Application Note

Quantitative in vivo imaging of molecular distances using FLIM-FRET

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1. Introduction

Genetically encoded labelling by fluorescent proteins has opened the possibility to observe protein distribution and localisation by fluorescence microscopy. A direct visualisation of these nanometre sized molecules is, however, not possible due to the Abbe limit of diffraction (about 200 nm in confocal microscopes). As an alternative, indirect methods such as Förster Resonance Energy Transfer (FRET) studies can be employed to enable distance measurements down to several nanometres. FRET is a non-radiative process whereby energy from an excited fluorescent molecule (Donor) is transferred to a second non-excited fluorescent molecule (Acceptor) in its direct vicinity. This donor quenching leads to changes in the fluorescence intensity and the fluorescence lifetimes of the two fluorophores. The process of energy transfer is strongly dependent on the distance between the molecules and can usually only occur in the range of several nanometres. Hence, this technique is used to measure intra- and intermolecular distances, thus providing a useful tool for a broad range of applications. Intermolecular interactions can be determined *in vitro* as well as *in vivo* by fusing the molecules of interest to appropriate fluorescent proteins. In addition, so-called FRET sensors allow the monitoring of environmental conditions such as pH and ion concentration by means of intramolecular FRET due to strong conformational changes.

One popular FRET pair in biological applications has been CFP and YFP. However, due to the low photo-stability and quantum yield of CFP and its bi-exponential fluorescence decay, other FRET molecules like e.g. EGFP and mRFP are used nowadays. With intensity-based microscopy, FRET can be determined by ratiometric techniques (sensitised emission) or by acceptor photo-bleaching. Ratiometric methods, however, require careful calibrations, while acceptor photo-bleaching can only be performed once in a given sample. Furthermore, sensitised emission FRET requires corrections for donor fluorophore emission bleed through in the acceptor emission channel. These limitations can be overcome by measuring the fluorescence lifetime of the FRET donor using Fluorescence Lifetime Imaging Microscopy (FLIM).

The fluorescence lifetime of a molecule is defined as the average time that a molecule remains in the excited state upon absorption of light prior to returning to the ground state by emitting a photon. The fluorescence lifetime of the donor is effectively decreased when it undergoes FRET with an acceptor molecule.

A FLIM-FRET measurement images the change in fluorescence lifetime of the donor via FRET, and therefore, under sufficient conditions directly visualises the proximity of the donor and the acceptor molecule. In a conventional FLIM-FRET measurement only the fluorescence lifetime of the donor molecule is used as a probe, and it is in a broad range, concentration independent. This is important since in biological systems like cells, the fluorophore concentration often cannot be accurately determined and compared amongst different cells. As mentioned above, the FRET process is identified by a decrease of the fluorescence lifetime (quenching) of the donor in comparison to the lifetime of the donor alone as a result of the energy transfer to the acceptor molecule. The FRET efficiency E is a measure of this process and is defined as the fraction of photons that is transferred from the donor to the acceptor. It can be calculated using the following equation:

$$E = 1 - \frac{\tau_{DA}}{\tau_{D}}$$

t_{DA} = *Lifetime of the donor in presence of the acceptor*

τ_D = *Lifetime of the donor without acceptor*

The fluorescence lifetime of many organic dyes and genetically encoded fluorescent proteins is typically in the range of several nanoseconds. It can in principle be determined using two different methods:

(a) Time-Correlated Single Photon Counting (TCSPC), which works in the time domain and is mainly applied on confocal laser scanning microscopes,

(b) the frequency domain method, that is used mainly on wide field fluorescence microscopes.

In terms of acquisition speed, the frequency domain approach is usually faster than TCSPC, as it

can be directly performed with imaging detectors such as CCD cameras (wide field), whereas TCSPC is based on single point measurements combined with scanning methods. However, in contrast to the frequency-domain method, TCSPC is a very intuitive approach with numerous advantages. It provides a higher sensitivity since it is based on counting of single photons which is much better suited for biological samples. Here moderate expression levels, comparable to endogenous protein concentrations, can be used despite the resulting relatively lower fluorescence intensities. Furthermore, a better timing resolution can be achieved by time domain FLIM. TCSPC measurements additionally provide a higher accuracy of multi-exponential decay analysis which is often essential to determine fluorescence lifetimes in the heterogeneous cellular environment. Finally the TCSPC set up of PicoQuant offers the possibility for other time resolved measurements such as FCS.

