Lifetime reloaded

DETERMINATION OF FLUORESCENCE LIFETIME MADE EASY.

Lifetime or decay rate relates to the phenomenon of fluorescence. This process, photoluminescence, is widely utilized across many areas, for tagging cells and cell fragments in order to observe metabolic processes, for differentiating between reaction products or for the characterization of any parameters that induce a change in the fluorescence.

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The term fluorescence is often used as a synonym for photoluminescence, although luminescence actually covers fluorescence and phosphorescence. Both of these terms describe the process of absorption of light at high energy and subsequent re-emission at a lower energy. In the following article, the terms fluorescence and photoluminescence will be considered interchangeable.

Depending on the dye – and its ability, in whatever form, to interact with its surroundings – then the absorption of photon energy by a dye molecule is not instantaneous, but instead requires a finite amount of time. The same also applies to the emission process, in that the dye continues to emit light for a short period of time after cessation of the excitation (**Figure 1**, left). Termed decay rate or lifetime, this emission decay process is a fundamental property that characterizes the dye (along with its specific spectral behavior).

The emission process can always be described by an exponential decay over time, although, as often as not, several exponential factors may be required to properly describe the observed behavior. The fluorescence lifetime can provide additional information about the dye or about its immediate environment, and the predictability of this behavior is often used as the basis for sensing applications. There are, for example, fluorescence dyes that, following excitation, transfer their excess energy non-optically in a subsequent collision with an oxygen molecule, resulting in a quenching of the emitted fluorescence. As this fluorescence quenching is proportional to the amount of oxygen present, the change in the fluorescence behavior can be used in many applications as an optical measure of the oxygen partial pressure.

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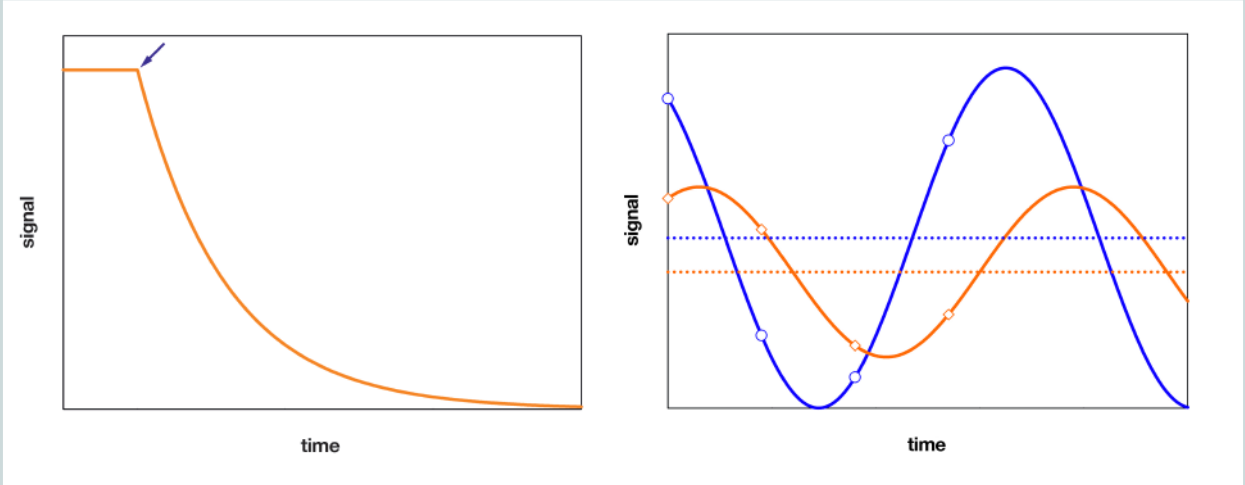
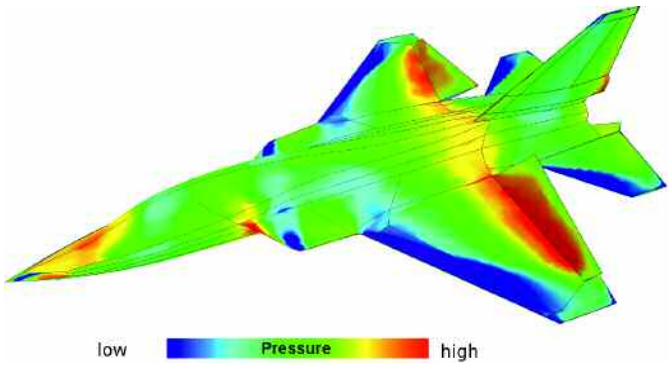
The use of dyes in this fashion requires calibration of the observed behavior against known oxygen partial pressures in order to provide an accurate conversion for values measured in real applications. Any potential falsification of the measurement caused by changes in the excitation intensity are obviated by measuring only the fluorescence lifetime, not the intensity. In this way, the number of dye molecules contributing to the signal measurement (assuming adequate signal to noise) is irrelevant.



