Phosphorescence Lifetime Imaging Microscopy (PLIM) Measurements: Practical Aspects

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Introduction

For many years, the characterization of luminescent materials including their luminescence lifetime has played an important role in the field of material science, especially in chemical sensing. Interest in characterization methods for phosphorescence imaging has been renewed over the past decade with the booming development of Organic Light Emitting Diode (OLED) technology, in which phosphorescent compounds are often a key component ("PHOLEDs")[1]. In life sciences, typical phosphorescent probes include metal ions complexed with organic ligands, which can be used to, e.g., image oxygen consumption in living tissue[2] or for energy transfer measurements. Furthermore, nanoparticles and quantum dots can also exhibit long fluorescence lifetime components and be thus imaged by Phosphorescence Lifetime Imaging Microscopy (PLIM)[3]. Analogous to Fluorescence Lifetime Imaging Microscopy (FLIM), the contrast in a PLIM image is based on excited state lifetime differences between individual fluorophores.

This situation is schematically shown in the simplified Jablonski diagram in Fig. 1. A molecule in the electronic ground state (S_0) can absorb (A) photons whose wavelength corresponds to the energy difference between the S_0 and the excited singlet states (S_1, S_2, and so on). In most cases, the excited molecule will relax via internal conversion (IC) to the
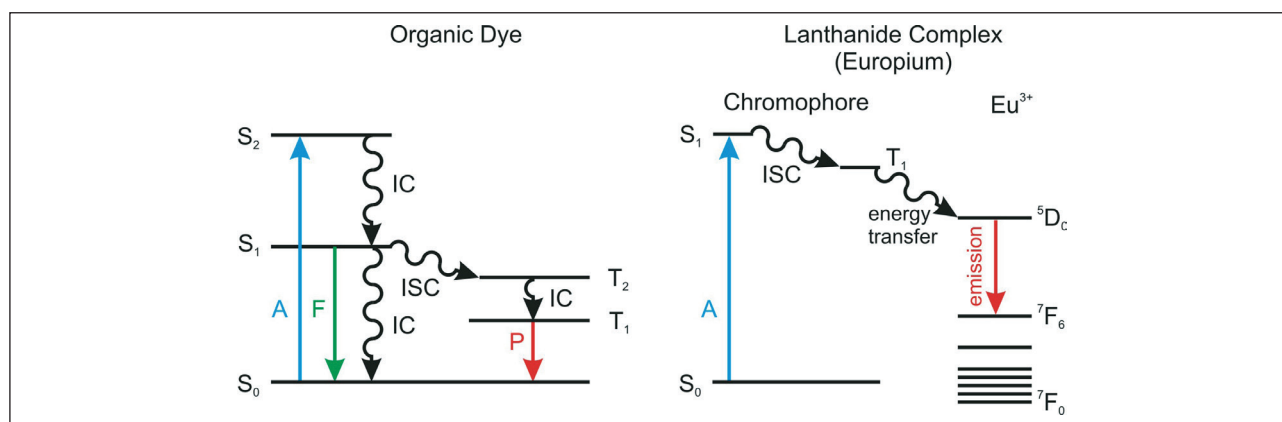

Figure 1: Simplified Jablonski diagrams summarizing possible electronic transitions in a molecule upon absorption of a photon. On the left, a the scheme for a classic dye-molecule is shown. The right diagram displays the transition scheme of a Eu^{3+} - complex. In this example, light is absorbed by the ligand and transferred via an energy transfer to the Eu^{3+} ion of the complex[4]. This example shows that luminescence from metal ions or other non purely organic dyes can be more complex than in the classic scheme. In PLIM measurements, only the measurable lifetime of the excited state is relevant, not the exact mechanisms involved in the whole process.

lowest emissive singlet state (i.e. S_1) from where it can return to the ground state by emitting a photon. This process is called fluorescence (F) and is spin-allowed. While being in the S_1 state, molecules can also either return to the ground state by transforming the excitation energy into vibrational energy (internal conversion, IC) or undergo an intersystem crossing (ISC) process. In this process, the excited electron transits into the energetically closest triplet state (T_2 in the example shown in Fig. 1). From there, the molecule will relax into the lowest triplet state via IC (T_1) and can return to the S_0 ground state by emitting a photon. In this case, the process is called phosphorescence (P) and is spin-forbidden. Therefore, the excited state lifetimes of the triplet state are much longer than those of the S_1 state in classic fluorescence.

The dominant relaxation pathway will depend on the material's structure as well as on it's environment. Efficient phosphorescence probes have a high intersystem crossing rate and a highly emissive T_1 state.

While fluorescence lifetimes are in the range of ns, phosphorescence lifetimes can range from hundreds of ns to ms. From a practical point of view, measurements of all lifetime transitions involving an emissive process with a lifetime >100 ns have similar requirements and are therefore covered here, even if the process in question may not be phosphorescence in a strict quantum mechanical sense.

