Theoretical investigation of the signal-to-noise ratio for different fluorescence lifetime imaging techniques

K. Carlsson[*a] and J. Philip[**b]
[a]Biomedical & X-ray physics, Royal Institute of Technology, SCFAB, SE-106 91 Stockholm, Sweden
[b]Dept. of Mathematics, Royal Institute of Technology, SE-100 44 Stockholm, Sweden.

ABSTRACT

Using Monte-Carlo methods, we have investigated the signal-to-noise ratio obtainable for different fluorescence lifetime imaging methods. Quantum noise limited performance and mono-exponential decays were assumed. We have also investigated the importance of parameter choice and implementation for the different methods. In addition, our simulations were in many cases compared with analytical theoretical investigations. The results from the simulations proved to be in good agreement with the theoretical results. It was found that all the investigated lifetime imaging methods have the potential to produce a high signal-to-noise ratio, but careful attention must be paid to implementation method and parameter choice in order to get optimal results.

Keywords: signal-to-noise ratio, lifetime imaging, fluorescence

1. INTRODUCTION

In recent years fluorescence lifetime imaging has found widespread use in the biomedical field. Its many uses include qualitative and quantitative imaging of ion concentrations such as Ca^{2+} and pH[1-3]. In many cases lifetime imaging is used in combination with light microscopy, both confocal and widefield[4-21]. Several methods can be used for measuring fluorescence lifetime (denoted by τ in this text). One possibility is to record the time decay function of the fluorescence intensity after excitation with a short pulse of light[14]. Instead of recording the entire decay function, the integrated light intensity in two or more time windows can be recorded and analyzed (time-gating)[22]. A third possibility is to illuminate the fluorescent sample with intensity-modulated (usually sinusoidal) light instead of using pulse excitation. In this case it is possible to obtain the fluorescence lifetime by measuring the phase-shift or demodulation of the fluorescent light[1].

An important aspect of fluorescence imaging is the signal-to-noise ratio (SNR). This is especially true in microscopy, where the total number of photons recorded per pixel is often very low (on the order of 100-1000). As a result, photon quantum noise limits the quality of the recorded information. An obvious remedy would be to collect more photons, but this often results in severe photobleaching, excessive recording times, and blurred images due to specimen movement. It is therefore important that the signals are recorded and processed in an efficient way, so that losses in SNR can be kept to a minimum.

Photon quantum noise is described by Poisson statistics. In an ideal photon-counting situation with an expectation value of $\overline{N}$, the standard deviation (= RMS noise) for repeated measurements would be $\sqrt{\overline{N}}$. Thus, the SNR = $\frac{\overline{N}}{\sqrt{\overline{N}}} = \sqrt{\overline{N}}$.

This is the ultimate SNR we can ever expect in a measurement employing photons. In fluorescence lifetime imaging, as well as in some other types of imaging, several measurements are combined to produce a result. This process tends to reduce the SNR, and the amount of reduction is a measure of the performance of the method. In analogy with Draaijer et al.[11], we will use a figure of merit, F, which is defined as the SNR in an ideal intensity measurement (i.e. $\sqrt{\overline{N}}$) divided by the SNR for the method under study. This means that high F-values represent poor performance, and the

[*] kjell.carlsson@biox.kth.se; phone +46 8 5537 8132; fax +46 8 5537 8466; http://www.biox.kth.se
[**] johan@math.kth.se; phone +46 8 790 6693; fax +46 8 790 7022; http://www.math.kth.se

closer to unity we get the better the performance will be. It should be noted that in order to compensate for a loss of a factor of two in F, it is necessary to collect four times as many photons.

The subject of SNR in lifetime imaging has been investigated in previous publications[11, 20-23]. These investigations include some implementations of the above-mentioned lifetime imaging techniques. However, many interesting possibilities were not investigated, and the optimum implementations were not always studied. Also, clear guidelines for parameter choice are needed. We therefore undertook the present study, employing Monte-Carlo simulation techniques, in order to quantify the SNR for different lifetime imaging techniques. In addition, we wanted to obtain guidelines for parameter choice. In some cases we also compared different implementations of the same technique. The simulated results were compared with analytical theoretical results, in cases where such results were available. Only photon quantum noise was considered, although in reality also other types of noise are present (electronic noise, multiplication noise in photomultipliers etc.). These types of noise are present in all imaging techniques, but often constitute a minor part of the total noise. For simplicity, we have also limited our investigations to mono-exponential decays.

It has been shown in previous investigations that techniques exist that can theoretically give F-values very close to unity, i.e. near-perfect utilization of the recorded photons. This does not mean that other and less well-performing methods are uninteresting. In reality one often has to make compromises, taking into account factors such as recording speed, cost and practical implementation when deciding on a suitable method. Such issues will not be discussed in detail here. We will mainly consider the performance potential and optimum parameter choice (where applicable) for different techniques, given an equal number of detected photons.

