Application Note

PicoQuant

Visualize dynamic processes with rapidFLIM^{HiRes}, the ultra fast FLIM imaging method with outstanding 10 ps time resolution

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Introduction

Time-resolved fluorescence microscopy is a very versatile characterization method where fluorescence lifetime information is combined with spatial localization in the sample, which allows investigating biochemical and physical processes. The fluorescence lifetime of a light emitting species (e.g., dye or fluorescent protein) indicates how long it remains in an excited state before returning to the ground state. The lifetime is independent of instrumental settings such as laser power or detector gain, but is characteristic for every species. Thus it can be used to identify and distinguish spectrally similar fluorophores. Furthermore, fluorescence lifetime is influenced by the fluorophore`s environment. By investigating lifetime changes of sensor fluorophores and combining it with spatial information, one can detect changes within the local environment of cell cultures or tissues (e.g., pH, temperature, or ion concentration). Molecular interactions or even conformational changes of proteins, RNA or DNA can be monitored by measuring lifetime changes due to changes in distance via Förster Resonance Energy Transfer (FRET).

Data acquisition on time-resolved fluorescence microscopes is typically based on Time-Correlated Single Photon Counting (TCSPC) electronics coupled with picosecond pulsed diode lasers or Titanium Sapphire (Ti:Sa) lasers as excitation sources and highly sensitive single photon counting detectors. Up to now users had to choose between high timing precision or fast data acquisition. Acquiring data with high precision TCSPC electronics was considered to be a somewhat slow process: Collecting the several thousand photons per pixel that are required for reliable data analysis took time. Typical acquisition times per frame were in the range of half a minute to achieve good photon statistics per pixel.

This made it difficult to employ Fluorescence Lifetime Imaging (FLIM) for following fast processes in biological samples, such as signal transduction pathways in cells, metabolic processes, fast moving sub-cellular structures (e.g., vesicles), dynamic FRET due to transient protein interaction, or cell movements such as in contracting heart muscle cells. We present here rapidFLIM^{HiRes}, a novel and elegant solution to tackle this challenge.

The rapidFLIM^{HiRes} approach allows recording several FLIM images per second with an outstanding temporal resolution of 10 ps. The method exploits recent hardware developments such as a TCSPC unit that combines ultra short dead times of 650 ps with extremely high timing precision of about 10 ps. Using this unit in combination with an appropriate hybrid photomultiplier detector, detection count rates up to ~78 Mcps (mega counts per second) can be achieved. Taking full advantage of these hardware improvements requires also an appropriate software package that can store and process all photon timing information collected during the measurement. Such a complete, unaltered data set is needed for a full analysis with various tools.

These improved hardware and software components make it possible to achieve excellent photon statistics in significantly shorter time spans: Depending on scanner speed, image size and sample brightness, one can capture up to 15 FLIM frames per second. This enables precise FLIM imaging for fast processes with high spatial and temporal reso-

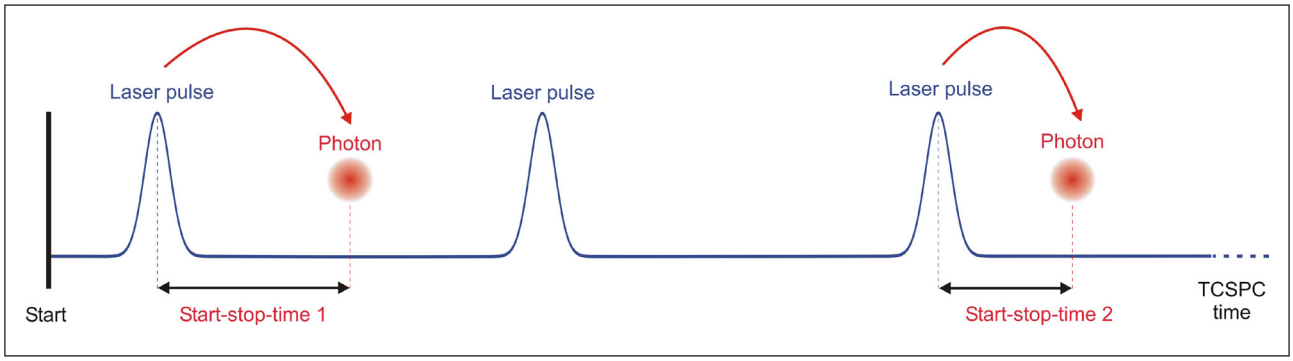

Figure 1: Recording photon arrival times in a time-resolved fluorescence measurement with TCSPC.

lution. As an additional benefit, a pattern matching approach can be applied as an easy and fast tool for analyzing FLIM data.