When using confocal set-ups with Time-Correlated Single Photon Counting (TCSPC), the requirements for performing PLIM are similar to classic FLIM, yet with slight differences in a few key points. This technical note will discuss some of the important aspects of PLIM.

Phosphorescence Compounds and their Applications

FLIM measurements are usually carried out with organic dyes or fluorescent proteins with excited state lifetimes of a few ns, and this imaging method has found a broad range of applications[5].

Typical compounds with luminescence lifetimes longer than 50 - 100 ns are metal ion complexes (e.g., based on Ru, Ir, Pd, Pt). These are often used as sensors to measure specific environmental properties such as, for example, oxygen concentrations in tissues and other specimen to analyze their effects on cell metabolism[6, 2, 7], or have been used to study aggregation of compounds related to Alzheimer's disease[8].

Metal complexes play an important role in material research and are used, e.g., in electronics[9]. Furthermore, semiconductor materials may exhibit relatively long lifetimes as, for example, in Cu_2ZnSnS_4 (CZTS)[10], Si, or SiC based materials[11, 12]. These
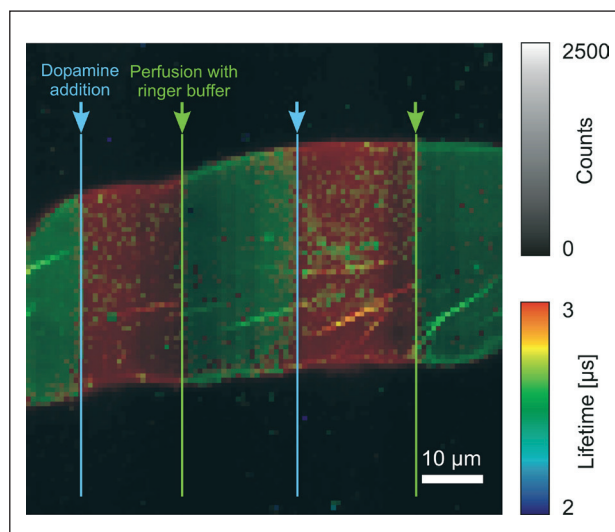

Figure 2a: PLIM imaging of an oxygen-sensitive Ru^{2+}-complex in a cockroach's salivary gland. The image is scanned with the fast axis from up to down and the slow axis from left to right. At certain time points dopamine was added to stimulate the metabolism, which results in a decrease of the oxygen concentration and thereby in an increase of the lifetime of the Ru^{2+}-complex KR341. After washing, the oxygen consumption decreases again as well as the lifetime of the KR341. The process was repeated during the scan. Data and figures reproduced from Jahn et al. Scientific Reports, Vol. 05, 2015[2], courtesy of Nature Publishing Group.
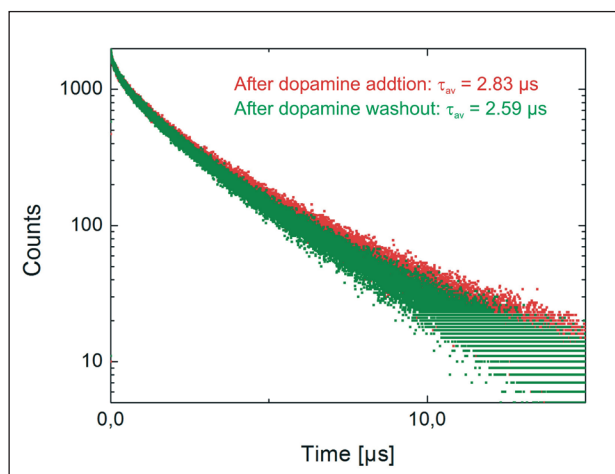

Figure 2b: Luminescent lifetime curves of 2a with and without dopamine added. Although the change in the luminescence lifetime is small, it is clearly measurable. Data and figures reproduced from Jahn et al. Scientific Reports, Vol. 05, 2015[2], courtesy of Nature Publishing Group.

long luminescence lifetimes make Time-Resolved Photoluminescence (TRPL) measurements very interesting when characterizing such materials for use as solar cells or in electronic devices, giving also insight into charge carrier diffusion lengths[13].

