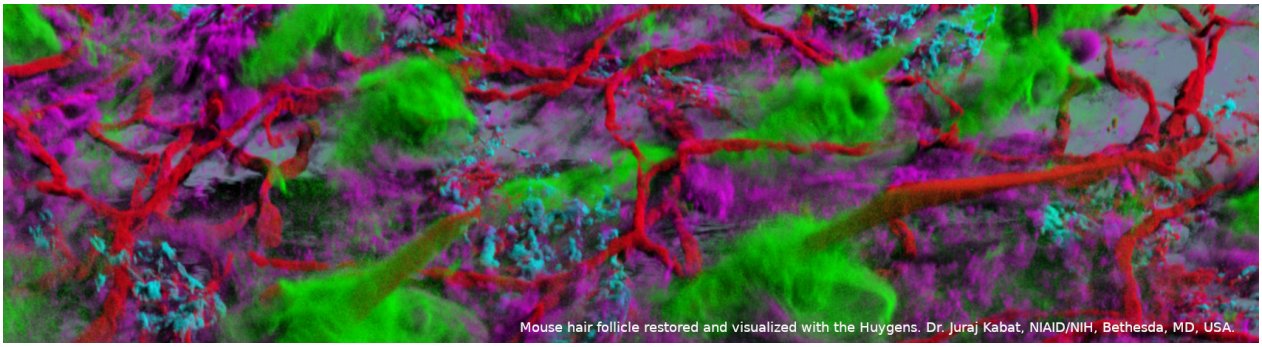

Analyzing photon limited fluorescence lifetime, are phasors a bad idea?

Hans van der Voort, Michel Ram, Frans van der Have, Nicolaas van der Voort
Scientific Volume Imaging B.V.
develop@svi.nl

1 Introduction

For over 30 years, Scientific Volume Imaging (SVI) offers high quality software packages for microscopic volume images to improve biological research around the globe. SVI is known for its high quality image restoration tools allowing researchers to study finer details in their images by improving resolution, reducing noise, and correcting artefacts. To obtain quantitative measurements from images, SVI also provides software to measure properties of objects, track and analyze movement of cells in 3D time series, and more. SVI's software 'Huygens' supports many kinds of optical microscopes, handling images with details on the molecular level to very large data sets encompassing small organs or entire organisms.

A particularly successful microscopic modality is fluorescence where dyes are coupled via highly specific molecular mechanisms to, for example, proteins in the cell. Dyes with different spectral properties can be used to simultaneously label and image multiple targeted proteins. Next to spectral properties, fluorescent dyes may differ in the typical time between getting excited by a photon and the subsequent emitting of a photon of slightly longer wavelength. This *fluorescent lifetime* may also be dependent on the chemical micro environment of the dye molecule, which opens up further possibilities for studies of molecular mechanisms in the cell or cell nucleus. For this project we are interested in computing fluorescence lifetime properties.

When the probability that an excited molecule emits a fluorescence photon is constant per unit of time, it follows that the decay of a large amount of such molecules is described by a first order differential equation with solution: $N(t) = N_0 \exp(-t/\tau)$, with N_0 the number of fluorophores in a certain volume at $t = 0$, N the number of fluorophores at time t after the laser pulse, τ the fluorescence lifetime. The fluorescent lifetime τ can be measured in various ways, for example by excitation with a sine wave modulated light source. Then, acting like a delay, the fluorescence decay will cause a phase shift of the resulting emitted light. This phase shift is fairly easy to measure using suitable electronics [1, 2].

[0]Top illustration: Mouse hair follicle restored and visualized with the Huygens. Green is epithelial cells, Red is blood vessels, Cyan is lymphatic vessels, Magenta is CD45, an antibody to label blood stem cells. Image courtesy Dr. Juraj Kabat, Biological Imaging Facility, NIAID/NIH, Bethesda, MD, USA

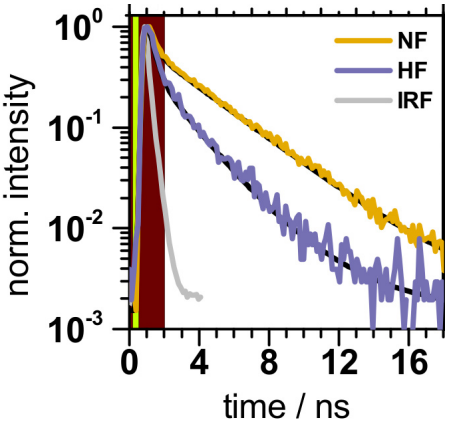

Figure 1: Fluorescence decay curves in a Fluorescence Resonance Energy Transfer experiment. Yellow: single exponent decay curve, purple: mixture of two decay curves, grey: Instrumental Response Function (IRF). Thesis N.T.M. van der Voort, HHU, Germany

More recently, very fast detectors have become available which are capable to register the arrival time of each photon with as short as 150 ps time resolution. With many fluorescent dyes having lifetimes more than 1 ns, their relaxation times can be measured with these so-called SPAD (Single Photon Avalanche Diode) detectors. Moreover, this can be done for each pixel or group of pixels in the image, see Fig 1.