The rapidFLIM^{HiRes} approach allows studying fast FRET processes such as protein interactions involved in endosome trafficking, observing dynamic changes in environmental parameters like ion flux, and metabolic imaging in a quantitative manner. This method also provides the opportunity for fast live cell FLIM observations, e.g., for high throughput FLIM screening. This is supported by the online FLIM visualization, which gives an additional contrast compared to standard intensity based imaging methods and thus a first impression of sample conditions.

We will provide here an outline of the general principles underlying rapidFLIM^{HiRes} as well as a series of application examples highlighting the usefulness of this method. A quick run-down of the hardware requirements for implementing rapidFLIM^{HiRes} rounds this application note out.

Time-correlated single photon counting – the underlying technology of rapidFLIM^{HiRes}

This section outlines the concepts of FLIM and Time-Correlated Single Photon Counting (TCSPC) upon which our novel approach rapidFLIM^{HiRes} relies. An in-depth introduction into both techniques as well as their implementation in PicoQuant's products can be found in their respective Technical Notes[1, 2].

Photons generated by sample fluorescence are separated from the excitation light by means of optical filters and are recorded with appropriate detection and timing electronics. A common method for obtaining FLIM data uses TCSPC electronics, which allows detecting photons with picosecond resolution. In a very simplified way, this method works by repeatedly recording the arrival of single photons at the detector with high temporal accuracy. The time difference between excitation laser pulse and arrival of these photons is determined by electronics acting like a highly precise stopwatch, as shown in Fig. 1.

This measurement step is repeated over multiple cycles and all recorded photon arrival times are sorted into a histogram, as can be seen in Fig. 2. From this histogram, the fluorescence lifetime information of the fluorophore can be extracted through mathematical methods. The FLIM image is acquired by scanning the pulsed excitation laser across the sample.

A histogram is built for each pixel or for a region of interest, which allows determining the fluorescence lifetime in this area. Carrying out a reliable FLIM data analysis requires collecting a large amount of photons, typically several thousand. Therefore, it can be beneficial to bin photons from several pixels or a region of interest.

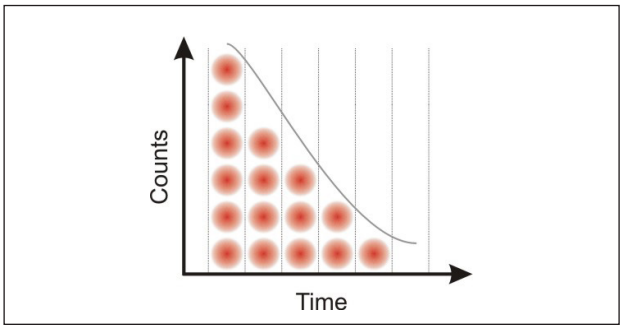

Figure 2: Histogram of start-stop times obtained from a TCSPC measurement. The discreet time bins (channels) are indicated by dashed lines.

Overcoming challenges of TCSPC at high count rates

The acquisition time of a FLIM image can be shortened by increasing the detection count rate. The maximum count rate is limited by the dead time of the detector and TCSPC electronics. Typically, the count rate should not exceed 1/10 of the inverse dead time. Recording more photons per second leads to a significant issue that affects the TCSPC histogram and fluorescence lifetime results: the pile-up effect.

The detector and electronics used in a TCSPC

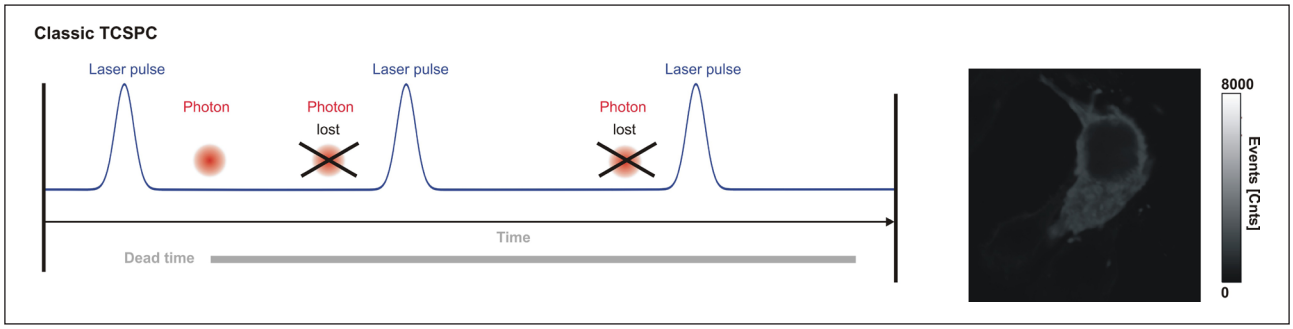

Figure 3A: Dead times in classic TCSPC electronics. Left: scheme of the interaction of laser pulses, dead times and detected photons. Right: FLIM intensity images of a cell recorded under classic TCSPC conditions.