Lanthanides are another class of compounds where TRPL experiments help in characterizing their excited state properties. Such compounds have been proposed in the 1990s as donors in Luminescence Resonance Energy Transfer (LRET) measurements, since they are nearly isotropic
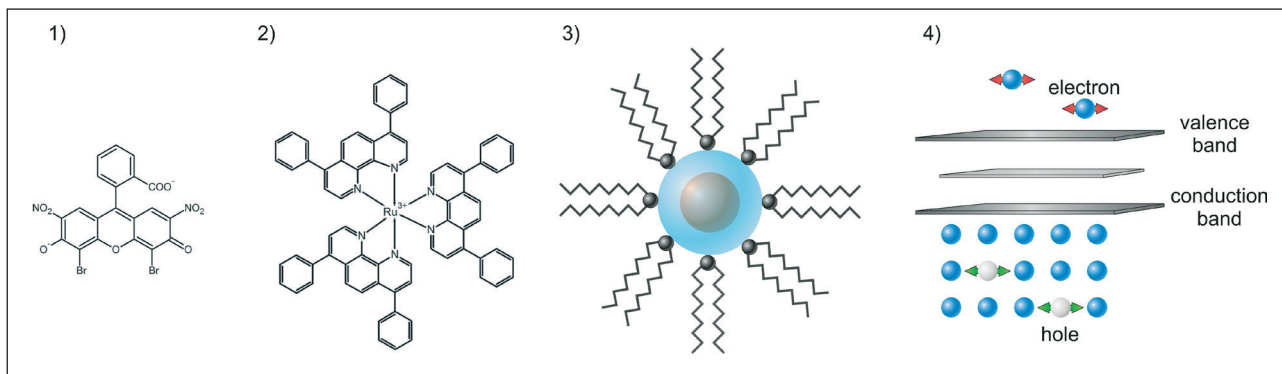
Figure 3: Structures of phosphorescent compounds.
1) Eosin B, a typical phosphorescent dye. Heavy atoms in the molecular structure favor intersystem crossing and thereby phosphorescence. Additionally, back-intersystem crossing to the S1 state can generate delayed fluorescence.
2) A very important group of compounds are metal-organic complexes that serve as, e.g., sensors or constituents of OLEDs.
3) Some quantum dots can have lifetime components of several tens to hundreds of ns[3].
4) Luminescence lifetimes of semiconductors can range from ns to ms, depending on the material. Here, Time Resolved Photoluminescence (TRPL) measurements can help to characterize the material[11].

donors[14, 15]. In mixtures, contributions from the donor-only fraction can be separated spectrally, while those from the acceptor-only fraction are removed by time gating. The acceptor is typically an organic fluorophore with a lifetime in the ns-range. The lifetime of the LRET excited acceptor equals that of the donor under LRET conditions, as the acceptor lifetime is negligible in this case. Thus, the method shows its full potential for the analysis of mixtures.

Lanthanide ions, mainly Tb^{3+} or Eu^{3+}, are used to form chelate complexes, which can be coupled to a biological sample in the same way as a standard organic dye. Lanthanides are also used in binding studies for Ca^{2+} and Mg^{2+} selective binding sites, As lanthanide ions have diameters similar to these ions, they can be used to titrate the binding sites e.g., on RNAs[16]. PLIM is also used to study the properties of quantum dots with long lifetimes.

A general advantage of phosphorescent compounds is that their luminescence can be easily separated from high fluorescent contributions, such as autofluorescence, by simple time-gating.

Signal Rate: Concentration and Pile-up in FLIM vs. PLIM-measurements

Both FLIM and PLIM data acquisition require an excitation rate where the time between two laser pulses is longer than the luminescence decay time. When measuring fluorescence lifetimes in the ns-range with a classical Time-Correlated Single Photon Counting (TCSPC) setup, individual photons are registered by very fast electronics (Fig. 1). As the dead time of electronics used in standard TCSPC setups are usually much longer than the lifetime of the dye (e.g., 25 ns for a standard TimeHarp 260P or 90 ns for a PicoHarp 300 TCSPC module), only a single photon can be registered at most after each laser pulse. Due to the dead time of the electronics, only the first photon reaching the detector after each pulse will be recorded. Should more than one photon arrive between two laser pulses, these additional photons will thus be lost.

This results in a statistical distortion of the recorded photon arrival times if the detector count rate gets too high. This phenomenon is known as pile-up effect where the collected photon statistic gets skewed towards shorter arrival times. Even if one accepts a low pile-up in order to decrease the measurement time, the maximum detection rate for standard lifetime measurements should not exceed 10% of the excitation rate to ensure the collection of proper photon statistics.

As the time between two excitation cycles should ideally not be shorter than 5 to 10 times the fluorescence or phosphorescence lifetime, it is easy to understand that strictly extending this concept to measuring long lifetimes in the μs range would result in extremely long acquisition times for a confocal setup.

Fortunately, phosphorescence lifetimes are significantly longer than the dead time of recently developed TCSPC electronics, which can be as short as 1 ns for optimized devices. In this case, many photons can be registered after a single laser pulse, and the pile-up effect is no longer present. Note that in order to exploit the short dead times of such TCSPC electronics, the detector also has to provide a short dead time. This is mainly the case for photomultiplier tubes (PMTs) and Hybrid-PMT detectors, such as the detectors from the PMA or PMA Hybrid series from PicoQuant.

