Measurements of Fluorescence Decay Time by the Frequency Domain Method

Enrico Gratton

Abstract Among the many contributions of Gregorio Weber to science and technology, the development of frequency domain technology in his lab in 1969 has caused a deep controversy, dividing scientist that will refuse using anything but the frequency domain approach to measure the fluorescence decay from the other camp that simply refuse anything but the time-correlated single photon counting (TCSPC) approach. Although at the time of the major contribution of Gregorio Weber and Richard Spencer in 1969, the TCSPC method was not yet invented, the basic controversy "frequency domain vs time domain" in the field continues today. We have made progress both in the scientific understanding and in describing the technical differences between the two approaches; still it is interesting how scientists continue to be divided. As for many of the contributions of Gregorio Weber that stirred controversy, I would like to mention a common theme of my conversations with Dr. Weber about refusing to follow a "god' and about the independence of the scientific thinking from "common believes" that ultimately slows scientific progress. In this chapter I would like to describe the scientific progress brought about by Weber's ideas in this specific "technological" area that should be judged by "pure" scientific analysis rather than by beliefs.

Keywords FLIMbox • Frequency domain • Gregorio Weber • Lifetime decay • Multifrequency • Parallel fluorometer

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E. Gratton (✉)
Laboratory for Fluorescence Dynamics, Department of Biomedical Engineering, University of California, Irvine, CL, USA
e-mail: egratton22@gmail.com

D.M. Jameson (ed.), *Perspectives on Fluorescence: A Tribute to Gregorio Weber*,
Springer Ser Fluoresc, DOI 10.1007/4243_2016_15,
© Springer International Publishing Switzerland 2016

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1 Time and Frequency Domain

Much has been said about measuring fluorescence lifetime decay in the time or in the frequency domain [1]. A common misconception is that the time-correlated single photon counting method (TCSPC) used in the time domain is more accurate and has better time resolution than the frequency domain approach [2]. From the statistics point of view the uncertainty of the TCSPC measurement depends on the number of photons collected which is considered the ultimate error possible. Also the dark noise, which are photons detected but not correlated with the decay, are minimal in TCSPC. However, the total number of photons collected in the TCSPC is not maximal. The TCSPC technique based on the TAC (time to amplitude converter) approach has a relatively large dead time and poor duty cycle which depends on the method used to measure the delay between the laser and the detection of a photon. Furthermore, when used in conjunction with high repetition rate lasers, which is the norm today, the entire laser period cannot be measured unless the duty cycle is reduced.

In the classical analog approach to frequency domain methods, the modulated detector photocurrent is directly used without the need of a discriminator, avoiding dead time and possibly detecting all photons including the dark counts from thermal emission of the dynodes [3]. In the classical frequency domain approach the detection and data processing are done in the analog mode. At a late stage the signal is converted in digital form after filtering for the desired light modulation frequency. Although it has been shown that the uncertainty in lifetime determination in the frequency domain method is also limited by the number of photons collected [4], a common criticism of the frequency domain method is that the system operates at a single frequency and it cannot resolve very short lifetimes. Another criticism is that the duty cycle is 50% or lower due to the modulation of the gain of the detector. A typical use of the frequency domain method is for cases where the photon flux is very high; a regime that the time domain method cannot keep up.

Clearly, both the TCSPC method and the traditional frequency domain method are far from being “ideal” and there is ample room for improvement in both camps.

From the mathematical point of view, the time domain or the frequency domain analyses of periodic signals are equivalent, being related by the Fourier transform. Therefore the differences between the two methods are a consequence of the way data are collected and processed rather than due to any profound reason. In this chapter we discuss the photon counting parallel frequency domain method, an evolution of Weber's original idea. This method has been previously proposed but never described in detail in the context of parallel frequency acquisition [5]. It is based on the "FLIMbox" approach which is an electronic circuit for producing the "frequency representation" of the fluorescence decay. Here we discuss the common features and the difference between the FLIMbox approach and the common TCSPC method. Importantly, in the FLIMbox method, a set of modulation frequencies are measured in parallel with a duty cycle of 100%.

One application we have in mind for this technique is fluorescence lifetime imaging microscopy (FLIM). This is a particularly demanding application since very few photons are collected at each pixel (typically less than 1,000) but a few bright pixels can have very large instantaneous counting rate. For this application the duty cycle of data acquisition must be as large as possible and the dead time for the detection of the photons emitted should be minimal to avoid saturation of the electronics at the bright pixels. Another application is for the collection of lifetime data at high speed as in stop flow experiments. In both applications we need to distinguish several molecular species at each spatial or temporal location.