1 Time response of the photoluminescence for varying excitation. Left: time domain – fluorescence over time following cessation of the excitation (blue arrow). Right: frequency domain – excitation light (blue) and fluorescence (orange) over time, and the four sample points used for imaged acquisition.



2 The first 360° measurement with pressure sensitive paint (PSP) in the DNW/HST wind tunnel in Amsterdam, with a projection of the pressure distribution on a CFD (›Computational Fluid Dynamics‹) grid following image processing. (Source: PSP Gruppe, DLR, Göttingen, Germany)

Lifetime imaging

Camera-based systems that are capable of measuring the spatial distribution of the fluorescence decay have been described in the literature since the start of the 1990's [1, 2]. In the meantime, the acronym FLIM has become the established term for ›Fluorescence Lifetime Imaging‹ or ›Fluorescence Lifetime Imaging Microscopy‹.

How does one acquire a fluorescence lifetime image? From a physics stand point this can be achieved either in the time or frequency domain, each approach exhibiting both advantages and disadvantages, depending on the type of light detector and acquisition method employed.

Time domain: Figure 1 (left) illustrates an ideal response of the fluorescence over time following cessation of the excitation. The fluorescence decay can be acquired via a variety of methods, and is then modeled with appropriate decay times. In general, one either already knows how many lifetimes are to be expected, or the model is reiterated until the best fit is obtained.

For imaging applications (that is, measurement of the spatial distribution of the lifetime), scanning methods are employed, whereby ›Time Correlated Single Photon Counting‹ systems, as marketed for example by Picoquant, Becker & Hickl and Horiba, is the established approach for weakly emitting dyes. Alternatively, a camera can be used to integrate the fluorescence emission over a short portion of the decay curve, where the (spatial distribution of the) lifetime is then calculated in combination with one or (ideally) many more such acquisitions over further portions of the decay curve.

Photon counting is a highly sensitive method and is best suited to weakly emitting dyes, although appropriate systems must exhibit adequate bandwidth so that the oftentimes very rapid light emission and correspondingly short lifetimes are measured accurately and are not distorted. Such measurement systems are available, for example, from Photonic Research Systems.

One application is the measurement of the distribution of the oxygen partial pressure across the wing of an airplane. In this ▶

approach, a model of the wing is covered with a pressure sensitive paint (PSP) whose emission is quenched in the presence of oxygen – it thus acts as an oxygen indicator. **Figure 2** shows the oxygen distribution following correction of the data for the temperature distribution.

Frequency domain: In this approach, dye fluorescence is excited with modulated light. Taking for simplicity's sake a sine wave modulation of the excitation, the fluorescence from the dye follows this modulation, although it is delayed as caused by the dye's excited state lifetime. **Figure 1** (right) depicts both the excitation (blue) and the fluorescence light (orange), and four typical sampling points are also illustrated. The fluorescence also exhibits a different ratio of the modulated component to the (constant) background intensity (compared to that of the excitation light). The delay is termed the phase angle (ϕ), while the aforementioned ratio is termed the modulation index (m). Both of these parameters can be determined by appropriate sampling of the signal.

By acquiring an image (taken over the same exposure time, and which can last over several modulation periods) at each of the four sampling points, then spatial distributions for the fluorescence intensity I, phase angle ϕ and modulation index m can be visualized:

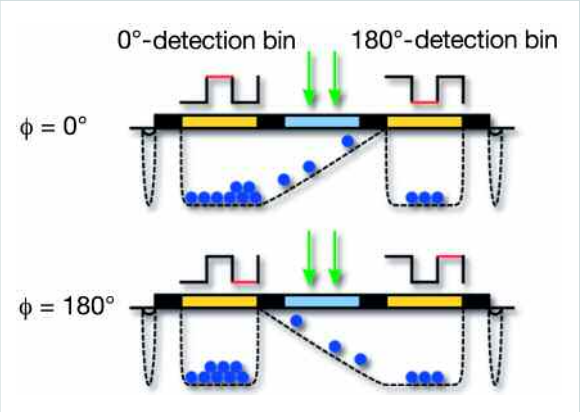
$$\phi = \arctan\left(\frac{I_1 - I_3}{I_0 - I_2}\right)$$

$$m = 2\,\frac{\sqrt{(I_1 - I_3)^2 + (I_0 - I_2)^2}}{I_0 + I_1 + I_2 + I_3}$$

$$I = \frac{I_0 + I_1 + I_2 + I_3}{4}$$

Assuming the zero phase position of the system is known, the lifetime distribution can be calculated directly from that of the phase angle and the modulation index. In contrast to measurements in the time domain, the excitation is continuously active and must thus be effectively optically filtered. Also, as the signal is modulated at a well defined frequency, the detection bandwidth can be appropriately limited in order to help reduce signal noise.

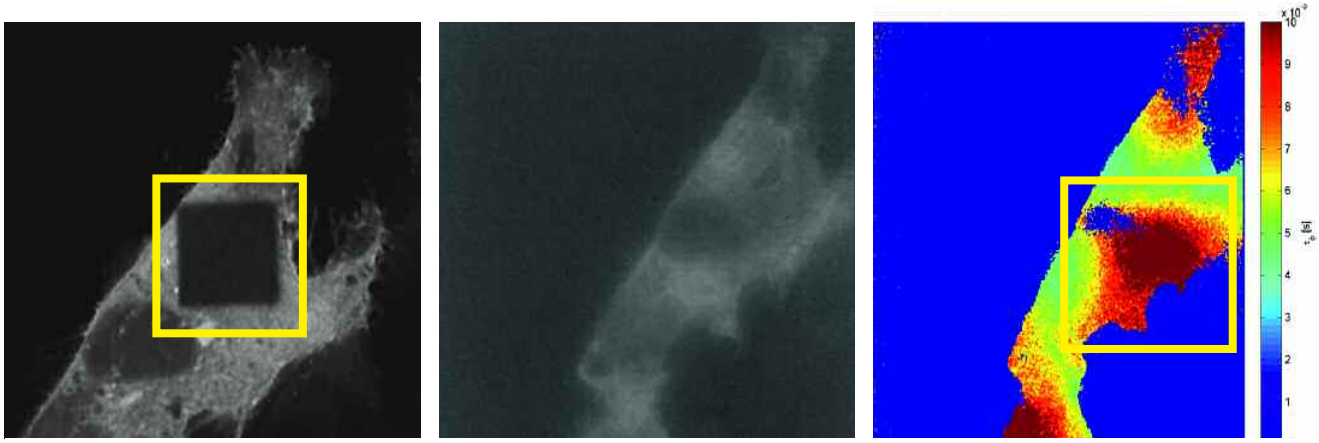
Camera-based systems are also available for measurements in the frequency domain. The greatest challenge here is the modulation required in the image acquisition, and until recently this was only possible via modulated amplification of an image intensifier tube placed in front of the camera. Such systems are available from Lambert Instruments and LaVision Biotec, and can also be found in a number of research laboratories. These are however complex systems, as on the one hand, having a modulation source with an amplitude of several hundred volts (for the image intensifier) is not exactly trivial, and on the other hand, an external frequency source must also be coupled to a modulation-capable light source. In the past, lasers could only be thus modulated via acousto-optic or electro-optic modulators. For these reasons, fluorescence lifetime measurements in frequency domain have remained the field of specialists, and it has not seen broad adoption as a general tool in laboratory testing and in research.



3 Schematic illustration of the charge carrier path in the pixels of the ›QMFLIM‹-CMOS image sensor. Phase-sensitive 0° and 180° images can be acquired by switching detection bins via a simple square wave

New image sensors and camera systems

New CMOS image sensors have been developed in the last ten years that permit modulation of the captured image. Essentially similar pixel structures have been developed both at the University of Siegen (Workgroup of Prof. R. Schwarte) and at the Centre Suisse d'Electronique et Microtechnique SA (CSEM, Zurich) that allow transportation of the light-induced carriers into one of two pixel bins. By controlling the direction of the generated charge carriers into one of the two detection bins with a simple square wave, phase-sensitive 0° and 180° images can be acquired (**Figure 3**).