2. General principle and set up of TCSPC measurements

The principle of TCSPC is based on the precise measurement of the time difference between the moment of excitation and the arrival of the first fluorescence photon at the detector.[1] TCSPC therefore requires a defined “start”, which is provided by exciting the donor fluorophore by a short laser pulse. A defined “stop” signal is realised by using special, single-photon sensitive detectors (e.g. Single Photon Avalanche Diodes, SPAD). The measurement of the time difference between laser pulse and detected photon is repeated several million times to account for the statistical nature of fluorescence emission. According to their arrival times, the photons are sorted into a TCSPC histogram that is then further analysed to extract the fluorescence lifetime.
To conduct FLIM measurements, PicoQuant offers an upgrade kit for all major Laser Scanning Microscopes (LSM). The set up is an external addition to the LSM and enhances its capabilities in an easy-to-use, maintenance-free and reasonably priced way. For more information about the FLIM and FCS upgrade kit, its components and possibilities, please refer to the Technical Note about the LSM Upgrade Kit.[2]

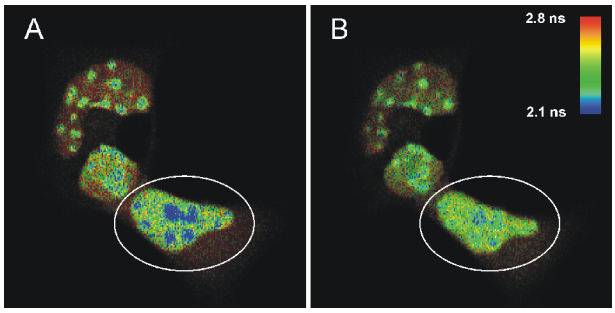

Fig. 1: FLIM-FRET measurements of living mouse adipocyte cells transfected with ECFPF46L, A206K-C/EBPα DBD and EYFPF46L, A206K-C/EBPα DBD before (A) and after photo-bleaching (B) of the FRET-acceptor EYFP in the cell indicated by a white circle. The donor fluorescence originating from ECFP is shown, the fluorescence lifetime is indicated by the the false colour representation. Image size 256 x 256 pixel.

3. FLIM-FRET measurement examples

3.1. Acceptor photo-bleaching FLIM-FRET experiment

In a first example we used FLIM to visualise the dimerisation process of the transcription factor CCAAT/enhancer binding protein alpha (C/EBPα) (samples provided by courtesy of Richard N. Day and Amasi Periasami, University of Virginia). The C/EBP family plays a key role in developmental gene expression. C/EBPα is known to form obligate dimers and to localise to regions of constitutive heterochromatin in mouse cells.[3,4] In order to visualise the distribution and dimerisation of C/EBFα, mouse adipocyte cells were transfected with plasmids encoding the DNA binding domain DBD of C/EBFα tagged with ECFP and EYFP that binds to certain regions of pericentric heterochromatin. The dimerisation of the proteins can be identified by the FRET process between ECFP and EYFP, which leads to a decreased fluorescence lifetime of the donor ECFP in comparison to a sample without EYFP staining.

FLIM images of living cells were taken using a Olympus FluoView FV1000 upgraded with FLIM and FCS capabilities with a dedicated kit from PicoQuant.[2] This set up contained a pulsed picosecond diode laser emitting at 440 nm, a single photon avalanche photo-diode (MPD), and a time-correlated single photon counting unit (PicoHarp 300).

Fig. 1A shows a heterogeneous FLIM image of a cell stained with ECFPA206K-C/EBPα DBD. Areas with high signal intensity represent pericentric heterochromatin regions. The FRET process can be identified in these images by the blue coloured areas. Here the fluorescence lifetime of the donor ECFP is decreased compared to the lifetime of a sample without EYFP staining, 2.1 ns compared to 2.4 ns on average, respectively. This strongly indicates that C/EBPα DBD dimerises specifically at pericentric heterochromatin. To prove this, EYFP was photo-bleached in one cell (marked by a circle in Fig. 1A) by repetitive scanning using a 514 nm cw laser. After photo-bleaching of the acceptor, it is obvious that the number of pixels corresponding to a fluorescence lifetime below 2.2 ns were significantly reduced, whereas the fluorescence lifetime of the donor in other cellular regions was unaffected (Fig. 1B). The average donor fluorescence lifetime distribution in the acceptor bleached cell is more homogeneous, with shorter components mainly disappeared; thus, indicating that the shorter measured average ECFP-lifetimes in pericentric heterochromatin have been caused by FRET. In addition, the FRET efficiency, as well as the distance between the donor and the acceptor molecules, could be determined by further analyses of these images.

