

On the performance of two types of Single Photon Avalanche Diodes (SPADs) for Fluorescence Lifetime Imaging



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Introduction

Fluorescence Lifetime Imaging (FLIM) adds another dimension to conventional intensity based imaging methods by looking for spatial variations in fluorescence decay properties of the fluorophore. FLIM permits to discriminate between fluorophores with similar emission spectra (like GFP and YFP), from autofluorescence and is not affected by fluctuations in the fluorescence intensity. It can be used to probe local environmental conditions (e.g. water concentrations, pH value), to determine ion concentrations (e.g. Ca^{2+}), to study intracellular signal transduction or to distinguish between DNA and RNA.



Fig. 1: SPAD module of the PDM series designed and manufactured by Micro-Photon-Devices (MPD) of Bolzano, Italy. The detection area can be selected to have a diameter of 20, 50 μm or 100 μm .

The technique of choice to record the necessary time-resolved data is Time-Correlated Single Photon Counting (TCSPC), which provides a high detection sensitivity down to the single molecule level combined with picosecond timing accuracy. The TCSPC unit is, however, not the only device one has to look at when judging the overall performance of a FLIM setup. In principle, the combination of high sensitivity (needed for e.g. single molecule experiments) and excellent timing performance in the picosecond range can only be achieved in combination with Single Photon Avalanche Photo Diodes (SPADs) and fast pulsed lasers. In terms of timing, the overall performance of a FLIM setup is usually described by the Instrument Response Function (IRF) which depends on the laser pulse width, the timing resolution of the photon counting detector as well as the TCSPC unit itself. Here, the most critical element is the detector, as even small and reliable pulsed diode lasers provide pulse widths well below 100 ps [1] and

state of the art TCSPC units like the PicoHarp 300 [2] offer a timing resolution of smaller than 12 ps rms. In contrast, the best detector modules on the market for the last decades, the SPAD modules SPCM-AQR from Perkin Elmer Inc. (Santa Clara, USA), have a timing response greater than 300 ps, which is much higher than that of good photomultipliers. Additionally, the temporal position of the detector pulse depends on the total number of detected photons ("count rate"), which is especially problematic for FLIM measurements since an image is per definition composed of pixels with highly variable intensity.

These limitations are now overcome by a new generation of SPAD detectors (PDM series), developed and manufactured by the Italian company Micro Photon Devices (MPD). They feature an excellent time response comparable to micro channel plate photomultiplier and a nearly count rate independent IRF [3] and are therefore extremely well suited for FLIM measurements, which is demonstrated in this application note.

Experimental details

As an example for FLIM the autofluorescence of daisy pollen grains was imaged, i.e. no staining was performed. The sample was excited with a picosecond pulsed diode laser emitting at 532 nm [4], and the fluorescence was collected through a detection bandpass set between 540 nm and 610 nm. All measurements were performed with the time-resolving confocal microscope MicroTime 200 [5], which allows to do FLIM measurements with fluorophore concentrations even down to the single molecule level. Two detection channels of the instrument were used and equipped with two different SPAD detectors: a MPD SPAD (PDM 50) with a sensitive detection area of 50 μm in diameter and a Perkin Elmer SPAD (SPCM-AQR-14) with a detection area of 180 μm in diameter. FLIM images were recorded using the PicoHarp 300 TCSPC module [2]. All data acquisition and analysis was performed with the SymPhoTime software [6].

