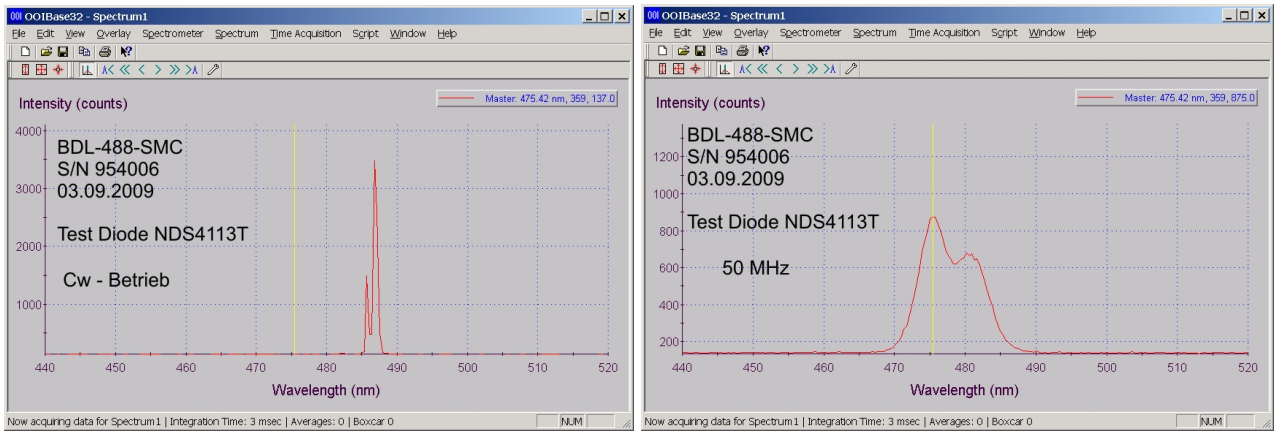

Fig. 5: Left: Optical spectrum in CW mode, power 30 mW. Right: Optical spectrum in picosecond mode, 50 MHz, average power 0.8 mW.

For one and the same diode, there can also be a spectral shift between CW operation and picosecond operation. For most diodes this shift is only a few nm. However, for the Nichia 488 nm diodes it can be almost 10 nm, see Fig. 5. Both diode-specific variations and shift between CW and ps operation have to taken into consideration when cleaning filters are specified.

Emission Path Filters

In a system with a dichroic beamsplitter it is normally sufficient to use an emission filter that reliably blocks the laser wavelength. For wideband systems this is not necessarily sufficient. For reasons explained above, the cleaning filter in the laser beam path has to leave room for laser wavelength tolerance, spectral width, and spectral shift. In practice, the cleaning filter transmission range must be about 20 nm wide. This has consequences to the selection of the filters in the detection beam path: The detection filters of a wideband system must be selected to block not only the laser wavelength, but the *whole transmission range of the cleaning filter*.

Filter issues are especially important for samples of low fluorescence yield and samples with strong scattering. A filter combination that delivers acceptable results for clear samples (see Fig. 8 and Fig. 9) does not necessarily work for thick tissue samples or other highly scattering objects. Please note that laser background has a waveform very similar to the fluorescence decay in the sample (see Fig. 4). Laser spectral background scattered back from the sample is therefore hard to identify. The only way of avoiding contamination with laser background is correct selection of filters.

Optical Reflections

Optical reflections from glass surfaces are much more troublesome in wideband systems than in systems with dichroic beamsplitters. Of course, in correctly designed scanner optics reflected laser light should not be focused into the pinholes. However, if the pinholes are opened wide reflected light may leak into the detectors. Reflected laser background can be identified by the fact that it arrives earlier than the fluorescence signal from the sample. Moreover, it is also detected when the sample is removed.

Fig. 6 shows an example. The data were recorded with a BDL-473SMC laser and a 475 ± 25 nm cleaning filter. The image shown left was recorded through a 485 nm long pass filter. This filter efficiently blocks the 473 m laser wavelength, but not the full transmission range of the cleaning filter. The waveform (shown in the middle) shows the decay function of the reflected laser background. Please note that the rising edge of the signal is not visible: It is outside the recording range because the reflected signal arrives earlier than the sample fluorescence. The image on the right was recorded with a 535 ± 25 nm bandpass filter in the detection path. This filter has no overlap with the cleaning filter. It removes the laser background signal entirely.

