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Single-photon avalanche diode (SPAD) array detector for high-throughput fluorescence lifetime flow cytometry

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Abstract: Time-domain measurement of the fluorescence lifetime in flow cytometry offers a complementary method to traditional flow cytometry, especially for the analysis of cells and biomolecules. Short measurement times due to high throughputs (cells/s) make a suited detector mandatory for a sufficient detection of the fluorescence signal and therefore the application of this method. We summarize the requirements for a suited detector for time resolved flow cytometry (TRFC) and present a new developed SPAD array detector with 100 pixels and 340 ns time between measurement windows, allowing a high-throughput of up to 60,000 cells/s. The functionality of the detector is shown with the measurement of a 22.2 ns laser pulse, making it applicable for an improved TRFC.

Keywords: flow cytometry, fluorescence lifetime, SPAD array detector

1 Introduction

Flow cytometry is a powerful tool for non-destructive, high-throughput analysis of cell populations [1, 2]. As a common approach in flow cytometry, cells are labeled by fluorophores with characteristic emission spectra and the fluorescence intensity is measured for different spectral ranges. This approach is limited by the spectral overlap of the fluorophore emission limiting the number of distinguishable fluorophores. In a complementary approach, fluorophores can be differentiated by their characteristic fluorescence lifetime τ instead of their spectral properties. This enables the differentiation of flu-

orophores with overlapping spectra. [3, 4]

While the measurement of fluorescence lifetimes with an amplitude-modulated laser excitation (frequency domain) is more popular, the measurement with an impulsive laser excitation (time domain) offers a higher temporal resolution and sensitivity [2]. This can be useful for measuring low fluorophore concentrations or reducing the excitation intensity to avoid photobleaching [3]. Moreover, time-domain measurements are able to determine multiexponential decays, which can be used to differentiate between multiple fluorophores measured at the same time [3, 5].

To determine the fluorescence lifetime in the time-domain, the exponential decay of the fluorescence is measured with time-correlated single photon counting (TCSPC) [6]. The precision of the lifetime determination scales with the number of counted photons [3, 7]. In flow cytometry, a desirable high throughput of up to 100,000 cells/s [8] and consequently a low measurement time per cell of 10 μ s restricts the number of counted photons with TCSPC and therefore the precision, limiting this approach [2, 4].

To overcome this limitation and make fluorescence lifetime measurement more applicable for flow cytometry, a suited detector should allow counting of a high number of photons in the short amount of time tolerable in flow cytometry. In this study, we highlight the requirements for a TRFC detector and present a new developed SPAD array detector meeting these requirements. Moreover, we present first functional tests with the detector, which will be further used in a compact, high-throughput TRFC.

2 Background

For a precise and accurate determination of fluorescence lifetimes in the time-domain, the fluorophore is excited multiple times with a laser pulse. Each excitation is followed by a measurement window, where the arrival times of single fluorescence photons are measured. This is known as TCSPC. The arrival times are sorted in a histogram and a decay curve is fitted to determine the fluorescence lifetime. [6]

The time needed to detect the required number of photons for

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the precise determination of the fluorescence lifetime limits the maximum throughput of the flow cytometer. The total measurement time t_{tot} can be described by the required number of measurement windows n_w multiplied by the time width of a measurement window Δt_w and the time between the measurement windows Δt_b (see Equation 1 [7]). The time between the measurement windows is required for resetting the detector, data readout, data evaluation and laser excitation. The number of required measurement windows is given by the fraction of the total number of counted photons N_C and the count rate \tilde{N}_C , specifying the number of photons counted per measurement window. [7]

$$t_{\text{tot}} = n_w \cdot (\Delta t_w + \Delta t_b) = \frac{N_C}{\tilde{N}_C} \cdot (\Delta t_w + \Delta t_b) \quad (1)$$

[3] and [7] evaluated the dependence of the precision of the lifetime determination on the number of counted photons. As seen in Equation 1, an increase in the number of photons also increases the total measurement time required and by that decreases the throughput for TRFC. Therefore, a suited number of detected photons for the desired precision enables a maximum throughput. In [7], a lifetime determination with a relative error of 5.5 % was achieved with 1000 photons detected.