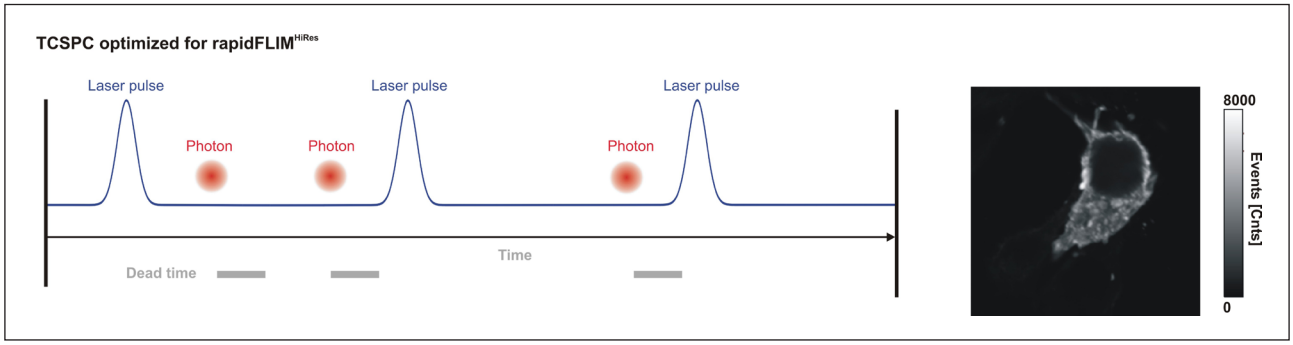

Figure 3B: Dead times in rapidFLIM^{HiRes} optimized TCSPC electronics. Left: scheme of the interaction of laser pulses, dead times and detected photons. Right: FLIM intensity images of a cell recorded under rapidFLIM^{HiRes} conditions.

measurement are not “ideal” instruments. After registering an incoming photon, the system is “busy” with data processing for a certain amount of time. This time period is called dead time and any photons reaching the detector and TCSPC electronics during that time will be lost as they cannot be detected and processed (see Fig. 3A, left).

This means when using “classic TCSPC” with a dead time of several nanoseconds, that, at most, only one photon can be counted per excitation pulse and the electronics can not process any photon for the next three to four excitation cycles. For example, at an excitation rate of 80 MHz, one excitation cycle takes 12.5 ns. These lost photons will lead to a distortion of the TCSPC histogram towards shorter lifetimes. This phenomenon is called the pile-up effect. Consequently, the photon flux must be attenuated, so that statistically only approximately one in 100 laser pulses generates a photon. This led to an increased image acquisition time.

Our rapidFLIM^{HiRes} approach exploits a series of hardware and software capabilities recently developed by PicoQuant to overcome the limitations outlined above. These components are available either individually, as part of a PicoQuant Laser Scanning Microscopy (LSM) Upgrade Kit, or as a special configuration of PicoQuant’s MicroTime 200 confocal microscopy platform.

The MultiHarp 150 4P – optimally suited for FLIM acquisition at high count rates and with 10 ps time resolution

The MultiHarp 150 4P is a TCSPC unit recently developed by PicoQuant that features a very short dead time of less than a 650 picoseconds, an extremely small time bin width of 10 ps and a sustained count rate of ~78 Mcps. This combination makes it the ideal choice for fast FLIM measurements: The short dead time allows detecting several photons within one excitation cycle, thereby avoiding the pile-up effect (see Fig. 3B, left part).[3] The ability to record and process more photons in a given time span when using rapidFLIM^{HiRes} can also be nicely seen in the intensity FLIM images shown in the right part of Fig. 3A and Fig. 3B: a single cell was imaged in classic and rapidFLIM^{HiRes} TCSPC regime, while maintaining all other imaging parameters. Thanks to the short dead time of rapidFLIM^{HiRes}, more photons can be collected in the same time span, which results in a much brigther image.

