Fluorescence Lifetime Determination for Application in Microscopy

Frank R. Boddeke[1], V.M. Erik Schenkeveld[1], Lambert K. van Geest[2] and Ian T. Young[1]

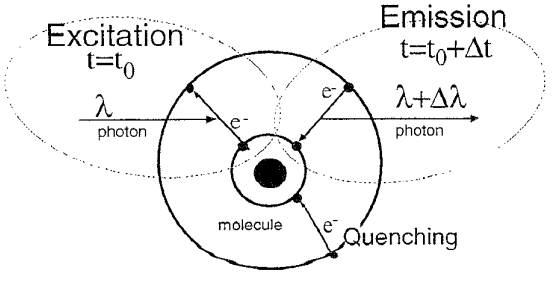
[1]Faculty of Applied Physics, Delft University of Technology, Lorentzweg 1, NL–2628 CJ Delft, The Netherlands. [2]Lambert Instruments B.V., Turfweg 4, NL–9313 TH Leutingewolde, The Netherlands.
e-mail: boddeke@ph.tn.tudelft.nl or vmes@ph.tn.tudelft.nl

Abstract

A recent development in fluorescence microscopy is fluorescence lifetime imaging microscopy. In this type of microscopy the lifetime of the excited state of the fluorescent molecules is of interest rather than the amount of light emitted. By evaluating the response –emission light– of the system –fluorescent molecules– to a specific input signal –excitation light–, the fluorescent lifetime can be determined. Different input signals are possible: light pulses, sinusoidally modulated light and white-noise modulated light. In this paper we will describe two systems under development in our laboratory based on the latter two input signals.

Introduction

Fluorescence microscopy [1,8] makes use of the fact that the light emitted by fluorescence molecules has a longer wavelength (Stokes' law) as depicted in figure 1.



Firgure 1: Fluorescence process

In a fluorescence microscopy, filters and dichroic mirror are choosen so that the excitation light can reach the specimen through the objective lens and only the fluorescence light is seen through the eye-pieces or is detected by the camera (see figure 2).

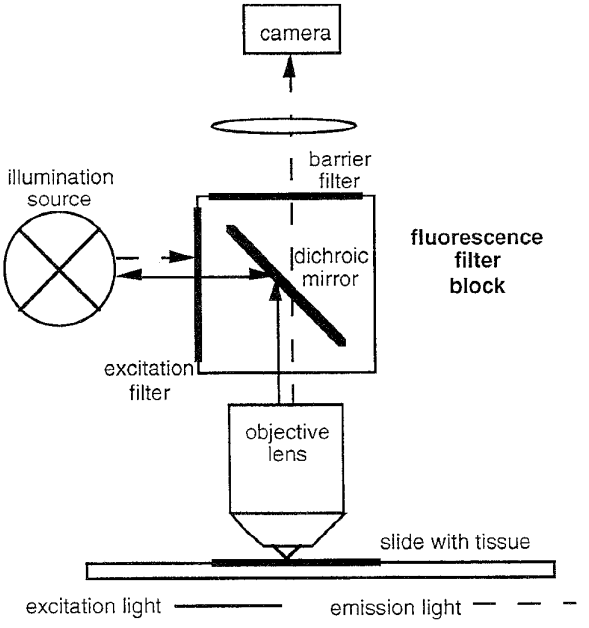


Figure 2: Fluorescence microscopy

In fluorescence lifetime imaging microscopy the lifetime of the excited state of the fluorescent molecules is of interest rather than the amount of light emitted. The lifetime of a fluorescent molecule is a physical property measuring the duration of the excited state of that molecule. The fluorescence lifetime –typical values of 1 to 100 nano seconds– depends on the molecule itself and on other molecules in the proximity. Therefore the fluorescent lifetime can be a probe for conjugation and molecular concentration (e.g. O_2, pH, pCO_2, Ca^{2+}, K^+, Mg^{2+}, Na^+, Cl^-), offering a wide range of enhancement