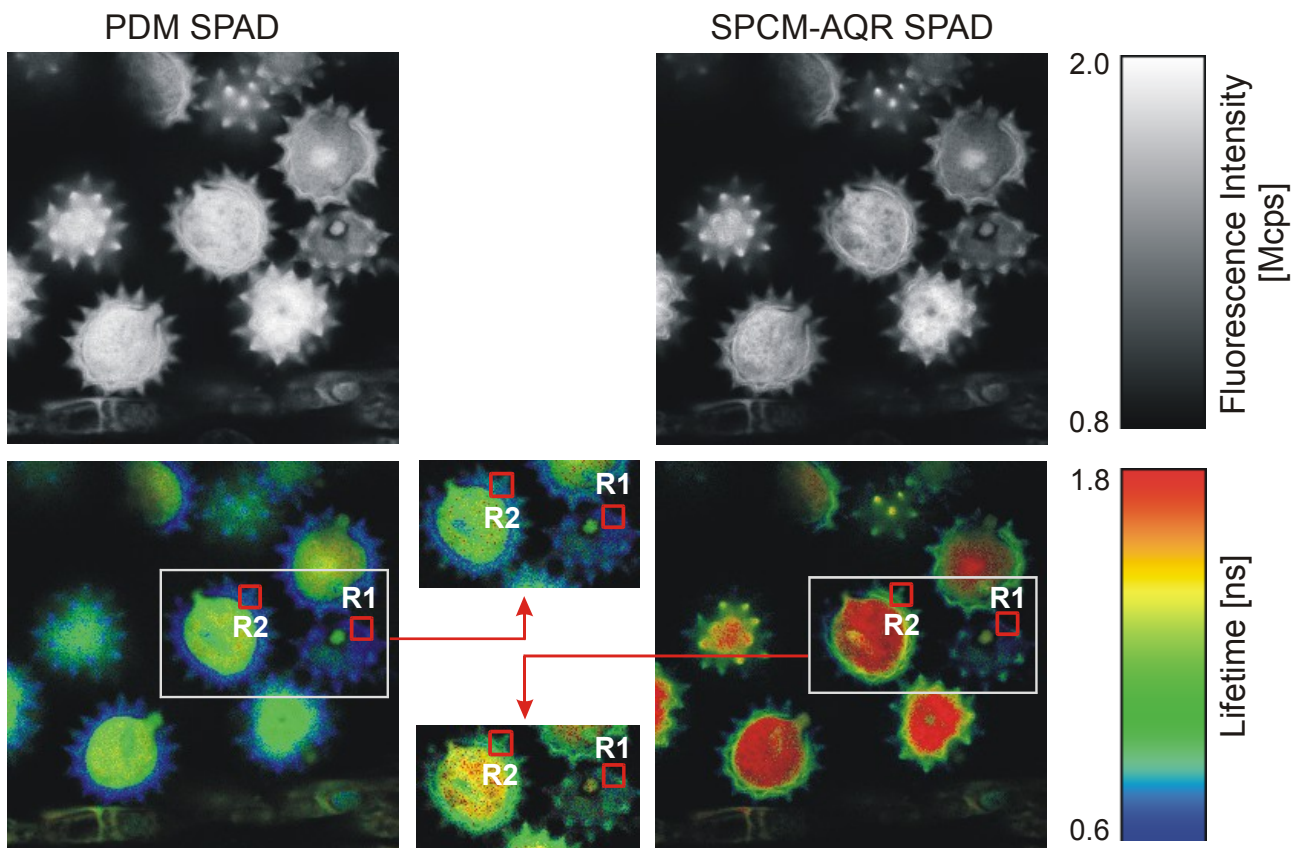


Fig. 2: FLIM images of the autofluorescence of daisy pollen grains. The images correspond to a region of $64 \mu\text{m} \times 64 \mu\text{m}$. First row: intensity images; Second row: lifetime images based on the mean photon arrival time per pixel (details see text). Regions R1 and R2 correspond to areas with similar lifetimes, but lower and higher count rates, respectively. The two small central images show a lifetime image of the marked region based on a detailed tail fit analysis of the fluorescence decay pixel by pixel. Left column: detected with a PDM SPAD. Right column: identical instrument settings but using a SPCM-AQR SPAD. The fluorescence intensity scales with the brightness of the image, the lifetime is color coded. The maximal count rate per pixel for both images was set to 2 Mcps.

Results

Figure 2 shows intensity and FLIM images that were recorded with identical instrument settings. For the images on the right the SPCM-AQR SPAD module was used while the images on the left were recorded using the PDM SPAD detector. The lifetime information in the FLIM images was obtained by calculating the mean arrival time of the photons per pixel in respect to the laser pulse. The latter was derived from the center of gravity of a IRF, recorded by detecting the scattered light from the lower side of the coverslip at low count rates (150 kcps).

Already on a first glance the differences between the images are clearly visible: The lifetime measured with the PDM SPAD detector (left image) is relatively homogeneously distributed in the range between 0.6 ns and 1.4 ns. In contrast, the regions in the right image that correspond to high fluorescence intensity show long lifetimes of up to 2.0 ns.

A closer look at two selected regions (R1 and R2) further demonstrates the difference in the performance of the two SPAD types: The fluorescence in these regions clearly shows different fluorescence intensities as can be seen in the upper two images. The photons originate, however, from similar parts of the pollen grain, therefore they

should have similar lifetimes. For the PDM module, both regions are drawn in the same color indicating similar lifetimes. In contrast region R2 shows a longer lifetime when using the SPCM-AQR SPAD compared to region R1. This discrepancy can be explained by looking at the total fluorescence intensity in these two regions. It is known for the SPCM-AQR modules that their temporal response shifts to later times for count rates exceeding 10^5 cps. If one wants to correct for this influence, it would be necessary to measure the IRF at every pixel. If, instead, the IRF is only measured once at a defined count rate (as typical), every shift in the temporal response influences the calculated lifetime. Looking at the intensity image, one can see that region R1 corresponds to a relatively low count rate, hence the temporal shift is comparably low with respect to the IRF measurements and the calculated lifetime is similar to the result of the PDM SPAD. In contrast region R2 corresponds to a very high count rate, so that the shift of the temporal response is large and leads to the difference in the calculated lifetime.