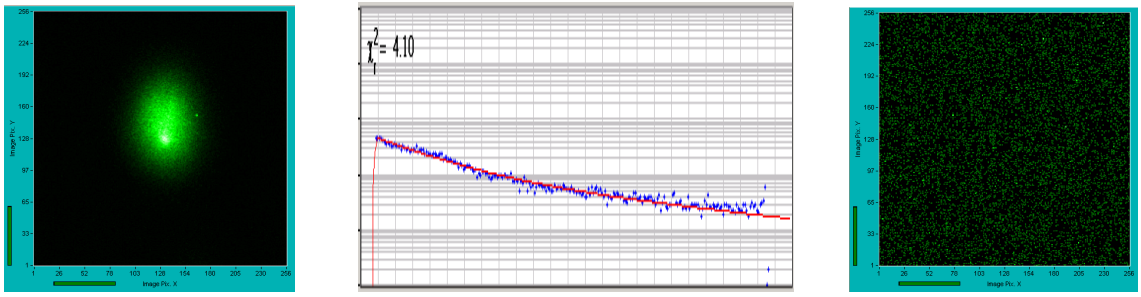

Fig. 6: Left: Laser background reflected at the scan lens, recorded with an emission filter that blocks only the laser wavelength. Middle: Waveform of signal, the rising edge is left of the recorded interval. Right: Non-overlapping filtering removes the problem entirely. DCS-120 with wideband beamsplitter, BDL-473 SMC laser, Pinhole 5 AU.

Polarisation

Laser background signals are highly polarised. Suppression of laser background is therefore especially important for fluorescence anisotropy and anisotropy decay measurements.

Moreover, it should be noted that a wideband beamsplitter is not entirely polarisation-independent. Polarisation-independent detection is, however, essential in order to cancel the influence of the anisotropy decay on the recorded decay functions. With a dichroic beamsplitter the anisotropy decay is cancelled by simply using high-NA objective lenses [1]. This is not exactly the case for a wideband beam splitter. Polarisation on a wideband beamsplitter is on the order of 10%. We believe that the effect of the anisotropy decay is small enough to have no noticeable influence on the fluorescence decay functions recorded. Detailed tests still have to be done.

Swapping Lasers

The intention of using a wideband beamsplitter is the ability to connect a large number of different lasers to the system. The DCS-120 uses precision fibre connectors of the Point-Source[1] type both at the laser and at the scanner side, see Fig. 7, left and right. The reproducibility of these connectors is so good that fibres can be swapped virtually without re-alignment. If necessary, fine confocal alignment can be done by the alignment screws of the fibre manipulators at the scanner input [2].

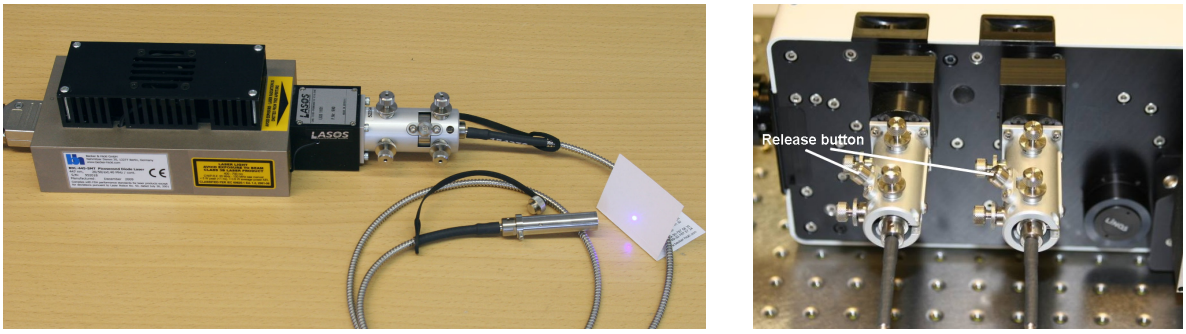

Fig. 7: Fibre coupling system used for BDL-SMC lasers and DCS-120 scanner

A few pitfalls do, however, exist also here. Single-mode fibres are designed for a single laser wavelength, or for a certain wavelength range. Moreover, unless lasers with integrated filters are used cleaning filters must be inserted in the collimator barrels of the fibres. For these reasons, fibres usually cannot be swapped at the laser side.

