DCS-120 Confocal Scanning FLIM System: Two-Photon Excitation with Non-Descanned Detection

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Abstract: This application note shows how the bh DCS-120 confocal FLIM system can be upgraded for multiphoton FLIM with non-descanned detectors. With the upgraded system, highly detailed lifetime images from unstained mammalian skin could be recorded from as deep as 90 µm below the surface. The efficiency of the system is excellent. An average count rate of 1 MHz could be obtained without detectable photobleaching within an acquisition time of two minutes. The quality of the data obtained within this time was adequate for triple-exponential decay analysis.

Two-Photon Excitation

Simultaneous absorption of two (or more) photons has been theoretically predicted by Maria Göppert-Mayer [7]. It has been suggested for laser scanning microscopy by Wilson and Sheppard in 1984 [8] and practically introduced by W. Denk and J.H. Strickler [6] in 1990. The efficiency of two-photon excitation increases with the square of the excitation power density. If a femtosecond Titanium-Sapphire laser is focused into a diffraction-limited volume by a microscope lens the excitation process becomes so efficient that no more than a about ten milliwatt of laser power are required to obtain clear fluorescence images with endogenous and exogenous fluorophores.

Two photon excitation has a number of advantages over one-photon excitation.

First, the absorption of the sample at the NIR wavelength of the Ti:Sa laser is low. Also the scattering coefficient is lower than at visible or ultraviolet wavelengths. Two-photon excitation therefore goes deeper into tissue than one-photon excitation, see Fig. 1, left and middle. Two-photon excitation can be used to excite fluorescence in tissue layers as deep as 100 um, in some cases even 1 mm.

Second, noticeable two-photon excitation is obtained only in the focus of the microscope lens (Fig. 1, middle). Thus, two-photon excitation is a second way to obtain depth resolution and suppression of out-of-focus fluorescence. Different from one-photon excitation with confocal detection, which avoids out-of focus *detection*, two-photon excitation avoids out-of-focus *excitation*.

Third, the fact that there is no substantial excitation outside the focal plane makes two-photon excitation save to live cells and tissue. Photodamage can occur only in the focal plane, and not, as in the case of one-photon excitation, in the entire irradiated sample volume. Detrimental effects on the viability of life cells and tissue are therefore smaller than for one-photon excitation.

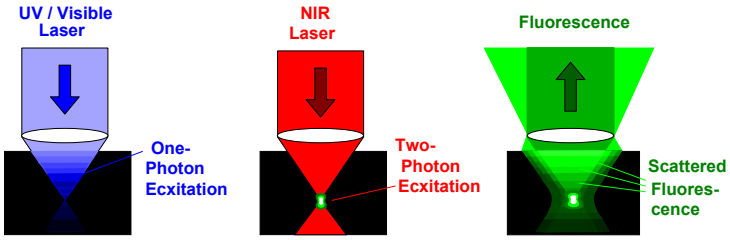

Fig. 1: Left: One-photon excitation. The effective excitation power decreases rapidly with increasing depth. Middle: Two-photon excitation. The NIR laser penetrates deeply into the sample. Right: The fluorescence from a deep focus is scattered on the way out of the sample. It leaves the back aperture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

The fact that the fluorescence signal comes only from the focus leads to a another advantage of two-photon excitation: A two-photon microscope is able to detect photons which are scattered on the way out of the sample. Scattered photons leave the back aperture of the microscope lens in a wide cone of light (Fig. 1, right). In a one-photon system with a descanned confocal beam path these photons would be lost. They can neither be fed through the narrow beam path of the scanner, nor can they be focused into a pinhole. In a two-photon microscope, however, there is no out-of focus excitation and, consequently, no need to use a pinhole. Two-photon microscopes therefore divert the fluorescence from the excitation beam directly behind the microscope lens and project it on a large-area detector [3]. The principle is called non-descanned (or 'direct') detection. A two-photon microscope with non-descanned detection not only excites fluorescence in deep sample layers, it also detects these photons efficiently.

Upgrading the DCS-120 Scanner for Multiphoton FLIM

Coupling the Ti:Sa Laser into the Scanner

Fibre coupling, as it is used for the visible lasers of the DCS -120 system, cannot be used for the Ti:Sa laser beam. The spectral dispersion in the glass would increase the pulse width so much that efficient multiphoton excitation would not be obtained. The Ti:Sa beam must therefore be free-beam coupled into the scanner. Fortunately, this does not require changes in the scanner optics because the laser inputs are designed for parallel beams [1]. 'Input 2', i.e. the input for the laser of longer wavelength, must be used for the Ti:Sa laser, see Fig. 2.