Less sensitive in this regard is the tail fit procedure since the lifetime calculated from the fluorescence decay should ideally not depend on a linear temporal shift of the detector response as long as the decay is mono exponential. The results of the

tail fit in selected areas is shown in the small center images in fig. 2. The results for the both SPADs now only show small difference in the fluorescence lifetime in the regions R1 and R2 as expected. However, the FLIM image of the SPCM-AQR SPAD still shows significant longer lifetimes even by using tail fitting. This observation can be explained by the lower time resolution of the SPCM-AQR SPAD module.

The time shift of the SPCM-AQR SPAD can also clearly be seen in the corresponding fluorescence decay curves in the image regions with lower and higher count rates (regions R1 and R2 respectively). In the decays shown in figure 3 and especially in the magnification of the peak area (right column), the peak of the curves for the region R2 (in red) is shifted in comparison to region R1 (in black) to later times. No such behaviour can be seen in the decay curve of the PDM SPAD – here, the two decay curves are nearly identical. The fast rising of the decay curve measured with the PDM SPAD additionally reflects the fast overall time response (incl. laser, detector and TCSPC unit) with an IRF width of only 70 ps FWHM (330 ps for the SPCM-AQR SPAD, respectively).

Figure 4 shows the temporal location of the IRF peak plotted against the count rate (PDM SPAD left and SPCM-AQR SPAD right). The shift of the peak of the SPCM-AQR SPAD module is typically in a range of around 1 ns for count rates up to 2.0 Mcps. Especially at high count rates above 1 Mcps this module shows a strong IRF shift towards later times. This behaviour together with the lower time resolution explains the artificial long life-

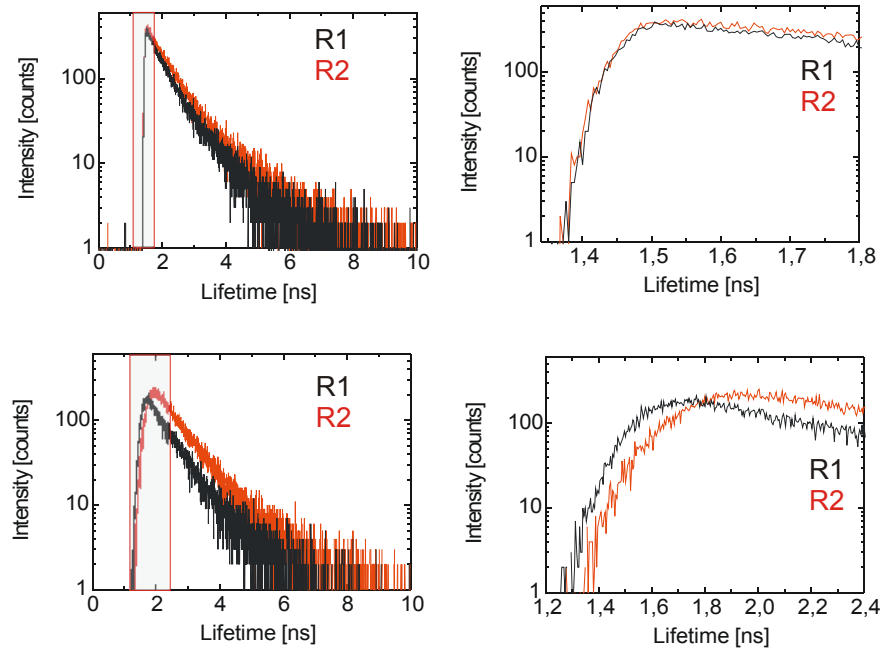


Fig.3: Fluorescence decays of a region with low (R1) and high (R2) count rates, but similar lifetimes. The right columns shows a magnification of the peak area of both curves (shaded area in the left image). Left: PDM SPAD Right: SPCM-AQR SPAD

times found in the FLIM measurement performed with the SPCM-AQR SPAD module. In contrast to this measurement the PDM SPAD shows an IRF shift in a range of only 0.015 ns. This is nearly two orders of magnitudes less compared to the SPCM-AQR SPAD module.