3.2. FLIM-FRET experiment using two-photon excitation (2PE)

For some applications it is essential to combine fluorescence lifetime imaging microscopy with two-photon excitation which is nowadays a well established technique in biological research (2P-FLIM).[5] Due to the non-linear process of 2PE, the generated fluorescence is restricted to a small focal volume. As a consequence, no pinhole is necessary to restrict the fluorescent light to the confocal plane. In addition the near infrared (NIR) excitation light used in 2PE is less scattered in biological tissues, allowing for high-resolution deep tissue imaging and penetration depths up to one millimetre. Finally, the low-energy NIR light and the highly localised excitation strongly reduce global photo-bleaching of the fluorophores as well as tissue damage.

The performance of 2P-FLIM is demonstrated in the following example using an upgraded Leica SP5 (Fig. 2). The sample (provided by courtesy of Dirk Daelemans and Thomas Vercruysse, Rega Institute for Medical Research, Katholieke Universiteit, Leuven) was excited by 2PE at 850 nm with 80 MHz repetition rate. The cells expressed a EGFP-RFP fusion construct where both fluorophores were separated only by a short linker. Such a donor-acceptor fusion serves as a positive control for FRET.

The FLIM image in Fig. 2A shows two cells with different average donor lifetimes: a FRET cell and a cell where the acceptor RFP was irreversibly bleached leading to a lifetime shift from approximately 2.1 ns towards 2.4 ns. This shift of the average lifetime could be clearly distinguished in the lifetime histogram by means of two peaks corresponding to both cells (Fig. 2B). The TCSPC histogram represents both fluorescence decays from the FRET and the bleached cell (Fig. 2C). By fitting them to a bi-exponential decay model, for each cell two lifetimes could be calculated (Table in Fig. 2D). The value of 2.4 ns corresponds to unquenched EGFP, whereas the second lifetime of 1.2 ns represents EGFP molecules that are quenched by FRET. A comparison of the relative amplitudes revealed that the ratio of both lifetime components in the FRET cell is almost 1 to 1 (amplitude 1 ~51%, amplitude 2 ~49%). This indicates that half of the EGFP-RFP fusion

