A low-cost high throughput microfluidics system for multi-thousand droplets per second sorting based on time resolved fluorescence measurement

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***Abstract*— Time-resolved fluorescence measurement screening and sorting has been proven to be interesting for biological applications. Fluorescence lifetime (FLT) measurement provides additional information to the measurement of the intensity or polarization of the fluorescence emission. Furthermore, droplet microfluidics enables higher throughput than well plate readers. Few researches have been recently done about the microfluidic droplet sorting based on the fluorescence lifetime measurement. Some of the proposed solutions support high throughput but they do not extract the FLT directly from the fluorescence decay. In this paper, we present an alternative low-cost system for the microfluidic droplets sorting. We implemented a FLT measurement system based on the time-correlated single photon counting (TCSPC) technique on a cyclone V SoC-FPGA. For the excitation light source, we use a simple pulsed laser diode and a single photon avalanche diode (SPAD) as a photodetector. The optical system is an ad hoc microscope. The droplet generation is done with flow focusing technique in a PDMS-based microfluidics chip. This system was successfully tested in real-time at a droplet rate of more than 3000 droplets per second.**

Keywords—Microfluidic, Droplet sorting, Screening, FPGA, Real-time, Time Correlated Single Photon Counting, TCSPC, SPAD, Fluorescence Lifetime, FLT.

I. INTRODUCTION

In biology, fluorescence measurement is currently used because the fluorescence emission of dyes or other molecules is affected by the reaction taking place. Indeed, fluorescence intensity depends on the analyzed reaction but at the same time it depends on the probing volume and many other factors such as the concentration and the excitation light intensity. To the contrary, fluorescence lifetime (FLT) is an intrinsic parameter that does not depend on these factors. Therefore, due to the fluctuation of the side factors, FLT detection is more robust than the fluorescence intensity measurement. It has been implemented in well plate readers as show in [1][2]. However, it takes about 0.5 second to read one well.

In fact, the analysis time can be enhanced by using microfluidic chips that support high throughput rates [3][4]. Furthermore, microfluidic chips allow to reduce the quantity of reactants and offer an easy solution to sort the targeted samples based on the fluorescence measurement results. If a sorting action is needed, the processing of lifetime measurement has to be done in real time. For example, in [5], a droplet rate of about 2.5 thousand droplets per second can be achieved. A real-time sorting was performed to enrich the population of fluorescent proteins expressed in bacteria. This system includes expensive parts such as the electro-optic modulator (EOM). The FLT is extracted from the phase between the emission and the excitation signals.

In [6], the authors of this paper demonstrated that time-correlated single photon counting (TCSPS) technique can be used for screening up to 1000 independent reactions per second. However, the setup proposed by [6] is not suitable for droplet sorting since the FLT measurement was post processed rather than processed in real time.

In [7], we proposed a robust TCSPC-based system for droplet sorters. We implemented this system using a low-cost SoC-FPGA platform. Furthermore, the laser has been replaced by a low-cost low-power pulsed laser diode. The high sensitivity of the single photon avalanche diode (SPAD) allows to work at very low power (about 80 µW). This is an advantage as compared to other proposed solutions, because the optical power can damage the samples. To validate the functionality of this system, it was tested on emulated droplets using a rotating wheel with segments of fluorescent paper separated by dark segments.

Here, in order to test the design proposed in [7] with real droplets, we integrated it in a larger system including a small microscope and a microfluidic chip that generates fluorescent droplets. The whole system is portable to be transported to the experiment place.

The experimental setup including the electronic system, the optical microscope and the microfluidic chip is described in section II. Section III presents the experimental results for two different droplet rates and proposes optimizations to improve the distribution of the measured FLT. Section IV discusses the discrimination of two population samples, as well as the SPAD defects and the ability to use the system for fluorescence labelled cells. Finally, section V presents the conclusion.