In the following I will present a personal view of the evolution in frequency domain phase fluorometry up to today's most current instruments starting with the state of the field when I joined Gregorio Weber lab during my first visit in April–June, 1974.

2 The Frequency Domain Method

In retrospective, major technical developments in light sources and detectors found in current fluorometers were just starting at that time and fast repetition pulsed laser sources became available only after many more years. Perhaps more importantly, the focus in the field was in using the intrinsic fluorescence of proteins and several dyes available in the 1970s that were absorbing in the ultraviolet-blue region of the spectrum. Nanosecond pulsed lasers were not available in the protein absorption region and the nitrogen laser emitting at 337 nm was too slow and not far enough in the UV for protein work. The available light sources were nanosecond low pressure gas discharge sources or modulated high pressure arc lamps. Weber's lab in the 1970s was one of the places where great developments in fluorescence were occurring on all areas, instrumentation for steady state and time resolved fluorescence, landmark work for the development of methods for the measurement and application of fluorescence anisotropy, synthesis of novel fluorescence probes, and applications of fluorescence in many areas of biophysics, biochemistry, and biology. In Fig. 1 we reproduce the basic design of the optics and electronics of Spencer and Weber cross-correlation frequency domain fluorometer [4].

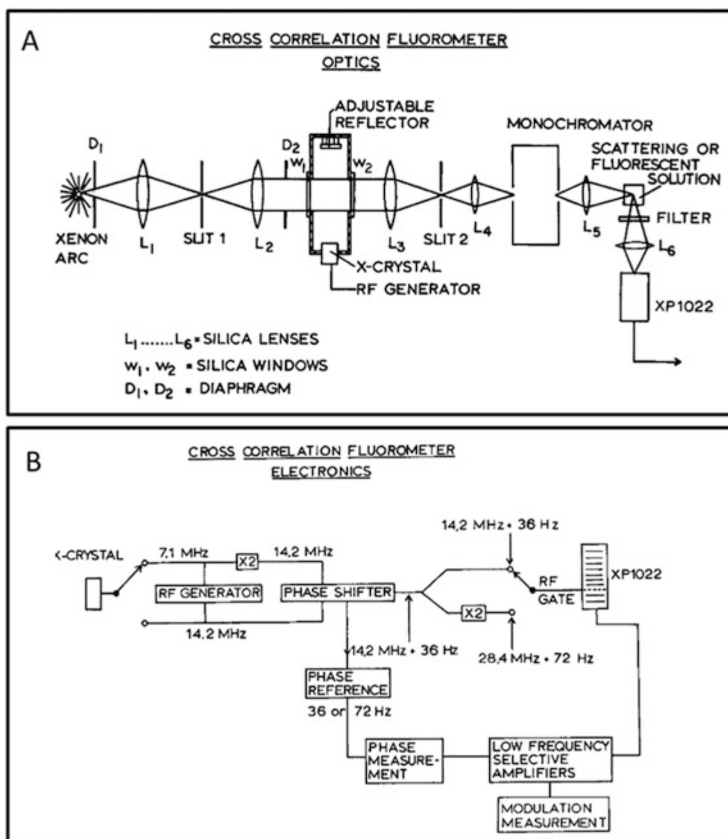

Fig. 1 Schematic of the original Spencer and Weber cross-correlation frequency domain fluorometer. (**a**) Schematic of the optical diagram. A high pressure xenon arc lamp is modulated by an ultrasonic Debye–Sears tank at high frequency generated by the X-crystal. The amplitude modulated light intensity is used to excite a fluorescence sample. (**b**) The X-crystal generator is shifted in frequency by the phase shifter and this shifted frequency is used to modulate the gain of the photomultiplier tube XP1022