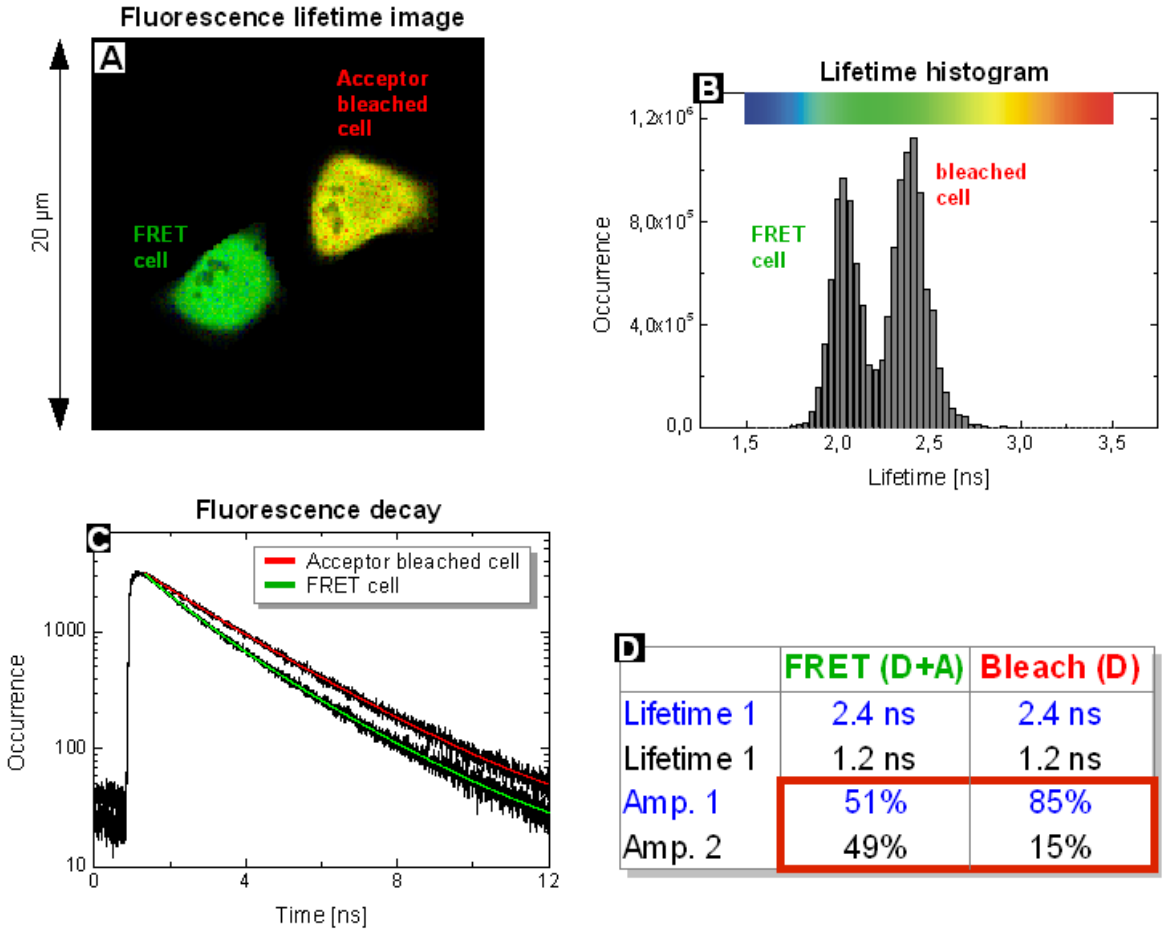

Fig. 2: FLIM-FRET experiment using two-photon excitation. The FLIM image displays the fluorescence lifetime indicated by a false colour representation of two cells transfected with a EGFP-RFP fusion protein (A). The fluorescence was restricted to the donor emission only by using a suited bandpass filter (500-540 nm). Image size 256 x 256 pixel. The fluorescence lifetime is false colour coded as indicated in (B). In the lifetime histogram the average lifetimes of the FRET and acceptor photo-bleached cell were plotted giving rise to two peaks at 2.1 ns and 2.4 ns (B). The results of bi-exponential fitting of the fluorescence decays measured in each cell (TCSPC histogram, C) are shown in (D).

proteins could adopt a proper conformation due to complete maturation allowing for FRET. When analysing the acceptor-bleached cell the situation was different. Only 15% of the EGFP molecules were quenched by energy transfer to some remaining acceptor molecules whereas the majority of donor molecules (85%) could not undergo FRET any more because an appropriate acceptor molecule was missing.

3.3. FLIM-FRET measurements by dual channel detection

An absence of FRET does not indicate that the fluorescently tagged molecules are not interacting. Amongst other reasons like unfavourable dipole orientation of the fluorophores, the acceptor molecule could be absent. Its presence within the sample can be directly proven by a dual-channel detection set up, thus allowing to exclude these artefacts. This is demonstrated in the following example where the extent of FRET between two human kinetochore proteins was determined.

The human centromere kinetochore complex is responsible for the correct chromosome segregation during cell division. It provides the attachment site for the spindle microtubules during mitosis and meiosis and is located at the primary constriction of each chromosome. This nucleo-protein complex consists of about 50 kinetochore proteins (CENPs) and the underlying DNA structure – the centromere. Malfunction of this complex can lead to aneuploidy and cancer.6 Although many components of this essential complex are known, its exact structure still remains unsolved. Therefore the neighbourhood relations of kinetochore proteins were determined in living human cells by FLIM-FRET.
Fig. 3 displays an experiment with the two kinetochore proteins CENP-A and -B (samples provided by courtesy of Sandra Orthaus, former Leibniz Institute for Age Research, Fritz Lipmann Institute, Jena). Both proteins have been tagged with the fluorophores Cerulean and EYFP at its C- and N-terminus respectively and display a punctual localisation at centromeres in the cell nucleus.

The measurements have been performed in transiently transfected living human cells using a Zeiss LSM510 equipped with a dedicated FLIM and FCS kit from PicoQuant.[2] The set up consisted of a 440 nm picosecond pulsed diode laser, two SPAD detectors and the PicoHarp300 TCSPC electronics.

