

Fluorescence lifetime biosensing with DNA microarrays and a CMOS-SPAD imager

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Abstract: Fluorescence lifetime of dye molecules is a sensitive reporter on local microenvironment which is generally independent of fluorophores concentration and can be used as a means of discrimination between molecules with spectrally overlapping emission. It is therefore a potentially powerful multiplexed detection modality in biosensing but requires extremely low light level operation typical of biological analyte concentrations, long data acquisition periods and on-chip processing capability to realize these advantages. We report here fluorescence lifetime data obtained using a CMOS-SPAD imager in conjunction with DNA microarrays and TIRF excitation geometry. This enables acquisition of single photon arrival time histograms for a 320 pixel FLIM map within less than 26 seconds exposure time. From this, we resolve distinct lifetime signatures corresponding to dye-labelled HCV and quantum-dot-labelled HCMV nucleic acid targets at concentrations as low as 10nM.

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1. Introduction

DNA microarrays are important tools for biomolecular detection. Widely used for gene expression profiling, disease screening, mutation and forensic analysis, they also hold much promise for the future development of personalized drugs and point of care testing devices [1,2].

In general terms, microarray technology exploits molecular recognition between a probe molecule attached to a substrate and a complementary target. The strength of the technique lies in its unique multiplexing capabilities, whereby a very large number of target molecules can be interrogated by a multitude of reporter probe spots printed on a single chip. The read out of microarrays can be achieved using a variety of detection techniques [3], however, fluorescence is the most commonly used transduction method [4]. Recently, fluorescence lifetime imaging (FLIM) has been reported as a powerful multidimensional technique that offers a number of benefits for microarray reading in term of sensitivity, specificity and multiplexing capabilities [5,6]. Nonetheless, the relative complexity of the method, combined with bulky and expensive equipment has yet prevented its integration into microarray based biosensing devices.

FLIM can be performed using various methods either in time or in frequency domain [7,8]. The respective advantages and disadvantages associated with different approaches have been reviewed in recent publications [9,10]. The choice of the given technique is driven by specific applications. In the case of DNA microarray reading, time-correlated single-photon counting (TCSPC) based methods present some critical advantages over other techniques that include single-photon sensitivity combined with unlimited dynamic range and high temporal resolution. The major draw back of TCSPC lies on its inherently low duty cycle and associated long data acquisition time, incompatible with real-time monitoring and high-throughput screening.

Improvements to wide-field photon counting cameras based on multi-channel plates have led to increased count rates up to 500 kHz with good spatial resolution [11] but the global detection scheme of one photon event at the time in the whole field of view limits the

scalability of this approach. The emergence of a new type of imager based on single-photon avalanche diode (SPAD) arrays built on CMOS technology [12,13] is enabling high parallelization of TCSPC acquisition in a compact and cost effective manner. Such a device has been utilized as an active array substrate to perform the time-resolved Forster resonance energy transfer detection of a DNA assay, demonstrating a reduction in the transduction signal dependence to the DNA probe surface density [14]. More recently our CMOS-SPAD device was used to demonstrate real-time FLIM based on an onboard lifetime estimation method [15] which works well for bright samples with mono-exponential decays, such as the reported mixing of highly concentrated dye solutions. However, samples encountered in the context of biosciences are typically much dimmer, which reduces the advantage of lifetime estimation both in terms of speed and data compression. Biological samples also often show more complex decays which require photon arrival time histograms for more detailed analysis. In the present article we show fast FLIM detection at low fluorophore concentrations relevant to DNA binding assays. By adding an onboard memory controller to our CMOS-SPAD device [12,15] we can capture the arrival time of every detected photon, allowing rapid FLIM based DNA microarray readout and the recording of arrival time histograms confirming multi-exponential decays. This study lays the foundations for high-throughput multiplex FLIM detection and fluorescence lifetime kinetic studies.

2. Material and methods

2.1 Microarray preparation

We prepared a DNA microarray spotted with two different DNA probes and hybridized with their distinct complementary artificial targets. The detailed procedure of production and incubation has been reported in a previous publication [5]. In short, the microarray consisted of 16 spots, incubated with a) hepatitis C virus (HCV) probe, b) human cytomegalovirus (HCMV) probe and c) a 1:1 molar ratio of HCV and HCMV probes.

The array was hybridized with a solution of complementary target containing 10 nM of Alexa Fluor 430 (Alexa430) labeled HCV target and 10 nM biotinylated HCMV target, which was further incubated with quantum dot (QDs) streptavidin-conjugate solution, Qdot525. Qdot525 and Alexa430 were chosen for their overlapping absorption and emission spectra combined with a distinctly different excited state lifetime, approximately 4.2 and 22 ns respectively. Those properties permitted FLIM measurements using both a single excitation wavelength (415 nm) and a single read out channel (515 to 565 nm band-pass).