Consequently, a much higher detection to excitation ratio is possible. It is crucial to consider that photons collected under classic TCSPC conditions may all stem from a single fluorophore after several excitation cycles, while in phosphorescence measurements, photons collected in the same time may be collected after a single pulse and therefore must originate from separate emitters. Therefore, in phos-
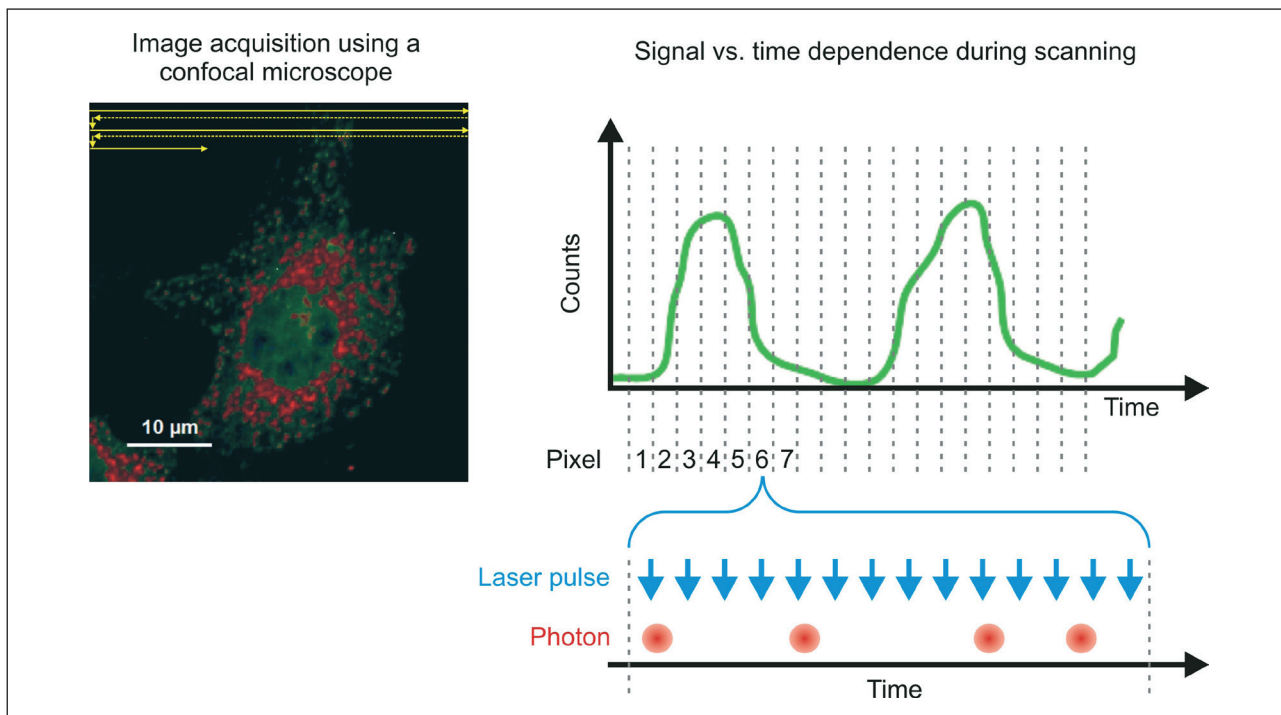
Figure 4: Principle of FLIM data acquisition with TCSPC equipment. In a confocal setup, an image is raster-scanned and detected photons are assigned to the different pixels based on their arrival time in relation to the scanner position. For lifetime determination, the sample is excited with a pulsed laser at a very high repetition rate (typically 10‑80 MHz), and the time difference between laser pulse and detected photon is recorded for every photon.

phorescence measurements, a significantly higher fluorophore concentration is needed to achieve a similar count rate. This concentration increase scales with the lifetime of the dye. Simply increasing the laser power (pulse energy) would rather induce artifacts such as bleaching than leading to higher detector count rates.

For example, approximately one molecule is in the focus of a confocal volume of ~1 fl, if the dye concentration is in the nano molar range. When measuring fluorescent dyes with nanosecond lifetimes, a useful parameter is the so-called molecular brightness, which is determined by the detector count rate divided by the number of molecules in the confocal volume. The latter value can be determined for freely diffusing dyes by Fluorescence Correlation Spectroscopy (FCS), and is dependent on excitation laser power, absorption cross section of the dye for this wavelength, quantum yield, and instrumental parameters like objective, filters, losses within the microscope system, detector sensitivity, etc. The molecular brightness can be maximized by optimizing laser power, filters, etc., and count rates in the range of several tens of kcps (kilocounts per second) can be achieved with decent fluorophores. A fluorophore with a lifetime in the order of nanoseconds can be repeatedly excited by a pulsed laser and consequently, many photons are emitted from the same molecule within a short time span. In contrast, the long lifetimes of phosphorescent compounds make achieving comparable photon count rates from a single luminescent complex impossible, and therefore also FCS mea-

surements. In order to get the same count rate from molecules with similar quantum yield and absorption coefficient, but a milisecond lifetime, the concentration needs to be in the order of mM instead of nM, since the photons must be emitted by several molecules.

