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Parametric Optimization for High Speed FLIM Implementation

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Abstract. FLIM(Fluorescence Lifetime Imaging Microscopy) has been considered an effective technique to investigate chemical properties of the specimens, especially of biological samples. Despite of this advantageous trait, researchers in this field have had difficulties applying FLIM to their systems because acquiring an image using FLIM consumes too much time. To increase the FLIM speed, many methodologies have been developed and applied to the system. One of the recent methodologies is an analogue mean delay based FLIM using a PMT and digitizer for image reconstruction. In this system, however, imaging time is largely dependent upon several parameters such as data transfer rate, sampling rate of an A/D converter, and signal width etc. In this paper, such parametric optimization method is introduced for faster acquisition of the image.

1. Introduction

In Microscopy field, a lot of efforts have been put forth to acquire information rather than just to increase magnification or acquisition speed. One of the accomplishments is FLIM (Fluorescence Lifetime Imaging Microscopy) based on TCSPC (Time Correlated Single Photon Counting). Traditionally TCSPC has enabled the acquisition of the information about the chemical composition and decomposition of biologically developing or decaying samples. With FLIM, researchers can start to evaluate the chemical variations, not simply just the physical ones which have been the major concerns before that.

Although TCSPC has been considered an effective tool to look into more than the mere surface or a few hundreds of microns, TCSPC has a tremendous disadvantage of long imaging time.

As its name implies, TCSPC operates well under extremely low intensity environment, namely dark surroundings that can only permit one photon at a single time event. It counts single photon at a time and accumulate and make a histogram chart that represents response curve.

TCSPC usually takes longer than one minute to acquire one 512 by 512 image with 10% accuracy, and longer than five minutes for 3% accuracy and for 1% accuracy, it takes over one hour [1].

Main reason why TCSPC takes such long time was the limited bandwidth of detector. When TCSPC was developed and established, the photo detector could not capture a signal faster than a few MHz, and by that reason, single photon was detected and counted and accumulated. But with the development and enhancement in the detector, fast signal as hundreds of MHz can be captured currently. AMD FLIM is an exertion to reconstruct a high frequency signal with a high-bandwidth PMT.

2. Principle and System Setup

Figure 1 shows schematic representation of AMD FLIM [2] and its principle. A photon emitted from pulse laser exhibits timing jitter which makes up Gaussian distribution represented in black in Figure 1. Some of the photons are struck on the fluorescence dye upon the specimen. These photons experience decaying process and are described in red. The rest of photons are simply reflected or scattered or absorbed. Reflected ones are detected in another path but don't experience decaying process. The reflected or decayed photons are going into separate PMT (Photomultiplier Tube) and experience time jitter called TTS (Transit Time Spread), namely, jittered detector response [3].

In timing chart of figure 1, large T means probability value, that is to say nondeterministic value for a single photon but can be described for a lot of photons stochastically. Small t means deterministic value even for a single photon. T_{ex} is an excitation time for a photon to be emitted from laser measured from the time reference of each pulse. t_{ex} is a photon's travelling time from laser to specimen, which is deterministic. T_{fl} is a fluorescence time for a photon hit on a dye and absorbed by the dye to be released again, which process can be represented by a decaying curve. t_{fl} is the time for a photon to travel from specimen to detector. T_{pd} is TTS mentioned above.

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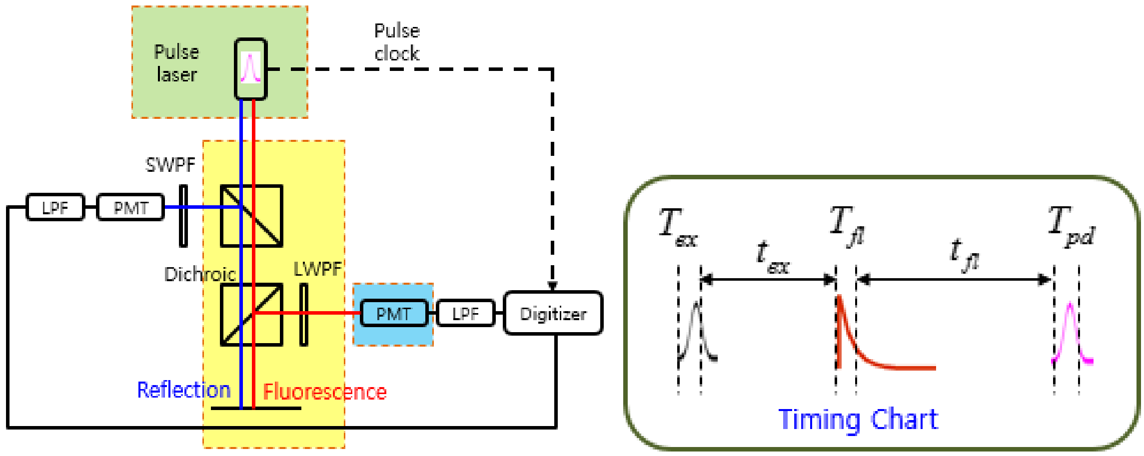