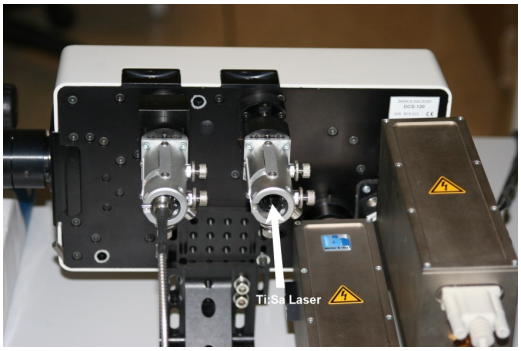

Fig. 2: Input of the Ti:Sa laser into the DCS-120 scanner

Alignment of the laser beam with respect to the scanner is critical, especially if also the confocal detectors are to be used for multiphoton FLIM. It is therefore recommended to place the laser and the microscope with the scanner on the same table. The scan head should be attached to the table by a metal bracket. The laser beam should be fed via two mirrors. One should be close to the laser, the other close to the scanner. Adjustments at the first mirror then mainly influences the lateral beam position, adjustment at the second mirror the beam angle.

Main Dichroic Beamsplitter of the Scanner

The main dichroic beamsplitter of the scanner must be replaced with one that reflects the Ti:Sa Laser. A suitable beamsplitter is available from bh. It reflects both the Ti:Sa laser and a 405 nm ps diode laser, see Fig. 3.

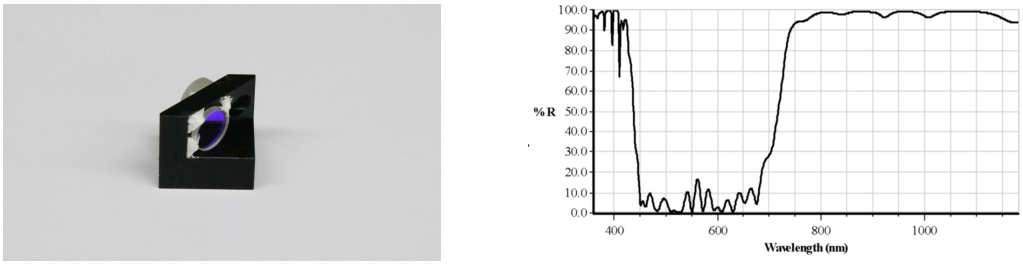

Fig. 3: Left: Main dichroic beamsplitter block of DCS-120. Right: Characteristics of multiphoton dichroic

Confocal Detectors

For reasons described above confocal detection is not the best solution for multiphoton excitation. If the confocal detectors are to be used for whatever reasons blocking filters for the Ti:Sa laser wavelengths must be placed in the detection beam paths. The filters can be screwed directly into the C-Mount adapters of the detectors, see Fig. 4. Suitable filters and adapter rings are available from bh.

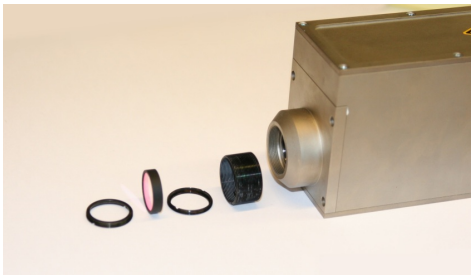

Fig. 4: Inserting a laser blocking filter in front of the detector

Non-Descanned Detectors

Non-descanned detectors are attached via an additional port of the microscope. The easiest solution is to use the fluorescence-lamp port of the microscope. The principle is shown in Fig. 5. A dichroic beamsplitter is inserted in the filter carousel of the microscope. This beamsplitter lets the Ti:Sa laser pass but diverts the fluorescence to the lamp port. The beamsplitter is switched into the beam path only for two-photon excitation with non-descanned detection.

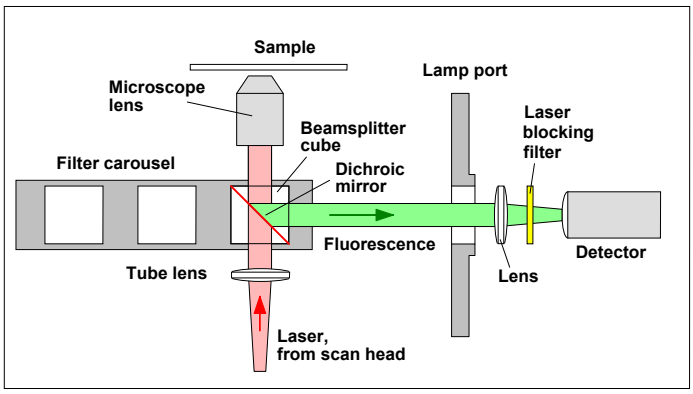

Fig. 5: Principle of non-descanned detection optics (inverted microscope)

A lens in the non-descanned detection path projects a de-magnified image of the microscope lens on the detector. The laser wavelength is blocked by a filter in front of the detector, see Fig. 4. Please note that this filter is absolutely required. The dichroic mirror in the beamsplitter cube is not sufficient to reject the laser light.