The small time bin width allows determining lifetimes of commonly used fluorescent probes (usually in the ns range)with an outstanding time resolution of 10 ps; a precision which cannot be achieved by TCSPC electronics featuring bin width of 97 ps or more.. Thus, the MultiHarp 150 4P can process much

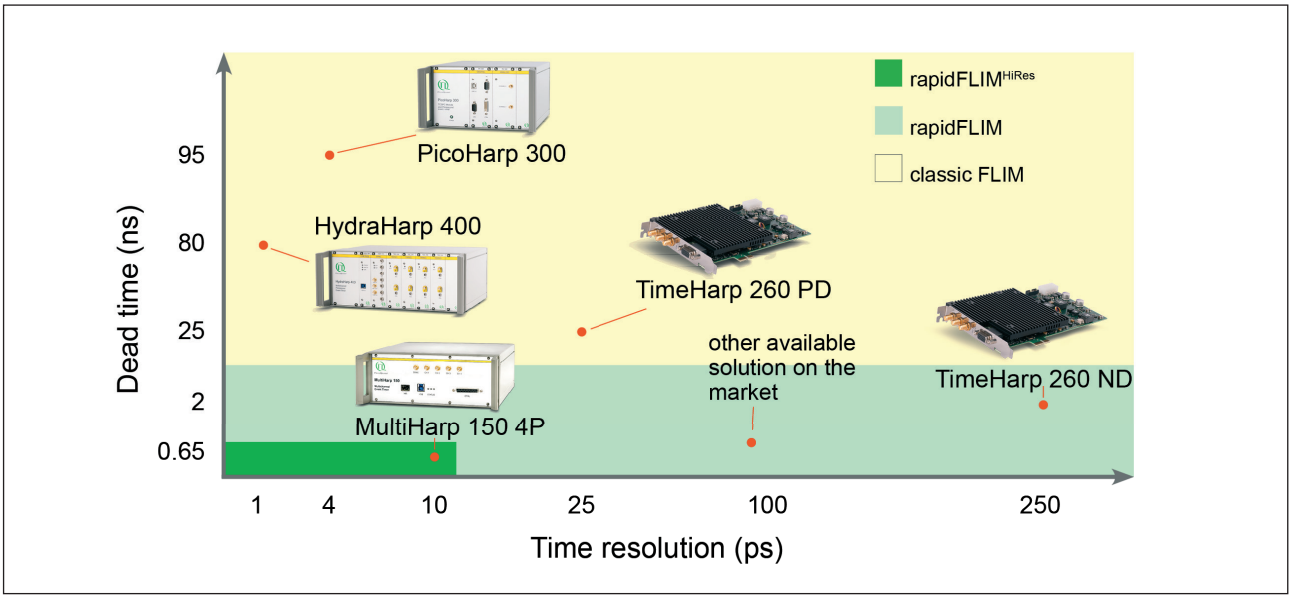

Figure 4: Dead times (y axis) and timing resolution (x axis) of PicoQuant's various TCSPC electronics and comparison with other commercially available solutions.

higher detector count rates while achieving highest timing precision in fluorescence lifetime measurements. The user no longer has to choose between high time resolution or fast data acquisition.

The MultiHarp 150 4P also features four detector input channels, which, in combination with multiple detectors, allows performing highly multiplexed imaging of many fluorescent markers simultaneously in both spectral and time domain as well as multicolor anisotropy imaging.

The PMA Hybrid detectors – matching the MultiHarp`s ultra short dead time

The rapidFLIM^{HiRes} approach requires using a detector with a short dead time matching that of the MultiHarp 150 4P. Hybrid photodetectors such as those from PicoQuant's PMA Hybrid Series have proven to be an excellent choice.[4] Such hybrid photodetectors combine the technology of photomultiplier tubes with that of semiconductor photodiodes. Thus they offer good timing resolution of about 120 ps as well as good sensitivity (< 45%) over the UV – VIS – NIR wavelength range (220 to 890 nm). Detectors from the PMA Hybrid Series can also sustain photon count rates up to ~78 Mcps, which can be fully utilized by the MultiHarp 150 4P.

Depending on sample brightness and image size, using a PMA Hybrid with the MultiHarp 150 4P allows acquiring FLIM data with more than 15 frames per second, enabling accurate observation of fast processes or rapidly moving species.