In a first measurement the fluorescence lifetime of the donor Cerulean fused to CENP-B was

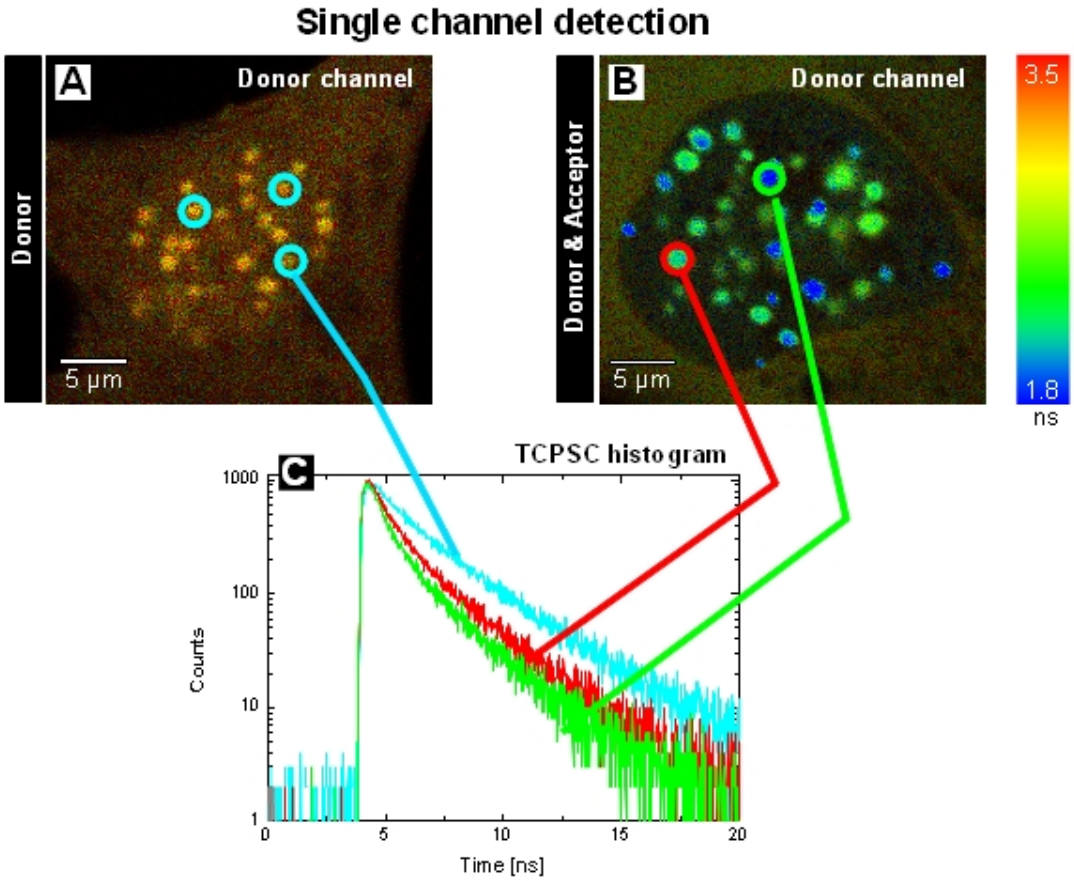

Fig. 3 (A, B, C): FLIM-FRET measurements of the human kinetochore proteins CENP-A and CENP-B. The FLIM images of human U2OS cells transfected with the donor CENP-B-Cerulean (A) or additionally with the acceptor EYFP-CENP-A (B) display the donor fluorescence lifetime (Single channel detection). The blue decay curve in the TCSPC histogram (C) corresponds to the three blue-circled centromeres of the control cell (A) whereas the green and red lines represent fluorescence decays of the indicated centromeres in the FRET cell (B).