During these years the idea of building a multifrequency phase fluorometer was a major discussion at the lab since in this type of future instrument the decay could be measured at many modulation frequencies. If available this instrument will advance the great innovation in the field at that time that was the frequency domain instrument invented in Weber's lab designed to modulate incoherent light sources at fixed frequencies, for example, at 14 and 28 MHz [4]. Of course, there were many predecessors of fluorometers using high frequency modulated light but one crucial innovation in Weber's work was the "cross-correlation" method first introduced in the Spencer and Weber instrument in which detection of the phase shift and of the modulation ratio was performed using the heterodyning or cross-correlation principle [4]. In this implementation of the technique, the detector gain was modulated

at a frequency which is slightly different from the frequency used to modulate the light source. Because of the detector gain modulation, this approach provided a maximum duty cycle of 50%. The gain modulation produces a difference frequency which is filtered and digitized for accurate phase and modulation determination. Using the heterodyning technique, phase shifts measurements as small as 0.1° were achieved [4]. For the purpose of comparing the phase histogram to the time bin histogram of the TCSPC, we note that 0.1° (which is the typical error in the phase value) in a period of 33 ns (30 MHz) corresponds to a time uncertainty of about 10 ps ($\delta\tau = \delta\phi/(2\pi f)$. Even for modern standards, the uncertainty obtained by frequency domain methods is remarkable since single exponential decay times of the order of one nanosecond could be measured with a precision of 10ps. However, one limitation of these early instruments was that complex exponential decays could not be resolved using only one or two light modulation frequencies. This explains the interest in developing the frequency domain technique but for multiple modulation frequencies.

The modulation frequency of the excitation light must be in a range that matches the rate of decay of the excited state [3, 6, 7]. For example, if the lifetime of the excited state is about 1ns, then the modulation frequency that has the highest sensitivity to changes around $\tau = 1$ ns must be in the range of 160 MHz:

$$f = \frac{1}{2\pi\tau} \approx 160\text{MHz}$$

Since it is technically difficult to measure the phase and modulation at very high frequencies and also the distortion of the waveform will render methods based on zero-crossing triggers subject to artifacts, the high frequency, at which the measurement is performed, is down converted to a very low frequency δf, generally on the order of 100–1,000 Hz where the signal is filtered and the phase and modulation of the emission with respect to the excitation is measured. The low frequency δf is called the heterodyning (or cross-correlation) frequency. The implementation of the cross-correlation method is achieved by generating two frequencies, one used for the modulation of the intensity of the light source at a frequency f and a second frequency at $f + \delta f$ used to modulate the gain of the detector where δf is in the 10–1,000 Hz range [4]. The detector in this case acts as a mixer by multiplying the signals at these two frequencies. The two signals are the modulated light impinging on the detector and the voltage used to modulate the gain of the detector. The output current of the detector is proportional to the light intensity times the gain of the detector. The product of two frequencies gives the sum and the difference of these frequencies. The sum is at very high frequency and it can be filtered from the difference δf using low pass frequency filters. Only the difference frequency is used.

For these early instruments, multifrequency referred to the possibility to sweep the frequency over a relatively large frequency range. True multifrequency was introduced later based on the principle of two locked frequency synthesizers [6] as shown in Fig. 2 where the light source was an argon ion laser.

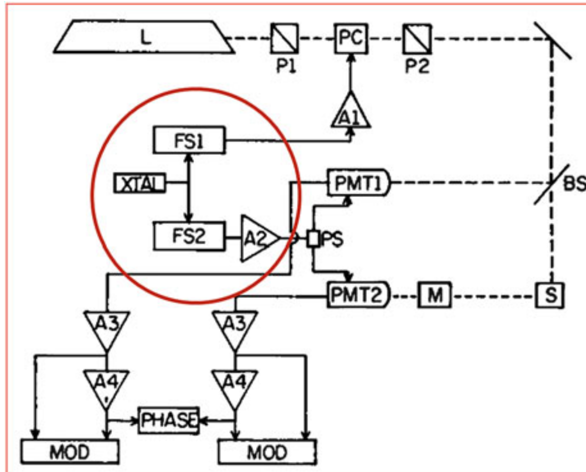

Fig. 2 Schematic and electronics of the Gratton–Limkeman multifrequency domain fluorometer. The *red circle* shows the two frequency-synthesizers FS1and FS2 that are phase locked to the same crystal. Similar to the Spencer and Weber design, the frequencyFS1 is used to modulate the light using a wide band Pockels cell and the frequency FS2 is used to modulated the gain of the PMT1 detectors

This technology is still used in commercial instruments. In the first multifrequency phase and modulation fluorometer [8], two phase-locked synthesizers generated the frequency f and $f + \delta f$. This approach provided a continuous range of modulation frequencies, limited only by the time response of the source or the detector. In this type of instrument, the operator selects the modulation frequencies generally in the range from 1 to 300 MHz and their number. The phase shift and the demodulation are measured for each frequency in a sequential fashion. An example of one of these commercial instruments is shown in Fig. 3 where the light source is a high pressure arc lamp emitting in the entire spectrum from 200 nm to above 800 nm.