SymPhoTime 64 – the tool box for rapidFLIM^{HiRes} experiments

Besides the advances achieved in TCSPC and detector hardware, the rapidFLIM^{HiRes} approach requires an appropriate software package to take advantage of the method's full potential. The SymPhoTime 64, PicoQuant's time-resolved microscopy software for data acquisition and analysis, has recently been updated with memory management and processing time improvements. These updates, coupled with the existing robust workspace handling, enable analysis of files containing several hundreds frames, as well as very large data files (10s of GB), which are typically acquired during rapidFLIM^{HiRes} measurements.

The TTTR file format collects and stores all photon timing information obtained during a measurement without discarding any details and keeping the raw data always accessible. Once a measurement has been completed, the stored, complete and unmodified data set can be subsequently analyzed with a variety of analysis routines to extract every bit of information, using not only with the powerful routines included in the SymPhoTime 64, but also with other commercial or open source tools. The well-documented file format ensures excellent data portability.

The SymPhoTime 64 provides a clean yet flexible interface for both data acquisition and analysis. For example, the user can easily adjust the laser repetition rate to optimally match the lifetime of the fluorophore. High laser repetition rates are desirable as they lead to higher count rates and shorter acquisition times. A new fitting model for rapidFLIM^{HiRes} data was also introduced that includes a correction for cyclic excitation. The automatic correction enables users to account for wraparound effects in their decay curves

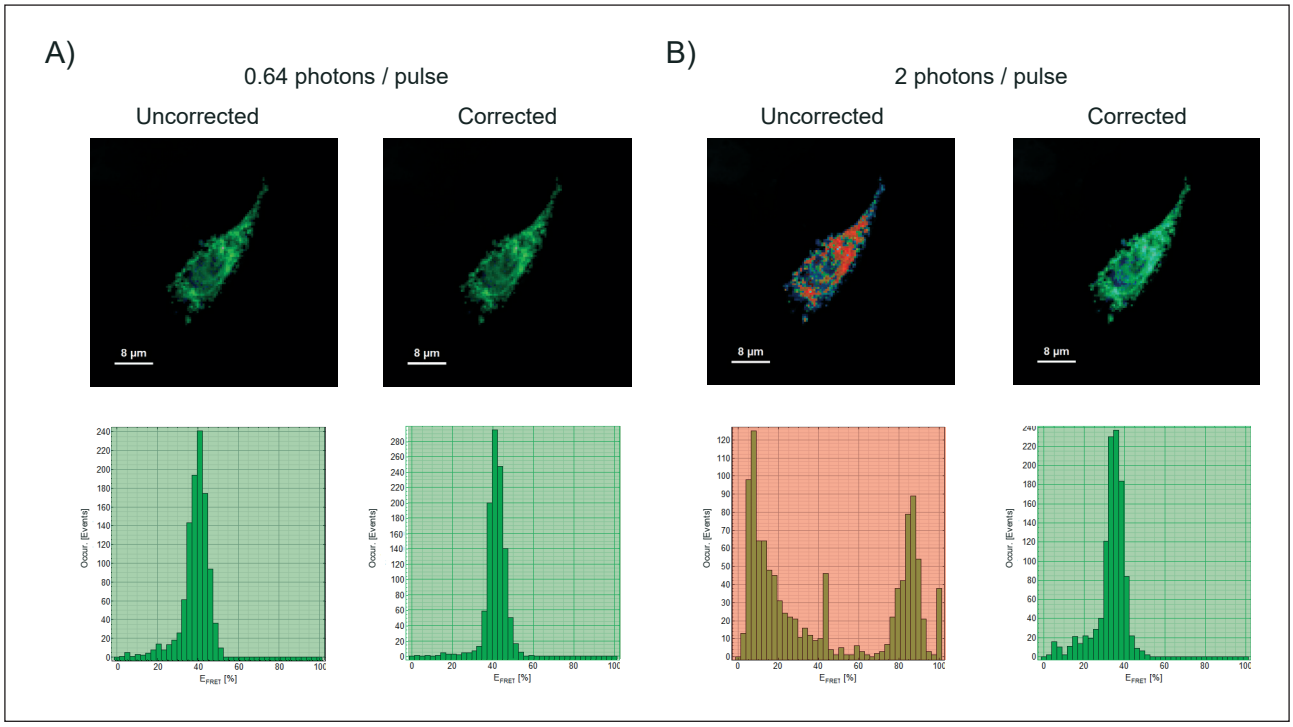

Figure 5: FLIM-FRET imaging of a CHO cell containing the FRET pair EGFP-N-WASP and mRFP-Toca-1 at different photon per excitation pulse rates. A) FLIM (top) and FRET efficiency histograms (bottom) acquired at 0.54 photons / pulse, without and with detector pulse pile-up correction. B) Same cell imaged at a rate of 2 photons per excitation pulse with and without pulse pile-up correction. Top: FLIM images, bottom: FRET efficiency histograms. Sample courtesy of T. Sudhaharan, Institute of Medical Biology, Singapore.

