Fluorescence (Luminescence) Lifetime
Imaging application simplified...
with the **pco.**flim

lifetimes from
100 ps to 100 µs

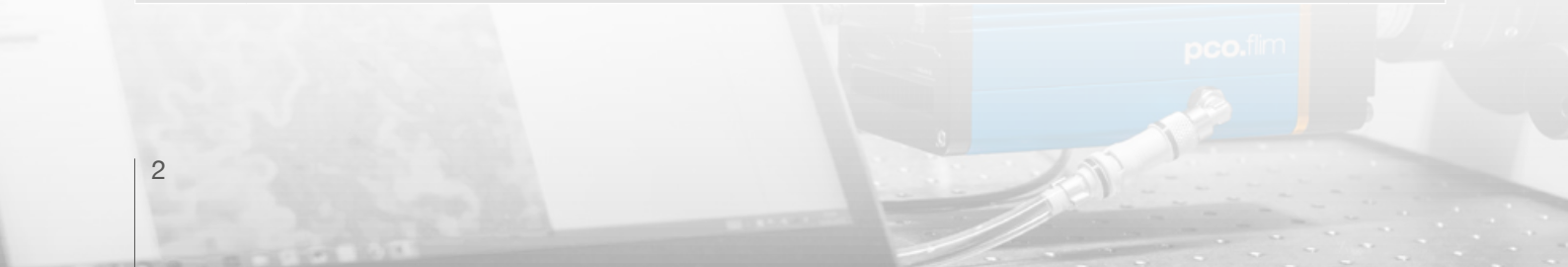
unique resolution
1008 x 1008 pixels

high frame rate
45 double frames/s

frequency synthesizer
5 kHz – 40 MHz

pco.

PCO.FLIM - FLUORESCENCE LIFETIME IMAGING CMOS CAMERA



1 PHOTOLUMINESCENCE

Photoluminescence describes an interaction of light and matter, when light is absorbed by certain compounds.

With this absorption of energy the molecules enter an excited state. Due to the fact that this excited state is not a stable state, several energy conversions take place. Some of these conversions are radiationless and some of them emit light with a lower energy than the absorbed light.

This radiative conversion is called luminescence (see fig. 1). Depending on the pathway of the physical process that causes the light emission the luminescence is either called fluorescence or phosphorescence. The energy states and conversions can be described by an energy level diagram, or Jablonski diagram, which is shown in figure 2.

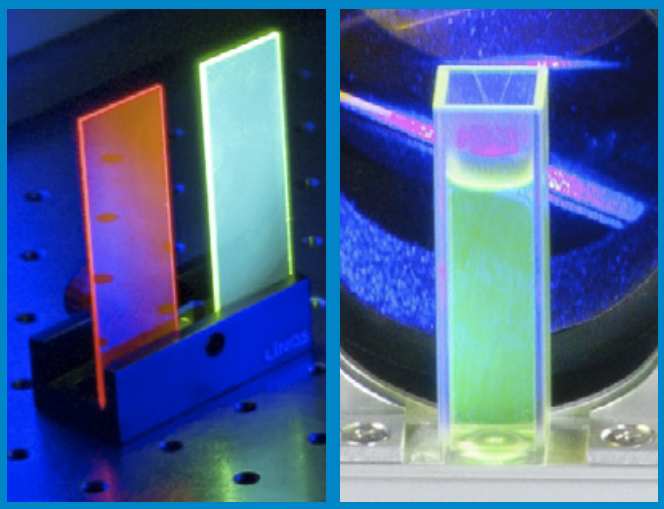
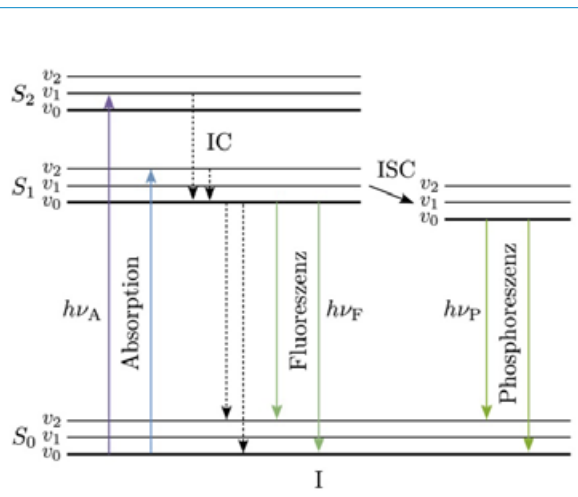


Figure 1: Images of fluorescing plastic slides and a cuvette with a fluorescine solution.

After absorption of light with the energy $h\nu_A$ (see fig. 2) the molecule turns from the ground energy level S_0 into an excited state S_1 or S_2 or other. Since these states are unstable the molecule tries to return to the stable ground state. During internal conversions (fig.2, IC) part of the absorbed light energy is converted into vibrational or thermal energy. From there it is possible that a radiationless conversion happens or light is emitted, with the energy $h\nu_F$, which is called fluorescence.

Another option involves an intersystem crossing (fig. 2, ISC) which includes a quantum mechanically less probable reversion of the electron spin and a consecutive emission of light with the energy $h\nu_P$, which is called phosphorescence. There are also energy transfers possible between the atoms of a molecule, but they are all radiationless and they are excluded from figure 2 for simplicity.

All of these conversions require time and therefore the radiative conversions like fluorescence and phosphorescence can be characterized and distinguished from each other by their corresponding time constants. Generally fluorescence takes less time, therefore the corresponding decay times are shorter in the range up to 10^{-8} s (10 ns). The intersystem crossing is less probable, which means, if it happens, it takes more time. Because of that the corresponding decay times are longer in the range of 10^{-6} – 10^{-3} s (µs – ms). These different decay times represent a good distinction whether the emitted light can be considered fluorescence or phosphorescence, if the underlying process is unknown.



Jablonski diagram, an energy level scheme for photoluminescence.

2 TIME RESPONSE OF PHOTOLUMINESCENCE

Assuming the availability of a light source, which rises and descends more than a hundred times faster than the luminescence, then figure 3 shows the temporal behavior of the excitation light and the luminescence that it has caused. When the excitation light is switched on (blue curve in fig. 3) the luminescence emission starts as well, but it takes some time until all radiative emission begins, therefore a delay is visible that in technical terms is called a rise time of a “lowpass” system. Then a steady state is reached with a constant luminescence emission and constant excitation (if photo bleaching and other loss effects are neglected).

When the excitation light is switched off, a decay behavior can be observed and measured, characterized by the same time constant as for the rise curve. It takes some time until all excited levels are empty again. This time constant is usually called luminescence decay time or luminescence lifetime. Together with the intensity (quantum efficiency) it is one of the characteristic parameters of each luminophore, which can be used for analytical or sensing purposes.

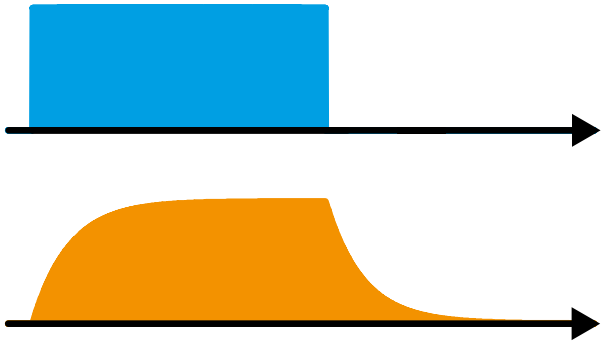


Figure 3: Temporal behavior of excitation light (blue) and corresponding luminescence (orange) emission (intensity vs. time).

3 LUMINESCENCE INTENSITY & LIFETIME

Both parameters, the luminescence intensity and the lifetime, are widely used for a large variety of analytical measurements. In Life Sciences the measurement of the luminescence intensity is a major tool for all kinds of microscopy and analysis, to visualize structures, metabolic processes, etc. If luminescent indicators are applied both parameters could be used for calibration purposes. Sometimes they contain the same information, but in many applications they are diverse, like in figure 4 where intensity and lifetime distribution show differences. While there are plenty of instruments to detect or measure luminescence images, only few exist to measure the 2D distributions of the corresponding luminescence lifetimes. In addition, if any luminophore should be used for sensing purposes (e.g. pressure sensitive paint, oxygen sensing) and a calibration curve has to be determined, every intensity based calibration strongly depends on the stability of the light field and the optical situation. In simpler words: For intensity based calibrations it matters whether 100 or 500 molecules are emitting light, but for lifetime based calibrations it is not relevant as long as they all have the same lifetime.

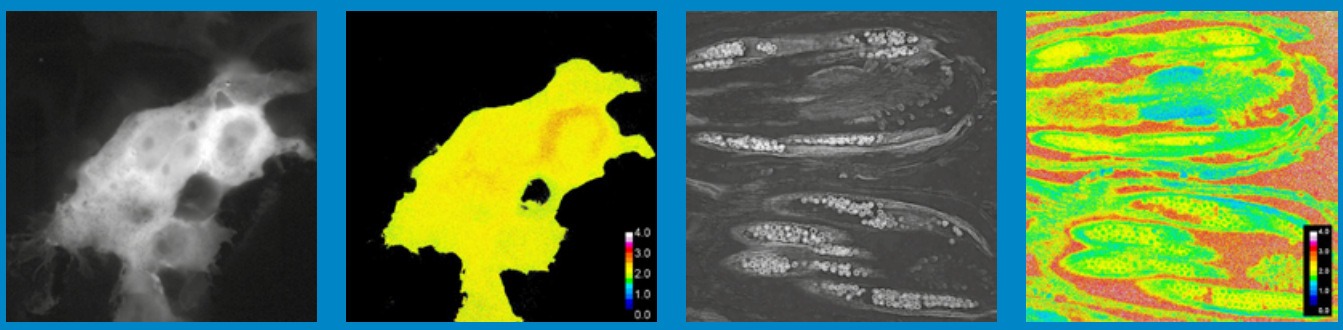


Figure 4: Two sets of fluorescence intensity images and their corresponding fluorescence lifetime distributions. Left: HEK-293 cells that expressed DJ-1/CFP, which was a control experiment. Right: an endogenous fluorescence image of a daisy slice sample and the corresponding lifetime distribution. The left image was recorded with a 60x oil immersion objective and the right image was obtained with 40x air objective. The modulation frequency was 30 MHz and the exposure time for one double image was 500 ms (left) and 100 ms (right). The lifetimes are color coded in units of ns. (courtesy of Prof. Dr. F.S. Wouters and Dr. G. Bunt, University Medicine Göttingen)

Authors: Gerhard Holst & Robert Franke

4 LUMINESCENCE LIFETIME & FLIM

As shown in chapter 2 the luminescence lifetime is one characteristic parameter of a luminophore, and it is either called a lifetime (describing the time, the excited molecules stay or "live" in an excited state) or decay time (the characteristic time that describes the luminescence decay curve after switching off of the excitation light). Both expressions are commonly used for the same time constant. Furthermore, the photoluminescence is split up into fluorescence and phosphorescence with their corresponding lifetimes, and in many cases the term fluorescence lifetime is used to name or describe most luminescence lifetime measurements.

When the first luminescence lifetime images were measured, this technique was called Fluorescence Lifetime Imaging Microscopy, because the measurements were made with microscope, so it was abbreviated to FLIM. Later this acronym was used for all types of fluorescence lifetime imaging. Recently some scientists recognize the differences in the underlying processes again and created the term PLIM for phosphorescence imaging, ignoring the origin of the "M" for microscopy. The literally correct term of "LLI" or "LLIM" for Luminescence Lifetime Imaging is not used, due to the simple fact that it does not sound good. Following the rule of common use the new camera system for luminescence lifetime imaging is called pco.flim.

5 HOW TO DO LUMINESCENCE LIFETIME IMAGING

5.1 Time Domain

In time domain, like figure 3 suggests, the ultimate goal is the acquisition of the decay curve. Since the quantum efficiency of photoluminescence is usually rather small, the light intensity of the luminescence signal is weak. Therefore the most common detection methods involve very sensitive detectors like photomultiplier tubes and low signal detection via photon counting. Hence for single point measurements and scanning applications the most common method is "Time Correlated Single Photon Counting" (TCSPC). Here many measuring points are collected in the time domain, when the excitation light is switched off (see fig. 5). The measuring systems require a large bandwidth, such that they do not alter the decay curve due to any bandwidth limitation.

When enough data points are collected, a predefined decay curve based on the assumption of one or more present decay times is fitted. The decay times resulting from this fitting process are the "measured data". For imaging purposes every point in the sample is scanned and processed in this way. This method gives good results for multi-exponential decay curves, but it is relatively slow for image uptake due to the involved scanning process. Another option for time domain imaging is the synchronized integration over different time slots or windows of the decay curve (also called boxcar integration). In this case one image is taken over a certain interval of the decay curve. A second image has to be taken over a different interval of the decay curve (see figure 6).