2.2 DNA oligonucleotide sequences

The sequences of the HCV probes (P1) and targets (T1) obtained from Metabion (Martinsried, Germany) are as follows: P1 5'-TGCACGGTCTACGAGACCTCCCGGGCACTCGCAAGCACCTATCAGGCAGTACCACAAGGCCTTTCGC-3'[5'] = SH, T1 5'-GCGAAGGCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGAGGTCTC GTAGACCGTGCA [5'] = Alexa 430. The sequences of the HCMV probes (P2) and targets (T2) obtained from Eurogentec (Seraing, Belgium) are as follows: P2 5'-CAAATACCGTGGGACGACACGCACCGGCAGTGCGCAGGCAGCGTCGGACACAACACGCTTACGGCCCTCAACACT-3'[5'] = SH(C6), T2 5'-AGTGTGAGGGCCGTAAGCGTGTGTGTCGACGCTGCCTGCGCACTGCCGGTGC GTGTCGTCCCACGGTATTTG - 3'[5'] = Biotin-TEG.

2.3 TIRF-SPAD array imaging Setup

The microarrays were imaged by combining a total internal reflection fluorescence (TIRF) setup with the SPAD imager (Fig. 1). The excitation source consisted of a Ti:Sapphire laser, pulse picker and frequency doubler (Coherent, Glasgow, Scotland) and produced femtosecond pulses at 415 nm wavelength with a repetition rate of 4.75 MHz. Note however that such a complex excitation system is not really required and could be replaced by a much more

compact picosecond diode laser. The TIRF setup used a quartz prism (Cairn Research, Faversham, UK) attached to the condenser of a Nikon TE300 inverted microscope and placed into contact with the undersurface of the DNA microarray using immersion oil. 2 mW of laser light was directed below critical angle, generating a local evanescent excitation of circa 1 mm^2 area on the DNA microarray. The resulting fluorescence was then collected with a $10\times$ microscope objective (Plan Apo, N.A. 0.45, Nikon, Japan), filtered with an emission band-pass filter (535/40 nm) and imaged onto the SPAD array imager. A de-magnifying image relay ($f_1 = 50 \text{ mm}$, $f_2 = 8.5 \text{ mm}$) was used to match the excitation area to the field of view of the sensor.

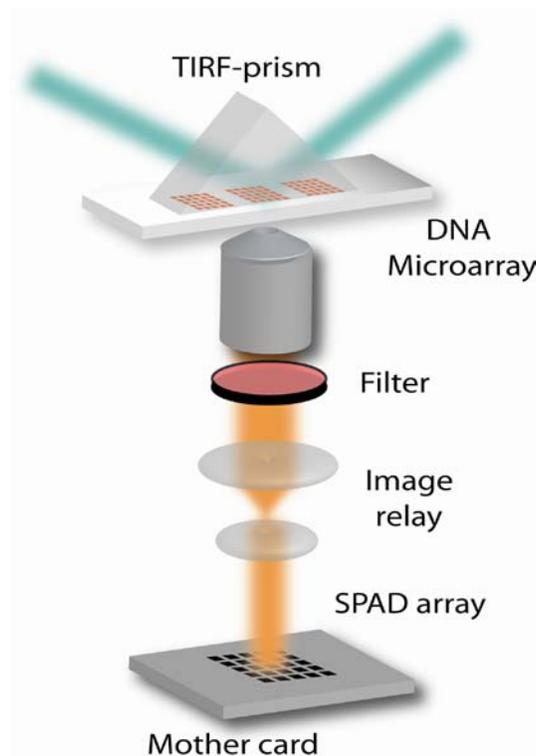


Fig. 1. Experimental setup of the TIRF-SPAD imager setup. (see text for details).

The camera is based on a 32×32 pixel array, fabricated in 130 nm CMOS technology, where each pixel contains its own SPAD and 10-bit time-to-digital converter (TDC). The two halves of this prototype chip have different implementations of the TDC, most notably leading to different time resolutions of 54 ps and 175 ps respectively (see ref [12,16]. for technical details and characterization of the chip). Detection of a photon starts the in-pixel TDC with the stop signal provided by a fast photo diode monitoring the excitation beam. The sensor is capable of supplying photon arrival time data at a variable frame rate up to 500 kHz (potentially over 500 million photon events per second), producing data rates of up to 5Gbit/s. In order to record full arrival time data a memory controller has been implemented on the device which can stream up to 100,000 frames of data directly to its local memory at any frame rate. Once the device memory has been filled up it can be transferred post-experiment to a PC.