Fluorescence : $T_e = (T_{ex} + t_{ex}) + (T_{fl} + t_{fl}) + (T_{pd})$

IRF : $T_e^0 = (T_{ex} + t_{ex}) + (\quad t_{fl}) + (T_{pd})$ –

$$\langle T_e \rangle - \langle T_e^0 \rangle = \langle T_{fl} \rangle = \tau$$

Figure 1. Schematic system setup and timing chart for AMD FLIM

While t_{ex} is the same both for fluorescence and reflection paths, t_{fl} differ a little depending on the paths. But, the difference is deterministic and so can be compensated. Likewise, although T_{pd} differs a little depending on the PMTs on different paths, this difference is deterministic and can be compensable. T_{pd} itself is non-deterministic and stochastic but the discrepancy is deterministic and thereby calibratable.

T_e^0 is the time taken for a photon to travel from a laser pulse reference time to the captured time as a signal by the detector on the reflection path. T_e is the time taken on the fluorescence path. When T_e^0 is subtracted from T_e as is shown in the Figure1, t_{ex} and t_{fl} can be cancelled because they are deterministic, but T_{ex} and T_{pd} cannot be offset because they are non-deterministic. However, large T values can be cancelled out on the stochastic basis, and that is why T_e^0 and T_e are bracketed which means time averaged value.

Then, on time-average basis, time constant of the decaying curve can be calculated by just subtraction. And, in detail, the decaying time constant is calculated by the following integration equation [2].

$$\tau = \langle T_e \rangle - \langle T_e^0 \rangle = \frac{\int t \cdot i_e(t)dt}{\int i_e(t)dt} - \frac{\int t \cdot i_{irf}(t)dt}{\int i_{irf}(t)dt}$$

3. Signal Optimization

AMD FLIM is basically based upon fast signal detection. But the signal bandwidth is band-limited by a few technological limitations. The major limitation is data transfer rate on the PC bus. Analogue integration is impossible for now, so A/D conversion is inevitable, which means digitizers are necessary for AMD FLIM. The digitized data should be transferred to PC RAM and processed fast enough in PC processor. From this point, the problem arises because for accuracy the faster the sampling frequency of digitizer the better, but that is limited by the PC bus. Currently, digitizers in business support PCIe Gen2 8lanes at best, and on the specification, that means 4GB/s data transfer rate. But in practice, it is not easy to accomplish 2GB/s, which means 1GB/s data transfer rate per channel (two channels are required, one for reflection and one for fluorescence) on PCIe bus is the maximum data transfer rate for real time processing.

Many digitizers support well over 1GB/s data transfer. For con-current transferring and subsequent processing, however, 1GB/s is the maximum that can be applied for AMD FLIM. Then a signal with narrow width cannot be A/D converted without loss of needed information. That situation is depicted in Figure 2.