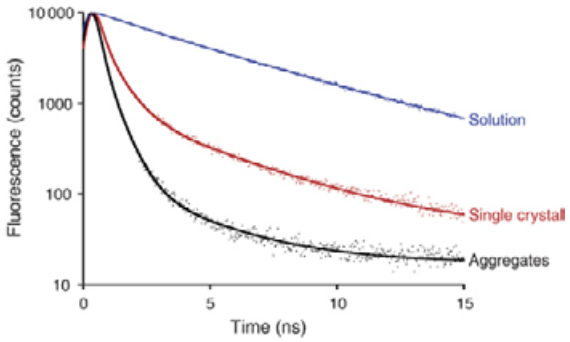


Figure 5: Typical TCSPC measurements (dots) with the corresponding fitted decay curves (lines).

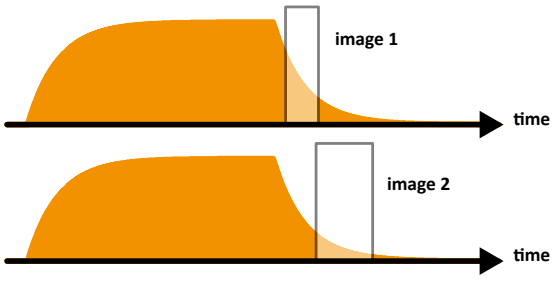


Figure 6: Integration of luminescence over two different intervals of the decay curve, resulting in two images.

5 HOW TO DO LUMINESCENCE LIFETIME IMAGING

If the signal is weak the procedure can be repeated many times while each portion of light generated charge carriers is integrated on the image sensor (sensicam sensimod, pco.1600, 2000, 4000 mod). Thus, in most cases the modulated light signal is synchronized with the image recording so, that the generated charge carriers are only accepted in the externally adjusted time interval, which is active only when the excitation light is off.

Note: Although in principle this type of measurement does not require excitation and emission filters to separate the radiation, they should be used anyway. The excitation light might be significantly brighter than the luminescence, exceeding the blocking efficiency of the image sensor, which integrates during the exposure time, but has to block the excitation light during the image sensor "off" phase.

Figure 7 illustrates the real integration on the image sensor for the two images.

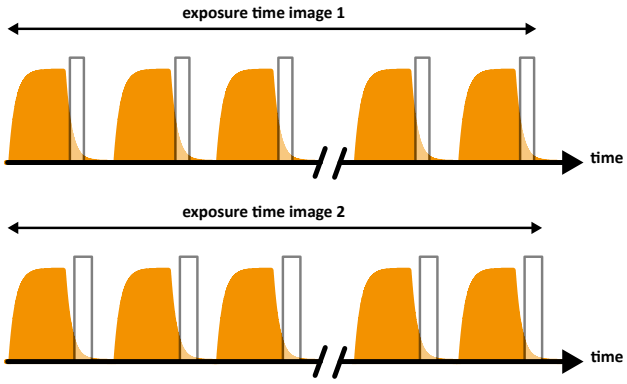


Figure 7: Integration of luminescence in two images over two different windows of the decay curve using a real camera. The integration on the chip is repeated numerous times until enough light has been collected for an image readout.

In contrast to the single point measurements, using this type of measurement with two images integrated over different "windows" only a single luminescence lifetime or decay time can be resolved. If more lifetimes are supposed to be present in the imaged sample, more integration windows have to be recorded, otherwise these lifetimes cannot be resolved. For short lifetimes and weak signals this detection scheme might require both, short exposure times and long total accumulation times. In case the real lifetime information is not required, but only a stable, reproducible signal is of interest, for example for sensing purposes when the luminescence lifetime unambiguously correlates to the change of an analyte, it is possible to simplify the detection scheme by a ratio type measurement called "Rapid Lifetime Determination"[1]. Instead of using two windows in the low light range, the first window is chosen in the rise phase of the luminescence signal and the second integration window is placed in the decay phase of the luminescence signal (see figure 8).

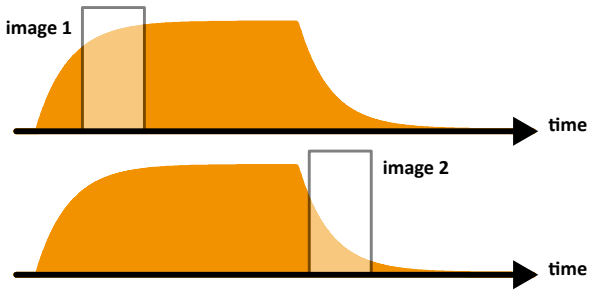


Figure 8: Integration of luminescence in two images over two different windows of the decay curve for the use of the Rapid Lifetime Determination (RLD) method (intensity vs. time).

Instead of evaluating the luminescence lifetime or decay time the ratio of both images is used as a measuring signal for calibration of the luminescence signal vs. an analyte concentration. This method has fewer requirements with respect to time resolution, and it delivers a simple decay time depending signal for measuring purposes.

[1] An error analysis of the rapid lifetime determination method for the evaluation of single exponential decays, Richard M. Ballew and J.N. Demas, Analytical Chemistry,1989, Vol. 61 (1), pp 30–33

Authors: Gerhard Holst & Robert Franke

5 HOW TO DO LUMINESCENCE LIFETIME IMAGING

5.2 Frequency Domain

In frequency domain a continuously modulated excitation signal is used instead of a pulse or repetitive pulse signal. This requires a good separation of excitation and luminescence light by proper optical filtering. The time response of such an excitation is shown in figure 9.

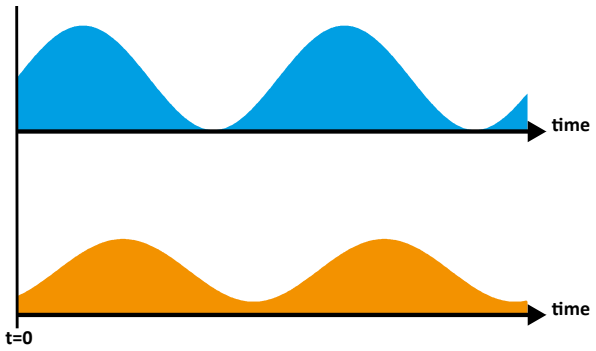


Figure 9: Time response of excitation light (blue) and luminescence (orange) emission if luminescence lifetime imaging is done in frequency domain (extract from continuous signals, intensity vs. time).

In the ideal case of a maximum modulation depth, the excitation signal changes between maximum light and darkness (zero light). The photoluminescent molecules respond by emitting luminescence. This decay time is not instantaneous and has a delay, with a smaller amplitude and changed amplitude. Changes in the delay, changed amplitude and constant component, can be measured and used to determine the luminescence lifetime of the molecule. Due to the known modulation frequency the time delay introduced by the luminescent molecules can also be expressed by an equivalent phase angle or phase shift.

In the technical world this is important, since luminophores can be considered as linear time-invariant (LTI) systems, which can equivalently be analyzed either in the time or the frequency domain. Figure 10 shows both the excitation and the luminescence emission signal in one graph and denotes the important characteristic parameters, which can be used for the lifetime determination.

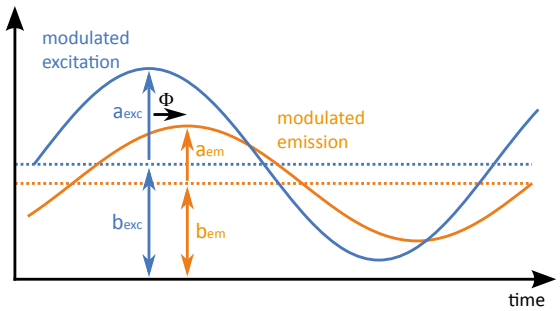


Figure 10: Time responses of excitation light (blue) and luminescence (orange) emission if luminescence lifetime imaging is done in frequency domain (extract from continuous signals). Amplitudes a_{exc} and a_{em}, constant or direct components b_{exc} and b_{em} and phase angle or phase shift Φ are denoted.

With the division of the amplitudes (a_{exc}, a_{em}) by their constant components (b_{exc}, b_{em}) a parameter is derived, which is independent of the intensity. It is called the modulation index (m_{exc}, m_{em}). The relation between the modulation indices and the luminescence lifetime τ_M in a mono-exponential decay is as follows:

$$m = \frac{m_{em}}{m_{exc}} = \frac{a_{em}/b_{em}}{a_{exc}/b_{exc}} = \frac{1}{\sqrt{1+(2\pi \cdot f_{mod})^2 \cdot \tau_M^2}}$$

The delay between both sinusoidal signals of the same frequency can be expressed by a phase shift with its corresponding phase angle Φ. This Φ also has a direct relation to the luminescence lifetime τ_P in a mono-exponential decay and the modulation frequency:

$$\tan(\Phi) = 2\,\pi\, f_{mod}\, \tau_P$$

For multi-exponential decays the relationships are more complex[2]. If the parameters modulation index and phase angle can be determined at multiple frequencies, it would therefore be possible to derive the corresponding luminescence lifetimes.

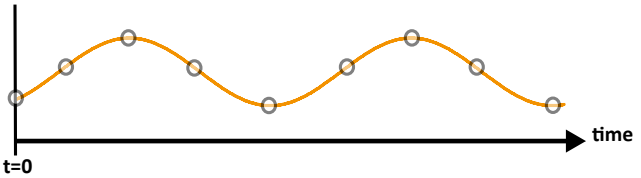


Figure 11: Sinusoidal signal (orange) with sampling points (grey circles)

For a proper measurement of these parameters, it is necessary to acquire the sinusoidal signal. In digital signal processing this is usually done by sampling and a subsequent "reconstruction" of the signal. Figure 11 shows a sinusoidal signal with some sample points. For a correct reconstruction it is necessary to sample at more than twice the frequency of the signal (Nyquist criterion), therefore four sample points or more per period should be sufficient.

For imaging applications it is not possible to acquire these sample points, since it would require extremely short exposure times with insufficient light, even for a repetition. Hence, instead of a discrete sampling the luminescence signal is integrated over a certain time window as illustrated in figure 12.

[2] See: "Principles of Fluorescence Spectroscopy", J.R. Lakowicz, Springer or "Hochintegriertes Kamerasystem für die Multifrequenz-Lumineszenzabklingzeitbildgewinnung", PhD thesis by R. Franke.

5 HOW TO DO LUMINESCENCE LIFETIME IMAGING

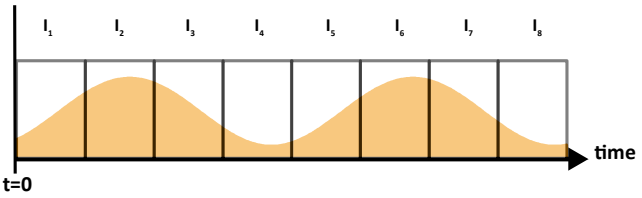


Figure 12: Sinusoidal luminescence signal (orange) with four sampling integration windows per period (grey rectangles), where the integrated light results in the intensity images I_1 .. I_8 (I_5..I_8 are the repetition of I_1..I_4)

In the example in figure 12 the first four integration windows have been chosen so that the intensity I_1 can be related to the sine value at $\Phi = 0°$, I_2 to $\Phi = 90°$, I_3 to $\Phi = 180°$ and finally I_4 to $\Phi = 270°$. From these four values I_1 .. I_4 the phase angle Phi, the modulation index m_{em} and the intensity of the signal can be calculated:

$$\Phi = \arctan\left(\frac{I_2 - I_4}{I_1 - I_3}\right)$$

$$m_{em} = 2 \cdot \frac{\sqrt{(I_2 - I_4)^2 + (I_1 - I_3)^2}}{I_1 + I_2 + I_3 + I_4}$$

$$\text{intensity} = b_{em} = \frac{I_1 + I_2 + I_3 + I_4}{4}$$

If typical readout times for one image in modern image sensors are considered, obviously for fluorescence lifetimes in the range of nanoseconds four integration windows have to be recorded in a sequence of 4 images one after the other. Further, assuming a realistic modulation frequency of 20 MHz for short luminescence lifetimes, a proper integration window would last for a fourth of a period resulting in an exposure time of 12.5 ns. This would not be enough time to integrate these weak light signals. Thus, the integration is repeated many times within the overall exposure time very similar to the time domain measurement shown in figure 7. This would look like the integration windows shown in figure 13. Still, each single integration time would be very short and difficult to achieve. For technical reasons a different evaluation or detection method based on the same assumptions and calculations can be chosen. The idea is to enlarge the integration window for the collection of more light to a half period and imitate the behavior of a two tap switch, meaning that two integrations in a sequence are performed. If we look at this situation, shown in figure 14, the integration windows cover half of a period of the sinusoidal light, and always one window is immediately followed by the other. Doing so, no photons are lost, and they all contribute to the overall acquired signal.

At first, the image I_1 is recorded, which corresponds to a phase angle of $\Phi = 0°$ and immediately after a second image I_3 is recorded, which corresponds to a phase angle of $\Phi = 180°$. In a second step the synchronization between the integration windows and the detected sinusoidal luminescence signal is changed by adding an artificial phase angle of 90°. Now, the integration is repeated in the same way, first the image I_2 is recorded, which corresponds to a phase angle of $\Phi = 90°$, and immediately after a second image I_4 is recorded, which corresponds to a phase angle of $\Phi = 270°$ (see fig. 14). A beneficial effect is, that the modulation signal, which controls the synchronization of the integration windows, has the same frequency as the sinusoidal luminescence signal. The next chapter will show why this method is convenient for FLIM.