A problem with these early instruments was that the zero-crossing detector used for the measurement of the phase was affected by the harmonic content of the low frequency signal. It was soon realized that the best measurement of the phase and modulation could be achieved if the signal at the heterodyning frequency was filtered from higher harmonics using a Fourier transform method. This method analyzes the harmonic content of the signal in separate orthogonal harmonics and required digitization of the low frequency signal [9]. The approach of analyzing the signal in Fourier components is still used today in most frequency domain instruments.

Fig. 3 Schematics of optics of the K1 multifrequency phase fluorometer built by ISS (Champaign, IL) circa 1987. A Pockels cell modulates the intensity from a high pressure xenon arc lamp. The gain of the 3 PMTs in this schematic are modulated at a slightly different frequency form the frequency used to modulate the intensity of the arc lamp

3 Pulsed Sources

However, another idea was discussed in Weber's lab at that time based on the availability of a new kind of light source: synchrotron radiation. Few synchrotrons were available in the 1980s but their characteristic time structure as well as their wavelength range was quite appealing for UV time resolved spectroscopy. The light emitted when the electrons circulating in a storage ring are deflected by a magnetic field is under the form of pulses at a relatively high repetition rate, which depends on the diameter of the storage ring and of the radiofrequency of operation used to accelerate the electrons. In an early design available at the time in Weber's lab (The Spencer design) the synchrotron radiation was used as a continuous light source to be modulated externally with a similar light modulator used in the original 2-frequency fluorometer in Weber's lab. Because of the experience in cross-correlation that we had acquired during these years we realized that we could cross-correlate directly with the pulsed light emitted at the synchrotron with our detection system and that no external modulators were required. The idea of using

the harmonic content of the synchrotron radiation was also discussed in a paper with Ricardo Lopez Delgado [10], but the synchronization with the radiofrequency of the synchrotron was our original idea. Based on the synchronization idea, we build the first multifrequency phase fluorometer that utilized fast repetition pulsed source, rather than sweeping the frequency at discrete values [11]. At this point, the way was paved for the future developments of the parallel phase fluorometer that makes use of the harmonic content of fast repetition lasers sources, in which all harmonic frequencies are collected and analyzed at the same time [12]. Still today, the synchronization with the source pulse train is the technique used with pulsed lasers including for the development of FLIM with multiphoton excitation. It is notable that in this paper of 1984 [11], it was discussed how to transform the analog acquisition in the instrument used at the synchrotron with a photon counting acquisition, a development that had to wait for about 20 years to be fully realized.

4 The Parallel Fluorometer Principle

As we described in the previous section, the conversion from the high frequency of the source repetition to the low frequency of the measurement is produced by the heterodyning process in which the output current of the detector which is at the frequency of light modulation is mixed (multiplied) by a slightly different frequency. If instead of using a sinusoidal signal to modulated the gain of the detector we use a signal which contains many harmonics, the multiplication generates not one, but a spectrum of harmonics that is the replica of the spectrum at high frequency.

In 1989 a “parallel multifrequency” fluorometer instrument was described by Feddersen et al. [9]. In this instrument, the excitation light is pulsed and the detector gain is modulated by a narrow pulse rather than by a sinusoidal signal. It was soon realized that although all harmonic frequencies were measured in parallel, the mixing scheme obtained by modulating the gain of the detector in the parallel multifrequency instrument was very ineffective. In fact, the operation of “pulsing” the detector gain is equivalent to turning the detector “on” for a very brief period, resulting in substantial decrease of the detector duty cycle. Feddersen et al. discussed this limitation [9] and they suggested keeping the detector “on” for 1/16 of the source period, providing about 16 frequencies in parallel. It was demonstrated that this is the optimal duty cycle that maximizes the speed of data acquisition and minimizes losses arising from turning “on” and ‘off” the detector [9]. This scheme has been used ever since in the so-called parallel frequency domain lifetime instruments. Table 1 shows the evolution of various frequency domain phase fluorometers developed in my lab.