2. MONTE-CARLO SIMULATIONS

Curves of the fluorescence emission as a function of time were used in the simulations. An example of such a curve, for the case of Dirac pulse excitation, is given in Fig. 1. Each period of the fluorescence emission curve was divided into a large number of short intervals Δt. For each Δt, we used a pseudo random number to determine if a fluorescence photon shall be generated. The probability for this to occur is proportional to Δt times the intensity of the fluorescence emission curve. The size of Δt was chosen so small that the probability of a fluorescence photon being generated in Δt was less than .01. In this way, we kept the probability of two photons occurring in Δt below .0001. The simulated train of fluorescence photons was then processed in different ways depending on the lifetime imaging method studied. When simulating timegating, the number of photons in each of two consecutive time windows was used for the estimation. In phase fluorometry, the pulse train was multiplied by signals representing the local oscillators in lock-in amplifiers, or the amplification in an image intensifier tube. The resulting signals were then low-pass filtered and used for estimating τ. For each case studied, this process was repeated 500-1000 times and the standard deviation calculated.

Simulations were carried out for two light levels, corresponding to averages of 240 and 2400 recorded photons per pixel. These values are representative of the situation in, say, confocal microscopy when the fluorescence brightness is normal and excellent. In the figures, only simulations corresponding to $\overline{N} = 2400$ are presented. Simulations and theoretical predictions for the case $\overline{N} = 240$ were very similar, except in some extreme cases with SNR < 3. Such low SNR values are not so interesting, because the image quality will then be extremely poor. Therefore, we feel that the figures presented are good representations of the F-numbers that will be obtained under widely different light conditions, excluding only extremely poor ones.

3. SNR FOR DIFFERENT LIFETIME IMAGING TECHNIQUES

In this section we will compare the SNR performance for the three techniques for measuring lifetime that were mentioned in the introduction. These techniques are 1) Recording of the time decay function of the fluorescence intensity after excitation with a short pulse of light, 2) Measurement of the integrated light intensity in two or more time windows after pulse excitation (timegating), 3) Illumination with modulated light and measurement of the phase-shift of the fluorescent light (phase fluorometry).

3.1 Recording of the time-decay function

Fluorescence light decay after pulse excitation can be recorded using, for example, time-correlated single photon counting (TCSPC)[24]. We did not include this technique in our study, because its SNR performance has already been investigated by another group[23]. It was found that F-values arbitrarily close to unity could be reached, provided that an appropriate number of recording channels were used, and that these channels were spread over a suitable time window. For example, a system with 100 channels can produce F-values below 1.1 for a wide range of different lifetimes. When fewer channels are used, the range of lifetimes that produce good (i.e. low) F-values will be smaller. If the number of channels is less than 8, F-values below 1.1 can no longer be obtained.

3.2 Timegating

Also timegating performance has been reported in previous publications by other groups[11, 22]. Ref. 22 includes an analytical theoretical investigation of the performance, valid for low noise levels. In our simulations of timegating, we found that these analytical results are a good approximation down to SNR levels below 10. In order to obtain optimum SNR the gate width should be 2.5τ, resulting in an F-value of 1.5, Fig. 2. Even better performance has recently been reported when using more than two gating windows[21], albeit at the price of increased complexity. This improvement is to be expected, since for a sufficiently large number of gating windows the difference between TCSPC and timegating disappears.

3.3 Phase fluorometry

Investigation of phase fluorometry performance will form the major part of this text (we did not include demodulation measurements in this study). Lifetime imaging according to this method is quite common, and can be implemented by using modulated excitation light and an HF-modulated image intensifier[1]. By collecting three different images, two with different phase angles for the modulation and one with the modulation off, a lifetime image can be calculated. It has been reported elsewhere that the best F-value for this method when using sine-modulated light is approximately 6 (optimum parameters were not given)[11]. We have simulated this case, and the result is seen in Fig. 3. In our simulations we found that an optimum F-value of 10 was obtained at a modulation frequency of $0.1/\tau$. At present we do not have an explanation for the discrepancy between the results of our investigation and the one previously reported. It has also been reported that by using a train of Dirac pulses, rather than sine-modulation of the light, an F-value of approximately 1.5 can be obtained[11]. Also in this case our simulations gave a different result. We obtained an optimum F-value of 4.3 at a modulation frequency of $0.1/\tau$, Fig. 4. In the simulations we assumed that 2400 photons per pixel were recorded for each of the three images recorded. One could argue that 2400/3 photons per pixel and image should be used, which would result in even higher F-values.