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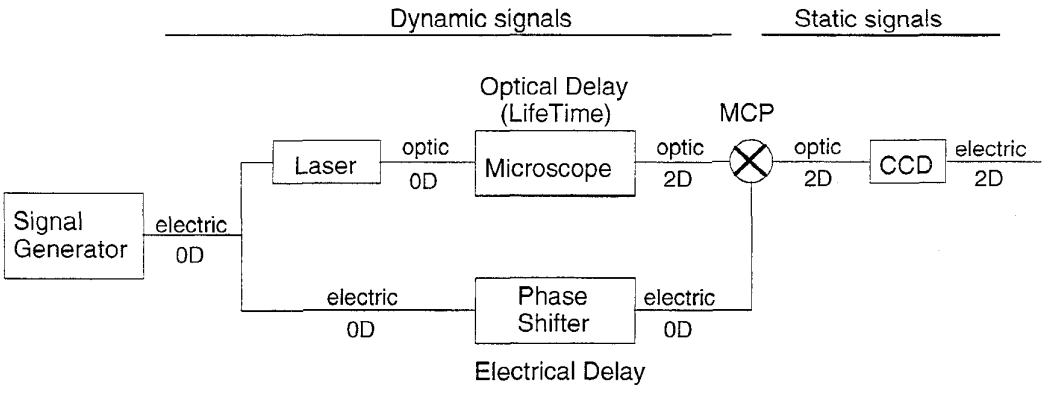

Figure 3: Schematic description of the FLIM system.

and monitoring applications.

In this paper we describe two methods in which fluorescence lifetime measurements can be performed in microscopy. The fluorescence lifetime is determined by observing the fluorescent light emitted by the molecules excited by (method 1) sinusoidally modulated light or (method 2) white-noise modulated light:

Method 1: This already existing method uses a homodyne detection scheme for the lifetime determination, i.e. the detected fluorescence light is demodulated with a phase shifted version of the original modulation signal. In this method, short delays due to the fluorescence lifetime are transformed into intensity differences. The use of image intensifiers allow demodulation of a 2D modulated signal, which makes it possible to measure (image) 2D distributions of fluorescence lifetimes at the same time. Due to the nature of this method, it is not possible to measure multiple lifetimes per pixel.

Method 2: In this new method the fluorescence lifetimes are determined by evaluating the fluorescence light after excitation with white-noise modulated excitation light. The method allows determination of multiple lifetimes and their relative contribution. We are currently building an experimental setup for these measurements. We have been working on numerical and electrical simulations to investigate the properties of noise signals. Some results of these simulations will be presented here.

Sinusoidally modulated light method

A method of fluorescence lifetime imaging (FLIM) base on sinusoidally modulated light is described by [2, 3 and 5]. This method allows 2D measurements (= imaging) of fluorescence lifetimes down to 1 nano second. Fluorescence lifetime imaging is done using a normal fluorescence microscope equipped with a special illumination source and image sensor. Lifetime retrieval is done according to a homodyne detection scheme: the illumination intensity and the image sensors' gain are modulated in time at the same frequency but with an adjustable measure phase shift. An schematic description of the system is depicted in figure 3.

The effect of the fluorescence process on the modulated excitation light is a phase shift and decrease of depth of modulation in the emission light compared to the excitation light. Demodulating this signal with the same frequency transforms lifetimes into intensity differences. The equation in figure 4 shows demodulation and low pass filtering with an image intensifier. For simplicity, the offsets to the input signal and detector sensitivity are ignored.

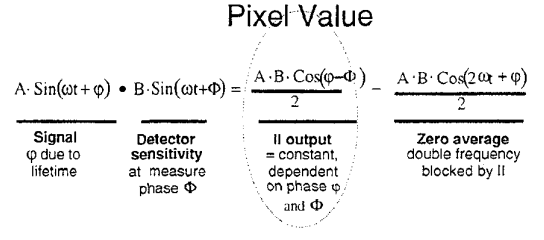

Figure 4: Demodulation equation

Digitizing images of this fluorescence light at different measure phase shifts between modulation and demodulation allows –by calculation [3]– estimating the spatial distribution of lifetimes. The calculated fluorescence lifetime image thus reveals the spatial distribution of lifetimes of the fluorescent molecules in the imaged object, instead of the amount of fluorescent light emitted as in normal steady-state fluorescence microscopy.

Our goal is to develop an affordable (using e.g. laser-diodes and normal image intensifiers), simple-to-use, advanced (automatic calibration and image acuisition)) fluorescence workstation which is capable of automatic acquisition of fluorescence lifetime images (lifetimes in the range of: 1 - 150 ns). The modulation frequencies of this system are set between 1 and 100 MHz, to be able to 'see' the desired lifetime range.