In some cases, e.g. for the Zeiss Axiovert and Axio Observer microscopes, the complete lamp port optics (with an additional lens in front of the detector) can be used to project the fluorescence signal on the detector. Adapters to the Zeiss lamp port are available for all bh detectors.

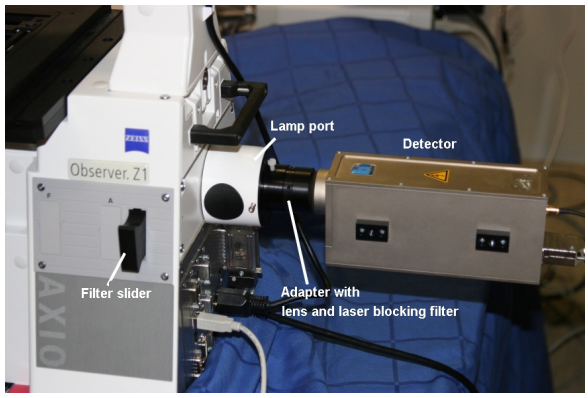

Fig. 6: HPM-100-40 detector attached to lamp ort of Zeiss Axio Observer

Emission filters can be inserted directly in front of the non-descanned detector. However, changing filters in this place may be awkward. Filters should therefore be better placed in the filter sliders some microscopes have in the path to the lamp port. If no such filter slider is available we recommend to insert the emission filter in the output of the two-photon beamsplitter cube in the microscope filter carousel. Several cubes, each with a two-photon dichroic and a different filter, can be placed in the filter carousel. If several non-descanned detectors are connected via a bh beamsplitter assembly no emission filter in the microscope is needed. In that case, filter cubes with different beamsplitter-filter combinations are used in the beamsplitter assembly.

Multiphoton FLIM Procedure

The general procedure of recording multiphoton FLIM is the same as for one-photon FLIM [1]. Put the sample in the microscope. Switch the microscope beam path to the eyepieces (see below, laser safety considerations). Turn the filter carousel to move the two-photon dichroic out of the beam path. Bring it into focus and move it in the desired position. Then turn the multiphoton dichroic in and switch the microscope beam path to the scanner. Start the ‘Preview’ mode. Note that the focus for the Ti:Sa laser wavelength may be not exactly the same as for visible light. Focus into the desired sample plane, and zoom into the desired image area. Adjust the laser power. Then stop the preview, and start a FLIM recording with the desired number of pixels. Let the acquisition run until you have reached a satisfactory signal-to-noise ratio.

Please note that a non-descanned detector detects from a much larger area than a confocal detector. It is therefore mandatory to keep daylight out of the microscope. Unfortunately, normal microscopes are not designed to for low daylight leakage. Therefore, cover the back of the sample with piece of black cardboard, cover the complete microscope with black cloth, and turn off the room lights before you start the FLIM acquisition.

Results

Multiphoton NDD FLIM was tested with a standard DCS-120 system attached to a Zeiss Axio Observer Z1. The NDD detector was attached to the lamp port of the Axio Observer as shown in Fig. 6 The scanner was modified with the dichroic beamsplitter shown in Fig. 3. The TCSPC electronics was the standard SPC-152 package of a bh Simple-Tau 152 system [4]. The NDD detector was a HPM-100-40 hybrid detector [4, 5].

Fig. 7 shows a two-photon NDD FLIM image of the Convallaria sample that is routinely delivered with the DCS-120 system. The convallaria is easy to use. It is easily visible in transmitted light through the eyepieces and excites brightly at any laser wavelength from 780 nm to about 900 nm. No more than a few percent of the laser power is needed to obtain a count rate on the order of 1 MHz. A typical result is shown in Fig. 7.