4 Fluorescence and lifetime images of cells tagged with FRET dye pairs. Left: Acceptor fluorescence following intentional bleaching (see yellow quadrant). Middle: Donor fluorescence following bleaching of the acceptor. Right: phase-delay-based lifetime distribution of the donor dye following the aforementioned bleaching (see yellow quadrant)

These image sensors – available for example from Mesa Imaging (Zurich) and PMDTec (Siegen) – have been developed for applications subject to a daylight environment, with the result that the requisite suppression of the ambient light leads to relatively large interference signals (compared to the measured fluorescence signal).

In recent years, a research program funded by the BMBF has led to the development of a specialized CMOS image sensor that ideally caters to the demands of lifetime imaging measurements. This image sensor has now been integrated into a prototype camera system.

The fluorescence lifetime camera system ›pco.flim‹ integrates both camera and modulation signal generation, and can thus be used with new, modulation-capable laser diode modules to provide a complete FLIM system. A selection of the technical specifications is given in **Table A**.

The one megapixel sensor is the highest resolution TOF (time-of-flight) image sensor yet available and also provides a very high frame rate. The latter is indeed reduced through the need to acquire four image pairs to an effective 20 frames/s, but is nonetheless faster than previous imaging lifetime measurement systems. The quantum efficiency is higher than for image intensifiers, al-

Parameter	pco.flim
Resolution	1024 × 1024 pixels
Pixel size	5.6 × 5.6 µm
Frame rate	80 frame pairs /s
Modulation frequency	5 kHz–40 MHz (50 MHz max.)
Quantum efficiency	39 %
Dynamic range	> 1 : 1096
Read-out noise	14 e
Frame exposure	100 ns–10 s
Cooled temperature	30 °C
Power requirement	approx. 60 W

A Technical parameter of ›pco.flim‹

though naturally without amplification. Despite this, the camera system has been used successfully with weakly emitting dyes.

Workflow

A measurement of the lifetime distribution is made as follows:

- Determination of the necessary excitation time for the sample.
- Acquisition of a sequence of reference images that are subsequently transferred into memory. A sequence comprises 2x2 frame pairs corresponding to the following phase positions: 0° – 180° and 90° – 270° as well as 180° – 0° and 270° – 90°. The re-acquisition of the frame pairs for reversed phase positions is necessary in order to minimize a remnant asymmetry in each pixel. An added benefit is that averaging in this way also improves the signal to noise.
- Acquisition of an image sequence from the sample.
- Data processing of both sequences in the computer.

As an example, measurements were performed on FRET (Förster Resonance Energy Transfer) dye pair, resulting in three images: the fluorescence intensity image, the spatial distribution of the modulation index and the spatial distribution of the phase delay. The last two can be converted into a lifetime distribution image (**Figure 4**, right), or be represented as a p

5 ›pco.flim‹ prototype mounted at an invers microscope

lase plot. **Figure 4, left** shows the fluorescence from the FRET acceptor dye that has been intentionally bleached over a small rectangle (over a somewhat smaller region). **Figure 4, central** depicts the donor fluorescence – although there is no evidence of the bleaching in this image, it is visible as a characteristic increase in lifetime in the equivalent lifetime distribution image. As the modulation frequency can be set anywhere between 5 kHz and 50 MHz, multiple measurements can be performed – although for each frequency a useable reference with known decay, or at least with a significantly shorter decay is required. Technical limitations in the sensor mean that read-out of each frame pair must be performed when the excitation light is off, and the necessary dark gate signal is generated within the camera system.

Figure 5 shows the ›pco.flim‹ prototype mounted to a microscope. Two coaxial cables transmit modulation and dark gate signal to the laser diode module. Manufacturers such as RGB Lasersysteme and Fisba both already have appropriate light sources with varying performance levels on the market. The two Camera-Link data cables transmit the image data to the computer for storage and data processing.

Summary

Initial results with the new FLIM camera system show that even this relatively simple set-up is capable of delivering fluorescence lifetime images and results with good resolution and at previously unattainable frame rates. This approach simplifies the process for specialists, opens up adoption of the technique for more routine studies, and will perhaps rejuvenate the field of fluorescence lifetime – ›lifetime reloaded‹, as it were. ■

Acknowledgement

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Literatur

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