2 The project

We are interested in developing optimal ways to analyze per pixel truncated decay curves to determine:

1. in the case of varying but mono exponential processes, the lifetime
2. in the case of two (or more) types of fluorophores each with constant lifetimes, the concentration of each type

In both cases, the first step in analyzing the data is a survey over all per-pixel decay curves. An example of this is the phasor scatter plot shown in Fig. 2. In the second case the aim is then to determine the relaxation constants of the fluorophores, and perhaps a classification criterion.

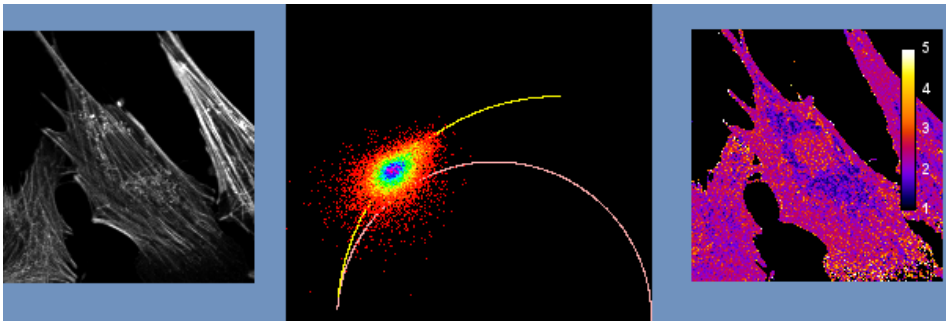

Figure 2: Cells stained with two fluorophores, Alexa 488 and Alexa 555. Left: intensity image, middle: phasor scatter plot, right: fluorescence lifetime. Phasors from different relaxtion times are located on a circle. Image from Wikipedia.

The second step is to determine the per-pixel properties. We suspect that an optimal method in each case would be Maximum Likelihood Estimation (MLE) of the parameters. In the first case the relaxation time, in the second case the concentration parameters. However, unless closed form solutions exist, this is likely computationally too challenging. In the literature, the following methods can be found:

- Moment based analysis. For example the lifetime of a single exponential process is found as the first order moment (the average arrival time) $\tau = \sum k_i t_i / N$, k_i the number of photon counts in time interval t_i. Higher order moments may be used to compute concentrations for case 2.

- Phasor based analysis. This method might have its roots in the frequency modulation and resulting phase shift scheme outlined above. Computationally, it is based on a one-frequency Fourier transform: $P_{\omega_0} = \sum k_i \exp(-i\omega_0 t_i)$, with ω_0 a suitable frequency (Fig.2).

- Instead of a Fourier transform a Laplace transform $L(s_0) = \sum k_i \exp(-s_0 t_i)$, $s_0 = \sigma_0 + i\omega_0$, again σ_0 and ω_0 suitable values.

- Direct curve fitting of the truncated series with MLE as mentioned above [3, 4].

Both the Fourier- and Laplace-based methods allow convenient inclusion of the IRF to model detector behavior. Gafni *et al* [5] point out that the Laplace transform has the beneficial property to reduce the weight of noisy data points at later arrival times.

In summary, some questions to consider:

- Is there a closed form MLE estimator for single component truncated decays, and if not, what is the error w.r.t. the average arrival time estimator?

- When the arrival times of each photon are available, given a certain maximum number of photons per pixel, and taking the IRF into account, what are optimal choices for binning the arrivals?

- Is there indeed a substantial advantage in using Laplace transforms to create a scatter plot like in Fig. 2, and in analyzing the decays?

- Lastly, do any of the transform-based methods offer advantages over moment-based methods?

References

[1] J. R. Lakowicz and A. Balter, "Theory of phase-modulation fluorescence spectroscopy for excited-state processes," *Biophysical chemistry*, vol. 16, no. 2, pp. 99–115, 1982.

[2] W. M. Lifetimes, "Which fluorescence lifetime system is best for you?,"

[3] S. J. Haberman, "Maximum likelihood estimates in exponential response models," *The annals of statistics*, vol. 5, no. 5, pp. 815–841, 1977.

[4] R. Fraile and E. García-Ortega, "Fitting an exponential distribution," *Journal of Applied Meteorology and Climatology*, vol. 44, no. 10, pp. 1620–1625, 2005.

[5] A. Gafni, R. L. Modlin, and L. Brand, "Analysis of fluorescence decay curves by means of the laplace transformation," *Biophysical journal*, vol. 15, no. 3, pp. 263–280, 1975.