II. EXPERIMENTAL SETUP

Figure.1 shows the setup used in the experiments. We implemented our designed TCSPC system on a cyclone V SoC-FPGA, namely the “Altera DE10-Nano” kit. We used a 405 nm pulsed laser diode driven by a fast pulse generator

described in [8]. A wavelength of 405 nm is not ideal for the excitation of the studied fluorescent dye which is the fluorescein in PBS at a concentration of 1 mM. However, the goal of these experiments is to prove the maximum detectable droplet rate. A 425-nm dichroic mirror separates the excitation beam from the emission signals. A x40 microscope objective is used to focus the excitation beam on the microfluidic channel and efficiently collect the fluorescence signal. As a photon detector, we used a commercial SPAD from IDQ coupled with an optical bandpass filter. The used microfluidic chip, shown in Figure.2, is fabricated with soft lithography of PDMS on glass. The flow focusing junction generates aqueous droplets containing 1 mM fluorescein in perfluorinated HFE-7500 oil with surfactant from Raindance company. The concentration is high because the excitation wavelength is not optimal as explained above. The sequence droplet-oil flows through a squared channel of 25 µm which is small enough to generate droplets rate up to 10 thousand droplets per second.

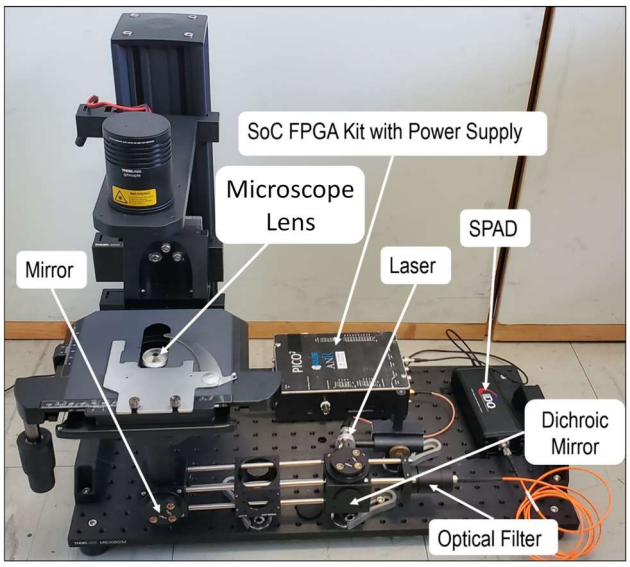

Figure.1 Experimental setup

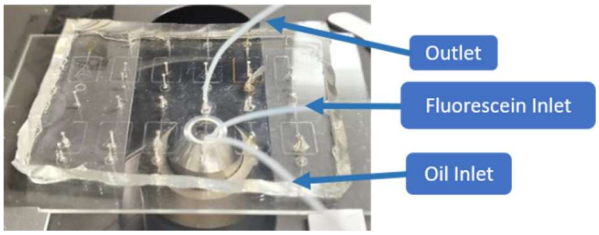

Figure.2 Microfluidic chip

III. Experimental Results

A. Maximal achieved droplet rate

By adjusting the oil and the fluorescein solution flow rates, a droplet rate of 3573 droplets per second was generated. The system presented in [7] was able to detect the droplets basing on the fluorescence intensity, as demonstrated in Figure.3, and to extract the FLT of the detected droplets in real-time. At this high droplet rate, the number of detected photons per droplet was only about 43 photons. With such a weak signal, the system was still able to extract the FLTs expected from literature on average (4.02 ns). To visualize the results, the resulting FLT values of 10000 droplets are stored and compiled in the histogram shown in Figure.4. The standard deviation of the extracted FLTs is 0.72 ns whereas the standard deviation caused by the photonic noise would be 0.6 ns. Thus, the noise of the system is only 20% above the photonic noise, meaning that the electronic and processing noise are negligible. To optimize the photonic noise, the detected photon rate can be multiplied by 5 by increasing the laser pulse rate from 8.5 MHz to 42.5 MHz. In this experiment, the instant photon rate during the passage of a droplet is 428 kphoton/s. With a laser pulse rate of 42.5 MHz, the photon rate would be 5% of the laser rate. If we accept this ratio to be 10%, we can increase the photon rate by another factor 2 by adjusting the laser wavelength. In doing so, the loss of precision on the FLT would be only 3% because of the pileup effect. Combining these two optimizations, the photon rate would be 10 times higher and the standard deviation would be divided by approximately 3.