The modulated light is derived by direct amplitude-modulation of a laser-diode (12 mW @ 635 nm). The

laser-diode is connected to a vibrating optical fiber to destroy coherency. A collimator on the other end of the fiber illuminates the object under study (using a regular fluorescence microscope). The microscope optics projects an image of the object onto the image sensor for demodulation and acquisition. This image sensor consists of a MCP (micro channel plate) image intensifier and a camera. The gain of the image intensifier is modulated which results in demodulation of the projected image. An electronic phase-shifter is used to get an adjustable measure phase difference between modulation and demodulation signals, allowing acquisition of images acquired at different phases. From a set of images acquired at different phase settings, the actual lifetime image can be calculated. The whole system is computer controlled to automatically set the measure frequency, acquire the images and calculate the lifetime image.

White-noise modulated light method

Theory

We consider a mixture of fluorescent lifetimes as a system with multiple poles. The transfer function of this system is a sum of exponentials. We want to get the parameters of these exponentials by exciting the system with a white noise signal. Such a signal has a flat frequency spectrum. By measuring the input and output signal we can get an estimation of the transfer function of the system. The system transfer function is:

$$N(t) = \sum_{k=1}^{P} N_k e^{-t/\tau_k} u(t) \tag{1}$$

In the Laplace domain this is:

$$H(s) = \sum_{k=1}^{P} \frac{N_k \tau_k}{1 + s\tau_k} \tag{2}$$

Since we sample input and output signal, we are working in the discrete domain. The transfer function in the discrete domain is:

$$\begin{aligned} N_D(t) &= \sum_{k=1}^{P} N_k e^{-nT/\tau_k} u(n) \\ &= \sum_{k=1}^{P} N_k \left(e^{-T/\tau_k}\right)^n u(n) \end{aligned} \tag{3}$$

and

$$\begin{aligned} H_D(z) &= \sum_{k=1}^{P} \frac{N_k}{1 - e^{(-T/\tau_k)} z^{-1}} \\ &= \sum_{k=1}^{P} \frac{N_k}{1 - a_k z^{-1}} \end{aligned} \tag{4}$$

with $a_k = e^{-T/\tau_k}$. and T is sampling interval.

For a 2-lifetime system, the transfer function is:

$$\begin{aligned} H_D(z) &= \frac{N_1 + N_2 - a_2 N_1 z^{-1} - a_1 N_2 z^{-1}}{\left(-1 + a_1 z^{-1}\right)\left(-1 + a_2 z^{-1}\right)} \\ &= \frac{z^{-1}\left(-a_2 N_1 - a_1 N_2\right) + N_1 + N_2}{a_1 a_2 z^{-2} - \left(a_1 + a_2\right) z^{-1} + 1} \\ &= \frac{q_0 + q_1 z^{-1}}{p_0 + p_1 z^{-1} + p_2 z^{-2}} \end{aligned} \tag{5}$$

When such a system is used in combination with a white noise input signal, we have made an Auto-Regressive Moving Average system [6,7]. The equation for such a system is:

$$\begin{aligned} &p_0 y(n) + p_1 y(n-1) + p_2 y(n-2) = \\ &q_0 u(n) + q_1 u(n-1) + e(n) \end{aligned} \tag{6}$$

with $u(n)$ is white noise and $e(n)$ additive noise. After measurement of input and output signal we use an ARMAX estimator to get an estimation of the system parameters p_n and q_n. Out of these parameters the lifetimes can be calculated.