This can be illustrated by a simple example: In an FCS measurement, the transit time of a single fluorescent molecule through the focus is e.g., 25 µs. If this molecule has a fluorescence lifetime of 2.5 ns, and an excitation rate of 40 MHz is used (corresponding to a laser pulse being emitted every 25 ns), the dye is excited 1000 times while passing through the focus. Given the numerical aperture of the objective, the quantum yield of the dye, the losses due to the optics and filters, the quantum efficiency of the detector will only be in the single digit percent range. If we assume that only 1% of the photons are detected, we obtain about 10 photons from this molecule during its transit. If we want to use a dye with lifetime of 2.5 µs, we would ideally have to decrease the repetition rate to 40 kHz, i.e. a pulse every 25 µs. So we could excite the dye only once while it is passing through the focus, if it has a similar size. Therefore, we could at most collect a single photon, which is not enough to get a correlation, as that requires several photons from the same molecule. Even if we use continuous wave excitation, which could increase the excitation efficiency, we still could go through the excitation cycle only once every 2.5 µs, i.e. 10 times during the molecular transit. As the detection efficiency of the whole system is significantly lower than 100%, we
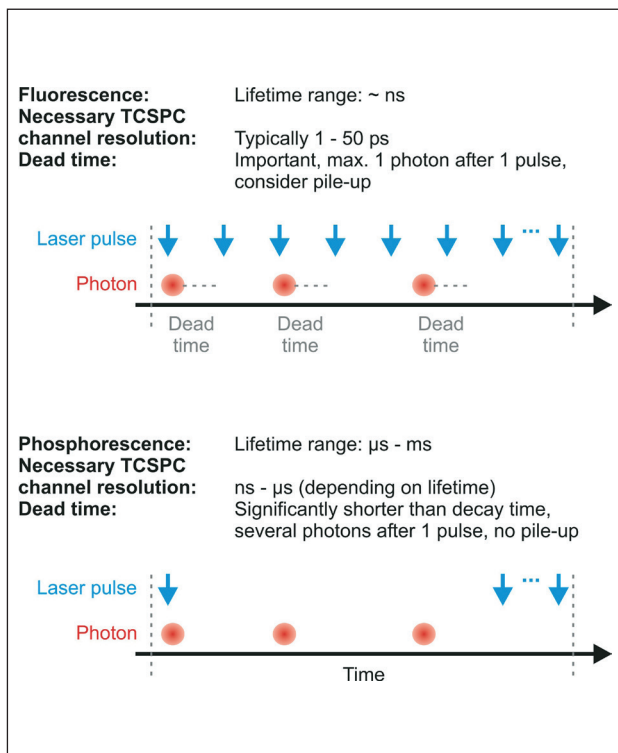
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Figure 5: Comparison of a typical FLIM vs. PLIM measurement, considering laser excitation rates and data acquisition.

would still get at most 1 photon from this molecule during its transit, still not sufficient for measuring a decent FCS curve.

Scan-Speed and Moiré-efffects

In addition to repetition rate, dye concentration, and dead time, the pixel dwell time is an important parameter in phosphorescence measurements, as this time is usually only a few multiples of the TCSPC window, which is defined as the time span between two laser pulses. In the simplest case, the pixel dwell time and the TCSPC window are independent from each other and result in interferences known as Moiré patterns[17]. In order to avoid this issue, the pixel dwell time should cover at least 10 excitation cycles. This phenomenon is similar to what can be observed when measuring fast lifetimes using resonant scanners with extremely short pixel dwell times. A detailed description of the latter effect on a fast time scale can be found in a separate technical note[18].

Systems equipped with piezo scanners can easily achieve the long pixel dwell times needed for Moiré free PLIM. Most microscopes using galvo scanners, however, have maximum pixel dwell times in the range of 100-200 µs, limiting the longest accessible luminescence lifetime using a standard approach to a few microseconds. This range can be extended, if the image is scanned with a very high pixel resolution e.g. using the maximum scan size of 4096 x 4096 pixels allowed by the SymPhoTime 64 and applying a pixel-binning afterwards. This leads to a significantly increased pixel dwell time in the binned image.

In conclusion, many parameters need to be considered in order to determine the ideal settings for PLIM imaging. Table 1 gives an overview of the dependence between fluorophore lifetime and measurement times.