2.4 Image acquisition and analysis

Data presented here only used 20×16 pixels on the ‘fast’ TDC half, which has a time range of 55 ns with 1024 bins of 54 ps resolution. Here, the low intensity fluorescence of the DNA

microarray samples required an increase in exposure time to 256 μ s, leading to a reduced frame rate of just under 4 kHz. The memory controller was used to acquire blocks of 100000 frames with TDC codes (\sim 26 s total exposure time), which were then transferred to the PC and combined into arrival-time histograms (\sim 12 s in total). Custom software written in Labview (National Instruments, Texas) was used to remove the background in noisy pixels and prepare the TCSPC data for import into commercial FLIM analysis software (SPCImage 3.1, Becker & Hickl GmbH, Germany).

3. Results and discussion

Initially, TCSPC data for the three different categories of spots ((a) HCV probes, (b) HCMV probes and (c) mixture of 50% HCV and 50% HCMV probes) was collected using subarrays containing four identical spots. Figure 2 shows representative decay curves for each category obtained from an individual pixel. Least square fitting using the SPCImage software clearly revealed multi-exponential decays for each category of spots, with bi-exponential models leading to good fits ($\chi^2 \sim 1.4$) with distinct average lifetimes.

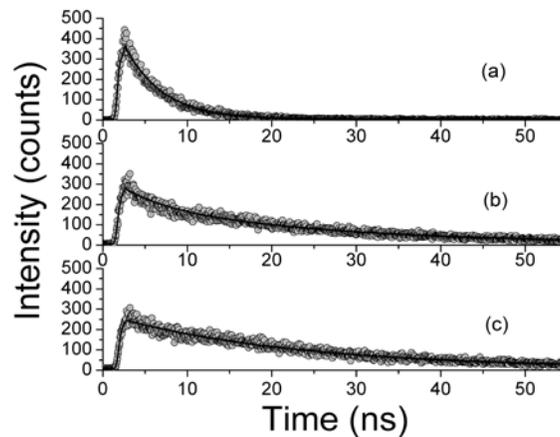


Fig. 2. Decays histograms (circles) with corresponding fit (solid lines) obtained for three individual pixels associated with the three categories of microspots, (a) HCV, (b) HCMV-HCV mixture and (c) HCMV.

Figure 3 shows the average lifetimes of four subframes (16x20 pixels each) as FLIM maps (left) and corresponding pixel lifetime distribution histograms (right). The fit of pure HCV and HCMV decay curves resulted in average lifetimes centered on 4.2 ns and 22 ns matching the reported emission decay values for Alexa430 and Qdot525 [5]. Both their distributions are fairly narrow with FWHM of around 10% of the mean lifetime (610 ps and 1.97 ns for HCV and HCMV, respectively). The distribution for HCV-HCMV mixture is markedly broader with a FWHM of 3.5 ns centered on a mean value of 11.3 ns. Dynamics of the QDs excited state follows complex mechanisms resulting in multi-exponential emission decays with up to 5 components [17,18] and Alexa in the complex environment of the microspot has several decay components [5]. Decays from the HCV-HCMV mixture are a superposition of these single label decays and the average lifetimes depend sensitively on the local relative concentration, giving raise to the observed much wider distribution. Repeating the fitting process with two globally fixed lifetimes ($\tau_1 = 4.2$ ns & $\tau_2 = 22.0$ ns) leads to good fits of comparable quality for all three types of probes, as demonstrated by the FLIM map with different spots displayed in Fig. 3(d). In this case, the relative contributions of the two lifetime components match the probe ratio of the spots (100:0, 50:50 or 0:100 resp.). A more detailed study is currently underway, as this might open the way for quantification of the probe ratios in individual spot, especially when combined with more rigorous global analysis of the lifetime images [19].