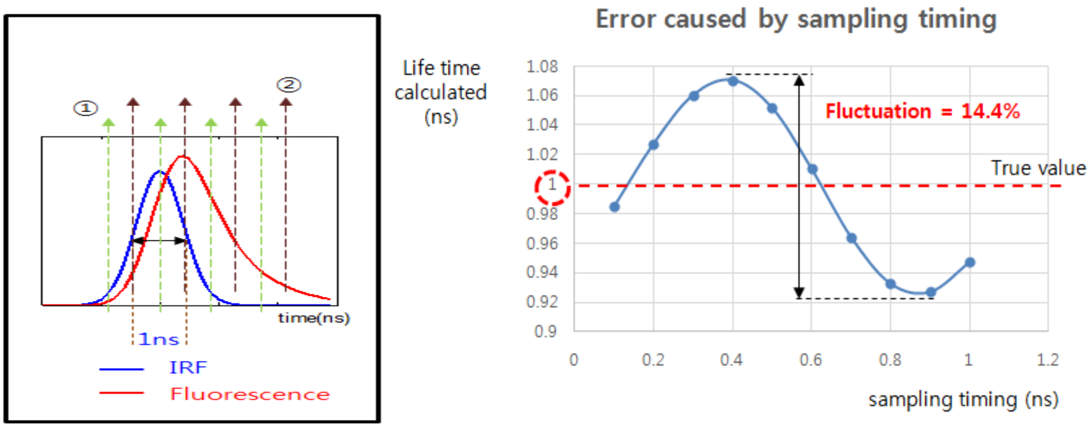

Figure 2. Error caused by sampling timing of a digitizer

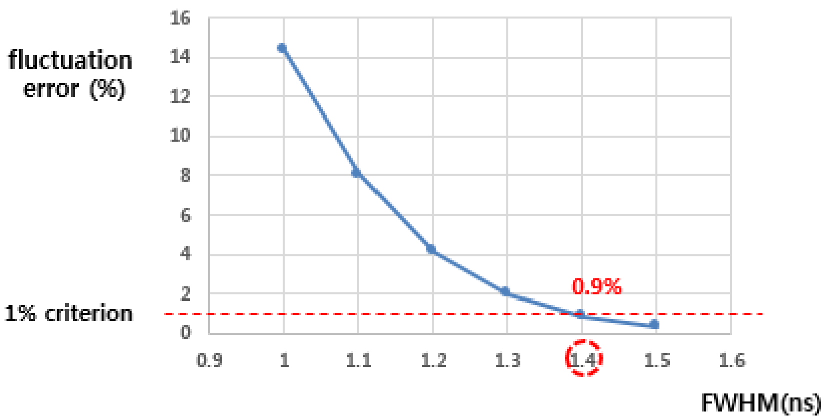

Figure 3. Fluctuation error caused by sampling timing is decreased as the signal gets broadened.

Simulated with dye with 1ns lifetime and 1GS/s digitizer and a signal of 1ns FWHM (Full Width Half Maximum), the error caused by sampling timing of digitizer is over 14 percent.

A signal of 1ns FWHM in the simulation is not presumed randomly. Pulse laser with 100ps FWHM and PMT with 0.9ns rise time will be used for experiment which constitutes around 1ns FWHM signal by RMS law [4].

The result showing a fluctuation error over 14% enforces the adoption of one of the following three options.

1. Increase the A/D sampling rate of a digitizer (impossible considering PCIe data transfer capability for real time processing)
2. Non-linear data processing to reconstruct the signal (impossible considering PC's con-current data processing capability)
3. Signal broadening with analogue low pass filter

While the first and second options cannot be selected on practical reason, third one (LPF) can be implemented with low cost. Considering the LPF implementation, two parameters should be evaluated. One is the fluctuation error caused by sampling timing mentioned above which gets smaller with a low cutoff frequency of LPF. Another is the accuracy of calculated lifetime which becomes degraded with a low cutoff frequency of LPF, because exceedingly broadened signal trespasses a following laser pulse window in time. As can be seen in Figure 3, the signal should be broadened up to 1.4ns FWHM under 1% accuracy criterion.

In the like manner, the minimum necessary FWHM of IRF signal for 1% lifetime accuracy error can be simulated with fluorophores of various lifetimes. In figure 4, the simulated results are charted. The point indicated by two blue dotted lines is the result from figure 3, which means with a fluorophore of 1ns lifetime, minimum 1.4 ns IRF FWHM is needed for 1% lifetime accuracy. The minimum required width is decreased as the lifetime of fluorophore is increased. If a IRF FWHM is placed under the curve in figure 4, the signal should be broadened to the upper area of the curve not to lose sufficient information under 1GS/s sampling rate condition.

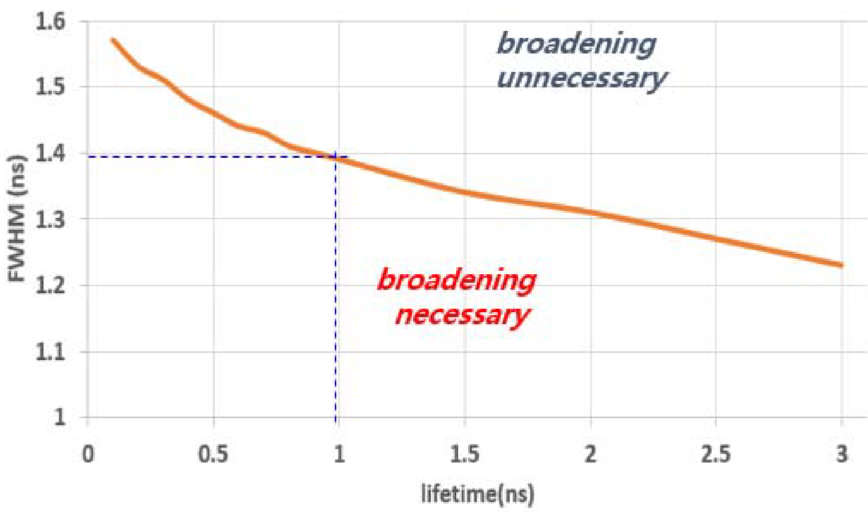

Figure 4. Minimum IRF FWHM with various lifetimes required for 1% lifetime accuracy error under 1GS/s sampling rate condition

4. Conclusion

With the help of a detector of high bandwidth, FLIM can be implemented on real time basis. To retain the sufficient information, however, the IRF signal should be adjusted to minimum necessary IRF because data transfer rate on PCIe is limited for real time FLIM system implementation. With a fluorophore of 1ns lifetime in use, minimum 1.4 ns FWHM IRF signal is necessary for 1% lifetime accuracy.

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