There are other methods for collecting and processing the data when using phase fluorometry. By using lock-in detection technique, rather than a modulated image intensifier tube, it is possible to simultaneously record two images with different phase settings[20]. Furthermore, there is no need to record an image with the modulation turned off. We have previously presented an analytical theory for this case, yielding an F-value of 3.7 for sine-modulated light when using optimum parameter settings[25]. In the present study we have performed Monte-Carlo simulation of this method, and the results were in good agreement with the analytical theory, Fig. 5. Although still quite a bit short of the ideal value of unity, these results represent an improvement in performance compared with using an image intensifier. Our investigations also show that by using Dirac pulse modulation of the light, F-values arbitrarily close to unity can be obtained with lock-in detection, Fig. 6. For pulse-repetition frequencies $< 0.1/\tau$ the F-value will be less than 1.2.

Trains of (approximately) Dirac pulses are expensive to produce, and it is interesting to investigate the use of other waveforms in phase fluorometry. We have therefore studied square-wave modulation with different duty cycles in combination with lock-in detection. Compared with sine-wave modulation, square-waves can produce significantly better results. In Fig. 7, the F-value as a function of modulation frequency is illustrated for different duty cycles. In analogy with the sine-modulated case, the F-value has a minimum at a certain modulation frequency (but the minimum is rather wide, and therefore the choice of frequency is not critical). From Fig. 7 we can also see that this minimum will occur at slightly different frequencies for different duty cycles. For a duty cycle of 0.5, the optimum F-value, 2.9, is obtained at a frequency of $0.11/\tau$, whereas a duty cycle of 0.1 will give an optimum F–value of 1.2 in a rather wide frequency region around $0.07/\tau$. Lower duty cycles will produce even better results. This behavior is to be expected, because when the duty-cycle approaches zero the pulses will approach Dirac pulses. In Fig. 8 the F-value as a function of duty cycle is illustrated for a modulation frequency of $0.1/\tau$. This is not exactly the optimal frequency for all duty cycles, but we nevertheless get a general impression of the variation o f F-value with duty cycle.

Although quite nice results can be obtained with square-wave light modulation, one can still argue that the steps in a square-wave pulse represent very high frequencies. This calls for fast (and expensive) modulators in combination with a CW laser, or a pulsed laser with a totally unrealistic pulse shape. We therefore investigated what happens if the pulses have a more rounded shape. The excitation used in this case consisted of square-waves with duty cycle 0.2, convolved with a kernel consisting of one period of a $\cos^2$ function with a width of $1/16^{th}$ of the period for the square-wave. This produced quite smooth, somewhat "Gaussian-like," pulses with a FWHM of 0.2, Fig. 9. The F-value as a function of pulse repetition frequency is shown in Fig. 10 for pulses of this type. Compared with using square-wave excitation with a duty cycle of 0.2, the F-values are nearly the same. At optimum frequency, the F-value is 1.55, i.e. only 7% worse than for square pulses. This suggests that the pulse shape is not critical.

A possibility that we have also studied is to use sinusoidal excitation in combination with non-sinusoidal modulation of the local oscillators in the lock-in amplifiers. This has previously been investigated for intensity measurements[26]. Simulations of lifetime imaging for this case showed that a square-wave local oscillator signal with 50% duty cycle produced higher (i.e. worse) F-values than when using sine-waves. At the optimum modulation frequency, an F-value of 4.1 was obtained when using square-waves. The corresponding value for sinusoidal local oscillator was 3.7. Duty cycles other than 0.5 were also tried, but this resulted in even worse performance. There are, of course, many other possibilities to explore concerning local oscillator signals, but the results so far are not encouraging.

When phase fluorometry is used, two images with different phase angle settings must be recorded. In most cases we have studied (the only exception being lock-in detection with square-wave local oscillator) both theory and simulations show that the choice of these phase angles does not influence the F-value. In a practical imaging situation, however, it is recommended to select phase angles that produce sufficiently large and non-negative pixel values in order to simplify digital storage and processing.

An important thing to keep in mind is that all results refer to ideal situations where the excitation light consists of infinitely short (Dirac) pulses, or 100% modulated sine- or square-waves. This is not the case in reality, of course, and therefore the SNR is expected to be lower. Just to give an example of the importance of these things, we illustrate how the F-value depends on the degree of modulation for sine-modulated excitation in phase fluorometry, Fig. 11.

In Table 1 we summarize the findings in this and previous studies concerning the SNR performance for different lifetime imaging methods. It should be noted that small F-values mean good performance.

Table 1.