In the “original” parallel fluorometer system described in 1989 by Feddersen et al. [9] the advantages of multifrequency acquisition were discussed vis-a-vis the reduction of the duty cycle needed in the analog system to achieve acquisition of several harmonic frequencies simultaneously. In the original parallel acquisition

Table 1 Parallel frequency domain history

Parallel fluorometer (1986)	Digital mixing (2000)	First FLIMBox (2007)	Parallel-FLIMBox (2009)
Analog mixing with pulsed external generator. Mixing using the detector gain	Digital mixing with external square wave generator. Detector gain not modulated	Digital mixing. Internal generator modulate signal after detector @48 MHz	Digital mixing. Internal generator produce pulsed modulating signal @10, 20 MHz
Duty cycle depends on harmonics. 6% for $n = 16$	Duty cycle is 50%	Duty cycle is 100%	Duty cycle is 100%
Parallel frequency domain lifetime instruments	FCS. System not ready to implement parallel acquisition	FLIM. System not ready to implement parallel acquisition	Parallel digital frequency domain for curvette, FLIM, and FCS
One input channel	One input channel	Two input channel	Up to four input channel
Average acquisition time takes several minutes			Average acquisition time takes seconds
		Frame synchronization and saturation problems. Limited number of windows, design instable	Flexible synchronization. Saturation control. Eight and 16 windows available. Stable design

system, it was discussed that a reduction of duty cycle to about 6.25% was giving optimal performance. Several commercial systems were produced to achieve the parallel frequency acquisition in which the duty cycle was sacrificed at the expensed of the harmonic content. With the invention of the FLIMbox approach this limitation was removed since the FLIMbox has 100% duty cycle.

5 Photon Histograms

From the point of view of the construction of the decay histogram, the TCSPC and the FLIMbox essentially do the "same thing" but with some notable technical differences. In the TCSPC the time axis is divided in "time bins" typically 1024 time channels or more. If the total time range is 12.5 ns (for a laser operating at 80 MHz repetition rate), each channel width (in time) assuming that we collect 256 time bins is about 49ps. In FLIM microscopy, due to the limited number of photons collected per pixel, the number of channels is generally reduced to 256 or 128 since the total counts per pixels is no more than 1,000 counts (Fig. 4).

The "same" concept of dividing the photons time of arrival in bins is also used in the FLIMbox, where the laser repetition period is divided in 256 phase intervals, but synchronized with the period of the laser (Fig. 5). For a laser operating at 80 MHz,

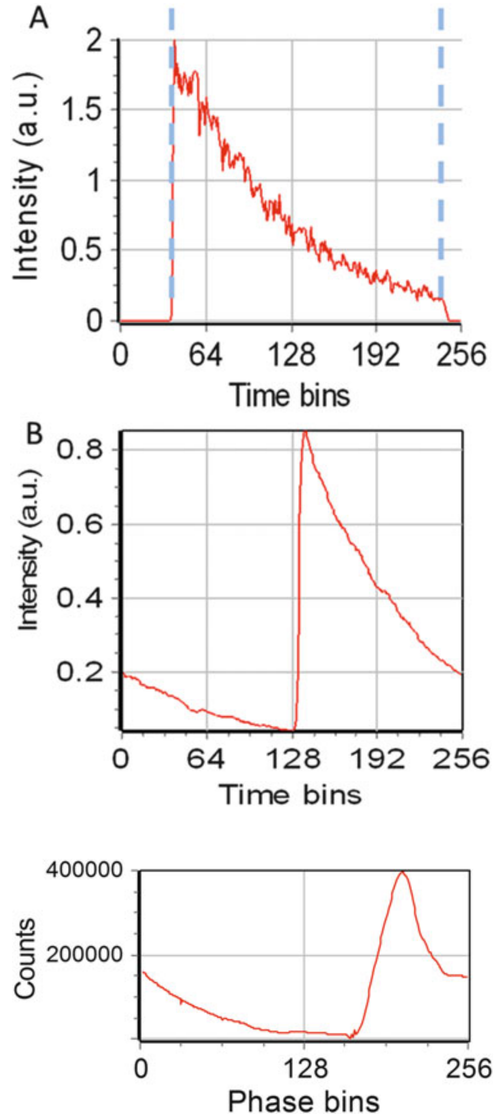

Fig. 4 Demonstration of the useful range of the Becker and Hickl 830 card. (**a**) For a 80 MHz repetition frequency laser using 50 ns total range at a TAC gain of 4 the total range is 12.5 ns. However the useful range is from channel 50 to 250 out of 256 (78%), and the time record is incomplete since the entire period of the laser is not used. (**b**) Using a TAC gain of 2 the total range is 25 ns of which a portion of 12.5 ns could be extracted. In this case the effective useful range is 50% but the record is complete since we cover an entire laser period