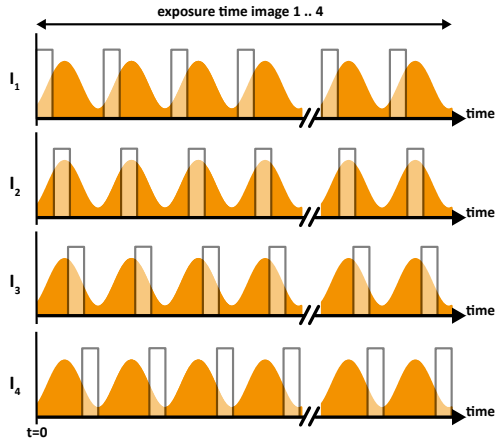


Figure 13: Sinusoidal luminescence signal (orange) with sampling integration windows (grey rectangles), where for each image the same part of the light waveform is collected within the same integration window. The example shows the repetitive integration at four different positions of the integration window, resulting in four images (theoretical consideration).

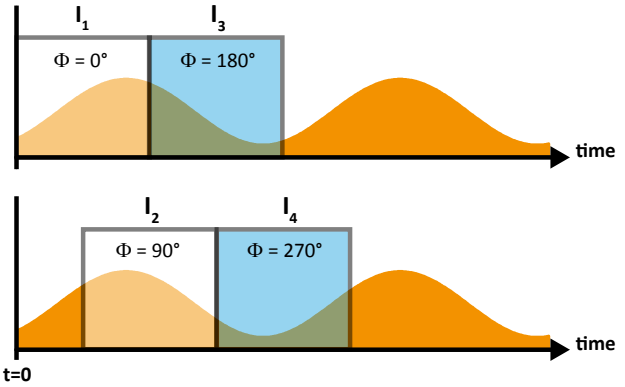


Figure 14: Sinusoidal luminescence signal (orange) with sampling integration windows (grey rectangles). At first for the first half of the period the image I_1 is integrated, which corresponds to $\Phi = 0°$, and subsequently the image I_3 is integrated, which corresponds to $\Phi = 180°$. For the next recording the synchronization is shifted by 90°, such that the first half period of integration covers I_2, which corresponds to $\Phi = 90°$, and subsequently the image I_4 is integrated, which corresponds to $\Phi = 270°$ (theoretical consideration).

Authors: Gerhard Holst & Robert Franke

6 QMFLIM2 - A DIRECTLY MODULATABLE CMOS IMAGE SENSOR

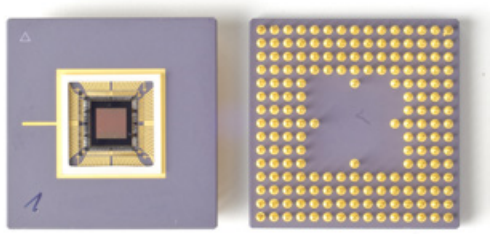


Figure 15: Top and bottom view of the package with the new QMFLIM2 CMOS image sensor

Within the research project FLI-Cam, from 01.03.2007 – 30.11.2012, a new CMOS image sensor, QMFLIM2 (see fig. 15), has been developed by the CSEM (Centre Suisse d'Electronique et de Microtechnique SA, Zürich, Switzerland) and PCO. Each pixel of the image sensor has two charge collection sites, called tap A and tap B, and a switch, which can be controlled by an external signal.

This configuration acts like a charge swing, which is illustrated in figure 16. The external two-level voltage signal, called modulation signal, selects whether tap A or tap B is active. If tap A is active, the photogenerated charge carriers are directed to the tap A charge bucket, when tap B is active, the carriers drift into the tap B bucket. If this switching of the signal corresponds to the zero-crossing of a sinusoidal or rectangular signal (with a duty cycle of 50%) at constant frequency, the tap A corresponds to a phase angle of 0°, while tap B corresponds to a phase angle of 180°. This is shown in figure 17, which is a repetition of figure 16 just with the additional information of the corresponding phase angle. Whenever the control signal for tap A (rectangular signal in figure 17 above the left bucket) is active (respectively high) the light generated charge carriers flow into the Φ = 0° (tap A) bucket, while tap B is inactive. Whenever the tap B signal is active, the charge carriers are collected in the Φ = 180 ° (tap B) bucket.

This mechanism can be used to integrate over a half period of the sinusoidal signal in the given example. The 0° information of the modulated light signal is collected during the first half period and the 180° information of the modulated light signal is collected during the second half period (see figure 14). The QMFLIM2 image sensor has following general characteristics. With a resolution of 1024 x 1024 pixels equipped with microlenses it is the only directly modulatable CMOS image sensor with such a high resolution. The pixel pitch of 5.6 µm is a good fit for a combination with a microscope, and the intra-scene dynamic of 10 bit is well suited for many luminescence imaging applications.

TABLE 1

PARAMETER	QMFLIM2
resolution	1024 x 1024 pixels
pixel size	5.6 µm x 5.6 µm
frame rate	45 double frames/s
modulation frequency range	0 - 40 MHz
quantum efficiency	appr. 39 %
dynamic range	1 : 1024
power consumption	4 W

When the image sensor is read out two images are generated simultaneously, a tap A and a tap B image. Due to the image sensor architecture the light signal has to be switched off or suppressed, when the recorded double images (frames) are read out, otherwise the light falling onto the sensor will cause additional noise in the read out images.

Any assymetry can be calibrated out. Alternatively the phase can alternate so that tap A recieves phase 0° half of the time and phase 180° degrees for the other half. This causes the assymetry to average out but the effective frame rate is cut in half. (described in detail in chapter 7.4).

[3] created after "All-solid-state lock-in imaging for wide-field fluorescence lifetime sensing", A. Esposito, 2005

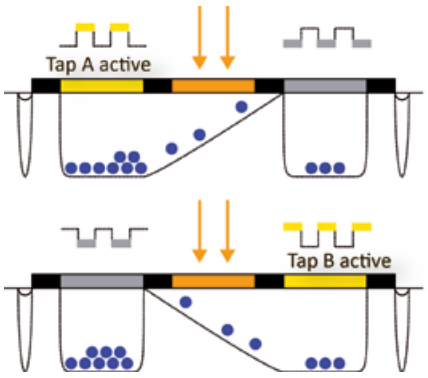


Figure 16: Schematical drawing[3] of the in-pixel charge swing of the QMFLIM2 image sensor.

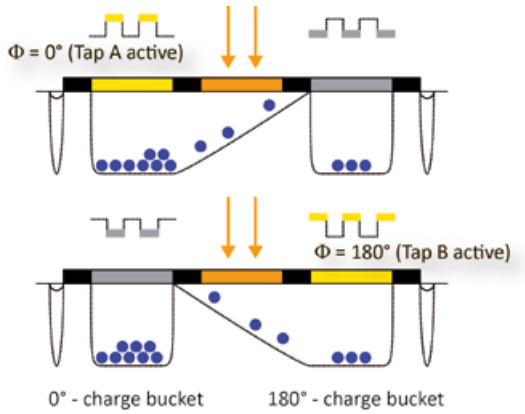


Figure 17: Schematical drawing of the in-pixel charge swing of the QMFLIM2 image sensor with control signal and phase angle added.

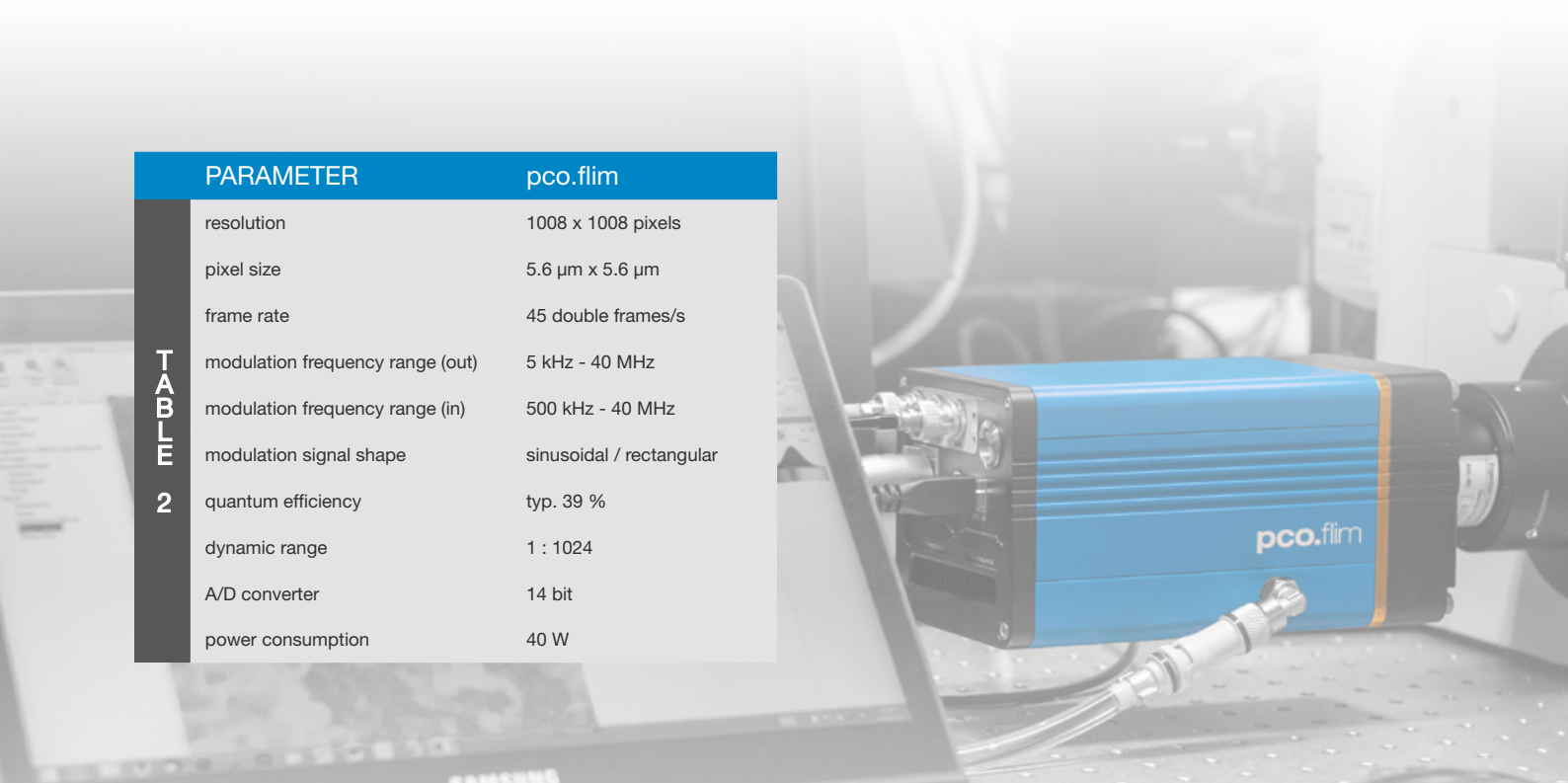
7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

7.1 Camera System

Figure 18: Different views of the pco.flim camera system, from left to right: front view without lens – side view without the water cooling connectors and back view.

TABLE 2

PARAMETER	pco.flim
resolution	1008 x 1008 pixels
pixel size	5.6 µm x 5.6 µm
frame rate	45 double frames/s
modulation frequency range (out)	5 kHz - 40 MHz
modulation frequency range (in)	500 kHz - 40 MHz
modulation signal shape	sinusoidal / rectangular
quantum efficiency	typ. 39 %
dynamic range	1 : 1024
A/D converter	14 bit
power consumption	40 W



The pco.flim camera system is the first luminescence lifetime imaging camera using the CMOS image sensor QMFLIM2. It offers all the required generation of frequency domain signals (5 kHz – 40 MHz) and also allows the use of external modulation signals in a limited frequency range (500 kHz – 40 MHz).

It has a USB 3.0 interface for image data transfer and control of all camera operation modes. Further, a variety of trigger input / output signals for integration of the camera into any application framework is available. Table 2 gives an overview of the performance data of the camera system.