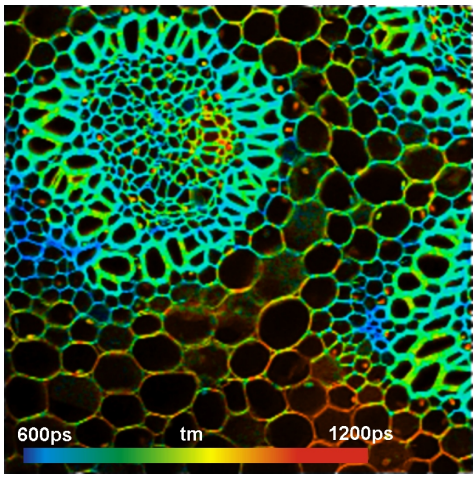

Fig. 7: Two-photon FLIM of a convallaria sample, mean lifetime of double-exponential decay model. 256x256 pixels, 256 time channels. Excitation 800 nm. Zeiss Axio Observer, x20 NA=0.6 lens, detection through lamp port, HPM-100-40 detector.

To demonstrate the capabilities of multiphoton NDD FLIM for realistic samples we used unstained samples of fresh pig skin. Skin is highly scattering. When looking at the sample through the eyepieces almost nothing can be seen. You may even have problems to find the surface of the sample. Nevertheless, the two-photon exited autofluorescence FLIM images are surprisingly rich in detail. Results for different depth in the sample are shown in Fig. 8. bh SPCImage data analysis software [1, 2] with a triple-exponential model was used to fit the decay data. The lifetimes shown are amplitude-weighted averages of the decay components. Each image was acquired at an average count rate of 1 MHz. The acquisition time was about two minutes per image. No decrease in the count rate was observed over this time, i.e. no photobleaching occurred.

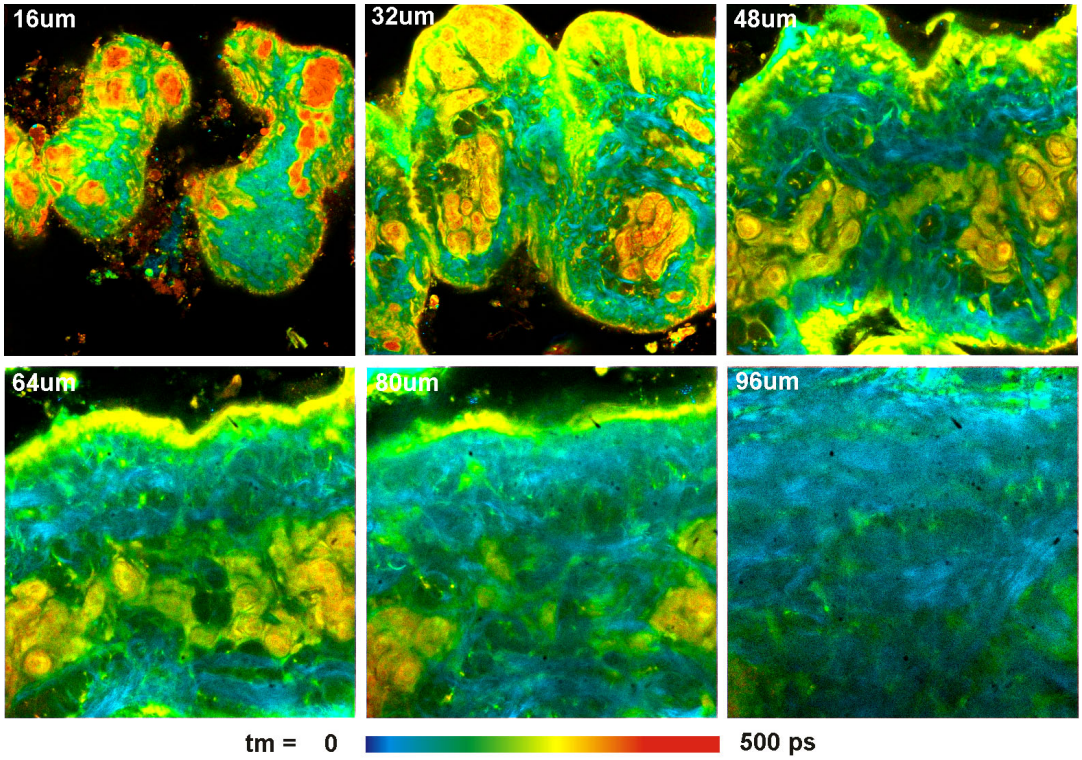

Fig. 8: Pig skin, autofluorescence, image in different depth in the sample. Amplitude-weighted lifetime of triple-exponential decay model. Excitation 805 nm, 512x512 pixels, 256 time channels. Zeiss Axio Observer Z1, Water C apochromate NA=1.2, detection through lamp port, HPM-100-40 hybrid detector.