Excitation Power and Repetition Rates

When exciting a dye with a pulsed diode laser, the mean excitation intensity correlates roughly with the number of pulses per second. Consequently, in PLIM measurements, the excitation intensity might become very low if just the laser repetition rate is lowered.
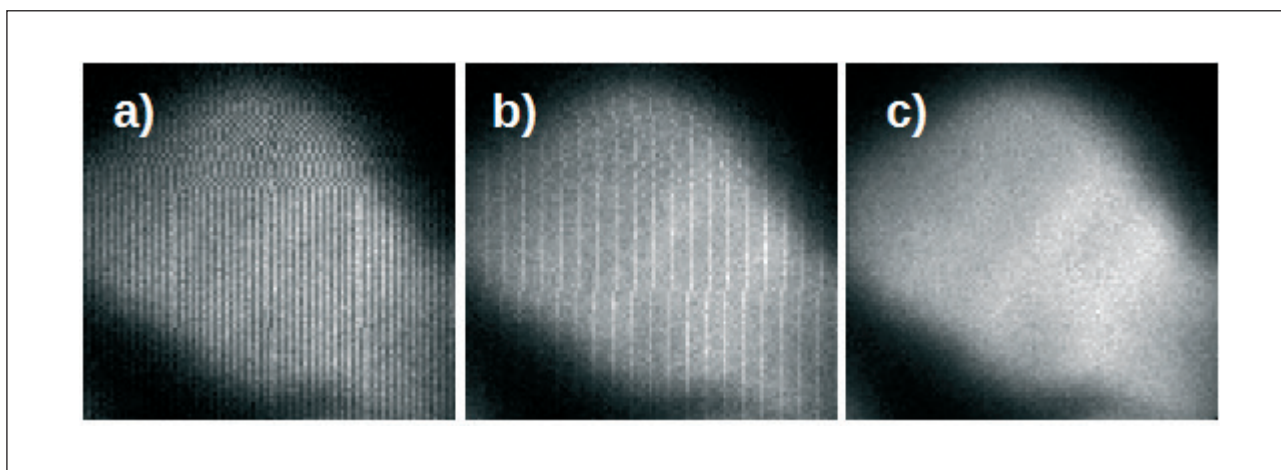
If a pulsed diode laser is used for excitation in

Figure 6: Moiré patterns observed when using different ratios of pixel dwell time to TCPSC window. a) 2.5:1, b) 5:1 c) 10:1
The interference stripes are no longer visible, when the pixel dwell time lasts at least 10 excitation cycles.

Table 1: Overview of the dependence between fluorophore lifetime and measurement times.

Lifetime of the fluorophore	Ideal Rep. Rate[1]	Max. Peak Count Rate	Min. total Pixel Dwell Time [to collect 1000 photons] (given highest countrate)	Measurement time for 100 x 100 pixels[7]
1 ns	100M Hz* * theoretical rate. Many lasers don't reach this repetition rate	10 Mcps[2] Pile-up limited[3]	100 µs one or several frames[5]	1 s
10 ns	10 MHz	1 Mcps Pile-up limited[3][8]	1 ms one or several frames[5]	10 s
100 ns	1 MHz	0.1 Mcps Pile-up limited[3][8]	10 ms one or several frames[5]	100 s
1 µs	100 kHz	Sample limited[4]	100 µs one frame only![6]	1 s
10 µs	10 kHz	Sample limited[4]	1 ms one frame only![6]	10 s
100 µs	1 kHz	Sample limited[4]	10 ms one frame only![6]	100 s
1 ms	100 Hz	Sample limited[4]	100 ms one frame only![6]	1000 s

[1] This repetition rate results in a time window 10x longer than the compound's lifetime. The luminescence intensity of the compound decreases to 1/e after a time period corresponding to its lifetime, Therefore, after a time period ten times longer, the intensity will have decreased by a factor of ~0.00004. For a typical decay with about 10000 photons in the peak maximum, the decay will have reached the background level at the end of the time window under these conditions.

[2] In practice, the registered count rate is usually less due to limitations of detector, board, or processing rate. Mcps = megacounts per second.

[3] Set to a maximum detection rate corresponding to 10% of the repetition rate. If small lifetime differences have to be measured, the maximum detection rate may need to be even significantly lower than this value.

[4] In order to reach such high count rates after one pulse, the fluorophore concentration needs to be sufficiently high. If a molecular brightness of 10 kcps is reached for a dye with a 1 ns lifetime, the concentration needs to be 1000 times higher if the same count rate has to be achieved for a dye with the same extinction coefficient and quantum yield, but with a 1 µs lifetime.

[5] As long as the pixel dwell time is significant longer than the fluorescence lifetime, the total illumination time may be calculated by summing up several frames. As a rule of thumb, the pixel dwell time should be at least 10 times larger than the TCSPC window, i.e. cover at least 10 decays.

[6] This is necessary in order to avoid Moiré patterns in the image.

[7] Nominal total acquisition time in which the real recording takes place, without acceleration/deceleration times after the last/before the first pixel and without the time needed to return to the beginning of the line for a monodirectional scan.