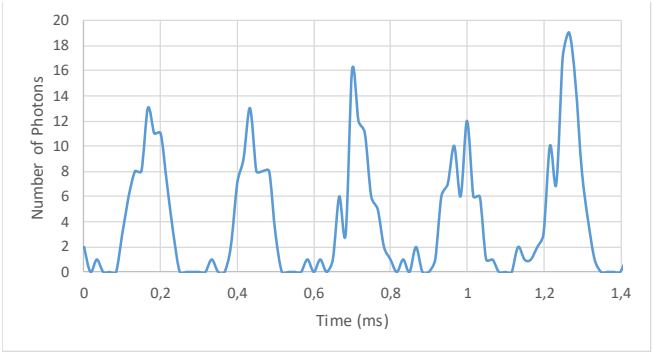

Figure.3 Chronogram of droplets fluorescence intensity

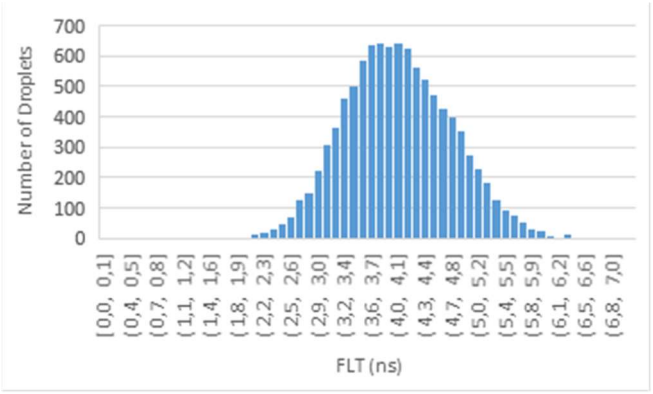

Figure.4 Resulting FLTs histogram at 3573 droplet/s

B. Practical droplet rate

In the second part of the experiments, the oil and the fluorescein solution flow rates were adjusted to generate a droplet rate of 1000 droplets per second. At this droplet rate, the number of detected photons per droplet was about 718 photons. The system was able to detect the droplets in real time and the average value of the extracted FLT of the detected droplets was 3.92 ns. This value is lower than the expected value because the laser pulse rate was about 8.5 MHz, i.e. the photon rate is about 16% of the laser rate, which leads to a high pileup effect and cause an underestimation in the measured FLT. The resulting FLT values of 2000 droplets are stored and compiled in the histogram shown in Figure.5. The standard deviation of the FLTs values is 0.18 ns whereas

the standard deviation due to the photonic noise would be 0.15 ns. However, the error in the average FLT value can be overcome by increasing the laser pulse rate to 42.5 MHz to have a photon rate 3.2% of the laser rate. Furthermore, if we accept this ratio to be 10 %, we can increase the photon rate by factor 3 and improve the photonic noise by factor 1.8 which accordingly decreases the standard deviation by the same factor to be about 0.1 ns.