7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

7.2 Principle Structure of an Experimental Set-Up

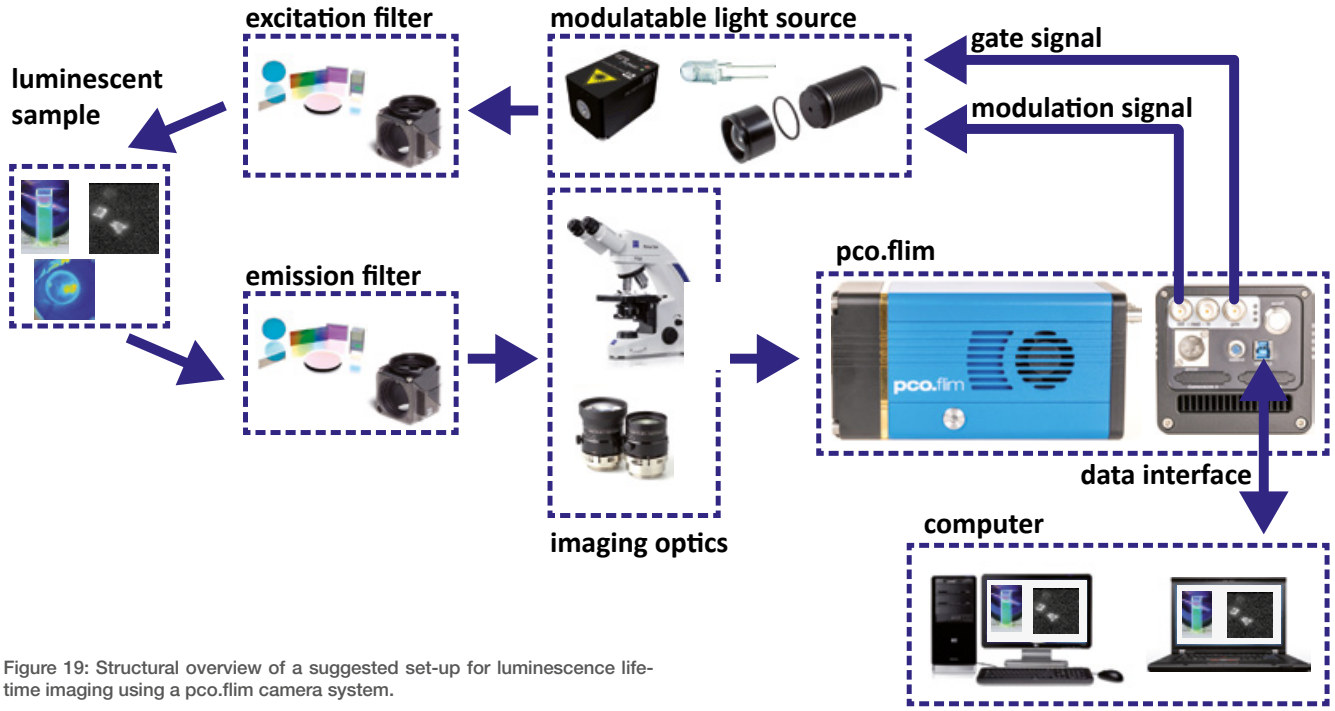


Figure 19: Structural overview of a suggested set-up for luminescence lifetime imaging using a pco.flim camera system.

In figure 19 an overview of a set-up for luminescence lifetime imaging using a pco.flim camera system is suggested, if the camera is the frequency master. The pco.flim camera sends the modulation signal and the "dark" gate signal to the light source, which must be capable of accepting both signals. While the modulation signal defines the shape of the excitation light, the gate signal controls whether the excitation light is generally switched on or off, because the light has to be switched off during the time of the image readout[4].

It depends on the application which modulatable light source is appropriate with respect to the required frequency range (fig. 19, modulatable light source). It can be anything from LED to laser diodes that can be properly modulated in the intended frequency range. The modulated light passes an optical excitation light filter (fig. 19, excitation filter) and will excite a luminophore in the sample of interest.

It might be necessary to use additional optics to guide and shape the light to the sample. These optics are not included in the overview. The luminescent sample in turn will emit luminescence light. This light has to pass appropriate optical emission filters (fig. 19, emission filter) and will be imaged by optics (fig. 19, imaging optics) to the image sensor of the pco.flim. It is not important whether the emission has to pass first the optics and then the filter or vice versa, in figure 19 just one version is shown. The kind of optics can range from lenses to microscopes, depending on the application. According to the operation modes and settings the camera system pco.flim will transfer the images to the controlling computer (fig. 19, computer) via the USB 3.0 data interface. The components shown in figure 19 are just examples to demonstrate the flexibility of the pco.flim system. Since the camera includes the generation and control of the modulation signals, the overall set-up is relatively simple.

Note: In principle the decay or lifetime based measurements are independent of any changes in intensity. Nevertheless, in frequency domain measurements care has to be taken to efficiently block the excitation light. In frequency domain FLIM (FD-FLIM) the phase angle equivalent to the time shift between excitation and emission signal is measured. Assuming that this signal is the superposition of the emission signal and for example 10% of the excitation signal (due to inefficient blocking), the phase angle of the superposed signal is measured. The phase angle of this signal depends to a large extend on the phase angle of the luminescent signal, but also on the ratio of the intensities of the unblocked excitation signal and the emission as well. Thus, any change in luminescence intensity, while the excitation usually stays constant, will introduce an additional phase angle and can cause false results.

[4] The QMFLIM2 image sensor catches additional noise, when the light is on during the readout of the image sensor

7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

7.3 Single Frequency Measurement

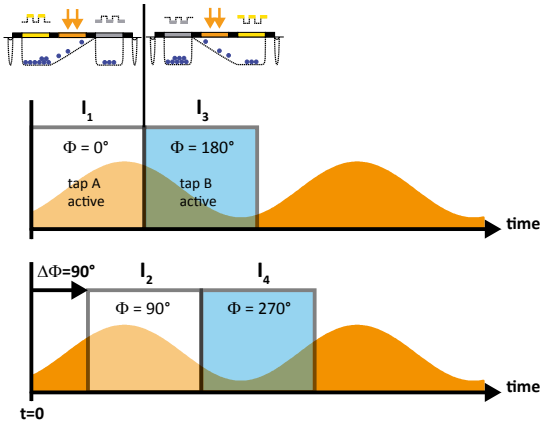


Figure 20: Sinusoidal luminescence signal (orange) with sampling integration windows (grey rectangles). At first, for Image I_1 the first half of the period is integrated, which corresponds to tap A is active and $\Phi = 0°$, and subsequently the image I_3 is integrated, which corresponds to tap B is active and $\Phi = 180°$. For the next recording the synchronization is shifted by $\Delta\Phi = 90°$, such that the first half period of integration covers I_2, which corresponds to tap A is active and $\Phi = 90°$, and subsequently the image I_4 is integrated, which corresponds to tap B is active and $\Phi = 270°$.

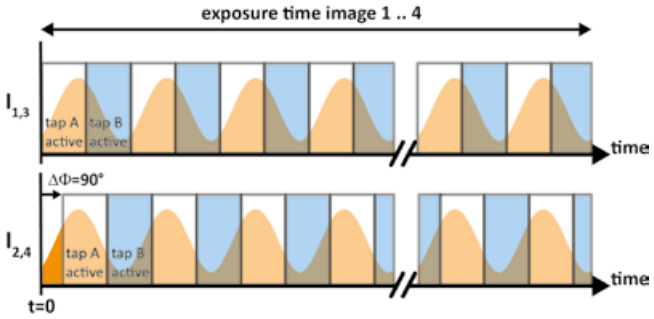


Figure 21: Sinusoidal luminescence signal (orange) with sampling integration windows (grey rectangles). Alternately, during the first half of the period Image I_1 is integrated (tap A is active $\Phi = 0°$), then image I_3 is integrated during the second half (tap B is active $\Phi = 180°$). This is repeated many times within the frame of the exposure time. For the next recording the synchronization is shifted by $\Delta\Phi = 90°$, such that the first half period of integration covers I_2 (tap A is active $\Phi = 90°$) and subsequently the image I_4 is integrated (tap B is active $\Phi = 270°$).

The two taps per pixel of the QMFLIM2 CMOS image sensor conducts the half period integration of a sinusoidal frequency signal as explained in chapter 5.2 and figure 14. For a better understanding this integration is combined with the charge swing schematic of the pixel in figure 20. When tap A is active it corresponds to $\Phi = 0°$ and subsequently tap B becomes active, which corresponds to $\Phi = 180°$. In both cases the sinusoidal luminescence is integrated over half a period.

This procedure is repeated for a second double image, but the recording starts with 90° phase shift, such that the integration windows of tap A and tap B now correspond to the phase angles Φ of 90° and 270°, respectively.

In most applications, the integration of half a period of a luminescence signal will not collect enough light, therefore figure 21 shows the time lapse of a real recording with four different images corresponding to $\Phi = 0° - 90° - 180° - 270°$, where the half period integration portions are accumulated in each pixel.

Note: There is no guaranty that the overall number number of integration periods is an integer and always the same, because the exposure time is independently adjusted and set from the frequency modulation. Usually, this can be neglected since the number of accumulated periods is much larger than a single period.

By this method 4 images are recorded, which represent the input data for further calculations. As explained in chapter 5.2 (and in more details in the Appendix) based on these images a phase angle, a demodulation index and an intensity value can be calculated per pixel, resulting in three computed images.

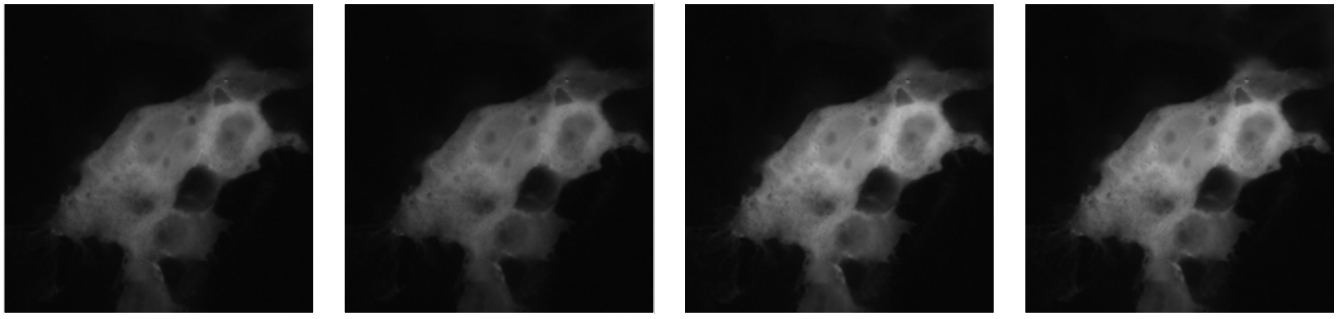


Figure 22: Four luminescence images corresponding to $\Phi = 0° - 90° - 180° - 270°$ are acquired.

Authors: Gerhard Holst & Robert Franke

7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

As an example from a research project four luminescence images, which have been recorded in the described manner, have been acquired (see fig. 22). Based on these four images the three images shown in figure 23 can be calculated. But at this point there are still some parameters unknown: the initial phase angle of the system including the influences of the application set-up (optics, path length of light etc.), the modulation index of the excitation light and the demodulation efficiency of the image sensor. Hence, a known (photoluminescent) reference, with similar brightness and comparable spectral behavior, has to be measured with similar settings. Subsequently the modulation index and phase angle data from figure 23 have to be referenced, which results in the false color coded distributions in figure 24. The referencing is done by subtraction of the phase angles and by division of the demodulation indices.

If it is assumed that each luminescent image point can be described by a single luminescence decay time or lifetime, both images in figure 24, the modulation index distribution as well as the phase angle distribution, can be converted into corresponding lifetime distributions, which corresponds to a different display of the same information (see fig. 25), and it represents the information which can be best interpreted by the user in most cases.

Note: In most applications this assumption will be inaccurate, because it is likely that the so-called apparent lifetime, which is calculated, is in reality the composition of different luminophores with different lifetimes. If several components have to be analyzed, it is necessary to repeat the measurement with different modulation frequencies and perform a proper multi-component analysis[5,6], which corresponds to the fit of multiple lifetime decay curves in the time domain.

[5] „Hochintegriertes Kamerasystem für die Multifrequenz-Lumineszenzabklingzeitbildgewinnung“, Dissertation, Robert Franke

[6] „Principles of Fluorescence Spectroscopy“, Joseph R. Lakowicz, Springer Verlag

[7] Courtesy of Fred Wouters & Gertrude Bunt, University Medicine Göttingen

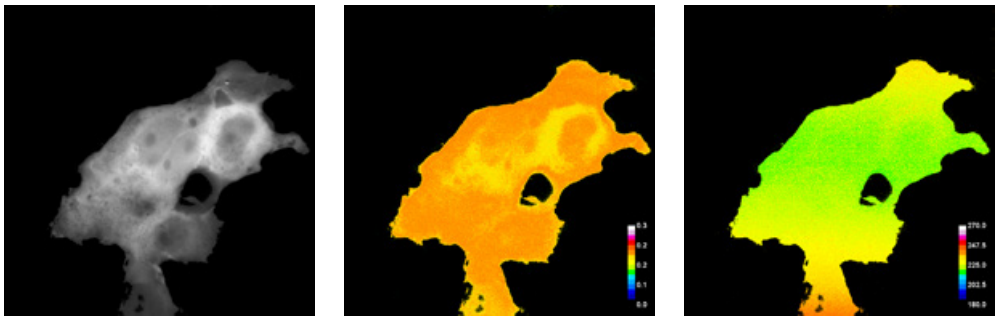


Figure 23: The three result images based on the recorded images shown in figure 22.

From left to right: intensity (normalized), modulation index (dimensionless), phase angle [degree] (false color coded).