[8] Here TCSPC modules with very short dead times can already be used, if a slightly worse TCSPC resolution is accepted. This results in significantly higher achievable count rates of several Mcps and correspondingly shorter acquisition times.

combination with a suitable laser driver, e.g., the PDL 828 SEPIA II from PicoQuant, the driver's master oscillator module can be programmed to generate a pulse train instead of a single pulse. This way, a more effective excitation is provided on a time scale of hundreds of ns, which does not interfere with the lifetime decay in a significant way, provided that the lifetime of the fluorophore is significantly longer than the excitation pulse train, as shown in Fig. 7. Consequently, by using efficient multi-pulse excitation, the PLIM acquisition time is shortened significantly due to the more efficient excitation, as long as the concentration is not a limiting factor of the detected signal.

Complex pulse patterns and pulse trains can be set up in the PDL 828 SEPIA II software. An example for generating a sequence with 50 pulses and a 19 times longer delay until the start of the next sequence is shown in Fig. 8. In this way, the sample can be efficiently excited for a certain time span (defined by the number of pulses) to pump the luminescent compounds into the excited state. The excitation laser is then switched off to detect the phosphorescence photons.
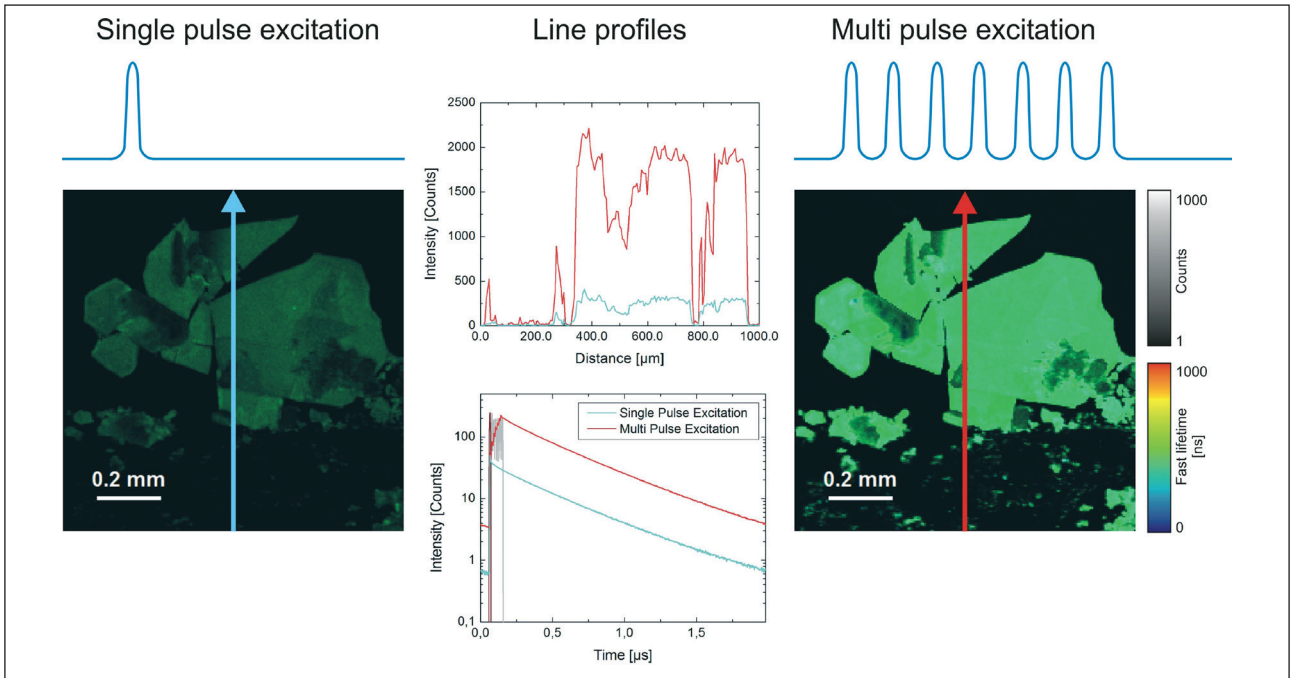
Figure 7: RuBiPy-crystals measured with single pulse and pulse train excitation. The intensity increase in the image correlates roughly with the pulse number (7 pulses in this example) as shown in the the line profiles. The phosphorescent decays are plotted below the line profiles.

Configuration: Electronics and Detectors

The requirements that have to be fulfilled by the TCSPC electronics and detectors depend mainly on the lifetime to be measured. All currently available PicoQuant TCSPC units operate in forward mode and have multi-stop capability, meaning that several photons can be registered following a single excitation pulse, if these photons arrive after the dead time of the system. The full time-span covered by the system can be calculated by multiplying the longest TCSPC bin width with the number of channels in the image recording mode called TTTR ("time-tagged time resolved", also usually called "t3-mode"[19], in which every photon is stored along with the time information from the beginning of the experiment and from the corresponding laser pulse). Information about maximum time bin width and channel number can be found in the data sheets for the corresponding boards available from the PicoQuant website as well as in the manuals of these boards.

