FLIM Systems for Zeiss LSM 710 Record Z Stacks

Abstract. The bh FLIM systems for the Zeiss LSM 710 and LSM 710 NLO microscopes [5] are able to record Z stacks of FLIM images. The LSM 710 subsequently scans a number of Z planes. Synchronisation of the FLIM recording with the Z scan is obtained via a z-plane trigger from the microscope. The FLIM system starts a FLIM acquisition for each plane trigger, and repeats the acquisition for the desired number of Z planes. After each plane the data are saved into a file. The result is a sequence of FLIM data files for consecutive planes of the sample.

Introduction

The recording of Z stacks of images, or 'Z scanning' has been early introduced into confocal and multiphoton laser scanning microscopes [11]. The microscope scans one image plane, saves the image data, changes the depth of the focus, and scans another plane. The process is continued until the desired number of planes have been scanned, see Fig. 1. Ideally, a sufficiently large number of closely spaced z planes would be scanned to allow the three-dimensional structure of the sample to be reconstructed.

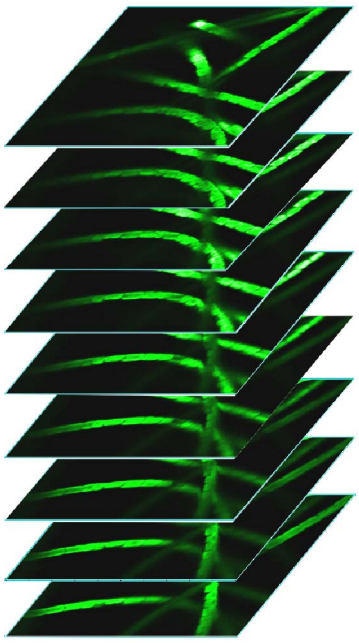

Fig. 1: Z stack of algae, recorded by bh FLIM system, intensity images

Z stack recording faces two major problems. First, the maximum depth in the sample from which a reasonable image can be obtained is limited by absorption, scattering, refractive-index mismatch and refractive index inhomogeneity. Second, scanning a large number of planes exposes the sample to a high excitation dose. Photobleaching is therefore a severe limitation. Both problems are mitigated by using two-photon excitation. Absorption at NIR wavelengths is usually low, and scattered excitation light can be collected by non-descanned detection (NDD) [8]. Different than for one-photon excitation, photobleaching is restricted to the focal plane. Although two-photon excitation causes increased photobleaching in the focal plane [9] the total amount of bleaching for a large number of Z planes is usually lower than for one-photon excitation [10].

Despite of these improvements, photobleaching has been a severe obstacle to the introduction of Z stack FLIM in the past 10 years. Although FLIM data are obtained from a given number of photons at the same accuracy as intensity data FLIM usually requires a larger number of photons per pixel [1, 2]. The reason is that the requirements to the lifetime accuracy in FLIM experiments are normally higher than to the intensity accuracy in steady-state experiments. Moreover, a moderate amount of photobleaching can usually be tolerated for the reconstruction of spatial structures. In

FLIM, however, photobleaching can cause unpredictable lifetime changes. Another obstacle to Z stack FLIM has been the synchronisation with the Z stepping of the microscope. The FLIM acquisition within one plane is synchronised via pixel, line, and frame clock pulses delivered by the microscope [1, 6]. However, until recently, laser scanning microscopes did not deliver a Z synchronisation pulse. Z scanning could therefore only be achieved by actively controlling the microscope from the FLIM system [2]. This approach has been employed in the bh DCS-120 confocal scanning FLIM system [3, 4] but cannot be used in other scanning microscopes because the Z control port is not accessible.

Z Stack FLIM with the Zeiss LSM 710

The Zeiss LSM 710 offers a number of advantages over previous laser scanning microscopes. The NDD light path of the multiphoton versions has been improved in efficiency, especially for scattered photons from deep sample layers. The efficiency of the LSM 710 scan head is near-perfect. The scan head has been equipped with an optional direct coupled confocal port ('DC port') which has a substantially higher efficiency than the fibre output of the LSM 510. Moreover, the microscopes have been complemented with a tuneable picosecond laser. This 'InTune' laser allows the optimum excitation wavelength to be selected for the fluorophores excited. All these features have resulted in a substantial increase in the efficiency of FLIM detection, and, consequently, in a reduction of photobleaching artefacts.

Another big leap in efficiency came with the introduction of the HPM-100-40 hybrid detector modules by bh [7]. The efficiency of these detectors is 5 to 10 times better than for conventional PMTs, and reaches or even surpasses the efficiency of single-photon avalanche photodiodes (SPADs). Different than SPADs, the hybrid detectors have active areas on the order of 7 mm^2. This is a perfect match to the NDD optics of the LSM 710, and thus provides unprecedented efficiency to deep-tissue imaging [5]. At the DC port of confocal systems, the large area avoids any alignment or focusing problems and collects light efficiently even from large pinholes [5]. With the new detectors photobleaching no longer prevents the use of Z scanning.