(cyclic excitation). Thus one can accurately measure fluorescence lifetimes that are twice as long without decreasing the laser repetition rate.

A new multi frame batch analysis method has been introduced in a recent update, which also benefits from the memory and processing time improvements mentioned above. This analysis method enables the selection of regions of interest based on the number of photons and range of fluorescence lifetimes. This novel way of setting thresholds is also available for the FLIM and FLIM-FRET analysis routines, allowing an intuitive analysis of e.g., time lapse FLIM experiments or multiple FLIM-FRET samples.

Our system software also features a novel correction method for our rapidFLIM^{HiRes} approach, which accounts for detectur pulse pile-up. Due to the extremely short dead time of the detectors, closely spaced detector pulses frequently merge at high count rates, which causes artifacts. This issue can be addressed with a data analysis method that corrects for detector pulse pile-up and the resulting systematic errors when fitting a decay histogram generated from a TCSPC measurement. The corresponding decay fit model was recently published in [5].

Due to the pending patent, the rapidFLIM^{HiRes} decay fit model, which allows for quantitative lifetime imaging even at high photon count rates of > 2 photons / pulse, is only available in the SymPhoTime 64 software. The effectiveness of the decay fit model can be seen in Fig. 5. In this example, a single Chinese Hamster Ovarian (CHO) cell containing the FRET pair EGFP-N-WASP and mRFP-Toca-1 was imaged with increasing detector count rates using a MicroTime 200. The sample was excited at 485 nm with a picosecond pulsed laser and emission lifetime data in the range from 500 to 540 nm was detected with a detector from PicoQuant's PMA Hybrid Series and a MultiHarp 150.

The collected FLIM data was then analyzed with either a conventional fitting algorithm (uncorrected) or with the rapidFLIM^{HiRes} decay fit model (corrected). At a rate of 0.64 emission photons per pulse (A), which is commonly used in classic FLIM measurements, both methods yield the same expected FRET efficiency of about 40%. However, when operating in the rapidFLIM^{HiRes} regime with 2 photons per excitation pulse (B), the conventional fit model results in a severely distorted FRET efficiency histogram with peaks at about 10%, 40% and 85%. The rapidFLIM^{HiRes} decay fit model can correct for these effects and produces the correct efficiency histogram even at such a high count rate (about 20x higher as in classic FLIM).

Application examples

In this section, we will illustrate the potential of the rapidFLIM^{HiRes} approach with two recent examples from research groups already using the method.