The shortest dead time (less than 2 ns) is currently achievable with the TimeHarp 260 N or the
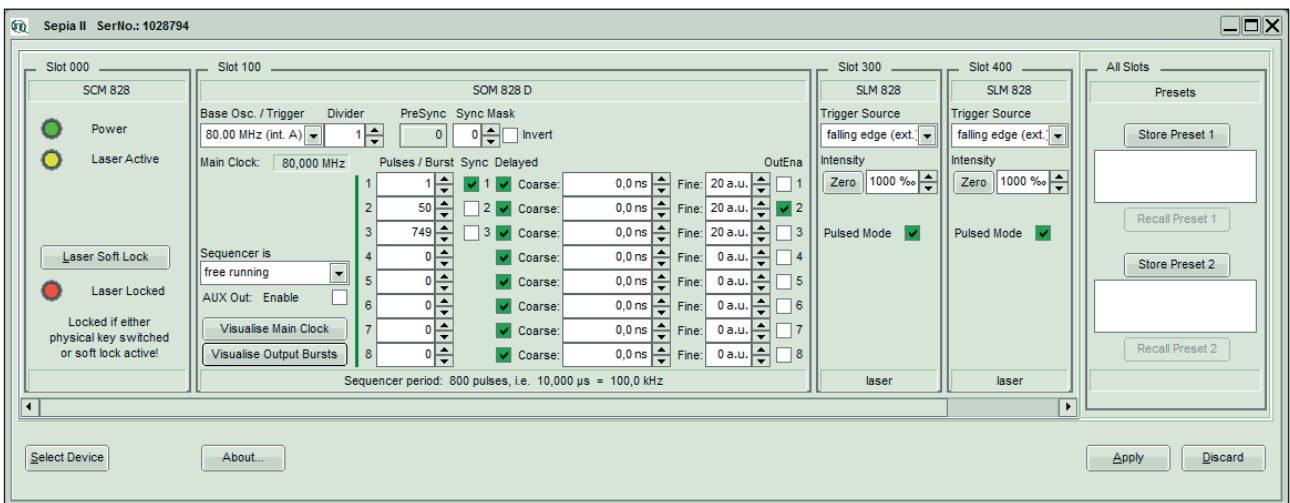

Figure 8: SEPIA II software graphical user interface (GUI) with a programmed excitation pulse sequence. In this example, the first output is only used to provide a SYNC signal and to initialize the sequence. The laser is attached to the oscillator output channel 2 and 50 pulses with a time delay of 12.5 ns (80 MHz clock) between each pulse are generated, which results in a total excitation time of 625 ns. The waiting time to the next pulse burst is defined by the 3rd output channel, which is just used as a counter delay. The entire sequence takes 10 µs to complete, corresponding to an excitation frequency of 100 kHz.

TimeHarp 260 P board when operated in the long-range mode[20]. This short dead time is only meaningful if the board is paired with a detector also having a very short dead time, i.e. PMTs or Hybrid-PMTs, as the dead time of SPAD detectors is typically in the range of several tens of ns.

Note that this is not problematic for the measurement of lifetimes in the ms range, as here even a dead time of 100 ns is still orders of magnitude shorter than the dye's lifetime, provided that the TCSPC board can span the complete time range between two pulses in the TTTR-mode.

Another important detector feature is the after-pulsing probability. The afterpulsing probability of most detectors is very low (mainly < 10%) and occurs on a time range of hundreds of ns, i.e. it is visible in the decay curve of a phosphorescence measurement. A reconvolution fit can be used to compensate for after-pulsing. However, it can be problematic especially in situations where a large contribution from fast fluorescence is combined with a small contribution from phosphorescence. In these cases, the after pulsing can appear as an additional bump on the decay curve at later times. If saturation effects occur during the recording of a short lived fluorescence, reconvolution fitting will not completely remove this artifact. There are two possible ways to avoid it: a) by using a detector with low after pulsing probability, like a Hybrid-PMT detector. Afterpulsing can also often be minimized for standard PMTs by illuminating the detector at a count rate of ~1 Mcps for a few hours in order to purge the tube.

b) The other possibility to remove the after pulsing artifact is to gate the detector, which is possible for certain single photon avalanche diode (SPAD) detectors. In this case, the detector is switched to an insensitive state during the times when bright fluorescence contributions arrive. Consequently, no after pulsing is generated in this case.

Conclusion

Modern detectors and TCSPC electronics open up new possibilities for significantly improving the performance of PLIM measurements. Also combinations of FLIM and PLIM measurements on a single instrument becomes feasible, as the fastest electronics provide TCSPC channel widths of 250 ps along with dead times of about 1 ns[20]. Nowadays it is possible to measure the oxygen concentration in living cells via a ruthenium-sensor along with the cells metabolism activity via the FAD-fluorescence in a single experiment[2]. The high achievable count rates combined with the lack of after pulsing make Hybrid-PMTs the detectors of choice for most PLIM applications[21]. Since some of the necessary components only have become available in recent years, the full potential of these techniques is still far from being exploited.

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