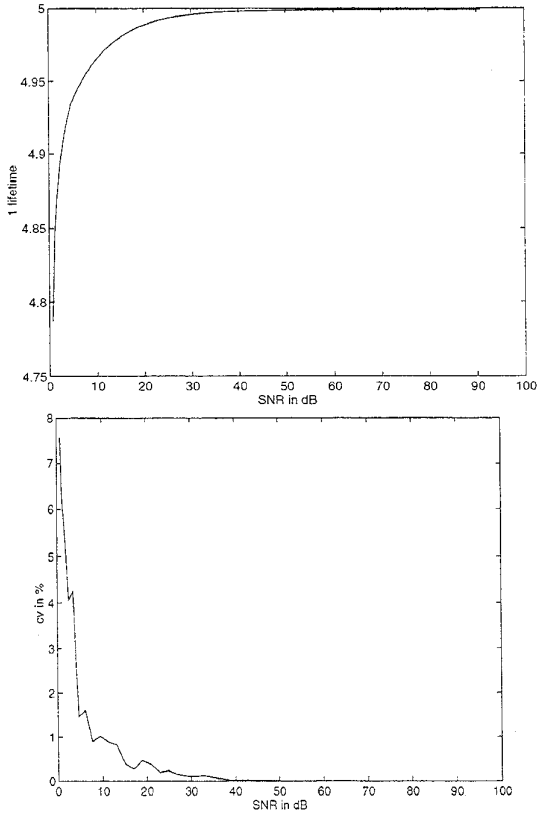

Figure 5: 1 lifetime

Results of simulation.

We have performed numerical and electrical simulations of ARMAX systems. We shall first give the results of the numerical simulations, then those of the electrical simulations.

Numerical simulations

We have investigated the sensitivity of the ARMAX estimation process to additive noise. In equation 6 we have varied the noise term $e(n)$ and calculated the estimation of the parameters p_n and q_n. Out of these parameters the lifetime is calculated. We did the experiment as follows. We took a lifetime (e.g. 5 nsec) and calculated the system parameters p_n and q_n that would give us a system with a lifetime of 5 nsec. We then took a random noise signal $u(n)$. With the parameters p_n and q_n we calculated signal $y(n)$. We added a small random noise signal $e(n)$. This resulting signal was put into the ARMAX estimator which gave us an estimation of p_n and q_n. From these estimates the lifetime is calculated. We have repeated this experiment for different noise signals $e(n)$. We have put the results in the figures 5 - 7, in which the SNR is defined as:

$$SNR = 10 * \log_{10}\left(\frac{\mathrm{var}(y(n)+e(n))}{\mathrm{var}(e(n))}\right) \qquad (7)$$

We can see from the previous figures that we need a minimum signal-to-noise ratio of 20 dB to estimate the parameter of a first order system. When the SNR is below 20 dB the estimation results become unacceptable. For a second order system we need a SNR value of 40 dB and for a third order system we need the high value of 80 dB. These high SNR values are needed to distinguish between different components of a multi-exponential model. When the SNR values are too low the bias of the estimation becomes too large and the variance of the estimator is too large as well.

Electrical simulations

After these numerical simulations we have performed electrical simulations. We have built an electrical filter that has the same transfer function as a multi-exponential system. We choose the values of the resistors and capacitors, so we know the location of the -3 dB points of the system. We want to recover these points using our ARMAX estimator. We have put a white noise signal from a Wavetek signal generator at the input of our filter, and sampled input and output signal using a LeCroy digitizing oscilloscope. These datasets are fed into the estimator. Out of the estimated parameters the -3 dB points are calculated. The electrical filters we used are shown in figure 8. The results are listed in table 1.