Courtesy of Prof. Dr. F.S. Wouters and Dr. G. Bunt, University Medicine Göttingen

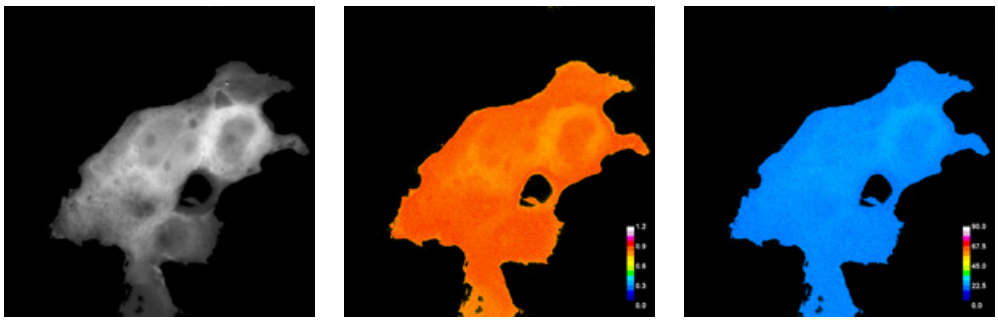


Figure 24: The three images out of figure 23, now referenced.

From left to right: intensity (normalized), modulation index (dimensionless), phase angle [degree] (false color coded).

Courtesy of Prof. Dr. F.S. Wouters and Dr. G. Bunt, University Medicine Göttingen

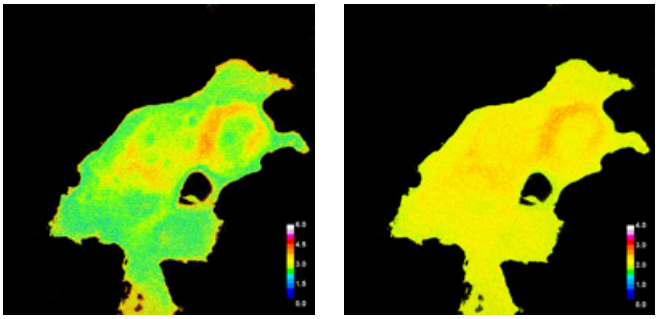


Figure 25: The referenced modulation index and phase angle distribution images from figure 24 are converted into luminescence lifetime distribution images.

From left to right: lifetime distributions based on modulation index and phase angle [ns] (false color coded).

Courtesy of Prof. Dr. F.S. Wouters and Dr. G. Bunt, University Medicine Göttingen

7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

7.3 Single Frequency Measurement

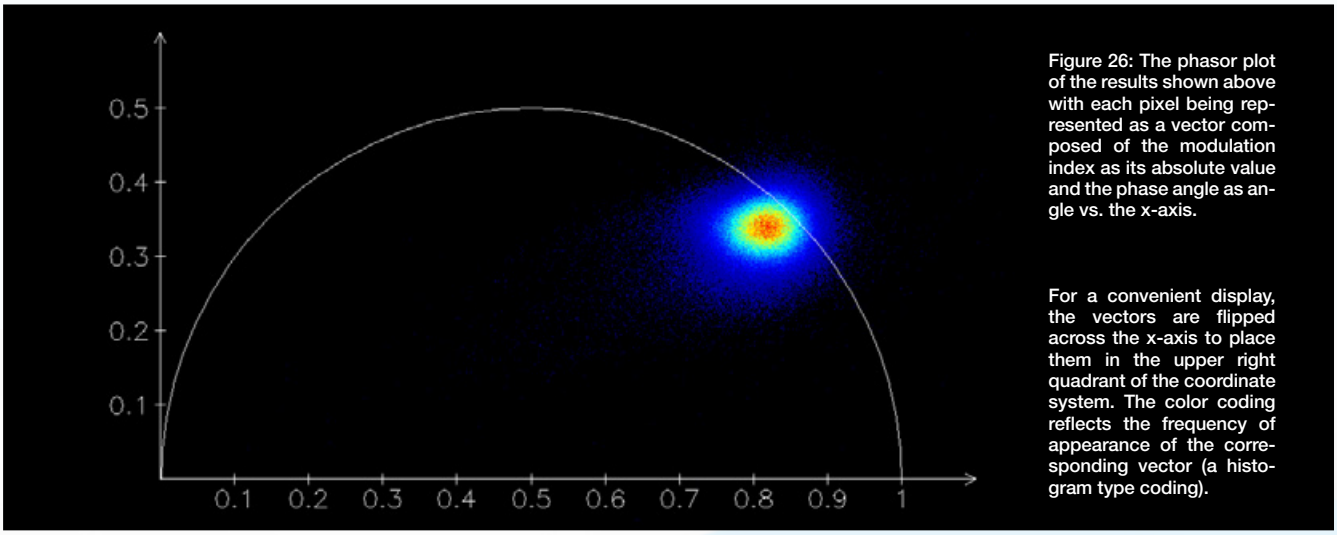


Figure 26: The phasor plot of the results shown above with each pixel being represented as a vector composed of the modulation index as its absolute value and the phase angle as angle vs. the x-axis.

For a convenient display, the vectors are flipped across the x-axis to place them in the upper right quadrant of the coordinate system. The color coding reflects the frequency of appearance of the corresponding vector (a histogram type coding).

Very often both luminescence lifetime distributions differ from each other, which can be a hint for the presence of multiple lifetimes as described in literature. Generally, the modulation index signal is more noise dependent than the phase angle signal.
Another option to display the results is the use of a Nyquist plot, in FLIM applications called “Phasor Plot”, where both results, the modulation index and the phase angle are combined to a vector, with the absolute value given by the modulation index and the angle given by the phase angle (see fig. 26). This allows to comparison between time domain and frequency domain results. The semi circle is parametrized by the fluorescence lifetimes and the modulation frequency.

7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

7.4 Pixel Asymmetry

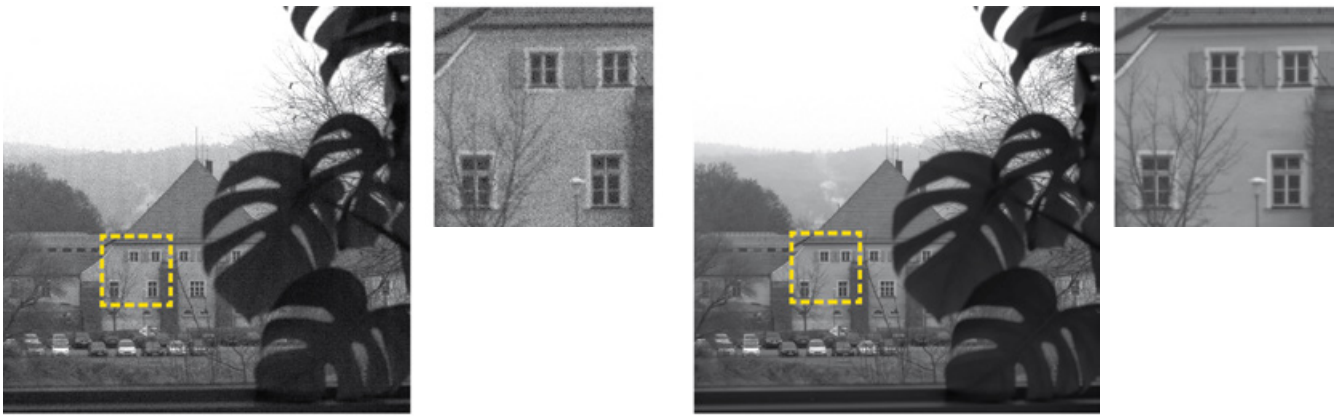


Figure 27: The left image shows an uncorrected image, obtained with tap A readout, while the right image shows the result image after an asymmetry correction, where the same image was read out from tap B as well, and both images have been averaged. The small images represent an extract of the area, which is denoted by the yellow square.

The left image in figure 27 shows a tap A readout image of a scene outside the lab. The right image is the result of the average of a tap A and a tap B readout, which corresponds to the second optional asymmetry correction, which is described below. The small image extracts in figure 27 show a zoom of the area within the yellow square in the images for better visibility of the differences. In principle there are two options to correct for the tap asymmetry.

Option 1 is an additional calibration, which should be done by the user since such a calibration would be dependent on frequency, potentially on wavelength and other parameters. Thus, it can be quite complex to foresee every possibility and store a lot of calibration data in the camera.

Option 2 is the extention of the additional phase shift to cover one whole period by both taps. The pitfall of this option is the reduction of the frame rate, because it requires to record twice as much images. On the other hand there is the advantage of improving the signal-to-noise-ratio, because two images containing the same information are averaged.

Figure 28 illustrates the acquisition procedure for asymmetry correction. First, as described before, two double images I_{1a} .. I_{4a} are recorded (fig. 28, 1 and 2) with the four phase angle integration windows at Φ = 0° - 90° - 180° - 270°. Then, the recording is extended by additional two double images with an additional phase shift beyond 180° and I_{1b} .. I_{4b} are recorded (fig. 28, 3 and 4) with the same four phase angle integration windows at Φ = 0° - 90° - 180° - 270°. Comparing the sampling windows of tap A and tap B, there are four pairs of tap A and tap B containing the same integral, i.e. phase information: I_{1a} / I_{1b}, I_{2a} / I_{2b}, I_{3a} / I_{3b}, I_{4a} / I_{4b}. If now these images are averaged, the asymmetry cancels out and the signal-to-noise ratio is improved.

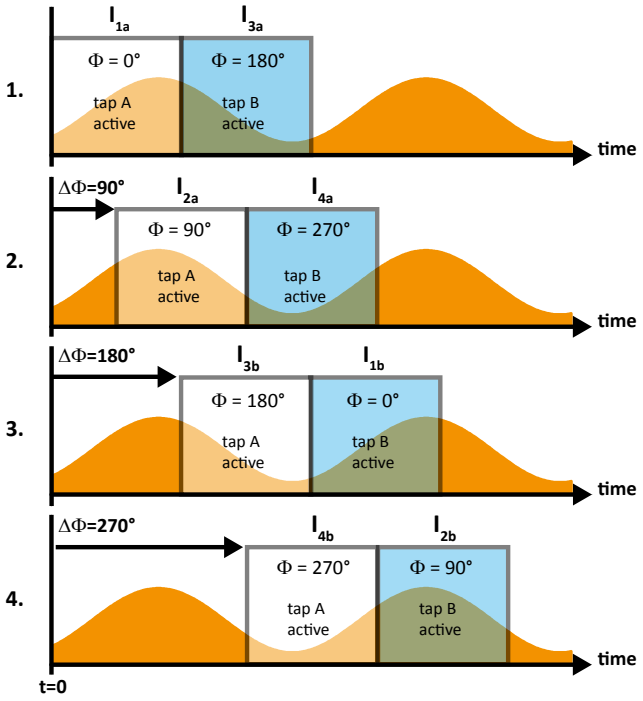


Figure 28: Schematically, 4 double images of a sinusoidal luminescence signal (orange) are recorded with sampling integration windows (grey rectangles). 1. Images I_{1a} and I_{3a} are recorded, which correspond to Φ = 0° (tap A active) and Φ = 180° (tap B active) 2. Images I_{2a} and I_{4a} are recorded, which correspond to Φ = 90° (tap A active) and Φ = 270° (tap B active) Now, the phase is further shifted beyond 180°, such that each tap sees all four phase integrals. 3. Images I_{3b} and I_{1b} are recorded, which correspond to Φ = 180° (tap A active) and Φ = 0° (tap B active) 4. Images I_{4b} and I_{2b} are recorded, which correspond to Φ = 270° (tap A active) and Φ = 90° (tap B active).

7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

7.5 Fluorescent Standards from Starna Scientific

To determine the initial phase of the optical system plus the camera system a reference measurement must be acquired using a fluorescence sample. This sample is excited by the same light, emits in a similar spectral range, and has a known mono-exponential fluorescence lifetime.

For this purpose we used reference materials from Starna Scientific[7], which were designed for molecular fluorescence spectrophotometry. Starna has "…dissolved the fluorescent materials in methylmethacrylate and the solution is then polymerized to produce a polymethylmethacrylate (PMMA) matrix, which provides a stable environment for the fluorescent compounds"[8]. By this means it is possible to create for example slide shaped samples, which can be placed on the microscope like the sample slides to record the reference values (see fig.29).

Starna Scientific offers seven different fluorophores of which we tested and used two dyes: Compound 610 (with λ_{exc} = 440 nm and λ_{em} = 480 nm) which is well suited for CFP type of dyes, and Rhodamine B (with λ_{exc} = 562 nm and λ_{em} = 573 nm). The corrected excitation and emission spectra are shown in figures 30 and 31 (source: Starna Scientific).