Lifetime imaging method	**Performance (F-value)**	**Parameter choice**
Time-correlated single photon counting	< 1.1	Ref. 23
Timegating:		
2 windows of equal width	1.5	Window width 2.5τ
8 windows of unequal width	1.2	Ref. 21
Phase fluorometry:		
Image intensifier: sine-wave excitation	10	Exc. modulation freq. = $0.1/\tau$
Image intensifier: Dirac pulse train exc.	4.3	Exc. modulation freq. = $0.1/\tau$
Lock-in: sine-wave local osc./sine-wave exc.	3.7	Exc. modulation freq. = $0.1/\tau$
Lock-in: sine-wave local osc./square-wave exc.	1.2	Exc. mod. freq. = $0.1/\tau$, duty cycle = 0.1
Lock-in: sine-wave local osc./Dirac pulse train exc.	< 1.1	Exc. modulation freq. < $0.1/\tau$
Lock-in: square-wave local osc./sine-wave exc.	4.1	Exc. modulation freq. = $0.1/\tau$

4. DISCUSSION

Extremely short laser pulses tend to be expensive, and the same is true for fast light modulators and electronics. It is therefore an advantage if the requirements in this respect can be relaxed. It is a common misconception that, for example, in phase fluorometry, the modulation frequency must be of the same order of magnitude as $1/\tau$. As seen from the results above, this is not true. A good frequency choice is $0.1/\tau$. This means that we can relax the requirements considerably concerning the bandwidth requirements. A typical fluorophore lifetime of, say, 3 ns, gives an optimum modulation frequency of approximately 30 MHz. Building modulators and electronics for this frequency region is considerably easier than for 300 MHz, so we are fortunate in this respect. When using square-wave (or more rounded pulse-shape) excitation in phase fluorometry, we have seen that a duty-cycle of 0.1 produces near ideal results. With a fluorophore lifetime of 3 ns, and a pulse repetition frequency of $0.1/\tau$, this requires a pulse width of approximately 3 ns. Thus, there is no need for extremely short pulses in the femto- or picosecond range.

In biological preparations, the fluorescence lifetime will, in general, be different in different parts of the specimen (this is the information we want to record!). Consequently parameters such as the window width in timegating, or the modulation frequency in phase fluorometry, cannot be optimized for all the lifetimes found in different specimen parts simultaneously. Therefore, the F-value should not increase too quickly as we move away from the optimum parameter setting. As we can see from the figures, this is not a great problem. Variations in τ by a factor of two or more generally produce only minor variations in F-value.

ACKNOWLEDGMENT

The Swedish Research Council for Engineering Sciences has financially supported this work.

REFERENCES

1. J.R. Lakowicz and K.W. Berndt, “Lifetime-selective fluorescence imaging using an rf phase-sensitive camera,” Rev. Sci. Instr. **62**, pp. 1727-1734, 1991
2. J.R. Lakowicz and H. Szmacinski, “Fluorescence lifetime-based sensing of pH, Ca^{2+}, K^{+} and glucose,” *Sens. Act. B* **11**, pp. 133-143, 1993.
3. G. Krishnamoorthy and A. Srivastava, “Intracellular dynamics seen through time-resolved fluorescence microscopy,” *Curr. Sci.* **72**, pp. 835-845, 1997.
4. C.G. Morgan, A.C. Mitchell, and J.G. Murray, “Nanosecond time-resolved fluorescence microscopy: principles and practice,” *Trans. Roy. Microsc. Soc.* **1**, pp. 463-466, 1990.
5. E.P. Buurman, R. Sanders, A. Draaijer, H.C. Gerritsen, J.J.F.van Veen, P.M. Houpt, and Y.K. Levine, “Fluorescence lifetime imaging using a confocal laser scanning microscope, *Scanning* **14**, pp. 155-159, 1992.
6. D.W. Piston, D.R. Sandison, and W.W. Webb, “Time-resolved fluorescence imaging and background rejection by two-photon excitation in laser scanning microscopy,” *Proc. SPIE* **1640**, pp. 379-389, 1992.
7. T.W.J. Gadella, T.M. Jovin, and R.M. Clegg, “Fluorescence lifetime imaging microscopy (FLIM): spatial resolution of microstructures on the nanosecond timescale,” *Biophys. Chem.* **48**, pp. 221-239, 1993.
8. T. Oida, Y. Sako, and A. Kusumi, “Fluorescence lifetime imaging microscopy (flimscopy),” *Biophys. J.* **64**, pp. 676-685, 1993.
9. C.J.R. van der Oord, H.C. Gerritsen, F.F.G. Rommerts, D.A. Shaw, I.H. Munro, and Y.K. Levine, “Micro-volume time-resolved fluorescence spectroscopy using a confocal synchrotron radiation microscope,” *Appl. Spectrosc.* **49**, pp. 1469-1473, 1995.
10. P.T.C. So, T. French, W.M. Yu, K.M.Berland, C.Y. Dong, and E. Gratton, “Time-resolved fluorescence microscopy using two-photon excitation,” *Bioimaging* **3**, pp. 49-63, 1995.
11. A. Draaijer, R. Sanders, and H.C. Gerritsen, ”Fluorescence lifetime imaging, a new tool in confocal microscopy,” in *Handbok of Biological Confocal Microscopy* (ed. J Pawley), pp. 491-505, Plenum, New York, 1995.