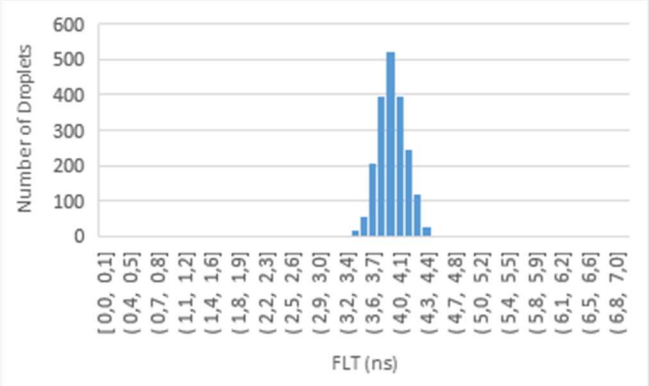

Figure.5 Resulting FLTs histogram at 1000 droplet/s

IV. Discussion

A. *Discrimination of two samples according to the FLT :*

In screening and sorting applications, the measured FLT is compared to a threshold to take a decision. Therefore, it is important to clearly distinguish between two population of samples having different FLT values. which means to have a completely separated histograms for the distribution of the two populations as illustrated in figure.6-a. However, due to large standard deviation values, the distribution of the two populations could overlap causing false positive and false negative decision as illustrated in figure.6-b. One method to evaluate the ability to discriminate between two populations is to estimate a factor known as ***Z'*** factor [9] calculated by equation.1.

$$Z' = 1 - \frac{3\sigma^+ + 3\sigma^-}{\tau^+ - \tau^-} \approx 1 - \frac{3}{\sqrt{N^+}(1-\sqrt{r})}$$

Where σ^+ and σ^- are respectively the standard deviation of the higher FLT population and the lower FLT population, τ^+ and τ^- are the FLT of these populations, r is the ratio between τ^- and τ^+, and $N+$ is the average number of photons in the droplet of the higher FLT population.

A Z factor of 0.5 guarantees that the average of the distribution of two populations are separated by 3 σ of the first population (σ^+) plus 3 σ of the other one (σ^-).

Assuming that the population mentioned in III-A is the higher FLT population, we can determine the maximum value of FLT (τ^-) for the second population with the lower FLT population that could be discriminated based on the criteria that Z factor should be at least 0.5. In this case, $N+$=43 photon, $\tau^+ = 4.02$ ns. From equation.1, the maximum accepted value of τ^- is 0.029 ns. This value is too low in practical situation because of the high photonic noise. However, considering the above mentioned optimization where ***N+*** is increased to 430 photons, the maximum value of $\boldsymbol{\tau}^-$ can be increased to 2.03 ns.

For the droplet rate of 1000 droplet/s where ***N+***=719 photons, $\boldsymbol{\tau}^+$=3.922 ns, the maximum accepted value of $\boldsymbol{\tau}^-$ would be 2.36 ns. Considering the mentioned optimization for this experiment, this value could be increased up to 2.69 ns

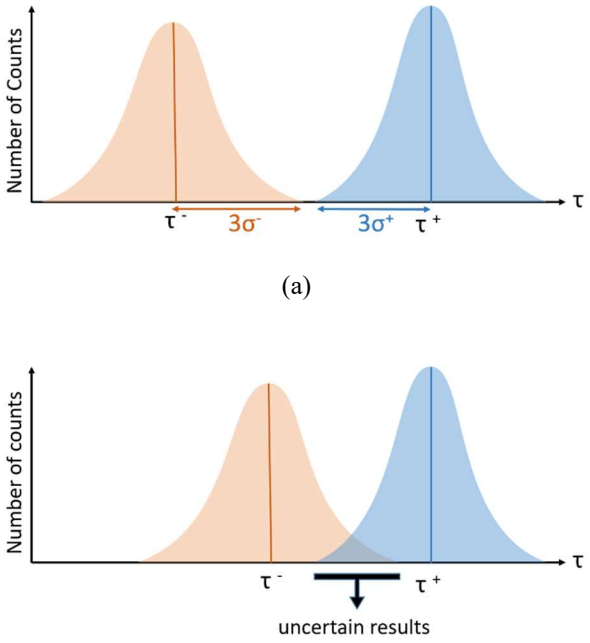

Figure.6 Discrimination of two samples population according to the FLT – (a) completely separated histograms for the distribution of the two populations. (b) the distribution of the two populations could overlap causing false positive and false negative decision

B. *SPAD defects*

1. Dark count rate (DCR):

Generally, the DCR of the SPAD used in our system is less than 100 Hz. The noise of the DCR is a white noise uniformly distributed along the temporal window of the measurement. Thus, the DCR counts can be considered as a part of the background signal and will be eliminated with the background signal when performing the background correction as described in [5]. At high droplet rates, higher than 1000 droplet per second, with a DCR of 100 Hz, the probability to have a parasite count in a droplet is less than 0.1 which is negligible.