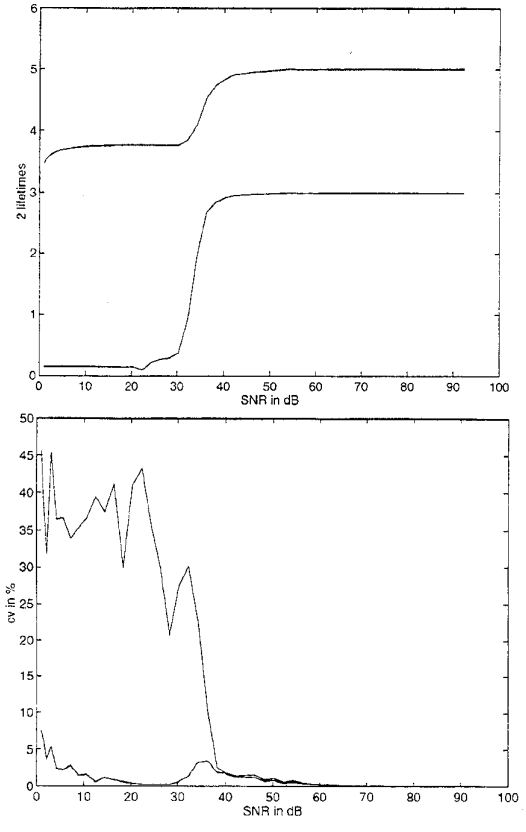

Figure 6: 2 lifetimes

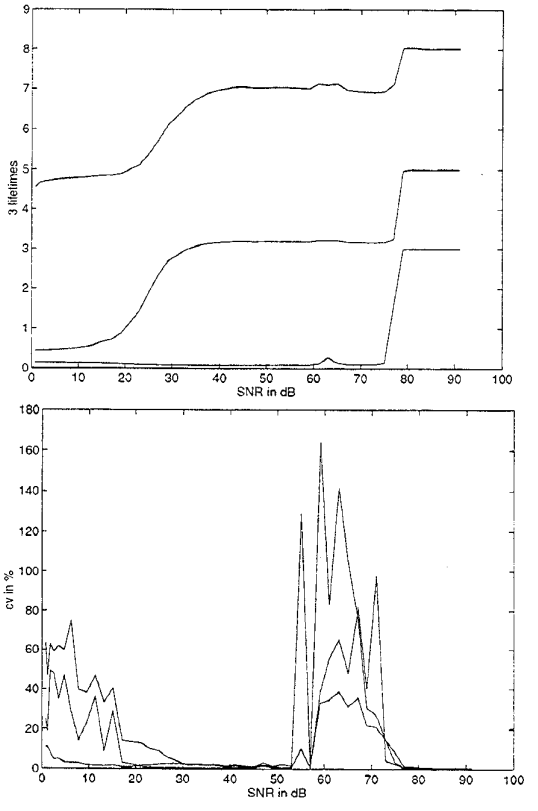

Figure 7: 3 lifetimes

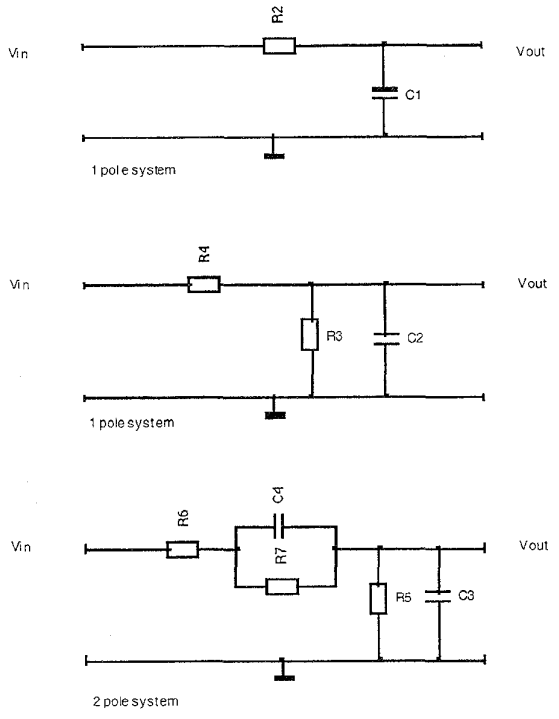

Figure 8: components layout

The values of the components used in figure 8 are printed below:

R2 = 820 Ω	C1 = 10 nF
R3 = 2 kΩ	C2 = 10 nF
R4 = 1 kΩ	C3 = 22 nF
R5 = 3 kΩ	C4 = 10 nF
R6 = 1 kΩ	
R7 = 2 kΩ	

poles	N	real values in kHz	mean in kHz	CV in %	bias in %
1	5000	19.4	19.6	9	1
1	5000	10	9.5	8	-5
1	5000	23.8	24.5	21	3
2	5000	3.8	3.6	15	-5
		29.6	24.8	14	-16

Table 1: Results of electrical simulations

In the column 'real values' the value of the -3 dB point of the filter is printed. This value is determined by the values of the components. 'mean' is the estimated value of this -3 dB point, and the CV is determined by repeating the experiment 10 times. The number of samples used in these experiments was 5000. We have also tested a three-lifetime system. The results were not acceptable at all. This is because the SNR value is not high enough. Sampling with a 8-bit quantizer gives a SNR value of 59 dB. In figure 7 we showed that this is not high enough for a three-lifetime system.

Conclusions

We conclude that the ARMAX estimator is capable of estimating the parameters of multi exponential systems in numerical simulations. When real data is used, the estimator can give an accurate estimation of single and double exponential systems. Higher order exponential systems can not be handled at this moment.

Acknowledgement

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