Swapping fibres at the scanner side bears another problem: The collimators at the fibre outputs must produce a perfectly collimated beam. Poor collimation is no problem for a permanently attached fibre - it can be corrected for by the divergence corrector of the DCS-120 scanner. However, if fibres are swapped the collimation state for different fibres must be identical. If the collimation is wrong the laser would no longer be focused into the same focal plane the pinhole is looking at. The result would be substantial loss in intensity, and poor optical resolution. Therefore, a DCS system that allows for swapping laser fibres must use especially specified fibres of similar collimation state.

[1] Since August 2010 Point Source Ltd. is Qioptiq Ltd.

Results

Fig. 8 and Fig. 9 show that the DSC-120 with a wideband beamsplitter delivers lifetime images of good quality. Fig. 3 shows a convallaria sample, excited with the 405 nm (left) and with the 473 nm (right) BDL-SMC laser. There is no intensity problem for this sample, count rates of several MHz are obtained at a fraction of the available laser power.

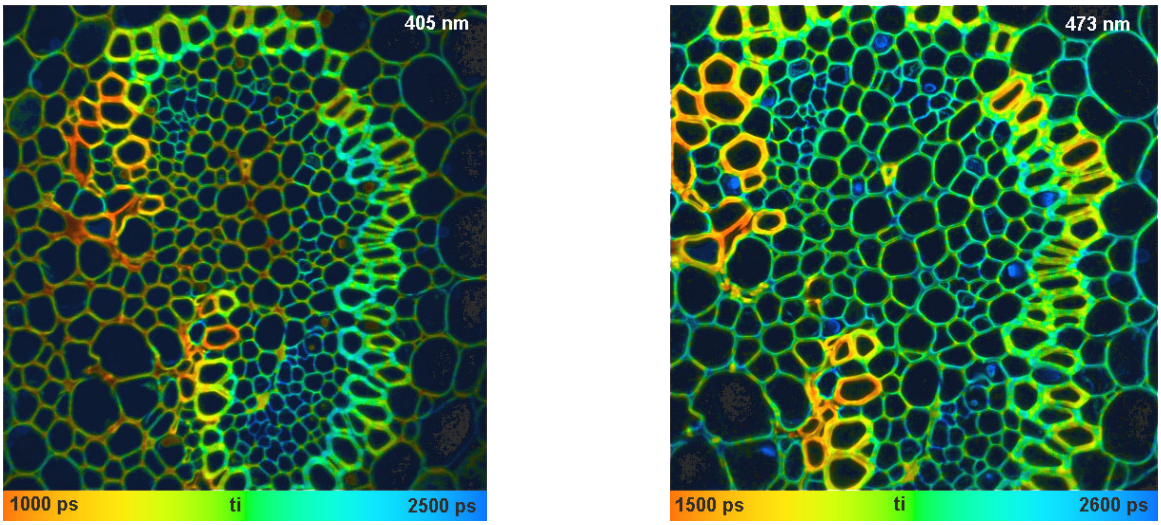

Fig. 8: Lifetime images of a Convallaria sample. DCS-120 system with wideband beamsplitter. Left: Excitation 405 nm, detection from 435 to 500 nm. Right: Excitation 473 nm, detection from 500 to 550 nm. Wideband beamsplitter 40/60, Nikon NA=1.3 oil immersion lens. Image format 512 x 512 pixels, 256 time channels.

Fig. 9 shows an autofluorescence image of a human epithelium cell. The 405 nm laser was used at a power of about 0.6 mW, where it still delivers near-gaussian pulse shape. The laser attenuator at the input of the DCS-120 was fully opened. Under these conditions, even standard PMC-100-20 PMT modules delivered a count rate on the order of 50,000 to 80,000 counts per second. The count rate with the more sensitive hybrid detectors is about 300,000 counts per second.

