Frequency-domain Phosphorescence Lifetime Imaging Microscopy (PLIM) with 2-photon excitation on commercial LSM systems

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1. Introduction

Phosphorescence is a special case of photoluminescence where an excited molecule decays between an excited triplet state and the singlet ground state. This transition is “forbidden” process and, as such, its probability is fairly low; thus, phosphorescence emissions are a slow process. The emissions occur in a significantly longer time scale (100ns and higher) than that of fluorescence (of the order of 1-10ns).

Although the phosphorescence phenomenon was first noted in the 17th century, the scientific explanation occurred in the 19th century. As the recent progress in research fields opens new application areas for phosphorescence materials, a new wave of interest is triggered to develop and characterize them. One relevant application in the life sciences encompasses using a suitable probe to study the oxygen concentration at set locations of a live-cell [1-3]. Such probes have been used for some time using single-photon excitation; only recently suitable and stable probes for 2-photon excitation have been introduced [4].

2. Instrumental Setup

PLIM experiments have two significant challenges. The first one is the repetition rate of the laser pulses. The laser's repetition period should be about five times longer than the phosphorescence lifetimes, or $T \sim 5\tau$, to assure that the decay is completed prior to additional photons impinging on the sample. While the repetition periods of a single-photon laser diode can be easily controlled by setting the modulation signal, the repetition rate of a 2-photon laser or of a supercontinuum laser is fixed. However, the confocal microscope controls the two-photon laser's intensity through an AOM (Acousto-Optic Modulator) and this device is used to select the excitation time. The rise and fall time of the AOM can be less than 50ns, which is an ideal value to measure any decay time longer than 500ns.

The second major challenge is the length of the pixel time. Generally, the microscope scanning mirror system is operated close to its maximum speed in order to acquire more frames in a given time. On the other hand, for phosphorescence measurements the scan speed has to be sufficiently slow.

ISS introduces a control unit between the AOM and the AOM controller. The unit has two operation modes: respectively the FLIM mode and the PLIM mode. In the FLIM mode, the control unit allows for the signal delivered by the AOM controller to reach the AOM without any interference; that is the

sample is irradiated with the train of pulses delivered by the 2-photon laser. In the PLIM mode, a signal sent by the ISS FastFLIM unit is superimposed to the signal from the AOM controller; this signal, allows for a set number of laser pulses to be delivered to the sample. For instance, let us say that we want to measure 1µs decay time; FastFLIM generates a TTL signal of the order of 100KHz and during the ON time about 400 pulses, each separated by 12.5 ns, are delivered to the sample. During the OFF time the phosphorescence signal is collected. The VistaVision software allows for the user to easily shape the modulation signal and set the time ON, TON, and the time OFF, TOFF (Figure 1.)

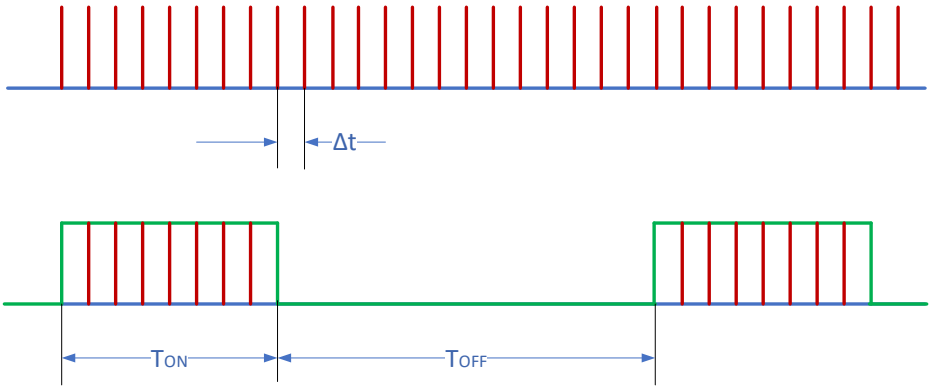

Figure 1. Schematics of the signal irradiating the sample in FLIM mode (top) where the laser pulses are separated by a time Δt and in PLIM Mode (bottom).