12. K. König, P.T.C. So, W.W. Mantulin, B.J. Tromberg, and E. Gratton, " Two -photon excited lifetime imaging of autofluorescence in cells during UVA and NIR photostress," *J. Microsc.* **183**, pp. 197-204, 1996.
13. R. Müller, C. Zander, M. Sauer, M. Deimel, D. -S. Ko, S. Siebert, J. Arden-Jacob, G. Deltau, N.J. Marx, K.H. Drexhage, and J. Wolfrum, "Time-resolved identification of single molecules in solution with a pulsed semiconductor diode laser, " *Chem. Phys. Lett*. **262**, pp. 716-722, 1996.
14. H. Brismar and B. Ulfhake, "Fluorescence lifetime measurements in confocal microscopy of neurons labeled with multiple fluorophores," *Nature Biotechn.* **15**, pp. 373-377, 1997.
15. A.H. Buist, M. Müller, E.J. Gijsbers, G,J. Brakenhoff, T.S. Sosnowski, T.B. Norris, and J. Squier, "Double-pulse fluorescence lifetime measurements, " *J. Microsc.* **186**, pp. 212-220, 1997.
16. T. French, P.T.C. So, D.J. Weaver, T. Coelho-Sampaio, and E. Gratton, "Two-photon fluorescence lifetime imaging microscopy of macrophage-mediated antigen processing," *J. Microsc.* **185**, pp. 339-353, 1997.
17. M. Sauer, J. Arden-Jacob, K.H. Drexhage, F. Göbel, U. Lieberwirth, K. Mühlegger, R. Müller, J. Wolfrum, and C. Zander, "Time-resolved identification of individual mononucleotide molecules in aqueous solution with pulsed semiconductor lasers," *Bioimaging* **6**, pp. 14-24, 1998.
18. J. Sytsma, J.M. Vroom, C.J. de Grauw, and H.C. Gerritsen, "Time-gated fluorescence lifetime imaging and microvolume spectroscopy using two-photon excitation," *J. Microsc.* **191**, pp. 39-51, 1998.
19. A. Squire and P.I.H. Bastiaens, "Three dimensional image restoration in fluorescence lifetime imaging microscopy," *J. Microsc.* **193**, pp. 36-49, 1999.
20. K. Carlsson, A. Liljeborg, R.M. Andersson, and H. Brismar, "Confocal pH imaging of microscopic specimens using fluorescence lifetimes and phase fluorometry: influence of parameter choice on system performance," *J. Microsc.* **199**, pp. 106-114, 2000.
21. C.J. de Grauw and H.C. Gerritsen, "Multiple time-gate module for fluorescence lifetime imaging," *Appl. Spectrosc.* **55**, pp. 670-678, 2001.
22. R.M. Ballew and J.N. Demas, "An error analysis of the rapid lifetime determination method for the evaluation of single exponential decays," *Anal. Chem.* **61**, pp. 30-33, 1989.
23. M. Köllner and J. Wolfrum, "How many photons are necessary for fluorescence lifetime measurements?," *Chem. Phys. Lett.* **200**, pp. 199-204, 1992.
24. D.V. O'Connor and D. Phillips, *Time-correlated single photon counting*, Academic Press, New York, 1984.
25. K. Carlsson and A. Liljeborg, "Simultaneous confocal lifetime imaging of multiple fluorophores using the intensity-modulated multiple-wavelength scanning (IMS) technique," *J. Microsc.* **191**, pp. 119-127, 1998.
26. K. Carlsson, N. Åslund, K. Mossberg, and J. Philip, "Simultaneous confocal recording of multiple fluorescent labels with improved channel separation," *J. Microsc.* **176**, pp. 287-299, 1994.

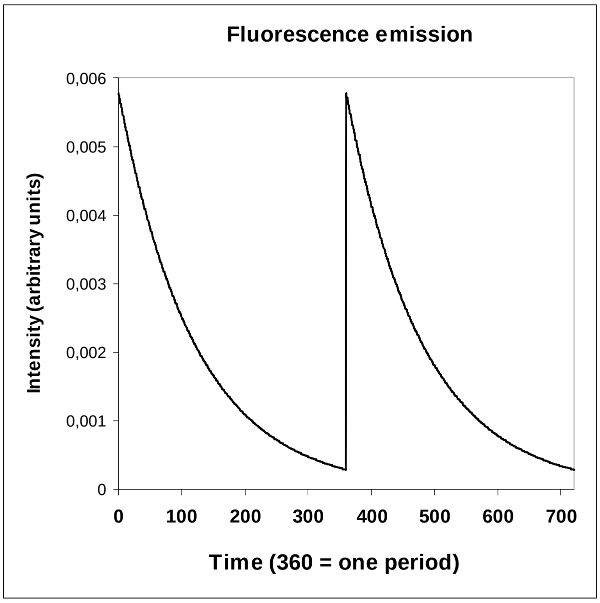

Fig. 1. Fluorescence light intensity as a function of time when using Dirac pulse excitation. Curves like this one were used in the simulations for calculating the probability for fluorophore photon emission.