In the first example, the group of Prof. Rose

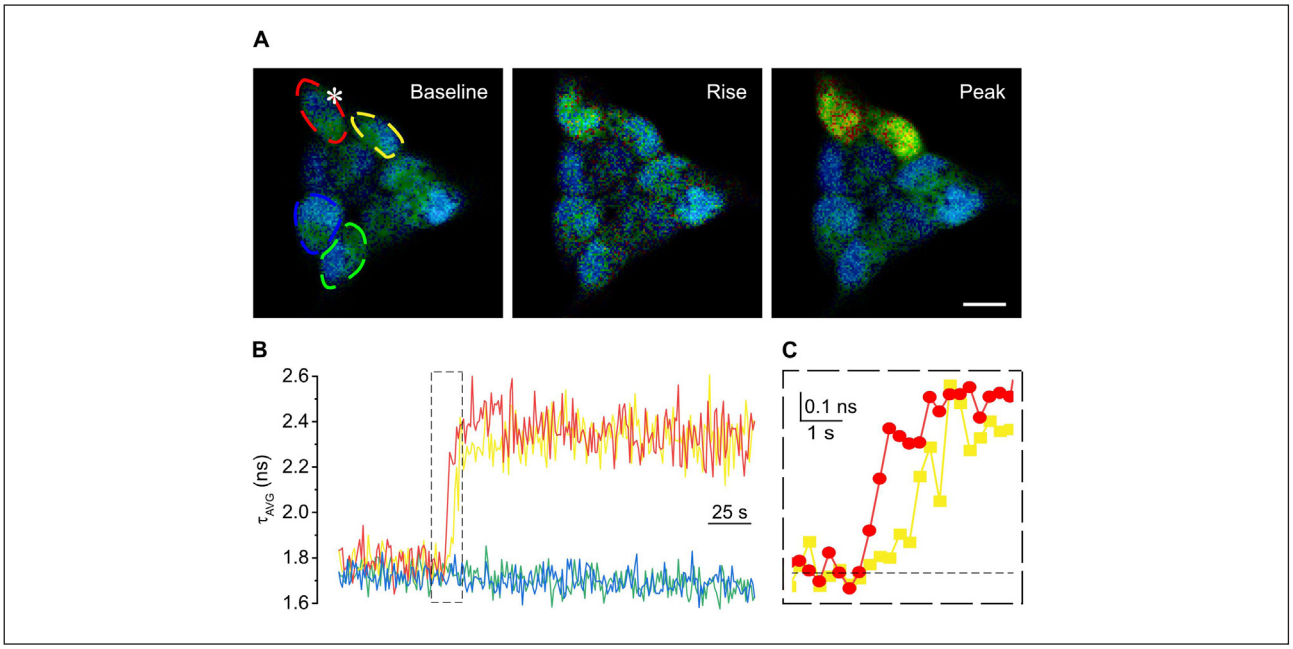

Figure 6: Intracellular Ca^{2+}-signals induced by mechanical stimulation of HEK-cells loaded with Oregon-Green-Bapta-1 (OGB-1 AM). A Left: Cells in artificial cerebrospinal fluid (ACSF). Site of mechanical stimulation is indicated with white star. Regions of Interest (ROI) shown in red, yellow, blue and green dashed lines. Image was averaged from frame 1-30 (0-6 seconds). Middle: Cells were mechanically stimulated at frame 120 for ~ 500 ms. Shown is an average of frames 121 to 125 (24.2 - 25 s). Right: Cells at the peak of the calcium signal (average of frames 126 - 155, 25.2 - 31 s). Scale: 50 µm. B Average fluorescence lifetime of the 4 ROIs depicted in A over a time course of 4 minutes. C Magnificitation of the rise in calcium. Baseline indicated with dashed line. Note the distinct temporal delay between the red and yellow ROI. Squares and dots indicate individual frames/datapoints. Imaging frame rate 5 Hz, Image size: 128 x 128 pixel, pixel dwell time 3.81 µs. Peak photon collection rate >20 Mcps. Sample and data courtesy of M.Sc. Jan Meyer/Prof. Dr. Christine R. Rose (Institute of Neurobiology, Heinrich Heine University Düsseldorf, Germany)

loaded Human Embryonic Kidney (HEK) cells with Oregon-Green-Bapta-1 (OGB-1 AM), a dye whose fluorescence lifetime is sensitive to Ca^{2+} concentration.[6, 7] These cells, kept in artificial cerebrospinal fluid, were imaged with the rapidFLIM^{HiRes} approach before, during, and after mechanical stimulation. The mechanical stimulation leads to an uptake of calcium ions in the cells. This increase in Ca^{2+} concentration results in a change of the fluorescence lifetime of OGB-1 AM, which can be tracked over time. The rapidFLIM^{HiRes} method enabled imaging a sample area of 128 x 128 pixels with a speed of 5 FLIM frames per second, which makes it possible to quantitatively observe the rise of calcium ion concentration in the cells over a short time period.

