Technical Note

PicoQuant

# FLIM: Scanning Speed vs. Excitation Rate

**Matthias Patting**

PicoQuant GmbH, Rudower Chaussee 29, 12489 Berlin, Germany, info@picoquant.com

## Introduction

Fluorescence Lifetime Imaging (FLIM) performed with the time correlated single photon counting technique requires pulsed excitation[1]. Typically the focus of a confocal microscope is scanned over the sample. In most cases the excitation rate is several orders of magnitude higher than the scanning speed, and for practical purposes the illumination of the focal volume can be regarded as pseudo-continuous. However, for confocal microscopes with fast scanning capabilities, for example Laser Scanning Microscopes (LSMs) with resonant scanning modes, the pixel sampling frequency can reach a similar order of magnitude as the excitation rate. This can produce characteristic artefacts, which shall be discussed in the following.

## Excitation Cycles, Markers, and Moiré Patterns

**Bias in a very short measurement**

Let us assume an excitation rate of 40 MHz. The time span between two subsequent excitation pulses would then be 25 ns. If we do a series of measurements with a very short accumulation time of 0.1 µs, each measurement will get the same 4 excitation cycles. Of course, each individual measurement would still acquire a different number of photons, due to the statistical nature of the process.

In a second experiment let the accumulation time be 0.12 µs. Then, on the average, every measurement would get 4.8 cycles. As there is no such thing as 0.8 cycles, we would observe the following: the first measurement gets 5 cycles, the second, third and fourth as well, but the fifth would get 4 cycles (assuming there is no gap between the measurements). Then the series repeats itself. Illustration 1 depicts this situation.

Again, the number of photons in each measurement shows statistical noise, but now we have a bias every fifth measurement, which in the average appears darker by 20 %. With a single measurement this bias is mostly buried in the statistical noise, but if we accumulate the results of enough series, we would see the bias.

**Image pixels and T3-mode**

In an image line scanned with a confocal volume, each pixel is a short measurement like in the previous example. If each image line is scanned just once, any bias with regard to the number of excitation cycles will be lost in the statistical noise. However, as an image scan with only a few photons per pixel cannot

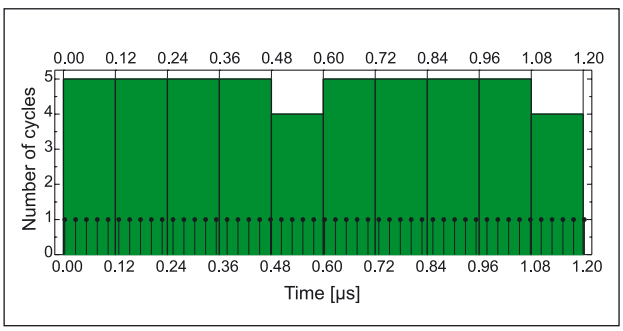


*Illustration 1: Bias in a series of short measurements.*

be analysed in terms of fluorescence lifetime, it may be necessary to scan the image repeatedly, especially if the scanning speed is high and the accumulation time per single scan is short. The statistics of the pixels is thus improved by repeated scanning, and the bias with regard to the number of excitation cycles becomes more and more visible.

A typical FLIM image will be recorded in T3-mode[2], i.e., for each individual photon from the sample two arrival times are recorded: The arrival time with regard to the beginning of the measurement and the arrival time with regard to the preceding laser pulse. The arrival time with regard to the beginning of the measurement is synchronized with the scanner movement and gives us the location, from which the photon has been emitted. As T3-mode can only be applied with pulsed excitation, the shortest meaningful time resolution is the time lapse between two excitation pulses. As the resolution is the same for both photon events as well as synchronization events (i.e. line markers), the beginning and the end of each single line are projected to the arrival time of the nearest excitation pulse. Consequentially, the biases of the pixels in all scanned lines are perfectly aligned along vertical lines.

As most scanning speeds will be incommensurable with the excitation rate, any image will show a bias of this kind, so the bias generally cannot be avoided, only suppressed. Let the average number of excitation pulses per pixel be near n, then the relative bias due to one skipped (or additional) excitation pulse is 1/n. To force it below 1 %, one would have to have at least 100 cycles per pixel.