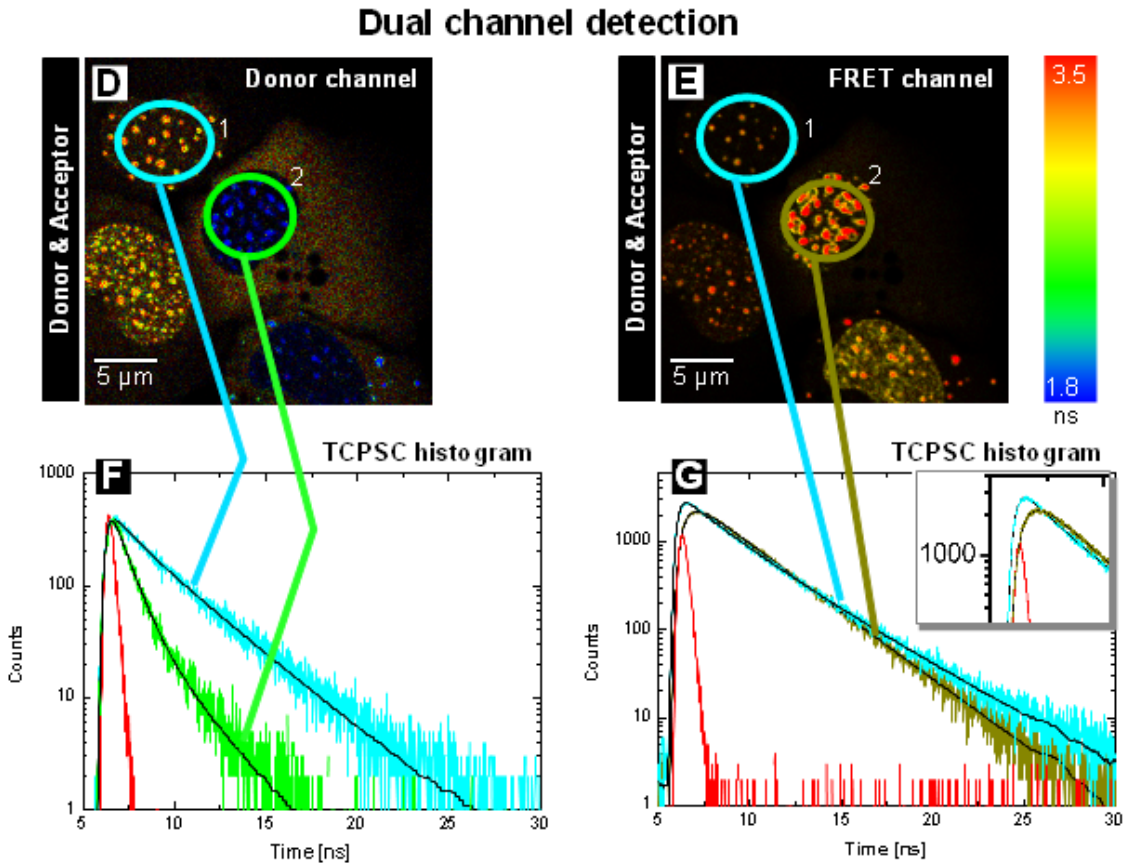

Fig. 3 (D, E, F, G): Dual channel detection using pulsed 440 nm excitation: Cell 1 was transfected only with the donor, and in cell 2 both kinetochore proteins were present. In the donor channel a bandpass filter BP 480/40 limited the detection to the Cerulean fluorescence only (D). In the FRET channel a longpass filter LP 530 allowed to record the acceptor fluorescence caused by FRET (E). The TCSPC histograms display the fluorescence decays of cell 1 and 2 in the corresponding channels (F, G). The enlargement in (G) shows the rise time of acceptor fluorescence. For data analysis the Instrumental Response Function (IRF, red curve) was used. The fluorescence lifetime is indicated by a false colour representation. Image size 256 x 256 pixel.

determined. By analysing three centromeres of a control cell (marked with blue circles) an average donor lifetime of 2.9 ns was calculated (Fig. 3A control cell and the blue trace in Fig. 3C).
In a cell containing both donor CENP-B-Cerulean and acceptor EYFP-CENP-A the donor lifetime was decreased due to FRET. At two single centromeres marked with green and red circles, average fluorescence lifetimes were shifted to 1.8 ns and 2.2 ns, respectively (Fig. 3B and green and red traces in Fig. 3C). Thus, one can conclude that both proteins are in direct vicinity in human kinetochores.
For this analysis, the fluorescence lifetime at the corresponding centromeres was calculated by fitting the fluorescence decay to a bi-exponential model. Whereas in the control cell, a homogeneous and longer average lifetime could be determined in all centromeres, in the double transfected cell, the fluorescence lifetimes varied amongst different complexes. These centromere specific lifetimes are most probably caused by different ratios of incorporated fluorescently labelled CENP A and -B molecules and do not reflect different complex structures at individual chromosomes.

A similar experiment was performed using two detectors to monitor the fluorescence lifetime of both the donor (Fig. 3D) and acceptor fluorophores simultaneously after excitation of the donor at 440 nm (Fig. 3E). Channel one displays the donor lifetime whereas in the second, so-called FRET channel, the acceptor fluorescence indirectly caused by energy transfer is shown. In this way the set up allows to directly prove the existence of FRET thus excluding artefacts.

Cell 1 marked with a blue circle contained only the donor CENP-B-Cerulean. At all centromeres, the analysis yielded an average fluorescence lifetime of 3.0 ns (see blue trace in Fig. 3F). The weak fluorescence seen in the acceptor channel was caused by bleed through.