In 2018, A. Colom *et al.* reported on a novel fluo-

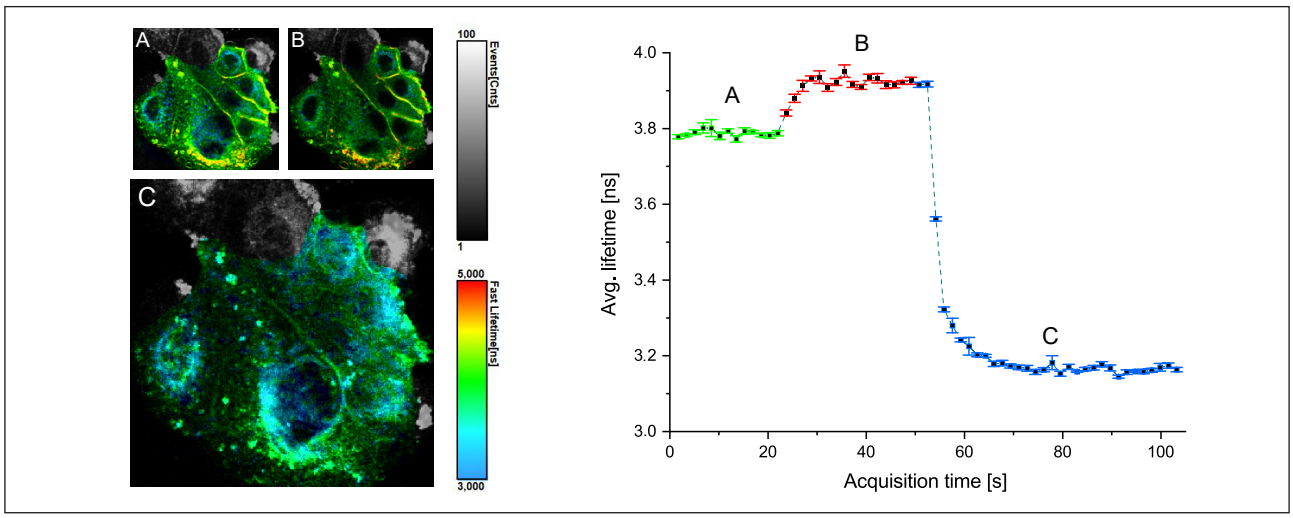

Figure 7: Monitoring changes in membrane tension in MDCK cells undergoing osmotic shocks using rapidFLIMHiRes and FliptR. Right: FLIM images of MDCK cells under isoosmotic conditions (A), hypoosmotic (B) and hyperosmotic conditions (C). Left: Plot of the FliptR lifetime as a function of acquisition time. Individual FLIM frames are shown as black squares and error bars are shown in green (isoosmotic), red (hypoosmotic) and blue (hyperosmotic). Image size is 256 x 256 pixels and the sample was imaged at a rate of 1.8 frames per second.

rescent probe called FliptR (Fluorescent lipid tensor Reporter).[8, 9] This fluorophore displays a significant change in lifetime when embedded in a confining environment, as the molecule will undergo a planarization if lateral pressure is applied to it.[6] Colom et al. demonstrated that this probe allows imaging lipid composition as well as membrane tension in both live cell and artificial membranes using fluorescence lifetime microscopy.

Fig.7 shows FLIM images of MDCK cells whose membranes were stained with FliptR as well as a plot of the average fluorescence lifetime versus acquisition time. Under isoosmotic conditions (A), FliptR exhibits an average fluorescence lifetime of ca. 3.8 ns. About ca. 25 s after the start of the experiment, the cells were subjected to hypoosmotic conditions by adding a water droplet. This leads to a slight dilatation of the cells and the observed average lifetime increases by less than 200 ps (B). As can be seen from the plot, this increase can be easily and quantitatively followed using rapidFLIMHiRes thanks to the outstanding time resolution.

Later in the experiment a hyperosmotic shock was induced by adding a drop of highly concentrated sucrose soultion, which lead to a rapid decrease in membrane tension. A corresponding shortening of the FliptR lifetime is observed. Imaging with the rapidFLIMHiRes approach is fast enough so that even this change can be recorded in real time with outstanding temporal precision as can be seen from the very small error bars in the plot of Fig. 7.

Conclusion

The rapidFLIM^{HiRes} approach, based on TCSPC electronics and detectors with extremely short dead times, allows performing fluorescence lifetime imaging with very short acquisition times without sacrificing the lifetime precision or the high spatial resolution of the confocal microscope. Thanks to PicoQuant's recent hardware and software developments, dynamic processes in living cells or other materials can now be quantitatively studied via fluorescence lifetime imaging at up to 15 frames per second and with an outstanding 10 ps time resolution. As an added benefit, every photon timing information is collected and stored by the SymPhoTime 64. The full, unmodified data set is always available for analysis thanks to the portable TTTR format.

The rapidFLIM^{HiRes} method can be integrated into many existing confocal laser scanning microscopes from major manufacturers such as Nikon, Olympus, Scientifica or Zeiss through LSM upgrade kits offered by PicoQuant. The required kits include a MultiHarp 150 4P with negligible dead time and one or more matched hybrid detector from the PMA Hybrid Series, along with a choice of pulsed laser sources and the system software SymPhoTime 64.

Further Reading

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[2] S. Trautmann, V. Buschmann, S. Orthaus, F. Koberling, U. Ortmann, R. Erdmann, Application Note on Fluorescence Lifetime Imaging (FLIM) in Confocal Microscopy Applications, PicoQuant (2012)

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