The commonly used detector technologies for detecting the photons in TCSPC are photo-multiplier tubes (PMT) and SPADs. While high-throughput TRFC requires a high photon count rate, both mentioned technologies limit the photon count rate by requiring a reset of the detector after the detection of one photon, resulting in a dead time before being able to detect a second photon. Typical dead times of SPADs [9] and PMTs [10] are in the order of or longer than typical fluorescence lifetimes, which range from hundreds of picoseconds to tens of nanoseconds [4, 11]. Because of this, if more than one photon is emitted in a measurement window only the first one is detected. This is known as pile-up effect and leads to an error in the determination of the lifetime, because later photons are not considered [7].

While PMTs consist of one photosensitive area, SPADs can be fabricated in CMOS processes and implemented in an array of SPAD-pixels. The pile-up effect can be circumvented using multiple detector pixels because each pixel is capable of detecting one photon per measurement window. The photon count rate of a pixelated detector is therefore defined by the product of the photon count rate of one pixel times the number of pixels. This enables the detection of multiple photons per measurement window before pile-up takes place and decreased total measurement time according to Equation 1. In [7], the maximum photon count rate without significant pile-up reducing the accuracy of lifetime determination is 0.31 per

pixel and per measurement window, achieving a relative error of 5.5 % with 30,000 measurement windows.

While a short duration of a measurement window would also decrease the total measurement time (see Equation 1), a duration of $\Delta t_w = 7\tau$ is required to detect 99 % of the emitted fluorescence photons [7]. Additionally, the time between measurement windows should be as short as possible to reduce the total measurement time (see Equation 1).

Another important parameter for the determination of the lifetimes is the temporal resolution of the detector, which defines the bin width of the histogram. While a high temporal resolution in the order of picoseconds seems to be necessary for an accurate determination of fluorescence lifetimes and is commonly used (e.g. 50 ps [6]), it was shown in [7] that a temporal resolution of $t_{\text{res}} = 4\tau$ can be sufficient to achieve a relative error of less than 10 %. Additionally, a lower temporal resolution requires lower data transfer rates. [7]

The required detector parameter, the pixel number and time between the measurement windows, for a throughput of 100,000 cells/s, which corresponds to a total measurement time of 10 μs , can be calculated with Equation 1. For a precise lifetime determination, the required duration of a measurement window equals 7τ , the photon count number equals 1000 photons/cell and the photon count rate equals 0.31 per pixel and per measurement window. For a lifetime of $\tau = 20$ ns and an exemplary pixel number of 100 pixels, a time between the measurement windows of less than 170 ns is required for a precise determination of the lifetime. Higher number of pixels would allow for a longer time between the measurement windows or higher measurement accuracy (e.g. 174 pixels for $\Delta t_b = 400$ ns).

As mentioned before, the most common technologies for TCSPC are PMTs and SPADs. Thereby, commercially available PMTs (e.g. H11870, Hamamatsu Photonics, Japan) offer linear counting up to 1 Mcps (mega counts per second) and one pixel SPAD detectors (e.g. C1120, Hamamatsu Photonics, Japan and FastGatedSPAD, Micro Photon Devices, Italy) offer photon count rates of up to 80 Mcps. This corresponds to throughputs of 1000 cells/s respective 8000 cells/s with a photon count number of 1000 photons per cell.