Starna Scientific offers to tailor the polymer material to the requested size and dimensions. We measured the following lifetimes of the compounds:

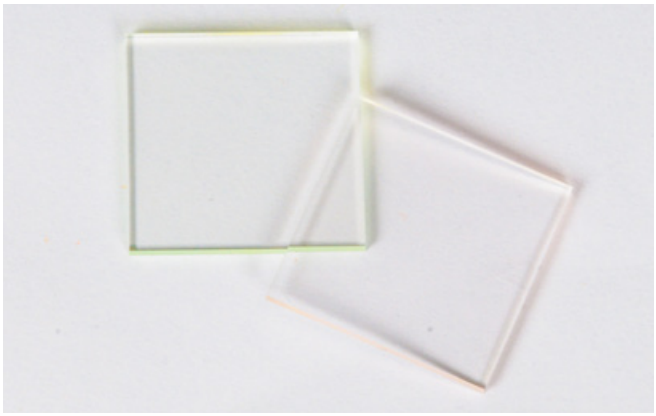


Figure 29: Sample slides with the fluorophores Compound 610 and Rhodamin B

Luminophore	Measured Monoexponential Lifetime [ns]	Excitation wavelength [nm] / Emission Wavelength [nm]
Compound 610	1.9	405[9] / 490
Rhodamine B	2.8	488[9] / 562

[7] http://www.starnascientific.com/
[8] taken from the „Reference materials for Molecular Fluorescence Spectrophotometry" brochure from Starna Scientific
[9] two photon excitation

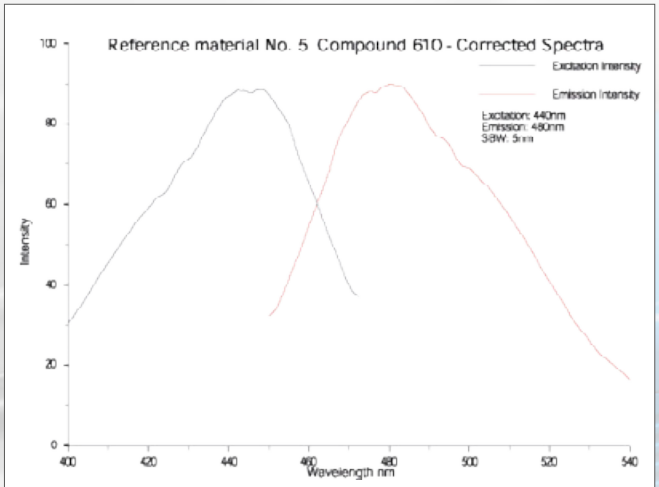


Figure 30: Corrected excitation and emission spectra of Compound 610

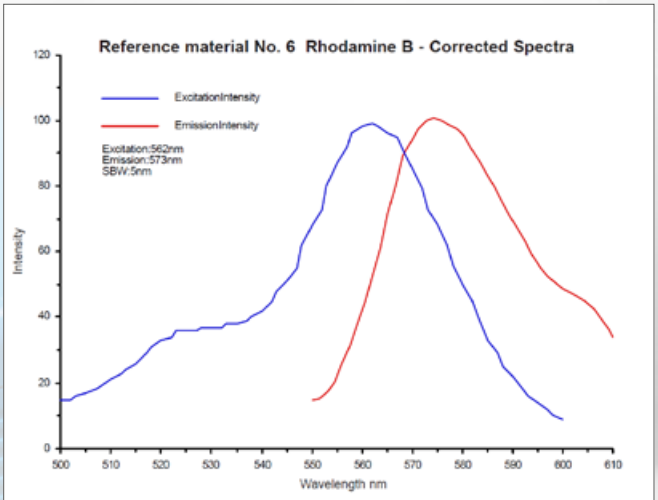


Figure 31: Corrected excitation and emission spectra of Rhodamine B

Authors: Gerhard Holst & Robert Franke

7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

7.6 Proper Modulation Frequency Selection

In many applications, when fluorescence lifetimes should be measured with a frequency domain FLIM system beside the requirement to select proper optical filters and a sufficiently fast excitation light source, the questions arises: What is an appropriate modulation frequency to measure the fluorescence lifetime of the sample? The relationship between the phase angle Φ, the modulation frequency f_{mod} and the fluorescence lifetime τ_P was already described in chapter 5.2 and it is:

$$\tan(\Phi) = 2 \pi f_{mod} \tau_P$$

If we have a look to the shape of the tangent function, we get the graph given in figure 32.

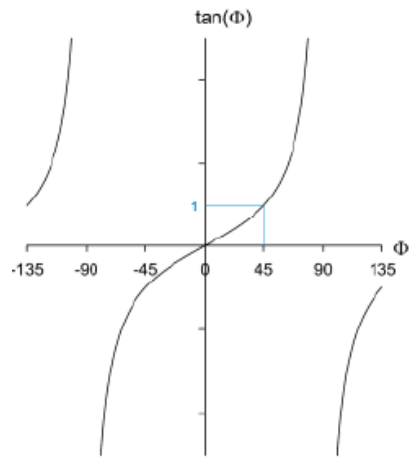


Figure 32: Graph of the tangent function

For Phi =45° the tangens function equals 1 and any lifetime change above and below has an equal change of phase angle. This is a good starting point for a frequency domain measurement. If the modulation frequency is selected such that the phase angles are either near to 0° or near to 90°, the sensitivity of the measurement towards changes or differences in fluorescence lifetimes is not optimum.

If the phase angle is close to 90°, a slight change in phase angle will cause huge changes in the tangens and therefore in the lifetime value. In turn near to 0°, larger changes in phase angle are required to get changes in the tangens value and therefore in the lifetime result. If the range of lifetimes or the lifetime itself is known, a good rule of thumb is to adjust the modulation frequency such that the phase angle is about 45°. This means:

$$\tan(45°) = 1 = 2 \pi f_{mod} \tau_P \Rightarrow f_{mod} = 1 / (2 \pi \tau_P)$$

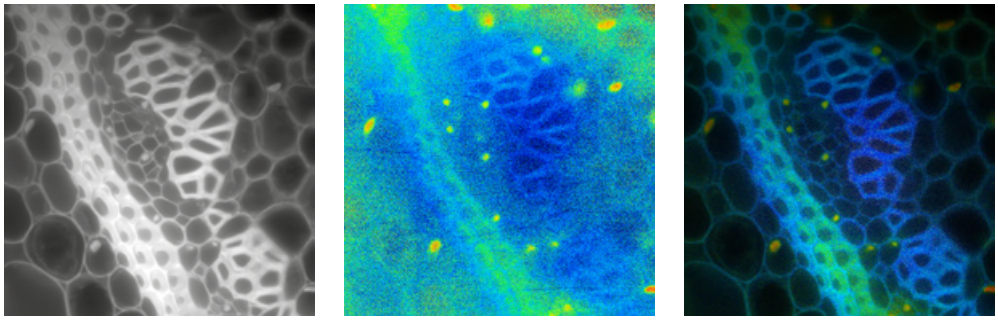
The next table shows some examples for a variety of fluorescence and phosphorescence lifetimes.

Luminescence Lifetime	Optimum Modulation Frequency
1 ns	159 MHz
5 ns	32 MHz
10 ns	16 MHz
50 ns	3 MHz
100 ns	1.6 MHz
500 ns	318 kHz
1 µs	159 kHz
10 µs	16 kHz
50 µs	3.2 kHz
100 µs	1.6 kHz

Obviously, modulation frequencies of 30 or 40 MHz are not optimum to measure lifetimes in the range of 0.1 – 2 ns, but the autofluorescence lifetime results (see figure 33) show that it is possible with a good sensitivity.

Figure 33: Autofluorescence measurement of a lily of the valley (convallaria) slice sample, from left to right:

fluorescence intensity – fluorescence lifetime distribution based on phase angle, the colorbar is given from 0.5 – 4 ns – fluorescence lifetime distribution with intensity weight



7 PCO.FLIM - CAMERA SYSTEM FOR LUMINESCENCE LIFETIME IMAGING

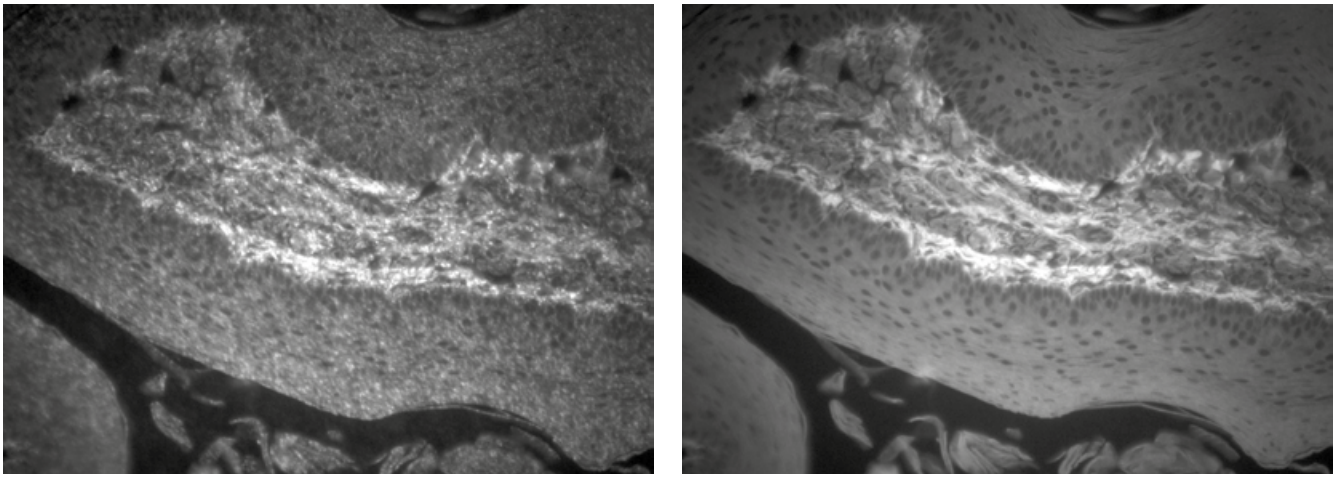


Figure 34: Fluorescence intensity image of a sample, left – de-speckling switched off, right – de-speckling switched on (courtesy of Rapp OptoElectronic)

7.7 Coherent Light and De-Speckling

When short fluorescence lifetimes in the range of a couple of nanoseconds is measured the modulation frequency is near to the upper limit of the pco.flim 20 – 40 MHz. This is often out of the useful application range of non-coherent light sources like LEDs. We tested a large number of LEDs and came to the conclusion that above 20 MHz LEDs should not be applied. Therefore it is likely that laser diodes have to be applied if the investigated fluorescence lifetimes are in the range of a few nanoseconds. If these coherent light sources are used, interference patterns (speckles) occur as can be seen in the left image of figure 34. If these patterns do not change during the recording of a pco.flim image stack, they have no influence on the phase angle and the modulation index distribution.

Nevertheless with the speckle pattern in the widefield illumination, the structural interpretation of the sample image (see fig. 34 comparison between left and right image) can be quite cumbersome. The original structure of the sample (see fig. 34 right image) can be better analyzed without the speckle pattern. The company Rapp OptoElectronic has developed an excellent combination of a de-speckling unit and an optimum optical coupling unit for laser light into widefield microscopes.

7.8 Optimum Optic Coupling of Excitation Light into a Microscope

Most of the available optical coupling units, which couple laser light into a microscope, usually try to illuminate spots for scanning purposes. The widefield illumination required for the use of the pco.flim system needs a homogenous illumination of the viewfield of the microscope without interference patterns. Rapp OptoElectronic has developed a laser light source and a very efficient optical coupling unit (see fig. 35), which is an optimum fit for microscopy applications of the pco.flim system. The light source has all protective features, which are required for safe laser applications. It accepts the modulation and dark gate signals from the pco.flim and it is connected via an optical fiber to the combined de-speckling and optical coupling unit at the back optical port of the microscope (see fig. 35).

Figure 35: Microscope set-up example (Zeiss AxioObserver Z1, inverse widefield fluorescence microscope, courtesy of Zeiss AG) with Rapp OptoElectronic laser light source and combined de-speckling and optical coupling unit, with a pco.flim camera connected to the microscope and the laser light source.

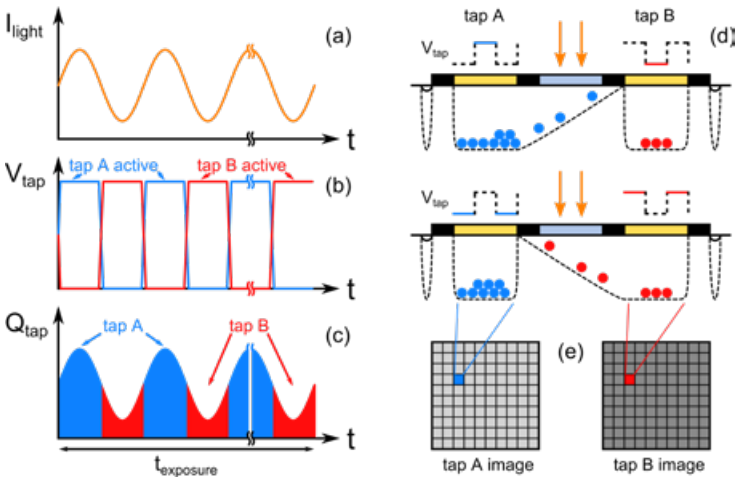
Authors: Gerhard Holst & Robert Franke

A1.1 LOCK-IN DETECTION AND CROSS-CORRELATION FUNCTION

Figure A1.1:

The sinusoidally modulated waveform of light incident on one pixel of the image sensor over time is shown in (a). The charge carriers (electrons) are generated by absorption of light in the photodiode and directed into one of two charge buckets, called tap A and B (d). The taps are alternately selected by applying a voltage waveform shown in (b). The rectangular modulation of the tap A control voltage (blue curve) is inverted for the opposite tap B (red curve).

The frequency equals that of the light modulation due to the homodyne detection. Figure (c) shows the charge carriers alternately accumulated in each tap over the entire exposure time, where each tap holds the complementary information of the other tap, i.e. phase shifted by $\pi \triangleq 180°$. During one exposure two intensity images (all taps A and B) are created simultaneously (e). Note that in the figure only one phase relation between sensor and light modulation is shown, resulting in two phase images with a phase difference of $\pi \triangleq 180°$.