In general, let $\nu_{Exc}$ be the excitation frequency, $n_{Pixel}$ be the number of pixels per line, $\nu_{Line}$ the line frequency resulting in $n_{Pixel}$ as the number of pixels per time unit and $n_{Syncs}$ the number of excitation cycles, then the following holds:

$$n_{Syncs}\,\nu_{Pixel} = n_{Syncs}\,\nu_{Line}\,n_{Pixel} \leq \nu_{Exc}$$

$$\Leftrightarrow \quad \nu_{Line} \leq \frac{\nu_{Exc}}{n_{Syncs}\,n_{Pixel}}$$

Illustration 2 shows the limits for some typical excitation rates. Above the curves, images will show vertical lines due to the bias, so these regions are “forbidden”.

A bias of 2 % means that above 2500 photons per pixel (a typical situation for FLIM) artefacts with the appearance of dark or bright vertical lines will be above noise level and will therefore be visible in the image. The visibility of the artefacts will appear even sooner if additional measures like a sinusoidal correction or the removal of acceleration regions are applied to the image.

## Countermeasures

Three courses of action can be taken: Decrease the line frequency $\nu_{Line}$, increase the excitation rate $\nu_{Exc}$ or decrease the number of pixels per line $n_{Pixel}$.

**Decreasing the number of pixels per line**
Less pixels per line mean either a reduction of the image size or a reduction of the resolution. Both effects are of course not desirable, so decreasing the number of pixels will in most cases not be an option. However, as this reduction can be achieved after the measurement by applying a binning to the image, it might be the method of choice to salvage an image that has been accidentally recorded within the forbidden area.

**Decreasing the line frequency**
From the mathematical viewpoint, a reduction of the line frequency is the best possibility, as it allows us to record more photons per pixel. Of course the acquisition time per frame increases. As FLIM requires a minimum amount of photons per pixel to generate meaningful contrast, usually a higher line frequency forces us to record more frames. In this case it is preferable to reduce both the number of frames as well as the line frequency. However, bleaching may occur if the excitation volume is dragged more slowly across the sample, which may force lower constraints onto the line frequency.

**Increasing the excitation rate**
To increase the number of shots per pixel the excitation rate $\nu_{Exc}$ can be increased. As more energy per time unit is deposited in the sample, it might be possible (or even necessary due to bleaching) to reduce the excitation intensity accordingly. However, there is an upper limit for $\nu_{Exc}$: The time window between two subsequent excitation pulses must be large enough to allow the fluorescence intensity to decrease.

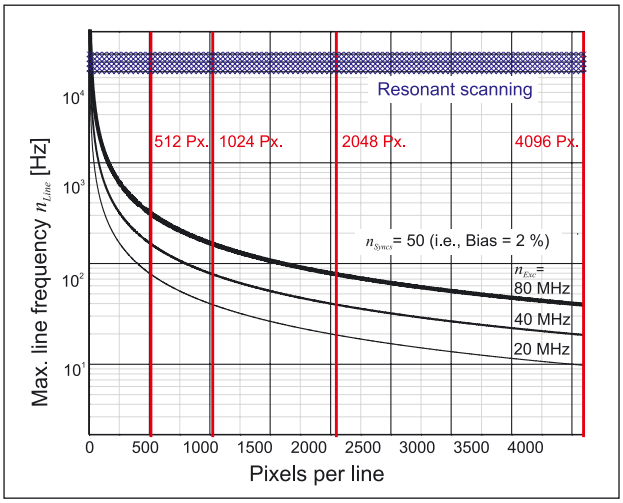


*Illustration 2: Limits for the line frequency. Note that resonant scanning conditions lie in the forbidden region except for very small resolutions.*

## Further Reading

[1] Susanne Trautmann, Volker Buschmann, Sandra Orthaus, Felix Koberling, Uwe Ortmann, Rainer Erdmann, Fluorescence Lifetime Imaging (FLIM) in Confocal Microscopy Applications: An Overview. Application Note, http://www.picoquant.com/images/uploads/page/files/7350/appnote_flim_overview.pdf (2012)

[2] M. Wahl, Time-Correlated Single Photon Counting. Technical Note, http://www.picoquant.com/technotes/technote_tcspc.pdf (2009)

PicoQuant GmbH
Rudower Chaussee 29 (IGZ)
12489 Berlin
Germany

Phone +49-(0)30-6392-6929
Fax +49-(0)30-6392-6561
Email info@picoquant.com
WWW http://www.picoquant.com