In cell 2 both the donor and acceptor molecules were present. In the donor channel the quenching of the lifetime down to 1.2 ns caused by FRET is indicated by the blue colour of all centromeres. A FRET efficiency of 60% could be calculated. In the FRET channel strong fluorescence of the acceptor EYFP-CENP-A was detected. The analysis yielded a value of approximately 2.8 ns corresponding to the fluorescence lifetime of EYFP (dark green trace in Fig. 3G). In addition a rise time of around 0.5 ns was observed (see enlargement in the

TCSPC histogram). This delay of acceptor emission was caused by energy transfer between the donor and acceptor.

With these experiments it could be demonstrated that both the N-terminus of CENP-A and the C-terminus of CENP-B are in very close vicinity in human centromeres.

3.4. FLIM-FRET analysis to separate quenched from unquenched donor species

Compared to intensity-based FRET measurements, FLIM can reveal sub-populations; thus, allowing to determine the fraction of free donors compared to associated donor molecules within a complex. If several molecules are forming a complex, intensity-based FRET is unable to distinguish if energy is transferred from all donor molecules with low FRET efficiency (e.g. due to distances larger than the Förster radius) or if only a few donor molecules are tightly bound into the complex enabling high efficiency of energy transfer between donor and acceptor.

If the donor fluorescence can be described by a mono-exponential decay, FLIM-FRET can distinguish between these two situations, because in the first case a single exponential decay is obtained, whereas in the second case, a multi-exponential fitting model must be applied to determine the fluorescence lifetime:

$$\tau_{average} = \frac{\sum_i A_i \tau_i}{\sum_i A_i}$$

From the relative amplitudes (A1, A2...Ai) one can get an estimate for the fraction of bound and unbound donor molecules, which will be demonstrated in the following example.

FLIM images of live 12V HC Red cells expressing a protein fused to EGFP (donor) and RFP (acceptor) separated by a short linker (sample courtesy of Philippe Bastiaens, MPI Dortmund) were acquired using an Olympus FluoView FV1000 equipped with the PicoQuant LSM Upgrade Kit for FLIM and FCS.2 The sample was excited by pulsed excitation at 470 nm with 40 MHz repetition. Photons were detected by a single channel SPAD set up. A fluorescence bandpass filter (500 - 540 nm) limited the detection to the donor (EGFP) fluorescence only.