As shown before, SPAD array detectors can be used to increase this throughput by combining a pixel array with a short time between the measurement windows. Nowadays, SPAD array detectors are often used in fluorescence imaging [11] and offer pixel numbers of more than 1000 pixels and time between measurement windows in the order of μs [6]. The time between measurement windows of 5.3 μs of the 1024 pixels detector used in [6] leads to an theoretical throughput of 58,000 cells/s, showing the potential of SPAD array detectors for high-throughput TRFC. In imaging, the pixel information of each detection needs to be transmitted, leading to an in-

creased data output with an increased number of pixels. This limits the system in [6] to a throughput of 300 cells/s due to its USB connection and shows another technical requirement for TRFC detectors. A suited detector is able to increase the throughput of commercial time resolved flow cytometers where actual systems offer throughputs of up to 1500 cells/s (e.g. Danube, Kinetic River, USA).

3 Materials and Method

3.1 SPAD array detector

To achieve a combination of a high number of pixels and short times between measurement windows, a new SPAD array detector was developed at the Fraunhofer Institute for Microelectronic Circuits and Systems IMS. It was fabricated in a XH018 180 nm CMOS-process at X-FAB Silicon Foundries SE. It is a front-side illumination silicon SPAD array and consists of a 10x10 pixel array to enable a high photon count rate. The rectangular pixel are 10x10 μm with an edge radius of 4.6 μm . This results in an active area of 82 μm^2 per pixel. The pixel pitch of the detector is 18 μm resulting in an array fill factor of 25.3 %. In opposition to imaging, the affiliation of a photon detection to the detecting pixel is not needed in TRFC. Therefore, the SPAD pixel signals are summed up and read-out with a 20 ns temporal resolution. This lowers the data output and enables a short time of 340 ns between measurement windows and therefore count rates up to 290 Mcps. The dark count rate for 80 % of the pixels is <3000 counts/s and the duration of the measurement windows is adjustable.

The temporal resolution limits the detector to lifetimes above 5 ns with a relative error of less than 10 %, according to [7]. Thereby, the total measurement time for the determination of a 20 ns fluorophore with an relative error of 5.5 % is

$$t_{\text{tot}} = \frac{1000 \text{ counts} \cdot (140 \text{ ns} + 340 \text{ ns})}{0.31 \frac{\text{counts}}{\text{px}} \cdot 100 \text{ px}} = 15.48 \mu\text{s}. \quad (2)$$

The total measurement time of 15.48 μs corresponds to a throughput of up to 64,583 cells per second, which is similar to the typical throughput of spectral flow cytometry of up to 100,000 cells per second.

3.2 Experimental setup

To evaluate the the functionality of the detector and its ability to acquire fast signals, a laser diode (PL450B, Osram, Premstaetten, Austria) is directed onto the detector. The laser diode has an average power of 100 mW and a central wavelength of

450 nm, which is detectable with the silicon-based SPAD array detector. To generate short laser pulses the diode is controlled by a laser driver (iC-HG (HG8M), iC-Haus, Bodenheim, Germany). The detector is connected to an field programmable gate array (FPGA). Besides controlling the detector, the FPGA generates the trigger signal for the laser driver to achieve a synchronized laser excitation and detection of fluorescence and reads out the data of the detector. In the experiment, 500 measurement windows with a duration of 400 ns are captured and added up in a histogram.

As a reference measurement, the laser is directed to a photodiode (HSA-X-S-1G4-SI-FS, Femto Messtechnik, Berlin, Germany) and the signal is recorded with an oscilloscope (MSO9404A, Agilent Technologies, Santa Clara, United States) with a time resolution of 0.1 ns. For direct comparison with the detector, the voltage values of the photodiode are divided in 20 ns bins and averaged within the bins. The binning start point is adjusted to match the detector start point so that the pulse is displayed in a comparable manner.

4 Results and Discussion

The measurement with the photodiode is shown in Figure 1A. The pulse exhibits a width of 22.2 ns (full width half maximum = FWHM). Additionally, the artificially binned pulse is displayed in Figure 1B. The pulse is mainly detected in three bins, with the main bin nearly in the middle of the pulse containing most of the signal. The rest of the signal is unsymmetrically detected in the bins before and after the main bin, with a higher signal in the bin before the main one. The other bins are not displayed in Figure 1B because of their low signal.