The quality of the data shown in Fig. 8 is so good that colour-coded images of individual parameters of the triple-exponential can be generated. Of particular interest are the contribution of the ultra-fast decay component, q1, and the ratio of the amplitudes of the medium and slow component, a1/a2. The fast component is dominated by SHG. SHG is generated by collagen and elastin. q1 therefore shows the amount of these tissue constituents. The slow and medium decay component are dominated by bound and unbound NADH. a1/a2 therefore gives insight into NADH binding. The corresponding images are shown in Fig. 9.

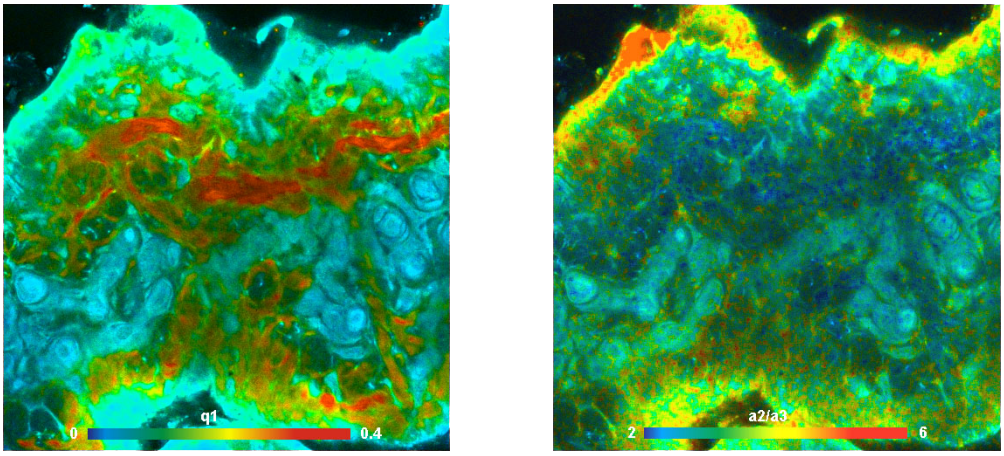

Fig. 9: Images of individual decay parameters of a triple-exponential fit to the data. Left: Relative intensity contribution, q1, of the fastest decay component. Right: Amplitude ratio, a2/a3, of the medium and slow decay component. Depth 48 um below the surface.

Laser Safety Considerations

When using the DCS-120 for multiphoton excitation, please obey to the usual laser safety rules. In particular, make sure that the laser light cannot be reflected back to the eyepieces. That means in practice that microscopes with 50/50 beamsplitters between the eyepiece and the scanner port should not be used, or the 50/50 position be blocked. Moreover, make sure that laser light is not reflected from optical surfaces into the environment. In particular, light reflected off the laser intensity regulators of the DCS-120 scan head should be blocked by a suitable beam stop. Moreover, we suggest to attenuate the laser power down to about 20% directly behind the laser output by a suitable beamsplitter. 20% are less dangerous than the full laser power, but are more than enough to obtain FLIM from any reasonable sample. The beam splitter has the side effect that the remaining 80% of the laser beam can be used for other applications without the need of switching any optical elements in the beam.

Concluding Remarks

The DCS-120 system can be upgraded with a Titanium-Sapphire laser and non-descanned detectors to obtain a multiphoton NDD FLIM system. The upgraded system records autofluorescence images from tissue layers as deep as 100 um within mammalian skin. The sensitivity of the system is sufficient to obtain a count rate of 1 MHz from unstained pig skin. No drop in the count rate was observed within a time of several minutes. This indicates that no photobleaching occurred at the excitation power used.

The pig skin images shown above were recorded within an acquisition time of two minutes. The long acquisition time was used to obtain high-quality data suitable for triple-exponential decay analysis. Please note also that the image format used was 512x512 pixels. For lower pixel numbers correspondingly shorter acquisition time is sufficient to obtain the same lifetime accuracy. For 256x256 pixels similar accuracy can be obtained in 30 seconds, for 128x128 pixels in about 8 seconds.

Please note that the efficiency of a two-photon microscope significantly depends on the microscope lens. Lenses of high NA should generally be preferred. High NA not only results in a smaller excited volume and thus in higher excitation efficiency, it also collects a larger fraction of the fluorescence. High NA also avoids distortion of the fluorescence decay functions by rotational depolarisation [4].

Please note that refractive-index mismatch has a strong influence on the excitation efficiency in deep sample layers. The excited volume remains small only if refractive-index mismatch is avoided. The refractive index of biological tissue or cells is closer to water than to immersion oil. Therefore, water immersion lenses usually give best results on live samples, oil immersion lenses are better for fixed samples.

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