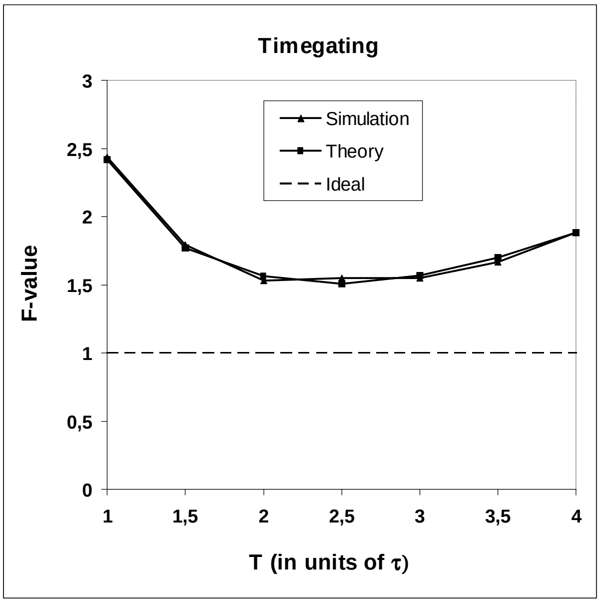

Fig. 2. Figure of merit, F, as a function of window width, T, for timegating (two windows of equal width). Low F-values represent high signal-to-noise ratios. The best F-value theoretically possible is unity.

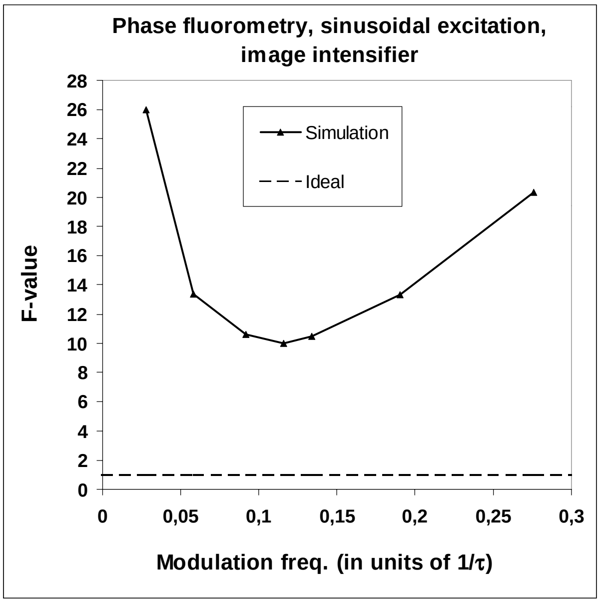

Fig. 3. F as a function of modulation frequency for phase fluorometry, using an image intensifier and sinusoidal excitation.

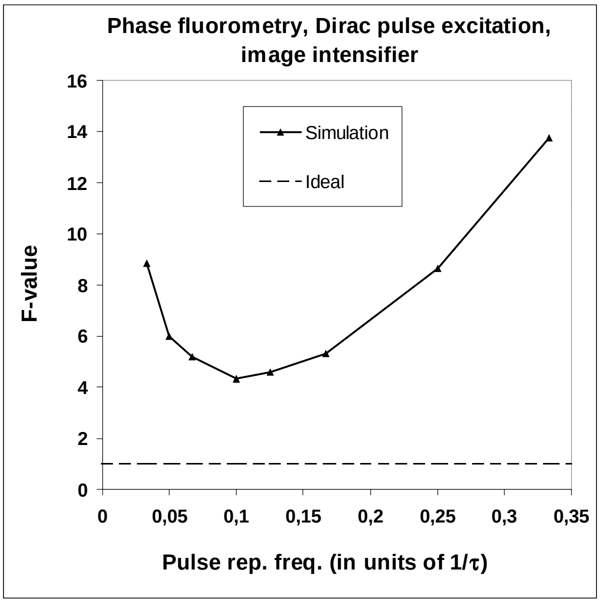

Fig. 4. F as a function of pulse repetition frequency for phase fluorometry using an image intensifier and Dirac pulse train excitation.