The last problem - the synchronisation of the FLIM recording with the Z stepping - has been solved by introducing a Z-plane trigger output into the Zeiss LSM 710 scan control system. The principle of the synchronisation is shown in Fig. 2.

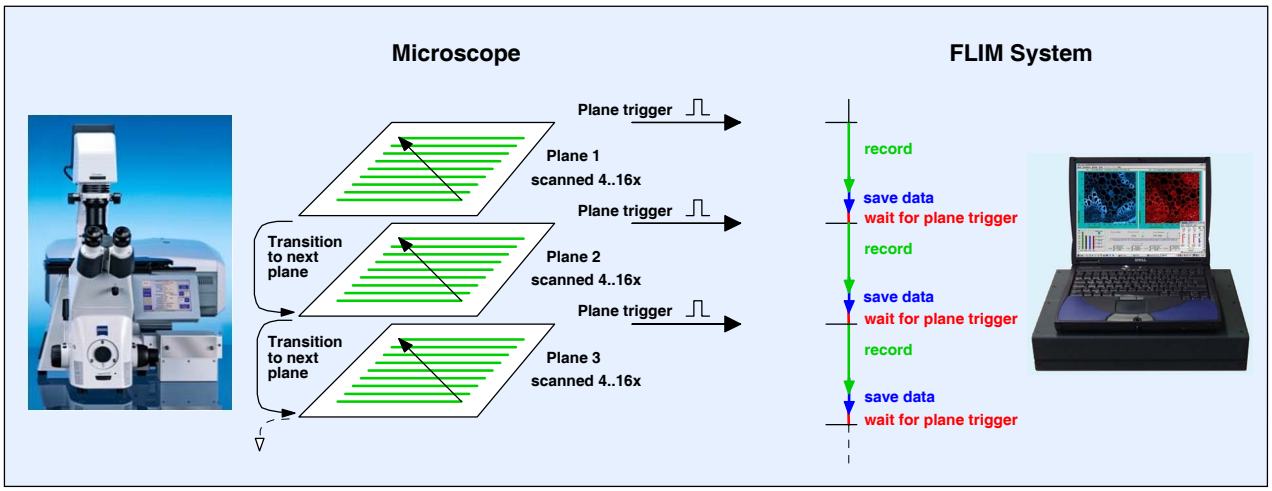

Fig. 2: Synchronisation of the FLIM system with the Z stepping

Stack acquisition is achieved by using the experiment trigger and autosave functions of the bh FLIM system in combination with the Z stepping and frame accumulation function of the Zeiss LSM 710. The LSM 710 real-time computer sends a plane trigger to the bh SPC-150 modules when it starts to scan a plane. Each plane is scanned repetitively, typically 4 to 16 times. The number of frames per plane, the frame time, the z step width, and the number of Z planes to be scanned is defined in the ZEN software of the LSM 710. For each plane, the FLIM system acquires FLIM data for a defined 'collection time'. The FLIM acquisition for the individual planes is synchronised in the usual way via the frame clock, line clock, and pixel clock pulses. When the collection time is over the FLIM system stops the acquisition, saves the data into a file, and waits for the next plane trigger. The acquisition continues for a number of Z planes defined by the 'cycles' parameter in the SPCM software of the FLIM system.

Typical results are shown in Fig. 3 and Fig. 4. Images of an (unstained) pig skin sample were recorded by a dual-channel FLIM system in an LSM 710 NLO multiphoton microscope. HPM-100-40 hybrid detectors were used at the 0° and the 90° output of the NDD T-Adapter of the LSM 710. A beamsplitter was used to record simultaneously images below and above 480 nm. The excitation wavelength was 800 nm, the laser power 2.4 %. A water apochromate 40 x, NA=1.1 was used; the scan area was 212 x 212 µm. The FLIM images of each plane were acquired at a resolution of 256 x 256 pixels and 256 time channels. The Z step interval chosen in the ZEN software was 5.09 µm. The total scan time per plane was 25 seconds. The collection time of the FLIM system was 20 seconds, i.e. 5 seconds were spared for saving the data and waiting for the next plane trigger.

Fig. 3 shows the FLIM image in the wavelength channel above 480 nm. The colour of images shows the average (intensity-weighted) lifetime of a double-exponential fit to the decay data. The intensity of the images was normalised to the intensity of the brightest pixel.