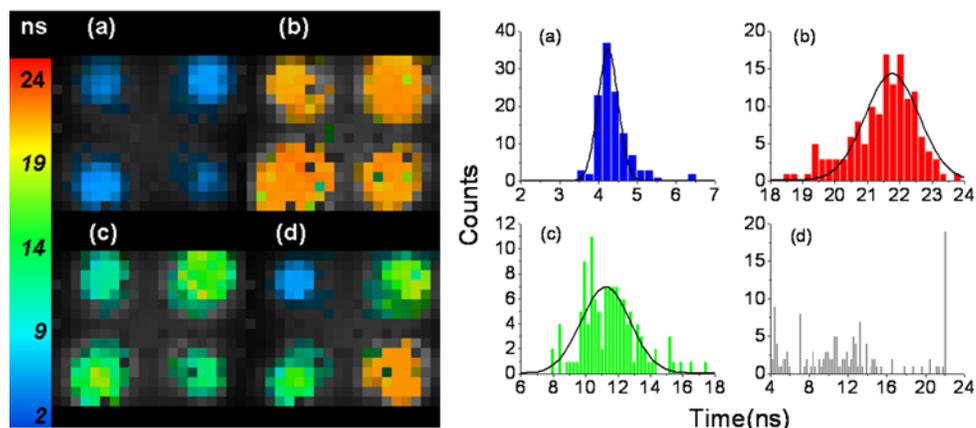


Fig. 3. (Left) FLIM map of DNA microarray spotted with a series of four sub-arrays corresponding to four subframes of 16x20 pixels each: (a) HCV probes, (b) HCMV probes, (c) four spots containing a mixture of 50% HCV and 50% HCMV probes, (d) HCV probes (top left), HCMV probes (bottom right) and 50% probe mixture (diagonal). The array was hybridized with a solution containing 10 nM of Alexa430 labeled HCV complementary target and 10nM of Qdot525 labeled HCMV complementary target. (Right) Fluorescence lifetime histograms and Gaussian fit, extracted from the four sub-arrays. Note that (d) used a bi-exponential model with $\tau_1 = 4.2$ ns & $\tau_2 = 22.0$ ns fixed.

Each individual FLIM map (320 pixels) presented in Fig. 3(a)–3(d) was obtained within 40 s. Assessment of different technologies is difficult as they often involve very different principle of operation, detector size, resolution and illumination. Compared with our recently published results, measured on similar samples and obtained with a state of the art TCSPC quadrant anode detector [5], the SPAD imager acquisition time was circa 200 times faster. With the quadrant anode detector it took up to 30 min to collect 500000 counts per spot of the microarray (imaging 9 to 12 of them simultaneously), whereas the SPAD imager could acquire 1.5M counts per spot in an exposure time of only 26 s.

Evidently, the SPAD array imager has a much lower spatial resolution than the device used in ref [5]. or other state-of-the-art FLIM systems but it is sufficient for use with microspot arrays. For the data presented in Fig. 3 there were about 20 pixels for each DNA microspot, sufficient to provide FLIM maps resolving the shape of individual spot as well as providing information on the variance of the lifetime estimate. For screening application this spatial detail is often not required and the throughput could be dramatically increased by mapping each microarray spot to fewer (ultimately individual) pixels.

A more severe limitation of the current prototype device is its low photon detection efficiency. The active area of the SPAD (8 μm diameter) is very small compared to the pixel pitch of $50 \times 50 \mu\text{m}$ mostly taken up by its timing and counting circuits, leading to a fill factor of only 2% [12]. The overall efficiency is therefore below 1% and the relatively high excitation light levels needed to achieve high count rates lead to noticeable photo bleaching of the Alexa labels.

To address this problem, mounting of micro-lenses onto the SPAD array is currently being investigated. This is expected to enhance light collection by at least 10-fold [20], making the overall efficiency directly comparable to PMT based devices. This will dramatically increase the sensitivity, making it feasible to work with lower sample concentrations and/or lower laser intensities. Alternatively this could further decrease the data acquisition time as the frame rate could be increased to 50 kHz (compared to ~4 kHz used for data presented here) without suffering from distortions through pile-up.

Additionally, acquisition speed could also be improved by streaming data directly to the PC using USB with data compression techniques (latest firmware/software updates can achieve continuous transfer of data to PC at about 20000 fps). However, the huge amount of raw data generated by such detector arrays requires large bandwidth and storage capacities,

and for better scalability with detector array size on-board data processing will be essential. By building arrival time histograms on the device the data transfer to the PC can be reduced dramatically while still retaining all information typically used for lifetime analysis [21]. Besides, for ultimate speed, software based lifetime determination can be replaced by an on-board method. The potential of such an approach was recently demonstrated by successfully monitoring the real-time mixing of dye solution in microfluidics using high-speed integration for extraction method (IEM) algorithms, implemented on field programmable gate arrays (FPGAs) [15]. Algorithms more suitable for dim samples, such as the centre-of-mass method [22], have since then been suggested. As the device can easily switch between full TCSPC data acquisition and lifetime estimation mode, verification/calibration can easily be achieved without changes to the experimental geometry. Finally, the production of a much larger SPAD plus TDC array will allow 256 microarray spots to be measured in a single detection event, opening true opportunities for high throughput FLIM detection.

4. Conclusion

In summary, we have demonstrated rapid TCSPC data acquisition for fluorescence lifetime imaging and multiplexing of a DNA microarray using a CMOS-SPAD array camera. Although results presented here were obtained using a complex excitation laser system, the required power levels (~2 mW) are easily accessible for compact picosecond pulsed diode lasers. The data presented has been obtained from fixed samples but the current system should be fast enough to monitor hybridization dynamics of DNA microspot arrays which constitute the focus of our future work. Ongoing upgrading of the SPAD imager will enable faster and more sensitive detection on a larger sensor area. Such improvements will permit high-throughput FLIM of DNA microarrays and open the possibility to integrate FLIM transduction on biosensing and point of care testing devices.

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