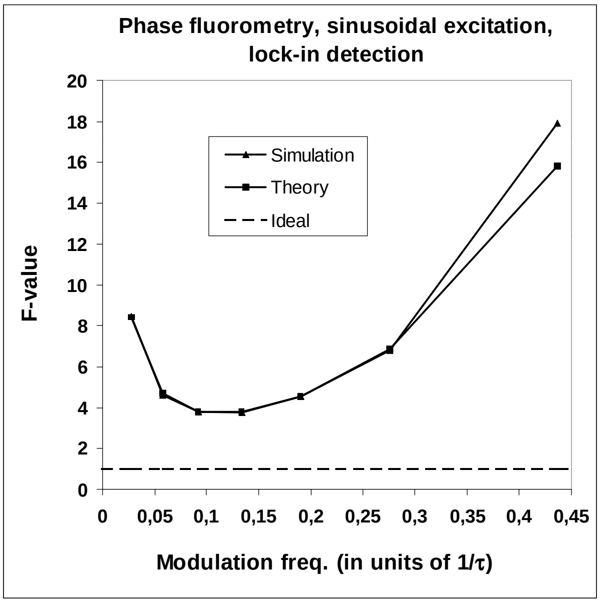

Fig. 5. F as a function of modulation frequency for phase fluorometry with lock-in detection. Sinusoidal excitation.

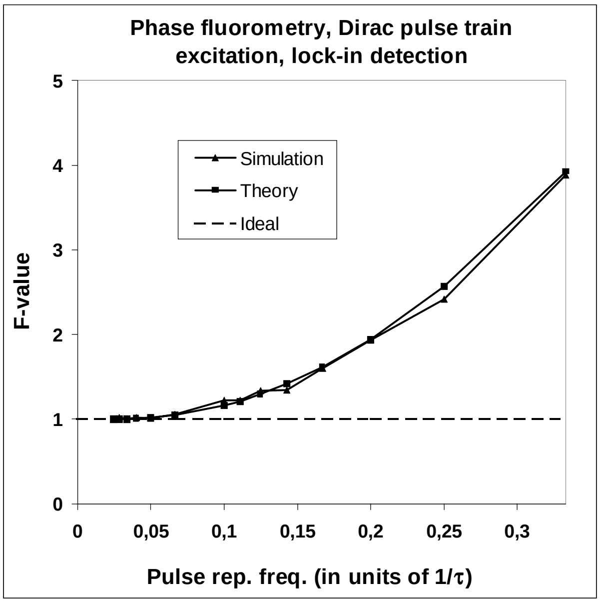

Fig. 6. F as a function of pulse repetition frequency for phase fluorometry with lock-in detection. Dirac pulse train excitation.

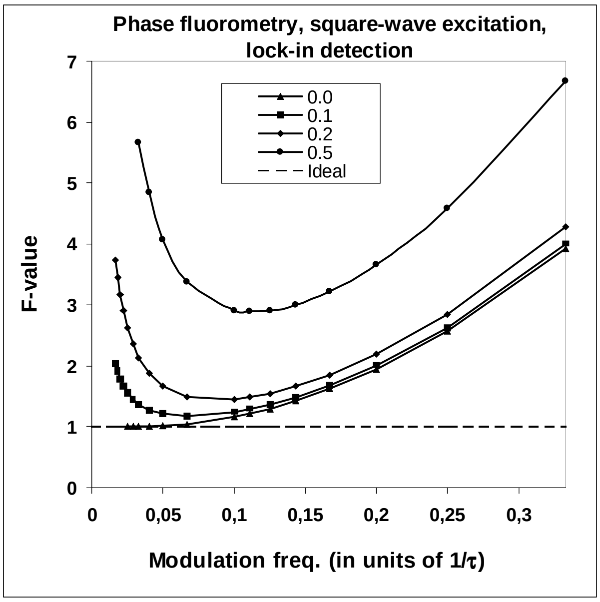

Fig. 7. F as a function of modulation frequency for phase fluorometry with lock-in detection. Theoretical curves are shown for square-wave pulse excitation with duty cycles 0 (= Dirac pulses), 0.1, 0.2, and 0.5. Results from simulations were very close to the theoretical values.

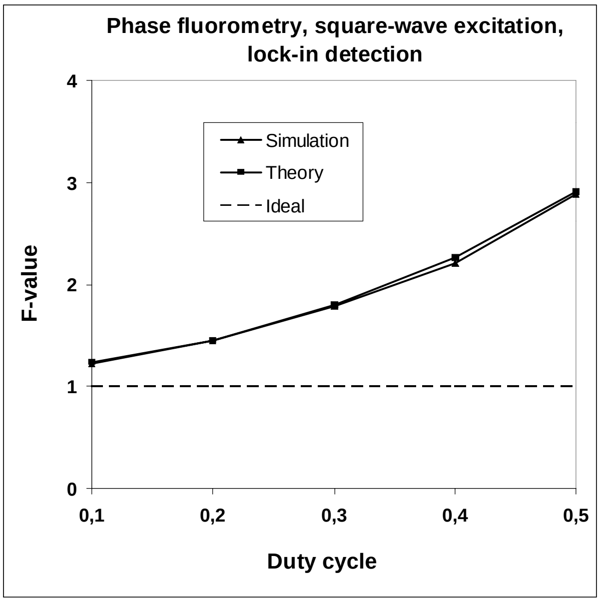

Fig. 8. F as a function of duty cycle for phase fluorometry with lock-in detection. Square-wave pulse excitation with frequency 0.1/τ, and duty cycles ranging from 0.1 to 0.5.