The pco.flim's image sensor is modulated by toggling the momentarily active tap using a rectangular waveform with a duty cycle of 50%. The outgoing analog modulation signal for the excitation light has the same frequency, meaning this detection scheme is a homodyne or lock-in detection. The incident light created by the excited luminophor which is assumed to have the same number of harmonics as the outgoing analog modulation signal is mixed (i.e. multiplied) with the rectangular sensor modulation signal and integrated over time in each pixel tap during the exposure.

During each integration the photogenerated charge carriers are integrated in both taps per pixel, resulting in two grey values per pixel and therefore in two grey value images per integration for a given phase relation between sensor and external light modulation (see figure A1.1). Due to the symmetric toggling of both taps each tap holds the complementary information of the other tap, i.e. the information at an additional phase shift of $\pi \triangleq 180°$.

Mathematically, this procedure is equivalent to the cross-correlation function, which is formed by phase shifting the sensor modulation signal relative to the external modulation signal. This phase shift is the independent variable of the cross-correlation function. The cross-correlation function has the property that it contains only the harmonics present in both sensor and external modulation signals. The external modulation waveform for the excitation light is often a pure sine wave which does not contain any higher harmonics. Therefore, the cross-correlation function is a sine wave as well. Due to the fact that the light intensity cannot become negative the cross-correlation function carries a constant component as well as the incident light does.

To reconstruct the sinusoidally shaped cross-correlation function it has to be sampled at three or more sampling points due to the Nyquist criterion. For that purpose at least three integrations at different phases have to be performed. The pco.flim camera supports powers of two as a number of equidistant phases, where 4, 8 or 16 phases are possible. For special applications (e.g. ratio imaging) a phase number of two is also available, simply halving the modulation period.

There are two possibilities to acquire the intensity images to cover all intended phases per modulation period. The first way is to use the first tap (called "tap A") to cover the first half period, whereas the second tap (called "tap B") automatically covers the second half period as it holds the complementary information as described above. Assuming a certain asymmetry between both taps A and B, which can actually be observed using the pco.flim, this method can lead to slight distortions of the computed results. To overcome this problem, the second method is to shift the phase throughout the complete modulation period, so that each tap covers all phase steps. By applying a subsequent averaging of taps A and B holding the same phase information the asymmetry can be compensated. A drawback of this method is the necessity of acquiring twice as many images resulting in half the effective frame rate of the computed results.

Note: Even if sinusoidally modulated light with the maximum modulation index of 1 (i.e. showing a possible momentary value of zero) is incident on the image sensor, the demodulation index of the cross-correlation (sine) function is theoretically $2/\pi \approx 64\%$, since each tap integrates over a half period of a sine wave with positive values only and cannot become zero. This reduction factor is automatically compensated during referencing (see Referencing).

A1.2 NUMERICAL HARMONIC ANALYSIS

If a pure sinusoidal excitation light waveform is used, the cross-correlation function mentioned above has a sinusoidal waveform as well, assuming a linear time-invariant photoluminescent sample.

Therefore the following formulae refer to the analysis of the first harmonic, i.e. the fundamental component, and the discrete complex Fourier transform is simplified. The modulation period $2\pi \triangleq 360°$ is divided into N equidistant phases indexed by $k = 0 \ldots N-1$. For the pco.flim camera valid values are $N \in \{2, 4, 8, 16\}$. For each phase step k the intensity (grey value) of each pixel $(x,\ y)$ is given by $I_{xy}[k]$.

The demodulation amplitude for each pixel $(x,\ y)$ of the image is given by

$$a_{xy} = \frac{2}{N}\left|\sum_{k=0}^{N-1} I_{xy}[k]\, \mathrm{e}^{-i\frac{2\pi}{N}k}\right| = \frac{2}{N}\left|\sum_{k=0}^{N-1} I_{xy}[k]\, w^k\right|$$

and the phase is given by

$$\phi_{xy} = \arg\left(\sum_{k=0}^{N-1} I_{xy}[k]\, \mathrm{e}^{-i\frac{2\pi}{N}k}\right) = \arg\left(\sum_{k=0}^{N-1} I_{xy}[k]\, w^k\right)$$

with the imaginary unit $i = \sqrt{-1}$ and the roots of unity which can also be written using Euler's formula to split the complex relation into two real equations, i.e. for the real and imaginary part

$$w^k = \mathrm{e}^{-i\frac{2\pi}{N}k} = \cos\left(\frac{2\pi}{N}k\right) - i\sin\left(\frac{2\pi}{N}k\right).$$

For a given N the roots of unity can be computed in advance for $k = 0 \ldots N-1$ and stored in an array:

$$w^k = w[k]$$

Then, the demodulation amplitude and phase can be computed by the sum of products:

$$a_{xy} = \frac{2}{N}\left|\sum_{k=0}^{N-1} I_{xy}[k]\, w[k]\right|$$

$$\phi_{xy} = \arg\left(\sum_{k=0}^{N-1} I_{xy}[k]\, w[k]\right)$$

The constant component, i.e. mean value, is given by

$$b_{xy} = \frac{1}{N}\sum_{k=0}^{N-1} I_{xy}[k].$$

To obtain the demodulation index the amplitude is normalized by the constant component

$$m_{xy} = \frac{a_{xy}}{b_{xy}}.$$

The computed two-dimensional arrays m and ϕ, containing real values only, can be displayed as images of the same size as the grey value raw images using an appropriate intensity and/or color coding. The constant component image b can be displayed as a normal grey value image.

Both real parameters, the demodulation index and the phase, can also be combined into a complex parameter in polar form, the so-called phasor

$$p_{xy} = m_{xy}\mathrm{e}^{i\phi_{xy}}.$$

Instead of displaying the computed images m and ϕ all complex phasors, whose quantity equals the number of pixels in the image, can be displayed by marking their complex value within the complex plane. Such a plot is called Nyquist plot or phasor plot. A color coding can be used to indicate the frequency of appearance within a certain proximity in the complex plane, creating a kind of density clouds.

Example

The following example demonstrates the computation of the parameters mentioned above for four phases per modulation period, i.e. $N = 4$.

The array containing the roots of unity is initialized with

$$w[0] = 1,\ w[1] = -i,\ w[2] = -1,\ w[3] = i.$$

The demodulation amplitude is computed by means of the sum of the intensity values weighted with the corresponding roots of unity

$$\begin{aligned} a_{xy} &= \frac{2}{N}\left|I_{xy}[0] - iI_{xy}[1] - I_{xy}[2] + iI_{xy}[3]\right| \\ &= \frac{2}{N}\left|I_{xy}[0] - I_{xy}[2] + i\left(I_{xy}[3] - I_{xy}[1]\right)\right| \\ &= \frac{1}{2}\sqrt{\left(I_{xy}[0] - I_{xy}[2]\right)^2 + \left(I_{xy}[3] - I_{xy}[1]\right)^2}. \end{aligned}$$

Authors: Gerhard Holst & Robert Franke

A1.2 NUMERICAL HARMONIC ANALYSIS

For simpler computation the real part is separated from the imaginary part to apply the Pythagorean theorem to obtain the magnitude of the complex sum. This separation can also be used to express $tan\ \phi_{xy}$ as the quotient of imaginary part divided by the real part, giving the following equation for the phase

$$\phi_{xy} = \arctan\left(\frac{I_{xy}[3] - I_{xy}[1]}{I_{xy}[0] - I_{xy}[2]}\right)$$

$$= -\arctan\left(\frac{I_{xy}[1] - I_{xy}[3]}{I_{xy}[0] - I_{xy}[2]}\right).$$

To be more precise, the signs of the real and imaginary part have to be examined to adjust the computed phase ϕ_{xy} to lie within all four possible quadrants of the complex plane since the principle values of the arctan function only cover the first and fourth quadrant, i.e. $-\pi / 2 \leq \phi_{xy} \leq \pi / 2$.

If the real part becomes negative the corresponding phase must be located in the second or third quadrant, depending on the sign of the imaginary part. In that case the adjustment can simply be done by adding π to the phase ϕ_{xy} computed in the above formula.

The constant component is computed by

$$b_{xy} = \frac{1}{4}\left(I_{xy}[0] + I_{xy}[1] + I_{xy}[2] + I_{xy}[3]\right),$$

giving the demodulation index

$$m_{xy} = \frac{2\sqrt{(I_{xy}[0] - I_{xy}[2])^2 + (I_{xy}[3] - I_{xy}[1])^2}}{I_{xy}[0] + I_{xy}[1] + I_{xy}[2] + I_{xy}[3]}.$$

A1.3 ORDER OF PHASE IMAGES USING THE PCO.FLIM CAMERA

The intensity images used in the computations mentioned above are supposed to be in an ascending order with respect to their phases as the roots of unity are indexed in an ascending order as well. However, the pco.flim camera delivers the intensity images in different possible phase orders, since both taps A and B, if both selected, are read out alternately. Due to various possible camera settings there are different resulting phase ordering schemes, which have to be considered in order to apply the above formulae correctly.

Because tap B automatically carries the phase information shifted by $\pi \triangleq 180°$ relative to tap A, it can be sufficient to choose a phase shifting mode, which lets tap A cover the first half period of one modulation period and tap B the second half. Each phase of one full modulation period is covered by one single tap only, calling this setting “singular”. As mentioned in section Lock-In Detection and Cross-Correlation Function a certain asymmetry between both taps can it make necessary to cover each phase information by both taps, which is done using the setting “twice”. Therefore, the resulting phase sequences are twice as long as in singular mode.

To ease the application of different calculations on the image sequence, the order of the different phase shifts can be altered. Using the “ascending” mode the phase is linearly shifted by one phase increment after both taps A and B have been integrated and read out, scanning the modulation period in an ascending manner. In order to easily apply an external or internal asymmetry correction by averaging two corresponding taps, the sequence order can be set to “opposite”. This mode will re-sort the image sequence in a way that two sequent pairs (taps A and B) carry information of two phases with inverted phases in the second pair, e.g. 0° (tap A), 180° (tap B), 180° (tap A), 0° (tap B), etc. Now, the averaging of taps A and B can be applied on the phase information 0°, 180°, etc. Note that both modes “ascending” and “opposite” have only effect if the first mode “twice” has been selected.

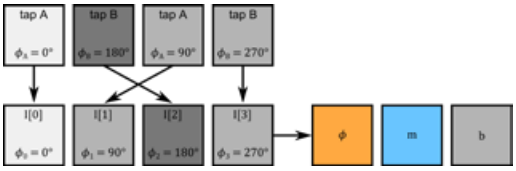
While both taps A and B are always integrated and read out of the image sensor, the user can select, whether tap A, tap B or both (alternately) are output at the camera interface via the options “tap A”, “tap B” or “both”. The following examples show several combinations of the parameters mentioned above resulting in different image sequences. The parameter designations are those used in the SDK description (see pco.flim SDK Manual for more information). All image sequences are re-sorted into a uniform ascending sequence of phase images for the further computation of phase, demodulation index and mean intensity images.

Example 1:

Parameter	Value
Phase Number	[4 phases]
Phase Symmetry	[singular]
Phase Order	[ascending]
Tap Select	[both]
Asymmetry Correction	[off]

A1.3 ORDER OF PHASE IMAGES USING THE PCO.FLIM CAMERA

Four phases per modulation period are selected, where each phase is covered by only one tap (“singular” mode). The phase order is set to “ascending”, putting each tap into an ascending order of phase increments, i.e. 0° and 90° for tap A and 180° and 270° for tap B. Due to the sensor readout scheme taps A and B are always output alternately, if both are selected.

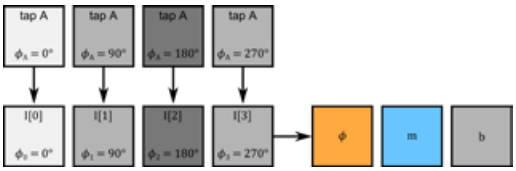


In the above figure the top row shows the sequence order of phase images out of the camera. The phase images have to be (virtually) re-sorted to an ascending phase sequence for the computation of the phase, demodulation index and intensity images (bottom row).

Example 2:

Parameter	Value
Phase Number	[4 phases]
Phase Symmetry	[twice]
Phase Order	[ascending]
Tap Select	[tap A]
Asymmetry Correction	[off]

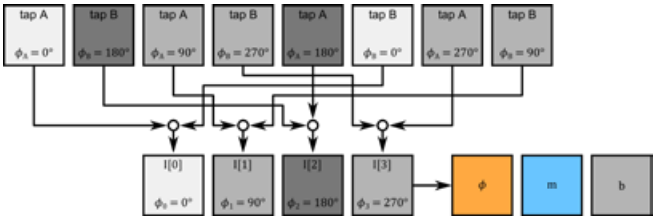
The mode “phase symmetry” is now set to “twice”, where each phase is covered twice by tap A and B, resulting in a sequence length of eight in case that both taps would be selected. In the first step, shown in this example, only tap A is picked, resulting in a sequence containing four tap A phase images in an ascending phase order.