2. After-pulsing:

At a laser rate of about 10 MHz, the temporal window of the measurement will be about 100 ns. With such a relatively short temporal window and considering the after-pulsing probability distribution of the commercial SPAD given in the datasheet (ID100 form IDQ), the counts caused by the after-pulsing effect can be also considered homogenously distributed along the measurement temporal window. For the SPAD used in our system, the after-pulsing probability is about 0.5%. The noise due to the after-pulsing effect is eliminated by reducing the number of photons in each bin by this ratio.

3. Pile-up effect at high photon rates:

At high droplet rates, the principal limitation of the system is the photon rate. A high photon rate is required in order to have a large number of detected photons for each droplet to increase the signal to noise ratio. In TCSPC application, a photon rate of up to 10 % of the laser pulse rate is accepted [6]. However, higher photon rates lead to a high pile-up effect causing an underestimation in the average value but at the same time a lower standard deviation of the measured FLT of each sample population [10]. In this case, the measured FLTs will not be accurate but the system resolution will be improved allowing to discriminate population samples with closer FLT values. Furthermore, the accurate FLT values can be measured at a low droplet rate.

C. *Fluorescence labelled cells:*

This setup is adapted for uniform droplets. However, in many applications, the goal is the sorting of fluorescence cells. This setup can be used for these applications if the cells are encapsulated in droplets [11][12]. In this case, the droplet would not be uniform and the laser spot may not excite the fluorescent cell as demonstrated in figure.7-a. a simple solution for this problem is to transform the laser spot into a line across the whole width of the channel figure.7-b. Considering a 3D perspective, this line is in fact a vertical sheet of light viewed from the top side and it covers the whole section of the channel. In this case, wherever the cell is inside the droplet, it would be excited by the laser line. furthermore, even if the excitation intensity is not uniform along the laser line there would no problem because the FLT in not sensitive to the excitation intensity.

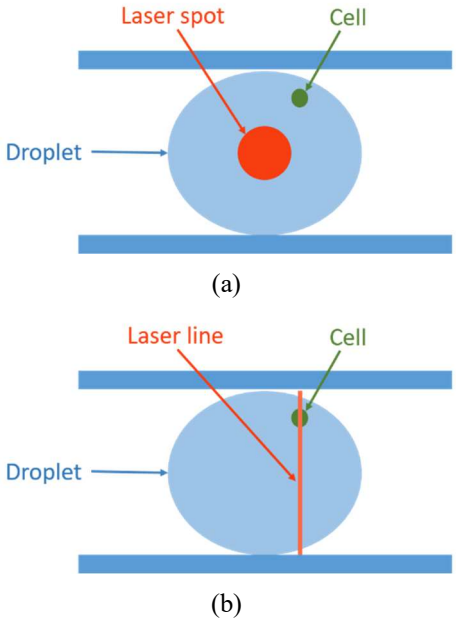

Figure.7 labled cell encapsulated in droplets – (a) the laser spot may not excite the fluorescent cell. (b) using a laser line across the whole width of the channel ensure the excitation of the cell.

V. Conclusion

In this paper, we demonstrated the possibility to detect and analyze 3573 droplets per second with the TCSPC technique. We also discussed how to optimize the resolution of the measurement which is an important factor to separate different populations in the sample based on the Z' factor. We also discussed the influence of the SPAD defects and how to overcome these defects. Finally, we proposed some amelioration to support the sorting of fluorescence cells.

Acknowledgment

We are thankful for financial support from the European InterregWPS Project, ITI Innovec IdEx (ANR-10-IDEX-0002), SFRI (ANR-20-SFRI-0012), the French National Research Agency (ANR-15-CE11-0006). We also want to thank PCBIS (UAR 3286) for their longstanding collaboration on microfluidics.

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