Besides the IRF shift also the width of the IRF shows a small dependence on the count rates. This difference is smaller for the PDM SPAD (0.02 ns) compared to the SPCM-AQR SPAD module with 0.08 ns for a count rate range up to 2.0 Mcps.

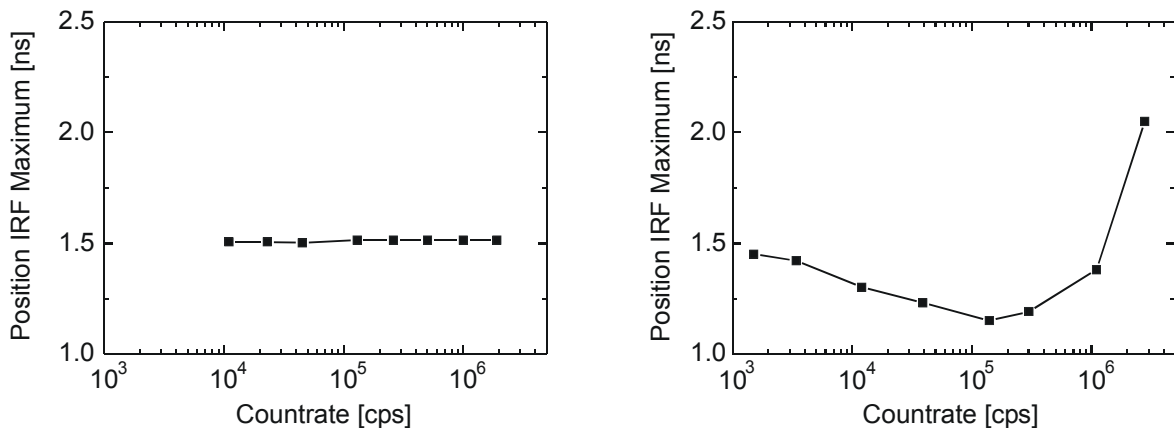


Fig.4: Dependence of the SPAD IRF peak position on the total count rate. Left: PDM SPAD Right: SPCM-AQR SPAD

Discussion

The measurements show that especially at high count rates the new PDM SPAD modules are superior regarding the timing performance (IRF width and shift) compared to SPCM-AQR modules. High count rates are important for fast FLIM imaging since the number of photons per pixel can be acquired in less time and hence the measurement time decreases. Nonetheless, even at moderate count rates a stable timing performance independent of the count rate is important for precise lifetime determinations.

For the SPCM-AQR SPAD the dependence of the calculated lifetimes on the measured count rate was clearly visible in the fast FLIM image based on the average photon arrival times. This dependence could be largely suppressed by using a tail fitting for the calculation of the FLIM image. However, this fitting procedure needs more computational time and the overall lifetimes were still shifted to longer times due to the lower time resolution of the SPCM-AQR SPAD.

Conclusion

The investigations presented in this application note demonstrate that the PDM SPAD modules are excellently suited for FLIM imaging. Since the time response of the module itself accounts to around 50 ps FWHM these modules can also be applied ideally for time resolved FRET measurements even in the case of short donor lifetimes. The PDM SPADs combine an excellent timing performance with a very high fluorescence detection efficiency of up to 48 % at 550 nm, which is comparable to conventional SPAD modules like the SPCM-AQR SPADs and sufficient for single molecule techniques like Fluorescence Correlation Spectroscopy [3].

Further reading

- [1] Pulsed picosecond diode laser heads of the LDH-Series – see <http://www.picoquant.com/products/ldh/ldhseries.htm>
- [2] PicoHarp 300 - Stand-alone TCSPC Unit with USB 2.0 Interface – see <http://www.picoquant.com/products/picoharp300/picoharp300.htm>
- [3] Technical Note: "Performance of the Micro Photon Devices PDM 50CT SPAD detector with PicoQuant TCSPC systems" – http://www.picoquant.com/products/pdm/technote_pdm.pdf
- [4] PicoTA - High Power Picosecond Diode Laser – see <http://www.picoquant.com/products/picota/picota.htm>
- [5] Wahl, M., Koberling, F., Patting, M., Rahn, H., Erdmann, R., Curr. Pharm. Biotech., Vol. 5, p. 299-308 (2004)
see also – <http://www.picoquant.com/products/microtime200/microtime200.htm>
- [6] SymPhoTime - Fluorescence Lifetime Imaging and Correlation Software – see http://www.picoquant.com/products/sw_mt/sw_mt.htm

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