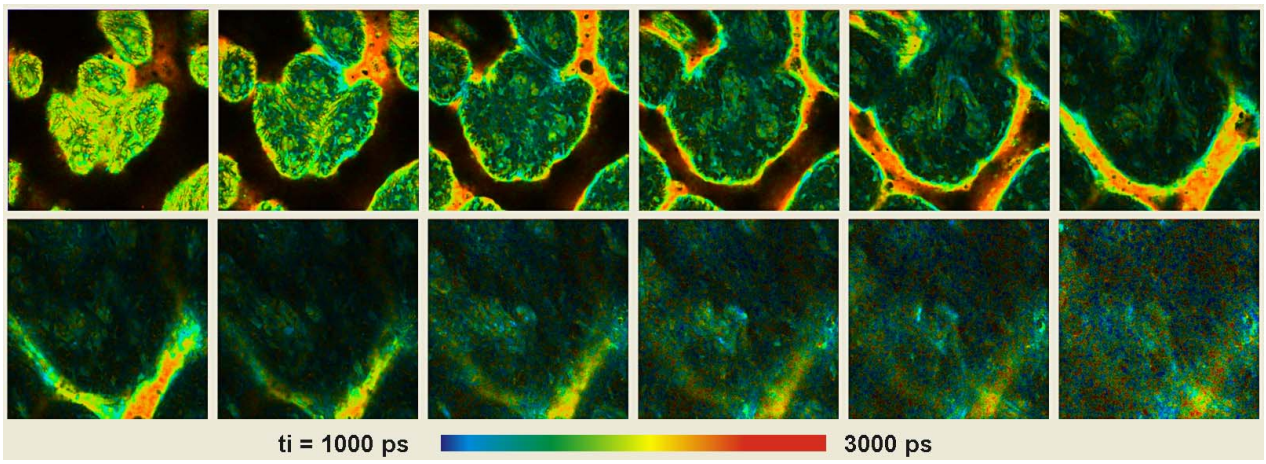

Fig. 3: FLIM Z stack recorded at a pig skin sample, excitation at 800 nm, emission above 480 nm. Z step width 5.09 µm, scan area 212 x 212 µm. Images from 5 µm to about 60µm below the surface. Colour represents average (intensity-weighted) lifetime of a double-exponential fit. FLIM data format 256 x256 pixels, 256 time channels.

No decrease in count rate was observed during the acquisition time of the individual Z planes. Moreover, after returning to the first plane the count rate had not changed. The stability of the count rate indicates that photobleaching was negligible.

Fig. 4 shows data recorded in the wavelength interval below 480 nm. The data recorded in this interval contain both fluorescence and SHG. The SHG signal can be extracted from these data by

selecting the signal component of infinitely short lifetime. Fig. 4 shows SHG images extracted from the FLIM data this way. The intensities were normalised to the intensity in the brightest pixel.

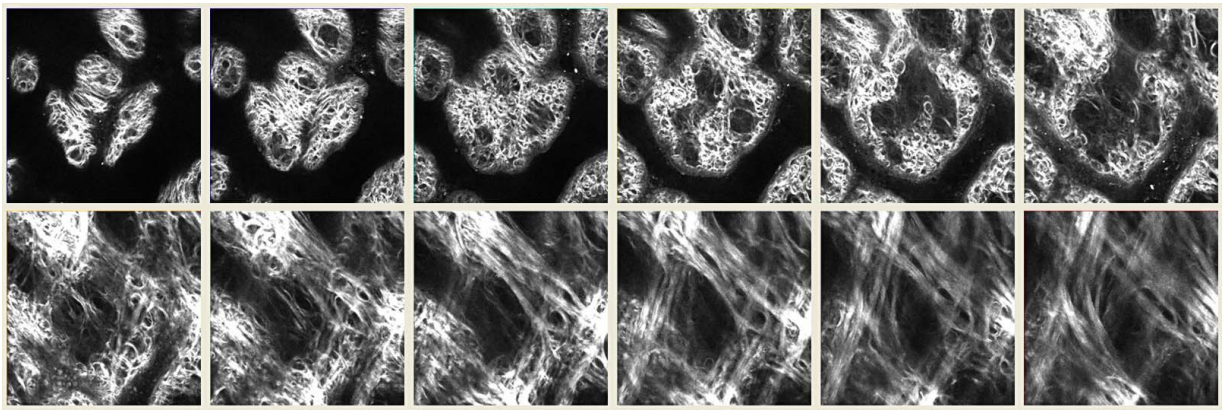

Fig. 4: Z stack recorded at a pig skin sample, excitation at 800 nm, emission below 480 nm, SHG signal, extracted by selecting photons in an early time window. Z step width is 5.09 µm, scan area 212 x 212 µm. Images from 5 µm to about 60µm depth.

Summary

We have demonstrated Z stack FLIM by synchronising the cycle and autosave functions of the bh FLIM systems with the Z stepping function of the Zeiss LSM 710 NLO microscopes. By using non-descanned detection and high-efficiency hybrid detectors autofluorescence and SHG images of biological tissue are obtained at low excitation power, negligible photobleaching, and within a reasonable acquisition time. Although Z stack FLIM is especially useful in combination with two-photon excitation it is in no way restricted to multiphoton systems. The FLIM system, the principle of synchronisation, and even the system setup parameters are the same for one-photon excitation by ps diode lasers and by the tuneable InTune laser of the LSM 710 confocals.

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