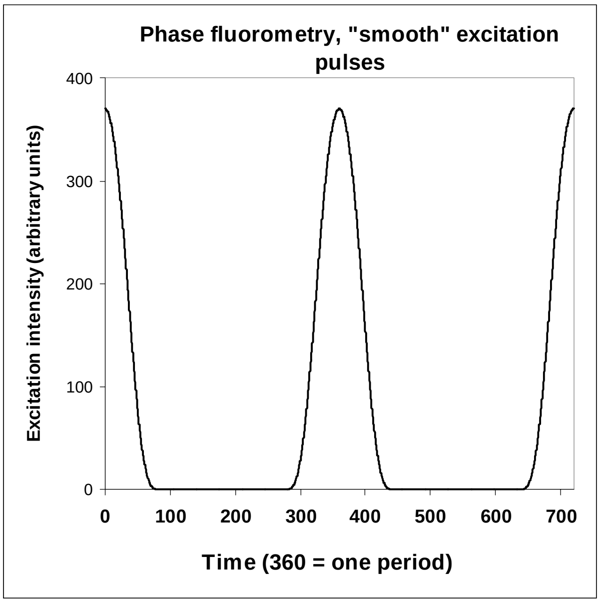

Fig. 9. "Smooth" excitation pulses with a FWHM of 0.2 of one period were simulated to investigate performance when using a non-square-wave pulse train (see text for details)

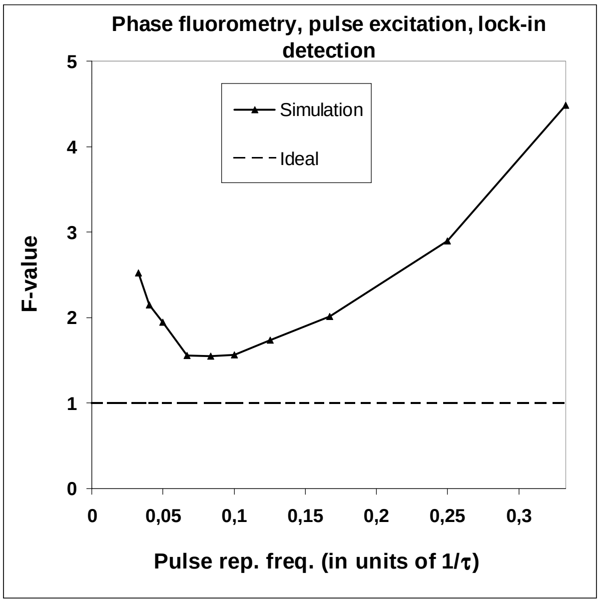

Fig. 10. F as a function of pulse repetition frequency for phase fluorometry with lock-in detection. Smooth, Gaussian-like pulses (see Fig. 9) were used for excitation.

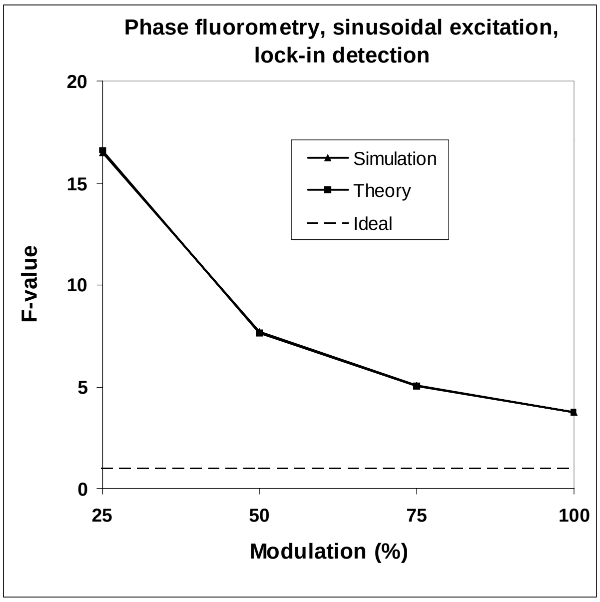

Fig. 11. F as a function of modulation depth for phase fluorometry with lock-in detection. Sinusoidal excitation with frequency = 0.13/τ.