The pulse, recorded using the SPAD array detector, is seen in Figure 1C. The results are similar to the artificially binned signal of the photodiode with differences in the ratio of the main bin to the adjacent bins and a detection of a decay after the pulse.

The difference in the ratio of the main bin to the adjacent bins is probably based on the pile-up effect. In the measurement, a total number of 19,904 photons has been counted. Therefore, the photon count rate with 500 measurement windows and 100 pixels is 0.4 photons per pixel in each measurement window, assuming an even distributed illumination among the detecting pixels. As seen before, this can lead to a significant pile-up, which reduces the counts in the main bin and results in a lower ratio to the bins before.

The reason for the detected decay could be an afterpulsing of the laser diode. Taking the maximum signal amplitude of the main bin and the maximum noise amplitude of the bin 200 ns behind the main bin, the peak-to-peak ratio of the photodiode

equals 14 and the one of the SPAD is 504. This demonstrates the low noise level of SPAD detectors, which enables the detection of low signals like fluorescence decays and also after-pulsing of lasers.

The measurement shows the functionality of the SPAD array detector by detecting a short laser pulse of 22.2 ns in a similar way to an artificially binned signal of a photodiode. Further tests need to prove the applicability of the detector in TRFC. Thereby, the detection characteristics of a SPAD array detector should be compared to the ones of a PMT detector presented in [2]. The potential of the combination of a high pixel number of 100 pixels and a short time between measurement windows of 340 ns was shown with the calculated maximum throughput of up to 64,583 cells/s. Thereby, the applicability of it is increased by a reduced data rate due to the circuit design. The maximum throughput is in the order of the desirable throughput of 100,000 cells/s and is superior to state-of-the-art TRFC detectors [6] and time resolved flow cytometers (Danube, Kinetic River, USA).

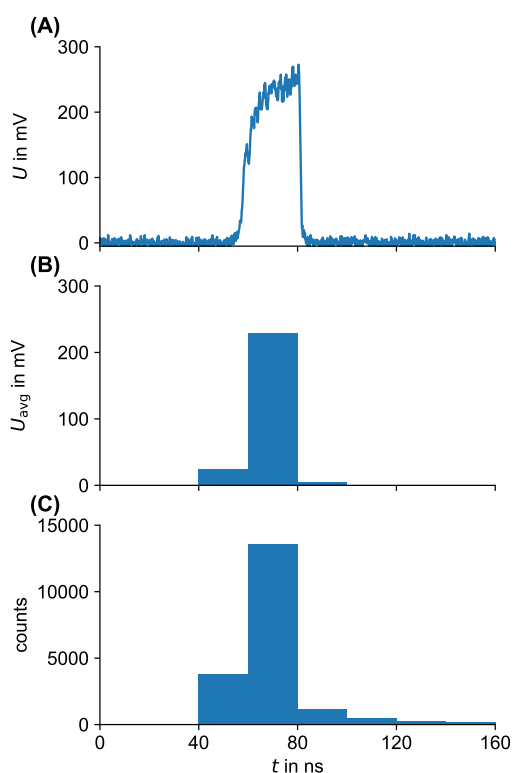


Fig. 1: Temporal pulse profile of a laser diode detected (A) with a photodiode. (B) The signal of the photodiode is separated in 20 ns bins and averaged within the bins to correspond to (C) the laser pulse detection with the developed SPAD with a time resolution of 20 ns. The starting point of the binning is shifted to align with the starting point of the SPAD detection.

5 Conclusion and Outlook

In this work we summarized the important SPAD detector requirements for high-throughput TRFC and presented a new developed detector which enables throughputs of up to 64,583 cells/s. After we have proven the functionality of the detector, we will evaluate it in an TRFC setup to determine the characteristics of lifetime determination with a SPAD array.

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