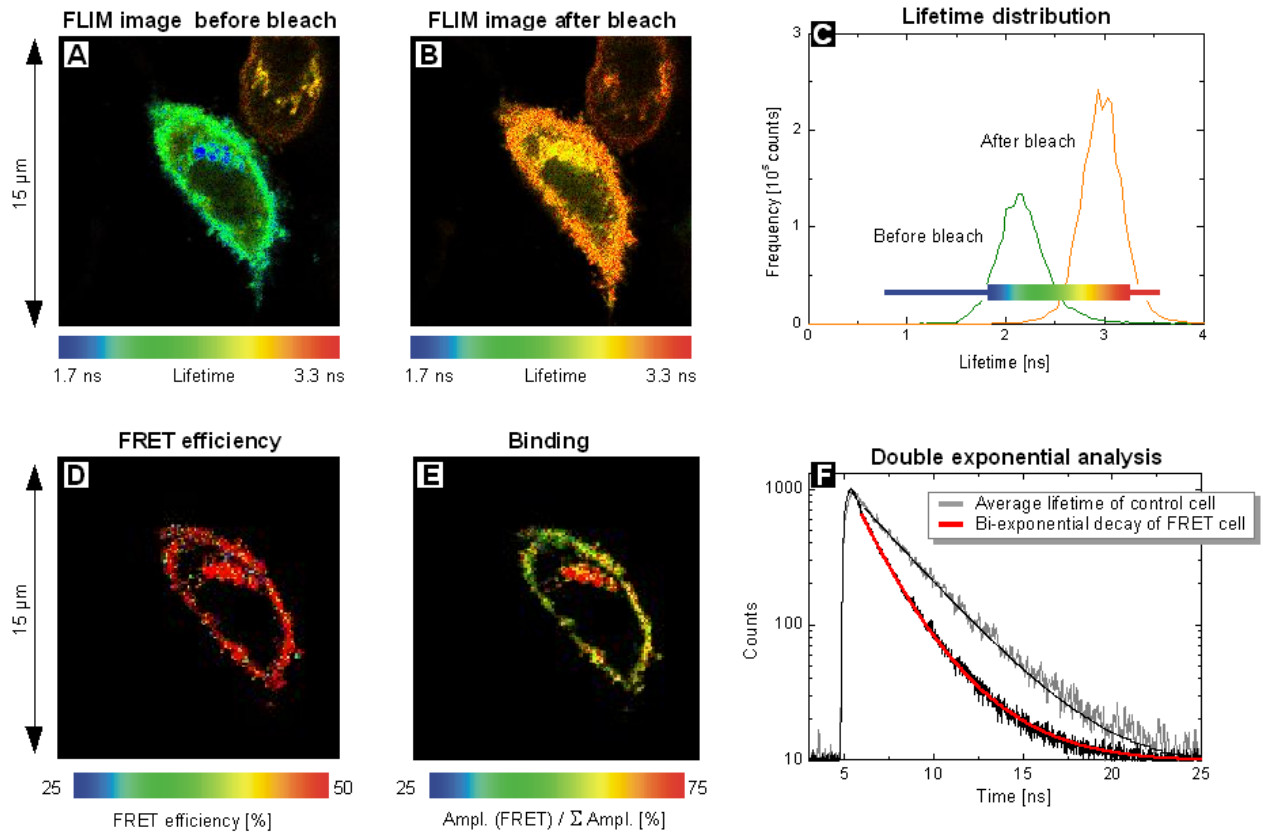

Fig. 4: FLIM-FRET measurements of living 12V HC Red cells expressing a EGFP-RFP fusion protein whereby both fluorophores are separated by a short peptide linker. The FLIM images monitor the donor fluorescence lifetime before (A) and after photo-bleaching of the acceptor RFP (B) using a 568 nm cw laser. The fluorescence lifetime is indicated by a false colour representation. The stronger FRET process can be identified in (A) in the blue coloured area. The lifetime distribution (C) reveals the shift of the average lifetime from 2.9 ns towards 2.2 ns due to FRET. Based on a double exponential decay analysis (TCSPC histogram, F) a FRET efficiency image (D) and an image of the amplitude ratio (E) are shown. Whereas the variation of the FRET efficiency was minimal, the ratios between quenched (bound) and unquenched (unbound) EGFP species differ. The colour code corresponds to the rainbow marker. This analysis was done with a special FRET script included in the system software SymPhoTime. Image size 256 x 256 pixel.

Fig. 4 displays FLIM images of the donor fluorescence lifetime before and after photo-bleaching of the acceptor RFP. By applying a mono-exponential decay model, an average lifetime of 2.2 ns could be obtained corresponding to quenched EGFP (Fig. 4A). After irreversibly destroying the acceptor the donor lifetime shifted towards 2.9 ns as depicted in (Fig. 4B) and in the lifetime distribution (Fig. 4C).
In the pre-bleach image, a cellular region with significantly shorter donor lifetimes as indicated by the blue coloured area can clearly be identified. To evaluate the reason for this stronger FRET quenching a scripting based FLIM-FRET analysis with a double exponential decay model was performed. The fitted shorter lifetime of 1.4 ns corresponds to FRET quenched donor molecules whereas the longer lifetime (3.0 ns) represents unbound donor only molecules (Fig. 4F).
A FRET efficiency image based on the short lifetime component revealed no variation within the cell (Fig. 4D). Thus the shortened lifetime could not be caused by different FRET efficiencies due to variable EGFP-RFP distances. Therefore, in a next step, an image of the amplitude ratio between the short (FRET quenched) and long (unbound donor) lifetime component was calculated according to the following formula:

$$\%\,Binding_i = \frac{A_i}{\sum_i A_i}$$

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It is clearly visible that in the region with shortened donor lifetimes, the ratio of quenched and unquenched EGFP molecules varied (Fig. 4E). Apparent FRET changes within the cell are therefore mainly due to different ratios between unbound donor molecules and FRET pairs with almost the same FRET efficiency.
Here it is demonstrated how FLIM-FRET can reveal the fraction of formed donor acceptor complexes.

Conclusion

The application note demonstrates several possibilities of FLIM-FRET measurements. The PicoQuant upgrade kit allows for the implementation of one- and two-photon excitation as well as for single and dual-channel detection. Results obtained by different systems have been presented, thus illustrating the performance of the FLIM and FCS upgrade using conventional LSMs.

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Further information

[1] Bibliography listing all publications with measurements based on PicoQuant instruments:

http://www.picoquant.com/_biblio.htm

[2] general download link of technical and application notes: http://www.picoquant.com/appnotes.htm

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