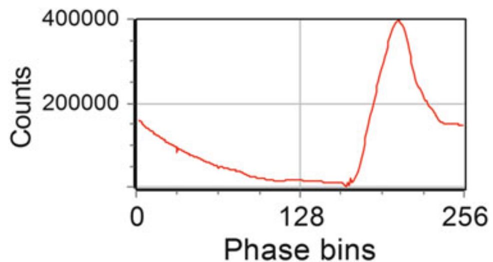

Fig. 5 The photon counting phase histogram produced by the FLIMbox. In this example, the laser repetition period is 12.5 ns. This interval is divided into 256 bins. The entire laser period is collected at 100% duty cycle

dividing the period in 256 parts results in a time bin of 48.9 ps or, referring to the period of 360°, each phase bin is 1.4°.

So the technical difference with respect to the TCSPC time domain approach is that the FLIMbox approach uses always 256 phase bins, it covers the entire laser repetition period and the bins size and phase are synchronized with the laser. In the TCSPC, generally the time range is set to be a part of the period, the size of the bin is dependent on an internal clock independent of the laser period and the phase of the bin sequence is synchronized with the laser by the start–stop clock of the Time

to Amplitude Converter (TAC) converter. In the frequency domain method, the phase bin size (in terms of time) is directly derived from the rep rate of the laser so that the time calibration depends on the laser repetition frequency.

Of course, if the laser repetition rate becomes much slower, the number of phase bins could be increased in the FLIMbox circuit. In few words, the "basic" difference between the FLIMbox and the TCSPC is the way the time bins are generated. In the FLIMbox, the time bins are derived by division of the laser repetition period. In the TCSPC the time bins are produced independently of the laser rep rate as they are determined by sampling a linear ramp using the TAC principle. This lack of synchronization with the laser rep rate results in several problems when high rep rate lasers are used, which is today the rule for FLIM.

5.1 Dead Time

The dead time of the FLIMbox technique is due to the discriminator rather than the internal FLIMbox circuit. The discriminator dead time used in this work is about 7 ns. The FLIMbox has two totally independent inputs so that two channels can be used simultaneously without loss of photons. The TCSPC dead time depends on the recovery time of the TAC. According to manufactures specifications, both PicoQuant and B&H quote figures in the order of 100–120 ns dead time, depending on the model of their data acquisition card. At high counting rates, this large dead time can strongly affect the linearity of the data collection in terms of time and intensity linearity.

5.2 Duty Cycle

This is an important difference between the FLIMbox frequency domain approach and the TCSPC method. The FLIMbox is always active, so that photons are collected irrespective of their time of arrival. In the TCSPC the time axis is limited to a percentage of the total period of the laser pulse. For example, if the laser repeats at 12.5 ns (typical of the Ti:Sa laser at 80 MHz) the usable range is generally on the order of 10 ns only (about 80% of the total range) as shown in Fig. 4a. However, in the tail of the distribution at longer delay times some photons are lost. To fix this problem, the TAC range could be set to collect data at twice the laser period, so that the total TAC range is 25 ns and it at least one complete period of the laser (Fig. 4b). In this case the losses are in terms of photons falling outside the range of the measurement and they can be substantial since part of the lost range occurs at times when the photon flux is large. If the losses are minimized using the 12.5 ns range (in this example), evidently the entire decay range is not available. The fitting routines will only use a smaller range with consequences about the accuracy of fitting longer lifetime components which contribute more in the lost region. Also,

since the entire period is not available, the phasor transformation cannot be done correctly, unless some assumptions are introduced about the behavior of the decay in the region that is not collected. If data are collected on a longer period (for example, 25 ns and only 12.5 ns are used for analysis) the duty cycle is only 50% (instead of 80%). However, the advantage of reducing the duty cycle is that an entire period is available; the phasor transformation can be used without assumptions about the missing parts and longer lifetime components could be properly analyzed.

Based on the dead time and duty cycle considerations, it appears that with the FLIMbox approach the frequency domain method is more effective than the TCSPC.