Example 3:

Parameter	Value
Phase Number	[4 phases]
Phase Symmetry	[twice]
Phase Order	[ascending]
Tap Select	[both]
Asymmetry Correction	[off]

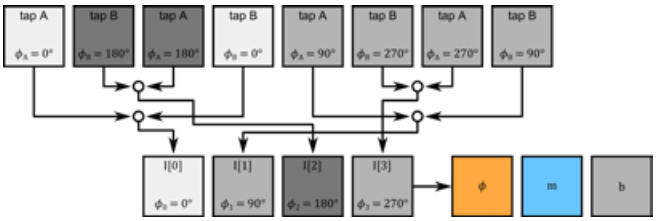
By selecting both taps in the example above, the resulting sequence length is eight. Each tap steps through the full modulation period in an ascending order, while taps A and B are output alternately as always. By externally averaging taps A and B containing the same phase information and re-sorting the original image sequence with four phase images is obtained.



Example 4:

Parameter	Value
Phase Number	[4 phases]
Phase Symmetry	[twice]
Phase Order	[opposite]
Tap Select	[both]
Asymmetry Correction	[off]

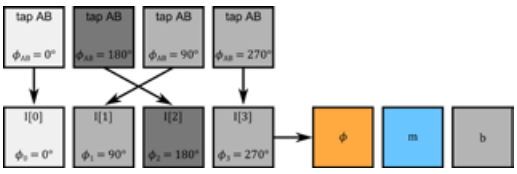
The phase order of the example above is now changed to „opposite“ instead of „ascending“, creating two sequential pairs of taps A and B with swapped phase information. The same averaging principle as mentioned above is externally applied.



Example 5:

Parameter	Value
Phase Number	[4 phases]
Phase Symmetry	[twice]
Phase Order	[opposite]
Tap Select	[both]
Asymmetry Correction	[average]

The settings of the last example are required to meaningfully use the internal averaging mode by changing the parameter “asymmetry correction” to “average”. Eight phase images per sequence are internally recorded and averaged in the same way as externally performed in the above examples. The resulting sequence length is four with the same phase sorting as in the first example.



Authors: Gerhard Holst & Robert Franke

A1.4 REFERENCING

A complete measuring setup, including the camera (image sensor, analog modulation circuitry), modulated light source and optical path length, exhibits initial values of the overall modulation index and phase, independent of the photoluminescent sample itself, which usually differ from the ideal initial values: a modulation depth of one (a theoretical demodulation depth of $2/\pi \approx 64\%$, see Lock-In Detection and Cross-Correlation Function) and a phase shift of zero (neglecting the delay induced by the optical path length and the finite velocity of light). To correct the deviations from the ideal values, a reference measurement has to be performed by replacing the sample of interest by a reference sample with known characteristics, i.e. with a known luminescent lifetime. By means of referencing, the influences of the whole measuring setup are compensated, since they affect the reference and sample measurements the same way.

The simplest reference would be a reflecting or scattering target which does not alter the modulation index and phase by intrinsic properties like a luminophore does. It would have a time constant or lifetime of zero. To calculate the referenced phasor p of the sample of interest, the phasor obtained by the sample measurement p_{em} is normalized by the phasor obtained by the scattered or reflected excitation p_{exc} by division of both phasors:

$$p = \frac{p_{\mathrm{em}}}{p_{\mathrm{exc}}} = \frac{m_{\mathrm{em}} \mathrm{e}^{i\phi_{\mathrm{em}}}}{m_{\mathrm{exc}} \mathrm{e}^{i\phi_{\mathrm{exc}}}} = \frac{m_{\mathrm{em}}}{m_{\mathrm{exc}}} \mathrm{e}^{i(\phi_{\mathrm{em}} - \phi_{\mathrm{exc}})} = m\mathrm{e}^{i\phi}$$

The demodulation indices are divided by each other and the phases are subtracted. This method is based on the assumption that the measuring system exhibits the same influences on the sample and reference (excitation) measurements, which is not true for most applications. The modulated image sensor shows wavelength dependent demodulation properties which are relevant, since excitation and luminescence usually are spectrally different.

The recommended reference method uses a reference luminophore with a mono-exponential behavior, a known luminescent lifetime τ_{ref} and a luminescence emission in a similar spectral range as the sample of interest. The referenced phasor for a given modulation frequency f is then computed by

$$p = \frac{1}{1 + i\omega\tau_{\mathrm{ref}}} \cdot \frac{p_{\mathrm{em}}}{p_{\mathrm{ref}}},$$

with the circular frequency $\omega = 2\pi f$. The first right-hand complex term describes a first-order low-pass system introducing an additional phase shift and decrease of the modulation index. This term is valid and constant for all pixels of the image, since it describes one single underlying model. The equation can be rewritten in Euler's form

$$p = \frac{\mathrm{e}^{-i\cdot\arctan(\omega\tau_{\mathrm{ref}})}}{\sqrt{1+\omega^2\tau_{\mathrm{ref}}^2}} \cdot \frac{m_{\mathrm{em}}}{m_{\mathrm{ref}}} \mathrm{e}^{i(\phi_{\mathrm{em}} - \phi_{\mathrm{ref}})}$$

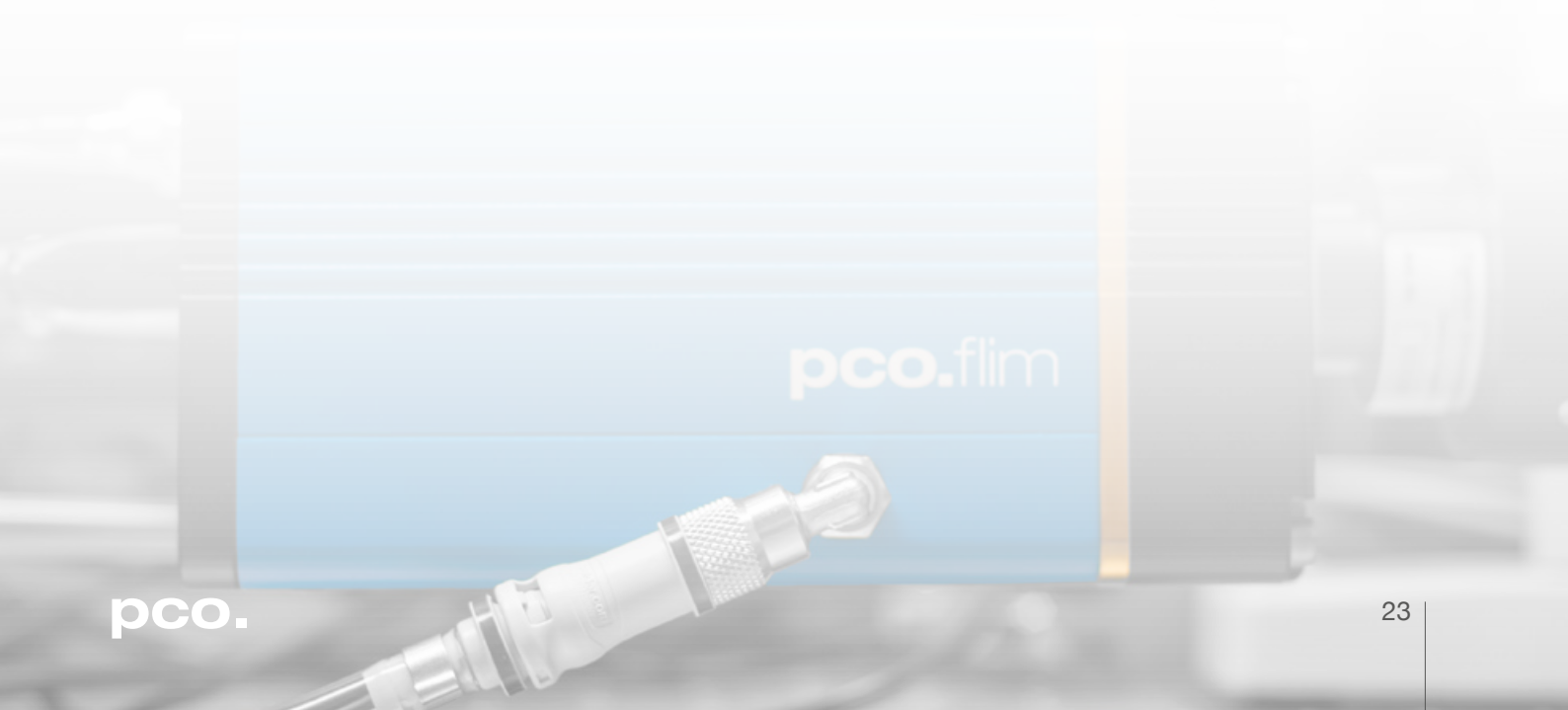
$$= \frac{m_{\mathrm{em}}}{m_{\mathrm{ref}}\sqrt{1+\omega^2\tau_{\mathrm{ref}}^2}} \mathrm{e}^{i(\phi_{\mathrm{em}} - \phi_{\mathrm{ref}} - \arctan(\omega\tau_{\mathrm{ref}}))},$$

with the referenced modulation index

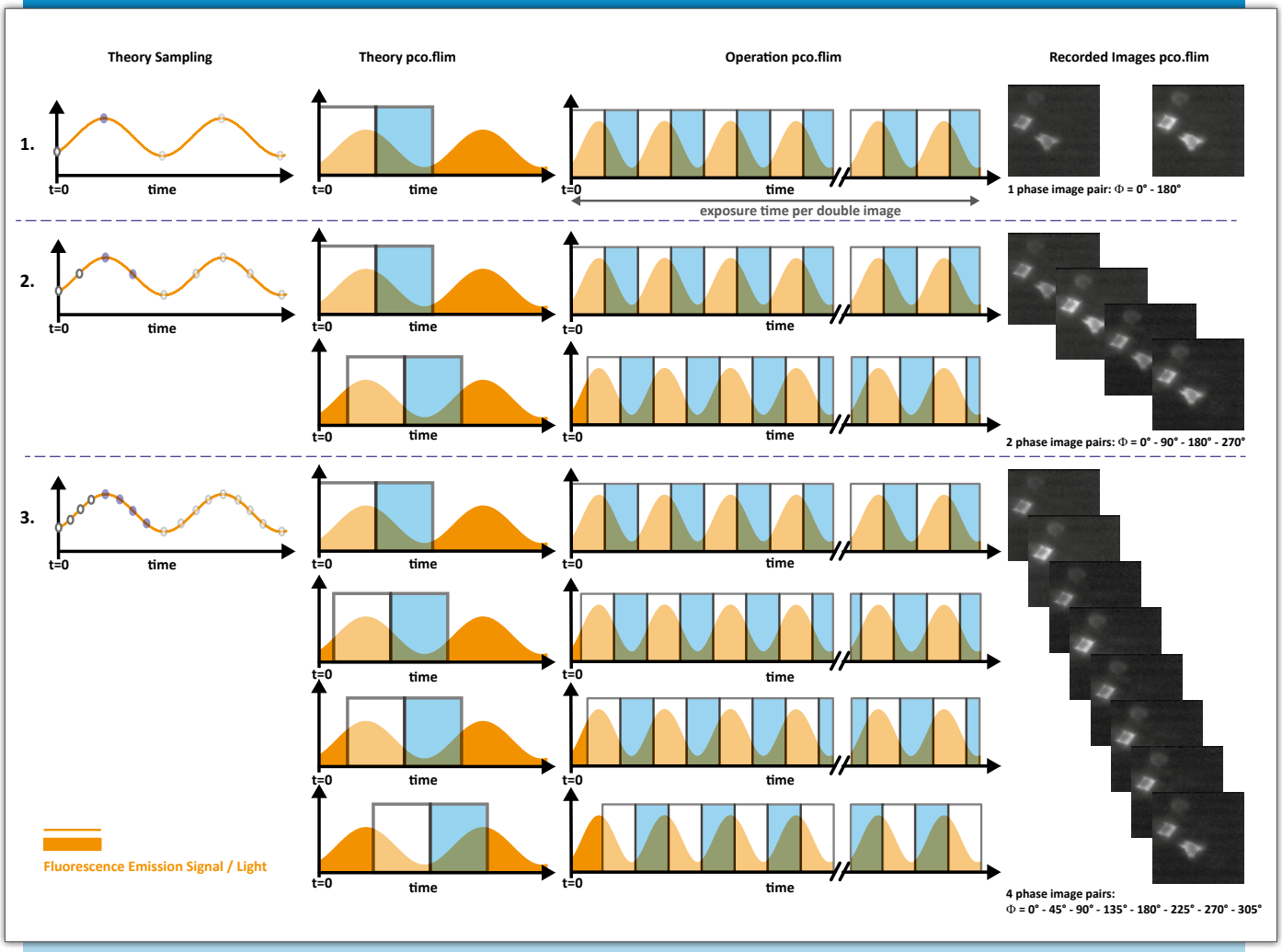
$$m = \frac{m_{\mathrm{em}}}{m_{\mathrm{ref}}\sqrt{1+\omega^2\tau_{\mathrm{ref}}^2}}$$

and the referenced phase

$$\phi = \phi_{\mathrm{em}} - \phi_{\mathrm{ref}} - \arctan\left(\omega\tau_{\mathrm{ref}}\right).$$



A1.5 SAMPLING THEORY - PCO.FLIM RESULT IMAGES



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