During an experiment, the sample can emit a photon through the fluorescence or phosphorescence process. Unfortunately, fluorescence is more likely to happen as phosphorescence is a "forbidden" process. The experiment should be repeated multiple times to collect enough photons conducive to analyze a phosphorescence event. The ISS control module signal is independent of the pixel time and can excite the sample multiple times for each pixel for each flyby. In addition to the repetition rate, the repetition rate's duty cycle can be controllable by the ISS software to achieve more efficient experiments.
Considering the rarity of events, ISS uses a multi-scalar data acquisition technique to record every single photon that arrives to the detector. This enables to shorten experimental times and reduces the illumination stress on samples.

3. The Phasor Plot

The collected data can be analyzed in VistaVision in two ways. The first approach uses the least squares analysis to fit an exponential decay curve to the data. The second approach is a novel method: the phasor plot.

A Phasor plot [5] is a graphical technique to display the decay rates in two dimensional Phasor space (G, S). The G_w and S_w are calculated as in Eq. 1 and Eq. 2 where ϕ is the phase, and m is the modulation for the signal with modulation frequency w (the basic frequency modulation is the repetition rate of the laser).

$$G_\omega = m_\omega \cdot \cos(\phi_\omega) \quad (1)$$

$$S_\omega = m_\omega \cdot \sin(\phi_\omega) \quad (2)$$

For each pixel, the raw PLIM data can be used to calculate the G_w and S_w without any assumption on the underlying decay models. The single exponential decay phasors (G_w,S_w) form a universal semicircle which is represented with light blue arc in Figure . The multi-exponential decay phasors can be represented by a linear combination of underlying single exponential decay phasors. Each component is weighted by the intensity contributions. Eq. 3 and Eq. 4 provide a general formula for the mixture. Eq. 5 ensures the normalization of the contributions.

$$G_\omega = \sum_{i=0}^{N} (f_i \cdot G_{\omega,i}) \quad (3)$$

$$S_\omega = \sum_{i=0}^{N} (f_i \cdot S_{\omega,i}) \quad (4)$$

$$\sum_{i=0}^{N} f_i = 1 \quad (5)$$

Figure 2 shows an example of the phasor plot. In this plot, red circles highlight data for the pure solutions with single component decays, and the other data are from the mixtures of the solutions with different ratios. Please note that the red circled data are on the cyan blue semicircle, a visual guide for single component decays. The solutions' mixing ratio is changed by 25% for each mixture during the experiment.

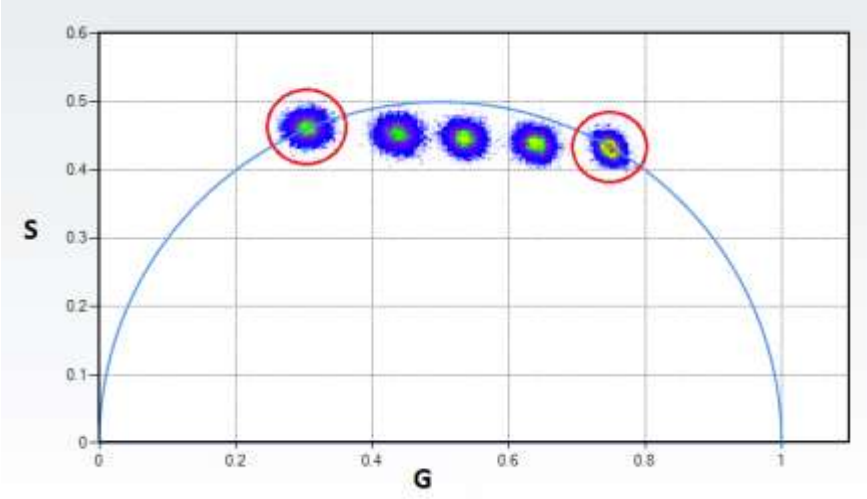

Figure 2. Phasor Plot representation; single exponential decays fall on the semicircle

4. Summary

An ISS control unit allows for control of the AOM that controls a two-photon laser's intensity in a commercial laser scanning confocal microscope. ISS uses the AOM to modulate the laser's intensity to perform a phosphorescence experiment. The emission signal is collected by the FastFLIM data acquisition card utilizing a multi-scalar data acquisition technique to increase the photon collection efficiency during the experiment. The VistaVision software allows for the user to select the optimal parameters for excitation. The VistaVision software offers both the fitting algorithm and the phasor plot to analyze the data.

The ISS control unit can be implemented on the commercial laser scanning microscopes made by major companies (Leica, Nikon, Olympus, Zeiss).

References

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