5.3 Time Resolution

As mentioned in the introduction, a common belief is that the TCSPC has better time resolution than the frequency domain approach. If time resolution refers to the pulse-to-pulse separation, this is true since the TCSPC uses a very small bin time. However, in the case of a "lifetime," which is an exponential process, photons are necessarily spread among bins. In this case the time resolution depends primarily on the number of photons collected in the decay curve. The FLIMbox is very efficient and maximize the photons collected.

6 Photon Counting Multifrequency Parallel Fluorometer

The FLIMbox concept is now at the second generation stage due to the availability of high frequency digital electronics in new FPGAs chips as well as the availability of programming languages for the FPGA that makes the code developed for a given chip, transportable to a next generation chip. We show in Fig. 6 a comparison of the bandwidth available in a current chip (FPGA Spartan 6, Xilinx) compared to the original FLIMB box chip (Spartan 3, Xilinx).

The first zeros of the original FLIMbox acquisition algorithm were at 160 MHz, 320 Mhz, repeating each even harmonics. Since the amplitude of these frequencies is zero, they are removed from the analysis. Figure 6 (green lines) shows that there're at least 20–30 harmonics that can be collected using the new generation FPGA. The high frequency limit is actually given by the lifetime of the probe. Fluorescein at basic pH has a lifetime of 4.04 ns. The absolute modulation of this sample is about 3% at 200 MHz.

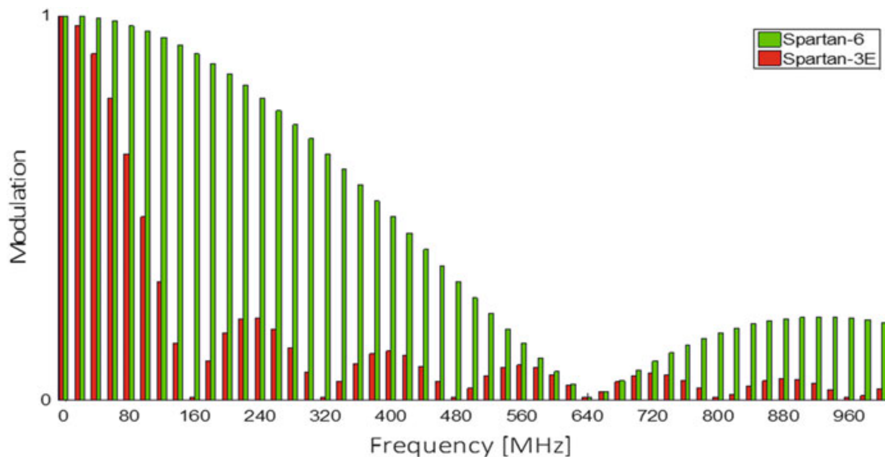

Fig. 6 Harmonic content of the original FLIMbox based on the Spart3 chip (in *red*) and of the new generation FLIMbox based on the Spartn6 chip. The new generation FLIMbox has a very good modulation (about 30%) in the GHz region

7 Conclusions

With the advent of the very inexpensive digital electronics available in current FPGA chips, a new technology is now available for the measurement of fluorescence lifetime decays at high speed and in many channels simultaneously. Since the frequency of operation of the FPGA chip is continuously increasing, it is possible that this technology on day will be used for all time domain or frequency domain instruments. Clearly, using this technology, the time and frequency domain is a distinction that is not needed any more. This result was anticipated in many of the discussions I had with Gregorio Weber starting in 1974 and continued until Dr Weber was alive.

The maximum frequency obtained in the FLIMbox is now limited to about 1GHz by the particular chip and technology we are using (Spartan 6, XILINX). However, faster and large chips are already in the market. It is the moment to further develop the digital technology so that the user can just choose which representation of the decay is preferable. At the inner core of the technology there should be no differences. The representation the user wants to see is determined by a click in the software.

The FLIMbox design can be synchronized with lasers that are intrinsically modulated or it can generate a frequency to amplitude modulate a laser diode or LED. In the most current implementation it provides up to 16 independent input channels, it has a saturation feedback control to avoid any time information loss, and it is only limited by the number of photons collected rather than by the sampling window implementation scheme.

This new design, in summary, is very stable, has very low power requirements, has high frequency capability and higher precision, and allows the multi exponential analysis to be performed on almost every photon detection based acquisition system (imaging microscopy, FLIM, FCS, multifrequency fluorometer, and tissue imaging).

Acknowledgments Part of the work described in this chapter was supported by the following grants NIH P41-GM103540 and NIH P50-GM076516

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