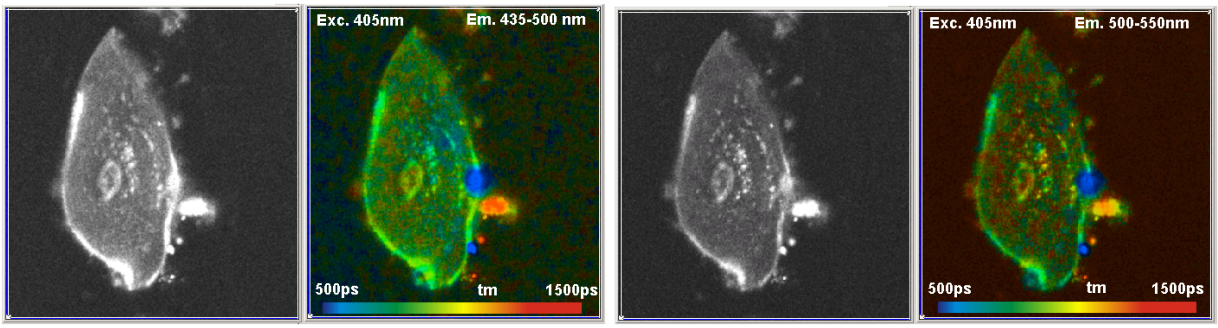

Fig. 9: Autofluorescence images of human epithelium cell, excitation with BDL-405SMC 405 nm ps diode laser. Left: Emission wavelength range 430 to 500 nm. Right: Emission wavelength range 500 to 550 nm. Intensity images and lifetime images. DCS-120 system with wideband beamsplitter, pinholes 1.5 AU, PMC-100-20 detectors.

An image recorded at 640 nm excitation wavelength is shown in Fig. 10. It shows cells stained with the dye Odyssys 680. An HPM-100-40 hybrid detector was used, the detection wavelength was 680 to 720 nm. Interestingly, the dye displays a slightly double-exponential decay function, and lifetime variations on the order of 200 ps. Whether the inhomomogeneity is caused by variation in the binding or by variation of other cell parameters is not known.

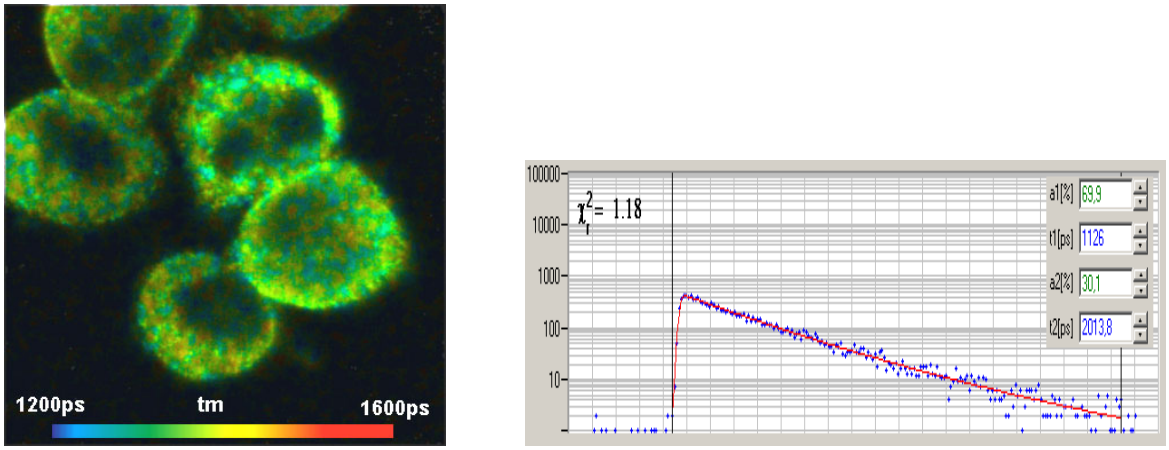

Fig. 10: Cells stained with red-absorbing dye, excitation with BDL-640 SMC (640 nm), detection from 680 to 720 nm. Right: Fluorescence decay function in selected spot of the cells.

Conclusions

The use of a wideband beamsplitter is a way to operate the bh DCS-120 confocal scanning FLIM system with more than the standard two excitation wavelengths. Different lasers can be connected to the DCS-120 by simply swapping the single-mode fibres at the input of the scan head. A few precautions are, however, recommended. Laser delivery fibres of tightly tolerated collimation state should be used, and cleaning filters must be inserted in the lasers or in the fibre collimators. The transmission band of the filters in the detection path should not overlap with the transmission band of the cleaning filters. With these precautions, high-quality FLIM results are obtained for a wide variety of laser wavelengths.

References

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